Accepted refereed manuscript of:

Salini MJ, Wade NM, Bourne N, Turchini GM & Glencross B (2016) The effect of marine and non-marine phospholipid rich oils when fed to juvenile barramundi (Lates calcarifer), *Aquaculture,* 455, pp. 125-135.

DOI: 10.1016/j.aquaculture.2016.01.013

The effect of marine and non-marine phospholipid rich oils when fed to juvenile barramundi (*Lates calcarifer*).

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Keywords: Phospholipid, Barramundi, Krill oil, Soybean oil, Soybean lecithin.

Abstract

An experiment was conducted to assess the response of juvenile barramundi (*Lates calcarifer*) to four diets containing either marine- or non-marine derived neutral lipid (NL) or polar lipid (PL) sources for eight weeks in a 2x2 factorial design. The four diets contained 8.2% added lipid composed of a 1% fish oil base with 7.2 % test lipid (n-3 NL: Fish oil, n-3 PL: Krill oil, n-6 NL: Soybean oil, n-6 PL: Soybean lecithin). The results demonstrated that the different lipid sources (either n-3 or n-6 omega series from either NL or PL class) had significant effects on growth performance and feed utilisation with some interaction terms noted. Growth was negatively affected in the n-6 NL fish and the feed conversion (FCR) was highest in the n-6 PL fish. Digestibility of total lipid and some specific fatty acids (notably 18:2n-6 and 18:3n-3) were also negatively affected in the n-6 PL fish. Analysis of the whole body neutral lipid fatty acid composition showed that these mirrored those of the diets and significant interaction terms were noted. However the whole body polar lipid fatty acids appeared to be more tightly regulated in comparison. The blood plasma biochemistry and hepatic transcription of several fatty acid metabolism genes in the n-6 PL fed and to a lesser extent in the n-6 NL fed fish demonstrated a pattern consistent with modified metabolic function. These results support that there are potential advantages in using phospholipid-rich oils however there are clear differences in terms of their origin.

1 Introduction

The phospholipids form the structural bilayer of cell membranes providing integrity and fluidity (Hazel and Williams, 1990; Tocher et al., 2008). Central to their biological importance is their structure with lipoproteins that assist in the extracellular transport of lipids thus improving parameters such as growth, survival and health throughout the organism (Tocher et al., 2008). However, the total lipid content in fish is mostly composed of neutral lipid in the form of triacylglycerol (TAG) which is a more readily available energy source (Glencross, 2009).

There is evidence to suggest that most larval and early juvenile fish have a dietary requirement for intact phospholipids as endogenous biosynthesis is not sufficient (Coutteau et al., 1997). Coutteau et al. (1997) reported that the phospholipid requirement of fish and crustaceans varied depending on the life stage and history. Freshwater fish generally have lower dietary requirements, of around 2% whereas marine fish generally had higher requirement ranging up to 7% however that gradually reduced as fish grew. Early studies found that in both rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*) the phospholipid requirement of first swim-up sized fish (<0.2 g) was 4% supplied in the form of soybean lecithin (Poston, 1990a; b). However, larger salmon (~7.5 g initial) showed no improvement in terms of growth suggesting that endogenous synthesis of phospholipid is sufficient to support the requirement of the fish and that high dietary levels had a negative effect on survival (Poston, 1990a). It should also be noted that the latter study, and possibly others, refer to a requirement of phospholipid containing ingredients rather than the precise phospholipid content which is often unclear.

With very few exceptions, provision of marine derived phospholipid to cultured fish is limited. Moreover, there are few studies on the effect of dietary phospholipids in juvenile fish greater than 5 g as it is generally accepted that they don't have a requirement based on the historical evidence presented for Atlantic salmon (Poston, 1990a). Recently, the influence of dietary phospholipid from either krill oil or soybean lecithin was investigated in Atlantic salmon from first feeding up to smolt (0 to 70 g range) (Taylor et al., 2015). These authors demonstrated a range of improvements among the parameters tested and concluded that Atlantic salmon have a dietary requirement for intact phospholipid particularly in early development. Therefore, with the continual reduction of fish meal (FM) and fish oil (FO) in commercial feeds and the complex biochemistry of the phospholipids particularly in juvenile

fish, further investigation is warranted. Moreover, the preferential incorporation and retention of phospholipid fatty acids are important in maintaining phospholipid quality and also to fulfil other downstream roles of the phospholipid classes (Linares and Henderson, 1991).

Recent *in*- and *ex-vivo* methods have so far demonstrated that barramundi or Asian seabass (*Lates* calcarifer) are not capable of any measureable long-chain polyunsaturated fatty acid (LC-PUFA) biosynthesis (Alhazzaa et al., 2011a; Mohd-Yusof et al., 2010; Tu et al., 2012). However, some notable effects on the phospholipid composition of tissues were identified, which may suggest that juvenile barramundi have a requirement for intact phospholipids in order to prevent the onset of deficiency (Alhazzaa et al., 2011b; Tu et al., 2013). It appears that when dietary PL are not sufficient then very selective retention of tissue phospholipids occurs in barramundi and other species, until depletion, this being a mechanism to prevent the onset of PL deficiency and secondary pathologies as a result (Skalli and Robin, 2004; Tocher et al., 2008; Tu et al., 2013).

Most phospholipid requirement studies to date have used soybean lecithin containing high levels of n-6 PUFA, while others have used egg lecithin or various other marine sources such as fish roe lecithin (Cahu et al., 2009). Recent studies have clearly demonstrated the potential of marine derived phospholipid sources to improve larval and juvenile fish performance (Betancor et al., 2012; Taylor et al., 2015). To date, information is scarce on the effect phospholipid in juvenile barramundi diets. Therefore, an experiment was designed to compare the metabolic effect of marine and non-marine neutral lipid (NL) and polar phospholipid (PL) sources using a two-by-two factorial approach in juvenile barramundi. The biochemical and molecular mechanisms underpinning the role of phospholipids was also investigated.

2 Materials and methods

2.1. Ingredient and diet preparation

The diets were formulated to provide digestible protein at ~55 %, lipid at ~12 % with a digestible energy value of ~19 MJ/kg. 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 ingredients were then thoroughly mixed in using an upright commercial mixer (Bakermix, Model 60 A-G, NSW, Australia). The chemical composition of the main dietary ingredients is presented in Table 1. A single batch of basal

diet was prepared then divided up and warmed aliquots of the oil mixtures were thoroughly mixed in. Water was added at approximately 30% of the mash weight and then mixed until consistent dough was formed. The pellets were extruded through a 4mm die attached to a screw-press pasta machine and cut off at lengths of 5 to 6mm. The pellets were dried overnight at 60˚C to a constant dry matter and stored in a freezer until required. The formulation and chemical composition of the four diets are presented in Table 2.

2.2. 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 (300L) with flow-through seawater (salinity =35 PSU; dissolved oxygen 4.6 ± 0.15 mg /L) maintained at 30.0 ± 0.01 ºC (mean ± SD) with a supply rate of about 3 L/min to each of the tanks. The tanks were maintained in an environment-controlled laboratory with the photoperiod set to a constant 12:12h cycle. At the beginning of the experiment, the tanks held 26 fish of 47.0 ± 0.3 g (mean ± SD, n =312 individually weighed fish). The four experimental diets were randomly distributed amongst the twelve tanks with each treatment having three replicate tanks. The fish were offered their respective diets to apparent satiety once daily except on the day of sampling.

2.3. Sample collection, preparation and digestibility analysis

Ethical clearance was approved for the experimental procedures by the CSIRO animal ethics committee A11/2013. 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 six fish were dissected and whole blood was removed from the caudal vein using 1 mL pre-heparinised syringes and an 18 G needle. Blood was pooled in a single Vacutainer™ tube and then centrifuged at 10,000 rpm for 5 min to settle the erythrocytes. The plasma was then drawn off and transferred to a 1.5 mL Eppendorf™ tube and frozen before being sent for analysis. A sample of liver tissue was then removed and placed into 1.5 mL screw-top vial and kept on dry ice before being transferred to a -80 ˚C freezer until analysis. The same sampling procedure occurred after 56 d with three fish from each treatment while the remaining fish were returned to their respective tank after

a short recovery. All sampling procedures occurred 24 h after the last feeding event (Wade et al., 2014).

