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Lipid and fatty acid digestibility in *Calanus* copepod and krill oil by Atlantic halibut (*Hippoglossus Hippoglossus* L.)

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1.0 ABSTRACT

Marine zooplankton represent a significant biomass of marine lipid that could supply lipid in diets for farmed marine fish. Digestibility of lipid and fatty acids of the copepod, *Calanus finmarchicus* and Antarctic krill, *Euphausia superba* by farmed juvenile Atlantic halibut (*Hippoglossus hippoglossus*) was investigated. Halibut were fed diets containing one of the following test oils at 15% inclusion level: fish oil (FO), *Calanus* copepod oil (CO) and *Euphausia* krill oil (KO). KO contained the highest level of saturates (SAT; 39%) and monounsaturates (MUFA; 38%), and was low in polyunsaturated fatty acids (PUFA; 24%) compared to CO (50%) and FO (43%). CO and FO contained lower levels of SAT (31% and 33%, respectively) and MUFA (19% and 24%, respectively). Lipid digestibility of the CO diet (81%) was significantly lower than that of KO (90%) and FO (93%) diets ($P<0.05$), likely due to wax esters in CO. Digestibility of SAT in the CO diet (70%) was significantly lower than FO (75%) and KO (77%) and MUFA in CO (84%) was significantly lower than KO (93%) and FO (93%). Digestibility of PUFA was significantly higher in FO (97%) than CO (94%) and KO (95%). Generally the CO diet was significantly less digestible than FO and KO diets.

*Keywords: *Calanus finmarchicus*, *Euphausia superba*, digestibility, Atlantic halibut

2.0 INTRODUCTION

Farmed Atlantic halibut (*Hippoglossus hippoglossus* L.) has become a key species in aquaculture and its demand as a high-quality seafood item is expected to increase in the future. Traditionally farmed halibut are fed dietary lipid of marine origin with high levels of n-3 fatty acids in order to achieve proper growth and maintain fish health. The n-3 fatty acids are also a benefit to human health as they have been shown to protect against heart disease (Drevon, 1992; Imazio et al., 2003; Holub & Holub, 2004) and are important in brain and eye development (Kris-Etherton et al., 2002; Mozaffarian & Rimm, 2006; Santerre, 2010). The world supply of fish oils however, is rapidly declining (FAO, 2009) and there is growing concern about the health of global fisheries stocks (Naylor et al., 2000). The demand for fish oil is expected to reach 40 million tonnes by 2030 (Tacon, 2004), therefore it is imperative to investigate alternative lipid sources for use in aquaculture.

A significant amount of research in this area has focused on the partial replacement of fish oil with terrestrial vegetable oils or animal fat. Most marine fish such as halibut require polyunsaturated fatty acids (PUFA) for cellular function. These must be provided in their diet because marine fish cannot synthesize them *de novo* due to limited activity of Δ5- and Δ6- desaturases (Owen et al., 1975; Cowey et al., 1976; Sargent et al., 2002). Both plant and animal sources may be used as an energy supply for marine fish, but plant oils are high in n-6 and n-9 fatty acids and animal fat is high in saturated fatty acids. In humans, vegetable oils and terrestrial animal fats have been linked to many metabolic disorders including heart disease, inflammatory disease and

cancer (Simopoulos, 2002). Neither of these vegetable oils or animal fat contain long chain n-3 PUFA which are significantly present in fish oils. Therefore, it is vital for the aquaculture industry to find new lipid resources that contain high amounts of n-3 PUFA for marine fish in aquaculture.

Under-utilized marine resources high in n-3 PUFA are ideal an source of lipid for use in marine fish diets. Low trophic marine zooplankton represent a significant harvestable biomass and have additional nutritional benefits over traditional fish meal. Marine zooplankton typically contain high levels of PUFA, including EPA and DHA. Since these organisms are not used directly in either human food or animal feeds, untapped zooplankton resources could contribute a significant quantity of marine lipids for human use and animal production. Lower trophic level aquatic organisms also have lower concentrations of lipophilic organic contaminants in particular those known to be persistent organic pollutants such as polychlorinated dibenzodioxins (PCDD) and polychlorinated dibenzofurans (PCDFs) and non-*ortho* and mono-*ortho* polychlorinated biphenyls (PCBs). Such organic contaminants are known to accumulate in both wild and cultured fish to levels considered undesirable for human consumption (Hites et al., 2004; Farrell et al., 2010).

As an unutilized resource by the fishing industry, these low trophic organisms are highly abundant, highly regenerative and available for harvest. In particular two abundant species of zooplankton are the Calanoid copepod, *Calanus finmarchicus* and Antarctic krill, *Euphausia* superba. The *Calanus* copepod is the most abundant herbivore in the Nordic seas, with an annual production of several hundred million

tonnes (Speirs et al., 2006). Copepods are viewed as a desirable lipid source as they have higher levels of lipid during part of the season (Falk-Petersen et al., 2009), however they predominantly store lipid as wax esters rather than the typical triacylglycerol (TAG) (Sargent et al., 1976; 1978). Due to the hydrophobic nature of wax esters, this lipid class is poorly digested in most mammals and the fatty alcohols have been found to accumulate in the intestine (Place, 1992). In the marine environment, copepods serve as the principal food for many fish species, including herring, salmon, and halibut (Place, 1992), and therefore these fish species have the ability to hydrolyze wax esters (Patton & Benson, 1975). It appears that salmonids such as Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) can effectively utilize and digest wax esters, but with a lower rate of hydrolysis than for TAG (Patton et al., 1975; Olsen et al., 2004; Bogevik et al., 2009). Studies have not yet determined the digestive efficiency of halibut fed wax ester-rich copepod lipid.

