Influence of dietary lipid composition on cardiac pathology in farmed Atlantic salmon, Salmo salar L.

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Abstract
The present study investigated the short-term (5 months) effect of replacing dietary marine oils with vegetable oils on the development of arteriosclerotic changes in the heart of Atlantic salmon, Salmo salar. The experiment was performed as a randomized observer-blinded and controlled trial. Farmed Atlantic salmon were randomly sampled from a study population containing 900 individuals. The salmon were divided into three groups and given diets with either 100% fish oil (Diet 1), a 50/50% mixture of fish oil and rapeseed oil (Diet 2) or 100% rapeseed oil (Diet 3). Ten sexually immature salmon from each dietary group were sampled in March and August 2002. Additionally, 47 sexually mature wild salmon were randomly collected in mid-September 2001. Serial histological sections were taken from the bulbus arteriosus and ventricle wall for histopathological evaluation of the coronary arteries and myocardium. No significant differences in mean coronary changes recorded by the main variable ‘mean range lesion’ (MRL) were detected between the groups in March or August. MRL increased significantly between March and August with Diet 2 (P < 0.01), was nearly significant with Diet 3 (P = 0.06) and was unchanged with Diet 1. This pattern coincided with the Diet 2 group having the highest increase in heart weight. MHC class II immunoreactive cells in the coronary changes were detected in sections from one individual in each group. Heart weight was the most dominant variable in the data set and explained linearly 15.5% of the variation in MRL. Body weight, fish length and heart weight were all significantly, positively and linearly correlated to MRL. The possible influence of diet composition on weight gain and MRL needs to be further elucidated. Increase in heart weight seems to be the dominating predictor of the appearance of MRL in Atlantic salmon. However, the present results cannot exclude the possibility that differences in fatty acid composition of fish feed can influence the development of arteriosclerotic changes in Atlantic salmon.

Keywords: Atlantic salmon, coronary arteriosclerosis, heart weight, histopathology, MHC class II, polyunsaturated fatty acids.

Introduction
Coronary arteriosclerosis in salmonids was first described by Robertson, Wexler & Miller (1961). Although several investigators have substantiated their work (Moore, Mayr & Hougie 1976a,b,c; House & Benditt 1981; Eaton, McConnell, Hivath, Black & Schwartz 1984; Saunders & Farrell 1988; Kubasch & Rourke 1990; Saunders, Farrell & Knox 1992; Farrell 2002) the pathogenesis of arteriosclerosis is still disputed. Vascular injury, resulting from overstrecthing of the superficial coronary artery during contraction and distention
of the bulbus arteriosus (Farrell 2002) is thought to be the initiating factor. Vascular injury caused by mechanical damage and increased mitotic activity of medial smooth muscle cells (SMC) has been observed in vitro (Gong & Farrell 1995). In mammals intimal SMC also proliferate in response to injury and are essential in atherogenesis (Schwartz, deBlois & O’Brien 1995). In addition, atherosclerosis is characterized by accumulation of lipid, mostly cholesterol and its ester, extracellular matrix and inflammatory cells, resulting in dramatic reduction in lumen diameter (Lusis 2000). In contrast to mammals, lipids, calcium or inflammatory cells have not been observed in coronary lesions in fish (Moore et al. 1976c; House & Benditt 1981).

Nutritional factors, especially very long-chain n-3 polyunsaturated fatty acids (VLC n-3 PUFAs), have attracted particular interest in the research of vascular lesion development. Increasing evidence suggests health benefits from dietary fish or fish oils in mammalian atherosclerosis (Mori & Beilin 2001), primarily associated with eicosapentaenoic acid (EPA) and decosahexaenoic acid (DHA) (Burr, Fehily, Gilbert, Rogers, Holliday, Sweetnam, Elwood & Deadman 1989; Anonymous 1999). A possible action of VLC n-3 PUFAs seems to be regulation of SMC proliferation in lesion formation in both fish (Gong, Townley & Farrell 1997) and humans (Libby, Schwarz, Brogi, Tanaka & Clinton 1992; Ross 1993). Based on in vitro studies of vascular explants from the coronary vessel from rainbow trout, Oncorhynchus mykiss (Walbaum), it was suggested that low doses of arachidonic acid could induce SMC proliferation in vitro but that EPA inhibited this effect (Gong et al. 1997). High levels of VLC n-3 PUFAs show a similar inhibitory effect in vivo for swine (Weiner, Ockene, Levine, Cuenoud, Fisher, Johnson, Daoud, Jarmolych, Hosmer, Johnson, Natale, Vaudreuil & Hoogasian 1986) and rhesus monkeys (Davis, Bridenstine, Vesselinovitch & Wissler 1987).

