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Effects of oral *Bt*-maize (MON810) exposure on growth and health parameters in normal and sensitised Atlantic salmon, *Salmo salar* L.

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Abstract

Responses to GM maize *Bt*-maize, MON810) expressing Cry1Ab protein from the soil bacterium *Bacillus thuringiensis* (*Bt*) in diets for both normal and immune-sensitised (with soyabean meal (SBM)-induced enteropathy) post-smolt Atlantic salmon were investigated following 33 and 97 d of exposure. Triplicate tanks of salmon were fed one of four diets, all containing 20 % whole-kernel meal maize, either *Bt*-maize or its near-isogenic maternal line, without or with 15 % extracted SBM inclusion. The fish fed *Bt*-maize utilised the feed less efficiently, as revealed by lower protein and mineral digestibilities and lower lipid and energy retention efficiencies. Higher intestinal weight, as well as increased interferon-γ and decreased sodium–glucose co-transporter mRNA expression, and a transient increase in T-helper cell presence, as measured by cluster of differentiation 4 (CD4) protein in the distal intestine (DI), may partly explain the lower nutrient digestibilities and retentions. The *Bt*-maize seemed to potentiate oxidative cellular stress in the DI of immune-sensitised fish, as indicated by increases in superoxide dismutase and heat shock protein 70 mRNA expression. The data suggest that Cry1Ab protein or other antigens in *Bt*-maize have local immunogenic effects in salmon DI. No systemic immune responses could be detected, as indicated by haematology, differential leucocyte counts, plasma clinical chemistry, as well as absence of Cry1Ab-specific antibodies and Cry1Ab protein in plasma. The responses to *Bt*-maize observed in the present study differed from results from earlier studies in salmon and other animals fed the same event *Bt*-maize. Longer-term experiments and more in-depth studies on intestinal physiology and immune responses are needed to evaluate health implications.

Key words: Cry1Ab: GM plants: Safety: Soyabean meal

GM maize is one of the major GM crops grown worldwide. In comparison to 2010, the field area of GM maize planted increased by 11 % to just under 51 million hectares in 2011, occupying 32 % of the total global maize cultivation area[(1)]. It is becoming increasingly difficult to obtain non-GM maize. Various maize products are used in commercial diets for farmed fishes. The carbohydrate in whole-kernel meal is an energy source and the starch serves as a feed binder. As a result of the increasing production of salmon and other fish species and decreasing availability of fishmeal, which has been the main dietary protein source, especially for piscivorous fish, plant protein sources are increasingly being used in diets to obtain a cost-efficient, sustainable aquaculture industry. Maize gluten meal, a by-product of starch extraction containing approximately 60 % protein, has increased in importance as a protein source in fish feeds. The wet-milling process used to extract starch and provide maize gluten meal as a by-product is carried out by steeping the maize kernels in water at 50°C, mildly acidified with sulphur dioxide (0·01 %) to prevent microbial growth, with subsequent grinding and mechanical separation of the starch and gluten. This mild treatment is not expected to denature the maize proteins, including any transgenic proteins (see later), although specific studies investigating this are apparently absent from the scientific literature. If they remain intact and biologically active, maize gluten meal would contain higher concentrations of any transgenic proteins than whole-kernel meal. Effects of maize gluten meal derived

Abbreviations: *Bt*, *Bacillus thuringiensis*; CD4, cluster of differentiation 4; DI, distal intestine; HSP70, heat shock protein 70; IFN, interferon; MI, mid intestine; nGM, non-GM; PCNA, proliferating cell nuclear antigen; PI, proximal intestine; SBM, soyabean meal; SGLT, sodium-dependent glucose transporter; SOD, superoxide dismutase.

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from GM maize as a feed ingredient for cultured fish have apparently not been investigated.

GM *Bt*-maize (MON810) expresses Cry1Ab protein, by transfer of a gene found in *Bacillus thuringiensis* (*Bt*) bacteria, which confers resistance to the European corn borer, *Ostrinia nubilalis*. The Cry proteins are endotoxins produced by the soil bacterium *Bt*, and each has putative species-specific toxicity against insects[2,3]. The high specificity of the three-domain crystal proteins towards target insects arises due to the need for alkaline pH (>9·5) for solubilisation, presence of serine proteases in the midgut for processing and presence of specific receptors in the insect midgut epithelial cells for binding[3]. The Cry protoxins are considered non-toxic to higher animals[4], putatively due to differing pH and enzymatic and binding conditions in the gastrointestinal tract. In one study, the Cry1Ab protein was reported to be rapidly digested in simulated gastric and intestinal fluids, as immune reactive bands were not identified[5], whereas another study concluded that under more physiologically relevant *in vitro* conditions (pH 2·5, pepsin-to-substrate ratio 1:20 and in the presence of phosphatidylcholine) Cry1Ab was stable[6]. Furthermore, at least some studies indicate that ingested Cry1A toxins may elicit humoral and mucosal immune responses in mice[7–9], suggesting that even fragments of the protein may be immunogenic, although this remains to be specifically investigated. Marked histological changes in rat liver and kidney by *Bt*-maize have been observed[10]. A review of rat feeding studies with three different commercialised GM maizes (NK603, MON810 and MON863) suggested sex- and often dose-dependent side effects, mostly affecting kidneys and liver. The effects differed between GM types. Moreover, effects on heart, adrenals, spleen and blood cells were also frequently identified[11].

Concerns regarding possible allergenicity of GM plant crops have also been raised[12,13]. Transgenic proteins have been suggested to be potentially antigenic or allergenic, possibly due to differences in post-translational modifications following integration of the transgenic DNA into a foreign organism's genome. It is not known whether Atlantic salmon or other fish have the ability to elicit a type I hypersensitivity reaction (classic allergic response), as they do not appear to have an IgE-analogue and their mast cells are devoid of histamine[14]. However, the putative T-cell-mediated type IV hypersensitivity reaction in the distal intestine (DI), caused by as little as 5–10 % inclusion of both full-fat and extracted soyabean meal (SBM)[15–17], appears to have similar histopathological and immunological characteristics to that of coeliac disease (gluten intolerance) or other inflammatory bowel diseases in human subjects. It may serve as a comparative model for studying intestinal pathophysiological and immunological responses in general, as well as investigating whether a pre-existing hypersensitivity reaction alters the fish's response to a potential antigen in the GM plant ingredient.

The purpose of the present study was to assess whether responses in Atlantic salmon to dietary inclusion of *Bt*-maize expressing Cry1Ab differed from the near-isogenic maternal line of maize and also when the fish were sensitised with 15 % SBM inclusion in diet (SBM-induced enteropathy). Initially, the intention was to test maize gluten meal made from the *Bt*-maize, as this is a more commonly used maize product in salmon diets. However, we were unable to locate a mill that could process the small quantities of *Bt*-maize and control non-GM maize that we had at our disposal within the financial and time restraints of the project. Thus, the feeding experiment was carried out with 20 % inclusion level of whole-kernel maize meal. Samples were taken from fish exposed to the diets following an initial 33 d period, as well as a longer period of 97 d. A wide range of physiological responses were assessed in an attempt to identify any changes that may serve as biomarkers for *Bt*-maize exposure, including (1) growth performance and feed utilisation; (2) health parameters such as haematology, plasma clinical chemistry and relative weights and histomorphology of main organs; (3) Cry1Ab protein and specific antibodies in plasma; (4) digestive and intestinal function and (5) distal intestinal cell proliferation, oxidative stress and immune responses.

Materials and methods

Feed ingredients and experimental diet

Two maize types, *Bt*-maize (MON810) and its near-isogenic maternal line (non-GM (nGM) maize), were derived from planted seed varieties PR34N44 and PR34N43, respectively, provided by Pioneer. They were grown simultaneously in neighbouring fields in Spain. All the maize grains were dried and ground just prior to diet preparation to obtain whole-maize meal. Feeds were prepared by Nofima AS. Diet formulations are given in Table 1. Yttrium oxide was added as a biological marker for apparent digestibility determinations. All diets were balanced regarding vitamins and minerals according to estimated requirements[18]. Protein, lipid and energy levels were kept as similar as possible and were comparable between each nGM diet and GM counterpart with and without SBM, respectively. The diets were extruded with a feed particle (pellet) size of 3·5 mm. Salmon feed production using extrusion is a common practice in Norwegian aquaculture. Rausell *et al.*[19] reported that heat up to 60°C did not denature the pore-forming domain of the Cry1Ab protein, although the receptor-recognising domains may be more heat sensitive, and Xu *et al.*[5] reported anti-Cry1Ab binding to Cry1Ab protein treated at 100°C for up to 60 min. Hence, Cry1Ab protein integrity is expected to be at least partially intact following extrusion.

Experimental design and facilities

The experiment was carried out using a 2 × 2 factorial design with four diet groups. The factors GM and SBM inclusion were tested separately and in combination. The feeding trial was conducted at the aquaculture research facilities of Nofima AS following the institutional and national guidelines for the care and use of animals, and approved by the National Animal Research Authority in Norway. Post-smolt Atlantic salmon of the Sunndalsøra breed with an initial average weight of 93·8 (SE 0·3) g were randomly allocated, 100 fish

Table 1. Formulation and proximate composition of the experimental diets on an as-fed basis

Formulation	nGM maize	*Bt*-maize	nGM maize + SBM	*Bt*-maize + SBM
Ingredient (g/kg)				
Fish meal (221/07)	486	486	382	382
nGM maize	196	–	196	–
GM maize (MON810)	–	196	–	196
Extracted SBM (180/07)	–	–	147	147
NorSalmOil	245	245	202	202
Krill meal (103/07)	49	49	49	49
Vitamin mix	196	196	196	196
Mineral mix	3·9	3·9	3·9	3·9
Yttrium oxide	0·1	0·1	0·1	0·1
Carophyll Pink 10 %	0·3	0·3	0·3	0·3
Proximate composition (g/kg)				
DM	948	943	943	912
Crude protein	402	392	394	389
Crude lipid	312	312	258	247
Starch	138	134	141	137
Ash	76	75	74	71
Residue*	20	30	76	68
Yttrium oxide	0·08	0·08	0·08	0·08
Gross energy (MJ/kg)†	24·2	23·9	21·9	21·3

nGM, non-GM; *Bt*, *Bacillus thuringiensis*; SBM, soyabean meal.
* Residue = DM−(protein + lipids + starch + ash).
† Gross energy was calculated using the energy concentrations of 39·5 for lipid, 23·6 for protein and 17·2 kJ/g for starch.

per tank, to twelve tanks. Triplicate tanks of fish were fed one of the four experimental diets. Water surface area in the tanks was 1 m^2 and water depth 50 cm. The tanks contained filtered running seawater with a temperature of 8·0 ± 0·5°C and salinity between 31 and 32‰. Fish were continuously fed by automatic disc feeders. During the trial, fish were subjected to a 24 h light photoperiod.