Prior to the termination of the growth assay, faeces were collected using established settlement protocols (Blyth et al., 2014; Glencross et al., 2005). Briefly, a collection chamber was filled with water and frozen then attached to the evacuation line of a swirl separator and left overnight. The following morning, the collection chamber was removed and the chilled faeces were captured in a plastic sample container and stored at -20 ˚C until analysis.

2.5. Chemical analysis

Prior to analysis the diets were each ground to a fine powder using a bench grinder (KnifeTec™ 1095, FOSS, Denmark). The 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. Total yttrium concentrations 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). 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 12 h. Gross energy was determined by adiabatic bomb calorimetry (Parr 6200 Calorimeter, USA). 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) 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} represent the yttrium content in both the diet and faeces, respectively and $Parameter_{diet}$ and $Parameter_{faeces}$ represent the nutritional parameter (dry matter, protein, lipid and energy) in the diet and faeces, respectively (Glencross et al., 2007).

Plasma samples were sent to the West Australian Animal Health Laboratories (South Perth, Western Australia) for enzyme and chemistry assessment. The assays were run on an Olympus AU400 automated chemistry analyser (Olympus Optical Co. Ltd). Each of the assays used was a standard kit developed for the auto-analyser. The tests performed included alanine aminotransferase (ALT, EC 2.6.1.2) (Olympus kit Cat. No. OSR6107), creatine kinase (CK, EC 2.7.3.2) (Olympus kit Cat. No. OSR6179), glutamate dehydrogenase (GLDH, EC 1.4.1.2) (Randox kit Cat. No. GL441), total protein (Olympus kit Cat. No. OSR6132), creatinine (Olympus kit Cat. No. OSR6178), alkaline phosphatase (Olympus kit Cat. No. OSR6004), glucose (Olympus kit Cat. No. OSR6121), haemoglobin (Randox kit Cat. No. HG1539) and haptoglobin (Randox kit Cat. No. HP3886). Trace elements were determined after mixed acid digestion using inductively coupled plasma mass spectrometry (ICP-MS).

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.

Lipid extracts were applied to silica Sep-Pak® (Waters, Massachusetts, USA) columns and separated into neutral (non-phosphorus) lipid and polar (phosphorus) lipid following Juaneda and Rocquelin (1985). Briefly, Sep-Pak® columns were pre-conditioned with 4ml of hexane before a 30 mg sample of lipid dissolved in chloroform was applied to each column. The neutral lipid was first eluted with 20 ml of chloroform and followed by 5 ml of chloroform:methanol (49:1). The polar lipid was then eluted with 30 ml of methanol. The

proportion of either neutral or polar lipid was quantified gravimetrically and then an aliquot was further esterified and separated into fatty acids following the protocol above.

2.6. Mass balance calculations

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 using the formula:

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

Where *Nf* and *Ni* are the final and initial nutrient composition (g/fish) of the fish on a wet basis, respectively, and *Nc* is the amount of the nutrient consumed (g/fish) during the study period (Maynard and Loosli, 1979). The computation of apparent *in vivo* fatty acid elongation, desaturation and β-oxidation was performed using the whole-body fatty acid balance method (WBFABM) following Turchini et al. (2007). Briefly, this involved determination of the appearance/disappearance of specific fatty acids by mass balance. The resulting values of net appearance/disappearance were then transformed to a molecular weight basis per gram of body weight per day (nmol/g fish/d). Subsequent back calculations along the known fatty acid bioconversion pathways were used to determine the fate of specific fatty acids.

2.7. RNA extraction and normalisation

Total RNA was extracted using Trizol reagent (Invitrogen) according to the manufacturer's instructions. RNA was precipitated using equal volumes of precipitation solution (1.2 M sodium chloride and 0.8 M disodium citrate) and isopropyl alcohol (Green and Sambrook, 2012). To eliminate any residual traces of DNA, total RNA was DNase digested with the Turbo DNA free kit (Applied Biosystems). To verify that RNA was not contaminated, an aliquot of DNase digested RNA from each sample was pooled (n=32) and later PCR amplified as a negative control. A NanoDrop spectrophotometer (NanoDrop Technologies) was used to asses RNA quantity and a Bioanalyser (Agilent Technologies) using RNA nanochips (Agilent #5067-1511) was used to asses RNA quality. All RNA samples were normalised to 200 ng/µl.

2.8. Quantitative real time RT-PCR

Expression of a range of genes involved in fatty acid metabolism was analysed by real-time qPCR as described below (Table 4). Of the genes examined in the present study, fatty acid elongation 5, fatty acid desaturation 6 and elongation factor 1α (GQ214180.1, GQ214179.1, GU188685) are contained within the published nucleotide database. The remaining genes were previously identified and reported following next generation sequencing of barramundi liver tissue and BLAST similarity searches (Wade et al., 2014). The raw sequence read data are made available online through the CSIRO Data Access Portal (http://doi.org/10.4225/08/55E799BA0F73E). For cDNA synthesis, 1 µg of total RNA was reverse transcribed using superscript III (Invitrogen) with 25 µM oligo $(dT)_{20}$ and 25 µM random hexamers (Resuehr and Spiess, 2003). Real-time PCR amplification reactions were carried out using 1X SYBR Green PCR Master Mix (Applied Biosystems), 0.2 µM of Real-Time PCR primers specific to each gene and the equivalent of 7.5 ng of reverse-transcribed RNA. Amplification cycle conditions were 2 min at 50 ˚C, 10 min at 95 ˚C followed by 40 cycles of 15 s at 95 ˚C and 40 s at 60 ˚C. After amplification, a melt curve analysis was routinely performed to verify the specificity of the target gene. Reactions were setup using the epMotion 5070 robot (Eppendorf) and run in triplicate on a Viia7 real-time PCR system (Applied Biosystems). Changes in expression levels of each gene were determined by normalising the cycle threshold values for each gene to elongation factor 1 alpha (*EF1α*) and Luciferase (*Luc*) reference genes, then to the cycle threshold of each gene in the initial fish samples. The variation in amplification of *EF1α* was 1.24 cycles and *Luc* was 0.58 cycles and this did not significantly change over time. The *EF1α* and *Luc* genes are routinely used as a reference in this species (De Santis et al., 2011; Wade et al., 2014).

2.9. Statistical analysis

All data are expressed as mean ± SEM unless otherwise specified. All data were checked for normal distribution and homogeneity of variance by qualitative assessment of residual and normal Q-Q plots. All data were analysed by two-way factorial ANOVA using the RStudio package v.0.98.501 (R Core Team, 2012). Any variables with only three treatments of data were analysed by one-way ANOVA. Any percentage data were arcsine transformed prior to analysis. Levels of significance were compared using Tukey's HSD *a posteriori* test with significance among treatments defined as $P < 0.05$.

3 Results

3.1 Growth and feed utilisation

In the present study the two levels of omega status were defined as n-3 and n-6 and the two levels of lipid class were defined as neutral lipid and phospholipid. There was no difference in the initial weight of the fish among the treatments; however there were significant differences in the growth and feed utilisation parameters (as final weight, feed intake and FCR) upon termination of the 56 d growth assay (Table 5). There were significant interaction terms indicating that the effectiveness of phospholipid-rich ingredients was found to be dependent on the omega status for the final weight, feed intake and FCR. The lowest growth performance was seen in the fish fed n-6 NL (soybean oil) and the FCR was reduced in the n-6 PL fed fish (soybean lecithin). There were no differences in terms of survival with only three fish removed from the system. There were no significant differences in either protein or lipid retention (Table 5).

There were no differences in apparent digestibility of dry matter, protein or gross energy (Table 6). There was a significant interaction effect indicating that the digestibility of total lipid in the phospholipid-rich treatments was dependent on the omega status with the lowest lipid digestibility in the n-6 PL (soybean lecithin) treatment. There was a significant interaction in the digestibility of 16:0 which showed that the highest digestibility in the n-6 NL treatment was dependent on the lipid class. There was also a significant interaction noted for the digestibility of 18:2n-6, total C_{18}PUFA and total n-6 showing that the digestibility of fatty acids from phospholipid-rich ingredients was dependent on the omega status. In each case the digestibility was lowest in the n-6 PL treatment (soybean lecithin). Similarly, the digestibility of 18:3n-3 was significantly lower in the n-6 PL fed fish.

