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

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23 **Highlights**

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

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

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

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

31

1 **Abstract**

2

3 An experiment was conducted to examine the potential for the complete replacement of fishmeal
4 (FM) and fish oil (FO) in diets for barramundi, *Lates calcarifer*. A series of diets were formulated to
5 the same digestible protein and energy specifications, but which were designed with FM inclusion
6 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
7 4 factorial design). Ricebran oil was the alternative oil used in the growth study, while soybean
8 meal and poultry meal were the main alternative protein sources used. For the growth study, fish
9 of an initial weight of 154.4 ± 1.1 g were randomly allocated across 48 tanks (three replicates per
10 treatment). After eight weeks, the average weight gain across all treatments was 187.7 ± 2.3 g/fish
11 and feed conversion across all treatments averaged 1.04 ± 0.01 feed/gain. A significant effect of
12 FM on both feed intake and weight gain was observed, and this was observed as early as within
13 the first few weeks, but no similar such effect was observed with FO. No effects were observed on
14 protein deposition efficiency, though both lipid and energy deposition efficiencies were affected
15 by FM level. The reduction in FO had a notable effect on the fatty acid composition of the diets
16 and subsequently the fish fatty acid composition. Expression of key LC-PUFA metabolism genes in
17 the liver of the fish was influenced by both FM and FO levels, but was only significant at the
18 extremes of the treatment ranges. The results from this study demonstrate that there is clear
19 potential to replace almost all the FM content of barramundi diets without loss of fish
20 performance, up to and including diets with as little as 100 g/kg fishmeal. Replacement of fish oil
21 was more successful with the ability to completely replace all FO demonstrated at all but the
22 lowest inclusion levels of FM. These results clearly demonstrate that the near complete
23 replacement of both FM and FO in barramundi diets is a technical reality.

1 **1 Introduction**

2

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

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

21 There has been less work on establishing the boundaries of fish oil replacement in feeds for
22 barramundi, though there has been much work done on other fish species (Borlongan and Parazo,
23 1991). It has recently been demonstrated that the requirement for the long-chain polyunsaturated
24 fatty acids (LC-PUFA) for barramundi is around 1% of the diet (Glencross and Rutherford, 2011;
25 Salini et al., 2015a). Other recent studies have demonstrated that it has been possible to replace
26 all the fish oil in barramundi diets, so long as high inclusions of fishmeal were maintained and this
27 level of LC-PUFA maintained (Alhazzaa et al., 2011).

28 In this study, it was hypothesised that the combined replacement of fishmeal and fish oil will be
29 problematic. But it was not known if the thresholds for this were singular or interactive. Therefore,
30 to address this question a factorial study of replacement in fishmeal (FM) and fish oil (FO) was
31 undertaken. To push the boundaries on this question FM inclusion was reduced from 30% to 0% at
32 10% increments, whilst FO has one inclusion level at 100%, but the other treatments were at 30%,

1 15% and 0% of the added oil components. Importantly, each of the dietary formulations were
2 based on the measured digestible value of each of the key raw materials that were used.

3

4 **2. Materials and Methods**

5 **2.1 Experiment concepts**

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

16 **2.2 Diet preparation**

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

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

12 **2.3 Chemical and digestibility analysis**

13 All samples were dried (or already dry) and milled to a fine powder prior to analysis. Faecal
14 samples were dried by freeze drying prior to being analysed for dry matter, yttrium, nitrogen and
15 gross energy content. In addition, diet samples were analysed for ash and total lipids and
16 carbohydrate content calculated. Dry matter was calculated by gravimetric analysis following oven
17 drying at 105°C for 12 h. Ash content was determined gravimetrically following the loss of mass
18 after combustion of a sample in a muffle furnace at 550°C for 4 h. Protein levels were calculated
19 from the determination of total nitrogen using a ThermoFlash Elemental Analyser, based on N x
20 6.25. Total starch content was measured using enzymatic methods with the Megazyme Total
21 Starch Kit, K-TSTA, following a modified AOAC Method 996.11. Amino acid analysis involved the
22 samples being hydrolysed at 110°C for 24 h in 6 M HCl with 0.05 % Phenol. Cystine was derivatized
23 during hydrolysis by the addition of 0.05 % 3-3-dithiodipropionic acid. The acid hydrolysis
24 destroyed tryptophan making it unable to be determined. Separation of the amino acids was
25 performed by HPLC on a Hypersil AA-ODS 5µM column using an 1100 series Hewlett Packard HPLC
26 system. Total lipid content of the diets was determined gravimetrically following extraction of the
27 lipids using chloroform:methanol (2:1). Gross energy was determined by isoperibol bomb
28 calorimetry. Carbohydrates were calculated based on the dry matter content of a sample minus
29 the protein, lipid and ash. Fatty acids were analysed as methyl ester derivatives based on the
30 method of O'fallon et al. (2007). Esterified lipids were separated by gas chromatography (GC) and
31 detected using flame ionisation detection according to standard methods (O'fallon et al., 2007).
32 Specific fatty acid peaks were identified by comparing retention times relative to standards.

