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Immunological, histological, and metabolic investigations in Japanese quail (*Coturnix coturnix japonica*) fed with diets containing maize with the Cry1Ab trait versus non-biotech counterparts for up to 20 generations

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Immunological, histological, and metabolic investigations in Japanese quail (*Coturnix coturnix japonica*) fed with diets containing maize with the Cry1Ab trait versus non-biotech counterparts for up to 20 generations

The aim of this dissertation was to advance the ongoing debate as to whether health is affected by the intake of diets containing maize with the Cry1Ab trait in comparison to non-biotech counterparts. For this purpose the Japanese quail was used as a model organism. Initially serum chemistry reference values were established from up to 125 male and 151 female adult quail. Most parameters observed showed sex-related differences.

In the following experiment, quail from generations 17 to 20 of a multigenerational comparison with animals fed diets containing either genetically modified *Bacillus thuringiensis*-maize (Bt), or isogenic maize (ISO) (n=40/feeding group/sex) were used. In addition, animals fed these maize varieties in first generation (n=30/feeding group/sex), as well as further control groups fed with two different isogenic hybrid reference maize cultivars (REF) (n=30/feeding group/sex) were included. At 16 wk of age, blood samples were analyzed for serum biochemical parameters and liver tissue was histomorphometrically evaluated.

Statistical differences between feeding groups occurred in few of the observed parameters; they were neither consistent nor analogous and were not limited to Bt vs. ISO, or REF comparisons.

In a further experiment, the effect of an active immunization against bovine serum albumin (BSA) was tested in feeding regimen including Bt or isogenic maize. After 16 wk on the experimental diets, one half of each feeding group was injected with BSA or NaCl, respectively. Thirty-six h after the injection, half of the BSA injected group (n=30) and half of the saline group (n=30) from both feeding groups were sacrificed and blood samples were analyzed for zinc concentrations that are indicative for an acute phase reaction. From the remaining animals, egg yolk samples were obtained biweekly from 0 to 6 wk following the injection and were analyzed for total IgY concentration and BSA-specific IgY titers. The response of both variables to the BSA injection did not differ between feeding groups. For serum zinc, no alterations related to the immunization against BSA were detectable. When pooling the BSA and saline injected quail within the Bt and the ISO feeding group, the Zn concentrations were slightly lower ($p < 0.01$) in the ISO animals than in the Bt group. The results indicated that feeding of Bt-maize does not impair the immune system of Japanese quail.

Analyses for nutrient composition and mycotoxins conducted in all feeds, yielded similar nutrient contents and no differences in the concentrations of zearalenone (ZON) and deoxynivalenol (DON); concentrations of DON and ZON were consistently below the limits of acceptance of the European Commission.

In conclusion, the present dissertation contributes to the general discussion of using genetically modified crops in animal nutrition, showing that no obvious adverse effects were observed in neither comparison. The results are of general importance to animal science and provide support for the comparability of Bt-maize to conventional reference maize varieties in terms of animal health.

Immunologische, histologische und metabolische Untersuchungen in Japanischen Wachteln (*Coturnix coturnix japonica*) deren Fütterung bis zu 20 Generationen mit gentechnisch verändertem Cry1Ab Mais oder isogenem Mais erfolgte

Ziel dieser Dissertation war es, die anhaltende Debatte, ob die Aufnahme von Futtermitteln mit Cry1Ab Mais im Vergleich zu isogenem Mais Auswirkungen auf die Gesundheit hat, voranzutreiben. Zu diesem Zweck wurde die Japanische Wachtel als Modellorganismus genutzt. Zunächst erfolgte die Etablierung klinisch-chemischer Referenzwerte von bis zu 125 männlichen und 151 weiblichen adulten Tieren. In der überwiegenden Anzahl der untersuchten Parameter traten erwartungsgemäß Unterschiede zwischen den Geschlechtern auf.

In dem darauffolgenden Mehrgenerationenversuch wurden Tiere der 17. bis 20. Generation verglichen, deren Fütterung mit *Bacillus thuringiensis*-Mais (Bt) oder isogenem Mais (ISO) erfolgte (n=40/Fütterungsgruppe und Geschlecht). Die Untersuchungen wurden ausgeweitet auf Tiere, die in der ersten Generation Bt-Mais erhielten (n=30/Fütterungsgruppe und Geschlecht) und auf zwei zusätzliche, mit isogenem Referenzmais (REF) (n=30/Fütterungsgruppe und Geschlecht) gefütterte Kontrollgruppen. Im Alter von 16 Wochen erfolgte die Analyse klinisch-chemischer Parameter sowie die histologische Betrachtung des Lebergewebes. Signifikante Unterschiede traten lediglich in einigen der betrachteten Parameter auf. Diese Unterschiede waren jedoch weder konstant noch analog und beschränkten sich nicht auf Vergleiche zwischen Bt vs. ISO, oder REF.

Abschließend wurde der Effekt einer aktiven Immunisierung mit Bovinem Serum Albumin (BSA) in Fütterungsversuchen mit Bt- oder isogenem Mais getestet. Nach 16-wöchiger Fütterung der Versuchsrationen erfolgte die Injektion mit BSA bzw. NaCl von jeweils der Hälfte der Tiere pro Fütterungsgruppe, wovon jeweils wiederum von der Hälfte (n=30) nach 36 h Blutproben gewonnen und auf ihren Zinkgehalt untersucht wurden. Von den verbleibenden Tieren wurden für insgesamt sechs Wochen nach der Injektion alle zwei Wochen Eidotterproben gesammelt und die Konzentrationen an Gesamt-IgY sowie BSA-spezifischem IgY gemessen. Bei beiden Parametern traten keine Unterschiede zwischen den Fütterungsgruppen auf. In der Gruppe Bt-Mais gefütterter Tiere konnte eine höhere Zinkkonzentration im Vergleich zu den isogen gefütterten Kontrolltieren ermittelt werden, nachdem die Proben von BSA und NaCl-injizierten Tieren der jeweiligen Fütterungsgruppe zusammengefasst wurden. Die Ergebnisse dieser Studie geben keine Hinweise darauf, dass die Fütterung von Bt-Mais das Immunsystem von Japanischen Wachteln beeinträchtigt.

Die Analyse der Nährstoffgehalte sowie der Konzentrationen der Mykotoxine Deoxynivalenol und Zearalenon in den verwendeten Futtermischungen zeigte keine Unterschiede zwischen den Fütterungsgruppen. Zudem lagen die Mykotoxinkonzentrationen durchweg unterhalb den von der EU-Kommission akzeptierten Höchstmengen.

Das Ausbleiben negativer Gesundheitseffekte in den durchgeführten Untersuchungen trägt zu der anhaltenden Diskussion bezüglich des Einsatzes gentechnisch veränderter Getreide in der Tierernährung bei. Die Ergebnisse sind von genereller Bedeutung für die Tierwissenschaft und unterstützen die Vergleichbarkeit von Bt-Mais mit konventionellen Maissorten im Hinblick auf die Tiergesundheit.

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II. List of Abbreviations

γ-GT	Gamma-Glutamyl Transferase
ALT	Alanine Aminotransferase
ANOVA	Analysis of Variance
AST	Aspartate Aminotransferase
BSA	Bovine Serum Albumin
Bt	Bacillus thuringiensis
BW	Body weight
ChE	Cholinesterase
CP	Crude protein
D.	Dark
DON.	Deoxynivalenol
DNA	Deoxyribonucleic Acid
ECB	European Corn Borer
EFSA	European Food Safety Authority
ELISA	Enzyme Linked Immunosorbant Assay
FLI	Friedrich-Loeffler-Institute
Hep. nuclear size	Hepatocyte nuclear size
HPLC	High Performance Liquid Chromatography
IgG	Immunoglobulin Gamma
IgY	Immunoglobulin Yolk
ISO	isogenic maize
L	Light
LP	Laying period
n	Number of animals
NaCl	Sodium Chloride
NS	Not Significant
p	Level of significance
PCR	Polymerase Chain Reaction
r	Correlation
REF	Reference group
SEM	Standard Error of the Mean
SD	Standard Deviation
vs	Versus
WSF	Water-Soluble Fraction

Zn ....Zinc
ZON. ....Zearalenone

1. INTRODUCTION

1.1 Basic principles of genetic modification within agriculture

All plants, animals, micro-organisms, and most viruses, contain a substance called deoxyribonucleic acid (DNA) which encodes the information for growth and development in an organism. The DNA can be divided into functional units called genes (Halford & Shewry, 2000). Genetic modification describes the process of manipulating the organisms' genes, outside of its normal reproductive process. This process involves the isolation, characterization, multiplication and novel combination of DNA within and beyond species borders (Halford & Shewry, 2000). The objectives of genetic modification within agriculture are to design new species, increase yield of an existing species, enhance disease resistance, altering taste and other quality parameters, and improve growth in adverse conditions such as drought and low temperatures (Rowland, 2002). Many of these benefits are similar to those that have been achieved in conventional breeding programs.

1.1.1 Production of genetically modified crops

The genetic modification of crop plants has been practiced for thousands of years by what is now called conventional plant breeding. Conventional breeding aims to make better use of the available natural resources and is based on the crossing of genotypes containing tens of thousands of expressed genes and the selection of progeny that combine the best features of the two parents (Halford & Shewry, 2000). In contrast, modern techniques of genetic modification allow to produce genetically modified organisms defined as plants, micro-organisms or animals into which foreign DNA coding one or more new genes have been integrated (Flachowsky et al., 2005a). The advantage of these techniques is the possibility of introducing a single gene, or a couple of genes into a plant, in a much more precise, controllable and predictable way that enables changes to be made to the genetic material of plants in much shorter time frames (Harlander, 2002). The two most commonly used methods to insert DNA into plant cells are the biolistic bombardment system, and *Agrobacterium*-mediated transformation, the latter being the most widely used system (Birch, 1997).

The biolistic bombardment method is based on a physical delivery of DNA-coated gold or tungsten microprojectiles into plant target tissue by acceleration (Cellini et al., 2004).

Agrobacterium-mediated transformation exploits the biological ability of this soil-born bacterium to copy and transfer a specific portion of DNA present on a tumour inducing plasmid into the plant cells nucleus, where it can be integrated into chromosomes, the organized structures of DNA (Cellini et al., 2004).

According to Flachowsky et al. (2005a), edible crops developed by modern biotechnology are classified into genetically modified plants from the 1st or the 2nd generation. The 1st generation of genetically modified plants includes crops with improved agronomic traits such as resistance to pests and diseases, and tolerance of specific chemical herbicides (Flachowsky et al., 2005a). The proteins produced which confer these benefits, occur in very low concentrations in the modified crops and thus do not significantly change the composition or feed value when compared to the isogenic lines (Flachowsky et al., 2005a). In contrast, the 2nd generation of genetically modified plants is characterized by targeted changes in nutrient composition or availability. This mode of genetic engineering allows both increasing the content of constituents which are desired (e.g. protein, amino acids, fat, fatty acids, minerals, vitamins, enzymes) as well as reducing the content of constituents which are detrimental to digestibility or feed safety (e.g. lignin, phytate, alkaloids, mycotoxins) (Flachowsky et al., 2005a).

The use of new techniques to modify the genetic makeup of plants offers a variety of benefits for agriculture. On the other hand, the use of ingredients and products from genetically modified plants in animal nutrition is discussed controversially, as many consumers' concerns are related to the uncertainty of the - potentially long-term - effects on human, animal, and plant health as well as environmental safety (Frewer & Salter, 2003; Miles & Frewer, 2001).

1.1.2 Commercial use of genetically modified feed

In 2008, the global area of biotech crops reached 125 million hectares planted by 13.3 million farmers in 25 countries worldwide (ISAAA Brief, 2008). The global area of biotech crops increased 74-fold between 1996 and 2008, making it the fastest

adopted crop technology in recent history. Biotech soybean continues to be the principal biotech crop (53 % of global biotech area), followed by maize (30 %), cotton (12 %) and canola (5 % of global biotech crop area). From the genesis of commercialisation in 1996 to 2008, herbicide tolerance has consistently been the predominant trait followed by insect resistance (ISAAA Brief, 2008).

The USA continued to be the principal adopters of biotech crops globally in 2008, with 62.5 million hectares planted (50 % of global biotech area) followed by Argentina (16.8 %), Brazil (12.6 %), Canada (6.1 %), India (6.1 %) and China (3.0 %). In 2008, seven out of 25 countries growing biotech crops belonged to the European Union (ISAAA Brief, 2008). The number of commercialised biotech crops in EU Member States is relatively low compared to their principal growers like e.g. the USA. Within the EU, consumers and their governments have been more wary of gene technology. The use of genetically modified organisms as food or food ingredients, as well as their release into the environment, is strictly regulated in the European Union (Rizzi et al., 2007). A risk assessment for human and animal health and the environment is required by the European Council Directive 2001/18/EC before genetically modified seeds can be imported or the plant itself can be cultivated in Europe (EU Council Directive, 2001). In addition, regulation (EC) No. 1829/2003 establishes a EU authorisation procedure for genetically modified feeds. To place a new product on the market, a request for authorisation has to be made to a national competent authority and forwarded to the European Food Safety Authority (EFSA) (Rizzi et al., 2007). Invoking a safeguard clause in EU legislation on genetically modified crops, the cultivation of insect resistant *Bacillus thuringiensis* (Bt)-maize (MON810) was recently banned in Germany assuming that genetically modified maize of the MON810 line constitutes a danger to the environment (BMELV, 2009).

1.2 Insect resistant *Bacillus thuringiensis*-maize

Insect control has been a priority target of agricultural biotechnology as great losses in maize plant yield are caused by insect pests and the annual costs of chemical control of maize plant insects are worldwide estimated at 3.5 billion US $ (Shah et al., 1995). To overcome this ecological as well as economical impact of chemical insecticides, genes encoding for the insecticidal protein of Bt have been inserted into

maize and other crop species by biotechnological methods to increase the resistance of genetically modified plants to insect pests (Ives, 1996).

Bacillus thuringiensis was first discovered in 1901 by the Japanese biologist Shigetane Ishiwata as the causative agent of a disease afflicting large populations of silkworms (*Bombyx mori*) (Roh et al., 2007). In 1911, ten years after the initial isolation, Ernst Berliner rediscovered the *Bacillus*, when he isolated it from the Mediterranean flour moth, *Anagasta kuehniella*. He named it after the province Thuringia in Germany where the infected moth was found. Bt products were first commercialised in France in the late 1930s as insecticidal sprays (Roh et al., 2007). In 1956, Fitz-James Hannay and Angus Hannay demonstrated that the insecticidal activity of *Bacillus thuringiensis* is based on crystalline protein inclusions produced by Bt in the course of sporulation. Bt was used commercially on a large scale in the 1980`s, when insects became increasingly resistant to the synthetic insecticides and scientists and environmentalists became aware of that the chemicals were harming the environment. Meanwhile Bt preparations account for 80-90 % of all biological pest agents worldwide (Bernhard et al., 1997). With the advancement in molecular biology, the opportunity was soon opened to isolate the gene encoding the toxic protein crystals and to introduce it into a plant. Accordingly, the first genetically modified maize plant was registered in 1995 (Kanglai et al., 2003).

