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Zebrafish (*Danio rerio*) as a model for investigating the safety of GM feed ingredients (soya and maize); performance, stress response and uptake of dietary DNA sequences

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A 20-d zebrafish (*Danio rerio*) feeding trial, in which a near doubling of fish weight was achieved, was conducted with GM feed ingredients to evaluate feed intake, growth, stress response and uptake of dietary DNA. A partial aim of the study was to assess zebrafish as a model organism in GM safety assessments. Roundup Ready® soya (RRS®), YieldGard® Bt maize (MON810) and their non-modified, maternal, near-isogenic lines were used in a 2 × 2 factorial design. Soya variety and maize variety were the main factors, both with two levels; non-GM and GM. Compared with fish fed non-GM maize, those fed GM maize exhibited significantly better growth, had lower mRNA transcription levels of *superoxide dismutase (SOD)-1* and a tendency (non-significant) towards lower transcription of *heat shock protein 70* in liver. Sex of the fish and soya variety had significant interaction effects on total RNA yield from the whole liver and transcription of *SOD-1*, suggesting that some diet component affecting males and females differently was present in different levels in the GM and the non-GM soya used in the present study. Dietary DNA sequences were detected in all of the organs analysed, but not all of the samples. Soya and maize *rubisco* (non-transgenic, multicopy genes) were most frequently detected, while MON810 transgenic DNA fragments were detected in some samples and RRS® fragments were not detected. In conclusion, zebrafish shows promise as a model for this application.

Zebrafish: GM feed: Roundup Ready® soya: Bt maize

The zebrafish (*Danio rerio*) is a commonly used model organism in developmental, molecular and toxicological studies[1–4]. This is not the case for nutrition studies; not even the basic nutritional requirements of zebrafish are published[5]. However, by using zebrafish as a model organism, duration and cost of trials can be greatly reduced. When working with GM feed ingredients, it is a challenge to obtain a proper control, such as the near-isogenic maternal line of the GM event[6]. Thus, it is advantageous that only small amounts are required to conduct a feeding trial. Furthermore, the fact that the zebrafish is omnivorous[7] means that more plant (GM) ingredients can be included in the diets than for carnivorous fish species such as Atlantic salmon (*Salmo salar*). Out of nineteen approved GM plants for use in animal feeds in Norway, only two, glyphosate-tolerant Roundup Ready® soya (RRS®, modification event GTS 40-3-2) and Bt toxin-producing YieldGard® maize (modification event MON810), have been subjected to fish-feeding trials. Thus, it makes sense to develop a model to reduce the costs of testing. Despite difficulty of procuring non-GM varieties of feed ingredients such as soyabean, the Norwegian aquaculture feed industry has so far avoided using GM ingredients[8].

Fish performance and health are issues of economical and animal welfare concern, and might be influenced by the newly expressed protein and/or unintended effects from the GM event. In a previous feeding trial, Atlantic salmon fed MON810 maize exhibited lowered feed intake and growth compared with fish fed the non-GM maternal line[9]. Red blood cell count, hepatosomatic index and distal intestine index were significantly higher in the GM group[9]. Furthermore, the fish fed GM maize had increased maltase activity in mid and distal intestine and increased Na-dependent D-glucose uptake in brush border membrane vesicles isolated from pyloric caeca[9]. Assessment of stress-related proteins in the same trial revealed that superoxide dismutase (SOD) activity in the liver and distal intestine was significantly higher in fish fed GM maize[10]. This group also exhibited decreased CAT (catalase) activity and a tendency towards increased heat shock protein (HSP) 70 levels in liver[10]. The authors interpreted this as a possible mild stress response in the GM-fed group. As the normal range of these parameters is not known, it is hard to conclude on the long-term biological significance of these findings. In another study in which two MON810 hybrids were fed to Atlantic salmon from first

Abbreviations: HSP, heat shock protein; PFU, PCR-forming units; RRS, Roundup Ready soya; SOD, superoxide dismutase.

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feeding onwards, no differences in growth or organ indices were detected[11].

Fish-feeding studies using GM soya have produced less differences between diet groups, and with no effect on growth[11–15]. Significant effects have been seen in these studies on plasma TAG levels and organ indices (spleen and intestine), but the results have been inconsistent or even contradictory between studies[13,14,16], thus can most likely be explained by other factors than the genetic modification *per se*. Differences in intestinal glucose uptake have been reported, but the control soyabean used was a commercial variety and heat treatments used differed between the non-GM and the GM soya[17]. Intestinal cell proliferation was higher in the non-GM-fed fish in a study on salmon parr, also conducted with a commercial soyabean line as control[18].

Transgenic DNA present in food/feed has been a topic of research interest, despite the fact that it does not seem to present safety issues other than conventional dietary DNA[19,20]. This includes studies investigating the fate of foreign DNA ingested by fish[21–25]. In a study where Atlantic salmon was force-fed high concentrations of DNA fragments, the uptake was observed in liver, kidney and blood, with the highest concentrations in liver and kidney[22]. Intravenously injected DNA fragments were detected in muscle, liver, kidney and blood in another study[23]. By means of *in situ* hybridisation, transgenic DNA was identified in intestinal cells of fish fed GM soya, although only in a few of the examined samples[25]. Chainark *et al.* [24] found DNA fragments of the transgenic promoter from RRS® in the leukocytes, head kidney and muscle of rainbow trout (*Oncorhynchus mykiss*) fed a GM soya diet.

In the present study, RRS® and MON810, as well as their non-modified lines were tested separately and in combination as feed ingredients in a 2 × 2 factorial design. The aims were to compare growth, performance and liver transcription of mRNA coding for different proteins involved in cellular and oxidative stress between fish fed the non-GM and GM varieties. Secondly, we aimed to detect transgenic DNA fragments and *rubisco*, a chloroplast gene present in plants in high copy numbers, in different tissues of the fish.

