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Fatty acid utilisation and metabolism in caecal enterocytes of rainbow trout (*Oncorhynchus mykiss*) fed dietary fish or copepod oil

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Abbreviations: CO, copepod oil; DAG, diacylglycerol; EFA, essential fatty acids; FO, fish oil; HUFA, highly unsaturated fatty acids ($\geq C_{20}$ ≥ 3 double bonds) LCFA, long-chain fatty acids; MAG, monoacylglycerol; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PL, phospholipid; PUFA, polyunsaturated fatty acids; TAG, triacylglycerol.

Abstract

A combined fatty acid metabolism assay was employed to determine fatty acid uptake and relative utilisation in enterocytes isolated from the pyloric caeca of rainbow trout. In addition, the effect of a diet high in long-chain monoenoic fatty alcohols present as wax esters in oil derived from *Calanus finmarchicus*, compared to a standard fish oil diet, on caecal enterocyte fatty acid metabolism was investigated. The diets were fed for 8 weeks before caecal enterocytes from each dietary group were isolated and incubated with [1-^{14}C]fatty acids: 16:0, 18:1n-9, 18:2n-6, 18:3n-3, 20:1n-9, 20:4n-6, 20:5n-3, and 22:6n-3. Uptake was measured over 2 h with relative utilisation of different [1-^{14}C]fatty acids calculated as a percentage of uptake. Differences in uptake were observed, with 18:1n-9 and 18:2n-6 showing the highest rates. Esterification into cellular lipids was highest with 16:0 and C_{18} fatty acids, accounting for over one-third of total uptake, through predominant incorporation in triacylglycerol (TAG). The overall utilisation of fatty acids in phospholipid synthesis was low, but highest with 16:0, the most prevalent fatty acid recovered in intracellular phosphatidylcholine (PC) and phosphatidylinositol (PI), although exported PC exhibited higher proportions of C_{20}/C_{22} polyunsaturated fatty acids (PUFA). Other than 16:0, incorporation into PC and PI was highest with C_{20}/C_{22} PUFA and 20:4n-6 respectively. Recovery of labelled 18:1n-9 in exported TAG was 3-fold greater than any other fatty acid which could be due to multiple esterification on the glycerol 'backbone' and/or increased export. Approximately 20-40% of fatty acids taken up were β-oxidised, and was highest with 20:4n-6. Oxidation of 20:5n-3 and 22:6n-3 was also surprisingly high, although 22:6n-3 oxidation was mainly attributed to retroconversion to 20:5n-3. Metabolic modification of fatty acids by elongation-desaturation was generally low at <10% of [1-^{14}C]fatty acid uptake. Dietary copepod oil had generally little effect on fatty acid metabolism in enterocytes,

although it stimulated the elongation and desaturation of 16:0 and elongation of 18:1n-9, with radioactivity recovered in longer n-9 monoenes. The monoenoic fatty acid, 20:1n-9, abundant in copepod oil as the homologous alcohol, was poorly utilised with 80% of uptake remaining unesterified in the enterocyte. However, the fatty acid composition of pyloric caeca was not influenced by dietary copepod oil.

1. Introduction

Calanoid copepods represent a significant proportion of zooplankton in the marine environment [1] and are an important natural dietary lipid source for many marine fish including anadromous salmonids [2]. The principal lipid class in copepods, constituting as much as two-thirds of copepod dry weight, is wax esters – esters of a fatty acid and a long-chain alcohol [1]. The most abundant copepod species in the North sea is recognised as *Calanus finmarchicus*, and like most calanoid copepods, the alcohol moiety is particularly rich in long-chain monoenes such as 20:1n-9 and 22:1n-11 [1]. Therefore, to effectively utilise wax esters, salmonids, and other marine fish, possess specialised anatomical and physiological features in the intestine, including blind-ending sacs termed pyloric caeca, to aid digestion and subsequent absorption of high lipid diets [3].

The rate of hydrolysis of wax esters in fish intestine is markedly slower than for triacylglycerols (TAG), partly attributed to the higher hydrophobicity of wax esters and associated lower biliary emulsification [2]. However, in fish, the wax ester hydrolase activity of a non-specific, bile salt-stimulated lipase appears sufficient for wax ester digestion [3]. Wax ester digestibility is also enhanced by the increased transit time and concomitant exposure to lipases for hydrolysis, and increased surface area for absorption, afforded by the pyloric caeca [4]. However, feeding studies have questioned the effectiveness of long-chain monoenoic alcohol utilisation, due to the appearance of 20:1n-9 and 22:1n-11 in faeces of fish fed wax esters [3]. Moreover, it is not clear how fish metabolise the high proportion of these dietary monoenes considering that they do not accumulate to the same extent in tissue or body lipids [5–7]. Therefore, due to their dietary abundance and low incorporation into phospholipids, it has been assumed that 20:1n-9 and 22:n-11 are selectively utilised as an

energy source in marine fish [3,8]. However, how much oxidation may occur within the enterocytes is unknown.

The pyloric caeca of salmonid fish is the most important site of uptake of the products of lipid digestion including fatty acids and alcohols [9]. However, upon uptake into the enterocytes, and before they can be utilised in the intestine, absorbed fatty alcohols are oxidised to the corresponding fatty acid [10]. Because of the lack of dietary glycerol 'backbones' for TAG synthesis during copepod consumption, it was suggested that the formation of glycerol via glyceroneogenesis may become critical [6]. Indeed it was concluded from an early study that the oxidation of fatty alcohols was closely linked to the reductive formation of TAG-glycerol, with a preference for glucose over amino acid precursors for glycerol and synthesis of TAG [11]. Thus, esterification into TAG may be a possible major fate for long chain monoenoic fatty acids, derived from fatty alcohols, within enterocytes.

Recent evidence has shown that appreciable fatty acid metabolism such as β-oxidation and elongation-desaturation activity occurs in enterocytes isolated from the pyloric caeca of salmonid fish, including Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) [12–14]. Therefore, we hypothesised that significant metabolic modification and utilisation of long-chain monoenoic fatty acids may occur in the intestine of salmonid fish. For this study, rainbow trout were used as a model salmonid as it was previously shown that this species could effectively digest wax esters when fed a diet of calanoid copepods [15]. Thus, rainbow trout were fed one of two dietary treatments differing only in their lipid source, being either standard fish oil (FO) or oil derived from calanoid copepods (CO). The primary objectives were to elucidate the relative utilisation of eight [1-^{14}C]-labelled fatty acids, including 20:1n-9, in isolated caecal enterocytes with respect to: a) fatty acid uptake across the enterocyte membrane, b) fatty acid oxidation for energy production, c) intracellular

esterification of fatty acids into TAG and phospholipids (PL), d) exportation of lipid classes, and e) modification by elongation-desaturation.

