Nutritional and environmental regulation of highly unsaturated fatty acid synthesis and fatty acid oxidation in Atlantic salmon (*Salmo salar* L.) enterocytes and hepatocytes

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Abbreviations: BHT - butylated hydroxytoluene; FAF-BSA - fatty acid-free bovine serum albumin; FO - fish oil; HBSS - Hanks balanced salt solution; HUFA - highly unsaturated fatty acids (carbon chain length ≥ C_{20} with ≥ 3 double bonds); PUFA - polyunsaturated fatty acids; VO - vegetable oil.

Abstract

The aim was to determine if highly unsaturated fatty acid (HUFA) synthesis and fatty acid oxidation in Atlantic salmon (*Salmo salar* L.) intestine was under environmental and/or seasonal regulation. Triplicate groups of salmon were grown through a full two-year cycle on two diets containing either fish oil (FO), or a diet with 75% of the FO replaced by a vegetable oil (VO) blend containing rapeseed, palm and linseed oils. At key points in the life cycle, fatty acyl desaturation/elongation (HUFA synthesis) and oxidation activities were determined in enterocytes and hepatocytes using [1-^{14}C]18:3n-3 as substrate. As observed previously, HUFA synthesis in hepatocytes showed peak activity at seawater transfer and declined thereafter, with activity consistently greater in fish fed the VO diet. In fish fed FO, HUFA synthesis in enterocytes in the freshwater stage was at a similar level to that in hepatocytes. However, HUFA synthesis in enterocytes increased rapidly after seawater transfer and remained high for some months after transfer before decreasing to levels that were again similar to those observed in hepatocytes. Generally, enterocyte HUFA synthesis was higher in fish fed the VO diet compared to the FO diet. Oxidation of [1-^{14}C]18:3n-3 in hepatocytes from fish fed FO tended to decrease during the freshwater phase but then increased steeply, peaking just after transfer before decreasing during the remaining seawater phase. At the peak in oxidation activity around seawater transfer, activity was significantly lower in fish fed VO compared to fish fed FO. In enterocytes, oxidation of [1-^{14}C]18:3 in fish fed FO showed a peak in activity just prior to seawater transfer. In fish fed VO, other than high activity at 9 months, the pattern was similar to that obtained in enterocytes from fish fed FO with a high activity around seawater transfer and declining activity in seawater. In conclusion, fatty acid metabolism in intestinal cells appeared to be under dual nutritional and environmental or seasonal regulation. The temporal patterns for fatty acid oxidation were generally similar in the two cell types, but HUFA synthesis in enterocytes peaked over the summer seawater phase rather than at transfer, as with hepatocytes, suggesting possibly different regulatory cues.

Introduction

Over-exploitation of wild fisheries and increased consumer demand has meant that an increasing proportion of fish for human consumption is now provided by aquaculture (Tidwell and Allan 2002). However, the high value fish farmed in Europe are basically carnivores feeding high in the food chain and so diets have been traditionally formulated using fishmeal and fish oil (FO) (Sargent and Tacon 1999; Pike 2005). Consequently, with aquaculture expanding worldwide at over 10% per year, the increasing demand for FO is predicted to outstrip global supplies within the next 5- 10 years (Barlow 2000; Tacon 2004). However, the only sustainable alternative to FOs are vegetable oils (VO) which are rich in C_{18} polyunsaturated fatty acids (PUFA), but devoid of the n-3HUFA abundant in FOs (Sargent et al. 2002). Atlantic salmon (*Salmo salar* L.) have all the enzymes necessary for the production of n-3HUFA from C_{18} PUFA and so much recent research has focussed on the regulation of the HUFA biosynthetic pathways in order to determine strategies for optimizing the conversion of the fatty acids in VO to HUFA (Tocher 2003).

Previously, we have shown that the intestine, specifically pyloric caeca, is a site of significant HUFA synthesis activity in salmonids, including rainbow trout (Bell et al. 2003; Tocher et al. 2004; Fonseca-Madrigal et al. 2005) and salmon (Tocher et al. 2002, 2003). These studies have also shown that the nutritional regulation of HUFA synthesis in caecal enterocytes showed similarities to HUFA synthesis in hepatocytes (Bell et al. 2001a, 2002). In general, HUFA synthesis in enterocytes could be increased in fish fed diets in which the dietary FO was replaced with VO (Tocher et al. 2003, 2004). Thus, in rainbow trout fed diets with crude palm oil replacing FO, HUFA synthesis in caecal enterocytes was significantly increased (Tocher et al. 2004; Fonseca-Madrigal et al. 2005). Similarly, in salmon fed a VO blend consisting of rapeseed and linseed oils in a 1:1 ratio, HUFA synthesis in caecal enterocytes was significantly higher in fish fed the VO blend compared to fish fed FO (Tocher et al. 2003). However, in another trial in which FO was substituted in a graded manner by linseed oil,

HUFA synthesis in caecal enterocytes was apparently inhibited at high levels (75 -100% replacement) of linseed oil whereas this effect was not observed in hepatocytes (Tocher et al. 2002). Although there was no direct evidence, it was later speculated that this inhibition was possibly an artifact of the assay whereby potentially high levels of intracellular 18:3n-3 were competing with the radiolabelled ^{14}C-18:3n-3 tracer (Tocher et al. 2004).

In addition to nutritional regulation, hepatocyte HUFA synthesis in Atlantic salmon has also been demonstrated to be under environmental regulation, particularly in relation to parr-smolt transformation (Bell et al. 1997; Tocher et al. 2000). Specifically, HUFA synthesis in hepatocytes increases during the freshwater phase, peaking around seawater transfer before declining again in the seawater phase (Tocher et al. 2002; Zheng et al. 2005). This increase in HUFA synthetic capacity in hepatocytes is part of the pre-adaptive processes of smoltification preparing the fish for the marine environment (Folmar and Dickhoff 1980), and may contribute to the increase in tissue HUFA levels observed in anadromous salmonids in advance of transfer to seawater (Sheridan et al. 1985; Li and Yamada 1992). As with other preadaptive responses, the environmental triggers are primarily day length and, to a lesser extent, water temperature, but the mechanisms whereby these affect hepatocyte HUFA synthesis are unknown (Wedermeyer et al. 1980; Duston and Saunders 1990; Tocher et al. 2000). It is not known if HUFA synthesis in the intestine is also similarly regulated during the salmon life cycle.

