Evaluation of stress- and immune-response biomarkers in Atlantic salmon, *Salmo salar* L., fed different levels of genetically modified maize (Bt maize), compared with its near-isogenic parental line and a commercial suprex maize

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Abstract

The present study was designed to evaluate if genetically modified (GM) maize (Bt maize, event MON810) compared with the near-isogenic non-modified (nGM) maize variety, added as a starch source at low or high inclusions, affected fish health of post-smolt Atlantic salmon, *Salmo salar* L. To evaluate the health impact, selected stress- and immune-response biomarkers were quantified at the gene transcript (mRNA) level, and some also at the protein level. The diets with low or high inclusions of GM maize, and its near-isogenic nGM parental line, were compared to a control diet containing GM-free suprex maize (reference diet) as the only starch source. Total superoxide dismutase (SOD) activity in liver and distal intestine was significantly higher in fish fed GM maize compared with fish fed nGM maize and with the reference diet group. Fish fed GM maize showed significantly lower catalase (CAT) activity in liver compared with fish fed nGM maize and to the reference diet group. In contrast, CAT activity in distal intestine was significantly higher for fish fed GM maize compared with fish fed reference diet. Protein level of heat shock protein 70 (HSP70) in liver was significantly higher in fish fed GM maize compared with fish fed the reference diet. No diet-related differences were found in normalized gene expression of SOD, CAT or HSP70 in liver or distal intestine. Normalized gene expression of interleukin-1 beta in spleen and head-kidney did not vary significantly between diet groups. Interestingly, fish fed high GM maize showed a significantly larger proportion of plasma granulocytes, a significantly larger sum of plasma granulocyte and monocyte proportions, but a significantly smaller proportion of plasma lymphocytes, compared with fish fed high nGM maize. In conclusion, Atlantic salmon fed GM maize showed some small changes in stress protein levels and activities, but none of these changes were comparable to the normalized gene expression levels analysed for these stress proteins. GM maize seemed to induce significant changes in white blood cell populations which are associated with an immune response.

Keywords: antioxidant enzymes, Atlantic salmon, Bt maize, gene expression, genetically modified, stress proteins.

Introduction

Studies on genetically modified (GM) maize in salmon diets are scarce, and exist only with low levels of inclusions (Sanden, Berntssen, Krogdahl, Hemre & Bakke-Mckellep 2005; Sanden, Krogdahl, Bakke-Mckellep, Buddington & Hemre 2006a). Previous studies have reported on proliferation of gastric mucosa in rats fed GM potatoes in the diet (Ewen & Pusztai 1999). Changed cell-morphology has also been observed in mice fed GM soybeans with irregularly shaped hepatocyte nuclei, which generally represent an index of high metabolic rate (Malatesta, Caporaloni, Gavaudan, Rocchi, Serafini, Tiberi & Gazzanelli 2002). A recent feeding study,

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evaluating GM soybean (Roundup Ready® Monsanto NV, Brussels, Belgium) in Atlantic salmon diet, indicated enlarged spleen and possible impaired spleen function as the number of smaller-sized red blood cells were simultaneously increased (Hemre, Sanden, Bakke-Mckellep, Sagstad & Krogdahl 2005).

The concerns of using GM material in foods and feed have focused largely on possible unintended effects caused by the gene modification process. Alterations in gene expression of existing genes, and/or formation of new genes coding for new proteins, are some unintended effects that have been suggested (Kuiper, Kleter, Hub Noteborn & Kok 2001; Cellini, Chesson, Colquhoun, Constable, Davies, Engel, Gatehouse, Karenlampi, Kok, Leguay, Lehesranta, Noteborn, Pedersen & Smith 2004). Several unintended effects have been observed with GM plants, such as metabolic changes (Shewmaker, Sheehy, Daley, Colburn & Ke 1999), phytotoxic effects (Murray, Llewellyn, Mcfadden, Last, Dennis & Peacock 1999) and increased amounts of the anti-nutritional component lignin (Gertz, Vencill & Hill 1999). Bt maize (event MON810) contains the transgenic protein Cry1A(b), which confers resistance to the European corn borer (ECB), *Ostrinia nubilalis*. The Cry proteins are endotoxins produced from the soil bacterium *Bacillus thuringiensis (Bt)* which exhibit insecticidal properties. These proteins act by binding to receptors of the mid-gut brush border membrane (Gill, Cowles & Pietrantonio 1992; Denolf, Jansens, Peferoen, Degheele & Van Rie 1993) and cause lethal damage to the intestinal tract of the ECB (Knowles 1994).

The use of GM ingredients is at present avoided by some feed producers due to uncertainties of how it affects fish health and because of the public resistance towards GM products in the human food chain (Ewen & Pusztai 1999). Transgenic DNA sequences of different sizes have proved to survive feed processing, and have been detected throughout the digestive tract of Atlantic salmon (Sanden, Bruce, Rahman & Hemre 2004), but also entered into mucosal cells in both rainbow trout and Atlantic salmon (Chainark, Satoh, Kiron, Hirono & Aoki 2004; Sanden *et al.* 2006a). Detection of dietary DNA in blood and vital organs of Atlantic salmon further proves absorption from the digestive tract (Nielsen, Berdal, Bakke-Mckellep & Holst-Jensen 2005). Transgenic DNA or possible new and unknown proteins originating during the gene modification process might be toxic at low levels of intake, or be potent allergens to the individuals ingesting GM feed.

