

**1The influence of temperature on the apparent lipid digestibility in Atlantic salmon**  
**2(*Salmo salar*) fed *Calanus finmarchicus* oil at two dietary levels**

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## Abstract

Oils extracted from the marine zooplankton, *Calanus finmarchicus*, have high levels of n-3 highly unsaturated fatty acids (HUFA) and are therefore of interest as an alternative lipid source in aquafeeds. Copepod lipid is composed mainly of wax esters (WE) with high levels of saturated fatty acids and monounsaturated fatty alcohols which are considered hard to digest, especially at low temperatures. This assumption has however not been verified and for this reason the present study examined the digestibility of diets containing high levels of WE and two fat levels in Atlantic salmon reared at 3 and 12 °C. The fish were acclimated for one month to 3 °C (485 g) and 12 °C (599 g) and then fed one of four diets, high fat fish oil (33% lipid, HFFO), high fat *Calanus* oil (32% lipid, HFCO), low fat fish oil (17% lipid, LFFO) and low fat *Calanus* oil (19% lipid, LFCO). The fish meal lipid content was lowered by the use of lipid-extracted fish oil (2.3% lipid). This enabled a level of 50% WE in the LFCO and HFCO oils, compared to 0% in the LFFO and HFFO diets. The fish were then allowed to grow to around 100% of initial weight (220 days at 3 °C and 67 days at 12 °C) and then analysed for faecal lipid digestibility, bile volume, bile composition and intestinal lipolytic activity. Differences were observed in all these parameters in relation to temperature, type of dietary oil and the lipid level in the diet. Faecal lipid content and lipid class composition were dependent on rearing temperature and the type of dietary lipid. Highest levels of undigested lipids were observed in the faeces of fish fed CO. Wax ester-derived fatty alcohols, particularly 20:1n-9 and 22:1n-11, were less extensively digested than corresponding fatty acids from FO at both fat levels and temperatures. Fish kept at 12 °C had a significantly higher bile volume than fish at 3 °C and higher volumes were found in fish fed CO diets compared to FO. Decreased faecal passage time at lower temperatures, was not sufficient to ensure high digestibility since the lower bile volume and enzyme activities at 3 °C in the present trial exerted a greater effect. Although the compensatory mechanisms of increased bile volume and lipolytic activity are initiated upon feeding WE at a level of 50% of dietary lipid, these are not sufficient to compensate lipid digestibility and growth as in FO diets. Low inclusion of CO in diets during winter has to be considered as saturated fatty acids and monounsaturated fatty alcohols were poorly digested at 3 °C in fish fed CO diets.

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## 641. Introduction

65 Salmonids are commonly reared at high latitudes where large fluctuations in sea  
66temperature occur. They have a growth optima at 12-17 °C (Brett, 1971; Koskela *et al.*,  
671997a; 1997b), but maintain feeding and growth even at temperatures approaching 0 °C  
68(Fraser *et al.*, 1993; Koskela *et al.*, 1997b). However, the digestive process is influenced by  
69temperature. In Arctic charr (*Salvelinus alpinus*) feed intake is reduced at low temperatures as  
70are the digestive processes and the gastrointestinal holding time in an attempt to maintain  
71optimal nutrient uptake (Olsen and Ringø, 1998). However, the results of studies with other  
72salmonid fish are inconclusive. Although most trials have shown increased macronutrient  
73digestibility with increasing temperature (Atherton and Aitken, 1970; Brauge *et al.*, 1995;  
74Olsen and Ringø, 1998; Bendiksen *et al.*, 2003), there are also reports showing no significant  
75effect on nutrient availability. For example, in rainbow trout (*Oncorhynchus mykiss*) reared at  
763 and 11 °C (Austreng, 1978) and 7, 11 and 15 °C (Windell and Norris, 1969), temperature  
77had no effect on lipid and fatty acid digestibility. However, rates of fatty acid digestibility are  
78known to decrease with increasing chain length, and increase with increasing unsaturation  
79(Sigurðsladóttir *et al.*, 1992; Johnsen *et al.*, 2000). This is to a large extent related to melting  
80point (Olsen and Ringø, 1997). Thus, some studies have shown that digestibility of saturated  
81fatty acids (SFA) is reduced at lower temperature, while the digestibility of monounsaturated  
82fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) is less affected (Olsen and  
83Ringø, 1998; Ng *et al.*, 2003; Ng *et al.*, 2004). This change in lipid digestibility may be at  
84least partly responsible for the maintenance of proper cell membrane fluidity, through  
85homeoviscous acclimation, that occur when ectotherms are exposed to low temperature  
86(Wallaert and Babin, 1994; Fodor *et al.*, 1995; Farkas *et al.*, 2001), since low temperature  
87acclimatised fish possess greater proportions of PUFA and reduced amounts of SFA in  
88membranes (Hagar and Hazel, 1985; Olsen and Henderson, 1997). As such, winter  
89performance might be affected when high levels of vegetable oils are included in feeds for  
90salmonids that are farmed at high latitudes, due primarily to the low concentrations of n-3  
91highly unsaturated fatty acid (HUFA) present in these oils (Bendiksen *et al.*, 2003).

92 Marine fauna from lower trophic levels could thus be a good alternative, as these sources  
93contain naturally high levels of n-3 HUFA. The zooplankton, *Calanus finmarchicus*, is  
94considered as an alternative lipid source in aquafeeds (Olsen *et al.*, 2004). These animals

95have a high level of wax esters (WE), compared to the triacylglycerols (TAG) that are the  
96most abundant neutral lipid in most fish species (Sargent *et al.*, 1976). Wax esters are esters  
97of long-chain fatty acids and fatty alcohols and are intrinsically more hydrophobic than TAG,  
98making them harder to digest than TAG (Bauermeister and Sargent, 1979). Several trials  
99have shown that WE levels above 40% of the dietary lipid (ca 30% lipid diets) reduce growth  
100and lipid digestibility in Atlantic salmon, while lower levels, allow the same growth and lipid  
101digestibility as fish fed fish oil diets (Olsen *et al.*, 2004; Bogevik *et al.*, 2009; Oxley *et al.*,  
1022009). These previous studies have, however, been carried out at single relative high  
103temperatures and did not make any comparisons with the situation at lower temperatures.  
104Consequently, the intention of the current study was to study the effect of low environmental  
105temperatures (3°) on WE utilisation and digestive capability (bile volume, bile composition,  
106midgut lipolytic activity) in Atlantic salmon.

## 1082. Materials and methods

### 1092.1. *Fish, diets and experimental design*

110 Three hundred and sixty Atlantic salmon (*Salmo salar* L., Mowi strain; Norwegian  
111 breeding programme, 13 month-old post-smolts) originally held at 9 °C, averaging 447 g were  
112 anaesthetized in 0.1% (w/v) MS-222 (tricaine methane sulphonate; Norwegian Medical  
113 Depot, Bergen, Norway) and measured for weight and length. The fish were then distributed  
114 equally between 24 1.5×1.5×1.0 m fibreglass tanks supplied with aerated seawater. The fish  
115 were then acclimatized to the experimental temperatures gradually over one month from 9 °C  
116 to either 3 or 12 °C, with twelve tanks in each temperature group. The fish grew through the  
117 acclimation period to an average of 485 g in the cold water group (3.1±0.4 °C) and 599 g in  
118 the warm water group (12.3±0.4 °C).

