

Accepted refereed manuscript of:

Poppi D, Moore SS & Glencross B (2017) Redefining the requirement for total sulfur amino acids in the diet of barramundi (*Lates calcarifer*) including assessment of the cystine replacement value, *Aquaculture*, 471, pp. 213-222.

DOI: [10.1016/j.aquaculture.2017.01.009](https://doi.org/10.1016/j.aquaculture.2017.01.009)

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1 **Redefining the requirement for total sulfur amino acids in the diet of barramundi (*Lates***
2 ***calcarifer*) including assessment of the cystine replacement value.**

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5 David A. Poppi^{*1,2}, Stephen S. Moore¹, Brett D. Glencross³.

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7 ¹ The University of Queensland, Queensland Alliance for Agriculture and Food Innovation,
8 Centre for Animal Science, St. Lucia, QLD 4067, Australia.

9 ² CSIRO Agriculture and Food, St. Lucia, QLD 4067, Australia.

10 ³ Institute of Aquaculture, Stirling University, FK9 4LA Stirling, United Kingdom.

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12 *Corresponding Author: david.poppi@uq.net.au

13 Postal Address: CSIRO Agriculture and Food, Building 80, Services Road, St. Lucia, QLD
14 4067, Australia.

15 Phone: +61 7 3833 5527

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18 **Keywords:** Barramundi, Methionine, Total Sulfur Amino Acids, Requirement, Response
19 Model.

20
21 **Highlights:**

- 22
- 23 • Barramundi require 10.5-13.6g kg⁻¹ Met in a diet with 6.6g kg⁻¹ Cys (17.1-20.2g kg⁻¹
24 TSAA; 1.8-2.3% CP Met + 1.1% CP Cys).
 - 25 • Cystine can constitute at least 40% of the TSAA content of the diet of barramundi
26 without significantly affecting growth.
 - 27 • TSAA requirement is considerably higher than previously estimated, depending on
28 the mode of expression.
 - 29 • Nine nutrient response models fitted to current and previous data and requirement
30 estimates compared.
 - 31 • Mode of expression and choice and interpretation of nutrient response model can
32 greatly affect requirement estimates.
- 33
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36 **Abstract**

37 This study was designed to confirm a previous estimate of the methionine (Met) and
38 total sulfur amino acid (TSAA) requirement of juvenile barramundi (*Lates calcarifer*)
39 (Coloso *et al.*, 1999) with a view for further study. Triplicate groups of fish (initial weight:
40 $18.3\text{g} \pm 1.5\text{g}$) were fed diets with graded levels of dietary Met ($7.2 - 12.8\text{g kg}^{-1}$ DM), centred
41 around a previously reported requirement, and a constant dietary cystine (Cys) inclusion
42 (5.9g kg^{-1} DM) over a 42 day period. At the termination of the experiment, a significant
43 linear increase ($p < 0.001$) in %BW gain was observed in response to increasing dietary
44 methionine, with no plateau in growth, suggesting the previous estimate of requirement may
45 have been inadequate. A second experiment was designed to re-evaluate the Met/TSAA
46 requirement in which a broader range of methionine inclusion levels were assessed ($8.6 -$
47 21.4g kg^{-1} diet DM Met). Triplicate groups of fish (initial weight: $36.4\text{g} \pm 8.3\text{g}$) were fed the
48 diets for a period of 49 days. A plateau and subsequent depression in growth, as well as
49 significant ($p < 0.05$) effects of dietary Met inclusion on %BW gain, feed conversion ratio
50 (FCR) and protein retention efficiency (PRE) were observed at the conclusion of this
51 experiment. The best fitting of nine nutrient response models, the Compartmental Model (R^2
52 $= 0.71$), predicted a requirement for Met of between 10.5 (95% of maximum response) and
53 13.6g kg^{-1} (99% of maximum response) in a diet with 592g kg^{-1} CP and 6.6g kg^{-1} Cys ($17.1-$
54 20.2g kg^{-1} TSAA; 1.8-2.3% CP Met + 1.1% CP Cys). This TSAA requirement is equivalent
55 to 43-51% of the lysine content of the diets. The applicability of this mode of expression and
56 its relation to the ideal protein concept is discussed as is the application of different response
57 models to the data. The impact of dietary Met:Cys ratio was also investigated with results
58 suggesting at least 40% of dietary Met can be replaced with Cys without significantly
59 affecting animal performance. It was concluded that disparity in the estimates of Met and
60 TSAA requirement between this study and that of Coloso *et al.* (1999) was likely the result of
61 a combination of model choice, experimental design and mode of expression of the
62 requirements.

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71 **1. Introduction**

72 Studies focusing on individual amino acid requirements of barramundi (*Lates*
73 *calcarifer*) are limited, with estimates made for only four of the ten amino acids known to be
74 essential to fish (Methionine/TSAA, Coloso *et al.* (1999); Lysine and Arginine, Murillo-
75 Gurrea *et al.* (2001) and Tryptophan, Coloso *et al.* (2004)).

76 Of these, methionine (Met) is often the first limiting amino acid in fish diets
77 containing high levels of plant proteins (Ahmed, 2014). The primary role of this amino acid
78 is as a constituent of proteins and as a precursor for the synthesis of the proteinogenic amino
79 acid cysteine (Cys). It is also, however, known to have several important metabolic
80 functions, including acting as the initiating factor in the synthesis of proteins in eukaryotic
81 organisms (Drabkin and RajBhandary, 1998) and inhibiting proteolysis (Métayer *et al.*,
82 2008). Dietary deficiency of this amino acid has been shown to be related to the
83 development of cataracts (Cowey *et al.*, 1992; Simmons *et al.*, 1999), as well as
84 compromising protein retention and feed efficiencies in a number of fish species (Zhou *et al.*,
85 2006; Nwana *et al.*, 2012; He *et al.*, 2013). Additionally, its metabolites, particularly S-
86 Adenosyl Methionine (SAM) and taurine, play important roles in many metabolic processes
87 (Mato *et al.*, 1997; Lunger *et al.*, 2007; Espe *et al.*, 2008).

88 Methionine requirements of other fish species have been reported to vary widely,
89 ranging from 4g kg⁻¹ of diet for Mossambique Tilapia (*Oreochromis mossambicus*) (Jackson
90 and Capper, 1982) up to 20.3g kg⁻¹ of diet reported by Zhou *et al.* (2011) for Black Sea
91 Bream (*Sparus macrocephalus*). Cysteine, a metabolite of methionine, and its dimer cystine
92 (both abbreviated as Cys), while not essential amino acids, are known to be capable of
93 replacing between 33% and 60% of the requirement for methionine in various fish species
94 (Harding *et al.*, 1977; Moon and Gatlin, 1991; Abidi and Khan, 2011). The inclusion level
95 which elicits peak growth in dose response studies based on variable methionine addition,
96 therefore, can be greatly influenced by the amount of Cys in the diet, potentially confounding
97 precise estimation of the methionine specific requirement (NRC, 2011). Consequently,
98 reported requirements for methionine are generally expressed as either a total sulfur amino
99 acid (TSAA) requirement (Met+Cys) or as a methionine specific requirement “in the
100 presence of (a certain proportion of) Cys”.

101 Despite its importance in carnivorous marine fish diets, only one study has so far
102 endeavored to determine the requirement of barramundi for methionine/TSAA. Using a
103 break point analysis on the growth response curve, Coloso *et al.* (1999) estimated the TSAA
104 requirement of juvenile barramundi to be 13.4g kg⁻¹ dry diet (10.3g kg⁻¹ Met+3.1g kg⁻¹ Cys)
105 (2.9% of protein in a 460 g kg⁻¹ protein diet). Uncertainty surrounding the calculated amino
106 acid composition of the diets and the choice of response model used suggested that

107 revisitation of this estimate was wise. Due to the limited abundance of Met in plant proteins,
108 it is imperative to accurately identify the minimum dietary requirement for this nutrient if the
109 use of cheaper and more sustainable plant protein sources is to become more widespread in
110 commercial diets for this species.

111 The primary objectives of this series of experiments were to provide an estimate of
112 the TSAA requirement for maximum growth in barramundi and to investigate the effect of
113 replacement of Met (limiting in non-cereal plant protein meals) with its metabolite Cys
114 (relatively abundant in plant proteins).

