Fat content and morphology of liver and intestine of Atlantic salmon (Salmo salar): Effects of temperature and dietary soybean oil

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Abstract

We investigate the effect of adding soybean oil (SO) to the diet of Atlantic salmon and water temperature on lipid composition and morphology of the liver and intestine. The fish were fed fish meal-based diets supplemented with either 100% fish oil (100% FO), 50% soybean oil (50% SO) or 100% soybean oil (100% SO) for 950 day degrees at 5 and at 12 °C. Fish fed the 50% SO and 100% SO diets had higher percentages of 18:2n-6 and 18:1n-9 in the triacylglycerol (TAG) fractions of liver and intestine than fish fed the 100% FO diet, at both temperatures. In addition, the percentages of 20:5n-3 and 22:6n-3 were considerably lower, while the percentages of 20:4n-6 and 20:4n-3 were higher in the phospholipids (PL) fractions of both liver and intestine of fish fed diets containing SO. Both morphological and chemical analyses revealed higher accumulation of fat both in the intestine and liver at 5 than at 12 °C. Diets, on the other hand, did not affect the fat content of the intestine on the contrary to what was found in the liver. Fish fed the 100% SO diet had higher accumulation of fat in the liver than fish fed the 100% FO diet at 5 °C. The higher fat accumulation in liver from 100% SO fed fish seemed to be mainly caused by a selective accumulation of 18:2n-6 and 18:1n-9.

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1. Introduction

Soybean oil (SO) is readily available at often lower prices than marine oils, and this oil is there-
the cell membranes (Røsjø et al., 1994; Hvattum et al., 2000) and the lipid content of the fish. The replacement of FO with SO results in higher levels of \( n-6 \), and generally, lower levels of \( n-3 \) FAs in tissues of Atlantic salmon (Hardy et al., 1987; Grisdale-Helland et al., 2002) and rainbow trout (Greene and Selivonchick, 1990; Finstad and Thomasen, 1991). Little is however known about how SO diets affect the lipid metabolism and lipid deposition in Atlantic salmon at very low water temperatures.

Changes in water temperature may cause changes in the lipid composition of fish membranes in a process known as homoviscous adaptation (Røsjø et al., 1994; Ruyter et al., 2003). Membranes become more rigid at low temperature, and this effect is offset by higher proportion of unsaturated FAs, which causes a disordering of the membrane (Cossins, 1994; Hazel, 1995). FAs with double bonds are much less rigid than saturated FAs. The enzyme that incorporates the first double bond into saturated FAs is \( \Delta^9 \) desaturase, and the activity of this enzyme is increased in cold-acclimatised carp liver (Trueman et al., 2000). The activities of other desaturases are also affected by temperature. One example is the higher activity of the \( \Delta^6 \) desaturase in rainbow trout kept at 5 °C than in trout kept at 20 °C (Hagar and Hazel, 1985).

A number of studies have investigated the effects of different dietary lipids on the morphology, including fat deposition of the intestine and liver of several fish species (Bell et al., 1995; Tucker et al., 1997; Olsen et al., 1999, 2000; Caballero et al., 2002, 2004). Tucker et al. (1997) found that large lipid droplets accumulated in the livers of red drum fed diets containing SO. Caballero et al. (2002) found that more lipid droplets accumulated in intestine and liver of rainbow trout (Oncorhynchus myskiss) fed different dietary vegetable oils than in fish fed a FO diet. Other studies have investigated the effects of temperature on the fat content of fish tissue. For example, Ingemansson et al. (1993) showed that the fat content in the dark muscle of rainbow trout increases in fish acclimatised to the cold. Similarly, Jobling and Bendiksen (2003) found that fat was deposited to a higher degree in the muscle and carcass of Atlantic salmon parr kept at 2 °C than in fish kept at 8 °C. The content of body fat is higher in cold-acclimatised carp than in warm-acclimatised carp (Shikata et al., 1995). It is however not known how a diet rich in SO affect the morphology of liver and intestine of Atlantic salmon kept at low water temperatures.

