Title: Influence of variation in dietary fatty acid and fasting on the hepatic lipid composition and gene expression by barramundi (*Lates calcarifer*)

Bruno C. Araújo[a,b]; Nicholas M. Wade[a]; Michael Salini[a,c], Brett D. Glencross[a*]

[a] CSIRO Agriculture Flagship – Aquaculture Program, 41 Boggo Rd, Dutton Park, QLD 4102, Australia

[b] Instituto de Biociências, Universidade de São Paulo, 321 Rua do Matão, SP 05508-090, Brazil

[c] Deakin University, Warrnambool Campus, Princess Hwy, Warrnambool, VIC 3280, Australia

*Address for correspondence:

(p) 61-7-3833-5926

(e) Brett.Glencross@csiro.au

This is the peer reviewed version of the following article: Araújo, B., Salini, M., Glencross, B. and Wade, N. (2017), The influence of dietary fatty acid and fasting on the hepatic lipid metabolism of barramundi (Lates calcarifer). ***Aquac Res*****, 48: 3879–3893, which has been published in final form at https://doi.org/10.1111/are. This article may be used for non-commercial purposes in accordance With Wiley Terms and Conditions for self-archiving.**

Abstract

This study investigated the changes in liver fatty acid profile and gene expression controlling the metabolism of fatty acids in barramundi (*Lates calcarifer*) after substitution of a rich source of long chain polyunsaturated fatty acids (LC-PUFA) (fish oil) by rich source of monounsaturated fatty acids (poultry oil), and in animals after a single feeding event. In general, the liver fatty acid profile reflected the diet composition, with some subtle exceptions supporting the enrichment of certain LC-PUFA in the liver. The fish from all experimental groups, retained preferentially more docosahexaenoic acid (22:6n3 – DHA) than eicosapentaenoic acid (20:5n3 – EPA) in the liver, suggesting a bioconversion of this fatty acid to intermediate fatty acids. The genes responsible for the synthesis and catabolism of LC-PUFA were upregulated in those fish fed with diets containing poultry oil, and these results were related to a higher percentage of monounsaturated acids (MUFA), mainly the oleic acid (18:1n9), in the livers of fish fed these diets. After a single feeding event, the gene expression in the barramundi liver were upregulated to favor fatty acid synthesis, whilst genes relating to fatty acid catabolism just showed a slight alteration after a feed event. The results demonstrated that diet composition significantly altered the lipid metabolism in barramundi and that there was a balance between direct dietary effects and endogenous synthetic capacity.

Keywords : Asian seabass, Gene expression, Liver, Fatty Acids, LC-PUFA

Introduction

Lipids are an important source of energy available for fish, with their metabolic degradation producing approximately twice as much energy compared with proteins or carbohydrates (Glencross 2009). In addition to their energetic value, lipids play essential roles in maintenance of the organism, acting in the structural composition of biomembranes, providing precursors for eicosanoid and hormone synthesis, and also influencing cognitive function (Sargent et al. 1999, 2002; Tocher 2003; Turchini et al. 2009; Glencross 2009). Fish oil has traditionally been an important nutrient source used in aquaculture, due especially to its high concentrations of long-chain polyunsaturated fatty acids (LC-PUFA). However, the utilization of fish oil is a limiting factor to the industry expansion due to increasing cost and a limited resource (Silva et al. 2011). Substitution of fish oil with vegetable oils and rendered terrestrial animal fats offer good alternatives, however this may influence many biochemical processes, such as; changing the fatty acid composition in the tissues, digestibility, catabolism, desaturation and elongation of fatty acids and the eicosanoids synthesis (Torstensen and Tocher 2011). Previous studies have reported that the incorporation of oils from other sources in the diet, is generally reflected, mainly in the liver and muscles (Torstensen et al. 2000; Bell et al. 2001, 2002; Higgs et al. 2006; Turchini et al. 2009; Glencross et al. 2003, 2011). Poultry oil has been used as an alternative lipid source in fish diets for many years due to comparative stable production and lower cost compared with fish oils. Experiments conducted with rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*) showed that partial, or even complete replacement of fish oil by poultry oil did not interfere with growth, feed intake, feed conversion and survival. However, tissues, such as muscle and liver, reflected the profile of the diets with high levels of monounsaturated fatty acids (MUFA) such as oleic acid (OLA: 18:1n9), and a drastic reduction of the important LC-PUFA such as eicosapentaenoic acid (EPA: 20:5n3) and docosahexaenoic acid (DHA: 22:6n3) (Greene and Selivonchek 1990; Higgs et al. 2006).

Beyond the fatty acid profile in tissues, diet composition can influence other metabolic processes such as synthesis and β-oxidation of fatty acids. In fish, results obtained for the expression of genes responsible for the fatty acids synthesis, such as; fatty acid desaturase (*FADS*), elongase (*elovl*), fatty acid synthase (*FAS*), stearoyl CoA desaturase (*SCD*), ATP citrate lyase (*acyl*) and acetyl CoA carboxylase (*ACC*) and for β-oxidation, such as; carnitine palmitoyltransferase (*CPT1*), acyl CoA dehydrogenase very long chain (*ACADVL*) and acyl-CoA Oxidase (*ACOX*), are still contradictory. Where some studies have shown an increased expression for animals fed with diets rich of LC-PUFA (Torstensen et al. 2009, Østbye et al. 2009), others showed the opposite pattern (Zheng et al. 2004; Jordal et al. 2005; Pratoomyot et al. 2008). These differences can potentially be justified by the influence of other variables, such as animal size, life stage, and maintenance conditions (Stubhaug et al. 2006). Other studies have also demonstrated that starvation and refeeding affects fatty acid synthesis and oxidation through the transcriptional regulation of genes, but few studies exist in fish (Ryu et al. 2005, Menningen et al. 2012; Seillez et al. 2013; Wade et al. 2014; Wade et al. 2015).

Barramundi, also known as Asian sea bass (*Lates calcarifer*), is a carnivorous species of economic importance in the Indo-Pacific region, (Glencross 2009). Understanding the molecular pathways that regulate lipid metabolism in barramundi can be considered a crucial step in understanding mechanisms for growth and tissue fatty acid composition. It was hypothesized that the replacement of FO by PO induces upregulation of genes related to fatty acid synthesis and oxidation, and results in a preferential retention of important LC-PUFA as DHA and EPA in the barramundi liver. In addition, we tested the hypothesis that even under fasting, barramundi can promote fatty acid synthesis in the liver, mainly modulated by the increase in the expression of lipid-relevant genes.

