



Atlantic salmon (*Salmo salar* L.) parr fed genetically modified soybeans and maize: Histological, digestive, metabolic, and immunological investigations

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Abstract

Physiological and health related responses to dietary inclusion of genetically modified (GM) full-fat soybean meal (Roundup Ready[®]; GM-soy) and maize (MON810[®] Bt-maize; GM-maize), as well as non-parental, untransformed lines (nGM-soy and nGM-maize D2), were evaluated in farmed Atlantic salmon (*Salmo salar* L.) parr during the first 8 months of feeding. Significant effects of dietary GM presence were only found in intestinal Na⁺-dependent D-glucose uptake and SGLT1 protein level in the region pyloric caeca in which the highest values were found in the GM-soy, intermediate in the nGM-soy, and lowest in the standard FM fed groups. Data from this study confirm that GM soybeans (RRS[®]) and maize (MON810[®]) at inclusion levels of about 6% appear to be as safe as commercially available nGM soy and maize in diets for Atlantic salmon parr. Results from studies with higher inclusion levels and with non-modified, isogenic or near-isogenic parental lines as control groups are pending.

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1. Introduction

The inclusion of vegetable ingredients in feeds for farmed Atlantic salmon (*Salmo salar* L.) and other carnivorous fish is now customary and likely to increase as the supply of fish meal and fish oil becomes more limited. Soybean meal and maize are two of the more commonly used vegetable ingredients in salmon feeds. The introduction of commercially available genetically modified (GM) crops and their ever-increasing share on the world market in

the last 10 years, especially marked in the soybean and maize supply, has led to difficulties in procuring exclusively non-GM crops. The use of GM crops in food, as well as feed for animal production including farmed fish, is controversial in Europe; at least until further safety assessments can be conducted (Kok and Kuiper, 2003).

Transgenic Roundup Ready soybeans (RRS[®]) is produced by the introduction of a gene coding for 5-enolpyruvyl shikimate 3-phosphate synthase (EPSPS) from *Agrobacterium* sp. The expressed enzyme delivers tolerance to glyphosate (Padgett et al., 1995), a broad-spectrum herbicide. Feeding trials performed on various animals have not revealed any important differences in growth performance, carcass traits, or animal product (i.e., meat or milk) composition in production animals (Hammond et al., 1996;

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Edwards et al., 2000; Cromwell et al., 2002; Hemre et al., 2005), nor has the transgenic DNA construct been found in the muscle, milk, or other tissue of animals fed GM soybeans (Jennings et al., 2003; Phipps et al., 2002, 2003; Zhu et al., 2004). This was also concluded in a feeding trial performed on Atlantic salmon, although smaller DNA fragments (120 base pairs) of the construct were found in the content (chyme) of all regions of the gastrointestinal tract (Sanden et al., 2004). Various health-related parameters such as gross necropsy findings, haematological and urine values, clinical serum parameters, hormone levels, organ weights and histopathology, some immunological responses, and/or soybean specific antibodies were evaluated in two long term (>90 day) trials performed on mice and rats (Teshima et al., 2000; Zhu et al., 2004) and in Atlantic salmon post-smolt (Hemre et al., 2005; Bakke-McKellep et al., 2007), and no important differences were observed between animals fed the conventional soybean and RRS meals. The transgene protein, CP4 EPSPS, appeared to degrade readily *in vitro* in gastric and intestinal fluids, and a high dose (572 mg/kg body weight; >1000-fold anticipated consumption level) did not appear to be acutely toxic to gavaged mice (Harrison et al., 1996). Additionally, RRS did not appear to have any negative effect on foetal, postnatal, pubertal or adult testicular development in a multigenerational study conducted in pregnant mice and their offspring (Brake and Evenson, 2004).

Transgenic *Bt*-maize is produced by the introduction of a gene coding for various crystal (Cry) delta-endotoxins from some strains of the soil bacterium *Bacillus thuringiensis*. The expressed proteins have insecticidal properties (reviewed by Schnepf et al., 1998). The endotoxins bind to the epithelium of the insects' midgut (Hofmann et al., 1988; Bravo et al., 1992) and cause pore formation (Schwartz et al., 1993; Lorence et al., 1995), osmotic dysfunction and cell lysis (Hendrickx et al., 1990; Knowles, 1994). As for RRS, a number of feeding trials have been performed to assess the safety of *Bt*-maize for animals in terms of nutritional, growth, and production parameters (Aulrich et al., 2001; Barriere et al., 2001; Reuter et al., 2002a,b; Donkin et al., 2003; Sanden et al., 2006), intestinal histomorphology (Sanden et al., 2005), and traceability of the transgenic DNA and its expressed protein (Chowdhury et al., 2003a,b,c, 2004; Phipps et al., 2003; Reuter and Aulrich, 2003; Tony et al., 2003). The results indicated there were no differences between *Bt*-maize and its conventional counterpart in nutritional, growth, production parameters, or intestinal histomorphology. The transgenic DNA and its expressed protein were often at least partially found in the contents of the intestinal tract, but not systemically.

Two reports from a long term feeding trial of GM soybeans and maize in diets for parr from the time of first feeding are published (Sanden et al., 2005, 2006). This is a third report of the above-mentioned study on parr, in which the long term effects on organ morphology, intestinal maltase, leucine aminopeptidase, and Na^+ -D-glucose cotransporter activity, hepatic fructose-1,6-bisphosphatase activity,

plasma insulin response, and on the immunological markers acid phosphatase, lysozyme, and immunoglobulin M in various tissues were examined. Additional data on the anti-nutritional factor content of the GM feed ingredients and on the functional characteristics of apical Na^+ -D-glucose cotransporter expressed by the Atlantic salmon intestine are also reported.

2. Materials and methods

2.1. Fish rearing conditions and experimental design

Animals were held and the experiment was conducted in accordance with the Norwegian Animal Welfare Act No. 73 of December 20th 1974 and the Regulation on Animal Experimentation of January 15th 1996. As reported by Sanden et al. (2005, 2006), 21,000 first-feeding Atlantic salmon parr at the late yolk sac stage (initial mean weight $0.21 \text{ g} \pm 0.02 \text{ SD}$) were randomly distributed into 21 indoor first feeding tanks (1 m^3) at the Institute of Marine Research's Matre Research Station, Matredal (61°N , Western Norway). The experiment was conducted over a period of eight months during which fish were subjected to two photoperiod regimes: LD 24:0 (L: light; D: dark) from March to September and LD12:12 during the last month. Water salinity was $2.0\text{--}2.3 \text{ g L}^{-1}$ and mean temperature $11 \pm 2^\circ\text{C}$. Throughout the experiment the rate of water flow was adjusted to maintain the oxygen content of the outlet water $>7 \text{ mg L}^{-1}$. Before the final growth period (August–October) the biomass of each tank was randomly decreased by sampling according to standard procedures from a density of 24.0 kg m^{-3} ($585 \text{ individuals m}^{-3}$) to a density of 9.0 kg m^{-3} ($220 \text{ individuals m}^{-3}$).