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116 **2. Materials and Methods**

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118 *2.1. Diets*

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120 *2.1.1. Formulation*

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122 *2.1.1.1. Experiment One*

123 A series of five isonitrogenous and isoenergetic diets were formulated (Table 1) with
124 variable Met inclusion ranging from 7.2 to 12.8g kg⁻¹ DM, centring around the requirement
125 of 10.3g kg⁻¹ DM established by Coloso *et al.* (1999), and with a constant Cys content (5.9g
126 kg⁻¹ DM). The non-essential amino acid glycine was substituted in place of DL-methionine
127 to maintain the total crystalline amino acid, protein and energy contents of the diets as has
128 been used in several other methionine/TSAA requirement studies with other species
129 (Simmons *et al.*, 1999; Liao *et al.*, 2014). These diets were used to determine the response of
130 barramundi to limitation and excess of methionine and TSAA and to estimate the requirement
131 for maximum growth.

132 All diets were supplemented with a mix of crystalline amino acids to ensure all
133 essential amino acids were provided in excess of requirements according to the ideal protein
134 concept based on the amino acid profile reported by Glencross *et al.* (2013).

135 Yttrium oxide was included in all diets at a concentration of 1g kg⁻¹ for the purposes
136 of digestibility assessment.

137 Finally, a commercial barramundi diet (6mm Marine Float, Ridley Aquafeed Pty Ltd),
138 proven to promote good growth in barramundi housed in the holding tanks at the Bribie
139 Island Aquaculture Research Centre, was used as a reference.

140

141

142 2.1.1.2. *Experiment Two*

143 Five isonitrogenous and isoenergetic diets were produced (Table 2) with a broader
144 range of methionine inclusion levels (8.6-21.4g kg⁻¹ DM) than that used in Experiment One
145 in order to find the break point in growth not seen in that earlier experiment (Diets 1-5).

146 In addition, five diets (Diets 6-10) were produced with a constant TSAA inclusion
147 level of 2.9% CP (marginally deficient of the anticipated maximum response, based on the
148 results of Experiment One), with Cys constituting between 22 and 54% of the dietary TSAA
149 content. These diets were designed to determine the proportion of the requirement for
150 methionine which can be spared by addition of dietary cystine (theorised to be a possible
151 contributing factor to an unexpected lack of a maximum response to methionine inclusion in
152 Experiment One).

153

154 2.1.2. *Diet manufacture*

155 Diets were prepared, manufactured on a laboratory-scale twin-screw extruder (APV
156 MFP19:25; APV-Baker, Peterborough, UK) and vacuum infused with fish oil according to
157 the protocol outlined in Glencross *et al.* (2016) with the exception that a 3mm die was used in
158 order to obtain pellets with a final diameter of 4mm.

159

160 2.2. *Fish management and faecal collection*

161 Experiments were conducted in accordance with the Australian Code of Practice for
162 the Care and Use of Animals for Scientific Purposes, under the approval of the CSIRO
163 Animal Ethics Committee (approval numbers: A13/2013 and A6/2014) and The University of
164 Queensland Animal Ethics Committee (approval number: CSIRO/QAAFI/391/14).

165 The experiments were run as six treatments (Experiment One) or 10 treatments
166 (Experiment Two), each being randomly assigned to tanks and replicated three times.

167 Forty juvenile hatchery-reared barramundi (*Lates calcarifer*) were individually
168 weighed from a pooled population to 0.1 g accuracy to obtain a population average weight.
169 Forty (*Experiment One*) or 25 (*Experiment Two*) fish within a weight range of (population
170 mean weight \pm 1 standard deviation) (18.3g \pm 1.5g – *Experiment One*; 36.4g \pm 8.3g –
171 *Experiment Two*) were randomly allocated to each of the 18 (*Experiment One*) or 30
172 (*Experiment Two*) 1000L tanks. A limited availability of suitably sized fish for Experiment
173 Two resulted in a reduced number of animals for this experiment. Fish were anaesthetised
174 using AQUI-S (~0.02mL/L) (AQUI-S New Zealand Ltd) prior to weighing and allowed to
175 recover in their allocated tank.

176 The experimental tanks were set up with ~3 L/min flow of continuously aerated
177 marine water (~35PSU) of $29.5^{\circ}\text{C} \pm 0.2^{\circ}\text{C}$ for the duration of the experiment. Photoperiod
178 was set at 12:12 (light:dark).

179 In order to avoid effects attributable to variation in feed intake and to focus on
180 responses to feed composition variation only (Glencross *et al.*, 2007), a restricted pair-fed
181 feeding strategy was employed in both experiments. Fish were fed a commercial barramundi
182 diet (4mm (*Experiment One*) or 6mm (*Experiment Two*) Marine Float, Ridley Aquafeed Pty
183 Ltd) to satiety twice daily for 7 days prior to the start of the experiment to establish a satiety
184 feeding rate. The average daily feed intakes were observed to be $0.7\text{g fish}^{-1}\text{ day}^{-1}$
185 (*Experiment One*) and $1.3\text{g fish}^{-1}\text{ day}^{-1}$ (*Experiment Two*), which compared well with the
186 expected intake for barramundi of this size (18.1g and 35.4g average weight respectively)
187 estimated by a published growth and feed utilisation model (Glencross and Bermudes, 2012).

188 Based on this, the initial rations were set at $0.6\text{g fish}^{-1}\text{ day}^{-1}$ (*Experiment One*) and
189 $1.0\text{g fish}^{-1}\text{ day}^{-1}$ (*Experiment Two*). These restricted rations were manually fed to each tank
190 twice daily at 0800 and 1600, seven days a week. The ration was increased by 0.2g fish^{-1}
191 day^{-1} weekly, except as needed (it was increased by $0.4\text{g fish}^{-1}\text{ day}^{-1}$ on Day 7 and Day 29 of
192 *Experiment Two* based on enthusiastic feeding response in all tanks).

193 The amount of feed fed was recorded daily for calculation of feed conversion and feed
194 efficiency ratios. Any uneaten feed was removed and weighed for consideration in these
195 calculations and an equivalent amount was added to the following feeding event. Feed intake
196 was equal for all tanks used in the experimental assessments.

197 All feed was kept in cold storage ($< 4^{\circ}\text{C}$) except for the purposes of feeding and
198 weighing.

199 At the conclusion of the growth trial, faeces were collected by stripping in order to
200 determine the digestible protein and energy contents of the feed. Fish were manually fed
201 their respective diets at 0800-1000 and faeces collected from all fish in the afternoon of the
202 same day (1600-1800). Fish were stripped on three separate, non-consecutive, days with the
203 intention of minimising stress and maximising feed intake on the collection days. Stripping
204 of faeces was undertaken in accordance with the procedures outlined in Glencross (2011). All
205 fish within each tank were transferred to a smaller tank containing aerated seawater with a
206 light dose of AQUI-S ($\sim 0.02\text{mL/L}$) until loss of equilibrium was observed. During
207 anesthesia, particular attention was paid to the relaxation of the ventral abdominal muscles to
208 ensure fish were removed from the tank and faeces collected before involuntary evacuation.
209 At this time, faeces were stripped from the distal intestine using gentle abdominal pressure,
210 collected in a plastic specimen jar (one pooled sample per tank) and frozen at -20°C . Hands

211 were rinsed between fish in order to minimise contamination of the faeces with urine or
212 mucous.

213

214 2.3. Sample collection

215 A random sample of five fish were euthanised by overdose of anaesthetic (AQUI-S)
216 at the commencement of the experiments for baseline proximate analysis and stored at -20°
217 C. At the conclusion of the experiments, all fish were lightly anaesthetised and individually
218 weighed for determination of growth rate and comparison of growth between treatments. A
219 random sample of five (Experiment One) or three fish (Experiment Two) from each tank was
220 also taken at this time. These animals were euthanised by overdose of anaesthetic (AQUI-S)
221 and stored at -20°C until processing. Feed was withheld for 24 hours prior to sampling.

222

223 2.4. Chemical and digestibility analyses

224 Whole animals, diets and ingredients were analysed for dry matter, ash, nitrogen,
225 lipid, gross energy and amino acid profiles. Diets and faeces were additionally analysed for
226 yttrium content. Faeces and minced carcass samples were freeze dried and all samples were
227 ground prior to analysis.