The aim of the present study was therefore to investigate lipid composition of liver and intestine of Atlantic salmon that had been fed either a diet based solely on marine oil \((n-6 \text{ to } n-3 \text{ ratio of 0.2})\) or a diet containing SO \((50\% \text{ or } 100\%) \text{ (n-6 to n-3 ratio of 1.6 and 4.2) for 3 months at 12 °C or for 7 months at 5 °C.}\) We also studied lipid deposition in the enterocytes of the intestinal folds and in the liver, using light microscopy.

2. Materials and methods

2.1. Chemicals

Metacaine (MS-222) was obtained from Norsk Medisinaldepot (Norway). Acetic acid, chloroform, petroleum ether and methanol were all obtained from Merck (Germany). Benzene was obtained from Rathburn Chemicals Ltd. (Scotland). Methanolic HCl and 2,2-dimethoxypropane were purchased from Supelco Inc. (USA). Glass-baked silica gel K6 plates were obtained from Whatman International Ltd. (England). Glutaraldehyde, Epon resin, copper grids and lead citrate were supplied by Electron Microscopy Sciences (USA).

2.2. Fish and experimental design

The feeding trial was performed primarily as described by Grisdale-Helland et al. (2002). Atlantic salmon (Salmo salar) in tanks containing seawater at 5 or 12 °C (three tanks per diet at each temperature, with 40 fish per tank) were fed for approximately 950 day-degrees one of three test diets (3 months for the 12 °C and 7 months for the 5 °C). The diets were all based on fish meal, and differed only in the type of supplemental oil that they contained (Table 1). One diet contained only fish oil (100% FO), another only crude soybean oil (100% SO), and the third diet a 50/50 mixture of the two sources (50% SO) (see Grisdale-Helland et al. (2002), for a detailed description of the diets). The salmon at both temperatures
grew from an initial weight of 113 ± 5 g to a final weight of 338 ± 19 g. The feed efficiency ratio (FER) in this trial was in average 1.4 kg gain per kg dry feed.

2.3. Final sampling

The fish were not fasted prior to sampling. All the fish in each tank were anaesthetized using MS-222, and individual weights and lengths were taken. Three fish from each tank were used for tissue FA composition and morphological studies. The fish were killed with a blow to the head, and samples of liver and intestine were taken for studying morphology. The remaining parts of the liver and intestine were pooled for each tank at each temperature, placed on dry ice, and stored at −80 °C until used for the determination of fat content and FA composition.

2.4. Lipid extraction and analysis of lipid classes and FA composition

The total lipids in the liver and mid intestine were extracted, and total lipid percentage and relative amounts of each FA present were determined using the methods described by Folch et al. (1957) and Røsjø et al. (1994). The chloroform phase that is produced by Folch’s method was dried under nitrogen gas and the residual lipid extract was redissolved in hexane. Phospholipids (PLs) and triacylglycerols (TAGs) were separated by TLC using a mixture of petroleum ether, diethyl ether and acetic acid (113:20:2 by vol) as the mobile phase. The lipids were visualized by spraying the TLC-plates with 0.2% (wt/vol) 2’,7’-dichlorofluorescein in methanol, and identified with known standards under UV-light (as described by Røsjø et al. (1994) and modified by Ruyter et al. (2000)). The spots corresponding to PLs and TAGs were scraped into glass tubes and transmethylated overnight with 2,2-dimethoxypropane, methanolic HCl and benzene at room temperature, as described by Mason and Waller (1964). The methyl esters of FAs were separated in a gas chromatograph (Perkin-Elmer Autosystem GC equipped with an autoinjector, and with a programmable split/splitless injector) with a CP Wax 52 column (length 25 m, internal diameter 0.25 mm and thickness of the film 0.2 μm), flame ionisation detector and 1022 data system. Helium was used as carrier gas, and the injector and detector temperatures were both 280 °C. The oven temperature was raised from 50 to 180 °C at the rate of 10 °C min⁻¹, and then raised to 240 °C at a rate of 0.7 °C min⁻¹. The relative quantity of each FA present was determined by measuring the area under the peak corresponding to that FA.

