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

Morten Poulsen [a,*], Stine Kroghsbo [a], Malene Schrøder [a], Andrea Wilcks [b], Helene Jacobsen [a], Andreas Miller [c], Thomas Frenzel [c], Jürgen Danier [d], Michael Rychlik [e], Qingyao Shu [f], Kaveh Emami [g], Duraialagraja Sudhakar [h], Angharad Gatehouse [g], Karl-Heinz Engel [c], Ib Knudsen [a]

[a] *Department of Toxicology and Risk Assessment, Danish Institute for Food and Veterinary Research, Mørkhøj Bygade 19, DK-2860 Søborg, Denmark*
[b] *Department of Microbiological Food Safety, Danish Institute for Food and Veterinary Research, Mørkhøj Bygade 19, DK-2860 Søborg, Denmark*
[c] *Lehrstuhl für Allgemeine Lebensmitteltechnologie, Technical University Munich, Am Forum 2, D-85350 Freising-Weihenstephan, Germany*
[d] *Bioanalytik Weihenstephan, Alte Akademie 10, D-85350, Freising-Weihenstephan, Germany*
[e] *Lehrstuhl für Lebensmittelchemie, Technical University Munich, Lichtenbergstr. 4, D-85748 Garching, Germany*
[f] *Institute of Nuclear Agricultural Sciences, Zhejiang University, Hangzhou 310029, China*
[g] *School of Biology, Institute for Research on Environment and Sustainability, University of Newcastle, Newcastle upon Tyne NE1 7RU, UK*
[h] *Tamil Nadu Agricultural University, Coimbatore 648 003, Tamil Nadu, India*

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**Abstract**

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


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

## 1. Introduction

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

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

* Corresponding author. Tel.: +45 7234 7540; fax: +45 7234 7699.
*E-mail address:* mop@dfvf.dk (M. Poulsen).

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

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

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

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

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

## 2. Materials and methods

### 2.1. Production and characterization of transgenic rice seed

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

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

### 2.2. Compositional analysis

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

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

### 2.3. Animals and housing

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

### 2.4. Experimental design

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

Table 1
Composition of diets

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

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

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

### 2.5. Blood biochemistry

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

### 2.6. Haematology

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

### 2.7. Immunology

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

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

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

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

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

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

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

### 2.8. Bacteriological quantification

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

### 2.9. Organ weights, gross necropsy and histopathology

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

### 2.10. Statistical analysis

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

## 3. Results

### 3.1. Agronomic parameters

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

### 3.2. Compositional analysis

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

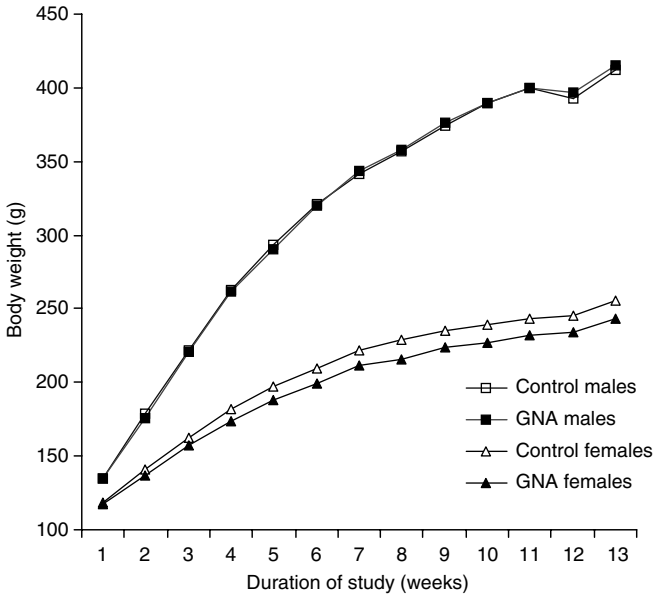


Fig. 1. Growth curves based on weekly measurements of body weight during the study. The curves show group means based on 16 rats/sex/group. SE bars not shown for clarity.

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

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

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

Table 8
Food consumption – group mean values ± SD

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

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

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

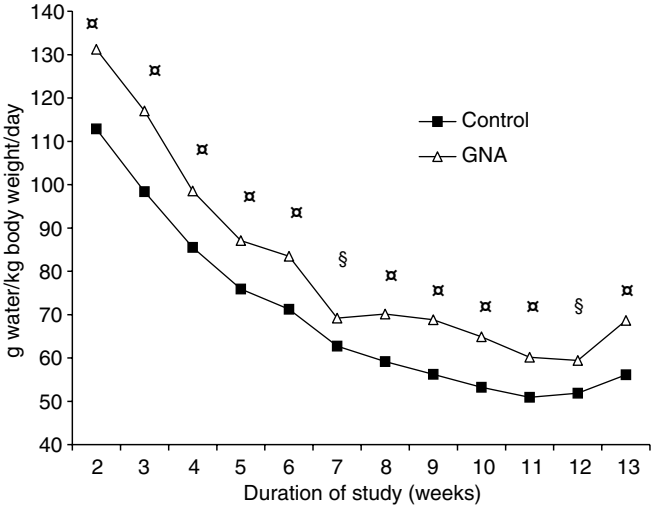


Fig. 2. Mean relative water intake of male rats. SE bars not shown for clarity. (§) and (¤) indicate significantly different from control group at $p < 0.01$ and $p < 0.001$, respectively. $n = 16$.

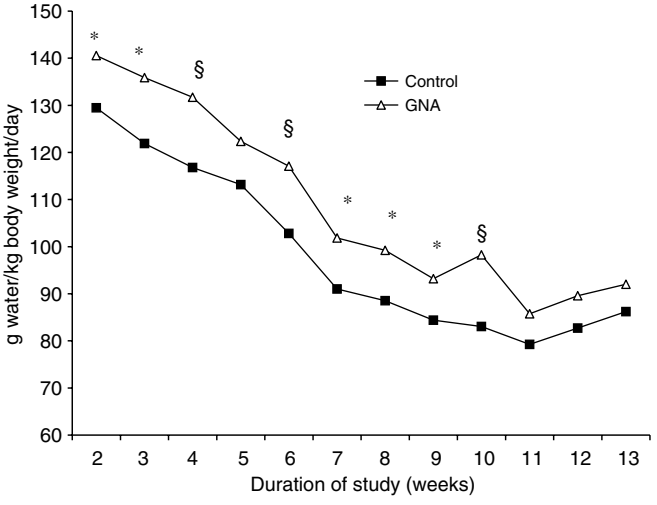


Fig. 3. Mean relative water intake of female rats. SE bars not shown for clarity. (*) and (§) indicate significantly different from control group at $p < 0.05$ and $p < 0.01$, respectively. $n = 16$.

### 3.4. Blood biochemistry

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

### 3.5. Haematology

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

### 3.6. Immunological parameters

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

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

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

### 3.7. Bacteriological quantification of faecal samples

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

Table 9
Blood biochemistry – group mean values ± SD

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

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

Table 10
Haematology – group mean values ± SD

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

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

Table 11
Immunological parameters – group mean values ± SD

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

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

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

### 3.8. Organ weights, gross necropsy and histopathology

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

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

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

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

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

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

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

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

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

Relative organ weights expressed as g/100 g body weight. Small intestinal length and relative length is expressed in cm and cm/g body weight. Data is presented as group mean values ± SD.

[a] Statistically significant different from control group ($p < 0.05$).

[b] Statistically significant different from control group ($p < 0.01$).

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

## 4. Discussion

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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