An analysis of the effects of different dietary macro-nutrient energy sources on the growth and energy partitioning by juvenile barramundi, *Lates calcarifer*, reveal a preference for protein derived energy.

Brett Glencross[1,2], David Blyth[1,2], Simon Irvin[1,2], Nicholas Bourne[1,2], Nick Wade[1,2]

1. CSIRO Food Futures Flagship, PO Box 120, Cleveland, QLD 4163, Australia
2. CSIRO Marine and Atmospheric Research, PO Box 120, Cleveland, QLD 4163, Australia

(p) 61-7-3826-7236
(f) 61-7-3826-7281
(e) Brett.Glencross@csiro.au

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Abstract

It is generally considered that fish respond to dietary energy densities on a consistent basis irrespective of what macronutrient source the dietary energy originates from. To test this assumption two experiments were undertaken to establish the different roles of protein, lipid and starch as energy sources in underpinning nutritional bioenergetics in juvenile barramundi, *Lates calcarifer*. To do this, a range of ingredients were evaluated for their digestible protein (DP) and digestible energy (DE) value. Following this, a series of diets were formulated to an equivalent DE basis, and observed a minimum DP:DE ratio required for fish of 80g. However, in each of the diets the proportion of DE available from protein, lipid or starch was varied to bias the contribution of each macronutrient on the origin that digestible energy when fed to the fish. Growth of fish fed the protein diet was better than those fed the lipid diet, which was better than those fed the starch diet. Feed intake was lower in the protein diet than the lipid diet, and both were lower than the starch diet. Feed conversion was most efficient in the protein diet fed fish, which was better than the lipid diet fed fish, which was better than the starch diet fed fish. Whole-fish composition varied among treatments, with differences observed in the dry matter composition, whole body lipid and gastrointestinal tract lipid content. Typically lipid and dry matter composition were in synchrony, and were usually higher in the starch fed fish and lower in the lipid fed fish. When flux of protein, lipid and energy was assessed in terms of deposition efficiencies some significant differences were observed. Protein deposition efficiency was relatively conservative, but ranged from 33% in the starch diet fed fish to 41% in the lipid diet fed fish. Lipid deposition efficiency was more dramatic; ranging from 40% in the lipid diet to 182% in the starch diet. Energy deposition efficiency was relatively conservative among treatments, ranging from 50% to 56% efficient. Overall the results from this study show that there is a clear hierarchy in preference for energy substrates by juvenile barramundi, such that protein > lipid > starch.

Introduction

Barramundi are an obligate carnivorous fish species that is the basis of a significant aquaculture industry in Southeast Asia and Australia (Glencross, 2006). Considerable work has been done to develop and optimise formulated, extruded feeds for barramundi and these are well established in the industry (Williams et al., 2003; 2006; Glencross, 2006; 2008). Underpinning recent development has been the establishment of a series of factorial bioenergetic nutritional models that not only serve as benchmarks for growth performance, but also provide estimations of feed demand and idealised feed compositions to support that growth performance (Bermudes et al. 2010; Glencross, 2008; Glencross & Bermudes, 2010; 2011; 2012). These modelling studies suggest that high-energy density feeds offer significant feed performance advantages for barramundi, provided nutrients are maintained at adequate levels. Assessments of these models have so far proven that they are relatively robust (Glencross et al., 2008; Glencross & Rutherford, 2010). However, these models rely on the assumption that the dietary DE source is irrelevant; that dietary DE derived from protein, lipid and starch is utilised with equal efficiency, provided key nutrients (e.g. protein) are provided at minimum critical ratios to energy supply (Boujard & Medale, 1994; Catacutan & Coloso, 1995; Lupatsch et al., 2003; Dumas et al., 2007; Glencross, 2008; Hua et al., 2010; Dumas et al., 2010; Glencross & Bermudes, 2012).

Utilisation of each of the different macronutrients for energy occurs by distinct metabolic pathways, and occurs with different levels of efficiency in terrestrial animals, resulting in the amendment of digestible values for diets and ingredients to metabolisable values (Azevedo et al., 2005; Hua et al., 2010). Such a transition, while examined in a few instances in fish nutrition has largely not gained much traction in the aquaculture feed sector (Bureau & Hua, 2008; Dumas et al., 2010). In addition, there is increasing evidence that the roles of gluconeogenesis, glycolysis and β-oxidation play substantially different relative roles in energy provision in fish compared to other vertebrates (Enes et al., 2009; Lansard et al., 2010; Saravanan et al., 2012; Schrama et al., 2012). This observation has important implications in the potential relative roles of each of the key macronutrients in terms of dietary energy supply.

This study examined the growth, feed utilisation and nutrient deposition of juvenile barramundi fed a series of different diet formulations based on supplying the same DE supply, whilst varying the macronutrient used to supply the energy. Furthermore, the effects of dietary DE density were examined using a control diet that was 20% lower in DE density (as a negative control). Therefore, this study proposes the hypothesis that there will be response effects (growth and intake) in juvenile barramundi in relation to changes in dietary energy density, and that the fish will also respond to different macronutrient sources based on their ability effectively metabolise each of those different macronutrients for energy.

Materials and Methods

Experiment 1 - design and fish management

The digestibility experiment design was based on the diet-substitution approach (reviewed by Glencross et al., 2007). The basal diet for this experiment was formulated and prepared to include approximately 500 g kg^{-1} protein, 100 g kg^{-1} lipid and included an inert marker (yttrium oxide at 1 g kg^{-1}) (Table 1). Each test ingredient was added at to the test diets at 300 g kg^{-1}inclusion to a reciprocal-sample of the basal mash (Table 1). Each of the supplied raw materials was milled using a RetschTM ZM200 rotor mill (Retsch Pty Ltd, North Ryde, NSW, Australia) with a 750 μm screen to create a flour prior to incorporation in the diet mashes. The composition and origin details of each ingredient are presented in Table 2. The diets were made by the addition of water (about 25% of mash dry weight) to the mash whilst mixing to form a dough which was subsequently screw pressed using a pasta maker through a 4 mm diameter die. The resultant moist pellets were then oven dried at 60 ºC for around 12 h before being allowed to cool to ambient temperature in the oven. The basal diet was prepared in a similar manner, but without the addition of any test ingredient.

