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An evaluation of the complete replacement of both fishmeal and fish oil in diets for juvenile Asian seabass, *Lates calcarifer*

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Highlights

1. A factorial study using diets balanced on a digestible protein and energy basis with combinations of four fish meal levels and four fish oil levels was undertaken with Asian seabass.

2. Variation in fish meal was observed to impact on feed intake and subsequently growth of the fish, but less so variations in fish oil.

3. Gene expression was more responsive to variation in fish oil than fish meal.

4. Fish meal was found to be the constraining factor in this experiment, having a clearer impact on overall performance than fish oil replacement

Abstract

An experiment was conducted to examine the potential for the complete replacement of fishmeal (FM) and fish oil (FO) in diets for barramundi, *Lates calcarifer*. A series of diets were formulated to the same digestible protein and energy specifications, but which were designed with FM inclusion levels at 300, 200, 100 or 0 g/kg and FO at 100%, 30%, 15% or 0% of the added oil in the diets (4 x 4 factorial design). Ricebran oil was the alternative oil used in the growth study, while soybean meal and poultry meal were the main alternative protein sources used. For the growth study, fish of an initial weight of 154.4 ± 1.1 g were randomly allocated across 48 tanks (three replicates per treatment). After eight weeks, the average weight gain across all treatments was 187.7 ± 2.3 g/fish and feed conversion across all treatments averaged 1.04 ± 0.01 feed/gain. A significant effect of FM on both feed intake and weight gain was observed, and this was observed as early as within the first few weeks, but no similar such effect was observed with FO. No effects were observed on protein deposition efficiency, though both lipid and energy deposition efficiencies were affected by FM level. The reduction in FO had a notable effect on the fatty acid composition of the diets and subsequently the fish fatty acid composition. Expression of key LC-PUFA metabolism genes in the liver of the fish was influenced by both FM and FO levels, but was only significant at the extremes of the treatment ranges. The results from this study demonstrate that there is clear potential to replace almost all the FM content of barramundi diets without loss of fish performance, up to and including diets with as little as 100 g/kg fishmeal. Replacement of fish oil was more successful with the ability to completely replace all FO demonstrated at all but the lowest inclusion levels of FM. These results clearly demonstrate that the near complete replacement of both FM and FO in barramundi diets is a technical reality.

1 Introduction

The reliance of aquaculture on fishmeal as a protein source and fish oil as a lipid source has been recognised for a long time as a significant risk for the industry (Tacon and Metian, 2008). Over the recent past decades there have been a multitude of studies examining a range of different raw materials that have potential application in reducing reliance on these resources for aquaculture (reviewed by Gatlin et al., 2007; reviewed by Glencross, 2009). In assessing new and different raw materials, a series of key knowledge elements is required to enable their effective utilisation by the feed production sector. Those being the characterisation of the raw material, the determination of its digestible nutrient and energy value, before assessing palatability and utilisation value parameters (Glencross et al., 2007).

For barramundi (*Lates calcarifer*), there has been a significant volume of work examining elements of the raw material assessment process. Much of this work has focussed on either rendered animal meals (Williams et al., 2001; 2003a; 2003b; Glencross, 2011; Glencross et al., 2011) or feed grains (Glencross, 2011; Glencross et al., 2011; 2012a; Irvin et al., 2015; Ngo et al., 2015). In both cases it has been demonstrated that either rendered animal meals or feed grains can replace substantial amounts of fishmeal in diets for this species. However, it has also been determined that a critical threshold of 15% fishmeal was pertinent to barramundi, based on a diet balanced for digestible protein, energy and amino acids using a plant protein concentrate as the alternative (Glencross et al., 2011).

There has been less work on establishing the boundaries of fish oil replacement in feeds for barramundi, though there has been much work done on other fish species (Borlongan and Parazo, 1991). It has recently been demonstrated that the requirement for the long-chain polyunsaturated fatty acids (LC-PUFA) for barramundi is around 1% of the diet (Glencross and Rutherford, 2011; Salini et al., 2015a). Other recent studies have demonstrated that it has been possible to replace all the fish oil in barramundi diets, so long as high inclusions of fishmeal were maintained and this level of LC-PUFA maintained (Alhazzaa et al., 2011).

In this study, it was hypothesised that the combined replacement of fishmeal and fish oil will be problematic. But it was not known if the thresholds for this were singular or interactive. Therefore, to address this question a factorial study of replacement in fishmeal (FM) and fish oil (FO) was undertaken. To push the boundaries on this question FM inclusion was reduced from 30% to 0% at 10% increments, whilst FO has one inclusion level at 100%, but the other treatments were at 30%,

15% and 0% of the added oil components. Importantly, each of the dietary formulations were based on the measured digestible value of each of the key raw materials that were used.

2. Materials and Methods

2.1 Experiment concepts

An experiment was conducted to examine the application of the complete replacement of fish meal and fish oil in diets for barramundi. Digestibility data from earlier studies using the same batches of raw materials were used to formulate the diets in this experiment (Glencross et al unpublished), with the nutrient composition of those ingredients shown in Table 1. The experiment examined the productivity of fish grown when fed diets formulated with fishmeal levels ranging from 0 g/kg to 300 g/kg and when the added oil was 100%, 30%, 15% or 0% fish oil, when replaced by ricebran oil (Table 2). This generated a 4x4 factorial design of fishmeal replacement against fish oil replacement. The experiments were conducted at the CSIRO Aquaculture Feed Technologies Laboratory at the Bribie Island Research Centre (Woorim, QLD, Australia) in a flow-through, aerated, heated seawater tank array.

2.2 Diet preparation

A laboratory-scale, twin-screw extruder (MPF24:25; Perkins-Baker, Peterborough, United Kingdom), with intermeshing, co-rotating screws was used to process each of the diets. The methodology was based on that reported in Glencross et al. (2012a). All dry ingredients were first mixed in a Hobart mixer (Hobart, Ohio, USA). The dry mash was delivered into the barrel at a feed rate of around 360 g/min. Barrel temperatures were set for each of the four zones from drive to die at 50°C, 80°C, 100°C and 120°C, respectively. The barrel of the extruder was a smooth-walled, open-clam design with twin-screws each with dimensions of 24 x 600 mm (diameter x length). The screw configuration was composed of a series of intermeshing feed screws (FS), forwarding paddles (FP) and lead screws (LS) arranged according to defined barrel diameters (D) such that overall configuration from the drive end was: 16D FS, 2D FP, 1D FS, 2D FP, 1D LS, 1D FP, 2D LS: to the die. A single 4.0 mm diameter cylindrical die tapered at a 67° angle with a land length of 3 mm was used. Each diet was extruded using the same temperature parameters. Water was peristaltically pumped (Watson-Marlow 504U, Falmouth, England) into the barrel at around 100 mL/min based on optimising the expansion of the pellet. Pre-conditioning and steam injection

were not used during the process. Pellets were cut into 5 to 6 mm lengths using a four-bladed variable speed cutter and collected on large aluminium oven trays (650 x 450 x 25 mm, L x W x D), which were subsequently used for drying of the pellets at 60°C for 12 h. Following drying the pellets were vacuum infused with their formulated allocation of oil. To infuse the oil an allocation (~5kg) of the warm, dried uncoated pellets were weighed into the mixing bowl of a Hobart mixer (Hobart, Ohio, USA) and the formulated allocation of warmed (60°C) oil slowly poured over the pellets whilst they were being mixed. Once all the pellets were evenly coated, the bowl was removed, a lid applied and the bowl chamber evacuated of air using vacuum pump. The vacuum was maintained until all signs of air escaping from the pellets were seen to stop. At this point the air pressure was slowly re-equilibrated, the lid removed and the pellets removed, bagged and stored at 4ºC ready for use. The composition of each diet is shown in Table 3.

2.3 Chemical and digestibility analysis

All samples were dried (or already dry) and milled to a fine powder prior to analysis. Faecal samples were dried by freeze drying prior to being analysed for dry matter, yttrium, nitrogen and gross energy content. In addition, diet samples were analysed for ash and total lipids and carbohydrate content calculated. Dry matter was calculated by gravimetric analysis following oven drying at 105°C for 12 h. Ash content was determined gravimetrically following the loss of mass after combustion of a sample in a muffle furnace at 550°C for 4 h. Protein levels were calculated from the determination of total nitrogen using a ThermoFlash Elemental Analyser, based on N x 6.25. Total starch content was measured using enzymatic methods with the Megazyme Total Starch Kit, K-TSTA, following a modified AOAC Method 996.11. Amino acid analysis involved the samples being hydrolysed at 110°C for 24 h in 6 M HCl with 0.05 % Phenol. Cystine was derivatized during hydrolysis by the addition of 0.05 % 3-3-dithiodipropoinic acid. The acid hydrolysis destroyed tryptophan making it unable to be determined. Separation of the amino acids was performed by HPLC on a Hypersil AA-ODS 5µM column using an 1100 series Hewlett Packard HPLC system. Total lipid content of the diets was determined gravimetrically following extraction of the lipids using chloroform:methanol (2:1). Gross energy was determined by isoperiobol bomb calorimetry. Carbohydrates were calculated based on the dry matter content of a sample minus the protein, lipid and ash. Fatty acids were analysed as methyl ester derivatives based on the method of O'fallon et al. (2007). Esterified lipids were separated by gas chromatography (GC) and detected using flame ionisation detection according to standard methods (O'fallon et al., 2007). Specific fatty acid peaks were identified by comparing retention times relative to standards.

