Defining essential fatty acid deficiency in juvenile barramundi *Lates calcarifer*

Michael J. Salini[1, 2]*, Giovanni M. Turchini[1], Nicholas M. Wade[2], Brett D. Glencross[2]

[1] School of Life and Environmental Sciences, Deakin University, Warrnambool, Princess Hwy, Sherwood Park, Victoria, Australia

[2] CSIRO Agricultural Productivity Flagship, 144 North Street, Woorim, Queensland, Australia.

[*] Email msalini@deakin.edu.au

*Telephone +61 (0)7 3410 3106

Shortened title: EFA deficiency in juvenile barramundi

Keywords: Essential fatty acid, deficiency, aetiology, barramundi

Abstract

Barramundi (*Lates calcarifer*), a catadromous teleost of significant and growing commercial importance, are reported to have limited fatty acid bioconversion capability, typical of marine species, and therefore require preformed long-chain polyunsaturated fatty acids (LC-PUFA) as dietary essential fatty acid (EFA). In this study, the response of juvenile barramundi (47.0 g/fish initial weight) fed isolipidic and isoenergetic diets with 8.2% added oil was tested. The experimental test diets were either devoid of any fish oil (FO), and thus with no n-3 LC-PUFA (FO FREE diet) or with a low inclusion of FO (FO LOW diet) and were compared against a control diet containing only FO (FO CTRL diet)as the added lipid source, over an eight week study period. Interim samples and measurements were taken every two weeks during the trial in order to define the onset and progression of the aetiology of EFA deficiency. After two weeks of feeding, the fish fed the FO FREE and FO LOW diets had significantly lower live-weights and after eight weeks significant differences were detected for all growth and feed utilisation parameters. The fish fed the FO FREE diet also had a significantly higher incidence of external abnormalities. Sub-clinical signs of deficiency were also noted in the plasma biochemistry with several parameters affected by dietary treatment. The transcription of several genes involved in fatty acid metabolism was affected after only two weeks of feeding with the experimental diets, showing a rapid nutritional regulation. This experiment documents the aetiology of the onset and the progression of EFA deficiency in juvenile barramundi and demonstrates that such deficiencies can be detected within two weeks in juvenile fish.

Introduction

Nutrient deficiency in any species can be difficult to determine accurately due to the difficulties in feeding a deficient diet. Early studies have specifically looked at fatty acid deficiency in fish [1] while others studying nutrient requirements have reached conclusions about deficiency signs and symptoms [2]. There is a vast amount of literature on lipids and their constituent fatty acids in fish diets and a focal point has been on their complexity, uniqueness and biological importance [3]. The barramundi or Asian sea bass (*Lates calcarifer*), are reported to have limited *de novo* capability to synthesise long-chain polyunsaturated fatty acids (LC-PUFA) [4, 5, 6]. Moreover, the requirement of total lipid and LC-PUFA is thought to be low based on the compositional status of wild caught specimens compared to that of cultured barramundi [7, 8].

Clinical signs of nutrient deficiency become an important indicator of fish health and productivity. If fish are fed a diet that is deficient in a particular nutrient that cannot be synthesised endogenously, then their physical condition will begin deteriorating once all body reserves become depleted or supply limits synthetic demands. Further, secondary pathological conditions may take hold as the animal's immunity is compromised and without treatment premature death often follows. To avoid this situation arising, commercially produced feeds need to ensure an adequate supply of all essential nutrients, often achieved by the addition of fishmeal (FM) and fish oil (FO). However, global supply of these resources is under increasing environmental and economic pressure. Therefore it is important to determine critical inclusion levels of all essential nutrients in dietary formulations for cultured species.

Several studies have induced essential fatty acid (EFA) deficiency in a range of fish species. One of the earliest was that of Castell *et al.* [1] who documented the feeding of rainbow trout (*Oncorhynchus mykiss*) over a prolonged time-course. These authors described a range of deficiency symptoms such as; poor growth, fin erosion, changing pigmentation, swollen livers and hearts and fainting or shock syndrome. A later study by Ruyter *et al.* [9] observed that Atlantic salmon (*Salmo salar*) exhibited poor growth, increased mortality and changed blood and liver condition after one month of feeding an EFA deficient diet. In other species such as channel catfish (*Ictalurus punctatus*) [10], red drum (*Sciaenops ocellatus*) [11], turbot (*Psetta maxima*) [12] and gilthead sea bream (*Sparus aurata*) [13], signs of EFA deficiency include a constant range of symptoms consistent with those described above, with the most prominent being reduced growth performance and survival.

The precursors to LC-PUFA, linoleic acid (18:2n-6) and linolenic acid (18:3n-3) are considered essential or at least conditionally essential in all vertebrates, including fish, as they lack the desaturase enzymes required for their synthesis [14, 15]. Typically, freshwater fish species, including the commercially important salmonids, can synthesise sufficient LC-PUFA from these precursor fatty acids to maintain biological function however most marine fish species have a pathway of fatty acid biosynthesis that is incomplete [15]. Distinct differences also exist between the marine fish in their ability to convert precursor fatty acids to longer chain derivatives. Cultured cell lines of the marine fish turbot were used to demonstrate that elongation activity was limiting the conversion of 18:3n-3 to EPA (eicosapentaenoic acid, 20:5n-3) whereas in gilthead sea bream delta-5 desaturase activity was limiting the conversion of 20:4n-3 to EPA [16, 17]. A recent study also concluded that barramundi (*Lates calcarifer*) lack significant delta-5 desaturase activity and are unable to convert 20:4n-3 to EPA, thus having an essential requirement for preformed dietary LC-PUFA including EPA, DHA (docosahexaenoic acid, 22:6n-3) and ARA (arachidonic acid; 20:4n-6) [6]. Therefore, this supports that *in vivo* this species should succumb to EFA deficiency due to this inability to produce the reputedly required LC-PUFA.

The lipid, and to a lesser degree the fatty acid requirements were examined in barramundi by a number of studies, yet there is still no clear documentation of the onset and progression of deficiency symptoms. Early studies demonstrated that performance of larval barramundi were improved by incorporating preformed long chain polyunsaturated fatty acid (LC-PUFA) into enriched live prey [18, 19]. Other juvenile barramundi studies have reported signs of EFA deficiency such as abnormal reddening of the fins and reduced growth performance associated with low levels of n-3 LC-PUFA [2, 20]. More recently, Williams *et al.* [21] demonstrated improvements in barramundi productivity with increasing n-3 LC-PUFA at either 20 or 29˚C. Williams *et al.* [21] described a 'fainting attack' from a small number of their fish and this effect has also previously been observed in rainbow trout fed EFA deficient diets [1]. Glencross and Rutherford [22] showed for the first time that barramundi performance was clearly affected by the presence or absence of certain EFA. These authors also reported symptoms such as reddening of the fins and the opercula region however this was attributed to increasing DHA in the absence of an equivalent increase of EPA.

Among these results, it is clear that EFA play an important physiological role in both larval and juvenile barramundi while less is known about the effects on larger fish. Despite the efforts of numerous studies so far, there remains to be a clear analysis of the onset and clinical signs of EFA deficiency in barramundi. Therefore, the aim of this experiment was to

document the aetiology of the onset of essential fatty acid deficiency in juvenile barramundi. It was hypothesised that once the endogenous reserves of LC-PUFA are progressively depleted then sub-clinical EFA deficiency symptoms will develop before further gross clinical signs become evident.

Materials and methods

Ingredient and diet preparation

A single basal diet was formulated and prepared without the addition of dietary lipids. The dry ingredients were passed separately through a hammermill (Mikro Pulverizer, type 1 SH, New Jersey, USA) such that the maximum particle size was less than 750 µm. All dry ingredients were then thoroughly mixed using an upright commercial mixer (Bakermix, Model 60 A-G, NSW, Australia). Fish meal was defatted prior to use by manually mixing hexane and fish meal (2:1) in a large drum. The mix was left to soak for 3h before draining the excess hexane and repeating the process a second time. The fish meal was oven dried overnight at 60 ˚C to a constant dry matter. The chemical composition of the main dietary ingredients is presented in Table 1. The basal diet was then separated into smaller batches and aliquots of lipid (8.2% diet) were added to form the three treatment diets. Fresh water was added at approximately 30% of dry mash weight and mixed to form consistent dough then the dough was subsequently screw pressed through a 4 mm die. The pellets were dried overnight at 60 ˚C to a constant dry matter. The dietary treatments provided protein at 60%, lipid at 13% with an energetic value of 22 MJ/kg. The three dietary treatments consisted of a control diet with added FO (designated as FO CTRL), a diet containing only 1% FO and a blend of olive and palm oil (designated as FO LOW) and a diet devoid of any FO and a blend of olive and palm oil (designated as FO FREE). The diets were then stored at -20 ˚C until required. The formulation and chemical composition of the three diets are presented in Table 2.

Barramundi husbandry and growth

Juvenile barramundi (*Lates calcarifer*) were sourced from the Betta Barra fish hatchery (Atherton, QLD, Australia), on-grown in a 10,000L tank and fed a commercial diet (Marine Float; Ridley Aquafeed, Narangba, QLD, Australia). Prior to commencement of the experiment the fish were transferred to a series of experimental tanks (300L) with flow-through seawater (salinity =35 PSU; dissolved oxygen 4.6 ± 0.15 mg /L) of 30.0 ± 0.01 ºC

(mean ± SD) at a flow rate of about 3 L/min being supplied to each of the tanks. At the beginning of the experiment, each of the tanks held 26 fish of 47.0 ± 0.3 g (mean ± SD, n = 624 individually weighed fish). The three experimental diets were randomly distributed amongst the nine tanks with each treatment having three replicate tanks.