Fishmeal and fish oils rich in VLC n-3 PUFAs are the major feedstock for fish farming. Because they are limited resources, future development of aquaculture will rely on the development of additional sustainable raw materials. Vegetable oils can partially replace fish oils in salmon feeds (Rosenlund, Obach, Sandberg, Standal & Tveit 2001; Torstensen, Frøyland & Lie 2004a). Although a substantial difference exists among fish oils and vegetable oils in their content of VLC PUFAs, many freshwater fish, including salmonids, have shown in vivo ability to convert 18:3n-3 found in vegetable oils to VLC n-3 PUFAs (20:5n-3 and 22:6n-3) (Sargent, Bell, Bell, Henderson & Tocher 1995; Buzzi, Henderson & Sargent 1997).

The influence of diet in the development of vascular lesions was suggested in the 1970s (Moore et al. 1976c). Altering the dietary ratios of n-3 to n-6 PUFAs has been shown to cause a marked cardiac muscle depletion of both the spongy and compact layers in salmonids (Bell, McVicar, Park & Sargent 1991). Limited information is available on how dietary lipid composition affects coronary changes in farmed salmon. In a 6-month feeding trial using diets enriched with marine oils, Saunders et al. (1992) were not able to detect any change in the progression of coronary lesions in salmon.

The objective of the present study was to investigate the short-term (5 months) effect of replacing dietary marine oils with vegetable oils on the development of arteriosclerotic and other histological changes in the heart in Atlantic salmon, Salmo salar L.

Materials and methods

The project population

In May 2001, a total of 1800 farmed Atlantic salmon (Mowi strain) with a mean body weight of 142 g (SD = 1) were randomly allocated to three sea net pens with 600 fish each. From sea transfer in May 2001 until March 2002, the fish were fed diets containing different lipid sources (Fig. 1, Table 1). Diet 1_FO contained 100% fish oil from northern hemisphere capelin (Mallotus villosus) oil (Norsildmel, Norway), Diet 2_FO/RO had a 50/50 mixture of fish oil and rapeseed (Brassica napus var. oleifera) oil (Oelmühle, Hamburg) and Diet 3_RO contained 100% rapeseed oil. The experimental conditions, including dietary fatty acid compositions followed during this period have been thoroughly described elsewhere (Torstensen, Frøyland, Ørnsrud & Lie 2004b).

The study population

In March 2002, 300 presumed healthy and sexually immature fish from each group were transferred to three different sea net pens (5 × 5 m). The average fish weight was 1.50, 1.61 and 1.32 kg in the diet groups 1, 2 and 3, respectively. The fish were fed on
the same three diets until August 2002 (Fig. 1). However, the northern hemisphere fish oil was replaced by South American anchovy (*Engraulis ringens*) oil in order to maximize the dietary range of VLC n-3 PUFAs (Table 1). Fish were kept at ambient conditions and were fed to satiation using automatic feeders adjusted to provide 20 g feed kg$^{-1}$ biomass day$^{-1}$. Dead fish were removed daily and weighed.

**Table 1** Composition of experimental diets containing three different lipid sources

<table>
<thead>
<tr>
<th>Raw material (g kg$^{-1}$)</th>
<th>Diet 1 (FO)</th>
<th>Diet 2 (FO/RO)</th>
<th>Diet 3 (RO)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fish meal$^a$</td>
<td>372.40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corn gluten$^b$</td>
<td>100.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extracted soybean meal$^c$</td>
<td>100.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wheat$^d$</td>
<td>102.40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamins, minerals and binders</td>
<td>25.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anchovy oil$^e$</td>
<td>300.20</td>
<td>150.10</td>
<td>0</td>
</tr>
<tr>
<td>Rapeseed oil$^e$</td>
<td>0</td>
<td>150.10</td>
<td>300.20</td>
</tr>
<tr>
<td>Composition of diets</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crude protein, %</td>
<td>39.1</td>
<td>38.1</td>
<td>39.5</td>
</tr>
<tr>
<td>Crude fat, %</td>
<td>33.8</td>
<td>35.6</td>
<td>34.5</td>
</tr>
<tr>
<td>Moisture</td>
<td>6.3</td>
<td>6.9</td>
<td>6.9</td>
</tr>
<tr>
<td>Total n-3 fatty acids$^f$, %</td>
<td>35.5 (15.8)</td>
<td>23.4 (13.3)</td>
<td>11.8 (10.6)</td>
</tr>
<tr>
<td>EPA, % of fat</td>
<td>13.2 (5.9)</td>
<td>7.2 (3.4)</td>
<td>1.5 (0.7)</td>
</tr>
<tr>
<td>DHA, % of fat</td>
<td>13.8 (4.6)</td>
<td>7.5 (3.0)</td>
<td>1.9 (1.0)</td>
</tr>
</tbody>
</table>

$^a$ Nordsildmel, Bergen, Norway.
$^b$ Cargill, Minneapolis, MN, USA.
$^c$ Denofa, Fredrikstad, Norway.
$^d$ Amylum, Ghent, Belgium.
$^e$ Oelmu¨hle, Hamburg, Germany.
$^f$ Values in brackets show levels in diets using capelin oil instead of anchovy oil.

