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Title: Effect of ration level and dietary eicosapentaenoic acid content on the requirements for docosahexaenoic acid by juvenile barramundi (*Lates calcarifer*).

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Running Title: Effect of ration and EPA on DHA requirements of barramundi

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Abstract

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Juvenile barramundi were fed one of six diets containing differing docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) levels. Fish were fed on a pair-fed feeding regime to eliminate
30 variability in feed intake, with two diets fed to satiety to examine the effects of fixed or variable feed rations on EFA requirements. Weight gain, feed intake, feed utilisation, and physical clinical signs were monitored. No effect of dietary DHA and EPA concentration, DHA:EPA ratio or total LC-
33 PUFA level was observed on weight gain, growth rate, feed conversion ratio (FCR), survival or physical clinical health signs. Satiety fed fish had higher feed intake, final weight, weight gain and growth rate compared to their respective restrictively fed treatments ($P<0.05$). No effect of ration
36 level on the responses to DHA concentration was observed. Body fatty acid composition was affected by diet, increasing dietary DHA resulted in higher body tissue DHA concentration, and a similar relationship was observed for EPA. Plasma haemoglobin increased with increasing DHA+EPA levels
39 ($P<0.05$) while glutamate dehydrogenase increased for fish fed DHA+EPA in a 1:1 ratio, regardless of total dietary LC-PUFA ($P<0.05$). Juvenile barramundi may be fed diets containing as low as 1 g kg^{-1} DHA without compromising growth or health status.

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1.1 Introduction

Traditionally, commercial aquaculture diets have relied on marine fishery oils for their lipid component as they were readily available, competitive in price and contained the essential fatty acids required by fish (reviewed by Turchini et al., 2009). Increasing aquaculture production continues to place demands on static global wild fish resources, making their continued use unsustainable (Tacon & Metian, 2008). To meet global aquaculture demands, a number of alternative lipid resources have been trialled including vegetable oils (such as palm oil, soybean oil, canola oil and rapeseed oil) and animal fats (such as lard, poultry oil and tallow; Turchini et al., 2009). However, all of these alternative lipid sources are non-marine and lack long-chain polyunsatured fatty acids (LC-PUFA).

Some freshwater species have little or no requirement for dietary LC-PUFA. However, the majority of aquatic marine species have a defined dietary requirement for LC-PUFA (reviewed by Glencross, 2009). This probably results from an evolutionary adaptation to naturally LC-PUFA rich diets, resulting in many marine species losing their ability to convert C18 n-6 and n-3 PUFA into the corresponding C20 and C22 n-3 and n-6 HUFA *in vivo* by alternating cycles of desaturation and elongation (Tocher, 2010). Marine and catadromous species, including barramundi, have been demonstrated to have an absolute dietary requirement for the longer, and more unsaturated 20:5n-3 (EPA) and 22:6n-3 (DHA; Buranapanidgit et al., 1988; Williams et al., 2006; Glencross and Rutherford, 2011). A total n-3 LC-PUFA inclusion level of between 10 - 17 g kg^{-1} (as a combination of 20:5 n-3 and 22:6 n-3) was sufficient to support growth of barramundi (Buranapanidgit et al., 1988, 1989 cited by Boonyaratpalin, 1997). Later work by Williams et al. (2006) found little effect of dietary LC-PUFA level on growth but observed that some fish exhibited a ‘fainting’ response, a reported sign of EFA deficiency in other fish when fed an LC-PUFA free diet containing 2 % lipid (made up of varying amounts of lauric, linoleic and linolenic acids; Castell et al., 1972). In a subsequent study, Glencross and Rutherford (2011) hypothesised a quantitative requirement of around 10 g kg^{-1} DHA for barramundi. The authors did not observe an effect of DHA inclusion levels on growth parameters, even at inclusion levels as low as 1 g kg^{-1}. Although in that study all diets were

pair-fed restrictively and it is unclear as to what effect that this feed ration regime had on the
72 outcomes of the study. However in that study, a subcutaneous haemorrhaging, observed as a reddening of the skin certain parts of in fish, occurred as dietary DHA levels increased from 1 to 18 g kg^{-1}, which was ameliorated by the inclusion of 10 g kg^{-1} EPA. These results highlighted the
75 importance not only of the absolute dietary inclusion level, but also the influence of other EFAs in the diet. The authors concluded, based on their study and that of Williams et al. (2006) that the total LC-PUFA inclusion level for barramundi could be reduced to at least 12 g kg^{-1} provided the DHA and
78 EPA are held in balance (i.e. a 1:1 ratio). However, it was suggested that further research was required to better define the EPA requirements, and the optimal ratio of DHA:EPA in the diets of barramundi.

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To address some of these questions an experiment was conducted to examine the effects of different DHA and EPA inclusion levels, total LC-PUFA inclusion levels and the effects of fixed or satietal
84 rations, to further refine the understanding of the requirements of LC-PUFA by barramundi.

1.2 Materials and Methods

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1.2.1 Experiment Overview

This study consisted of six dietary treatments of a range of inclusion levels of DHA both with and
90 without EPA (Table 1). Each of these diets was restrictively pair-fed to eliminate effects of feed intake variation as a secondary variable. Two additional treatments consisted of those diets containing 1 g kg^{-1} DHA + 1 g kg^{-1} EPA and 10 g kg^{-1} DHA + 1 g kg^{-1} EPA, which were each fed to apparent
93 satiety. These additional treatments were included to measure the effect of varying feed intake on the results achieved relative to the pair-fed strategy. All the procedures described herein were approved by CSIRO's Animal Ethics Committee (A3/2012) and performed in accordance with the Australian
96 Code of Practice for Care and Use of Animals for Scientific Purposes.

1.2.2 Animal management

Juvenile barramundi (*Lates calcarifer*) were sourced from Betta Barra (Cairns, QLD, Australia) and on-grown in a 10,000 L tank and fed a commercial diet (Marine Float, Ridley Aquafeed, Narangba, Qld, Australia). At the commencement of the experiment, fish (n=480; 16.5 ± 0.1 g, mean ± S.D.) were anesthetised with AQUI-S^{TM} (0.02 mL L^{-1}; AQUI-S New Zealand, Lower Hutt, New Zealand), weighed on an electronic top-loading balance to 0.1 g accuracy and allocated to one of 24 aquaria (300 L; n=20 fish per aquaria) housed at the Aquaculture Feed Technology Laboratory at the Bribie Island Research Centre. Aquaria were supplied with flow-through seawater (salinity = 35 PSU; dissolved oxygen 7.0 ± 0.01 mg L^{-1}) at a rate of 4 L min^{-1}, maintained at 28.9 ± 0.6ºC (mean ± S.D.) for the duration of the experiment (28 days). Treatments were randomly allocated to 24 tanks, with each treatment having three replicates.

