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Elucidation of the roles and requirements of sulphur amino acids in the diet of barramundi (*Lates calcarifer*).

David Alido Poppi

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Abstract

With the increasing use of plant protein meals to replace fishmeal in aquafeeds, there is a concomitant need to properly characterise the requirements for individual amino acids in the diet of carnivorous fish species such as barramundi (*Lates calcarifer*). This may be especially important for methionine (Met) and taurine (Tau) which are known to be limiting in these ingredients. This project aimed to define the requirements for these nutrients for barramundi, as well as to elucidate some of the mechanisms underpinning any observed stimulatory effect on growth.

The aim of the first experiment was to evaluate the methionine (Met) and total sulphur amino acid (TSAA) requirements of juvenile barramundi to act as a baseline for further study. 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) was established. Additionally, it was concluded that at least 40% of dietary Met can be replaced by Cys. The impact of nutrient response model choice and the mode of expression of requirements on interpretation of these figures is discussed in Chapter Two.

The experiment presented in Chapter Three was designed to elucidate some of the metabolic roles of Met. Results suggested that expression of genes of the sulphur amino acid turnover pathways may be chiefly regulated by feed ingestion and not, as was expected, by the amino acid profile of the feed. Taurine biosynthesis was apparent, as was the conservation of previously described markers of proteolytic pathways. Significantly, two forms of Methionine Adenosyltransferase (MAT) appeared to be active. A number of important genes were investigated for the first time in this species and shown to be nutritionally regulated.

Whether Tau is required in the diet of juvenile barramundi, and what effect dietary Met has on this requirement, was investigated and is presented in Chapter Four. The best-fit response model predicted a Tau requirement of 5.5g kg^{-1} DM (0.96% CP), similar to reported values for several other species. The fit of this model, however was relatively weak ($R^2 = 0.183$). The response to variable dietary Met was more pronounced, highlighting its importance to this species. It was concluded that taurine appears to be conditionally essential to barramundi.

An experiment investigating the role of dietary Tau in affecting protein and SAA turnover in juvenile barramundi was undertaken, the results of which are presented in Chapter Five. Most significantly, two pathways of Tau biosynthesis were observed to be active in this species with the sulphinoalanine pathway being more responsive to feeding. Changes in the utilisation of Cys when dietary Tau was in excess were also suggested, along with an apparent link between adequate supply of taurine and both somatotropic index and TOR pathway-mediated growth stimulation.

Unexpectedly, the expression profile of several genes suggested that excessive Tau may have a negative impact on protein accretion and growth.

Calculation of protein and amino acid loss exponents, and their application to predicting requirements for Met for maintenance and growth in barramundi, was the focus of Chapter 6. Contrary to previous assumptions used in nutritional models, the exponents derived for the proteinaceous amino acids were significantly different to that of protein. An influence of fish size on the utilisation of non-protein nitrogen was also suggested. Similarly, predicted partitioning of ingested Met was proposed to change with increasing fish size, with weight gain suggested to drive the requirement for this amino acid in smaller fish and maintenance requirements representing a greater proportion of the total requirement in larger fish. Consequently, it was concluded that the use of the Met-loss-adjusted metabolic body weight exponent derived in this study may provide a more accurate representation over the protein weight exponent in modelling Met utilisation during growth in this species.

The main findings of this thesis were that juvenile barramundi do have a defined requirement for Met, a proportion of which can be spared by dietary Cys, and that the mode of expression and choice of nutrient response model can have a significant impact on the interpretation and application of this requirement. As well, several genes associated with SAA and protein metabolism in this species were investigated, and shown to be nutritionally regulated, for the first time in this species. Significantly, two forms of MAT appear to be active and it was suggested that barramundi possess the capacity to synthesise Tau from precursor SAA through two separate pathways. Thus, Tau was concluded to be conditionally essential in this species. Finally, the utilisation of individual amino acids was shown to differ significantly from that of protein as a whole but, in the case of Met, consideration of this in a published nutrient utilisation model appeared not to significantly improve the accuracy of utilisation efficiency predictions.

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

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Publications during candidature

Conference Abstracts

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Contributor	Statement of contribution
Poppi, D.A. (Candidate)	Designed experiments (70%) Performed experiments (100%) Data collection (95%) Data analysis and interpretation (90%) Drafted the manuscript (100%) Final drafting and submission (60%)
Moore, S.S.	Edited manuscript (30%) Final drafting and submission (10%)
Glencross, B.D.	Designed experiments (30%) Data collection (5%) Data analysis and interpretation (10%) Edited manuscript (70%) Final drafting and submission (30%)

Contributions by others to the thesis

Professor Stephen Moore (UQ principal advisor. Director, Centre for Animal Science, QAAFI, University of Queensland): Facilitated the coordination of this project through the University of Queensland, allowing me to undertake the work presented within this thesis. Provided comments during the planning stages of each experiment as well as feedback to written reports and oral presentations throughout the course of my degree; comments which were incorporated into the final document. Provided feedback on early drafts of each chapter and final drafts of the complete thesis.

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Keywords

Barramundi, Sulphur amino acids, Methionine, Taurine, Nutrient requirements, Allometric scaling, Nutrient response modelling, Nutrigenomics, Gene Expression, Postprandial.

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List of Abbreviations used in the Thesis

%	Percent
^{0}C	Degrees Cesius
AD	Apparent Digestibility
ADO	Cysteamine Dioxygenase
ALAT	Alanine transaminase
ANOVA	Analysis of Variance
AQC	6-aminoquinolyl-N-hydroxysuccinimidyl
AU$	Australian Dollars
BCAA	Branched Chain Amino Acid
BHMT	Betaine:Homocysteine Methyltransferase
BW	Body Weight
CAA	Crystalline Amino Acids
CDO	Cysteine Dioxygenase
CDO	Cysteine Dioxygenase
CGL	Cystathionine Gamma Lyase
CHNS	Carbon, Hydrogen, Nitrogen, Sulphur
CL	Cysteine Lyase
CP	Crude Protein
CSAD	Cysteine Sulphinic Acid Decarboxylase
CSD	Cysteinesulphinate Decarboxylase
CSIRO	Commonwealth Scientific and Industrial Research Organisation
DE	Digestible Energy
DHA	Docosahexaenoic Acid
DM	Dry Matter
DM	Dry Matter
DNA	Deoxyribonucleic Acid
DP	Digestible Protein
EAA	Essential Amino Acids
EF1a	Elongation Factor 1 alpha
EPA	Environmental Protection Agency; Eicosapentaenoic Acid
ERE	Energy Retention Efficiency
EU	Efficiency of Utilisation
FAO	Food and Agriculture Organization of the United Nations
FAS	Fatty Acid Synthase
FCR	Feed Conversion Ration
FML	Fishmeal-based diet
g	Grams
g kg^{-1}	Grams per Kilogram
GAD	Glutamic Acid Decarboxylase
GE	Gross Energy
GH	Growth Hormone
GHR-II	Growth Hormone Receptor Two
GMW	Geometric Mean Weight

HSD	Honestly Significant Difference
HTDeHase	Hypotaurine Dehydrogenase
IAA	Indispensible Amino Acids
ICP-MS	Inductively Coupled Plasma Mass Spectrometer
IGF-I	Insulin-like Growth Factor One
IGF-II	Insulin-like Growth Factor Two
Lc CS	Citrate Synthase
Lc FXR	Farnesoid X Receptor
Lc LXR	liver X receptor
Lcal	*Lates calcarifer*
LPC	a Lupin Protein Concentrate Meal-based diet
LPCM	a Lupin Protein Concentrate Meal-based diet with supplemental DL-Met
Luc	Luciferase
M	Molar
MA	Massachusetts
MAT	Methionine Adenosyltransferase
MAT-1	Methionine Adenosyltransferase - variant One
MAT-2a	Methionine Adenosyltransferase - variant Two a
MetBW	Body Met Loss Exponent-Transformed Geometric Mean Body Weight
MetADQ	Methionine Adequate Diet
MetDEF	Methionine Deficient Diet
MetEXC	Methionine Excess Diet
MI	Michigan
MJ	Megajoules
MJ kg^{-1}	Megajoules per Kilogram
MS	Methionine Synthase
MUL-1	Mitochondrial Ubiquitin Ligase Activator of NF-κβ -1
NA	Not Applicable
NADH	Nicotinamide Adenine Dinucleotide Hydrate
NF-κβ	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NPN	Non-Protein Nitrogen
NRC	National Research Council
PBW	Body Protein Loss Exponent-Transformed Geometric Mean Body Weight
PDH	Pyruvate Dehydrogenase
PPA	Postprandial Plasma Ammonia
PRE	Protein Retention Efficiency
QAAFI	Queensland Alliance for Agriculture and Food Innovation
qPCR	Quantitative Polymerase Chain Reaction
RNA	Ribonucleic Acid
RQ	Relative Quantification
RT-PCR	Realtime Polymerase Chain Reaction
SAA	Sulphur Amino Acids
SAD	Sulphinoalanine Decarboxylase
SAHH	S-Adenoslyhomocysteine Hydrolase

SAM	S-adenosyl Methionine
SBM	Soybean Meal
SEM	Standard Error of the Mean
SKM	Saturation Kinetics Model
SPC	Soy Protein Concentrate
SPI	Soy Protein Isolate
SSE	Sum of Squared Errors
TauADQ	Taurine Adequate Diet
TauDEF	Taurine Deficient Diet
TauEXC	Taurine Excess Diet
TCA	Tricarboxylic Acid
TGF-β1	Transforming Growth factor beta 1
TOR	Target of Rapamycin
TSAA	Total Sulphur Amino Acids
ug	Microgram
UHPLC	Ultra High Performance Liquid Chromatography
UK	United Kingdom
µl	Microliter
µM	Micromolar
x *g*	Times the Force of Gravity
ZFAND-5	Zinc Finger AN1-type domain-5

Amino Acid Abbreviations

Ala	Alanine
Arg	Arginine
Asp	Aspartic Acid
Cys	Cyst(e)ine
Glu	Glutamic Acid
Gly	Glycine
His	Histidine
Ile	Isoleucine
Leu	Leucine
Lys	Lysine
Met	Methionine
Phe	Phenylalanine
Pro	Proline
Ser	Serine
Ser	Serine
Tau	Taurine
Thr	Threonine
Trp	Tryptophan
Tyr	Tyrosine
Val	Valine
SumAA	Sum of all Proteinaceous Amino Acids

Chapter 1 - General Introduction

1.1 Introduction

Traditionally, fishmeal has been the major source of protein in diets for carnivorous fish species such as barramundi, mainly due to its favourable amino acid profile in relation to that of the culture species and its ready acceptance by fish (Espe *et al.*, 2006). In the last 20 years, with the rapid expansion of intensive aquaculture moving increasingly towards the use of nutrient dense diets, demand for fishmeal has risen dramatically (Asche *et al.*, 2013). The use of this ingredient, however, is becoming increasingly economically and environmentally unsustainable, stimulating a focus on replacement with more sustainable alternatives such as those derived from plant materials or animal processing waste (Ayadi *et al.*, 2012). These alternative protein sources, however, are often deficient in one or more essential nutrients (Gatlin *et al.*, 2007) so careful formulation of diets including these ingredients is paramount.

Determining the optimal inclusion of essential nutrients in diets for fish requires a range of parameters be considered. Primarily, the requirement of the species for these individual nutrients must be ascertained. Early research in barramundi established the requirements for crude nutrients (lipid and protein) (Cuzon *et al.*, 1989; Catacutan and Coloso, 1995) while more recent research has focussed on individual nutrients such as amino and fatty acids (Murillo-Gurrea *et al.*, 2001; Coloso *et al.*, 2004; Glencross and Rutherford, 2011; Salini *et al.*, 2015b).