228 Carcass and diet dry matter contents were determined by gravimetric analysis following
229 drying at 105°C for 16h. Gross ash contents were similarly determined based on mass change
230 after combustion in a muffle furnace at 550°C for 16 hours. The lipid portion of the samples
231 was extracted according the method proposed by Folch *et al.* (1957) and used to determine
232 crude lipid contents. Measurement of total nitrogen content was undertaken using a CHNS
233 auto-analyser (Leco Corp., St. Joseph, MI, USA) and used to calculate sample protein content
234 based on $N \times 6.25$. Gross energy was determined by isoperibolic bomb calorimetry in a Parr
235 6200 oxygen bomb calorimeter (Par Instrument Company, Moline, IL, USA). Amino acid
236 compositions were determined by mass detection after reverse-phase ultra high-performance
237 liquid chromatography with pre-column derivatisation with 6-aminoquinolyl-N-
238 hydroxysuccinimidyl (AQC). Analyses were undertaken on a Shimadzu Nexera X2 series
239 UHPLC (Shimadzu Corporation, Kyoto, Japan) with quaternary gradient module, coupled
240 with a Shimadzu 8030 Mass Spectrometer using the Waters AccQ·tag system (Waters
241 Corporation, Milford, MA). Samples were prepared according to the protocol for complex
242 feed samples outlined by Waters Corp. (1996) following hydrochloric acid hydrolysis.
243 Cyst(e)ine is known to be destroyed during acid hydrolysis and methionine can be oxidized to
244 methionine sulfone (Rutherford and Gilani, 2009). These amino acids were determined
245 independently as cysteic acid and methionine sulfone respectively, after oxidation with
246 performic acid according to an adaptation of the protocol of Chavali *et al.* (2013) (using

247 11mL glass vials and drying by Speedivac vacuum drier), followed by HCl hydrolysis as
248 previously described. Correction factors were also applied in the conversion of cysteic acid
249 to Cys and methionine sulfone to Met, to account for differences in molecular weights.

250 Yttrium concentrations in the feed and faeces were determined by inductively coupled
251 plasma mass spectrometry (ICP-MS) after microwave digestion in 5mL HNO₃ based on a
252 modification of EPA method 3051 (EPA, 1994). The apparent digestibilities (AD_{Parameter}) of
253 individual nutritional parameters (DM, protein and gross energy) were calculated by the
254 differences in the ratios of the parameter of interest in the diets and faeces based on the
255 following formula (Maynard and Loosli, 1969):

256

$$257 \quad AD_{\text{Parameter}} = \left[1 - \left(\frac{Y_{\text{diet}} \times \text{Parameter}_{\text{faeces}}}{Y_{\text{faeces}} \times \text{Parameter}_{\text{diet}}} \right) \right] \times 100$$

258

259 Where: Y_{diet} and Y_{faeces} represent the yttrium content of the diet and faeces, respectively, and
260 $\text{Parameter}_{\text{diet}}$ and $\text{Parameter}_{\text{faeces}}$ represent the nutritional parameter of interest (DM, protein or
261 energy) content of the diet and faeces, respectively. These digestibility values were then used
262 to calculate digestible protein and energy values of the diets.

263

264 2.5. Statistical analysis

265 The trends of the responses (linear, quadratic or cubic) to variable methionine
266 inclusion in both experiments were analysed by orthogonal polynomial contrast analysis. Due
267 to inequality of the spacing of the Cys replacement treatments in Experiment Two, the linear,
268 quadratic and cubic effects of this series of treatments was analysed by multiple regression
269 analysis. All parameters of interest within each experiment (Final Body Weight, % Body
270 Weight Gain, FCR, Feed Intake and Protein and Energy Retention Efficiencies) were
271 analysed by One-Way ANOVA with *post hoc* comparison of treatment group means by
272 Tukey's HSD multiple range test in order to illustrate the magnitude of the differences. All
273 statistical tests were conducted in the R-project statistical environment (R Core Team, 2014).
274 Effects were considered significant at $p < 0.05$.

275 Data for percent body weight gain in response to variable dietary TSAA in
276 Experiment Two was analysed using regression response models as described by Vedenov
277 and Pesti (2008). Eight models (Table 5) previously applied to the estimation of animal
278 nutrient requirements (linear and quadratic ascending broken line, four-parameter Saturation
279 Kinetics, three- and four-parameter logistics models, a compartmental model, a sigmoidal
280 model and an exponential model) and subsequently developed in Excel workbooks by those
281 authors were applied to the data. The fit of each of the models was optimised through the

282 iterative adjustment of each model parameter using the solver function of Excel to minimise
283 the sum of squared errors (SSE). A five-parameter Saturation Kinetics Model was also
284 developed in Excel and fitted in the same way. The coefficient of determination (R^2) was
285 calculated for each of the models according to Pesti *et al.* (2009) and compared, along with
286 the SSE, as a measure of the goodness of fit of each model. Estimates of Met requirement
287 were also derived from each model for comparison.

288

289 **3. Results**

290

291 *3.1. Experiment One*

292

293 *3.1.1. Response to increasing dietary methionine content*

294 Highly significant ($p < 0.001$) linear effects on final body weight, percent body weight
295 gain, feed conversion ratio (FCR), energy and protein retention efficiencies (ERE, PRE) and
296 carcass crude protein content and significant ($p < 0.05$) linear effects on ERE and carcass lipid
297 and gross energy compositions were observed in response to increasing dietary Met inclusion
298 (Table 3 and Fig. 1).

299 The ERE and carcass DM, lipid and GE content responses had significant ($p < 0.05$)
300 quadratic components to their response. Significant ($p < 0.05$) improvements in FCR, %BW
301 gain and final weight were seen between diets with 7.2, 9.8 and 12.8g kg^{-1} Met. The
302 efficiency of protein retention (PRE) was observed to only differ significantly between fish
303 fed the diets with the lowest two methionine inclusion levels and three highest levels and
304 ERE between the lowest and three highest methionine inclusion treatments. Carcass crude
305 protein content was significantly higher in fish fed Diet 5 (12.8g kg^{-1} Met) compared with
306 those fed Diets One and Two (7.2 and 8.4g kg^{-1} Met). Conversely, fish fed Diet 5 had
307 significantly lower lipid and gross energy contents than those fed Diet 2. Carcass dry matter
308 and ash contents were not significantly different between treatments. Survival was 100% in
309 all treatments.

310

311 *3.1.2. Experiment Two.*

312

313 *3.1.2.1. Response to increasing dietary methionine content*

314 In the second experiment, there was observed to be highly significant ($p < 0.001$) linear
315 effects of dietary methionine content on final weight, FCR, PRE and carcass crude protein
316 contents and significant ($p < 0.05$) linear effects on %BW gain and ERE (Table 4). Final
317 weight, FCR and PRE responses were also determined to have highly significant ($p < 0.001$)
318 quadratic and significant ($p < 0.05$) cubic components. The %BW gain response had a

319 significant ($p < 0.05$) quadratic component. Significant improvements in final weight, %BW
320 gain, FCR and PRE were seen between those fish fed the diet with the lowest methionine
321 content and those fed all other diets. Carcass compositions were not significantly different,
322 with the exception of the crude protein content which fluctuated.

323 Preliminary evaluation of data assessing the effect of variable dietary methionine
324 content on various indicators of growth (final weight, body weight gain, %BW gain, Specific
325 Growth Rate) suggested that percent BW gain was the most appropriate response variable
326 with which to fit the models. This decision was based on statistical significance and, given
327 the relatively small numerical variation in final body weights, its consideration of the small
328 variations in initial body weight. Nine models (Table 5) were fitted to the data using dietary
329 methionine content as the independent and average weight gain as a percent of initial weight
330 of each replicate tank as the response variable. All models fit the data well but, based on
331 maximum R^2 and lowest SSE, the Compartmental Model (Fig. 1) was deemed the most
332 appropriate model, explaining 71% of the variation in percent body weight gain (Table 6) and
333 predicting a dietary methionine requirement of between 10.5 (± 1.30 g 95% confidence
334 intervals) (95% of maximum response) and 13.6g kg^{-1} DM (± 0.88 g 95% confidence
335 intervals) (99% of maximum response) methionine in a diet with 592g kg^{-1} CP and 6.6g kg^{-1}
336 Cys (1.8-2.3% CP Met + 1.1% CP Cys).
337 Survival was 100% in all treatments.

338

339 3.1.2.2. Response to variable proportions of Met:Cys in the diet.