1.2.3 Diet preparation and management

A series of six diets, three with different DHA and three with different DHA+EPA inclusion levels were created by blending a range of ingredients including defatted fishmeal, an algal derived DHA source, fish oil, olive oil and butter fat to provide the lipid (Table 1).

Diets were formulated to provide approximately 540 g kg^{-1} protein and 100 g kg^{-1} of lipid at a gross energy level of 20.0 MJ kg^{-1} (estimated digestible protein and energy of 500 g kg^{-1} and 17.0 MJ kg^{-1} respectively; Table 2). Diets were manufactured by making a single 40 kg batch of extruded pellets before an oil coating was applied under vacuum to each treatment. Dry ingredients were mixed using an upright planetary mixer (Robo Coupe, Vincennes Cedex, France).

Following dry mixing, the base diet was extruded using an APV MFP24 laboratory scale twin-screw extruder (APV-Baker, Peterborough, UK) using the operational configuration as described in Glencross et al. (2012). The pellets were produced by delivering the dry mash into the barrel at a feed rate of around 20 kg h^{-1}. Barrel temperatures were set for each of the four zones from drive to die at 50, 80, 100 and 110°C respectively. Water was peristaltically pumped (Baoding Longer Precision Pump Co., Ltd, Hebei, China) into the barrel at 82 mL min^{-1}. Feeds were extruded through a 4mm Ø

die with the machine running at *c.* 470 rpm. Pellets were cut into 6-7 mm lengths using two-bladed variable-speed cutter and collected on large aluminium oven trays (650 x 450 x 25 mm, length x
129 width x depth). Approximately 40 kg of a single basal formulation of the resulting pellets were collected and dried at 65°C prior to coating with oil.

132 Specific lipid allocations, consisting of blends of butter, olive oil, DHA oil and fish oil (Table 1), were vacuum-infused into the pellets after warming both the oils and pellets in an oven at 60°C for 1 h. Warmed pellets were then placed in a Hobart mixer and the prescribed oil blend for each treatment
135 was poured over the pellets whilst mixing. After mixing, the pellets were then exposed to a vacuum through the application of a perspex lid with a rubber seal to the mixing bowl and a vacuum pump connected to a valve in the lid. After all pellets were vacuum-infused with their respective oil blend,
138 the pellets were stored at -20°C until required for feeding.

Fish were fed once daily between 0900 and 1030 each day. The fish were fed diets in a restricted pair-
141 fed regime to minimise feed intake variability among treatments, thereby ensuring that any effects seen on the fish were due solely due to diet composition and not possible confounding intake variability effects. Any uneaten feed was accounted for to accurately determine feed intake by each
144 tank of fish (Helland et al., 1996). A correction factor was applied to recovered uneaten pellets to account for soluble losses incurred on the pellet between feeding and collection to make a more accurate feed intake assessment. The initial restrictive rations were defined based on the feed demand
147 as estimated for a 17.0 MJ kg^{-1} diet and fish of 16 g at 30ºC using a published bioenergetic model for this species (Glencross, 2008).

150 1.2.4 Sample preparation and analysis

At the beginning of the study, five fish, representative of the initial population, were euthanized by an overdose of AQUI-S^{TM} and frozen at -20°C until subsequent composition analysis. At the conclusion
153 of the experiment, after the final weighing, three fish from each tank were euthanized as described above before being stored at -20°C until subsequent composition analysis. An additional three fish per

tank were also euthanized. Blood samples were collected from the caudal vein using a 3 mL syringe and 18G needle pre-loaded with 0.5 M EDTA. Whole blood was then split into two aliquots, transferred into a 1.5 mL Eppendorf™ tube and stored at 4°C (prior to haematological analysis), or transferred into a 1.5 mL Eppendorf™ tube and centrifuged (1000 x g; 3 mins). After centrifugation, the plasma was transferred to a new Eppendorf™ tube prior to being snap-frozen at -80°C.

1.2.5 Chemical and compositional analysis

All chemical and compositional analyses were performed in-house. Diet and whole-fish samples were analysed for dry matter, ash, nitrogen, total lipids, fatty acids and gross energy content. Dry matter was calculated by gravimetric analysis following oven drying at 105ºC for 24 h. Protein levels were calculated from the determination of total nitrogen by CHNOS auto-analyser, based on N x 6.25. Total lipid content of the diets was determined gravimetrically following extraction of the lipids using chloroform: methanol (2:1) described by Folch et al. (1957). Gross ash content was determined gravimetrically following loss of mass after combustion of a sample in a muffle furnace at 550°C for 12 h. Gross energy was determined by adiabatic bomb calorimetry. Fatty acids were analysed as methyl ester derivatives. Lipids were esterified and analysed by gas chromatography (GC) using flame ionisation detection. Peaks were identified by comparing relative retention times to standards.

Samples of whole fish combined by tank, were dried of residual surface moisture and minced together. For mincing, samples were passed twice through an industrial food processor to ensure sample homogeneity. A sub-sample of mince was taken for dry matter analysis and an additional mince sub-sample was then frozen prior to being freeze-dried. Freeze-dried mince samples were milled and then analysed for dry matter, nitrogen, ash, total lipid, fatty acids and energy as described above.

1.2.6 Haematology and plasma biochemistry

Whole blood and plasma samples were sent to an independent laboratory for analysis (West Australian Animal Health Laboratories, South Perth, Western Australia). Haematological analysis

included packed cell volume (PCV) and erythrocyte fragility. Packed cell volume was determined using standard pathology laboratory procedures and erythrocyte fragility measured by percent haemolysis when red cells were placed in a 0.6 g/L sodium chloride solution. Plasma samples subjected to the clinical chemistry panel, with assays 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 Aminotransferance (ALT; Olympus kit Cat. No. OSR6107), Glutamate Dehydrogenase (GLDH; Randox kit Cat. No. GL441), Total Protein (Olympus kit Cat. No. OSR6132), Creatinine (Olympus kit Cat. No. OSR6178), and Haem (Randox test kit Cat. No. HG1539). Trace elements were determined after mixed acid digestion using inductively coupled plasma atomic emission spectrophotometry (ICP-AES).