Materials and methods

Diets and experimental design

The GM varieties Bt maize, event MON810, and RRS®, event GTS 40-3-2, and the non-modified lines from which the GM varieties were originally derived, maize cultivar Hi-II and soya cultivar A5403 (the maternal, near-isogenic lines), were grown under identical conditions (all kindly supplied by the Monsanto Company, St Louis, MO, USA). The non-GM varieties were grown side by side with the GM varieties, in order to constitute the best possible control for safety assessment of the GM plants[26,27]. Analyses of proximate composition and pesticide residues in these are listed in Table 1; there were minor differences in the analysed nutrients and

Table 1. Proximate composition and pesticide residue levels of the raw materials used in the diets

	Non-GM maize	GM maize	Non-GM soya	GM soya
Composition (g/100 g)				
DM	91·0	91·0	91·1	92·9
Lipid	4·2	4·4	20·5	18·2
Protein	9·5	9·1	35·1	38·2
Starch	62·5	61·9	NA	NA
Ash	1·4	1·3	4·6	5·0
Residue*	13·4	14·3	39·8	38·6
Pesticides (μg/kg)				
Aldrin	<0·6	<0·6	<0·6	<0·6
α-Endosulfan	<0·5	<0·5	<0·3	<0·3
Endosulfan sulphate†	NA	NA	<0·5	<0·5
β-Endosulfan	NA	NA	<0·3	<0·3
cis-Chlordane	<0·5	<0·5	<0·5	<0·5
cis-Nonachlor	<0·7	<0·7	<1·5	<1·5
Dieldrin	<0·3	<0·3	NA	NA
Endrin	<1	<1	NA	NA
Heptachlor	<2·5	<2·5	<2·5	<2·5
Heptachlor-A	<0·5	<0·5	<0·5	<0·5
Oxychlordane	<1·3	<1·3	<1·3	<1·3
Toxaphene 26	<2·5	<2·5	<1·0	<1·0
Toxaphene 32	<1·5	<1·5	<0·7	<0·7
Toxaphene 50	<2·5	<2·5	<2·5	<2·5
Toxaphene 62	<1·5	<1·5	<1·5	<1·5
trans-Chlordane	<0·7	<0·7	<0·7	<0·7
trans-Nonachlor	<0·5	<0·5	<0·5	<0·5
α-Hexachlorocyclohexane	NA	NA	<0·6	<0·6
Hexachlorobenzene	NA	NA	0·13	0·88
γ-Hexachlorocyclohexane	NA	NA	<2·0	<2·0

NA, not available.

* Residue for maize was calculated as DM − (protein + lipid + starch + ash), residue for the soyabean meal also includes the starch fraction.

† Differences in limit of detection for some of the pesticides between analysis on the maize and soya and some differences in what pesticides were analysed are due to changes in the method between the time when the maize and the soya samples were processed.

British Journal of Nutrition

none in the analysed pesticides. Additionally, we have previously analysed the soya batch used in this trial for a range of anti-nutritional factors[14], while maize is known to have a low content of these[28]. Four experimental diets were designed with the same composition, the only difference between them being whether the soya and maize included were GM or not. In the first diet, maize and soya were both included as non-modified varieties (the non-GM diet); in the second, only the maize was GM (mGM); in the third, only the soya was GM (sGM); while in the fourth diet, both soya and maize were GM (smGM). Thus, these four diets constituted a 2×2 factorial design with soya and maize variety as the two factors, each with two levels, non-GM and GM. Each diet was fed to three replicate, randomly allocated, fish tanks. The diets contained 20 % maize, which was the major source of starch, and 25 % full fat soyabean meal supplying protein and lipid. Additional protein came from cod fillet and squid (squid was also added as a feed attractant), and additional lipid was provided from cod liver oil. Vitamin and mineral mixes were added according to NRC[57]. The formulation of the feeds is given in Table 2. In addition to the four experimental diets, a commercial diet (Teklad adult zebrafish diet, Harlan Teklad, Madison, WI, USA) was fed to triplicate tanks. This diet had other ingredients (fishmeal, high gluten wheat starch, blood meal, maize gluten, fish oil, wheat, liquid fish soluble as well as various additives) and a different proximate composition. The purpose of including this diet was not related to GM plant evaluation, but was simply to evaluate the feed production process and ensure that an acceptable growth rate was achieved with the experimental diets. It was not checked whether or not this diet contained GM material, as this was considered irrelevant. This diet was also crushed and sieved (see below) to obtain the same size fraction as the experimental diets.

Heat-coagulated experimental diets were made. All ingredients were blended to a visually homogenous mixture in a standard food processor, and the resulting feed dough was made into 1·5 cm thick strings. These were heat treated for 12 min at 83°C to denature the protein, with 100 % humidity to avoid the formation of a crust, in a convection oven (SCC 202, Rational AG, Landsberg am Lech, Germany). The feed was subsequently dried in the same oven for 24 h with a maximum temperature of 45°C and 0 % humidity. All four feeds were made at the same time to prevent any differences in time, temperature etc in the processing of the diets. The feeds were then ground (Mg 1-314, Frewitt, Granges-Paccot, Switzerland) and sieved (As200 basic, Retsch, Düsseldorf, Germany) to obtain the desired particle size fraction. Particles between 400 and 560 μm were used. The feed was kept refrigerated at 4°C until use.

Table 2. Formulation of the four experimental diets; the composition was the same for all diets, the only difference was whether the maize and soya were GM or not

Ingredient	DM in diet (%)
Maize*	20
Cod fillet†	39·0
Cod liver oil‡	7·6
Squid†	4·4
Full fat soyabean meal*	25·0
Vitamin mix§	1·0
Mineral mix§	3·0

* Monsanto Company (St Louis, MO, USA).
† Lerøy, Bergen, Norway.
‡ Peter Möller, Lysaker, Norway.
§ Harlan Teklad, Madison, WI, USA.

Dietary moisture was analysed by drying at 103°C for 24 h, ash by weight after burning at 540°C and lipid after extraction with ethyl acetate[29]. Nitrogen was measured with a nitrogen determinator (LECO, FP-428, Leco Corporation, St Joseph MI, USA) according to Association of Official Agricultural Chemists official methods of analysis[30] and protein calculated as $N \times 6{\cdot}25$. Starch was measured after enzymatic degradation as described by Hemre *et al.* [31]. Vitamin B_6 was determined by HPLC[32]. Multielement determination in the feed was done by inductively coupled plasma MS[33]. Pesticides were determined by GCMS on a Trace GC 2000 series and Trace DSQ single quadrupole (Thermo Fisher Scientific, Waltham, MA, USA)[34,35]. Both the maize and soya used as diet ingredients and the four experimental diets were tested for the presence of the transgenic proteins Cry1Ab and EPSPS expressed in MON810 and RRS®, respectively, using lateral flow strip assays (QuickStrix™ Kit for Cry1Ab bulk grain/for Roundup Ready® bulk soyabeans, EnviroLogix™, Portland, ME, USA). The test for each ingredient/diet was run in triplicate with 0·25 g sample material each time.