340 Percent body weight gain responded in a significantly linear fashion ($p < 0.05$) in
341 response to increasing replacement of dietary Met with Cys, with a significantly quadratic
342 component ($p < 0.05$) (Table 7). Protein retention efficiency and carcass lipid content followed
343 significantly ($p < 0.05$) quadratic and linear trends respectively. While significant trend effects
344 were seen, no significant differences in any parameter were observed between treatments.
345 Survival was 100% in all treatments.

346

347 3.1.3. Re-evaluation of Coloso *et al.* (1999) Data.

348 The nine models previously described were also fitted to the %BW gain data of
349 Coloso *et al.* (1999) for the purpose of assessment of the validity of the model chosen by
350 those authors to estimate the methionine requirement of barramundi. This re-assessment
351 demonstrated that the Broken-Line with Linear Ascending Line Model may not have been the
352 most appropriate model. Based on high R^2 and low SSE, three models with a quadratic
353 component (5-SKM, Broken-Line with quadratic ascending line and the compartmental
354 model) were shown to describe the response more accurately. Of these three, the model

355 which fit the data most closely was the five-parameter Saturation Kinetics Model (Table 8)
356 which estimated a methionine requirement of between 8.9 (95% of the maximum response)
357 and 10.3g kg⁻¹DM (99% of the maximum response) compared with 10.1g kg⁻¹ by the reported
358 model (reported by the authors as 10.3g kg⁻¹).

359

360 3.1.4 Essential amino acid composition of juvenile barramundi.

361 The analysed EAA composition (+Cys) of juvenile barramundi of a similar size to
362 that used in the present study is presented in Table 9 for comparison with that of the diets.

363

364 4. Discussion

365

366 The results of the first experiment (Experiment One) suggested that the previous
367 estimate, provided by Coloso *et al.* (1999), may have underestimated the true requirement for
368 Met. In that experiment, despite the two diets with the highest inclusion of Met (and, by
369 extension, TSAA) being well above the requirement estimated by Coloso *et al.* (1999) for this
370 species, as well as for other carnivorous fish species (Sveier *et al.*, 2001; NRC, 2011), there
371 was no apparent leveling off in growth, with percent body weight gains of fish increasing in a
372 significantly linear fashion in response to increasing dietary TSAA. This may be the result of
373 a number of factors. Firstly, the one-slope break-point (broken-line model) analysis used by
374 Coloso *et al.* (1999) to estimate the requirement may have been inappropriate, resulting in
375 underestimation of the requirement. Non-linear models, such as the four- and five-parameter
376 saturation kinetics models, derived from the Michaelis-Menten model for enzyme-catalyzed
377 reaction velocity (Michaelis and Menten, 1913) and developed and described by Mercer and
378 others in a series of reports (Mercer *et al.*, 1975; Mercer, 1980; Mercer *et al.*, 1986; Mercer *et al.*
379 *et al.*, 1989), are considered to be more accurate representations of biological responses
380 compared with those which “force responses to conform to straight lines” (Pesti *et al.*, 2009).
381 In a re-evaluation of the Coloso *et al.* (1999) data outlined in Table 8, the most complex
382 model (the 5-SKM) best described the observed response but nevertheless predicted a
383 requirement similar to that estimated by the two-slope Broken-Line model. This result,
384 however, may be confounded by the presence of only one data point after the asymptotic
385 response. It may be that more points are required on the downward aspect of the slope (as
386 seen in the result of the present study) to establish a clearer pattern of growth decline after the
387 asymptotic response in order to accurately estimate the growth inhibition component of the
388 model. The Broken-Line with Quadratic Ascending Line model fitted the data almost
389 equally as well and estimated a requirement for methionine of 11.8g kg⁻¹. This figure is 140%
390 of the requirement predicted by the Broken-Line with Linear Ascending Line model used by

391 Coloso *et al.* (1999), highlighting the effect of model choice in nutrient requirement
392 estimates. Perhaps it may have been prudent to conduct this re-evaluation prior to designing
393 Experiment One, in which case higher levels of TSAA would have been evaluated, possibly
394 resulting in emergence of a plateau in the response. This re-analysis, however, was
395 conducted using only the mean percent body weight gains of each treatment and should be
396 considered a representation only. It is not clear whether Coloso *et al.* (1999) used the
397 individual experimental units or averages for their analysis, however, consideration of all
398 replicates and the variation within may have yielded a different result.

399 Based on this hypothesis, a greater range of dietary methionine inclusion levels were
400 investigated in Experiment Two and, as expected, the response in body weight gain (as a
401 percentage of initial weight) appeared to reach a peak and declined thereafter. Of the nine
402 nutrient response models fitted to this data, the Compartmental Model accounted for the
403 greatest amount of variation in the data ($R^2=71$), estimating the requirement of juvenile
404 barramundi for methionine to be between 10.5 (95% of maximum response) and 13.6g kg⁻¹
405 DM (99% of maximum response) methionine in a diet with 592g kg⁻¹ CP and 6.6g kg⁻¹ Cys
406 (1.8-2.3% CP Met + 1.1% CP Cys; 2.9 – 3.4% CP TSAA). It has been suggested previously,
407 though not proven experimentally to our knowledge, that amino acid requirements may be
408 affected by, among other things, fish size (Twibell *et al.*, 2000). This is likely based on the
409 observation that fish in general have a reduced requirement for dietary protein with
410 increasing size (Wilson, 2002). Perhaps, then, it may be wise to consider the current
411 requirement figures as being so only for barramundi of the size investigated (18-120g).

412 If the output of the Compartmental Model is to be used to establish the requirement
413 level, it must be decided whether it is more appropriate to consider the requirement as being
414 the input (dietary Met level) which elicits the response at 95% or 99% of the maximum
415 output (growth) predicted by the model (which are considerably different in this case). This
416 may depend on the purpose for which the figure is required. While statistically little gain is
417 predicted to be made above the 95% level (a significant observation for commercial feed
418 formulators), it is important to report the asymptote of the response for the purposes of
419 further scientific investigation into the effects of Met supplementation. This may be
420 especially relevant when it comes to the application of more sensitive molecular techniques
421 as a means of assessing the impact of dietary amino acid supply. As such, both estimates are
422 presented for consideration and the figure is shown with indications of the Met levels
423 eliciting 95% and 99% of the maximum response. The Met requirement estimate of 10.3g
424 kg⁻¹ proposed by Coloso *et al.* (1999) is within the lower 95% confidence interval of the
425 prediction by the compartmental model for the Met level eliciting 95% of the asymptotic

426 response in the present study, however, is well outside predictions for maximising growth in
427 this species (99% of the asymptotic response).

428 This disparity, when considered on a g kg^{-1} basis, is amplified when TSAA
429 requirements are calculated. This is due to the fact that the proportion of Met:Cys was
430 significantly higher in the diets of Coloso *et al.* (1999) than in those in this study, possibly
431 due to underestimation of the dietary Cys content in that study. Whilst variability in soybean
432 meal (SBM) quality has been widely reported (Dale, 1996; Thakur and Hurburgh, 2007), the
433 analysed Cys content of this ingredient used for formulation of the diets (and ultimately
434 interpretation of the results) is somewhat low when compared with other published SBM
435 composition data, such as that of Cromwell *et al.* (1999). Amino acid composition was
436 determined using “automated amino acid analysis”, however the authors did not elaborate on
437 the procedure used. It is well documented that sulfur amino acids can be degraded during the
438 acid hydrolysis step of amino acid analysis, requiring either pre-hydrolysis oxidation with
439 performic acid (Fountoulakis and Lahm, 1998; Rutherford and Gilani, 2009) or a correction
440 factor be applied. If neither of these was applied in this case, underestimation of the content
441 of these amino acids may have occurred (particularly for Cys of which a large proportion is
442 readily destroyed by HCl hydrolysis).

443 It has been reported that Cys can replace between 33% (Abidi and Khan, 2011) and
444 60% (Harding *et al.*, 1977) of the dietary Met requirement of various fish species. Some
445 authors, however, have suggested that the Met sparing effect of Cys may be limited to 3 g kg^{-1}
446 (NRC, 2011). It was on the basis of this question that the Met replacement value of Cys in
447 diets for barramundi was investigated as part of Experiment Two.