Materials and methods

Ingredient preparation and diet manufacture

The diet formulation and chemical compositions of the diets are presented in Table 1. The dry ingredients were passed separately through a hammermill (Mikro Pulverizer, type 1 SH, New Jersey, USA) such that the maximum particle size was less than 750 µm, and were mixed in 30 kg batches using a commercial mixer (Bakermix, model 60A-G, New South Wales, Australia). In total 150 kg of a basal mash (53% protein, 16% lipid, energetic value of 22 MJ/Kg), was pelletized using a laboratory-scale twin-screw extruder with intermeshing, co-rotating screws (MPF24, Baker Perkins, Peterborough, United Kingdom). Pellets were cut to 5-6mm lengths using a variable speed 4-blade cutter, and were dried at 60 °C until a constant dry weight was achieved. The pellets were warmed in a drying oven at 60 °C for 1 h prior to being mixed using Hobart mixer. The experimental oils were added to the pellets (8.5% of the diet total weight) during the mix process, a vacuum pump was attached to evacuate the air from the pellets, and the oil was infused into the pellets when the atmospheric pressure was re-equilibrated. Three diets were formulated by the addition of 100% of fish oil (FO group); 30% of fish oil and 70% of poultry oil (FO:PO group) and 100% poultry oil (PO group). Finally the pellets were stored at -20 °C until required. The diet for the postprandial response experiment (PR) was made using the same protocol with 9.4% of the diet total weight, using 100% fish oil.

Fish handling and experimental protocol

Nutritional Experiment

Juvenile barramundi (*Lates calcarifer*) were obtained from a commercial hatchery (Betta Barra, Atherton, Australia) initially were maintained in a 10,000-L tank and fed once a day *ad libitum* with commercial diet (Marine Float; Ridley Aquafeed, Narangba, QLD, Australia). Prior to the experiment, the animals were transferred to 600-L experimental tanks with a continuous seawater supply at 3 L min^{-1} flow and with an average temperature of 29.5 ± 0.09 °C, salinity =35 PSU and dissolved oxygen 4.5 ± 0.17 mg \ L^{-1}. Each tank held 20 fish with an average weight of 209 ± 26 g (mean ± SD). The animals were fed once a day *ad libitum* with the respective diets for a total of 6 weeks. Upon termination of the experiment the fish were dissected, and liver tissue of eight fish from each treatment was collected and snap frozen on dry ice then transferred to a -80 °C freezer until analysis. The biometric data of this trial, such as growth performance, fatty acid profile of the fillet and digestibility are presented in Salini et al. (2015).

Postprandial Experiment

In the postprandial experiment, 24 fish were divided between two 200-L tanks (with mean weights ± SD of 99.3 ± 7.8 g and 99.4 ± 11.8 g, respectively) with a continuous seawater supply at 2 L min^{-1} flow with an average temperature of 30 °C, salinity of 35 PSU and dissolved oxygen of 6.3 ± 0.17 mg / L. The fish were fed to excess (with uneaten feed collected to allow determination of intake, data not presented) twice daily using the same experimental diet (PR) during 6 days, and after kept under starvation for 24 h. Average fish weight on day seven was 113.2 ± 11.8 and 109.7 ± 16.5 g respectively. After 24 h fasting, three fish were sampled from each tank as a pre-feeding control, then 100 g of diet was offered to each of the two tanks over 10-min feeding event. Then three fish were sampled from each tank at 30 min, 1, 2, 4, 8, 12 and 24 h intervals post feeding. The fish were dissected and the liver was stored as in the first experiment.

In both experiments, the fish collected were euthanized by placing them in seawater containing an overdose of 0.2 ml L^{-1} AQUI-S (AQUI-S New Zealand Ltd). The experiments were performed in accordance with Australian code of practice for the care and use of animals for scientific purposes and were approval by the CSIRO Animal Ethics Committee (approval number: A8/2010).

Fatty acid analysis

The fatty acid profile of diets (Table 2) and liver were determined using an adapted protocol described by Coutteau and Sorgeloos (1995). An aliquot of 50 mg liver tissue was homogenized in a Precellys bead beater (Bertin Technologies) and directly esterified by an acid-catalyzed methylation, and to each sample was added to 0.3 mg of an internal standard (21:0 Supelco, PA, USA). The fatty acids were identified by gas chromatography (GC) using flame ionization detection and an Agilent Technologies 6890N GC system (Agilent Technologies, California, USA) fitted with DB-23 capillary column. The carrier gas used was hydrogen at a flow rate of 40 mL min^{-1}. The GC was programmed with the following temperature, 50 – 175 ºC at 25 ºC min then 175 – 230 ºC at 2.5 ºC min. The injector and detector temperatures were set at 250 ºC and 320 ºC, respectively. The fatty acids were identified by peaks comparing retention times to known standards (37 Comp. FAME mix, Supelco, PA, USA).

RNA extraction and normalization

RNA was extracted using Trizol® reagent (Invitrogen) according to the manufacturer's instructions. RNA was precipitated by adding 0.5 volumes of isopropyl alcohol and 0.5 volume of RNA precipitation solution (1.2 M sodium chloride, 0.8 M disodium citrate). Total RNA was DNase digested using Turbo DNA-free kit (Life Technologies). Total RNA was quantified using a NanoDrop spectrophotometer (NanoDrop Technologies), and RNA quality was verified using a Bioanalyzer (Agilent Technologies) using RNA nanochips (Agilent #5067-1511). All samples were diluted to 200 ng μl^{-1}.

Quantitative real-time PCR

Reverse transcription was done on 1 µg of total RNA using Superscript III (Invitrogen) with a mastermix containing 25 µM of oligo $(dT)_{20}$ and 25 µM of random hexamers (Resuehr and Spiess 2003). Real-time PCR primers for each gene (Table 3) were designed using PerlPrimer v1.1.17 (Marshall 2004). PCR amplification was performed for a pool of DNAse-treated RNA samples using each gene-specific primer, to verify that there was no contamination with genomic DNA. For all genes, PCR efficiency was optimized to be between 95 and 105% using the slope of a standard curve over a five-fold serial dilution of a pooled cDNA sample containing all samples analyzed. Real-time PCR amplification reactions were performed using 1X SYBR Green PCR Master Mix (Applied Biosystems), 0.2 µM of each primer and an equivalent of 7.5 ng of the reverse transcribed RNA.