Fish husbandry and sampling

Tubingen × AB wild-type zebrafish of 60 d old were supplied by the SARS centre (Bergen, Norway). The experiment was conducted in an AHAB multiple rack zebrafish system (Aquatic habitats, Aquatic Eco-Systems, Apopka, FL, USA) with reverse osmosis followed by automatic salt dosing of intake water, UV, mechanical and carbon filtration of the water. The fish were distributed in seven 10 l tanks where they were allowed to acclimatise for 6 d, while being fed a commercial zebrafish diet (Teklad adult zebrafish diet that was also used as a reference diet in the experiment). At the start of the experiment, the fish (mean weight 149 mg, SD 22) were distributed in the fifteen experimental tanks (3 l), with seventeen fish each. All fish were examined during the distribution, and fish that were especially big or small, or had spinal or jaw deformities, were not used in the experiment to achieve a material that was as homogenous as possible. Temperature, pH, salinity and conductivity were monitored on a daily basis. The mean temperature was 27·2°C (SD 0·9), the pH 7·12 (SD 0·29), salinity 0·3 (SD 0) and conductivity 601uS (SD 99). Ammonia (NH_3/NH_4), nitrate (NO_3^-), nitrite (NO_2^-) and oxygen were measured weekly. The fish were hand fed to satiation three times a day, and feed consumption per tank was recorded daily.

The experiment was terminated after 20 d of feeding, when the fish had near doubled their weight and would presumably have been affected by the diet ingredients. As zebrafish reach sexual maturity within 3–4 months after fertilisation, this represents a not insignificant part of the life cycle. A time lag in the last feeding ensured that each tank was sampled at the same time interval after their last meal to avoid differences in nutritional status. The fish were killed by a sharp

object to the head, before weight and length were measured. On the fish used for mRNA analysis, the head and tail were cut off and the remainder of the fish was fixed in a 10 × volume of RNA*later*® (Ambion, Austin, TX, USA) and stored in the fridge at 4°C until liver was dissected under a dissecting microscope. The sex of the fish was determined upon dissection. For tracing of dietary DNA sequences, fish were flash frozen whole in liquid nitrogen. Tracing and mRNA analysis were only done on fish fed the four experimental diets, not the commercial diet.

The feeding trial was approved by the National Animal Research Authority in Norway, case no. 07/16 836.

Transcriptional (mRNA) analysis

The RNeasy mini kit (Qiagen, Hilden, Germany) was used for RNA extraction according to the manufacturer's instructions. Tissue was homogenised in buffer from the kit on the bead grinder homogenizer Precellys 24 (Bertin Technologies, Montigny-le-Bretonneaux, France) for 3 × 10 s at 6000 rpm. DNase treatment (DNA free, Ambion) was applied to eliminate any DNA contamination and ethanol precipitation to remove salts. RNA quantity and quality were assessed with Nanodrop® ND-1000 UV–Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and the Agilent 2100 Bioanalyzer with the RNA 6000 Nano LabChip® kit (both Agilent Technologies, Pao Alto, CA, USA). Samples with RNA integrity number lower than 6 were discarded. The average RNA integrity number value of the samples used was 8·1 (SD 0·9). Samples were stored at −80°C for further use, and three samples per tank (nine per experimental diet) were used.

Primer sequences were obtained from the literature for the target genes *HSP90α*[36], *DNA repair protein RAD51*[37], *SOD-1* and *glutathione peroxidase-1*[38] and the potential reference genes *β-actin*, *elongation factor 1α*, *hypoxanthine guanine phosphoribosyl transferase* 1 and *ribosomal protein L13α*[39]. For *HSP70*, forward and reverse primers were designed using Primer Express® 2.0 (Applied Biosystems, Foster City, CA, USA). Sequences of all primer pairs (Invitrogen, Life Technologies, Carlsbad, CA, USA) are given in Table 3. Samples were run in a 96-well format, and all samples fit into one cDNA (RT) plate, but separate real-time plates were run for each gene. A twofold serial dilution curve (500–31·25 ng RNA) of a pooled sample was run with six dilutions in triplicate for calculation of PCR efficiency. Non-template and non-amplification controls were also used, in addition to the experimental samples in duplicate (125 ng RNA). Reverse transcription was performed on a GeneAmp® PCR 9700 machine (Applied Biosystems), using the TaqMan® Reverse Transcriptase kit with oligo(dT) primers (Applied Biosystems) in 50 µl reactions. For real-time PCR, 18 µl SYBR® Green I Mastermix (Roche Applied Science, Indianapolis, IN, USA) with forward and reverse primers (0·5 µM of each) and 2 µl of the cDNA were mixed in 96-well plates by Biomek® 3000 Laboratory automation workstation (Beckman Coulter, Fullerton, CA, USA). Thermal cycling was performed on a LightCycler® 480 System (Roche Applied Science) according to the following protocol: pre-incubation at 95°C; 45 cycles of amplification with 10, 20 and 30 s at 95, 20 and 72°C, respectively; and finally melting curve analysis.

C_t values were calculated using the second maximum derivative method in the Lightcycler® software. Twofold dilution curves were used to determine the efficiency with the formula $E = 10 \wedge (-1/\text{slope})$, with the slope of the linear curve of C_t values plotted against the log dilution[40]. The geNorm VBA applet[41] and NormFinder[42] were used to investigate reference gene stability. Based on the results, all four of the tested reference genes were included to achieve the most stable normalisation index. The software package GenEx 4.3.5 (MultiD Analyses AB, Gothenburg, Sweden) was used for efficiency correction of the *Ct* values for all genes, normalisation to reference genes, averaging of the RT repeats, calculation of quantities relative to the average and log(2) transformation of the numbers.