Sampling

Sampling was conducted following 33 and 97 d of dietary exposure to the experimental diets. The fish were not fasted before the samplings, as this reduces potential diet-induced inflammatory changes in the intestine[15] as well as changes in physiological parameters[20]. A total of ten randomly selected fish per tank were anaesthetised, individually weighed and body (fork) length measured. Blood was collected from the caudal vein by means of a heparinised medical syringe. Organs including liver, spleen, head kidney, gonads (the latter only large enough to be weighed at 97 d) and the different regions of the intestine were removed and weighed for calculation of organosomatic indices. Prior to weighing, the intestinal tract was cleared of visceral fat and luminal content, and then divided into proximal intestine (PI), mid intestine (MI) and DI, as described earlier[21]. From four of the ten fish per tank, samples from the PI, MI, DI, spleen, head kidney and liver for histological examination were collected, fixed in 4 % buffered formaldehyde solution for 24 h and subsequently stored in 70 % ethanol until further processing. For mRNA expression investigations, DI tissue samples were rinsed in sterile PBS, transferred to RNA later for 24 h and stored at −20°C. Head kidney, spleen and intestinal samples for protein expression and/or digestive enzyme analyses were frozen in liquid N_2 and stored at −80°C. Intestinal contents of MI (at 97 d only) and DI (at both 33 and 97 d) were gathered from ten fish, weighed and freeze-dried. Faeces were collected from thirty fish by stripping from the DI, pooled and frozen on dry ice for digestibility measurements. For composition analyses, whole fish and livers from an additional five fish per tank at the 97 d sampling time were pooled, frozen in liquid N_2 and stored at −80°C.

Chemical composition analyses

Diets and faeces were analysed for DM, crude protein, crude lipid, starch, ash (mineral) and yttrium oxide (Y_2O_3), the latter by inductivity coupled plasma mass spectroscopy, as previously described[22]. Whole fish and livers were analysed for DM, crude protein and crude lipid. Liver glycogen was also analysed. DM was measured by drying at 103°C for 24 h with the exception of faeces, which were freeze-dried. For ash, samples were weighed before and after burning at 540°C. Fat in liver and whole body was determined gravimetrically after ethyl-acetate extraction and the fat in feed and faeces after acid hydrolysis and extraction with diethyl ether. N was measured with a nitrogen determinator (LECO FP-428; Väsby) according to Association of Official Analytical Chemists (AOAC) official methods of analysis[23] and crude protein calculated as N × 6·25. Starch and glycogen were measured by enzymatic degradation[24].

Haematology, plasma clinical chemistry and detection of plasma Cry1Ab protein and specific antibodies

Blood smears of whole blood on microscope slides were prepared for differential blood cell counts using standard techniques. Haematocrit was immediately measured using microhaematocrit tubes centrifuged at 13 000 rpm for 5 min. Erythrocyte counts and Hb were measured on a Cell-Dyn 400 (Sequoia-Turner) according to the manufacturer's instructions, using Para 12 control blood (Streck) for calibration.

The rest of the whole blood was centrifuged at 3000 ***g*** for 10 min to obtain the plasma fraction. Plasma clinical chemistry

was performed at the Central Laboratory of the Norwegian School of Veterinary Science using Advia® 1650 (Bayer Healthcare), an automated analysis system for clinical chemistry. Total protein, globulins, albumin, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, amylase, lipase, TAG, total bilirubin, total bile acids, NEFA and cholesterol were analysed using standard techniques.

Cry1Ab protein levels and specific antibodies in plasma were determined as previously described[25] and modified for salmon. Briefly, plasma Cry1Ab protein levels were analysed by 'sandwich' ELISA using a commercially available kit QuantiPlate for Cry1Ab/Cry1Ac (Envirologix) following manufacturer's instructions. A standard curve was prepared by using purified Cry1Ab protein standards (0–5 μg/ml) provided in the kit. The mean absorbance read at 450 nm against reference wavelength 630 nm was calculated and used to determine sample concentrations. Positive and negative controls were prepared with the kit Cry1Ab-positive control solution as internal standards. The matrix-specific limit of detection of the adapted kit was determined by interpolation at optical density units (ODmatrix) from the Cry1Ab standard curve. ODmatrix units were determined to be 3 SD from the mean of a population of negative samples measured for a specific matrix. The criteria for positive samples was to exceed the matrix-specific limit of detection.

For detection of specific antibodies against Cry1Ab in plasma, ELISA plates were coated overnight at 4°C with 1 mg/ml of purified Cry1Ab toxin (a fermentation product of *B. thuringiensis* var. *Kurstaki* from Dipel, produced by Valent BioSciences Corporation) in 0·05 M-carbonate–bicarbonate buffer (pH 9·6). The plates were blocked for 1 h at 37°C with 0·01 M-PBS (pH 7·4), containing 1 % gelatine. PBS alone or serial dilutions (from 1:5 to 1:78 125) of fish plasma were added to the plates and incubated for 1 h at 37°C. Plates were then incubated with anti-fish IgM mouse IgG1 (1:16 000) antibodies for 1 h at 37°C. Following this, biotin-labelled anti-mouse IgG1 antibody conjugate (1:5000) was added to the wells and the plates were incubated for 1 h at 37°C. Horse-radish peroxidise-labelled avidin conjugate was subsequently added and the incubation step was repeated. For colour development, H_2O_2/*o*-phenylenediamine-containing substrate solution (pH 5) was added and the plates were developed for 5 min in the dark. The enzyme reaction was stopped by the addition of 4 M-H_2SO_4. The absorbance was read at 492/630 nm. Samples were analysed in duplicate and the washing procedure repeated after each step involved manually washing the plates twice with PBS containing 1 % gelatine and decanting. Antibody titre was defined as the maximum serum dilution with an absorbance measurement higher than the blank signal plus three times the standard deviation.

Histology

All formalin-fixed tissues were routinely dehydrated in ethanol, equilibrated in xylene and embedded in paraffin according to standard histological procedures. Sections of 5 μm were stained with haematoxylin and eosin and blindly evaluated under a light microscope. Distal intestinal morphology was scored according to the criteria previously described in Atlantic salmon with SBM-induced enteritis[15]: (1) widening and shortening of the intestinal folds; (2) loss of the supranuclear vacuolisation in the absorptive cells (enterocytes) in the intestinal epithelium; and (3) cellular infiltration of a mixed leucocyte population in the central lamina propria within the intestinal folds as well as in the submucosa. The degree of histological changes was assessed as normal, mild, moderate or severe.

Digestive enzyme activities and bile acid concentrations

Leucine aminopeptidase activity in the PI, MI and DI tissue was analysed to assess intestinal function following the method described previously[26]. Activities are related to mmol substrate hydrolysed per unit time in the whole tissue per kg body weight (enzymatic capacity) and per mg protein (specific activity). Protein was analysed using the Bio-Rad Protein Assay (Bio-Rad Laboratories).

For trypsin activity analysis, 50 mg freeze-dried intestinal content was homogenised and suspended in 2 ml dH_2O. Trypsin activity was measured colorimetrically according to Kakade *et al.*[27], using the substrate benzoyl-arginine-*p*-nitroanilide (Sigma-Aldrich). The trypsin activity is expressed as the change in optical density units (U) and related to mg DM. The bile acid concentrations in the intestinal contents were analysed using the Enzabile® kit from Nycomed Pharma AS Diagnostics.

Quantitative real-time PCR

RNA purification and quality control, DNase treatment, complementary DNA synthesis, primer optimisation and quantitative PCR assays were performed as described in detail elsewhere[28]. Quantitative PCR primers were obtained from the literature or designed using Primer3 software (http://frodo.wi.mit.edu/primer3). See Table 2 for details. β-Actin (*ACTB*), RNA polymerase II (*RNAPOLII*), hypoxanthine phosphoribosyltransferase 1 (*HPRT1*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) were evaluated for use as reference genes by ranking relative gene expression according to their stability, as described previously[28]. A combination of GAPDH and RNAPOLII was used as normalisation factor. Distal intestinal mean normalised expression of the target genes was calculated from raw C_q values using a plate calibrator-normalised relative quantification[29].

Western blot analysis

A measure of 50 mg tissues samples from DI, head kidney and spleen were homogenised with 400 μl cold radioimmunoprecipitation assay lysis buffer (50 mM-Tris–HCl (pH 8·0), 150 mM-NaCl, 0·1 % SDS, 1 % Igepal CA-630, 0·5 % sodium deoxycholate, 2·5 mM-phenylmethylsulfonyl fluoride (PMSF), 2·5 mM-EGTA, 4 mM-ethylene glycol tetraacetic acid (EGTA), 5 mM-sodium orthovanadate, 5 μg/ml aprotinin, 50 μg/ml leupeptin, 2 μg/ml pepstatin, 0·2 mg/ml benzamidine and

10 mM-sodium fluoride). The homogenates were centrifuged at 16 000 ***g*** at 4°C for 20 min and supernatants were collected. Protein content was quantified using the Bradford assay (Bio-Rad) with bovine serum albumin (BSA) as a standard.

Samples of 10 μg protein were prepared for SDS-PAGE and transferred to a nitrocellulose membrane (Invitrogen). Membranes were incubated with primary antibodies: rabbit polyclonal anti-GAPDH (1:250; Abcam); mouse monoclonal anti-CD4 (1:50; provided by Karsten Skjødt, University of Southern Denmark); and mouse monoclonal anti-proliferating cell nuclear antigen (PCNA) (1:2000; Dako) in 1× Tris-buffered saline with Tween-20 (TBST) (50 mM-Tris–HCl (pH 7·5), 150 mM-NaCl, 0·1 % Tween 20) with 5 % non-fat dry milk and subsequently incubated with the alkaline phosphatase-conjugated secondary antibodies. The targeted proteins were visualised with fluorescence by ECF™ Western Blotting reagent Pack (GE Healthcare) and Typhoon 9200 imager system (Amersham Biosciences). ImageQuant software (Amersham Biosciences) was applied to quantify the band intensities. GAPDH was used as a reference for normalisation of the target protein expressions.

Calculations

$$\text{Condition factor } (K) = (\text{body weight}/\text{fork length}^3) \times 100.$$
$$\text{Organosomatic index} = (\text{organ weight}/\text{body weight}) \times 100.$$

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

$$\text{Feed efficiency} = \text{weight gain (g)}/\text{feed intake (g).}$$

$$\text{Apparent digestibility coefficient (\%)} = 100 - (100 \times (\text{nutrient in faeces}/\text{yttrium oxide in faeces}) \times (\text{yttrium oxide in feed}/\text{nutrient in feed})).$$

$$\text{Nutrient retention efficiency} = 100 \times \text{nutrient gain}/\text{nutrient intake.}$$

$$\text{Mean corpuscular volume} = (\text{haematocrit}/\text{erythrocytes}) \times 10.$$

$$\text{Mean corpuscular Hb} = (\text{Hb}/\text{erythrocytes}) \times 10;\ \text{Hb} = \text{Hb (g/100 ml).}$$

$$\text{Mean corpuscular Hb concentration} = (\text{Hb}/\text{haematocrit}) \times 100.$$

Statistics

The data were statistically evaluated using JMP version 9.0 (2010) Statistical Discovery™ (SAS Institute, Inc.). Normal distribution of data was tested using a plot of actual *v.* predicted residuals and the Shapiro–Wilk *W*-statistic on the residuals. The results were subjected to two-way ANOVA, with GM and SBM inclusion as the class variables. The data for mRNA expression (n 7 or 8) and protein levels (n 3) were analysed based on individual fish, while for other data tank means (n 3) were used. For the histological evaluation, results were compared using the χ^2 test. All reported P values are two-sided and significance was set at $P<0{\cdot}05$.

