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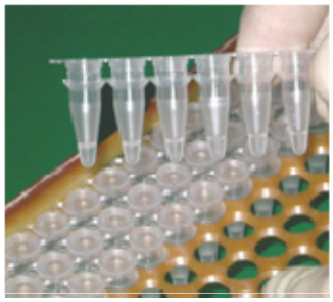

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Contents

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).

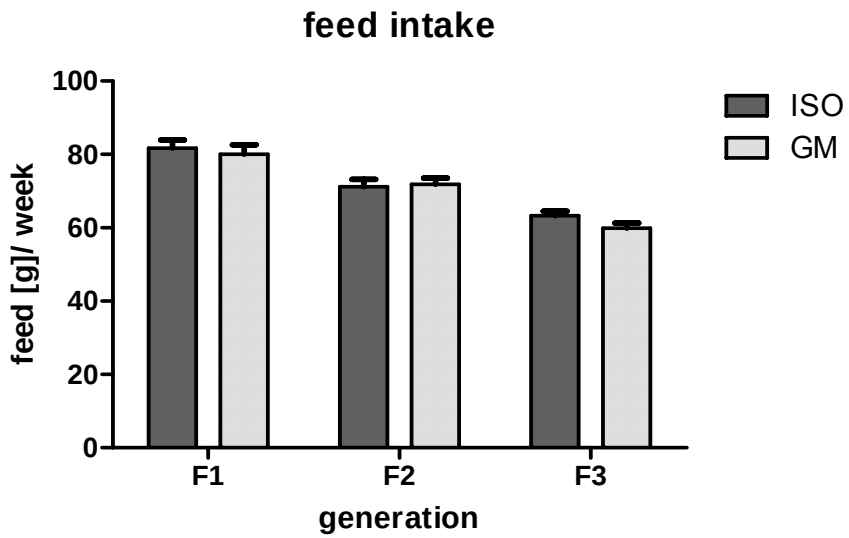


Figure 1: **Feed intake of adult mice fed 33% isogenic (ISO) or genetically modified (GM) corn in their diet in several generations**

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 ($p < 0.05$)

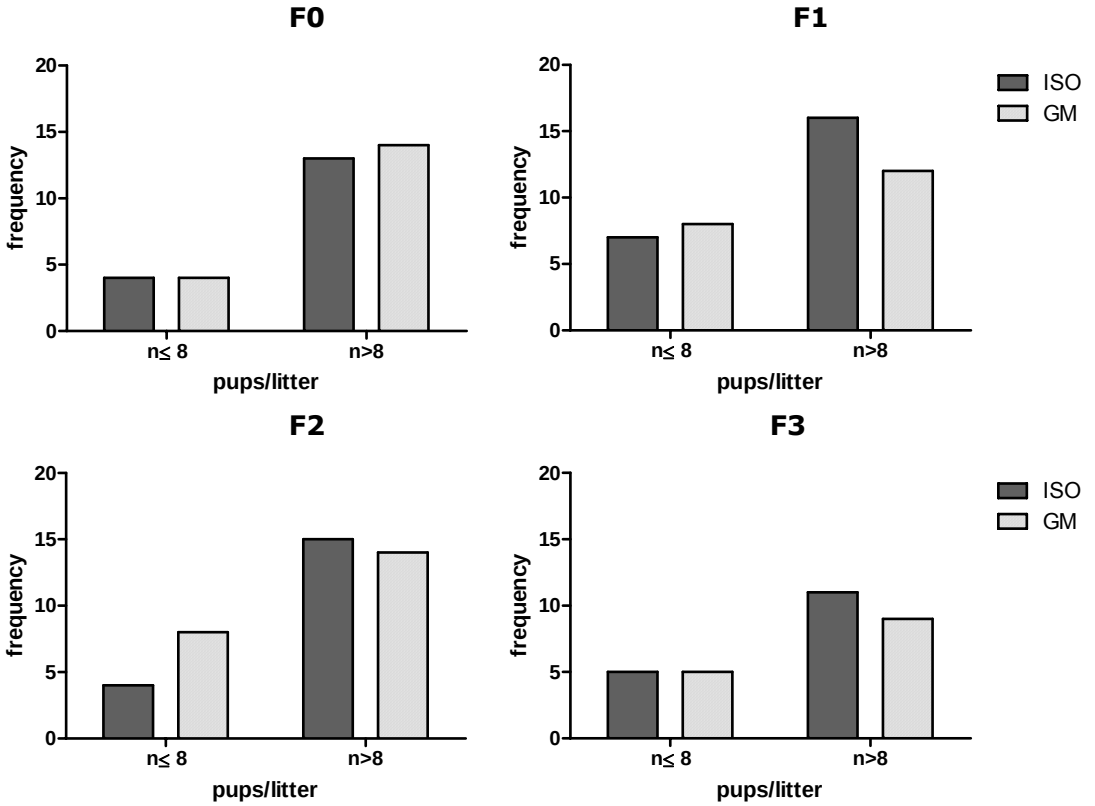


Figure 2: **Distribution of litter size at delivery from females fed 33% isogenic (ISO) or genetically modified (GM) corn in their diet in several generations**

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).

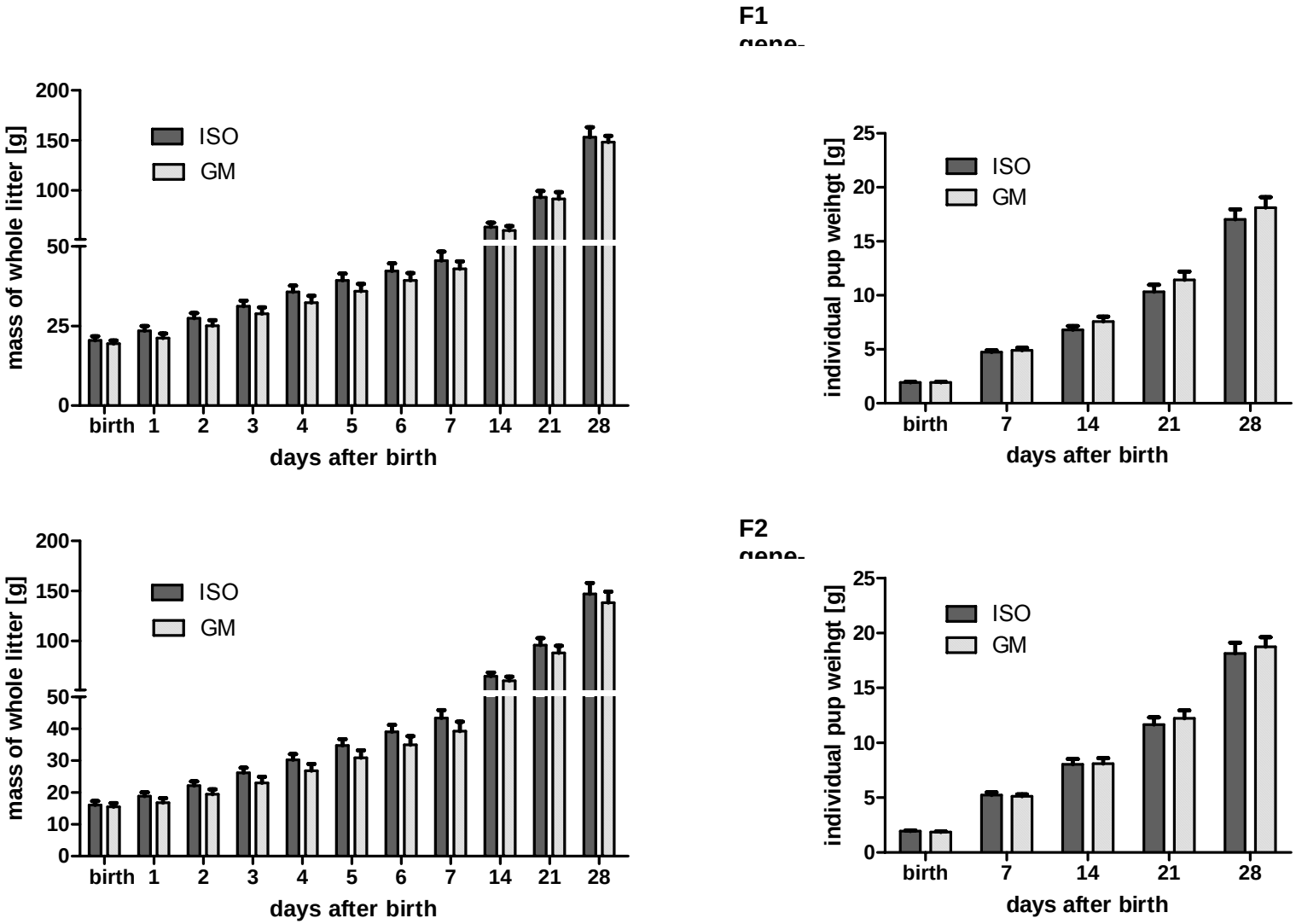


Figure 3 a-b and 3 a'-b': **Mass of whole litter [g] and individual pup weight [g] at several timepoints of the F1 and F2 generation after birth of offspring from parental mice fed 33% isogenic (ISO) or genetically modified (GM) corn in their diet**