2.2. Feed ingredients

Four maize varieties, two traditional – Dekalb 2 (D2) and Pioneer 2 (P2) – and two genetically modified – Dekalb 1 (D1) and Pioneer 1 (P1) – were obtained from Mississippi State University (USA). The GM-maizes D1 and P1 were both products of MON810® events, a registered trademark of Monsanto Technology LLC, which express the protein Cry1Ab to confer resistance to target insects. The two GM-maize varieties were hybrids between MON810® and the two traditional maize varieties (D2 and P2), and the concentration of MON810® maize was 51% in D1 and 59% in P1 (analysed at the National Veterinary Institute, Oslo, Norway). The conventional maize varieties D2 and P2, grown side-by-side with the GM-maize D1 and P1, were used as reference groups. No transgenic event was detected in either conventional maize variety (National Veterinary Institute, Oslo, Norway). All maize grains were ground separately prior to diet preparation (no heat treatment) to obtain maize flour. Proximate and chemical compositions of the maize grains are shown in Table 1.

The raw GM soybeans were kindly supplied by Mississippi State University (USA) and the non-genetically mod-

Table 1

Proximate composition and antinutritional factor levels in the test ingredients: soybeans nGM-soy and GM-soy (Round-up Ready®) and the maize varieties nGM maize D2, GM maize D1 (Dekalb maize, D1 a hybrid of MON810®; *Bt*-maize), nGM maize P2, and GM maize P1 (Pioneer maize, P1 a hybrid of MON810®; *Bt*-maize)

	nGM-soy	GM-soy	nGM-maize D2	GM-maize D1	nGM-maize P2	GM-maize P1
Proximate analysis (g kg ⁻¹)						
Dry matter	910	893	881	877	884	881
Protein	344	366	82	83	72	77
Lipid	186	182	32	32	32	32
Ash	47	50	534	514	551	546
Starch ^a	4	7	12	12	11	11
Residue ^b	329	288	221	236	218	215
Low molecular carbohydrates ^c (g kg ⁻¹)						
Glucose	0.6	0.9	0.6	0.6	1.0	0.8
Fructose	0.1	0.4	0.2	0.2	0.4	0.3
Sucrose	21.0	15.3	7.6	9.6	6.8	6.3
Raffinose	4.2	3.7	0.7	0.8	0.3	0.2
Stachyose	15.5	14.8	0.1	0.2	0.1	0.1
Total	41.4	35.1	9.2	11.4	8.6	7.7
Monosaccharide subunits in total NSP ^d (g kg ⁻¹)						
Rhamnose	5	5	2	2	2	2
Fucose	3	3	1	1	1	1
Arabinose	30	28	36	43	36	36
Xylose	22	15	50	59	47	47
Mannose	11	7	4	3	4	3
Galactose	44	43	16	19	15	14
Glucose	70	42	55	62	50	56
Total monosaccharides	184	143	164	190	154	158
Monosaccharide subunits in insoluble NSP ^d (g kg ⁻¹)						
Rhamnose	3	3	1	2	2	2
Fucose	2	2	0	1	1	1
Arabinose	21	17	35	41	35	34
Xylose	16	10	53	61	49	49
Mannose	6	4	3	2	3	2
Galactose	28	26	9	11	8	7
Glucose	51	30	45	54	38	35
Total monosaccharides	127	92	146	172	136	131
Phytoestrogens (g kg ⁻¹)						
Daidzein	0.25	0.10	<0.01	<0.01	<0.01	<0.01
Genistein	0.41	0.47	<0.01	<0.01	<0.01	<0.01
Phytates (g kg ⁻¹)						
IP6 ^e	5.83	7.02	5.13	5.58	4.73	5.28
IP5 ^e	0.80	0.60	0.30	0.30	0.30	0.30
IP4 ^e	0.15	0.10	0.00	0.00	0.05	0.05
Trypsin inhibitors (g kg ⁻¹)						
Total bovine trypsin inhibited ^f	6.7	35.7	1.4	1.4	1.2	1.1
Kunitz trypsin inhibitor	0.3	11.6	–	–	–	–
Bowman–Birk inhibitor	0.003	0.8	–	–	–	–
Lectin (mg kg ⁻¹)	58.6	6.7	–	–	–	–
Saponins (mM kg ⁻¹)						
Saponin V	0.00005	0.00016	–	–	–	–
Saponin I	0.785	0.665	–	–	–	–
Saponin II	0.470	0.275	–	–	–	–
Saponin alfaG	0.445	0.275	–	–	–	–
Saponin betaG	2.405	1.720	–	–	–	–
Saponin betaA	1.495	0.825	–	–	–	–
Total Saponin	5.605	3.765	–	–	–	–

Roundup Ready® soy and MON810® maize registered trademark of Monsanto Technology LLC (not heat treated prior to extrusion). Conventional soy (nGM-soy), SoyaX-aqua LF®, Schouten Industries B.V., Glessen, the Netherlands (heat-treated prior to extrusion).

^a Digestible carbohydrates; glucose hydrolysed from starch and oligosaccharides.

^b Residue was calculated as 1000 – (moisture + lipid + starch + ash).

^c Total monosaccharides (rhamnose, fucose, arabinose, xylose, mannose, galactose, uronic acid, and glucose).

^d Non-starch polysaccharides.

^e Hexa- penta- and tetra-phosphates of *myoinositol*.

^f Trypsin inhibitor activity; g bovine trypsin inhibited per kg sample.

ified (nGM; heat treated) soybeans (SoyaX-aqua LF®, Schouten Industries B.V., Glessen, The Netherlands) were from Argentina. Both soybean varieties were ground prior

to diet preparation to obtain full-fat soybean meal (FFSBM). The GM soybeans were Roundup Ready®, a registered trademark of Monsanto Technology LLC, mod-

ified to express the protein CP4 EPSPS which confers resistance to glyphosate, the active ingredient in the herbicide Roundup. The concentration of GM soy in the batch was 50% (analysed at the National Veterinary Institute, Oslo, Norway). Proximate and chemical compositions of the soybeans are shown in Table 1.

Ingredients and feeds were analyzed for proximate composition using standard reference methods (Tables 1 and 2). Crude protein was calculated as $N \times 6.25$. Total trypsin inhibitor activity was measured according to Hamerstrand et al. (1981). Bowman–Birk inhibitor and Kunitz trypsin inhibitor (Hajós et al., 1995) and soybean lectin were analysed at the Central Food Research Institute in Budapest by the research groups of Eva Gelencsér and Gyöngyi Hajós. For lectin detection a competitive indirect ELISA assay for quantification of SBA (soybean agglutinin, *Glycine max* lectin) was performed as previously described (Hajós et al., 1995) with some modifications. Plates were coated overnight at 4 °C with 0.5 $\mu\text{g mL}^{-1}$ SBA (Sigma) diluted in PBS (in 0.01M phosphate-buffered saline, pH 7.4). Standard SBA (Sigma) diluted in GPBS (0.01 M PBS containing 0.1 M galactose, pH 7.4) or soybean samples with unknown content of lectin were added, followed by anti-SBA rabbit IgG antibody (CFRI) diluted 1:600 v/v with GPBS. After incubation (30 °C 30 min), anti-rabbit goat IgG horseradish peroxidase conjugate (Jackson)

diluted with GPBS (1:3000 v/v) was added. Following the incubation (30 °C 30 min) a substrate solution of OPD (*o*-phenylene-diamine)– H_2O_2 (Sigma Fast, pH 5.0) was added. After 15 min incubation at room temperature the reaction was stopped by adding 3 M H_2SO_4 and the optical density was measured at 492 nm against a reference filter at 630 nm. Between each step the plates were washed three times with PBST (0.01 M phosphate-buffered saline containing 0.1% Tween 20, pH 7.4). Results were calculated on the basis of a half logarithmic regression fitting to the calibration curve (10^{-3} – $10^3 \mu\text{g mL}^{-1}$ SBA).