One of the more widely used and studied alternatives to fishmeal protein in feeds for carnivorous fish species is soy protein concentrate (SPC). It is more cost effective, compared to fishmeal and has a generally acceptable nutrient profile, having a generally favourable amino acid profile and protein which is relatively highly digestible (Glencross *et al.*, 2004; Walker *et al.*, 2010). It is, however, low in methionine in comparison to fishmeal (Mambrini *et al.*, 1999; Dersjant-Li, 2002), making accurate assessment of the requirement for this nutritional component a priority if SPC inclusion in diets for carnivorous fish is to be optimised.

Methionine (Met) is the only amino acid in the sulphur amino acid group which is considered indispensable to the growth and development of barramundi, on the basis that it cannot be synthesised by the animal from other amino acids in the required quantities so is an essential component of the diet. While its requirement has been established in other species (Rollin *et al.*, 1994; Rodehutscord *et al.*, 1995; Luo *et al.*, 2005), only one study has estimated this for barramundi (Coloso *et al.*, 1999). In addition to its primary role as a component of protein, and the associated effect on protein synthesis when it is limiting, Met has been shown in other animals to be involved in the initiation of protein synthesis (Drabkin and RajBhandary, 1998) and so deficiencies in the

diet can greatly impact the growth, development and health of the fish. It also acts as a precursor for S-adenosylmethionine (SAM), cysteine (Cys) and taurine (Tau) which are known in terrestrial animals to be involved in other important metabolic functions such as enzyme activity regulation, protein structure, osmoregulation and regulation of oxidative stress (Brosnan and Brosnan, 2006; Lunger *et al.*, 2007; Jong *et al.*, 2012). These functions are assumed, but yet to be confirmed, to also be active in aquatic species. While the roles of sulphur amino acids are relatively consistent across species, their specific roles in barramundi are unknown (and cannot be deduced from gross compositional changes in currently published growth response studies).

Growth response studies with fish have been used extensively in the past to determine the value of inclusion of individual amino acids in the diet of various fish species (Twibell *et al.*, 2000). The design of these studies was generally limited to the evaluation of changes in the gross composition of the animal and overall growth parameters in response to the dietary manipulation. While some studies incorporated enzyme assays (Walton *et al.*, 1986; Dabrowski *et al.*, 2005) and metabolite measurement (Conceição *et al.*, 1997; Luo *et al.*, 2005; Helland and Grisdale-Helland, 2011), until recently, the interpretation of the growth response was discussed primarily with reference to the role of the amino acid as a component of protein (i.e. in terms of balance and imbalance of the amino acid profile of the diet vs. that of the body protein). In these studies, little reflection was given on the exact mechanism of the response and thus offered no indication of whether these underlying processes could be regulated through other means.

One approach which does allow consideration of the effect of an individual nutrient on the multitude of metabolic pathways which control growth of the animal is assessment of the differential expression of the genes which code for the various regulators of these pathways. The utility of this approach has gained recent attention in the fish nutrition field, being applied to studies investigating lipid (Richard *et al.*, 2006; Panserat *et al.*, 2008; Martinez-Rubio *et al.*, 2013), and amino acid metabolism (Gómez-Requeni *et al.*, 2003; Lansard *et al.*, 2011; Belghit *et al.*, 2014). It has, however, only thus far been used in barramundi-specific studies to evaluate lipid metabolism (Mohd-Yusof *et al.*, 2010; Alhazzaa *et al.*, 2011; Tu *et al.*, 2012a) and, recently, energy (Wade *et al.*, 2014) and fatty acid (Salini *et al.*, 2015b; Salini *et al.*, 2016) metabolic pathways. Amino acid metabolism in barramundi is poorly understood and represents a significant gap in our knowledge which can be addressed through the application of such nutrigenomic techniques. This review is intended to outline the current knowledge in fish and barramundi in particular, with a focus on amino acid utilisation. A major aim was to identify knowledge gaps and emerging techniques for the evaluation of amino acid metabolism, leading to highlighted opportunities for further investigation.

As a footnote: for the purpose of consistency (and to avoid any confusion), *Lates calcarifer* will herein be referred to by the Australian common name of 'barramundi', regardless of the name given it in the respective publication. By the same token, the 16th element on the periodic table (and its derivatives) will be spelt 'sulphur', however the alternate spelling is recognised (e.g. transulphuration/transulfuration). Additionally, when discussing sulphur amino acid requirements, these are referred to as being in relation to requirements for cystine (and not cysteine). There seems to be little consensus in the literature on the form reported to be "required" by the animal. Cysteine is considered to be the standard amino acid incorporated into proteins, however a high degree of crosslinking between cysteine molecules occurs in the protein, forming the dimer cystine, an important process in determining protein structure. Free cystine, however, is readily reduced to cysteine under certain conditions. As such, the two are essentially interchangeable and each can fulfil the requirements for the other. The dimer (cystine) will be referred to in this document as this is generally the standard in the literature. In addition, the processing conditions for amino acid determination of the feed and carcass samples converts both cysteine and cystine to cysteic acid so any reported compositions are by effect a combination of both forms.

1.2 Barramundi (*Lates calcarifer*)

1.2.1 Biology

Lates calcarifer, commonly known as barramundi, giant perch, giant sea perch or Asian sea bass, is a euryhaline fish species with a natural habitat ranging throughout the Indo-Pacific region from eastern Australia, through south-east Asia, Japan and eastern Africa (Keenan, 1994). They have been reported to grow to sizes up to 1.8m in length and 60kg in weight (Harrison *et al.*, 2013) and are protandrous hermaphrodites (changing sex from male to female at around 5 years of age). The catadromous nature of this species dictates that the adults migrate from their usual fresh or brackish water habitat to spawn in the saltwater estuaries (Pender and Griffin, 1996). The larvae and early juveniles, therefore, are classified as marine fish with the associated implications of more active osmoregulatory mechanisms, higher metabolic demands and reliance on a marine food source nutritional profile (Houde, 1994; Sargent *et al.*, 1999; Evans, 2008). In addition, these fish are top-order carnivores (Tanimoto *et al.*, 2012), deriving the majority of their energetic needs from protein and lipids, with a limited capacity to metabolise dietary carbohydrates (Glencross, 2006).

1.2.2 Aquaculture production statistics

Commercial culture of this species began in Thailand in the mid- 1970s and in Australia in the mid-1980s (Boonyaratpalin and Williams, 2002). By 2014, worldwide production was estimated by the FAO to be 71,581 tonnes (http://www.fao.org/fishery/species/3068/en). Rapid expansion of this industry in Australia saw growth in production of over 530% between 2000 and 2011 (Harrison *et al.*, 2013). In that year, Australian production, at only 4,352 tonnes, represented a small proportion of global production volume (estimated at 68,557t) but, with a value of AU$35.7 million (Skirtun *et al.*, 2012), represented 14% of the global production value for barramundi (Harrison *et al.*, 2013). The latest statistics put this production at 3,772 tonnes in 2014-15 at a value of AU$37.1 million (Savage, 2016).

1.2.3 Nutrition research

Compared to more widely cultured species such as the salmonids, the nutritional requirements of barramundi are relatively poorly understood. Early studies into the feed conversion rates of barramundi fed trash fish were undertaken in Thailand as early as 1971 (Dhebtaranon *et al.*, 1979) but it wasn't until the mid to late 1980s that efforts to determine the requirements for individual nutrients were made (e.g. Cuzon *et al.*, 1989). Since that time, a number of studies have investigated the protein, individual amino and fatty acid, lipid and energy requirements and utilisation by this species (See review by Glencross *et al.*, 2013; Salini *et al.*, 2015a; Salini *et al.*, 2015b; Salini *et al.*, 2016).

1.2.3.1 Protein

The protein requirement of juvenile barramundi (1.34±0.01g) was determined by Catacutan and Coloso (1995) to be 42.5% of the diet (at a protein:energy ratio of 30.6g protein MJ^{-1}). Weight gain was observed to be numerically, but not significantly, higher at the 50% protein inclusion level; however, condition factor, protein efficiency ratio and apparent protein retention were significantly reduced. This compares to an earlier estimate of 45-50% by Cuzon *et al.* (1989) (at a comparable protein:energy ratio of 31.8g protein MJ^{-1}) and reported values of between 40 and 50% outlined by Boonyaratpalin and Williams (2002). This variation in estimates could be due to the lifestage of the fish (which differed among studies) or the composition of the diets used. Cuzon *et al.* (1989), for example, used increasing levels of fishmeal with decreasing corn content to achieve increasing protein level so it is not possible to separate the effect of compositional change from that of crude protein level. While the requirement for some amino acids have been defined for this species (reviewed below), further work is needed in this area.

1.2.3.2 Lipid

The requirement of barramundi for lipid has been investigated in a number of studies, initially with the assumption that it plays a similar role in protein sparing and/or as an energy source to that seen in other fish species. Williams *et al.* (2003), investigated the protein-sparing effect of lipid through two experiments (one with plate size fish – 230g initial body weight – and one with fingerlings – 80g initial body weight) assessing the effect of feeding diets with differing crude protein (CP) to lipid ratios on growth, feed conversion and body composition. They observed a linear increase in feed conversion ratio (FCR) and growth in response to increasing dietary protein (within each lipid level) and a similar step-wise improvement with increasing dietary lipid content (within diets with the same crude protein content) in both experiments, all the way up to the diet with the highest protein and lipid level (18.8% lipid/52.4% CP in Experiment 1; 18% lipid/60.3% CP in Experiment 2). It was calculated that, in the plate size fish, every 1% increase in dietary lipid content allowed a reduction in CP inclusion of 0.7% without affecting weight gain (reported as average daily gain – ADG) or FCR and, in fingerlings, this was improved to a protein-sparing potential of 1.1% per 1% dietary lipid. This sparing effect, however, was based on total weight gain of the fish which was later attributed to an increased deposition of body lipid. Lean gain was examined and found to be primarily a function of CP and not lipid content of the diets, leading to the conclusion by these authors that barramundi have a limited capability to utilise lipid as an energy source, in agreement with observations by Catacutan and Coloso (1995). A recent study with barramundi by Glencross *et al.* (2017), however, found equivalent efficiencies of energy utilisation in this species from both dietary protein and lipid. As lipid was also reported to be deposited with a high efficiency in that study (with an energetic cost of as little as 0.65kJ per kJ of deposited lipid), this may support the prospect of protein sparing for deposition by lipid as reported in other carnivorous fish species (Cho, 1992; Li *et al.*, 2012). Either way, barramundi do have a minimum requirement for lipid for other functions. The maximum weight gain and significantly higher FCR reported by Williams *et al.* (2003) for fingerling barramundi fed a diet with 18% (analysed content) dietary lipid inclusion (with 60.3% CP), compares to estimates of 10% (with 42.5% CP) (Catacutan and Coloso, 1995) and 12% (with 20% carbohydrate) (Catacutan and Coloso, 1997), with the latter authors, similarly to Williams *et al.* (2003), finding no significant difference in growth between fish fed diets containing 12/13% and 18% dietary lipid at the same level of the other nutrient in question (CP or carbohydrate), indicating a minimum dietary requirement in the vicinity of 12% of dietary dry matter. Inclusion over this level appears to be surplus to requirement and is deposited mainly as lipid, resulting in undesirably fatty flesh (Williams *et al.*, 2003). Demands for lipid, however, can be highly size-dependent with projections

of optimal inclusions of crude fat in the diet of barramundi made by the model of Glencross (2008) ranging from 75 g kg^{-1} (7.5%; for 50g fish) up to 325 g kg^{-1} (32.5%; for 2000g fish).