448 In that part of the experiment, significant linear and quadratic effects on percent
449 weight gain of increasing replacement of Met with Cys (suggesting a quadratic response with
450 a shortened tail), taken with the numerical depression in this parameter in fish fed the diet
451 with the highest level of methionine replacement (Diet 10), indicate that the limit of the
452 ability of Cys to replace dietary Met in diets for juvenile barramundi may lie between 40 and
453 54% of TSAA. The depression observed may also be due to a lower crude and digestible
454 protein content measured in Diet 10, allowing for the possibility that replacement of Met by
455 Cys at this high level is also feasible. Protein retention efficiency and %BW gain responded
456 in a significant manner (significant ($p < 0.05$) quadratic effect on PRE and significant ($p < 0.05$)
457 linear and quadratic trends in the %BW gain data) with numerical, but not significant,
458 increases in these parameters with increasing Cys up to Diet 9 (40% of TSAA as Cys) which
459 suggests that up to 6.1 g kg^{-1} Cys may be usable by barramundi. This figure is only slightly
460 below that used in the diets in Experiments One and Two (6.6 g kg^{-1}), suggesting that the

majority of the resulting combined TSAA component of the diets was usable and that excess dietary Cys can be excluded as a confounding factor in the response to increasing dietary TSAA. The TSAA requirement estimate can then be considered to be reliable in this case. If it is accepted that the Cys included in the diets in the present study was completely usable, this lends more credence to the theory that the true Cys value of the diets in the study of Coloso *et al.* (1999) may have been underestimated. Confirmation of the results using diets with lower Cys inclusion may answer this question.

Another confounding factor in the comparison of the results of this study with those of Coloso *et al.* (1999) is the differences in the crude protein (CP) content of the diets (~590 g kg⁻¹ in this study compared with ~460 g kg⁻¹ in that of Coloso). The higher CP content used in the present study is in line with the recommendations of the feed utilisation model of Glencross and Bermudes (2012) for the ideal protein to energy ratio for barramundi of this size. The consequence of the higher CP content being, for example, that the diet containing the “adequate” level of methionine in Experiment One (Diet 3), around which the other diets were formulated, was similar in Met content to the requirement estimated by Coloso *et al.* (1999) on a g kg⁻¹ basis, however due to the higher crude protein content, this proportion on a unit of protein basis was lower. It has been argued in the past that EAA requirements may be linked to the dietary protein content due to a need to maintain a balance in the dietary amino acid profile (Cowey and Cho, 1993). Given the similarities in estimates of Met requirements between this study and that of Coloso *et al.* (1999) when compositions are expressed on a percentage of crude protein basis, it appears that this may have been a more appropriate foundation on which to formulate the diets (at least the Met levels) or that the differences in CP contents should have been taken into consideration. This however, is in disagreement with the assertions of the NRC (2011) who cite the findings of several studies on Lys requirements across species where similar estimates of requirement were reported in spite of highly variable dietary CP contents. Perhaps the circumstance is different for EAAs other than lysine, although there is no published literature comparison to this effect.

An additional implication of the variance in crude protein contents of the diets is the differences this creates in the dietary lysine compositions. If the ideal protein concept is held as true, whereby individual dietary EAA requirements may be considered proportional to the provision of Lys, the requirement for Met (and, by extension, TSAA), will be affected by the dietary Lys level. The requirement for Met estimated by Coloso *et al.* (1999) was approximately 29.2% that of the Lys content of the diets and that estimated in the present study was between 28.4% (95% of asymptote) and 32.8% (99% of asymptote) that of the dietary Lys level. As dietary Lys content in the present study (39.9g kg⁻¹ or 6.7% CP) was

496 considerably higher than the requirement of 20.6g kg⁻¹ (4.5% CP) estimated by Murillo-
497 Gurrea *et al.* (2001) and the Lys contents of the diets in the two experiments differed
498 considerably, the similarity of the Met requirement figures, when expressed as a proportion
499 of dietary Lys, supports the concept of amino acid balance in dietary formulation. This ideal
500 proportion of dietary Met:Lys (Met \approx 30% of dietary Lys content) is also reflected in the
501 whole body amino acid profile of the fish, suggesting the “ideal protein” on this basis is an
502 accurate approximation of the essential amino acid requirements of barramundi (at least for
503 Met) as has been suggested for other species (NRC, 2011).

504 Calculations for predicted TSAA requirement as a function of Lys content yielded
505 figures of 38% of Lys content for the Coloso *et al.* (1999) data and 43-48% of the Lys
506 content in the present study, highlighting the major contribution of the variation in dietary
507 Cys content to the overall differences in TSAA requirement estimates between the two
508 studies. Similarly to the requirement for Met, this calculated requirement for TSAA (43-48%
509 of the Lys content) in the present study is similar to the whole body TSAA content (44% of
510 Lys), further suggesting that the level of Cys used in the present study was appropriate. This
511 relationship is also seen between the Met requirement of Channel Catfish (*Ictalurus*
512 *punctatus*) (Harding *et al.*, 1977) and the TSAA requirement of Rainbow Trout
513 (*Oncorhynchus mykiss*) (Bae *et al.*, 2011) and their respective contents in the carcasses
514 (according to the data of Wilson and Cowey (1985)).

515

516 **5. Conclusion**

517

518 This study represents a comprehensive reassessment of the TSAA requirement of
519 juvenile barramundi. The results confirm the established requirement for Met by juvenile
520 barramundi, when expressed as a proportion of dietary crude protein, of 1.8-2.3% CP
521 (reported as 2.24% CP by Coloso *et al.* (1999)). An updated requirement for TSAA of 2.9-
522 3.4% CP was also established. The impact of selection of an appropriate model for
523 estimation of amino acid requirements, proper interpretation of the outputs of that model and
524 choice of the mode of expression of amino acid requirements are highlighted in this study.
525 Establishment of reliable estimates of requirement for individual essential amino acids is
526 paramount to the proper design of further studies for advancement of our understanding of
527 amino acid metabolism in fish (i.e. investigation into the metabolic effects of nutrient
528 deficiency, sufficiency and excess). In order to get a better understanding of the mechanisms
529 behind the stimulating effect of amino acid supply on growth in fish, it is important to define
530 the roles they play in the various protein and energy metabolism pathways.

531

532 **Acknowledgments**

533 The authors wish to acknowledge assistance provided in sample collection by staff of
534 the Bribie Island Aquaculture Centre (BIRC): Mr. Simon Irvin, Ms. Kinam Salee and Ms.
535 Natalie Habilay. As well, that of Mr. David Blyth in the preparation of the diets and Mr.
536 Nicholas Bourne and Ms. Sue Cheers for advice regarding analyses. This research did not
537 receive a specific grant but was funded by the Commonwealth Scientific and Industrial
538 Research Organisation (CSIRO). David Poppi received support through an Australian
539 Government Research Training Program Scholarship.

540

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Table 1. Formulations and analysed compositions of Experiment One diets.

	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5
<i>Ingredients (g kg⁻¹)</i>					
Fishmeal ¹	150	150	150	150	150
SPC	490	490	490	490	490
Fish oil ²	100	100	100	100	100
Cellulose	79	79	79	79	79
Pregel Starch	53	53	53	53	53
CaHPO ₄	20	20	20	20	20
Vit. and Min. Premix ³	6	6	6	6	6
Choline chloride ⁴	1	1	1	1	1
Marker (Y ₂ O ₃)	1	1	1	1	1
DL-Met	-	1.0	2.0	3.5	5.0
Tau	5	5	5	5	5
Gly	10.0	9.0	8.0	6.5	5
EAA Premix ⁵	85	85	85	85	85
<i>Composition as determined (g kg⁻¹DM unless otherwise stated)</i>					
Dry matter (g kg ⁻¹ as is)	962	966	967	966	960
Crude Protein	602	580	579	591	592
Digestible Protein	551	523	527	534	539
Lipid	98	115	103	106	107
Ash	61	60	60	62	61
Gross Energy (MJ kg ⁻¹ DM)	22.5	23.1	22.7	22.5	22.4
DE (MJ kg ⁻¹ DM)	17.3	17.4	17.0	16.8	16.6
<i>EAA's</i>					
Arg	45.5	45.2	46.2	45.9	45.2
His	16.5	15.9	16.4	15.6	16.4
Ile	29.1	29.0	29.5	29.3	29.7
Leu	51.7	50.2	51.5	51.1	51.2
Lys	40.7	40.9	41.4	40.8	41.8
Met	7.2	8.4	9.8	10.6	12.8
Cys	6.0	5.7	5.9	5.7	5.7
Phe	31.1	31.5	33.1	32.8	32.4
Thr	31.2	30.3	31.1	30.6	31.6
Val	35.1	35.2	35.7	36.0	35.5
Tau	6.2	6.2	6.2	6.2	6.2

697 ¹ Fishmeal: Chilean anchovy meal, Ridley Aquafeeds, Narangba, QLD, Australia.