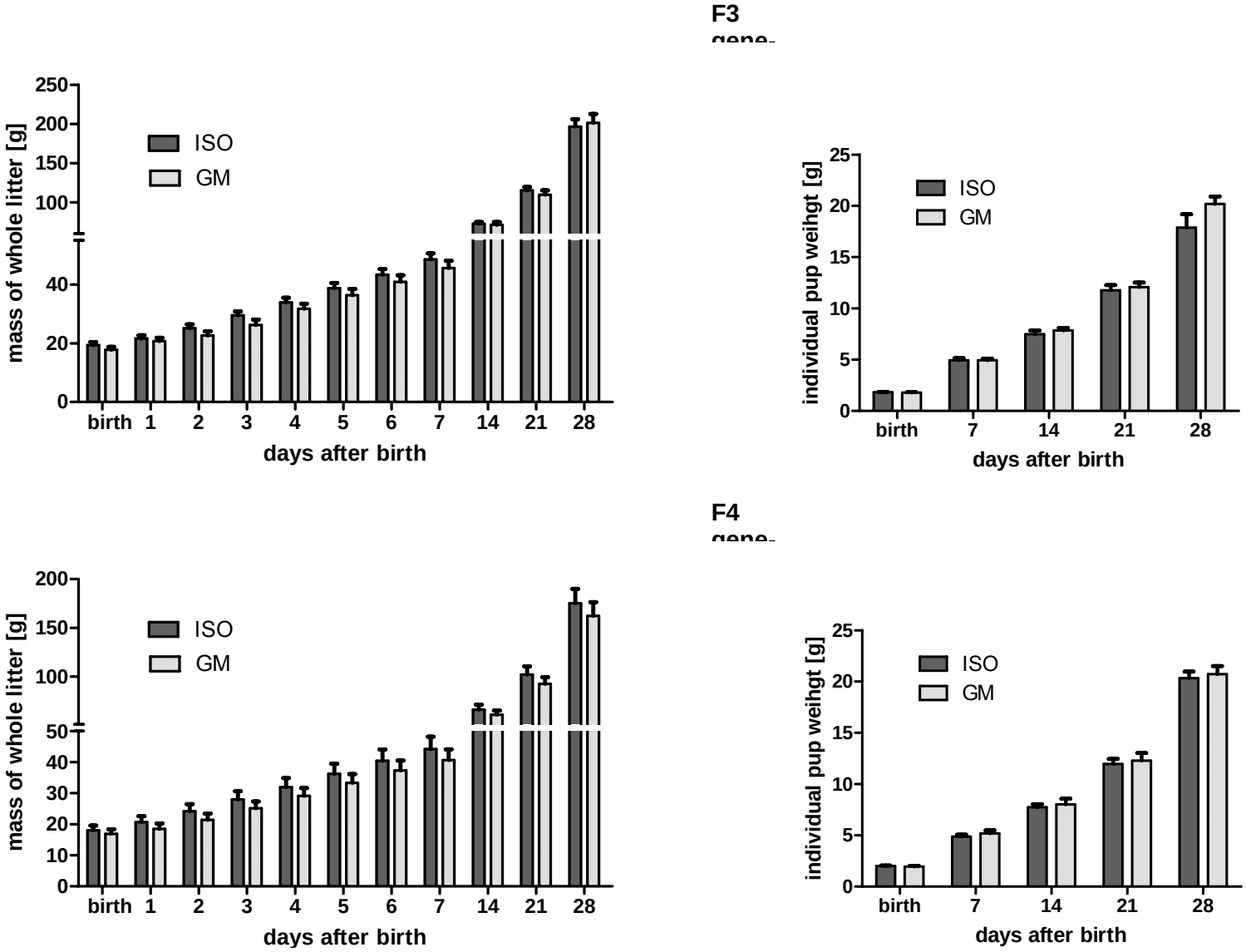


Figure 3 c-d and 3 c'-d': **Mass of whole litter [g] and individual pup weight [g] at several timepoints of the F3 and F4 generation after birth of offspring from parental mice fed 33% isogenic (ISO) or genetically modified (GM) corn in their diet**

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).

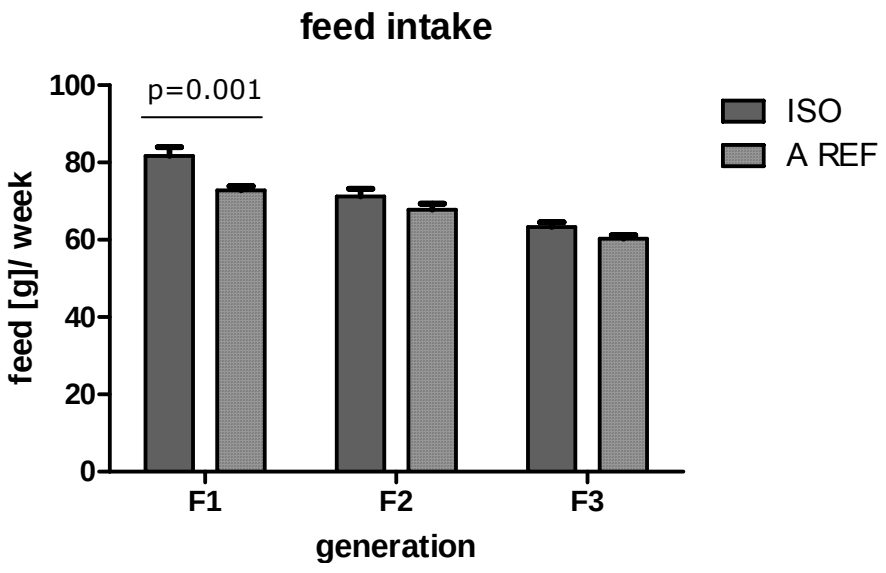


Figure 4: **Feed intake of adult mice fed 33% isogenic (ISO) or Austrian reference (A REF) corn in their diet in several generations**

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).