Juvenile barramundi (*Lates calcarifer*) were obtained from the Gladstone Water Board Hatchery (Gladstone, QLD, Australia), and grown in a 10,000L tank being fed a commercial feed (Marine Float; Ridley Aquafeed, Narangba, QLD, Australia). In preparation for this experiment, the fish were transferred to a series of experimental tanks (300 L) with flow-through seawater (salinity =35 PSU; dissolved oxygen 6.4 ± 0.18 mg L^{-1}) of 28.8 ± 0.22ºC (mean ± S.D.) at a flow rate of about 3 L min^{-1} being supplied to each of the tanks. Each of the tanks were stocked with 20 fish of 397 ± 69 g (mean ± S.D.; n = 40 from a representative sample of the population). Treatments were randomly assigned amongst 10 tanks, with each treatment having four replicates. The experiment was conducted over two block events to achieve this level of replication. The same batch of fish was used for both blocks, but a complete randomised design applied to each block to ensure experimental validity. The fish were allowed to acclimatise to their allocated dietary treatment for at least seven days before faecal collection commenced.

For faecal collection the barramundi were manually fed the diets once daily to apparent satiety as determined over three separate feeding events between 0800 and 0900 each day. Faeces were collected in afternoon (1600 – 1800) from each fish within each tank using stripping techniques based on those reported by Glencross (2011). Prior to any handling, the fish were sedated using AQUI-S™. The fish were then allowed to regain consciousness and equilibrium before being placed within their designated tank. The hands of the person collecting the faeces were rinsed between handling each fish to ensure that the faeces were not contaminated by urine or mucous. Fish were also not stripped on consecutive days in order to minimise stress on the animal and maximise feed intake prior to faecal collection. Faecal sample were stored at -20 ºC prior to freeze drying and milling in preparation for chemical analysis.

Chemical and digestibility analysis

Diet, ingredient, faecal and whole fish samples were collected and their moisture content determined by oven drying at 105 ºC for 24 h. For the whole fish a second sample freeze-dried prior to chemical analysis. Faeces were also freeze dried prior to analysis. Freeze-dried samples were milled prior to analysis for dry matter, ash, fat, nitrogen, amino acid and gross energy content. Protein levels were calculated from the determination of total nitrogen by CHNOS elemental auto-analyser, based on N x 6.25. Carbohydrates were calculated based on the dry matter content of a sample minus the protein, lipid and ash. Total starch content was measured using enzymatic methods with the Megazyme Total Starch Kit, K-TSTA, following a modified AOAC Method 996.11. Amino acid analysis involved the samples being hydrolysed at 110 ºC for 24 h in 6 M HCl with 0.05 % Phenol. Cystine was derivatized during hydrolysis by the addition of 0.05 % 3-3-dithiodipropoinic acid. The acid hydrolysis destroyed tryptophan making it unable to be determined. Separation of the amino acids was performed by HPLC on a Hypersil AA-ODS 5µm column using an 1100 series Hewlett Packard HPLC system. Total lipid content of the diets was determined gravimetrically following extraction of the lipids using chloroform:methanol (2:1). Gross ash content was determined gravimetrically following the 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.

Differences in the ratios of dry matter, protein or gross energy to yttrium, in the feed and faeces in each treatment were calculated to determine the apparent digestibility (AD_{diet}) for each of the nutritional parameters examined in each diet (Table 3) based on the following formula (reviewed in Glencross et al., 2007):

$$AD_{diet} = \left(1 - \left(\frac{Y_{diet} \times Parameter_{faeces}}{Y_{faeces} \times Parameter_{diet}} \right) \right) \times 100$$

where Y_{diet} and Y_{faeces} represent the yttrium content of the diet and faeces respectively, and $Parameter_{diet}$ and $Parameter_{faeces}$ represent the nutritional parameter of concern (dry matter, protein or energy) content of the diet and faeces respectively. The digestibility values for each of the test ingredients in the test diets examined in this study were calculated according to the formulae:

$$Nutr.AD_{ingredient} = \frac{\left(AD_{test} \times Nutr_{test} - \left(AD_{basal} \times Nutr_{basal} \times 0.7 \right) \right)}{\left(0.3 \times Nutr_{Ingredient} \right)}$$

where $Nutr.AD_{ingredient}$ is the digestibility of a given nutrient from the test ingredient included in the test diet at 30%. AD_{test} is the apparent digestibility of the test diet. AD_{basal} is the apparent digestibility of the basal diet, which makes up 70% of the test diet. $Nutr_{Ingredient}$, $Nutr_{test}$ and $Nutr_{basal}$ are the level of the nutrient of interest in the ingredient, test diet and basal diet respectively (reviewed in Glencross et

al., 2007). All raw material inclusion levels were also corrected for dry matter contribution and the effects that this may have had on the actual ratio of reference diet to test ingredient. All ingredient digestibilities are reported in Table 1 and digestible nutrient and energy values in Table 2.