119 Four diets were prepared at NOFIMA (Bergen, Norway) as outlined in detail previously  
120 (Olsen *et al.*, 2004) and contained 0.01% yttrium oxide as a marker of digestibility (Table 1).  
121 The only exception being that the fish meal was lipid extracted fishmeal obtained from  
122 TripleNine Fish protein amba (Esbjerg, Denmark) containing 2.3% lipid. The diets were  
123 designed to be low (ca 18%) and high (33%) in lipid. One low lipid diet contained oils  
124 extracted from the marine copepod *Calanus finmarchicus* (termed LFCO) while the other  
125 contained fish oil (LFFO). Likewise, the high lipid diets were either added *Calanus* oil  
126 (HFCO) or fish oil (HFFO). Further details on the composition are given in Table 1.

127 The fish were then fed the four diets in triplicate tanks at both temperatures. In order to  
128 attain a fairly similar end weight, fish at low temperature were fed for 220 days, while those  
129 in the high temperature groups were fed for 67 days. All fish were fed to satiation twice a day  
130 using ArvoTec TD2000 feeders (Huutokoski, Finland). After the experimental period had  
131 elapsed, fish were anaesthetised in 0.1% MS-222 and measured for weight and length. Faeces  
132 were stripped from fish according to Ringø (1991), the samples from tanks pooled, and stored  
133 at -80 °C prior to analysis. Five fish from each tank were killed by a sharp blow to the head.  
134 The luminal content of the midgut regions was then collected for analysis of lipolytic enzyme  
135 activity (Tocher and Sargent, 1984; Bøgevik *et al.*, 2008). The remaining fish were starved for  
136 72 h. Then, five fish from each tank were anaesthetized and killed as above, and bile collected  
137 from the gall-bladder with a 5 mL syringe with 0.1 mL resolution. After recording the

138volume, the bile was stored at -80 °C for analysis of bile salts and osmolality. Remaining fish  
139from each triplicate group were then pooled and cross-fed with the opposite dietary fat source.  
140Thus, fish previously fed HFCO where now fed HFFO, and those previously fed HFFO were  
141now fed HFCO. The same was done for low fat fed fish, i.e. LFCO fed fish were now given  
142LFFO and those previously fed LFFO were now fed LFCO. After one week, the fish were  
143anaesthetized, and faeces collected as described above.

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## 1452.2. Analysis of diets and faeces

146 Diets and faeces were freeze-dried to obtain dry weight, followed by analysis of yttrium  
147oxide according to Otterå *et al.* (2003). Yttrium was determined in feed and faeces by use of  
148an ICP-MS (inductive-coupled plasma – mass spectrometry) method after wet digestion in a  
149microwave oven (Otterå *et al.*, 2003). Total lipid of diets and faeces was extracted with  
150chloroform/methanol (2:1, v/v) according to Folch *et al.* (1957). Dilute HCl (3 M, 30% of  
151original faecal weight) was added prior to the last extraction. The organic solvent phase was  
152evaporated to dryness *in vacuo* at room temperature before resuspending the lipid residue in  
15310 mg mL<sup>-1</sup> of chloroform/methanol (2:1, v/v) containing 0.05% (w/v) BHT. Extracted lipid  
154was stored under N<sub>2</sub> at -80 °C prior to further analysis.

155 Lipid class composition of total lipid was determined by double-development high-  
156performance thin-layer chromatography (HPTLC) coupled with scanning densitometry,  
157essentially as described by Olsen and Henderson (1989). HPTLC plates were initially  
158developed to halfway in methyl acetate/isopropanol/chloroform/methanol/0.25% aqueous KCl  
159(25:25:25:10:9, v/v) before developing fully with hexane/diethyl ether/acetic acid (85:15:1,  
160v/v). Lipid classes were visualised by spraying the plate with 3% copper acetate (w/v) in 8%  
161phosphoric acid (v/v) and charring at 160 °C for 15 min (Olsen and Henderson, 1989) with  
162subsequent quantification using a CAMAG TLC Scanner 3 and WinCATS software  
163(CAMAG, Muttens, Switzerland). Identities of individual lipid classes were confirmed by  
164running authentic standards alongside samples on HPTLC plates.

165 To determine fatty acid and long-chain fatty alcohol composition of diets and faeces,  
166extracted lipid was subjected to acid-catalysed transesterification using 1% (v/v) H<sub>2</sub>SO<sub>4</sub> in  
167methanol with 17:0 fatty acid and fatty alcohol added as internal standards. Resultant fatty

168 acid methyl esters (FAME) were extracted and purified by TLC on 20×20 cm plates as  
169 described previously (Tocher and Harvie, 1988). Long-chain fatty alcohols present in  
170 extracted lipid from CO diets and faeces of fish fed these diets were identified on TLC plates  
171 as a single component and recovered from silica by elution with chloroform/methanol (2:1,  
172 v/v) before conversion to acetate derivatives by reaction with acetic anhydride/pyridine (1:2,  
173 v/v) (Farquhar, 1962). Prior to GC analysis, fatty alcohol acetates were purified on TLC  
174 plates as described for FAME.

175 Fatty acid methyl esters and fatty alcohol acetates were separated and quantified by gas  
176 liquid chromatography using a 60 m x 0.32 mm i.d. fused silica capillary column coated with  
177 ZB-Wax (Phenomenex, Macclesfield, UK) and a Thermo Finnigan Trace gas chromatograph.  
178 Hydrogen was used as carrier gas and temperature programming was from 50 to 150 °C at a  
179 rate of 40 °C min<sup>-1</sup>, from 150 to 170 °C at a rate of 2 °C min<sup>-1</sup>, from 170 °C to 199 °C at a  
180 rate of 0.5 °C min<sup>-1</sup>, and then to a final temperature of 220 °C at 40 °C min<sup>-1</sup>. Individual  
181 components were identified by comparison with known standards. The absolute amounts of  
182 individual fatty acids and long-chain alcohols present were calculated by reference to the  
183 internal standard (Olsen *et al.*, 2004).

184

### 185 2.3. Analysis of bile and lipolytic activity

186 Analysis of conjugated bile salts was performed essentially according to Coca *et al.* (1994)  
187 and Bogevik *et al.*, (2009). Conjugated bile salts were separated using a Waters Alliance  
188 HPLC system with 2690 Separation Module and a LiChrospher RP<sub>18</sub> column (4.6 × 250 mm,  
189 id 5 µm) (Supelco, Inc., Bellefonte, USA) with methanol/0.5 M acetate buffer pH 4.3 (70:30,  
190 v/v) as mobile phase. Detection was via a Waters 996 Photodiode Array Detector set at λ =  
191 205 nm with reference to authentic standards (Coca *et al.*, 1994).

192 Bile osmolality was measured using a Fiske one-ten osmometer (Fiske Associates, MA,  
193 USA) with calibration and reference solutions (290 mOsm L<sup>-1</sup>) from the manufacturer.