Low molecular carbohydrates (Bach Knudsen, 1997), monosaccharide in total and insoluble non-starch polysaccharides (Englyst et al., 1992) and phytic acid (Carlsson et al., 2001) were analysed by the research group of Stephan Sahlström at Matforsk (Ås, Norway). Saponins analysis (according to Hu et al., 2002) was carried out by the research group of Patricia A. Murphy at Department of Food Science and Human Nutrition at Iowa State University.

Phytoestrogens (isoflavones) were analyzed according to Greenfield et al. (2000), Chiang et al. (2001), and Müllner and Sontag (2000), but modified with regards to optimization of the acid hydrolysis of the samples, sample clean up and chromatographic conditions. Chromatographic analysis were carried out on a Gilson HPLC system consisting of

Table 2
Formulation and proximate composition of the experimental diets

Diet code	Standard fish meal (FM)	nGM-soy	GM-soy	nGM-maize D2	GM-maize D1
<i>Formulation (g kg⁻¹)</i>					
Fish meal	742	686	686	745	745
nGM-FFSBM ^d	–	125	–	–	–
GM-FFSBM ^{dd}	–	–	125	–	–
nGM-maize D2	–	–	–	121	–
GM-maize D1 ^e	–	–	–	–	121
Norseoil	74	79	79	70	70
Soy oil	23	–	–	23	23
Suprex maize ^{ee}	121	78	78	–	–
Vitamin/mineral mix ^a	20	20	20	20	20
Other ingredients ^{bc}	20	11	11	20	20
<i>Proximate analysis (g kg⁻¹)</i>					
Dry matter	924	919	920	921	922
Protein	556	551	557	550	552
Lipid	174	171	172	169	166
Starch	90	62	61	81	77
Residue ^f	104	135	130	121	127
Gross energy (kJ g ⁻¹)	21.5	20.8	21.0	21.0	20.9
Trypsin inhibition ^g (g kg ⁻¹)	0.0	0.3	0.7	0.9	1.1

^a Vitamins and minerals were added according to NRC's (1993) recommendations.

^b Lecithin (10 g kg⁻¹), inositol (0.3 g kg⁻¹) and betafin (1 g kg⁻¹) were added to all diets.

^c Sucrose (9 g kg⁻¹) was added to the diets except for the soy-based diets.

^d SoyaX-aqua LF[®], Schouten Industries B.V., Glessen, the Netherlands (heat-treated prior to extrusion).

^{dd} Full fat soybean meal (FFSBM) – Roundup Ready[®] soy, registered trademark of Monsanto Technology LLC.

^e MON810[®] maize, registered trademark of Monsanto Technology LLC.

^{ee} Suprex maize (Condrico, the Netherlands).

^f Residue was calculated as 1000 – (moisture + protein + lipid + starch).

^g Trypsin inhibitor activity; g bovine trypsin inhibited per kg diet.

a 232 XL sampling injector, 402 syringe pump, 321 pump and 864 degasser (all units from Gilson Inc., Middleton, USA) for continuous degassing of the mobile phase. A C-18 Nova-Pak (150 × 3.9 mm I.D, 4 µm; Waters, Milford, USA) column equipped with a Phenomenex Security Guard C-18 precolumn (3 × 4 mm) was used. For detection a diode-array detector, Series 1100 from Hewlett-Packard (Waldbronn, Germany) was utilized. The analytes were separated by using a solvent gradient system consisting of acetonitrile and water, at a flow rate of 0.8 mL min⁻¹. The detection limits for the isoflavones daidzein and genistein were found to range from 9 to 11 µg, calculated on a 3:1 signal-to-noise ratio monitored at each analytes' absorption maximum. Homogenised samples were extracted by acid hydrolysis with hydrochloric acid and by soxhlet extraction at 50 °C. Further sample clean up was performed on 500 mg BondElute LRC-C18 cartridges (Varian, Middelburg, The Netherlands) prior to the HPLC analysis. The isoflavone standards (daidzein and genistein) were purchased from Fluka (Buchs, Switzerland). Methanol was obtained from Rathburn (Walkerburn, Scotland), whilst acetonitrile, dimethyl sulfoxide (DMSO), fuming hydrochloric acid and sodium hydroxide were purchased from Merck (Darmstadt, Germany).

2.3. Experimental diets

Formulations and proximate compositions of the diets are shown in Table 2. Maize was used as the main source of carbohydrate in all diets. A standard fish meal (FM) diet containing fish meal as the sole protein source was used as the reference diet. Two diets were formulated containing 125 g kg⁻¹ of either non-GM FFSBM (nGM-soy) or GM FFSBM (GM-soy) and four diets containing 121 g kg⁻¹ of one of the maize varieties; nGM-maize (nGM-maize D2), GM-maize (GM-maize D1), nGM-maize (nGM-maize P1) and GM-maize (GM-maize P2). However, since inclusion level of purely GM materials was 50–59% (see above), levels of the GM ingredients in the experimental diets were only 6–7%.

Diets were supplemented with lecithin, choline (phospholipids) and betaine and were approximately isoenergetic. Sucrose and soybean oil were included in the reference diet to match the contents in the soybean diets. Gross energy was calculated according to Tacon (1987) using the following energy densities for lipid (39.5 kJ g⁻¹), protein (23.6 kJ g⁻¹) and carbohydrate (17.2 kJ g⁻¹). All diets were produced at the Norwegian Institute of Fisheries and Aquaculture Research, Bergen, Norway. Each diet was fed in excess of expected feed intake to fish in three replicate tanks.

2.4. Sampling procedure

At the end of the feeding trial, 20 fish were randomly sampled from each tank given the experimental diets FM, nGM-soy, GM-soy, nGM-maize D2, and GM-maize D1.

Fish fed the diets nGM-maize P2 and GM-maize P1 were not sampled since both the Pioneer and DeKalb GM maize varieties were found to be products of the same transgenic event. Table 1 does, however, include proximate analysis and added antinutritional factor levels for nGM-maize P2 and GM-maize P1 to supplement information given in Sanden et al. (2005, 2006).

The fish were anesthetized with metacaine (MS-222™; Norsk Medisinaldepot AS, Bergen, Norway; dosage 50 mg L⁻¹ water). Their body weights and fork lengths were measured. The tissues sampled from each fish were the gastrointestinal tract – stomach, pyloric caeca with adjacent diffuse pancreatic tissue, mid intestine, and distal intestine – and the organs liver, spleen, kidney, head kidney, and thymus. The gastrointestinal tract was opened and contents gently removed – with the exception of the pyloric caeca – and the tract divided into the various regions as described previously (Nordrum et al., 2000).