The present study was designed with GM Bt maize (event MON 810) and its near-isogenic nGM parental line at two inclusion levels, in diets for Atlantic salmon. The study evaluates selected biomarkers on toxic and immunological stress, and aims to elucidate possible secondary effects of GM maize compared with nGM maize, included in diets for Atlantic salmon in a 3-month feeding study.

Materials and methods

Feed ingredients

Three maize types, Suprex corn (suprex maize) (Condrico AB, Den Haag, The Netherlands), GM maize (GM-maize-event MON810) and the untransformed near-isogenic parental line of MON810 (nGM-maize) were used in this study. GM maize and nGM maize were kindly supplied by the Monsanto Company (St Louis, MO, USA). Maize ingredients were dried and ground to the correct particle size prior to feed production. Low-temperature quality of fish meal and fish oil contributed to most of the protein and lipid fractions. National Research Council (1993) recommendations were followed for vitamin and mineral additions. Rovimix, which contains 8% astaxanthin was added as a pigment source.

Experimental diets and feeding

Five experimental diets were made at the Norwegian Institute of Fisheries and Aquaculture Research (Bergen, Norway): a reference diet with suprex maize as the only source of starch (reference diet), two nGM maize diets where half (low) or all (high) of the suprex maize was replaced with nGM maize (low nGM maize and high nGM maize) and two GM maize diets where half (low) or all (high) of the suprex maize was replaced with GM maize (low GM maize and high GM maize). Formulation and analysed composition of diets are shown in Table 1. Diets were aimed to be compositionally equivalent in nutrients, with regard to protein, lipid and starch levels, fatty acid and amino acid composition, and vitamin and mineral content. Each diet was fed in triplicate and in excess from automated feeders running in intervals of 20 s intervened by 200 s (5 AM to 8 AM and 2 PM to 3 AM).

Table 1 Formulation and chemical composition of the experimental diets (g kg^{-1})

Diet code	Reference diet	nGM maize diet		GM maize diet	
		Low	High	Low	High
Fish meal	532	523	524	514	515
nGM maize		150	300		
GM maize				150	300
Fish oil	184	178	177	171	170
Suprex maize	269	135		135	
Yttrium oxide Y_2O_3	0.1	0.1	0.1	0.1	0.1
Constant ingredients	15	15	15	15	15
Proximate analysis (g kg^{-1})					
Dry matter	933	937	932	933	930
Protein[a]	403	401	398	401	395
Lipid[a]	227	226	225	223	228
Starch	163	165	161	153	166
Ash[a]	93	94	94	92	92
Residue[b]	47	51	54	64	49
Gross energy (kJ g^{-1})	21.3	21.2	21.2	20.9	21.2

[a]Fatty acid composition (g kg^{-1} of total fatty acids) and total amino acids were equivalent in all diets. Calcium varied from 18.6 to 19.0 g kg^{-1}, phosphorous from 12.7 to 13.1 g kg^{-1}, zinc from 206 to 214 mg kg^{-1}, iron from 154 to 166 mg kg^{-1} and selenium from 1.4 to 1.6 mg kg^{-1}.
[b]Residue was calculated as 1000 − (moisture + protein + lipid + starch + ash).

Fish and facilities

The feeding experiment took place at the Norwegian Institute of Fisheries and Aquaculture Research facilities (61°N, Austevoll, Norway). Atlantic salmon smolts, *Salmo salar* L. (Aqua Gen breed, Sunndalsøra, Norway), were individually tagged with a personal integrated transponder (PIT), Glass Tag Unique 2 12 × 12 mm (Jojo Automasjon AS, Sola, Norway), 2 weeks before onset of the experiment. During the 2 weeks of acclimatization to PIT tags and experimental tank facilities, the fish were fed a commercial 3 mm salmon pellet 'Ewos Transfer Boost' (Ewos AS, Dirdal, Norway). Prior to the start of the experiment, fish were weighed and randomly distributed to 15 dark green fibreglass tanks (1.5 × 1.5 × 0.9 m), each containing 45 fish. Initial weight averaged 155 ± 3 g. The feeding trial lasted for 82 days, from 17 June 2004 to 9 September 2004, whereas sampling took place at the start (day 1) and for 2 days at the end of the experiment (days 82 and 83). During the experiment fish were subjected to a 24-h light photoperiod, water salinity varied between 31‰ and 32‰, and water temperature averaged 8 ± 0.5 °C. A flow-through system of 50–55 L min^{-1} ensured high water quality, maintaining average water oxygen content at 7.8 mg L^{-1} (88% saturation).

Sampling procedure

Before the start of the feeding trial, all fish were weighed and length-measured, and clinical haematology screened (Hemre *et al.*, unpublished data), to assume the fish were healthy and of similar size. Besides the initial sampling, a major final sampling took place at the end of the feeding trial. Before netting the fish during the samplings, the fish were pre-anaesthetized with Aqui-S^{TM} isoeugenol (540 g L^{-1}) (Scan Aqua, Årnes, Norway) and thereafter fully anaesthetized with metacainum (50 mg L^{-1}; MS-222^{TM}; Norsk Medisinaldepot AS, Bergen, Norway) before blood withdrawal and dissection of organs. Blood was sampled from the caudal vessel into sterile, heparinized syringes, from five individual fish per tank. Tissues from liver, distal intestine, head-kidney and spleen were sampled for gene expression analysis from three individual fish per tank (nine fish per diet), and immediately put on RNA Later^{TM} (Ambion, Warrington, UK) and stored as recommended by the manufacturer. From the same fish, tissue samples for enzyme activity and protein measurements were collected from liver and distal intestine, immediately frozen in liquid nitrogen and stored at −80 °C until analysed. Samples were withdrawn continuously, tank for tank, under similar conditions. The reference diet groups and nGM maize groups were sampled first, followed by the GM maize diet groups, to avoid contamination from GM feed. All fish were fed until sampling, ensuring the fish to be in an absorptive phase when sampled.