In addition to supplying essential fatty acids, dietary lipid is also a major energy source for fish, including salmon (Frøyland et al. 1998, 2000). Indeed, *in vivo* stable isotope studies in rainbow trout have shown that the vast majority of dietary 18:3n-3 was oxidized rather than utilized for HUFA synthesis (Bell et al. 2001b; Bell and Dick 2004). Consistent with this, our previous studies with isolated cells have shown that there may be competition between HUFA synthesis and oxidation for the ^{14}C-labelled 18:3 tracer in both hepatocytes and enterocytes (Tocher et al. 2002). In general, a greater proportion of the added tracer was oxidized rather than utilized for HUFA synthesis in both cell types (Tocher et al. 2004; Fonseca-Madrigal et al. 2005), consistent with the *in vivo* studies (Bell et al.

2001b). However, fatty acid oxidation had not been determined in either hepatocytes or enterocytes in the previous studies investigating seasonal and environmental regulation of HUFA synthesis.

In the present study our primary aim was to determine if HUFA synthesis in intestine, specifically caecal enterocytes, was under environmental and/or seasonal regulation in Atlantic salmon similar to that observed in hepatocytes. In addition, we aimed to determine if fatty acid oxidation was also under environmental and/or seasonal regulation in either liver (hepatocytes) or intestine (enterocytes). Triplicate groups of Atlantic salmon were grown through a full two-year cycle on two diets, either a control, FO diet, or a diet with 75% of the FO replaced by a VO blend containing rapeseed, palm and linseed oils (3.7:2:1). At various points throughout the trial, fatty acyl desaturation/elongation (HUFA synthesis) and oxidation activities were determined in isolated hepatocytes and intestinal enterocytes using [1-^{14}C]18:3n-3 as substrate.

Materials and methods

2.1. Animals and diets

The effect of replacing dietary fish oil with vegetable oil (75%) was investigated in Atlantic salmon in a trial conducted over an entire two-year production cycle. The diets were fed to triplicate tanks/cages during the trial that was carried out at Marine Harvest Ltd. facilities at Invergarry (freshwater) and Loch Duich, Lochalsh (seawater) on the west coast of Scotland. Atlantic salmon fry were distributed randomly into 6 tanks (3m x 3m, depth 0.5m) at a stocking level of 3000/tank, and weaned onto extruded feeds containing 20% added oil which was either fish oil (FO; capelin oil) or a VO blend, containing rapeseed, palm and linseed oils in a 3.7 : 2 : 1 ratio, replacing 75% of the FO. Fish were fed the diets described above for around one year until seawater transfer, during which time they were held at ambient temperature (Fig.1) and under natural photoperiod. The precise timing of seawater

transfer was determined by subjecting samples of fish to a seawater challenge test to assess their ability to osmoregulate. Briefly, at weekly intervals from early March, twenty fish per dietary treatment were transferred into standardised seawater at 35 parts per thousand in a static tank, with aeration, for a period of 24 h. Artificial seawater was produced by adding the appropriate volume of "Seamix" sea salts (Peacock Salt Ltd., Glasgow, Scotland) to freshwater. After 24 h the fish were euthanased, blood collected via the caudal vein into heparinised tubes, plasma isolated and plasma chloride concentrations measured (Jenway Chloride Meter, Model PCLM3). At seawater transfer, fish (average weight ~ 50g) were transferred into 5m x 5m net pens at 700 fish/pen. The smolts were fed the same diet in seawater as in freshwater although the dietary oil levels were increased to 25% (3mm pellet) rising to 32% (9mm pellets) through the year-long seawater phase. The practical-type diets were formulated and manufactured by Nutreco ARC, Stavanger, Norway according to current practices in the salmon feed industry. All diets were formulated to satisfy the nutritional requirements of salmonid fish (US National Research Council, 1993). The dietary ingredients and formulation are given in Table 1, and the measured proximate and fatty acid compositions of the diets are given in Table 2. There was no significant difference in final weights between fish fed 100% FO (2.54 ± 0.14 Kg) and fish fed 75% VO (2.37 ± 0.13 Kg).

2.2.Proximate and fatty acid compositions of diet

Dry matter in the diets was measured gravimetrically after oven drying of homogenised samples for 24 hours at 105ºC to constant weight. Ash was determined by dry ashing in a muffle furnace at 600ºC for 24 hours. Crude protein was determined by combustion using the Kjeldahl method. Total lipid in diets was measured gravimetrically following acid hydrolysis and exhaustive Soxhlet extraction with petroleum ether according to Tecator application notes 92/87 and 67/83. All proximate analysis methods were conducted as described by the Association of Official Analytical Chemists (1990). Total

lipid for fatty acid analysis was extracted from diets by homogenization in chloroform/methanol (2:1, v/v) containing 0.01 % butylated hydroxytoluene (BHT) as antioxidant, basically according to Folch et al. (1957). Fatty acid methyl esters (FAME) were prepared from total lipid by acid-catalyzed transesterification, with FAME extracted and purified as described previously (Tocher and Harvie 1988). FAME were separated and quantified by gas-liquid chromatography with on-column injection using a Thermo Finnegan, Trace 2000 GC (Thermoquest, Hemel Hempstead U.K.) equipped with a fused silica capillary column (ZB-WAX; 30m x 0.32 mm id.; Phenomenex, Macclesfield, U.K) with hydrogen as carrier gas and temperature programming from 50ºC to 150ºC at 40ºC/min and then to 195ºC at 1.5ºC/min then to 205ºC at 0.5ºC/min and finally to 220ºC for 2 min. Individual methyl esters were identified by comparison to known standards and by reference to published data (Ackman 1980). Data were collected and processed using the Chromcard for Windows (version 1.19) computer package (Thermoquest Italia S.p.A., Milan, Italy).

Preparation of isolated hepatocytes and enterocytes

The methodology for preparing hepatocytes and enterocytes was developed for use in the field (at fish farms) and has been successfully used in previous studies (Tocher et al. 2002, 2003). Briefly, isolated caecal hepatocytes and enterocytes were prepared by collagenase treatment of chopped liver and intestinal caeca tissue and sieving through 100 μm nylon gauze essentially as described in detail previously for salmonids including Atlantic salmon (Tocher et al. 2003; Fonseca-Madrigal et al. 2005). The intestinal caeca cell preparation was predominantly enterocytes although some secretory cells were present. Viability of both hepatocytes and enterocytes, as assessed by Trypan blue exclusion, was > 95% at isolation and decreased by only 5 – 10% over the incubation period. One hundred μl of the cell suspensions were retained for protein determination according to the method of Lowry et al. (1951) following incubation with 0.4 ml of 0.25% (w/v) SDS/1M NaOH for 45 min at 60 ºC.

