EPA, ARA and eicosanoid metabolism in juvenile barramundi *Lates calcarifer*.

Michael J. Salini [1,2,3], Nicholas M. Wade [3], Bruno C. Araújo [4], Giovanni M. Turchini [1], Brett D. Glencross [5]

1 Deakin University, Geelong, Australia. School of Life and Environmental Sciences, Warrnambool Campus, Princess Hwy, Warrnambool, Vic., Australia.

2 CSIRO Agriculture, 144 North Street, Woorim, Qld, Australia.

3 CSIRO Agriculture, QLD Biosciences Precinct, Services Rd, St Lucia, Qld, Australia.

4 Instituto de Biociências, Universidade de São Paulo, 321 Rua do Matão, SP 05508 090, Brazil.

5. Institute of Aquaculture, Stirling University, FK9 4LA Stirling, United Kingdom

***Email** Michael.salini@gmail.com

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Abstract

The potential benefits of dietary long-chain polyunsaturated fatty acids to fish include improved growth, survival and stress resistance, reproduction and regulation of immune function. The metabolism of eicosapentaenoic acid (EPA; 20:5n-3) and arachidonic acid (ARA; 20:4n-6) into short-lived hormones know as eicosanoids play central roles in the regulation of these responses. However, little is known about the EPA and ARA requirements and their effect on fatty acid and eicosanoid metabolism in barramundi (*Lates calcarifer*). Therefore, a two part experiment was conducted to assess the response of juvenile barramundi (initial weight = 10.3 ± 0.03 g; mean ± S.D.) fed one of five diets with graded levels of EPA (diets EPA 1.2, EPA, 4.5, EPA 9.5, EPA 17.0, EPA 20.1) or one of four diets with graded levels of ARA (diets ARA 1.1, ARA 5.3, ARA 11.5 and ARA 16.4) compared against a fish oil control diet. A six week feeding trial demonstrated that the addition of EPA or ARA did not have any impact on growth performance or feed utilisation. Analysis of the whole body fatty acid composition showed that these fatty acids reflected those of the diets. The retention and marginal utilisation efficiency suggests that dietary ARA and to a lesser extent EPA are required in juvenile barramundi; however, a modified metabolic response was observed. The partial cDNA sequences of genes regulating eicosanoid biosynthesis were identified in barramundi tissues, namely cyclooxygenase 1 (*Lc COX1a, Lc COX1b*), cyclooxygenase 2 (*Lc COX2*) and lipoxygenase (*Lc ALOX-5*). Both *COX2* and *ALOX-5* expression in the liver tissue were elevated in response to increasing dietary ARA, meanwhile expression levels of *Lc COX2* and the mitochondrial fatty acid oxidation gene *Lc CPT1a* were elevated in the kidney. Dietary EPA levels had little effect on the expression of eicosanoid biosynthesis genes in the liver or kidney. The present study demonstrates that consideration should be given to the ARA supply for juvenile barramundi in light of pro-inflammatory and inducible nature of the cyclooxygenase and lipoxygenase enzymes.

Introduction

Eicosapentaenoic (EPA; 20:5n-3) and arachidonic acid (ARA; 20:4n-6) are important for many metabolic and physiological functions, and are precursor molecules for the production of eicosanoid hormones that play a role in the inflammatory response, immune function and regulation as well as ionic regulation and reproduction in fish and mammals (Calder, 2004; Rowley et al., 1995). In humans, the incidence of diseases involving inflammatory processes can be related to the production these eicosanoids, for example prostaglandin E2 (PGE_2) and leukotriene B4 (LTB_4) that are derived from high cellular concentrations of ARA (Tocher, 2015; Wall et al., 2010). The cyclooxygenase (COX; prostaglandin G/H synthase) and lipoxygenase (LOX; arachidonate 5-lipoxygenase) enzymes interact and compete for the EPA and ARA substrates and as such the eicosanoids produced are determined by their availability (Calder, 2012; Tocher, 2003).

The COX enzyme catalyses 20-carbon chain fatty acids (ARA or EPA) through *bis*-dioxygenation and subsequent reduction to produce 2 - and 3 - series prostaglandins ($PGG_{2/3}$ and $PGH_{2/3}$) that are substrates for the synthesis of biologically active PGs, prostacyclins and thromboxanes (Calder, 2012; Rouzer and Marnett, 2009; Rowley et al., 1995). While the LOX enzyme catalyses the same substrates to yield biologically active metabolites of hydroperoxy-eicosatetraenoic acid (HPETE) such as leukotrienes and lipoxins (Matsumoto et al., 1988; Rowley et al., 1995). Unlike most vertebrate organisms, the COX enzyme system in teleost fish is further complicated by an evolutionary duplication event that led to alternative chromosomal regions of COX genes, often identified as ‘a and b’ isoforms of either the COX-1 or -2 series, but rarely both (Ishikawa and Herschman, 2007; Ishikawa et al., 2007b). There is also an accepted paradigm that COX-1 is constitutively expressed whereas COX-2 is inducible, however this is now widely viewed as an oversimplified as inducible COX-1 genes and constitutively expressed COX-2 genes have been identified (Breder et al., 1995; Cha et al., 2006; Olsen et al., 2012; Rouzer and Marnett, 2009).

It has been shown in rainbow trout (*Oncorhynchus mykiss*) cell lines that fatty acids such as 18:4n-3 and 20:4n-3, can reduce the production of 2-series prostanoids and 4-series leukotrienes by conversion to EPA and subsequent competition with ARA (Ghioni et al., 2002). In many vertebrate and fish species, EPA and ARA are required for important metabolic and physiological functions, and the optimal as well as dietary requirements are well understood (Bell and Sargent, 2003; Das, 2006; Glencross, 2009; Izquierdo, 1996; Tocher, 2010; Tocher, 2015). In addition, there is great potential for dietary ARA to affect growth, stress response, immune response and survival,

particularly at early life stages (Atalah et al., 2011a; Atalah et al., 2011b; Bell and Sargent, 2003; Castell et al., 1994; Montero et al., 2015b; Norambuena et al., 2016; Yuan et al., 2015).

Information on the EPA and ARA requirements in the barramundi or Asian seabass (*Lates calcarifer*) are scarce. In the only study available thus far, increased levels of dietary ARA in the absence of EPA showed negative effects on fish health, including disproportionate tissue LC-PUFA retention and pathophysiological effects such as subcutaneous haemorrhaging and disrupted ionic regulation (Glencross and Rutherford, 2011). There is evidence to suggest that wild barramundi (either from fresh or salt water) can contain ARA levels more than 5-fold higher than EPA in their tissues (Nichols et al., 2014). The increasing use of alternative oils in farmed fish, often containing high levels of omega 6 fatty acids which can act as ARA precursors (eg. soybean oil, sunflower oil) may present issues associated with modified lipid metabolism (Brown and Hart, 2011).

Based on previous observations, the present study hypothesised that the effect of changing the dietary ARA or EPA level may significantly modify fatty acid metabolism in barramundi. A two part, dose-response experimental design was used to determine the effect of increasing the dietary EPA and ARA. To achieve this, commercial preparations of an EPA rich fish oil and an ARA rich fungal oil were incrementally added to a series of barramundi diets. It was also hypothesised that there may be effects on the transcription of genes involved in eicosanoid synthesis. The genes regulating eicosanoid synthesis have not been identified in barramundi, and no information is available on their nutritional regulation.

Materials and methods

Ingredient and diet preparation

A single basal diet was formulated and prepared without the addition of dietary oils (lipids). 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. All dry ingredients were then thoroughly mixed using an upright commercial mixer (Bakermix, Model 60 A-G, NSW, Australia). Fish meal was defatted prior to use by manually mixing n-hexane and fish meal (2:1 respectively) in a large drum. The mix was left to soak for 3h before draining the excess hexane and repeating the process a second time. The fish meal was oven dried overnight at 60 ˚C to a constant dry matter. The chemical composition of the main dietary ingredients is presented in Table 1. The single batch of basal diet was produced using a laboratory-scale twin-screw extruder with intermeshing, co-rotating

screws (MPF24, Baker Perkins, Peterborough, United Kingdom). The pellets were extruded through a 2 mm tapered die and obtained a 1.5-fold increase in diameter by expansion. The pellets were cut off at lengths of 3-4 mm using a variable speed 4-blade cutter and dried overnight at 60 ˚C to a constant dry matter. The dietary treatments were generated by vacuum-infusion of the different lipid sources (lipid added at 8.2% diet) to batches of dried pellets. Five dietary treatments were formulated with increasing eicosapentaenoic acid (added via; Incromega™ TG500, CRODA, United Kingdom) up to 20.1 g/kg inclusion (EPA 1.2, EPA, 4.5, EPA 9.5, EPA 17.0, EPA 20.1) and four treatments were formulated with increasing arachidonic acid (added via; ARASCO®, Martek Biosciences, USA) up to 16.4 g/kg inclusion (ARA 1.1, ARA 5.3, ARA 11.5 and ARA 16.4). A diet containing only fish oil was used as a unique control for both experiments (FO CTRL). The diets were then stored at -20 ˚C until required. The formulation and chemical composition of the ten diets are presented in Table 2.