1 Differences in the ratios of dry matter, protein or gross energy to yttrium, in the feed and faeces in
2 each treatment were calculated to determine the apparent digestibility (AD_{diet}) for each of the
3 nutritional parameters examined in each diet based on previously published methods (Blyth et al.,
4 2014).

5 **2.4 Fish management**

6 Fish were obtained from BettaBarra (Cairns, QLD, Australia), and on-grown to 154.4 ± 1.08 g/fish
7 (mean \pm SD, $n=768$) in preparation for the experiment. During the on-growing period all fish were
8 fed the same diet (MarineFloat 4mm, Ridley Aquafeeds, Australia) and kept in 3 x 5,000L seawater
9 tanks. Water temperature was maintained at $30.1 \pm 0.10^\circ\text{C}$ (mean \pm S.D.) and dissolved oxygen at
10 6.1 ± 0.05 mg/L (mean \pm SD.) for the 56-day duration of the experiment. At the initiation of the
11 trial 40 fish were weighed on an electronic top-loading balance to 0.1 g accuracy to determine the
12 mean and standard deviation of the population. Following this, 16 fish were randomly allocated to
13 each of 48 tanks (24 x 300L or 24 x 600L) based on having to be within the mean \pm 1 x SD. Five fish
14 were euthanized from the population at the beginning of the experiment as a representative initial
15 sample.

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

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

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

1 caudal tail vein from each of the five fish from each tank using a 1mL Li-Heparinised syringe and an
2 18G needle. Blood from each of the fish was pooled within a single Vacutainer™ tube. The tube
3 was then centrifuged at 1000 x g for 5 min to settle the erythrocytes and the plasma transferred to
4 a new Eppendorf™ tube prior to it being frozen and sent for plasma analysis. Samples of liver were
5 collected separately from each of the other two fish from each tank and frozen on dry-ice before
6 being stored at -80°C.

7 **2.5 Plasma chemistry**

8 Samples of plasma were sent to the West Australian Animal Health Laboratories (South Perth,
9 Western Australia) for plasma enzyme and chemistry assessment. The assays were run on an
10 Olympus AU400 automated chemistry analyser (Olympus Optical Co. Ltd). Each of the assays used
11 was a standard kit developed for the auto-analyser (absbiomedical.com). The tests performed
12 included Alanine Aminotransferase (ALAT) (ALT2-125), Creatine Kinase (CK) (OSR6179),
13 Glutamate Dehydrogenase (GLDH) (Randox kit Cat. No. GL441), Total Protein (TPT2-125),
14 Creatinine (CRE2-125), Cholesterol (CHO2-125), Calcium (CAA2-125), Magnesium (MAG2-125),
15 Phosphorus (PHO2-125), Iron (IRO2-125), Urea (BUN2-125), Albumin (ALB2-125) and Haem
16 (Randox test kit Cat. No. HG1539). Trace elements were determined after mixed acid digestion
17 using inductively coupled plasma atomic emission spectrophotometry (ICP-AES). Data are
18 presented in Table 6.

19 **2.6 Gene expression analysis**

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

1 Primer optimisation and expression of a range of genes that regulate various metabolic pathways
2 was analysed by real-time PCR as described previously (Wade et al., 2014). Verification that there
3 was no gDNA contamination was carried out by PCR amplification of a pool of DNase-treated RNA
4 samples using gene-specific primers. Real-time PCR amplification reactions were carried out using
5 SYBR Green PCR Master Mix (Applied Biosystems) and run in triplicate on a Viia7 real-time PCR
6 system (Applied Biosystems). Normalization was performed using the ΔCq method by normalizing
7 the cycle threshold values for each gene to Ef1 α , then using the average cycle threshold of all
8 genes at each time point to express relative transcript abundance. The variation in amplification of
9 Ef1 α across all samples was 0.68 cycles and did not significantly change over time (data not
10 shown).