194 Lipolytic activity of midgut content on triacylglycerol (TAG), wax esters (WE) and sterol  
195 esters (SE) were performed essentially according to Tocher and Sargent (1984) and Bogevik  
196 *et al.* (2008). In brief, a solution of 0.2 ml extracted midgut enzyme was added to 0.1 ml of  
197 the substrate suspension of either TAG, WE or SE and 0.1 ml of 80 mM sodium taurocholate

198(Sigma-Aldrich, St. Louis, USA), giving a total volume of 0.4 ml. The mixture was then  
199incubated for 4 h at 10 °C and the reaction terminated by the addition of 1.5 ml of  
200chloroform/methanol/ toluene (2:2.4:1, by vol.) containing 0.3 mM oleic acid as a carrier.  
201Fifty µl of 1 M NaOH was then added to ensure partitioning of all free fatty acids into the  
202aqueous phase. The tubes were vortexed for 15 s and centrifuged at 3220 x g for 30 min  
203before 400 µl of the aqueous phase was transferred to scintillation vials. After addition of 2.5  
204ml of scintillation fluid (Opi-fluor, PerkinElmer, Wellesley, MA, USA), radioactivity was  
205determined in a Packard Tri-Carb 2300TR liquid scintillation spectrophotometer. Results  
206were calculated and expressed as pmol oleic acid produced min<sup>-1</sup> mg protein<sup>-1</sup> as described  
207previously (Tocher and Sargent, 1984).

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#### 2092.4. Calculations and statistical treatment

210 Specific growth rate (SGR) and apparent digestibility coefficient (ADC) were calculated  
211using the equations given in Olsen *et al.* (2004). Fatty acid and long-chain alcohol  
212digestibilities were calculated based on the concentration of individual components in total  
213lipid (µg mg<sup>-1</sup>). Data are given as means ± standard derivation (S.D.) for replicate tanks  
214where *n*=3. All statistical analyses were performed using STATISTICA software for  
215Windows (Louisiana, USA). Data were checked for homogeneity of variances by the Levene  
216test and, where necessary, transformed via arcsin (percentage data) or Ln functions. Effects of  
217diet or temperature were assessed by multivariate analysis using standard general linear model  
218(GLM) methods. Differences were analysed via three-way ANOVA for mean effect of  
219temperature, fat level and oil source. Significance was accepted at a level of *P*<0.05 in all  
220cases.

221



## 2223. Results

### 2233.1. *Growth*

224 The fish almost doubled their body weight at both temperatures. Fish at 3.1 °C grew from 225485±15 g to 899±40 g over 220 days, while fish at 12 °C grew from 599±26 g to 985±45 in 22667 days (Figure 1A). The fish thus had specific growth rates (SGR) of 0.28±0.02 and 2270.74±0.06, respectively, at water temperatures of 3 °C and 12 °C (Figure 1B). The diets also 228had significant effects on growth. Fish fed diets added *Calanus* oil (HFCO and LFCO) had an 229overall lower SGR than those fed fish oil as the main lipid source (HFFO and LFFO) (Figure 2301B). This was especially noticeable at 12 °C for fish fed the low fat diet where SGR was 231significantly lower in the LFCO group (0.65±0.02) compared to the LFFO group (0.76±0.05). 232There was also a noticeable effect of fat level with growth generally higher in high fat groups 233compared to low fat groups (Figure 1B). There was thus an overall significantly effect of 234temperature and diet on growth.

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### 2363.2. *Dietary lipid composition*

237 The major differences in lipid class composition between the diets were in the neutral lipid 238fraction with a predominance of TAG (62% and 55% of lipid in the HF and LF diets 239respectively) in the FO diets, and WE (53% and 50% of lipid in the HF and LF diets 240respectively) in the CO diets. The content of cholesterol was roughly double in the FO diets 241(8-9%) compared to that in the CO diet (4-5%). Furthermore, the level of phospholipids in 242the LF diets (11.5-11.6%) was twice that of the HF diets (6.4-6.6%), due to the generally 243larger contribution from the fish meal. The major phospholipids were in all cases 244phosphatidylcholine (PC) and phosphatidylethanolamine (PE). No free long-chain fatty 245alcohols (FFAlc) were detected in the CO diets (Table 1).

246 The absolute levels of fatty acids (FA) in dietary lipid were 881 and 757 µg mg lipid<sup>-1</sup>, 247respectively for the HFFO and the LFFO diets, compared to 508 and 499 µg mg lipid<sup>-1</sup> in the 248HFCO and the LFCO diets. These latter diets contained additionally 344 and 310 µg mg 249lipid<sup>-1</sup> of long-chain FAlc (Table 2). The FO dietary lipid was comprised predominantly of 250MUFA (42-45%), followed by n-3 PUFA (27%), SFA (23%) and n-6 PUFA (5-7%). The

major fatty acids being, 22:1n-11 (14-15%), 16:0 (13-14%), 22:6n-3 (11-12%), 20:1n-9 (9-10%) and 18:1n-9 (9-10%) (Table 2). The CO diets contained a relatively lower level of MUFA (26%), but a higher level of n-3 PUFA (35-38%), SFA (28-29%) and n-6 PUFA (6-9%). This was due to a higher level of 18:4n-3, 20:5n-3, 14:0 and 20:4n-6 (Table 2). In contrast, the FALC of the CO diets were dominated by monounsaturates, particularly 22:1n-11 (36-37%) and 20:1n-9 (28-29%). The FALC moiety was accordingly low in saturates (14%) and polyunsaturates (5-6%).

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### 3.3. *Faecal lipid composition*

Faecal lipid content was always (except HFCO, 91 g kg<sup>-1</sup> independent of temperature) lower in fish kept at 12 °C (14-46 g kg<sup>-1</sup>) than those kept at 3 °C (38-56 g kg<sup>-1</sup>). This was particularly notable in fish fed the LF diets, where lipid constituted 14-22 g kg<sup>-1</sup> of the faecal dry matter at 12 °C compared to 38-56 g kg<sup>-1</sup> at 3 °C (Table 3). Furthermore, the faecal lipid contents of fish fed the HF diet (46-91 g kg<sup>-1</sup>) was significantly higher than those fed the LF diet (14-56 g kg<sup>-1</sup>), and in fish fed the CO diet (22-91%) compared to those fed the FO diet (14-53%).

The faeces of fish held at 12 °C had higher level of the lipolytic products FFA and FALC and consequently lower level of TAG and WE compared to those maintained at 3 °C. This was especially observed in fish fed the HFFO diet with 10% and 32% TAG and 51% and 31% FFA, respectively in faecal contents of fish fed at 12 °C and 3 °C (Table 3). Fish fed the HF diets had a significantly higher level of FFA and lower level of TAG compared to fish fed the LF diets. Furthermore, high levels of faecal TAG and FFA were observed with fish fed the FO diets, whereas high levels of faecal WE and FALC were found in fish fed the CO diets (Table 3). The highest levels of undigested faecal lipids were thus observed in fish at 3 °C fed either HFCO (48% WE and 4% TAG) or LFCO (41% WE and 6% TAG). Faecal cholesterol was significantly higher in fish maintained at 12 °C compared to 3 °C, in fish fed FO compared to CO and significantly lower in fish fed HF compared to LF diets (Table 3).