1.2.1 *Bacillus thuringiensis*: Ecology and taxonomy

Bacillus thuringiensis is a gram-positive sporulating soil bacterium that produces a crystalline inclusion known as parasporal crystal. These inclusions can be distinguished as distinctively shaped crystals consisting of proteins known as crystal proteins, Cry proteins, or δ-endotoxins, which are highly toxic to a wide variety of important agricultural and health related insect pests as well as other invertebrates (Roh et al., 2007).

Several thousands natural Bt strains, which synthesize different insecticidal crystal proteins have been obtained from numerous geographical areas worldwide (Lecadet et al., 1999). The classification and identification of these strains is based on the differentiation into serovarieties, developed on the basis of flagellar antigens. To

date, up to 69 different serotypes and 13 sub-antigenic groups have been defined (Lecadet et al., 1999).

1.2.2 Mode of action of *Bacillus thuringiensis* Cry proteins

The first step of the infection cycle following the ingestion of Bt parasporal crystal bodies is the solubilisation of the crystalline protein in the alkaline midgut of the insect. In a second step the solubilised protein or protoxin is proteolytically processed to produce the actual toxic fragment (Visser et al., 1993). This toxin binds to specific receptors on the midgut of susceptible larvae and results in the formation of pores and disturbance of osmotic balance and control (Meadows, 1993). As a consequence, K^+ conductance is increased in the midgut apical epithelium, causing a decrease in the electrical gradient and dissipation of the pH gradient in the gut (Gringorten, 1999). Subsequent, the gut is paralysed and the insect dies due to starvation and bacterial sepsis (Visser et al., 1993).

In addition to the insect defence mechanism obtained through introduction of Cry genes, Bt-maize profits from lower insect damage which hinders penetration of germs and fungi, e.g. *Fusarium* species through perforated plant surfaces (Ariño et al., 2009).

1.2.3 Fusarium infestation and mycotoxin contamination in cultivation of maize

The cultivation of maize is often accompanied by infections with *Fusarium* species, which can reduce yields and quality, and result in mycotoxin accumulation in grain (Munkvold et al., 1997). *Fusarium* species are among the most common fungal associates of maize plants, causing diseases of seedlings, roots, stalks, and kernels (Munkvold et al., 1997). Infected grains are often contaminated with mycotoxins, such as deoxynivalenol (DON) and zearalenone (ZON) (Lysøe et al., 2006). Deoxynivalenol and zearalenone play an important role in animal feeding since they might occur in concentrations which can cause adverse effects in farm animals (Dänicke et al., 2007).

Deoxynivalenol is a mycotoxin produced by *Fusarium graminearum* under conditions of high moisture and low temperature. It is considered to have a worldwide economic

influence on the agricultural industry (Awad et al., 2006). Reviewed by Awad et al. (2006), swine are among the livestock species most susceptible to DON toxicity, mainly characterized as feed refusal or reduced feed intake. In livestock production, moderate to low levels of deoxynivalenol can cause a number of effects associated with poor performance and altered immunity. In contrast, poultry appear to be considerably more tolerant to DON in comparison with other species (Awad et al., 2006). Diets containing more than 10 mg DON/kg were observed to reduce immune function and cause detrimental effects on the performance of broilers as well as changes in haematology and serum chemistry (Harvey et al., 1991; Dänicke et al., 2003). Feeding of grains naturally or artificially contaminated with moderate concentrations of DON had no effect on feed intake, body-weight gain and feed efficiency of broiler chicken (Awad et al., 2006).

Zearalenone, produced by *Fusarium graminearum*, is one of the most prevalent mycotoxins in maize and maize products but also in soybean and various cereal grains, representing a major component of human food and animal feed (Kolf-Clauw et al., 2008). Because of its estrogenic activity, crop contamination with ZON constitutes a potential risk for human and animal health (Lysøe et al., 2006). In a large number of animal species, hyperestrogenism results in reproductive disorders and decreased fertility with marked variations of sensitivity (Kolf-Clauw, 2008). Pigs are generally considered the animal species most sensitive to ZON, whereas ruminants and poultry show a lower responsiveness (Thieu et al., 2008). Reviewed by Dänicke et al. (2002), laying performance as well as reproductive performance of chicken, including growth of progeny was not influenced by increasing dietary ZON-concentrations up to 800 mg/kg. However, the co-occurrence of ZON and DON, which is very common, increased the incidence of chick developmental anomalies when hens were fed these mycotoxins, although fertility, hatchability or perinatal mortality were not influenced (Dänicke et al., 2002).

The European corn borer (ECB) (*Ostrinia nubilalis*) is one of the most important pests in cultivation of maize (Quinton et al., 2005). Several types of host-insect-pathogen interactions increase the risk for the occurrence of maize diseases. One type of interaction is a vector relationship, where the ECB larvae carry spores of *Fusarium*

species from plant surface to the surfaces of damaged kernels or to the interior of stalks where the infection takes place. Another type of interaction is based on the formation of entry wounds for the fungi at the feeding sites of larvae on stalks or kernels. Additionally, these damages caused by insects imply stress in plants that predisposes stalk rot development (Munkvold & Hellmich, 2000).

Maize hybrids genetically engineered with Bt genes encoding for the δ-endotoxin Cry1Ab show a high resistance against the ECB. In hybrids expressing Cry1Ab in kernels, incidence and severity of Fusarium ear rot and the incidence of symptomless kernel infection is reduced compared with near-isogenic hybrids lacking Cry1Ab genes. A positive correlation between disease incidence and ECB damage to kernels was shown in various studies in 1994 (r = 0.88), 1995 (r = 0.53) and 1996 (r = 0.66); insecticide applications also reduce *Fusarium* symptoms and infection when applied to isogenic plants (Munkvold et al., 1997).

1.3 Studies with feeds containing *Bacillus thuringiensis*-maize

For Bt-maize, a number of studies in livestock and poultry has estimated its nutrient value in comparison to their conventional counterparts and some have additionally followed the fate of DNA and novel protein (reviewed by Flachowsky et al., 2005a). The results available are reassuring and indicate no risk for health when considering nutritional assessment and performance parameters (Aumaitre et al., 2002; Clark & Ipharaguerre, 2001; Flachowsky et al., 2005a). Even long-term studies in livestock, poultry, and rats did not reveal significant influences on animal development after feeding of genetically modified maize. Guertler et al. (2009) recently investigated the fate of Cry1Ab DNA and Cry1Ab protein in a long-term study of 25 months during digestion and metabolism of dairy cows. The absence of Cry1Ab DNA and Cry1Ab protein in milk of cows fed genetically modified maize (MON810) classify milk as being not different from milk of cows fed non-transgenic maize. In a four-generation study with laying hens, laying intensity (83.5 and 83.3 % at the age of 23 – 30 wk) and hatchability (86.8 and 88.0 %) was not significantly influenced by Bt-maize (Bt176) feeding in comparison with isogenic fed control animals (Halle et al., 2006). Feeding diets containing genetically modified maize (Bt176) to Japanese quail for 10 generations did not significantly influence animal health, feed intake, feed efficiency,

laying performance, or hatchability. Meat and egg quality were also not affected (Flachowsky et al., 2005b). Similarly, performance values were not influenced in a three-generation study with rats fed with genetically modified Bt-maize (Kilic & Akay, 2008).

During the past ten years, a large number of feeding studies has been carried out in accordance with the increased use of genetically modified foods for human and livestock, (Kilic & Akay, 2008). Nevertheless, the evidence is still far from proving whether the mid- or long-term exposure to Bt-maize varieties implies a possible danger for animal health, especially when investigating more detailed pathophysiological traits in multigenerational comparisons. In short-term feeding studies with Atlantic salmon, genetically modified maize (MON810) seemed to induce significant changes in white blood cell populations which are associated with an immune response (Sagstad et al., 2007). Preliminary feeding studies with layer quail fed on Bt-maize revealed significant differences in various serum biochemical parameters. Changes were observed for triglyceride concentration and the enzyme activities of alanine aminotransferase and γ-glutamyltransferase being elevated in Bt-maize fed animals. In contrast, glucose and lactate dehydrogenase activity were lower in these quail compared to isogenic control animals (Scholtz et al., 2006).

In the following, studies were conducted to advance the ongoing debate as to whether health is affected by the intake of diets containing maize with the Cry1Ab trait in comparison to non-biotech counterparts. For this purpose, three different experimental approaches have been conducted:
As a laboratory model organism the Japanese quail was used, justified by the fact that it is well adapted to laboratory conditions and possesses several advantages, such as rapid growth, early sexual maturity, high rate of egg production, easy handling of adult size, and a short generation interval (Ichilcik and Austin, 1978). Even so the Japanese quail has been widely used as a laboratory animal for the past 50 years (Huss et al., 2008), to date, serum chemistry reference values for this species are limited. Reference values may provide useful information about the physiological condition of individuals, making them a valuable tool in differentiating

normal and healthy animals from abnormal and diseased states (Pérez-Rodríguez et al., 2008). In a first study, serum chemistry reference ranges were established for adult Japanese quail.

The first study provided the opportunity to interpret serum chemistry values in a following multigenerational comparison with quail fed diets containing genetically modified Bt-maize. In combination with the analysis of histological variables, the aim of this experiment was to close the gap of more detailed pathophysiological trials investigating the mid- or long-term exposure to Bt-maize varieties. To clarify the biological range of all variables tested, further controls with isogenic hybrid reference maize grain were included.

To date, no publications exist investigating whether a deflection of the regulatory physiological system might yield divergent dynamic responses in animals fed diets containing transgenic crops. Studies observing the effects of feeding diets containing genetically modified maize were all done in homeostatic situations. The immune system, considered to be a functional system, defends the body producing lymphocytes and antibodies. For this reason, the effect of an active immunization against BSA was tested in the third study, to clarify, whether a deflection of the regulatory physiological systems might yield divergent dynamic responses in different feeding regimen with or without Bt-maize using quail as a model organism.

2. MANUSCRIPT 1 (published in: Poultry Science 2009; 88(6):1186-1190)

Serum chemistry reference values in adult Japanese quail (*Coturnix coturnix japonica*) including sex-related differences

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ABSTRACT Serum chemistry reference values may provide useful information about the physical condition of individuals, making them a useful tool in differentiating normal and healthy animals from abnormal or diseased states. For Japanese quail that are used for producing eggs and meat for human consumption and also as laboratory animals, we aimed to extend the available array of reference values and to compare 16-wk-old adult male versus female birds. In the present study, clinical chemistry data (albumin, total protein, glucose, uric acid, cholesterol, bilirubin, cholinesterase, creatinine, triglycerides, alanine aminotransferase, aspartate aminotransferase, and γ-glutamyltransferase) in blood serum from up to 125 male and 151 female Japanese quail were established. Statistical comparisons were made between male and female birds. Aspartate aminotransferase, alanine aminotransferase, glucose, cholinesterase, and bilirubin values were higher ($P < 0.01$) in males, whereas females had higher ($P < 0.05$) concentrations of albumin, total protein, γ-glutamyltransferase, total cholesterol, and triglycerides. No significant sex-based differences were observed for creatinine and uric acid. The reference values provided are relevant in particular for the use of quail as laboratory animals when responses to specific treatments have to be monitored and appraised.

Key words: quail, reference value, serum biochemistry, sex-related difference

INTRODUCTION

The *Coturnix coturnix* or common quail are birds originating from Asia, Africa and Europe. Species or subspecies of the genus *Coturnix* are native to all continents except the Americas. One of the domesticated subspecies, *Coturnix coturnix japonica,* is called Japanese quail (Faqi et al., 1997). These animals are mainly produced for their eggs and meat, but are also used as laboratory animals (Fitzgerald, 1969). The preference for the Japanese quail is justified by the fact that it is well adapted to laboratory conditions and possesses several advantages, such as rapid growth, early sexual maturity, high rate of egg production, easy handling of adult size and a short generation interval (Ichilcik and Austin, 1978).

The Japanese quail is fairly resistant to diseases but clinical chemistry data can be useful aids for diagnosis and monitoring responses in birds which often show no clinical signs (Fudge, 1997). The range of avian species for which reference values are published is mostly limited to racing pigeons, to the most common psittacine species, and to peregrine falcons (Lumeij, 1997). For those species used as poultry, blood chemistry was established to some extent for quail (Faqi et al., 1997), chicken (Ross et al., 1976, 1978), duck (Farhat and Chavez, 2000), Turkey (Huff et al., 2008) and ostrich (Verstappen et al., 2002). The aim of the present paper was to provide physiological reference values for serum chemistry parameters of adult Japanese quail and to investigate the variation in these values arising from differences between male and female animals.

MATERIALS AND METHODS

Bird Housing and Feeding

Up to 125 male and 151 laying female Japanese quail (*Coturnix coturnix japonica*) were used to determine the reference range for clinical chemistry parameters. At 1 d of age, the chicks were randomly allocated into stainless steel wire cages (60 x 100 x 50 cm) in groups of 40 animals in a windowless room. The target room temperature was initially set at 38°C and was then gradually decreased during the first 20 d of life to 20°C. The animals were initially exposed to a photo period of 24 h L that was then gradually changed to 18L:6D during the breeding phase. Starting at 6 wk of age, the adult male and female quail were housed pairwise (1 male and 1 female) in stainless steel wire cages (20 x 23 x 17 cm) under constant temperature (19°C ± 1°C) and 8L:16D, with the light phase starting 0745 h every day. The birds were fed *ad libitum* with a ration containing wheat, peas, soybean extraction meal, green meal and minerals. The diets were fed to the quail in three phases: starter (d 0 to 21), grower (d 22 to 42) and adult (d 42 to end). Depending upon the phase, maize (*Zea mays*) was contained in concentrations of 40 to 50 % (i.e. 40 % in starter and 50 % in grower and adult diets). The CP content was decreased from 26 % in the starter diet to 15 % in the diet for adults.

Clinical Chemistry Parameters

All animals were killed at 16 wk of age in the morning about 2 h after start of the light phase without a preceding fasting period. The animals were removed from their cages in groups of 10 animals and were then transferred in boxes to a dissection room. After weighing, they were euthanized by cervical dislocation and were then decapitated. Blood samples were collected into 1.5 mL tubes immediately thereafter. The time interval between the removal of the birds from their cages and feed and the time of blood sampling was 8 to 12 min. Contamination of blood samples with crop content was avoided by accurate conduction of the sampling procedure. After overnight clotting at 4°C, the samples were centrifuged for 20 min at 4,000 *g*. The separated serum was transferred to a commercial laboratory (Synlab Vet Laboratory, Cologne, Germany) and was analyzed for albumin, total protein, glucose, uric acid, cholesterol, bilirubin, cholinesterase, creatinine and triglyceride concentration and the enzyme activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and gamma-glutamyltransferase (γ-GT) using a clinical chemistry analyzer Olympus AU 640 (Olympus Deutschland GmbH, Hamburg, Germany).