2. Materials and Methods

2.1. Experimental fish, diets and sampling

One hundred and fifty rainbow trout with a mean body mass of 74.9 ± 3.4 g were obtained from a local hatchery (Almondbank, Perth, Scotland) and were randomly distributed into 6 circular tanks of 100 L capacity at the Institute of Aquaculture, University of Stirling, Scotland, at 25 fish per tank. The tanks were supplied with dechlorinated freshwater at a flow rate of 1 L min^{-1} with additional air supplied through an air stone. Water temperature was ambient and varied from 5 to 7°C during the experimental period and photoperiod was constant at 12 h light: 12 h dark. After acclimatization for one week, the fish were fed with two fishmeal-based diets of 47% protein and 29% lipid with the added oil (21%) being either fish oil (FO) or oil derived from the calanoid copepod *Calanus finmarchicus* (CO). Diet formulation and preparation was as described in detail previously [7]. The lipid class composition, and fatty acid and alcohol compositions of the diets are presented in Table 1. The diets were fed to triplicate tanks of fish until satiation three times a day for 8 weeks. Prior to sampling, the fish were starved for 48 hrs to clear the intestinal tract, anaesthetised in benzocaine, and bulk weighed. There was no significant difference in growth of fish with respect to dietary treatment, with final mean weights being 109.6 ± 10.8 g for fish oil-fed fish and 102.5 ± 4.4 g for copepod oil-fed fish. Six fish per tank were killed by a blow to the head, the intestinal tract removed, and pyloric caeca dissected and used for the preparation of enterocytes as described below. In addition, the intestinal tract from a further three fish per tank was dissected out and immediately frozen on dry ice, and stored at -80°C, for subsequent fatty acid composition analysis.

2.2. Lipid extraction and fatty acid analysis

Total lipid was extracted and fatty acid, long-chain alcohol, and lipid class compositions of the diets determined as described in detail previously [7]. Intestine samples were thawed, surrounding mesenteric fat removed, and 0.5 g of pyloric caeca homogenised in 10 ml of chloroform/methanol (2:1, v/v) with total lipid extracted according to the method of Folch et al. [16]. The total lipid extract was resuspended in toluene and the fatty acid methyl esters (FAME) prepared by acid-catalysed transmethylation in methanol-sulphuric acid (1%) according to the method of Christie [17]. FAME were purified, separated and quantified by gas chromatography as described in detail previously [7]. Total lipid was extracted and fatty acid, long-chain alcohol, and lipid class compositions of the diets determined as described in detail previously [7].

2.3. Isolation of caecal enterocytes

Single enterocyte suspensions were prepared using the pyloric caeca pooled from all six fish per tank resulting in three enterocyte preparations per dietary treatment. Caecal enterocytes were prepared essentially as described previously [12,13]. In brief, pyloric caeca were cleaned of adherent adipose tissue, finely chopped with scissors, and incubated with 20 ml of calcium and magnesium-free Hank's balanced salt solution (HBSS) supplemented with 10 mM HEPES and 1 mM EDTA (solution A), containing 1 mg ml^{-1} collagenase for 45 min in a shaking water bath at 20°C. Cells were isolated by filtering the digested caecal tissue through a 100 µm nylon gauze and washing with solution A containing 1% (w/v) fatty acid-free bovine serum albumin (FAF-BSA). Enterocytes were pelleted by centrifuging at 500 × g for 5 min and washed again in solution A. The final cell pellet was resuspended in 25 ml of

Medium 199 containing 10 mM HEPES and 2 mM glutamine. For protein determination, 100 µl aliquots of the cell suspension were taken in triplicate and assayed by the method of Lowry et al. [18]. Previously it was shown that >90% of the isolated trout caecal enterocytes remain viable after 2 hours under these conditions [13].

2.4. Incubation of enterocytes with [1-^{14}C]fatty acids

A combined fatty acid metabolism assay was employed to determine fatty acid uptake, β-oxidation, metabolic interconversion (desaturation-elongation), esterification into caecal enterocytes lipids, and also exportation of lipid classes into the culture medium. Eight stock solutions of radiolabelled long-chain fatty acids (LCFAs) complexed with FAF-BSA were individually prepared using [1-^{14}C]16:0, [1-^{14}C]18:1n-9, [1-^{14}C]20:1n-9, [1-^{14}C]18:2n-6, [1-^{14}C]20:4n-6, [1-^{14}C]18:3n-3, [1-^{14}C]20:5n-3 and [1-^{14}C]22:6n-3 according to the method of Ghioni et al. [19] resulting in a BSA:FA molar ratio of 8:1 for all [1-^{14}C]fatty acids. Each pooled enterocyte suspension was distributed into eight 25 cm^2 cell culture flasks (Nunclon, Nunc A/S, Denmark) in three ml aliquots and 50 µl (0.25 µCi) of the respective fatty acid-BSA complex added and incubation continued for 2 h at 20ºC. The final fatty acid concentration in incubations was ~2µM. Cells were harvested by gentle resuspension and 1 ml withdrawn for estimation of β-oxidation as described below. The remaining 2 ml of cell suspension was centrifuged in a conical glass test tube at 500 × g for 5 min. The supernatant was transferred to a clean test tube and 10 ml of ice-cold chloroform/methanol (2:1, v/v) containing 0.01% (w/v) butylated hydroxytoluene (BHT) added and total lipid extracted according to Folch et al. [16], and used for determining the export of fatty acids from the enterocytes after esterification into lipid classes. The cell pellet was homogenised in 5 ml of ice-cold chloroform/methanol (2:1, v/v) containing BHT and the extracted lipid [16] divided

equally into two portions for determination of incorporation into cellular lipids and fatty acid elongation-desaturation as described below.

2.5. Determination of enterocyte β-oxidation activities

Oxidation of the radiolabelled fatty acids in enterocytes was estimated by determining radioactivity in acid-soluble products by a method developed for rat isolated hepatocytes [20,21] which has been successfully applied to salmonid enterocytes [12,13]. The 1 ml of cell suspension withdrawn after incubation with [1-^{14}C]-labelled fatty acids was homogenised (Ultra-Turrax T8/S8N-5G probe, IKA-Werke GmbH & Co., Slaufen, Germany), the homogenate centrifuged at 2000 × g for 10 min, and 500 µl of supernatant transferred to a clean microcentrifuge tube. One hundred µl of 6% FAF-BSA in water was added and protein precipitated by addition of 1 ml of ice-cold 4M perchloric acid followed by centrifugation at 5000 × g for 10 min. A 500 µl aliquot of the supernatant was then transferred to a scintillation vial containing 4 ml scintillation fluid and radioactivity determined in a TRI-CARB 2000CA scintillation counter (United Technologies, Packard, UK). Results were corrected for counting efficiency and quenching of ^{14}C.

2.6. Determination of esterification of fatty acids into lipid classes

The incorporation of [1-^{14}C]-labelled fatty acids into lipid classes was determined by single-dimension, double-development high-performance thin-layer chromatography (HPTLC) [22]. The total lipid extracts prepared as above from both the cell pellets and the medium were resuspended in 50 µl of chloroform/methanol (2:1, v/v). Ten µl of total lipid extract were applied to HPTLC plates as a 1 cm streak. Plates were initially developed to

half-way in a methyl acetate/isopropanol/chloroform/methanol/0.25% aqueous KCl (25:25:25:10:9, by vol.) solvent system, dried briefly by vacuum desiccation, and subsequently fully-developed in an isohexane/diethyl ether/acetic acid (85:15:1, by vol.) solvent system. Lipid classes were visualised by exposure to iodine, and individually scraped into scintillation vials, and radioactivity determined as stated previously.