**Feeds**

Three different lipid sources were added to a standard extruded 9 mm salmon feed pellet (Nutreco Technology Centre, Stavanger, Norway). The dietary fatty acid profile varied with the lipid source added (Table 1). All diets were designed to satisfy the nutritional requirements of salmonid fish including the minimum requirement of...
salmonids for n-3 PUFAs (National Research Council 1993).

Trial design

The study was performed as a randomized observer-blinded controlled trial with parallel group design. The fish were randomly sampled 1:1:1 from the study population after crowding the fish with a sweep-net.

Sampling of fish was performed at the start in March 2002 and at the end of the study period in August 2002. The fish were not fed for 24 h prior to sampling. On each of two occasions, 10 individuals from each dietary group were randomly selected. For histological comparison, 47 sexually mature wild Atlantic salmon (1–5 kg) were randomly collected from the Namsen River, Norway. The wild salmon were sampled by netting during their spawning migration in mid-September 2001.

Study procedure

The farmed salmon were anaesthetized using a saturated benzocaine (Rikshospitalets apotek, Oslo, Norway) in alcohol solution (75 mg L⁻¹) in water, measured and weighed before blood (10 mL EDTA vacuum vacutainers, Terumo®; Venoject, Terumo Europe N.V Leuven, Belgium) was drawn from the caudal vein. Blood plasma was separated by centrifugation at 2200 g for 10 min, immediately frozen on dry ice and stored at −80 °C before fatty acid analyses. After blood sampling, the anaesthetized fish were killed by a sharp blow to the head, bled by severance of the gill arteries and necropsied. The heart with the bulbus arteriosus was excised, weighed and placed in 10% phosphate-buffered formalin, and processed for histological analysis using standard procedures (Bancroft & Gamble 2002). The bulbus arteriosus from five fish in each diet group was sampled in both March and August, frozen in liquid nitrogen and stored at −20 °C prior to lipid staining.

Histological analysis

Coronary arteries located on the ventral surface of the bulbus arteriosus, cranial to the major bifurcation, were examined. For microscopic examination, a minimum of five serial histological cross-sections of the coronary arteries were taken at each of two locations (Fig. 2a). These measurements were used to estimate mean intimal coronary changes. The chosen main variable for this purpose was the mean range lesions (MRL) for each individual (Seierstad, Poppe & Larsen 2005). The MRL for each fish is defined as the mean value of at least 10 scored sections from the same coronary artery.

To detect possible vascular changes in smaller intramyocardial branches, horizontal sections of the ventricle were also performed. Sections were processed routinely and stained with haematoxylin and eosin (H&E), van Gieson and elastin-van Gieson. Additional frozen sections were stained with oil red O to identify lipids (Bancroft & Gamble 2002). Vessels were blindly evaluated for intimal changes (Moore et al. 1976b) in accordance with the modified method described by Seierstad et al. (2005). With this system, grades 2–4 represent a progressive increase in intimal change severity and grade 5 was assigned to severe vascular changes. Arterial sections without any identifiable intimal cell proliferation were classified as grade 1 (Fig. 2b). Grade 2 changes indicated early intimal proliferations with less than six cells in a single cluster, involving a maximum 10% of the entire vessel circumference. Grade 3 was an intimal proliferation with more than six cells, involving 10–20% of the...
entire vessel circumference. Grade 4 was a combination of several multifocal intimal proliferations involving 20–50% of the entire vessel circumference and grade 5 was a change involving more than 50% of the intima in the vessel circumference.

As cross-sections were taken at a minimum of 10 locations along the length of the main coronary artery, the MRL for a given fish was a reasonable estimate of the distribution of the coronary intimal changes along the length of this part of the artery.

**Evaluation of ventricular myocardium**

Transverse sections taken from six equal locations through the long axis of the ventricle determined the proportion of the compact layer of the ventricular myocardium. The calculation in μm of the relative thickness of the compact component of the myocardium was measured using a Leica DME microscope (Leica Microsystems, Inc., Buffalo, NY, USA) with a measuring graticule attached to the eyepiece. The thickness of the compact layer was defined as the layer between the epicardium and the interface towards the spongiosa.

**Immunohistochemistry**

Coronary arteries from nine individuals, three from each group in the final sampling, were examined for major histocompatibility complex (MHC) class II expression using polyclonal antisera against the β chain (Koppang, Hordvik, Bjerkås, Torvund, Aune, Thevarajan & Endresen 2003) and visualized using avidin–biotin complex/horseradish peroxidase (ABC/HRP-complex, PK-4000; Vector Laboratories Inc., Burlingame, CA, USA) according to the manufacturer’s instructions. The immunohistochemical procedure has been described elsewhere (Koppang, Haugarvoll, Hordvik, Poppe & Bjerkås 2004).