Statistical Analyses

Statistical analyses were carried out using SPSS 15.0 software for Windows (SPSS Inc., Chicago, Illinois, USA). Male and female animals were analyzed separately since sex may affect the parameters studied (Pérez-Rodríguez et al., 2008). The results were tested for Gaussian distribution by the Kolmogorov-Smirnov test and are displayed both as the inner limits of the 2.5 and 97.5 percentiles and as means ± SEM. Differences between male and female birds were compared using Student's t-test, if the data were normally distributed. For serum biochemical data which did not have a Gaussian distribution, i.e. ALT, γ-GT, glucose, triglycerides, and cholinesterase, the non-parametric Mann-Whitney U test was used. The level of significance was set at $P \leq 0.05$.

RESULTS AND DISCUSSION

At the time of killing, the mean BW of the female quail (185.7 ± 16.4 g) was 1.2 fold higher compared to the male animals (160.9 ± 15.2 g). This sexual dimorphism in body weight observed herein confirms previous reports in Japanese quail (Karabayir and Tolu, 2008). The serum chemistry reference values established in adult Japanese quail are presented in Table 1. The concentrations of albumin, total protein, glucose, total cholesterol, cholinesterase and uric acid as well as the activities of AST, ALT, γ-GT were similar to those reported for other bird species [i.e. for ostriches, peregrine falcons, pigeons, African greys, amazons, cockatoos and macaws (Lumeij, 1997; Verstappen et al., 2002)], for chicken (Ross et al., 1978; 16 wk of age) and for Japanese quail (Faqi et al., 1997). However, creatinine concentrations reported previously (Lumeij, 1997; Faqi et al., 1997) were higher than in the present study; serum bilirubin concentrations as well as AST activities were higher than those previously reported elsewhere (Faqi et al., 1997).

There was evidence for sex-related differences in several biochemical parameters: The AST, ALT, glucose, cholinesterase and bilirubin values were higher in males, whereas the female animals had higher values of albumin, total protein, γ-GT, total cholesterol and triglycerides.

Several sex-related differences may be explained by the physiological changes in metabolism in female birds due to egg laying: during the laying period, the hepatic synthesis of triglycerides, phospholipids, and cholesterol is increased (Walzem et al., 1999). These lipids are incorporated into lipoproteins that are secreted into the blood and are incorporated into the oocytes of the ovary. Laying hens therefore have extraordinarily high circulating concentrations of triglycerides and cholesterol in contrast to male birds (König et al., 2007). In addition, females of oviparous species demonstrate a marked increase in total protein concentration just before egg production; this estrogen-induced hyperproteinemia is associated with an increase in the yolk precursors vitellogenin and lipoproteins (Lumeij, 1997). Avian total protein consists mainly of albumin and α-, β- and γ-globulin (Lumeij, 1997), thus elevated concentrations of total protein in egg-laying birds are accompanied by significant increases in levels of serum albumin and globulin (Hunt and Hunsaker, 1965).

Sex-related differences are also observed for γ-GT which is a good mirror for the overall body enzymatic and metabolic process in broiler chickens (Tony et al., 2003). γ-Glutamyltransferase is involved in the transamination of glucogenic amino acids in order to produce glucose. In birds, high levels of serum γ-GT are commonly used as an index of liver disease, as well as damages in biliary ducts and renal epithelium (Lewandowski et al., 1986), but it also increases in female birds, apparently reflecting increased liver metabolism due to egg laying (Alonso-Alvarez et al., 2007).

A generalized pattern of sex as a source of variation for avian cholinesterase (ChE) levels seems equivocal since the studies published are inconsistent (Maul and Farris, 2004). Serum cholinesterase is used as an indicator for the acetyl ChE activity at the myoneural junction; serum ChE activity is reportedly decreased during acute infection, muscular dystrophy, chronic renal disease and organophosphate insecticide intoxication (Kramer and Hoffmann, 1997). Recording ChE activity has been suggested as a strategy for biomonitoring environmental background levels of organophosphate contamination and establishment of local reference values was recommended (Halbrook et al., 1992). Moreover, among the hepatic proteosynthetic function indices, ChE activity has been described to be a sensitive and appropriate parameter to assess the functional ability of impaired liver (Kupcová et al., 1994). Estimation of serum ChE activity is useful in diagnosis of diseases of the hepatobiliary system (Turecky et al., 2005) and decreased activity in liver dysfunction is reportedly due to reduced synthesis (Ogunkeye and Roluga, 2006). In contrast, increased serum ChE concentrations are reported for humans with fatty liver from man (Koda et al., 2007). In view of the latter relationship, the lower ChE activity we observed in females compared to males seems contradictory since fat liver content is knowingly increased in egg laying birds. However, other factors such as sex steroids may also affect ChE synthesis as demonstrated in rats (Illsley and Lamartiniere, 1981). In nonpasserine birds, cholinesterase enzyme activity reportedly differs between sexes in Japanese quail (*Coturnix coturnix japonica*) (Ludke et al., 1975; Hill, 1989), northern bobwhite (*Colinus virginianus*) (Hill and Murray, 1987), and American kestrel (*Flaco sparverius*) (Rattner and Franson, 1984). Maul and Farris (2004) examined the sex-dependent variability of cholinesterase enzyme activity in the free-living northern cardinal (*Cardinalis cardinalis*) with females having a lower

cholinesterase activity than males. Other studies have tested for the effect of sex in passerines and report no difference between male and female total cholinesterase activity (Hill and Murray, 1987).

Not all significant data obtained in this study can be explained by metabolic changes during the laying period; nevertheless, analogous findings are reported. AST is often associated with liver parenchymal cells, but is also present in red blood cells, and cardiac and skeletal muscle. Like γ-GT, AST enzymes are involved in the transamination of glucogenic amino acids in order to produce glucose (Lewandowski et al., 1986). Our results for AST are in accordance with those in a study of pigeons (Gylstorff and Grimm, 1998) and Siamese fighting fowls (Sribhen et al., 2006) demonstrating higher values in males compared to females. Significant elevations in bird AST concentrations would be indicative of hepatocellular disease (Brugère-Picoux et al., 1987; Harr, 2002).

For glucose, similar results are reported by Pérez-Rodríguez et al. (2008) in red-legged partridges with males showing higher values than females. In contrast, a previous descriptive study in red-legged partridges reported sexual differences in various parameters but not in glucose (Rodriguez et al., 2004, 2006). Inconsistency between studies may be attributed to the hour of the day at which samples are collected, as many blood parameters show daily patterns of variation (e.g. Garcia-Rodriguez et al., 1987; Ferrer et al., 1994). For glucose, diurnal variations are well known depending on the effect of the eat-fast cycle and its interference with internal rhythms (Pérez-Rodríguez et al., 2008). Furthermore, not all published studies are directly comparable because the distribution and concentration of the diagnostic clinical chemistry parameters and intracellular enzymes often is bird species-specific (Lumeij, 1994). For ALT, Lierz and Hafez (2005) reported significantly higher activities in female stone curlews compared to male animals. ALT is a cytoplasmic enzyme that catalyzes the transamination of alpha-ketoglutarate and L-alanine, forming glutamate and pyruvate (Kraft and Dürr, 1999). For the sex difference we observed for bilirubin, a conclusive explanation can not be provided; bilirubin is the major breakdown product of haemoglobin and is excreted by the liver as part of the bile (Gilmore and Garvey, 2009). Increases in Bilirubin indicate inefficient lymphatic or liver/gallbladder function (Lumeij, 1994). In avian plasma bilirubin occurs only in

scant quantities, therefore bilirubin assays were regarded as not useful for clinical information (Fudge, 2000). However, bilirubin is reportedly affected by genetic selection in turkeys (Bayyari et al., 1997) and the reports of sex differences in red blood picture from chicken (Vo et al., 1978) indicate that changes in haemoglobin turn-over might be the underlying cause of the difference. This notion is supported by Mercke and Lundh (1976) showing the changes of haeme catabolism during the human menstrual cycle. A more recent study in turkeys contradicts sex differences for several indicators of the red blood picture (Suchý et al., 1995) and in view of the conflicting data both the cause and the function of sex differences in bilirubin have to remain speculative. No sex differences were observed for creatinine which is a useful indicator of renal injury (Mahesh et al., 2008) and thus give no indications for sex differences in nitrogen metabolism, growth and muscle mass, the latter being positively correlated with creatinine (Szabó and Milisits, 2007). For uric acid being the major end product of nitrogen metabolism, again no sex-related differences were observed.

In conclusion, we found that many of the evaluated parameters differed significantly between sexes. The effect of sex should therefore be considered to avoid undesirable sources of variation and thus misjudgement at least for some blood parameters. Furthermore, other factors, such as circadian variation and the handling of birds, should also be taken into account, as they may affect various parameters. Capture and restraint of birds may also change some variables (Lierz and Hafez, 2005). However, it is inevitable in practice for birds to be restrained for blood sampling or treatment in general.

Clinical chemistry reference values are often not fully comparable between avian genera and species because the analysed parameters are mostly bird species-specific. However, the clinical chemistry reference values of adult Japanese quail obtained in this study can be considered as representative values for this species since the number of animals used herein clearly exceeded the limit of 40 animals required to estimate the 2.5^{th} and 97.5^{th} percentiles (Solberg, 1994).

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Table 1. Serum chemistry serum reference values of 16-wk-old male and female Japanese quail

	Males			Females			
Parameters	n	$P_{2.5}$ to $P_{97.5}$	Mean ± SEM	n	$P_{2.5}$ to $P_{97.5}$	Mean ± SEM	Sex effect
Albumin (g/L)	124	11.4 to 15.2	13.3 ± 0.2	150	12.6 to 18.0	15.3 ± 0.2	***
Total protein (g/L)	124	6.2 to 57	31.6 ± 2.3	151	29.7 to 43.3	36.5 ± 0.6	***
AST[1] (U/L)	125	315 to 528	422 ± 9.5	151	243 to 562	402 ± 13.0	**
ALT[2] (U/L)	121	2.7 to 16.5	9.6 ± 0.6	145	4.5 to 8.5	6.5 ± 1.1	***
γ-GT[3] (U/L)	121	<1 to 3.5	1.7 ± 0.2	145	<1 to 3.2	1.9 ± 0.1	**
Glucose (mmol/L)	121	10.4 to 24.2	17.3 ± 0.6	139	7.5 to 21.3	14.4 ± 0.6	***
Total cholesterol (mmol/L)	118	4.8 to 7.4	235 ± 4.5	146	4.1 to 9.9	269 ± 9.1	***
Triglyceride (mmol/L)	120	0.4 to 5.6	3.0 ± 0.2	146	3.3 to 49.7	23.5 ± 2.2	***
Cholinesterase (KU/L)	60	3.7 to 5.9	4.8 ± 0.1	54	1.9 to 3.7	2.8 ± 0.1	***
Bilirubin (µmol/L)	60	12.4 to 28.4	20.4 ± 1.0	54	3.6 to 14.2	8.9 ± 0.7	***
Creatinine (µmol/L)	60	1.1 to 6.7	4.0 ± 0.4	54	1.1 to 6.7	4.5 ± 0.3	NS
Uric acid (µmol/L)	60	158 to 422	324 ± 21.5	54	158 to 422	320 ± 13.9	NS

[1]Aspartate aminotransferase.

[2]Alanine aminotransferase.

[3]γ-Glutamyltransferase.

**: P ≤ 0.01; ***: P ≤ 0.001; NS = P > 0.05.

3. MANUSCRIPT 2 (to be submitted to: Food and Chemical Toxicology)

Blood chemistry and histological investigations in Japanese quail (*Coturnix coturnix japonica*) fed diets containing genetically modified maize: a multigenerational comparison

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Abstract

To evaluate the effects of feeding genetically modified maize expressing the insecticidal Cry1Ab protein from *Bacillus thuringiensis*, a multigenerational study with male and female Japanese quail fed either Bt-maize (Bt), or isogenic maize (ISO) of the same cultivar for up to 20 generations was performed. In multigenerational comparisons, genetic drift might be an issue and therefore the examinations were extended to animals fed Bt-maize, or isogenic maize in first generation (generation 0). In addition, two further control groups fed with two other non-Bt-maize control varieties (REF1, REF2) were included. At 16 wk of age, all animals were sacrificed, liver tissue was histomorphometrically evaluated and blood samples were analyzed for serum biochemistry.

Expectedly, sex differences were observed for most of the recorded variables. Comparing Bt-maize fed animals versus quail fed the non-transgenic control diets, differences were observed for body weight at 6 wk of age, as well as for the enzyme activity of γ-GT from generation 0 and generations F17 to F20. Changes in these parameters were also determined when comparing animals from Bt and ISO vs. REF groups. Comparing quail from generation 0 with REF1 and 2, differences in body weight at 6 wk of age, relative liver weight, hepatocyte nuclear size, AST, ALT, γ-GT, and glucose were observed for Bt vs. ISO and REF, as well as ISO vs. REF comparisons.

The statistical differences observed throughout the study were thus not limited to Bt vs. ISO, or REF comparisons; they were neither consistent nor analogous and thus give no indication for targeted pathophysiological alterations induced by Bt feeding.

Key words: Bt-maize; Japanese quail; Multigenerational study; Blood chemistry; Histology

1. Introduction

The use of transgenic crops, claimed to produce higher yields with minimal effort, has worldwide rapidly increased during the past decade. Nevertheless, substantial scientific and societal concerns have emerged, in particular in Europe, and have occasionally led to a ban on cultivating such crops, e.g. for genetically modified

maize expressing the insecticidal Cry1Ab protein from *Bacillus thuringiensis* (Bt-maize) in April 2009 in Germany (BMELV, 2009). Besides potentially adverse effects on social structures and insect biodiversity, there are consumers` concerns that diets containing transgenic plants may exert negative effects on animal and human health. Previous feeding studies with diets containing Bt-maize indicated no risk for health when considering nutritional assessment and performance parameters (Aumaitre et al., 2002, Clark & Ipharaguerre, 2001, Flachowsky et al., 2005a). Even long-term studies in birds (laying hens, 4 generations, Halle et al., 2006; quail, 10 generations, Flachowsky et al., 2005b) and rats (3 generations, Kilic and Akay, 2008) did not reveal significant influences on animal development after feeding of genetically modified Bt-maize. However, the effect of mid- or long-term exposure to Bt-maize varieties in multigenerational studies has rarely been investigated on more detailed pathophysiological trials. Preliminary feeding studies with layer quail fed on Bt-maize revealed significant differences in various serum biochemical parameters (Scholtz et al., 2006); it remained open whether the deflections observed might result from the maize variety in the diet, or from naturally occurring variance.