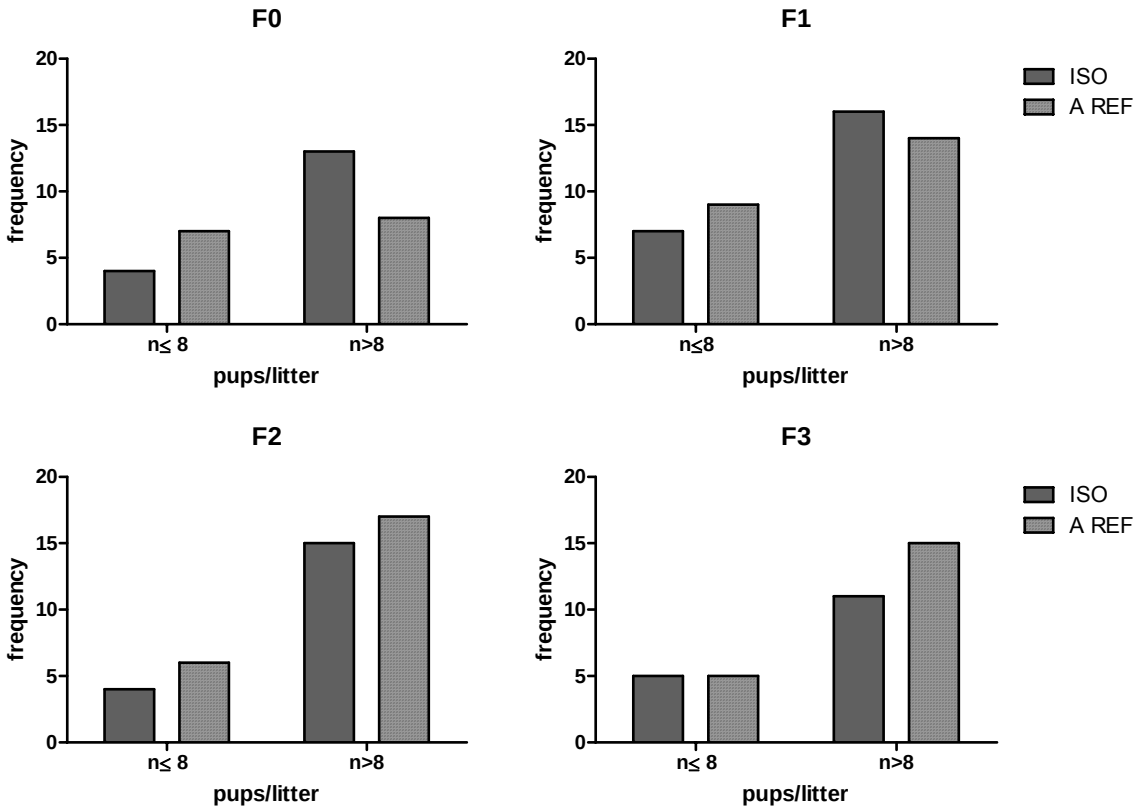


Figure 5: **Distribution of litter size at delivery from females fed 33% isogenic (ISO) or Austrian reference corn in their diet in several generations**

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 ($p < 0.05$)

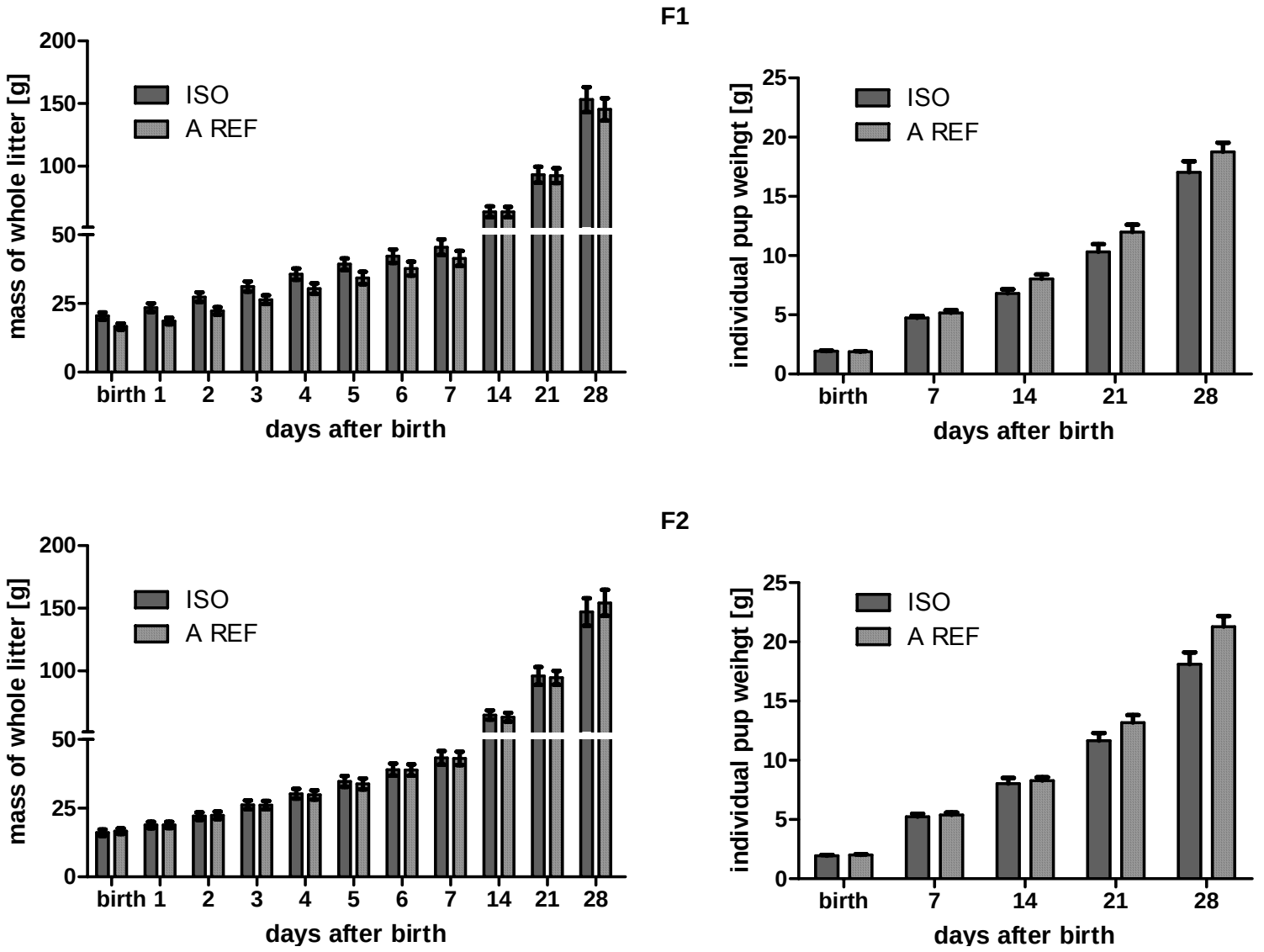
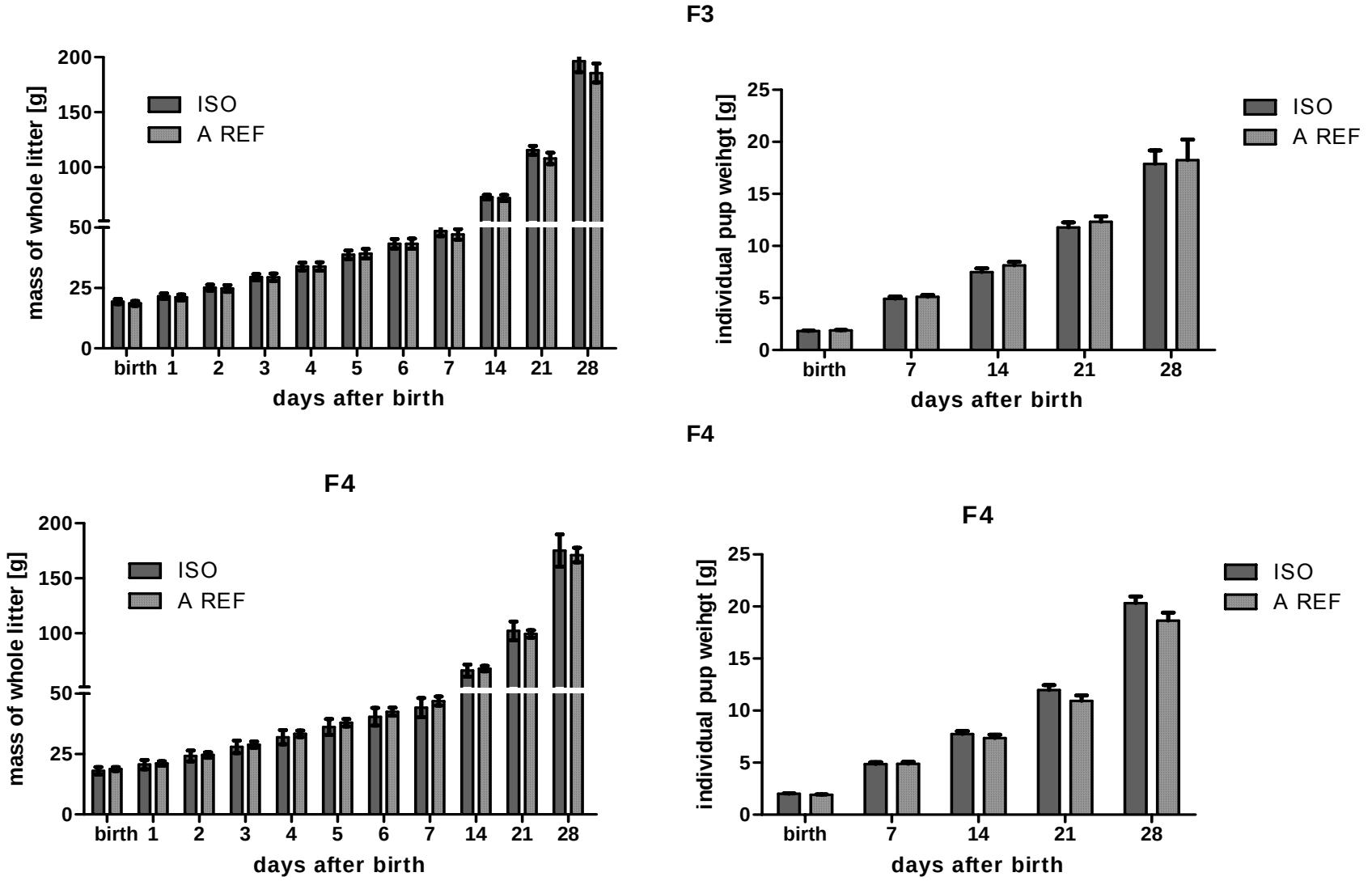