Few studies have investigated the requirement of barramundi for specific individual fatty acids. Estimates of demand for several essential fatty acids (EFA) for maintenance, gain and growth, over a range of fish sizes, were derived by Salini *et al.* (2016a), based on the predictive growth model for this species (Glencross, 2008; Glencross and Bermudes, 2012) and utilisation efficiencies for these fatty acids from published studies (Salini *et al.*, 2015a; Salini *et al.*, 2015b; Salini *et al.*, 2017), although many of these are yet to be experimentally validated. In a study, by Glencross and Rutherford (2010), it was observed that a dietary inclusion of 10g kg^{-1} docosahexaenoic acid (DHA) elicited numerically, but not significantly, higher weight gain in barramundi, compared to diets with varying (higher and lower) levels of this EFA. A similar lack of response was also seen by Salini *et al.* (2016c) to variable dietary inclusion of two other long chain polyunsaturated fatty acids (lc-PUFA), eicosapentaenoic acid (EPA) and arachidonic acid (ARA). Significantly improved growth was, however, observed by Glencross and Rutherford (2010) when this level of DHA was accompanied by an equivalent amount of EPA. These authors noted the similarity between this result and that of Williams *et al*. (2006) who observed maximum growth in response to diets containing 19g kg^{-1} lc-PUFA (in that case, DHA+EPA combined) provided in similar proportion to each other. This growth, however, was not significantly different to that of fish fed a diet at 12 g kg^{-1} n-3 lc-PUFA, suggesting that either the EPA:DHA ratio or combined n-3 lc-PUFA level is more important than the requirement for the individual fatty acids, provided they are supplied at a minimum combined level (12 g kg^{-1}), although this is theory is not supported by the findings of Morton *et al.* (2014) who reported no significant effect of DHA:EPA ratio on barramundi performance. More work is required to elucidate whether one EFA can spare a proportion of the other at the observed minimum requirement for *n*-3 lc-PUFA or whether the ratio of EPA:DHA is truly the most important consideration. Clearly, deficiencies in these dietary components can have substantial effects on the health and growth of these fish with Salini *et al.* (2015b) reporting significantly reduced growth and significantly higher prevalence of external abnormalities (such as inflammation and erosion of fins and skin) after as little as two weeks feeding on diets containing low levels of *n*-3 lc-PUFAs. This class of lipids, however, has been shown able to be spared from oxidation by supplementation of additional dietary monounsaturated and saturated fatty acids (Salini *et al.*, 2017), which may be especially significant since these were derived primarily from plant oils (olive oil and refined palm oil, respectively) in that study, sparing the use of more expensive, and potentially less environmentally sustainable, fish oils. The utility of

these ingredients for replacing fish oil, however, may differ depending on their origin (Salini *et al.*, 2016b), suggesting assessment of individual oils may be necessary.

1.2.3.3 Carbohydrate

While, due to the diminished capacity of marine fish to process complex carbohydrates (Wilson, 1994), it is traditionally included in barramundi feeds primarily for its pellet-binding properties, there is some evidence that this species does possess an, albeit limited, ability to utilise this nutrient for energetic purposes. In a study by Catacutan and Coloso (1997), it was observed that replacement of dietary lipid with carbohydrate (reductions of lipid by 6%, accompanied by an increase in carbohydrate of 5%) in isonitrogenous diets, did not significantly affect specific growth rate or weight gain, despite an overall reduction in dietary energy content. Similarly, feed conversion ratio was significantly improved when an additional 5% carbohydrate was supplemented to the diet with the lowest inclusion of dietary lipid. On this basis, these authors recommended a carbohydrate content of 20% in diets containing between 6 and 18% lipid and 42.5% CP. No higher levels of carbohydrate were tested, however, so it cannot be said if they are suggesting this as an ideal or a minimum level.

Several studies by Glencross and others, however, argue that starch is an extremely poor source of energy for this species, with significantly reduced efficiency of energy utilisation reported in fish fed a high starch diet by Glencross *et al.* (2017), for example, compared to those with high inclusions, as a proportion of dietary digestible energy, of either lipid or protein. While a proportion of dietary starch was proposed to have been efficiently deposited as lipid in that study, suggesting it may spare some dietary lipid for other functions, it was also observed that increasing dietary starch had a negative impact on protein utilisation, significantly impacting growth in these fish. This effect was also seen in barramundi in an earlier study by Glencross *et al.* (2014) who reported a drop in protein deposition efficiency from 41% in fish fed a diet high in lipid to 33% in those fed a high starch diet, contributing to a conclusion that this species has a clear hierarchy for energetic substrate preference, with protein being most preferable, followed by lipid, then carbohydrate/starch. A proportion of this poor utilisation may be related to an apparently limited capacity of barramundi to digest starch, even from a range of sources (Glencross *et al.*, 2012). As a result, Glencross *et al.* (2014) recommend a formulation strategy whereby starch inclusion in the diet is minimised and replaced by protein, though they concede that this may cause difficulties for extruded diets which require a minimum inclusion of starch for binding of the pellets.

1.3 Amino acid utilisation by fish

Since the advent of semi-purified diets in the 1950s, a major topic in fish nutrition has been determining the requirement for individual essential amino acids/indispensable amino acids (EAA/IAA). In recent years, this has become especially important given the variation in the amino acid profile of emerging alternative sources of dietary protein, particularly the plant meals, to that of fishmeal, historically the primary source of protein in aquafeeds (Gatlin *et al.*, 2007).

The principal role of the standard 20 proteinogenic amino acids is in the synthesis of body proteins which have a defined balance of each amino acid in relation to one another (Trushenski *et al.*, 2006). This concept of amino acid balance and imbalance is also regarded as an important consideration in dietary formulation for fish. Aragão (2004) observed that amino acid oxidation was higher in Senegalese sole (*Solea senegalensis*) fed a diet with an imbalanced amino acid profile than in those supplemented with a dipeptide solution containing leucine and phenylalanine (which were deficient in the imbalanced diet). They attributed this to the fact that, since free amino acid pools are relatively small, any amino acid which is present in excess of its proportion in the balanced profile would not be used in the synthesis of protein and is more likely to be catabolised (most likely oxidised via the TCA cycle) rather than retained. As a result, it has been argued that amino acid requirements should be expressed as a proportion of the dietary protein supply and in relation to each other to ensure this balance is maintained. This mode of expression, however, has been debated, with arguments made that requirements be expressed as a proportion of dietary energy or as a proportion of the whole diet (see review in NRC, 2011 and arguments presented below). The different modes of expression, and the arguments surrounding them, are presented here.

In addition to their role as building blocks of protein, amino acids, and their metabolites, play a number of important functions in fish metabolism including transport of long chain fatty acids from the cytosol into the mitochondria for β-oxidation (carnitine from lysine, (lysine, Li *et al.*, 2009)), osmoregulation (taurine, Lunger *et al.*, 2007), initiation of protein synthesis (methionine, Kolitz and Lorsch, 2010), eye lens protein turnover and prevention of cataracts (histidine, Remo *et al.*, 2014) and appetite stimulation (alanine and glycine, Shamushaki *et al.*, 2007). Additionally, while their excess is not considered a stimulant, deficiencies in some individual EAAs (arginine, leucine, lysine and tryptophan) has been shown to depress feed intake (De la Higuera, 2001; Tibaldi and Kaushik, 2005; Kaushik and Seiliez, 2010). Consideration of their supply, then, is an important component of dietary formulations for fish.

1.3.1 Estimation of essential amino acid requirements

Traditional amino acid requirement studies involve observation of the growth responses to variable dietary inclusion of the amino acid of interest and then repetition of this process for each of the 10 essential amino acids. This approach, however, is time consuming, especially given the rate at which novel species are being introduced (with an associated need for nutrient requirements to be established). Several methods have been used in the past to provide more rapid estimates for these requirements. While these estimates have several limitations and may not completely represent the true values, they serve as a starting point for more detailed analysis and as rough approximations where exact values are not required.

1.3.1.1 The Ideal Protein concept

One efficient method of estimating these requirements involves incorporating the ideal protein concept. This approach entails empirical assessment of the requirement for a single amino acid (usually lysine) and subsequent estimation of the requirement of the remaining essential amino acids based on their relative proportions to lysine in the whole body amino acid profile of fishmeal or of the fish itself.

This profile (considered to be the "ideal" amino acid ratio) shows a general homology with experimentally derived requirement figures (Mambrini and Kaushik, 1995; Kaushik, 1998), however, it has been observed that the intake requirements for some EAA may be under- or overestimated (Rollin *et al.*, 2003). Twibell *et al.* (2003) found that threonine, isoleucine and tryptophan may have been inadequate in diets formulated to reflect the balance (the proportions relative to each other) of the whole body amino acid profile of hybrid striped bass and adjusted to meet the requirement for lysine. Those authors postulated that this may be a reflection of the higher turnover rate of these amino acids relative to lysine. A similar effect may have been seen in a study undertaken by Hart *et al.* (2010) who found significantly improved weight gain of juvenile yellow perch (*Perca flavescens*) fed a diet with an EAA profile reflecting that of the whole body profile of that species but supplied at a level 20% in excess of the reported Met requirement (i.e. the "ideal protein" relative to Met). This suggests one or more of the EAA may have been required in the diet at a level exceeding that predicted by the carcass profile. No consideration was made, however, of the protein:lipid/energy ratio so it is hard to deduce if the extra 20% was catabolised to meet energetic demands.

1.3.2 The amino acid deletion model

The amino acid deletion model is another technique which can be used to rapidly estimate the ideal amino acid profile for a particular species. It was first established for swine by Wang and Fuller (1989) and has been used subsequently in fish to estimate ideal dietary amino acid profiles for rainbow trout (*Oncorhynchus mykiss*) (Green and Hardy, 2002), Atlantic salmon (*Salmo salar*) (Rollin *et al.*, 2003) and gilthead seabream (*Sparus aurata*) (Peres and Oliva-Teles, 2009). This method assumes that each essential amino acid is equally limiting for protein synthesis. On this basis, a basal diet is formulated to reflect either the whole body profile of the fish (Green and Hardy, 2002) or of fishmeal (Peres and Oliva-Teles, 2009) and a series of diets are designed with levels of the test amino acid 40-45% lower than that found in the basal diet (one amino acid deletion per treatment). Nitrogen retention for each treatment is then compared to the control and the proportions of each EAA required in the ideal amino acid profile are calculated based on the degree of difference in nitrogen retention between fish fed the limiting and basal diets. While Peres and Oliva-Teles (2009) noted that the EAA profile estimated by the deletion method in their study corresponded well (R^2=0.99; p<0.001) with that estimated by Kaushik (1998) using the ideal protein concept, they, along with the other two authors, acknowledged several limitations of the use of this method. Several assumptions required for this model (linearity of the nitrogen retention response, independence of the EAA utilisation efficiency from its dietary inclusion level and identical maintenance requirement for each EAA) are not always true and are therefore susceptible to artefact from experimental conditions. Green and Hardy (2002) further point out that, as all EAAs are estimated in a single experiment, the scope of the data is reduced in comparison to that generated by dose-response studies incorporating several levels of dietary inclusion. This was illustrated by the lack of improvement in nitrogen utilisation of fish fed a diet formulated to this profile compared to that of the whole body profile in a follow-up experiment.

