The influence of temperature on the apparent lipid digestibility in Atlantic salmon (*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 ºC had a significantly higher bile volume than fish at 3 ºC 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.

1. Introduction

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

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

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

2. Materials and methods

2.1. Fish, diets and experimental design

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

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

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

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

2.2. Analysis of diets and faeces

Diets and faeces were freeze-dried to obtain dry weight, followed by analysis of yttrium oxide according to Otterå *et al*. (2003). Yttrium was determined in feed and faeces by use of an ICP-MS (inductive-coupled plasma – mass spectrometry) method after wet digestion in a microwave oven (Otterå *et al.*, 2003). Total lipid of diets and faeces was extracted with chloroform/methanol (2:1, v/v) according to Folch *et al.* (1957). Dilute HCl (3 M, 30% of original faecal weight) was added prior to the last extraction. The organic solvent phase was evaporated to dryness *in vacuo* at room temperature before resuspending the lipid residue in 10 mg mL^{-1} of chloroform/methanol (2:1, v/v) containing 0.05% (w/v) BHT. Extracted lipid was stored under N_2 at -80 ºC prior to further analysis.

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

To determine fatty acid and long-chain fatty alcohol composition of diets and faeces, extracted lipid was subjected to acid-catalysed transesterification using 1% (v/v) H_2SO_4 in methanol with 17:0 fatty acid and fatty alcohol added as internal standards. Resultant fatty

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

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

2.3. Analysis of bile and lipolytic activity

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

Bile osmolality was measured using a Fiske one-ten osmometer (Fiske Associates, MA, USA) with calibration and reference solutions (290 mOsm L^{-1}) from the manufacturer.

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

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

2.4. Calculations and statistical treatment

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

3. Results

3.1. Growth

The fish almost doubled their body weight at both temperatures. Fish at 3.1 ºC grew from 485±15 g to 899±40 g over 220 days, while fish at 12 ºC grew from 599±26 g to 985±45 in 67 days (Figure 1A). The fish thus had specific growth rates (SGR) of 0.28±0.02 and 0.74±0.06, respectively, at water temperatures of 3 ºC and 12 ºC (Figure 1B). The diets also had significant effects on growth. Fish fed diets added *Calanus* oil (HFCO and LFCO) had an overall lower SGR than those fed fish oil as the main lipid source (HFFO and LFFO) (Figure 1B). This was especially noticeable at 12 ºC for fish fed the low fat diet where SGR was significantly lower in the LFCO group (0.65±0.02) compared to the LFFO group (0.76±0.05). There was also a noticeable effect of fat level with growth generally higher in high fat groups compared to low fat groups (Figure 1B). There was thus an overall significantly effect of temperature and diet on growth.

3.2. Dietary lipid composition

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

The absolute levels of fatty acids (FA) in dietary lipid were 881 and 757 µg mg lipid^{-1}, respectively for the HFFO and the LFFO diets, compared to 508 and 499 µg mg lipid^{-1} in the HFCO and the LFCO diets. These latter diets contained additionally 344 and 310 µg mg lipid^{-1} of long-chain FAlc (Table 2). The FO dietary lipid was comprised predominantly of MUFA (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%).