1.2.7 Statistical analyses

All figures are mean ± SEM unless otherwise specified. Statistical differences were determined by ANOVA and means compared using least significant differences using GenStat (Release 14, VSN International, Hemel Hempstead, UK) with $P<0.05$ considered significant. Additionally, the effect of ration level and diet were also analysed by two-way ANOVA using GenStat (Release 14, VSN International, Hemel Hempstead, UK) with $P<0.05$ considered significant.

The retention of specific fatty acids (DHA and EPA) was calculated using the formula described by Glencross et al. (2003). Briefly, the percentage retention of fatty acids was calculated using the average feed intake per fish in each tank and the average gain in mass of each fatty acid by fish using the formula:

$$Fatty\ Acid\ Retention\ (\%) = \left(\frac{FAf - FAi}{FAc} \right) \times 100$$

Where *FAf* is the absolute amount of a specific fatty acid in the fish at the end of the study, *FAi* is the absolute amount of that specific fatty acid in the fish at the beginning of the study, and *FAc* is the amount of that specific fatty acid that the fish consumed over the study period.

1.3 Results

1.3.1 Fish growth and feed utilisation

Initial and final fish weights, growth rate, feed intake, feed conversation ratio and survival parameters are presented in Table 3. The poorest performing treatment increased their weight by 171% and the best performing treatment increased their weight by 245% (Table 3). Among the restricted fed groups, there was no effect of dietary DHA or EPA level on final weight, weight gain or growth rate. However, final weight, weight gain and growth rate were higher ($P<0.05$) for the two satiety fed groups, compared with the restricted fed groups, reflective of their greater ($P<0.05$) feed intake. There was no interaction between feeding regime (restricted or satiety) and dietary DHA levels ($P>0.05$). Feed conversion and survival were all unaffected ($P>0.05$) by dietary DHA and EPA levels and feeding regime (restricted or satiety).

1.3.2 Tissue composition and fatty acid retention

Dietary treatment significantly affected whole body fatty acid composition in the fish ($P<0.05$; Table 4). Compared with the initial fish, the majority of dietary treatment groups showed a reduction in whole body DHA ($P<0.05$) although fish fed diet D10E1 showed an increase in whole body DHA compared with the initial fish ($P<0.05$). For restricted fed fish, increasing dietary DHA resulted in an increase in the observed whole body DHA, with the highest levels observed in fish fed diet containing D10E1 ($P<0.05$), followed by fish fed diet D5E1 ($P<0.05$) while the fish fed diets D3E1 and D1E1 did not differ from each other in whole body DHA content ($P>0.05$). However, the whole body DHA content of these two groups was significantly lower than the D10E1 and D5E1 diet fed fish ($P<0.05$). Fish from the D10E1 satiety fed treatment contained less ($P<0.05$) tissue DHA than the D10E1 restricted fed group, although similar whole body DHA levels were observed for the restricted and satiety fed D1E1 ($P>0.05$).

A similar relationship was observed for EPA, in that most dietary treatments resulted in lower tissue EPA compared with the initial fish ($P<0.05$). The highest ($P<0.05$) tissue EPA levels were observed

237 in fish fed diets containing E10D10, which was increased compared with the initial fish ($P<0.05$). The next highest tissue EPA levels were observed in fish fed the D3E3 and D5E5 treatments that had similar tissue EPA levels ($P>0.05$). The lowest tissue EPA was observed in fish fed the D1E1 diet and
240 was similar regardless of the DHA content of the diet ($P>0.05$) and feeding regime (restricted vs satiety; $P>0.05$).

243 Total tissue SFA increased compared with the initial fish ($P<0.05$) and was similar across most treatment groups (range: 38.8 – 40.1%) except that tissue SFA was significantly increased for the D10E1 restricted and satiety fed groups as well as the D1E1 satiety fed group ($P<0.05$). Total tissue
246 MUFA was similar for the initial fish and the D1E1 and D3E3 restricted fed groups ($P>0.05$), but was increased ($P<0.05$) for the D5E1, D10E10, D5E5 and both satiety fed groups (D1E1 and D10E10).

249 Significant differences were observed in whole body PUFA between treatments ($P<0.05$), with PUFA concentration increasing with dietary DHA and EPA content for fish on the restricted feeding regime. Compared with the initial fish, both satiety fed groups (D1E1 and D10E10) showed reduced levels of
252 whole body PUFA along with most of the restricted fed fish, with the only group containing significantly more PUFA than the initial fish being the D10E10 diet fed fish. The total LC-PUFA content ranged from 9.4 – 14.6% ($P<0.05$), and increased with increasing dietary LC-PUFA content.
255 Compared with the initial fish, only the D10E1 and D10E10 dietary treatments showed an increase ($P<0.05$) in tissue LC-PUFA content, all other groups showed a significant reduction in tissue LC-PUFA content ($P<0.05$).

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Tissue n-3 levels were significantly lower for all dietary treatments compared with the initial fish ($P<0.05$). Tissue n-3 levels were increased ($P<0.05$) by increasing dietary DHA, as well as the
261 combined concentration of DHA + EPA concentration in the diet ($P<0.05$). However, feeding regime (restricted vs satiety) did not affect tissue n-3 levels ($P>0.05$). Tissue n-6 levels were similar between the initial fish and all fish fed diets with greater than 1 g kg^{-1} DHA (D1E1). However, the addition of
264 10 g kg^{-1} EPA to the 10 g kg^{-1} DHA diet (D10E10) reduced ($P<0.05$) the total tissue n-6. Compared

with the initial fish, all fish fed diets containing greater than 1 g kg^{-1} EPA had reduced tissue n-6 levels ($P<0.05$). Again, feeding regime (restricted vs satiety) did not affect tissue n-6 levels ($P>0.05$).