1.3.3 Expression of individual amino acid requirements

There has, in the past, been a great deal of debate over the most appropriate mode of expression of individual amino acid requirements for fish. The general standard has been to express these figures as a percent of the diet dry matter content. The utility of this, however, is reliant upon the assumption that these requirements will be independent of the dietary digestible nutrient density (NRC, 2011), a condition which is largely invalid given the wealth of evidence of the effect of variable protein (Rodehutscord *et al.*, 2000; Kim and Lee, 2005; Wang *et al.*, 2006a) and overall energetic composition (Lupatsch *et al.*, 2001; Azevedo *et al.*, 2004) of the diet on nutrient utilisation in various fish species. As such, it has been proposed that individual amino acid

requirements be expressed on the basis of either the digestible energy (i.e. as g/ MJ DE) or digestible protein (i.e. g/kg DP) content of the diet.

1.3.3.1 Digestible energy basis

Expression of the requirement on a proportion of digestible energy basis has been advocated by several authors (Cho, 1990; Rodehutscord *et al.*, 1997; Tibaldi and Kaushik, 2005). This approach is thought to be preferable due to the wide variation in reported digestible energy contents of diets used in studies predicting amino acid requirements and would thus act as a correcting factor. It is also considered to be a suitable foundation for normalisation on the basis that fish have been shown to adjust feed intake in response to dietary DE content (presumably to meet an energetic requirement) (Cho, 1992; Lupatsch *et al.*, 2001; Yamamoto *et al.*, 2002). It was, however, shown by Encarnação *et al.* (2004) that the lysine requirement of rainbow trout (*Oncorhynchus mykiss*) was not significantly affected by dietary DE content. In this study, fish were observed to reduce diet intake with increasing dietary DE, as reported by others, but the efficiency of utilisation of the ingested nutrients for protein deposition was increased, negating the effect of intake and suggesting that it may not be appropriate to express individual amino acid requirements in this way.

1.3.3.2 Percentage of protein basis

An alternative basis for expression is to consider the requirement for individual amino acids as a proportion of dietary protein (g $16g^{-1}$ N or g kg^{-1} DP/CP). This school of thought is based on the foundation that the relationship between amino acid requirement and protein intake is constant. However, this is only true up to the level at which the DP requirement of the animal is met (Cowey and Cho, 1993). This is supported by the findings of Cheng *et al.* (2003) who found no effect on performance of feeding rainbow trout diets with protein levels varying from 37% to 42% CP and a constant lysine level, suggesting that, at least at levels approaching the CP requirement of the animal, dietary protein level does not significantly affect the requirement for individual amino acids. After this point, the limiting factor in amino acid utilisation becomes the proportion of the dietary amino acids which are catabolised for energy, as dictated by the digestible protein to digestible energy ratio (DP:DE). The higher the DP:DE, the more amino acids are catabolised for energy and the lower the utilisation efficiency of the ingested amino acids. It is not known, however, whether amino acids are catabolised at equal rates, leading to uncertainty about the extent to which EAA are spared by non-essential amino acids (NRC, 2011) and confounding the estimate of requirement for maximum growth at higher dietary protein levels. A study by Encarnação *et al.*

(2006), for example, found that non-essential amino acids provided at high levels of inclusion have limited nutritive value for rainbow trout and concluded that they may not preferentially spare EAA when the rate of amino acid catabolism is high.

Additionally, a comparison made by NRC (2011) between the studies of Encarnação *et al.* (2004) and Rodehutscord *et al.* (1997) illustrated that growth rates and lysine requirement estimates were similar when compared on a dietary concentration basis but considerably different when expressed as a proportion of dietary protein content, lending further doubt to the suitability of this mode of expression.

While neither of these approaches are completely satisfactory, what is clear is that some consensus needs to be reached as to a standardised mode of expression (or for expression on multiple bases to be reported) to allow simpler comparison between studies.

1.3.4 Protein-bound vs. crystalline amino acid utilisation

Supplementation of alternative protein sources in fish feeds with crystalline amino acids (CAA) is one method to redress the imbalance (in relation to the requirements of the target species) of the amino acid profiles of these ingredients. Earlier studies into plant protein utilisation in fish (Steffens, 1994; Gomes *et al.*, 1995), where serial replacement of fishmeal with plant protein meals resulted in variable amino acid profiles between diets, routinely used CAAs to restore this balance. Due to differences in utilisation between the two sources of EAA, however, it has been recommended that closer attention needs to be paid to the proportions of free to protein-bound amino acid inclusion in the diet (Rønnestad *et al.*, 2000).

Several studies have suggested that protein-bound amino acids are more efficiently utilised by fish than free amino acids (Zarate and Lovell, 1997; Webb and Gatlin, 2003), introducing a confounding factor when different levels of each source are used between diets. El-Haroun and Bureau (2007), for example, observed significantly greater weight gain and retained nitrogen in fish fed a diet containing 1.8% lysine from flash-dried blood meal than those fed a diet with the equivalent level of lysine provided by L-lysine HCl, suggesting the protein-bound lysine was more available for protein synthesis. This is likely due the fact that CAAs are more readily absorbed than those that are bound in proteins (Rønnestad *et al.*, 2000). Ambardekar *et al.* (2009), in a study investigating the time-course of absorption of purified amino acids by channel catfish (*Ictalurus punctatus*) compared to those in practical feedstuffs, suggested that the utilisation efficiency of dietary CAAs is reduced when used in conjunction with slowly digested proteins since the rapidly absorbed CAAs, which if being used to supplement amino acids deficient in the protein source will be out of balance with that recommended for protein synthesis, may be catabolised instead of

stored. The solution then lies in either increasing the rate of digestion of the protein source, the use of rapidly digested proteins, or reducing the rate of absorption of CAAs. Reductions in the rate of absorption can be achieved through coating of the CAAs with agar (Fournier *et al.*, 2002; Peres and Oliva-Teles, 2005; 2009; Kaushik and Seiliez, 2010; Helland and Grisdale-Helland, 2011). Alam *et al.* (2004) observed improved feed conversion efficiency, protein efficiency and specific growth rate of kuruma shrimp (*Marsupenaeus japonicas*) fed with diets where the CAAs were coated in either carboxymethyl cellulose, zein, *k*-carrageenan or agar, compared to those containing uncoated CAAs and concluded that was due to the effect of retardation of intestinal absorption and/or leaching of the CAAs into the water before ingestion.

There has been shown, however, to be a threshold, below which CAAs can effectively replace a proportion of protein. Peres and Oliva-Teles (2005) found that CAAs could replace up to 19% of the dietary protein without negatively affecting performance or feed utilisation by turbot (*Scophthalmus maximus*) juveniles, in diets with an amino acid profile reflecting that of the whole-body of that species. This compares to estimates of around 20% (reported as 10% of the diet) for Atlantic salmon (*Salmo salar*) (Espe *et al.*, 2006) and up to 50% for Senegalese sole (*Solea senegalensis*) (Pérez-Jiménez *et al.*, 2014). Williams *et al.* (2001), however, suggested that the ideal inclusion level of CAAs is dependent on the total protein level of the diet, finding that crystalline lysine was utilised as efficiently as protein-bound lysine at levels up to 3.3g L-lysine HCl kg^{-1} in a high protein diet (540g protein kg^{-1} dry matter) while up to 6g L-lysine HCl kg^{-1} could be supplemented in a low protein diet (390g protein kg^{-1} dry matter) without affecting utilisation efficiency compared to protein-bound lysine. This may be due to the fact that as dietary protein level increases, more amino acids are catabolised for energy (NRC, 2011). As previously discussed, CAAs are absorbed and catabolised at a higher rate than protein bound amino acids so at the higher protein level, a greater proportion of the supplied CAAs would have been catabolised. It is for this reason that a number of researchers advocate the statement of individual amino acid requirements on a “percent of dietary protein” basis to allow comparison between studies.

1.3.5 Incorporation of amino acid requirements into nutrient utilisation models

A number of models exist for the prediction of fish growth in response to variable dietary nutrient supply for the purposes of feed formulation optimisation. These have traditionally been primarily bioenergetic-based models (e.g. Cho, 1992; Cho and Bureau, 1998) where biomass gain is predicted based on the relationship between energy intake and the requirements for energy for various metabolic processes (Bureau *et al.*, 2002). This approach, however, has several weaknesses which limit its application within the dynamic nature of commercial aquaculture. Firstly, they are

focussed primarily on the conversion of ingested to retained energy, ignoring the sources of that energy (protein vs. lipid vs. carbohydrate) and the associated variation in the efficiencies of utilisation of these different inputs (Azevedo *et al.*, 2004) as well as the differing impact deposition of these nutrients has on the live weight of the animal (Bureau and Hua, 2008). Additionally, they do not provide any indication of the composition of the biomass gain. This is an especially important consideration for the aquaculture industry which is often interested in manipulating the final composition of the animal (Johnston *et al.*, 2006). As a result, nutrient-based models are becoming more widely used and are considered to be more representative of true biological metabolic functioning (Dumas *et al.*, 2010).

While mechanistic models (e.g. Machiels and Henken, 1986) have been developed in the past, and are often considered to be more theoretically correct (Glencross, 2008), it has been argued that less complex forms such as the factorial models proposed, and later developed further, by Glencross (2008) and Lupatsch *et al.* (1998), and their associates, adequately describe these responses in fish. These models, however, do not account directly for individual amino acid requirements or utilisation. Rather, the utilisation efficiency and maintenance requirement for crude protein, derived from dose-response studies, is incorporated into these models, allowing calculation of the requirement for maximal growth. Subsequent dietary formulations are then derived on the basis of meeting this protein demand, with the individual amino acid composition established according to inferential requirements, such as those based on the ideal protein, according to the assumption that the sum of the amino acid requirements is equal to that of protein (Lupatsch *et al.*, 1998). This assumption may not, however, be entirely accurate. Fish are thought not to have a specific requirement for protein but rather for individual amino acids (NRC, 2011). As conceded by (Lupatsch and Kissil, 2005), the protein (i.e. nitrogen) utilisation value used in these models may be dependent on the amino acid profile of the "protein" used to derive this requirement. In the event that this profile is "imbalanced" compared with that required by the fish, efforts to meet requirements for the first limiting amino acid may lead to oxidation of superfluous amino acids (Wilson, 2002; Aragão, 2004) and reduced protein synthesis (Hepher, 1988) resulting in overestimation of the requirements for crude protein. Additionally, individual amino acid requirements may change as the animal grows, as evidenced by the surmised changes in amino acid profile of the carcass of African catfish (*Clarias gariepinus*) throughout the larval and juvenile stages of development reported by Conceição *et al.* (2003), possibly due to the rate of development of individual organs which are known to have varying amino acid profiles (Ng and Hung, 1995). The percentage of whole body protein has also been shown to change during the ontogeny of salmonid species (Shearer, 1994), however, it has not been shown whether these changes occur at

the same rate as that of individual amino acids. Allowing for the differing utilisation of individual amino acids, particularly as fish size changes, may be a valuable development of growth and nutrient utilisation models. In this way, consideration of amino acids as individual dietary components, allowing incorporation of updated requirements as they become available, may improve the accuracy of model predictions.

