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Dietary alpha-linolenic acid does not enhance accumulation of omega-3 long-chain polyunsaturated fatty acids in barramundi (*Lates calcarifer*)

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ABSTRACT

This study examined the effects of substituting fish oil and fish meal with a blend of alpha-linolenic acid (ALA, 18:3 n-3) rich vegetable oils (14%, w/w) and defatted poultry meal (34%, w/w) in a formulated diet, on growth and tissue fatty acid profiles in barramundi fingerlings. Results indicated that on average, while the ALA levels of the barramundi liver and fillet increased with increasing dietary ALA, there was no corresponding increase in the levels of the omega-3 (n-3) long chain polyunsaturated fatty acid (LCPUFA). Compared to fish consuming a commercial feed, which contained fish meal and fish oil, fish on the ALA diets grew slower, had a lower feed intake and lower n-3 LCPUFA levels in the tissues. Hepatic mRNA expression of Δ6 desaturase (FADS2) and elongase (ELOVL5/2) were ~10 fold and ~3 fold higher, respectively, in all the ALA dietary groups, relative to those fed the commercial feed. However, the level of expression of the two genes was not different between fish fed differing ALA levels. These data demonstrate that increasing the ALA level of the diet is not an appropriate strategy for replacing marine sources of n-3 LCPUFA in barramundi. It was also noted, however, that within the different ALA dietary groups there was a large amount of variation between individual fish in their tissue DHA levels, suggesting a significant heterogeneity in their capacity for conversion of ALA and/or retention of n-3 LCPUFA. When dietary ALA intakes were greater than 0.8%en, tissue DHA levels were inversely related to ALA intake, suggesting that high intake of dietary ALA may inhibit DHA synthesis.

Keywords: alpha-linolenic acid (ALA, 18:3n-3), desaturase (FADS), elongase (ELOVL), long chain polyunsaturated fatty acid (LCPUFA), aquaculture nutrition

1. Introduction

Fish have long been considered as a major source of high quality protein and long-chain polyunsaturated fatty acids (LCPUFA) for both human consumption and as feed ingredients for farmed fish and other food animals (Howe et al. 2002; Sioutis et al. 2008; Tacon et al. 2008). Although aquaculture is expected to meet much of the growing consumer demand for fish supply in the future, the substantial use of fish oils in this industry has raised concerns that the rapid expansion of the aquaculture industry will put more pressure on already declining marine resources (Tidwell et al. 2001). Unless alternatives for fish meal and fish oil are developed, aquaculture will continue to use vast quantities of fish meal and fish oil derived from wild-catch fish (Bostock et al. 2010). Thus, replacing fish oil in aquaculture feed with vegetable oils rich in 18-carbon α-linolenic acid (ALA, 18:3 n-3), a precursor of eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3), may help to reduce the dependence of the aquaculture industry on wild fisheries.

It has been reported that feed for freshwater and herbivorous or omnivorous farmed fish species such as Murray cod (*Maccullochella peelii*) and tilapia (*Oreochromis niloticus*) does not need to contain fish meal or fish oil, because these fish have the capacity to convert 18-carbon PUFA to their long-chain derivatives, and are able to maintain a consistent n-3 LCPUFA status when fed with diets high in 18-carbon PUFA (de Souza et al. 2007; Francis et al. 2007). In contrast, carnivorous marine species such as gilthead sea bream (*Sparus aurata*) and turbot (*Scophthalmus maximus*) are unable to produce LCPUFA from ALA to any physiologically relevant extent, due to apparent deficiencies

in one or more steps of the fatty acid metabolic pathway (Ghioni et al. 1999; Tocher et al. 1999). Diadromous fish such as barramundi (*Lates calcarifer*) are of particular interest in relation to LCPUFA biosynthesis because of the known differences in dietary PUFA requirements and enzyme capabilities of converting PUFA to LCPUFA between marine and freshwater species. Studies have been undertaken to examine the nutritional requirements of farmed barramundi and most of these studies suggest that this species requires high amounts of protein (crude protein 40−50%), consistent with the carnivorous/piscivorous nature of the fish (Catacutan et al. 1995; Williams et al. 2003; Glencross 2006). Fish meal can be replaced by meat meal as the predominant protein source in diets for juvenile barramundi (Williams et al. 2003) and part of the fish oil can be replaced by vegetable oils without compromising growth, feed conversion ratio or body protein and fat contents (Raso et al. 2003). However, all previous feeding trials included at least some fish meal or fish oil in diets, making it difficult to draw clear conclusions as to the ability of these fish to derive n-3 LPCUFA from 18-carbon PUFA precursors.

To eliminate the interference effects of LCPUFA in fish by-products on the endogenous LCPUFA synthesis of barramundi fingerlings, non-marine proteins and ALA-rich vegetable oils were used in this study to determine 1) effects of ALA-based, non-marine diets on fish growth and 2) whether barramundi are capable of converting ALA to n-3 LCPUFA.

2. Materials and methods

2.1. Fish management

All experimental procedures were performed in accordance with institutional guidelines for the use of animals and the Australian code of practice for the care and use of animals for scientific purpose. The protocol was approved by the Animal Ethics Committee, University of Adelaide (Ethics number S-28-08). Australian barramundi fingerlings at ~5 g were obtained from a commercial supplier (W. B. A. Hatcheries, SA, Australia). All fish were kept in a 1000 L tank provided with flow through temperature controlled (28°C) seawater and situated within an environmentally controlled aquarium room. Fish were allowed 3 weeks for acclimation to these conditions prior to being fed the experimental diets. During the acclimation phase, all fish were fed with the washout diet (5% total n-6 and 0.4% total n-3 of total fatty acids; LA:ALA ratio of 15.7) (Tables 1 and 3) to dilute tissue LCPUFA levels and ensure that these were consistent in all fish before initiation of the experiment. After the acclimation, the fish were sampled from the holding tank and relocated into the experimental tanks in batches according to their body weight.

All diets were fed *ad libitum* to apparent satiation by hand twice daily (9:00 am and 3:30 pm). The trial was performed at a constant water temperature of 28°C. The water in each tank was monitored daily for temperature, pH and dissolved oxygen content and weekly for salinity to assess water quality. Photoperiod was held to a constant 12 hr light/12 hr dark cycle. The seawater had a salinity of 37 g/L and a pH of 8 and the oxygen level was 95% saturation throughout the trial.

2.2. Diets

The formulation, nutrient and fatty acid composition of the diets are given in Tables 1, 2 and 3, respectively. The diets used in the study were designed based on recommended levels of protein, lipid and micronutrients included in the standard barramundi diet used commercially (Glencross 2006). The nutrient composition of the diets was analysed by Agrifood Technology Pty Ltd (Victoria, Australia). The fatty acid composition of the feeds and tissues were determined using procedures described previously (Tu et al. 2010). The energy values were calculated based on the standard physiological fuel values for protein, fat and carbohydrate of 4, 9 and 4 kcal/g, respectively. The ALA% en and linoleic acid (LA, 18:2 n-6) % en were calculated as: fatty acid (% en) = [(% fatty acid in diets) × fat% en] × 100. Thus, LA provided 2.4% en and ALA provided 0.1−3.2% en among all five ALA diets used in the study. The vegetable oil blends (14%, w/w) of the diets were prepared by mixing varying proportions of macadamia oil (2.13% LA and 0.2% ALA of total fatty acids; Suncoast Gold Macadamias Limited, Australia), flaxseed oil (18.64% LA and 51.61% ALA of total fatty acids; Melrose Laboratories Pty Ltd, Australia) and/or sunflower oil (60.9% LA and 0.04% ALA of total fatty acids; Buona Cucino, Italy). The three major protein ingredients of the diets were defatted poultry meal (34%, w/w) (Poultry BP meal refined grade, Skretting, TAS, Australia), soybean protein concentrates (25%, w/w) (Inpak foods, SA, Australia) and low fat wheat gluten (12%, w/w) (Inpak foods, SA, Australia). Other ingredients used for formulating the diets were gelatinised wheat starch (10%, w/w) (Inpak foods, SA, Australia), choline chloride (3%, w/w) and vitamin min/premix (2%, w/w) (Lienert, SA, Australia). All ingredients were

dry-mixed thoroughly to form the feed dough. The soft feed dough was cold extruded, pelleted and dried. The commercial barramundi feed (Grobest Corporation Co., Ltd., Thailand) was purchased from W. B. A. Hatcheries and used as a reference diet. The barramundi were maintained on the washout diet for 3 weeks and the test diets for 4 weeks.