The reaction was incubated 2 min at 50 °C, 10 min at 95 °C followed by 40 cycles of 15 s at 95 °C and 40 s at 60 °C. At the end of these cycles the melt curve analysis was performed to test the specificity of reaction. Reactions were set up using the epMotion 5070 robot (Eppendorf) and run in triplicate on a Viia7 real-time PCR system (Applied Biosystems). EF1α and Luciferase genes were used as endogenous and exogenous references for analyzing the relative expression of other genes. Gene expression for the nutrient experiment was normalized to a pooled reference sample containing cDNA from all samples and log2 transformed, while for the postprandial experiment expression was normalized to the T0 expression and log2 transformed.

Statistical analyses

Data are presented as mean ± SEM (standard error of the mean). Comparisons between fish from the FO, FO:PO and PO groups, and different times after feeding were performed by one-way analysis of variance (ANOVA), followed by the Tukey's HSD test allowing 5% error. The significance level adopted was 95% ($P < 0.05$). Statistical analyses were performed using software SigmaStat for Windows version 3.5 (SyStat Software, San Jose, CA, USA).

Results

Nutritional Experiment

The liver fatty acid composition from the fish in the nutritional experiment is presented in Table 4. The liver fatty acid profile reflected the diet fatty acid composition in all experimental groups. Fish fed with diets containing poultry oil (FO:PO and PO) showed a lower percentage of total PUFA and LC-PUFA in the liver and these changes were mainly influenced by the lower percentage of 20:5n3 (EPA) and 22:6n3 (DHA). However, for total SFA and MUFA an inverse pattern was observed, with a high percentage in the animal's liver of the PO:FO and PO groups compared with FO group, mainly due to a elevation of 18:1n9 and 18:2n6, respectively. Due to alterations in PUFA concentration in the liver among the experimental groups, a gradual increase in n3/n6 ratio was observed, highest for the FO group, followed by FO:PO group and finally for the PO group. In the liver of animals from all groups, low levels of certain fatty acids were detected, such as 18:3n6, 20:3n6, 18:4n3 and 22:5n3, that were much less abundant in the diets or even not observed (Table 4). The 18:3n6 percentage was significantly higher in the FO:PO and PO groups compared with FO group, and proportion of 20:3n6 gradually increased among the FO, FO:PO and PO groups, respectively. For 18:4n3 and 22:5n3, a gradual decrease was observed complementary to increase in 18:3n6 and 20:3n6 levels (Table 4).

Using a combination of data from all treatments, the proportion of fatty acids in the liver relative to the diet was calculated, with a dashed line indicating an equal proportion between the liver and the diet (Figure 1). In general, each of the fatty acid classes (SFA, MUFA, PUFA and LC-PUFA) were maintained close to the proportion supplied in the diet (Figure 1A). A subtle elevation was recorded in SFA levels in the liver of animals fed the PO diet, with a complementary decrease in PUFA levels but LC-PUFA levels were unaffected (Figure 1A). Despite being supplied in higher levels in the FO diet, levels of n6 fatty acids were under-represented in the liver of these animals (Figure 1B). Meanwhile, n6 levels were over-represented in the livers of PO fed fish relative to the levels in the diet. The largest alteration was observed with EPA, which was under-represented in the livers of fish from all experimental groups, and most evident in the FO group. Meanwhile, ARA and DHA were maintained close to the linear function (Figure 1C).

Significant changes in the hepatic expression of all genes related with fatty acid biosynthesis were observed (Figure 2A). A significantly higher level of elongase 5 (*Lc elovl5*) and ATP citrate lyase (*Lc acyl*) expression was observed in the PO group compared with FO:PO group, but neither were significantly different from the FO group. The fatty acid **Δ**6-desaturase (*Lc FADS2*) expression was higher in the PO group compared with the FO. A similar expression profile was observed for acetyl CoA carboxylase (*Lc ACC*), fatty acid synthase (*Lc FAS*) and stearoyl CoA desaturase (*Lc SCD*), with significantly increased expression in the PO group compared with other experimental groups. Significant alterations were also observed in the expression of all genes related to the fatty acid β-oxidation (Figure 2B). Expression of carnitine palmitoyltransferase (*Lc CPT1a*), acyl CoA dehydrogenase very long chain (*Lc ACADVL*), acyl-CoA oxidase (*Lc ACOX1*), β-hydroxybutyrate dehydrogenase (*Lc HADHB)* and 3-ketoacyl-CoA thiolase (*Lc pACAA1*) were all significantly upregulated in the liver of animals from PO group compared with the FO:PO group (Figure 2B). The expression of enoyl CoA hydratase (*Lc ECH)* was significantly lower in the FO:PO group that either the FO or PO groups. Overall, for most genes regulating both fatty acid synthesis and β-oxidation, higher expression levels were observed in animals fed with poultry oil (PO group) compared with animals fed with other diets, especially the FO:PO diet.

Postprandial Experiment

Significant differences in the levels of hepatic gene expression were observed in the postprandial experimental (Figure 3). Expression of *Lc ACC*, *Lc FADS2* and *Lc* HADHB had significantly increased by 3-5 fold after 1 hour or 2 hours post feeding, and remained elevated 12 hours after feeding until returning to basal levels (Figure 3). The expression pattern of these genes showed a significant positive correlation with each other (Figure 4). The expression of *Lc elovl5* significantly increased until 2 h after feeding, and had returned to pre-feeding levels after 4 hours This expression pattern was positively correlated with .*Lc ACC* and *Lc ACOX1* was positively correlated with *Lc elovl5*, *Lc ACOX1* and *Lc HADHB*; whereas *Lc FADS2* with *Lc ECH*, *Lc HADHB* and *Lc pACAA1* (Fig. 4). The other genes *Lc ACADVL*, *Lc ACOX1*, *Lc ECH* and *Lc pACAA1* showed very little post-prandial regulation, although the small fold change increase in expression between 4 and 8 hours resulted in a significant correlation between the expression of several of these genes, such as *Lc ACOX1* with *Lc elovl5* and *Lc ACC*, *Lc pACAA1* with *Lc FADS2*, *Lc ECH* and *Lc HADHB.*