1.4 Amino acid requirements of barramundi

Studies focussing on individual amino acid requirements of barramundi are limited, with estimations made for only four of the ten amino acids known to be essential to fish. The earliest of these studies, that of Coloso *et al.* (1999) determined the Met requirement to be 10.3g kg^{-1} dry diet (2.24% of protein). This equates to 13.4g kg^{-1} dry diet (2.9% of protein) TSAA (Met+Cys) in a diet containing 46% crude protein, 10.5% lipid and a constant 0.31% Cys level. More recent studies from the same authors have determined the requirement for lysine and arginine to be 20.6g kg^{-1} dry diet (4.5% of protein) and 18.2g kg^{-1} dry diet (3.8% of protein) respectively (Murillo-Gurrea *et al.*, 2001) and that of tryptophan to be 2.1g kg^{-1} dry diet (0.41% of protein) (Coloso *et al.*, 2004).

All of these studies employed a broken-line assay (according to Zeitoun *et al.*, 1976 and Robbins *et al.*, 1979). Coloso *et al.* (2004) and Coloso *et al.* (1999) incorporated linear equations derived from the linear regression method but it is unclear which models were used in Murillo-Gurrea *et al.* (2001), who reported using "the most appropriate model". Selection of the model(s) used to estimate nutrient requirements is an important consideration. As discussed in Robbins *et al.* (2006), the response to nutrient dose is generally not linear, with the magnitude of the response at each step decreasing as the requirement is approached, and so in most cases is best described by a quadratic equation. In this case (but not all), a straight-line single-breakpoint model may underestimate the requirement. Shearer (2000) undertook a re-evaluation of data from a number of nutrient does-response studies, applying different models to determine that which best fit the response. This author found that, out of 30 studies, 18 were best described by a second-order polynomial model, a 4 parameter saturation kinetics model (SKM) provided the best fit for 8 data sets and a 5 parameter SKM for the remaining four cases. Perhaps "the most appropriate model" used in Murillo-Gurrea *et al.* (2001) did indeed have a quadratic component (or such a model was applied and found not to best describe the response) and does therefore estimate the requirement to a high degree of accuracy. It would, however, have been useful for the purposes of repeatability, if the model used was stated.

1.5 The sulphur amino acids

One of the main limitations of the use of plant proteins in diets for carnivorous fish species is the departure from the amino acid profile of fishmeal (Table 1.1), long considered to have the ideal profile for marine fish culture due to its similarity to that of the culture species (hence its use as a reference protein in some amino acid requirement studies adhering to the ideal protein concept). Of the alternative protein sources being investigated for use in barramundi diets, soy products (SPC, SBM etc), are the most widely used. While the composition of these products differ from fishmeal in a number of ways (e.g. lipid content and profile), the most marked difference is in the amino acid profile, in particular the sulphur amino acid content. The content of Met, an "indispensible" or "essential" sulphur amino acid (i.e. one which is required to be included in the diet due to the inability/greatly reduced ability of fish to synthesis it *de novo*), for example, is considerably lower in plant protein meals such as soy products than in fishmeal and is generally considered one of the first two limiting amino acids to fish (along with Tau – another sulphur amino acid) in these products (Takagi *et al.*, 2001; Jirsa *et al.*, 2013). This group of amino acids play significant roles in protein and energy metabolism in fish and, as such, careful consideration needs to be taken of their inclusion in the diet.

Table 1.1. Essential amino acid compositions (+Cys) of some commonly used protein sources for carnivorous fish species (g kg^{-1} of ingredient dry matter).

	Essential Amino Acids										Source
	Arg	His	Ile	Leu	Lys	Met	Phe	Thr	Val	Cys	
Plant Proteins											
Soybean Meal	35	14	20	40	31	10	26	20	22	10	Riche and Williams (2011)
Soy Protein Concentrate	52	17	33	59	41	10	39	27	35	10	Deng *et al.* (2006)
Soy Protein Isolate	60	17	42	75	54	11	47	35	41	8	Riche and Williams (2011)
Wheat gluten	14	8	30	40	3	7	22	28	30	48	Reigh and Williams (2013)
Canola Meal	25	11	17	29	23	7	16	19	32	2	Cheng *et al.* (2010)
Lupin Meal[1]	39	7	14	25	13	3	14	14	14	8	Tabrett *et al.* (2012)
Animal Proteins											
Fishmeal	38	21	28	51	50	22	27	29	33	8	Tabrett *et al.* (2012)
Feather Meal	5	8	37	63	20	6	32	28	58	31	Wang *et al.* (2006b)
Meat and Bone Meal	37	12	21	41	37	10	20	18	27	4	Wang *et al.* (2006b)

[1]*Lupinus angustifolius* cv. Myallie

1.5.1 Structures of the sulphur amino acids

The sulphur amino acid class comprises those amino acids containing a sulphur atom in the side-chain (Fig. 1.1). These include Met (one of the ten essential amino acids for fish) and cysteine, two of the 20 proteinogenic amino acids (those which are incorporated into polypeptides), as well as two non-proteinogenic amino acids: homocysteine and taurine, which play important roles in many metabolic functions. The presence of the sulphur atom in the side-chain, due to its low electronegative nature, gives rise to many of the distinguishing features of these amino acids. Methionine, for example, is highly hydrophobic, an attribute which is thought to be central to its role in initiating eukaryotic protein synthesis.

Cysteine, while generally classed as a polar amino acid due to chemical similarities between its thiol group and the hydroxyl group of other polar amino acids, is considered to be equivalently hydrophobic to Met (Nagano *et al.*, 1999). As a result, these amino acids are most commonly found on the interior of proteins. Unlike most other amino acids, cysteine is rarely present in cells in its free form. It is more commonly found in the form of cystine (two cysteine residues covalently bound), due to its tendency to ionise, forming the thiolate anion and subsequently oxidising and forming disulphide linkages with other cysteine residues (Houghland *et al.*, 2013), another function of the low electronegativity of sulphur.

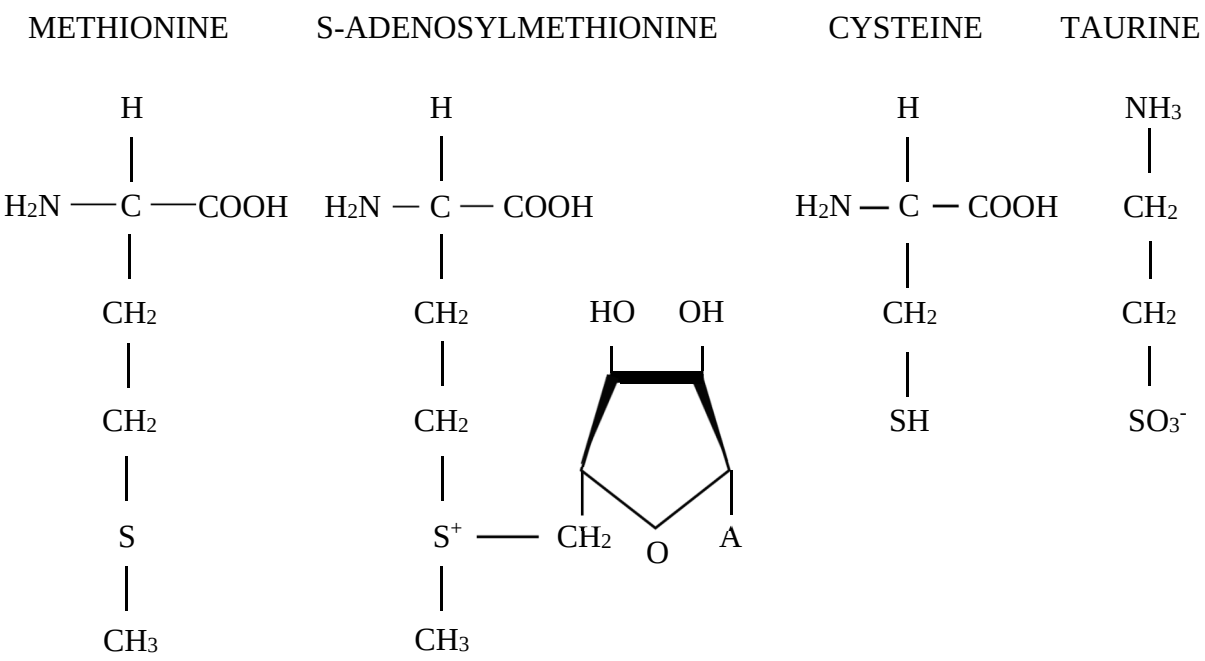

Figure 1.1. Molecular structures of the sulphur amino acids.

1.5.1.1 Metabolism of the sulphur amino acids

The sulphur containing amino acids are metabolised through a number of pathways (Fig. 1.2). The first of these, transmethylation (Step 1), involves the activation of Met to S-adenoslymethionine (SAM) in a reaction catalysed by the enzyme Met Adenosyltransferase (MAT), before SAM is converted to S-Adenosylhomocysteine by methyl transferases (Step 2) and finally homocysteine by S-Adenoslyhomocysteine Hydrolase (SAHH) (Step 3).
From this point, remethylation of homocysteine to Met can occur by either methionine synthase (MS) or betaine:homocysteine methyltransferase (BHMT)(Step 4)(the latter occurring only in the liver and, in some species, the kidney). In the absence of a need for remethylation, homocysteine is catabolised via the transsulphuration pathway, whereby it is converted to cystathionine (Step 5) and then on to Cys by cystathionine β-synthase and cystathionine γ-lyase respectively (Step 6).

This is an irreversible chain of reactions and illustrates why Met is considered a dietary essential amino acid while Cys (which can be synthesised from Met but not the reverse) is not, provided sufficient Met is available (Brosnan and Brosnan, 2006).

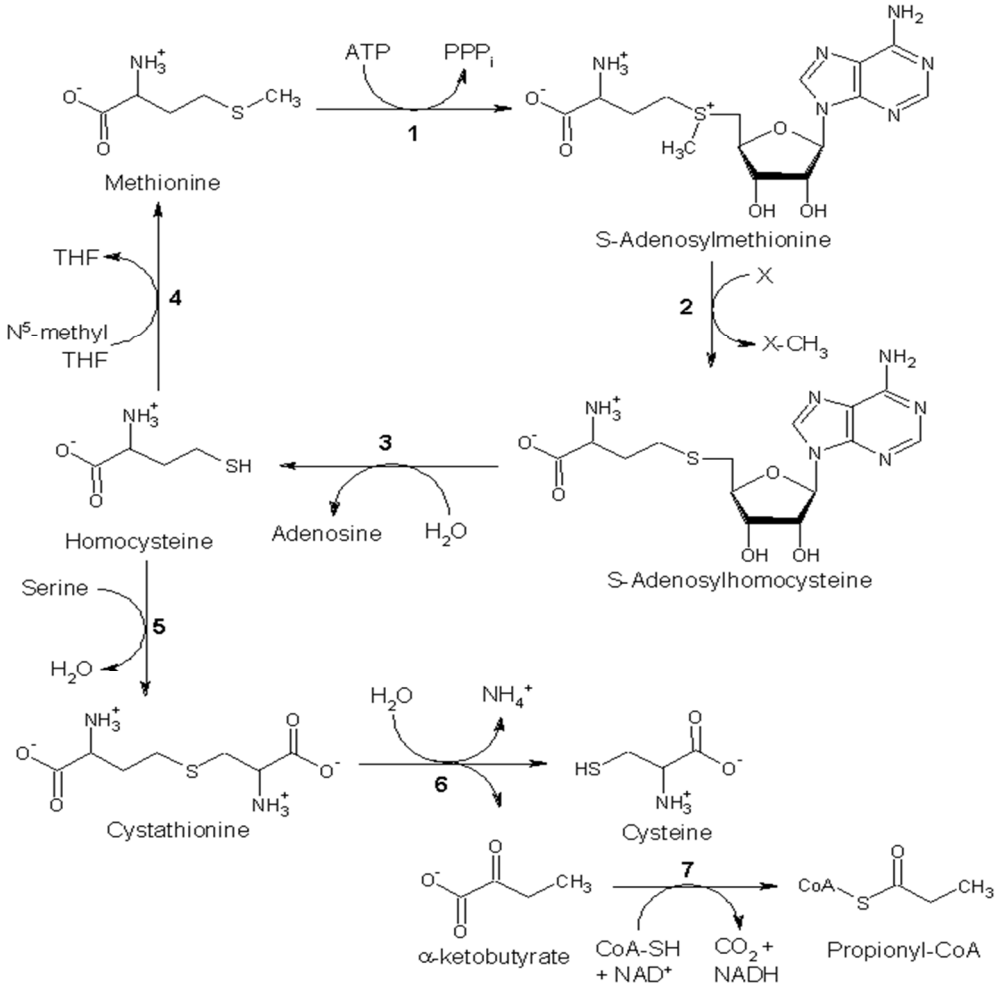

Figure 1.2. Pathways of sulphur amino acid metabolism (Inositol, 2005).