Primers and probes

The polymerase chain reaction (PCR) primer and TaqMan® probe sequences for β-actin, 18S rRNA, catalase (CAT) and Cu/Zn-superoxide dismutase (SOD) used in this study have been previously described (Olsvik, Kristensen, Waagbø, Rosseland, Tollefsen, Bæverfjord & Berntssen 2005a). Primer and probe sequences for heat shock protein 70 (HSP70) were obtained from GenBank accession no. BG933934, and similar to CAT and SOD primers, did not span exon–exon borders as they were made from mRNA sequences. Amplified PCR product of HSP70 was sequenced, and a BLAST conducted at the homepage of the National Center for Biotechnical Information (NCBI) at the National Library of Medicine (http://www.ncbi.nlm.nih.gov/BLAST) to identify and ensure that the desired mRNA sequence was quantified. Sequencing of PCR product was

performed with a Big Dye version 3.1 sequencing kit (Applied Biosystems, Foster City, CA, USA) using an ABI PRISM® 377 Sequencer (Applied Biosystems), and performed at the University of Bergen Sequencing Facility (Norway).

RNA extraction and isolation

Total RNA from liver and distal intestine was isolated and extracted from 50–70 mg tissue using TRIZOL® reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Liver and distal intestine were homogenized with MagNA Lyser Green Beads (Roche, Indianapolis, USA), liver tissue using a MM301 shaker (Anders Pihl AS Sunnfjord, Norway) at full speed for 4 min and distal intestinal tissue using a Polytron (Kinematica, Newark, NJ, USA) until a homogeneous sample was achieved. For distal intestinal tissue, the procedure was modified with an additional step of 100 μL chloroform extraction. The RNA-isolated samples were diluted in RNase-free double distilled water (ddH2O) and treated with DNA-free™ kit (Ambion) according to the manufacturer's descriptions, and stored at −80 °C until further processing. For head-kidney and spleen tissues, disruption and homogenization of samples (15–25 mg) were performed with 5 mm stainless steel beads using a Mixer Mill MM 301 (Retsch, Haan, Germany). Total RNA from spleen and head-kidney was prepared from the lysate using a RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and treated on-column with RNase-free DNase (Qiagen). All steps were in accordance with the RNeasy® Mini Handbook for animal tissue.

Quantity and quality of all RNA samples were assessed with a NanoDrop® ND-1000 UV–Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) using an RNA 6000 Nano LabChip® kit (Agilent Technologies). Samples with optical density (OD) ratio $OD_{260}:OD_{280}$ within the range 1.8–2.1, 28S:18S ratios above 1.0, and RNA integrity number between 8 and 10 were accepted.

Real-time reverse transcriptase (RT)-PCR

A two-step RT-PCR protocol was used to measure the gene expression levels (mRNA) of selected target genes in liver (CAT, SOD and HSP70) and distal intestine (HSP70) of Atlantic salmon. Twofold dilutions of total RNA at four different concentrations (250, 125, 62.5 and 31.25 ng) were made to monitor the PCR amplification efficiency of each gene (a 1000-fold dilution of these concentrations was used for 18S rRNA) in each tissue, and run on the same PCR plates as the samples to be analysed through the entire two-step RT-PCR protocol. The complementary DNA (cDNA) of samples was synthesized from RNA (125 ng ± 5%) in triplicates on 96-well PCR plates. The RT-PCR runs were performed on a GeneAmp PCR 9700 (Applied Biosystems) using a TaqMan® Reverse Transcription Reagents kit (Applied Biosystems), following a modified protocol (Jordal, Torstensen, Tsoi, Tocher, Lall & Douglas 2005) but with a 20-μL total reaction volume and accordingly adjusted volumes of reaction reagents, to maintain the same reagent concentrations. Complementary DNA synthesis of 18S rRNA was carried out using random hexamer primers (2.5 μM), while oligo dT primers were used for cDNA synthesis of CAT, SOD, HSP70 and β-actin. Negative template controls (ntc: no template) and negative amplification controls (nac: no reverse transcriptase) were run for each PCR plate. The RT-PCR was performed as described by Jordal *et al.* (2005). The cDNA plates were stored at −20 °C after running the RT-PCR.

The real-time PCR step was performed on an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Oslo, Norway) as described by Jordal *et al.* (2005), but with modified concentrations of forward and reverse primers (400 nM each) and TaqMan MGB probes (100 nM), and the PCR program modified to run 50 cycles at 95 °C for 15 s (denaturation) followed by 1 min at 60 °C (annealing and extension). The PCR amplification efficiencies ranged from 0.95 to 1.00 for the transcribed genes.