Results

Proximate composition, as well as pesticide and mycotoxin levels in the two maize types are reported in Walsh *et al.*[30]. Cry1Ab protein levels in the *Bt* MON810 maize was 0·6 parts per million (0·00 006 %), while the non-GM counterpart did not contain detectable levels of Cry1Ab. Analysed proximate compositions of the experimental diets (Table 1) were similar to calculated compositions. All diets were close to equivalent in composition, similar in protein, starch and ash. However,

Table 2. Primer pair sequences, amplicon size (AS), annealing temperature (AT), efficiency (*E*) and Genbank accession number for genes used for quantitative real-time PCR

	5′-3′ primer sequence					
Gene	Forward	Reverse	AS (bp)	AT (°C)	*E*	Genbank accession no.
CD4	GAGTACACCTGCGCTGTGGAAT	GGTTGACCTCCTGACCTACAAAGG	123	60	1·85	[DQ867018]
IL-1β	GCTGGAGAGTGCTGTGGAAGA	TGCTTCCCTCCTGCTCGTAG	73	60	1·92	[AY617117]
IL-17a	TGGTTGTGTGCTGTGTGTCTATGC	TTTCCCTCTGATTCCTCTGTGGG	136	60	2·00	[GW574233]
TGF-β	AGTTGCCTTGTGATTGTGGGA	CTCTTCAGTAGTGGTTTGTCG	191	60	1·90	[EU082211]
IFN-γ	CTAAAGAAGGACAACCGCAG	CACCGTTAGAGGGAGAAATG	159	60	1·93	[FJ263446]
PCNA	TGAGCTCGTCGGGTATCTCT	GTCCTCATTCCCAGCACACT	170	55	2·00	[BT056931]
HSP70	CCCCTGTCCCTGGGTATTG	CACCAGGCTGGTTGTCTGAGT	121	60	1·97	[BG933934]
CAT	CCGACCGTCCGTAAATGCTA	GCTTTTCAGATAGGCTCTTCATGTAA	140	58	2·00	[BG935638]
SOD	CCACGTCCATGCCTTTGG	TCAGCTGCTGCAGTCACGTT	140	55	1·88	[BG936553]
PEPT	GGCTTTCTGCTCTGTGAAGG	TAGGGGGACACAACAAGACC	89	55	1·94	[EB174326]
SGLT	TCGTGGGATCTTTCATCCTCA	CCATGTAGCCCGTCTGGAAG	78	60	2·00	[NM_001171787]
ACTB	CAAAGCCAACAGGGAGAAGATGA	ACCGGAGTCCATGACGATAC	133	60	1·86	[AF012125]
RNA-POLII	CCAATACATGACCAAATATGAAAGG	ATGATGATGGGGATCTTCCTGC	157	60	1·80	[BG936649]
HPRT1	CCGCCTCAAGAGCTACTGTAAT	GTCTGGAACCTCAAACCCTATG	255	60	1·99	[BT043501]
GAPDH	AAGTGAAGCAGGAGGGTGGAA	CAGCCTCACCCCATTTGATG	96	60	1·85	[BT050045]

CD4, cluster of differentiation 4; *TGF-β*, transforming growth factor β; *IFN-γ*, interferon-γ; *PCNA*, proliferating cell nuclear antigen; *HSP70*, heat shock protein 70; *CAT*, catalase; *SOD*, superoxide dismutase; *PEPT*, peptide transporter; *SGLT*, sodium-dependent glucose transporter; *ACTB*, β-actin; *RNA-POLII*, RNA polymerase II; *HPRT1*, hypoxanthine phosphoribosyltransferase 1; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

Table 3. Mean growth performance and feed utilisation of Atlantic salmon (initial body weight 93·8 (SE 0·3) g) fed non-GM (nGM) maize or GM *Bt*-maize without or with soyabean meal (SBM) for 33 and 97 d†

	Normal (nSBM)		Sensitised (SBM)			Two-way ANOVA		
	nGM maize	*Bt*-maize	nGM maize	*Bt*-maize	Pooled SE	nGM/GM: *P*	nSBM/SBM: *P*	GM–SBM interaction: *P*
Day 33								
BW (g)	137·4	136·6	131·5	130·6	2·5	0·75	0·0329*	0·99
BL (cm)	22·0	22·2	22·1	22·3	0·2	0·28	0·64	0·64
BWG (g) (0–33 d)	43·9	41·8	38·2	37·2	2·3	0·48	0·0402*	0·82
SGR (0–33 d)	1·17	1·11	1·04	1·01	0·05	0·40	0·0481*	0·75
CF	1·29	1·28	1·22	1·24	0·02	0·60	0·0152*	0·44
FI (g) (0–33 d)	30·1	30·2	29·6	30·7	0·9	0·52	0·97	0·63
FE (0–33 d)	1·46	1·38	1·29	1·21	0·05	0·12	0·0040*	0·97
Day 97								
BW (g)	212·4	211·5	197·2	202·2	5·5	0·72	0·0450*	0·61
BL (cm)	25·2	25·2	24·9	25·0	0·3	0·83	0·31	0·42
BWG (g) (0–97 d)	118·9	116·7	104·0	108·8	5·3	0·80	0·0512	0·53
SGR (0–97 d)	0·85	0·83	0·77	0·79	0·03	0·93	0·0639	0·42
CF	1·38	1·35	1·35	1·33	0·02	0·36	0·28	0·83
FI (g) (0–97 d)	93·0	92·3	89·8	95·3	2·5	0·33	0·96	0·16
FE (0–97 d)	1·28	1·26	1·16	1·14	0·03	0·58	0·0021*	0·99

nSBM, non-SBM; *Bt*, *Bacillus thuringiensis*; BW, body weight; BL, body length; BWG, body weight gain; SGR, specific growth rate; CF, condition factor; FI, feed intake; FE, feed efficiency.
*$P<0·05$.
† The *P* values are given for nGM/GM and nSBM/SBM inclusion, respectively, as well as for interactions between *Bt*-maize and SBM inclusion by two-way ANOVA analysis.

SBM-containing diets were slightly lower in lipid and gross energy and higher in residue levels.

During the entire feeding trial, all the fish appeared normal and no mortalities were observed. The fish fed the 15 % SBM inclusion diets were successfully sensitised (see Effects of soyabean meal section).

Effects of Bt*-maize and interactions between GM and soyabean meal inclusion*

Growth performance and feed utilisation. No differences in initial or final body weight, body weight gain, body length, specific growth rate, condition factor, feed intake or feed efficiency were observed between nGM maize- and *Bt*-maize-fed fish (Table 3). Nor were interactions between *Bt*-maize and SBM observed.

Proximate composition of whole body and liver. *Bt*-maize caused lower crude lipid deposition (Table 4). No effects on whole-body crude protein or DM, or on liver crude lipid, glycogen or DM were observed in *Bt*-maize-fed fish. No interactions between *Bt*-maize and SBM were observed.

Apparent nutrient digestibilities, faecal DM and nutrient retention efficiencies. Crude protein and mineral digestibility of the *Bt*-maize diets were reduced ($P=0·0456$ and 0·0261, respectively; Fig. 1). Decreased crude lipid retention ($P=0·0203$) and a tendency toward decreased energy retention ($P=0·0550$) were observed in the fish fed *Bt*-maize (Fig. 2). No interactions between *Bt*-maize and SBM were found in any of these parameters.

Organosomatic indices. A transient (detected at 33 but not at 97 d) trend ($P<0·10$) towards higher relative weights of whole intestine ($P=0·0924$) and PI ($P=0·0752$) were observed

Table 4. Mean composition of whole body and liver of Atlantic salmon fed non-GM (nGM) maize or GM *Bt*-maize without or with soyabean meal (SBM) for 97 d†

	Normal (nSBM)		Sensitised (SBM)			Two-way ANOVA		
	nGM maize	*Bt*-maize	nGM maize	*Bt*-maize	Pooled SE	nGM/GM: *P*	nSBM/SBM: *P*	GM–SBM interaction: *P*
Whole body (g/100 g)								
Crude protein	16·3	16·4	16·3	16·3	0·1	0·56	0·91	0·74
Crude lipid	13·8	12·8	13·1	11·9	0·2	0·0012*	0·0054*	0·72
DM	32·3	31·7	31·0	30·8	0·4	0·30	0·0195*	0·57
Liver (g/100 g)								
Crude protein	13·7	13·4	13·6	13·3	0·2	0·19	0·72	0·90
Crude lipid	4·2	4·6	4·4	4·1	0·2	0·76	0·49	0·41
Glycogen	7·3	8·1	7·7	8·2	0·5	0·15	0·58	0·91
DM	25·9	26·6	25·9	26·3	0·4	0·22	0·81	0·86

nSBM, non-SBM; *Bt*, *Bacillus thuringiensis*.
*$P<0·05$.
† The *P* values are given for nGM/GM and nSBM/SBM inclusion, respectively, as well as for interactions between *Bt*-maize and SBM inclusion by two-way ANOVA analysis.

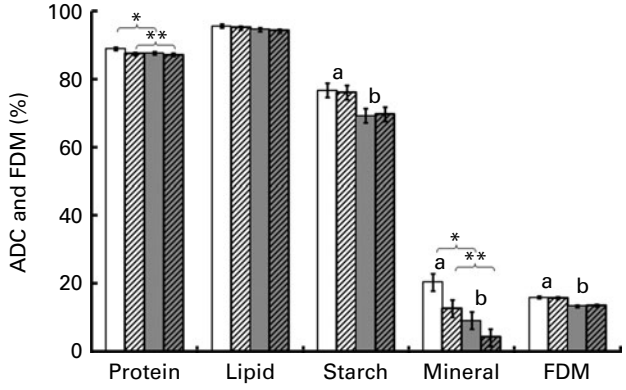

Fig. 1. Apparent digestibility coefficients (ADC (%)) of crude protein, crude lipid, starch and mineral, as well as faecal DM (FDM) in fish fed the four experimental diets for 97 d. Values are means, with their pooled standard errors represented by vertical bars (n 3). *,**Significant effect of GM inclusion (two-way ANOVA). [a,b] Significant effect of soyabean meal (SBM) inclusion ($P<0.05$; two-way ANOVA). □, non-GM maize; ▨, GM *Bt*-maize; ■, non-GM maize + SBM; ▨, GM *Bt*-maize + SBM.

in fish fed *Bt*-maize diets (Table 5). Moreover, an interaction, albeit not significant ($P=0·0936$), indicated that *Bt*-maize tended to increase PI weight reduced by SBM at 33 d. At 97 d, no differences were observed, nor were interactions between *Bt*-maize and SBM observed.

Haematology, leucocyte counts, plasma clinical chemistry and Cry1Ab-specific antibodies and protein in plasma. The *Bt*-maize did not cause any differences in haematological values or differential leucocyte counts (Table 6), or in plasma clinical chemistry (Table 7) at either 33 or 97 d. Nor were interactions between *Bt*-maize and SBM observed. Cry1Ab protein plasma concentrations were below detection limits and no specific antibodies (IgM) against Cry1Ab could be detected (data not shown).