Discussion

Liver reflects diet fatty acid profiles

This study examined the influence of diluting the LC-PUFA with principally monounsaturated fatty acids in the content of diets fed to juvenile barramundi, through the replacement of fish oil with poultry oil. It was hypothesized that this reduction in LC-PUFA in the diet would result in a concomitant decrease in LC-PUFA in the liver fatty acids and also induces an upregulation of the fish's endogenous capacity to synthesize these fatty acids. We also proposed to further explore the broader regulation of lipid metabolism, through the examination a range of lipogenic and lipolytic genes, some of which are reported for the first time in this species. As observed in previous studies with fish, the liver fatty acid profile directly reflected the diet profile (Greene and Selivonchek 1990; Torstensen et al. 2000; Bell et al. 2001, 2002; Zheng et al. 2004; Pratoomyot et al. 2008; Mohd-Yusof et al. 2010). It is known that changes in the diet fatty acid profile generally influence the fatty acid composition of tissues, and also affect processes like digestibility, oxidation, elongation and desaturation of fatty acids, lipid transport and signaling and synthesis of eicosanoids (Glencross, 2009; Torstensen and Tocher 2011). This pattern was observed for most all fatty acids particularly for the MUFA in fish fed the PO diet, and LC-PUFA, such as DHA and EPA in fish fed with FO diets.

The complete substitution of fish oil by poultry oil resulted in a marked reduction of LC-PUFA percentage in the liver, with a slight increase in the expression of *Lc FADS2* (compared with FO group) and *Lc elovl5* expression (compared with FO:PO group). The slight increase in the percentage of n6 and n3 intermediate fatty acids, such as 18:3n6 and 20:3n6, 18:4n3 and 22:5n3 between the three experimental groups suggests the increased activity of **Δ**6-desaturase and elongase 5 enzymes (modulated by *Lc FADS2* and *Lc elovl5* respectively), that are responsible for biosynthesis of fatty acids from their respective precursors as 18:2n6, 18:3n3 and 20:5n3. This suggestion is consistent with the lower ratio liver/diet found to EPA in all experimental groups, suggesting that this fatty acid was elongated to 22:5n3. In studies conducted with seabream (*Sparus aurata*) and seabass (*Dicentrarchus labrax*) also found a similar profile, with an increase of several n3 and n6 intermediate PUFA in the fishes fed with diets containing low levels of LC-PUFA, as EPA and DHA, possibly due to **Δ**6-desaturase and elongase 5 activity (Izquierdo et al. 2003). Despite this, there was no measurable influence on important fatty acids levels, such as that of DHA, mainly due a lower or absent activity of **Δ**5-desaturase in barramundi (modulated by the *FADS1* gene that has not been identified in this species). Thus far, no barramundi ortholog of any *FADS1-like* **Δ**5-desaturase, or LC-PUFA ecosanoid pathway has been identified, genes such as cyclooxygenase (*COX-1* or *COX-2*) or lipooxygenase (*ALOX5*). The ratio between levels of fatty acids in the liver or diet showed that DHA, as well total LC-PUFA, with exception of EPA, are largely preserved (Figure 1C). Previous data from the same treatment showed a same profile in the fillet and whole fish fatty acid profile, with a higher preservation of DHA compared with EPA (Salini et al. 2014). Experiments realized with barramundi juveniles (Mohd-Yusof et al. 2010; Tu et al. 2012), were observed as well a preference for elongation of 18 carbon n6 PUFA (18:3n6 more than 18:4n3), and a preference for 20 carbon n3 PUFA (22:5n3 more than 22:4n6) corroborating with the results found in the present study.

Pathways modified by FO substitution with PO

The results of gene expression showed that the fish fed with poultry oil had a higher level of expression of both lipogenic genes (*Lc acyl*; *Lc FAS*; *Lc SCD*; *Lc ACC*) and lipolytic genes (*Lc CPT1a*, *Lc ACADVL*, *Lc HADHB*, *Lc ACOX1* and *Lc pACAA1*) compared with the other groups. These effects on gene expression can be potentially explained by 2 distinct mechanisms: PUFA-specific inhibition of lipogenesis or MUFA-specific stimulation of β-oxidation. Results obtained in fish and mammals, to different tissues, showed a significant reduction of gene expression in the animals fed with diets containing high PUFA percentage (Blake and Clarke 1990; Fukuda et al. 1992; Panserat et al. 2008; Torstensen et al. 2009). Similar than observed in this study, the regulation of gene expression by a diet with a higher PUFA percentage inhibited the expression of *FAS* in rats and fish, and also suppressed the activity and expression of *acyl* (Fukuda and Iritani 1999), *ACC* (Toussant et al. 1981) and *SCD* (Ntambe 1992; Figueiredo-Silva et al. 2012; Wade et al. 2015). Different profile was observed in study realized with Atlantic salmon juveniles, with an increase in the SCD expression in fish fed with LC-PUFA diets (Jordal et al. 2005; Berge et al. 2004).

This study also identified several barramundi orthologs of genes that regulate β-oxidation, and each of the genes examined (*Lc CPT1a*, *Lc ACADVL*, *Lc ECH*, *Lc HADHB*, *Lc ACOX1* and *Lc pACAA1*) were significantly upregulated in fish fed with poultry oil. Similar results, as a lower *CPT1a* expression in response to elevated PUFA levels in the diet were observed by Wade et al. (2015) in experiments using the same species. Previous studies with Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) showed high levels of oxidation of fatty acids such as 18:1n9, 18:2n6 and 18:3n3 in those fish fed diets containing vegetable oils rich in these fatty acids (Henderson and Sargent 1984; Bell et al. 2003; Torstensen et al. 2004, Stubhaug et al. 2005, Turchini and Mailer, 2011). Certain types of fatty acids may be readily oxidized for energy production, although others such as LC-PUFAs may be preserved because of their important physiological functions (Sargent 1999, 2002; Glencross 2009). Thus, the increase the expression of β-oxidation genes in barramundi fed a high concentration of these fatty acids in PO diet, in particular due to the high percentage of 18:1n9. However, some reports of gene expression related to β-oxidation are contradictory indicating that the expression of these genes can be altered under the influence of several variables. Atlantic salmon fed diets containing a high percentage of MUFA, especially 18:1n9, did not show significant alterations in *CPT1a* expression compared with animals fed with diets containing high LC-PUFA percentage (Leaver et al. 2008). *CPT1a* is responsible for the transport of long-chain fatty acids into the mitochondrial matrix to be oxidized, and can also be considered the limiting step of mitochondrial β-oxidation (Power and Newsholme, 1997, Frøyland et al. 1998, Turchini et al. 2003). Alterations in the *CPT1a* expression can change all of the mitochondrial β-oxidation pathway, since the initial step, performed by very-long-chain acyl-CoA dehydrogenase (ACADVL) and the subsequent, enoy-CoA hydratase (ECH) and 3 -hydroxyacyl-CoA dehydrogensae (HADHB) mediated reactions, and this observation can explain the high level of expression of these genes for fish fed with poultry oil (Reddy and Hashimoto 2001).