For head-kidney and spleen, preparation of cDNA for gene expression analysis of interleukin-1 beta (IL-1β), β-actin and elongation factor 1-alpha (EF1-α) was carried out as described for confirmation of transcripts from subtractive libraries by Haugland, Torgersen, Syed & Evensen (2005). Complementary DNA was diluted 1:2 before use in real-time PCR. The real-time PCR step was run on a LightCycler 2.0 instrument (Roche Diagnostics, Basel, Switzerland) using LightCycler® TaqMan Master (Roche Diagnostics). The real-time PCR runs were performed in duplicate and in a total volume of 10 μL. The crossing point values were determined by use of the maximum-second-deriv-

ative function on the LightCycler software. The cycling conditions were 95 °C for 10 min to activate hot-start polymerase followed by 45 cycles of 95 °C for 10 s, 62 °C for 30 s and 72 °C for 10 s. Each reaction contained 2.0 μL LightCycler TaqMan Master (5X), 0.5 μL of forward and reverse primer (10 μM), 0.08 μL TaqMan probe (25 μM) and 4.92 μl PCR grade water; 2.0 μL diluted cDNA was used for template in each reaction. Products from both assays were tested by agarose gel electrophoresis to confirm one product of correct size.

Two reference genes were transcribed for each tissue, except for distal intestine where only 18S was used due to its higher stability in tissues with high cell turnover, as reported with tumour cell lines (Aerts, Gonzales & Topalian 2004). For liver, β-actin and 18S were transcribed, while EF1-α and β-actin were chosen for head-kidney and spleen tissue. The choice of reference genes was based on results from Olsvik, Lie, Jordal, Nilsen & Hordvik (2005b). The geNorm VBA applet was used to investigate reference gene stability (Vandesompele, De Preter, Pattyn, Poppe, Van Roy, De Paepe & Speleman 2002) and evaluate the most proper reference gene in each tissue, to calculate normalized expression of target genes. Consequently, β-actin was used for gene expression normalization in liver, 18S rRNA in distal intestine and EF1-α in head-kidney and spleen.

Total protein measurement

Total protein concentrations were measured in homogenates of liver and distal intestine samples which were subjected to enzyme activity or protein measurements of CAT, SOD and HSP70. Total protein was analysed by the bicinchoninic acid assay (BCA) method (Smith, Krohn, Hermanson, Mallia, Gartner, Provenzano, Fujimoto, Goeke, Olson & Klenk 1985). A complete BCA assay kit (Sigma, St Louis, MO, USA) was used for this measurement, following the manufacturer's instructions. Bovine serum albumin was used as standard and different dilutions made to generate a standard curve. Tissue sample dilutions were made according to the absorbance values, which were within the limits of the linearity range on the standard curve.

Enzyme activity measurement of CAT and SOD

CAT activity in liver and distal intestine was measured by recording the decrease in H_2O_2

concentration at 240 nm (Aebi 1984). Approximately 0.3 g tissue was homogenized in 3 mL 0.05 M sodium-phosphate buffer (pH 7.0) with 1% Triton X-100 (Sigma-Aldrich), using a Potter-Elvehjem homogenizer for liver tissue and ultra-turrax for distal intestinal tissue. The homogenates were centrifuged at 11 000 *g* for 30 min at 4 °C. The supernatants were incubated in ethanol and kept on ice for 30 min, to avoid the formation of an inactive complex of CAT with H_2O_2 (Cohen, Dembiec & Marcus 1970). Enzyme solutions from liver and distal intestines were diluted 1:401 and 1:68, respectively, in 0.05 M sodium-phosphate buffer, pH 7.0. One millilitre of substrate solution (30 mM H_2O_2) was added to 2 mL of enzyme solution (sample), and the decrease in absorbance was measured at 240 nm, every 5 s for 30 s using a UV-1601PC spectrophotometer (Shimadzu, Kyoto, Japan) and the software program UVProbe (Shimadzu). One unit (U) CAT was defined as the amount of enzyme needed to eliminate 1 μmol H_2O_2 min^{-1} (Aebi 1984). Values of CAT activity are expressed as U mg^{-1} total protein.

SOD activity in liver and distal intestine was assayed by the ferricytochrome *c* reduction method (Flohè & Otting 1984). Approximately 0.3 g tissue was homogenized in 3 mL 0.05 M sodium-phosphate buffer (pH 7.4) with 0.1 mM EDTA and 1% Triton X-100 (Sigma). The homogenates were sonicated for 2×15 s with 20 s interval to release mitochondrial SOD, before centrifugation at 13 000 *g* for 30 min at 4 °C. Samples were diluted 1:5 in 0.05 M sodium-phosphate buffer (pH 7.4). The xanthine–xanthine oxidase system was utilized as the source of superoxide anion radical O_2^-, and prepared by dissolving 5 μmol crystalline xanthine (Sigma-Aldrich) in 1.0 mM NaOH and 2 μmol cytochrome *c* from horse heart (Sigma-Aldrich) in 0.05 M sodium-phosphate buffer, and mixing the respective solutions in 1:11 dilution (substrate solution). The enzyme solution of xanthine oxidase (Sigma-Aldrich) had a concentration equivalent to the reduction of cytochrome *c* of 0.025 absorbance units min^{-1} (ca. 0.1 U mL^{-1}), without the presence of SOD (blank). Fifty microlitres of sample was added to 2.9 mL of substrate solution and the reaction initiated by adding 50 μL of enzyme solution. The reduction of cytochrome *c* was recorded by measuring the increase in absorbance units at 550 nm every 5 s for 2 min, using a UV-1601PC spectrophotometer (Shimadzu). One unit (U) of SOD was defined as the amount of enzyme

needed to inhibit the rate of cytochrome *c* by 50% (Flohè & Otting 1984). Values of SOD activity are expressed as U mg^{-1} total protein.

