EXHIBIT J – PART 2

Why We Need Labeling of Genetically Engineered Food

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Overview

Food is different from other consumer products. It's something we literally take inside ourselves, it's necessary on a daily basis for growth and life, and bound up in our cultures and traditions, so we care about it intensely. Consumers, therefore, have a fundamental right to know what they are eating, and that it is safe. Most developed countries have adopted laws that reflect this view, requiring labelling, to show ingredients (e.g. broccoli, beef), processing (e.g. frozen, homogenized, irradiated), conformance to standards of identity (e.g. peanut butter must be made from peanuts), and additives (e.g. sulfites, preservatives). Some countries require fat, protein, carbohydrate and vitamin content of food to be labeled as well.

All of this labelling serves the consumer's right to know, and is above and beyond underlying national programs to assure the safety of food from such things as hazardous pesticides residues and additives, and disease-causing bacteria.

Consumers want to know what they are eating both as a matter of taste and preference, and for many health-related reasons. They may want to eat fish to improve their chances of avoiding heart disease, or avoid fish because they are concerned about depletion of certain species in the oceans or about mercury contamination. They may seek out carbohydrates because they are training for a marathon, or avoid them because they want to lose weight. They may eat bananas because they want a good source of potassium, or may avoid bananas because even one bite causes them to go into anaphylactic shock (as is the case with some people with severe food allergies). Body builders may want red meat, vegetarians will avoid it, and Muslims will avoid pork but not lamb. A mother may look for apple juice for her child because it is a natural drink, or avoid it because it gives the child a stomach ache. Every day, millions of consumers world-wide read millions of food labels and make millions of decisions like this for themselves and their families.

Consumers also have a right to know if food is genetically engineered. In this case too, it may be for taste and preference, or for important health related reasons. Some food producers claim that genetically engineered food is basically the same ('substantially equivalent' is the description used) as conventionally produced food. But this is not the case; some individuals can have unpredictable mild to severe allergic reactions; it can have unanticipated toxic effects; and it can change the nutrition in food. In addition consumers express a wide variety of religious, ethical and environmental preferences in their food choices, and they cannot do this without comprehensive labelling.

The countries of the European Union have recognized this, and have introduced regulations requiring labelling of all genetically engineered food. In the United States, where genetically engineered corn, soybeans and potatoes are being grown commercially, repeated public opinion surveys show consumers overwhelmingly want labelling, but thus far the government has failed to require it. In 1997, a survey sponsored by Novartis found that more than 90 per cent of

Americans want labelling (Feder, 1997). Most countries have not considered the issue yet. Of the large chemical/biotechnology companies that are developing these foods, some, like Novartis, support labelling, but most, like Monsanto and other major developers, oppose it.

The Codex Alimentarius Commission, an agency of the United Nations World Health Organization and Food and Agriculture Organization, has been considering whether to adopt a guideline recommending that all countries require labelling of genetically engineered food. Codex guidelines are not binding, but are often adopted by developing countries and can be used to settle trade disputes (if a country adopts a Codex standard, that standard cannot be challenged as protectionist). Consumers International is urging the Codex Alimentarius to recommend full mandatory labelling of all genetically engineered foods. This paper discusses eight important reasons why.

1. Genetically Engineered Food is Different

A strawberry can be given a flounder gene that makes it frost resistant, a bacterial gene that confers antibiotic resistance, and a virus gene that "turns on" the other added genes. Under normal circumstances, a strawberry can only acquire genetic material from other strawberries--that is, plants of the same or closely related species. With genetic engineering, however, scientists can give strawberries genetic material from trees, bacteria, fish, pigs, even humans if they chose to. Consumers International believes that any plant or animal food to which genes have been added from a source other than the species to which the food belongs, should be required to be labeled, to tell the consumer that this has been done.

Some people, mostly scientists and corporations involved in the development of genetically engineered food, argue that the strawberry with the foreign genes is not really different but "substantially equivalent" in the language Codex and international regulation and therefore needs no label.

Consumers, however, through their organizations, through comments to regulators, and through opinion surveys, have repeatedly expressed the view that this strawberry, and all other genetically engineered foods, are not "substantially equivalent," but sufficiently different that, like irradiated foods, and foods containing additives, they should be labeled. Since labelling laws are created to meet consumer needs, consumer opinion should be respected.

2. Genetically Engineered Food Can Cause Toxic Effects

The fact that genetic engineering can go seriously wrong was shown by one of the very first products introduced into the market. An amino acid (a protein building block) called tryptophan is sold in a number of countries including the United States as a dietary supplement. In the late 1980s, the Showa Denko company of Japan began making tryptophan by a new process, using genetically engineered bacteria, and selling it in the United States.

Within months thousands of people who had taken the supplement began to suffer from eosinophilia myalgia syndrome, which included neurological problems. Eventually at least 1500 were permanently disabled and 37 died (Mayeno and Gleich, 1994).

As doctors encountered this syndrome, they gradually noticed that it seemed linked to patients taking tryptophan produced by Showa Denko. However, it took months before this was taken off the market. Had it been labeled as genetically engineered, it might have accelerated the identification of the source of the problem.

Showa Denko refused to cooperate in any U.S. government efforts to investigate the cause of the problem. However, the tryptophan that caused the problem was determined to contain a toxic contaminant which appears to have been a by-product of the increased tryptophan production of the genetically engineered bacteria (Mayeno and Gleich, 1994).

There are many ways besides this in which genetic engineering could go awry and result in hazardous toxins in food. Many common plant foods such as tomatoes and potatoes produce highly toxic chemicals in their leaves, for example. Any responsible company working with such plants would check for changes in toxin levels. But not all companies are equally responsible, and as the Showa Denko example shows, and a serious hazard can be missed.

Government agencies cannot be counted on to prevent unexpected problems. World-wide, government premarket

safety reviews of genetically engineered products range from relatively thorough in the European Union to no review at all in much of the world. In the United States, premarket safety reviews are voluntary.

We can expect that in the future genetically engineered food will be developed and grown in many countries with no premarket safety reviews. Unless all such products are labeled, it will be difficult to determine the source of any toxin problems originating in such food.

3. Genetically Engineered Food Can Cause Allergic Reactions

In the United States, about a quarter of all people report that they have an adverse reaction to some food (Sloan and Powers, 1986). Studies have shown that 2 percent of adults and 8 percent of children have true food allergies, mediated by immunoglobin E (IgE) (Bock, 1987; Sampson et al., 1992).

People with IgE mediated allergies have an immediate reaction to certain proteins, ranging from itching to potentially fatal anaphylactic shock. The most common allergies are to peanuts, other nuts and shellfish.

Genetic engineering can transfer allergies from foods to which people know they are allergic, to foods that they think is safe. In March 1996, researchers at the University of Nebraska in the United States confirmed that an allergen from Brazil nuts had been transferred into soybeans. The Pioneer Hi-Bred International seed company had put a Brazil nut gene into soybeans to improve their protein content for animal feed. In an in-vitro and a skin prick test, the engineered soybeans reacted with the IgE of individuals with a Brazil nut allergy in a way that indicated that the individuals would have had an adverse, potentially fatal reaction to the soybeans (Nordlee et al., 1996).

This case has a happy ending. As Marion Nestle, the head of the Nutrition Department at New York University summarized in an editorial in the respected New England Journal of Medicine "In the special case of transgenic soybeans, the donor species was known to be allergenic, serum samples from persons allergic to the donor species were available for testing and the product was withdrawn" (Nestle, 1996: 726). However, for virtually every food, there is someone allergic to it. Proteins are what cause allergic reactions, and virtually every gene transfer in crops results in some protein production. Proteins will be coming into food crops not just from known sources of common allergens, like peanuts, shellfish and dairy, but from plants of all kinds, bacteria and viruses, whose potential allergenicity is uncommon or unknown. Furthermore, there are no fool-proof ways to determine whether a given protein will be an allergen, except tests involving serum from individuals allergic to the given protein. Nestle continues, "The next case could be less ideal, and the public less fortunate. It is in everyone’s best interest to develop regulatory policies for transgenic foods that include premarketing notification and labelling" (Nestle, 1996: 727).

To protect consumer health from the effects of unrecognized or uncommon allergens, all genetically engineered food must be labeled. Otherwise there will be no way for sensitive individuals to distinguish foods that cause them problems from ones that do not. This need is particularly urgent, since one of the potential consequences is sudden death, and children are the part of the population most at risk.

4. Genetic Engineering Can Increase Antibiotic Resistance

Despite the precise sound of its name, genetic engineering, is actually a messy process, and most attempts end in failure. While the gene to be transferred can be identified fairly precisely, the process of inserting it in the new host can be very imprecise. Genes are often moved with something that is the molecular equivalent of a shotgun. Scientists coat tiny particles with genetic material and then "shoot" these into thousands of cells in a petri dish before they get one where the desired trait "takes" and is expressed.

Because the transferred trait, such as ability to produce an insecticide in the leaves of the plant, is often not immediately apparent, scientists generally also insert a "marker gene" along with the desired gene into the new plant. The most commonly used marker is a bacterial gene for antibiotic resistance. Most genetically engineered plant food contains such a gene.

Widespread use of antibiotic resistance marker genes could contribute to the problem of antibiotic resistance. The genes may move from a crop into bacteria in the environment, and since bacteria readily exchange antibiotic resistance

genes, move into disease-causing bacteria and make them resistant too. Antibiotic resistance genes could even be transferred in the digestive tract to bacteria. An example of this is the genetically engineered Bt maize plant from Novartis which includes an ampicillin-resistance gene. Ampicillin is a valuable antibiotic used to treat a variety of infections in people and animals. A number of European countries, including Britain, have refused to permit the Novartis Bt corn to be grown, because of concern that the ampicillin resistance gene could move from the corn into bacteria in the food chain, making ampicillin a less effective weapon against bacterial infections.

But there are already foods in the market made using plants with antibiotic resistance marker genes. Without labelling, consumers cannot choose not to buy them.

5. Genetic Engineering Can Alter Nutritional Value

Genetic engineering can alter nutritional value of foods in positive ways. For example, canola oil has been engineered to have a different profile of fatty acids, so that they contain less of the fat molecules that tend to build up in people's arteries. Scientists are also working on increasing the vitamin C content in some foods. However, it is also possible that nutritional content could be reduced as an unexpected side effect of some other genetic engineering. Labelling is needed to make sure consumers are properly informed.

6. Genetically Engineered Food Can Create Environmental Risk

The most widely grown genetically engineered crops, accounting for 99 percent of the land under transgenic cultivation world-wide, are engineered for herbicide tolerance, insect resistance, and virus resistance (James, 1997). Each of these poses environmental risks.

Herbicide-tolerant crops are varieties on which herbicides can be used to kill weeds, without killing the crop itself. These varieties encourage farmers to use more herbicides, which frequently pollute groundwater and can cause various other forms of ecological damage.

Insect-resistant crops almost all contain a gene from the bacterium Bacillus thuringiensis (Bt) which causes the plant to produce an endotoxin throughout the plant, including leaves and fruit. Bt corn, cotton, potatoes tomatoes and rice are all being grown in various parts of the world.

While Bt crops at first glance appear to be ecologically sound, because they need less chemical pesticides, they have serious drawbacks. Crops that continuously produce Bt endotoxin quickly speed up the process of the spread of resistance to the Bt endotoxin among the pests feeding on the crops.

A recent computer model developed by a scientist at the University of Illinois in the U.S. predicted that if all U.S. farmers grew Bt corn, resistance would develop in a single year! Scientists at the University of North Carolina in the U.S. have already found Bt resistance genes in wild populations of a moth pest that feeds on corn. (Gloud et. al, 1997)

The Bt endotoxin, produced by the Bt bacteria, is a staple of organic farming since it is a relatively harmless natural pesticide. It is also widely used by conventional farmers who use integrated pest management to minimize the use of more toxic chemicals. Scientists predict that Bt will become less and less useful, however, within a few years of widespread planting of Bt crops.

The Bt crops may also be toxic to beneficial insects. Researchers from Swiss Federal Research Station for Agroecology and Agriculture found, for example, over 60% mortality of green lacewings, that ate moth larvae that had fed on Bt corn.

Virus-resistant crops almost all contain genes that can mix with genes from other viruses that naturally infect the plant to create new gene combinations, some of which can give rise to new or deadlier viruses. US and Canadian work has shown that wild viruses can hijack genes from engineered crops at rates far higher than previously suspected. The concern was great enough that the U.S. Department of Agriculture held a meeting in October, 1997 to discuss possible restrictions aimed at reducing the risk of creating harmful new plant viruses due to the use of virus-resistant crops (Kleiner, 1997).

Another serious concern is "gene pollution". If the gene for herbicide tolerance escapes into wild relatives of crop plants that are weeds, it could result in a new generation of herbicide-tolerant superweeds. In fact, researchers in both Norway (Jorgensen and Andersen, 1995) and the United States (Hileman, 1995) have already demonstrated that the gene for herbicide tolerance moved from cultivated canola to close relatives in nearby fields, such as wild mustard.

If the gene for the production of the Bt endotoxin moves into wild plants, they could become resistant to butterfly, moth and beetle pests, just like the Bt crops. This could upset established ecological balances by either causing the wild plant to flourish excessively and become a plant pest, or by reducing the butterfly or moth population that previously fed on the newly toxic plant.

Gene pollution would be especially problematic in many developing countries where the center of origin for many crops is.

In these areas, traditional crop varieties could become "polluted" with genes from the genetically engineered crops and biological diversity will suffer. The rate of gene flow between genetically engineered plants and their wild relatives may be higher than previously thought. Researchers in the southern United States demonstrated that more than 50% of the wild strawberries growing within 50 meters of a strawberry field contained marker genes from the cultivated strawberries.

Researchers in central U.S. found that after ten years more than a quarter of the wild sunflowers growing near fields of cultivated sunflowers had a marker gene from the cultivated sunflowers. (Kling, 1996)

These problems illustrate the need for great caution in introducing and using genetically modified plants. But even with this, consumers have a right to know about the environmental impact of the foods they buy so that, if they wish they can exercise their own preferences and avoid - or choose to buy - food that has been produced in a particular way.

7. Genetic Engineering Can Affect Dietary Preferences

Consumers make decisions about what they eat for a wide variety of religious, ethical, philosophical and emotional reasons. Most major world religions have some rules or traditions as to food. Jews and Muslims do not eat pork; Christians often avoid meat on Fridays or during Lent, many Buddhists are vegetarians.

Many other individuals have food preferences that are not related to an organized religion but which reflect deeply held personal beliefs, such as wanting to protect the environment.

Consumers International supports labelling of genetically engineered food in order to allow consumers the opportunity to exercise their religious and ethical preferences. For example, some people will want to avoid lamb which contains pig genes (a product which is not yet on the market, but is well within the current capabilities of science). For this, labelling would be essential.

8. Science is Fallible

When a new technology of food production emerges, all the problems it may cause may not be foreseen. When pesticides were first synthesized and used widely in the 1950s, they were heralded as a miracle cure for pest problems. Only later did we discover that some of them could also cause birds to lay eggs with shells that collapsed, humans to get cancer, and insects to become resistant to them.

Genetic engineering is shuffling the deck of genes in ways that are entirely new, and creating living things that have never before existed. Consumers International believes consumers have a right to be cautious about using these, if they wish. The right to choose can be exercised only if proper information is provided — on labels or the food itself.

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STATE-OF-THE-SCIENCE ON THE HEALTH RISKS OF GM FOODS

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We all know stories of tobacco, asbestos, and DDT. Originally declared safe, they caused widespread death and disease. Although their impact was vast, most of the population was spared. The same cannot be said for sweeping changes in the food supply. Everyone eats; everyone is affected. The increase in several diseases in North America may be due to the profound changes in our diet. The most radical change occurred a little over a decade ago when genetically modified (GM) crops were introduced. Their influence on health has been largely ignored, but recent studies show serious problems. Genetically modified organisms (GMOs) have been linked to thousands of toxic or allergic-type reactions, thousands of sick, sterile, and dead livestock, and damage to virtually every organ and system studied in lab animals.[1] Nearly every independent animal feeding safety study shows adverse or unexplained effects.

GM foods were made possible by a technology developed in the 1970s whereby genes from one species are forced into the DNA of other species. Genes produce proteins, which in turn can generate characteristics or traits. The promised traits associated with GMOs have been sky high—vegetables growing in the desert, vitamin fortified grains, and highly productive crops feeding the starving millions. None of these are available. In fact, the only two traits that are found in nearly all commericialized GM plants are herbicide tolerance and/or pesticide production.

Herbicide tolerant soy, corn, cotton, and canola plants are engineered with bacterial genes that allow them to survive otherwise deadly doses of herbicides. This gives farmers more flexibility in weeding and gives the GM seed company lots more profit. When farmers buy GM seeds, they sign a contract to buy only that seed producer's brand of herbicide. Herbicide tolerant crops comprise about 80% of all GM plants. The other 20% are corn and cotton varieties that produce a pesticide in every cell. This is accomplished due to a gene from a soil bacterium called *Bacillus thuringiensis* or Bt, which produces a natural insect-killing poison called Bt-toxin. In addition to these two traits, there are also disease resistant GM Hawaiian papaya, zucchini and crook neck squash, which comprise well under 1% of GMO acreage.

THE FDA'S "NON-REGULATION" OF GM FOODS

Rhetoric from the United States government since the early 1990s proclaims that GM foods are no different from their natural counterparts that have existed for centuries. The Food and Drug Administration (FDA) has labeled them "Generally Recognized as Safe," or GRAS. This status allows a product to be commercialized without any additional testing. According to US law, to be considered GRAS the substance must be the subject of a substantial amount of peer-reviewed published studies (or equivalent) and there must be overwhelming consensus among the scientific community that the product is safe. GM foods had neither. Nonetheless, in a precedent-setting move in 1992 that some experts contend was illegal, the FDA declared that GM crops are GRAS as long as their producers say they are. Thus, the FDA does not require *any* safety evaluations or labeling of GMOs. A company can even introduce a GM food to the market without telling the agency.

Such a lenient approach was largely the result of the influence of large agricultural corporations According to Henry Miller, who had a leading role in biotechnology issues at the FDA from 1979 to 1994, "In this area, the US government agencies have done exactly what big agribusiness has asked them to do and told them to do." The Ag biotech company with the greatest influence was clearly Monsanto. According to the *New York Times*, "What Monsanto wished for from Washington, Monsanto and, by extension, the biotechnology industry got. . . . When the company abruptly decided that it needed to throw off the regulations and speed its foods to market, the White House quickly ushered through an unusually generous policy of self-policing."

This policy was heralded by Vice President Dan Quayle on May 26, 1992. He chaired the Council on Competitiveness, which had identified GM crops as an industry that could boost US exports. To take advantage, Quayle announced "reforms" to "speed up and simplify the process of bringing" GM products to market without "being hampered by unnecessary regulation."[2] Three days later, the FDA policy on non-regulation was unveiled.

The person who oversaw its development was the FDA's Deputy Commissioner for Policy, Michael Taylor, whose position had been created especially for him in 1991. Prior to that, Taylor was an outside attorney for both Monsanto and the Food Biotechnology Council. After working at the FDA, he became Monsanto's vice president. The Obama administration has put Talyor back into the FDA as the US Food Safety Czar.

THE FDA COVERS UP HEALTH RISKS

Taylor's GMO policy needed to create the impression that unintended effects from GM crops were not an issue. Otherwise their GRAS status would be undermined and they would need the extensive testing and labels that are normally required for food additives. But internal memos made public from a lawsuit showed that the overwhelming consensus among the agency scientists was that GM crops can have unpredictable, hard-to-detect side effects. Various departments and experts spelled these out in detail, listing allergies, toxins, nutritional effects, and new diseases as potential dangers. They urged superiors to require long-term safety studies.[3] In spite of the warnings, according to public interest attorney Steven Druker who studied the FDA's internal files, "References to the unintended negative effects of bioengineering were progressively deleted from drafts of the policy statement (over the protests of agency scientists)."[4]

FDA microbiologist Louis Pribyl, PhD, wrote about the policy, "What has happened to the scientific elements of this document? Without a sound scientific base to rest on, this becomes a broad, general, 'What do I have to do to avoid trouble'-type document. . . . It will look like and probably be just a political document. . . . It reads very pro-industry, especially in the area of unintended effects."[5]

The scientists' concerns were not only ignored, their very existence was denied. The official FDA policy stated, "The agency is not aware of any information showing that foods derived by these new methods differ from other foods in any meaningful or uniform way."[6] In sharp contrast, an *internal* FDA report stated, "The processes of genetic engineering and traditional breeding are different and according to the technical experts in the agency, they lead to different risks."[7] The FDA's deceptive notion of no difference was coined "substantial equivalence" and formed the basis of the US government position on GMOs.

Many scientists and organizations have criticized the US position. The National Academy of Sciences and even the pro-GM Royal Society of London[8] describe the US system as inadequate and flawed. The editor of the prestigious journal *Lancet* said**,** "It is astounding that the US Food and Drug Administration has not changed their stance on genetically modified food adopted in 1992. . . . The policy is that genetically modified crops will receive the same consideration for potential health risks as any other new crop plant. This stance is taken despite good reasons to believe that specific risks may exist. . . . Governments should never have allowed these products into the food chain without insisting on rigorous testing for effects on health."[9] The Royal Society of Canada described substantial equivalence as "scientifically unjustifiable and inconsistent with precautionary regulation of the technology."[10]

GMOs ARE INHERENTLY UNSAFE

There are several reasons why GM plants present unique dangers. The first is that the *process* of genetic engineering itself creates unpredicted alterations, irrespective of which gene is transferred. The gene insertion process, for example, is accomplished by either shooting genes from a "gene gun" into a plate of cells, or using bacteria to infect the cell with foreign DNA. Both create mutations in and around the insertion site and elsewhere.[11] The "transformed" cell is then cloned into a plant through a process called tissue culture, which results in additional hundreds or thousands of mutations throughout the plants' genome. In the end, the GM plant's DNA can be a staggering 2-4% different from its natural parent.[12] Native genes can be mutated, deleted, or permanently turned on or off. In addition, the insertion process causes holistic and not-well-understood changes among large numbers of native genes. One study revealed that up to 5% of the natural genes altered their levels of protein expression as a result of a single insertion.

The Royal Society of Canada acknowledged that "the default prediction" for GM crops would include "a range of collateral changes in expression of other genes, changes in the pattern of proteins produced and/or changes in metabolic activities."[13] Although the FDA scientists evaluating GMOs in 1992 were unaware of the extent to which GM DNA is damaged or changed, they too described the potential consequences. They reported, "The possibility of unexpected, accidental changes in genetically engineered plants" might produce "unexpected high concentrations of plant toxicants."[14] GM crops, they said, might have "increased levels of known naturally occurring toxins," and the "appearance of new, not previously identified" toxins.[15] The same mechanism can also produce allergens, carcinogens, or substances that inhibit assimilation of nutrients.

Most of these problems would pass unnoticed through safety assessments on GM foods, which are largely designed on the false premise that genes are like Legos that cleanly snap into place. But even if we disregard unexpected changes in the DNA for the moment, a proper functioning inserted gene still carries significant risk. Its newly created GM protein, such as the Bt-toxin, may be dangerous for human health (see below). Moreover, even if that protein is safe in its natural organism, once it is transferred into a new species it may be processed differently. A harmless protein may be transformed into a dangerous or deadly version. This happened with at least one GM food crop under development, GM peas, which were destroyed before being commercialized.

FDA scientists were also quite concerned about the possibility of inserted genes spontaneously transferring into the DNA of bacteria inside our digestive tract. They were particularly alarmed at the possibility of antibiotic resistant marker (ARM) genes transferring. ARM genes are employed during gene insertion to help scientists identify which cells successfully integrated the foreign gene. These ARM genes, however, remain in the cell and are cloned into the DNA of all the GM plants produced from that cell. One FDA report wrote in all capital letters that ARM genes would be "A SERIOUS HEALTH HAZARD," due to the possibility of that they might transfer to bacteria and create super diseases, untreatable with antibiotics.

Although the biotech industry confidently asserted that gene transfer from GM foods was not possible, the only human feeding study on GM foods later proved that it does take place. The genetic material in soybeans that make them herbicide tolerant transferred into the DNA of human gut bacteria and continued to function[16]. That means that long after we stop eating a GM crop, its foreign GM proteins may be produced inside our intestines. It is also possible that the foreign genes might end up inside our own DNA, within the cells of our own organs and tissues.

Another worry expressed by FDA scientists was that GM plants might gather "toxic substances from the environment" such as "pesticides or heavy metals,"[17] or that toxic substances in GM animal feed might bioaccumulate into milk and meat products. While no studies have looked at the bioaccumulation issue, herbicide tolerant crops certainly have higher levels of herbicide residues. In fact, many countries had to increase their legally allowable levels—by up to 50 times—in order to accommodate the introduction of GM crops.

The overuse of the herbicides due to GM crops has resulted in the development of herbicide resistant weeds. USDA statistics show that herbicide use is rapidly accelerating. Its use was up by 383 million pounds in the first 13 years of GM crops.[18] But the rate of application is accelerating due in large part to the emergence of herbicide tolerant weeds. According to a study by Charles Benbrook, "Crop years 2007 and 2008 accounted for 46% of the increase in herbicide use over 13 years across the three HT [herbicide tolerant] crops. Herbicide use on HT crops rose a remarkable 31.4% from 2007 to 2008." And as Roundup becomes less effective, farmers are now using more toxic herbicides, such as 2-4D, which increased by 237% from 2004 to2006.[19]

All of the above risks associated with GM foods are magnified for high-risk groups, such as pregnant women, children, the sick, and the elderly. The following section highlights some of the problems that have been identified.

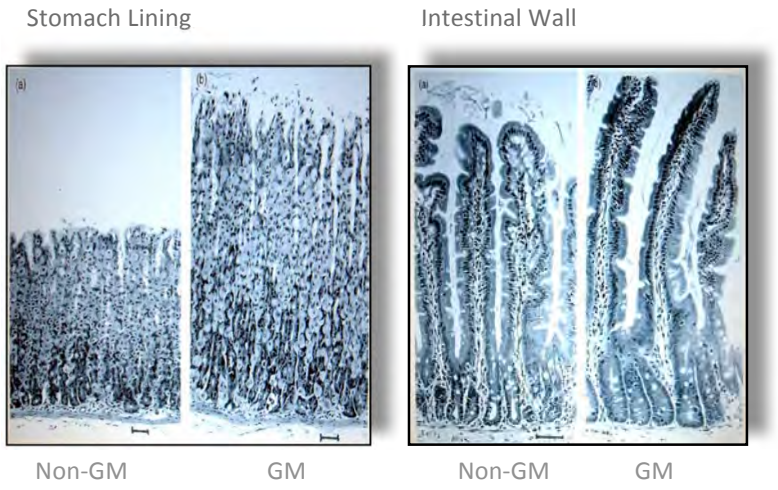
GM DIET SHOWS TOXIC REACTIONS IN THE DIGESTIVE TRACT

The very first crop submitted to the FDA's voluntary consultation process, the FlavrSavr tomato, showed evidence of toxins. Out of 20 female rats fed the GM tomato, 7 developed stomach lesions.[20] The director of FDA's Office of Special Research Skills wrote that the tomatoes did not demonstrate a "reasonable certainty of no harm,"[21] which is their normal standard of safety. The Additives Evaluation Branch agreed that "unresolved questions still remain."[22] The political appointees, however, did not require that the tomato be withdrawn.[1]

According to Arpad Pusztai, PhD, one of the world's leading experts in GM food safety assessments, the type of stomach lesions linked to the tomatoes "could lead to life-endangering hemorrhage, particularly in the elderly who use aspirin to prevent [blood clots]."[23] Dr. Pusztai believes that the digestive tract, which is the first and largest point of contact with foods, can reveal various reactions to toxins and should be the first target of GM food risk assessment. He was alarmed, however, to discover that studies on the FlavrSavr never looked passed the stomach to the intestines. Other studies that did look found problems.

Mice fed potatoes engineered to produce the Bt-toxin developed abnormal and damaged cells, as well as proliferative cell growth in the lower part of their small intestines (ileum).[24] Rats fed potatoes engineered to produce a different type of insecticide (GNA lectin from the snowdrop plant) also showed proliferative cell growth in both the stomach and intestinal walls (see photos).[25] Although the guts of rats fed GM peas were not examined for cell growth, the intestines were mysteriously heavier; possibly as a result of such growth.[26] Cell proliferation can be a precursor to cancer and is of special concern.

Rats fed GM potatoes showed proliferative cell growth in the stomach and intestines.



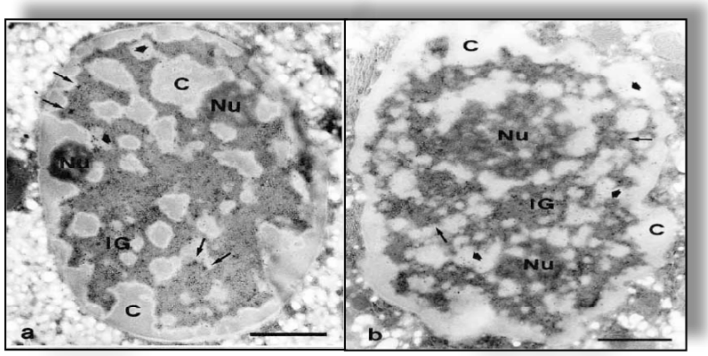
GM DIETS CAUSE LIVER DAMAGE

[1] Calgene had submitted data on two lines of GM tomatoes, both using the same inserted gene. They voluntarily elected to market only the variety that was not associated with the lesions. This was not required by the FDA, which did not block approvals on the lesion-associated variety. The FlavrSavr tomato has since been taken off the market. After the FlavrSavr, no other biotech company has submitted such detailed data to the FDA.

The state of the liver—a main detoxifier for the body—is another indicator of toxins.

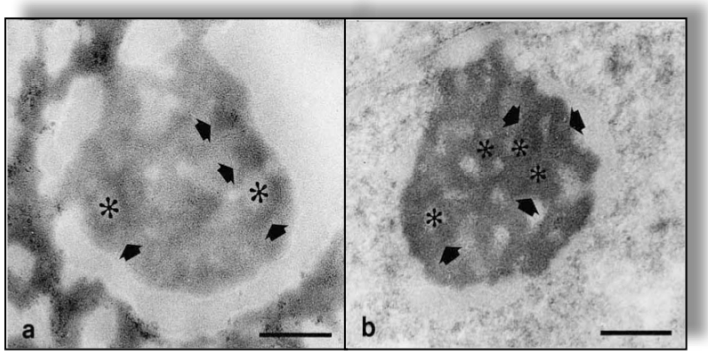
- Rats fed the GNA lectin potatoes described above had smaller and partially atrophied livers.[27]
- Rats fed Monsanto's Mon 863 corn, engineered to produce Bt-toxin, had liver lesions and other indications of toxicity.[28]
- Rabbits fed GM soy showed altered enzyme production in their livers as well as higher metabolic activity.[29]
- The livers of rats fed Roundup Ready canola were 12%–16% heavier, possibly due to liver disease or inflammation.[30]
- Microscopic analysis of the livers of mice fed Roundup Ready soybeans revealed altered gene expression and structural and functional changes (see photos).[31] Many of these changes reversed after the mice diet was switched to non-GM soy, indicating that GM soy was the culprit. The findings, according to molecular geneticist Michael Antoniou, PhD, "are not random and must reflect some 'insult' on the liver by the GM soy." Antoniou, who does human gene therapy research in King's College London, said that although the long-term consequences of the GM soy diet are not known, it "could lead to liver damage and consequently general toxemia."[32]
- Rats fed Roundup Ready soybeans also showed structural changes in their livers. [33]

The liver cells from soy-fed mice showed anomalies.



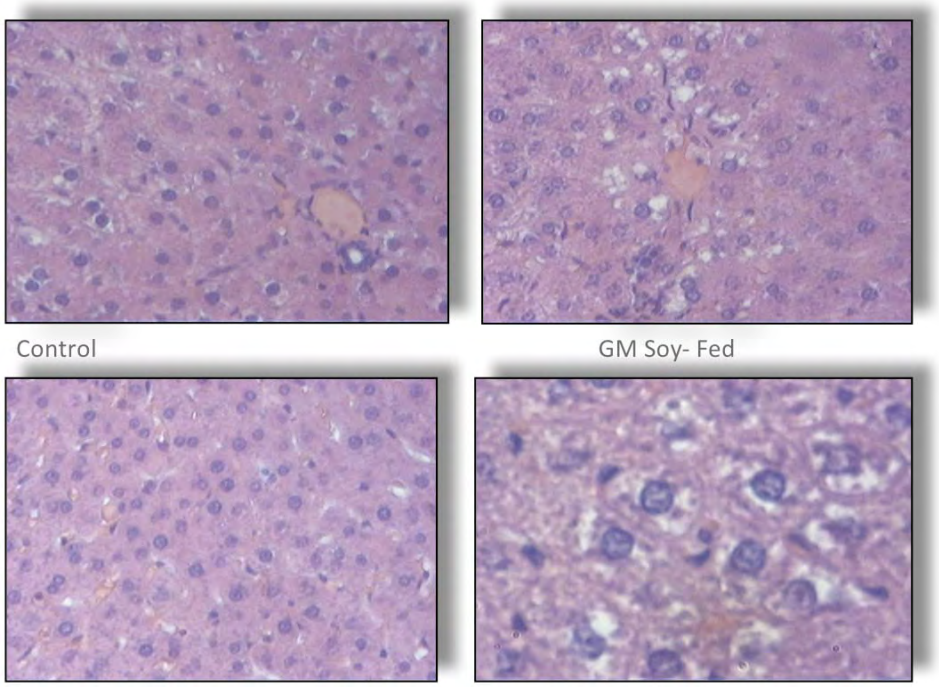
Liver cell nuclei

Control GM Soy Fed



Liver cell nucleoli

The livers of soy-fed rats showed unique changes.



GM FED ANIMALS HAD HIGHER DEATH RATES AND ORGAN DAMAGE

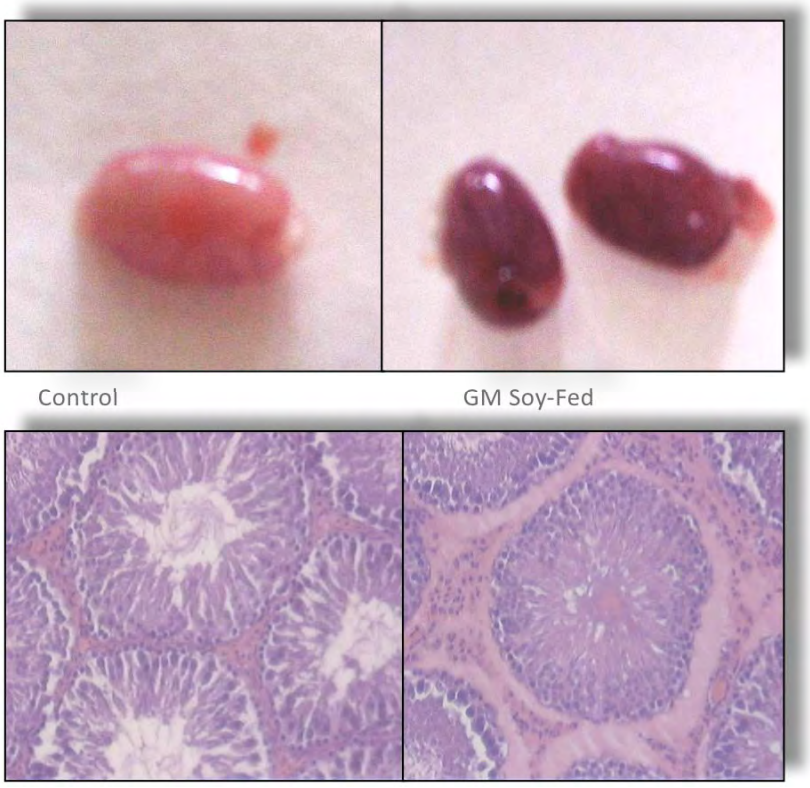
In the FlavrSavr tomato study, *a note in the appendix* indicated that 7 of 40 rats died within two weeks and were replaced.[34] In another study, chickens fed the herbicide tolerant "Liberty Link" corn died at twice the rate of those fed natural corn.[35] But in these two industry-funded studies, the deaths were dismissed without adequate explanation or follow-up.

In addition, the cells in the pancreas of mice fed Roundup Ready soy had profound changes and produced significantly less digestive enzymes;[36] in rats fed a GM potato, the pancreas was enlarged.[37] In various analyses of kidneys, GM-fed animals showed lesions, toxicity, altered enzyme production or inflammation.[38,39] Enzyme production in the hearts of mice was altered by GM soy.[40] And GM potatoes caused slower growth in the brain of rats.[41] A team of independent scientists re-analyzed the raw data in three Monsanto 90-day rat feeding studies and saw signs of toxicity in the liver and kidneys, as well as effects in the heart, adrenal glands, spleen, and blood.[42]

REPRODUCTIVE FAILURES AND INFANT MORTALITY

The testicles of both mice and rats fed Roundup Ready soybeans showed dramatic changes. In rats, the organs were dark blue instead of pink (see photos on next page).[43] In mice, young sperm cells were altered.[44] Embryos of GM soy-fed mice also showed temporary changes in their DNA function, compared to those whose parents were fed non-GM soy.[45]

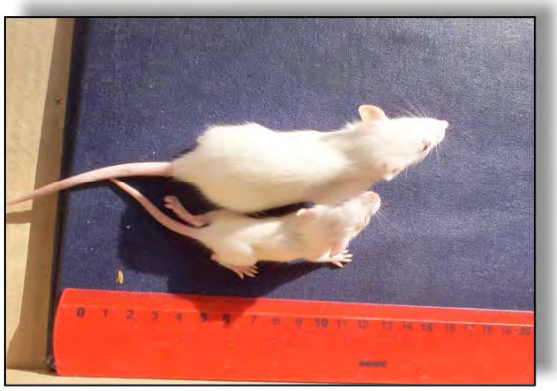
The testicles of rats fed Roundup Ready soybeans were dark blue instead of pink, and the structure of the cells were significantly altered.



An Austrian government study showed that mice fed GM corn (Bt and Roundup Ready) had fewer babies and smaller babies.[46] More dramatic results were discovered by a leading scientist at the Russian National Academy of sciences. Female rats were fed GM soy, starting two weeks before they were mated.

- Over a series of three experiments, 51.6 percent of the offspring from the GM-fed group died within the first three weeks, compared to 10 percent from the non-GM soy group, and 8.1 percent for non-soy controls.
- "High pup mortality was characteristic of every litter from mothers fed the GM soy flour."[47]
- The average size and weight of the GM-fed offspring was quite a bit smaller (see photo on next page).[48]
- In a preliminary study, the GM-fed offspring were unable to conceive.[49]

After the three feeding trials, the supplier of rat food used at the Russian laboratory began using GM soy in their formulation. Since all the rats housed at the facility were now eating GM soy, no non-GM fed controls were available for subsequent GM feeding trials; follow-up studies were canceled. *After two months on the GM soy diet, however, the infant mortality rate of rats throughout the facility had skyrocketed to 55.3 percent (99 of 179).*[50]



The 20 day old smaller rat, born of a mother fed GM soy, is quite a bit smaller than the 19 day-old rat from the control group.

FARMERS REPORT LIVESTOCK STERILITY AND DEATHS

About two dozen farmers reported that their pigs had reproductive problems when fed certain varieties of Bt corn. Pigs were sterile, had false pregnancies, or gave birth to bags of water. Cows and bulls also became sterile. Bt corn was also implicated by farmers in the deaths of cows, horses, water buffaloes, and chickens.[51]

When Indian shepherds let their sheep graze continuously on Bt cotton plants, within 5-7 days, one out of four sheep died. There was an estimated 10,000 sheep deaths in the region in 2006, with more reported in 2007. Post mortems on the sheep showed severe irritation and black patches in both intestines and liver (as well as enlarged bile ducts). Investigators said preliminary evidence "strongly suggests that the sheep mortality was due to a toxin. . . . most probably Bt-toxin."[52] In a small feeding study, 100% of sheep fed Bt cotton died within 30 days. Those fed natural plants had no symptoms.

Buffalo that grazed on natural cotton plants for years without incident react to the Bt variety. In one village in Andhra Pradesh, for example, 13 buffalo grazed on Bt cotton plants for a single day. All died within 3 days.[53] Investigators in the state of Haryana, India, report that most buffalo that ate GM cottonseed had reproductive complications such as premature deliveries, abortions, infertility, and prolapsed uteruses. Many young calves and adult buffaloes died.

GM CROPS TRIGGER IMMUNE REACTIONS AND MAY CAUSE ALLERGIES

Allergic reactions occur when the immune system interprets something as foreign, different, and offensive, and reacts accordingly. All GM foods, by definition, have something foreign and different. And several studies show that they provoke reactions. Rats fed Monsanto's GM corn, for example, had a significant increase in blood cells related to the immune system.[54] GM potatoes caused the immune system of rats to respond more slowly.[55] And GM peas provoked an inflammatory response in mice, suggesting that it might cause deadly allergic reactions in people.[56]

It might be difficult to identify whether GM foods were triggering allergic responses in the population, since very few countries conduct regular studies or keep careful records. One country that does have an annual evaluation is the UK. **Soon after GM soy was introduced into the British diet, researchers at the York Laboratory reported that allergies to soy had skyrocketed by 50% in a single year**.[57] Although no follow-up studies were conducted to see if GM soy was the cause, there is evidence showing several ways in which it might have contributed to the rising incidence of allergies:

- The only significant variety of GM soy is Monsanto's "Roundup Ready" variety, planted in 89% of US soy acres. A foreign gene from bacteria (with parts of virus and petunia DNA) is inserted, which allows the plant to withstand Roundup herbicide. The protein produced by the bacterial gene has never been part of the human food supply. Because people aren't usually allergic to a food until they have eaten it several times, it would be difficult to know in advance if the protein was an allergen. Without a surefire method to identify allergenic GM crops, the World Health Organization (WHO) and others recommend examining the properties of the protein to see if they share characteristics with known allergens. One method is to compare the amino acid sequence of the novel protein with a database of allergens. If there is a match, according to the WHO, the GM crop should either not be commercialized or additional testing should be done. Sections of the protein produced in GM soy *are* identical to shrimp and dust mite allergens,[58] but the soybean was introduced before WHO criteria were established and the recommended additional tests were not conducted. If the protein does trigger reactions, the danger is compounded by the finding that the Roundup Ready gene transfers into the DNA of human gut bacteria and may continuously produce the protein from within our intestines.[59]
- In addition to the herbicide tolerant protein, GM soybeans contain a unique, unexpected protein, which likely came about from the changes incurred during the genetic engineering process. Scientists found that this new protein was able to bind with IgE antibodies, suggesting that it may provoke dangerous allergic reactions. The same study revealed that one human subject showed a skin prick immune response only to GM soy, but not to natural soy.[60] These results must be considered preliminary,

as the non-GM soy was a wild type and not necessarily comparible to the GM variety. Another study showed that the levels of one known soy allergen, called trypsin inhibitor, were as much as seven times higher in cooked GM soy compared to a non-GM control.[61] This was Monsanto's own study, and did use comparable controls.

- GM soy also produces an unpredicted side effect in the pancreas of mice—the amount of digestive enzymes produced is dramatically reduced.[62] If a shortage of enzymes caused food proteins to breakdown more slowly, then they have more time to trigger allergic reactions. Thus, digestive problems from GM soy might promote allergies to a wide range of proteins, not just soy.
- The higher amount of Roundup herbicide residues on GM soy might create reactions in consumers. In fact, many of the symptoms identified in the UK soy allergy study are among those related to glyphosate exposure. [The allergy study identified irritable bowel syndrome, digestion problems, chronic fatigue, headaches, lethargy, and skin complaints, including acne and eczema, all related to soy consumption. Symptoms of glyphosate exposure include nausea, headaches, lethargy, skin rashes, and burning or itchy skin. It is also possible that glyphosate's breakdown product AMPA, which accumulates in GM soybeans after each spray, might contribute to allergies.]

It is interesting to note that in the five years immediately after GM soy was introduced, US peanut allergies doubled. It is known that a protein in natural soybeans cross-reacts with peanut allergies, i.e. soy may trigger reactions in some people who are allergic to peanuts.[63] Given the startling increase in peanut allergies, scientists should investigate whether this cross-reactivity has been amplified in GM soy.

BT-TOXIN, PRODUCED IN GM CORN AND COTTON, MAY CAUSE ALLERGIES

For years, organic farmers and others have sprayed crops with solutions containing natural Bt bacteria as a method of insect control. The toxin creates holes in their stomach and kills them. Genetic engineers take the gene that produces the toxin in bacteria and insert it into the DNA of crops so that the plant does the work, not the farmer. The fact that we consume that toxic pesticide in every bite of Bt corn is hardly appetizing.

Biotech companies claim that Bt-toxin has a history of safe use, is quickly destroyed in our stomach, and wouldn't react with humans or mammals in any event. Studies verify, however, that natural Bt-toxin is *not* fully destroyed during digestion and *does* react with mammals. Mice fed Bt-toxin, for example, showed an immune response as potent as cholera toxin, [64,] became immune sensitive to formerly harmless compounds,[65] and had damaged and altered cells in their small intestines.[66] A 2008 Italian government study found that Bt corn provoked immune responses in mice.[67] Moreover, when natural Bt was sprayed over areas around Vancouver and Washington State to fight gypsy moths, about 500 people reported reactions—mostly allergy or flu-like symptoms.[68,69] Farm workers and others also report serious reactions[70][71][72][73][74] and authorities have long acknowledged that "people with compromised immune systems or preexisting allergies may be particularly susceptible to the effects of Bt."[75]

The Bt-toxin produced in GM crops is "vastly different from the bacterial [Bt-toxins] used in organic and traditional farming and forestry."[76] The plant produced version is designed to be more toxic than natural varieties,[77] and is about 3,000-5,000 times more concentrated than the spray form. And just like the GM soy protein, the Bt protein in GM corn varieties has a section of its amino acid sequence identical to a known allergen (egg yolk). The Bt protein also fails other allergen criteria recommended by the WHO, i.e. the protein is too resistant to break down during digestion and heat.

If Bt-toxin causes allergies, then gene transfer carries serious ramifications. *If Bt genes relocate to human gut bacteria, our intestinal flora may be converted into living pesticide factories, possibly producing Bt-toxin inside of us year after year.* The UK Joint Food Safety and Standards Group also described gene transfer from a different route. They warned that genes from inhaled pollen might transfer into the DNA of bacteria in the respiratory system.[78] Although no study has looked into that possibility, pollen from a Bt cornfield appears to have been responsible for allergic-type reactions.

In 2003, during the time when an adjacent Bt cornfield was pollinating, virtually an entire Filipino village of about 100 people was stricken by mysterious skin, respiratory, and intestinal reactions.[79] The symptoms started with those living closest to the field and spread to those further away. Blood samples from 39 individuals showed antibodies in response to *Bt*-toxin, supporting—but not

proving—a link. When the same corn was planted in four other villages the following year, however, the symptoms returned in all four areas—only during the time of pollination.[80]

Bt-toxin might also trigger reactions by skin contact. In 2005, a medical team reported that hundreds of agricultural workers in India are developing allergic symptoms when exposed to Bt cotton, but not when
axposed to natural varieties.[81] They say reactions come from picking the cotton, cleaning it in factories, loading it onto trucks, or even leaning against it. Their symptoms are virtually identical to those described by the 500 people in Vancouver and Washington who were sprayed with Bt.

GOVERNMENT EVALUATIONS MISS MOST HEALTH PROBLEMS

Although the number of safety studies on GM foods is quite small, it has validated the concerns expressed by FDA scientists and others. Unfortunately, government safety assessments worldwide are not competent to even *identify* most of the potential health problems described above, let alone protect its citizens from the effects.[82]

A 2000 review of approved GM crops in Canada by professor E. Ann Clark, PhD, for example, reveals that 70% (28 of 40) "of the currently available GM crops . . . have not been subjected to any actual lab or animal toxicity testing, either as refined oils for direct human consumption or indirectly as feedstuffs for livestock. The same finding pertains to all three GM tomato decisions, the only GM flax, and to five GM corn crops." In the remaining 30% (12) of the other crops tested, animals were *not* fed the whole GM feed. They were given just the isolated GM protein that the plant was engineered to produce. But even this protein was not extracted from the actual GM plant. Rather, it was manufactured in genetically engineered bacteria. This method of testing would never identify problems associated with collateral damage to GM plant DNA, unpredicted changes in the GM protein, transfer of genes to bacteria or human cells, excessive herbicide residues, or accumulation of toxins in the food chain, among others. Clark asks, "Where are the trials showing lack of harm to fed livestock, or that meat and milk from livestock fed on GM feedstuffs are safe?"[83]

Epidemiologist and GM safety expert Judy Carman, PhD, MPH, shows that assessments by Food Safety Australia New Zealand (FSANZ) also overlook serious potential problems, including cancer, birth defects, or long-term effects of nutritional deficiencies. [84]

"A review of twelve reports covering twenty-eight GM crops - four soy, three corn, ten potatoes, eight canola, one sugar beet and two cotton—revealed no feeding trials on people. In addition, one of the GM corn varieties had gone untested on animals. Some seventeen foods involved testing with only a single oral gavage (a type of forced-feeding), with observation for seven to fourteen days, and only of the substance that had been genetically engineered to appear [the GM protein], not the whole food. Such testing assumes that the only new substance that will appear in the food is the one genetically engineered to appear, that the GM plant-produced substance will act in the same manner as the tested substance that was obtained from another source [GM bacteria], and that the substance will create disease within a few days. All are untested hypotheses and make a mockery of GM proponents' claims that the risk assessment of GM foods is based on sound science. Furthermore, where the whole food was given to animals to eat, sample sizes were often very low—for example, five to six cows per group for Roundup Ready soy—and they were fed for only four weeks."[85]

Dr. Carman points out that GM "experiments used some very unusual animal models for human health, such as chickens, cows, and trout. Some of the measurements taken from these animals are also unusual measures of human health, such as abdominal fat pad weight, total de-boned breast meat yield, and milk production." In her examination of the full range of submittals to authorities in Australia and New Zealand, she says that there was no proper evaluation of "biochemistry, immunology, tissue pathology, and gut, liver, and kidney function."[86] Writing on behalf of the Public Health Association of Australia, Dr. Carman says, "The effects of feeding people high concentrations of the new protein over tens of years cannot be determined by feeding 20 mice a single oral gavage of a given high concentration of the protein and taking very basic data for 13-14 days."[87]

THE FDA'S FAKE SAFETY ASSESSMENTS

Submissions to the US Food and Drug Administraion (FDA) may be worse than in other countries, since the agency doesn't actually require *any* data. Their policy says that biotech companies can determine if their own foods are safe. Anything submitted is voluntary and, according to former Environmental Protection Agency scientist Doug Gurian-Sherman, PhD, "often lack[s] sufficient detail, such as necessary statistical analyses needed for an adequate safety evaluation." Using Freedom of Information Requests, Dr. Gurian-Sherman analyzed more than a fourth of the data summaries (14 of 53) of GM crops reviewed by the FDA. He says, "The FDA consultation process does not allow the agency to require submission of data, misses obvious errors in company-submitted data summaries, provides insufficient testing guidance, and does not require sufficiently detailed data to enable the FDA to assure that GE crops are safe to eat."[88] Similarly, a Friends of the Earth review of company and FDA documents concluded:

"If industry chooses to submit faulty, unpublishable studies, it does so without consequence. If it should respond to an agency request with deficient data, it does so without reprimand or follow-up. . . . If a company finds it disadvantageous to characterize its product, then its properties remain uncertain or unknown. If a corporation chooses to ignore scientifically sound testing standards . . . then faulty tests are conducted instead, and the results are considered legitimate. In the area of genetically engineered food regulation, the 'competent' agencies rarely if ever (know how to) conduct independent research to verify or supplement industry findings."[89]

At the end of the consultation, the FDA doesn't actually approve the crops. Rather, they issue a letter that includes a statement such as the following:

"Based on the safety and nutritional assessment you have conducted, it is our understanding that Monsanto has concluded that corn products derived from this new variety are not materially different in composition, safety, and other relevant parameters from corn currently on the market, and that the genetically modified corn does not raise issues that would require premarket review or approval by FDA. . . . As you are aware, it is Monsanto's responsibility to ensure that foods marketed by the firm are safe, wholesome and in compliance with all applicable legal and regulatory requirements."[90]

COMPANY RESEARCH IS SECRET, INADEQUATE, AND FLAWED

The unpublished industry studies submitted to regulators are typically kept secret based on the claim that it is "confidential business information." The Royal Society of Canada is one of many organizations that condemn this practice. They wrote:

"In the judgment of the Expert Panel, the more regulatory agencies limit free access to the data upon which their decisions are based, the more compromised becomes the claim that the regulatory process is 'science based.' This is due to a simple but well-understood requirement of the scientific method itself—that it be an open, completely transparent enterprise in which any and all aspects of scientific research are open to full review by scientific peers. Peer review and independent corroboration of research findings are axioms of the scientific method, and part of the very meaning of the objectivity and neutrality of science."[91]

Whenever private submissions *are* made public through lawsuits or Freedom of Information Act Requests, it becomes clear why companies benefit from secrecy. The quality of their research is often miserable, incompetent, and unacceptable for peer-review. In 2000, for example, after the potentially allergenic StarLink corn was found to have contaminated the food supply, the corn's producer, Aventis CropScience, presented wholly inadequate safety data to the EPA's scientific advisory panel. One frustrated panel member, Dean Metcalfe, MD,—the government's top allergist—said during a hearing, "Most of us review for a lot of journals. And if this were presented for publication in the journals that I review for, it would be sent back to the authors with all of these questions. It would be rejected."[92]

UNSCIENTIFIC ASSUMPTIONS ARE THE BASIS OF APPROVALS

Professor Clark, who analyzed submissions to Canadian regulators, concluded, "Most or all of the conclusions of food safety for individual GM crops are based on inferences and assumptions, rather than on actual testing." For example, rather than actually testing to see if the amino acid sequence produced by their inserted gene is correct, "the standard practice," according to research analyst William Freese, "is to sequence just 5 to 25 amino acids,"[93] even if the protein has more than 600 in total. If the short sample matches what is expected, they assume that the rest are also fine. If they are wrong, however, a rearranged protein could be quite dangerous.

Monsanto's submission to Australian regulators on their high lysine GM corn provides an excellent example of overly optimistic assumptions used in place of science. The gene inserted into the corn produces a protein that is naturally found in soil. Monsanto claimed that since people consume small residues of soil on fruits and vegetables, the protein has a history of safe consumption. Based on the amount of GM corn protein an average US citizen would consume (if all their corn were Monsanto's variety), they would eat up to 4 trillion times the amount normally consumed through soil. In other words, "for equivalent exposure" of the protein from soil "people would have to eat . . . nearly as much as 10,000kg [22,000 pounds, every] second 24 hours a day seven days a week."[94]

STUDIES ARE RIGGED TO AVOID FINDING PROBLEMS

In addition, to relying on untested assumptions, industry-funded research is often designed specifically to force a conclusion of safety. In the high lysine corn described above, for example, the levels of certain nutritional components (i.e. protein content, total dietary fiber, acid detergent fiber, and neutral detergent fiber) were far outside the normal range for corn. Instead of comparing their corn to normal controls, which would reveal this disparity, Monsanto compared it to obscure corn varieties that were also substantially outside the normal range *on precisely these values*. Thus, their study found no statistical differences *by design*.

When independent researchers published a study in July 1999 showing that GM soy contains 12%-14% less cancer-fighting phytoestrogens, Monsanto responded with its own study, concluding that soy's phytoestrogen levels vary too much to even carry out a statistical analysis. Researchers failed to disclose, however, that they had instructed the laboratory to use an obsolete method of detection—one that had been prone to highly variable results.[95]

When Aventis prepared samples to see if the potential allergen in StarLink corn remained intact after cooking, instead of using the standard 30-minute treatment, they heated corn for two hours.[96]

To show that pasteurization destroyed bovine growth hormone in milk from cows treated with rbGH, scientists pasteurized the milk 120 times longer than normal. Unable to destroy more than 19%, they then spiked the milk with a huge amount of the hormone and repeated the long pasteurization, destroying 90%.[97] (The FDA reported that pasteurization destroys 90% of the hormone.[98])

To demonstrate that injections of rbGH did not interfere with cow's fertility, Monsanto apparently added cows to the study that were pregnant prior to injection.[99]

And in order to prove that the protein from their GM crops breaks down quickly during simulated digestion, biotech companies used thousands of times the amount of digestive enzymes and a much stronger acid compared to that recommended by the World Health Organization.[100]

Other methods used to hide problems are varied and plentiful. For example, researchers:

- Use highly variable animal starting weights to hinder detection of food-related changes
- Keep feeding studies short to miss long-term impacts

- Test effects of Roundup Ready soybeans that have not been sprayed with Roundup
- Avoid feeding animals the actual GM crop, but give them instead a single dose of the GM protein that was produced inside GM bacteria
- Use too few subjects to derive statistically significant results
- Use poor statistical methods or simply leave out essential methods, data, or statistics
- Use irrelevant control groups, and employ insensitive evaluation techniques

ROUNDUP READY SOYBEANS: CASE STUDY OF FLAWED RESEARCH

Monsanto's 1996 *Journal of Nutrition* studies on Roundup Ready soybeans[101,102] provide plenty of examples of scientific transgressions. Although the study has been used often by the industry as validation for safety claims, experts working in the field were not impressed. For example, Dr. Arpad Pusztai was commissioned at the time by the UK government to lead a 20 member consortium in three institutions to develop rigorous testing protocols on GM foods—protocols that were never implemented. Dr. Pusztai, who had published several studies in that same nutrition journal, said the Monsanto paper was not "up to the normal journal standards." He said, "It was obvious that the study had been designed to avoid finding any problems. Everybody in our consortium knew this." Some of the flaws include:

- Researchers tested GM soy on mature animals, not young ones. Young animals use protein to build their muscles, tissues, and organs. Problems with GM food could therefore show up in organ and body weight. But adult animals use the protein for tissue renewal and energy. "With a nutritional study on mature animals," says Dr. Pusztai, "you would never see any difference in organ weights even if the food turned out to be anti-nutritional. The animals would have to be emaciated or poisoned to show anything."
- If there were an organ development problem, the study wouldn't have picked it up since the researchers didn't even weigh the organs.
- In one of the trials, researchers substituted only one tenth of the natural protein with GM soy protein. In two others, they diluted their GM soy six- and twelve-fold. [103] Scientists Ian Pryme, PhD, of Norway and Rolf Lembcke, PhD, of Denmark wrote, the "level of the GM soy was too low, and would probably ensure that any possible undesirable GM effects did not occur."
- Pryme and Lembcke, who published a paper in *Nutrition and Health* that analyzed all published peer-reviewed feeding studies on GM foods (10 as of 2003), also pointed out that the percentage of protein in the feed used in the Roundup Ready study was "artificially too high." This "would almost certainly mask, or at least effectively reduce, any possible effect of the [GM soy]." They said it was "highly likely that all GM effects would have been diluted out." [104]
- Proper compositional studies filter out effects of weather or geography by comparing plants grown at the same time in the same location. Monsanto, however, pooled data from several locations, which makes it difficult for differences to be statistically significant. Nonetheless, the data revealed significant differences in the ash, fat, and carbohydrate content. Roundup Ready soy meal also contained 27% more trypsin inhibitor, a potential allergen. Also, cows fed GM soy produced milk with a higher fat content, demonstrating another disparity between the two types of soy.
- One field trial, however, did grow GM and non-GM plants next to each other, but this data was not included in the paper. Years after the study appeared, medical writer Barbara Keeler recovered the data that had been omitted. It showed that Monsanto's GM soy had significantly lower levels of protein, a fatty acid, and phenylalanine, an essential amino acid. Also, toasted GM soy meal contained nearly twice the amount of a lectin—a substance that may interfere with the body's ability to assimilate other nutrients. And the amount of trypsin inhibitor in cooked GM soy was as much as seven times higher than in a cooked non-GM control.

- The study also omitted many details normally required for a published paper. According to Pryme and Lembcke, "No data were given for most of the parameters."
- And when researchers tested the effects of Roundup Ready protein on animals, they didn't extract the protein from the soybeans. Instead, they derived it from GM bacteria, claiming the two forms of protein were equivalent. There are numerous ways, however, in which the protein in the soy may be different. In fact, nine years after this study was published, another study showed that the gene inserted into the soybeans produced unintended aberrant RNA strands, meaning that the protein may be quite different than what was intended.[105]

In Pryme and Lembcke's analysis, it came as no surprise that this Monsanto study, along with the other four peer-reviewed animal feeding studies that were "performed more or less in collaboration with private companies," reported no negative effects of the GM diet. "On the other hand," they wrote, "adverse effects were reported (but not explained) in [the five] independent studies." They added, "It is remarkable that these effects have all been observed after feeding for only 10–14 days."[106]

TOXIC GM FOODS COULD HAVE BEEN APPROVED

Two GM foods whose commercialization was stopped because of negative test results give a chilling example of what may be getting through. Rats fed GM potatoes had potentially precancerous cell growth in the stomach and intestines, less developed brains, livers, and testicles, partial atrophy of the liver, and damaged immune systems.[107] GM peas provoked an inflammatory response in mice, suggesting that the peas might trigger a deadly anaphylactic shock in allergic humans.[108] Both of these dangerous crops, however, could easily have been approved. The problems were only discovered because the researchers used advanced tests that were never applied to GM crops already on the market. Both would have passed the normal tests that companies typically use to get their products approved.

Ironically, when Monsanto was asked to comment on the pea study, their spokesperson said it demonstrated that the regulatory system works. He failed to disclose that none of his company's GM crops had been put through such rigorous tests.

RAMPANT, UNRELENTING INDUSTRY BIAS

Industry-funded research that favors the funders is not new. Bias has been identified across several industries. In pharmaceuticals, for example, positive results are four times more likely if the drug's manufacturer funds the study.[109] When companies pay for the economic analyses of their own cancer drugs, the results are eight times more likely to be favorable.[110] Compared to drug research, the potential for industry manipulation in GM crop studies is considerably higher. Unlike pharmaceutical testing, GM research has no standardized procedures dictated by regulators. GM studies are not usually published in peer-reviewed journals and are typically kept secret by companies and governments. There is little money available for rigorous independent research, so company evidence usually goes unchallenged and unverified. Most importantly, whereas drugs *can* show serious side-effects and still be approved, GM food cannot. There is no tolerance for adverse reactions; feeding trials *must* show no problems.

Thus, when industry studies show problems (in spite of their efforts to avoid them), serious adverse reactions and even deaths among GM-fed animals are ignored or dismissed as "not biologically significant" or due to "natural variations." In the critical arena of food safety research, the biotech industry is without accountability, standards, or peer-review. They've got bad science down to a science.

PROMOTING AND REGULATING DON'T MIX

While such self-serving behavior may be expected from corporations, how come government bodies let such blatant scientific contortions pass without comment? One reason is that several regulatory agencies are also charged with promoting the interests of biotechnology. This is the official position of the FDA and other US government bodies, for example. Suzanne Wuerthele, PhD, a US EPA toxicologist, says, "This technology is being promoted, in the face of concerns by respectable scientists and in the face of data to the contrary, by the very agencies which are supposed to be protecting human health and the environment. The bottom line in my view is that we are confronted with the most powerful technology the world has ever known, and it is being rapidly deployed with almost no thought whatsoever to its consequences."

Canadian regulators are similarly conflicted. The Royal Society of Canada reported that, "In meetings with senior managers from the various Canadian regulatory departments . . . their responses uniformly stressed the importance of maintaining a favorable climate for the biotechnology industry to develop new products and submit them for approval on the Canadian market. . . . The conflict of interest involved in both promoting and regulating an industry or technology . . . is also a factor in the issue of maintaining the transparency, and therefore the scientific integrity, of the regulatory process. In effect, the public interest in a regulatory system that is 'science based'. . . is significantly compromised when that openness is negotiated away by regulators in exchange for cordial and supportive relationships with the industries being regulated."[111]

Many scientists on the European Food Safety Authority (EFSA) GMO Panel are personally aligned with biotech interests. According to Friends of the Earth (FOE), "One member has direct financial links with the biotech industry and others have indirect links, such as close involvement with major conferences organized by the biotech industry. Two members have even appeared in promotional videos produced by the biotech industry. . . . Several members of the Panel, including the chair Professor Kuiper, have been involved with the EU-funded ENTRANSFOOD project. The aim of this project was to agree [to] safety assessment, risk management, and risk communication procedures that would 'facilitate market introduction of GMOs in Europe, and therefore bring the European industry in a competitive position.' Professor Kuiper, who coordinated the ENTRANSFOOD project, sat on a working group that also included staff from Monsanto, Bayer CropScience, and Syngenta." In a statement reminiscent of the deceptive policy statement by the FDA, the FOE report concludes that EFSA is "being used to create a false impression of scientific agreement when the real situation is one of intense and continuing debate and uncertainty."[112]

The pro-GM European Commission repeats the same ruse. According to leaked documents obtained by FOE, while they privately appreciate "the uncertainties and gaps in knowledge that exist in relation to the safety of GM crops, . . . the Commission normally keeps this uncertainty concealed from the public whilst presenting its decisions about the safety of GM crops and foods as being certain and scientifically based." For example, the Commission privately condemned the submission information for one crop as "mixed, scarce, delivered consecutively all over years, and not convincing." They said there is "No sufficient experimental evidence to assess the safety."[113]

With an agenda to promote GM foods, regulators regularly violate their own laws. In Europe, the law requires that when EFSA and member states have different opinions, they "are obliged to co-operate with a view to either resolving the divergence or preparing a joint document clarifying the contentious scientific issues and identifying the relevant uncertainties in the data."[114] According to FOE, in the case of *all* GM crop reviews, none of these legal obligations were followed.[115] The declaration of GRAS status by the FDA also deviated from the Food and Cosmetic Act and years of legal precedent. Some violations are more blatant. In India, one official tampered with the report on Bt cotton to increase the yield figures to favor Monsanto.[116] In Mexico, a senior government official allegedly threatened a University of California professor, implying "We know where your children go to school," trying to get him not to publish incriminating evidence that would delay GM approvals.[117] In Indonesia, Monsanto gave bribes and questionable payments to at least 140 officials, attempting to get their genetically modified (GM) cotton approved.[118]

MANIPULATION OF PUBLIC OPINION

When governments fail in their duty to keep corporations in check, the "protector" role should shift to the media, which acts as a watchdog to expose public dangers and governmental shortcomings. But mainstream media around the world has largely overlooked the serious problems associated with GM crops and their regulation. The reason for this oversight is varied and includes contributions from an aggressive public relations and disinformation campaign by the biotech industry, legal threats by biotech companies, and in some cases, the fear of losing advertising accounts. This last reason is particularly prevalent among the farm press, which receives much of its income from the biotech industry.

Threatening letters from Monsanto's attorneys have resulted in the cancellation of a five-part news series on their genetically engineered bovine growth hormone scheduled for a Fox TV station in Florida, as well as the cancellation of a book critical of Monsanto's GMO products. A printer also shredded 14,000 copies of the *Ecologist* magazine issue entitled "The Monsanto Files," due to fear of a Monsanto lawsuit. (See the chapter "Muscling the Media" in *Seeds of Deception*[119] for more examples.)

The methods that biotech advocates use to manipulate public opinion research has become an art form. Consumer surveys by the International Food Information Council (IFIC), for example,whose supporters include the major biotech seed companies, offers conclusions such as "A growing majority of Americans support the benefits of food biotechnology as well as the US Food and Drug Administration's (FDA) labeling policy." But communications professor James Beniger, who was past president of the American Association for Public Opinion Research, described the surveys as "so biased with leading questions favoring positive responses that any results are meaningless."[120] The 2003 survey, for example, included gems such as:

"All things being equal, how likely would you be to buy a variety of produce, like tomatoes or potatoes, if it had been modified by biotechnology to taste better or fresher?" and

"Biotechnology has also been used to enhance plants that yield foods like cooking oils. If cooking oil with reduced saturated fat made from these new plants was available, what effect would the use of biotechnology have on your decision to buy this cooking oil?"[121]

A similar tactic was used at a December 11, 2007 focus group in Columbus, Ohio "designed" to show that consumers wanted to make it illegal for dairies to label their milk as free from Monsanto's genetically engineered bovine hormone rBST. The facilitator said, "All milk contains hormones. There is no such thing as hormone-free milk. The composition of both types of milk is the same in all aspects. Now what do you think of a label that says 'no added hormones?' Don't you think it is deceiving and inappropriate to put 'rBST-free' on labels?" Not only was the facilitator "leading the witness," he presented false information. Milk from cows treated with rBST has substantially higher levels of Insulin-like Growth Factor-1,[122] which has been linked to higher risk of cancer,[123] and higher incidence of fraternal twins.[124] It also has higher levels of bovine growth hormone, pus, and in some cases, antibiotics.

Another example of manipulated consumer opinion was found in a 2004 article in the *British Food Journal*, authored by four advocates of genetically modified (GM) foods.[125] According to the peer-reviewed paper, when shoppers in a Canadian farm store were confronted with an informed and unbiased choice between GM corn and non-GM corn, most purchased the GM variety. This finding flew in the face of worldwide consumer resistance to GM foods, which had shut markets in Europe, Japan, and elsewhere. It also challenged studies that showed that the more information on genetically modified organisms (GMOs) consumers have, the *less* they trust them.[126] The study, which was funded by the biotech-industry front group, Council for Biotechnology Information and the industry's trade association, the Crop Protection Institute of Canada (now Croplife Canada), was given the Journal's prestigious Award for Excellence for the Most Outstanding Paper of 2004 and has been cited often by biotech advocates.

Stuart Laidlaw, a reporter from Canada's *Toronto Star*, visited the farm store several times during the study and described the scenario in his book *Secret Ingredients*. Far from offering unbiased choices, key elements appeared rigged to favor GM corn purchases. The consumer education fact sheets were entirely pro-GMO, and Doug Powell, the lead researcher, enthusiastically demonstrated to Laidlaw how he could convince shoppers to buy the GM varieties. He confronted a farmer who had already

purchased non-GM corn. After pitching his case for GMOs, Powell proudly had the farmer tell Laidlaw that he had changed his opinion and would buy GM corn in his next shopping trip.

Powell's interference with shoppers' "unbiased" choices was nothing compared to the effect of the signs placed over the corn bins. The sign above the non-GM corn read, "Would you eat wormy sweet corn?" It further listed the chemicals that were sprayed during the season. By contrast, the sign above the GM corn stated, "Here's What Went into Producing Quality Sweet Corn." It is no wonder that 60% of shoppers avoided the "wormy corn." In fact, it may be a testament to people's distrust of GMOs that 40% still went for the "wormy" option.

Powell and his colleagues did not mention the controversial signage in their study. They claimed that the corn bins in the farm store were "fully labelled"—either "genetically engineered Bt sweet corn" or "Regular sweet-corn." When Laidlaw's book came out, however, Powell's "wormy" sign was featured in a photograph,[127] exposing what was later described by Cambridge University's Dr. Richard Jennings as "flagrant fraud." Jennings, who is a leading researcher on scientific ethics, says, "It was a sin of omission by failing to divulge information which quite clearly should have been disclosed."[128]

In his defence, Powell claimed that his signs merely used the language of consumers and was "not intended to manipulate consumer purchasing patterns." He also claimed that the "wormy" corn sign was only there for the first week of the trial and was then replaced by other educational messages. But eye witnesses and photographs demonstrate the presence of the sign long after Powell's suggested date of replacement.[129]

Several scientists and outraged citizens say the paper should be withdrawn, but the Journal refused. In fact, the Journal's editor has not even agreed to reconsider its Award for Excellence. A blatant propaganda exercise still stands validated as exemplary science.

Critics and Independent Scientists are Attacked

One of the most troubling aspects of the biotech debate is the attack strategy used on GMO critics and independent scientists. Not only are adverse findings by independent scientists often suppressed, ignored, or denied, researchers that discover problems from GM foods have been fired, stripped of responsibilities, deprived of tenure, and even threatened. Consider Dr. Pusztai, the world's leading scientist in his field, who inadvertently discovered in 1998 that unpredictable changes in GM crops caused massive damage in rats. He went public with his concerns, was a hero at his prestigious institute for two days, and then, after the director received two phone calls allegedly from the UK Prime Minister's office, was fired after 35 years and silenced with threats of a lawsuit. False statements were circulated to trash his reputation, which are recited by GMO advocates today.

After University of California Professor Ignacio Chapela, PhD, published evidence that GM corn contaminated Mexico's indigenous varieties, two fictitious internet characters created by Monsanto's PR firm, the Bivings Group, initiated a brutal internet smear campaign, lying about Dr. Chapela and his research.

Irina Ermakova, PhD, a leading scientist at the Russian National Academy of Sciences, fed female rats GM soy and was stunned to discover that more than half their offspring died within three weeks—compared to only 10% from mothers fed non-GM soy. Without funding to extend her analysis, she labeled her work "preliminary," published it in a Russian journal, and implored the scientific community to repeat the study. Two years later, no one has repeated it, but advocates use false or irrelevant arguments to divert attention from the shocking results and have tried to vilify Dr. Ermakova.

A New Zealand MP testified at the 2001 Royal Commission of Inquiry on Genetic Modification, "I have been contacted by telephone and e-mail by a number of scientists who have serious concerns . . . but who are convinced that if they express these fears publicly. . . or even if they asked the awkward and difficult questions, they will be eased out of their institution." Indeed this year, after Professor Christian Velot, PhD, raised the difficult questions on GMOs at public conferences, his 2008 research funds were confiscated, his student assistants were re-assigned, and his position at the University of Paris-Sud faces early termination.

WE'RE THE GUINEA PIGS

Since GM foods are not properly tested before they enter the market, consumers are the guinea pigs. But this doesn't even qualify as an experiment. There are no controls and no monitoring. Given the mounting of evidence of harm, it is likely that GM foods are contributing to the deterioration of health in the United States, Canada, and other countries where it is consumed. But without post-marketing surveillance, the chances of tracing health problems to GM food are low. The incidence of a disease would have to increase dramatically before it was noticed, meaning that millions may have to get sick before a change is investigated. Tracking the impact of GM foods is even more difficult in North America, where the foods are not labeled.

Regulators at Health Canada announced in 2002 that they would monitor Canadians for health problems from eating GM foods. A spokesperson said, "I think it's just prudent and what the public expects, that we will keep a careful eye on the health of Canadians." But according to CBC TV news, Health Canada "abandoned that research less than a year later saying it was 'too difficult to put an effective surveillance system in place.'" The news anchor added, "So at this point, there is little research into the health effects of genetically modified food. So will we ever know for sure if it's safe?"[130]

Not with the biotech companies in charge. Consider the following statement in a report submitted to county officials in California by pro-GM members of a task force. "[It is] generally agreed that long-term monitoring of the human health risks of GM food through epidemiological studies is not necessary because there is no scientific evidence suggesting any long-term harm from these foods."[131] Note the circular logic: Because no long-term epidemiological studies are in place, we have no evidence showing long-term harm. And since we don't have any evidence of long-term harm, we don't need studies to look for it.

What are these people thinking? Insight into the pro-GM mindset was provided by Dan Glickman, the US Secretary of Agriculture under President Clinton.

"What I saw generically on the pro-biotech side was the attitude that the technology was good, and that it was almost immoral to say that it wasn't good, because it was going to solve the problems of the human race and feed the hungry and clothe the naked. . . . And there was a lot of money that had been invested in this, and if you're against it, you're Luddites, you're stupid. That, frankly, was the side our government was on. Without thinking, we had basically taken this issue as a trade issue and they, whoever 'they' were, wanted to keep our product out of their market. And they were foolish, or stupid, and didn't have an effective regulatory system. There was rhetoric like that even here in this department. You felt like you were almost an alien, disloyal, by trying to present an open-minded view on some of the issues being raised. So I pretty much spouted the rhetoric that everybody else around here spouted; it was written into my speeches."[132]

Fortunately, not everyone feels that questioning GM foods is disloyal. On the contrary, millions of people around the world are unwilling to participate in this uncontrolled experiment. They refuse to eat GM foods. Manufacturers in Europe and Japan have committed to avoid using GM ingredients. And the US natural foods industry, not waiting for the government to test or label GMOs, is now engaged in removing all remaining GM ingredients from their sector using a third party verification system. The Campaign for Healthier Eating in America will circulate non-GMO shopping guides in stores nationwide so that consumers have clear, healthy non-GMO choices. With no governmental regulation of biotech corporations, it is left to consumers to protect ourselves.

For a guide to avoiding GMOs, go to www.NonGMOShoppingGuide.com.

International bestselling author and independent filmmaker Jeffrey M. Smith is the Executive Director of the Institute for Responsible Technology and a leading spokesperson on the health dangers of GMOs. His first book, *Seeds of Deception,* is the world's bestselling book on the subject. His second, *Genetic Roulette*: *The Documented Health Risks of Genetically Engineered Foods*, identifies 65 risks of GMOs and demonstrates how superficial government approvals are not competent to find *most* of them. Mr. Smith has pioneered the Campaign for Healthier Eating in America, designed to create the tipping point of consumer rejection against GMOs. See www.ResponsibleTechnology.org, www.NonGMOShoppingGuide.com.

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A three generation study with genetically modified Bt corn in rats: Biochemical and histopathological investigation

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Abstract

For the last ten years, in accordance with the increased use of genetically modified (GM) foods for human and livestocks, a large number of feeding studies have been carried out. However, the evidence is still far from proving whether the long-term consumption of GM foods posses a possible danger for human or animal health. Therefore, this study was designed to evaluate the effects of transgenic corn on the rats that were fed through three generations with either GM corn or its conventional counterpart. Tissue samples of stomach, duodenum, liver and kidney were obtained for histopathological examinations. The average diameter of glomeruli, thickness of renal cortex and glomerular volume were calculated and number of affected animals/number of examined animals for liver and kidney histopathology were determined. Amounts of urea, urea nitrogen, creatinine, uric acid, total protein, albumin and globulin were determined; enzyme activities of aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, gamma glutamyltransferase, creatine kinase and amylase were measured in serum samples. No statistically significant differences were found in relative organ weights of rats within groups but there were some minimal histopathological changes in liver and kidney. Changes in creatinine, total protein and globulin levels were also determined in biochemical analysis.

Keywords: Transgenic Bt corn; Three generation study; Histopathology; Biochemical analysis; Wistar albino rat

1. Introduction

The global area of approved genetically modified (GM) crops such as soybean, corn, cotton, rice, canola, tomato have risen from 1.7 million hectares in 1996 to 102.0 million hectares in 2006 and the number of countries planting GM crops increased to 22. GM corn is the second principal biotech crop occupying 25.2 million hectares, after the GM soybean (James, 2006). The views endorsing the development of GM crops created with recombinant DNA technology, are based on improving the yield and quality of crops, solving the famine that would be a dangerous risk in the next 25 years, founding renewable sources for vaccines, drugs and bioplastics (Coghlan, 1995). Meanwhile, this technology poses scientific, technological, environmental, social, ethical, economical and political issues as well as health risks (Jones, 1999). In order to assess the potential risks of transgenic organisms, International Food Biotechnology Council (IFBC) has initially reported the safety evaluation of GMOs and then Organisation for Economic Cooperation and Development (OECD), Food and Agriculture Organisation of the United Nations (FAO), World Health Organisation (WHO) and International Life Science Institute (ILSI) have established safety assessment guidelines. OECD, developed the concept of substantial equivalence defined as comparison between GM organism and its traditional counterpart and used the data for future safety assessments (Kuiper et al., 2001).

European corn borer (*Ostrinia nubilalis*) and Southwestern corn borer (*Diatraea grandiosella*) have caused significant yield losses in corn (*Zea mays* L.) agriculture

Abbreviations: Bt, *Bacillus thuringiensis*; Cry, crystal protein; GM, genetically modified.

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(MacKenzie et al., 2007; Williams et al., 2005). Therefore, for over 40 years an insecticidal spray that includes a mixture of spores and associated protein crystals belonging to a Gram (–) bacterium *Bacillus thuringiensis* (Bt) has been being worldwide used (Nester et al., 2002). Intracellular crystal inclusions produced during sporulation of Bt are called parasporal crystals (*crystal protein*) which have insecticidal activity on insects midgut epithelium. After applications of recombinant DNA technology, more direct and controlled ways have been employed, namely genetically modified Bt crops, to fight with the aforesaid pests. Bt toxin encoded by bacterial cry gene, transferred to crops and expressed in their specific tissues. During the process of obtaining Bt protein from cultures in order to reach sufficient amounts, unwanted processes may cause toxicity in different organisms (Kuiper et al., 2001). Various nutritional analysis and short term feeding studies have been performed to demonstrate the possible effects of GM Bt corn on human and animal health (Brake and Evenson, 2004; Hammond et al., 2006; El Sanhoty et al., 2004; Netherwood et al., 2004). However, no reports on the possible health effects of GM crops through multigeneration in rats were obtained. Therefore, this three generation study in which rats fed with transgenic Bt corn was designed to clarify and enlighten the safety of long-term Bt corn consumption.

2. Materials and methods

2.1. Animals and housing

Eighteen female and nine male Wistar albino rats, obtained from Experimental Animals Production Center of Başkent University, in Ankara, Türkiye were 10 weeks of age at the beginning of the study. The study conforms the National Research Council guidelines for animal experimentation (National Research Council, 1996). The rats were allowed to acclimate to the housing conditions for 1 week during which they kept on basal diet. All rats were provided with tap water ad libitum through the study and were housed singly in polycarbonate cages with stainless steel cover. Laboratory conditions were maintained 12 h light/dark schedule, at temperature of 23 ± 3 °C and a relative humidity of 47 ± 5. Animals were observed two times daily for general wholesomeness and care.

2.2. Experimental diets

Bt corn that has insect resistance trait for the most invasive corn borers and its reference (*same genetic and breeding background but lack of the Bt transgene*) were obtained by the agency of Turkish Ministry of Agriculture and Rural Affairs. Besides, standard rat diet were purchased from Dokuz Tuğ Yem A.Ş., Ankara, Türkiye. Composition list of experimental diets are given at Table 1. Rats in Group I were fed only with standard diet, those in Group II with standard diet containing 20% reference corn and the ones in Group III with standard diet containing 20% transgenic Bt corn. These percentages were chosen in order to maintain a balanced rodent diet in this long-term feeding study. Corn grains and standard food were ground weekly to have a homogenous-mixed diet. Experimental diets were kept at 4 °C to preserve their protein content. Mixing ratios of standard and experimental diets are given at Table 2. The amount of dam's diet was 25 g/rat/day during gestation and lactation on the other hand offspring's diet was 20 g/rat/day from the beginning of 1.5 months after the birth.

Table 1
Composition of experimental diets for rats (%)

Ingredients	Bt corn	Non-Bt corn
Water	11.00	11.50
Dry nutrient	89.00	88.50
Crude protein	9.28	9.37
Crude fat	3.39	3.10
Starch	60.70	54.20
Sugar	2.33	2.10

2.3. Experimental design and treatment

Animals were randomly assigned to three groups, depending on their body weight means. Eighteen female Wistar albino rats (6 rats/each group) were mated with 9 male rats (one male for two female rats) overnight. Vaginal lavages were examined on light microscopy and the day that sperm was detected was considered to be the first day of pregnancy. Then, pregnant rats (F_0) were started to feed with either the diet containing 20% transgenic corn or 20% reference corn or standard rat diet depending on their groups. Dams and their offsprings were fed with the diets during the periods of mating, gestation, lactation, offspring care and pubescence. The offsprings of different dams in a group of each generation were mated among themselves throughout three generations. F_1, F_2 and F_3 generations were acquired by the same procedures described above. F_3 rats were also fed with either standard diet or experimental diets until they reached to 3.5 months age. The male rats that were used in mating were out of study. Male and female gender ratio of F_1, F_2, F_3 rats were compared.

2.4. Processing of tissues for histopathology

All F_3 rats were weighed and sacrificed by cervical dislocation at the end of the treatment. Their tissues of stomach (*corpus*), small intestine (*duodenum*), liver and kidney were removed, weighed and immediately fixed in Bouin's fixative for 8 h or 10% formaldehyde for 10 h. After the routine procedure, the fixed tissues were embedded in paraffin and 5 μm thick tissue sections were stained with routine haemotoxylin and eosin (H&E) or periodic acid schiff (PAS) in order to examine under light microscopy. All tissue sections were observed; only liver and kidney tissues were photographed. For histopathological changes "affected number of animals/examined number of animals" of liver and kidney, and their percentages were calculated. Forty glomeruli for each kidney specimen were selected and the maximum diameter and the thickness of cortex of selected glomeruli in serial sections was measured by Bs200prop program in Olympus BX51 system light microscope. The diameters were calculated as the mean of the longest and shortest diameters (Yamashita et al., 2002). The glomerular volume was calculated from the mean glomerular diameter, $d(G)$, using the formula: $4\pi(d(G)/2)3/3$ (Sugimoto et al., 1998).

2.5. Biochemical analysis

Blood samples were taken from heart of F_3 rats under ether anaesthesia at the end of the study. After centrifugation at 3000 rpm for 15 min, serum was separated. Serum samples were analysed for determination of the amounts of urea, urea nitrogen (BUN), creatinine, uric acid, total protein, albumin and globulin and for the measurement of enzyme activities of aspartate aminotransferase (AST), alanine aminotransferase

Table 2
Ratios of standard and experimental diets (%)

Groups	Diets (%)		
	Transgenic corn	Reference corn	Standard diet
Group I	0	0	100
Group II	0	20	80
Group III	20	0	80

(ALT), alkaline phosphatase (ALP), gamma-glutamyltransferase (GGT), creatine kinase (CK) and amylase by closed system of Olympus Au autoanalyser in Ölçüm Tıp Laboratory in Ankara, Türkiye.

2.6. Statistical analysis

Statistical analysis were performed using a SPSS 11.5 program for Windows. Data were expressed as mean ± standard error (SE) and statistical significance was assigned at the $p \leqslant 0.05$ level. The homogeneity of variance and normal distribution between groups was evaluated by General Linear Model procedure and Kolmogorov–Smirnov *nonparametric* test. Serum parameters were analysed by two-way ANOVA in male and female rats separately. To identify the sources of significant main effect, *post hoc* comparisons (Games–Howell, Tukey) were used. Body and relative organ weights were examined by one-way ANOVA and Games–Howell *post hoc* test (Sokal and Rohlf, 1995). Besides, histopathological findings were compared by using Fisher's exact test as described by Gad and Weil (1989).

3. Results

3.1. Clinical observations, number of offspring, body and relative organ weights

No signs of adverse effects were seen in clinical appearance of new borns in all three generation. The dams gave fertile progeny and successfully continued their strips. Number of offsprings in F_1, F_2, F_3 generations are shown in Table 3.

The final body weights, relative kidney and liver weights of female and male F_3 rats are given at Table 4. There were no significant differences in final body weights of rats in all groups whereas relative liver weights of female rats in Groups II and III and also relative kidney weights in Group II showed decreases. A statistically significant decrease was determined in the relative kidney weight of male rats in Group II.

3.2. Histopathology

3.2.1. Stomach and duodenum

No histopathological finding was observed in the stomach and duodenum of Bt rats in Group III. Gastric glands, surface epithelium preserved their structure in stomach. Villi and microvilli were continuous and there were no deformations in lacteals and goblet cells in duodenum.

3.2.2. Liver

Different levels of minimal granular degeneration were seen in all groups. The degrees and percentages of granular degeneration among groups are shown in Fig. 1. Granular degeneration level in 10% of examined sections was maximum (level 4) in Group III while no degeneration was observed at level 4 in Groups I and II. Additionally, degeneration level 3 was seen in 6.6% of Group II. Besides, focal mononuclear cell infiltration, congestion and nuclear border changes were determined locally in some ratios among groups and some of them were statistically significant (Table 5; Fig. 2B and C).

Table 3
Number of female and male offsprings in Groups I–III for three generation

Generations										
Groups	F0	F1			F2			F3		
	♀	♀	♂	Total	♀	♂	Total	♀	♂	Total
Group I	6	10	16	26	8	14	22	5	14	19
Group II	6	12	10	22	15	22	37	10	22	32
Group III	6	18	6	24	18	16	34	14	16	30

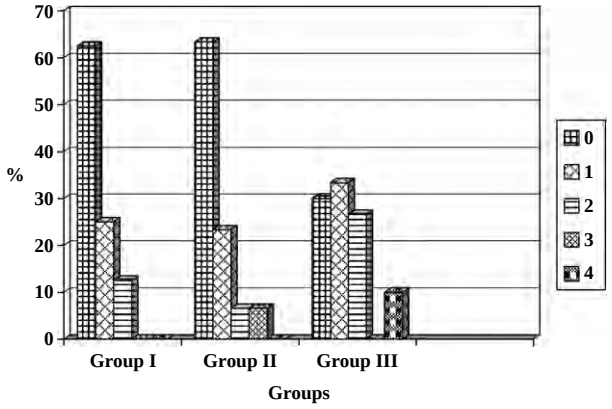
Table 4
Final body weights, relative liver and kidney weights of F_3 rats

	Groups		
	Group I	Group II	Group III
n	5	10	14
Females			
Body weight (g)	204.30 ± 4.35	246.15 ± 1.69	251.33 ± 6.43
Liver ($g \times 10^{-3}$)	33.46 ± 0.65	6.70 ± 0.78[a]	25.11 ± 0.34[a]
Kidney ($g \times 10^{-3}$)	3.36 ± 0.075	2.68 ± 0.053[a]	2.84 ± 0.002
n	14	22	16
Males			
Body weight (g)	294.20 ± 6.22	289.12 ± 6.30	299.89 ± 4.76
Liver ($g \times 10^{-3}$)	28.43 ± 0.25	30.10 ± 0.42	29.13 ± 0.53
Kidney ($g \times 10^{-3}$)	3.04 ± 0.04	2.97 ± 0.06[a]	2.99 ± 0.10

Each value is mean ± SE.
n: number of rats.
[a] Significantly different from control group, $P \leqslant 0.05$.

3.2.3. Kidney

Enlargements in parietal layer of Bowman's capsule and minimal tubular degenerations were observed at different ratios in groups (Table 5; Fig. 2E and F). The decreases in average short and long diameter of glomeruli and glomerular volume in Groups II and III were statistically different from controls while changes in the thickness of cortex was not significant among groups (Table 6).



Cumulative severity score on a 4-point scale: 0, no lesions; 1, slight severity; 2, minimal severity; 3, moderate severity; 4, marked severity.

Fig. 1. Levels and percentages of granular degenerations in liver of rats in control, reference and Bt group.

Table 5
Incidence of histopathological changes observed in liver and kidney of rats in control and experimental groups

Tissue	Finding	Groups					
		Female			Male		
		Group I	Group II	Group III	Group I	Group II	Group III
Liver	Focal infiltration	1/5	2/10	7/14	2/16	3/20	8/16[b]
	Congestion	0/5	2/10	10/14[a,b]	2/16	4/20	7/16
	Granular degeneration	1/5	2/10	9/14	3/16	6/20	13/16[a,b]
	Nuclear border change	0/5	3/10	10/14[a]	3/16	6/20	13/16[a,b]
Kidney	Enlargement in parietal layer of Bowman's capsule	0/5	1/10	6/14	2/14	1/15	5/11[a,b]
	Tubular degeneration	0/5	3/10	13/14[a,b]	2/14	4/15	9/11[a,b]

Data are expressed as number of affected/number of examined animals. Each value is mean ± SE.
[a] Significantly different from Group I (control) group, $P \leqslant 0.05$ (Fisher's exact test).
[b] Significantly different from Group II (reference) group, $P \leqslant 0.05$ (Fisher's exact test).

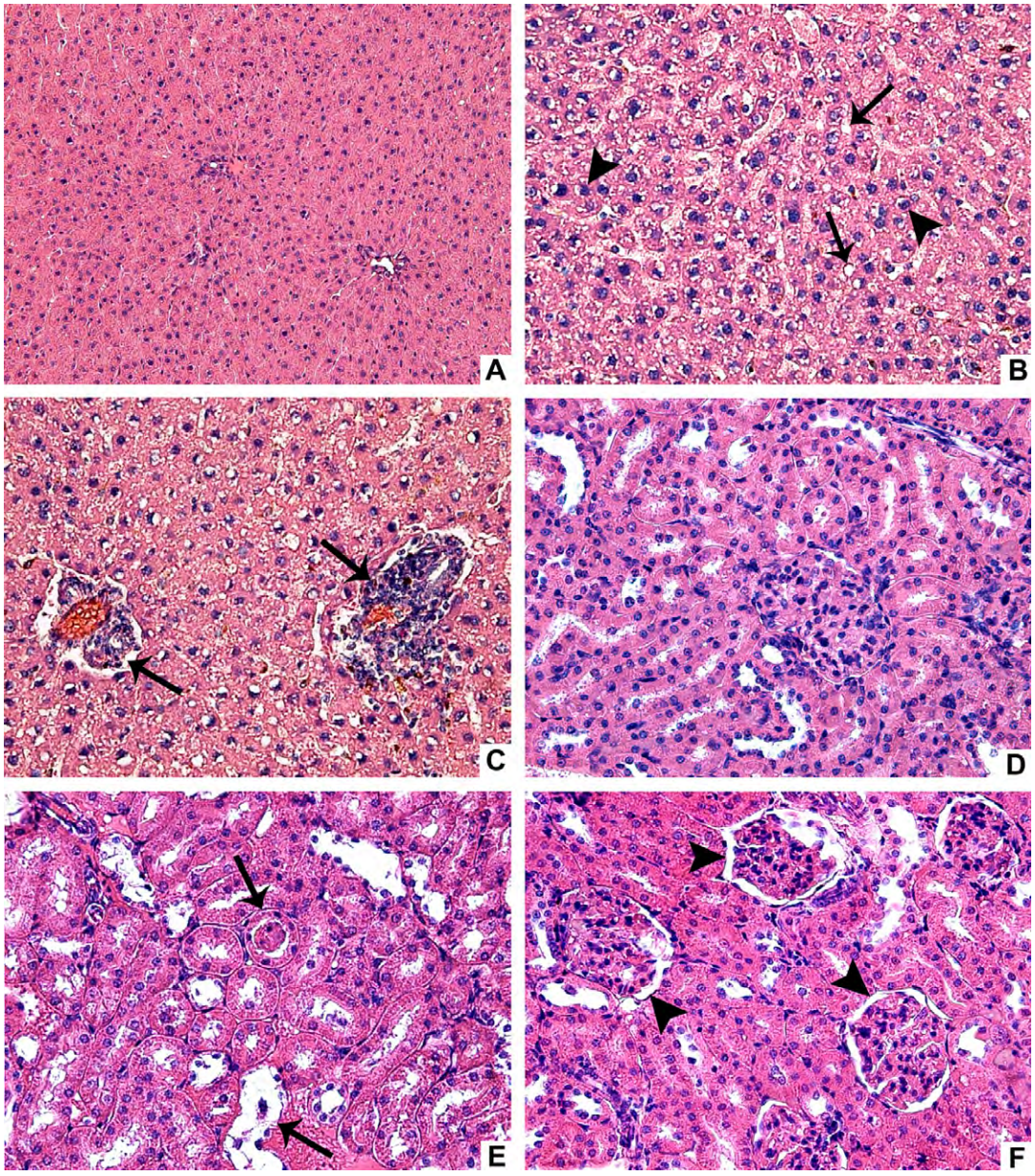


Fig. 2. Photomicrographs of liver (A–C) and kidney (D–F) tissues of rats stained with H&E. A and D are of control groups; B, C, E and F are of Bt corn groups. In liver, minor granular degeneration (arrows), nuclear border changes (B) (arrow heads) and (C) focal mononuclear cell infiltration (arrows) are shown. In kidney, (E) minimal tubular degeneration (arrows), (F) enlargements in parietal layers of Bowman's capsule (arrow heads) (magnification: A, 100×; B–F, 200×).

Table 6
Measurements of glomerular diameter, glomerular volume and thickness of cortex of rats in control and experimental groups

Groups	Diameter of glomerulus		Glomerular volume (10^4 μm^3)	Thickness of cortex (μm)
	x-axis	*y*-axis		
Group I	193.73 ± 38.88	187.96 ± 24.96	360	389.53 ± 27.50
Group II	172.89 ± 27.67[a]	184.89 ± 30.04	299	370.89 ± 41.04
Group III	168.19 ± 29.15[a]	164.73 ± 30.65 [a,b]	241	370.38 ± 44.70

Each value is mean ± SE.
[a] Significantly different from Group I (control) group, $P \leqslant 0.05$.
[b] Significantly different from Group II (reference) group, $P \leqslant 0.05$.

Table 7
Serum analysis values of female rats in control and experimental groups

Parameter	Groups		
	Group I	Group II	Group III
Urea (mg/dl)	38.50 ± 4.50	36.00 ± 3.00	36.25 ± 1.31
Urea nitrogen (mg/dl)	18.00 ± 2.10	16.73 ± 1.43	16.95 ± 0.61
Creatinine (mg/dl)	0.47 ± 0.005	0.54 ± 0.003[a]	0.50 ± 0.01
Uric acid (mg/dl)	1.69 ± 0.60	1.75 ± 0.14	1.63 ± 0.10
Total protein (g/dl)	6.07 ± 0.07	6.86 ± 0.15[a]	6.25 ± 0.13[b]
Albumin (g/dl)	3.15 ± 0.07	3.31 ± 0.008	3.42 ± 0.14
Globulin (g/dl)	2.92 ± 0.14	3.55 ± 0.16[a]	2.98 ± 0.14[b]
AST (U/L)	250.70 ± 53.20	152.50 ± 12.71	285 ± 22.98
ALT (U/L)	80.55 ± 17.55	50.06 ± 3.88	61.00 ± 5.54
ALP (U/L)	105.80 ± 12.62	63.98 ± 9.00	86.88 ± 14.75
GGT (U/L)	2.65 ± 0.15	1.76 ± 0.27	4.35 ± 0.91
Creatine kinase (U/L)	1368.00 ± 144.0	833.33 ± 90.87	1560.00 ± 100.51
Amylase (U/L)	443.5 ± 23.50	355.0 ± 7.09	369.2 ± 5.64

Each value is mean ± SE and each group consists of five rats.
[a] Significantly different from Group I (control) group, $P \leqslant 0.05$.
[b] Significantly different from Group II (reference) group, $P \leqslant 0.05$

Table 8
Serum analysis values of male rats in control and experimental groups

Parameter	Groups		
	Group I	Group II	Group III
Urea (mg/dl)	40.80 ± 2.63	39.00 ± 1.63	34.33 ± 2.18
Urea nitrogen (mg/dl)	19.06 ± 1.23	18.22 ± 0.75	16.03 ± 1.02
Creatinine (mg/dl)	0.47 ± 0.005	0.49 ± 0.017	0.41 ± 0.006[a]
Uric acid (mg/dl)	1.76 ± 0.14	2.05 ± 0.34	1.33 ± 0.05
Total protein (g/dl)	6.09 ± 0.19	6.62 ± 0.18[a]	6.37 ± 0.05[b]
Albumin (g/dl)	3.10 ± 0.09	3.19 ± 0.04	3.27 ± 0.07
Globulin (g/dl)	2.98 ± 0.10	3.42 ± 0.14[a]	3.10 ± 0.12[b]
AST (U/L)	299.12 ± 63.20	240.35 ± 23.02	547.03 ± 35.43
ALT (U/L)	81.52 ± 9.28	76.80 ± 8.32	122.10 ± 35.20
ALP (U/L)	115.21 ± 15.71	99.53 ± 16.02	120.99 ± 7.47
GGT (U/L)	3.56 ± 0.65	2.65 ± 0.32	2.00 ± 0.05
Creatine kinase (U/L)	1296.20 ± 200.51	1329.50 ± 167.7	823.33 ± 89.09
Amylase (U/L)	521.60 ± 31.46	559 ± 34.84	632.33 ± 84.33

Each value is mean ± SE and each group consists of five rats.
[a] Significantly different from Group I (control) group, $P \leqslant 0.05$.
[b] Significantly different from Group II (reference) group, $P \leqslant 0.05$.

3.3. Biochemical analysis

Results of biochemical analysis for female and male rats are presented in Tables 7 and 8. Alterations in the amounts of creatinine, globulin and total protein were statistically significant in treatment groups. Creatinine level differed depending on the group and also on gender. There were increases in the amount of creatinine in Group II females, on the other hand decreases in Group III males. Amounts of globulin and total protein were statistically different from controls in Group II but not in Group III. No statistically significant differences were noted for other parameters.

4. Discussion

GM technology have the advantage of improvement in productivity and quality of crops which express 0.01–0.1% protein of host's total protein (first generation-transgenic foods) and also prevention of various disease such as diabetes, hypertension, hypercholesterolemia and corpulence with novel crop (second generation-transgenic foods) having more than 1–10% expressing level (Hashimoto et al., 1999). Maize is one of the most widely used crop producing high-fructose corn syrup, glycose, dextrose, starch, oil, flour and meal. By means of transferring insecticidal trait to maize (Bt corn) it is possible to combat with demolishing pests that cause 7% loss of maize products (Kuiper et al., 2001).

The results of sub-chronic feeding studies on rats showed no histopathological, and biochemical effects but caused some minor changes observed suggesting long-term studies (Akay et al., 2003; Seralini, 2005). The reports on long-term feeding studies and comprehensive analysis with transgenic Bt corn are rare so this current study was planned in which rats fed with 20% Bt or reference corn (below the safety margin 33%) containing diets in order to restrain from one way and unbalanced feeding.

In our study, final body weights were not considered to be significant and decreases in relative weights of liver and kidney appeared randomly among all groups and sex, so differences were diet independent. Likewise no differences were observed in body weights and weights of kidney and liver in a recently published 90-day feeding study with Bt (Cry1Ab protein) corn in rats (Schrøder et al., 2007).

Results of a 13 week feeding study in rats with 11% or 33% Roundup Ready corn containing diets showed few increases in weight gain of males (Hammond et al., 2004) and no statistically significant differences were found in body or organ weights in a 90-day feeding study in rats with 11% or 33% MON 810 corn containing diets (Hammond et al., 2006). Similar body weights and increase in relative weight of small intestine and adrenal were found in another 90-day safety study with *Galanthus nivalis* expressing GM rice in rats (Poulsen et al., 2007).

Throughout our study, no adverse behavioural or clinical effects on F_1, F_2, F_3 generation animals were observed. Besides, birthrate and survival of the offsprings did not change among groups demonstrating successful reproduction. Polat (2005), reported no apparent differences were found in histopathological examinations in male and female rat reproductive system that were fed with transgenic Bt corn throughout two generation. Conversely, high level of mortality (55.6%) and decreases in weights of offsprings were reported in GM soybean feeding study in which female rats were fed before mating, during mating and pregnancy (Ermakova, 2005).

Our histopathological examinations in stomach and duodenum pointed out that Bt toxin did not cause deformations in gastrointestinal system. A 105-day feeding study supporting our findings was with Brown Norway rats and mice in which they fed with GM soybean and no histopathological abnormalities in mucosa of small intestine were detected (Teshima et al., 2000). In the only reported study on humans, seven volunteers fed with meal containing GM soybean and low levels of transgene survival were detected in small intestine only in three ileostomists with using molecular biology techniques (Netherwood et al., 2004). On the other hand, increase in hyperplastic cell was observed in the ileum of mice fed with *Bacillus thuringiensis* var. *kurstaki* delta-endoxin treated potatoes through 14 days (Fares and El Sayed, 1998).

The changes in the liver, as a site responsible for biotransformation and detoxification, suggest alterations in the metabolic processes. Markedly severity level of granular degeneration was seen in Bt diet containing groups in our study but not in control and reference groups. Hepatocyte nuclear size change related to both age and food (Schmucker, 1990). Therefore diets containing Bt may cause excess fatty supply for animals. But, we also observed granular degeneration at lower levels in rats of control and reference groups not showing health problems. Granular degeneration was statistically significant only in male rats in Group III. Additionally, nuclear border changes found statistically significant in female and male rats in Group III. Malatesta et al. (2002) observed irregular shaped hepatocyte nuclei and increase in number of nuclear pore at electron microscopy in offspring's of GM soybean fed pregnant mice. Thirty-five-day feeding study with GM corn in porcine showed the presence of transgene Cry1A(b) in tissues of liver, spleen, kidney and in blood but not in muscle (Mazza et al., 2005).

One of the most important processes in kidneys is excretion of toxic metabolic waste products by glomerular and tubular filtration so we examined parietal layer of Bowman's capsule and tubular changes. These findings were statistically different in males and females in Group III from control and reference groups in our study. Glomerular diameters and volume reflecting renal functions decreased in experimental groups. Decreases in short glomerular diameter in Group II also short and long diameter in Group III were statistically significant. These alterations were minor changes and parallel to the enlargements in parietal layers. The thickness of renal cortex did not changed significantly among groups. Besides, thickness in Bowman's capsule, basal membrane and glomerular mesangium were not seen at PAS stained sections. In a short term safety assessment in rats fed with GM potato showed neither pathological nor histopathological finding in liver and kidney (Hashimoto et al., 1999). Another feeding study in rats with MON 863 Bt corn demonstrated inflammation in kidney and lesions in liver and kidney (Smith, 2005). Seralini (2005) observed decreases in weight of kidney, tubular changes and inflammation in male rats fed with 33% MON 863 Bt corn in a 90-day study.

According to the results of biochemical analysis, sex-dependent creatinine levels were detected. Significant lower plasma level of creatinine in Group III may refer to anomaly in working of muscles but we did not encounter any abnormal situation during the study (Vural et al., 1986). Creatinine levels of female serum samples in Group II significantly increased from Groups I and III, depending on individual alterations and diets. Significant differences were observed in amounts of globulin and total protein in reference groups besides, findings in Group III were statistically significant from Group II. Other parameters like AST, ALT, ALP reflecting liver function and like urea, urea nitrogen, uric acid reflecting renal function did not change. Histopathological changes in liver and kidney were in accordance with our biochemical results, showing damages were minor but not critical on animal health. Parallel to our findings, Poulsen et al. (2007) pointed out lower creatinine levels but increased in plasma activity of ALT in female rats fed on GM rice. Slight reduction of albumin/globulin ratio was observed in male rats fed with 33% MON 810 GM corn through 90 day but individually albumin and globulin were not different from control groups (Hammond et al., 2006). In another 90-day study, higher concentration of urea and reduction in concentration of protein was reported in male rats fed with Bt rice (Schrøder et al., 2007).

In conclusion, although the results obtained from this study showed minor histopathological and biochemical effects in rats fed with Bt corn, long-term consumption of transgenic Bt corn throughout three generation did not cause severe health concerns on rats. Therefore, long-term feeding studies with GM crops should be performed on other species collaboration with new improving technologies in order to assure their safety.

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Induction of Mortality and Malformation in *Scinax nasicus* Tadpoles Exposed to Glyphosate Formulations

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On the global scale several factors have been suggested as possible causes of amphibians declines, including ultraviolet radiation related to ozone layer depletion, climate change, and virulent pathogens (Wake 1991; Blaustein et al. 1994; Pounds et al. 1997). Similarly, there is an extensive literature on the toxic effects on larval amphibians of metals and chemicals used in insecticides and herbicides (Power et al. 1989). On a regional scale there have been cases of frog mortality and cessation of frog choruses following the application of glyphosate based herbicides (Tyler and Willams 1996). *Scinax nasicus* was selected to carry out the present study. This hylid has an extensive distribution on Neotropical region. Moreover it is frequently found in agricultural lands and urban territories.

Glyphosate (N-(phosphonomethyl)glycine) (GLY) is a broad spectrum, non-selective systemic herbicide that will kill most plants. For the agricultural practice, the application of GLY are 0.3-5.8 kg a.i./ha and are dependent on the type of use (WHO 1994). Approximately up to 3 % of applied GLY may recuperate in aquatic environments (Urban and Cook 1986). In a field experiment in a temperate coastal rainforest in British Columbia, Canada, the highest concentration of GLY in water was 162 µg/L (Feng et al. 1990). The presence of GLY in surface water is most likely to occur as a result of heavy rainfall after recent application, with subsequent rapid dissipation into stream sediment (WHO 1994). Some of the surfactants used in agricultural formulations have been found to be significantly more toxic to fish, amphibians and aquatic invertebrates than the herbicide itself (Mitchell et al. 1987; Servizi et al. 1987; Wan et al. 1989; Mann and Bidwell 1999). The objective of this study was to investigate, under laboratory conditions, the acute toxicity of commercial glyphosate formulations (GLY-F) in *S. nasicus* tadpoles, through their survival and larvae malformation.

MATERIALS AND METHODS

S. nasicus tadpoles (250 individuals, stage 18-24: Gosner 1960) were collected from a temporary pond in the Floodplain Paraná River (31° 42´S; 60° 34´O, Paraná, Argentina) and maintained under laboratory conditions. The tadpoles were acclimatized to a 12 h-12 h light-dark cycles in glass tanks with artificial

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pond water (APW) of pH 6.8, conductivity 149 μmhos/cm^{-1}, dissolved oxygen concentration 5.5 ± 1 mg/L, hardness 66.6 mg/L of CO_3Ca at 22 ± 2 °C for 7 days.

The 96-h acute toxicity test was conducted according to USEPA (1975; 1989) standard methods, with prometamorphic larvae (from stages 25-26) (Gosner 1960). The glass tanks (35 cm diameter and 60 cm high) with 4 L of APW and 10 tadpoles (average weight: 0.01 ± 0.001 g) per tank were used in the experiments. The assayed product was the herbicide GLYFOS®, commercial formulation containing 48 % GLY as isopropylamine salt and inert ingredients POEA (polyoxethylamine) (ESP 2001). In the acute toxicity survival test, the concentrations used were: 3.07, 3.84, 4.8, 6 and 7.5 mg of GLY-F/L. Tests were conducted at 22 ± 2 °C and 12:12 light:dark. Both control and test solutions were in triplicate. Solutions were renewed daily. Mortality was recorded every 24 h. The LC50 with confidence limits ($p \leq 0.05$) were estimated by using an analysis program based on Finney (1971). Data from control and experimental groups were analyzed by one-way analysis of variance in conjunction with LSD test.

Control and treated tadpoles that survived acute tests at 24, 48, 72 and 96 h, were fixed in 10 % formalin solution. Following fixation, the tadpole's external morphology was examined with binocular microscopy. Tadpoles were stained with Alcian blue for cartilage visualization and cleared according to Wassersug (1976). Their branchial skeletons were then examined with a binocular Olympus SZX9 microscope equipped with a Olympus SC 35 camera.

RESULTS AND DISCUSSION

At 3.07 mg GL-F/L, tadpole mortality at 48-h was lower than controls but not significantly (Fig 1). Within the range of 3.84 to 7.5 m GLY-F/L survival was lower at 48 h. An elevated mortality rate was detected at all concentration at 96-h. The 96-h LC50 was 1.8 times lower than the 24-h LC50, indicating an increase of GLY-F toxicity when exposure time was prolonged (Table 1). In accordance with Pauli and Berril (1996) the low concentration of GLY-F that causes toxicity in the tadpoles to mimic concentrations that might occur in water following typical agricultural applications.

The 48 h-LC50 for tadpoles treated in this study with GLY-F/L was 3.62 mg GLY/L. Mann and Bidwell (1999) found a concentration higher than the values for tadpoles of Australian frogs (*Crinia insignifera*, *Heleioporus eyrei*, *Limnodynastes dorsalis*, and *Litoria moorei*) (48-LC50: 8.1-32.2 mg GLY/L). Several authors indicated their significant variation among and within amphibian species with respect to pesticide tolerance (Bridges and Semlitsch 2000).

Larval maldevelopment (craniofacial and mouth deformities, eye abnormalities and bent curved tails) (Fig 2) occurred in all tests and increased with time and GLY-F concentration. These effects were combined into all percent external malformation and tabulated in Table 2. Malformations were minimal at 3.07 mg/L

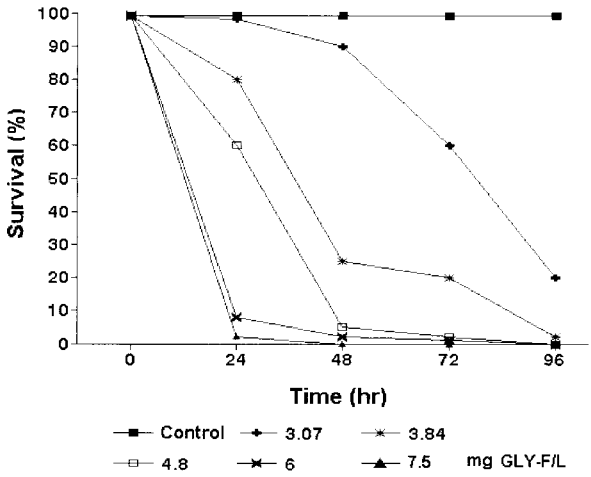

Figure 1. Survival curves for *Scinax nasicus* tadpoles.

Table 1. Acute toxicity response (LC50) of *Scinax nasicus* tadpoles exposed by glyphosate formulation.

Time	LC50	Confidence Limits 95 %	
(h)	(mg GLY/L)	Lower	Upper
24	4.78	4.23	5.35
48	3.62	3.28	5.02
72	3.23	3.07	3.36
96	2.64	2.19	2.84

n = 30

exposed for one day, whereas greater that 90 % were malformed at a GLY-F level of 7.5 mg/L. The current test confirmed the malformation effects of GLY-F on tadpoles.

Schultz et al. (1985) and Riggin and Schultz (1986) hypothesized that various pesticides may alters the synthesis of collagen in amphibian. Lajmanovich et al. (1998) found that the gills of *S. nasicus* tadpoles were very sensitive to different herbicides. The hyobranchial skeletons of *S. nasicus* tadpoles exposed to GLY-F show alterations in their cartilage structure consistent with disruption of collagen formation. The dispersant of GLY may be the culprit. This agent reduces surface tension on the leaves, allowing spray droplets to completely cover the surface. Such detergents interfere with the ability of frogs to breathe through their skin and tadpoles to breathe through their gills (Tyler 1997). This reduction in branchial cartilage is more marked in the individuals exposed to concentrations of 4.8 and 6 mg GLY-F/L - 48 h (Fig 3). In the extreme the ceratobranchials cartilages appeared as thin sheets stained. In the exposed individuals to a concentration of 6

mg/L, besides the ceratobranchials I to IV, the ceratohyals and the hypohyals were also reduced. Higher dose of GLY-F cause partial destruction of branchial arches and notable reduction of the structure of the branchial apparatus.

Table 2. Percent mortality and external morphology alterations in *S. nasicus* exposed to glyphosate formulation in static-renewal larval tests.

Days Exposure	GLY-F, mg/L	% mortality	% malformations [a]
1	3.07	2	5
	3.84	20	55
	4.8	40	70
	6	92	70
	7.5	98	70
2	3.07	10	55
	3.84	75	70
	4.8	95	70
	6	98	70
	7.5	100	90
3	3.07	40	60
	3.84	80	75
	4.8	98	75
	6	99	75
	7.5	100	-
4	3.07	80	75
	3.84	98	90
	4.8	100	90
	6	100	90
	7.5	100	-

[a] Includes both dead and alive

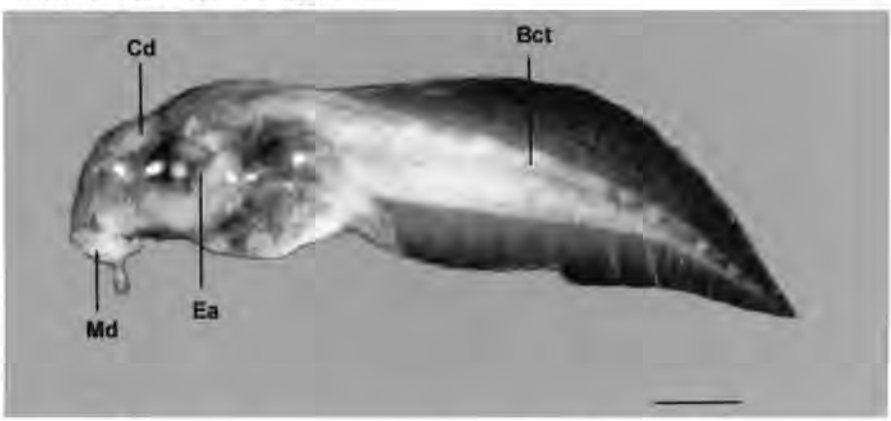

Figure 2. External malformation of *Scinax nasicus* tadpoles after 3.84 mg GLY-F/L - 24 h. Cd: Cranial deformities, Md: Mouth deformities, Ea: eye abnormalities and Bct: Bent curved tails. (X 12) (Bar, 1.5 mm).

Mitchell et al. (1987), McComb et al. (1990) and Giesy et al. (2000) considered that under normal usage, GLY herbicide did not present hazards for aquatic environment and aquatic fauna, because both the GLY and surfactant would be diluted in the water body. However, Mann and Bidwell (1999) found that in lentic, or ephemeral water bodies, at normal application rates, the concentration of

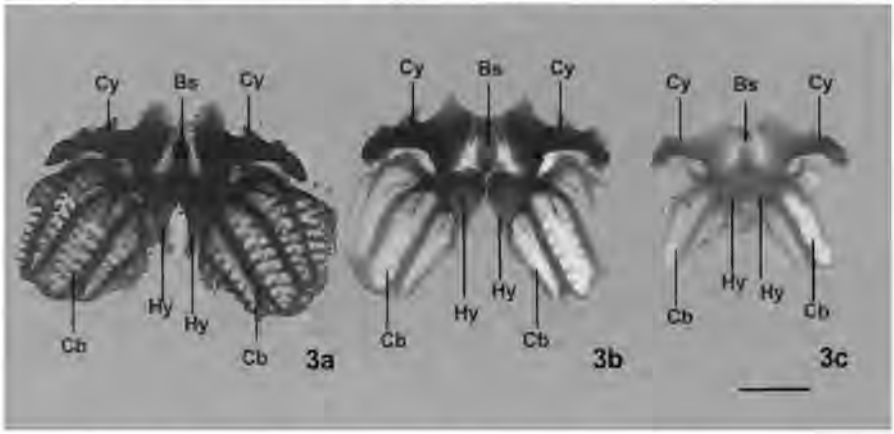

Figure 3. Hyobranchial skeletons alterations of *Scinax nasicus* tadpoles. (a) Control, observe the normal branchial arches. Cb: Ceratobranchials, Cy: Ceratohyals, Hy: Hypohyals and Bs: Basibranchial. (b) 4.8 mg GLY-F/L - 48 h. Note the size loss of that cartilaginous arc. (c) 6 mg GLY-F/L - 48 h. (X 30) (Bar, 0.75 mm).

surfactant may reach toxic levels. Considering that most amphibians are dependent on seasonal bodies of waters for their life cycles, GLY and surfactants may indeed reach harmful levels (Berger 1989). Given the effects of GLY-F on tadpole morphology we suggest that its use in the proximity of temporary pond tadpole habitats should be regulated.

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ORIGINAL PAPER

A long-term study on female mice fed on a genetically modified soybean: effects on liver ageing

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Abstract Liver represents a suitable model for monitoring the effects of a diet, due to its key role in controlling the whole metabolism. Although no direct evidence has been reported so far that genetically modified (GM) food may affect health, previous studies on hepatocytes from young female mice fed on GM soybean demonstrated nuclear modifications involving transcription and splicing pathways. In this study, the effects of this diet were studied on liver of old female mice in order to elucidate possible interference with ageing. The morpho-functional characteristics of the liver of 24-month-old mice, fed from weaning on control or GM soybean, were investigated by combining a proteomic approach with ultrastructural, morphometrical and immunoelectron microscopical analyses. Several proteins belonging to hepatocyte metabolism, stress response, calcium signalling and mitochondria were differentially expressed in GM-fed mice, indicating a more marked expression of senescence markers in comparison to controls. Moreover, hepatocytes of GM-fed mice showed mitochondrial and nuclear modifications indicative of reduced metabolic rate. This study demonstrates that GM soybean intake can influence some liver features during ageing and, although the mechanisms remain unknown, underlines the importance to investigate the long-term consequences of GM-diets and the potential synergistic effects with ageing, xenobiotics and/or stress conditions.

Keywords Ageing · Cell nucleus · Genetically modified soybean · Liver · Mitochondria

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Introduction

Diet is considered one of the most important environmental factors affecting lifespan. Genetically modified (GM) crops, in which new genes have been inserted into the original genome, are nowadays distributed all over the world, thus frequently becoming part of human and animal diets (Sanvido et al. 2007). The fact that GM food may affect human or animal health is debated: actually, no consensus exists neither on the test designs nor on the criteria to be assumed for assessing the presence of possible pathological signs (Doull et al. 2007; Séralini et al. 2007). However, it cannot be ignored that some scientific reports have described structural and molecular modifications in different organs and tissues of GM-fed animals (e.g. Ewen and Pustzai 1999; Malatesta et al. 2002a, b, 2003a, 2005, Vecchio et al. 2004; Tudisco et al. 2006; Trabalza-Marinucci et al. 2008). These observations suggest that the risk of genetically modified crops cannot be ignored and deserves further investigations in order to identify possible long-term effects, if any, of GM food consumption that might help in the post market surveillance (Kuiper et al. 2004).

Within this context, it seems of prime importance to elucidate whether a GM-containing diet may interfere with the ageing process, since senescence is characterized by progressive changes in several cellular functions that eventually result in disease and/or the loss of the ability to successfully respond to stress and xenobiotics (Jameson 2004). In fact, to test GMO-related effects on laboratory mammals, experiments have usually been performed for some months only, thus making impossible to detect long-term consequences (Séralini et al. 2007).

Liver represents an especially suitable model for monitoring the effects of a diet, since it is a multifunctional organ exerting a key role in controlling the whole metabolism and in detoxifying toxic compounds. It is known that liver sensitivity to xenobiotics is gender-related, many detoxification pathways being hormone-regulated (e.g. Voss et al. 2003); for this reason, our studies were carried out only on female animals, which seem to be more capable to cope with exogenous stress conditions (e.g. Lin et al. 2003; Dai et al. 2006; Patel et al. 2008).

In previous studies on hepatocytes from young and adult (2–8 months of age) female mice fed on GM soybean we demonstrated nuclear modifications involving structural constituents of the transcription and splicing processing pathways (Malatesta et al. 2002a).

In the present study, we have investigated the morpho-functional characteristics of the liver of 24-month-old female mice, fed from weaning on control or GM soybean, by combining a proteomic approach with ultrastructural, morphometrical and immunocytochemical analyses.

Materials and methods

Animals and treatments

Ten female Swiss mice were fed on a laboratory chow (Malatesta et al. 2002a) containing 14% GM soybean, a percentage corresponding to that usually present in the standard diet of this mouse strain and also included in the range (11–33%, Séralini et al. 2007) generally used in the regulatory tests for GMOs. This GM soybean has been obtained by insertion of the bacterial CP4 EPSPS (5-enolpyruvylshikimate-3-phosphate synthase) gene conferring a high level of tolerance to glyphosate, the active ingredient of the herbicide Roundup (GTS 40-3-2; Padgette et al. 1995). In parallel, ten female mice were fed on the same diet with commercial, non-GM soybean (controls). Both animal groups started their respective diets at weaning and were grown in standard cages under constant environmental conditions (21 ± 1°C, 50 ± 5% moisture, 12L:12D daylight cycle) until 24 months of age. During treatment, mice were weighed every 2 months. After death by cervical dislocation, liver was quickly removed, weighed and the right lobe of the organ was partly processed for microscopical analyses, and partly stored in liquid nitrogen for proteome analysis.

All animals received humane care and this study protocols comply with the institution's guidelines.

Proteome analysis

Sample preparation Liver samples were obtained from three mice for each experimental condition and were kept separate during all experiments. The tissue was homogenized and immediately resuspended in lysis buffer (8 M urea, 2% CHAPS, 65 mM dithioerythritol, 2% pharmalyte pH 3–10 and trace amount of bromophenol blue). Protein concentration was determined according to Bradford (1976).

2-DE 2-DE was performed in two independent assays, where samples from all animals were run in triplicate. Samples containing 60 μg (analytical gels) or 1 mg (preparative gels) of protein underwent 2-DE using the Immobiline/polyacrylamide system (Bjellqvist et al. 1993). Isoelectric focusing was performed on IPGphor system (GE-Healthcare, Uppsala, Sweden) at 16°C using two different protocols. For analytical gels: passive rehydration for 16 h, 500 V for 1 h, 500–2,000 V for 1 h, 3,500 V for 3 h, 5,000 V for 30 min and 8,000 V for 12 h. For preparative gels a preliminary step at 200 V constant for 12 h was added. Thereafter, immobilized pH gradient strips were reduced (2% dithioerythritol) and alkylated (2.5% iodoacetamide) in equilibration buffer (6 M urea, 50 mM Tris–HCl, pH 6.8, 30% glycerol, 2% SDS). Subsequently, strips were loaded onto 12% acrylamide vertical gels using an Ettan DALTsix electrophoresis unit (GE-Healthcare, Uppsala, Sweden). Analytical gels were stained with ammoniacal silver nitrate (Hochstrasser et al. 1988), whereas preparative gels for mass spectrometric analysis were silver-stained (Shevchenko et al. 1996).