Antarctic krill is the most abundant zooplankton species in the Southern Ocean with a standing biomass of 44 million tonnes (Hewitt et al., 2002). Antarctic krill lipid is high in phospholipid, and particularily high in n-3 PUFA like eicosapentaenoic acid (EPA) and does not contain wax esters (Storebakken, 1988; Ju & Harvey, 2004). Whole krill meal has previously been investigated as a protein source in diets for marine fish because of its high protein content and desirable feed attractant properties (Storebakken, 1988; Karlsen, 2006; Olsen et al., 2006; Suontama et al., 2007; Tibbetts et al., 2010).

Their nutritional values as a potential lipid source in diets for marine fish, particularly its digestion and absorption by Atlantic halibut have yet to be investigated.

Lipid digestibility is an important measure to evaluate the nutritional value of any new lipid source for farmed fish. Determination of the digestibility coefficients can show the absorption of total lipid and fatty acids from these zooplankton sources. The main objectives of the present study were: (1) to determine the digestibility coefficients of total lipid and fatty acids of the copepod, *Calanus finmarchicus* and the Antarctic krill, *Euphausia superba*; (2) to determine digestibility coefficients of fatty alcohols from the wax esters of *Calanus finmarchicus* and (3) to establish suitability of these dietary oils as lipid supplements for use in commercial feed for Atlantic halibut.

3.0 MATERIALS AND METHODS

3.1 TEST INGREDIENTS

Calanus finmarchicus oil was supplied from the Institute of Marine Research (IMR), Bergen, Norway in April 2009. Copepods were caught in four trawls in Andfjorden, Norway during the first week of June 2006. *Calanus* oil was produced in September 2006 (NOFIMA; Bergen, Norway) by separation of the thawed copepod biomass on a tricanter at 25°C, which yielded 93% of total lipid. The press fluid was heated to 90°C and re-separated to produce the final oil product. Wax esters constituted 80% of total lipid. *Euphausia superba* krill oil was supplied from Aqion (Colorado Springs, CO USA). Both *Calanus* and krill oils were stored at -20°C under nitrogen

upon arrival at the National Research Council, Institute for Marine Biosciences, Halifax, Nova Scotia.

3.2 EXPERIMENTAL DIET

Experimental diets were formulated using a mixture of practical and purified ingredients (Table 1). A basal mixture was initially produced using casein, corn gluten meal and fish meal as the main sources of protein. Three experimental diets were formulated to contain one of the following test oils at 15% inclusion level: fish oil (FO), *Calanus* copepod oil (CO) and *Euphausia* krill oil (KO). Both chromic oxide and cholestane were used as inert digestion markers for the determination of apparent digestibility coefficients of total lipid and fatty acid digestibility. Vitamin and mineral pre-mixes were finely ground with wheat as a base and thoroughly mixed in a twin-shell blender (Paterson-Kelly, East Stroudsburg, PA, USA). Ingredients were mixed in a Hobart mixer (Model H600T, Rapids Machinery Co., Troy, OH, USA) and steam pelleted into 4.0 mm pellets (California Pellet Mill Co., San Francisco, CA, USA). The pellets were dried in a forced-air drier at 80°C for 50 min and screened to remove fine particles. All diets were stored at -20°C in air tight plastic bags.

3.3 EXPERIMENTAL FISH

Three hundred and eighty Atlantic halibut (*Hippoglossus hippoglossus*) were obtained from Scotian Halibut Ltd (Clark's Harbour, Nova Scotia, Canada) and were held in a single circular fiberglass tank 2000 L capacity at the National Research Council, Institute for Marine Biosciences, Marine Research Station in Sandy Cove, Nova Scotia, Canada. The tanks were supplied with 12°C filtered (60-80μm) seawater

(salinity, 28-30 ppt) at a flow rate of 2.5 L min-1. The fish were acclimatized to the tanks (described below) for 6 days prior introduction of the experimental diets and fed Corey Aqua Sea (salmonid feed) 3.0 and 5.0 mm pellet (Corey Feed Mills, Fredericton, NB, Canada) twice daily at 0900 and 1530. The proximate composition of the experimental diets is reported in Table 1.

3.4 DIGESTIBILITY SYSTEM AND FECAL COLLECTION

Fifteen fish (95.5±1.0 g fish-1) were randomly distributed into each of 9 digestibility tanks (120 L capacity). Each digestibility tank was equipped with a fecal collection column, which was a modification of the Guelph system (Cho et al., 1982) in order to increase the rate and quantity of fecal recovery. Filtered (60-80 μm) seawater (salinity 28-30 ppt) was supplied to each tank at a flow rate of 3 L min-1 in a flow-through system and continuously aerated (8.6 mg L-1 dissolved oxygen, 91% gas saturation) at 12°C. Each of the three experimental diets was provided to three separate tanks in order to achieve triplicate replication. Fish were fed twice daily to satiation at 0900 and 1530 for 26 days including the acclimation period. Each day after the second feeding, the tanks and collections columns were thoroughly scrubbed and columns rinsed with warm fresh water to remove any residual particulate matter such as feces and uneaten feed. The following morning prior to feeding, fecal matter which settled overnight in the column was collected in 250 mL plastic bottles for each tank and centrifuged (4000 rpm; 2750 x g) for 20 min at 4°C (Centra CL3R, Thermo IEC). The supernatant was decanted and discarded and the weight of the fecal sample was recorded. This procedure was repeated until approximately 12 g of fecal matter was

collected for the first period. It was then repeated a second time to obtain an additional 12 g of fecal matter for the second period. All fecal matter was collected over a period of 20 days. Fecal samples were stored under nitrogen at -20°C to prevent lipid oxidation for the duration of the collection period. Fecal matter was lyophilized, finely ground and stored at -20°C until further analyses.