Retention of DHA and EPA is presented in Table 4. Retention efficiency was affected by dietary treatment for both DHA and EPA ($P<0.05$). For DHA, retention efficiency was highest (80%) for satiety fed fish receiving the lowest DHA inclusion level (1 g kg^{-1}) followed by the restricted fed fish receiving diets with 1g kg^{-1} and 5 g kg^{-1} DHA ($P<0.05$) and then the fish receiving the highest DHA inclusion level (10 g kg^{-1}; $P<0.05$). Feeding regime (restricted vs satiety) affected DHA retention, which was higher for satiety than restricted fed fish at the lower DHA inclusion level (1 g kg^{-1}) but was similar ($P>0.05$) for fish receiving diets containing 10 g kg^{-1} DHA. DHA retention was also reduced when DHA and EPA were included at a 1:1 ratio, and the retention efficiency decreased with increasing total DHA + EPA concentration ($P<0.05$).

The retention efficiency of EPA was also affected by dietary treatment. EPA retention was highest for groups containing 1 g kg^{-1} EPA, regardless of the DHA inclusion level, although it did differ between these groups ($P<0.05$). EPA retention was affected by feeding regime in a similar way to DHA; retention was increased by satiety feed at the 1 g kg^{-1} DHA + 1 g kg^{-1} EPA inclusion (D1E1) but was not different for the 10 g kg^{-1} DHA + 1 g kg^{-1} EPA inclusions levels (diet D10E1). EPA retention was also affected by DHA: EPA ratio, as well as total DHA + EPA content. When EPA is included at a 1:1 ratio with DHA, the retention efficiency of EPA is reduced ($P<0.05$).

1.3.3 Plasma chemistry

Haematology and plasma chemistry parameters are presented in Table 5. Dietary DHA and EPA inclusion levels did not affect haematological parameters such as PCV or erythrocyte fragility. However, glutamine dehydrogenase and haemoglobin levels were affected by dietary treatment. Glutamate dehydrogenase was significantly increased in fish that were fed DHA and EPA in a 1:1 ratio, regardless of the total dietary PUFA content ($P<0.05$) as well as being increased by satiety feeding compared with the restricted fed groups ($P<0.05$).

294 For fish fed diets containing 1 g kg^{-1} EPA, increasing DHA content resulted in an increase in haemoglobin ($P<0.05$). However, for fish fed DHA and EPA at a 1:1 ratio, the picture is less clear with the 10: 10 g kg^{-1} inclusion diet resulting in the highest haemoglobin levels, the D5E5 diet with
297 the lowest and the D3E3 diet with an intermediary level. Haemoglobin levels were not affected by feeding regime ($P>0.05$) and there were no significant differences in haptoglobin levels.

300 1.4 Discussion

1.4.1 Fish growth and feed utilisation

303 Weight gain, growth rate and feed conversion in the present study were not affected by dietary DHA content, DHA: EPA ratio or the total LC-PUFA content for fish fed a restricted ration. Fish fed to satiation showed an increased weight gain and growth rate, reflecting their higher feed intake rather
306 than ratio of DHA: EPA or total LC-PUFA content. Despite having different DHA inclusion levels (1 g kg^{-1} and 10 g kg^{-1}) the satiety fed groups had similar feed intake, weight gain and growth rate. These results are in agreement with previous studies that show no link between dietary DHA content and
309 growth in restricted (Glencross and Rutherford, 2011) or satiety fed fish (Williams et al., 2006).

Glencross and Rutherford (2011) found that the addition of EPA to the diet (10 g kg^{-1} EPA) resulted
312 in significantly higher growth rates than the two lowest (1 and 3 g kg^{-1}) and two highest DHA inclusion levels (15 and 18 g kg^{-1}). However, in the present study there was no difference in growth rates observed when fish were fed diets containing 10 g kg^{-1} DHA in the absence of EPA (D10E1), or
315 with a 1:1 ratio of EPA (D10E10). This lack of effect of EPA addition on growth could be a result of the shorter duration of the present study (28 days) compared with Glencross and Rutherford (2011; 42 days), reflecting a reduction in the biological (mass) turnover. However, despite the shorter duration,
318 both trials used fish of a similar initial size (present study: 16.2-16.4 g, or 17.3-17.6 g for Glencross and Rutherford, 2011) and grew them until approximately three times their initial weight. Growth rates in the present study (range: 3.6 - 3.7%) were higher than those observed (range 2.1 - 3.1%) by

Glencross and Rutherford (2011), suggesting that differences in biological turnover are not responsible for differences between the studies. Using post-smolt Atlantic salmon, Glencross et al. (2013) demonstrated that the fish fed on an EFA-free diet (1 g kg^{-1} inclusion level) maintained performance for five weeks before a dramatic and sudden decline in survival. This suggests that the effects of dietary EFA could be a function of absolute time rather than just biological turnover.

One of the more novel aspects of the present study was the examination of various DHA: EPA ratios, as well as varying total LC-PUFA content. The majority of studies examining EFA requirements have focussed on the total EFA level relative to lipid content of the diet, or the total n-3 LC-PUFA requirements (reviewed by Glencross, 2009). For barramundi, the optimal total n-3 LC-PUFA level has been suggested by Glencross and Rutherford (2011), based on the studies of Williams et al. (2006) to be 12 g kg^{-1} provided that DHA and EPA are held in balance (1:1 ratio). However, when the DHA: EPA were in balance (1: 1 ratio), the total LC-PUFA could be reduced to 4 g kg^{-1} without detrimental effects on growth, suggesting that when DHA and EPA are in balance, the LC-PUFA requirement is considerably lower than 12 g $kg^{-1.}$

When the DHA: EPA ratio was extended to 5: 1 (total LC-PUFA 7 g kg^{-1}) no effect on growth was observed, and for higher LC-PUFA levels (greater than 12 g kg^{-1}), again no effect on growth was observed, even when the ratio was 10: 1. In juvenile Gilthead seabream (Kalogeropoulos et al., 1992; Ibeas et al., 1996) and European seabass (Skalli and Robin, 2004) the best growth occurs within a narrow range of DHA: EPA ratios (1:1 or 1:2). However, the results of the present study clearly show little effect on growth of either the DHA: EPA ratio or total dietary LC-PUFA concentration, possibly reflecting species differences. For two closely related species (Atlantic salmon and Rainbow trout) considerable variation in the LC-PUFA requirements have been demonstrated; Atlantic salmon grew best with 10 g kg^{-1} LC-PUFA (Ruyter et al 2000), while Rainbow trout, grew best with 3 g kg^{-1} LC-PUFA (Watanabe and Takeuchi, 1976). However, any species influence on the optimal DHA: EPA ratio remains unknown.