Tissues from 3 fish from each tank were used for histological screening. Circa 0.5 cm pieces from the stomach, adipose tissue surrounding the pyloric caeca containing diffusely distributed pancreatic tissue, liver, spleen, kidney, head kidney, and thymus were placed in 4% buffered formalin. Samples from muscle tissue were not taken because (1) whole body proximate analysis during the course of the feeding trial did not reveal significant differences between feeding groups (Sanden et al., 2006) and (2) histological differences were not detected in a previous three-month study on post-smolt salmon fed RRS (Bakke-McKellep et al., 2007). Sampling for and results of the evaluation of intestinal histology were reported in Sanden et al. (2005).

From another 7 fish from each tank, each gastro-intestinal region and the other organs were frozen in their entirety in liquid nitrogen and stored at –80 °C for subsequent analysis for immunological factors and intestinal transport studies. From the remaining 10 fish, blood was collected from the caudal vessels using evacuated glass test tubes (Vacutainers®) containing EDTA. The blood was stored on ice until plasma was separated by centrifugation at 3000g for 10 min, frozen, and stored at –80 °C. After blood sampling, these fish were likewise dissected, and their whole tissues and organs, as well as faeces, were frozen in liquid nitrogen. Prior to freezing the regions of the gastrointestinal tract from these fish, surrounding extraneous fat and connective tissue were carefully removed. The tissues were stored at –80 °C.

2.5. Processing of tissue for histological screening

All formalin-fixed tissues were routinely dehydrated in ethanol, equilibrated in xylene and embedded in paraffin according to standard histological techniques. Sections of ≈5 µm were cut and stained with haematoxylin and eosin (H and E) before blinded examination by two evaluators under a light microscope. Organ morphology was evaluated according to Amin et al. (1992).

2.6. Enzyme activities

Enzyme activities of maltase, leucine aminopeptidase (LAP), and acid phosphatase (AcP) were analyzed in tissue homogenates. AcP was measured in intestine, stomach, liver, kidney, and head kidney while LAP and maltase were measured on intestinal mucosa homogenate. The tissues were thawed and homogenized (1:20 w/v) in ice-cold 2 mM Tris/50 mM mannitol, pH 7.1, containing phenylmethylsulphonyl fluoride (Sigma No. P-7626) as serine protease inhibitor. Aliquots of homogenates were frozen in liquid nitrogen and stored at -80°C prior to analysis. The AcP and LAP activities were measured colorimetrically by standardized methods (Sigma procedure Nos. 104 and 251, respectively) – using *p*-nitrophenyl phosphate as the substrate for AcP and *L*-leucyl-*b*-naphthylamide as the substrate for LAP. Disaccharidase (maltase) activity was analyzed according to the methods described by Dahlquist (1970), using maltose as substrate. Incubations were performed at 37°C . The high incubation temperature was justified by the simple, positive relationship between disaccharidase activities and temperatures between 5 and 37°C (Brudeseth, 1996).

Fructose-1,6-bisphosphatase activity (F1,6bisP) of hepatic tissue was measured as described by Borrebaek et al. (1993), in the presence of 2 mM NADP + 2 mM fructose-1,6-bisphosphate and 0.65 U mL^{-1} of both purified glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. Activity was assessed spectrophotometrically by the formation of NADPH at 30°C .

Enzyme activities are expressed as amount of substrate (mol) hydrolysed as related to time and g tissue, kg body weight, and mg protein in the homogenate. Protein was analysed using Bio-Rad Protein Assay (Bio-Rad Laboratories, Munich, Germany).

2.7. Transport activity and protein expression in intestinal brush border membrane vesicles

Brush-border membrane vesicles (BBMVs) were prepared from the thawed intestinal regions pyloric caeca, mid and distal intestine as described previously (Storelli et al., 1986; Verri et al., 2000) from fish fed the standard FM, nGM-soy and GM-soy diets. The preparation was based on the selective precipitation, in the presence of EGTA and 12 mM MgCl_2 , of all cellular components with the exception of brush-border membranes. After the final centrifugation step, BBMVs were re-suspended in buffer, centrifuged at 50,000g for 30 min and re-suspended by passing them through a 21-gauge needle in a suspension solution containing (in mM) 100 mannitol, 100 KCl, and 20 HEPES, pH adjusted to 7.4 by Tris. Protein concentration was measured using the Bio-Rad DC protein assay kit, using lyophilised bovine plasma γ -globulin as a standard. Intra- and extravesicular buffers were prepared to the same ionic strength, anion concentration and osmolarity. Alkaline phosphatase (EC 3.1.3.1.) activity was measured by

monitoring spectrophotometrically (410 nm) *p*-nitrophenyl phosphate hydrolysis at a constant pH of 10.0 (Berner et al., 1976).

Uptake of $\text{D-}[^{14}\text{C}]\text{glucose}$ was measured by a rapid filtration technique (Maffia et al., 1996). The uptake reaction was initiated by addition of 5 μL (150–250 μg protein) of vesicle suspension to 95 μL of incubation medium containing: 1 mM KCl, 99 mM NaCl, 100 mM mannitol, 20 mM HEPES, 0.1 mM D-glucose , as well as 0.1 μCi of $\text{D-}[^{14}\text{C}]\text{glucose}$ (Amersham Biosciences Europe GMBH), and valinomycin ($6.5\text{ }\mu\text{g mg}^{-1}$ protein), pH adjusted to 7.4 with Tris (experimental conditions inducing a transient inside negative membrane potential), with or without 0.05 mM phlorizin, a specific inhibitor of the $\text{Na}^{+}\text{-D-glucose}$ cotransporter. At the end of the respective incubation intervals at 15°C , isotopic uptake was stopped by injection of 3 mL ice-cold stop solution (100 mM mannitol, 100 mM KCl, and 20 mM HEPES, pH adjusted to 7.4 by Tris) into the reaction tube and rapidly filtering the mixture through a Millipore filter (HAWP pore size = $0.45\text{ }\mu\text{m}$) under vacuum. Filters were washed twice with 3 mL of stop solution and assayed for radioactivity (PACKARD 1900 TR β -counter; Ecolite – MP Biomedicals scintillation cocktail).

Western blot analysis was performed to verify SGLT1 protein expression. Aliquots of BBMVs suspension (equivalent to 100 μg of protein) from pyloric caeca of fish fed the standard FM, nGM-soy and GM-soy diets were solubilised in Laemmli sample buffer and resolved by 10% SDS-PAGE. Proteins were electrotransferred onto nitrocellulose membranes (Amersham Biosciences Europe GMBH) for 2 h at constant intensity current 200 mA at 4°C . Filter was blocked with 5% BSA in phosphate buffered saline (PBS) containing 0.1% Tween 20 and probed with rabbit polyclonal antibody (Alpha Diagnostics, San Antonio, USA) raised against a 19 aa peptide from an extracellular region of mouse SGLT1. The filter was washed 6 times for 4 min in PBS with 0.1% Tween 20. The bound antibody was detected using horseradish peroxidase conjugated goat anti-rabbit IgG as secondary antibody (Sigma, St. Louis, USA) again followed by 6 washings with PBS plus 0.1% Tween 20. Activation of X-ray film was done by means of an enhanced chemiluminescence (ECL) reaction within 2–10 min. For the molecular weight determination Prestained SDS PAGE Standards Broad Range (Bio-Rad Laboratories Inc., USA) were used and signals on X-ray film were quantified in arbitrary units by Scion Image software.