Histology. The histomorphology of head kidney, spleen, liver, PI, MI (data not shown) and DI (Fig. 3 and Table 8; see also Effects of soyabean meal section), as assessed by light microscopy, did not differ between nGM maize- and *Bt*-maize-fed fish, nor were any interactions between *Bt*-maize and SBM observed.

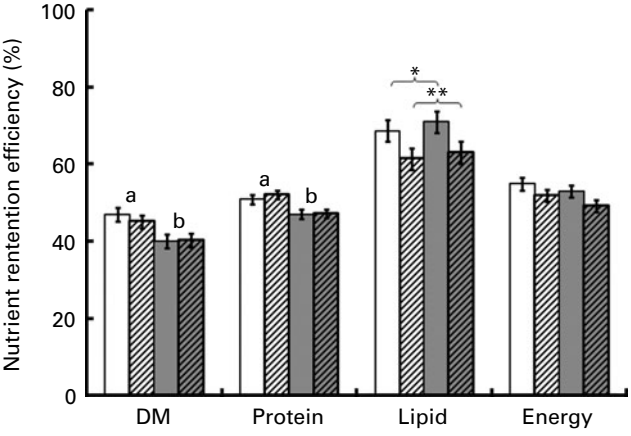

Fig. 2. Nutrient retention efficiencies (%) of DM, crude protein, crude lipid and energy in fish fed the four experimental diets for 97 d. Values are means, with their pooled standard errors represented by vertical bars (n 3). *,**Significant effect of GM inclusion (two-way ANOVA). [a,b] Significant effect of soyabean meal (SBM) inclusion ($P<0.05$; two-way ANOVA). □, non-GM maize; ▨, GM *Bt*-maize; ■, non-GM maize + SBM; ▨, GM *Bt*-maize + SBM.

Digestive enzyme activities and bile acid concentration. *Bt*-maize significantly decreased leucine aminopeptidase-specific activity ($P=0·0368$; Fig. 4(B)) and tended to decrease enzymatic capacity ($P=0·0724$; Fig. 4(A)) of PI tissue at 97 d. No differences in either trypsin activity or bile acid concentration in MI and/or DI content were caused by *Bt*-maize (Table 9). No interactions between *Bt*-maize and SBM were observed for any of these parameters.

mRNA expression in distal intestine tissue. Real-time quantitative PCR analyses were limited to the DI, where possible interactions in immune-sensitised salmon would be expected. The relative mRNA expression analyses of CD4, IL-1β, IL-17a, transforming growth factor-β, IFN-γ, PCNA, heat shock protein 70 (HSP70), catalase, superoxide dismutase (SOD), peptide transporter and sodium-dependent glucose transporter (SGLT) in DI are shown in Fig. 5(A)–(K), respectively.

Significant interactions between *Bt*-maize and SBM inclusion were observed for SOD ($P=0·0271$) at 33 d (Fig. 5(I)), as well as IFN-γ ($P=0·0340$; Fig. 5(E)), HSP70 ($P=0·0366$; Fig. 5(G)) and SGLT ($P=0·0070$; Fig. 5(K)) at 97 d. *Bt*-maize caused increased SOD (Fig. 5(I)) at 33 d and HSP70 (Fig. 5(G)) at 97 d only in SBM-fed fish. At 97 d, increased IFN-γ (Fig. 5(E)) was caused by *Bt*-maize feeding in normal fish, but this response appeared to be abolished or masked with SBM inclusion in the diet. For SGLT, the *Bt*-maize decreased its expression in normal fish, but not in fish fed SBM inclusion diets (Fig. 5(K)).

Western blot analyses of cluster of differentiation 4 and proliferating cell nuclear antigen. Western blot analyses of protein isolated from DI were analysed for CD4 and PCNA as local immune and toxicity indicators, respectively. Furthermore, protein isolated from head kidney and spleen were analysed for PCNA as an indicator of toxicity that may have escaped detection by plasma clinical chemistry, organosomatic indices or histological evaluation.

Single immunoreactive CD4 bands at approximately 51 kDa in DI (Fig. 6) and PCNA bands at approximately 31 kDa in head kidney, spleen and DI (Fig. 7) were detected. An increase in CD4 protein was transiently caused by *Bt*-maize at 33 d ($P=0·0436$; Fig. 6). PCNA expression was transiently decreased in DI by *Bt*-maize at 33 d ($P=0·0330$), but not at 97 d. The *Bt*-maize did not have any effect on PCNA protein levels in either HK (Fig. 7(A) and (B)) or spleen (Fig. 7(C) and (D)). No interactions between *Bt*-maize and SBM were observed for any of these parameters.

Effects of soyabean meal

The fish fed 15 % SBM inclusion diets were sensitised, as indicated by mild-to-moderate histopathological signs of inflammation in DI (Fig. 3 and Table 8). These were characterised by increased width of the lamina propria and submucosa, infiltration of inflammatory cells, decreased enterocyte

Table 5. Mean organosomatic indices (g/kg body mass) of head kidney (HKSI), spleen (SPSI), liver (LISI), whole intestine (ISI), proximal intestine (PISI), mid intestine (MISI), distal intestine (DISI) and gonad (GSI) of Atlantic salmon fed non-GM (nGM) maize or GM *Bt*-maize without or with soyabean meal (SBM) for 33 and 97 d†

	Normal (nSBM)		Sensitised (SBM)			Two-way ANOVA		
	nGM maize	Bt-maize	nGM maize	Bt-maize	Pooled SE	nGM/GM: *P*	nSBM/SBM: *P*	GM–SBM interaction: *P*
Day 33								
HKSI	1·5	1·6	1·5	1·6	0·1	0·30	0·62	0·87
SPSI	0·6	0·6	0·5	0·6	0·02	0·14	0·0647	0·11
LISI	18·6	19·0	17·7	18·2	0·3	0·19	0·0184*	0·91
ISI	52·4	52·7	45·7	48·8	0·8	0·0924	0·0002*	0·11
PISI	43·8	44·0	38·6	41·6	0·7	0·0752	0·0012*	0·0936
MISI	3·0	3·1	2·6	2·7	0·1	0·33	0·0002*	0·62
DISI	5·6	5·6	4·5	4·5	0·1	0·91	<0·0001*	0·78
GSI	–	–	–	–		–	–	–
Day 97								
HKSI	1·5	1·5	1·3	1·4	0·1	0·64	0·15	0·63
SPSI	0·9	0·9	0·7	1·0	0·1	0·47	0·68	0·29
LISI	16·4	16·5	15·4	14·8	0·4	0·70	0·0474*	0·60
ISI	59·6	60·5	56·1	58·1	1·7	0·40	0·10	0·74
PISI	51·4	52·0	49·3	51·2	1·1	0·44	0·38	0·72
MISI	2·6	2·7	2·1	2·3	0·1	0·28	0·0109*	0·99
DISI	5·7	5·7	4·7	4·7	0·2	0·93	0·0016*	0·93
GSI	1·3	1·3	1·3	1·3	0·1	0·79	0·34	0·84

nSBM, non-SBM; *Bt*, *Bacillus thuringiensis*.
*$P < 0·05$.
† The *P* values are given for nGM/GM and nSBM/SBM inclusion, respectively, as well as for interactions between *Bt*-maize and SBM inclusion by two-way ANOVA analysis.

vacuolisation, increased goblet cell frequency and a shift of enterocyte nuclei from basal to a more apical position. With time, the inflammation became exacerbated and fusion of mucosal folds was also observed. Moreover, the SBM-fed fish also showed reduced faecal DM (diarrhoea; Fig. 1), decreased intestinal and liver weights (Table 5), increased trypsin activity in DI content (Table 9) and increased CD4 mRNA levels in the DI at 97 d (Fig. 5(A)).

Other results also indicated reduced performance and organ malfunction associated with the hypersensitivity reactions,

Table 6. Mean haematological parameters including haematocrit (Hct), erythrocyte count, Hb, mean corpuscular volume (MCV), mean corpuscular Hb (MCH) and MCH concentration (MCHC) and differential leucocyte counts including lymphocytes, granulocytes and monocytes of Atlantic salmon fed non-GM (nGM) maize or GM *Bt*-maize without or with soyabean meal (SBM) for 33 and 97 d†

	Normal (nSBM)		Sensitised (SBM)			Two-way ANOVA		
	nGM maize	Bt-maize	nGM maize	Bt-maize	Pooled SE	nGM/GM: *P*	nSBM/SBM: *P*	GM–SBM interaction: *P*
Day 33								
Hct (%)	44·2	43·1	42·1	42·7	0·8	0·75	0·15	0·35
Erythrocytes (10^{12}/litres)	1·5	1·5	1·5	1·6	0·1	0·77	0·37	0·78
Hb (g/100 ml)	11·2	11·3	11·3	11·4	0·4	0·75	0·88	1·00
MCV (10^{-15} litres)	292	295	277	279	7	0·71	0·0390*	0·99
MCH ($\times 10^{-6}$ g)	74·6	75·7	73·1	72·6	1·1	0·77	0·0644	0·48
MCHC (g/100 ml)	25·5	25·7	26·5	26·0	0·5	0·81	0·21	0·52
Lymphocytes (%)	87·7	91·0	90·3	93·3	3·0	0·30	0·41	0·97
Granulocytes (%)	10·4	7·0	8·1	5·3	2·3	0·18	0·39	0·90
Monocytes (%)	1·9	2·0	1·6	1·4	0·8	0·99	0·52	0·84
Day 97								
Hct (%)	44·9	45·7	44·9	40·4	2·3	0·44	0·28	0·29
Erythrocytes (10^{12}/litres)	1·2	1·3	1·2	1·1	0·1	0·34	0·24	0·27
Hb (g/100 ml)	10·0	10·0	9·5	8·5	0·5	0·31	0·0650	0·28
MCV (10^{-15} litres)	370	370	364	385	11	0·37	0·70	0·38
MCH ($\times 10^{-6}$ g)	80·6	81·1	77·6	80·4	1·9	0·39	0·33	0·57
MCHC (g/100 ml)	22·0	22·1	21·5	21·0	0·5	0·71	0·15	0·59
Lymphocytes (%)	87·5	89·8	91·3	88·9	2·1	0·99	0·50	0·29
Granulocytes (%)	10·7	8·8	7·8	9·5	2·1	0·93	0·61	0·42
Monocytes (%)	1·8	1·4	0·9	1·6	0·4	0·63	0·41	0·23

nSBM, non-SBM; *Bt*, *Bacillus thuringiensis*.
*$P < 0·05$
† The *P* values are given for nGM/GM and nSBM/SBM inclusion, respectively, as well as for interactions between *Bt*-maize and SBM inclusion by two-way ANOVA analysis.