3.2 Biochemical analysis

As intended, the neutral and polar composition of the diets was reflective of the lipid composition of the ingredients used (Table 3).There were no significant differences in the neutral and polar composition of the whole fish with all treatments in the range of 89.4 to 94.0 % neutral lipid (Table 7). Fatty acid analysis of the neutral and polar fractions revealed several significant differences among the treatments. Significant interaction terms were noted for many of the neutral lipid fatty acids. Most notably, the effect of phospholipid-rich ingredients on LC-PUFA (specifically 22:6n-3, 22:5n-3 and 20:5n-3) composition in the whole fish were found to be dependent on the omega status with the highest levels present in

the n-3 treatments. Of the polar lipid fatty acids, the18:2n-6 composition was significantly higher in the n-6 treatments whereas the 16:0, 20:5n-3 and 22:6n-3 composition was highest in the n-3 treatments.

There was several significant interaction terms noted on the apparent *in-vivo* β-oxidation activity (Table 8). The phospholipid-rich ingredients were found to be effective in modifying the β-oxidation of MUFA in the n-6 PL fed fish however this was not the case for the n-3 PL fish. There was a significant interaction term noted in the β-oxidation C_{18}PUFA and Total n-6 with the highest activity in the phospholipid-rich lipid being dependent on the omega status. There was also a significant interaction for the β-oxidation of LC-PUFA and Total n-3 which was dependent on the omega status with the highest activity in the n-3 NL treatments.

There was a significant interaction indicating that elongation activity of SFA was highest in the n-6 NL treatment and lowest activity was in the n-3 NL treatment (Table 8). There was no detectable elongation activity of the LC-PUFA or Total n-3 in the n-3 NL fed fish; however, when analysed by one-way ANOVA there was significantly greater activity in the n-3 PL fed fish compared to both of the n-6 treatments. There was also a significant interaction in the delta-9 (SCD) desaturation activity with greatest activity recorded in the n-6 NL and the lowest activity in the n-3 NL treatment.

There was a significant interaction in the plasma GLDH enzyme with an elevated level present in the n-6 PL treatment which was dependant on the omega status (Table 9). Similarly, there was a significant interaction noted on plasma creatinine levels with an elevated level in the n-6 PL fish which was dependent on the omega status. The plasma cholesterol level was significantly elevated in the n-3 PL treatment and lowest in the n-6 NL and n-6 PL treatments. Circulating protein levels in the plasma were also significantly higher in the phospholipid class treatments. Among the haematological parameters, only minor numerical differences were observed with Hb significantly elevated in the n-3 NL fish and haptoglobin significantly elevated in the n-6 PL fish (Table 9).

3.3 Gene expression

Several significant differences were observed in the expression of genes related to fatty acid metabolism (Figure 1). There was a significant interaction in the relative gene expression of *Lc ACYL*, *Lc FAS* and *Lc FADS2* with increased expression in the n-6 PL treatment that was

dependent on the lipid class. The expression of these genes was at least 1.5-fold higher in the n-6 PL compared to the n-3 PL treatments. There was also a significant interaction effect in the modification of *Lc CPT1α* expression by the phospholipid-rich ingredients was dependent on the omega status. The expression of *Lc CPT1α* was down regulated by approximately 1.5-fold in the n-3 PL compared to the control. Similarly, the expression of *Lc SCD* was significantly up regulated in the n-6 treatments compared to the n-3 treatments however there was no interaction effect observed. The expression of *Lc ELOVL5* in all treatments was down regulated compared to the initial fish levels however there were no significant differences among the treatments.

4 Discussion

It is well established that larval and early juvenile fish have a dietary requirement for intact phospholipids that can lead to long term improvements in many growth performance parameters (Coutteau et al., 1997; Tocher et al., 2008). Historically most phospholipid studies were conducted with commercial phospholipid preparations of commonly available emulsifying agents such as lecithin from soybeans or corn (Tocher et al., 2008). However, in some cases the use of these products has potentially lead to unclear requirement data as the composition of polar lipid and polar lipid classes is seldom reported and there are other potential interacting effects of the ingredients themselves. The aims of the present study were to provide an up to date assessment of the physiological and metabolic effect of commercially available preparations of phospholipid-rich ingredients such as krill oil and soybean lecithin against neutral lipid-rich ingredients such as fish oil and soybean oil in juvenile barramundi.

Based on the performance data of the present study, it is clear that for barramundi in the range of ~47 to ~238 g, that a response to the lipid ingredients was evident. It should be noted that each of the diets were prepared with a minimum (1% diet) inclusion of FO in order to prevent the onset of essential fatty acid deficiency (Salini et al., 2015). The growth, feed intake and FCR of the n-3 NL and n-3 PL fed fish were nearly identical. Despite the n-6 PL fed fish being statistically the same weight as the two n-3 diets they consumed significantly more feed which resulted in a higher FCR. Many studies have clearly demonstrated that growth potential is driven by the demand for energy (derived mostly from protein and lipid) in this species (Glencross et al., 2013). However, the diets in the present study were equivalent in digestible energy and the n-6 PL fish consumed more feed to maintain growth,

comparable to the control (n-3 NL) fish suggesting that the difference in FCR is attributable to the lipid ingredients. Therefore this suggests that the lecithin was poorly utilised by the fish as also supported by numerically lower lipid retention in this treatment. There may be other features of the soybean lecithin that contributed to the results observed however investigation of these was beyond the objectives of the present study.

A recent study on dietary phospholipids in Atlantic salmon over a range of sizes (from first feeding to ~60 g) found that at 2.6% phospholipid in the form of soybean lecithin reduced growth performance and FCR (Taylor et al., 2015). However the most profound negative effect of soybean lecithin on growth rate (SGR) was in early phase (first feeding to 2.5 g) rather than the latter stages of growth studied. It should be clearly noted that the diets used in the present study included 7.2 % added lipid to a base of 1% fish oil. Based on the analysed composition of the diets used in the present study, the determined level of phospholipid in the lecithin diet was around 5.8 % and the krill diet was around 4.0%. It is unclear whether this level of inclusion could have influenced the growth performance of the n-6 PL fed fish. In the study of Taylor et al. (2015), the inclusion of krill oil above 2.6% phospholipid led to a decrease in the growth of Atlantic salmon however no effect of krill oil was seen in the present study. They reasoned that the difference could be due to the reduced energy availability of phospholipid-rich oils compared to that of neutral lipid oils that are mostly triacylglycerol (Taylor et al., 2015).

In larval fish, the digestibility of diets containing phospholipids is consistently better than control diets, mostly owing to their emulsifying effect leading to better absorption by the developing gut system (Coutteau et al., 1997; Tocher et al., 2008). In contrast, the juvenile barramundi fed soybean lecithin in the present study had the lowest total lipid digestibility potentially owing to its physical characteristics rather than its chemical composition. However, the most abundant fatty acid in the phospholipid fraction of the n-6 PL diet was 18:2n-6 and this was also significantly less digestible leading to the lowest total C_{18}PUFA digestibility. These reductions in digestibility most likely also led to the poor FCR observed in that treatment group. However it is unclear whether these effects are caused by the phospholipid composition or another feature of the ingredient as the same effects were not replicated in the krill oil fed fish.

A further interesting result of the present study was that the n-6 NL fed fish ate less and consequently grew less than the n-3 treatments with no difference in FCR. The digestibility

was also high for the total lipid and all the dominant fatty acids in fish fed the n-6 NL diet. This reduction in growth might be explained by several mechanisms. Firstly, soybean oil lacks any measureable n-3 LC-PUFA and to some extent is also likely to be pro-inflammatory due to the high proportion of omega-6 fatty acids (Brown and Hart, 2011; Turchini et al., 2009). However, the reduced growth response should also have been expected in the n-6 PL fed fish as the fatty acid composition of the raw ingredients is quite similar.