Barramundi husbandry and growth

Juvenile barramundi (*Lates calcarifer*) were sourced from the Betta Barra fish hatchery (Atherton, QLD, Australia), on-grown in a 10,000L tank and fed a commercial diet (Marine Float; Ridley Aquafeed, Narangba, QLD, Australia). Prior to commencement of the experiment the fish were transferred to a series of experimental tanks (1000L) with flow-through seawater (salinity =38 PSU; dissolved oxygen 5.4 ± 0.01 mg /L) of 28.9 ± 0.01 ºC (mean ± SD) at a flow rate of about 3 L/min being supplied to each of the tanks. At the beginning of the experiment, each of the tanks held 30 fish of 10.3 ± 0.1 g (mean ± SD, n = 900 individually weighed fish). The two experiments were nested within one and conducted simultaneously utilising a common control diet for both experiments. The ten experimental diets were therefore randomly distributed amongst thirty tanks with each treatment having three replicate tanks. The fish were restrictively fed a sub-satietal (approximately 80%) pair-feeding regime in order to avoid the potentially confounding issue of unregulated feed intake (Glencross et al., 2003a).

Sample collection, preparation and digestibility analysis

Ethical clearance was approved (Approval A05/2014) for the experimental procedures by the CSIRO animal ethics committee. Six fish of similar size from the original stock were euthanized by an overdose of AQUI-S™ (Lower Hutt, New Zealand) at the beginning of the experiment and stored at -20 ºC until analysis. A further three fish from each treatment were euthanized upon termination of the experiment and they were frozen at -20 ºC until analysis.

Upon termination of the growth assay, faeces were collected using established abdominal stripping protocols (Blyth et al., 2014). Briefly, the fish were netted from their tanks and anesthetised, then gentle abdominal pressure was applied to the distal intestine to extract the faeces. Care was taken by the operator to avoid contamination of the sample with foreign material and hands were rinsed after each stripping. The faecal sample was placed into a small plastic vial on ice before being stored in a freezer -20˚C until analysis.

Chemical analysis

Prior to analysis the diets were each ground to a fine powder using a bench grinder (KnifeTec™ 1095, FOSS, Denmark). The initial and final fish were processed using the following method. The frozen whole fish were passed through a commercial meat mincer (MGT – 012, Taiwan) twice to obtain a homogeneous mixture. A sample was taken for dry matter analysis and another sample was freeze-dried along with the faecal samples until no further loss of moisture was observed (Alpha 1-4, Martin Christ, Germany). Dry matter was calculated by gravimetric analysis following oven drying at 105ºC for 24 h. Crude protein was calculated after the determination of total nitrogen by organic elemental analysis (CHNS-O Flash 2000, Thermo Scientific, USA), based on N x 6.25. Total lipid content was determined gravimetrically following extraction of the lipids using chloroform:methanol (2:1) following Folch et al. (1957). Gross ash content was determined gravimetrically following loss of mass after combustion of a sample in a muffle furnace at 550 ˚C for 24 h. Gross energy was determined by adiabatic bomb calorimetry (Parr 6200 Calorimeter, USA). Total yttrium concentrations in the diets and faeces were determined after nitric acid digestion in a laboratory microwave digester (Ethos One, Milestone, Italy) using inductively coupled plasma-mass spectrophotometry (ICP-MS) (ELAN DRC II, Perkin Elmer, USA).

Fatty acid composition was determined following the methods of Christie (2003). Lipids were esterified by an acid-catalysed methylation and 0.3 mg of an internal standard was added to each sample (21:0 Supelco, PA, USA). The fatty acids were identified relative to the internal standard following separation by gas chromatography (GC). An Agilent Technologies 6890N GC system (Agilent Technologies, California, USA) fitted with a DB-23 (60m x 0.25mm x 0.15 μm, cat 122-2361 Agilent Technologies, California) capillary column and flame ionisation detection was used. The temperature program was 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 column head pressure was set to constant pressure mode at 170 kPa using hydrogen as the carrier gas. The peaks were identified by comparing retention times to the internal standard and further referenced against known

standards (37 Comp. FAME mix, Supelco, PA, USA). The resulting peaks were then corrected by the theoretical relative FID response factors (Ackman, 2002) and quantified relative to the internal standard.

Cloning of putative prostaglandin G/H synthase (COX) and arachidonate 5-lipoxygenase (LOX) genes

Sequences of the prostaglandin G/H synthase and arachidonate 5-lipoxygenase (*COX1a, COX1b, COX2 andALOX-5*) genes from several teleost species were identified in the Genbank database. Highly conserved regions from protein alignments across species were used to design pairs of degenerate primers that were subsequently synthesised by Sigma-Aldrich (Table 3). Pooled barramundi liver cDNA (1000 ng) was amplified in reactions, with each degenerate primer pair (F and R; 10 μm) using platinum TAQ mix (Thermofisher). Polymerase chain reaction (PCR) conditions including an initial denaturation step at 90°C for 2 min followed by 35 cycles of 94°C/10 s, 50°C/30 s (50% ramp speed) and 72°C/30 s with a final extension step of 72°C/5 min were used. The amplification products were separated by size using electrophoresis on a 1% agarose gel and then excised and extracted using the QIA quick gel extraction kit (QIAGEN). The target was then ligated using the pGEM-T Easy vector system (Promega). Ligation reactions were then transformed onto One Shot TOP10 chemically competent *Escherichia coli* cells (Thermofisher) which were then cultured overnight on LB ampicillin plates (100 μg/mL). Positive clones were selected by PCR amplification with primers flanking the multiple cloning site (M13F and M13R), using an initial denaturation step at 94°C/5 min followed by 35 cycles of 94°C/20 s, 55°C/30 S, 72°C/1.5 min with a final extension step of 72°C/5 min. Randomly selected positive clones were prepared for sequencing with a QIAprep Miniprep kit (QIAGEN) and then the plasmid insert was sequenced using the BigDye® Terminator V3.1 sequencing kit (Applied Biosystems) with an ABI 3130 Automated Capillary DNA Sequencer (Applied Biosystems).

Sequencing analysis

Multiple alignments of the target genes (*Lc COX-1a, Lc COX-1b, Lc COX-2 and Lc ALOX-5*) were made using the CLC Genomics Workbench software (CLC Bio, Aarhus, Denmark). The ORF and amino sequence alignment and analysis were conducted using the Create Alignment tool, and confirmed as the target gene sequence using the BLASTX algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequence analysis of the previously unreported *Lates calcarifer* COX and LOX genes identified expressed sequence tags (EST) of between 752 and 999

base pairs, open reading frame protein alignments showing similarity with other teleost fish are presented in supplementary figures 1 a-d.

RNA extraction, cDNA synthesis and quantitative real-time RT-PCR

Total RNA was extracted from the livers and kidneys of fish from the FO CTRL, EPA 1.2, EPA 20.1, ARA 1.1 and ARA 16.4 treatments using TRIzol® reagent (Invitrogen) according to the manufacturer's instructions. Only the two extreme dietary treatments of each experiment were used in this analysis as it was anticipated that only minor differences in gene expression would be observed. RNA extraction and cDNA synthesis were performed following previously reported methods from the same laboratory (Salini et al., 2015b; Wade et al., 2014). Real-time PCR primers specific to each target gene (Table 4) were designed using PerlPrimer v.1.1.17 (Marshall, 2004). Quantitative real-time RT-PCR was performed using 2X SYBR Green PCR master mix (Applied Biosystems), 0.2 µm RT-PCR primers for each gene and the equivalent of 7.5 ng of reverse transcribed RNA following previously reported cycling and processing conditions from the same laboratory (Salini et al., 2015b; Wade et al., 2014). Changes in expression levels were normalised for each gene to the endogenous reference gene elongation factor 1 alpha (EF1α) and the exogenous reference gene Luciferase. The variation in amplification across all samples was very small, 0.57 cycles and 0.40 cycles for EF1α Luciferase, respectively. The EF1α and Luciferase genes have been routinely used as a reference in this species (De Santis et al., 2011; Wade et al., 2014).

