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Dietary fatty acids and inflammation in the vertebral column of Atlantic salmon, *Salmo salar* L., smolts: a possible link to spinal deformities

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Abstract

Vegetable oils (Vo) are an alternative to fish oil (Fo) in aquaculture feeds. This study aimed to evaluate the effect of dietary soybean oil (Vo diet), rich in linoleic acid, and of dietary fish oil (Fo diet) on the development of spinal deformities under bacterial lipopolysaccharide (LPS)-induced chronic inflammation conditions in Atlantic salmon, *Salmo salar* L. Fish [25 g body weight (BW)] were fed the experimental diets for 99 days. On day 47 of feeding (40 g BW), fish were subjected to four experimental regimes: (i) intramuscular injections with LPS, (ii) sham-injected phosphate-buffered saline (PBS), (iii) intraperitoneally injected commercial oil adjuvant vaccine, or (iv) no treatment. The fish continued under a common feeding regime in sea water for 165 more days. Body weight was temporarily higher in the Vo group than in the Fo group prior to immunization and was also affected by the type of immunization. At the end of the trial, no differences were seen between the dietary groups. The overall prevalence of spinal deformities was approximately 14% at the end of the experiment. The Vo diet affected vertebral shape but did not induce spinal deformities. In groups injected with LPS and PBS, spinal deformities ranged between 21% and 38%, diet independent. Deformed vertebrae were located at or in proximity to the injection point. Assessment of inflammatory markers revealed high levels of plasma prostaglandin E_2 (PGE_2) in the Vo-fed and LPS-injected groups, suggesting an inflammatory response to LPS. Cyclooxigenase 2 (COX-2) mRNA expression in bone was higher in fish fed Fo compared to Vo-fed fish. Gene expression of immunoglobulin M (IgM) was up-regulated in bone of all LPS-injected groups irrespective of dietary oil. In conclusion, the study suggests that Vo is not a risk factor for the development of inflammation-related spinal deformities. At the same time, we found evidence that localized injection-related processes could trigger the development of vertebral body malformations.

Keywords: arachidonic acid: eicosapentanoic acid (AA: EPA), Atlantic salmon, fatty acids, inflammation, prostaglandins, spinal deformities.

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Introduction

Vertebral column deformities are a common problem in salmon farming (Waagbø, Kryvi, Breck & Ørnsrud 2005; Witten, Gil Martens, Hall, Huysseune & Obach 2005; Fjelldal, Hansen & Berg 2007; Sullivan, Hammond, Roberts & Manchester 2007; Waagbø 2008). Deformities cause down-grading of the product (Michie 2001) and constitute a problem for animal welfare (Hansen, Fjelldal, Yurtseva & Berg 2009). The most common type of deformity is compression of vertebrae (platyspondyly) (Witten *et al.* 2005), in the caudal part of the spine (Fjelldal, Hansen,

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Breck, Sandvik, Waagbø, Berg & Ørnsrud 2009). The aetiology of platyspondyly appears to be multifactorial (Vågsholm & Djupvik 1998; Waagbø *et al.* 2005; Waagbø 2008). Risk factors that may promote the development of spinal deformities in salmon are genetics (McKay & Gjerde 1986; Gjerde, Pante & Bæverfjord 2005), nutrition (Vielma & Lall 1998; Waagbø, Hamre & Maage 2001; Ornsrud, Gil & Waagbø 2004; Lall & Lewis-McCrea 2007; Waagbø 2008; Fjelldal *et al.* 2009), production conditions of under-yearling smolts (Fjelldal, Lock, Grotmol, Totland, Nordgarden, Flik & Hansen 2006), environmental factors (Wargelius, Fjelldal & Hansen 2005; Gil Martens, Witten, Fivelstad, Huysseune, Sævareid, Vikeså & Obach 2006) and vaccination (Berg, Rødseth, Tangerås & Hansen 2006; Aunsmo, Guttvik, Midtlyng, Larssen, Evensen & Skjerve 2008).

It is known from biomedical research that inflammation can alter the normal pattern of bone growth (Raisz 1999, 2005; Hughes, Turner, Belibasakis & Martuscelli 2006) and can lead to spinal deformities (Gratacos, Collado, Fillella, Sanmarti, Llena, Molina, Ballesta & Munoz-Gomez 1994). Inflammation as a risk factor for spinal deformities in salmon was suggested by Kvellestad, Høie, Thorud, Tørud & Lingøy (2000) but has not yet been investigated experimentally. Inflammation can be defined as the reaction of a living vascularized tissue to a localized damage and plays a role in both normal repair reactions and the pathogenesis of disease (De Caterina & Basta 2001). Inflammation affects bone development at two levels in mammals: (i) systemic inflammation alters the animals' hormone and mineral homeostasis as well as its nutritional metabolism, (ii) local inflammation influences growth and remodelling of bone as a result of prostaglandin and cytokine release (Raisz 2005; Hughes *et al.* 2006). It is not known whether bone of teleost fish is affected in a similar way as in mammals (Witten & Huysseune 2009). In commercial farming, handling of fish during vaccination, grading, pumping and transport may affect the integrity of the spine and may induce local inflammation. Fast-growing under-yearling smolts (so-called 0^+ smolts or autumn smolts) have a reduced bone mineral content and lower mechanical strength. These features have been associated with increased predisposition to develop spinal deformities in sea water (Fjelldal *et al.* 2006, 2007).

Vegetable oils are currently used as alternative lipids in aquaculture feeds. The effects of vegetable oils on the physiology of fish have been reviewed by Turchini, Mentasti, Frøyland, Orban, Caprino, Moretti & Valfre (2003). Evidently, partial replacement of fish oil in fish feed formulations does not compromise growth (Turchini *et al.* 2003; Bell 2008; Izquierdo, Robaina, Juarez-Carrillo, Oliva, Hernandez-Cruz & Afonso 2008; Berge, Witten, Baeverfjord, Vegusdal, Wadsworth & Ruyter 2009) or health (Waagbø, Sandnes, Jørgensen, Engstad, Glette & Lie 1993; Bell, Torstensen & Sargent 2005). Vegetable oils that are rich in n-6 polyunsaturated fatty acids have been associated with chronic inflammatory diseases in humans (Watkins, Li, Allen, Hoffmann & Seifert 2000; Gil 2002; Plumb & Aspden 2004; Calder 2005; Pilbeam & Raisz 2005; Cashman 2008). Soybean oil, sunflower oil, rapeseed oil and cottonseed oil contain high levels of linoleic acid (18:2n-6, LA), while crude palm oil, olive oil and coconut oil are rich in monounsaturated fatty acids (MUFA) (Turchini, Torstensen & Ng 2009). LA serves as a precursor for arachidonic acid (AA, 20:4n-6), a substrate for eicosanoid synthesis. Eicosanoids are signalling molecules made from either n-3 or n-6 essential fatty acids. They are involved in many processes, particularly in inflammation and immunity. Among the eicosanoids are the biologically active prostaglandins that are potent regulators of inflammation (Henderson & Tocher 1987; Rowley, Knight, Lloyd-Evans, Holland & Vickers 1995; Calder 2005). Studies on Atlantic salmon suggest a close correlation between dietary fatty acids and tissue fatty acids (Bell & Raynard 1990; Bell, Ashton, Secombes, Weitzel, Dick & Sargent 1996b; Sargent, Tocher & Bell 2002; Ruyter, Moya-Falcon, Rosenlund & Vegusdal 2006; Berge *et al.* 2009; Turchini *et al.* 2009; Petropoulos, Thompson, Morgan, Dick, Tocher & Bell 2009) including in immune competent organs like spleen and head kidney (Waagbø *et al.* 1993; Waagbø, Hemre, Holm & Lie 1995). The dietary fatty acid composition influences the eicosanoid production (Bell, Farnadale, Dick & Sargent 1996a; Gjøen, Obach, Røsjø, Grisdale-Helland, Rosenlund, Hvattum & Ruyter 2004; Gjøen, Kleveland, Moya-Falcon, Frøystad, Vegusdal, Hvattum, Berge & Ruyter 2007). By feeding vegetable oils, tissue n-6 PUFA levels increase together with the amount of eicosanoids produced from AA (Gjøen *et al.* 2004). According to Sargent, Bell, McEvoy, Tocher & Estevez (1999), the arachidonic acid: eicosapentanoic acid (AA: EPA) ratio determines the type of eicosanoids, because EPA competitively inhibits the