SDS-PAGE and Western blot

Liver tissue (0.2 g) was homogenized in 100 mM Tris–HCl buffer (pH 7.5) containing 0.1% sodium dodecyl sulphate (SDS) (Sigma-Aldrich), and Complete Mini protease inhibitor cocktail tablets (Roche Diagnostics) added immediately before homogenization to avoid protein degradation. Homogenization, sonication and centrifugation were carried out as previously described for SOD analysis. An aliquot of the supernatant was withdrawn for total protein measurement, and the remainder was stored at −80 °C until further analysis.

The proteins of the samples were separated in size using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method described by Laemmli (1970). Samples were diluted 1:1 in Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA, USA) containing β-mercaptoethanol (Sigma-Aldrich), and denatured at 95 °C for 5 min before loading onto the gels. Fifty microlitres of total protein (300 μg protein) was loaded on 10 wells of 7.5% Tris–HCl Ready Gel (Bio-Rad Laboratories) and the electrophoresis run in running buffer [25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS, pH 8.3] for 35–40 min at 200 V, using a Mini Protean III Cell (Bio-Rad Laboratories).

The relative concentration of HSP70 in each sample was determined by Western blotting using the method of electrophoretic transfer of proteins onto a nitrocellulose membrane (Towbin, Staehelin & Gordon 1979), but using Immun-Blot^{TM} PVDF (polyvinylidene difluoride) membrane (Bio-Rad Laboratories) to increase the binding of protein. The electrophoretic transfer was accomplished using a Mini Protean III Cell (Bio-Rad Laboratories) running for 60 min at 100 V in which the membrane was soaked in transfer buffer [25 mM Tris, 192 mM glycine, 20% (v/v) methanol, pH 8.3]. After blotting, the PVDF membranes were washed for 5 min in Tris-buffered saline (TBS) (20 mM Tris, 500 mM sodium chloride, pH 7.5) containing 0.05% Tween-20 (TBS-T) and incubated for 15 min in blocking solution (3% skim milk in TBS-T) before repeated washing in TBS-T for 2 × 5 min. The membranes were further incubated for 1 h with polyclonal antibody rabbit anti-HSP70 (SPA-763E; Stressgen Bioreagents, Ann Arbor, MI, USA) diluted 1:1000 in blocking solution. After primary antibody incubation, the membrane was washed for 2 × 5 min in TBS-T and incubated for 1 h in alkaline phosphatase-conjugated anti-rabbit IgG secondary antibody (SAB-301; Stressgen Bioreagents) diluted 1:10 000 in blocking solution. Colorimetric detection using premixed nitroblue tetrazolium (NBT)/5-bromo-4chloro-3-indolyl phosphate (BCIP) solution (0.48 mM NBT, 0.56 mM BCIP, 10 mM Tris, 59.3 mM MgCl$_2$, pH 9.2) (Bio-Rad Laboratories) was used to detect the HSP70 protein on the membranes. The membranes were photographed with GelDoc 2000 (Bio-Rad Laboratories) and density of the bands quantified using the software program Quantity One (Bio-Rad Laboratories), based on a standard curve generated from recombinant Chinook salmon HSP70 (SAB-301), covering the range between 10 and 150 ng HSP70. The recombinant Chinook salmon HSP70 was run on each gel as a positive control.

Differential white blood cell count

Blood was sampled as previously described and blood films were prepared on glass microscope slides (superfrost slides) from five fish per tank (15 fish per diet). The blood films were air-dried and fixed in absolute methanol for 5 min. Within 24 h all blood films were stained with May–Grünwald stain (Merck & Co. Inc., Whitehouse Station, NJ, USA) for 5 min and Giemsa stain (Merck & Co. Inc.) for 15 min. One hundred white blood cells were counted for each fish sampled and classified either as lymphocytes, granulocytes or monocytes, and expressed as a percentage of the total number of leucocytes examined. Each sample was blinded and examined randomly with an Olympus light microscope (BX 51) (Olympus, Center Valley, PA, USA) at 400×.

Statistics

Calculations of PCR amplification efficiencies of each gene and mean normalized expression of target mRNAs were performed using the Microsoft Excel-based software tool Q-Gene version 384 according to Muller, Janovjak, Miserez & Dobbie (2002). Gene expression data from individual fish were statistically tested using a relative expression soft-

ware tool (REST©2005), applying a Pair-Wise Fixed Reallocation Randomisation Test© with 50 000 randomizations, as described by Pfaffl, Horgan & Dempfle (2002). Except for gene expression data, all other data were tested statistically using Statistica version 7.0 (Statsoft Inc., Tulsa, OK, USA). Data displaying normality (Kolmogorov–Smirnov test) and homogeneity of variance (Levene test) were subjected to one-way ANOVA or nested ANOVA, and significant differences revealed with Tukey HSD tests. Data failing normality tests and displaying heterogeneity of variance were tested statistically applying the nonparametric Kruskal–Wallis ANOVA and nonparametric multiple comparison tests to reveal differences. A significance level of $P < 0.05$ was used for all statistical tests.

Results

The enzyme activity of CAT (U mg^{-1} protein) in liver (Table 2) was found to be significantly lower for fish fed the GM maize diets (low and high levels grouped together) compared with fish fed nGM maize (low and high levels grouped together), and compared with the reference diet group. Normalized gene expression of CAT in liver showed no significant variations between diet groups (Table 3). Enzyme activity of CAT in distal intestine (Table 2) was significantly higher for fish fed high and low GM maize (grouped together), when compared with fish fed the reference diet. No

significant correlations were found between enzyme activity and normalized gene expression of CAT in liver, for any diet group.