Table 7. Mean plasma clinical chemistry including total protein (T_{prot}), globulins, albumin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (AP), amylase, lipase, TAG, total bilirubin, total bile acids, NEFA and cholesterol in Atlantic salmon fed non-GM (nGM) maize or GM *Bt*-maize without or with soyabean meal (SBM) for 33 and 97 d†

	Normal (nSBM)		Sensitised (SBM)			Two-way ANOVA		
	nGM maize	Bt-maize	nGM maize	Bt-maize	Pooled SE	nGM/GM: *P*	nSBM/SBM: *P*	GM−SBM interaction: *P*
Day 33								
T_{prot} (g/l)	32·5	30·8	28·0	33·2	3·2	0·60	0·74	0·32
Globulins (g/l)	17·0	15·8	14·5	17·2	1·8	0·69	0·76	0·32
Albumin (g/l)	15·5	15·0	13·5	16·0	1·4	0·50	0·74	0·32
ALT (IU/l)	13·8	15·3	14·0	13·0	1·2	0·84	0·39	0·32
AST (IU/l)	215	213	201	218	32	0·81	0·89	0·77
AP (IU/l)	160	144	147	193	32	0·65	0·58	0·36
Amylase (IU/l)	819	825	919	930	131	0·95	0·43	0·99
Lipase (IU/l)	11·5	11·2	10·8	11·8	0·6	0·59	1·00	0·30
TAG (mM)	4·6	3·1	3·1	2·8	0·7	0·23	0·25	0·46
Total bilirubin (μM)	5·3	4·8	3·7	4·5	0·8	0·84	0·22	0·42
Bile acids (μM)	21·0	9·0	8·8	13·5	5·7	0·56	0·54	0·18
Cholesterol (mM)	11·8	10·6	8·2	10·1	1·7	0·84	0·25	0·38
NEFA (mM)	0·57	0·58	0·53	0·47	0·10	0·79	0·43	0·68
Osmolality (mOsm/k)	334	330	332	332	1·0	0·20	0·89	0·13
Day 97								
T_{prot} (g/l)	39·5	37·0	38·3	38·0	1·6	0·38	0·95	0·51
Globulins (g/l)	20·8	19·2	19·7	19·7	0·7	0·30	0·67	0·29
Albumin (g/l)	18·7	17·8	18·7	18·3	0·9	0·50	0·77	0·78
ALT (IU/l)	59·0	47·7	32·3	36·8	20·0	0·86	0·35	0·70
AST (IU/l)	290	362	278	348	55	0·20	0·81	0·99
AP (IU/l)	166	164	186	219	15	0·34	0·0371*	0·28
Amylase (IU/l)	688	606	765	674	72	0·24	0·32	0·95
Lipase (IU/l)	13·8	13·5	13·5	13·8	0·4	1·00	1·00	0·46
TAG (mM)	5·3	5·4	4·8	4·1	0·7	0·69	0·24	0·55
Total bilirubin (μM)	5·8	5·8	5·2	5·2	0·5	1·00	0·16	1·00
Bile acids (μM)	13·0	16·8	6·0	7·5	3·0	0·37	0·0179*	0·70
Cholesterol (mM)	14·8	13·6	11·7	12·4	0·8	0·74	0·0246*	0·27
NEFA (mM)	0·62	0·62	0·48	0·47	0·04	0·85	0·0079*	0·85
Osmolality (mOsm/k)	337	336	340	339	3·0	0·83	0·31	0·98

nSBM, non-SBM; *Bt*, *Bacillus thuringiensis*.
*$P < 0·05$.
† The *P* values are given for nGM/GM and nSBM/SBM inclusion, respectively, as well as for interactions between *Bt*-maize and SBM inclusion by two-way ANOVA analysis.

such as decreased growth performance and feed and nutrient utilisation and retention (Tables 3 and 4; Fig. 1), erythrocyte mean corpuscular volume (Table 6) at 33 d and plasma alkaline phosphatase, bile acid, cholesterol and NEFA concentrations (Table 7), despite no difference in feed intake (Table 3). The fish exhibited maldigestion, as indicated by lower macronutrient digestibilities (Fig. 1), digestive enzyme activities (Fig. 3) and nutrient transporter mRNA levels (peptide transporter and SGLT; Fig. 5(J) and (K), respectively), as well as changes in trypsin activity and bile acid concentration in the intestinal content (Table 9). Increased PCNA mRNA and protein (Figs. 5(F), and 7(E)–(F), respectively), HSP70 mRNA (Fig. 5(G)) and decreased catalase mRNA (Fig. 5(H)) in DI are also indicative of malfunction of the inflamed tissue.

Discussion

Despite somewhat lower protein and mineral digestibility of the *Bt*-maize containing diets, growth and feed efficiency after 97 d of feeding did not differ between fish fed *Bt*-maize or nGM maize. However, *Bt*-maize appeared to cause changes in nutrient metabolism, either locally in the intestine or systemically, as indicated by the reduced whole-body lipid content and lipid retention efficiency, a response not previously observed in *Bt*-maize-fed salmon[31]. The battery of analyses conducted did not reveal any remarkable differences that unequivocally explain this finding, although tendencies of higher intestinal weights and increased CD4 protein, albeit transient, as well as relative mRNA expression of IFN-γ in DI may indicate that local changes in tissue growth and immune responses in the intestinal tissue may have had a metabolic cost. Maintenance of the intestine has been estimated to require as much as 20–25 % of an organism's daily nutrient and energy requirements[32], and changes in intestinal growth may therefore account for reduced body lipid stores. The *Bt* (Cry) protein has been characterised as lectin-like[33–35] and the data suggest that this protein or other components in the *Bt*-maize may act as growth factor(s) in the intestine of salmon, leading to an increase in relative intestinal weights. Some lectins, such as phytohaemagglutinin, have been demonstrated to function as growth factors for the intestinal mucosa[36–38]. Correspondingly, lower activity of the brush-border enzyme leucine aminopeptidase in PI may indicate that the growth led to an increase in immature epithelial cells that had not developed full functionality[39].

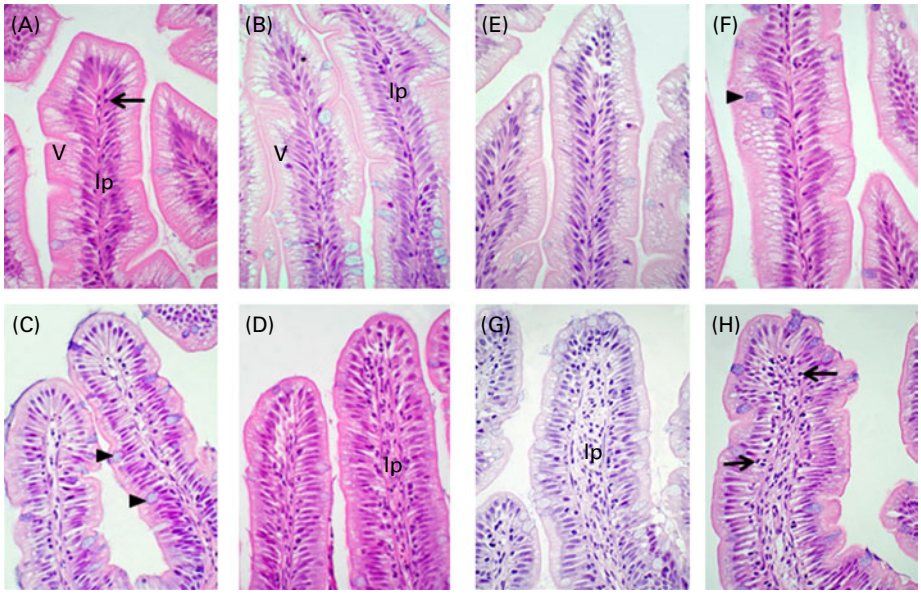

Fig. 3. Histological details of distal intestinal simple folds of Atlantic salmon fed (A and E) non-GM (nGM)-maize, (B and F) GM *Bt*-maize, (C and G) GM *Bt*-maize with soyabean meal (SBM) and (D and H) *Bt*-maize with SBM diets for 33 and 97 d (haematoxylin and eosin; × 400). No differences between nGM and *Bt*-maize-fed fish were observed. The SBM-fed fish, however, showed reduced supranuclear vacuolisation (v) in enterocytes, widened lamina propria (lp) due to cellular infiltration and increased numbers of intra-epithelial lymphocytes (←) and goblet cells (►). The SBM-induced changes became exacerbated with time. (A colour version of this figure can be found online at http://www.journals.cambridge.org/bjn).

This again may explain the lower nutrient digestibility in fish fed *Bt*-maize-containing diets.

The histological evaluation indicated that the presence of *Bt*-maize in diets neither caused damage to any organs or tissues, nor did it appear to change the SBM-induced hypersensitivity reaction in salmon DI. This was supported by the general lack of differences in haematological and plasma clinical chemistry values. This is in agreement with results of earlier studies in salmon[40,41]. Similarly, light microscopy investigation in liver and pancreas of sheep following oral exposure to Bt176-maize did not reveal histological changes. However, at higher magnification with electron microscopy, smaller, irregularly shaped cell nuclei containing increased amounts of granules in hepatocytes and pancreatic acinar cells were caused by Bt176-maize[42]. Moreover, electron microscopy also revealed that nuclei in pancreatic acinar cells, hepatocytes and testes cells were modified by GM soyabean[43–45]. Thus, electron microscopy analysis may be necessary for further investigation of *Bt*-maize effects on subcellular structures in salmon tissues.

The transient increase in CD4 mRNA expression and protein level in DI caused by *Bt*-maize indicated presence of $CD4^+$ T effector cell. The reason for the transient nature of these responses is unknown. It does, however, imply development of tolerance to the *Bt*-maize following a period of oral exposure. Cry1Ab protein immunoreactivity may be

Table 8. Distal intestinal histomorphological changes†‡

	Healthy (nSBM)		Sensitised (SBM)		Two-factor χ^2 test		
	nGM maize	*Bt*-maize	nGM maize	*Bt*-maize	nGM/GM: *P*	nSBM/SBM: *P*	GM–SBM interaction: *P*
Day 33							
Normal structures	11	10	1	4	0·91	<0·0001*	0·40
Mild changes	1	2	6	5			
Moderate changes	0	0	5	3			
Day 97							
Normal structures	11	11	0	0	1·00	<0·0001*	1·00
Mild changes	1	1	4	3			
Moderate changes	0	0	8	9			

nGM, non-GM; nSBM, non-soyabean meal; SBM, soyabean meal; *Bt*, *Bacillus thuringiensis*.
*$P < 0·05$
† The values signify the number of Atlantic salmon per dietary treatment that displayed the various degrees of morphological changes (*n* 12) following 33 and 97 d of feeding nGM maize or GM *Bt*-maize without or with soyabean meal (SBM). The changes were classified according to the criteria previously described[14].
‡ The *P* values are given for nGM/GM and nSBM/SBM inclusion, respectively, as well as for interactions between *Bt*-maize and SBM inclusion.