2.8. Insulin

Endocrine pancreatic response was determined by analyzing plasma insulin levels by radioimmunoassay as described by Plisetskaya et al. (1986).

2.9. Lysozyme activity

Lysozyme levels were determined in homogenate of distal intestine, kidney, and head kidney using a turbidimetric

microplate technique with the Gram-positive bacteria *Micrococcus lysodeikticus* (Sigma No. M-3770) suspension (0.2 mg mL^{-1}) as previously described (Ellis, 1990) and calibrated with hen egg white lysozyme (VWR no. 67016-1). Lysozyme activity was calculated relative to tissue weight.

2.10. Immunoglobulin M

The total amount of immunoglobulin M (IgM) was determined in homogenate from pyloric caeca, mid intestine, distal intestine, kidney, head kidney, spleen, and plasma as described by Lund et al. (1995). Enzyme-linked immunosorbent assay (ELISA) plates were coated with a monoclonal mouse anti-salmonid Ig antibody raised against rainbow trout and Atlantic salmon immunoglobulins (MCA2182; Serotec Ltd, Hamar, Norway) diluted $1:6000$ in 0.05 mol L^{-1} carbonate buffer (pH 9.6). The plates were rinsed and incubated with rinsing buffer containing 1% bovine serum albumin (BSA) for 45 min at 37°C . After a further rinse, mucosa homogenate (diluted $1:20$ in phosphate buffer containing 0.5 mL L^{-1} Tween 20 and 4% horse serum) was added to the wells and incubated overnight at 4°C . The plates were then rinsed and incubated with the monoclonal anti-salmonid Ig, diluted in rinsing buffer, for 1 h at room temperature. After rinsing, peroxidase-labelled anti-mouse Ig (Amersham, Uppsala, Sweden) diluted $1:3000$ in rinsing buffer, was added and rinsed. The substrate *o*-phenylenediamine was added and the plates were incubated for 10 min at room temperature, followed by addition of $100 \mu\text{L } 1 \text{ mol L}^{-1} \text{ H}_2\text{SO}_4$. The optical density was read spectrophotometrically at 492 nm.

2.11. Statistical analysis

Analysis of variance and Duncan's multiple range test were used in the evaluation of the results (tank means; $n = 3$) with Diet and Tank location (block) as class variables. For most variables the analysis was run separately for each intestinal section and organ due to significant differences in variances. Therefore, transformation of the results to obtain similar variances was not performed. The software package SAS (Release 8.02 TS Level 02MO) was used in the evaluation. The software Graph-Pad-Prism 4 was used to estimate the uptake of D-glucose, while the statistical evaluation of transport data was performed by means of the ANOVA and Bonferroni post tests. Differences between means were considered statistically significant when $p < 0.05$. Critical range was used where applicable to indicate the minimum difference required between means across all experimental groups for them to be significantly different.

3. Results

The chemical analysis of the GM soy and maize varieties and their non-GM counterparts (Table 1) indicates that there were differences in composition of the raw mate-

rials. The most noteworthy differences were the higher starch, genistein, trypsin inhibitor activity, and total saponin levels, but lower mono and oligosaccharides in low molecular carbohydrate fraction, total monosaccharides in total as well as insoluble non-starch polysaccharide (NSP) fractions, daidzein, phytates, and lectin in the GM-soy. Trypsin inhibition was also higher in the formulated diet containing GM-soy (Table 2) but levels were very low in all the extruded diets.

No apparent, diet-related histological changes were observed in any of the sections from the stomach, diffuse pancreatic tissue adjacent to the pyloric caeca, liver, spleen, kidney, head kidney, or thymus. Histological findings of the intestinal regions pyloric caeca, mid and distal intestines were reported in Sanden et al. (2005).

Enzyme activities related to g tissue, whole tissue weight and kg body weight (BW), and mg protein in the different gastro-intestinal regions (LAP, maltase, and AcP) and liver (AcP and F-1,6bisP), as well as AcP in kidney and head kidney are shown in Table 3. Significant effects of tank location were often detected, but since this was included as a class variable it has been taken into consideration in the statistical model and we report only diet effects.

In the pyloric caeca, significant diet effects ($p < 0.05$) were found for LAP activity. In all cases, lowest enzyme activities were observed in fish fed the standard FM diet, intermediate levels in the nGM and GM-soy-fed fish, and highest values in those fed the nGM and GM-maize diets. No significant effects of genetic modification were detected. No significant effects were detected for maltase or AcP activities in the pyloric caeca. In the mid intestine, a significant effect was found for maltase and AcP activities per mg protein due to diet. Activity of maltase and AcP were highest in fish fed the standard FM diet whereas no significant differences were found between the soy and maize diets whether genetically modified or not. Similar trends were observed for maltase and AcP, as well as LAP activities relative to g tissue and kg BW. In the distal intestine, tissue enzyme (LAP, maltase, and AcP) activities were not significantly affected by diet.

In the liver, AcP activity per g tissue was significantly affected by diet but not when activity was related to mg protein or kg BW. The fish fed the maize and soy-containing diets had higher AcP activity per g liver tissue than the fish fed the standard FM diet. The same trend was observed for AcP per kg BW as well as per mg protein (Table 3). Liver fructose-1,6-bisphosphatase activities were not significantly affected by diet. In the stomach, AcP activities were not significantly affected by diet.

Fig. 1 presents the time course of D-[^{14}C -glucose] uptake by brush border membrane vesicles isolated from different regions (pyloric caeca, mid and distal intestine) of the intestines taken from fish fed the standard FM, nGM-soy and GM-soy diets. Fig. 1a indicates that in the presence of an inwardly directed Na-gradient, D-glucose uptake was rapid in vesicles from pyloric caeca of fish fed the standard FM diet, reaching a maximal intra-

Table 3

Mean activities in homogenates of different tissues of the enzymes leucine aminopeptidase (LAP; in mmol h⁻¹), maltase (in μ mol min⁻¹), acid phosphatase (in μ mol h⁻¹), and fructose-1,6-bisphosphatase (in pmol min⁻¹) as related to g tissue, whole tissue and kg body weight (kg BW), and mg protein