Data acquisition and analysis To detect significant differences in protein abundance between the two experimental conditions, all silver-stained gel images were digitalized at 400 dpi resolution using ImageScanner (GE-Healthcare, Uppsala, Sweden) and analysed using Melanie 3.0 software (GE-Healthcare, Uppsala, Sweden). After background subtraction, protein spots were automatically defined and quantified with the feature detection algorithm (Hochstrasser et al. 1988). Spot intensities were expressed as percentages (vol %) of relative volumes by integrating the optical density (OD) of each pixel in the spot area (vol) and dividing with the sum of volumes of all spots detected in the gel. Only those spots, within the same experimental condition, exhibited the same trend of

expression in all gels underwent further quantitative analysis. Mean values, standard deviations and coefficients of variation were calculated. Statistical data were obtained using GraphPad software (San Diego, CA, USA) and compared by the unpaired *t* test. Differences between treatments were considered significant at $P < 0.05$. For MS analysis only those spots whose expression appeared significantly changed upon GM soybean treatment have been selected.

In-gel destaining and digestion of protein samples Spots of interest were manually excised from preparative silver-stained 2-DE gels. Silver-stained gel pieces were destained as described by Gharahdaghi et al. (1999). All excised spots were incubated with 12.5 ng/µl sequencing grade trypsin (Roche Molecular Biochemicals, Basel, Switzerland) in 25 mM AmBic overnight at 37°C. Peptide extraction was carried out twice using 50% ACN, 1% TFA and then 100% ACN. All extracts were pooled, and the volume was reduced by SpeedVac.

Mass spectrometry

Peptides were resuspended in aqueous 5% formic acid and subsequently eluted onto a 150 mm × 75 µm Atlantis C18 column analytical (Waters, Milford, MA, USA) and separated with an increasing ACN gradient from 10 to 85% in 30 min using a Waters CapLC system. The analytical column (estimated flow approximately 200 nL/min) was directly coupled, through a nanoES ion source, to a Q-TOF Ultima Global mass spectrometer (Waters, Milford, MA, USA). Multicharged ions (charge states 2, 3 and 4) were selected for fragmentation and the acquired MS/MS spectra were searched against the SWISS-PROT/TrEMBL non-redundant protein and NCBI database using the Mascot (www.matrixscience.com) MS/MS search engine. Initial search parameters were the follows: enzyme, trypsin; maximum number of missed cleavages, 1; fixed modification, carbamidomethylation of cysteines; variable modification parameters, oxidation Met; peptide tolerance, 0.5 Da; MS/MS tolerance, 0.3 Da; charge state, 2, 3, or 4. We basically selected the candidate peptides with probability-based MOWSE scores that exceeded its threshold, indicating a significant (or extensive) homology ($P < 0.05$), and referred to them as "hits". The criteria were based on the manufacturer's definitions (Matrix Science, Boston, MA, USA) (Honore et al. 2004). Proteins identified with at least two peptides were validated without any manual processing, when score higher than 40, whereas were systematically checked and/or interpreted manually to confirm or cancel MASCOT suggestions, when score was lower than 40 but higher than 20.

Light microscopy

For conventional histological observations, liver samples were fixed by immersion in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 6 h at 4°C, then dehydrated with ethanol and embedded in paraffin. Five-µm-thick sections were stained with either haematoxylin–eosin or Mallory's connective tissue stain. Samples were observed in an Olympus BX51 light microscope.

Electron microscopy

For conventional ultrastructural morphology and mitochondria morphometrical evaluations, liver samples were fixed with 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M Sörensen phosphate buffer, pH 7.4 for 3 h, washed, post-fixed with 1% OsO_4 at 4°C for 1 h, dehydrated and embedded in Epon.

For morphometrical and immunocytochemical studies on cell nuclei, samples were fixed with 4% paraformaldehyde in 0.1 M Sörensen buffer at 4°C for 2 h, dehydrated and embedded in LRWhite resin.

Epon-embedded ultrathin sections were conventionally contrasted with uranyl acetate and lead citrate, while LRWhite-embedded sections were stained with the EDTA method (Bernhard 1969). This procedure entails three steps: first, the sections are contrasted with uranyl acetate, then they are exposed to EDTA to bleach condensed chromatin, whose contrast generally masks the ribonucleoprotein (RNP) constituents involved in RNA processing, and, finally, lead citrate is briefly applied to increase RNP contrast. Specimens were observed in a Philips Morgagni TEM equipped with a Megaview II camera for digital image acquisition.

Morphometry

Morphometrical analyses were carried out by using the AnalySIS Image processing software (Soft Imaging System GmbH, Germany). Cellular and nuclear areas were measured (440×) on 30 hepatocytes/animal; then, the nucleus/cytoplasm (N/C) ratio was calculated. Further morphometrical evaluations (11,000×) were made on ten hepatocyte nuclei/animal: nucleolar areas, percentages of fibrillar centres (FCs), dense fibrillar component (DFC) and granular component (GC) per nucleolus, FC area, index of nuclear shape irregularity (the ratio between the measured perimeter and the circumference of the equivalent circle), percentage of nuclear area occupied by condensed chromatin, perichromatin granule density (PG/μm^2 of nucleoplasm) and nuclear pore frequency (NP/µm of perimeter) were considered. Finally, the sectional area as well as inner and outer membrane profile length were

measured (18,000×) in 20 mitochondria/animal, then the inner/outer membrane length ratio was calculated in order to estimate the inner membrane length independently from mitochondrial size.

Immunoelectron microscopy

For immunocytochemical analyses, mouse monoclonal antibodies directed against phosphorylated polymerase II (Research Diagnostics Inc., Flanders, NJ, USA) and the splicing factor SC-35 (Sigma-Aldrich, Buchs, Switzerland) were used and revealed by secondary gold-conjugated probes (Malatesta et al. 2002a, b). Labelling density (number of gold grains/μm^2) over nucleoplasm and nucleolus was evaluated on ten nuclei/animal (18,000×). Areas of interest were measured as described above and gold grains counted manually.

Data for each variable were pooled according to the experimental group and expressed as mean ± standard error (SE). Statistical comparisons were performed by the one-way ANOVA test ($P \leq 0.05$).

Results

Mice body weight was quite similar in all animals during the whole experiment. At sacrifice, animal's weight varied from 21 to 29 g, whereas liver's weight ranged from 0.6 to 1.5 g, without significant differences between control and GM soybean-fed animals. No macroscopic alterations or pathologic lesions were observed in any organ of all animals.

Proteomics

The total protein content of the liver did not reveal any significant difference between control and GM-fed mice, as evaluated by the Bradford assay. Similarly, the number of proteins separated by 2-DE was approximately of 1,400 from each sample, independently from the experimental condition.

In order to exclude the influence of possible high intra-sample variability, the coefficient of variation (CV) (standard deviation of normalized spot volume divided by mean) was evaluated for each sample from triplicate parallel preparations.

As reported (Molloy et al. 2003), we considered for further analysis spots with CV values of normalized volumes lower than 30%. Consistently with the observation that the great majority of proteins gave reproducible results in terms of sample preparation, extraction procedures and 2-DE, CV values of normalized volumes higher than 30% were only obtained for very faint spots and for spots located close to the gel edges.

In the liver of GM-fed mice we demonstrated significant changes in the expression of 49 spots, as indicated by labels on two representative gels obtained from control (Fig. 1a) and GM-fed animals (Fig. 1b). In particular, 39 proteins appeared significantly more expressed in GM-fed mice, whereas 10 proteins were significantly decreased.

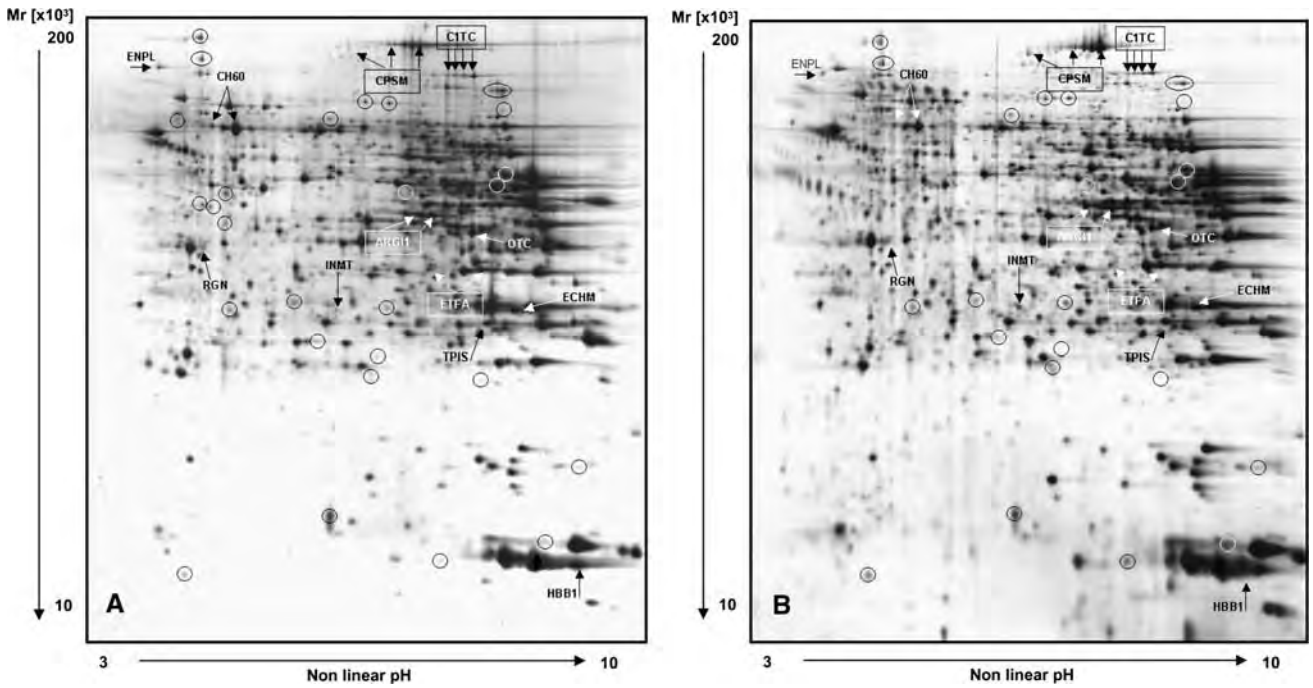

Fig. 1 Representative silver-stained 2-D electropherograms of liver from control (**a**) and GM-fed mice (**b**). Differentially expressed proteins are indicated by *arrows* and *symbol name* when identified, or by *open circles* when unidentified

By mass spectrometry we identified 20 differentially expressed proteins in the whole tissue lysate (Table 1). The remaining proteins were either in insufficient amount to be analysed by MS or MS/MS or the MS-compatible staining procedure failed to reveal them.

Protein distribution into functional categories indicates that the majority of differentially expressed proteins identified by MS belong to hepatocyte metabolism (namely, nitrogen, carbohydrate and lipid metabolism), stress response and calcium signalling pathways, as well as to mitochondria.

Morphology

The general structure of the liver parenchyma showed similar organization of hepatocytes, blood vessels, bile ducts, and extracellular matrix in the periportal areas in control and GM soybean-fed mice.

Accordingly, hepatocyte cytoplasmic organelles showed similar features in all animals: abundant rough and smooth endoplasmic reticulum, well-developed Golgi apparatus, ovoid mitochondria with transversal cristae, glycogen deposits, lipid droplets and some residual bodies (not shown).

Conversely, hepatocyte nuclei of GM-fed mice showed some morphological differences in comparison to controls (Fig. 2a, b). Hepatocyte nuclei from control mice showed a roundish shape characterized by little irregularities appearing as a fine waving and contained clumps of condensed chromatin distributed both at the periphery and inside the nucleus. In the nucleoplasm, perichromatin fibrils (PF) and perichromatin granules (PG) were distributed along the borders of the condensed chromatin, while interchromatin granules (IG) occurred as clusters in the interchromatin space (Fakan 2004). Nucleoli exhibited easily recognizable, intermingled, dense fibrillar (DFC) and granular components (GC), whereas fibrillar centres (FC) were not prominent (Fig. 2a) (nucleolar nomenclature in Schwarzacher and Wachtler 1993). Hepatocyte nuclei from GM-fed mice differed from controls because of more regular contour, large clumps of condensed chromatin, high number of PG, and smaller and compact nucleoli rich in GC (Fig. 2b). Interestingly, many PG were observed inside the condensed chromatin areas.

Morphometry

Cellular and nuclear areas were generally smaller in GM-fed than in control mice, without modifying the N/C ratio.

Table 1 Differentially expressed proteins identified in the liver of GM-fed mice compared to controls

Symbol[a]	Protein name and accession number	Theoretical MW(kDa)/p*I*	Fold-change ± SD[b]	Identification method, % coverage, no. matched peptides
ARGI1	Arginase-1 (EC 3.5.3.1) (Q61176)	34.8/6.52	+2.1 ± 0.5*	MS/MS, 49%, 10
ARGI1	Arginase-1 (EC 3.5.3.1) (Q61176)	34.8/6.52	+1.6 ± 0.3*	MS/MS, 38%, 8
C1TC	C-1-tetrahydrofolate synthase, cytoplasmic (EC 1.5.1.5) (Q922D8)	101.2/6.68	+3.6 ± 1.0*	MS/MS, 10%, 9
C1TC	C-1-tetrahydrofolate synthase, cytoplasmic (EC 1.5.1.5) (Q922D8)	101.2/6.68	−1.8 ± 0.4*	MS/MS, 2%, 3
C1TC	C-1-tetrahydrofolate synthase, cytoplasmic (EC 1.5.1.5) (Q922D8)	101.2/6.68	−3.4 ± 1.1*	MS/MS, 5%, 5
C1TC	C-1-tetrahydrofolate synthase, cytoplasmic (EC 1.5.1.5) (Q922D8)	101.2/6.68	−4.4 ± 1.3**	MS/MS, 5%, 5
CH_60	60 kDa heat shock protein, mitochondrial (P63038)	60.9/5.91	−2.0 ± 0.5*	MS/MS, 34%, 13
CH_60	60 kDa heat shock protein, mitochondrial (P63038)	60.9/5.91	+1.9 ± 0.3*	MS/MS, 43%, 15
CPSM	Carbamoyl-phosphate synthase (EC 6.3.4.16) (Q8C196)	164.6/6.48	+ 3.1 ± 1.1**	MS/MS, 17%, 21
CPSM	Carbamoyl-phosphate synthase (EC 6.3.4.16) (Q8C196)	164.6/6.48	+2.1 ± 0.7*	MS/MS, 6%, 2
CPSM	Carbamoyl-phosphate synthase (EC 6.3.4.16) (Q8C196)	164.6/6.48	+2.6 ± 1.0*	MS/MS, 11%, 16
ETFA	Electron transfer flavoprotein subunit alpha, mitochondrial (Q99LC5)	35/8.62	−2.0 ± 0.3*	MS/MS, 18%, 4
ETFA	Electron transfer flavoprotein subunit alpha, mitochondrial (Q99LC5)	35/8.62	−2.2 ± 0.5*	MS/MS, 5%, 1
ENPL	Endoplasmin (P08113)	92.4/4.74	−2.0 ± 0.6*	MS/MS, 7%, 6
ECHM	Enoyl-CoA hydratase, mitochondrial (E C 4.2.1.17) (Q8BH95)	31.5/8.76	+1.9 ± 0.2*	MS/MS, 4%, 1
HBB1	Haemoglobin subunit beta-1 (P02088)	15.8/7.13	+2.0 ± 0.6*	MS/MS, 29%, 4
INMT	Indolethylamine N-methyl-transferase (EC 2.1.1.49) (P40936)	29.5/6	+2.1 ± 0.9*	MS/MS, 5%, 1
OTC	Ornithine carbamoyl-transferase, mitochondrial (EC 2.1.3.3) (P11725)	39.8/8.81	−3.2 ± 1.1**	MS/MS, 29%, 9
RGN	Regucalcin (Q64374)	33.4/5.16	−2.3 ± 0.6*	MS/MS, 38%, 9
TPIS	Triosephosphate isomerase (EC 5.3.1.1) (P17751)	26.7/6.9	−2.2 ± 0.9*	MS/MS, 40%, 5

[a] Symbols correspond to the entry name of the sequence and are reported on gels in Fig. 1

[b] Data represent the mean fold change variation ("+" increase and "−"decrease) in GM-fed mice versus control animals. * $P < 0.05$, ** $P < 0.01$

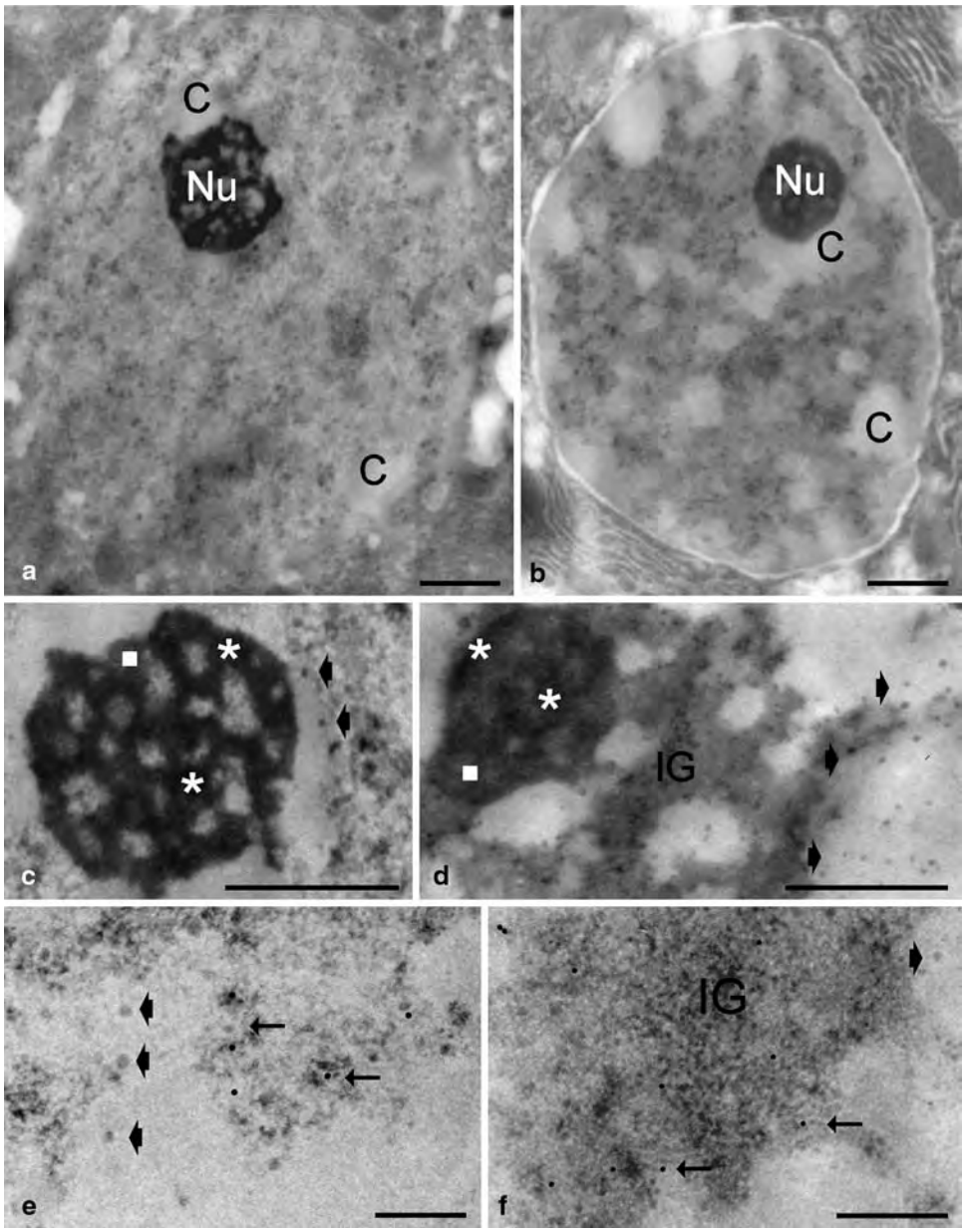

Fig. 2 Hepatocyte nuclei from a control mouse (**a**, **c**) and from a GM-fed mouse (**b**, **d**); LRWhite-embedded EDTA-stained samples. The general aspect of the nucleus is similar; however, in GM-fed mice the condensed chromatin (**c**) is quite abundant, PGs (*arrowheads*) are very numerous and occur also inside condensed chromatin areas, the nucleoli (*Nu*) appear smaller and more compact. *Asterisks*: DFC; *squares*: GC; IG: interchromatin granules. *Bars*: 1 μm. **e** Immunolabelling with anti-polymerase II antibody; the signal is specifically located on pericromatin fibrils (*arrows*), whereas PG (*arrowheads*) are devoid of labelling. **f** Immunolabelling with anti-SC-35 antibody; the gold grains occur on perichromatin fibrils (*arrows*) and on interchromatin granules (*IG*). The *arrowhead* indicates an unlabelled PG. *Bars*: 0.2 μm. The gold grain contrast was digitally enhanced using Adobe Photoshop

In GM-fed mice, shape index, nuclear pore frequency and nucleolar area decreased, while condensed chromatin percentage, PG density and GC percentage significantly increased (Table 2).

In mitochondria of GM-fed mice, a significant decrease in the inner/outer membrane length ratio (2.35 ± 0.05 vs. 3.19 ± 0.04, respectively, $P < 0.001$) was observed, although the mitochondrial area remained unchanged (0.23 ± 0.01 μm^2 vs. 0.27 ± 0.01 μm^2, respectively, $P = 0.199$).

Immunoelectron microscopy

No difference in the localization of polymerase II and SC-35 was found between GM-fed and control mice. As expected, polymerase II was mainly associated with PF (Fig. 2e), while SC-35 specifically occurred on PF and IG (Fig. 2f). By contrast, quantitative evaluation of immunolabelling revealed a significantly weaker labelling for polymerase II and SC-35 in GM-fed mice (Table 3).

Discussion

The present study was performed to investigate the effects on female mouse liver of a 2-year-long diet containing 14% GM soybean and its potential impact on the physiological ageing process; this is, to our knowledge, the longest test so far performed on laboratory mammals fed on a commercially available GMO.

Table 2 Mean ± SE values of variables considered in hepatocytes of the two groups of animals

	Cell area	Nuclear area	N/C ratio	Shape index	Condensed chromatin (%)	PG density	Pore frequency	Nucleolar area	FC area	FC (%)	DFC (%)	GC (%)
Control	405.77 ± 13.86*	45.14 ± 1.95*	0.11 ± 0.001	1.38 ± 0.07*	27.55 ± 1.44*	0.76 ± 0.04*	0.69 ± 0.03*	2.25 ± 0.13*	0.05 ± 0.01	6.92 ± 0.71	29.65 ± 1.35	63.48 ± 1.28*
GM-fed	291.26 ± 9.42*	36.95 ± 1.42*	0.13 ± 0.006	1.19 ± 0.02*	41.05 ± 1.06*	1.73 ± 0.06*	0.55 ± 0.02*	1.70 ± 0.10*	0.05 ± 0.01	5.40 ± 0.90	25.63 ± 0.98	68.97 ± 1.41*
P values	<0.001	0.05	0.071	0.02	<0.001	<0.001	0.002	0.007	0.877	0.285	0.060	0.022

DFC dense fibrillar component; *FC* fibrillar centre; *GC* granular component; *N/C* nucleus/cytoplasm; *PG* perichromatin granule

The effect of gender on hepatic metabolism has been extensively examined for a number of drugs and xenobiotics, and female rats seem to be less sensitive to many xenobiotic treatments as demonstrated for DCPT (Patel et al. 2008), APAP (Dai et al. 2006, 2008), or clivorine (Dai et al. 2006, 2003, 2008). Gender-associated variations may be related to differences in the hepatic transport (Torres 1996), in the fluidity of the liver sinusoidal membranes (i.e. phosphatidylethanolamine to phosphatidylcholine ratio), and in higher levels of total *mdr* gene products in female rat livers compared to males (Morris et al. 2003). It has also been suggested that differences in the hepatic antioxidant defence mechanisms could be responsible for the higher resistance of females to some hepatotoxicants (Sverko et al. 2004; Justo et al. 2005). Other gender differences are hormone-dependent: i.e. the arginine metabolism (Kumar and Kalyankar 1984; Tipton 2001) and the expression of heat shock proteins (HSPs) (Voss et al. 2003) are related to the estrogen availability. By considering these all, we carried out our experiments only on female mice.

We performed a complementary interdisciplinary approach which allowed us to reveal changes in liver protein profile as well as in structural cellular components that may suggest morpho-functional changes in the organ.

The macroscopical analysis did not reveal evident differences between control and GM-fed mice; moreover, no significant difference in the mortality rate was observed between the two animal groups. The liver weight reduction observed, independently from treatment, in old animals compared to young and adult mice (Malatesta et al. 2002a) is a well-known phenomenon occurring during ageing (Schmucker 1990; Anantharaju et al. 2002).

Proteome analysis demonstrated in GM-fed mice a differential expression of a number of proteins mostly related to metabolic pathways (i.e. lipid and carbohydrate) and to the urea cycle. In particular, as for the urea cycle, arginase and carbamoyl-phosphate synthetase were significantly increased, whereas ornithine transcarbamoylase was markedly downregulated. Arginase is a cytosolic enzyme responsible for the cleavage of arginine, an amino acid particularly required during growth, stress and injury acting, and also involved in the metabolism of biologically active compounds (Tong and Barbul 2004). Arginine cleavage generates urea and ornithine, the latter being necessary for tissue repair processes (Witte and Barbul 2003; Durante et al. 2007) as well as for citrullin production in the mitochondrial matrix, where ornithine transcarbamoylase catalyzes the condensation of ornithine with carbamoyl phosphate.

Since nitric oxide (NO) synthase and arginase compete for arginine, it could be speculated that increased expression of arginase might be associated to a decrease in NO synthase activity, thus influencing the redox grading which

Table 3 Mean ± SE values of labelling densities obtained with anti-RNA polymerase II, and anti-SC35 antibodies on hepatocyte nuclei of the two animal groups. Background: 0.14 ± 0.01 gold grains/μm^2

	Anti-pol II nucleoplasm	Anti-pol II nucleolus	Anti-SC35 nucleoplasm	Anti-SC35 nucleolus
Control	1.15 ± 0.10	0.31 ± 0.08	2.52 ± 0.10	0.31 ± 0.08
GM-fed	0.74 ± 0.06	0.24 ± 0.06	2.01 ± 0.08	0.28 ± 0.07
P values	<0.001	0.576	<0.001	0.815

contributes to dual activation of proliferating and proapoptotic cascades (Carreras and Poderoso 2007), ultimately modulating senescence (Gilca et al. 2007). Consistently, it is known that NO synthase activity undergoes reduction with increasing age (Valdez et al. 2004; Numao et al. 2007). Interestingly, NO synthase also occurs in the inner mitochondrial membrane, functioning as a regulatory factor of respiration (Kato and Giulivi 2006), and reduced mitochondrial inner membrane as well as decreased expression of some respiratory enzymes have been found in GM-fed mice.

In GM-fed mice, we also found a significant downregulation of the senescent marker regucalcin. Regucalcin plays a regulatory role in intracellular signalling systems (Yamaguchi 2005) maintaining intracellular Ca(2+) homeostasis through activation of Ca(2+) pump enzymes in plasma membrane, endoplasmic reticulum and mitochondria, beside its activatory effect on SOD in the liver cytosol (Fukaya and Yamaguchi 2004). A reduced expression of regucalcin could imply an impaired ratio between pro-oxidant and anti-oxidant molecules. It is known that regucalcin levels significantly decrease in aged rats, thus favouring the age-dependent deterioration of liver (Tobisawa et al. 2003; Fujita 1999). It seems therefore that senescence pathways are significantly activated in GM-fed mice.

Furthermore, proteome analysis revealed a downregulation of several HSPs in GM-fed animals. HSPs are expressed in response to a wide variety of physiological and environmental insults, acting as molecular chaperones for nascent and stress-accumulated misfolded proteins, or mediating immunological functions, thus exerting a protective function (Multhoff 2006; Schmitt et al. 2007). However, it is known that during ageing a reduction in the expression of HSPs occurs (e.g. Rea et al. 2001; Zhang et al. 2002), thus contributing to the lower capability of elderly to cope against xenobiotics and stress conditions. Again, hepatocytes of GM-fed animals seem to be characterized by a more pronounced senescence than controls.

Several findings also suggest a lower metabolic activity in hepatocytes from GM-fed mice in comparison to controls. First, smaller cellular and nuclear areas, without alteration of the N/C ratio, generally indicate decreased metabolic rate (Hildebrand 1980). When compared to previous data (Malatesta et al. 2002a, 2005), cellular and nuclear areas from both control and GM-fed old mice show dimensions more similar to young than adult animals, according to previous data demonstrating an increase in size during development and maturation and a decline during ageing (Schmucker 1990). Nucleoli also decrease in size and show prominent GC, suggesting a downregulation of ribosomal RNA transcription/processing and export rate (Schwarzacher and Wachtler 1993). In addition, more regular nuclear contour and lower nuclear pore frequency imply reduced nucleus–cytoplasmic molecular trafficking (e.g. Malatesta et al. 1998); weaker labelling for RNA polymerase II and SC-35, and higher amounts of condensed chromatin indicate reduced transcriptional activity; higher densities of PG, site of intranuclear storage and transport of already spliced (pre)-mRNA (Fakan 2004), suggest altered pre-mRNA processing and/or impaired intranuclear or nucleus–cytoplasmic transport (e.g. Lafarga et al. 1993).

Interestingly, most of these findings represent a peculiar response of aged GM-fed animals; in fact, until the 12th month of age, hepatocyte nuclei of GM-fed mice show features typical of high metabolic rate (Malatesta et al. 2002a, 2005). It is known that a general decrease in transcription and splicing factors (Frasca et al. 2003; Malatesta et al. 2003b, 2004), in nucleocytoplasmic transport factors (Pujol et al. 2002) and in nuclear pore number (Galy et al. 2000) occur in hepatocyte nuclei during ageing, together with a PG accumulation (Malatesta et al. 2003b). However, in old mice fed on GM soybean these age-related alterations appear to be more pronounced than in controls. It could be hypothesized that the high metabolic rate observed in younger mice fed on GM soybean may accelerate the ageing process, possibly favouring the accumulation of reactive oxygen species (ROS) (e.g. Hallen 2002; Roijkind et al. 2002), thus contributing to the expression of senescent markers.

At present, we do not know which could be the factor(s) present in the GM soybean capable of inducing such modifications. The changes of regucalcin as well as of other differentially expressed liver proteins observed in the present study are comparable to that reported after exposure of several xenobiotics (Yamaguchi et al. 2002; Pastorelli et al. 2006; Wei et al. 2008), thus suggesting the involvement of similar pathways activated in response to different toxic compounds. The soybean used in this study has been

treated in the field with the herbicide Roundup; although the treatment conditions used were not specified by the manufacturer, it is worth considering the possible presence in the chow of traces of glyphosate (Granby et al. 2003), i.e. the active ingredient of the herbicide Roundup to which the soybean has been rendered tolerant (Padgette et al. 1995). It has been demonstrated that Roundup slows down transcription (Marc et al. 2005), interferes with estrogen synthesis (Richard et al. 2005) and depresses respiratory activity (Peixoto 2005), inducing alterations of the mitochondria inner membrane (Szarek et al. 2000). Although the respiratory activity of mitochondria declines during ageing (Schmucker 1990), in GM-fed old mice such a decrease appeared to be significantly more pronounced than in controls. Again, this phenomenon becomes evident only in aged animals: in fact, measurements performed on 8 and 12-month-old mice revealed no modification of mitochondrial membrane length (unpublished results).

At present, no data are available on the effects of this GM-containing diet on old male mice, but microscopical observations on livers of 3-month-old male mice (unpublished results) revealed a situation comparable to that found in females of the same age (Malatesta et al. 2002a), thus suggesting a limited influence of the gender on the effects of this GM soybean. On the other hand, the comparison of the features of hepatocytes from young and old GM-fed female mice seems indicate the occurrence of cumulative long-term effects: GM soybean would first enhance liver metabolism, and this prolonged activation may then accelerate the ageing process with increased expression of senescent markers.

In conclusion, the present work demonstrate that GM soybean intake can influence the liver morpho-functional features during the physiological process of ageing and, although the mechanisms responsible for such alterations are still unknown and some data have been discussed on a speculative basis, there are several findings underlining the importance to further investigate the long-term consequences of a GM-diet and the potential synergistic effects with ageing, xenobiotics and/or stress conditions.

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DEPARTMENT OF HEALTH & HUMAN SERVICES Public Health Service

Memorandum

Date October 28, 1991

From Edwin J. Matthews, Ph.D. (HFF-156)

Subject Analysis of the Major Plant Toxicants

To Toxicology Section of the Biotechnology Working Group

Through R. Daniel Benz, Ph.D. (HFF-156)

Background

Safety of Whole Food Plants Transformed by Technology Methods

E. An analysis of all major toxins that have been identified to occur naturally in the edible part of the plant that has been transformed, or any of its close relatives (*i.e.* same genus), should be done to show that no change has occurred as compared to the natural parent or relatives. The levels of the toxins should be compared using an appropriate statistical test if the normal range of any of the toxin levels is unknown. If the normal range of any toxin has been established and published, then the edible portion of the transformed plant should be within this range providing that all levels within this range have been shown to be safe.

F. Results from an appropriately designed 28-day feeding study in swine that show that the edible portion of the transformed plant causes no acute toxicity. Endpoints to be examined should include the usual general screen done for 28-day animal studies, These include, but are not limited to effects on: 1) weight gain, 2) organ function, 3) electrolyte levels, 4) metabolism and 5) gastrointestinal tract.

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Analysis of Major Plant Toxins

A genetically engineered plant may contain an identical profile of expected plant toxicant levels (*i.e.* expected toxicants) as is normally found in a closely related, natural plant. However, genetically modified plants could also contain unexpected high concentrations of plant toxicants. The presence of high levels of toxicants in the bioengineered plant food could occur by two or more mechanisms. For example, normal levels of toxicants could be amplified through enhancement of toxicant gene transcription and translation. This might occur as a result of up-stream or down-stream promotion of gene activities in the modified plant DNA. In addition, plant toxicant genes which were normally inactive could be expressed in the modified plant gene as a result of insertion of the new genetic material (*i.e.* positional mutagenesis). Thus, the task of analysis of all major toxins in genetically engineered plant food includes the assessment of both expected toxicants and unexpected toxicants that could occur in the modified plant food. The unexpected toxicants could be closely related chemicals produced by common metabolic pathways in the same pant genus/species; however, unexpected toxicants could also be uniquely different chemicals that are usually expressed in unrelated plants.

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The task of assessing the presence or the absence of expected and unexpected plant toxicants in genetically modified plants and the control plant could be very difficult, because thousands of plant biochemicals have been shown to have toxic effects on animals and microorganisms. While all of these plant toxicants could conceivably be harmful to man by direct ingestion of plant food, or indirectly by ingestion of animal by-products that had consumed plants containing toxicants, the agency's primarily concern is for plant toxicants that could be present in common plant foods. Based upon our current knowledge of plant toxicants which groups all toxicants into one of four categories, we can rank-order the types of toxicants that we would most likely encounter in bioengineered plant foods:

Glycosides > Proteins and Protein By-Products > Alkaloids > Phenolics[1]

Thus, the most common toxicants in plant foods will be glycosides, and least likely toxicants will be phenolics. A more complete discussion of the four types of toxicant chemicals has been provided below.

Analyses for expected and unexpected plant toxicants can be achieved using either chemical/biochemical methods or toxicological bioassays. Chemical/biochemical methods have a high sensitivity for detecting the level of an individual toxicant, but their quantification of toxicant levels require extraction and purification of toxicants from plant cell homogenates. Unfortunately, purification procedures permit the detection of one toxicant while simultaneously destroying or excluding the detection of additional toxicants. Furthermore, current technology has purification procedures for only a fraction of the known plant toxicants.

Alternatively, toxicological bioassays could be used to simultaneously detect both expected and unexpected toxicants. Based upon our current knowledge, if elevated levels of toxicants occurred in genetically modified common plant food, these toxicants should elicit toxic effects in two types of assays, assuming the toxicants were present in the food product at concentrations several-fold higher than normally present in non-stressed, natural plants. First, a portion of the plant toxicants would be expected to be mutagenic in the *Salmonella typhimurium* reverse mutagenesis assay, including: alkaloids, certain glycoside functional groups (isothiocyanates and nitriles) and nitro alkanes. Second, rats and swine would be expected to be sensitive to the toxicological effects of most plant glycoside-, alkaloid-, protein-, and phenolic-toxicants. For some plant foods, the oral short-term study might have to be modified to include a different route of exposure, such as *gavage* for green, leafy foods and food extracts or *i.v.* to overcome the adsorption and solubility problems for saponins. Furthermore, the 28-day study should be optimized to detect hepatotoxicity, toxicity to certain sensitive organs (*i.e.* gastrointestinal tract, pancreas, spleen, and thyroid), anti-nutritive effects (*e.g.* growth retardation), and specific clinical chemistry tests (anemia, electrolytes *etc.*).

Plant Toxicant Addendum

Glycosides

Glycosides are plant toxicants that contain a sugar attached to one of several different functional groups. Important plant food glycoside toxicants include glycosinolates, cyanogenic glycosides, and saponins.

Glycosinolates [n > 100, *e.g.* glucoiberverin]: Toxicant structure includes a sugar linked to an isothiocyanate functional group [R-C(-S-glucose)=NOSO$_3$]. Glycosinolates (EDI = 46 mg) contribute to the flavor of cruciferous vegetables, relishes and condiments, and they are contained in plant foods (*i.e.* brussel sprouts, cabbage, cauliflower, horseradish, mustard, and radishes) and animal feedstuffs (*i.e.* cabbage, kale, rapeseed, swedes, and turnips). Toxic effects include: anti-nutritive (inhibition of growth), impairment of reproduction, blocking of thyroid function, and hepatotoxicity. Glycosinolate-induced toxicity is very common in certain animal feeds (*e.g.* rapeseed).

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Research Article Open Access

Hematotoxicity of *Bacillus thuringiensis* as Spore-crystal Strains Cry1Aa, Cry1Ab, Cry1Ac or Cry2Aa in Swiss Albino Mice

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Abstract

Formulated and sporulated cultures of *Bacillus thuringiensis* (*Bt*) have been widely used against insect pests, but after the advent of genetically modified plants expressing δ-endotoxins, the bioavailability of Cry proteins has been increased. For biosafety reasons their adverse effects should be studied, mainly for non-target organisms. Thus, we evaluated, in Swiss albino mice, the hematotoxicity and genotoxicity of four *Bt* spore-crystals genetically modified to express individually Cry1Aa, Cry1Ab, Cry1Ac or Cry2A, administered alone by gavage with a single dose of 27 mg/Kg, 136 mg/Kg or 270 mg/Kg, 24 h, 72 h or 7 days before euthanasia. Binary combinations of these four spore-crystal proteins were also assayed at 270 mg/Kg with a single administration 24 h before euthanasia. Control mice received filtered water or cyclophosphamide at 27 mg/kg. For hematotoxicity evaluations, blood samples were drawn by cardiac puncture and processed in a multiple automated hematology analyzer; for genotoxicity analyses, micronucleus test was carried out in mice bone marrow cells. Spore-crystal administrations provoked selective hematotoxicity for the 3 exposure times, particularly for erythroid lineage. A significant reduction in bone marrow cell proliferation demonstrated cytotoxic but not genotoxic effects. These effects persisted for all exposure times, becoming more evident at 7 days. Similar results were observed for binary combinations at 24 h, suggesting that further studies are required to clarify the mechanism involved in the hematotoxicity found in mice, and to establish the toxicological risks to non-target organisms, especially mammals, before concluding that these microbiological control agents are safe for mammals.

Keywords: *Bacillus thuringiensis*, δ-endotoxins, Cry, Biosafety, Non-target organisms, Bioinsecticide

Introduction

Agricultural production has been boosted by continued and indiscriminate applications of pesticides, mainly from the 1940s to the 1960s [1]. Unfortunately, this pest control management resulted in harmful outcomes such as the selection of resistant populations of insect pests, compromising the efficiency of control and forcing farmers to use increasingly high doses of pesticides [2-4]. Moreover, these chemical insecticides have harmful effects on human health and wildlife, leaving residues in food and the environment [2,3].

Among the viable alternatives for the replacement of these synthetic pesticides, entomopathogenic biological agents show potential for use in biological control programs and integrated production, because they leave few human side effects and have low impact on natural enemies and the environment [3,5,6]. In this context, *Bacillus thuringiensis* (*Bt*), a gram-positive, rod-shaped, spore-forming bacterium, is the most important biopesticide sold worldwide [7,8], having been used for over 40 years by organic farmers who spray it as a foliar insecticide [7,9].

Bt is a microbial control agent (MCA) that produces a range of entomopathogenic toxins [10,11]. The most prominent feature of *Bt* is that during sporulation it synthesizes δ-endotoxins or insecticidal crystal proteins (ICPs), which are parasporal crystalline protein inclusions containing crystal proteins (Cry proteins or Cry toxins) as their major constituent [12–15]. These are toxic to larvae of susceptible insects and small invertebrates [1,16], and their use in combating predators from the Hymenoptera, Homoptera, Orthoptera, Coleoptera, Diptera and Lepdoptera Orders, the main cause of damage to agriculture, has been effective [7,17].

Apart from the wide use of formulated and sporulated cultures of *Bt* as foliar sprays, forming part of integrated pest management strategies against insect pests of agricultural crops [11,18], advances in biotechnology have allowed the development of many genetically modified plants expressing *Bt* δ-endotoxins [8,19,20]. Consequently, this gene has been widely cloned in different crops and then large amounts of such toxins are released into the environment. However, its adverse effects on non-target organisms are poorly understood [7,9,20].

The primary threat to the effectiveness of long-term use of *Bt* toxins is the evolution of resistance by pests [21], and one of the strategies to delay the emergence of resistant pests is the combined use of Cry toxins that are effective for the same target species. The simultaneous expression of binary combinations of Cry toxins minimizes the chance of insect resistance to Bt-plants [22]. In addition to the binary combinations, advances in genetic engineering promise the expression of multiple Cry toxins in *Bt*-plants, known as gene pyramiding [23]. Therefore, studies on non-target species are requirements of international protocols to verify the adverse effects of these toxins, ensuring human and environmental biosafety [8].

Due to its growing use in agricultural activities, *Bt* presence has already been detected in different environmental compartments such as soil and water [8]. Consequently, the bioavailability of Cry proteins has increased, and for biosafety reasons their adverse effects might be studied, mainly for non-target organisms. Studies are therefore needed to evaluate (i) *Bt* toxicity to non-target organisms [7,9]; (ii) the

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persistence of *Bt* toxin and its stability in aquatic environments [24]; and (iii) the risks to humans and animals exposed to potentially toxic levels of *Bt* through their diet [25].

Thus, we aimed to evaluate, in Swiss albino mice, the hematotoxicity and genotoxicity of four *Bt* spore-crystals genetically modified to express individually Cry1Aa, Cry1Ab, Cry1Ac or Cry2A, administered alone by gavage with a single dose of 27 mg/Kg, 136 mg/Kg or 270 mg/Kg, 24 h, 72 h or 7 days before euthanasia, as well as their Cry binary combinations at 270 mg/Kg with a single administration 24 h before euthanasia.

Materials and Methods

Bt spore-crystal toxins

The spore-crystals Cry1Aa, Cry1Ab, Cry1Ac and Cry2Aa from *B. thuringiensis* var. *kurstaki* were obtained in lyophilized form from the Germplasm Bank of the Brazilian Agricultural Research Corporation (Embrapa) through its National Genetic Resource and Biotechnology Research Center (Cenargen), Brasilia/DF, Brazil. These strains were genetically modified to express individually Cry1Aa, Cry1Ab, Cry1Ac or Cry2A, and bioassays using the purified proteins from these genetically modified (GM) *Bt* spore-crystals have been published [26].

Animals and experimental design

Swiss albino mice of both genders obtained from the animal facilities of the Faculty of the University of São Paulo (Ribeirão Preto/SP, Brazil) were kept in the animal facility of the Laboratory of Genetics of the University of Brasilia (Brasília/Brazil), housed in plastic cages at room temperature (22°C ± 2°C) in a 12 h light/dark cycle with lights on at 6 a.m., and with free access to food and water. The period of acclimatization of the animals was at least seven days. Because quantitative differences in micronucleus induction have been identified between the sexes, but no qualitative differences have been described [27], and hematology reference values for mice in the veterinary therapeutic guidelines do not differentiate sexes [28], a sample size (N) of 6 mice aged approximately three months, 50% male and 50% female, was used.

Four strains of lyophilized *Bt* spore-crystals, Cry1Aa, Cry1Ab, Cry1Ac and Cry2Aa, were resuspended in distilled water at 37°C, agitated for 10 minutes and administered orally by gavage, with a single dose of 27 mg/Kg, 136 mg/Kg or 270 mg/Kg, 24 h, 72 h or 7 days before euthanasia. Cry binary combinations (Cry1Aa+1Ab, Cry1Aa+1Ac, Cry1Aa+2Aa, Cry1Ab+1Ac, Cry1Ab+2Aa, Cry1Ac+2Aa) were also assayed at 270 mg/Kg with a single administration 24 h before euthanasia. Control mice received filtered water (negative control) or cyclophosphamide (CP, positive control) at 27 mg/kg. The minimum dose of *Bt* spore-crystal toxins was of 27 mg/Kg; the maximum dose (270 mg/Kg) was 10 times greater than the minimum dose, while the intermediate dose (136 mg/Kg) was equivalent to about half the maximum dose. In our previous experiments, exposures greater than 270 mg/Kg had caused signs of toxicity and death, so this concentration was considered the maximum tolerated.

The animals were anesthetized by an intraperitoneal administration of ketamine (80 mg/kg) plus xylazine (10 mg/kg). Blood samples collected by cardiac puncture (400 µL), using an insulin syringe containing EDTA as anticoagulant, were used to carry out hemogram in a multiple automated hematologic analyzer for veterinary use, Sysmex pocH-100iV Diff (Curitiba/Paraná, Brazil) calibrated for mice. Blood smear slides were also prepared and stained with Giemsa for visual assessments of anisocytosis (variation in size), poikilocytosis (change in shape of red blood cells - RBC), polychromasia (variation in erythrocyte coloration related to the maturation of RBC), hemagglutination and erythrocyte rouleaux. After euthanasia by cervical dislocation, bone marrow cells were surgically removed and the slides for the micronucleus (MN) test were prepared according to a standard method [29]. The genotoxic potential of spore-crystal toxins was assessed by quantification of MN in polychromatic erythrocytes (PCE) and normochromatic erythrocytes (NCE), and their possible cytotoxicity was evaluated by calculating the percentage of PCE. A total of 4000 cells was analyzed in light microscopy (1000X magnification), using a Zeiss Axioskop 2 microscope. For the 24-hour exposure, circulating blood and bone marrow of the mice were collected; for the 72-hour and 7-day exposure, only circulating blood was collected.

All procedures were reviewed and approved by the institutional Ethics Committee for Animal Research (Institute of Biological Science, University of Brasília), number 32942/2009.

Colony Forming Units (CFU)

In order to quantify the number of viable *Bt* spore-crystals, the colony forming units test (CFU) was performed according to Alves and Moraes (1998) [30]. For this, 0.1 g of each lyophilized spore-crystal was diluted and homogenized with a vortex in 10 mL of sterile distilled water. Thenceforward, five successive dilutions were made, using in each one 0.1 mL of previous dilution to 9.9 mL of sterile distilled water. Then, 0.1 mL of each one of the last three dilutions was plated in three replicates on a conventional culture medium (NYSM) and placed in an incubator at 30°C for 14 hours. The colonies of each replicate were counted and the average was calculated, with the result given in cells/mL (Table 5).

Statistical analysis

Statistical analysis was carried out using SPSS (Statistical Package for the Social Sciences) version 15.0. Data were expressed as mean ± SEM (standard error of mean) and values of $p<0.05$ were considered statistically significant. The continuous variables were tested for normal distribution with Shapiro-Wilk. Possible differences among the groups analyzed were investigated through Kruskal-Wallis test, since the data were not normally distributed. For significant Kruskal-Wallis results, Mann-Whitney U test was performed to verify differences between the treatments (2-to-2 comparisons). P-values with statistical significance ($p<0.05$) were only considered when they also presented biological significance, according to the following criteria: (1) group C (negative control) compared to all groups; (2) group CP (positive control) compared to the treatments with the same exposure time or between CP of different exposure times; (3) the same toxins compared in the same exposure time but in different doses (dose-effect) or at 270 mg/Kg in different exposure times (24 h, 72 h, 7 days); (4) different toxins compared to each other at 270 mg/Kg in the 24 h, 72 h and 7-day exposure; (5) binary combinations compared to single doses of those Cry present in the combinations at 270 mg/Kg, at 24 h of exposure; (6) binary combinations compared to each other.

Results

Erythrogram (Table 1)

24 hours of exposure: Oral administrations of single doses of Cry1Ab 136 mg/Kg ($p=0.006$), Cry1Ac 270 mg/Kg ($p=0.011$) and Cry2Aa 27 mg/Kg ($p=0.006$) significantly reduced MCH values, while Cry1Aa 27 mg/Kg ($p=0.034$) increased MCHC values. All *Bt* spore-crystals promoted significant reductions in MCV values ($p=0.004$ for Cry1Aa 27 mg/Kg, $p=0.003$ for Cry1Ac 27 mg/Kg, and $p=0.000$ for the

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G	Treatment	RBC (x 10^6/μL)	HGB (g/dL)	HCT (%)	MCH (pg)	MCHC (g/dL)	MCV (fL)	RDW (%)
1	Filtered water,	7.53 ± 0.28	11.90 ± 0.44	30.38 ± 1.02	15.83 ± 0.09	39.12 ± 0.41	40.40 ± 0.40	17.83 ± 0.41
2	CP 27 mg/Kg, 24 h	7.60 ± 0.12	12.05 ± 0.26	30.57 ± 0.66	15.85 ± 0.16	39.45 ± 0.75	40.22 ± 0.46	16.80 ± 0.60
3	Cry1Aa 27 mg/Kg, 24 h	8.25 ± 0.13	12.78 ± 0.21	30.90 ± 0.36	15.50 ± 0.25	41.36 ± 0.38*	37.50 ± 0.43**,•	14.94 ± 0.54**,•
4	Cry1Aa 136 mg/Kg, 24 h	7.92 ± 0.14	12.19 ± 0.31	29.84 ± 0.68	15.37 ± 0.19	40.81 ± 0.32	37.64 ± 0.28**,•	14.16 ± 0.38**,•
5	Cry1Aa 270 mg/Kg, 24 h	8.35 ± 0.20	12.40 ± 0.29	31.08 ± 0.71	14.85 ± 0.16	39.88 ± 0.34	37.22 ± 0.12**,•	15.38 ± 0.21**,‡
6	Cry1Ab 27 mg/Kg, 24 h	8.17 ± 0.22	12.55 ± 0.42	31.13 ± 0.89	15.35 ± 0.22	40.30 ± 0.38	38.12 ± 0.21**,•	15.35 ± 0.31*
7	Cry1Ab 136 mg/Kg, 24 h	7.69 ± 0.09	11.23 ± 0.25	28.55 ± 0.62	14.62 ± 0.21**,•	39.35 ± 0.26	37.12 ± 0.42**,•	16.65 ± 0.56**
8	Cry1Ab 270 mg/Kg, 24 h	8.19 ± 0.16	12.63 ± 0.20	30.77 ± 0.53	15.45 ± 0.18	41.07 ± 0.33	37.60 ± 0.17**,•	15.70 ± 0.36**
9	Cry1Ac 27 mg/Kg, 24 h	7.98 ± 0.12	12.25 ± 0.24	30.42 ± 0.56	15.33 ± 0.20	40.28 ± 0.56	38.08 ± 0.22**,•	16.93 ± 0.36
10	Cry1Ac 136 mg/Kg, 24 h	8.11 ± 0.32	12.12 ± 0.40	29.92 ± 1.02	14.98 ± 0.22	40.53 ± 0.47	36.93 ± 0.23**,•	16.18 ± 0.67*
11	Cry1Ac 270 mg/Kg, 24 h	8.17 ± 0.35	11.98 ± 0.39	29.68 ± 1.07	14.67 ± 0.21*	40.42 ± 0.32	36.37 ± 0.35**,•	16.17 ± 0.67
12	Cry2Aa 27 mg/Kg, 24 h	8.24 ± 0.18	12.02 ± 0.15	29.95 ± 0.53	14.62 ± 0.22**,•	40.15 ± 0.35	36.40 ± 0.36**,•	15.02 ± 0.52**
13	Cry2Aa 136 mg/Kg, 24 h	7.86 ± 0.23	11.68 ± 0.33	29.05 ± 0.77	14.87 ± 0.10	40.22 ± 0.18	36.97 ± 0.31**,•	15.47 ± 0.30**
14	Cry2Aa 270 mg/Kg, 24 hk	7.75 ± 0.27	11.82 ± 0.37	28.97 ± 0.89	15.27 ± 0.17	40.82 ± 0.42	37.42 ± 0.29**,•	15.62 ± 0.34**
15	Cry1Aa+1Ab 270 mg/Kg, 24 h	8.30 ± 0.18	12.45 ± 0.30	31.15 ± 0.78	15.00 ± 0.10 •	40.00 ± 0.23	37.53 ± 0.28**,•	14.87 ± 0.12**,•,a
16	Cry1Aa+1Ac 270 mg/Kg, 24 h	8.47 ± 0.42	12.93 ± 0.63	31.78 ± 1.25	15.30 ± 0.13	40.60 ± 0.48	37.63 ± 0.39**,•	14.85 ± 0.46**,•
17	Cry1Aa+2Aa 270 mg/Kg, 24 h	8.46 ± 0.14	12.70 ± 0.09	31.77 ± 0.24	15.03 ± 0.19	39.98 ± 0.27	37.57 ± 0.47**,•	14.95 ± 0.32**,•
18	Cry1Ab+1Ac 270 mg/Kg, 24 h	8.03 ± 0.15	12.17 ± 0.29	29.63 ± 0.78	15.15 ± 0.21	41.08 ± 0.28	36.88 ± 0.47**,•	15.48 ± 0.39**
19	Cry1Ab+2Aa 270 mg/Kg, 24 h	8.20 ± 0.16	11.95 ± 0.10	29.83 ± 0.35	14.58 ± 0.23**	40.07 ± 0.24	36.42 ± 0.48**,•	15.38 ± 0.43**
20	Cry1Ac+2Aa 270 mg/Kg, 24 h	8.33 ± 0.17	11.95 ± 0.21	30.50 ± 0.47	14.37 ± 0.33**,•	39.18 ± 0.48	36.70 ± 0.53**,•	15.82 ± 0.37**
	P-values 24 h	0,075	0.072	0.179	0.000	0.002	0.000	0.000
21	CP 27 mg/Kg, 72 h	7.72 ± 0.28	11.65 ± 0.28	29.30 ± 0.76	15.13 ± 0.34	39.75 ± 0.27	38.03 ± 0.60*,•	15.02 ± 0.22**,•
22	Cry1Aa 270 mg/Kg, 72 h	6.86 ± 0.47[a]	10.77 ± 0.65[a]	26.73 ± 1.59[a]	15.77 ± 0.29	40.28 ± 0.45	39.15 ± 0.54	16.57 ± 0.67
23	Cry1Ab 270 mg/Kg, 72 h	7.92 ± 0.29	11.87 ± 0.26[b]	29.83 ± 0.81	15.02 ± 0.31	39.82 ± 0.44	37.75 ± 0.47**	15.40 ± 0.62**
24	Cry1Ac 270 mg/Kg, 72 h	8.17 ± 0.19	11.97 ± 0.44	30.28 ± 0.88	14.63 ± 0.27*	39.48 ± 0.46	37.07 ± 0.56**	16.40 ± 0.36*,○
25	Cry2Aa 270 mg/Kg, 72 h	8.30 ± 0.22	12.38 ± 0.31	30.67 ± 0.78	14.92 ± 0.16	40.38 ± 0.22	36.95 ± 0.52**	15.82 ± 0.10**,○
	P-values 72 h	0,027	0.300	0.213	0.014	0.211	0.002	0.004
26	CP 27 mg/Kg, 7 days	8.10 ± 0.28	12.25 ± 0.34	30.82 ± 0.87	15.15 ± 0.13	39.75 ± 0.25	38.13 ± 0.41*,•	16.32 ± 0.59*
27	Cry1Aa 270 mg/Kg, 7 days	7.12 ± 0.30	10.77 ± 0.44[a]	26.88 ± 0.99[a]	15.13 ± 0.26	40.00 ± 0.25	37.80 ± 0.57**	16.38 ± 0.46*,a
28	Cry1Ab 270 mg/Kg, 7 days	6.53 ± 0.38[b]	9.68 ± 0.58**,b,b'	24.55 ± 1.36**,b,b'	14.83 ± 0.15**	39.38 ± 0.40	37.63 ± 0.37**	17.02 ± 0.54
29	Cry1Ac 270 mg/Kg, 7 days	8.16 ± 0.10	12.17 ± 0.26	30.38 ± 0.48	14.92 ± 0.26*	40.03 ± 0.31	37.27 ± 0.47**	16.03 ± 0.33**
30	Cry2Aa 270 mg/Kg, 7 days	7.99 ± 0.20	11.90 ± 0.31	30.25 ± 0.77	14.88 ± 0.09*	39.33 ± 0.34	37.85 ± 0.28**	15.92 ± 0.41**
	P-values 7 days	0.001	0.000	0.000	0.004	0.293	0.000	0.059
	Total p-values	0.000	0.002	0.007	0.000	0.001	0.000	0.000

The data correspond to the means and to the standard error of mean (SEM). RBC=Red Blood Cells; HGB=Hemoglobin; HCT=Hematocrit; MCV=Mean Corpuscular volume; MCH=Mean Corpuscular hemoglobin; MCHC=Mean corpuscular hemoglobin concentration; RDW=Red cell distribution width (represents an indication of the amount of variation − anisocytosis − in cell size); g/dL=grams per deciliter; fL=fentoliters; pg=picograms. P-values were generated by the Kruskall-Wallis test. Asterisks indicate significant (p<0.05) and highly significant (p<0.01) differences detected by the Mann Whitney U test in the comparisons with the negative control. The following symbols represent significant differences in respect to: ●=CP 24 h; ○=CP 72 h; †= dose-effect for the dose of 27 mg/Kg; ‡=dose-effect for the dose of 136 mg/Kg; a= Cry1Aa 270 mg/Kg, 24 h; b=Cry1Ab 270 mg/Kg, 24 h; b'=Cry1Ab 270 mg/Kg, 72 h

Table 1: Results of erythrogram of Swiss albino mice treated with *Bt* spore-crystal Cry1Aa, Cry1Ab, Cry1Ac and Cry2Aa administered orally (*per os*, p.o.) 24 h, 72 h and 7 days before euthanasia. Control mice received filtered water (negative control) or cyclophosphamide (CP – positive control) at 27 mg/Kg.

others). For the visually assessed slides, microcytosis and hypochromia particularly increased for the treatments with Cry1Ab at 27 and 136 mg/Kg, with hypochromia being more severe for the dose of 136 mg/Kg; spherocytes were also very prevalent in these slides. Microcytosis with spherocyte presence was also observed in slides of Cry2Aa 270 mg/Kg. Significantly decreased RDW were also observed for most Cry treatments, except for Cry1Ac at 27 and 270 mg/Kg (p=0.041 for Cry1Ab and Cry1Ac at 136 mg/Kg, p=0.002 for the other treatments). In the dose-effect evaluations, values for RDW were significantly higher for Cry1Aa at 270 mg/Kg than at 136 mg/Kg (p=0.004). Binary combinations containing Cry1Ab+Cry2Aa (p=0.004) and Cry1Ac+Cry2Aa (p=0.000) caused significant reductions in MCH values, and all Cry combinations promoted significant reductions in VCM and RDW. For the visually assessed slides, a higher hypochromia to Cry1Ac+2Aa was observed. Among different binary combinations, Cry1Ac+Cry2Aa showed a higher RDW compared to Cry1Aa+Cry1Ab and Cry1Aa+Cry1Ac (p=0.041), which also occurred with Cry1Aa in respect to Cry1Aa+Cry1Ab (p=0.041).

72 hours of exposure: Compared to negative control, Cry1Ac 270 mg/Kg continued to significantly reduce MHC values (p=0.040), while MCV and RDW were significantly reduced after treatment with CP, Cry1Ab, Cry1Ac and Cry2Aa. Among toxins, Cry1Aa showed a significant reduction in RBC (p=0.030), HGB (p=0.041) and HCT (p=0.015) values compared to Cry2Aa, and increased MCV compared to Cry2Aa (p=0.009) and Cry1Ac (p=0.026).

7 days of exposure: Cry1Ab 270 mg/Kg resulted in significantly reduced HGB (p=0.009) and HCT (p=0.002) values, while all Cry administrations significantly reduced MCH and MCV; the same occurred with MCV after CP treatment and with RDW after CP and all Cry administrations, except the Cry1Ab. For the visually assessed slides

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a higher hypochromia was observed for Cry1Aa, Cry1Ac, and Cry2Aa, more pronounced in the two first treatments.

Comparing toxins, RBC, HGB and HCT were significantly lower for Cry1Ab than for Cry1Ac and Cry2Aa.

Leukogram (Table 2)

24 hours of exposure: In single doses only Cry1Ab 27 mg/Kg promoted a significant increase in lymphocyte frequency in comparison to the negative control (p=0.041). Cry1Ab significantly increased total leukocytes (white blood cells or WBC) and lymphocytes for the dose of 27 mg/Kg in respect to the doses of 136 mg/Kg (p=0.015 for WBC, p=0.002 for lymphocytes) and 270 mg/Kg (p=0.026 for total WBC, p=0.015 for lymphocytes), and increased neutrophils+monocytes for 136 mg/Kg in respect to 270 mg/Kg (p=0.009).

72 hours of exposure: Compared to negative control, only Cry1Aa caused significantly increased neutrophils+monocytes (p=0.003). Cry1Aa also showed increased WBC compared to Cry1Ab (p=0.015) and Cry1Ac (p=0.009), as well as neutrophils+monocytes in respect to Cry1Ab (p=0.016), Cry1Ac (p=0.004) and Cry2Aa (p=0.002).

7 days of exposure: Compared to the negative control, CP (p=0.026), Cry1Ac and Cry2Aa (p=0.041 for both) promoted a significant increase in WBC, and this result was related to increased neutrophils+monocytes (p=0.004 for CP; p=0.026 for Cry1Ac and Cry2Aa). Cry1Ab (p=0.002) caused a significant reduction in lymphocytes, while Cry2Aa (p=0.026) resulted in a significant increase in their number. Also, Cry1Aa (p=0.041) and Cry1Ab (p=0.026) caused a significant increase in eosinophils. Cry1A (p=0.009) and Cry1Ab (p=0.015) also showed significantly increased eosinophils compared to Cry2Aa. WBC and lymphocytes were also significantly reduced for Cry1Ab in respect to Cry1Ac and Cry2Aa.

G	Treatment	WBC (× 10^3/μL)	Lymphocytes (× 10^3/μL)	Neutrophils + Monocytes (× 10^3/μL)	Eosinophils (× 10^3/μL)
1	Filtered water	4.63 ± 0.74	3.35 ± 0.55	1.22 ± 0.20	0.07 ± 0.05
2	CP 27 mg/Kg, 24 h	5.37 ± 0.77	3.73 ± 0.53	1.58 ± 0.34	0.05 ± 0.03
3	Cry1Aa 27 mg/Kg, 24 h	6.32 ± 0.85	4.94 ± 0.73	1.38 ± 0.14	0.00 ± 0.00
4	Cry1Aa 136 mg/Kg, 24 h	5.53 ± 0.52	4.20 ± 0.45	1.27 ± 0.11	0.06 ± 0.04
5	Cry1Aa 270 mg/Kg, 24 h	5.47 ± 0.77	3.93 ± 0.77	1.47 ± 0.31	0.07 ± 0.03
6	Cry1Ab 27 mg/Kg, 24 h	6.82 ± 0.62	5.13 ± 0.33*	1.68 ± 0.33	0.00 ± 0.00
7	Cry1Ab 136 mg/Kg, 24 h	4.60 ± 0.49†	2.60 ± 0.42†	1.92 ± 0.45	0.08 ± 0.03
8	Cry1Ab 270 mg/Kg, 24 h	4.60 ± 0.47†	3.53 ± 0.43†	0.98 ± 0.10‡	0.08 ± 0.04
9	Cry1Ac 27 mg/Kg, 24 h	6.90 ± 0.89	4.25 ± 0.45	2.52 ± 0.66	0.13 ± 0.10
10	Cry1Ac 136 mg/Kg, 24 h	6.78 ± 1.66	4.00 ± 0.97	2.48 ± 0.56	0.30 ± 0.24
11	Cry1Ac 270 mg/Kg, 24 h	5.17 ± 0.66	3.55 ± 0.66	1.60 ± 0.37	0.02 ± 0.02
12	Cry2Aa 27 mg/Kg, 24 h	4.38 ± 0.63	2.43 ± 0.36	1.90 ± 0.53	0.05 ± 0.02
13	Cry2Aa 136 mg/Kg, 24 h	4.68 ± 1.06	3.05 ± 0.91	1.50 ± 0.32	0.13 ± 0.06
14	Cry2Aa 270 mg/Kg, 24 h	5.03 ± 0.78	2.98 ± 0.28	2.02 ± 0.73	0.03 ± 0.02
15	Cry1Aa+1Ab 270 mg/Kg, 24 h	5.97 ± 0.52	4.28 ± 0.54	1.67 ± 0.08^{b}	0.02 ± 0.02
16	Cry1Aa+1Ac 270 mg/Kg, 24 h	4.10 ± 0.69	2.85 ± 0.58	1.20 ± 0.28	0.05 ± 0.02
17	Cry1Aa+2Aa 270 mg/Kg, 24 h	4.92 ± 0.84	3.38 ± 0.84	1.43 ± 0.52	0.10 ± 0.05
18	Cry1Ab+1Ac 270 mg/Kg, 24 h	4.72 ± 0.59	3.18 ± 0.51	1.42 ± 0.13^{b}	0.12 ± 0.08
19	Cry1Ab+2Aa 270 mg/Kg, 24 h	4.78 ± 0.54	3.47 ± 0.48	1.28 ± 0.24	0.03 ± 0.02
20	Cry1Ac+2Aa 270 mg/Kg, 24 h	4.63 ± 0.70	3.13 ± 0.51	1.48 ± 0.40	0.02 ± 0.02
	P-values 24 hours	0.454	0.095	0.758	0.486
21	CP 27 mg/Kg, 72 h	3.37 ± 0.78	2.40 ± 0.46	0.95 ± 0.41	0.02 ± 0.02
22	Cry1Aa 270 mg/Kg, 72 h	6.53 ± 0.82°	3.42 ± 0.41	2.98 ± 0.43$^{**,\circ,a}$	0.13 ± 0.08
23	Cry1Ab 270 mg/Kg, 72 h	4.27 ± 0.36	2.72 ± 0.16	1.48 ± 0.29	0.07 ± 0.03
24	Cry1Ac 270 mg/Kg, 72 h	3.72 ± 0.35	2.37 ± 0.35	1.25 ± 0.13	0.10 ± 0.06
25	Cry2Aa 270 mg/Kg, 72 h	4.80 ± 0.57	3.58 ± 0.46	1.18 ± 0.15	0.03 ± 0.02
	P-values 72 hours	0.059	0.331	0.000	0.557
26	CP 27 mg/Kg, 7 days	7.22 ± 0.68$^{*,\circ}$	4.33 ± 0.40°	2.82 ± 0.35$^{**,\bullet,\circ}$	0.07 ± 0.03
27	Cry1Aa 270 mg/Kg, 7 days	6.70 ± 1.83	3.30 ± 0.77	3.15 ± 1.20	0.25 ± 0.08$^{*,\lozenge,a}$
28	Cry1Ab 270 mg/Kg, 7 days	3.90 ± 0.58$^{\lozenge}$	1.85 ± 0.10$^{**,\lozenge,b,b'}$	1.77 ± 0.42	0.28 ± 0.08*
29	Cry1Ac 270 mg/Kg, 7 days	6.58 ± 0.50$^{*,c'}$	3.85 ± 0.62	2.63 ± 0.60*	0.10 ± 0.04
30	Cry2Aa 270 mg/Kg, 7 days	7.85 ± 1.37*	5.33 ± 0.89^{*,d}	2.48 ± 0.55$^{*,d'}$	0.03 ± 0.02
	P-values 7 days	0.034	0.006	0.092	0.015
	Total P-values	0.015	0.004	0.034	0.024

G= group. WBC= White Blood Cells. The data correspond to the means and to the standard error of mean (SEM). P-values were generated by the Kruskall-Wallis test. Asterisks indicate significant (p<0.05) and highly significant (p<0.01) differences detected by the Mann Whitney U test in the comparisons with the negative control. The following symbols represent significant differences in respect to: ●=CP 24 h; ○=CP 72 h; ◊=CP 7 days; †=dose-effect for the dose of 27 mg/Kg; ‡=dose-effect for the dose of 136 mg/Kg; a=Cry1Aa 270 mg/Kg, 24 h; b=Cry1Ab 270 mg/Kg, 24 h; b'=Cry1Ab 270 mg/Kg, 72 h; c=Cry1Ac 270 mg/Kg, 24 h; c'=Cry1Ac 270 mg/Kg, 72h; d=Cry2Aa 270 mg/Kg, 24 h; d'=Cry2Aa 270 mg/Kg, 72 h.

Table 2: Results of leukogram of Swiss albino mice treated with *Bt* spore-crystal Cry1Aa, Cry1Ab, Cry1Ac and Cry2Aa administered orally (*per os*, p.o.) 24 h, 72 h, and 7 days before euthanasia. Control mice received filtered water (negative control) or cyclophosphamide (CP – positive control) at 27 mg/Kg.

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Plateletgram (Table 3)

24 hours of exposure: In comparison with negative control, CP promoted a significant fall in platelet number (PLT, p=0.026), while a single dose of Cry1Aa 27 mg/Kg significantly increased PLT (p=0.009), decreased mean platelet volume (MPV, p=0.015) and platelet large cell ratio (P-LCR, p=0.026); the same decreased P-LCR occurring at 136 mg/Kg (p=0.041). Cry1Ac 136 mg/Kg significantly increased MPV (p=0.019) and P-LCR (p=0.038), while Cry2Aa at 270 mg/Kg significantly decreased platelet distribution width (PDW, p=0.026). Regarding dose-effect, Cry1Aa at 136 mg/Kg showed reduced PLT (p=0.015) compared to 27 mg/Kg and reduced PDW (p=0.009) compared to 270 mg/Kg. Significantly increased values of MPV, P-LCR and PDW were verified for Cry1Ac at 136 mg/Kg compared to 27 mg/Kg and 270 mg/Kg (at 27 mg/Kg, p=0.010; at 270 mg/Kg, p=0.029). Cry1Ac at 136 mg/Kg also showed a significantly increased PDW in respect to the dose of 270 mg/Kg (p=0.038). Among toxins, at 270 mg/Kg, PLT was significantly higher after treatment with Cry1Aa compared to Cry2Aa (p=0.041), while PDW was significantly higher for Cry1Ab than for Cry1A (p=0.041).

As regards the negative control, binary combinations significantly reduced levels as follows: Cry1Aa+Cry2Aa for PLT (p=0.002), Cry1Ab+Cry2Aa for MPV (p=0.024), and Cry1Ab+Cry1Ac for PDW (p= 0.030). Between combinations, Cry1Aa+Cry2Aa showed a lower PLT compared to Cry1Ab+Cry1Ac (p=0.015), and Cry1Aa+Cry2Aa presented higher MPV and RDW (p=0.036) in comparison with Cry1Ab+Cry2Aa.

Cry1Aa+Cry2Aa showed significantly reduced PLT (p=0.009) and increased PDW (p=0.009) compared to Cry1Aa, which also presented lower PDW compared to Cry1Aa+Cry1Ab and Cry1Aa+Cry1Ac (p=0.015 for both), and Cry1Ab showed significantly higher PDW

Group	Treatment	PLT (× 10^3/μL)	MPV (fl)	P-LCR (%)	PDW (fl)
1	Filtered water	1219.00 ± 56.64	6.93 ± 0.10	10.88 ± 0.81	6.95 ± 0.11
2	CP 27 mg/Kg, 24 h	978.67 ± 93.57*	7.10 ± 0.30	11.23 ± 2.33	7.25 ± 0.24
3	Cry1Aa 27 mg/Kg, 24 h	1431.60 ± 27.40$^{**\bullet}$	6.38 ± 0.15$^{*\bullet}$	7.32 ± 1.15*	6.74 ± 0.05$^{\bullet}$
4	Cry1Aa 136 mg/Kg, 24 h	1243.57 ± 113.56†	6.56 ± 0.14	7.91 ± 1.14*	6.77 ± 0.07
5	Cry1Aa 270 mg/Kg, 24 h	1292.00 ± 74.24$^{\bullet}$	6.75 ± 0.15	11.15 ± 1.68	6.53 ± 0.07$^{\bullet\ddagger}$
6	Cry1Ab 27 mg/Kg, 24 h	1168.00 ± 73.22	6.62 ± 0.17	9.93 ± 1.10	6.63 ± 0.10$^{\bullet}$
7	Cry1Ab 136 mg/Kg, 24 h	1076.00 ± 148.54	6.85 ± 0.22	10.73 ± 1.74	6.75 ± 0.16
8	Cry1Ab 270 mg/Kg, 24 h	1020.50 ± 113.38	7.05 ± 0.16	12.33 ± 1.35	6.87 ± 0.11
9	Cry1Ac 27 mg/Kg, 24 h	1224.67 ± 58.93$^{\bullet}$	6.90 ± 0.09	9.63 ± 0.88	7.03 ± 0.10
10	Cry1Ac 136 mg/Kg, 24 h	955.83 ± 183.68	7.48 ± 0.10*†	15.35 ± 0.80*†	7.13 ± 0.09†
11	Cry1Ac 270 mg/Kg, 24 h	1213.50 ± 133.20	6.83 ± 0.16‡	10.78 ± 0.78‡	6.63 ± 0.08$^{\bullet\ddagger}$
12	Cry2Aa 27 mg/Kg, 24 h	1282.33 ± 110.10$^{\bullet}$	6.90 ± 0.16	10.24 ± 0.87	6.92 ± 0.14
13	Cry2Aa 136 mg/Kg, 24 h	1146.83 ± 62.99	6.97 ± 0.09	11.13 ± 0.94	6.9 ± 0.07
14	Cry2Aa 270 mg/Kg, 24 h	1057.50 ± 65.31	7.00 ± 0.10	11.78 ± 0.42	6.80 ± 0.13*
15	Cry1Aa+1Ab 270 mg/Kg, 24 h	1090.33 ± 69.82	6.80 ± 0.09	10.22 ± 0.61	6.83 ± 0.08^{a}
16	Cry1Aa+1Ac 270 mg/Kg, 24 h	1187.33 ± 156.68	6.77 ± 0.13	9.78 ± 1.04	6.80 ± 0.06^{a}
17	Cry1Aa+2Aa 270 mg/Kg, 24 h	942.67 ± 44.98^{**,a}	7.00 ± 0.11	11.78 ± 1.17	6.88 ± 0.07^{a}
18	Cry1Ab+1Ac 270 mg/Kg, 24 h	1171.67 ± 60.58	6.78 ± 0.16	11.16 ± 1.45	6.54 ± 0.10*
19	Cry1Ab+2Aa 270 mg/Kg, 24 h	1110.50 ± 69.8	6.57 ± 0.03^{*,b}	9.23 ± 0.38^{b,d}	6.57 ± 0.09
20	Cry1Ac+2Aa 270 mg/Kg, 24 h	1164.67 ± 105.33	7.00 ± 0.16	12.35 ± 1.34	6.75 ± 0.18
	P-values 24 hours	0.048	0.015	0.044	0.005
21	CP 27 mg/Kg, 72 h	1112.17 ± 178.48	6.58 ± 0.15	8.14 ± 1.19	6.78 ± 0.12
22	Cry1Aa 270 mg/Kg, 72 h	1152.33 ± 96.43	6.65 ± 0.10*	7.67 ± 0.43**	6.78 ± 0.15
23	Cry1Ab 270 mg/Kg, 72 h	928.67 ± 92.55	6.93 ± 0.22	11.83 ± 1.55	6.58 ± 0.16
24	Cry1Ac 270 mg/Kg, 72 h	1095.50 ± 96.69	7.13 ± 0.23	12.80 ± 1.39	7.00 ± 0.16
25	Cry2Aa 270 mg/Kg, 72 h	1196.00 ± 67.53	7.16 ± 0.21	12.28 ± 2.12	7.04 ± 0.18
	P-values 72 hours	0.454	0.077	0.010	0.316
26	CP 27 mg/Kg, 7 dias	1345.50 ± 158.38	6.67 ± 0.10	8.80 ± 0.81*	6.72 ± 0.09
27	Cry1Aa 270 mg/Kg, 7 dias	1002.50 ± 101.89^{a}	7.23 ± 0.18$^{a'}$	12.07 ± 0.96$^{a'}$	7.05 ± 0.22
28	Cry1Ab 270 mg/Kg, 7 dias	589.50 ± 92.13$^{b,b'}$	7.47 ± 0.34	15.03 ± 2.23*	6.93 ± 0.29
29	Cry1Ac 270 mg/Kg, 7 dias	1289.00 ± 97.91	6.72 ± 0.10	9.85 ± 0.44	6.68 ± 0.09
30	Cry2Aa 270 mg/Kg, 7 dias	1205.33 ± 88.27	6.92 ± 0.09	9.98 ± 0.82	6.95 ± 0.10
	P-values 7 days	0.000	0.006	0.034	0.388
	Total P-values	0.005	0.002	0.002	0.020

The data correspond to the means and to the standard error of mean (SEM). Platelet indices: platelet count (PLT), mean platelet volume (MPV), platelet large cell ratio (P-LCR) and platelet distribution width (PDW); fl=fentoliters. P-values were generated by the Kruskall-Wallis test. Asterisks indicate significant (p<0.05) and highly significant (p<0.01) differences detected by the Mann Whitney U test in the comparisons with the negative control. The following symbols represent significant differences in respect to: ●=CP 24 h; ○=CP 72 h; †=dose-effect for the dose of 27 mg/Kg; ‡=dose-effect for the dose of 136 mg/Kg; a=Cry1Aa 270 mg/Kg, 24 h; a'=Cry1Aa 270 mg/Kg, 72 h; b=Cry1Ab 270 mg/Kg, 24 h; b'=Cry1Ab 270 mg/Kg, 72 h; d=Cry2Aa 270 mg/Kg, 24 h

Table 3: Results of platelet gram of Swiss albino mice treated with *Bt* spore-crystal Cry1Aa, Cry1Ab, Cry1Ac and Cry2Aa administered orally (*per os*, p.o.) 24 h, 72 h and 7 days before euthanasia. Control mice received filtered water (negative control) or cyclophosphamide (CP – positive control) at 27 mg/Kg.

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compared to Cry1Ab+Cry2Aa (p=0.024). Similar results were observed for P-LCR, whose values were significantly higher for Cry1Ab (p=0.048) and Cry2Aa (p=0.024) in comparison to the binary combination Cry1Ab+Cry2Aa.

72 hours of exposure: Cry1Aa promoted significantly decreased MPV (p=0.026) and P-LCR (p=0.002) compared to negative control; for the latter, values presented for Cry1Aa were also lower in respect to those presented for treatments with Cry1Ab, Cry1Ac (p=0.010) and Cry2Aa (p=0.017).

7 days of exposure: Compared to negative control, CP caused a significant reduction in P-LCR (p=0.041), while Cry1Ab promoted a significant increase in its values (p=0.048). Cry1Ab also presented a reduced PLT number compared to Cry1Ac (p=0.001) and Cry2Aa (p=0.003), and increased MVP compared to Cry1Ac (p=0.036).

Micronucleus (MN) test (Table 4)

None of the tested Bt-toxins induced MN. Single doses of Cry1Aa 136 mg/Kg (p=0.041), Cry1Ab at 27 (p=0.009) and 136 mg/Kg (p=0.026), Cry1Ac 136 mg/Kg and Cry2Aa 27 mg/Kg (p=0.009 for both) significantly decreased cell proliferation in mice bone marrow (%PCE) compared with negative control. Binary combinations of Cry1Aa+Cry2Aa, Cry1Ab+Cry2Aa and Cry1Ac+Cry2Aa (p=0.026 for all) also decreased the %PCE index.

Colony Forming Units (CFU) (Table 5)

CFU varied with *Bt* strain. Thus, although animals received Cry1Aa, Cry1Ab, Cry1Ac or Cry2Aa at 27 mg/Kg, 136 mg/Kg or 270 mg/Kg, the number of viable *Bt* spore-crystals ingested by animals varied according to *Bt* strain.

Discussion

Since the late 1960's, spore-crystals from *B. thuringiensis* have been an important ally in combating insect pests in agriculture, against insect vectors of human diseases and in biological pest control, through their use as a foliar insecticide [1,31] and, more recently, by inserting δ-endotoxin genes in transgenic *Bt*-plants [5]. A major environmental advantage of microbial *Bt* preparations and of genetically engineered insect-resistant plants expressing genes encoding δ-endotoxins is the greater specificity of δ-endotoxins to target species, compared with use of many synthetic chemical insecticides. However, despite their more targeted specificity, there may still be insects and other non-target organisms potentially affected by the δ-endotoxins, and extended exposure might affect their populations [8]. Thus, the Brazilian Collegiate Board of Directors of the National Sanitary Surveillance Agency (ANVISA) Nº 194/02 advocates evaluations of toxicity and pathogenicity of microbiological control agents (MCAs), given that little is known about their toxicological potential [32].

Group	Treatment	MN-NCE	Polychromatic erythrocytes (PCE)	
			MN-PCE	Cellular proliferation index (%PCE)
1	Filtered water	2.00 ± 1.44	2.50 ± 1.43	52.61 ± 1.01
2	CP 27 mg/Kg	2.17 ± 0.48	3.50 ± 0.62	45.97 ± 1.21**
3	Cry1Aa 27 mg/Kg	0.40 ± 0.25•	3.00 ± 0.55	48.55 ± 1.54
4	Cry1Aa 136 mg/Kg	0.43 ± 0.20•	2.29 ± 0.36	49.28 ± 1.36*
5	Cry1Aa 270 mg/Kg	1.00 ± 0.37	3.67 ± 0.56	52.90 ± 1.57•
6	Cry1Ab 27 mg/Kg	1.67 ± 0.49	4.67 ± 0.88	45.92 ± 1.54**
7	Cry1Ab 136 mg/Kg	1.50 ± 0.43	3.33 ± 0.33	47.78 ± 1.28*
8	Cry1Ab 270 mg/Kg	1.00 ± 0.37	2.17 ± 0.95	52.91 ± 1.55•,†,‡
9	Cry1Ac 27 mg/Kg	1.33 ± 0.21	3.33 ± 0.76	49.49 ± 2.14
10	Cry1Ac 136 mg/Kg	2.50 ± 0.96	5.17 ± 0.98	44.92 ± 1.87**
11	Cry1Ac 270 mg/Kg	2.17 ± 0.40	4.00 ± 0.73	48.69 ± 1.73
12	Cry2Aa 27 mg/Kg	1.00 ± 0.52	0.83 ± 0.31•	47.06 ± 1.25**
13	Cry2Aa 136 mg/Kg	0.50 ± 0.22•	2.00 ± 0.45	47.93 ± 1.89
14	Cry2Aa 270 mg/Kg	0.17 ± 0.17•	0.50 ± 0.22•,‡	48.63 ± 1.39
15	Cry1Aa+1Ab 270 mg/Kg	0.50 ± 0.22•	0.67 ± 0.33	45.02 ± 2.27a
16	Cry1Aa+1Ac 270 mg/Kg	0.33 ± 0.21•,c	0.83 ± 0.31•,a,c	46.72 ± 2.10
17	Cry1Aa+2Aa 270 mg/Kg	0.17 ± 0.17•	0.83 ± 0.31•,a	48.88 ± 0.56*
18	Cry1Ab+1Ac 270 mg/Kg	0.50 ± 0.22•,c	1.50 ± 0.43•,c	51.46 ± 1.43•
19	Cry1Ab+2Aa 270 mg/Kg	1.00 ± 0.45	2.83 ± 0.48d	46.50 ± 2.50*
20	Cry1Ac+2Aa 270 mg/Kg	0.50 ± 0.34•,c	1.83 ± 0.48c,d	46.51 ± 2.08*
	P-values	0.001	0.000	0.026

The data correspond to the means and to the standard error of mean (SEM). MN-NCE and MN-PCE = micronucleus test results for normochromatic erythrocytes (NCE) and polychromatic erythrocytes (PCE), respectively. P-values were generated by the Kruskall-Wallis test. Asterisks indicate significant ($p<0.05$) and highly significant ($p<0.01$) differences detected by the Mann Whitney U test in the comparisons with the negative control. The following symbols represent significant differences in respect to: ●= CP; †=dose-effect for the dose of 27 mg/Kg; ‡=dose-effect for the dose of 136 mg/Kg; a=Cry1Aa 270 mg/Kg; c=Cry1Ac 27 mg/Kg; d=Cry2Aa 270 mg/Kg.

Table 4: Micronucleus evaluation of bone marrow cells from Swiss white mice treated with *Bt* spore-crystal Cry1Aa, Cry1Ab, Cry1Ac and Cry2Aa administered orally (*per os*, p.o.) 24 h before euthanasia. Control mice received filtered water (negative control) or cyclophosphamide (CP – positive control) at 27 mg/Kg.

	Cry1Aa	Cry1Ab	Cry1Ac	Cry2Aa
at 27 mg/Kg (cells/mL)	2×10^7	3×10^7	4×10^7	2×10^7
at 136 mg/Kg (cells/mL)	1×10^8	1×10^8	2×10^8	1×10^8
at 270 mg/Kg (cells/mL)	2×10^8	3×10^8	4×10^8	2×10^8

Table 5: Results of Colony Forming Units (CFU) for Cry1Aa, Cry1Ab, Cr1Ac and Cry2Aa and quantification of the viable *Bt* spore-crystals ingested by the animals at the different concentrations used (27 mg/Kg, 136 mg/Kg and 270 mg/Kg).

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It has been reported that Cry toxins exert their toxicity when activated at alkaline pH of the digestive tract of susceptible larvae, and, because the physiology of the mammalian digestive system does not allow their activation, and no known specific receptors in mammalian intestinal cells have been reported, the toxicity these MCAs to mammals would negligible [8,22,23]. However, our study demonstrated that *Bt* spore-crystals genetically modified to express individually Cry1Aa, Cry1Ab, Cry1Ac or Cry2A induced hematotoxicity, particularly to the erythroid lineage. This finding corroborates literature that demonstrated that alkali-solubilized *Bt* spore-crystals caused *in vitro* hemolysis in cell lines of rat, mouse, sheep, horse, and human erythrocytes and suggested that the plasma membrane of susceptible cells (erythrocytes, in this case) may be the primary target for these toxins [33].

It has been reported that strains of Cry toxins can be solubilized by alkaline buffer or a combination of alkaline buffer and reducing conditions, and that, although intravenous and subcutaneous administrations of 15-30 µg per gram body weight (0.4-0.9 mg per animal) of these alkali-soluble crystal proteins in Balb-C mice resulted in death, there were no toxic effects when orally administered [33]. In our study, lyophilized *Bt* spore-crystals resuspended in distilled water (and not in alkaline and/or reducing conditions) and orally administered at higher doses than the foregoing ones presented cytotoxic effects, particularly to the erythroid lineage of mice. Considering the increased risk of human and animal exposures to significant levels of these toxins, especially through diet, our results suggest that further evaluations are needed, with longer exposure of mammals to these diets, and involving clinical observations, before concluding that these microbiological control agents are safe to mammals. Cry1Ab induced microcytic hypochromic anemia in mice, even at the lowest tested dose of 27 mg/Kg, and this toxin has been detected in blood of non-pregnant women, pregnant women and their fetuses in Canada, supposedly exposed through diet [34]. These data, as well as increased bioavailability of these MCA in the environment, reinforce the need for more research, especially given that little is known about spore crystals' adverse effects on non-target species.

Because of its high mitotic index, hematopoietic tissue becomes the target of the adverse effects of many chemical substances entering the body. Some substances that act on bone marrow may have a selective effect, that is, they may be toxic to a given cell line [35]. Our study found selective cytotoxicity for the erythroid lineage and showed differences in the dose response curves of *Bt* spore-crystals genetically modified to express individually Cry1Aa, Cry1Ab, Cry1Ac or Cry2A. After 24 hours of exposure, Cry1Ac and Cry2Aa showed a non-monotonic dose response curve, where Cry2Aa presented a U-shaped dose response curve, with high responses at both low and high levels of contamination, while the Cry1Ac response curve was shaped like an inverted U with the greatest response in intermediate ranges. On the other hand, Cry1Ab seemed to have an effect similar to hormesis, where lower toxin doses (such as 27 and 136 mg/Kg) increased the body's tolerance for greater toxicity (such as 270 mg/Kg), while Cry1Aa seemed to maintain almost the same behavior, regardless of the dose. These differences among Cry toxins were already somewhat expected, since they have a defined spectrum of insecticidal activity, each of which is specific for some species belonging to a particular order of insects, according to their affinities for specific receptors on their target organisms [10,36]. Additionally, although results of CFU showed fewer viable spores/mL for Cry2Aa and Cry1Aa than for Cry1Ab and Cry1Ac, at 270 mg/mL the cytotoxic effects of Cry2Aa and Cry1Aa seemed higher compared to Cry1Ab and Cry1Ac.

Knowledge of the genotoxic potential of chemical industrial agents or those naturally present in the environment is essential information for regulatory agencies, regarding the establishment of risk for humans [37]. Since micronuclei in interphase cells result from chromosomal breaks or chromosomal lagging, the MN test is most widely used for the detection of clastogenic and aneugenic agents [29,38,39]. For the fish species *Danio rerio*, it has been reported that Cry1Aa significantly increased the frequency of micronuclei in peripheral blood of erythrocytes, while Cry1Ab, Cry1Ac, and Cry2A did not present genotoxicity [40]. In our study, all evaluated spore-crystals in single or binary combinations were hematotoxic and cytotoxic to the mice bone marrow, but not genotoxic, and this could indicate differences among vertebrates.

In the biological control of pests, a combination of different mixtures of *Bt* spore-crystal strains is also used. Also, a second generation of *Bt*-plants expressing two different Cry toxins has been developed to avoid insect resistance. There are several examples of transgenic plants with dual *Bt* gene insertion, such as Bollgard® II RR Flex cotton (Cry1Ac+Cry2Ab) and maize (Cry1Ac+Cry2Ab) [41]. Consequently, various interactive processes may occur, such as additivity, synergism, potentiation or antagonism [41,42]. However, to date, there are few studies on the cytotoxicity of conjugated *Bt* toxins for different organisms, and no studies were found in the literature evaluating the potential toxic and genotoxic effects of binary combinations of Cry toxins for non-target organisms. Our results demonstrated that the binary combinations of Cry1Ac+Cry2Aa and Cry1Ab+Cry2Aa were also hematotoxic to the erythroid lineage in particular. Furthermore, these binary combinations and also Cry1Aa+Cry2Aa were cytotoxic to the bone marrow cells in that they reduced the %PCE.

Literature has shown that *Bt* toxins are generally nontoxic and do not bioaccumulate in fatty tissue or persist in the environment [23], but our study demonstrated that all Cry at 270 mg/Kg showed a more pronounced cytotoxic effect on the erythroid lineage from 72 hours of exposure onwards, and that these effects were more pronounced after 7 days of exposure. After 7 days of exposure, Cry1Ab was toxic showing alterations in the hematological parameters of the exposed mice. It is well known that processes or substances that cause damage in the hematopoietic stem cell or bone marrow stroma of mice can also cause a decrease in WBC count [43]. Indeed, Cry1Ab significantly decreased MCH, MCV, and RDW and also decreased the number of PLT, which was non-significant in relation to the negative control but was substantially lower than the reference values for mice (900-1600 $\times 10^3$/µL) [43], as well as significantly increased P-LCR and decreased lymphocyte number. The profile of observed cytotoxic effects of these Cry toxins can be related to their high concentrations and the exposure time. Such exposures at these high concentrations are not commonly found in the environment.

In mice, the inflammatory response is often associated with both increased lymphocytes and neutrophils, and small changes in the number of neutrophils may be biologically significant and reflected in the total leukocyte count [43]. In this context, our study showed a higher inflammatory response for Cry1Aa 270 mg/Kg after 72 hours of exposure and for Cry1Ac and Cry2Aa at 270 mg/Kg after 7 days of exposure. Immunophenotypic changes have been demonstrated in the intestine and peripheral sites of young and old mice after ingestion of *Bt* corn MON810 encoding the active form of Cry1Ab [44], and intragastric administration of Cry1Ac prototoxin has induced secretion of mucosal antibodies in mice [45]. Our results corroborate these findings for Cry1Ac and also demonstrate leukogenic activity for other spore-crystals not yet reported in the literature.

Citation: Mezzomo BP, Miranda-Vilela AL, Freire IdS, Barbosa LCP, Portilho FA, et al. (2013) Hematotoxicity of *Bacillus thuringiensis* as Spore-crystal Strains Cry1Aa, Cry1Ab, Cry1Ac or Cry2Aa in Swiss Albino Mice. J Hematol Thromb Dis 1: 104. doi: 10.4172/2329-8790.1000104

In conclusion, results showed that the *Bt* spore-crystals genetically modified to express individually Cry1Aa, Cry1Ab, Cry1Ac or Cry2A can cause some hematological risks to vertebrates, increasing their toxic effects with long-term exposure. Taking into account the increased risk of human and animal exposures to significant levels of these toxins, especially through diet, our results suggest that further studies are required to clarify the mechanism involved in the hematotoxicity found in mice, and to establish the toxicological risks to non-target organisms, especially mammals, before concluding that these microbiological control agents are safe for mammals.

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REVIEW ARTICLE

An overview of the last 10 years of genetically engineered crop safety research

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Abstract

The technology to produce genetically engineered (GE) plants is celebrating its 30th anniversary and one of the major achievements has been the development of GE crops. The safety of GE crops is crucial for their adoption and has been the object of intense research work often ignored in the public debate. We have reviewed the scientific literature on GE crop safety during the last 10 years, built a classified and manageable list of scientific papers, and analyzed the distribution and composition of the published literature. We selected original research papers, reviews, relevant opinions and reports addressing all the major issues that emerged in the debate on GE crops, trying to catch the scientific consensus that has matured since GE plants became widely cultivated worldwide. The scientific research conducted so far has not detected any significant hazards directly connected with the use of GE crops; however, the debate is still intense. An improvement in the efficacy of scientific communication could have a significant impact on the future of agricultural GE. Our collection of scientific records is available to researchers, communicators and teachers at all levels to help create an informed, balanced public perception on the important issue of GE use in agriculture.

Keywords

Biodiversity, environment, feed, food, gene flow, –omics, substantial equivalence, traceability

History

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Introduction

Global food production must face several challenges such as climate change, population growth, and competition for arable lands. Healthy foods have to be produced with reduced environmental impact and with less input from non-renewable resources. Genetically engineered (GE) crops could be an important tool in this scenario, but their release into the environment and their use as food and feed has raised concerns, especially in the European Union (EU) that has adopted a more stringent regulatory framework compared to other countries (Jaffe, 2004).

The safety of GE crops is crucial for their adoption and has been the object of intense research work. The literature produced over the years on GE crop safety is large (31 848 records up to 2006; Vain, 2007) and it started to accumulate even before the introduction of the first GE crop in 1996. The dilution of research reports with a large number of commentary papers, their publication in journals with low impact factor and their multidisciplinary nature have been regarded as negative factors affecting the visibility of GE crop safety research (Vain, 2007). The EU recognized that the GE crop safety literature is still often ignored in the public debate even if a specific peer-reviewed journal (http://journals.cambridge.org/action/displayJournal?jid=ebs) and a publicly accessible database (http://bibliosafety.icgeb.org/) were created with the aim of improving visibility (European Commission, 2010).

We built a classified and manageable list of scientific papers on GE crop safety and analyzed the distribution and composition of the literature published from 2002 to October 2012. The online databases PubMed and ISI Web of Science were interrogated to retrieve the pertinent scientific records (Table S1 – Supplementary material). We selected original research papers, reviews, relevant opinions and reports addressing all the major issues that emerged in the debate on GE crops. The 1783 scientific records collected are provided in .xls and .ris file formats accessible through the common worksheet programs or reference manager software (Supplementary materials). They were classified under the scheme given in Table 1, according to the major issues emerging from the literature. Beyond a numerical analysis of the literature, we provide a short explanatory summary of each issue.

General literature (GE gen)

Here we group all the reviews and critical comments offering a broad view of the issues concerning the release of the GE crops into the environment and their use as food and feed, including the regulatory frameworks and risk assessment procedures.

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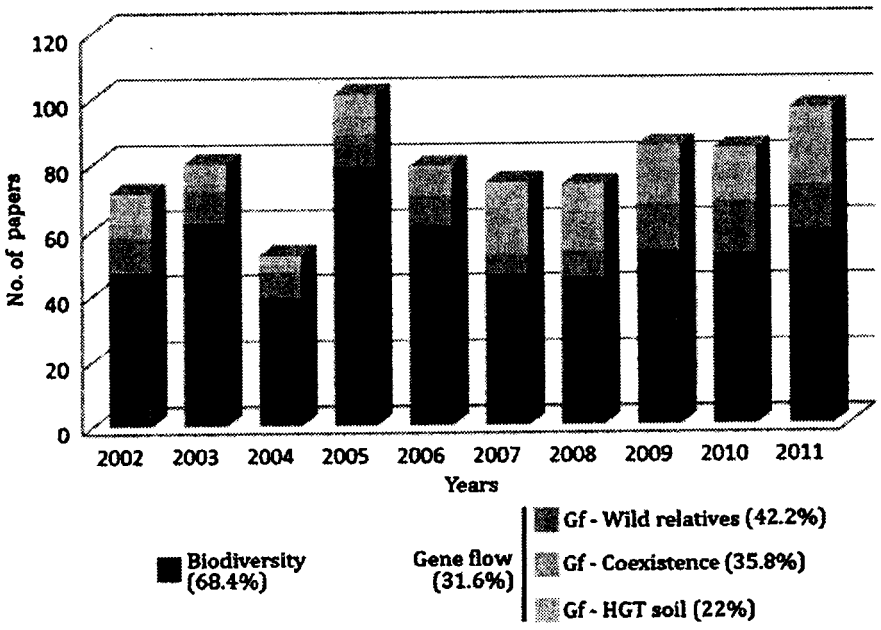
Table 1. Classification of 1783 scientific records on GE crop safety published between 2002 and 2012.

Topic	No. of papers	%*
General literature (GE gen)	166	9.3
Interaction of GE crops with the environment (GE env)	847	47.5
Biodiversity	579	32.5
Gene flow	268	15
Gf – Wild relatives	113	6.3
Gf – Coexistence	96	5.4
Gf – Horizontal gene transfer in soil	59	3.3
Interaction of GE crops with humans and animals (GE food&feed)	770	43.2
Substantial equivalence	46	2.6
Non-targeted approaches to equivalence assessment	107	6
GE food/feed consumption	312	17.5
Traceability	305	17.1

*Percentage of the total number of collected papers.

Figure 1. Main topics of the scientific papers belonging to the GE env group.



The weight of the **GE gen** section, in terms of number or records, is low in our database (9.3% – 166/1783) compared to **GE env** (47.5% – 847/1783) and **GE food&feed** (43.2% – 770/1783) (Table 1). The literature grouped in **GE gen** reflects the difference between the EU and the US regulatory frameworks: the former is based on the evaluation of the process by which the GE crop is obtained and the application of the precautionary principle, the latter is based on the evaluation of the product. The adoption of such different concepts resulted in the need for new legislation and new authorities in the EU, whereas in the US new regulations were integrated into the existing legislation and institutions (Jaffe, 2004).

Other countries have been inspired by these two systems in developing their own regulatory framework (Ramessar et al., 2008). As a result, the regulations on the release of GE crops into the environment and their use as food and feed are not uniform (Gómez-Galera et al., 2012; Jaffe, 2004; McHughen & Smyth, 2008; Ramessar et al., 2008). This lack of harmonization, and the frequent non-scientific disputes in the media that are not balanced by an effective communication from the scientific and academic world, greatly contribute to enhance the concerns on GE crops.

The EU funded more than 50 research programs in 2001–2010, for a total budget of 200 million euros, with the intent to gain new scientific evidence addressing the public concern on the safety of GE crops. A summary report of these programs highlighted that the use of biotechnology and of GE plants *per se* does not imply higher risks than classical breeding methods or production technologies (European Commission, 2010).

Interaction of GE crops with the environment (GEenv)

Biodiversity

Biodiversity preservation is unanimously considered a priority by the scientific community and society at large. This topic is predominant in **GE env** (68.4%) throughout the decade (Table 1; Figure 1). The literature is highly heterogeneous, since the potential impact of GE crops on biodiversity can be investigated at different levels (crop, farm and landscape) and different organisms or microorganisms (target and non-target) can be considered.

The GE crops commercialized so far are herbicide and/or pest resistant. Glyphosate tolerance obtained by

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expressing an *Agrobacterium tumefaciens* enolpyruvyl shikimate 3-phosphate synthase (EPSPS), and the production of insecticidal proteins derived from *Bacillus thuringiensis* (Bt), are by far the most widespread GE traits.

The literature considering the effects on biodiversity of non-target species (birds, snakes, non-target arthropods, soil macro and microfauna) is large and shows little or no evidence of the negative effects of GE crops (Carpenter, 2011 and references therein; Raven, 2010; Romeis et al., 2013). Two reviews about pest resistant GE crops published by Lövei et al. (2005, 2009) reported negative impacts on non-target arthropods; however, these reports have been criticized mainly for the statistical methods and the generalizations between crops expressing Bt proteins (commercialized), proteinase inhibitors (only a transgenic cotton line SGK321 present in the Chinese market) and lectins (not commercialized) (Gatehouse, 2011; Shelton et al., 2009). Negative impacts of Bt plants on non-target arthropods and soil microfauna have not been reported in recent papers (e.g. de Castro et al., 2012; Devos et al., 2012; Lu et al., 2012; Verbruggen et al., 2012 Wolfenbarger et al., 2011). Indeed, the positive impacts have been emphasised.

If we consider the effect of GE crops on the target species, weeds or pests, a reduction of biodiversity is obviously expected and necessary for the success of the crop. For instance, cases of area-wide pest suppression due to the adoption of Bt crops (where also the non-adopters of GE crops received beneficial effects), have been reported (Carpenter, 2011 and references therein). This is also the case of the UK Farm Scale Evaluations (FSE), a series of studies which highlighted that the adoption of a management system based on herbicide tolerant GE crops generally resulted in fewer weeds and weed seeds. These results have been used as proof of the negative environmental impact of herbicide tolerant crops, but indeed they demonstrate the effectiveness of such a management system (Carpenter, 2011 and references therein). On the other hand, higher reductions on biodiversity is generally expected with non-GE crops and herbicide/insecticide applications, because the chemicals used are often more toxic and persistent in the environment (Ammann, 2005).

Concerns have been raised about possible outbreak of resistant populations of target species due to the high selection pressures produced by the repetitive sowing of GE herbicide and pest resistant crops. Glyphosate resistant weeds have been reported (Shaner et al., 2012), as well as Bt resistant pests (Baxter et al., 2011; Gassman et al., 2011). Glyphosate tolerance appears more relevant because, while new Bt proteins are available which can be combined in strategies of stacking, or pyramiding, to reduce the risks of insect resistance (Sanahuja et al., 2011), it seems difficult to find herbicides equivalent to glyphosate in terms of efficacy and environmental profile; therefore, proper management of weed control is necessary (Shaner et al., 2012).

Gene flow

In an agricultural context, gene flow can be defined as the movement of genes, gametes, individuals or groups of individuals from one population to another, and occurs both spatially and temporally (Mallory-Smith & Sanchez Olguin, 2011). For instance, GE crop plants may be capable of surviving through seed or asexual propagules for years in the field, or they may be able to fertilize sexually compatible non-GE plants (non-GE crop or wild relative plants). The occurrence of gene flow may lead to the spread and persistence of transgenes into the environment or the market.

We have subdivided this topic into three subgroups: gene flow to wild relatives (Gf – Wild relatives), to other crops (Gf – Coexistence) or to microorganisms (Gf – Horizontal gene transfer in the soil). The literature on *Gene flow* makes up 31.6% of the **GEenv** literature and is clearly a ''hot topic'' because its share increased considerably after 2006 (Table 1; Figure 1).

Gf – Wild relatives

This topic represents 42.2% of the *Gene flow* literature (Table1; Figure 1). For estimating the gene flow to wild relatives, the knowledge of several factors is necessary: the reproductive biology of the GE crop, the presence or absence of sexually compatible wild relatives within the reach of GE pollen, and the reproductive biology and the fitness of any hybrid.

The formation of hybrids between GE crops and wild relatives is possible and documented (Londo et al., 2010; Mizuguti et al., 2010). Hybrid fitness determines the chance of transgene introgression, that is, permanent incorporation into the wild receiving population, which was reported in some cases (Reichman et al., 2006; Schoenenberger et al., 2006; Warwick et al., 2008). The risk of introgression should be evaluated case-by-case, considering the features of the transgene(s) incorporated into the GE crop.

The presence of spontaneous populations of GE canola with multiple herbicide resistance genes, probably due to multiple events of hybridization, has been reported (Schafer et al., 2011). Zapiola and Mallory-Smith (2012) recently described a new herbicide tolerant intergeneric hybrid of transgenic creeping bentgrass. Other cases have been reviewed (Chandler & Dunwell, 2008). Pest-resistant GE crops (i.e. Bt crops) may pose more risks than herbicide-resistant crops, because the introgression of a pest resistance transgene may confer fitness advantages to wild plants. Pest resistant wild plant populations may in turn exert selective pressure on the pest populations even in the absence of transgenic crops.

Strategies to mitigate the effect of the transgene(s) in pre- and post -hybridization phases have been proposed (e.g. male sterility, delayed flowering, genes that reduce fitness). However, none of them can be considered completely effective for transgene containment and complete segregation of GE crops is not possible. In any case, there is no evidence of negative effects of transgene introgression so far (Kwit et al., 2011).

It should be kept in mind that the gene flow between cultivated and wild species and its impact on biodiversity is an issue that exists independently of GE crops. The literature is rich in examples of natural invasive hybrids, disappearance of local genotypes (genetic swamping) and resistance to herbicides appearing in wild populations due to natural mutation (Kwit et al., 2011).

Gf – Coexistence

Gene flow from a GE to a non-GE crop can lead to an unwanted presence of the transgene in non-GE products. This issue involves not only the movement of pollen, but also the seeds that could remain in the field and give rise to volunteers, and the mechanical admixture of materials occurring during harvest, transportation and storage. The establishment of populations becoming partially wild (ferals) functioning as a natural reservoir of the transgene must also be considered, as well as the survival chances of the GE crops in the wild.

The coexistence issue goes beyond the matter of gene flow and involves several social and economic aspects, such as the manageability of complex agricultural scenarios where different agricultural systems (organic, conventional and biotech) coexist and a full traceability system is in force.

The collected records on coexistence account for 35.8% of the *Gene flow* literature and their number increased significantly after 2006 (Table 1; Figure 1). Even in the US, the coexistence issue is becoming actively discussed (http://www.gmo-compass.org/eng/news/548.docu.html).

Strategies of coexistence have been investigated for several species, such as maize (Devos et al., 2008; Langhof et al., 2010; Rühl et al., 2011), canola (Colbach, 2008; Gruber et al., 2005), soybean (Gryson et al., 2009), flax (Jhala et al., 2011), wheat (Foetzki et al., 2012), potato, cotton and sugar beet (European Commission, 2006). Maize has been the most intensively studied crop, followed by canola and wheat. Isolation distances, harvesting and post-harvesting practices have been proposed in order to avoid unwanted mixing of GE and non-GE-crop.

The feasibility of a coexistence plan is not only evaluated from a scientific point of view but also considering the extra economic costs due to the containment practices; such extra costs must find compensation in extra income from GE crops (Demont & Devos, 2008). In the EU, the scenario on coexistence is very poor currently, considering that only three GE crops are authorized for cultivation (MON 810 and T25 maize and "Amflora" potato), with only MON810 actually commercialized, and Spain accounting for 87% of the entire cultivated surface with GE crops (James, 2011).

Gf – Horizontal gene transfer in soil

Soil microorganisms may uptake the transgene(s) present into the GE crop. In fact, bacteria are naturally capable of acquiring genetic material from other organisms through horizontal gene transfer (HGT). To obtain a GE plant it can be necessary to introduce a gene that makes it possible to select the transgenic cells in tissue culture, by giving them an advantage over the non-transgenic cells. This is frequently achieved with bacterial antibiotic resistance genes that play the role of selectable marker genes (SMGs, recently reviewed by Rosellini, 2012). SMG presence in GE crops is not necessary in the field, and it has raised concerns about the spread of antibiotic resistance genes into the environment and their consumption as food or feed (see below).

The transfer of these genes to bacteria and the possible outbreak of "super pathogenic bacteria" resistant to antibiotics has been a matter of detailed investigation by the scientific community. The number of publications on this topic accounts for 22% of the *Gene flow* literature, with a stable presence in recent years (Table 1; Figure 1).

The results obtained so far clearly indicate that soil bacteria can uptake exogenous DNA at very low frequency (10^{-4} to 10^{-8}) in laboratory experiments (Ceccherini et al., 2003; de Vries et al., 2003), whereas experiments in the field did not show any evidence of HGT (Badosa et al., 2004; Demanèche et al., 2008, 2011; Ma et al., 2011). Moreover, in the unlikely event that soil bacteria acquired the resistance to an antibiotic among those currently used in the laboratory to select GE plants, this would not affect the population of natural antibiotic resistant bacteria already present in the soil (D'Costa, 2006; Forsberg et al., 2012) or imply any additional risk for human and animal health.

The substitution of antibiotic SMGs with plant-derived genes (Rosellini, 2011, 2012), their elimination (Ferradini et al., 2011 and references therein) and in general the elimination of any unwanted DNA sequence in the final GE crop is recommended (EFSA, 2011), as proposed with new approaches to plant genetic engineering such as the so-called intragenic (Nielsen, 2003; Rommens, 2004) or cisgenic (Jacobsen & Schouten, 2007) techniques.

Interaction of GE crops with humans and animals (GE food&feed)

Substantial equivalence

One of the crucial aspects of the risk assessment procedure for a GE crop is to verify if the insertion and/or the expression of the transgene produces alterations in the host organism. The concept of substantial equivalence implies that the GE crop be compared with an isogenic counterpart, that is, the same genotype without the transgene(s).

The demonstration of substantial equivalence is a two-step procedure. First, the GE crop is assessed for agronomic, morphological and chemical characteristics, such as macro- and micro-nutrients, anti-nutrients and toxic molecules. The results of this analysis will provide information on the necessity for further analysis of the nutritive value. Any difference which falls within the range of the normal variability for the crop is considered safe (Colquhoun et al., 2006; EFSA, 2011). This methodology has been agreed internationally (Codex, FAO, OECD, WHO) and involves the quantification of selected molecules, in a so-called "targeted approach" (Kok & Kuiper, 2003). If compositional differences are detected, then they have to be assessed with respect to their safety (Ramessar et al., 2007; EFSA, 2011).

The principle of substantial equivalence has been used for risk assessment of the GE crops commercialized so far (Kier & Petrick, 2008; König et al., 2004) and the results support the fact that these crops are equivalent to their non-transgenic counterparts (Parrot et al., 2010).

Concerns have been expressed about the efficacy of the method for detecting unintended effects. Field comparisons in multiple locations have been recommended in order to minimize the differences due to the environmental effects and large data collections have been created (www.cropcomposition.org).

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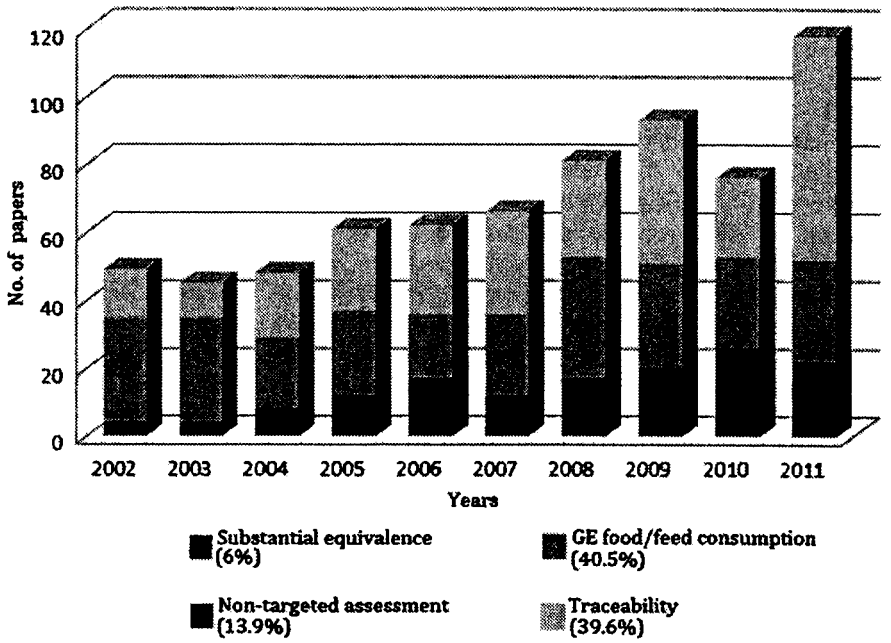
Figure 2. Main topics of the scientific papers belonging to the GE food&feed group.



It is noteworthy that substantial equivalence represents an important common ground of the process-based and product-based regulatory frameworks. This clearly indicates a large consensus amongst scientists worldwide on GE crop evaluation (Kok et al., 2008). Substantial equivalence accounts for 6% of the scientific records collected in **GE food&feed** (Table 1; Figure 2). The literature is composed mainly by the publications produced by the companies that developed the GM cultivars, as part of the authorization process for commercialization. Public availability of the data on which these studies are based should be guaranteed.

Nontargeted approaches to equivalence assessment

The targeted approach to substantial equivalence assessment has an obvious limitation in the number of compounds that are analyzed. On the contrary, the so-called ''–omic'' approaches (transcriptomics, proteomics, metabolomics) can analyze a larger number of molecules (Kier & Petrick, 2008). Several GE crops were compared to their isogenic counterparts using –omic approaches and in some cases differences were observed. However, the interpretation of these results is difficult due to the non-homogeneity of the experimental designs. Moreover, the differences emerging from the –omic analyses have to be cleaned up from the environmental effects and their biological relevance weighted in terms of food and feed safety (Ricroch et al., 2011 and references therein).

It appears that the application of the –omics methods as standard procedure in the risk assessment of GE crop does not actually provide manageable information, and needs further development and validation. In this scenario, the substantial equivalence concept remains a robust and safe reference to determine the presence of unintended effects (European Commission, 2010). The weight of the nontargeted assessment topic increased significantly over the years, especially in 2009–2011 leading to a significant number of publications (13.9%) (Table 1; Figure 2).

GE food/feed consumption

The scientific records grouped under this topic are numerous and constitute 40.5% of the **GE food&feed** literature, clearly indicating the importance of the human health issues. The distribution over the year is uniform, but a peak was observed in 2008, probably due to the scientific fervors that followed the publication of experimental studies conducted by the private companies after 2006 (Table 1; Figure 2). According to the literature, the concerns about GE food/feed consumption that emerge from the scientific and social debates can be summarized as follows: safety of the inserted transgenic DNA and the transcribed RNA, safety of the protein(s) encoded by the transgene(s) and safety of the intended and unintended change of crop composition (Dona & Arvanitoyannis, 2009; Parrot et al., 2010).

Safety of the inserted transgenic DNA and the transcribed RNA

DNA. It is estimated that, with a normal diet, humans consume between 0.1 and 1 g of DNA/day from different sources (e.g. meat, vegetables) (Parrot et al., 2010). This DNA is partly digested, but it can also stimulate the immune-system or promote bacterial biofilm formation (Rizzi et al., 2012). The DNA sequences that drive the expression of the transgenes in the plant cell are generally derived from viruses or bacteria. Concerns have been expressed on the possibility that the transgenic DNA may resist the digestion process, leading to HGT to bacteria living in the gastrointestinal (GI) tract, or translocation and accumulation into the human body and food products from livestock animals. Some considerations can help to put this issue in context:

(a) transgenic DNA is enormously diluted by the total amount of ingested DNA (from 0.00006% to 0.00009%) and is digested like any other DNA (Parrot et al., 2010). In addition, food processing (e.g. baking, frying, boiling)

usually results in DNA degradation (Gryson, 2010; Rizzi et al., 2012) further reducing the amount of intact DNA;

(b) HGT of transgenic DNA to GI bacteria of human and animals is estimated to be an extremely rare event, as confirmed by all the experiments conducted so far (Rizzi et al., 2012). In the unlikely case that this event occurs, the worst scenario is characterized by the HGT of antibiotic resistance genes to GI bacteria, making them resistant to clinical therapies. However, the antibiotic resistance genes found into GE crops today do not present any significant risk to human or animal health (Ramessar et al., 2007), and they are already naturally present into the environment and/or the human/animal GI (EFSA, 2011; Wilcks & Jacobsen, 2010).

(c) DNA fragments can be transferred across the GI barrier. This natural phenomenon has been demonstrated only for high-copy-number genes that have been detected in internal organs, tissues and blood of different animals and even in cow milk (Parrot et al., 2010; Rizzi et al., 2012; van de Eede et al., 2004 and references therein). In humans, the transfer through the GI tract of a high-copy-number gene from rabbit meat has been reported (Forsman et al., 2003).

(d) Transgenic DNA transfer through the GI tract has been reported in the literature in pig, lamb and rainbow trout (Chainark et al., 2006, 2008; Mazza et al., 2005; Sharma et al., 2006;), but in micro quantities and in the case of pigs and lambs with questionable reproducibility due to possible cross contamination (Walsh et al., 2011).

(e) In most studies conducted so far, no fragments of transgenic DNA were detected in any animal-derived products (ILSI, 2008). Only in one case, the presence of transgenic DNA in both "organic" and "conventional" cattle milk has been reported (Agodi et al., 2006).

(f) No evidence has been obtained to date that DNA absorbed through the GI tract can be integrated into the cells of the host organism and lead to a germ line transfer.

It can be concluded that transgenic DNA does not differ intrinsically or physically from any other DNA already present in foods and that the ingestion of transgenic DNA does not imply higher risks than ingestion of any other type of DNA (European Commission, 2010).

RNA. Along with the DNA also the corresponding transcribed RNAs are ingested and in general the content of DNA and RNA in foods are roughly comparable (Parrot et al., 2010). In the light of recent scientific evidence (Zhang et al., 2012a discussed below) concerns have been expressed about the potential effects that certain types of RNA (small double-strand RNAs, dsRNAs) introduced in some GE crops (e.g. virus resistant, altered oil composition) could have on human/animal health.

The function of such dsRNAs is not to be translated into proteins but to mediate gene regulation through a mechanism termed RNA interference (RNAi). The general mechanism of RNAi is conserved across eukaryotes and is triggered by different types of dsRNAs including small interfering RNA (siRNAs) and microRNAs (miRNAs) (Melnyk et al., 2011).

Recently, Zhang et al., (2012a) reported the first evidence of transfer, through the mouse GI tract, of a food-derived exogenous miRNA (MIR168a) naturally abundant in rice and previously detected also in human blood. This study highlights the unexpected resistance of the rice MIR168a to heat treatment during cooking and to digestion during the transit through the GI tract in the mouse. Moreover, the authors showed significant activity of the MIR168a on the RNAi-mediated regulation of a protein involved in the removal of low-density lipoprotein (LDL) in liver cells (Zhang et al., 2012a). This evidence is still the object of debate at the scientific level and a summary of the major issues are reported here:

(a) miRNAs are naturally present in both animal and plant derived foods/feeds and with a reported similarity to human genes (Ivashuta et al., 2009; Petrick et al., 2013);

(b) Petrick et al. (2013) pointed out that previous studies on feeding rats with rice (Zhou et al., 2011, 2012) failed to provide evidence on any alteration on LDL. However, such studies may be difficult to compare as they were conducted on another species of rodent and with different methodological approaches (e.g. different fasting of the animals and composition of the diet);

(c) although the systemic transmission of dsRNAs has been demonstrated in plants, worms and insects, such transport in mammals is still largely unknown (Melnyk et al., 2011). In humans, the presence of endogenous miRNAs has been documented in microvesicles circulating in the bloodstream and their role in intercellular communication is currently under investigation (Mittelbrunn & Sánchez-Madrid, 2012 and references therein);

(d) the results presented by Zhang et al. (2012a) are not in agreement with that documented in numerous clinical trials involving oral delivery of small RNA molecules. The stability of the dsRNAs in the GI tract and an efficient absorption through the mucosa in order to reach the active concentration of the molecule in the bloodstream, are still the limiting factors in this therapeutic approach (Petrick et al., 2013 and references therein);

(e) some miRNAs are active even at low concentrations and plant miRNAs seem to differ structurally from mammalian miRNAs (Yu et al., 2005; Zhang et al. 2012a; http://www.the-scientist.com/?articles.view/articleNo/31975/title/Plant-RNA-Paper-Questioned/);

(f) interestingly, Zhang et al. (2012b) detected the MIR168a sequence as predominant or sole plant miRNA in public animal small RNA datasets including insects. The authors point out that this may be an artifact due to the sequencing methodology employed (i.e. cross-contamination of the multiplexed libraries).

It can be concluded, that the RNA in general has the same "history of safe use" as DNA, since it is a normal component of the diet (Parrot et al., 2010). However, further investigations are necessary to clarify whether the evidence about the MIR168a is due to its unique properties or such conclusions can also be extended to other dsRNAs molecules contained in food/feed.

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Safety of the proteins encoded by the transgenes

The expression of the introduced gene(s) leads to biosynthesis of one or more proteins. The ingestion of transgenic proteins has posed some questions about their possible toxic or allergenic effects in humans and animals. The safety of each transgenic protein is evaluated by means of the following analyses:

- bioinformatic analysis to assess the similarity with known allergens, toxic proteins and bioactive peptides;
- functional stability to pH and temperature;
- *in vitro* digestibility using simulate mammalian gastric fluid and simulated mammalian intestinal fluid, following the principle that a digested protein is less likely to be allergenic and absorbed in a biologically active form;
- protein expression level and dietary uptake, to estimate exposure of humans or animals to the protein;
- single dose (acute) toxicity testing and repeated dose (sub-chronic) toxicity testing in rodents using the purified transgenic protein, to predict *in vivo* possible toxic outcome in humans (Delaney et al., 2008; EFSA, 2008).

The results of these analyses are usually part of the documentation that GE crops developers submit to the competent authorities during the approval phase (risk assessment) that precede the commercialization of a GE crop. These data are not always made accessible by the companies or the competent authorities or published on peer-reviewed journals (Jaffe, 2004). However, as indicated by the significant increment of the publications after 2006, it seems that the GE crop developers acknowledged the necessity of an improved transparency (Domingo & Bordonaba, 2011). The experimental data collected so far on authorized GE crops can be summarized as follows:

(a) there is no scientific evidence of toxic or allergenic effects;
(b) some concern has been raised against GE corn MON 810, MON863 and NK603 (de Vendômois et al., 2009; Séralini et al., 2007, 2012), but these experimental results have been deemed of no significance (EFSA 2007, 2012; Houllier, 2012; Parrot & Chassy, 2009);
(c) only two cases are known about the potential allergenicity of transgenic proteins, the verified case of the brazil-nut storage protein in soybean, which has not been marketed (Nordlee et al., 1996) and the not verified case of maize Starlink (Siruguri et al., 2004);
(d) during the digestion process the proteins generally undergo degradation that leads to the loss of activity (Delaney et al., 2008);
(e) even though there are examples of some ingested proteins that are absorbed in minute quantities in an essentially intact form (e.g. ovalbumin, ovomucoid, β-lactoglobulin) (Kier & Petrick, 2008) or proteins that are hydrolyzed into smaller absorbed bioactive peptides (Udenigwe & Aluko, 2012), the consumption of transgenic proteins contained in the authorized GE crop does not result in any detectable systemic uptake (Kier & Petrick, 2008) and transgenic proteins are usually rapidly degraded and not detectable in animal derived products (e.g. milk, meat, eggs) (Ramessar et al., 2007);
(f) pre-screening of transgenic proteins through bioinformatic analyses contributes to avoid the introduction of potentially toxic, allergenic or bioactive proteins into food and feed crops (Delaney et al., 2008; Gibson, 2006; Ladics et al., 2011);
(g) the application of the concept of ''history of safe use'' to the choice the transgene donor organisms may increase intrinsic safety and simplify safety assessment procedures.