Sample collection, preparation and digestibility analysis

Ethical clearance was approved for the experimental procedures by the CSIRO animal ethics committee A10/2013. Six fish of similar size from the original stock were euthanized by an overdose of AQUI-S™ (Lower Hutt, New Zealand) at the beginning of the experiment and stored at -20 ºC until analysis. A further six fish were dissected and a sample of whole blood was removed from the caudal vein using 1 mL pre-heparinised syringes and an 18 G needle. Blood from three fish was pooled in a single Vacutainer™ tube and then centrifuged at 10,000 rpm for 5 min to settle the erythrocytes. The plasma was then drawn off and transferred to a 1.5 mL Eppendorf™ tube and frozen ay -80 ˚C before being sent for analysis. A sample of liver tissue was then removed and placed into 1.5 mL screw-top vial and kept on dry ice before being transferred to a -80 ˚C freezer until analysis. All sampling procedures occurred 24 h post feeding [23]. Interim samples of individual fish (n=3) were collected in the same fashion after each 14 d period and upon termination after 56 d and the remaining fish returned to their respective tank after a short recovery.

Prior to the termination of the growth assay, faeces were collected using established settlement protocols [24] . Briefly, a collection chamber was filled with water and frozen then attached to the evacuation line of a swirl separator and left overnight. The following morning, the collection chamber was removed and the chilled faeces were captured in a plastic sample container and stored at -20 ˚C until analysis.

Abnormalities and behaviour assessment

The physical condition of individual fish was recorded at each fortnightly sampling event. Any fish that had symptoms such as erosion of the fins, reddening of the fins and extremities, gross lesions and physical deformities were recorded. These symptoms were pooled and a percentage score given to each tank and each tank was used as a replicate within each treatment. The behaviour of each tank of fish was assessed by an operator holding a hand over the corner of a tank to simulate commencement of a feeding event. Fish activity was scored as being either cryptic (0), ambivalent to the hand (1) or actively searching for food (2). This assessment was carried out prior to feeding on the same day of each week by the

same operator to maintain consistency and the results were averaged across time to give a repeated measures response to each tank. Each tank was used as a replicate within each treatment [22].

Chemical analysis

Prior to analysis the diets were each ground to a fine powder using a bench grinder (KnifeTec™ 1095, FOSS, Denmark). The initial and final fish were processed using the following method. The whole fish were passed through a commercial meat mincer (MGT – 012, Taiwan) twice to obtain a homogeneous mixture. A sample was taken for dry matter analysis and another sample was freeze-dried along with the faecal samples until no further loss of moisture was observed (Alpha 1-4, Martin Christ, Germany). Dry matter was calculated by gravimetric analysis following oven drying at 105ºC for 24 h. Total yttrium concentrations were determined after nitric acid digestion in a laboratory microwave digester (Ethos One, Milestone, Italy) using inductively coupled plasma-mass spectrophotometry (ICP-MS) (ELAN DRC II, Perkin Elmer, USA). Crude protein was calculated after the determination of total nitrogen by organic elemental analysis (CHNS-O Flash 2000, Thermo Scientific, USA), based on N x 6.25. Total lipid content was determined gravimetrically following extraction of the lipids using chloroform:methanol (2:1) following Folch *et al.* [25]. Gross ash content was determined gravimetrically following loss of mass after combustion of a sample in a muffle furnace at 550 ˚C for 24 h. Gross energy was determined by adiabatic bomb calorimetry (Parr 6200 Calorimeter, USA).

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. The tests performed included alanine aminotransferase (ALT, EC 2.6.1.2) (Olympus kit Cat. No. OSR6107), creatine kinase (CK, EC 2.7.3.2) (Olympus kit Cat. No. OSR6179), glutamate dehydrongenase (GLDH, EC 1.4.1.2) (Randox kit Cat. No. GL441), total protein (Olympus kit Cat. No. OSR6132), creatinine (Olympus kit Cat. No. OSR6178), alkaline phosphatase (Olympus kit Cat. No. OSR6004), glucose (Olympus kit Cat. No. OSR6121) and haem (Randox test kit Cat. No. HG1539). Trace elements were determined after mixed acid digestion using inductively coupled plasma mass spectrometry (ICP-MS).

Fatty acid composition was determined following the methods of Christie [26]. Lipids were esterified by an acid-catalysed methylation and 0.3 mg of an internal standard was added to

each sample (21:0 Supelco, PA, USA). The fatty acids were identified relative to the internal standard following separation by gas chromatography (GC). An Agilent Technologies 6890N GC system (Agilent Technologies, California, USA) fitted with a DB-23 capillary column and flame ionisation detection was used. The temperature program was 50–175 ºC at 25 ºC /min then 175–230 ºC at 2.5 ºC /min. The injector and detector temperatures were set at 250 ºC and 320 ºC, respectively. The carrier gas was hydrogen at a flow rate of 40 mL/min. The peaks were identified by comparing retention times to the internal standard and further referenced against known standards (37 Comp. FAME mix, Supelco, PA, USA). The resulting peaks were then corrected by the theoretical relative FID response factors [27] and quantified relative to the internal standard.

RNA extraction and normalisation

Total RNA was extracted using Trizol reagent (Invitrogen) according to the manufacturer's instructions. RNA was precipitated using equal volumes of precipitation solution (1.2 M sodium chloride and 0.8 M disodium citrate) and isopropyl alcohol [28]. To eliminate any residual traces of DNA, total RNA was DNase digested with the Turbo DNA free kit (Applied Biosystems). To verify that RNA was not contaminated, an aliquot of DNase digested RNA from each sample was pooled (n=54) and later PCR amplified as a negative control. A NanoDrop spectrophotometer (NanoDrop Technologies) was used asses RNA quantity and a Bioanalyser (Agilent Technologies) using RNA nanochips (Agilent #5067-1511) was used to asses RNA quality. All RNA samples were normalised to 200 ng/µl.

Quantitative real time RT-PCR

Reverse transcription was performed on 1 µg of total RNA using Superscript III (Invitrogen) with 25 µM oligo $(dT)_{20}$ and 25 µM random hexamers [29]. Expression of a range of genes involved in fatty acid metabolism was analysed by real-time PCR as described below. Real-time PCR amplification reactions were carried out using 1X SYBR Green PCR Master Mix (Applied Biosystems), 0.2 µM of Real-Time PCR primers specific to each gene (Table 3) and the equivalent of 7.5 ng of reverse-transcribed RNA. Amplification cycle conditions were 2 min at 50 ˚C, 10 min at 95 ˚C followed by 40 cycles of 15 s at 95 ˚C and 40 s at 60 ˚C. After amplification, a melt curve analysis was routinely performed to verify the specificity of the target gene. Reactions were setup using the epMotion 5070 robot (Eppendorf) and run in triplicate on a Viia7 real-time PCR system (Applied Biosystems). Changes in expression levels of each gene over the eight week trial (denoted WK0, WK2, WK4, WK6 and WK8) were determined by normalising the cycle threshold values for each gene to elongation factor

1 alpha (EF1α) and Luciferase reference genes, then to the cycle threshold of each gene at time zero (WK0). The variation in amplification of EF1α was 1.24 cycles and Luciferase was 0.58 cycles and this did not significantly change over time. The EF1α and Luciferase genes are routinely used as a reference in this species [23, 30].

Calculations and statistical analysis

Differences in the ratio of dry matter, protein, lipid and energy to yttrium in the diet and faeces were calculated to determine the apparent digestibility coefficients (ADC) following Maynard and Loosli [31]. Nutrient retention efficiencies were calculated as the ratio of the nutrient or specific fatty acid gained relative to their respective consumption during the study period following Maynard and Loosli [31]. The computation of apparent *in vivo* fatty acid β-oxidation was performed using the whole body fatty acid balance method (WBFABM) following Turchini *et al.* [32].

All data were checked for normal distribution and homogeneity of variance by qualitative assessment of residual and normal Q-Q plots using the RStudio package v.0.98.501 [33]. All performance data were analysed by one-way analysis of variance (ANOVA) using the RStudio package v.0.98.501 [33]. Live-weight data were analysed by analysis of covariance (ANCOVA) between the week two and week eight measurements using the RStudio package v.0.98.501[33]. Gene expression data were analysed by t-test. Any percentage data were arcsine transformed prior to analysis. Levels of significance were compared using Tukey's HSD *a posteriori* test with significance among treatments defined as $P < 0.05$.

Results

Growth and feed utilisation

During the 56 d growth assay, the fish responded readily to the experimental diets and growth in the control group was consistent with the predicted model growth, achieving 106% of the modelled potential [34]. When analysed by ANOVA there was a significant difference in live-weight after two weeks of feeding on the experimental diets, with the fish fed the FO LOW and FO FREE diets being smaller from those fed the FO CTRL diet. By six weeks of feeding there was a significant difference among all three groups of fish and this remained the same at eight weeks. The same results were found with weight gain. When the live-weight data were analysed by ANCOVA there was a significant effect of the diets after controlling for

week two measurements (F=1739, *df 5,30*, P<0.001). There was no difference in feed intake among the groups of fish however there was a significant difference in FCR between the FO CTRL and FO FREE fed fish (Table 4). There were no differences in terms of survival with only one fish removed from the system.

The total lipid was significantly more digestible in the FO CTRL fed fish than the FO FREE fed fish (Table 5). There were several significant differences in specific fatty acid digestibility. The n-3 LC-PUFA including EPA and DHA were all completely digested by the fish fed FO LOW and FO FREE diets whereas the FO CTRL fed fish digested less of these fatty acids (Table 5). Although significant, the numerical differences were minor. The digestibility of total saturated fatty acids (SFA) was significantly different with fish fed the FO FREE diet able to digest more than the fish fed the FO CTRL diet.

Biochemical analysis

There were significant differences in macro nutrient retention of the fish after eight weeks of feeding with the control fed fish retaining more protein, lipid and gross energy than the FO LOW and FREE fed fish (Table 4). Generally, the fatty acid composition of the diets was reflected in the whole fish and also the liver tissue (Table 6). There were significant differences in most of the dominant fatty acids in the whole-body with the only exceptions being 18:3n-3 and 16:0 (Table 6). Similarly, in the liver, there were significant differences in most of the dominant fatty acids with the exception of 18:2n-6, 18:3n-3 and 16:0 (Table 6).