Calculations and statistical analysis

Differences in the ratio of dry matter, protein, lipid and energy to yttrium in the diet and faeces were calculated to determine the apparent digestibility coefficients (ADC) following Maynard and Loosli (1979) using the formula:

$$ADC = \left(1 - \left(\frac{Y_{diet} \times Parameter_{faeces}}{Y_{faeces} \times Parameter_{diet}}\right)\right) \times 100$$

Where Y_{diet} and Y_{faeces} are the yttrium content in the diet and faeces respectively and $Parameter_{faeces}$ and $Parameter_{diet}$ are the nutritional parameter of concern in the diet and faeces respectively.

Nutrient retention efficiencies were calculated as the ratio of the nutrient or specific fatty acid gained relative to their respective consumption during the study period following Maynard and Loosli (1979) using the formula:

$$Retention\ (\%) = \left(\frac{Nf - Ni}{Nc}\right) \times 100$$

Where *Nf* and *Ni* are the nutrient composition in the final and initial fish (g/fish) on a live-basis and *Nc* is the amount of the nutrient consumed (g/fish) during the study period.

To provide a size independent function for the calculation of maintenance demands and utilisation efficiencies of specific fatty acids, modelling of the specific fatty acid retention efficiency data was initially carried out (unpublished laboratory study). Live-weight exponents of 0.679 and 0.857 were recorded for EPA (20:5n-3) and ARA (20:4n-6) respectively as size independent functions for the determination of maintenance demands. Maintenance demands and utilisation efficiencies were then determined from the regression of marginal fatty acid intake against marginal fatty acid gain on a transformed live-weight basis following (Glencross, 2008) using the formula:

$$Marginal\ Intake\ or\ Gain = Intake\ or\ Gain\ _{FA}\ /GMW^{x}/d$$

Where *Intake* $_{FA}$ or *Gain* $_{FA}$ is the specific fatty acid consumed or gained (g/fish) on a weight specific (geometric mean live-weight g/fish) basis then transformed to a fatty acid specific exponent. The duration of the study period in days is defined as *d.*

All data are expressed as mean with pooled standard error mean unless otherwise specified. All data were checked for normal distribution and homogeneity of variance by qualitative assessment of residual and normal Q-Q plots. Growth performance data were analysed using polynomial contrasts and all other data were analysed by one-way ANOVA. Levels of significance were compared using Tukey's HSD a posteriori test. The RStudio package v.0.98.501was used for all statistical analyses (R Core Team, 2012). Any percentage data were arcsine transformed prior to analysis. Significance among the treatments defined as $P < 0.05$.

Results

Growth performance and feed utilisation

The results of the 42 d growth assay demonstrated that the barramundi responded well to the experimental diets in both experiments exceeding over 400% of their original weight at a rate of more than 1 g/d. The growth parameters for each experiment were analysed separately using polynomial contrasts (Table 5 and 6). When the fish were fed increasing EPA there were no significant contrasts in any of the growth parameters (Table 5). Similarly, the barramundi did not show any significant contrasts in response to the increasing ARA in the second experiment (Table 6). The only exception being a slight improvement in FCR with the addition of ARA however the numerical differences were minor. There was also a linear improvement in survival with increasing ARA. Several moribund fish were removed from the system after a weight check at day 28. These fish were considered non-contributing as they had not consumed any feed. Additionally, the growth performance of the fish fed different EPA and ARA treatments were compared by one-way ANOVA against the control. There were no significant differences in any of the growth parameters measured (data not reported).

Digestibility analysis of the diets

While no differences were recorded in the digestibility of dry matter, protein or energy across the treatments, the digestibility of lipid was significantly higher in the control (FO CTRL) fed fish (Table 7). Significantly lower digestibility levels of MUFA and C_{18}PUFA were recorded in the FO CTRL compared with other treatments, predominantly from reduced 18:1 and 18:2n-6. Increased levels of dietary EPA or DHA resulted in a significantly reduced digestibility of SFA fatty acids, predominantly from reduced 18:0. There were numerically minor differences in the digestibility of LC-PUFA (20:4n-6, 20:5n-3, 22:5n-3, 22:6n-3).

Whole-body composition.

When compared against the control (FO CTRL), there were significant differences in whole body composition for many parameters (Table 8). Dry matter was significantly reduced in the EPA 1.2 treatment compared with all other treatments. This change was reflected in the lipid and energy composition, with significantly reduced levels recorded in the EPA 1.2 treatment. Protein composition was unaffected by the treatments. As expected, significant elevation of ARA (20:4n-6) and EPA (20:5n-3) levels were recorded in the fish fed the ARA 16.4 and EPA 20.1 diets, respectively. Increased levels of EPA (20:5n-3) in the EPA 20.1 treatment led to a significant

increase in DPA levels (22:5n-3) The FO CRTL treatment had significantly elevated DHA (22:6n-3) and total SFA.

Nutrient retention efficiency

Fatty acid retention efficiency in the control fed fish (FO CTRL) did not differ from the quadratic trendlines (Figure 1a-j). There was a quadratic effect on the retention efficiency of 20:4n-6 when dietary EPA or ARA increased (Fig1 c, h; Table 9).There was a significant quadratic effect on 20:5n-3 retention in response to increasing dietary EPA diets with a decrease in retention before increasing at the highest inclusion whereas increasing ARA had no effect (Fig.1d, i; Table 9). Retention efficiency of 22:6n-3 was not affected by increasing dietary EPA however there was a significant quadratic effect in response to increasing dietary ARA with a decrease in retention before increasing at the highest inclusion (Fig.1e, j; Table 9).

Marginal utilisation efficiencies

The marginal utilisation efficiencies are presented for both EPA and ARA fed fish (Fig. 3a, 3b respectively). Based on the linear assessment of marginal EPA intake against marginal EPA gained, a significant difference was observed ($P<0.01$). The positive *y* intercept value indicates that no maintenance demand could be established for 20:5n-3. Using the live-weight exponent value of 0.679 the EPA fed fish had a 20:5n-3 utilisation efficiency of 62.1% described by the linear equation of $y = 0.621x + 0.0003$, $R^2 = 0.975$ (Fig. 3a). Similarly, there was a significant linear relationship of marginal ARA intake against marginal ARA gain ($P<0.05$). The negative *y* intercept value suggests that a maintenance value of around 0.01 $g/kg^{0.85}/d$ could be determined for 20:4n-6. Using the live-weight exponent of 0.85 the ARA fed fish had a 20:4n-6 utilisation efficiency of 91.9% described by the linear equation of $y = 0.919x – 0.011$, $R^2 = 0.965$ (Fig. 3b). A summary of the maintenance demand and utilisation efficiencies is presented in Table 9.

Gene identification and quantitative expression

Partial cDNA sequences of *Lc COX1a, Lc COX1b, Lc COX2 and Lc ALOX-5* were identified through degenerate PCR. BLAST similarity searches showed that barramundi gene orthologs shared between 88 and 91 % similarity with other teleost fish at the amino acid level, and protein alignments showed similarity with other teleost fish (Supplementary Figure 1a-d). The expression of eicosanoid pathway genes (*Lc COX1a, Lc COX1b, Lc COX2 and Lc ALOX-5*), as well as the mitochondrial ß-oxidation gene (*Lc CPT1a*) were analysed in the liver and kidney by quantitative real-time RT-PCR.

The expression of all genes analysed was up regulated in the liver of fish fed the EPA 1.2 diet compared with the FO CTRL fed fish (Fig. 3). There was a significant difference in the expression of *Lc COX1b* among the EPA 1.2 and EPA 20.1 treatments. No other genes were nutritionally regulated in the liver or kidney tissue of fish fed increasing EPA. In the case of the ARA fed fish, expression of *Lc COX1b*, *Lc COX2* and *Lc CPT1α* in the kidney tissue was lowest in the ARA 1.1 and highest in the ARA16.4 treatments. Similarly, the expression of *Lc COX2* was also significantly up regulated in the liver tissue (Fig. 4).