Assay of hepatocyte and enterocyte HUFA synthesis activity

Six ml of each hepatocyte and enterocyte suspension were dispensed into 25 cm^2 tissue culture flasks and incubated at 20 ºC for 2 h with 0.3 μCi (~ 1 μM) [1-^{14}C]18:3n-3, added as a complex with fatty acid-free bovine serum albumin (FAF-BSA) in phosphate buffered saline prepared as described previously (Ghioni et al. 1997). After incubation, hepatocytes and enterocytes were harvested and 1 ml of each incubation taken for determination of fatty acid oxidation activity as described below. The remaining hepatocytes and enterocytes were washed, and lipid extracted as described previously (Tocher et al. 2003; Fonseca-Madrigal et al. 2005), and total lipid transmethylated and FAME prepared as described above. The FAME were separated by argentation (silver nitrate) thin-layer chromatography (Wilson and Sargent, 1992), located on the plate by autoradiography for 6 days, and quantified by liquid scintillation after being scraped from the plates all as described in detail previously (Fonseca-Madrigal et al. 2005).

Assay of hepatocyte and enterocyte fatty acid oxidation activities

The assay of fatty acid oxidation in intact salmon hepatocytes and enterocytes has been demonstrated previously (Tocher et al. 2002, 2003) and requires the determination of acid-soluble radioactivity as described in detail previously (Frøyland et al. 2000; Torstensen et al. 2000). Briefly, 1 ml of each cell suspension was homogenized with a hand-held tissue disrupter (Ultra-Turrax T8/S8N-5G probe, IKA-Werke GmbH & Co., Slaufen, Germany) and centrifuged at 2000 x g for 10 min in a microcentrifuge. Five hundred μl of the supernatant was taken into a clean 2 ml microcentrifuge and 100 μl of ice-cold 6% FAF-BSA solution in water was added. After mixing thoroughly, the protein was precipitated by the addition of 1 ml of ice-cold 4M perchloric acid ($HClO_4$). After vortexing, the tubes were

centrifuged at 5000 g for 10 min in a microcentrifuge. Five hundred μl of the supernatant was carefully transferred to a scintillation vial, 4 ml of scintillant added and radioactivity in the acid-soluble fraction determined as described above for the assay of HUFA synthesis.

Materials

[1-^{14}C]18:3n-3 (50-55 mCi/mmol) was obtained from NEN (Perkin Elmer LAS (UK) Ltd., Beaconsfield, U.K.). HBSS, Medium 199, HEPES buffer, glutamine, collagenase (type IV), FAF-BSA, BHT, silver nitrate and perchloric acid were obtained from Sigma-Aldrich Co. Ltd. (Poole, U.K.). Thin-layer chromatography (TLC) plates, precoated with silica gel 60 (without fluorescent indicator) were obtained from Merck (Darmstadt, Germany). All solvents were HPLC grade and were obtained from Fisher Scientific UK Ltd., Loughborough, England.

Statistical analysis

All the data are presented as means ± SD (n = 6). Effects of diet and time on HUFA synthesis and fatty acid oxidation in each cell type were analysed by two-way ANOVA with Bonferroni post-tests for effects of diet at each time point. Data which were identified as non-homogeneous (Bartlett's test) were subjected to either arcsine or log transformation before analysis. All statistical analyses were performed using Prism 3 (Graphpad Software, Inc., San Diego, USA). Differences were regarded as significant when $P < 0.05$ (Zar 1984).

Results

Dietary fatty acid compositions

The control diet (FO) was formulated with 100% fish oil and in freshwater contained approximately 20% saturates, mainly 16:0 and 14:0, almost 60% monounsaturated fatty acids over half of which were the long chain monoenes, 20:1 and 22:1, 4.4% n-6 fatty acids predominantly 18:2n-6, and 15% n-3 fatty acids, predominantly the n-3HUFA, 20:5n-3 and 22:6n-3 in approximately equal amounts, and less than 1% 18:3n-3 (Table 2). In the VO diet, 75% of the FO was replaced with a vegetable oil blend resulting in increased percentages of 18:3n-3, 18:2n-6 and 18:1n-9, with decreased proportions of n-3HUFA, and the long-chain monoenes, 20:1, 22:1. Although the FO diet in seawater showed lower 20:1 and higher 22:6n-3 compared to the freshwater phase due to seasonal variation in batches of capelin oil, replacement of FO with the VO blend had similar effects in seawater diets as in the freshwater diets other than total monoene levels, which were unchanged by substitution (Table 2).

Effects of diet on HUFA biosynthesis from [1-^{14}C]18:3n-3 in hepatocytes and enterocytes during the growth cycle

The activity of the HUFA biosynthetic activity in both hepatocytes and enterocytes was determined at eight points throughout the growth cycle. In hepatocytes, HUFA synthesis activity showed a peak in activity around the point of seawater transfer and declined after transfer (Fig.2). HUFA biosynthetic capacity was consistently greater in fish fed the VO diet, significantly so in the period around seawater transfer and also at all points in seawater. In fish fed the FO diet, HUFA biosynthetic pathway in isolated enterocytes showed a similar level of activity to that in hepatocytes during much of the freshwater stage except that it decreased immediately prior to seawater transfer (Fig.3). However HUFA synthesis in enterocytes rapidly increased after seawater transfer and peaked around 4 months after transfer before decreasing to levels that were again similar to those observed in hepatocytes in the later seawater phase. Although variation between fish in HUFA synthesis was higher in enterocytes

than in hepatocytes, enterocyte HUFA synthesis was affected by diet with significantly higher activities in fish fed the VO diet compared to fish fed the FO diet at both 9 months, and just after seawater transfer (Fig.3). The significant effects of diet and time (sampling point) on both hepatocyte and enterocyte HUFA synthesis were confirmed by the results of two-way ANOVA (Table 3). There was also significant interaction between the two variables which could be understood with enterocytes where the effect of diet was variable at different time points (cf. time point 11), but the interaction was less clear with hepatocytes where the effect of diet was consistent at all time points.