**Diet and fatty acids analysis**

Crude protein and fat contents in the diets were determined by standard procedures (Torstensen et al. 2004a). Fatty acid profiles of plasma and diets were analysed according to Lie & Lambertsen (1991).

**Thiobarbituric acid reactive substances**

At the end of the study period, cardiac muscles from five fish in each dietary group were analysed using a colorimetric method based on a malondialdehyde standard and 2-thiobarbituric acid solution (Hamre, Ness, Esp, Holm & Lie 2001). The results are given as nmol g⁻¹ wet tissue.

**Vitamin E (α-tocopherol)**

At the end of the trial, cardiac muscle from five fish in each dietary group was analysed using high-pressure liquid chromatography (Lie, Sandvin & Waagbø 1994).

**Statistical analysis**

The results are expressed as mean values with 95% confidence intervals constructed by using the Student’s t-test (Altman 1991). As an index of dispersion, standard deviations are used. The MRL is known to be lognormally distributed (Seierstad et al. 2005). All analyses of this variable were performed on the transformed data and the results retransformed for presentation. Both changes within and differences between groups were performed two-tailed with a significance level of 5%. Analysis of variance (ANOVA) with body weight as covariate was performed for comparison between groups with regard to the MRL scores (Kleinbaum, Kupper, Muller & Nizam 1998). Comparison between and change within groups with regard to the other observed continuously distributed variables were performed by using a simple ANOVA model. The Pearson correlation model was used in the correlation analysis (Kleinbaum et al. 1998).

**Results**

The final average body weights in the total study population were 3.48, 3.83 and 3.37 kg in the diet groups 1, 2 and 3, respectively. This corresponds to specific growth rates of 0.58, 0.58 and 0.55% per day. Except for fish fed diet 2_FO/RO in March, the sampled fish were heavier compared with the average study population on both occasions (Table 2). The mortality was negligible and no clinical disease was recorded.

In the first sampling in March, coronary intimal proliferations were detected in all fish in all the three groups. The MRL was 2.32 (95% CI: 1.79–3.00) in the Diet 1_FO group, 2.28 (95% CI: 1.87–2.76) in the Diet 2_FO/RO group and 2.22 (95% CI: 1.69–2.92) in the Diet 3_RO group (Fig. 3). Corrected for body weight no significant
difference between the groups was detected \((P = 0.95)\). However, the ventricle wall was significantly thinner \((P < 0.01)\) in fish fed Diet 2 \_FO/RO compared with the other diets (Table 2).

Additionally, both fish length \((P = 0.06)\) and body weight \((P = 0.17)\) were smaller in the Diet 2 \_FO/RO group.

In the second sampling in August, intimal proliferations were also detected in all the examined fish. The MRL was 2.81 (95% CI: 2.29–3.44) in Diet 1 \_FO, 3.39 (95% CI: 2.79–4.13) in Diet 2 \_FO/RO and 2.98 (95% CI: 2.38–3.72) in Diet 3 \_RO (Fig. 3). Corrected for body weight, the difference between the groups was not significant \((P = 0.24)\). No significant differences were found between the three diet groups with respect to body weight \((P = 0.26)\), fish length \((P = 0.44)\), heart weight \((P = 0.14)\), or ventricle wall thickness \((P = 0.14)\) (Table 2).

The MRL did not increase significantly \((P = 0.31)\) in the Diet 1 \_FO group from March to August (Table 3). During the same period, the MRL increased significantly in the Diet 2 \_FO/RO group \((P < 0.01)\) and nearly significantly \((P = 0.06)\) in the Diet 3 \_RO group. Body weight, fish length, heart weight and the ventricle wall thickness increased significantly \((P < 0.01)\) in all the three diet groups from March to August (Table 3). The increase in all these four variables was largest in the Diet 2 \_FO/RO group, whereas the increases in the diet groups 1 and 3 were similar.

Body weight, fish length and heart weight were all significantly, positively and linearly correlated to MRL. The correlation between body weight, fish length and MRL partially disappeared and was explained by the heart weight. Consequently, heart weight was found to be the most dominant variable in the set and explained linearly 15.5% of the variation in MRL.