3.3. Faecal lipid composition

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

3.4. Digestibility of FA and FAlc

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

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

3.5. Bile volume and composition

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

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

3.6. Lipase activity in midgut

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

4. Discussion

Fish kept at 3 °C in the present study had a 5 month longer feeding period, and required a total of over 7 months to double their weight compared to fish reared at 12 °C that took approximately 2 months. The specific growth was thus significantly lower in salmon kept at 3 °C than 12 °C. This is due to reduced feed intake and slower metabolism at low temperatures (Olsen and Ringø, 1997). It is generally assumed that decreases in temperatures will increase faecal passage time and thereby increase the digestive capacity (Fauconneau *et al.*, 1983; Olsen and Ringø, 1997). However, in the present experiment, fish at 3 °C, and in particular those fed CO diets, exhibited significantly reduced digestibility leading to increases in faecal WE and TAG. This shows that the compensation of increased passage time was not sufficient to ensure a high lipid digestibility, as shown for Arctic charr (*Salvelinus alpinus*) previously by Olsen and Ringø (1998). Higher temperature increases the general metabolism including higher enzyme activities (Chiu and Benitez, 1981), faster absorption (Stokes and Fromm, 1964) and increased enzyme affinity (Somero, 1969). The increased *in vitro* hydrolysis of TAG and WE at 12 °C compared to 3 °C in the present trial confirms this. This is most likely due to an increased amount of lipases in the midgut from fish kept at 12°C. The higher feed intake at 12 °C was dependent not only on higher enzyme activity, but also on better solubilisation and absorption in bile to utilize the ingested energy efficiently. The higher bile volume at 12 °C would therefore be necessary for high digestibility and to compensate increased feed intake to render more energy for growth. Fish kept at 12 °C had thus a significantly higher amount of hydrolysed lipid in the faeces and showed a higher lipid digestibility compared to fish at 3 °C.

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

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

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

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

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

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Tables

Table 1.
Formulation (g kg $^{-1}$ diet) and gross composition (%) of the Atlantic salmon experimental diet.

	HFFO	HFCO	LFFO	LFCO
Triple Nine fish meal[a]	417.0	417.0	575.0	575.0
Fish oil[b]	289.0	0.0	131.0	0.0
Calanus oil	0.0	289.0	0.0	131.0
Soyalecitin	5.0	5.0	5.0	5.0
Soya protein[c]	60.0	60.0	60.0	60.0
Wheat 230/05[d]	140.0	140.0	140.0	140.0
Wheat gluten 156/05[e]	80.0	80.0	80.0	80.0
Vitamin mixture[f]	10.0	10.0	10.0	10.0
Mineral mixture[g]	4.0	4.0	4.0	4.0
Charophyll Pink (10%)	0.3	0.3	0.3	0.3
Yttrium oxide (Y_2O_3)	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[h]	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

Feed codes are as follows: LF, low fat; HF, high fat; FO, fish oil; CO, *Calanus* oil.
[a] Fish meal: low fat, Tripple Nine, Denmark, (89,1% dry matter, 76,5% crude protein, 2,3% fat (soxhlet) and 13,1% ash).
[b] Fish oil: NorSalmOil, Norsildmel, Bergen, Norway
[c] Soya protein: Soya protein consentrate (SPC 70) , Sopropeche, Boulogne, France.
[d] Wheat: Norgesmøllene, Bergen, Norway
[e] Wheat gluten: Received from Ewos Innovation, Dirdal, Norway
[f] Diets supplied with following vitamins per kg diet: vitamin D3, 3000 I.E; vitamin E (Rovimix, 50%), 160 mg; thiamine, 20 mg; riboflavin, 30 mg; pyridoxine-HCl, 25 mg; vitamin C (Riboflavin Stay C 35%), 200 mg; calcium pantothenate, 60 mg; biotin, 1 mg; folic acid, 10 mg; niacin, 200 mg; vitamin B12, 0.05 mg; menadione bisulphite, 20 mg.
[g] Diets supplied with following minerals per kg diet: magnesium, 500 mg; potassium, 400 mg; zinc, 80 mg; iron, 50 mg; manganese, 10 mg; copper, 5 mg.
[h] May contain some diacylglycerol.

607Table 2.
608Total lipid content (ug mg^{-1}) 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[a]	FA[a]	FAlc[b]	FA[a]	FA[a]	FAlc[b]
14:0	6.4	11.9	1.2	5.9	11.1	1.2
16:0	**13.4**	**13.2**	**12.1**	**14.1**	**14.5**	**11.7**
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
18:1n-9	**9.0**	**6.3**	**3.9**	**9.6**	**7.5**	**3.8**
20:1n-9	**10.4**	**4.8**	**29.4**	**9.4**	**4.0**	**28.3**
22:1n-11	**15.2**	**7.1**	**37.3**	**13.7**	**6.2**	**36.3**
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	
20:5n-3	**7.1**	**9.9**		**7.0**	**8.9**	
22:6n-3	**11.4**	**10.0**		**11.7**	**10.0**	
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[a]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. [b]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^{-1} 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[a]	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±653	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	-

[a] 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.