Differences in dietary lipid levels in the aforementioned studies also confounds the interpretation of the absolute LC-PUFA requirements as dietary lipid level is known to influence EFA and therefore
351 LC-PUFA requirements in fish (Rainbow trout: Takeuchi and Watanabe, 1977a,b) and shrimp (Glencross et al., 2002). Nevertheless, the results of the present study highlight the possibility that the total LC-PUFA requirement and the ratio of DHA: EPA may not be as important for the growth of
354 juvenile barramundi as previously suggested in the literature. Further studies, with a duration of longer than six weeks, examining further reductions in the dietary LC-PUFA concentration in conjunction with differing DHA: EPA ratios are required to fully elucidate the roles of both LC-
357 PUFA content and DHA: EPA ratio on the growth of juvenile barramundi. Indeed a more comprehensive study better defining the aetiology of essential fatty acid deficiency in this species is warranted given the vagaries in observations seen among a range of studies (Glencross and
360 Rutherford, 2011; Williams et al., 2006).

1.4.2 Tissue composition and fatty acid retention

363 Tissue fatty acid composition was altered by diet, which has been previously demonstrated for a number of fish species (reviewed by Glencross, 2009). Compared with the initial fish, the majority of dietary treatments showed a reduction in tissue DHA and tissue EPA which is in contradiction to
366 previous studies in barramundi (Glencross and Rutherford, 2011) but consistent with results in post-smolt Atlantic salmon fed diets containing similar DHA and EPA levels (Glencross et al., 2013).

369 Examination of the mass-balance relationship between dietary and tissue fatty acids shows a contrast with previous studies in barramundi (Glencross and Rutherford, 2011) and Atlantic salmon (Glencross et al., 2013). Both studies demonstrated high (>100%) DHA retention levels when fish
372 were fed diets low in DHA, suggesting high-level conservation and or possible synthesis of DHA was occurring. Atlantic salmon possess this capability (Tocher, 2003) although it is generally believed that barramundi are not able to synthesise DHA and the results of the present study support this.

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Despite differences in the magnitude of DHA and EPA retention between the present study and those of Glencross and Rutherford (2011) and Glencross et al. (2013), a similar pattern of retention was observed. In all three studies, DHA retention was highest at the lowest DHA inclusion level, reducing as dietary DHA increased, consistent with previous studies (Bell et al., 2002; Torstensen et al., 2004; Stubhaug et al., 2007).

The pattern of EPA retention is less clear, with retention being higher at dietary DHA levels of 5 g kg^{-1} or less, contrasting with previous studies (Glencross and Rutherford, 2011; Glencross et al., 2013). EPA retention improved substantially with increasing dietary DHA (Glencross and Rutherford, 2011) but this was not observed in the present study. In the present study, increasing dietary DHA, decreased EPA retention and the addition of EPA to the diet further reduced EPA retention. Glencross et al. (2013) demonstrated reduced EPA retention levels when EPA was added to the diet but observed increasing EPA retention until dietary DHA reached 10 g kg^{-1}, in contrast with the present study. Negative EPA retention values at both low and high DHA inclusion levels, suggests that chain elongation and desaturation was occurring (Glencross et al., 2013). However, low levels of EPA retention suggest that DHA is not being retroconverted to EPA nor are shorter chain fatty acids being elongated to form EPA, contrasting with previous studies (Glencross and Rutherford, 2011) but agreeing with Glencross et al. (2003) who demonstrated EPA retention in Red seabream did not exceed 60% regardless of dietary EPA (Glencross et al., 2003).

Higher levels of DHA and EPA in the initial fish may explain differences between the present study and that of Glencross and Rutherford (2011). Fish of a similar starting size (17 g) but the initial fish in the present study contained 7.6% DHA and 3.4% EPA compared with 3% DHA and 2% EPA for Glencross and Rutherford (2011). Glencross et al. (2013) suggested that the effects of EFAs on growth may be a function of time rather than just mass turnover (discussed previously), it is not surprising that the results of the present study differ to that of Glencross and Rutherford (2011) considering that the endogenous reserves of DHA and EPA were almost double in the present study.

1.4.3 Plasma chemistry

Essential fatty acids, specifically DHA and EPA play a role in the membranes of red blood cells and diets deficient in EFAs have been associated with perturbations in red cell function including increased red cell fragility (Montero et al., 2004). No effect of dietary DHA or EPA concentration or total LC-PUFA concentration was observed on the haematological parameters measured (PCV and erythrocyte fragility). However, there was a large variation between individual fish from the same groups suggesting that individual variation is considerably larger than any dietary effect, or that there is no effect of dietary fatty acids in these parameters.

Of the biochemical markers assessed only glutamate dehydrogenase and haemoglobin were significantly affected by dietary treatment. Glutamate dehydrogenase is an enzyme, present in the mitochondria that converts glutamate to α-ketoglutarate and has roles in transdeamination as well as osmoregulation in aquatic animals (by synthesis of proline and alanine; Li et al., 2011). Glutamate dehydrogenase activity was not affected by DHA concentration when fish were fed diets containing 1 g kg^{-1} of EPA agreeing with previous research (Glencross and Rutherford, 2011). When fish were fed diets containing greater than 1 g kg^{-1} EPA glutamine dehydrogenase activity was increased, irrespective of the total LC-PUFA inclusion level. Glutamate dehydrogenase activity was also increased in the satiety fed vs restricted groups, possibly a reflection of the increased feed intake, and therefore increased EPA intake.

Glutamate dehydrogenase plays an important role in the diagnosis of liver disease, in combination with aminotransferases (also examined in this study). Liver disease involving necrosis of the hepatocytes resulted in elevated GLDH in conjunction with elevated aminotransferases (Ozer et al., 2008). A concomitant increase in alanine aminotransferase was not observed in this study, suggesting that the increase in GLDH was not owing to liver damage, but reflective of the enzymes role in osmoregulation. The role of EPA in osmotic regulation and hydration balance has already been highlighted (Glencross and Rutherford, 2011) with the results of the present study supporting this.