3.5 ANALYSES

The proximate composition of test ingredients, diets and lyophilized fecal samples were performed as follows. Moisture content was determined by weight loss using a convection oven (Precision Model STM 80) at 110°C for 18 hours; ash was determined by incineration in a muffle furnace at 550°C for 18 hours. Crude protein (% nitrogen x 6.25) was analyzed by the Dumas method (Ebeling, 1968) using the LECO FP-528 Nitrogen Analyzer (Model FP-528, Leco Corporation, St. Joseph, MI, USA). Gross energy was determined using the Parr Isoperibol Oxygen Bomb Calorimeter (model 6200, Parr Instrument Company, Moline, IL, USA). Lipids were extracted using a modification of the Folch method (Folch et al., 1957), and taken to dryness under a stream of nitrogen and constant weight in an SPD Savant SpeedVac (Model PD131DDA). Lipid extracts were stored under nitrogen at -80°C until subsequently analyzed.

To determine the fatty acid composition of lipid, fatty acid methyl esters (FAMES) were prepared by transesterification using 7% boron trifluoride in methanol (Ackman, 1989). The resulting FAMEs from samples containing *Calanus* oil were isolated using thin layer chromatography (TLC) on 5 x 2.5 cm HPTLC plates coated

with silica gel 60 F_{254} (EMD Chemicals Inc. Gibbstown, NJ, USA) using hexane/ethyl ether/ glacial acetic acid (80:20:1 v/v/v). The FAME and cholestane bands were scraped from the TLC plate and recovered from the adsorbent by elution with hexane:chloroform (1:1 v/v) and evaporated under nitrogen.

To determine the fatty alcohol composition from wax esters in *Calanus* oil, the resulting alcohols from wax ester transesterification were separated using thin layer chromatography (TLC) on 5 x 2.5 cm HPTLC plates coated with silica gel 60 F_{254} (EMD Chemicals Inc. Gibbstown, NJ, USA) using hexane/ethyl ether/ glacial acetic acid (70:30:1 v/v/v). The alcohol band was scraped and isolated alcohols were acetylated using acetic anhydride-pyridine (3:6 v/v) (Farquhar, 1962) to form acetate derivatives.

All FAMES and acetates were separated using gas chromatography equipped with a flame-ionization detector (Agilent 6890 GC system, Wilmington, DE, USA) on an Omegawax 250 capillary column (30 m x 0.25 mm x 0.25 μm; Supelco, Bellefonte, PA, USA). FAMEs were identified by comparison of retention times with those of known standards (Supelco 37 and PUFA-3 menhaden oil, Supelco, Bellefonte, PA, USA) and acetates were identified by comparison of retention times of known acetate standards (Nu-Chek Prep, Inc, Elysian, MN, USA).

To determine fatty acid and fatty alcohol absorption, cholestane was used as a digestibility marker. The chromatogram peak areas of cholestane, fatty acids and alcohols in the feed were compared directly to those of cholestane, fatty acids and

alcohols in the feces. Digestibility was calculated using the equation (Sigurgisladottir et al., 1992):

$$D(\%) = 100 - \frac{\text{(area cholestane-feed)}}{\text{(area cholestane-feces)}} \times \frac{\text{(area fatty acid-feces)}}{\text{(area fatty acid-feed)}} \times 100$$

Individual fatty acids were grouped according to chain length in order to summarize the total saturates, monounsaturates and polyunsaturates. Certain fatty acids reported in the results excluded unknowns and fatty acids of insignificant amounts. The summation of saturates, monounsaturates and polyunsaturates reported in the results included all fatty acids that were identified on the chromatogram. To calculate the digestibility of saturates, monounsaturates and polyunsaturates, the total aggregate value was used in the digestibility equation instead of individual fatty acid values.

3.6 STATISTICAL ANALYSIS

Statistical analysis followed methods outlined by Zar (1996). Digestibility coefficients were calculated from the average of three replicate tanks receiving each experimental diet, for the two collection periods for each tank, creating a sample size of n=6. Statistical analyses were performed using a one-way analysis of variance, ANOVA (Minitab 15 Statistical Software) with 95% confidence to detect significant differences of digestibility coefficients between collection periods. A one-way ANOVA was also used to detect significant differences between digestibility coefficients for total lipid and individual fatty acids in dietary groups. A pair-wise

comparisons test (Tukey's HSD) was performed when significant differences among diets were found.

4. 0 RESULTS

The proximate composition of the experimental diets (Table 1) showed crude protein, lipid and energy levels were consistent at 45%, 15% and 22 MJ/kg, respectively. The fatty acid composition of the dietary oils (Table 2) reflected that of the diets (Table 3). Krill oil (KO) had the highest level of saturated fatty acids (SAT) (39%) and monounsaturated fatty acids (MUFA) (38%) of the dietary oils, and was particularly high in 14:0, 16:0 and 18:1. The PUFA content of copepod oil (CO) and fish oil (FO) were 50 and 43% respectively and nearly twice as high as that of KO (24%). The proportion of SAT, MUFA and PUFA in the dietary oils varied. CO had the highest levels of n-3 fatty acids (46%), and was particularly high in 18:4n-3 (18%) compared to FO, although this excludes the fatty alcohol fraction of the total lipid in CO. KO had one third less the amount of n-3 fatty acids compared to CO (15%). CO and FO had comparable levels of highly unsaturated fatty acids, however FO showed a higher level of eicosapentaenoic acid (EPA; 20:5n-3) (16.5%) compared to CO (11.9%). The EPA content of KO (6.4%) was less than half that of FO and CO, and docosahexaenoic acid (DHA; 22:6n-3) (2.3%) was less than a quarter compared to FO (11%) and CO (10%). All three experimental oils contained low amounts of arachidonic acid (AA, 20:4n-6) (<1%) resulting in low accumulation of the n-6 series, ranging from 2-3%.

The fatty acid composition of experimental diets and feces is presented in Table 3. An increase of a particular fatty acid in fecal lipid compared to that of the diet

indicated poor digestion. In the fecal lipid of all groups, there was a notable increase in the proportion of SAT and a reduction of PUFA compared to the dietary lipid. Saturated fatty acids, 14:0, 16:0 and 18:0 tended to increase in fecal lipid compared to dietary lipid. Lower levels of a fatty acid in fecal lipid compared to the corresponding dietary lipid, as exhibited in the PUFA group, indicated digestion of a particular fatty acid.