Safety of the intended and unintended changes of crop composition

Safety of the introduced change in the GE crop is usually evaluated during the determination of compositional equivalence (Section ''Substantial equivalence''). However, on a case-by-case basis, additional analyses can be requested, such as that of processed foods or feeds, nutritional equivalence and 90-day rodent feeding tests with whole GE food or feed (EFSA, 2008, 2011).

A useful distinction can be introduced here between GE crops modified for input traits (e.g. herbicide or insect resistance) and GE crops with enhanced nutritional characteristics (e.g. increased vitamin content). For the former, the experience suggests that, once the compositional equivalence has been verified, little can be added by the other types of analysis, and nutritional equivalence can be assumed (EFSA, 2011).

On the contrary, for GE crops with improved nutritional characteristics, the nutritional equivalence cannot be assumed, and a nutritional animal feeding test using rapidly growing animals (e.g. broilers) should be conducted to demonstrate the intended nutritional effect. The high sensitivity of rapidly growing animals to toxic compounds may also help to detect unintended effects. The 90-day rodent feeding test is generally performed when the composition is modified substantially or if there are indications of potential unintended effects.

Only GE crops modified for agronomic traits have been authorized for commercialization so far, with the only exception of the ''Amflora'' potato (event EH92-527-1), intended for industrial purpose but authorized also for feed and nonintended consumption (http://ec.europa.eu/food/dyna/gm_register/gm_register_auth.cfm?pr_id=39).

It is noteworthy that, at the moment, the route to the authorization of GE crops intended only for industrial purposes is not fully clarified by the legislation. However, the results of animal tests are routinely presented to the European safety assessment authorities, even if not explicitly required (http://www.gmo-compass.org/eng/safety/human_health/41.evaluation_safety_gm_food_major_undertaking.html).

Recently, Podevin & Jardin (2012) pointed out that the viral promoter P35S, isolated from the cauliflower mosaic virus (CaMV) and used in several GE crops to achieve strong and constitutive expression of the transgene/s, partially overlaps with the CaMV viral gene VI. In some long variants of the P35S promoter this could potentially lead to the production of a residual viral protein. The use of the short version of the promoter is therefore recommended, even if the

bioinformatics analysis of the viral protein has not revealed any relevant similarity with known allergens (Podevin & Jardin, 2012).

An issue emerged about whether the combination of more GE traits in a single crop (GE stacks) may introduce changes that require additional safety assessment. Once safety of the single traits has been established independently, their combination should be evaluated in terms of stability, expression and possible interactions (EFSA, 2011). Weber et al. (2012) pointed out that GE stacks do not impose any additional risks in terms of transgene stability and expression, whereas attention should be focused only on the possible interactions between different traits.

Traceability

This is clearly a "hot topic" in **GE food&feed** (39.6%) (Table 1), with the publication rate after 2005 being high and constant (Figure 2). Traceability is defined in the EU General Food Law Regulation 178/2002/EC, inspired to the ISO standard, as the "ability to trace and follow food, feed, food producing animals and other substances intended to, or expected to, be incorporated into food or feed, through all stages of production, processing and distribution".

Traceability is a concept already widely applied to non-GE food/feed and it is not connected with their safety (Davison & Bertheau, 2007). It may include mandatory or voluntary labeling for the foods or feeds that contain or consist of GE crops or derived products. Labeling implies the definition of a threshold value, above which the food/feed is labeled according to the regulations in force.

The EU developed the most stringent regulatory framework for traceability of GE crops food/feed and derived products in the world. They have adopted mandatory labeling for unintentional presence of GE material in food or feed, with the lowest threshold value (0.9% based on the number of haploid genomes) compared to other countries (Davison & Bertheau, 2007; Ramessar et al., 2008). Labeling requires the detection and quantification of the GE food/feed or derived product in the tested food/feed or seeds or any other product when applicable. The scientific literature compiled about traceability largely deals with the following issues:

(a) sampling procedures – there are no universally acknowledged sampling procedures (Davison & Bertheau, 2007); this has been the object of a EU funded research programme (Paoletti et al., 2006);
(b) detection method – a large consensus has been established on qPCR (real-time quantitative PCR) -based methodologies that allows detection and quantification at the same time. Other experimental strategies and analytical methods have been proposed (e.g. microarray, Luminex XMAP), but they need further evaluation (Querci et al., 2010);
(c) definition of reference systems – the measurement unit of the GE product concentration depends on the unit used for the certified reference material (CRM) chosen for the analysis. At the moment, in the EU, mass fraction percentages are used for the CRMs, whereas a later recommendation from the EU suggested to use the "copy number of transgenic DNA in relation to haploid genomes", the unit of the legal threshold, so the development of suitable CRMs is necessary (Trapmann et al., 2009);
(d) detection of transgenes in mixtures composed by different ingredients, stacked transgenes and unauthorized events: all these issues require specific approaches and strategies have been proposed. The detection of the unauthorized events is very complex, because it could involve an already known transgene that did not receive authorization or a totally unknown GE event. Unfortunately, asynchronous authorization of GE crops or derived products in different countries does not improve this scenario: a higher degree of international harmonization would be beneficial (Holst-Jensen et al., 2012).

Conclusions

The technology to produce GE plants is celebrating its 30th anniversary. It has brought about a dramatic increase in scientific production over the years leading to high impact findings either in basic research (such as RNAi-mediated gene silencing) and applied research (GE crops), but the adoption of GE plants in the agricultural system has raised issues about environmental and food/feed safety.

We have reviewed the scientific literature on GE crop safety for the last 10 years that catches the scientific consensus matured since GE plants became widely cultivated worldwide, and we can conclude that the scientific research conducted so far has not detected any significant hazard directly connected with the use of GM crops. The analysis of the record list shows that the Biodiversity topic dominated, followed by Traceability and GE food/feed consumption, which contributed equally in terms of the number of records (Table 1; Figure 3).

It is noteworthy that the number of papers on Traceability has increased over the years, overcoming those on Biodiversity in 2011, clearly indicating an increasing demand for methods and protocols for transgene detection (Figure 3). The Gene flow issue also received increasing attention by the scientific community, as a response to the demands of the consumers connected with the coexistence of different productive systems (Figure 3).

It appears that knowledge on Gene flow and GE food/feed consumption would have benefited from a higher number of publications considering their high impact on both environmental and food/feed risk assessment. The difficulties of experimental design and, in the case of Gene flow, the public opposition to field trials, may have discouraged researchers, at least in the EU.

The literature about Biodiversity and the GE food/feed consumption has sometimes resulted in animated debate regarding the suitability of the experimental designs, the choice of the statistical methods or the public accessibility of data. Such debate, even if positive and part of the natural process of review by the scientific community, has frequently been distorted by the media and often used politically and inappropriately in anti-GE crops campaigns. In this regard, Houllier (2012) pointed out that, when

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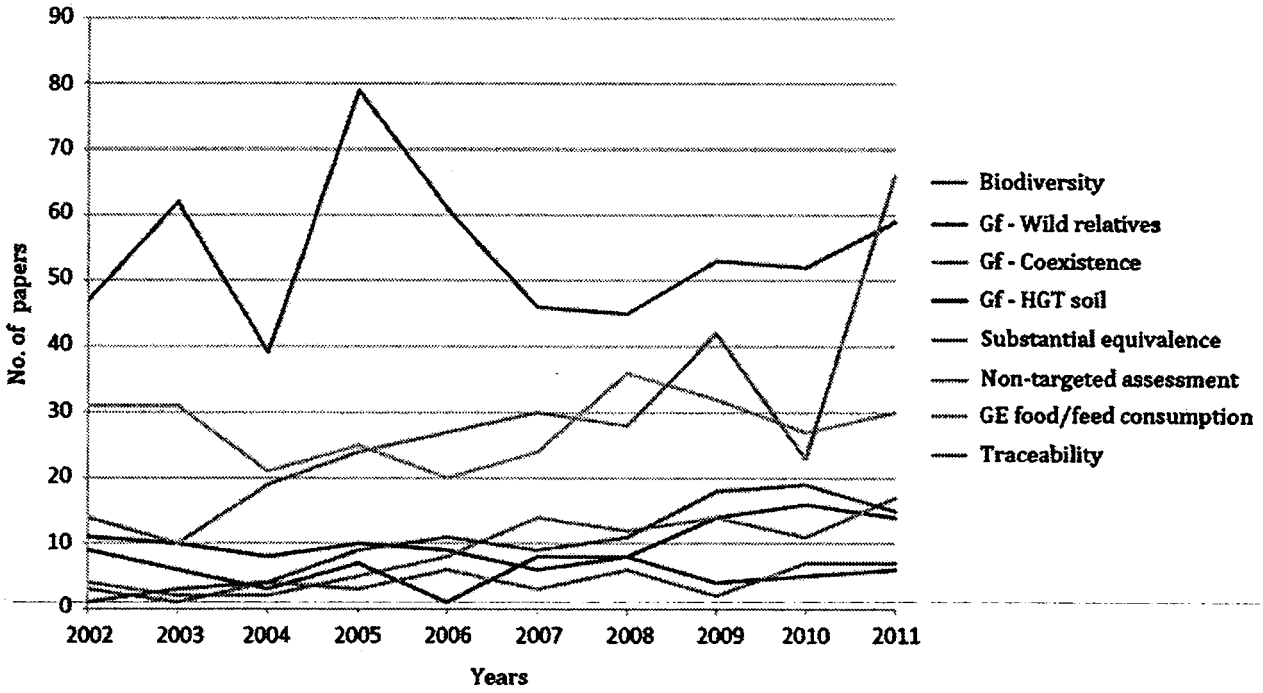

Figure 3. Distribution of the collected scientific papers. Records classified under the *General literature* are not shown.

dealing with ''hot issues'', researchers should take special care in following rigorous scientific standards, avoiding the publication of data not sufficiently peer reviewed by the scientific community.

It is interesting to note that the recent increase of scientific publications about Traceability and Non-targeted assessment (Figure 3) indicates considerable attention to the detection systems and the search for new safety evidence about a relatively low number of new approved GE crops. This likely reflects the consolidation of a situation in which the EU plays mainly the role of the importer of GE crop products from other countries, and enforces a stringent regulatory system.

In the EU, the regulatory burdens for GE crop approval are extremely heavy (Kalaitzandonakes et al., 2007), *de facto* excluding the public sector and minor crops from the development of GE technology. As a result, the number of experimental releases of GE crops is rapidly decreasing (Löchte, 2012) and even large companies are abandoning GE (Dixelius et al., 2012; Laursen, 2012). This scenario is the result of the interaction of complex sociological and psychological factors, risk/benefit ratios, political aspects and an unbalanced scientific communication.

All these factors have to be considered globally and taken into account in a constructive debate on whether the GE crops represent a strategic resource for the future. An improvement in the efficacy of the scientific communication to stakeholders, as clearly demonstrated in the case of the recent case of GE wheat field trials in the UK (Löchte, 2012), could have a significant impact on the future of agricultural GE.

We believe that genetic engineering and GE crops should be considered important options in the efforts toward sustainable agricultural production. Our collection of scientific records is available to researchers, communicators and teachers at all levels to help create an informed and balanced public perception on the hot issue of GE use in agriculture.

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Supplementary material available online

Supplementary Table S1

Chem. Res. Toxicol. **XXXX,** *xxx,* 000

Glyphosate-Based Herbicides Produce Teratogenic Effects on Vertebrates by Impairing Retinoic Acid Signaling

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The broad spectrum herbicide glyphosate is widely used in agriculture worldwide. There has been ongoing controversy regarding the possible adverse effects of glyphosate on the environment and on human health. Reports of neural defects and craniofacial malformations from regions where glyphosate-based herbicides (GBH) are used led us to undertake an embryological approach to explore the effects of low doses of glyphosate in development. *Xenopus laevis* embryos were incubated with 1/5000 dilutions of a commercial GBH. The treated embryos were highly abnormal with marked alterations in cephalic and neural crest development and shortening of the anterior−posterior (A-P) axis. Alterations on neural crest markers were later correlated with deformities in the cranial cartilages at tadpole stages. Embryos injected with pure glyphosate showed very similar phenotypes. Moreover, GBH produced similar effects in chicken embryos, showing a gradual loss of rhombomere domains, reduction of the optic vesicles, and microcephaly. This suggests that glyphosate itself was responsible for the phenotypes observed, rather than a surfactant or other component of the commercial formulation. A reporter gene assay revealed that GBH treatment increased endogenous retinoic acid (RA) activity in *Xenopus* embryos and cotreatment with a RA antagonist rescued the teratogenic effects of the GBH. Therefore, we conclude that the phenotypes produced by GBH are mainly a consequence of the increase of endogenous retinoid activity. This is consistent with the decrease of Sonic hedgehog (Shh) signaling from the embryonic dorsal midline, with the inhibition of *otx2* expression and with the disruption of cephalic neural crest development. The direct effect of glyphosate on early mechanisms of morphogenesis in vertebrate embryos opens concerns about the clinical findings from human offspring in populations exposed to GBH in agricultural fields.

Introduction

The broad-spectrum glyphosate based herbicides (GBHs) are widely used in agricultural practice, particularly in association with genetically modified organisms (GMO) engineered to be glyphosate resistant such as soy crops. Considering the wide use of GBH/GMO agriculture, studies of the possible impacts of GBH on environmental and human health are timely and important. Given the intensive use of this technological package in South America, studies of the possible impacts on environment and human health are absolutely necessary, together with adequate epidemiological studies. The need for information about the developmental impact of GBH is reinforced by a variety of adverse health effects on people living in areas where GBH is extensively used, particularly since there is a paucity of data regarding chronic exposure to sublethal doses during embryonic development.

It is important to note that the bulk of the data provided during the evaluation stages of GBH/GMO safety were provided by the industry. Given the recent history of the endocrine disruptor field with low dose effects observed in numerous academic laboratories but not in industry-funded studies (*1*, *2*), it is clear that a reasonable corpus of independent studies is necessary to fully evaluate the effects of agrochemicals on human health. This is particularly important when significant economic interests are concerned.

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There is growing evidence raising concerns about the effects of GBH on people living in areas where herbicides are intensively used. Women exposed during pregnancy to herbicides delivered offspring with congenital malformations, including microcephaly, anencephaly, and cranial malformations (*3*).

Relevant contributions to the subject were made by Seralini's group, among others (*4*). They showed that a GBH acts as an endocrine disruptor in cultures of JEG3 placental cells, decreasing the mRNA levels of the enzyme CYP19 (an essential component of cytochrome p450 aromatase) and inhibiting its activity. CYP19 is responsible for the irreversible conversion of androgens into estrogens. The GBH Roundup is able to disrupt aromatase activity. Importantly, the active principle glyphosate interacts with the active site of the purified enzyme and its effects in cell cultures, and microsomes are facilitated by other components in the Roundup formulation that presumably increase the bioavailability of glyphosate (*4*). Glyphosate penetration through the cell membrane and subsequent intracellular action is greatly facilitated by adjuvants such as surfactants (*5*, *6*).

In addition, both glyphosate and the commercial herbicide severely affect embryonic and placental cells, producing mitochondrial damage, necrosis, and programmed cell death by the activation of caspases 3/7 in cell culture within 24 h with doses far below those used in agriculture. Other effects observed include cytotoxicity and genotoxicity, endocrine disruption of the androgen and estrogen receptors, and DNA damage in cell lines (*7*, *8*).

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More recently, rats fed with a glyphosate-resistant genetically modified corn showed functional alterations in two detoxificants organs: kidney and liver, and the heart and the hematopoietic system (*9*).

Another line of evidence supporting adverse effects of glyphosate was provided by Bellé's group. They suggested that glyphosate and its principal metabolite, AMPA, alter cell cycle checkpoints by interfering with the physiological DNA repair machinery. Several GBHs were assayed, and they induced cell-cycle dysfunction from the first cell division in sea urchin embryos (*10, 11*). The threshold concentration for this effect is 500- to 4000-fold lower than that sprayed on crops in the field. Eight millimolar glyphosate induces a delay in the kinetics of the first cell cleavage of sea urchins, altering the entry into S-phase by interfering with the activation of the CDK1/cyclin B complex (*6, 12*). This failure of cell-cycle checkpoints is known to lead to genomic instability and the possible development of cancer. In agreement with these findings, genotoxicity studies of glyphosate or its metabolites suggest that the irreversible damage in the DNA may increase the risk of carcinogenesis (*13, 14*).

Aside from the previously reported teratogenic effects of glyphosate-based formulations on cephalic structures in amphibians (*15*), there is almost no information available about the molecular mechanisms associated with GBH or glyphosate teratogenesis. Reports of neural defects and craniofacial malformations from regions where GBHs are used heavily led us to an embryological approach to explore the effects of low doses of glyphosate in *Xenopus* and chicken embryogenesis.

We show here that sublethal doses are sufficient to induce reproducible malformations in *Xenopus* and chicken embryos treated with a 1/5000 dilution of a GBH formulation (equivalent to 430 μM of glyphosate) or in frog embryos injected with glyphosate alone (between 8 and 12 μM per injected cell). GBH treated or glyphosate injected frog embryos showed very similar phenotypes, including shortening of the trunk, cephalic reduction, microphthalmy, cyclopia, reduction of the neural crest territory at neurula stages, and craniofacial malformations at tadpole stages. These defects suggested a link with the retinoic acid (RA) signaling pathway. Reporter gene assays using a RA-dependent reporter revealed that GBH treatment increases endogenous RA activity. Strikingly, we demonstrate that Ro 41-5253 (Ro), an antagonist of RA (*16, 17*), rescues the phenotype produced by GBH. We propose that at least some of the teratogenic effects of GBH are mediated by increased endogenous RA activity in the embryos. This is consistent with the very well-known syndrome produced by an excess of RA, as described by the epidemiological study of Lammer et al. in humans (*18*) and in vertebrate embryos (*19*−*25*).

Experimental Procedures

Embryo Culture and Treatments. *Xenopus laevis* embryos were obtained by in vitro fertilization, incubated in 0.1× modified Barth's saline (MBS) (*26*) and staged according to Nieuwkoop and Faber (*27*). The GBH used was Roundup Classic (Monsanto), containing 48% w/v of a glyphosate salt. Treatments were performed from the 2-cell stage with GBH dilutions of 1/3000, 1/4000, and 1/5000 prepared in 0.1× MBS. For rescue experiments, 0.5 or 1 μM Ro-415253 was added at stage 9. Cyclopamine (Sigma C4116) was used at 100 μM concentration in 0.1× MBS and was applied from the 2-cell stage until fixation. Embryos were fixed in MEMFA (*28*) when sibling controls reached the desired stage.

***Xenopus* Embryo Injections, Whole Mount in Situ Hybridization and Cartilage Staining.** Embryos were injected with 360 or 500 pg of glyphosate (*N*-(phosphonomethyl) glycine (Sigma 337757) per cell into one or both cells at the 2-cell stage. Glyphosate was coinjected with 10 ng of Dextran Oregon Green (DOG, Molecular Probes) to identify the injected side as previously described (*29*). Embryos were cultured in 0.1× MBS and fixed in MEMFA when sibling controls reached the desired stage. Whole-mount in situ hybridization (WMISH) with digoxigenin-labeled antisense RNA probes was performed as previously described (*30*) except that the proteinase K step was omitted. For cartilage visualization, embryos were fixed in MEMFA at stages 45−47, washed with PBS, and stained overnight in 0.04% Alcian blue, 20% acetic acid, and 80% ethanol. After extensive washing with ethanol and bleaching with 2% KOH, embryos were washed with 20% glycerol and 2% KOH, and dehydrated through a glycerol/2% KOH series until 80% glycerol was reached.

Detection of RA Activity. Embryos were injected into one cell at the 2-cell stage with 320 pg of the plasmid RAREhplacZ (RAREZ) (*31, 32*) and placed immediately in 1/3000, 1/4000, and 1/5000 GBH dilutions. Basal luminiscence was detected in uninjected and untreated embryos. The endogenous RA activity was measured in embryos injected with RAREZ and left untreated. As positive controls, embryos were injected with the RAREZ plasmid and incubated at late blastula stage with 0.5 or 5 μM all-*trans*-retinoic acid (RA, Sigma R2625). For rescue experiments, embryos injected with the reporter plasmid were incubated in a 1/4000 dilution of GBH from the 2-cell stage, and when they reached the blastula stage, 1 μM of Ro 41-5253 was added. Finally, when sibling controls reached the neurula stages (*14, 15*), all embryos were processed for chemiluminiscent quantitation of the reporter activity by using the β-gal reporter gene assay (Roche). Protein extracts and enzymatic reactions were performed as previously reported (*33*). Luminiscence was measured on duplicate samples in FlexStation 3 equipment (Molecular Devices), and values were normalized by protein content (*32*). A two-tailed *t* test was employed to analyze the significance in the difference of the means. The experiment was repeated three times.

Treatments of Chicken Embryos. After opening a small window in the shell, fertilized chicken eggs (White Leghorn strain) were injected above the air chamber in the inner membrane with 20 μL of 1/3500 or 1/4500 dilutions of GBH. Control embryos were injected only with 20 μL of H_2O. After injection, the window was sealed with transparent adhesive tape, and eggs were placed with their blunt end up at room temperature for 30 min. Then, eggs were incubated in darkness at 38 °C in a humidified incubator (56−58% humidity) and rotated at regular intervals. After appropriate incubation times, embryos were isolated and staged according to Hamburger and Hamilton (*34*).

Whole-Mount Inmunofluorescence and WMISH of Chicken Embryos. Embryos were fixed 2−4 h in freshly prepared 4% paraformaldehyde, rinsed, and processed for analysis. For immunofluorescence, embryos were blocked overnight at 4 °C in blocking solution (5% normal goat serum, 0.3% Triton X-100, 0.01% NaN3, and Tris buffer saline (TBS) at pH 7.4). Then, they were incubated with a 1/50 dilution of a mouse anti-Pax6 monoclonal primary antibody (Developmental Hybridoma Bank) in TBS at pH 7.4 and 0.3% Triton X-100 for 48 h at 4 °C. Embryos were washed three times with TBS and incubated at 4 °C with the secondary antibody (1/1000 fluorescein-conjugated (FITC) antimouse IgG, Jackson ImmunoResearch) in TBS at pH 7.4, 0.3% Triton X-100, and 3% normal goat serum for at least 12 h. Finally, embryos were washed with TBS, placed in a glass culture dish with 80% v/v of glycerol in water, and photographed. WMISH was performed as described for *Xenopus* embryos, using a *c-shh* probe.

Results

GBH and Glyphosate Alter Neural Crest Markers, Rhombomeric Patterning, and Primary Neuron Differentiation. In order to examine whether GBH treatment can affect neural crest development, rhombomeric patterning, and neuronal differentiation, 2-cell stage *Xenopus laevis* embryos were

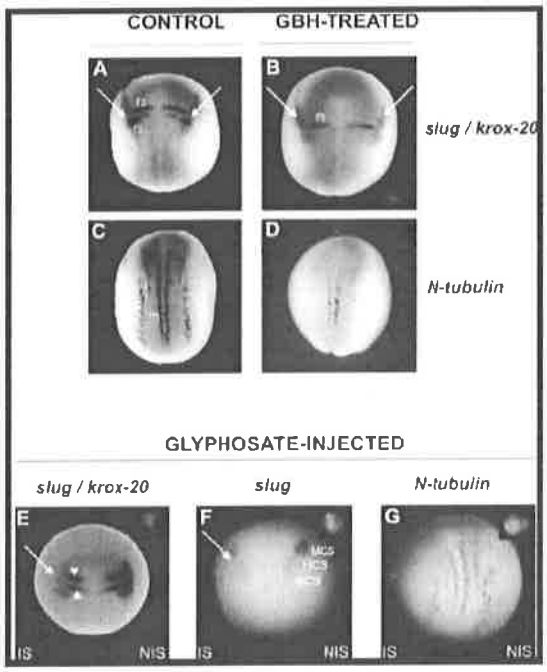

Figure 1. GBH and glyphosate disturb neural crest formation, rhombomeric patterning and primary neuron differentiation. (A−G) Embryos were analyzed at neurula stage by WMISH with different markers. All are dorsal views (anterior is up). (A,C) Control embryos. (B,D) Embryos treated with 1/5000 dilution of GBH. (B) Impairment of neural crest formation as revealed by the specific marker *slug* (arrows). Notice the down-regulation of the *krox-20* domain in the r3 rhombomere. *Slug* and *krox-20* were down-regulated in 87% of treated embryos (n = 30). (D) Suppression of primary neuron formation as seen with the differentiation marker *N-tubulin*. The number of primary neurons was decreased in 83% of treated embryos (n = 30). (E−G) Embryos unilaterally injected with 500 pg of glyphosate per cell plus DOG as the tracer. The injected side is demarcated by the green fluorescence in the insets and is oriented to the left. IS, injected side. NIS, noninjected side. (E,F) Abolishment of *slug* expression in the cranial neural crest domains (arrow; 77%, n = 31) and diminution of *krox-20* expression in r3 and r5 (arrowheads; 71%, n = 21) on the IS. (G) Reduction of *N-tubulin* expression on the IS (81%, n = 16). r3, is the third rhombomere; r5, fifth rhombomere. m, i, and s, are primary motor neurons, interneurons, and sensory neurons, respectively. MCS, HCS, and BCS, are mandibular crest segment, hyoid crest segment, and branchial crest segment, respectively.

exposed to GBH, as described in Experimental Procedures, and assayed by whole mount in situ hybridization (WMISH) at the neurula stage (stage 14−15). The neural crest marker *slug* begins its expression early, where neural crest induction takes place. At neurula stage, it is expressed in the neural crest territory (Figure 1A, arrows) (*35*). Treated embryos show an important down-regulation of *slug* in the neural crest territory (Figure 1B, arrows) in comparison with that of sibling controls. To study the effects on hindbrain patterning, we analyzed the expression of *krox-20*. This zinc finger transcription factor is expressed in rhombomeres r3 and r5 (Figure 1A) and has been shown to play an important role in controlling rhombomere identity (*36*). The r3 stripe was lost in GBH-treated embryos (Figure 1B). This resembles the progressive loss from anterior to posterior rhombomeres associated with increasing concentrations of RA treatments in *Xenopus* and mouse embryos (*37, 38*).

Then we investigated primary neurogenesis at the neural plate stage. At this time, *N-tubulin* is normally expressed in differentiated primary neurons organized in three longitudinal domains in the posterior neural plate: medial, intermediate, and lateral, which correspond to motor neurons (m), interneurons (i), and sensory neurons (s), respectively (Figure 1C) (*39*). Treated embryos showed a down-regulation in the three stripes of primary neurons (Figure 1D).

To corroborate if the effect is specifically due to the active principle of the herbicide and not to adjuvants present in formulations, glyphosate was injected into one cell at the 2-cell stage and *slug*, *krox-20*, and *N-tubulin* were revealed at stages 14−15, as before. These embryos showed an important down-regulation of *slug* (Figure 1E, arrow), resembling the effects of GBH on this marker at this stage of development. Although *Krox-20* did not completely disappear from r3 as in GBH-treated embryos, the expression clearly decreased in this rhombomere as well as in r5, indicating that glyphosate also alters rhombomeric patterning (Figure 1E; arrowheads).

Normally, at stage 18, the neural crest has formed three premigratory blocks from which three different segments segregate: mandibular crest segment, hyoid crest segment, and branchial crest segment (MCS, HCS, and BCS; Figure 1F). The first segment contributes to the Meckel, quadrate, and ethmoid-trabecular cartilages; the hyoid crest segment to the ceratohyal cartilage, and the branchial segment to the cartilages of the gills (*40*). Glyphosate-injected embryos showed that the segregation process clearly affected the injected side (Figure 1F, arrow), suggesting that the derived cartilages may be affected at later stages during development. When hybridized with *N-tubulin*, these embryos showed a decrease in the number of primary neurons in the three stripes corresponding to motor neurons, interneurons, and sensory neurons (Figure 1G, arrows), resembling the effects of GBH treatments, although with milder consequences for this marker.

In conclusion, the effects of GBH-treated and glyphosate-injected embryos represent equivalent phenotypes despite the fact that they are not identical. The adjuvant present in the commercial formulation may explain the differences. Taken together, these results indicate that both GBH and glyphosate impair neuronal differentiation, rhombomeric formation, and the pattern of the neural crest during induction and segregation.

GBH and Glyphosate Produce Head Defects and Impair the Expression of Dorsal Midline and Cephalic Markers. Because craniofacial defects were observed in humans residing in areas chronically exposed to GBH, we decided to explore whether genes involved in head development are altered as a consequence of treatment with GBH or injection of glyphosate. Shh acts as a morphogen controlling multiple developmental processes. During early vertebrate embryogenesis, *shh* expressed in midline structures such as the notochord, prechordal mesoderm, and floor plate controls left−right asymmetry, neuron identity, neural survival, and dorso-ventral patterning of the neural tube (*41, 42*). Moreover, Shh secreted by the prechordal mesoderm is responsible for resolving the brain and the retina field into two separate hemispheres and eyes, preventing cyclopia (*43*).

Shh expression was dramatically reduced in the dorsal midline at neurula stages, especially in the prechordal mesoderm in GBH-treated embryos. The anterior limit of the *shh* expression domain is moved caudally in treated embryos, in relation to the *pax6* domain (compare green arrowheads, Figure 2A−C).

Pax6 is essential for eye formation in a wide range of species. It is expressed in the eye primordia of vertebrates such as the mouse, chicken, *Xenopus*, zebrafish, and humans, as well as in invertebrates such as *Drosophila* (*44−47*). Embryos incubated with GBH showed a distinct down-regulation of the *pax6* territory (compare white arrowheads; Figure 2A−C). Moreover, in treated embryos, the *pax6* domain is not divided in the eye field (light blue arrowheads; Figure 2B,C). These results suggest

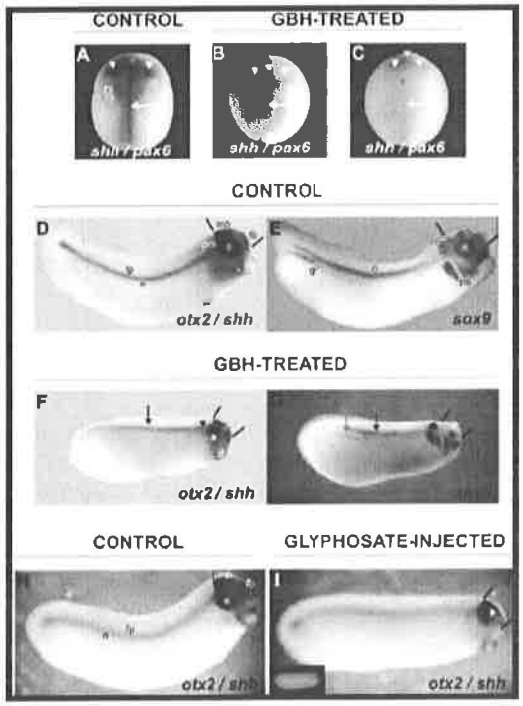

Figure 2. GBH and glyphosate produce A-P truncations and impair the expression of dorsal midline and neural crest markers. (A−I) WMISH analysis at neurula (A−C) and tailbud (D−I) stages. (A) Control embryo hybridized with *shh* (arrow) and *pax6* (white arrowheads). (B−C) Embryos exposed to 1/5000 dilution of GBH. Notice the dramatic reduction of *shh* expression in the embryonic dorsal midline (arrows) and the caudal displacement of the anterior limit (green arrowheads) (85%, $n = 33$). The expression of *pax6* is reduced, and the domain is not properly resolved in the eye field (light blue arrowheads) (85%, $n = 33$). (D,E) Control embryos. (D) Normal expression of *shh* in the notochord (n), floor plate (fp), and prechordal mesoderm (pm) and of *otx2* in the eye (e), forebrain (fb), and midbrain (mb). The space between bars indicates the size of the brain. (E) Normal expression of *sox9* in the pharyngeal arches (pa), otic placode (op), eye (e), genital ridge (gr), and notochord (n). (F,G) 1/5000 GBH-treated embryos. (F) Reduced expression of *shh* and *otx2* (92%, $n = 24$) in dorsal midline cells (*shh*, arrow), prechordal mesoderm (*shh*, black arrowhead), eye (*otx2*, yellow arrowhead), and brain structures (*otx2*, space between bars). (G) Diminution of *sox9* expression in the notochord (black arrow), genital ridge (green arrow), and eyes (yellow arrowhead) (87%, $n = 30$). Notice the delay in the migration of neural crest cells toward the pharyngeal arches (red arrowheads). Treated embryos (F and G) showed microphtalmy, microcephaly (compare the space between bars in D−G), and a shortened A-P axis (89%, $n = 54$). (H,I) Embryos hybridized with *shh* and *otx2*. (H) Control embryo showing the same structures as those in D. (I) Embryo bilaterally injected with 360 pg of glyphosate per cell at the 2-cell stage plus DOG as tracer (green fluorescence in the inset). Similar to that in GBH-treated embryos, *shh* and *otx2* expression was reduced (62%, $n = 16$), and this was accompanied by microcephaly (compare space between bars) and microphtalmy (yellow arrowhead).

that a down-regulation of *shh* expression in the prechordal mesoderm together with a diminution of *pax6* expression may underlie the defects in the resolution of the retina field and the brain hemispheres in embryos treated with GBH.

To test whether these molecular alterations were associated with defects at later stages, we analyzed the expression of *shh/otx2* (Figure 2D,F) and *sox9* (Figure 2E,G) in embryos treated with GBH as before but fixed at tailbud stages. *Otx2* is a homeobox-containing gene expressed in retinal and lens components of the eye and telencephalic, diencephalic, and mesencephalic regions and plays an important role in specifying anterior structures (*48, 49*). Exposed embryos showed a decrease of anterior *shh* expression with concomitant microphtalmy and microcephaly, as revealed by the reduction of the *otx2* domain (Figure 2, compare the space between bars in the control embryo in D with the treated embryo in F). Also, there is a pronounced shortening of the A-P axis (compare control embryos in Figure 2D,E with treated embryos in F,G). In control embryos, the transcription factor *sox9* is expressed in the cranial neural crest cells as they populate the pharyngeal arches, the otic placode, the developing eye, the genital ridges, and also the notochord (Figure 2E) (*50*). Embryos treated with GBH showed reduced eyes and genital ridges, and developed abnormal pharyngeal arches. The migration of neural crest cells to these structures was delayed, as revealed by a more dorsal position (compare Figure 2G with E).

To analyze the effects of glyphosate alone on dorsal midline development, we performed bilateral injections at the 2-cell stage. Embryos were fixed when sibling controls reached stage 28−30, and the expression of *shh* was analyzed. To better understand cephalic defects, the pattern of *otx2* was also examined. Similar to embryos treated with GBH, we observed reduced prechordal *shh* expression accompanied by strong microcephalic and microphtalmic phenotypes. This is likely due to a decrease of midline-derived signals (Figure 2H,I). Taken together, all of these results indicate that GBH as well as glyphosate alone cause cephalic defects that probably result from a reduction of *shh* and *otx2* expression in anterior structures. The delay in the migration of cranial neural crest cells in the tailbud stage embryos together with the inhibition of *slug* expression at earlier stages led us to next examine whether craniofacial development would be impaired in older embryos.

GBH and Glyphosate Disrupt the Development of the Craniofacial Skeleton. The pattern of neural crest derivatives in the cranial skeleton of the *Xenopus* embryo was previously established (*40*). Briefly, in the first pharyngeal arch, neural crest cells contribute to the upper (quadrate, Qu) and lower (Meckel's, Me) jaws; in the second arch, they contribute to the cerathoyal cartilage (Ce), while in the third and fourth arches, neural crest cells contribute to the anterior and posterior regions of the branchial/gills cartilage (Br), respectively (Figure 3C).

To address if the effects seen at neurula and tailbud stages are correlated with craniofacial malformations, embryos treated with GBH and embryos unilaterally or bilaterally injected with glyphosate at the 2-cell stage were allowed to develop up to stage 47 and processed with Alcian Blue staining for skeletal analysis. The gross morphology of GBH-treated embryos revealed an overall reduction of cranial structures and microphthalmy (compare Figure 3A,C with B,D). All affected embryos displayed a reduction of the quadrate and Meckel's cartilages (asterisks, Figure 3D), while the branchial and cerathoyal cartilages were mildly affected.

Unilateral glyphosate injections resulted in a general decrease of Alcian blue staining and in a reduction of the Meckel's and quadrate cartilages on the injected side (asterisks, Figure 3E,F). In some embryos, the eye practically disappeared from the injected side (arrow, Figure 3H). Moreover, bilaterally injected embryos exhibited cyclopia (Figure 3I, arrow), consistent with the loss of Shh signaling from the prechordal mesoderm observed at earlier stages. Similar results were obtained in frog embryos treated with cyclopamine (Figure 3J), a known inhibitor of the Hedgehog pathway which leads to developmental malformations and holoprosencephaly-like abnormalities, including cyclopia in the most severe cases (*51−53*). Unilateral injections of cyclopamine produced cartilage alterations similar to those obtained with glyphosate injections (not shown).

In summary, our results are compatible with the malformations observed in the offspring of women chronically exposed

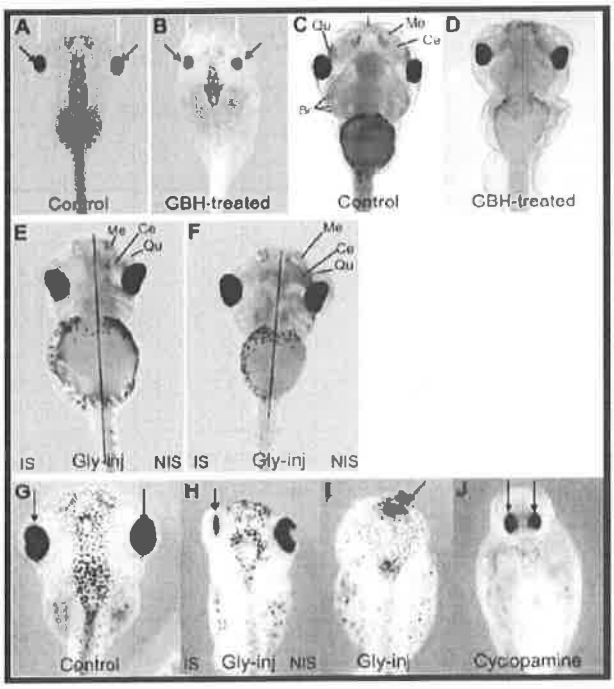

Figure 3. GBH treatment and glyphosate injection result in cephalic malformations and abnormal development of the craniofacial skeleton. (A–D) 1/5000 GBH-treated embryos analyzed at stage 45–47. (A,B) Gross morphology. (A) Control embryo; eyes (arrows); head size (space between yellow bars). (B) Embryo exposed to GBH showing reduced eyes (arrows) and head structures (89%, $n = 38$) (compare the space between yellow bars in A and B). (C,D) Embryos stained with Alcian blue. (C) Control embryo, showing facial cartilages: Meckel (Me), ceratohyal (Ce), infrarrostral (I), quadrato (Qu), and branchial (Br). (D) Reduction of Me and Qu cartilages (asterisks) in GBH-exposed embryos (77%, $n = 39$). (E–I) Embryos injected with 360 pg of glyphosate per cell in one or both cells at the 2-cell stage and analyzed at stage 47 by Alcian blue staining (E,F) or gross morphology (H,I), which was compared with that of sibling controls (G). (E,F) Unilaterally injected embryos showing reduced Alcian blue staining and smaller Qu and Me cartilages (asterisks) on the IS (56%, $n = 16$). (G) Control embryo. Arrows indicate the position of the eyes. (H) Notice the reduction of the eye in the IS (arrow) (54%, $n = 13$). (I) Bilaterally injected embryo exhibiting cyclopia (arrow) (38%, $n = 8$). (J) Cyclopamine-treated embryo. Observe the proximity of both eyes (arrows), due to midline defects (compare with the control embryo in G). IS, injected side. NIS, noninjected side. Gly-inj, embryo injected with glyphosate.

to GBH during pregnancy (see Discussion). These malformations suggest the loss of midline signaling, accompanied by defects in neural crest migration (or increased apoptosis) with aberrant development of mandibular and maxillary structures.

Phenotype Induced by GBH Is at Least Mediated by Changes in RA Signaling. It was previously reported that increasing concentrations of RA caused progressive truncation of anterior and posterior structures in *Xenopus laevis* (*20, 21*). The most severely affected embryos lacked eyes, nasal pits, forebrain, midbrain, and otic vesicles, and displayed truncations of the tail. The phenotypes produced by GBH and glyphosate resemble the teratogenic effects of embryos treated with RA; therefore, we theorized that the RA pathway could be associated with the morphogenetic effects of glyphosate during early embryogenesis.

The RA signal is transduced through nuclear retinoic acid receptors (RARs), which control the expression of target genes involved in vertebrate pattern formation, organogenesis, and tissue homeostasis (*54*). Ro 41-5253 (Ro) is an antagonist of the RARα receptor, which is expressed during early development in *Xenopus* (*16, 17, 55, 56*). Ro was previously used as a tool to block retinoid-mediated signaling, producing a variety of morphological changes in the frog embryo. The most severe phenotypes showed anterior and posterior truncations, a reduction or loss of eyes and otic vesicles, and a general disorganization of branchial arches (*22*). Moreover, maternal insufficiency of vitamin A (the precursor of RA) or RA in excess in vertebrates cause a wide range of teratologic effects (*18, 57, 58*). All this evidence demonstrates that vertebrates require a precisely regulated supply of retinoids during embryogenesis.

Considering that the phenotypes obtained in our analysis predominantly resemble those of RA excess, we wondered if GBH treatments are able to increase endogenous RA activity. To answer this question, we measured the levels of RA signaling by taking advantage of the reporter plasmid RAREZ (*32*), as described in the Experimental Procedures section. Figure 4A shows that GBH treatment significantly increased the level of RA signaling in the embryo in a concentration-dependent manner. Importantly, the RA receptor antagonist Ro rescued the effect of GBH since the level of the RA output, as measured by the reporter assay, was not significantly different from that in RAREZ-injected, untreated controls (Figure 4A). Together, these observations strongly suggest that GBH increases endogenous retinoid activity.

If an increase of RA signaling underlies the phenotype produced by GBH treatments, antagonizing the RA pathway should rescue the effect of GBH. To examine this hypothesis, embryos were incubated at the 2-cell stage with GBH alone or with GBH together with 0.5 or 1 μM Ro added when sibling controls reached stage 9 (*22*). Frog embryos were analyzed by their morphological aspect and also were hybridized with *shh* and *otx2* probes.

Control embryos showed an expression of *otx2* in the forebrain, midbrain, and optic vesicle, while *shh* transcripts are distributed along the embryonic dorsal midline (Figure 4B). Embryos treated continuously with GBH showed a down-regulation of *shh* and *otx2*, reduced head structures, and shortened A-P axis (Figure 4C). Similar results were obtained after treating frog embryos with 0.1 or 1 μM RA (*21, 59, 60*). As previously reported (*22*), embryos incubated with 0.5 or 1 μM Ro alone also displayed a concentration-dependent shortening of the A-P axis and reduction of head structures, which was confirmed by a reduction of the *otx2* domain (Figure 4D,E; compare the space between bars with B). We also observed a more diffuse staining of *shh*, mainly in the prechordal mesoderm, in comparison with that of sibling controls (Figure 4D,E; arrows). When 0.5 or 1 μM Ro was added at stage 9 to embryos continuously exposed to 1/5000 dilution of GBH, the elongation of the A-P axis was recovered as well as the normal expression pattern of *otx2* and *shh* (Figure 4F,G). We conclude that the ability of Ro treatment to rescue the teratogenic effect of the GBH supports the idea that RA activity is elevated in GBH-treated embryos.

GBH Produces Similar Teratogenic Effects in Chick Embryos. To test whether the teratogenic effects of GBH are reproducible in an amniote vertebrate, we chose the chick model. Embryos were incubated with 1/3500 or 1/4500 dilutions of GBH and analyzed at the HH stage 9 (8 somites) by immunofluorescence with an anti-Pax6 antibody and by WMISH with a *c-shh* probe (*61*). As was previously demonstrated for Pax6 mRNA (*62*), the Pax6 protein is normally distributed in the optic vesicle; in the distinctive comet-like shape in the ectoderm, posterior to the region of the optic vesicle; in the hindbrain in rhombomeres r3 and r5 and along the spinal cord (Figure 5A,D). GBH treatments produced a concentration-dependent reduction

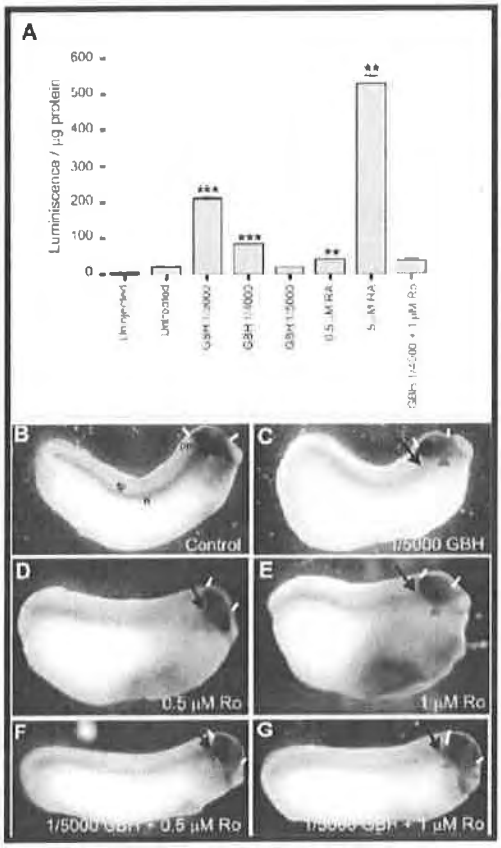

Figure 4. Phenotype induced by GBH is mediated by an increase of RA signaling (A). Analysis of RA activity with the reporter plasmid RAREZ. All embryos were injected with the reporter plasmid RAREZ, except for uninjected controls, and left untreated or were treated as indicated in the figure until stage 14−15, when they were processed. Results are expressed as arbitrary luminiscence units per μg of protein. A two-tailed *t* test was employed to analyze the significance in the difference of the means. ** $p < 0.01$; *** $p < 0.0001$. (B-G) WMISH for *shh* and *otx2* at tailbud stages. (B) Control embryo. Notochord (n); floor plate (fp); brain (space between bars), eye (arrowhead). (C) Embryo treated with 1/5000 GBH manifesting microcephaly (space between bars), reduced eyes (arrowhead), diminished Shh signaling from the prechordal mesoderm (arrow), and shortened A-P axis (78%, $n = 9$). (D,E) Embryos incubated with 0.5 and 1 μM RA antagonist Ro 41-5253, displaying a reduction in the *otx2* domain accompanied by microcephaly (bars) and microphtalmy (arrowhead), and more diffuse expression of *shh* (arrows) (80%, $n = 15$ for 0.5 μM Ro; 87%, $n = 15$ for 1 μM Ro). (F,G) Embryos treated with 1/5000 GBH at the 2-cell stage; 0.5 μM Ro (F) or 1 μM Ro (G) was added at stage 9, and phenotypes were analyzed at the tailbud stage. Notice that Ro reverts the phenotype produced by GBH, rescuing the A-P axis elongation and the expression of *shh* and *otx2* (compare with the control embryo in B) (88%, $n = 17$ for 0.5 μM Ro, which gives the best rescue effect since the effect of the retinoid antagonist begins to prevail with 1 μM Ro). All embryos are oriented with the anterior end toward the right.

of the optic vesicles, as revealed by a reduction of the corresponding Pax6 domain, and this was accompanied by microcephaly (compare the space between bars in Figure 5B,C with A). We also observed a gradual loss of the r3 and r5 domains in embryos treated with GBH (compare Figure 5E,F with D), which resembles the results observed in frog embryos in the *krox-20* domains (Figures 1B and 2E). Hybridization with the *c-shh* probe showed that, as in *Xenopus*, the prechordal mesoderm domain is preferentially lost in GBH-treated chick embryos (compare Figure 5G with H,I). As the GBH concentration increases, the expression along the embryonic dorsal midline also gradually disappears (Figure 5H,I).

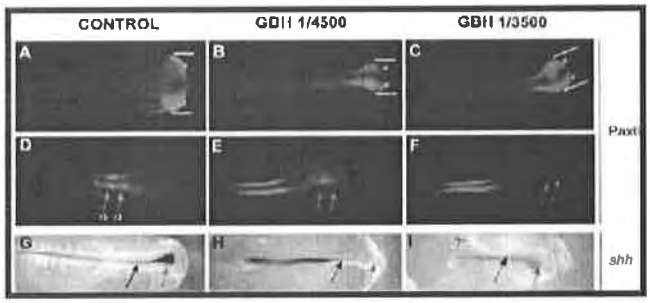

Figure 5. Teratogenic effects of GBH in chicken embryos. (A−C) Whole-mount inmunofluorescence analysis of Pax6 at 8 somites. (A,D) Control embryo showing Pax6 expression in the optic vesicles (arrowheads in A) and in rhombomeres r3 and r5 (blue arrows in D). (B,E and C,F) graded reduction of Pax6 expression in embryos treated with 1/4500 and 1/3500 dilutions of GBH, respectively. Notice the progressive microcephaly (compare space between bars with D) and the loss of Pax6 expression corresponding to rhombomeres r3 and r5 (red arrows). The remaining fluorescence corresponds to specific Pax6 expression that is normally found in the spinal cord but is out of focus in the control embryo in D. (G−I) WMISH with *c-shh*. (G) Control embryo. *Shh* transcripts are seen in dorsal midline cells (black arrow) and in the prechordal mesoderm (green arrow). (H,I) Embryos treated with 1/4500 and 1/3500 dilutions of GBH, respectively. Notice the abolishment of *shh* expression in the prechordal mesoderm (dotted green arrow) and the progressive decrease of *shh* expression in the midline cells in a concentration-dependent manner (dotted black arrows).

Therefore, our experiments with chick embryos further extend conclusions from studies about the teratogenic effects of GBH in amphibians to other vertebrate species.

Discussion

The results presented above argue that both GBH and glyphosate itself interfere with key molecular mechanisms regulating early development in both *Xenopus* and chicken embryos, leading to congenital malformations. Sublethal doses of the herbicide (430 μM of glyphosate in 1/5000 dilutions of GBH) and injections leading to a final concentration of 8 to 12 μM of glyphosate in the injected side of the embryo were sufficient to induce serious disturbances in the expression of *slug*, *otx2*, and *shh*. These molecular phenotypes were correlated with a disruption of developmental mechanisms involving the neural crest, embryonic dorsal midline formation, and cephalic patterning. Because glyphosate penetration through the cell membrane requires facilitation by adjuvants present in commercial formulations (*5, 6*), we tested the effects of glyphosate alone by directly microinjecting it into *Xenopus* embryos. The similarity of the phenotypes obtained in both situations suggests that they are attributable to the active principle of GBH and not to the adjuvants.

We will discuss our results in the following context: (1) the correlation of our phenotypes with those observed in animal models with an impairment of RA signaling or deficits in the expression of critical genes that control embryonic development; (2) the probable mechanisms underlying the phenotypes induced by GBH and glyphosate; (3) possible correlations with clinical cases of human offspring exhibiting malformations in zones exposed to GBH.

Misregulation of RA, *shh*, and *otx2* Are Involved in Cephalic Malformations and Neural Crest-Derived Phenotypes Reminiscent of the Effects of GBH and Glyphosate. The phenotypes obtained after GBH treatments or injections of glyphosate alone are strikingly reminiscent of those observed as a consequence of an excess of RA signaling in vertebrates and humans. Acute or chronic increase of RA levels leads to teratogenic effects during human pregnancy and in experimental

models. The characteristic features displayed by RA embryopathy in humans include brain abnormalities such as microcephaly, microphthalmia, and impairment of hindbrain development; abnormal external and middle ears (microtia or anotia); mandibular and midfacial underdevelopment; and cleft palate. Many craniofacial malformations can be attributed to defects in cranial neural crest cells (*19, 24*).

This spectrum is consistent with the phenotypes obtained in rodent models exposed to RA. When administered during gastrulation in mice, RA severely impairs the development of the anterior neural plate, resulting in ocular, brain, and facial malformations. Exposure at critical stages of neural crest cell migration induces craniofacial malformations comparable to those seen in Di-George syndrome. Later exposure, when the epibranchial placodes are active, results in mandibulofacial dysostosis-like syndromes (*19*). These authors suggest that excessive cell death in regions where apoptosis normally takes place may underlie a general mechanism for craniofacial malformations associated with teratogens.

An excess of RA signaling is able to down-regulate *shh* expression in the embryonic dorsal midline in *Xenopus* (*60, 63*). *Shh* deficiency is associated with holoprosencephaly syndrome (HPE), a CNS malformation with a frequency of 1/250 of pregnancies and 1/10000 of live births. HPE is a defect generated by the deficiency of the embryonic dorsal midline, leading to a failure in the division of the brain hemispheres. This results in unilobar brain, cyclopia, and defects in the closure of the dorsal neural tube, accompanied by other defects including microcephaly, abnormally decreased distance between the eyes (hypotelorism), proboscis, and cebocephaly (a simple nose) (*51*−*53*). Moreover, Shh signaling is necessary for the development of the cranial neural crest derivatives. In the mouse, specific removal of Shh responsiveness in the neural crest cells that give rise to skeleton and connective tissue in the head increases apoptosis and decreases proliferation in the branchial arches, leading to facial truncations (*64*). In zebrafish, the cranial neural crest requires Shh signaling emanating from the embryonic dorsal midline and the oral ectoderm to achieve correct migration and chondrogenesis (*65*). In chicken embryos, development of the lower jaw skeleton requires Shh signaling from the foregut endoderm to prevent apoptosis of the neural crest cells that migrate to the first branchial arch (*66*). Shh signaling from the ventral midline is necessary, as an antiapoptotic agent, for the survival of the neural epithelium, and it is also essential for the rapid and extensive expansion of the early vesicles of the developing midbrain and forebrain (*67*−*69*).

An excess of RA signaling also down-regulates *otx2* expression in *Xenopus*, chicken, and mouse embryos (*24*). Knockout mice for *otx2* lack all the brain structures anterior to rhombomere 3. Interestingly, heterozygous mutants showed craniofacial malformations including the loss of the eyes and lower jaw (agnathia). These phenotypes are reminiscent of otocephaly reported in humans and other animals and suggest that *otx2* plays an essential role in the development of cranial skeletons of mesencephalic neural crest origin (*70*−*72*).

Otx2, in turn, is necessary for the expression of *shh* in the ventral midbrain (*73*). All this evidence indicates that RA signaling, *otx2*, and *shh* are part of a genetic cascade critical for the development of the brain and craniofacial skeleton of neural crest origin. Glyphosate inhibits the anterior expression of *shh*, reduces the domain of *otx2*, prevents the subdivision of the eye field, and impairs craniofacial development, resembling aspects of the holoprensecephalic and otocephalic syndromes. This prompted us to investigate whether an increase of RA signaling could be mediating the effects of GBH treatments.

GBH Increases the Activity of the Morphogen RA, Leading to Teratogenic Effects. In *Xenopus* embryos, the endogenous activity of retinoids gradually increases during early embryogenesis and is finely regulated in space. At late gastrula, a rostral−caudal gradient from 0.01 to 0.16 μM RA is established, with the highest levels at the posterior end of the embryo. The gradient persists at the early neurula stage (stage 13−14). Synthesis and degradation of RA seem to be the mechanisms that lead to this uneven distribution (*74*). This gradient explains why low doses of applied RA primarily affect the cephalic region and increasing the doses begins to affect the trunk (*20, 21*). Moreover, maintaining a normal endogenous distribution of RA is important for axes patterning and organogenesis not only in *Xenopus* (*74, 22, 38*) but also in other Vertebrates such as zebrafish (*75*−*77*), chicken (*78*−*80*), and mouse embryos (*81*).

In this study, GBH treatments or glyphosate injections mostly reproduce the morphological phenotype obtained after treatments of *Xenopus* embryos with RA concentrations from 0.1 to 10 μM (*21*). The fact that GBH treatments increase endogenous RA activity, as measured by the RAREZ reporter, and that the GBH-induced phenotypes are rescued by the antiretinoid Ro strongly suggest that augmented RA activity is a major cause of the molecular and morphological phenotypes described in this work.

GBHs are considered endocrine disruptors because of their ability to impair the synthesis of steroid hormones (*82*). Glyphosate inhibits the activity of aromatase, a member of the cytochrome P450 family crucial for sex steroid hormone synthesis (*4*). Retinoid activity is regulated by degradation of RA by the CYP26 enzymes, which are members of the cytochrome P450 family and are present in all vertebrates from early stages of embryogenesis. Transcription of CYP26 is developmentally and spatially regulated. Deficiencies of this enzyme produce serious malformations in different vertebrate models consistent with an important increase in RA signaling. These phenotypes include cephalic defects, abnormalities of the eye and the forebrain, agnathia, and caudal truncations (*83*−*90*). In this context, it will be interesting to elucidate in the future if the increase of RA signaling induced by GBH could be a consequence of inhibiting the activity of CYP26 enzymes responsible for maintaining a normal RA distribution by specific territorial degradation.

In *Xenopus laevis*, RA favors the differentiation of primary neurons (*39, 60, 91*). Since GBH increases retinoid signaling, the reduction in the number of primary neurons in GBH-treated and glyphosate-injected embryos is paradoxical. Other biochemical mechanisms could be triggering the inhibition of neurogenesis. For example, we cannot rule out that apoptosis of neural precursors could be involved in this process since GBH and glyphosate have a toxic effect on mitochondrial membranes and activate caspases3/7 (*7*). Both GBH and glyphosate inhibit *shh* expression, and the Shh protein is known to have an antiapoptotic function, necessary for the survival of the neuroepithelium (*67, 68*). Abnormal induction of cell death is one of the crucial mechanisms of malformations associated with different teratogenic agents such as ethanol, RA, hypoxia, and chemicals herbicides (*19, 92*).

Assuming a linear response of the luminescence system with the RAREZ reporter used to measure RA signaling, we estimate that the endogenous concentration of RA available for activity in *Xenopus* embryos is around 0.2 μM (Figure 4A, compare

RA bars with the RAREZ bar). This is very similar to the average concentration of 0.15 μM previously measured by HPLC (*20*). Importantly, treatments with 1/5000 dilution of GBH do not show a significant increase of RA activity when compared to that of untreated controls, as measured by the reporter system (Figure 4A). However, this dilution clearly produces cephalic and trunk phenotypes and craniofacial malformations, as shown throughout this work, which are rescued by Ro treatments. Therefore, the RAREZ reporter does not seem to be sensitive enough to detect minimal variations in the levels of RA activity. This reinforces the importance of using vertebrate embryos as biosensors for testing possible teratogens.

Moreover, it has been recently reported that Triadimefon, a systemic fungicide with teratogenic effects in rodent models, produces craniofacial malformations in *Xenopus laevis* by altering endogenous RA signaling (*93*). Arsenic, another endocrine disruptor, also increases RA signaling at low, noncytotoxic doses, in human embryonic NT2 cells (*94*). RA signaling is one of the finest pathways to tune up gene regulation during development, and all this evidence raises the possibility that disturbances in RA distribution may be a more general mechanism underlying the teratogenic effects of xenobiotics in vertebrates. Since mechanisms of development are highly conserved in evolution among vertebrates (*95*), we would like to stress that they could be useful as very sensitive biosensors to detect the undesirable effects of new molecules.

Clinical Approaches. In Argentina, the extension of soil devoted to transgenic soy reached 19 million hectares. Two hundred million liters of glyphosate-based herbicide is used for a production of 50 million tons of soy beans per year (*96, 97*). The intensive and extensive agricultural models based on the GMO technological package are currently applied without critical evaluation, rigorous regulations, and adequate information about the impact of sublethal doses on human health and the environment, leading to a conflicting situation. In this work, we focused on sublethal doses of GBH to arrive at the thresholds for teratogenic phenotypes instead of lethality.

In the last 10 years, several countries in Latin America have initiated studies about the environmental consequences of the use of herbicides and pesticides. In Paraguay, an epidemiological study in the offspring of women exposed during pregnancy to herbicides showed 52 cases of malformations (*3*), which strikingly resemble the wide spectrum phenotypes resulting from a dysfunctional RA or Shh signaling pathway. In Argentina, an increase in the incidence of congenital malformations began to be reported in the last few years (Dr. Hugo Lucero, Universidad Nacional del Nordeste, Chaco; personal communication). In Córdoba, several cases of malformations together with repeated spontaneous abortions were detected in the village of Ituzaingó, which is surrounded by GMO-based agriculture. These findings were concentrated in families living a few meters from where the herbicides are regularly sprayed. All of this information is extremely worrying because the risk of environmentally-induced disruptions in human development is highest during the critical period of gestation (2 to 8 weeks) (*98*). Moreover, the mature human placenta has been shown to be permeable to glyphosate. After 2.5 h of perfusion, 15% of administered glyphosate is transferred to the fetal compartment (*99*).

All of the evidence reported in the scientific literature and the clinical observations in the field were not sufficient, however, to activate the precautionary principle of the environmental legislation in order to realize the depth of the impact on human health produced by herbicides in GMO-based agriculture. To our knowledge, the results presented in this work show for the first time that at least some of the malformations produced by GBH in vertebrate embryos are due to an increase of endogenous RA activity, consistent with the well-known syndrome produced by an excess of RA.

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Short Communication

Evaluation of DNA damage in an Ecuadorian population exposed to glyphosate

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Abstract

We analyzed the consequences of aerial spraying with glyphosate added to a surfactant solution in the northern part of Ecuador. A total of 24 exposed and 21 unexposed control individuals were investigated using the comet assay. The results showed a higher degree of DNA damage in the exposed group (comet length = 35.5 μm) compared to the control group (comet length = 25.94 μm). These results suggest that in the formulation used during aerial spraying glyphosate had a genotoxic effect on the exposed individuals.

Key words: comet assay, DNA damage, Ecuador, genotoxicity, glyphosate.

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Glyphosate is a non-selective herbicide which is the main chemical component in many systemic herbicides used to control most annual and perennial plants. It controls weeds by inhibiting the synthesis of aromatic amino acids necessary for protein formation, which link primary and secondary metabolism in susceptible plants (Carlisle and Trevors, 1988; U.S. Forest Service, 1997).

According to some reports glyphosate shows no adverse effects on soil microorganisms, it is relatively non-toxic to fish (U.S. Forest Service, 1997) and is of relatively low toxicity to birds and mammals, including humans (Batt *et al.*, 1980; Evans and Batty, Williams *et al.*, 2000; Goldstein *et al.*, 2002). However, Lioi *et al.*, (1998) reported de induction of oxidative stress and mutagenic effects for some pesticides, including glyphosate, in bovines and Paz-y-Miño *et al.*, (2002a) reported that some pesticides were associated with genetic damage in human populations subjected to high pesticide exposure levels due intensive use, misuse or failure of control measures.

Since January 2001, the northern area of Ecuador (mainly Sucumbíos district) has been subjected to aerial spraying by the Colombian Government with Roundup-Ultra, a herbicide formulation containing glyphosate, polyethoxylated tallowamine surfactant (POEA) and the adjuvant Cosmoflux 411F which is a propriety Colombian component probably included to aid the adherence or absorption of the herbicide (Ministerio de Relaciones Exteriores, Ecuador (MREE), 2003). According to the National Narcotic Council for air spraying of illicit cultures the load of the airplane was 1137 to 1705 liters and the effective unloading with Roundup Ultra (43.9% of glyphosate) was 23.4 liters ha^{-1} equivalent to 10.3 L ha^{-1} of glyphosate (Acción Ecológica, 2003, Nivia, 2001). The main purpose of spraying glyphosate in this formulation is to eradicate illicit crops grown in this area, and several research projects have been carried out to investigate the consequences of the use of this formulation in Ecuador (MRE, Ecuador, 2003; Acción Ecológica, 2003).

The comet assay can be used to evaluate DNA damage and provides a useful tool for estimating the genetic risk from exposure to complex mixtures of chemicals (Paz-y-Miño *et al.*, 2002b), this assay having been widely applied in genotoxicity studies of factors such as X-rays and pesticides (Singh *et al.*, 1988; Tice *et al.*, 1990; Scarpato *et al.*, 1996; Slamenová *et al.*, 1999; Blasiak *et al*; 1999; Garaj-Vrhovac and Zeljezic, 2000; Paz-y-Miño *et al.*, 2002a; Paz-y-Miño *et al.*, 2002b; Acción Ecológica, 2003).

The aim of the study described in this paper was to determine the possible influence of the formulation of

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glyphosate used during aerial spraying in northern Ecuador on the genetic material of exposed individuals.

The exposed (E) group consisted of 24 randomly selected individuals (Table 1) who lived 3 km or less from an area on the border between Ecuador and Colombia where aerial spraying with a glyphosate-based herbicide had occurred continuously during three days between December 2000 and March 2001, sporadic aerial spraying continuing for three weeks following continuous spraying (MREE, 2003, Acción Ecológica 2004). Exposed group individuals manifested symptoms of toxicity after several exposures to aerial spraying, with half of the individuals in this group having received spraying directly over their houses and the other half living within 200m to 3 km from the sprayed areas.

A clinical history was completed for each of the exposed individuals and a wide-range of reactions were noted, including intestinal pain and vomiting, diarrhea, fever, heart palpitations, headaches, dizziness, numbness, insomnia, sadness, burning of eyes or skin, blurred vision, difficulty in breathing and blisters or rash (MREE, 2003; Acción Ecológica 2003).

Venous blood (5 mL) was taken from the exposed individuals between two weeks and two months after their exposure to aerial spraying and processed immediately after collection.

The blood samples analyzed in this study were provided by Dr. Adolfo Maldonado, a specialist in tropical medicine and a member of the Ecological Action foundation and part of the group of investigators of the International Commission on the Impact on Ecuadorian Territory of Aerial Fumigations in Colombia. This study was approved by the Bioethics Committee of the Pontifical Catholic University of Ecuador, according to the international guidelines. Each individual completed a personal and biomedical survey and gave their informed consent to be part of this study. In the case of the adolescents involved in the study (14-17 year-olds) their legal guardians, as well as themselves, gave their informed consent.

All of the individuals included in this study combine their activities mainly in the house and sometimes cultivating and harvesting. This persons neither used herbicides, pesticides nor similar substances in the named activities (Acción Ecológica, 2004).

Table 1 - DNA damage assessed by the comet assay in individuals exposed (E) to glyphosate and unexposed (U) control individuals. Note that the same numbers (1, 2, 3 etc.) for the individuals does not indicate that the exposed and control individuals were matched.

Exposed to glyphosate								Unexposed controls							
Individual (gender, age)[a]	Number of cells scored in each group					DNA migration (μm)		Individual (gender, age)[a]	Number of cells scored in each group					DNA migration (μm)	
	A	B	C	D	E	Mean	Median		A	B	C	D	E	Mean	Median
1E (F, 53)	2	120	76	5	3	39.5	32.5	1U (F, 17)	150	59	3	0	0	26.2	25.0
2E (F, 37)	13	92	82	14	0	44.1	32.5	2U (F, 40)	164	43	4	0	0	25.4	25.0
3E (F, 40)	2	64	62	77	4	56.6	52.5	3U (F, 26)	165	40	2	0	0	25.7	25.0
4E (M, 27)	8	75	64	47	8	49.2	37.5	4U (M, 14)	111	96	6	0	0	27.3	26.5
5E (F, 44)	9	138	63	3	0	34.6	30.0	5U (M, 32)	165	38	3	0	0	25.9	25.0
6E (F, 50)	51	113	30	3	0	30.8	27.5	6U (M, 21)	171	35	1	0	0	25.7	25.0
7E (F, 38)	21	139	48	3	0	33.2	30.0	7U (M, 16)	177	25	6	0	0	25.8	25.0
8E (F, 46)	21	116	72	4	0	35.2	30.0	8U (F, 47)	176	25	3	0	0	25.7	25.0
9E (F, 55)	26	100	84	1	0	32.8	30.0	9U (F, 15)	190	14	1	0	0	25.2	25.0
10E (F, 50)	26	100	84	1	0	34.2	30.0	10U (F, 36)	179	25	1	0	0	25.4	25.0
11E (F, 22)	28	123	60	0	0	32.0	27.5	11U (F, 21)	150	46	9	0	0	26.3	25.0
12E (F, 27)	11	130	63	6	0	33.7	30.0	12U (F, 43)	148	49	15	0	0	26.8	25.0
13E (F, 28)	40	132	40	2	0	31.0	30.0	13U (F, 53)	161	27	10	0	0	26.1	25.0
14E (F, 59)	10	96	99	1	0	36.4	32.5	14U (F, 35)	164	23	21	0	0	27.0	25.0
15E (F, 55)	35	110	62	1	0	32.7	30.0	15U (F, 38)	169	28	11	0	0	26.4	25.0
16E (F, 17)	60	101	44	1	0	31.3	37.5	16U (F, 22)	183	15	8	0	0	25.1	25.0
17E (F, 34)	7	114	57	2	0	33.4	30.0	17U (F, 71)	191	8	5	0	0	25.0	25.0
18E (F, 45)	10	150	50	4	0	33.0	30.0	18U (F, 39)	195	13	6	0	0	25.5	25.0
19E (F, 28)	13	160	44	0	0	31.1	27.5	19U (F, 21)	179	20	8	0	0	25.9	25.0
20E (F, 21)	1	153	47	3	0	33.2	30.0	20U (F, 50)	190	14	2	0	0	25.3	25.0
21E (F, 34)	2	130	25	1	0	31.8	30.0	21U (F, 43)	150	56	9	0	0	26.4	25.0
22E (F, 23)	0	29	173	2	0	39.3	37.5								
23E (F, 34)	2	88	115	1	0	35.5	37.5								
24E (F, 42)	93	103	9	0	0	27.6	27.5								
Mean age = 38 ± 12.2[b]						35.5 ± 6.4[c]	30 ± 5.4[d]	Mean age = 33 + 15 [b]						25.94 ± 0.6[c]	25 ± 0.3[d]

[a]F = female; M = male, [b,c]Mean ± standard deviation (SD), [d]Mean median value ± SD.

The unexposed (U) control group consisted of 21 unrelated healthy individuals living 80 km away from the spraying area. They were similar to the exposed group regarding their demographic characteristics and occupation but were not matched controls. Blood samples were collected and processed as for the exposed group, but not concomitantly.

None of the individuals analyzed in this study (neither the exposed group nor the control group) smoked tobacco, drank alcohol, took non-prescription drugs or had been exposed to pesticides during the course of their normal daily lives. All of the individuals included in this study mainly worked at home, sometimes cultivating and harvesting crops without the use of herbicides, pesticides or similar substances in the named activities and their windowed houses did not contain asbestos in the ceilings or roofs (Acción Ecológica, 2004).

The Comet assay is a rapid and sensitive method for the detection of DNA damage induced *in vivo* (Singh *et al.*, 1988, McKelvey-Martin *et al.*, 1993, Monroy *et al.*, 2005) or after environmental and occupational exposures (Albertini *et al.*, 1996, Leroy *et al.*, 1996).

The blood samples were assayed using the alkaline comet assay as described by Singh *et al.*, (Singh *et al.*, 1988) with the modifications implemented in our laboratory (Paz-y-Miño *et al.*, 2002). The comet assay slides were analyzed at 400x magnification using a Zeiss fluorescence microscope equipped with a calibrated ocular micrometer and a 50 W mercury lamp with and excitation filter of 515-560nm and a 590nm barrier filter.

Cells were visually allocated to classified one o five predefined categories (A-E) according to the amount of DNA in the comet's tail, tail and a rank-number of from 0 (A) to 400 (E) was assigned to quantify the damage in each cell and calculate a mean of the amount of DNA damage (Anderson *et al.*, 1994).

To measure the head-to-tail comet length randomly-selected cells from the center of the gel were measured using a calibrated scale and DNA migration was determined by measuring the nuclear DNA and the migrating DNA (Singh *et al.*, 1988).

An average of 200 cells per individual was scored and the mean and median comet length from each individual was used for statistical analysis by the Mann-Whitney U test, which was applied to determine the differences between exposed and control group in the comet assay.

We found that individuals in the group which had been exposed to spraying with the glyphosate-containing herbicide showed higher DNA migration levels than controls ($p < 0.001$), the exposed group having a mean total migration level of 35.50 μm as compared with 25.94 μm for the control group (Table 1). Comet types D and E were not observed in the control group (Table 1).

This work reports the results of the cytogenetic monitoring and DNA damage assessment of individuals exposed to aerial spraying of glyphosate in the northern part of Ecuador. A study of the genotoxicity of chemicals, such as glyphosate is important because of their possible consequences on human health and their association with cancer, as has been published in similar studies with pesticides (Paz-y-Miño *et al.*, 2002a). The Alaska Community Action on Toxics (ACAT, 1998) factshhet, other studies like Arbuckle *et al.*, (2001) and Richard *et al.*, (2005) reported that when people ingest or absorb glyphosate through their skin or bathe or drink in water contaminated with this herbicide a wide range of symptoms can occur, such as headaches or reactions which affect the eyes, skin, lungs, heart, blood cells and genitals and gonads. Ecuadorian governmental data confirms the existence of health problems associate with such symptoms in the spraying zone (MREE, 2003).

Published data showed that chromosomal damage induced by pesticides appears to be transient transient in acute or discontinuous exposure but cumulative in continuous exposure to complex agrochemical mixtures (Bolognesi, 2003).

Formulated herbicides containing glyphosate are more potent mutagens to animals and humans than pure glyphosate, most probably due to the concomitant effects of additional toxic components, such as surfactants (ACAT, 1998). The aerial spraying on the border between Ecuador and Colombia used 44% of Roundup-Ultra (see above) but the recommended application rate of this formulation in the USA is 1.6% to 7.7% up to a maximum concentration of 29% (MREE, 2003) and according to Acción Ecológica (2003) the application rate of the formulated product must not exceed 0.95 L ha^{-1}. In the area of our study the application rate was 23.4 L ha-1 (10.3 L ha^{-1} with respect to glyphosate) and therefore more than 20 times the maximum recommended application rate for the formulated product, which may explain our comet assay results (Table 1) (Acción Ecológica, 2003, Nivia, 2001).

The analysis of genes implicated in the process of DNA detoxification, would be useful to understand the genetic influence of some chemicals like glyphosate. In our study factors such us age and DNA damage were not found to be related and because most members of the exposed and control groups were female we cannot conclude anything regarding the influence of sex on the results of the comet assay. Similar results have been reported in other investigations, which report that in general terms sex and age seem to have little, if any, effect in pesticide exposed populations (Carbonell *et al.*, 1993, Steenland *et al.*, 1986).

However, we did find a higher degree of DNA damage in the exposed group compared to the control group (Table 1). The significant increase in DNA damage levels observed seem to reflect a general response to the formulation used during aerial spraying, since none of the individuals in the exposed group smoked tobacco or drank alcohol

or had been exposed to other pesticides when the samples were taken.

Our findings suggest the existence of a genotoxic risk for glyphosate exposure in the formulation used during the aerial sprayings and indicate the need for further studies on individuals exposed to glyphosate to determine its possible influence on genetic material.

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A 90-day safety study in Wistar rats fed genetically modified rice expressing snowdrop lectin *Galanthus nivalis* (GNA)

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Abstract

Genetically modified plants expressing insecticidal traits offer a new strategy for crop protection, but at the same time present a challenge in terms of food safety assessment. The present 90-day feeding study was designed to assess the safety of a rice variety expressing the snowdrop *Galanthus nivalis* lectin (GNA lectin), and forms part of a EU-funded project where the objective has been to develop and validate sensitive and specific methods to assess the safety of genetically modified foods. Male and female Wistar rats were given a purified diet containing either 60% genetically modified or parental rice for 90 days. This corresponds to a mean daily GNA lectin intake of approximately 58 and 67 mg/kg body weight for males and females, respectively. Prior to the animal study comprehensive analytical characterization of both rice materials was performed. The chemical analyses showed a number of statistically significant differences, with the majority being within the ranges reported in the literature. In the animal study a range of clinical, biological, immunological, microbiological and pathological parameters were examined. A number of significant differences were seen between groups fed the two diets, but none of them were considered to be adverse. In conclusion, the design of the present animal study did not enable us to conclude on the safety of the GM food. Additional group(s) where the expressed gene products have been spiked to the diet should be included in order to be able to distinguish whether the observed effects were due to the GNA lectin *per se* or to secondary changes in the GM rice.

Keywords: Genetically modified rice; Safety assessment; Animal study; GNA lectin; SAFOTEST

1. Introduction

Rice (*Oryza sativa* L.) is one of the most important cereal crops in the world. Worldwide it comprises approximately 23% of all calories consumed; in some countries more than 60% of the dietary calories are derived from this cereal (Khush, 2003).

The ever-increasing demands on yield are responsible for the development of many different high yielding varieties of rice. However, whilst the extensive cultivation of modern high yielding varieties has on the one hand resulted

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in a significant increase in yield of most food crops, including rice, on the other hand it has augmented the development towards monocultures, which often favour a drastic increase in the insects that feed upon these crops (Gatehouse and Gatehouse, 1998). Despite the extensive use of pesticides it has been estimated that 37% of all crop production is lost worldwide to pests and diseases, with at least 13% directly to insects (Gatehouse and Gatehouse, 1998). Thus, better and more sophisticated forms of crop protection are sought to ensure a stable food supply to meet the demands from an ever-increasing global population. During the past decade genetic transformation has resulted in a number of crop varieties expressing transgene(s) from related or unrelated taxa, as a novel, and often more effective, way of crop protection to ensure higher yields.

Genetically modified (GM) crops represent a challenge in terms of food safety assessment. In general, the safety of food for human consumption is based on the concept that there should be a reasonable certainty that no harm will result from intended uses under the anticipated conditions of food consumption (OECD, 1993). In Europe, the placing on the market of genetically modified foods is governed by Regulation (EC) 1829/2003 on genetically modified food and feed, and before that by the so-called novel food regulation (258/97 of January 1997). Documents giving guidance on the safety assessment of GM foods have been developed (SCF, 1996; FAO/WHO, 2000; EFSA, 2005). However, for issues concerning the safety no specific instructions on how to perform the requested investigations are given.

The present study forms part of a EU-funded research project 'New methods for the safety testing of transgenic food' (SAFOTEST), where the objective has been to develop and validate sensitive and specific methods to assess the safety of genetically modified foods. A rice variety with a novel gene inserted expressing the snowdrop *Galanthus nivalis* lectin (GNA lectin) was selected as one of the model crops to be tested in a 90-day feeding study in rats. GNA is a promising candidate as an inserted trait in GM-food since it is toxic to several species of insect pests due to its binding capacity to their gut epithelia (Powell et al., 1998), whilst at the same time considered non-toxic to mammals, due to the low binding capacity in the jejunum. A gene encoding GNA has now been inserted and expressed in a number of different food plants including rice, wheat, potato and sugarcane (Sudhakar et al., 1998; Rao et al., 1998; Stoger et al., 1999; Birch et al., 1999; Setamou et al., 2002) so as to increase the inherent resistance of these crops to insect pests.

Transgenic GNA rice and its parental control (ASD16) were assessed in a subchronic 90-day feeding study in rats; this duration is considered to be sufficient to provide data for use in evaluating safety or determining whether further studies are required (Howlett et al., 2003). To our knowledge, this is the first time a transgenic rice variety expressing GNA lectin has been subject to a safety assessment study in experimental mammals. Prior to the 90-day feeding study the two rice lines were subjected to a comprehensive analytical characterization. The compositional analyses measured a total of more than 50 parameters including major constituents and amino acids, fatty acids, minerals, vitamins, steryl ferulates and phytic acid. In addition the material was screened for contaminants (heavy metals, pesticides), and the microbiological quality was controlled through screening for mycotoxins and by bacterial/fungal counts. On the basis of these data, similarities and differences between the parental and the GM rice material used for the feeding study should be demonstrated. The results were also expected to assist in the interpretation of effects detected in the subchronic 90-day study.

The aim of the 90-day feeding study was to compare the safety of a novel insect-resistant rice variety expressing the snowdrop *G. nivalis* lectin (GNA lectin) to its parental variety. Furthermore, the outcome of the study and the overall experience gained should provide valuable lessons for the future safety assessment of genetically modified food crops.

2. Materials and methods

2.1. Production and characterization of transgenic rice seed

Transgenic rice (*O. sativa* L.) expressing the snowdrop lectin (*G. nivalis*; GNA) was generated by particle bombardment of immature rice embryos (cultivar ASD16) as previously described (Sudhakar et al., 1998). Transgene expression levels in mature seeds were estimated by immunoassay using Western blots (Gatehouse et al., 1997). The average GNA lectin content of mature seeds from the transgenic line was estimated to be 1.25% of the total soluble protein (unpublished data). Southern blot analysis of the selected transgenic line revealed the presence of a single copy of the transformation construct in the rice genome (unpublished data). Using inverse PCR and similarity studies (Altschul et al., 1997) of the cloned PCR product sequence, the transgene was shown to have integrated into a non-protein coding region of the rice genome.

Rice seeds of the transgenic (T_6 generation) and parental line used in the animal studies were bulked up at the Experimental Farm of Zhejiang University at Jiande County, Zhejiang Province of China. Rice seeds of both transgenic and parent were sown concurrently in the same field, and 30 days after sowing, seedlings were transplanted in adjacent fields. Regular practice of field management, fertilizer application and pest control were applied. A pesticide with Triazophos as the major active component was used to control stem borer damage at the vegetative (2 weeks after transplantation), the booting and the heading stages. Rice seeds were harvested in about 4 weeks after heading. The rice seeds were later sent from China to the Danish Institute for Food and Veterinary Research (DFVF), Denmark as whole rough rice. After arrival the rice was dehulled using a testing husker THU 35B (Sakate Corporation, Japan) and milled with a hammer mill SB-89 (United Milling Systems, Denmark). The resulting flour from the brown rice was kept at −20 °C until use.

2.2. Compositional analysis

Proximates (moisture, starch, fiber, sugars, protein, fat, and ash), amino acids, fatty acid distribution and minerals were determined using validated standard protocols (VDLUFA, 1996, 1997). The content of protein was calculated using a nitrogen to protein conversion factor of 5.95. Vitamin B_1 was measured by the AOAC method (AOAC, 2000). Extraction and HPLC analysis of vitamin B_6 were performed according to Reitzer-Bergaentzle et al. (1993). To measure total vitamin B_6 contents including pyridoxol glucosides, extracts were treated with β-glucosidase

(Bognar and Ollilainen, 1997). Niacin was extracted according to Ward and Trenerry (1997) and determined via HPLC analysis (Wills et al., 1977). Folate vitamers and total pantothenic acid were quantified by stable isotope dilution assays based on LC/MS/MS (Freisleben et al., 2003; Rychlik, 2003). A method based on on-line coupled liquid chromatography–gas chromatography was used for determination of γ-oryzanol contents and steryl ferulate distributions (Miller et al., 2003). Phytic acid was measured using a colorimetric method (Latta and Eskin, 1980). Heavy metals (cadmium, lead, mercury) were measured by AAS (VDLUFA, 1996). Analysis for mycotoxins included aflatoxins (B_1, B_2, G_1, G_2), ochratoxin A, zearalenon and deoxynivalenol (VDLUFA, 1997). Bacterial and fungal counts were measured using validated standard protocols (VDLUFA, 1997). Pesticides were determined according to DFG (1991).

2.3. Animals and housing

Sixty-four male and female Wistar rats (SPF) were obtained from M&B Breeding Center, Ll. Skensved, Denmark. The rats were 4–5 weeks old at the start of treatment. They were kept in stainless steel wire cages (two/cage) at 22 ± 1 °C, relative humidity 55 ± 5%, air change 10 times/h, and electric light from 09.00 to 21.00. The guidelines formulated in Council of Europe's 'Convention for the protection of vertebrate animals used for experimental and other scientific purposes' were strictly followed. Housing and treatments of the rats followed procedures approved by the Danish Animal Experimentation Inspectorate.

2.4. Experimental design

The animals were randomly sorted into two experimental groups each comprising 16 males and 16 females. The rats were fed diets of defined composition containing either 60% GNA rice or parental ASD16 rice for 13 weeks (Table 1). Ingredients used in large amounts like the rice flour were added directly and thoroughly mixed into the purified diet to ensure homogeneity. Vitamins and minerals were added via premixes. All ingredients were ground to a similar particle size to ensure a homogeneous mixture. The purified diet used in the study is produced in house (Poulsen et al., 2002), based on the rodent diet AIN-93 (Reeves et al., 1993). Diets and acidified water (adjusted to pH 3.5 by citric acid to prevent growth of microorganisms) were provided *ad libitum*. During the experimental period all animals were inspected twice daily. Body weight, and food and water consumption were recorded weekly. Two weeks before sacrifice, blood was collected from all animals. Six days before sacrifice eight male and eight female rats from each group were immunized with sheep red blood cells (SRBC). At termination, all animals were anaesthetised by carbon dioxide inhalation and killed by exsanguinations for gross and histopathological examination.

Table 1
Composition of diets

Ingredients (%)	Group 1	Group 2
Control rice	60	0
GNA rice	0	60
Na-caseinate	14	14
Corn starch	5.2	5.2
Sucrose	6.8	6.8
Soybean oil	5	5
Cellulose	5	5
Mineral mixture[a]	2.8	2.8
Vitamin mixture[b]	1.2	1.2

[a] Containing in mg/kg diet: 5000 Ca; 3100 P; 3600 K; 300 S; 2500 Na; 1500 Cl; 600 Mg; 34 Fe; 30 Zn; 10 Mn; 7 Cu; 0.20 I; 0.15 Mo; 0.15 Se; 2.5 Si; 1.0 Cr; 1.0 F; 0.5 Ni; 0.5 B; 0.1 Li; 0.1 V; 0.07 Co.

[b] Containing in mg/kg diet: 5000 (IU) vitamin A; 1000 (IU) vitamin D_3; 50 (IU) vitamin E; 5 thiamin; 6 riboflavin; 8 pyridoxol; 2 folic acid; 0.3 D-biotin; 0.03 vitamin B-12; 20 pantothenate; 2600 cholinhydrogentartrate; 400 inositol; 40 nicotinic acid; 1 phylloquinine; 40 *p*-aminobenzoic acid; 1000 methionine; 2000 L-cystine.

2.5. Blood biochemistry

Blood samples were taken under Hypnorm/Dormicum anaesthesia from the tail vein in the penultimate week of treatment. The animals were fasted overnight to minimise fluctuations in the parameters measured. The samples were stabilised using heparin. The following plasma biochemical parameters were measured: urea, alanine aminotranferase (ALAT), sodium, potassium, cholesterol, protein, albumin, creatinine and glucose using a Cobas Mira S analyser (Roche, Switzerland).

2.6. Haematology

Blood samples were taken under Hypnorm/Dormicum anaesthesia from the tail vein in the penultimate week of treatment and stabilised using EDTA. The following characteristics were assessed using a Vet ABC, Animal Blood Counter (Analysis instruments AB, Stockholm, Sweden): hematocrit (HCT), haemoglobin concentration (HC), erythrocyte count (RBC), mean cell haemoglobin concentration (MCHC), mean cell haemoglobin (MCH), mean cell volume (MCV), platelet count (PLT), and total and differential leucocyte count (WBC). The differential count distinguishes neutrophils (N), lymphocytes (L), eosinophils (E), basophils (B), monocytes (M) and a small proportion of large unstained cell (LU).

2.7. Immunology

To determine the primary antibody response against sheep red blood cells (SRBC), one of the most sensitive immunotoxicological parameters P (Luster et al., 1992), rats were immunized with an intravenous injection of 2×10^8 SRBC in 0.5 ml of sterile saline in the tail vein 6 days prior to sacrifice. SRBC from a single animal source (Statens Serum Institut, Copenhagen, Denmark) were used for all experiments. Sera samples obtained at sacrifice were analysed in enzyme-linked immunosorbent assay (ELISA) for anti-SRBC IgM as well as for anti-GNA and concentrations of total IgM, IgG and IgA.

For quantification of total IgM, IgG and IgA, 96-well microtitre plates (MaxiSorp, Nunc, Roskilde, Denmark) were coated with anti-rat IgM, IgG, or IgA in carbonate buffer (0.05 M, pH 9.6) for 1 h at RT followed by blocking of remaining active sites for 1 h at RT with PBS/Tween buffer. Thereafter plates were incubated with serially dilutions of rat sera and standards (purified rat IgM, IgG, or IgA) for 1 h at RT and then incubated for 1 h at RT with horseradish peroxidase-labelled (HRP-labelled) anti-rat IgM, IgG, or IgA. Plates were developed with TMB-one substrate for 10 min in the dark, the reaction terminated by the addition of 0.2 M sulphuric acid and read at 450 nm with a reference wavelength of 630 nm, using a microtitre plate reader (Bio-Tek Instruments, Winooski, VT, USA). Concentrations of IgM, IgG, or IgA in serum samples were interpolated from the linear part of the third-degree polynomial standard curves for purified IgM, IgG, and IgA, respectively. The average concentration of at least two serum dilutions was used for final calculation and expressed as mg/ml serum.

Anti-SRBC IgM-specific ELISA was performed as previously described by Temple et al. (1993, 1995). Briefly, haemoglobin-free SRBC membranes were prepared by wash of defibrinated SRBC in saline and removal of the buffy coat. Then SRBC were lysed and haemoglobin removed by repeated washing with Tris–EDTA buffer followed by suspension to 0.5 mg/ml in PBS with 0.1% SDS, dialysed for 24 h at RT and stored at −20 °C. For detection of anti-SRBC IgM, plates were coated overnight at 4 °C with 0.5 μg/ml of SRBC membranes in carbonate buffer then blocked with PBS/Tween buffer for 1 h at 37 °C followed by incubation with serially dilutions of rat sera (starting at 1:16) for 1 h at RT. After incubation with HRP-labelled goat anti-rat IgM antibody for 1 h at RT, plates were developed for 10 min in the dark as described above. Known positive and negative serum pool controls were included in each plate. Positive controls were obtained from rats immunized intravenously

(2×10^8 SRBC/rat) six days before blood sampling. Serum from untreated rats was used as negative control. The antibody titres were expressed as $\log_2$ titres and defined as the interpolated dilution of a serum sample leading to an absorbance of 0.5.

For detection of GNA-specific IgG1, IgG2a and IgA, plates were coated for 2–3 days at 4 °C with 2 μg/ml of GNA lectin in carbonate buffer, blocked for 1 h at 37 °C and then incubated with serial dilutions of rat sera (starting at 1:4) for 1 h at RT. Thereafter plates were incubated with HRP-labelled anti-rat IgG1, IgG2a or IgA antibodies for 1 h at RT and then developed for 10 min in the dark as described above. Known positive and negative serum pool controls were included in each plate. Positive controls were obtained from rats immunized intraperitoneally with 100 μg/0.5 ml/rat of GNA lectin absorbed on 12 mg $Al(OH)_3$ at day 1 and with 10 μg/0.5 ml/rat of GNA lectin at day 21, 35 and 49. Serum from untreated rats was used as the negative control. The antibody titres were expressed as $\log_2$ titres, defined as the interpolated dilution (3-parameter analysis) of a serum sample leading to an absorbance of 0.2.

To prepare the spleen cells, spleens were aseptically removed and weighed and transferred into sterile 50 ml tubes with Hank's balanced salt solution and kept on ice until preparation. Single-cell suspensions of splenocytes from each rat were prepared and suspended in culture medium (RPMI 1640 with phenol red supplemented with 2 mM L-glutamine 10% heat-inactivated FBS, 100 μg/ml of penicillin and 100 IU/ml of streptomycin). Cell numbers were determined for each splenocyte suspension and viability was verified by eosin exclusion.

A modified non-radioactive assay was used for T-cell dependent proliferation as previously described (Ahmed et al., 1994; Zhi-Jun et al., 1997). Spleen lymphocytes were cultured in quadruplicates in a 96-well black flat-bottomed tissue culture plate with 0 (basal proliferation), 0.04, 0.2 or 0.4 μg/well of concanavalin A (Con A) or phytohemagglutinin (PHA-L, Sigma). The plates were incubated at 37 °C in a 5% humidified atmosphere of CO_2. After 48 h of culture, the non-radioactive fluorescent dye alamarBlue (BioSource International, Camarillo, CA, USA) were added to each well and 24 h later plates were read on a fluorometer with excitation at 530 and emission at 590 nm. The mitogenic responsiveness was calculated as the average of the absolute fluorescence units (FLU) of replicates with mitogen minus the average of FLU of replicates without mitogen (ΔFLU ± SD).

2.8. Bacteriological quantification

During the experimental period, fresh faecal samples were taken from 10 animals (5 males and 5 females) from each of the two groups by provoked defaecation for microbial analysis at day 0, 30 and 60, and at termination of the study. Furthermore, at sacrifice, samples from ileum and duodenum were taken from the same 10 animals from each group. The faecal and intestinal samples were homogenized in saline supplemented with 0.1% peptone to 10^{-1} dilution. Ten-fold serial dilutions were prepared in the same buffer and samples were applied to appropriate selective media. The total aerobic and anaerobic populations were enumerated on RCA plates (Reinforced Clostridial Agar, Oxoid), and incubated for 72 h aerobically and anaerobically, respectively. Rogosa agar plates (Oxoid) were used to determine the number of *Lactobacillus* in the samples. Bifidobacteria were counted on RCA plates containing supplement according to Munoa and Pares (1988). *Lactobacillus* and Bifidobacteria were counted after anaerobic growth for 72 h. MacConkey (MacConkey Agar no. 3, Oxoid) and Slanetz (Slanetz & Bartley Medium, Oxoid) were used for the detection of Enterobacteria and Enterococci, respectively. The plates were incubated aerobically for 24 and 48 h, respectively. On the MacConkey plates all intense violet–red colonies were counted. On the Slanetz plates all red or maroon colonies were counted. All plates were incubated at 37 °C.

2.9. Organ weights, gross necropsy and histopathology

A thorough necropsy was performed and the following organs were excised and weighed: testes, epididymis, ovaries, uterus, small intestine, liver, kidneys, adrenals, pancreas, spleen, mesenterial lymph nodes, heart, thyroid, brain. Tissues from these organs and macroscopically evident lesions were fixed in 4% buffered formaldehyde for histological processing. Tissue samples were embedded in paraffin and sections, 4–6 μm thick, stained with standard hematoxylin–eosin for light microscopy. In addition, the intact small intestines were flushed with a 0.09% NaCl solution and the length measured.

2.10. Statistical analysis

Compositional data are presented as means ± confidence intervals ($p < 0.05$). Means are considered as statistically significantly different if their confidence intervals are not overlapping. Data obtained from the animal studies were analysed separately for each sex and presented as mean ± SD where appropriate. The homogeneity of variance between groups was evaluated by judgement of standard residual plots (General Linear Model procedure). Statistical comparisons of body weight, food and water consumption, bacterial counts, clinical biochemistry, haematology, immunology and organ weights between control rats and GNA fed rats were performed by one-way analysis of variance and if significant a *t*-test was performed. Data not showing homogeneity of variance and normal distribution was transformed and the analysis of variance procedure was repeated. If the homogeneity of variance still not was obtained, data was subjected to a Kruskall–Wallis test followed by Wilcoxons test for pair-wise comparisons if significant. Differences were considered significant at $p < 0.05$. All statistical analyses were carried out using SAS release 8.1 (SAS Institute Inc., Cary, NC).

3. Results

3.1. Agronomic parameters

The number of days from sowing to heading of the transgenic rice is about three weeks shorter than its parent, and the former was also shorter in plant height and less in biomass than the parent. However, the parent had a lower seed-set (<50%) compared to GNA rice, probably because it experienced a period of high temperature (higher than 35 °C) before heading.

3.2. Compositional analysis

Transgenic (GNA) and parental (ASD16) brown rice tested in the 90-day study were subjected to comprehensive analytical characterization. Compositional data were compared to data reported for brown rice (Latta and Eskin, 1980; Juliano, 1985; Scherz and Senser, 2000; Møller et al., 2002; USDA, 2004; OECD, 2004) and differences between the lines were assessed for statistical significance ($p < 0.050$).

Contents of proximates are presented in Table 2. No statistically significant differences between transgenic and parental rice were observed for moisture and fat content. However, GNA rice exhibited statistically significant higher contents of fiber (+29%), sugars (+100%), protein (+19%) and ash (+35%), and a statistically significant lower starch content (−8%). Compared to data presented in the literature (Juliano, 1985; Scherz and Senser, 2000; Møller et al., 2002; USDA, 2004; OECD, 2004) both lines exhibited high contents of protein, fat and ash.

Table 2
Proximate composition of brown rice material from transgenic line GNA and its corresponding parental line ASD16 (mean ± confidence interval, $n = 4$, $p < 0.05$)

Component (%)	GNA	ASD16	Literature data[a]
Moisture	12.9 ± 0.2	12.8 ± 0.3	9.1–14.1
Starch	63.9 ± 0.7[b]	69.3 ± 1.8	57–77
Fiber	1.8 ± 0.1[b]	1.4 ± 0.3	0.5–3.5
Sugars	0.6 ± 0.1[b]	0.3 ± 0.1	0.6–1.3
Protein	12.6 ± 0.2[b]	10.5 ± 0.3	6.1–9.5
Fat	3.23 ± 0.44	3.47 ± 0.10	1.4–2.9
Ash	2.01 ± 0.03[b]	1.49 ± 0.03	0.9–1.5

[a] Ranges from minimum to maximum reported values (Juliano, 1985; Scherz and Senser, 2000; Møller et al., 2002; USDA, 2004; OECD, 2004).
[b] Statistically significant different from parental line ($p < 0.05$).

Table 3
Amino acid levels in brown rice material from transgenic line GNA and its corresponding parental line ASD16 (g/100 g; mean ± confidence interval, $n = 3$, $p < 0.05$)

Amino acid	GNA	ASD16	Literature data[a]
Alanine	0.75 ± 0.09[b]	0.57 ± 0.05	0.46–0.58
Arginine	1.03 ± 0.04[b]	0.88 ± 0.07	0.44–0.91
Aspartic acid	1.55 ± 0.04[b]	1.39 ± 0.10	0.74–0.87
Cystine	0.33 ± 0.02	0.31 ± 0.01	0.06–0.19
Glutamic acid	2.28 ± 0.21[b]	1.77 ± 0.16	1.52–1.76
Glycine	0.64 ± 0.07	0.60 ± 0.03	0.39–0.49
Histidine	0.50 ± 0.07[b]	0.38 ± 0.04	0.12–0.27
Isoleucine	0.55 ± 0.04[b]	0.44 ± 0.05	0.26–0.57
Leucine	1.06 ± 0.09[b]	0.88 ± 0.02	0.50–0.93
Lysine	0.63 ± 0.05[b]	0.46 ± 0.04	0.10–0.42
Methionine	0.38 ± 0.03	0.35 ± 0.05	0.05–0.31
Phenylalanine	0.77 ± 0.15	0.58 ± 0.07	0.30–0.55
Proline	0.95 ± 0.04[b]	0.45 ± 0.01	0.37–0.40
Serine	0.63 ± 0.05[b]	0.51 ± 0.05	0.41–0.50
Threonine	0.47 ± 0.05[b]	0.35 ± 0.07	0.19–0.62
Tryptophan	0.16 ± 0.00[b]	0.13 ± 0.01	0.03–0.11
Tyrosine	0.72 ± 0.08	0.62 ± 0.15	0.21–0.47
Valine	0.78 ± 0.03[b]	0.67 ± 0.05	0.40–0.76

[a] Ranges from minimum to maximum reported values (Scherz and Senser, 2000; USDA, 2004).
[b] Statistically significant different from parental line ($p < 0.05$).

The difference in protein content was also reflected in the amino acid levels (Table 3). GNA rice exhibited statistically significant higher contents of almost all amino acids. In particular the content of proline was very high in GNA rice compared to the parental rice (+111%). Except for isoleucine, threonine and valine, data for GNA rice exceed data reported in the literature (Scherz and Senser, 2000; USDA, 2004).

Fatty acid distributions of the two lines were similar (Table 4). Minor but statistically significant differences were detected for proportions of myristic acid (+33%) and stearic acid (+26%). Patterns of both lines were in agreement to data reported in literature (Scherz and Senser, 2000; USDA, 2004; OECD, 2004; Kitta et al., 2005).

Mineral compositions are presented in Table 5. No statistically significant differences were observed for contents of calcium, magnesium, molybdenum and zinc. However,

Table 4
Fatty acid distribution in brown rice material from transgenic line GNA and its corresponding parental line ASD16 (mean ± confidence interval, $n = 4$, $p < 0.05$)[a]

Fatty acid	GNA	ASD16	Literature data[b]
Myristic acid	0.4 ± 0.0[c]	0.3 ± 0.0	0.4–3.0
Palmitic acid	20.2 ± 0.2	19.9 ± 0.1	18–31
Stearic acid	2.4 ± 0.0[c]	1.9 ± 0.0	1.6–2.6
Oleic acid	39.0 ± 0.1	39.4 ± 0.1	27–41
Linoleic acid	33.2 ± 0.2	33.2 ± 0.1	31–40
Linolenic acid	1.4 ± 0.0	1.4 ± 0.0	0.9–1.7

[a] Proportions of total fatty acids (%).
[b] Ranges from minimum to maximum reported values (Scherz and Senser, 2000; USDA, 2004; OECD, 2004; Kitta et al., 2005).
[c] Statistically significant different from parental line ($p < 0.05$).

Table 5
Contents of minerals in brown rice material from transgenic line GNA and its corresponding parental line ASD16 (mean ± confidence interval, $n = 4$, $p < 0.05$)

Mineral	GNA	ASD16	Literature data[a]
Calcium (g/kg)	0.3 ± 0.0	0.2 ± 0.0	0.1–0.5
Copper (mg/kg)	2.5 ± 0.2[b]	3.1 ± 0.1	1–6
Iron (mg/kg)	44 ± 4[b]	18 ± 2	2–52
Magnesium (g/kg)	1.6 ± 0.0	1.5 ± 0.0	0.2–1.7
Manganese (mg/kg)	24.6 ± 0.3[b]	21.4 ± 0.3	2–37
Molybdenum (mg/kg)	1.3 ± 0.1	1.2 ± 0.1	0.3–1.0
Phosphorous (g/kg)	4.3 ± 0.0[b]	3.6 ± 0.0	1.7–4.4
Potassium (g/kg)	3.4 ± 0.1[b]	2.6 ± 0.1	0.6–2.8
Zinc (mg/kg)	24.5 ± 11.2	28.0 ± 0.3	6–28

[a] Ranges from minimum to maximum reported values (Juliano, 1985; Scherz and Senser, 2000; Møller et al., 2002; USDA, 2004).
[b] Statistically significant different from parental line ($p < 0.05$).

GNA rice exhibited statistically significant higher contents of iron (+144%), manganese (+15%), phosphorous (+19%) and potassium (+31%), and a statistically significant lower copper content (−19%). Given the very large variation previously reported for mineral levels in brown rice (Juliano, 1985; Scherz and Senser, 2000; Møller et al., 2002; USDA, 2004), these differences observed between GNA and ASD16 were considered as low.

Statistically significant differences were found for important vitamins of the B-complex (Table 6). GNA rice exhibited higher contents of vitamin B_1 (+28%) and B_6 (+50%). No statistically significant difference was observed for the niacin content. Whereas the content of total pantothenic acid was higher in GNA rice (+52%), the content of total folic acid was higher in the parental rice (+129%). In both lines 5-methyl-H_4 folate was the major folate vitamer. Vitamin contents for both lines were in agreement with data reported for brown rice (Juliano, 1985; Scherz and Senser, 2000; Møller et al., 2002; USDA, 2004).

γ-Oryzanol comprises a mixture of steryl ferulates found in rice (Xu and Godber, 1999). They exhibit antioxidative (Xu et al., 2001) and cholesterol-lowering properties (Rong et al., 1997). No statistically significant difference was observed for the γ-oryzanol contents (Table 7). Data from

Table 6
Contents of vitamins in brown rice material from transgenic line GNA and its corresponding parental line ASD16 (mg/kg; mean ± confidence interval, $p < 0.05$)

Vitamin	GNA	ASD16	Literature data[a]
B_1[b]	4.1 ± 0.5[c]	3.2 ± 0.2	2.9–6.1
B_6[b]	1.8 ± 0.2[c]	1.2 ± 0.3	2–10
Niacin[b]	51 ± 2	57 ± 10	35–58
Total pantothenic acid[d]	13.7	9.0	9–17
Total folic acid [d]	0.14	0.32	0.1–0.5
5-Methyl-H_4 folate[d,e]	0.12	0.29	
5-Formyl-H_4 folate[d,e]	0.02	0.03	

[a] Ranges from minimum to maximum reported values (Juliano, 1985; Scherz and Senser, 2000; Møller et al., 2002; USDA, 2004).
[b] $n = 3$.
[c] Statistically significant different from parental line ($p < 0.05$).
[d] $n = 2$.
[e] Calculated as folic acid.

the present study were within the range reported in the literature (Miller et al., 2003). Apart from minor, although statistically significant differences, both lines exhibited similar steryl ferulate distributions. Compared to data presented in the literature, both lines exhibited low proportions of cyclartenyl ferulate but high proportions of 24-methylenecycloartanyl ferulate.

Phytic acid is known to be an anti-nutritient of rice, and has been shown to limit the bioavailability of minerals (Saha et al., 1994). Both transgenic rice and parental rice exhibited similar contents of phytic acid (0.88% and 0.87%, respectively; $n = 2$) and were in agreement with the literature (0.6–1.6%; Latta and Eskin, 1980).

To evaluate the microbiological quality of the rice material, bacterial and fungal counts were measured and the material screened for the presence of mycotoxins. Both materials exhibited similarly low bacterial ($<0.3 \times 10^6$/g) and fungal counts ($<5.4 \times 10^3$/g). No mycotoxins were detected in the material.

As regards contaminants, no lead was detected in either of the lines (<0.05 mg/kg) although low levels of mercury were found in both GNA rice (0.019 ± 0.001 mg/kg, $n = 4$) and parental rice (0.027 ± 0.001 mg/kg). Whilst the transgenic rice line only exhibited a low cadmium content (0.02 ± 0.01 mg/kg), the parental line contained high levels of cadmium (0.17 ± 0.01 mg/kg). However, for both lines the cadmium levels were below the limit set for rice by Commission Regulation (EG) 466/2001.

The pesticide Triazophos was detected in both the GNA rice (0.12 ± 0.07 mg/kg) and the control rice (0.41 ± 0.11 mg/kg). The contents of all other pesticides analysed were below the detection limit of the method applied.

3.3. Clinical observations, body weight and food and water intake

During the course of the experiment no treatment-related signs of adverse effects in clinical appearance of the animals were observed. The body weights of male rats were comparable throughout the study period, whereas female rats given the GNA rice tended to have a lower body weight ($p = 0.065$) compared to rats in the control group (Fig. 1). The reduction in body weight seen in week 12 was due to the blood sampling procedure and fasting of

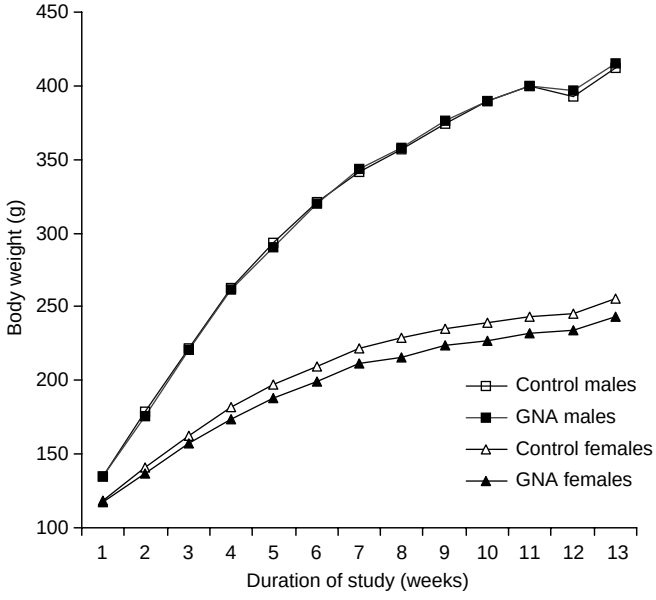

Fig. 1. Growth curves based on weekly measurements of body weight during the study. The curves show group means based on 16 rats/sex/group. SE bars not shown for clarity.

Table 7
γ-Oryzanol (steryl ferulates) in brown rice material from transgenic line GNA and its corresponding parental line ASD16 (mean ± confidence interval, $n = 3$, $p < 0.05$)

	GNA	ASD16	Literature data[a]
γ-Oryzanol (mg/100 g)	32 ± 3	28 ± 4	31–63
Steryl ferulate distribution[b]			
Campesteryl ferulate	13.5 ± 0.2[c]	15.5 ± 0.4	7–19
Campestanyl ferulate	5.9 ± 0.2[c]	7.2 ± 0.3	6–13
β-Sitosteryl ferulate	7.3 ± 0.2[c]	8.8 ± 0.3	5–10
Cycloartenyl ferulate	20.6 ± 0.4[c]	25.0 ± 0.4	33–47
24-Methylenecycloartanyl ferulate	52.8 ± 0.9[c]	43.7 ± 0.6	27–36

[a] Ranges from minimum to maximum values reported in Miller et al. (2003).
[b] Proportions of total γ-oryzanol content (%).
[c] Statistically significant different from parental line ($p < 0.05$).

Table 8
Food consumption – group mean values ± SD

Group	Week 2–7 (g/animal/week)	Week 8–13 (g/animal/week)
1M	130 ± 9	135 ± 14
2M	132 ± 13	134 ± 18
1F	94 ± 7	97 ± 13
2F	89 ± 8	93 ± 14

1M: control rice-males, 2M: GNA rice-males, 1F: control rice-females, 2F: GNA rice-females.

the animals. There was no statistically significant difference in food consumption between groups, although consumption was slightly lower in females fed the diet containing GNA rice (Table 8). However, a marked significantly higher relative water intake was seen in both males and females given GNA rice (Figs. 2 and 3). The absolute water intake was significantly higher for males, but not females, fed GNA rice.

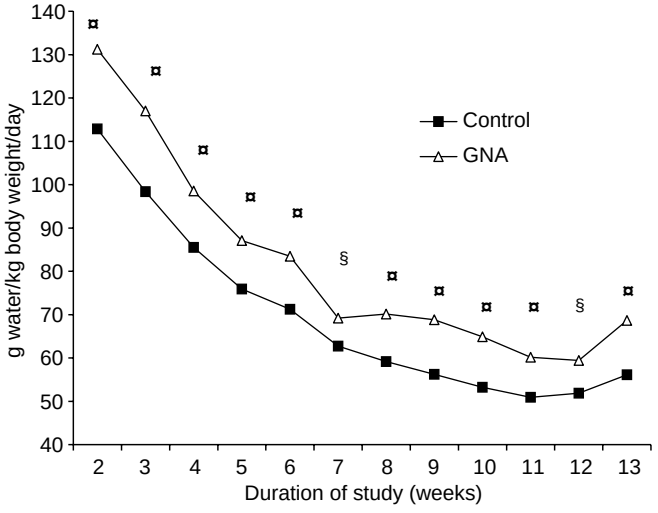

Fig. 2. Mean relative water intake of male rats. SE bars not shown for clarity. (§) and (¤) indicate significantly different from control group at $p < 0.01$ and $p < 0.001$, respectively. $n = 16$.

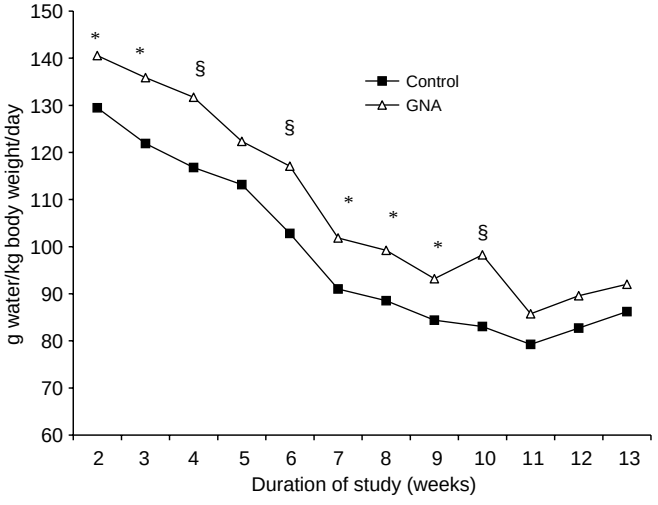

Fig. 3. Mean relative water intake of female rats. SE bars not shown for clarity. (*) and (§) indicate significantly different from control group at $p < 0.05$ and $p < 0.01$, respectively. $n = 16$.

3.4. Blood biochemistry

Data on blood biochemistry are presented in Table 9. Male rats given GNA rice had a significantly lower plasma concentration of potassium, and levels of protein and albumin were significantly lower in both males and females given GNA rice. Levels of creatinine were significantly lower in female rats fed GNA rice. Furthermore, significantly higher plasma activities of alanine aminotransferase were seen in female rats fed the GNA rice.

3.5. Haematology

Only a few statistically significant differences occurred in the haematological parameters between rats fed GNA rice and the parental control (Table 10). In male rats the platelet count (PLT) was slightly higher in the group fed GNA rice, whereas the mean cell haemoglobin concentration (MCHC) was slightly lower compared to the control group. Female rats given GNA rice had a lower number of large unstained cells (LU) compared to the female control group.

3.6. Immunological parameters

No statistical differences in the basal level of total IgM, IgG and IgA nor the anti-SRBC IgM response were observed between groups within the same sex (Table 11).

Immunization with SRBC statistically increased the concentration of total IgM in male and female rats ($p \leqslant 0.001$ and $p \leqslant 0.05$, respectively), total IgG in female rats ($p \leqslant 0.01$), and total IgA in male rats ($p \leqslant 0.01$) in the control group. In addition, immunization with SRBC generally increased the concentration of total IgM, IgG, and IgA in rats fed GNA rice although this was only found to be statistically different for the total IgM concentration in females (data not shown). Feeding rats with GNA rice did not induce a detectable GNA-specific antibody response for any of the tested antibody isotypes IgG1, IgG2a and IgA (data not shown).

GNA rice statistically reduced the mitogen-induced proliferative response at optimal concentrations of Con A in female rats, whereas a tendency to an increased response was found in male rats at the same concentrations (Table 11). Generally, the group fed GNA rice did not statistically differ in PHA lectin-induced proliferative response from the control group, although a statistical increase at the suboptimal concentration of PHA lectin was found in female rats (Table 11).

3.7. Bacteriological quantification of faecal samples

For the faecal samples no significant difference could be detected between the group fed GNA rice and the control

Table 9
Blood biochemistry – group mean values ± SD

Group	*N*	Urea (mmol/l)	ALAT (U/l)	Sodium (mmol/l)	Potassium (mmol/l)	Cholesterol (mmol/l)	Protein (g/l)	Albumin (g/l)	Creatinine (μmol/l)	Glucose (mmol/l)
1M	15	5.2 ± 0.6	28.6 ± 7.3	146.7 ± 16.2	4.8 ± 0.7	1.4 ± 0.2	61.0 ± 2.1	39.0 ± 0.9	32.4 ± 8.9	7.8 ± 0.9
2M	16	5.6 ± 0.6	31.4 ± 7.5	146.0 ± 17.5	4.2[a] ± 0.5	1.3 ± 0.3	59.4[a] ± 1.8	37.9[a] ± 1.3	36.3 ± 6.4	8.4 ± 1.7
1F	16	7.2 ± 0.9	20.8 ± 4.6	139.1 ± 23.8	4.1 ± 1.0	1.2 ± 0.3	65.5 ± 4.2	45.6 ± 3.7	52.1 ± 9.6	7.8 ± 1.2
2F	16	6.7 ± 1.1	24.8[a] ± 3.4	142.6 ± 10.7	4.0 ± 0.5	1.1 ± 0.2	62.2[a] ± 3.2	42.7[a] ± 2.4	44.1[a] ± 10.1	7.4 ± 1.0

1M: control rice-males, 2M: GNA rice-males, 1F: control rice-females, 2F: GNA rice-females.
[a] Statistically significantly different from control group within same sex when a Students *t*-test was performed ($p \leqslant 0.05$).

Table 10
Haematology – group mean values ± SD

Group	*N*	RBC 10^{12}/L	PLT (10^9/l)	HC (mmol/l)	HCT (%)	MCV FL	MCH (fmol)	MCHC (mmol/l)	Total (10^9/l)	L (%)	N (%)	WBC			
												M (%)	E (%)	B (%)	LU (%)
1M	15	8.64 ± 0.4	527 ± 135	15.5 ± 0.5	45.8 ± 1.3	53 ± 2	17.9 ± 0.7	33.7 ± 0.7	4.1 ± 1.2	75.5 ± 10.2	20.4 ± 9.3	2.8 ± 1.3	1.1 ± 0.8	0.0 ± 0.1	0.1 ± 0.2
2M	16	8.70 ± 0.3	617[a,1] ± 83	15.4 ± 0.6	46.5 ± 1.6	53 ± 2	17.6 ± 0.6	33.0[a,1] ± 0.3	4.1 ± 1.4	80.8 ± 5.5	16.0 ± 4.8	1.7 ± 1.6	1.2 ± 0.6	0 ± 0	0.1 ± 0.2
1F	16	8.12 ± 0.4	611 ± 142	14.8 ± 0.6	43.6 ± 1.6	54 ± 1	18.2 ± .0.6	33.8 ± 0.4	2.7 ± 1.0	78.2 ± 6.8	19.2 ± 6.6	1.2 ± 1.0	1.3 ± 0.8	0 ± 0	0.2 ± 0.2
2F	16	8.00 ± 0.3	566 ± 147	14.6 ± 0.5	43.6 ± 1.4	55 ± 2	18.3 ± 0.6	33.6 ± 0.6	2.1 ± 0.5	82.1 ± 8.2	15.5 ± 8.0	0.8 ± 0.5	1.6 ± 1.5	0 ± 0	0[a,2] ± 0

1M: Control rice-males, 2M: GNA rice-males, 1F: control rice-females, 2F: GNA rice-females.
[a] Statistically significantly different from control group within same sex ($p < 0.05$). Statistical analysis; 1: *t*-test, 2: Wilcoxon two-sample test.

Table 11
Immunological parameters – group mean values ± SD

Analysis	Group			
	1M	2M	1F	2F
Total IgM[a]	0.225 ± 0.06	0.282 ± 0.07	0.265 ± 0.09	0.282 ± 0.09
Total IgG[a]	1.977 ± 0.67	2.232 ± 0.63	2.991 ± 0.73	3.493 ± 1.12
Total IgA[a]	0.029 ± 0.01	0.038 ± 0.01	0.028 ± 0.01	0.032 ± 0.01
Anti-SRBC IgM[b]	8.98 ± 0.76	7.98 ± 1.79	8.65 ± 2.07	8.28 ± 0.79
Con A (0.04 μg/well)[c]	22.4 ± 12.5	17.3 ± 12.7	29.8 ± 10.5	21.7 ± 11.4
Con A (0.2 μg/well)[c]	42.8 ± 17.7	55.6 ± 12.5	60.2 ± 3.8	53.7 ± 3.9[d]
Con A (0.4 μg/well)[c]	52.9 ± 17.4	70.1 ± 15.0	75.9 ± 7.9	64.1 ± 6.0[d]
PHA (0.04 μg/well)[c]	11.2 ± 6.6	10.1 ± 3.9	4.9 ± 4.1	11.0 ± 6.4[d]
PHA (0.2 μg/well)[c]	26.2 ± 7.7	26.5 ± 7.1	19.6 ± 3.3	20.5 ± 3.2
PHA (0.4 μg/well)[c]	29.1 ± 5.0	30.4 ± 4.4	26.1 ± 5.8	25.1 ± 3.1

[a] Concentrations of total IgM, IgG and IgA are expressed as mg/ml.
[b] The anti-SRBC IgM response was measured as $\log_2$ titres.
[c] The proliferative responsiveness of splenocytes to Con A and PHA, respectively, is expressed as $\Delta FLU \times 10^3$ (see materials and methods).
[d] Statistically significant different from the control group ($p \leqslant 0.05$).

group (Table 12). However, in samples from the duodenum a statistically significant increase in the total anaerob, the Lactococcal and the Enterococcal population was observed in the GNA group compared to the control group (Table 13). This was not observed in the ileal samples, where a decrease in Enterobacteria was observed in the GNA group compared to the control group ($P < 0.005$).

3.8. Organ weights, gross necropsy and histopathology

The absolute and relative mean organ weights are presented in Table 14. A statistically significant increase in the relative weight of the small intestine (+10%) was observed in female rats fed on GNA rice, as well as an increase in absolute and relative weight of the adrenals

Table 12
Bacterial counts – faecal samples – means $\log_{10}$ cfu g^{-1} faeces ± SD

Group	Total aerob	Total anaerob	Lactobacilli	Bifidobacteria	Enterobacteria	Enterococci
Bacterial counts – day 0						
1	8.35 ± 0.59 (8)[a]	8.91 ± 0.44 (8)	7.05 ± 0.65	8.3 ± 0.4	7.93 ± 0.5	7.84 ± 0.55
2	8.46 ± 0.63 (9)	9.48 ± 0.42 (9)	7.44 ± 0.24	8.45 ± 0.48 (9)	8.02 ± 0.64	7.75 ± 0.64
Bacterial counts – day 30						
1	9.59 ± 0.39 (9)	9.5 ± 0.41 (7)	7.66 ± 1.01	8.91 ± 0.11	9.12 ± 0.53 (7)	8.52 ± 0.18
2	9.36 ± 0.7 (9)	9.46 ± 0.59	7.06 ± 0.68	8.83 ± 0.36	8.78 ± 1.13 (8)	8.54 ± 0.4
Bacterial counts – day 60						
1	7.96 ± 0.6 (7)	8.08 ± 0.52 (8)	7.73 ± 0.66	7.99 ± 0.55 (9)	7.33 ± 0.75	7.74 ± 0.8
2	7.94 ± 0.52	8.17 ± 0.89 (3)	7.67 ± 0.63	8.16 ± 0.62	7.39 ± 0.61	7.54 ± 0.58
Bacterial counts – day 90						
1	8.41 ± 0.41 (2)	8.31 ± 0.48 (8)	8.02 ± 0.89 (8)	8.02 ± 0.45 (8)	6.79 ± 0.69 (8)	7.49 ± 0.57 (3)
2	7.65 ± 0.27 (3)	8.5 ± 0.5 (7)	8.19 ± 0.46 (7)	8.17 ± 0.24 (7)	7.08 ± 0.53 (7)	8.25 ± 1.2 (5)

Group 1: Control rice, Group 2: GNA rice.
[a] The numbers in brackets indicate the number of animals, no number means 10 animals.

Table 13
Bacterial counts – intestinal samples – means $\log_{10}$ cfu g^{-1} intestinal content ± SD

Group	Total aerob	Total anaerob	Lactobacilli	Bifidobacteria	Enterobacteria	Enterococci
Bacterial counts – duodenum						
1	7.02 ± 0.22 (4)[a]	4.48 ± 0.39 (6)	6.71 ± 0.55 (7)	4.42 ± 0.49 (7)	3.8 ± 0.55 (8)	3.78 ± 0.28 (4)
2	6.6 ± 1.27 (4)	5.09 ± 0.71[b] (7)	7.22 ± 0.36[b] (8)	4.97 ± 0.76 (9)	3.88 ± 0.9 (6)	4.28 ± 0.34[b] (6)
Bacterial counts – ileum						
1	7.31 (1)	6.81 ± 0.78 (6)	7.46 ± 0.4 (4)	6.76 ± 0.55 (9)	6.69 ± 0.79	6.44 ± 0.75 (8)
2	6.34 ± 0.8 (6)	6.63 ± 0.45 (9)	7.41 ± 0.14 (3)	6.33 ± 0.62 (8)	5.72 ± 0.62[b] (10)	5.93 ± 0.68 (9)

Group 1: Control rice, Group 2: GNA rice.
[a] The numbers in brackets indicate the number of animals, no number means 10 animals.
[b] Statistically significant different from the control group ($p \leqslant 0.05$).