Effects of diet on oxidation of [1-^{14}C]18:3n-3 in hepatocytes and enterocytes during the growth cycle

Oxidation of the [1-^{14}C]18:3n-3 substrate was estimated by determining the production of acid soluble products at the same eight time points as for HUFA synthesis. Recovery of acid soluble products in hepatocytes from fish fed the FO diet tended to decrease during the freshwater phase but then increased steeply, peaking just after seawater transfer before decreasing during the remaining seawater phase (Fig.4). This temporal effect was highly significant (Table 3). At the peak in oxidation activity around seawater transfer, activity was significantly lower in fish fed the VO diet whereas there was no effect of diet at any other time point other than at 11 months when activity was higher in hepatocytes from fish fed the VO diet (Fig.5; Table 3). In enterocytes, the recovery of radioactivity in acid-soluble products was more variable than in hepatocytes. However, the temporal pattern in enterocytes from fish fed the FO diet was generally similar to that in hepatocytes in that the oxidation activity showed a peak in activity around seawater transfer although in enterocytes it was prior to actual transfer rather than after as in hepatocytes (Fig.4). In fish fed the VO diet, oxidation of the 18:3 substrate showed a high activity at 9 months but other than this, the pattern was broadly similar to that obtained in enterocytes from fish fed the FO diet with a high activity around seawater transfer and declining activity in seawater (Fig.5). The oxidation activity in enterocytes around seawater transfer was lower in fish fed the VO diet than in

fish fed the FO diet. The two-way ANOVA indicated there was no overall significant effect of diet on oxidation of 18:3 in enterocytes although, as with HUFA synthesis activity, this was due to the significant interaction and the effect of diet differing at different time points (Table 3).

Discussion

Our primary aim in this study was to determine if HUFA synthesis in intestine, specifically caecal enterocytes, was under environmental and/or seasonal regulation in Atlantic salmon similar to that observed previously, and in the current study, in hepatocytes. The results show that HUFA synthesis in intestine is also under apparent seasonal control but, interestingly, the relationship between activity and time is out of phase with hepatic HUFA synthesis suggesting that the physiological significance of the variation in activity in the two tissues is different.

Investigations into the seasonal and environmental regulation of HUFA synthesis activity in Atlantic salmon derived primarily from interest in the process of parr-smolt transformation. There is now a significant body of work, accrued over several seasons, that indicates hepatic HUFA synthesis is under seasonal/environmental control with a peak in activity around seawater transfer. However, recently it has been appreciated that intestine is also a site of significant HUFA synthesis activity in salmonids (Bell et al. 2003). As there is much interest in alternatives to FO in aquaculture and, as intestine is the first tissue to experience altered dietary composition, we have focused on nutritional regulation of intestinal fatty acid metabolism (Tocher et al. 2002, 2003; Fonseca-Madrigal et al. 2005). These studies showed HUFA synthesis in enterocytes was regulated by nutritional factors, specifically dietary fatty acid composition, in a generally similar manner to that in hepatocytes (Tocher et al. 2002, 2003; Fonseca-Madrigal et al. 2005). However, it does not appear that the variation in HUFA synthesis activity in enterocytes during the growth cycle of Atlantic salmon can have the same physiological function or have necessarily the same environmental cues. Indeed it is not certain if the variation in

intestinal HUFA synthesis is driven by environmental cues at all. In hepatocytes, HUFA synthesis activity peaks clearly around seawater transfer with increasing activity in the late freshwater stage (Bell et al. 1997; Tocher et al. 2000, 2003). The precise timing of transfer is, of course, an artifact in a farming situation, but in general the pattern suggests the role of increased hepatic HUFA synthesis activity is an integral part of the smoltification process, with a specific physiological role to increase tissue HUFA levels in preparation for entry to seawater (Sheridan et al. 1985; Li and Yamada 1992). As such, we can be confident that the cues for these changes in activity are ultimately environmental and primarily driven by day length (Wedermeyer et al. 1980). In contrast, the pattern of activity in enterocytes cannot be interpreted as a consequence of, or related to, smoltification. Although activity may increase slightly during the freshwater stage it declined to a minimum just prior to transfer, and the main increase in activity clearly appeared to occur after transfer and it remained high for at least 4 months (May-September) but declined to a low level by mid winter (20 months). Therefore, it appears that in salmon, high HUFA synthesis activities in enterocytes occur during the summer season in seawater. As this is the most active growth phase with maximum feed intake and accumulation of fat stores it is tempting to speculate that the increased HUFA synthesis activity in enterocytes is a response to feeding activity rather than an environmental cue such as day length. However, this is the period both of longest day length and warmest water temperatures so environmental factors in the mechanism of increased activity cannot be ruled out.

With the importance of lipid as an energy source in most fish species, particularly salmonids and marine fish, there have been several studies on fatty acid oxidation in fish (Crockett and Sidell 1993; Kiessling and Kiessling 1993; Frøyland et al. 1998, 2000; Fjermestad et al. 2000; Torstensen et al. 2000; Nanton et al. 2003; Vegusdal et al. 2004; Oxley et al. 2005; Stubhaug et al. 2005a,b,c, 2006). Thus, our secondary aim in this study was to determine if fatty acid oxidation in either liver (hepatocytes) or intestine (enterocytes) was also under environmental or seasonal regulation. As with HUFA synthesis it is clear that fatty acid oxidation also showed a pattern of variation throughout the

growth cycle and, again, the activity in the two tissues was not entirely in phase, although in the case of oxidation it was not so obviously different as in HUFA synthesis. In hepatocytes, fatty acid oxidation activity tended to increase during the latter freshwater stage, peaked just after transfer, and then declined quite rapidly throughout the seawater phase. In a similar study investigating salmon in Norway over a two-year cycle, total β-oxidation activity in liver increased during the freshwater stage, peaking just prior to transfer (Stubhaug et al. 2005a), and then rapidly declined just after transfer (Stubhaug et al. 2006). Therefore, the two studies using either isolated hepatocytes with 18:3 as substrate, as in the present study, or liver homogenates with palmitoyl Co-A substrate as in the Norwegian study, gave remarkably consistent results. The only difference was whether the peak in oxidation activity occurred just prior to, or just after, transfer but, as mentioned previously, the exact point of transfer is an artifact, and so its timing in relation to fish physiology could vary slightly between trials. Consistent with this, in an earlier trial with salmon post-smolts in Norway, β-oxidation activity in liver (expressed per mg protein) was high just after seawater transfer and was significantly lower at subsequent samplings during the seawater phase (Stubhaug et al. 2005b).