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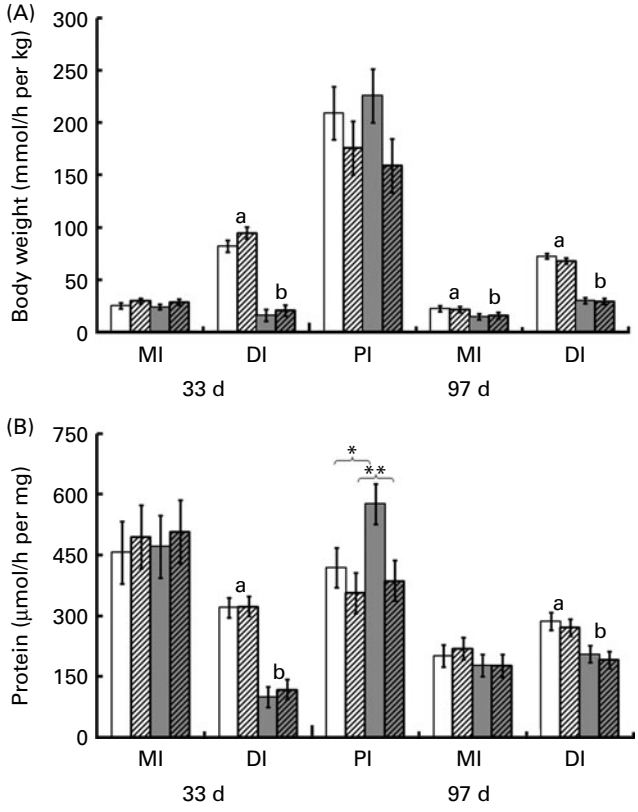

Fig. 4. Activities of leucine aminopeptidase in intestinal tissue, as related to mmol substrate hydrolysed per unit time in the whole tissue per kg body weight (capacity; A) and per mg protein (specific activity; B), in proximal intestine (PI), mid intestine (MI) and distal intestine (DI) of Atlantic salmon fed the four experimental diets for 33 and 97 d. Values are means, with their pooled standard errors represented by vertical bars (n 3). *,** Significant effect of GM inclusion (two-way ANOVA). [a,b] Significant effect of soyabean meal (SBM) inclusion ($P<0.05$; two-way ANOVA). □, non-GM maize; ▨, GM *Bt*-maize; ■, non-GM maize + SBM; ▨, GM *Bt*-maize + SBM.

conserved as it passed through the digestive tract, as indicated by *in vitro* digestion trials, in which the protein was only slightly degraded at pH 2 even at high pepsin-to-substrate ratio[6]. The protein or immunoreactive fragments of the protein was also found in the digesta of pigs[25] fed the same batch of *Bt*-maize as the salmon in the present study. The pH range along the gastrointestinal tracts of Atlantic salmon is 4·5–8·6[21,46], thus higher than most monogastric mammals. Therefore, it is likely that the Cry1Ab protein escapes full digestion also in the salmon gastrointestinal tract. The elevated DI IFN-γ expression observed in the *Bt*-maize-fed fish is an indication that a local immune response is elicited following more than 3 months of dietary exposure. The cytokine is produced by CD4 and CD8 effector T cells once antigen-specific immunity develops[47]. It has been reported that IFN-γ and other cytokines, such as IL-12, IL-10 and IL-4, were induced in cultured mice spleen cells by Cry1A protein[8]. Thus, the CD4 and IFN-γ transcriptional responses merit further investigations as biomarker candidates for exposure to GM maize.

With few exceptions, responses to *Bt*-maize exposure were generally not influenced by the SBM-induced hypersensitivity reaction. The only significant interactions detected were with

Table 9. Mean activities of trypsin (change in optical density units/mg DM) and concentration of bile acid (mg/g DM) in content of mid intestine (MI) and distal intestine (DI) of Atlantic salmon fed non-GM (nGM) maize or GM *Bt*-maize without or with soyabean meal (SBM) for 33 and 97 d†

	Normal (nSBM)		Sensitised (SBM)			Two-way ANOVA		
	nGM maize	Bt-maize	nGM maize	Bt-maize	Pooled SE	nGM maize/GM maize: *P*	nSBM/SBM: *P*	GM–SBM interaction: *P*
Day 33								
Trypsin activity								
MI	–	–	–	–	–	–	–	–
DI	20·1	9·1	50·3	50·5	7·0	0·45	0·0005*	0·45
Bile acid concentration								
MI	–	–	–	–	–	–	–	–
DI	22·1	16·6	14·5	13·5	4·3	0·45	0·22	0·60
Day 97								
Trypsin activity								
MI	141·5	138·8	108·7	105·4	12·8	0·81	0·0229*	0·98
DI	36·1	38·8	53·1	55·7	5·5	0·62	0·0098*	0·99
Bile acid concentration								
MI	105·4	99·8	76·6	79·7	6·3	0·84	0·0031*	0·51
DI	36·6	41·2	22·1	21·4	3·0	0·53	0·0002*	0·41

nSBM, non-SBM; *Bt*, *Bacillus thuringiensis*.
*$P<0.05$
† The *P* values are given for nGM/GM and nSBM/SBM inclusion, respectively, as well as for interactions between *Bt*-maize and SBM inclusion by two-way ANOVA analysis.

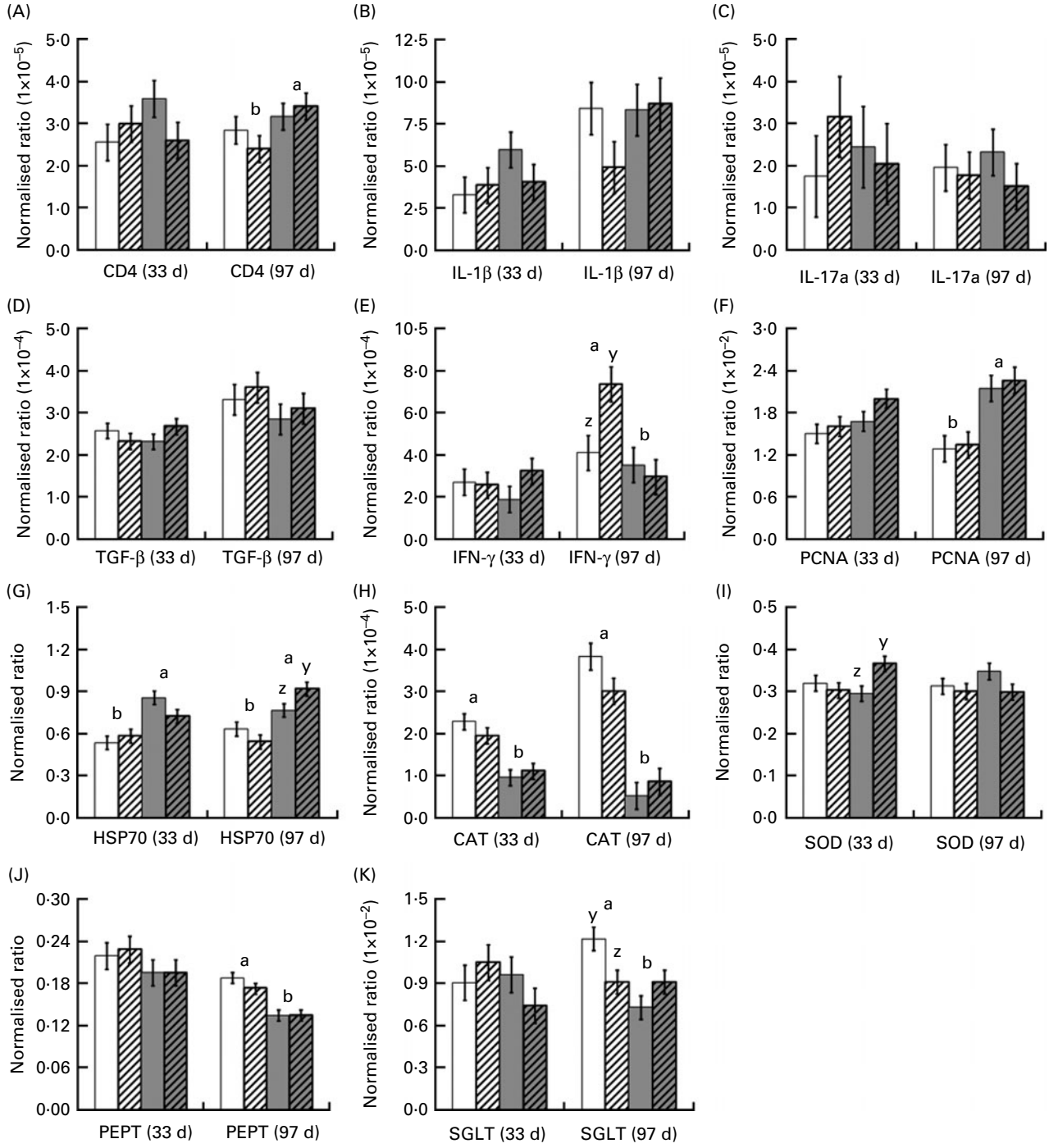

Fig. 5. Normalised ratio of relative mRNA expression levels (quantitative real-time PCR) of genes in the distal intestine of salmon fed the four diets for 33 and 97 d: (A) cluster of differentiation 4 (CD4), (B) IL-1β, (C) IL-17a, (D) transforming growth factor-β (TGF-β), (E) interferon-γ (IFN-γ), (F) proliferating cell nuclear antigen (PCNA), (G) heat shock protein 70 (HSP70), (H) catalase (CAT), (I) superoxide dismutase (SOD), (J) proton-coupled peptide transporter (PEPT) and (K) sodium-dependent GLUT (SGLT). Values are means, with their pooled standard errors represented by vertical bars (n 7–8). *,** Significant effect of GM inclusion (two-way ANOVA). ^{a,b} Significant effect of the soyabean meal (SBM) inclusion ($P<0.05$; two-way ANOVA). ^{y,z} Significant interaction between GM and SBM inclusion ($P<0.05$; two-way ANOVA). □, non-GM maize; ▨, GM *Bt*-maize; ■, non-GM maize + SBM; ▨, GM *Bt*-maize + SBM.

quantitative PCR analyses from the DI. SBM-inclusion in the diets appeared to increase the DI response to *Bt*-maize regarding HSP70, SOD (transient effect) and possibly SGLT mRNA levels. For the latter, IFN-γ has been shown to decrease ion transporter presence or activity in intestinal epithelium in higher vertebrates[48–50] and result in malabsorption of ions[50,51]. Thus, the elevated IFN-γ expression in DI may explain the tendency of decreased SGLT mRNA expression, and also help explain the reduced mineral digestibility observed in *Bt*-maize-fed salmon. For HSP70 and SOD, the

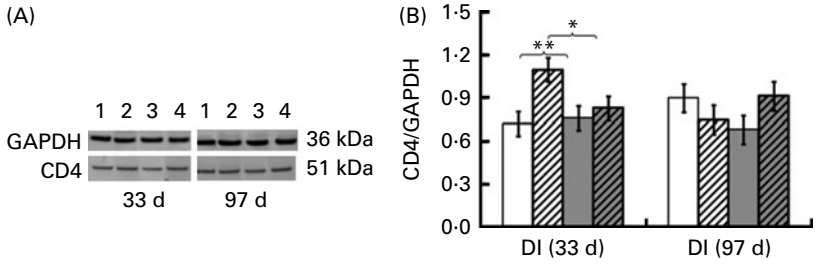

Fig. 6. Western blot analysis of cluster of differentiation 4 (CD4) protein in distal intestinal (DI) tissue of fish fed the four diets for 33 and 97 d. (A) Representative immunoblots including glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control: lane 1 – non-GM (nGM) maize; lane 2 – GM *Bt*-maize; lane 3 – nGM maize + soyabean meal (SBM); and lane 4 – GM *Bt*-maize + SBM. Values are means, with their pooled standard errors represented by vertical bars (*n* 3). The respective relative immunostaining intensities (B) are also shown. *,** Significant effect of GM inclusion (two-way ANOVA). □, nGM maize; ▨, GM *Bt*-maize; ■, nGM maize + SBM; ▨, GM *Bt*-maize + SBM.

dependency of the *Bt*-maize effects on sensitised fish indicates a potentiating effect on cellular stress responses. On the other hand, the opposite was seen with IFN-γ, in which SBM appeared to ameliorate the increase caused by *Bt*-maize alone. However, the dependency of the *Bt*-maize effects on sensitised fish for all these genes were generally low, so that the implications for the general health of the fish appeared to be marginal, as confirmed by other findings in the fish following 97 d of exposure to the *Bt*-maize.