formation of eicosanoids from AA. A high AA: EPA ratio enhances synthesis of PGE_2, a highly active pro-inflammatory product. In contrast, a low AA: EPA ratio leads to the production of series 3 eicosanoids that are considered less potent compounds. The effect of vegetable oils on the development of inflammation associated with fish skeletal tissues is presently not known, but intraperitoneal injection with oil-adjuvanted vaccines has been linked to the development of spinal deformities in fish (Berg *et al.* 2006; Aunsmo *et al.* 2008). Vaccines are indeed known to cause inflammation-like tissue reactions adjacent to the injection site (Midtlyng 1996).

The present study was designed to investigate whether LPS-induced inflammation located close to the spine can cause spinal malformations and whether such inflammation-related deformities develop more or less frequently in animals that have been fed different dietary AA/EPA ratios derived from Vo and Fo, respectively.

Materials and methods

Experimental design and rearing conditions

Atlantic salmon, *Salmo salar* L., juveniles (n = 720, initial body weight 25 g), Aquagen strain, spawned in November 2007, were distributed randomly to six fibreglass tanks (1 × 1 × 0.43 m diameter) at the Institute of Marine Research, Matre, Norway. Fish were anaesthetized with 40 mg benzocaine L^{-1} (Benzoak® Vet; A.C.D. SA) and intra-abdominally tagged (ID-100A Microtransponder; Trovan Ltd.) 14 days before starting the experiment. On 27 October, triplicate groups of fish were fed two experimental diets for 99 days. At day 47 (40 g), each dietary group was subjected to four experimental regimes with 25 fish in each: (i) single injection of bacterial lipopolysaccharide (LPS), (ii) single injection of phosphate-buffered saline (PBS, placebo), (iii) commercial vaccination, or (iv) no treatment. LPS and PBS injections were performed intramuscularly at a standardized point resulting from the intersection between the lateral line and a vertical line demarcated by the anterior edge of the anal fin. This point corresponds to vertebrae 39–41 and is located in spinal region 3 (as described by Kacem, Meunier & Bagliniere 1998). Bacterial lipopolysaccharide (LPS, *Escherichia coli 0111:B4*, phenol extracted; SIGMA) was injected at a dose of approximately 30 mg kg^{-1} fish (1.14–1.26 mg LPS per fish) in suspension with 0.2 mL PBS. The placebo group was injected with 0.2 mL PBS. One syringe with a detachable sterile needle was used per individual for LPS and PBS treatments. A third group was injected intraperitoneally with 0.2 mL commercial oil-adjuvanted vaccine (Compact 6 Vet, Norvax®; Intervet) using a vaccination pistol (Dosys™ 173 *classic*; Socorex Isba S.A.) and following a standard in-house vaccination procedure for experimental fish (IMR; Matre).

The water temperature was 8.3 ± 1.1 °C (days 1–46) and 7.1 ± 1.0 °C (days 47–99). Fish were transferred to sea water on day 99 (2 February, 2008). Oxygen was kept above 9 mg L^{-1}. The light regime was in accordance with that used for under-yearling smolt production: 12 h light and 12 h darkness regime for 6 weeks continued by 24 h light for 3 weeks until smoltification. Fish were fed to satiation using automatic feeders. The tanks were checked daily for mortalities.

Diets

Two diets containing 490 g kg^{-1} protein (a mix of marine and vegetable protein) and 190 g kg^{-1} lipids were formulated based on the same basal mix of raw materials, differing only in the type of supplemental oil: fish oil (Fo) (Nordsildmel) and refined soybean oil (Vo) (COOP Norden) (Table 1). The feed was produced in two pellet sizes (2 and 3 mm) at Ewos Innovation facilities. Fish were fed the experimental diets until the artificially induced smoltification (day 99). Subsequently, all fish were transferred to one 5 × 5 m sea cage, reared under natural light regime, and fed a common commercial diet for 165 days. In total, the trial lasted for 264 days.

Table 1 Feed formulation in two pellet sizes (2 and 3 mm) of the 100% Fo (100% fish oil) and 100% Vo (100% soybean oil) diets fed to Atlantic salmon under-yearlings for a 99-day period in fresh water (g kg^{-1} feed)

	100% Fo	100% Vo
Fish meal	280	280
Soy protein concentrate	300	300
Wheat gluten	80	80
Wheat grain	134.7	134.7
Rovimix Stay-C 35%	0.3	0.3
Vitamin premix	1	1
Mineral premix	1	1
Northern hemisphere fish oil	203	0
Soybean oil	0	203

TBARS values in the diet were 17.43 and 12.64 nmol g^{-1} feed and vitamin E levels (α-Tocopherol) were 253 and 225 mg kg^{-1} feed in the Fo and Vo diets, respectively.

Fish sampling

Ninety individuals per experimental group (30 fish per tank) were individually weighed at the start of the trial (day 0) and one day before injection (day 46). All remaining fish were measured at the end of both the freshwater and the seawater periods (days 99 and 264, respectively). Fish sampled on days 0 and 46 were returned to the experiment; tissue samples and X rays (n = 98) were taken on day 99 (fish were removed from the experiment), while the rest of the population was followed up in sea water until the final sampling (day 264).

The specific growth rate (SGR %) was calculated according to the formula:

$$\mathrm{SGR}\% = \left(\left(\frac{\mathrm{FinalW}}{\mathrm{InitialW}}\right)^{1/\mathrm{days}} - 1\right) \times 100$$

where InitialW and FinalW are the initial and the final fish weights for a given period (triplicated tanks), and days is the number of feeding days for the period.

The condition factor (CF) was calculated as:

$$\mathrm{CF} = \left[\frac{W(g)}{\mathrm{FL}(\mathrm{cm}^3)}\right] \times 100$$

where W and FL correspond to body weight and fork length of the individual fish.