Production of Tau from Cys can then occur via one of two pathways (Fig. 1.3). The first involves the oxidation of Cys to 3-sulphinoalanine by cysteine dioxygenase (CDO), then on to hypotaurine (by either glutamic acid decarboxylase – GAD – or sulphinoalanine decarboxylase – SAD) before final alteration to Tau by hypotaurine dehydrogenase (HTDeHase). Alternately, a shorter pathway can be used whereby Cys is converted to cysteate glutamic acid by cysteine lyase (CL). The final step, conversion of cysteate glutamic acid to Tau can be catalysed by either glutamic acid decarboxylase (GAD), sulphinoalanine decarboxylase (SAD) or cysteine sulphinic acid decarboxylase (CSAD) (Turano *et al.*, 2012).

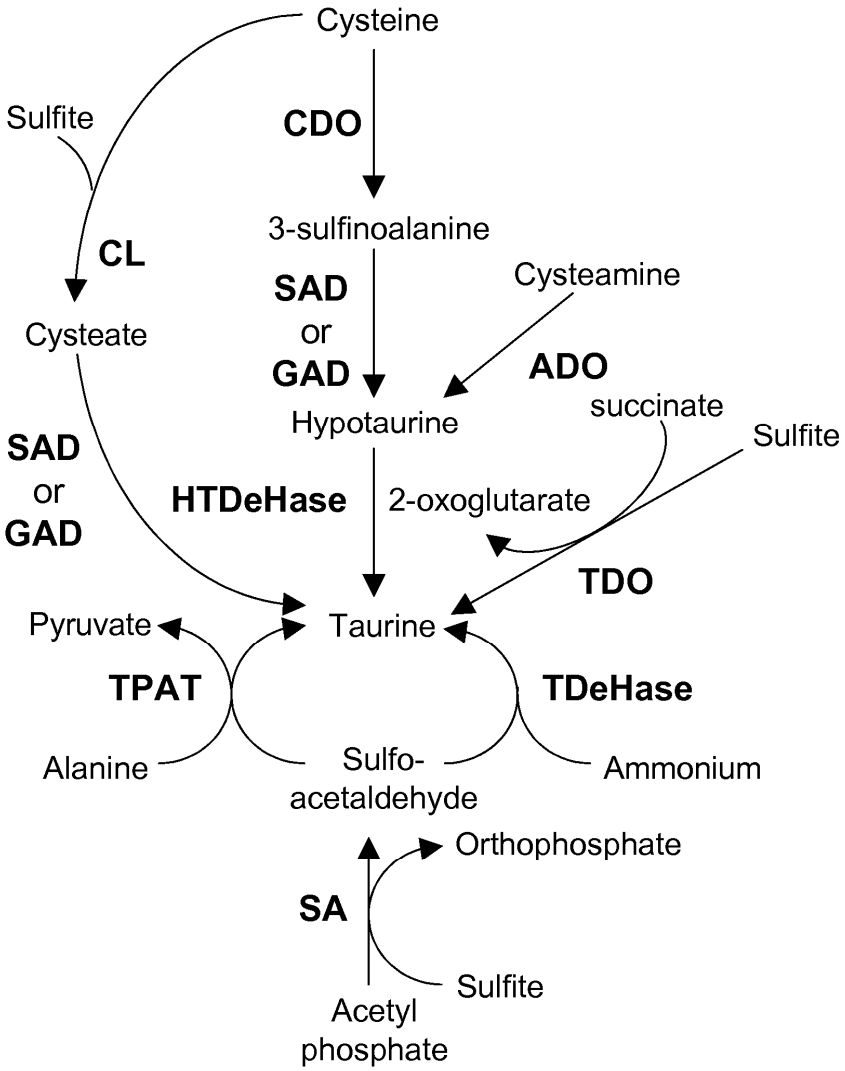

Figure 1.3. Pathways of taurine biosynthesis (Turano *et al.*, 2012)

1.5.2 Metabolic roles of the sulphur amino acids

1.5.2.1 Methionine

In addition to the incorporation of Met and Cys into proteins, the sulphur amino acids, and their metabolites, play vital roles in many metabolic processes. Methionine, as suggested previously, is an initiating factor in the synthesis of proteins in eukaryotic organisms. This process is most commonly instigated when the AUG codon of the initiator Met tRNA binds to the eukaryotic initiation factor EIF2. Drabkin and RajBhandary (1998) showed that the mutant tRNAs AGG (with Met) and GUC (with valine) could both initiate protein synthesis in mammalian COS1 cells, however, they maintain that Met is still "probably the best". Deficiencies in Met have been reported to result in the formation of cataracts in both rainbow trout (*Oncorhynchus mykiss*) (Cowey *et al.,* 1992) and Arctic charr (*Salvelinus alpinus*) (Simmons *et al.,* 1999).

S-adenosylmethionine (SAM), an intermediate in the transmethylation of Met to homocysteine, acts as the principle methyl donor in a large number of metabolic reactions, including transmethylation (transfer of methyl groups), transulphuration (Cys ↔ homocysteine) and aminopropylation (leading to the synthesis of polyamines)(Mato *et al.,* 1997). It is estimated that there are around 300 Class 1 SAM-dependent methyltransferases alone in humans (Katz *et al.,* 2003; Brosnan and Brosnan, 2006). Methyl group donation by SAM is involved in the synthesis of nucleic acids, proteins, phospholipids and biogenic amines (Espe *et al.,* 2008) and is known to regulate enzyme activity, inhibiting methylene tetrahydrofolatereductase (an enzyme involved in remethylation reactions) and activating cystathionine β-synthase (stimulating transsulphuration). This functional diversity is largely a factor of the activation, by its sulphonium ion, of the electrophilic carbons adjacent to the sulphur atom for nucleophilic attack (Lieber and Packer, 2002). Additionally, SAM can donate sulphur atoms, methylene, amino and aminoisopropyl groups for the synthesis of a range of compounds from fatty acids to vitamins.

1.5.2.2 Cysteine

The main biological function of cysteine is in its role in the folding and stability of many proteins, due to the ease with which it forms covalent disulphide bridges with other cysteine molecules (forming cystine). Disulphide bonding between Cys residues is important in both the rigidity of proteins (when bonds are crosslinking proteins) and the tertiary structure of individual polypeptides (when bonding occurs within the polypeptide) as well as minimising proteolysis due to the resistance of these bonds to proteases. Liu *et al.* (2012), for example, found that adding a disulphide bond to a recombinant immunotoxin enhanced its resistance to trypsin degradation.

1.5.2.3 Taurine

Taurine (Tau) is not an amino acid *per se*, as it lacks a carboxyl group, possessing a sulphonate group in its place (so, technically, should be classed as an amino sulphonyl acid) but is generally referred to as a sulphur amino acid. It is not incorporated into proteins and yet, in its free form, is one of the most abundant amino acids in the brain, retina, muscle tissue and various organs (Ripps and Shen, 2011). It is also found in the cytosol but accumulates in "excitable tissues" (those which are able to go through rapid change in the membrane potential of their cells) (Huxtable, 1992). Tau is perhaps the most intriguing of the sulphur amino acids due to the variety of biological functions in which it is involved and the fact that the exact mechanism of its action is poorly understood. In mammals, it is known to play a role in the control of neurotransmitter and synaptic activity, calcium uptake and antioxidant protection of retinal rod outer segments (Pinto *et al.*, 2013) as well as modulating osmoregulation, bile acid conjugation, membrane stabilisation and hormone release (Lunger *et al.*, 2007). Its bile acid conjugating action is important in the absorption of nutrients (Espe *et al.*, 2010). Due to its antioxidant properties, Tau is considered a significant cytoprotectant and plays a pivotal role in the regulation of oxidative stress. Jong *et al.* (2012) concluded that this was due to Tau serving as a regulator of mitochondrial protein synthesis. In doing so, it stabilises the electron transport chain and inhibits excessive superoxide generation (Ripps and Shen, 2011). Schaffer *et al.* (2000) stated that many of the processes affected by cellular Tau content are associated with fluctuations in ion transport and that the three most common methods of manipulating cellular ion transporter activity (influencing expression, protein phosphorylation status and cytoskeletal changes) are all altered by osmotic stress, a condition directly related to Tau's major role as an organic osmolyte. In addition to affecting cell volume through influencing fluid fluctuation, Tau has also been shown to regulate the influx and efflux of a number of important electrolytes such as K^+ and Na^+ through stimulation of the activity of Na^+/K^+ ATPase (the 'Na^+/K^+ pump') (Nandhini and Anuradha, 2003), another process of cell volume regulation. Ribeiro *et al.* (2009) showed that Tau supplementation significantly increased Ca^{2+} uptake and expression of the L-type β_2 subunit Ca^{2+} channel. Ca^{2+} is known to activate a phosphatase which dephosphorylates pyruvate dehydrogenase (PDH), increasing its activity (Pizzo *et al.*, 2012). This enzyme (PDH) catalyses the transformation of pyruvate to acetyl-CoA, linking the glycolytic and citric acid cycle energetic metabolic pathways, releasing NADH in the process. Thus, it can be said that cellular Tau level may have a direct influence on energy metabolism.

1.6 Sulphur amino acid requirement studies in fish species

1.6.1 Methionine and cystine (TSAA) requirement

All fish species, as is the case for all monogastric animals, have a defined dietary requirement for Met, given that it cannot be synthesised, or at least is not synthesised efficiently in the required quantities, by the animal (Webster and Lim, 2002). Cys is able to be synthesised from Met, provided sufficient quantities are present in the diet. Therefore it is not considered an indispensible or essential amino acid. However, it is known to have a sparing effect on the requirement for Met (i.e. if adequate Cys is provided, a lower proportion of dietary Met is required for Cys synthesis)(Wang *et al.*, 2014). As a result, Met requirements of fish are generally reported as either a total sulphur amino acid (TSAA) requirement (Met+Cys) or as a Met specific requirement but "in the presence of (a certain proportion of) Cys". For the purposes of comparison in this review, I will report all values in "g kg^{-1}" (they can be reported in the literature as % of dry diet, % of protein, g $16g^{-1}$ N or g kg^{-1} diet dry matter).