Differences in the ratios of dry matter, protein or gross energy to yttrium, in the feed and faeces in each treatment were calculated to determine the apparent digestibility (AD_{diet}) for each of the nutritional parameters examined in each diet based on previously published methods (Blyth et al., 2014).

2.4 Fish management

Fish were obtained from BettaBarra (Cairns, QLD, Australia), and on-grown to 154.4 ± 1.08 g/fish (mean ± SD, n=768) in preparation for the experiment. During the on-growing period all fish were fed the same diet (MarineFloat 4mm, Ridley Aquafeeds, Australia) and kept in 3 x 5,000L seawater tanks. Water temperature was maintained at 30.1 ± 0.10°C (mean ± S.D.) and dissolved oxygen at 6.1 ± 0.05 mg/L (mean ± SD.) for the 56-day duration of the experiment. At the initiation of the trial 40 fish were weighed on an electronic top-loading balance to 0.1 g accuracy to determine the mean and standard deviation of the population. Following this, 16 fish were randomly allocated to each of 48 tanks (24 x 300L or 24 x 600L) based on having to be within the mean ± 1 x SD. Five fish were euthanized from the population at the beginning of the experiment as a representative initial sample.

Each diet was manually fed to each tank of fish once daily (0900 – 1000) to slight excess, based on observation of several pellets being ignored by the fish during the feeding process, seven days a week for 56-days. All feed fed and all uneaten feed was accounted for and correction factors applied to the collected uneaten feed to allow the determination of feed consumption within each tank (Helland et al., 1996). This also ensures potential feed palatability effects could be evaluated (Glencross et al., 2011). Feeding once a day has already been proven to be suitable for this species at this size (Williams et al., 2001).

At the end of the experiment (day 56) all fish in each tank were anesthetised prior to weighing and tissue sample collection. A total of five fish were euthanized by AQUI-S™ overdose. Three of the fish from each tank were towel dried to remove excess water and then frozen whole for total carcass analysis. The other two fish were dissected for liver samples. The remaining fish were stripped of faeces using the methods reported by Blyth et al. (2014).

Frozen whole fish samples were minced by two passes through an industrial food processor to ensure sample homogeneity. Samples were then collected and their moisture content determined by oven drying at 105°C for 24 h and a second sample freeze-dried for chemical analysis. Freeze-dried samples were milled to a powder prior to analysis. Blood samples were collected via the

caudal tail vein from each of the five fish from each tank using a 1mL Li-Heparinised syringe and an 18G needle. Blood from each of the fish was pooled within a single Vacutainer™ tube. The tube was then centrifuged at 1000 x g for 5 min to settle the erythrocytes and the plasma transferred to a new Eppendorf™ tube prior to it being frozen and sent for plasma analysis. Samples of liver were collected separately from each of the other two fish from each tank and frozen on dry-ice before being stored at -80°C.

2.5 Plasma chemistry

Samples of plasma were sent to the West Australian Animal Health Laboratories (South Perth, Western Australia) for plasma 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 (absbiomedical.com). The tests performed included Alanine Aminotransferance (ALAT) (ALT2-125), Creatine Kinase (CK) (OSR6179), Glutamate Dehydrongenase (GLDH) (Randox kit Cat. No. GL441), Total Protein (TPT2-125), Creatinine (CRE2-125), Cholesterol (CHO2-125), Calcium (CAA2-125), Magnesium (MAG2-125), Phosphorus (PHO2-125), Iron (IRO2-125), Urea (BUN2-125), Albumin (ALB2-125) and Haem (Randox test kit Cat. No. HG1539). Trace elements were determined after mixed acid digestion using inductively coupled plasma atomic emission spectrophotometry (ICP-AES). Data are presented in Table 6.

2.6 Gene expression analysis

Total RNA extractions from the liver samples, cDNA synthesis and quantitative PCR analysis were conducted as described previously (Wade et al., 2014). Briefly, RNA was extracted using Trizol reagent (Invitrogen, Scoresby, VIC, Australia) according to the manufacturer's instructions, including DNase digestion with the Turbo DNA-free kit (Applied Biosystems, Scoresby, VIC, Australia). RNA quantity was assessed on a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and RNA quality was assessed using a Bioanalyser (Agilent Technologies, Santa Clara, CA, USA) and RNA nanochips (Agilent #5067-1511). Reverse transcription was performed on 1 µg of total RNA using Superscript III (Invitrogen). Real-time PCR primers specific to each gene of interest (Table 7) were designed with PerlPrimer v1.1.17 (Marshall, 2004).

Primer optimisation and expression of a range of genes that regulate various metabolic pathways was analysed by real-time PCR as described previously (Wade et al., 2014). Verification that there was no gDNA contamination was carried out by PCR amplification of a pool of DNAse-treated RNA samples using gene-specific primers. Real-time PCR amplification reactions were carried out using SYBR Green PCR Master Mix (Applied Biosystems) and run in triplicate on a Viia7 real-time PCR system (Applied Biosystems). Normalization was performed using the **Δ**Cq method by normalizing the cycle threshold values for each gene to Ef1α, then using the average cycle threshold of all genes at each time point to express relative transcript abundance. The variation in amplification of Ef1α across all samples was 0.68 cycles and did not significantly change over time (data not shown).

2.7 Statistical analysis

All values are means unless otherwise specified. Effects of inclusion level of soybean meal on digestibility parameters were examined using linear regression analysis. Limits for all critical ranges were set at $P < 0.05$. A MANOVA (two-way ANOVA) design was used for analysis of the factorial design study. Levels of significance were determined using a Fishers Least Significant Difference (LSD) test. The MANOVA and LSD tests were undertaken using Statistica™ (Statsoft®, Tulsa, OK, USA). Co-expression analysis was undertaken using a correlation analysis of the various treatments between each of the genes. The fish-in fish-out ratio for each diet were calculated based on: (%diet fishmeal + %diet fishoil) / 29% * FCR (Crampton et al 2010).

3. Results

3.1 Fish growth and feed utilisation

All treatments showed substantial increases in weight during the trial (Table 4), and all treatments more than doubled their initial weight (Table 4; Figure 1). Weight gain was poorest by those fish fed the FM:20-FO:0 diet (gain of 167.1 g/fish) and best by those fish fed the FM:30-FO:15 diet (gain of 221.0 g/fish). However, the weight gain by the poorest performing fish was not significantly different from that of each of the FM:0 diets, which all performed sub-optimally relative to the remainder of the treatments. Weight gain was significantly affected by fishmeal inclusion, but not by fish oil inclusion and there was no interaction term. Feed intake by fish was significantly affected by fishmeal content, but not fish oil content (Figure 1 and 2). Overall there was a close relationship between weight gain and feed intake across all treatments (R^2=0.838). Daily feed intake was consistently lower in the FM:0 series of diets compared to the FM:30 series of diets (Figure 2A). However, no such differences were observed with different inclusions of fish oil (Figure 2B). Feed conversion ratio values ranged from 0.99 (diet FM:20-FO:15) to 1.13 (diet FM:0-FO:0) and averaged 1.04 ± 0.01 (Table 4, Figure 1), and was affected by fishmeal content (P=0.040). Fish survival was high (>95%) across all treatments with no significant effects attributable to either fishmeal of fish oil inclusion (Table 4).

3.2 Fish composition and nutrient deposition

Fish proximate composition was relatively consistent across all treatments (Table 5). Virtually all treatments increased their lipid content relative to the initial fish sample. Similarly, there was also a minor increase in protein content relative to the initial sample. However, there were no significant effects on carcass dry matter, protein or lipid content relative to dietary fishmeal or fish oil content. There was substantially more variability in the fatty acid content of the fish from each treatment. The SFA and MUFA were the least variable of the total fatty acids. The total SFA ranged from 31.8% of total fatty acids to 38.0% of total fatty acids (Table 5). Total MUFA ranged from 39.4% of total fatty acids to 45.6% of total fatty acids (Table 5). Variability was greater in the total PUFA, which ranged from 9.8% of total fatty acids to 19.6% of total fatty acids (Table 5). Similarly, the total LC-PUFA was also quite variable, ranging from 3.3% of total fatty acids to 13.2% of total fatty acids (Table 5).