In the present study, fatty acid oxidation in enterocytes increased during the freshwater phase and peaked just prior to transfer, before declining throughout the seawater phase. Thus, in contrast to HUFA synthesis, the pattern of fatty acid oxidation in salmon through the growth cycle was, other than the slight difference in peak timing, rather similar in hepatocytes and enterocytes. This perhaps suggests that the regulatory mechanisms are not fundamentally different in the two tissues and that, irrespective of exact timings of peak activities, the physiological significance of the changes are similar in both tissues. That is, increased oxidation activity for provision of energy during smoltification, the period of high energy demand but low growth. This is the first study to measure fatty acid oxidation in salmon intestine or enterocytes throughout a growth cycle so there are no data to compare. However, β-oxidation in red muscle was low just prior to seawater transfer but was high during the summer phase possibly reflecting higher levels of activity with higher water temperatures (Stubhaug et al. 2005b). In

contrast, fatty acid oxidation in white muscle showed little variation throughout the growth cycle (Stubhaug et al. 2005b). Differences in patterns of fatty acid oxidation regulation between tissues is of further interest as β-oxidation is primarily mitochondrial in muscle tissues, but peroxisomal in liver (Frøyland et al. 2000). Only total β-oxidation was determined in the present study and so it is not known whether fatty acid oxidation in enterocytes was primarily mitochondrial or peroxisomal.

In the present trial, the only consistent effect on fatty acid oxidation of feeding VO was that at the peak of oxidation activity in both hepatocytes and enterocytes, activities were higher in fish fed FO compared to fish fed VO. This was opposite to what was observed with HUFA synthesis where activities were higher in fish fed VO. This reciprocal effect had been observed previously and led us to speculate that there was competition between the pathways for available fatty acid substrate, at least in these whole cell metabolism studies using a common fatty acid substrate (Tocher et al. 2002). However, it would be wrong to dismiss this as an artifact of the assay as a similar situation must occur *in vivo*. Higher β-oxidation activity in the FO group may, at least partly, be related to the long-chain monoene fatty acids levels of the diets, as the FO diet contained more than twice the amount of long-chain monoenes compared to the VO diet. This is based on studies measuring β-oxidation capacity using subcellular fractions, in which long-chain monenes were preferred over PUFA as substrates in fish (Henderson and Sargent 1985; Kiessling and Kiessling 1993; Henderson 1996; Frøyland et al. 2000). However, recent intact cell studies, which measure relative fates of fatty acids taken up by the cells, have suggested that PUFA can be relatively more oxidized than 18:1n-9 in salmonid hepatocytes or enterocytes (Oxley et al. 2005; Stubhaug et al. 2005c). In contrast, in the trial with salmon post-smolts mentioned above, there was a tendency for β-oxidation in liver in salmon fed VOs to be higher than in fish fed FO in the autumn after transfer, but by the following spring there was no difference in β-oxidation capacity in liver between fish fed FO or VO (Stubhaug et al. 2005a). In contrast, in a study investigating fatty acid metabolism in isolated hepatocytes from 2-year-old salmon fed either FO or VO over the whole trial, the proportion of incorporated fatty acids oxidized was greater in hepatocytes from

the fish fed VO compared to fish fed FO (Stubhaug et al. 2005c). Mitochondrial β-oxidation of [1-^{14}C]palmitoyl-CoA in red muscle homogenates of Atlantic salmon smolts was not significantly affected by feeding a range of VOs as replacement of FO after 21 weeks of feeding (Torstensen et al. 2000).

The molecular mechanisms underpinning the regulation of fatty acid desaturation and oxidation will require considerably more investigation. Nutritional regulation by dietary PUFA has a variety of possibilities including direct affects at a membrane level through alterations in fluidity or membrane microenvironments, or effects on gene expression. For instance, the expression of fatty acid desaturase genes in Atlantic salmon was shown to be increased in fish fed VOs compared to fish fed FO (Zheng et al. 2004, 2005). PUFA can potentially affect gene transcription by a number of direct and indirect mechanisms and are known to bind and directly influence the activities of a variety of transcription factors (Jump et al. 1999), which consequently can affect desaturase genes (Nakamura and Nara 2004). Activation of transcription factors may also offer a mechanism whereby environmental cues such as day length and temperature can affect gene transcription and, although the ligands require to be investigated, they could include a number of hormones involved in smoltification such as melatonin, growth hormone, insulin-like growth factor (IGF) and prolactin (Agustsson et al. 2003). However, little is known about the possible interaction of these hormones and their tissue receptors with transcription factors especially in fish (Gillespie et al. 2004).

In conclusion, fatty acid metabolism in intestinal cells appeared to be under dual nutritional and environmental or seasonal regulation in Atlantic salmon. For fatty acid oxidation, the temporal pattern was generally similar to that in hepatocytes suggesting similar regulatory mechanisms but the pattern for HUFA synthesis was rather different suggesting different regulatory factors and/or mechanisms in the two tissues.

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Legends to Figures:

FIG. 1. Daily water temperature throughout the two-year growth cycle. Water temperature was recorded both AM and PM and the data represent the means. In seawater the temperature was recorded at a depth of 2 m.

FIG.2. HUFA synthesis activity in isolated hepatocytes from Atlantic salmon throughout the two-year growth cycle. Results are means ± S.D. (n = 6) and represent the rate of conversion (pmol.h^{-1}.mg protein^{-1}) of [1-^{14}C]18:3n-3 to all desaturated products (sum of radioactivity recovered as 18:4n-3, 20:4n-3, 20:5n-3, 22:5n-3 and 22:6n-3). The dotted line indicates the time of seawater transfer. An asterisk indicates that the value at a particular time point for fish fed the vegetable oil (VO) diet was significantly different to the value for fish fed fish oil (FO) as determined by two-way ANOVA with Bonferroni post-tests.

FIG. 3. HUFA synthesis activity in isolated enterocytes from Atlantic salmon throughout the two-year growth cycle. Legend as for Fig.2.

FIG. 4. Fatty acid oxidation activity in isolated hepatocytes from Atlantic salmon throughout the two-year growth cycle. Results are means ± S.D. (n = 6) and represent the rate of oxidation (pmol.h^{-1}.mg protein^{-1}) of [1-^{14}C]18:3n-3 to acid soluble products. The dotted line indicates the time of seawater transfer. An asterisk indicates that the value at a particular time point for fish fed the vegetable oil (VO) diet was significantly different to the value for fish fed fish oil (FO) as determined by two-way ANOVA with Bonferroni post-tests.

FIG. 5. Fatty acid oxidation activity in isolated enterocytes from Atlantic salmon throughout the two-year growth cycle. Legend as for Fig.4.

Table 1. Ingredients and formulation (g/100g diet) of representative experimental diets.