The aim of the present study was to extend histological and serum biochemical examinations on further generations of male and female Japanese quail fed on diets containing Bt-maize, in order to elucidate if animal health is affected by the intake of the maize variety, or if differences between feeding groups arise from naturally occurring variances. The preference for the Japanese quail as a model organism is justified by the fact that it is well adapted to laboratory conditions and possesses several advantages, such as rapid growth, early sexual maturity, high rate of egg production, easy handling of adult size, and a short generation interval (Ichilcik & Austin, 1978). To clarify the biological range of all variables tested, further controls with isogenic hybrid reference maize grain were included and feeds were analysed for their composition. Potential differences between animals fed Bt-maize or isogenic maize might also be attributable to differing contents of mycotoxins; for example it is known that the mycotoxins zearalenone and deoxynivalenol produced by *Fusarium* species have negative effects on health and performance of animals (Cerveró et al., 2007). The protection of maize plants against insect damage (European corn borer and pink stem borer) through the use of Bt technology aims to reduce the

contamination of maize by fungi and the toxins they produce (Bakan et al., 2002). To address this issue, we quantified different mycotoxins in the diets used.

2. Material and Methods

2.1 Animals and housing

Four hundred male and female Japanese quail (*Coturnix coturnix japonica*) were used to determine the effects of genetically modified feeds on animal health.

Multigenerational comparison

To avoid inbreeding, male and female parental animals from 16 different strains and a rotation crossbreeding system were used to produce progeny. The parental generation was randomly divided into groups fed on either an experimental diet containing transgenic (Bt)-maize or isogenic maize (ISO). In total, 20 generations were bred and generations F17 to F20 animals were used for this study.

Generation Zero experiment (generation 0):

In multigenerational comparisons, genetic drift might nevertheless be an issue and therefore the examinations were extended to animals fed ad libitum with diets containing 50 % Bt-maize or isogenic maize of the same cultivar in first generation (generation 0). To elucidate naturally occurring variance of all parameters tested, two further controls with commercial hybrid reference maize [REF1 (“Gavot”; KWS Saat AG, Einbeck, Germany); REF2 (“Seiddi”; Caussade Saaten GmbH, Hamburg, Germany)], were included. Variables measured in quail fed these reference varieties can be considered to approximate the normal range of biological responses for the larger population of control quail. Table 1 presents the different feeding groups of this multigenerational comparison and gives the actual numbers of animals for each generation and treatment.

After hatching, the animals were housed in stainless steel wire cages (60 x 100 x 50 cm) in groups of 40 animals each. The target room temperature was initially set at 38°C and was then gradually decreased during the first 20 days of life to 20°C. The animals were initially exposed to a photo period of 24 h L that was then gradually changed to 18L:6D during the breeding phase.

Starting at six wk of age, the adult male and female quail were housed pair wise (1 male and 1 female) in stainless steel wire cages (20 x 23 x 17 cm) under constant temperature (19°C ± 1°C) and 8L:16D, with the light phase starting 0745 h every day. Water and feed were available *ad libitum* throughout the experiment.

2.2 Experimental diets

The insect resistant *Bacillus thuringiensis* transgenic (Bt)-maize expressing the Cry1Ab gene, its non-transgenic near-isogenic control (ISO), and two commercially available non-transgenic hybrid reference maize cultivars (REF1, REF2) were grown at field test sites in Braunschweig, Germany during the 2005-2007 growing seasons.

Diets containing test, control, and reference grain were based on a ration containing wheat, peas, soybean extraction meal, green meal and minerals. The diets were fed to the quail in 3 phases: starter (d 0 to 21), grower (d 22 to 42), and adult (d 42 to end). Depending upon the phase, maize (*Zea mays*) was contained in concentrations of 40 to 50 % (i.e., 40 % in starter and 50 % in grower and adult diets).

Following diet preparation, nutrient composition was analyzed in samples of all diets at the Institute of Animal Nutrition, Friedrich-Loeffler-Institute (FLI), Federal Research Institute for Animal Health, Braunschweig, Germany. The analyses included dry matter, crude protein, crude fat, crude fiber, and crude ash, carbohydrates, amino acids, and minerals (calcium, and phosphor). The chemical composition of the different maize diets was generally comparable as determined by proximate analysis (Table 2).

The presence and quantity of the Cry1Ab protein was evaluated at the Center of Life and Food Sciences Weihenstephan, Research Department Animal Sciences, Physiology, Technical University Munich, Freising, Germany using a sensitive and highly specific ELISA as described by Guertler et al. (2009).

2.3 Analysis of mycotoxins

Mycotoxin analysis of all diets was performed using HPLC-analysis (high performance liquid chromatography) with fluorescence detection for the investigation of deoxynivalenol (DON) and zearalenone (ZON) as described by Dänicke et al. (2007). The limits of detection were 0.1 mg/kg for DON and 0.1 µg/kg for ZON.

2.4 Performance recordings and sample collection

Body weight was measured for each quail at 6 wk of age when being feather-sexed, and at study termination at 16 wk of age. About 2 h after start of the light phase in the morning, the animals were removed from their cages in groups of 10 animals and were then transferred in boxes to a dissection room. They were weighed, sacrificed by cervical dislocation and were subsequently decapitated. Blood samples were collected immediately thereafter. All blood samples were obtained within a time interval of 8 to 12 min following removal of the birds from cages and feed. Contamination of blood samples with crop content was avoided by accurate conduction of the sampling procedure. In the following, the livers were carefully removed and immediately weighed. Organ weight was recorded and calculated relative to body weight. Laying intensity in female quail (7–10 wk of age) and the feed intake combined for males and females were recorded during starter and grower period (wk 1-6) and in adult animals (wk 7-10).

2.5 Preparation of liver sections and histological evaluation

Immediately after removal from the body, three small fragments (2 x 2 mm) from the medial lobe of the liver were dissected. For conventional ultrastructural morphology, these samples were fixed in Karnovsky's fixative (Karnovsky, 1965), and then postfixed in 1 % osmium tetroxide, dehydrated with degraded series of ethanol concentrations, and embedded in Spurr resin according to Spurr (1969). From each liver fragment, five sections (0.5 µm in thickness) were cut with glass knives on a Reichert Ultracut E ultramicrotome (Reichert; C., Optische Werke AG, Vienna, Austria), collected on glass slides and stained with 1 % toluidine blue. After staining, the slides were cleared in Xylol (Applechem Inc. Newark, New Jersey, USA), covered under coverslips using Entellan (Merck, Darmstadt, Germany) and were evaluated with a Leica DML light microscope (Leica Microsystems GmbH, Wetzlar, Germany) and a JVC KY-F75U digital camera (JVC, Tokyo, Japan). Histological analyses were performed at light microscopic level on Sparr resin-embedded liver samples by using a computerised image analysis system (Discus, C. H. Hilgers, Königswinter, Germany). Hepatocyte nuclear areas were measured with a grid within a total area of 7.1 mm^2 at a final magnification of 100x in all 15 sections from each animal.

2.7 Serum chemistry

After overnight clotting at 4°C, the blood samples were centrifuged for 20 min at 4,000 x *g*. The separated serum was transported to a commercial laboratory (Synlab Vet Laboratory, Cologne, Germany) and was analyzed for albumin, total protein, glucose, cholesterol, and triglyceride concentration and the enzyme activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and γ-glutamyltransferase (γ-GT) using a clinical chemistry analyzer Olympus AU 640 (Olympus Deutschland GmbH, Hamburg, Germany).

2.8 Statistical analyses

All analyses were carried out using SPSS 17.0 software for Windows (SPSS Inc., Chicago, Illinois, USA). An univariate analysis of variance (ANOVA) with a post hoc Bonferroni correction was performed for each biological variable; generation, feeding group, sex, and the respective two and three-way interactions were considered to be fixed factors. Differences were considered significant, if both the ANOVA and the Bonferroni adjustment were significant ($p \leq 0.05$). For analyses of mycotoxin concentrations in the diets, as well as laying intensity, and feed intake no individual animal data, but only pooled data were available, and thus comparison between Bt, ISO, and REF groups were conducted by using the Student's t-test.

3. Results

3.1 Mycotoxin and Cry1Ab protein contents in the diets

The mycotoxin concentrations of DON and ZON in the diets were below acceptable limits for animal feed according to guidance of the European Commission concerning levels of mycotoxins in animal feeds (EU-Kommission, 2006), and did not differ between diets containing Bt-maize in comparison to diets containing isogenic or reference control maize, respectively (Table 3).

Using a highly specific ELISA, Cry1Ab protein (46.9 ng/g diet) was detectable only in one of the transgenic Bt-maize diets.

3.2 Performance

All variables recorded were analysed and are presented in the following order:

- comparison of data in quail from generation 0 and generations F17 to F20 fed on Bt or isogenic control maize, respectively (Table 4);
- comparison of data in quail from generation 0 and generations F17 to F20 fed on Bt or isogenic control maize vs. two commercial reference diets;
- comparison of data in quail from generation 0 fed on Bt or isogenic control maize vs. two commercial reference diets (Table 5)

Most variables recorded were significantly influenced by generation throughout the study, though these differences were not consistent and showed irregular alterations. Comparing male and female animals, most performance variables showed sex differences. In contrast, only few variables were affected by the maize variety fed in the different groups.

Bt-maize fed male and female quail from generation 0 and generations F17 to F20 had significantly lower ($p < 0.001$) body weights at 6 wk of age when compared to isogenic control animals (Table 4). Comparing these animals with quail fed the reference control diets, differences between Bt-maize fed animals and the REF1 group occurred. Analogous differences were recorded when comparing solely generation 0 vs. the reference controls (Table 5). In contrast, final body weight ranging from 154 to 166 g in adult males and from 180 to 188 g in females did not differ between feeding groups in either comparison (Tables 4, 5).

No macroscopic alterations of liver were observed; the absolute liver weight and relative liver weight was not different between diet groups with the only exception of generation 0, in which both male and female Bt-maize fed quail as well as isogenic fed male animals had higher relative liver weights than the REF2 animals. In contrast female quail from the isogenic feeding group had lower relative liver weight than the REF2 animals (Table 5).

On a g/animal/day basis, feed intake ranged from 15.7 to 16.3 g during the grower phase from hatch until 6 wk of age, and from 21.4 to 24.6 g during 7 to 10 wk of age without differences between feeding groups. Similarly, there were no differences in

the laying intensity in female quail, ranging from 69.4 to 79.0 % during the time of observation from 7–10 wk of age.

3.2 Histological examinations

Light microscopic examinations of Sparr resin-embedded liver samples showed similar hepatocyte nuclear size in both male and female Japanese quail when comparing Bt-maize fed animals from generation 0 and generations F17 to F20 with isogenic control animals (Table 4), as well as in comparison to different REF diets. Differences in hepatocyte nuclear size were observed for the Bt vs. ISO and for Bt vs. REF2 comparison in generation 0 with increased sizes in Bt-maize fed quail (Table 5).

3.3 Serum chemistry parameters

Most serum chemistry values were physiological influenced by sex. Observing the influence of different diets only few differences were recorded, when comparing Bt-maize fed animals from generation 0 and generations F17 to F20 with isogenic control animals (Table 4), and with different REF diets. Differences occurred solely in the enzyme activity of γ-GT between Bt- vs. ISO fed animals (Table 4), Bt-maize fed animals vs. REF1 and 2, as well as between isogenic control animals vs. REF1 and 2. Comparing serum chemistry parameters in male and female quail from generation 0 with both REF diets, differences between the enzyme activities of AST, ALT, and γ-GT, as well as glucose were observed (Table 5). AST and ALT values were higher in Bt and isogenic fed quail in comparison to the REF1 feeding group. The enzyme activity of and γ-GT was higher in male and female Bt-maize fed animals in comparison to both the isogenic group and the REF2 feeding group. Differences in serum chemistry parameters were not limited to Bt vs. ISO comparisons, but also occurred between Bt and isogenic control groups vs. REF groups; no differences were observed between the different REF groups (Table 5). Furthermore, no feeding effect was found for serum values of albumin, total protein, total cholesterol, and triglycerides in either comparison.

4. Discussion

The reports on long-term feeding studies and comprehensive analyses with transgenic Bt-maize are limited, therefore this study was conducted with adult male and female Japanese quail fed with diets containing 50 % Bt, isogenic control maize, or two commercially available reference maize grains for up to 20 generations. The reference diets were comprised to characterize the biological range of all parameters. For all diets, compositional equivalency was obtained, supplying all animals with similar amounts of macro- and micronutrients, and energy. Accordingly, the feeding value of different diets was not altered by genetic incorporation of *Bacillus thuringiensis* Cry proteins into the maize. This finding was consistent with results obtained in previous studies reviewed by Flachowsky et al. (2007), in which different feeds from genetically modified plants were analysed for their composition and undesirable substances. Undesirable substances, e.g. the F*usarium* toxins zearalenone and deoxynivalenol, are of special importance under European production conditions because of their high frequency and because they have been shown to cause a variety of toxic effects in both experimental animals and livestock (European Commission, 1999). Maximum limits of these *Fusarium* toxins in animal feed are controlled by law throughout the European Union. In the present study, concentrations of DON and ZON were below the acceptable limits for animal feed according to guidance of the European Commission (EU-Kommission, 2006), they were not different between the different maize varieties and thus gave no reason for concerns of adverse effects. Furthermore, poultry is regarded as very resistant to DON and ZON (Dänicke, 2002).

Thermal treatment, in terms of feed processing (e.g. drying of maize) with temperatures above 75°C, is known to cause protein denaturation of the Cry1Ab protein (De Luis et al., 2008), and thus might be the reason why only one Bt-maize diet was found positive for the Cry1Ab protein.

There were, however, differences between feeding groups in body weight of growing male and female quail at 6 wk of age. These differences appeared to be randomly distributed among several groups, they were restricted to the growing phase and were not present in adult animals. Furthermore, Bt-maize fed animals did not consistently show a lighter body weight compared to isogenic, or reference control

animals. Earlier studies evaluating feed effects of genetically modified Bt-maize over several generations, gave no indication of impairments in gross performance and health of laying Japanese quail (Flachowsky et al., 2005b) and rats (Schrøder et al., 2007; Kilic and Akay, 2008). Likewise no differences were observed in feed intake, laying intensity, as well as absolute liver weight and relative liver weight, with one exception; relative liver weight in Bt and isogenic fed animals from generation 0 differed from REF2 fed quail. Differences were considered to be diet independent as they were not limited to Bt-maize fed animals.

Blood chemistry values obtained in this study were mostly sex-dependent. This sexual dimorphism in serum biochemical parameters observed herein confirms previous reports in Japanese quail (Scholtz et al., 2009) and other bird species (Sribhen et al., 2006; Pérez-Rodríguez et al., 2008). In contrast, only few parameters were affected by feeding group, like e.g. γ-GT. γ-Glutamyltransferase which is involved in the transamination of glucogenic amino acids to produce glucose is a good mirror for the overall body enzymatic and metabolic process in broiler chicken (Tony et al., 2003). In birds, high levels of serum γ-GT are commonly used as an index of liver disease, as well as damages in biliary ducts and renal epithelium (Lewandowski et al., 1986). The liver is a primary site for biotransformation of the products of digestion, and it degrades and detoxifies toxic compounds received from the intestines or from the general circulation and excretes them in the bile. Furthermore, it synthesizes many protein components of blood plasma and exerts an important control over the general metabolism (Malatesta et al., 2002). However, macroscopic and microscopic observations gave no hints for alterations in liver tissue of animals from different feeding groups in the present study. Other parameters like the enzyme activities of AST and ALT reflecting liver function did not vary between animals from generation 0 and generations F17 to F20 in comparison to both reference groups and were in accordance with light microscopic examinations on liver tissue.