2.5. Tissue preparation

Samples for morphological examination were taken from three fish from each dietary group, both at 5 and 12 °C. After washing the gut in a 0.9% sodium chloride solution, the middle part of intestine was removed and tissue for morphological studies dissected. Samples from the central region of the liver were washed briefly in 0.1 M cacodylate buffer (pH 7.4). All tissue samples were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer for 24 h. The samples were rinsed in this buffer, and then post-fixed for 1 h in 2% OsO₄ in 0.1 M cacodylate buffer containing 1.5% potassium ferrocyanide. They were then stained en bloc in 1.5% uranyl acetate. Upon dehydration in ethanol series (70-90-

Table 1

Formulation of the diets

<table>
<thead>
<tr>
<th>Ingredients (g·kg⁻¹)</th>
<th>Diets</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100% FO</td>
</tr>
<tr>
<td>Fish meal</td>
<td>409.4</td>
</tr>
<tr>
<td>Capelin oil</td>
<td>278</td>
</tr>
<tr>
<td>Soybean oil</td>
<td></td>
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<tr>
<td>Maize gluten</td>
<td>215</td>
</tr>
<tr>
<td>Wheat</td>
<td>76.4</td>
</tr>
<tr>
<td>Lysine (78%)</td>
<td>11.83</td>
</tr>
<tr>
<td>Mineral + vitamin + pigment</td>
<td>9.28</td>
</tr>
<tr>
<td>Yttrium oxide</td>
<td>0.11</td>
</tr>
</tbody>
</table>

Chemical composition

| Dry matter (%)                | 97.2    | 97.5   | 96.1    |
| Crude protein                 | 51.9    | 51.9   | 51.3    |
| Crude fat                     | 29.6    | 28.8   | 30.8    |
| Ash                           | 6.1     | 6.1    | 5.9     |
| Gross energy (MJ/kg)          | 26.13   | 26.1   | 25.94   |

Diets: 100% FO (100% fish oil diet); 50% SO (50% fish oil and 50% soybean oil diet); 100% SO (100% soybean oil added).
96-4×100%) and propylene oxide, the specimens were embedded in Epon resin and polymerized at 60 °C for 12 h. Semithin sections (1.5 μm) were cut with a diamond knife on a Reichert Ultracut E ultramicrotome, dried onto polylysine-coated slides and stained with a mixture of toluidine blue O and alkaline fuchsin dissolved in PEG 200 (polyethylene glycol) as described by Alsop (1974). This staining procedure colours fat green, and stains the surrounding tissue purple to red. All sections were examined in a Leica DC 100 light microscope (Leica, Germany) and a Leica DC 100 camera integrated with the microscope was used to capture digitized images.

2.6. Statistics

The data were analysed by two-way ANOVA for the factors “diet” and “water temperature”. Significant differences among means were analysed using Duncan’s test in the software package UNISTAT (London, England). The significance level was set at 5%.

3. Results

3.1. FA compositions of diets

The FO diet was characterised by relatively high percentages of the long-chain n-3 FAs, 20:5n-3 and 22:6n-3, and low percentages of C18 FAs. The 50% SO and the 100% SO diets, on the other hand, contained about 25% and 45% of 18:2n-6, respectively, and relatively low percentages of 20:5n-3 and 22:6n-3 (Table 2). The n-6 FAs to n-3 FAs ratios of the diets were 0.2 for the diet based purely on marine oil, 1.6 for the 50% SO diet and 4.2 for the 100% SO diet.