2.7. Determination of enterocyte fatty acid desaturation-elongation activities

The desaturation-elongation products of [1-^{14}C]-labelled fatty acids in the cellular lipid were determined by argentation thin-layer chromatography (TLC) [23]. FAME were prepared from enterocyte total lipid by acid-catalysed transmethylation as described above. After addition of 2 ml of 2% $KHCO_3$, FAME were extracted twice in 5 ml of isohexane/diethyl ether (1:1, v/v) containing 0.01% BHT and resuspended in 100 µl of isohexane (including BHT). TLC plates were prepared by impregnating with 2 g silver nitrate dissolved in 20 ml acetonitrile and activation at 110ºC for 30 min. FAME were applied as 2 cm streaks on the TLC plates, and the plates fully developed in toluene/acetonitrile (95:5, v/v). Autoradiography was used to visualise the FAME by exposing the plate to Kodak MR2 film for 7-10 days. The areas of silica corresponding to individual FAME were scraped into scintillation vials, 2.5 ml of scintillation fluid added, and radioactivity determined as above.

2.8. Materials

All [1-^{14}C]fatty acids (50-55 mCi/mmol) were purchased from NEN (DuPont Ltd., Stevenage, UK) except for [1-^{14}C]22:6n-3 which was obtained from American Radiolabeled Chemicals Inc. (Laborel, Oslo, Norway). BHT, HBSS, Medium 199, HEPES buffer,

glutamine, collagenase (type IV), FAF-BSA, silver nitrate, and perchloric acid were obtained from Sigma Chemical Co. (Poole, UK). HPTLC (10 cm x 10 cm x 0.15 mm) and TLC (20 cm x 20 cm x 0.25 mm) plates pre-coated with silica gel 60 were obtained from Merck (Darmstadt, Germany. All solvents were HPLC grade and were obtained from Fisher Scientific (Loughborough, UK).

2.9. Calculations and statistical analysis

[1-^{14}C]fatty acid uptake (Fig. 1) is expressed in nmol mg $^{-1}$ protein for a 2 hr period and was calculated as the sum of dpm recovered from cellular lipid classes, cellular unesterified fatty acids, cellular metabolic interconversion (elongation-desaturation) products, total β-oxidation, and exported lipid classes (excluding free fatty acids) compared to the specific activity of the individual [1-^{14}C]LCFA-BSA stock solution added to cell incubations, with subsequent normalisation for amount of protein in the different pooled enterocyte suspensions where each pooled cell suspension was considered as n = 1 for statistical analysis. Utilisation of [1-^{14}C]fatty acids, for various cellular metabolic processes (Figs 2 – 5), is expressed as a percentage of fatty acid uptake to display, proportionally, the differences in respective intracellular fatty acid fate independent of uptake rate.

Unless otherwise stated, all data are presented as means ± SD (n = 3). The significance of differences between different fatty acids were determined by one-way analysis of variance (ANOVA) followed, where appropriate, by Tukey's post hoc test. Effects of diet were determined by the Student t-test. Percentage data and data which were identified as non-homogeneous (Bartlett's test) were subjected to arcsine transformation before analysis. All statistical analyses were performed using STATISTICA 6.1 software. Differences were regarded as significant when $P < 0.05$ [24].

3. Results

3.1. Dietary lipid composition

The FO diet contained 56% of total lipid as TAG with the principal lipid class constituents of the CO diet being both wax esters (38%) and TAG (24%) (Table 1). In absolute terms, the concentration of fatty acids in the FO diet was 887 μg mg $^{-1}$ of lipid compared to 559 μg mg $^{-1}$ for the CO diet which also contained 198 μg mg $^{-1}$ as long-chain alcohols. The majority of the long-chain alcohol moieties in the CO diet were monoenoic alcohols (80%), mostly 22:1n-11 and 20:1n-9 (Table 1). Only 18:3n-3 was detectable as an alcohol of the n-3 series at 1.3%. Regarding the fatty acid moieties, there was relatively more n-3 PUFA in the CO diet, although this was mainly attributable to the high level of 18:4n-3 (Table 1). The CO diet also contained proportionally less total monoenoic fatty acids due to lower levels of 18:1n-9, 20:1n-9, and 22:1n-11. Both diets possessed high n-3/n-6 PUFA ratios, being 6.6 and 7.6 for FO and CO respectively.

3.2. Fatty acid composition of pyloric caeca

There was no significant effect of dietary treatment on the fatty acid composition of pyloric caeca with similar proportions of total saturates, monoenes, n-6 PUFA and n-3 PUFA, and individual fatty acids (Table 1). Thus, the major fatty acids in pyloric caeca, for both dietary groups, were 16:0 = 22:6n-3 > 18:1n-9 > 22:1n-11 > 20:1n-9. Therefore, the fatty acid composition of the caeca did not reflect the high proportion of dietary 22:1n-11 and 20:1n-9 fatty alcohols in the CO diet.

3.3. Uptake of [1-^{14}C]fatty acid into caecal enterocytes

The uptake of radiolabelled fatty acids in enterocytes from fish fed FO was significantly higher for 18:1n-9 and 18:2n-6 than for 18:3n-3, 20:1n-9, 20:4n-6, and 20:5n-3 (Fig. 1). Uptake of 16:0 and 22:6n-3 was intermediate between these two groups. [1-^{14}C]18:1n-9 exhibited the highest total uptake into enterocytes at 0.9 nmol mg protein $^{-1}$ over a 2 hr period, which was approximately double the amount of [1-^{14}C]20:4n-6 uptake – the fatty acid taken up the least. With respect to long-chain monoenoic fatty acids, the uptake of [1-^{14}C]20:1n-9 was poor (0.5 nmol mg protein $^{-1}$) compared to [1-^{14}C]18:1n-9. There was no significant difference in the uptake of individual fatty acids in enterocytes derived from fish fed FO or CO.

The metabolic fates of fatty acids absorbed by the pyloric caeca in rainbow trout was estimated by determining the relative proportions of individual fatty acids taken up by the enterocytes that were oxidised, esterified into lipid classes and then incorporated into cellular lipids or exported from the cell (Figs. 2-4). Further conversion of the fatty acids by desaturation and/or elongation was also determined (Fig. 5). All these data were expressed as a percentage of the total [1-^{14}C]fatty acid taken up by the cells that eliminates the influence of differences in fatty acid uptake.

3.4. β-oxidation of [1-^{14}C]fatty acids in enterocytes

Surprisingly, recovery of radioactivity in acid-soluble (fatty acid oxidation) products was greatest for the HUFA. Specifically, recovery of radioactivity in acid-soluble products was greatest for [1-^{14}C]20:4n-6, with approximately 40% of the fatty acid taken up being utilised as a substrate for β-oxidation (Fig. 2). Around 20 – 30% of the radioactivity from the

n-3 HUFAs, [1-^{14}C]20:5 and [1-^{14}C]22:6, was recovered in acid-soluble products. In contrast, less than 30% of the [1-^{14}C]16:0, [1-^{14}C]18:2n-6 and [1-^{14}C]18:3n-3 that were taken up were oxidised, with the monoenes [1-^{14}C]18:1n-9 and [1-^{14}C]20:1n-9 being especially poor substrates. With respect to dietary treatment, there was a trend for decreased proportions of most fatty acids (other than [1-^{14}C]20:1n-9) to be oxidised in enterocytes from fish fed CO, although this was only statistically significant with [1-^{14}C]16:0 and [1-^{14}C]18:1n-9 (Fig. 2).

3.5. Incorporation and esterification of [1-^{14}C]fatty acids into enterocyte cellular lipids

There was a highly significant accumulation of an intracellular unesterified fatty acid 'pool' when enterocytes were incubated with [1-^{14}C]20:1n-9, with almost 80% of this fatty acid taken up by the cells being located in this pool (Fig. 2). In contrast, the next largest accumulation of intracellular unesterified fatty acids was around 35% of fatty acid uptake for labelled 18:2n-6, 18:3n-3, and 22:6n-3. The lowest recovery of radioactivity in the unesterified fatty acid pool was with [1-^{14}C]16:0. Dietary CO had no significant effect on recovery of fatty acids in the unesterified fatty acid pool (Fig. 2). This pool is termed "unesterified" rather than "free" when regarding fatty acids in enterocytes due to them most likely being protein-bound as a cellular response to prevent cytotoxic effects.