Protein deposition efficiency (as defined by the protein gain relative to protein consumed) was poorest in those fish fed the FM:30-FO:15 diet, but generally there were no significant effects attributable to fishmeal or fish oil inclusion levels (Figure 3; P>0.05). Lipid deposition was more responsive to fishmeal inclusion level (P=0.001), with a decline in lipid deposition efficiency seen at complete replacement of fishmeal (FM:0 series of diets). However, there was no effect attributable to fish oil inclusion level (Figure 3; P>0.05). Energy deposition mirrored that of the lipid deposition (P=0.003), being poorest in those fish fed the FM:0 series of diets, but only marginally so. The effect of fishmeal level on energy deposition was not as dramatic as that of the lipid deposition (Figure 3).

Deposition efficiency of the n-3 fatty acids was poorest in those fish fed the FM:0-FO:0 diet, but most of the FM:0 diets had lower levels of n-3 deposition efficiency (Figure 4). However significant effects were attributable to both fishmeal (P=0.003) and fish oil (P=0.033) inclusion levels, but there was no interaction term (Figure 4; P>0.05). Deposition of n-6 fatty acids was also responsive to both fishmeal (P=0.001) and fish oil (P=0.001) inclusion levels, but there was no interaction between the two (Figure 4). Deposition of LC-PUFA was poorest in those fish fed the FM:0-FO:0 diet, and there were significant effects attributable to both fishmeal (P=0.001) and fish oil (P=0.011) inclusion levels, but in this case there was also an interaction between the two (P=0.015; Figure 4).

3.3 Plasma chemistry

There were very few significant differences among the plasma chemistry parameters based on the different dietary treatments (Table 6). The only notable effect was attributable to fish oil replacement on the plasma cholesterol levels. Cholesterol levels declined significantly in response to fish oil replacement, from peak values of 6.2 mmol/L in the FO:100 diets to 4.9 mmol/L in the FO:0 diets. An effect of fishmeal inclusion was also noted, though not significant (P=0.071), and there was no interaction between fishmeal and fish oil inclusion levels (P=0.950). Despite some large numerical differences in other plasma chemistry parameters there were no other significant differences.

3.4 Liver gene expression

There was a significant effect of treatment on the expression of lipid metabolism genes (Table 7). In particular *FADS2* was significantly (P=0.044) up-regulated in fish fed the FM:0-FO:0 diet relative

to the other treatments which by comparison where all mildly down regulated. A similar relationship among the treatments and the expression of *ELOVL5* was also seen, but this was not significant (P= 0.173). Fatty acid synthase (*FAS*) also had a similar response among treatments as the other two lipid metabolism genes, in that there was an increased level of *FAS* expression in the liver of fish fed the FM:0-FO:0 diet, but it was down regulated in the other diets. These effects were also non-significant (P=0.363; Table 7). Other genes analysed for their expression in the liver included C-reactive protein (*CRP*), heat shock protein 70 (*HSP70*) and cytosolic alanine aminotransferase (*cALAT*). Variability among and within the different treatments was substantial with these genes and as such no significant effects (P>0.05) were observed (Table 7).

There was a high degree of similarity in the manner in which *cALAT* and *FAS* were expressed among the different treatments. Co-expression analysis indicated that there was a significant positive correlation of R^2=0.606 (P=0.001) between these two genes. Significant co-expression correlations were also observed between *ELOVL5* and *FADS2* (R^2=0.498; P=0.006), *FAS* and *ELOVL5* (R^2=0.608; P=0.001), *cALAT* and *ELOVL5* (R^2=0.579; P=0.001), and *FADS2* and *FAS* (R^2=0.714; P=0.001).

4. Discussion

This study examined the co-replacement of both fishmeal and fish oil in diets for juvenile barramundi (*Lates calcarifer*). The diets were all formulated on an equal digestible nutrient and energy basis, which has previously been shown to enable effective use of a wide range of alternatives (Glencross et al., 2011). Based on the diets being formulated on an equivalent digestible energy and nutrient basis, it was hypothesised that the main response by the juvenile barramundi would be towards the threshold values for fishmeal inclusion for palatability reasons and to fish oil for essential fatty acid reasons (Glencross and Rutherford, 2011; Glencross et al., 2011). This added degree of rigour in the diet formulation process additionally sets this piece of work apart from others attempting the replacement of either fishmeal or fish oil to date (Boonyaratpalin et al., 1998; Raso and Anderson, 2002; Williams et al., 2003b; Tantikitti et al., 2005; Alhazzaa et al., 2011). No other studies examining the joint replacement of either fishmeal or fish oil in this species, or indeed any other species, were found in the literature.

4.1 Growth and feed utilisation

It is important to note that the fish in this study grew close to their predicted potential (90% of modelled optimal growth (Glencross and Bermudes, 2012). Many other studies report growth studies of fish where growth was clearly suboptimal and therefore it can be questioned as to how much influence the dietary treatments alone are having if other factors are affecting performance. The performance of fish at moderate levels of fishmeal replacement (e.g. FM:30, FM:20 and FM:10) shows that there is clear utility to replace this ingredient without introducing growth performance problems, subject to the diets being balanced for digestible protein, essential amino acids and digestible energy. The results for replacement of fish oil were even more conclusive and showed that virtually all the fish oil can be replaced and not affect performance (based on weight gain and feed conversion). However, at the higher levels of fishmeal replacement (FM:0) there was a clear effect on feed intake, weight gain and also feed conversion. These results demonstrate that the primary apparent response by barramundi to the replacement of fishmeal is to reduce their feed intake, presumably for palatability reasons. Feed intake was notably lower in the FM:0 series of diets compared to the FM:30 series of diets within the first few weeks (Figure 2A). This observation supports a potential palatability effect rather than a feedback effect from growth, given the response time involved. Despite this suggestion, it cannot be ruled out that there is a

potential deficiency which has a feedback effect of reducing growth, which in turn results in reduced feed intake and poorer feed conversion. Similar such results to a decline in fishmeal inclusion were reported by Glencross et al. (2011), who examined the effects of a serial dilution of fishmeal against an amino acid balanced plant protein concentrate diet. In that study, a critical threshold of 150 g/kg of fishmeal was reported after which the authors saw a dramatic decline in feed intake. The present study obtained good feed intake and growth results for diets incorporating 100 g/kg of fishmeal, in contrast to the work of Glencross et al. (2011). A potential explanation for this difference between the diets in each case was the use of an animal protein (poultry meal) and the supplementation of taurine to the diets in the present study.

Studies by Williams et al, (2003a) also examined the replacement of fishmeal using rendered bovine and ovine meals. These authors found that there was no reduction in feed intake with the complete replacement of fishmeal using these rendered animal meals. However, comparison of the growth data against the growth model of Glencross and Bermudes (2012) identified that these fish only grew at 66% of their potential. Studies on fishmeal-free diets for Atlantic salmon (*Salmo salar*) found a small but a significant decline in feed intake (Espe et al. 2006), similar to the effect observed in the present study. However, a study by Hansen et al. (2007) examined diets fed to cod (*Gadus morhua*) where there was the serial replacement of fishmeal with a suite of plant proteins (wheat gluten, soy meal and soy protein concentrate). These authors observed a significant decline in feed intake and growth with complete replacement of fishmeal, but no significant decline at 180 g/kg fishmeal inclusion.

In the present study the "fish-in-fish-out ratio" ranged from 1.33 for the FM:30-FO:0 diet to 0.00 for the FM:0-FO:0 diet (Table 4). The additional use of FO replacement strategies in this study, implemented concurrently with the FM replacement, has clearly shown that it is possible to obtain a "fish-in-fish-out ratio" considerably less than one (Tacon and Metian, 2008; Kaushik and Troell, 2010). If fish production from those diets in the FM:20 and FM10 range are considered acceptable then the findings from this study demonstrate that there is clear scope for the production of barramundi to be "fish-production-positive", with more fish being produced than that being used to provide the feed.

4.2 Body composition and nutrient deposition

It was observed that the fish proximate composition was relatively consistent across all treatments in this study. The protein content of the fish was not affected by treatment and lipid content was

generally related to fish size and weight gain as has been shown in other studies with this species (Glencross and Bermudes, 2012). Importantly, there were no significant effects on carcass dry matter, protein or lipid content that could be attributed to diet fishmeal or fish oil content. However, there was substantially more variability in the fatty acid content of the fish from each treatment and this is typical from studies where the fatty acid content of the diet has been manipulated (Raso and Anderson, 2002; Alhazzaa et al., 2011; Glencross and Rutherford, 2011). There was a trend with increasing FM replacement towards a reduction in total SFA, although the replacement of FO produced a greater reduction in SFA. The total MUFA increased with FM replacement and a similar degree of MUFA enrichment was also noted due to the FO replacement. In this study the FO was replaced by ricebran oil which is relatively low in SFA and has a moderate level of MUFA, and as such the composition of the fatty acids in the carcass of each treatment can be seen to be largely reflecting those of their respective diets.