Digestibility coefficients were obtained for total lipid and fatty acids using cholestane as an indigestible marker (Table 4). Total lipid digestibility of the CO diet (81%) was significantly lower than that of KO (90%) and FO (93%) diets ($P<0.05$). Digestibility of SAT was lower than MUFA and PUFA for all diets, and digestibility of SAT in the CO diet (70%) was significantly lower than FO (75%) and KO (77%). KO exhibited the highest level of SAT in the dietary oils and correspondingly for the diets more SAT was digested than in FO and CO. Digestibility of SAT was found to decrease with increasing chain length. For example, digestibility of 18:0 was the least digested of all fatty acids in all diets, and was significantly lower in the FO diet (66%). MUFA was better digested than SAT by fish in all dietary groups (84-93%). Digestibility of MUFA in the CO diet was significantly lower than FO and KO diets. Apparent digestibility coefficient of 22:1n-11 in particular was notably lower in the CO diet (74%) than FO and KO. PUFA digestibility was over 94% for all diets, and was significantly higher in FO (97%). Total n-3 and n-6 fatty acids were highly digestible (>91%), but digestibility of n-3 fatty acids was higher (>95%) than total n-6 fatty acids. The n-3 fatty acids in the FO diet were significantly more digested than in the CO and KO diets. Similarly, digestibility of the n-6 series was significantly higher in FO (94%) than CO (91%).

Highly unsaturated fatty acids, such as AA, DHA and EPA in all diets showed high digestibility values. EPA was >99% digestible in the FO diet and it was significantly more digested than in CO and KO diets. No significant differences in the digestibility of fatty acids were detected between the two collection periods in all the experimental diets.

The fatty alcohols composition of *Calanus* oil, *Calanus* oil diets and feces is presented in Table 5. *Calanus* oil was high in the fatty alcohols 16:0 (14%), 18:3 (9%), 20:1 (25%), 22:1 (21%) and 24:1 (14%). Similarly, the *Calanus* oil diet was high in fatty alcohols 16:0 (11%), 18:3 (9%), 20:1 (28%), 22:1 (30%), and 24:1 (10%). Fatty alcohol digestibility ranged 77% to 96%. Digestibility of 22:1 was the lowest of the reported fatty alcohols (77%) whereas 16:1 was the most digestible (96%).

5.0 DISCUSSION

Digestibility of total lipid and fatty acids was significantly affected by dietary lipid source. Fish, krill and copepod oils have markedly different lipid compositions, including different lipid classes and varying proportions of saturated and unsaturated fatty acids, which significantly affected lipid digestion and utilization. Fish oil, composed mainly of triacylglycerol, is easily hydrolyzed and well digested, while krill oil is largely made up of phospholipids (Fricke et al., 1984), which may be even more easily digested (Shields et al., 1999). For example, early lipid digestibility studies with Atlantic halibut fed diets containing fish oil showed that total lipid digestibility of diets ranged between 78-94% (Berge & Storebakken, 1991). In the present study, total lipid

digestibility was higher for fish oil than the zooplankton oils; however digestibility of all the experimental diets were within the same range of values reported earlier for halibut (Berge & Storebakken, 1991). Lipid digestibility of *Calanus* oil was significantly lower than fish oil and krill oil. Although lipid digestibility values higher than 80% are considered relatively high, lower values for the digestion of *Calanus* lipid appears to be affected in some way by lipid class composition. Calanoid copepods primarily store lipid as wax esters composed of a fatty acid esterified to a long-chain alcohol (Sargent, 1978). This neutral lipid component is known to be poorly digested due to its hydrophobic nature (Place, 1992). However salmonids are able to feed on several species of crustaceans such as copepods that contain high proportions of wax ester in their storage lipids, thus some capacity to digest and utilize these lipids must exist in salmonid fishes (Place, 1992; Olsen et al., 2004; 2010). Previous studies that have measured lipid digestibility of *Calanus* oil fed to species such as Atlantic salmon (*Salmo salar)* and rainbow trout (*Onchorhyncus mykiss*) showed no adverse effects to growth and digestion of lipid (Patton & Benson, 1975; Patton et al., 1975; Olsen et al., 2004; Oxley et al., 2005). Similar to the findings of this study, Bogevik et al. (2009) also reported a lipid digestibility of 84% when Atlantic salmon were fed diets containing *Calanus finmarchicus* oil, which was significantly lower than diets containing fish oil. In addition, Lie & Lambertsen (1991) observed up to 6% lipid remained in the gut of Atlantic cod (*Gadus morhua)* fed a wax ester diet compared to less than 2% lipid detected in the gut of cod fed diet containing triacylglycerol. This indicates that wax esters are poorer substrates than triacylglycerol for hydrolysis by

lipases in the digestive tract of cod. It has been suggested that digestion of *Calanus* copepod oil is somewhat limited due to lower hydrolytic activity in the gut in the presence of wax esters compared to triacylglycerol (Patton et al., 1975; Olsen & Ringø, 1997; Bogevik et al., 2008a; Bogevik et al., 2009). In contrast, Fricke et al. (1984) reported that Antarctic krill oil and fish oil contained only negligible amounts of wax esters (Fricke et al., 1984) which may explain the significantly higher total lipid digestibility values of krill and fish oil compared to *Calanus* oil found in the present study.