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### 3.4. *Digestibility of FA and FALC*

280 The apparent digestibility coefficient (ADC) of total lipid was generally higher at 12 °C  
281 compared to 3 °C. But the difference was often quite marginal (Table 4). The same was true  
282 for the total FA digestibility varying between 93-98% at 12 °C and falling by a few percent in  
283 average to between 91-96% at 3 °C. There was also a general effect of lipid source as ADC of  
284 total FA was higher in fish fed FO diets compared to CO diets (Table 4). The lower ADC of  
285 FA in CO fed fish at 3 °C was particularly due to poor utilisation of SFA. For total FAIc,  
286 ADC were generally lower than for the fatty acids being 81% and 94%, for fish fed the  
287 HFCO and LFCO diets respectively at 12 °C. Lowering the temperature to 3 °C dramatically  
288 reduced ADC to 73% and 74%, for fish fed the HFCO and LFCO diets respectively.  
289 Although there was a general reduction in ADC for all alcohols, the reduction was most  
290 dramatic for the very long chain ones 20:1n-9 (74%) and 22:1n-11 (55-57%) (Table 4).  
291 However, the FAIc digestibility of SFA was also significantly lower at 3 °C. The level of fat  
292 in the diets had little effect on the digestibility of lipids.

293 Cross-fed fish previously fed dietary CO for 100 days showed increased digestibility when  
294 fed dietary FO for one week, and conversely fish fed FO initially showed lowered ADC when  
295 fed CO. This was especially seen at 12 °C for fish fed the HF diets. Atlantic salmon  
296 previously fed the HFCO diet had a total lipid digestibility of 89% at the end of the trial, and  
297 after one week on the HFFO diet the total lipid digestibility was increased to 96%. This was  
298 also higher than the total lipid digestibility (94%) in fish fed HFFO for 100 days in the trial  
299 (Figure 2).

300

### 301 3.5. Bile volume and composition

302 Fish kept at 12 °C had a significantly higher bile volume (ml kg fish<sup>-1</sup>) than fish at 3 °C  
303 (Figure 3a). There was also a significant effect of dietary oil source on bile volume, with  
304 higher volumes in fish fed CO diets compared to FO. This was especially seen at 12 °C where  
305 the bile volume was 2.0±0.5 ml kg fish<sup>-1</sup> in the HFCO and the LFCO groups compared to  
306 1.7±0.3 and 1.8±0.5 ml bile kg fish<sup>-1</sup> in the HFFO and LFFO groups respectively. Dietary  
307 lipid level had no significant effect on the bile volume, although fish kept at 3 °C tended to  
308 have lower bile volumes in the low fat groups (LFFO, 1.3±0.3 and LFCO, 1.2±0.3) compared  
309 to the high fat groups (HFFO, 1.4±0.4 and HFCO, 1.7±0.4) (Figure 3a). Bile osmolality was  
310 not affected by lipid type and amount. Temperature did however significantly affect

311osmolality being higher at 3 °C compared to 12 °C (346 and 324 mOsm respectively) (Figure  
3123b). The composition of the major bile salt, taurocholate (TC), was increased in bile of fish  
313kept at 3 °C compared to fish kept at 12 °C (351 vs. 323 mM), but was unaffected by dietary  
314lipid source and amounts (Figure 3c). There was however a tendency to be lower level of  
315taurocholate in fish fed FO diets. This was especially seen in fish kept at 3 °C, with  
316taurocholate levels at  $349 \pm 39$  and  $342 \pm 46$  mM respectively in the HFFO and LFFO group  
317compared to  $355 \pm 41$  and  $357 \pm 45$  mM respectively in the HFCO and LFCO group. The  
318concentration of the minor bile salt, taurochenodeoxycholate (TCDC), was unaffected by  
319temperature and lipid source, but appeared to increase significantly in fish fed low fat diets  
320( $67 \pm 18$  mM) compared to high fat diets ( $57 \pm 20$  mM) (Figure 3d).

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### 3223.6. *Lipase activity in midgut*

323 The lipolytic activity of the midgut extract towards TAG and WE was higher in fish kept  
324at 12 °C (2007 and 699 pmol oleic acid/min/mg protein, respectively) compared to fish kept  
325at 3 °C (1556 and 284 pmol oleic acid/min/mg protein, respectively) (Figure 4a,c). Dietary  
326lipid source or level had no significant effect on the hydrolysis of TAG (TAGH) and wax  
327esters (WEH). There was however a tendency for better hydrolysis in intestinal contents of  
328fish fed CO compared to FO in that all three substrates were better hydrolysed in midgut from  
329fish kept at 3 °C and fed HFCO (TAGH, 1854; SEH, 327 and WEH, 337 pmol oleic  
330acid/min/mg protein) compared to HFFO (TAGH, 1527; SEH, 235 and WEH, 301 pmol oleic  
331acid/min/mg protein) (Figure 4).

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#### 3344. Discussion

335 Fish kept at 3 °C in the present study had a 5 month longer feeding period, and required a  
336total of over 7 months to double their weight compared to fish reared at 12 °C that took  
337approximately 2 months. The specific growth was thus significantly lower in salmon kept at 3  
338°C than 12 °C. This is due to reduced feed intake and slower metabolism at low temperatures  
339(Olsen and Ringø, 1997). It is generally assumed that decreases in temperatures will increase  
340faecal passage time and thereby increase the digestive capacity (Fauconneau *et al.*, 1983;  
341Olsen and Ringø, 1997). However, in the present experiment, fish at 3 °C, and in particular  
342those fed CO diets, exhibited significantly reduced digestibility leading to increases in faecal  
343WE and TAG. This shows that the compensation of increased passage time was not sufficient  
344to ensure a high lipid digestibility, as shown for Arctic charr (*Salvelinus alpinus*) previously  
345by Olsen and Ringø (1998). Higher temperature increases the general metabolism including  
346higher enzyme activities (Chiu and Benitez, 1981), faster absorption (Stokes and Fromm,  
3471964) and increased enzyme affinity (Somero, 1969). The increased *in vitro* hydrolysis of  
348TAG and WE at 12 °C compared to 3 °C in the present trial confirms this. This is most likely  
349due to an increased amount of lipases in the midgut from fish kept at 12°C. The higher feed  
350intake at 12 °C was dependent not only on higher enzyme activity, but also on better  
351solubilisation and absorption in bile to utilize the ingested energy efficiently. The higher bile  
352volume at 12 °C would therefore be necessary for high digestibility and to compensate  
353increased feed intake to render more energy for growth. Fish kept at 12 °C had thus a  
354significantly higher amount of hydrolysed lipid in the faeces and showed a higher lipid  
355digestibility compared to fish at 3 °C.

356 Atlantic salmon fed CO diets have previously been shown to display growth rates fairly  
357similar to fish fed FO diets provided that the WE level is below 40% of the dietary lipid  
358(Olsen *et al.*, 2004; Bogevik *et al.*, 2009). In the present study, WE was approximately 50%  
359of the dietary lipid level both in the LF and HF group. Thus, as expected, growth was  
360significantly lower in these groups compared to the FO groups. However, the growth  
361differences were smaller than expected when based on previous literature. The reason may  
362reside in size differences. In earlier studies where WE comprised 48% of the dietary lipid,  
363fish end size was around 500 g (Bogevik *et al.*, 2009; Oxley *et al.*, 2009), while fish in the  
364present study were around 1000 g. As larger fish may have a better capacity to utilize WE

365(higher hydrolytic activity) (Bogevik *et al.*, 2008), they may tolerate higher levels of WE  
366without affecting growth. In CO-fed fish, decreased lipid digestibility and growth were  
367associated with higher proportions of WE remaining in faecal lipid in fish kept at 3 °C with a  
368resultant decreased FAlc fraction. The higher amount of bile and enzymes at 12 °C would  
369increase hydrolysis of WE to FFA and FFAlc. However, the increased gastric evacuation and  
370reduction in lipid absorption which are observed at higher temperatures in *Dicentrarchus*  
371*labrax* (Santulli *et al.*, 1993), concomitant with a high dietary WE level, might explain the  
372high level of esterified lipid in the faeces also found at 12 °C in fish fed the HFCO diet.