Esterification of labelled fatty acids in enterocytes from trout fed FO varied between different fatty acids with around 15 to 40% of radioactivity taken up being recovered in cellular lipid classes (Fig. 2). Esterification was greatest for 16:0 and the C_{18} fatty acids with approximately one-third of the fatty acids taken up being recovered in cellular lipids (Fig. 2). Approximately 20% of the radioactivity from the n-3 HUFAs, [1-^{14}C]20:5n-3 and [1-^{14}C]22:6n-3, was found to be esterified, with the poorest recovery of radioactivity in intracellular lipid classes being observed with [1-^{14}C]20:1n-9 and [1-^{14}C]20:4n-6.

Esterification into intracellular lipid classes was generally unaffected by dietary treatment for six of the [1-^{14}C]fatty acids examined, however, [1-^{14}C]20:4n-6 and [1-^{14}C]20:5n-3 exhibited significantly higher incorporation into lipid classes in enterocytes from fish fed dietary CO (Fig. 2).

The greatest proportion of all the [1-^{14}C]fatty acids was esterified into TAG, such that the pattern of recovery of radioactivity in TAG (Fig. 3) very much reflected the pattern of intracellular [1-^{14}C]fatty acid esterification in caecal enterocytes (Fig. 2). Less than 7% of the labelled fatty acids was recovered in phospholipids (PL) with between approximately 1 – 3% recovered in phosphatidylcholine (PC), with labelled 16:0, 20:4n-6 and 20:5n-3 being incorporated into PC to the greatest extent (Fig.3). Incorporation into phosphatidylethanolamine (PE) did not exceed 1% of fatty acid uptake in trout fed FO, and incorporation into phosphatidylinositol (PI) was around 0.2 to 0.3% except for [1-^{14}C]16:0 and [1-^{14}C]20:4 where incorporation into PI was 3- to 4-fold higher (Fig. 3). With respect to dietary treatment, CO induced a significant decrease in 16:0 and 18:1n-9 esterification into enterocytic PC (Fig. 3). Enterocytes from CO-fed fish also exhibited a tendency of increased incorporation of certain fatty acids into PE, although only significantly for 18:1n-9 (Fig. 3).

3.6. Export of [1-^{14}C]fatty acids in lipid classes from enterocytes

Recovery of radioactivity in extracellular lipid classes was greatest with [1-^{14}C]18:1n-9, with approximately 40% of the total being recovered in this fraction (Fig. 2). Recovery of the labelled substrate in this fraction was similar for most other fatty acids at around 10 – 15%, with around half this amount being recovered in this fraction with [1-^{14}C]20:1n-9. Dietary CO had no significant effect on export of lipids from the enterocytes as estimated by the proportion of radioactivity recovered in extracellular lipids (Fig. 2). With all the labelled

fatty acids, the majority of the radioactivity in extracellular lipids was recovered in TAG, but the proportion was significantly greater with [1-^{14}C]18:1n-9 than any other fatty acid (Fig. 4). Recovery of radioactivity in extracellular PLs was greatest with PC, with [1-^{14}C]20:5n-3 incorporated at a significantly higher rate than any other labelled fatty acid, followed by [1-^{14}C]22:6n-3, [1-^{14}C]20:4n-6, and [1-^{14}C]16:0. No dietary effect was observed with respect to the distribution of labelled fatty acids within extracellular lipid classes other than reduced proportions of [1-^{14}C]20:1n-9, [1-^{14}C]20:4n-6, and [1-^{14}C]22:6n-3 in PC (Fig. 4).

3.7. Conversion of [1-^{14}C]fatty acids by desaturation and/or elongation in caecal enterocytes

Conversion of incorporated fatty acids by desaturation and/or elongation was typically <10% of labelled fatty acid uptake in caecal enterocytes from trout fed FO (Fig. 5). The fatty acid with the highest metabolic conversion was [1-^{14}C]22:6n-3 with approximately 12.5% of fatty acid incorporated being converted (Fig. 5), although mostly by retroconversion to 20:5n-3 (Table 2). With the labelled C_{18} PUFA, 18:3n-3 and 18:2n-6, around 75 and 60%, respectively, were converted to products of the HUFA synthesis pathway with the remaining being elongated to the so-called "dead-end" products 20:3n-3 and 20:2n-6 (Table 2). Similarly with [1-^{14}C]20:5n-3 and [1-^{14}C]20:4n-3, the major products were elongation to the corresponding C_{22} HUFA (and C_{24} in the case of 20:5n-3), and desaturation to 22:5n-6 and 22:6n-3, respectively. The main products of conversion of the labelled saturated and monounsaturated fatty acids were elongation with around a third of [1-^{14}C]16:0 also being desaturated by Δ9 stearoyl CoA desaturase. With respect to dietary treatment, the recovery of radioactivity in conversion products was significantly increased with [1-^{14}C]16:0, [1-^{14}C]18:1n-9 and [1-^{14}C]18:3n-3 (primarily 18:4), but decreased with [1-^{14}C]22:6n-3, in enterocytes from CO-fed fish (Fig. 5).

4. Discussion

Two major barriers to cellular LCFA uptake exist. Firstly, the solubilisation of fatty acids in an aqueous environment for delivery to the cell surface and, secondly, the plasma membrane itself [25]. Although it has been shown in the CaCo-2 human enterocyte model that fatty acid absorption is increased when presented as bile salt micelles rather than bound to BSA [26], trout primary enterocyte cultures are highly sensitive to the presence of bile salts resulting in impaired membrane integrity over prolonged exposure [27]. Therefore, BSA was employed as the fatty acid solubilising vehicle in this study. Based solely on passive diffusion, uptake across the membrane favours lipophilicity [28,29]. Increasing uptake with increasing chain length and saturation was previously shown in trout enterocytes [27]. This was also observed to some extent in the present study with uptake of 18:1n-9 > 18:2n-6 > 18:3n-3 > 20:4n-6, although uptake of 18:1n-9 was also greater than 16:0 and 20:1n-9. It is believed that LCFA uptake across the brush border membrane in enterocytes occurs by a combination of two processes that are concentration dependent: facilitated transport at low luminal fatty acid concentrations (μM), via putative plasma membrane LCFA transporters (e.g. $FABP_{pm}$, FAT/CD36, FATP), and passive diffusion predominating at high luminal fatty acid concentrations (mM) [30]. Considering the low concentrations of fatty acids used in this study (2 μM) and the inconsistent patterns in uptake with regard to fatty acid permeability coefficients, it appears that the rate of LCFA uptake was not exclusively dependent on passive diffusion. Indeed, mammalian research has shown there are many factors that determine and cellular LCFA uptake including protein-mediated translocation across the membrane and cytoplasmic channelling of LCFA or LCFA-CoAs towards specific metabolic pathways by $FABP_c$/ACBP [31], all of which are regulated by peroxisome proliferator-activated receptors (PPAR) [32].