Table 14
Absolute and relative organ weights for rats fed on GNA rice diet and control rice diet

	Males		Females	
	GNA rice	Control	GNA rice	Control
Absolute weight				
Body weight	422 ± 33	417 ± 40	244 ± 22	257 ± 16
Adrenals	0.0576 ± 0.007	0.0600 ± 0.012	0.0759 ± 0.015[a]	0.0666 ± 0.008
Brains	2.02 ± 0.07	2.00 ± 0.09	1.88 ± 0.08	1.86 ± 0.10
Epididymis	1.176 ± 0.10	1.178 ± 0.16	–	–
Heart	1.14 ± 0.11	1.12 ± 0.09	0.805 ± 0.07	0.818 ± 0.08
Kidneys	2.40 ± 0.28	2.32 ± 0.25	1.52 ± 0.16	1.57 ± 0.16
Liver	12.7 ± 1.4	12.7 ± 1.7	7.54 ± 0.88	7.78 ± 0.72
Mesenterial ln.	0.109 ± 0.03	0.108 ± 0.03	0.092 ± 0.03[b]	0.131 ± 0.04
Ovaries	–	–	0.133 ± 0.03	0.122 ± 0.03
Pancreas	1.370 ± 0.43	1.284 ± 0.36	1.050 ± 0.23	1.047 ± 0.14
Small intestine	8.05 ± 1.05	8.07 ± 1.03	6.14 ± 0.84	5.91 ± 0.59
Spleen	0.776 ± 0.07	0.762 ± 0.10	0.555 ± 0.079	0.552 ± 0.051
Testes	3.91 ± 0.34	3.92 ± 0.34	–	–
Thymus	0.393 ± 0.08	0.385 ± 0.06	0.334 ± 0.078	0.365 ± 0.075
Uterus	–	–	0.500 ± 0.12	0.482 ± 0.14
Length small int.	112.8 ± 7.2	111.5 ± 8.0	100.8 ± 4.4	100.8 ± 3.1
Relative weight				
Adrenals	0.0137 ± 0.002	0.0145 ± 0.003	0.0313 ± 0.006[a]	0.0261 ± 0.003
Brains	0.481 ± 0.03	0.491 ± 0.04	0.777 ± 0.06	0.727 ± 0.04
Epididymidis	0.280 ± 0.02	0.284 ± 0.05	–	–
Heart	0.270 ± 0.02	0.269 ± 0.02	0.331 ± 0.02	0.319 ± 0.03
Kidneys	0.569 ± 0.04	0.557 ± 0.03	0.625 ± 0.04	0.614 ± 0.06
Liver	3.00 ± 0.19	3.04 ± 0.22	3.09 ± 0.26	3.04 ± 0.30
Mesenterial ln.	0.0258 ± 0.006	0.0260 ± 0.009	0.0379 ± 0.011[b]	0.0509 ± 0.013
Ovaries	–	–	0.0547 ± 0.013	0.0478 ± 0.011
Pancreas	0.324 ± 0.10	0.310 ± 0.09	0.433 ± 0.09	0.410 ± 0.06
Small intestine	1.95 ± 0.28	1.91 ± 0.26	2.52 ± 0.34[a]	2.30 ± 0.20
Spleen	0.185 ± 0.02	0.183 ± 0.02	0.228 ± 0.027	0.215 ± 0.019
Testis	0.931 ± 0.09	0.945 ± 0.10	–	–
Thymus	0.093 ± 0.02	0.093 ± 0.01	0.137 ± 0.028	0.142 ± 0.024
Uterus	–	–	0.206 ± 0.05	0.189 ± 0.05
Length small int.	0.269 ± 0.018	0.259 ± 0.023	0.416 ± 0.036	0.395 ± 0.026

Relative organ weights expressed as g/100 g body weight. Small intestinal length and relative length is expressed in cm and cm/g body weight. Data is presented as group mean values ± SD.
[a] Statistically significant different from control group ($p < 0.05$).
[b] Statistically significant different from control group ($p < 0.01$).

(+14% and +20%, respectively). Furthermore, this group had a significantly reduced absolute (−30%) and relative (−26%) weight of the mesenterial lymph node compared with the female control group. No macroscopic or histological findings were observed.

4. Discussion

Even though the two rice varieties were grown under almost identical environmental conditions, chemical analyses revealed a number of statistically significant differences between transgenic and parental rice. Differences were detected for proximates (starch, fiber, sugars, protein, and ash), amino acids, minerals (copper, iron, manganese, phosphorous, and potassium) and vitamins (B_1, B_6, pantothenic acid, folic acid). Minor, but statistically significant differences were also observed for distributions of fatty acids and steryl ferulates. Additional field trials would be necessary to determine whether the differences detected are due to the genetic modification or due to biological variability in the field.

The compositional data for transgenic rice were within the ranges reported in the literature except for protein, amino acids, ash and potassium. One has to keep in mind that existing food composition databases do not necessarily reflect the complete natural variation (Burlingame, 2004). In the present case, for example, protein contents exceed literature data for both the transgenic and the parental line. To assess the overall relevance of statistically significant differences in the light of natural variability within species, more comprehensive databases for the different plant species are necessary, which include samples with different genetic and/or environmental backgrounds. Recently, the International Life Science Institute released a comprehensive crop composition database that provides information on the natural variability in compositions of maize, soybean and cotton (Ridley et al., 2004). The intended extension of the database to other crops including rice will assist

in the assessment of compositional data like those generated in the present study. Further experiments are needed to ascertain the actual reasons for lower concentrations of cadmium and Triazophos in GNA rice compared to the control. These effects might be partially explained by the more vigorous vegetative growth, but lower seed-set, of the control: the more vigorous the vegetative growth of the plants the more cadmium might be taken up from the soil, and the more pesticide might be accumulated; however, it might also be argued that the lower the seed-set, the greater the concentrations of these contaminants in the seeds. The level of Triazophos in the control rice diet was below the no-observed-adverse-effect level (NOAEL) found in a study where rats were given Triazophos in the diet for 104 weeks (WHO, 2003). However, the levels found in the parental and the GM rice were above the maximum residue level of 0.02 mg/kg.

The rice tested in the 90-day feeding study was given to the animals as raw brown ground rice. To test the rice in a standardised way it was decided to use raw rice because cooking may affect the activity of many proteins, including the expressed lectin; furthermore, cooking recipes differ markedly. The inclusion level of 60% was found to be fully acceptable to the rat after testing different inclusion levels of rice meal in a preliminary 28-day study (data not published). The inclusion level of 60% GNA rice in the diet corresponds to a mean daily GNA lectin intake of approximately 58 and 67 mg/kg body weight for males and females, respectively.

To take into account the inclusion of rice at a level of 60%, the diet in the present study was balanced to ensure an adequate supply of macro- and micro-components (Table 1). It can be argued as to whether to balance the animal diet within an individual study where both the intended effects, as well as unintended effects resulting from the genetic modification, are being evaluated. In the present study no adjustments were made to balance differences between parental and GNA rice as identified in the course of the chemical analyses. Despite the fact that the chemical characterization revealed markedly significant differences in the levels of some macro- and micro-nutrients between parental and GNA rice, the differences of these compounds in the final diet were below 10%, with the exception of iron, which was 35% higher in feed containing the GNA rice. However, the iron level of 60 mg/kg diet in the GNA rice diet is still lower than the 75 mg/kg diet which is the level recommended for rats during pregnancy and lactation (NRC, 1995).

The similar body weights and similar levels of feed intake throughout the study of rats given control or GNA rice, and the absence of observed clinical effects, demonstrated the nutritional adequacy within and between diets. The significantly increased water intake of both male and female rats fed GNA rice could be explained by the GNA lectin present in the diet, however the underlying mechanism for this observation is not known. One possible hypothesis could be that the higher iron content in the GNA rice diet could have increased the water intake of the rats in order to excrete the excess iron. However, no further measurements of the urine were taken in order to elucidate this.

The significantly lower plasma concentrations of potassium and protein in the GNA-fed male and female rats could not be explained by the levels in the diet. In as much as the concentration of plasma albumin represents a fraction of the protein fraction, the decrease in plasma albumin appears to be responsible for the observed decrease in plasma protein. The decreased plasma albumin concentration seen in males and females fed GNA rice as well as the decreased potassium concentration in males fed GNA rice could be due to a dilution effect resulting from the significantly higher water intake of these animals. However, no decreases in concentrations were observed for the other plasma solutes. The minor but significantly higher level of ALAT seen in females fed GNA rice could indicate some kind of effect on the liver (Hoffmann et al., 1989; Moss and Henderson, 1994). However, no effect on liver weight and no histopathological findings in the liver were observed, and accordingly, the increased ALAT activity was not considered adverse. Although renal diseases often result in an elevated plasma concentration of creatinine (Whelton et al., 1994), the observed change in the level of creatinine between female rats in the two groups was not considered as an adverse finding, but could rather be related to the increased water consumption of the rats fed GNA rice. Enlargement of the lymph nodes could be indicative of an immune toxic response (Haschek and Rousseaux, 1998). The decreased weight of the mesenteric lymph nodes seen in rats given a diet based on GNA rice is therefore not considered as an adverse effect. Furthermore, no differences were observed in the histopathological examinations of these organs. The few significant differences in the other immunological parameters measured were considered of only minor biological importance and the feeding of GNA rice was considered to cause no adverse immunological response in the present study.

Haematology data from the present study were within the normal range for this rat strain and the few statistically significant findings seen, in one sex only, were considered to be of negligible biological importance.

The absolute and relative increase in adrenal weight after intake of GNA rice was seen in females only. In as much as no differences were observed by the histopathological examination we do not consider the effect to be adverse. It cannot be excluded that the higher content of cadmium in the control diet could have affected the adrenal weight as observed earlier (Selypes et al., 1986), however, in this study cadmium was given i.p. to female mice at relatively high doses.

Female rats fed GNA rice had an increased relative, but not absolute, weight of the small intestine, which could result from an effect of GNA lectin. As no effect was seen on the small intestine at the macroscopic and histopathological examination the increased relative weight was not

considered as an adverse effect. Pusztai et al. (1990) found no increase in the relative weight of the jejunum after 10 days exposure to GNA, despite an observed strong binding of the GNA to the epithelial surface of the small intestine.

An effect of GNA on bacterial counts was only observed in samples taken from the small intestine. An increase in total anaerob bacteria, Lactobacilli and Enterococci in the duodenum was observed for the group fed GNA rice. However, corresponding increases were not detected in the ileum samples where reduced numbers of Enterobacteria were found in the GNA group. Pusztai et al. observed that feeding rats with 42 mg GNA for six days had no effect on the number of lactose fermenters, non-lactose fermenters or lactobacilli (Pusztai et al., 1993). In contrast, GNA was able to block the PHA-induced *Escherichia coli* overgrowth probably because of mannose-specific binding of GNA, which is in keeping with the decrease in enterobacteria in the ileum samples observed in our study.

The present 90-day feeding study was designed to detect both intended and unintended effects of the genetic modification of the rice. However, it can be argued as to whether the animal model selected was sufficiently sensitive to detect these effects and distinguish between them. One way to demonstrate the sensitivity and specificity of the animal model could be to include additional groups, which received the parental or genetically modified rice spiked with the transgene product from the inserted trait in a concentration known to induce effects within the test animals. Another way could be to include groups, which received a mixture of the GM rice and the parental rice to obtain a dose–response relationship. In the present study, the increased water intake of the GNA fed rats appeared to be a key finding which could account for the observed differences in other parameters investigated; a urine analysis might have explained some of these observed differences.

The results of the study demonstrated that the combination of a thorough chemical analysis of the rice with the *in vivo* testing of the same rice enables us to better evaluate whether significant effects observed in the animal study were due to biological variation of the animals, or differences in the rice varieties resulting from the genetic modification.

In the present study, several significant differences were observed between rats fed diets with genetically modified and parental rice. Most of these differences appeared to be related to the increased water intake of the rats fed GM rice, which probably relates to the GNA lectin content, but none of the effects were considered to be adverse. The lesson from this study is that a 90-day study with the present design of one control group and one group given the GM food is not sufficient enough for the safety assessment of this GM food crop. The addition of groups given parental or genetically modified rice spiked with the expressed gene product could have elucidated whether the observed findings were caused by GNA lectin *per se* or by secondary changes in the GM rice due to the genetic modification and could thereby have improved the comparative safety assessment of parental rice and the GM rice.

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Transgenic Expression of Bean α-Amylase Inhibitor in Peas Results in Altered Structure and Immunogenicity

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The development of modern gene technologies allows for the expression of recombinant proteins in non-native hosts. Diversity in translational and post-translational modification pathways between species could potentially lead to discrete changes in the molecular architecture of the expressed protein and subsequent cellular function and antigenicity. Here, we show that transgenic expression of a plant protein (α-amylase inhibitor-1 from the common bean (*Phaseolus vulgaris* L. cv. Tendergreen)) in a non-native host (transgenic pea (*Pisum sativum* L.)) led to the synthesis of a structurally modified form of this inhibitor. Employing models of inflammation, we demonstrated in mice that consumption of the modified αAI and not the native form predisposed to antigen-specific $CD4^+$ Th_2-type inflammation. Furthermore, consumption of the modified αAI concurrently with other heterogeneous proteins promoted immunological cross priming, which then elicited specific immunoreactivity of these proteins. Thus, transgenic expression of non-native proteins in plants may lead to the synthesis of structural variants possessing altered immunogenicity.

KEYWORDS: α-Amylase inhibitor; transgenic plant; animal model; Th2 inflammation; mass spectrophotometry

INTRODUCTION

Genetically modified (GM) plants are designed to enhance agronomic productivity or product quality and are being increasingly employed in both agricultural and livestock industries (*1*, *2*). Recently, peas (*Pisum sativum* L.) expressing a gene for α-amylase inhibitor-1 (αAI) from the common bean *(Phaseolus* vulgaris L. cv. Tendergreen) were generated to protect the seeds from damage by inhibiting the α-amylase enzyme in old world bruchids (pea, cowpea, and azuki bean weevils) and are currently undergoing risk assessments (*3*−*6*).

The present study was initiated to (1) characterize the proteolytic processing and glycopeptide structures of αAI when transgenically expressed in peas (pea-αAI) and (2) evaluate in an in vivo model system the immunological consequence of oral consumption of pea-αAI. We demonstrate that expression of αAI in pea leads to a structurally modified form of this inhibitor. Employing experimental models, we show that the structural modification can lead to altered antigenicity. These investigations reveal that expression of proteins in non-native hosts can lead to the synthesis of a protein variant with altered immunogenicity.

MATERIALS AND METHODS

Nontransgenic and Transgenic Plants. Seed meal was obtained from nontransgenic peas, genetically modified peas expressing bean α-amylase inhibitor-1 (αAI) (*5*), genetically modified narrow leaf lupin (*Lupinus angustifolius* L.) expressing sunflower seed albumin protein (SSA) in the seeds (SSA-lupin) (*7*), and from nontransgenic Pinto bean. Seeds were ground into fine flour in liquid N_2 using a mortar and pestle. This seed meal was then suspended in PBS (0.166 g meal/mL), homogenized, sieved through a 70 μm mesh, and stored at −70 °C. In some experiments, seed meal homogenates were cooked at 100 °C for 30 min before administration to mice (indicated in text).

Purification of SSA from Transgenic Lupin and αAI from Common Beans and from Transgenic Peas. αAI was purified from the common beans (Pinto and Tendergreen) and transgenic peas and SSA from genetically modified narrow leafed lupin (SSA-lupin) as previously described (*7*, *8*). Purified proteins were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 15−

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25% gradient, 1 mm thick, mini-gel format) and MALDI-TOF mass spectrometry.

Western Immunoblot Analysis. αAI polypeptide composition was determined in protein extracts from common bean and transgenic peas as previously described (*3*). Protein was extracted from seeds with 0.5 M NaCl, 1 mM EDTA, and 0.1 M *N*-tris(hydroxymethyl)methylaminoethanesulfonic acid at pH 7.8. Aliquots of reduced protein (20 μg by Bradford assay) were fractionated by SDS-PAGE and electroblotted onto nitrocellulose membrane. αAI polypeptides were detected with an αAI antiserum from rabbit and goat anti-rabbit IgG conjugated to alkaline phosphatase (*3*). The concentration of αAI in transgenic peas was determined as 4% of total protein as previously described (*3*).

Structural Analysis of Purified αAI from the Pinto and Tendergreen Beans and from Transgenic Peas. Purified αAI from the common beans, Pinto and Tendergreen, and from transgenic peas were analyzed by matrix-assisted laser desorption/ionization-time-of-flight-mass spectrometry (MALDI-TOF-MS). The proteins were dissolved in water (approximately 1 μg/μL), and then 1 μL was mixed with 1 μL of matrix solution (saturated sinapinic acid in 50% acetonitrile/0.1% trifluoroacetic acid) on the sample plate of a Voyager Elite MALDI-TOF mass spectrometer (Perseptive Biosystems) and allowed to dry. Spectra were collected in linear mode with myoglobin used for close external calibration (Sigma, Cat. No. M-1882, 16952.6 $[M + H]^+$, 8476.8 $[M + 2H]^{2+}$).

Mice and Intragastric Administration of Seed Meal from Nontransgenic and Transgenic Plants. BALB/c mice were obtained from specific pathogen-free facilities at the Australian National University. Mice were intragastrically administered 250 μL of seed meal suspension (~100 mg/mL) containing either transgenic peas, nontransgenic peas, SSA-lupin, or Pinto bean twice a week for 4 weeks. In some experiments, serum was taken from the mice at the start of the third and fifth weeks during feeding. The serum antibody titers were determined as previously described (*9*).

Mice and Delayed Type Hypersensitivity Responses. BALB/c mice were administered seed meal as described above. Seven days following the final intra-gastric challenge, mice were subcutaneously injected with 25 μL of the appropriate antigen [Tendergreen-αAI, pea α-AI, or lupin SSA (1 mg/mL in PBS)] into the footpad. The positive control [(+) control] is mice immunized by i.p. injection of 200 μL containing 50 μg of Tendergreen-αAI dissolved in PBS with Alum (1 mg/mL) and subsequently receiving 25 μL of purified Tendergreen-αAI (1 mg/mL PBS). The negative control [(−) control] is mice immunized by i.p. injection of 200 μL containing 50 μg of Tendergreen-αAI dissolved in PBS with Alum (1 mg/mL) and subsequently receiving 25 μL of PBS. DTH responses were assessed by measuring the specific increase in footpad thickness using a digmatic calliper (Mitutoyo, Kawasaki, Japan) 24 h following the challenge. Serum was collected on day 14, and antibody titers were determined as previously described (*9*).

Murine Model of $CD4^+$ Th2 Cell-Mediated Inflammation. BALB/c WT mice were administered seed meal as indicated in the text. Seven and nine days following the final intra-gastric challenge, mice were anesthetized with an intravenous injection of 100 μL of Saffan solution (1:4 diluted in PBS). Mice were intubated with a 22 gauge catheter needle, through which purified αAI from Tendergreen bean or transgenic pea (1 mg/mL PBS), or vehicle control (PBS), was instilled. Airway responsiveness (AHR), mucus production, and eosinophilia were measured 24 h following the final intra-tracheal challenge. AHR to methacholine was assessed in conscious, unrestrained mice by barometric plethysmography, using apparatus and software supplied by Buxco (Troy, NY) as previously described (*9*). This system yields a dimensionless parameter known as enhanced pause (Penh), reflecting changes in waveform of the pressure signal from the plethysmography chamber combined with a timing comparison of early and late expiration, which can be used to empirically monitor airway function. Measurements were performed as previously described (*9*). Lung tissue representing the central (bronchi-bronchiole) and peripheral (alveoli) airways was fixed, processed, and stained with Alcian Blue-PAS for enumeration of mucin-secreting cells or Charbol's chromotrope-Haematoxylin for identification of eosinophils as previously described (*9*).

Intragastric Administration of Purified αAI and OVA. Mice were administered 200 μL of affinity purified Tendergreen- or transgenic pea-αAI (5 μg) with ovalbumin (OVA, 1 mg/mL) in a PBS suspension three times a week for 2 weeks. One week following feeding, the mice were intubated with a 22 gauge catheter needle, through which 25 μL of OVA (1 mg/mL PBS), or vehicle control (PBS), was instilled and the $CD4^+$ Th2-inflammation indices determined as described above. Serum was taken from the mice 1 day after the final intra-tracheal challenge, and serum antibody titers were determined as described (*9*).

Antigen Specific $CD4^+$ T-Cell Response. Peribronchial lymph nodes (PBLN) were subjected to pea-αAI or αCD3/αCD28 stimulation as previously described (*9*). In brief, 5×10^5 PBLN cells/mL were cultured with αAI (50 μg/mL) or αCD3 (5 μg/mL)/αCD28 (1 μg/mL) for 96 h. IL-4, IL-5, IFNγ levels were determined in supernatants from stimulated PBLN homogenates by using the OptEIA Mouse IL-4, IL-5, and IFNγ kits (Pharmingen).

Statistical Analysis. The significance of differences between experimental groups was analyzed using Student's unpaired *t*-test. Values are reported as the mean ± SEM. Differences in means were considered significant if $p < 0.05$.

RESULTS

MALDI-TOF-MS Analysis of αAI. To assess the consequences of transgenic expression of the bean αAI in peas, we initially performed a structural analysis of the transgenically expressed protein (pea-αAI). Pea-αAI was compared by Western blot analysis and MALDI-TOF-MS with natively expressed αAI from the common beans, cvs. Pinto (Pinto-αAI) and Tendergreen (Tendergreen-αAI) (collectively termed bean-αAI). Previous studies have shown that bean-αAI is synthesized as a pre-pro-αAI polypeptide that is cleaved following Asn^{77} to form two peptide chains (α and β), both of which are glycosylated and have one or more amino acid residue(s) removed from their C-termini (*8*). This post-translational processing results in major forms of the α and β chains with masses of 11 646 and 17 319, respectively, and minor forms containing alternative glycans (*10*−*12*). Western immunoblot analysis of Tendergreen-αAI and pea-αAI revealed immunoreactive bands in the 11 000−18 000 mass range consistent with the reported structure (*10*−*13*). Detailed comparison of Tendergreen-αAI with pea-αAI revealed differences in the banding profile, suggesting possible differences in the molecular structure of natively and transgenically expressed αaI (**Figure 1A**).

To better resolve the differences between pea-αAI and bean-αAI, affinity purified αAI was analyzed by MALDI-TOF-MS (**Figure 1B**). The mass spectra of Tendergreen-αAI and Pinto-αAI closely matched a previously published spectrum (*10*) of a bean-αAI (*Phaseolus vulgaris* L. cv. Greensleeves) confirming that both Tendergreen- and Pinto-αAI possess similar well-characterized post-translational modifications and very similar relative abundance of minor processing variants (*10*, *11*). Alignment of our spectra with the previously published data (*10*) allowed identification of peaks in the pea-, Tendergreen-, and Pinto-αAI spectra. The major form of the α-chain (11 646 Da) of bean-αAI contains residues 1−76 by cleavage of the pro-protein following Asn^{77}, removal of Asn^{77}, and the addition of sugar residues ($Man_6GlcNAc_2$ at Asn^{12} and $Man_9GlcNAc_2$ at Asn^{65}). Minor forms of the α-chain of bean-αAI differed by having one to three fewer mannose residues resulting in a series of peaks in the MALDI-TOF spectrum that differ by 162 mass units. In contrast, less heavily glycosylated forms dominated for the α-chain of pea-αAI. In particular, an α-chain with two fewer mannose residues (11 322 Da) was the most abundant for pea-αAI but the least abundant for Tendergreen-αAI (**Figure 1C(i)**). A further difference in the pea-αAI spectrum was a series of minor peaks differing from the main α-chain peaks by either

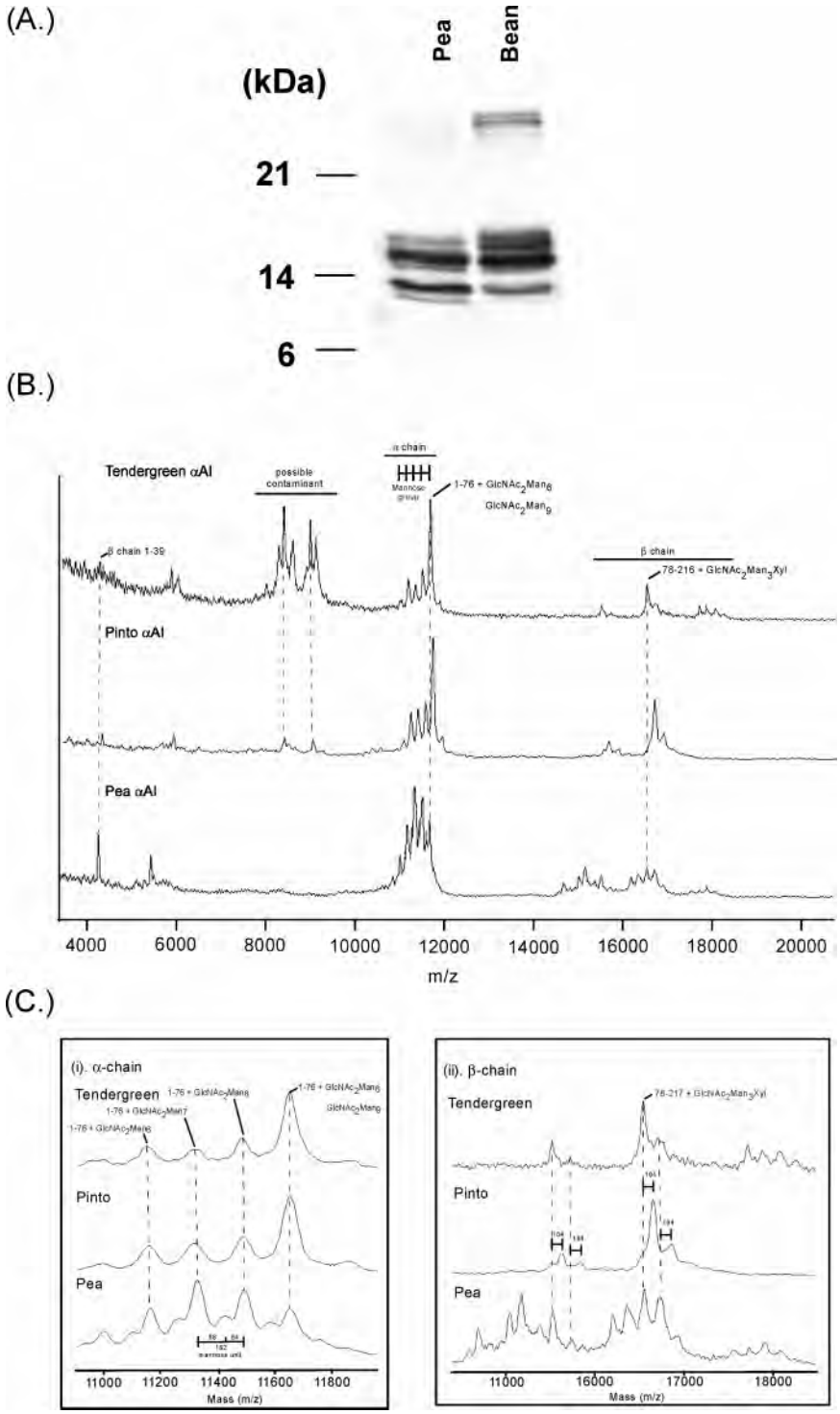

Figure 1. Western immunoblot and MALDI-TOF-MS analysis of common bean-derived-αAIs and αAI from transgenic peas. (**A**) Western blot analysis of αAI protein in extracts of transgenic peas and the Tendergreen variety of common bean. The masses of standard proteins are indicated. (**B**) Aligned MALDI-TOF mass spectra of purified αAI from transgenic pea and the common beans, Tendergreen and Pinto. (**C**) Detail from the spectra in panel B showing the regions of the α-chain (**i**) and the *β*-chain (**ii**).

+98 or −64 mass units, indicating another modification of some of the pea-αAI α-chains (**Figure 1C(i)**).

The major form of the *β*-chain of Greensleeves-αAI (16527 Da) contains residues 78−216 by cleavage of the pro-protein following Asn^{77}, the removal of the seven C-terminal residues following Asn^{216}, and the addition of sugar residues (Man_3-$GlcNAc_2Xyl_1$ at Asn^{140}) (*10*−*13*). The *β*-chain region of the Tendergreen-αAI spectrum closely aligned with that of Greensleeves-αAI (**Figure 1C**). The *β*-chain region of the Pinto-αAI spectrum also closely resembled that of Greensleeves-αAI except that both major and minor peaks of Pinto-αAI were shifted by approximately +104 mass units. This mass discrepancy is consistent with five amino acid residue differences between the *β*-chains of Tendergreen-αAI and Pinto-αAI as predicted by gene sequence comparison (see Supporting Information Figure 1). Further, there are also three predicted residue differences between the Tendergreen-αAI and Pinto-αAI α-chains that result in a difference of +1 mass unit, which would not be

detected by our methods. These sequence differences are consistent with previous reports of αAI polymorphisms among bean cultivars (*12*, *13*). The pea-αAI spectrum showed major peaks corresponding to the two major and minor forms of the *β*-chain found in Tendergreen-αAI; however, the pea-αAI spectrum also showed a number of other peaks (**Figure 1C(ii)**). DNA sequencing of the transgene in pea and comparison with the published sequence (*14*) confirmed that the nucleotide sequences were identical, establishing that the observed further forms of the pea-αAI are related by variations in post-translational modifications including glycosylation (**Figure 1C(ii)**).

Analysis of the spectra of pea- and bean-αAI also revealed several other differences. First, a number of peaks at ~8−9000 and 5824 mass units and below were observed in the bean-αAI spectrum, which are consistent with a previously reported protein that copurifies with bean-αAI (*10*) and doubly charged ($(MH_2)^{2+}$) forms of the α-chain, respectively. Further, a peak at 4223 mass units was detected in the pea-αAI spectrum, which has not been previously reported. While this peak is barely detected in the bean-αAI spectrum presented here, the peak was observed in a number of other bean-αAI preparations (results not shown). The mass of this peak is consistent with the first 39 residues of the *β*-chain, which could be obtained by cleavage following an Asn residue, the same protease specificity that provides the reported processing of αAI at Asn^{77}. Consistent with this hypothesis, a small peak was detected in some preparations at about 12 304 mass units that could correspond to the remainder of the *β*-chain.

While pea-αAI has not yet been characterized as thoroughly as the bean-αAI, it is clear that the transgenic expression of the bean αAI gene in the pea led to differences of glycosylation and possibly other differences in both the α- and the *β*-chains.

Immunological Consequence of Oral Consumption of Beans. Peas are used as a feed component in the livestock industry and also in human diets. Generally, dietary protein antigens undergo gastric digestion leading to the formation of nonimmunogenic peptides and the induction of a state of specific immunological unresponsiveness termed oral tolerance (*15*, *16*). However, the demonstration of structural differences between the transgenic αAI in pea and the natively expressed bean forms raised the concern that the tolerance mechanism may be perturbed, possibly leading to enhanced immunoreactivity.

The induction of oral tolerance results in the failure of the immune system to elicit an active immune response to subsequent exposure to the same antigen in the skin (delayed type hypersensitivity [DTH] response) or lung ($CD4^+$ T-helper [Th_2] cell-mediated inflammation). To examine potential differences in immunological responsiveness following oral consumption, mice were fed Pinto bean, which expresses a native form of αAI and subsequently received purified Tendergreen-αAI in the skin and lung. Most varieties of common beans such as Red Kidney or Tendergreen contain high levels of phytohemagglutinin (PHA), an anti-nutritional factor that induces dietary toxicity in rodents and birds. We therefore used the Pinto variety that contains very low levels of PHA (*17*, *18*) as the appropriate control for oral exposure. Oral consumption of native uncooked Pinto bean seed flour followed by intra-tracheal (i.t.) challenge with Tendergreen-αAI or phosphate buffered saline (PBS) failed to induce an αAI-specific IgG_1 antibody response (**Figure 2A**). Similarly, sub-cutaneous (s.c.) challenge of the footpad or i.t. challenge of Pinto bean-fed mice with Tendergreen-αAI also failed to promote a DTH response (results not shown) or a pulmonary Th_2-inflammatory response [pulmonary eosinophilia, mucus hypersecretion, and enhanced AHR to a bronchoconstrictive agents], respectively (**Figure 2B−D**). While the level of AHR in the Pinto bean-fed αAI-challenged mice was higher than PBS-challenged mice, the level of responsiveness is not significantly different from that of naïve mice i.t. challenged with Tendergreen-αAI (**Figure 2D**). As a positive control, mice were sensitized by intra-peritoneal (i.p.) injection and subsequently challenged via the airways with bean-derived αAI to induce immunological responsiveness (**Figure 2A−D**). Collectively, these data showed that oral consumption of the native bean form of αAI followed by respiratory exposure to bean-αAI did not promote immunological responsiveness or inflammation.

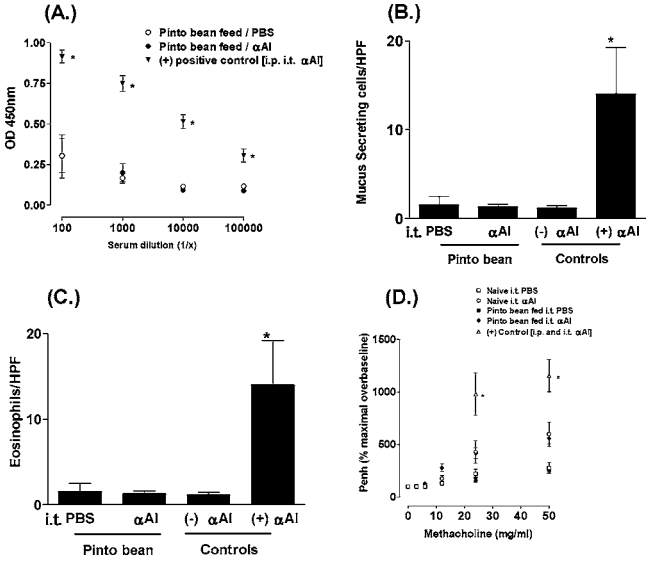

Figure 2. Experimental consumption of bean (cv. Pinto) seed meal does not predispose to inflammation. (**A**) αAI-specific IgG_1 in serum and (**B**) mucus-secreting cell numbers and (**C**) eosinophil levels in lung tissue from Pinto bean-fed mice i.t. challenged with PBS or Tendergreen-αAI. (**D**) AHR in Pinto bean-fed mice i.t. challenged with PBS or Tendergreen-αAI. Data are expressed as the (**A**–**D** and **F**) mean ± SEM and (**E**) mean O.D. of the serum dilution 1/10 ± SEM from 4 to 6 mice per group from duplicate experiments. (**A**–**D**) * $p < 0.05$ as compared to Pinto bean-fed i.t. αAI.

Immunological Consequence of Oral Consumption of Transgenic Peas. To determine whether oral consumption of the transgenic αAI (from pea) elicited an immunological response, mice were orally administered transgenic pea seed meal and αAI; serum antibody titers and DTH responses were examined. Interestingly, in mice that were fed transgenic pea, but not nontransgenic pea, αAI-specific IgG_1 was detected at 2 weeks and at significant levels after 4 weeks of oral exposure (**Figure 3A**). Consistent with the antibody findings, mice fed nontransgenic pea seed meal did not develop DTH responses following footpad challenge with purified pea-αAI (**Figure 3B**). In contrast, mice fed transgenic pea seed meal exhibited a significant DTH response as compared to the nontransgenic pea exposed group when purified pea-αAI was injected into the footpad (**Figure 3B**). As a control for any general effect of genetic modification, we repeated the experiment with material from two other genetically modified plants, lupin (*Lupinus angustifolius* L.) expressing sunflower seed albumin (SSA) [transgenic lupin] (*9*) and chickpeas (*Cicer arietinum* L.) expressing bean derived αAI. Mice were orally administered lupin or transgenic lupin or chickpea or transgenic chickpea seed meal and subsequently footpad challenged with SSA or αAI and DTH responses were examined. In contrast to transgenic pea, mice fed transgenic lupin or chickpea did not develop

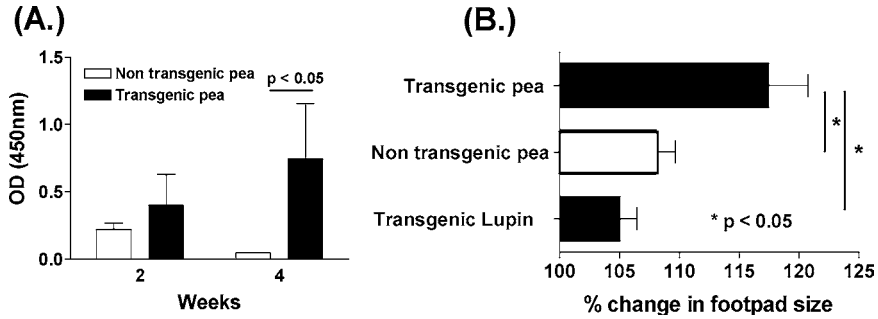

Figure 3. Experimental consumption of transgenic pea seed meal predisposed to antigen-specific IgG_1 and DTH responses. (**A**) Antigen-specific IgG_1 and (**B**) DTH responses in pea nontransgenic and pea transgenic-fed mice. Data are expressed as the (**F**) mean ± SEM and (**E**) mean O.D. of the serum dilution 1/10 ± SEM from 4 to 6 mice per group from duplicate experiments. (**A**–**C**) * $p < 0.05$ as compared to nontransgenic pea or transgenic lupin fed mice i.t. αAI.

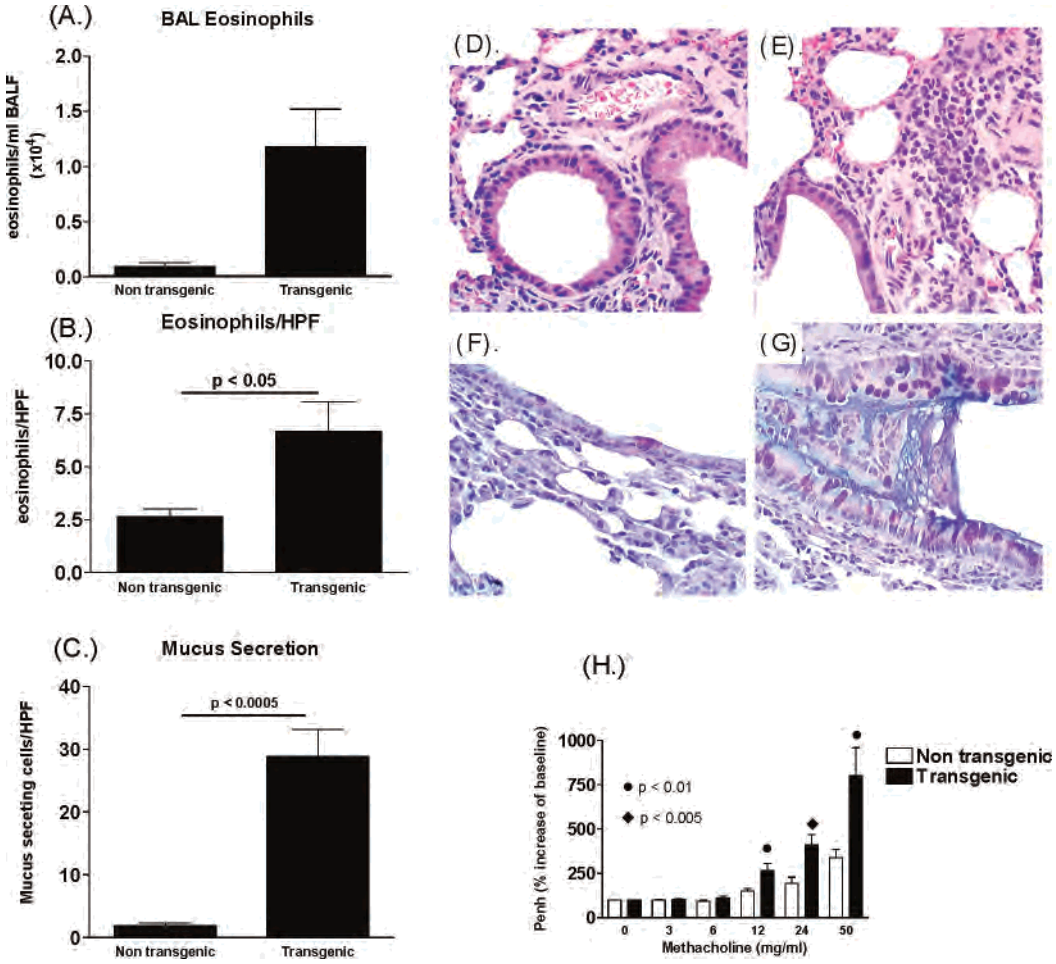

Figure 4. Consumption of transgenic pea seed meal predisposed to $CD4^+$ Th_2-type inflammatory response. Eosinophil accumulation in bronchoaveolar lavage fluid (BAL) (**A**), tissue (**B**), and mucus-secreting cell numbers (**C**) in lung tissue from nontransgenic and transgenic pea-fed mice i.t. challenged with αAI purified from pea. (**D**–**G**) Representative photomicrographs of eosinophil accumulation in lung of (**D**) nontransgenic and (**E**) pea transgenic-fed mice and mucus-secreting cell numbers in lung tissue of (**F**) nontransgenic and (**G**) pea transgenic-fed mice i.t. challenged with αAI from pea. (**H**) Airways hyperresponsiveness (AHR) in nontransgenic and pea transgenic-fed mice i.t. challenged with αAI from pea. Data are expressed as the mean ± SEM from 3 to 6 mice per group from duplicate experiments. Statistical significance of differences ($p < 0.05$) was determined using Student's unpaired *t*-test. (**D**–**G**) ×400 magnification.

DTH responses following footpad challenge with the transgenically expressed and purified SSA or αAI protein (**Figure 3B**; results not shown). Thus, consumption of transgenic pea containing αAI promoted αAI-specific immunological responsiveness.

To characterize the type of immune response elicited against pea-αAI following oral consumption of transgenic pea, we employed a well-characterized murine model of $CD4^+$ Th_2 cell-mediated inflammation (*19*). Mice were orally administered transgenic pea seed meal and subsequently i.t. challenged with purified pea-αAI, and key features of Th_2-inflammation [pulmonary eosinophilia, mucus hypersecretion, and AHR] were examined. I.t. challenge of nontransgenic pea-fed mice with purified pea-αAI failed to induce features of Th_2-inflammation (**Figure 4A**−**G**). Furthermore, airways responsiveness to the cholinergic spasmogen, methacholine, was not induced in these

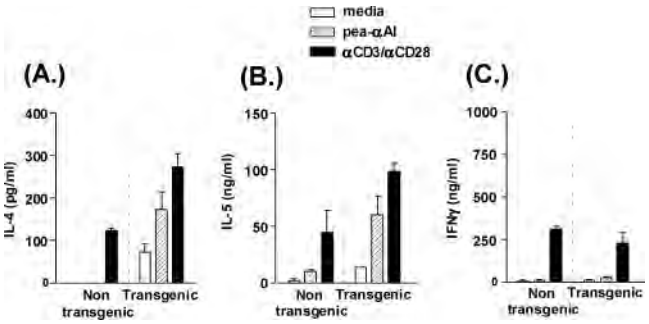

Figure 5. Consumption of transgenic pea seed meal predisposed to $CD4^+$ T-cell derived Th_2-type cytokine production. IL-4 (**A**), IL-5 (**B**), and IFNγ (**C**) levels in supernatants from αCD3/αCD28 or pea-αAI or media alone stimulated PBLN cells from nontransgenic and transgenic pea-fed mice i.t. challenged with αAI from pea. Data are expressed as the mean ± SEM from 3 to 6 mice per group from duplicate experiments. Statistical significance of differences ($p < 0.05$) was determined using Student's unpaired *t*-test.

mice (**Figure 4H**). However, instillation of pea-αAI into the lungs of mice fed transgenic pea induced key features of Th_2-type inflammation including pulmonary eosinophilia, mucus hypersecretion, and AHR (**Figure 4A−H**).

Pulmonary eosinophilia, mucus hypersecretion, and AHR are critically linked to the effector function of the Th_2 cytokines (*20*). To examine whether consumption of transgenic pea promoted a αAI-specific $CD4^+$ Th_2-type T-cell response, $CD4^+$ T-cells in peribronchial lymph node (PBLN) cultures from mice fed nontransgenic pea or transgenic pea seeds challenged with pea-αAI were stimulated with pea-αAI and cytokine profiles determined. Stimulation of $CD4^+$ T-cells in peribronchial lymph node (PBLN) cultures from nontransgenic pea-fed mice challenged with pea-αAI did not elicit Th_2 (interleukin (IL)-4 and IL-5)- or Th_1-type (gamma interferon, IFNγ) cytokine production in response to pea-αAI stimulation (**Figure 5A−C**). By contrast, stimulation of PBLN cultures with pea-αAI from i.t. challenged mice fed transgenic pea resulted in the significant production of Th_2 cytokines (**Figure 5A−C**). Thus, oral exposure of mice to transgenic pea, but not nontransgenic seed meal, predisposed to systemic immunological responsiveness characterized by a Th_2-type immune profile.

Pea-αAI Promotes Immune Responses to Other Oral Antigens. Previous investigations have demonstrated that various plant-derived proteins such as tomatine possess immunomodulatory activity and potentiate and polarize immune responses (*21−23*). We have demonstrated that consumption of transgenic pea in the presence of a large number of potential dietary antigens in the gastrointestinal tract induces an active systemic Th_2-immune response against pea-αAI. In light of these findings, we were next interested in determining whether consumed pea-αAI possessed immunomodulatory activity for Th_2 immune responses and could sensitize mice to heterogeneous nongenetically modified food antigens. Thus, we intragastrically (i.g.) administered purified Tendergreen- or pea-αAI with the well-characterized dietary antigen, chicken egg white protein OVA, or OVA alone and subsequently i.t. challenged mice with OVA. I.g. administration of OVA alone did not systemically sensitize mice to OVA (**Figure 6A**). Further, subsequent OVA challenge in the airways did not promote Th_2-inflammation (mucus hypersecretion, pulmonary eosinophilia, or AHR). Similarly, i.g. administration of bean-αAI and OVA did not systemically sensitize mice or predispose to Th_2-inflammatory processes. However, consumption of pea-αAI and OVA promoted a strong OVA-specific Th_2-type antibody

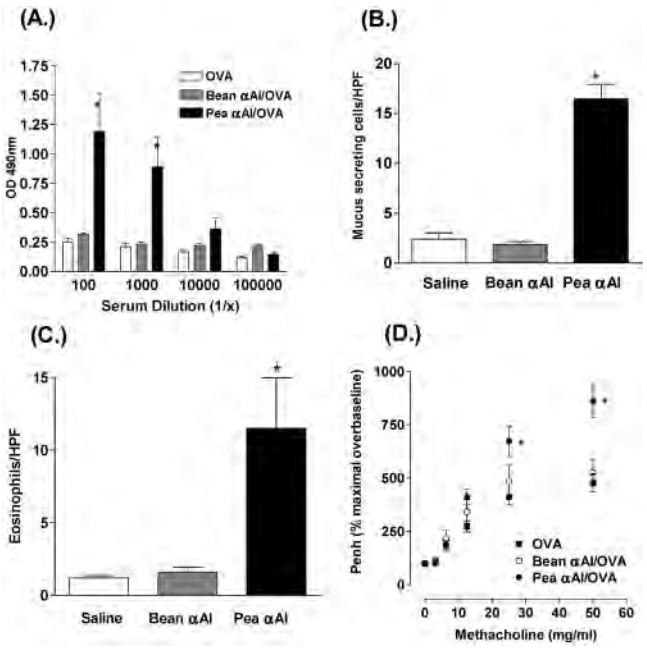

Figure 6. Intra-gastric administration of αAI from pea induces cross-priming of heterogeneous food antigens. OVA-specific IgG_1 levels (**A**) and the Th_2-inflammation phenotype (mucus hypersecretion) (**B**), pulmonary eosinophilia (**C**), and airways hyperreactivity (**D**) in mice that were fed (i.g. challenged) ovalbumin (OVA) alone (the control) or in combination with natively expressed Tendergreen bean-αAI or transgenically expressed (pea) αAI and subsequently intra-tracheal challenged with purified OVA. Data are expressed as the mean ± SEM from 4 to 6 mice per group. * $p < 0.05$ as compared to OVA and bean αAI/OVA.

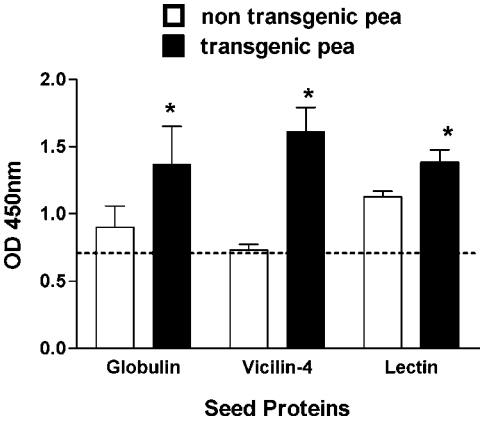

Figure 7. αAI from pea induces cross-priming of pea proteins. Pea globulin-, vicilin-4, and lectin-specific IgG_1 levels in serum from mice that were intragastrically administered 250 μL (~100 mg/mL) of either nontransgenic or transgenic pea seed meal twice a week for 4 weeks. Data are expressed as mean ± SEM from 4 to 5 mice per group. * $p < 0.05$ as compared to nontransgenic pea.

response (**Figure 6A**) and predisposed mice to OVA-induced Th_2-inflammation (**Figure 6B−D**). To support this observation, we examined serum levels of antigen-specific IgG_1 against pea seed proteins (pea globulins, lectin, and vicilin-4) in transgenic pea and nontransgenic pea-fed mice. Interestingly, levels of antigen-specific IgG_1 against pea globulins, lectin, and vicilin-4 in serum of transgenic pea fed mice were significantly higher than those of nontransgenic pea-fed mice, suggesting a heightened immune responsiveness to dietary proteins due to pea-αAI (**Figure 7**). Thus, these studies demonstrate that modified αAI possesses immunodulatory activity and that consumption

of the modified αAI concurrently with heterogeneous proteins can promote immunological cross priming, which predisposes to specific immunoreactivity to these proteins.

DISCUSSION

Recently, peas expressing a gene for αAI from the common bean were generated for protection against field and storage pests (*3*−*6*). Characterization of αAI by structural analysis has demonstrated that transgenic expression of this protein in peas led to the synthesis of a modified form of αAI. Further, we show that the modified form of αAI possessed altered antigenic properties and consumption of this protein by mice predisposed to αAI-specific $CD4^+$ Th_2-type inflammation and elicited immunoreactivity to concurrently consumed heterogeneous food antigens.

Bean-αAI undergoes significant post-translational modification including variable glycosylation and proteolytic processing leading to the synthesis of a mature functional protein (*8*, *11*). We demonstrate that differences in glycosylation and/or other modifications of the pea-αAI lead to altered antigenicity. Consistent with our observations, investigators have previously demonstrated that differential glycosylation of subunits of a cereal α-amylase-inhibitor family (unrelated to legume αAIs) enhances IgE-binding capacity (*24*). Moreover, glycosylated cereal αAI subunits have been shown to possess significantly enhanced IgE-binding affinity when compared to the unglycosylated forms (*24*). These cereal proteins possess identical amino acid sequences and only differ in their carbohydrate moieties, indicating that glycosylation can confer IgE-binding capacity and Th_2-inflammation. In particular, recent investigations have demonstrated that glycan side chains linked to high mannose-type N-glycans on plant-derived glycoproteins can confer immunogenicity and are IgE binding determinants (*25*, *26*). Moreover, α(1,3)-fucose and *β*(1,2)-xylose linkage to high mannose-type N-glycans ($Man_5GlcNAc_2$−$Man_9GlcNAc_2$) promote immunogenicity and IgE binding. The *β*-chain of pea-αAI possesses *β*(1,2)-xylose linked high mannose-type N-glycans, and other complex glycoforms and the α-chain may possess an as yet undefined glycoform variant, and it remains to be determined how these modifications alter pea-αAI immunogenicity.

Functional and structural properties of pea-αAI may contribute to its ability to circumvent immune tolerance and elicit inflammatory responses. Bean-αAI is a potent inhibitor of human α-amylase activity and can induce gastrointestinal dysfunction (*27*). Comparison of bean- and pea-derived αAI activity revealed no difference in enzymatic activity between the two proteins (results not shown). Furthermore, we examined the gastrointestinal tract of pea and transgenic pea-fed mice and observed no histological abnormalities to the gastrointestinal tissue in either group (results not shown). Bean-αAI is also a heat-stable protein and partially resistant to proteolytic degradation (*28*, *29*). Extensive boiling (100 °C for 20 min), while significantly reducing α-amylase inhibitory activity, failed to alter the ability of the transgenic pea to prime for Th_2-inflammation when challenged in the lung [results not shown: see Supporting Information Figure 2]. These findings are consistent with previous demonstrations that cooking of plant material such as lentils and peanuts does not diminish the allergenic potential of certain proteins (*30*, *31*). Furthermore, these studies suggest that the altered immunogenicity of αAI is unrelated to its properties as an amylase inhibitor.

We demonstrate that the immune response elicited against pea-αAI following oral consumption of transgenic pea is characterized by $CD4^+$ Th_2 cell-mediated inflammation, in particular, the presence of IL-4 and IL-5. To examine whether the immune response was dependent on IL-5 and eosinophils, we employed IL-5 and eotaxin-deficient mice. IL-5/eotaxin-deficient mice were i.g. administered nontransgenic and transgenic seed meal and subsequently i.t. challenged with purified αAI. We show that i.t. challenge of transgenic pea fed IL-5/eotaxin-deficient mice induced Th_2-inflammation that was significantly elevated over nontransgenic fed mice (*32*). These investigations suggest that the immune response elicited against pea-αAI following oral consumption of transgenic pea is not dependent on IL-5 and eosinophils.

In this study, we have demonstrated that transgenic expression of αAI in a pea can lead to the synthesis of a modified form of the protein with altered antigenic properties. Furthermore, we show that concomitant exposure of the gastrointestinal tract to modified αAI and heterogeneous food antigens cross primes and elicits immunogenicity. Currently, we do not know the frequency at which alterations in structure and immunogenicity of transgenically expressed proteins occur or whether this is unique to transgenically expressed αAI. These investigations, however, demonstrate that transgenic expression of non-native proteins in plants may lead to the synthesis of structural variants with altered immunogenicity.

ABBREVIATIONS USED

αAI, α-amylase inhibitor-1; pea (*Pisum sativum* L.), transgenic pea; *Phaseolus vulgaris* L. cv. Tendergreen, *Pisum sativum* L. expressing α-amylase inhibitor-1 from the common bean; MALDI-TOF-MS, matrix-assisted laser desorption/ionization-time-of-flight-mass spectrometry.

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Supporting Information Available: Amino acid sequence of αA1 from common bean and consumption of pea seed meal predisposed to Th_2-type inflammation. This material is available free of charge via the Internet at http://pubs.acs.org.

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CAN SCIENCE GIVE US THE TOOLS FOR RECOGNIZING POSSIBLE HEALTH RISKS OF GM FOOD?†

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ABSTRACT

Nearly ten years after the introduction of GM foodcrops there are still only a handful of published studies about their safety. Independent studies are even fewer, moreover, no peer-reviewed publications exist in which the results of clinical investigations on the possible effects of GM food on human health are described. Even though the evaluation of the safety or possible toxicity of GM foodstuffs is more difficult than that of drugs or food additives, this scarcity of data and the lack of a scientific database is curious particularly as descriptions of the results of chemical, nutritional and biological testing in some early (unpublished) studies or some more recent publications demonstrate the feasibility of carrying out proper and scientifically valid health risk assessment on GM foods. In this review, after critically examining some of the basic principles, past results and possible novel methods of future health safety assessment of GM foodstuffs, the conclusion appears to be that as the tools for the recognition and indeed for the elimination of the risks GM foods may present for us are available or can be developed, it is the will and the funding for such work that needs to be found.

INTRODUCTION

Lectins are a class of proteins which occur widely in nature and are capable of specific recognition of and binding to carbohydrate ligands. In plants they appear to function as nitrogen storage compounds but they may also have a defensive role and protect the plant against pests and predators (Gatehouse *et*

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†The McCarrison Society Scottish Group organized a half day symposium entitled *GM Foods—Right or Wrong?* in Edinburgh on 31st March 2001. Two speakers, Professor Anthony Trewavas FRS, FRSE of the Plant Biochemistry Department in the University of Edinburgh, and Dr Arpad Pusztai, FRSE, formerly of the Rowett Research Institute, Aberdeen, gave lectures, and were invited to submit written versions. This is one of them. Professor Trewavas declined owing to shortage of time.

al., 1995; Pusztai and Bardocz, 1997). Lectins are thus regarded as natural insecticidal proteins. Based on this recognition, by transferring insecticidal lectin genes using recombinant gene methodologies, many novel genetically modified (GM) agronomically and commercially important crop plants have recently been developed which are resistant to their most devastating pests. Currently one of the best known examples of insecticidal lectin genes from higher plants is the snowdrop (*Galanthus nivalis*) bulb lectin, GNA. From bacteria the lectin-endotoxins of *Bacillus thuringiensis* are the most widely used protective agents in GM plants. However, as the protective effect of the lectin is passive the plant tissues first need to be ingested before the released constitutive lectin could harm the pest/predator by interfering with nutrient assimilation in their digestive system. It also needs to be recognized that if the same plant is eaten by mammals this lectin may also react with their gut and harm their health. Thus, there is an inextricable link between lectin-mediated plant protection and potential nutritional and health problems for the consumers.

The antinutritive effects of some lectins in traditional crops have long been recognized (Liener, 1980), particularly in animal nutrition. In contrast, the possibility that novel GM foods expressing lectin genes may represent health hazards for mammalian consumers does not appear to have been generally recognized let alone rigorously excluded. Thus, as has been noted there are many opinions on the safety of GM food but very few data published in peer-reviewed science journals (Domingo, 2000). This has recently been confirmed in a comprehensive scientific review (Pusztai, 2001). The almost total dependence of GM food regulation on the so-called principle of "substantial equivalence" and lack of independently verified safety testing is particularly regrettable. The apparent similarity of macro/micro nutrient components in GM crops and their conventional counterparts established by chemical analysis, regardless of how sophisticated this may be, cannot reveal the presence of unknown toxic/allergenic components resulting from unintended effects of the insertion of a novel gene into the plant's genome. Thus, GM food regulation is presently based on inadequate and potentially dangerous principles.

There is therefore a growing realization, particularly in medical and nutritional science that the presently available safety testing methods are inadequate and need to be further developed. Better chemical diagnostic methods are needed, including mRNA* fingerprinting, proteomics and secondary metabolite profiling (Kuiper *et al.*, 2000). However, and most importantly, novel toxicological/nutritional methods need to be developed or present methods improved to be able to screen for the potential harmful consequences on human/animal health of GM food crops before these are allowed

*messenger Ribonucleic acid: The DNA code is transcribed within the cell nucleus into complementary mRNA, which in the cell protein synthesizing apparatus is then translated into the appropriate protein.

to enter the human food chain either directly or indirectly (Ewen & Pusztai, 1999a; Pusztai, 2000). However, before exploring the efficacy of possible novel biological testing methods it may be instructive to review some studies which influenced the development of testing.

SAFETY STUDIES WITH GM TOMATOES

The importance of toxicological/nutritional testing using laboratory animals was already recognized by the FDA (Food and Drug Administration in the USA) in 1992 by requesting Calgene# to commission the safety evaluation of FLAVR-SAVR™ tomato, the first GM crop, before it could be released. This was despite the fact that the results of chemical analyses showed that there were no significant alterations in the composition of the tomatoes and therefore the GM and parent tomatoes were "substantially equivalent". According to the FDA's evaluation the commissioned biological tests, in full agreement with the results of the chemical analyses, supported the safety of the GM tomatoes as no toxic effects were observed in the study. The weight gain, food consumption and clinical and haematology parameters of well-fed male/female rats which were also tube-fed with homogenized GM or conventional tomatoes, were claimed not to be statistically significantly different. On the basis of all these tests the FDA decided that FLAVR-SAVR™ tomatoes were as safe as conventional tomatoes and, furthermore, that no toxicological testing of other GM foodstuffs will in future be required.

The FLAVR-SAVR™ safety studies and their results have not been peer-reviewed or published. However, as they are now on the internet (Alliance for Biointegrity, 1998) it was possible to subject the study to independent critical scrutiny. From this it is difficult to understand how the FDA came to the conclusion that these GM tomatoes were safe and needed no further studies. The fact that the tomatoes used in the different experiments were from different locations and harvested at different times should have at least put a serious question mark to the validity of the compositional comparisons in these studies and to the substantial equivalence of the GM and non-GM tomatoes (Pusztai, 2001). Furthermore, the acute toxicity experiments were poorly designed and some of the methods used in the study were flawed. Thus, in a major nutritional study of such importance more attention should have been given to selecting groups of rats whose starting weights were more closely matched. When differences in rat weights are close to twofold, as in these experiments, the large standard deviations in the data make it difficult to draw valid conclusions whether the weight gain, diet consumption, organ weights and other parameters of rats given GM or non-GM tomatoes were

#Calgene Inc. is a US based biotechnology company that applied to the Food & Drugs Administration (FDA) to sanction the release of the first commercial GM crop, the FLAVR-SAVR tomato developed by them.

similar or different. Even more importantly, no histology was done on the intestines although some of the female rats developed erosive/necrotic stomach lesions (Table 1). However, these were dismissed by the FDA because the lesions were claimed to be spontaneous/transitory and unrelated to GM tomatoes but probably due to mucolytic agents, food restriction and/or stress resulting from animal restraint. However, tomatoes are not known to contain mucolytic agents, feed was provided *ad lib*, rats were not restrained or stressed and all test and control rats were treated the same way. As they were only tested once at the end of the experiment, it is difficult to maintain that the lesions were spontaneous or transitory. It also needs to be stressed that in these tests the rats were used as models for humans. However, in human pathology it is not legitimate to use the term "mild erosion/necrosis" because glandular stomach erosion can lead to life-threatening haemorrhage, particularly in the elderly and in patients on non-steroidal anti-inflammatory agents. In the FDA study there was also an unexplained mortality of rats, apparently seven out forty rats given GM tomatoes died within two weeks of the experiment. The conclusion appears to be inescapable that as the whole FLAVR-SAVR™ study was poorly designed and executed and, most importantly, led to flawed conclusions, the claim that these GM tomatoes were as safe as conventional ones is at best premature and, at worst, faulty.

SAFETY STUDIES WITH GM SOYA

The feeding value of GM soybean was compared with that of its non-GM parent line in a comprehensive study in which rats were *ad lib* fed for 105 days a diet containing heat-treated glyphosate-tolerant GM soybeans (at 30% soybean meal incoporation; the final dietary protein concentration in the diet

TABLE 1

Incidence of stomach erosion/necrosis in rats tube-fed with GM or non-GM tomatoes

Study 677–004			Study 677–005 (different tomatoes)		
Tomatoes	Rats affected/tested		Tomatoes	Rats affected/tested	
Non-GM	male	0/20	Non-GM	male	1/20
Non-GM	female	0/20	Non-GM	female	0/19
GM	male	0/20	GM	male	0/20
GM	female	4/20	GM	female	2/15
GM	female	7/20*			

*Independently re-scored by a different group of pathologists.
Histological stomach sections were scored for erosion/necrosis on an arbitrary scale from 1 to 4. Most lesions were mild/moderate; scored 1–2.

between 17–18%) expressing the bacterial 5–7 enolpyruvylshikimate-3-phosphate synthase in comparison with a control diet containing the same cultivar non-GM parent line. It was claimed that the growth, feeding value, the histopathology of immune related organs and the amounts of soybean-specific immunoglobulins showed no significant differences between animals fed GM and non-GM lines and no immunotoxic activity was found in GM soybean-fed rats or mice. Unfortunately, although the design of this long-term study was acceptable its execution was poor. Thus, the growth of rats was unacceptably low and only amounted to just over 20 g over 105 days and the growth of mice was zero. For comparison in the much-criticised GM potato study (temporarily displayed on the Rowett Research Institute website in 1999 against my expressed wishes) in which the dietary protein concentration was only 8%, the rats grew close to 300 g during the same period of 105 days (Figure 1). Thus, these unphysiological, basically, starvation conditions may have invalidated all the results. In fact, this study gave a good example of how under starvation conditions most physiological/metabolic/immunological parameters could become unreliable, as demonstrated by the large variability and standard deviation of the mean values obtained for soybean-specific IgE and IgG in both rat and mice sera (Table 2). Despite these shortcomings, this study made a significant contribution to an eventual risk assessment protocol for GM foodcrops that could be generally accepted.

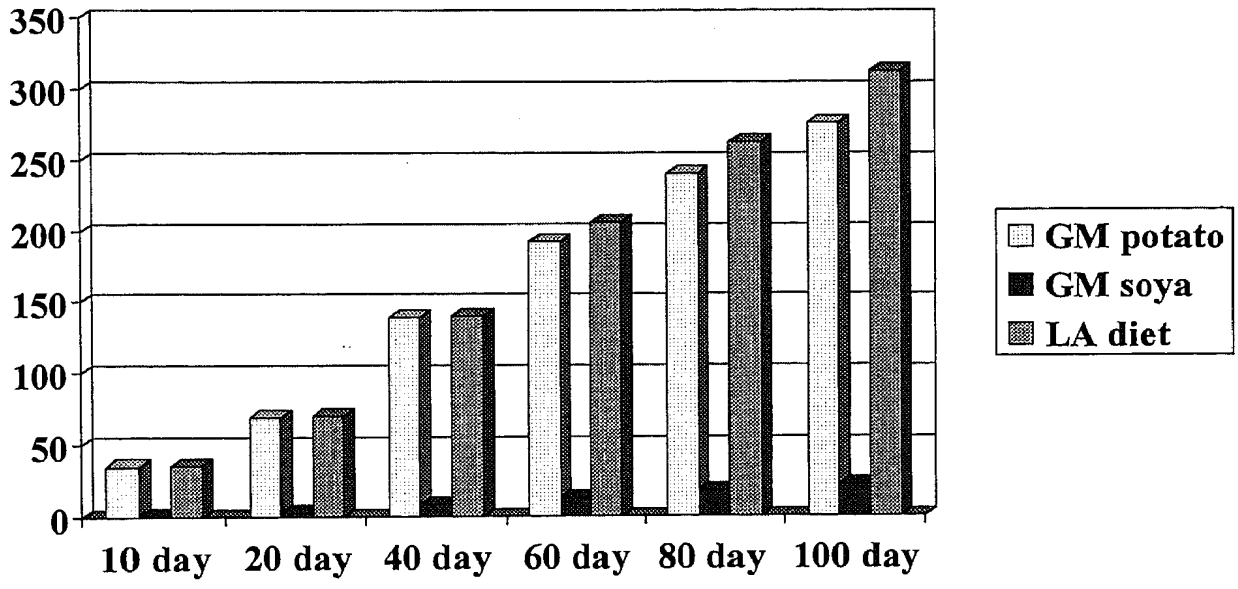


Figure 1. Growth rates (g/day) of rats fed GM potato or GM soya diets for 105 days. The data are from two different experiments. The GM potato experiments were published on the Rowett website (Rowett Research Institute, 1999) and the feeding studies with GM soya were by Teshima *et al.*, 2000. For comparison, the growth rate of control rats fed on a high-quality diet containing lactalbumin (LA) are also included.

TABLE 2

Soybean-specific IgE and IgG production in (a) rats or (b) mice fed heat-treated GM or non-GM soybean diets

(a)

Diet fed	ELISA titre (n = 5)	
	IgE	IgG
GM soybean	143 (100)	1576 (1207)
Non-GM soybean	152 (149)	1950 (348)
Diet control	66 (37)	643 (511)
(b)		
GM soybean	61 (12)	100 (112)
Non-GM soybean	88 (71)	57 (16)
Diet control	51 (0.9)	173 (226)

Standard ELISA method was used with sera obtained from the animals at the end of the feeding study; the values are means (sd). Taken from Teshima et al. (2000).

SUGGESTED PROTOCOL FOR COMPOSITIONAL AND NUTRITIONAL/TOXICOLOGICAL TESTING OF GM FOOD

Selection of suitable genes

Candidate genes for transgenic applications need to be screened for safety by subjecting their isolated products to a thorough nutritional/toxicological evaluation in advance of the gene transfer. Obviously, only those genes can be accepted whose products have no apparent toxic/antinutritive effects in these biological tests. However, the lack of toxicity of this gene product as expressed in the transgenic plant must also be established. Thus, the gene product will have to be isolated from the transgenic plant and its biological, hormonal and immunological properties and allergenicity tested. The present practice of using an *E. coli* recombinant gene product for the testing is not acceptable because post-translational modifications, which are different in prokaryotes and eukaryotes, can have major effects on the biological properties of the gene product proteins.

Compositional analysis

Prior to any nutritional evaluation, GM foodcrops must be subjected to meticulous chemical compositional analyses. For this, in addition to quantitatively measuring the macronutrient (protein, DNA/RNA, carbohydrate, fat, etc) composition of the GM foodcrops and their parent lines, novel methods

need to be developed to pinpoint any differences in minor constituents between GM and their parent lines (Kuiper *et al.*, 1999). However, for a rigorous and meaningful compositional comparison, the GM plant must be grown together with its non-GM parent line under identical and favourable conditions. For example, when this was done with GM and parent line potatoes significant differences were observed in a number of tuber constituents of nutritional importance (Rowett Research Institute, 1999). Moreover, the differences were not only significant between the parent and GM lines but also between the two GM lines developed using the same vector construct and at the same time (Table 3). It may also be prudent to grow the same GM and non-GM plants under non-ideal conditions because it is possible that compositional changes will occur in stressed plants and the GM and non-GM lines could be differently affected. Thus, "substantial equivalence" must be established under a variety of conditions and when it is claimed that the GM and non-GM crops are equivalent, the conditions of the comparison must be declared.

Nutritional/toxicological testing

After the gene selection is done as outlined above, the resulting GM foodcrops are unlikely to be poisonous. Thus, toxicity is an unhelpful concept and would also be difficult to assay at the low doses of the GM products found in GM food. In contrast, when nutritional studies are carried out in which diets containing the GM crop at high and variable concentrations are fed first to young rapidly growing laboratory animals in comparison with controls, these should reveal the possible harmful effects of the GM crop on animal metabolism, the development of their organs and immune and endocrine functions and gut flora. As the normality of these together determine the development of the young animal into healthy adults, this also may guarantee the short-term safety of the GM crop.

TABLE 3

Compositional values for "Desiree" potato tubers and two GM lines expressing the snowdrop (*Galanthus nivalis*) bulb lectin, GNA, derived from them

Constituent	Parent line	GM lines	
		Line 71	Line 74
Protein (% w/w)	7.2[a]	7.2[a]	5.6[b]
Lectin (μg/g)	6.7 (0.4)[b]	7.9 (< 0.1)[a]	5.8 (0.8)[c]
Trypsin inhibitor (mg/g)	3.4 (< 0.1)[a]	3.1 (0.1)[b]	2.7 (0.1)[c]
Chymotrypsin inhibitor (mg/g)	2.7 (0.1)[a]	2.6 (0.1)[a]	2.2 (0.1)[b]

The plants were grown side-by-side in field tunnels. The values are means (sd) of analyses at least four determination of each constituent independently carried out by two workers. Values with different superscripts are significantly different ($p < 0.05$).

It is of paramount importance that the nutritional testing is rigorously standardized. Thus, all diets must be *iso*-proteinic and *iso*-energetic and fully supplemented with vitamins and essential minerals. The composition of the control diet containing the parent line should be as close to the GM diet as possible. In a second control diet, the parent line should be supplemented with the gene product isolated from the GM crop whose concentration should be the same as in the GM crop. The GM crop should be fed both raw and after heat-treatment.

Groups of young rapidly growing animals (5/6 in each group) closely matched in weight (less than ± 2% w/w), housed separately should be fed these diets in short- and long-term experiments. The progress of the animals should be closely monitored, urine and faecal samples collected throughout the experiment and the nutritional performance of the animals and the nutritional value of the diets assessed by Net Protein Utilization (NPU), and measurements of Nitrogen and dry weight balances and feed utilization ratios. The animals should be weighed daily and any abnormalities observed. Blood samples should be taken before, during and at the end of the feeding experiments for immune studies (immune responsiveness assays, Elispot, etc), hormone assays (insulin, CCK, etc) and determination of blood constituents. At the end of the experiments the animals are killed, dissected, their guts rinsed and the contents saved for further studies (enzyme contents, GM products, DNA, etc), gut sections taken for histology, the wet and dry (after freeze-drying) weights of organs recorded, and the organs subjected to compositional analyses. All these data can comprehensively characterise the health and metabolic status of the animals and the behaviour of the GM fed animals can be directly compared with that of the controls.

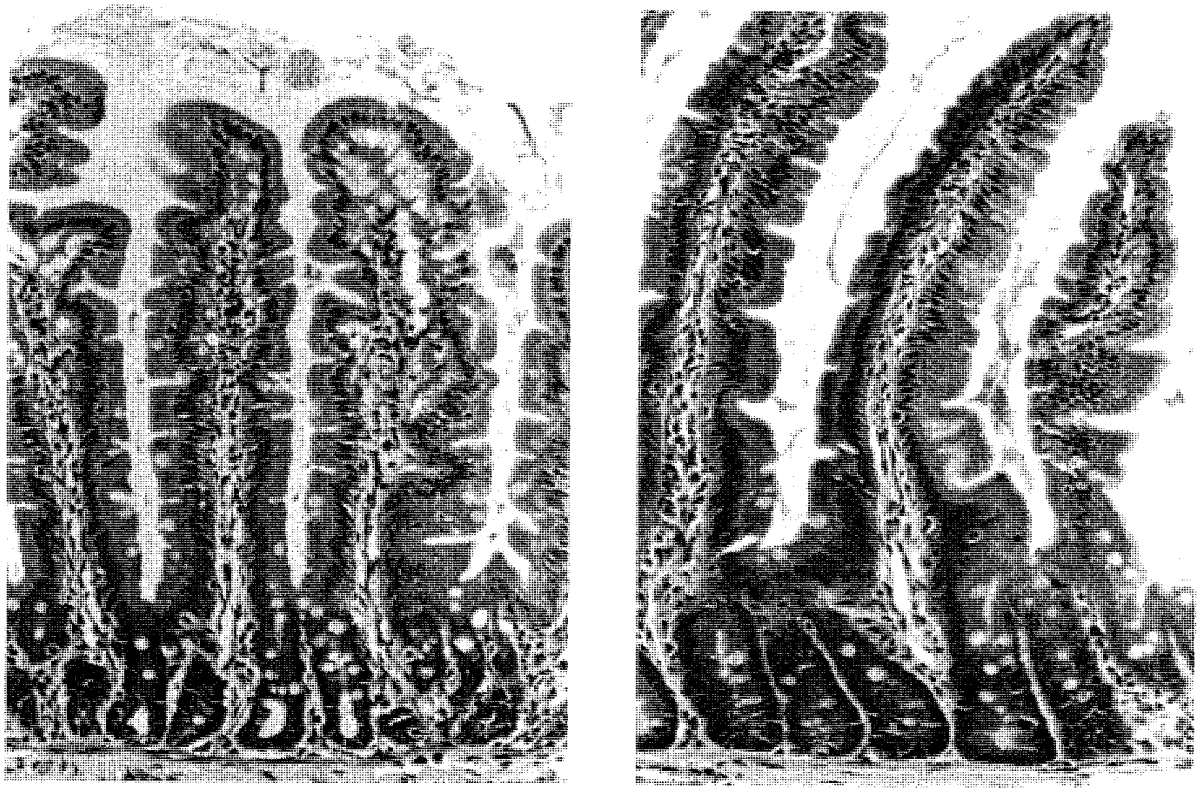
Assessment of potential deviations in the normal development of key organs (Table 4) is of great diagnostic value as shown in our of our GM potato feeding studies (Rowett Research Institute, 1999). Thus, a follow up gut histology study (Ewen & Pusztai, 1999b, Figure 2) indicated the possibility that, due to the trophic effect of an unknown growth/proliferative signal of the GM potatoes, the small intestine of the GM potato-fed rats underwent hyperplastic growth. This was of particular significance because the jejunum was not enlarged when the parent line diet was supplemented with the gene product. This was in line with previous observations which showed that GNA, the gene product, had negligible growth factor effect on the jejunum even when included in the diet at several hundredfold concentration compared with that expressed in the GM potato lines (Pusztai *et al.*, 1990). In a different study ileal sections of mice fed GM potatoes expressing *Bacillus thuringiensis* var. *kurstaki* Cry 1 toxin gene or the toxin itself similar hypertrophic and other changes in gut ultrastructure were shown (Fares & El-Sayed, 1998), suggesting that GM potatoes may have common trophic effects on the gut. Organ weight changes could therefore be useful indicators of metabolic events after feeding with diets containing GM foodstuffs, particularly if followed up by histological examinations in the safety assessment of GM crops.

TABLE 4

Relative dry organ weights of rats significantly affected by feeding with diets containing raw or boiled GM potatoes and/or parent potatoes spiked with the gene product (GNA, *Galanthus nivalis* agglutinin)

	Raw potatoes			Boiled potatoes
Diet	Pancreas	Jejunum	Prostate	Liver
Parent	0.68 (0.08)	0.62 (0.06)	0.24 (0.08)	3.78 (0.14)
GM	0.81 (0.05)	0.72 (0.07)	0.16 (0.02)	3.28 (0.21)
Parent + GNA	0.70 (0.08)	0.67 (0.04)	0.18 (0.02)	3.40 (0.28)
Significance ($p <$)				
Parent vs GM	0.01	0.03	0.05	0.001
Parent + GNA vs GM	0.03	ns	ns	ns

Rats were fed with the diets for 10 days. The values of relative dry organ weights (g organ weight/100 g dry body weight) are means (sd), n=6, by multivariate statistical analysis.



(a) (b)

Figure 2. Histology of jejunal sections of rats on GM potatoes. Jejunal crypt length and cells exhibit marked enlargement after feeding rats raw GM potato diets for 10 days (*b*) in comparison with that of rats given parental line potato diets (*a*). The villus length is similar in both but intraepithelial lymphocyte cell counts appear to be increased on GM potato diet (14 mm bar = 100 μm).

Measurement of immune responsiveness could also be a useful indicator of potential hazards of the ingestion of GM foodstuffs as shown in our GM potato studies (Table 5), particularly as this method is in general clinical use and could therefore easily be carried out with humans. Although in the GM potato study no hormone assays were performed on rats fed GM or non-GM diets the consistent strong pancreatic growth stimulated by the GM potato diets in the feeding studies suggests that this possibly was the result of the release of CCK (cholecystokinin) or some other humoral growth factor from the duodenum by an unknown growth/proliferative signal only found in the GM potato diets. Again, GNA could not be responsible for this because GNA does not stimulate the enlargement of the pancreas (Pusztai *et al.*, 1990). It would also be a good indicator to measure circulating insulin levels after ingestion of GM diets, particularly as insulin assays can easily be done on humans.

Evaluation

With suitable statistical analyses (ANOVA, multivariate analysis, multiple comparisons, etc) the statistical significance of any differences should be established.

Differences in any of the parameters measured between diets containing GM crops and their corresponding parent line should indicate that the genetic modification had led to significant effects in the utilisation and nutritional value of the crop. Accordingly, the GM crop cannot be accepted for inclusion in the human/animal diet unless the differences were minor and the benefits

TABLE 5

Results of lymphocyte proliferation assays in rats fed for 10 days diets containing raw GM-, control/non-GM potatoes, or control/non-GM potatoes supplemented with the gene product, GNA.

	µg Con A/well				
Diet	0.3	1.0	3.0	6.0	9.0
Parent	10.3 (13.4)	16.0 (18.5)	4.4 (4.9)	1.9 (1.0)	1.6 (1.6)
Parent + GNA	2.5 (4.3)	2.6 (3.5)	2.0 (3.6)	1.1 (0.5)	0.9 (0.6)
GM	1.5 (0.9)	1.7 (1.1)	1.0 (0.4)	1.6 (1.1)	1.6 (1.5)
Significance ($p <$)					
Parent vs					
Parent + GNA	ns	$p < 0.05$	ns	$p < 0.05$	ns
Parent vs GM	$p < 0.05$	$p < 0.05$	$p < 0.05$	ns	ns

Rats were fed on different diets for 10 days. At the end of the experiment blood samples were taken and subjected to standard lymphocyte stimulation assay with Concanavalin A (Con A) as the mitogenic signal. The results are expressed as stimulation indexes vs control. Values are means (sd) and significance was assessed by Student *t* test.

deemed to outweigh the potential harmful effects of the GM foodstuff. However, such favourable risk/benefit analysis must be confirmed by further independent research with animals closer to humans and any final decision should be made by concensus openly, transparently and inclusively and not secretly by companies which have developed the GM crop.

If the measured parameters are established to be different between rats fed parent line diets spiked with the gene product and parent line control diets, the use of this gene in GM food is not acceptable.

If the negative effects of the GM foodstuffs are not observed with the diet containing the parent line supplemented with the gene product, it is likely that the harm of the GM foodstuff is due to the use of the particular construct or caused by an unintended and unforeseen effect of the foreign gene insertion into the plant genome. Accordingly, this method of gene transfer and the resulting GM crop is unacceptable. Thus, further research is needed into other, more precise and safer methods of genetic modification.

FURTHER CONSIDERATIONS AND PERSPECTIVES

Laboratory animal testing is but the first step in the risk assessment process and the suggested protocol can, and indeed needs to be, further developed. Since there were indications that the GM potatoes affected some of the male secondary sex organs in rats, similar studies also ought to be routinely performed with female small animals and extended to studies into the effects of GM foodstuffs on reproductive performance. Furthermore, as some of the potential effects of the consumption of GM foods is likely to be manifested in the long-term, these reproductive studies should be coupled to nutritional/toxicological tests in which offsprings of successive generations brought up on GM food should be tested in comparison with those reared on comparable non-GM diets.

In the next step of the risk assessment procedure if there were no indications of harm to laboratory animals, the results will have to be validated with human volunteers in clinical, double-blind, placebo-controlled drug-type tests. However, we must keep it in mind that any harm could be most acute in the young, the elderly, the sick, particularly those suffering from HIV, hepatitis and other viral diseases and those with diseases of the alimentary tract.

The only hope for GM foods is that the present crude methods of genetic modification will be replaced with novel and more refined methods. Unlike the present methodology where the effects of the various constructs, promoters, plasmids, marker genes, etc on the mammalian gastrointestinal tract have never been properly investigated, in future recombinant gene technologies every step should be thoroughly tested before the development of novel GM crops. However, even these will have to be subjected to rigorous animal testing as outlined above and then followed by drug-type certification

tests before allowing them into the food chain. Some may think that the studies envisaged in this protocol are excessive but when it comes to the health and long-term prospects of the human race, nothing can be excessive. Moreover, the methods for starting up testing are available and many scientists are ready. It is only the political will and the money which needs to be found.

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Short Communication

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Cytotoxicity on human cells of Cry1Ab and Cry1Ac Bt insecticidal toxins alone or with a glyphosate-based herbicide

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ABSTRACT: The study of combined effects of pesticides represents a challenge for toxicology. In the case of the new growing generation of genetically modified (GM) plants with stacked traits, glyphosate-based herbicides (like Roundup) residues are present in the Roundup-tolerant edible plants (especially corns) and mixed with modified *Bt* insecticidal toxins that are produced by the GM plants themselves. The potential side effects of these combined pesticides on human cells are investigated in this work. Here we have tested for the very first time Cry1Ab and Cry1Ac *Bt* toxins (10 ppb to 100 ppm) on the human embryonic kidney cell line 293, as well as their combined actions with Roundup, within 24 h, on three biomarkers of cell death: measurements of mitochondrial succinate dehydrogenase, adenylate kinase release by membrane alterations and caspase 3/7 inductions. Cry1Ab caused cell death from 100 ppm. For Cry1Ac, under such conditions, no effects were detected. The Roundup tested alone from 1 to 20 000 ppm is necrotic and apoptotic from 50 ppm, far below agricultural dilutions (50% lethal concentration 57.5 ppm). The only measured significant combined effect was that Cry1Ab and Cry1Ac reduced caspases 3/7 activations induced by Roundup; this could delay the activation of apoptosis. There was the same tendency for the other markers. In these results, we argue that modified *Bt* toxins are not inert on nontarget human cells, and that they can present combined side-effects with other residues of pesticides specific to GM plants. Copyright © 2012 John Wiley & Sons, Ltd.

Keywords: Roundup; *Bt* toxins; Cry1Ab; Cry1Ac; GMOs; mixtures; glyphosate; human cells

INTRODUCTION

The real effects of mixtures of chemical pollutants are a major concern for public health (Monosson, 2005). Humans are exposed to hundreds of compounds on a daily basis. The commercialized combinations could be a first matter of concern. Agricultural genetically modified organisms (GMOs) are steadily increasing worldwide, and they need to be carefully assessed (Séralini *et al*., 2009, 2011; Spiroux de Vendômois *et al*., 2010). Nowadays 99.9% of GMOs can be described as pesticide plants, designed for herbicide tolerance and/or modified insecticide production (James, 2010). Thus pesticides residues co-occur in the plant, synthesized by the plant itself, by the expression of the inserted transgene (modified *Bt* from *Bacillus thuringiensis*) or through external pesticide treatment facilitated by the transgene-dependent tolerance to herbicides (Roundup in most instances). In turn, such residues exert their effects upon consumption or release into the environment (Arregui *et al*, 2004; Tank *et al*., 2010). Owing to their key role in intensive agriculture, potential side effects of such combined pesticides residues should be assessed. *In vitro* tests are frequently recommended as a first step to replace animal models in toxicity studies. Here, we have tested for the first time the effects of Cry1Ab and Cry1Ac alone and combined with Roundup on human cells.

Modified toxins from *Bt* are Cry proteins forming pores in insect cell membranes (Then, 2010); they account for 39% of edible plant GMOs worldwide (James, 2010). Since natural *Bt* toxins have long been used, their modified counterparts are often compared with them. However, the latter derivatives are truncated, adapted and modified synthetic sequences; consequently their activity is possibly quite different from the natural ones (Séralini *et al*., 2011). Also, *Bt* toxins are claimed and believed to be safe. Yet prions, hormones and venoms are also proteins, and are far from being innocuous. To date, *Bt* toxins have not been tested on human cells. However, *Bt* corns are regularly consumed by humans in America and their residues have even been found in maternal and fetal cord serum at around 0.2 ppb (Aris and Leblanc, 2011), which does not take into account the tissue levels. Nontarget toxicity of natural *Bt* toxins has been detected in mammals, for instance at a 50% lethal concentration (LC_{50}) from around 10 to 520 ppb (Ito *et al*., 2004; Nagamatsu *et al*., 2010; Rani and Balaraman, 1996).

Roundup formulations are mixtures of glyphosate and adjuvants such as ammonium sulfate, benzisothiazolone, glycerine, isobutane, isopropylamine, polyethoxylated alkylamines and

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sorbic acid (Cox, 2004). Glyphosate-based herbicides are the object of an increasing number of studies, which reveal, in combination with adjuvants, endocrine-disrupting effects, and tumor-promoting or teratogenic effects on numerous nontarget species (Gasnier *et al.*, 2009; George *et al.*, 2010; Paganelli *et al.*, 2010), for instance from 0.5 ppm on androgen receptors. We have used human embryonic kidney cell line HEK293 as a sensitive model (Benachour and Séralini, 2009). The kidney model was used also because a body of evidence suggests kidney dysfunctions as endpoints of GMO diet effects (Séralini *et al.*, 2011), and thus kidney cells could be a target for GMOs. We first measured the mitochondrial respiration level, by succinate dehydrogenase (SD) activity assessment in order to test cytotoxicity. Then, as *Bt* proteins act as pore forming toxins (Then, 2010), we determined adenylate kinase (AK) activity when released in the medium, revealing possible membrane alterations. In association, we assayed caspase 3 and 7 activities in order to separate the apoptotic and necrotic actions involved in cytotoxic effects. Moreover, human cell lines allow the study of unintended side effects on nontarget species of GMO-associated pesticides.

MATERIALS AND METHODS

Chemicals

Cry1Ab and Cry1Ac were prepared as described previously by two different laboratories (Székács *et al*, 2010; Pusztai-Carey *et al.*, 1994). The glyphosate-based herbicide tested was commercially available Roundup® GT Plus formulation, approval no. 2020448 (Monsanto, Anvers, Belgium). It contains 450 g l^{-1} glyphosate acid (*N*-phosphonomethyl-glycine). Successive dilutions were prepared in Eagle's modified minimum essential medium (EMEM; Abcys, Paris, France). 3-(4,5-Dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) and all other compounds were obtained from Sigma-Aldrich (Saint-Quentin Fallavier, France), unless specified. MTT was prepared as a 5 mg ml^{-1} stock solution in phosphate-buffered saline, filtered through a 0.22 μm filter before use, and diluted to 1 mg ml^{-1} in EMEM.

Toxin Preparations

The Cry1Ab and Cry1Ac toxins are cloned from the natural *Bacillus thuringiensis* subspecies kurstaki HD-1 strain and expressed in *Escherichia coli* as single gene products. The inclusion bodies, containing the protoxins, were solubilized at pH 10.5 in the presence of β-mercaptoethanol and treated with commercial bovine trypsin (Sigma, USA). The 65 kDa activated toxins were isolated by ion exchange HPLC and the pure toxin fractions were desalted and lyophilized and stored at −80 °C. After storage, toxins were diluted in a 50 mM Na-carbonate–HCl buffer at 1 mg ml^{-1} (pH 9.5), and then diluted in EMEM.

Cell Lines

The human embryonic kidney 293 cell line (ECACC 85120602) was provided by Sigma-Aldrich (Saint-Quentin Fallavier, France). Cells were grown in phenol red-free EMEM (Abcys, Paris, France) containing 2 mM glutamine, 1% nonessential amino acid, 100 U ml^{-1} of antibiotics (a mixture of penicillin, streptomycin and fungizone; Lonza, Saint Beauzire, France), 10 mg ml^{-1} of liquid kanamycin (Dominique Dutscher, Brumath, France) and 10% fetal bovine serum (PAA, les Mureaux, France). Cells were grown at 37 °C (5% CO_2, 95% air) during 24 h to 80% confluence, washed with serum-free EMEM and then exposed to various chemicals, since the serum delayed the cell necrosis by about 48 h in the presence of toxic compounds (Benachour *et al.*, 2007). The control cells grow normally in serum-free medium up to 96 h.

Cell Treatments and Cytotoxicity Biomarkers

Cells at 80% confluence in 48- or 96-well plates (Dominique Dutscher, Brumath, France) were washed with serum-free EMEM, in order to avoid other combined effects, and then exposed to various concentrations of *Bt* toxins or Roundup GT Plus in EMEM serum-free medium for 24 h. *Bt* toxins were used from 10 ppb to 100 ppm (in the range of GM plant production). Concerning Roundup, 50% lethal concentrations (LC_{50}) were assessed from 1 to 20 000 ppm (the latter is the agricultural dilution). Combined effects were measured by mixing LC_{50} of Roundup with three doses of each *Bt* toxin. After treatments, the following tests were applied: mitochondrial respiration assay (MTT) through the succinate dehydrogenase activity measurement (Mosmann, 1983). The optical density was measured at 570 nm using a Mithras LB 940 luminometer (Berthold, Thoiry, France). The bioluminescent ToxiLight bioassay (Lonza, Saint Beauzire, France) was applied for the membrane degradation assessment, by the intracellular AK release in the medium; this is described as a necrosis marker (Crouch *et al.*, 1993). Finally, the apoptotic cell death was evaluated with the Caspase-Glo 3/7 assay (Promega, Paris, France). Luminescence was measured using a Mithras LB 940 luminometer (Berthold, Thoiry, France). These methods were previously described by our group (Benachour and Séralini, 2009).

Statistical Analysis

The experiments were repeated at least three times in different weeks on three independent cultures ($n = 9$). LC_{50} values were calculated by a nonlinear regression using a sigmoid (five-parameter) equation with the GraphPad Prism 5 software. All data were presented as the means ± standard errors (SEs). Statistical differences were determined by Student *t*-test using significance levels at $P < 0.01$ (**) and $P < 0.05$ (*).

RESULTS

We measured for the first time cytotoxic effects of *Bt* toxins, alone or in combination with a glyphosate-based herbicide, on HEK293 cells. First of all, we confirmed that the buffer was not cytototoxic for the cells. The mitochondrial succinate dehydrogenase activity of treated cells significantly decreased at 100 ppm of Cry1Ab alone (by 11%, Fig. 1A). Even if it was limited, this phenomenon was undetected for Cry1Ac. Lower doses were tested from 10 ppb to 10 ppm, but significant effects were not observed. We obtained similar effects with a Cry1Ab toxin provided by another laboratory that was prepared and stored independently (Fig. 1A). We measured AK activity after its release in the medium in order to evaluate plasma membrane integrity. A concentration of Cry1Ab of 100 ppm increased AK leakage in the medium 2-fold, revealing plasma membrane alterations. This was performed for the two Cry1Ab toxins. Apoptotic effects of Cry1Ab and Cry1Ac by means of caspase 3/7 activities were studied; no effects on HEK293 cells were visible. We can therefore confirm that Cry1Ab can induce cytotoxic effects via a necrotic mechanism in these conditions at 100 ppm.

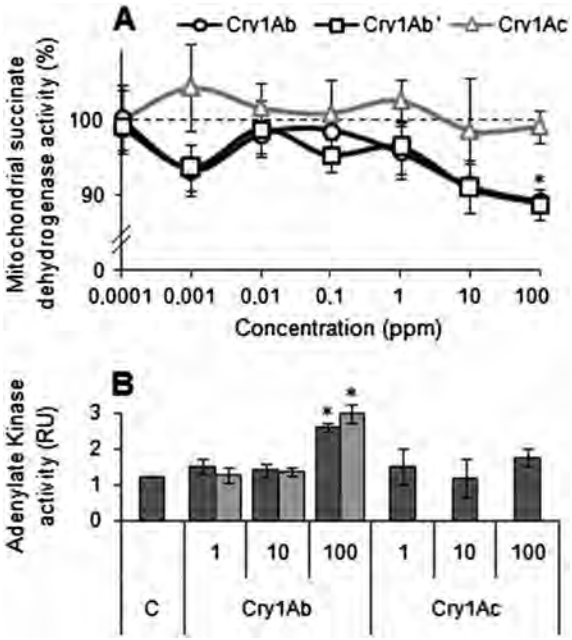

Figure 1. Cytotoxic effects of modified *Bt* toxins Cry1Ac and Cry1Ab on HEK293 cell line. (A) Cell death has been measured on mitochondrial succinate dehydrogenase after 24 h exposure to two Cry1Ab toxins up to 100 ppm prepared from different sources (A, black curves with circles and squares) and Cry1Ac (grey curve). (B) Cell membrane degradation was measured by adenylate kinase release (RU, relative units) provoked by Cry1Ab and Cry1Ac toxins (1–100 ppm) in comparison to control (C). No effect was detected on caspase 3/7 activities; therefore results are not displayed. Standard errors of the mean are indicated in all instances ($n = 9$; * $P < 0.05$).

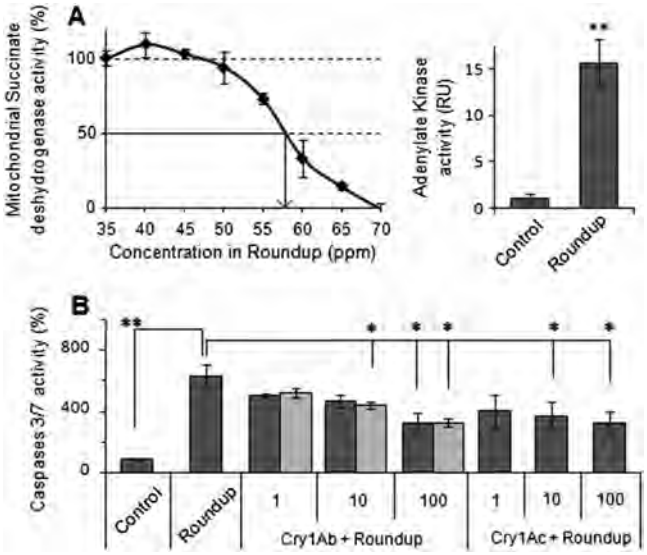

Figure 2. Effects of Roundup alone and with modified *Bt* toxins Cry1Ab and Cry1Ac on HEK293 cell line. (A) Cells were treated with Roundup (1–20 000 ppm) and the mitochondrial respiration was measured through succinate dehydrogenase activity (left). The LC_{50} for Roundup was determined as 57.5 ppm by nonlinear regression (arrow). On the right, the adenylate kinase release showing membrane alterations by Roundup alone at its LC_{50} in comparison to control. (B) Combined effects of Roundup at its LC_{50} with *Bt* toxins measured on caspase 3/7 activities (%). The same tendency for the combined effects but with no significant results was detected on adenylate kinase release and succinate dehydrogenase; results not shown. Standards errors of the mean are indicated in all instances ($n = 9$). Significance of the effects (* $P < 0.05$; ** $P < 0.01$) is tested against the negative control C (medium alone) or the positive Roundup control (R).

Closer to the reality of exposure, we then tested combined effects of *Bt* toxins with Roundup (Fig 2). According to our previous results, Roundup is cytotoxic by inhibition of mitochondrial respiration activity, far below agricultural dilutions (around 200 times less) with an LC_{50} of 57.5 ppm (Fig. 2A). At this concentration, Roundup also induced necrosis evidenced by a 15-fold increase of an AK release. Apoptosis induction was measured by a 6.7-fold caspase 3/7 activities enhancement (Fig. 2B). However, concerning combined effects we observed significant effects on apoptosis; both *Bt* toxins from 10 ppm reduced caspase 3/7 activities (by around 50%) when they were induced in Roundup at its LC_{50} (Fig. 2B). Similarly, there was a non significant tendency for both toxins (data not shown) to reduce AK leakage and mitochondrial respiration inhibition induced by Roundup.

DISCUSSION

Few studies have been performed on nontarget effects of *Bt* toxins, and none with modified *Bt* toxins extracted from plants, or together with Roundup residues, even in regulatory files. For natural *Bt* toxins, their mechanisms of action and insect resistance are not fully understood (Singh and Sivaprasad, 2009), and the metabolism of these proteins in mammals is unknown (Séralini *et al*., 2011; Chowdhury *et al*., 2003). They may even interact with extrinsic factors (Then, 2010). Billions of people and wildlife could be exposed to modified *Bt* toxins; therefore understanding their potential side effects is crucial.

On two biomarkers of cell death, Cry1Ab exposure led to respiration inhibition and plasma membrane alterations, by contrast to Cry1Ac. This could be consistent with the fact that the consumption of MON810 maize producing Cry1Ab (in the ppm range) induced signs of hepatorenal alterations in a subchronic feeding study on rats (Spiroux de Vendômois *et al*., 2009). It is known that both toxins differ significantly in their domain III structure (Karim and Dean, 2000), which is the only one to be involved at the same time in ion channel function, receptor binding and insertion into the membrane (Dean *et al*., 1996). This occurred at relatively high concentrations (100 ppm) in comparison to the concentrations produced in GM plants (1–20 ppm, Székács *et al*., 2010). The content can differ greatly according to the GM variety and environmental conditions (Then and Lorch, 2008). The exposure during consumption can appear low enough to avoid side effects, and whether this occurs *in vivo* remains to be checked. However, the bioaccumulation in tissues, or bioaccumulative or long-term effects, has to be taken into account since *Bt* residues were recently claimed to be measured in pregnant women's serum at around 0.2 ppb (Aris and Leblanc, 2011). In addition, high quantities of *Bt* crops can be consumed by mammals. The procedure for GMO market authorizations for crops such as MON810 (EFSA, 2009) does not require *in vitro* tests on human cells of Bt toxins, nor on its combinatorial effects, thus our results are raising new questions about the safety of these toxins and the *Bt* crops in general. Although *in vitro* studies suggest degradation in human gastric

secretions, digestion is never a complete process and insecticide toxins cannot be fully degraded *in vivo* (Paul *et al*., 2010). This is known to be the case for Cry1Ab (Chowdhury *et al*., 2003). It must be underlined that the insecticidal proteins produced by the GM plants are in soluble forms, and thus already biochemically activated, unlike those produced by the microorganism *Bt*, secreted as inactive precursors or protoxins (Hilbeck and Schmidt, 2006). The importance of *Bt* toxin activation has been demonstrated in relation to *in vitro* membrane damages of human erythrocytes, by solubilized *Bt* toxins, but not by the intact form (Rani and Balaraman, 1996). Cellular response to *Bt* toxins does not elicit apoptosis; it induces necrotic effects via a plasma membrane disruption for Cry1Ab within only 24 h. This may be due to pore formation like in insect cells owing to binding to specific receptors or membrane lipid rafts (Then, 2010; Soberón *et al*., 2009).

We also demonstrated that Cry1Ab and Cry1Ac exposures slightly reduced caspase 3/7 activations induced by Roundup. This could be related at least in part to the properties of Roundup compounds, especially adjuvants. We observed previously, in our group, that serum delayed the cytotoxic effects induced by Roundup. This was probably due to serum binding proteins (Benachour *et al*., 2007). Here we can assume that physico-chemical properties of proteins may give them the ability to bind and form complexes with Roundup adjuvants that have tendencies to form vesicles, buffering their bioavailability to cells. Similarly, a nonsignificant tendency of reduction of the cytotoxic effects of Roundup was observed on mitochondrial respiration and membrane degradation when the toxins were added. The apoptosis induction appeared to be the most sensitive impact of combined effects. This does not exclude other intracellular targets such as endocrine disruption, since Roundup is antiandrogenic from 0.5 ppm, below toxic levels and close to human serum levels (0.1–0.2 ppm in Acquavella *et al*., 2004).

Here we documented that modified *Bt* toxins are not inert on human cells, but can exert toxicity, at least under certain *in vitro* conditions. *In vivo* implications should be now assessed. Our results raise new questions in the risk assessment of food and feed derived from genetically engineered plants.

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A 90-day safety study of genetically modified rice expressing Cry1Ab protein (*Bacillus thuringiensis* toxin) in Wistar rats

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Abstract

An animal model for safety assessment of genetically modified foods was tested as part of the SAFOTEST project. In a 90-day feeding study on Wistar rats, the transgenic KMD1 rice expressing Cry1Ab protein was compared to its non-transgenic parental wild type, Xiushui 11. The KMD1 rice contained 15 mg Bt toxin/kg and based on the average feed consumption the daily intake was 0.54 mg Bt toxin/kg body weight.

No adverse effects on animal behaviour or weight gain were observed during the study. Blood samples collected one week prior to sacrifice were analyzed and compared for standard haematological and biochemical parameters. A few parameters were significantly different, but all within the normal reference intervals for rats of this breed and age and not in relation to any other findings, thus not considered treatment related. Upon sacrifice a large number of organs were weighed, macroscopic and histopathological examinations were performed with only minor changes to report.

The aim of the study was to use a known animal model in performance of safety assessment of a GM crop, in this case KMD1 rice. The results show no adverse or toxic effects of KMD1 rice when tested in the design used in this 90-day study. Nevertheless the experiences from this study lead to the overall conclusion that safety assessment for unintended effects of a GM crop cannot be done without additional test group(s).

Keywords: Genetically modified rice; *Bacillus thuringiensis*; Cry1Ab protein; Animal study; Wistar rats; SAFOTEST; Food safety; Feeding trial

1. Introduction

Bt rice is rice that has been genetically modified to express insecticidal genes (*cry* genes) from *Bacillus thuringiensis* (Bt). The transgenic rice is resistant to major lepidopteran insect pests of rice and thus has the potential to significantly decrease yield losses, reduce the use of broad-spectrum chemical insecticides, and furthermore reduce levels of mycotoxins, one of the unexpected benefits of reducing larval attacks (Cheng et al., 1998; Papst et al., 2005). The Bt rice line, KMD1, since its development in 1998 has been characterized thoroughly at the molecular level, and in numerous field trials has shown evidence of

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affording the farmer a high level of resistance against at least eight different insect pest species (Shu et al., 2000; Ye et al., 2001, 2003).

In spite of the extensive research that has been conducted in developing this pest resistant rice, as summarized by High et al. (2004), Bt rice is not yet grown commercially. Other Bt crops, expressing a range of different *cry* genes, are commercially grown in many parts of the world including Bt corn, Bt cotton, Bt canola and Bt potatoes. Bt toxins (Cry proteins) have been used as microbial pesticides for many years and have a long history of safe use (Mendelsohn et al., 2003; Betz et al., 2000).

Cry proteins show highly species-specific toxicity against certain insects and only a few insect species are affected by each of the Cry proteins. The mode of action in the insect is through specific receptors in the gut, which is highly alkaline, with binding of the toxin resulting in pore-formation, osmotic imbalance, cell lysis and subsequently death of the insect (Betz et al., 2000).

The Cry proteins are regarded harmless or nontoxic to mammals, including humans, probably due to acidified gut pepsinolysis and the lack of Cry protein binding-sites on mammalian gut epithelial cells. Numerous data from toxicity studies show no significant adverse effects of the Cry proteins on body weight gain or clinical observations. Furthermore, no signs of pathogenicity to mammals, including humans, have been reported (McClintock et al., 1995).

Investigations on the effects of the Cry1Ab protein on mammalian cells have revealed no significant effect on bovine hepatocyte morphology or on albumin secretion *in vitro* (Shimada et al., 2003). In animal studies no significant differences were observed in general health or growth rate in pigs fed a Bt corn diet (Chowdhury et al., 2003), although in 1998 Fares and El Sayed observed fine structural microscopic changes in the ileum of mice fed Cry1 potato diet. Bt toxin released by the crop root or from the biomass of Bt corn has been found nontoxic to soil bacteria (Saxena and Stotzky, 2001).

This study is part of the EU-project SAFOTEST, designed to develop scientific methodologies for assessing the safety of genetically modified (GM) crops. The aim of the present 90-day study in Wistar rats was to perform a comparative safety assessment study of the genetically modified Bt rice, KMD1, expressing Cry1Ab in an animal model, when compared to the parental wild-type rice, Xiushui 11, and to furthermore monitor changes in major aerobe and facultative anaerobe bacterial populations in the intestines of the rats.

The study design includes two test groups given comparable diets containing 60% raw brown rice flour from parental and transgenic rice, respectively, to be tested in a directly comparative 90-day feeding study without spiking of the recombinant protein.

The objective was to have identical cohorts of male and female rats in a sub-chronic 90-day exposure to 60% rice diets, which contained realistic and meaningful levels of the transgene-expressed Cry protein, Cry1Ab. The focus was first and foremost on the tissues and organs in initial contact with the diets. These are the digestive tract and related organs, including a detailed veterinarian and pathological assessment of the whole animals' well-being and behaviour.

The rice materials tested in the 90-day toxicity study were subjected to comprehensive analytical characterization before the study so that the compositional data could provide the basis for the interpretation of any possible effects detected in the feeding studies. Every effort was made to provide a consistent and well-characterized GMO diet to the test animals, in a universally adoptable and approvable manner, for a study based on the OECD Guideline no. 408 (OECD, 1995).

2. Materials and methods

2.1. Test material

Bt rice KMD1 and the corresponding parental rice Xiushui 11, were accessed from University of Ottawa (Canada) and Zhejiang University (China), respectively. Seeds of KMD1 and its parental line, Xiushui 11, were produced in the late season of 2000 in Hangzhou, China. Wu et al. (2001) have described generation and selection of the transformant rice. During multiplication of rice seeds, the performance of these materials was consistent with previous years' observations. Neither leaf folders nor stem borers damaged plants of KMD1, while Xiushui 11 was infested by both, leading to curled leaves (caused by leaf folders), dead-hearts and white-heads (by stem borers at vegetative and heading stage, respectively) in the field. All shipping and handling was conducted to protect the freshness and quality of the rice grains. On arrival by air courier at the Danish Institute for Food and Veterinary Research (DFVF, Søborg, Denmark) the rice was stored at 5 °C, before dehulling, grinding and subsequent storage at −18 °C until use. Samples of intact rice grains representative for the bulk material were shipped to Technical University Munich (Germany).

2.2. Characterization of test material

Rice plants were generated by *Agrobacterium*-mediated transformation and positive transformants selected on the basis of hygromycin resistance (Wu et al., 2001). The presence of the Cry1Ab transformation cassette was confirmed by PCR and Southern blot analysis using standard protocols (Sambrook and Russell, 2002). Transgene expression of Cry1Ab in mature seeds of line KMD1 was verified by immunological assay (Western blotting after analysis of total protein by SDS-PAGE) using rabbit polyclonal antibodies raised against Cry1Ab as the primary antibody, with HRP-conjugated goat anti-rabbit IgG (Bio-Rad) as the secondary antibody. The protein was visualized using ECL (chemiluminescence) detection (Amersham) as previously described (Gatehouse et al., 1997) and quantified by densitometric scanning using Bio-Rad Molecular Analyst software. The final concentration of Cry1Ab in the animal diet was also determined by immunoassay.

2.3. Compositional analyses of test material

Intact rice grains were manually dehulled by means of a wooden rice dehuller and ground using a cyclone mill equipped with a 500-μm sieve. The rice flour obtained was immediately frozen and stored at −20 °C until analysis.

Proximates (moisture, starch, fibre, sugars, protein, fat, ash), amino acids, fatty acid distribution and minerals were determined using validated standard protocols (VDLUFA, 1996; VDLUFA, 1997). The content of

protein was calculated using nitrogen to protein conversion factor of 5.95. Vitamin B_1 was measured by the AOAC method (AOAC, 2000). Extraction and HPLC analysis of vitamin B_6 were performed according to Reitzer-Bergaentzle et al. (1993). To measure total vitamin B_6 contents including pyridoxol glucosides, extracts were treated with β-glucosidase (Bognar and Ollilainen, 1997). Niacin was extracted according to Ward and Trenerry (1997) and determined via HPLC analysis (Wills et al., 1977). Folate vitamers and total pantothenic acid were quantified by stable isotope dilution assays based on LC/MS/MS (Freisleben et al., 2003; Rychlik, 2003). A method using on-line coupled liquid chromatography–gas chromatography was used for determination of γ-oryzanol contents and steryl ferulate distributions (Miller et al., 2003). Phytic acid was measured using a colorimetric method (Latta and Eskin, 1980). Heavy metals (cadmium, lead, mercury) were measured by AAS (VDLUFA, 1996). Analysis for mycotoxins included aflatoxins (B_1, B_2, G_1, G_2), ochratoxin A, zearalenon and deoxynivalenol (VDLUFA, 1997). Bacterial and fungal counts were measured using validated standard protocols (VDLUFA, 1997). Pesticides were determined according to DFG, 1991.

2.4. Animals and housing

Sixty-four SPF Wistar rats [mol:Wist] (32 male and 32 female) were obtained from M&B Breeding Center, Ll. Skensved, Denmark. The rats were 6–7 weeks old at the initiation of the study. The animals were housed pair wise in stainless steel wire cages at 22 ± 1 °C, relative humidity 55 ± 5%, air change 10 times/h and electric light from 09.00 to 21.00. Animal experiments and housing procedures were performed in accordance to the Danish Animal Experimentation act on a license granted by the Ministry of Legal Affairs and the Convention ETS 123 of the Council of Europe and the Danish Animal Experimental Inspectorate approved the study.

2.5. Diet formulation and feeding

The purified, or semi-synthetic, rat diet used in the study is produced in house (Poulsen et al., 2002), based on the rodent diet AIN-93 (Reeves et al., 1993). The purified diet is based on cornstarch and does not contain rice. In this study, both test diets contained 60% ground rice flour, either Xiushui 11 for the controls or KMD1 expressing Cry1Ab protein from the *cry1Ab* gene. Mixing procedures were performed as described by Poulsen et al. (2006); see Table 1 for diet composition.

Both diets were adjusted identically to assure an adequate supply of macronutrients and vitamins after substitution with 60% rice, but no adjustments were made to outbalance the differences in the constitution of the rice, observed by the compositional/chemical analyses. The rats were allowed free access to both food and water.

Table 1
Composition of diets

Ingredients (%)	Group 1	Group 2
Control rice (Xiushui 11)	60	0
Bt rice (KMD1)	0	60
Corn starch	5.2	5.2
Sucrose	6.8	6.8
Soybean oil	5	5
Cellulose	5	5
Mineral mixture[a]	2.8	2.8
Vitamin mixture[b]	1.2	1.2

[a] In mg/kg diet: Ca: 5000, P: 3100, K: 3600, S: 300, Na: 2500, Cl: 1500, Mg: 600, Fe: 34, Zn: 30, Mn: 10, Cu: 7, I: 0.20, Mo: 0.15, Se: 0.15, Si: 2.5, Cr: 1.0, F: 1.0, Ni: 0.5, B: 0.5, Li: 0.1, V: 0.1, Co: 0.07.

[b] In mg/kg diet: Vit. A.: 5000 (IU); Vit. D_3.: 1000 (IU); Vit. E.: 50 (IU); Thiamin: 5; Riboflavin: 6; Pyridoxol: 8; Folic acid: 2; D-biotin: 0.3; Vit. B_{12}.: 0.03; Panthothenate: 20; Cholinhydrogentartrat: 2600; Inositol: 400; Nicotinic acid: 40; Phylloquinine: 1; p-aminobenzoic acid: 40; Methionine: 1000; L-cystine: 2000.

2.6. Experimental design

Animals were randomly assigned to two experimental groups of 16 males and females, based on body weight means. The animals were observed twice daily; body weight, food and water consumption were measured once weekly. During the last week of treatment, blood samples were taken from the tail vein and collected in EDTA and heparin coated tubes for hematology and blood biochemistry, respectively. Blood samples were taken under Hypnorm–Dormicum anaesthesia and the animals were fasted overnight to minimize fluctuations in the parameters measured.

At terminal sacrifice, the animals were anaesthetized by CO_2 inhalation and killed by decapitation and exsanguination followed by examination for gross and histopathological changes.

2.7. Blood biochemistry and haematology

Following biochemical parameters were measured in plasma: urea (BUN), alanine aminotransferase (ALAT), sodium, potassium, cholesterol, protein, albumin, creatinine and glucose. All analyses on blood plasma were performed on a Cobas Mira S analyzer (Roche Diagnostic Systems, Switzerland) using the relevant kits for each parameter.

Haematology characteristics were assessed using a Twincounter 187 Hematology Analyser (Analysis Instruments AB, Stockholm, Sweden) on the following parameters: White blood cells (WBC), red blood cells (RBC), platelets (PLT), haemoglobin (HGB), haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC). The differential count was performed manually on blood smears for neutrophilic, eosinophilic and basophilic granulocytes, lymphocytes, monocytes and large unstained cells (LU). The smears were stained with May-Grünwald and Giemsa and two times 100 cells were counted.

2.8. Bacterial counts

During the experimental period, fresh faecal samples were taken for microbial analysis from ten animals (5 males and 5 females) of each of the two groups by provoked defecation at day 30 and 60 of the experiment, and at termination of the study. Furthermore, at terminal sacrifice samples from ileum and duodenum were taken from the same ten animals of each group. The faecal and intestinal samples were treated as described by Poulsen et al. (2006).

2.9. Gross necropsy and histopathology

A complete necropsy was performed and the following organs were excised and weighed: adrenals, brains, epididymis, heart, kidneys, liver, mesenterial lymph nodes, ovaries, pancreas, small intestine, spleen, stomach, testes, thyroid gland and uterus. Paired organs (adrenals, epididymides, kidneys, ovaries and testis) were weighed as a total of left and right. Sections from the above organs including the axillary lymph nodes, skin with mammary glands, bones, spine and other organs and tissues with macroscopically visible lesions were fixed for a minimum of 24 h in 4% buffered formaldehyde before histological processing. Tissue samples were embedded in paraffin and sections, 4–6 μm thick, were then stained routinely with hematoxylin–eosin (H&E) for light microscopy.

The main focus of the histopathological examination was on the intestinal tract and the related organs. From a total of 10 males and females per group the following tissues were selected for histological examination: forestomach, glandular stomach, duodenum, jejunum and ileum of the small intestine, cecum, colon, rectum, the mesenterial lymph node, liver (sections from both right and left lateral lobes), pancreas (exocrine and endocrine), adrenal cortex and medulla, kidneys, axillary lymph node, heart, skeletal muscle (*m. biceps femoris*), spleen, and thymus.

2.10. Statistical analysis

Compositional data are presented as means ± confidence intervals ($p < 0.05$). Means are considered as statistically significantly different if their confidence intervals are not overlapping. All statistical calculations on data obtained from the feeding study were carried out using SAS release 8.1 (SAS Institute Inc., Cary, NC). Homogeneity of variance among groups and normality distribution were investigated. Two-way analysis of variance with repeated measures on one factor was used to analyze food and water consumption, body weight and faecal and intestinal microflora. A Least Significant Difference test, or Duncan multiple-range test if significant, followed the analyses. Organ weights, data on hematology and blood biochemistry were analyzed by ANOVA (general linear model), and where the overall *F*-test was significant, least square means was used to compare the exposed group to the control group.

In cases where data was not normally distributed a non-parametrical test was performed, using Kruskal–Wallis test followed by Wilcoxon Two-Sample test. $P \leqslant 0.05$ was in all cases considered significant, data on males and females were always analyzed separately. Data is mainly presented as group mean values ± SEM (standard error of the mean).

3. Results

3.1. Compositional analysis

Bt (KMD1) brown rice and parental (Xiushui 11) brown rice tested in the 90-day feeding study were subjected to comprehensive analytical characterization. More than fifty rice constituents were measured including proximates, amino acids, fatty acids, minerals, vitamins, steryl ferulates and phytic acid. In addition the material was screened for contaminants (heavy metals, pesticides), and the microbiological quality was evaluated by screening for mycotoxins and bacterial/fungal counts. Compositional data were compared to data reported for brown rice (Juliano, 1985; Latta and Eskin, 1980; Scherz and Senser, 2000; Møller et al., 2002; USDA, 2004; OECD, 2004; Kitta et al., 2005) and differences between the lines were assessed for statistical significance ($p < 0.05$).

Contents of proximates are presented in Table 2. Compared to the parental rice, KMD1 exhibited a statistically significantly higher protein content (+8%) and a statistically significantly lower fat content (−18%). However, data for both lines are within literature range (Juliano, 1985; Scherz and Senser, 2000; Møller et al., 2002; USDA, 2004; OECD, 2004). No statistically significant difference between the lines was detected for contents of moisture, starch, fibre, sugars, and ash.

The difference between KMD1 and Xiushui 11 in protein content is also reflected in the amino acid levels (Table 3). The transgenic rice exhibited statistically significantly higher contents of all amino acids except cystine and proline. In particular, levels of arginine and histidine were very high in KMD1 compared to Xiushui 11 (+98% and +123%, respectively). Whereas the level of arginine in KMD1 is within the data range reported in the literature (Scherz and Senser, 2000; USDA, 2004), the level of histidine in KMD1 significantly exceeds the data range reported for brown rice.

Statistically significant differences were observed for the fatty acid distribution (Table 4). A statistically significantly higher proportion of linoleic acid was found for the transgenic rice (+13%), whereas the parental rice exhibited a statistically significant higher proportion of oleic and stearic acid (+9% and +38%, respectively). For stearic acid the amount in KMD1 was below the minimum value reported in literature. For myristic and palmitic acid the proportions in both parental and GM material were slightly below the minima described in literature whereas for oleic acid the amount in the parental line was slightly above the maximum reported. Despite these minor differences, the overall fatty acid patterns of both lines with in agreement with the data reported for rice in the literature (Scherz and Senser, 2000; USDA, 2004; OECD, 2004; Kitta et al., 2005).

Contents of minerals are presented in Table 5. No statistically significant difference between the transgenic and the parental rice was observed for contents of calcium, magne-

Table 2
Proximate composition of brown rice material from transgenic KMD1 and its corresponding parental line Xiushui 11 (mean ± confidence interval, $n = 4$, $p < 0.05$)

Component (%)	Xiushui 11	KMD1	Literature data[a]
Moisture	12.5 ± 0.1	12.5 ± 0.4	9.1–14.1
Starch	72.5 ± 0.4	72.7 ± 0.2	57–77
Fibre	1.1 ± 0.2	1.2 ± 0.3	0.5–3.5
Sugars	0.6 ± 0.2	0.5 ± 0.1	0.6–1.3
Protein	8.7 ± 0.1	9.4 ± 0.1[b]	6.1–9.5
Fat	2.99 ± 0.05	2.46 ± 0.08[b]	1.4–2.9
Ash	1.30 ± 0.05	1.25 ± 0.03	0.9–1.5

[a] Ranges from minimum to maximum reported values (Juliano, 1985; Møller et al., 2002; Scherz and Senser, 2000; USDA, 2004; OECD, 2004).
[b] Statistically significantly different from parental line ($p < 0.05$).

Table 3
Amino acid levels in brown rice material from transgenic KMD1 and its corresponding parental line Xiushui 11 (g/100 g; mean ± confidence interval, $n = 3$, $p < 0.05$)

Amino acid	Xiushui 11	KMD1	Literature data[a]
Alanine	0.48 ± 0.02	0.60 ± 0.02[b]	0.46–0.58
Arginine	0.44 ± 0.05	0.87 ± 0.02[b]	0.44–0.91
Aspartic acid	0.81 ± 0.04	1.00 ± 0.01[b]	0.74–0.87
Cystine	0.20 ± 0.01	0.22 ± 0.07	0.06–0.19
Glutamic acid	1.40 ± 0.03	1.76 ± 0.00[b]	1.52–1.76
Glycine	0.40 ± 0.00	0.47 ± 0.01[b]	0.39–0.49
Histidine	0.26 ± 0.01	0.58 ± 0.07[b]	0.12–0.27
Isoleucine	0.33 ± 0.03	0.39 ± 0.01[b]	0.26–0.57
Leucine	0.70 ± 0.01	0.85 ± 0.00[b]	0.50–0.93
Lysine	0.30 ± 0.01	0.35 ± 0.01[b]	0.10–0.42
Methionine	0.19 ± 0.01	0.27 ± 0.01[b]	0.05–0.31
Phenylalanine	0.44 ± 0.01	0.56 ± 0.02[b]	0.30–0.55
Proline	0.46 ± 0.02	0.50 ± 0.05	0.37–0.40
Serine	0.37 ± 0.01	0.44 ± 0.00[b]	0.41–0.50
Threonine	0.23 ± 0.00	0.32 ± 0.01[b]	0.19–0.62
Tryptophan	0.10 ± 0.00	0.12 ± 0.00[b]	0.03–0.11
Tyrosine	0.42 ± 0.02	0.56 ± 0.01[b]	0.21–0.47
Valine	0.48 ± 0.00	0.63 ± 0.01[b]	0.40–0.76

[a] Ranges from minimum to maximum reported values (Scherz and Senser, 2000; USDA, 2004).
[b] Statistically significantly different from parental line ($p < 0.05$).

Table 4
Fatty acid distribution in brown rice material from transgenic KMD1 and its corresponding parental line Xiushui 11 (mean ± confidence interval, $n = 4$, $p < 0.05$)[a]

Fatty acid (%)	Xiushui 11	KMD1	Literature data[b]
Myristic acid	0.3 ± 0.1	0.3 ± 0.1	0.4–3.0
Palmitic acid	16.9 ± 0.2	16.2 ± 0.6	18–31
Stearic acid	1.8 ± 0.1	1.3 ± 0.1[c]	1.6–2.6
Oleic acid	42.2 ± 0.1	38.4 ± 0.5[c]	27–41
Linoleic acid	35.5 ± 0.1	40.1 ± 0.3[c]	31–40
Linolenic acid	1.5 ± 0.1	1.7 ± 0.1	0.9–1.7

[a] Proportions of total fatty acids (%).
[b] Ranges from minimum to maximum reported values (Scherz and Senser, 2000; USDA, 2004; OECD, 2004; Kitta et al., 2005).
[c] Statistically significantly different from parental line ($p < 0.05$).

Table 5
Contents of minerals in brown rice material from transgenic KMD1 and its corresponding parental line Xiushui 11 (mean ± confidence interval, $n = 4$, $p < 0.05$)

Mineral	Xiushui 11	KMD1	Literature data[a]
Calcium (g/kg)	0.2 ± 0.0	0.5 ± 0.3	0.1–0.5
Copper (mg/kg)	5.1 ± 0.1	2.2 ± 0.3[b]	1–6
Iron (mg/kg)	22 ± 2	16 ± 1[b]	2–52
Magnesium (g/kg)	1.2 ± 0.1	1.2 ± 0.1	0.2–1.7
Manganese (mg/kg)	35.0 ± 0.4	21.6 ± 0.2[b]	2–37
Molybdenum (mg/kg)	0.6 ± 0.1	0.4 ± 0.1[b]	0.3–1.0
Phosphorous (g/kg)	3.0 ± 0.1	3.0 ± 0.1	1.7–4.4
Potassium (g/kg)	2.3 ± 0.1	2.4 ± 0.1	0.6–2.8
Zinc (mg/kg)	22.4 ± 0.3	15.7 ± 0.7[b]	6–28

[a] Ranges from minimum to maximum reported values (Juliano, 1985; Møller et al., 2002; Scherz and Senser, 2000; USDA, 2004).
[b] Statistically significantly different from parental line ($p < 0.05$).

sium, phosphorous and potassium. However, the transgenic rice exhibited statistically significantly lower contents of copper (−57%), iron (−27%), manganese (−38%), molybdenum (−33%), and zinc (−30%). For both lines contents of minerals were in agreement with literature data (Juliano, 1985; Møller et al., 2002; Scherz and Senser, 2000; USDA, 2004).

Table 6 presents contents of important rice vitamins, which were in agreement with literature data (Juliano, 1985; Møller et al., 2002; Scherz and Senser, 2000; USDA, 2004).