Different postprandial responses according the pathway

The liver is largely a lipogenic tissue with a high potential to catabolise lipid through mitochondrial or peroximal β-oxidation (Crockett & Sidell 1993). In mammals, lipid production is enhanced and lipid breakdown is slowed so that excess energy is stored in the form of fat, with this process reversed during times of starvation. In barramundi, the post feeding expression patterns of different lipogenic genes (*Lc acyl*; *Lc ACC*; *Lc FAS*; *Lc SCD*; *Lc FADS2*; *Lc elovl5*) were strongly correlated with each other. However, this expression was also correlated with the expression of key regulators of mitochondrial β-oxidation, *Lc CPT1a* (Wade et al. 2014) and *Lc HADHB*, but not regulators of peroxisomal β-oxidation, *Lc pACAA1* and *Lc ACOX*.

The acetyl CoA carboxylase (*ACC*) gene is considered a critical regulator of synthesis and β-oxidation of fatty acids because it produces a precursor molecule of fatty acid synthesis, malonyl CoA, which in turn acts as an inhibitor of *CPT1a* expression (McGarry et al. 1977; Hillgartner et al. 1995; Kim 1997; Bonnefont et al. 1999; Ryu et al. 2005). Different to mammals, barramundi presented a significant increase in the *Lc ACC* expression. In rats, the expression of *ACC* underwent a significant decrease following starvation, and consequently there was an increase in the expression of *CPT1a* suggesting an inverse action among fatty acid synthesis and β-oxidation process when compared with barramundi (Ryu et al. 2005). The results found in the present study for barramundi suggest that high *Lc ACC* expression may have influenced the low *Lc ACADVL*, *Lc ECH* and *Lc pACAA1* expression, based on the observed negative correlation between *Lc ACC* and these genes.

In summary, the results of the present study demonstrated that even under fasting conditions the expression of *Lc ACC*, *Lc elovl5* and *Lc FADS2* were regulated to promote fatty acid synthesis in the liver, the same pattern was verified to genes related with β-oxidation processes, as *Lc ACADVL*, *Lc ECH*, *Lc ACOX1* and *Lc pACAA1*, but for this genes was observed just a slight increase, supporting that in barramundi under fasting, β-oxidation processes supposedly can be mainly realized in non-lipogenic tissues as muscle.

Acknowledgements

This work was funded by CSIRO’s Agricultural Productivity Flagship. We acknowledge the technical support of Natalie Habilay, Nick Polymeris, Dylan Rylatt and Kinam Salee. Thanks to Greg Coman and Michael Salini for constructive comment and editorial on an early draft.

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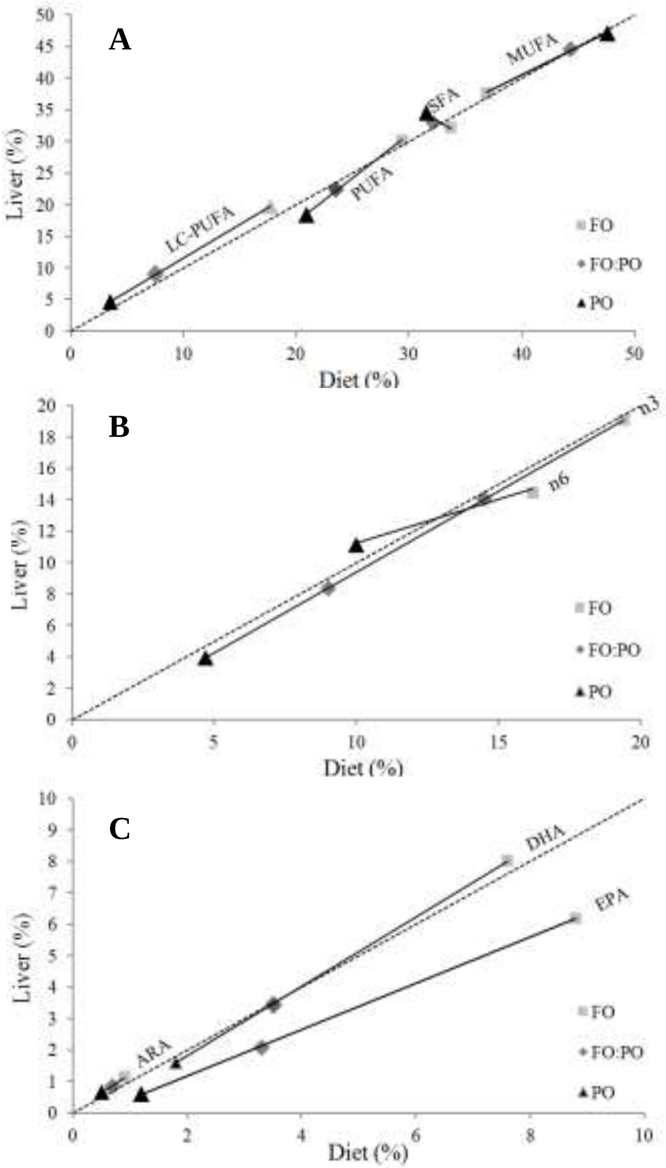
Figure Legends

Fig.1 Liver fatty acids (%) as a function of diet fatty acids (%) for different fatty acid classes (A), n-3 or n-6 PUFAs (B), or specific LC-PUFAs (C). A linear function was used to show how liver fatty acids changed after feeding diets containing fish oil (FO), poultry oil (PO) or an equal proportion of fish oil and poultry oil (FO:PO). A linear function (dashed line) indicates equal fatty acid levels in the liver and in the diet.