2.3. Fish sampling

Fish were fasted for one day prior to weighing and sampling and were killed with an overdose of the anaesthetic, benzocaine. Eight fish were randomly selected from the 1000 L tank prior to the introduction of the washout diet and are referred to as the initial fish (before washout period). A further 8 fish were sampled immediately following the 3-week washout period and the remaining fish (8 fish per tank in triplicate for each experimental diet) were sampled at the end of the 4-week experimental period. Growth was determined by measuring following parameters: body length (mm) was measured from the tip of the snout to the end of the tail; body height (mm) was measured the distance from the dorsal fin to the ventral surface. Body weight (g) was measured after washout (AWB) and at the end of the experimental feeding trial (final body weight; FWB); weight gain (%) was calculated based on [(FWB − AWB) / AWB] × 100. Daily feed intake (g/day/fish) was calculated as (dry feed intake per fish (g) / feeding duration (day)). The daily feed intake of fish in a tank was calculated as the difference between the amount fed and the amount of waste feed collected and air-dried at 105°C overnight. The experimental set up comprised of 18 conical-based aqua research tanks (70 L) (3 tanks for each diet) with identical and independent recirculation systems, netting and a bottom

filter for uneaten feed collection as measurement of the amount of waste feed. Between 9:00−9:30 am and 3:30−4:00 pm each day, the uneaten feed was collected by manipulating the water current so that the waste feed and feces was collected on a mesh at the base of the tank. The feces were removed and only waste pellets were collected. Feed conversion ratio (FCR) was calculated as [dry feed intake (g) / (FBW − AWB)]. Survival (%) was calculated as [(initial fish number before feeding − dead fish number after feeding) / initial fish number before feeding] × 100. Hepatosomatic index (HSI, %) was calculated as [liver weight (g) / FBW (g)] × 100.

2.4. Fatty acid analyses of fillet and liver tissues

Total lipids were extracted from whole fillet (skin off) and liver using chloroform/methanol (2:1, v/v) solvent system. The extracted total lipids were weighed, phospholipid and triglyceride fractions separated from lipid extracts by thin layer chromatography (TLC) on silica gel plates (Silica gel 60H, Merck, Darmstadt, Germany). The solvent system for all TLC was petroleum spirit/diethyl ether/glacial acetic acid (180:30:2, v/v). Lipid classes were visualized with fluorescein 5-isothiocyanate against TLC standard 18-5 (Nuchek Prep Inc, MN). The phospholipids and triglycerides were methylated in 1% H_2SO_4 in methanol at 70°C for 3 hours. When cooled, the resulting methyl esters were extracted into n-heptane and transferred to vials containing anhydrous Na_2SO_4 as the dehydrating agent. Fatty acid methyl esters were separated and quantified using a Hewlett-Packard 6890 gas chromatography (Hewlett Packard, CA, USA) equipped with a 50 m capillary column (0.32 mm ID) coated with BPX-70 (0.25 μm film thickness, SGE Pty Ltd, Ringwood, Victoria, Australia). The injector temperature was set

179 at 250°C and the detector (flame ionization) temperature at 300°C. The initial oven
180 temperature was 140^{o}C and was programmed to rise to 220°C at 5°C per minute. Helium
181 was used as the carrier gas at a velocity of 35 cm per second. Fatty acid methyl esters
182 were identified based on the retention time to authentic lipid standards obtained from
183 Nuchek Prep Inc. (Elysian, MN).
184
185 **2.5. RNA isolation and quantitative real-time PCR (qRT-PCR)**
186 Total RNA was isolated from 10 mg of fish liver using a Qiagen RNeasy kit (Qiagen,
187 Victoria, Australia) following the protocol provided by the manufacturer with the tissue
188 initially disrupted using a Tissue Lyser (Mixer MM 300; F. Kurt Retsch GmbH & Co.
189 KG, Haan, Germany). The quality of the RNA was determined by measuring the ratio of
190 OD 260/280 and 260/230. The RNA concentration was determined by measuring the
191 absorbance at 260 and 280 nm and RNA integrity was confirmed by 1.5% agarose gel
192 electrophoresis. Procedures for liver tissue disruption, RNA isolation and RNA quality
193 determination were as described previously (Tu et al. 2012a; Tu et al. 2012b). Relative
194 gene expression levels were determined by one-step qRT-PCR using SYBR green
195 fluorescence. The abundance of each mRNA transcript was measured and expression
196 relative to β-actin calculated using the comparative Ct method (Q-gene qRT-PCR
197 analysis software), which provides a quantitative measure of the relative abundance of a
198 specific transcript in the liver by the comparative Ct method which takes into account any
199 differences in the amplification efficiencies of the target and reference genes. The qRT-
200 PCR conditions, primer validation, quality control and detection procedures were as
201 described previously (Tu et al. 2012a; Tu et al. 2012b). Gene expression internal control

β-actin (GenBank accession no. **GU188683**) was selected as the most stable reference gene from barramundi reference gene candidates including 18S ribosomal RNA (18S rRNA; GenBank accession no. **GU188686**), elongation factor 1α (EF1α; GenBank accession no. **GU188685**), ribosomal protein LP1 (RPLP1α; GenBank accession no. **GU188684**) and glyceraldehydes-3-phosphate dehydrogenase (GAPDH; GenBank accession no. **GQ507430**) using geNorm software (http://medgen.ugent.be/~jvdesomp/genorm/#housekeepers). Primers used for assessing β-actin, FADS2 and ELOVL5/2 mRNA abundance were F5'TGCGTGACATCAAGGAGAAG3' and R5'AGGAAGGAAGGCTGGAAGAG3' with an amplicon size 175 bp for β-actin; F5'CTGGTCATCGATCGAAAGGT3' and R5'CTGCGCACATAAAGAGTGGA3' (GenBank accession no. **GU047383**) with an amplicon size 249 bp for FADS2; and F5'GTGCGTCCCTAAACAGCTTC3' and R5'GCACACATTGTCTGGGTCAC3' (GenBank accession no. **GU047382**) with an amplicon size 154 bp for ELOVL5/2. Primers were designed using the primer design software Primer3 (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi).

2.6. Statistical analysis

All fatty acid composition data and mean normalised expression data between the different dietary groups were tested using one-way ANOVA followed by the Tukey-Kramer multiple comparisons test if *P* value was less than 0.05. Data were sampled from populations which had identical SD and followed Gaussian distributions. Kruskal-Wallis post test with Dunn's multiple comparison test was applied for non-parametric analyses of groups with $N < 3$ or data which did not pass the normality test. An unpaired t-test was

used to examine differences between growth measurements and fatty acid compositions between initial and after washout period fish. Correlation results are presented as Pearson correlation coefficients (r). A probability level of 0.05 ($P < 0.05$) was used in all tests. All analyses were performed using GraphPad InStat 3.10 software.