Glencross and Rutherford (2011) demonstrated a relationship between plasma urea concentration and dietary DHA content, which was absent in the present study but may be reflective of the lower plasma urea concentrations in the present study (range: 1.0-1.6 mmol L^{-1}) compared with the former (2.3-3.0 mmol L^{-1}).

Plasma haemoglobin was affected by DHA concentration but not by total LC-PUFA concentration or feeding regime (restricted vs. satiety), contradicting Glencross and Rutherford (2011). In Sea bream an n3-LC-PUFA deficient diet increased total haemoglobin attributing the increase to a reduction in erythrocyte volume (Montero et al., 2004). However, haemoglobin increased with increasing DHA content in the present study, suggesting that n3-LC-PUFA deficiency was not responsible for this observation.

Catacutan and Coloso (1995) observed abnormal reddening of the fins, a sign of essential fatty acid deficiency, in fish fed diets containing less than 5% lipid. Glencross and Rutherford (2011) also observed this reddening of fins, identifying it as mild subcutaneous haemorrhaging resulting from a reduced ability of the fish to ionically regulate. In this study it was identified that this was linked to the absence of EPA in the diet, with high levels of DHA exacerbating the problem. In the present study this reddening was not observed, suggesting that the EPA inclusion levels were sufficient to facilitate ionic regulation, or that residual levels in the fish's body reserves were adequate to prevent any subcutaneous haemorrhage (and associated perturbations to ionic regulation; Glencross and Rutherford, 2011). Inclusion levels of EPA (1.5 g kg^{-1}) were similar to those of Glencross and Rutherford (2011), which ranged from 1.5-3.1 g kg^{-1} in the diets as were ARA levels (present study range: 0-1.0 g kg^{-1}; Glencross and Rutherford, 2011 range: 0.2-0.9 g kg^{-1}), suggesting that either the ARA content was sufficient to produce sufficient prostaglandin H_2 in the present study but not that of Glencross and Rutherford (2011). Fish in the present study contained substantially higher tissue levels of ARA and EPA than those used by Glencross and Rutherford (2011) and utilisation of these stored reserves may have contributed to the production of sufficient prostaglandin H_2 for ionic regulation. Conversely, as the dietary EPA + ARA concentration is less than that of Glencross and Rutherford

(2011), it raises the possibility that the majority of ionic regulation was mediated through pathways other than the prostaglandin hormones, such as gills, kidneys or intestine (Glencross, 2006). Further research examining the activity of these pathways is required to confirm this.

1.4.4 Implications and conclusions

Diets containing DHA inclusions levels as low as 1 g kg^{-1} did not compromise growth or survival of juvenile barramundi. In contrast to previous studies, the absence of EPA (i.e. levels as low as 1 g kg^{-1}) did not result in a range of physiological problems suggesting that further research is required to elucidate differences between this study and previous ones. It is generally suggested that DHA and EPA should be provided in balance although the results of the present study suggest that is less important than previously thought. Given the lack of deleterious effects on growth and health of fish fed the D1E1 diet, further studies are warranted and should examine lower levels of DHA and EPA inclusion as well as examining the effect of altering the DHA: EPA ratio beyond 10:1 or below 1:1. Given that the response of the juvenile barramundi to low-LC-PUFA diets may be related to time and/or turnover, studies should be lengthened and the range of health parameters expanded to fully assess the longer-term implications on growth and health.

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Table 1. Formulations and composition of the experiment diets (all values are g kg^{-1}).

	Formulated DHA: EPA Content (g kg^{-1})					
Ingredient	D1E1	D5E1	D10E1	D10E10	D5E5	D3E3
Pregelled wheat starch	50	50	50	50	50	50
Wheat gluten	80	80	80	80	80	80
Wheat flour	182	182	182	182	182	182
Casein	100	100	100	100	100	100
Soy Protein Isolate	200	200	200	200	200	200
Fish oil	0	0	0	75	30	15
Olive Oil	41	37	31	4	26	34
DHASCO	0	8	20	0	0	0
Defatted Fish meal	300	300	300	300	300	300
Butter fat	41	37	31	4	26	34
Yttrium oxide	1	1	1	1	1	1
Pre-mix vitamins*	5	5	5	5	5	5
Cellulose	0	0	0	0	0	0
	Fatty acids as a per cent of total fatty acids					
Diet composition						
Dry matter	871	963	873	964	967	968
Crude protein	578	613	574	609	609	632
Ash	55	65	57	65	66	66
Lipid	98	85	94	99	105	85
Gross energy	22.6	22.0	22.3	22.2	22.3	22.2
14:0	5.8%	5.9%	6.2%	7.1%	6.4%	6.1%
16:0	19.8%	21.4%	20.4%	20.2%	20.9%	21.4%
18:0	7.0%	6.0%	5.6%	4.4%	5.3%	5.7%
SFA	38.4%	38.4%	36.3%	35.2%	36.6%	36.5%
16:1n7	1.8%	2.2%	2.1%	7.4%	4.7%	3.7%
18:1n-9	43.9%	36.6%	35.6%	18.9%	29.7	33.4%
MUFA	46.3%	39.3%	37.9%	26.9%	34.7%	37.4%
18:2 n6	8.7%	10.4%	9.0%	8.6%	10.1%	11.6%
18:3n3	1.0%	1.2%	1.0%	1.2%	1.2%	1.3%
PUFA	15.3%	22.3%	25.9%	37.9%	28.7%	26.2%
20:4n6	0.2%	0.3%	0.0%	0.9%	0.6%	0.0%
20:5n3	1.8%	2.7%	2.7%	12.2%	7.3%	5.4%
22:6n3	2.6%	5.9%	9.8%	11.9%	7.5%	6.5%
LC-PUFA	4.6%	9.8%	15.2%	25.0%	15.4%	11.8%
n-3	5.9%	10.4%	14.1%	27.3%	17.4%	14.2%
n-6	9.1%	11.8%	11.8%	9.5%	10.7%	11.5%

*Vitamin and mineral premix includes (IU kg^{-1} or g kg^{-1} of premix): Vitamin A, 2.5MIU; Vitamin D3, 0.25 MIU; Vitamin E, 16.7 g; Vitamin K3, 1.7 g; Vitamin B1, 2.5 g; Vitamin B2, 4.2 g; Vitamin B3, 25 g; Vitamin B5, 8.3; Vitamin B6, 2.0 g; Vitamin B9, 0.8; Vitamin B12, 0.005 g; Biotin, 0.17 g; Vitamin C, 75 g; Choline, 166.7 g; Inositol, 58.3 g; Ethoxyquin, 20.8 g; Copper, 2.5 g; Ferrous iron, 10.0 g; Magnesium, 16.6 g; Manganese, 15.0 g; Zinc, 25.0 g.