Experiment 2 - design and fish management

A second experiment was conducted to compare the performance of barramundi fed a range of diets varying in macronutrient concentrations, whilst providing equivalent DE densities (Tables 3 and 4). An additional control diet with a lower digestible energy density was also included. Fish were obtained from the Gladstone Water Board Hatchery (Gladstone, QLD, Australia), and on-grown to 81.2 ± 1.48 g (mean ± SD, n=480) in preparation for the experiment. During the on-growing period all fish were fed the same diet (Nova-LE; Skretting Australia, Cambridge, TAS, Australia) and kept in 3 x 1000L seawater tanks. At the initiation of the trial 40 fish were weighed on an electronic top-loading balance to 0.1 g accuracy to determine the mean and standard deviation of the population. Following this 20 fish were allocated to each of 15 x 300L tanks based on having to be within the mean ± 1 x S.D. The experiment was conducted at the CSIRO Marine Research Laboratories at Cleveland in a flow-through, aerated, heated seawater tank array. Water temperature was maintained at 27.8 ± 0.45 ºC (mean ± S.D.) and dissolved oxygen 5.6 ± 0.18 mg L^{-1} (mean ± S.D.) for the 84 days of the experiment. At the end of the 84 day period faeces were stripped from the fish for digestibility assessment of each of the diets as per the methods described earlier.

Each diet was fed by an autofeeder suspended above each tank. Feed was fed to each tank of fish twice daily (0900 – 0930 and 1630 - 1700) to slight excess, seven days a week for 84-days. All feed fed and all uneaten feed was accounted for and correction factors applied to the collected uneaten feed to allow the determination of solubilisation losses and pellet dry matters and therefore of actual feed consumption within each tank (based on methods reported by Helland et al., 1996). This also allowed the potential effects of dietary digestible energy density or macronutrient source on feed intake to be evaluated (Glencross et al., 2007).

For Experiment 2 all diets (Tables 3 and 4) were formulated to be isoenergetic (15.3 MJ DE kg^{-1}) on a digestible nutrient basis. Most diets were also isoproteic (475 g kg^{-1}) on a digestible basis, with the exception of the 'Protein' diet in which the digestible protein was 562 g kg^{-1} and the control diet which was lower in both digestible protein (379 g kg^{-1}) and energy (12.3 MJ DE kg^{-1}). All diets, except the 'Protein' diet maintained approximately the same protein to energy ratios (~30 g MJ-DE^{-1}). For fish of ~80 g an ideal DP : DE ratio of 28.4 g MJ DE^{-1} is recommended (Williams et al., 2003; Glencross, 2008). Diets were made by mixing all the dry ingredients and then processed by the addition of the oil component and water (about 30 % of mash dry weight) to all ingredients while mixing to form dough. The dough was then screw-pressed through a 4 mm diameter die using a pasta maker. The resultant moist pellets were oven dried at 70 ºC for about 12 h before being air-cooled,

bagged and stored at –20 ºC. Formulations and composition of the diets are presented in Tables 3 and 4 respectively.

Sample preparation and chemical analysis

Five fish were euthanized from the population at the beginning of the experiment as a representative initial sample. At the end of Experiment 2, three whole fish from each tank were euthanized by immersion in an overdose of AQUI-S™ before then being placed in iced-seawater slurry. Another three fish were also euthanized and blood and tissue samples taken for compositional and molecular analysis (see Wade et al., 2013). All of these fish from the end of the experiment were sampled 2 h post-feeding. Following sample collection, each whole fish sample was frozen prior to being minced by two passes through an industrial food processor to ensure sample homogeneity. A sample was then analysed for dry matter content as described previously. Another sample was then frozen prior to being freeze-dried in preparation for chemical analysis as also described previously.

Nutrient and energy balance and deposition assessment

The net balance for Protein (as N), lipid (L) and energy (E) were calculated based on the data derived in this study. Gross intake levels were determined based on total feed intake for each tank by the composition of the feed being fed. Digestible intake levels were measured based on the digestibility of N and E, with the starch free diet used to determine the lipid digestibility (86 %) from the residual of the energy digestibility not accounted for from protein digestibility. Faecal losses were determined as the reciprocal of the digestible levels. Retained nutrient and energy were determined based the net gain in nutrients and energy between the fish at the end of the trial and those from the initial sample. Brachial and urinary nitrogen (BUN) were determined based on the difference between digestible nitrogen intake and retained nitrogen with energy values defined based on 24.85 kJ x brachial and urinary nitrogen (Saravanan et al., 2012). Metabolisable energy intake (MEI) was determined based on digestible energy intake minus the brachial and urinary energy losses. Heat production (HP) was determined based on the difference between metabolisable energy and retained energy (RE). Basal metabolism (HeE) was calculated based on fasting energy losses of 34.4 kJ $kg^{-0.8}$ d^{-1} (Glencross, 2008). The Heat increment (HiE) was determined based on the MEI minus the RE and the HeE. Net energy (NE) was determined based on ME minus HiE (Bureau et al., 2002).

Protein (P), lipid (L) and energy (E) deposition were determined based on the mass gain in P, L and E over the course of the growth study, against the respective consumption of P, L and E. All values were calculated according to the following formula (reviewed in Glencross et al., 2007):

$$Nutrient\ Deposition\ (\%) = \left(\frac{Nt - Ni}{Nc} \right) \times 100$$

225 Where N_t is the nutrient/energy content of the fish in a specific replicate at time *t* and N_i is the
226 mean initial nutrient/energy content of the fish at the beginning of the study (n=3 replicates of 3
227 representative fish). N_c is the amount of nutrient/energy consumed by the fish from the time of initial
228 assessment to time *t*. In this study these values were determined based on both gross and digestible
229 intake data (Table 2).
230
231 *Statistical analysis*
232 All figures are mean ± SEM unless otherwise specified. Effects of diet for each experiment
233 were examined by ANOVA using the software package Statistica (Statsoft™, Tulsa, OA, USA).
234 Levels of significance were determined using an LSD planned comparisons test, with critical limits
235 being set at $P < 0.05$.