3. Results

3.1. Growth performance and feed efficiency

At the end of the 3-week washout diet, the fish had an average body weight of 6.7 g, length of 83 mm and height of 21.1 mm. During the 4-week experimental period, the overall survival rate ranged between 73.3% (ALA diet 2) and 97.8% (commercial feed) and did not differ significantly between any of the dietary groups (Table 4). The mean final body weight did not differ between the fish fed the various ALA diets but the fish fed the commercial feed were ~2.1-fold heavier ($P < 0.05$) than fish fed the ALA diets. A similar pattern was observed for other growth parameters including weight gain, length and height. However, these parameters were not different between fish fed the different ALA diets. A significant difference in feed intake between fish on the ALA diets (~0.1 g/fish/day) and the commercial diet (~0.4 g/fish/day) was also observed. Feed conversion ratio, however, was not significantly different between fish on the ALA diets and the commercial feed. Hepatosomatic index also showed no significant difference between the diets (Table 4).

3.2. Effect of washout diets, ALA diets and commercial feed on tissue phospholipid fatty acid compositions

In barramundi fingerlings that had previously been fed on a fish oil- and fish meal-based commercial diet, the fatty acid composition of the liver phospholipids was significantly altered after being maintained on the washout diet for 3 weeks (Table 5). Compared to the pre-washout fatty acid composition, the level of n-3 LCPUFA particularly EPA, DPA and DHA decreased significantly, while total monoenes (16:1 n-7, 18:1 n-9 and 18:1 n-7), which were present at high levels in the washout diet (macadamia oil) increased by 2.8 fold (Table 5). ALA in liver phospholipids was below the detection level after the washout diet period. Levels of EPA, DPA and DHA decreased by by 2.4−1.6 fold (Table 5). Similar fatty acid changes were also observed in fillet phospholipids (data not shown).

There were differences in levels of saturated fatty acids in fillets (~29−30% for fish fed the ALA diets and 35% for commercial diet fed fish; Table 6) and liver (~32−33% for fish fed ALA diets and 38% for commercial diet fed fish; Table 7) for fish fed different diets. On the other hand, the level of monounsaturates in ALA treated fish was higher in both fillet (~35−40% versus commercial feed 20%) and liver (~29−32% versus commercial feed 10%) (Tables 6 and 7). No differences were observed in total n-6 in fillet phospholipids with increasing dietary ALA, but in liver phospholipids the total n-6 was significantly lower in fish fed ALA diet 5. The fillet and liver LA and total n-6 levels were lower still in the fish fed the commercial diet relative to fish fed any of the ALA diets, while AA levels were higher in the fish fed the commercial diet compared to the ALA-based diets (Tables 6 and 7).

The ALA level of fillet and liver phospholipids increased progressively with increasing dietary ALA content but this did not translate into increased levels of n-3 LCPUFA. No significant differences were observed in the level of n-3 LCPUFA in the fillet and liver phospholipids among fish on the ALA diets (Tables 6 and 7). The 18:4 n-3 level of liver phospholipid increased with increasing dietary ALA however the levels were all below 0.5% of fatty acids and 18:4 n-3 was not detected in fillet phospholipid (data not shown). Fish fed the commercial feed had, on average, 2.4-, 1.7- and 2-fold higher levels of EPA, docosapentaenoic acid (DPA, 22:5 n-3) and DHA, respectively, in their fillet phospholipids than fish fed the ALA diets (Table 4). Similar results were observed in liver phospholipids in which the EPA, DPA and DHA levels were 4.7-, 2- and 2.7-fold higher respectively, in fish on the commercial feed (Table 7).

3.3. Effect of ALA diets and commercial feed on mRNA expression level of FADS2 and ELOVL5/2 in the liver

The effect of dietary ALA level on the mRNA expression of Δ6 desaturase (FADS2) and elongase (ELOVL5/2) is shown in Figure 1. FADS2 (Figure 1A) and ELOVL5/2 (Figure 1B) mRNA expression did not differ between fish on the ALA dietary groups, however, the mRNA expression of both genes was lower in fish on the commercial feed compared to those on all of the ALA diets (Figure 2). On average, the relative expression of the FADS2 gene was 10-fold (Figure 2A) and ELOVL5/2 gene was 3.3-fold (Figure 2B) lower in fish on the commercial feed than in the fish on the ALA diets.

3.4. Effect of ALA diets and commercial feed on liver phospholipid DHA composition in individual fish

Scatter plots of liver DHA, the major n-3 LCPUFA, of the individual fish on the ALA diets and the commercial feed are presented in Figure 3. The results show higher levels of variability in the DHA in individual fish being fed the ALA diets compared to fish fed the commercial feed (Figure 3). In fish on the commercial feed, the DHA ranged from 26.7−34% of total fatty acids in livers (Figure 3). In contrast, DHA level varied from 5.9−21.2% in liver phospholipids of the fish on the ALA diet 1 and 4.2−34.3% in fish on the ALA diet 5. Similar ranges were also observed in liver phospholipid DHA content among fish on the ALA diets 2−4 (Figure 3). When the differences between individual fish in liver phospholipid DHA contents were expressed as coefficient of variation (CV%) values, the range of CV% was from 36.1 to 62.0 ± 5% for fish in the ALA diets compared to a CV% of only 5.6% for fish on the commercial feed.

To further analyse the possible underlying mechanisms of DHA synthesis in barramundi, we examined the correlation between ALA and DHA levels in fish liver phospholipids. Increases in dietary ALA from 0.1−0.5%en were associated with an increase in the hepatic ALA content, but there was no relationship between the level of ALA and DHA in the liver in the fish consuming diets within this ALA range (Figure 4A and 4B). However when dietary ALA levels were increased above 0.8%en, there was a significant negative correlation between liver ALA and DHA levels ($P < 0.0001$) (Figure 4C−E). There was no correlation between liver ALA and DHA levels in fish fed with the commercial diet (Figure 4F).

4. Discussion

There have been numerous studies indicating that most aquatic species have some form of demand for n-3 LCPUFA, particularly EPA and DHA, as essential dietary nutrients. However, the amount of these n-3 fatty acids in the diet and how the n-3 fatty acids are influenced by the presence of other fatty acids in the diets appear to vary among different fish species (Glencross et al. 2011). Previous studies have suggested that diadromous species may be able to obtain their fatty acid requirements from 18-carbon PUFA or have a reduced requirement for n-3 LCPUFA and some freshwater fish appear to have no demand for n-3 LCPUFA at all (Castell et al. 1972; Bell et al. 1986; Tocher et al. 1989). The differences between marine and freshwater fish can be accounted for by considering their natural diets in the food chain and particularly whether a species is carnivorous, omnivorous or herbivorous. In the case of barramundi, studies from the 1980s by Buranapanidgit *et. al.* (Buranapanidgit et al. 1988; Buranapanidgit et al. 1989) as cited by Glencross and Rutherford (Glencross et al. 2011) indicated that dietary LCPUFA levels, primarily as a mix of EPA and DHA, of 1−1.7% (w/w) of the diets were adequate to maintain good growth. Early studies by Dhert *et. al*. suggested that barramundi larvae fed on a n-3 LCPUFA fortified feed had a superior physiological condition which was reflected by significantly lower mortality figures during a stress test (Dhert et al. 1990). It has been reported that the diet-induced growth and tissue fatty acid changes were not affected by salinity or the interaction between salinity and diet, suggesting that it was primarily the species fatty acid metabolism/dietary lipid combination, rather than any effect of salinity per se, that determines barramundi growth and lipid synthesis (Alhazzaa

et al. 2011b). In our study, growth retardation was significant in fish fed the ALA diets (n-3 LCPUFA-free) compared to fish fed the commercial feed (n-3 LCPUFA-rich). Results from this study suggest that the n-3 LCPUFA are indeed essential for optimal growth and development, and that this requirement for n-3 LCPUFA is independent of the total dietary fat content.