373 As in a previous trial, feeding WE induced an increase in bile volume and lipolytic  
374enzyme activity (Bogevik *et al.*, 2009). Thus, WE feeding significantly increased bile volume  
375at both temperatures and increased enzyme activity in midgut in fish kept at 3 °C. The  
376hydrolytic activity in the gut is known, however, to be much lower towards WE than TAG  
377(Patton *et al.*, 1975; Tocher and Sargent, 1984; Olsen and Ringo, 1997). This was observed in  
378both *in vivo* and *in vitro* studies, where bile salt dependent lipase (BSDL) was reported to  
379hydrolyze at rates of 1-2 orders of magnitude slower than TAG in anchovy (*Engraulis*  
380*mordax*) and rainbow trout (Patton *et al.*, 1975; Tocher and Sargent, 1984), and 5-fold slower  
381than TAG in Atlantic salmon (Bogevik *et al.*, 2008). Whether the slower hydrolysis is due to  
382the greater hydrophobicity of WE, associated with lower biliary emulsification, or the  
383specificity of the lipase is still unclear. Nonetheless, the increased ability to hydrolyse lipid  
384upon WE feeding did not decrease the level of esterified lipid of TAG and WE, especially at  
3853 °C where WE was at the same level in diet and faeces. Fish fed the FO diets had thus a  
386faster hydrolysis of TAG to FFA, and thus a higher digestibility of total lipid. However, fish  
387feeding on CO have a larger digestive capacity that causes improved digestibility when these  
388fish are cross-fed with FO diets compared to fish only fed FO diets. In the present trial this  
389mechanism was especially pronounced in fish fed HF diets and kept at 12 °C, while slower  
390digestion at 3 °C and less lipid in LF diets slowed down the compensation.

391 In conclusion, the present study showed reduced bile content and lipolytic activity  
392combined to lower hydrolysis rates, digestibility and growth at 3 °C compared to 12 °C in  
393Atlantic salmon. Even though compensatory mechanisms in lipid digestion were initiated  
394upon WE feeding at a level of 50% of dietary lipid, these mechanisms were not sufficient to  
395maintain lipid digestibility and growth as observed with FO diets. Nevertheless, these CO-fed  
396fishes had better digestive capacity to maintain higher lipid digestibility when cross-fed with

397FO for 1 week than fish fed FO for 100 days. The lower growth and lipid digestibility were  
398associated with increased faecal lipid load and poor digestibility of SFA through poor lipid  
399hydrolysis. As the present study demonstrated poor lipid digestion upon WE feeding, it is  
400important to carefully consider the inclusion levels of WE to optimise feeds. Further studies  
401are required to evaluate the effects of inclusion levels of WE during cold acclimatisation in  
402the winter season.

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# 574Tables

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576Table 1.

577Formulation (g kg<sup>-1</sup> diet) and gross composition (%) of the Atlantic salmon experimental diet.

	HFFO	HFCO	LFFO	LFCO
Triple Nine fish meal <sup>a</sup>	417.0	417.0	575.0	575.0
Fish oil <sup>b</sup>	289.0	0.0	131.0	0.0
<i>Calanus</i> oil	0.0	289.0	0.0	131.0
Soyalecitin	5.0	5.0	5.0	5.0
Soya protein <sup>c</sup>	60.0	60.0	60.0	60.0
Wheat 230/05 <sup>d</sup>	140.0	140.0	140.0	140.0
Wheat gluten 156/05 <sup>e</sup>	80.0	80.0	80.0	80.0
Vitamin mixture <sup>f</sup>	10.0	10.0	10.0	10.0
Mineral mixture <sup>g</sup>	4.0	4.0	4.0	4.0
Charophyll Pink (10%)	0.3	0.3	0.3	0.3
Yttrium oxide (Y <sub>2</sub> O <sub>3</sub> )	0.1	0.1	0.1	0.1
Dry matter	93.0	93.4	91.3	90.7
Protein	43.0	44.4	54.8	55.3
Lipid	33.3	32.4	17.3	18.6
Total polar lipid	6.4	6.6	11.5	11.6
Cholesterol <sup>h</sup>	8.6	4.2	8.1	5.1
Free fatty acids	12.5	15.4	12.5	13.9
Triacylglycerol	62.3	21.2	55.0	19.8
Wax esters/Sterol esters	10.1	52.7	12.8	49.6

578Feed codes are as follows: LF, low fat; HF, high fat; FO, fish oil; CO, *Calanus* oil.

579<sup>a</sup> Fish meal: low fat, Tripple Nine, Denmark, ( 89,1% dry matter, 76,5% crude protein, 2,3% fat (soxhlet) and 58013,1% ash).

581<sup>b</sup> Fish oil: NorSalmOil, Norsildmel, Bergen, Norway

582<sup>c</sup> Soya protein: Soya protein concentrate (SPC 70) , Sopropêche, Boulogne, France.

583<sup>d</sup> Wheat: Norgesmøllene, Bergen, Norway

584<sup>e</sup> Wheat gluten: Received from Ewos Innovation, Dirdal, Norway

585<sup>f</sup> Diets supplied with following vitamins per kg diet: vitamin D3, 3000 I.E; vitamin E (Rovimix, 50%), 160 mg;

586thiamine, 20 mg; riboflavin, 30 mg; pyridoxine-HCl, 25 mg; vitamin C (Riboflavin Stay C 35%), 200 mg;

587calcium pantothenate, 60 mg; biotin, 1 mg; folic acid, 10 mg; niacin, 200 mg; vitamin B12, 0.05 mg; menadione

588bisulphite, 20 mg.

589<sup>g</sup> Diets supplied with following minerals per kg diet: magnesium, 500 mg; potassium, 400 mg; zinc, 80 mg; iron,

59050 mg; manganese, 10 mg; copper, 5 mg.

591<sup>h</sup>May contain some diacylglycerol.

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607Table 2.

608Total lipid content (ug mg<sup>-1</sup>) and composition of fatty acids and fatty alcohols of diets fed to Atlantic salmon in  
609the present experiment.