Detection of dietary DNA fragments

These analyses were conducted at the National Veterinary Institute (Oslo, Norway), which is an accredited laboratory for GMO detection. Frozen zebrafish were thawed on ice before isolation of organs in the following order: intestinal organs; liver; brain; muscle. Intestinal organs included the stomach and the whole intestine. One fish from each tank from the four experimental diets was investigated. The equipment was sterilised using 70 % EtOH and flaming before isolation of each organ. A fresh sheet of single-use bench paper was used for each fish. The organs were transferred to sterile test-tubes, and DNA isolated using the DNeasy Blood and Tissue kit (Qiagen GmbH, Germany) according to the manufacturer's procedures and eluted in 100 µl elution buffer. DNA was isolated from 200 mg of the four experimental

Table 3. Sequences and accession numbers of the primer pairs used

Gene	Forward primer	Reverse primer	Accession no.
EF1α	CTGGAGGCCAGCTCAAACAT	ATCAAGAAGAGTAGTACCGCTAGCATTAC	ENSDART00000023156
HPRT	ATCAGCGAAACAGGAAAGGAG	CTGCGGTGAGCTGCACTACT	NM_212986
β-actin	CGAGCTGTCTTCCCATCCA	TCACCAACGTAGCTGTCTTTCTG	ENSDART00000055194
Rpl13α	TCTGGAGGACTGTAAGAGGTATGC	AGACGCACAATCTTGAGAGCAG	NM_212784
HSP70	CATCGACGCCAACGGG	CCAGGGAGTTTTTAGCAGAAATCTT	AF210640
HSP90α	AGCTGGCGGATCGTTCACTGTC	AAAACTCGCCGTACTCCTCATTGG	AF068773
DNA-rep. prot	CGTCTCGCTGATGAGTTTGG	CCACCTGTGCTACAACCTGGTT	AW184428
SOD1 (Cu–Zn)	CGCATGTTCCCAGACATCTA	GAGCGGAAGATTGAGGATTG	Y12236
GPx-1	AGATGTCATTCCTGCACACG	AAGGAGAAGCTTCCTCAGCC	AW232474

EF1α, elongation factor 1α; HPRT, hypoxanthine guanine phosphoribosyl transferase; Rpl13α, ribosomal protein L13α; HSP, heat shock protein; DNA-rep. prot, DNA repair protein RAD51; SOD, superoxide dismutase; GPx, glutathione peroxidase.

British Journal of Nutrition

diets using a slightly modified version of the CTAB method[43] and resolved in 120 μl TE buffer. The DNA concentration of each sample (μg/μl) was measured using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies).

Quantitative PCR was used to detect the GM insert in RRS® soya and MON810 maize, and to detect the chloroplast multicopy *rubisco* gene from soya (sRubisco) and maize (mRubisco). Event specific primers and probes were used for RRS®[44] and MON810[45], while primer and probe sequences were constructed for sRubisco (accession number: Z95552) and mRubisco (accession number: Z11973) using Primer3 (http://primer3.sourceforge.net/). Primer and probe sequences are given in Table 4. Probes were labelled as 5′Fam–3′Tamra. The PCR volume was 25 μl, containing 5 μl of DNA, 0·3 μM of each primer and 0·15 μM of probe in 1 × TaqMan Universal PCR Master mix (#4304437, Applied Biosystems). Amplification reactions were performed using Stratagene Mx3005P real-time cycler (Stratagene, La Jolla, CA, USA), with the amplification programme: 2 min at 50°C; 10 min at 95°C; 50 cycles of 15 s at 95°C; 60 s at 60°C (MON810, sRubisco and mRubisco) or 61°C (RRS®). Fluorescence measurements were analysed using the MxPro 3005 QPCR software (Stratagene). Two parallels were used when analysing for *rubisco*, and the number of PCR-forming units (PFU)/μl in the samples was calculated using a standard in the range of 1×10^5–1×10^1 PFU/μl. Limit of quantification for sRubisco and mRubisco was 50 PFU, while the limit of detection was 5 PFU. When analysing for RRS® and MON810, PFU/μl were calculated according to the most probable number method (SIMQUANT)[46–48], using eight parallels. When analysing for RRS® and MON810 in the diets, PFU/μl were calculated using a standard curve in the range of $5{\cdot}12 \times 10^2$–8 PFU/μl. The limit of detection and limit of quantification for quantification of RRS® and MON810 in the diets were 40 and 5 PFU, respectively. PFU/μl detected in the samples were used to calculate PFU/μg of DNA.

Statistical analysis

Statistical analyses were performed in Statistica™ 8.0 (Statsoft Inc., Tulsa, OK, USA). For the parameters obtained on a tank basis (SGR, FI and FCR), classical ANOVA was used. However, for parameters measured on individual fish, mixed-effects ANOVA was used, including the fish tanks as a random factor in the model. This gave a nested (hierarchical) model as the different tanks were nested within the diet treatments, and thus individual fish from the same tank were only pseudoreplicates. Nested ANOVA maintains both the between-tank and within-tank variabilities in the analysis, the latter is lost when the individual measurements are pooled and tank means calculated[49,50]. The nested test will often be more powerful in resolving treatment differences, as statistical power in this design can be increased either by increasing the number of tanks or by increasing the number of individual measurements made from each tank, albeit to different extents[49,51].

The commercial reference diet was compared with all the experimental diets (pooled), as our only interest here was simply to compare a commercial diet to the 'home-made' diets. In the experimental diets, the soya and maize ingredients were used in a 2 × 2 factorial design, with two factors (maize variety and soya variety) and two levels within each factor (non-GM and GM). All of the possible combinations (each level of each factor combined with every other) were used in each of our four experimental diets. Thus, although each experimental diet only was fed to three replicate tanks, each ingredient being studied (e.g. GM soya) was fed to six replicate tanks. In the comparisons of fish fed the experimental diets, the variables used for testing were maize and soya varieties, the four individual diet groups were not used as factors in the statistical testing. Sex was included as a factor in the model if found to exert a significant effect on that particular parameter/gene. The mRNA transcription data were log(2) transformed to obtain normality, assessed by the Kolmogorov–Smirnov test. Correlation between the RNA integrity number values and normalised expression levels in the samples was tested for each target gene. Three of the genes showed a positive correlation (significant or close to significant); meaning that better quality RNA resulted in apparent higher expression (Table 5), as reported by others[52–54]. To correct for this, the RNA integrity number value was included as a covariate in the statistical model for these genes. The significance threshold was set at $P < 0{\cdot}05$, but all P values $< 0{\cdot}10$ are given in the tables.