Of all the studies investigating the use of soybean oil, growth reduction is rarely reported in fish (Brown and Hart, 2011; Glencross, 2009). In a study by Raso and Anderson (2003) they found that although juvenile barramundi fed soybean oil grew slightly less than the controls it was not confirmed statistically and the FCR also remained unchanged. However, in a select group of marine species, such as the black sea bream (*Acanthopagrus schlegeli*), Japanese flounder (*Paralichthys olivaceous*), red sea bream (*Pagrus auratus*), silver bream (*Rhabdosargus sarba*) and cobia (*Rachycentron canadum*) feeding exclusively with soybean oil has lead to differences in growth performance, however most simply put, they were probably caused by essential fatty acid (EFA) deficiency rather than another unique feature of the soybean oil itself (Brown and Hart, 2011; Trushenski et al., 2012).

Another more recent hypothesis is that certain fatty acids including 18:2n-6 may influence feed intake by modulating the expression of 'satiety' and 'hunger' hormones via various signalling pathways, for example neuropeptide Y or Agouti-related protein (NPY and AGRP respectively), however, this is yet to be thoroughly explored in teleost fish (Coccia et al., 2014; Liland et al., 2013; Schwartz et al., 2000). Therefore, in light of the many possibilities, the fish fed the n-6 NL (soybean oil) diet in the present study were likely to be EFA deficient. However, the same effect did not manifest itself in the n-6 PL fish, indicating a marginal improvement owing to the phospholipid content of the diet.

In the present study, the whole body fatty acid composition mostly resembled the diet profiles as previously reported in the vast majority of studies (Rosenlund et al., 2011). Despite the varied NL and PL composition of the diets used in the present study, it was clear that the proportion of NL and PL in the whole body was tightly regulated. However there were some changes to the fatty acid composition of both the NL and PL fraction of the whole body, consistent with other studies. In larval fish such as sea bream, increasing marine derived PL led to better assimilation of n-3 FA whereas soybean lecithin PL increased assimilation of n-6 FA (Saleh et al., 2015). Alhazzaa et al. (2011b) correlated the up regulation of fatty acid

synthesis genes (*FADS2* and *ELOVL5*) with highly selective fatty acid retention in muscle and liver PL composition of juvenile barramundi fed vegetable oils. Other studies have also demonstrated that the LC-PUFA composition of the PL fraction of certain tissues is tightly regulated in the absence of adequate dietary supply (Skalli et al., 2006). In the present study, this effect was seen with the n-6 NL (soybean oil) fish able to retain numerically more n-3 LC-PUFA than the n-6 PL fish. Moreover, 18:2n-6 was preferentially retained in the n-6 NL fed fish.

In the present study, the apparent *in vivo* whole body mass-balance of specific fatty acids was calculated to determine discrete differences in metabolism (Turchini et al., 2007). The LC-PUFA β-oxidation activity was highest in the n-3 NL followed by the n-3 PL fish, typical of when barramundi and other species are fed excess LC-PUFA (Salini et al., 2015; Stubhaug et al., 2007). The high elongation of SFA and delta-9 desaturation activity in the n-6 NL fish suggests an attempt to generate 18:1n-9 as an available energy source. However, there was no corresponding increase in β-oxidation of MUFA and moreover the same effect was not recorded in the n-6 PL fish. It is unclear as to why they would invest energy into elongation and delta-9 desaturation processes with no further downstream effect; however, it clearly indicates a modified metabolic function that could be part of a compensatory mechanism when EFA deficient. Moreover, it is difficult to correlate the mass-balance computations with the quantitative gene expression analysis used in the present study as different enzymes are involved throughout the fatty acid metabolic pathway. However, the mass-balance results do correlate with those previously reported in barramundi and other species fed alternative oils (Francis et al., 2007; Salini et al., 2015; Turchini et al., 2013).

The biochemical analysis of the plasma and the hepatic gene expression potentially indicate a modified metabolic pattern, particularly as a result of the n-6 lipids, soybean lecithin and to a lesser extent soybean oil. However, there were no dramatic sub-clinical pathologies noted indicating the potential relevance of these ingredients. Creatinine kinase (CK) levels were however numerically highest in the n-3 NL fed fish and although there is a general lack of data in this and other teleost species it could be argued that these fish were in a diseased state (Nanji, 1983; Sandnes et al., 1988). However, it may also be reasoned that these are the normal enzyme levels as they are the control group of this study. The elevated glutamate dehydrogenase (GLDH) activity, creatinine level and total protein in the plasma of the n-6 PL fish are potentially indicative of organ failure or dehydration that can be characterised by the

depletion of hepatic LC-PUFA stores (Van Waes and Lieber, 1977; Videla et al., 2004). However, the plasma enzyme markers (eg GLDH and ALT) are typically used in combination to confirm a clinical diagnosis and in the present study there is not adequate evidence to support this. In contrast, none of the plasma markers were elevated in the n-6 NL fed fish. In addition, several authors have reasoned that together elevated GLDH and plasma urea are implicated in osmoregulatory processes leading to clinical pathologies such as subcutaneous haemorrhaging (Glencross and Rutherford, 2011; Morton et al., 2014). However, in the present study these pathologies were not present and moreover the plasma urea content was unaffected suggesting that the lipid sources used were nutritionally adequate unlike those of the previously mentioned studies. Further work is clearly warranted in this area to resolve some of the discrepancies relating to clinical diagnosis in barramundi and other teleosts.

The hepatic expression of genes related to fatty acid initial synthesis (*Lc ACYL* and *Lc FAS*) and delta-6 and delta-9 desaturation (*Lc FADS2* and *Lc SCD*), were also significantly up regulated in n-6 PL fed fish. These transcriptional changes suggest that there was potentially a metabolic modification liver and the fish have a limited ability to respond to the soybean lecithin. Recent studies have shown similar nutritional regulation of fatty acid metabolism related genes with soybean oil (Li et al., 2016); however the same effect of soybean oil (diet n-6 NL) was not replicated to the same extent in the present study. Interestingly, the expression of *Lc CPT1α*, which is considered to be the initial step in the mitochondrial β-oxidation of fatty acids, was dependent on the omega status being significantly down regulated in the n-3 PL fish compared to the n-3 NL fish (Frøyland et al., 1998). Moreover, the apparent *in vivo* β-oxidation of SFA, MUFA and PUFA was also significantly lower in the n-3 PL treatment which may indicate an active anti-oxidant effect of phospholipid rich lipids of marine origin such as krill (Saito and Ishihara, 1997).

5 Conclusions

Studies have consistently demonstrated a range of advantages when using phospholipid-rich lipid sources in diets for larval and juvenile fish. In this study, we report for the first time the use of phospholipid-rich krill oil and also soybean lecithin compared to neutral lipid sources including fish oil and soybean oil. In support of the vast majority of studies, we demonstrated that the inclusion of either marine or non-marine phospholipid maintains performance of

juvenile barramundi equivalent to a fish oil based control diet. An interesting result of the present study was the differences observed between both the n-6 diets. The fish fed soybean lecithin avoided gross signs on EFA deficiency which was in contrast to the soybean oil fed fish. However, the dietary soybean lecithin affected the FCR and some sub-clinical markers potentially indicated a modified metabolic function.

Acknowledgements

The authors wish to acknowledge the technical assistance provided by Bruno Araujo, David Blyth, Natalie Habilay, Simon Irvin, Kinam Salee and Richard Thaggard of the Bribie Island Research Centre (BIRC), Queensland, Australia. The authors wish to acknowledge the CSIRO Agricultural Productivity Flagship for financial support.

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Figure 1. Hepatic gene expression of selected lipid metabolism genes in juvenile barramundi (*Lates calcarifer*) after eight weeks of feeding. Relative expression is calculated for each gene using cycle threshold values, normalised to control genes (Elongation factor 1a and Luciferase). Values are shown as log-2 fold change relative to the initial fish. Letters above error bars indicate significant differences between the treatments. Analysed by two-way factorial ANOVA, *df 1,1,1,8*, post-hoc Tukey's HSD.