Results

Experiment 1 - Digestibility of experimental ingredients

There were subtle differences among the digestibility parameters of the ingredients studied in this experiment (Table 1). Ingredient protein digestibility ranged from of 93.2% for the fishmeal to 100% for both the casein and gluten (starch had no protein content to viably assess). However, ingredient digestibilities for energy ranged from of 86.3% for the starch to 98.1% for the wheat gluten.

Experiment 2 - Growth and feed utilisation

Growth, feed intake, feed utilisation and composition data for fish fed the control, protein, lipid, starch and negative control diets are presented in Table 5. Growth of fish fed the 'Control' diet was consistent with high-performing juvenile barramundi (Table 5). Fish fed the 'Protein' diet grew significantly better than those fed the 'Control' with a lower feed intake and lower FCR. The 'Lipid' diet fed fish grew the same as the 'Control' with a similar feed intake and similar FCR. Fish fed the 'Starch' diet grew at a poorer rate than those fed the 'Control', with a marginally higher feed intake and higher FCR. Fish fed the 'Negative' control diet grew significantly slower than all other diets, despite a higher feed intake, which led to a higher FCR than all other diets.

Digestible energy (DE) intake was relatively consistent amongst most treatments (~4450kJ $fish^{-1}$), with only the negative control (3874 kJ $fish^{-1}$) being significantly different from any of the other treatments. Digestible protein (DP) intake was more variable amongst the treatments (range 117.1 to 152.7 g $fish^{-1}$), being lowest in the 'Negative' diet fed fish and highest in the 'Protein' diet fed fish. Intake of DP was significantly higher in the 'Protein' diet fed fish compared to both the 'Lipid' and 'Starch' diets, which had almost identical levels of DP intake. Survival was high in all treatments and not significantly different.

Body composition

There were a range of differences in whole body composition of the fish from each of the treatments (Table 5). There were several differences in lipid content, which was the most variable compositional parameter measured. Total lipid content of the carcass was highest in those fish fed the 'Starch' diet (9.7%) and lowest in those fish fed the 'Protein' diet (6.2%). Gross energy content was also significantly different among the treatments with the 'Starch' diet (8.0 MJ kg^{-1}) highest and the 'Protein' diet (7.5 MJ kg^{-1}) lowest.

The variation in lipid and gross energy content observed in the whole carcasses of the fish from each treatment could also been seen in greater detail by examination of the composition of head-on-gutted (HOG) and the gastrointestinal tract (GIT) compositions. The dress-out 'yield' of the head-on-gutted carcass was variable and significantly highest for the fish fed the 'Lipid' treatment (89.5%) and lowest for fish fed the 'Negative' control diet (87.6%), but typically averaged around 88.5%

across all treatments (Table 5). Lipid content of the HOG was highest for fish fed the 'Negative' diet (7.4%) and lowest for fish fed the 'Lipid' diet (5.3%). Average lipid content across all treatments was 6.8%. The HOG gross energy content had little variability with samples ranging from 6.9 to 7.3 kJ g^{-1}.

In contrast, significant variation in the dry matter content of the GIT composition was observed (range from 60.4% to 67.7%). Lipid composition of the GIT averaged 40.4% but also varied significantly from 30.4% in the 'Protein' diet fed fish to 45.5% in the 'Control' diet fed fish, though this was not significantly different from those fish fed the 'Starch' and 'Negative' diets. Gross energy content of the GIT was largely consistent with the variation in lipid content of the GIT samples ranging from 18.0 to 21.8 MJ kg^{-1} and an average of 20.1MJ kg^{-1}. Protein content of the GIT was also variable ranging from 13.9% to 17.7% with an average of 15.4%.

Protein, lipid and energy deposition efficiencies

Protein deposition efficiencies were relatively conservative, but ranged from 33.3% for fish fed the 'Starch' diet to 41.0% for fish fed the 'Lipid' diet (Table 6). Average protein deposition efficiency across all treatments was 36.3%. Lipid deposition was much more variable ranging from 40.1% for the 'Lipid' diet to 182.8% for the 'Starch' diet. Average efficiency of lipid deposition was 92.1% across all treatments. Gross energy deposition was also much more conservative, ranging from 49.8% in the fish fed the 'Lipid' diet to 55.6% in fish fed the 'protein' diet. Across all treatments energy deposition efficiency averaged 51.9%.

Nitrogen, lipid and energy balance

There were a range of significant differences in nitrogen balance among the different diets (Table 7). Gross nitrogen intake ranged from 20.5 g $fish^{-1}$ for fish fed the 'Negative' diet to 26.9 g $fish^{-1}$ for fish fed the 'Protein' diet and a similar consistent pattern was seen in brachial and urinary nitrogen losses, and retained nitrogen levels.

Lipid balance was more variable, with lipid intakes ranging from 19.6 g $fish^{-1}$ for the 'Starch' diet to 62.7 g $fish^{-1}$ for the 'Lipid' diet (Table 7). Retained lipid was highest in the fish fed the 'Starch' diet (30.8 g $fish^{-1}$) and lowest in those fish fed the 'Protein' diet (20.3 g $fish^{-1}$).

Energy balance was more conservative, with gross energy intakes (GEI) ranging from 5819 kJ $fish^{-1}$ in the fish fed the 'Protein' diet to 6304 kJ $fish^{-1}$ in fish fed the 'Negative' diet (Table 7). Similar effects were also seen in faecal energy losses (FE) which meant that the digestible energy intake (DEI) was basically the reciprocal, with the highest DEI in those fish fed the 'Protein' diet and lowest in those fish fed the 'Negative' diet. Brachial and urinary energy (BUE) losses were lowest in those fish fed the 'Negative' diet and highest in those fed the 'Protein' diet. The metabolisable energy intake (MEI) was lowest in the fish fed the 'Negative' and 'Protein' diets and highest in the 'Lipid' diet fed fish. Retained energy (RE) was relatively consistent across the treatments, except those fish fed the 'Negative' diet which had a significantly lower RE. Heat increment energy (HiE) was lowest

in fish fed the ‘Protein’ diet and highest in those fish fed the ‘Lipid’ diet, though there were no significant differences between the fish fed the ‘Lipid’, ‘Starch’ and ‘Control’ diets. Net energy intake (NEI) was lowest in those fish fed the ‘Negative’ diet and highest in those fish fed the ‘Control’ diet.