In a preliminary comparison within the 13th generation of this long-term feeding experiment altered ALT and γ-GT concentrations were observed in female quail compared to animals fed diets containing isogenic control maize (Scholtz et al., 2006). Changes in ALT and γ-GT and, additionally in glucose and the enzyme activity

of AST were also observed in animals from generation 0 compared to animals from both REF groups. However, differences were not limited to Bt vs. ISO, or REF comparisons. In addition, hepatocyte nuclear size differed between Bt-maize fed quail and isogenic control from generation 0 and REF2 animals. Hepatocytes are involved in numerous metabolic activities (Velimirov, 2008); modifications of hepatocyte nuclear size are related to both age and food, and it is known that cell nuclei become progressively larger as age increases (Schmucker, 1990). Altogether, differences in serum chemistry parameters and hepatocyte nuclear size were of small magnitude and mostly fell within ± 2 standard deviations of the reference population mean and the inner limits of the reference ranges of serum chemistry parameters in adult Japanese quail (Scholtz et al., 2009). Accordingly, the differences were considered to be incidental findings and not related to Bt-feeding, as furthermore, differences were not limited to Bt vs. ISO and REF comparisons.

In conclusion, feeding of Bt-maize did not elicit any consistent and analogous alterations in any of the variables recorded. The few statistical differences observed herein were not limited to Bt vs. ISO and REF comparisons and thus give no indication for targeted pathophysiological alterations induced by Bt feeding and encourage the suspicion of naturally occurring variances. The absence of any meaningful differences in the measured parameters provides support for the comparability of Bt-maize to conventional reference control maize varieties. The fact that no adverse effects were revealed in the Japanese quail is consistent with results seen in feeding studies with e.g. rats (Hammond et al., 2004; 2006a, b; Kilic and Akay, 2008), mice (Brake et al., 2004), birds (broiler, Taylor et al., 2004; laying hens, Halle et al., 2006; quail, Flachowsky et al., 2005b), and Atlantic salmon (Sanden et al., 2006). The lack of obvious adverse effects in our quail studies is of general importance to animal science.

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Table 1. Number of animals in the different feeding groups

Treatment	Quail from F17, F18, F19 & F20		Quail from "generation 0"	
	Males n	Females n	Males n	Females n
Bt-maize	40	40	30	30
ISO	40	40	30	30
REF1	-	-	30	30
REF2	-	-	30	30

Table 2. Nutrient composition of starter, grower and adult diets containing Bt, non-transgenic control (ISO), or commercial reference maize grains (REF1, REF2)

	Starter diet (d 0-21)				Grower diet (d 22-42)				Adult diet (d > 42)			
	Bt	ISO	REF1	REF2	Bt	ISO	REF1	REF2	Bt	ISO	REF1	REF2
Dry mass (g/kg)	880	882	877	877	875	877	874	874	880	883	883	883
Crude protein (%)	28	28	26	26	21.0	21.0	20.7	20.7	15.5	15.5	15.0	15.0
Crude ash (%)	7.15	7.16	7.92	7.92	5.65	5.67	7.03	7.03	12.0	11.6	13.1	13.1
Crude fiber (%)	5.06	4.94	4.05	4.05	4.33	4.23	3.47	3.47	4.08	4.07	2.74	2.74
Crude fat (%)	4.39	4.35	3.87	3.87	2.47	2.54	2.77	2.77	2.04	2.02	2.68	2.68
Starch (%)	26.9	26.7	29.4	29.4	40.6	40.5	39.8	39.8	43.4	43.5	44.4	44.4
Glucose (%)	5.01	4.96	4.9	4.9	3.72	3.65	3.96	3.96	2.78	2.71	2.95	2.95
Calcium (%)	1.2	1.2	1.1	1.1	1.1	1.1	1.0	1.0	3.98	3.84	3.73	3.73
Phosphorus (%)	0.9	0.9	0.8	0.8	0.7	0.7	0.7	0.7	0.45	0.45	0.55	0.55
Lysin (%)	1.65	1.65	1.5	1.5	1.3	1.3	1.2	1.2	0.68	0.68	0.78	0.78
Methionin (%)	0.5	0.5	0.5	0.5	0.4	0.4	0.4	0.4	0.34	0.34	0.34	0.34
Energy (MJ ME/kg)	10.9	10.9	10.9	10.9	11.3	11.3	11.3	11.3	10.7	10.7	11.0	11.0

Table 3. Deoxynivalenol (DON) and zearalenone (ZON) concentrations in Bt (generations 0 & 17th-20th) or REF diets (generations 0 & 17th–20th; ISO & REF1, REF2) (mean ± SD)

	Bt n = 5	REF n = 7	Recommendations for maximum limits[1]
DON mg/kg	0.66 ± 0.64	0.97 ± 0.73	8.0
ZON mg/kg	0.02 ± 0.01	0.03 ± 0.02	2.0

[1]EU-Kommission (2006)

Table 4a. Comparison of performance and blood chemistry data (mean ± SD) in male and female quail from generation 0 and generations F17 to F20 fed on Bt or isogenic control maize, respectively

	Bt		ISO	
	Males n = 69	Females n = 68	Males n = 66	Females n = 70
Body weight 6wk (g)	153 ± 17.3[a]	167 ± 21.7[a]	156 ± 18.5[b]	171 ± 23.0[b]
Body weight 16 wk (g)	165 ± 15.1	182 ± 16.1	159 ± 14.4	185 ± 16.4
Absolute liver weight (g)	2.6 ± 0.4	6.1 ± 1.4	2.5 ± 0.3	5.8 ± 1.4
Relative liver weight (%)	1.6 ± 0.2	3.4 ± 0.8	1.6 ± 0.2	3.2 ± 0.7
Hep. nuclear size (μm^2)	19.1 ± 2.5	21.0 ± 2.7	18.4 ± 2.9	20.4 ± 2.9
Albumin (g/L)	12.0 ± 1.9	15.3 ± 0.2	12.5 ± 1.8	15.4 ± 2.4
Total protein (g/L)	28.5 ± 3.1	37.2 ± 4.7	33.6 ± 35.3	38.7 ± 5.8
AST[1] (U/L)	477 ± 228	413 ± 252	448 ± 117	456 ± 160
ALT[2] (U/L)	20.8 ± 99.4	10.7 ± 15.7	8.9 ± 9.5	9.9 ± 18.8
γ-GT[3] (U/L)	2.0 ± 1.5[a]	2.3 ± 1.7[a]	2.4 ± 2.4[b]	2.0 ± 1.5[b]
Glucose (mmol/L)	15.6 ± 8.6	13.4 ± 7.3	16.0 ± 9.3	10.2 ± 8.6
Total cholesterol (mmol/L)	6.3 ± 1.2	7.0 ± 2.3	6.0 ± 1.5	6.9 ± 2.9
Triglyceride (mmol/L)	3.2 ± 1.2	26.5 ± 15.3	3.0 ± 1.8	22.1 ± 14.6

Means within rows with different superscript letters indicate significant differences between feeding groups

Table 4b. Statistical results

	p			p			
	Generation	Group	Sex	Generation x Group	Generation x Sex	Group x Sex	Generation x Group x Sex
Body weight 6wk (g)	< 0.001	< 0.001	< 0.001	NS	< 0.001	NS	< 0.001
Body weight 16 wk (g)	< 0.001	NS	< 0.001	NS	NS	0.04	NS
Absolute liver weight (g)	< 0.001	NS	< 0.001	NS	< 0.001	NS	NS
Relative liver weight (%)	< 0.001	NS	< 0.001	NS	< 0.001	NS	NS
Hep. nuclear size (μm^2)	< 0.001	NS	< 0.001	0.03	0.003	NS	NS
Albumin (g/L)	< 0.001	NS	< 0.001	NS	0.002	NS	NS
Total protein (g/L)	NS	NS	0.001	NS	NS	NS	NS
AST[1] (U/L)	0.04	NS	NS	NS	0.03	NS	NS
ALT[2] (U/L)	NS	NS	NS	NS	0.04	NS	NS
γ-GT[3] (U/L)	< 0.001	0.04	NS	0.002	< 0.001	NS	NS
Glucose (mmol/L)	< 0.001	NS	0.02	NS	NS	NS	NS
Total cholesterol (mmol/L)	< 0.001	NS	NS	NS	0.001	NS	NS
Triglyceride (mmol/L)	< 0.001	NS	< 0.001	NS	< 0.001	NS	NS

[1]Aspartate aminotransferase; [2]Alanine aminotransferase; [3]γ-Glutamyltransferase; NS = not significant

Table 5a. Comparison of performance and blood chemistry data (mean ± SD) in quail from generation 0 fed on Bt or isogenic control maize, respectively, vs. two commercial reference diets (REF1, 2)

	Bt		ISO		REF1		REF2	
	Males n = 30	Females n = 30	Males n = 30	Females n = 30	Males n = 30	Females n = 30	Males n = 30	Females n = 30
Body weight 6wk (g)	143 ± 15.0^{a}	169 ± 21.2^{a}	145 ± 14.1^{ab}	180 ± 17.6^{ab}	147 ± 15.0^{b}	182 ± 18.6^{b}	149 ± 12.3^{ab}	183 ± 14.2^{ab}
Body weight 16 wk (g)	163 ± 14.9	180 ± 16.6	154 ± 10.8	182 ± 16.9	160 ± 14.4	187 ± 18.0	166 ± 17.1	188 ± 15.0
Absolute liver weight (g)	2.6 ± 0.4	6.4 ± 1.1	2.6 ± 0.4	5.8 ± 1.1	2.5 ± 0.5	6.1 ± 1.0	2.3 ± 0.4	6.1 ± 0.8
Relative liver weight (%)	1.6 ± 0.2^{a}	3.6 ± 0.6^{a}	1.7 ± 0.3^{a}	3.2 ± 0.6^{a}	1.6 ± 0.3^{ab}	3.3 ± 0.6^{ab}	1.4 ± 0.2^{b}	3.3 ± 0.5^{b}
Hep. nuclear size (µm^{2})	18.1 ± 1.2^{a}	20.6 ± 2.1^{a}	17.1 ± 1.3^{b}	19.2 ± 2.0^{b}	17.7 ± 1.2^{ab}	19.7 ± 1.8^{ab}	17.4 ± 1.4^{b}	19.7 ± 1.5^{b}
Albumin (g/L)	13.3 ± 1.7	16.5 ± 2.2	13.5 ± 2.0	16.3 ± 2.3	14.1 ± 1.6	16.8 ± 2.6	14.0 ± 1.6	15.4 ±1.3
Total protein (g/L)	29.0 ± 3.3	38.6 ± 5.1	40.2 ± 55.2	38.2 ± 5.7	29.5 ± 3.6	36.3 ± 9.5	29.3 ± 3.0	34.5 ± 2.8
AST[1] (U/L)	493 ± 134^{a}	421 ± 362^{a}	419 ± 105^{a}	467 ± 174^{a}	377 ± 83.8^{b}	332 ± 114^{b}	407 ± 85.3^{ab}	369 ± 178^{ab}
ALT[2] (U/L)	14.8 ± 6.3^{a}	4.8 ± 6.4^{a}	13.6 ± 6.6^{a}	6.2 ± 6.8^{a}	9.9 ± 2.7^{b}	3.6 ± 3.3^{b}	10.5 ± 1.8^{ab}	3.7 ± 3.1^{ab}
γ-GT[3] (U/L)	1.0 ± 0.2^{a}	2.0 ± 1.7^{a}	1.0 ± 0.0^{b}	1.2 ± 0.5^{b}	1.0 ± 0.1^{ab}	1.3 ± 1.3^{ab}	1.0 ± 0.1^{b}	1.0 ± 0.1^{b}
Glucose (mmol/L)	10.6 ± 9.2^{a}	10.1 ± 7.6^{a}	9.7 ± 9.9^{b}	4.1 ± 7.0^{b}	19.0 ± 2.3^{c}	17.3 ± 2.0^{c}	18.3 ± 2.1^{c}	16.4 ± 1.5^{c}
Total cholesterol (mmol/L)	6.0 ± 1.0	8.1 ± 2.5	5.5 ± 1.0	7.2 ± 2.8	5.9 ± 1.2	8.6 ± 3.1	6.4 ± 0.8	7.0 ± 1.2
Triglyceride (mmol/L)	2.7 ± 0.9	30.0 ± 14.6	2.2 ± 0.8	24.0 ± 11.0	3.1 ± 4.3	34.4 ± 53.3	2.8 ± 1.6	24.8 ± 6.9

Means within rows with different superscript letters indicate significant differences between feeding groups

Table 5b. Statistical results

	p		
	Group	Sex	Group x Sex
Body weight 6wk (g)	< 0.001	NS	< 0.001
Body weight 16 wk (g)	NS	< 0.001	NS
Absolute liver weight (g)	NS	< 0.001	NS
Relative liver weight (%)	0.025	< 0.001	0.041
Hep. nuclear size (µm^{2})	0.001	< 0.001	NS
Albumin (g/L)	NS	< 0.001	NS
Total protein (g/L)	NS	NS	NS
AST[1] (U/L)	0.006	NS	NS
ALT[2] (U/L)	< 0.001	< 0.001	NS
γ-GT[3] (U/L)	0.004	< 0.001	0.01
Glucose (mmol/L)	< 0.001	0.003	NS
Total cholesterol (mmol/L)	NS	< 0.001	0.043
Triglyceride (mmol/L)	NS	< 0.001	NS

[1]Aspartate aminotransferase; [2]Alanine aminotransferase; [3]γ-Glutamyltransferase; NS = not significant

4. MANUSCRIPT 3 (submitted to: Poultry Science 2010)

Effects of an active immunization on the immune response of laying Japanese quail (*Coturnix coturnix japonica*) fed with or without genetically modified *Bacillus thuringiensis*-maize

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ABSTRACT Potentially adverse effects of diets containing transgenic plants are a concern for many consumers, particularly in Europe. For *Bacillus thuringiensis* (Bt)-maize, a number of studies in livestock and poultry showed that the zootechnical data provide no indication for such adverse effects. These studies were all done in homeostatic situations; it remained open whether a deflection of the regulatory physiological systems might yield divergent dynamic responses in Bt-maize fed animals. We therefore tested the effect of an active immunization using Bovine Serum Albumin (BSA) as antigen in feeding regimen with or without Bt-maize using quail as a model organism.

Newly hatched Japanese quail were randomly allocated to two groups (n=120 per group) fed with diets containing either Bt-maize or isogenic maize (ISO) of the same cultivar. After 16 wk on the experimental diets, one half of each group was immunized against BSA. The remaining animals were injected with saline. Thirty-six h after the injection, half of the BSA injected subgroup (n=30) and half of the saline subgroup (n=30) from Bt-maize and isogenic fed animals were killed, blood samples were collected and analyzed for serum zinc levels, indicative for acute phase response. For determining IgY mediated immune responses, eggs were collected biweekly for 6 wk after the injections from the remaining birds and total IgY concentrations and BSA-specific IgY titers were measured in egg yolk.