3.2. FA compositions of the liver and intestine at the two temperatures

There were significant differences in the FA compositions of the PL fraction and the TAG fraction of liver and intestine of the different dietary fish groups kept at 5 and at 12 °C (Tables 3 and 4). The percentages of n-6 FAs were approximately twice as high in the PLs of the liver and intestine in the 50% SO group and approximately four times as high in the 100% SO group than they were in the 100% FO group. Not only did the percentage of 18:2n-6 increase, but also the percentage of 20:4n-6, which is the “dead end” elongation product of 18:2n-6, and the percentage of 20:4n-6, which is the desaturation and elongation product of 18:2n-6. The percentages of 20:5n-3 and 22:6n-3 were significantly lower in the PL fraction of fish fed the SO diets than in fish fed the FO diet. The dietary effects were more pronounced in the TAG fractions of the liver and intestine than in the PL fraction. There was a major decrease in the percentages of 20:1n-9 and 22:1n-11 in the TAG of liver and intestine with increased inclusion of SO. The percentages of the n-6 FAs were approximately three times higher in the TAGs of liver and intestine in the 50% SO group and six

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Fatty acid composition of the diets (percent of total fatty acids)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diet</td>
<td>100% FO</td>
</tr>
<tr>
<td>----------</td>
<td>----------</td>
</tr>
<tr>
<td>Fatty acids (percentage of total)</td>
<td></td>
</tr>
<tr>
<td>14:0</td>
<td>5.7</td>
</tr>
<tr>
<td>16:0</td>
<td>14.0</td>
</tr>
<tr>
<td>18:0</td>
<td>1.6</td>
</tr>
<tr>
<td>ΣSaturated¹</td>
<td>21.6</td>
</tr>
<tr>
<td>16:1n-7</td>
<td>7.5</td>
</tr>
<tr>
<td>18:1n-7</td>
<td>2.8</td>
</tr>
<tr>
<td>18:1n-9</td>
<td>10.3</td>
</tr>
<tr>
<td>24:1n-9</td>
<td>0.7</td>
</tr>
<tr>
<td>20:1 (sum isomers)</td>
<td>12.8</td>
</tr>
<tr>
<td>22:1 (sum isomers)</td>
<td>15.9</td>
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<tr>
<td>ΣMonounsaturated¹</td>
<td>50.5</td>
</tr>
<tr>
<td>16:2n-6</td>
<td>0.5</td>
</tr>
<tr>
<td>18:2n-6</td>
<td>4.4</td>
</tr>
<tr>
<td>20:4n-6</td>
<td>0.2</td>
</tr>
<tr>
<td>18:3n-3</td>
<td>0.8</td>
</tr>
<tr>
<td>18:4n-3</td>
<td>3.2</td>
</tr>
<tr>
<td>20:4n-3</td>
<td>0.4</td>
</tr>
<tr>
<td>20:5n-3</td>
<td>9.0</td>
</tr>
<tr>
<td>22:5n-3</td>
<td>0.6</td>
</tr>
<tr>
<td>22:6n-3</td>
<td>7.0</td>
</tr>
<tr>
<td>Σn-6¹</td>
<td>5.3</td>
</tr>
<tr>
<td>Σn-3¹</td>
<td>21.3</td>
</tr>
<tr>
<td>n-6/n-3 ratio</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Diets: 100% FO (100% fish oil diet); 50% SO (50% fish oil and 50% soybean oil diet); 100% SO (100% soybean oil diet).
¹ Includes fatty acids not listed.
nd=not detectable.
times higher in the 100% SO group than they were in the 100% FO group. The increased percentage of the $n = -6$ FAs was mainly due to an increase in 18:2n-6 and (to a lesser extent) an increase in 20:2n-6. Increased levels of SO in the diets also led to higher percentages of 18:n-9 particularly in the PL fraction and the TAG fraction of liver and intestine in the low temperature group. However, 18:1n-9 was incorporated to greater extent in the TAGs of both tissues.

