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

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5 Brett Glencross^{1,2}, David Blyth^{1,2}, Simon Irvin^{1,2}, Nicholas Bourne^{1,2}, Nick Wade^{1,2}

6
7
8 1. CSIRO Food Futures Flagship, PO Box 120, Cleveland, QLD 4163, Australia

9 2. CSIRO Marine and Atmospheric Research, PO Box 120, Cleveland, QLD 4163, Australia

10
11
12 (p) 61-7-3826-7236

13 (f) 61-7-3826-7281

14 (e) Brett.Glencross@csiro.au

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22 **Abstract**

23

24 It is generally considered that fish respond to dietary energy densities on a consistent basis
25 irrespective of what macronutrient source the dietary energy originates from. To test this assumption
26 two experiments were undertaken to establish the different roles of protein, lipid and starch as energy
27 sources in underpinning nutritional bioenergetics in juvenile barramundi, *Lates calcarifer*. To do this,
28 a range of ingredients were evaluated for their digestible protein (DP) and digestible energy (DE)
29 value. Following this, a series of diets were formulated to an equivalent DE basis, and observed a
30 minimum DP:DE ratio required for fish of 80g. However, in each of the diets the proportion of DE
31 available from protein, lipid or starch was varied to bias the contribution of each macronutrient on the
32 origin that digestible energy when fed to the fish. Growth of fish fed the protein diet was better than
33 those fed the lipid diet, which was better than those fed the starch diet. Feed intake was lower in the
34 protein diet than the lipid diet, and both were lower than the starch diet. Feed conversion was most
35 efficient in the protein diet fed fish, which was better than the lipid diet fed fish, which was better than
36 the starch diet fed fish. Whole-fish composition varied among treatments, with differences observed
37 in the dry matter composition, whole body lipid and gastrointestinal tract lipid content. Typically lipid
38 and dry matter composition were in synchrony, and were usually higher in the starch fed fish and
39 lower in the lipid fed fish. When flux of protein, lipid and energy was assessed in terms of deposition
40 efficiencies some significant differences were observed. Protein deposition efficiency was relatively
41 conservative, but ranged from 33% in the starch diet fed fish to 41% in the lipid diet fed fish. Lipid
42 deposition efficiency was more dramatic; ranging from 40% in the lipid diet to 182% in the starch
43 diet. Energy deposition efficiency was relatively conservative among treatments, ranging from 50% to
44 56% efficient. Overall the results from this study show that there is a clear hierarchy in preference for
45 energy substrates by juvenile barramundi, such that protein > lipid > starch.

46

47 **Introduction**

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

64 Utilisation of each of the different macronutrients for energy occurs by distinct metabolic
65 pathways, and occurs with different levels of efficiency in terrestrial animals, resulting in the
66 amendment of digestible values for diets and ingredients to metabolisable values (Azevedo et al.,
67 2005; Hua et al., 2010). Such a transition, while examined in a few instances in fish nutrition has
68 largely not gained much traction in the aquaculture feed sector (Bureau & Hua, 2008; Dumas et al.,
69 2010). In addition, there is increasing evidence that the roles of gluconeogenesis, glycolysis and β -
70 oxidation play substantially different relative roles in energy provision in fish compared to other
71 vertebrates (Enes et al., 2009; Lansard et al., 2010; Saravanan et al., 2012; Schrama et al., 2012). This
72 observation has important implications in the potential relative roles of each of the key macronutrients
73 in terms of dietary energy supply.