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%	HFFO	HFCO		LFFO	LFCO	
	FA <sup>a</sup>	FA <sup>a</sup>	FAIc <sup>b</sup>	FA <sup>a</sup>	FA <sup>a</sup>	FAIc <sup>b</sup>
14:0	6.4	11.9	1.2	5.9	11.1	1.2
<b>16:0</b>	<b>13.4</b>	<b>13.2</b>	<b>12.1</b>	<b>14.1</b>	<b>14.5</b>	<b>11.7</b>
Total SFA	22.8	28.3	14.2	23.1	28.9	13.8
16:1n-7	5.1	4.6	2.1	4.8	4.3	2.2
<b>18:1n-9</b>	<b>9.0</b>	<b>6.3</b>	<b>3.9</b>	<b>9.6</b>	<b>7.5</b>	<b>3.8</b>
<b>20:1n-9</b>	<b>10.4</b>	<b>4.8</b>	<b>29.4</b>	<b>9.4</b>	<b>4.0</b>	<b>28.3</b>
<b>22:1n-11</b>	<b>15.2</b>	<b>7.1</b>	<b>37.3</b>	<b>13.7</b>	<b>6.2</b>	<b>36.3</b>
Total MUFA	44.7	26.1	80.4	42.4	25.7	80.0
18:2n-6	4.0	5.2	2.3	6.1	7.9	2.5
20:4n-6	0.3	0.3		0.4	0.3	
Total n-6 PUFA	5.1	6.2	2.3	7.2	9.2	2.5
18:3n-3	2.0	3.2	3.1	2.1	3.1	3.7
18:4n-3	4.2	12.9		4.0	10.9	
<b>20:5n-3</b>	<b>7.1</b>	<b>9.9</b>		<b>7.0</b>	<b>8.9</b>	
<b>22:6n-3</b>	<b>11.4</b>	<b>10.0</b>		<b>11.7</b>	<b>10.0</b>	
Total n-3 PUFA	26.7	38.1	3.1	26.6	34.6	3.7
Total PUFA	32.5	45.6	5.4	34.5	45.3	6.2
Total of lipid (ug mg)	881	508	344	757	499	310

611<sup>a</sup>Some minor fatty acids are not shown, including 15:0, 18:0, 16:1n-9, 16:2, 16:3, 18:1n-7, 18:2n-3, 18:3n-6,  
61220:0, 20:1n-7, 20:1n-11, 20:2n-6, 20:3n-3, 20:3n-6, 20:4n-3, 22:0, 22:1n-9, 22:4n-6, 22:5n-6, 22:5n-3, 24:0and  
61324:1n-9. <sup>b</sup>Some minor fatty alcohols are also not shown, including 18:0, 20:0, 18:1n-7

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Table 3.

Total lipid (g kg<sup>-1</sup> of dry weight) and lipid class composition of faeces collected from Atlantic salmon fed four diets at two temperatures for 100 days.

	HFFO		HFCO		LFFO		LFCO		ANOVA			
	3	12	3	12	3	12	3	12	Temp	Fat	Oil	T*F*O
LPC	0.3±0.0	0.4±0.1	0.3±0.0	0.7±0.3	0.6±0.2	0.6±0.1	0.7±0.0	0.6±0.3	-	0.03	-	-
SM	0.5±0.3	0.4±0.2	0.4±0.3	0.4±0.2	0.9±0.3	0.8±0.5	0.6±0.1	0.9±0.3	-	0.01	-	-
PC	2.1±0.2	2.5±0.2	1.2±0.2	1.3±0.3	3.1±0.6	3.7±0.8	2.6±0.3	2.4±0.6	-	0.00	0.00	-
PE	3.1±0.1	4.5±0.8	2.5±1.0	3.2±1.4	2.6±0.5	3.5±0.3	2.7±0.5	3.9±0.8	0.00	-	-	-
UPL	10.2±0.6	16.8±1.6	5.8±0.6	12.0±4.6	9.2±2.2	20.2±4.5	9.5±1.5	20.1±3.4	0.00	0.01	-	-
C <sup>a</sup>	9.7±0.9	11.9±1.7	5.7±0.6	6.5±1.2	11.3±1.0	14.9±1.9	7.8±0.9	12.7±1.0	0.00	0.00	0.00	-
FFAlc			27.5±1.3	39.6±3.0			23.1±1.1	31.2±2.5	0.00	0.00		
FFA	31.0±5.0	50.7±1.3	3.9±0.3	12.8±3.5	27.8±3.7	31.8±5.8	5.3±0.8	8.7±2.4	0.00	0.00	0.00	-
UNL	1.1±0.3	0.9±0.9	0.6±0.2	0.1±0.1	0.7±0.4	1.6±0.2	0.7±0.1	2.0±0.5	-	0.00	-	-
TAG	32.1±5.8	9.8±5.6	3.7±1.4	1.5±1.3	33.9±3.6	19.1±2.4	6.2±2.4	1.8±0.1	0.00	0.03	0.00	-
WE/SE	9.8±1.3	2.5±2.7	48.3±1.8	22.0±7.9	10.0±2.2	3.8±1.5	40.7±5.2	16.3±1.8	0.00	-	0.00	-
Lipid	53.5±6.5	46.1±28.0	90.5±7.6	91.3±9.4	38.2±10.5	14.0±0.3	56.3±8.8	22.2±2.6	0.00	0.00	0.00	-

may contain some diacylglycerol.

Abbreviations: LPC, lysophosphatidylcholine; SM, sphingomyelin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; UPL, unidentified polar lipids; C, cholesterol; FFAlc, free fatty alcohols; FFA, free fatty acids; UNL, unidentified neutral lipid; TAG, triacylglycerol; WE/SE, wax ester/sterol ester.

Values are significantly different ( $P<0.05$ ) with respect to temperature (temp), dietary fat level (fat), dietary fat source (oil) and interactions of these (T\*F\*O) as determined by three-way ANOVA.



651Table 4.

652Digestibility of fatty acids (FA), long-chain fatty alcohols (FAIc), total lipid, and total dry matter in Atlantic salmon fed four diets at two temperatures for 100 days.

