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

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Highlights:

- Barramundi require 10.5-13.6g kg^{-1} Met in a diet with 6.6g kg^{-1} Cys (17.1-20.2g kg^{-1} TSAA; 1.8-2.3% CP Met + 1.1% CP Cys).
- Cystine can constitute at least 40% of the TSAA content of the diet of barramundi without significantly affecting growth.
- TSAA requirement is considerably higher than previously estimated, depending on the mode of expression.
- Nine nutrient response models fitted to current and previous data and requirement estimates compared.
- Mode of expression and choice and interpretation of nutrient response model can greatly affect requirement estimates.

Abstract

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

1. Introduction

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

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

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

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

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

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

2. Materials and Methods

2.1. Diets

2.1.1. Formulation

2.1.1.1. Experiment One

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

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

Yttrium oxide was included in all diets at a concentration of 1g kg^{-1} for the purposes of digestibility assessment.

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

2.1.1.2. Experiment Two

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

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

2.1.2. Diet manufacture

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

2.2. Fish management and faecal collection

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

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

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

The experimental tanks were set up with ~3 L/min flow of continuously aerated marine water (~35PSU) of 29.5°C ± 0.2°C for the duration of the experiment. Photoperiod was set at 12:12 (light:dark).

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

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

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

All feed was kept in cold storage (< 4ºC) except for the purposes of feeding and weighing.

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

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

2.3. Sample collection

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

2.4. Chemical and digestibility analyses

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

Carcass and diet dry matter contents were determined by gravimetric analysis following drying at 105^0C for 16h. Gross ash contents were similarly determined based on mass change after combustion in a muffle furnace at 550^0C for 16 hours. The lipid portion of the samples was extracted according the method proposed by Folch *et al.* (1957) and used to determine crude lipid contents. Measurement of total nitrogen content was undertaken using a CHNS auto-analyser (Leco Corp., St. Joseph, MI, USA) and used to calculate sample protein content based on *N* x 6.25. Gross energy was determined by isoperibolic bomb calorimetry in a Parr 6200 oxygen bomb calorimeter (Par Instrument Company, Moline, IL, USA). Amino acid compositions were determined by mass detection after reverse-phase ultra high-performance liquid chromatography with pre-column derivatisation with 6-aminoquinolyl-N-hydroxysuccinimidyl (AQC). Analyses were undertaken on a Shimadzu Nexera X2 series UHPLC (Shimadzu Corporation, Kyoto, Japan) with quaternary gradient module, coupled with a Shimadzu 8030 Mass Spectrometer using the Waters AccQ·tag system (Waters Corporation, Milford, MA). Samples were prepared according to the protocol for complex feed samples outlined by Waters Corp. (1996) following hydrochloric acid hydrolysis. Cyst(e)ine is known to be destroyed during acid hydrolysis and methionine can be oxidized to methionine sulfone (Rutherfurd and Gilani, 2009). These amino acids were determined independently as cysteic acid and methionine sulfone respectively, after oxidation with performic acid according to an adaptation of the protocol of Chavali *et al.* (2013) (using

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

Yttrium concentrations in the feed and faeces were determined by inductively coupled plasma mass spectrometry (ICP-MS) after microwave digestion in 5mL HNO_3 based on a modification of EPA method 3051 (EPA, 1994). The apparent digestibilities ($AD_{Parameter}$) of individual nutritional parameters (DM, protein and gross energy) were calculated by the differences in the ratios of the parameter of interest in the diets and faeces based on the following formula (Maynard and Loosli, 1969):

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

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

2.5. Statistical analysis

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

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

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

3. Results

3.1. Experiment One

3.1.1. Response to increasing dietary methionine content

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

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

3.1.2. Experiment Two.

3.1.2.1. Response to increasing dietary methionine content

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

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

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

Survival was 100% in all treatments.

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

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

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

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

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

3.1.4 Essential amino acid composition of juvenile barramundi.

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

4. Discussion

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

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

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

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

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

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

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

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

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

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

5. Conclusion

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

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

	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5
Ingredients (g kg^{-1})					
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_2O_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
Composition as determined (g kg^{-1}DM unless otherwise stated)					
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
EAAs					
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

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

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

[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.

[4] Choline chloride 70% corn cob

[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, 176.5g; L-Phenylalanine, 82.4g; L-Threonine, 141.2g; L-Lysine, 176.5g; L-Arginine, 176.5g.

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**
Ingredients (g kg^{-1})										
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_2O_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
Composition as determined (g kg^{-1}DM unless otherwise stated)										
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

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

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

[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.

[4] Choline chloride 70% corn cob

[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.

[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 One[1].**

	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
Carcass composition as determined (g kg^{-1}as is unless otherwise stated)										
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 Two[1].**

	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}	100^{a}	100^{a}	100^{a}	100^{a}	0.00	ns	ns	ns
Carcass composition as determined (g kg^{-1}as is unless otherwise stated)										
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 consumed
736 [4] PRE: protein retention efficiency = g protein gain * 100/g protein consumed

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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Table 6. Comparison of goodness of fit and dietary methionine requirements predicted by each of the nine models based on %BW Gain data from Experiment Two (data is ranked according to R^2).

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

[1] SSE: Sum of Squared Errors
[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 Two[1].**

	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]	100[a]	100[a]	100[a]	100[a]	0.00	ns	ns	ns
Carcass composition as determined (g kg^{-1}as is unless otherwise stated)										
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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Table 8. Comparison of goodness of fit and dietary methionine requirements predicted by each of the nine models based on the %BW Gain data of Coloso et al. (1999) (data is ranked according to R^2).