After eight weeks, there was a significant decrease in the retention of SFA, MUFA and PUFA in the FO LOW and FO FREE fed fish compared to the FO CTRL fed fish while the LC-PUFA retention was highest in the FO LOW fed fish (Figure 1). In terms of calculated β-oxidation values there was significantly less oxidation of total SFA and MUFA in the FO CTRL fed fish. There was no difference in PUFA oxidation however significantly more LC-PUFA was oxidised in the FO CTRL fed fish (Figure 1).

Clinical observations

A range of abnormalities were observed during the experiment. These included red fins (typically caudal and pectoral), red skin, fin erosion, lesions and non-contributing (feed refusal) fish. The fish fed the FO FREE diet were significantly more affected by a range of abnormalities than the fish fed the FO LOW and FO CTRL diets (Table 4). There was no

significant difference in the behavioural response of fish assessed by the methods described earlier (Table 4).

Sub-clinical parameters

A range of plasma chemistry parameters were assessed and some significant differences were observed among the dietary treatments (Figure 2). Only those parameters with significant differences are reported. There was a significant time effect on alanine aminotransferase (ALT) activity among the dietary groups decreasing dramatically from the initial level until week four and increasing thereafter. Creatine kinase (CK) activity were affected by time in a similar fashion to that of ALT however the FO FREE fish levels did not increase beyond four weeks which lead to a significant diet effect. Plasma cholesterol was significantly elevated in the FO CTRL fish at each time point. There was a significant diet and time effect on the glutamate dehydrogenase (GLDH) activity, decreasing from the initial sample until week six then rapidly rising in the FO LOW and FO FREE groups. There was an effect between the diets over time on the haemoglobin levels leading to a significantly lower level in the FO FREE fed fish at the final sample. There was also a significant time effect on haptoglobin with levels rapidly rising between the sixth and final sampling events. Similarly, the plasma magnesium level increased between the sixth and final sampling event and urea decreased following the initial sample however it gradually increased after each consecutive sampling event.

Expression of lipid metabolism related genes in the liver of the FO CTRL and FO FREE fish were analysed by real-time quantitative PCR and several significant differences were observed (Figure 3). There was no significant diet effect for CPT1a and ELOVL5. There was a significant diet effect on the expression of ACYL with the highest expression in the FO FREE fed fish at week four. Similarly, the expression of FAS was approximately 2.5-fold higher in the FO FREE fish after two weeks of feeding and significant differences were observed at each time point. Similarly, the expression of SCD was significantly affected at each time point by the dietary treatment with levels of expression in the FO FREE fish approximately 3- to 4-fold higher than the FO CTRL fish. There was a significant difference in the expression of FADS2 at each sampling time point with approximately 2-fold higher expression among the FO FREE fed fish.

Discussion

The onset of EFA deficiency on growth and feed utilisation in juvenile barramundi was evident after only two weeks of feeding the experimental diets. After eight weeks growth was clearly different among all three groups suggesting that a primary feature of EFA deficiency is growth potential. In addition, there was strong evidence of a difference in the rate of live-weight change between the diet treatments showing a clear progression towards EFA deficiency. This is in agreement with other studies showing reduced growth when carnivorous marine fish were fed with high levels of FO substitution [35, 36, 37]. In addition, consistent with the maintenance of feed intake but with reduced growth, the feed conversion of FO FREE fed fish was significantly poorer. The ability to define a response to a nutrient relies on being able to define any symptoms that may occur in its absence. The defatted fish meal used in the present study contained trace amounts of lipid and LC-PUFA. However, the inclusion of 15% defatted fish meal was based on previous recommendations to achieve what is considered normal growth for this species [34, 38].

Early studies with barramundi demonstrated that decreased dietary lipid is associated with reduced growth and in some cases EFA deficiency was inferred [2, 20, 21, 39]. In the present study, the macro nutrient levels were kept constant as barramundi, like most other species, have an interdependent demand for energy and specific nutrients [40]. The total lipid level was formulated to be lower than the known optimal requirement (>14%) for fish of this size (<100 g) so that in this experimental design the lipid would theoretically be limiting and therefore cause the supply of this nutrient (and vagaries in its composition) to be the point of sensitivity in the study [40]. Therefore, the differences in the present study are directly attributable to the fatty acid profile of the diets, resulting in EFA deficiency.

In the present study, the digestibility of total lipid was lowest in the FO FREE fed fish consistent with other studies investigating FO replacement [41, 42, 43]. The apparent digestibility of specific fatty acids was not greatly modified by the dietary treatments with the exception of the saturated fatty acids. In the study of Olsen *et al.* [44], 18:0 was reported to be less digestible than other saturates such as 16:0 and 14:0 and in agreement, the reduction of total lipid digestibility of the present study is likely to be caused by the 18:0 composition of the feeds. Several studies have concluded that changes in lipid or fatty acid digestibility can result in reduced growth [45, 46, 47] whereas others have found no such effect [41, 42]. The whole-body and liver fatty acid profiles in the present study showed some clear differences in terms of their composition, however the tissue composition largely resembled that of the fed diet, also consistent with other studies [48, 49, 50]. A caveat of the present study was that the initial EFA composition of the fish was reflective of the commercial diet fed prior to

experimentation. There was a slight reduction of total SFA in the whole body and a lack of any change in the liver, despite the different digestibility values and growth. Moreover, the LC-PUFA composition of the tissues in the FO FREE fed fish was almost entirely depleted after eight weeks suggesting that the reduced digestibility of total SFA had no bearing on the overall composition of the fish.

Examination of the mass-balance relationship of dietary nutrients or indeed specific fatty acids in the tissues was used reveal discrete differences in their utilisation. Improved protein retention and consequently weight gain was evident in the control group, consistent with previous studies on barramundi [(21)]. The disproportionately low lipid and energy retention of the FO LOW and FO FREE fed fish is a response to the diets without adequate LC-PUFA. The FO CTRL fed fish, selectively retained more of the dietary SFA, MUFA and PUFA than the other fish however LC-PUFA were catabolised when in surplus to optimal tissue concentration, similar to observations from other carnivorous species such as Atlantic salmon, European sea bass, Murray cod and rainbow trout [(48, 51, 52, 53)].

The aetiology of deficiency was less apparent in the FO LOW fed fish. The total LC-PUFA concentration in the FO LOW diet with 1% added FO was equivalent to 4.3 g/kg of the diet compared to 25.6 g/kg in the control diet. Currently available requirement data for barramundi suggest that an adequate dietary LC-PUFA supply should be at least 12 g/kg in growing barramundi [(21)]. In agreement, Glencross and Rutherford [(22)] reasoned that provided EFA are kept in balance the total LC-PUFA requirement could be further revised. Despite the very low inclusion of LC-PUFA (FO LOW and FO FREE diets) there were no apparent effects of the diets on survival or behavioural changes. However, a range of physical abnormalities were observed in the FO FREE fed fish including; erosion of the fins, reddening of the fins and extremities, gross lesions and physical deformities, symptoms which are associated with EFA deficiency in fish [(1)]. This observation suggests that even at very low inclusion levels of FO there was a marked improvement in the physical health of fish despite the reduced growth. Moreover, the whole-body composition data suggest that the FO LOW fed fish maintained a proportion of LC-PUFA similar to that of the initial fish fed a commercially available diet. Therefore in support of the previous studies on this species, we too support that it is possible to reduce the LC-PUFA to levels below 12 g/kg without pathological evidence of EFA deficiency however below this level is not considered adequate to support ideal growth of the fish.

A range of plasma biochemical markers were used to assess the aetiology of EFA deficiency over the eight week time course. The results suggest that aminotransferase activity, including glutamate dehydrogenase (GLDH) and alanine aminotransferase (ALT), generally increased after six weeks in the EFA deficient fish. Consistent with other species, GLDH activity in barramundi is considered a reliable marker of liver cell necrosis which can be also characterised by the depletion of hepatic LC-PUFA stores [54, 55]. In addition, elevated creatine kinase (CK) activity levels, indicative of muscle damage and often linked to myocardial infarction [56], also increased after six weeks in the FO CTRL and FO LOW fish. Similarly, plasma magnesium and urea, a marker for renal dysfunction which has been related to EFA deficiency [57], responded in much the same fashion. Both haemoglobin and haptoglobin showed a similar response where levels increased after six weeks. The same effect has been linked to a mild anemia in rainbow trout [58]. The plasma markers are consistently affected after six weeks of feeding which potentially indicates a point of sensitivity for the dilution of body EFA reserves in barramundi. This suggests that a temporal scale is an important consideration in defining the aetiology of EFA deficiency in barramundi. Other studies found no difference in growth performance parameters after feeding a deficient diet for four weeks [59] or six weeks [22], while longer term studies demonstrated clear signs of EFA deficiency, including changes in plasma biochemistry, with rainbow trout after 18 weeks [58] and with Atlantic salmon between 12 -16 weeks [9].

Consistent with other studies, nutritionally regulated gene expression in the FO CTRL fed fish appear to follow a normal progression of desaturation and elongation of FA to their longer chain and less-saturated derivatives [6, 23, 60]. In the present study, there was no significant diet effect in the expression of elongation (ELOVL5), ATP citrate lyase (ACYL) or carnitine palmitoyltransferase (CPT1a) however there was a tendency towards higher expression in the FO FREE fed fish. Similarly, Araujo *et al.* [60] found that there was a tendency towards higher expression of each of these genes in barramundi fed a FO deficient diet. Mixed results from previous studies have shown that CPT1a expression can be down regulated in response to vegetable oils [61] whereas others have shown an up-regulation [62]. This study and that of Araujo *et al.* [60] found that steroyl CoA desaturase (SCD) expression in barramundi was significantly up-regulated in fish fed a FO deficient diet as they attempt to convert available stearic acid to more energetically available oleic acid. Moreover, in agreement with other studies, on a range of species, the expression of desaturase enzymes (including both SCD and FADS2) increased in response to reduced FO [60, 63, 64, 65, 66]. Fatty acid synthase (FAS) expression in the present study was also significantly up-regulated in fish fed the FO FREE diet. Although the FAS enzyme system has rarely been investigated in

fish in response to FO replacement, it is an important step in the initial synthesis of palmitate from acetyl-CoA.