Figure 6 a-b and 6 a'-b': **Mass of whole litter [g] and individual pup weight [g] at several timepoints of the F1 and F2 generation after birth of offspring from parental mice fed 33% isogenic (ISO) or Austrian reference(A REF) corn in their diet**



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				
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).

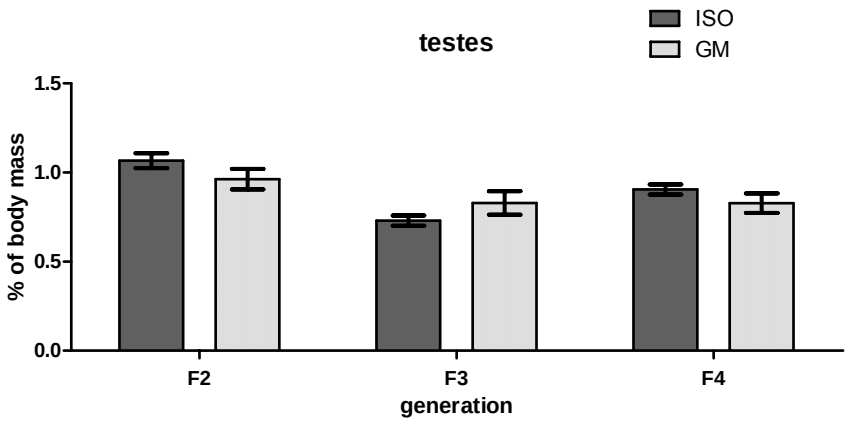


Figure 7**: Relative mass of testes of 5 week old mice fed 33% isogenic (ISO) or genetically modified (GM) corn in their diet**

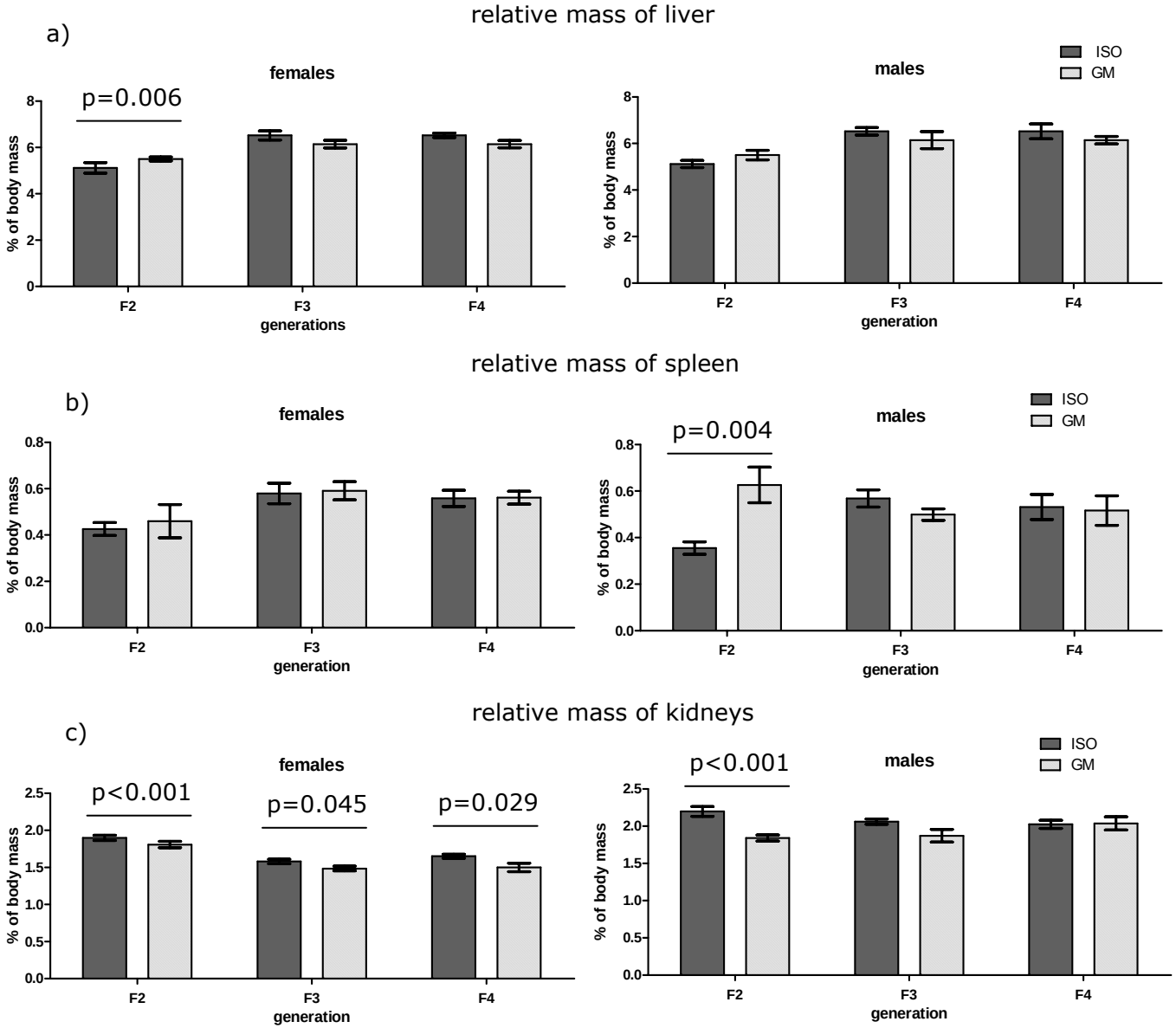


Figure 8: **Relative mass of liver (a), spleen (b) and kidneys(c) of 5 week old mice fed 33% isogenic (ISO) or genetically modified (GM) corn in their diet**

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.

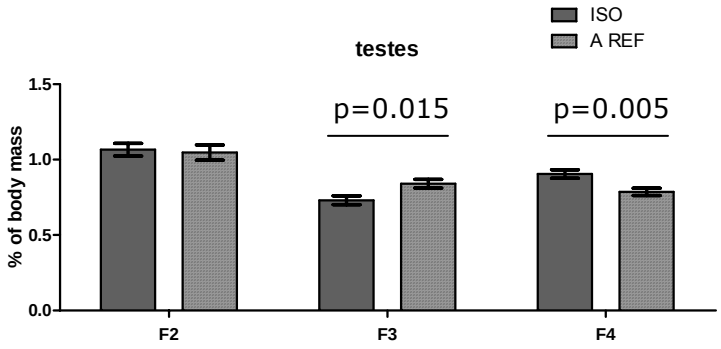


Figure 9: **Relative mass of testes of 5 week old mice fed 33% isogenic (ISO) or Austrian reference (A REF) corn in their diet**