Calculations

$$\text{Condition factor (K)} = (\text{weight}/\text{length}^3) \times 100$$

$$\text{Specific growth rate} = (\ln W_f - \ln W_i \times 100)/t \; (W_f, \text{ final weight; } W_i, \text{ initial; } t, \text{ time in d})$$

$$\text{Feed conversion ratio} = \text{feed intake}/\text{weight gain.}$$

Results

When comparing the macro- and micronutrient composition of the four experimental diets (the non-GM, mGM, sGM and smGM diets; Table 6), these were compositionally very similar. The transgenic protein Cry1Ab was detected in the

Table 4. Primer and probe sequences for detection of dietary DNA fragments from feeds and zebrafish tissues

	Forward primer	Reverse primer	Probe
RRS®	TAGCATCTACATATAGCTTC	GACCAGGCCATTCGCCTCA	ACAAAACTATTTGGGATCGGAGAAGA
MON810	TCGAAGGACGAAGGACTCTAACGT	GCCACCTTCCTTTTCCACTCTCTT	AACATCCTTTGCCATTGCCCAGC
sRubisco	GGGCTTACCAGTCTTGATCG	TGATTTTCTTCCCCAGCAAC	GGGCGATGCTACGGCCTTGA
mRubisco	GCCTGTGTACAAGCTCGTAACG	CACTCCATTTGCAAGCTGCTT	AGGGCGCGATCTTGCTCGTGAA

RRS, Roundup Ready soya; sRubisco, *rubisco* gene from soya; mRubisco, *rubisco* gene from maize.

Table 5. Correlation between RNA integrity number value of the RNA samples and normalised relative mRNA transcription

Gene	R^2	*P* value
HSP70	−0·05	NS
HSP90	0·04	NS
SOD-1 (Cu–Zn)	0·48	0·005
DNA-rep. prot.	0·34	NS, *P*=0·054
GPx-1	0·44	0·011

HSP, heat shock protein; SOD, superoxide dismutase, DNA-rep. prot, DNA repair protein RAD51; GPx, glutathione peroxidase.

GM maize and EPSPS in the GM soya (three out of three samples positive for both), but all diet samples were negative, presumably due to denaturation of protein during heat treatment of the diets[55]. The reference diet had higher protein and energy levels and a lower residue content (Table 6); this was unavoidable as we wanted to include high levels of the GM plant materials. Most minerals were present in higher levels in the reference diet, and there was also a difference in vitamin B_6 (the only vitamin measured). All the feeds were accepted by the fish, although feed intake was significantly higher ($P=0·0005$) on the reference diet compared with the four experimental diets (Table 7). The reference diet resulted in better growth compared with the experimental diets, observed by a higher growth rate and final weight ($P=0·005$). The experiment passed without unexpected events, mortality was low, and there were no differences between the diet groups in this regard. The remainder of the results is based on comparisons between the four experimental diets only, as the reference diet is of no relevance to the GM ingredients investigated in this trial.

There was a significant ($P=0·02$) reduction of feed intake in the groups fed GM soya compared with non-GM (Table 7). However, there were no corresponding effects observed on growth or feed conversion ratio. A significantly higher ($P=0·045$) weight was obtained with GM maize compared with non-GM maize. There was also a tendency towards a difference in length between these groups ($P=0·08$), but the condition factor was similar. Highly significant sex differences in final weight, condition factor and total RNA yield from the whole liver (presumably correlating to the size of the liver) were observed (all with $P<0·0001$); thus, sex was included in the statistical model for these parameters. Furthermore, there was a significant interaction effect of soya variety (GM or non-GM) and sex on whole-liver RNA yield ($P=0·003$), and also a nearly significant effect ($P=0·06$) of GM soya by itself on this parameter (Fig. 1).

Transcription of *SOD-1* was found to be significantly affected by GM maize ($P=0·03$) and by sex ($P=0·03$; Table 8; Fig. 2). Transcription of this gene was higher in fish fed non-GM maize compared with GM maize, and higher in female than in male fish. There was also a significant interaction effect between soya variety and sex ($P=0·004$). For *HSP70*, there was a nearly significant difference between GM and non-GM maize ($P=0·08$). The fold difference was

Table 6. Analyses of the four experimental diets and the commercial reference

	Non-GM	mGM	sGM	smGM	Commercial*
Proximate composition					
DM (g/100 g)	91·7	93·5	90·0	93·3	93·3
Ash (g/100 g)	7·2	7·2	7·0	7·1	7·7
Protein (g/100 g)	42·9	43·4	41·6	43·3	51·7
Lipid (g/100 g)	13·5	13·3	13·2	13·2	14·7
Starch (g/100 g)	13·3	13·7	13·0	13·3	13·6
Residue (g/100 g)†	14·8	15·9	15·2	16·4	5·6
Energy (kJ/g)‡	17·7	17·9	17·3	17·7	20·3
Vitamins (mg/kg)					
Vitamin B_6	22	23	20	17	74
Elements (mg/kg)					
V	0·10	0·12	0·12	0·11	1·10
Mn	58	63	58	61	91
Fe	93	110	110	99	620
Co	0·03	0·04	0·11	0·08	1·2
Cu	7·3	9·3	8·4	7·1	47
Zn	49	52	48	51	120
As	1·8	2·1	2·0	7·5	3·3
Se	0·85	1·0	0·79	0·83	2·4
Sr	9·8	7·0	5·8	4·6	31
Mo	0·77	1·1	1·6	0·98	0·59
Ag	<0·01	<0·01	<0·01	<0·01	<0·01
Cd	0·02	0·03	0·03	0·02	0·09
Sn	0·04	0·04	0·04	0·10	0·04
Ba	4·4	5·1	3·2	3·3	2·5
Hg	0·13	0·20	0·19	0·19	0·04
Pb	0·04	0·04	0·04	0·04	0·15

The results are presented as the average of two analytical parallels.
* Commercial zebrafish diet (Harland Teklad, MD, USA).
† Residue was calculated as DM − (ash + protein + lipid + starch).
‡ Gross energy was calculated according to Tacon[88] using the energy content of 39·5 kJ/g for lipid, 23·6 kJ/g for protein and 17·2 kJ/g for starch.

British Journal of Nutrition

Table 7. Growth and feed performance

(Mean values with their standard errors of each diet group)

	non-GM		mGM		sGM		smGM		Commercial		
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	ANOVA
Weight (mg)	257	8	271	9	253	11	269	8	315	15	NS†, P=0·005*, P=0·05‡
Length (cm)	2·85	0·02	2·91	0·02	2·85	0·04	2·93	0·03	2·98	0·04	NS*†‡ (P=0·06*, P=0·08†)
K	1·11	0·03	1·09	0·02	1·09	0·03	1·09	0·04	1·16	0·04	NS*†‡
SGR	2·84	0·24	3·14	0·07	2·79	0·36	3·12	0·12	3·95	0·41	NS†‡ (P=0·005*)
FI (mg)	186	3	189	6	183	2	174	2	212	9	NS†‡ (P=0·0005*, P=0·02†)
FCR	1·78	0·18	1·56	0·04	1·82	0·27	1·45	0·08	1·30	0·14	NS*†‡ (P=0·08*)

SGR, specific growth rate; FI, feedintake; FCR, feed conversion ratio.