The deposition efficiency (as defined by the gain relative to nutrient consumed) was largely unaffected by diet for protein deposition, but was so for both lipid and accordingly energy deposition (Figure 3). This effect on lipid and energy deposition was clearly responsive to fishmeal inclusion but not to fish oil inclusion level (Figure 3). It can be argued that these effects are consistent with those expected based on fish size differences, but also the marginal effect of feed intake relative to daily energy demands (Glencross, 2006; Glencross and Bermudes, 2012).

Deposition efficiency of the different fatty acids was slightly more variable than that of the macro-nutrients and energy. Notably n-3 deposition efficiency declined significantly with decreasing levels of FM and also somewhat with decreasing levels of FO, but not to the same extent (Figure 4). Deposition of n-6 fatty acids also declined with decreasing FM inclusion levels, but in contrast improved with increasing FO replacement (Figure 4). However, most dramatic was the deposition of the LC-PUFA which not only showed highly significant effects attributable to both FM and FO replacement and an interaction term, but also presented a negative deposition efficiency, implying a loss of LC-PUFA from those fish fed the FM:0-FO:0 diet (Figure 4). This observation is in direct contrast to earlier work of Glencross and Rutherford (2011), who observed an increase in the deposition (retention) efficiencies of DHA, EPA and ARA in diets largely devoid of LC-PUFA. However, it is notable that these low LC-PUFA diets still contained 8.4% LC-PUFA (equivalent to ~ 9 g/kg) in their total fatty acids in contrast to the FM:0-FO:0 diets in the present study which had 0.2% LC-PUFA. This difference in deposition efficiency therefore perhaps highlights that at low levels of LC-PUFA that barramundi are capable of scavenging the small amounts available, thereby

improving their utilisation. This is consistent with observations in other studies on a wide range of species (Francis et al., 2007; Stubhaug et al., 2007). However, in absolute (or near) absence of LC-PUFA in the diet then there is a net loss as the animal turns over those essential nutrients through its growth and maintenance processes. Although the SFA and MUFA were the least variable of the total fatty acids in the carcass of the animals, there was still a clear pattern in the deposition efficiencies of these fatty acids in mirroring the pattern of weight gain response to varying FM and FO levels.

4.3 Plasma chemistry

Despite some large numerical differences in a range of the plasma chemistry parameters there were few significant effects attributable to the treatments in this study. This suggests that the health of the fish being fed diets with high levels of replacement of both FM and FO is not being compromised. In an earlier study the authors had noted a range of sub-clinical effects due to replacement of fishmeal on the juvenile barramundi relative to the reference diet (Glencross et al., 2011). In contrast to the present study, in the earlier study noted effects in levels of creatinine kinase (CK), alanine aminotransferase (ALAT) and glutamate dehydrogenase (GLDH) which are markers of muscle and liver damage respectively (Glencross et al., 2011). In the earlier study, it was suggested that the hard physical properties of some of the pellets in that study were responsible for physical trauma to the gastrointestinal tract and this presented as the elevated levels of these plasma marker enzymes. However, similar such physical extremes in the pellet properties were not seen in the present study.

The only significant effect on plasma chemistry in the present study was attributable to fish oil replacement on the plasma cholesterol levels (Table 6). It was noted that cholesterol levels declined significantly in response to fish oil replacement. However, although effects associated fishmeal inclusion were also notable, they were not significant. Earlier studies examining FM or FO replacement in barramundi have not examined plasma cholesterol content (Glencross and Rutherford, 2011; Glencross et al., 2011), so a direct comparison was not possible. However, studies examining the replacement of FO with rapeseed oil in Atlantic salmon found no significant changes in plasma cholesterol levels with increasing FO replacement (Torstensen et al., 2004). However, it was unclear in this study as to what the FM inclusion level was and this may have been a pertinent factor. Interestingly, other studies have shown that modification of the dietary essential fatty acid profiles can affect the plasma chemistry, immune response and the gut and

liver structure (Montero et al., 2003; Glencross and Rutherford, 2011). It is likely that the observed changes in plasma enzymes are indicative of such changes in the test fish.

4.4 Liver gene expression

In the present study, a focus was made on the expression of genes in the liver involved in the LC-PUFA synthesis pathways and accessory lipid metabolism (*FADS2*, *ELOVL5*, *FAS*), and also a range of stress and inflammatory responses (*HSP70*, *CRP*, *cALAT*). Of those six genes, the only significant expression response to treatment was the significant up-regulation of the fatty acid metabolism gene FADS2 in those fish fed the FM:0-FO:0 diet, relative to the other treatments which were all down regulated. It can be reasoned that this up-regulation of the FADS2 gene (a delta-6-desaturase) occurred in fish fed the diet devoid of any LC-PUFA as those fish are attempting at 'all costs' to produce the essential fatty acids of EPA and DHA from whatever substrates it can access (Mohd-Yusof et al., 2010; Tu et al., 2012; Betancor et al., 2014). However, at even low levels of LC-PUFA present in the other diets the expression of the gene is maintained at low levels suggesting that barramundi have a tolerance for very low levels of these nutrients, just not complete absence (Salini et al., 2015b). Other studies examining *FADS2* and *ELOVL5* (Elongase of very long-fatty acids – 5) in barramundi have also found that the expression of these two genes is up-regulated when the levels of the LC-PUFA become low (Alhazzaa et al., 2011; Salini et al., 2015b). The expression of fatty acid synthase (*FAS*) was also similar to the other two lipid metabolism genes (*FADS2*, *ELOVL5*), in that there was an increase level of FAS in the liver of fish fed the FM:0-FO:0 diet, but it was down regulated in the other diets. Of more significant note though, was that there was a significant level of co-expression of *FAS* with *FADS2* and also *FAS* with *ELOVL5*. This co-expression suggesting that the up-regulation of the desaturation (*FADS2*) and elongation (*ELOVL5*) genes is consistent with a stimulation of other aligned growth pathways (Wade et al., 2014).

The other genes analysed for their expression in the liver in this study (CRP, HSP70 and cALAT) where those primarily directed at examining potential inflammation effects. However, consistent with the observations from the plasma chemistry data there were no significant effects attributable to any inflammation events due to the replacement of either FM or FO (Figure 5). However, substantial temporal variation in the expression of some of these genes has been previously observed (Wade et al., 2014). This may explain the large level of variance seen around some of these genes which makes it difficult to define a significant effect, where as some genes, like *FAS* are much more stable in their expression over a longer time period.

The co-expression observed between *cALAT* and *FAS* expression may be linked to the fact that these two genes are generally up-regulated in those fish growing well (Wade et al., 2014). However, in the earlier study of Wade et al. (2014) there was only a moderate linkage between *FAS* and *cALAT*, but there were strong relationships between FAS and other similar enzymes like *ASAT*.

4.5 Conclusions

The findings from this study demonstrate that there is clear potential to reduce both the FM and FO content of barramundi diets to as little as 10% FM and 0% FO without loss in productivity. However, at this level of inclusion of FM and FO there are substantial impacts on the fatty acid profile of the fish, most notably the LC-PUFA levels which are about one third of those of the fish fed the FM:30-FO:100 diet. It would be of value to consider a sensory evaluation of the impact that this has on the fish product produced (Williams et al., 2003c). Importantly, for most of the inclusion levels of both FM and FO used in this study, the production of barramundi was demonstrated to be "fish-production-positive", with more fish being produced than that being used to provide the feed. This therefore provides solid support for notion of sound feed sustainability for production of barramundi.

However, the present study relies on three key ingredients (poultry meal, soybean meal, ricebran oil). To reduce feed risk further, additional raw materials need evaluation and development and this remains one of the highest priorities to provide enhanced flexibility for formulation options for use in barramundi feeds (Glencross et al., 2007). To follow from this work therefore further effort needs to be spent on defining those factors that affect the nutritional value of a broader range of raw materials, most notably by defining their digestible value (Glencross, 2011; Glencross et al., 2007; 2011).

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Figures

Figure 1. Heat maps of each of the performance parameters for each of the treatments in the study. Green is identified in the top 80% -100% percentile while red is identified in the lowest 20% to 0% percentile of performance in each criterion.