Rice contains a mixture of steryl ferulic acid esters named γ-oryzanol (Xu and Godber, 1999). γ-Oryzanol was shown to exhibit antioxidative (Xu et al., 2001) and cholesterol-lowering properties (Rong et al., 1997). No statistically significant difference between transgenic and parental rice was observed for contents of total γ-oryzanol (Table 7). Data were within the range reported in literature (Miller et al., 2003). Despite minor but statistically significant differences, steryl ferulate distributions were similar in both lines. Except for the proportion of 24-methylenecycloartanyl ferulate in the transgenic rice, steryl ferulate distributions were in agreement with literature data (Miller et al., 2003).

Table 6
Contents of vitamins in brown rice material from transgenic KMD1 and its corresponding parental line Xiushui 11 (mg/kg; mean ± confidence interval, $p < 0.05$)

Vitamin	Xiushui 11	KMD1	Literature data[a]
B_1[b]	3.4 ± 0.4	3.8 ± 0.9	2.9–6.1
B_6[c]	1.1 ± 0.2	1.1 ± 0.1	2–10
Niacin[b]	54 ± 2	57 ± 3	35–58
Total pantothenic acid[d]	8.0	9.6	9–17
Total folic acid[a]	0.15	0.13	0.1–0.5
5-Methyl-H_4folate[a,e]	0.09	0.06	
5-Formyl-H_4folate[a,e]	0.06	0.07	

[a] Ranges from minimum to maximum reported values (Juliano, 1985; Møller et al., 2002; Scherz and Senser, 2000; USDA, 2004).
[b] $n = 3$.
[c] $n = 5$.
[d] $n = 2$.
[e] Calculated as folic acid.

Phytic acid is known as an anti-nutritive rice constituent. It has been shown to limit bioavailability of minerals (Saha et al., 1994). No statistically significant difference was detected between phytic acid content of the transgenic (0.90 ± 0.03%, mean ± confidence interval, $p < 0.05$, $n = 4$) and the parental rice (0.88 ± 0.04%). Data were in agreement with literature data (0.6–1.6%; Latta and Eskin, 1980).

To evaluate the microbiological quality of the rice material bacterial and fungal counts were measured and the materials were screened for mycotoxins. Analyses followed standard methods with limits of detection of 0.0003 mg/kg for aflatoxins (B_1, B_2, G_1, G_2), 0.00025 mg/kg for ochratoxin A, 0.0025 mg/kg for zearalenon and 0.025 mg/kg for deoxynivalenol (VDLUFA, 1997). No mycotoxins were detected in the material. Both materials exhibited similarly low bacterial ($<0.07 \times 10^6$/g) and fungal counts ($<0.1 \times 10^3$/g).

As regards contaminants, 149 pesticides from different classes were analyzed in each line following standard methods. They were shown to be below their respective detection limits ranging from 0.0025 mg/kg for PCBs (7) and chlorinated hydrocarbons (13) to 0.5 mg/kg for cymoxanil, pyridaben and thiobendazol; for the majority of the pesticides the detection limits were 0.005 mg/kg (59) and 0.05 mg/kg (42), respectively. Levels of heavy metals (lead, cadmium and mercury) were low. Contents of lead and cadmium were below limits set for rice by the Commission Regulation (EG) 466/2001 (Table 8).

The level of transgene expression of Cry1Ab in mature KMD1 rice seeds was shown to be 0.015–0.018% of the total soluble protein, while no Cry1Ab protein was detected in the parental control rice.

3.2. Clinical observation, body weight and food and water intake

Throughout the study, no adverse effects on animal behaviour were observed. The animals were observed twice

Table 7
γ-Oryzanol (steryl ferulates) in brown rice material from transgenic KMD1 and its corresponding parental line Xiushui 11 (mean ± confidence interval, $n = 3$, $p < 0.05$)

	Xiushui 11	KMD1	Literature data[a]
γ-Oryzanol (mg/100 g)	31 ± 1	32 ± 3	31–63
Steryl ferulate distribution[b]			
Campesteryl ferulate	19.3 ± 0.3	21.1 ± 1.4[c]	7–19
Campestanyl ferulate	6.8 ± 0.2	6.0 ± 0.2[a]	6–13
β-Sitosteryl ferulate	9.5 ± 0.3	10.5 ± 0.2[a]	5–10
Cycloartenyl ferulate	37.7 ± 0.6	44.2 ± 0.6[a]	33–47
24-Methylenecycloartanyl ferulate	26.6 ± 0.6	18.3 ± 0.4[a]	27–36

[a] Ranges from minimum to maximum values reported in Miller et al. (2003).
[b] Proportions of total γ-oryzanol content (%).
[c] Statistically significantly different from parental line ($p < 0.05$).

Table 8
Contents of heavy metals in brown rice material from transgenic KMD1 and its corresponding parental line Xiushui 11 (mg/kg; mean ± confidence interval, $n = 4$, $p < 0.05$)

Heavy metal	Xiushui 11	KMD1	Limit[a]
Lead	0.15 ± 0.09	0.14 ± 0.05	0.2
Cadmium	0.03 ± 0.02	0.02 ± 0.01	0.2
Mercury	0.006 ± 0.001	0.006 ± 0.001	–

[a] Commission Regulation (EG) 466/2001.

daily for well-being. Body weight, food and water consumption was measured weekly and the relative food consumption calculated. Statistically significant differences were seen on the food consumption in single weeks for the males, where the rats fed KMD1 had a slightly lower food intake, but no overall effect was seen when comparing the two groups (Table 9). There were no differences observed on water consumption (data not shown).

Growth curves are included for males and females in Fig. 1. They illustrate normal and similar growth patterns within and between the two groups. The slight reduction in body weight at week 12 in both groups was due to an overnight fasting period prior to blood sampling and possibly also stress, related to the blood sampling procedure.

3.3. Blood biochemistry and haematology

Male rats fed KMD1 had a significantly higher plasma concentration of urea (+10%; $p < 0.05$) and glucose (+13%, $p < 0.05$), whereas the concentration of protein was significantly reduced by 5% ($p < 0.05$) compared to the control group. For female rats fed KMD1, the only statistically significant observation was a 1% increase in plasma concentration of sodium ($p < 0.05$). See Table 10 for details on group values.

Regarding haematology only a few differences were observed between the two groups. In male rats statistically significant differences were observed on MCH, which was 3.5% lower in males fed on KMD1 ($p < 0.05$) and on the WBC, which was reduced by 17% in the same group ($p < 0.05$). See Table 11 for details about hematological measurements.

Table 9
Food consumption (in g/animal/week) presented as group mean values ± SD

	Males		Females	
	Xiushui 11	KMD1	Xiushui 11	KMD1
Weeks 2–7	134 ± 13	134 ± 12	95 ± 5	97 ± 5
Weeks 8–13	141 ± 14	137 ± 8	102 ± 10	100 ± 9

3.4. Microbiology

For the faecal samples no significant differences in the bacterial micro flora could be found between the two groups ($p < 0.05$) (data not shown). Results of the significant microbiological findings in the small intestines are summarized in Table 12. In the samples from the duodenum a 13% decrease in the Bifidobacterial population was observed in the dosed group compared to the control group ($p < 0.05$). In samples from ileum an increase was observed in the coliform population, which was 23% higher in the KMD1 group ($p < 0.05$).

3.5. Organ weights

Only few significant differences in organ weights were observed in this study, namely on adrenal, testis and uterus weight. A statistically significantly reduced absolute weight of the adrenals (−15%) ($p < 0.05$) was detected in male rats fed the KMD1. The absolute weight of the testis from male rats fed the KMD1 was increased (+10%) ($p < 0.05$), as was the relative weight (+12%) ($p < 0.01$). The absolute weight of the uterus in KMD1 fed females was increased (+19%) ($p < 0.05$). There was no statistical difference in the relative weight of the uterus. Details regarding organ weights and minimum and maximum weight for relevant organs are summarized in Table 13.

3.6. Gross necropsy and histopathology

During the necropsy there were no gross pathological findings, nor did the histopathological examination reveal

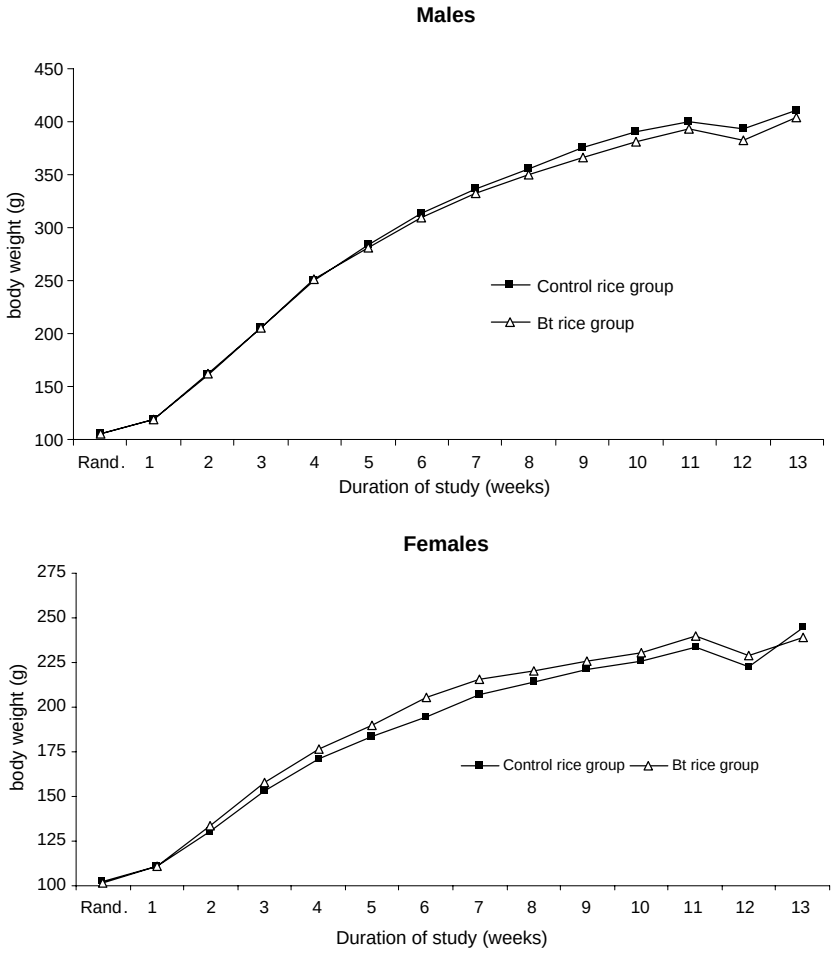

Fig. 1. Growth curves based on weekly measurements of body weight during the study. The curves show group means based on 16 rats/sex/group.

Table 10
Blood biochemical findings in rats fed on Xiushui 11 (control) diet and KMD1 diet

	Males		Females	
	Xiushui 11	KMD1	Xiushui 11	KMD1
BUN (μmol/l)	5.4 ± 0.6	6.0 ± 0.5[a]	6.6 ± 0.7	6.7 ± 1.1
ALAT (U/l)	35.3 ± 4.7	32.4 ± 5.4	24.3 ± 8.1	25.3 ± 3.4
CREA (μmol/l)	33.5 ± 7.4	33.9 ± 6.8	40.1 ± 7.7	37.9 ± 7.5
CHOL (mmol/l)	1.7 ± 0.3	1.5 ± 0.3	1.3 ± 0.3	1.2 ± 0.3
PROT (g/l)	64.4 ± 2.2	61.2 ± 2.4[a]	63.9 ± 3.1	63.9 ± 3.5
ALB (g/l)	40.8 ± 1.5	39.9 ± 1.6	45.5 ± 3.2	45.2 ± 3.0
GLUC (mmol/l)	7.5 ± 0.8	8.6 ± 1.7[a]	10.5 ± 13.5	6.8 ± 0.6
Na^+ (mmol/l)	145.1 ± 1.3	145.9 ± 1.1	143.9 ± 1.3	145.5 ± 1.1[a]
K^+ (mmol/l)	4.3 ± 0.3	4.2 ± 0.3	4.1 ± 0.4	4.2 ± 0.5

The number of animals was 16 rats/sex/group; data is presented as group mean values ± SD.
[a] $p < 0.05$.

any dose-related changes in the intestinal tract or the related organs; in general no pathologically relevant changes were found to explain the identified differences in organ weights between the two groups.

Due to the observed difference in the weight of the testis a thorough histological examination was performed revealing unilateral testicular degeneration in different stages in both groups.

Macroscopically the testis were more or less swelled or atrophied which was directly related to the observed different stages of degeneration in the seminiferous tubules; 2 of 16 control males had mainly atrophic degeneration,

Table 11
Haematological findings in rats fed on Xiushui 11 (control) diet and KMD1 diet

	Males		Females	
	Xiushui 11	KMD1	Xiushui 11	KMD1
WBC (10^9/l)	5.5 ± 0.2	4.7 ± 0.2[a]	3.0 ± 0.2	2.7 ± 0.2
RBC (10^{12}/l)	8.4 ± 0.1	8.7 ± 0.1	7.9 ± 0.1	7.8 ± 0.07
PLT (10^9/l)	640 ± 24	642 ± 19	663 ± 11	709 ± 32
HGB (mmol/l)	15.1 ± 0.1	15.1 ± 0.2	14.4 ± 0.2	14.0 ± 0.09
HCT (%)	45.5 ± 0.5	46.2 ± 0.6	43.5 ± 0.6	42.5 ± 0.3
MCV (fL)	53.9 ± 0.5	53.1 ± 0.4	54.9 ± 0.3	54.8 ± 0.5
MCH (fmol)	17.9 ± 0.2	17.3 ± 0.2[b]	18.2 ± 0.2	18.0 ± 0.2
MCHC (mmol/l)	33.1 ± 0.2	32.7 ± 0.2	33.2 ± 0.2	32.9 ± 0.2
Differential count				
Lymphocytes (%)	79.5 ± 1.5	79.1 ± 1.9	81.8 ± 1.5	77.6 ± 2.0
Neutrophils (%)	17.8 ± 1.6	17.3 ± 1.8	14.7 ± 1.4	18.8 ± 1.9
Eosinophils (%)	1.4 ± 0.3	1.5 ± 0.2	1.7 ± 0.3	1.5 ± 0.3
Basophils (%)	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Monocytes (%)	1.4 ± 0.2	1.4 ± 0.2	1.4 ± 0.2	1.7 ± 0.2
Other cells (%)	0.4 ± 0.2	0.2 ± 0.06	0.4 ± 0.09	0.5 ± 0.1

The number of animals was 16 rats/sex/group; data is presented as group mean values ± SEM.
[a] *F*-value: 5.71, $p = 0.024$.
[b] $\chi^2 = 3.92$, $p = 0.048$ (non-par).

Table 12
Bacterial counts in the small intestine of rats (top: duodenum, bottom: ileum) fed Xiushui 11 (control) diet and KMD1 diet

	Xuishui 11	KMD1
Bacterial counts in duodenum (log_{10} cfu/g intestinal content)		
Total aerobe	5.75 ± 1.16 (9)	5.9 ± 1.05 (9)
Total anaerobe	6.18 ± 0.85 (9)	5.52 ± 1.34
Lactobacilli	6.87 ± 1.44 (8)	6.31 ± 0.66 (9)
Bifidobacteria	6.49 ± 0.87[a] (9)	5.72 ± 0.57[b] (9)
Coliforms	3.39 ± 0.69 (6)	2.85 ± 0.21 (2)
Enterococci	4.35 ± 1.15 (9)	4.45 ± 1.27 (8)
Bacterial counts in ileum (log_{10} cfu/g intestinal content)		
Total aerobe	6.78 ± 1.0 (9)	7.01 ± 0.78 (8)
Total anaerobe	7.53 ± 0.69	7.15 ± 0.59
Lactobacilli	7.55 ± 1.39 (9)	7.58 ± 1.25
Bifidobacteria	7.01 ± 0.96	7.02 ± 0.81
Coliforms	5.73 ± 0.93[a]	7.03 ± 0.59[b]
Enterococci	6.43 ± 0.98	6.04 ± 0.58 (9)

Data are presented as group mean values ± SEM for 10 animals; figures in brackets indicate the number of animals, if different from 10. Different superscripts in a row indicate significant difference in the bacterial counts between the two groups ($p < 0.05$).

whereas 3 of 16 males in the KMD1 group has more pronounced swelling associated with the degeneration.

4. Discussion

Compositional analysis of KMD1 and the parental rice Xiushui 11 tested in the feeding studies revealed statistically significant differences between the two. These significant differences were observed for contents of protein, fat, and minerals (copper, iron, manganese, molybdenum, zinc). The higher protein content in KMD1 was also reflected in higher levels of amino acids. Minor, although statistically significant differences were detected for the distribution of fatty acids and steryl ferulates. Compositional data on GM rice KMD1 and its parental rice Xiushui 11 has been published previously (Wang et al., 2002). The material investigated in that study had also been grown in Hangzhou (China), however at a different site and in another year. The spectrum of the constituents analyzed was more limited than in the present study and for some of the parameters investigated, the results were different from those found in the study presented here. However, Wang et al. (2002) reported no statistically significant differences between the GM and parental rice in their study. This indicates that the differences detected between KMD1 and Xiushui 11 in our study might be due to biological variability rather than to the genetic modification. Additional field trials would be necessary to confirm this conclusion, and field trial permission must be sought well enough in advance to have all the requisite rice lines grown in randomized block design in the same filed station sector in one optimal rice growing season (sow April–October harvest).

The concentration of Bt toxin present in mature rice seeds was estimated to 0.0165% of total soluble protein in the KMD1, which equates to approx. 15 mg Bt toxin/kg rice. With an estimated daily feed consumption of 15 g/rat/day and a mean body weight of 250 g, the daily feed consumption is approx. 60 g/kg body weight. In the 60% rice diet the Bt toxin concentration was 9 mg/kg feed giving a mean daily dosage of Bt toxin of 0.54 mg/kg body weight. For comparison sub-chronic oral toxicity studies have shown a NOEL (no-observed-effect-level) of up to 8400 mg Bt product/kg body weight/day when feeding Bt microbial toxins to rats (Betz et al., 2000). This means that possible toxicological findings in the present study with 0.54 mg/kg body weight/day most likely will derive from unintended changes introduced in the GM rice and not from toxicity of Bt toxin.

Table 13
Absolute and relative organ weights for rats fed on Xiushui 11 (control) diet and KMD1 diet

	Males		Females	
	Xiushui 11	KMD1	Xiushui 11	KMD1
Absolute weight (g)				
Body weight	419 ± 13	408 ± 7	249 ± 4	250 ± 6
Adrenals	0.0694 ± 0.003	0.0602 ± 0.002[a]	0.0611 ± 0.002	0.0673 ± 0.003
Brains	1.97 ± 0.03	1.87 ± 0.11	1.84 ± 0.02	1.89 ± 0.03
Epididymides	1.13 ± 0.03	1.15 ± 0.01		
Heart	1.15 ± 0.03	1.16 ± 0.02	0.813 ± 0.01	0.831 ± 0.03
Kidneys	2.27 ± 0.07	2.25 ± 0.04	1.53 ± 0.05	1.51 ± 0.03
Liver	13.04 ± 0.50	12.59 ± 0.25	8.18 ± 0.14	8.20 ± 0.25
Mesenterial ln.	0.222 ± 0.016	0.189 ± 0.017	0.183 ± 0.015	0.167 ± 0.014
Ovaries			0.0967 ± 0.007	0.1071 ± 0.007
Pancreas	1.43 ± 0.07	1.38 ± 0.05	1.06 ± 0.18	1.01 ± 0.26
Small intestine	7.79 ± 0.21	7.73 ± 0.18	6.16 ± 0.82	6.04 ± 0.94
Spleen	0.744 ± 0.03	0.714 ± 0.02	0.589 ± 0.105	0.532 ± 0.080
Testis	3.57 ± 0.09	3.94 ± 0.10[b]		
Thymus	0.435 ± 0.04	0.413 ± 0.02	0.414 ± 0.067	0.392 ± 0.101
Uterus			0.437 ± 0.05	0.519 ± 0.04[d]
Length small int.	107.5 ± 1.4	109.4 ± 1.1	99.3 ± 1.1	99.3 ± 1.9
Relative weight (g/100 g BW)				
Adrenals	0.0167 ± 0.001	0.0148 ± 0.001	0.0248 ± 0.001	0.0270 ± 0.001
Brains	0.473 ± 0.01	0.461 ± 0.03	0.740 ± 0.01	0.760 ± 0.02
Epididymides	0.271 ± 0.01	0.283 ± 0.005		
Heart	0.275 ± 0.005	0.286 ± 0.015	0.327 ± 0.005	0.333 ± 0.010
Kidneys	0.542 ± 0.009	0.553 ± 0.023	0.612 ± 0.014	0.607 ± 0.012
Liver	3.10 ± 0.05	3.09 ± 0.05	3.29 ± 0.06	3.28 ± 0.08
Mesenterial ln.	0.054 ± 0.005	0.046 ± 0.004	0.073 ± 0.006	0.067 ± 0.006
Ovaries			0.0390 ± 0.003	0.0425 ± 0.002
Pancreas	0.342 ± 0.01	0.340 ± 0.01	0.429 ± 0.02	0.405 ± 0.03
Small intestine	1.87 ± 0.06	1.90 ± 0.04	2.48 ± 0.09	2.41 ± 0.08
Spleen	0.177 ± 0.003	0.176 ± 0.005	0.236 ± 0.009	0.213 ± 0.008
Testis	0.860 ± 0.03	0.967 ± 0.02[c]		
Thymus	0.102 ± 0.009	0.101 ± 0.004	0.167 ± 0.006	0.158 ± 0.011
Uterus			0.177 ± 0.021	0.212 ± 0.021
Length small int.	25.9 ± 0.6	26.9 ± 0.4	40.4 ± 1.0	39.9 ± 1.0

Small intestinal length and relative length is expressed in cm and cm/100 g BW. Data is presented as group mean values ± SEM.
[a] Adrenals: *F*-value: 5.89, $p = 0.0216 \sim$ reduced absolute weight. Xiushui 11: min. 0.055 g, max. 0.103 g; KMD1: min. 0.043 g, max. 0.073 g.
[b] Testis: *F*-value: 7.43, $p = 0.011 \sim$ increased absolute weight. Xiushui 11: min. 2.76 g, max. 4.18 g; KMD1: min. 3.44 g, max. 5.27 g.
[c] Testis: *F*-value: 8.94, $p = 0.006 \sim$ increased relative weight. Xiushui 11: min. 0.585 g/100 g BW, max. 0.999; KMD1: min. 0.878, max. 1.18.
[d] Uterus: *F*-value: 7.09, $p = 0.013 \sim$ increased absolute weight. Xiushui 11: min. 0.24 g, max. 1.14 g; *KMD1*: min. 0.32 g, max. 0.82 g.

On analysis of hematological parameters a significantly reduced amount of white blood cells was observed in the male rats of the group fed with KMD1. This could be indicative of immuno-suppression, but neither the differential count nor other significant findings on clinical observations, organ weights or pathology of the immune organs (thymus and spleen) support this possibility. With respect to blood biochemistry the observed differences in glucose, urea, protein and sodium were minor and the measured values were all within the normal reference intervals for rats of this breed and age. The observed differences are not related to other clinical or pathological findings, and were thus considered insignificant.

Only minor effects were recognized in samples taken from the small intestine for bacteriological quantification in the dosed group. The faecal samples did not reveal any differences in bacterial counts in the animals fed KMD1 compared to animals fed the wild type rice. A recently published study investigated bacterial changes in the rumen of cattle fed Bt corn, where no significant influence of Bt corn could be found on the composition of the microbial population (Einspanier et al., 2004). In the current study reduced amounts of Bifidobacteria in the duodenum and increased amounts of coliforms in the ileum were observed in the KMD1 group. The mechanisms behind these changes are unknown, and further studies are required to clarify whether these findings are biologically significant.

The adrenal weight was significantly reduced in the KMD1 group, but due to the lack of histopathological changes in the organ, this was considered an insignificant finding. As to the observed testicular degeneration, the difference in testis weight was explained by the finding of different stages of degeneration in the seminiferous tubules. The incidence of these findings is not significantly higher in the KMD1 group and thus found not to be related to the GM rice. The conclusion from the present study suggesting

that expression of Cry 1Ab in transgenic rice was not responsible for the observed changes in the testis are supported by Wang et al. (2002) who carried out a comparable feeding study on Sprague–Dawley rats without any observed changes in the testis. In addition, Brake et al. (2004) evaluated the effects of Bt corn on mouse testicular development and with special emphasis on the effect of Bt toxins on the germ cell population, neither short-term nor multigenerational studies showed any apparent toxic effects on the reproductive system.

The inclusion of an additional test group to the present study, where the rats were fed on a diet spiked with pure recombinant protein Cry1Ab would have been desirable and probably the most suitable model for testing the safety of the GM crop, since this would have enabled a more comprehensive assessment of the observed differences in organ weights and the minute pathological changes in the reproductive organs of the male rats. An additional group spiked with Cry1Ab could have increased the specificity of the study to detect specific compound-related effects in order to furthermore ascertain whether the pathological findings and increased organ weights were indeed insignificant or related to either the Bt toxin in the rice or unintended changes in the rice genome. As tested in this present 90-day study the genetically modified Bt rice, KMD1, exhibited no toxicological effects on Wistar rats when fed as 60% of the diet, in comparison with the wild type parental rice, Xiushui 11.

The design may be limited in its ability to detect unintended toxic or nutritional effects of the genetic modification but in large measure this 90-day feeding trial with male and female rats proved that such a model is feasible and successful for safety assessment purposes when the incoming GMO grain diet contains a verifiable level of Cry1Ab in mature KMD1 rice at 0.015–0.018% of the total soluble protein, with no Cry1Ab protein detected in the Xiushui 11 parental control. There were no adverse findings that led to the conclusion that Bt rice is not safe to eat, but nevertheless greater certainty could have been obtained by the inclusion of an additional test group.

In the actual situation where recombinant protein was not available in 100 g quantities sufficient to perform neither a 28-day toxicity study nor to add a spiked group to the 90-day study it is relevant to discuss different possibilities for addition of a group to the study. Additional groups with different levels of KMD1 rice would have been helpful to assess the observed differences between the groups that were found.

It is important to keep in mind that the original aim of this study was not to perform a safety assessment study on KMD1 *per se*, but to test the suitability of the well-known 90-day study for safety assessment of GM crops. Based on the results of this study as presented here, the conclusion to be drawn concerning the model is that in order to thoroughly assess the safety of the GM crop an additional group is desirable, not to say necessary.

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New Analysis of a Rat Feeding Study with a Genetically Modified Maize Reveals Signs of Hepatorenal Toxicity

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Abstract. Health risk assessment of genetically modified organisms (GMOs) cultivated for food or feed is under debate throughout the world, and very little data have been published on mid- or long-term toxicological studies with mammals. One of these studies performed under the responsibility of Monsanto Company with a transgenic corn MON863 has been subjected to questions from regulatory reviewers in Europe, where it was finally approved in 2005. This necessitated a new assessment of kidney pathological findings, and the results remained controversial. An Appeal Court action in Germany (Münster) allowed public access in June 2005 to all the crude data from this 90-day rat-feeding study. We independently re-analyzed these data. Appropriate statistics were added, such as a multivariate analysis of the growth curves, and for biochemical parameters comparisons between GMO-treated rats and the controls fed with an equivalent normal diet, and separately with six reference diets with different compositions. We observed that after the consumption of MON863, rats showed slight but dose-related significant variations in growth for both sexes, resulting in 3.3% decrease in weight for males and 3.7% increase for females. Chemistry measurements reveal signs of hepatorenal toxicity, marked also by differential sensitivities in males and females. Triglycerides increased by 24–40% in females (either at week 14, dose 11% or at week 5, dose 33%, respectively); urine phosphorus and sodium excretions diminished in males by 31–35% (week 14, dose 33%) for the most important results significantly linked to the treatment in comparison to seven diets tested. Longer experiments are essential in order to indicate the real nature and extent of the possible pathology; with the present data it cannot be concluded that GM corn MON863 is a safe product.

Very little data have been published on mid- or long-term feeding studies with genetically modified plants, approved and commercialized, in equilibrated diets, given to mammals, with numerous blood and organs parameters measured (Domingo 2000; Meningaud *et al.* 2001) and only one study with the MON 863 maize in such conditions. It has been performed under the responsibility of Monsanto Company and was recently published after the authorities' assessment (Hammond *et al.* 2006). The crude data at first kept confidential were subjected to questions from regulatory reviewers in Europe, where it was finally approved in 2005. This necessitated, in particular, a new assessment of kidney pathological findings, and because the study was claimed afterwards to provide an assurance of safety (Hammond *et al.* 2006), we independently re-analyzed these data here obtained after a Court action. The whole approval was based on the statement that all the significant differences were not biologically meaningful. To assess this hypothesis, we wanted to link the statistical differences per organ and to apply new methods of analysis. This transgenic maize was modified to produce in its cells a new artificial insecticidal and modified toxin Cry3Bb1 (49-97 μg/g) that was exempted from subchronic toxicity *in vivo* studies (Hammond *et al.* 2006), and its mechanism of action is not known in mammals, because it was not tested, and the target receptor has not been characterized precisely in insects.

Most, if not all, of the commercialized genetically modified organisms (GMOs) in open fields contain pesticide residues that they tolerate and/or produce (Clive 2006). Regulatory rules do not require 3-month tests with three mammalian species, then with a mammal for 1 year and yet another for 2 years, such as those employed for the testing of pesticides or drugs. This is why it appears crucial to analyze carefully the longest toxicity tests available only in one mammalian species, where numerous parameters have been measured for 400 rats, according to Organisation for Economic Co-operation and Development (OECD) standards during only 90 days. Other independent studies over 8 months with mice fed a GM Roundup tolerant soy were very detailed but only at an ultrastructural level, and showed nuclear transcription abnormalities in hepatocytes during the feeding (Malatesta *et al.* 2002), in pancreas (Malatesta *et al.* 2003), and testes (Vecchio *et al.* 2004), and hypothesized that these changes might be due to Roundup herbicide (Monsanto) toxic effects, similar to those observed on mammalian cells (Richard *et al.* 2005), but

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the parameters measured in these longest toxicity tests published on GMOs did not concern almost all organs and blood and urine chemistry, as in the present experiment.

Materials and Methods

Biological Context: The In Vivo Protocol of Monsanto

All OECD standards were claimed to be followed by the Monsanto Company: individual cages, animal randomly distributed in each group after a 1-week stabilization period, standard and validated measurement methods, and so on. This feeding study served to authorize the MON863 maize by the European and American authorities. It included young adult Sprague-Dawley-derived rats (Crl:CD®(SD)IGS BR, Charles River laboratories, NY), approximately 6 weeks old separated in 10 groups of 20 males and 10 of 20 females analyzed in details (organ weights and histology), but the biochemical parameters were measured only for half of these at weeks 5 and 14. For each sex, two groups were fed with GMOs, one with 11% and the second with 33% of MON 863 in the equilibrated diet, and two with the closest control line and regimen, grown in the same location (Hawaii), called control herein, indicated to be substantially equivalent (Hammond *et al.* 2006), in similar proportions. The closest control plant possible will then be the equivalent isogenic or parental nontransformed line, grown in similar conditions. In this article, the control is called the LH82 × A634 line. The six other groups were given diets without GMOs but that did not have the same final chemical composition, even if these diets also met PMI specifications for Certified 5002 Rodent Diet. They contained 33% of conventional different maize lines (MON 847 Repl, Asgrow RX-770, LH235 × LH185, LH200 × LH172, B73Ht × LH82, Burrus BX-86). These were not grown in the same locations (Illinois or other places in Hawaii), and were not demonstrated to be substantially equivalent to the GMO and control diet, but were supposed to mimic the variability of regular reference regimens, called reference herein, and other details have been described (Hammond *et al.* 2006).

The genetic modification in the maize tested here was inserted by chance by particle bombardment in the plant genome of immature cells. This may cause insertional mutagenesis effects, which may not be directly visible by compositional analysis; the latter can then be only partially compared for a nonexhaustive list of substances to conventionally bred lines, for instance, to test "substantial equivalence." The genetic construction itself comprises a transgene with an ubiquitous adapted 35S promoter encoding a modified toxin directed against the coleopteran insect Diabrotica. This dangerous parasite was probably introduced several times by airplane in Europe from the late 1990s (Miller *et al.* 2005). The problem apparently has been anticipated by the first trials of MON 863 or similar GMOs in Europe. This maize also contains a neomycin phosphotransferase II marker gene, coding for antibiotic resistance, to facilitate the selection of the transformed plants.

Statistical Methods

The present feeding experiment was designed and statistically assessed by Monsanto Company (St. Louis, MO), but animals were analyzed by Covance Laboratory (Vienna, VA). We first repeated the same statistical analysis as that of Monsanto to verify descriptive statistics (sample size, means, standard deviations) and one-way analysis of variance (ANOVA) by sex and by variable. For that, the normality of the residues was tested using the Shapiro test and the homoscedasticity (homogeneity of the variances) using the Bartlett test. In the case where the Shapiro and Bartlett tests were nonsignificant ($*p > 0.05$ and $**p > 0.01$, respectively) we performed an ANOVA, and in the case of heteroscedasticity the approximate Welch method was used. In the case where the Shapiro test was significant, we performed the Kruskal-Wallis rank sum test.

In addition, we undertook a multivariate analysis of the growth curves and the consumption of the rats. For the weight growth curve of the rats, after linear regression, the weekly relative increase rate can be considered proportional to the logarithm of the weight, and thus we used a Gompertz model (Ratkowsky 1990; Huet *et al.* 2004), $Y = a \cdot \exp(-\exp(-b(X-c)))$. The parameter a represents the top of the curve, b is related to the growth rate, and c is a position parameter with the X axis. These parameters were estimated by nonlinear regression. In order to see whether the growth curves are significantly different, we compared the models by testing the null hypothesis (which would give the same curves with identical parameters for both groups) against the alternative (different curves). For that, we used the F test to compare the sum of square errors under the two hypotheses. The Akaike's Information Criteria (AIC, Akaike 1974) was also used to evaluate the probability of differences.

We then analyzed the GMO effects for each sex and each diet by pairwise comparisons of the parameters of GMO-fed rats to the control groups and after to the reference groups. In order to select the appropriate two-tailed comparison test (Crawley 2005), we again studied first normality (Shapiro test) and variance equality (F test). According to the results, we performed the adapted test, i.e., an unpaired t test, a Welch corrected t test or a Mann-Whitney test (which is generally more appropriate with a sample size of 10).

We used the R language (Crawley 1995) version 2.2.1 for statistical computations (Comprehensive R Archive Network, CRAN - http://cran.r-project.org), except for the weight growth curves statistical study, for which nonlinear regressions were performed using GraphPad Prism (version 4.02 for Windows, GraphPad Software, San Diego, CA, www.graphpad.com).

Results

We first checked all the crude data, and we noticed a concordance for descriptive statistics (sample size, means, standard deviations) and one-way ANOVA by sex and by variable between our calculated values and those published by Hammond *et al.* (2006) from Monsanto Company.

Body Weights

Our study consisted of a multivariate analysis of the growth curve and the consumption of the rats for the four groups receiving GMOs or equivalent diets. If the animal consumption was not noticeably changed, it appeared for the growth curves that the variations for the two controls for each sex are superimposed, whereas the GMO feeding trials provoked different growths (Fig. 1). The 11% GMO groups were always under the 33% groups for both sexes. All the males are growing less than the controls from week 2, and all the females more. This sex- and dose-related effect resulted in the fact that the growth variations of the 11% GMO males are highly statistically lower than their controls, and 33%-GM fed females higher (Table 1). All p values of different groups versus controls are <0.01. This results in 3.3% decrease in weight for males and 3.7% increase for females.

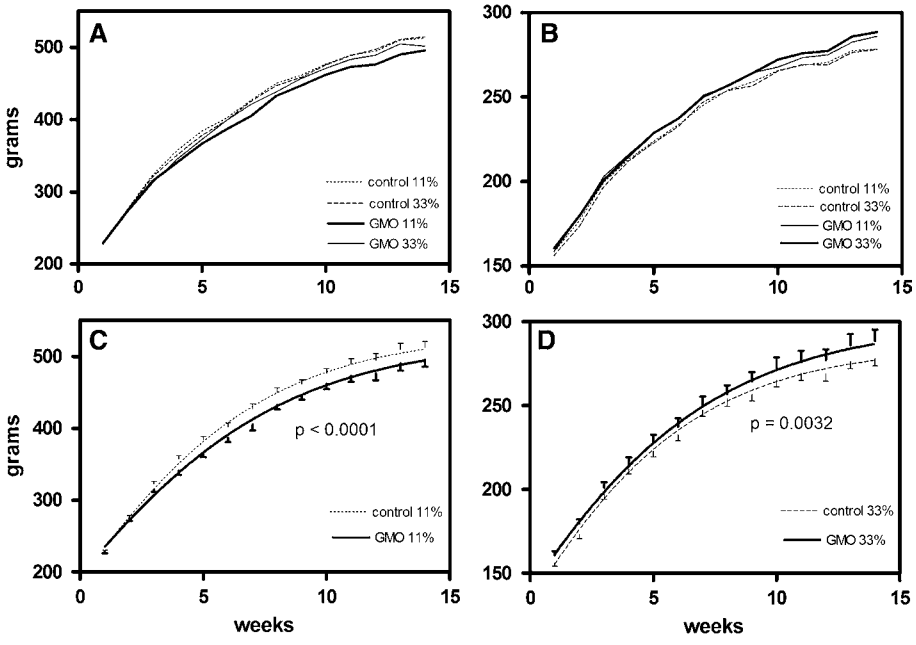

Fig. 1. Body weight growth for males **(A, C)** and females **(B, D)** over a period of 14 weeks. The experimental (A, B) and corresponding theoretical curves according to Gompertz models **(C, D)** are presented. The most important effects in each sex are in bold lines and statistically different from controls (see Materials and Methods)

Table 1. Statistical differences between weight curves

a

	Gompertz models for males		
Par.	Control 11%	GMO 11%	One model
a	533.6	524.6	528.8
b	0.2240	0.2011	0.2126
c	0.1251	−0.0939	0.0185
	Gompertz models for females		
	Control 33%	GMO 33%	One model
a	286.1	300.1	292.9
b	0.2272	0.2016	0.2142
c	−1.185	−1.376	−1.282

b

Sex	*F* test	AIC
Males	$p < 0.0001$	Prob. > 99.99%
	$F = 11.73$	Diff. 28.34
Females	$P = 0.0032$	Prob. = 98.04%
	$F = 4.66$	Diff. 7.83

The parameter estimates for Gompertz models have been calculated (a) for parameters (Par.) a, b and c and tested for statistical differences (b, F test column 2) with p values and the F ratio. The Akaike's Information Criteria (AIC) and the probabilities (Prob.) for differences (Diff.) in curves are precised (b, column 3).

Other Parameters

We then studied first the GMO effects in comparison to the isogenic, nontransgenic, equivalent maize (control) in Table 2, then the effects of different nonequivalent maize compositions on rat physiology (six different reference groups versus controls. Finally, we studied the GMO effects versus all different diets (double frame, Table 2). In total, 58 biochemical parameters reflecting most physiological functions were measured two times (week 5 and 14), in particular through serum and urine chemistry, and hematology. Organ weights and relative ratios were added. We thus performed 494 comparisons: 40 differences (8%) were statistically significant ($*p < 0.05$); 25 would have been expected under the global null hypothesis of no differences between GMO and control diet effects. Among the 40 significant differences, we retained only the 33 with a relatively ≥±5% difference to the mean; this most probably also excluded potential incidental differences, if any. Table 2 summarizes only the list of significantly disturbed parameters at least for one sex or one treatment, and also shows the percentage of variations of the means. The same Table 2 is obtained if we systematically use the

Table 2. Differences between GMO−fed rats and controls

	Week	m 11%	m 3 %	f 11%	f 33%
Liver parameters					
Albumin/globulin ratio	5	11*	-3	-9	4
Albumin/globulin ratio	14	6	-2	-18**	7
Albumin	5	-3	-2	-2	5*
Albumin	14	-2	3	-6*	5
Globulin	5	-12*	2	9*	1
Globulin	14	-8	7	15*	-2
Alanine aminotransferase	14	-30*	-8	37	4
Total protein	14	-5*	5*	1	3
Triglycerides	5	22	-2	-11	40**
Triglycerides	14	15	-1	24*	6
Liver weight	14	-1	-2	7**	6
Liver/brain ratio	14	-1	-3	6*	4
Kidney parameters					
Creatinin	14	-7	13*	13*	-2
Urine sodium	14	-23	-25*	11	-26
Urine sodium excretion	14	3	-35*	35	-24
Urine chloride excretion	5	35	3	50*	67*
Urine potassium	5	35*	-20	-3	-13
Urine phosphorus	5	3	-35*	24	-15
Urine phosphorus	14	-34	-31*	12	-8
Urea nitrogen	14	-8	4	17*	-1
Kidney weight	14	-3	-7*	3	2
Kidney/brain ratio	14	-3	-7*	1	1
Kidney % body weight	14	-1	-5*	-1	-1
Pancreas					
Glucose	14	-4	9	9*	10**
Bone marrow					
Neutrophils	5	5	22*	-14	3
Eosniophils	14	32	54*	20	0
Reticulocytes	14	15	-17	-35	-52*
Reticulocytes % RBC	14	16	-16	-36	-55*

Study of the GMO effects indicated by mean differences (%) for each parameter with the corresponding control group per sex and per dose. The significant differences *versus* controls (*$p < 0.05$, **$p < 0.01$), for all the parameters measured in the subchronic feeding tests, are presented. The parameters were grouped by organs according to the sites of synthesis or classical indicators of dysfunction. They were indicated for all groups only if they showed at least for one sex or one diet a significant and relatively ≥ ± 5% difference to the mean. The animals were male (m) or female (f) young adult rats fed during 5 or 14 weeks with GMO (MON 863, 11 or 33% in the diet) and compared with controls fed with a ''substantially equivalent'' isogenic maize line (LH82 × A634) grown in the same location (Hawaï). The parameters were measured for 10 rats, except for the organ weights (20 rats), obtained only at the end of the experiment. In single−boxed numbers, we indicate the statistical differences between GMO−fed rats and controls, which are not found between the mean of the six reference groups and controls. A difference between reference and control groups could indicate an effect of the diet per se. In double−boxed numbers, among the effects due to the GMO, are indicated the statistical differences between the GMO groups and the mean of the six reference groups (which have not even eaten the same composition as the control and the GMO treated groups).

Mann-Whitney test for all the biological parameters, except for albumin–14–f11%, urine phosphorus–5-m33%, and urea nitrogen–14-f11%; the *p* values in this case are comprised between 6.3% and 10.6%; these were not considered below. Table 3 corresponds to physiological values of the significantly disturbed parameters in GMO-fed rats in comparison to their corresponding controls. It emphasizes the impressive quantity of abnormalities.

Table 2 indicates that GMO-linked variations in comparison to controls were concentrated mostly on five male and nine female liver parameters, and nine and four kidney parameters for males and females, respectively, on all organs studied. We then measured the significant variations between the six reference groups and controls (isogenic to GMO), which allowed us to study the potential effects of the diet composition alone. The parameters that were also disturbed in this case were deducted from the first ones, and still three and five liver parameters and seven and one kidney parameters at least appeared to be specifically linked to the GMO diet. We consecutively compared the parameters of GMO-fed rats to the six reference groups given other diets, focusing on the GMO effects as being more important than any other diet effects, and

Table 3. Effects of GMO treatments classified by organs

Parameters	Week	Sex	Dose	Control mean ± sem	GMO mean ± sem	Units
Liver parameters						
Albumin / Globulin Ratio	5	m	11%	1.782 ± 0.053	1.974 ± 0.043	Ratio
Albumin / Globulin Ratio	14	f	11%	2.334 ± 0.085	1.914 ± 0.083	Ratio
Albumin	5	f	33%	4.600 ± 0.054	4.850 ± 0.056	g/dl
Albumin	14	f	11%	5.130 ± 0.104	4.830 ± 0.091	g/dl
Globulin	5	m	11%	2.450 ± 0.090	2.150 ± 0.072	g/dl
Globulin	5	f	11%	2.110 ± 0.041	2.300 ± 0.080	g/dl
Globulin	14	f	11%	2.220 ± 0.080	2.560 ± 0.097	g/dl
Alanine aminotransferase	14	m	11%	67.100 ± 11.078	47.300 ± 1.422	u/l
Total protein	14	m	11%	7.140 ± 0.092	6.810 ± 0.099	g/dl
Total protein	14	m	33%	6.860 ± 0.090	7.1778 ± 0.112	g/dl
Triglycerides	5	f	33%	39.300 ± 1.578	54.900 ± 3.743	mg/dl
Triglycerides	14	f	11%	40.900 ± 3.889	50.900 ± 2.479	mg/dl
Liver weight	14	f	11%	7.250 ± 0.116	7.789 ± 0.163	g
Liver / brain ratio	14	f	11%	3.664 ± 0.059	3.890 ± 0.085	Ratio
Kidney parameters						
Creatinin	14	m	33%	0.520 ± 0.013	0.589 ± 0.031	mg/dl
Creatinin	14	f	11%	0.560 ± 0.016	0.630 ± 0.021	mg/dl
Urine sodium	14	m	33%	26.980 ± 3.487	20.122 ± 5.699	meq/l
Urine sodium excretion	14	m	33%	0.290 ± 0.028	0.189 ± 0.020	meq/time
Urine chloride excretion	5	f	11%	0.220 ± 0.025	0.330 ± 0.042	meq/time
Urine chloride excretion	5	f	33%	0.150 ± 0.022	0.250 ± 0.037	meq/time
Urine potassium	5	m	11%	112.210 ± 13.860	151.000 ± 10.039	meq/l
Urine phosphorus	5	m	33%	166.970 ± 24.719	108.310 ± 7.922	mg/dl
Urine phosphorus	14	m	33%	119.120 ± 13.479	81.822 ± 10.468	mg/dl
Urea nitrogen	14	f	11%	13.200 ± 0.742	15.500 ± 0.792	mg/dl
Kidney weight	14	m	33%	3.446 ± 0.070	3.201 ± 0.078	g
Kidney / brain ratio	14	m	33%	1.600 ± 0.030	1.483 ± 0.034	Ratio
Kidney % body weight	14	m	33%	0.705 ± 0.015	0.667 ± 0.009	%
Pancreas						
Glucose	14	f	11%	103.300 ± 2.495	112.600 ± 3.497	mg/dl
Glucose	14	f	33%	105.300 ± 2.432	115.800 ± 2.476	mg/dl
Bone marrow						
Neutrophils	5	m	33%	0.860 ± 0.058	1.050 ± 0.054	$\times 10^3/\mu l$
Eosinophils	14	m	33%	0.130 ± 0.015	0.200 ± 0.024	$\times 10^3/\mu l$
Reticulocytes	14	f	33%	0.085 ± 0.015	0.041 ± 0.008	$\times 10^6/\mu l$
Reticulocytes % RBC	14	f	33%	1.040 ± 0.201	0.470 ± 0.092	%

Based on Table 2, all the parameters significantly different between GMO-fed rats and corresponding controls are represented by their crude means ± SEM in exactly corresponding units. The differences were always $p < 0.05$ or <0.01 to controls according to one or two asterisks in Table 2. The controls are submitted to a substantially equivalent isogenic maize with the same diet, with all other conditions (genetic, temperature, light, space of caging, and so on) are identical. The time of exposure (weeks 5 and 14 corresponding, respectively, to 4 and 13 weeks of GMO diet), the sexes (males: m, females: f), and the dose (11 or 33% of GM Bt maize MON 863 in the equilibrated diet) are indicated.

always for males and females, respectively, four and zero kidney parameters and one and two liver parameters remained significantly different in all cases.

The significant liver changes in the 11% GMO-fed male rats that had the lowest growth rate was a total serum protein decrease (5%), possibly linked to a globulin decrease (12%). In females, the triglycerides were specifically enhanced in the animals that had liver and body weight increases above normal. In fact, triglycerides increased by 24–40% in females (either at week 14, dose 11% or at week 5, dose 33%, respectively).

At the kidney level, phenomena corresponding to urine phosphorus and sodium excretions diminished in males by 31–35% (week 14, dose 33%) for the most important results significantly linked to the treatment in comparison to seven diets tested, whereas other diets enhanced sodium excretion in some instances (data not shown).

Moreover, for males, none of these significantly changed parameters were similar to the variations due to the composition of the diet. The effect of the GMO diet was concomitant with a kidney weight decrease.

Other sporadic effects on serum glucose, urine chloride excretion, or reticulocytes, depending on the sex or the dose, are apparent.

Discussion

The statistical analysis used in the conclusion of Hammond *et al.* (2006) was only carried out for this experiment by the Monsanto statistics center. The goal of this experiment is to study the possible toxicological effects of introducing the genetic construction producing an insecticide into the maize; thus, it should be guaranteed that the only variability sources in

the results are related to the presence, or not, of this transgene apart from purely random effects. In a sense, the presence of the 6 reference groups fed with other commercial varieties of corn, which are not substantially equivalent (with more or less salts or sugars), introduces the simultaneous study of other parameters. Moreover, the reference groups representing 60 rats per sex, measured for their biological parameters, have been compared to 10 rats fed with 33% GMO, by Monsanto. We think that this difference in size favors the uncertainties. We thus preferred to separate the analysis first between the GMO groups and the control ones, and then between GMO groups and the reference groups, in contrast to Monsanto analysis.

Moreover, a study with 20 animals per group already has a limited power of discrimination. Consequently, we could consider possible toxic effects if several parameters are disturbed for the same organ in a non-negligible manner. Unfortunately, besides controls and references, only 40 rats per sex in a total of 400 animals have been given GMOs in this study, and only half of those have been analyzed for biochemical parameters, *i.e.*, 10 per dose and per sex after 5 and 14 weeks, as indicated.

The body weight growth variations, usually hardly modified by a normal diet with very little quantities of toxin, represent an important factor to follow. This study was absent from the statistical report of Monsanto. The significant variations were not tested by Hammond *et al.* (2006), although the 11% GMO males form the lowest curve after week 2. However, we clearly proved very significant differences in weight growths for both males and females, with a lower effect with the 11% diet in comparison to 33% and controls. This increase was over controls in females with the 33% diet, and under controls for the 11% diet given to males. This may be not only an indication of the dysfunction of several organs as shown in Table 3, but also a sex-dependent effect related to endocrine disruption and/or hormonal metabolism differences. Surprisingly, sexual hormones were not measured in these regulatory tests. This could have explained some of these observations. In fact, the results of Table 2 concur with signs of possible hepatorenal toxicity with a greater kidney sensitivity in males and liver sensitivity in females. A differential sensitivity for toxicants among sexes is usual, the hepatic detoxification being hormone-dependent, for instance.

The differences were significant even if the reference diets had specific effects between them, such as 8–23% differences in liver alkaline phosphatase, alanine or aspartate aminotransferase activities, or small different sodium chloride exchanges and urine volume, probably due to different lipid or salt contents in the diets (data not shown).

The GMO-linked differences are illustrated at an hepatic level by a protein or triglyceride metabolism disruption. It is known that some hepatotoxics, such as the drug metabolite hydrazine, may cause liver necrosis and steatosis with hypertriglyceridemia in the blood (Sarich *et al.* 1996). These changes may have differential thresholds according to the sex or hormonal status, as with classical reactions to hepatocarcinogens (Castelli *et al.* 1986; Pitot *et al.* 1989). Moreover, nothing in the protocol allowed the conclusion that the 11% or 33% GMO proportions chosen in the diets were in the linear portion of a dose–response curve, after intoxication by the Bt protein, for instance. Some Bt toxins may cause human hepatotoxicity by a nonapoptotic mechanism (Ito *et al.* 2004), or hepatic lipid peroxidation in rats (Shaban *et al.* 2003). However, it should be emphasized that a pleiotropic metabolic effect due to insertional mutagenesis and independent of the new insecticide produced in the GMO cannot be excluded.

To interpret the kidney data, although we did not have access to the kidney slices after the Appeal Court, Hammond *et al.* (2006) from Monsanto published that there were small increases of focal inflammation, and tubular regenerative changes in this group, in comparison to controls. They commented on a small decrease of serum chloride. After questions from the regulators in Europe, two board-certified pathology experts, proposed by Monsanto and who re-examined the slides, concluded that a classic chronic progressive nephropathy, for which male rats are sensitive (Hard and Khan 2004), had an incidence of 18/20 in the MON863 male group, higher than in controls (14/20), even if this was not considered as relevant by Hammond *et al.* (2006). If all the data are taken together, and overall in regard to the specifically disturbed urine chemistry parameters at weeks 5 and 14 (Table 2), which were not indicated by Hammond *et al.* (2006), it could be concluded that a GM-linked male renal toxicity is observed in this work.

To explain the sporadic results observed in the blood, we have little data. However, it is known in some instances that Bt toxins may also perforate blood cells (Rani and Balaraman 1996).

In conclusion, the two main organs of detoxification, liver and kidney, have been disturbed in this study. It appears that the statistical methods used by Monsanto were not detailed enough to see disruptions in biochemical parameters, in order to evidence possible signs of pathology within only 14 weeks. Moreover, the experimental design could have been performed more efficiently to study subchronic toxicity, in particular with more rats given GMOs in comparison to other groups. Considering that the human and animal populations could be exposed at comparable levels to this kind of food or feed that has been authorized in several countries, and that these are the best mammalian toxicity tests available, we strongly recommend a new assessment and longer exposure of mammals to these diets, with cautious clinical observations, before concluding that MON863 is safe to eat.

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Genetically modified crops safety assessments: present limits and possible improvements

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Abstract

Purpose: We reviewed 19 studies of mammals fed with commercialized genetically modified soybean and maize which represent, per trait and plant, more than 80% of all environmental genetically modified organisms (GMOs) cultivated on a large scale, after they were modified to tolerate or produce a pesticide. We have also obtained the raw data of 90-day-long rat tests following court actions or official requests. The data obtained include biochemical blood and urine parameters of mammals eating GMOs with numerous organ weights and histopathology findings.

Methods: We have thoroughly reviewed these tests from a statistical and a biological point of view. Some of these tests used controversial protocols which are discussed and statistically significant results that were considered as not being biologically meaningful by regulatory authorities, thus raising the question of their interpretations.

Results: Several convergent data appear to indicate liver and kidney problems as end points of GMO diet effects in the above-mentioned experiments. This was confirmed by our meta-analysis of all the *in vivo* studies published, which revealed that the kidneys were particularly affected, concentrating 43.5% of all disrupted parameters in males, whereas the liver was more specifically disrupted in females (30.8% of all disrupted parameters).

Conclusions: The 90-day-long tests are insufficient to evaluate chronic toxicity, and the signs highlighted in the kidneys and livers could be the onset of chronic diseases. However, no minimal length for the tests is yet obligatory for any of the GMOs cultivated on a large scale, and this is socially unacceptable in terms of consumer health protection. We are suggesting that the studies should be improved and prolonged, as well as being made compulsory, and that the sexual hormones should be assessed too, and moreover, reproductive and multigenerational studies ought to be conducted too.

Background, aim, and scope

Recently, an ongoing debate on international regulation has been taking place on the capacity to predict and avoid adverse effects on health and the environment for new products and novel food/feed (GMOs, chemicals, pesticides, nanoparticles, etc.). The health risk assessments are often, but not always, based on the study of blood analyses of mammals eating these products in subchronic tests, and more rarely in chronic tests. In particular, in the case of GMOs, the number and nature of parameters assessed, the length of the necessary tests, the statistics used and their interpretations are the subject of controversies, especially in the application of Organization of Economic Cooperation and Development (OECD) norms. Confusion is perceived even in regulatory agencies, as in the European Food Safety Authority (EFSA) GMO panel working group and its guidelines. Doubt has arisen on the role and necessity of animal feeding trials in safety and nutritional assessments of GM plants and derived food and feed [1]. Based on the literature data, EFSA first admitted (p. S33) that for other tests than GMOs: "For 70% (57 of 81) of the studies evaluated, all toxicological findings in the 2-year tests were seen in or predicted by the 3-month subchronic tests". Moreover, they also indicated (p. S60) that "to detect effects on reproduction or development [...] testing of the whole food and feed beyond a 90-day rodent feeding study may be needed." We fully agree with these assumptions. This is why we think that in order to protect large populations from unintended effects of novel food or feed, imported or cultivated crops on a large scale, chronic 2-year and reproductive and developmental tests are crucial.

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However, they have never been requested by EFSA for commercial edible crops. We therefore wish to underline that in contrast with the statements of EFSA, all commercialized GMOs have indeed been released without such tests being carried out, and as it was the case recently with maize stacked events without 90-day *in vivo* mammalian tests being conducted. GM stacked events have the cumulated characteristics of first generation of GMOs (herbicide tolerance and insecticide production), which are mostly obtained by hybridization. For instance, Smarstax maize contains two genes for herbicide tolerance and six genes for insecticide production. In fact, this contradictory possibility was already highlighted in the same review by EFSA (p. S60), when substantial equivalence studies and other analyses were performed: "animal feeding trials with rodents [...] adds little if anything [...], and is not recommended." This is why, in this work we will analyze and review deficiencies in GMO safety assessments, not only performed by biotech companies, but also by regulatory agencies.

We will focus on the results of available 90-day feeding trials (or more) with commercialized GMOs, in the light of modern scientific knowledge. We also suggest here an alternative to conventional feeding trials, to understand the biological significance of statistical differences. This approach will make it possible to avoid both false negative and false positive results in order to improve safety assessments of agricultural GMOs before their commercialization for cultivation and food/feed use and imports.

Overview of the safety studies of GMOs performed on mammals

Our experience in scientific committees for the assessment of environmental and health risks of GMOs and in biological, biostatistical research, and medicine, as well as in the research relative to side effects [2-6] allowed us to review and criticize mammalian feeding trials with GMOs and make new proposals. Mammalian feeding trials have been usually but not always performed for regulatory purposes in order to obtain authorizations or commercialization for GM plant-derived foods or feed. They may have been published in the scientific literature afterwards; however, without public access to the raw data.

We have obtained, following court actions or official requests, the raw data of several 28- or 90-day-long safety tests carried out on rats. The thing we did was to thoroughly review the longest tests from both a biostatistical and a biological point of view. Such studies often analyze the biochemical blood and urine parameters of mammals eating GMOs, together with numerous organ weights and histopathology. We have focused our review on commercialized GMOs which have been cultivated in significant amounts throughout the world since 1994 (Table 1). We observe and emphasize that all the events in Table 1 correspond to soybean and maize which constitute 83% of the commercialized GMOs, whilst other GMOs not displayed in the table, but still commercialized, are canola or cotton. However, they are not usually directly consumed [7]. Only Sakamoto's and Malatesta's studies have been more than 90 days long (104 weeks and 240 days with blood analyses in Japanese for the first one). Moreover, such tests are not obligatory yet for all GMOs. No detailed blood analysis is available for Malatesta's study, as it mostly includes histochemistry at the ultrastructural level; moreover, the latter tests have not been used to obtain the commercial release by the firm. However, this work has been performed by researchers independent from the GMO industry; it is an important element to take into account for an objective interpretation of the facts, as pointed out in the case of the risk assessments conducted by regulatory agencies with Bisphenol A. For instance in the latter case, it was observed that none of the industry-funded studies showed adverse effects of Bisphenol A, whereas 90% of government-funded studies showed hazards at various levels and various doses [8]. However, regulatory agencies still continue to refer only to industry-funded studies because they are supposed to follow OECD norms, even if such standards are not always appropriate for the detection of environmental hazards [9]. In this paper, Myers et al. showed that hundreds of laboratory animals and cell culture studies were rejected by regulatory authorities because they did not follow the Good Laboratory Practices (GLP). The Food and Drug Administration and EFSA have based their final decision on two industry-funded studies, claiming that they were superior to the others because they followed GLP. Yet, GLP are based on ancient paradigms. They have serious conceptual and methodological flaws, and do not take into account the latest knowledge in environmental sciences. For example, in the case of Bisphenol A assessment, the animal models used are known to be insensitive to estrogen (CD-1 mouse). Also, assays and protocols in some OECD guidelines are out of date and insensitive. It is obvious that new product assessments should be based on adapted studies using state-of-the-art experiments. The significant gap between scientific knowledge and regulations should be filled also in the case of GMOs [9]. Therefore, some tests presented here show controversial results or statistically significant results that were not considered as biologically significant by EFSA, raising the question of their interpretation.

First of all, the data indicating no biological significance of statistical effects in comparison to controls have been published mostly by companies from 2004 onwards, and at least 10 years after these GMOs were

Table 1 Review of the longest chronic or subchronic toxicity studies in mammals fed with commercialized GM soybean and maize representing more than 80% of edible GMOs (2010)

References	Plant	Pesticide contained	Name of event	Species	Duration	Main observations
[17,38,39,19,15]	Soybean	Roundup herbicide	mCP4 EPSPS	Mouse	240 days	Ultrastructural histochemistry disturbed
[14]	Soybean	Roundup herbicide	mCP4 EPSPS	Rat	91 days	Weight problems
[40]	Soybean	Roundup herbicide	Optimum GAT DP-356Ø43-5	Rat	93 days	Statistical differences[a]
[41]	Soybean	Roundup herbicide	Not precise	Rat	104 weeks	Statistical differences[a]
[42]	Maize	Roundup herbicide	Optimum GAT DP-Ø9814Ø-6	Rat	91 days	Statistical differences[a]
[43,5]	Maize	Roundup herbicide	NK603	Rat	90 days	Controversial results
[44,5]	Maize	mCry1Ab insecticide	MON810	Rat	90 days	Controversial results
[25,2,4,5]	Maize	mCry3Bb1 insecticide	MON863	Rat	90 days	Controversial results
[16]	Maize	mBt insecticide	not indicated	Rat	Multi-generational (F3)	Histopathological, biochemical, organ weights alterations
[45]	Maize	mCry1F insecticide - glufosinate ammonium-based herbicide	DAS-Ø15Ø7-1	Rat	91 days	Statistical differences[a]
[46,47]	Maize	mCry34Ab1, mCry35Ab1 insecticides - glufosinate ammonium-based herbicide	DAS-59122-7	Rat	90 days	Statistical differences[a]
[48]	Maize	mCry1F, mCry34Ab1, mCry35Ab1 insecticides - glufosinate ammonium-based herbicide	DAS-Ø15Ø7-1 × DAS-59122-7	Rat	92 days	Statistical differences[a]

[a]Statistical differences are not biologically meaningful for the authors; however, this can be debated. Oilseed rape and cotton have been excluded because they are not directly edible and not primarily grown for feed. This table includes authorized events for food and feed at least in the European Union and America.

first commercialized round the world. This is a matter of grave concern. Moreover, only three events were tested for more than 90-days in feeding experiments or on more than one generation. This method was not performed by industries which conducted 90-day tests (with blood and organ analyses), but it was in some cases only. However, a 90-day period is considered as insufficient to evaluate chronic toxicity [1,5]. All these commercialized cultivated GMOs have been modified to contain pesticides, either through herbicide tolerance or by producing insecticides, or both, and could therefore be considered as "pesticide plants." Almost all GMOs only encode these two traits despite claims of numerous other traits. For instance, Roundup ready crops have been modified in order to become insensitive to glyphosate. This chemical together with adjuvants in formulations constitutes a potent herbicide. It has been used for many years as a weed killer by blocking aromatic amino acid synthesis by inhibition of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS). Most Roundup ready plants have been modified thanks to the insertion of a mutated EPSPS gene coding for a mutated enzyme, which is not inhibited by glyphosate. Therefore, GM plants exposed to glyphosate-based herbicides such as Roundup do not specifically degrade glyphosate. They can even accumulate Roundup residues throughout their life, even if they excrete most of such residues. Glyphosate and its main metabolite AMPA (with its own toxicity) are found in GMOs on a regular and regulatory basis [10,11]. Therefore, such residues are absorbed by people eating most GM plants (as around 80% of these plants are Roundup tolerant). On the other hand, about 20% of the other GMOs do synthesize new insecticide proteins through the insertion of mutated genes derived from *Bacillus thuringiensis* (Bt).

Usually, pesticides are tested over a period of 2 years on a mammal, and this quite often highlights side effects. Additionally, unintended effects of the genetic modification itself cannot be excluded, as direct or indirect consequences of insertional mutagenesis, creating possible unintended metabolic effects. For instance, in the MON810 maize, the insertion of the transgene in the ubiquitine ligase gene caused a complex recombination event, leading to the synthesis of new RNA products encoding unknown proteins [12]. Thus, genetic modifications can induce global changes in the genomic, transcriptomic, proteomic, or metabolomic profiles of the host. The frequency of such events in comparison to classical hybridization is by nature unpredictable. In addition, in a plant producing a Cry1Ab-modified toxin, a metabolomic study [13] revealed that the transgene introduced indirectly 50% changes in osmolytes and branched amino acids.

Review of statistical effects after GMO consumption

Some GMOs (Roundup tolerant and MON863) affect the body weight increase at least in one sex [2,14]. It is a parameter considered as a very good predictor of side

effects in various organs. Several convergent factors appear to indicate liver and kidney problems as end points of GMO diet effects in these experiments [2,5,15,16]. This was confirmed by our meta-analysis of all *in vivo* studies published on this particular topic (Table 2). The kidneys are particularly affected, concentrating 42% of all parameters disrupted in males. However, other organs may be affected too, such as the heart and spleen, or blood cells [5].

Liver parameters

For one of the longest independent tests performed, a GM herbicide-tolerant soybean available on the market was used to feed mice. It caused the development of irregular hepatocyte nuclei, more nuclear pores, numerous small fibrillar centers, and abundant dense fibrillar components, indicating increased metabolic rates [17]. It was hypothesized that the herbicide residues could be responsible for that because this particular GM plant can absorb the chemicals to which it was rendered tolerant. Such chemicals may be involved in the above-mentioned pathological features. This became even clearer when Roundup residues provoked similar features in rat hepatic cells directly *in vitro* [18]. The reversibility observed in some instances for these parameters *in vivo* [19] might be explained by the heterogeneity of the herbicide residues in the feed [20]. Anyway, these are specific parameters of ultrastructural dysfunction, and the relevance is clear. The liver is reacting. The Roundup residues have been also shown to be toxic for human placental, embryonic, and umbilical cord cells [21-23]. This was also the case for hepatic human cell lines in a comparable manner, inducing nuclei and membrane changes, apoptosis and necrosis [24].

The other major GMO trait has to do with the mutated (mBt) insecticidal peptidic toxins produced by transgenes in plants. In this case, some studies with maize confirmed histopathological changes in the liver and the kidneys of rats after GM feed consumption. Such changes consist in congestion, cell nucleus border changes, and severe granular degeneration in the liver [16]. Similarly, in the MON810 studies, a significantly lower albumin/globulin ratio indicated a change in hepatic metabolism of 33% of GM-fed male rats (according to EFSA opinion on MON810 and [5]). Taken together, the results indicate potential adverse effects in hepatic metabolism. The insecticide produced by MON810 could also induce liver reactions, like many other pesticides. Of course, the mCry1Ab and other mBt (mutated Bt toxins derived from native *Bacillus thuringiensis* toxins) in GMOs are proteic toxins; however, these are modified at the level of their amino acid sequence by biotechnologies and introduced by artificial vectors, thus these could be considered as xenobiotics (i.e., a molecule foreign to life). The liver together with the kidneys are the major reactive organs in case of food chronic intoxication.

Kidney parameters

In the NK603 study, statistically significant strong urine ionic disturbances and kidney markers could be explained by renal leakage [5], which is well correlated with the effects of glyphosate-based herbicides (like Roundup) observed on embryonic kidney cells [23]. This does not exclude metabolic effects indirectly due to insertional mutagenesis linked to the plant transformation. Roundup adjuvants even stabilize glyphosate and allow its penetration into cells, which in turn inhibit estrogen synthesis as a side effect, cytochrome P450 aromatase inhibition [21]. This phenomenon changes the androgen/estrogen ratio and may at least, in part, explain differential impacts in both sexes.

Kidney dysfunctions are observed with mBt maize producing mutated insecticides such as in MON863. For instance, we quote the initial EFSA report: "Individual kidney weights of male rats fed with the 33% MON863 diet were statistically significantly lower compared to those of animals on control diets", "small increases in the incidences of focal inflammation and tubular regenerative changes in the kidneys of 33% MON863 males." This was confirmed by the company tests [25] and another counter analysis revealed disrupted biochemical

Table 2 Meta-analysis of statistical differences with appropriate controls in feeding trials

All parameters measured *in vivo* in GMO toxicity studies	Measured by organ (%)/Total (694-698)		Disturbed in each organ (%)/Total disrupted parameters (approximately 9%)	
	Females	Males	Females	Males
Liver	22.9	22.9	**30.8**	26.1
Kidney	23.7	23.7	26.4	**43.5**
Bone marrow	29.5	29.5	29.7	22.8
Total for 3 tissues	76.1	76.1	86.9	92.4

Commercialized soybean and maize GMOs were fed to rats and their blood analyses were obtained. The different parameters are classified according to the tissue [2] to which they are related (e.g., liver, kidney, bone marrow). Of the total parameters measured 76.1% are related to these three organs. The percentages of significantly different parameters to the controls are called "disrupted parameters." There are in total 9% of disrupted parameters and, for instance, 43.5% of these are concentrated in kidneys in males. The bold values are significantly over the parameters measured per organ.

markers typical of kidney filtration or function problems [2]. The first effects were not always but sometimes greater than the ones with non-isogenic maize (called reference lines), which contain different salts, lipids, or sugars. Moreover, both results described are different between males and females; this is quite usual in liver or kidney pesticide reactions. These facts do not exclude that such effects can be considered as treatment-related. Other studies also confirmed effects on kidneys. Tubular degeneration and not statistically significant enlargement in parietal layer of Bowman's capsules were also observed with GM maize fed rats [16].

Last but not least, a total of around 9% of parameters were disrupted in a meta-analysis (Table 2). This is twice as much as what could be obtained by chance only (generally considered as 5%). Surprisingly, 43.5% of significant different parameters were concentrated in male kidneys for all commercialized GMOs, even if only around 25% of the total parameters measured were kidney-related. If the differences had been distributed by chance in the organs, not significantly more than 25% differences would have been found in the kidney. Even if our own counter analysis is removed from the calculation, showing numerous kidney dysfunctions [2], around 32% of disturbances are still noticed in kidneys.

Discussion

Need for chronic tests and other tests

Chronic toxicity tests (both with males and females) and reproductive tests with pregnant females and then with the developing progeny over several generations (none of these steps exist at present) are called as a whole the Toxotest approach (or Risk management test, see "Details on the new suggested Toxotest approach"). This could address the long-term physiological or pathological relevance of the previous observations. The physiological interpretations of 90-day-based effects are otherwise somewhat limited. These studies should be complementary to the present regulations or the Safotest and the sentinel test suggested by EFSA [1]. The Toxotest could provide evidence of carcinogenic, developmental, hormonal, neural, and reproductive potential dysfunctions, as it does for pesticides or drugs. Additionally, it is obvious that the 90-day-long trials on mature animals performed today cannot scientifically replace the sensitivity of developmental tests on neonates. A good example is the gene imprinting by drugs that will be revealed only at maturity; this is an important subject of current research, and many findings have been reported for some chemicals such as bisphenol A [26,27]. Even transgenerational effects occur after epigenetic imprinting by a pesticide [28]. These effects cannot be detected by classical 90-day feeding trials and will be visible after many decades by epidemiology in humans if any, as illustrated in the case of diethylstilbestrol, which induced female genital cancers among other problems in the second generation [29]. The F3 multigenerational study for a GMO (Table 1) was too rarely performed. This is why, because of the number of parameters disrupted in adult mammals within 90 days, the new experiments should be systematically performed to protect the health of billions of people that could consume directly or indirectly these transformed products.

The acute toxicity approach (less than a month of investigations on rodents with high doses) may give effects which are more proportional to the dose, as it might correspond to a rapid poisoning of the animals, generally with force-fed experiments. However, for many pesticide studies in the scientific literature, some long-term side effects of pesticides at environmental doses are described, which are not apparent in short-term experiments [30]. Classical toxicology is quite often based on the concept of revealing linear dose-responses as defined by Paracelsus, which generally fails to evidence U or J curves observed after hormonal sex-specific disruptions. Moreover, the effects of mixtures are also neglected in long-term studies, when supposed active principles of pesticides are not assessed with their adjuvants, which also are present as residues in GMOs. Such pesticides may have the capacity to disrupt the "cell web", i.e., to interfere with a signaling pathway, and this could be unspecific. For instance Roundup is known to disrupt the EPSPS in plants, but is also known to interact with the mammalian ubiquist reductase [21] common and essential to cytochromes P450, a wide class of detoxification enzymes. The so-called Roundup active principle, glyphosate, acts in combination with adjuvants to increase glyphosate-mediated toxicity [21,31], and this may apply to other environmental pollutants [22]. Moreover, all new metabolites in edible Roundup ready GMOs, as acetyl-glyphosate for the new GAT GMOs, have not been assessed for their chronic toxicity [11], and we consider this as a major oversight in the present regulations.

Therefore, as xenobiotic effects are complex, the determination of their toxic effects cannot be determined using a single method, but rather converging pieces of evidence. In GMO risk assessment, the protocols must be optimized to detect side effects, in particular for herbicide-treated GM plants. These cannot be reduced to GM assessment on one side and herbicide residues with any diet on the other side, but unfortunately this has been the case, and this approach has been promoted up to now by regulatory authorities.

In fact, it is impossible, within only 13 weeks, to conclude about the kind of pathology that could be induced by pesticide GMOs and whether it is a major pathology or a minor one. It is therefore necessary to prolong the

tests, as suggested by EFSA, since at least one third of chronic effects visible with chemicals are usually new in comparison to the ones highlighted in subchronic studies [1]. The so-called Toxotests, which are supposed to include the studies of chronic pathologies in particular, should be performed on three mammalian species, with at least one non-rodent, similar to the type of rodents used for pesticides and drugs. However, the chronic feeding tests for GMOs cannot be based on the *no observed adverse effect level*, nor on the *lowest observed adverse effect level* approach, as in classical toxicology. There are several reasons for that. There is not only one chemical, but also several unknown metabolites and components, in Roundup tolerant varieties for instance, and therefore toxicity is enhanced thanks to the fact that they are mixed together. There is also no possibility of increasing the doses of GMOs in an equilibrated diet over an acceptable level. The diets should be rather representative of an equilibrated diet with GMOs like it could be the case in a real population in America. To prolong 90-day subchronic tests with three normal doses of GM in the diet (11%, 22%, 33% for instance) is the solution.

Sex- or dose-specific pathological effects are common

When there is a low or environmental dose impregnation of the feed (with a pesticide GM plant for instance), the chronic effects could be more differentiated according to the sex, the physiological status, the age, or the number of intakes over such and such a period of time in the case of a drug. These parameters (chronic intake, age of exposure, etc.) are more decisive for pathologies like cancers, than the actual quantity of toxin ingested in one intake. This is in part because the liver, kidney, and other cytochrome P450-rich organs are concerned for long-term metabolism and detoxification, and this phenomenon is hormone dependent. It is also due to the process of carcinogenesis or hormone-sensitive programming of cells [32]. The liver for instance is a sex differentiated organ as far as its enzymatic equipment is concerned [4]. An effect in subchronic or chronic tests cannot be disregarded on the rationale that it is not linear to the dose (or dose-related) or not comparable in genders. This would not be scientifically acceptable. However, this reasoning was adopted both by companies and EFSA for several GMOs, as underlined by Doull et al. [33]. Indeed, most xenobiotics or pollutants may have non-linear effects, and/or may have sex- and age-specific impacts.

One of the pivotal requirements for regulators nowadays, in order to interpret a significant difference as biologically relevant, is to observe a linear dose-response. This allows them to deduce a causality. However, this dose-response cannot be studied with only two points, which is nonetheless the case for all major commercial GMOs today, which are given in the diet in 11% and 33% concentrations only, in subchronic tests. This is true overall if no preliminary data has been obtained to choose the given doses, which is the case in regulatory files. As we have already emphasized, most of pathological and endocrine effects in environmental health are not directly proportional to the dose, and they have a differential threshold of sensitivity in both sexes [34]. This is, for instance, the case with carcinogenesis and endocrine disruption.

Improving the knowledge on impacts of modified Bt toxins

One of the interpretations of the side effects observed (Tables 1 and 2) would be that the insecticide toxins in maize lines may have more pleiotropic or specific actions than originally supposed. The toxins could generate particular metabolites, either in the GM plant or in the animals fed with it. The Bt toxins in GMOs are new and modified, truncated, or chimerical in order to change their activities/solubility in comparison to wild Bt. For instance, there is at least a 40% difference between the toxin in Bt176 and its wild counterpart [10]. None of the modified Bt toxins have been authorized separately for food or feed, neither has the wild Bt, and neither have they been tested by themselves on animal or human health to date. Even if some studies were performed, the receptors have not been cloned and the signaling pathways have not been identified as yet, nor required for authorizations, and the metabolism of these proteins in mammals are unknown [35]. Thus, the argument about "safe use history" of the wild Bt protein (not designed for direct consumption, in contrast to several GMOs) cannot, on a sound scientific basis, be used for direct authorizations of the above-cited GM corns, overall without *in vivo* chronic toxicity tests (or Toxotest approach), as it is requested for a pesticide. Some improvements may even be included with regard to pesticide legislation, since these human modified toxins considered as xenobiotics are continuously produced by the plants devoted to consumption.

The proteins usually compared (modified Bt toxins and wild ones) are not identical, and the tests on human cells of Bt proteins are not performed nor are they requested by authorities. Their stability has been assessed *in vitro*, and GM insecticide toxins are never fully digested in vivo [36]. If some consumers suffer from stomach problems or ulcers, the new toxins will possibly act differently; the digestion in children could be affected too; however, these GMOs could be eaten anywhere and all proteins are never fully decomposed in amino acids by the digestive tract.

Details on the new suggested Toxotest approach

The suggested Toxotest would basically include an extension of the existing 90-day tests, but with at least three doses plus controls (0%, 11%, 22%, 33% GMOs for instance; today the equilibrated diets tested contain 0%, 11%, and 33% GMOs in the best regulatory tests). The purpose would be to characterize scientifically the dose-response approach. The latter cannot be taken seriously with only two GM doses. The final goal is the best health protection for the population without really possible clinical trials, in our case for practical and ethical reasons. There is also no epidemiological follow-up for lack of traceability and labeling in GM-producing American countries. In addition, the fact that the Toxotest includes the best possible toxicological approach will also be in favor of the biotechnology economy and the European Community because it is more expensive to address an issue concerning a whole population afterwards, rather than to work with laboratory animals beforehand; it is also more ethical to work on rats and other mammalian experiments, in order to get the relevant information, rather than to give pesticide plants directly to humans on a long-term basis.

As previously underlined, the health effects such as those suggested in Table 2 (if any, are revealed by adapted studies, such as Safotests or Toxotests), could only be due to two possibilities:

Firstly, the side effects may be directly or indirectly due to a pesticide residue and/or its metabolites. The direct effect is about the pesticide effect on the consumer, and the indirect one is about a metabolism disruption that it has provoked within the plant first. This could not be visible by a detailed compositional analysis, such as the one performed to be assessed by a substantial equivalence study. This concept is not a well-defined one (how many cultivations of crops, over how many years, under which climate, and to measure what precise parameters).

Secondly, the pathological signs may be due to the genetic transformation itself, its method provoking either insertional mutagenesis or a new metabolism by genetic interference. This is the reason why separating intended effects (the direct genetic trait consequence itself) from unintended effects (linked to biotechnology, e.g., insertional mutagenesis), such as spiking the control diet with the purified toxin in the Toxotest approach, is clearly inadequate. It could work in the case of a direct action of the toxin in mammals, but conversely one could not conclude, between an insertional mutagenesis and a specific metabolic action in the plant due to the toxin. However, this is more a research question about the mode of genesis of an effect on health, and new research avenues could be, for instance, to compare the GM diet with or without herbicide treatment in long-term tests with the isogenic control diet including herbicide residues added. This is only necessary for the understanding of the potential signs of toxicity and not for a conclusion of the Safotest or the Toxotest, which would rather suggest, if positive, excluding immediately the corresponding GMO from food and feed.

Improvement of statistical analysis

A serious experimental design is based on a proper choice of the groups, with only one question studied per experiment if possible, and balanced sample sizes. In several authorized GMOs, the sample sizes appear inadequate in 90 days: ten animals per group for the measurement of biochemical parameters out of 20, as performed by the major stakeholders, and accepted by EFSA for MON863, MON810, or NK603 for instance. This is too limited a size to ensure that parametric statistical methods used by the company are reliable. Moreover, an important discrepancy between GMO-treated rats (40 measured out of 80) and the total number of animals (400) renders more difficult the evidencing of relevant effects, and confusion factors are brought in at the same time with six different reference diets in addition to the two normal control groups as performed in three commercialized GMOs at least [5,6]. This introduces new uncontrolled sources of variability about the effects of the diets and new unnecessary questions not relevant to the GMO safety. The representation of a standard diet with multiple sources could have been studied with only one control group of the same size than the GMO group, eating a mix of six different regular non-GM diets.

Several questions have been raised by companies and authorities as well as comments on statistically significant effects that would supposedly not be biologically meaningful. A subjective part is introduced at this level because it is necessary to take into account the context and the general and detailed knowledge of toxicology and endocrine disruption, as EFSA underlines. This might be highly expert dependent. This is why, to avoid or prevent any misunderstanding, we suggest, in addition to a new statistical approach based on classical methods, to analyze the 90-day tests, even with control and reference diets called the “SSC method” (according to the initials of the authors in [2]).

Briefly, following the necessity to model and analyze the growth curves, multivariate data analysis and data mining of all parameters can be used to correlate, cluster, and select meaningful variables. This kind of approach is not performed at all today. Thereafter, the detailed comparison between GM-treated and control groups, fed with the near isogenic line (because the real isogenic line does not often exists anymore), will necessarily be followed by the study of specific diet effects,

when there are non-substantially equivalent diets for reference groups. For that purpose, the controls will be first compared using multivariate inference with reference groups, and thereafter, similarly GMO-treated groups with reference groups. The significant differences linked to the GMO and/or the composition of the diet will be classified according to organ and function. The results will appear more clearly than with the simple statistics accepted today by the authorities (that is, comparison of the highest GM dose group with the mean value of all six control groups), and will reveal in addition new information, as it can be demonstrated.

As recommended by EFSA, an appropriate and relevant statistical analysis is crucial. It should follow the following series of steps, allowing the use of several methods depending on the questions raised:

- Obtaining and modeling the growth curves and feed consumption, assessed by non-linear regression, validation, and statistical comparisons in order to test if the curves are significantly different, thus taking into account individual variability. This necessitates the use of time series analysis, selection models, and non-parametric tests, Akaike Information Criteria and related methods. Water consumption should also be an important factor to follow-up and therefore better understand kidney and urine data.
- The study of dose-response predictions using non-linear regression should be the goal, but the only two doses generally used in these tests do not make it possible to evidence linearity as we indicated. Moreover, in the cases where there are not dose-related trends or relationships using the two doses mentioned, the absence of linear dose-response curves cannot be a reason to neglect the effects. For instance, as previously cited, U or J curves may be characteristic of endocrine effects [37], and spiky irregular curves may be detected in carcinogenesis.
- Simultaneous analysis of all observed variables: multivariate data analysis, principal component analysis, correlations analysis, factorial analysis and clustering
- Multivariate comparisons of the different variables: hypothesis testing, multiple ways ANOVA, MANOVA, and others to determinate if the groups differ relative to the different questions: specific GMO effect or diet effect per se. To evidence a detail, when comparing two mean values, SEM should be calculated to determine confidence intervals; however, SD have been used up to now by the company for MON863 and NK603 files for instance.

Apart from empirical curves in some instances, ANOVA and univariate hypothesis testing only the GMO effect, none of the other statistical approaches is currently used nor requested by the authorities.

Human tests and post-market monitoring

For the record, it must be said that very few tests on humans have been carried out up to now. Moreover, epidemiological studies are not feasible in America, since there is no organized traceability of GMOs anywhere on the continent, where, by far, most of edible GMOs are cultivated (97%). As a consequence, a post-market monitoring (PMM) is offered to the population. The Cartagena Biosafety Protocol identifying GMOs at the borders of a country has now been signed by over 150 countries, including the member states of the European Union. PMM may have some value in detecting unexpected adverse effects. It could therefore be considered as a routine need. This approach makes it possible to collect information related to risk management. It can be relied upon as a technique for monitoring adverse events or other health outcomes related to the consumption of GM plant-derived foods, provided that the Toxotest approach, together with the SSC method, should have already been applied. The PMM should be linked with the possibility of detecting allergenicity reactions to GMOs in routine medicine, thanks to the very same routine cutaneous tests that should be developed prior to large-scale commercialization. A screening of serum banks of patients with allergies could be also put forward in order to search for antibodies against the main GMOs and not only their transgenic proteins, since they may induce secondary allergenic metabolites in the plant not visible in the substantial equivalence study.

The traceability of products from animals fed on GMOs is also crucial. The reason for this is because they can develop chronic diseases which are not utterly known today. Such possible diseases could be linked to the hepatorenal toxicity observed in some GMO-related cases (Table 1).

Moreover, labeling animals fed on GMOs is therefore necessary because some pesticide residues linked to GMOs could pass into the food chain and also because nobody would want to eat disabled or physiologically modified animals after long-term GMOs ingestion, even if pesticides residues or DNA fragments are not toxic nor transmitted by themselves.

Conclusion

Transcriptomics, proteomics and other related methods are not ready yet for routine use in the laboratories, and moreover they may be inappropriate for studying toxicity in animals, and could not in any way replace *in vivo* studies with all the physiological and biochemical parameters that are measured with organs weight,

appearance, and histology. By contrast, afterwards, new approaches could well help to explain pathological results or action mechanisms of pesticides present in the GM plants or GM-fed animals, if found.

To obtain the transparency of raw data (including rat blood analyses) for toxicological tests, maintained illegally confidential, is crucial. It has also become crucial to apply objective criteria of interpretation like the criteria described here: sex-specific side effects or non-linear ones. Such data can be put online on the EFSA website with a view to provide a fuller review to the wider scientific community, and in order to better inform the citizen to make biotechnologies more socially acceptable. Since fundamental research is published on a regular basis, it should be the same for this kind of applied research on long-term health effects, as suggested by the CE/2001/18 and the corresponding 1829/2003 regulations.

We can conclude, from the regulatory tests performed today, that it is unacceptable to submit 500 million Europeans and several billions of consumers worldwide to the new pesticide GM-derived foods or feed, this being done without more controls (if any) than the only 3-month-long toxicological tests and using only one mammalian species, especially since there is growing evidence of concern (Tables 1 and 2). This is why we propose to improve the protocol of the 90-day studies to 2-year studies with mature rats, using the Toxotest approach, which should be rendered obligatory, and including sexual hormones assessment too. The reproductive, developmental, and transgenerational studies should also be performed. The new SSC statistical method of analysis is proposed in addition. This should not be optional if the plant is designed to contain a pesticide (as it is the case for more than 99% of cultivated commercialized GMOs), whilst for others, depending on the inserted trait, a case-by-case approach in the method to study toxicity will be necessary.

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Authors' contributions

GES designed and coordinated the review. RM participated in the drafting of the manuscript and final version. EC, SG, JSV and DC helped the writing, compiling the literature, revising in details and proofreading the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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Long term toxicity of a Roundup herbicide and a Roundup-tolerant genetically modified maize

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ABSTRACT

The health effects of a Roundup-tolerant genetically modified maize (from 11% in the diet), cultivated with or without Roundup, and Roundup alone (from 0.1 ppb in water), were studied 2 years in rats. In females, all treated groups died 2–3 times more than controls, and more rapidly. This difference was visible in 3 male groups fed GMOs. All results were hormone and sex dependent, and the pathological profiles were comparable. Females developed large mammary tumors almost always more often than and before controls, the pituitary was the second most disabled organ; the sex hormonal balance was modified by GMO and Roundup treatments. In treated males, liver congestions and necrosis were 2.5–5.5 times higher. This pathology was confirmed by optic and transmission electron microscopy. Marked and severe kidney nephropathies were also generally 1.3–2.3 greater. Males presented 4 times more large palpable tumors than controls which occurred up to 600 days earlier. Biochemistry data confirmed very significant kidney chronic deficiencies; for all treatments and both sexes, 76% of the altered parameters were kidney related. These results can be explained by the non linear endocrine-disrupting effects of Roundup, but also by the overexpression of the transgene in the GMO and its metabolic consequences.

1. Introduction

There is an ongoing international debate as to the necessary length of mammalian toxicity studies in relation to the consumption of genetically modified (GM) plants including regular metabolic analyses (Séralini et al., 2011). Currently, no regulatory authority requests mandatory chronic animal feeding studies to be performed for edible GMOs and formulated pesticides. However, several studies consisting of 90 day rat feeding trials have been conducted by the biotech industry. These investigations mostly concern GM soy and maize that are rendered either herbicide tolerant (to Roundup (R) in 80% of cases), or engineered to produce a modified *Bt* toxin insecticide, or both. As a result these GM crops contain new pesticide residues for which new maximal residual levels (MRL) have been established in some countries.

If the petitioners conclude in general that there is no major change in genetically modified organism (GMO) subchronic toxicity studies (Domingo and Giné Bordonaba, 2011; Hammond et al., 2004, 2006a,b), significant disturbances have been found and may be interpreted differently (Séralini et al., 2009; Spiroux de Vendômois et al., 2010). Detailed analyses have revealed alterations in kidney and liver functions that may be the signs of early chronic diet intoxication, possibly explained at least in part by pesticide residues in the GM feed (Séralini et al., 2007; Spiroux de Vendômois et al., 2009). Indeed, it has been demonstrated that R concentrations in the range of 10^3 times below the MRL induced endocrine disturbances in human cells (Gasnier et al., 2009) and toxic effects thereafter (Benachour and Seralini, 2009), including *in vivo* (Romano et al., 2012). After several months of consumption of an R-tolerant soy, the liver and pancreas of mice were affected, as highlighted by disturbances in sub-nuclear structure (Malatesta et al., 2008a, 2002a,b). Furthermore, this toxic effect was reproduced by the application of R herbicide directly to hepatocytes in culture (Malatesta et al., 2008b).

Abbreviations: GM, genetically modified; R, Roundup; MRL, maximal residual levels; GMO, genetically modified organism; OECD, Organization for Economic Co-operation and Development; GT, glutamyl-transferase; PCA, principal component analysis; PLS, partial least-squares; OPLS, orthogonal partial least-squares; NIPALS, Nonlinear Iterative Partial Least Squares; OPLS-DA, Orthogonal Partial Least Squares Discriminant Analysis; G, glycogen; L, lipid droplet; N, nucleus; R, rough endoplasmic reticulum (on microscopy pictures only); U, urinary; UEx, excreted in urine during 24 h; APPT, Activated Partial Thromboplastin Time; MCV, Mean Corpuscular Volume; PT, Prothrombine Time; RBC, Red Blood Cells; ALT, alanine aminotransferase; MCHC, Mean Corpuscular Hemoglobin Concentration; A/G, Albumin/Globulin ratio; WBC, White Blood Cells; AST, aspartate aminotransferase.

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Since then, long-term and multi-generational animal feeding trials have been performed with some possibly providing evidence of safety, while others conclude on the necessity of further investigations because of metabolic modifications (Snell et al., 2011). However, none of these studies have included a detailed follow-up of the animals with up to 11 blood and urine samples over 2 years, and none has investigated the NK603 R-tolerant maize.

Furthermore, toxicity evaluation of herbicides is generally performed on mammalian physiology through the long-term study of only their active principle, rather than the formulation used in agriculture, as was the case for glyphosate (Williams et al., 2000), the active herbicide constituent of R. It is important to note that glyphosate is only able to efficiently penetrate target plant organisms with the help of adjuvants present in the various commercially used R formulations (Cox, 2004). When R residues are found in tap water, food or feed, they arise from the total herbicide formulation, which is the most commonly used mixture in agriculture; indeed many authors in the field have strongly emphasized the necessity of studying the potential toxic effects of total chemical mixtures rather than single components (Cox and Surgan, 2006; Mesnage et al., 2010; Monosson, 2005). Even adjuvants and not only glyphosate or other active ingredients are found in ground water (Krogh et al., 2002), and thus an exposure to the diluted whole formulation is more representative of an environmental pollution than the exposure to glyphosate alone in order to study health effects.

With a view to address this lack of information, we have performed a 2 year detailed rat feeding study. The actual guideline 408 of the Organization for Economic Co-operation and Development (OECD) was followed by some manufacturers for GMOs even if it was not designed for that purpose. We have explored more parameters and more frequently than recommended in this standard (Table 1) in a long-term experiment. This allowed us to follow in details potential health effects and their possible origins due to the direct or indirect consequences of the genetic modification itself in GMOs, or due to the formulated herbicide mixture used on GMOs (and not glyphosate alone), or both. Because of recent reviews on GMOs (Domingo and Giné Bordonaba, 2011; Snell et al., 2011) we had no reason to settle at first for a carcinogenesis protocol using 50 rats per group. However we have prolonged the biochemical and hematological measurements or disease status recommended in combined chronic studies using 10 rats per group (up to 12 months in OECD 453). This remains the highest number of rats regularly measured in a standard GMO diet study. We have tested also for the first time 3 doses (rather than two in the usual 90 day long protocols) of the R-tolerant NK603 GM maize alone, the GM maize treated with R, and R alone at very low environmentally relevant doses starting below the range of levels permitted by regulatory authorities in drinking water and in GM feed.

2. Materials and methods

2.1. Ethics

The experimental protocol was conducted in accordance with the regulations of our ethics in an animal care unit authorized by the French Ministries of Agriculture and Research (Agreement Number A35-288-1). Animal experiments were performed according to ethical guidelines of animal experimentations (CEE 86/609 regulation). Concerning field studies of plant species, no specific permits were required, nor for the locations/activities. The maize grown (MON-00603-6 commonly named NK603) was authorized for unconfined release into the environment and use as a livestock feed by the Canadian Food Inspection Agency (Decision Document 2002-35). We confirm that the location is not privately-owned or protected in any way and that the field studies did not involve endangered or protected species. The GM maize was authorized for import into the European Union (CE 258/97 regulation).

2.2. Plants, diets and chemicals

The varieties of maize used in this study were the R-tolerant NK603 (Monsanto Corp., USA), and its nearest isogenic non-transgenic control. These two types of maize were grown under similar normal conditions, in the same location, spaced at a sufficient distance to avoid cross-contamination. The genetic nature, as well as the purity of the GM seeds and harvested material, was confirmed by qPCR analysis of DNA samples. One field of NK603 was treated with R at 3 L ha^{-1} (WeatherMAX, 540 g/L of glyphosate, EPA Reg. 524-537), and another field of NK603 was not treated with R. Corns were harvested when the moisture content was less than 30% and were dried at a temperature below 30 °C. From these three cultivations of

Table 1
Protocol used and comparison to existing assessment, and to non-mandatory regulatory tests.

Treatments and analyses	In this work	Hammond et al., 2004	Regulatory tests
Treatments + controls	GMO NK603, GMO NK603 + Roundup, Roundup, and closest isogenic maize	GMO NK603 + Roundup, closest isogenic maize, and six other maize lines non substantially equivalent	GMOs or chemicals (in standard diet or water)
Doses by treatment	3	2	At least 3
Duration in months	24 (chronic)	3 (subchronic: 13 weeks)	3
Animals measured/group/sex	10/10 SD rats (200 rats measured)	10/20 SD rats (200 rats measured/total 400)	At least 10 rodents
Animals by cage (same sex)	1–2	1	1 or more
Monitoring/week	2	1	1 or more
Feed and water consumptions	Measured	For feed only	At least feed
Organs and tissues studied			For high dose and controls
Histology/animal	34	17/36	At least 30
Organs weighted	10	7	At least 8
Electronic microscopy	Yes	No	No
Behavioral studies (times)	2	1 (no protocol given)	1
Ophtalmology (times)	2	0	2
Number of blood samples/animal	11, each month (0–3) then every 3 months	2, weeks 4 and 13	1, at the end
Blood parameters	31 (11 times for most)	31 (2 times)	At least 25 (at least 2 times)
Plasma sex steroids	Testosterone, estradiol	No	No, except if endocrine effects suspected
Liver tissue parameters	6	0	0
Number of urine samples	11	2	Optional, last week
Urine parameters studied	16	18	7 if performed
Microbiology in feces or urine	Yes	Yes	No
Roundup residues in tissues	Studied	Not studied	Not mandatory
Transgene in tissues	Studied	Not studied	Not studied

The protocol used in this work was compared to the regulatory assessment of NK603 maize by the company (Hammond et al., 2004), and to non mandatory regulatory *in vivo* tests for GMOs, or mandatory for chemicals (OECD 408). Most relevant results are shown in this paper.

maize, laboratory rat chow was made based on the standard diet A04 (Safe, France). The dry rat feed was made to contain 11, 22 or 33% of GM maize, cultivated either with or without R, or 33% of the non-transgenic control line. The concentrations of the transgene were confirmed in the three doses of each diet by qPCR. All feed formulations consisted in balanced diets, chemically measured as substantially equivalent except for the transgene, with no contaminating pesticides over standard limits. All secondary metabolites cannot be known and measured in the composition. However we have measured isoflavones and phenolic acids including ferulic acid by standard HPLC-UV. All reagents used were of analytical grade. The herbicide diluted in the drinking water was the commercial formulation of R (GT Plus, 450 g/L of glyphosate, approval 2020448, Monsanto, Belgium). Herbicides levels were assessed by glyphosate measurements in the different dilutions by mass spectrometry.

2.3. Animals and treatments

Virgin albino Sprague-Dawley rats at 5 weeks of age were obtained from Harlan (Gannat, France). All animals were kept in polycarbonate cages (820 cm^2, Genestil, France) with two animals of the same sex per cage. The litter (Toplit classic, Safe, France) was replaced twice weekly. The animals were maintained at 22 ± 3 °C under controlled humidity (45–65%) and air purity with a 12 h-light/dark cycle, with free access to food and water. The location of each cage within the experimental room was regularly moved. This 2 year life-long experiment was conducted in a GPL environment according to OECD guidelines. After 20 days of acclimatization, 100 male and 100 female animals were randomly assigned on a weight basis into 10 equivalent groups. For each sex, one control group had access to plain water and standard diet from the closest isogenic non-transgenic maize control; six groups were fed with 11, 22 and 33% of GM NK603 maize either treated or not with R. The final three groups were fed with the control diet and had access to water supplemented with respectively 1.1×10^{-8}% of R (0.1 ppb of R or 50 ng/L of glyphosate, the contaminating level of some regular tap waters), 0.09% of R (400 mg/kg, US MRL of glyphosate in some GM feed) and 0.5% of R (2.25 g/L, half of the minimal agricultural working dilution). This was changed weekly. Twice weekly monitoring allowed careful observation and palpation of animals, recording of clinical signs, measurement of any tumors that may arise, food and water consumption, and individual body weights.

2.4. Biochemical analyses

Blood samples were collected from the tail vein of each rat under short isoflurane anesthesia before treatment and after 1, 2, 3, 6, 9, 12, 15, 18, 21 and 24 months: 11 measurements were obtained for each animal alive at 2-years. It was first demonstrated that anesthesia did not impact animal health. Two aliquots of plasma and serum were prepared and stored at −80° C. Then 31 parameters were assessed (Table 1) according to standard methods including hematology and coagulation parameters, albumin, globulin, total protein concentration, creatinine, urea, calcium, sodium, potassium, chloride, inorganic phosphorus, triglycerides, glucose, total cholesterol, alanine aminotransferase, aspartate aminotransferase, gamma glutamyl-transferase (GT), estradiol, testosterone. In addition, at months 12 and 24 the C-reactive protein was assayed. Urine samples were collected similarly 11 times, over 24 h in individual metabolic cages, and 16 parameters were quantified including creatinine, phosphorus, potassium, chloride, sodium, calcium, pH and clairance. Liver samples at the end made it possible to perform assays of CYP1A1, 1A2, 3A4, 2C9 activities in S9 fractions, with glutathione S- transferase and gamma-GT.

2.5. Anatomopathology

Animals were sacrificed during the course of the study only if necessary because of suffering according to ethical rules (such as 25% body weight loss, tumors over 25% body weight, hemorrhagic bleeding, or prostration), and at the end of the study by exsanguination under isoflurane anesthesia. In each case, the following organs were collected: brain, colon, heart, kidneys, liver, lungs, ovaries, spleen, testes, adrenals, epididymis, prostate, thymus, uterus, aorta, bladder, bone, duodenum, esophagus, eyes, ileum, jejunum, lymph nodes, lymphoreticular system, mammary glands, pancreas, parathyroid glands, Peyer's patches, pituitary, salivary glands, sciatic nerve, skin, spinal cord, stomach, thyroid and trachea. The first 14 organs (at least 10 per animal depending on the sex, Table 1) were weighted, plus any tumor that arose. The first nine organs were divided into two parts and one half was immediately frozen in liquid nitrogen/carbonic ice. The remaining parts including other organs were rinsed in PBS and stored in 4% formalin before anatomopathological study. These samples were used for further paraffin-embedding, slides and HES histological staining. For transmission electron microscopy, kidneys, livers and tumors were cut into 1 mm^3 fragments. Samples were fixed in pre-chilled 2% paraformaldehyde/2.5% glutaraldehyde in 0.1 M PBS pH 7.4 at 4 °C for 3 h and processed as previously described (Malatesta et al., 2002a).

2.6. Statistical analysis

Biochemical data were treated by multivariate analysis with the SIMCA-P (V12) software (UMETRICS AB Umea, Sweden). The use of chemometrics tools, for example, principal component analysis (PCA), partial least-squares to latent structures (PLS), and orthogonal PLS (OPLS), are robust methods for modeling, analyzing and interpreting complex chemical and biological data. OPLS is a recent modification of the PLS method. PLS is a regression method used in order to find the relationship between two data tables referred to as X and Y. PLS regression (Eriksson et al., 2006b) analysis consists in calculating by means of successive iterations, linear combinations of the measured X-variables (predictor variables). These linear combinations of X-variables give PLS components (score vectors t). A PLS component can be thought of as a new variable – a latent variable – reflecting the information in the original X-variables that is of relevance for modeling and predicting the response Y-variable by means of the maximization of the square of covariance (Max cov^2(X,Y)). The number of components is determined by cross validation. SIMCA software uses the Nonlinear Iterative Partial Least Squares algorithm (NIPALS) for the PLS regression. Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) was used in this study (Weljie et al., 2011; Wiklund et al., 2008). The purpose of Discriminant Analysis is to find a model that separates groups of observations on the basis of their X variables. The X matrix consists of the biochemical data. The Y matrix contains dummy variables which describe the group membership of each observation. Binary variables are used in order to encode a group identity. Discriminant analysis finds a discriminant plan in which the projected observations are well separated according to each group. The objective of OPLS is to divide the systematic variation in the X-block into two model parts, one linearly related to Y (in the case of a discriminant analysis, the group membership), and the other one unrelated (orthogonal) to Y. Components related to Y are called predictive, and those unrelated to Y are called orthogonal. This partitioning of the X data results in improved model transparency and interpretability (Eriksson et al., 2006a). Prior to analysis, variables were mean-centered and unit variance scaled.

3. Results

3.1. Mortality

Control male animals survived on average 624 ± 21 days, whilst females lived for 701 ± 20, during the experiment, plus in each case 5 weeks of age at the beginning and 3 weeks of stabilization period. After mean survival time had elapsed, any deaths that occurred were considered to be largely due to aging. Before this period, 30% control males (three in total) and 20% females (only two) died spontaneously, while up to 50% males and 70% females died in some groups on diets containing the GM maize (Fig. 1). However, the rate of mortality was not proportional to the treatment dose, reaching a threshold at the lowest (11%) or intermediate (22%) amounts of GM maize in the equilibrated diet, with or without the R application on the plant. It is noteworthy that the first two male rats that died in both GM treated groups had to be euthanized due to kidney Wilm's tumors that were over 25% of body weight. This was at approximately a year before the first control animal died. The first female death occurred in the 22% GM maize feeding group and resulted from a mammary fibroadenoma 246 days before the first control. The maximum difference in males was 5 times more deaths occurring during the 17th month in the group consuming 11% GM maize, and in females 6 times greater mortality during the 21st month on the 22% GM maize diet with and without R. In the female cohorts, there were 2–3 times more deaths in all treated groups compared to controls by the end of the experiment and earlier in general. Females were more sensitive to the presence of R in drinking water than males, as evidenced by a shorter lifespan. The general causes of death represented in histogram format (Fig. 1) are linked mostly to large mammary tumors in females, and other organic problems in males.

3.2. Anatomopathological observations

All rats were carefully monitored for behavior, appearance, palpable tumors, infections, during the experiment, and at least 10 organs per animal were weighted and up to 34 analyzed post mortem, at the macroscopic and/or microscopic levels (Table 1).

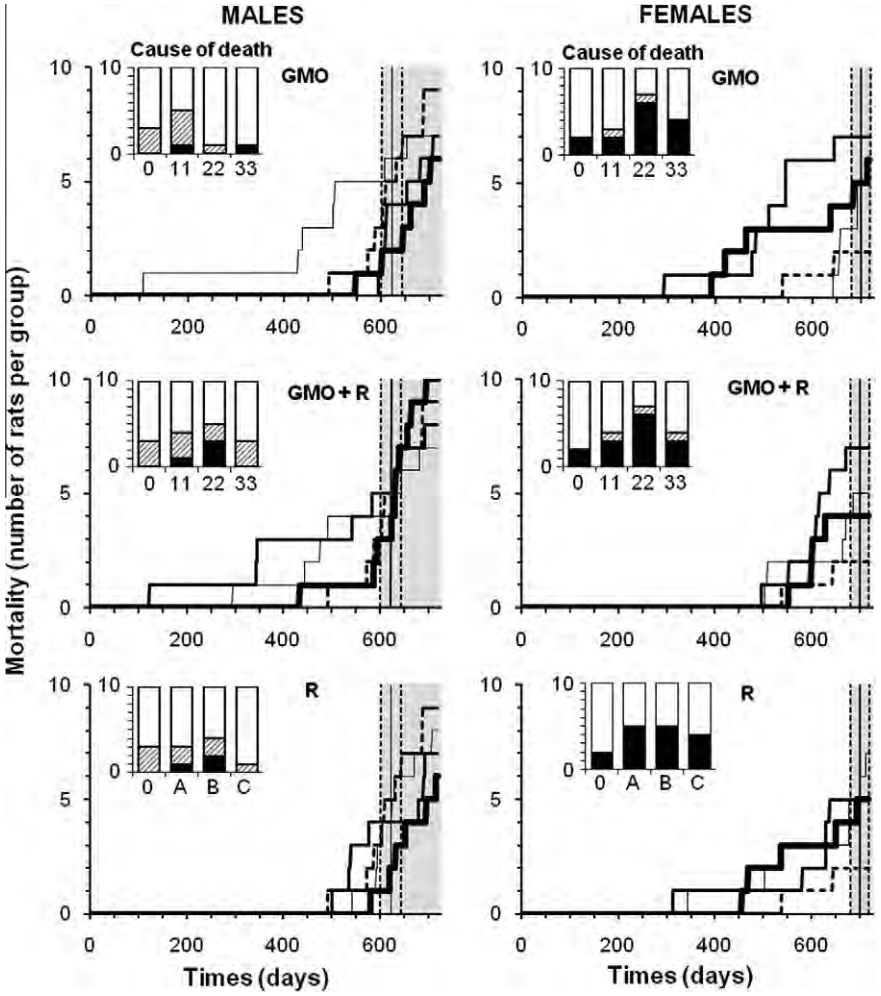

Fig. 1. Mortality of rats fed GMO treated or not with Roundup, and effects of Roundup alone. Rats were fed with NK603 GM maize (with or without application of Roundup) at three different doses (11, 22, 33% in their diet: thin, medium and bold lines, respectively) compared to the substantially equivalent closest isogenic non-GM maize (control, dotted line). Roundup was administrated in drinking water at 3 increasing doses, same symbols (environmental (A), MRL in agricultural GMOs (B) and half of minimal agricultural levels (C), see Section 2). Lifespan during the experiment for the control group is represented by the vertical bar ± SEM (grey area). In bar histograms, the causes of mortality before the grey area are detailed in comparison to the controls (0). In black are represented the necessary euthanasia because of suffering in accordance with ethical rules (tumors over 25% body weight, more than 25% weight loss, hemorrhagic bleeding, etc.); and in hatched areas, spontaneous mortality.

All data cannot be shown in one report, and the most relevant are described here. There was no rejection by the animals of the diet with or without GMOs, nor any major difference in the body weight.

The largest palpable growths (above a diameter of 17.5 mm in females and 20 mm in males) were found to be in 95% of cases non-regressive tumors, and were not infectious nodules. These growths progressively increased in size and number, but not proportionally to the treatment dose over the course of the experiment (Fig. 2). As in the case of rates of mortality, this suggests that a threshold in effect was reached at the lowest doses. They were rarely equal but almost always more frequent than in controls for all treated groups, often 2–3 times more in both sexes. Tumors began to reach a large size on average 94 days before in treated females, and up to 600 days earlier in 2 male groups eating the GM maize (11 and 22% with or without R).

In female animals, the largest tumors were in total 5 times more frequent than in males after 2 years, with 93% being mammary tumors. Adenomas, fibroadenomas and carcinomas were deleterious to health due to a very large size, rather than the grade of the tumor itself. Large tumor size caused impediments to either breathing or nutrition and digestion because of their thoracic or abdominal location and also resulted in hemorrhaging. In addition, one metastatic ovarian cystadenocarcinoma and two skin tumors were identified. Metastases were observed in only 2 cases; one in a group fed with 11% GM maize, and another in the highest dose of R treatment group.

Up to 14 months, no animals in the control groups showed any signs of tumors whilst 10–30% of treated females per group developed tumors, with the exception of one group (33% GMO + R). By the beginning of the 24th month, 50–80% of female animals had developed tumors in all treated groups, with up to 3 tumors per animal, whereas only 30% of controls were affected. The R treatment groups showed the greatest rates of tumor incidence with 80% of animals affected with up to 3 tumors for one female, in each group. A summary of all mammary tumors at the end of the experiment, independent of the size, is presented in Table 2. The same trend was observed in the groups receiving R in their drinking water; all females except one (with metastatic ovarian carcinoma) presented, in addition mammary hypertrophies and in some cases hyperplasia with atypia (Table 2).

The second most affected organ in females was the pituitary gland, in general around 2 times more than in controls for most treatments (Table 2). At this level again, adenomas and/or hyper-

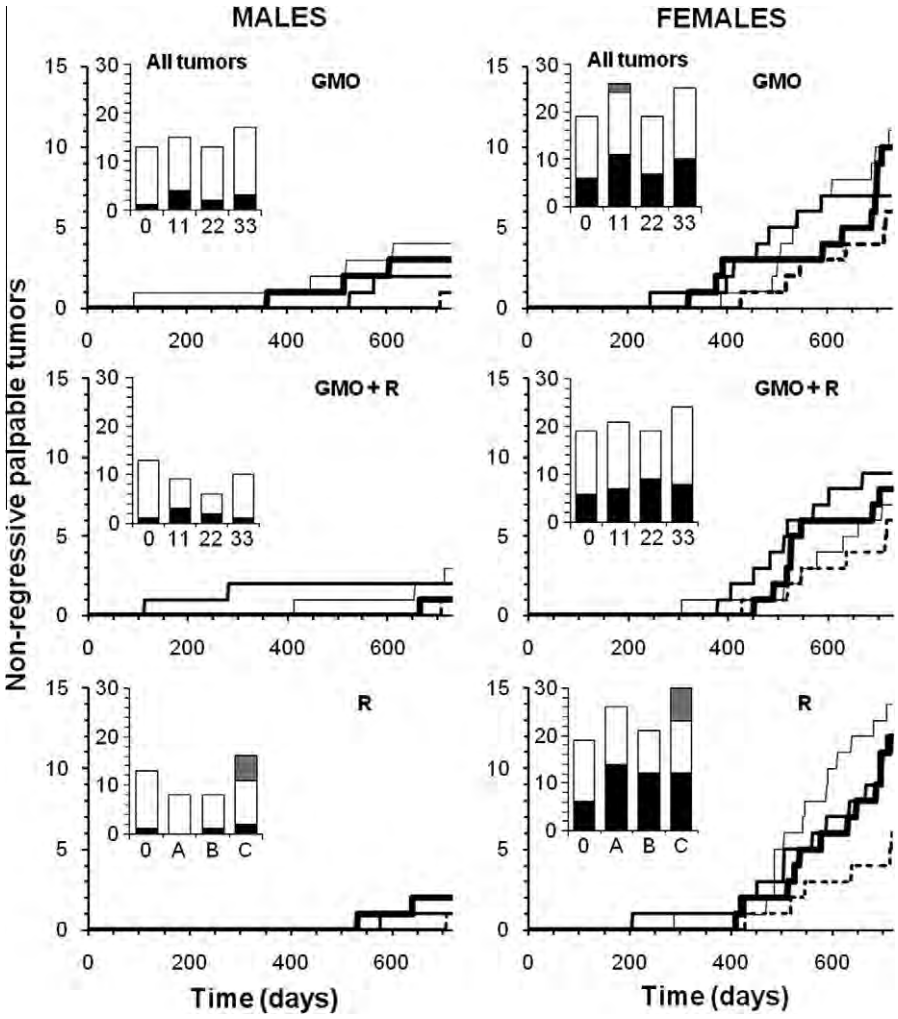

Fig. 2. Largest non-regressive tumors in rats fed GMO treated or not by Roundup, and effects of Roundup alone. The symbols of curves and treatments are explained in the caption of Fig. 1. The largest tumors were palpable during the experiment and numbered from 20 mm in diameter for males and 17.5 mm for females. Above this size, 95% of growths were non-regressive tumors. Summary of all tumors are shown in the bar histograms: black, non regressive largest tumors; white, small internal tumors; grey, metastases.

Table 2
Summary of the most frequent anatomical pathologies observed.

Organs and associated pathologies	Controls	GMO 11%	GMO 22%	GMO 33%	GMO 11% + R	GMO 22% + R	GMO 33% + R	R (A)	R (B)	R (C)
Males, in liver	2 (2)	5 (4)	11 (7)	8 (6)	5 (4)	7 (4)	6 (5)	11 (5)	9 (7)	6 (5)
In hepatodigestive tract	6 (5)	10 (6)	13 (7)	9 (6)	9 (6)	13 (6)	11 (7)	23 (9)	16 (8)	9 (5)
Kidneys, CPN	3 (3)	4 (4)	5 (5)	7 (7)	5 (5)	4 (4)	4 (4)	6 (6)	5 (5)	3 (3)
Females, mammary tumors	8 (5)	15 (7)	10 (7)	15 (8)	10 (6)	11 (7)	13 (9)	20 (9)	16 (10)	12 (9)
In mammary glands	10 (5)	22 (8)	10 (7)	16 (8)	17 (8)	16 (8)	15 (9)	26 (10)	20 (10)	18 (9)
Pituitary	9 (6)	23 (9)	20 (8)	8 (5)	19 (9)	9 (4)	19 (7)	22 (8)	16 (7)	13 (7)

After the number of pathological abnormalities, the number of rats reached is indicated in parentheses. In male animals pathological signs are liver congestions, macroscopic spots and microscopic necrotic foci. Hepatodigestive pathological signs concern the liver, stomach and small intestine (duodenum, ileum or jejunum). Only marked or severe chronic progressive nephropathies (CPN) are listed, excluding two nephroblastomas in groups consuming GMO 11% and GMO 22% + Roundup. In females, mammary fibroadenomas and adenocarcinomas are the major tumors detected; galactoceles and hyperplasias with atypia are also found and added in mammary glands pathological signs. Pituitary dysfunctions include adenomas, hyperplasias and hypertrophies. For details of the various treatment groups see Fig. 1.

plasias and hypertrophies were noticed. For all R treatment groups, 70–80% of animals presented 1.4–2.4 times more abnormalities than controls in this gland.

The big palpable tumors in males (in kidney, and mostly skin) were by the end of the experimental period on average twice as frequent as in controls, in which one skin fibroma appeared during the 23rd month. At the end of the experiment, internal non-palpable tumors were added, and their sums were lower in males than in females. They were not really different from controls, although slightly above in females (Histograms Fig. 2).

The most affected organs in males were the liver, together with the hepatodigestive tract and kidneys (Table 2 and Fig. 3). Hepatic congestions, macroscopic and microscopic necrotic foci were 2.5–5.5 times more frequent in all treatments than in control groups. Gamma GT hepatic activity was increased in particular for GMO + R groups (up to 5.4 times), this being probably due to a liver disorder.

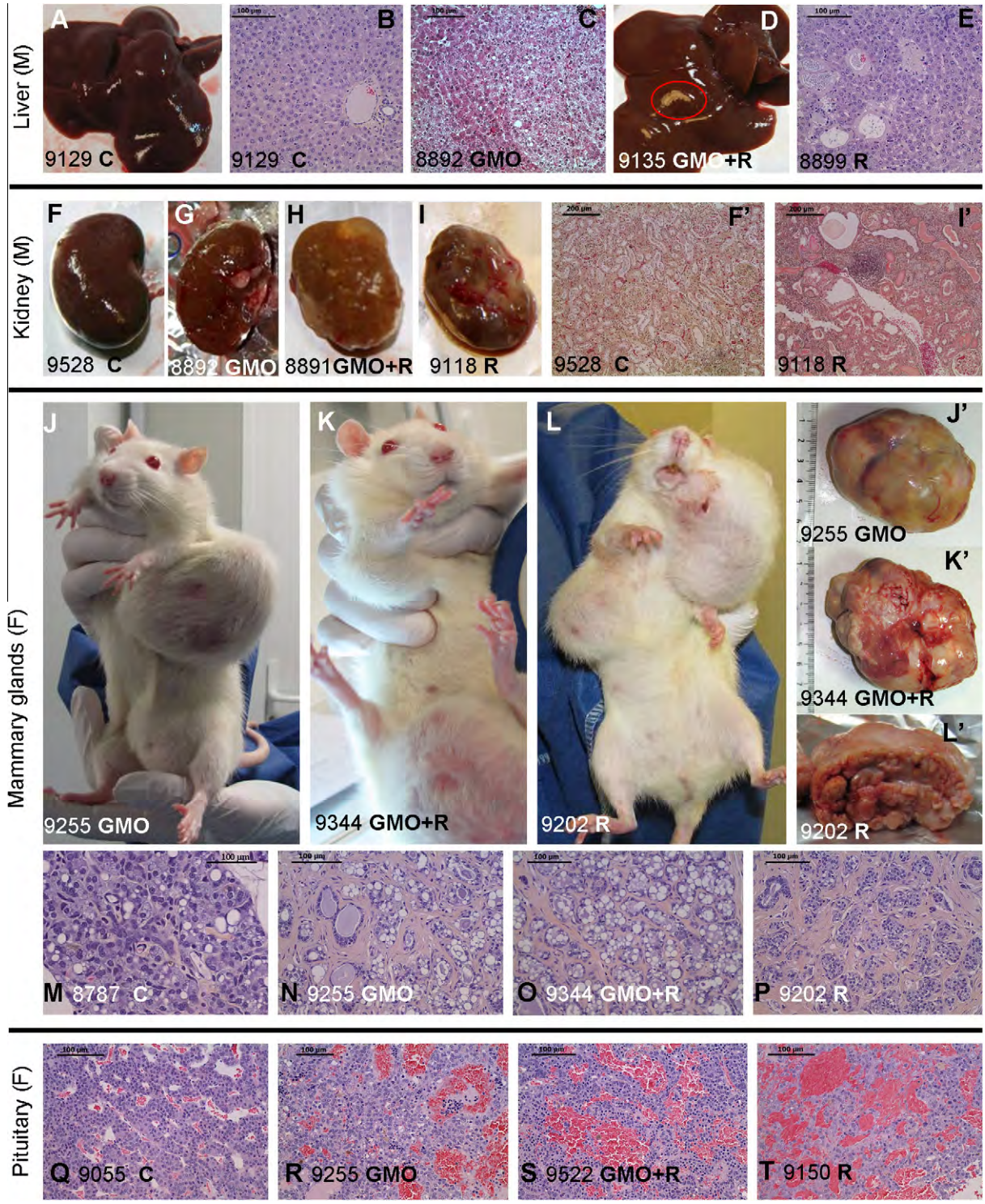

Fig. 3. Anatomopathological observations in rats fed GMO treated or not by Roundup, and effects of Roundup alone. Macroscopic and microscopic photographs show male livers (A–E) and left kidneys (F–I′), female mammary glands (J–P) and pituitaries (Q–T), according to Table 2. The number of each animal and its treatment is specified. Macroscopic pale spots (D) and microscopic necrotic foci in liver (C clear-cell focus, E basophilic focus with atypia), and marked or severe chronic progressive nephropathies, are illustrated. In females, mammary tumors (J,J′,N adenocarcinoma and K,K′,L,L′,O,P fibroadenomas) and pituitary adenomas (R–T) are shown and compared to controls (C after the rat number).

In addition, cytochrome activities also generally increased in the presence of R (in drinking water or GM diet) according to the dose up to 5.7 times at the highest dose. Transmission electron microscopic observations of liver samples confirmed changes for all treated groups in relation to glycogen dispersion or appearance in lakes, increase of residual bodies and enlargement of cristae in mitochondria (Fig. 4). The GM maize fed groups either with or without R application (in plants) showed a reduced transcription in mRNA and rRNA because of higher heterochromatin content, and decreased nucleolar dense fibrillar components. In the GMO + R group (at the highest dose) the smooth endoplasmic reticulum was drastically increased and nucleoli decreased in size,

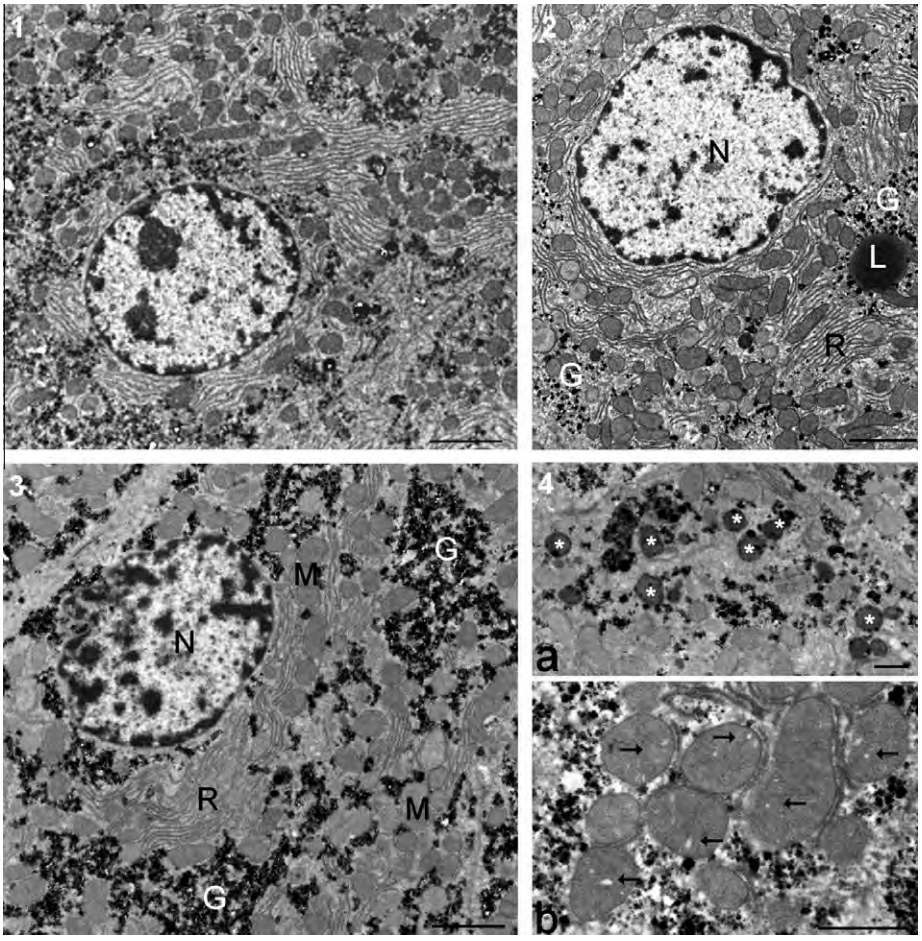

Fig. 4. Ultrastructure of hepatocytes in male rats from groups presenting the greatest degree of liver pathology. (1) Typical control rat hepatocyte (Bar 2 μm except in 4). (2) Effects with Roundup at the lowest dose. Glycogen (G) is dispersed in the cytoplasm. L, lipid droplet; N, nucleus; R rough endoplasmic reticulum. (3) Hepatocytes of animal fed GM maize (GMO) at 22% of total diet. Large lakes of glycogen occur in the cytoplasm. M, mitochondria. (4) Details of treatment effects with 22% dietary GMO (Bar 1 μm). (a) Cluster of residual bodies (asterisks). (b) Mitochondria show many enlarged cristae (arrows).

becoming more compact. For R treatment alone similar trends were observed, with a partial resumption of nucleolar activity at the highest dose.