Discussion

The growth performance of juvenile barramundi in the present study, using a pair-fed feeding regime, was not affected by increasing dietary EPA or ARA content. Moreover, the response was compared to a control diet containing only fish oil and no significant differences in growth performance were reported. Although it is an unlikely real-world scenario to include high levels of any one LC-PUFA, these observations and those of a previous study, confirm that there are no growth stimulatory effects owing to any individual LC-PUFA in juvenile barramundi (Glencross and Rutherford, 2011). In many species, EPA can exert cardio-protective benefits such as lowering triglycerides and low-density lipoprotein levels (Aarsland et al., 1990; Cahill et al., 1988; Cottin et al., 2011; Wei and Jacobson, 2011; Weiner et al., 1986). Whereas ARA on the other hand, is an essential and necessary precursor to the 2-series and 4-series eicosanoids that mediate homeostasis during times of environmental or physiological stress (Bell and Sargent, 2003).

Studies examining the dose-response of EPA in juvenile or growing fish are relatively scarce and mostly concentrate on larval fish requirements. However, many studies have investigated interactions between DHA, EPA and ARA or n-3 to n-6 ratios in fish and the general consensus is that size and species differences exist and that an appropriate balance between these essential fatty acids is critical (Sargent et al., 1999; Tocher, 2015). Consistent with the present study, several studies in larval and juvenile fish such as the Atlantic salmon *Salmo salar* (Thomassen et al., 2012), cobia *Rachycentron canadum* (Trushenski et al., 2012), gilthead sea bream *Sparus aurata* (Atalah et al., 2011a), Senegalese sole *Solea senegalensis* (Villalta et al., 2008), striped jack *Pseudocaranx dentex* (Watanabe et al., 1989) and turbot *Scophthalmus maximus* (Bell et al., 1995) all concluded that EPA does not stimulate an improved growth response. However, larval and juvenile red seabream *Pagrus major* were found to have an EPA requirement with linear improvements in growth and survival observed (Furuita et al., 1996; Takeuchi et al., 1990).

Also in agreement with the present study, a range of larval and juvenile marine species such as Atlantic cod Gadus morhua (Bransden et al., 2005), gilthead sea bream Sparus aurata (Alves Martins et al., 2012; Atalah et al., 2011a; Fountoulaki et al., 2003), Senegalese sole (Villalta et al., 2005) and turbot (Estévez et al., 1999), were able tolerate a wide range of dietary ARA levels with growth and survival found to be independent of ARA inclusion. In agreement, several recent studies have further demonstrated that balanced ARA and EPA are more critical than either individual FA in terms of growth and other metabolic processes further highlighting the need for understanding the optimal LC-PUFA balance (Norambuena et al., 2015; Norambuena et al., 2016).

Many studies, including the present study, have demonstrated that the fatty acid composition of the tissues is representative of the profile of the fed diet (Rosenlund et al., 2011). However, the efficiency by which fatty acids are retained may represent a more metabolic or biological importance to the fish. The DHA retention of the fish in the present study fed increasing EPA did not change. However, the EPA and ARA retention were inversely related and responded in a curvilinear fashion. The effect was not dramatic however it indicated a point of sensitivity as EPA in the diet increased and the barramundi retained more of the endogenous ARA. Glencross and Rutherford (2011) reported disproportionate EPA and ARA retention in barramundi however this was likely an effect of the increasing DHA level in combination with either EPA or ARA. Atlantic salmon were also shown to conserve ARA (Norambuena et al., 2015) while Senegalese sole attempt to synthesise ARA when dietary supply is limited (Norambuena et al., 2013).

A recent study has already suggested that the marginal efficiency of LC-PUFA utilisation are low compared to other fatty acids in growing barramundi (Salini et al., 2015a). The present study describes a more up to date and relevant assessment of the marginal efficiency of EPA and ARA assessed in a dose-response manner, while simultaneously controlling for other LC-PUFA (rather than FO substitution). On closer examination of dietary ARA demands in juvenile barramundi, we report the maintenance requirement, albeit low, of this fatty acid to be 0.01 g/kg $LW^{0.85}$/d. This confirms that ARA is perhaps unusual in its characteristic metabolic requirement for this species. EPA on the other hand is apparently not required for maintenance in juvenile barramundi suggesting that they have a very low dietary requirement for this fatty acid and that their endogenous reserves are sufficient for maintenance. In addition, the EPA retention figures were modest with a calculated intake to gain ratio of 1.6:1 confirming this. This is consistent with other studies on barramundi and red sea bream in that EPA is only modestly retained unless there is a gross imbalance of other LC-PUFA (Glencross et al., 2003b; Glencross and Rutherford, 2011).

There is a very large body of research into the mechanisms of action of n-3 LC-PUFA on inflammatory pathways (Rangel-Huerta et al., 2012). The data from the present study build upon this in barramundi by presenting the nutritional regulation of key eicosanoid pathway enzymes. The current work verifies that barramundi possess two variants of the cyclooxygenase 1 enzyme (COX1a, COX1b) and a single cyclooxygenase 2 (COX2). The fish in the present study were not exposed to any form of physical or environmental stressors (apart from being in research aquaria) that could cause an inflammatory response and therefore the only trigger for a response is from the diets.

The gene expression analysis in the present study demonstrated that EPA supplied in excess mostly did not affect the gene expression between the treatments in either the liver or kidney tissue. However, the slight exception to this was the nutritional regulation of the COX1b isoform in the liver of the EPA fed fish. COX1 isoforms are widely distributed and generally constitutively expressed however this homeostatic function is now considered to be an oversimplified view (Rouzer and Marnett, 2009). In agreement with the present study, there were non-significant yet quite measurable differences in COX1b expression in larval tongue sole *Cynoglossus semilaevis* (Yuan et al., 2015). The results of the present study confirm that the COX1b gene in barramundi is inducible however the differences warrant further investigation into the potential for pathophysiological effects. Some explanation for these inconsistencies may be found in whole-body composition of wild barramundi collected from different environments (Nichols et al., 2014; Sinclair et al., 1983). These authors found that Northern Australian barramundi, maintain a characteristically high ARA content regardless of their environment. Therefore, it is suggested that barramundi can modify the transcription of these genes depending on the potentially transient nature of dietary ARA supply.

The EPA or anti-inflammatory derived eicosanoids are known to be cross-linked with changes in transcription of fatty acid synthesis and beta-oxidation genes (Aarsland et al., 1990; Calder, 2007; Chen et al., 2014; Sijben and Calder, 2007) . However, the CPT1α expression data in the present study are inconsistent in that they are not affected by the inclusion level of either EPA or ARA albeit with a slight decrease in the kidney tissue of the ARA1.1 fish. However, our data showed that CPT1α expression is elevated in both low and high EPA treatments compared to the control fish. Additionally, the ALOX-5 expression was affected in much the same way as CPT1α and this may suggest that some other characteristic, perhaps of the fish oil base used in the control diet caused the response. Further exploration of these responses is warranted as the optimal ratio of EPA to ARA to DHA is likely to be species and potentially size specific in barramundi (Sargent et al., 1999).

In agreement with many vertebrates, nutritionally inducible COX2 genes have been identified in several teleost species (Ishikawa and Herschman, 2007; Ishikawa et al., 2007a; Montero et al., 2015a; Olsen et al., 2012; Rouzer and Marnett, 2009; Yuan et al., 2015; Zuo et al., 2015). The role of COX2 as an active and rapidly inducible gene has undoubtedly played a role in the modified metabolic function of the ARA fed fish during the present study. The COX2 expression was significantly increased in both liver and kidney tissues suggesting that the highest inclusion of ARA was excessive or that the EPA to ARA ratio was unbalanced.

To conclude, the present study supports that there is no phenotypic response by barramundi to the addition of either EPA or ARA to the diets. However, there are some metabolic changes to the retention and marginal utilisation efficiencies as a result of the diets. Increasing the dietary EPA or ARA level also modulated the expression of several eicosanoid metabolism and fatty acid oxidation genes. Investigation is warranted into the most appropriate balance of dietary LC-PUFA in light of the current levels of fish oil substitution in aquafeeds. Further refinements could also be made to factorial growth and feed utilisation models for barramundi with respect to specific fatty acids and their marginal utilisation efficiencies.

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Table 1. Composition of key ingredients used in diet formulations (g/kg DM). Fatty acids are percentages of total fatty acids (%).