This study confirmed that the fatty acid composition of the fillet and liver are directly influenced by the dietary fats as described previously by others (Mourente et al. 2006; Ji et al. 2011). The major fatty acids in the ALA diets in this present study were monounsaturated fatty acids and 18-carbon PUFA (LA and ALA) and while these fatty acids were shown to accumulate in fish tissues there was no increase in tissue n-3 LCPUFA levels. Nevertheless, despite the absence of fish oil and fish meal in the ALA diets, the tissues of fish on the ALA diets still contained low levels of EPA, DPA and DHA. This suggests that these fish may have capacity for retaining tissue n-3 LCPUFA or/and converting some small fraction of dietary ALA to LCPUFA (Schlechtriem et al. 2007). It has been reported that when barramundi were fed diets containing fish oil, echium oil (rich in ALA and 18:4 n-3) or rapeseed oil (rich in monounsaturates and LA), a fatty acid mass balance of fish whole body showed that barramundi on 18:4 n-3-rich feed bypassed the first rate-limiting step in n-3 LCPUFA biosynthesis pathway and 18:4 n-3 was elongated to 20:4 n-3 (Alhazzaa et al. 2011a). The fish, however, showed no accumulation of EPA and DHA but largely increases in whole body ALA and 18:4 n-3 levels, and to a lesser extent 20:4 n-3 (Alhazzaa et al. 2011a).

In our previous study (Tu et al. 2012b), we observed that the barramundi ELOVL5/2 showed efficient activity at elongating the homologous pairs 18-carbon to their 20-carbon metabolites. The recombinant barramundi ELOVL5/2 also showed high efficiency for utilising the 20-carbon substrates 20:5 n-3 and 20:4 n-6 to produce 22-carbon products but weak activity towards 22-carbon LCPUFA, indicating a high degree of activity with ELOVL5. In this current study, the hepatic mRNA expression of FADS2 and ELOVL5/2 genes was lower in fish on the commercial diet compared with those consuming ALA diets but this was not concentration dependent. This is consistent with a report suggesting that juvenile barramundi on diets high in monounsaturates and PUFA (ALA and LA) had a higher apparent Δ6 desaturase activity than the barramundi on fish oil diets (Alhazzaa et al. 2011a); however, no increases in the n-3 LCPUFA content of tissues was observed when the fish were fed vegetable oil diets (Alhazzaa et al. 2011a). Results from the present study are also consistent with the results of a study in European sea bass, in which 60% substitution of dietary fish oil by rapeseed oil or flaxseed oil resulted in a 5-fold increase of the FADS2 gene compared with fish which were on 100% fish oil diets (González-Rovira et al. 2009). Similar increased FADS2 mRNA expression levels were also observed in vegetable oil fed marine gilthead sea bream when compared with fish consuming fish oil-based diets (Izquierdo et al. 2008). In contrast, feeding freshwater zebra fish and Nile tilapia with vegetable oil diets that provided 1% (w/w) of LA and ALA resulted in increases of hepatocyte Δ6 desaturase activity and LCPUFA levels. In zebra fish, the main effect of the vegetable oil diet was to increase Δ6 desaturase activity and to produce the ALA desaturation product, 18:4 n-3. The same diet in tilapia induced activity of both enzymes in the fatty acid biosynthetic pathway and resulted in increases

of EPA and DHA. These differences suggested that the mechanism of LCPUFA biosynthesis is complex and highly species and habitat dependent.

Another important observation in our study was the significant variability within treatments in n-3 LCPUFA levels between individual fish in the ALA dietary groups. This suggests a high degree of heterogeneity between individuals in their capacity for metabolising ALA, incorporating tissue fatty acid and/or retaining n-3 LCPUFA. Further examination of the relationship between liver ALA and DHA levels revealed that when dietary ALA levels were above 0.8%en, a significant inverse relationship between hepatic ALA and DHA levels emerged. The reason for this are unclear, however, one possibility is that at high levels of ALA intake, the increased uptake of ALA into liver phospholipids acts to inhibit DHA synthesis and/or tissue incorporation.

A previous study has also shown appreciable levels of inter-individual variation between Atlantic salmon smolts fed on the same diets in the level of n-3 LCPUFA accumulating in the flesh (Schlechtriem et al. 2007). The variation in tissue n-3 LCPUFA levels within the ALA diet fed barramundi in the current study indicates that some fish may be suitable for selection for genetic improvement of aquaculture stocks to increase the capacity of these fish to convert ALA to n-3 LCPUFA and is an area that warrants further research.

In this study, the growth rate, length, height, weight gain and specific growth rate were all significantly lower in fish on the ALA diets than that of fish on the commercial feed; this decreased growth performance occurred despite the fact that total fat levels in the ALA

diets were ~3% higher than in the commercial feed. This implies, therefore, that fat or energy content may not be the major determinant for barramundi growth and performance, and that the type of lipid in the diet or the palatability also plays an important role. Therefore, it would be worthwhile to examine the effects of different proportions of fish oil with the balance of vegetable oil in fish diets on the ALA to LCPUFA conversion. Also, whether the poultry meal or other meat meal with the balance of fish meal provides the best amino acid composition for barramundi needs to be investigated.

In summary, 100% fish-oil and fish meal-based substitution with ALA-rich vegetable oil is not suitable for barramundi as the absence of fish oil and fish meal from the diet resulted in retardation in fish growth and decreases of all major n-3 LCPUFA in tissues. Although the ALA diets induced increases in hepatic mRNA abundances of FADS2 and ELOVL5/2 genes when compared to fish on the commercial feed, the increasing ALA level in the ALA diets showed no additional effect on the regulation of expression of FADS2 and ELOVL5/2 genes or the accumulation of tissue n-3 LCPUFA. However, it is noteworthy that the substantial variations between individual fish in their n-3 LCPUFA levels after exposure to the ALA diets, suggests significant heterogeneity in the capacity of barramundi for converting dietary ALA to DHA. Also, the negative relationship between liver ALA and DHA levels in fish fed the ALA-only diets suggests that DHA synthesis and/or incorporation may actually be inhibited by high intake of dietary ALA. Whilst it appears that replacing n-3 LCPUFA with ALA in the diets of barramundi is unlikely to be an effective strategy for increasing n-3 LCPUFA content, and is

detrimental to fish growth and survival, the heterogeneity in the response to the ALA diets and the suppression of DHA synthesis/incorporation at high levels of ALA intakes suggests that further studies are warranted.

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FIGURE CAPTIONS

Figure 1. The relative hepatic gene expression of FADS2 (A) and ELOVL5/2 (B) of fish fed the ALA diets 1−5. Relative transcript (mRNA) levels were determined by qRT-PCR and normalised by reference gene β-Actin. Abundance of mRNA is expressed as the mean normalised expression value. No significant differences were found between groups. Data are mean ± SEM (n = 21−24).

Figure 2. The relative hepatic gene expression of FADS2 (A) and ELOVL5/2 (B) of fish fed the ALA diets (mean value of the 5 ALA diets) and the commercial feed. Relative transcript (mRNA) levels were determined by qRT-PCR and normalised by reference gene β-Actin. Abundance of mRNA is expressed as the mean normalised expression value. Values with different superscripts are significantly different from each other ($P < 0.05$). Data are mean ± SEM (n = 21−24).