Weight, length and condition factor (K) are measured on individual fish (43–48 per diet), but nested within the respective tanks in the ANOVA to account for pseudoreplication. The growth rate (SGR), FI and FCR are calculated on a tank basis (n 3 per diet). Note that the statistical testing was not performed to compare individual diet groups, but on the factors 'soya variety' and 'maize variety' for the experimental diets. The reference diet was tested against all the experimental diets together.

Initial values: weight 149 (SD 22) mg, length 2·6 (SD 0·2) cm and condition factor 0·83 (SD 0·10).

* Commercial reference *v.* the four experimental diets.

† Soya variety (GM *v.* non-GM).

‡ Maize variety (GM *v.* non-GM).

large, with a 6·5 times higher mean expression level in the fish fed non-GM maize compared with GM, but due to large variation this difference was not significant. In comparison, the observed fold difference in *SOD-1* was only 1·2, but this mRNA exhibited much more consistent expression levels with low variability within groups. For the remainder of the genes, no significant differences were observed.

The concentration of DNA isolated from samples for detection of dietary DNA was in the range 68–184 ng/μl for intestinal organs, 47–114 ng/μl for liver, 5–25 ng/μl for brain, 5–16 ng/μl for muscle and 90–190 ng/μl in the feed samples. DNA fragments of RRS® and MON810 were detected in feed samples from all the experimental diets, although only in amounts just above the detection level in the diets not formulated with that GM ingredient (Table 9). The concentration in the diet samples of DNA fragments from RRS® was approximately seven times higher than the concentration of fragments from MON810. Soya and maize *rubisco* were present at high levels in all diets. Transgenic DNA from RRS® was not detected in any of the analysed organs (data not shown). MON810 fragments could be detected in some samples of intestinal organs and in one liver sample, all in fish fed GM maize diets (Table 10).

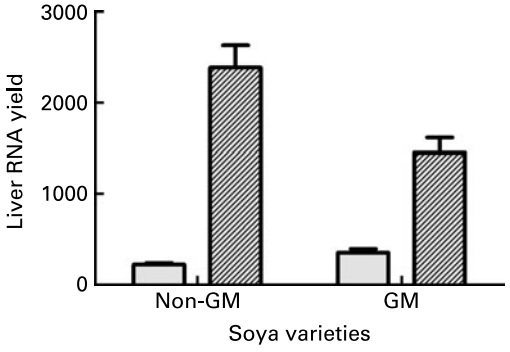

Fig. 1. Total liver RNA yield. RNA yield is given as ng/μl when the total RNA is diluted in 50 μl. The data are given as the means with their standard errors as the error bar (□, male; ▨, female). Soya variety: P=0·06; sex: P>0·0001; interaction: P=0·003.

Soya and maize *rubisco* (Tables 11 and 12) could be detected in several samples of each of the analysed organs. *Rubisco* sequences could be detected in the intestinal samples of all individuals, but in other organs the presence was more variable, it could only be detected at low levels and not in all of the fish. The level of *rubisco* in all organs, except the intestinal organs, was near the detection limit and below limit of quantification. There were no differences in the level of *rubisco* when comparing the different diets.

Discussion

Better growth of the fish fed the reference diet compared with our experimental diets seems to have been caused mainly by the highly significant difference in feed intake. This suggests that the main difference was in organoleptic properties or in the technical quality of the feed, rather than in the nutrient composition. The commercial diet contained less residue, indicating less plant material of unrefined qualities than our diets, which could affect palatability. The close to significant better FCR in the same reference diet group is often seen as a consequence of improved growth[56]. Furthermore, higher energy and protein levels could have a positive effect on FCR. Using requirements for other fish species for Fe, Zn, Mn, Cu and vitamin B_6, our experimental diets were above these[57], despite being lower than the commercial feed. The nutritional requirements of zebrafish are largely unknown[5], and despite differences from the commercial diet, our experimental diets supported satisfactory growth for the purpose of the present experiment.

The higher weight in fish fed GM maize compared with non-GM was only revealed when the sex of the fish was taken into account. The highly significant sex differences resulted in large variations within diet groups and thus low statistical power when this variability was unaccounted for. Zebrafish females are larger and allocate energy into eggs rather than muscle as they go into maturation[58]. A possible explanation for the effect of maize variety could be by different levels of mycotoxins. Large variability in mycotoxin is observed between crops and years in both GM and non-GM maize, but the general trend is reduced levels in Bt maize

Table 8. Transcription of mRNA

(Mean values with their standard errors of diet group)

	non-GM		mGM		sGM		smGM				
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Soya	Maize	Sex
HSP70	8·31	4·34	1·24	0·61	7·53	4·82	1·18	0·40	NS	NS (P=0·08)	NS
HSP90α	3·70	1·56	2·62	1·37	1·18	0·45	3·70	2·76	NS	NS	NS
DNA-r.p	0·93	0·19	1·63	0·34	1·17	0·18	1·27	0·32	NS	NS	NS
SOD-1	1·24	0·19	1·13	0·15	1·26	0·16	0·94	0·22	NS	P=0·03	P=0·03
GPx-1	1·24	0·23	1·84	0·25	1·37	0·31	0·84	0·20	NS	NS	NS

HSP, heat shock protein; DNA-r.p., DNA repair protein; SOD, superoxide dismutase; GPx, glutathione peroxidase; min, minimum; max, maximum.
The statistical testing was done on the log(2) of the normalised quantities, not with the individual diet groups, but with soya variety and maize variety as factors (GM *v.* non-GM). Three fish from each tank (nine from each diet group) were used. Sex and RNA integrity number value were included in the model if significant. *SOD-1* had a GM soya–sex interaction effect, significant with a p value of 0·004.

compared with conventional maize varieties, due to better resistance against *Fusarium* spp.[59–62]. This has been used to explain higher weight gain in the GM-fed group in three studies with broilers, broiler chicks and piglets, respectively (reviewed by Flachowsky *et al.* [63], although, in these studies, the hypothesis was supported by mycotoxin results that we do not have. In a study with Atlantic salmon fed the exact same batch of GM maize as in the present study, reduced growth was seen in the GM group, caused by a somewhat lower feed intake[9].