Enzyme		Standard FM	nGM-soy	GM-soy	nGM-maize D2	GM-maize D1	Critical range	ANOVA <i>p</i> -value
Tissue	Units							
<i>Leucine aminopeptidase</i>								
Pyloric intestine	g tissue	19.3 ^c	24.0 ^b	23.0 ^b	28.3 ^a	27.0 ^a	3.1	0.0007
	kg BW	308.0 ^b	381.0 ^{ab}	377.7 ^{ab}	479.7 ^a	454.3 ^a	112.1	0.0321
	mg protein	539.3 ^b	669.7 ^{ab}	583.0 ^b	735.7 ^a	660.0 ^{ab}	135.5	0.0448
Mid intestine	g tissue	16.7	15.0	14.7	15.3	18.0	4.8	n.s.
	kg BW	30.7	19.7	18.0	18.7	23.0	14.1	n.s.
	mg protein	580.7	475.7	431.3	445.7	505.3	162.6	n.s.
Distal intestine	g tissue	29.3	25.3	20.7	22.3	25.7	16.2	n.s.
	kg BW	104.0	97.3	80.0	78.0	93.3	66.7	n.s.
	mg protein	625.0	533.7	399.0	451.3	528.3	304.9	n.s.
<i>Maltase</i>								
Pyloric intestine	g tissue	3.0 ^{abc}	3.5 ^a	2.7 ^c	2.8 ^{bc}	3.3 ^{ab}	0.7	0.0505
	kg BW	47.3	55.9	43.1	46.5	55.8	13.9	n.s.
	mg protein	82.4 ^{ab}	99.2 ^a	69.0 ^b	74.9 ^{ab}	81.2 ^{ab}	26.0	0.1397
Mid intestine	g tissue	1.3 ^a	1.1 ^{ab}	1.1 ^{ab}	1.0 ^b	1.1 ^{ab}	0.2	0.0595
	kg BW	3.0	1.6	1.4	1.3	1.5	1.8	n.s.
	mg protein	48.5 ^a	33.3 ^b	32.7 ^b	28.1 ^b	31.5 ^b	14.1	0.0474
Distal intestine	g tissue	1.4	1.2	0.8	0.8	1.0	0.8	n.s.
	kg BW	4.9	4.5	3.3	2.8	3.7	3.4	n.s.
	mg protein	29.1	25.5	16.0	16.1	19.9	15.6	n.s.
<i>Acid phosphatase</i>								
Stomach	g tissue	0.08	0.08	0.08	0.08	0.08	0.01	n.s.
	kg BW	0.40 ^{ab}	0.39 ^b	0.46 ^a	0.43 ^{ab}	0.42 ^{ab}	0.07	0.1921
	mg protein	0.74	0.79	0.82	0.81	0.77	0.09	n.s.
Pyloric intestine	g tissue	0.21	0.20	0.21	0.20	0.22	0.02	n.s.
	kg BW	3.23	3.24	3.42	3.33	3.49	0.79	n.s.
	mg protein	2.30	2.26	2.15	2.15	2.11	0.25	n.s.
Mid intestine	g tissue	0.18	0.16	0.18	0.16	0.17	0.03	n.s.
	kg BW	0.39	0.22	0.22	0.19	0.23	0.23	n.s.
	mg protein	2.50 ^a	2.04 ^b	2.13 ^{ab}	1.79 ^b	1.91 ^b	0.43	0.0285
Distal intestine	g tissue	0.13 ^{ab}	0.12 ^{ab}	0.14 ^a	0.13 ^{ab}	0.11 ^b	0.03	0.1752
	kg BW	0.45 ^{ab}	0.44 ^b	0.55 ^a	0.45 ^{ab}	0.41 ^b	0.10	0.0874
	mg protein	1.10	0.96	1.11	1.03	0.95	0.26	n.s.
Liver	g tissue	0.17 ^b	0.20 ^a	0.21 ^a	0.21 ^a	0.21 ^a	0.02	0.0158
	kg BW	2.08	2.20	2.13	2.16	2.33	0.46	n.s.
	mg protein	0.95	0.95	0.95	1.02	1.01	0.09	n.s.
Kidney	g tissue	0.20	0.20	0.21	0.21	0.21	0.02	n.s.
	kg BW	0.99	0.98	1.06	1.09	1.13	0.19	n.s.
	mg protein	1.15 ^{ab}	1.07 ^b	1.17 ^a	1.21 ^a	1.20 ^a	0.11	0.0619
Head kidney	g tissue	0.06	0.06	0.06	0.05	0.05	0.01	n.s.
	kg BW	0.06	0.06	0.06	0.06	0.05	0.03	n.s.
	mg protein	1.36	1.31	1.28	1.43	1.31	0.36	n.s.
<i>Fructose-1,6-bisphosphatase</i>								
Liver	g tissue	4.3	3.9	4.4	4.4	4.5	1.9	n.s.
	kg BW	13.9	12.2	12.6	12.5	13.1	4.8	n.s.
	mg protein	24.6	19.3	20.6	21.9	22.3	9.9	n.s.

Critical range indicates the minimum difference required between values for them to be significantly different. n.s. = not significant ($p > 0.05$).

^{a-c} Mean values within a row with different superscript letters indicate significant differences ($p < 0.05$).

vesicular sugar accumulation (overshoot) between 40 s and 2 min of incubation, which was about 3 times that at equilibrium (90 min). Under the same conditions the labelled D-glucose uptake overshoot by the mid intestine

(Fig. 1b) was similar to that observed by the pyloric caeca, while in the distal intestine (Fig. 1c) the sugar accumulation was significantly lower, reaching a value of 1.1 times that at equilibrium after 40 s. In fish fed diets con-

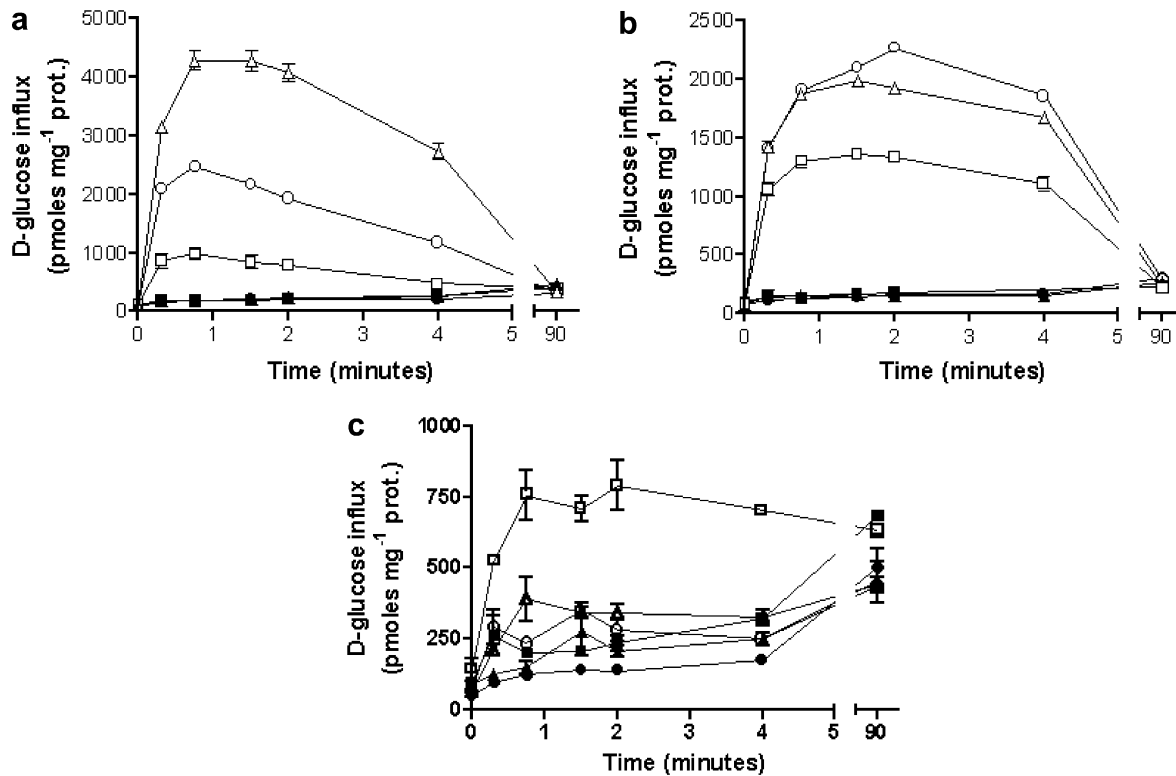


Fig. 1. D-Glucose transport (pmoles mg⁻¹ protein; mean $\pm$ S.E.M.) as a function of incubation time in brush border membrane vesicles (BBMV) from (a) pyloric caeca, (b) mid intestine, and (c) distal intestine of Atlantic salmon fed standard FM (empty squares), nGM-soy (empty circles), or GM soy (empty triangles). The filled points signify BBMV D-glucose transport in the presence of phlorizin.