Figure 3. Individual variation in the DHA composition of liver phospholipids (PL) in fish on the ALA diets and the commercial feed. Scatter plots were plotted using DHA data from 20−24 fish per dietary treatments.

Figure 4. Pearson correlations (*r*) of ALA and DHA between liver phospholipids (PL) of fish fed the ALA diets (diets 1−5) (A−E) and the commercial feed (F), n=20−24.

Table 1. Formulation of the diets.

	Diets						
	Washout	ALA 1	ALA 2	ALA 3	ALA 4	ALA 5	Commercial [a]
Ingredient composition (% dry weight) [b]							
Defatted poultry meal	34	34	34	34	34	34	Protein Min 43% (w/w)
Soybean protein concentrate	25	25	25	25	25	25	Carbohydrate Max 15% (w/w)
Wheat gluten	12	12	12	12	12	12	Ash Max 13% (w/w)
Gelatinised wheat starch	10	10	10	10	10	10	Fibre Max 2% (w/w)
Vegetable oil	14	14	14	14	14	14	Fat Min 9% (w/w)
Oil proportion (% w/w in 14% fat)							
Macadamia oil	100	94	92	90	83	76	
Flaxseed oil	0	0	3	5.5	15	24	
Sunflower oil	0	6	5	4.5	2	0	
Choline chloride	3	3	3	3	3	3	
Vitamin/mineral premix	2	2	2	2	2	2	

[a] Commercial diet (Grobest Corporation Co., Ltd., Thailand) was purchased from W. B. A. Hatcheries.

[b] Macronutrients of commercial diet were provided by the Grobest Corporation Co., Ltd., Thailand.

Table 2. Nutrient composition of the diets.

	Diets						
	Washout	ALA 1	ALA 2	ALA 3	ALA 4	ALA 5	Commercial
Ingredient composition (% dry weight) [a]							
Crude protein (N × 6.25)	50.0	50.6	50.0	50.4	48.6	50.0	48.7
Crude fat (by acid hydrolysis)	12.7	13.7	12.5	13.0	12.7	11.4	9.9
Crude carbohydrate (by difference)	20.9	23.1	21.2	20.8	19.3	20.0	20.0
Total energy (kcal/kg)	3930.8	3976.3	3975.5	4019.9	3856.9	3825.6	3640.3
Energy (% en) [b]							
Carbohydrate	21.0	22.1	21.3	20.7	20.0	20.9	22.0
Fat	28.7	29.5	28.3	29.2	29.6	26.8	24.5
Protein	50.3	48.4	50.4	50.1	50.4	52.3	53.5

[a] Ingredient composition was analysed by Agrifood Technology Pty Ltd (Victoria, Australia).

[b] The calculation of calorie content was based on the standard physiological fuel values for carbohydrate, fat and protein of 4, 9 and 4, respectively.

Table 3. Fatty acid composition of the diets. Values are presented as % of total fatty acids. Data are means ± SEM of n = 3.

	Diets						
	Washout	ALA 1	ALA 2	ALA 3	ALA 4	ALA 5	Commercial
Fat% en	28.7	29.5	28.3	29.2	29.6	26.8	24.5
LA% en	1.4	2.5	2.3	2.4	2.5	2.3	3.3
ALA% en	0.1	0.1	0.5	0.8	2.2	3.2	0.4
LA:ALA Ratio	15.7	21.9	4.7	2.9	1.2	0.7	8.8
Fatty acids (%)							
16:0	9.2±0.0	9.1±0.0	8.8±0.0	8.8±0.0	8.8±0.0	8.5±0.0	22.1±0.4
18:0	3.8±0.0	3.7±0.0	3.9±0.0	3.9±0.0	4.0±0.0	4.1±0.0	5.2±0.1
Total saturates	17.5±0.0	17.0±0.0	16.9±0.0	16.9±0.0	16.6±0.0	16.1±0.0	33.1±0.4
16:1 n-7	15.0±0.1	13.8±0.1	14.4±0.1	13.7±0.1	12.6±0.1	11.9±0.1	3.9±0.1
18:1 n-9	55.4±0.2	54.2±0.2	52.8±0.1	52.2±0.2	49.3±0.2	46.4±0.1	16.8±0.2
18:1 n-7	3.6±0.0	3.6±0.1	3.6±0.1	3.5±0.0	3.3±0.1	3.1±0.1	2.5±0.1
Total monoenes	76.9±0.1	74.2±0.1	73.2±0.1	71.8±0.0	67.4±0.1	63.4±0.0	25.6±0.2
18:2 n-6 (LA)	5.0±0.0	8.3±0.0	8.0±0.0	8.3±0.0	8.5±0.0	8.6±0.0	13.3±0.2
20:4 n-6 (AA)	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	1.1±0.0
Total n-6	5.0±0.0	8.3±0.0	8.0±0.0	8.3±0.0	8.5±0.0	8.6±0.0	14.7±0.1
18:3 n-3 (ALA)	0.3±0.0	0.4±0.0	1.7±0.0	2.9±0.0	7.4±0.0	11.8±0.0	1.5±0.0
20:5 n-3 (EPA)	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	7.3±0.1
22:5 n-3 (DPA)	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	1.3±0.0
22:6 n-3 (DHA)	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	14.7±0.2
Total n-3	0.4 ± 0.0	0.4 ± 0.0	1.8 ± 0.0	3.0 ± 0.0	7.4 ± 0.0	11.8±0.0	25.2±0.1

N.D. = not detected.

Table 4. Growth measurement of barramundi on the ALA diets and the commercial feed. Values with different letters indicate significantly different from each other ($P < 0.05$) Data are means ± SEM of n = 24 per group, except FCR and survival where n=3 (tanks) per group.

	Diets					
Growth parameters	ALA 1	ALA 2	ALA 3	ALA 4	ALA 5	Commercial
Pre-washout body weight (g) [a]	5.2±0.3	5.2±0.3	5.2±0.3	5.2±0.3	5.2±0.3	5.2±0.3
After-washout body weight (g) [a]	6.7±0.3	6.7±0.3	6.7±0.3	6.7±0.3	6.7±0.3	6.7±0.3
Final body weight (g)	10.5±0.4 [a]	10.6±0.7 [a]	10.2±0.4 [a]	11.3±0.5 [a]	10.0±0.5 [a]	21.9±0.7 [b]
Length (mm)	95.0±1.0 [a]	95.0 ± 2.0 [a]	94.0±2.0 [a]	98.0±2.0 [a]	95.0±2.0 [a]	124.0±2.0 [b]
Height (mm)	23.8±0.3 [a]	23.7±0.5 [a]	23.3±0.4 [a]	24.9±0.4 [a]	23.7±0.5 [a]	30.4±0.3 [b]
Weight gain (%)	56.7±6.2 [a]	58.3±9.9 [a]	52.4±6.5 [a]	68.4±8.2 [a]	49.8±7.8 [a]	227.5±9.9 [b]
Feed intake (g/fish/d)	0.1±0.0 [a]	0.1±0.0 a	0.1±0.0 [a]	0.1±0.0 [a]	0.1±0.01 [a]	0.4±0.0 b
FCR [b]	0.9±0.0	1.0±0.0	1.0±0.2	0.8±0.1	1.0±0.1	0.7±0.0
Survival (%)	86.7±7.7	73.3±3.8	88.9±5.9	77.8±8.0	86.6±6.7	97.8±2.2
HSI (%) [c]	1.5±0.1	1.5±0.1	1.5±0.1	1.3±0.1	1.5±0.1	1.4±0.1

[a] Pre-washout and after-washout data are means ± SEM of n=8.