Fig.2 Relative expression levels of genes regulating fatty acid synthesis (A) and β-oxidation (B) after feeding diets containing fish oil (FO), poultry oil (PO) or an equal proportion of fish oil and poultry oil (FO:PO). Transcript levels of each gene were calculated relative to one another using raw cycle threshold values for each gene, normalized to Ef1α. Values shown are log2-fold change relative to the average Ct value for all genes. Superscripts denote significant ($P<0.05$) differences between the different diets.

Fig. 3 The relative change in the expression of several genes regulating aspects of fatty acid synthesis (*Lc Elovl5*, *Lc FADS2*, *Lc ACC*) or β-oxidation (*Lc ACADVL*, *Lc ECH*, *Lc HADHB*, *Lc pACAA1*, *Lc ACOX1*) over time after a single feeding event. Relative expression was calculated for each gene independently using raw cycle threshold values for each gene, normalized to an endogenous control (EF1*α*) and an exogenous control (*Luc*). Values shown are log2-fold change relative to the average Ct value for that gene prior to feeding. Different superscripts indicate significant differences between time points ($P \leq 0.05$).

Fig. 4 Person's correlation coefficient matrix showing potential relationships between the expression of genes regulating fatty acid synthesis(*Lc Elovl5*, *Lc FADS2*, *Lc ACC*) or β-oxidation (*Lc ACADVL*, *Lc ECH*, *Lc HADHB*, *Lc pACAA1*, *Lc ACOX1*). The colour of each *box* shows the relationship between the different parameters analysed in this study, with *dark red* highlighting positive relationships and *dark green* highlighting negative relationships. *Asterisks* denote significant ($P < 0.05$) correlations between two parameters.



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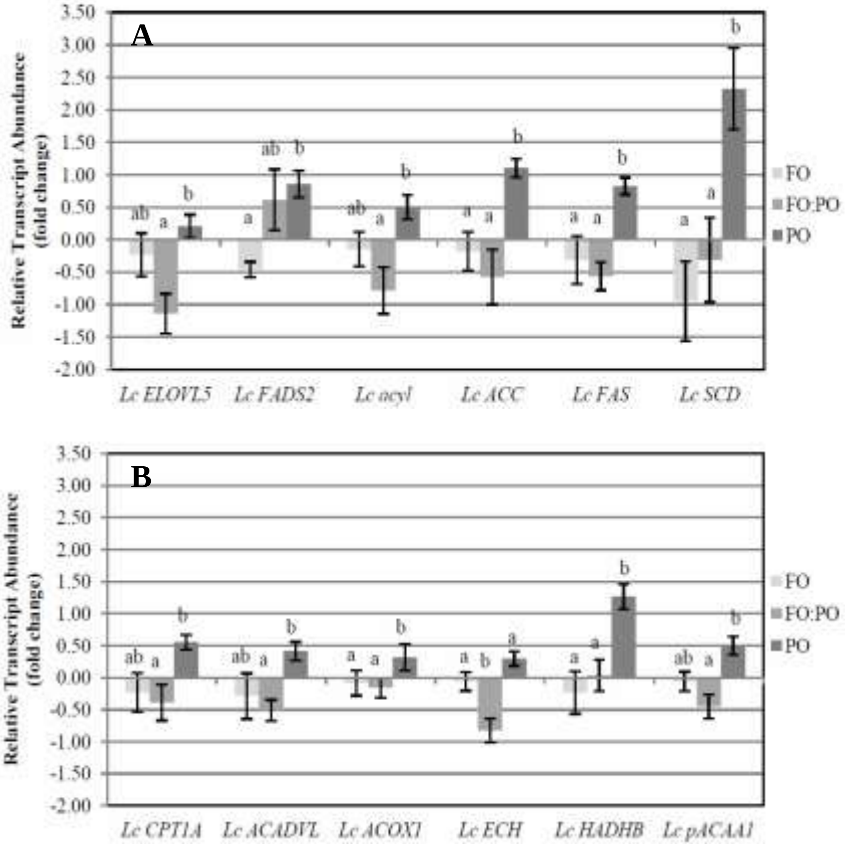

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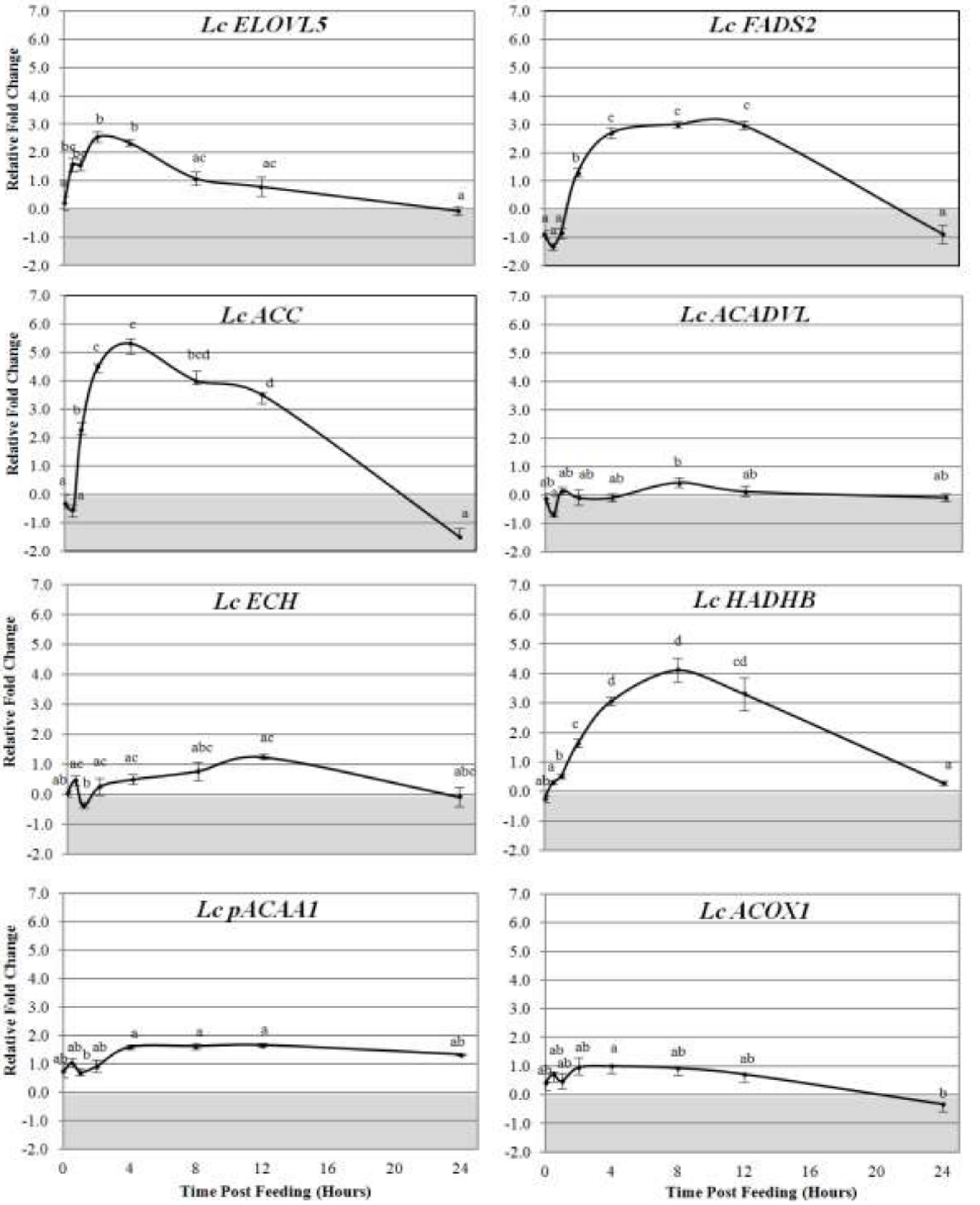