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

82 **Materials and Methods**

83 *Experiment 1 - design and fish management*

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

96 Juvenile barramundi (*Lates calcarifer*) were obtained from the Gladstone Water Board
97 Hatchery (Gladstone, QLD, Australia), and grown in a 10,000L tank being fed a commercial feed
98 (Marine Float; Ridley Aquafeed, Narangba, QLD, Australia). In preparation for this experiment, the
99 fish were transferred to a series of experimental tanks (300 L) with flow-through seawater (salinity
100 =35 PSU; dissolved oxygen 6.4 ± 0.18 mg L⁻¹) of 28.8 ± 0.22°C (mean ± S.D.) at a flow rate of about
101 3 L min⁻¹ being supplied to each of the tanks. Each of the tanks were stocked with 20 fish of 397 ± 69
102 g (mean ± S.D.; n = 40 from a representative sample of the population). Treatments were randomly
103 assigned amongst 10 tanks, with each treatment having four replicates. The experiment was conducted
104 over two block events to achieve this level of replication. The same batch of fish was used for both
105 blocks, but a complete randomised design applied to each block to ensure experimental validity. The
106 fish were allowed to acclimatise to their allocated dietary treatment for at least seven days before
107 faecal collection commenced.

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

118

119 *Chemical and digestibility analysis*

120 Diet, ingredient, faecal and whole fish samples were collected and their moisture content
121 determined by oven drying at 105 °C for 24 h. For the whole fish a second sample freeze-dried prior
122 to chemical analysis. Faeces were also freeze dried prior to analysis. Freeze-dried samples were
123 milled prior to analysis for dry matter, ash, fat, nitrogen, amino acid and gross energy content. Protein
124 levels were calculated from the determination of total nitrogen by CHNOS elemental auto-analyser,
125 based on N x 6.25. Carbohydrates were calculated based on the dry matter content of a sample minus
126 the protein, lipid and ash. Total starch content was measured using enzymatic methods with the
127 Megazyme Total Starch Kit, K-TSTA, following a modified AOAC Method 996.11. Amino acid
128 analysis involved the samples being hydrolysed at 110 °C for 24 h in 6 M HCl with 0.05 % Phenol.
129 Cystine was derivatized during hydrolysis by the addition of 0.05 % 3-3-dithiodipropionic acid. The
130 acid hydrolysis destroyed tryptophan making it unable to be determined. Separation of the amino
131 acids was performed by HPLC on a Hypersil AA-ODS 5µm column using an 1100 series Hewlett
132 Packard HPLC system. Total lipid content of the diets was determined gravimetrically following
133 extraction of the lipids using chloroform:methanol (2:1). Gross ash content was determined
134 gravimetrically following the loss of mass after combustion of a sample in a muffle furnace at 550 °C
135 for 12 h. Gross energy was determined by adiabatic bomb calorimetry.

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

$$140 \quad AD_{diet} = \left(1 - \left(\frac{Y_{diet} \times Parameter_{faeces}}{Y_{faeces} \times Parameter_{diet}} \right) \right) \times 100$$

143 where Y_{diet} and Y_{faeces} represent the yttrium content of the diet and faeces respectively, and
144 $Parameter_{diet}$ and $Parameter_{faeces}$ represent the nutritional parameter of concern (dry matter, protein or
145 energy) content of the diet and faeces respectively. The digestibility values for each of the test
146 ingredients in the test diets examined in this study were calculated according to the formulae:

$$147 \quad Nutr.AD_{ingredient} = \frac{(AD_{test} \times Nutr_{test} - (AD_{basal} \times Nutr_{basal} \times 0.7))}{(0.3 \times Nutr_{ingredient})}$$

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

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

158

159 *Experiment 2 - design and fish management*

160 A second experiment was conducted to compare the performance of barramundi fed a range
161 of diets varying in macronutrient concentrations, whilst providing equivalent DE densities (Tables 3
162 and 4). An additional control diet with a lower digestible energy density was also included. Fish were
163 obtained from the Gladstone Water Board Hatchery (Gladstone, QLD, Australia), and on-grown to
164 81.2 ± 1.48 g (mean \pm SD, n=480) in preparation for the experiment. During the on-growing period all
165 fish were fed the same diet (Nova-LE; Skretting Australia, Cambridge, TAS, Australia) and kept in 3
166 x 1000L seawater tanks. At the initiation of the trial 40 fish were weighed on an electronic top-
167 loading balance to 0.1 g accuracy to determine the mean and standard deviation of the population.
168 Following this 20 fish were allocated to each of 15 x 300L tanks based on having to be within the
169 mean \pm 1 x S.D. The experiment was conducted at the CSIRO Marine Research Laboratories at
170 Cleveland in a flow-through, aerated, heated seawater tank array. Water temperature was maintained
171 at 27.8 ± 0.45 °C (mean \pm S.D.) and dissolved oxygen 5.6 ± 0.18 mg L⁻¹ (mean \pm S.D.) for the 84 days
172 of the experiment. At the end of the 84 day period faeces were stripped from the fish for digestibility
173 assessment of each of the diets as per the methods described earlier.