11 **2.7** *Statistical analysis*

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

1 **3. Results**

2 **3.1 *Fish growth and feed utilisation***

3 All treatments showed **substantial** increases in weight during the trial (Table 4), and all treatments
4 more than doubled their initial weight (**Table 4**; Figure 1). Weight gain was poorest by those fish
5 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
6 (gain of 221.0 g/fish). However, the weight gain by the poorest performing fish was not
7 significantly different from that of each of the FM:0 diets, which all performed sub-optimally
8 relative to the remainder of the treatments. Weight gain was significantly affected by fishmeal
9 inclusion, but not by fish oil inclusion and there was no interaction term. Feed intake by fish was
10 significantly affected by fishmeal content, but not fish oil content (Figure 1 and 2). Overall there
11 was a close relationship between weight gain and feed intake across all treatments ($R^2=0.838$).
12 Daily feed intake was consistently lower in the FM:0 series of diets compared to the FM:30 series
13 of diets (Figure 2A). However, no such differences were observed with different inclusions of fish
14 oil (Figure 2B). Feed conversion ratio values ranged from 0.99 (diet FM:20-FO:15) to 1.13 (diet
15 FM:0-FO:0) and averaged 1.04 ± 0.01 (Table 4, Figure 1), and was affected by fishmeal content
16 ($P=0.040$). Fish survival was high (>95%) across all treatments with no significant effects
17 attributable to either fishmeal or fish oil **inclusion** (Table 4).

18 **3.2 *Fish composition and nutrient deposition***

19 Fish proximate composition was relatively consistent across all treatments (Table 5). Virtually all
20 treatments increased their lipid content relative to the initial fish sample. Similarly, there was also
21 a minor increase in protein content relative to the initial sample. However, there were no
22 significant effects on carcass dry matter, protein or lipid content relative to **dietary** fishmeal or fish
23 oil content. There was substantially more variability in the fatty acid content of the fish from each
24 treatment. The SFA and MUFA were the least variable of the total fatty acids. The total SFA ranged
25 from 31.8% of total fatty acids to 38.0% of total fatty acids (Table 5). Total MUFA ranged from
26 **39.4%** of total fatty acids to 45.6% of total fatty acids (Table 5). Variability was greater in the total
27 PUFA, which ranged from 9.8% of total fatty acids to 19.6% of total fatty acids (Table 5). Similarly,
28 the total LC-PUFA was also quite variable, ranging from 3.3% of total fatty acids to 13.2% of total
29 fatty acids (Table 5).

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

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

19 **3.3 Plasma chemistry**

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

28 **3.4 Liver gene expression**

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

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

10 There was a high degree of similarity in the manner in which *cALAT* and *FAS* were expressed
11 among the different treatments. Co-expression analysis indicated that there was a significant
12 positive correlation of $R^2=0.606$ ($P=0.001$) between these two genes. Significant co-expression
13 correlations were also observed between *ELOVL5* and *FADS2* ($R^2=0.498$; $P=0.006$), *FAS* and *ELOVL5*
14 ($R^2=0.608$; $P=0.001$), *cALAT* and *ELOVL5* ($R^2=0.579$; $P=0.001$), and *FADS2* and *FAS* ($R^2=0.714$;
15 $P=0.001$).

16

1 **4. Discussion**

2

3 This study examined the co-replacement of both fishmeal and fish oil in diets for juvenile
4 barramundi (*Lates calcarifer*). The diets were all formulated on an equal digestible nutrient and
5 energy basis, which has previously been shown to enable effective use of a wide range of
6 alternatives (Glencross et al., 2011). Based on the diets being formulated on an equivalent
7 digestible energy and nutrient basis, it was hypothesised that the main response by the juvenile
8 barramundi would be towards the threshold values for fishmeal inclusion for palatability reasons
9 and to fish oil for essential fatty acid reasons (Glencross and Rutherford, 2011; Glencross et al.,
10 2011). This added degree of rigour in the diet formulation process additionally sets this piece of
11 work apart from others attempting the replacement of either fishmeal or fish oil to date
12 (Boonyaratpalin et al., 1998; Raso and Anderson, 2002; Williams et al., 2003b; Tantikitti et al.,
13 2005; Alhazzaa et al., 2011). No other studies examining the joint replacement of **either fishmeal**
14 **or fish oil** in this species, or indeed any other species, were found in the literature.