698 ² Fish (anchovy) oil: Ridley Aquafeeds, Narangba, QLD, Australia.

699 ³ Vitamin and mineral premix includes (IU/kg or g/kg of premix): retinol, 2.5 MIU; cholecalciferol, 0.25 MIU; α-tocopherol, 16.7g; Vitamin
700 K3, 1.7g; thiamin, 2.5g; riboflavin, 4.2g; niacin, 25g; pantothenic acid, 8.3g; pyridoxine, 2.0g; folate, 0.8g; Vitamin B12, 0.005g; Biotin,
701 0.17g; Vitamin C, 75g; Inositol, 58.3g; Ethoxyquin, 20.8g; Copper, 2.5g; Ferrous iron, 10.0g; Magnesium, 16.6g; Manganese, 15.0g; Zinc,
702 25.0g.

703 ⁴ Choline chloride 70% corn cob

704 ⁵ Essential amino acid premix consisting of (g kg⁻¹ of premix): L-Isoleucine, 70.6g; L-Valine, 117.6g; L-Histidine, 58.8g; L-Leucine,
705 176.5g; L-Phenylalanine, 82.4g; L-Threonine, 141.2g; L-Lysine, 176.5g; L-Arginine, 176.5g.

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708 **Table 2. Formulations and analysed compositions of Experiment Two diets.**

	Met Requirement					Cys Replacement				
	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7	Diet 8	Diet 9	Diet 10
<i>Ingredients (g kg⁻¹)</i>										
Fishmeal ¹	150	150	150	150	150	150	150	150	150	150
SPC	490	490	490	490	490	-	-	-	-	-
Casein	-	-	-	-	-	130	130	130	130	130
Wheat	-	-	-	-	-	40	40	40	40	40
SPI	-	-	-	-	-	150	150	150	150	150
Fish oil ²	100	100	100	100	100	100	100	100	100	100
Cellulose	76	76	76	76	76	198	198	198	198	198
Pregel Starch	53	53	53	53	53	30	30	30	30	30
CaHPO ₄	20	20	20	20	20	20	20	20	20	20
Vit. and Min. Premix ³	6	6	6	6	6	6	6	6	6	6
Choline Cl ⁴	1	1	1	1	1	1	1	1	1	1
Marker (Y ₂ O ₃)	1	1	1	1	1	1	1	1	1	1
DL-Met	-	3.5	6.5	10.0	13.0	7.0	6.0	5.0	3.0	-
L-Cys	-	-	-	-	-	-	1.0	2.0	4.0	7.0
Tau	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
L-Gly	13.0	9.5	6.5	3.0	-	-	-	-	-	-
EAA Premix 1 ⁵	85	85	85	85	85	-	-	-	-	-
EAA Premix 2 ⁶	-	-	-	-	-	162	162	162	162	162
<i>Composition as determined (g kg⁻¹DM unless otherwise stated)</i>										
DM (g kg ⁻¹ as is)	960	956	954	955	957	967	975	972	980	979
CP	600	595	582	604	589	555	548	556	526	515
DP	530	526	519	529	520	500	510	516	482	468
Lipid	116	116	117	115	114	120	121	121	125	118
Ash	67	67	67	67	67	63	63	63	62	63
GE (MJ kg ⁻¹ DM)	22.5	22.4	22.5	22.3	22.2	22.2	22.3	22.3	22.3	22.3
DE(MJ kg ⁻¹ DM)	14.87	15.20	15.71	14.69	13.90	13.06	15.13	13.95	15.34	14.00
Arg	44.3	44.3	44.3	43.5	44.0	44.2	45.0	44.8	45.5	44.8
His	17.3	16.8	16.7	16.4	16.4	13.3	13.5	13.5	13.1	13.3
Ile	28.6	28.9	29.0	28.3	28.7	31.6	31.8	31.9	31.7	31.9
Leu	46.1	47.1	29.2	44.5	44.7	48.6	49.2	46.7	47.8	46.5
Lys	39.4	40.3	40.7	38.9	40.0	40.0	36.2	41.8	36.5	41.0
Met	8.6	12.4	14.9	18.2	21.4	13.0	12.0	10.9	9.1	6.7
Cys	6.6	6.6	6.8	6.5	6.6	3.7	4.3	4.8	6.1	7.9
Phe	34.9	33.7	34.4	34.2	34.8	37.3	37.3	35.0	36.3	37.2
Thr	29.7	29.5	31.1	29.5	29.7	32.0	33.0	32.2	32.8	32.7
Val	35.4	35.8	35.6	35.1	35.2	39.1	39.5	39.5	39.6	39.2
Tau	6.4	6.6	6.5	6.2	6.3	6.5	6.2	6.2	5.5	5.9

709 ¹ Fishmeal: Chilean anchovy meal, Ridley Aquafeeds, Narangba, QLD, Australia.710 ² Fish (anchovy) oil: Ridley Aquafeeds, Narangba, QLD, Australia.711 ³ Vitamin and mineral premix includes (IU/kg or g/kg of premix): retinol, 2.5 MIU; cholecalciferol, 0.25 MIU; α-tocopherol, 16.7g; Vitamin K3, 1.7g; thiamin, 2.5g; riboflavin, 4.2g; niacin, 25g; pantothenic acid, 8.3g; pyridoxine, 2.0g; folate, 0.8g; Vitamin B12, 0.005g; Biotin, 0.17g; Vitamin C, 75g; Inositol, 58.3g; Ethoxyquin, 20.8g; Copper, 2.5g; Ferrous iron, 10.0g; Magnesium, 16.6g; Manganese, 15.0g; Zinc, 25.0g.714 ⁴ Choline chloride 70% cob715 ⁵ Essential amino acid premix 1 consisting of (g kg⁻¹ of premix): Taurine, 55.6g; L-Isoleucine, 66.7g; L-Valine, 111.1g; L-Histidine, 55.6g; L-Leucine, 166.7g; L-Phenylalanine, 77.8g; L-Threonine, 123.3g; L-Lysine, 166.7g; L-Arginine, 166.7g.716 ⁶ Essential amino acid premix 2 consisting of (g kg⁻¹): Taurine, 29.9g; L-Isoleucine, 89.8g; L-Valine, 113.8g; L-Histidine, 35.9g; L-Leucine, 173.7g; L-Phenylalanine, 101.8g; L-Threonine, 119.8g; L-Lysine, 143.7g; L-Arginine, 191.6g.

720 **Table 3. Response of fish to variable dietary methionine content in Experiment One¹.**

	Initial Fish	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Pooled SEM	Polynomial Contrasts		
								Linear	Quadratic	Cubic
Dietary Met (g kg ⁻¹ DM)		7.2	8.4	9.8	10.6	12.8	-	-	-	-
Dietary Met (% CP)		1.2	1.5	1.7	1.8	2.2	-	-	-	-
Initial Weight (g fish ⁻¹)		18.2	18.2	18.1	18.1	18.2	0.03	ns	ns	ns
Final Weight (g fish ⁻¹)		74.9 ^a	76.5 ^{ab}	79.2 ^{bc}	80.6 ^{cd}	83.9 ^d	0.91	<0.001	ns	ns
BW Gain (%)		312.2 ^a	321.7 ^{ab}	335.3 ^{bc}	344.4 ^{cd}	361.1 ^d	4.95	<0.001	ns	ns
FCR ²		0.92 ^a	0.93 ^{ab}	0.89 ^{bc}	0.86 ^{cd}	0.82 ^d	0.01	<0.001	ns	ns
Feed Intake (g/fish)		54.6	54.6	54.6	54.6	54.6	0.00	ns	ns	ns
ERE ³		39.5 ^a	42.5 ^{ab}	44.1 ^b	44.8 ^b	44.5 ^b	0.60	<0.001	0.02	ns
PRE ⁴		28.0 ^a	31.0 ^a	34.2 ^b	34.8 ^b	37.2 ^b	0.91	<0.001	ns	ns
Survival (%)		100 ^a	100 ^a	100 ^a	100 ^a	100 ^a	0.00	ns	ns	ns
<i>Carcass composition as determined (g kg⁻¹ as is unless otherwise stated)</i>										
DM	245	294	306	305	305	292	0.22	ns	<0.01	ns
CP	166	158 ^a	163 ^a	170 ^{ab}	172 ^{ab}	174 ^b	0.18	<0.001	ns	ns
Lipid	28	87 ^{ab}	96 ^a	90 ^{ab}	86 ^{ab}	78 ^b	0.21	<0.05	<0.05	ns
Ash	40	35	33	32	33	32	0.07	ns	ns	ns
GE (MJ kg ⁻¹ as is)	5.0	7.4 ^{ab}	8.0 ^a	7.8 ^{ab}	7.7 ^{ab}	7.3 ^b	0.08	ns	<0.01	ns

721 ¹ values sharing a common superscript letter are not significantly different (p<0.05).