Top					Bottom
100%	80%	60%	40%	20%	0%

1A. Weight Gain

	30%FM	20%FM	10%FM	0%FM	mean
FO 100%	189.8	196.4	175.6	167.5	182.3
FO 30%	198.2	199.0	189.5	172.5	189.8
FO 15%	221.0	190.0	199.0	167.6	194.4
FO 0%	201.9	167.1	197.1	171.9	184.5
mean	202.7	188.1	190.3	169.9	

p-value Fishmeal

	30% vs 0%	20% vs 0%	10% vs 0%
	0.182	0.096	0.621
	0.144	0.126	0.323
	0.005	0.219	0.087
	0.160	0.788	0.129

p-value Fishoil

100% vs 0%	0.593	0.091	0.202	0.810
30% vs 0%	0.895	0.072	0.602	0.977
15% vs 0%	0.265	0.194	0.996	0.844

Summary Statistics	*F*	*p value*
Fishmeal (FM)	4.839	**0.007**
Fishoil (FO)	0.714	0.551
FM x FO	0.857	0.572

1B. Intake

	30%FM	20%FM	10%FM	0%FM	mean
FO 100%	195.2	194.5	184.6	181.1	188.9
FO 30%	204.7	201.0	192.7	182.0	195.1
FO 15%	230.8	187.3	201.4	176.2	198.9
FO 0%	215.6	174.1	198.4	193.9	195.5
mean	211.6	189.2	194.3	183.3	

p-value Fishmeal

	30% vs 0%	20% vs 0%	10% vs 0%
	0.340	0.365	0.813
	0.127	0.199	0.464
	0.001	0.450	0.092
	0.145	0.183	0.759

p-value Fishoil

100% vs 0%	0.170	0.170	0.836	0.233
30% vs 0%	0.460	0.073	0.700	0.418
15% vs 0%	0.303	0.371	0.350	0.387

Summary Statistics	*F*	*p value*
Fishmeal (FM)	5.623	**0.003**
Fishoil (FO)	0.667	0.578
FM x FO	1.285	0.283

1C. Feed Conversion

	30%FM	20%FM	10%FM	0%FM	mean
FO 100%	1.03	0.99	1.05	1.08	1.04
FO 30%	1.03	1.01	1.02	1.06	1.03
FO 15%	1.04	0.99	1.01	1.05	1.02
FO 0%	1.07	1.04	1.01	1.13	1.06
mean	1.04	1.01	1.02	1.08	

p-value Fishmeal

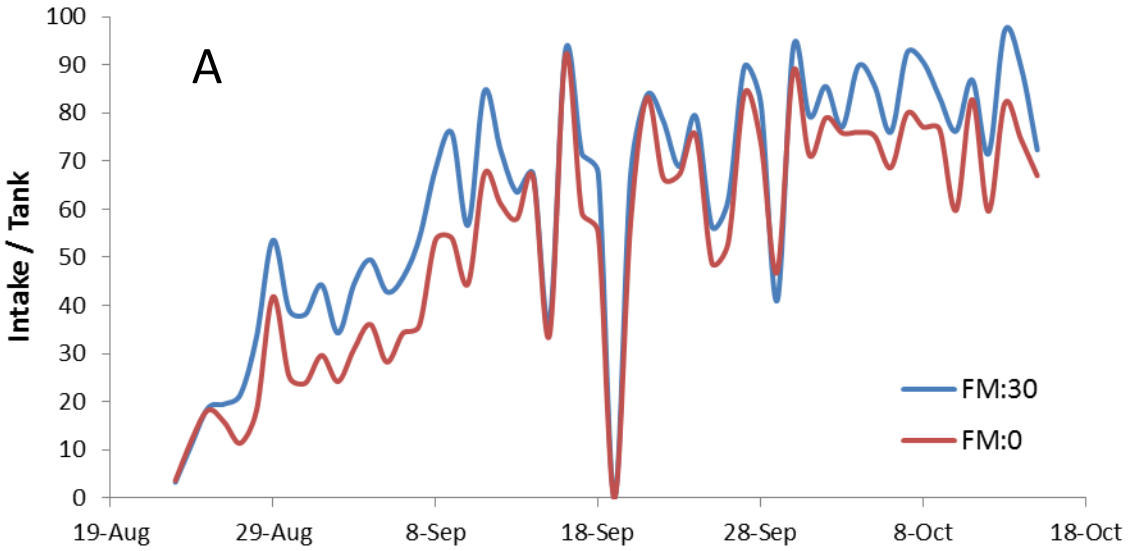
	30% vs 0%	20% vs 0%	10% vs 0%
	0.072	**0.019**	0.234
	0.679	0.460	0.511
	0.706	0.206	0.549
	0.548	0.191	0.054

p-value Fishoil

100% vs 0%	0.333	0.399	0.298	0.787
30% vs 0%	0.347	0.582	0.850	0.261
15% vs 0%	0.437	0.341	0.706	0.318

Summary Statistics	*F*	*p value*
Fishmeal (FM)	3.114	**0.040**
Fishoil (FO)	0.809	0.498
FM x FO	0.478	0.879

Figure 2. Daily feed intake (g/tank) of pooled FM:30 series and FM:0 series treatments (A) over the full eight weeks. Fish were not fed on the day they were weighed in week 4. Note that the FM:0 series are nearly always lower than the FM:30 series. Daily feed intake (g/tank) of pooled FO:100 series and FO:0 series treatments (B) over the full eight weeks. Note that there is essentially no difference between these two treatments.



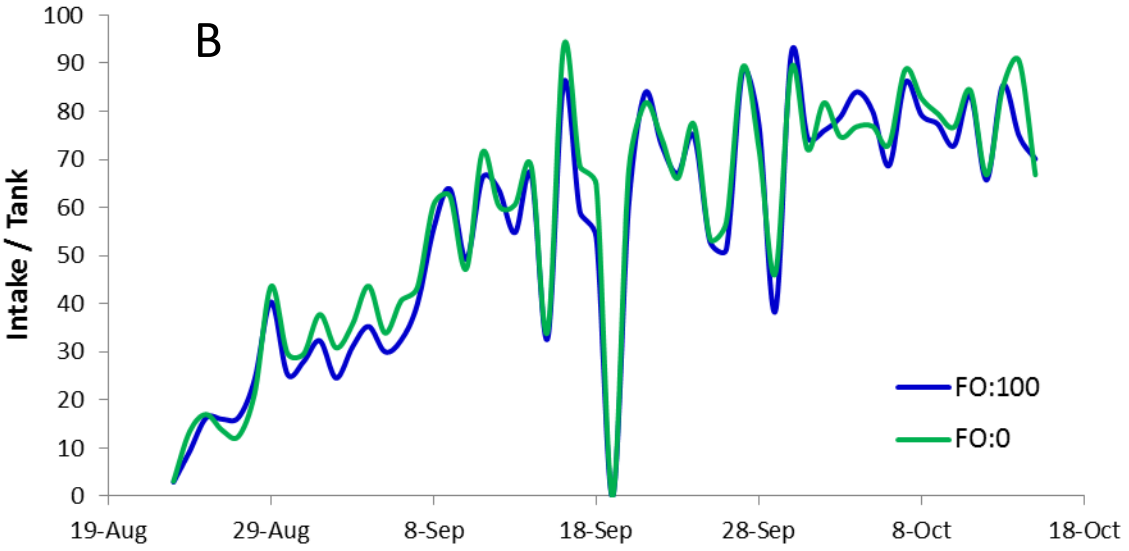


Figure 3. Heat maps of each of the protein, lipid and energy deposition efficiency parameters for each of the treatments in the study. Green is identified in the top 80% -100% percentile while red is identified in the lowest 20% to 0% percentile of performance in each criterion.

Top					Bottom
100%	80%	60%	40%	20%	0%

2A. Protein deposition

	30%FM	20%FM	10%FM	0%FM	mean
FO 100%	38%	45%	43%	36%	40%
FO 30%	45%	41%	44%	43%	43%
FO 15%	35%	46%	41%	40%	41%
FO 0%	45%	40%	50%	37%	43%
mean	41%	43%	45%	39%	

p-value Fishmeal

30% vs 0%	20% vs 0%	10% vs 0%
0.679	0.093	0.195
0.728	0.622	0.799
0.304	0.187	0.769
0.092	0.484	**0.011**

p-value Fishoil

100% vs 0%	0.158	0.378	0.139	0.899
30% vs 0%	0.905	0.953	0.242	0.215
15% vs 0%	**0.037**	0.221	0.080	0.546

Summary Statistics	*F*	*p value*
Fishmeal (FM)	2.034	0.129
Fishoil (FO)	0.783	0.512
FM x FO	1.428	0.218

2B. Lipid deposition

	30%FM	20%FM	10%FM	0%FM	mean
FO 100%	69%	59%	63%	41%	58%
FO 30%	60%	60%	61%	39%	55%
FO 15%	71%	69%	60%	46%	61%
FO 0%	67%	69%	52%	38%	56%
mean	67%	64%	59%	41%	

p-value Fishmeal

30% vs 0%	20% vs 0%	10% vs 0%
0.000	**0.008**	**0.002**
0.002	**0.002**	**0.001**
0.000	**0.001**	**0.037**

p-value Fishoil

100% vs 0%	0.766	0.148	0.099	0.591
30% vs 0%	0.252	0.172	0.143	0.896
15% vs 0%	0.601	0.944	0.238	0.237

Summary Statistics	*F*	*p value*
Fishmeal (FM)	26.624	**0.000**
Fishoil (FO)	1.429	0.252
FM x FO	0.968	0.484

2C. Energy deposition

	30%FM	20%FM	10%FM	0%FM	mean
FO 100%	38%	39%	34%	33%	36%
FO 30%	36%	39%	37%	36%	37%
FO 15%	40%	40%	37%	34%	38%
FO 0%	40%	38%	37%	35%	37%
mean	39%	39%	36%	34%	

p-value Fishmeal

30% vs 0%	20% vs 0%	10% vs 0%
0.034	**0.013**	0.694
0.883	0.190	0.672
0.025	**0.026**	0.300
0.127	0.373	0.626

p-value Fishoil

100% vs 0%	0.654	0.535	0.240	0.278
30% vs 0%	0.184	0.626	0.996	0.954
15% vs 0%	0.756	0.342	0.927	0.643

Summary Statistics	*F*	*p value*
Fishmeal (FM)	5.695	**0.003**
Fishoil (FO)	0.825	0.490
FM x FO	0.629	0.763

Figure 4. Heat maps of each of the n-3 and n-6 fatty acid deposition efficiency parameters for each of the treatments in the study. Green is identified in the top 80% -100% percentile while red is identified in the lowest 20% to 0% percentile of performance in each criterion.