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

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

191 bagged and stored at -20°C . Formulations and composition of the diets are presented in Tables 3 and
192 4 respectively.

193

194 *Sample preparation and chemical analysis*

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

204

205 *Nutrient and energy balance and deposition assessment*

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

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

$$\text{Nutrient Deposition (\%)} = \left(\frac{N_t - N_i}{N_c} \right) \times 100$$

224

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 \pm 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 $P < 0.05$.

236 **Results**

237 *Experiment 1 - Digestibility of experimental ingredients*

238 There were subtle differences among the digestibility parameters of the ingredients studied in
239 this experiment (Table 1). Ingredient protein digestibility ranged from of 93.2% for the fishmeal to
240 100% for both the casein and gluten (starch had no protein content to viably assess). However,
241 ingredient digestibilities for energy ranged from of 86.3% for the starch to 98.1% for the wheat
242 gluten.

243

244 *Experiment 2 - Growth and feed utilisation*

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

253 Digestible energy (DE) intake was relatively consistent amongst most treatments (~4450kJ
254 fish⁻¹), with only the negative control (3874 kJ fish⁻¹) being significantly different from any of the
255 other treatments. Digestible protein (DP) intake was more variable amongst the treatments (range
256 117.1 to 152.7 g fish⁻¹), being lowest in the ‘Negative’ diet fed fish and highest in the ‘Protein’ diet
257 fed fish. Intake of DP was significantly higher in the ‘Protein’ diet fed fish compared to both the
258 ‘Lipid’ and ‘Starch’ diets, which had almost identical levels of DP intake. Survival was high in all
259 treatments and not significantly different.

260

261 *Body composition*

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

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

273 across all treatments (Table 5). Lipid content of the HOG was highest for fish fed the ‘Negative’ diet
274 (7.4%) and lowest for fish fed the ‘Lipid’ diet (5.3%). Average lipid content across all treatments was
275 6.8%. The HOG gross energy content had little variability with samples ranging from 6.9 to 7.3 kJ g⁻¹.

276 In contrast, significant variation in the dry matter content of the GIT composition was
277 observed (range from 60.4% to 67.7%). Lipid composition of the GIT averaged 40.4% but also varied
278 significantly from 30.4% in the ‘Protein’ diet fed fish to 45.5% in the ‘Control’ diet fed fish, though
279 this was not significantly different from those fish fed the ‘Starch’ and ‘Negative’ diets. Gross energy
280 content of the GIT was largely consistent with the variation in lipid content of the GIT samples
281 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
282 variable ranging from 13.9% to 17.7% with an average of 15.4%.

283

284 *Protein, lipid and energy deposition efficiencies*

285 Protein deposition efficiencies were relatively conservative, but ranged from 33.3% for fish
286 fed the ‘Starch’ diet to 41.0% for fish fed the ‘Lipid’ diet (Table 6). Average protein deposition
287 efficiency across all treatments was 36.3%. Lipid deposition was much more variable ranging from
288 40.1% for the ‘Lipid’ diet to 182.8% for the ‘Starch’ diet. Average efficiency of lipid deposition was
289 92.1% across all treatments. Gross energy deposition was also much more conservative, ranging from
290 49.8% in the fish fed the ‘Lipid’ diet to 55.6% in fish fed the ‘protein’ diet. Across all treatments
291 energy deposition efficiency averaged 51.9%.