Table 1 Chemical composition of ingredients used in experimental diets, all values are g/kg DM unless otherwise stated

	Fish meal[#]	Poultry meal	Soy isolate	Wheat gluten	Wheat flour	Casein	Wheat starch	Fish oil	Krill oil	Soybean oil	Soybean lecithin
Composition											
Dry matter (g/kg)	98.4	95.8	95.8	92.7	83.9	92.4	83.6	99.2	99.9	100.0	98.0
Protein	78.9	64.1	89.5	82.3	11.2	87.0	0.5	0.4	4.5	1.0	7.5
Ash	16.3	13.8	4.6	0.1	0.6	1.1	0.3	0.1	2.9	ND	9.8
Lipid	4.6	15.1	5.7	12.1	2.2	0.5	ND	95.6	92.6	94.6	75.7
Carbohydrate	0.1	7.0	0.2	5.5	86.0	11.3	99.2	3.9	ND	4.5	7.1
Gross energy (mJ/kg)	18.9	20.4	21.8	21.2	15.3	21.9	14.5	39.3	36.3	39.5	29.7
*Fatty acids (mg/g lipid)^*											
16:0	149.0	161.7	NA	NA	NA	NA	NA	128.4	107.3	93.7	111.5
18:0	50.9	55.4	NA	NA	NA	NA	NA	29.0	6.3	35.4	23.7
18:1	89.8	277.0	NA	NA	NA	NA	NA	104.0	90.0	220.7	53.3
18:2n-6	10.4	71.4	NA	NA	NA	NA	NA	11.7	12.2	430.6	326.1
18:3n-3	4.7	7.1	NA	NA	NA	NA	NA	5.8	7.1	49.7	41.8
20:4n-6	16.4	4.5	NA	NA	NA	NA	NA	9.1	3.2	ND	ND
20:5n-3	57.6	3.7	NA	NA	NA	NA	NA	70.3	144.5	ND	ND
22:5n-3	13.0	ND	NA	NA	NA	NA	NA	12.4	0.0	ND	ND
22:6n-3	152.2	ND	NA	NA	NA	NA	NA	105.3	94.8	ND	ND
SFA	231.5	230.1	NA	NA	NA	NA	NA	205.5	175.3	137.1	135.8
MUFA	129.1	322.0	NA	NA	NA	NA	NA	163.2	135.5	221.7	53.3
C_{18}PUFA	20.0	78.5	NA	NA	NA	NA	NA	27.5	39.6	483.6	367.9
LC-PUFA	244.4	8.2	NA	NA	NA	NA	NA	200.5	242.6	ND	ND
Total n-3	222.7	3.7	NA	NA	NA	NA	NA	198.0	259.7	ND	ND
Total n-6	36.7	83.0	NA	NA	NA	NA	NA	29.9	22.5	483.6	367.9

Fish meal was defatted using hexane. Please see methods for details. NA, Not analysed; ND, Not detected.

^ 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 (as analysed) of experimental diets, all values are g/kg unless otherwise stated

	n-3 NL (Fish)	n-3 PL (Krill)	n-6 NL (Soybean)	n-6 PL (Lecithin)
Formulation				
Fish meal [a]	150	150	150	150
Poultry meal [a]	150	150	150	150
Soy protein isolate [b]	150	150	150	150
Wheat gluten [b]	150	150	150	150
Wheat flour [b]	109	109	109	109
Casein [c]	100	100	100	100
Pregelled wheat starch [b]	80	80	80	80
DL-Methionine	10	10	10	10
Di-calcium phosphate	10	10	10	10
Pre-mix vitamins [d]	8	8	8	8
Yttrium oxide [e]	1	1	1	1
Fish oil [a]	82	10	10	10
Krill Oil [f]	0	72	0	0
Soy oil [g]	0	0	72	0
Soy lecithin [g]	0	0	0	72
Composition				
Dry matter (g/kg)	940	933	959	952
Protein(g/kg DM)	598	613	597	593
Digestible protein (g/kg DM)	547	561	559	555
Ash (g/kg DM)	64	65	67	70
Lipid (g/kg DM)	122	124	122	118
Carbohydrate (g/kg DM)^	212	198	214	219
Gross energy (mJ/kg)	21.5	21.1	21.8	21.0
Digestible energy (mJ/kg)	18.9	18.4	19.8	18.6

a Ridley aquafeeds, Narangba, QLD, Australia. Fish meal defatted with hexane (see methods)
b Manildra Group, Rocklea, QLD, Australia.
c Bulk Powders, Moorabbin, Victoria, Australia.

d Vitamin and mineral premix (g/kg of premix): vitamin A, 0.75 mg; vitamin D3, 6.3 mg; 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 g; vitamin B6, 2.0 g; vitamin B9, 0.8 g; 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.
e Yttrium oxide; Stanford Materials, Aliso Viejo, California, United States.
f Swisse, Collingwood, Victoria, Australia.
g Sydney Essential Oil Co. Sydney, NSW, Australia.
^ Calculated by difference.

Table 3 Neutral and polar lipid composition of experimental diets, all values are mg/g lipid unless otherwise stated

	Diets			
	n-3 NL (Fish)	n-3 PL (Krill)	n-6 NL (Soybean)	n-6 PL (Lecithin)
Lipid class (% total lipid)				
Neutral	91.5	67.5	89.7	51.1
Polar	8.5	32.5	10.3	48.9
Neutral lipid fatty acids ^				
16:0	190.9	186.7	137.7	207.6
18:0	45.9	34.0	44.0	52.3
18:1n-9	194.6	223.6	252.9	225.4
18:2n-6	48.0	69.7	351.5	183.5
18:3n-3	10.2	10.8	40.3	22.2
20:4n-6	10.9	5.6	0.0	6.2
20:5n-3	79.4	92.6	13.1	35.0
22:5n-3	14.4	5.0	3.6	7.3
22:6n-3	117.5	71.8	19.7	54.7
SFA	298.6	304.0	195.1	291.5
MUFA	284.0	293.1	274.7	278.4
C_{18}PUFA	58.3	97.7	395.7	210.9
LC-PUFA	222.2	175.1	36.4	103.3
Total n-3	211.2	186.7	40.2	102.3
Total n-6	69.2	86.1	391.9	211.9
Total fatty acids	863.1	869.9	901.8	884.0
Polar lipid fatty acids ^				
16:0	139.5	147.3	121.4	118.7
18:0	44.1	18.4	35.9	27.7
18:1n-9	101.8	87.0	109.0	68.1
18:2n-6	121.4	55.3	150.9	317.6
18:3n-3	7.7	7.0	11.6	37.3

20:4n-6	10.1	ND	6.9	ND
20:5n-3	27.5	87.8	29.3	5.9
22:5n-3	7.4	4.4	4.9	ND
22:6n-3	60.0	68.2	41.6	12.1
SFA	204.8	188.9	166.1	146.4
MUFA	144.9	110.4	128.5	71.6
C_{18}PUFA	129.1	71.6	162.5	354.9
LC-PUFA	105.0	160.4	82.6	18.0
Total n-3	94.9	169.7	75.7	18.0
Total n-6	139.2	62.3	169.4	354.9
Total fatty acids	583.8	531.2	539.7	591.0

n-3 NL, Fish oil; n-3 PL, Krill oil; n-6 NL, Soybean oil; n-6 PL, Soybean lecithin.
ND, not detected.
^ Refer to Table 1 for details.

Table 4 Real time quantitative PCR primer pairs for fatty acid metabolism and control genes.