Fatty acid utilisation in caecal enterocytes from trout fed FO was dependent on chain length/unsaturation. The C_{16} and C_{18} LCFAs were preferentially esterified into lipid classes (cellular and exported), with pattern of esterification reflecting prevalence of [1-^{14}C]fatty acid in TAG. Conversely, the esterification of C_{20} HUFAs into lipid classes was lower with relatively more oxidation. These trends were independent of differential uptake, as utilisation was calculated as a percentage of [1-^{14}C]fatty acid uptake, revealing specificities of intracellular enterocyte LCFA metabolism. Previous work on esterification rates in trout enterocytes also revealed higher incorporation of labelled 18:1n-9, 18:2n-6, and 18:3n-3 compared to C_{20} HUFAs [27]. Highest esterification in the present study was obtained with 18:1n-9, amounting to 80% of uptake, half of which was recovered in extracellular TAG. The high incorporation of 18:1n-9 in TAG corresponded with the observed fatty acid composition of salmonid tissues *in vivo*, with 18:1n-9 usually the most abundant fatty acid in TAG body lipids in rainbow trout [6].

The monoacylglycerol (MAG) pathway is the predominant intestinal TAG biosynthetic pathway in mammals [33]. Recently, it was shown that the MAG pathway is active in salmonid intestinal microsomes with respective monoacylglycerol acyltransferase (MGAT) and diacylglycerol acyltransferase (DGAT) enzymes sharing similar homologies with equivalent mammalian intestinal enzymes [34]. Therefore, the high incorporation of 18:1n-9 could reflect preferential utilisation by MGAT and DGAT. In mammalian tissues, DGAT displays a high specificity towards dioleoylglycerol, although direct substrate specificity studies using subcellular fractions are hampered by inadequate and differential solubilisation of diacylglycerols (DAG) [35]**.** However, when *sn*-2-MAG is not available for fatty acid esterification, the *de novo* phosphatidic acid (PA) pathway dictates TAG synthesis [33]. Considering that fish in this study were unfed prior to sampling, and that enterocytes were not supplemented with 2-MAG, the PA pathway probably contributed to TAG

synthesis. The high level of 18:1n-9 in exported TAG suggested that either it was esterified in more positions than other fatty acids, or there was increased export of TAG rich in 18:1n-9. A study on salmon hepatocytes showed increased TAG secretion in 18:1n-9 stimulated cells [36]. Further, there was no overall effect of dietary treatment on fatty acid specificities for esterification in TAG, even though another long-chain monoene, 20:1n-9, was abundant in CO. Therefore, it seems that 18:1n-9, along with 16:0, 18:2n-6, and 18:3n-3, are important for TAG synthesis and export also in salmonid enterocytes.

A major function of LCFAs is for membrane biogenesis in the form of PLs which, in fish, predominantly contain 16:0 and 18:1n-9 at the *sn*-1 position and 20:5n-3 and 22:6n-3 at the *sn*-2 position [8]. Results from this study show [1-^{14}C]fatty acid esterification in PLs being < 7%, predominantly in PC. Previously in trout enterocytes, Perez et al. [27] described that 5-25% of labelled fatty acids were recovered in PLs after 15 min incubation. This could indicate that choline availability may have become limiting over a 2 h period, thereby increasing the TAG:PL ratio. Nevertheless, the importance of esterification of LCFAs in PC for efficient chylomicron/VLDL formation and subsequent export from enterocytes is well documented in both mammals and fish, particularly the requirement for long-chain saturates such as 16:0 [37–39]. PC displayed the highest incorporation of [1-^{14}C]fatty acid into PL classes, with preferential esterification, and export, of 16:0 and C_{20} PUFA. This trend in incorporation was observed previously in trout enterocytes, although individual PL classes were not identified [27]. The TAG:PL ratio in trout VLDL was around 2.5:1.0 with 80% of the PL being PC, predominantly containing 16:0 and 22:6n-3, although liver will contribute to this pool [40]. In the intestinal mucosa, PC can by synthesised from dietary lyso-PC or via the *de novo* PA pathway where a branch-point of DAG into TAG, PC, or PE occurs. In mammalian intestine, evidence supports a common DAG pool for TAG and PC synthesis [41], with DAG containing long-chain PUFA in the sn-2 position being preferentially utilised

for PL synthesis [35]. The preferential esterification of 20:4n-6 into PI was also noteworthy as this is a common feature of PI in fish [6,8].

In the present study, 80% of [1-^{14}C]20:1n-9 taken up remained unesterified, presumably protein-bound, compared to 20–40% with other fatty acids. Mammalian cytosolic fatty acid binding proteins ($FABP_c$) bind LCFA and acyl-CoAs with high affinity, with two forms present in the intestine [42]. It is speculated that L-$FABP_c$ acts as a cytosolic buffer for unesterified fatty acids preventing cytotoxic effects, whereas I-$FABP_c$ modulates intracellular lipid metabolism by targeting specific enzymes located in the cytosol or in organelles [43]. The poor utilisation of 20:1n-9 in trout enterocytes was surprising considering the high proportion of the homologous fatty alcohol in the diet of fish fed CO, and that 20:1n-9 did not increase in caecal fatty acid composition. Moreover, previous studies have demonstrated effective utilisation of dietary CO in salmonid fish [7,11,44] with the assumption that 20:1n-9 is an important source of metabolic energy [10]. The significance of 20:1n-9 accumulation in an unesterified pool has to be determined.

It was previously hypothesised that 22:1n-11 and 20:1n-9 long-chain monoenes, present as fatty alcohols in CO, were preferentially utilised for energy in fish liver [5]. Another hypothesis is that fatty acid catabolism in salmonid liver is up-regulated when fatty acid is in surplus and is a function of uptake into the cell [45]. Previously, feeding a diet rich in 18:3n-3 increased oxidation of [1-^{14}C]18:3n-3 [12] in salmon enterocytes, whereas high dietary 16:0 (and low 18:3n-3) decreased oxidation of [1-^{14}C]18:3n-3 [13]. However, similar relationships between levels of specific dietary fatty acids and oxidation were not observed in the present study. For instance, 20:1n-9 was a very poor substrate and there was no dietary effect on its oxidation despite its high level (as the alcohol) in CO. Therefore, it would seem that oxidation in caecal enterocytes was not a simple function of dietary fatty acid abundance. Indeed, the highest level of β-oxidation measured in the present trial in trout enterocytes was

with 20:4n-6, at 40% of uptake. Mitochondrial β-oxidation is chiefly responsible for the production of metabolic energy in salmonid liver and muscle [46], displaying a broad spectrum for utilisation of long-chain monoenes and PUFA in trout liver [47]. This was true for n-6 and n-3 PUFA in the present study, but surprisingly not for 18:1n-9 and 20:1n-9 which were oxidised at the lowest rate. Earlier work showed that the monoenes 18:1n-9, 20:1n-9, and 22:1n-11 were oxidised at similar rates in salmonid liver mitochondria [47]. Hepatic mitochondrial β-oxidation utilised 18:1n-9, 18:2n-6, and 18:3n-3 at similar rates, compared to red muscle where 18:1n-9, 20:1n-9, 22:1n-11, and 22:6n-3 were oxidised at comparable rates [48]. Vegusdal et al. [49] reported that 50% of both 18:1n-9 and 20:5n-3 added to cultured white muscle were oxidised after 48 h, with this tissue having the greatest overall β-oxidation capacity in fish [46]. Thus, fatty acid specificity of mitochondrial β-oxidation in fish appears to be tissue-dependent.