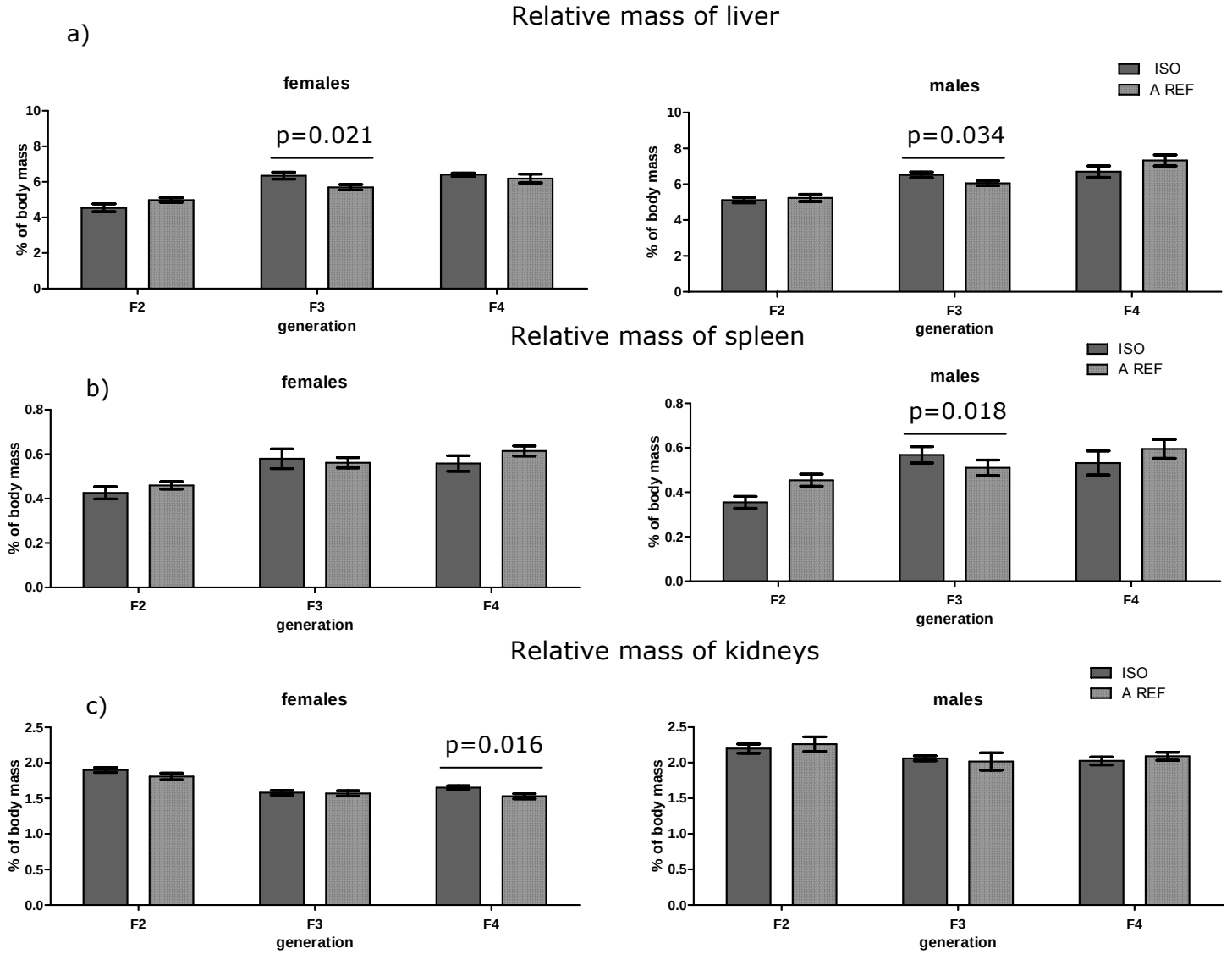


Figure 10: **Relative mass of liver (**a**), spleen (**b**) and kidneys (**c**) of 5 week old mice fed 33% isogenic (ISO) or Austrian reference (A REF) corn in their diet**

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.

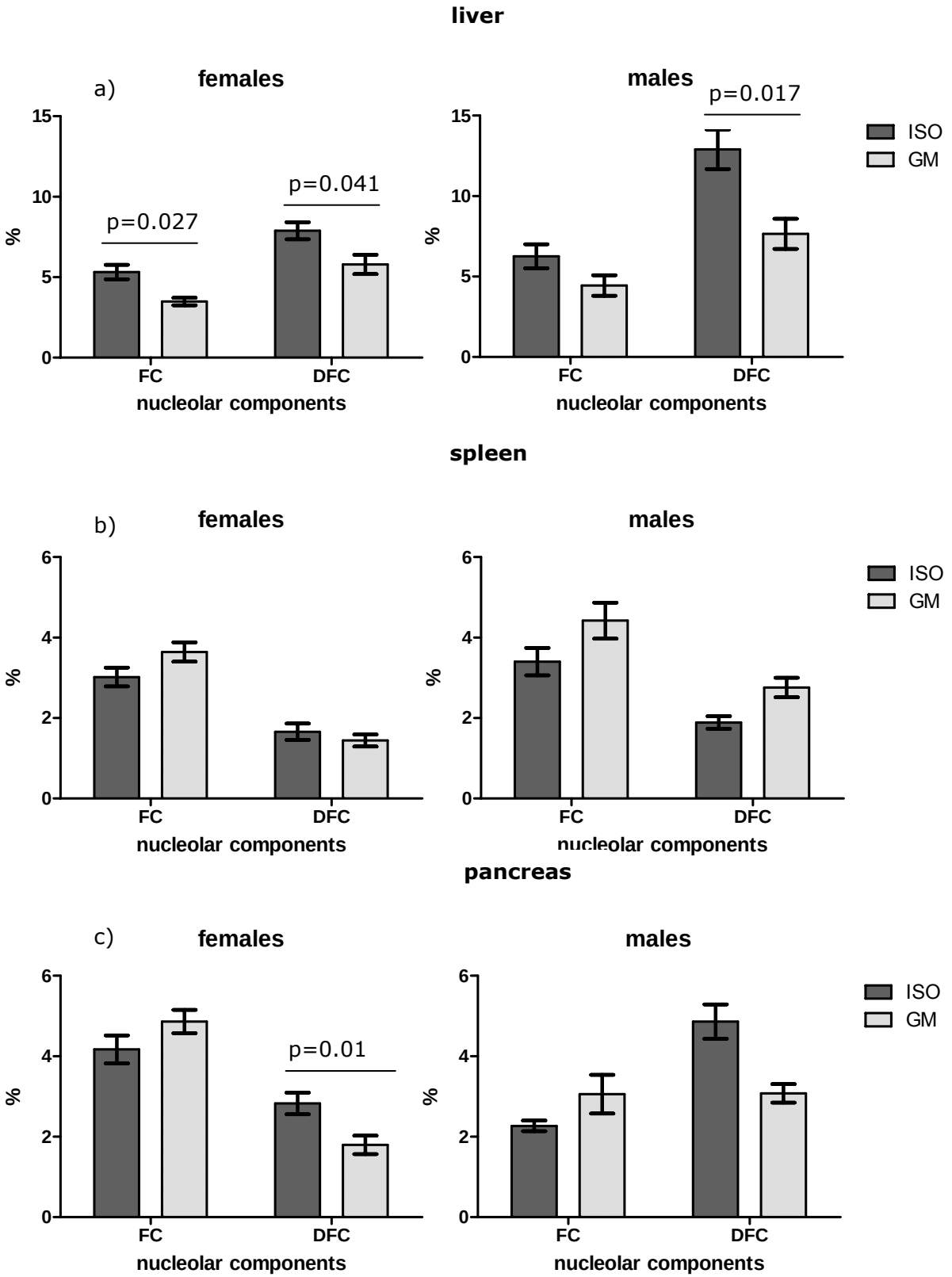


Figure 11: **Fibrillar centres (FC) and dense fibrillar components (DFC) of liver (a) (hepatocytes), spleen (b) (lymphocytes) and pancreatic acinar cells (c) from the F3 generation of mice fed 33% genetically modified (GM) or isogenic (ISO) corn.**

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).