	Fish meal*	Poultry meal	Fish oil	Palm flake	Olive oil	ARASCO®	Incromega EPA TG500	Incromega DHA TG500
Dry matter (g/kg)	974	966	985	997	992	991	990	992
Lipid	44	157	946	958	911	972	971	975
Ash	191	151	ND	ND	ND	ND	ND	ND
Protein	684	660	7	7	5	10	6	5
Energy (MJ/kg)	18.6	20.9	35.6	39.5	39.7	40.1	39.1	40.1
16:0	27.0	23.8	22.5	47.4	10.4	9.3	ND	1.7
18:0	6.3	8.6	5.2	51.3	4.0	8.3	ND	4.2
18:1	16.1	41.9	19.5	ND	72.3	23.0	1.2	8.7
18:2n-6	1.3	10.6	2.6	ND	10.8	7.2	ND	0.8
18:3n-3	ND	1.4	1.2	ND	1.0	ND	ND	ND
20:4n-6	1.1	1.2	1.6	ND	ND	42.5	3.5	2.7
20:5n-3	13.4	0.6	11.6	ND	ND	ND	70.2	9.2
22:5n-3	3.3	0.4	2.1	ND	ND	0.9	2.3	2.8
22:6n-3	14.6	0.8	15.4	ND	ND	ND	17.5	64.6
SFA	39.9	33.9	35.2	100.0	14.9	22.5	2.1	6.5
MUFA	25.8	49.9	29.4	ND	73.3	23.0	4.6	13.3
C_{18} PUFA	1.3	12.1	3.8	ND	11.8	7.2	ND	0.8
LC-PUFA	32.5	4.0	30.8	ND	ND	47.3	93.4	79.4
n-3	31.3	3.3	30.3	ND	1.0	0.9	89.9	76.6
n-6	2.4	12.8	4.2	ND	10.8	53.6	3.5	3.6

* Fish meal was defatted prior to use. See methods for details. ND, not detected; GE, gross energy. 18:1, sum of 18:1n-7, 18:1n-9 cis, 18:1n-9 trans; saturated fatty acids (SFA), sum of 12:0, 14:0, 16:0, 18:0, 20:, 22:0, 24:0; monounsaturated fatty acids (MUFA), sum of 14:1n-5, 16:1n-7, 18:1n-7, 18:1n-9 (cis and trans), 20:1n-7, 20:1n-9, 22:1n-9, 24:1n-9; polyunsaturated fatty acids, with 18 carbon atoms (C_{18} PUFA), sum 18:2n-6 (cis and trans), 18:3n-6, 18:3n-3, 18:4n-3; long chain polyunsaturated fatty acids, with 20 or more carbon atoms (LC-PUFA), sum 20:2n-6, 20:3n-6, 20:4n-6, 22:4n-6, 20:3n-3, 20:5n-3, 22:5n-3, 22:6n-3; n-3, sum of omega 3 C_{18} PUFA and LC-PUFA; n-6, sum of omega 6 C_{18} PUFA and LC-PUFA.

Table 2. Formulation and composition of experimental diets (g/kg DM). Fatty acids are percentages of total fatty acids (%)

	CTRL FO	EPA 1.2	EPA 4.5	EPA 9.5	EPA 17.0	EPA 20.1	ARA 1.1	ARA 5.3	ARA 11.5	ARA 16.4
Fish meal [1]	150	150	150	150	150	150	150	150	150	150
Poultry meal [1]	97	97	97	97	97	97	97	97	97	97
Casein [2]	150	150	150	150	150	150	150	150	150	150
Soy protein isolate [3]	150	150	150	150	150	150	150	150	150	150
Wheat gluten [3]	150	150	150	150	150	150	150	150	150	150
Wheat flour [3]	150	150	150	150	150	150	150	150	150	150
Pregel wheat starch [3]	50	50	50	50	50	50	50	50	50	50
Methionine [2]	5	5	5	5	5	5	5	5	5	5
Premix [4]	6	6	6	6	6	6	6	6	6	6
Dicalcium phosphate	5	5	5	5	5	5	5	5	5	5
Choline chloride	2	2	2	2	2	2	2	2	2	2
Stay-C	2	2	2	2	2	2	2	2	2	2
Yttrium [5]	1	1	1	1	1	1	1	1	1	1
Fish oil [1]	82.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Palm Flake [6]	0.0	31.0	28.5	25.4	22.0	19.0	28.5	23.5	17.2	11.0
Olive oil [6]	0.0	38.7	36.0	31.9	28.0	24.0	36.0	30.0	21.9	13.7
ARASCO® [7]	0.0	2.3	1.8	1.2	0.5	0.0	1.8	13.0	27.6	42.2
Incromega™ EPA TG500 [8]	0.0	0.0	7.7	18.2	29.0	39.0	7.7	7.5	7.1	6.9
Incromega™ DHA TG500 [8]	0.0	10.0	8.0	5.3	2.5	0.0	8.0	8.0	8.2	8.2
Composition										
Dry matter (g/kg)	959	961	959	955	946	950	959	952	961	963
Lipid	90	100	96	106	96	94	96	92	97	96
Ash	69	67	67	66	67	63	67	107	68	68
Protein	577	586	566	584	563	559	566	568	577	596
GE (MJ/kg)	21.8	21.6	21.7	21.5	21.4	21.3	21.7	21.6	21.7	21.7
FA (mg/g lipid)^	866.3	832.7	876.3	828.2	824.0	854.4	876.3	823.8	848.9	866.9
16:0	22.4	25.8	25.6	18.9	18.7	17.7	25.6	20.3	17.7	15.8

18:0	5.6	17.8	17.1	16.3	15.2	12.9	17.1	14.9	13.6	11.3
18:1	21.4	33.6	31.4	29.4	29.3	26.8	31.4	31.3	29.1	26.4
18:2n-6	9.9	10.3	10.4	11.7	6.7	9.5	10.4	11.7	9.9	10.6
18:3n-3	1.5	0.8	0.8	1.2	0.7	1.0	0.8	1.0	0.8	0.8
20:4n-6	1.5	1.3	1.1	1.3	1.3	1.9	1.1	5.3	11.5	16.4
20:5n-3	8.9	1.2	4.5	9.5	17.0	20.1	4.5	5.7	5.3	5.6
22:5n-3	1.7	0.5	0.5	0.9	0.8	0.9	0.5	0.6	0.8	0.9
22:6n-3	11.5	4.8	5.2	5.6	6.3	5.8	5.2	5.4	6.5	5.6
SFA	34.3	45.1	44.3	37.6	35.1	32.0	44.3	36.5	33.0	29.1
MUFA	29.9	35.0	33.2	31.9	31.0	28.9	33.2	32.9	30.6	28.3
C_{18} PUFA	11.4	11.6	11.3	13.1	7.4	10.5	11.3	13.1	11.5	12.5
LC-PUFA	23.7	8.3	11.3	17.3	26.5	28.7	11.3	17.5	24.9	30.1
n-3	23.7	7.3	11.0	17.3	26.0	27.8	11.0	12.7	13.3	12.8
n-6	11.4	12.5	11.5	13.2	8.0	11.4	11.5	17.9	23.1	29.8

^ Refer to Table 1 for definitions of fatty acids.

1 Ridley Aquafeed, Narangba, QLD, Australia.

2 Bulk Powders, www.bulkpowders.com.au

3 Manildra Group, Rocklea, QLD, Australia

4 Vitamin and mineral premix (IU kg-1 or g/kg of premix): vitamin A, 2.5MIU; vitamin D3, 0.25 MIU; vitamin E, 16.7 g; vitamin K3, 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

5 Yttrium oxide, Stanford materials, Aliso Viejo, CA,
USA.

6 Sydney Essential Oil Co., Sydney, NSW, Australia

7 ARASCO®, Martek Biosciences Co., Columbia, MD, USA.

8 CRODA™, Snaith, East Yorkshire, UK.

Table 3. Forward and reverse primer pairs (5′ – 3′) used the cloning of eicosanoid metabolism genes in barramundi.