[b] Feed conversion ratio.

[c] Hepatosomatic index.

Table 5. Effects of 3-week washout diets on fatty acid compositions of barramundi liver phospholipids (PL). Fatty acid (%) is presented as % of total fatty acids. Values denoted with asterisk superscripts were significantly different from each other ($^{*}P < 0.05$ and $^{***}P < 0.001$) as determined by a two-tailed, unpaired t-test. Data are means ± SEM of n = 9−10.

Liver PL	Before washout	After washout
Fatty acids (%)		
16:0	18.5±0.3	15.0±0.7***
18:0	15.8±0.5	14.7±0.5
Total saturates	37.9±0.4	32.0±0.5***
16:1 n-7	< 0.05	1.3±0.4***
18:1 n-9	7.0±0.1	22.6±2.0***
18:1 n-7	1.9±0.0	3.1±0.1***
Total monoenes	10.3±0.1	28.7±2.4***
18:2 n-6 (LA)	7.6±0.3	8.8±0.3*
20:4 n-6 (AA)	5.4±0.1	4.2±0.2***
Total n-6	14.3±0.3	15.6±0.6
18:3 n-3 (ALA)	0.3±0.0	N.D.
20:5 n-3 (EPA)	5.8±0.1	2.4±0.4***
22:5 n-3 (DPA)	2.1±0.1	1.4±0.1***
22:6 n-3 (DHA)	28.5±0.4	18.0±2.2***
Total n-3	36.8±0.3	22.3±2.8***
Total PUFA	51.2±0.3	37.9±2.3***

Table 6. Effects of 4-week experimental diets on fatty acid composition of fish fillet phospholipids (PL). Fatty acid (%) is presented as % of total fatty acids. Different letters within a row indicate values are significantly different from each other ($P < 0.05$). Data are means ± SEM of n = 24 per group.

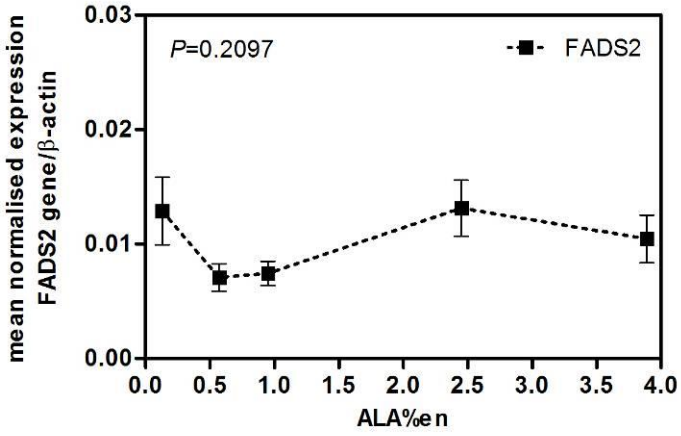
	Diets					
	ALA 1	ALA 2	ALA 3	ALA 4	ALA 5	Commercial
Fillet PL fatty acids (%)						
16:0	17.2±0.2 [a]	17.2±0.2 [a]	16.7±0.2 [a]	16.6±0.2 [a]	16.9±0.3 [a]	20.5±0.2 [b]
18:0	8.5±0.1 [a]	8.6±0.1 [a]	9.0±0.1 [b]	9.1±0.1 [b]	9.3±0.1 [b]	10.7±0.1 [c]
Total saturates	29.2±0.3 [a]	29.4±0.3 [a]	29.5±0.3 [a]	29.4±0.4 [a]	30.0±0.4 [a]	35.3±0.3 [b]
16:1 n-7	3.4±0.1 [a]	3.4±0.1 [a]	3.2±0.1 [a]	3.1±0.1 [ab]	2.7±0.1 [b]	1.3±0.1 [c]
18:1 n-9	30.9±0.4 [a]	30.2±0.5 [a]	29.3±0.5 [ab]	29.0±0.4 [ab]	27.0±0.6 [b]	14.8±0.2 [c]
18:1 n-7	3.3±0.1 [a]	3.3±0.1 [a]	3.3±0.1 [a]	3.3±0.1 [a]	3.0±0.1 [a]	2.5±0.1 [b]
Total monoenes	40.0±0.6 [a]	39.5±0.7 [a]	38.3±0.7 [ab]	38.0±0.6 [ab]	35.3±0.9 [b]	20.6±0.2 [c]
18:2 n-6 (LA)	10.1±0.2 [a]	10.1±0.3 [a]	9.9±0.2 [a]	10.0±0.2 [a]	10.4±0.2 [a]	7. 3±0.2 [b]
20:4 n-6 (AA)	2.0±0.1 [a]	2.09±0.1 [a]	2.2±0.1 [ab]	2.1±0.1 [ab]	2.5±0.1 [b]	2.8±0.0 [c]
Total n-6	14.7±0.2 [a]	14.6±0.4 [a]	14.5±0.2 [a]	14.5±0.2 [a]	15.2±0.3 [a]	12.5±0.2 [b]
18:3 n-3 (ALA)	0.2±0.0 [a]	0.5±0.0 [bc]	0.7±0.0 [cd]	1.7±0.1 [d]	2.2±0.2 [d]	0.3±0.0 [ab]
20:5 n-3 (EPA)	1.8±0.1 [a]	1.8±0.1 [a]	2±0.1 [a]	1.8±0.1 [a]	2.2±0.2 [a]	4.7±0.1 [b]
22:5 n-3 (DPA)	1.2±0.0 [a]	1.2±0.1 [a]	1.3±0.1 [a]	1.2±0.1 [a]	1.3±0.1 [a]	2.1±0.0 [b]
22:6 n-3 (DHA)	11.3±0.4 [a]	11.5±0.4 [a]	12.4±0.4 [a]	12.1±0.4 [a]	12.7±0.5 [a]	24.0±0.2 [b]
Total n-3	14.6±0.5 [a]	15.1±0.5 [a]	16.5±0.6 [ab]	16.9±0.4 [ab]	18.5±0.5 [b]	31.1±0.2 [c]
Total PUFA	29.2±0.5 [a]	29.7±0.6 [a]	31.0±0.5 [ab]	31.4±0.4 [ab]	33.7±0.7 [b]	43.7±0.2 [c]

Table 7. Effects of 4-week experimental diets on fatty acid composition of fish liver phospholipids (PL). Fatty acid (%) is presented as % of total fatty acids. Different letters within a row indicate values are significantly different from each other ($P < 0.05$). Data are means ± SEM of n = 24 per group.