Enzyme activity of SOD (U mg^{-1} protein) in liver and distal intestine (Table 2) was significantly higher for fish fed GM maize (low and high grouped together) compared with fish fed nGM maize (low and high grouped together), and compared with the reference diet groups. No significant differences were observed in normalized gene expression of SOD (Table 3) in liver or distal intestine between any of the diet groups. Further, no significant correlations were found between enzyme activity and normalized gene expression of SOD in liver for any diet group.

Protein level of HSP70 in liver was found to be significantly higher for fish fed GM maize (low and high grouped together) compared with fish fed reference diet (Table 2). No significant differences were observed in normalized gene expression of HSP70 in liver (Table 3), between any diet groups. The protein level of HSP70 in distal intestine was not detectable using Western blot analysis. No significant correlations were found between protein level and normalized gene expression of HSP70 in liver for any diet group.

Normalized gene expression of CAT in liver correlated positively with normalized gene expression of SOD in liver in fish fed high GM maize ($R^2 = 0.74$, $P < 0.05$), but also in fish fed high nGM maize ($R^2 = 0.95$, $P < 0.05$), but not for any other diet groups.

Table 2 Enzyme activities of CAT and SOD in fractions of liver and distal intestine, and protein level of HSP70 in liver of Atlantic salmon fed different levels of GM and nGM maize, and a reference diet

	Reference diet	nGM maize diet		GM maize diet		Statistics (Kruskal–Wallis ANOVA)		Range	CV (%)[1], protein activity/ level
		Low	High	Low	High	Diet	GM/nGM		
Liver									
CAT (U mg^{-1} protein)	6317 (229)[b]	5694 (708)[ab]	6113 (180)[ab]	4768 (397)[a]	5328 (440)[ab]	s	s[2,3]	1400–7572	12
totSOD (U mg^{-1} protein)	93 (9)[a]	101 (6)[a]	110 (1)[ab]	131 (8)[b]	134 (11)[b]	s	s[2,3]	70–166	10
HSP70 (ng mg^{-1} protein)	348 (102)	285 (26)	391 (32)	551 (79)	406 (43)	ns	s[3]	36–298	39
Distal intestine									
CAT (U mg^{-1} protein)	230 (19)	279 (20)	307 (66)	335 (30)	331 (36)	ns	s[3]	108–615	20
TotSOD (U mg^{-1} protein)	155 (14)[a]	150 (5)[a]	153 (12)[a]	231 (28)[b]	184 (22)[ab]	s	s[2,3]	117–295	15
HSP70	nd	nd	nd	nd	nd	–	–	–	–

Values are given as mean ($n = 3$) with standard error of mean (SEM) in parenthesis. Mean values in the same row displaying different subscripts are significantly different from each other ($P < 0.05$). nd, not detected; ns, non-significant; (assumptions for statistical test were not fulfilled).
[1]CV = [(SD$_{mean}$/mean) × 100].
[2]Significant between GM maize diets vs. nGM maize diets.
[3]Significant between GM maize diets vs. reference diet.

Table 3 Mean normalized expression (MNE) of CAT, SOD and HSP70 in liver and HSP70 in distal intestine (DI), and IL-1β in spleen and head-kidney (HK) of Atlantic salmon fed different levels of GM and nGM maize in diet, and a reference diet

Enzyme/ protein	Reference diet	nGM maize diet		GM maize diet		Statistics (REST 2005)		Range MNE	CV^1 (%) MNE
		Low	High	Low	High	Diet	GM/nGM		
Liver									
CAT	0.227 ± 0.011	0.254 ± 0.050	0.199 ± 0.016	0.189 ± 0.018	0.165 ± 0.024	ns	ns	0.074–0.418	19.7
SOD	1.069 ± 0.123	1.221 ± 0.207	1.086 ± 0.071	1.110 ± 0.055	0.983 ± 0.106	ns	ns	0.642–1.574	17.6
HSP70	3.890 ± 0.197	4.297 ± 0.534	3.614 ± 0.375	4.052 ± 0.247	3.735 ± 0.152	ns	ns	1.859–6.759	13.2
DI									
HSP70	0.001 (<0.001)[a]	0.001 ± <0.001[ab]	0.002 ± <0.001[b]	0.002 ± <0.001[ab]	0.002 ± <0.001[ab]	s	ns	0.00006–0.00449	25.7
Spleen									
IL-1β	1.56 ± 0.01	1.52 ± 0.02	1.54 ± 0.02	1.57 ± 0.03	1.55 ± 0.01	ns	ns	1.416–1.682	2.2
HK									
IL-1β	1.58 ± 0.02	1.57 ± 0.02	1.58 ± 0.02	1.62 ± 0.04	1.60 ± 0.02	ns	ns	1.439–1.758	2.4

Values are given as mean ($n = 3$) ± standard error of mean (SEM). Mean values in the same row displaying different subscripts are significantly different from each other ($P < 0.05$). s, significant; ns, non-significant.
[1]CV = [(SD_mean/mean) × 100].

Normalized gene expression of IL-1β in spleen and head-kidney did not vary significantly between diet groups (Table 3). For fish fed high GM maize, normalized gene expression of IL-1β in spleen correlated positively with normalized gene expression of IL-1β in head-kidney ($R^2 = 0.71$, $P < 0.05$).