Fish were anaesthetized as described earlier and subsequently killed by a blow to the head. Blood samples were taken from the caudal vein using heparinized syringes and needles and were stored on ice until isolation of plasma by centrifugation. Plasma samples were frozen on dry ice and stored at −80 °C until analysis. Six vertebrae anterior to the injection point were removed and frozen on dry ice for gene expression and bone fatty acid analyses. Six vertebrae posterior to the injection point were fixed for histological examination in 10% buffered formalin (formaldehyde solution at 37%), followed by 80% ethanol after 24 h.

All fish were examined for external signs of deformities at the end of the freshwater and the seawater periods. Ninety-eight individuals and 240 fish were individually measured, weighed and radiographed at the end of the freshwater and the seawater periods.

Analytical procedures

On day 99, blood samples from six fish per experimental group were taken for the assessment of systemic inflammation markers. Plasma lysozyme was analysed with a turbidometric microtitre assay according to Ellis (1990). Plasma protein was analysed in a multipurpose diagnostic autoanalyzer (Maxmat PL Analyzer), based on a colorimetric biuret protein assay according to standardized reagents and procedures from the manufacturer.

PGE_2 was analysed by mass spectrometry. The prostaglandin extraction was performed as previously described by Araujo & Frøyland (2006).

Bone fatty acid composition was analysed in three bone samples per experimental diet, each one consisting of pooled samples from four fish taken from the Fo and Vo untreated control groups. The samples comprised five vertebrae (vertebrae 38–42), which were homogenized with N_2 in a mortar. Lipids were extracted from diets and bone samples with chloroform: methanol (2:1, v/v) and analysed by gas chromatography (GC) as described by Torstensen, Bell, Rosenlund, Henderson, Graff, Tocher, Lie & Sargent (2005).

For mRNA expression analysis, the first six vertebrae prior to the injection point (vertebrae 33–39) were sampled 52 days post-injection (day 99). Samples were dissected, cleaned from surrounding tissue, frozen in liquid nitrogen and kept at −80 °C until analyses. Individual samples were crushed and homogenized. Total RNA was extracted and isolated from the samples using FastPrep and TRI reagent® (Sigma-Aldrich Norway AS) according to the manufacturer's instructions. Genomic DNA was eliminated from the samples by RQ DNase I (Promega GmbH) treatment and stored at −80 °C until analysis. Quantity and quality of the isolated RNA was assessed by a NanoDrop® spectrophotometer (NanoDrop Technologies). Only samples with a 260/280 nm absorbance ratio of 1.8:2.0 were approved. The RNA integrity was evaluated by an Agilent 2100 Bioanalyzer (Agilent Technologies) using a RNA 6000 Nano LabChip® Kit (Agilent Technologies). Only samples showing no sign of RNA degradation were used. Stranded cDNA was reverse transcribed from 250 ng RNA using a Reverse Transcription Core Kit (RT-RTCK-05; Eurogentec).

Primers for amplification and detection of mRNA for *IgM* (forward primer 5′-TGGGA AAATGACAATG GAAAGA-3′, nt961, reverse primer 5′-GTGATGTCAAGTATGGC AATTT-TGT-3′, nt1034) (Hordvik, Berven, Solem, Hatten & Endresen 1997), *Cox2* (forward primer 5′-AT-CACCTTTGTGCGAAACGC-3′ (nt42), reverse

primer 5′-CCTC CCAGCTCTTGTAGCCATA-3′, nt151) (Ingerslev, Cunningham & Wergeland 2006) and *MMP-13* (forward primer 5′-GTTT-CTTTGGCCTCCAGGTG-3′, nt209), reverse primer 5′-TCGTC ACAGTCT CAGCGTCC-3′, nt260) (Skugor, Glover, Nilsen & Krasnov 2008) in addition to the control gene elongation factor alpha (*ef1α*) (Olsvik, Lie, Jordal, Nilsen & Hordvik 2005) were designed using the Primer Express 2.0 software (Applied Biosystems).

Real-time PCR was carried out on an ABI 7900 Fast Real-Time PCR System (Applied Biosystems) using SYBR® Green PCR Master Mix (Applied Biosystems), with the following thermal cycling conditions: 50 °C for 2 min followed by 98 °C for 10 min, and then 40 cycles of 95 °C for 15 s followed by 60 °C for 1 min. The samples were run in triplicate in a 96-well PCR plate. No-template controls (NTC) for each gene were run on each PCR plate. To determine the efficiency of targets in relation to reference (*ef1α*), a standard curve and a validation experiment described in ABI were used (User Bulletin #2; ABI 7700 sequence detection system). In the validation experiment, 250, 125, 62.5 and 31.25 ng of RNA were used for cDNA synthesis, and the slope of log input amount of RNA versus delta Ct was for *IgM*/*ef1α* = −0.04, for *Cox2*/*ef1α* = −0.1 and for *MMP-13*/*ef1α* = −0.03 which is < 0.1. This demonstrates that the efficiency between target and reference genes was approximately equal. The relative expression level was calculated using the Comparative Ct method (ABI User Bulletin #2; ABI 7700 sequence detection system).

Radiographs were taken with a portable X-ray apparatus (HI-Ray 100; Eickenmeyer Medizintechnik für Tierärzte e.K.) and 30×40 cm film (FUJIFILM IX 50; FUJIFILM Corp.). The film was exposed twice for 50 mA s and 72 kV and developed using a manual developer (Cofar Cemat C56D) with Kodak Professional manual fixer and developer (KODAK S.A.). The pictures were digitalized by scanning (Epson Expression 10000 XL; Seiko Epson Corp.). The vertebral length and dorso-ventral diameter were measured by means of image analysing software (Image-Pro Plus, version 4.0; Media Cybernetics). The vertebral column was divided into four main regions based on Kacem *et al.* (1998): region 1 (cranial trunk), comprising vertebrae 1–8 (V1–8); region 2 (caudal trunk) V9–30; region 3 (tail), V31–49 and region 4 (tail fin) V50–58. Observed vertebral deformations were classified according to Witten, Gil Martens, Huysseune, Takle & Hjelde (2009).

For histological analyses, the vertebrae were fixed in 10% buffered formalin for 24 h and preserved in 70% ethanol. The samples were decalcified in a 10% EDTA solution buffered with 0.1 M Tris–HCl, pH 7.0 for a period of 8 weeks. After decalcification, the samples were stepwise dehydrated and embedded in paraffin blocks. Five-micrometer (μm) serial sections (80 sections per block) were taken in the sagittal plane of the vertebrae, from the periphery to the middle plane of the spine. Sections were stained with haematoxylin/eosin and Masson's trichrome as described by Witten & Hall (2002). Histological sections were examined to evaluate the presence of inflammatory cells and bone microstructure.

Statistical analysis

Growth parameters, inflammatory markers and fatty acid composition of bone were statistically evaluated using a one-way (period days 1–45) or two-way ANOVA (period days 46–264) followed by Tukey's HSD test. To evaluate differences in the frequency of deformities, arcsine-transformed values were used for further statistical analysis (Sokal & Rohlf 1995). The data were analysed with the program Statistica (version 8.0; StatSoft Inc.). The significance level was set to $P < 0.05$, and the data are presented as mean ± SD.