Target	Abbreviation	Genbank*	Sequence (F/R)	Product
Degenerate primers				
Prostaglandin G/H synthase	*Lc COX-1a*	KU188276	TTTGGGAATGTACGCTACGC GTGATAGAGGAGTATGTGCAGCA	752 bp
Prostaglandin G/H synthase	*Lc COX-1b*	KU188277	TCAGTGTGCGTTTCCAGTACAG GGATTCTTTCTCCAGACAGC	999 bp
Prostaglandin G/H synthase	*Lc COX-2*	KU188278	GTGATGTGCTGAAGGAGGTG AGGATTGCGGACATTTCTTTCTC	997 bp
Arachidonate 5-lipoxygenase	*Lc ALOX-5*	KU188279	TTTACCATCGCCATCAACAC GAGATGACGGCTACAGGGTG	976 bp

* Genbank accession numbers

Table 4. Forward and reverse primer pairs (5′ – 3′) used in real-time qPCR expression analysis of eicosanoid metabolism genes

Target	Abbreviation	EC number	Sequence (F/R)	Length
RT- qPCR primers				
Prostaglandin G/H synthase	*Lc COX-1a*	1.14.99.1	AACCGAGTCTGTGACATCCT	20
			CAACGTGGGATCAAACTTCAG	21
Prostaglandin G/H synthase	*Lc COX-1b*	1.14.99.1	CAGCCCTTCAATCAGTACAG	20
			TCTCACCGAATATGCTACCA	20
Prostaglandin G/H synthase	*Lc COX-2*	1.14.99.1	AGTTTGTCTTCAACACCTCTG	21
			ATTTCTCTGCTGTTCTCAATGG	22
Arachidonate 5-lipoxygenase	*Lc ALOX-5*	1.13.11.34	TTTACCATCGCCATCAACACC	21
			CTCTTCCTTGCTGTCCACAC	20
Carnitine palmitoyltransferase	*Lc CPT1a*	2.3.1.21	TGATGGTTATGGGGTGTCCT	20
			CGGCTCTCTTCAACTTTGCT	20
Luciferase	*Luc*	NA	GGTGTTGGGCGCGTTATTTA	20
			CGGTAGGCTGCGAAATGC	18
Elongation factor 1 alpha	*EF1α*	NA	AAATTGGCGGTATTGGAAC	19
			GGGAGCAAAGGTGACGAC	18

NA, Not available

Table 5. Growth performance and feed utilisation of barramundi fed increasing EPA analysed using polynomial contrasts.

	Diets						Polynomial contrasts ^		
	EPA 1.2	EPA 4.5	EPA 9.5	EPA 17.0	EPA 20.1	P SEM	Linear	Quadratic	Cubic
Initial weight (g)	10.0	10.4	10.3	10.3	10.3	0.00	0.815	0.556	0.553
Final weight (g)	55.6	53.8	54.2	53.6	53.6	0.40	0.126	0.437	0.550
Weight gained (g)	45.3	43.5	43.9	43.3	43.3	0.40	0.126	0.413	0.526
BW gain (%)	439.5	419.7	426.0	419.5	420.3	3.80	0.132	0.329	0.444
Daily gain rate (g/d)	1.08	1.03	1.05	1.03	1.03	0.01	0.118	0.417	0.496
Feed conversion	0.70	0.73	0.72	0.74	0.73	0.01	0.132	0.654	0.820
Survival (%)	91.1	93.3	93.3	92.2	93.3	0.40	0.649	0.754	0.639

^ Degrees of freedom 4, 10; linear, quadratic and cubic values are P values at an alpha level of 0.05.

BW, Body weight, P SEM, Pooled standard error mean.

Table 6. Growth performance and feed utilisation of barramundi fed increasing ARA analysed using polynomial contrasts.

	Diets					Polynomial contrasts ^		
	ARA 1.1	ARA 5.3	ARA 11.5	ARA 16.4	P SEM	Linear	Quadratic	Cubic
Initial weight (g)	10.4	10.2	10.4	10.4	0.02	0.466	0.230	0.062
Final weight (g)	53.8	53.7	53.2	54.8	0.51	0.435	0.209	0.357
Weight gained (g)	43.5	43.5	42.8	44.4	0.52	0.466	0.239	0.299
BW gain (%)	419.7	424.9	412.6	428.7	4.96	0.625	0.427	0.153
Daily gain rate (g/d)	1.03	1.04	1.02	1.06	0.01	0.466	0.239	0.299
Feed conversion	0.73	0.73	0.72	0.70	0.01	0.014	0.053	0.698
Survival (%)	93.3	93.3	97.8	98.9	1.65	0.017	0.730	0.301

^ Multiple R2 0.30, df 3,8; linear, quadratic and cubic values are P values at an alpha level of 0.05.
BW, Body weight; P SEM, Pooled standard error mean.

Table X. Growth performance and feed utilisation of barramundi fed EPA and ARA diets analysed by one-way ANOVA.

	EPA 1.2	EPA 9.5	EPA 20.1	ARA 1.1	ARA 16.4	FO CTRL	P SEM	F Value	P Value ^
Initial weight (g)	10.3	10.3	10.3	10.4	10.4	10.3	0.01	0.57	0.717
Final weight (g)	55.6	54.2	53.6	53.8	54.8	56.1	0.31	2.29	0.112
Weight gained (g)	45.3	43.9	43.3	43.5	44.4	45.8	0.31	2.33	0.106
BW gain (%)	439.5	426.0	420.3	419.7	428.7	442.3	2.99	2.41	0.098
Daily gain rate (g/d)	1.08	1.05	1.03	1.03	1.06	1.09	0.01	2.33	0.106
Feed conversion	0.70	0.72	0.73	0.73	0.70	0.69	0.01	2.09	0.137
Survival (%)	91.1	93.3	93.3	93.3	98.9	91.1	0.01	1.85	0.178

^ Degrees of freedom 5, 12.

BW, Body weight, P SEM, Pooled standard error mean.

23 Table 7. Apparent digestibility (%) of nutrients and fatty acids in diets analysed by one-way ANOVA.

	EPA 1.2	EPA 9.5	EPA 20.1	ARA 1.1	ARA 16.4	FO CTRL	P SEM	F value	P Value^
Dry matter	70.2	69.1	74.0	70.8	67.3	69.2	1.1	0.46	0.800
Protein	92.3	91.7	93.1	92.0	91.1	92.4	0.3	1.05	0.435
Lipid	79.3 ^{a}	85.2 ^{a}	89.3 ^{ab}	78.3 ^{a}	86.4 ^{a}	94.9 ^{b}	1.6	11.12	0.001
Energy	82.3	79.2	84.7	80.0	81.1	83.3	0.8	1.27	0.340
16:0	70.1	52.7	51.4	63.6	51.8	63.8	2.7	1.64	0.223
18:0	64.9 ^{a}	42.1 ^{cd}	28.2 ^{d}	53.8 ^{abc}	30.7 ^{d}	56.8 ^{abc}	3.5	16.56	0.000
18:1	92.1 ^{ab}	93.1 ^{a}	94.7 ^{a}	89.3 ^{ab}	93.3 ^{a}	84.1 ^{b}	1.0	4.87	0.014
18:2n-6	95.9 ^{ab}	94.9 ^{ab}	97.0 ^{a}	94.4 ^{ab}	95.3 ^{ab}	92.5 ^{b}	0.4	4.31	0.017
18:3n-3	100.0	100.0	100.0	100.0	100.0	100.0	0.0	NA	NA
20:4n-6	100.0	100.0	99.0	100.0	96.9	100.0	0.3	NA	NA
20:5n-3	100.0	97.8 ^{ab}	99.2 ^{b}	96.1 ^{a}	97.6 ^{ab}	96.7 ^{a}	0.4	8.70	0.002
22:5n-3	100.0	100.0	100.0	100.0	95.3	90.4	1.1	NA	NA
22:6n-3	95.6	96	97.9	92.9	95.8	95.7	0.5	2.74	0.071
SFA	71.3 ^{a}	54.5 ^{ab}	49.6 ^{b}	63.9 ^{ab}	50.7 ^{b}	66.9 ^{ab}	2.4	4.89	0.011
MUFA	92.4 ^{a}	93.6 ^{a}	96.0 ^{a}	89.8 ^{ab}	93.3 ^{a}	83.7 ^{b}	1.1	8.10	0.002
C_{18} PUFA	96.3 ^{a}	95.4 ^{ab}	97.3 ^{a}	94.9 ^{ab}	96.0 ^{ab}	93.5 ^{b}	0.4	5.07	0.010
LC-PUFA	98.2 ^{a}	98.2 ^{a}	98.9 ^{a}	94.5 ^{c}	96.9 ^{ab}	95.9 ^{bc}	0.4	10.16	0.000
n-3	96.9 ^{ab}	98.2 ^{a}	98.9 ^{a}	94.4 ^{c}	96.7 ^{ab}	95.9 ^{bc}	0.4	7.88	0.002
n-6	97.6 ^{a}	95.6 ^{ab}	97.4 ^{a}	95.1 ^{ab}	96.6 ^{a}	93.5 ^{b}	0.4	7.60	0.002

^ Degrees of freedom 5, 12.