Conclusions

In conclusion, the results of this study report comprehensively the aetiology of the onset and progression of EFA deficiency in barramundi. The EFA deficient diets clearly impacted growth performance and feed utilisation in as little as two weeks. Discrete differences in the utilisation of dietary lipid and also specific fatty acids suggest that in the absence of EFA, signs of deficiency were evident substantiating its essentiality in barramundi. In addition, a range of clinical abnormalities manifested in the fish fed the FO FREE diet. Sub-clinical diagnostics of plasma biochemistry and gene expression consistently demonstrated the negative effect on those fish receiving the FO FREE and in some cases FO LOW diet and these appeared to occur after six weeks of feeding, lending support to the hypothesis that assessment on a temporal basis is critical in fatty acid studies on fish.

6. Acknowledgements

We would like to thank and acknowledge the technical assistance provided by Bruno Araujo, David Blyth, Natalie Habilay, Simon Irvin, Kinam Salee and Richard Thaggard at the Bribie Island Research Centre (BIRC), Queensland, Australia.

Authors' responsibilities were as follows: MJS, GMT and BDG conceived and resourced the experiment, MJS conducted the experiment, laboratory analyses and analysed the data. NMW provided valuable instruction on molecular techniques and analysis and MJS wrote the manuscript. All authors read and approved the final version of the manuscript. There are no conflicts of interest.

References

1. Castell JD, Sinnhuber RO, Wales JH *et al.* (1972) Essential fatty acids in the diet of rainbow trout (*Salmo gairdneri*): Growth, feed conversion and some gross deficiency symptoms. *J Nutr* **102**, 77-85.
2. Catacutan MR & Coloso RM (1995) Effect of dietary protein to energy ratios on growth, survival, and body composition of juvenile Asian seabass, *Lates calcarifer*. *Aquaculture* **131**, 125-133.
3. Sargent JR, Tocher D & Bell G (editors) (2002) *The Lipids*. San Diego, California: Academic Press.
4. Alhazzaa R, Bridle AR, Nichols PD *et al.* (2011) Replacing dietary fish oil with Echium oil enriched barramundi with C18 PUFA rather than long-chain PUFA. *Aquaculture* **312**, 162-171.
5. Tu WC, Muhlhausler BS, James MJ *et al.* (2013) Dietary alpha-linolenic acid does not enhance accumulation of omega-3 long-chain polyunsaturated fatty acids in barramundi (*Lates calcarifer*). *Comp Biochem Physiol B Comp Biochem* **164**, 29-37.
6. Mohd-Yusof NY, Monroig O, Mohd-Adnan A *et al.* (2010) Investigation of highly unsaturated fatty acid metabolism in the Asian sea bass, *Lates calcarifer*. *Fish Physiol Biochem* **36**, 827-843.
7. Nichols PD, Glencross B, Petrie JR *et al.* (2014) Readily available sources of long-chain omega-3 oils: is farmed Australian seafood a better source of the good oil than wild-caught seafood? *Nutrients* **6**, 1063-1079.
8. Nichols P, Elliott NG, Mooney B *et al.* (1998) *Nutritional Value of Australian Seafood II: Factors Affecting Oil Composition of Edible Species*: CSIRO Division of Marine Research.
9. Ruyter, Røsjø, Einen *et al.* (2000) Essential fatty acids in Atlantic salmon: time course of changes in fatty acid composition of liver, blood and carcass induced by a diet deficient in n-3 and n-6 fatty acids. *Aquac Nutr* **6**, 109-117.
10. Satoh S, Poe WE & Wilson RP (1989) Studies on the essential fatty acid requirement of channel catfish, *Ictalurus punctatus*. *Aquaculture* **79**, 121-128.
11. Lochmann R & Gatlin D, III (1993) Essential fatty acid requirement of juvenile red drum (Sciaenops ocellatus). *Fish Physiol Biochem* **12**, 221-235.
12. Bell MV, Henderson RJ, Pirie BJS *et al.* (1985) Effects of dietary polyunsaturated fatty acid deficiencies on mortality, growth and gill structure in the turbot, *Scophthalmus maximus*. *J Fish Biol* **26**, 181-191.
13. Ibeas C, Cejas J, Gómez T *et al.* (1996) Influence of dietary n − 3 highly unsaturated fatty acids levels on juvenile gilthead seabream (*Sparus aurata*) growth and tissue fatty acid composition. *Aquaculture* **142**, 221-235.
14. Cunnane SC (2003) Problems with essential fatty acids: time for a new paradigm? *Prog Lipid Res* **42**, 544-568.
15. Tocher DR (2003) Metabolism and functions of lipids and fatty acids in teleost fish. *Rev Fish Sci* **11**, 107-184.
16. Tocher D & Ghioni C (1999) Fatty acid metabolism in marine fish: Low activity of fatty acyl Δ5 desaturation in gilthead sea bream (*Sparus aurata*) cells. *Lipids* **34**, 433-440.
17. Ghioni C, Tocher DR, Bell MV *et al.* (1999) Low C18 to C20 fatty acid elongase activity and limited conversion of stearidonic acid, 18:4(n–3), to eicosapentaenoic acid, 20:5(n–3), in a cell line from the turbot, *Scophthalmus maximus*. *Biochim Biophys Acta* **1437**, 170-181.
18. Dhert P, Lavens P, Duray M *et al.* (1990) Improved larval survival at metamorphosis of Asian seabass (*Lates calcarifer*) using ω3-HUFA-enriched live food. *Aquaculture* **90**, 63-74.
19. Rimmer MA, Reed AW, Levitt MS *et al.* (1994) Effects of nutritional enhancement of live food organisms on growth and survival of barramundi, *Lates calcarifer* (Bloch), larvae. *Aquac Res* **25**, 143-156.
20. Buranapanidgit J & Boonyaratpalin M (1988) Essential fatty acid requirement of juvenile seabass, *Lates calcarifer*. *[Seminar on Fisheries 1988], Bangkok (Thailand), 21-23 Sep 1988*.
21. Williams KC, Barlow CG, Rodgers LJ *et al.* (2006) Dietary composition manipulation to enhance the performance of juvenile barramundi (*Lates calcarifer* Bloch) reared in cool water. *Aquac Res* **37**, 914-927.
22. Glencross BD & Rutherford N (2011) A determination of the quantitative requirements for docosahexaenoic acid for juvenile barramundi (*Lates calcarifer*). *Aquac Nutr* **17**, e536-e548.

23. Wade N, Skiba-Cassy S, Dias K *et al.* (2014) Postprandial molecular responses in the liver of the barramundi, *Lates calcarifer*. *Fish Physiol Biochem* **40**, 427-443.
24. Blyth D, Tabrett S, Bourne N *et al.* (2014) Comparison of faecal collection methods and diet acclimation times for the measurement of digestibility coefficients in barramundi (*Lates calcarifer*). *Aquac Nutr*, DOI: 10.1111/anu.12159.
25. Folch J, Lees M & Sloane-Stanley G (1957) A simple method for the isolation and purification of total lipids from animal tissues. *J. biol. Chem* **226**, 497-509.
26. Christie WW (2003) *Lipid analysis, isolation, separation, identification and structural analysis of lipids, 3rd edition.*: PJ Barnes and Associates.
27. Ackman RG (2002) The gas chromatograph in practical analysis of common and uncommon fatty acids for the 21st century. *Anal Chim Acta* **465**, 175-192.
28. Green MR & Sambrook J (2012) *Molecular cloning: a laboratory manual*: Cold Spring Harbor Laboratory Press New York.
29. Resuehr D & Spiess A-N (2003) A real-time polymerase chain reaction-based evaluation of cDNA synthesis priming methods. *Anal Biochem* **322**, 287-291.
30. De Santis C, Smith-Keune C & Jerry DR (2011) Normalizing RT-qPCR data: are we getting the right answers? An appraisal of normalization approaches and internal reference genes from a case study in the finfish Lates calcarifer. *Mar Biotechnol* **13**, 170-180.
31. Maynard LA & Loosli JK (1979) *Animal Nutrition*. 6th edn. ed. New York, NY: McGraw-Hill Book Co.
32. Turchini GM, Francis DS & De Silva SS (2007) A whole body, in vivo, fatty acid balance method to quantify PUFA metabolism (desaturation, elongation and beta-oxidation). *Lipids* **42**, 1065-1071.
33. R Core Team (2012) R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing.
34. Glencross BD & Bermudes M (2012) Adapting bioenergetic factorial modelling to understand the implications of heat stress on barramundi (*Lates calcarifer*) growth, feed utilisation and optimal protein and energy requirements – potential strategies for dealing with climate change? *Aquac Nutr* **18**, 411-422.
35. Glencross BD, Hawkins W & Curnow J (2003) Evaluation of canola oils as alternative lipid resources in diets for juvenile red seabream, *Pagrus auratus*. *Aquac Nutr* **9**, 305-315.
36. Izquierdo MS, Montero D, Robaina L *et al.* (2005) Alterations in fillet fatty acid profile and flesh quality in gilthead seabream (*Sparus aurata*) fed vegetable oils for a long term period. Recovery of fatty acid profiles by fish oil feeding. *Aquaculture* **250**, 431-444.
37. Montero D, Robaina L, Caballero MJ *et al.* (2005) Growth, feed utilization and flesh quality of European sea bass (*Dicentrarchus labrax*) fed diets containing vegetable oils: A time-course study on the effect of a re-feeding period with a 100% fish oil diet. *Aquaculture* **248**, 121-134.
38. Glencross BD, Rutherford N & Jones B (2011) Evaluating options for fishmeal replacement in diets for juvenile barramundi (*Lates calcarifer*). *Aquac Nutr* **17**, E722-E732.
39. Williams KC, Barlow CG, Rodgers L *et al.* (2003) Asian seabass *Lates calcarifer* perform well when fed pelleted diets high in protein and lipid. *Aquaculture* **225**, 191-206.
40. Glencross BD (2006) The nutritional management of barramundi, *Lates calcarifer* - a review. *Aquac Nutr* **12**, 291-309.
41. Caballero MJ, Obach A, Rosenlund G *et al.* (2002) Impact of different dietary lipid sources on growth, lipid digestibility, tissue fatty acid composition and histology of rainbow trout, *Oncorhynchus mykiss*. *Aquaculture* **214**, 253-271.
42. Torstensen BE, Lie O & Froyland L (2000) Lipid metabolism and tissue composition in Atlantic salmon (*Salmo salar* L.) - Effects of capelin oil, palm oil, and oleic acid-enriched sunflower oil as dietary lipid sources. *Lipids* **35**, 653-664.
43. Ng W-K, Sigholt T & Bell JG (2004) The influence of environmental temperature on the apparent nutrient and fatty acid digestibility in Atlantic salmon (*Salmo salar* L.) fed finishing diets containing different blends of fish oil, rapeseed oil and palm oil. *Aquac Res* **35**, 1228-1237.
44. Olsen RE, Henderson RJ & Ringø E (1998) The digestion and selective absorption of dietary fatty acids in Arctic charr, *Salvelinus alpinus*. *Aquac Nutr* **4**, 13-21.