651 Table 4.
652 Digestibility of fatty acids (FA), long-chain fatty alcohols (FAlc), 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		12		3	12	3		12					
	FA	FA	FA	FAlc	FA	FAlc	FA	FA	FA	FAlc	FA	FAlc	Temp	Fat	Oil	T*F*O
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	-
Total SFA	**90.8±0.3**	**93.1±4.2**	**76.8±1.2**	**88.6±1.8**	**84.8±2.4**	**94.0±2.0**	**89.4±3.3**	**96.6±0.8**	**77.9±3.3**	**85.3±3.4**	**92.2±0.1**	**97.7±0.3**	**0.00**	**-**	**0.00**	**-**
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	-
20:1n-9	**96.3±0.3**	**96.5±2.3**	**96.4±0.6**	**74.4±2.6**	**90.0±2.3**	**80.4±4.8**	**94.0±3.0**	**98.7±0.4**	**93.9±1.9**	**74.1±3.8**	**97.7±0.2**	**93.7±0.6**	**-**	**-**	**0.00**	**-**
22:1n-11	**95.6±0.3**	**95.6±2.7**	**95.8±1.0**	**55.1±3.6**	**87.1±2.4**	**71.9±5.1**	**93.4±2.9**	**98.5±0.5**	**94.0±1.8**	**57.1±5.2**	**97.4±0.4**	**89.7±0.9**	**-**	**-**	**0.00**	**-**
Total MUFA	**96.5±0.2**	**96.5±2.2**	**96.6±0.6**	**69.0±2.6**	**92.4±1.4**	**78.2±4.5**	**94.0±3.0**	**98.7±0.4**	**94.1±1.9**	**70.2±4.1**	**97.9±0.2**	**92.7±0.7**	**-**	**-**	**0.01**	**0.01**	**-**
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	-	-	-
20:5n-3	**99.2±0.2**	**99.4±0.4**	**99.7±0.2**		**99.6±0.1**		**96.7±3.0**	**99.5±0.1**	**97.9±1.1**		**99.6±0.1**		**0.03**	**0.04**	**-**	**-**
22:6n-3	**98.8±0.2**	**99.1±0.6**	**99.3±0.5**		**98.7±0.4**		**95.7±3.7**	**99.3±0.1**	**97.1±1.4**		**99.0±0.4**		**0.04**	**-**	**-**	**-**
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	-	-	-	-
Total FA	**95.9±0.2**	**96.5±2.2**	**92.1±0.6**	**73.3±2.3**	**93.2±1.0**	**81.3±4.0**	**93.6±3.2**	**98.4±0.4**	**90.8±1.9**	**73.8±3.9**	**96.9±0.1**	**93.8±0.6**	**0.02**	**-**	**0.00**	**-**
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	-	-

653 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
654 three-way ANOVA.

Figures

(a) (b)