Estimates of the Met requirement for salmonid fishes vary widely. Sveier *et al.* (2001) estimated the Met requirement for maximum growth of Atlantic salmon (*Salmo salar*) to be 9.2 g kg^{-1} of diet dry matter (2.24% of CP), derived from a linear best-fit model of Met intake vs. mean weight increase (kg $fish^{-1}$). If it is assumed that feed, and thus Met, intake was comparable between diets (data which was not reported but can be estimated based on the feed conversion ratio (FCR) and growth data provided), and considering the model predicted maximum growth response as occurring at a Met inclusion level between that of the diets with 8.5 and 10.6 g kg^{-1} of Met (which contained 12.9 and 15.3 g kg^{-1} TSAA respectively), it can be estimated that the TSAA requirement estimate would have been approximately 13.7g kg^{-1} (i.e. the requirement for TSAA would be equal to 9.2 g kg^{-1} Met in the presence of 4.5 g kg^{-1} Cys). This estimate compares to other experimentally derived reported values for Atlantic salmon Met requirement of 11.4 g kg^{-1} (2.85% of CP) (Scott, 1998) and 11 g kg^{-1} (2.4% of CP) by Rollin *et al.* (1994). Rollin *et al.* (2003) estimated the TSAA requirement of Atlantic salmon to be 15.3 g kg^{-1} (3.4% of CP) from a model calculating the optimum indispensible amino acid balance, but did not provide individual figures for Met and Cys.

TSAA requirement figures for rainbow trout (*Oncorhynchus mykiss*) vary from 8g kg^{-1} DM (2.3% CP)(Kim *et al.*, 1992) to 14 g kg^{-1} (3.5% CP) (Hardy, 2002). Earlier figures based on dose-response growth studies, such as that proposed by Rodehutscord *et al.* (1995), have been used as the basis for the formulation of experimental rainbow trout diets for many years and little research has been done recently to confirm these estimations with alternative techniques. One study which has attempted to do so is that by Bae *et al.* (2011) who used plasma Met and ammonia concentrations in surgically modified (dorsal aorta canulated) rainbow trout to re-evaluate the dietary requirement for

Met. They found that post-prandial and post-absorptive plasma free Met (PPmet and PAmet) concentrations were significantly elevated when fish were fed diets with 6 g kg^{-1} or more (of dietary dry matter) Met, with the assumption that dietary Met in excess of the requirement would be present in the free amino acid pools, either stored or en route to the liver for excretion, rather than incorporated into protein or immediately catabolised for other metabolic functions. Similarly, post-prandial plasma ammonia (PPA) concentrations were measured as excess amino acids are catabolised in the liver and excreted as ammonia. These levels were seen to remain low and constant in fish fed the diets with up to 6g kg^{-1} Met, after which point (from 7 g kg^{-1}), they increased significantly. A broken-line model analysis on these three parameters (PPmet, PAmet and PPA), indicated the dietary requirement to be between 5.9 and 6.7 g kg^{-1} Met in the presence of 5 g kg^{-1} Cys (TSAA = 10.9 – 11.7 g kg^{-1}) (3.1 – 3.3% CP). In a study by Belghit *et al.* (2014), the authors considered 8.2 g kg^{-1} Met in the presence of 4.3 g kg^{-1} Cys (12.5 g kg^{-1} (3.15% CP) TSAA) as an "adequate" level as part of a comparison of the effect of dietary Met level (deficient vs. adequate vs. excess) on growth and the expression of genes involved in the regulation of mRNA translation and the ubiquitin-proteosomal and Autophagy-Lysosomal proteolytic systems. They found that fish fed diets with a deficient level of Met (5.6 g kg^{-1} Met + 4.7 g kg^{-1} Cys = 10.3 g kg^{-1} (2.61% CP) TSAA) had a significantly lower body weight and feed efficiency than those fed a diet with an adequate or excess level of Met. Additionally, a decreased activation of ribosomal protein s6 and eIF2α, translation initiation factors was observed in fish fed the Met deficient diets. This suggests that a Met level of 8.2 g kg^{-1} (in the presence of 4.3 g kg^{-1} Cys = 12.5 g kg^{-1} (2.07% CP) TSAA) is adequate for rainbow trout but it cannot be known whether a lower level would produce the same result so an accurate estimation of minimum requirement based on this data is not possible. A replicated study including a greater range of dietary TSAA levels would allow this and would be extremely useful for comparison to growth studies.

Estimates of the TSAA requirement of other fish species vary widely based on species and assessment criteria (Table 1.2). Disparity in requirements of fish for protein and amino acids such as this may be the result of several variables, including differences in the ecological niches of the species (natural habitat affecting the turnover of specific amino acids and/or trophic level influencing the composition of natural food sources or transport of amino acids in the gut) (Ferraris and Ahearn, 1984; Auerswald *et al.*, 1997; Hertrampf and Piedad-Pascual, 2012), fish size or age (Tacon and Cowey, 1985) or experimental design (dietary crude protein level and source (Cowey and Cho, 1993; NRC, 2011), range and spacing of inclusion levels of the amino acid of interest and/or model used to define the requirement (Shearer, 2000); proportions of dietary Met:Cys (as discussed previously), feeding regime (Cowey, 1995), water temperature (Bermudes *et al.*, 2010);

and the use of crystalline amino acids and whether these were bound or encapsulated prior to feed production (as also referred to earlier in this chapter)).

The response parameter on which the requirement is based, in particular, can have a major impact on the requirement value reported. Twibell *et al.* (2000) showed that Cys can spare up to 51% of the Met requirement of juvenile yellow perch (*Perca flavescens*). In a follow up experiment, these authors used this maximum replacement value of Cys (i.e. a ratio of 51:49% Cys:Met) in diets with graded levels of TSAA. They determined, by broken line analysis, the dietary TSAA requirement to be between 8.5 (based on weight gain) and 10g kg^{-1}(based on feed efficiency)(4.2 – 4.9 g kg^{-1} Met + 4.3-5.1 g kg^{-1} Cys).

Table 1.2. Selected estimates of total sulphur amino acid requirements of various fish species. Figures are g kg-1 of diet with % of crude protein in brackets, unless otherwise stated.

Species	CP	TSAA	Met	Cys	Reference
Yellow Perch (*Perca flaviscens*)	336	8.5 – 10 (2.5 – 3.0%)	4.2 – 4.9 (1.3 – 1.5%)	4.3 – 5.1 (1.3 – 1.5%)	Twibell *et al.* (2000)
Indian Catfish (*Heteropneustes fossilus*)	400	14.9 (3.7%)	10.9 (2.7%)	4.0 (1.0%)	Ahmed (2014)
Stinging Catfish (*Heteropneustes fossilus*)	380	14.7 (3.8%)	7.1 (1.9%)	7.6 (2.0%)	Khan (2014)
Black Sea Bream (*Sparus macrocephalus*)	380	20.2 – 20.3 (5.3%)	17.1 – 17.2 (4.5%)	3.1 (0.8%)	Zhou *et al.* (2011)
Rohu (*Labeo rohita*)	400	9.6 – 11.9 (2.5 – 3.1%)	5.8 – 7.1 (1.5 – 1.9%)	3.8 – 4.8 (1.0 – 1.3%)	Abidi and Khan (2011)
Golden Pompano (*Trachinotus ovatus*)	430	14.6 (3.4%)	12.9 (3.0%)	1.7 (0.4%)	Niu *et al.* (2013)
Atlantic Salmon (*Salmo salar*)	412	13.7 (3.3%)	9.2 (2.2%)	4.5 (1.1%)	Sveier *et al.* (2001)
Rainbow Trout (*Oncorhynchus mykiss*)	340	11 (3.2%)	8 (2.4%)	3 (0.8%)	Rodehutscord *et al.* (1995)
Blunt Snout Bream (*Megalobrama amblycephala*)	340	10.6 – 10.7 (3.2%)	8.4 – 8.5 (2.5%)	2.2 (0.7%)	Liao *et al.* (2014)
Cobia (*Rachycentron canadum*)	438	18.6 (4.3%)	11.2 (2.6%)	6.7 (1.5%)	Zhou *et al.* (2006)

Table 1.2 (cont.)

Yellowtail (*Seriola quinqueradiata*)	434	14.2 (3.3%)	11.1 (2.6%)	3.1 (0.7%)	Ruchimat *et al.* (1997)
Grouper (*Epinephelus coioides*)	485	15.7 (3.2%)	13.1 (2.7%)	2.6 (0.5%)	Luo *et al.* (2005)
European Sea Bass (*Dicentrarchus labrax*)	440	13.1 (3.0%)	12 (2.7%)	1.1 (0.3%)	Tulli *et al.* (2010)
Indian Major Carp (*Cirrhinus mrigala*)	396	22 (5.5%)	12 (3.0%)	10 (2.5%)	Ahmed *et al.* (2003)
Large Yellow Croaker (*Pseudoscianena crocea*)	428	16.8 – 17.3 (3.9 – 4.0%)	13.9 – 14.4 (3.2 – 3.3%)	2.9 (0.7%)	Mai *et al.* (2006)
Arctic Charr (*Salvelinus alpinus*)	400	17 (4.3%)	7 (1.8%)	10 (2.5%)	Simmons *et al.* (1999)
Japanese Flounder (*Paralichthys olivaceous*)	500	15.0 – 15.6 (3.0 – 3.1%)	14.4 – 14.9 (2.9 – 3.0%)	0.6 (0.1%)	Alam *et al.* (2000)
Channel Catfish (*Ictalurus punctatus*)	240	5.6 (2.3%)	5.6 (2.3%)	0.0 (0.0%)	Harding *et al.* (1977)
Red Drum (*Sciaenops ocellatus*)	350	10.6 (3.0%)	9.4 (2.7%)	1.2 (0.3%)	Moon and Gatlin (1991)
Mossambique Tilapia (*Saratherodon mossambicus*)	400	12.7 (3.2%)	5.3 (1.3%)	7.4 (1.9%)	Jackson and Capper (1982)
Nile Tilapia (*Oreochromis niloticus*)	280	9.4 (3.4%)	4.9 (1.8%)	4.5 (1.6%)	Nguyen and Davis (2009)
Barramundi (*Lates calcarifer*)	460	13.4 (2.9%)	10.3 (2.2%)	3.1 (0.7%)	Coloso *et al.* (1999)

The estimates by Zhou *et al.* (2006 and 2011) and Ahmed (2014) of the TSAA requirements of Cobia, Black Sea Bream and Indian major carp respectively stand out as elevated in comparison to reported values for other fish species. There seems no obvious reason, however, to conclude that these estimates are not correct. Fish of between 0.50g (Ahmed *et al.*, 2003) and 14.21g (Zhou *et al.*, 2011) starting weight were used in these experiments which is within the size range used in several other TSAA requirement studies concluding much lower requirement estimates (Jackson and Capper, 1982; Moon and Gatlin, 1991; Nguyen and Davis, 2009). Similarly, fish in all experiments were fed to satiation, second-order polynomial or quadratic regression was used to estimate requirements (a valid statistical approach) and there were no dietary or environmental conditions differing significantly from studies with similar fish which may point to erroneous conclusions. It must, therefore, be accepted that the figures presented are, in fact true representations of the requirement of these species for TSAA. Another species of Indian carp, with

a similar lifestyle to Indian major carp (freshwater omnivore), rohu (*Labeo rohita*), was shown by Abidi and Khan (2011) to have a comparatively low requirement for TSAA of between 9.6 to 11.9 g kg^{-1} DM (Table 1.2), despite similar starting weights of the fish in the study to those studied by Ahmed *et al.* (2003) (0.66g and 0.50g, respectively), suggesting lifestyle alone is not a reliable indicator of the metabolic needs for this dietary component.