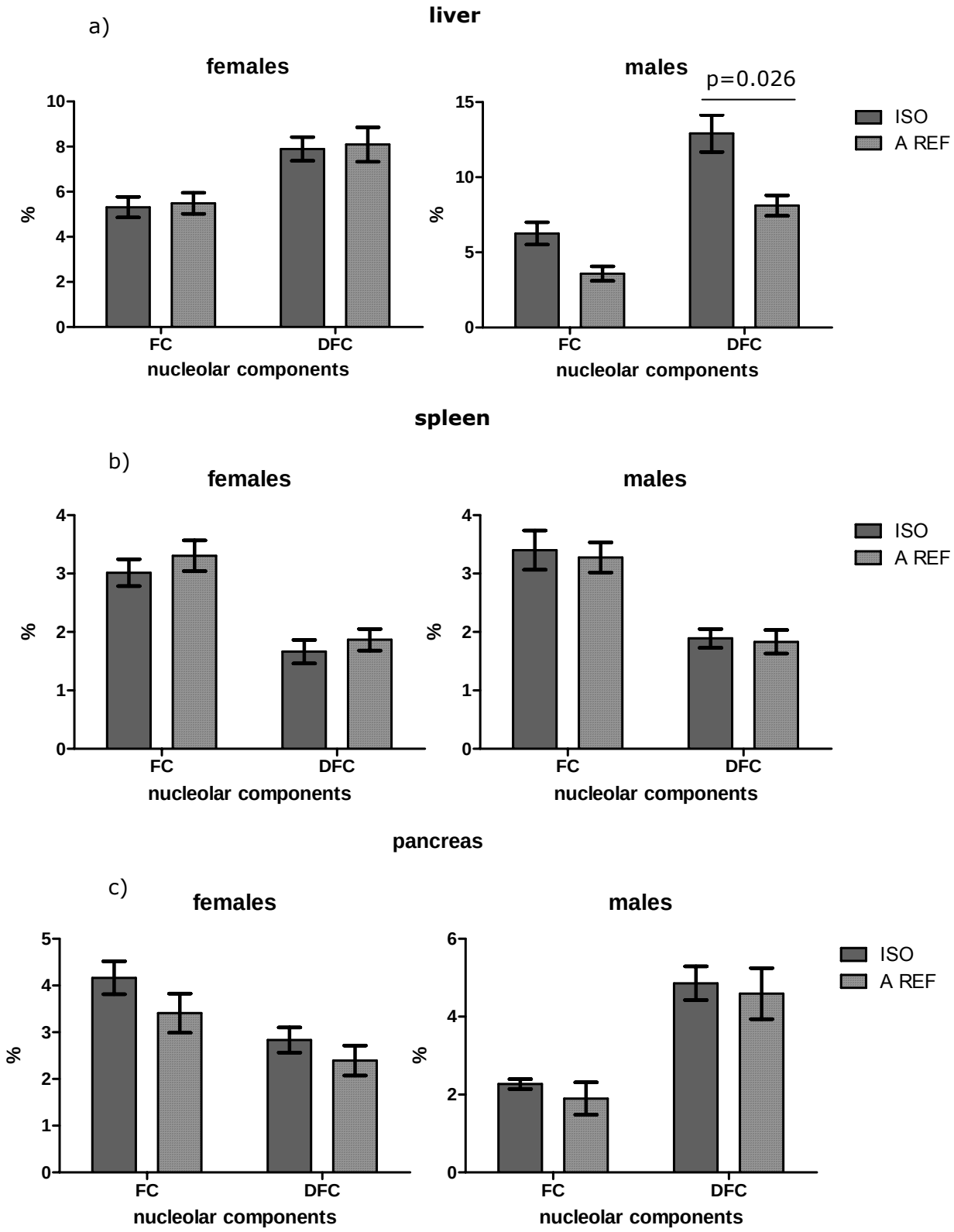


Figure 12 a-c**: Fibrillar centres (FC) and dense fibrillar components (DFC) of liver (**a**), spleen (**b**) and pancreatic acinar cells (**c**) from the F3 generation of mice fed 33% isogenic (ISO) or Austrian Reference (A REF) corn.**

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.

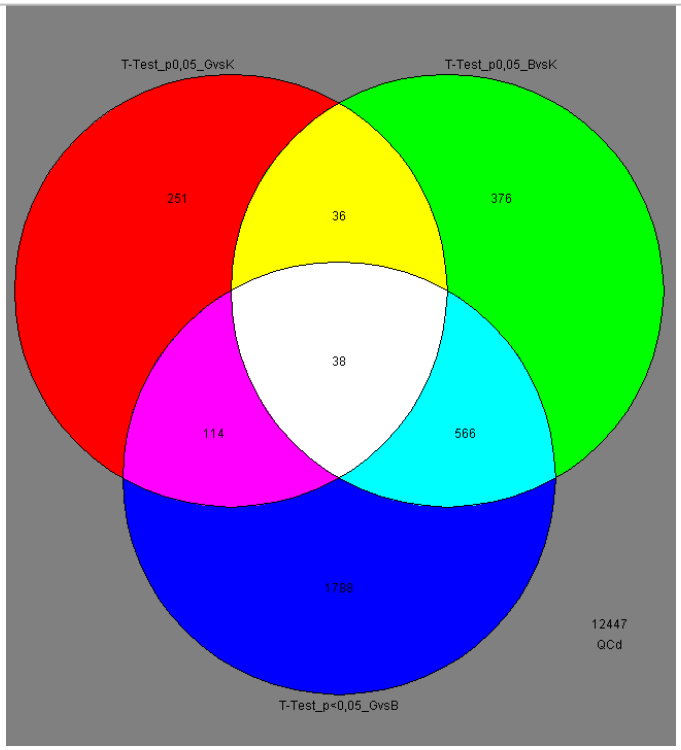


Figure 13: **Venn diagram depicting the number of deregulated genes in the F3 generation of mice fed 33% genetically modified (GM) corn. Isogenic (ISO) and Austrian reference (A REF) corn were used as controls**

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.

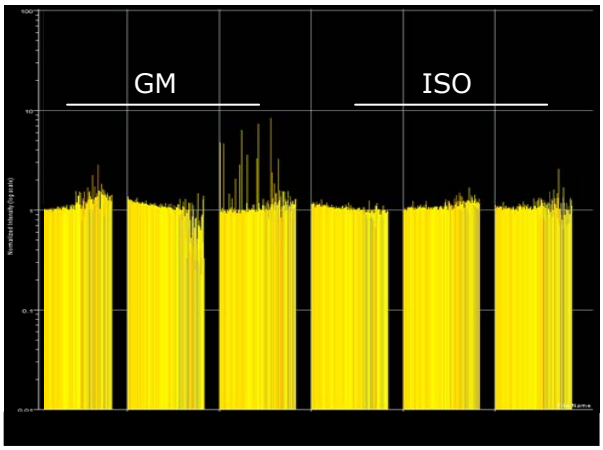


Figure 14: **Bar graphs of normalized data of jejunal miRNAs (each bar represents one miRNA) of the 3rd generation (F3) from mice fed 33% isogenic(ISO) or transgenic(GM) corn**

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.

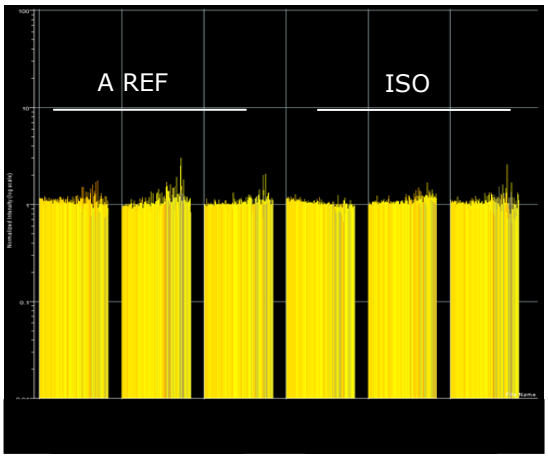


Figure 15: **Bar graphs of normalized data of jejunal miRNAs (each bar represents one miRNA) of the 3rd generation (F3) from mice fed 33% isogenic(ISO) or Austrian reference corn (A REF)**

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).