In the present study using [1-^{14}C]-labelled substrates, recovery in acid-soluble products represents one cycle of β-oxidation. Thus, although similar levels of acid-soluble products were recovered from 20:5n-3 and 22:6n-3, in the case of 22:6 much was due to retroconversion to 20:5n-3 rather than complete oxidation. In rat hepatic mitochondria, ten times more acid-soluble products were recovered from [1-^{14}C]20:5 compared to [1-^{14}C]22:6 [50]. In mammals, the oxidation of PUFA requires induction of auxiliary enzymes, such as 2,4-dienoyl-CoA reductase and Δ^3,Δ^2-enoyl-CoA isomerase, which are rate-limiting for mitochondrial oxidation of long-chain PUFAs [51]. Therefore, it was suggested that fish, in response to a diet rich in n-3 PUFA, have an active 2,4-dienoyl-CoA reductase [47].

The CO diet provided less n-3HUFA and more long chain monoenes compared to the FO diet, but this had a few significant effects on metabolic conversions of the fatty acids. One reason may be that enterocyte metabolism of the [1-^{14}C]fatty acids to other fatty acid products was low (typically < 10%), but 16:0 was converted to 18:1n-9/20:1n-9 and 18:1n-9 to 20:1n-

9, and dietary CO increased these conversions. Considering that the CO diet was not EFA-deficient and already contained a high proportion of monoenes, the mechanism for increased desaturation of 16:0 to monoenoic fatty acids, and the elongation of 18:1n-9 to higher monoenes, is unclear. Significant portions of radioactivity from 16:0, 18:1 and, to a lesser extent, 20:1, were also recovered in products labelled as a result of recycling of labelled acetyl-CoA released by one round of β-oxidation. This recycling may have contributed to the lower recovery of radioactivity in acid-soluble products with these fatty acids compared to PUFA. Dietary CO also increased conversion of 18:3n-3, although over 60% was recovered in 18:4n-3 and 20:4n-3 (Δ6 desaturase products) and relatively little as n-3 HUFA. Increased elongation-desaturation of [1-^{14}C]18:3n-3 in hepatocytes is associated with feeding salmonids vegetable oil diets lower in n-3 HUFA [52-54]. However in enterocytes, elongation-desaturation of [1-^{14}C]18:3n-3 decreased with increasing levels of dietary linseed oil rich in 18:3n-3 [12]. It was speculated that this may be due to enterocytes containing high levels of 18:3n-3 that competed in the desaturation assay [55]. The present study provides evidence consistent with that hypothesis, as much of the fatty acid taken up by enterocytes remains in an unesterified intracellular pool whereas this does not happen with hepatocytes [55].

In conclusion, the present study has shown that after uptake, LCFAs are differentially utilised in salmonid enterocytes, having several fates including joining a large unesterified intracellular pool, or oxidation, as well as esterification into cellular lipids and/or export. Moreover, based on evidence from previous work, such as in salmonid hepatocytes and myocytes, preferential channelling of LCFAs into metabolic pathways appears to be tissue-specific. In general, a diet rich in wax esters derived from calanoid copepods, and thus high in monoenoic long-chain fatty alcohols such as 20:1n-9, had no obvious physiologically detrimental effects.

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Legends to Figures

Fig. 1. The total uptake of individual [1-^{14}C]-labelled fatty acids over a 2 hour incubation period by trout caecal enterocytes isolated from fish fed either a diet containing fish oil (FO) or *Calanus* oil (CO) as lipid source. Columns represent the mean of 3 replicate measurements ±SD, values not having the same superscript letter are significantly different ($p < 0.05$) as determined by one-way ANOVA followed by the Tukey post-hoc test. With respect to dietary treatment, significant differences were determined by a Student's t-test ($p < 0.05$) and are indicated by an asterisk.

Fig. 2. Comparison of differential [1-^{14}C]fatty acid metabolism in isolated trout enterocytes over a 2 hour incubation period from fish fed a diet containing fish oil (FO) or copepod oil (CO) as lipid source: fatty acid oxidation (oxidation), intracellular unesterified fatty acid pool (unesterified), intracellular fatty acid incorporation into lipid classes (esterified), and exportation of fatty acids into the medium as lipid classes (exported). Columns represent the mean of 3 replicate measurements ±SD, values not having the same superscript letter are significantly different ($p < 0.05$) as determined by one-way ANOVA followed by the Tukey post-hoc test. With respect to dietary treatment, significant differences were determined by a Student's t-test ($p < 0.05$) and are indicated by an asterisk.

Fig. 3. Esterification of [1-^{14}C]fatty acids in triacylglycerol (TAG), phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylinositol (PI) lipid classes, over a 2 hour incubation period, in isolated trout enterocytes from fish fed either a diet containing fish oil (FO) or copepod oil (CO) as lipid source. Columns represent the mean of 3 replicate measurements ±SD, values not having the same superscript letter are significantly different (p

< 0.05) as determined by one-way ANOVA followed by the Tukey post-hoc test. With respect to dietary treatment, significant differences were determined by a Student's t-test ($p < 0.05$) and are indicated by an asterisk.

Fig. 4. Export of [1-^{14}C]fatty acids as triacylglycerol (TAG) or phosphatidylcholine (PC), over a 2 hour incubation period, from isolated trout enterocytes derived from fish fed either a diet containing fish oil (FO) or copepod oil (CO) as lipid source. The results for phosphatidylethanolamine (PE) and phosphatidylinositol (PI) are omitted due to incorporation of [1-^{14}C]fatty acids <0.5% in these lipid classes. Columns represent the mean of 3 replicate measurements ±SD, values not having the same superscript letter are significantly different ($p < 0.05$) as determined by one-way ANOVA followed by the Tukey post-hoc test. With respect to dietary treatment, significant differences were determined by a Student's t-test ($p < 0.05$) and are indicated by an asterisk.

Fig. 5. Elongation-desaturation of [1-^{14}C]fatty acids, over a 2 hour incubation period, from isolated trout enterocytes derived from fish fed either a diet containing fish oil (FO) or copepod oil (CO) as lipid source. Columns represent the mean of 3 replicate measurements ±SD, values not having the same superscript letter are significantly different ($p < 0.05$) as determined by one-way ANOVA followed by the Tukey post-hoc test. With respect to dietary treatment, significant differences were determined by a Student's t-test ($p < 0.05$) and are indicated by an asterisk.

Table 1

Total fatty acid (FA) and long-chain alcohol (LCA) composition (percentage of total fatty acids/alcohols by weight) of diets, containing either fish oil or copepod oil, and pyloric caeca from fish fed the respective diets.