	Diets					
	ALA 1	ALA 2	ALA 3	ALA 4	ALA 5	Commercial
Liver PL fatty acids (%)						
16:0	14.4±0.3 [a]	14.3±0.4 [a]	14.5±0.4 [a]	14.4±0.4 [a]	15.2±0.4 [a]	19.7±0.2 [b]
18:0	14.6±0.2	14.3±0.3	14.9±0.3	14.9±0.3	14.9±0.4	15.0±0.3
Total saturates	33.2±0.4 [a]	32.5±0.4 [a]	33.4±0.4 [a]	33.4±0.3 [a]	33.8±0.8 [a]	38.5±0.3 [b]
16:1 n-7	2.4±0.1 [a]	2.6±0.1 [a]	2.3±0.1 [a]	2.3±0.1 [a]	2.3±0.1 [a]	0.72±0.0 [b]
18:1 n-9	24.9±0.8 [a]	24.8±1.1 [a]	24.1±0.9 [a]	23.2±0.8 [a]	21.8±1.1 [a]	6.3±0.1 [b]
18:1 n-7	2.6±0.0 [a]	2.7±0.1 [a]	2.6±0.1 [a]	2.5±0.0 [a]	2.4±0.1 [a]	1.6±0.0 [b]
Total monoenes	32.1±0.9 [a]	32.5±1.3 [a]	31.4±1.1 [a]	30.9±0.9 [a]	29.5±1.3 [a]	10.3±0.2 [b]
18:2 n-6 (LA)	11.2±0.2 [a]	11.1±0.3 [a]	11.2±0.3 [a]	11.20.3 [a]	10.5±0.3 [a]	4.6±0.1 [b]
20:4 n-6 (AA)	3.0±0.1 [a]	2.9±0.1 [a]	3.0±0.1 [a]	2.9±0.1 [a]	2.9±0.1 [a]	4.8±0.1 [b]
Total n-6	19.2±0.2 [a]	18.7±0.4 [ab]	18.5±0.3 [ab]	18.2±0.4 [ab]	17.6±0.4 [b]	12.2±0.2 [c]
18:3 n-3 (ALA)	0.1±0.0 [a]	0.5±0.0 [bc]	0.7±0.0 [cd]	1.9±0.1 [d]	2.8±0.2 [d]	0.2±0.0 [ab]
20:5 n-3 (EPA)	1.1±0.1 [a]	1.0±0.1 [a]	1.3±0.2 [a]	1.2±0.1 [a]	1.2±0.2 [a]	5.5±0.1 [b]
22:5 n-3 (DPA)	1.0±0.1 [a]	1.0±0.1 [a]	1.0±0.1 [a]	1.0±0.0 [a]	1.0±0.1 [a]	2.0±0.1 [b]
22:6 n-3 (DHA)	10.8±0.8 [a]	11.5±1.5 [a]	11.7±1.0 [a]	11.4±1.2 [a]	11.9±1.6 [a]	30.8±0.4 [b]
Total n-3	13.1±1.0 [a]	14.0±1.6 [a]	14.7±1.2 [a]	15.6±1.2 [a]	17.2±1.6 [a]	38.6±0.3 [b]
Total PUFA	32.3±0.8 [a]	32.7±1.3 [a]	33.3±1.0 [a]	33.8±0.9 [a]	34.7±1.3 [a]	50.8±0.3 [b]

Figure 1.

A


mean normalised expression
FADS2 gene/β-actin
P=0.2097
FADS2
0.03
0.02
0.01
0.00
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
ALA%en

B

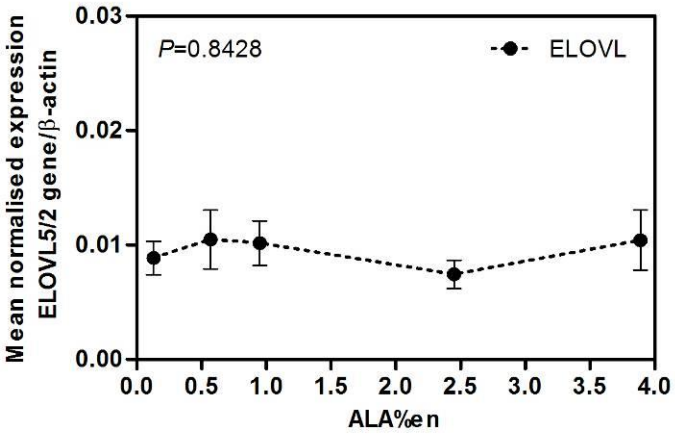
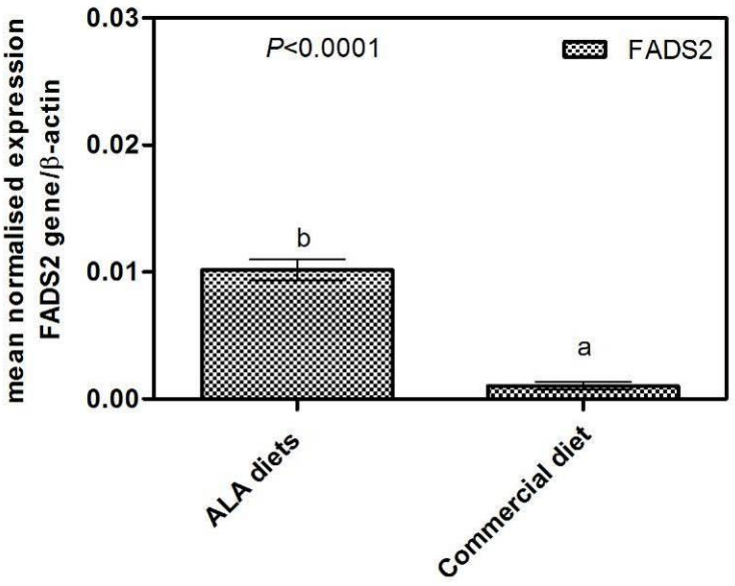

Mean normalised expression
ELOVL5/2 gene/β-actin
P=0.8428
ELOVL
0.03
0.02
0.01
0.00
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
ALA%en

Figure 2.

A


mean normalised expression
FADS2 gene/β-actin
0.03
0.02
0.01
0.00
P<0.0001
FADS2
b
a
ALA diets
Commercial diet

B

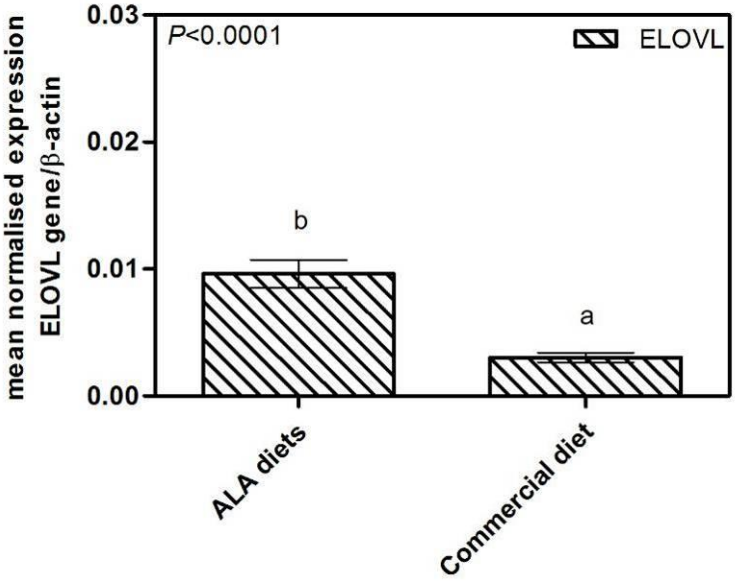

mean normalised expression
ELOVL gene/β-actin
0.03
0.02
0.01
0.00
P<0.0001
ELOVL
b
a
ALA diets
Commercial diet

Figure 3.