573 Table 2. Nutrient composition of key experimental ingredients (all values are g kg^{-1} DM unless otherwise indicated).

Nutrient	Defatted Fishmeal[a]	Olive Oil[b]	Casein[c]	Butter fat[d]	Anchovy Oil[e]	DHASCO[f]	Soy Protein Isolate[g]
Dry matter content (g kg^{-1})	943	999	995	999	996	999	995
Crude protein	713	9.6	939	6.8	78.5	83.2	901
Total lipid	48	912	8.6	846	929	964	62
Ash	170	0	48	0	0	0	44
Gross energy (MJ kg^{-1} DM)	21.0	43.1	21.6	41.0	38.5	39.1	22.1
Fatty acids as a per cent of total fatty acids							
14:0	6.7%	0.0%	11.7%	14.1%	8.3%	9.6%	6.9%
16:0	22.7%	10.3%	34.4%	32%	18.7%	25.5%	22.3%
18:0	5.3%	3.2%	14.3%	14.1%	3.4%	0.9%	0.0%
22:0	0.0%	0.0%	0.0%	0.0%	0.0%	0.1%	11.2%
SFA	38.2%	13.9%	61.9%	68%	35.2%	38%	46.9%
16:1n-7	7.1%	0.6%	3.2%	0.2%	10.2%	1.8%	6.5%
18:1n-9	14.3%	74.5%	31.1%	28.7%	12.5%	1.9%	5.4%
20:1n-9	0.0%	0.3%	0.0%	0.2%	0.0%	0.0%	0.0%
MUFA	21.3%	77.3%	24.9%	29%	24.2%	4.2%	45.3%
18:2 n-6	1.8%	8.2%	3.6%	3%	1.4%	0.5%	3.1%
18:3n-6	0.0%	0.6%	0.0%	1%	0.4%	0.4%	0.0%
18:3n-3	0.0%	0.0%	0.0%	1.1%	0.7%	0.1%	0.0%
PUFA	40.5%	8.7%	3.9%	2%	40.6%	57.8%	7.8%
20:3n-6	0.0%	0.0%	0.0%	0.0%	0.0%	1.0%	0.0%
20:4n-6	1.5%	0.0%	0.0%	0.0%	1.1%	0.9%	0.0%
20:3n-3	0.0%	0.0%	0.0%	0.0%	0.1%	0.1%	1.30%
20:5n-3	13.2%	0.0%	0.0%	0.1%	16.9%	1.9%	0.0%
22:4n-6	0.0%	0.0%	0.0%	0.0%	0.8%	0.0%	0.0%
22:5n-6	0.0%	0.0%	0.0%	0.0%	0.0%	1.6%	2.3%
22:6n-3	21.2%	0.0%	0.0%	0.0%	14.5%	50.8%	0.0%
LC-PUFA	35.9%	0.0%	0.0%	0.1%	33.7%	56.3%	4.1%
n-3	36.2%	0.0%	0.0%	1%	35.1%	55.1%	4.1%
n-6	3.3%	0.6%	0.0%	2%	3.9%	2.8%	3.7%

576 [a]Fish meal (prior to being defatted): Chilean anchovy meal, Skretting Australia, Cambridge, TAS, Australia; [b]Refined olive oil: Conga Foods, Coburg North, VIC, Australia; [c]Casein: Vitamin-free Casein, MP Biomedicals Australia, Seven Hils, NSW, Australia; [d]Butterfat: Woolworths Dairies, Bella Vista, NSW, Australia. [e]Anchovy oil, Skretting Australia,
579 Cambridge, TAS, Australia; [f]DHASCO: HuaTai BioPharm, Deyang, Sichuan, Szechuan, China; [g]Soy protein isolate: ADM, Decatur, IL, USA.

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Table 3. Growth and feed utilisation of juvenile barramundi fed diets containing different DHA and EPA levels.

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Parameter	Formulated DHA: EPA Content (g kg^{-1})								Pooled SEM
	D1E1	D5E1	D10E1	D10E10	D5E5	D3E3	D1E1 Sat	D10E1 Sat	
Initial weight (g fish^{-1})	16.4	16.5	16.4	16.5	16.7	16.3	16.6	16.2	0.05
Final weight (g fish^{-1})	45.0^{a}	45.5^{a}	46.2^{a}	44.9^{a}	45.7^{a}	44.6^{a}	56.5^{b}	55.7^{b}	0.4
Weight gain (g fish^{-1})	28.6^{a}	29.0^{a}	29.8^{a}	28.4^{a}	29.1^{a}	28.2^{a}	39.9^{b}	39.6^{b}	0.5
Growth rate* (% d^{-1})	3.6^{a}	3.7^{a}	3.7^{a}	3.6^{a}	3.6^{a}	3.6^{a}	4.6^{b}	4.6^{b}	0.15
Feed intake (g fish^{-1})	24.3^{a}	24.2^{a}	24.4^{a}	23.9^{a}	24.8^{a}	24.1^{a}	34.5^{b}	35.0^{b}	0.3
Feed Conversion (feed gain^{-1})	0.85	0.83	0.82	0.84	0.85	0.85	0.86	0.89	0.02
Survival (%)	100	100	98.3	98.3	98.3	98.3	100	100	0.3

*Growth rate is daily growth coefficient = (Wf $^{1/3}$ – Wi $^{1/3}$)/*t* x 100, where Wf is the final weight Wi is the initial weight and *t* is time. Different superscripts indicate significant differences between means among treatments ($P<0.05$) and lack of superscripts denotes no significant differences between means among treatments.