15 **4.1 Growth and feed utilisation**

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

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

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

21 In the present study the “fish-in-fish-out ratio” ranged from 1.33 for the FM:30-FO:0 diet to 0.00
22 for the FM:0-FO:0 diet (Table 4). The additional use of FO replacement strategies in this study,
23 implemented concurrently with the FM replacement, has clearly shown that it is possible to obtain
24 a “fish-in-fish-out ratio” considerably less than one (Tacon and Metian, 2008; Kaushik and Troell,
25 2010). If fish production from those diets in the FM:20 and FM10 range are considered acceptable
26 then the findings from this study demonstrate that there is clear scope for the production of
27 barramundi to be “fish-production-positive”, with more fish being produced than that being used
28 to provide the feed.

29 **4.2 Body composition and nutrient deposition**

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

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

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

19 Deposition efficiency of the different fatty acids was slightly more variable than that of the macro-
20 nutrients and energy. Notably n-3 deposition efficiency declined significantly with decreasing
21 levels of FM and also somewhat with decreasing levels of FO, but not to the same extent (Figure
22 4). Deposition of n-6 fatty acids also declined with decreasing FM inclusion levels, but in contrast
23 improved with increasing FO replacement (Figure 4). However, most dramatic was the deposition
24 of the LC-PUFA which not only showed highly significant effects attributable to both FM and FO
25 replacement and an interaction term, but also presented a negative deposition efficiency, implying
26 a loss of LC-PUFA from those fish fed the FM:0-FO:0 diet (Figure 4). This observation is in direct
27 contrast to earlier work of Glencross and Rutherford (2011), who observed an increase in the
28 deposition (retention) efficiencies of DHA, EPA and ARA in diets largely devoid of LC-PUFA.
29 However, it is notable that these low LC-PUFA diets still contained 8.4% LC-PUFA (equivalent to ~ 9
30 g/kg) in their total fatty acids in contrast to the FM:0-FO:0 diets in the present study which had
31 0.2% LC-PUFA. This difference in deposition efficiency therefore perhaps highlights that at low
32 levels of LC-PUFA that barramundi are capable of scavenging the small amounts available, thereby

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

8 **4.3 Plasma chemistry**

9 Despite some large numerical differences in a range of the plasma chemistry parameters there
10 were few significant effects attributable to the treatments in this study. This suggests that the
11 health of the fish being fed diets with high levels of replacement of both FM and FO is not being
12 compromised. In an earlier study the authors had noted a range of sub-clinical effects due to
13 replacement of fishmeal on the juvenile barramundi relative to the reference diet (Glencross et al.,
14 2011). In contrast to the present study, in the earlier study noted effects in levels of creatinine
15 kinase (CK), alanine aminotransferase (ALAT) and glutamate dehydrogenase (GLDH) which are
16 markers of muscle and liver damage respectively (Glencross et al., 2011). In the earlier study, it
17 was suggested that the hard physical properties of some of the pellets in that study were
18 responsible for physical trauma to the gastrointestinal tract and this presented as the elevated
19 levels of these plasma marker enzymes. However, similar such physical extremes in the pellet
20 properties were not seen in the present study.

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

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

3 **4.4** *Liver gene expression*

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

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

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

6 **4.5** *Conclusions*

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

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

24

25

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6

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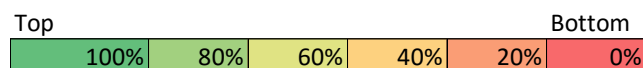
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Figures

- 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**.
- Figure 2. Daily feed intake (g/tank) of pooled FM:30 series and FM:0 series treatments (A) over the full eight weeks. Note that the FM0 series are nearly always lower than the FM30 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 essential no difference between treatment series.
- 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**.
- 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**.

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**.



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%
FO 100%	0.182	0.096	0.621
FO 30%	0.144	0.126	0.323
FO 15%	0.005	0.219	0.087
FO 0%	0.160	0.788	0.129

p-value Fishoil

	30%FM	20%FM	10%FM	0%FM
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%
FO 100%	0.340	0.365	0.813
FO 30%	0.127	0.199	0.464
FO 15%	0.001	0.450	0.092
FO 0%	0.145	0.183	0.759

p-value Fishoil

	30%FM	20%FM	10%FM	0%FM
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%
FO 100%	0.072	0.019	0.234
FO 30%	0.679	0.460	0.511
FO 15%	0.706	0.206	0.549
FO 0%	0.548	0.191	0.054

p-value Fishoil

	30%FM	20%FM	10%FM	0%FM
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.