Results

Feed composition

The fatty acid profiles of the experimental feeds are presented in Table 2. The Fo diet contained higher levels of saturates, monoenes and *n*-3 fatty acids than the Vo diet. Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) nutritional requirements (Ruyter 1998) were also met in the Vo diet (1.3% DHA and 0.9% EPA). The n3/n6 ratios in the Fo and Vo diets were 4.1 and 0.2, respectively, with respective AA/EPA ratios of 0.04 and 0.1.

Growth

Fish fed Vo showed a significantly higher body weight and condition factor than the group fed Fo before the injection was performed (day 46)

(Table 3). No differences in body length were observed in the same period. Fish fed Vo and subjected to LPS injection presented a significantly lower weight and SGR compared to other experimental groups 52 days after LPS injection. No differences in growth rate during the period in sea water or weight at the final sampling were observed. Mortality rate was below 2% and was not related to any experimental group.

External examination and radiology

At the end of the grow-out period (day 264), all fish were externally normal, and the overall condition factor was high (1.4 ± 0.14). Few visible signs of spinal deformities were observed in either of the experimental groups.

Using radiology, the overall prevalence of fish with one or more deformed vertebrae was 8.9% and 13.9% at the end of freshwater and seawater periods, respectively. Three different types of vertebral malformations were observed: multiple fusions located in the trunk (types 6 and 8; Witten

Table 2 Fatty acid compositions in the experimental diets and in bone from Atlantic salmon smolts fed either fish oil (Fo) or vegetable oil (Vo) for a period of 99 days

Fatty acids (mg FA g^{-1})	Diet		Bone	
	Fo	Vo	Fo	Vo
14:0	7.5	0.6	6.9 ± 0.7^{a}	3.3 ± 0.5^{b}
16:0	13.1	11.0	18.5 ± 1.8	16.4 ± 2.0
18:0	1.5	3.6	3.8 ± 0.3	5.2 ± 0.5
Saturated FA	22.9	16.3	30.4 ± 3.0	26.0 ± 3.4
16:1n-7	4.5	0.6	6.8 ± 0.8^{a}	3.8 ± 0.5^{b}
18:1n-7	1.5	1.5	3.2 ± 0.3	2.8 ± 0.3
18:1n-9	7.9	21.8	18.9 ± 2.0	23.9 ± 2.7
18:1n-11	0.4	0.0	1.3 ± 0.1^{a}	0.4 ± 0.1^{b}
20:1 (sum isomers)	12.9	3.0	10.0 ± 1.0^{a}	3.8 ± 0.7^{b}
22:1 (sum isomers)	19.9	28.9	10.5 ± 1.1^{a}	3.4 ± 0.8^{b}
Monoenes FA	47.4	26.9	3.0 ± 5.6^{b}	39.6 ± 4.9^{a}
18:2n-6	4.5	47.7	7.1 ± 0.6^{b}	24.1 ± 3.8^{a}
20:4n-6	0.3	0.1	0.8 ± 0.1^{b}	1.0 ± 0.1^{a}
n-6 FA	5.0	47.7	8.8 ± 0.7^{b}	28.1 ± 4.2^{a}
18:3n-3	1.5	5.4	1.6 ± 0.2^{b}	2.4 ± 0.3^{a}
20:4n-3	0.6	0.0	1.1 ± 0.1^{a}	0.7 ± 0.1^{b}
20:5n-3	6.6	0.9	6.0 ± 0.6^{a}	3.7 ± 0.4^{b}
22:5n-3	0.6	0.0	2.4 ± 0.3^{a}	1.6 ± 0.2^{b}
22:6n-3	6.8	1.3	13.8 ± 1.6^{a}	9.1 ± 0.9^{b}
n-3 FA	20.3	7.9	28.6 ± 3.4^{a}	19.8 ± 2.2^{b}
n-3/n-6	4.1	0.2	3.2 ± 0.2^{a}	0.7 ± 0.0^{b}
AA:EPA	0.1	0.2	0.1	0.6

Significant effects between dietary groups (control fish) were determined by one-way ANOVA.

n.s., no significant effects, no letters indicate significant effects ($P < 0.05$).

Table 3 Body weight (mean ± SD), specific growth rate (SGR %), condition factor (CF) at start, days 46 and 99 of feeding and at the end of seawater period (day 264) in Atlantic salmon fed fish oil (Fo) and vegetable oil (Vo) – containing diets for 99 days and subjected to different treatments on day 47 of feeding: (a) intramuscular injections of bacterial lipopolysaccharide (LPS), (b) sham-injected phosphate-buffered saline (Placebo), (c) intraperitoneally injection of oil adjuvant vaccine (Vaccinated), or (d) no treatment (Control)

	Fo			Vo			ANOVA		
	Control	Placebo	LPS	Control	Placebo	LPS	Vaccine	Diet	Injection	Interaction
Body weight, g										
Day 0	25.6 ± 1.8			26.0 ± 0.8				n.s.		
Day 46	39.6 ± 0.8^{b}			42.8 ± 1.9^{a}				$P < 0.05$		
Day 99	57.1 ± 6.2^{a}	58.4 ± 3.1^{a}	53.0 ± 2.7^{ab}	52.3 ± 2.2^{ab}	60.2 ± 4.6^{a}	57.1 ± 0.6^{a}	44.5 ± 5.4^{b}	56.7 ± 3.5^{a}	n.s.	$P < 0.05$	n.s.
Day 264	1038 ± 109	1102 ± 5.5	1028 ± 11	931 ± 89	1134 ± 109	1060 ± 126	1000 ± 162	1012 ± 78	n.s.	n.s.	n.s.
SGR %											
Day 0–46	0.96 ± 0.2			1.08 ± 0.1				n.s.			
Day 47–99	0.59 ± 0.2	0.51 ± 0.2	0.57 ± 0.2	0.48 ± 0.3	0.60 ± 0.1	0.57 ± 0.1	0.55 ± 0.2	0.55 ± 0.3	n.s.		
Day 100–264	1. 02 ± 0.2	1.07 ± 0.2	1.10 ± 0.1	1.02 ± 0.2	1.09 ± 0.1	1.10 ± 0.2	1.06 ± 0.3	1.08 ± 0.1	n.s.	n.s.	n.s.
Condition factor											
Day 0	1.39 ± 0.01			1.38 ± 0.01				n.s.			
Day 46	1.29 ± 0.00^{b}			1.35 ± 0.01^{a}				$P < 0.001$			
Day 99	1.29 ± 0.01^{a}	1.28 ± 0.01^{a}	1.28 ± 0.02^{a}	1.25 ± 0.10^{b}	1.29 ± 0.1^{a}	1.31 ± 0.1^{a}	1.31 ± 0.1^{a}	1.30 ± 0.1^{a}	$P < 0.05$	$P < 0.05$	n.s.
Day 264	1.38 ± 0.1	1.40 ± 0.01	1.35 ± 0.10	1.33 ± 0.10	1.40 ± 0.01	1.37 ± 0.10	1.31 ± 0.1	1.30 ± 0.1	n.s.	n.s.	n.s.