591 Table 4. Whole body fatty acid composition and retention of DHA and EPA for juvenile barramundi (data are percent of total fatty acids)

Fatty Acid	Initial Fish	Formulated DHA: EPA content (g kg^{-1}) in diets								Pooled
		D1E1	D5E1	D10E1	D10E10	D5E5	D3E3	D1E1 Sat	D10E1 Sat	SEM
14:0	3.2^{a}	4.1^{b}	4.1^{b}	3.9^{b}	4.2^{d}	4.1^{b}	4.0^{b}	4.0^{b}	4.0^{b}	0.1
16:0	21.8	24.7	24.7	24.7	24.8	24.9	24.8	25.2	25.8	0.2
18:0	6.4^{a}	6.8^{b}	6.5^{a}	6.5^{a}	6.5^{a}	6.5^{a}	6.7^{b}	6.9^{b}	6.8^{b}	0.1
SFA	34.9^{a}	$39.2^{b,c}$	38.8^{b}	38.8^{b}	39.9^{c}	$39.5^{b,c}$	$39.3^{b,c}$	39.7^{c}	40.1^{d}	0.5
16:1n-7	6.3^{a}	5.0^{b}	5.1^{b}	5.3^{c}	6.7^{d}	5.9^{e}	5.5^{f}	5.1^{b}	5.1^{b}	0.1
18:1n-9	30.6^{a}	32.6^{b}	31.3^{c}	29.4^{a}	24.9^{d}	28.9^{e}	31.3^{c}	32.9^{b}	30.1^{a}	0.0
20:1n-9	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.0
MUFA	$38.3^{a,b}$	38.9^{a}	$37.6^{b,e}$	35.9^{c}	32.9^{d}	36.2^{b}	38.0^{a}	39.2^{e}	36.3^{b}	0.7
18:2n-6	10.9^{a}	9.7^{b}	9.9^{b}	9.9^{b}	9.5^{c}	9.9^{b}	$9.7^{b,c}$	9.6^{c}	9.4^{c}	0.7
PUFA	26.8^{a}	21.9^{b}	23.6^{c}	25.4^{d}	27.3^{e}	24.3^{c}	$22.7^{b,c}$	21.2^{b}	23.6^{c}	0.7
20:4n-6	0.9^{a}	0.6^{b}	0.6^{b}	0.6^{b}	0.8^{c}	0.6^{b}	0.6^{b}	0.5^{d}	0.5^{d}	0.02
20:5n-3	3.4^{a}	2.5^{b}	2.6^{b}	$2.8^{b,e}$	4.9^{c}	3.6^{d}	3.0^{e}	2.4^{b}	2.4^{b}	0.2
22:6n-3	7.6^{a}	5.6^{b}	6.7^{c}	7.8^{d}	8.1^{d}	6.5^{c}	5.9^{b}	5.2^{b}	7.2^{c}	0.2
LC-PUFA	12.7^{a}	9.4^{b}	11.0^{c}	12.7^{a}	14.6^{d}	11.5^{c}	$10.2^{b,c}$	8.9^{e}	11.7^{b}	0.6
n-3	13.3^{a}	9.8^{b}	11.1^{c}	12.5^{d}	15.2^{e}	$12.1^{c,d}$	$10.8^{b,c}$	9.4^{b}	11.3^{c}	0.6
n-6	13.2^{a}	11.9^{b}	12.2^{a}	12.7^{a}	11.8^{b}	$12.0^{b,c}$	11.7^{b}	11.6^{b}	12.1^{a}	0.2
DHA retention	n/a	63.1^{a}	67.3^{a}	33.0^{b}	35.5^{b}	37.3^{c}	40.8^{c}	80.0^{d}	38.4^{c}	4.1
EPA retention	n/a	40.2^{a}	45.7^{b}	32.6^{c}	25.6^{d}	24.5^{d}	28.1^{d}	51.7^{e}	39.2^{a}	3.0

Different superscripts indicate significant differences between means among treatments ($P<0.05$) and lack of superscripts denotes no significant differences between means among treatments.

Table 5. Haematology and plasma chemistry parameters for juvenile barramundi

Parameter	Units	Formulated DHA: EPA content (g kg^{-1}) in diets								Pooled
		D1E1	D5E1	D10E1	D10E10	D5E5	D3E3	D1E1 Sat	D10E1 Sat	SEM
PCV	%	33.0	33.7	27.7	28.7	29.3	23.3	27.0	31.7	1.25
Erythrocyte fragility	%	72.0	15.0	100	16.3	87.0	57.7	60.0	51.0	4.8
Total Protein	g L^{-1}	39.0	39.3	40.3	43.3	45.0	37.3	40.7	43.3	1.2
Alanine Aminotransferase	U L^{-1}	58.5	98.5	83.5	164.5	145.0	87.0	91.0	50.67	10.1
Glutamate Dehydrogenase	U L^{-1}	$10.3^{a,c}$	9^{a}	$14.7^{a,c}$	$15.0^{c,d}$	$15.0^{c,d}$	20.0^{d}	17.7^{d}	33.7^{e}	2.0
Urea	mmol L^{-1}	1.5	1.4	1.43	1.0	1.3	1.3	1.5	1.6	0.1
Creatinine	umol L^{-1}	11.7	11.3	12.0	13.0	12.7	11.7	12.7	13.3	0.3
Cholesterol	mmol L^{-1}	2.75	3.1	3.2	2.9	3.0	3.0	2.9	2.8	0.1
Albumin	g L^{-1}	2.8	3.6	3.8	4.1	4.2	3.7	3.1	3.1	0.2
Calcium (Ca)	mmol L^{-1}	1.4	1.4	1.4	0.9	1.1	1.3	1.5	1.4	0.1
Glycerol	mmol L^{-1}	0.14	0.12	0.19	0.06	0.13	0.13	0.20	0.20	0.01
Iron (Fe)	umol L^{-1}	101.9	115.9	80.6	80.2	46.2	124.6	101.8	87.6	7.0
Haem	mg mL^{-1}	232.5^{a}	$489.7^{b,d}$	$449.3^{b,d}$	993.0^{c}	243.3^{a}	544.7^{d}	$351.0^{a,b}$	$383.7^{a,b}$	44.2
Haptoglobin	mg mL^{-1}	0.12	0.15	0.16	0.25	0.13	0.14	0.13	0.11	0.02

597 Different superscripts indicate significant differences between means among treatments ($P<0.05$) and lack of superscripts denotes no significant differences between means among treatments.