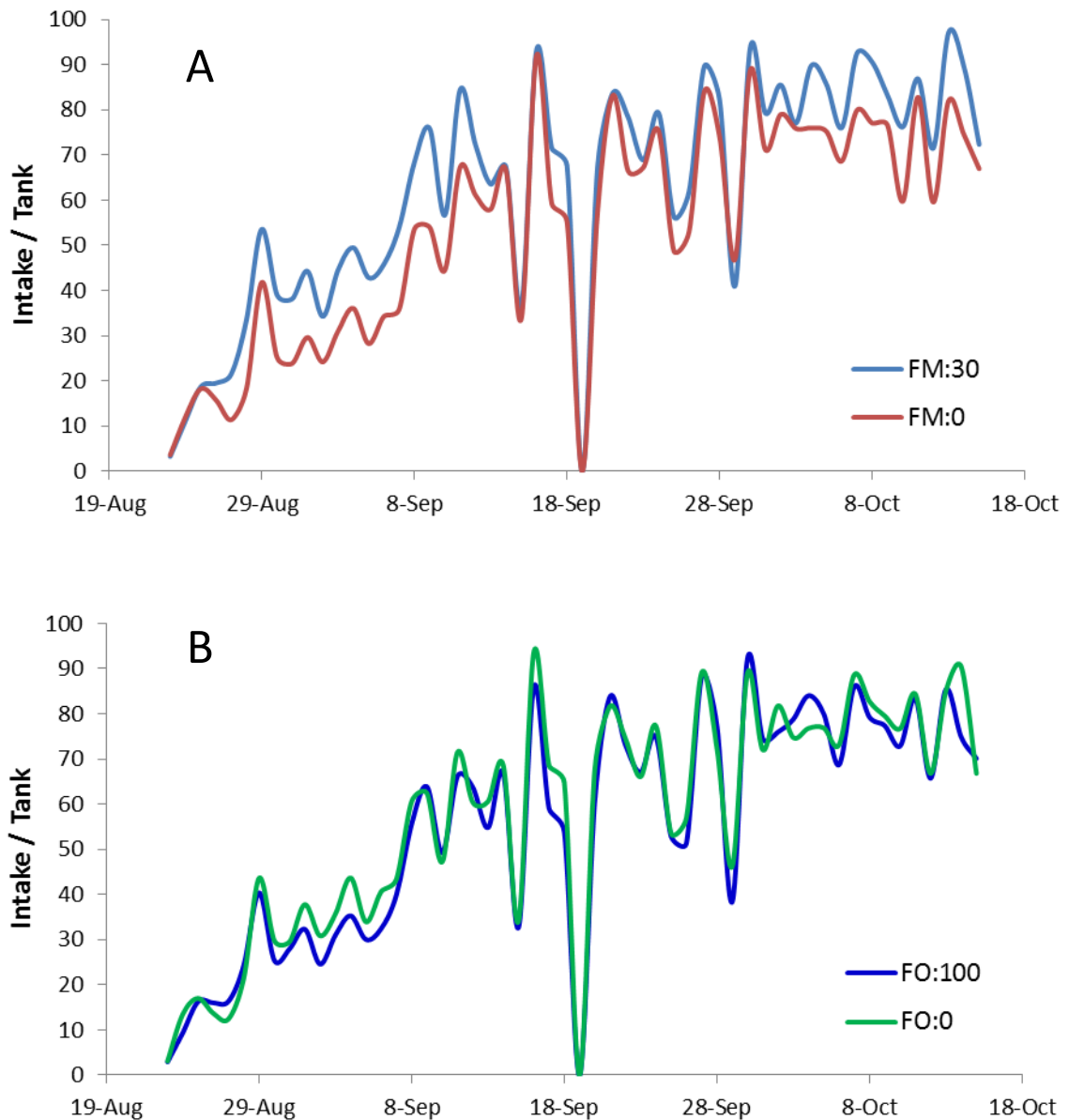


Figure 3. Heat maps of each of the protein, lipid and energy deposition efficiency parameters for each of the treatments in the study. Green is identified in the top 80% -100% percentile while red is identified in the lowest 20% to 0% percentile of performance in each criterion.