Degenerating kidneys with turgid inflammatory areas demonstrate the increased incidence of marked and severe chronic progressive nephropathies, which were up to 2-fold higher in the 33% GM maize or lowest dose R treatment groups (Table 2 and Fig. 3).

3.3. Biochemical analyses

For the different corns and diets, the study of the standard chemical composition revealed no particular difference; this is why they were classified as substantially equivalent, except for transgene DNA quantification. For instance, there was no difference between total isoflavones. In addition, other specific compounds not always requested for substantial equivalence establishment were assayed. Among phenolic acids, the only consistent and significant ($p < 0.01$) results concerned ferulic acid that was decreased in both GM and GM + R diets by 16–30% in comparison to the control diet (889 ± 107, 735 ± 89 respectively vs control 1057 ± 127 mg/kg) and caffeic acid by 21–53% (17.5 ± 2.1, 10.3 ± 1.3 vs control 22.1 ± 2.6 mg/kg).

For biochemical measurements in rats, statistical analysis was performed on the results obtained from samples taken at the 15th month time point, as this was the last sampling time when most animals were still alive (in treated groups 90% males, 94% females, and 100% controls). OPLS-DA 2-class models were built between each treated group per sex and controls. Only models with an explained variance $R^2(Y) \geqslant 80\%$, and a cross-validated predictive ability $Q^2(Y) \geqslant 60\%$, were used for selection of the discriminant variables (Fig. 5A), when their regression coefficients were significant at 99% confidence level. Thus, in treated females, kidney failures appeared at the biochemical level (82% of the total disrupted parameters). Ions (Na, Cl) or urea increased in urine. Accordingly, the same ions decreased in serum (Fig. 5B) as did the levels of P, K and Ca. Creatinine or clairance decreased in urine for all treatment groups in comparison to female controls (Table 3). In GM maize treated males (with or without R), 87% of discriminant variables were kidney related, but the disrupted profiles were less obvious because of advanced chronic nephropathies and deaths. In summary, for all treatments and both sexes, 76% of the discriminant variables versus controls were kidney related.

Moreover, in females (Table 3) the androgen/estrogen balance in serum was modified by GM maize and R treatments (at least 95% confidence level, Fig. 5B), and for male animals at the highest R-treatment dose, levels of estrogens were more than doubled.

4. Discussion

This report describes the first life-long rodent (rat) feeding study investigating possible toxic effects rising from an R-tolerant

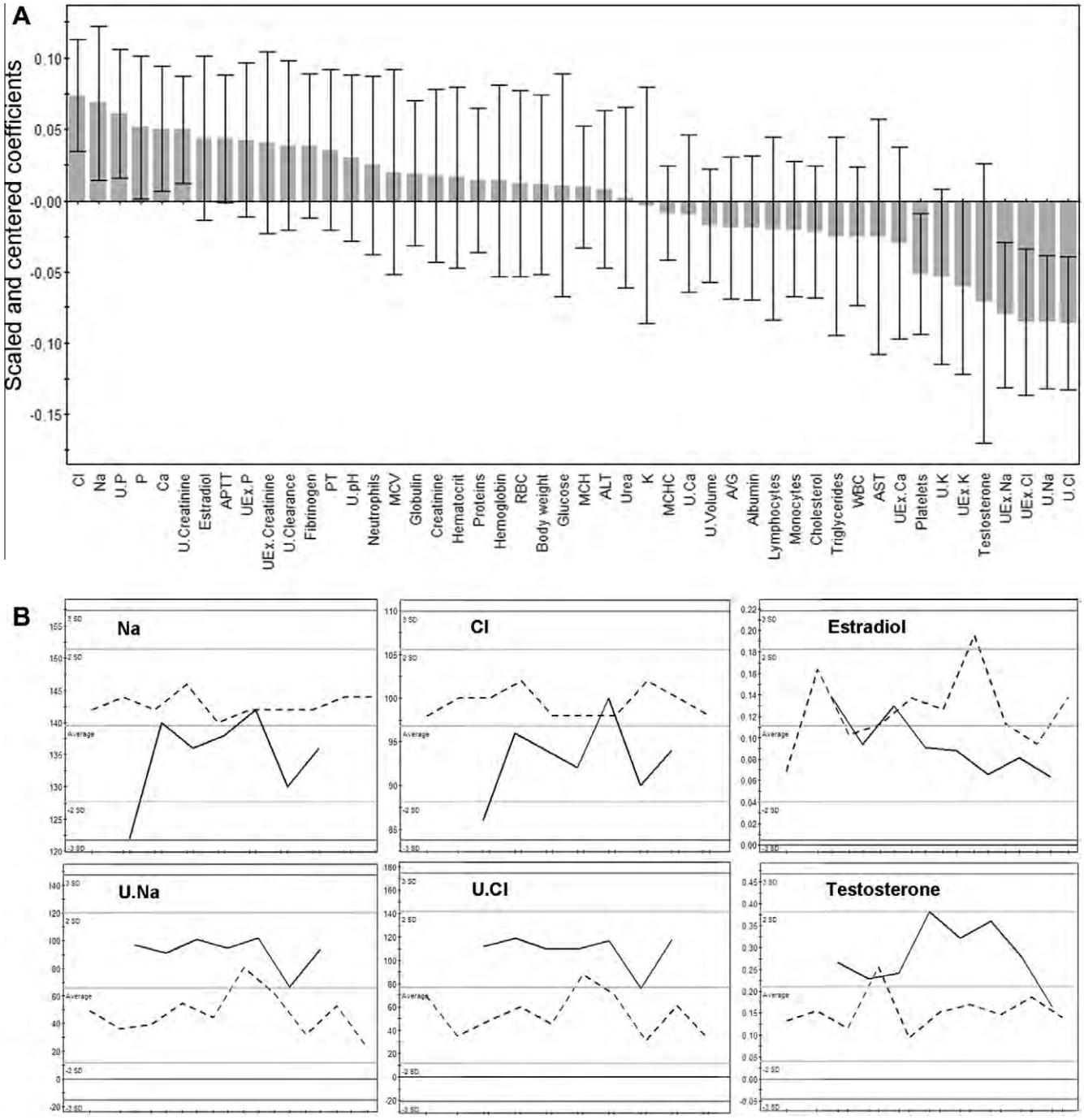

Fig. 5. Orthogonal Partial Least Squares-Discriminant Analysis (OPLS-DA) for biochemical data (females fed 33% GMO versus controls). (A) OPLS-DA regression coefficients for predictive component, with jack-knifed confidence intervals at 99% confidence level, indicate discriminant parameters versus controls at month 15 (Abbreviations: U Urinary, UEx Excreted in urine during 24 h, APPT Activated Partial Thromboplastin Time, MCV Mean Corpuscular Volume, PT Prothrombine Time, RBC Red Blood Cells, ALT ALanine aminoTransferase, MCHC Mean Corpuscular Hemoglobin Concentration, A/G Albumin/Globulin ratio, WBC White Blood Cells, AST aspartate aminotransferase). (B) In this case, detailed examples of significant discriminant variables distribution between females fed 33% GMO (bold line) and controls (dotted line). On *x* axis: animals; on *y* axis: serum or urine biochemical values for Na, Cl, estradiol, testosterone. Profiles evidence kidney ion leakages and sex hormonal imbalance versus controls.

GM maize (NK603) and a complete commercial formulation of R-herbicide.

Our data show that, as is often the case for hormonal diseases, most observed effects in this study were not proportional to the dose of the treatment (GM maize with and without R application; R alone), non-monotonic and with a threshold effect (Vandenberg et al., 2012). Similar degrees of pathological symptoms were noticed in this study to occur from the lowest to the highest doses suggesting a threshold effect. This corresponds to levels likely to arise from consumption or environmental exposure, such as either 11% GM maize in food, or 50 ng/L of glyphosate in R-formulation as can be found in some contaminated drinking tap waters, and which fall within authorized limits.

The lifespan of the control group of animals corresponded to the mean rat lifespan, but as is frequently the case with most mammals including humans (WHO, 2012), males on average died before females, except for some female treatment groups. All treatments in both sexes enhanced large tumor incidence by 2–3-fold in com-

Table 3
Percentage variation of parameters indicating kidney failures of female animals.

Discriminant variables		GMO 11% + R	GMO 22% + R	GMO 33% + R	GMO 11%	GMO 22%	GMO 33%	R (A)	R (B)	R (C)
Urinary decrease	Clairance	−4	−11	−20	**−20**	**−20**	−19	**−20**	**−24**	**−40**
	Creatinine	−5	**−32**	**−37**	−19	−37	**−36**	−43	−23	−1
	Creatinine ex	−5	−11	**−19**	−18	**−17**	−21	**−21**	**−22**	**−39**
Urinary increase	Urea	12	**18**	15	15	12	−1	0	13	**32**
	Na	25	33	30	52	−2	**95**	**62**	65	**91**
	Na ex	24	50	68	50	24	**125**	**108**	51	7
	Cl	14	35	28	46	5	**101**	**67**	56	**94**
	Cl ex	20	63	70	51	31	**138**	**121**	48	13
Serum decrease	Na	2	1	1	−1	**−4**	**−6**	−7	0	−3
	Cl	−1	−2	−2	−5	−7	**−6**	**−8**	−1	−4
	P	−6	−11	−13	−17	**−18**	**−20**	**−32**	−9	−13
	K	4	5	10	2	−4	0	−4	8	**−5**
	Ca	4	3	3	**2**	−2	**−5**	−6	3	**−6**
Gonads	Estradiol	8	−1	2	5	−2	−25	−26	**−73**	39
	Testosterone	5	−9	27	**56**	17	81	**97**	**−72**	10

OPLS-DA was performed on 48 variables at month 15. Here we showed mean differences (%) of variables (discriminant at 99% confidence level, in bold character) indicating kidney parameters of female animals, together with sex hormones. Male kidney pathologies are already illustrated in Table 2.

parison to our controls but also for the number of mammary tumors in comparison to the same Harlan Sprague Dawley strain (Brix et al., 2005), and overall around 3-fold in comparison to the largest study with 1329 Sprague Dawley female rats (Chandra et al., 1992). In our study the tumors also developed considerably faster than the controls, even though the majority of tumors were observed after 18 months. The first large detectable tumors occurred at 4 and 7 months into the study in males and females respectively, underlining the inadequacy of the standard 90 day feeding trials for evaluating GM crop and food toxicity (Séralini et al., 2011).

Suffering inducing euthanasia and deaths corresponded mostly in females to the development of large mammary tumors. These appeared to be clearly related to the various treatments when compared to the control groups. These tumors are generally known to be mostly estrogen-dependent (Harvell et al., 2000). We observed a strikingly marked induction of mammary tumors by R alone, a major formulated pesticide, even at the very lowest dose administered. R has been shown to disrupt aromatase which synthesizes estrogens (Richard et al., 2005), but to also interfere with estrogen and androgen receptors in cells (Gasnier et al., 2009). In addition, R appears to be a sex endocrine disruptor *in vivo*, also in males (Romano et al., 2010). Sex steroids are also modified in treated rats. These hormone-dependent phenomena are confirmed by enhanced pituitary dysfunction in treated females. An estrogen modified feedback mechanism may act at this level (Popovics et al., 2011; Walf and Frye, 2010). The similar pathological profiles provoked by the GM maize containing R residues may thus be explained at least by R residues themselves, knowing that the medium dose of the R treatment corresponds to acceptable levels of this pesticide residues in GMOs.

Interestingly, in the groups of animals fed with the NK603 without R application, similar effects with respect to enhanced tumor incidence and mortality rates were observed. A possible explanation for this finding is the production of specific compound(s) in the GM feed that are either directly toxic and/or cause the inhibition of pathways that in turn generate chronic toxic effects. This is despite the fact that the variety of GM maize used is this study was judged by industry and regulators as being substantially equivalent to the corresponding non-GM closest isogenic line. As the total chemical composition of the GM maize cannot be measured in details, the use of substantial equivalence is insufficient to highlight potential unknown toxins and therefore cannot replace long-term animal feeding trials for GMOs. A cause of the effects of the effects could be that the NK603 GM maize used in this study is engineered to overexpress a modified version of the *Agrobacterium tumefaciens* 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) (Hammond et al., 2004) allowing the R tolerance. The modified EPSPS is not inhibited by glyphosate by contrast to the wild enzyme. This enzyme is known to drive the first step of aromatic amino acid biosynthesis in the plant shikimate pathway; in addition estrogenic isoflavones and their glycosides are also products of this pathway (Duke et al., 2003). They were not disturbed in our study. By contrast, the levels of caffeic and ferulic acids in the GM diets, which are also secondary metabolites from this pathway, but not always measured in regulatory tests, are significantly reduced. This may lower their protective effects against carcinogenesis and even mammalian tumors (Kuenzig et al., 1984; Baskaran et al., 2010). Moreover, these phenolic acids and in particular ferulic acid may modulate estrogen receptors or the estrogenic pathway in mammalian cells (Chang et al., 2006). This does not exclude the action of other unknown metabolites. This explanation also corresponds to the fact that the observed effects of NK603 and R are not additive and reached a threshold. This implies that both the NK603 maize and R may cause hormonal disturbances in the same biochemical and physiological pathway.

As expected, mammary tumors in males occurred far less frequently than in females. Death in male rats was mostly due to the development of severe hepatorenal insufficiencies, confirming the first signs of toxicity observed in 90 day feeding trials with NK603 maize (Spiroux de Vendômois et al., 2009). In females, kidney ion leakages were evidenced at the biochemical levels at month 15, when severe nephropathies were evidenced in dead male animals afterwards, at the anatomopathological level. Early signs of toxicity at month 3 in kidney and liver were also observed for 19 edible GM crops containing pesticide residues (Séralini et al., 2011). As a matter of fact, only elderly male rats are sensitive to chronic progressive nephropathies (Hard and Khan, 2004). The disturbed kidney parameters may have been induced by the reduction of phenolic acids in our study, since caffeic and ferulic acids are beneficial in the kidney as they prevent oxidative stress (Srinivasan et al., 2005; U Rehman and Sultana, 2011). Accordingly, we previously demonstrated that plant extracts containing ferulic and caffeic acids were able to promote detoxification of embryonic kidney cells after R contamination (Gasnier et al., 2011). It is thus possible that NK603 consumption by reducing these compounds may well provoke an early aging of kidney physiology in this study, like R by oxidative stress.

Disturbances that we found to occur in the male liver are characteristic of a chronic intoxication, confirmed by alterations

in biochemical liver and kidney function parameters. The observation that liver function in female animals is less affected may be due to their physiology being better adapted to estrogen metabolism. Furthermore, liver enzymes have been clearly demonstrated as sex-specific in their expression patterns, including in a 90-day rat feeding trial of NK603 maize (Spiroux de Vendômois et al., 2009). However, in a long-term study, evidence of early liver aging was observed in female mice fed with R-tolerant GM soy (Malatesta et al., 2008a). In the present investigation, deeper analysis at an ultrastructural level revealed evidence of impediments in transcription and other defects in cell nuclear structure that were comparable in both sexes, and dose-dependent in hepatocytes in all treatments. This is consistent with the well-documented toxic effect of very low dilutions of R on apoptosis, mitochondrial function, and cell membrane degradation inducing necrosis of hepatocytes, and other cell lines (Benachour and Seralini, 2009; Benachour et al., 2007; Gasnier et al., 2010; Peixoto, 2005).

The disruptions of at least the estrogen-related pathways and/or enhancement of oxidative stress by all treatments need further investigations. This can be addressed through the application of transcriptomic, proteomic and metabolomic methods to analyze the molecular profiles of kidneys and livers, as well as the GM NK603 maize (Jiao et al., 2010; Zhou et al., 2009; Zolla et al., 2008). Other possible causes of observed pathogenic effects may be due to disturbed gene expression resulting from the transgene insertional, general mutagenic or metabolic effects (Latham et al., 2006; Wilson et al., 2006) as has been shown for MON810 GM maize (Rosati et al., 2008). A consequent disruption of general metabolism in the GMO cannot be excluded, which could lead, for example, to the production of other potentially active compounds such as miRNAs (Zhang et al., 2012) or leukotoxin diols (Markaverich et al., 2005).

In conclusion, it was previously known that glyphosate consumption in water above authorized limits may provoke hepatic and kidney failures (EPA). The results of the study presented here clearly demonstrate that lower levels of complete agricultural glyphosate herbicide formulations, at concentrations well below officially set safety limits, induce severe hormone-dependent mammary, hepatic and kidney disturbances. Similarly, disruption of biosynthetic pathways that may result from overexpression of the EPSPS transgene in the GM NK603 maize can give rise to comparable pathologies that may be linked to abnormal or unbalanced phenolic acids metabolites, or related compounds. Other mutagenic and metabolic effects of the edible GMO cannot be excluded. This will be the subject of future studies, including transgene and glyphosate presence in rat tissues. Reproductive and multigenerational studies will also provide novel insights into these problems. This study represents the first detailed documentation of long-term deleterious effects arising from the consumption of a GM R-tolerant maize and of R, the most used herbicide worldwide.

Altogether, the significant biochemical disturbances and physiological failures documented in this work confirm the pathological effects of these GMO and R treatments in both sexes, with different amplitudes. We propose that agricultural edible GMOs and formulated pesticides must be evaluated very carefully by long term studies to measure their potential toxic effects.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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Reply to letters to the editor

Answers to critics: Why there is a long term toxicity due to a Roundup-tolerant genetically modified maize and to a Roundup herbicide

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ABSTRACT

Our recent work (Séralini et al., 2012) remains to date the most detailed study involving the life-long consumption of an agricultural genetically modified organism (GMO). This is true especially for NK603 maize for which only a 90-day test for commercial release was previously conducted using the same rat strain (Hammond et al., 2004). It is also the first long term detailed research on mammals exposed to a highly diluted pesticide in its total formulation with adjuvants. This may explain why 75% of our first criticisms arising within a week, among publishing authors, come from plant biologists, some developing patents on GMOs, even if it was a toxicological paper on mammals, and from Monsanto Company who owns both the NK603 GM maize and Roundup herbicide (R). Our study has limits like any one, and here we carefully answer to all criticisms from agencies, consultants and scientists, that were sent to the Editor or to ourselves. At this level, a full debate is biased if the toxicity tests on mammals of NK603 and R obtained by Monsanto Company remain confidential and thus unavailable in an electronic format for the whole scientific community to conduct independent scrutiny of the raw data. In our article, the conclusions of long-term NK603 and Roundup toxicities came from the statistically highly discriminant findings at the biochemical level in treated groups in comparison to controls, because these findings do correspond in an blinded analysis to the pathologies observed in organs, that were in turn linked to the deaths by anatomopathologists. GM NK603 and R cannot be regarded as safe to date.

1. Introduction

Our recent publication of research evaluating the long term toxicity of a NK603 Roundup-tolerant genetically modified (GM) maize and of a Roundup (R) herbicide (Séralini et al., 2012) has provoked numerous positive and negative reactions throughout the world. This is the way science moves forward and here we provide a response to this intense debate. Our work is the most detailed study involving the life-long consumption of an agricultural genetically modified organism (GMO), and especially on NK603 for which only a 90-day safety test was previously conducted and using the same rat strain (Hammond et al., 2004). It is also the first long term detailed research on mammals exposed to a highly diluted pesticide in its total formulation with adjuvants. These adjuvants help to stabilize the active principles of pesticides, and promote a better penetration into organisms, and thus more side-effects. R is the most widely used herbicide in the world, which we tested from levels arising in tap water. Indeed in our study, its active principle glyphosate (G) was not studied alone, contrasting with the long term experiments conducted by the manufacturer as part of its application for regulatory approval. As such, the debate in question here is at the cornerstone of science and regulatory issues on this topic. This fact has major economic ramifications for the development of such products, which can explain the severe comments posted within hours of our publication becoming available online. This may explain why 75% of our first criticisms arising within a week, among publishing authors, come from plant biologists, some developing patents on GMOs, even if it was a toxicological paper on mammals, and from Monsanto Company who owns both the NK603 GM maize and R herbicide.

We must firstly focus on science. Our work is a research study; it has not a direct regulatory purpose and should not be considered as a final point in knowing the toxicological effects of NK603 and R. This is a first step in the iterative investigation of the long-term health effects on mammals of these commercial products that should be replicated independently, as well as on developing mammals. It has limits like any study, and here we carefully answer to all criticisms from agencies, consultants and scientists, that

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were sent to the Editor of *Food & Chemical Toxicology* or to ourselves. These challenged our results and the validity of our protocol, some letters even requested the withdrawal of the publication from the journal. All remarks and answers are summarized in Table 1 and with some explanatory details given below.

At this level, a full debate is biased if the toxicity tests on mammals of NK603 and R obtained by Monsanto Company remain confidential and thus unavailable for the scientific community to conduct independent scrutiny of their raw data. This is why, after several exchanges, we requested again from the European Food Safety Agency (EFSA) on September 20th and October 18th 2012 the release on a public website of the raw data on health risks on the basis of which commercialization of these products was granted, in particular results from the longest study of NK603 and Roundup on mammals (Hammond et al., 2004). We ask for a free and transparent exchange of scientific findings, mainly when these are related to public health and environmental risks (Schreider et al., 2010). Examination of industry raw data previously evidenced divergence of regulatory decisions from scientific evidence underestimating toxicological features of G (Antoniou et al., 2012). We recall that the tests on rats are usually considered as a model for mammalian health before clinical trials (for example for pharmaceuticals) or for a direct market release (for novel food and feed, pesticides or chemicals). Moreover, tests on rats are also models for environmental risk assessment, since they are models for other wildlife mammals. The public release of these raw data will reveal if significant differences observed between test and control groups in both studies are coherent and if the statistics are of sufficient power in both cases, thereby allowing the design of appropriate follow-up experiments by others, perhaps through a publically discussed and agreed protocol.

2. Relevance of the scientific context

Some remarks emphasize a lack of context, claiming that the study was performed for non-scientific reasons. The establishment of this protocol was however the consequence of an intense debate about the biological relevance of numerous statistically significant differences compared to controls revealed and admitted to in 90-day feeding studies with agricultural GMOs (Spiroux de Vendomois et al., 2010). This is highly controversial, with companies and regulatory agencies having refuted findings, which were validated by a peer reviewed process in international journals (EFSA, 2007; Séralini et al., 2007). Indeed, regulatory agencies such as EFSA appear to have their own criteria to judge the biological relevance of research findings (Doull et al., 2007), which is markedly at odds with some recent knowledge. This includes cases of sex specific non-linear endocrine disruptions, which were not admitted to as valid at a regulatory level although accepted at a scientific research level (Myers et al., 2009b). In order to overcome the divergence in biological interpretation of early signs of toxicity in blood biochemistry for GMOs, one solution was to prolong 90-day feeding tests to chronic periods. We therefore chose the R tolerant NK603 GM maize because R tolerance is the trait present in approximately 80% of agricultural GMOs (James, 2011) and because statistical differences in the 90-day feeding trial with this maize were admitted to by both the petitioner and regulatory agencies (EFSA, 2009).

3. Originality and limits of the experimental design

Due to the economic and regulatory issues of this topic, it is not surprising that our research study was confounded with pre-commercial regulatory assessments. This is why the most common criticism questions the following of Organization for Economic Co-operation and Development (OECD) guidelines. However, no guidelines exist for GMO toxicity studies in vivo, which are still not mandatory. Published reviews have confirmed that most of the studies conducted to date did not follow specific guidelines or were contradictory (Domingo, 2007; Domingo and Giné Bordonaba, 2011). We compared our design (Table 1 of Séralini et al., 2012) to Hammond et al. (2004) inspired from OECD guideline 408 for chemicals. We have replicated, extended and thus improved the experiments conducted by Hammond and colleagues (Hammond et al., 2004) by measuring outcomes from 3 instead of 2 feed doses and more crucially for a period 8 times longer in duration (90-days vs 2 years), with 11 blood and urine measures of around 50 parameters, 34 organs instead of 17, etc., in order to ascertain if the statistical findings (observed at 90 days; Hammond et al., 2004), were biologically relevant or not in the long term. We thus biochemically measured 10 rats per sex per group as performed by Monsanto. Even for a study of up to two years, we had no reason to monitor biochemical effects on more than 10 animals per sex per group as this is the number recommended in OECD guideline 452 for chronic toxicity testing (OECD 1981 was in application when the study started in 2008), even if 20 animals per group or more are possible.

The purpose of the addition of R treated groups was not to assess R long term carcinogenesis, which needs to follow OECD 453 guideline with at least 50 rats per sex per group (even if 10 rats are then still measured at a biochemical level). The aim of our study was to test R under similar conditions to the GM maize in order to try and understand if residues of R in the feed could explain the possible pathologies that may arise. There were two main potential sources of harm tested in our study: (i) effects from the GM maize itself, treated or not with R, and (ii) herbicide residues alone in drinking water, using 3 doses for each treatment. We recall that the initial investigation published by Hammond and colleagues (Hammond et al., 2004) used 2 doses for each treatment group despite that fact that 3 doses are recommended by OECD guideline 408, which they reported to have followed.

In addition, one of the criteria for biological relevance employed by Monsanto and other critics of our study is the linearity or lack thereof in response to the dose. Such a dose–response relationship cannot be claimed from a trial using only 2 doses of test material as employed in the initial NK603 assessment (Hammond et al., 2004). We therefore find it surprising that the relevance of Monsanto's and the agencies' conclusion of safety was not challenged due to such protocol insufficiencies. A recent review of the literature is often cited as a proof of the safety of GMO consumption on a long-term basis (Snell et al., 2012). However, of the 24 studies they evaluated, only 2 are long-term on rodents, since a 2 year feeding period with pigs or cows do not constitute a life-long experiments. The 2 rodent studies quoted by Snell and colleagues are from Sakamoto et al. (2008) where not all rats fed transgenic soy were analyzed, and Malatesta et al. (2008a) in mice fed again GM soy, which showed at an electronic microscopy level effects of this product on hepatic function. Moreover, of the 24 studies cited, 16 did not mention the use of the closest isogenic non-GM line as a control, many did not describe the methods in detail, and contained additional deficiencies (Snell et al., 2012). However, all these studies were accepted as proof of safety regardless of the inadequacies highlighted here. It would appear that conclusions of safety seem to need fewer requirements than conclusions of toxicity. However, scientifically it is easier to conclude an outcome of toxicity than safety. This was not the first time regulatory agencies used such double standards to minimize independent research findings in regard to industry findings (Hilbeck et al., 2012; Myers et al., 2009a). Our control groups were also questioned and this needs some clarification. Some claimed that controls are lacking for all 4 test groups (GMO+R and GMO alone at 11% and 22%). We compared

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Table 1
Summary of criticisms and responses on Séralini et al. long-term NK603 GM maize and Roundup toxicity rat study.

Criticisms	Answers
Relevance of the scientific context	
No scientific context	This study addresses biological interpretations of early signs of toxicity in biochemistry after 90-day feeding trials (Spiroux de Vendomois et al., 2010)
OECD guidelines not respected	No guidelines exist for GMO animal studies. Protocol based and adapted from OECD 408 and 452
Protocol not adapted to tumor findings	This is not a carcinogenesis study, but a long term full toxicological study
GLP violation because of amendments	Research protocols not adapted to GLP agreement because of amendments. The experiment was conducted under a GLP environment and conditions
History of flaw by the authors which are not toxicologists. Previous studies of the group rejected	More than 26 international scientific peer reviewed papers by the team with the lead author on the topic in the last 5 years, and 11 in toxicological journals on the same period only in PubMed. One author, Malatesta, has also published on GMO/pesticide health risks. None of the papers was considered as flawed by the scientific community. Regulatory agencies or Monsanto are not scientific peer reviewed journal systems
Lack of signs in 90 days	Statistical differences in biochemical parameters of liver and kidney function recognized by both industry and agencies
Not the first long term study	First chronic investigation with NK603 GM maize; others of two years in farm animals are not over the entire lifespan; the most detailed study for all agricultural GMOs and a formulated pesticide
Originality and limits of the experimental design	
Choice of the rat strain (sensitivity to mammary tumors and nephropathies in males)	Necessity to have sensitive strains, recommended by the US National Toxicology Program (King-Herbert et al. 2010). Rats and mice have been preferred experimental models because of their susceptibility to tumor induction (OECD guidelines) Relevant comparisons to controls in this work
Number of rats per group	OECD 408 (90-day toxicity study) 10 animals per group OECD 452 (Chronic toxicity study) 20 animals per group but at least 10 animals per group are studied for hematological and clinical biochemical function
Missing data: diet composition and process, PCR analysis of batches, contaminants (mycotoxins, pesticides), storage (R in water, BPA, feed), isogenic line, culture conditions	Normally included in GLP environment studies. No possibility to detail all these data in this scientific study in this journal – in process of publication. Diet equilibrated for substantial equivalence between GMO and the closest isogenic line and other compounds. Other points detailed in the text
No blinding, not the knowledge to interpret tumors, no morphometric analyses, no use of PETO codes, no classification	Independent and blinded analysis by GLP performed by professional regulatory anatomopathologists. Nature of most frequent tumors in Fig. 3 legend and results. A professional report for each rat indicates the cause of mortality
R formulations are different	Depends on the country
Controls not sufficient (number of rats per group, 4 groups 11 and 22%, no drinking water control group)	Number of rats approved in guidelines, best in the world at this level of details for these products. All the animals have eaten 33% of maize and substantially equivalent diets. Only R treated rats had received R in water
No reference groups, no lab historical data	Reference groups add irrelevant variability with non-substantially equivalent diets; historical data contain diets not controlled for pesticides and GMOs, thus not relevant
Ad Libitum feeding	In accordance to guidelines and usual practices
Diurnal variations	All samplings were taken at the same time
Focus on statistics	
Not enough statistical power No Kaplan Meier's curves Variability expected by chance Only raw data in Figs. 1–3 and Table 2	Statistics do not tell the truth, but may help in understanding results. The biological interpretations and the crossing of methodologies are the key. Enough and high statistical power for OPLS-DA, and this is why raw data only were presented in Figs. 1 and 2 and Table 2; no statistical power of Kaplan Meier's analyses for a conclusion demonstrating effects or no effects.
No means and standard deviations in Table 3	OPLS-DA is not a method to compare mean differences which were presented for understanding of biochemical measurements with highly discriminant parameters in bold
Pertinence of the results	
Missing data (Behavioral studies, ophthalmology, microbiology in feces and in infectious nodules, G in tissues, body and organ weights, feed and water consumptions, transgene in tissues, time effects)	All measures cannot be presented in one paper and will be the subject of other publications. The other analyses are not relevant for the conclusions presented
No isoflavones in maize	Testing the diets for phytoestrogens is relevant because the equilibrated diet (non-GM) contains other components
Phenolic acids in the normal range	Used as biomarkers indicative of change in the metabolism of the GMO. This does not exclude the presence of unknown toxic compounds
No incidence / severity Lack of histopathology data	Taken into account as indicated in the legend of Table 2 which consists in a summary of the most relevant data
Endocrine disruption not sufficiently supported	Convergent body of evidence stemming from mammary tumors, pituitary dysfunctions, histopathology and sex hormone biochemistry
Wilm's tumors are only of genetic origin	Promotion by pesticide exposure is plausible and as evidenced by gestational exposure described in the literature
Feeding state explains glycogen in electron microscopy	No difference in feed consumption; experience in the domain by M. Malatesta
Pictures of control rat not shown	Rats representative of each group shown, controls do not present tumors in majority during the experiment, pictures non necessary
Discussion: findings in regard with the contradictory hypotheses	
R is not a sex endocrine disruptor	This is still true at a regulatory but not at a scientific research level. R endocrine disrupting properties are described in vivo and in vitro (references in the text).

(*continued on next page*)

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Table 1 (*continued*)

Criticisms	Answers
	Regulatory classification should be in process
G is not toxic in two-year tests	G is never used alone in agriculture, but in formulations with G far more toxic than G alone; G tests are not relevant, we used R
G is close in structure to amino acids and surfactant exposure is as soap exposure	This is not supported by the scientific literature; the structural and activity comparisons are not scientifically relevant to predict with certainty toxicological effects or safety
No effects on farm animals and in human population of the USA	No epidemiology, no life-long experimental studies; farms animals are generally killed too young to show development of long term diseases. No traceability and labeling of GMOs in USA, no epidemiological survey can be performed
Sakamoto et al. 2008 not reported	This study does not use the same GMO (soy vs maize) and neither the same strain of rat. No effect for GM soy in F344 rats is claimed but does not imply the same for NK603 GM maize in SD rats
Raw data expected for our study	Raw data also expected for regulatory accepted tests for this GMO and this pesticide to scientifically discuss details
Ethical issues and deontology	
Maize illicitly grown	Not at all; grown and imported with appropriate authorizations
Animal welfare problems, a veterinarian would not authorize such tumor development	The work follows GLP conditions. All rats followed by veterinarians on the site, applying the rules of the ethical committee and guidelines
Conflicts of interests	No conflict for us. Conflicts of interests for companies testing their own products
Role of funders	See acknowledgments, funders identified. No interference in study or results; confidential up to the embargo
Publication released before for journalists	Everything was released on the same day (September 19th), in accordance with the conditions set by the FCT editorial board.
Confidentiality agreement unusual	The confidentiality of the work is a usual practice before embargo
The authors should alert agencies from the end of experiment instead of waiting for a publication	The publication and reviewing of the work is the guarantee of quality with no interference

all treated groups to the control group containing 33% of the closest available isogenic maize, as all diets were equilibrated to 33% maize; that is, for example the 11% GM maize diet was supplemented with non-GM control maize to reach 33%. More accurately the closest available isogenic line was the DKC2675 variety compared to the DKC2978 GM maize (NK603). Regulatory agencies also questioned the conditions under which the maize was grown. One R treatment was applied 4 months before harvest. Fungicides were applied similarly. We were unable to use the same R formulation in the field (Canada) and in the drinking water of the rats (France) because authorized formulations vary between nations. The diet was nutritionally equilibrated from substantially equivalent maize and was then checked by PCR for GMO content. A major concern was the potential presence of mycotoxins. Fumonisin B1 and B2, zearalenone, deoxynivalenol (DON), nivalenol, 3-acetyl-DON, 15-acetyl-DON, fusarenone X, T2 toxin, HT2 toxin and diacetoxyscirpenol were all under recommended limits in food/feed used in this study. We did not present details of each of these substances when no particular changes affecting the understanding of the results were noticed.

As a research protocol, Good Laboratory Practice (GLP, OECD, 1997; 2004/10/EC regulation) was followed, meaning that housing conditions, manufacturing process, diet composition and storage, stability of solutions and dietary contaminants were assessed by approved laboratories. Anatomopathology was performed in a blind manner (without knowing the treatments) by professional anatomopathologists approved for regulatory purposes. An electronic chip was inserted in each rat for identification. However, the technicians employed for the care and sampling of the animals did not know either the nature of the diets or the drinking water prepared independently, or which was the control group. The cages housing the animals were moved within holding rooms regularly and similarly for all animals. The blood (1 mL) and urine samples were coded and the measurement of biochemical parameters also blind, as were the decisions of euthanasia to avoid suffering in accordance with precise regulatory ethical rules (hemorrhages, impossibility to drink and eat, large tumors over 25% body weight because they provoke mortality). All the animals were monitored during the experiment by professional veterinarians. The statistical analysis was also undertaken on coded groups. However, we have made research amendments adding additional analyses (tissue and biochemical parameters) adapted to the findings in order to improve the understanding of the pathologies, thus we are only in a GLP environment. Generally, it is standard practice that a regulatory agency does not take note of research studies because they are not conducted under GLP conditions (Myers et al., 2009a). By its very nature, a research protocol is rarely compatible with GLP agreements. GLP agreement is a good tool to normalize regulatory assessment but research studies need a greater degree of freedom, in test protocols, models, etc.

4. Rat strain

We would like to explain the choice of the strain of rat. This is another redundant remark made by critics of our study design. We recall that OECD norms (408, 452 and 453) are not prescriptive for the strain of rat to be used. Sprague Dawley (SD) rats are subject to spontaneous neoplasms and this property is supposed to invalidate them being used as a model for carcinogenesis. However, on the contrary, the fact that the SD strain develops tumors, hence has led to it is preferentially used by some agencies such as for the National Toxicology Program using it for 2-year carcinogenicity and other long-term studies (King-Herbert et al., 2010). Indeed, it would be a non-sense to study pathologies in a strain insensitive to tumor formation. Long-term OECD guideline 452 even states that rats and mice have been preferred as experimental model systems because of their susceptibility to tumor induction. The same reasoning is used for chronic progressive nephropathies (CPN) developed by SD rats. The fact that the strain developed spontaneous CPN with age (Hard and Khan, 2004) does not invalidate the model as we looked at the difference in the chronology, age, number and severity of CPN in comparison to controls.

To assess the biological relevance of results, many authors make comparisons with historical data of control rats, either within the laboratory or the breeding company from which animals are

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sourced. However, this clearly greatly enhances control variability and heightens the risk of false negative findings (Cuffe, 2011). It is now established that this concept should be used with caution. There are several reasons for this. Control diets for rats are generally not monitored, neither for pesticides (Hayes, 2004), nor for chemicals leaching from cages or other environmental sources (Howdeshell et al., 2003). This artificially enhances background effects. The supplier even recognizes that their historical data come from rats potentially fed GMOs since this was not controlled for (Harlan communication), except in our experiment. Thus, it was not appropriate for us to use historical control data. This is also the reason why we did not use reference groups fed different non-substantially equivalent diets, as they increase the standard deviation of the control groups, hiding differential effects due to treatments.

Many non-relevant remarks have also been noticed. Among others, some criticized the use of *ad libitum* feed to explain the increase of tumor incidence. Guidelines on the design and conduct of chronic toxicity studies state that rodents should be fed and watered *ad libitum* (OECD, Guidance Document No. 116). The hormonal imbalances were criticized to be due to diurnal or cyclic variations. However, sampling was performed at the same time each day in the morning.

5. Focus on statistical analytical methods and outcomes

Statistics do not tell the truth, but may assist in our understanding of experimental outcomes. The biological interpretations and the crossing of methodologies are the key (Cooper and Kavlock, 1997). We have applied the most modern statistical methods (OPLS-DA, see below) for multivariate data analysis of approximately 50 parameters measured 11 times for 200 rats. This allowed, in a blinded manner, to obtain results significantly discriminant at 99% confidence levels. These discriminant biochemical markers were, for example in the case of sexual hormones (at 95% for females at month 15), when the differences in hormone-dependent tumor incidence with the control group began. Disability in pituitary function was characteristic of this second most affected organ as certified independently by the pathologists in a blinded manner in treated female groups in comparison to controls. Such a disturbance in hormonal function is known to elicit mammary tumors in rats with the pituitary being a target of endocrine disrupting chemicals (Wozniak et al., 2005). The pathologists employed in our study explained that most of the mortality in females resulted from tumors, which led to euthanasia independently of the grade of cancer. This is why we did not detail the grade of tumors in our research but with the cancerous nature of the major tumor growths described in our study (Fig. 3 legend and results section (Par. 3.2)). These observations together with microscopic analysis reinforced our conclusions.

We believe all this was more pertinent than the study of statistically non-powerful Kaplan–Meiers' curves on survival (because of the groups of 10 animals per sex dying progressively) that cannot allow any conclusion on mortality linked or not to the treatment. Taking into account these limits, we decided to be simply factual in our presentation and thus describe the chronology and incidence of tumors and deaths. In comparison, statisticians from agencies could evaluate the power of the statistical analyses of the tests conducted by Hammond et al. (2004), which gave a score of safety, and that were used for market release. For us, the power of statistics used in Hammond et al. (2004) is extremely low to conclude to safety.

In our study, case PLS-regression (Projections to Latent Structures by means of partial least squares) is of particular relevance because, unlike conventional multivariate data analytical methods, it can analyze data sets with variables more numerous than observations, which can be strongly correlated (Wold et al., 2001). In the case of Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) there is separation between the inter-group variation (represented on the predictive component) and the intra-group variation (variability of the samples, represented on the first Y-orthogonal components). OPLS-DA is thus not an appropriate method with which to compare mean differences. However, for providing biochemical understanding, we have presented and highlighted those in Table 3 of our study, with highly discriminant parameters in bold text. OPLS-DA renders it possible to identify which variables are responsible for the separation of the groups. For instance, we also indicate in Fig. 5B that estradiol and testosterone are significantly discriminant at 95% confidence levels in some groups (not at 99% like other parameters presented).

Moreover, the SIMCA-P (V12) software (UMETRICS AB Umea, Sweden) for the multivariate analysis of biochemical data uses a method of validation of models, which is a k-fold cross-validation. The $Q^2(Y)$ parameter which measures the predictive ability of the models is calculated according to this cross-validation method. Only valid models with a satisfactory predictive quality Q^2 index were retained for the selection of the discriminant variables (bold in figures, Table 3). Furthermore, all models retained are significant (CV-ANOVA test with p-value <5%). One of the authors of our paper (D.H.) previously used this method and published their results in international peer-reviewed journals (Ledauphin et al., 2010; Malzert-Freon et al., 2010a; Malzert-Freon et al., 2010b).

6. Pertinence of the results

The first major criticisms that were raised concerned the results and their format of presentation. A scientific publication is by necessity limited in figures/tables and only shows the data necessary to understand and discuss the conclusions. This is why behavioral studies, ophthalmology, microbiology in feces and in infectious nodules, G in tissues, body and organ weights, feed and water consumptions, transgene in tissues, time effects will be the subject of future publications. The inclusion of these data at this stage would neither add to the main message nor would it improve the understanding of this first publication. Indeed, the peer review process has controlled the logic of the body of data presented. Additional sets of results were included in the revision of the manuscript in response to issues raised by the reviewers prior to publication.

The second major criticism of the results is that we attached too much importance to findings related to mortality and tumor relative to their scientific significance. We are aware of the limitations of these findings as discussed above in relation to the statistical analysis undertaken. The body of evidence for our conclusions comes from the converging methodologies and data (see Focus on Statistics). The variability in rates of mortality can indeed, if looked at in isolation, arise in principle by chance. However, statistical analysis for Figs. 1 and 2 is not of sufficient power to conclude that this is the case or the contrary. This is why we have presented the raw data for these sets of observations. For instance, males presented up to 4 times (2 times of the mean) more large palpable tumors than controls, similarly to that observed in female animals. As these observations may represent a potential risk for the human population, this cannot simply be disregarded so rapidly with non-potent statistics. This is also why we emphasized statistically discriminant biochemical effects at the 15th month, when most of animals were still alive (in treated groups 90% males, 94% females, and 100% controls). The significantly discriminant biochemical markers disrupted do correspond to the organic markers linked to the pathologies in a blinded analysis for the pathologists, who

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in turn linked that to the deaths. The two nephroblastomas in GMO fed groups linked to premature deaths was criticized for bringing confusion to the results, because these tumors are often of embryonic and/or genetic origin. However, these tumors are also known to be promoted by pesticide exposure (Fear et al., 1998).

The summary of the major histopathological findings in Table 2 was subject to the same criticisms. In fact, we indicated the severities of the CPN and only marked or severe CPN were shown. Indeed, elderly rats are subject to CPN and taking into account all CPN could hide interesting and important differences. The power of statistics may be discussed as for Figs. 1 and 2. However, all these data need to be seen in the context of all the significant results presented in the paper, as previously underlined.

For the findings obtained from the electron microscopy analysis, it is important to compare our results with those reported previously. Several studies have shown ultrastructural abnormalities in the liver of mice fed with GM soy (Malatesta et al., 2002) and that this structural disturbance was reproduced by adding the herbicide R directly to rat hepatocytes (Malatesta et al., 2008b). We thus wanted to test if the same disruptions can be seen in the liver of the rats in our experiment. This was indeed the case, and furthermore these observations conform with ours and others published in vitro effects of R (Gasnier et al., 2010, 2011). Glycogen dispersion or appearance in lakes found by electron microscopy was attributed to the feeding state by some critics. However, differences in feed consumption were not observed during the course of our study. Not only appearance of glycogen in lakes was noticed, but also a reduced rate of transcription of mRNA and rRNA, which is not normally known to be due to the feeding state, but rather to a toxic insult. Ultrastructural patterns revealed by of electron microscopy were coherent with an increase in detoxifying activity in liver, and this is corroborated by differences in cytochrome enzyme activities.

A major gap in some toxicological assessments is the lack of measurements investigating endocrine disrupting effects (Birnbaum, 2012). As noted previously, the central dogma in toxicology is that effects vary linearly to dose. This is true for standard poison intoxication. However, toxins with endocrine disruptive properties can give response curves that are U, inverted U or J in shape and are frequently observed in the case of exposure to environmental pollutants (Vandenberg et al., 2012). Endocrine disturbance is supported by observations in human (Gasnier et al., 2009) and rat testicular cells for R residues (Clair et al., 2012). In our study it is demonstrated by statistically significant sex hormone imbalances and disabled pituitary function. Moreover, doses varied from 50 ng/L to 2.5 g/L of glyphosate in R; that is, a factor 50 million, from which we cannot expect linear effects with such a wide range of doses tested, characteristic of the range of different kinds of environmental exposures (tap water, GM food and feed, diluted agricultural use). The kidneys and liver are also sensitive to endocrine disruptors. As the two major detoxifying organs, containing cytochrome P450 or other enzymes involved in xenobiotic or sex steroid metabolism, they often react with steroid sex hormone and related compounds (Pascussi et al., 2008).

Last but not least, we have identified phenolic acids as potential biomarkers of metabolic disturbances in the GM diet. We have also measured isoflavones in the diet even though maize does not produce these compounds. Rats indeed did not eat only maize but also other plants in an equilibrated diet. Even OECD 452 guidelines on chronic toxicity ask for testing phytoestrogen content of the diet. Importantly, decrease in phenolic acids is a good indicator of change in the metabolism of the GMO that could in turn lead to a reduced protection against the pathologies observed in the animals fed the NK603 GM maize. However, this does not exclude the possibility of other toxic effects of the GMO alone, which have not been identified in the experiment.

7. Discussion

7.1. Findings in regard with the contradictory hypotheses

Critics have claimed that no argument exists for R to be a sex hormone endocrine disruptor, which is based on a review by Williams et al. (2000), where most of the studies cover G effects alone and not R. We wish to draw attention again to the fact that G is never used as such, but in formulations with other substances allowing toxicity, both of target and non-target species. This is extensively described for G-based herbicides, but also for other pesticides (Eddleston et al., 2012). This is why, in our opinion, all discussion of our study referring to testing of G alone is not relevant. Furthermore, we find it incomprehensible that non-scientific assertions justify R innocuousness by the structural homology of G with non-toxic amino acids. In addition adjuvants in the R formulation cannot be judged harmless by a comparison of their activity to soap. There is no scientific basis to use these assertions to predict with certainty toxicological effects or safety. The fact that G alone is neither a carcinogen nor an endocrine disruptor in regulatory tests is not a proof of the safety of whole R formulations, especially when some formulations contained toxic compounds (Cox, 2004). The unexpected finding of new active principles with human cell toxicity capabilities in G-based herbicides has challenged the relevance of testing G alone as the active principle in R (Mesnage et al., 2012). R has already been demonstrated to be an endocrine disruptor in vivo (Dallegrave et al., 2007; Oliveira et al., 2007; Romano et al., 2010, 2012) with the underlying mechanism understood in vitro.

Several studies have shown significant endocrine disrupting effects of R, such as decrease in progesterone production, decreased levels of Steroidogenic Acute Regulatory (StAR) mRNA production in MA-10 mouse Leydig cells (Walsh et al., 2000), decrease in aromatase mRNA and activity levels in JEG3 cells and placental and equine testicular microsomes (Richard et al., 2005; Benachour et al., 2007), inhibition of transcriptional activities of androgens and of both α- and β-estrogen receptors in cells (Gasnier et al., 2009), and a decrease in testosterone production in rat Leydig cells (Clair et al., 2012). All these studies reinforce the biological relevance of our findings.

Some critics have emphasized that no adverse effects have been reported on either farm animals or in the human population of the USA who have consumed an unknown mixture GMO crop derived food. Such claims are scientifically unsound for the following reasons. First, it is important to note that there have been neither epidemiological studies of the human population nor monitoring of farm animals in an attempt to correlate any ill-health observed with the consumption of a given GM crop. Second, it should be recalled that farm animals are not reared to live for the entire duration of their natural lifespan, and thus usually do not live long enough to develop long-term chronic diseases, which contrasts with the rats in our life-long experiment. If any studies in lactating cows are conducted, biological analyses performed are far less complete than those done in regulatory tests using rodents including in our study. Third, as there is no labeling of GMO food and feed in the USA, the amount consumed is unknown, and no "control group" exists. Thus, without a clear traceability or labeling, no epidemiological survey can be performed.

7.2. Ethical issues

Many critics argue against our refusal to release all the raw data generated in our study. This is a very unusual request when we clearly stated that we plan several other papers out of this data set. Our study was not performed for regulatory purposes. How-

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ever, due to the social impact and for full scientific understanding of the potential risks associated NK603 GM maize and R, we will release our raw data if the regulatory agencies that have taken industry data into account in their approval of their products also release the data pertinent for environmental and health risk assessments, in particular their longest toxicological tests on mammals, as we have indicated in our correspondence with EFSA. As a first step to this end, we have communicated the raw data underlying the data presented in Figs. 1 and 2 to the French food safety agency (ANSES), and answered their questions on experimental design and results, including analysis of food composition and mycotoxin content, etc.

Most of the criticisms on the topic of ethical conduct relate to animal welfare, some thinking that we overpassed the threshold in size of tumors above which animals should be euthanized, with the purpose of taking shocking photographs. However, it should be recalled that in a GLP environment, animal welfare is of major concern and that we fully respected the threshold in tumor size before euthanasia. Pictures of every animal and organ were taken. We presented those related to the most observed pathologies, including those of a microscopic nature, for illustrative purposes in Fig. 3, with rats representative of each group.

Some critics raised concerns about the role of the funders of this work, and possible conflicts of interest. Of course, the funders neither played a role in the design and conduct of the experiment, and nor in its interpretation. The data remained confidential to the funders. We recall that in the regulatory assessment of GMOs, chemicals and medicines, tests are conducted by the applying companies themselves, often in their own laboratories. As a result, conflicts of interest exist in these cases. These are even not claimed by authors from the company defending the safety of the tested products (Hammond et al., 2012). Our study does not aim to request commercialization of a new product. In contrast, we wanted to estimate the health risk of these products. It is the most detailed test conducted to date that is also independent from biotechnology and pesticide companies. We encourage others to replicate such chronic experiments, with greater statistical power. What is now urgently required is for the burden of proof to be obtained experimentally by studies conducted independent from industry. This was recommended by regulatory agencies in France that have assessed our work, even though their objective is more to regulate products than to review research. GM NK603 and R cannot be regarded as safe to date.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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Glyphosate induces human breast cancer cells growth via estrogen receptors.

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Abstract

Glyphosate is an active ingredient of the most widely used herbicide and it is believed to be less toxic than other pesticides. However, several recent studies showed its potential adverse health effects to humans as it may be an endocrine disruptor. This study focuses on the effects of pure glyphosate on estrogen receptors (ERs) mediated transcriptional activity and their expressions. Glyphosate exerted proliferative effects only in human hormone-dependent breast cancer, T47D cells, but not in hormone-independent breast cancer, MDA-MB231 cells, at 10(-12) to 10(-6)M in estrogen withdrawal condition. The proliferative concentrations of glyphosate that induced the activation of estrogen response element (ERE) transcription activity were 5-13 fold of control in T47D-KBluc cells and this activation was inhibited by an estrogen antagonist, ICI 182780, indicating that the estrogenic activity of glyphosate was mediated via ERs. Furthermore, glyphosate also altered both ERα and β expression. These results indicated that low and environmentally relevant concentrations of glyphosate possessed estrogenic activity. Glyphosate-based herbicides are widely used for soybean cultivation, and our results also found that there was an additive estrogenic effect between glyphosate and genistein, a phytoestrogen in soybeans. However, these additive effects of glyphosate contamination in soybeans need further animal study.

KEYWORDS: Estrogenic effect, Genistein, Glyphosate, Human breast cancer, T47D, T47D-KBluc

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A three-year longitudinal study on the effects of a diet containing genetically modified Bt176 maize on the health status and performance of sheep

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Abstract

This study shows that a diet including insect-resistant Bt176 maize, fed to 53 ewes and their progeny for 3 years, did not have adverse effects on their health or performance and that no horizontal gene transfer to ruminal microorganisms or animal tissues was detected. No differences were observed regarding performance, reproductive traits, haematological parameters, antioxidant defences, lymphocyte proliferative capacity, phagocytosis and intracellular killing of macrophages, and ruminal microbial population characteristics between control and genetically modified (GM) maize-fed animals. Immune response to *Salmonella abortus ovis* vaccination was more efficient in GM maize fed sheep. No modifications of histological features of tissues were found; however, cytochemical analyses of ruminal epithelium by Ki67 staining provided evidence of proliferative activation of basal cells in all GM maize-fed ewes. Preliminary electron microscopy analyses of the liver and pancreas revealed smaller cell nuclei containing increased amounts of heterochromatin and perichromatin granules in GM maize-fed lambs. Meat protein content and water loss by cooking were slightly affected by the dietary treatment. No transgenic DNA was detected in tissues, blood, and ruminal fluid or ruminal bacteria. Longitudinal studies should be included in evaluation of food safety whenever possible and sheep may be a useful animal model for toxicological assessment.

Keywords: Bt176 maize; Sheep; Health status; Transgene detection

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1. Introduction

Genetically modified (GM) plants, as well as feed and food derived from these plants, have to undergo a risk assessment prior to market authorization in the EU. The particular requirements for risk assessment have been and still are particularly contested issues and only recently attempts have begun to specify requirements in greater detail (Kleter and Kuiper, 2002; König et al., 2004; Jank et al., 2005). A recent review of a number of applications that have been submitted and approved at the European level (Spök et al., 2005), suggests that there are shortcomings in the risk assessment approach, in particular with issues revolving around the concept of substantial equivalence.

The agronomic improvements afforded by genetic modification of crops have resulted in a dramatic increase in their use. Thus, as adoption of GM crops continues to increase, so will their consumption by animals reared for food. However, despite rigorous approval standards for transgenic plants, little is known about the fate of recombinant DNA following ingestion of GM crops by animals and exposure of the plant DNA to microorganisms within the digestive tract (Duggan et al., 2003; Einspanier et al., 2004). Scientific knowledge on horizontal gene transfer from plant cells to mammalian or bacterial cells is still incomplete (van den Eede et al., 2004). The ruminal microbial population is considered one of the most likely targets for natural transformation, when the diet contains GM organisms (GMO). There is a lack of long-term studies, performed on a high number of animals over several generations, aimed at evaluating the effects of genetically modified (GM) feeds on livestock species.

In this context, a 3-year longitudinal study was carried out to evaluate possible effects of a diet containing GMO (Bt176 maize) on a group of meat sheep and their progeny. The study was aimed at assessing: (a) animal welfare and health status through periodic evaluation of metabolic and haemato-chemical profiles as well as immune response following vaccination; (b) performance and reproductive traits; (c) nutritional and organoleptic properties of meat; (d) the presence of transgenes in ruminal microorganisms and animal tissues.

2. Material and methods

2.1. Animals and feeds

One hundred and six Bergamasca × Appenninica ewes, aged approximately 10 months, were divided in 2 groups, treated (T) and control (C), balanced for body weight (BW: 36.9 (±0.9) vs. 37.1 (±1.2) kg) and body condition score (BCS: 1.9 (±0.03) vs. 1.9 (±0.04)) (Russel et al., 1969). During the first 8 months of the trial, animals were fed a diet based on non-GM mixed hay (crude protein 115.5 (±5.3) g/kg; crude fibre 323.0 (±6.1) g/kg), non-GM maize grains and a non-GM mineral-vitamin supplement ("PRE" period). For the following 36 months of the experiment, starting in May 2002 ("EXP" period), group T had the non-GM maize replaced with the maize hybrid NK COMPA CB (event Bt176). The amount of hay and maize varied according to physiological state (hay: 1700 to 2500 g/day; maize: 100 to 600 g/day, for dry period and lactation, respectively) (NRC, 1981). The animals had free access to water. Compositional analyses were conducted to measure proximate, fibre and mineral content (AOAC, 2000) of the maize (Table 1). Rumen degradability parameters (Ørskov and McDonald, 1979; McDonald, 1981) were assessed using 2 ruminal cannulated ewes (Table 1).

The sheep were subjected to natural mating in June 2002, November 2003 and August 2004 and presented

Table 1
Composition of maize lines (as-fed basis) used in the experiment

Item	Non-GM maize	SEM	GM maize	SEM	*P*-value
Chemical composition (g/kg)					
Dry matter	877.8	1.6	879.6	1.8	0.50
Ether extract	28.4	4.5	40.7	1.1	0.07
Ash	10.3	1.3	10.9	0.1	6.5
Crude protein	74.3	1.0	75.1	1.1	0.63
Neutral detergent fibre	97.4	0.4	82.7	2.2	<0.01
Acid detergent fibre	18.1	1.4	13.7	0.8	0.06
Acid detergent lignin	4.8	0.5	4.0	0.4	0.31
Starch	630.2	1.8	644.6	3.1	0.06
Minerals (μg/g)					
Calcium	383.80	118.63	157.25	91.05	0.18
Phosphorus	2352.10	208.20	2833.30	185.60	0.04
Magnesium	1088.30	26.83	1056.77	23.43	0.42
Zinc	19.92	1.42	16.89	0.81	0.09
Copper	3.74	0.21	2.69	0.20	<0.01
Iron	24.75	2.40	19.47	0.55	0.06
Manganese	7.80	0.55	7.03	0.62	0.37
Rumen dry matter degradation parameters					
ED (%)	80.50	1.66	81.12	1.66	0.80
Degradation rate (h^{-1})	0.06	0.006	0.06	0.006	0.65

ED: effective degradability at k=0.02 h^{-1} (where k=rate of outflow from the rumen).

normal pregnancies and deliveries. Fertility and twin rate, the lambs' BW at birth, the lambs' mortality and daily weight gain up to weaning (90 days of age) were evaluated.

Sheep BW and BCS were assessed at 180 day intervals. The lambs' diet before weaning was supplemented with a commercial mixture of non-GM steam-rolled cereals and faba bean (crude protein: 210.3 g/kg; crude fibre 57.6 g/kg), which progressively increased from 50 (30 days of age) to 300 g/day (60 days of age).

At weaning, 46 ewes and their lambs (14, 20 and 12 after the first, second and third lambing, respectively), equally distributed between the 2 experimental groups, were slaughtered approximately 12 h after feeding. Samples of ruminal contents, venous blood, gastrointestinal organs and muscles were collected and processed for chemical analyses and recombinant DNA fragment detection.

The present study was carried out in accordance with the guidelines of animal care and experimentation of the Istituto Zooprofilattico Sperimentale dell'Umbria e delle Marche.

2.2. Welfare status

Animals were periodically subjected to clinical examinations to assess cardiorespiratory and reproductive functions as well as motory, sensory and/or reflex changes.

Haematological analyses were conducted at 4-month intervals, starting 4 months before the end of the PRE period. Blood samples were taken in K3 EDTA vacutainer tubes to analyse the common haematological profile by a semi-automatic electric impedance analyser (Hemacomp 10, SEAC, Florence, Italy). Leukogram was determined through microscopic observation of blood smears stained using the May-Grunwald Giemsa technique. For the determination of biochemistry parameters, blood was collected in heparinized tubes. Plasma samples were stored at −20 °C prior to the following analyses: total protein, aspartate aminotransferase, alanine amino transferase, gamma glutamyltransferase, alkaline phosphatase, albumin, bilirubin, creatinkinase, glucose, calcium, chloride, potassium, sodium, urea nitrogen, creatinine, iron, magnesium, and phosphorus. Analyses were performed using standard enzymatic methods (Boehringer Mannheim GmbH, Mannheim, Germany) with a fully automated Hitachi 704 analyser (Hitachi Ltd., Tokyo, Japan).

Analyses for oxidative stress markers were conducted, as for haematological indexes, at 4-month intervals. Blood samples were taken in EDTA vacutainer tubes. The malonildialdeide content, end product of lipid peroxidation, was assayed in plasma by separating the malondialdehyde/thiobarbituric acid adduct by reverse-phase HPLC and quantified by using fluorescence detection (Draper et al., 1993). Total scavenger capacity of plasma was evaluated by ABTS (2,2′-azinobis-3-ethyl-benzothiazoline-6-sulphonic acid) test according to Rice-Evans and Miller (1994). Antioxidant defences were assessed in vitro by treating erythrocytes with 2 mmol/l *tert*-butyl-hydroperoxide incubation; BHT (0.1 mmol/l) was used to interrupt the peroxidative chain reaction at 1, 2, 5, and 10 min and the percentage of methaemoglobin, as haemoglobin oxidative product, was calculated (Winterbourn, 1990).

2.3. Immune response

The immunological response to vaccination against *Clostridium* spp. and *Salmonella abortus ovis* was evaluated by an optimized indirect ELISA assay. Two vaccine preparations without adjuvants were used. The first was composed of formaldehyde-inactivated (0.05% v/v) *C. perfringens* Type A, B, C and D, *C. septicum*, *C. chauvoei*, and *C. novy* A, at a total concentration of 3×10^9 CFU/ml with 20 LD_{50} mouse/ml toxins of *C. perfringens* and 2000 LD_{50} Guinea pig/ml toxins of *C. septicum*. The second one consisted of *S. abortus ovis* bacteria inactivated with formaldehyde (0.05% v/v) at a concentration of 1.8×10^9 CFU/ml. The time schedule of the experiment is reported in Fig. 1.

Blood samples were collected at 4-month periods from 7 animals per experimental group for cell-mediated immunity evaluation (Fig. 1). Peripheral blood mononuclear cells (PBMC) were obtained from EDTA blood by separating cells on Histopaque (Sigma, St. Louis, MO, USA). Monocytes were separated from lymphocytes by adherence overnight to plastic dishes and distributed into 24-well plates (10×10^6/well) for killing assessment or in trak tubes (5×10^6/tube) for phagocytosis study and cultured at 37 °C in 5% CO_2 for 10 days, at the end of which the monocytes had matured into macrophages (M/M). Phagocytic activity of M/M was assessed by using both latex fluorescent microparticles (Molecular Probes, Inc., Eugene, USA) and bacterial suspensions of *S. abortus ovis* (Antonelli et al., 1997). Latex beads and bacterial cells were pre-opsonized in RPMI medium, 10%-supplemented with a pool of sheep sera, by incubation at 37 °C for 30 min. Bacteria and beads were sonicated to disperse clumps and added to cellular monolayers, maintaining a 1:50 effector/target ratio. After 1 h incubation at 37 °C, 5% CO_2, the cultures were washed 3 times with HBSS to remove any extracellular nonphagocytosed microorganism or particle. Phagocytosis and the phagocytic index were

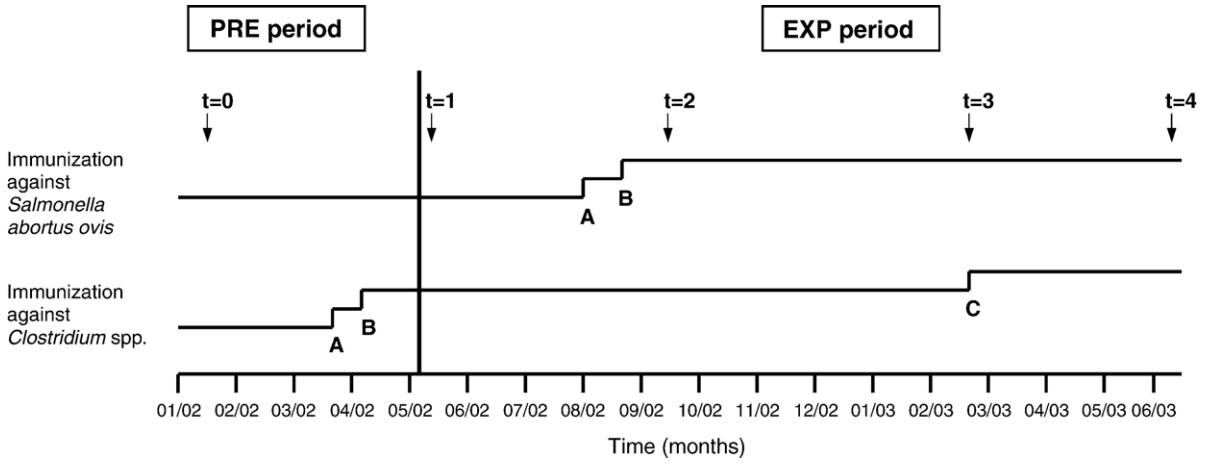

Fig. 1. Time schedule for the accomplishment of the different events of the experiment, referred as months of the year. Vaccination for *Clostridium* spp. and *Salmonella abortus ovis* are indicated with elevated lines, while sera samplings are indicated with arrowheads. PRE period=pre-experimental period, EXP period=experimental period with animals divided in 2 groups (control and GM-maize fed), A=I immunization – first dose, B=I immunization – booster dose, C=II immunization.

evaluated as previously reported (Miliotis, 1991; Roilides et al., 1990).

Intracellular killing was performed with *S. abortus ovis* grown under starvation conditions, preopsonized as described for phagocytosis tests and added to cellular monolayers at a 1/50 ratio. After 30 min incubation, to allow bacterial internalization by macrophages, the cultures were washed three times to eliminate extracellular microorganisms and processed as reported elsewhere (Roilides et al., 1990). The bactericidal activity was calculated as the percentage of killed intracellular bacteria after 60 min, with respect to time zero.

The proliferation capacity of lymphocytes was assessed by monitoring the incorporation of [^{3}H]thymidine into newly synthesized DNA after concanavalin A, phytohaemagglutinin, and pokeweed stimulation (Chiaradia et al., 2002).

2.4. Ruminal metabolism and microbial population

Samples obtained from the slaughtered ewes (n=46) were used. Immediately after slaughter, whole ruminal contents were mixed, the pH was measured and composite samples were collected for microbiological and chemical analyses. A sub-sample of ruminal contents (50 g) was anaerobically processed for total, amylolytic and cellulolytic bacterial numbers evaluation according to the MPN procedure (Dehority, 1969; Dehority et al., 1989). The amylolytic and cellulolytic sub-populations were chosen because adherent to feed particles and more likely to get in close proximity with the plant DNA. A second sub-sample of ruminal contents (50 ml) was processed for generic counts of protozoa (Dehority, 1984). A further sub-sample of ruminal contents was filtered through 4 layers of cheesecloth and then processed for chemical analyses as follows:

(a) Volatile fatty acids (VFA)—A gas chromatograph (Carlo Erba GC 6000 Vega Series 2, Milan, Italy), equipped with a flame ionization detector, was used. After filtration and acidic precipitation, samples were centrifuged and the liquid fraction was analysed in a 2 m×2 mm glass column i.d. packed with Carbograph 1 80–120 mesh, AL acid washed +4% Carbowax 20 M (Laboratori Analitici di Ricerca Associati, Rome, Italy).

b) NH_3-N—After filtration of ruminal contents and precipitation with 1 N cloridric acid, NH_3-N content was evaluated in the supernatant fluid using a colorimetric assay (Beecher and Whitten, 1970).

c) Lactate—Filtered ruminal contents were mixed with 8% perchloric acid in a 5:1 ratio and centrifuged at 2000×g for 15 min at 4 °C. Analyses on the supernatant fluid were carried out following a colorimetric method (L-Lactate PAP, Randox Laboratories Ltd., Co Antrim, UK).

2.5. Histological analyses

Twenty-six (13 C+13 T) ewes and 32 (16 C+16 T) lambs, distributed between first and second lambing, were used. Samples of liver, spleen, pancreas, duodenum, cecal appendix, mesenteric lymph nodes, rumen and abomasum were fixed in a 10% neutral buffered

formalin solution and routinely embedded in paraffin wax. For histological evaluations, 4 μm-thick sections were stained with haematoxylin–eosin and observed with an Olympus BX51 (Hamburg, Germany) light microscope.

In addition, the ewes' ruminal epithelium from the dorsal sac was submitted to immunohistochemical procedures using a monoclonal antibody against the Ki-67 nuclear protein, a proliferating marker (Gerdes et al., 1984). Four μm-thick tissue sections were deparaffinised, rehydrated, treated with 0.3% H_2O_2–methanol (v/v) and then microwaved in 10 mmol/l citrate buffer. The antibody for Ki-67 protein (Clone MIBI, Dakocytomation, Denmark) was incubated with the sections overnight at 4 °C. Detection of immunoreactive staining was carried out by streptavidin–biotinilated–peroxidase complex using the LSAB kit (Dako, Copenhagen, Denmark). Finally, 3.3 diaminobenzidine was used as the chromogen and Mayer's haematoxylin for counterstaining. Positive controls consisted of normal lymph node tissue, while negative controls were obtained by omitting the primary antibody. Scoring of immuno-reactivity was assessed by counting the total number of positively stained basal cell nuclei (40×) in 5 randomly selected sections per sample and values were expressed as the number of labelled cells/mm^2.

For transmission electron microscopy, samples of liver and pancreas from the lambs and ewes were processed (Malatesta et al., 2003). Ultrathin sections were stained with the EDTA technique (Bernhard, 1969) to visualize the ribonucleoprotein structural constituents and observed in a Zeiss EM 902 (Thornwood, NY, USA) electron microscope.

2.6. Meat quality

Samples of meat from 20 sheep (10 T and 10 C) and 14 lambs (7 T and 7 C), distributed between first and second lambing, were analysed 24 h following slaughtering. One hundred and fifty gram portions of *Longissimus dorsi* between the 10th and the 15th rib and *Biceps femoris* of each carcass were sampled and chilled for 24 h before analysis. Analyses for moisture, fat, protein and ash content (AOAC, 2000) and for pH, water loss by cooking and tenderness (AMSA, 1995) were carried out for each muscle sample.

2.7. Transgene detection

Transgene detection was performed on: (a) blood collected periodically from 20 ewes, at 4-month intervals, beginning 18 months after the start of the EXP period; (b) blood, liver, spleen, pancreas, jejunum and rumen sampled at slaughter from 12 ewes; (c) ruminal fluid collected from 24 ewes at slaughter and ruminal bacteria grown in liquid anaerobic media as described above. Samples were equally distributed between the group T and C and between first and second lambing.

a) *Animal tissues*—Tissue samples and blood with EDTA were quick-frozen and stored at −20 °C until analysis. Total DNA was extracted using a commercial kit for tissue and blood extraction (QIAamp® DNA Mini Kit, QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions. The yield and purity of the extracted DNA were determined following measurement of UV absorption at 260 and 280 nm. DNA extraction was shown using primers for amelogenin gene amplification. During each extraction procedure an extraction control (duplicate) was also carried out using only the same buffers/reagents. A pair of primers was used in PCR detection of transgene sequences: Cry03 (5′-CTCTCGCCGTTCATGTCCGT-3′) and Cry04 (5′-GGTCAGGCTCAGGCTGATGT-3′) (GenBank accession no. I41419). These primers amplify the last 73 bp region of the CDPK promoter and the first 138 bp of the N-terminus of the CryIA(b) (Hupfer et al., 1998). When assembling each PCR plate, a positive (5 μl of Bt176 maize) and a negative control (5 μl of water) were routinely set up. PCR was performed with a Thermal Mastercycler (Eppendorf) in a 25 μl final volume. Five microliters of each DNA sample were added to 20 μl containing 1× PCR reaction buffer, 1.5 mmol/l $MgCl_2$, 200 μmol/l dNTP (each), 0.6 μmol/l of each primer and 1 unit of AmpliTAQ Gold DNA Polymerase (Applied Biosystems, Foster, CA, USA). The amplification for transgene sequences was carried out under the following cycling conditions: 30 s at 95 °C, 30 s at 63 °C, and 30 s at 72 °C for 38 cycles and a final extension at 72 °C for 6 min. Amplification products (25 μl) were electrophoresed in 2% (w/v) Tris-borate-EDTA agarose gel stained with ethidium bromide.

b) *Ruminal fluid and bacteria*—Total DNA was extracted from the ruminal fluid as well as from the microorganisms grown in liquid media for total, amylolitic and cellulolytic bacterial species using Dynabeads® DNA DIRECT™ (Dynal Biotech, Oslo, Norway), following the manufacturer's instruction. PCR analyses were performed to reveal the presence or absence of two different fragments of the delta-endotoxin CryIA(b) using 2 pairs of primers: Cry1 (5′-ACCATCAACAGCCGCTACAACGACC-3′) and Cry2 (5′-TGGGGAACAGGCTCACGATGTCCAG-3′), designed to amplify a 184 bp fragment of transgene sequence (Hurst et al., 1995), and the

Cry03/Cry04 described above (Hupfer et al., 1998). To exclude false positive results, 2 intrinsic maize genes were assayed as a control for maize DNA contamination in the samples, using the primer pair IVR1/2 (IVR1 5′-CCGCTGTATCACAAGGGCTGGTACC-3′; IVR2 5′-GGAGCCCGTGTAGAGCATGAC-GATC-3′) as primer for the maize invertase gene (Chiter et al., 2000) and the F1/B1 (F1 5′-TACGGC AACAAGAAGTTCGAGAC-3′; B1 5′-AACATG GGCAGCTTCCACTGG-3′) designed to amplify a 200 bp fragment of the ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) small subunit, which is normally present in plant DNA (Hurst et al., 1995). The universal bacterial primer set 27f (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1525r (5′-AAGGAGGTGWTCCARCC-3′) were used for the amplification of a 1500 bp of the bacterial 16S rDNA to ensure the quality and suitability of the DNA extracts for PCR.

All PCR were performed with a GeneAmp PCR system 2700 thermocycler (Applied Biosystems, Foster, CA, USA) in a 25 µl final volume. Four microliters of each DNA sample was added to 21 µl containing 1× PCR reaction buffer, 2.5 mmol/l $MgCl_2$, 200 µmol/l (each) dNTP, 400 nmol/l of each primer, bovine serum albumin 200 ng/µl, and 0.625 U/reaction of Hot-Rescue DNA Polymerase (DIATHEVA s.r.l, Fano, Italy). The amplifications for transgene sequences and maize endogenous genes were carried out under the same cycling conditions: after 1 cycle at 95 °C for 10 min a 3-step PCR procedure, consisting of 20 s at 95 °C, 15 s at 64 °C and 20 s at 68 °C for 50 cycles and a final extension at 68 °C for 5 min, was used. Finally, 1/5 of the PCR mixture was subjected to an ulterior 50 PCR cycles. For the bacterial 16S rDNA amplification, the temperature program consisted of denaturation at 95 °C for 10 min, followed by 35 cycles consisting of 95 °C for 20 s, annealing at 60 °C for 15 s, and extension at 68 °C for 80 s and a final extension at 72 °C for 7 min. For each amplification, 10-fold dilutions (1:8 and 1:80 from extracted DNA) were tested to ensure the absence of PCR-inhibiting contaminants in the samples.

When assembling each PCR plate, a positive control (GM maize standard) and a negative control (water) were routinely set up. Amplification products (25 µl) were electrophoresed in 2% (w/v) Tris-borate-EDTA agarose gel stained with ethidium bromide. All the PCR products were cloned into the pCR 2.1 plasmid vector (Invitrogen, San Diego, CA), following the manufacturer's instructions and directly sequenced. The limit of detection of the PCR assay established, using negative control DNA, spiked with known quantities of plasmids containing the transgene or endogenous maize sequences, was 2 molecules (Fig. 2).

2.8. Statistical analysis

Statistical analyses were performed using the Statistical Analysis Systems statistical software package version 8.2 (SAS, 2001). Performance, reproductive traits and ruminal characteristics were evaluated by ANOVA using the GLM procedure. The main independent factors were dietary treatment (C and T), year of birth and sampling period. Bacterial numbers were log-transformed prior to analysis to satisfy the requirement for constant variance. For BW and BCS data of the adult animals, values obtained during the PRE period were used as a covariate.

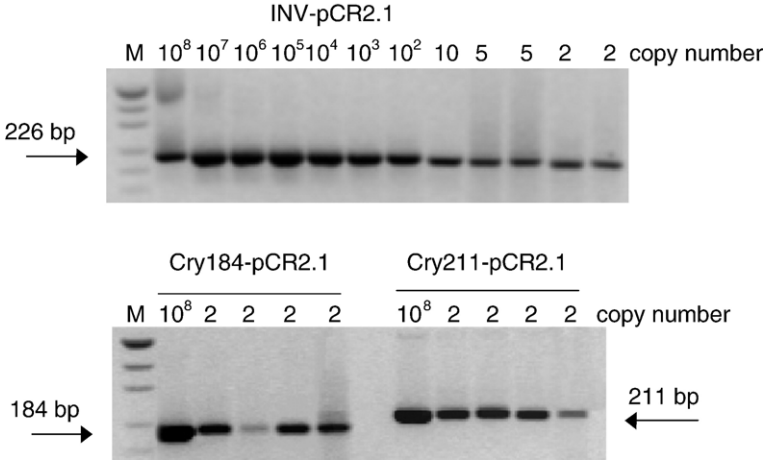

Fig. 2. Sensitivity assay. Negative control DNA was spiked with known quantities of each plasmid and subjected to PCR analyses. INV=maize invertase gene, Cry=Bt176 maize transgene sequences.

The lambs' BW was analysed by taking into consideration sex, dietary treatment (C and T) and year as fixed factors and BW at birth as a covariate. Interactions between factors were included if found significant at ANOVA.

A repeated measures design with sheep within diet as the error term, and diet and period as main effects, was carried out to assess the dietary effect on haematological parameters and oxidative stress markers. Multiple comparisons among treatments were by the Tukey test if significance had been indicated by the analysis of variance. As for the immune response to vaccination, absorbance values obtained for 1:100 serum dilution for *Clostridium* spp. ELISA assay and 1:500 for *S. abortus ovis* assay were used in an ANOVA model for repeated measures. Post hoc comparisons were performed using the Tukey least significant difference test. Cell-mediated immunity data were statistically analysed by the *t* test in case of equal variance assumption.

The histological data were analysed by an ANOVA model which included animal category (ewe and lamb) and dietary treatment (C and T) as main factors.

Factors considered in the ANOVA model for meat quality traits were dietary treatment (C and T), type of muscle (*L. dorsi* and *B. femoris*) and animal category (ewe and lamb). Interactions between factors were not included in the model because they were found not significant at ANOVA.

3. Results

3.1. Animals and feeds

The data in Table 1 indicate that the chemical composition and the ruminal degradability of the 2 maize hybrids were similar. Minor differences between lines were related to neutral detergent fibre, ether extracts,

Table 2
Effect of dietary treatment and sampling period on haematological parameters in ewes

Item	Dietary treatment				*P*-value		
	GM	SEM	Control	SEM	Dietary treatment	SP	INT
Albumin (mmol/l)	36.53	0.51	35.64	0.54	0.239	<0.001	0.006
Alk phos (U/l)	242.66	10.16	230.64	11.03	0.428	<0.001	0.629
ALT (U/l)	30.10	0.68	28.98	0.75	0.053	<0.001	0.040
AST (U/l)	111.72	2.45	107.05	2.64	0.202	<0.001	0.676
Bilirubin (μmol/l)	3.04	0.16	3.22	0.16	0.431	<0.001	0.177
Glucose (mmol/l)	2.47	0.05	2.42	0.05	0.545	<0.001	0.640
Urea nitrogen (mmol/l)	6.36	0.17	6.43	0.18	0.794	<0.001	<0.001
Creatinine (μmol/l)	90.54	1.24	91.16	1.36	0.735	<0.001	0.973
Total protein (g/l)	59.59	0.60	59.07	0.64	0.557	<0.001	0.211
GGT (U/l)	44.49	0.91	40.19	0.98	0.003	<0.001	0.440
Calcium (mmol/l)	2.46	0.03	2.42	0.03	0.307	<0.001	0.399
Chloride (mmol/l)	104.07	0.66	103.11	0.70	0.324	<0.001	0.028
Potassium (mmol/l)	5.98[a]	0.18	6.68[b]	0.20	0.012	<0.001	0.074
Sodium (mmol/l)	154.47	1.48	153.31	1.67	0.605	<0.001	<0.001
Iron (mmol/l)	24.49	0.39	23.79	0.42	0.236	<0.001	0.203
Magnesium (mmol/l)	0.91[a]	0.03	0.76 [b]	0.03	<0.001	<0.001	<0.001
Phosphorus (mmol/l)	1.73	0.04	1.75	0.05	0.848	<0.001	0.260
Haematocrit (%)	31.38	0.25	31.00	0.26	0.290	<0.001	0.023
Haemoglobin (g/dl)	10.92[a]	0.11	10.46[b]	0.11	0.005	<0.001	0.067
MCH (pg)	9.57	0.10	9.47	0.11	0.518	<0.001	0.084
MCHC (%)	30.89	0.19	31.16	0.19	0.323	<0.001	0.179
MCV (fl)	31.54	0.23	30.96	0.24	0.090	<0.001	<0.001
Platelet (10^3/μl)	195.44	5.77	179.08	6.18	0.060	<0.001	0.575
Basophil (%)	0.10	0.03	0.10	0.03	0.980	0.119	0.185
Eosinophil (%)	3.95	0.17	4.15	0.17	0.412	<0.001	0.067
Lymphocyte (%)	52.25	0.44	51.60	0.46	0.313	<0.001	0.030
Monocyte (%)	4.74	0.18	4.60	0.19	0.597	<0.001	0.322
Neutrophil (%)	41.95	0.54	40.56	0.57	0.085	<0.001	<0.001
RBC (10^6/ml)	11.33	0.12	11.22	0.13	0.564	<0.001	0.352
WBC (10^3/μl)	12.91	0.35	13.89	0.36	0.056	<0.001	0.002

SP = sampling period; INT = interaction between treatment and SP; Alk phos: alkaline phosphatase; ALT: alanine aminotransferase; AST: aspartate aminotransferase; GGT: gamma glutamyltransferase; MCH: mean corpuscular haemoglobin; MCHC: mean corpuscular haemoglobin concentration; MCV: mean corpuscular volume; RBC: red blood cells; WBC: white blood cells.
[a,b]Means values ($n = 327$) within a row with unlike superscript letters were significantly different ($P < 0.05$).

copper and iron contents. No differences were detected between the two groups of ewes, in all generations studied, as far as BW, BCS, fertility, and twin rate were concerned (62 (±1.0) vs. 62 (±1.0) kg, 2.8 (±0.04) vs. 2.8 (±0.05), 79 (±1.6) vs. 72 (±1.9) %, and 25 (±1.2) vs. 24 (±1.1) % for T and C ewes, respectively). The lambs' BW at birth, mortality and daily weight gain up to weaning (5 (±0.1) vs. 5 (±0.1) kg, 4 (±0.4) vs. 4 (±0.5) %, and 288 (±5.3) vs. 293 (±4.8) g, for T and C lambs, respectively) were not affected by dietary treatment.

3.2. Welfare status

Clinical examinations did not detect any alterations of cardiorespiratory and reproductive functions, or changes in motory, sensory and/or reflex behaviour, associated to dietary treatment.

Analysis of variance carried out on haematological findings and least square means of dietary treatments are presented in Table 2. All values, except for the percentage of basophil, were affected by the sampling period. Four (gamma glutamyltransferase, potassium, magnesium, and haemoglobin) of the 30 haematological traits evaluated were affected by diet.

Values recorded for malondialdehyde and in vitro oxidative stress did not show any significant effect due to dietary treatment. The sampling period was able to affect the oxidation of Hb to MetHb by *t*-BuOOH. Values obtained during lactation (second sampling period) were lower than those recorded at the end of the experiment.

Table 3
Statistical analysis of ELISA absorbance values

Phase of analysis*		*Clostridium* spp.		*Salmonella abortus ovis*	
		Mean	SEM	Mean	SEM
$t=0$	C	0.274	0.022	0.07809	0.014
	GM	0.285	0.019	0.07508	0.013
$t=1$	C	0.955[a]	0.068	–	
	GM	1.151[b]	0.059	–	
$t=2$	C	0.938	0.055	1.38	0.053
	GM	1.007	0.048	1.517	0.046
$t=3$	C	0.714	0.053	0.525[a]	0.054
	GM	0.839	0.046	0.729[b]	0.047
$t=4$	C	1.074	0.066	0.466[a]	0.045
	GM	1.119	0.058	0.639[b]	0.039

C: Control ewes; GM: GM maize-fed ewes.
[a,b]Within a phase of analysis, means values ($n=70$) in a column with unlike superscript letters were significantly different ($P<0.01$).
*Phases of analysis are: $t=0$, 3 months before immunization; $t=1$, first immunization against *Clostridium* spp.; $t=2$, immunization against *Salmonella abortus ovis*; $t=3$, second immunization against *Clostridium* spp.; $t=4$, 4 months after $t=3$.

Table 4
Rumen microbial numbers and metabolism as affected by dietary treatment

	Control	GM	SEM
Bacteria (n/ml, log)			
Total	9.03	9.05	0.08
Amylolytic	8.76	8.96	0.11
Cellulolytic	7.75	8.04	0.11
Protozoa			
Total (n/ml × 10^4)	16.06	14.65	1.70
Entodinium (%)	84.72	84.55	1.64
Diplodiniinae (%)	8.45	8.50	0.82
Isotricha (%)	1.87	2.17	0.40
Dasytricha (%)	3.08	2.58	0.62
Ophryoscolex (%)	1.88	2.20	0.72
pH	6.57	6.54	0.14
NH_3-N (mg/dl)	14.96	13.48	1.15
Lactate (mmol/l)	0.32	0.45	0.05
Volatile fatty acids			
Acetate (%)	58	57	0.9
Propionate (%)	23	24	1.0
Butyrate (%)	19	19	1.3

Values are means of 46 measurements. GM: GM maize-fed ewes.

3.3. Immune response

A low titer of anti-*Clostridium* spp. and anti-*S. abortus ovis* antibodies was detected by ELISA assays in the pre-immune sera ($t=0$) of sheep from both groups T and C. The statistical analysis showed that the dietary treatment played a highly significant role in the variations of anti-*S. abortus ovis* titer following vaccination ($P=0.008$). With respect to the anti-*Clostridium* spp. titer, a quasi-significant role of the dietary treatment ($P=0.077$) was detected. For both types of investigations, the mean absorbance values were higher in T animals in every phase of the study. In spite of this, the confidence intervals for differences of means showed significant differences only in $t=1$ sampling for clostridiosis and in $t=3$ and $t=4$ sampling for salmonellosis vaccination (Table 3).

As for cell-mediated immunity, M/M yields obtained at the established periods (4th, 8th, 12th and 16th month) from peripheral blood of both groups C and T were 2.20 (±0.3) % of the total mononucleated blood cells (Knoll, 2000). The number of phagocytosed bacteria per cell ranged from 3.29 (±1.68) to 5.67 (±0.11) for C sheep and from 3.83 (±2.58) to 6.07 (±0.56) for T sheep. Phagocytosis of latex beads resulted to be in a range from 5.82 (±1.78) to 10.9 (±3.3) particles for C and from 5.79 (±1.13) to 10.8 (±5.63) for T samples. Intracellular killing of *S. abortus ovis* resulted to be 18.53% in C sheep vs. 19.6% in T. However, when the data of the phagocytic index and killing obtained from C

and T samples at any sampling time were compared, the differences resulted to be not significant ($P>0.05$).

The response of T and B peripheral lymphocytes to 66 h of stimulation with ConA, PKW, or PHA was unaffected by diet (5 animals per group) and no differences were observed among the periods considered. The percentage of proliferation of the stimulated vs. resting (without mitogen) cells was extremely variable, ranging from 500 to up to 2000.

3.4. Ruminal metabolism and microbial population

Total, amylolytic and cellulolytic bacterial concentrations, as well as protozoal numbers and composition, did not differ between groups (Table 4). The ruminal metabolism indicators taken into consideration (pH, VFA, NH_3-N, and lactate) were not affected by dietary treatment.

3.5. Histological analyses

Light microscopy observations of liver, spleen, pancreas, duodenum, cecal appendix, mesenteric lymph nodes, rumen and abomasum sections did not reveal histological differences between groups C and T, for both sheep and lambs.

Immunocytochemical analyses of ruminal epithelium by Ki-67 staining provided evidence of the proliferative activation of basal cells in T ewes: the values of anti-Ki-67 labelled cells/mm^2 of ruminal epithelium were significantly higher in T animals than in the controls at all ages ($P<0.001$) (Fig. 3). Moreover, preliminary EM analyses of hepatocytes and pancreatic acinar cells revealed smaller, irregularly shaped cell nuclei containing increased amounts of heterochromatin and perichromatin granules (ribonucleoprotein structural components involved in transport and/or storage of already spliced pre-mRNA) (Fakan et al., 1984) in T lambs (Fig. 3).

3.6. Meat quality

Except for protein content (C: 20.3 (±0.2); T: 19.7 (±0.2); $P<0.05$) and water loss by cooking (C: 37.9 (±0.8); T: 40.3 (±0.7); $P<0.05$), the parameters examined were not affected by the dietary treatment. Other factors

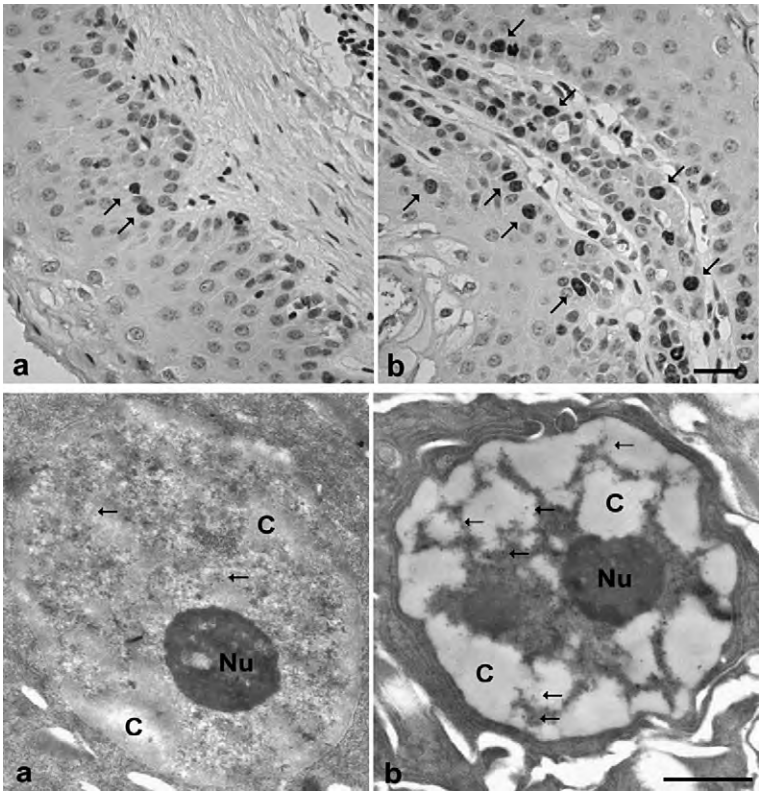

Fig. 3. Top: immunocytochemical detection of Ki-67 protein on control (a) and GM maize-fed (b) ewes. The number of immunolabelled cell nuclei (arrows) is markedly higher in the samples from GM maize-fed animals; bar=10 μm. Bottom: ultrastructural features of pancreatic acinar cell nuclei from control (a) and GM maize-fed (b) 3 month-old lambs. The cell nuclei from GM-fed animals show smaller size and larger amounts of heterochromatin (C) and perichromatin granules (arrows). Nu=nucleolus, bar=1 μm.

such as type of muscle (*L. dorsi*/*B. femoris*) and animal category (ewe/lamb) were found to be significant at ANOVA (data not shown).

3.7. Transgene detection

No transgenic DNA was detected in any of the tissue and blood samples taken from both C and T groups. While bacterial DNA was detected in all ruminal fluid and bacterial culture samples at all time points, neither transgenic maize nor intrinsic maize fragments were detected.

4. Discussion

The differences among the maize hybrids used in the study were so minor that they are unlikely to be of any biological significance. Trace mineral content of the two maize lines was possibly influenced by agronomic traits and geographical origin (Ipharraguerre et al., 2003). The relative lack of chemical composition differences between the Bt and non-Bt hybrid agrees with literature data (Sidhu et al., 2000; Folmer et al., 2002). On the contrary, the crude protein content of the Rh208 maize hybrid was found to be higher than that of its near isogenic line (Rh289) in a study where maize silage composition was examined (Barrière et al., 2001).

In this study, the animals' BW and BCS were not affected by treatment in all generations studied and were similar to that indicated in the literature for animals of the same genotype (Valusso et al., 1998) or physiological state (Bocquier et al., 1988). Other authors have shown the nutritional equivalence of transgenic maize fed to monogastric and poligastric animals, but there is a lack of information concerning the ovine species. Moreover, no multi-generational studies have been published so far. The absence of differences between transgenic maize Bt176 (Rh208Bt) and its near isogenic hybrid (Rh208), fed as silage to Texel ewes, was demonstrated as far as neutral detergent fibre, crude fibre and organic matter digestibility was concerned (Barrière et al., 2001). Similarly, no differences in either digestibility or energy content between Bt-maize and its conventional counterpart, used in a series of trials with poultry, pigs and wethers, were found (Aulrich et al., 2001).

Most of the haematological indexes examined in this study fell within the range normally observed in healthy ewes of similar age (Kaneko, 1989). At present, there is no plausible explanation for the differences found between the groups regarding gamma glutamyltransferase, potassium, magnesium, and haemoglobin. Other authors have reported no differences between the haematological and clinical serum parameters of rats fed with GM glyphosate-tolerant soybean meal and controls (Zhu et al., 2004). No effects on haematology and blood biochemical indexes between rats or mice, fed either GM sweet pepper or tomato diets, were found when compared with those fed the non-GM diets (Zhang-Liang et al., 2003). With reference to malondialdehyde and oxidative stress in vitro, results are indicative of a lack of effect by dietary treatment on the antioxidant defences of the animals. No data are available in the literature on this subject. Data from the immune response study indicated that GM maize did not impair two important functions of M/M, such as phagocytosis and intracellular killing. These findings are in agreement with the results obtained regarding the lymphocyte proliferative capacity. The immune response against *S. abortus-ovis* was more efficient in T animals; however, more extensive research is needed in order to reach definite conclusions on this aspect.

Values recorded for ruminal pH, VFA, NH_3-N, and lactate in samples obtained from both C and T groups were within the physiological ranges and compatible with the diet being fed at slaughter (third month of lactation, characterized by moderate supplementation with maize grain, i.e. 27% of total DM intake). Similar results were obtained with dairy cows fed glyphosate-tolerant soybean (Hammond et al., 1996). The lack of differences observed in protozoal and bacterial populations is in agreement with results indicated in previous studies (Einspanier et al., 2004). In a 4-week feeding experiment, during which Bt176 maize was fed to cows, no significant variations in the microbial population sampled from different organs of the gastrointestinal tract were reported.

The diet containing GM maize did not seem to affect the histological features of the examined tissues. However, cytochemical analyses revealed that functional modifications took place without inducing evident histological alterations. In fact, the basal cells of the ruminal epithelium of T ewes showed a higher expression of Ki-67 in comparison to controls. It is known that sheep rumen is sensitive to age and diet (Lane et al., 2000). The higher expression of Ki-67 in the ruminal cells was possibly induced by some components of the GM maize; it has been reported that Cry1 is able to bind the intestinal mucosal surface, influencing some epithelial cell functions (Vazquez-Padron et al., 2000).

The cell nuclear modifications observed in the pancreatic acinar cells and hepatocytes could also be due to some direct or indirect effect of Bt 176 maize. The changes in chromatin arrangement and ribonucleoprotein constituents observed in T animals are suggestive of functional modifications concerning transcriptional/

post-transcriptional events. However, the significance of this phenomenon is unclear and is presently under investigation. Fine structural modifications of cellular components in relation to GM feed intake have already been described, although without any consequences on organ functions or animal health (Fares and El-Sayed, 1998; Malatesta et al., 2002).

There are no available explanations that could support the effects of diet on the meat chemical traits observed in the present study and no data concerning the effects of GM maize on sheep meat quality traits are available in the scientific literature. Stanford et al. (2003), in a study involving Arcott lambs slaughtered at 45 kg BW, did not observe changes in carcass composition and characteristics except for the moisture content of loin tissue, which was higher in the group fed glyphosate-tolerant (Roundup Ready®) rapeseed compared to the controls. As for studies conducted with monogastrics, Hyun et al. (2004) compared glyphosate-tolerant Roundup Ready® (event NK603) corn with its nontransgenic genetically similar control corn (RX670) in an experiment carried out with barrows and gilts and found no differences in muscle quality traits, composition measurements and *L. dorsi* colour and marbling scores. In a 38-d feeding trial with broiler chickens, in which Bt176 maize was compared with a non-Bt counterpart, minor differences on feed conversion ratio, breast skin, and *Pectoralis minor* yield, not directly associated to dietary treatment, were reported (Brake and Vlachos, 1998).

The present transgene detection study only focused on the presence or absence of the amplicon resulting from the last 73 bp of the CDPK promoter and the first 138 bp of the N-terminus of the CryIA(b) gene. These results are in agreement with most data from the literature, that do not report the presence of transgenic DNA fragments in tissues of either mono- and poligastric animals. In contrast, Mazza et al. (2005) detected a small fragment of the CryIA(b) gene in blood, liver, spleen and kidney of piglets fed for 35 day with a diet containing GM (MON810) maize. Sharma et al. (2006) reported the presence of fragments of the *cp4 epsps* transgene in the gastrointestinal tissues of sheep and pigs fed Roundup Ready® rapeseed. It has been assumed that, when compared with non-GM plant DNA, the exposure to DNA of GMO material is negligible. Most plant DNA is likely to be degraded by DNase activity within the gastrointestinal tract. Furthermore, low pH conditions of the abomasum contribute to denaturize most adenine and guanine bases from naked DNA fragments (Beever and Kemp, 2000).

Although it is not possible to conclusively prove that transgene sequences were not transferred to the ruminal bacterial species, uptake of transgenic DNA fragments is probably precluded or time-limited by rapid degradation of plant DNA upon plant cell lysis (Sharma et al., 2004). Plasmid DNA exposed to ovine saliva in vitro was able to transform competent *Escherichia coli* cells to ampycillin resistance even after 24 h (Duggan et al., 2000). In contrast, free maize chromosomal DNA was rapidly destroyed within 1 min of incubation in ruminal fluid or silage effluent (Duggan et al., 2000). Stability of DNA fragments in the gastrointestinal tract can be affected by the type of feed being used, with processed feedstuffs being more degradable than the untreated grains (Chiter et al., 2000). Fragments of the Rubisco gene were found in bovine digesta samples obtained from the rumen and duodenum and from faeces and milk, but single-copy genes such as cp4epsps and Cry1A(b), from GM-soybean and maize, were only detected in the solid phase of ruminal and duodenal digesta (Phipps et al., 2003). These data are consistent with the results obtained in the current study, where no transgenic DNA was detected in rumen fluid. To our knowledge, the only evidence of gene transfer from GM-soya to gut microorganisms was reported in mixed cultures of human intestinal bacteria (Netherwood et al., 2005).

5. Conclusions

Overall welfare status indexes could confirm substantial equivalence with conventional hybrid as far as nutritional and safety characteristics are concerned. These results were partly expected, considering the genetic transformation involved, and in agreement with previous studies conducted in other monogastric and poligastric species (Aumaitre et al., 2002).

Our findings confirm that transgenes from maize are unlikely to survive in the ruminant prestomachs for 12 h or longer and provide a source of transforming DNA fragments for microorganisms. No transgenic DNA was found in the animals' tissues, which supports the opinion that intact genes from foods are unlikely to be absorbed through the gastrointestinal tract and integrated into the DNA of eukaryotic cells (Schubbert et al., 1997, 1998; Hohlweg and Doerfler, 2001; Einspanier et al., 2001).

However, more extensive research is needed to clarify some of the metabolic aspects under investigation, to improve safety assessments for GM organisms used for feed and food. In particular, the cytochemical modifications of the gastrointestinal organs and the immune response mechanisms that take place in GM-fed animals should deserve special emphasis and priority in future investigations.

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Genetically modified soya bean in rabbit feeding: detection of DNA fragments and evaluation of metabolic effects by enzymatic analysis

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Abstract

The presence of DNA fragments in tissues from rabbits given genetically modified (GM) soya-bean meal (solvent extracted) was investigated by using the polymerase chain reaction (PCR) approach. Moreover, the possible effects on cell metabolism were evaluated by determination of several specific enzymes in serum, heart, skeletal muscle, liver and kidney. The chloroplast sequence for tRNA Leu by using the Clor1/Clor2 primers designed on chloroplast trnL sequence was clearly detected. On the contrary, two couples of species specific primers for conventional (Le1-5/Le 1-3 which amplifies the soya bean lectin gene) and genetically modified (35S1/35S2 which amplifies the 35S CMV promoter that is present in the genomic structure of GM soya bean) soya bean were not found in all samples. No differences in enzyme levels were detected in serum, but a significant increase of lactic dehydrogenase, mainly concerning the LDH1 isoenzyme was found in particular in kidney and heart but not in the muscle, thus suggesting a potential alteration in the local production of the enzyme. Finally, no significant differences were detected concerning body weight, fresh organ weights and no sexual differences were detected.

Keywords: *genetic modification, polymerase chain reaction, rabbits, soya-bean oil meal.*

Introduction

Several genetically modified (GM) plants have been produced and approved by regulatory agencies worldwide for cultivation and commercialization. The insertion of new genes or the repression of endogenous gene expression can be in fact an useful tool to obtain specific characteristic which can lead to an improvement of agronomically relevant traits or food quality. Resistance to insects and tolerance to herbicides are the most recurrent agronomic traits modified in GM crops approved for feeding.

Nowadays, a number of GM plants have been approved for animal and human consumption but concerns over safety persist in the public. Allergenicity and toxicity, which can be related to novel foods are a major concern (HINO, 2002). Potential toxicological risks of a GM plant as whole food are evaluated on laboratory and target animals according to the classical methods used for drugs: blood and urine chemistry, organ weight and gross histo-pathological examination (Food and Agriculture Organisation-World Health Organisation, 2000; Organisation for Economic Co-operation and Development, 2003, novel foods OECD no. 9; European Food Safety Authority, 2004). It has also suggested finding specific biomarkers of early effects in order to increase diagnostic value and sensitivity of toxicity tests on food.

Animal nutritionists have evaluated in several studies the nutritional equivalence and the efficacy of the new feeds, in comparison with near isogenic or conventional varieties of plants (Aumaitre *et al.*, 2002; Cromwell *et al.*, 2002) and no direct evidence that GM plants may represent a possible danger for animal health has been reported so far (for a review, see Aumaitre (2004)). Another aspects that has been studied is the fate and integrity of forage plant DNA in the gastro-intestinal tract (GIT) of various animal models. Some authors have shown that highly fragmented plant DNA can be isolated from animal organs and tissues, thus suggesting that plant DNA is not completely degraded during animal digestion (Chowdhury *et al.*, 2003; Duggan *et al.*, 2003; Einspanier *et al.*, 2004).

The aims of this research have been the evaluation, by the polymerase chain reaction (PCR) approach, of the presence of plant DNA fragments in rabbit tissues to follow the fate of plant fed and the possible health effects of a GM diet by studying the activity of organ specific enzymes in rabbits.

Material and methods

Animal and diets

Twenty weaned 30-day-old New Zealand rabbits (10 males and 10 females) individually caged were equally assigned to control (C) and treated (T) groups. The animals were given (130 g/day) a diet constituted per kg of 800 g pelleted concentrate (165 g crude protein and 155 g crude fibre, as fed; dehydrated lucerne meal, sunflower meal, wheat, carob, soft wheat middlings, sugar-beet pulp, barley) and 200 g soya-bean meal (solved extracted) (SBM) which was from conventional or genetically modified (Roundup Ready®) beans, for group C and T, respectively. Roundup Ready® (RR) is tolerant to the glyphosate family of herbicides by expressing transgenic DNA from the CP4 strain of *Agrobacterium tumefaciens*, encoding 5-enolpyruvilshikimate-3-phosphate synthase protein (cp4 epsps).

The proximate composition of conventional and GM SBM was determined according to Association of Official Analytical Chemists (1990); the fibre fraction was analysed as suggested by Van Soest *et al.* (1991).

Water was given *ad libitum*. The rabbits were slaughtered at 70 ± 5 days of age (2 ± 0·2 kg live weight), food was available until 12 h before slaughtering. Body weights were taken before the onset of the experiment and immediately before slaughtering, organ weights were taken soon after slaughtering.

Sampling

Blood was withdrawn just before slaughtering and put in two different plastic tubes, with or without sodium citrate 9:1. Small pieces of liver, muscle, kidney and heart were washed in saline. All samples were stored at −20°C. As controls, conventional and transgenic SBM were used.

DNA extraction

Plant samples were extracted according to the Wizard extraction method (Promega, Madison, Wisconsin). One hundred milligrams of SBM were resuspended by careful vortexing in 860 μl of extraction buffer (10 mmol/l Tris HCl (pH 8·0), 150 mmol/l NaCl, 2 mmol/l EDTA, 1% (w/v) SDS), 100 μl guanidine hydrochloride (5 mol/l) and 40 μl of proteinase K (20 mg/ml). Samples were then incubated at 58°C for at least 3 h on a shaking incubator and then centrifuged at 20 000 **g** for 10 min. Five hundred microlitres of the supernatant were incubated with 5 μl RNase (10 mg/ml) at 37°C for 10 min. One millilitre of Wizard DNA Purification Resin (Promega) was added to the supernatant and mixed by gently inversion. A 2-ml syringe was mounted on the column and the mixture was pushed with the plunger through the column. The DNA-resin mixture was washed with 2 ml 80% (v/v) isopropyl alcohol following by centrifugation at 20 000 **g** for 1 min. After drying at 70°C for 10 min, the DNA was eluted with 50 μl of 70°C elution buffer (10 mmol/l tris HCl (pH 9·0), 0·1 mmol/l EDTA) and centrifuged at 20 000 **g** for 1 min.

Tissue (25 mg) and blood (200 μl) samples were extracted by using the 'nucleo-spin tissue' and 'blood-spin tissue' (Macherey-Nagel, Duren, Germany), respectively, according to users' manual. Briefly, 25 mg of ground tissue were incubated with 180 μl buffer T1 and 25 μl proteinase K solution at 56°C for at least 3 h on a shaking incubator. After digestion, the lysates were again incubated with 200 μl buffer B3 at 70°C for 10 min. About the blood samples, they were slowly defrosted (in ice-water bath) and then 200 μl of whole blood were incubated with 25 μl proteinase K solution and 200 μl lysis buffer B3 at 70°C for 15 min on a shaking incubator. To both samples (tissue and blood) were added 210 μl ethanol (96 to 100%), and all of the precipitate was loaded on the column placing into a 2 ml collecting tube and then centrifuged at 11 000 **g** for 1 min. The silica membrane was washed with 500 μl buffer BW and 600 μl buffer B5 following by centrifugation at 11 000 **g** for 1 min. After drying by centrifugation at 11 000 **g** for 1 min, the DNA was eluted with 100 μl pre-warmed elution buffer BE (70°C), incubating for 1 min, and centrifuged the column at 11 000 **g** for 1 min.

The DNA concentration was determined by measuring the UV absorption at 260 nm, then its quality was checked from 260/280 nm UV absorption ratios. All extracted DNA was stored at −20°C until used.

Primers

The quality of DNAs extracted has been checked in a PCR reaction with UNIV P/UNIV Q primers to amplify a conserved portion of animal mtDNA 16S rRNA gene (Sawyer *et al.*, 2003). Therefore, samples have been monitored for the presence of the chloroplast sequence for tRNA Leu by using the Clor1/Clor2 primers designed on chloroplast trnL sequence (Terzi *et al.*, 2004). Finally, two couples of species specific primers for conventional and GM soya bean were used: Le1-5/Le 1-3 which amplifies the soya-bean lectin gene (Kuribara *et al.*, 2002) and 35S1/35S2 which amplifies the 35S CMV promoter that is present in the genomic structure of GM soya bean (Lipp *et al.*, 1999). The sequence of all the primers is shown in Table 1.

Table 1 *Sequence, annealing temperature and amplicon size (bp) of primer pairs used in the study*

Primers	Sequence (5′ to 3′)	Annealing temperature(°C)	Amplicon size (bp)	Reference
Clor1	TTCCAGGGTTTCTCTGAATTTG	60	100	Terzi *et al.* (2004)
Clor2	TATGGCGAAATCGGTAGACG			
UNIV P	GGTTTACGACCTCGATGTT	55	104	Sawyer *et al.* (2004)
UNIV Q	CCGGTCTGAACTCAGATCAC			
Le1-5	GCCCTCTACTCCACCCCCA	59	118	Kuribara *et al.* (2002)
Le1-3	GCCCATCTGCAAGCCTTTTT			
35S-1	GCTCCTACAAATGCCATCA	54	195	Lipp *et al.* (1999)
35S-2	GATAGTGGGATTGTGCGTCA			

The primer pairs have been selected among those reported in literature (Jennings *et al.*, 2003) with the aim of obtaining short amplicons (118 bp), compatible with highly fragmented DNA samples.

PCR analysis

PCR reactions were performed in 20 μl reaction volumes containing 20 mmol/l Tris HCl pH 8·4, 1·5 mmol/l $MgCl_2$, 50 mmol/l KCl, 100 mmol/l of each dNTPs, 900 nmol/l forward and reverse primers (see Table 1), 100 ng of genomic DNA templates, and 1U of Taq polymerase (Invitrogen, Carlsbad, CA). Amplifications were performed using an Applied Biosystems Gene Amp PCR System 2400 programmed as follows: one step of 5 min at 94°C; 35 cycles of 30 s at 94°C, 30 s at annealing temperature (see Table 1), 1 min at 72°C; and one step of 3 min at 72°C. The PCR products were separated on 2·5% agarose gels in TBE buffer.

Enzyme assay

Aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatine kinase (CK), lactic dehydrogenase (LDH), gamma glutamyltransferase (GGT) and alkaline phosphatase (ALP) were assayed in serum and in homogenates from liver, kidney, heart and skeletal muscle. Briefly, one gram of tissue was put into an ice-cold homogenization buffer (in mmol/l): 280 mannitol, 10 KCl 1 $MgCl_2$, 0·2 pefabloc SC, 10 hepes, pH 7·0 adjusted with tris. Samples were homogenized by an Ultra-Turrax homogenizer and then centrifuged in a Beckman L7 ultracentrifuge at 10 000 **g** for 10 min, the upper layer was used for analysis.

Enzyme activity was determined spectrophotometrically by using reagents from Spinreact SA, Sant Esteve de Bas, Spain. Since significant differences were found for LDH between control and treated groups, in order to assess the isoenzymatic distribution of LDH, electrophoretic separation was performed on each sample. Briefly, 20 μl of sample were applied on cellulose acetate membranes and electrophoresis was performed under undenaturing conditions at 200 V for 50 min in barbital buffer. Following electrophoresis, the membranes were stained to reveal the LDH isoenzymes by using the ISO-LAD commercial kits (Chemetron Chimica S.p.A., Milan, Italy). Quantification of isoenzymes fractions was done by using a densitometer (CGA, Florence, Italy). The relative distribution of the isoenzymes in the samples was expressed as a percentage of total enzymatic activity.

Statistics

Results were expressed as mean ± standard deviation. Differences in enzyme levels between groups were analysed by the Student *t* test (Statistical Packages for the Social Sciences (SPSS), 1999). Diet and sexual differences within groups were analysed by analysis of variance (ANOVA) using the model: $y_{ijk} = \mu + D_i + S_j + D \times S_{ij} + \varepsilon_{ijk}$, where $y =$ single observation; $\mu =$ general mean; $D =$ diet effect ($i =$ control or treated); $S =$ sex effect ($j =$ male or female); $D \times S =$ interaction between diet and sex effects; $\varepsilon =$ error (SPSS, 1999).

Results

Detection of DNA fragments

The chemical compositions of conventional and GM SBM were superimposable (Table 2), in agreement with the results summarized by Aumaitre (2004).

Figure 1 shows the DNA amplification in one rabbit from each group by using the UNIV P/UNIV Q primers; as seen, a 104 base pair (bp) band was detected in all the samples thus showing the good quality of extracted DNA.

A similar representative example is reported in Figure 2 for Clor1/Clor2 primers, where a 100 bp band was found in many tissues thus showing the presence of chloroplast DNA in tissues and blood from both control and treated groups. The Clor1/Clor2 primers were not detected in all samples, in particular, percentages of positive samples were: 50% (blood), 70% (muscle), 80% (heart), 70% (liver) and 80% (kidney).

In Figure 3 a representative example obtained by using Le1-5/Le1-3 soya-bean specific primers shows how the signal could not be detected in all samples and the band (118 bp) was seen only in the plant sample.

A similar aspect can be seen in Figure 4 where also the 35S1/35S2 primers gave undetectable results (195 bp) in all samples except for the GM soya bean.

Table 3 shows body weight before and after the end of the experiment and the organ weights in control and treated

Table 2 *Composition (g/kg dry matter) of conventional and genetically modified (GM) soya-bean meal (SBM)*

	Component†						
	CP	EE	CF	Ash	NDF	ADF	ADL
Conventional SBM	544	250	41	71	157	140	20
GM SBM	536	248	43	69	152	125	38

† CP: crude protein; EE: ether extract; CF: crude fibre; NDF: neutral detergent fibre; ADF: acid detergent fibre; ADL: acid detergent lignin.

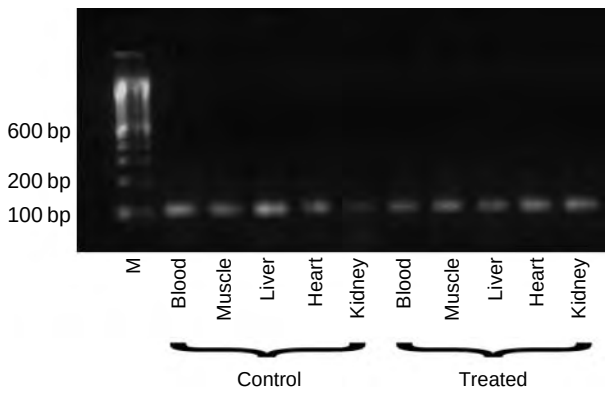

Figure 1 Electrophoretic analysis of DNA amplification in one rabbit from each group by using the UNIV P/UNIV Q primers. M = 100 bp DNA ladder.

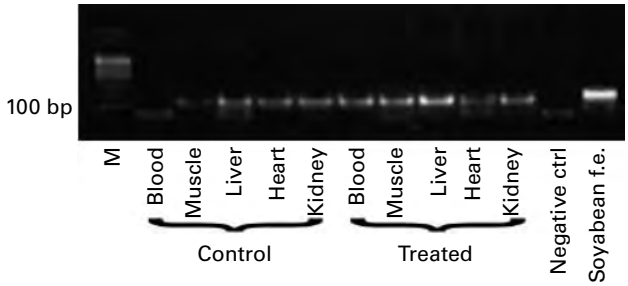

Figure 2 Electrophoretic analysis of DNA amplification in one rabbit from each group by using the Clor1/Clor2 primers. M = 100 bp DNA ladder.

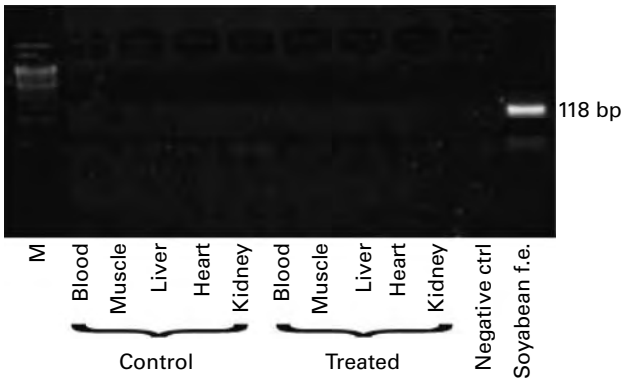

Figure 3 Electrophoretic analysis of DNA amplification in one rabbit from each group by using the Le1-5/Le1-3 conventional soya bean specific primers. M = 100 bp DNA ladder.

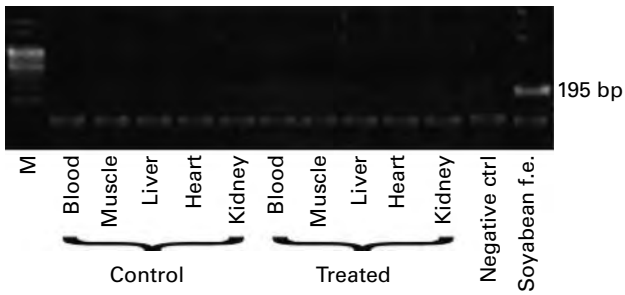

Figure 4 Electrophoretic analysis of DNA amplification in one rabbit from each group by using the 35S-1/35S-2 GM soya bean specific primers. M = 100 bp DNA ladder.

animals, no statistical difference was detected. Finally, no statistical differences were found between sexes.

Enzyme activity

Figure 5 shows the differences in enzyme activity in heart, skeletal muscle, kidney, liver and serum. Statistical differences ($P < 0{\cdot}05$) were detected in kidney for ALT, GGT and LDH whereas in the heart such result was seen only for LDH. No statistical differences were found for serum, liver and skeletal muscle. No statistical differences were found between sexes (data not shown).

Table 4 shows the relative distribution of LDH isoenzymes in serum and in tissues. Significant differences ($P < 0{\cdot}05$) between control and treated animals were detected for heart LDH1 and LDH2 and for kidney LDH1, thus confirming the significant increase of the enzyme in these tissues. Moreover, despite no significant differences were found for LDH total activity in liver, a significant increase (LDH1) and decrease (LDH4) were found also in this organ.

Discussion

DNA

Our findings confirm that, despite chloroplastic plant DNA can be fragmented by technological processes for food preparation and by digestion, multicopy gene can be found in rabbit tissues by using the Clor1/Clor2 primers.

The persistence of short DNA sequences from plant tissues offered has been shown in the GIT of ruminants, from the oral cavity of sheep to rumen and abomasum ingesta of cattle, differing in the case of maize silage and grain (Duggan *et al.*, 2003; Einspanier *et al.*, 2004). The high level of degradation of ubiquitous plant chloroplast DNA in the last section of cattle GIT (jejunum and colon) has been demonstrated by Einspanier *et al.* (2004). In the GIT of monogastrics, plant DNA is detectable in pigs (Klotz *et al.*, 2002; Chowdury et al., 2003; Reuter and Aulrich, 2003), in chickens (Chambers *et al.*, 2002) and in humans (Martìn-Orùe *et al.*, 2002; Netherwood *et al.*, 2004).

In blood, muscular tissues and organs the presence of residual plant DNA has been demonstrated in poultry but not in pig (Klotz *et al.*, 2002; Jennings *et al.*, 2003; Reuter and Aulrich, 2003). Contrasting results have been reported also for ruminants, such as cattle and sheep (Einspanier *et al.*, 2001; Duggan *et al.*, 2003; Phipps *et al.*, 2003).

Also concerning the research of specific DNA fragments the data in the literature are controversial. Indeed, the fragment of invertase gene (ivr, 226 bp) from corn was found in the liver, spleen and muscle of poultry by Aeschbacher *et al.* (2002) but not by Tony *et al.* (2003). In the pigs, while Chowdhury *et al.* (2003) detected fragments of zeina (242 bp), ivr (226 bp) and cry1A(b) (110–437 bp) gene from conventional and GM maize in the gastric and intestinal contents, Jennings *et al.* (2003) did not find fragments (198 bp) of le1 gene for soybean lectin in the muscle. In the present trial, by using the same gene sequence (gi170005/gbK00821·1) of Jennings *et al.* (2003) in the same sample where we found the chloroplast fragment it was not possible to detect neither a shorter fragment (118 bp) of the lectin gene nor the 35S promoter thus confirming that plant low-abundance genes are not detectable in animal tissues.

This finding agrees with the results obtained by Phipps *et al.* (2003) in cow blood and milk, confirming that single copy

Table 3 *Organ fresh weights and body weights in control and treated animals*

		Weight (g)†									
		Liver		Kidney		Muscle		Heart		Body	
	No.	Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d	Mean	s.d.
Diet											
Control	10	89·0	5·2	14·7	1·2	127·0	12·6	8·7	0·7	2063·1	114·9
Treated	10	86·9	7·2	14·5	1·5	125·5	10·0	8·8	0·7	2040·0	203·5
Sex											
Males	10	87·0	7·2	14·2	1·6	124·2	11·7	8·5	0·7	2070·3	135·0
Females	10	88·9	5·2	15·0	0·9	128·3	10·7	9·0	0·7	2030·4	191·0

† Two-way ANOVA: main effects diet, sex and interaction between diet and sex, revealed no significant differences ($P > 0{\cdot}05$).

gene are difficult to identify (Artim *et al.*, 2001). However, in a previous trial we found the single copy gene of barley (data not yet published) in the tissues and organs of rabbits.

Our negative results in detecting single copy gene from SBM could be affected by its processing. Indeed, according to Forbes *et al.* (1998) and Chiter *et al.* (2000), the oil extraction or the heat treatment can cause fragmentation of food DNA.

The persistence of transgenic proteins in the GIT and tissues of animal models have been evaluated in monogastrics.

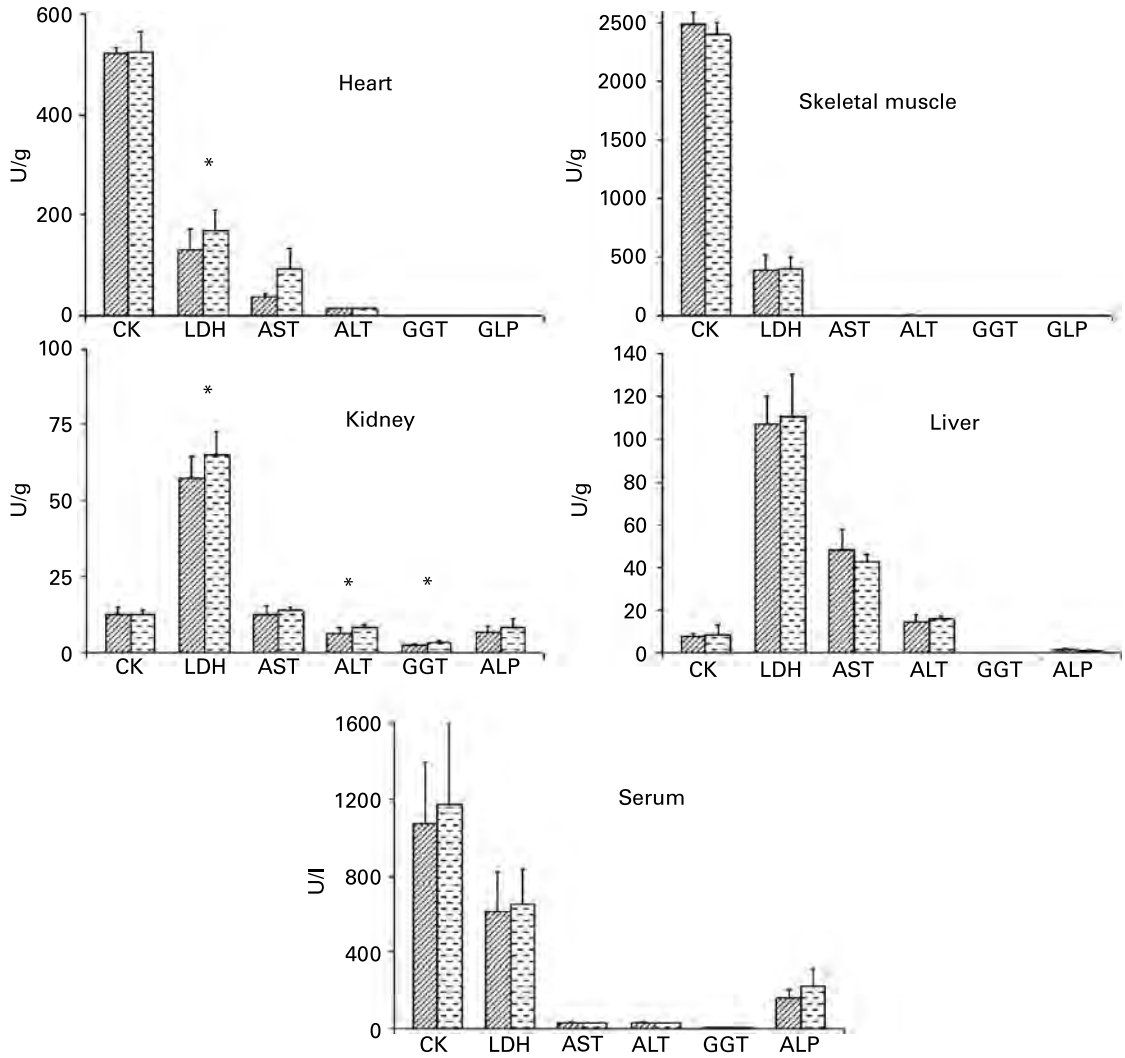

Figure 5 Levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatine kinase (CK), lactic dehydrogenase (LDH), gamma glutamyltransferase (GGT) and alkaline phosphatase (ALP) in serum and in homogenates from heart, skeletal muscle, kidney and liver from control (▨) and treated (⊡) rabbits.