45. Karalazos V, Bendiksen EÅ & Bell JG (2011) Interactive effects of dietary protein/lipid level and oil source on growth, feed utilisation and nutrient and fatty acid digestibility of Atlantic salmon. *Aquaculture* **311**, 193-200.
46. Hansen JØ, Berge GM, Hillestad M *et al.* (2008) Apparent digestion and apparent retention of lipid and fatty acids in Atlantic cod (*Gadus morhua*) fed increasing dietary lipid levels. *Aquaculture* **284**, 159-166.
47. Menoyo D, Lopez-Bote CJ, Diez A *et al.* (2007) Impact of *n*−3 fatty acid chain length and *n* - 3/*n* - 6 ratio in Atlantic salmon (*Salmo salar*) diets. *Aquaculture* **267**, 248-259.
48. Turchini GM, Francis DS, Senadheera SPSD *et al.* (2011) Fish oil replacement with different vegetable oils in Murray cod: Evidence of an "omega-3 sparing effect" by other dietary fatty acids. *Aquaculture* **315**, 250-259.
49. Pratoomyot J, Bendiksen EÅ, Bell JG *et al.* (2008) Comparison of effects of vegetable oils blended with southern hemisphere fish oil and decontaminated northern hemisphere fish oil on growth performance, composition and gene expression in Atlantic salmon (*Salmo salar* L.). *Aquaculture* **280**, 170-178.
50. Glencross BD, Tocher D, Matthew C *et al.* (2014) Interactions between dietary docosahexaenoic acid and other long-chain polyunsaturated fatty acids on performance and fatty acid retention in post-smolt Atlantic salmon (*Salmo salar*). *Fish Physiol Biochem* **40**, 1213-1227.
51. Turchini GM & Francis DS (2009) Fatty acid metabolism (desaturation, elongation and β-oxidation) in rainbow trout fed fish oil- or linseed oil-based diets. *Br J Nutr* **102**, 69-81.
52. Eroldogan TO, Yilmaz AH, Turchini GM *et al.* (2013) Fatty acid metabolism in European sea bass (*Dicentrarchus labrax*): effects of n-6 PUFA and MUFA in fish oil replaced diets. *Fish Physiol Biochem* **39**, 941-955.
53. Stubhaug I, Lie Ø & Torstensen BE (2007) Fatty acid productive value and β-oxidation capacity in Atlantic salmon (*Salmo salar* L.) fed on different lipid sources along the whole growth period. *Aquac Nutr* **13**, 145-155.
54. Van Waes L & Lieber CS (1977) Glutamate dehydrogenase: a reliable marker of liver cell necrosis in the alcoholic. *British Medical Journal* **2**, 1508-1510.
55. Videla LA, Rodrigo R, Araya J *et al.* (2004) Oxidative stress and depletion of hepatic long-chain polyunsaturated fatty acids may contribute to nonalcoholic fatty liver disease. *Free Radical Biology and Medicine* **37**, 1499-1507.
56. Nanji AA (1983) Serum creatine kinase isoenzymes: a review. *Muscle Nerve* **6**, 83-90.
57. Hjelte L, Larsson M, Alvestrand A *et al.* (1990) Renal function in rats with essential fatty acid deficiency. *Clinical Science* **79**, 299-305.
58. Castell JD, Sinnhuber RO, Lee DJ *et al.* (1972) Essential fatty acids in the diet of rainbow trout (*Salmo gairdneri*): physiological symptoms of EFA deficiency. *J Nutr* **102**, 87-92.
59. Morton KM, Blyth D, Bourne N *et al.* (2014) Effect of ration level and dietary docosahexaenoic acid content on the requirements for long-chain polyunsaturated fatty acids by juvenile barramundi (*Lates calcarifer*). *Aquaculture* **433**, 164-172.
60. Araujo B, Wade NM, Salini MJ *et al.* (2015) Influence of variation in dietary fatty acid and fasting on the hepatic lipid composition and gene expression by barramundi (*Lates calcarifer*). *Fish Physiol Biochem* **Submitted**.
61. Østbye T-K, Kjær MA, Røra AMB *et al.* (2009) High n-3 HUFA levels in the diet of Atlantic salmon affect muscle and mitochondrial membrane lipids and their susceptibility to oxidative stress. *Aquac Nutr* **17**, 177-190.
62. Menoyo D, Diez A, Lopez-Bote CJ *et al.* (2006) Dietary fat type affects lipid metabolism in Atlantic salmon (*Salmo salar* L.) and differentially regulates glucose transporter GLUT4 expression in muscle. *Aquaculture* **261**, 294-304.
63. Alhazzaa R, Bridle AR, Nichols PD *et al.* (2011) Up-regulated desaturase and elongase gene expression promoted accumulation of polyunsaturated fatty acid (PUFA) but not long-chain PUFA in *Lates calcarifer*, a tropical euryhaline fish, fed a stearidonic acid- and γ-linoleic acid-enriched diet. *J Agric Food Chem* **59**, 8423-8434.

64. González-Rovira A, Mourente G, Zheng X *et al.* (2009) Molecular and functional characterization and expression analysis of a Δ6 fatty acyl desaturase cDNA of European Sea Bass (*Dicentrarchus labrax* L.). *Aquaculture* **298**, 90-100.
65. Tocher D, Zheng X, Schlechtriem C *et al.* (2006) Highly unsaturated fatty acid synthesis in marine fish: Cloning, functional characterization, and nutritional regulation of fatty acyl Δ6 desaturase of Atlantic cod (*Gadus morhua* L.). *Lipids* **41**, 1003-1016.
66. Betancor MB, Howarth FJE, Glencross BD *et al.* (2014) Influence of dietary docosahexaenoic acid in combination with other long-chain polyunsaturated fatty acids on expression of biosynthesis genes and phospholipid fatty acid compositions in tissues of post-smolt Atlantic salmon (*Salmo salar*). *Comp Biochem Physiol Part B Biochem Mol Biol* **172–173**, 74-89.

Figure legends

Figure 1 (a-d) Mass-balance computations of fatty acid retention and β-oxidation in juvenile barramundi, mean (SEM) n=3. Saturated fatty acid (a; SFA) retention F=82.5***, β-oxidation F=5.2*; Monounsaturated fatty acid (b; MUFA) retention F=44.5***, β-oxidation F=7.0*; Polyunsaturated fatty acid (c; PUFA) retention F=10.8*, β-oxidation F=1.4, P=0.32; Long-chain polyunsaturated fatty acid (d; LC-PUFA) retention F=6.1*, β-oxidation F=241.5***. Significant differences are indicated by a star (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$), one – way ANOVA *df 2,6*., *post-hoc* Tukey's HSD.

Figure 2 (a-h) Plasma chemistry parameters of juvenile barramundi. Different symbols represent ○ FO CTRL ● FO LOW Δ FO FREE treatment diets, mean (SEM) n=3. Significant differences at each time point are indicated by a star (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$), one–way ANOVA *df 2,6*.

Figure 3 (a-f) Expression of lipid metabolism genes in the liver of juvenile barramundi. All data are normalised to EF1α and Luc reference genes, log 2 transformed and expressed relative to the initial fish (WK 0), mean (SEM) n=3. The FO CTRL (control) groups are indicated by light bars and the FO FREE groups are indicated by dark bars. Significant differences are indicated by a star (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$) above a sampling point, two-tailed t-test *df* 10. *Lc* ACYL, *Lates calcarifer* ATP citrate lyase; *Lc* CPT1a, *L. calcarifer c*arnitine palmitoyltransferase; *Lc* FAS, *L. calcarifer* fatty acid synthase, *Lc* SCD, *L. calcarifer* steroyl CoA desaturase, *Lc* ELOVL5, *L. calcarifer* fatty acid elongation 5; *Lc* FADS2, *L. calcarifer* fatty acid desaturase 6.