Other responses to *Bt*-maize observed in the present study may be of lower value as biomarkers, as they are quite unspecific and differed from results in earlier studies in salmon and other animals fed the same GM-event maize or other crop plants. For example, the lack of effects on feed intake and growth was not in agreement with the earlier

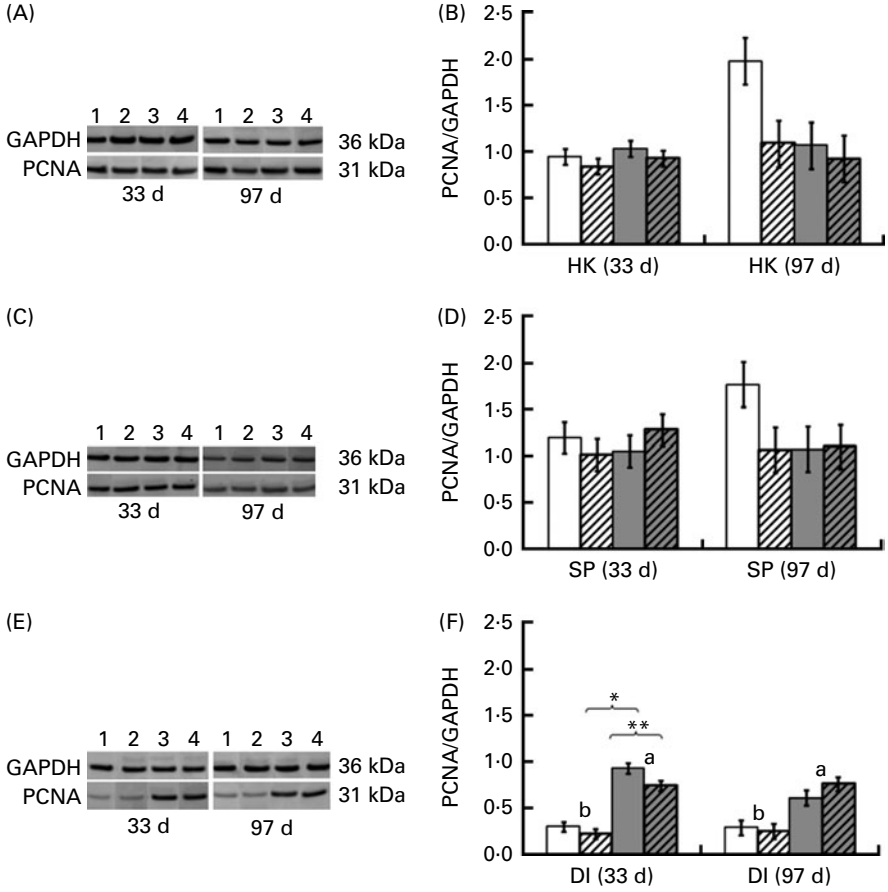

Fig. 7. Western blot analysis of proliferating cell nuclear antigen (PCNA) protein in (A and B) head kidney (HK), (C and D) spleen (SP) and (E and F) distal intestine (DI) of fish fed the four experimental diets for 33 and 97 d. Representative immunoblots (A, C and E) including glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control: lane 1 – non-GM (nGM)-maize; lane 2 – GM *Bt*-maize; lane 3 – nGM maize + Soyabean meal (SBM) and lane 4 – GM *Bt*-maize + SBM. The respective relative immunostaining intensities are also shown (B, D and F). Values are means, with their pooled standard errors represented by vertical bars (*n* 3). *,** Significant effect of GM inclusion (two-way ANOVA). [a,b] Significant effect of the soyabean meal (SBM) inclusion ($P<0.05$; two-way ANOVA). □, nGM maize; ▨, GM *Bt*-maize; ■, nGM maize + SBM; ▨, GM *Bt*-maize + SBM.

studies in salmon, in which the same GM-event maize significantly decreased both[31]. Nor were the significantly decreased protein and ash digestibilities, lipid retention, lipid content in filet and unchanged starch digestibility observed in the present study in line with results reported by Hemre *et al.*[31]. A 3-year study on sheep, however, reported decreased plasma potassium levels caused by *Bt*-maize[41]. It has been reported earlier that *Bt*-maize induced increased liver and DI weights and decreased spleen and head kidney weights in Atlantic salmon[31] and enlarged spleen in rats[11]. However, no effects of *Bt*-maize on these organs were currently observed in salmon. Nor were unchanged peptide transporter 1 mRNA in DI in agreement with an earlier study[52]. Previous studies showed that plasma total protein or albumin tended to decrease in rats and broiler chickens fed *Bt*-rice, *Bt*-maize or *Bt*-cottonseed meal[10,53–55]. However, *Bt*-maize did not cause any changes in plasma protein level or osmolality in the present study, which indicate that the decreased protein and mineral digestibilities of the *Bt*-maize diets were not enough to negatively influence salmon health during the timespan encompassed by this feeding trial.

Furthermore, changes in cellular proliferation (PCNA) in DI, spleen or head kidney do not appear to be of value as biomarkers for *Bt*-maize exposure. Changes in normal intestinal cell renewal have been suggested as early indicators for abnormal or toxic conditions in the gastrointestinal tract[39,56–60]. It has been reported that Cry1A can bind to the intestinal mucosal surfaces, influencing some epithelial cell functions[61]. The expression of a proliferation marker, Ki-67, was significantly higher in the basal cells of the ruminal epithelium of sheep fed *Bt*-maize[41]. As the DI was the main region showing histomorphological changes in the present study, PCNA mRNA and protein expression were determined in this tissue as well as in the immunologically important tissues, spleen and head kidney. PCNA was increased both at gene and protein expression level in the DI of fish with SBM-induced hypersensitivity, as reported in other studies[39,40]. Analyses in more proximal intestinal regions, where tendencies of increased tissue weights were observed in the *Bt*-maize-fed fish, would be useful in future studies to assess PCNA as a potential biomarker for *Bt*-maize exposure.

Conclusions

In the present study, an overall evaluation of health and growth performance indicated that *Bt*-maize (MON810) in diets for normal and SBM-sensitised Atlantic salmon resulted in lower protein and mineral digestibility as well as lipid and energy retention, indicating that these fish utilised and metabolised feed less efficiently compared to those fed the nGM maize. The increased IFN-γ associated with the tendency of decreased SGLT expression may partly explain the lower nutrient and mineral digestibilities in *Bt*-maize-fed salmon. Metabolic costs associated with higher intestinal growth and immune responses, as indicated by elevated distal intestinal IFN-γ mRNA expression levels and transient CD4 protein level, may explain lower lipid retention. Furthermore, *Bt*-maize may potentiate oxidative cellular stress in the DI of fish afflicted with an intestinal hypersensitivity reaction, as indicated by increases in SOD mRNA and HSP70 mRNA. The increases in CD4 protein and IFN-γ mRNA in the DI of *Bt*-maize-fed fish suggest that Cry1Ab protein or other antigens produced due to genetic modification have potential local immunogenic effects in the gastrointestinal tract and may function as biomarkers for MON810-event maize exposure for this species. However, long-term observations and more in-depth studies on immune responses and nutrient utilisation may be needed to confirm these results. Testing maize gluten meal derived from GM maize, a more commonly used, protein-rich maize product in salmonid diets, would also be of practical value for the aquaculture industry.

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References

1. James C (2012) Global Status of Commercialized Biotech/GM crops: 2012. ISAAA Briefs, no. 43. http://www.isaaa.org/resources/publications/briefs/42/default.asp
2. Knowles BH (1994) Mechanism of Action of *Bacillus Thuringiensis* Insecticidal Delta-Endotoxins. *Adv Insect Phys* **24**, 275–308.
3. Bravo A, Gill SS & Soberón M (2007) Mode of action of *Bacillus thuringiensis* Cry and Cyt toxins and their potential for insect control. *Toxicon* **49**, 423–435.
4. Betz FS, Hammond BG & Fuchs RL (2000) Safety and advantages of *Bacillus thuringiensis*-protected plants to control insect pests. *Regul Toxicol Pharmacol* **32**, 156–173.
5. Xu W, Cao S, He X, *et al.* (2009) Safety assessment of Cry1Ab/Ac fusion protein. *Food Chem Toxicol* **47**, 1459–1465.
6. Guimaraes V, Drumare MF, Lereclus D, *et al.* (2010) *In vitro* digestion of Cry1Ab proteins and analysis of the impact on their immunoreactivity. *J Agric Food Chem* **58**, 3222–3231.
7. Adel-Patient K, Guimaraes VD, Paris A, *et al.* (2011) Immunological and metabolomic impacts of administration of Cry1Ab protein and MON 810 maize in mouse. *PLoS ONE* **6**, e16346.
8. Guerrero GG, Russell WM & Moreno-Fierros L (2007) Analysis of the cellular immune response induced by *Bacillus thuringiensis* Cry1A toxins in mice: effect of the hydrophobic motif from diphtheria toxin. *Mol Immunol* **44**, 1209–1217.