Table 4 *Relative distribution of lactic dehydrogenase (LDH) isoenzymes in serum and in homogenates from heart, skeletal muscle, kidney and liver from control and treated rabbits*

				Relative distribution of LDH isoenzyme (U/g)									
		Total LDH activity		LDH-1		LDH-2		LDH-3		LDH-4		LDH-5	
Tissue	Group†	Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.
Serum (U/l)	C	614·8	204·2	15·6	6·4	31·8	10·3	37·8	10·8	139·0	33·2	390·6	146·4
	T	646·8	189·6	27·3	4·3	34·9	12·6	40·2	11·0	154·9	42·6	399·5	122·8
Heart (U/g)	C	129·9	34·6	122·5	31·9	3·9	2·1	1·5	1·1	0·9	0·5	1·1	0·5
	T	168·8*	19·2	153·5*	18·0	6·6*	1·4	2·7	1·6	1·9	2·2	1·9	1·7
Skeletal muscle (U/g)	C	385·3	127·3										
	T	398·9	107·1										
Kidney (U/g)	C	57·5	6·8	34·4	4·0	13·4	1·2	6·1	0·9	1·6	0·9	1·6	0·9
	T	65·2*	7·3	41·4*	4·6	15·1	2·3	5·0	1·6	1·3	0·5	1·3	0·5
Liver (U/g)	C	107·5	12·6	54·9	5·9	20·4	2·5	13·1	3·5	9·9	3·9	9·3	3·9
	T	110·5	19·8	63·7*	10·3	19·6	4·6	12·5	3·3	5·3*	3·5	7·4	4·1

† C = control; T = treated. *Significant differences are indicated for T *v.* C groups ($P < 0·05$).

Yonemochi *et al.* (2002) for example evaluated transgenic event CBH 351 (StarLink) corn in broiler chicks feeding, finding that both cry9C gene and cry9C protein were not detected in blood, liver and muscle.

Enzymes

Another interesting aspect of our results concerns the enzyme activity in serum and organs. The levels of the enzyme tested in serum did not show significant differences thus suggesting that no adverse effects were induced by GM soya bean in treated animals. Such result is in agreement with Yonemochi *et al.* (2003), who found no effects of maize GM on serum LDH levels in dairy cows. By contrast, the analysis of enzyme relative activities in tissues gave a different picture. As depicted in Figure 5, significant differences in enzyme levels concerned mainly the kidney, showing higher levels of LDH, ALT and GGT in treated animals. Such a result seems to indicate that some alteration occurred in kidney even if serum levels were not affected. Moreover, LDH was significantly increased also in the heart thus showing that the local production of LDH altered in two of the most important organs of the body. The relative distribution of LDH isoenzymes confirms this hypothesis showing significant differences for heart LDH1 and LDH2 and for kidney LDH1. LDH1 was the dominant isoenzyme in both organs and, additionally, a significant increase of this isoenzyme and a decrease of LDH4 were also shown in the liver, despite no significant differences were found for total LDH activity in this organ. Such a result means that, since LDH is a tetrameric enzyme made up of M and H subunits, a different combination of H and M subunits occurred in the liver ($LDH\text{-}H_4 > LDH\text{-}H_1M_3$). The reason for that shift is not clear, substrate specificity is greater for pyruvate than for alpha-hydroxybutyrate, but a higher amount of H subunit is thought to mean a higher specificity for the reduction of alpha-hydroxybutyrate to alpha-oxobutyrate. Anyway, such shift supports the hypothesis that some metabolic changes occurred in the liver. Therefore, an increased activity of LDH1 occurred in three organs from GM-fed rabbits. The reason for such increase is not fully understood and confirmed but such result suggests that even slight modifications occurred in the local production of the LDH1 isoenzyme in GM-fed rabbits. Moreover, it is known that serum enzyme activities are a reflection of the relative contribution of each tissue to the serum pool. From the lactate dehydrogenase isoenzymatic pattern of the serum and in comparison to the various tissue patterns, it seems that most lactate dehydrogenase activity in the rabbit serum originates from the skeletal muscle which is the major mass component of the body. It is also known that the dominant isoenzyme in muscle is the LDH5 and, for these reasons, it is possible that the LDH1 increase was not detected in serum. Moreover, since LDH1 is known to be involved in cell metabolism by favouring the reaction of lactate to pyruvate (Van Hall, 2000), our results should indicate a general increase of cell metabolism. Such hypothesis is in agreement with other authors who showed significant modifications of some nuclear features in GM-fed mice suggesting a high metabolic rate and an intense molecular trafficking (Malatesta *et al.*, 2002). Anyway, since no diseases were detected in treated animals and serum activities of all the enzymes showed similar levels between the groups, it should be overspeculative to assess that the GM diet is responsible for that but it is a fact that the synthesis of LDH changed in more than one organ and such results should be taken into account for future research.

In any event, our results suggest that an accurate enzymatic analysis can be useful to detect the effects of the diet on cell metabolism even in absence of clinical and biochemical signs. Since the techniques for enzyme assay are well established, enzymes can represent an additional tool to evaluate the risks of GM feeding for animal and human health.

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Cry1Ac Protoxin from *Bacillus thuringiensis* sp. *kurstaki* HD73 Binds to Surface Proteins in the Mouse Small Intestine

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***Bacillus thuringiensis* (Bt), considered a safe insecticide, produces insecticidal proteins named Cry during sporulation, which possess exceptional immunological properties. In this work using an immunohistochemical test we demonstrated that Cry1Ac protoxin (pCry1Ac) binds to the mucosal surface of the mouse small intestine. Ligand blot assay allowed us to detect, under denaturing conditions, six pCry1Ac-binding polypeptides present in brush border membrane vesicles isolated from the small intestine. Moreover, this protein induced *in situ* temporal changes in the electrophysiological properties of the mouse jejunum. The data obtained indicate a possible interaction *in vivo* of Cry proteins with the animal bowel which could induce changes in the physiological status of the intestine.** © 2000 Academic Press

Bacillus thuringiensis produces inclusion bodies during sporulation, which are formed by proteins toxic to a group of important pests. These are called Cry proteins. The biochemical properties of Cry proteins are very peculiar: they have a high molecular weight, are resistant to proteolysis and are soluble at alkaline pH (1). Little is known about the physiological or immunological effects of Cry proteins on vertebrate organisms, despite the proven homology of Bt with the pathogenic *Bacillus cereus* species (2).

The few studies related with the immunological properties of Cry proteins have been limited to the protoxin. In previous reports, Prasad *et al.* suggested that these proteins have antitumoral activity against Yoshida ascites sarcoma in rats (3), and that they enhance the immune response to sheep red blood cells (4). Recently, we demonstrated that recombinant Cry1Ac protoxin (pCry1Ac) administered to mice by intraperitoneal or intragastric route induces systemic and mucosal antibody responses similar to those obtained with cholera toxin (5). Moreover, in adjuvanticity studies, pCry1Ac elicited serum antibodies to hepatitis B surface antigen and BSA when these antigens were co-administered via intragastric, and IgG antibodies in the intestinal fluid when administered by the intraperitoneal route (6).

Transgenic maize containing Cry proteins is being used in food elaboration (7). In this way, animals and man will be in contact with these toxins at the intestinal epithelium; however, we do not know if Cry1Ac produces any physiological effect on the mammalian bowels. In this report, we show that pCry1Ac from Bt HD73 binds *in situ* to the intestinal epithelium of mice and induces a transient hyperpolarization of the mucosal tissue.

MATERIALS AND METHODS

Organisms and culture conditions. Dr. Donald H. Dean from Ohio State University, Columbus, gently provided *Escherichia coli* JM103 [pOS9300] strain. The recombinant strain was grown in LB medium containing 50 μg of ampicillin per ml, and the induction of Cry1Ac protein production was performed using isopropyl-β-D-thlogalactopyranoside (IPTG) (8).

Purification of Cry1Ac protein. Recombinant Cry1Ac protoxin was purified from IPTG-induced *E. coli* JM103 [pOS9300] cultures (8). The cell pellet harvested by centrifugation was resuspended in TE buffer (50 mM Tris–HCl, pH 8, 50 mM EDTA) and sonicated (Fisher Sonic Dismembrator Model 300, U.S.A.) three times for 5 min on ice. Inclusion bodies were collected by centrifugation at 10,000*g* for 10 min. The pellets were washed twice with TE buffer and pCry1Ac was solubilized in CBP buffer (0.1 M Na_2CO_3 pH 9.6, 1% 2-mercaptoethanol, 1 mM PMSF). The particulate material was discarded by centrifugation at 10,000*g* for 10 min. Purified protoxin was examined by SDS–PAGE (9) and protein concentration was determined using the method of Bradford (10).

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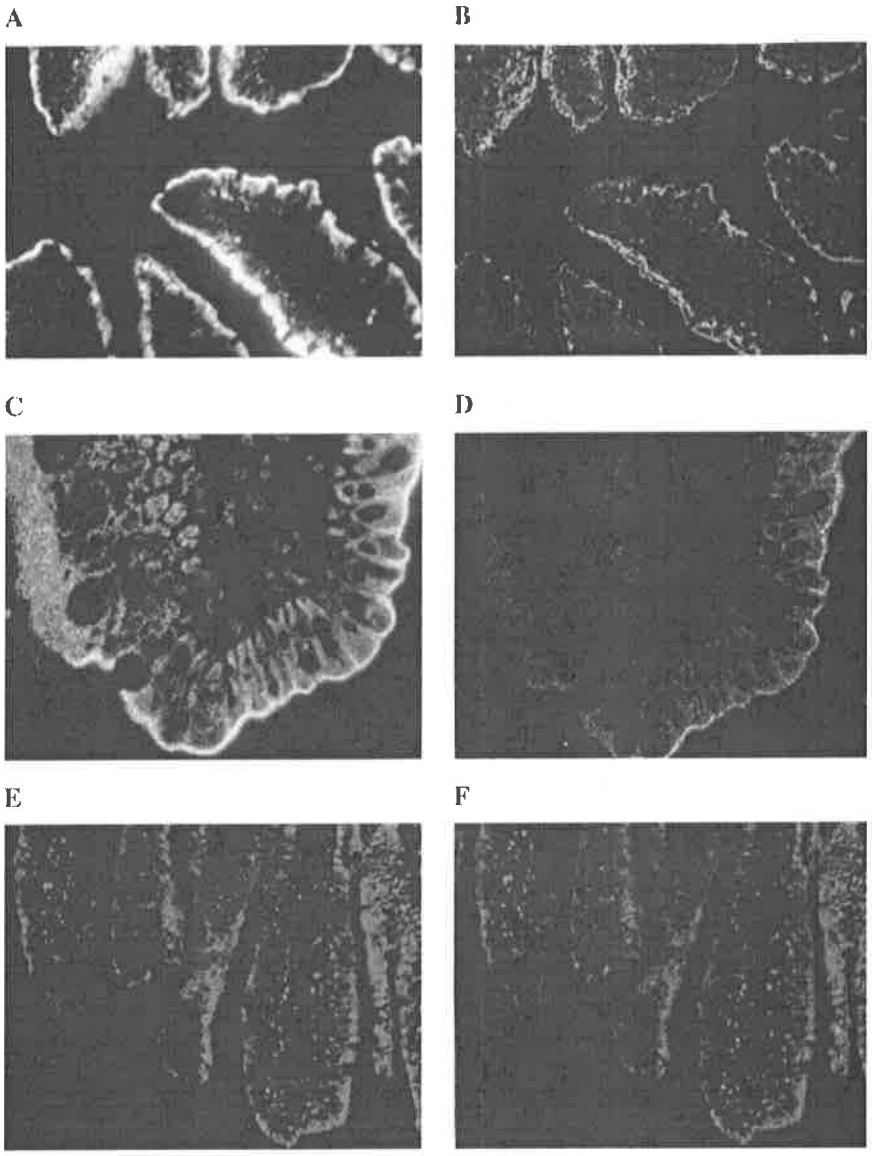

FIG. 1. Immunolocalization of pCry1Ac bound to mouse jejunum sections. The fixed small intestine from anesthetized mouse was extracted and cryosection into the thin layers was performed. The protoxin was added on the tissue section and the protein bound detected using an immuno-purified anti-Cry1 polyclonal antibody. A and C showed different intestine preparations treated with the protoxin. The same images converted to pseudocolor scale are shown in B and D. The image obtained from the section not treated with pCry1Ac is shown in E and F (pseudocolor scale).

Indirect immunofluorescence assay. The mouse intestines were fixed *in vivo* using cardiac perfusion with 4% formaldehyde in PBS (11). The jejunum of treated mice was then extracted, flushed out with ice-cold PBS and immersed into an osmotic solution (2.3 M sucrose in PBS). Frozen cut sections from fixed intestine were obtained using an IEC Minotone cryomicrotome (International Equipment Co., U.S.A.) and picked up on gelatin-coated slides. The preparations were stored at −20°C until use. For *in situ* binding assays intestine sections were thawed in PBS solution for 1 h at 37°C. Cry1Ac protoxin was added on the tissue at 0.1 mg/ml in 0.1 M Na_2CO_3, pH 9.6, during 2 h at 37°C. Unbound protein was removed by washing six times with PBS and the slides were incubated for 1 h at 37°C with an anti-Cry1Ac polyclonal antibody at 10 μg/ml in PBS (12). The anti-rabbit monoclonal antibody conjugated with rhodamine (Sigma Chemical Co., U.S.A.) was used at a 1:100 dilution. Immunofluorescence was recorded using a MRC-6000 confocal microscope (Bio-Rad, U.S.A.). Cry1Ac protoxin was omitted in the negative controls. The images were analyzed in its original form or were transformed to pseudocolor scale.

Electrophysiological experiments. The small intestine was removed from adult Balb/c male mice under sodium pentobarbital anesthesia. Segments of jejunum were placed on ice-cold Ringer's solution at 4°C and gassed with an O_2–CO_2 (95:5) mixture. Each segment was cut open along its mesenteric border, rinsed clean of luminal contents, and extended and kept in gassed Ringer's solution. Full-thickness segments were divided into two and mounted between the circular openings of two adjacent Ussing hemichambers (13). Each hemichamber was filled with 2.5 ml of gassed Ringer's solution and kept at 37°C while bubbling with the O_2–CO_2 mixture. The

protoxin (7.5 μg) was applied on the mucosal side of the preparation that had been mounted ten minutes before, and the transmural potential difference (PD) and short-circuit currents (Isc) were recorded each minute for sixteen minutes. Transmural resistance (R) values were obtained from PD/Isc ratios at each time point (13). Decay rates were calculated by regression analysis of the corresponding PD and Isc values.

Brush border membrane vesicles (BBMV) purification. Mouse intestinal BBMVs were prepared according the methodology reported by Biber *et al.* (1980) with some modifications (14). Briefly, small intestine from five anesthetized mice was extracted, rinsed with PBS and cut to small pieces. Intestinal fragments were immersed in 15 ml of ice-cold BBMV isolation buffer (300 mM D-mannitol, 5 mM EGTA, 12 mM Trls–HCl, pH 7.4) and homogenized at 4°C using a potter (B. Braund, Germany). The homogenate was mixed with 21 ml of ice-cold water and $MgCl_2$ was added to 12 mM. The mixture was incubated 15 min on ice and part of the precipitate was discarded by centrifugation at 4500*g*. The BBMVs suspended in the supernatant were then collected by centrifugation at 16,000*g* during 15 min at 4°C and stored at −70°C. The quality of BBMVs was tested by measuring the activity of alkaline phosphatase and β-glucoronidase enzymes (14). Protein concentration was determined by Bradford's method (10).

Ligand blot assay. Ligand blot assays were performed following the protocol reported by Hoffman *et al.* (15) for insect BBMVs (15). Different amounts (5, 10, 15, 20 μg) of mouse BBMV were dissolved in sample buffer (1% 2-mercaptoethanol, 1% SDS, 10% glycerol, 100 mM Tris–HCl pH 8) and applied on SDS–PAGE (9). The resolved proteins were immobilized onto Hybond C+ (Amersham, UK) using a submarine transfer (Bio-Rad, USA). Membranes were blocked with 5% dry milk in PBS for 1 h at 37°C, and incubated under similar conditions with 10 μg of pCry1Ac alone or with 10 mM of glucose, mannose, *N*-acetyl-galactosamine, or biotin. Cry1Ac protein binding to the membrane was detected using an anti-Cry1Ac polyclonal antibody (12). The immune complexes were revealed with an ECL Western blot kit (Amersham, UK).

RESULTS

Immunohistochemical detection of Cry1Ac binding after in situ exposure. Cry1Ac protoxin under alkaline conditions bound to the jejunum epithelium, being more intensive the binding to enterocytes. The processing of confocal images with pseudocolor scale showed that the protoxin binds mainly to the apical surface, including the brush border. The tissue sections to which pCry1Ac was not added did not emit intense red fluorescence (Fig. 1).

Study of Cry1Ac interaction with BBMV proteins from mouse small intestine. BBMVs isolated from the small intestine were separated and immobilized onto a nitrocellulose membrane. Six polypeptides with molecular weights of 102, 87, 62, 55, 51, and 39 kDa bound pCry1Ac in ligand blot assays (Fig. 2). The signal was saturated with more than 10 μg of BBMVs per lane. The assay performed with no protoxin did not show signal, which evidences the lack of antibody immunoreactivity with the membrane proteins.

A second experiment was performed to characterize the binding of pCry1Ac to intestinal surface proteins. The presence of biotin, glucose, mannose and *N*-acetyl-glucosamine in the reaction buffer of ligand blot assay did not inhibit the protoxin interaction with the BBMV proteins (Fig. 2).

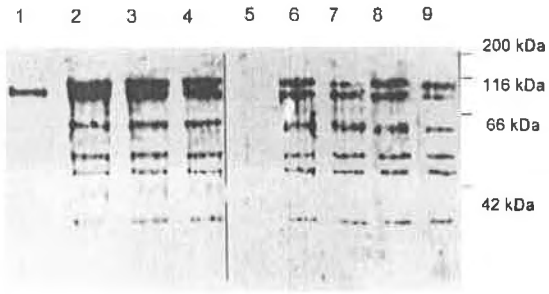

FIG. 2. Ligand blotting assay performed with BBMVs isolated from mouse small intestine. (Left) Amounts of BBMV proteins of 5 (1), 10 (2), 15 (3), and 20 μg (4) were loaded onto a SDS–PAGE and blotted to a nitrocellulose membrane. The membrane was incubated with 10 μg of pCry1Ac and the bound protoxin was detected using a polyclonal antibodies. (Right) Strips of nitrocellulose membrane, contained 15 μg of resolved proteins from mouse BBMVs, were incubated with pCry1Ac mixed with glucose (6), mannose (7), *N*-acetyl-galactosamine (8), or biotin (9). Line 5 was treatment without pCry1Ac and was used as a negative control.

Cry1Ac effect on electric properties of mouse small intestine. The PD and Isc values of full-thickness jejunum fragments mounted in the Ussing chamber decayed very slow with a rate of -0.015 mV min^{-1} and -0.016 μA cm^2 min^{-1}, respectively (Fig. 3). The R values of this tissue in Ringer's solution were constant during the experiments. Treatment of mice jejunum with pCry1Ac raised ΔIsc in 37% and ΔPD in 31% during the first 5 min, with the increased rates of 0.092 μA cm^2 min^{-1} and 0.096 mV min^{-1}, respectively. Seven minutes later, these electrical parameters decayed with a rate of -0.040 μA cm^2 min^{-1} and -0.020 mV min^{-1}. The resistance of treated jejunum did not change during the experiments (Fig. 3).

DISCUSSION

Cry1Ac protoxin induced a high immune response in mice by both oral and intraperitoneal route (5), and had an immunostimulant effect when coadministered with other antigen (6). In this study, we demonstrate that a Cry binding protein exists in mice, which could be related with an efficient capture and processing of Cry proteins by the antigen presenting cells in the mucosal tissue. Ligand blot and immunohistochemical assays allowed us to detect six Cry1Ac binding proteins probably placed in the apical membrane of the small intestine. The data obtained by us do not discard the possibility that Cry1Ac protein could bind to another molecule in the intestine mucosal surface. Other bacterial toxins with exceptional immunological properties like cholera toxin and *E. coli* labile enterotoxin from *E. coli* (LT), bind to gangliosides presents on the intestinal surface of vertebrates (16).

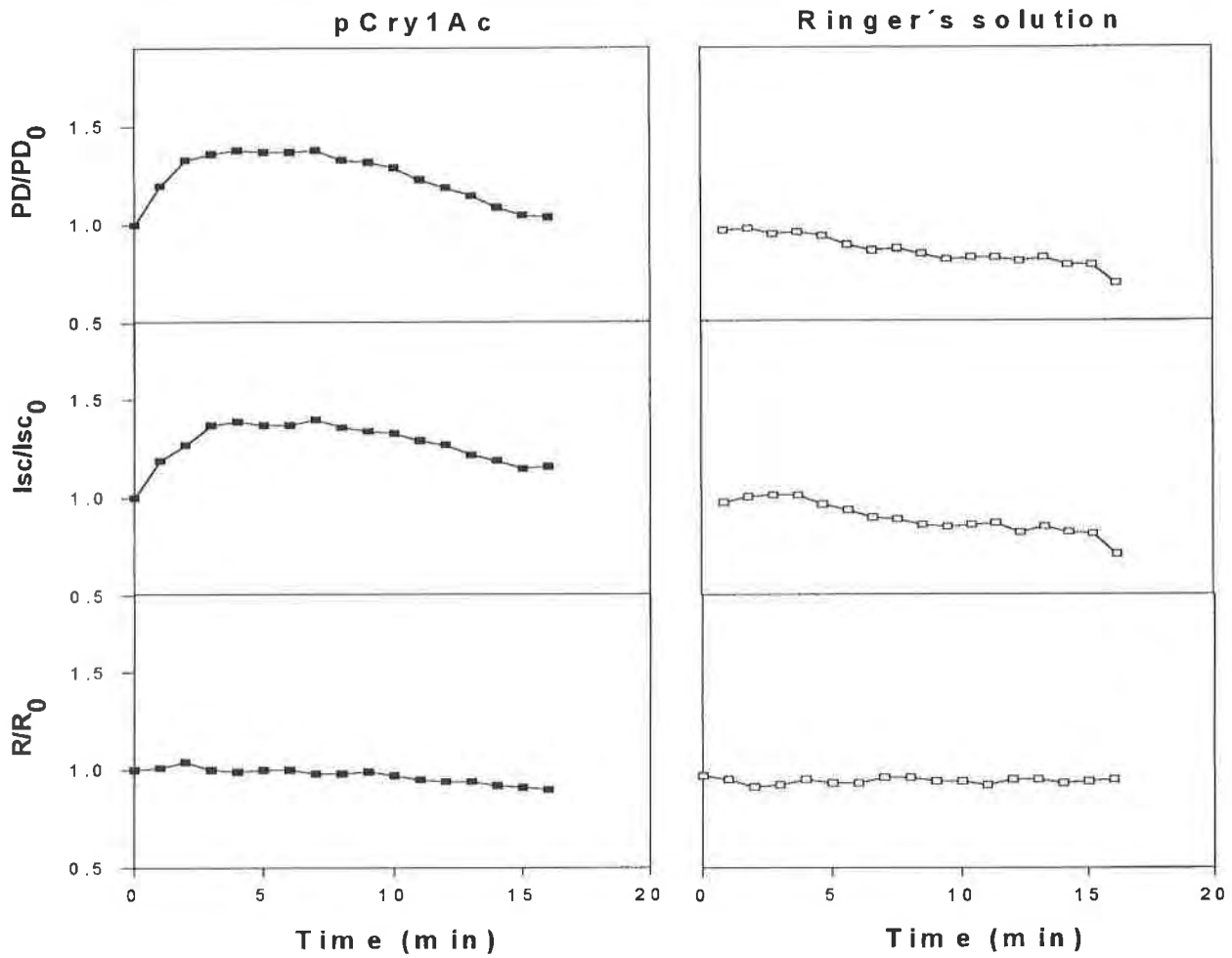

FIG. 3. Effects of pCry1Ac protoxin on the electrophysiological properties of mouse jejunum preparation. Both hemichambers contained Ringer's solution gassed with 95% O_2–5% CO_2, and 15 min later 7.5 μg of protoxin were added to each preparation on the mucosal side. The symbols represent the average normalized values of PD/PD_0, Isc/Isc_0, and R/R_0 ratios, i.e., (value for each time)/(initial values). Each point represents the mean of four experiments.

Putative Bt toxin receptors have been found in the apical membrane of the columnar cells present in the insect midgut, and have been identified in many cases as either 100- or 120-kDa N-aminopeptidases (17) or 200- to 220-kDa E-cadherine-like proteins (18). Similar proteins are present in vertebrate bowel playing important roles in nutrient absorption (19) and cellular adhesion (20). In contrast with the data reported for insect BBMVs, the interaction of Cry binding protein with the protoxin is not mediated neither by sugars nor biotin, thus electrostatic interactions involving other structures may occur (17, 18).

Cry1Ac not only binds to the intestinal surfaces, but also induces a temporal hyperpolarization of the intestine without apparent tissue damage. This phenomenon could be explained by supposing that pCry1Ac, or a locally produced toxin, form cationic channels like it happens in the insect midgut (21). In this way, a net ion flux could be generated across the enterocytes increasing the positive charges on the intestinal serose side. Many substances, from simple organic molecules to complex proteins, induce changes on the electric parameters of animal intestine (22). In contrast with LT and ciguatoxins, which produce hyperpolarization on the intestinal tissue, pCry1Ac does not induce electrolyte secretions (23, 24).

For many years, Bt-based formulations containing high concentrations of Cry proteins have been used as an example of totally safe insecticides. This idea is based on three important facts: (i) Bt-based insecticides have been used for many years with no reports of consistent hypersensitivity or toxicity (25); (ii) the toxicological tests performed to support registration of Bt-based insecticides show that several strains of this microorganism are innocuous when administered to animals and man by systemic and mucosal routes (26); and (iii) Bt strains isolated from food and human infected wounds are unable to produce illness in animals

(27, 28). However, there are no histopathological or immunotoxicological assays probing the absence of a microscopic effect on animal physiology.

The results obtained by us indicate that pCry1Ac could induce temporal changes in the intestinal membrane of mice without affecting the macroscopic physiological signals, perhaps due to the existence of a mechanism that eliminates or inhibits the toxin effect. We think that previous to commercialization of food elaborated with self-insecticide transgenic plants it is necessary to perform toxicological tests to demonstrate the safety of Cry1A proteins for the mucosal tissue and for the immunological system of animals. If pCry1Ac is a totally harmless protein that binds to the intestinal surface of animals it could be now used as a carrier of vaccinal antigens for oral immunization.

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Biological effects of transgenic maize NK603xMON810 fed in long term reproduction studies in mice



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Contents

Abstract

The aim of the study was to examine effects of the stacked GM crop NK603 x MON810 in different models of long term feeding studies. So far no negative effects of GM corn varieties have been reported in peer-reviewed publications. But the hypothesis, that effects after long term exposure might become evident in multi-generation studies has rarely been investigated.
In this study three designs were used, including a multi-generation study (MGS), a reproductive assessment by continuous breeding (RACB) and a life-term feeding study (LTS), all performed with laboratory mice (strain OF1). The test diets differed only as to the inclusion of 33% NK603 x MON810 corn (GM) versus non-GM corn of a near isogenic line (ISO), both grown under identical conditions in Canada. The MGS also included one group with a non GM corn cultivated in Austria (A REF). All corn varieties used in the MGS and LTS were harvested in 2005, the transgenic and isogenic corn for the RACB were harvested in Canada in 2007. No Austrian corn was used in this case. In the MGS microscopic and ultrastructural investigations were performed to detect changes at the organ and cell level. Gene expression patterns were compared by micro array expression profiles of the intestine as feed-animal interface and by real time PCR.
The results of the MGS showed no statistically significant differences concerning parental body mass. The number of females without litters decreased with time in the GM and ISO group, especially in the 4th generation. In the group fed with A REF corn fewer females were without litters, and accordingly more pups were weaned. The production parameters average litter size and weight as well as number of weaned pups were in favour of the ISO group. These differences were also seen in the RACB design and were statistically significant in the 3rd and 4th litters. In addition, the inter-individual variability was higher in the GM group as compared to the other groups.
The LTS showed no statistically significant differences in the survival of 3 groups of mice fed the different maize varieties.
In the MGS the continuative investigations revealed differences between the GM and ISO groups. The comparison of organ weights did not indicate directed dietary effects, except for kidneys. The electron histological investigation of the cell nuclei revealed differences as to fibrillar centres, dense fibrillar components and the pore density in hepatocytes. This could point to an effect of the GM crop on metabolic parameters. Immunohistochemistry revealed no systematic differences in CD3, CD20 positive cells and macrophages in gut tissue. The microarrays showed differences between the feeding groups. When the data of both non-GM feeding groups from MGS were combined and compared to the GM feeding group, the discrimination became more evident. Analyses of metabolic pathways indicated, that the groups differed regarding some important pathways, including interleukin signalling pathway, cholesterol biosynthesis and protein metabolism.

Summarizing the findings of this study it can be concluded, that multi-generation studies, especially based on the RACB design are well suited to reveal differences between feeds. The RACB trial showed time related negative reproductive effects of the GM maize under the given experimental conditions. The RACB trial with its specific design with the repeated use of the parental generation is a demanding biological factor for the maternal organism. Compared to the findings in the RACB trials it can be assumed that the physiological stress was considerably lower in the MGS trial. The trial design of using “new” parental generations instead of continuous breeding with the same generation has to be considered as being obviously less demanding. This might have masked the impact of dietary

factors on reproductive performance. However, this part of the experiment is valuable as such because it underlines the need for different experimental designs for the assessment of dietary effects that have an unknown impact on animals. The outcome of this study suggests that future studies on the safety of GM feed and food should include reproduction studies. Physiological and genomic traits and depending on the nature of the genetic modification proteomic and metabolomic methods might be taken into consideration as additional tools to the tests performed in this study.

1. Introduction

Transgenic crops are playing an increasing role in the EU. It is often, but arbitrarily distinguished between the 1st generation of transgenic plants, with the characteristics of pest or insect resistance, the 2nd generation that has modified nutritional quality and the 3rd generation plants that are used for plant made pharmaceuticals, vaccines, or plant made industrials. Today, the 1st generation is used in animal nutrition either as source of feed protein, such as soy, or as energy source, such as corn. The demand especially for high value protein feedstuffs for the nutrition of food producing animals is high but the perception of transgenic crops in the public is quite controversial (Finucane 2002; Schiermeier 2004). Discussions on food and feed safety, precaution measures and ethical aspects have been conducted since many years and there seems to be no clear direction.

One important aspect is the potential impact of transgenic feed on the metabolism of animals. The effects on digestion, metabolism and health in farm animals have been addressed in many studies. Most feeding experiments have been short term feeding trials using conventional designs such as digestibility experiments or growth trials until slaughter. Adverse effects of GM crops have not been reported in peer-reviewed publications related to animal feed. Compositional analyses have demonstrated substantial equivalence, feeding trials could show that the 1st generation of transgenic crops is nutritionally equivalent to conventional feedstuffs (Flachowsky et al. 2005; Flachowsky et al. 2007). A certain variability of nutrient composition has been described in almost every study comparing trans- with the isogenic substrates. However, these fluctuations have also been well known for conventional plants because seasonal, environmental and agricultural factors can have severe impact on the nutritional properties of crops. Nutrients in transgenic plants have always been described to be within the variation of the reference lines, representing the “normal range of agronomic variability” (Kuiper et al. 1999; Aulrich et al. 2001; Aumaitre et al. 2002). The variability of feed composition may affect the utilization of feedstuffs by farm animals. In conventional feedstuffs considerable variation of nutrient concentration occurs and has been shown to affect apparent digestibility of the organic matter in several domestic animals. Comparisons have been made for iso- and transgenic maize in monogastric animals as pigs or poultry and ruminants. A certain variability of feed utilization was obvious in some studies, but the experiment-related variability has to be taken into account when discussing the potential impact of the newly introduced events on animal performance. In all, the published data do not give clear indications for a systematic effect of genetic engineering on nutrient digestibility so far. Nutritional and anti-nutritional properties of transgenic crops depend on the geographical position, the growth conditions, soil and fertilization and climate variations, as in isogenic feedstuffs.

Only few studies have been conducted to assess “toxicity” and “long term effects” of transgenic crops in warm blooded animals. An advanced safety evaluation process has to address several important issues and should include genomic and postgenomic technologies, DNA microarrays, proteomics and advanced methods of metabolic profiling (Kuiper et al. 1999; Kleter and Kuiper 2002). *In vitro* studies using rumen epithelia could not demonstrate an impact by Cry1Ab toxin (Bondzio et al. 2008). But Vasquez-Padron et al. (2000) demonstrated that Cry1Ac protoxin binds to the mucosal surface of the mouse intestine and induces

in situ temporal changes in the electrophysiological properties of the mouse jejunum thus, indicating potential changes in the physiological status of the intestine.
The risk of allergenicity seems to be limited in farm animals in comparison to the situation in humans. The effects on reproduction are important because breeding and growing animals should have a higher susceptibility to adverse effects if present compared to animals with lower performance. There are only few long-term chronic or multigenerational studies in laboratory or domestic animals. Up to now, no negative effects were described in peer-reviewed publications on reproduction and testicular development in mice or rats (Brake and Evenson 2004; Brake et al. 2004; Rhee et al. 2005; Kilic and Akay 2008), or on animal health, feed intake, feed efficiency, laying performance, or hatchability, DNA-transfer and quality of meat and eggs of 10 generations of quails compared with the isogenic counterparts (Flachowsky et al. 2005). The outcome of one study in rats was discussed controversially because of differing interpretations of the effects of the transgenic maize MON 863 (Hammond et al. 2006; Doull et al. 2007; Seralini et al. 2007).

Whether there is a risk under specific conditions, for instance disorders of the digestive tract, has not been widely studied. Rats did not show adverse reaction when gastrointestinal injury was induced and purified Bt protein Cry1Ab from *B. thuringiensis var. Kurustaki HD-1* was applied orally (Onose et al. 2008).

The comprehensive characterization of novel transgenic plants will be most important for the future evaluation process. The biological response of animals has to be described in the best possible way according to the scientific state of the art. Traditional feeding and digestive trials appear in many regards less suitable for a well-founded risk evaluation. This should be defined based on the analysis of the physiological reaction of the body, the modification of the intestinal microflora, the interactions with the immune system, the fate of DNA and protein in the organism and the potential appearance in animal products. Reproductive function and efficiency might be considered as important research area.
The present study in mice uses advanced approaches to assess biological effects of the transgenic maize NK603 x MON810 in a multigenerational study in mice. Two different designs have been applied to assess the impact of different maize varieties on reproduction traits, a Reproductive Assessment by Continuous Breeding design and a Multigeneration Reproductive Trial have been used. Data related to the reproductive function were analyzed and the interaction with the animal organism was studied in relation to intestinal gene expression, and histological studies. In addition a life term study was performed with a limited number of animals.

2. Material and Methods

2.1. Crop production and choice of comparators

Crop Production
The crop production for all feeding studies was carried out by the Organic Agriculture Centre of Canada (Prof. R. Martin, Organic Agriculture Centre of Canada, Nova Scotia Agricultural College, P.O. Box 550, Truro, NS B2N 5E3, Tel: 902-893-7256; Fax: 902-896-7095). Both test crops, GM and control, were grown on loamy soil in Canada, Nova Scotia, in 2005 and 2007.

Corn production sites 2005:
The comparators were grown on 4 locations, two for each variant. The distance between the fields in Plumdale (non-GM corn) and Interval (GM corn) was 2 km and between Masstown (non-GM corn) and Hamilton-Onslow (GM corn) 10 km. There were 20 km between the two test field layouts.

Corn production sites 2007:
The second feed lot was grown on two locations in the Marsh region, Nova Scotia, Canada. The treatments were arranged in a manner to reduce the possibility of cross contamination of GM with non-GM corn by planting according to prevailing wind direction and growing a 75 m buffer zone of non-GM corn between the two test variants.

Soil samples were taken from all fields and analysed in the state laboratory of the Nova Scotia Department of Agriculture and Fisheries to compare soil qualities and determine the fertilisation management.

The fertility management differed between 2005 and 2007 insofar as liquid dairy manure was used additionally to the mineral fertilizers in 2007 only. In both cultivation years the same herbicides containing the active ingredients dicamba, atrazine and s-metalochlor for the non-GM variant and glyphosate for the GM corn, were used.

The corn was hand harvested when the moisture content was less than 30%. After shelling in a small threshing machine designed for this purpose the corn was dried to < 14% moisture in separate bulk drying bins at a low temperature (<30°C) to ensure good feeding quality. The corn was shipped in 25 kg bags on treated pallets.

Choice of comparators
Since it was not possible to obtain a genetically modified test crop plus parental line from the agro-business companies, two comparators where chosen according to availability on the Canadian market. The trade names are DKC 26-79, genetically modified corn hybrid, and DKC 26-75, NK 603-near isogenic line. The test corn represents the stacked event NK 603 x MON 810 and contains three gene cassettes, conveying herbicide tolerance and insect resistance. The hybrid was produced by traditional breeding of the two genetically modified parental inbred lines derived from maize transformation events NK 603 and MON 810. The two parental lines were modified using the particle acceleration method.

Description of the test corn NK 603 x MON 810

NK 603 has been modified to tolerate the broad spectrum herbicide glyphosate *(N-phosphonomethyl-glycine)* by introducing two gene cassettes containing the CP4 EPSPS (5-enolpyruvyl-shikimate-3-phosphate synthase) gene derived from *Agrobacterium tumefaciens,* strain CP 4 (Table 1 and Table 2).

Table 1: **1st cp4 EPSPS gene cassette**

genetic element	derived from	size	function
P-ract1/ ract1 intron	*Oryza sativa*	1.4 kb	promotor, transcription start site, 1st intron
ctp 2	*Arabidopsis thaliana*	0.2 kb	chloroplast transit peptide
cp4-epsps	*Agrobacterium sp.*	1.4 kb	glyphosate tolerant CP4 EPSPS enzyme
NOS 3`	*Agrobacterium tumefaciens*	0.3 kb	ends transcription and directs polyadenylation of the mRNA

Table 2: **2nd cp4 EPSPS gene cassette**

genetic element	derived from	size	function
e35S	*Cauliflower mosaic virus*	0.6 kb	promotor
Zmhsp70	*Zea mais L.*	0.8 kb	stabilizes level of gene transcription
ctp 2	*Arabidopsis thaliana*	02 kb	chloroplast transit peptide
cp4-epsps l214p	*Agrobacterium sp*	1.4 kb	glyphosate tolerant CP4 EPSPS L214P enzyme
NOS 3`	*Agrobacterium tumefaciens*	0.3 kb	ends transcription, and directs polyadenylation of the mRNA

The transgenic EPSPS enzyme is not inhibited by glyphosate and can therefore substitute the sensitive plant-derived EPSPS enzyme in the shikimic acid pathway for the biosynthesis of aromatic amino acids. Thus the continued function of the aromatic amino acid pathways is ensured, even in the presence of glyphosate.

MON 810 contains one gene cassette (Table 3) to import insect resistance by producing the Cry1Ab protein, which targets specific lepidopteran insect pests including the European Corn Borer (*Ostrinia nubilalis*) and pink borers (*Sesamia spp*.). After activation by proteolytic processing in the target insect´s gut the toxin binds to receptors on the surface of midgut epithelial cells generating pores in the membrane. Resulting electrolyte imbalance and pH changes paralyze the gut, which entails the insect to stop eating and die (Sacchi et al. 1986).

Table 3: **Gene cassette of MON 810**

genetic element	derived from	size	function
e35S	*Cauliflower mosaic virus*	0.32 kb	promotor
Zmhsp70	*Zea mais L.*	0.8 kb	stabilizes level of gene transcription
Cry1Ab	*Bacillus thuringiensis*	3.5 kb	Cry1Ab protein

By means of traditional breeding methods NK 603 and MON 810 inbred lines, homozygous for the respective insert, were produced and crossed to obtain the stacked event NK 603 x MON 810, now containing both traits of the parental lines.

A slight GM contamination of the isoline of harvest 2005 was detected and therefore a further reference group (A REF) was introduced. This corn was grown in Austria under conventional conditions. Since it was not possible to obtain the same variety, a substantially equivalent cultivar (Sarastro) was chosen.

2.2. Diet

2.2.1. Diet composition

A purified diet with 33.0% maize content was chosen as a common level of maize in commercial rodent diets (Table 4). For all diets a standardized diet for laboratory mice in reproduction in accordance with Nutrient Requirements of Laboratory Animals (National Research Council, NRC, 1995) was used (Table 5). All three diets were produced by Ssniff Spezialdiaeten GmbH, Ferdinand-Gabriel-Weg 16, D-59494 Soest.

Table 4: **Overview about diets used**

Abbreviation	Diet
GM group	test diet with 33.0% of the transgenic corn (NK603 x MON810)
ISO group	control diet with the 33.0% isoline
A REF group	reference diet with 33.0% GM free Austrian corn

Table 5: **Diet composition according to Ssniff**

Ingredient	%
Corn (according to group)	33.0
Potato protein vD (No1)	17.0
Caseinacid, ssniff (Na- poor) 86% XP	5.0
Barm vD (No 9006)	1.0
Dry whey, suss VD (1009; kaasweip.)	5.0
Saccharose	3.5
Cellulose (Arbocell) ssniff	6.0
Tarwevoerbloem (wheat sort) (No. 92) Okt.05	18
(H) CaCO3 vanDijck (No. 23)	0.6
(H) MCP (Aliphos) vanDijck (No. 228)	1.0
(H) NaCl, salt (Zout) vanDijck (N0. 351)	0.2
(H) Magnesiumoxid, MgO van Dijck (No. 28)	0.1
(H) Cholinchlorid (50%) van Dijck (No. 1015)	0.3
(H) Ca-Propionate	1.0
(H) DL-Methionin 99 % van Dijck (No. 36)	0.2
(H) L-Tryptophan van Dijck (Nr. 702)	0.1
Ssniff EF 1/0 Vitamin-VM	1.0
Ssniff EF 1/0 Mineralstoff-VM	3.0
Sunflower oil	4.0
	100

2.2.2. Analyses of corn and diets

2.2.2.1. Test on the genetic modification

Test at protein level
For the determination of Cry1Ab and EPSPS the ELISA technique was used. Corn was tested with a Roundup Ready® Cry1Ab and Roundup Ready® CP4 EPSPS (Agdia Incorporated, Indiana, USA) ELISA system according to the manufacturers protocol.

Test at DNA level
Corn was first screened on the presence of the 35S-promoter and nos-terminator using PCR (Oesterreichische Agentur fuer Gesundheit und Ernaehrungssicherheit GmbH, AGES, 1226 Vienna, Spargelfeldstrasse 191) and if positive analysed for the event specific region.

2.2.2.2. Crude nutrients and gross energy

Corn and diets were analysed according to Weende (Institut fuer Tierernaehrung, Freie Universitaet Berlin, Bruemmerstrasse 34, 14195 Berlin, Germany) for dry matter (DM), crude protein (XP), crude fat (EE), crude fibre (XF) and crude ash (XA). Nitrogen free extracts (NfE) which include α-glycosidic polysaccharides, soluble sugars and soluble parts of cellulose, lignins and pectins, were calculated: NfE = DM - (XA+XF+EE+XP); (VDLUFA Methodenbuch, Bd. III – Futtermittel, Untersuchung von Futtermitteln, VDLUFA-Verlag Darmstadt).
Gross energy (GE) was calculated: GE (MJ/ kg)= 0.0239 XP + 0.0398 EE + 0.0201 XF + 0.0175 NfE.

2.2.2.3. Minerals and trace elements

Atom absorption spectrometry (AAS) was used for the quantification of calcium (Ca), sodium (Na), manganese (Mg), copper (Cu), zinc (Zn) and iron (Fe). Phosphorus (P) was determined photometrically in corn and diets (Institut fuer Tierernaehrung, Freie Universitaet Berlin).

2.2.2.4. Vitamins

Vitamin A, β-Carotene and Vitamin E were investigated as they play a major role in reproduction. Samples were analyzed with high liquid chromatography (HPLC, Institut fuer Physiologische Chemie, Stiftung Tieraerztliche Hochschule Hannover, Buenteweg 17, 30559 Hanover, Germany).

2.2.2.5. Fatty acids

For the determination of fatty acids gas-chromatography was performed (Institut fuer Tierernaehrung, Freie Universitaet Berlin, Germany).

2.2.2.6. Amino acids

Amino acids were determined by ion exchange chromatography after acid hydrolysis.

2.2.2.7. Hygienic evaluation

Total microbial count, yeasts and moulds were determined by cultivation techniques (VDL LUFA, Methodenbuch). HPLC-mass spectrometry (MS/MS) was used for the investigation of deoxynivalenol (DON) and zearalenon (ZON) (LUFA-ITL GmbH, Dr.-Hell-Str. 6, 24107 Kiel, Germany).

2.2.2.8. Herbicides

Glyphosate and its derivative aminomethylphosphonic acid (AMPA) were determined by liquid chromatography (LC)-MS/MS (LUFA Nord-West, Jaegerstraße 23-27, 26121 Oldenburg, Germany). Dicamba, S-Metolachlor, Atrazin were determined by the QuEChERS method which is a rapid multiresidue method that is established for herbicide analysis.

2.2.3. Diet processing

The feed was offered in feeding troughs (Tecniplast, Hohenpreißenberg, Germany) as meal diet to avoid structural changes in the proteins by the pelleting process, where high temperature and pressure are applied. It has been shown that delta- endotoxins are heat instable (EPA 2003).

2.3. Animals and housing

The animal trials were conducted at the Institute of Nutrition, University of Veterinary Medicine Vienna, and were approved by the ethics committee of the University of Veterinary Medicine of Vienna and the national ethics committee for animal experiments (GZ: 68.205/0042 – BrGT/2006).

In risk assessment feeding studies, both, inbred and outbred mouse strains have been used. In some cases the possibility is addressed that a less fertile strain should be the strain of choice for testing potential effects on reproduction, since strains with low fertility are more susceptible to potential effects on reproduction. On the other hand a good breeding performance is necessary especially for the RACB design to ensure enough offspring for data collection and for continuing multigeneration studies. Therefore the fertile outbred mouse strain OF1/SPF was chosen to ensure good breeding success and provide a diverse genetic background for potential feed impacts beyond fertility parameters. For the Life Term Study the same strain was used.

The mice were kept in macrolon cages III in an animal experimental unit of the Institute of Nutrition, University of Veterinary Medicine, Vienna. Average room temperature was 23°C. A light program was installed for a 12 hour day/night cycle. Health status of mice was checked daily by a veterinarian.

2.4. Multigeneration study (MGS)

2.4.1. Performance and reproduction data

2.4.1.1. Data collection

The parental generation (F0) was fed since birth with either 33% genetically modified diet or the 33% isogenic maize variety and 4 generations were bred (Table 6). Eighteen -24 pairs from these groups were randomly paired at the age of 7 weeks. After one week of mating the males were sacrificed.
The offspring stayed with the parents and were weaned after 3 weeks. Then male and female pups were separated and raised until sexual maturity. Again random allotment of pairs and mating occurred at the age of 7 weeks.
Feed was given ad libitum throughout the study. The collected data were divided into parental data (Table 7) and data from offspring (Table 8)

The animals were weighed on a high precision analytical balance with animal weighing modus (AND GF 300EC, Ehret, Tulln, Austria).

Table 6: **Overview of succession of generations**

F0 parents
F1 offspring
F1 parents
F2 offspring
F2 parents
F3 offspring
F3 parents
F4 offspring

Table 7: **Data collection of parental mice per generation**

Performance data	**Reproduction data**
Feed intake [g]	Deliveries/group
Females body mass [g]	at birth
mating	Litter size distribution
1 week after mating	Number of pups
delivery	at birth/pair
1 week after delivery	at birth/group
2 weeks after delivery	at weaning/ pair
3 weeks after delivery	at weaning/ group
Males body mass [g]	Pup losses/group
mating	birth to weaning
1 week after mating	Pup losses/group [%]
	Birth to weaning

Table 8: **Data collection of the offspring per generation**

Performance data
Litter mass [g]
birth
1/2/3/4/5/6 d after birth
7/14/21 d after birth
Individual pup mass [g]
Birth
7/14/21 d after birth
Individual female pup mass [g]
4/5 week after birth
Individual male pup mass [g]
4/5 week after birth
Weight gain [g]
Birth to weaning

2.4.1.2. Statistics

Normal distribution was tested with Kolmogorov-Smirnov-test and *t* test was performed for feed intake, individual body mass, litter mass, number of pups, pup losses and weight gain comparison. Frequencies of deliveries per group were assessed using χ^2-test. The homogeneity of variances was evaluated by the Levene test. Further comparison of intra-group performance over the generations was done by two way analysis of variance and Duncan's post hoc test.
A difference was considered statistical significant at $p < 0.05$. Statistical significance was tested between the groups GM versus ISO and ISO versus A REF.

2.4.2. Organ weights

2.4.2.1. Data collection

Five male and 5 female pups of the F2, F3 and F4 generation were randomly chosen at the age of approximately 5 weeks and sacrificed for organ weight. Thus the mice were 3 weeks suckling to the dam and 2-3 weeks consuming the test diet.
After fasting for 2 hours mice body weights were recorded and they were killed by vertebral dislocation. Liver, spleen, kidneys and testes were carefully removed, connective tissue dissected and organs immediately weighed on a high precision analytical balance (measures masses to within 0.0001 g) (AND GF 300EC, Ehret, Tulln, Austria).
Absolute organ weight was recorded and relative organ weight was calculated:
Relative organ weight [%] = (absolute organ weight/ body weight) * 100

2.4.2.2. Statistics

Normal distribution was tested with Kolmogorov-Smirnov-test and *t* test was used for group comparison.
A difference was considered statistically significant at $p < 0.05$. Statistical significance was tested between the groups GM versus ISO and ISO versus A REF.

2.4.3. Histology

2.4.3.1. Data collection

Sample collection
The organs for histological investigation included the intestine, kidneys, liver, pancreas, spleen, lung and testes of 5 males and females per group from randomly chosen mice of the F3 generations at the age of 7 weeks.

Fixation and staining method
The tissues were processed in 4 steps to obtain microscopic sections. Immediately after removal from the body the organs were fixed in formalin (Sigma-Aldrich, Steinheim, Germany) to prevent autolysis. The tissue is fixed by cross-linkages formed in the proteins without harming the structure of proteins, so that antigenicity is not lost and the same tissue preparations can also be used for immune histochemistry. To remove the water the tissues were placed in a series of alcohols (70% to 95% to 100%). The dehydrant was then substituted by limolene, which is miscible with the embedding medium paraffin. The dehydration steps were done in an automated tissue processor. The tissues that came off the tissue processor were still in the cassettes and had to be put manually into the blocks by picking them out of the cassette and pouring molten paraffin over them. Now the tissues could be aligned properly in the paraffin block. After cooling the paraffin blocks were ready for sectioning. On a microtome (Mikrom HM 400, Mikrom, Heidelberg, Germany) with disposable knives the tissues were cut into sections (3 µm) which were floated in a warm water bath to remove wrinkles. The slices were picked up and placed on slides.
The slides were placed over night in a 37°C warm oven to dry and help the sections adhere to the slides. The embedding process had to be reversed to remove the paraffin and allow water soluble dyes to penetrate the section. Therefore before staining was done the slides were deparaffinised by running them through limolene to alcohols to water. The routine stain of haematoxylin and eosin (H and E) was used. Haematoxylin is a basic dye and has an affinity to the nucleic acids of the cell nucleus. Eosin is an acidic dye with an affinity to cytoplasmic components of the cell. Nuclei appear blue, the cytoplasm pale red, muscle fibres and erythrocytes red. The stained slide was again taken through a series of alcohol solutions to remove the water and through clearing agents before covered with a thin glass cover slip. Pieces of the gut tissues were snap frozen in liquid nitrogen.

Histological evaluation
Slides were investigated with light microscope (Reichert-Jung Polyvar, Nussloch, Germany) and traits investigated are shown in table 9.

Table 9: **Histological evaluation of the different anatomical sides**

Intestine	
	Villus, crypt structure and enterocytes
	Infiltration of leukocytes
	Pathological changes (ulceration, oedema, fibrosis, hyperplasia)
Kidneys	
	Epithelia of tubuli
	Mineralisation
	Infiltration of leukocytes
	Pathological changes (ulceration, oedema, fibrosis, hyperplasia)
Liver	
	Hepatocytes
	Glycogen accumulation
	Bile ducts
	Infiltration of leukocytes
	Pathological changes (necrosis, oedema, fibrosis, hyperplasia)
Spleen	
	Pathological changes (necrosis, oedema, fibrosis, hyperplasia)
Pancreas	
	Pathological changes (necrosis, oedema, fibrosis, hyperplasia)
Lung	
	Pathological changes (necrosis, oedema, fibrosis, hyperplasia)
Testes	
	Pathological changes (necrosis, oedema, fibrosis, hyperplasia)

2.4.4. Immunohistochemistry

2.4.4.1. Data collection

For the investigation of the intestinal immune system, immunohistochemical staining of CD3+ lymphocytes (representing the T cell line) was performed.

Sample collection
Same mice used for microscopic evaluation were used for immunohistochemical evaluation. Samples were taken immediately after slaughter. The small intestine (duodenum and jejunum, excl. Ileum) was dissected and divided into four segments of the same length. From each angular point a 1 cm segment was placed in 4% paraformaldehyde.

Fixation and staining method
CD3
The tissue samples of the small intestine were embedded in paraffin wax and cut with a slide microtome (Mikrom HM 400, Mikrom, Heidelberg, Germany) into slices of 2 µm and mounted on coated slides (Superfrost®, Menzel, Braunschweig, Germany). After deparaffinisation with NeoClear (Merck, Darmstadt, Germany) and ethanol (Sigma-Aldrich, Steinheim, Germany) antigen retrieval was performed by placing the slides in a microwave oven (2 x 5 min at 750 W) submerged in a sodium citrate buffer (0.01 M, pH 6, Sigma-Aldrich). After washing with phosphate buffered saline (PBS, Sigma-Aldrich) the endogen peroxidase was blocked by immersion in 1.5 % H2O2 in methanol (both Sigma-Aldrich) for 30 min. After the application of the normal serum (Normal goat serum, Vector, Burlingame, USA), diluted 1:10, the samples were left in a humidified chamber at room temperature for 45 min. The serum was then removed and the primary antibody (polyclonal rabbit anti-human T cell CD3, Code No. A 452, DakoCy-

tomation, Glostrup, Denmark) was applied (dilution 1:200). Incubation was done overnight in a humidified chamber at 4 °C.
On the next day the slides were washed with PBS and then incubated with the secondary antibody (biotinylated goat anti rabbit IgG, Vector, Burlingame, USA) diluted 1:200. After 30 min the streptavidin-peroxidase (Vectastain ABC Kit, Vector, Burlingame, USA) was applied and the slides were left in the humidified chamber at room temperature for 60 min. For detection of bound antibodies, a diaminobenzidine (DAB) kit was used (Vector, Burlingame, USA). Slides were counterstained with Mayer's haemalaun (Merck), dehydrated with ethanol (Sigma-Aldrich) and NeoClear (Merck), dried and sealed with a cover slip.

CD20
Preparation and staining were performed as described for CD3. The blocking serum was obtained from goat (Normal goat serum, Vector, Burlingame, USA). As primary antibody the polyclonal goat anti mouse CD20 (M-20): sc-7735 (Santa Cruz Biotechnology, Inc., Santa Cruz, USA) was used in a dilution of 1:100, as the secondary antibody a biotinylated rabbit anti goat IgG (DakoCytomation, Glostrup, Denmark), diluted 1:200, was used.

Macrophages
Preparation of slides was done as described for CD3. For antigen retrieval, samples were incubated with 1 mg Protease (Sigma-Aldrich, Steinheim, Germany P5147) /ml PBS* for 20 min at room temperature.
For staining, MAC387 was used as the anti- macrophage antibody (Thermo Fisher Scientific, Fremont, CA, USA: diluted 1:75). After incubation with the secondary antibody, 100 µl ABC Elite working solution (Vector, Burlingame, USA) were applied and left for 30 min. For development of staining, diaminobenzidine (DAB) was used. Samples were counterstained with haemalaun, dehydrated and sealed with a cover slip.

Analysis
The stained samples were checked microscopically (Reichert-Jung Polyvar, Nussloch, Germany). Digital pictures of all specimens were taken with a Nikon DN 100 Digital Net Camera with control unit (Nikon Corporation, Chiyoda-ku, Tokyo, Japan) using the program EclipseNet, version 1.16.3 (Laboratory Imaging, Praha, Czech Republic). Analysis was performed with Ellipse 2.0.6.1 (ViDiTo Systems, Kosice, Slovakia) using stereological counting rules.

2.4.4.2. Statistics

Normal distribution was tested with Kolmogorov-Smirnov-test. Statistical significance was tested with t test between the group GM versus ISO and ISO versus A REF.
Further comparison of intra-group performance over the different anatomical intestinal sites was done by two way analysis and Duncan's post hoc test. A difference was considered statistical significant at $p < 0.05$.

2.4.5. Ultrastructural investigations

2.4.5.1. Data collection

Liver, pancreas and spleen were taken from 5 male and 5 female mice of the F3 generation to perform ultrastructural comparisons. For the morphometric analyses of the nuclear components liver, pancreas and spleen were cut in 0.5 mm pieces. The samples were immersed in 2.5% glutaraldehyde and 2% paraformaldehyde with 0.1 M Soerensen phosphate buffer (pH 7.4) at 4°C for 3 h, washed in buffer solution, postfixed with 1% osmium-tetroxide and 1.5% potassium-ferrocyanide at 4°C for 1 h, dehydrated in a graded series of ethanol and embedded in Epon. Ultrathin sections (60-80 nm) were mounted on cupper slot grids coated with formvar in dioxane, stained with uranyle acetate and lead citrate (Reynolds 1963) and examined in a TEM Zeiss EM 902.
Morphometrical measurements were carried out on 270 micrographs of nuclei (3 per animal and organ) using the image analysis program ImageJ 1.38X. Area and perimeter of the nuclei were measured and opposed to the circumference of the equivalent circle to calculate the nuclear shape irregularity (the radius r of the equivalent circle is given by $r = \sqrt{A/\pi}$, where A is the measured area; the nuclear shape irregularity I follows from $I = P/2\pi r$, where P is the observed perimeter). Areas of nucleoli and nucleolar components - fibrillar centres (FCs), dense fibrillar component (DFC) and granular component (GC) - where measured to calculate the percentages of FC, DFC, GC per nucleolus. Moreover the nuclear pores where counted and the pore density (pores per µm membrane length) was assessed.

2.4.5.2. Statistics

Normal distribution was tested with Kolmogorov-Smirnov-test. Statistical significance was tested with t test between the group GM versus ISO and ISO versus A REF. A difference was considered statistical significant at $p < 0.05$.

2.4.6. Microarray analyses

These analyses were conducted in collaboration with Dr. Christian Guelly, Core Facility Molecular Biology, Medical University of Graz and with the scientific advisory of Prof. Ralf Steinborn, Vetomics Core Facility, University of Veterinary Medicine, Vienna.

2.4.6.1. Data collection

Sample collection
Twelve males per group of the F3 generation were randomly at the age of 7 weeks. Males were preferred to females to minimize hormonal influences. Mice were fasted for 3 hours and then sacrificed by vertebral dislocation.
The small intestine (beginning from the pylorus to the ileocaecal junction) was dissected immediately and parted into 2 fragments of the same length. To avoid any pancreatic tissue, approximately 4 cm distal the pyloric edge, a 2 cm segment of the intestine was dissected, representing the duodenal/proximal jejunal section. A 2 cm segment was also dissected 1 cm proximal and 1 cm distal of the angular point representing the distal jejunum. Finally a 2 cm sample 2 cm proximal to the ileo-caeco-colic junction comprised the ileum. These tissues include a

variety of cell lineages (epithelial, immune, endothelial, etc.) and were already described in ABC transporter expression analyses along the intestinal tract (Mutch et al. 2004).
Every tissue sample was immediately shock frozen in methyl-butane (Merck, Darmstadt, Germany) on liquid nitrogen. Then the samples were stored in cryo tubes (Bertoni, Vienna, Austria) at liquid nitrogen until further analyses. Simultaneously intestinal samples close to the sampling side were taken for microscopic reassurance and fixed in 4 % paraformaldehyde.

mRNA and miRNA purification
Prior to RNA purification, 50mg intestinal tissue samples were homogenised in 700µL Qiazol Lysis Reagent (Qiagen, Hilden, Germany) with ceramic beads (Magna Lyser Green Beats, Roche, Basel, Switzerland) for 20 s in a Magna Lyser (Roche). Intermittent storage of the homogenate was done in a deep-freezer at -80 °C.

The extraction of mRNA and miRNA occurred in one step by using a column-based RNA isolation kit (miRNEasy Kit, Qiagen) according to the manufacturer's instructions. Briefly, after storage 700 µL frozen homogenized lysates were incubated at 37°C for 2 min in a water bath (Julabo, Seelbach, Germany) so that lysates were completely thawed and salts dissolved. After adding 140 µL chloroform (Merck) to the lysate, the tube was shaken vigorously for 15 s and incubated for 2 min at room temperature. Next the tubes were centrifuged for 15 min at 12.000 x g at 4 °C (Centrifuge 5417 R, Eppendorf, Hamburg, Germany). The upper aqueous phase containing the RNA was carefully transferred into a new collection tube. Then the purification of RNA was automated on the QIAcube (Qiagen). Sample preparation using the QIAcube follows the same steps as the manual procedure (i.e., lyse, bind, wash, and elute). Total RNA including miRNA was finally diluted in 40 µL RNAse free water.
RNA concentrations were determined in a BioPhotometer (Eppendorf) and yields ranged from 0.5- 2 µg/µL. Only samples with an extraction value of ratio 260/280 at 1.9 were accepted. Subsequently, samples were examined for RNA integrity with an Agilent Bioanalyzer (Agilent Technologies, Santa Clara, USA) as previously described. Three biological-technical replicates per group from distal jejunum were selected with similar RIN between the groups ranging from 8.5 to 10 for the microarrays.

cDNA synthesis
DIG-labelled cDNA probes were generated by reverse transcription of 40µg total RNA using the chemiluminescent RT-Labelling kit (Applied Biosystems, Foster City, CA, US) as described by the protocol. Array hybridization, chemiluminescence detection, image acquisition and analysis were performed using Applied Biosystems Chemiluminescence Detection Kit and Applied Biosystems 1700 Chemiluminescence Microarray Analyzer following the manufacturer's instructions.

Array hybridisation
Briefly, each microarray was first pre-hybridized at 55°C for 1hr in hybridization buffer with blocking reagent. Oligo-dT-primed, DIG-labelled cDNA targets were fragmented mixed with internal control target and then hybridized to the equilibrated microarrays in a volume of 1.5ml at 55°C for 16 hrs. After hybridization, the arrays were washed with hybridization wash buffer and chemiluminescence rinse buffer. Enhanced chemiluminescent signals were generated by incubating

arrays with Alkaline Phosphatase conjugated anti-digoxigenin antibody followed by incubation with chemiluminescence Enhancing Solution and a final addition of chemiluminescence substrate. Four images were collected for each microarray using the ABI 1700 Chemiluminescent Microarray Analyzer. Images were auto-gridded and the chemiluminescence signals were quantified, corrected for back-ground and spot and spatially normalized.

Data Analysis
Data analysis was performed using GeneSpring 7.3.1 software (Agilent Tech-nologies). Normalization and data transformation: Per Chip normalization was done to the 50th percentile followed by median Per Gene normalization. Only if a certain gene tag was found to be expressed in all 3 biological replicates of a group (Signal to Noise >3 in 3 of 3 experiments) the gene was considered as "expressed" in the referred group. The final gene set used for statistical analysis contained the combined "expressed" gene sets derived from both of the treat-ment groups. Groups were compared pair wise using Welch t-test. The following pair wise comparisons were performed: GM vs ISO and A REF vs ISO. A p-value <0.05 was considered significant. Additionally, a minimum fold-change filtering with a cut-off value of 2 was applied. Comparisons drawn were:
1. GM vs ISO
2. ISO vs A REF
3. GM vs. ISO and A REF. This is regarded as a pilot study and is justified by the criteria that both, ISO and A REF do not contain the genetic modification. Para-metric test was performed with variances not assumed to be equal (Welch t-test) with a p-value of 0.05 and without multiple testing correction. Default Interpreta-tion - Genes from SN>3_QCd with statistically significant differences among the following groups based on values of 'Experiment Type': ISO ("ISO+A REF"=> n=6), GM.

2.4.6.2. Statistics

Further analysis in PANTHER
For further analyses, the gene list with the differentially expressed genes ($p < 0.05$) was compared in the PANTHER database (http://www.pantherdb.org) and genes were allocated to biological processes and pathways.

The first column contains the name of the PANTHER classification category. The second column contains the number of genes in the reference list (Mouse AB 1700 genes) that map to this particular PANTHER classification category. The third column contains the number of genes in the uploaded list that map to this PANTHER classification category.
The fourth column contains the expected value, which is the number of genes expected in the list for this PANTHER category, based on the reference list. The fifth column has either a + or -. A plus sign indicates over-representation of this category in the experiment: more genes are observed than expected based on the reference list (for this category, the number of genes in the list is greater than the expected value). Conversely, a negative sign indicates under-representation. The sixth column is the p-value as determined by the binomial statistic. This is the probability that the number of genes observed in this cate-gory occurred by chance (randomly), as determined by the reference list. A low p-value indicates that the number observed is significant and potentially interest-ing. A cut-off of 0.05 was used as a starting point.

2.4.7. q-RT-PCR

Gene expression was analysed with custom TaqMan low density arrays (TLDA) (Applied Biosystems). The genes ($p<0.05$) for the TLDAs were selected according to higher fold changes (cut off 2) and for a possible group classification. Out of those genes 17 genes could be clustered to the Protein Metabolism and Modification and 3 genes to the signal transduction, further selected genes belonged to not specified pathways (Table 10). Endogenous controls were *Hprt1*, *Tbp* and *18s-rRNA*.
The same gene setup was used for the RACB intestinal samples.

Table 10: **Genes on the TLDA fulfilling with a 2-fold deregulation ($p<0.05$) between ISO vs GM groups**

Biological process	**Deregulated genes**
Protein Metabolism & Modification	*1810064L21Rik, Ntrk2, Gga1, Pum1, Sgta, Clk3, 2610529C04Rik, Eef1b2, Gsk3b, Herc3, Rpl22, Eef2k, Gspt2, Prkcn, Dnajc1, Trim47, Fkbp5*
Signal Transduction	*Ramp1, Ift140, Nphp1*
Other diverse pathways (not specified)	*Igtp, Adpn, Itga5, Bcar3,Elmo2, Gpr39, Shc1, Socs1, LOC433259|,Csf3r, Irs1, Aatf, Cd40, Bmyc, Per3, Stat5b, Mapk10, Sntb2, Fbxw7, Cd68, Hmg20a, Ccnh, Btla, Foxq1, Kif3c, Rgs6*

Samples (n=6, only male mice) with a RNA Integrity Number (RIN) > 7 were used for qRT-PCR. The RNA was diluted in water to a concentration of 0.2µg/µl. The cDNA was synthesised using the High-Capacity cDNA reverse Transcription Kit (Applied Biosystems) according to the manufacturer's protocol. The samples were incubated at 25 °C for 10 min followed by 120 min at 37 °C. The reaction was stopped by exposing the samples to 85 °C for 5 s.
For validation of the reverse transcription a qPCR targeting HPRT was performed using the fluorescent dye EvaGreen (Biotium, Hayward, USA), to determine whether the reverse transcription worked or not. A ΔCt range of two was chosen as an arbitrary quality cut-off. 2 µl of cDNA was mixed with 3.5 mM MgCl2, 200 nM of each primer 0.4 x EvaGreen, 1 U Hot Firepol® DNA polymerase (Solis Biodyne, Tartu, Estonia), 0.2 mM dNTP mix and 0.08 M Tris-HCl, 0.02 M (NH4)2SO4, 0.02% w/v Tween-20. Thermocycling was done on a RotorGene 6000 (Corbett Life Science, Sydney, Australia). After a hot start phase of 15 min at 95 °C, 37 cycles of 95 °C for 10 s, 60 °C for 40 s and 70 °C for 15 s were run. After thermocycling a melting curve was measured starting at 65 °C and using the parameters given by the Rotor-Gene-6000-Series-Software 1.7. The sample cDNA was amplified in duplicate, the minus reverse transcription control was run in unicate. The ct values of all samples lay within 1.78 cycles.

cDNA samples were diluted to 100 ng/µl in nuclease-free water. 50µl of the diluted cDNA was mixed with 50µl of TaqMan Gene Expression Master Mix (Applied Biosystems) and transferred into the fill-ports of the TLDA (1 sample/ port). The micro fluidic cards were then centrifuged for one minute at 331 x g twice in a Heraeus Multifuge 3S-R Centrifuge (DJB Labcare Ltd, Newport Pagnell, England). TLDA were run on an ABI 7900HT Sequence Detection System (Applied Biosystems).
The conditions of the qRT-PCR were given by the supplied run-template file. 2 min at 50°C were followed by10 min at 94.5°C. This hot start was followed by 45 cycles of 30 s at 97°C and 1 min at 59.7°C.

After setting the cut-offs and thresholds for each gene separately the Cts were loaded into MS excel for further processing. First the most stable genes were determined using the GeNorm software (Vandesompele et al. 2002). The Cts were calibrated gene-wise by subtracting the lowest Cts from the individual Cts. Raw expression values were obtained by using the formula:

$$REV = \frac{1}{2^{Ct}}$$

The raw expression values were loaded into GeNorm and the most stable genes and the suggested number of genes for normalization were calculated. The Cts of the appropriate genes for Normalization were loaded into the BestKeeper software, to obtain an artificial gene that could be used as a normaliser, called BestKeeper (Pfaffl et al. 2004). The Cts of the genes and the BestKeeper were copied into the REST 2005 software tool (Pfaffl et al. 2002). By using a Pair Wise Fixed Reallocation Randomisation Test REST creates a much sharper statistic than the commonly used ANOVA test, allowing one to minimize the chance of type I errors.

2.4.8. miRNA analyses

2.4.8.1. Data collection

The same RNA samples as in 2.5.6. were used for miRNA profiling.

miRNA profiling
An extern company which is specialized in miRNA profiling was contracted. Array synthesis and validation, hybridisation and detection as well as data analysis were performed by febit biotech gmbh (Heidelberg, Germany) using the companies Geniom Biochip for murine miRNA detection. The chip targeted all 460 major mature miRNA of mouse contained in the latest version of the miRBase 10 database (http://microrna.sanger.ac.uk, visited September 2007).

2.5. Life term study (LTS)

2.5.1. Feed intake and body mass survey

2.5.1.1. Data collection

10 female mice per group were randomly allotted from the F1 generation, thus being already born from dams that were fed either 33% genetically modified corn or transgenic corn in the diet from the onset of pregnancy. Feed intake was recorded weekly and body mass was measured biweekly.

2.5.1.2. Statistics

Normal distribution was tested with Kolmogorov-Smirnov-test and *t* test was used for inter group comparison. A difference was considered statistically significant at a $p < 0.05$. Statistical significance was tested between the groups GM versus ISO and ISO versus A REF.

2.5.2. Survival

2.5.2.1. Data collection

Death was noted per group and cross necropsy as well as histopathology was performed by a pathologist from the Institute of Pathology and Forensic Veterinary Medicine, University of Veterinary Medicine Vienna.

2.5.2.2. Statistics

Descriptive statistics and Kaplan-Meier survival test were performed.

2.6. Reproductive Assessment by Continuous Breeding (RACB)

2.6.1. Performance and reproduction

2.6.1.1. Data collection

Data from Reproductive Assessment by Continuous Breeding (RACB) studies are used for risk assessment processes. The RACB design has been used for instance by the US National Toxicology Program (NTP) since 15 years, so far to establish any possible adverse effects of chemical compounds on the reproductive performance of the test animals. In these cases a short dose-finding-range study is performed in advance. According to the Environmental Protection Agency (EPA), risk assessments of the plant pesticide Bt delta-endotoxin needed no threshold regulation, since it revealed no toxic effects in acute toxicity testing and its exposure level is very low. Therefore no dose-related diets have been tested.

For the RACB test in this study 24 breeding pairs of mice per group were chosen at random. Exposure started 1 week prior to cohabitation to allow for diet adaption. Then the animals were housed as breeding pairs until the end of the experiment after 20 weeks. During this time 4 litters (Table 10) were bred approximately 3-4 weeks apart, which were left with the parents until weaning after 3 weeks. This approach differs from the established RACB, where all newborns are killed immediately after birth except for the last litter. The pups of all litters were kept alive in this study to obtain more data on pup development during lactation. These data present valuable information since the susceptibility of growing organisms to potentially adverse dietary effects is known to be much higher than in adults.
Data collected were divided into parental data (Table 11) and offspring data (Table 12).

Weighing was done using a high precision analytical balance with animal weighing modus (AND GF 300EC, Ehret, Tulln, Austria).

Table 11: **Succession of generation in the RACB**

F0 parents
1. litter
2. litter
3. litter
4. litter

Table 12: **Data of parental mice collected in the RACB**

Performance data	**Reproduction data**
Feed intake [g]	Deliveries/group
Females body mass [g]	at birth
mating	Litter size distribution
1 week after mating	Number of pups
delivery	at birth/pair
1 week after delivery	at birth/group
2 weeks after delivery	at weaning/ pair
3 weeks after delivery	at weaning/ group
Males body mass [g]	Pup losses/group
mating	birth to weaning
1 week after mating	Pup losses/group [%]
Delivery of female	Birth to weaning
1 week after delivery of female	Birth interval
2 weeks after delivery of female	Period from one delivery to the next
3 weeks after delivery of female	

Table 13: **Data of offspring collected in the RACB**

Performance data
Litter mass [g]
birth
1/2/3/4/5/6 d after birth
7/14/21 d after birth
Individual pup mass [g]
Birth/7/14/21 d after birth
Weight gain [g]
Birth to weaning

2.6.1.2. Statistics

Normal distribution was tested with Kolmogorov-Smirnov-test and for inter group comparison of feed intake, individual body mass, litter mass, number of pups, pup losses and weight gain the *t* -test was used. Frequencies of deliveries per group were assessed using χ^2-test. Homogeneity of variances was investigated with Levene test.
Further comparison of intra group performance over the generations was done by two way analysis of variance and Duncan's post hoc test.
A difference was considered statistical significant at $p < 0.05$. Statistical significance was tested between the groups GM versus ISO.

2.6.2. q-RT-PCR

The RACB intestinal samples were investigated by q-PCR technique. The same protocols as described in part 2.4.7. were applied. For q-RT-PCR samples from male and female mice of the distal section of jejunum were used.

3. Results

3.1. Diet

3.1.1. Harvest in 2005 (diets for MGS and LTS)

3.1.1.1. Test on the genetic modification

Test at protein level
The transgenic corn was positive for the genetic modifications whereas the control and reference corn were negative. Quantification was difficult as the ELISA was not designed for quantitative approach. Semiquantitative analysis revealed 0.11-0.24 µg Cry1Ab / g corn (fresh weight).

Test at DNA level
The NK603 x MON810 maize was tested positive on the presence of 35S and nos in the screening. The positive sequences were specific for the maize line NK603 and MON810. Furthermore the control maize was slightly positive for 35S. The A REF corn was tested negative (Table 14).

Table 14: **Test on genetic modification with PCR**

sequence	corn		
	ISO	GM	A REF
35S-Promotor	0.25% pos.	100% pos.	neg.

3.1.1.2. Crude nutrients and gross energy

There was no difference in the content of crude nutrients and energy (Table 15 and 16). Additionally, the analyses of the diets meet the nutritional standards for mice in reproduction and crude nutrients are in accordance to the manufacturers' declaration.

Table 15: **Crude nutrients and gross energy in the corn**

	corn		
%	ISO	GM	A REF
DM	90.4	89.8	88.9
XA	1.4	1.2	0.9
EE	3.6	3.7	3.9
XP	9.4	9.9	9.3
XF	3.4	3.4	3.3
NfE	72.6	71.6	71.5
GE/kg	17.0	17.0	17.0

Table 16: **Crude nutrients and gross energy in the diets**

	diet		
%	ISO	GM	A REF
DM	90.5	90.6	90.3
XA	6.8	6.4	5.8
EE	5.0	5.1	5.1
XP	25.8	23.4	24.5
XF	6.0	5.8	6.3
NfE	46.9	50	48.6
GE/kg	17.5	17.5	17.7

3.1.1.3. Minerals and trace elements

Minerals and Trace elements are shown in table 17 and table 18.

Table 17: **Minerals and trace elements in the corn**

		corn		
	Unit	ISO	GM	A REF
Ca	%	0.02	0.03	0.04
P	%	0.23	0.21	0.28
Na	%	0.07	0.08	0.12
Mg	%	0.10	0.11	0.10
Zn	mg/kg	15.0	16.7	26.9
Cu	mg/kg	2.5	2.9	not invest.
Fe	mg/kg	28.5	29	55.3

Table 18: **Minerals and trace elements in the diets**

		diet		
	Unit	ISO	GM	A REF
Ca	%	1.17	1.13	1.04
P	%	0.93	0.98	0.91
Na	%	0.46	0.37	0.34
Mg	%	0.21	0.24	0.20
Zn	mg/kg	37.90	44.40	44.2
Cu	mg/kg	12.90	9.50	not invest.
Fe	mg/kg	102.20	90.60	137.5

3.1.1.4. ß-Carotene, Vitamins A and E

Carotene and vitamin analyses are shown in table 19 and table 20.

Table 19: **Carotene and vitamin analyses in the corn**

		Corn		
	Unit	ISO	GM	A REF
ß-Carotene	mg/kg	1.8	1.7	1.3
Vit. E	mg/kg	1.9	1.2	2.4

Table 20: **Vitamin analyses in the diets**

		diet		
	unit	ISO	GM	A REF
Vit. A	IU/kg	7049.0	9857.0	17773.0
Vit. E	mg/kg	114.2	91.2	140

3.1.1.5. Fatty acids

Fatty acid analyses of the corn are shown in table 21.

Table 21: **Fatty acid profile of corn (g 100g^{-1} total fatty acids)**

	corn		
Fatty acid	ISO	GM	A REF
C 16:0	11.26	10.03	7.66
C 18:0	2.68	1.62	1.45
C 18:1 *n*-9	23.40	24.50	17.16
C 18:2 *n*-6	48.52	48.14	45.67
C 18:3 *n*-3	1.00	0.98	1.04

3.1.1.6. Amino acids

Amino acids are shown in table 22.

Table 22: **Amino acids of the diet**

%	diet ISO	GM	A REF
Aspartic acid	19.05	20.18	20.28
Threonine	9.75	10.29	10.63
Serine	11.07	11.62	11.67
Glutamic acid	26.14	28.92	28.74
Glycine	7.70	8.21	8.43
Alanine	9.01	9.49	9.58
Cystine	3.70	3.68	3.66
Valine	10.44	11.31	11.70
Methionine	7.44	8.02	8.32
Isoleucine	8.86	9.39	9.84
Leucine	17.87	19.31	19.33
Tyrosine	8.43	9.19	9.03
Phenylalanine	10.99	11.56	11.81
Histidine	5.59	5.95	5.88
Lysine	12.42	13.16	13.39
Arginine	7.49	7.48	8.18
Proline	13.36	14.05	14.21
Sum	189.3	201.8	204.7

3.1.1.7. Hygienic evaluation

Total microbial counts, yeast and moulds (Table 23 and Table 24) were within limits according to the standards for mixed feed of the VDLUFA (Bucher, 2003). The mycotoxins deoxynivalenol and zearalenone were within acceptable limits for animal feed and there were no concerns of adverse effects according to guide-lines for the quality-assured production of laboratory animal diets of the Society for Laboratory Animal Science (GV-SOLAS, 2002).

Table 23: **Investigation of feed hygiene**

		corn		
	unit	ISO	GM	A REF
Total microbial count	CFU*/g	2.200.000	1.000.000	2.300
Yeasts	CFU/g	16.000	35.000	<200
Moulds	CFU/g	36.000	18.000	1.300
Deoxynivalenol	mg/kg	<0.05	0.23	<0.05
Zearalenone	mg/kg	<0.005	<0.005	<0.005

*colony forming units

Table 24: **Investigation of feed hygiene**

		diet		
	unit	ISO	GM	A REF
Total microbial count	CFU*/g	23.000	160.000	< 2000
Yeasts	CFU/g	2.400	1.600	<200
Moulds	CFU/g	3.600	36.000	<200
Deoxynivalenol	mg/kg	<0.05	0.18	<0.05
Zearalenone	mg/kg	0.01	0.01	<0.011

*colony forming units

3.1.1.8. Herbicides

No residual levels of herbicides were found in the diets, analytical procedures were targeted to the herbicides that were actually used in the production process (Table 25).

Table 25: **Evaluation of herbicide levels in the diet**

		diet		
	unit	ISO	GM	A REF
Glyphosate	mg/kg	Not tested	<0.010	Not tested
AMPA	mg/kg	Not tested	<0.010	Not tested
Dicamba	mg/kg	<0.01	Not tested	Not tested
S-Metolachlor	mg/kg	<0.01	Not tested	<0.01
Atrazine	mg/kg	<0.01	Not tested	Not tested
Terbuthylazin	mg/kg	Not tested	Not tested	<0.01

3.1.2. Harvest in 2007 (diets for RACB)

3.1.2.1. Test on genetic modification

Test at protein level
The transgenic corn was tested positive for the genetic modifications whereas the control corn was negative. Quantification was difficult as the ELISA was not designed for quantitative approach. Semiquantitative analysis revealed 0.13-0.26 µg Cry1Ab / g corn (fresh weight).

Test at DNA level
The MON810 x NK603 maize was tested positive on the presence of 35S and nos. Furthermore the control maize was positive for 35S which was slightly above the detection limit of 0.02% and limit of quantification 0.01% (Table 26). The positive sequences were characteristic for the maize line NK603 and MON810 in the declared transgenic and for MON810 in the isogenic corn. Since the isogenic corn showed insignificant traces of obviously cross polluted transgenic corn a second control group was not necessary.

Table 26: **Test on genetic modification with PCR**

	corn	
sequences	ISO	GM
35S-Promotor	<0.02% pos.	100% pos.

3.1.2.2. Crude nutrients and gross energy

There was no difference in the content of crude nutrients and energy between isogenic and transgenic corn and diet (Table 27).

Table 27: **Crude nutrients and gross energy in the corn and diets**

	corn		diet	
%	ISO	GM	ISO	GM
DM	86.1	84.7	90.1	89.1
XA	1.5	1.3	6.7	6.3
EE	3.7	3.3	5.5	4.8
XP	8.1	8.5	25.0	24.9
XF	6.8	6.1	7.8	7.3
NfE	70.3	70.6	45.5	47.4
GE/kg	17.0	16.9	17.6	17.6

3.1.2.3. Minerals and trace elements

Minerals and trace elements are shown in table 28.

Table 28: **Minerals and trace elements in the corn and diets**

		corn		diet	
	unit	ISO	GM	ISO	GM
Ca	%	0.1	0.2	1.2	1.2
P	%	0.35	0.30	0.92	0.84
Na	%	0.014	0.012	0.34	0.33
Mg	%	0.13	0.12	0.24	0.21
Zn	mg/kg	19.70	18.9	66.1	50.8
Cu	mg/kg	3.0	2.9	12.8	16.5
Fe	mg/kg	30.2	31.6	147.0	134.0

3.1.2.4. ß-Carotene, Vitamins A and E

Carotene and vitamin levels are shown in table 29.

Table 29: **Carotene and vitamins in the corn and diets**

		corn		diet	
	unit	ISO	GM	ISO	GM
ß-Carotene	mg/kg	1.2	1.4	-	-
Vit. A	IU/kg	-	-	9.365.0	13.198.0
Vit. E	mg/kg	7.4	7.8	125.0	127.0

3.1.2.5. Fatty acids

No difference of fatty acid levels was seen between the two corn lines (Table 30).

Table 30: **Fatty acid profile of corn (g 100g^{-1} total fatty acids)**

Fatty acid	ISO	GM
C 16:0	10.89	10.85
C 18:0	3.61	3.62
C 18:1 *n*-9	24.79	24.17
C 18:2 *n*-6	59.38	59.82
C 18:3 *n*-3	0.45	0.62

3.1.2.6. Amino acids

Table 31 shows the amino acids of the diet.

Table 31: **Amino acids of the diet**

	diet	
%	ISO	GM
Aspartic acid	2.4	2.5
Threonine	1.2	1.2
Serine	1.3	1.3
Glutamic acid	3.6	3.7
Glycine	0.9	1.0
Alanine	1.1	1.2
Cystine	0.3	0.3
Valine	1.4	1.4
Methionine	0.8	0.7
Isoleucine	1.2	1.2
Leucine	2.4	2.4
Tyrosine	1.2	1.2
Phenylalanine	1.4	1.4
Histidine	0.7	0.6
Lysine	1.7	1.7
Arginine	1.0	1.0
Proline	1.7	1.8
Sum	24.2	24.8

3.1.2.7. Hygienic evaluation

Total microbial count, yeast and mould were within acceptable limits according to the guidance levels for mixed feed of the VDLUFA (Bucher 2003). Zearalenone concentrations were within limits for animal feed and there were no concerns of adverse effects according to guidelines for the quality-assured production of laboratory animal diets of the Society for Laboratory Animal Science (GV-Solas 2002)(Table 32).

Table 32**: Investigation of feed hygiene**

		ISO		GM	
	unit	corn	diet	corn	diet
Total microbial count	CFU*/g	11.000.000	10.000.000	140.000	1.300.000
Yeast	CFU/g	250.000	220.000	25.000	14.000
Mould	CFU/g	150.000	50.000	24.000	3.800
Deoxynivalenol	mg/kg	0.87	0.25	0.64	0.42
Zearalenone	mg/kg	0.03	<0.005	0.048	0.02

*CFU colony forming units

3.1.2.8. Herbicides

No residual levels of herbicides were found in the diets (Table 33).

Table 33: **Evaluation of herbicide residues in the diet**

		diet	
	unit	ISO	GM
Glyphosate	mg/kg	-	<0.010
AMPA	mg/kg	-	<0.010
Dicamba	mg/kg	<0.01	Not tested
S-Metolachlor	mg/kg	<0.01	Not tested
Atrazine	mg/kg	<0.01	Not tested

3.2. Multi Generation Study

3.2.1. Performance and reproduction

The trial was conducted from November 2006 to July 2007 (Table 34).

Table 34: **Overview of succession of generation and season when performed**

Generation	pairs/group	date
F0 parents	n= 18	November 2006
F1 offspring		December 2006/January 2007
F1 parents	n= 24	January 2007
F2 offspring		February/March 2007
F2 parents	n= 24	March 2007
F3 offspring		April/May 2007
F3 parents	n= 22	Mai 2007
F4 offspring		June/July 2007

3.2.1.1. GM versus ISO

Parental performance

From the F1 parents 1 female of the ISO group and 3 females of the GM group died and in the F2 parents 1 female of the ISO group died before delivery for unknown reasons.
No difference in feed intake was seen between the two groups. The feed intake differed significantly between the generations, being highest in the F1 generation and lowest in the F3 generation (Figure 1).

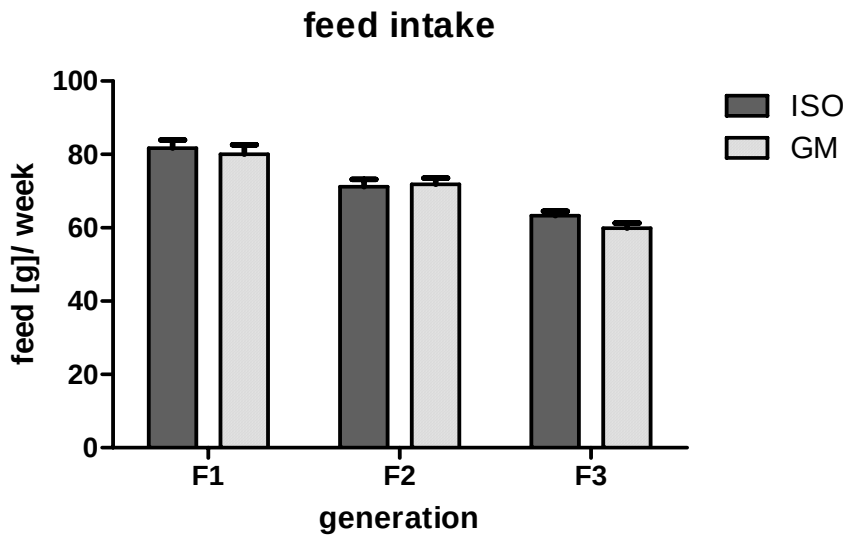


Figure 1: **Feed intake of adult mice fed 33% isogenic (ISO) or genetically modified (GM) corn in their diet in several generations**

No differences were seen in performance of the parental mice in all generations (Table 35). In the ISO group body mass of females and males at mating (F1 < F3, F2 < F0), females at delivery (F1, F3 < F0, F2) and 3 weeks after delivery (F1, F3 < F3, F2, F0) differed significantly over several generations. A similar pattern was seen in the GM group with a significant influence of generations on female body mass at mating (F1 < F0, F2, F3), at delivery (F0, F3 < F0, F1, F2) and male body mass at mating (F1, F2, F3 < F0).

Parental reproduction

No statistically significant differences were seen in reproduction data between the two feeding groups (Table 36), but litter size was influenced by generation (Figure 2). More litters with n> 8 were seen in the ISO compared to the GM group.
Within the ISO group F3 delivered significantly smaller litters than F0 and within the GM group significantly more pups were delivered in the F0 and F2 than in the F3 generation.

The number of pups at birth (except in F2 generation) and at weaning (all generations) were always lower and pup losses were always higher in the GM group but not on a significantly different level. All data showed high variations.
Over all generations about twice as many pups were lost in the GM group as compared to the ISO group (14.59% vs 7.4%).

Table 35: **Performance of mice fed 33% isogenic (ISO) or genetically modified (GM) corn in their diet over several generations**

	Parental performance							
	F0		F1		F2		F3	
Traits	ISO	GM	ISO	GM	ISO	GM	ISO	GM
Females body mass [g]								
Mating	27.21 ± 0.452	26.42 ± 0.469	20.26 ± 0.572	21.47 ± 0.590	25.87 ± 0.345	26.04 ± 0.494	25.77 ± 0.433	25.47 ± 0.465
1 week after mating	29.74 ± 0.431	29.14 ± 0.462	26.92 ± 0.451	27.25 ± 0.468	- -	- -	27.85 ± 0.418	26.73 ± 0.428
Delivery	35.86 ± 0.571	34.62 ± 0.749	33.27 ± 0.533	36.02 ± 1.332	35.65 ± 0.642	35.64 ± 0.577	33.54 ± 0.699	32.10 ± 0.977
1 week after delivery	37.00 ± 0.919	38.00 ± 0.782	36.62 ± 0.675	36.94 ± 0.659	38.55 ± 0.699	38.01 ± 0.746	36.66 ± 0.724	34.88 ± 1.671
2 weeks after delivery	37.31 ± 1.207	37.88 ± 1.360	37.24 ± 0.993	38.06 ± 0.683	37.35 ± 0.974	38.80 ± 0.635	36.83 ± 0.578	35.59 ± 0.676
3 weeks after delivery	36.77 ± 1.134	35.61 ± 0.791	33.77 ± 0.789	35.07 ± 0.720	36.55 ± 0.592	36.80 ± 0.727	34.47[a] ± 0.893	36.36 ± 0.733
Males body mass [g]								
Mating	34.02 ± 0.741	34.52 ± 0.703	29.02 ± 0.535	29.73 ± 0.565	31.13 ± 0.474	30.36 ± 0.694	32.27 ± 0.435	31.77 ± 0.552
1 week after mating	33.31 ± 0.533	33.59 ± 0.573	30.74 ± 0.514	31.36 ± 0.499	- -	- -	31.74 ± 0.297	30.40 ± 0.661

Values represent means and standard error

Table 36: **Reproduction data of mice fed 33% isogenic (ISO) or genetically modified (GM) corn in their diet over several generations**

	Parental reproduction							
	F0		F1		F2		F3	
traits	ISO	GM	ISO	GM	ISO	GM	ISO	GM
Pairs/group	18	18	24	24	24	24	22	22
Deliveries/group	17	18	23	22	19	21	16	14
Non deliveries/group	5.6%	0.0%	4.2%	8.3%	20.8%	12.5%	27.3%	36.4%
Number of pups at birth/pair	10.28 ± 0.980	10.22 ± 0.629	8.25 ± 0.778	7.88 ± 0.779	8.42 ± 1.025	8.92 ± 0.875	6.59 ± 1.046	5.68 ± 1.10
Sum of pups at birth/group	185	184	198	189	202	208	145	125
Number of pups at weaning/ pair	8.39 ± 0.936	7.67 ± 0.792	8.00 ± 0.766	6.96 ± 0.786	7.96 ± 0.928	7.63 ± 0.850	6.45 ± 1.040	5.23 ± 1.03
Sum of pups at weaning/ group	151	138	192	167	191	183	142	115
Sum of pup losses/group	34	46	6	22	11	25	3	10
Pup losses/group	2.06 ± 0.683	2.61 ± 0.837	0.26 ± 0.157	1.00 ± 0.510	0.58 ± 0.289	2.95 ± 0.631	0.19 ± 0.136	0.71 ± 0.322

Values represent means and standard error, means that do not share a common superscript are significantly different ($p < 0.05$)

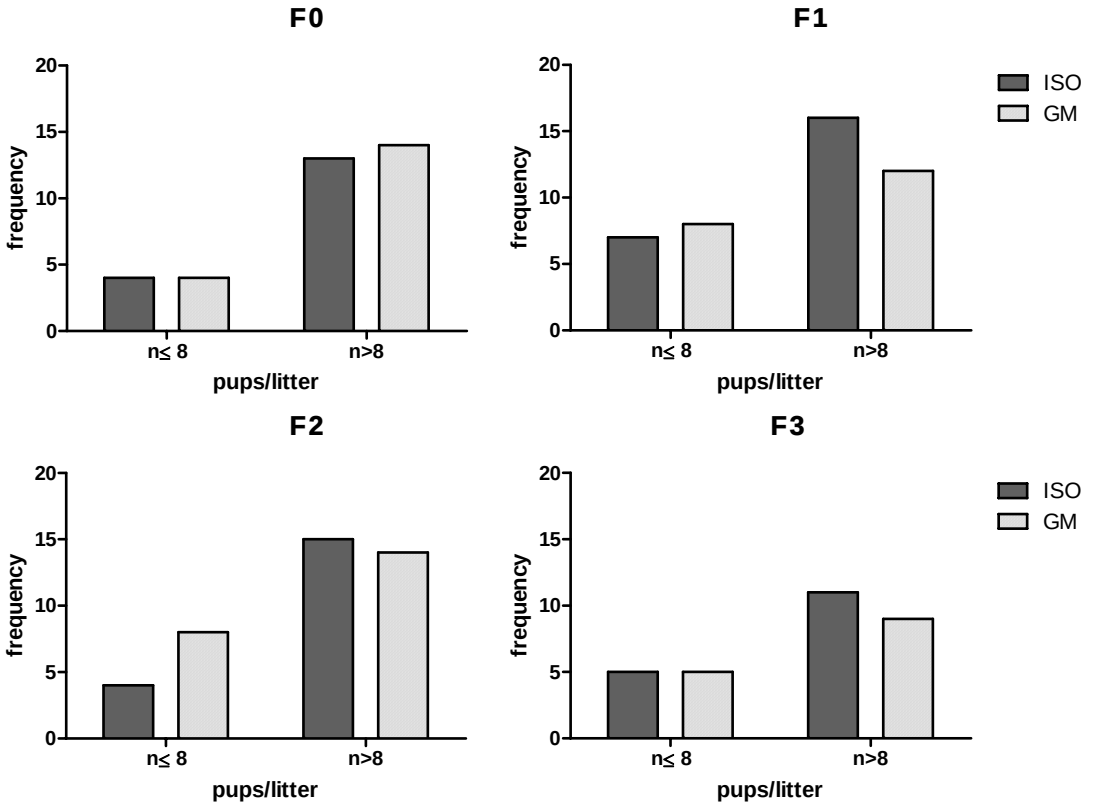


Figure 2: **Distribution of litter size at delivery from females fed 33% isogenic (ISO) or genetically modified (GM) corn in their diet in several generations**

Offspring performance

Although the average mass of whole litters was always lower in the GM group as compared to the ISO group, no statistically significant difference could be registered (Figure 3 a-c). The individual pup weights remained not significantly different (Figure 3 a'-3c') but when clustered to litter size n ≤ 8 and n > 8, significant differences were found in the individual pup mass of the small litters. Differences were inconsistent in the generations. In the F1 generation the individual pup mass at 7d was higher (p= 0.024) in the GM group, whereas in the second generation the pup weight at birth and 7d lower (p=0.027) in the GM group. Further significant differences in individual pup mass of litters n ≤ 8 were seen in the F4 generation 1, 2 and 3 weeks after birth. The average individual pup weight was lower (p= 0.055, p=0.003 and p=0.015, respectively) in the ISO than in the GM group (Table 37).

Litters > 8 pups did not differ in individual pup mass except of the F3 generation at birth with the individual pup mass being significantly higher (p=0.038) in the ISO group.

No differences were seen in individual pup mass after weaning (Table 38).

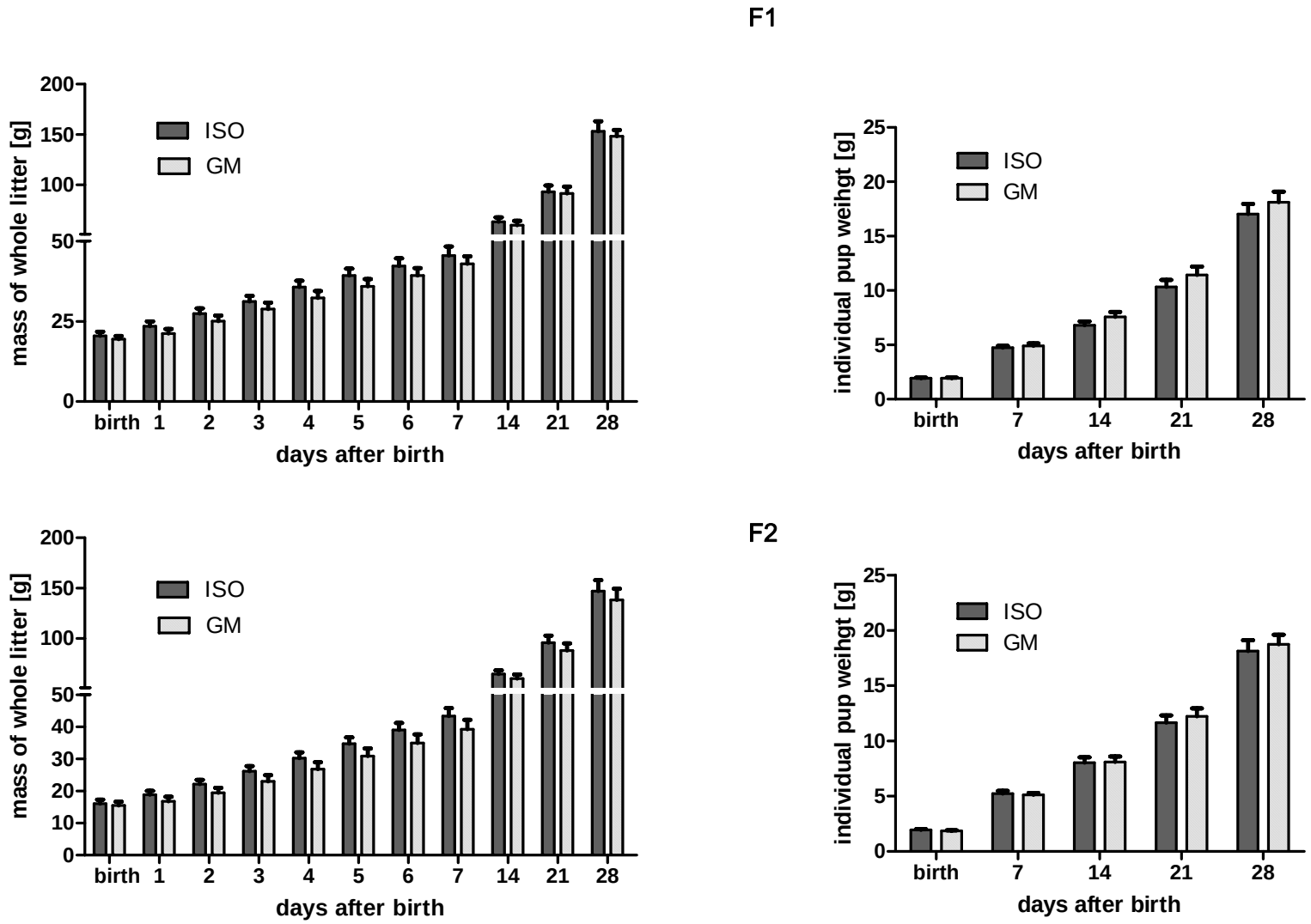
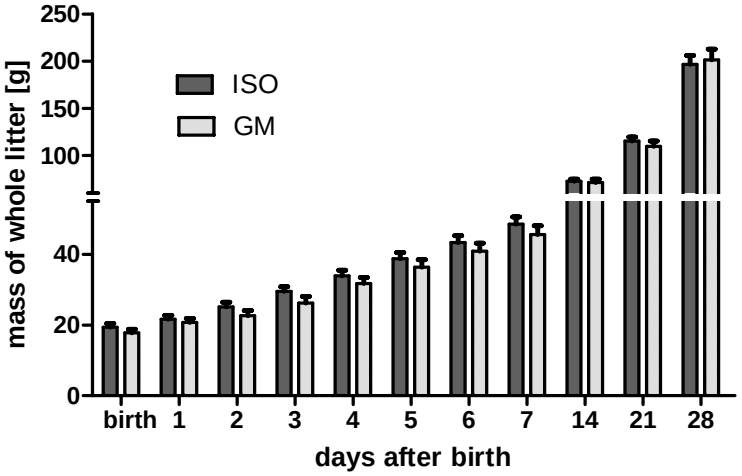
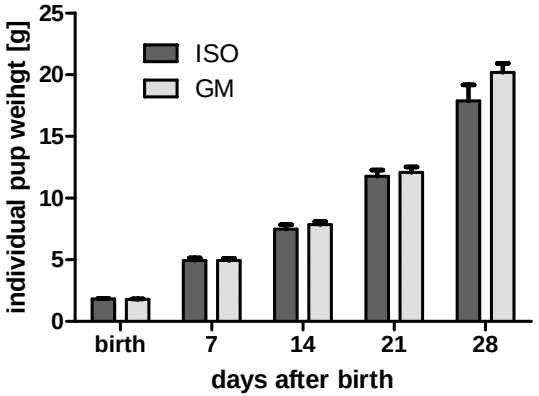


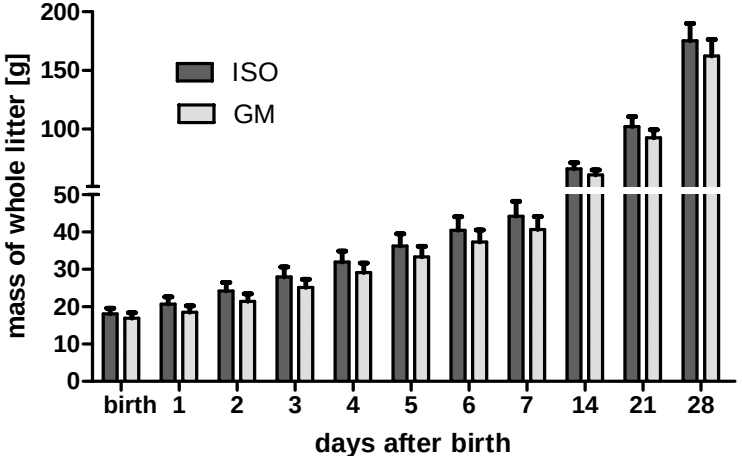
Figure 3 a-b and 3 a'-b': **Mass of whole litter [g] and individual pup weight [g] at several timepoints of the F1 and F2 generation after birth of offspring from parental mice fed 33% isogenic (ISO) or genetically modified (GM) corn in their diet**

F3





F4



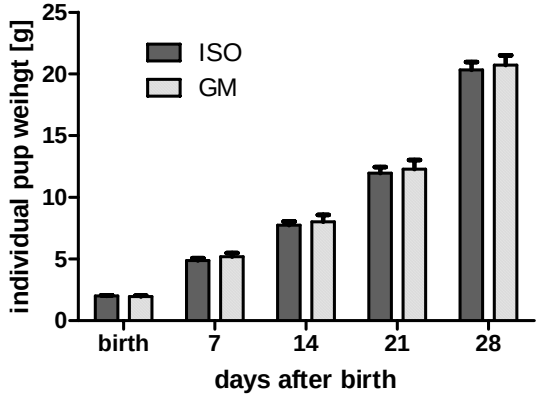


Figure 3 c-d and 3 c'-d': **Mass of whole litter [g] and individual pup weight [g] at several timepoints of the F3 and F4 generation after birth of offspring from parental mice fed 33% isogenic (ISO) or genetically modified (GM) corn in their diet**

Table 37: **Individual pup mass [g] per group at birth, 7, 14, 21 and 28 days after birth from parents with 33% of near isogenic (ISO) or transgenic(GM) corn in their diet**

	group			
	ISO n≤ 8	**GM n≤ 8**	**ISO n> 8**	**GM n> 8**
			F1 generation	
Birth	2.13 ± 0.113	2.09 ± 0.046	1.87 ± 0.059	1.88 ± 0.063
7d	5.21[a] ± 0.277	5.94[b] ± 0.104	4.54 ± 0.175	4.38 ± 0.208
14 d	7.48 ± 0.678	8.77 ± 0.702	6.51 ± 0.399	6.51 ± 0.301
21 d	12.16 ± 1.084	13.20 ± 1.275	9.50 ± 0.688	9.85 ± 0.512
28 d	19.95 ± 1.506	21.33 ± 0.928	15.70 ± 0.970	15.62 ± 0.940
			F2 generation	
Birth	2.27[a] ± 0.114	1.96[b] ± 0.059	1.81 ± 0.036	1.81 ± 0.051
7d	6.70[a] ± 0.431	5.59[b] ± 0.183	4.69 ± 0.140	4.80 ± 0.241
14 d	10.67 ± 1.002	9.75 ± 0.738	7.04 ± 0.275	6.99 ± 0.430
21 d	14.88 ± 0.878	14.08 ± 1.162	10.44 ± 0.619	10.88 ± 0.717
28 d	22.17 ± 1.261	20.54 ± 1.376	16.51 ± 1.048	17.42 ± 1.024
			F3 generation	
Birth	1.84 ± 0.101	1.92 ± 0.045	1.82[a] ± 0.039	1.70 [b] ± 0.034
7d	5.89 ± 0.468	5.41 ± 0.236	4.67 ± 0.188	4.65 ± 0.165
14 d	9.66 ± 0.822	8.75 ± 0.258	6.90 ± 0.222	7.28 ± 0.287
21 d	14.44 ± 1.077	13.66 ± 0.508	11.06 ± 0.387	11.09 ± 0.491
28 d	19.15 ± 0.763	20.37 ± 0.620	18.88 ± 0.506	19.60 ± 0.838
			F4 generation	
Birth	2.19 ± 0.097	2.13 ± 0.104	1.93 ± 0.045	1.85 ± 0.079
7d	5.14[a] ± 0.495	6.44[b] ± 0.298	4.75 ± 0.152	4.49 ± 0.108
14 d	8.91[a] ± 0.325	10.61[b] ± 0.257	7.20 ± 0.279	6.57 ± 0.185
21 d	13.40[a] ± 0.738	15.77[b] ± 0.201	11.30 ± 0.525	10.34 ± 0.293
28 d	22.44 ± 0.497	24.05 ± 0.584	19.37 ± 0.757	18.88 ± 0.467

Values represent means and standard error, [a,b] means that have a superscript are significantly different ($p < 0.05$)

Table 38: **Individual pup mass [g] per group 5 and 6 weeks after birth from parents with 33% of near isogenic (ISO) or transgenic (GM) corn in their diet**

Weeks after birth	F1 generation	
5 w	**ISO**	**GM**
Males & Females	22.07 ± 0.636	22.86 ± 0.727
	F2 generation	
	ISO	**GM**
5 w		
Females	20.36 ± 0.768	21.92 ± 0.758
Males	24.01 ± 0.742	23.98 ± 0.789
6 w		
Females	26.93 ± 1.554	24.97 ± 0.564
Males a	28.73 ± 0.561	28.51 ± 0.803
	F3 generation	
	ISO	**GM**
5 w		
Females	22.92 ± 0.440	23.30 ± 0.475
Males	26.38 ± 0.551	25.39 ± 0.980
6 w		
Females	25.21 ± 0.525	24.88 ± 0.537
Males a	30.26 ± 0.427	30.35 ± 0.948
	F4 generation	
	ISO	**GM**
5 w		
Females	22.58 ± 0.429	22.91 ± 0.512
Males	25.50 ± 0.588	24.75 ± 2.004
6 w		
Females	24.58 ± 0.630	23.93 ± 0.495
Males	28.45 ± 0.685	27.36 ± 0.823

3.2.1.2. ISO versus A REF

Parental performance

One female died in the F1 generation in the ISO and A REF group and in the F2 generation 1 female from the ISO group before delivery for unknown reasons.

In the F1 generation the A REF group had a lower ($p=0.001$) feed intake than the ISO group (Figure 4). Further feed intake was significantly different ($p<0.001$) between the generations but similar in succession (F1>F2>F3).

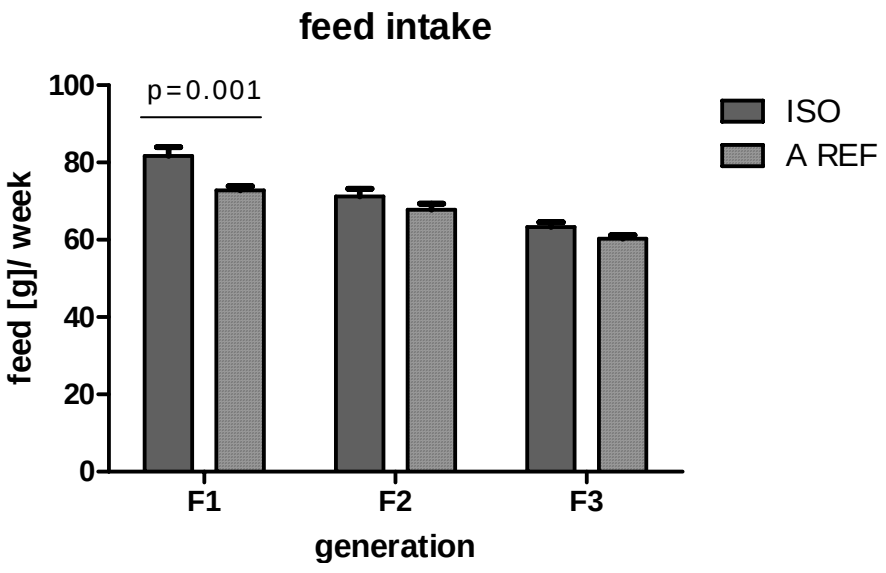


Figure 4: **Feed intake of adult mice fed 33% isogenic (ISO) or Austrian reference (A REF) corn in their diet in several generations**

Differences in performance (Table 39) were seen in body mass of females at delivery in the F1 and F3 generation where females from the ISO group were significantly lighter than females from the A REF group (p= 0.000 and p=0.004, respectively). In the aforementioned generations also the body mass of females 3 weeks after delivery was significantly different and again females from the ISO group were significantly lighter than females from the A REF group (p= 0.016 and p=0.019, respectively). Differences in body mass of males was seen in the F2 generation and males from the ISO group were significantly (p= 0.01) lighter than males from the A REF group.
In the ISO group body mass of females and males at mating (F1 < F3, F2 < F0), females at delivery (F1, F3 < F0, F2) and 3 weeks after delivery (F1, F3 < F3, F2, F0) differed significantly over several generations. In the A REF body mass differed significantly at mating in the F2 > F1 in females and F3>F1 in males.

Parental reproduction

In general the breeding success of the A REF group defined by number of deliveries, number of pups and average litter weight was lower in the first two and higher in the last two generations as compared to the ISO group (Table 40).
The number of deliveries per group decreased in the succession of generations in the ISO group but remained constant in the A REF group. Accordingly in the ISO group the number of weaned pups in the 4th generation was 14% lower.
Except for the first generation the loss of pups until weaning was higher in the A REF group but all findings were not at a significant level. So was the frequency of number of pups per litter n ≤ 8 that was always lower in the ISO group (Figure 5).

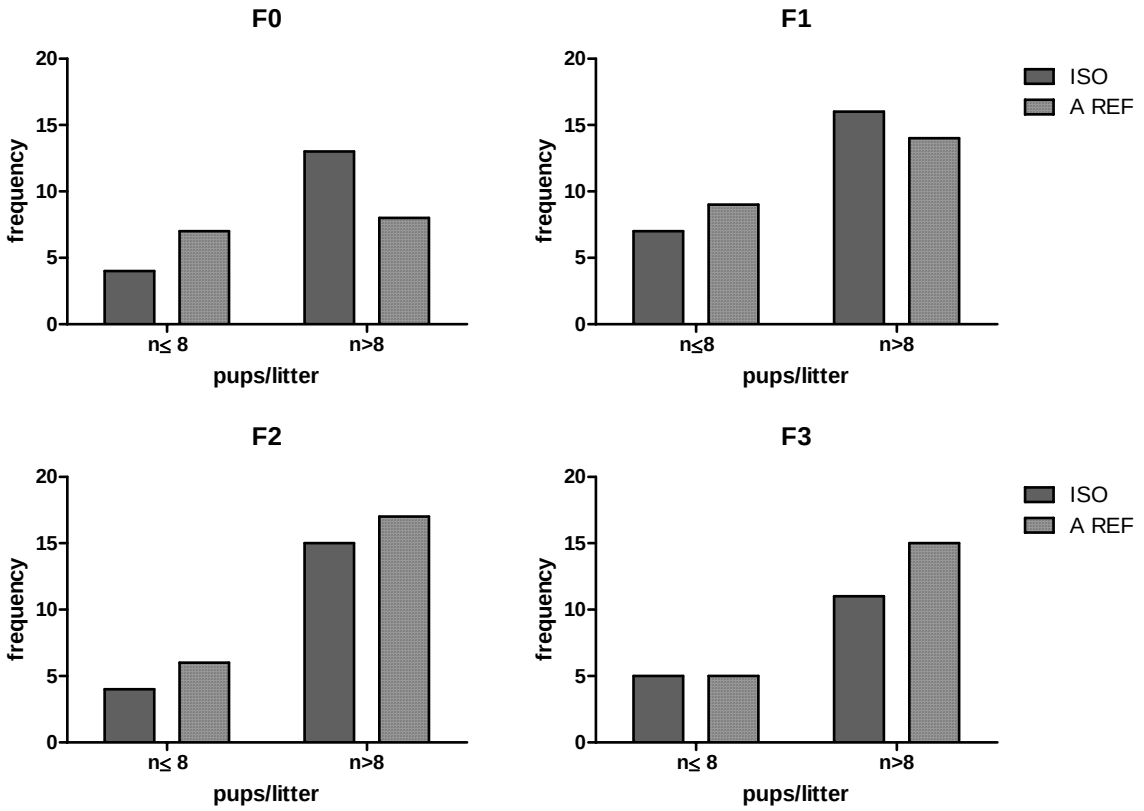


Figure 5: **Distribution of litter size at delivery from females fed 33 % isogenic (ISO) or Austrian reference corn in their diet in several generations**

Offspring performance

No differences were seen in litter weight (Figure 6 a-c) and individual pup weight (Figure 6 a'-c' and table 41). Except on a single time point in the F3 generation the A REF pups in the small litters were significantly (p=0.011) heavier (Table 41). At the age of 5 weeks the A REF male and female pups were significantly heavier in the F1 (p=0.021) and F2 (p=0.06 and p=0.01, respectively) generation as compared to the ISO group (Table 42). There was no weight difference at the age of 6 weeks.

Table 39: **Performance of mice fed 33% isogenic (ISO) or Austrian reference (A REF) corn in their diet over several generations**

	Parental performance							
	F0		F1		F2		F3	
Traits	ISO	A REF	ISO	A REF	ISO	A REF	ISO	A REF
Females body mass [g]								
mating	27.21	26.25	20.26	24.44	25.87	28.75	25.77	26.37
	± 0.452	± 0.312	± 0.572	± 0.644	± 0.345	± 1.408	± 0.433	± 0.489
1 week after mating	29.74	28.98	26.92	27.91	-	-	27.85	27.79
	± 0.431	± 0.324	± 0.451	± 0.465	-	-	± 0.418	± 0.507
delivery	35.86	35.01	33.27[a]	34.45[b]	35.65	36.00	33.54[a]	36.44[b]
	± 0.571	± 0.587	± 0.533	± 0.490	± 0.642	± 0.562	± 0.699	± 0.658
1 week after delivery	37.00	36.58	36.62	37.93	38.55	37.93	36.66	38.01
	± 0.919	± 0.892	± 0.675	± 0.534	± 0.699	± 0.754	± 0.724	± 0.626
2 weeks after delivery	37.31	36.61	37.24	38.53	37.35[a]	40.14[b]	36.83	38.08[b]
	± 1.207	± 0.913	± 0.993	± 0.649	± 0.974	± 0.671	± 0.578	± 0.495
3 weeks after delivery	36.77	36.52	33.77[a]	36.37[b]	36.55	38.14	34.47[a]	37.27[b]
	± 1.134	± 0.573	± 0.789	± 0.683	± 0.592	± 0.530	± 0.893	± 0.720
Males body mass [g]								
mating	34.02	35.01	29.02	29.21	31.13[a]	33.37[b]	32.27	33.09
	± 0.741	± 0.635	± 0.535	± 0.709	± 0.474	± 0.677	± 0.435	± 0.559
1 week after mating	33.31	33.71	30.74	30.93	-	-	31.74	31.91
	± 0.533	± 0.679	± 0.514	± 0.407	-	-	± 0.297	± 0.425

Values represent means and standard error, [a,b] means that have a superscript are significantly different ($p < 0.05$)

Table 40: **Reproduction data of mice fed 33% isogenic (ISO) or genetically modified (GM) corn in their diet over several generations**

	Parental reproduction							
	F0		F1		F2		F3	
traits	ISO	A REF	ISO	A REF	ISO	A REF	ISO	A REF
Pairs/group	18	18	24	24	24	24	22	22
Deliveries/group	17	16	23	21	19	22	16	20
Non deliveries/group	5.6%	11.1%	4.2%	12.5%	20.8%	8.0%	27.3%	9.1%
Number of pups at birth/pair	10.28 ± 0.980	7.67 ± 1.042	8.25 ± 0.778	7.46 ± 0.736	8.42 ± 1.025	9.20 ± 0.735	6.59 ± 1.046	9.05 ± 0.774
Sum of pups at birth/group	185	138	198	194	202	230	145	199
Number of pups at weaning/ pair	8.39 ± 0.936	6.72 ± 0.928	8.00 ± 0.766	6.77 ± 0.705	7.96 ± 0.928	8.36 ± 0.709	6.45 ± 1.040	8.59 ± 0.732
Sum of pups at weaning/ group	151	121	192	176	191	209	142	189
Sum of pup losses/group	34	17	6	18	11	21	3	10
Pup losses/group	2.06 ± 0.683	1.06 ± 0.322	0.26 ± 0.157	0.78 ± 0.281	0.58 ± 0.289	0.91 ± 0.266	0.19 ± 0.136	0.50 ± 0.212

Values represent means and standard error, ^{a,b} means that have a superscript are significantly different ($p < 0.05$)

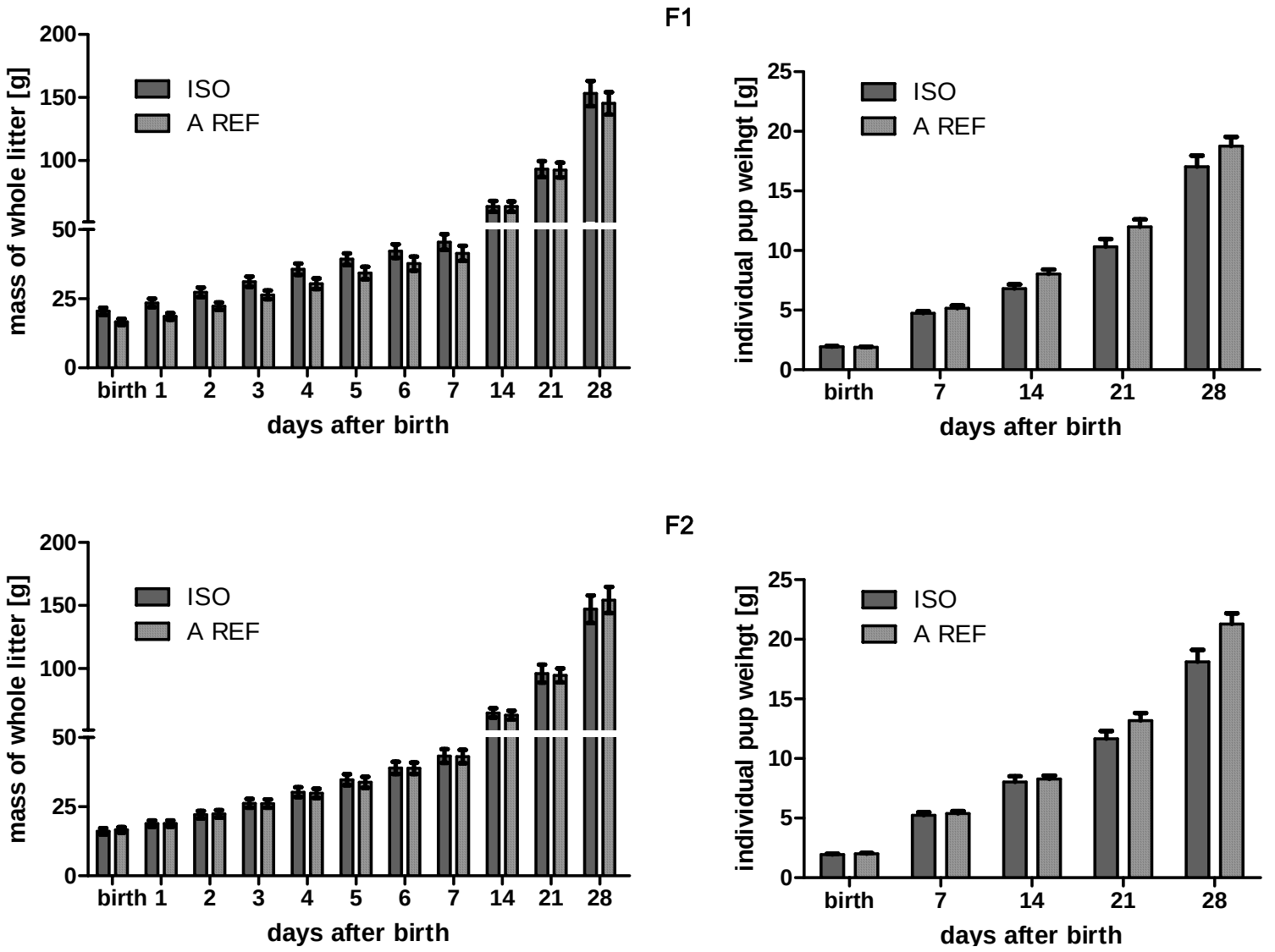
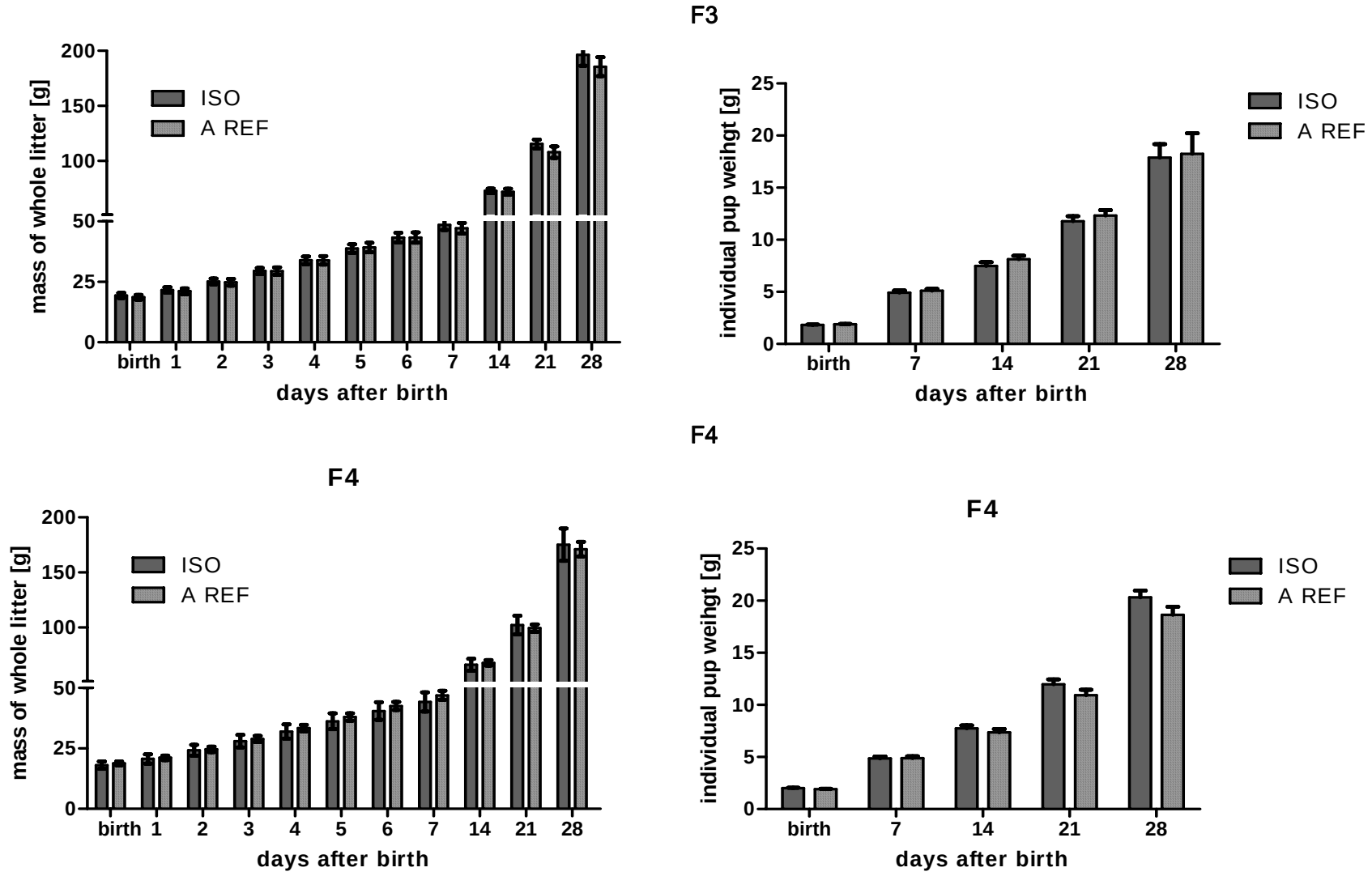


Figure 6 a-b and 6 a'-b': **Mass of whole litter [g] and individual pup weight [g] at several timepoints of the F1 and F2 generation after birth of offspring from parental mice fed 33% isogenic (ISO) or Austrian reference(A REF) corn in their diet**



Figures 6 c-d and 6 c'-d'**: Mass of whole litter [g] and individual pup weight [g] at several timepoints of the F3 and F4 generation after birth of offspring from parental mice fed 33% isogenic (ISO) or Austrian reference (A REF) corn in their diet**

Table 41: **Individual pup mass [g] per group at birth, 7, 14, 21 and 28 days after birth from parents with 33% of near isogenic (ISO) or Austrian reference (A REF) corn in their diet**

	group			
	ISO n≤ 8	**A REF n≤ 8**	**ISO n> 8**	**A REF n> 8**
		F1 generation		
Birth	2.14 ± 0.114	2.03 ± 0.066	1.84 ± 0.054	1.74 ± 0.065
7d	5.33 ± 0.323	5.65 ± 0.253	4.54 ± 0.192	4.60 ± 0.242
14 d	7.52 ± 0.873	8.87 ± 0.494	6.75 ± 0.359	7.06 ± 0.271
21 d	12.23 ± 1.397	13.50 ± 0.701	9.50 ± 0.688	10.27 ± 0.536
28 d	20.64 ± 1.731	20.96 ± 0.553	15.70 ± 0.970	16.24 ± 0.734
		F2 generation		
Birth	2.28 ± 0.114	2.17 ± 0.089	1.81 ± 0.036	1.91 ± 0.052
7d	6.70 ± 0.431	5.67 ± 0.305	4.69 ± 0.141	5.12 ± 0.242
14 d	10.67 ± 1.002	8.92 ± 0.445	7.04 ± 0.276	7.59 ± 0.298
21 d	14.88 ± 0.878	14.62 ± 0.983	10.44 ± 0.619	11.56 ± 0.556
28 d	22.17 ± 1.261	23.05 ± 1.188	16.51 ± 1.048	19.35 ± 1.174
		F3 generation		
Birth	1.84 ± 0.101	2.00 ± 0.099	1.82 ± 0.039	1.83 ± 0.044
7d	5.89 ± 0.468	5.68 ± 0.377	4.67 ± 0.188	4.85 ± 0.183
14 d	9.66 ± 0.822	9.67 ± 0.512	6.90 ± 0.222	7.29 ± 0.245
21 d	14.44 ± 1.077	13.89 ± 0.856	11.06 ± 0.387	11.32 ± 0.478
28 d	19.15[a] ± 0.763	21.86[b] ± 0.691	18.88 ± 0.506	18.37 ± 1.137
		F4 generation		
Birth	2.19 ± 0.097	2.09 ± 0.086	1.93 ± 0.045	1.85 ± 0.030
7d	5.14 ± 0.495	5.65 ± 0.323	4.75 ± 0.152	4.64 ± 0.164
14 d	8.91 ± 0.325	9.17 ± 0.523	7.20 ± 0.279	6.76 ± 0.246
21 d	13.40 ± 0.738	13.83 ± 0.630	11.30 ± 0.525	9.95 ± 0.443
28 d	22.44 ± 0.497	22.62 ± 0.731	19.37 ± 0.757	17.30 ± 0.708

Values represent means and standard error, [a,b] means that have a superscript are significantly different ($p < 0.05$

Table 42: **Individual pup mass [g] per group 5 and 6 weeks after birth from parents with 33% of near isogenic (ISO) or Austrian reference (A REF) corn in their diet**

Weeks after birth				
	F1 generation			
5 w	**ISO**		**A REF**	
Males & Females	22.07[a]	± 0.636	24.18[b]	± 0.584
	F2 generation			
5 w				
Females	20.36[a]	± 0.768	23.66[b]	± 0.488
Males	24.01[a]	± 0.742	27.16[b]	± 0.773
6 w				
Females	26.93	± 1.554	25.82	± 0.518
Males	28.73	± 0.561	29.83	± 0.762
	F3 generation			
5 w				
Females	22.92	± 0.440	23.64	± 0.491
Males	26.38	± 0.551	27.08	± 0.612
6 w				
Females	25.21	± 0.525	25.74	± 0.428
Males	30.26	± 0.427	31.00	± 0.566
	F4 generation			
5 w				
Females	22.58	± 0.429	22.41	± 0.428
Males	25.50	± 0.588	25.14	± 0.684
6 w				
Females	24.58	± 0.630	24.36	± 0.559
Males	28.45	± 0.685	27.09	± 0.627

Values represent means and standard error, [a,b] means that have a superscript are significantly different ($p < 0.05$)

3.2.2. Organ weights

3.2.2.1. GM versus ISO

Relative organ weights showed significant differences between groups that were not consistent through the investigation. No differences were seen in the relative weight of testes (Figure 7). Relative liver weight differed in females of the F2 and F4 generation, being higher in the GM group of the F2 generation ($p=0.006$) but lower in the F4 generation ($p=0.035$). No differences were found in relative liver weight of males over all generations (Figure 8 a).
In the F2 generation males of the GM group had higher ($p=0.004$) relative spleen weight than the ISO group. No further differences were observed in the successive generations nor in the other sex (Figure 8 b).
Relative kidney weights of females differed significantly in the F2, F3 and F4 generation. Females of the GM group had lower ($p<0.001$, $p=0.045$ and $p=0.029$, respectively) kidney weights than females from the ISO group. Additionally, males in the GM group of the F2 generation showed lower ($p<0.001$) kidney weights (Figure 8 c).