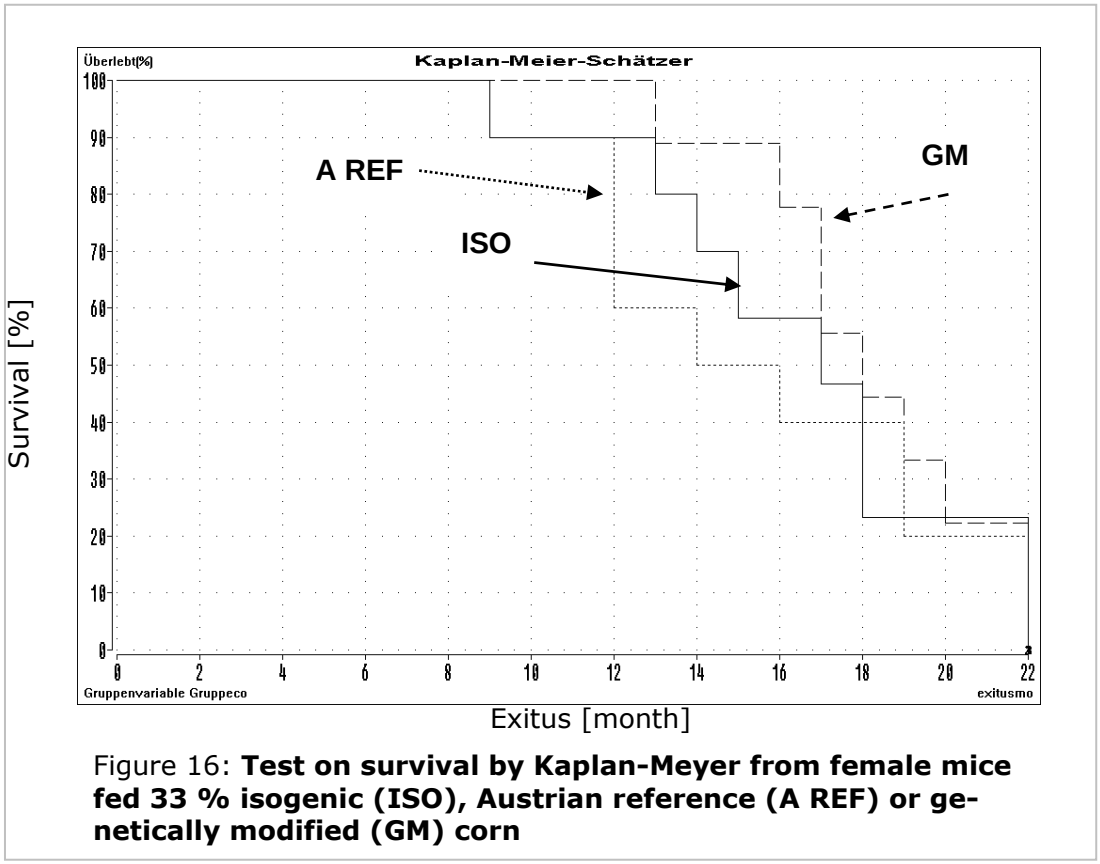


Figure 16: **Test on survival by Kaplan-Meyer from female mice fed 33 % isogenic (ISO), Austrian reference (A REF) or genetically modified (GM) corn**

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).

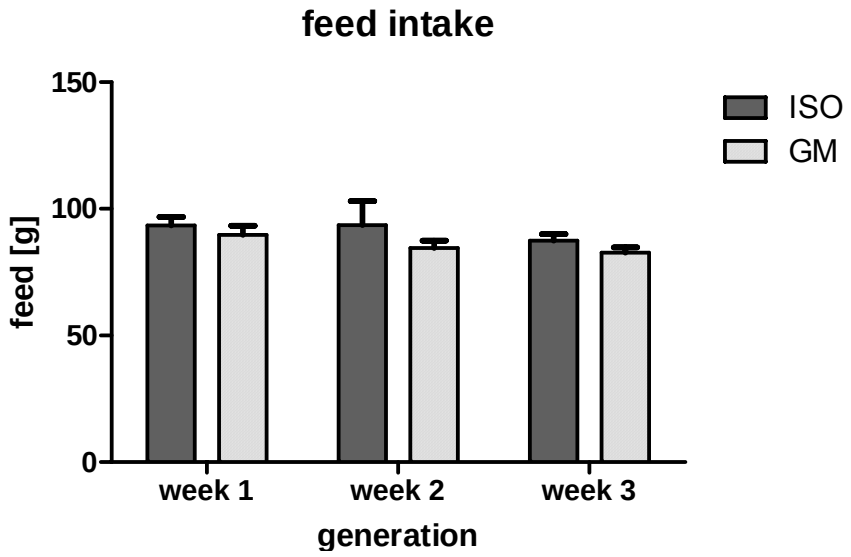


Figure 17: **Feed intake within the first 2 weeks of the RACB of the mating pairs fed 33% isogenic (ISO) or genetically modified (GM) corn in their diet**

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

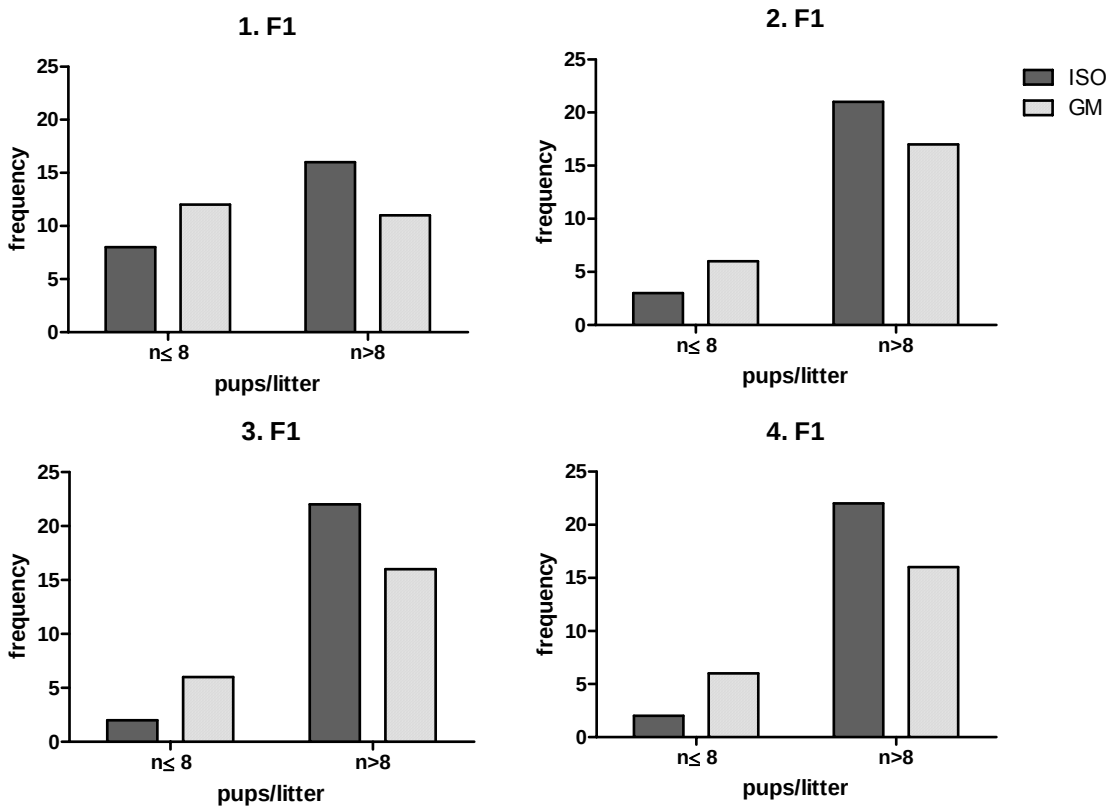


Figure 18: **Distribution of litter size at delivery from females fed 33% isogenic (ISO) or genetically modified (GM) corn in their diet over 4 litters**

Table 59: **Reproduction data of mice fed 33% isogenic (ISO) or genetically modified (GM) corn in their diet over several generations**

traits	Parental reproduction							
	1st litter		2nd litter		3rd litter		4th litter	
	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 ($n \leq 8$) was once significantly (p=0.043) lighter as compared to GM pups in the 4^{th} 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.