Discussion

This study used a series of two experiments to examine the effects of the three primary macronutrient sources (protein, lipid and starch) on the bioenergetic value of diets fed to a carnivorous fish. The study initially sought to define the digestible nutrient and energy value of the ingredients to be used so as to enable a more accurate formulation of the experimental diets. Those digestible nutrient and energy specifications where then used to formulate diets where the total digestible energy was kept constant, but the relative proportions of the macronutrient suppling that digestible energy varied. This has enabled an insight into the roles that these macronutrients play in contributing to energy supply in this species.

Effects of digestible energy density on growth and feed utilisation

Classic bioenergetic dogma dictates that fish will eat to an energetic demand to grow to a target weight, subject to being able to consume enough feed to provide that energy and the diets including minimum levels of essential nutrients (Boujard & Medale, 1994; Bureau et al., 2002; Dumas et al., 2010). A classic test of this hypothesis is reinforced in the present study where two diets of the same ratios of protein:lipid:starch ratios were fed, each with the same DP to DE ratio, but one about 20% lower in DE than the other. In the present study, not only did the fish fed the lower DE diet consume more, but they were also unable to consume enough feed to compensate fully for the lower energy density and therefore also grew less than their counterparts fed the higher DE diet. These results show that aspects of the basic dogma of bioenergetic theory are clearly right. However, this also assumes that the ratio between protein:lipid:starch is kept constant and therefore the roles of each of the macronutrients in energy supply does not vary.

Effects of macronutrient source on growth and feed utilisation

The main focus in the present study was the observation that there were substantial effects of different dietary macronutrients on the growth and feed utilisation by barramundi. Despite being fed diets that were isoenergetic on a digestible basis, it was clear that there was a preference for energy in the order of protein > lipid > starch. This can be seen by the subtle differences in growth and the clearer effects on FCR of the ‘Protein’, ‘Lipid’ and ‘Starch’ diet treatments. It could be argued that this demonstrates that the metabolisable energy value (or more specifically the net energy value) of protein is greater than lipid which is greater than starch. However, the observation that a greater level of lipid deposition but an equivalent level of energy deposition occurs between protein and starch diet fed fish suggest that it is primarily the metabolic ‘fate’ of these nutrients that differs. Protein, whilst being able to be metabolised for both energy and as a nutrient source, clearly differs from starch which has only energetic value. Furthermore, in a species evolved to derive its energy almost exclusively from protein and lipid, the supply of energy from starch clearly causes metabolic complications. Analysis of gene expression levels of key rate limiting enzymes in energy metabolism

pathways supports this notion (Wade et al., 2013). Further examination of the fatty acid composition of the lipids deposited in each treatment should also provide further support for this hypothesis, given that barramundi have limited ability to elongate and desaturate fatty acids (Mohd-Yusof et al., 2010) there should be a skewing of fatty acids towards deposition of saturates and monounsaturates.

A number of studies on carnivorous fish have demonstrated that the digestible value for starch by these species can be substantial (Bergot & Breque, 1983; Enes et al., 2008; Glencross et al., 2012). However, few studies have followed up to examine the metabolisable energy value of this energy source (Saravanan et al., 2012). A range of studies have endeavoured to examine the 'ratios of lipid to starch' in diets for fish though usually this has not been done on a DE basis (Catactuan & Coloso, 1997). The present study demonstrates that, despite the starch content of the diet being highly digestible, that this starch energy it not translated into efficient 'growth' as defined by improved efficiencies of protein deposition. Instead, what occurred was a large increase in the lipid deposition efficiency but only a marginal increase in the energy deposition efficiency. What this indicates is that a large portion of the starch is being converted to lipid, but little of it is directly used to sustain energy needs for protein deposition within the animal. Indeed, the contrast of the 'Starch' diet fed fish to the 'Lipid' diet fed fish show that there are clearly problems with the effective metabolism of starch/glucose in this species. Similar observations have been reported before in other carnivorous fish (Enes et al., 2009).

A bias towards supply of energy by lipid did result in an increase in the efficiency of protein deposition, though the relative lipid deposition efficiency declined substantially. This can be easily interpreted by the fact that with the other diets the other macronutrients (which are in greater relative supply) are being actively converted to lipid as energy reserves. In contrast, fish fed the 'Lipid' diet, do not need to synthesise lipids from either starch or protein, as there is adequate supplies provided as dietary lipids. This effect has also been noted in other carnivorous fish (Dias et al., 1998).

The results reported by Saravanan et al. (2012) with rainbow trout indicated that the inclusion of starch as an energy source depressed growth and also feed intake. In the present study, in diets balanced for DE intake we also saw a depression in growth from the fish fed the 'Starch' diet, but in contrast an increase in feed intake was observed. Therefore, in contrast to rainbow trout, barramundi in this study attempted to compensate for the differences in the diets, despite the diets having been formulated at equivalent DP and DE levels.