The BSA injections did not elicit significant decreases of serum zinc concentrations. Expectedly, total IgY as well as BSA-specific IgY titers increased with time in the BSA-immunized quail. The response of both variables to the BSA injection did not differ between the feeding groups. Our results indicate that feeding of Bt-maize does not impair the immune system of Japanese quail and thus gives no indication for respective concerns.

Key words: active immunization, Bt-maize, immune response, quail

INTRODUCTION

Interest in genetically modified crops is continuously increasing due to the possibility of higher yields with more efficient cultivation without the use of pesticides. Although several studies have been conducted to evaluate the safety of feeding genetically modified crops (Flachowsky et al., 2005a), potentially adverse effects of diets containing transgenic plants are still a concern for many consumers, particularly in Europe. For *Bacillus thuringiensis* (Bt)-maize, a number of studies in livestock and poultry is available, showing that zootechnical data provide no indication for such adverse effects (Flachowsky et al., 2007). These studies were all done in homeostatic situations; it remained open whether setting a stimulus to the regulatory physiological systems might yield divergent dynamic responses in Bt-maize fed animals when compared with animals fed without transgenic feed stuffs. The dynamic response of the immune system is important in protecting animals from infectious diseases and facilitating cell-mediated immune responses to clear pathogens. Antibodies are one group of humoral mediators that accomplish immune responses. Immunoglobulin gamma (IgG) is the major class of antibodies produced and the primary antibody circulating in the blood system (Wang et al., 2004). In laying hens, IgG is efficiently transferred across the follicular epithelium of the ovary and accumulated in the yolk during oogenesis (Rose and Orlans, 1981). The concentration of egg yolk IgG, which is more commonly called IgY (Leslie and Clem, 1969), is higher than that in the serum (Rose et al., 1974; Larsson et al., 1993). The use of yolk antibodies instead of serum is very attractive because these antibodies can be measured by simple collection of eggs without restraining the birds for blood sampling (Schade et al., 1996). Japanese quail was used as a model organism; it is well adapted to laboratory conditions and possesses several advantages, such as rapid growth, early sexual maturity, high rate of egg production, and easy handling of adult size (Ichilcik & Austin, 1978).

In addition to the specific immune response, non-specific defence mechanisms play an important role in protecting the organism when exposed to infections or inflammatory agents. One part of these mechanisms is known as the acute phase response which includes, e.g., hepatic synthesis of acute phase proteins and

alterations in trace mineral metabolism (Klasing et al., 1987). Changes in trace mineral metabolism are usually reflected in increased serum Cu and decreased serum Fe and Zn levels. Decreased serum Zn levels apparently result from the synthesis of the acute phase protein metallothionein in liver and other tissues (Klasing, 1988). Thus, serum Zn is a useful indicator of the acute phase response (Koutsos & Klasing, 2001).

The aim of the present study was to determine the effect of an active immunization with bovine serum albumin (BSA) on the specific immune defence and the acute phase response in feeding regimen with or without Bt-maize using quail as a model organism. Potential differences between animals fed Bt-maize or isogenic maize might also be attributable to differing contents of mycotoxins: Fungi that produce mycotoxins are frequently associated with insect damage to the plants and thus in Bt-maize variants that are protected against lepidopteran insect pests, lower mycotoxin contents can be expected (Bakan et al., 2002; Munkvold et al., 1999). To address this issue, we quantified different mycotoxins in the diets used.

MATERIALS AND METHODS

Experimental design

A total of 240 newly hatched female Japanese quail (*Coturnix coturnix japonica*) were randomly divided into two different feeding groups, fed on either an experimental diet containing transgenic (Bt)-maize or isogenic maize of the same cultivar. The animals were housed in stainless steel wire cages in groups of 40 animals. The target room temperature was initially set at 38°C and was then gradually decreased during the first 20 days of life to 20°C. The animals were initially exposed to a photo period of 24 h L that was then gradually changed to 18L:6D during the breeding phase.

Starting at six wk of age, the quail were housed individually in stainless steel wire cages (25 x 21 x 23 cm) under constant temperature (19°C ± 1°C) and 8L:16D, with the light phase starting 0745 h every day. Water and feed were available *ad libitum* throughout the experiment. The laying intensity was recorded during the laying phase from eight wk of age until study termination at 22 wk of age and was expressed as

percent eggs per hen whereby one egg per hen per day throughout the laying phase would be 100%.

Diets

Diets containing Bt-maize or isogenic maize were based on a ration containing wheat, peas, soybean extraction meal, green meal and minerals. The diets were fed to the quail in three phases: starter (d 0 to 21), grower (d 22 to 42), and adult (d 42 to end). Depending upon the phase, maize (*Zea mays*) was contained in portions of 40 to 50% (i.e., 40% in starter and 50% in grower and adult diets).

Following diet preparation, nutritional and contaminant analyses were conducted on samples of all diets at the Institute of Animal Nutrition, Friedrich-Loeffler-Institute (FLI), Federal Research Institute for Animal Health, Braunschweig, Germany. Analyses of nutrient composition included percent composition of proximates (dry matter, crude protein, crude fat, crude fiber, and crude ash), carbohydrates, amino acids, and minerals (calcium, and total phosphorus) (Table 1). Mycotoxin analysis was performed using HPLC-analysis (high performance liquid chromatography) with diode array and fluorescence detection for the investigation of deoxynivalenol (DON) and zearalenone (ZON), respectively, after clean-up of the sample extracts by using immuno-affinity columns as described by Dänicke et al. (2007). The limits of detection were 0.1 mg/kg for DON and 0.1 µg/kg for ZON, respectively.

Immunization of quail with BSA

At 16 wk of age, 60 quail per feeding group were randomly selected and immunized with 1 mg of BSA (low endotoxin; Sigma-Aldrich, St. Louis, MO, USA) by injection into the large breast muscle. BSA was dissolved in 0.9% sterile NaCl solution and Freund's complete adjuvant emulsion at a concentration of 1 mg/100 µL. The BSA immunization was conducted under the approval of the local authority committee (AZ 8.87-50.10.45.08.015). The control groups consisted of the other half of the quail from each feeding group and received 100 µL saline solution in the breast muscle, in order to discern whether the handling of the animals *per se* affects the immune response. 36 h after injection, half of the BSA immunized group (n=30) and half of the saline group (n=30) from both feeding groups were killed by cervical dislocation

and subsequently decapitated for blood sampling. After overnight clotting at 4°C, serum was obtained after centrifugation at 4,000 x *g* for 20 min and stored at -20°C until use.

Eggs were collected from the remaining birds (n=60/feeding group) on day 0, prior to the injection, as well as 2, 4 and 6 wk thereafter.

Preparation of the Water-Soluble Fraction from Egg Yolk

The water-soluble fraction (WSF) from egg yolk containing IgY, was prepared as described by Wang et al. (2004) with minor modifications. After separation from the albumen using a small spoon, the egg yolk was gently rolled on a paper towel to remove the attached albumen. The yolk membrane was pierced with a pipette and the yolk aspirated. After recording the yolk volume, it was diluted (1:7, vol/vol) with acidified deionized water (pH 2.5), mixed well and kept at 4°C overnight. The solution was centrifuged at 12,000 x *g* at 4°C for 15 min, and the supernatant (WSF) was stored at -20°C until analysis for total IgY and BSA-specific IgY titers.

Enzyme-linked immunsorbent assays (ELISA) for total IgY and for BSA-specific IgY titers

Total IgY. IgY concentrations in WSF were analyzed using an in-house competitive ELISA. Microtiter plates (Corning Incorporated, Lowell, MA, USA) were coated with 10 ng quail IgY/well (Alpha Diagnostic International, San Antonio, TX, USA) in 100 µL coating buffer (0.05 M carbonate buffer, pH 9.6). After incubation overnight at 4°C in a moist chamber, the plates were blocked with 2.5% casein (pH 7.3; 300 µL/well) for 2 h at room temperature. The wells were washed five times with 300 µL PBS, 0.05% Tween20 (AppliChem GmbH, Darmstadt, Germany) and were then stored at -20°C until use.

WSF was diluted 1:5,000 with assay buffer (0.12 M NaCl, 0.02 M Na_2HPO_4, 0.01 M EDTA, 0.005% Chlorhexidine, 0.1% Gelatin, 0.05% Tween20, pH 7.3). As standard series, quail IgY was used in 6 dilutions covering a range of 0.64 to 2000 ng/mL. The prediluted standard series and the WSF samples were added to the wells (50 µL/well). A pooled WSF sample was used as control for assay variation. Fifty µL of peroxidase-conjugated rabbit anti-chicken IgY antibody (0.1 µg/mL; Sigma, St. Louis,

MO, USA) were dispensed into each well and incubated at room temperature for 2 h. The wells were washed five times as described above, followed by the addition of 140 µL of substrate solution containing 0.05 M citric acid, 0.055 M Na_2HPO_4, 0.05% urea hydrogen peroxide, and 2% of a tetramethylbenzidine solution (12.5 mg/mL dimethyl sulfoxide). The reaction was stopped after 30 min incubation by adding 50 µL of 1 M oxalic acid and the absorbance was determined at 450 nm using an ELISA microplate reader (EL800, Bio-Tek Instruments, Winooski, VT, USA). The cross-reactivity of the antibody against chicken IgY with quail IgY was initially tested: analogous dilutions of chicken IgY (Rockland, Gilbertsville, PA, USA) yielded identical binding curves indicating a 100% crossreactivity and thus confirming the applicability of this antiserum for quail. The recovery of quail IgY spiked into WSF was 99.5%. The detection limit of the total IgY assay was 0.003 µg/mL, the intra and inter assay coefficients of variation were 4.5% and 5.2%, respectively.

***BSA-specific antibody IgY titers*.** Microtiter plates were coated with 100 µL of BSA solution (10 µg/mL coating buffer) to each well. Blocking was done as described for the total IgY ELISA. The samples were incubated for 2 h at room temperature in log_2 series [each 100 µL per well; in assay buffer (0.12 M NaCl, 0.02 M Na_2HPO_4, 0.01 M EDTA, 0.005% Chlorhexidine, 0.1% Gelatine, 0.05% Tween 20, pH 7.3)] starting at a dilution of 1:50 and ending at 1:51,200. Subsequently, plates were washed three times and binding of specific antibodies to the immobilized antigen was determined by 1 h incubation at room temperature with a peroxidase-conjugated rabbit anti-chicken IgY antibody (0.16 µg/mL; 100 µL/well; Sigma, St. Louis, MO, USA), followed by five times washing. All washings, substrate reaction and readings were done as described above for total IgY. The antibody titers were obtained using the Gen 5.0 software (Bio-Tek Instruments, Winooski, VT, USA). Positive titers were defined at the dilution for which the optical density measured could be differentiated from the background, i.e. at 0.05 OD. The intra and inter assay coefficients of variation were 4.9% and 10.0%, respectively.

Serum Zinc

Serum zinc concentrations were analyzed by a colorimetric method (WAKO Chemicals GmbH, Neuss, Germany) on a Dimension RxL Max (Siemens Healthcare

Diagnostics, Eschborn, Germany) at the Institute of Clinical Chemistry and Pharmacology, University Hospital Bonn, Germany. The detection limit of the zinc assay was 10 µg/dL, the intra-assay coefficient of variation was 3.2%, and the interassay coefficient of variation was 3.4%.

Statistical analysis

Statistically comparisons were designed to determine whether differences were attributable to consumption of diets produced with Bt-maize compared to diets containing isogenic maize. All analyses were carried out using SPSS 17.0 software for Windows (SPSS Inc., Chicago, IL, USA). A mixed model with a post hoc Bonferroni correction was performed for analyzing total IgY, BSA-specific IgY, and laying intensity; fixed effects included time of sampling, feeding group, immunization mode, and their respective interactions. An univariate analysis of variance (ANOVA) with a post hoc Bonferroni correction was performed for analyzing serum zinc levels; feeding group, injection mode, and the interaction term were considered to be fixed factors. Differences were considered statistically significant, at $p \leq 0.05$.

RESULTS AND DISCUSSION

In the present study, we evaluated as to whether transgenic Bt-maize consumption might affect the immune response as compared to its isogenic control maize, by considering the specific and non-specific immune response of laying Japanese quail. Feed analyses yielded similar nutrient contents (Table 1) and no differences in the concentrations of the F*usarium* toxins ZON and DON between the Bt and the isogenic diets; concentrations of DON and ZON were consistently lower than the guidance values for critical concentrations of complete diets (EU-Commission, 2006) (Table 2). Although the pure maize batches were not analysed for mycotoxins, it can be further deduced that the DON and ZON concentrations were by far lower than the corresponding guidance values for cereal grains when the maize proportions of 40 to 50 % of the complete diets were considered. DON might exert immuno-modulating effects especially in mice and rats (Rotter et al., 1996), but such influences can

presumably be excluded for the present experiment in view of the low DON concentrations and the negligible differences between both experimental diets.

Throughout the laying phase, the laying intensity ranging from 70.2 to 79.0%, was neither influenced by feeding group nor by injection with NaCl or BSA, respectively (Figure 1). Similarly, feeding of Bt-maize did not affect the laying intensity in a ten-generation study with laying quail (Flachowsky et al., 2005b) and in a four-generation study with laying hens (Halle et al., 2006). The majority of feeding studies with Bt-maize gave no indication for adverse health effects when considering nutritional assessment and performance variables (Aumaitre et al., 2002, Clark & Ipharaguerre, 2001). Only few publications report effects of feeding transgenic crops on animal health when considering more detailed pathophysiological trials (Hammond et al., 2006a,b; EFSA, 2005a,b). Regardless of the target variables investigated and the lack or presence of Bt-attributable effects, it must be noted that all studies were done in homeostatic situations. To our knowledge no publications exist investigating whether challenging the regulatory physiological systems might yield divergent dynamic responses in animals fed diets containing transgenic crops. To evaluate the effect of Bt-maize feeding on the immune response of Japanese quail, both unspecific (serum Zn) and specific, antibody-mediated responses to BSA were assessed. The changes of total IgY in egg yolk are shown in Figure 2. Expectedly, an activation of the immune system was indicated by increasing total IgY concentrations with time in the BSA-immunized quail, reaching a plateau at 4 wk post injection. Total IgY concentrations in BSA-immunized quail differed significantly from NaCl-injected control animals without differences between feeding groups. However, the antibody response to BSA was affected by a time point of sampling x feeding interaction. Total IgY concentration varied with time between feeding groups showing significantly higher values in the Bt-group at time point 0. At wk 2 and 4 post injection, total IgY concentrations were higher in control animals, and equal concentrations were observed at study termination. An interaction was also recorded for time point of sampling x injection mode with total IgY concentrations being higher in BSA-immunized quail throughout the different time points of sampling.