Fig. 3 The relative change in the expression of several genes regulating aspects of fatty acid synthesis (*Lc Elovl5*, *Lc FADS2*, *Lc ACC*) or β-oxidation (*Lc ACADVL*, *Lc ECH*, *Lc HADHB*, *Lc pACAA1*, *Lc ACOX1*) over time after a single feeding event. Relative expression was calculated for each gene independently using raw cycle threshold values for each gene, normalized to an endogenous control (EF1*α*) and an exogenous control (*Luc*). Values shown are log2-fold change relative to the average Ct value for that gene prior to feeding. Different superscripts indicate significant differences between time points ($P \leq 0.05$).

	Lc FADS2	Lc ELOVL5	Lc ACC	Lc ACADVL	Lc ACOX1	Lc ECH	Lc HADHB
Lc FADS 2							
Lc ELOVL5	0.32						
Lc ACC	0.85 *	0.71 *					
Lc ACADVL	0.59	-0.14	0.50				
Lc ACOX1	0.64	0.76 *	0.80 *	0.11			
Lc ECH	0.77 *	0.08	0.46	0.16	0.54		
Lc HADHB	0.97 *	0.30	0.80 *	0.60	0.63	0.78 *	
Lc pACAA1	0.78 *	-0.05	0.41	0.34	0.21	0.78 *	0.82 *

Fig. 4 Person's correlation coefficient matrix showing potential relationships between the expression of genes regulating fatty acid synthesis(*Lc elovl5*, *Lc FADS2*, *Lc ACC*) or β-oxidation (*Lc ACADVL*, *Lc ECH*, *Lc HADHB*, *Lc pACAA1*, *Lc ACOX1*). The colour of each *box* shows the relationship between the different parameters analysed in this study, with *dark red* highlighting positive relationships and *dark green* highlighting negative relationships. *Asterisks* denote significant ($P < 0.05$) correlations between two parameters.

Table 1 The formulations and chemical composition of experimental diets.

	Diet (g kg^{-1} dry matter)			
Ingredients	FO	FO:PO	PO	PR[*]
Fishmeal	150.0	150.0	150.0	655.0
Fish oil	85.0	25.5	0.0	94.0
Poultry oil	0.0	59.5	85.0	-
Wheat flour	119.0	119.0	119.0	145.0
Wheat gluten	85.0	85.0	85.0	100.0
Lupin kernel	100.0	100.0	100.0	-
Poultry meal	455.0	455.0	455.0	-
Premix vitamins[a]	5.0	5.0	5.0	5.0
Tapioca	0.0	0.0	0.0	-
Yttrium oxide	1.1	1.1	1.1	1.0
Chemical Composition				
Dry matter	908.2	977.6	981.4	909.0
Crude protein	518.8	543.8	539.4	547.0
Total lipid	149.7	157.0	161.9	150.0
Ash	92.9	91.3	93.8	102.0
Gross Energy (MJ/Kg^{-1} DM)	21.9	22.6	22.6	20.51

[*] Diet formulated to postprandial experiment.
All values are g kg^{-1} dry matter (DM) unless otherwise detailed
[a] Vitamin and mineral premix includes (IU kg^{-1} or g kg^{-1} of premix): vitamin A, 2.5MIU; vitamin D3, 0.25 MIU; vitamin E, 16.7 g; vitamin K,3, 1.7 g; vitamin B1, 2.5 g; vitamin B2, 4.2 g; vitamin B3, 25 g; vitamin B5, 8.3; vitamin B6, 2.0 g; vitamin B9, 0.8; vitamin B12, 0.005 g; biotin, 0.17 g; vitamin C, 75 g; choline, 166.7 g; inositol, 58.3 g; ethoxyquin, 20.8 g; copper, 2.5 g; ferrous iron, 10.0 g; magnesium, 16.6 g; manganese, 15.0 g; zinc, 25.0 g

Table 2 Experimental diets fatty acid composition (%).

	Diets (% of Total Fatty Acids)		
Fatty acids	FO	FO:PO	PO
14:0	4.7	2.4	1.4
16:0	21.1	22.7	22.9
18:0	5.6	6.5	6.7
16:1 (n7)	7.7	6.0	5.4
18:1(n7)	2.9	2.6	2.4
18:1 (n9)c	24.0	34.8	38.9
18:2 (n6)c	8.9	13.9	15.6
18:3 (n6)	0.3	ND	ND
20:3 (n6)	ND	ND	ND
20:4 (n6)	0.9	0.7	0.5
18:3 (n3)	1.1	1.5	1.7
18:4 (n3)	1.5	0.6	ND
20:5 (n3)	8.8	3.3	1.2
22:5 (n3)	ND	ND	ND
22:6 (n3)	7.6	3.5	1.8
Total (n3)	19.4	9.0	4.7
Total (n6)	10.0	14.5	16.2
Total (n9)	25.5	35.4	39.4
SFA	33.7	32.1	31.5
MUFA	36.9	44.3	47.6
PUFA	29.4	23.5	20.9
LC-PUFA	17.7	7.5	3.5
n3/n6	1.9	0.6	0.3

Table 3 Target genes of lipid synthesis and β-oxidation in barramundi, and the primer sequences used to analyse their expression.