Values presented as mean ± SD ($n = 3$ tanks). Significant effects determined by two-way ANOVA are indicated by $P < 0.01$, $P < 0.05$ and n.s., not significant.
Tukey's multiple comparison test was used where appropriate. 30 fish per tank ($n = 6$) were measured at the start of the experiment (day 0) and one day before the injection (day 46). Weight and length from all remaining fish were recorded on days 99 and 264 (no < 15 fish per experimental group per tank).

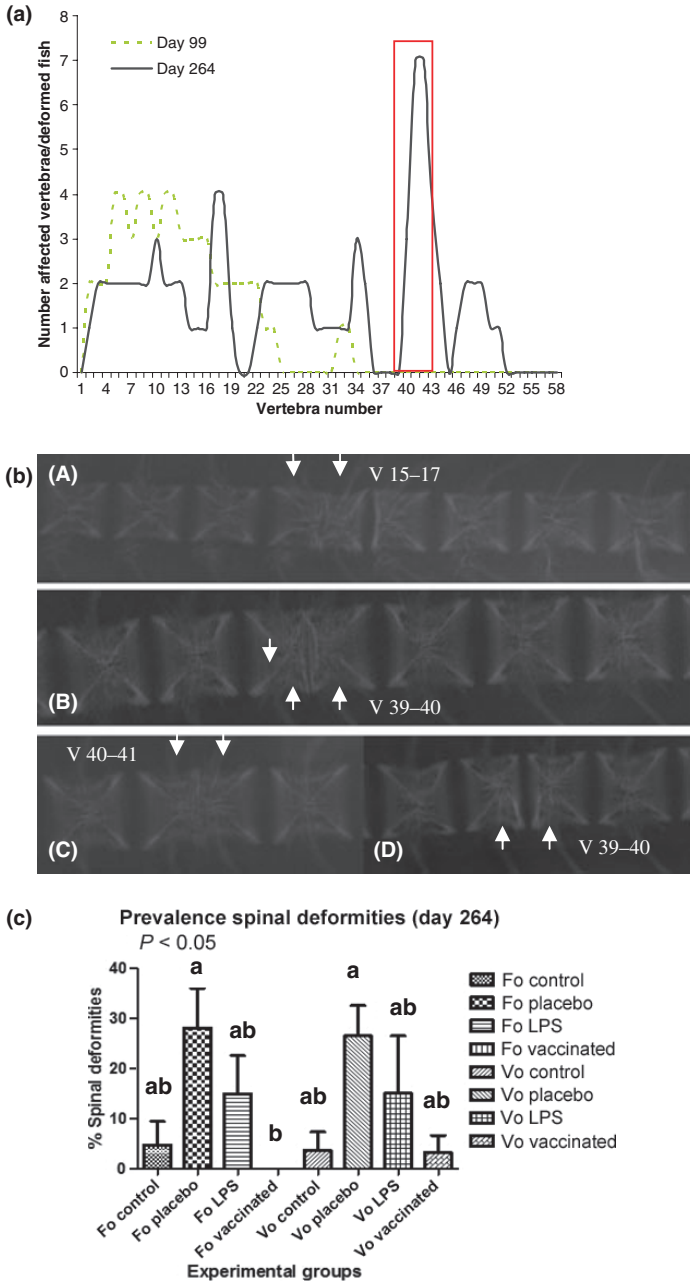

Figure 1 Spinal deformations observed in Atlantic salmon. (a) Number and location of deformities along the entire spinal column observed for fish with deformities at smolt stage (day 99) and end of seawater period (day 264). (b) Type of vertebral malformations observed at smolt stage (A, B) and end of seawater period (C, D). (c) Relative percentage of spinal deformities at the end of seawater period (day 264) in Atlantic salmon fed fish oil (Fo) and vegetable oil (Vo) – containing diets for 99 days and subjected to different treatments on day 47 of feeding: (a) intramuscular injections of bacterial lipopolysaccharide (LPS), (b) sham-injected phosphate-buffered saline (Placebo), (c) intraperitoneal injection of oil adjuvant vaccine (Vaccinated) or (d) no treatment (Control).

et al. 2009) and one-sided vertebral compression in the tail (Type 5; Witten *et al.* 2009) (Fig. 1a). Type 6 involved 6–8 vertebrae and was the predominant type at the smolt stage (Fig. 1a). Type 5 involved between two and three vertebral bodies and emerged during the seawater period (Fig. 1b).

In fish fed either Fo or Vo and injected with LPS or PBS, the prevalence of one or more deformed

vertebrae ranged between 21% and 38% (Fig. 1c). No significant differences were observed in relation to diet. However, fish injected with either PBS or LPS displayed a high prevalence of deformities in region 3 (tail region, vertebrae 31–49). Most of the one-sided vertebral malformations were co-located with the injection point (vertebrae 39–41).

Vertebral morphology

At the end of the experiment (day 264), the ratio between vertebral length /dorso-ventral diameter was significantly lower in region 3 in the fish fed Vo (Table 4). A local effect from the injection was observed in vertebrae 39–41 in fish that were PBS and LPS injected. These fish had a significantly lower (1-way ANOVA, $P = 0.0139$) vertebral length/dorso-ventral diameter compared to fish that were not intramuscularly injected (Vaccinated and Control in Fig. 2).

No evidence of vertebral malformations was observed at day 99 of feeding. All groups displayed numerous large granulocytes (Fig. 3a–d) adjacent to the spine. Granulocytes in the vicinity of the vertebral body growth zone were observed on sagittal sections. Granulocytes were located in the connective tissue that surrounded the spinal cord. Cells ventral to the spinal cord were in proximity to the dorsal vertebral body growth zone. Abundance and location of granulocytes were similar in animals from all experimental groups. No other signs of chronic inflammation were observed at day 99 of feeding since no infiltration of melano-macrophages, fibrosis or granulomatous foci was seen in bone tissue.

Bone fatty acid composition

Bone total lipid fatty acid composition mirrored that from the diet (Table 2). Fish fed soybean oil had higher levels of both linoleic acid and AA, and the AA/EPA ratio in bone was 0.1 and 0.3 in the Fo and Vo groups, respectively.

Systemic inflammation markers in plasma

Plasma lysozyme values (Fig. 4a) ranged from 37.3 to 295 U mL^{-1}. No dietary differences were observed between groups; however, treated fish (placebo, LPS and vaccinated) tended to have higher plasma levels compared to untreated control groups, but these were not statistically different (*P*-value, 0.25).