Marine oils are known to be high in PUFA, particularly in the n-3 series. The proportion of SAT, MUFA and PUFA varied between dietary oils tested in this study, and thus digestion of these fatty acids also significantly differed between dietary groups. *Calanus* oil contained the highest proportion of PUFA (50%) and n-3 fatty acids (46%) and the lowest proportion of SAT and MUFA. However these proportions were determined from fatty acids of the total lipid, and does not include fatty alcohol which can account for 40% of the lipid when wax esters constitute 80% of the total lipid. Therefore the contribution of PUFA and n-3 fatty acids is likely a fraction of this since the total lipid contains only a portion of fatty acids. Although krill oil is considered as an excellent source of PUFA and n-3 fatty acids by the pharmaceutical industry (Sampalis et al., 2003; Deutsch, 2007), it contains only about half the amount of PUFA and n-3 fatty acids compared to *Calanus* oil and fish oil. About 60% of krill lipid was unsaturated; however this is only about 10% lower than other reported values (Shibata, 1983; Suzuki & Nobukazu, 1990). The lipid composition data from this study falls

within ranges reported by Hertrampf & Piedad-Pascual (2000), where high variability in SAT (20-42%), MUFA (35-50%), and PUFA (18-39%) of krill oil existed. EPA and DHA were present in similar amounts in fish oil (17% EPA; 11% DHA) and *Calanus* oil (12% EPA; 10% DHA), but were very low in krill oil (6% EPA; 2% DHA). These findings do not agree with results reported by Bustos et al. (2002) of 21% EPA and 20% DHA in KO. These results also do not agree with concentration of EPA and DHA reported by Deutsh (2007) who found only 10% DHA in NKO^{TM} oil tablets from Antarctic krill. It is well known that both the amount and composition of krill lipid may show wide variations (Storebakken, 1988; Shibata, 1983). This high variability observed in various reports may be associated with seasonal variation caused by a combination of environmental and physiological factors that influence the composition of natural food organisms, *de novo* synthesis, and metabolic demands; for example during starvation there may be a selective mobilization of PUFA (Clarke, 1980). Marine invertebrates may synthesize *de novo* only saturated and monoenoic acids (Sargent, 1976; Holland, 1978). In addition to environmental and physiological factors, the level of PUFA will depend on the method of oil extraction. Increased levels of PUFA in the oil is usually attributed to high levels of hydrolysis to form free fatty acids (FFA), mainly from phospholipid. Also, since krill TAG is very low in PUFA in adult animals and high in phospholipid, solvent extraction where the phospholipid is also extracted may show variable results (Olsen et al., 2010). Despite lower PUFA concentration, krill oil contains a sufficient amount of essential fatty acids for most marine fish species including halibut, which require at least 2% EPA and DHA (NRC, 1993). Among

PUFA's, higher amount of 18:4n-3 (18%) was detected in *Calanus* oil as compared with either fish oil (2%) or krill oil (4%). This has also been reported by other investigators (Lie & Lambertsen, 1991; Olsen et al., 2004; Oxley et al., 2005; Bogevik et al., 2009). SAT and MUFA proportions also varied between oils. Krill oil was higher in 14:0, 16:0, 16:1n-7, and 18:1n-9 fatty acids than *Calanus* and fish oils.

Digestibility of fatty acids tended to decrease with increasing chain length and increase with the degree of unsaturation of fatty acids as reported previously (Austreng et al., 1979; Lie & Lambertsen, 1985; Sigurgisladottir et al., 1992; Olsen & Ringø, 1997; Olsen et al., 1998; Olsen et al., 2004; Martins et al., 2009). Lipases in fish lumen are known to have high specificity toward PUFA, followed by MUFA and SAT (Patton et al., 1975; Lie & Lambertsen, 1985; Bogevik et al., 2008b). Digestibility is also linked to the melting point of the fatty acid because absorption increases with lower melting points and *vice versa* (Olsen & Ringø, 1997; Olsen et al., 1998). This may explain why digestibility of SAT was lowest compared to MUFA and PUFA for all three dietary groups in this study. Digestibility of SAT in krill oil was significantly higher than *Calanus* oil. As mentioned earlier, low digestibility of *Calanus* oil may be due to high levels of wax esters. On the other hand, SAT in krill oil may be significantly better digested due to high concentrations of phospholipids, which may be more digestible on due to their polarity and natural hydrophilic properties (Shields et al., 1999). Saturates with high melting points may be efficiently absorbed when their polarity is high (Olsen et al., 1998) due to the enhancement of passing from an oil phase to a micellar phase in

the lumen (Carlier et al., 1991). Among the saturated fatty acids, digestibility of 18:0 was the lowest of all fatty acids. This agrees with previous studies that reported low absorption of 18:0 in various fish species (Austreng et al., 1979; Ringø, 1991; Sigurgisladottir et al., 1992; Olsen et al. 1998; Torstensen et al., 2000; Martins et al., 2009). As expected, saturated fatty acids 14:0 and 16:0 were also poorly digested because these saturates are known to be more resistant to lipolysis (Lie & Lambertsen, 1985).

Among the MUFA fatty acids, digestibility of *Calanus* oil was consistently and significantly lower than the fish and krill oil groups. This observation is consistent with other wax ester digestibility studies that showed significantly lower digestibility of MUFA due to high levels of wax esters in *Calanus* oil (Bogevik et al., 2009). This is likely due to a lower efficiency of digestive lipases towards wax esters which causes reduced lipid absorption by fish. The slow hydrolysis of wax esters as compared to triacylglycerol has also been observed in other fish species such as anchovy (*Engraulis mordax*), rainbow trout and cod (Patton et al., 1975; Tocher & Sargent, 1984; Lie & Lambertsen, 1985). Digestibility of 22:1n-11 was significantly different among all dietary oils, showing a digestibility of fish oil > krill oil > *Calanus* oil. A relatively poor digestion of 22:1n-11 alcohol of *Calanus* wax esters has also been reported, however the results may vary for the fatty acid moiety ranging between 69 to 94% (Olsen et al., 2004; Bogevik et al., 2009). Despite the variation in digestion and absorption, it has been observed that 22:1n-11 accumulates in the digesta of fish (Lie et al., 1987; Lie &

Lambertsen, 1991; Sigurgisladottir et al., 1992; Olsen et al., 1998). Lipases in the digestive tract of fish are thought to have a lower affinity towards 22:1n-11 and the rate of absorption is less efficient due to its long chain length and single double bond, thus making it even less efficiently hydrolyzed when esterified to a fatty alcohol.