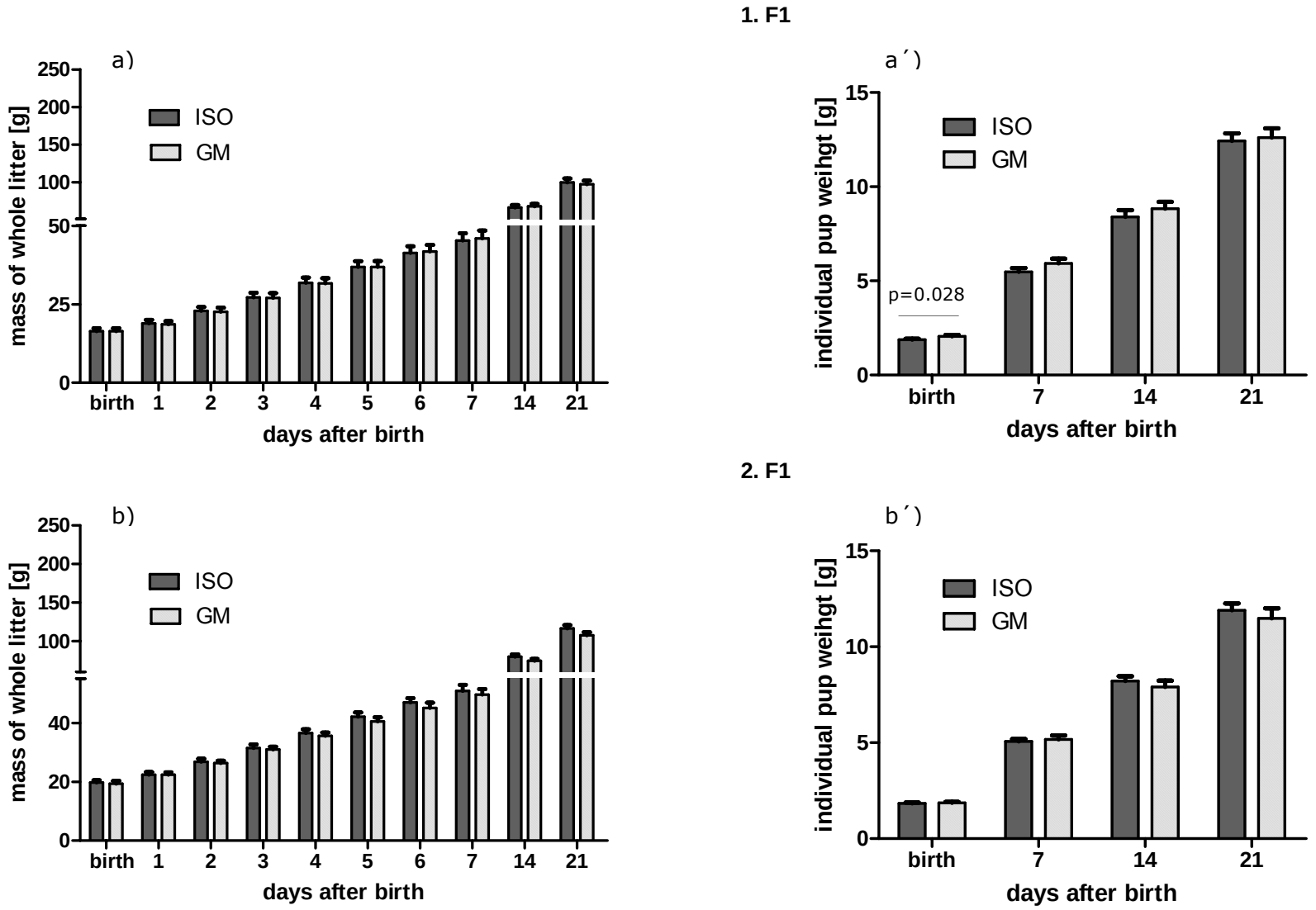
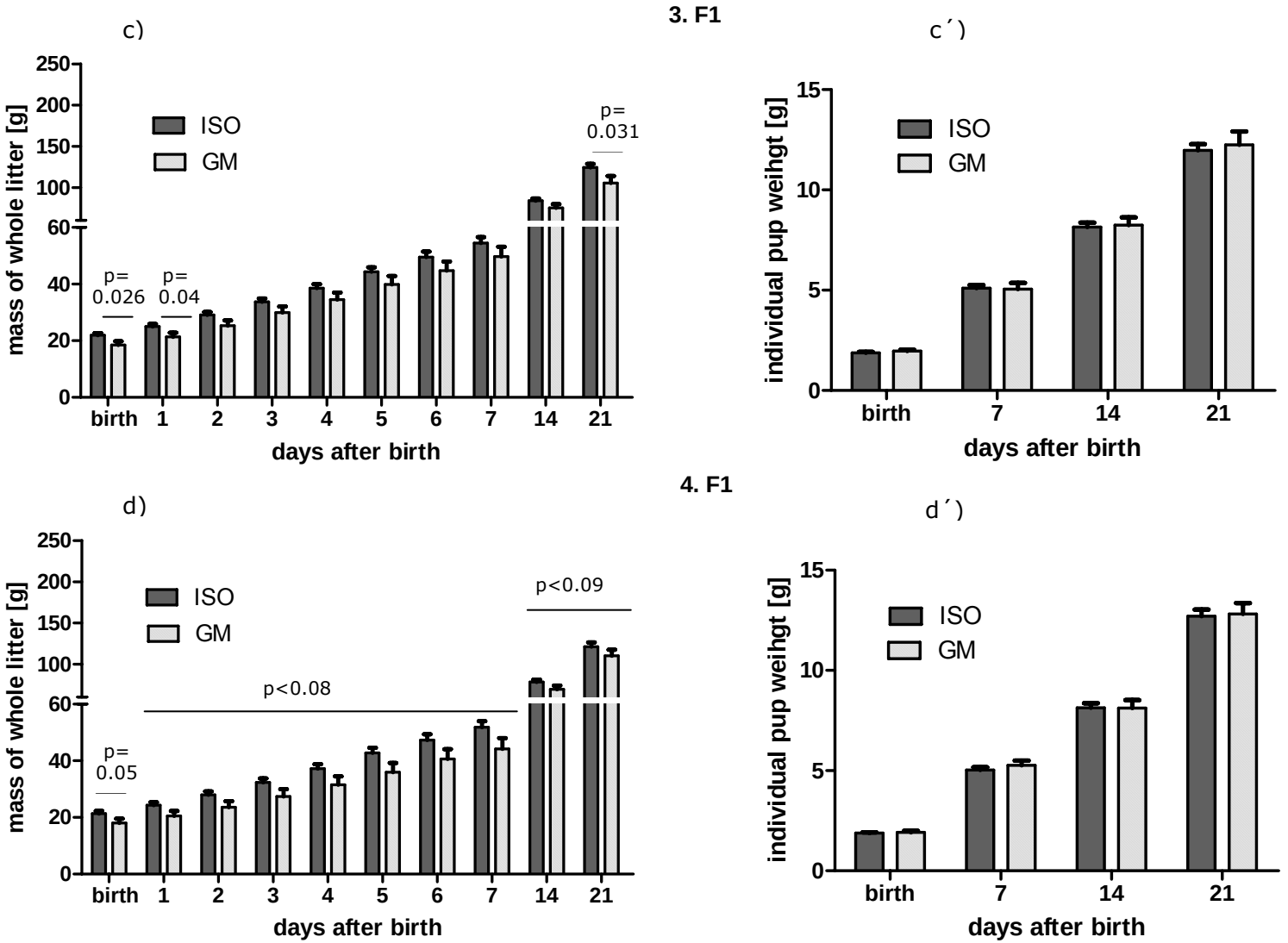


Figure 19 a-b and 19 a'-b': **Mass of whole litter [g] and individual pup weight [g] at several time-points of the 1st and 2nd F1 generation after birth of offspring from parental mice fed 33% isogenic (ISO) or genetically modified (GM) corn in their diet**



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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Ziel der Studie war die Überprüfung von Futtereffekten der gentechnisch veränderten Maissorte NK603 x MON810 nach Langzeitexposition in Mäusen. Reproduktion und Laktation sowie Wachstum und Überlebensrate der Nachkommen stellen sehr sensible Parameter dar. Daher ist es dringend notwendig, den Einfluss der Futtermittel auf die Zucht detailliert zu untersuchen.

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 mit 4 Mäusegenerationen. Das zweite Versuchsdesign war die Methode der fortlaufenden Zucht (RACB) mit 4 Würfen. Zusätzlich wurden mikroskopische Untersuchungen (Histologie, Elektronenmikroskopie, Immunhistochemie) und Microarray Analysen sowie q-RT-PCR durchgeführt, um mögliche Effekte des transgenen Maises auf Zellebene und Genomebene zu untersuchen.