Top					Bottom
100%	80%	60%	40%	20%	0%

3A. n-3 deposition

	30%FM	20%FM	10%FM	0%FM	mean
FO 100%	62%	50%	44%	27%	46%
FO 30%	55%	55%	57%	51%	55%
FO 15%	52%	53%	38%	26%	42%
FO 0%	48%	40%	35%	24%	37%
mean	54%	50%	43%	32%	

p-value Fishmeal

30% vs 0%	20% vs 0%	10% vs 0%
0.004	0.058	0.149
0.689	0.685	0.609
0.030	**0.024**	0.319
0.052	0.186	0.344

p-value Fishoil

100% vs 0%	0.214	0.406	0.459	0.819
30% vs 0%	0.511	0.190	0.075	**0.029**
15% vs 0%	0.699	0.258	0.845	0.887

Summary Statistics	*F*	*p value*
Fishmeal (FM)	5.602	**0.003**
Fishoil (FO)	3.304	**0.033**
FM x FO	0.558	0.820

3B. n-6 deposition

	30%FM	20%FM	10%FM	0%FM	mean
FO 100%	26%	25%	22%	12%	21%
FO 30%	38%	30%	42%	32%	35%
FO 15%	51%	47%	46%	32%	44%
FO 0%	51%	54%	43%	29%	44%
mean	42%	39%	38%	26%	

p-value Fishmeal

30% vs 0%	20% vs 0%	10% vs 0%
0.057	0.066	0.168
0.414	0.775	0.152
0.012	**0.038**	0.058
0.004	**0.002**	0.059

p-value Fishoil

100% vs 0%	**0.001**	**0.001**	**0.006**	**0.024**
30% vs 0%	0.073	**0.002**	0.930	0.693
15% vs 0%	0.976	0.403	0.702	0.709

Summary Statistics	*F*	*p value*
Fishmeal (FM)	7.335	**0.001**
Fishoil (FO)	17.644	**0.000**
FM x FO	1.028	0.440

3C. LC-PUFA deposition

	30%FM	20%FM	10%FM	0%FM	mean
FO 100%	61%	52%	46%	26%	46%
FO 30%	50%	46%	59%	33%	47%
FO 15%	58%	58%	37%	20%	43%
FO 0%	44%	44%	40%	-125%	1%
mean	53%	50%	46%	-11%	

p-value Fishmeal

30% vs 0%	20% vs 0%	10% vs 0%
0.254	0.406	0.516
0.588	0.670	0.395
0.215	0.225	0.581
0.000	**0.000**	**0.000**

p-value Fishoil

100% vs 0%	0.574	0.795	0.837	**0.000**
30% vs 0%	0.853	0.935	0.524	**0.000**
15% vs 0%	0.642	0.654	0.924	**0.000**

Summary Statistics	*F*	*p value*
Fishmeal (FM)	8.117	**0.000**
Fishoil (FO)	4.358	**0.011**
FM x FO	2.816	**0.015**

Table 1. Nutrient composition of key experimental ingredients (all values are g/kg DM unless otherwise indicated. Fatty acid data is percent of total fatty acids)

	Fish Meal [a]	Poultry Meal [b]	Wheat Flour [c]	Wheat Gluten [c]	Soybean Meal [a]	Fish oil [b]	Ricebran Oil [e]
Dry matter	934	974	866	929	877	1000	997
Protein	657	530	121	823	515	4	6
Digestible protein	466	461	121	823	350	-	-
Lipid	85	179	15	53	27	993	912
Carbohydrates	7	138	856	114	386	3	80
Ash	234	149	6	6	68	0	0
Energy*	19.4	22.6	18.8	23.7	20.1	38.6	39.7
Digestible energy*	10.9	14.7	12.2	23.0	7.0	35.5	36.6
Alanine	41	41	4	21	22	-	-
Arginine	38	45	4	27	36	-	-
Aspartic acid	59	52	6	30	57	-	-
Cysteine	9	13	3	20	9	-	-
Glutamic acid	78	83	39	294	89	-	-
Glycine	44	58	4	27	20	-	-
Histidine	17	12	3	14	14	-	-
Isoleucine	26	26	4	28	21	-	-
Leucine	48	48	8	56	38	-	-
Lysine	47	33	2	12	26	-	-
Methionine	19	15	2	15	8	-	-
Phenylalanine	26	28	6	40	26	-	-
Proline	29	46	13	113	24	-	-
Serine	28	39	6	41	28	-	-
Taurine	1	2	0	0	0	-	-
Threonine	30	27	4	21	21	-	-
Tyrosine	20	20	3	29	18	-	-
Valine	30	31	4	30	21	-	-
C14:0	3.0	1.2	0.0	0.0	0.0	8.2	0.5
C16:0	25.3	24.6	19.8	19.6	17.4	18.9	19.8
C16:1	3.6	6.9	1.0	0.0	0.0	10.2	0.0
C18:0	9.6	9.1	1.9	1.5	4.9	3.7	2.2
C18:1	17.7	43.4	15.2	14.5	15.6	13.6	41.8
C18:2n-6	2.8	11.3	56.0	59.7	53.4	1.9	32.4
C18:3n-3	0.0	1.5	3.7	2.9	8.2	0.7	1.2
C20:4n-6	2.6	1.3	0.0	0.0	0.0	1.1	0.0
C20:5n-3	4.6	0.0	1.3	0.8	0.0	17.6	0.0
C22:6n-3	23.5	0.8	1.3	0.0	0.0	13.9	0.0
SFA	40.8	34.9	21.7	21.1	22.8	33.1	23.7
MUFA	24.4	50.2	16.1	15.5	15.6	26.2	42.3
PUFA	2.8	12.8	59.6	62.6	61.6	5.9	33.6
LC-PUFA	32.0	2.1	2.5	0.8	0.0	34.9	0.5
n-3	29.3	2.3	6.2	3.7	8.2	37.4	1.6
n-6	5.5	12.6	56.0	59.7	53.4	3.0	32.4

*Gross energy and digestible energy data is in MJ/kg. [a] Fish meal - Tuna meal, Solvent extracted soybean meal : BEC Feed Solutions, Carole Park, QLD, Australia. [b] Fish oil – anchovy and Poultry meal – Pet food grade : Ridley Aquafeeds, Narangba, QLD, Australia. [c] Wheat flour - whole wheat and Wheat gluten: Manildra, Auburn, NSW, Australia. [d] Ricebran oil: Alfaone, Condell Park, NSW, Australia.

Table 2. Formulations of the diets (all values are g/kg).

Treatment	30-100	20-100	10-100	0-100	30-30	20-30	10-30	0-30	30-15	20-15	10-15	0-15	30-0	20-0	10-0	0-0
Fishmeal	30%	20%	10%	0%	30%	20%	10%	0%	30%	20%	10%	0%	30%	20%	10%	0%
Fish oil	100%	100%	100%	100%	30%	30%	30%	30%	15%	15%	15%	15%	0%	0%	0%	0%
Fishmeal	300	198	102	0	300	198	102	0	300	198	102	0	300	198	102	0
Soybean Meal	150	196	239	285	150	196	239	285	150	196	239	285	150	196	239	285
Poultry Meal	280	338	392	450	280	338	392	450	280	338	392	450	280	338	392	450
Fish oil	60	61	61	62	18	18	18	19	9	9	9	9	0	0	0	0
Ricebran Oil	0	0	0	0	42	42	43	43	51	52	52	53	60	61	61	62
Vitamin Premix	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6
Wheat Flour	142	138	134	130	142	138	134	130	142	138	134	130	142	138	134	130
Wheat Gluten	50	47	43	40	50	47	43	40	50	47	43	40	50	47	43	40
Ca_2PO_4	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
DL-Methionine	7	9	12	14	7	9	12	14	7	9	12	14	7	9	12	14
L-Lysine	0	2	5	7	0	2	5	7	0	2	5	7	0	2	5	7
L-Taurine	0	0	1	1	0	0	1	1	0	0	1	1	0	0	1	1

Table 3. Composition of the diets (all values are g/kg, except fatty acids which are percent of total fatty acids).