Fish fed high GM maize showed a significantly higher proportion of granulocytes, and a lower proportion of lymphocytes, compared with fish fed high nGM maize (Table 4). The proportion of monocytes in the high GM maize groups was markedly higher (not significant) when compared with the high nGM maize groups. Moreover, the sum of granulocytes and monocytes were significantly higher for fish fed high GM maize compared with fish fed high nGM maize.

Normalized gene expression of IL-1β in spleen and head-kidney did not correlate significantly with the proportion of white blood cells for any of the populations investigated.

Discussion

Fish performance in the present study was very good, where fish from all experimental groups grew from an initial 155 g to final weights varying from 560 to 624 g, showing specific growth rates around 1.6 independent of diet treatment (Hemre *et al.*, unpublished data). Slightly lower growth rates were observed for fish fed GM maize compared with nGM maize regardless of inclusion level, but this coincided with slightly lower feed intake for the same diet groups (Hemre *et al.*, unpublished data), suggesting a possible secondary effect such as changes in taste of the feed, perhaps resulting from changes in composition of the GM maize such as secondary metabolites or other substances that were not analysed. Small differences were found in phytic acid content between the nGM maize compared with GM maize, but the range was within natural variation between conventional varieties (Francis, Makkar & Becker 2001). The experimental diets were isocaloric and provided the same nutrient profile. The fish experienced good feed utilization (feed conversion rates ranging 0.87–0.91) with no diet-related differences (Hemre *et al.*, unpublished data), indicating that all diets were well utilized by the salmon.

Due to increased liver SOD and decreased liver CAT enzyme activities only in the GM maize groups, there might have been some component in the GM maize resulting in a secondary effect that

Table 4 Differential counts of white blood cells (granulocytes, monocytes and lymphocytes) in Atlantic salmon fed high levels of GM maize and nGM maize, and reference diet

White blood cell population	Reference diet	nGM maize diet (high)	GM maize diet (high)	Statistics (ANOVA)
Granulocytes (%)	6.7 (1.5)[ab]	3.9 (0.7)[a]	9.2 (1.2)[b]	s
Monocytes (%)	12.1 (1.5)	8.2 (0.8)	13.5 (1.8)	ns
Lymphocytes (%)	81.0 (2.7)[ab]	88.5 (1.3)[a]	77.4 (2.3)[b]	s
Sum granuloyctes plus monocytes (%)	18.8 (2.7)[ab]	12.1 (1.2)[a]	22.7 (2.2)[b]	s

Values are given as mean ($n = 3$) with standard error of mean (SEM) in parenthesis. Mean values in the same row displaying different subscripts are significantly different from each other ($P < 0.05$). s, significant; ns, non-significant.

can be linked to the antioxidant system of liver cells (Armstrong 2002). The SODs are a group of metalloenzymes and catalyse the conversion of the free radical superoxide anion into hydrogen peroxide and O_2 (Fridovich 1974; Villafranca, Yost & Fridovich 1974), reducing the level of this reactive oxygen species. Hydrogen peroxide is subsequently detoxified by CAT to O_2 and water (Aebi 1984). In the present study, lower feed intake was recorded in the GM maize groups (Hemre *et al.*, unpublished data). Food deprivation has been linked to lowered liver CAT activity in *Sparus aurata* L. (Pascual, Pedrajas, Toribio, Lopez-Barea & Peinado 2003). However, all fish showed good appetite and fish growth was better in the present study compared with many earlier studies (Hemre, Sandnes, Lie, Torrisen & Waagbø 1995; Hemre, Waagbo, Hjeltnes & Aksnes 1996). Therefore, food deprivation can only to a minor extent explain the variable SOD and CAT enzyme activities and HSP70 protein level observed in the liver of fish fed GM maize. In mice, irregular-shaped hepatocyte nuclei were found after feeding GM soybeans, indicating a high metabolic rate (Malatesta *et al.* 2002). In the same study, a higher number of hepatocyte nuclear pores was also observed in the mice fed GM soybeans, possibly caused by an intense molecular trafficking (Malatesta *et al.* 2002). An altered hepatosomatic index was observed in the present study (Hemre *et al.*, unpublished data), showing an increased relative size in fish fed GM maize, compared with fish fed nGM maize, giving a stronger indication of possible secondary effects on liver metabolism due to GM presence in the feed. Furthermore, HSP70, a protective protein reported to respond to a variety of stressors (Iwama, Thomas, Forsyth & Vijayan 1998; Iwama, Vijayan, Forsyth & Ackerman 1999; Basu, Todgham, Ackerman, Bibeau, Nakano, Schulte & Iwama 2002), was also found to be significantly higher in the GM maize diet groups. Thus, there seems to be some indication of altered liver metabolism in the present

study. However, these changes might not have been strong enough to result in morphological changes, as histological examination of liver recorded a normal liver morphology (Hemre *et al.*, unpublished data).

Recent published work has shown that dietary DNA is absorbed into the bloodstream and taken up by vital organs in Atlantic salmon (Nielsen *et al.* 2005). As already mentioned, the antioxidant defence systems of SOD and CAT are closely related to activity level and metabolic rate in animals, including fish (Filho 1996), where levels of SOD and CAT in liver and blood are positively correlated with fish activity. Different individual activity levels might therefore affect the activities of SOD and CAT, perhaps explaining the large individual variability observed. Liver activities of CAT and SOD are also affected by dietary starch sources, and to increase with raw carbohydrates and high lipid levels in the diet (Rueda-Jasso, Conceicao, Dias, De Coen, Gomes, Rees, Soares, Dinis & Sorgeloos 2004). The diets of the present experiment were extruded and heat-treated, and high starch digestibility was obtained for all three maize qualities, with no differences between diet groups (Hemre *et al.*, unpublished data).