Component	Diet	
	Freshwater	Seawater
Fishmeal[1]	55.5	38.5
Corn gluten[2]	10.0	10.0
Soybean meal[3]	0.0	10.0
Wheat[4]	16.7	9.9
Oil[5]	15.3	29.1
Micronutrients[6]	2.5	2.5

[1]LT Nordsilmel, Norway; [2]Cargill, USA; [3]Extracted, Denofa, Norway; [4]Statkorn, Norway; [5]Capelin oil (Nordsilmel, Norway) for fish oil based diet, or a blend of 55% rapeseed (Oelmuhle, Germany) 30% palm (Denofa, Norway) and 15% linseed (NV Oliefabriek, Belgium) oils for vegetable oil based diet; [6]Vitamin and mineral supplementation formulated to meet requirements according to NRC (1993).

Table 2. Proximate compositions (percentage of diet weight) and fatty acid compositions (percentage by weight of total fatty acids) of represenatative diets used in freshwater (3mm) and seawater (9mm pellet).

	Freshwater		Seawater	
	FO	VO	FO	VO
Proximate composition				
Protein	46.5 ± 0.4	45.8 ± 0.3	42.1 ± 0.2	41.2 ± 0.4
Lipid	19.6 ± 0.1	18.2 ± 0.2	30.2 ± 0.8	32.8 ± 0.3
Moisture	8.4 ± 0.0	6.8 ± 0.1	7.0 ± 0.3	6.4 ± 0.2
Ash	7.8 ± 0.0	7.9 ± 0.0	7.0 ± 0.1	7.1 ± 0.0
Fatty acid composition				
14:0	6.1 ± 0.1	2.7 ± 0.1	6.2 ± 0.1	2.2 ± 0.2
16:0	12.4 ± 0.2	15.5 ± 0.5	14.5 ± 0.4	16.1 ± 0.2
18:0	1.5 ± 0.0	2.4 ± 0.1	2.4 ± 0.6	3.0 ± 0.5
Total saturated[1]	20.3 ± 0.3	20.8 ± 0.6	23.6 ± 0.9	21.5 ± 0.5
16:1n-7[2]	7.9 ± 0.1	3.2 ± 0.0	4.9 ± 0.2	1.8 ± 0.3
18:1n-9	11.9 ± 0.4	30.6 ± 0.7	13.2 ± 0.4	35.2 ± 0.0
18:1n-7	3.3 ± 0.1	2.6 ± 0.1	2.4 ± 0.1	2.5 ± 0.2
20:1n-9[3]	19.9 ± 0.4	7.4 ± 0.1	11.1 ± 1.0	3.8 ± 0.3
22:1n-11[4]	15.8 ± 0.3	6.4 ± 0.1	16.5 ± 1.9	5.1 ± 0.4
24:1n-9	0.7 ± 0.0	0.4 ± 0.0	0.7 ± 0.0	0.2 ± 0.0
Total monoenes	59.4 ± 1.3	50.6 ± 0.8	48.8 ± 2.5	48.6 ± 0.6
18:2n-6	3.9 ± 0.1	11.7 ± 0.3	3.6 ± 0.6	12.7 ± 1.2
20:4n-6	0.2 ± 0.0	0.2 ± 0.0	0.5 ± 0.1	0.2 ± 0.1
Total n-6 PUFA[5]	4.4 ± 0.1	12.2 ± 0.3	4.6 ± 0.7	13.0 ± 1.1
18:3n-3	0.6 ± 0.0	6.8 ± 0.3	1.2 ± 0.1	9.0 ± 0.7
18:4n-3	1.9 ± 0.1	1.0 ± 0.1	2.5 ± 0.1	0.8 ± 0.0
20:4n-3	0.3 ± 0.0	0.2 ± 0.0	0.7 ± 0.0	0.2 ± 0.0
20:5n-3	5.8 ± 0.6	3.6 ± 0.3	6.5 ± 0.2	2.4 ± 0.5
22:5n-3	0.4 ± 0.1	0.2 ± 0.0	0.9 ± 0.2	0.3 ± 0.2
22:6n-3	5.9 ± 0.6	4.2 ± 0.4	10.0 ± 0.6	3.7 ± 1.1
Total n-3 PUFA[6]	14.9 ± 1.4	15.9 ± 1.1	21.8 ± 0.8	16.5 ± 1.1
Total PUFA[7]	20.3 ± 1.6	28.6 ± 1.3	27.6 ± 1.6	29.8 ± 0.1

Results are means ± SD (n=3 for proximates and n = 2 for fatty acids).

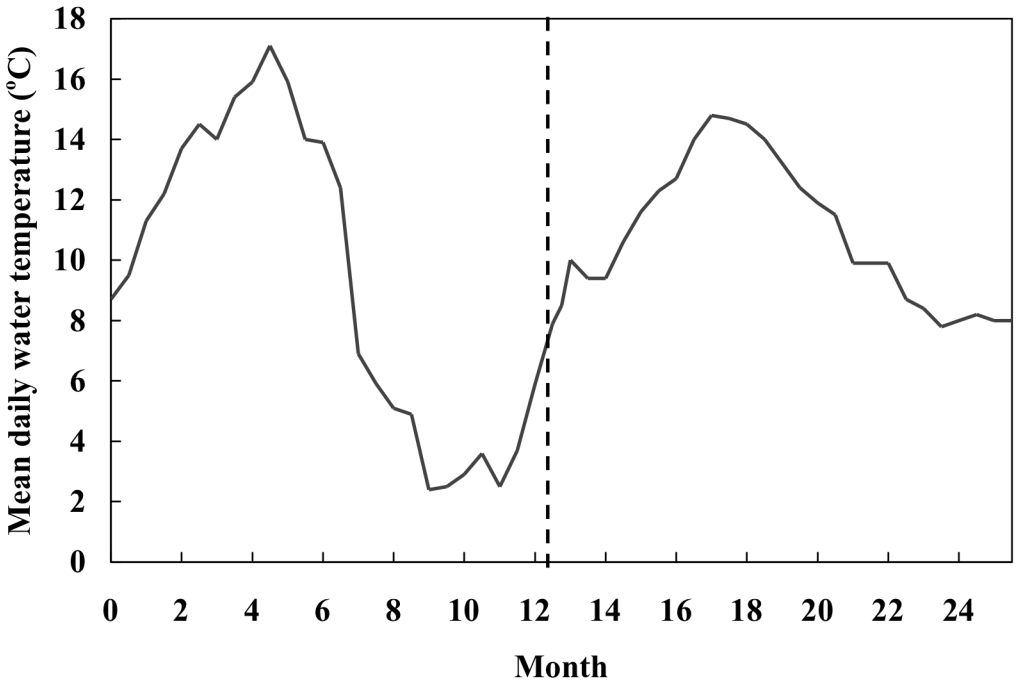
[1]totals include 15:0 present at up to 0.5%; [2]also contains n-9 isomer; [3]also contains n-11 and n-7 isomers; [4]also contains n-9 and n-7 isomers; [5]totals include 18:3n-6, 20:2n-6 and 22:5n-6 present at up to 0.2%; [6]totals include 20:3n-3 present at up to 0.1%; [7]totals include C_{16} PUFA present at up to 1.1% in FO diets and up to 0.5% in VO diets.