Treatment	30-100	20-100	10-100	0-100	30-30	20-30	10-30	0-30	30-15	20-15	10-15	0-15	30-0	20-0	10-0	0-0
Fishmeal	30%	20%	10%	0%	30%	20%	10%	0%	30%	20%	10%	0%	30%	20%	10%	0%
Fish oil	100%	100%	100%	100%	30%	30%	30%	30%	15%	15%	15%	15%	0%	0%	0%	0%
Dry matter	961	979	984	976	966	978	982	980	929	976	976	981	924	975	979	975
Protein	523	532	536	515	530	541	525	515	518	515	534	519	529	505	471	514
Digestible Protein*	479	478	485	448	465	483	448	431	460	455	464	460	480	456	427	457
Lipid	125	134	123	146	132	139	127	130	132	132	131	130	133	129	129	129
Carbohydrates	224	218	235	246	207	200	242	261	219	235	228	258	207	247	294	265
Ash	127	116	107	93	130	120	106	94	131	118	107	93	131	119	106	92
Energy	21.5	21.8	21.9	22.4	21.6	21.7	21.9	22.3	21.7	21.9	21.9	22.4	21.6	21.7	21.9	22.2
Digestible Energy*	17.1	16.5	16.8	15.8	15.1	16.0	14.1	13.9	15.9	15.9	14.9	16.5	16.6	16.9	15.5	16.5
C14:0	4.5	4.7	4.6	4.6	2.2	2.1	2.0	2.1	1.7	1.6	1.8	1.3	1.3	1.5	0.0	0.9
C16:0	23.4	22.0	22.7	23.2	23.9	23.3	23.3	24.1	22.9	22.8	24.4	22.3	22.5	22.8	24.0	22.6
C18:0	6.5	6.2	6.4	6.5	6.1	5.8	5.9	6.5	5.6	5.7	6.1	5.4	5.5	5.6	6.0	5.4
SFA	34.4	32.9	33.7	34.2	32.3	31.2	31.1	32.7	30.2	30.1	32.3	29.5	29.3	29.8	29.9	29.5
C16:1	7.1	7.6	7.8	8.0	4.2	4.3	4.4	4.7	3.4	3.6	4.0	3.8	3.0	3.5	3.5	3.2
C18:1	26.7	24.6	26.8	28.5	34.1	35.4	36.0	36.1	35.7	36.2	37.4	38.4	36.2	36.9	38.8	40.4
MUFA	33.8	32.2	34.6	36.5	38.2	39.8	40.4	40.7	39.1	39.8	41.4	42.7	39.2	40.4	42.2	43.7
C18:2n-6	10.9	9.2	10.9	11.3	18.7	20.7	20.8	20.4	21.8	22.3	20.4	22.9	22.4	22.5	24.0	25.1
C18:3n-3	0.0	1.3	1.5	1.5	1.3	0.0	1.6	1.7	1.4	1.5	1.4	1.7	1.4	1.5	1.6	1.8
PUFA	12.2	13.4	13.9	14.2	20.0	20.7	22.4	22.1	23.2	23.8	21.8	24.6	23.8	24.1	25.6	26.8
C20:4n-6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C20:5n-3	7.9	8.7	8.4	7.7	2.9	3.0	2.6	2.4	2.2	2.0	1.8	1.4	1.4	1.8	1.2	0.1
C22:6n-3	10.3	9.9	8.2	6.1	5.2	5.3	3.4	2.0	5.4	4.3	2.7	1.2	4.9	3.9	2.2	0.1
LC-PUFA	19.5	20.0	17.8	15.0	9.5	8.3	6.0	4.4	7.5	6.3	4.5	3.2	7.7	5.6	3.4	0.2
n-3	20.8	22.7	20.8	17.9	9.4	8.3	7.6	6.1	8.9	7.8	5.9	4.9	7.8	7.2	5.0	1.8
n-6	10.9	9.2	10.9	11.3	20.1	20.7	20.8	20.4	21.8	22.3	20.4	22.9	23.7	22.5	24.0	25.1

*Digestible protein and energy values are those as measured.

Table 4. Performance parameters of *Lates calcarifer* from each treatment.

Treatment		30-100	20-100	10-100	0-100	30-30	20-30	10-30	0-30	30-15	20-15	10-15	0-15	30-0	20-0	10-0	0-0				
Fishmeal		30%	20%	10%	0%	30%	20%	10%	0%	30%	20%	10%	0%	30%	20%	10%	0%	Pooled	P-value		
Fish oil	units	100%	100%	100%	100%	30%	30%	30%	30%	15%	15%	15%	15%	0%	0%	0%	0%	SEM	FM	FO	FM x FO
Initial	(g/fish)	155.2	154.7	154.2	153.5	153.9	154.2	153.9	153.3	153.4	153.8	153.8	154.3	152.5	153.5	155.5	153.4	0.18	0.431	0.485	0.229
Week 8	(g/fish)	345.0 ^{ab}	351.1 ^{a}	329.8 ^{b}	321.0 ^{b}	352.1 ^{a}	353.2 ^{a}	343.4 ^{ab}	325.8 ^{b}	374.3 ^{a}	343.8 ^{ab}	352.7 ^{a}	321.8 ^{b}	354.4 ^{a}	320.5 ^{b}	352.6 ^{a}	325.3 ^{b}	3.39	0.007	0.550	0.572
Survival	%	100	100	98	96	100	100	100	100	100	100	98	100	98	100	100	98	0.01	0.485	0.485	0.685
FIFO	(g/g)	1.28	0.88	0.59	0.23	1.13	0.75	0.42	0.07	1.11	0.70	0.39	0.03	1.10	0.71	0.35	0.00	0.06			
FMIFO	(g/g)	1.29	0.82	0.45	0.00	1.29	0.83	0.43	0.00	1.31	0.81	0.43	0.00	1.33	0.86	0.43	0.00	0.07			
FOIFO	(g/g)	1.23	1.20	1.29	1.34	0.37	0.37	0.37	0.39	0.19	0.18	0.19	0.20	0.00	0.00	0.00	0.00	0.07			

FIFO : Fish-in fish out ratio. FMIFO : Fishmeal-in fish out ratio. FOIFO : Fish oil-in fish out ratio. FIFO, FMIFO and FOIFO values not statistically analysed as they derived values. Parameters with a P-value >0.05 are not discriminated.

Table 5. Whole body composition of *Lates calcarifer* from each treatment at the end of the experiment