### 3.3. Chemically determinations of fat content of the liver and intestine at the two temperatures

There was a general tendency to higher fat content of liver at 5 than at 12 °C irrespective of dietary
The quantity of each fatty acid is given in percent of total fatty acids. Data are means ± SEM. nd=not detectable. n=3. *abcd Values marked with different superscripts are significantly different.

1 Includes fatty acids not listed. 100% FO=100% fish oil diet; 50% SO=50% soybean oil diet; 100% SO=100% SO diet.
affected by the temperature, with more lipid droplets accumulated at 5 than at 12 °C. Fig. 3 shows images of intestinal tissues from fish fed the two diets.

The lipid staining (green colour) in enterocytes from fish kept at 12 °C was weak and only limited to small areas close to the nuclei and lipid droplets were also present to some extent in lamina propria (Fig. 3A–B). On the contrary to what was found at 12 °C, enterocytes of the folds from fish kept at 5 °C were strongly stained (Fig. 3C–E). Fat appeared as numerous small droplets that almost completely filled the cytoplasm in enterocytes from both feeding groups (Fig. 3C–D). In addition, large amounts of lipid were located in the lamina propria (Fig. 3E).

3.4.2. Liver

There were no differences in the pattern and amount of lipid staining in the tissue of livers from fish kept at 12 °C and fed different diets. Fig. 4A shows a representative image with almost no lipid staining of a liver section from fish fed the SO diet and kept at 12 °C. In contrast, the amount of lipid staining in liver from fish kept at 5 °C was higher than in liver from fish kept at 12 °C and was also substantially affected by the diet: Lipid accumulated to a greater extent in liver from fish fed 100% SO diet (Fig. 4B) than it did in liver from fish fed the 100% FO diet (Fig. 4C).

4. Discussion

Feeding Atlantic salmon with different dietary oils, clearly influenced the FA composition of the PL and TAG lipid fractions of the liver and intestine. SO diets increased considerably the percentages of 18:1n−9, 18:2n−6 and total n−6 FAs in the TAG fraction and, to a lesser extent, in the PL fraction. These increases were accompanied by decreases in the percentages of n−3 FAs, especially 20:5n−3 and 22:6n−3, in the monounsaturated FAs 20:1n−9 and 22n−11 and in saturated FAs. The observed changes in FA composition agree mainly with those obtained in previous studies in which vegetable oils have been used in the diets of salmonid species (Thomassen and Røsjø, 1989; Røsjø et al., 1994; Guillou et al., 1995; Ruyter and Thomassen, 1999; Bell et al., 2001). The FA composition of the diets affected the FA composition of the tissue TAGs more than that of the PLs. This agrees with previous work showing that the FA composition in TAGs depends closely on the FA composition of the dietary lipids, whereas the FA composition of the PLs does not depend as closely (Xia et al., 1993; Jobling and Bendiksen, 2003). This is probably due to the fact that a number of FAs, such as 20:5n−3 and
22:6n−3, are preferentially conserved in the PLs (Henderson and Tocher, 1987; Sargent et al., 2002; Moya-Falcón et al., 2004), while the FAs 18:1n−9 and 18:2n−6 are mainly found in the TAG fractions. The percentage of 20:4n−6 was significantly higher in the PL fraction of liver and intestine in fish fed the 100% SO diet, than in fish fed the 100% FO diet and the 50% SO diet, at both tempera-
tures. This may be a result of increased desaturation and elongation of 18:2n-6 from the diet to 20:4n-6 in the SO dietary groups. High desaturation and/or elongation activities have been reported in hepatocytes of salmon fed rapeseed oil (Bell et al., 2001), palm oil (Tocher et al., 2004) and SO oil diets (Tocher et al., 1997; Ruyter et al., 2003; Ruyter and Thomassen, 1999). An increase in the percentage of 20:4n-6 may also be caused by removal of product inhibition as a result of decreased levels of 20:5n-3 and 22:6n-3 (Ruyter et al., 2003). The physico-chemical properties of membrane PLs change significantly when a single double bond is introduced into a saturated FA (Farkas et al., 1980; Hazel and Williams, 1990). In the present study, the percentages of the monounsaturated FAs, 18:1n-9 and (to a lesser extent) 16:1n-7, were higher in the PLs of the liver and intestine of salmon kept at 5 °C than they were in fish kept at 12 °C, while the percentages of 16:0 and 18:0 were slightly lower. These results suggest that the activity of the Δ⁹ desaturase activity was higher in fish kept at 5 °C than in fish kept at 12 °C. This agrees with Trueman et al. (2000) who reported that cold acclimatisation induces the activity of acyl CoA Δ⁹ desaturase in carp liver.