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

	Fish meal[#]	Poultry meal	Soy isolate	Wheat gluten	Wheat flour	Casein	Wheat starch	Fish oil	Olive oil	Palm oil	Palm flake
Composition											
Dry matter	984	958	958	927	839	924	836	992	987	999	994
Protein	789	641	895	823	112	870	5	4	4	3	4
Ash	163	138	46	1	6	11	3	1	ND	ND	ND
Lipid	46	151	57	55	22	5	1	956	973	963	986
Carbohydrate	2	70	2	121	860	113	992	39	23	34	10
Gross energy (MJ/kg)	18.9	20.4	21.8	21.2	15.3	21.9	14.5	39.3	39.5	39.5	39.3
Fatty acids (%) ^											
Total FA (mg/g lipid)	625.0	638.8	-	-	-	-	-	596.4	879.5	867.8	749.8
22:6n-3	19.9	ND	-	-	-	-	-	14.2	ND	ND	ND
22:5n-3	2.2	ND	-	-	-	-	-	2.1	ND	ND	ND
20:5n-3	9.0	0.5	-	-	-	-	-	11.3	ND	ND	ND
20:4n-6	2.5	0.6	-	-	-	-	-	1.5	ND	ND	ND
18:3n-3	0.8	1.0	-	-	-	-	-	1.0	1.0	ND	ND
18:2n-6	1.7	11.0	-	-	-	-	-	2.0	11.0	6.7	ND
18:1	15.5	43.8	-	-	-	-	-	18.6	73.8	34.6	0.4
18:0	8.7	8.6	-	-	-	-	-	5.1	3.0	4.6	51.3
16:0	25.8	25.4	-	-	-	-	-	22.9	9.9	51.9	46.2
SFA	39.8	36.1	-	-	-	-	-	36.4	13.4	58.6	99.6
MUFA	22.2	50.8	-	-	-	-	-	29.1	74.6	34.7	0.4
PUFA	3.4	12.0	-	-	-	-	-	4.9	12.0	6.7	ND
LC-PUFA	34.5	1.2	-	-	-	-	-	29.7	ND	ND	ND
Total n-3	32.7	1.6	-	-	-	-	-	30.5	1.0	ND	ND
Total n-6	5.2	11.6	-	-	-	-	-	4.1	11.0	6.7	ND

Any values <0.01 are reported as 0.1; NA, Not analysed; ND, Not detected

Fish meal was defatted using hexane.

^ All fatty acids are presented as a percentage of the total fatty acids. Quantitative data can be obtained by multiplying the total FA (mg/g lipid) by specific fatty acids (%). 18:1, sum of 18:1n-7, 18:1n-9 cis, 18:1n-9 trans; saturated fatty acids (SFA), sum of 12:0, 14:0, 16:0, 18:0, 20:, 22:0, 24:0; monounsaturated fatty acids (MUFA), sum of14:1n-5, 16:1n-7, 18:1n-7, 18:1n-9 (cis and trans), 20:1n-7, 20:1n-9, 22:1n-9, 24:1n-9; polyunsaturated fatty acids (PUFA), sum 18:2n-6 (cis and trans), 18:3n-6, 18:3n-3, 18:4n-3; long chain polyunsaturated fatty acids (LC-PUFA), sum 20:2n-6, 20:3n-6, 20:4n-6, 22:4n-6, 2-:3n-3, 20:5n-3, 22:5n-3, 22:6n-3; n-3, sum of omega 3 PUFA and LC-PUFA; n-6, sum of omega 6 PUFA and LC-PUFA.

Table 2 Formulation and composition of experimental diets, all values are g/kg unless otherwise stated.

	FO CTRL	FO LOW	FO FREE
Formulation			
Defatted fish meal [a]	150	150	150
Poultry meal [a]	150	150	150
Soy protein isolate [b]	150	150	150
Wheat gluten [b]	150	150	150
Wheat flour [b]	109	109	109
Casein [c]	100	100	100
Pregelled wheat starch	80	80	80
DL-Methionine	10	10	10
Di-calcium phosphate	10	10	10
Pre-mix vitamins [d]	8	8	8
Yttrium oxide [e]	1	1	1
Fish oil [a]	82	10	0
Olive oil [f]	0	36	41
Palm Oil [f]	0	18	20
Palm Flake [f]	0	18	20
Composition			
Dry matter	940	939	945
Protein	598	588	603
Ash	64	64	66
Lipid	126	123	122
Carbohydrate	204	219	202
Gross energy (MJ/kg)	21.5	21.3	21.6
Fatty acids (% total) ^			
Total FA (mg/g lipid)	681.8	708.4	717.4
22:6n-3	10.6	2.0	0.9
22:5n-3	1.6	0.1	0.1
20:5n-3	8.1	1.3	0.5
20:4n-6	1.2	0.3	0.1
18:3n-3	1.3	1.1	1.0
18:2n-6	10.0	13.0	13.2
18:1	22.7	38.8	41.1
18:0	5.5	11.9	12.6
16:0	22.9	27.0	27.6
SFA	34.5	41.2	41.8
MUFA	31.3	41.1	42.5
PUFA	12.7	14.1	14.3
LC-PUFA	21.5	3.7	1.4
Total n-3	22.9	4.4	2.4
Total n-6	11.3	13.4	13.2

^ Please refer to Table 1 for details.

a Ridley aquafeeds, Narangba, QLD, Australia. Fish meal defatted with hexane (see methods)

b Manildra Group, Rocklea, QLD, Australia

c Bulk Powders, www.bulkpowders.com.au

d Vitamin and mineral premix (g/kg of premix): vitamin A, 0.75 mg; vitamin D3, 6.3 mg; vitamin E, 16.7 g; vitamin K3, 1.7 g; vitamin B1, 2.5 g; vitamin B2, 4.2 g; vitamin B3, 25 g; vitamin B5, 8.3 g; vitamin B6, 2.0 g; vitamin B9, 0.8 g; vitamin B12, 0.005 g; biotin, 0.17 g; vitamin C, 75 g; choline, 166.7 g; inositol, 58.3 g; ethoxyquin, 20.8 g; copper, 2.5 g; ferrous iron, 10.0 g; magnesium, 16.6 g; manganese, 15.0 g; zinc, 25.0 g

e Yttrium oxide; Stanford Materials, Aliso Viejo, California, United States

f Sydney Essential Oil Co. (Sydney, NSW, Australia)

Any values <0.01 are reported as 0.1; NA, Not analysed; ND, Not detected

Table 3 Real-time qPCR primer pairs for target genes involved in fatty acid metabolism.

Target gene	Abbreviation	EC number	Primer name	Sequence	Length
Fatty acid metabolism					
Fatty acid synthase	*Lc FAS*	EC 2.3.1.85	FAS qPCR.For 1	TGAATCTCACCACGCTTCAG	20
			FAS qPCR.Rev 1	AGGCAGCAATAGAACCCTCA	20
Steroyl CoA desaturase	*Lc SCD*	EC 1.14.19.1	SCD qPCR.For 1	CCTGGTACTTCTGGGGTGAA	20
			SCD qPCR.Rev 1	AAGGGGAATGTGTGGTGGTA	20
Carnitine palmitoyltransferase	*Lc CPT1a*	EC 2.3.1.21	CPT1A qPCR.For 1	TGATGGTTATGGGGTGTCCT	20
			CPT1A qPCR.Rev 1	CGGCTCTCTTCAACTTTGCT	20
ATP citrate lyase	*Lc ACYL*	EC 2.3.3.8	Lcal ACYL F1	CAACACCATTGTCTGTGCTC	20
			Lcal ACYL R1	GAAATGCTGCTTAACAAAGTCC	21
Fatty acid elongation 5	*Lc ELOVL5*	EC 2.3.1.n8	Lcal ELOVL5 F1	ATCCAGTTCTTCTTAACCGT	20
			Lcal ELOVL5 R1	GGTTTCTCAAATGTCAATCCAC	22
Fatty acid desaturase 6	*Lc FADS2*	EC 1.14.19.-	Lcal FADS2 F1	TCATACTACCTTCGCTACTTCTC	23
			Lcal FADS2 R1	ACAAACCAGTGACTCTCCAG	20
Reference					
Luciferase	*Luc*	Na	Luc qPCR F	GGTGTTGGGCGCGTTATTTA	20
			Luc qPCR R	CGGTAGGCTGCGAAATGC	18
Elongation factor 1 alpha	*EF1α*	Na	Lcal EF1α F	AAATTGGCGGTATTGGAAC	19
			Lcal EF1α R	GGGAGCAAAGGTGACGAC	18

Table 4 Growth performance and feed utilisation of barramundi fed experimental diets for eight weeks.

	FO CTRL	FO LOW	FO FREE	P SEM	ANOVA
Live-weight initial (g)	46.9	46.9	46.8	0.07	F=0.1, P=0.89
Live-weight WK2 (g)	92.7^{b}	85.8^{a}	87.4^{a}	1.18	F=10.8*
Live-weight WK4 (g)	139.2^{b}	130.6^{a}	125.9^{a}	2.07	F=23.7**
Live-weight WK6 (g)	185.0^{c}	173.9^{b}	162.9^{a}	3.40	F=22.3**
Live-weight WK8 (g)	238.3^{c}	218.9^{b}	202.8^{a}	5.26	F=44.6***
Gain (g/fish)	191.4^{c}	172.1^{b}	156.0^{a}	5.24	F=48.7***
Feed intake (g/fish)	209.6	196.3	207.9	3.91	F=1.2, P=0.36
FCR	1.10^{a}	1.14^{ab}	1.33^{b}	0.04	F=11.7 **
Protein retention (%)	34.1^{a}	30.3^{b}	27.8^{b}	1.01	F=13.7 **
Lipid retention (%)	74.8^{a}	61.7^{a}	44.8^{b}	4.61	F=24.2 **
Energy retention (%)	37.8^{a}	34.5^{a}	29.8^{b}	1.30	F=11.4 **
Survival (%)	98	100	100	0.01	NA
Abnormalities (%)	4.0^{a}	2.0^{a}	41.0^{b}	0.08	F=6.8*
Behaviour index	1.9	1.7	1.8	2.8	F=4.0, P=0.08

P SEM, Pooled standard error mean, FCR, Feed conversion ratio

P<0.001*** P<0.01** P<0.05*

One-way ANOVA *df 2,6*, post-hoc Tukey's HSD

Percentage data were arcsine transformed prior to analysis

Any values <0.01 are reported as 0.1; NA, Not analysed; ND, Not detected

Table 5 Apparent digestibility of macro nutrients and fatty acids present in the experimental diets.