Treatment	Initial	30-100	20-100	10-100	0-100	30-30	20-30	10-30	0-30	30-15	20-15	10-15	0-15	30-0	20-0	10-0	0-0	Pooled
Fishmeal		30%	20%	10%	0%	30%	20%	10%	0%	30%	20%	10%	0%	30%	20%	10%	0%	SEM
Fish oil		100%	100%	100%	100%	30%	30%	30%	30%	15%	15%	15%	15%	0%	0%	0%	0%	
Composition																		
Dry matter (%)	26.9	30.5	31.0	30.7	29.9	31.0	30.8	30.2	30.8	31.6	31.4	31.0	30.3	30.5	30.4	30.9	30.7	0.12
Protein (%)	16.6	18.4	20.2	20.3	18.3	20.6	19.1	20.2	19.9	17.2	20.1	19.5	19.1	20.7	18.6	19.8	18.9	0.25
Lipid (%)	6.7	7.9	7.1	7.7	6.7	6.9	7.8	7.5	7.3	7.6	8.0	7.4	6.6	7.7	7.9	7.4	7.8	0.11
Ash (%)	2.9	3.2	3.7	4.5	3.3	3.6	3.5	3.7	4.2	3.3	4.0	3.5	4.1	3.3	3.8	3.8	3.3	0.08
Gross energy (kJ/g)	6.34	7.51	7.63	7.33	7.22	7.37	7.53	7.43	7.51	7.81	7.66	7.39	7.35	7.70	7.51	7.61	7.71	0.04
C14:0	1.9	4.1	4.1	3.9	3.9	2.4	2.8	2.5	2.2	2.2	2.2	2.0	2.1	1.9	1.9	1.7	1.8	0.13
C16:0	24.1	25.5	24.7	24.7	25.4	23.7	27.0	24.1	23.3	24.1	24.7	24.9	24.2	23.7	25.2	23.9	24.1	0.16
C18:0	6.6	7.6	7.0	7.0	7.4	6.7	7.4	6.6	6.3	6.3	6.6	7.0	6.6	6.2	6.7	6.5	6.4	0.07
Total SFA	32.6	38.0	36.4	36.0	36.7	33.1	37.6	33.3	31.8	32.9	33.6	34.1	32.9	32.1	33.9	32.1	32.4	0.33
C16:1	4.0	7.3	7.3	7.3	7.5	4.9	5.4	5.1	5.0	4.6	4.6	4.5	4.8	4.1	4.1	4.1	4.3	0.18
C18:1	37.1	31.3	31.5	32.6	35.0	36.0	39.4	37.1	37.4	38.3	38.1	38.8	39.6	38.8	39.1	40.0	39.6	0.41
C20:1	16.9	0.9	0.8	0.7	0.8	0.8	0.8	0.7	0.6	0.8	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.01
Total MUFA	41.9	39.4	39.6	40.8	43.3	41.6	45.6	43.0	43.1	43.7	43.5	44.0	45.0	43.5	43.9	44.8	44.6	0.27
C18:2n-6	16.9	8.0	8.7	9.4	10.2	14.6	12.7	15.2	16.2	15.7	15.5	15.8	16.4	17.0	16.5	17.6	17.6	0.47
C18:3n-3	1.1	0.9	1.0	1.1	1.1	1.1	0.8	1.2	1.3	1.0	1.0	1.0	1.2	1.0	1.0	1.1	1.2	0.02
Total PUFA	18.6	9.8	10.8	11.6	11.8	16.1	13.4	16.9	18.6	17.0	17.0	17.4	17.9	18.6	17.9	19.4	19.6	0.47
C20:4n-6	0.7	0.9	0.9	0.8	0.3	0.7	0.0	0.4	0.6	0.6	0.4	0.3	0.3	0.6	0.4	0.4	0.2	0.04
C20:5n-3	1.5	4.3	4.6	4.1	3.3	2.4	1.1	2.1	2.0	1.5	1.5	1.2	1.3	1.1	1.0	0.9	1.0	0.18
C22:5n-3	0.9	1.6	1.7	1.6	1.3	1.2	0.6	1.1	1.1	0.9	0.9	0.8	0.8	0.8	0.5	0.5	0.6	0.02
C22:6n-3	3.9	6.0	6.0	5.1	3.4	4.8	1.7	3.3	2.7	3.5	3.1	2.2	1.8	3.3	2.4	1.9	1.5	0.21
Total LC-PUFA	7.0	12.8	13.2	11.6	8.2	9.1	3.3	6.9	6.5	6.5	5.9	4.5	4.2	5.8	4.2	3.7	3.3	0.46
Total n-3	7.4	13.7	14.3	12.8	9.6	9.7	4.1	7.8	7.7	6.9	6.6	5.2	5.1	6.2	4.8	4.4	4.4	0.47
Total n-6	18.2	8.9	9.7	10.4	10.5	15.6	12.7	15.9	17.5	16.6	16.4	16.7	17.0	18.1	17.3	18.6	18.6	0.49
n-3 : n-6	0.41	1.55	1.47	1.22	0.91	0.62	0.32	0.49	0.44	0.41	0.40	0.31	0.30	0.34	0.28	0.24	0.24	0.11

All fatty acid data are % of total fatty acids.

Table 6. Plasma chemistry of *Lates calcarifer* fed each of the experimental diets

Treatment		30-100	20-100	10-100	0-100	30-30	20-30	10-30	0-30	30-15	20-15	10-15	0-15	30-0	20-0	10-0	0-0				
Fishmeal		30%	20%	10%	0%	30%	20%	10%	0%	30%	20%	10%	0%	30%	20%	10%	0%	Pool	P-values		
Fish oil	units	100%	100%	100%	100%%	30%	30%	30%	30%	15%	15%	15%	15%	0%	0%	0%	0%	SEM	FM	FO	FM x FO
CK	U/L	2564	660	2586	2875	566	768	1655	3792	4730	2891	1982	2584	905	1655	1216	1018	312	0.671	0.225	0.619
ALT	U/L	5.7	3.7	8.3	6.3	3.7	5.3	5.7	8.3	14.3	11.3	5.0	11.7	12.0	6.0	7.0	4.7	0.8	0.660	0.128	0.482
GLDH	U/L	12.3	9.3	7.7	12.3	6.7	9.7	8.0	10.0	11.0	8.3	10.0	11.0	8.7	13.7	10.0	14.3	0.7	0.582	0.587	0.948
Urea	mmol/	3.2	3.6	4.0	3.4	3.1	3.8	4.0	3.8	2.8	3.9	3.1	4.1	3.2	4.0	3.7	4.4	0.1	0.101	0.744	0.902
Creatinine	umol/L	16.0	17.3	16.3	16.7	15.0	19.0	18.3	15.0	17.0	18.7	13.7	17.3	16.7	18.3	16.7	16.0	0.4	0.210	0.992	0.699
Calcium	mmol/	2.0	3.1	2.1	2.2	2.1	3.3	3.1	2.2	2.1	3.1	1.1	2.2	3.1	3.1	3.1	3.2	0.2	0.394	0.268	0.921
Magnesium	mmol/	0.7	1.1	0.7	0.7	0.7	1.0	1.0	0.7	0.9	1.1	0.4	0.8	1.1	1.1	1.0	1.1	0.1	0.446	0.431	0.895
Phosphorus	mmol/	2.5	2.9	2.7	2.6	2.7	3.0	2.9	2.6	2.8	3.1	2.6	2.8	2.7	3.0	2.9	2.8	0.0	0.071	0.621	0.964
Iron	umol/L	13.5	17.8	12.4	11.9	8.0	16.1	16.4	10.9	10.4	18.0	5.9	10.3	16.4	15.5	12.8	18.5	1.0	0.348	0.503	0.804
Cholesterol	mmol/	6.0^{a}	6.8^{a}	6.1^{a}	6.0^{a}	5.5^{ab}	6.4^{a}	5.8^{ab}	4.9^{b}	5.6^{ab}	5.6^{ab}	5.0^{ab}	4.8^{b}	5.5^{ab}	5.4^{ab}	4.5^{b}	4.4^{b}	0.2	0.071	0.017	0.950
Total Protein	g/L	42.5	49.5	44.8	48.6	48.1	53.8	51.9	46.4	47.4	51.6	43.2	49.3	50.1	50.1	49.9	52.3	0.9	0.434	0.408	0.891
Albumin	g/L	12.3	13.5	13.2	14.5	13.8	15.0	14.8	13.9	13.7	14.0	13.2	14.7	14.1	14.3	13.8	15.1	0.2	0.231	0.223	0.808
Haem	mg/dL	6.3	1.3	8.3	3.7	1.3	2.0	2.3	6.0	16.3	31.3	2.3	9.0	16.0	2.7	4.3	3.7	2.1	0.729	0.228	0.593

Parameters with a P-value >0.05 are not discriminated.

Table 7. Hepatic gene expression from fish fed experimental diets across the factorial array of treatments. Positive relative expression values mean that those genes were upregulated relative to the reference genes. While those values that were negative were down-regulated relative to the reference genes.

Gene Abbreviation	Name	EC Number	Primers	Relative Expression					Pooled SEM	P-value
				FM:30 FO:100	FM:0 FO:100	FM:10 FO:15	FM:30 FO:0	FM:0 FO:0		
Lipid metabolism genes										
FADS2	Delta 6 desaturase	EC 1.14.19.-	F-TCATACTACCTTCGCTACTTCTC R-ACAAACCAGTGACTCTCCAG	-1.001[a]	-0.749[a]	-0.537[ab]	-0.402[ab]	0.545[b]	0.159	0.044
ELOVL5	Elongase of very long fatty acids	EC 2.3.1.n8	F-ATCCAGTTCTTCTTAACCGT R-GGTTTCTCAAATGTCAATCCAC	-0.952	-0.024	-0.135	0.075	0.329	0.157	0.173
FAS	Fatty acid synthase	EC 2.3.1.85	F-TGAATCTCACCACGCTTCAG R-AGGCAGCAATAGAACCCTCA	-0.887	-1.067	-0.210	-0.657	0.309	0.215	0.363
Amino acid metabolism genes										
ALAT	Alanine aminotransferase	EC 2.6.1.2	F-GATGAACCCTCCTACACCAC R-TTTGAGGTAGAGTGATGCGG	-0.541	-0.659	-0.176	-0.422	0.053	0.150	>0.5
Inflammation response genes										
HSP70	Heat shock protein 70	n/a	F-CAAGGTGATTTCAGATGGAGG R-CTTCATCTTCACCAGGACCA	-0.388	-0.025	-0.355	-0.520	-0.071	0.110	>0.5
CRP	C-reactive protein	n/a	F-ATGGTGTTTCCGATTGAGAC R-CTAGCGAGGTATAAGGACAG	-0.459	0.216	-0.997	-0.956	-1.086	0.194	>0.5
Reference genes										
EF1a	Elongation factor 1 alpha	n/a	F- AAATTGGCGGTATTGGAAC R- GGGAGCAAAGGTGACGAC							
Luc	Luciferase	n/a	F-GGTGTTGGGCGCGTTATTTA R-CGGTAGGCTGCGAAATGC							

Parameters with a P-value >0.05 are not discriminated.