Target name	Abbreviation	*EC* number	Primer name	Sequence	Length
Fatty acid synthase	*Lc FAS*	*EC* 2.3.1.85	FAS qPCR.For1	TGAATCTCACCACGCTTCAG	20
			FAS qPCR.Rev1	AGGCAGCAATAGAACCCTCA	20
Steroyl CoA desaturase	*Lc SCD*	*EC* 1.14.19.1	SCD qPCR.For1	CCTGGTACTTCTGGGGTGAA	20
			SCD qPCR.Rev1	AAGGGGAATGTGTGGTGGTA	20
Carnitine palmitoyltransferase	*Lc CPT1α*	*EC* 2.3.2.21	CPT1a qPCR.For1	TGATGGTTATGGGGTGTCCT	20
			CPT1a qPCR.Rev1	CGGCTCTCTTCAACTTTGCT	20
ATP citrate lyase	*Lc ACYL*	*EC* 2.3.3.8	Lcal acyl F1	CAACACCATTGTCTGTGCTC	20
			Lcal acyl R1	GAAATGCTGCTTAACAAAGTCC	21
Fatty acid elongation 5	*Lc ELOVL5*	*EC* 2.3.1.n8	Lcal Fads2 F1	ATCCAGTTCTTCTTAACCGT	20
			Lcal Fads2 R1	GGTTTCTCAAATGTCAATCCAC	22
Fatty acid desaturase 6	*Lc FADS2*	*EC* 1.14.19	Lcal ELOVL5 F1	TCATACTACCTTCGCTACTTCTC	23
			Lcal ELOVL5 R1	ACAAACCAGTGACTCTCCAG	20
Luciferase	*Luc*	NA	Luc qPCR For	GGTGTTGGGCGCGTTATTTA	20
			Luc qPCR Rev	CGGTAGGCTGCGAAATGC	18
Elongation factor 1α	*EF1α*	NA	Lcal EF1a F	AAATTGGCGGTATTGGAAC	19
			Lcal EF1a R	GGGAGCAAAGGTGACGAC	18

NA, Not analysed.

Table 5 Growth and feed utilisation parameters of juvenile barramundi fed experimental diets for eight weeks. Data (n=3) are presented as mean ± SEM.

	Diets				Test †		
	n-3 NL (Fish)	n-3 PL (Krill)	n-6 NL (Soy)	n-6 PL (Lecithin)	Omega	Class	Interaction
Initial weight (g)	46.9 ± 0.1	46.7 ± 0.2	47.1 ± 0.1	46.9 ± 0.2	NS	NS	NS
Final weight (g)	238.3 ± 1.2 [a]	237.1 ± 1.1 [a]	217.5 ± 1.6 [b]	233.4 ± 3.8 [a]	***	*	**
Feed intake	209.6 ± 2.7 [b]	211.1 ± 2.6 [b]	189.4 ± 0.7 [c]	222.2 ± 0.9 [a]	*	***	***
FCR	1.10 ± 0.1 [a]	1.11 ± 0.1 [a]	1.11 ± 0.1 [a]	1.19 ± 0.1 [b]	*	*	*
Survival (%)	98.0	98.0	100.0	98.0	NS	NS	NS
Ret. Protein (%)	34.1 ± 1.1	34.1 ± 1.5	31.7 ± 0.9	28.4 ± 0.5	NS	NS	NS
Ret. lipid (%)	48.6 ± 4.5	49.8 ± 1.4	49.2 ± 1.4	43.1 ± 3.6	NS	NS	NS

n-3 NL; Fish oil, n-3 PL; Krill oil, n-6 NL; Soybean oil, n-6 PL; Soybean lecithin; Ret. nutrient = (nutrient final - nutrient initial / nutrient consumed) * 100.
‘ * ’ < 0.05, ‘ ** ’ < 0.01, ‘ *** ’ < 0.001; superscript letters indicate significant differences among means.
† Two-way factorial ANOVA, df 1,1,1,8, post-hoc Tukey's HSD; Elongation analysed by one-way ANOVA, df 2,6, post-hoc Tukey’s HSD.
NS, not significant P > 0.05.

Table 6 Apparent digestibility (%) parameters of the diets fed to juvenile barramundi. Data (n=3) are presented as mean ± SEM

	Diets				Test †		
	n-3 NL (Fish)	n-3 PL (Krill)	n-6 NL (Soybean)	n-6 PL (Lecithin)	Omega	Class	Interaction
Diet							
Dry matter	64.3 ± 1.6	67.4 ± 1.6	63.7 ± 4.1	66.6 ± 2.2	NS	NS	NS
Protein	91.1 ± 0.7	91.2 ± 1.8	92.6 ± 0.3	92.8 ± 0.4	NS	NS	NS
Lipid	90.7 ± 0.9 ^{ab}	91.2 ± 1.2 ^{ab}	94.2 ± 0.4 ^{a}	89.3 ± 0.6 ^{b}	NS	*	*
Energy	87.3 ± 1.5	86.6 ± 1.3	89.3 ± 0.7	87.5 ± 0.8	NS	NS	NS
Fatty acids ^							
16:0	81.5 ± 1.9 ^{a}	84.2 ± 1.0 ^{ab}	87.7 ± 0.8 ^{b}	84.4 ± 0.8 ^{ab}	*	NS	*
18:0	76.7 ± 3.0	77.5 ± 2.9	84.6 ± 1.2	79.5 ± 1.1	NS	NS	NS
18:1n-9	92.1 ± 0.8	90.4 ± 2.7	95.0 ± 0.3	90.5 ± 0.4	NS	NS	NS
18:2n-6	95.8 ± 0.2 ^{ab}	94.9 ± 1.0 ^{ab}	97.3 ± 0.1 ^{a}	90.3 ± 1.6 ^{b}	NS	**	*
18:3n-3	98.6 ± 1.2 ^{a}	100.0 ± 0.0	98.3 ± 0.1 ^{a}	92.5 ± 1.4 ^{b}	NA	NA	NA
20:4n-6	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	NA	NA	NA
20:5n-3	99.0 ± 0.4	97.7 ± 0.2	100.0 ± 0.0	100.0 ± 0.0	NA	NA	NA
22:5n-3	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	NA	NA	NA
22:6n-3	98.5 ± 0.2	96.0 ± 0.2	92.9 ± 0.6	98.0 ± 2.0	NS	NS	NS
SFA	82.3 ± 1.8	85.3 ± 1.1	87.3 ± 1.0	84.1 ± 0.6	NS	NS	NS
MUFA	92.6 ± 0.8	91.3 ± 2.3	94.7 ± 0.4	91.1 ± 0.4	NS	NS	NS
C_{18}PUFA	96.5 ± 0.2 ^{a}	96.1 ± 0.7 ^{a}	97.5 ± 0.1 ^{a}	90.5 ± 1.5 ^{b}	*	**	*
LC-PUFA	98.9 ± 0.2	97.1 ± 0.2	96.2 ± 0.3	98.9 ± 1.1	NS	NS	NS
Total n-3	98.9 ± 0.2	97.5 ± 0.1	97.6 ± 0.1	95.2 ± 1.5	NS	NS	NS
Total n-6	96.3 ± 0.1 ^{a}	95.2 ± 0.9 ^{ab}	97.2 ± 0.1 ^{a}	91.1 ± 1.4 ^{b}	NS	**	*

‘ * ’ < 0.05, ‘ ** ’ < 0.01, ‘ *** ’ < 0.001; superscript letters indicate significant differences among means.

† Two-way factorial ANOVA, df 1,1,1,8, post-hoc Tukey's HSD; 18:3n-3 analysed by one-way ANOVA df 3,7, P<0.01, post-hoc Tukey's HSD.

NA, not analysed; NS, not significant P > 0.05.

^ Refer to Table 1 for details.

Table 7 Neutral and polar lipid composition in the whole body of juvenile barramundi fed experimental diets. All values are mg/g lipid unless otherwise stated. Data (n=3) are presented as mean ± SEM.