Table 3. Significance of effects of diet (fish oil, FO v. vegetable oil, VO) and time (sampling point) as determined by two-way ANOVA analyses of the data in Figures 2-5 for highly unsaturated fatty acid (HUFA) synthesis and fatty acid oxidation in isolated hepatocytes and enterocytes from Atlantic salmon (*Salmo salar* L.).

	Diet		Time		Interaction	
Data	p value	F value	p value	F value	p value	F value
HUFA synthesis						
Hepatocytes	< 0.0001	227.40	< 0.0001	179.80	< 0.0001	37.94
Enterocytes	< 0.0001	47.95	< 0.0001	241.50	< 0.0001	13.12
Fatty acid oxidation						
Hepatocytes	< 0.0001	18.74	< 0.0001	246.3	< 0.0001	20.14
Enterocytes	0.3748	0.7965	< 0.0001	86.22	< 0.0001	22.75

Results of Bonferroni post-tests for significance of differences between values for fish fed FO compared to fish fed VO are shown in Figs. 2-5.

Fig.1


Mean daily water temperature (°C)
18
16
14
12
10
8
6
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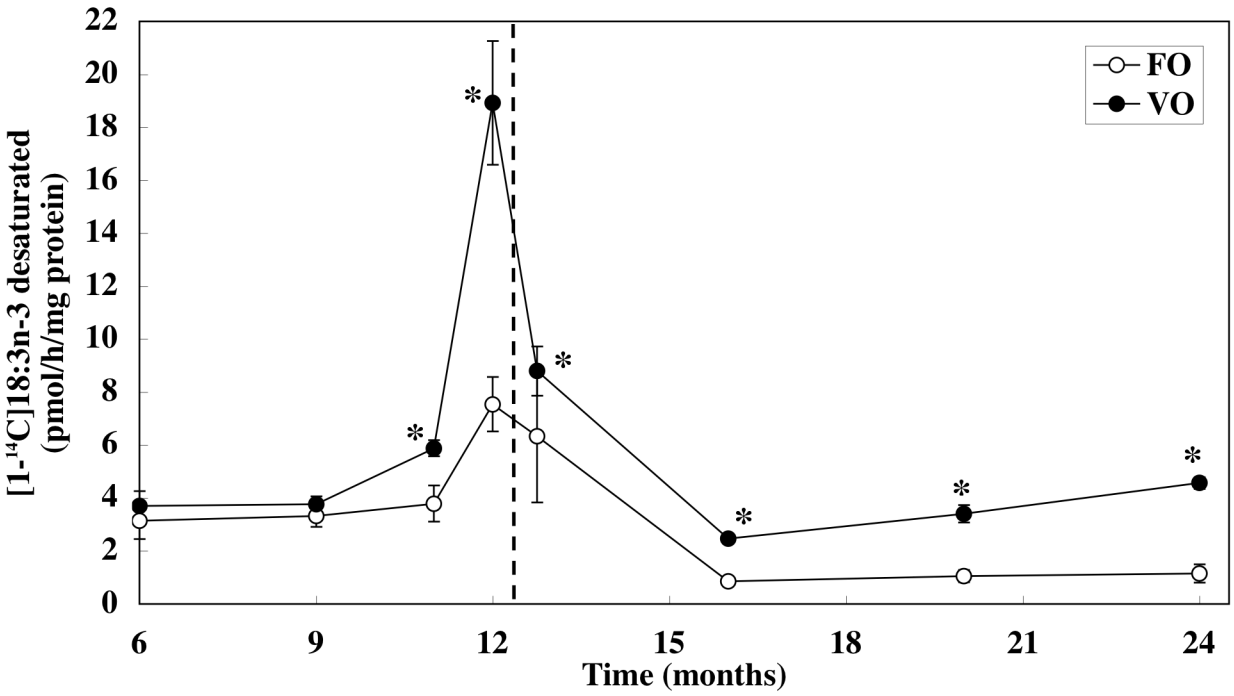
22
24
Month

Fig.2


[1-14C]18:3n-3 desaturated (pmol/h/mg protein)
Time (months)
FO
VO
0
2
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8
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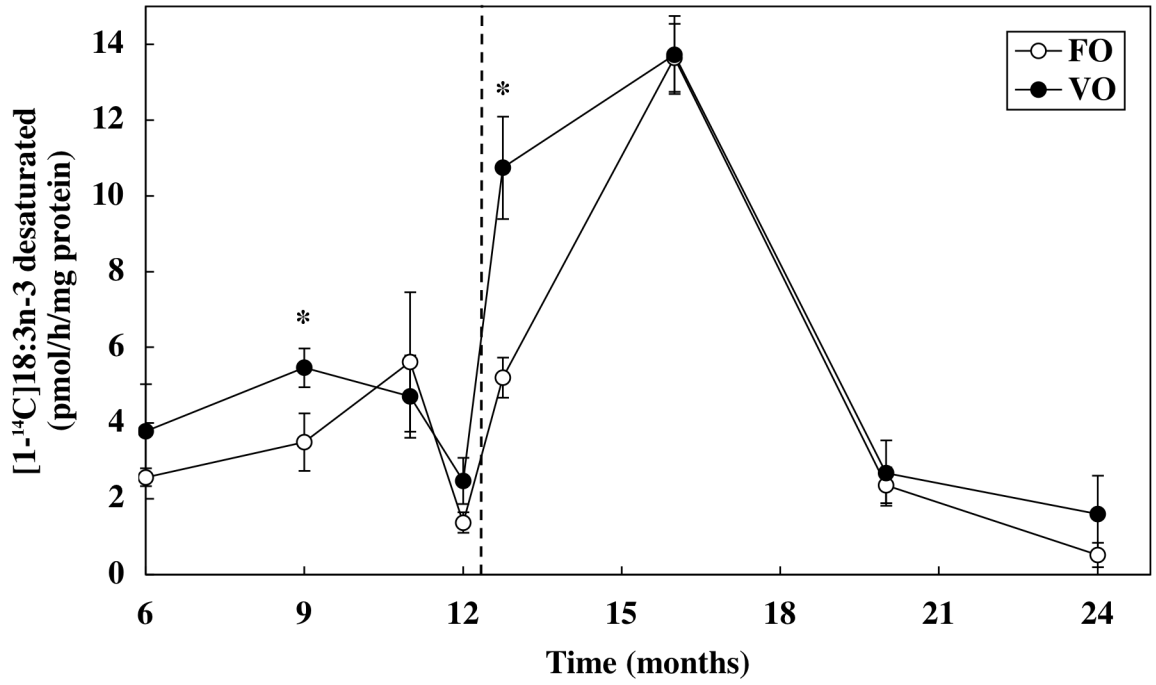
Fig.3