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

[1] SSE: Sum of Squared Errors

[2] requirement predicted by the abscissa of the breakpoint of the curve

Table 9. Essential amino acid composition of juvenile (average weight = 82.1g) 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

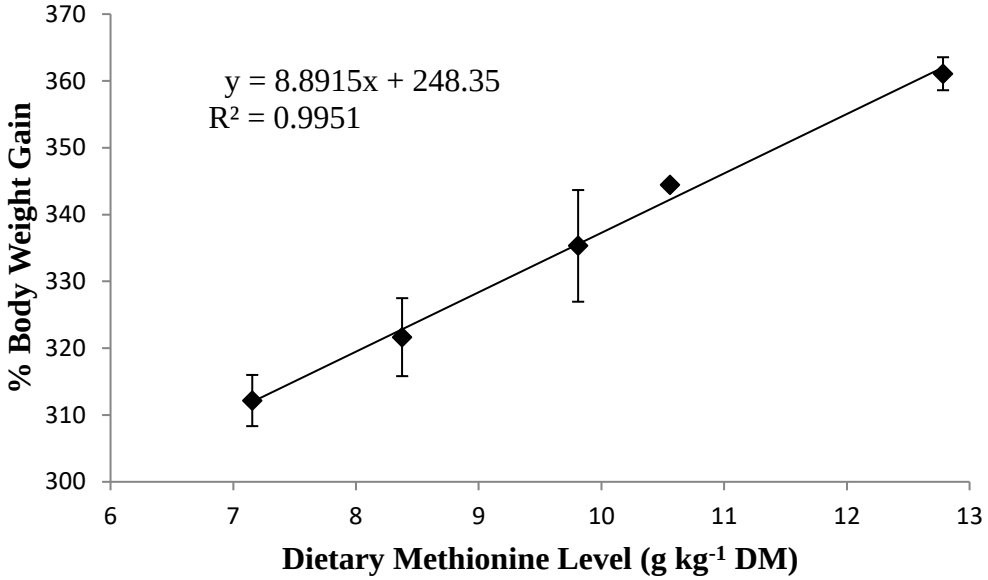

Figure 1. Percent Weight Gain (± S.E.M.) of fish fed diets with variable methionine content in Experiment One (mean initial weight = 18.1g).

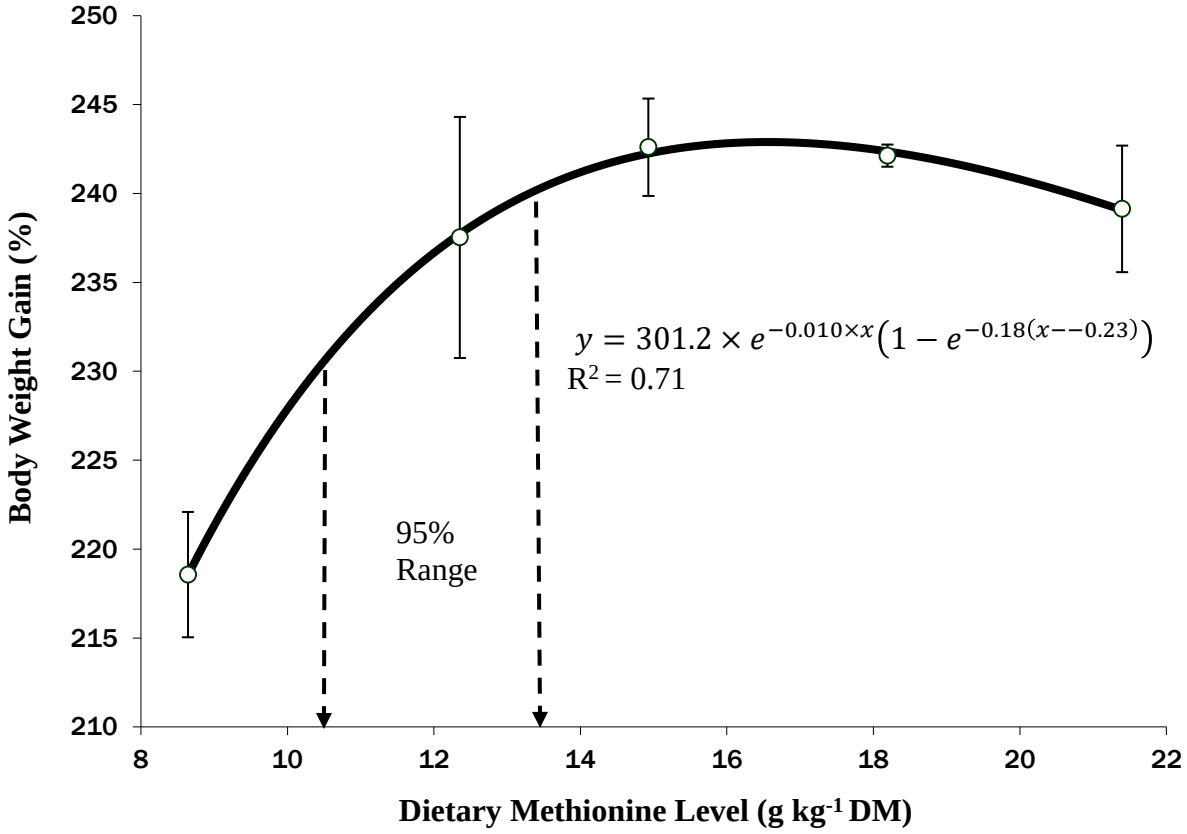

Figure 2. Percent Weight Gain (±S.E.M.) (mean initial weight = 35.0g) of fish in Experiment Two with Met requirement as predicted by the Compartmental model (arrows indicate 95% and 99% of the asymptote)