Similarly, immunization with BSA resulted in the appearance of specific anti-BSA antibodies in egg yolk, which gradually increased until study termination (Figure 3). The response of BSA-specific IgY titers to the BSA injection was not affected by the feeding treatment. As expected, BSA-specific IgY titers in NaCl-injected control animals were below the limit of detection.

For serum zinc, no alterations related to the immunization against BSA were detectable. Decreasing serum Zn is a component of the acute phase response resulting from an increase in metallothionein synthesis in the liver and other tissues (Klasing, 1984). Zinc concentrations are known to be decreased maximally by 52 % at 12 h and 15.7 % at 48 h after the injection with inflammatory stimuli (Klasing, 1984). Serum zinc values were not affected by the injection mode in the present study, indicating that a single intramuscular immunization with BSA was not effective eliciting an acute phase response in Japanese quail. The serum Zn concentrations in the quail used herein were about 3-fold higher than reported previously for quail (Sahin & Kucuk, 2003; Sahin et al., 2006) and for various psittacine species (Puschner et al., 1999). When compared to broiler chicken (Herzig et al., 2009), the serum Zn concentrations we observed in quail were even 6-fold higher. These differences might be attributable to the different methods used. However, the variation within the Zn concentrations we recorded herein (coefficient of variation: 22.5 %) was relatively smaller than reported for normal values in broiler chicken 43.5 % (Herzig et al., 2009) and in psittacines 26.7 % (Puschner et al., 1999), respectively. When pooling the BSA and saline injected quail within the Bt-maize and the isogenic feeding group, the Zn concentrations were lower ($p < 0.01$) in the isogenic fed animals (594 ± 22.4 µg/dL) than in the Bt group (674 ± 17.8 µg/dL) and amounted to 88 % of the concentrations recorded in the Bt group mode (Figure 4). This difference is low compared to the alterations of serum Zn reported for acute phase responses in poultry with reductions to 60 % of the serum Zn values recorded in control animals (Baert et al., 2005; Takahashi et al., 1995). Possible causes for the higher Zn serum concentrations in Bt fed quail might rather be attributable to differences in feed intake and/or Zn content of the diets than to immune mediated regulatory processes. Most of Zn contained in the diets was probably provided by the premix included and the Zn content in the maize portion thus is neglible albeit

differing concentrations might occur in Bt versus isogenic maize as indicated by differences in lignin content (Poerschmann et al., 2005). However, comparisons of insect-protected and glycophosphate-tolerant maize with their isogenic counter parts yielded no differences in Zn contents (McCann et al., 2007). Accordingly, the difference in serum Zn concentrations between the Bt and the isogenic group presumably reflects random differences in Zn intake and probably ranges within the normal variation of Zn serum concentrations in Japanese quail. In addition, the observation that Zn concentrations in the Bt group were slightly higher implies that consumption of Bt maize per se does not impose inflammatory stimuli.

In conclusion, our results indicate that feeding of Bt-maize does not impair the laying intensity, as well as the specific and non-specific immune response of Japanese quail and thus gives no indication for respective concerns. The absence of meaningful differences in the measured parameters provides support for the equivalence of Bt-maize and isogenic control maize. These observations done in our quail study are of general importance not only for avian species but for animal science in general.

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Table 1. Nutrient composition of starter, grower and adult diets containing Bt or isogenic control (ISO) maize, respectively.

	Starter diet (d 0-21)		Grower diet (d 22-42)		Adult diet (d > 42)	
	Bt	ISO	Bt	ISO	Bt	ISO
Dry matter (g/kg)	880	882	875	877	880	883
Crude protein (%)	28	28	21.0	21.0	15.5	15.5
Crude ash (%)	7.15	7.16	5.65	5.67	12.0	11.6
Crude fiber (%)	5.06	4.94	4.33	4.23	4.08	4.07
Crude fat (%)	4.39	4.35	2.47	2.54	2.04	2.02
Starch (%)	26.9	26.7	40.6	40.5	43.4	43.5
Glucose (%)	5.01	4.96	3.72	3.65	2.78	2.71
Calcium (%)	1.2	1.2	1.1	1.1	3.98	3.84
Phosphorus (%)	0.9	0.9	0.7	0.7	0.45	0.45
Lysine (%)	1.65	1.65	1.3	1.3	0.68	0.68
Methionine (%)	0.5	0.5	0.4	0.4	0.34	0.34
ME (kcal/kg)	2,605	2,605	2,700	2,700	2,557	2,557

Table 2. Deoxynivalenol (DON) and zearalenone (ZON) concentrations (means ± SD) in Bt or reference control maize adult diets (ISO)

			Guidance values for critical concentrations (EU-Commission, 2006)	
	Bt n = 5	ISO n = 7	In complete feeds for poultry[1]	In maize
DON mg/kg	0.66 ± 0.64	0.97 ± 0.73	5.0	8.0
ZON mg/kg	0.02 ± 0.01	0.03 ± 0.02	-[2]	2.0

[1] Farm poultry

[2] No guidance value necessary according to the present knowledge

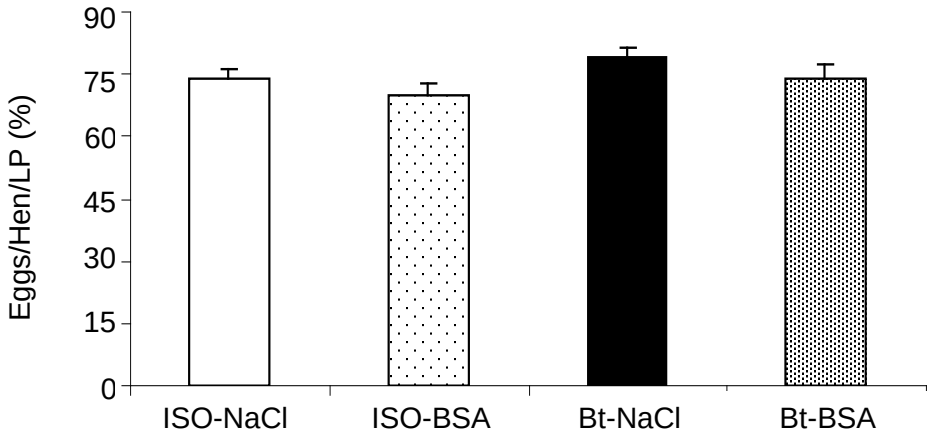

Figure 1. Percent eggs per hen (means ± SEM) in quail fed Bt-maize or isogenic control maize, injected with NaCl or BSA, respectively.

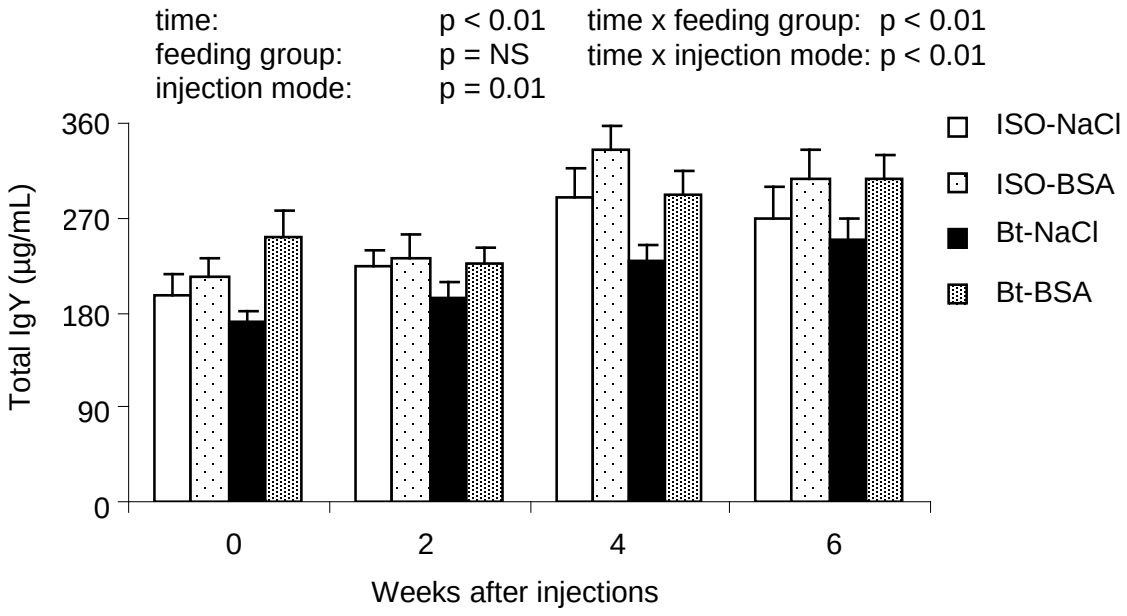

Figure 2. Total IgY concentrations (means ± SEM) in WSF from quail fed on Bt or isogenic control maize, injected with NaCl or BSA, respectively.

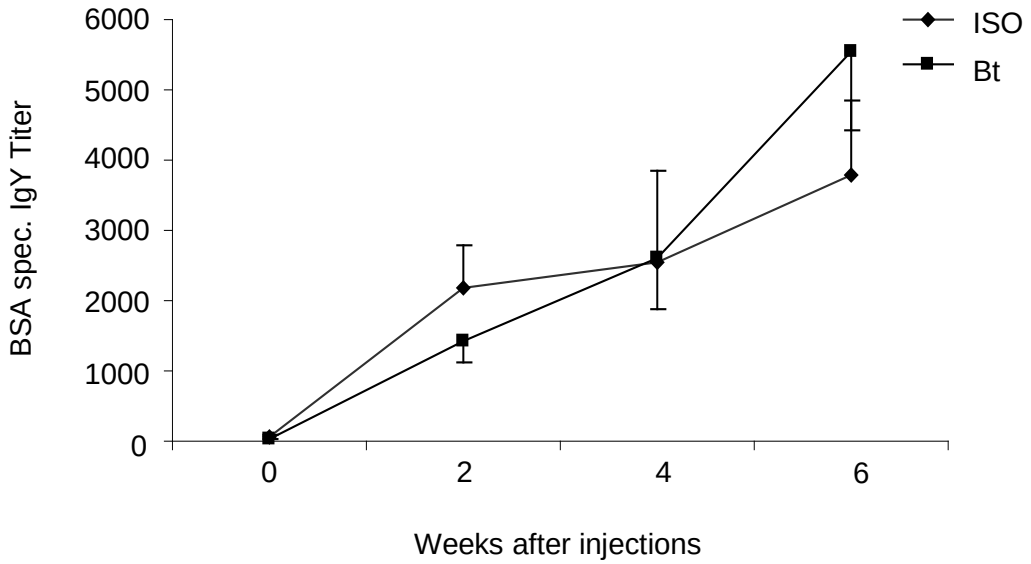

Figure 3. BSA-specific IgY titers (means ± SEM) after injection of BSA in egg yolk from quail fed on diets containing Bt or isogenic control maize, respectively. BSA-specific IgY titers in NaCl-injected control animals remained below the limit of detection throughout the study.

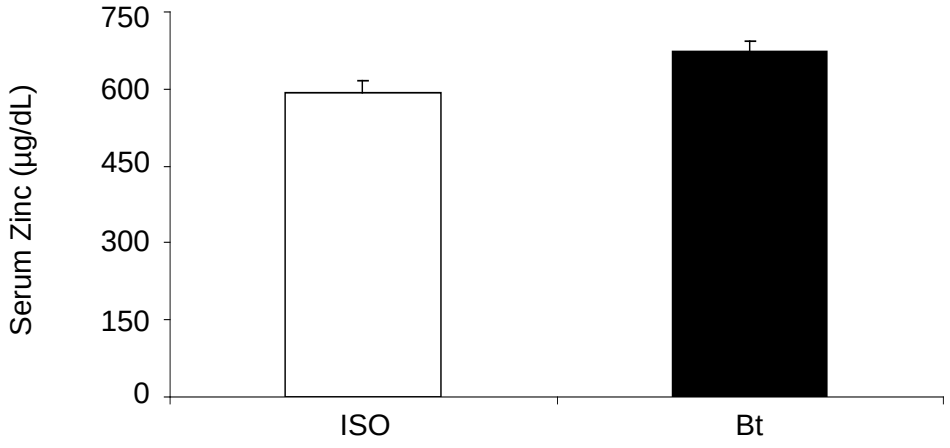

Figure 4. Serum zinc concentrations (means ± SEM) in quail fed on Bt or isogenic control maize, respectively.

5. CONCLUSIONS

The aim of this dissertation was to investigate the effect of diets containing maize with the Cry1Ab trait versus non-biotech counterparts in Japanese quail in a multigenerational comparison. Until now, long-term studies were limited to nutritional assessments and evaluation of performance; the effect of mid- or long-term exposure to Bt-maize varieties has rarely been investigated with respect to more detailed pathophysiological trials.

When considering potentially adverse effects on health exerted by xenobiotic agents, it is essential to take the physiological status of the observed organism into consideration which is at least partially reflected in serum chemistry values. The serum chemistry reference values established throughout this dissertation can be considered as representative values for Japanese quail because the number of animals used herein clearly exceeded the limit of 40 animals required to estimate the 2.5^{th} and 97.5^{th} percentiles.

According to the observations conducted throughout the multigenerational comparison and the evaluation of the immune response, the present studies contribute to the general discussion of using genetically modified crops in animal nutrition, since neither consistent nor analogous long-term effects were recorded when observing serum chemistry values and liver histology. In addition, Bt-maize feeding did not impair the specific and non-specific immune response of Japanese quail.

These conclusions concur with numerous observations in previous short-term studies, indicating that animal health is not affected by feeding of genetically modified crops. The lack of obvious adverse effects in these quail studies is of general importance to animal science and provides support for the comparability of Bt-maize to conventional reference maize varieties in terms of animal health.

Nevertheless, further research on the safety of genetically modified crops should be carried out, especially in terms of environmental safety concerning the potential impact on farmland insect wildlife. In addition, the dependence of farmers from seed companies when using genetically modified seeds and plants needs careful consideration.

Altogether, the most important factor for the acceptance and the use of genetically modified crops, particularly in Europe where consumers express major concerns, is to provide adequate information about the background and the intention of genetically modified crops, and to increase transparency on production methods, enabling the consumer to get a better understanding and to form an opinion which is science based and not vectored by the different stake holders involved.

6. SUMMARY

In recent years, there has been a rapid global increase in the use of transgenic crops. Although several studies have been conducted to evaluate the safety of genetically modified crops, potentially adverse effects of diets containing transgenic plants are a concern for many consumers, particularly in Europe. For *Bacillus thuringiensis* (Bt)-maize, a number of studies in livestock and poultry is available, showing that both nutritional assessment as well as performance data give no indication for such adverse effects. Even long-term studies in birds and rats did not reveal significant influences on animal development after feeding of genetically modified Bt-maize. Nevertheless, the evidence is still far from proving, whether the mid- or long-term exposure to Bt-maize varieties implies a possible danger for animal health, especially when investigating more detailed pathophysiological traits in multigenerational comparisons.