Notably, the diets used in the present study differed substantially from those used by Saravanan et al. (2012) in that none of the diets were protein limiting. By ensuring that the DP:DE ratio exceeded the established requirements for this species at the size of animal being fed (Glencross, 2008, Glencross & Bermudes, 2012), it can be assured that the responses observed are solely due to energetic constraints and not potential nutrient limitation constraints. The results from the study by Saravanan et al. (2012) indicate that diets of equivalent DE, but limiting in DP result in growth depression and are supported by the observations from the present study. In other words, the

metabolisable energy value of the different macronutrients is not consistent with their DE basis and that this difference could also explain some of their observations. Indeed, the authors stated that they believe "control of DE intake might be a function of heat production". However, based on our results we observed an improved relationship as we moved the focus from DE Intake against HP ($R^2 = 0.59$) to NEI ($R^2 = 0.63$) of the diets, suggesting that perhaps it is more the NE value of the diet that dictates both performance and feed intake. Furthermore, the observation that there was no compensation for DP difference between the diets in the study of Saravanan et al. (2012) supports the notion that the fish are not eating to a DP demand, but rather an energy demand. These authors also asserted that changes in levels of plasma triglycerides or glucose did not exert an effect on DE intake. In addition, observations from the present study also reaffirm the lack of a 'lipostatic' effect, with the relationship between body lipid content and DE intake being very poor ($R^2 = 0.02$).

Conclusions and future directions

The outcomes of this study demonstrate that each of the three key macronutrient classes, protein, lipid and starch, clearly have different net energy values, which means that simplistic digestible energy based models need some reconsideration based on the actual metabolic fate of that energy. To assess the discrete energy values of each macronutrient, and to determine the partial efficiencies of utilisation of each energy source is the obvious next step in this regard.

The observation that the fish fed the 'Starch' diet are depositing substantial amounts of lipid could be further confirmed by assessing the fatty acid composition of the fat deposited in the fish, or even from discrete tissues in the animal like the liver, the dominant site of lipid synthesis. The observation that performance can be substantially improved through the increasing of protein content of the diet (notably the 'lipid' diet also had no starch) raises some considerations for improving commercial diet formulations, though putting this into practice in modern extruded feed designs will be a challenge. Further exploration in the use of cereals with high amylose contents relative to amylopectin provides some scope in this regard (Glencross et al., 2012).

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Tables and Figures

Table 1. Formulations and digestibility parameters of the key experimental diets and ingredients from experiment 1. All values are g kg^{-1}as is unless otherwise detailed.

Ingredient	Basal	Fishmeal	Starch	Casein	Gluten
Fishmeal	640.0	448.0	448.0	448.0	448.0
Fish oil	100.0	70.0	70.0	70.0	70.0
Cellulose	124.0	86.8	86.8	86.8	86.8
Wheat gluten	130.0	91.0	91.0	91.0	91.0
Fishmeal#		300			
Pregelatinised Starch			300		
Vitamin-Free Casein				300	
Wheat gluten					300
Vitamin-mineral premix*	5.0	3.5	3.5	3.5	3.5
Yttrium oxide	1.0	0.7	0.7	0.7	0.7
TOTAL	1000.0	1000.0	1000.0	1000.0	1000.0
Diet Apparent Digestibilities (%)					
ADC-Dry Matter	66.3±0.3	73.9±0.2	71.5±1.8	72.0±5.2	73.5±2.6
ADC-Protein	93.5±1.0	91.8±0.9	88.6±2.5	94.0±1.3	95.4±0.3
ADC-Energy	82.6±0.6	85.5±1.1	81.2±1.4	84.1±3.1	85.4±1.0
Ingredient Digestibilities (%)					
ADC-Dry Matter		91.8±0.8	84.0±6.0	84.8±16.8	90.5±8.6
ADC-Protein		93.2±2.6	0.0±340	100.0±3.4	100.0±1.0
ADC-Energy		95.2±3.8	86.3±5.9	87.1±9.6	98.1±3.5
Digestible Protein and Energy					
Digestible Protein (g kg^{-1}DM)		672	n/c	811	710
Digestible Energy (MJ kg^{-1} DM)		19.9	14.7	20.7	22.4

#same as fishmeal in row 1, but identified here to clarify its addition as a 'test' ingredient. * Vitamin and mineral premix includes (IU/kg or g/kg of premix): Vitamin A, 2.5MIU; Vitamin D3, 0.25 MIU; Vitamin E, 16.7 g; Vitamin K,3, 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. n/c : not calculated.

572 Table 2. Composition of the key ingredients used in each of the experiment diets. All values
573 are g kg^{-1}dry basis unless otherwise specified.
574

	Gluten[a]	Starch[a]	Cellulose[b]	Casein[b]	Fishmeal[c]
Dry matter (g kg^{-1} as is)	924	907	927	955	920
Protein	710	10	7	811	721
Digestible Protein	710	n/a	n/a	811	672
Lipid	46	1	1	1	85
Ash	8	3	2	13	158
Carbohydrates*	236	986	991	175	36
Starch	225	983	0	0	14
Energy (MJ kg^{-1}DM)	22.9	17.1	17.0	23.7	20.9
Digestible Energy (MJ kg^{-1}DM)	22.4	14.7	n/a	20.7	19.9
Alanine	20	0	0	31	43
Arginine	27	0	0	36	39
Aspartate	27	0	0	76	62
Cysteine	22	0	0	5	10
Glutamate	289	0	0	227	87
Glycine	26	0	0	18	40
Histidine	12	0	0	25	20
Isoleucine	28	0	0	50	29
Leucine	54	0	0	98	52
Lysine	10	0	0	74	49
Methionine	12	0	0	29	21
Phenylalanine	41	0	0	53	28
Proline	84	0	0	110	37
Serine	40	0	0	62	28
Taurine	0	0	0	0	7
Threonine	22	0	0	45	31
Tyrosine	28	0	0	58	22
Valine	29	0	0	64	32