PUFA are known to be good substrates for fish digestive lipases (Lie & Lambertsen, 1985; Lie et al., 1987; Olsen & Ringø, 1997). Digestibility of PUFA was greater than 90% for all dietary treatments; however both n-3 and n-6 groups in fish oil were absorbed significantly higher than *Calanus* and krill oils for most fatty acids, with the exception of linoleic acid and arachadonic acid which did not differ between dietary treatments. These findings are not consistent with that of Olsen et al. (2004) who reported that digestibility and feed efficiency of diets containing *Calanus* oil fed to salmon was not significantly different to salmon fed diets containing fish oil. Digestibility of PUFA in *Calanus* oil was only marginally lower than fish oil despite the wax ester content. As with salmon, halibut must adapt their digestion in several ways to maintain a high PUFA digestibility. Salmon showed increased available bile salts and heightened midgut lipolytic activities as adaptations to diets with elevated wax esters in salmon (Bogevik et al., 2009). However, species and size difference affect lipase activity and effectiveness. Although it was found that hydrolysis of WE was lower than TAG in both salmon and rainbow trout, hydrolysis of WE in salmon was significantly greater than in rainbow trout due to differences in intestinal lypolytic activity (Bogevik et al., 2008a). Furthermore, higher lipase activity was found in 300 g salmon than 1500

g salmon (Bogevik et al., 2008a). Lipase activity toward wax esters has not been studied in halibut. Therefore it is possible that halibut lipase activity and effectiveness toward hydrolysation of wax esters may not be as efficient as salmon, particularly if there is a difference in size between animals. Increased lipolytic activity has also been suggested as a response to increased dietary lipid content (Borlongan, 1990; Bazaz & Keshavanath, 1993). Since the PUFA content of *Calanus* oil was 50% and PUFA is preferentially hydrolyzed, it is apparent that PUFA in *Calanus* diets is almost as efficiently absorbed as in fish oil diets because of physiological adaptations and high PUFA levels. Although PUFA levels in krill oil were much lower than *Calanus* and fish oils (24% vs. 43% and 50%, respectively), digestibility over 90% was probably due to its high phospholipid content and the preferential retention of PUFA as a possible physiological adaptation to absorb essential fatty acids required for metabolism.

Generally the digestibility of fatty alcohols was comparable to the corresponding fatty acid, in that digestibility decreased with increasing chain length, which suggests that utilization of both fatty alcohols and acids is similar. However, there were some observable differences with regards to wax ester hydrolysation and digestibility of the alcohols. Saturated fatty alcohols were better digested than the corresponding fatty acids. In the wax esters of *Calanus*, long chain monounsaturated fatty alcohols such as 20:1 and 22:1 tend to esterify to short chain fatty acids such as 14:0, while medium chain alcohols such as 16:0 tend to esterify to PUFA, such as 18:4n-3 (Sargent & Henderson, 1986). Fish digestive lipases preferentially hydrolyze linkages of

unsaturated fatty acids, especially PUFA, therefore short chain alcohols esterified to long chain PUFA are preferentially hydrolyzed and better digested than long chain alcohols. This is apparent in fatty alcohols 14:0, 16:0 which were better digested than their 14:0 and 16:0 fatty acid counterparts. Therefore this observation does not correspond well to the tendency that long chain molecular species are not as well digested due to increasing chain length which is observed in fatty acids. In addition, wax esters containing short chain saturated fatty acids esterified to long chain fatty alcohols would not be hydrolyzed as efficiently, and this is clearly observed in the 20:1 and 22:1 alcohols which are poorly digested relative to the saturated alcohols. The increase in proportion of 20:1 and 22:1 alcohols in fecal lipid and the decrease of 14:0 and 16:0 was also noted by Olsen et al. (2004) and Bogevik et al. (2009) who evaluated alcohol digestibility by Atlantic salmon. Digestibility of 22:1 was particularly low, similarly to the corresponding fatty acid. This particular fatty alcohol and fatty acid has been known to accumulate in the digestive tract of Atlantic salmon (Bogevik et al., 2009; Olsen et al., 2004), rainbow trout (Sargent et al., 1979), and cod (Lie & Lambertsen, 1991). The poor absorption indicates that this particular fatty acid and alcohol are a poor substrate for digestive lipases across several species, including halibut.

Calanus copepod and *Euphausia* krill oils were well digested by juvenile halibut. Saturates and monounsaturates in both lipid supplements were not as well digested as polyunsaturates, however this trend was also observed in the fish oil control

and has been well documented by several other studies. The wax esters present in *Calanus* copepod oil may have inhibited the absorption efficiency of lipids; however the physiological adaptations to wax ester digestion may have allowed for increased digestibility compared to the fish oil diet. Polyunsaturated fatty acids were generally low in the krill oil, which is likely due to seasonal variations, however digestibility of this lipid source was relatively high. Due to the high variability of PUFA content though, it may not be suitable to use krill oil as the main lipid supplement in fish diets. Storebakken (1988) made a similar recommendation. The findings of this study recommend the use of lipid from *Calanus* copepod oil as an alternate lipid supplement to fish oil in halibut feeds for aquaculture of this fish species. *Calanus* oil showed high digestibility of two long chain essential fatty acids, namely EPA and DHA. Additional studies are needed to better understand the biochemical mechanisms involved in the digestion and absorption of wax esters, particularly the fatty alcohols in *Calanus* oil by Atlantic halibut.