722 ² FCR: feed conversion ratio (g dry feed/g wet weight gain)

723 ³ ERE: energy retention efficiency = MJ energy gain * 100/MJ energy consumed

724 ⁴ PRE: protein retention efficiency = g protein gain * 100/g protein consumed

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732 **Table 4. Response of fish to variable dietary methionine content in Experiment Two¹.**

	Initial Fish	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Pooled SEM	Polynomial Contrasts		
								Linear	Quadratic	Cubic
Dietary Met (g kg ⁻¹ DM)		8.6	12.4	14.9	18.2	21.4		-	-	-
Dietary Met (% CP)		1.4	2.1	2.6	3.0	3.6		-	-	-
Initial Weight (g fish ⁻¹)		35.2	35.3	34.6	35.1	34.7	0.18	ns	ns	ns
Final Weight (g fish ⁻¹)		112.2 ^a	119.1 ^b	118.4 ^b	119.0 ^b	117.5 ^b	0.76	<0.001	<0.001	<0.05
BW Gain (%)		218.6 ^a	237.5 ^b	242.6 ^b	242.1 ^b	239.1 ^b	2.99	<0.05	<0.05	ns
FCR ²		0.98 ^a	0.90 ^b	0.90 ^b	0.90 ^b	0.91 ^b	0.01	<0.001	<0.001	<0.05
Feed Intake (g/fish)		75.5	75.5	75.5	75.5	75.5	0.00	ns	ns	ns
ERE ³		41.87	45.4	44.9	46.1	45.8	0.58	<0.05	ns	ns
PRE ⁴		32.2 ^a	39.0 ^b	38.4 ^b	39.9 ^b	39.4 ^b	0.82	<0.001	<0.001	<0.05
Survival (%)		100 ^a	0.00	ns	ns	ns				
<i>Carcass composition as determined (g kg⁻¹ as is unless otherwise stated)</i>										
DM (g kg ⁻¹ as is)	270	290	295	291	296	295	0.16	ns	ns	ns
CP	184	183 ^a	195 ^b	190 ^{ab}	200 ^c	197 ^{bc}	0.21	<0.001	ns	ns
Lipid	45	96	89	88	90	87	0.15	ns	ns	ns
Ash	45	23	23	23	23	22	0.03	ns	ns	ns
GE (MJ kg ⁻¹ as is)	5.9	7.7	7.7	7.6	7.8	7.7	0.06	ns	ns	ns

733 ¹ values sharing a common superscript letter are not significantly different (p<0.05).734 ² FCR: feed conversion ratio (g dry feed/g wet weight gain)735 ³ ERE: energy retention efficiency = MJ energy gain * 100/MJ energy consumed736 ⁴ PRE: protein retention efficiency = g protein gain * 100/g protein consumed

Table 5. Nutrient response models fitted to the data from Experiment Two.

Broken line spline with ascending linear segment model (Robbins, 1986)

$$y = \begin{cases} \text{Maximum}, & \text{If } x > \text{Requirement} \\ \text{Maximum} + \text{Rate Constant} \times (\text{Requirement} - x) & \text{If } x \leq \text{Requirement} \end{cases}$$

Broken line spline with ascending quadratic segment model (Vedenov and Pesti, 2008)

$$y = \begin{cases} \text{Maximum}, & \text{If } x > \text{Requirement} \\ \text{Maximum} + \text{Rate Constant} \times (\text{Requirement} - x)^2 & \text{If } x \leq \text{Requirement} \end{cases}$$

4-Parameter Saturation Kinetics Model (Morgan *et al.*, 1975)

$$y = \frac{(\text{Intercept} \times \text{Rate Constant}) + (\text{Maximum} \times x^{\text{Kinetic Order}})}{\text{Rate Constant} + x^{\text{Kinetic Order}}}$$

5-Parameter Saturation Kinetics Model adapted from Mercer *et al.* (1989)

$$y = \frac{(\text{Intercept} \times \text{Rate Constant}) + (\text{Maximum} \times x^{\text{Kinetic Order}}) + \text{Intercept} \times x^{2 \times \text{Kinetic Order}} \div \text{Inhibition Constant}^{\text{Kinetic Order}}}{\text{Rate Constant} + x^{\text{Kinetic Order}} + x^{2 \times \text{Kinetic Order}} \div \text{Inhibition Constant}^{\text{Kinetic Order}}}$$

Three-parameter logistic model (SAS Institute Inc, 1990)

$$y = \frac{\text{Maximum} \times \text{Intercept} \times e^{-\text{Scale} \times x}}{\text{Maximum} \times \text{Intercept} \times (e^{-\text{Scale} \times x} - 1)}$$

Four-parameter logistic model (Gahl *et al.*, 1991)

$$y = \frac{\text{Maximum} + [\text{Intercept} \times (1 + \text{Shape}) - \text{Maximum}] e^{-\text{Scale} \times x}}{1 + \text{Shape} \times e^{-\text{Scale} \times x}}$$

Sigmoidal model (Robbins *et al.*, 1979)

$$y = \text{Lower Asymptote} + \frac{\text{Range}}{1 + e^{r+s \times x}}$$

Exponential model (Robbins *et al.*, 1979)

$$y = \text{Intercept} + \text{Range} \times (1 - e^{c \times X})$$

Compartmental model (Pesti *et al.*, 2009)

$$y = \text{Maximum} \times e^{-\text{Intercept} \times x} (1 - e^{-\text{Nutrient Rate Constant} \times (x - \text{Kinetic order})})$$

739 **Table 6. Comparison of goodness of fit and dietary methionine requirements predicted by**
 740 **each of the nine models based on %BW Gain data from Experiment Two (data is ranked**
 741 **according to R²).**

Model	SSE ¹	R ²	Met concentration (g kg ⁻¹ DM) at 99% of asymptotic response	Met concentration (g kg ⁻¹ DM) at 95% of asymptotic response
Compartmental	472.7	0.71	13.6	10.5
Broken-Line (Linear Ascending)	492.5	0.70	13.1 ²	N/A
Broken-Line (Quadratic Ascending)	492.5	0.70	14.6 ²	N/A
4-SKM	496.3	0.69	12.8	10.7
5-SKM	496.3	0.69	12.8	10.7
Logistics, 3 Parameters	501.3	0.69	12.6	9.8
Logistics, 4 Parameters	501.8	0.69	12.6	9.8
Exponential	502.1	0.69	12.9	9.8
Sigmoidal	521.6	0.68	9.0	8.7

742 ¹ SSE: Sum of Squared Errors

743 ² requirement predicted by the abscissa of the breakpoint of the curve

744 **Table 7. Response of fish to variable dietary Met:Cys content in Experiment Two¹.**

	Initial Fish	Diet 6	Diet 7	Diet 8	Diet 9	Diet 10	Pooled SEM	Regression			
								Linear	Quadratic	Cubic	
Dietary Met (g kg ⁻¹ DM)		13.0	12.0	10.9	9.1	6.7		-	-	-	
Dietary Cys (g kg ⁻¹ DM)		3.7	4.3	4.8	6.1	7.9		-	-	-	
Proportion of TSAA as Cys (%)		22	26	31	40	54		-	-	-	
Initial Weight (g fish ⁻¹)		35.6	35.6	36.0	35.4	36.4	0.17	ns	ns	ns	
Final Weight (g fish ⁻¹)		114.7	113.9	115.8	114.7	112.3	0.45	ns	ns	ns	
BW Gain (%)		219.8	219.9	221.8	224.3	208.5	2.03	<0.05	<0.05	ns	
FCR ²		0.96	0.97	0.97	0.95	0.99	0.01	ns	ns	ns	
Feed Intake (g/fish)		75.5	75.4	75.5	75.5	75.4	0.04	ns	ns	ns	
ERE ³		39.9	41.8	42.2	42.0	41.0	0.45	ns	ns	ns	
PRE ⁴		37.6	37.7	38.5	40.8	37.1	0.50	ns	<0.05	ns	
Survival (%)		100 ^a	0.00	ns	ns	ns					
<i>Carcass composition as determined (g kg⁻¹ as is unless otherwise stated)</i>											
DM		270	282	292	292	293	293	0.21	ns	ns	ns
CP		184	190	191	193	195	185	0.15	ns	ns	ns
Lipid		45	82	89	85	89	93	0.16	<0.05	ns	ns
Ash		45	25	24	25	24	25	0.03	ns	ns	ns
GE (MJ kg ⁻¹ as is)		5.9	7.3	7.6	7.5	7.6	7.6	0.07	ns	ns	ns

745 ¹ values sharing a common superscript letter are not significantly different (p<0.05).