Table 4 Vertebral length/dorso-ventral diameter ratio (mean ± SD) at day 264 in Atlantic salmon fed fish oil (Fo) and vegetable oil (Vo) – containing diets for 99 days and subjected to different treatments on day 47 of feeding: (a) intramuscular injections of bacterial lipopolysaccharide (LPS), (b) sham-injected phosphate-buffered saline (Placebo), (c) intraperitoneally injection of oil adjuvant vaccine (Vaccinated), or (d) no treatment (Control)

	Fo				Vo				ANOVA[b]		
Vertebral region[a]	Control	Placebo	LPS	Vacc	Control	Placebo	LPS	Vacc	Diet	Injection	Interaction
1	0.73 ± 0.04	0.73 ± 0.05	0.74 ± 0.02	0.73 ± 0.04	0.74 ± 0.05	0.73 ± 0.03	0.73 ± 0.03	0.73 ± 0.04	n.s.	n.s.	n.s.
2	0.83 ± 0.02	0.84 ± 0.02	0.84 ± 0.03	0.84 ± 0.02	0.84 ± 0.04	0.83 ± 0.02	0.84 ± 0.02	0.83 ± 0.03	n.s.	n.s.	n.s.
3	0.92 ± 0.02	0.92 ± 0.02	0.92 ± 0.03	0.92 ± 0.02	0.91 ± 0.05	0.92 ± 0.02	0.90 ± 0.02	0.92 ± 0.03	$P < 0.05$	n.s.	n.s.
4	0.81 ± 0.02	0.81 ± 0.05	0.81 ± 0.02	0.80 ± 0.03	0.80 ± 0.06	0.81 ± 0.03	0.79 ± 0.03	0.81 ± 0.04	n.s.	n.s.	n.s.
1–4	0.84 ± 0.02	0.85 ± 0.02	0.85 ± 0.02	0.85 ± 0.02	0.84 ± 0.03	0.84 ± 0.01	0.84 ± 0.01	0.84 ± 0.02	n.s.	n.s.	n.s.
Injection site[c]	0.93 ± 0.03	0.91 ± 0.06	0.91 ± 0.03	0.93 ± 0.03	0.92 ± 0.03	0.92 ± 0.05	0.90 ± 0.03	0.92 ± 0.04	n.s.	n.s.	n.s.

[a]1 = Vertebral (V) number 1–8, 2 = V number 9–30, V number 31–49, 4 = V number 50–58 (Kacem *et al.* 1998). Injection site = the injection site of LPS and PBS (vertebrae number 39–41).
[b]In vertebral regions 1, 2, 3 and 4, the statistical analysis (2-way ANOVA) is based on mean values per region per individual, while '1–4' and 'injection site' are based on mean values per individual.
[c]The vertebral length/dorso-ventral diameter ratio was taken from a total of 240 individuals ($n = 15–25$ fish per experimental group per tank).

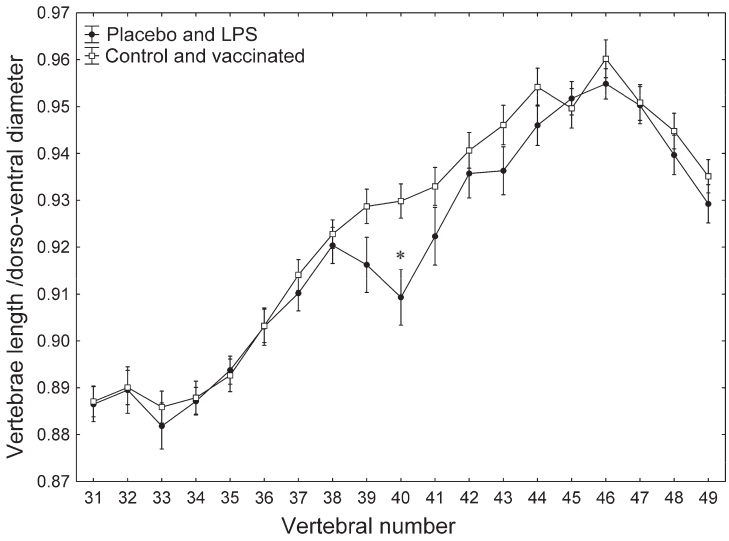

Figure 2 Vertebral morphometrics measured as ratio between vertebral length and dorso-ventral diameter at the end of seawater period (day 264) in Atlantic salmon fed fish oil (Fo) and vegetable oil (Vo) – containing diets for 99 days and subjected to different treatments on day 47 of feeding: (a) intramuscular injections of bacterial lipopolysaccharide (LPS), (b) sham-injected phosphate-buffered saline (Placebo), (c) intraperitoneal injection of oil adjuvant vaccine (Vaccinated) or (d) no treatment (Control). Data presented as mean ± SEM.

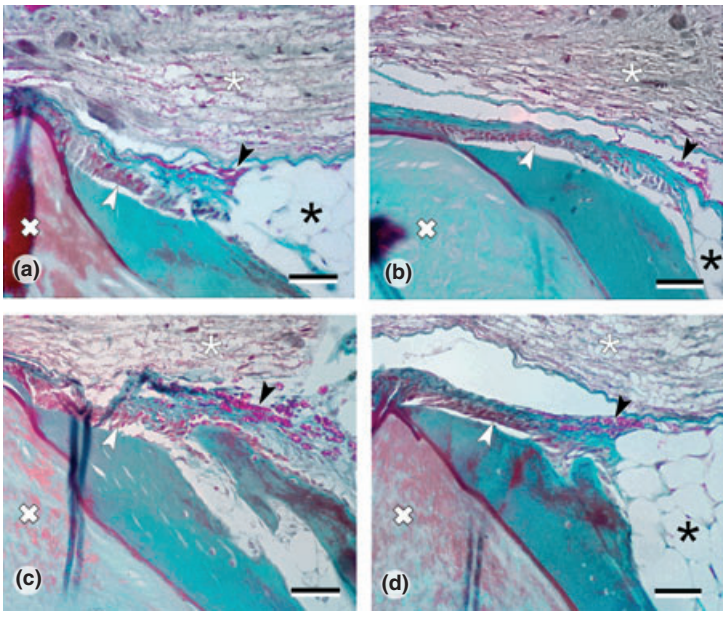

Figure 3 (a–d) Histological markers of inflammation in bone at day 99 of feeding in Atlantic salmon subjected to different treatments on day 47 of feeding: (a) intramuscular injections of bacterial lipopolysaccharide (LPS), (b) sham-injected phosphate-buffered saline (Placebo), (c) intraperitoneal injection of oil adjuvant vaccine (Vaccinated) or (d) no treatment (Control). Black arrowhead, granulocytes. White arrowhead, osteoblasts located in the vertebral body growth zone. White cross, notochord sheath separating two adjacent vertebral bodies. Black asterisk, adipose tissue. White asterisk, spinal cord (Masson's trichrome, bars = 50 μm).

Fish fed vegetable oil and subjected to LPS injection had significantly higher PGE_2 levels in plasma ($P < 0.05$) compared to the other experimental groups (Fig. 4b).