P SEM, Pooled standard error mean.

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Table 8. Whole body and fatty acid composition of barramundi analysed by one-way ANOVA (g/kg live-basis).

	Initial fish	EPA 1.2	EPA 9.5	EPA 20.1	ARA 1.1	ARA 16.4	FO CTRL	P SEM	F statistic	P value^
Dry matter	225.4	266.2[a]	280.6[ab]	284.6[b]	275.3[ab]	280.4[ab]	287.5[b]	2.0	4.95	0.011
Protein	144.4	173.0	182.3	181.1	182.2	178.0	177.4	1.5	0.95	0.482
Lipid	32.9	55.0[a]	63.9[b]	66.0[b]	61.2[ab]	67.5[bc]	73.5[c]	1.5	12.7	0.000
Energy (MJ/kg)	47.2	59.9[a]	67.0[b]	66.4[b]	65.2[ab]	66.6[b]	67.3[b]	0.8	4.50	0.015
16:0	5.5	9.5[a]	10.2[a]	9.9[a]	10.2[a]	9.7[a]	13.7[b]	0.4	36.0	0.000
18:0	1.8	2.9[a]	3.4[b]	3.0[ab]	3.1[ab]	3.5[c]	3.5[c]	0.1	7.51	0.002
18:1	7.7	15.1	16.4	15.1	16.5	14.5	14.4	0.3	3.32	0.041
18:2n-6	2.4	4.8	5.1	5.1	5.2	5.3	4.9	0.1	1.28	0.333
18:3n-3	0.3	0.4[a]	0.4[a]	0.5[b]	0.4[ab]	0.4[a]	0.6[c]	0.0	33.05	0.000
20:4n-6	0.2	0.4[a]	0.5[a]	0.5[a]	0.5[a]	5.3[b]	0.5[a]	0.5	1638.9	0.000
20:5n-3	0.7	0.5[a]	2.7[c]	5.6[d]	1.3[b]	1.5[b]	2.4[c]	0.5	186.1	0.000
22:5n-3	0.3	0.4[a]	1.0[c]	1.3[d]	0.7[b]	0.7[b]	1.2[d]	0.1	108.9	0.000
22:6n-3	1.4	1.9[a]	2.3[a]	2.4[a]	2.1[a]	2.4[a]	4.2[b]	0.2	82.93	0.000
SFA	8.2	13.1[a]	14.3[a]	13.9[a]	14.1[a]	13.9[a]	19.4[b]	0.5	38.90	0.000
MUFA	9.3	16.6	18.0	16.6	18.1	16.0	18.0	0.3	2.92	0.059
PUFA	2.8	5.5	5.8	5.9	5.9	6.3	5.8	0.1	2.08	0.139
LC-PUFA	2.5	3.3[a]	6.6[c]	9.8[e]	4.8[b]	10.9[e]	8.3[d]	0.7	108.47	0.000
n-3	2.6	3.1[a]	6.4[c]	9.8[e]	4.6[b]	4.9[b]	8.3[d]	0.6	98.78	0.000
n-6	2.7	5.7[a]	6.0[a]	5.9[a]	6.1[a]	12.3[b]	5.7[a]	0.6	152.3	0.000

^ Degrees of freedom 5, 12.

P SEM, Pooled standard error mean.

Table 9. Retention efficiency of selected fatty acids in barramundi fed either increasing EPA or ARA analysed by polynomial contrasts.

	EPA Polynomial contrasts ^			ARA Polynomial contrasts ^		
	Linear	Quadratic	Cubic	Linear	Quadratic	Cubic
18:2n-6 retention	0.000	0.242	0.006	0.000	0.660	0.001
18:3n-3 retention	0.020	0.989	0.104	0.001	0.094	0.001
20:4n-6 retention	0.057	0.006	0.209	0.911	0.000	0.178
20:5n-3 retention	0.167	0.047	0.165	0.303	0.339	0.682
22:6n-3 retention	0.965	0.201	0.061	0.004	0.006	0.180

^ Degrees of freedom EPA 4, 10; ARA 3, 8; linear, quadratic and cubic values are P values at an alpha level of 0.05.

Table 10. Summary of maintenance demand and utilisation efficiencies by barramundi fed either increasing EPA or ARA.

	Maintenance demand (g/kg LW^x/t)	Efficiency constant *k*	Intake:Gain ratio	R^2	P value
20:5n-3; EPA	0.000	0.621	1.610	0.975	<0.01
20:4n-6; ARA	0.011	0.919	1.088	0.966	<0.05

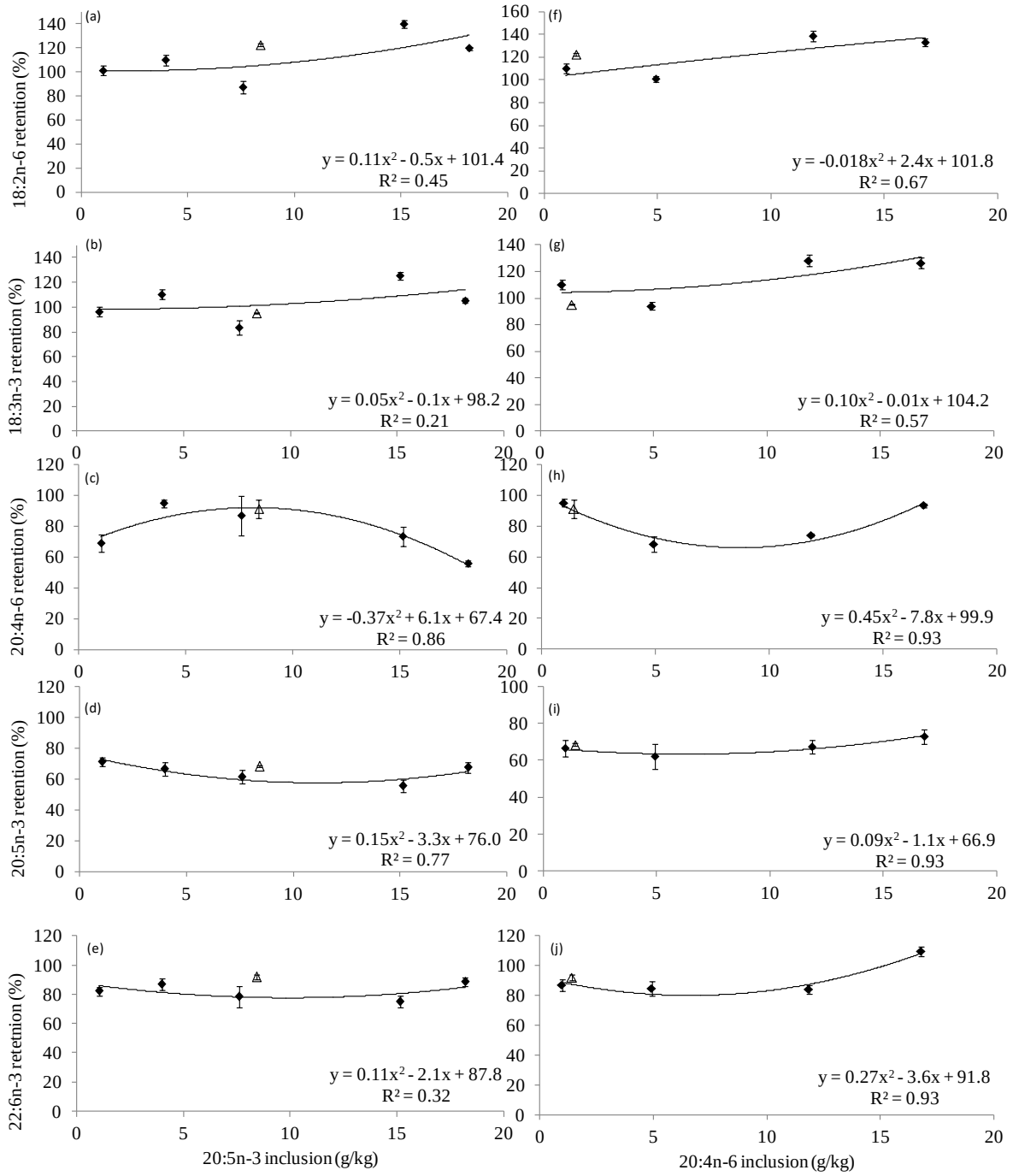
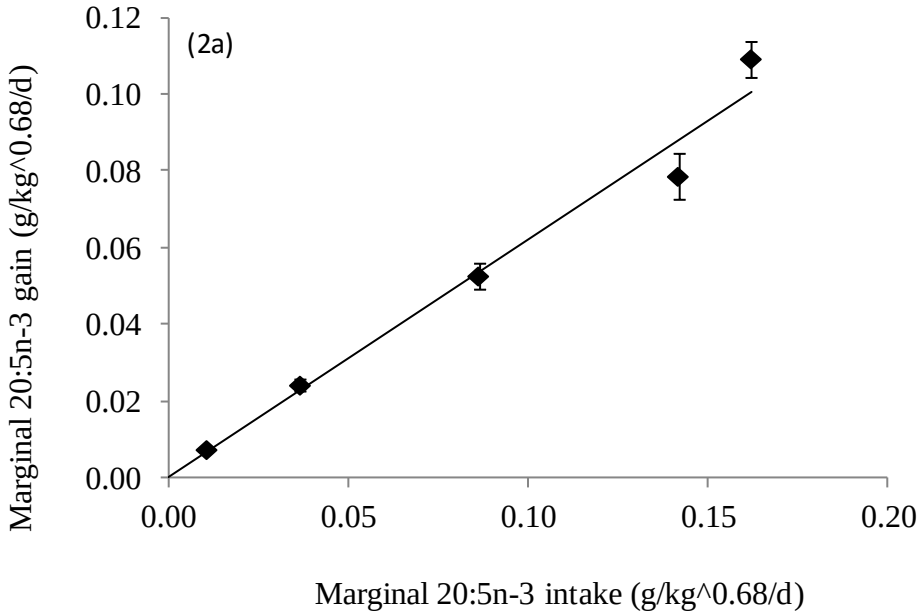
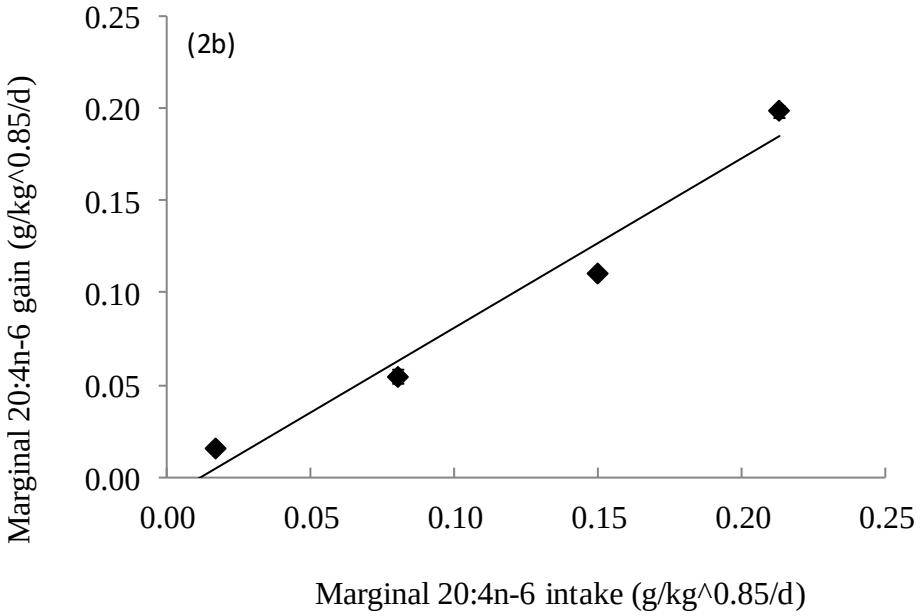