The effect on feed intake of soya variety (non-GM or GM) was not supported by changes in growth or feed conversion ratio, thus seems to be of little biological relevance. The difference is possibly due to the difficulty of making sure there was never excess feeding, making the feed intake estimates inaccurate.

The interaction effects between soya variety and sex on total liver RNA yield and *SOD-1* expression indicate that there is a difference between the non-GM and GM soya that affects male and female fish differently. Soya isoflavones are phyto-oestrogens known to affect sex hormones and reproduction in mammals[64–67], and have been shown to induce vitellogenesis in sturgeon but not in rainbow trout[68,69]. Isoflavones are products of the shikimate pathway, which is the target of the herbicide glyphosate. It has been hypothesised that isoflavone content may be reduced in glyphosate-tolerant soya[70]. However, differences have not been found when comparing glyphosate-treated to hand-weeded RRS®[70], nor when comparing RRS® to its non-modified maternal line[71–73]. Isoflavone levels were not measured in our diets, and thus it remains a speculation whether they are to blame for the observed effects. Significantly higher *SOD-1* liver transcription in zebrafish fed non-GM maize is not supported by further results from the previously mentioned salmon study[10]. In that study, *SOD-1* transcription in liver was equal between the diet groups, but total SOD enzyme activity (includes both the soluble isoform, SOD-1 and SOD-2 in mitochondria) was higher in both the liver and distal intestine in the GM group. Higher expression in females than males is consistent with what has been observed in other animals such as rats and hamsters[74,75]. Furthermore, our increase (although not significant) in *HSP70* in the non-GM maize groups differs from the salmon study[10]. In salmon, no differences were seen at the mRNA level, while the HSP70 protein was increased in the liver of fish fed the GM maize compared with a reference diet, while the non-GM maize diet exhibited intermediate levels. Maybe species differences can explain the opposite effects found in salmon and zebrafish, and further there was no maturation in the salmon study. Neither our difference in growth nor in *SOD-1* would not have been revealed without taking sex into account.

Furthermore, it must be taken into account that the difference in *SOD-1* transcription between the diet groups is minor (1·2-fold) and smaller than the difference between male and female fish. It is not known whether the difference by diet has any physiological relevance, although the corresponding effect on growth might suggest that it does. Given the effect on growth, other molecular markers including genes involved in metabolism might have given interesting results. However, limited sample material was available, and a focus on stress-related genes was chosen, because stress response had been singled out as a potential effect of GM feed[10] and to obtain comparative data with salmon to evaluate the utility of zebrafish as a model.

The results on the uptake of dietary DNA sequences support those studies referred to in the introduction, reporting that dietary DNA can withstand feed processing and be taken up by the fish intestine and be distributed to various tissues of the fish. There are similar reports of the same from various other animals[76–84]. This is, however, not specific to transgenic DNA, as the same happened with *rubisco* both in the

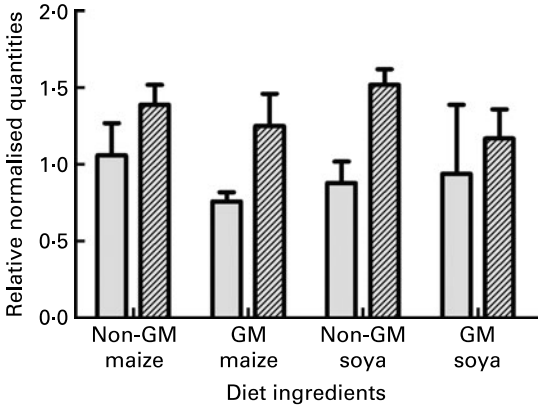

Fig. 2. *Superoxide dismutase (SOD)-1* transcription in liver. The data are given as the means with their standard errors as the error bar (□, male; ▨, female). Sex: P=0·03; maize variety: P>0·03; soya–sex interaction: P=0·004.

present study and others[85]. *Rubisco* is present in plants cells in high copy numbers, which is likely the cause why this sequence was detected much more frequently than the transgenic DNA. The present results give no reason to suspect that transgenic sequences should be taken up more frequently than regular plant DNA. However, it is interesting to see that dietary DNA is detectable in all tissues analysed also in zebrafish.

The presence of MON810 DNA fragments in intestinal organs could be explained by residual feed in the intestine, and it cannot be ruled out that the presence of MON810 DNA fragments in one liver sample could be explained by

Table 9. DNA fragments from Roundup Ready® soya (RRS®), MON810 and *rubisco* detected the four diets
(Mean values and standard deviations)

	RRS®		MON810		sRubisco		mRubisco	
Diet	PFU[1]/μg DNA	± min/max	PFU/μg DNA	± min/max	PFU/μg DNA	SD	PFU/μg DNA	SD
non-GM	12·6	8·4	3·8	0·7	$2{\cdot}64 \times 10^{7}$	$4{\cdot}43 \times 10^{6}$	$4{\cdot}49 \times 10^{6}$	$2{\cdot}51 \times 10^{5}$
mGM	18·9	8·9	8809·4	432·5	$3{\cdot}73 \times 10^{7}$	$9{\cdot}44 \times 10^{6}$	$6{\cdot}68 \times 10^{6}$	$8{\cdot}47 \times 10^{4}$
sGM	$7{\cdot}16 \times 10^{4}$	7783·1	38·5	7·1	$6{\cdot}69 \times 10^{7}$	$2{\cdot}01 \times 10^{6}$	$8{\cdot}64 \times 10^{6}$	$8{\cdot}58 \times 10^{5}$
smGM	$7{\cdot}33 \times 10^{4}$	7176·8	$1{\cdot}29 \times 10^{4}$	587·5	$9{\cdot}05 \times 10^{7}$	$1{\cdot}77 \times 10^{7}$	$1{\cdot}13 \times 10^{7}$	$1{\cdot}37 \times 10^{6}$

sRubisco, *rubisco* gene from soya; mRubisco, *rubisco* gene from maize; PFU, PCR-forming unit.
Rubisco was quantified by qPCR using a standard curve (two parallels and two dilutions of DNA). RRS® and MON810 were quantified by qPCR using a standard curve (one parallel and two dilutions of DNA).