	Fish oil		Copepod oil		
	Diet FA[a,b]	Caecal FA	Diet FA[a,c]	Diet LCA[a,d]	Caecal FA
14:0	6.8	2.8 ± 1.2	10.6	1.7	3.9 ± 0.6
16:0	13.4	18.9 ± 4.5	14.5	14.6	17.6 ± 1.2
18:0	1.6	4.3 ± 0.8	1.7	0.9	3.7 ± 0.4
Σ saturates	22.6	26.1 ± 6.3	27.7	17.2	25.2 ± 1.3
16:1n-7	4.6	3.9 ± 0.6	4.3	1.6	3.6 ± 0.3
18:1n-7	1.8	2.6 ± 0.2	1.5	2.0	2.3 ± 0.1
18:1n-9	11.3	14.7 ± 2.9	8.8	3.8	13.7 ± 1.8
20:1n-9	11.8	9.6 ± 0.9	7.0	29.2	9.4 ± 0.2
22:1n-9	1.2	–	0.8	1.6	–
22:1n-11	18.1	11.0 ± 2.0	10.6	40.3	9.1 ± 0.2
24:1n-9	1.0	–	0.9	1.3	–
Σ monoenes	51.1	41.8 ± 3.1	35.3	80.1	38.0 ± 1.9
18:2n-6	2.6	3.6 ± 0.9	3.2	1.5	3.6 ± 0.3
20:4n-6	0.3	0.9 ± 0.1	0.4	–	1.2 ± 0.3
Σ n-6 PUFA	3.3	4.5 ± 0.9	4.2	1.5	4.8 ± 0.3
18:3n-3	1.1	1.1 ± 0.3	2.5	1.3	1.8 ± 0.2
18:4n-3	3.5	1.7 ± 0.4	10.9	–	3.7 ± 0.6
20:4n-3	0.6	1.2 ± 0.3	1.1	–	1.3 ± 0.1
20:5n-3	6.8	4.6 ± 0.9	7.6	–	4.7 ± 0.6
22:5n-3	0.6	1.5 ± 0.2	0.6	–	1.5 ± 0.0
22:6n-3	9.1	17.5 ± 2.6	8.9	–	18.9 ± 2.4

Σ n-3 PUFA	21.9	27.5 ± 3.9	31.8	1.3	32.0 ± 1.8
Total PUFA	26.3	32.1 ± 4.5	37.0	2.8	36.8 ± 1.6
n-3/n-6	6.6	6.1 ± 1.0	7.6	0.9	6.7 ± 0.7

[a]Lipid class composition (% of total lipid) of the fish oil/copepod oil diet was: 56.1/24.0 triacylglycerol, 0.0/37.5 wax ester, 13.5/14.9 free fatty acid, 10.4/7.8 cholesterol, 6.0/4.8 phosphatidylcholine, 5.6/4.6 phosphatidylethanolamine, 2.3/2.4 phosphatidylinositol + phosphatidylserine.

[b]Total concentration = 886.5 μg mg $^{-1}$ lipid.

[c]Total concentration = 558.7 μg mg $^{-1}$ lipid.

[d]Total concentration = 198.3 μg mg $^{-1}$ lipid.

Table 2.

Products of metabolic conversions of the radiolabelled fatty acids as a percentage of the total radioactivity recovered in converted fatty acid products in caecal enterocytes from trout fed fish oil.

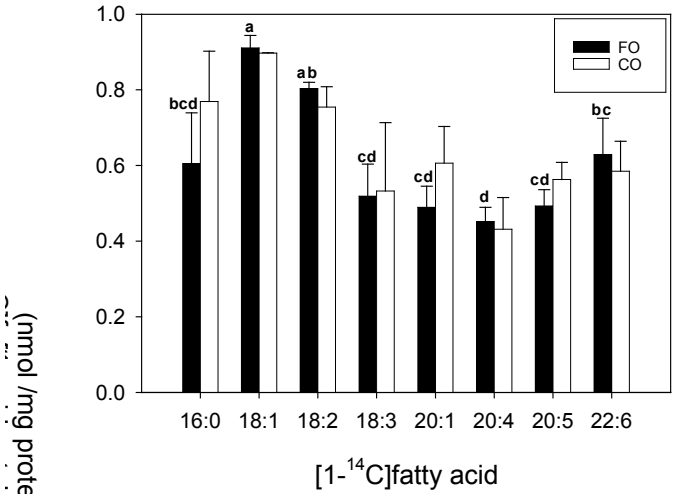
	[1-^{14}C]fatty acid substrate							
	Saturated	Monounsaturated		n-6 PUFA		n-3 PUFA		
Products	16:0[1]	18:1n-9	20:1n-9	18:2n-6	20:4n-6	18:3n-3	20:5n-3	22:6n-3
18:1n-9	13.2 ± 0.4							
20:1n-9	18.2 ± 0.3	29.5 ± 5.5						
22:1n-9			26.0 ± 4.0					
24:1n-9			55.9 ± 5.0					
20:2n-6				39.0 ± 4.5				
18:3n-6				22.0 ± 4.4				
20:3n-6				10.2 ± 1.8				
20:4n-6								
22:4n-6				14.8 ± 0.3	77.6 ± 3.3			
22:5n-6				14.1 ± 1.4	22.4 ± 3.3			
20:3n-3						25.7 ± 4.1		
18:4n-3						51.3 ± 7.0		
20:4n-3						11.0 ± 4.5		
20:5n-3								73.3 ± 4.5
22:5n-3						6.6 ± 1.4	63.3 ± 3.7	17.8 ± 5.0
24:5n-3							28.8 ± 3.9	8.5 ± 2.0
22:6n-3							7.9 ± 2.3	
Other[2]	68.6 ± 8.9	70.5 ± 9.7	18.2 ± 2.4			5.5 ± 1.0		

[1]A major product of 16:0 metabolism will be conversion by elongation to 18:0 but this cannot be separated from 16:0 by the solvent system used and so cannot be included in the analyses.

[2]Recovery of radioactivity from areas of more unsaturated fatty acids and most likely represents recycling of ^{14}C-labeled acetyl-CoA released as a result of one round of β-oxidation of the substrate fatty acid although conversion to n-9 PUFA cannot be excluded.

[3]Brackets denote fatty acids which could not be unequivocally resolved in the methodology.

Fig. 1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18:1
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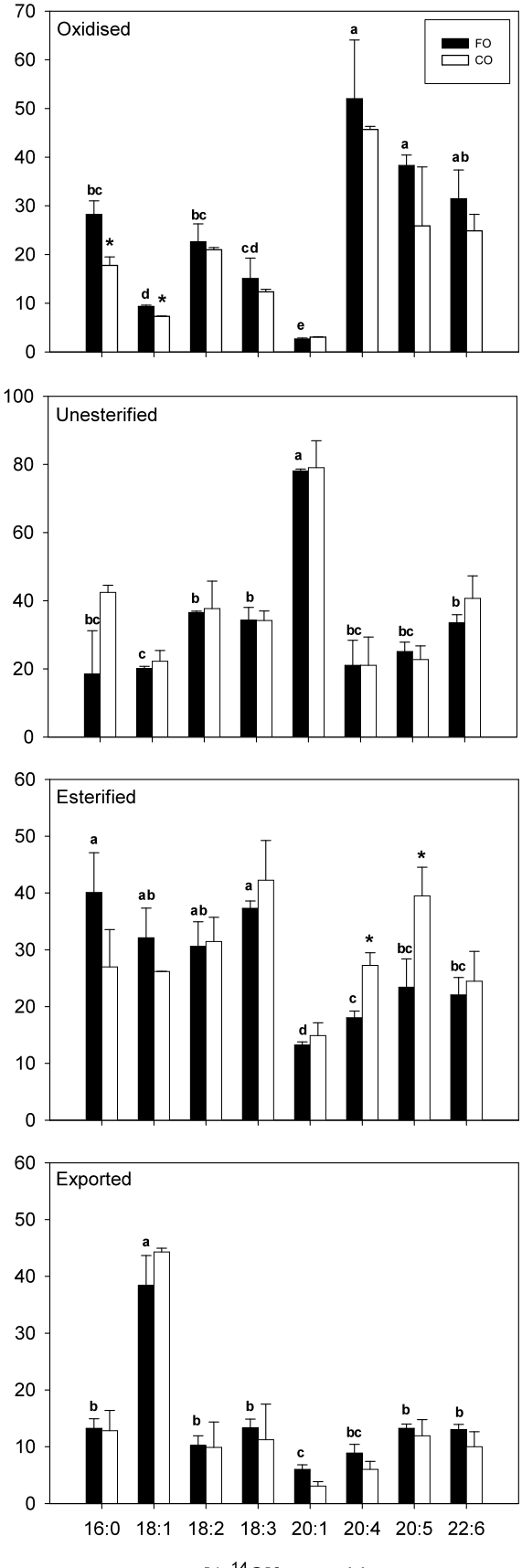
18:3
20:1
20:4
20:5
22:6
[1-14C]fatty acid