taining soy, D-glucose accumulation by pyloric caeca was significantly increased; particularly in fish fed GM-soy in which the overshoot observed was about 4 times that of standard FM-fed fish. In the mid intestine, D-glucose overshoot was comparable between the two soy-diets, being 1.6 times higher than that observed in fish fed the standard FM diet. In the distal part of salmon intestine, the presence of soy in the diet completely abolished overshoot phenomenon in the presence of Na-gradient, significantly decreasing D-glucose uptake in the first 2 min to values not significantly different than those observed in the presence of phlorizin, a specific inhibitor of Na⁺-D-glucose cotransport. Addition of 0.05 mM phlorizin to the external medium abolished the ability of vesicles from all intestinal samples to accumulate the radiolabelled sugar against a concentration gradient in the presence of an inwardly directed Na⁺-driving force. D-Glucose uptakes at equilibrium were approximately the same for vesicles from all the fish diets, under control and inhibited conditions, suggesting that similar-sized vesicles were present at all treatments, ruling out transport differences due to variable vesicular volumes. These results demonstrated that a Na-dependent, phlorizin-sensitive D-glucose transport mechanism is present in the epithelial brush borders of salmon intestine, quantitatively less expressed in the distal portion, and that the presence of soy in the diet modifies the sugar transporting capabilities differently in the various intestinal regions.

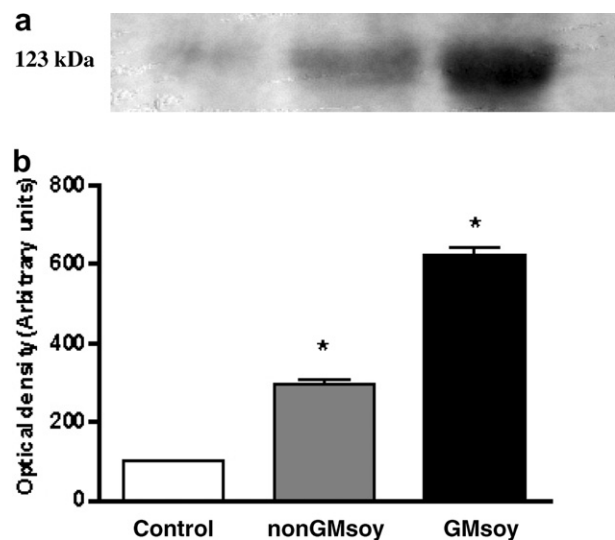


Fig. 2. (a) Immunoblots for SGLT1 protein expression in pyloric caeca of salmon fed control (left), nGM-soy (center) and GM-soy (right) diet. Primary antibody was raised in rabbit against a 19 aa peptide from an extracellular region of mouse SGLT1 and used in a 1:2000 dilution. Each lane represents an immunoblot of 100 μ g protein extracted from pyloric caeca brush border membrane protein. Molecular weights are shown on the left. (b) Intensity (expressed in arbitrary units, control = 100) of immunoblots on X-ray film for SGLT1 protein expression in pyloric caeca as a function of diet. Results show means $\pm$ S.E.M. of 3 experiments. *Significantly different ($p < 0.01$).

Fig. 2a shows an immunoblot carried out using a polyclonal anti-SGLT1 antibody. This antibody recognizes a single band around 123 kDa in pyloric caeca BBMV, the intestinal region where the D-glucose flux is highest. Fig. 2b shows the densitometric analysis of the three separate assays.

Insulin levels did not vary significantly between the feeding groups. The means in ng mL⁻¹ for the various groups were: nGM-soy group 26.3; GM maize D1 group 14.3; control group 14.0; GM-soy group 11.3; nGM maize D2 group 10.2 (critical range 19.2).

Some immunological responses, as measured by AcP activity, lysozyme and IgM in tissue homogenates, are shown in Table 3 (AcP in kidney and head kidney) and Table 4 (lysozyme and IgM). Significant models were found for several of the parameters but they were not due to the diet, but rather to tank location.

4. Discussion

The results reported here support and augment the results presented previously from this experiment (Sanden et al., 2005, 2006) as well as results from similar analyses performed on tissues from a feeding trial conducted with Atlantic salmon post-smolt in seawater fed 172 g kg⁻¹ GM FFSBM (Bakke-McKellep et al., 2007). In this long term feeding trial spanning first-feeding, early stages of development of parr, and maturation toward parr-smolt transformation of Atlantic salmon, low inclusion levels (6–7%) of genetically modified full-fat soybean meal and maize in the diets did not appear to have any deleterious effects on histomorphology of stomach, pancreatic tissue, liver, spleen, kidney, head kidney, or thymus, digestive enzymes activity in the gastro-intestinal tract, liver metabolism, or intestinal or systemic immunological response. Only intestinal D-glucose uptake mediated by the apical Na⁺ dependent sugar transport was significantly affected, differently in the different intestinal regions, by the presence of GM and nGM-soy in the fish diet. Since we were unable

to procure the non-GM parent line of the GM-soy used in this experiment, it is not possible to distinguish these differences as effects of the genetic modification, lack of heat treatment of the GM-soy compared to the heat-treated nGM-soy before addition to the experimental diets, and/or differences in nutrients and anti-nutritional factor levels in the two soybean varieties. Particularly the higher saponin level in the nGM-soy may have led to increased levels in the corresponding extruded diet since saponins are considered relatively heat resistant. In any case, this diet effect did not appear to affect the overall growth of the fish, as reported by Sanden et al. (2006). Although the GM and non-GM maize varieties used in the diets were related and grown side-by-side, they were not GM and parent line and thus not optimal counterparts. No effects of the different maize varieties at a dietary inclusion level of 121 g kg⁻¹ were detected on nutritional, physiological, or histomorphological parameters reported herein or in the other papers (Sanden et al., 2005, 2006). However, due to the high variability between individuals within each tank, as well as mean values between parallel tanks, a higher number of replicates are needed to lower variation. With the methods employed, this appears to be particularly relevant for the immunological responses. The critical ranges presented may be used as a basis for power analysis and thereby the number of replicates needed to identify valid differences in future studies.