[1-14C]18:3n-3 desaturated
(pmol/h/mg protein)
Time (months)
FO
VO
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0
2
4
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8
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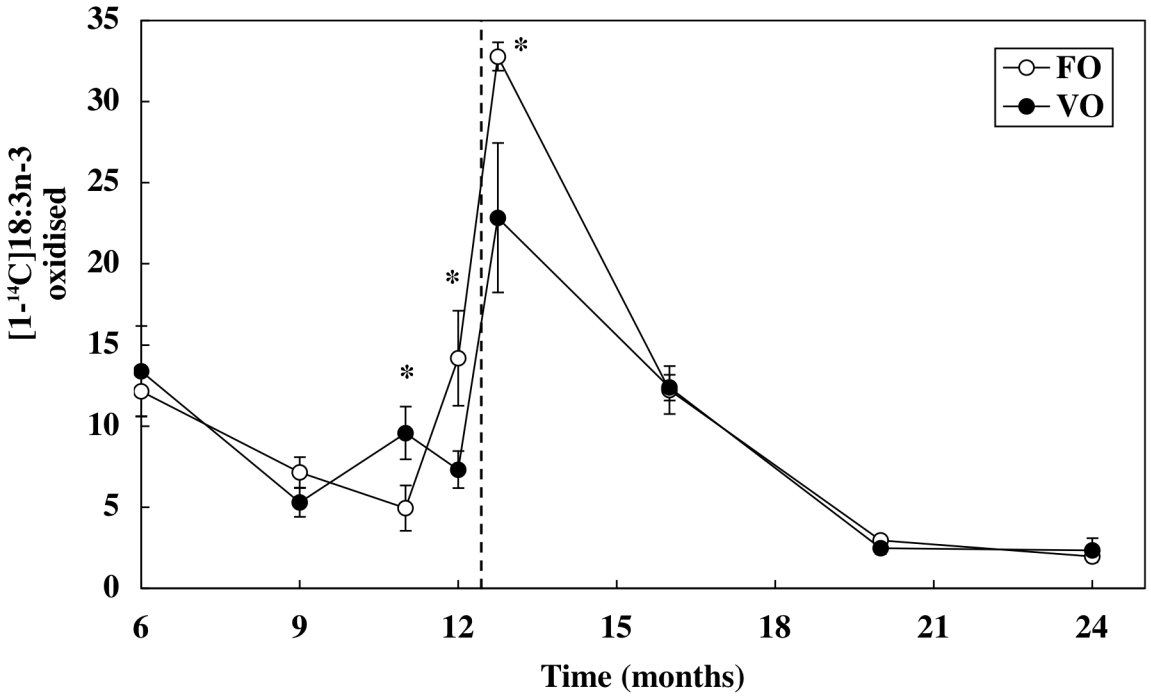
Fig.4


[1-14C]18:3n-3 oxidised
35
30
25
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9
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Time (months)
FO
VO
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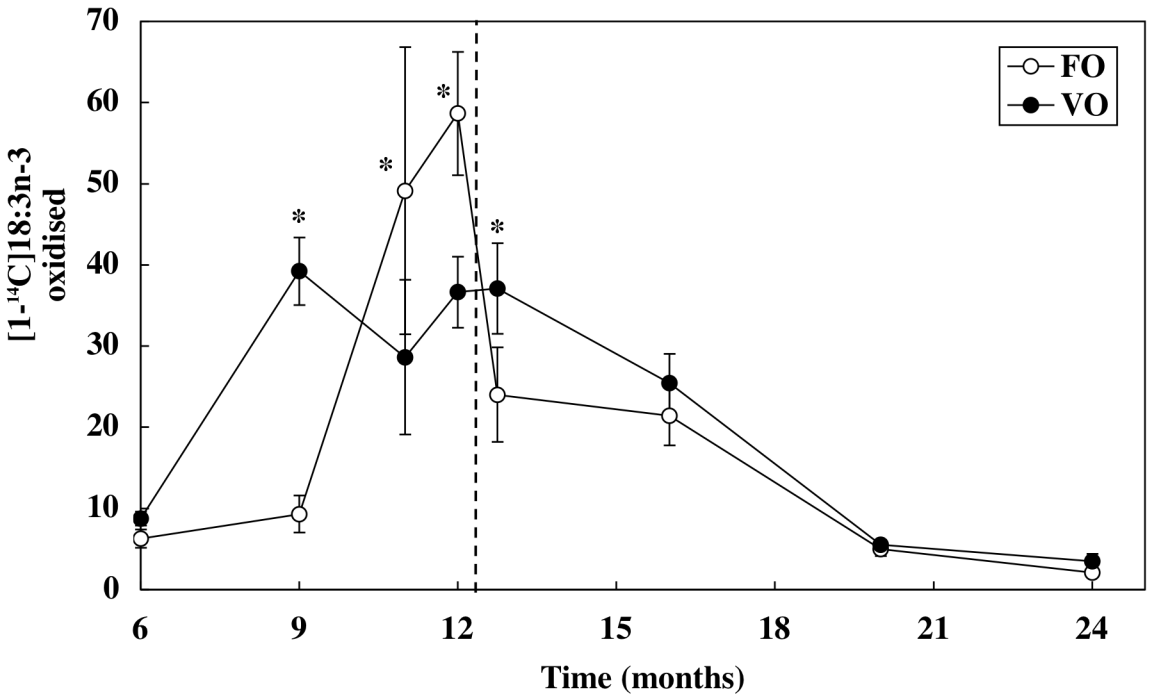
Fig.5


70
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Time (months)
[1-14C]18:3n-3 oxidised
FO
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