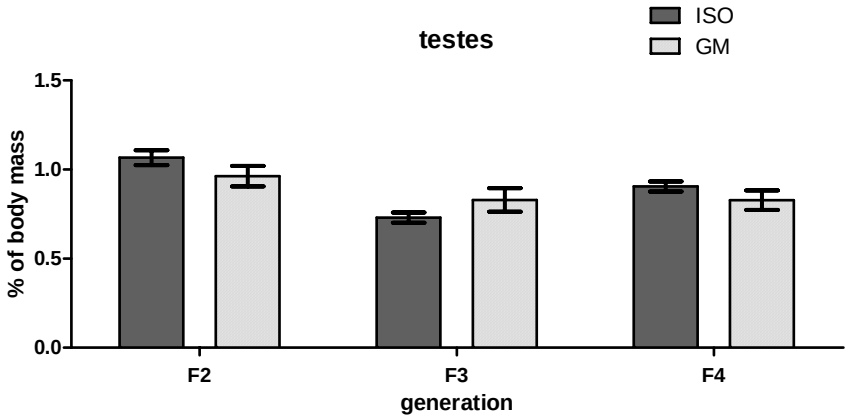


Figure 7: **Relative mass of testes of 5 week old mice fed 33% isogenic (ISO) or genetically modified (GM) corn in their diet**

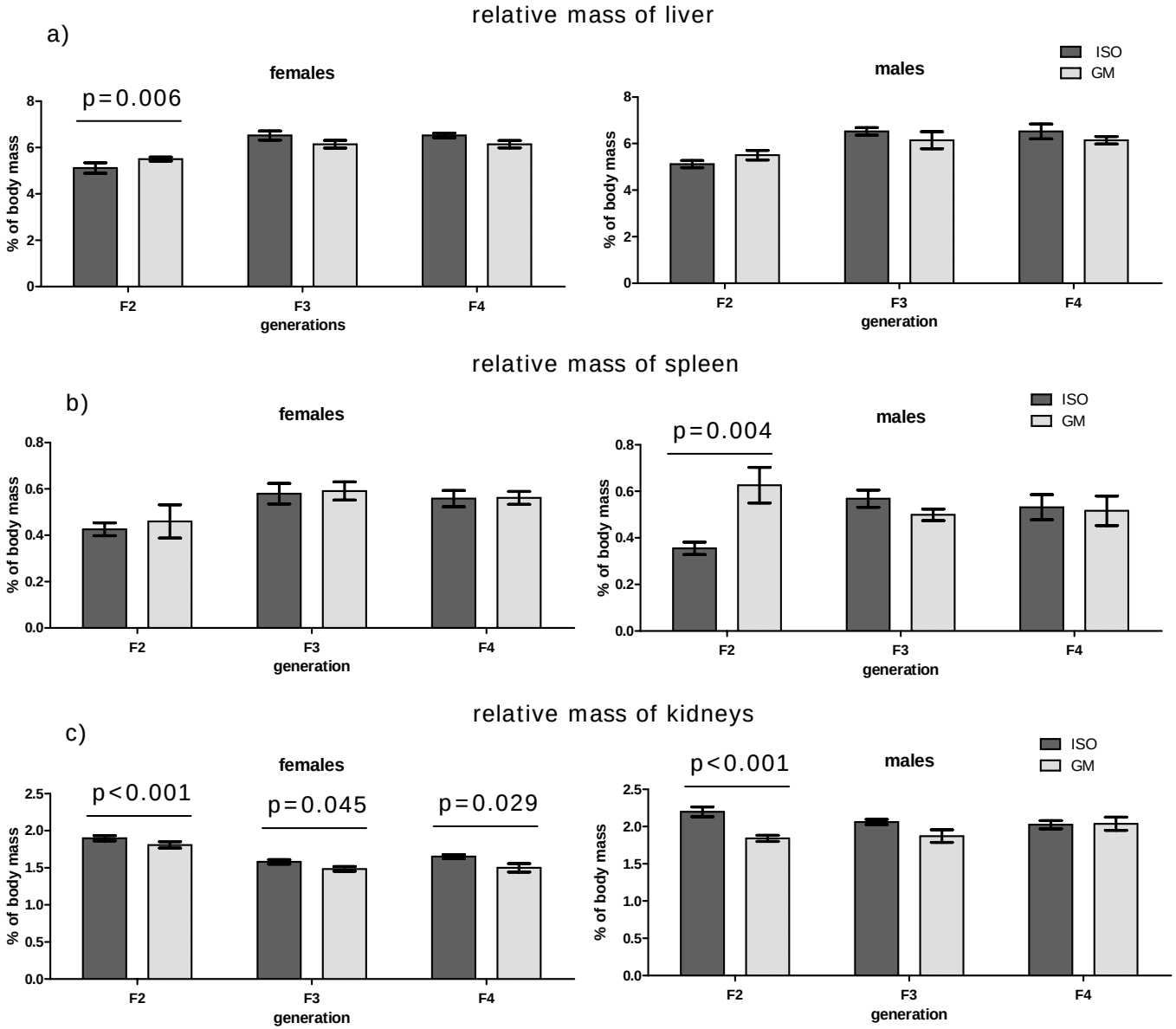


Figure 8: **Relative mass of liver (a), spleen (b) and kidneys(c) of 5 week old mice fed 33 % isogenic (ISO) or genetically modified (GM) corn in their diet**

3.2.2.2. ISO versus A REF

Differences were seen in several organs of different generations. Males showed significant differences (p=0.015 and p=0.005) of the relative organ weight of testes in the F3 and F4 generation (Figure 9). These were inconsistent insofar as the ISO males had lower relative testes weight in the F3 and higher relative testes weight in F4 than the A REF group.
In the F3 generation the ISO mice of both sexes had significantly higher relative liver weights (p= 0.021 for females and p=0.034 for males, Figure 10 a).
The males of the A REF group showed a significantly (p=0.018) higher relative spleen weights in the F2 generation only (Figure 10 b)
Finally a further single statistically significant difference was seen for kidney weight of females (p=0.016) in the F4 generation (Figure 10 c) being higher in the ISO group.

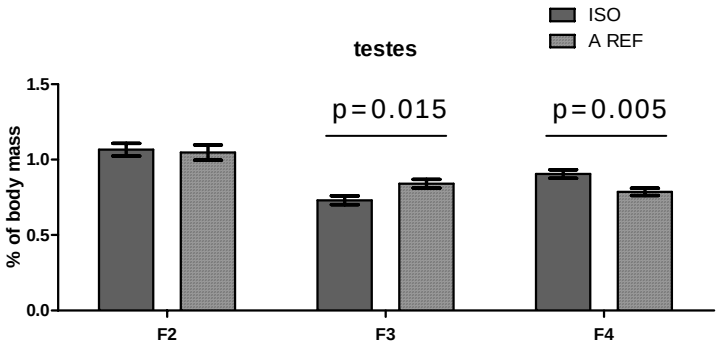


Figure 9: **Relative mass of testes of 5 week old mice fed 33% isogenic (ISO) or Austrian reference (A REF) corn in their diet**

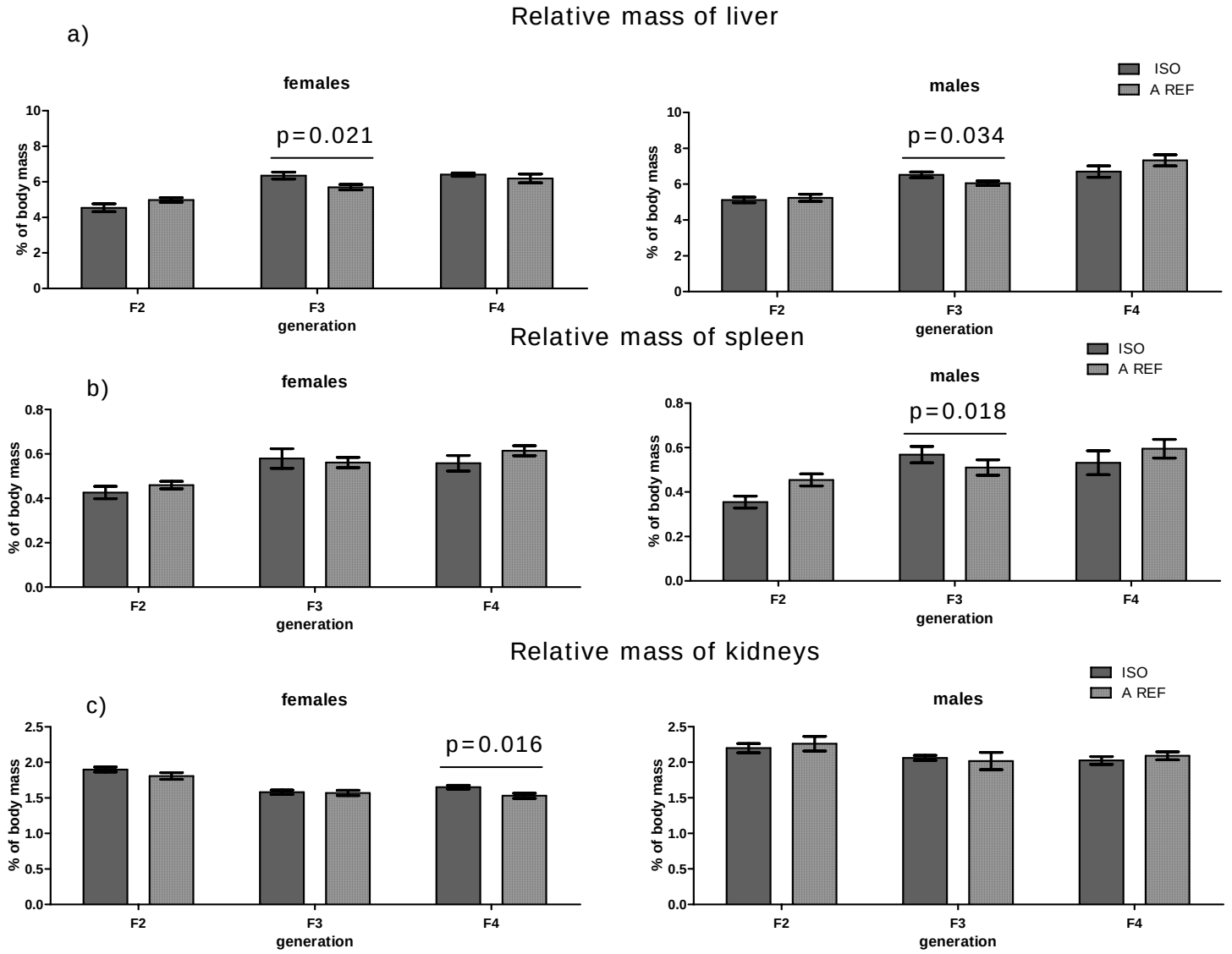


Figure 10: **Relative mass of liver (a), spleen (b) and kidneys (c) of 5 week old mice fed 33% isogenic (ISO) or Austrian reference (A REF) corn in their diet**

3.2.3. Histology

The histological comparison included the gastrointestinal tract, liver, pancreas, kidney, spleen, lung and testes of 5 male and 5 female test mice of the F3 generation of the multigeneration study at the age of 7 weeks.
The organs were examined for pathological changes such as necrosis, oedema, fibrosis, hyperplasia and ulceration.
Although a number of significant differences concerning relative organ weights had been found, these differences could not be corroborated by the microscopic comparisons between the feeding groups in any of the above mentioned organs.

- **Gastrointestinal Tract**

  The comparison of villi and crypt structure showed no difference between the feeding groups. There was no infiltration of lymphocytes seen on any of the investigated slides. The test mouse strain is prone to develop ulcers, but in this case the animals were probably too young for this pathological change to occur.
- **Liver**

  Although size differences in hepatocytes were observed these were randomly distributed among all samples and therefore not feed dependent. There were also slight differences in the accumulation of glycogen in the liver cells. But glycogen aggregates vary depending on the feed uptake shortly before the animal is sacrificed. The bile duct epithelia did not show any pathological changes in all groups and no infiltration of leucocytes was observed.
- **Pancreas**

  No acute pancreatitis or infiltration of leucocytes was seen in any of the slides. No necrosis of adipose tissue associated with pancreatic damage was found. The accumulation of zymogen granula in the acinus cells was low and comparable between the groups indicating a similar feed intake, since zymogen granula point at the nutritional status of the animal.
- **Kidney**

  No differences were seen by histological comparisons. Renal tubules (deposition of calcium phosphate) were not mineralized and the epithelia were without pathological findings.
- **Spleen**

  No lymphocyte accumulation was seen in the white pulpa of the compared spleens. All spleen samples showed dense accumulations of megacaryocytes that is known for young animals.
- **Lungs**

  No accumulation of eosinophilic granulocytes representing control mechanisms associated with allergy and asthma was found in any of the lung samples and no other abnormality was detected.
- **Testes**

  The developmental state of the testes was comparable between the groups, since mature spermatozoa were found equally in the seminiferous tubules. The testes of all groups were without any pathological findings.

In conclusion, there was no evidence of diet related changes in the tissues of the gastrointestinal tract, liver, pancreas, kidneys, spleen, lungs and testes.

3.2.4. Immunohistochemistry

$CD3^+$ T-lymphocytes

In general, the highest density of $CD3^+$ intraepithelial lymphocytes was seen in the two proximal segments of the small intestine and in the rectum, the lowest in the colon. Statistically significant differences were found between the ISO and the GM group in the 2nd intestinal segment of the male mice ($p=0.021$) and in the 3rd intestinal segment of the female mice ($p=0.009$) with contradictory results (table 43). GM females showed higher but GM males lower $CD3^+$ density. In the A REF less ($p=0.003$) $CD3^+$ cells were seen than in the ISO males.
The differences are inconsistent between the two sexes and were not found in all segments. For the $CD3^+$ immune population the impact of feed seems rather low.

Table 43: **$CD3^+$ Intraepithelial lymphocytes in the gut tissue (per 0.1 mm^2)**

	Group		
	ISO	**GM**	**A REF**
		Male	
small intest_1	0.68 ± 0.199	0.16 ± 0.165	0.44 ± 0.093
small intest_2	1.13[a] ± 0.070	0.67[b] ± 0.068	0.30[b] ± 0.106
small intest_3	0.37 ± 0.096	0.46 ± 0.051	0.15 .
small intest_4	0.36 ± 0.146	0.56 ± 0.256	0.21 ± 0.068
caecum	0.48 ± 0.156	0.22 ± 0.053	0.16 ± 0.034
colon	0.08 ± 0.015	0.20 ± 0.107	0.05 ± 0.001
rectum	1.08 ± 0.416	0.37 ± 0.023	0.07 ± 0.035
		Female	
small intest_1	1.26	1.00 ± 0.144	1.05 ± 0.050
small intest_2	0.90 ± 0.162	0.81 ± 0.070	0.51 ± 0.123
small intest_3	0.57[a] ± 0.056	1.25[b] ± 0.031	0.75 ± 0.009
small intest_4	0.60	0.76	0.51 ± 0.169
caecum	0.23	0.12	0.13 ± 0.080
colon	0.30	0.29 ± 0.095	0.10 ± 0.013
rectum	0.85	0.12	0.36 .

[a,b] means that have a superscript are significantly different ($p < 0.05$)

$CD20^+$ B-lymphocytes

In the male animals, the highest density of $CD20^+$ cells (> 1.1/0.1 mm^2) was seen in the most distal segment of the small intestine. In the female mice, distribution of $CD20^+$ cells was more inhomogeneous and ranged from 0.63 to 2.23 $CD20^+$ cells/0.1 mm^2.
Due to a high inter-individual variability of the results, statistically significant differences between the feeding groups could not be found (Table 44).

Table 44: **CD20$^+$ cells in the lamina propria of the small intestine (per 0.1 mm$^{2)}$**

	Group					
	ISO		GM		A REF	
			Male			
small intest_1	0.59	± 0.094	0.92	± 0.479	1.16	± 0.354
small intest_2	0.60	± 0.182	0.58	± 0.047	1.05	± 0.572
small intest_3	0.51	± 0.149	0.37	± 0.124	0.87	± 0.266
small intest_4	1.99	± 0.802	1.10	± 0.395	2.15	± 0.621
			Female			
small intest_1	0.99	± 0.357	0.95	± 0.113	1.60	± 0.113
small intest_2	2.23	± 0.819	0.82	± 0.206	1.40	± 0.206
small intest_3	1.46	± 0.482	0.63	± 0.185	0.81	± 0.185
small intest_4	1.10	± 0.802	1.50	± 0.718	1.16	± 0.718

Macrophages

With the available antibody against macrophage clone MAC387 only the samples from the female mice could be stained. The density of macrophages in the lamina propria of the small intestine ranged from 1.44 to 3.7 cells/0.1 mm^2. No statistically significant differences were seen between the groups (Table 45).

Table 45: **Macrophages in the lamina propria of the small intestine of female mice (per 0.1 mm^2)**

	group					
	ISO		GM		A REF	
small intest_1	2.44	± 0.762	3.28	± 0.671	2.67	± 0.477
small intest_2	3.50	± 0.901	2.47	± 0.613	1.71	± 0.477
small intest_3	2.65	± 0.425	2.87	± 0.777	1.50	± 0.246
small intest_4	1.44	± 0.460	1.56	± 0.460	3.70	± 0.951

3.2.5. Ultrastructural investigation

3.2.5.1. ISO versus GM

The ultrastructural observations showed comparable nuclear shape irregularity in the ISO and the GM group in hepatocytes, lymphocytes and pancreas acinar cells.
Fibrillar centres (FC) and dense fibrillar components (DFC) of hepatocytes were significantly lower in females ($p=0.027$ and $p= 0.041$) and DFC in males ($p=0.017$) in the GM group in contrast to the ISO group (Figure 11 a). FC was also lower in males but not at a significant level.

No differences were seen in characteristics of spleen lymphocytes.

In pancreatic acinar cells DFC were significantly less abundant in females ($p=0.01$) but not in males of the GM group (Figure 11 c). FC of both females and males were higher in the GM group but not at a significant level.

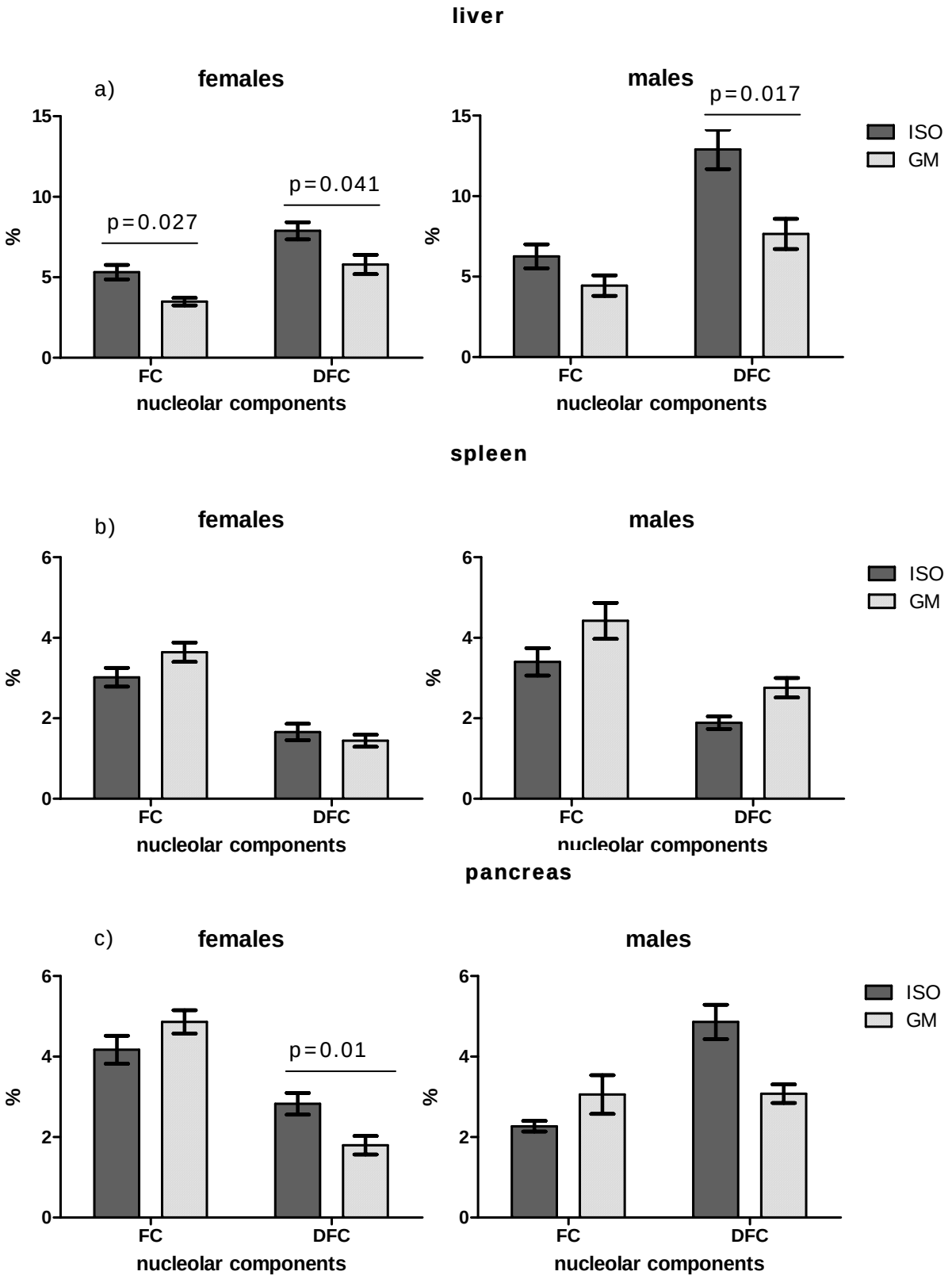


Figure 11: **Fibrillar centres (FC) and dense fibrillar components (DFC) of liver (a) (hepatocytes), spleen (b) (lymphocytes) and pancreatic acinar cells (c) from the F3 generation of mice fed 33% genetically modified (GM) or isogenic (ISO) corn.**

Differences were seen in the pore density of hepatocytes of males ($p<0.001$) but not of females.

Table 46: **Pore density (pores/µm nuclear membrane length) from different tissue of mice fed 33% genetically modified (GM) or isogenic (ISO) corn**

		ISO	GM
Liver	females	0.74 ± 0.068	0.54 ± 0.051
	males	0.68[a] ± 0.045	0.36[b] ± 0.038
Spleen	females	0.35 ± 0.043	0.37 ± 0.029
	males	0.36 ± 0.035	0.24 ± 0.035
Pancreas	females	0.51 ± 0.043	0.62 ± 0.056
	males	0.49 ± 0.039	0.50 ± 0.039

No significant divergences could be found in the spleen and pancreatic cells.

3.2.5.2. ISO versus A REF

A significant variation regarding the nuclear shape irregularity was only ascertained in liver cells of female mice, which was lower ($p=0.025$) in the A REF group in comparison to the ISO group (Table 47).

Table 47: **Nuclear shape irregularity of different anatomical sites from mice fed 33% isogenic (ISO) or Austrian reference (A REF) corn**

		ISO	A REF
Liver	females	1.04 ± 0.008	1.08 ± 0.016
	males	1.05 ± 0.016	1.04 ± 0.017
Spleen	females	1.13 ± 0.021	1.09 ± 0.015
	males	1.18 ± 0.031	1.16 ± 0.026
Pancreas	females	1.09 ± 0.018	1.09 ± 0.018
	males	1.07 ± 0.010	1.08 ± 0.028

Dense fibrillar components (DFC) but not fibrillar centers (FC) of hepatocytes were significantly lower in males ($p= 0.026$) in the A REF group than in the ISO group, values of females where comparable in both groups (Fig. 12 a). Calculations of nucleolar components made on spleen and pancreas tissue gave no significant results (Figure 12 b-c).

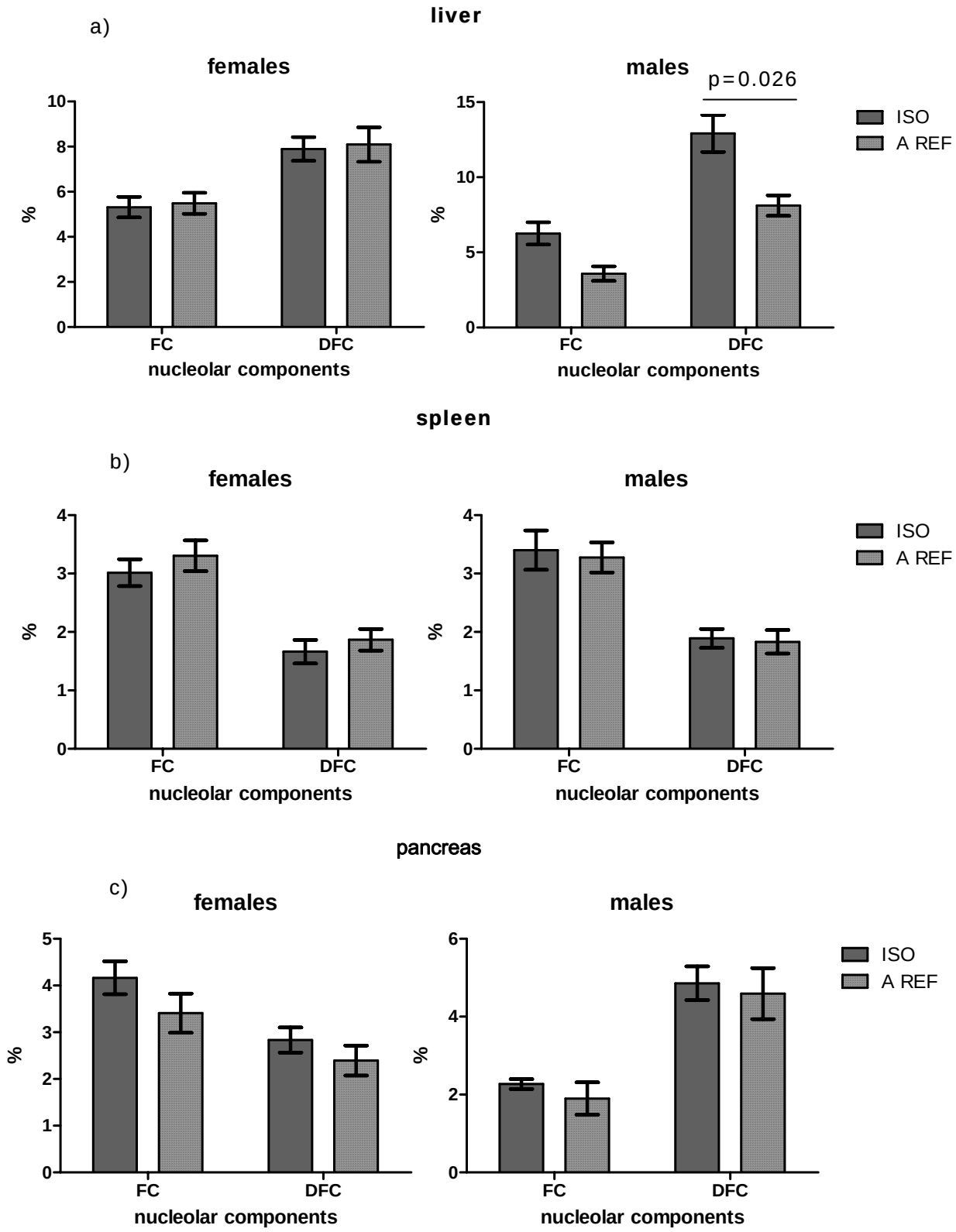


Figure 12 a-c: **Fibrillar centres (FC) and dense fibrillar components (DFC) of liver (a), spleen (b) and pancreatic acinar cells (c) from the F3 generation of mice fed 33 % isogenic (ISO) or Austrian Reference (A REF) corn.**

The pore density of lymphocyte nuclei in the spleen was significantly lower in males (p=0.026) in the A REF group than in the ISO Group. The other values were not significant (Table 48).

Table 48: **Pore density (pores/µm nuclear membrane length) of different anatomical sites of mice fed 33% isogenic (ISO) or Austrian reference (A REF) corn**

		ISO		A REF	
Liver	females	0.74	± 0.068	0.91	± 0.059
	males	0.68	± 0.045	0.56	± 0.050
Spleen	females	0.35	± 0.043	0.39	± 0.042
	males	0.36[a]	± 0.035	0.24[b]	± 0.031
Pancreas	females	0.51	± 0.043	0.62	± 0.058
	males	0.49	± 0.039	0.50	± 0.039

3.2.6. Microarray analyses

ISO versus GM

In total 439 genes were found to be expressed differentially ($p<0.05$) using the *t* test approach for gene expression of mice fed transgenic versus isogenic corn. The minimum fold-change with a cut-off value of 2 revealed 43 genes being up-regulated and 98 down-regulated.
Gene Set Enrichment Analysis (GSEA) using PANTHER database tool was performed to extract biological information from the data set. Annotation and classification of the differentially expressed genes due to their biological context revealed significant alterations between the following biological processes: protein biosynthesis ($p=2.4*10^{-15}$), G-protein mediated signalling ($p=1.6*10^{-04}$) and protein metabolism and modification ($p=2.3*10^{-04}$). The biological processes ranking on top of the classification (p-values$<1.4*10^{-02}$) are listed in table 49.

Table 49: **GSEA of deregulated genes (2fold change) between GM and ISO groups describing biological processes**‡

Biological Process	Genes on Mouse AB 1700 genes	Number of genes	Expected by chance	over/under represented	P-value
Protein biosynthesis	446	38	7.80	+	$2.4*10^{-15}$
G-protein mediated signalling	1251	7	21.88	-	$1.6*10^{-04}$
Protein metabolism and modification	2720	72	47.56	+	$2.3*10^{-04}$
Ion transport	574	3	10.04	-	$9.4*10^{-03}$
Sensory perception	873	7	15.27	-	$1.4*10^{-02}$
Cation transport	442	2	7.73	-	$1.6*10^{-02}$
Other receptor mediated signalling pathway	200	8	3.50	+	$2.6*10^{-02}$
Proteolysis	924	9	16.16	-	$3.7*10^{-02}$
Translational regulation	71	4	1.24	+	$3.7*10^{-02}$
Exocytosis	141	6	2.47	+	$3.9*10^{-02}$

‡ see 2.4.6.2. for further explanation

Biological processes involved in protein biosynthesis reflect the most significantly affected set of genes differentially expressed in the distal jejunum from male mice in the 3rd generation fed either 33% genetically modified or isogenic corn.

Furthermore, pathway analysis revealed interleukin signalling pathway, cholesterol biosynthesis and insulin/IGF pathway-protein kinase B signalling cascade as prominent differentiators of the two groups (see table 50).

Table 50: **GSEA of deregulated genes (2fold change) between GM and ISO groups describing pathways**‡

Pathway	Genes on Mouse AB 1700 genes	Number of genes	Expected by chance	over/under represented	P-value
Interleukin signalling pathway	157	5	0.87	+	$1.9*10^{-03}$
Cholesterol biosynthesis	12	2	0.07	+	$2.1*10^{-03}$
Insulin/IGF pathway-protein kinase B signalling cascade	84	3	0.46	+	$1.2*10^{-02}$
PI3 kinase pathway	110	3	0.61	+	$2.4*10^{-02}$
Notch signalling pathway	49	2	0.27	+	$3.0*10^{-02}$
Integrin signalling pathway	212	4	1.17	+	$3.1*10^{-02}$
TGF-beta signalling pathway	146	2	0.81	+	$2.0*10^{-01}$
Synaptic_vesicle_trafficking	42	1	0.23	+	$2.1*10^{-01}$
Insulin/IGF pathway-mitogen activated protein kinase /MAP kinase cascade	42	1	0.23	+	$2.1*10^{-01}$
Integrin signalling pathway	212	6	0.96	+	$1.7*10^{-01}$

‡ see 2.4.6.2 for further explanation

ISO versus A REF

In total, 1016 genes were found to be differentially expressed ($p<0.05$) using the *t* test approach for gene expression of mice fed 33% isogenic (ISO) versus Austrian reference (A REF) corn. Out of these genes 186 were greater than 2-fold down regulated (182 genes) or up regulated (4 genes).
The gene list containing all the 1016 differentially expressed genes was selected for Gene Set Enrichment Analysis using the PANTHER database ($p<0.05$). Genes were allocated according to their biological processes and pathways. This approach identified biological process like G-protein signalling pathway, the cell surface receptor mediated signal transduction and the signal transduction (Table 51) processes as significantly altered between the study groups. Furthermore pathway analyses with gene tags deregulated at least two fold indicated differences in genes of the circadian clock system, the T cell activation and the FAS signalling pathway (Table 52).

Table 51**: GSEA of deregulated genes (2fold change) between ISO and A REF groups describing biological processes**‡

Biological Process	Mouse AB 1700 genes	genes.txt	expected	over/under	P-value
G-protein mediated signalling	1251	14	54.05	-	$3.9*10^{-11}$
Sensory perception	873	8	37.72	-	$3.3*10^{-09}$
Cell surface receptor mediated Signalling transduction	1978	41	85.46	-	$2.3*10^{-08}$
Chemosensory perception	463	1	20.00	-	$3.6*10^{-08}$
Signal transduction	3590	100	155.11	-	$2.3*10^{-07}$
Protein biosynthesis	446	43	19.27	+	$1.7*10^{-06}$
Intracellular protein traffic	878	66	37.94	+	$1.5*10^{-05}$
Tricarboxylic acid pathway	28	8	1.21	+	$3.8*10^{-05}$

‡ see 2.4.6.2 for further explanation

Table 52: **GSEA of deregulated genes (2fold change) between ISO and A REF groups describing pathways**‡

Pathway	Mouse AB 1700 genes	genes.txt	expected	over/under	P-value
Circadian clock system	15	4	0.11	+	$6.4*10^{-06}$
T cell activation	115	5	0.88	+	$2.1*10^{-03}$
FAS signalling pathway	36	3	0.28	+	$2.8*10^{-03}$
Histamine H1 receptor mediated signalling pathway	40	3	0.31	+	$3.8*10^{-03}$
Oxytocin receptor mediated signalling pathway	57	3	0.44	+	$9.9*10^{-03}$
Wnt signalling pathway	315	7	1.41	+	$1.1*10^{-02}$
Thyrotropin-releasing hormone receptor signalling pathway	60	3	0.46	+	$1.1*10^{-02}$
Phenylalanine biosynthesis	2	1	0.02	+	$1.5*10^{-02}$
5HT2 type receptor mediated signalling pathway	67	3	0.51	+	$1.5*10^{-02}$
Apoptosis signalling pathway	132	4	0.01	+	$1.9*10^{-02}$

‡ see 2.4.6.2 for further explanation

ISO & A REF versus GM

When the mRNA gene expression data of mice fed genetically modified corn with the Cry1Ab and CP4 EPSPS event was compared to gene expression data without any Cry1Ab and CP4 EPSPS exposure 2,374 genes were significantly ($p<0.05$) deregulated and 421 out of these had a fold change of at least 2 (Figure 13). In total 13,034 genes were expressed in the distal jejunum.

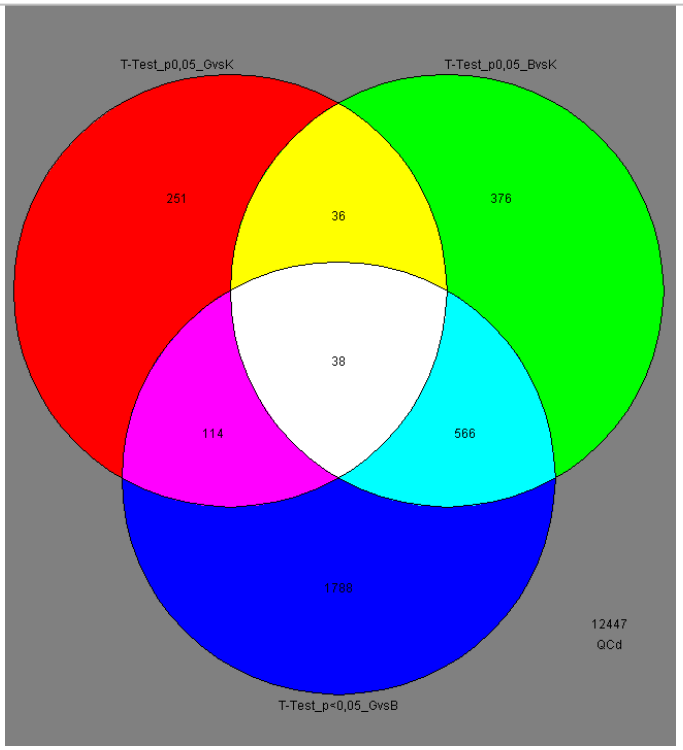


Figure 13: **Venn diagram depicting the number of deregulated genes in the F3 generation of mice fed 33% genetically modified (GM) corn. Isogenic (ISO) and Austrian reference (A REF) corn were used as controls**

For further analyses, the 2374 differentially expressed gene tags ($p<0.05$) were functionally classified using the PANTHER database and were allocated to biological processes and pathways (Table 53 and 54). Expression data became even more pronounced and the level of significance increased in protein biosynthesis from $p= 2.30*^{-15}$ to $p=1.26*^{-148}$ and protein metabolism and modification from $p= 2.35*^{-04}$ to $p=5.34*^{-61}$.

Table 53: **GSEA of deregulated genes between GM and ISO+A REF groups describing biological processes** ‡

Biological Process	Genes on Mouse AB 1700 genes	Number of genes	Expected by chance	over/under represented	P-value
Protein biosynthesis	446	301	44.09	+	$1.3*10^{-148}$
Protein metabolism and modification	2720	552	268.88	+	$5.3*10^{-61}$
Nucleoside, nucleotide and nucleic acid metabolism	2779	397	274.71	+	$5.3*10^{-14}$
G-protein mediated signalling	1251	56	123.67	-	$2.5*10^{-12}$
Signal transduction	3590	248	354.88	-	$5.9*10^{-11}$
Sensory perception	873	37	86.30	-	$1.0*10^{-09}$
Cell surface receptor mediated signal transduction	1978	123	195.53	-	$4.7*10^{-09}$
Transport	1203	63	118.92	-	$6.2*10^{-09}$
mRNA transcription	1569	227	155.10	+	$1.1*10^{-08}$
Olfaction	457	13	45.18	-	$1.4*10^{-08}$

‡ see 2.4.6.2 for further explanation

Table 54: **GSEA of deregulated genes between GM and ISO+A REF groups describing pathways**‡

Pathway	Genes on Mouse AB 1700 genes	Number of genes	Expected by chance	over/under represented	P-value
Circadian clock system	15	3	0.25	+	$2.0*10^{-03}$
Cholesterol biosynthesis	12	2	0.20	+	$1.7*10^{-02}$
Hypoxia response via HIF activation	33	3	0.54	+	$1.7*10^{-02}$
Carnitine metabolism	2	1	0.03	+	$3.2*10^{-02}$
Carnitine and CoA metabolism	2	1	0.03	+	$3.2*10^{-02}$
Interleukin signalling pathway	157	6	2.56	+	$4.6*10^{-02}$
PDGF signalling pathway	173	6	2.83	+	$6.7*10^{-02}$
Salvage pyrimidine deoxyribonucleotides	5	1	0.08	+	$7.8*10^{-02}$
PI3 kinase pathway	110	4	1.80	+	$1.1*10^{-01}$

‡ see 2.4.6.2 for further explanation

3.2.7. q-RT-PCR

From 45 genes that were identified as deregulated by microarray screening, some could be confirmed by q-RT-PCR (Table 55) using a larger cohort. With *t* test 19 genes were proofed to be significantly deregulated ($p<0.05$) in the MGS and 11 genes ($p<0.05$) in the RACB. By using REST procedure, a pair wise fixed reallocation randomisation test providing a much sharper statistical evaluation, 9 ($p<0.05$) and 3 ($p<0.01$) genes were found to be deregulated depending on study design, sex and anatomical site (Table 55).

Table 55: **Fold changes of deregulated genes confirmed by q-RT-PCR in the MGS and the RACB**

	MGS GM vs. ISO		RACB GM vs. ISO	
Gene symbol	prox. Jejunum	dist. Jejunum	dist. Jejunum	
	male	male	female	male
Fkbp5	4.7[a]	2.9[a]	0.9	0.9
Foxq1	1.3	1.3	2.1	0.6[a]
Gspt2	0.7[a]	1.0	1.5[b]	1.0
Hmg20a	1.0	0.9	1.2[b]	0.9
Igtp	0.6	0.3[a]	1.2	2.2
Mapk10	1.0	0.8	1.5[a]	1.0
Per3	1.8	1.7	1.3	4.9[a]
Pnpla3	1.6	0.2[a]	1.8	0.6
Socs1	0.9	0.5[a]	1.2	1.2
Trim47	1.2	1.0	1.0	0.7[b]

[a]$p < 0.05$, [b]$p < 0.1$

Increasing the sample size by pooling the data of MGS and RACB, sexes as well as sampling sites indicated statistically significant differences ($p < 0.05$) in 3 genes (Table 56).

Table 56**: Fold changes of deregulated genes confirmed by q-RT-PCR of the individual and pooled studies (GM versus ISO)**

	MGS		RACB		Pooled
Gene Symbol	prox. Jejunum	dist. Jejunum	dist. Jejunum		
	male	male	female	male	
Fkbp5	4.7[b]	2.9[a]	0.9	0.9	1.9[a]
Per 3	1.8	1.7	1.3	4.9[a]	2.4[a]
Pnpla3	1.6	0.2[a]	1.8	0.6	0.4[a]

[a]$p<0.05$

The deregulated genes can be allotted to different pathways. *Fkbp5* is a member of the Immunophilin family and functions as a peptidyl-prolyl-isomerase. It acts in the AR-signal transduction pathway and it works as a cochaperone for Hsp90. High *Fkbp*-levels block activation of NFATc and therefore inhibit T-cell activation (Magee et al. 2006).
Socs-1 acts in the feedback inhibition of the Jak-Stat signal transduction pathway by ubiquitination of NFκB. It is also thought to inhibit insulin receptors. By blocking Irs it can mediate type II diabetes (Chung et al. 2007; Gagnon et al. 2007).
Igtp is thought to be mainly produced by activated macrophages and limits bacterial growth. Its detailed function is unknown. It might be involved in the traf-

ficking and processing of immunological active proteins, and might mediate cell survival (Lapaque et al. 2006; Zhang et al. 2003).
Pnpla3 also known as adiponutrin is induced during adipogenesis and is highly enriched in adipose tissue. Adiponutrin may participate primarily in triglyceride/NEFA recycling rather than in net lipolysis making its function different from human adiponutrin (Kershaw et al. 2006).
Gspt2 is involved in cell proliferation and in the termination of protein synthesis (Royland and Kodavanti 2008). *Mapk10* is a member of the MAP kinase family and is thought to activate apoptosis in neurons (Bruckner et al. 2001).
Per3 as member of the Period gene family is part of the circadian clock system. It only exhibits a DBPE one of three transcriptional factors found in circadian clock genes, while other genes exhibit up to all three transcriptional factors. The combination of expressed transcription factors is thought to control the functions of the circadian rhythm (Yamamoto et al. 2004).

Until now, no general conclusion can be drawn in aspects of molecular analyses of intestinal mRNA. Differences between ISO and GM fed mice detected by microarray analysis were observed in several pathways. Within the chosen set of deregulated genes selected from the initial microarray screen and further analyzed by the use of TLDA, several genes could be confirmed by RT-PCR, which is considered the gold standard for mRNA quantification.
But it still has to be investigated whether the quantitative changes seen at genetic level are also seen at protein level. Proteins represent the functionally active substances in the host metabolism.

3.2.8. miRNA profiling

ISO versus GM

No significantly different expression of miRNAs was seen in the array profiling when using a p-value cut-off as high as 0.2 (Figure 14). These data do not indicate differential miRNA expression between the groups fed either the transgenic or isogenic corn.

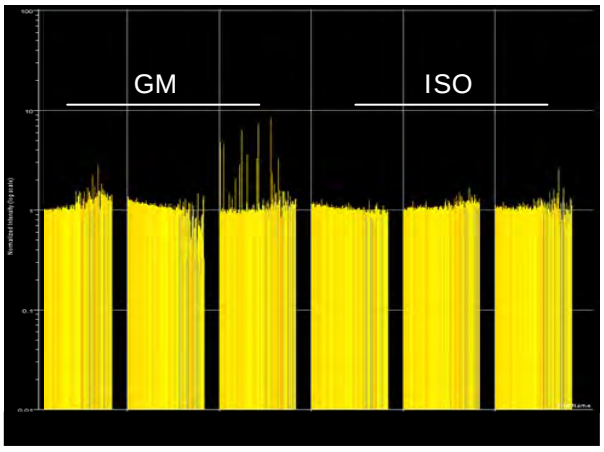


Figure 14: **Bar graphs of normalized data of jejunal miRNAs (each bar represents one miRNA) of the 3rd generation (F3) from mice fed 33 % isogenic(ISO) or transgenic(GM) corn**

ISO versus A REF

No significantly different expression of miRNAs was seen in the array profiling even when a cut-off as high as p=0.2 was used (Figure 15). This data set does not indicate differential miRNA expression between the groups fed either the isogenic corn or the A REF corn.

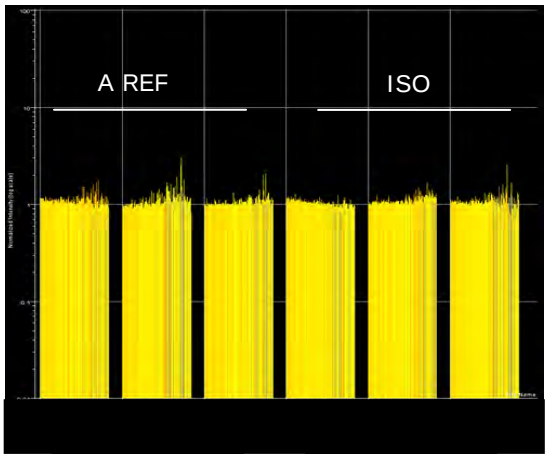


Figure 15: **Bar graphs of normalized data of jejunal miRNAs (each bar represents one miRNA) of the 3rd generation (F3) from mice fed 33 % isogenic(ISO) or Austrian reference corn (A REF)**

3.3. Life term study

The test period was terminated after almost 22 months, when 2 mice of each group were still alive.

3.3.1. Feed intake and body mass investigation

No significant difference concerning feed intake were seen. The feed consumed within the investigated period was approx. 23kg in the ISO, 22kg in the GM and 21,5kg in the A REF group.
Body mass development was not statistically calculated as the clinical manifest cancer form biased the investigation. In times without any obvious clinical disease the body mass was not undergoing considerable fluctuations.

3.3.2. Survival rate

The average life time of mice was 16.3 month in the ISO, 15.7 month in the A REF and 17.0 month in the GM group but was not significantly different (Figure 16).

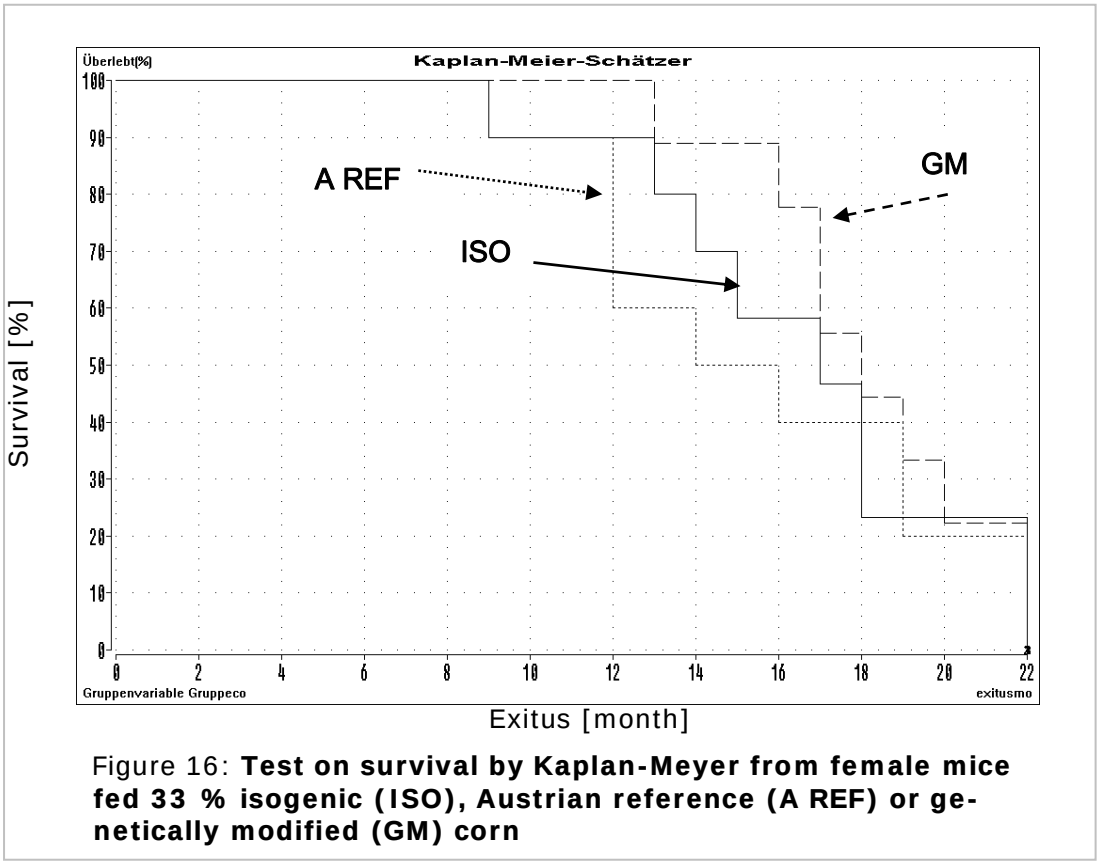


Figure 16: **Test on survival by Kaplan-Meyer from female mice fed 33 % isogenic (ISO), Austrian reference (A REF) or genetically modified (GM) corn**

The common causes of death were cancer (leucosis) that is considered to be triggered by the activation of a murine retrovirus inherent in the genome of the test mice. Typical pathological findings were cachexia, spleno- and hepatomegaly with diffuse or local infiltration with abnormal leukocytes.
No differences were found in the inter group comparison. Long term studies may have limitations as the majority of outbred or inbred strains develop different forms of cancer. Thus diet related differences could be masked and not really assessed.

3.4. Reproductive Assessment by Continuous Breeding (RACB)

3.4.1. Performance and reproduction

The RACB was performed with 24 breeding pairs per feeding group and lasted for 20 weeks. During this time 4 litters were produced and weaned at the end of the test (Table 57).

Table 57: **Overview of litters in the RACB**

Generation	pairs/group	date
F0 parents	n= 24	
1. litter		February 2008
2. litter		March 2008
3. litter		April/May 2008
4. litter		May/June 2008

Parental performance

No statistically significant differences were seen in the prefeeding period and feed intake of breeding pairs within the first weeks of the RACB (Figure 17).

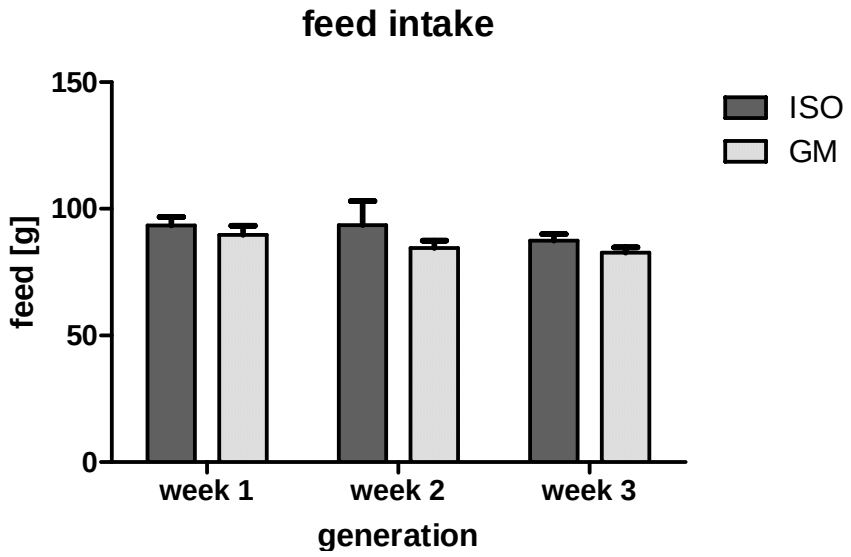


Figure 17: **Feed intake within the first 2 weeks of the RACB of the mating pairs fed 33% isogenic (ISO) or genetically modified (GM) corn in their diet**

At mating the F0 breeding pairs did not differ in weight. All females and males gained weight throughout the test. But no statistically significant differences between the two groups were seen in body mass of females or males (Table 58).

Parental reproduction

During the 20 week period of the RACB 4 litters were bred. From 24 pairs assigned to the ISO and GM group, all females of the ISO group (100%) delivered 4 litters (Table 59). In the GM group the number of deliveries declined with time. In the 4th litter only 20 deliveries occurred (p=0.055). The average number of pups born was always lower in the GM group but not significant before the 3rd delivery. There were significantly fewer pups born in the GM group in the 3rd (p= 0.011) and 4th (p=0.010) delivery and weaned in the 4th litter (p=0.025).
Regarding all deliveries per group more pups were born in the ISO than in the GM group (1035 versus 844). Furthermore females of the GM group always had smaller litters (n≤ 8) as compared to females of the ISO group (Figure 18).
At weaning the GM group had significantly fewer pups weaned in the 4th litter, though less pups were lost during weaning in all generations (only significantly in the 3rd litter p=0.025). Litters with a high number of pups tended to lose more pups. No difference was seen in the birth interval of 1st, 2nd and 3rd litters (data not shown).

Inter-litter comparison within the ISO group showed significantly less pups born in the 1st than in the other three litters and in the GM group significantly less pups were born in the 1st and 4th litters.

Table 58: **Performance of mice fed 33% isogenic (ISO) or genetically modified (GM) corn in their diet over several deliveries**

	Parental performance							
	1st litter		2nd litter		3rd litter		4th litter	
	ISO	GM	ISO	GM	ISO	GM	ISO	GM
body mass [g] female								
delivery	33.11 ± 0.440	32.86 ± 0.548	36.09 ± 0.455	36.17 ± 0.604	38.37 ± 0.572	37.36 ± 0.607	39.97 ± 0.797	38.39 ± 0.751
1 week after delivery	34.97 ± 0.526	34.67 ± 0.600	36.76 ± 0.504	35.91 ± 0.685	38.65 ± 0.427	38.67 ± 0.606	40.47 ± 1.801	41.60 ± 0.963
2 week after delivery	36.94 ± 0.698	35.98 ± 0.706	39.47 ± 0.798	38.59 ± 0.887	42.37 ± 0.696	42.05 ± 0.849	42.36 ± 0.508	41.55 ± 0.957
3 week after delivery	43.42 ± 2.113	47.29 ± 2.417	50.92 ± 1.675	47.43 ± 2.178	53.66 ± 1.481	54.73 ± 1.702	38.38 ± 0.601	40.99 ± 2.207
body mass [g] male								
delivery	32.80 ± 0.436	32.78 ± 0.603	34.54 ± 0.481	34.90 ± 0.649	34.96 ± 0.439	35.64 ± 0.721	-	-
1 week after delivery	33.41 ± 0.469	34.15 ± 0.600	34.53 ± 0.426	35.30 ± 0.581	35.56 ± 0.526	35.91 ± 0.738	-	-
2 week after delivery	34.31 ± 0.475	34.79 ± 0.765	35.65 ± 0.439	36.12 ± 0.682	37.02 ± 0.805	36.35 ± 0.873	-	-
3 week after delivery	34.67 ± 0.432	34.74 ± 0.683	35.96 ± 0.474	36.77 ± 0.745	37.49 ± 0.703	37.30 ± 0.893	-	-

Values represent means and standard error

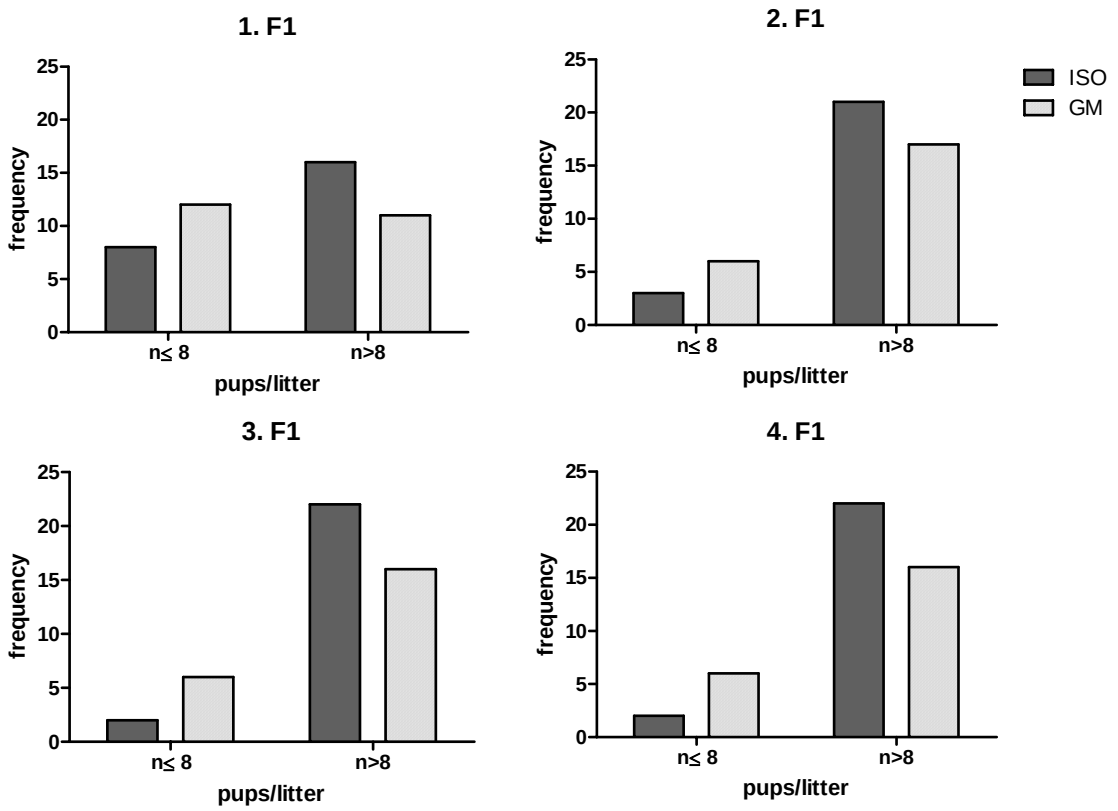


Figure 18: **Distribution of litter size at delivery from females fed 33% isogenic (ISO) or genetically modified (GM) corn in their diet over 4 litters**

Table 59: **Reproduction data of mice fed 33% isogenic (ISO) or genetically modified (GM) corn in their diet over several generations**

	Parental reproduction							
	1st litter		2nd litter		3rd litter		4th litter	
traits	ISO	GM	ISO	GM	ISO	GM	ISO	GM
Pairs/group	24	24	24	24	24	24	24	24
Deliveries/group	24	23	24	23	24	22	24	20
Non deliveries/group	0	1	0	1	0	2	0	4
Weaned litters/group	24	23	24	23	24	17	24	19
Number of pups at birth/pair	9.00 ± 0.614	8.22 ± 0.590	10.83 ± 0.473	10.65 ± 0.649	11.92[a] ± 0.496	9.68[b] ± 0.688	11.38[a] ± 0.462	8.21[b] ± 1.077
Sum of pups at birth/group	216	189	260	245	286	213	273	197
Number of pups at weaning/ pair	8.33 ± 0.560	8.13 ± 0.560	10.04 ± 0.480	9.83 ± 0.550	10.58 ± 0.454	9.06 ± 0.820	9.79[a] ± 0.525	7.21[b] ± 0.985
Sum of pups at weaning/ group	200	187	241	226	254	207	235	173
Pup losses/group	0.67 ± 0.305	0.09 ± 0.060	0.79 ± 0.289	0.83 ± 0.375	1.33[a] ± 0.433	0.12[b] ± 0.081	1.58 ± 0.371	1.00 ± 0.376
Sum of pup losses/group	16	2	19	19	32	2	38	24

Values represent means and standard error, means that do not share a common superscript are significantly different ($p < 0.05$)

Offspring performance

In the first two litters no significant differences were found between the groups. In the third litters the average litter weights were statistically significantly lower in the GM group at birth and on the second day (at birth p=0.026; 2 d p=0.040) as well as at weaning (p=0.031). In the fourth litter the average litter weight was significantly lower in the GM group (p=0.05), but all the consecutive average litter weights failed to meet the level of significance by a small margin (2 d p=0.053; 3 d p=0.070; 4 d p=0.080; 5 d p=0.082; 6 d p=0.064; 7 d 0.088 and 14 d p= 0.088).

The individual pup masses in litters with a high number of pups are commonly lower. In the ISO group the average individual pup mass of the small litters (n≤ 8) was once significantly (p=0.043) lighter as compared to GM pups in the 4th litter (Table 60). The average individual pup weights were not higher in the GM group, although this group had significantly smaller litter sizes (p<0.001).

Data in figure 19 a-d and 19 a'-d' and table 60.

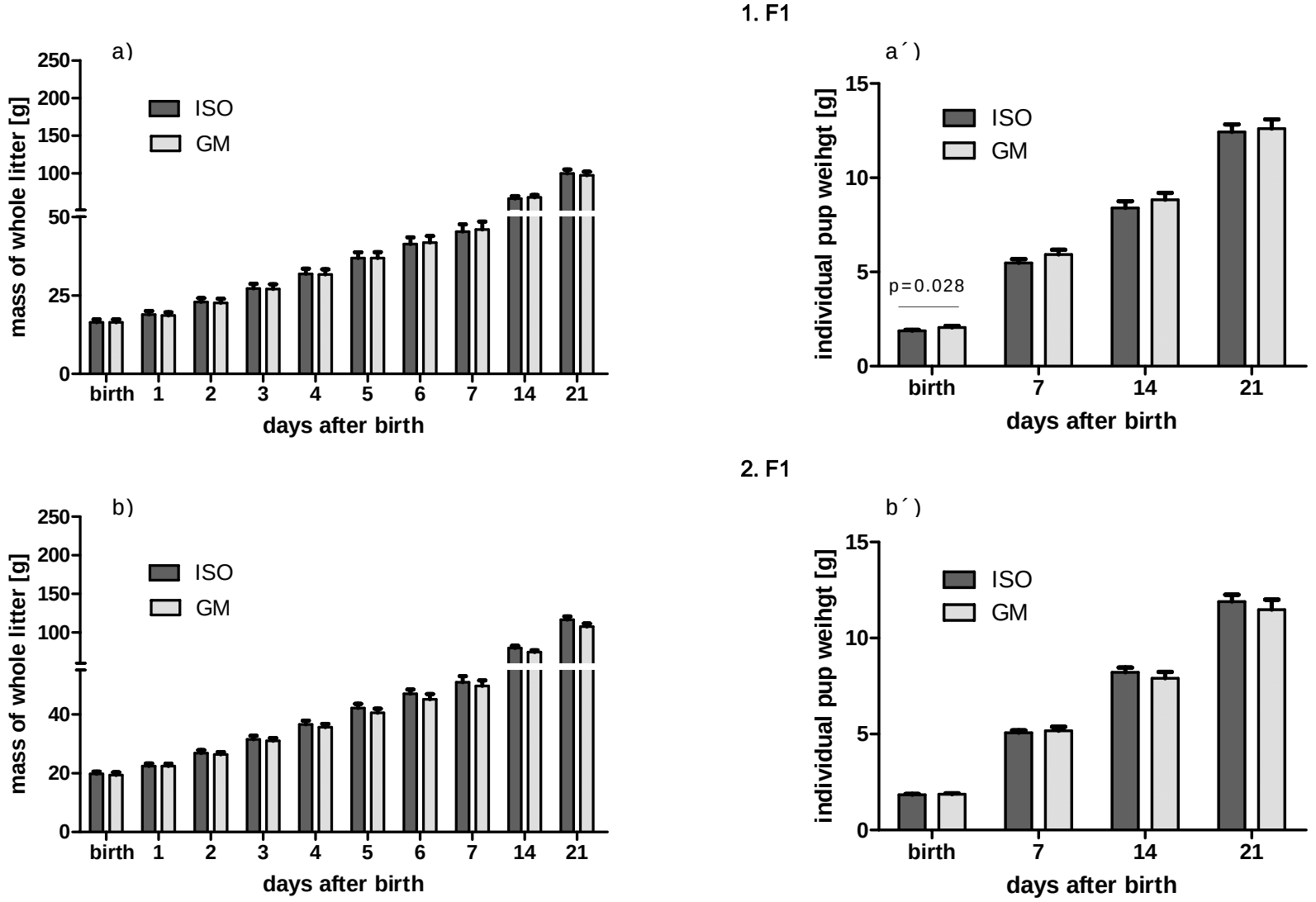
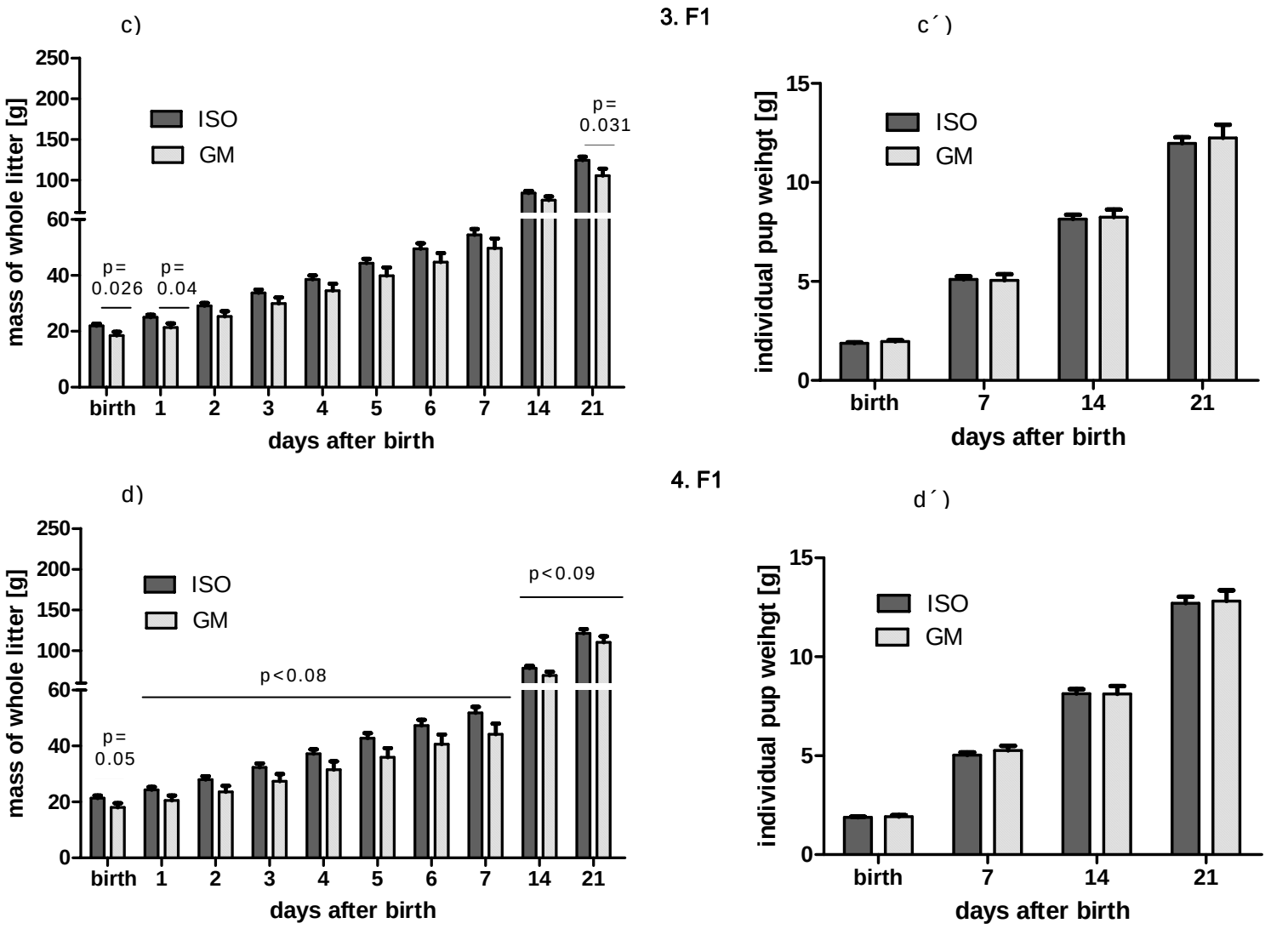


Figure 19 a-b and 19 a'-b': **Mass of whole litter [g] and individual pup weight [g] at several time-points of the 1st and 2nd F1 generation after birth of offspring from parental mice fed 33 % isogenic (ISO) or genetically modified (GM) corn in their diet**



Figures 19 c-d and 19 c'-d': Mass of whole litter [g] and individual pup weight [g] at several timepoints of the 3rd and 4th F1 generation after birth of offspring from parental mice fed 33% isogenic (ISO) or genetically modified (GM) corn in their diet

Table 60: **Individual pup mass [g] per group at birth, 7, 14 and 21 days after birth from parents with 33% of near isogenic (ISO) or transgenic (GM) corn in their diet**

	generation			
	ISO n≤ 8	**GM n≤ 8**	**ISO n> 8**	**GM n> 8**
		1. F1		
Birth	2.08 ± 0.060	2.21 ± 0.083	1.77 ± 0.051	1.89 ± 0.078
7d	6.36 ± 0.326	6.44 ± 0.372	4.95 ± 0.124	5.38 ± 0.221
14 d	9.92 ± 0.624	9.87 ± 0.493	7.48 ± 0.209	7.68 ± 0.263
21 d	14.03 ± 0.588	14.23 ± 0.505	11.47 ± 0.346	10.82 ± 0.445
		2. F1		
Birth	1.99 ± 0.102	2.17 ± 0.057	1.81 ± 0.044	1.75 ± 0.027
7d	5.55 ± 0.240	6.16 ± 0.255	4.93 ± 0.141	4.73 ± 0.209
14 d	9.63 ± 0.314	9.54 ± 0.457	7.83 ± 0.251	7.17 ± 0.293
21 d	13.62 ± 0.343	13.96 ± 0.321	11.44 ± 0.385	10.38 ± 0.536
		3. F1		
Birth	2.21 ± 0.308	2.20 ± 0.121	1.83 ± 0.050	1.87 ± 0.064
7d	5.66 ± 0.555	6.26 ± 0.226	5.00 ± 0.132	4.60 ± 0.360
14 d	9.696 ± 0.563	9.95 ± 0.874	7.83 ± 0.183	7.57 ± 0.242
21 d	13.72 ± 0.973	14.48 ± 0.928	11.62 ± 0.280	11.02 ± 0.650
		4. F1		
Birth	1.94 ± 0.033	2.15 ± 0.178	1.87 ± 0.037	1.82 ± 0.058
7d	5.4[a] ± 0.305	6.49[b] ± 0.330	4.89 ± 0.134	4.83 ± 0.185
14 d	9.31 ± 0.246	10.30 ± 0.643	7.65 ± 0.192	7.34 ± 0.279
21 d	14.36[a] ± 0.356	15.93[b] ± 0.629	12.00 ± 0.321	11.71 ± 0.391

Values represent means and standard error, means that do not share a common superscript are significantly different ($p < 0.05$)

3.4.2. q-RT-PCR

See point 3.2.7.

4. Discussion

Aim of the study

The aim of the study was to examine chronic feed effects of the stacked GM maize NK603 x MON810 in mice. A short term broiler study showing no effects had been conducted with the event in question, but no rodent feeding study was performed, since both parental GM lines had been declared safe and the new event was obtained by conventional breeding. No further transgene has been introduced.
Toxicological risks of GM plants are currently assessed by 90 day feeding studies with rodents. A 90 day study is considered as sufficient to detect adverse effects and the duration is considered as long enough by the EFSA GMO Panel. However, chronic effects might only become evident in longer lasting multi-generation studies, since reproduction and lactation as well as growth and survival rate of the offspring are very sensitive parameters. Furthermore almost all present GM crops are used for the nutrition of breeding animals.

Therefore the impact of dietary factors on fertility needs to be investigated in more detail. This is the first study investigating a stacked event in a multigeneration study focussing on mice in reproduction and development. Additionally microscopic investigations (histology, electron microscopy and immunohistochemistry) were performed to investigate possible effects of transgene maize at cellular level and microarray analyses for possible impacts at molecular level.

Methods

In this project two breeding designs were applied for the evaluation of the stacked event NK603 x MON 810 to highlight and compare the suitability of different study designs for risk assessment. The first experimental design was a multigeneration study (MGS) with 4 generations of mice. The second breeding scheme was a reproductive assessment by continuous breeding including 4 litters (RACB). Traits investigated were body mass development of parents and offspring as well as the fertility parameters litter size and survival rate until weaning.
To corroborate the results of the feeding studies additional investigations have been included in the MGS. Organ weights, histological and electron microscopic ultrastructural investigations were performed to detect changes at the organ and cellular level. Focus was laid on the intestine as a primary indicator of feed-animal interface. Immunohistochemistry was applied for the investigation of immune cells in the small intestine and finally gene expression profiles of the jejunum were performed by microarray analyses and q-RT-PCR.
Finally a life term study was performed with focus on mortality of mice allotted to the feeding groups. This design turned out to be less suitable for risk assessment studies.

For all trial designs, animals from an outbred mouse strain were chosen. The results obtained from an outbred strain can be considered as basis with a wider

range of various mouse genotypes. The alternative would have been to use inbred mice strains. Due to a lower genetic variability the results might have been less variable. However, the disadvantages of such an approach are also obvious. Inbred lines may be more or less susceptible to certain external stimuli and may therefore give a biased insight into the nutrition host interaction. This might happen in both directions, making the assessment of nutritional factors more complicated.

The results presented in the study provide a wide range of differences between the feeding groups that appear higher than natural variations normally expected. The total spectrum of methods is broad and should allow a valid conclusion about the potential impact of the different corn varieties on the animal. However, even with such a broad approach subtle effects might have been missed. On the other hand, some methods would need to be explored in more detail in future studies to evaluate the background and the variability under varying dietary conditions and with a broader spectrum of different mice strains or animal species, ideally covering several nutritional types (omnivorous, herbivorous, carnivorous).

Corn used for the feeding trials

In the MGS three feeding groups were established with diets containing the stacked corn NK603 x MON810 (GM group), the near isogenic line (ISO group) grown in Canada and an additional Austrian GM free reference corn (A REF). The addition of A REF corn was prompted by a slight contamination of the ISO corn and fulfilled the criteria of substantial equivalence. All different varieties were harvested in 2005. The RACB investigation included only the GM and ISO corn from a second harvest in Canada in 2007. All corn varieties were substantially equivalent in both harvests. The diets were offered as meal instead of pellets in order to avoid potential changes of feed components due to the application of heat and pressure. This is an important fact because for GMO crops the heat sensibility and in general the susceptibility to feed and food processing methods has hardly been addressed up to now.

Reproduction and performance- MGS

The MGS over 4 generations did not show significant differences between the feeding groups ISO and GM. The number of pups weaned, the average litter size and weight at weaning tended to be lower in the GM group as compared to the ISO group. At the same time the pup losses were higher in the GM group. These differences were consistent over the generations, but not significant, since the intra-group variability was very high.
It might be speculated that not all mice were compromised by the GM feed because of the high genetic variation between the test animals. The effects on litter size and weight became more notable in the 4th generation. In terms of production profit the ISO group had more weaned pups, 9% more females with litters (64% vs 73%) and slightly higher average litter weights at weaning in the ISO group (92.6 g vs 102.1 g).

The additional A REF group excelled in number of females with litters (91%) and accordingly more pups weaned as well as a 35% higher body mass production as compared to the GM group. Within four generations bred in the MGS no adverse effects on overall health and reproduction as well as performance were seen. Feed intake, fertility rate and number of pups born and weaned as well as body weight gain showed no statistically significant ($p<0.05$) differences.

Reproduction and performance- RACB

The 1st litters in the RACB displayed no differences between the GM and ISO feeding groups. Comparing the 2nd litters a very slight tendency towards smaller litter size and accordingly lower average litter weight in the GM group could be observed. In the 3rd and 4th litters the aforementioned traits became significant ($p<0.05$). Apart from a decline of deliveries, in the 3rd and 4th litters significantly fewer pups were born and in the 4th litter also significantly fewer pups were weaned in the GM group. The average litter weights were in favour of the ISO group with significant results in the 3rd litters at birth and weaning as well as in the 4th litters at birth. But in contrast to the MGS the loss of pups was higher in the ISO group. These results substantiate the assumption that long term feeding studies with more generations are useful in studying chronic diet related effects. According to our data the RACB design was better suited than the MGS, since the differences between the feeding groups were at significant levels. The biological phenomenon observed in the RACB trial cannot be explained by different nutrient intakes, because both diets were covering the energy and nutrient requirements and fulfilled the prerequisite of nutritional equivalence. Lower reproduction performance can be considered as indicator for a dietary effect. It can be speculated, that this effect was caused by a factor beyond nutrient supply. Whether this can be related to one of the two genetic modifications in the transgenic material or whether this is an unintended effect in the strict sense related to the stacked events has to be further evaluated.

Compared to the findings in the RACB trials it can be assumed that the physiological stress was considerably lower in the MGS trial. The trial design of using “new” parental generations instead of continuous breeding with the same generation has to be considered as being obviously less demanding. This might have masked the impact of dietary factors on reproductive performance. However, this part of the experiment is valuable as such because it underlines the need for different experimental designs for the assessment of dietary effects that have an unknown impact on animals.

The genomic work that was performed in the gut tissue of the mice of both groups is not indicative. However, the high number of deregulated genes that has been identified as difference between both groups could indicate a complex nutrition-host-interaction. This has to be further evaluated and gene expression profiles need to be considered in other organs and especially in the reproductive system. To date, trials have not been performed on that issue in feeding studies with genetically modified corn to our best knowledge.

Reproduction and performance in other trials

It is surprising that despite the long use of Bt corn since 1996 and the many controversial discussions about its safety, partly fuelled by anecdotal evidence, only few peer-reviewed multi-generation studies investigating potential effects of delta endotoxins on rodents have been conducted so far. Brake et al. (2004) used mouse testes as a sensitive indicator of potential toxic effects of diets containing Bt corn. The type of delta-endotoxin was not mentioned nor the conditions under which the diet was processed. This is an essential point when comparing different studies. When heat is applied during feed processing (e.g. pelleting), the danger of denaturing the transprotein is high and the outcome might be completely different compared to the raw material.
In the aforementioned Brake-study different mouse strains were used and crossed. For a short term study the mice were obtained at the age of 5 weeks and kept for 3 weeks on a conventional mouse chow. Only at the time of breeding the test diets were given. For the long term study with four generations 16 randomly chosen males and females (2 of each sex and strain for each diet) were used at the start and fed the test diets before mating. To produce the 2nd and 3rd generation 6 females and 3 males were paired for each strain and diet.
No diet related differences in the sperm development were found in this study. Significant differences occurring during the spermatogenesis were attributed to age differences. The progeny born within the same 24 hours was considered the same age. The authors also mentioned effects on litter sizes and weights. In the 4th generation they found significant differences in body weight comparing 3 animals / treatment at day 26 in favour of the GM diet ($p=0.001$) and on day 63 in favour of the conventional diet ($p=0.005$). It is also stated that litter sizes were similar in both feeding studies, suggesting that the Bt diet is not a factor impairing reproductive performance. The results are not corroborated by the present study. Data cannot be directly compared to the present results since inbred mouse strains have smaller litters and often have lower body weights.
A three generation study with Bt corn was also conducted with laboratory rats. Apart from some significant histopathological changes in liver and kidney no differences were found between the feeding groups (Kilic and Akay 2008). No differences concerning developmental performance were reported. But the number of offspring was generally very low in this 3 generation study, 4-5 pups / dam, whereas 10-12 pups / dam can be expected from Wistar Albino rats.
Many short term feed conversion studies with GM crops conducted with farm animals showed no negative effects (Aumaitre 2002; Flachowsky et al. 2005). The number of feeding studies with rodents is small, and inconsistent differences make it difficult to draw an overall conclusion on the tested GM feed (Hammond et al. 2006). Thus the safety of NK603 x MON810 is based on one poultry study performed by the applicant with the parental lines including 90 day rodent studies, and one poultry study with the stacked event (ACRE 2004). The GMO Panel of EFSA considers it unlikely that NK603 x MON810 maize will have any adverse effect on human and animal health (Opinion of GM Panel, 2005)

Regarding the weight development of the parental mice in the present study the short term feeding results can be corroborated, since the weight differences observed were very small and inconsistent. Chronic effects are difficult to measure and cannot be assessed by feeding trials in non performing animals. To ascertain that no chronic health impacts are caused by GM feed components the animal homeostatic system has to be challenged, since health is defined by the ability to

handle and overcome challenges, e.g. infections or stress, successfully. In the present study reproduction was chosen as a high performance status in a long-term feeding study encompassing several generations (MGS) and continuous reproduction of several litters (RACB). The RACB test design is normally applied for testing xenobiotic substances such as pesticides to define safety limits and has never been used before in connection with GM assessment to our knowledge. Since in toxicity tests, the LD50 for Cry1Ab showed no dose related deaths at an amount of 4000mg/kg (oral), the EPA has established the rule of an exemption from the requirement of a tolerance for residues of the plant pesticide active ingredients Bacillus thuringiensis Cry1Ab delta-endotoxin and the genetic material necessary for its production in all plants (EPA, 2001). But no multigenerational studies with the toxins have been performed to exclude any possible chronic effects.
The present RACB has been designed as whole feed study. The interpretation poses difficulties since it does not concern one single compound in different concentrations, but whole feed effects. On the other hand realistic conditions are reflected. Further studies are needed comparing GM corn producing the Bt toxin with non-GM corn spiked with corresponding amounts of Bt toxin to investigate whether the method of GM and/or the toxin are responsible for the outcome.

Organ weights and microscopic investigations (histology, immunohistochemistry, ultrastructural investigations)

Organ weights were recorded as potential indicator of a dietary effect on the organism. Liver and kidneys are central metabolic organs and are important for metabolic and excretory processes and are therefore often regarded as indicator organs for toxic effects. Therefore differences in liver and kidney weights are considered as sensitive risk parameters. Kilic and Akay (2008) also referred to significant differences in these organs.
Significantly lower relative kidney weights were found in GM females (F2, F3, F4) and in GM males (F3). Hammond et al. (2006) also mentioned lower relative kidney weights for MON863 (Cry3Bb1) fed males compared to the controls, but not at a statistically significant level. Microscopic pathological changes were described earlier in kidneys from rats from a 90day feeding trial, but they were not considered being feed related. A revision of these data indicated the possibility of GM-linked renal toxicity in male animals (Séralini et al. 2007), however, these results were critically discussed by several other authors including EFSA. Increased liver weights in females fed a GM diet were discussed as potential risk indicator (Séralini et al. 2007). In the present study liver weights were different between feeding groups in GM females, however, this was not unidirectional and therefore not interpretable. No differences in liver weight were seen in males.
The spleen is an important immunological organ and thus may also reflect dietary impacts. In the present study the relative spleen weights were significantly higher in the GM males of the F2 generation, in the other trial periods no such differences were found. No histological changes were seen in these organs.

The investigation of T- and B- lymphocytes as well as macrophages by immunohistochemistry did not reveal differences between the groups.

The ultrastructural investigation revealed some statistically significant differences between the groups. The fibrillar centres (FC) and dense fibrillar components (DFC) and the pore density are linked to the metabolic rate of cells. Increasing

metabolic rate leads to higher values of these parameters (Schwarzacher and Wachtler 1993; Dzidziguri et al. 1994). The nuclear shape irregularity, a way to detect enlarged surface areas, sometimes appears with rapid nucleus activity enhancement (Malatesta et al. 1998). Regarding the main test groups ISO and GM some differences were found. The lower nuclear pore density and the lower quantity of the nucleolar components FC and DFC in both females and males, found in hepatocytes of GM mice, indicate a lower liver metabolic rate in animals fed the GM feed. Similar findings were reported previously (Malatesta 2002). Since hepatocytes are involved in numerous metabolic activities, the cause of these observations is not clear.
The spleen lymphocytes in male mice showed higher DFC values in the GM group compared to the ISO group, suggesting an increased activity. Females seemed not to be affected. The DFC in pancreatic cells was decreased in males of the GM group, the FC was slightly increased.

Therefore, a generalizing conclusion about cell activities is not possible. The comparison of the ISO group to the REF A group showed only few differences. Only the decreased FC and DFC values in hepatocytes of male mice in the A REF group as compared to the ISO group were significant. The other findings showed comparable values.
Although the ISO and A REF diets were based on different corn varieties, the ultrastructural data of these two groups are closer together than those obtained from comparing the ISO and the GM group. Possibly, these parameters are less influenced by the maize variety than by the genetic modification.

Molecular analyses- Microarray and q-RT-PCR

Differences in gene expression in the intestinal tissue were seen in a number of biological processes when the different groups were compared. The corn might have contributed to that because the substantial equivalence was given, however, minor differences might have acted as extrinsic factors. The inter-individual differences generated by the outbred strain (intrinsic factors) may have amplified noise of the expression data. Microarray data display phenotypic variability through noise from intrinsic or extrinsic sources and can make those data difficult to interpret (Raser and O'Shea 2005). When ISO and A REF were pooled to one group and compared to the GM group, the expression data from ISO and GM comparison became more pronounced and the level of significance increased in the pathways protein biosynthesis as well as protein metabolism and modification. In addition to difficult data interpretation through noise the majority of differences found in the array data were under a fold change of 2 which is rather low and near detection limit of microarray analyses. Moreover, the dynamic range/sensitivity of microarrays limits their use in detecting changes in mRNA levels of those genes expressed at low abundance (Lord et al. 2006). Clustering into biological processes and pathways was used in our study to overcome this limitation.

Influence of the variety but also of the genetic modification were observed in microarray analyses of jejunal tissue. The intestine is considered as "feed-host interface" and until now no effects of Cry1Ab on mammalian intestinal cells were reported (Bondzio et al. 2008). As there are no previous pathways identified whole transcriptome microarray analyses covering the whole murine genomic

profile were used as a pre-screening tool. Significant ($p<0.001$) differences in gene expression were identified in a number of biological processes and pathways between the GM and non-genetically modified diets.

Q-RT PCR, the gold standard for gene expression analysis, was done with a set of 45 out of 400 deregulated genes previously identified by microarray analyses. From the 45 genes investigated on the TLDA, 9 ($p<0.05$) genes were classified as deregulated with influences by the trial, but also by sex and anatomical site. Differences between ISO and GM fed mice detected by microarray analysis were observed in several pathways. Clearly more work needs to be done on those analyses to get further insights into natural variation of gene expression and potential impact of dietary modifications. In a next step more work is necessary regarding the normalization of expression data and extending set of target genes that can contribute to the list of deregulated genes by GMO food. The networks around the marker genes identified are a promising issue of further research. Further the sampling strategy has to be improved to circumvent high intra-group variability.

Variability trough noise is coming from extrinsic e.g. the corn varieties and intrinsic, e.g. outbred strain sources that make array data difficult to interpret (Raser and O'Shea 2005). Further work is necessary to confirm and identify the full set of deregulated genes, identify involved pathways and especially to proof the deregulation at protein level. So far the genes detected represent only a proof of principle that differences can be seen between the ISO and GM group but no statement about the meaning of those genes can be discussed.
Due to the high technical demands and costs of such trials it will be difficult to set up these approaches in the future regarding the high number of new applications for the import of transgenic foods and feeds into the EU that are expected to come. However, based on the experience of this study it seems to be feasible to establish new test models that would increase consumer safety in this important area of risk assessment at least in selected GM feed and food materials.

5. Conclusion

Feeding mice with diets containing the transgenic corn NK603 x MON810 in different models of multi-generation studies indicated that the RACB trial design was sensitive and could therefore be better suited compared to the MGS model for the evaluation of reproductive traits. Reproductive traits were not statistically different over 4 generations in the MGS, but in differences between the groups became obvious in the 3rd and 4th litters of the RACB.

RNA microarrays and q-RT-PCR indicated differences between the groups. The findings were weak and need confirmation. However, a dietary impact on gene expression cannot be excluded. The high intra group variance could be due to different sensitivity of genotypes within the outbred mouse strain OF1 used in this study. For further investigation an RACB including several inbred strains could be useful. Some data obtained from the assessment of selected traits in organs by electron microscopy indicate a diet-host interaction that should be further evaluated.

The trial indicates that dietary interactions with the host organism have to be further evaluated. Regarding the sensitivity of the topic, studies are needed to extend the database using standardized feeding trials with clear endpoints such as reproductive performance and a backup by genomic, proteomic and metabolomic traits.

Summarising the study, the maize with the stacked event NK603 x MON810 affected the reproduction of mice in the RACB trial. Whether similar findings could be expected for other animals, needs to be evaluated in studies including reproductive traits. Future studies are necessary to determine the impact of normal and transgenic dietary ingredients on the organism.

6. Diskussion

Ziel der Untersuchung

Das Ziel der Untersuchung war die Überprüfung von möglichen Effekten der gentechnisch veränderten Maissorte NK603 x MON810 auf Reproduktionsparameter in Fütterungsversuchen bei Mäusen. Ein Kurzzeitfütterungsversuch mit Hühnern zeigte keine nachteiligen Effekte. Fütterungsstudien mit Nagern sind nicht durchgeführt worden. Beide Elternlinien des untersuchten stacked event Produkts sind als sicher eingestuft worden.
Toxikologische Risiken gentechnisch veränderter Pflanzen werden derzeit mittels 90-Tage Fütterungsversuchen mit Nagern bewertet. Eine 90-Tage Studie wird als ausreichend eingestuft, um nachteilige Effekte zu erfassen. Es stellt sich aber die Frage, inwieweit länger dauernde Multigenerationenversuche in diesem Zusammenhang sinnvoll sein können. Reproduktion und Laktation sowie Wachstum und Überlebensrate der Nachkommen stellen sensible Parameter dar. Weiters werden fast alle derzeit vermarkteten gentechnisch veränderten Pflanzen für landwirtschaftliche Nutztiere eingesetzt, die Reproduktionsleistungen erbringen müssen. Daher erschien es notwendig, den Einfluss der Futtermittel auf die Zucht detailliert zu untersuchen.
Die vorliegende Studie untersucht einen stacked event in einem Multigenerationenversuch mit den Schwerpunkten Reproduktion und Jungtierentwicklung bei Mäusen. Zusätzlich wurden mikroskopische Untersuchungen von Darm und Organen (Histologie, Elektronenmikroskopie und Immunhistochemie) und in Darmgewebe Microarrayanalysen durchgeführt, um mögliche Effekte des transgenen Maises weitergehend zu untersuchen.

Methoden

In diesem Projekt wurden für die Evaluierung des stacked events NK603 x MON810 zwei Versuchsdesigns angewendet, um die Eignung verschiedener Ansätze für die Risikoforschung zu beleuchten und zu vergleichen. Das erste Versuchsdesign war eine Multigenerationenstudie (MGS) mit vier Mäusegenerationen. Das zweite Versuchsdesign war die Methode der fortlaufenden Zucht (Reproductive Assessment by Continuous Breeding; RACB) mit vier Würfen. Untersuchte phänotypische Parameter waren die Gewichtsentwicklung der Eltern und Nachkommen sowie Wurfgröße und Überlebensrate bis zum Absetztermin.
In der Multigenerationenstudie wurden neben der phänotypischen Erfassung der Leistungen ergänzende Untersuchungen durchgeführt. Diese umfassten die Erhebung der Organgewichte sowie histologische und elektronenmikroskopische Untersuchungen. Der Fokus lag dabei auf dem Darm im Sinne eines Grenzflächenorgans zwischen Nahrung und Tier. Immunhistochemische Methoden wurden für die Untersuchung von Lymphozytenpopulationen im Dünndarm angewendet und Genexpressionsprofile des Jejunums wurden mittels Microarrayanalysen und anschließender q-RT-PCR erstellt. Eine Lebensdauerstudie stellte den dritten Versuchsansatz dar, um mögliche Einflüsse der untersuchten Maisvarianten zu erfassen.
Für alle Versuchsdesigns wurden Mäuse eines Auszuchtstamms (OF 1) verwendet. Die Verwendung eines Auszuchtstamms bedingt eine tierexperimentelle Ba-

sis mit relativ heterogenen Mausgenotypen. Als Alternative hätte ein Inzuchtstamm verwendet werden können, der möglicherweise den Vorteil einheitlicherer Ergebnisse gehabt hätte. Nachteil dieses Ansatzes sind nicht vorhersehbare unterschiedliche Sensitivitäten des jeweiligen Testkollektivs, die das Risiko einer gerichteten, positiven oder negativen Beeinflussung der Ergebnisse erhöht hätte.

Die Ergebnisse dieser Studie zeigen einige Unterschiede zwischen den Fütterungsgruppen an, die zum Teil statistisch abzusichern waren. Das Methodenspektrum war so gewählt, dass eine Erfassung potentieller Interaktionen zwischen den verfütterten Maisvarianten und dem Organismus möglich war. Die gewählten Methoden sollten in zukünftigen Studien weiter evaluiert werden, um deren Sensitivität und die Variabilität in Abhängigkeit von Fütterungsbedingungen, Mausstämmen oder anderen Tierarten zu überprüfen. Die Einbeziehung anderer Ernährungstypen (omnivor, herbivor, karnivor) wäre sinnvoll.

Maisvarianten der Fütterungsstudien

Für die MGS wurden drei Fütterungsgruppen verwendet. Die drei entsprechenden Diäten enthielten entweder 33% NK603 x MON810 (GM Gruppe), eine isogene Maislinie (ISO Gruppe) – beide angebaut unter kontrollierten Bedingungen in Kanada – und Mais aus Österreich (A REF Gruppe). Die Diät mit österreichischem Mais wurde auf Grund einer leichten Verunreinigung der aus Kanada bezogenen isogenen Maisvariante eingeführt. Der Mais für die MGS und den Lebensdauerversuch wurde 2005 geerntet. Für den RACB Versuch wurden nur die beiden kanadischen Testsorten (GM und ISO) aus einer Ernte von 2007 verwendet. Alle Maissorten waren weitgehend substanziell äquivalent. Die Diäten wurden in Schrotform, nicht pelletiert und nicht thermisch behandelt angeboten, um mögliche Veränderungen der Futterkomponenten durch die Einwirkung von Druck und Hitze, wie es beim Pelletieren der Fall ist, zu vermeiden. Eine Temperatursensitivität der Deltaendotoxine ist nach Literaturangaben vorhanden.

Reproduktion und Leistung - MGS

Die MGS zeigte über 4 Generationen keine statistisch signifikanten Unterschiede zwischen den Fütterungsgruppen GM und ISO. Die Anzahl abgesetzter Junge, die durchschnittliche Wurfgröße und das durchschnittliche Wurfgewicht waren im Vergleich zur ISO Gruppe in der GM Gruppe geringfügig niedriger, während der Verlust an Jungen in dieser Gruppe etwas höher war. Diese Unterschiede waren konsistent in den Generationen, aber nicht auf einem signifikanten Niveau.
Tierindividuelle Reaktionen sind nicht auszuschließen, da nicht alle Mäuse durch die GM Diät beeinträchtigt waren und vermutlich eine hohe genetische Variation zwischen den Mäusen bestand. Die Einflüsse auf Wurfgröße und –masse wurden in der vierten Generation deutlicher. Im Hinblick auf die Produktion erbrachte die ISO Gruppe eine 20% höhere Gesamtleistung als die GM Gruppe, was auf das Zusammentreffen mehrerer Faktoren zurückzuführen war. Die ISO Gruppe hatte mehr abgesetzte Junge, 9% mehr Weibchen mit Würfen und ein geringfügig höheres Durchschnittsgewicht bei den abgesetzten Würfen.
Die zusätzliche A REF Gruppe übertraf die beiden anderen Gruppen sowohl bezüglich der Weibchen mit Würfen und dementsprechend höheren Anzahl ange-

setzter Junge als auch einer 35% höheren Gesamtproduktionsleistung verglichen mit der GM Gruppe.
Während der vier Generationen wurde kein Einfluss auf die Gesundheit beobachtet. Die Futteraufnahme, die Fruchtbarkeitsrate sowie die Anzahl an Jungen und deren Gewichtszunahme zeigten keine statistisch signifikanten Unterschiede.

Reproduktion und Leistung - RACB

Die ersten Würfe in dem RACB Versuch zeigten keine Unterschiede zwischen den Fütterungsgruppen. Während des zweiten Wurfes konnte eine geringfügige Veränderung zu kleineren Würfen und niedrigeren durchschnittlichen Wurfgewichten in der GM Gruppe beobachtet werden. Die Ergebnisse des dritten und vierten Wurfes ergaben deutlichere Unterschiede ($p<0,05$). Neben der Abnahme an Würfen pro Gruppe (im vierten Wurf nahe der Signifikanzgrenze, $p=0,055$), wurden in der GM Gruppe bei den dritten und vierten Würfen weniger Welpen geboren und bei den vierten Würfen auch weniger Jungtiere abgesetzt. Die durchschnittlichen Wurfgewichte waren in der ISO Gruppe in den dritten Würfen bei Geburt und Absetzen abgesichert höher, bei den vierten Würfen bei der Geburt. Im Unterschied zu der MGS war der Verlust an Jungtieren in der ISO Gruppe gegenüber der GM Gruppe höher.
Diese Ergebnisse unterstützen die Annahme, dass Langzeitfütterungsversuche für die Erfassung von ernährungsbedingten Effekten geeignet sind. Entsprechend unserer Ergebnisse war das RACB Versuchsdesign offenbar sensitiver als das MGS Design und zeigte abzusichernde Unterschiede. Die unterschiedlichen Reproduktionsdaten im RACB Versuch können nicht plausibel durch unterschiedliche Nährstoffaufnahmen erklärt werden, da beide Diäten den Energie- und Nährstoffbedarf der Tiere abdeckten und den Anforderungen an Äquivalenz sehr weitgehend entsprachen.
Eine geringere Reproduktionsleistung kann als Indikator für diätetische Effekte angesehen werden. Es ist nicht auszuschließen, dass diese Effekte auf Einflussfaktoren außerhalb der Nährstoffversorgung beruhen. Ob diese Beobachtung auf die genetische Modifikation oder auf einen unbeabsichtigten Effekt des geprüften Produkts zurückzuführen ist, sollte in künftigen Arbeiten untersucht werden. Die genomischen Untersuchungen des Darmgewebes der Mäuse beider Gruppen können die Ursache der Veränderungen in den Reproduktionsparametern nicht erklären. Aber die große Zahl an deregulierten Genen, die als Unterschied zwischen den beiden Gruppen identifiziert wurden, könnte auf eine komplexe Interaktion von Nahrungsfaktoren mit dem Organismus hindeuten. Diese Analysen wären zu erweitern und sollten auf andere Körpersysteme, besonders solche mit Relevanz für die Reproduktion, ausgedehnt werden. Bis heute gibt es nach unserem Wissen keine solchen Untersuchungen im Zusammenhang mit gentechnisch verändertem Mais.

Der direkte Vergleich der Ergebnisse des RACB Versuches mit denen des MGS legt die Hypothese nahe, dass die physiologische Stressbelastung in der MGS im Vergleich zum RACB erheblich geringer war. Das Versuchsdesign, bei welchem für jede Generation „neue“ Eltern aus den Nachkommen der vorherigen Generation herangezogen werden anstatt dieselben Eltern in mehreren konsekutiven Phasen zu verwenden, ist offensichtlich für den Organismus weniger fordernd und belastend. Diese Tatsache könnte den Futtereinfluss auf die Reproduktion in der MGS maskiert haben. Das MGS Design stellt trotzdem einen wichtigen Beitrag in der Diskussion um die Bewertung von genetisch modifizierten Futtermitteln dar,

da es die Notwendigkeit der vergleichenden Prüfung verschiedener Versuchsdesigns bei der Beurteilung von unbekannten Diätfaktoren verdeutlicht.

Reproduktion und Leistung in anderen Untersuchungen

Es ist festzustellen, dass trotz der langjährigen Anwendung von Bt Mais seit 1996 und vieler kontroversieller Sicherheitsdiskussionen, die teilweise ohne wissenschaftlich belegte Aussagen geführt werden, bisher kaum Multigenerationenstudien zu möglichen Effekten von Deltaendotoxinen bei Nagern durchgeführt wurden. Brake et al. (2004) verwendeten Hoden von Mäusen für die Evaluierung potenziell Ernährungseffekte von Bt Mais. Weder die Art des Endotoxins noch die Futterzubereitung wurden explizit dargestellt. Diese Informationen sind für den Vergleich verschiedener Studien von grundlegender Bedeutung. Bei Hitzeanwendung, z. B. beim Pelletieren, besteht die Gefahr der Denaturierung von Proteinen. Es ist daher nicht auszuschließen, dass Ergebnisse anders ausfallen als bei Rohprodukten. In der zitierten Studie wurden verschiedene Mauszuchtlinien verwendet und gekreuzt. Für einen Kurzzeitversuch wurden fünf Wochen alte Mäuse über einen Zeitraum von drei Wochen mit kommerziellem Mischfutter ernährt. Erst zur Paarungszeit wurde die Testdiät verabreicht. Für die Langzeitstudie mit vier Generationen wurden nach dem Zufallsprinzip 16 Weibchen und Männchen (jeweils zwei Tiere je Geschlechts und Zuchtlinie / Testdiät) ausgewählt und bereits vor der Paarung mit der Testdiät gefüttert. Für die Produktion der zweiten und dritten Generation wurden für jede Zuchtlinie und Diät jeweils sechs Weibchen mit drei Männchen gepaart. In dieser Studie wurden keine Unterschiede in der Spermatogenese gefunden. Signifikante Unterschiede, die während der Spermatogenese auftraten, wurden auf das differierende Alter der Tiere zurückgeführt. Die innerhalb von 24 Stunden geborenen Nachkommen waren als gleichaltrig eingestuft worden. Die Autoren erwähnen auch Wurfgrößen und –gewichte. In der vierten Generation am 26. Tag waren die Tiere der GM Gruppe signifikant schwerer ($p = 0{,}001$), während am 63. Tag das Ergebnis umgekehrt war ($p = 0{,}005$). Allerdings wurden jeweils nur drei Tiere für die Gewichtsbestimmung herangezogen. Die Wurfgrößen waren ähnlich, so dass die Bt Diät keine negativen Einflüsse auf die Reproduktion hatte. Diese Ergebnisse können mit der hier präsentierten Untersuchung nicht direkt verglichen werden, da sich Inzuchtlinien durch kleinere Würfe und geringere Körpermassezunahmen auszeichnen und auch die Futtervarianten unterschiedlich waren.

Eine weitere Studie mit Laborratten und Bt Mais wurde über drei Generationen durchgeführt. Abgesehen von histopathologischen Unterschieden in Leber und Niere wurden keine Effekte gefunden, auch nicht in der Reproduktionsleistung (Kilic und Akay 2008). Die Anzahl an Nachkommen war in dieser Generationenstudie sehr niedrig, 3-4 Junge pro Weibchen, während 10-12 Junge pro Weibchen bei dieser Zuchtlinie (Wistar Albino Ratten) normalerweise hätten erwartet werden können.

Publizierte Kurzzeitfütterungsversuche mit genetisch modifiziertem Mais an Nutztieren zeigten keine negativen Einflüsse der Bt Produkte (z.B. Aumaitre 2002; Flachowsky et al. 2005). Die Anzahl der Fütterungsversuche mit Nagern ist zu gering und inkonsistente Ergebnisse erschweren eine allgemeine Schlussfolgerung über mögliche Effekte der Testfutter (Hammond et al. 2006).

Die Sicherheitsbewertung von NK603 x MON810 beruht auf den Ergebnissen einer Fütterungsstudie mit Hühnern und einer 90-Tagesstudie mit Laborratten mit den Elternlinien der stacked event Maissorte sowie einer Untersuchung mit dem kombinierten Mais an Hühnern, die von den Antragsstellern durchgeführt wurden.

Das GMO Panel der EFSA betrachtet es daher als unwahrscheinlich, dass NK603 x MON810 negative Effekte auf die Gesundheit von Mensch und Tier haben wird (Opinion of GMO Panel 2005).
In der hier präsentierten Studie können die Ergebnisse der Kurzzeitversuche bestätigt werden, so weit es die Gewichtsentwicklung der Elterntiere betrifft, da die Gewichtsunterschiede sehr gering und inkonsistent waren. Mögliche chronische Effekte sind schwerer erfassbar, vermutlich erlauben reproduzierende Tiere eine genauere Erfassung. Langfristige Gesundheitseffekte erscheinen unter dem Aspekt einer Belastung des homöostatischen Systems von Interesse, da Gesundheit auch durch die Fähigkeit, mit Herausforderungen wie z.B. Infektionen oder Stress erfolgreich umzugehen und diese zu bewältigen, definiert wird. In der vorliegenden Studie wurde diesem Aspekt in Langzeitversuchen zur Reproduktion Rechnung getragen, wobei mehrere reproduzierende Generationen (MGS) bzw. mehrere Würfe einer Elterngeneration (RACB) eingeschlossen waren.
Das RACB Versuchsdesign wird bei der Sicherheitsbewertung von xenobiotischen Substanzen wie Pestiziden angewendet und wurde nach unserem Wissen nie zur Überprüfung von GM Produkten benützt. Da toxikologische Untersuchungen zur Bestimmung des LD50 Wertes für Cry1Ab bis zu einer Menge von 4000mg/kg keine dosisabhängigen Effekte (Todesfälle) ergaben, wurden in den USA von der zuständigen Behörde (Environmental Protection Agency) die Deltaendotoxine sowie alle Pflanzen, die solche exprimieren, von der Notwendigkeit einer Grenzwertbestimmung ausgenommen (EPA 2001). Mit den Toxinen wurden allerdings keine Mehrgenerationenstudien durchgeführt, um mögliche chronische Effekte auszuschließen. In dem vorliegenden RACB Versuch wurden Maiskörner und nicht das isolierte Endotoxin als Testprodukt gewählt. Die Interpretation solcher Versuche ist naturgemäß schwierig, da nicht ein Einzelstoff in verschiedenen Konzentrationen untersucht werden kann, sondern die Effekte des Testprodukts insgesamt erfasst werden. Im Sinne der Verwendung als Futtermittel werden dadurch aber realistische Bedingungen reflektiert. Weitere Studien mit genetisch modifiziertem und mit isogenem Mais, der mit einer äquivalenten Menge an Deltaendotoxin versetzt wurde, könnten für die Differenzierung von möglichen Effekten der genetischen Modifikation bzw. der Toxine hilfreich sein.

Organgewichte sowie mikroskopische Untersuchungen (Histologie, Immunhistochemie und ultrastrukturelle Untersuchungen)

Als mögliche Indikatoren für diätetische Effekte wurden die Organgewichte erhoben. Leber und Niere werden als mögliche Indikatoren angesehen, da sie für die Metabolisierung und Ausscheidung zahlreicher Substanzen verantwortlich sind. Daher werden Unterschiede im Leber- und Nierengewicht als empfindliche Risikoparameter gewertet. Kilic und Akay (2008) erwähnten signifikante Unterschiede bei diesen Organen zwischen den Fütterungsgruppen.
In der vorliegenden Studie wurden bei den Weibchen (F2, F3, F4) und Männchen (F3) der GM Gruppe signifikant niedrigere relative Nierengewichte nachgewiesen.
Hammond et al. (2006) erwähnten ebenfalls niedrigere relative Nierengewichte bei Männchen, die mit einer MON863 Diät (Cry3Bb1) gefüttert wurden, aber nicht auf signifikantem Niveau. Auch histopathologische Veränderungen in Nieren von Laborratten in einem 90-Tage Fütterungsversuch wurden beschrieben, aber als biologisch bedeutungslos eingestuft. Eine Evaluierung dieser Daten indizierte die Möglichkeit einer nierentoxischen Wirkung der GV Fütterung in männlichen Laborratten (Séralini et al., 2007), diese Interpretation wurde allerdings von anderen Autoren einschließlich der EFSA kritisch hinterfragt. Auch erhöhte Leberge-

wichte bei Rattenweibchen wurden als mögliche Indikatoren für toxische Wirkungen diskutiert (Séralini et al. 2007). In der vorliegenden Studie waren die Lebergewichte bei den Weibchen unterschiedlich zwischen den Fütterungsgruppen, diese Unterschiede waren jedoch nicht gleich gerichtet und sind daher nicht interpretierbar. In den Lebergewichten der Männchen wurden keine Unterschiede gefunden.
Die Milz hat neben anderen auch immunologische Funktionen und könnte daher ebenfalls Diäteinflüsse reflektieren. In der vorliegenden Studie war das relative Milzgewicht bei den Männchen der F2 Generation der GM Gruppe signifikant höher, in den anderen Generationen wurde das wiederum nicht bestätigt.
Die ultrastrukturellen Untersuchungen zeigten einige Unterschiede zwischen den Gruppen. Die Fibrillarzentren (FC) und die dichten Fibrillarkomponenten (DFC) sowie die Porendichte werden mit der metabolischen Aktivität der Zelle in Zusammenhang gebracht, wobei eine zunehmende Aktivität zu höheren Werten dieser Parameter führen soll (Schwarzacher und Wachtler, 1993; Dzidziguri et al., 1994). Eine Unregelmäßigkeit der Kernform mit vergrößerter Oberfläche wurde ebenfalls mit einer Aktivitätszunahme in Zusammenhang gebracht (Malatesta et al., 1998). Zwischen den Haupttestgruppen GM und ISO wurden einige Unterschiede gefunden. Die niedrigere Porendichte und geringere Ausprägung der FC und DFC in den Leberzellen bei Männchen und Weibchen deutet auf eine geringere Stoffwechselaktivität in der GM Gruppe hin. Ähnliche Ergebnisse wurden auch in einer früheren Studie berichtet (Malatesta, 2002). Da Leberzellen in viele metabolische Aktivitäten involviert sind, ist der Grund für diese Veränderungen allerdings nicht klar.
Die Milzlymphozyten der männlichen Mäuse zeigten hohe DFC Werte in der GM Gruppe, was demnach eine erhöhte Aktivität andeuten könnte. Die Weibchen zeigten keine Unterschiede. Die DFC Werte in den Pankreaszellen waren bei den Männchen der GM Gruppe niedriger, während die FC Werte leicht erhöht waren. Daher kann eine Schlussfolgerung über Zellaktivitäten im Zusammenhang mit GM Futter auf Basis der Ergebnisse nicht erfolgen.
Der Vergleich zwischen der ISO und AREF Gruppe zeigte geringere Unterschiede. Nur FC und DFC Werte in den Leberzellen der Männchen der A REF Gruppe im Vergleich zur ISO Gruppe ergaben signifikante Daten. Obwohl die ISO und AREF Diäten verschiedene Maissorten enthielten, waren die ultrastrukturellen Daten dieser beiden Gruppen ähnlicher zueinander verglichen mit den Daten der GM Gruppe.

Molekularbiologische Analysen – Microarray und q-RT-PCR

In den Genexpressionsuntersuchungen am Darmgewebe ergaben sich in Abhängigkeit von den Gruppen Unterschiede. Die substantielle Äquivalenz konnte in den Nährstoffuntersuchungen bestätigt werden, trotzdem scheint die Maislinie als extrinsischer Faktor in die Daten einzufließen. Auch intrinsische Faktoren wie die interindividuelle Variabilität in dem Auszuchtstamm führen zu biologischem Rauschen. Die Summe dieser Faktoren erhöht die Variabilität in derartigen Experimenten und kann zu Schwierigkeiten in der Datenanalyse führen (Raser and O´Shea 2005). Wenn die beiden nicht transgenen Maislinien (ISO und A REF) zusammengefasst und gegen die transgene Linie verglichen wurden, konnten Unterschiede zur GM Gruppe im Rahmen der biologischen Prozesse ebenfalls und sogar deutlicher bestätigt werden. Die Daten zeigen eine Erhöhung der Anzahl an deregulierten Genen im Rahmen von Prozessen der Proteinbiosynthese sowie des Proteinmetabolismus bzw. der –modifikation. Eine Schwierigkeit stellen die gerin-

gen Expressionsunterschiede dar. Nur wenige Gene über wurden oberhalb des Faktors zwei differentiell exprimiert. Für die Detektion von geringen Expressionsunterschieden sind Arrays auf Grund einer geringeren Sensitivität und eines geringeren dynamischen Bereichs eingeschränkt geeignet (Lord et al. 2006). In dieser Studie wurden die Microarrayanalysen durchgeführt, um eine Vorauswahl an Genen zu treffen, die in weiterer Folge durch Clusteranalysen und q-RT-PCR untersucht wurden.

Unterschiede in der Genexpression im Dünndarmgewebe konnten der Maissorte und der gentechnischen Veränderung zugeordnet werden. An Darmzellen von Mäusen wurden solche Untersuchungen nicht durchgeführt bzw. es wurden keine Effekte von Cry1Ab beobachtet (Bondzio et al. 2006). In der vorliegenden Studie ergaben sich zwischen den Fütterungsgruppen Hinweise auf Unterschiede in verschiedenen biologischen Prozessen.

In einem folgenden Schritt wurden 45 vorselektierte Gene aus mehreren Stoffwechselwegen mittels q-RT-PCR als „Referenzmethode“ für Expressionsanalysen untersucht. Von diesen selektierten Genen konnten 9 ($p<0.05$) als unterschiedlich zwischen ISO und GM in Abhängigkeit von Versuchsdesign, Geschlecht und anatomischer Lokalisation bestätigt werden. Angesichts fehlender Vergleichsstudien zu diätetischen Einflüssen von GMOs auf die Genexpression ist eine breitere Datenbasis in diesem Bereich erforderlich. Methodisch konnten die geringen Unterschiede in der Genexpression dargestellt werden, in weiteren Schritten sollte die Methode noch dahingehend optimiert werden, bessere Normalisierungsstrategien zu finden und somit potentielle Kandidatengene mit höherer Wahrscheinlichkeit auffinden und bestätigen zu können.

Die Variabilität von extrinsischen Faktoren, z.B. der Maissorte, und intrinsischen Faktoren, z.B. dem verwendeten Auszuchtstamm, erschwert die Datenanalyse. Weitere Arbeiten scheinen notwendig um die betroffene biologische Prozesse und Gene mit höherer Sicherheit zu identifizieren, auch unter Einbeziehung der Proteinebene. Im Moment können die aufgezeigten Unterschiede zeigen, dass die Interaktionen zwischen Futter und Darm zwischen GM und ISO unterschiedlich sind, die Bedeutung ist jedoch weiter abzuklären.

Auf Grund der hohen technischen Ansprüche und damit verbundenen Kosten wird die Anwendung der Methode auf die zahlreichen GMO Produkte mit Vorbehalt gesehen. Anhand dieser Studie konnte gezeigt werden, dass die Etablierung neuer Methoden sinnvoll wäre, zumindest bei ausgewählten GM Produkten, um den Verbraucherschutz zu erhöhen.

7. Schlussfolgerung

Die Fütterung von Labormäusen mit dem GV Mais NK603 x MON810 in zwei Mehrgenerationenstudien mit unterschiedlichen Ansätzen, MGS und RACB, zeigte, dass der RACB Versuch die sensiblere Methode darstellt und daher besser geeignet erscheint, potenzielle Effekte von genetisch modifizierten Produkten zu überprüfen. Die Reproduktionsparameter waren nicht signifikant unterschiedlich in vier Generationen im Rahmen der MGS, zeigten aber signifikant negative Effekte der GV Diät im dritten und vierten Wurf des RACB.

Die Genexpressionsanalyse und q-RT-PCR deuten ebenfalls Unterschiede zwischen den Gruppen an. Die Differenzen bedürfen der weiteren Bestätigung, Effekte der GV Diät können nicht ausgeschlossen werden. Die hohe interindividuelle Variabilität könnte auf unterschiedlich sensitive Genotypen bei den Versuchsmäusen des Auszuchtstamms OF1 hinweisen. Weitere Untersuchungen mit Inzuchtstämmen könnten hier Aufschluss geben. Einige Ergebnisse der elektronenmikroskopischen Untersuchungen geben Hinweise auf eine Interaktion der Futterzusammensetzung mit dem Organismus.

Zusammenfassend weisen die Ergebnisse dieser Studie darauf hin, dass Interaktionen zwischen den Testtieren und den verwendeten Maissorten bestehen, bei Verwendung des stacked event NK603 x MON810 zeigten sich geringere Reproduktionsleistungen im Verlaufe des RACB. Ob ähnliche Befunde für andere Stämme oder auch Spezies reproduzierbar sind, muss in entsprechenden Ansätzen untersucht werden. Diese sollten Reproduktionsparameter und eine weitergehende Untersuchung der möglichen Wirkmechanismen umfassen.

8. Overview of tables

9. Overview of figures

10. Reference List

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