575 [a] Wheat gluten and pregelatinised wheat starch: Manildra, Auburn, NSW, Australia. [b] Cellulose and Vitamin-free casein :
576 Sigma, St Louis, Missouri, United States. [c] Peruvian anchovetta fishmeal : Skretting Australia, Cambridge, TAS,
577 Australia.*Carbohydrates determined by 1000-(protein+ash+lipid). n/a : not applicable.
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580 Table 3. Formulations of the diets for Experiment 2
581

Ingredient	Control	Protein	Lipid	Starch	Negative
Fishmeal	560	640	560	560	450
Gluten	100	100	100	100	80
Casein	50	100	50	50	40
Fish oil	50	40	100	0	40
Pregelatinised Starch	120	0	0	240	95
Yttrium Oxide	2	2	2	2	2
Vitamin-mineral premix	5	5	5	5	5
Cellulose	113	113	183	43	288

582
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584 Table 4. Composition and digestible protein and energy parameters of the diets as
585 measured from experiment 2. All values are g kg^{-1}dry matter (DM) basis unless otherwise
586 detailed.
587

	Control	Protein	Lipid	Starch	Negative
Dry Matter (g kg^{-1} as is)	903	930	930	890	918
Crude Protein	527	633	510	502	402
Digestible Protein	475	575	476	448	368
Total Lipid	129	117	223	66	113
Ash	93	90	91	115	64
Total Carbohydrates	251	161	176	317	421
Total Starch	150	16	12	325	134
Gross Energy (kJ g^{-1} DM)	21.2	21.3	21.7	20.8	19.8
Digestible Energy (kJg^{-1} DM)	15.9	15.9	16.2	15.2	12.1
Alanine	30	35	28	28	21
Arginine	28	33	27	27	22
Aspartate	44	51	42	43	33
Cysteine	7	8	7	7	5
Glutamate	94	110	91	92	73
Glycine	28	33	27	27	21
Histidine	17	20	16	17	12
Isoleucine	23	28	22	23	18
Leucine	41	48	39	39	30
Lysine	32	40	34	31	23
Methionine	16	18	15	15	11
Phenylalanine	25	29	24	24	19
Proline	35	42	33	30	28
Serine	25	29	25	24	19
Taurine	4	5	4	4	2
Threonine	23	27	22	22	17
Tyrosine	20	22	19	19	15
Valine	26	31	24	25	20
Total amino acids	518	610	496	494	388

588 Table 5. Performance and carcass composition parameters of fish fed each of the
589 diets over the 84-day period.
590

	Control	Protein	Lipid	Starch	Negative	Pooled SEM
Initial weight (g fish^{-1})	82.0	80.9	81.6	81.5	80.3	0.11
Final weight (g fish^{-1})	370.6^{d}	389.7^{e}	368.6^{cd}	357.1^{c}	324.3^{b}	10.61
Gain (g fish^{-1})	288.6^{d}	308.8^{e}	287.0^{cd}	275.6^{c}	244.0^{b}	10.60
Gain Rate (g d^{-1})	3.48^{d}	3.72^{e}	3.46^{cd}	3.32^{c}	2.94^{b}	0.13
Survival (%)	100.0 ^{a}	100.0 ^{a}	98.3^{ab}	100.0 ^{a}	95.0^{b}	0.4%
Feed Intake (g fish^{-1} dry basis)	287.9^{bc}	265.6^{b}	281.0^{bc}	297.7^{bc}	318.3^{c}	7.63
DE Intake (kJ fish^{-1} dry basis)	4578^{c}	4223^{c}	4562^{c}	4537^{c}	3874^{b}	155.6
DP intake (g fish^{-1} dry basis)	136.7^{c}	152.7^{d}	133.9^{c}	133.3^{c}	117.1^{b}	4.1
FCR (feed gain^{-1} dry basis)	1.00^{b}	0.86 ^{a}	0.98^{b}	1.08^{bc}	1.31^{d}	0.03
Whole body composition						
DM (g kg^{-1})	334^{b}	329^{ab}	320 ^{a}	334^{b}	328^{ab}	1.3
Lipid (g kg^{-1})	84^{bc}	62 ^{a}	70^{ab}	97^{c}	83^{bc}	3.4
Protein (g kg^{-1})	172 ^{a}	170 ^{a}	188^{b}	165 ^{a}	179^{ab}	1.8
GE (MJ kg^{-1})	8.0^{b}	7.5 ^{a}	7.7 ^{a}	8.0^{b}	7.8^{ab}	0.6
Gastrointestinal tract composition						
DM (g kg^{-1})	677^{b}	608 ^{a}	639^{ab}	634^{ab}	672^{b}	11.2
Lipid (g kg^{-1})	455^{c}	304 ^{a}	369^{ab}	442^{bc}	454^{c}	15.6
Protein (g kg^{-1})	177^{b}	160^{ab}	174^{b}	139 ^{a}	151^{ab}	5.9
GE (MJ kg^{-1})	21.4^{b}	18.0 ^{a}	19.6^{ab}	19.9^{ab}	21.7^{b}	4.5
Head-On-Gutted composition						
Yield (%)	88.5^{ab}	89.2^{b}	89.5^{b}	88.7^{ab}	87.6 ^{a}	0.17
DM (g kg^{-1})	314 ^{a}	310 ^{a}	318^{b}	305 ^{a}	318^{b}	2.7
Lipid (g kg^{-1})	63^{b}	66^{b}	53 ^{a}	66^{b}	74^{c}	2.5
Protein (g kg^{-1})	177^{ab}	180^{ab}	185^{b}	168^{a}	178^{ab}	2.1
GE (MJ kg^{-1})	7.2	7.0	6.9	6.9	7.3	0.07

591 Superscripts denote significant (P<0.05) differences among dietary treatments within a parameter. Lack of any superscripts
592 within a row indicate that there were no significant differences among any of those treatments for that parameter.
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Table 6. Nutrient and energy deposition characteristics of fish from each treatment