Target gene	Gene abbreviation	EC number		Sequence
Synthesis				
Elongase 5	*Lc ELOVL5*	EC 2.3.1.n8	For	ATCCAGTTCTTCTTAACCGT
			Rev	GGTTTCTCAAATGTCAATCCAC
Fatty Acid Desaturase 6	*Lc FADS2*	EC 1.14.19.-	For	TCATACTACCTTCGCTACTTCTC
			Rev	ACAAACCAGTGACTCTCCAG
ATP Citrate Lyase	*Lc ACYL*	EC 2.3.3.8	For	CAACACCATTGTCTGTGCTC
			Rev	GAAATGCTGCTTAACAAAGTCC
Acetyl CoA Carboxylase	*Lc ACC*	EC 6.4.1.2	For	TTGATAGCTTCCCACCTTCC
			Rev	ATCCTGACCACCTGATTACT
Fatty Acid Synthase	*Lc FAS*	EC 2.3.1.85	For	TGAATCTCACCACGCTTCAG
			Rev	GGTTTCTCAAATGTCAATCCAC
Stearoyl CoA Desaturase	*Lc SCD*	EC 1.14.19.1	For	CCTGGTACTTCTGGGGTGAA
			Rev	AAGGGGAATGTGTGGTGGTA
β-Oxidation				
Carnitine palmitoyltransferase	*Lc CPT1A*	EC 2.3.1.21	For	TGATGGTTATGGGGTGTCCT
			Rev	CGGCTCTCTTCAACTTTGCT
Acyl CoA dehydrogenase (very long chain)	*Lc ACADVL*	EC 1.3.8.9	For	GTGCCATTGACATCTACTCC
			Rev	AAACCACTCCTCCATTCTCC
Acyl-CoA Oxidase	*Lc ACOX1*	EC 1.3.3.6	For	CATTGTGGTCGGAGATATTGG
			Rev	CACCTTGGCGTATTTCATCAG
Enoyl CoA Hydratase	*Lc ECH*	EC 4.2.1.17	For	ACAAGAAGCCAAGCAATCAG
			Rev	CTTTAGCCATAGCAGAGACC
ß-hydroxybutyrate dehydrogenase	*Lc HADHB*	EC 1.1.1.30	For	TCTGTCTTGCCATGAAATCC
			Rev	AAAGGTGTTGTGAATCGGTG
3-ketoacyl-CoA thiolase	*Lc pACAA1*	EC 2.3.1.16	For	CCTGATGTTATGGGTATTGGA
			Rev	GCCTCGTTGATTTCAAACAC

Table 4 Liver tissue fatty acid composition.

	Liver (% of Total Fatty Acids)		
Fatty acids	FO	FO:PO	PO
14:0	2.96 ± 0.17^{a}	1.58 ± 0.09^{b}	1.14 ± 0.07^{c}
16:0	20.31 ± 0.19^{a}	21.71 ± 0.34^{ab}	22.95 ± 0.77^{b}
18:0	7.71 ± 0.27^{a}	8.88 ± 0.38^{ab}	9.47 ± 0.46^{b}
16:1 (n7)	5.53 ± 0.25^{a}	4.18 ± 0.22^{b}	3.55 ± 0.19^{b}
18:1 (n7)	3.27 ± 0.03^{a}	2.91 ± 0.05^{b}	2.71 ± 0.06^{b}
18:1 (n9)c	26.76 ± 0.32^{a}	35.94 ± 0.32^{b}	39.29 ± 0.61^{c}
18:2 (n6)c	7.16 ± 0.35^{a}	9.22 ± 0.34^{b}	9.20 ± 0.58^{b}
18:3 (n6)	0.89 ± 0.17^{a}	1.61 ± 0.10^{b}	1.93 ± 0.16^{b}
20:3 (n6)	0.34 ± 0.02^{a}	0.57 ± 0.03^{b}	0.71 ± 0.06^{c}
20:4 (n6)	1.14 ± 0.07^{a}	0.85 ± 0.04^{b}	0.67 ± 0.03^{b}
18:3 (n3)	0.72 ± 0.06^{a}	0.81 ± 0.07^{ab}	0.73 ± 0.08^{b}
18:4 (n3)	0.93 ± 0.05^{a}	0.47 ± 0.02^{b}	0.30 ± 0.02^{c}
20:5 (n3)	6.19 ± 0.10^{a}	2.09 ± 0.08^{b}	0.60 ± 0.04^{c}
22:5 (n3)	3.07 ± 0.15^{a}	1.30 ± 0.06^{b}	0.56 ± 0.03^{c}
22:6 (n3)	8.01 ± 0.45^{a}	3.43 ± 0.15^{b}	1.61 ± 0.06^{c}
Total (n3)	19.05 ± 0.54^{a}	8.35 ± 0.30^{b}	3.95 ± 0.17^{c}
Total (n6)	11.14 ± 0.14^{a}	14.06 ± 0.21^{b}	14.46 ± 0.50^{b}
Total (n9)	28.20 ± 0.34^{a}	37.10 ± 0.33^{b}	40.42 ± 0.61^{c}
SFA	32.13 ± 0.24	33.00 ± 0.50	34.51 ± 1.23
MUFA	37.68 ± 0.48^{a}	44.60 ± 0.34^{a}	47.08 ± 0.69^{b}
PUFA	30.19 ± 0.56^{a}	22.40 ± 0.50^{b}	18.41 ± 0.66^{c}
LC-PUFA	19.77 ± 0.69^{a}	9.00 ± 0.31^{b}	4.65 ± 0.19^{c}
n3/n6	1.71 ± 0.05^{a}	0.59 ± 0.01^{b}	0.27 ± 0.01^{c}