The occurrence of coronary changes in the main coronary artery was significant in both farmed and wild Atlantic salmon. In total, 98% of the fish had at least one identifiable proliferation in one or more cross-sections. Vascular changes in smaller branches

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**Table 2** Comparison of diets and sampling time with regard to external variables

<table>
<thead>
<tr>
<th>March 2002</th>
<th>August 2002</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Diet 1 (FO)</td>
</tr>
<tr>
<td></td>
<td>(n = 10)</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>2.13 (0.80)</td>
</tr>
<tr>
<td></td>
<td>95% CI 1.56–2.70</td>
</tr>
<tr>
<td>Fish length (cm)</td>
<td>55.42 (4.09)</td>
</tr>
<tr>
<td></td>
<td>95% CI 52.5–58.4</td>
</tr>
<tr>
<td>Heart weight (g)</td>
<td>2.98 (0.60)</td>
</tr>
<tr>
<td>Ventricle walla</td>
<td>34.90 (3.64)</td>
</tr>
<tr>
<td></td>
<td>95% CI 32.3–37.5</td>
</tr>
</tbody>
</table>

*1 mm = 42 units.

The results are expressed as mean values with standard deviation (SD) in brackets and 95% confidence intervals.
of the coronary artery in the ventricular wall were seen in both farmed and wild fish, but were considered negligible compared with the changes in the main artery and therefore not interpreted in the main variable MRL.

The fish length and the thickness of the ventricle wall were significantly larger \((P < 0.01)\) in the wild fish compared with the farmed fish in March (Table 4). No significant differences were detected between the wild and the farmed salmon in March in MRL, body weight and circumference. Comparison of the wild and the farmed salmon in August showed a significantly larger \((P < 0.01)\) MRL, fish weight and fish length in the farmed group (Table 4). However, after correction for fish length the MRL was still significantly larger \((P < 0.01)\) in the farmed group in August.

**Histological analysis**

The vascular changes varied from small aggregates of cells within the intima to larger changes, occluding a large part of the arterial lumen (Fig. 4a). Larger intimal changes were characterized by a disrupted and sometimes reduplicated inner elastic lamina (Fig. 4b), but in lesions ranging from...

### Table 3 Comparison between and development in the external variables within diet groups from March to August 2002

<table>
<thead>
<tr>
<th></th>
<th>Diet 1 (FO) ((n = 10 &amp; 10))</th>
<th>Diet 2 (FO/RO) ((n = 10 &amp; 11))</th>
<th>Diet 3 (RO) ((n = 10 &amp; 10))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean range lesion</td>
<td>0.46</td>
<td>1.13</td>
<td>0.75</td>
</tr>
<tr>
<td>95% CI</td>
<td>-0.32–1.23</td>
<td>0.37–1.90</td>
<td>-0.03–1.53</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>1.73</td>
<td>2.46</td>
<td>1.50</td>
</tr>
<tr>
<td>95% CI</td>
<td>1.10–2.35</td>
<td>1.77–3.15</td>
<td>0.95–2.06</td>
</tr>
<tr>
<td>Length (cm)</td>
<td>12.53</td>
<td>16.85</td>
<td>10.68</td>
</tr>
<tr>
<td>95% CI</td>
<td>9.09–15.97</td>
<td>12.50–21.20</td>
<td>7.16–14.20</td>
</tr>
<tr>
<td>Heart weight (g)</td>
<td>1.78</td>
<td>2.89</td>
<td>1.90</td>
</tr>
<tr>
<td>95% CI</td>
<td>1.27–2.29</td>
<td>1.84–3.93</td>
<td>1.02–2.78</td>
</tr>
<tr>
<td>Ventricle walla</td>
<td>1.49</td>
<td>12.19</td>
<td>2.81b</td>
</tr>
<tr>
<td>95% CI</td>
<td>3.04–6.02</td>
<td>8.40–15.98</td>
<td>-2.91–8.54</td>
</tr>
</tbody>
</table>

* 1 mm = 42 units.

b Three observations missing.

The results are expressed as mean values with 95% confidence intervals.

### Table 4 Comparison of farmed and wild Atlantic salmon with regard to external variables

<table>
<thead>
<tr>
<th></th>
<th>Farmed salmon</th>
<th>Wild salmon</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>March 2002 ((n = 31))</td>
<td>August 2002 ((n = 30))</td>
</tr>
<tr>
<td>Mean range lesion</td>
<td>2.25 (0.75)</td>
<td>3.05 (1.33)</td>
</tr>
<tr>
<td>95% CI</td>
<td>2.07–2.46</td>
<td>2.75–3.39</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>1.97 (0.57)</td>
<td>3.87 (0.76)</td>
</tr>
<tr>
<td>95% CI</td>
<td>1.76–2.18</td>
<td>3.59–4.16</td>
</tr>
<tr>
<td>Length (cm)</td>
<td>54.4 (4.0)</td>
<td>67.8 (4.4)</td>
</tr>
<tr>
<td>95% CI</td>
<td>53.0–55.9</td>
<td>66.2–69.5</td>
</tr>
<tr>
<td>Circumference (cm)</td>
<td>28.49 (2.43)</td>
<td>–a</td>
</tr>
<tr>
<td>95% CI</td>
<td>27.60–29.30</td>
<td>26.72–29.84</td>
</tr>
<tr>
<td>Ventricle wall</td>
<td>31.25 (5.48)</td>
<td>37.12 (5.14)</td>
</tr>
<tr>
<td>95% CI</td>
<td>29.24–33.26</td>
<td>35.09–39.15</td>
</tr>
</tbody>
</table>

* The variable was not recorded.