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6.0 TABLES AND FIGURES

Table 1. Formulation and proximate composition of the experimental diets

	Diet 1 Fish oil	Diet 2 *Calanus* oil	Diet 3 Krill oil
Ingredient	**(% of diet)**		
Casein[a]	31	31	31
Corn gluten meal[b]	10	10	10
Fish meal[c]	8	8	8
Herring oil[c]	15	-	-
Calanus oil[d]	-	15	-
Krill oil[e]	-	-	15
Wheat middlings[f]	15	15	15
Whey powder[g]	6	6	6
Pregelatinized corn starch[h]	9.13	9.13	9.13
Squid meal[i]	3	3	3
Vitamin mix[j]	1	1	1
Mineral mix[k]	1	1	1
Choline chloride[a]	0.3	0.3	0.3
Chromic oxide[l]	0.5	0.5	0.5
5α-Cholestane[m]	0.07	0.07	0.07
Proximate composition on as-fed basis, (n=2)			
Moisture %	11.0	11.7	11.8
Ash %	3.5	3.5	3.4
Crude protein %	44.7	45.2	44.5
Gross energy (MJ/kg)	21.9	22.1	21.6
Lipid %	15.5	15.3	15.3
Carbohydrate[n] %	36.3	36.5	36.8

[a]USB Corporation (Cleveland, OH, USA)
[b]Northeast Nutrition (Truro, NS, Canada)
[c] Corey Feed Mills (Fredericton, NB, Canada)
[d] Institute of Marine Research (Bergen, Norway)
[e] Aqion (Colorado Springs, CO, USA)
[f] Dover Mills (Halifax, NS, Canada)
[g] Farmers Cheese Division (Truro, NS, Canada)
[h] National Starch and Chemical Company (Bridgewater, NJ, USA)
[i] Produced in lab from whole frozen freeze dried squid
[j] Vitamin mix (IU per kg); Vitamin A, 8000 IU; Vitamin D_3, 4500 IU; Vitamin E 300 IU, Vitamin K_3 Rovomix 33, 40 mg kg^{-1}; Thiamin 50 mg kg^{-1}, Riboflavin, 70 mg kg^{-1}, Pantothenate 200 mg kg^{-1}, Biotin, 1.5 mg kg^{-1}, Folic acid, 20 mg kg^{-1}; Vitamin B_{12} 0.15 mg kg^{-1}; Niacin, 300 mg kg^{-1}; Pyridoxine, 20 mg kg^{-1}; Ascorbic acid, 300 mg kg^{-1}; Inositol, 400 mg kg^{-1}; Butylated hydroxy toluene, 15 mg kg^{-1}; Butylated hydroxy anisole, 15 mg kg^{-1}
[k]Mineral mix (per kg); Manganous sulfate, 40 mg kg^{-1} ; ferrous sulfate, 30 mg kg^{-1}; copper sulfate, mg kg^{-1}, zinc sulfate, 75 mg kg^{-1}, sodium selenite 1 mg kg^{-1}, cobalt chloride 2.5 mg kg^{-1}, sodium fluoride 4 mg kg^{-1}
[l] Fisher Scientific (Fair Lawn, NJ, USA)
[m] Sigma-Aldrich Inc (St.Louis, MO, USA)
[n]Carbohydrates = 100 – (protein + lipid + ash)

Table 2. Fatty acid composition[1] in dietary oils

	Dietary Oils		
Fatty acid	**Fish Oil**	***Calanus* Oil**	**Krill Oil**
14:0	7.9±0.01	14.1±0.09	16.3±0.01
16:0	17.9±0.04	11.3±0.4	19.0±0.01
18:0	3.5±0.01	0.9±0.03	1.4±0.01
16:1n-7	8.4±0.02	2.3±0.05	11.2±0.01
18:1n-9	9.8±0.02	3.9±0.1	15.8±0.01
18:1n-7	2.7±0.01	0.4±0.02	7.8±0.01
20:1n-9	1.0±0.06	5.1±0.3	1.4± 0.01
22:1n-11	0.9±0.01	6.5±0.3	-
22:1n-9	0.2±0.01	-	0.3±0.01
18:2n-6	1.1±0.01	1.5±0.04	1.6±0.01
20:4n-6	0.9±0.01	0.2±0.01	0.1±0.01
18:3n-3	0.5±0.01	3.9±0.1	0.8±0.01
18:4n-3	2.3±0.01	17.6±0.7	3.6±0.02
20:4n-3	0.8±0.01	1.4±0.07	0.2±0.01
20:5n-3	16.5±0.01	11.9±0.5	6.4±0.01
22:5n-3	1.9±0.01	0.6±0.03	0.2±0.01
22:6n-3	10.9±0.02	9.8±0.5	2.3±0.01
∑SAT	33.1±0.05	30.5±0.2	38.6±0.01
∑MUFA	24.0±0.02	19.4±0.7	38.1±0.01
∑PUFA	42.9±0.03	49.9±0.4	23.7±0.3
∑n-3 FA	32.1±0.03	46.4±0.3	14.6±0.2
∑n-6 FA	3.3±0.01	2.1±0.04	2.1±0.01

[1] Data expressed as mean area percentage of FAME ± standard error, n=2

Table 3. Fatty acid composition in experimental diets[1] and feces[2]