	Diets				Test †		
	n-3 NL (Fish)	n-3 PL (Krill)	n-6 NL (Soybean)	n-6 PL (Lecithin)	Omega	Class	Interaction
Lipid class (% total lipid)							
Neutral	91.7 ± 1.1	91.4 ± 1.4	94.0 ± 1.5	89.4 ± 0.6	NS	NS	NS
Polar	8.3 ± 1.1	8.6 ± 1.4	6.0 ± 1.5	10.6 ± 0.6	NS	NS	NS
Neutral lipid fatty acids ^							
16:0	227.1 ± 2.1 ^{a}	229.6 ± 1.1 ^{a}	198.0 ± 4.5 ^{b}	238.4 ± 3.9 ^{a}	*	***	***
18:0	61.2 ± 0.6 ^{ab}	56.5 ± 0.7 ^{a}	64.6 ± 1.9 ^{b}	71.3 ± 0.9 ^{c}	***	NS	**
18:1n-9	254.8 ± 3.3 ^{a}	257.1 ±0.6 ^{a}	286.7 ±8.0 ^{b}	244.1 ± 1.6 ^{a}	NS	**	***
18:2n-6	76.4 ± 1.6 ^{a}	82.5 ± 0.8 ^{a}	264.5 ± 8.9 ^{b}	202.0 ± 0.4 ^{c}	***	***	***
18:3n-3	9.0 ± 0.3 ^{a}	10.0 ± 0.1 ^{a}	27.0 ± 1.2 ^{b}	21.2 ± 0.2 ^{c}	***	**	***
20:4n-6	5.6 ± 0.1	1.2 ± 1.2	ND	ND	NA	NA	NA
20:5n-3	32.1 ± 0.9 ^{a}	53.2 ± 1.1 ^{b}	8.7 ± 0.7 ^{c}	10.5 ± 0.2 ^{c}	***	***	***
22:5n-3	13.4 ± 0.4 ^{a}	11.6 ± 0.2 ^{b}	5.9 ± 0.3 ^{c}	6.5 ± 0.1 ^{c}	***	NS	**
22:6n-3	54.7 ± 2.4 ^{a}	47.4 ± 1.4 ^{b}	18.0 ± 1.1 ^{c}	21.1 ± 0.7 ^{c}	***	NS	**
SFA	335.3 ± 1.8 ^{a}	331.4 ± 0.8 ^{a}	286.7 ± 7.0 ^{b}	338.3 ± 4.7 ^{a}	**	***	***
MUFA	322.9 ± 3.9 ^{a}	315.3 ± 0.6 ^{a}	319.1 ± 8.3 ^{a}	284.7 ± 2.0 ^{b}	**	**	*
C_{18}PUFA	97.7 ± 1.8 ^{a}	109.7 ± 0.8 ^{a}	297.4 ± 10.0 ^{c}	231.3 ± 0.3 ^{b}	***	***	***
LC-PUFA	105.8 ± 3.8 ^{a}	113.3 ± 3.0 ^{a}	37.5 ± 2.3 ^{b}	43.1 ± 1.0 ^{b}	***	*	NS
Total n-3	106.4 ± 3.9 ^{b}	123.1 ± 2.7 ^{a}	32.5 ± 2.1 ^{c}	38.1 ± 0.9 ^{c}	***	**	NS
Total n-6	97.0 ± 1.7 ^{a}	99.9 ± 1.9 ^{a}	302.3 ± 10.2 ^{c}	236.2 ± 0.3 ^{b}	***	***	***
Total fatty acids	861.6 ± 5.7 ^{a}	869.7 ± 3.8 ^{a}	940.7 ± 25.6 ^{b}	897.3 ± 5.4 ^{ab}	**	NS	NS
Polar lipid fatty acids ^							
16:0	171.4 ± 5.7 ^{a}	163.6 ± 0.2 ^{a}	135.8 ± 2.4 ^{b}	143.8 ± 2.8 ^{b}	**	NS	NS
18:0	65.9 ± 4.5	62.0 ± 1.2	69.9 ± 0.6	61.7 ± 3.5	NS	NS	NS
18:1n-9	155.0 ±1.0	145.2 ± 3.4	139.1 ± 4.5	143.3 ± 8.9	NS	NS	NS
18:2n-6	42.9 ± 0.9 ^{a}	39.5 ± 1.7 ^{a}	129.7 ± 3.2 ^{b}	120.3 ± 9.9 ^{b}	***	NS	NS
18:3n-3	ND	ND	ND	9.8 ± 1.6	NA	NA	NA
20:4n-6	12.2 ± 2.6	6.9 ± 1.3	8.9 ± 0.1	5.7 ± 0.5	NS	NS	NS

20:5n-3	26.5 ± 3.2 [ab]	35.2 ± 5.4 [a]	11.1 ± 0.1 [b]	11.4 ± 0.7 [b]	**	NS	NS
22:5n-3	11.8 ± 1.6	10.8 ± 1.8	10.0 ± 0.3	7.0 ± 0.1	NS	NS	NS
22:6n-3	66.8 ± 13.0 [a]	57.6 ± 11.9 [a]	42.1 ± 0.9 [ab]	24.5 ± 1.7 [b]	*	NS	NS
SFA	254.9 ± 6.4 [a]	244.9 ± 3.2 [ab]	205.7 ± 3.0 [c]	221.2 ± 5.3 [bc]	**	NS	NS
MUFA	189.9 ± 6.0	175.7 ± 6.3	156.5 ± 4.8	172.7 ± 8.3	NS	NS	NS
C_{18}PUFA	48.4 ± 3.6 [a]	43.2 ± 4.7 [a]	133.3 ± 6.1 [b]	135.0 ± 12.2 [b]	***	NS	NS
LC-PUFA	117.3 ± 20.4 [a]	110.5 ± 20.4 [a]	83.9 ± 1.4 [ab]	56.0 ± 1.8 [b]	*	NS	NS
Total n-3	106.7 ± 16.4 [a]	100.4 ± 17.7 [a]	63.2 ± 1.3 [ab]	42.9 ± 1.1 [b]	*	NS	NS
Total n-6	59.0 ± 0.3 [a]	48.3 ± 2.0 [a]	154.1 ± 6.0 [b]	148.1 ± 11.5 [b]	***	NS	NS
Total fatty acids	610.6 ± 17.2	569.4 ± 6.1	579.4 ± 12.5	584.8 ± 13.4	NS	NS	NS

‘ * ’ < 0.05, ‘ ** ’ < 0.01, ‘ *** ’ < 0.001; superscript letters indicate significant differences among means.

† Two-way factorial ANOVA, df 1,1,1,8, post-hoc Tukey's HSD

NA, not analysed; ND, not detected; NS, not significant P > 0.05.

^ Refer to Table 1 for details.

Table 8 Whole body fatty acid balance calculations of β-oxidation, elongation and desaturation of juvenile barramundi fed experimental diets for eight weeks. All values are presented as nmol/g fish/d. Data (n=3) are presented as mean ± SEM.

	Diets				Test †		
	n-3 NL (Fish)	n-3 PL (Krill)	n-6 NL (Soybean)	n-6 PL (Lecithin)	Omega	Class	Interaction
β-Oxidation ^							
SFA	339.7 ± 18.2	ND	ND	45.7 ± 0.3	NA	NA	NA
MUFA	554.1 ± 43.9^{b}	120.4 ± 5.7^{c}	8.1 ± 6.1^{c}	1338.2 ± 113.2^{a}	***	***	***
C_{18}PUFA	661.6 ± 34.9^{b}	411.9 ± 22.9^{bc}	46.5 ± 1.5^{c}	3376.6 ± 208.4^{a}	***	***	***
LC-PUFA	1695.1 ± 102.7^{a}	1049.5 ± 53.3^{b}	171.5 ± 5.9^{c}	136.9 ± 22.8^{c}	***	***	***
Total n-3	1688.5 ± 103.5^{a}	1101.8 ± 56.7^{b}	125.2 ± 5.8^{c}	116.7 ± 20.9^{c}	***	**	**
Total n-6	668.1 ± 34.3^{b}	359.5 ± 19.9^{bc}	92.8 ± 1.8^{c}	3396.9 ± 209.2^{a}	***	***	***
Elongation ^							
SFA	73.0 ± 10.1^{d}	3154.3 ± 297.9^{b}	4894.0 ± 205.1^{a}	1301.4 ± 438.2^{c}	***	NS	***
MUFA	ND	ND	17.8 ± 3.5	ND	NA	NA	NA
C_{18}PUFA	ND	ND	ND	ND	NA	NA	NA
LC-PUFA	ND	118.5 ± 7.1^{a}	55.4 ± 1.0^{b}	65.1 ± 2.9^{b}	NA	NA	NA
Total n-3	ND	118.5 ± 7.1^{a}	55.4 ± 1.0^{b}	65.1 ± 2.9^{b}	NA	NA	NA
Total n-6	ND	ND	ND	ND	NA	NA	NA
Desaturation							
SCD (Δ-9 Des.)	2.4 ± 2.4^{c}	466.7 ± 60.9^{b}	1138.0 ± 41.2^{a}	19.2 ± 14.0^{c}	**	***	***
FADS2 (Δ-6 Des.)	ND	ND	29.1 ± 1.2	40.1 ± 1.6	NA	NA	NA

SCD, Steroyl CoA desaturase; FADS2, Fatty acid desaturase 6. FADS1 (Δ-5 Des.) and chain shortening were not detected or reported.
‘ * ’ < 0.05, ‘ ** ’ < 0.01, ‘ *** ’ < 0.001; superscript letters indicate significant differences among means.
† Two-way factorial ANOVA, df 1,1,1,8, post-hoc Tukey's HSD; Elongation LC-PUFA and total n-3 were analysed by one-way ANOVA df 3,7, post-hoc Tukey's HSD. NA, not analysed; ND, not detected; NS, not significant P > 0.05.
^ Refer to Table 1 for details.