The results are expressed as mean values with standard deviation (SD) in brackets and 95% confidence intervals.
grade 2 to 3, this phenomenon was rarely observed (Fig. 4c,d). The changes in the coronary artery in farmed and wild fish were identical and characterized by intimal accumulations of spindle-shaped cells with an appearance resembling SMC (Fig. 4d,e). The cells were structurally heterogeneous.
ous and had triangular to round nuclei. In farmed fish, the cells appeared ballooned, large and transparent with cytoplasmic vacuoles (Fig. 4a,b,e). In both farmed and wild salmon, advanced intimal proliferations affected the underlying media layer (Fig. 4a). The smooth muscle layer was thinner with fewer, but larger heterogeneous cells and displayed increased collagenous material compared with the normal media layer. In the most complex intimal proliferations (grade 5), the underlying medial layer was often absent. In these vascular changes, the underlying adventitia with loose connective tissue extended into the affected intima (Fig. 4a). Beneath the elastic lamina in proliferated areas, cells with a histological appearance different from that of the medial SMC were seen (Fig. 4e). Oil red O staining provided no indication of lipid accumulation in the affected intima.

MHC class II immunoreactive cells in the coronary changes were detected in three individuals, one from each group (Fig. 4f). Scattered immunopositive cells were embedded within the proliferative changes. A weak endothelial reaction was seen (Fig. 4f).

Plasma fatty acid profile

The fatty acid profile of the diets was mirrored in the plasma. The plasma level of the total monoenes was lowest in the FO group compared with the FO/RO and the RO group in both March and August (Table 5). Additionally, the decrease in monoenes from March to August was most pronounced in the FO group. This reflects the switch from capelin oil rich in long chain monoenes to South American fish oil rich in VLC n-3 PUFAs. The plasma levels of α-linolenic acid (18:3n-3) and total n-6 were lowest in the FO group compared with the other two groups in both March and August. The total n-3 fatty acid level in plasma was higher in the FO group in both March and August compared with the other two groups. Additionally, the increase in n-3 fatty acids was most pronounced in this group, again reflecting the change in dietary fish oil (Table 5). A similar pattern was also found with respect to the sum of saturated fatty acids, the level of EPA (20:5 n-3), DHA (22:6n-3) and the ratio (n-3)/(n-6).

Vitamin E and thiobarbituric acid reactive substances (TBARS) were measured in the heart muscle of five randomly selected salmon in each group in the August sample. The mean vitamin E concentrations and TBARS in the FO group were 15.7 µg g⁻¹ (SD = 18.3) and 155.8 nmol g⁻¹ (SD = 117.8), respectively. In the FO/RO and the RO groups the mean vitamin E concentrations were 22.5 µg g⁻¹ (SD = 15.4) and 41.2 µg g⁻¹ (SD = 4.9), respectively. The corresponding mean TBARS were 70.4 nmol g⁻¹ (SD = 41.4) and 10.4 nmol g⁻¹ (SD = 1.9), respectively.

Discussion

There was an increase in coronary proliferations over the 5-month study period in both diet groups that were given different levels of rapeseed oils. These changes had a significant and positive correlation with heart weight, which was the most

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>March 2002</th>
<th>August 2002</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Diet 1 (FO)</td>
<td>Diet 2 (FO/RO)</td>
</tr>
<tr>
<td>Sum saturates</td>
<td>19.8 (1.1)</td>
<td>19.3 (0.9)</td>
</tr>
<tr>
<td>Oleic (18:1n-9)</td>
<td>10.2 (0.4)</td>
<td>18.9 (0.4)</td>
</tr>
<tr>
<td>Total monoenes</td>
<td>26.7 (0.9)</td>
<td>31.3 (0.8)</td>
</tr>
<tr>
<td>Linoleic (18:2n-6)</td>
<td>2.2 (0.2)</td>
<td>5.3 (0.3)</td>
</tr>
<tr>
<td>Arachidonic (20:4n-6)</td>
<td>1.2 (0.1)</td>
<td>1.3 (–)</td>
</tr>
<tr>
<td>Total n-6</td>
<td>4.2 (0.3)</td>
<td>8.2 (0.2)</td>
</tr>
<tr>
<td>α-linolenic (18:3n-3)</td>
<td>0.4 (–)</td>
<td>1.6 (0.1)</td>
</tr>
<tr>
<td>EPA (20:5n-3)</td>
<td>11.5 (0.8)</td>
<td>9.5 (0.6)</td>
</tr>
<tr>
<td>DHA (22:6n-3)</td>
<td>23.2 (1.6)</td>
<td>20.8 (0.3)</td>
</tr>
<tr>
<td>Total n-3</td>
<td>40.8 (2.3)</td>
<td>36.8 (0.8)</td>
</tr>
<tr>
<td>n-3/n-6</td>
<td>9.9 (0.7)</td>
<td>4.5 (0.1)</td>
</tr>
<tr>
<td>Rest FA</td>
<td>8.5 (2.6)</td>
<td>4.4 (1.7)</td>
</tr>
<tr>
<td>Total identified</td>
<td>91.5 (2.6)</td>
<td>95.6 (1.7)</td>
</tr>
</tbody>
</table>