292

293 *Nitrogen, lipid and energy balance*

294 There were a range of significant differences in nitrogen balance among the different diets
295 (Table 7). Gross nitrogen intake ranged from 20.5 g fish⁻¹ for fish fed the ‘Negative’ diet to 26.9 g
296 fish⁻¹ for fish fed the ‘Protein’ diet and a similar consistent pattern was seen in brachial and urinary
297 nitrogen losses, and retained nitrogen levels.

298 Lipid balance was more variable, with lipid intakes ranging from 19.6 g fish⁻¹ for the ‘Starch’
299 diet to 62.7 g fish⁻¹ for the ‘Lipid’ diet (Table 7). Retained lipid was highest in the fish fed the ‘Starch’
300 diet (30.8 g fish⁻¹) and lowest in those fish fed the ‘Protein’ diet (20.3 g fish⁻¹).

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

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

313 **Discussion**

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

322

323 *Effects of digestible energy density on growth and feed utilisation*

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

335

336 *Effects of macronutrient source on growth and feed utilisation*

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

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

354 A number of studies on carnivorous fish have demonstrated that the digestible value for starch
355 by these species can be substantial (Bergot & Breque, 1983; Enes et al., 2008; Glencross et al., 2012).
356 However, few studies have followed up to examine the metabolisable energy value of this energy
357 source (Saravanan et al., 2012). A range of studies have endeavoured to examine the 'ratios of lipid to
358 starch' in diets for fish though usually this has not been done on a DE basis (Catactuan & Coloso,
359 1997). The present study demonstrates that, despite the starch content of the diet being highly
360 digestible, that this starch energy is not translated into efficient 'growth' as defined by improved
361 efficiencies of protein deposition. Instead, what occurred was a large increase in the lipid deposition
362 efficiency but only a marginal increase in the energy deposition efficiency. What this indicates is that
363 a large portion of the starch is being converted to lipid, but little of it is directly used to sustain energy
364 needs for protein deposition within the animal. Indeed, the contrast of the 'Starch' diet fed fish to the
365 'Lipid' diet fed fish show that there are clearly problems with the effective metabolism of
366 starch/glucose in this species. Similar observations have been reported before in other carnivorous
367 fish (Enes et al., 2009).

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

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

380 Notably, the diets used in the present study differed substantially from those used by
381 Saravanan et al. (2012) in that none of the diets were protein limiting. By ensuring that the DP:DE
382 ratio exceeded the established requirements for this species at the size of animal being fed (Glencross,
383 2008, Glencross & Bermudes, 2012), it can be assured that the responses observed are solely due to
384 energetic constraints and not potential nutrient limitation constraints. The results from the study by
385 Saravanan et al. (2012) indicate that diets of equivalent DE, but limiting in DP result in growth
386 depression and are supported by the observations from the present study. In other words, the

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

398

399 *Conclusions and future directions*

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

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

413

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415

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419

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556 **Tables and Figures**

557

558 Table 1. Formulations and digestibility parameters of the key experimental diets
559 and ingredients from experiment 1. All values are g kg⁻¹ as is unless otherwise detailed.

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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
<i>Diet Apparent Digestibilities (%)</i>					
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
<i>Ingredient Digestibilities (%)</i>					
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
<i>Digestible Protein and Energy</i>					
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

561 #same as fishmeal in row 1, but identified here to clarify its addition as a 'test' ingredient. * Vitamin and
562 mineral premix includes (IU/kg or g/kg of premix): Vitamin A, 2.5MIU; Vitamin D3, 0.25 MIU; Vitamin E,
563 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;
564 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;
565 Inositol, 58.3 g; Ethoxyquin, 20.8 g; Copper, 2.5 g; Ferrous iron, 10.0 g; Magnesium, 16.6 g; Manganese,
566 15.0 g; Zinc, 25.0 g. n/c : not calculated.

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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.
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	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
 583
 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

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

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

		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.