Table 9 Plasma chemistry of barramundi fed experimental diets for eight weeks. Data (n=3) are presented as mean ± SEM.

	Diets				Test †		
	n-3 NL (Fish)	n-3 PL (Krill)	n-6 NL (Soybean)	n-6 PL (Lecithin)	Omega	Class	Interaction
CK (U/L)	7486.3 ± 3156.8	3394.0 ± 1048.1	3667.7 ± 631.9	3591.0 ± 1076.3	NS	NS	NS
ALT (U/L)	8.7 ± 4.3	4.3 ± 1.5	5.7 ± 4.3	9.0 ± 3.9	NS	NS	NS
GLDH (U/L)	10.0 ± 2.6 [a]	9.3 ± 6.5 [a]	11.3 ± 3.0 [a]	24.7 ± 8.3 [b]	***	***	***
Urea (mmol/L)	2.9 ± 0.6	2.2 ± 0.4	3.1 ± 0.5	3.0 ± 0.3	NS	NS	NS
Creatinine (umol/L)	22.7 ± 0.3 [a]	23.3 ± 1.1 [a]	21.7 ± 1.0 [a]	28.0 ± 1.2 [b]	*	***	**
Ca (mmol/L)	3.2 ± 0.1	3.0 ± 0.2	3.3 ± 0.2	3.3 ± 0.1	NS	NS	NS
Mg (mmol/L)	1.8 ± 0.2	1.4 ± 0.6	1.6 ± 0.2	1.7 ± 0.3	NS	NS	NS
Phosphate (mmol/L)	3.6 ± 0.2	3.3 ± 0.2	3.7 ± 0.4	4.0 ± 0.3	NS	NS	NS
Cholesterol (mmol/L)	5.6 ± 0.3 [ab]	6.8 ± 0.2 [a]	4.4 ± 0.6 [b]	4.4 ± 0.3 [b]	***	*	*
Total Protein (g/L)	48.1 ± 1.2 [a]	49.9 ± 2.8 [ab]	48.1 ± 0.9 [a]	54.3 ± 0.8 [b]	NS	**	NS
Albumin (g/L)	13.6 ± 0.5	13.8 ± 0.9	14.1 ± 0.4	16.0 ± 0.4	NS	NS	NS
Fe (umol/L)	21.5 ± 0.7	21.7 ± 1.0	19.6 ± 2.6	27.1 ± 2.6	NS	NS	NS
Hb (mg/ml)	0.26 ± 0.5 [b]	0.10 ± 0.1 [a]	0.07 ± 0.7 [a]	0.14 ± 0.9 [ab]	NS	NS	**
Haptoglobin (mg/ml)	1.3 ± 0.1 [a]	1.3 ± 0.1 [a]	1.3 ± 0.1 [ab]	1.4 ± 0.1 [b]	**	NS	NS

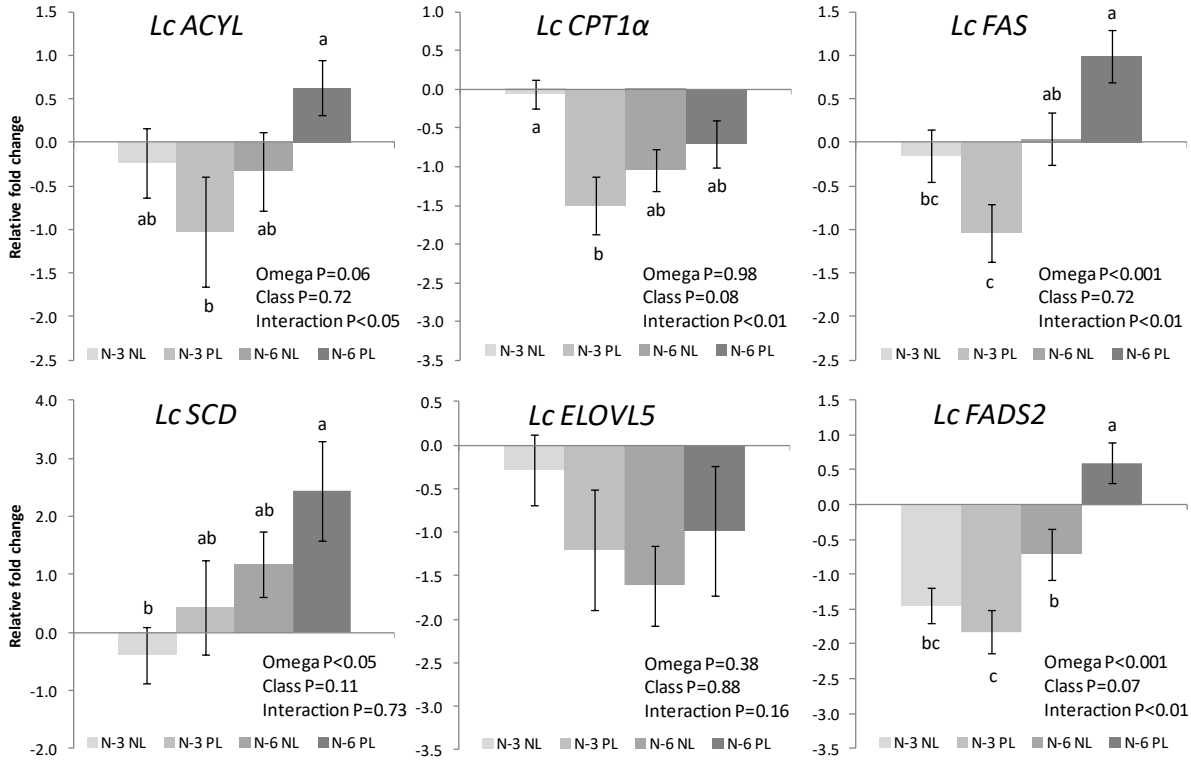
CK, creatine kinase; ALT, alanine aminotransferase; GLDH, glutamate dehydrogenase.

‘ * ’ < 0.05, ‘ ** ’ < 0.01, ‘ *** ’ < 0.001; superscript letters indicate significant differences among means.

† Two-way factorial ANOVA, df 1,1,1,8, post-hoc Tukey's HSD

NS, not significant P > 0.05.

Figure 1


Lc ACYL
Relative fold change
a
ab
b
ab
Omega P=0.06
Class P=0.72
Interaction P<0.05
N-3 NL N-3 PL N-6 NL N-6 PL
Lc CPT1α
a
b
ab
ab
Omega P=0.98
Class P=0.08
Interaction P<0.01
N-3 NL N-3 PL N-6 NL N-6 PL
Lc FAS
bc
c
ab
a
Omega P<0.001
Class P=0.72
Interaction P<0.01
N-3 NL N-3 PL N-6 NL N-6 PL
Lc SCD
Relative fold change
b
ab
ab
a
Omega P<0.05
Class P=0.11
Interaction P=0.73
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Lc ELOVL5
Omega P=0.38
Class P=0.88
Interaction P=0.16
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Lc FADS2
bc
c
b
a
Omega P<0.001
Class P=0.07
Interaction P<0.01
N-3 NL N-3 PL N-6 NL N-6 PL