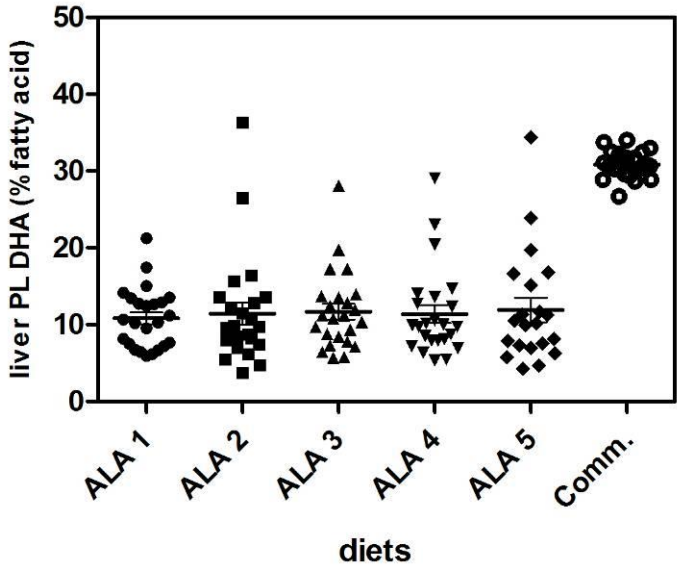
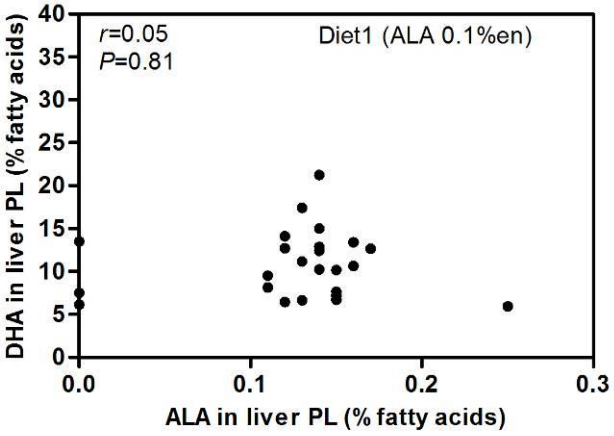
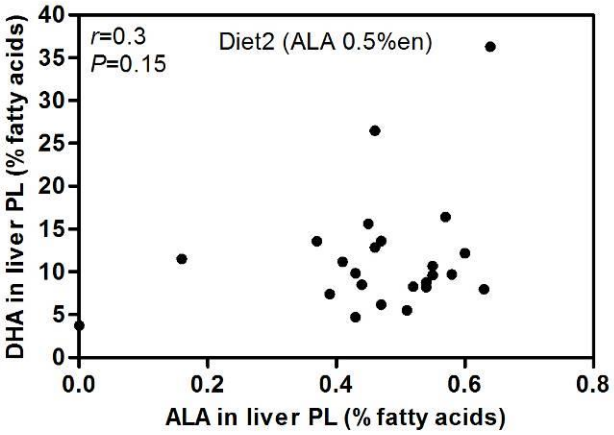

liver PL DHA (% fatty acid)
50
40
30
20
10
0
ALA 1
ALA 2
ALA 3
ALA 4
ALA 5
Comm.
diets

Figure 4.

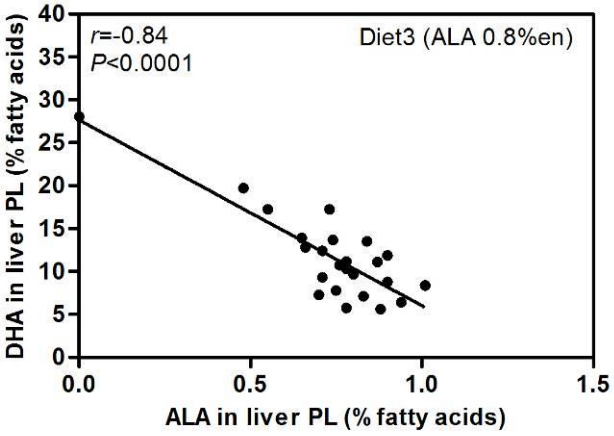
A


r=0.05
P=0.81
Diet1 (ALA 0.1%en)
DHA in liver PL (% fatty acids)
ALA in liver PL (% fatty acids)
0
5
10
15
20
25
30
35
40
0.0
0.1
0.2
0.3

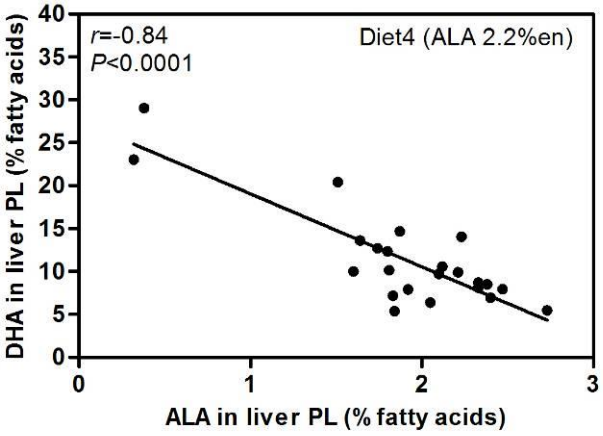
B


r=0.3
P=0.15
Diet2 (ALA 0.5%en)
DHA in liver PL (% fatty acids)
ALA in liver PL (% fatty acids)
0
5
10
15
20
25
30
35
40
0.0
0.2
0.4
0.6
0.8

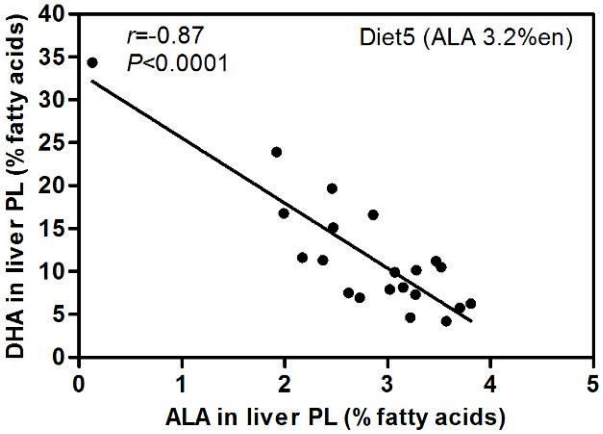
C


r=-0.84
P<0.0001
Diet3 (ALA 0.8%en)
DHA in liver PL (% fatty acids)
ALA in liver PL (% fatty acids)
0
5
10
15
20
25
30
35
40
0.0
0.5
1.0
1.5

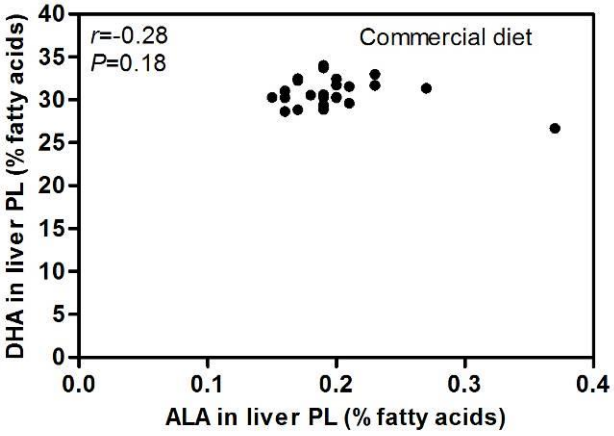
D


r=-0.84
P<0.0001
Diet4 (ALA 2.2%en)
DHA in liver PL (% fatty acids)
ALA in liver PL (% fatty acids)
0
5
10
15
20
25
30
35
40
0
1
2
3

E


r=-0.87
P<0.0001
Diet5 (ALA 3.2%en)
DHA in liver PL (% fatty acids)
ALA in liver PL (% fatty acids)
0
5
10
15
20
25
30
35
40
0
1
2
3
4
5

F


r=-0.28
P=0.18
Commercial diet
DHA in liver PL (% fatty acids)
ALA in liver PL (% fatty acids)
0
5
10
15
20
25
30
35
40
0.0
0.1
0.2
0.3
0.4