9. Vázquez-Padrón RI, Moreno-Fierros L, Neri-Bazán L, *et al.* (1999) Intragastric and intraperitoneal administration of Cry1Ac protoxin from *Bacillus thuringiensis* induces systemic and mucosal antibody responses in mice. *Life Sci* **64**, 1897–1912.
10. Kiliç A & Akay MT (2008) A three generation study with genetically modified Bt corn in rats: biochemical and histopathological investigation. *Food Chem Toxicol* **46**, 1164–1170.
11. de Vendômois JS, Roullier F, Cellier D, *et al.* (2009) A comparison of the effects of three GM corn varieties on mammalian health. *Internat J Biol Sci* **5**, 706–721.
12. Prescott VE & Hogan SP (2006) Genetically modified plants and food hypersensitivity diseases: usage and implications of experimental models for risk assessment. *Pharmacol Ther* **111**, 374–383.
13. Prescott VE, Campbell PM, Moore A, *et al.* (2005) Transgenic expression of bean a-amylase inhibitor in peas results in altered structure and immunogenicity. *J Agric Food Chem* **53**, 9023–9030.
14. Reite OB & Evensen Ø (2006) Inflammatory cells of teleostean fish: A review focusing on mast cells/eosinophilic granule cells and rodlet cells. *Fish Shellfish Immunol* **20**, 192–208.
15. Baeverfjord G & Krogdahl Å (1996) Development and regression of soybean meal induced enteritis in Atlantic salmon, *Salmo salar* L., distal intestine: A comparison with the intestines of fasted fish. *J Fish Dis* **19**, 375–387.
16. Bakke-McKellep AM, Frøystad MK, Lilleeng E, *et al.* (2007) Response to soy: T-cell-like reactivity in the intestine of Atlantic salmon, *Salmo salar* L. *J Fish Dis* **30**, 13–25.
17. Lilleeng E, Penn MH, Haugland Ø, *et al.* (2009) Decreased expression of TGF-β, GILT and T-cell markers in the early stages of soybean enteropathy in Atlantic salmon (*Salmo salar* L.). *Fish Shellfish Immunol* **27**, 65–72.
18. NRC (1993) *Nutrient Requirements of Fish*. Washington DC, USA: National Academy Press.
19. Rausell C, Pardo-López L, Sánchez J, *et al.* (2004) Unfolding events in the water-soluble monomeric Cry1Ab toxin during transition to oligomeric pre-pore and membrane-inserted pore channel. *J Biol Chem* **279**, 55168–55175.
20. Krogdahl Å & Bakke-McKellep AM (2005) Fasting and refeeding cause rapid changes in intestinal tissue mass and digestive enzyme capacities of Atlantic salmon (*Salmo salar* L.). *Comp Biochem Physiol Part A Mol Integr Physiol* **141**, 450–460.
21. Nordrum S, Krogdahl Å, Røsjø C, *et al.* (2000) Effects of methionine, cysteine and medium chain triglycerides on nutrient digestibility, absorption of amino acids along the intestinal tract and nutrient retention in Atlantic salmon (*Salmo salar* L.) under pair-feeding regime. *Aquaculture* **186**, 341–360.
22. Refstie S, Helland SJ & Storebakken T (1997) Adaptation to soybean meal in diets for rainbow trout, *Oncorhynchus mykiss*. *Aquaculture* **153**, 263–272.
23. Soderberg DL (1995) Meat and meat products. In *Official Methods of Analysis of AOAC International*, 16th ed., pp. 1–23 [P Cunniff, editor]. Arlington, VA: AOAC International.
24. Hemre GI, Lie Ø, Lied E, *et al.* (1989) Starch as an energy source in feed for cod (*Gadus morhua*): Digestibility and retention. *Aquaculture* **80**, 261–270.
25. Walsh MC, Buzoianu SG, Gardiner GE, *et al.* (2011) Fate of transgenic DNA from orally administered Bt MON810 maize and effects on immune response and growth in pigs. *PLoS One* **6**, e27177.
26. Krogdahl Å, Bakke-McKellep AM & Baeverfjord G (2003) Effects of graded levels of standard soybean meal on intestinal structure, mucosal enzyme activities, and pancreatic response in Atlantic salmon (*Salmo salar* L.). *Aquac Nutr* **9**, 361–371.
27. Kakade ML, Hoffa DE & Liener IE (1973) Contribution of trypsin inhibitors to deleterious effects of unheated soybeans fed to rats. *J Nutr* **103**, 1772–1778.
28. Kortner TM, Valen EC, Kortner H, *et al.* (2011) Candidate reference genes for quantitative real-time PCR (qPCR) assays during development of a diet-related enteropathy in Atlantic salmon (*Salmo salar* L.) and the potential pitfalls of uncritical use of normalization software tools. *Aquaculture* **318**, 355–363.
29. Muller PY, Janovjak H, Miserez AR, *et al.* (2002) Processing of gene expression data generated by quantitative real-time RT-PCR. *BioTechniques* **32**, 1372–1374.
30. Walsh MC, Buzoianu SG, Gardiner GE, *et al.* (2012) Effects of short-term feeding of Bt MON810 maize on growth performance, organ morphology and function in pigs. *Br J Nutr* **107**, 364–371.
31. Hemre GI, Sagstad A, Bakke-McKellep AM, *et al.* (2007) Nutritional, physiological, and histological responses in Atlantic salmon, *Salmo salar* L. fed diets with genetically modified maize. *Aquac Nutr* **13**, 186–199.
32. Ferrell CL (1988) Contribution of visceral organs to animal energy expenditures. *J Anim Sci* **66**, 23–34.
33. Burton SL, Ellar DJ, Li J, *et al.* (1999) *N*-acetylgalactosamine on the putative insect receptor aminopeptidase N is recognised by a site on the domain III lectin-like fold of a *Bacillus thuringiensis* insecticidal toxin. *J Mol Biol* **287**, 1011–1022.
34. Saraswathy N & Kumar PA (2004) Protein engineering of d-endotoxins of *Bacillus thuringiensis*. *Electron J Biotechnol* **7**, 178–188.
35. Knowles BH, Thomas WE & Ellar DJ (1984) Lectin-like binding of *Bacillus Thuringiensis* Var. Kurstaki lepidopteran-specific toxin is an initial step in insecticidal action. *FEBS Lett* **168**, 197–202.
36. Linderoth A, Prykhod'ko O, Ahrén B, *et al.* (2006) Binding and the effect of the red kidney bean lectin, phytohaemagglutinin, in the gastrointestinal tract of suckling rats. *Br J Nutr* **95**, 105–115.
37. Otte JM, Chen CX, Brunke G, *et al.* (2001) Mechanisms of lectin (phytohemagglutinin)-induced growth in small intestinal epithelial cells. *Digestion* **64**, 169–178.
38. Bardocz S, Grant G, Ewen SWB, *et al.* (1995) Reversible effect of phytohemagglutinin on the growth and metabolism of rat gastrointestinal tract. *Gut* **37**, 353–360.
39. Bakke-McKellep AM, Penn MH, Salas PM, *et al.* (2007) Effects of dietary soyabean meal, inulin and oxytetracycline on intestinal microbiota and epithelial cell stress, apoptosis and proliferation in the teleost Atlantic salmon (*Salmo salar* L.). *Br J Nutr* **97**, 699–713.
40. Sanden M, Berntssen MHG, Krogdahl Å, *et al.* (2005) An examination of the intestinal tract of Atlantic salmon, *Salmo salar* L., parr fed different varieties of soy and maize. *J Fish Dis* **28**, 317–330.
41. Bakke-McKellep AM, Sanden M, Danieli A, *et al.* (2008) Atlantic salmon (*Salmo salar* L.) parr fed genetically modified soybeans and maize: histological, digestive, metabolic, and immunological investigations. *Res Vet Sci* **84**, 395–408.
42. Trabalza-Marinucci M, Brandi G, Rondini C, *et al.* (2008) A three-year longitudinal study on the effects of a diet containing genetically modified Bt176 maize on the health status and performance of sheep. *Livest Sci* **113**, 178–190.

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43. Malatesta M, Caporaloni C, Gavaudan S, *et al.* (2002) Ultrastructural morphometrical and immunocytochemical analyses of hepatocyte nuclei from mice fed on genetically modified soybean. *Cell Struct Funct* **27**, 173–180.
44. Malatesta M, Boraldi F, Annovi G, *et al.* (2008) A long-term study on female mice fed on a genetically modified soybean: effects on liver ageing. *Histochem Cell Biol* **130**, 967–977.
45. Vecchio L, Cisterna B, Malatesta M, *et al.* (2004) Ultrastructural analysis of testes from mice fed on genetically modified soybean. *Eur J Histochem* **48**, 449–453.
46. Nordrum S, Olli JJ, Røsjø C, *et al.* (2003) Effects of graded levels of medium chain triglycerides and cysteine on growth, digestive processes and nutrient utilization in sea water reared Atlantic salmon (*Salmo salar* L.) under *ad libitum* feeding regime. *Aquac Nutr* **9**, 263–274.
47. Schoenborn JR & Wilson CB (2007) Regulation of interferon-γ during innate and adaptive immune responses. *Adv Immunol* **96**, 41–101.
48. Bertelsen LS, Eckmann L & Barrett KE (2004) Prolonged interferon-g exposure decreases ion transport, NKCC1, and Na^+-K^+-ATPase expression in human intestinal xenografts *in vivo*. *Am J Physiol Gastrointest Liver Physiol* **286**, G157–G165.
49. Rocha F, Musch MW, Lishanskiy L, *et al.* (2001) IFN-γ downregulates expression of Na^+/H^+ exchangers NHE2 and NHE3 in rat intestine and human Caco-2/bbe cells. *Am J Physiol Cell Physiol* **280**, C1224–C1232.
50. Woo AL, Gildea LA, Tack LM, *et al.* (2002) *In vivo* evidence for interferon-gamma-mediated homeostatic mechanisms in small intestine of the NHE3 Na^+/H^+ exchanger knockout model of congenital diarrhea. *J Biol Chem* **277**, 49036–49046.
51. Musch MW, Clarke LL, Mamah D, *et al.* (2002) T cell activation causes diarrhea by increasing intestinal permeability and inhibiting epithelial Na^+/K^+-ATPase. *J Clin Invest* **110**, 1739–1747.
52. Frøystad-Saugen MK, Lilleeng E, Bakke-McKellep AM, *et al.* (2009) Distal intestinal gene expression in Atlantic salmon (*Salmo salar* L.) fed genetically modified maize. *Aquac Nutr* **15**, 104–115.
53. Schroder M, Poulsen M, Wilcks A, *et al.* (2007) A 90-day safety study of genetically modified rice expressing Cry1Ab protein (*Bacillus thuringiensis* toxin) in Wistar rats. *Food Chem Toxicol* **45**, 339–349.
54. Wang ZH, Wang Y, Cui HR, *et al.* (2002) Toxicological evaluation of transgenic rice flour with a synthetic *cry1Ab* gene from *Bacillus thuringiensis*. *J Sci Food Agric* **82**, 738–744.
55. Elangovan AV, Tyagi PK, Shrivastav AK, *et al.* (2006) GMO (*Bt-Cry1Ac* gene) cottonseed meal is similar to non-GMO low free gossypol cottonseed meal for growth performance of broiler chickens. *Anim Feed Sci Technol* **129**, 252–263.
56. Ortego LS, Hawkins WE, Walker WW, *et al.* (1995) Immunohistochemical detection of proliferating cell nuclear antigen (PCNA) in tissues of aquatic animals utilized in toxicity bioassays. *Mar Environ Res* **39**, 271–273.
57. Uni Z, Platin R & Sklan D (1998) Cell proliferation in chicken intestinal epithelium occurs both in the crypt and along the villus. *J Comp Physiol B, Biochem Syst Environ Physiol* **168**, 241–247.
58. Sheen-Chen SM, Ho HT, Chen WJ, *et al.* (2003) Obstructive jaundice alters proliferating cell nuclear antigen expression in rat small intestine. *World J Surg* **27**, 1161–1164.
59. Berntssen MHG, Hylland K, Julshamn K, *et al.* (2004) Maximum limits of organic and inorganic mercury in fish feed. *Aquac Nutr* **10**, 83–97.
60. Hemre GI, Deng DF, Wilson RP, *et al.* (2004) Vitamin A metabolism and early biological responses in juvenile sunshine bass (*Morone chrysops* × *M. saxatilis*) fed graded levels of vitamin A. *Aquaculture* **235**, 645–658.
61. Vázquez-Padrón RI, Moreno-Fierros L, Neri-Bazán L, *et al.* (2000) Characterization of the mucosal and systemic immune response induced by Cry1Ac protein from *Bacillus thuringiensis* HD 73 in mice. *Braz J Med Biol Res* **33**, 147–155.