Local inflammation markers in bone

An up-regulation of IgM mRNA was observed in the group that were fed the Fo diet and injected

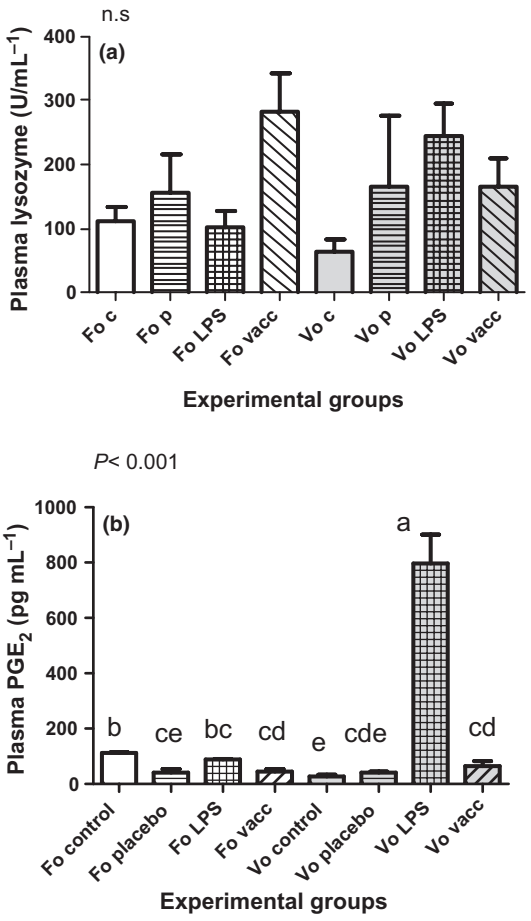

Figure 4 Markers of systemic inflammation measured as (a) plasma lysozyme (U mL^{-1}) and (b) plasma prostaglandin E2 (pg mL^{-1}) at day 99 of feeding in Atlantic salmon subjected to different treatments on day 47 of feeding: (a) intramuscular injections of bacterial lipopolysaccharide (LPS), (b) sham-injected phosphate-buffered saline (Placebo), (c) intraperitoneal injection of oil adjuvant vaccine (Vaccinated), or (d) no treatment (Control). Data presented as mean ± SEM.

with LPS (Fig. 5a). No significant differences in COX-2 mRNA expression were observed between the experimental treatment groups; however, fish fed fish oil presented consistently higher levels of COX-2 mRNA expression than the Vo fed fish (Fig. 5b). No significant differences in MMP13 mRNA abundance were observed (Fig. 5c). The

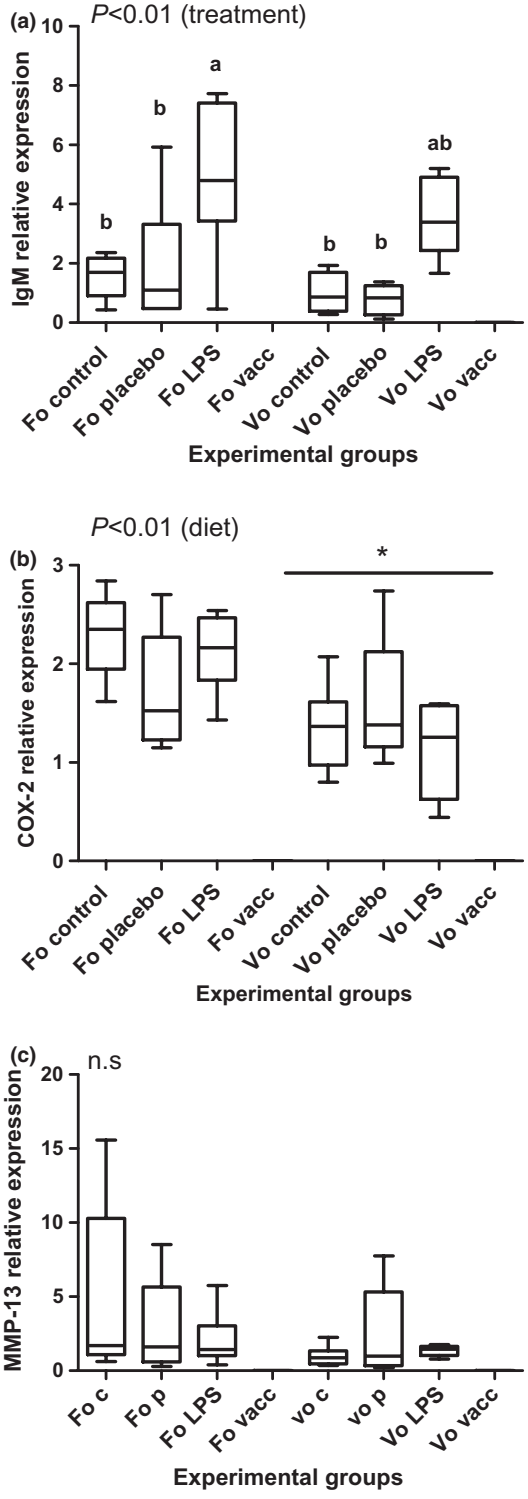

Figure 5 Markers of inflammation in bone measured as gene expression of (a) immunoglobulin M (IgM) (b) cycloxigenase-2 (COX-2) (c) metalloproteinase-13 (MMP13) relative to elongation factor 1A (*ef1α*) at day 99 of feeding in Atlantic salmon subjected to different treatments on day 47 of feeding: (a) intramuscular injections of bacterial lipopolysaccharide (LPS), (b) sham-injected phosphate-buffered saline (Placebo), (c) intraperitoneal injection of oil adjuvant vaccine (Vaccinated) or (d) no treatment (Control). Data presented as median (horizontal line), 95% confidence interval (box) and min–max values (whiskers).

vaccinated groups were not analysed for these bone inflammation markers.

Discussion

Spinal deformities are a recurring problem in Atlantic salmon aquaculture, and many fish farming-related variables have been suggested as risk factors. The presence of inflammatory cells in connection with abnormal bone and cartilage development has been reported in intensively farmed salmonids (Ostland, McGrogan & Ferguson 1997; Kvellestad *et al.* 2000) and in wild cyprinid fish (Kent, Watral, Whipps, Cunningham, Criscione, Heidel, Curtis, Spitsbergen & Markle 2004). Therefore, we proposed that inflammation could be a physio-pathological mechanism that disrupts the normal pattern of bone growth.

The overall prevalence of spinal deformities at the end of the trial was 8.4% at the smolt stage and 13.9% at the end of the experiment. This prevalence was similar to that observed by Fjelldal *et al.* (2007), who investigated the development of spinal deformities in individually tagged Atlantic salmon and reported a 7% and 12.4% prevalence of spinal deformities at the parr stage and 10 months after smoltification, respectively, suggesting that our results could be a normal background level of deformities. The character of vertebral deformations observed at the smolt stage (day 99) differed consistently with those observed at day 264 in sea water, although it should be pointed out that these samplings were not performed on the same individuals (Fig. 1a). At the smolt stage, deformities consisted of multiple vertebral fusions, compressions and vertebral dislocations (Fig. 1b) involving 6–8 vertebrae in the trunk region (regions 1 and 2, types 6 and 8; as described by Witten *et al.* (2009)). The type of deformities observed on day 264 represented a different deformity phenotype. This phenotype consisted of vertebral compressions with different degrees of ankylosis involving only two or three vertebral bodies, equivalent to type 5 as described by Witten *et al.* (2009). These deformities were observed at or in proximity to the injection point. As stated by Fjelldal, Nordgarden, Berg, Grotmol, Totland, Wargelius & Hansen (2005) and Fjelldal *et al.* (2006, 2007, 2009), the vertebral column of Atlantic salmon displays different patterns of regional growth suggesting that the observed deformities at the smolt stage and in sea water could result from different pathogenic mechanisms.