Table 10. MON810 detected in organs of zebrafish using SIMQUANT (eight parallels)

		Brain		Muscle		Liver		Intestinal organs	
Diet	Tank	PFU/μg DNA	95 % CI	PFU/μg DNA	95 % CI	PFU/μg DNA	95 % CI	PFU/μg DNA	95 % CI
non-GM	3	–*		ND		ND		ND	
non-GM	8	ND		ND		ND		ND	
non-GM	11	ND		ND		ND		ND	
mGM	12	ND		ND		ND		0·36	0·03, 0·73
mGM	13	ND		ND		0·32	0·02, 0·64	3·89	2·56, 6·56
mGM	15	ND		ND		ND		ND	
sGM	1	ND		ND		ND		ND	
sGM	4	ND		ND		ND		ND	
sGM	7	ND		ND		ND		ND	
smGM	2	ND		ND		ND		0,15	0·01, 0·30
smGM	6	ND		ND		ND		ND	
smGM	10	ND		ND		ND		0,25	0·02, 0·51

PFU, PCR-forming unit; ND, not detected.
* No sample available.

Table 11. Soya *rubisco* detected in organs of zebrafish using a standard curve (two parallels)

		Brain		Muscle		Liver		Intestinal organs	
Diet	Tank	PFU/μg DNA	± min/max	PFU/μg DNA	± min/max	PFU/μg DNA	± min/max	PFU/μg DNA	± min/max
non-GM	3	–*		11·7		50·6	1·4	7580·0	847·0
non-GM	8	62·8	11·8	148·0	91·4	ND		5560·0	1210·0
non-GM	11	116·0	98·8	117·0	16·0	27·0	1·9	$1{\cdot}14 \times 10^{4}$	940·0
mGM	12	ND		46·7	35·4	55·4	25·6	2490·0	412·0
mGM	13	37·9		420·0	12·3	124·0	21·5	$3{\cdot}28 \times 10^{4}$	3670·0
mGM	15	20·0		ND		8·7	1·6	7130·0	1430·0
sGM	1	29·6	2·8	296·0	21·6	ND		1120·0	56·0
sGM	4	ND		115·0		$3{\cdot}9 \times 10^{4}$	4820·0	2370·0	188·0
sGM	7	ND		88·3	32·8	593·0	74·7	$9{\cdot}11 \times 10^{4}$	5370·0
smGM	2	258·0	95·3	ND		19·0	4·0	$1{\cdot}05 \times 10^{4}$	202·0
smGM	6	15·3		ND		4·2		2170·0	49·2
smGM	10	77·1	19·8	27·2		ND		$2{\cdot}81 \times 10^{4}$	2540·0

PFU, PCR-forming unit; ND, not detected.
In wells without min/max values, soya *rubisco* could only be detected in one out of the two parallels. When only one parallel was positive, the PFU of that parallel is presented in the table.
* No sample available.

British Journal of Nutrition

Table 12. Maize *rubisco* detected in organs of zebrafish using a standard curve (two parallels)

		Brain		Muscle		Liver		Intestinal organs	
Diet	Tank	PFU/mg DNA	± min/max	PFU/mg DNA	± min/max	PFU/mg DNA	± min/max	PFU/mg DNA	± min/max
non-GM	3	–*		4·7	4·3	ND		50·4	7·5
non-GM	8	5·3	4·2	ND		ND		180·0	4·6
non-GM	11	ND		ND		2·3		224·0	11·7
mGM	12	ND		ND		2·2		412·0	47·2
mGM	13	27·2		106·0	77·4	675·0	53·8	$1{\cdot}34 \times 10^4$	148·0
mGM	15	ND		ND		ND		31·3	0·17
sGM	1	8·1		4·1	1·2	ND		33·0	3·4
sGM	4	ND		ND		22·9	8·6	52·1	2·3
sGM	7	ND		8·1		16·2	4·1	577·0	20·4
smGM	2	ND		0·04		1·1	0·85	122·0	3·7
smGM	6	ND		8·2	0·57	0·4		140·0	13·1
smGM	10	37·6		26·1		ND		367·0	9·3

PFU, PCR-forming unit; ND, not detected.
In wells w/o min/max values, mRubisco could only be detected in one out of two parallels. When only one parallel was positive, the PFU of that parallel is presented in the table.
* No sample available.

contamination from the intestinal organs during sampling. The fact that we were not able to detect transgenic DNA in any of the other organs could be due to limitations in sensitivity, and the presence of these DNA fragments in the organs can therefore not be ruled out. No difference in the uptake of *rubisco* between the diet groups shows that the transgenic DNA does not modify the uptake of other dietary DNA fragments. This is in agreement with Mazza *et al.*[82] who found no difference in the uptake of specific maize genes when comparing piglets fed non-GM or MON810 maize. Similar findings have also been done by others[80,83]. Contrary to a higher level of the RRS® than the MON810 fragments detected in the diets, only MON810 and not RRS® could be detected in the intestinal organs. There are two possible explanations to this. Either there is some sequence specificity in the DNA uptake, which would also indicate that the detected DNA fragments in intestinal organs have been taken up by the fish and not from residual feed. Alternatively, there might be differences in the stability of different DNA fragments in the intestine, dependent on DNA sequence, methylation patterns or similar. The feed matrix can also influence the persistence and stability of DNA in the intestine[86,87].

Conclusions

Differences were apparent between zebrafish fed non-GM and GM maize in the present study. The fish fed GM maize showed better growth and lower transcription levels of *SOD-1* and possibly also *HSP70*, which could indicate a stress response in the fish fed non-GM maize. All these effects were inconsistent with an earlier salmon trial.

Interaction effects between soya variety and sex were found in RNA yield from liver and *SOD-1* transcription, both parameters with marked sex differences. This suggests that some diet component that affects males and females differently was present in different levels in the GM and the non-GM soya used in the present study.

Dietary DNA sequences were detected in low levels in fish tissues. The multicopy gene *rubisco* was detected much more frequently than the transgene fragments, and the RRS® sequence was not detected at all despite being higher than MON810 in the diets, suggesting that there was not a linear relationship between amount present in feed and the amount of detected in the fish.

The present study also provides support for the feasibility of using zebrafish as a model organism, not only in relation to chemical toxicology, but also to study the safety of whole foods. Dietary DNA seemed to behave similarly to what has been reported from other species, while the different responses to GM maize compared with salmon are hard to explain.

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