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

2A. Protein deposition					p-value Fishmeal			
	30%FM	20%FM	10%FM	0%FM	mean	30% vs 0%	20% vs 0%	10% vs 0%
FO 100%	38%	45%	43%	36%	40%	0.679	0.093	0.195
FO 30%	45%	41%	44%	43%	43%	0.728	0.622	0.799
FO 15%	35%	46%	41%	40%	41%	0.304	0.187	0.769
FO 0%	45%	40%	50%	37%	43%	0.092	0.484	0.011
mean	41%	43%	45%	39%				

p-value Fishoil					Summary Statistics		
					F	p value	
100% vs 0%	0.158	0.378	0.139	0.899	Fishmeal (FM)	2.034	0.129
30% vs 0%	0.905	0.953	0.242	0.215	Fishoil (FO)	0.783	0.512
15% vs 0%	0.037	0.221	0.080	0.546	FM x FO	1.428	0.218

2B. Lipid deposition					p-value Fishmeal			
	30%FM	20%FM	10%FM	0%FM	mean	30% vs 0%	20% vs 0%	10% vs 0%
FO 100%	69%	59%	63%	41%	58%	0.000	0.008	0.002
FO 30%	60%	60%	61%	39%	55%	0.002	0.002	0.001
FO 15%	71%	69%	60%	46%	61%	0.000	0.001	0.037
FO 0%	67%	69%	52%	38%	56%			
mean	67%	64%	59%	41%				

p-value Fishoil					Summary Statistics		
					F	p value	
100% vs 0%	0.766	0.148	0.099	0.591	Fishmeal (FM)	26.624	0.000
30% vs 0%	0.252	0.172	0.143	0.896	Fishoil (FO)	1.429	0.252
15% vs 0%	0.601	0.944	0.238	0.237	FM x FO	0.968	0.484

2C. Energy deposition					p-value Fishmeal			
	30%FM	20%FM	10%FM	0%FM	mean	30% vs 0%	20% vs 0%	10% vs 0%
FO 100%	38%	39%	34%	33%	36%	0.034	0.013	0.694
FO 30%	36%	39%	37%	36%	37%	0.883	0.190	0.672
FO 15%	40%	40%	37%	34%	38%	0.025	0.026	0.300
FO 0%	40%	38%	37%	35%	37%	0.127	0.373	0.626
mean	39%	39%	36%	34%				

p-value Fishoil					Summary Statistics		
					F	p value	
100% vs 0%	0.654	0.535	0.240	0.278	Fishmeal (FM)	5.695	0.003
30% vs 0%	0.184	0.626	0.996	0.954	Fishoil (FO)	0.825	0.490
15% vs 0%	0.756	0.342	0.927	0.643	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 **critierion**.

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 Fishoil

	30%FM	20%FM	10%FM	0%FM
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

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

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 Fishoil

	30%FM	20%FM	10%FM	0%FM
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

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

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 Fishoil

	30%FM	20%FM	10%FM	0%FM
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

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

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 ₂ PO 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	Pooled	P-value				
Fishmeal		30%	20%	10%	0%	30%	20%	10%	0%	30%	20%	10%	0%	30%	20%	10%	0%	SEM	FM	FO	FM x FO		
Fish oil	units	100%	100%	100%	100%	30%	30%	30%	30%	15%	15%	15%	15%	0%	0%	0%	0%						
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%	
<i>Composition</i>																		
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	Pool	P-values		
Fishmeal		30%	20%	10%	0%	30%	20%	10%	0%	30%	20%	10%	0%	30%	20%	10%	0%	SEM	FM	FO	FM x FO
Fish oil	units	100%	100%	100%	100%	30%	30%	30%	30%	15%	15%	15%	15%	0%	0%	0%	0%				
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		
<i>Lipid metabolism genes</i>										
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-TGAATCTCACCAGCTTCAG R-AGGCAGCAATAGAACCCTCA	-0.887	-1.067	-0.210	-0.657	0.309	0.215	0.363
<i>Amino acid metabolism genes</i>										
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
<i>Inflammation response genes</i>										
HSP70	Heat shock protein 70	n/a	F-CAAGGTGATTCAGATGGAGG R-CTTCATCTTCACCAGGACCA	-0.388	-0.025	-0.355	-0.520	-0.071	0.110	>0.5
CRP	C-reactive protein	n/a	F-ATGGTGTTCCGATTGAGAC R-CTAGCGAGGTATAAGGACAG	-0.459	0.216	-0.997	-0.956	-1.086	0.194	>0.5
<i>Reference genes</i>										
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.