Diet	HFFO		HFCO				LFFO		LFCO				ANOVA			
	Temp		3		12		3		3		12		Temp	Fat	Oil	T*F*O
	FA	FA	FA	FAIc	FA	FAIc	FA	FA	FA	FAIc	FA	FAIc				
14:0	92.4±0.2	94.3±3.6	68.0±1.1	93.8±1.0	83.0±3.0	95.7±1.4	91.2±2.7	97.5±0.6	71.3±3.7	89.8±3.2	91.6±0.3	98.2±0.3	0.01	-	0.02	-
16:0	90.4±0.3	92.8±4.4	82.2±1.3	88.3±1.8	85.7±2.1	94.1±2.0	89.0±3.4	96.4±0.9	81.6±3.2	85.1±3.4	92.5±0.2	97.7±0.3	0.00	0.05	0.00	-
<b>Total SFA</b>	<b>90.8±0.3</b>	<b>93.1±4.2</b>	<b>76.8±1.2</b>	<b>88.6±1.8</b>	<b>84.8±2.4</b>	<b>94.0±2.0</b>	<b>89.4±3.3</b>	<b>96.6±0.8</b>	<b>77.9±3.3</b>	<b>85.3±3.4</b>	<b>92.2±0.1</b>	<b>97.7±0.3</b>	<b>0.00</b>	-	<b>0.00</b>	-
16:1n-7	98.4±0.2	98.2±1.3	98.2±0.3	97.1±0.4	97.5±0.6	95.0±2.0	95.9±2.8	99.3±0.1	95.7±1.7	94.5±2.6	98.9±0.0	98.8±0.3	0.02	-	-	-
18:1n-9	97.9±0.2	97.7±1.6	97.0±0.3	96.4±1.1	96.9±0.6	91.5±3.0	94.9±3.1	99.0±0.3	93.9±2.1	93.5±3.1	98.3±0.0	98.3±0.5	0.05	-	0.03	-
<b>20:1n-9</b>	<b>96.3±0.3</b>	<b>96.5±2.3</b>	<b>96.4±0.6</b>	<b>74.4±2.6</b>	<b>90.0±2.3</b>	<b>80.4±4.8</b>	<b>94.0±3.0</b>	<b>98.7±0.4</b>	<b>93.9±1.9</b>	<b>74.1±3.8</b>	<b>97.7±0.2</b>	<b>93.7±0.6</b>	-	-	<b>0.00</b>	-
<b>22:1n-11</b>	<b>95.6±0.3</b>	<b>95.6±2.7</b>	<b>95.8±1.0</b>	<b>55.1±3.6</b>	<b>87.1±2.4</b>	<b>71.9±5.1</b>	<b>93.4±2.9</b>	<b>98.5±0.5</b>	<b>94.0±1.8</b>	<b>57.1±5.2</b>	<b>97.4±0.4</b>	<b>89.7±0.9</b>	-	-	<b>0.00</b>	-
<b>Total MUFA</b>	<b>96.5±0.2</b>	<b>96.5±2.2</b>	<b>96.6±0.6</b>	<b>69.0±2.6</b>	<b>92.4±1.4</b>	<b>78.2±4.5</b>	<b>94.0±3.0</b>	<b>98.7±0.4</b>	<b>94.1±1.9</b>	<b>70.2±4.1</b>	<b>97.9±0.2</b>	<b>92.7±0.7</b>	-	-	<b>0.01</b>	<b>0.01</b>
18:2n-6	97.8±0.4	98.0±1.3	98.2±0.6	97.4±0.8	97.1±0.5	93.1±2.5	94.4±3.8	98.9±0.2	95.1±1.9	94.9±2.4	98.6±0.1	98.7±0.4	0.04	-	-	-
20:4n-6	97.9±0.2	98.4±1.0	100.0±0.0		99.4±1.0		94.8±4.1	98.9±0.2	96.1±1.3		98.6±0.4		0.03	0.01	-	-
Total n-6 PUFA	97.9±0.3	98.1±1.2	98.5±0.5	97.4±0.8	97.3±0.5	93.1±2.5	94.7±3.6	98.9±0.2	95.5±1.8	94.9±2.4	98.6±0.1	98.7±0.4	0.05	-	-	-
18:3n-3	98.7±0.3	98.7±0.9	99.5±0.2	97.3±0.8	98.9±0.2	94.2±1.7	96.1±3.0	99.4±0.1	97.4±1.4	95.1±2.2	99.4±0.1	98.8±0.3	-	-	-	-
18:4n-3	99.3±0.2	99.5±0.3	99.8±0.1		99.7±0.0		97.2±2.6	99.7±0.1	98.2±1.1		99.8±0.0		0.02	-	-	-
<b>20:5n-3</b>	<b>99.2±0.2</b>	<b>99.4±0.4</b>	<b>99.7±0.2</b>		<b>99.6±0.1</b>		<b>96.7±3.0</b>	<b>99.5±0.1</b>	<b>97.9±1.1</b>		<b>99.6±0.1</b>		<b>0.03</b>	<b>0.04</b>	-	-
<b>22:6n-3</b>	<b>98.8±0.2</b>	<b>99.1±0.6</b>	<b>99.3±0.5</b>		<b>98.7±0.4</b>		<b>95.7±3.7</b>	<b>99.3±0.1</b>	<b>97.1±1.4</b>		<b>99.0±0.4</b>		<b>0.04</b>	-	-	-
Total n-3 PUFA	99.0±0.2	99.2±0.5	99.2±0.5	97.3±0.8	99.1±0.2	94.2±1.7	96.2±3.3	99.4±0.1	97.6±1.1	95.1±2.2	99.4±0.2	98.8±0.3	-	-	-	-
Total PUFA	98.8±0.2	99.0±0.6	99.1±0.5	97.3±0.8	98.9±0.2	93.7±2.0	95.9±3.3	99.3±0.1	97.2±1.2	95.0±2.3	99.3±0.1	98.8±0.3	-	-	-	-
<b>Total FA</b>	<b>95.9±0.2</b>	<b>96.5±2.2</b>	<b>92.1±0.6</b>	<b>73.3±2.3</b>	<b>93.2±1.0</b>	<b>81.3±4.0</b>	<b>93.6±3.2</b>	<b>98.4±0.4</b>	<b>90.8±1.9</b>	<b>73.8±3.9</b>	<b>96.9±0.1</b>	<b>93.8±0.6</b>	<b>0.02</b>	-	<b>0.00</b>	-
Total lipid	93.7±0.3	94.5±3.4	89.2±0.8		88.8±1.5		91.4±3.8	97.2±0.2	84.9±3.4		94.7±0.4		0.00	-	0.00	-
Dry matter	94.4±0.1	95.0±0.4	94.5±0.2		94.4±0.2		93.5±1.4	94.2±0.2	92.3±0.7		94.4±0.3		0.00	0.00	-	-

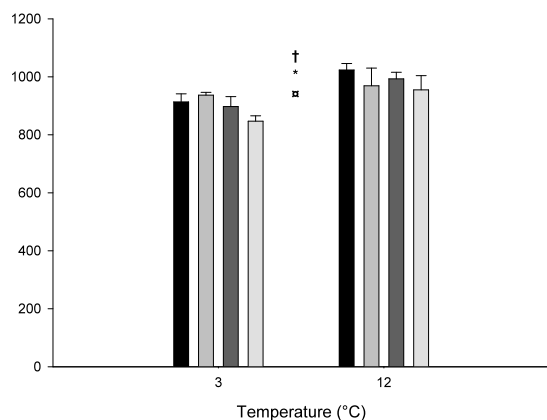
653Values are significantly different ( $P<0.05$ ) with respect to temperature (temp), dietary fat level (fat), dietary fat source (oil) and interactions of these (T\*F\*O) as determined by  
654three-way ANOVA.

## 655 Figures

656

(a)

(b)



657

658 Figure 1.

659 Growth performance data of Atlantic salmon fed four diets at two temperatures for 100 days. Means  $\pm$  S.D.  
 660 ( $n=3$ ). A) Final weight after 100 days feeding. B) Specific growth rate (SGR). Values are significantly different  
 661 ( $P<0.05$ ) with respect to dietary fat source (\*), dietary fat level (□) and temperature (†) as determined by  
 662 multivariate analysis.