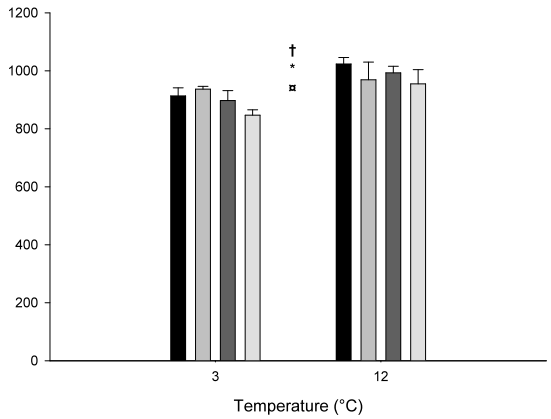
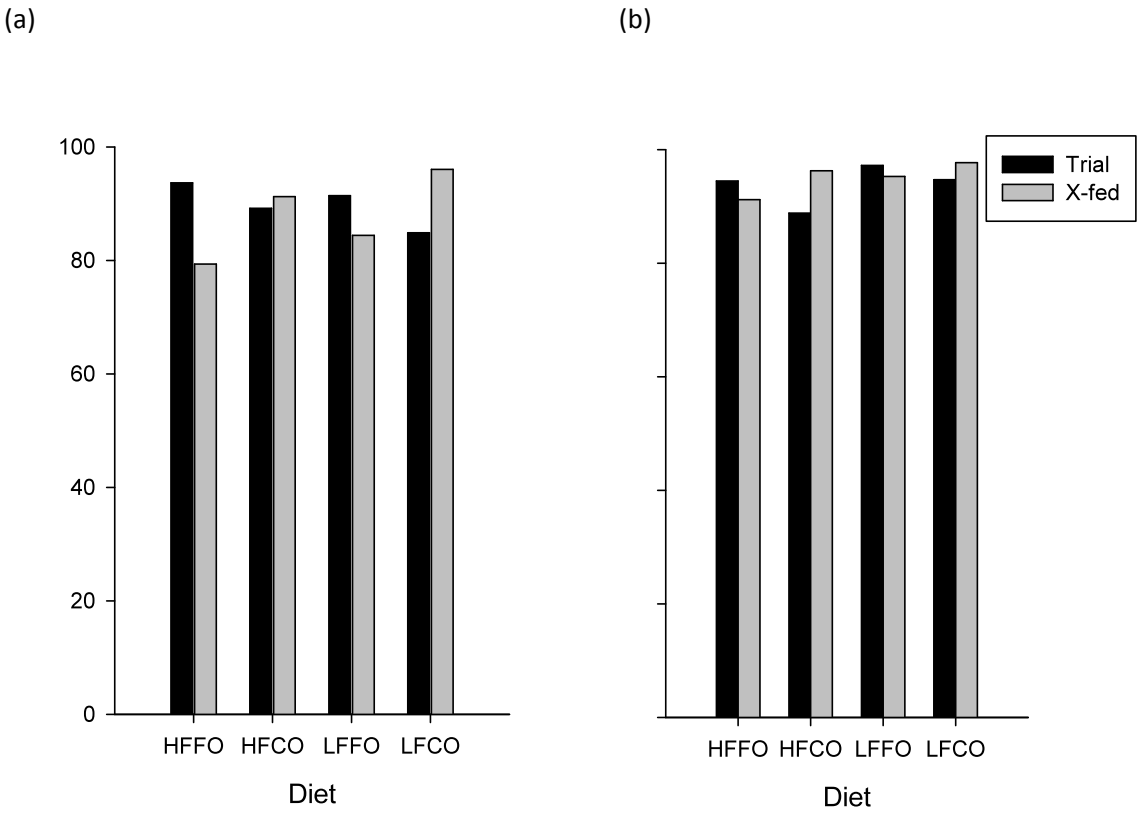


Figure 1.

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



676

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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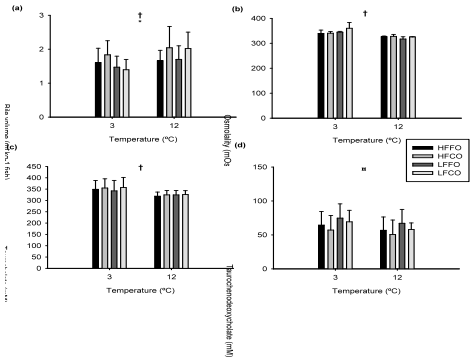