There were no significant differences in the fat levels in livers of fish kept at 5 and at 12 °C in the 100% FO and 50% SO dietary groups. On the contrary, livers from fish fed the 100% SO diet, had a 1.5 fold higher fat percentage at 5 than at 12 °C. The higher fat percentage at the lower temperature was mainly due to increased percentages of 18:1n-9 and 18:2n-6 in the liver TAG. The increased accumulation of 18:1n-9 and 18:2n-6 in the 100% SO
group than compared to the other dietary groups, may be related to the lower level of dietary long chain n-3 FAs available for fish in this group. We have recently shown that 18:1n-9, given to cultured Atlantic salmon hepatocytes, is mainly esterified in TAG VLDL for secretion, while 22:6n-3 is primarily esterified in the PL of VLDL, indicating that long chain n-3 PUFAs are important for the production of VLDL in Atlantic salmon liver cells (Vegusdal et al., 2005). If there is a lack of n-3 PUFAs for production of VLDL PL, then the available 18:1n-9 and 18:2n-6 will most probably be stored in cell lipids. Dietary long chain n-3 FAs are also previously found to be important for synthesis of lipoprotein PL in fish intestine (Olsen et al., 1999; Olsen et al., 2000, Caballero et al., 2003) The higher lipid accumulation in the 100% SO livers were however only observed at 5°C and not at 12°C in our study, indicating that low temperature may also reduce the activity of the enzymes involved in the esterification of FFA into TAG and PL for VLDL production, and thereby lead to increased lipid deposition in the liver.

We observed no significant effect of dietary inclusion of SO on lipid accumulation in the intestine of Atlantic salmon at the two temperatures. On the contrary, several studies with other fish species like Arctic char (Olsen et al., 1999, 2000), Atlantic cod (Lie and Lambertsen, 1987), rainbow trout (Caballero et al., 2002), turbot (Koven et al., 1997) and sea bream (Caballero et al., 2004) have all shown increased lipid accumulation in the intestines of fish fed diets rich in vegetable oils. The reason why there was no increase in lipid accumulation in Atlantic salmon fed SO than compared to fish fed the FO diet, we do not know. The temperature however, had a pronounced effect on the lipid accumulation in the intestine, with a 1.8 fold increase in fat percentage at 5°C than compared to at 12°C. During light microscopy examination we also observed a large accumulation of lipid in the intestinal folds of fish kept at 5°C, especially in supranuclear locations of the enterocytes. These droplets probably form during the absorption of diets with high lipid content (Sire et al., 1981; Fontagné et al., 1998) and are thought to be temporary storage forms that are used when the rate of absorption exceeds the rate of synthesis of lipoproteins (Iwai, 1969; Noaillac-Depeyre and Gas, 1974; Deplano et al., 1991; Kjorsvik et al., 1991), rather than pathological formations (Spisni et al., 1998). Probably the low temperature delays the lipoprotein synthesis and the further transport of lipoproteins from the apical parts of the enterocytes, resulting in high accumulation of lipid.

In conclusion, we have shown that low temperature and high levels of SO in the diet for Atlantic salmon lead to an accumulation of fat in the liver, probably due to a selective deposition of 18:1n-9 and 18:2n-6. The diet did not affect the lipid deposition in the intestine, but lowering of temperature, significantly increased the amount of intracellular lipid droplets.

Acknowledgements

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