The aim of this dissertation was to advance the ongoing debate as to whether health is affected by the intake of diets containing maize with the Cry1Ab trait in comparison to non-biotech counterparts. For this purpose, three different experimental approaches have been conducted using Japanese quail as a model organism:

Even so the Japanese quail has been widely used as a laboratory animal for the past 50 years, to date, the available array of serum chemistry reference values for this species was limited. Serum chemistry reference values may provide useful information about the physiological condition of individuals, making them a useful tool in differentiating normal and healthy animals from abnormal or diseased states, e.g. when responses to specific treatments have to be monitored and appraised. Thus, clinical chemistry data [albumin, total protein, glucose, uric acid, cholesterol, bilirubin, cholinesterase, creatinine, triglycerides, alanine aminotransferase (ALT), aspartate aminotransferase (ALT), and γ-glutamyltransferase (γ-GT)] from up to 125 male and 151 female 16-wk-old Japanese quail were established. Statistical comparisons were made between male and female birds.

AST, ALT, glucose, cholinesterase, and bilirubin values were higher ($p < 0.01$) in males, whereas females had higher ($p < 0.05$) concentrations of albumin, total

protein, γ-GT, total cholesterol, and triglycerides. No significant sex-based differences were observed for creatinine and uric acid.

These newly established reference ranges provided the opportunity to interpret serum chemistry values in a following multigenerational comparison with quail fed diets containing either genetically modified *Bacillus thuringiensis*-maize (Bt), or isogenic maize (ISO) of the same cultivar for up to 20 generations. At 16 wk of age, a total of 140 male and 140 female animals were sacrificed, blood samples were analyzed for serum biochemistry and in addition, liver tissue was histomorphometrically evaluated. In multigenerational comparisons, genetic drift might be an issue and thus, the examinations were extended to animals fed diets containing Bt-maize or isogenic maize in first generation (generation 0) (n=30/feeding group and sex). To clarify the biological range of all variables tested, further controls with two different isogenic hybrid reference maize cultivars (REF1, REF2) (n=30/feeding group and sex) were included.
Expectedly, sex differences were observed for most of the recorded variables. Comparing Bt-maize fed animals versus quail fed the non-transgenic control diets, differences were observed for body weight at 6 wk of age as well as for the enzyme activity of γ-GT from generation 0 and generations F17 to F20. Changes in these parameters were also determined when comparing animals from Bt and ISO vs. REF groups. Comparing quail from generation 0 with REF1 and 2, differences in body weight at 6 wk of age, relative liver weight, hepatocyte nuclear size, AST, ALT, γ-GT, and glucose were observed for Bt vs. ISO and REF as well as ISO vs. REF comparisons.
The statistical differences observed throughout the study were thus not limited to Bt vs. ISO, or REF comparisons, they were neither consistent nor analogous and thus give no indication for targeted pathophysiological alterations induced by Bt-maize feeding. These findings are consistent with numerous studies observing the effects of feeding diets containing genetically modified maize.

To date, these studies were all done in homeostatic situations; it remained open whether a deflection of the regulatory physiological systems might yield divergent

dynamic responses in Bt-maize fed animals. Thus, the effect of an active immunization against bovine serum albumin (BSA) was tested in feeding regimen including Bt or isogenic maize. After 16 wk on the experimental diets, one half of each feeding group was immunized intramuscularly with BSA. The remaining animals were injected with saline. Thirty-six h after the injection, half of the BSA immunized group (n=30) and half of the saline group (n=30) from both feeding groups were sacrificed, blood samples were collected and analyzed for serum zinc levels, indicative for acute phase response. For determining IgY mediated immune responses, eggs were collected biweekly for 6 wk after the injections from the remaining birds and total IgY concentrations and BSA-specific IgY titers were measured in egg yolk using in-house ELISAs.

For serum zinc, no alterations related to the immunization against BSA were detectable. When pooling the BSA and saline injected quail within the Bt-maize and the isogenic feeding group, the Zn concentrations were lower ($p < 0.01$) in the ISO animals (594 ± 22.4 µg/dL) than in the Bt group (674 ± 17.8 µg/dL) and amounted to 88 % of the concentrations recorded in the Bt group mode.

Expectedly, total IgY as well as BSA-specific IgY titers increased with time in the BSA-immunized quail. The response of both variables to the BSA injection did not differ between feeding groups. The results indicate that feeding of Bt-maize does not impair the immune system of Japanese quail and thus gives no indication for respective concerns.

Due to the fact that potential differences between animals fed Bt-maize or isogenic maize of the same cultivar might also be attributable to differing contents of mycotoxins and variations in nutritional composition, nutritional and contaminant analyses were conducted in all feeds used throughout these experiments. Feed analyses yielded similar nutrient contents and no differences in the concentrations of the F*usarium* toxins zearalenone (ZON) and deoxynivalenol (DON) between the Bt and the isogenic diets; concentrations of DON and ZON were consistently below the limits of acceptance of the European Commission.

In conclusion, the present dissertation contributes to the general discussion of using genetically modified crops in animal nutrition, since neither consistent nor analogous long-term effects were recorded throughout this multigenerational comparison which is consistent with numerous observations in previous short-term studies. The lack of obvious adverse effects in these quail studies is of general importance to animal science and provides support for the comparability of Bt-maize to conventional reference maize varieties in terms of animal health.

7. ZUSAMMENFASSUNG

Der Einsatz von gentechnisch verändertem Getreide hat in den vergangenen Jahren weltweit zugenommen. Obwohl die Produktsicherheit dieser transgenen Getreide in zahlreichen Studien untersucht wurde, bestehen auf Seiten der Verbraucher, insbesondere in Europa, erhebliche Bedenken in Bezug auf potentiell negative Effekte von Nahrungs- und Futtermitteln, die gentechnisch veränderte Pflanzen oder Pflanzenteile enthalten. Etliche Studien mit Nutztieren und Geflügel, deren Fütterung mit *Bacillus thuringiensis* (Bt)-Mais erfolgte lieferten keine Hinweise auf nachteilige Effekte hinsichtlich der Nährstoffgehalte sowie der Leistungsdaten. Auch in Langzeitstudien mit Geflügel und Ratten wurden keine signifikanten Einflüsse auf die Entwicklung der Tiere in Folge der Fütterung von gentechnisch verändertem Bt-Mais erkennbar. Bislang bleibt jedoch ungeklärt, ob die mittel- bis langfristige Aufnahme von Bt-Mais-Sorten eine mögliche Gefahr für die Gesundheit von Tieren birgt; insbesondere bei der Betrachtung von umfassenderen pathophysiologischen Merkmalen in Mehrgenerationenversuchen.

Ziel dieser Dissertation war es, die anhaltende Debatte, ob die Aufnahme von Futtermitteln mit Cry1Ab Mais im Vergleich zu isogenem Mais Auswirkungen auf die Gesundheit hat, voranzutreiben. Für diesen Zweck wurden drei verschiedene Versuchsansätze realisiert, bei denen jeweils die Japanische Wachtel als Modellorganismus genutzt wurde:

Die Japanische Wachtel findet zwar seit über 50 Jahren Verwendung als Labortier, trotzdem sind nur begrenzt Referenzwerte für klinisch-chemische Parameter dieser Spezies verfügbar. Klinisch-chemische Parameter liefern nützliche Informationen über den physiologischen Zustand von Individuen und dienen so der Unterscheidung zwischen normalen und gesunden von abnormalen oder erkrankten Organismen, z.B. bei der Überwachung und Beurteilung von Reaktionen auf spezifische Behandlungen. Aufgrund dieser Tatsache erfolgte die Ermittlung klinisch-chemischer Referenzwerte [Albumin, Gesamtprotein, Glukose, Harnsäure, Cholesterin, Bilirubin, Cholinesterase, Kreatinin, Triglyzeride, Alanin-Aminotransferase (ALT), Aspartat-Aminotransferase (AST) und γ-Glutamyltransferase (γ-GT)] von bis zu 125

männlichen und 151 weiblichen, 16 Wochen alten Japanischen Wachteln. Statistische Vergleiche wurden zwischen männlichen und weiblichen Tieren vorgenommen.
Die ALT-, AST-, Glukose-, Cholinesterase- und Bilirubin-Werte lagen bei den männlichen Tieren oberhalb den Werten der weiblichen Wachteln ($p < 0,01$), wobei die weiblichen Tiere höhere ($p < 0,05$) Konzentrationen an Albumin, Gesamtprotein, γ-GT, Cholesterin und Triglyzeriden aufwiesen. Bei Kreatinin und Harnsäure waren keine Geschlechtsunterschiede zu erkennen.

Die somit etablierten Referenzwerte ermöglichten in einem darauffolgenden Mehrgenerationenversuch mit Wachteln, deren Fütterung bis zu 20 Generationen mit *Bacillus thuringiensis*-Mais (Bt) oder isogenem Mais (ISO) erfolgte, die Interpretation klinisch-chemischer Parameter. Im Alter von 16 Wochen konnten von 140 männlichen und 140 weiblichen Wachteln Blutproben auf ihre klinisch-chemischen Parameter analysiert werden; darüber hinaus wurde die histomorphometrische Beurteilung des Lebergewebes vorgenommen. Da in Mehrgenerationenversuchen eine genetische Drift in Betracht gezogen werden muss, wurden die Untersuchungen auf Tiere ausgedehnt, deren Fütterung in der ersten Generation (Generation 0) mit Bt- oder isogenem Mais erfolgte (n=30/Fütterungsgruppe und Geschlecht). Zur Klärung der biologischen Variabilität der getesteten Parameter wurden zusätzliche Kontrollgruppen mit zwei verschiedenen isogenen Referenzmais-Sorten (REF1, REF2) (n=30/Fütterungsgruppe und Geschlecht) gefüttert und in die Untersuchung mit einbezogen.
Erwartungsgemäß konnten in der überwiegenden Anzahl der untersuchten Parameter Unterschiede zwischen den Geschlechtern beobachtet werden. Unterschiede im Vergleich zwischen transgen und isogen gefütterten Tieren zeigten sich in Bezug auf das Körpergewicht im Alter von 6 Wochen sowie die Enzymaktivität der γ-GT in Tieren der Generation 0 und den Generationen 17 bis 20. Unterschiede in diesen Parametern zeigten sich auch beim Vergleich sowohl der Bt- als auch der isogen gefütterten Tiere mit den Tieren der beiden Referenzmais-Gruppen. Bei dem Vergleich von Tieren der Generation 0 mit REF1 und REF2 traten Unterschiede im Körpergewicht im Alter von 6 Wochen, dem relativen Lebergewicht, der Größe der

Leberzellkerne, den Enzymaktivitäten der AST, ALT und der γ-GT, sowie der Glukose sowohl zwischen Bt vs. ISO und REF als auch zwischen ISO vs. REF auf.
Die statistischen Unterschiede, die im Verlauf der Untersuchungen beobachtet wurden, beschränkten sich nicht nur auf Vergleiche zwischen Bt vs. ISO, oder REF. Darüber hinaus waren sie weder konsistent noch analog und lieferten daher keinen Hinweis für gerichtete pathophysiologische, durch die Bt-Mais Fütterung ausgelöste Alterationen. Diese Beobachtungen stimmen mit zahlreichen Studien überein, welche den Effekt der Fütterung von Diäten mit gentechnisch verändertem Mais untersucht haben.

Da die bislang publizierten Studien alle in homeostatischen Situationen durchgeführt wurden, blieb bislang ungeklärt, ob eine Auslenkung der regulativen physiologischen Systeme abweichende dynamische Reaktionen in Bt-Mais-gefütterten Tieren auslöst. Diesbezüglich wurde der Effekt einer aktiven Immunisierung mit Bovinem Serum Albumin (BSA) in Fütterungsversuchen mit Bt- im Vergleich zu isogenem Mais getestet. Nach 16-wöchiger Fütterung der Versuchsrationen erfolgte die intramuskuläre Immunisierung mit BSA von jeweils der Hälfte der Tiere pro Fütterungsgruppe. Die verbleibenden Tiere erhielten eine NaCl-Injektion. Sechsunddreißig Stunden nach der Injektion erfolgte die Schlachtung von jeweils der Hälfte der BSA immunisierten Tiere (n=30) und der NaCl-Gruppe (n=30) beider Fütterungsgruppen. Blutproben wurden gewonnen und auf ihren Zinkgehalt, als Indikator der Akute-Phase-Reaktion analysiert. Zur Bestimmung der IgY-vermittelten Immunreaktion wurden für insgesamt sechs Wochen nach der Injektion, alle zwei Wochen Eidotterproben von den verbleibenden Tieren gesammelt und mit Hilfe von eigens etablierten ELISAs Gesamt-IgY sowie BSA-spezifische IgY-Konzentrationen gemessen.
Die Immunisierung mit BSA löste keine signifikante Reduktion der Zinkkonzentration im Serum aus. Jedoch konnte in der Gruppe Bt-Mais gefütterter Tiere eine 1,13-fach höhere Zinkkonzentration im Vergleich zu den isogen gefütterten Kontrolltieren ermittelt werden nachdem die Proben von BSA- und NaCl-immunisierten Tieren der jeweiligen Fütterungsgruppe gepoolt wurden.

Erwartungsgemäß stieg mit der Zeit sowohl die Konzentration an Gesamt-IgY als auch an BSA-spezifischem IgY bei den gegen BSA immunisierten Tieren an. Zwischen den Fütterungsgruppen traten keine Unterschiede in der Reaktion beider Parameter auf die BSA-Injektion auf. Die Ergebnisse dieser Untersuchung geben keine Hinweise darauf, dass die Fütterung von Bt-Mais das Immunsystem von Japanischen Wachteln beeinflusst.

Da potentielle Unterschiede zwischen transgen oder isogen gefütterten Tieren auch auf unterschiedliche Mykotoxingehalte und Variationen in der Nährstoffzusammensetzung der Futtermitteln zurückzuführen sein könnten, erfolgte zusätzlich die Analyse auf die Mykotoxine Deoxynivalenol (DON) und Zearalenon (ZON) sowie auf die Nährstoffzusammensetzung aller im Laufe dieser Versuche eingesetzten Futtermittel. Zwischen den Fütterungsgruppen traten hierbei keine Unterschiede in der Nährstoffzusammensetzung und den Konzentrationen an DON und ZON auf. Zudem lagen die Konzentrationen der Mykotoxine durchweg unterhalb den von der EU-Kommission akzeptierten Höchstmengen.

Die hier gezeigten Ergebnisse tragen zu der anhaltenden Diskussiom bezüglich des Einsatzes gentechnisch veränderter Getreide in der Tierernährung bei, da im Laufe dieses Mehrgenerationenvergleiches keine konsistenten und analogen Langzeiteffekte festgestellt werden konnten. Diese Beobachtungen stimmen mit zahlreichen bislang durchgeführten Kurzzeitstudien überein. Das Ausbleiben negativer Effekte in den an Wachteln realisierten Versuchen ist von genereller Bedeutung für die Tierwissenschaft und unterstützt die Vergleichbarkeit von Bt-Mais mit konventionellen Maissorten im Hinblick auf die Tiergesundheit.

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