	Fish Oil Diet		*Calanus* Oil Diet		Krill Oil Diet	
Fatty acid	**Diet**	**Feces**	**Diet**	**Feces**	**Diet**	**Feces**
14:0	7.2±0.01	9.3±0.1	11.5±0.4	19.5±0.2	14.6±0.04	23.8±0.1
16:0	17.9±0.05	41.8±0.4	12.3±0.3	24.5±0.3	19.4±0.04	34.8±0.3
18:0	3.4±0.03	10.9±0.1	1.5±0.04	2.8±0.04	1.7±0.01	8.4±0.04
16:1n-7	7.5±0.02	2.7±0.07	2.4±0.20	1.5±0.2	9.8±0.02	4.0±0.07
18:1n-9	10.7±0.03	6.2±0.1	6.5±0.10	4.4±0.1	15.9±0.03	8.4±0.1
18:1n-7	2.6±0.02	1.8±0.02	0.7±0.01	0.7±0.01	6.9±0.01	4.3±0.04
20:1n-9	1.5±0.01	1.4±0.01	4.7±0.08	5.9±0.09	1.9±0.01	1.5±0.02
22:1n-11	1.5±0.02	1.8±0.03	6.1±0.08	10.2±0.2	0.9±0.02	1.3±0.02
22:1n-9	0.3±0.01	0.4±0.02	-	-	0.4±0.01	0.6±0.01
18:2n-6	5.3±0.2	3.1±0.05	7.2±0.3	3.6±0.07	5.3±0.08	3.2±0.06
20:4n-6	0.9±0.02	0.2±0.02	0.2±0.01	0.2±0.04	0.2±0.01	0.07±0.08
18:3n-3	0.7±0.01	0.3±0.01	3.4±0.04	1.3±0.05	0.9±0.01	0.4±0.09
18:4n-3	2.0±0.01	0.3±0.02	12.3±0.2	2.4±0.1	3.0±0.01	0.5±0.02
20:4n-3	0.6±0.01	0.1±0.01	1.0±0.04	0.6±0.2	0.2±0.01	0.08±0.01
20:5n-3	14.6±0.01	2.3±0.1	8.4±0.4	1.9±0.2	5.7±0.07	1.3±0.04
22:5n-3	1.6±0.01	0.5±0.02	0.4±0.01	0.1±0.01	0.2±0.01	0.2±0.01
22:6n-3	10.2±0.06	4.9±0.2	8.4±0.2	4.5±0.08	2.8±0.05	1.4±0.04
∑SAT	30.7±0.06	70.7±0.4	30.5±1.0	58.1±0.6	38.0±0.03	67.0±0.5
∑MUFA	25.0±0.10	15.6±0.1	24.0±0.02	23.5±0.9	37.0±0.1	21.1±0.3
∑PUFA	44.3±0.16	13.9±0.3	45.0±0.2	17.0±0.7	24.8±0.1	11.9±0.3
∑n-3 FA	30.7±0.04	8.1±0.2	33.2±0.04	11.5±0.5	13.6±0.09	4.5±0.2
∑n-6 FA	7.0±0.01	4.1±0.09	7.7±0.3	4.0±0.1	5.9±0.04	3.4±0.07

[1] Data expressed as mean area percentage of FAME ± standard error, n=2
[2] Data expressed as mean area percentage of FAME ± standard error, n=6

Table 4. Digestibility[1] of total lipid and fatty acids in halibut fed diets with fish oil, *Calanus* oil and krill oil

Fatty acid	Fish Oil Diet	*Calanus* Oil Diet	Krill Oil Diet
Total Lipid	92.7±0.2^{a}	81.0±0.5^{b}	89.7±0.3^{a}
14:0	86.2±0.3^{a}	73.9±1.8^{b}	79.1±0.5^{c}
16:0	75.0±0.4^{a}	69.4±1.5^{b}	77.9±0.5^{a}
18:0	65.8±0.6^{a}	72.1±1.6^{b}	74.8±0.5^{b}
16:1n-7	96.1±0.1^{a}	90.4±2.3^{b}	94.7±0.2^{a}
18:1n-9	93.8±0.1^{a}	89.7±0.9^{b}	93.1±0.2^{a}
18:1n-7	92.4±0.1^{a}	84.8±1.2^{b}	92.1±0.2^{a}
20:1n-9	89.9±0.2	84.0±2.8	89.4±0.2
22:1n-11	96.2±0.08^{a}	74.3±1.5^{b}	82.0±0.4^{c}
18:2n-6	93.6±0.03	92.4±0.6	92.4±0.2
20:4n-6	97.5±0.05	92.5±2.4	95.1±1.2
18:3n-3	95.6±0.08^{a}	94.0±0.6^{b}	94.0±0.2^{b}
18:4n-3	98.5±0.05^{a}	96.9±0.4^{b}	97.9±0.2^{a}
20:4n-3	99.0±0.01^{a}	91.1±3.5^{b}	95.1±0.2^{ab}
20:5n-3	99.9±0.01^{a}	96.6±0.3^{b}	97.0±0.08^{b}
22:5n-3	97.1±0.07^{a}	96.8±0.9^{a}	89.3±0.4^{b}
22:6n-3	95.7±0.07^{a}	91.8±0.6^{b}	93.6±0.3^{c}
∑SAT	75.0±0.5^{a}	70.0±0.2^{b}	77.0±0.4^{a}
∑MUFA	93.3±0.1^{a}	83.5±0.03^{b}	92.6±0.2^{a}
∑PUFA	96.6±0.02^{a}	93.8±0.08^{b}	94.5±0.2^{b}
∑n-3 FA	97.1±0.02^{a}	95.0±0.5^{b}	95.7±0.1^{b}
∑n-6 FA	93.7±0.06^{a}	91.4±0.5^{b}	92.3±0.2^{ab}

[1] Data expressed are mean ± standard error, n=6. Different superscript letters in the same row indicate significant differences between values, P<0.05

Table 5. Fatty alcohol composition in *Calanus* oil and *Calanus* oil diet[1], and digestibility of *Calanus* oil diet[2].

Fatty alcohol	Oil	Diet	Feces	Digestibility
14:0	2.59±0.73	1.65±0.04	0.47±0.02	95.4±0.50
16:0	14.2±0.57	11.5±0.58	4.87±0.11	93.2±0.59
16:1	2.05±0.52	1.29±0.10	0.33±0.05	95.9±0.81
18:0	4.12±0.02	3.50±0.21	2.31±0.02	89.5±0.80
18:1	1.90±0.01	1.62±0.10	1.16±0.01	88.6±0.89
18:2	4.50±0.52	3.23±0.12	1.82±0.02	91.0±0.69
18:3	8.89±1.78	5.75±0.10	2.56±0.23	93.0±0.83
20:1	25.4±3.2	28.3±1.50	28.7±0.23	83.9±1.08
22:1	21.3±2.9	30.5±1.00	44.8±1.04	76.8±1.33
24:1	14.4±2.0	10.1±0.13	9.62±0.06	84.9±1.01

[1] Data expressed as area percentage of FAME, n=2
[2] Data expressed as area percentage of FAME, n=6

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