	FO CTRL	FO LOW	FO FREE	P SEM	ANOVA
Dry matter (%)	64.3	70.8	65.4	1.5	F=4.3, P=0.10
Protein (%)	91.6	93.0	94.1	0.4	F=4.4, P=0.08
Total lipid (%)	90.7[a]	87.1[ab]	84.6[b]	1.5	F = 8.1 *
Energy (%)	87.3	85.7	88.7	0.7	F=2.4, P=0.19
22:6n-3 (%)	98.5	100.0	100.0	0.6	NA
22:5n-3 (%)	100.0	100.0	100.0	0.0	NA
20:5n-3 (%)	99.0	100.0	100.0	0.2	NA
20:4n-6 (%)	100.0	100.0	100.0	0.0	NA
18:3n-3 (%)	98.6	100.0	100.0	0.6	NA
18:2n-6 (%)	95.8	96.0	95.6	0.6	F=0.2, P=0.84
18:1 (%)	92.1	93.9	92.9	0.6	F=1.6, P=0.30
18:0 (%)	76.7[a]	70.9[ab]	65.6[b]	2.6	F=9.4*
16:0 (%)	81.5	77.0	73.7	2.1	F=4.7, P=0.9
SFA (%)	82.3[a]	76.0[ab]	71.8[b]	2.4	F = 8.0 *
MUFA (%)	92.6	94.0	92.8	0.5	F=1.1, P=0.41
PUFA (%)	96.5	96.3	96.0	0.6	F=0.5, P=0.66
LC-PUFA (%)	98.9	100.0	100.0	0.4	NA
Total n-3 (%)	98.9	100.0	100.0	0.4	NA
Total n-6 (%)	96.3	96.1	95.6	0.5	F=0.5, P=0.64

P SEM, Pooled standard error mean
P<0.001*** P<0.01** P<0.05*
One-way ANOVA *df 2,6*, post-hoc Tukey's HSD
Percentage data were arcsine transformed prior to analysis
Any values <0.01 are reported as 0.1; NA, Not analysed; ND, Not detected

Table 6 Initial and final fatty acid composition of whole-body and liver tissue from juvenile barramundi. All data are presented as percentage of total fatty acids (%) unless otherwise stated.

	Whole-body						Liver					
	Initial	FO CTRL	FO LOW	FO FREE	P SEM	ANOVA	Initial	FO CTRL	FO LOW	FO FREE	P SEM	ANOVA
Total lipid (wet weight %)	5.1	9.5	9.4	8.7	0.2	F=2.9, P=0.14	NA	NA	NA	NA	NA	NA
Total FA (mg/g Lipid) ^	681.0	616.6^{a}	777.2^{b}	729.6^{ab}	25.8	F=17.4 **	NA	NA	NA	NA	NA	NA
22:6n-3	2.1	5.5^{a}	2.0^{b}	1.3^{c}	0.7	F=39.2 ***	4.4	4.8^{a}	2.2^{b}	0.9^{c}	0.6	F=202.2 ***
22:5n-3	0.8	1.6^{a}	0.7^{b}	0.1^{c}	0.2	F=88.9 ***	1.1	1.4^{a}	0.5^{b}	0.3^{c}	0.2	F=167.2 ***
20:5n-3	1.4	3.7^{a}	1.0^{b}	0.6^{b}	0.5	F=70.4 ***	1.7	2.7^{a}	0.7^{b}	0.2^{c}	0.4	F=440.8 ***
20:4n-6	0.4	0.7^{a}	0.1^{b}	0.1^{b}	0.1	F=34.5 ***	0.7	0.8^{a}	0.5^{b}	0.2^{c}	0.1	F=94.9 ***
18:3n-3	1.0	1.0	0.9	0.9	0.1	F=0.9, P=0.44	0.6	0.5	0.4	0.5	0.1	F=1.25, P=0.35
18:2n-6	9.7	8.8^{a}	11.2^{b}	11.4^{b}	0.4	F=136.9 ***	6.4	4.8	6.0	6.8	0.4	F=3.0, P=0.12
18:1	37.9	30.1^{a}	41.4^{b}	43.9^{c}	2.1	F=475.1 ***	34.8	31.1^{a}	37.9^{b}	40.7^{c}	1.4	F=116.1 ***
18:0	7.8	7.4^{a}	9.2^{b}	8.9^{b}	0.3	F=94.3 ***	11.5	10.6^{a}	12.9^{b}	11.8^{ab}	0.4	F=14.1 **
16:0	26.1	26.9	25.8	25.7	0.2	F=3.9, P=0.08	27.8	30.8	31.3	31.0	0.4	F=0.2, P=0.85
SFA	38.5	39.3^{a}	37.3^{b}	36.5^{b}	0.5	F=10.8 *	42.9	45.5	46.9	45.1	0.6	F=0.7, P=0.53
MUFA	45.7	37.7^{a}	45.0^{b}	47.3^{c}	1.5	F=153.3 ***	40.0	37.9^{a}	41.3^{b}	44.1^{c}	0.9	F=47.6 ***
PUFA	11.0	11.7^{a}	14.0^{b}	14.3^{b}	0.4	F=60.2 ***	8.5	6.2	7.1	8.4	0.5	F=2.6, 0.15
LC-PUFA	4.7	11.4^{a}	3.8^{b}	2.0^{b}	1.5	F=57.2 ***	8.6	10.4^{a}	4.7^{b}	2.4^{c}	1.2	F=349.0 ***
Total n-3	4.3	11.9^{a}	4.4^{b}	2.7^{b}	1.5	F=54.0 ***	8.6	9.7^{a}	4.1^{b}	2.6^{c}	1.2	F=361.0 ***
Total n-6	11.4	11.2^{a}	13.4^{b}	13.5^{b}	0.5	F=92.0 ***	8.5	6.8	7.7	8.2	0.4	F=2.3, P=0.19

^ Please refer to Table 1 for details.

P SEM, Pooled standard error mean

P<0.001*** P<0.01** P<0.05*

One-way ANOVA *df 2,6*, post-hoc Tukey's HSD

Percentage data were arcsine transformed prior to analysis

Any values <0.01 are reported as 0.1; NA, Not analysed; ND, Not detected

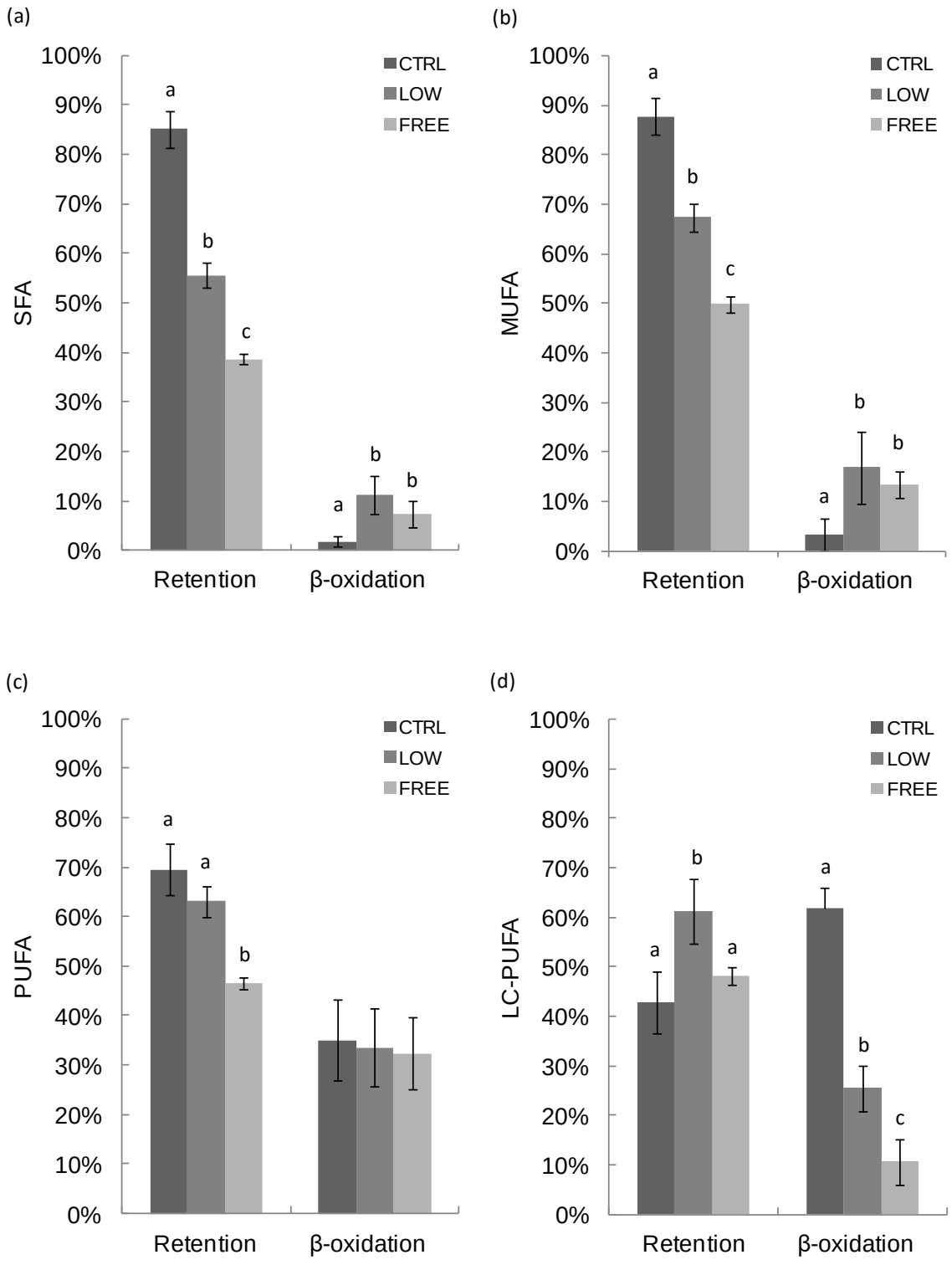
(a)
SFA
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
CTRL
LOW
FREE
a
b
c
a
b
b
Retention
β-oxidation
(b)
MUFA
a
b
c
a
b
b
(c)
PUFA
a
a
b
(d)
LC-PUFA
a
b
a
a
b
c