The increased SOD and CAT enzyme activities in distal intestine of salmon fed GM maize might be linked to possible presence of 'δ-endotoxin' in the MON810 maize. The level of δ-endotoxin was not measured in the feed or any organs in the present study. However, feeding rats with potatoes treated with isolated 'δ-endotoxin' from a Bt strain carrying the Cry1 gene, resulted in structural changes, such as hypertrophied and multinucleated enterocytes and development of hyperplastic cells in mice ileum (Fares & El-Sayed 1999). Proliferation of gastrointestinal mucosa of rat intestine is reported after feeding rats with GM potatoes expressing the *Galanthus nivalis* lectin (Ewen & Pusztai 1999). The gastrointestinal tract is the portal of entry and first contact site with foreign

DNA and proteins (Palka-Santini, Schwarz-Herzke, Hosel, Renz, Auerochs, Brondke & Doerfler 2003). A possible stress response occurring from feed components would most likely be observed here. It might also be that large transgenic DNA sequences act as stressors. Quite large transgenic DNA sequences survive feed processing and can be found in all parts of the digestive tract, and can be absorbed (Chainark et al. 2004; Sanden et al. 2004; Sanden, Berntssen & Hemre 2006b). The slight but still significant increased SOD and CAT enzyme activity observed in the distal intestine in the present study might therefore be partly due to an induced stress response from the transgenic protein or secondary products from the gene modification process, present in the GM maize used.

Profiling methods such as DNA and RNA microarray technologies, proteomics and metabolomics have been suggested as useful methods to detect unintended effects in GM safety assessments (Kuiper, Kok & Engel 2003). Normalized gene expression of the stress proteins CAT, SOD and HSP70 in liver and distal intestine showed no differences between diet groups. No significant correlations were observed between the normalized mRNA expression levels and the enzyme activity or protein levels, which agrees with some previous studies (Misra, Crance, Bare & Waalkes 1997; Hansen, Rømma, Garmo, Olsvik & Andersen 2006), but not with other studies (Kawamoto, Matsumoto, Mizuno, Okubo & Matsubara 1996; Anderson & Seilhamer 1997). Protein turnover has been suggested to be more slowly regulated than the regulation of mRNA levels, as observed with hypoxia exposure in blue crab (Brouwer, Larkin, Brown-Peterson, King, Manning & Denslow 2004). The gene expression profile reflects a snapshot of cell activity at the moment of sample withdrawal. The amount of transcribed genes might be rapidly regulated, e.g. due to fluctuations of unknown and uncontrollable extrinsic or intrinsic factors, which might explain some of the large variability observed in normalized gene expression of target genes in the present study. Lack of a positive stress control group for CAT, SOD and HSP70 might be significant, where individual variations in the ability to resist stress and recover from stress could be recorded. However, controls for tank effects ($n = 3$) were included, and the experimental conditions were as identical as possible for all diet groups, except for the different ingredients in the experimental diets. All animals were

checked for feed remnants in the gastrointestinal tract before sampling, ensuring the sampled fish were in an absorptive state and exposed to the experimental diets at the time of sampling.

Variations in relative spleen size were observed when feeding salmon GM soybeans (Hemre et al. 2005; Sagstad et al., unpublished data) and in spleen and head-kidney relative sizes when feeding GM maize in the present study (Hemre et al., unpublished data). The uncertainties of the meaning of these results lend to investigations of gene expression of IL-1β in these two important immune organs. Cytokines are small secreted proteins that control and coordinate the innate and acquired immune responses (Magnadottir 2006). IL-1β is a pro-inflammatory cytokine and acts on T helper cells providing a co-stimulatory signal for activation following antigen recognition (Kuby 1997), and is part of the acute phase responses (Decker 2006). Ingested foreign DNA has earlier been traced and found in spleen, leucocytes and white blood cells of mice (Schubbert, Renz, Schmitz & Doerfler 1997), and an incorporation of transgenic DNA into these organs, or the presence of potent allergens, might affect the immune system. Normalized gene expression of IL-1β was, however, not different between diet groups in this study. However, significant differences found in the proportion of some white blood cell populations, agrees with a study on rats fed GM cucumber, whereas an increase in neutrophilic granulocytes was observed (Kosieradzka, Sawosz, Pastuszewska, Szwacka, Malepszy, Bielecki & Czuminska 2001). The present findings of an increased granulocyte population, and a significant increase in the total granulocyte and monocyte populations, in fish fed high GM maize compared with fish fed high nGM maize, possibly indicates some immune response resulting from the presence of GM maize in the diet. It might be that the gene modification process has resulted in the formation of new and unknown proteins that act as weak allergens. Further studies, applying advanced proteomic methods to screen for new proteins, might provide more answers to the possible immune responses observed in the present study.

Thus, this study has shown that feeding Atlantic salmon GM maize resulted in small changes in CAT and SOD enzyme activities in liver and distal intestine, and HSP70 protein level in liver. These signs were indicative of a mild stress response, but were not correlated with changes in normalized gene expressions of these stress proteins. Differential

leucocyte counts showed altered proportions of white blood cell populations, suggestive of an immune response taking place in the blood as a response to the GM maize in the diet.

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