The results are expressed in weight % as mean of n = 3 with standard deviation (SD) in brackets. Results below 0.1 are denoted by dashes.
dominant variable in the observed set to predict MRL. The increase in heart weight was significant in all the three diet groups and, as for MRL, highest in the group fed Diet 2_FO/RO. The same trend has also been observed in experiments with rainbow trout where intimal changes increased with heart size (Davie & Thorarensen 1996). Heart weight correlated positively with body weight. However, the body weights in the study samples were substantially larger than that found in the total study population. Additionally, the increase in both heart weight and body weight was largest in the Diet 2_FO/RO group. This was also the group with the largest discrepancy in body weight between the study sample and the study population. To what extent the findings in the experimental groups reflect changes in the population can therefore be questioned.

Except for a slight tendency for a higher MRL level in the two rapeseed oil groups compared with the 100% fish oil group at the final sampling date, no significant differences in MRL were detected between the three groups. If the maximum observed difference of 0.58 in MRL between the groups in August sample is the true difference, the probability of detecting this in the present study was only 14%. To obtain a power of 90%, the number of salmon within each group would have to be increased from 10 to 56 individuals. Although the biological significance of MRL differences at this level is difficult to ascertain, the sample size of 10 fish per group in the present study is obviously small and only large differences between groups might therefore be detected. In order to achieve a complete picture of the diet influence on coronary change in Atlantic salmon, studies involving a larger number of individuals should be performed.

The positive linear correlation pattern between MRL, body weight, fish length and heart weight is consistent with previous results (Saunders et al. 1992). The farmed salmon in the August sample displayed higher levels of MRL at a given length compared with their wild counterparts. This indicates that sexual maturation and body length per se as seen in wild salmon, may not be the only factor responsible for development of intimal proliferation in farmed salmon. The observed rapid increase in body weight of farmed fish has to be compensated for by increasing heart weight and vessel calibre. This might be due to the need for greater contractile forces in the myocardium of a large heart to generate sufficient blood pressure. Mammalian studies have shown that arteries generally respond to an increased demand for blood flow by rearrangement of existing wall components rather than synthesis of new mass or cell replication (Mulvany 1992). Such remodelling results in uniform changes in lumen and vessel wall size rather than localized proliferations. A variety of arterial stimuli or injuries induce local intimal proliferations in laboratory animals. The initial vascular stimuli in salmon have been suggested to occur as a consequence of stretching or dilation of the coronary arteries due to their location on the highly elastic bulbus arteriosus (Saunders et al. 1992).

It is relevant whether the changes observed in the coronary artery actually are pathologic lesions or merely a ‘physiological phenomenon’ with little or no relevance for the function of the vessel and heart as discussed by Farrell (2002).

This is, however, dependent on an otherwise normally functioning heart. Several production-related abnormalities occur frequently in modern fish farming and many of these are associated with the cardiovascular system (Ferguson, Poppe & Speare 1990; Sande & Poppe 1995; Poppe, Midlyng & Sande 1998; Mercier, Aubin, Lefrancois & Claireaux 2000; Poppe & Taksdal 2000; Brocklebank & Raverty 2002; Poppe, Johansen & Tørud 2002). Such conditions may impair cardiac function leading to increased mortality, particularly during stressful operations like grading, netting, transportation and medicinal baths. The presented morphological changes are not easily interpreted as providing benefits for the artery wall or for the individual.

The significance of coronary changes may be increased by aggravation through reduced blood flow in hearts already compromised by other subclinical conditions. However, mortality was low and not influenced by diet in the present study. The lack of clinical manifestation might suggest that the observed vascular changes are ‘normal abnormalities’, and may therefore be acknowledged as not pathological.

The intimal proliferations in salmonids are similar to the SMC proliferations involved in early human atherosclerosis. In humans, the coronary changes can progress and after years cause significant arterial stenosis resulting in tissue ischaemia or acute occlusive events.