Figure 1. Specific fatty acid retention efficiency by barramundi fed increasing EPA (a-e) or ARA (f-j). The control (FO CTRL) fed fish are represented in each figure with a triangle (Δ). Bars indicate standard error means (n=3).





42

43 Figure 2. Marginal utilisation efficiency assessments of 20:5n-3 (a) and 20:4n-6 (b) gain with
44 varying intake by juvenile barramundi. Efficiency functions are described by the linear
45 regression for 20:5n-3 gain $y = 0.621x + 0.0003$, $R^2 = 0.975$ and 20:4n-6 gain $y = 0.919x -$
46 0.011, $R^2 = 0.965$).
47

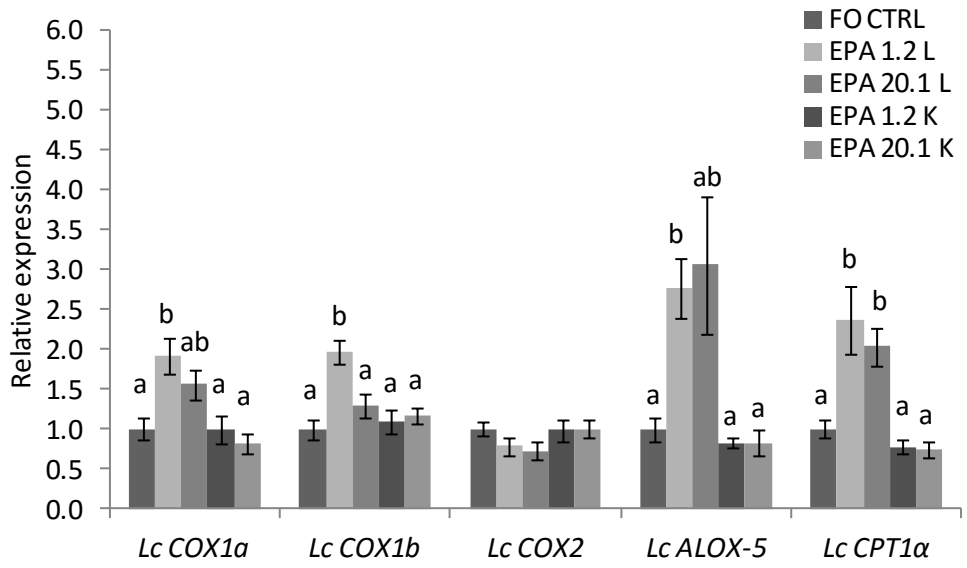


Figure 3. Eicosanoid pathway and mitochondrial fatty acid oxidation gene expression in the liver (L) and kidney (K) of juvenile barramundi fed increasing EPA. Gene expression is normalised to the EF1α and Luc reference genes. Data were analysed by one-way ANOVA (*df* 4,28) with letters above bars indicating differences defined as P<0.05.

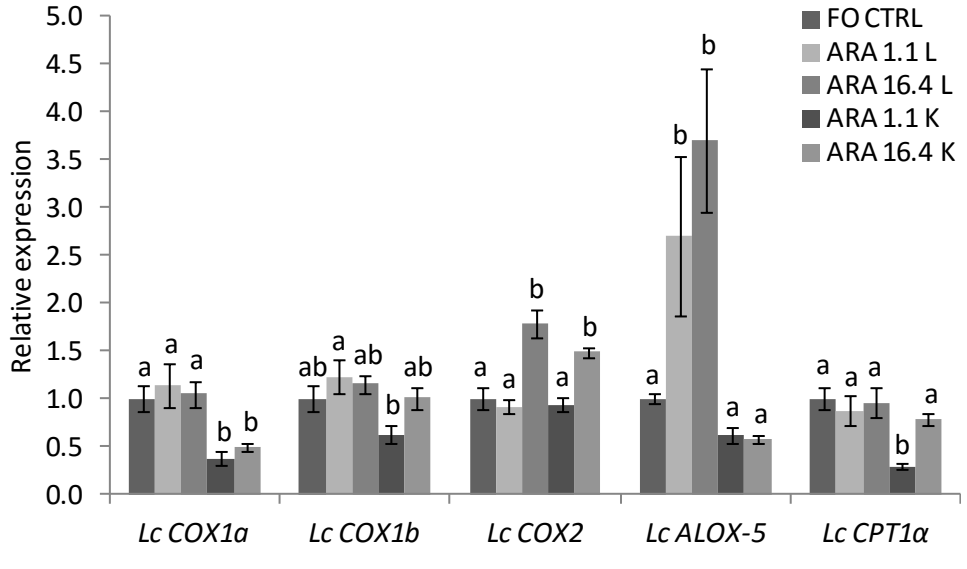


Figure 4. Eicosanoid pathway and mitochondrial fatty acid oxidation gene expression in the liver (L) and kidney (K) of juvenile barramundi fed increasing ARA. Gene expression is normalised to the EF1α and Luc reference genes. Data were analysed by one-way ANOVA (*df* 4,28) with letters above bars indicating differences defined as P<0.05.