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686

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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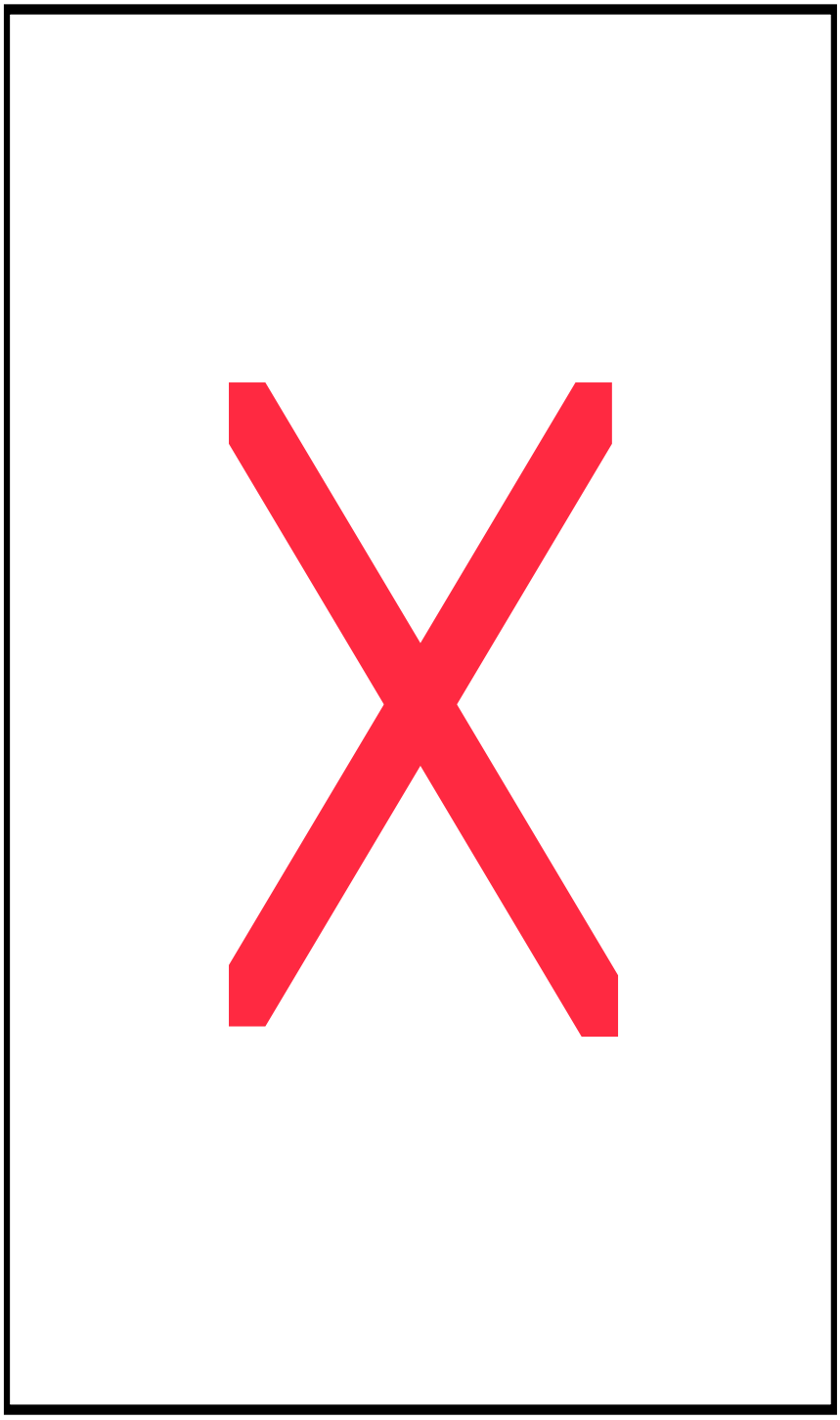

695

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 ±S.D. of 3 replicates (each from 3 individuals). Values are significantly different (P<0.05) with
700respect to dietary fat level (¤) and temperature (†) as determined by multivariate analysis.