In this short-term study, increase in MRL correlated largely with heart weight. In spite of
similar increases in heart weight in the FO and RO groups during the 5-month experimental period, a tendency for higher MRL values in the RO group was noted at the last sampling. This might indicate that the fatty acid composition of the fish feed is of some importance for the development of arteriosclerotic changes in farmed salmon, and would also be in accordance with observations on the modulating effect of VLC n-3 PUFAs on SMC proliferation during vascular change formation in both fish (Gong et al. 1997) and humans (Libby et al. 1992; Ross 1993).

If, or to what extent, the intimal proliferations will develop and tissue changes occur in fish on prolonged vegetable oil diets is speculative. It is tempting to presume that an intense growth triggers arteriosclerosis in salmonids by inducing an abnormal SMC proliferation as an adaptive response to increased cardiovascular demand. The mechanism is not understood in any detail, but mechanical injury can result in activation of cascades involved in inflammatory responses and the observed presence of MHC class II+ cells in the coronary vessel wall might be an indication of this. In mammals, proportions of SMC in coronary lesions have also been found to be MHC class II+, probably following an induction by pro-inflammatory cytokines (Jonasson, Holm, Skalli, Gabbiani & Hansson 1985). In relation to the possible inflammatory mechanisms involved, it has been shown in mammals that the products of the n-6 PUFA family are more pro-inflammatory than their n-3 counterparts. The balance in the intake of the n-3 and n-6 PUFA will determine the amounts of various eicosanoids in the body, potentially affecting their inflammatory profiles (Lopez-Huertas, Baro, Cerrero, Fonolla, Jimenez & Boza 2003).

The groups of fish in the present study that were fed vegetable oils had higher plasma levels of n-6 fatty acids and lower levels of n-3 PUFA and in particular, EPA and DHA, compared with fish fed a diet with fish oil very rich in n-3 PUFAs. Endothelial cells can also play a role in this respect and may be activated to secrete growth factors by haemodynamic and systemic stimuli. n-3 PUFA and especially EPA inhibit SMC proliferation in rainbow trout (Gong et al. 1997).

A thinner ventricular wall in the farmed compared with wild fish might indicate that the lesions are related to similar adaptive responses. However, despite the very large variation in dietary n-3 PUFA levels, ventricle wall thickness was not different among groups at the end of the study.

Taken together, the effects of n-3 fatty acids in the present study are not clear but the indications are that replacement of fish oil by rapeseed oil is not an independent predictor of arteriosclerotic changes, although it cannot be excluded. This is also in line with previous work by Saunders et al. (1992) where it was suggested that diets rich in n-3 or n-6 have no influence on the development of arteriosclerosis in farmed salmon.

Polyunsaturated fatty acids are prone to peroxidation and generated free radicals may be cytotoxic (Steinberg, Parthasarathy, Carew, Khoo & Witzum 1989). In the present study, lower levels of vitamin E were found in cardiac tissue from fish fed Diet 1_OFO compared with the other diet groups. This might be explained by the high level of n-3 PUFAs in the fish oil and their susceptibility to oxidation. Higher levels of TBARS in cardiac tissue from fish fed Diet 1_OFO compared with the other diet groups supports the hypothesis of peroxidation caused by the high levels of n-3 PUFAs. The present results indicate increased oxidative stress, demonstrated by a consumption of vitamin E and increased TBARS. However, there was no significant change in MRL from March to August in Diet group 1_OFO indicating that lipid peroxidation is of limited importance in vascular change development in salmon.

Histological examination revealed coronary intimal proliferations in both farmed and wild salmon which displayed the same appearance with local intimal thickenings consisting of SMC, intercellular matrix and reduplication and disruption of the inner elastic lamina. Larger coronary changes that compress media to a thin layer consisting of heterogeneous cells and collagenous intercellular matrix have also been observed in dogfish, Scorpaenichthys canicula (L.) (Muñoz-Chápuli, García-Garrido & de Andrés 1991).

MHC class II positive cells were demonstrated in situ in coronary proliferations. However, the classification of the cells detected is not easy. In mammals, specialized antigen-presenting cells, such as macrophages and dendritic cells are constitutively MHC class II+. In addition, expression may be induced in a number of other cells through influence of inflammatory cytokines (Glimcher & Kara 1992) and activated T-lymphocytes and early B-lymphocytes can express MHC class II. Further investigations are warranted to elucidate
the pathogenesis and to determine the immune involvement in coronary change formation in Atlantic salmon.

In conclusion, the variation in MRL was best explained by heart weight. The underlying mechanisms have not been studied in any detail but MHC class II+ cells are found in conjunction with changes in the intimal vessel wall, possibly pointing to an immune involvement. Further, replacing fish oil with vegetable oil in the diet results in n-6 bias in plasma content, which might possibly drive pro-inflammatory responses. Finally, the present results cannot exclude the possibility that differences in fatty acid composition of fish feed can influence the development of arteriosclerotic changes in Atlantic salmon.

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References


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