Figure 1 (a-d)

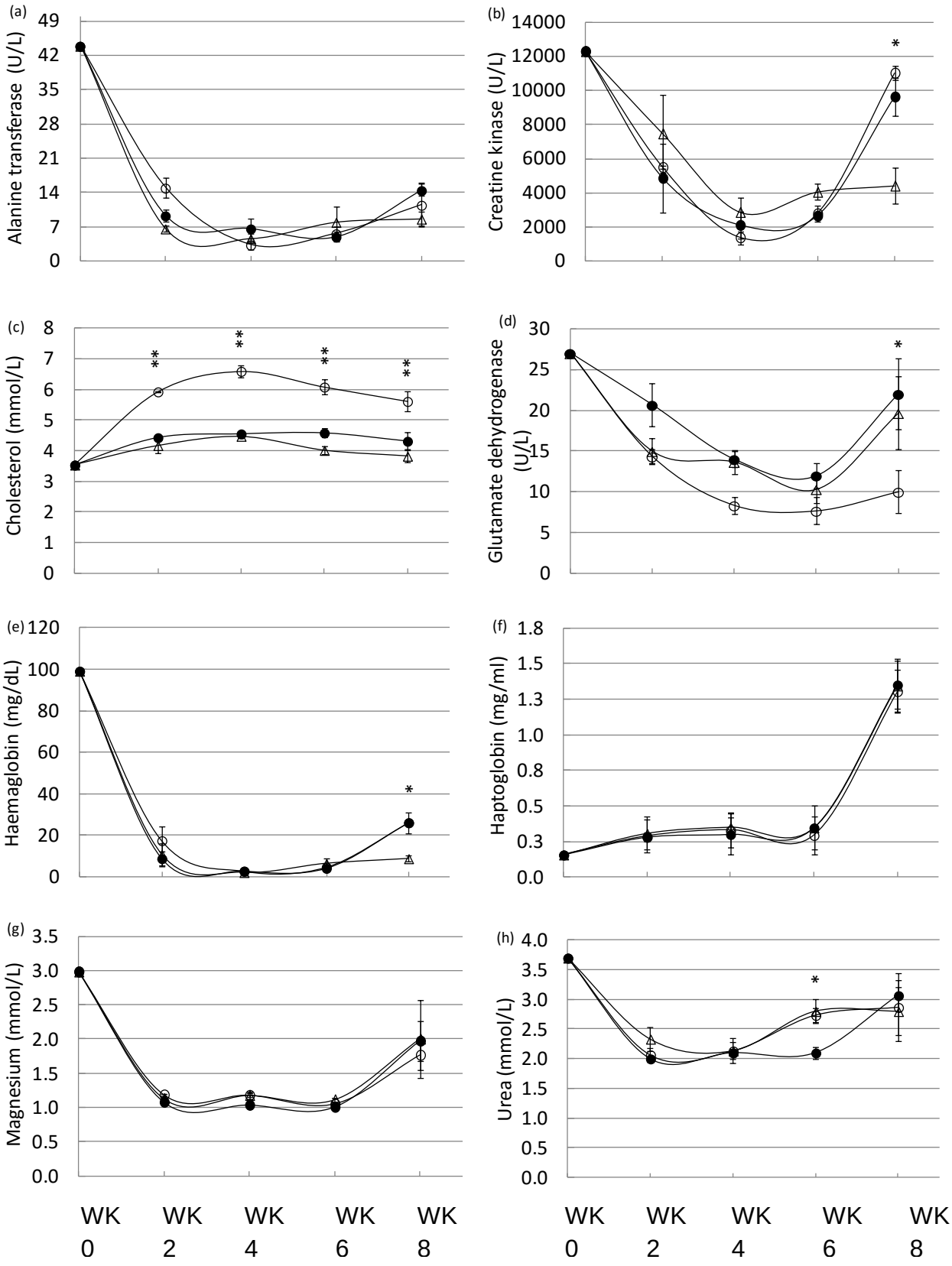
(a)
Alanine transferase (U/L)
49
42
35
28
21
14
7
0
(b)
Creatine kinase (U/L)
14000
12000
10000
8000
6000
4000
2000
0
*
(c)
Cholesterol (mmol/L)
8
7
6
5
4
3
2
1
0
**
**
**
**
(d)
Glutamate dehydrogenase (U/L)
30
25
20
15
10
5
0
*
(e)
Haemaglobin (mg/dL)
120
100
80
60
40
20
0
*
(f)
Haptoglobin (mg/ml)
1.8
1.5
1.3
1.0
0.8
0.5
0.3
0.0
(g)
Magnesium (mmol/L)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
(h)
Urea (mmol/L)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
WK 0
WK 2
WK 4
WK 6
WK 8
WK 0
WK 2
WK 4
WK 6
WK 8

Figure 2 (a-h)

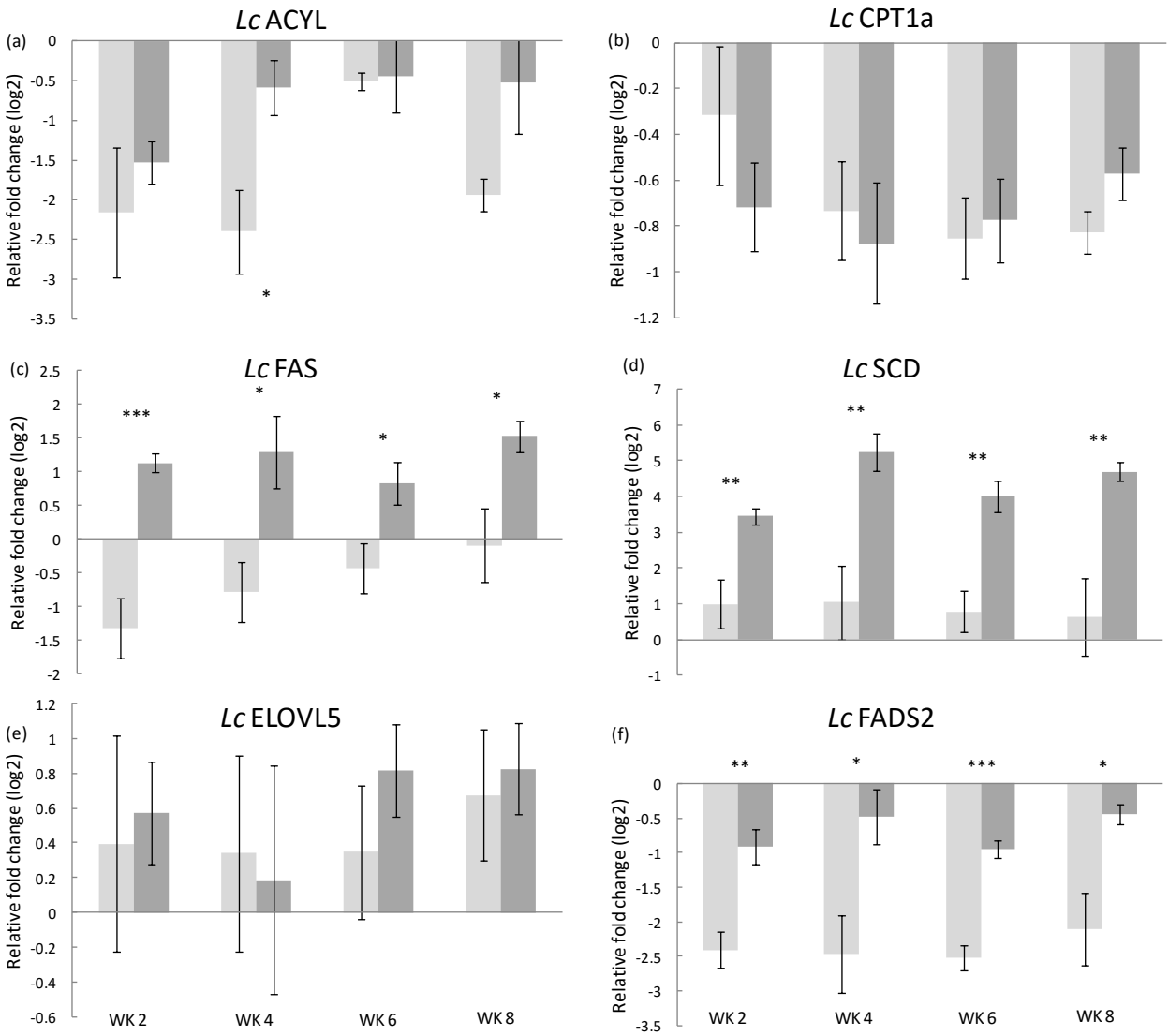
(a)
Lc ACYL
Relative fold change (log2)
0
-0.5
-1
-1.5
-2
-2.5
-3
-3.5
*
(b)
Lc CPT1a
Relative fold change (log2)
0
-0.2
-0.4
-0.6
-0.8
-1
-1.2
(c)
Lc FAS
Relative fold change (log2)
2.5
2
1.5
1
0.5
0
-0.5
-1
-1.5
-2
***
*
*
*
(d)
Lc SCD
Relative fold change (log2)
7
6
5
4
3
2
1
0
-1
**
**
**
**
(e)
Lc ELOVL5
Relative fold change (log2)
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
WK 2
WK 4
WK 6
WK 8
(f)
Lc FADS2
Relative fold change (log2)
0
-0.5
-1
-1.5
-2
-2.5
-3
-3.5
**
*
***
*
WK 2
WK 4
WK 6
WK 8

Figure 3 (a-f)