Fig. 2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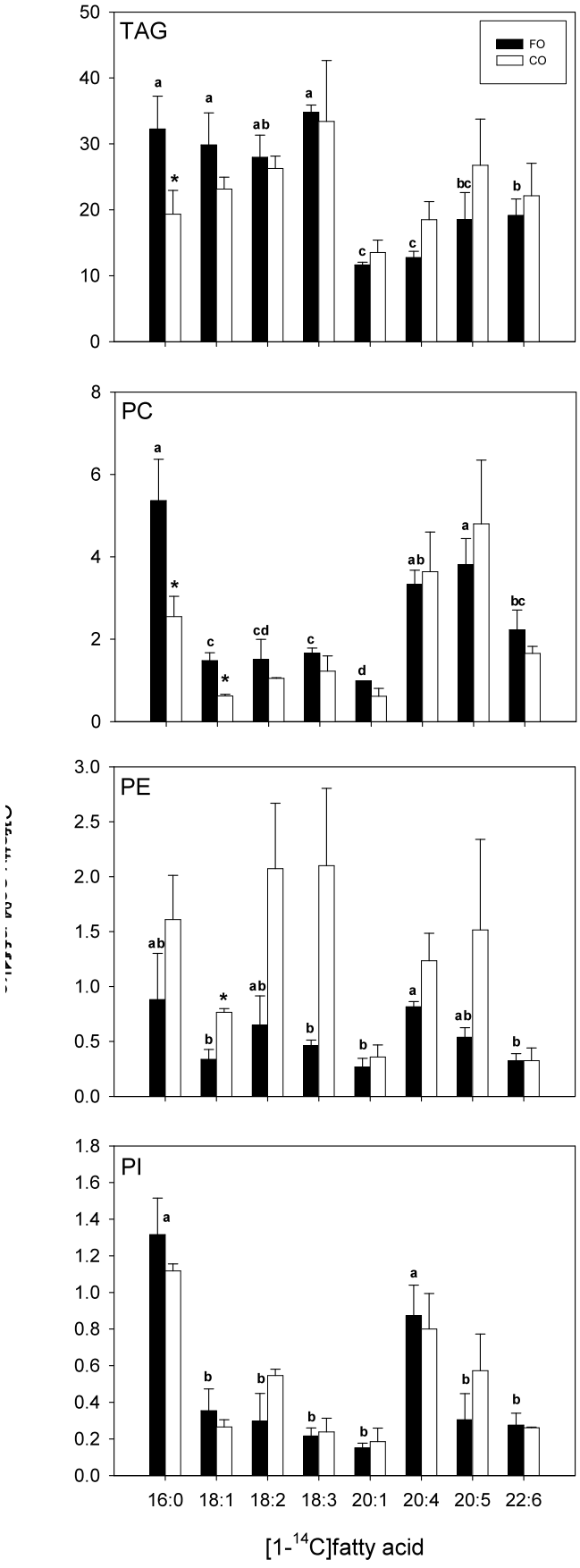
22:6
[1-14C]fatty acid

Fig. 3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22:6
[1-14C]fatty acid

Fig. 4

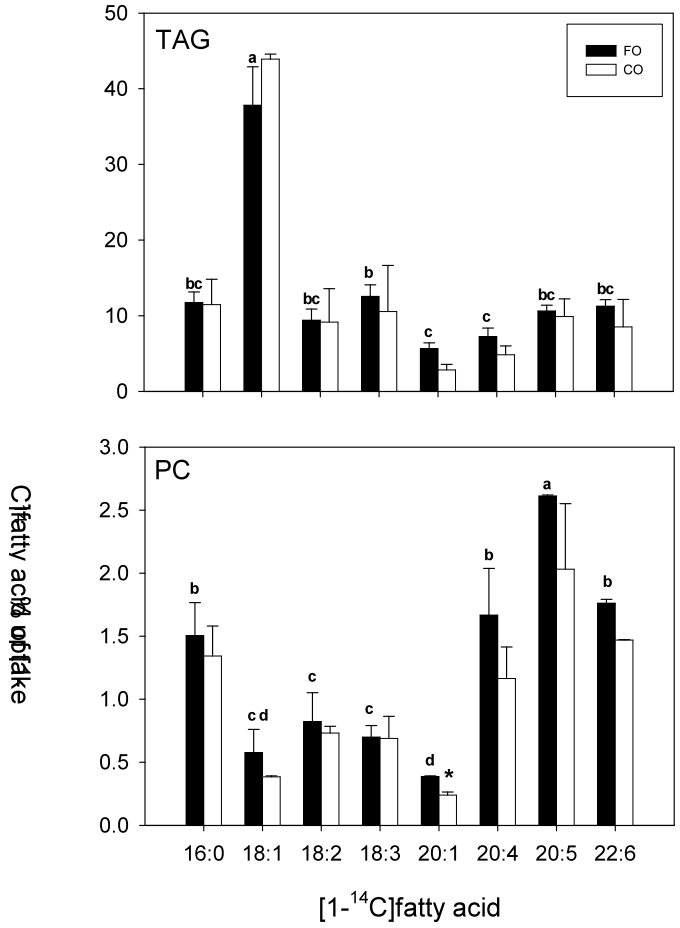
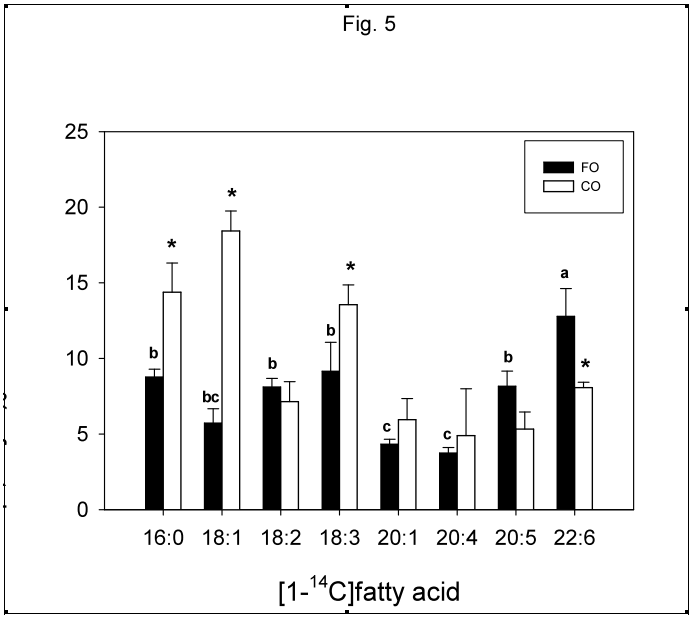


Fig. 5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16:0
18:1
18:2
18:3
20:1
20:4
20:5
22:6
[1-14C]fatty acid