An additional important finding suggested from results reported here, as well as those in the other two reports (Sanden et al., 2005, 2006), is that Atlantic salmon parr appear to tolerate a 12.5% inclusion level of full-fat soybean meal (FFSBM) in their diets from first feeding and through their development toward smoltification. Growth was not significantly affected (Sanden et al., 2006) and only 3 of the 18 FFSBM-fed fish sampled demonstrated any histological changes in the distal intestine (Sanden et al., 2005) typical of soybean meal-induced enteritis (Baeverfjord and Krogdahl, 1996). Associated with this, distal intestinal somatic index (Sanden et al., 2005) and brush border enzyme activities were not significantly reduced (Table 3),

Table 4
Mean lysozyme (units g⁻¹ tissue) and IgM (absorbance) in tissue homogenates

	Standard FM	nGM-soy	GM-soy	nGM-maize D2	GM-maize D1	Critical range	ANOVA <i>p</i> -value
<i>Lysozyme (units g tissue⁻¹)</i>							
Distal intestine	4184	4925	5701	5272	4953	1908	n.s.
Kidney	6554	6774	6504	6133	6298	1332	n.s.
Head kidney	10998	9591	7551	8099	8708	2667	n.s.
<i>Immunoglobulin M (optical density)</i>							
Pyloric intestine	n.d.	n.d.	n.d.	n.d.	n.d.	–	–
Mid intestine	0.123	0.039	0.074	0.133	0.042	0.230	n.s.
Distal intestine	n.d.	n.d.	n.d.	n.d.	n.d.	–	–
Kidney	0.018	0.044	0.070	0.028	0.054	0.085	n.s.
Head kidney	0.329	0.483	0.484	0.234	0.344	0.439	n.s.
Spleen	0.521	0.429	0.506	0.392	0.412	0.339	n.s.
Plasma	0.103	0.102	0.181	0.156	0.122	0.114	n.s.

Critical range indicates the minimum difference required between values for them to be significantly different. n.d. = none detected; n.s. = not significant (*p* > 0.05).

nor lysozyme or IgM content increased (Table 4), as has been reported earlier in post-smolt Atlantic salmon following dietary solvent-extracted SBM exposure at 24–30% inclusion levels (Krogdahl et al., 2000, 2003). Therefore, feeding trials with higher inclusion levels are needed to further investigate parr's sensitivity toward full-fat as well as extracted SBM.

On the other hand, soybean meal had a significant effect on the activity of the Na-dependent, phlorizin-sensitive D-glucose transport mechanism occurring in the epithelial brush borders of salmon intestine (Fig. 1), which generally has characteristics similar to those observed in other teleosts (Storelli et al., 1986; Maffia et al., 1996; Sala-Rabanal et al., 2004). In particular, SBM appeared to cause a decrease in Na-dependent sugar transporter number and/or activity in the brush border membrane vesicles from distal intestine of the SBM-fed salmon. This supports our findings in an earlier study of transport of glucose, as well as amino acids and dipeptide, measured in whole tissue preparations (everted intestinal sleeves) from SBM-fed post-smolt Atlantic salmon (Nordrum et al., 2000). However, in the other regions of salmon intestine the sugar absorption capabilities are considerably increased by SBM. In particular, in the pyloric caeca compartment, Na-dependent sugar influx was about four and 1.6 times higher in fish fed GM-soy than in fish fed the control diet and non GM-soy, respectively. The simple diffusion pathway of D-glucose uptake did not seem to be significantly influenced by SBM, since the phlorizin-inhibited sugar influxes were not modified by the presence of soy in any intestinal region under investigation. The observed increase of Na-dependent sugar transport in pyloric caeca and mid intestine could be explained by the presence of a feedback mechanism that causes increased transporter numbers and/or activities in the more proximal intestinal regions, when transport is impaired in the distal intestine, as previously suggested (Nordrum et al., 2000). A feedback mechanism may also explain the higher leucine aminopeptidase activity in the pyloric caeca of fish fed the diets containing higher levels of plant ingredients. The cause of the differing glucose transport in the pyloric caeca between GM and nGM-soy fed fish can again not be distinguished between effects of the genetic modification, differential heat treatment, and/or differing nutrient and anti-nutritional factor levels of the two FFSBM.

Immunoblots, using anti-SGLT1 antibody, identified a protein in pyloric caeca with a molecular weight around 123 kDa. In a previous study (Sala-Rabanal et al., 2004) using a different antibody against SGLT1, a protein SGLT1 type with a MW of 75 kDa was observed in gilt-head sea bream (*Sparus aurata*) intestine. At the moment, nothing is known about the molecular weight of SGLT1 type protein in salmon. A different glycosylation profile could explain the differences in molecular weights of Na-dependent glucose transporters between *S. salar* and *Sparus aurata*. The influences of the experimental diets on signals from the densitometric analysis support the trend observed in the time course transport experiments. Hence

the dietary influence on the D-glucose fluxes may be caused by different levels of SGLT1 protein expression.

The generally higher acid phosphatase and lysozyme (not significant) activities in the GM-soy fed salmon suggest that even intermediate levels of FFSBM may cause increased macrophage infiltration in the lamina propria and submucosa of the distal intestine. A previous study (Bakke-McKellep et al., 2000) showed that AcP activity in supranuclear vacuoles of the distal intestinal enterocytes was reduced while the number of AcP-containing macrophages in the lamina propria and submucosa were apparently increased in salmon fed 30% extracted SBM (Bakke-McKellep et al., 2000). Since the presence of supranuclear vacuoles was reportedly only diminished in two of nine GM-soy-fed fish (Sanden et al., 2005) in this study, the increased AcP activity reported in GM-soy-fed fish here was most likely due to increased macrophage infiltration. The results also suggest that macrophage infiltration occurs before histomorphological changes of the epithelium can be detected. Acid phosphatase has been characterized as a membrane-associated enzyme in lysosomes of almost all cells studied (Hauser et al., 1988), including macrophages, as indicated by AcP-knockout mice (Hayman and Cox, 2003) and histochemical studies of Atlantic salmon spleen, head kidney (Press et al., 1994, 1998), and distal intestine (Bakke-McKellep et al., 2000). In the present study, acid phosphatase activity appeared to be a more sensitive parameter than lysozyme activity as an indication of an inflammatory response in the distal intestine of Atlantic salmon parr fed SBM. Whether macrophage recruitment is associated with changes caused by the GM-soy directly or indirectly, for example, by changes it may cause in the composition of products of digestion or intestinal microflora in the distal intestine, cannot be differentiated.

In contrast to findings from a similar study in Atlantic salmon post-smolt held in seawater and fed 172 g kg⁻¹ GM FFSBM (Bakke-McKellep et al., 2007), acid phosphatase and lysozyme activities in head kidney were not increased by feeding 125 g kg⁻¹ FFSBM from either soybean varietal to parr in the current study. Whether this was due to differences in the GM soybean batches, their inclusion levels in the diets, anti-nutritional factor levels, developmental stage of the fish, water quality, or other factors remains to be elucidated.

Differences in origin as well as heat treatment of the soybean varieties used in this study may have caused disparities in the nGM and GM FFSBM compositions due to genetic, environmental, and/or processing influences. This prevents any conclusions being made concerning the causes of the differences in physiological and immunological parameters in the fish reported here. However, when all the data from this study (present results; Sanden et al., 2005, 2006) are considered as a whole, genetically modified RRS[®] soybeans and MON810[®] maize at inclusion levels of up to 6% appear to be as safe as traditionally bred counterparts in diets for Atlantic salmon parr. Further reports from this feeding trial regarding DNA tracing as well as

reports from other feeding trials with higher inclusion levels of genetically modified soybean meal and maize, and their non-transgenic parent line counterparts grown side-by-side, are pending.

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