In our experiment, spinal deformities in groups injected with LPS and PBS ranged between 21% and 38% independent of the dietary oil source (Fig. 1c). We did, however, observe a dietary effect on vertebral shape in terms of a lower ratio between the vertebral length and dorso-ventral diameter in fish fed vegetable oil compared to the fish fed fish oil. A low ratio between the length and diameter of the vertebrae indicates a compressed phenotype and can be used as an early indicator of ensuing deformity (Fjelldal *et al.* 2009). However, the total prevalence of deformities between Fo and Vo fed fish was similar within the timeframe of the present experiment. The prevalence and type of vertebral malformations observed in the vertebrae close to the area of injection in the LPS and placebo groups (Figs. 1a, c and 2) could have resulted from mechanical damage caused by the needle or from hydrostatic tension exerted by the injected solutions. In the present study, inter-vertebral tissue could have been physically injured during the injection and may have caused inflammatory-induced deformities, especially since the prevalence of spinal deformities in i.p. vaccinated groups was low. Possibly, the subsequent inflammatory processes in the visceral cavity associated with vaccination (Midtlyng 1996; Berg *et al.* 2006; Mutoloki, Alexandersen, Gravningen & Evensen 2008), normally referred to as vaccine side-effects, did not represent a risk for spinal deformities under the present experimental conditions.

The feed supplemented with soybean oil contained a high level of linoleic acid (18:2*n*-6), a precursor for AA (20:4 n-6), and different AA/EPA ratios were achieved between the Vo and Fo diets. These dietary values correlated with the fatty acid profile in bone and are consistent with the findings reported by Watkins *et al.* (2000) and Berge *et al.* (2009) in which linoleic acid was markedly increased in bone total lipids in the Vo group when compared to individuals fed Fo. Vegetable oil had no negative effect on growth, a result in accordance with Berge *et al.* (2009), Turchini *et al.* (2009) and Izquierdo *et al.* (2008). In fact, fish fed Vo presented a higher body weight than the fish fed Fo at day 46. Similar findings have been reported by Torstensen *et al.* (2005). The condition factor was higher in the Vo group before immunostimulation at day 47 which may indicate body lipid accumulation. Turchini *et al.* (2003) observed increased fat deposition in carcass and fillets of brown trout, *Salmo trutta* L., fed Vo compared to Fo fed control

fish. The low body weight observed in fish injected with LPS and fed Vo might have been the result of a rapid fat mobilization as a consequence of LPS injection-related stress. Stress increases the metabolic rate of fish (Sloman, Metcalfe, Taylor & Gilmour 2000). Linoleic acid has the capacity to inhibit the catabolism of cortisol by lymphocytes and may thus enhance the strength of the cortisol response (Welker & Congleton 2003).

A systemic pro-inflammatory condition in LPS-injected and Vo-fed fish was indicated by high plasma PGE_2 levels 52 days after the injection. PGE_2 is an eicosanoid produced by monocytes and thrombocytes from AA, by the action of the inducible COX-2 enzyme. PGE_2 is involved in a number of immune responses such as vasodilation, activation and migration of leucocytes to the site of inflammation (Fast, Ross & Johnson 2005). PGE_2 has also been linked to stress related to diets rich in linoleic acid (Bell, McVicar, Park & Sargent 1991; Sloman *et al.* 2000). Petropoulos *et al.* (2009) reported no dietary effects and high individual variation in plasma AA-derived PGE_2 levels after the substitution of dietary fish oil with a blend of vegetable oils in three strains of Atlantic salmon. Low concentrations of circulating PGE_2 ($< 10^{-9}$ M) are associated with a normal immune function and T-cell differentiation, while concentrations of $PGE_2 > 10^{-8}$ M are reported to be immunosuppressive (Kinsella & Lokesh 1990). High plasma PGE_2 levels were observed at 9, 33 and 40 days post-infection in a model of induced infection with *Lepeophtheirus salmonis* in Atlantic salmon, indicating a stress response and the activation of the immune system (Fast, Muise, Easy, Ross & Johnson 2006). However, no significant differences in plasma lysozyme or in total plasma protein (data not shown) were observed between groups, indicating no systemic inflammation. There is a wide individual variation in lysozyme activity in teleosts (Lie, Evensen, Sørensen & Frøysadal 1989; Balfry & Iwama 2004). However, the effect of LPS on the fish immunoresponse seems to depend on LPS strain, exposure time, dose of the toxin, and time after exposure, among other factors (Swain, Nayak, Nanda & Dash 2008), and that may explain the lack of responsiveness observed in our trial.

The observation that gene expression of COX-2 was higher in fish fed Fo might be explained by a PGE_2-mediated negative feedback mechanism in the control of prostaglandins through down-regulation of COX-2 in the Vo group (Fast *et al.* 2005). It may also be explained by the high EPA (20:5n-3) content in fish oil, since EPA also serves as substrate for COX-2 although at a lower priority than AA (Calder & Grimble 2002). On the contrary, it has been reported that both PGE_2 and PGE_3 can induce COX-2 mRNA expression by using similar pathways, although in mammals PGE_3 seems to be less efficient in inducing COX-2 gene expression than PGE_2 (Bagga, Wang, Farias-Eisner, Glaspy & Reddy 2003). In fish, dietary oils rich in n-6 fatty acids seem to induce over-expression of COX-2, pro-inflammatory cytokines such as TNF and IL-1β and other systemic inflammation markers after pathogen challenge (Montero, Grasso, Izquierdo, Ganga, Real, Tort, Caballero & Acosta 2007). The AA: EPA ratio determines the synthesis of different series eicosanoids in fish since EPA competitively inhibits the formation of eicosanoids from AA (Sargent *et al.* 1999). That may explain contradictory results observed in immune parameters in relation to the use of Vo's in different experiments (Montero *et al.* 2007; Mourente, Good, Thompson & Bell 2007; Bell 2008; Seierstad, Haugland, Larsen, Waagbø & Evensen 2009). LPS can be a strong immunogenic compound since both the 'O' polysaccharide chain and the core region of the molecule can act as antigenic determinants (Jakobsen, Gutierrez & Wergeland 1999). Likewise, LPS is a T-independent antigen and can directly stimulate B cells to induce an antibody-mediated immune response without activation of T cells or development of immunological memories (Elkins, Stashak & Baker 1989). At the bone level, gene expression of IgM revealed that bacterial LPS had a stimulatory effect on the lymphocyte population, however, with no effect by dietary oil source.

Matrix metalloproteinase 13 (MMP13) was assessed as a marker of cartilage degradation in the spinal samples. The activity of MMPs has been associated with chronic inflammatory bone diseases (Hernández, Martínez, Tejerina, Valenzuela & Gamonal 2007) and bone deformities in salmon (Wargelius, Fjelldal, Grini, Gil Martens, Kvamme & Hansen 2009). However, no significant differences in MMP-13 gene expression were found in our study 52 days after injection. Granulocytic leucocytes that surrounded the spinal cord and thus also appeared in the vicinity of the bone of the vertebral bodies were observed in bone sections from all experimental groups. Thus, it is difficult to assess whether any of the experimental treatments led to an inflammatory condition in bone.

The present study was designed to investigate the effects of two different dietary AA/EPA ratios on the development of LPS-induced inflammation in Atlantic salmon bone as a risk factor for spinal deformities. The results of this study suggest that Vo is not a risk factor for the development of inflammation-related spinal deformities. At the same time, we found evidence that localized injection-related processes trigger a diet-independent development of vertebral body malformations.

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