746 ² FCR: feed conversion ratio (g dry feed/g wet weight gain)

747 ³ ERE: energy retention efficiency = MJ energy gain * 100/MJ energy consumed

748 ⁴ PRE: protein retention efficiency = g protein gain * 100/g protein consumed

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751 **Table 8. Comparison of goodness of fit and dietary methionine requirements predicted**
 752 **by each of the nine models based on the %BW Gain data of Coloso et al. (1999) (data is**
 753 **ranked according to R²).**

Model	SSE ¹	R ²	MET concentration (g kg ⁻¹ DM) at 99% of asymptotic response	MET concentration (g kg ⁻¹ DM) at 95% of asymptotic response
5-SKM	492.817	98.2	10.3	8.9
Broken-Line (Quadratic Ascending)	528.235	98.0	11.8 ²	N/A
Compartmental	590.684	97.8	10.6	8.9
Broken-Line (Linear Ascending)	642.012	97.6	10.1 ²	N/A
Logistic, 4 Parameter	728.444	97.3	11.7	9.1
Sigmoidal	728.445	97.3	11.7	9.1
4-SKM	765.395	97.1	12.8	9.4
Logistic, 3 Parameter	776.269	97.1	13.1	9.6
Exponential	812.662	97.0	14.0	9.8

754 ¹ SSE: Sum of Squared Errors

755 ² requirement predicted by the abscissa of the breakpoint of the curve

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758 **Table 9. Essential amino acid composition of juvenile (average weight = 82.1g)**
 759 **barramundi whole carcass (g 16g N⁻¹) and its relationship to whole body Lysine content.**

Amino Acid	Whole Body	Whole Body Relative to Lys (%)
Arg	5.8	86
His	1.5	22
Ile	5.6	83
Leu	3.2	48
Lys	6.7	100
Met	2.3	35
Cys	0.7	10
Phe	3.3	50
Thr	3.5	52
Val	3.5	52
TSAA (Met+Cys)	3.0	44

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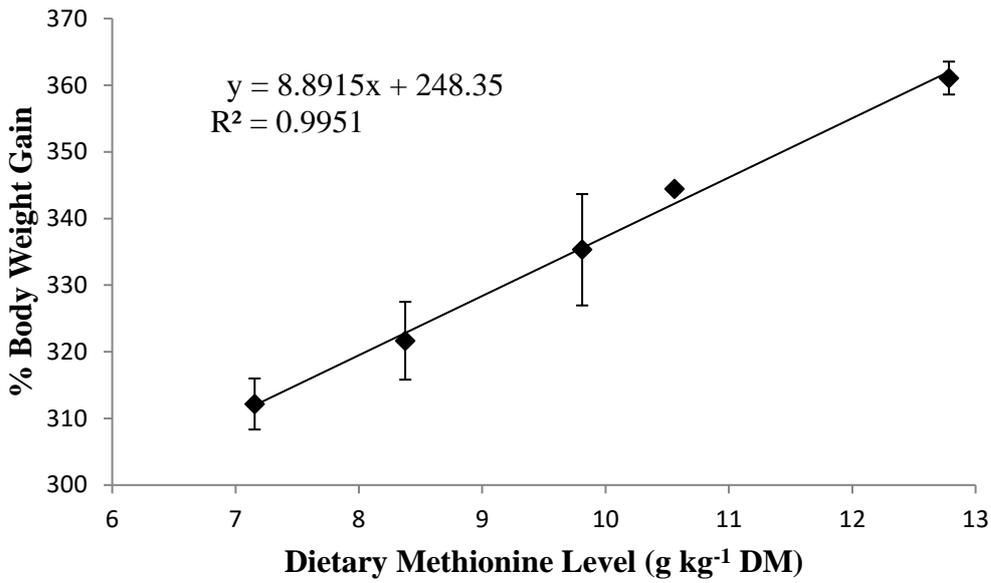
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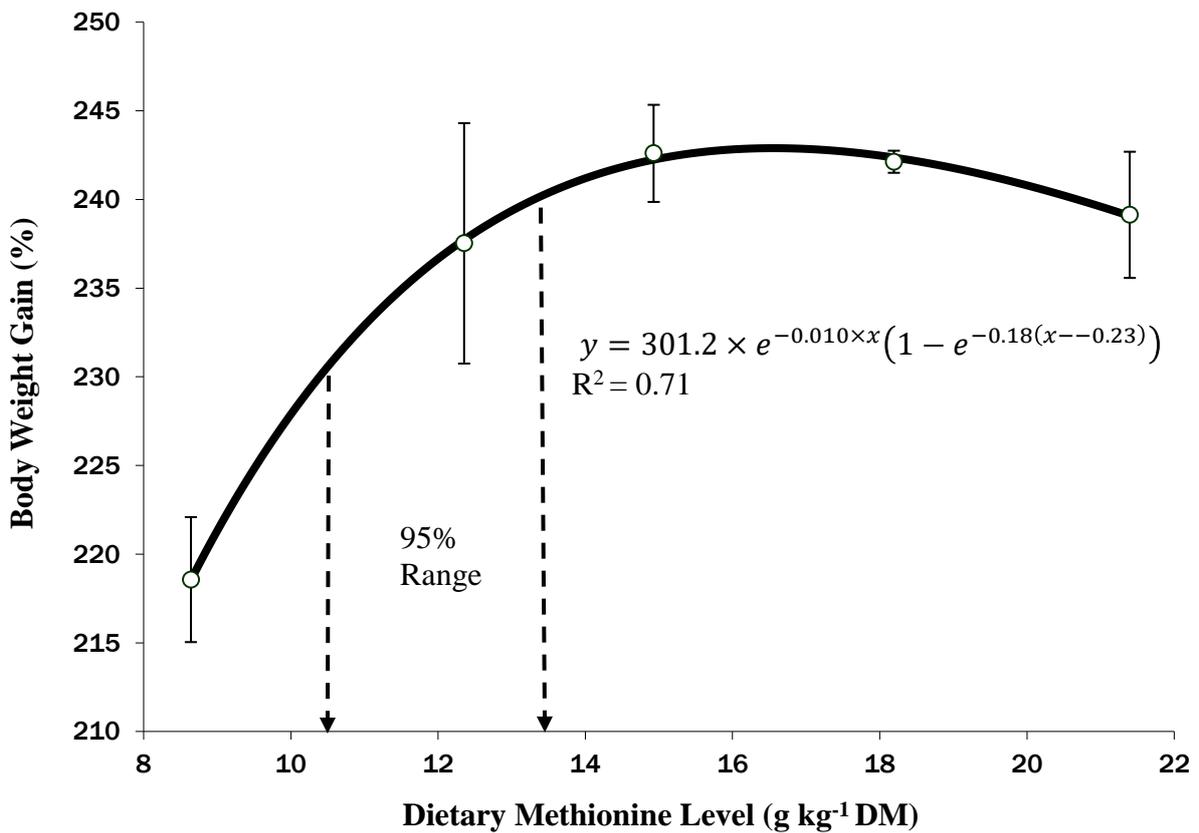
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Figure 1. Percent Weight Gain (\pm S.E.M.) of fish fed diets with variable methionine content in Experiment One (mean initial weight = 18.1g).



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Figure 2. Percent Weight Gain (\pm S.E.M.) (mean initial weight = 35.0g) of fish in Experiment Two with Met requirement as predicted by the Compartmental model (arrows indicate 95% and 99% of the asymptote)