		Control	Protein	Lipid	Starch	Negative	Pooled SEM
Final	Body DM (g kg^{-1})	334^{b}	329^{ab}	320^{a}	334^{b}	328^{ab}	1.3
	Body Protein (g kg^{-1})	172^{a}	170^{a}	188^{b}	165^{a}	179^{ab}	1.8
	Body Lipid (g kg^{-1})	84^{bc}	62^{a}	70^{ab}	97^{c}	83^{bc}	3.4
	Body Energy (MJ kg^{-1})	8.0^{b}	7.5^{a}	7.7^{a}	8.0^{b}	7.8^{ab}	0.06
Gain	Body DM (g)	98^{cd}	103^{d}	93^{bc}	94^{cd}	81^{b}	3.49
	Body Protein (g)	49^{bc}	52^{c}	55^{c}	44^{b}	44^{b}	1.77
	Body Lipid (g)	27^{bc}	20^{b}	22^{b}	31^{c}	23^{b}	1.35
	Body Energy (kJ)	2369^{c}	2348^{c}	2263^{c}	2291^{c}	1969^{b}	79.67
Efficiency	Protein deposition (%)	36.0^{b}	34.0^{a}	41.0^{c}	33.3^{a}	37.3^{b}	0.7
	Lipid deposition (%)	85.0^{b}	77.3^{b}	40.1^{a}	182.8^{c}	75.4^{b}	8.8
	Energy deposition (%)	51.8^{ab}	55.6^{c}	49.8^{a}	50.6^{a}	51.7^{ab}	1.0

Superscripts denote significant ($P<0.05$) differences among dietary treatments within a parameter. Lack of any superscripts within a row indicate that there were no significant differences among any of those treatments for that parameter.

609 Table 7. Nitrogen (protein), lipid and energy balance over the 84-day period
610

		units	Control	Protein	Lipid	Starch	Negative	Pooled SEM
Nitrogen	GNI	(g fish^{-1})	24.3^{c}	26.9^{d}	22.9^{bc}	23.9^{c}	20.5^{b}	0.7
	FN	(g fish^{-1})	2.4^{bc}	2.5^{c}	1.5^{a}	2.6^{c}	1.7^{ab}	0.1
	DNI	(g fish^{-1})	21.9^{c}	24.4^{d}	21.4^{c}	21.3^{c}	18.8^{b}	0.7
	BUN	(g fish^{-1})	14.0^{c}	16.1^{d}	12.7^{b}	14.3^{c}	11.8^{b}	0.5
	RN	(g fish^{-1})	7.8^{bc}	8.3^{c}	8.8^{c}	7.1^{b}	7.0^{b}	0.3
	RN/DNI	%	36.0^{b}	34.0^{a}	41.0^{c}	33.3^{a}	37.3^{b}	0.7
Lipid	GLI	(g fish^{-1})	37.2^{cd}	31.0^{bc}	62.7^{e}	19.6^{a}	35.9^{c}	2.5
	FL	(g fish^{-1})	5.2^{c}	4.3^{b}	8.8^{d}	2.7^{a}	5.0^{bc}	0.3
	DLI	(g fish^{-1})	32.0^{bc}	26.6^{b}	53.9^{d}	16.9^{a}	30.9^{bc}	2.1
	RL	(g fish^{-1})	27.2^{bc}	20.3^{b}	21.7^{b}	30.8^{c}	23.2^{b}	1.3
	RL/DLI	%	85.0^{b}	77.3^{b}	40.1^{a}	182.8^{c}	75.4^{b}	8.8
Energy	GEI	(kJ fish^{-1})	6113^{bc}	5819^{b}	6091^{bc}	6182^{bc}	6304^{c}	153.4
	FE	(kJ fish^{-1})	1535^{a}	1595^{a}	1529^{a}	1645^{a}	2430^{b}	74.8
	DEI	(kJ fish^{-1})	4578^{c}	4223^{c}	4562^{c}	4537^{c}	3874^{b}	155.6
	BUE	(kJ fish^{-1})	349^{c}	401^{d}	315^{b}	354^{c}	293^{b}	12.1
	MEI	(kJ fish^{-1})	4229^{d}	3823^{bc}	4247^{d}	4183^{cd}	3581^{b}	146.3
	RE	(kJ fish^{-1})	2369^{c}	2348^{c}	2263^{c}	2291^{c}	1969^{b}	79.7
	HP	(kJ fish^{-1})	1860^{cd}	1475^{b}	1984^{d}	1891^{cd}	1612^{bc}	84.1
	HeE	(kJ fish^{-1})	706^{b}	716^{b}	703^{b}	694^{ab}	664^{a}	9
	HiE	(kJ fish^{-1})	1154^{c}	758^{a}	1281^{c}	1198^{c}	949^{b}	78
	NEI	(kJ fish^{-1})	3075^{c}	3064^{c}	2966^{b}	2985^{bc}	2632^{a}	43
	RE/DEI	%	51.8^{ab}	55.6^{c}	49.8^{a}	50.6^{a}	51.7^{ab}	1.0

611 GNI: Gross Nitrogen Intake. FN : Faecal Nitrogen. DNI :Digestible Nitrogen Intake. BUN : Brachial and Urinary Nitrogen. RN :
612 Retained Nitrogen. GLI : Gross Lipid Intake. FL : Faecal Lipid. DLI : Digestible Lipid Intake. RL : Retained Lipid. GEI : Gross
613 Energy Intake. FE : Faecal Energy. DEI : Digestible Energy Intake. BUE : Brachial and Urinary Energy. MEI : Metabolisable Energy
614 Intake. RE : Retained Energy. HP : Heat Production. HeE : Basal Metabolism. HiE : Heat Increment Energy. NEI : Net Energy
615 Intake.