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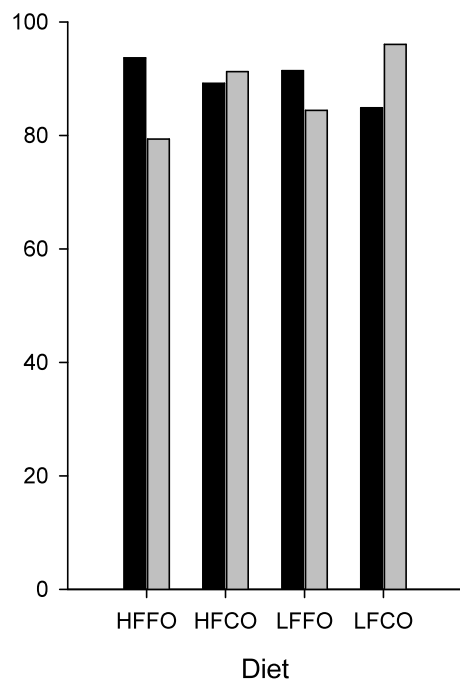
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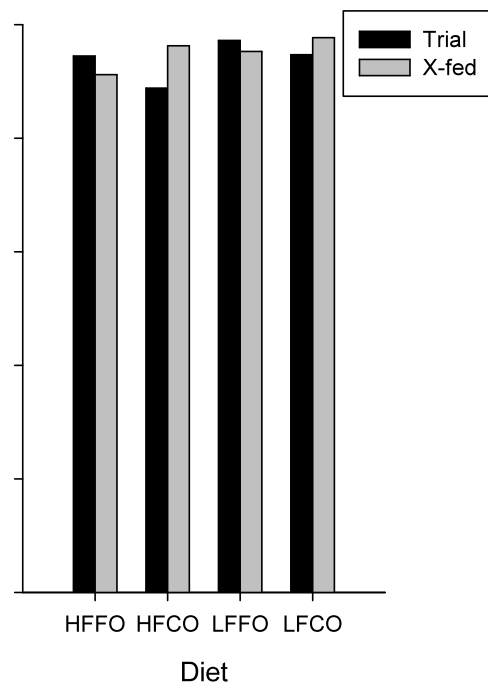
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(a)



(b)



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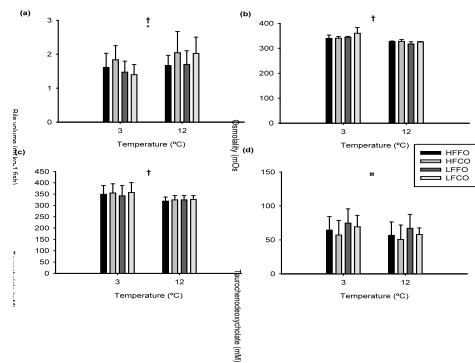
677Figure 2.

678Apparent digestibility coefficient (ADC) of total lipid in fish fed four diets at 3°C (a) and 12°C (b) for 100 days  
679(Trial) and cross-feeding where fish previous fed HFFO were now fed HFCO, HFCO group were now fed  
680HFFO, LFFO group were now fed LFCO and LFCO group were now fed LFFO at 3°C (a) and 12°C (b) for one  
681week (X-fed).

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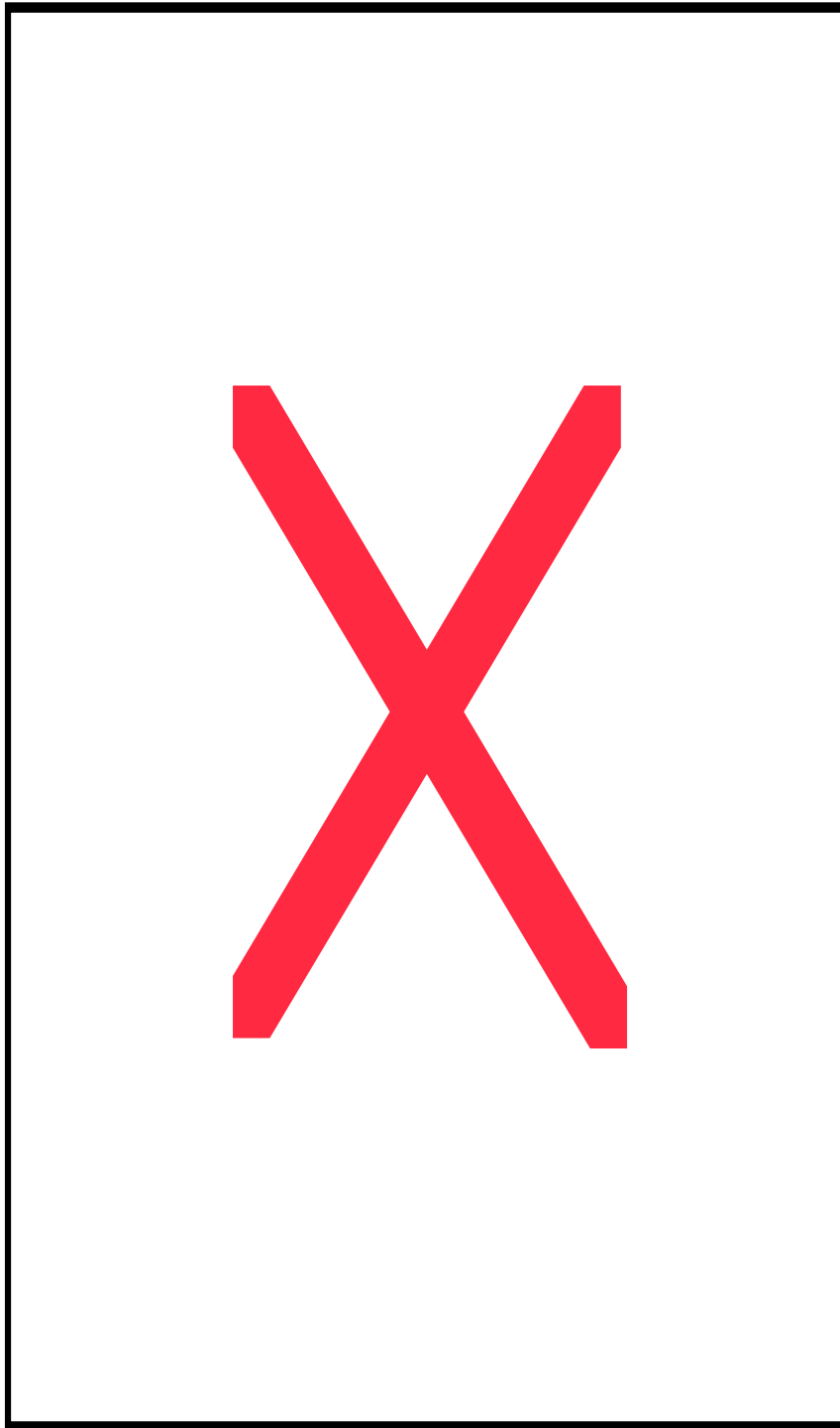
686

687Figure 3.

688Bile volume (a), osmolality (b), taurocholate concentration (c), and taurochenodeoxycholate concentration of  
 689Atlantic salmon fed four diets at two temperatures for 100 days. Means ±S.D. of 3 replicates (each from ten  
 690individuals for bile volume and each from three individuals for bile composition). Values are significantly  
 691different ( $P<0.05$ ) with respect to dietary fat source (\*), dietary fat level (□) and temperature (†) as determined  
 692by multivariate analysis.

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696Figure 4.

697Enzyme activity of a) triacylglycerol hydrolase (TAGH), b) sterol ester hydrolase (SEH) and c) wax ester  
698hydrolase (WEH) in desalted midgut extract from Atlantic salmon fed four diets at two temperatures for 100  
699days. Means  $\pm$  S.D. of 3 replicates (each from 3 individuals). Values are significantly different ( $P < 0.05$ ) with  
700respect to dietary fat level ( $\square$ ) and temperature ( $\dagger$ ) as determined by multivariate analysis.