1.6.2 Taurine Requirement

The Tau requirements of fish have not received a great deal of attention in the past due to the relatively high level of this amino acid in the traditional main (or often, sole) source of protein in aquaculture feeds, fishmeal. However, the recent trend towards reducing fishmeal inclusion in fish dietary formulations, and associated increases in the use of plant meals, which are devoid of Tau, has provided a great impetus for determining a minimum requirement for various species.

As Tau can be synthesised from Met or Cys, it has not traditionally been considered an essential amino acid. Recent work, however, has shown that the activity of cysteinesulphinate decarboxylase (CSD), otherwise known as sulphinoalanine (or cysteine sulphinic acid) decarboxylase (CSAD/SAD), the enzyme which catalyses the conversion of cysteinesulphinate to hypotaurine, varies widely between fish species. For those species with a reduced activity of this enzyme, and thus a reduced capacity to biosynthesise Tau, this amino acid may be considered to be conditionally indispensible (i.e. it is required during conditions where Tau synthesis from Met or Cys does not meet the metabolic demand). Species with this reduced activity include red sea bream (*Pagrus major*) and yellowtail (*Seriola quinqueradiata*), the latter having no detectable activity of CSD at all (Yokoyama *et al.*, 2001), while salmonids are known to have a high capacity for Tau biosynthesis. According to Yokoyama *et al.* (2001), Japanese flounder (*Paralichthys olivaceus*) also have little capacity to synthesis Tau, however, those authors surmised that this was related less to CSD activity (despite the fact it was measured to be one third to one half that of rainbow trout) than to a possible preference for the transamination of cysteinesulphinate to α-sulphinylpyruvate rather than decarboxylation to hypotaurine.

These variations in Tau biosynthetic capacity may not have been significant in fish fed diets with high inclusion of fishmeal as the major source of protein, however, the increasing use of Tau deficient plant meals has prompted a number of groups to investigate the effect of supplementing plant protein based diets with Tau on the performance of various fish species with the view to allowing maximum inclusion of these more sustainable and economical ingredients. Lunger *et al.* (2007) showed that supplementation of 0.5g Tau $100g^{-1}$ of diet dry matter to a diet containing high levels of fishmeal replacement with a yeast-based protein source significantly improved weight gain

in juvenile cobia (*Rachycentron canadum*). In a second experiment, where fishmeal was increasingly replaced by the yeast-based protein in a series of diets, these authors again observed weight gain, specific growth rate and feed efficiency ratios to be improved by Tau supplementation, although these parameters decreased with increasing replacement of fishmeal. Tau supplementation level remained at 0.5g Tau $100g^{-1}$ of diet dry matter for all diets, although it was not clear why this level was chosen and no discussion was made as to the possible effect of increased supplementation.

The beneficial effects of Tau supplementation of plant-based diets on growth has also been seen in rainbow trout (*Oncorhynchus mykiss*) fed Met deficient soy protein concentrate-based diets (Gaylord *et al.*, 2006; Gaylord *et al.*, 2007), red sea bream (*Pagrus major*) (Matsunari *et al.*, 2008b; Matsunari *et al.*, 2008a; Takagi *et al.*, 2010), larval cobia (*Rachycentron canadum*) (Salze *et al.*, 2012), yellowtail (*Seriola quinqueradiata*) (Takagi *et al.*, 2008) and juvenile turbot (*Scophthalmus maximus* L.) (Qi *et al.*, 2012) with the latter authors going on to give an estimate of dietary Tau requirement of 1.0% (10g kg^{-1}) for turbot of 6.3±0.01 g weight and 0.5% (5g kg^{-1}) for larger turbot (165.9±5.01 g weight) fed the same diets. Taurine was also seen to benefit gilthead seabream (*Sparus auratus*) larvae through an apparent sparing of Met for other metabolic processes (increasing Met retention in body tissue and free amino acid fractions with a related reduction in Met catabolism) (Pinto *et al.*, 2013).

1.6.3 Studies with barramundi

To date, only one study has been undertaken to determine the sulphur amino acid requirements of barramundi (*Lates calcarifer*). Using a break point analysis on the basis of growth response, Coloso *et al.* (1999) estimated the TSAA requirement of juvenile barramundi to be 13.4g kg^{-1} dry diet (10.3 g kg^{-1} Met+3.1 g kg^{-1}Cys) (2.24% of protein in a 46% protein diet). As is standard for studies estimating TSAA requirements, variable Cys level was not examined so the Met requirement can only be stated as being so at that level of Cys. One factor which makes comparison of the results of this study with any future estimations of Met or TSAA requirement in barramundi difficult is the calculated amino acid composition of the diets, in particular the analysed Cys content.

The level of Cys in the SBM used in this study was reported to be 0.46% of dry weight (based on a back-calculation of Table 2 in that publication, which outlines the amino acid contribution of each of the ingredients). This compares to an average of 0.7% reported by Cromwell *et al.* (1999) from 31 samples of SBM and 0.91% in a recent sample of locally sourced SBM in our lab (Bourne, 2012 *pers comm.*). Accurate measurement of this amino acid is reliant on

the use of an additional oxidation step prior to hydrolysis of the protein (Rutherfurd and Gilani, 2009). The procedure for amino acid analysis was not reported so it cannot be known if this protocol was used, lending doubt to the figures derived. If, indeed, the Cys content of the SBM used in this study was underestimated, so too would be the requirement, suggesting that revisitation of this estimate would be wise.

1.7 Nutrigenomics

Nutrient metabolism is a complex process, involving the combined action of a large number of factors through a myriad of pathways. In order to determine the true metabolic requirement for individual nutrients, we must further understand the impact of the provision of these compounds on the various processes which control the growth of the animal. This is particularly important in fish nutrition presently given the pressing need to properly assess the suitability of alternative sources of protein to fishmeal.

Traditional growth response studies are limited to observations of the effect of feeding these products on growth, feed intake and body composition, with little consideration of the metabolic basis of how and why these changes occur. One approach which allows the simultaneous quantification of the effect of individual nutrient intake on the various metabolic pathways (and thus the mechanisms behind the observed manifestations of these processes) is the assessment of its effect on expression of the multitude of genes associated with their control.

1.7.1 Health-related gene expression

Optimal growth of an animal is achieved primarily through maximising utilisation of dietary nutrients. This is a two-fold consideration whereby the nutrient profile of the diet needs to meet or exceed the requirement of the animal for energy, maximum protein synthesis and other biological functions as well as maintaining the health (i.e. immune function) of the animal. A lack of consideration of the latter may lead to an undesirable proportion of dietary energy, otherwise sparing protein for muscle gain, being utilised in combating disease and, in chronic cases, may even lead to catabolism of these protein stores, resulting in an animal that is less saleable and economical to produce. Understanding the mechanism of the effect of dietary composition on health-related markers of response may provide vital clues to ameliorating these issues, whether it be characterising the importance of the stimulating or depressant effect of certain nutrients on the immune response or identifying dietary components which have direct detrimental effects on the health of the animal (e.g. causative agents of SBM-induced enteritis).

One example of the complementary nature of combining growth and pathology studies with examination of gene expression levels is that of Hernández *et al.* (2013). These authors investigated the effect of feeding juvenile rainbow trout diets with varying levels of lupin (*Lupinus albus*), pea (*Pisum sativum*) and rapeseed (*Brassica rapus*) cake meals on growth performance and expression of immune-regulatory genes. They observed that lupin inclusion up to 250g kg^{-1} and pea up to 50 g kg^{-1} had no significant effect on growth parameters (weight gain and feed efficiency) or protein efficiency compared to the control diet. Inclusion over these levels were reported to cause significant reductions in these growth parameters. This result was reflected in the molecular response. RT-qPCR was used to determine the expression of the genes for the immunological proteins 'transforming growth factor β1' (TGF-β1), 'nuclear factor-κβ' (NF-κβ) and Mx-1 isolated from head kidney tissue, an important immune organ in teleost fish (Chen *et al.*, 2012). No significant effect of protein source or level was seen on the constitutive gene expression of TGF-β1 or NF-κβ. However, the Mx-1 gene in fish fed those same diets showing significant reductions in growth was determined to be significantly downregulated in comparison to the control group. The Mx-1 protein has been linked to protection against infectious pancreatic necrosis in Atlantic salmon and viral haemorrhagic septicaemia and hirame rhabdovirus in Japanese flounder (Caipang *et al.*, 2003; Larsen *et al.*, 2004). No histological examination (other than hepatosomatic index – liver weight:body weight ratio which was not significant) or immune challenge was performed in Hernández *et al.* (2013), so no link between plant meal-induced Mx-1 downregulation and incidence of viral infection could be made, nor any causative effect of expression of this gene on the depressed growth rate (or vice versa), which might have added considerably to the findings. The observation that there appears to be an effect of type and level of plant meal inclusion on Mx-1 gene expression, which is likely unrelated to the observed growth depression, is nonetheless interesting and would benefit from further investigation. Direct links between plant protein inclusion, histological observations and molecular changes have, however been made in Atlantic salmon in response to soybean meal inclusion in the diet. Sahlmann *et al.* (2013) observed that salmon fed diets containing 200g kg^{-1} SBM started to show the first histological signs of inflammation associated with SBM-induced enteritis after five days of feeding the SBM diet. Significantly, this macroscopic response was preceded by changes in expression of a number of genes associated with the immune response as early as one day after first feeding of this diet, highlighting the utility of this tool as a means of rapid assessment of a diet or ingredient's suitability. Without listing every gene which was differentially expressed in response to feeding the SBM diet, compared to the control diet, it should be noted that, by day three after first feeding, this list included 37 genes of varying functions in addition to eight specific to immune regulation. Most of the observed

molecular responses were directly linked by the authors to known histological responses to SBM (e.g. upregulation of the p100 sub-unit of NF-κβ which is involved in the inflammatory response). While a wealth of information can be extracted from this study (which also included investigation of response of protein and lipid metabolic genes amongst others), a similar study isolating the response to specific antinutritional factors, while excluding the effects of others could be very useful. Similar results were reported in Atlantic salmon in an earlier study by Marjara *et al.* (2012), who highlighted the significant upregulation of IL-17A, the proinflammatory cytokine responsible for regulating the activity of NF-κβ.

1.7.2 Protein metabolism gene expression

Studies focussing on the molecular response of fish to varying nutritional stimuli are important in the confirmation of the roles individual nutrients play in affecting overall growth and body composition and in the identification of alternate pathways in which these nutrients are involved, through which they may indirectly influence these parameters. This is particularly important when novel ingredients are being fed to the animal, where the form of the individual nutrients can vary according to processing technique or metabolism of those same nutrients can be confounded by the presence of other factors.

A study undertaken by Wacyk *et al.* (2012) investigated the effect of either replacing a proportion of the protein in a fishmeal-based diet with soy protein isolate (SPI) or supplementing increasing amounts of branched-chain-amino acids (BCAA) on growth, nutrient utilisation, plasma variables and hepatic gene expression in rainbow trout (*Oncorhynchus mykiss*). They observed a significant reduction in growth performance of fish fed the SPI diets, independent of BCAA content, as well as significant elevation of plasma indispensable amino acid, BCAA and alanine levels. This response was accompanied by significant effects on the regulation of genes involved in hepatic intermediary protein metabolism. These included reductions in the expression levels of alanine aminotransferase (hence the elevated plasma concentration of alanine) and glutamine synthetase 2 (an enzyme which catalyses the production of glutamine from glutamate, a reaction which binds toxic ammonia as well as producing an energy substrate so could potentially be a very important element). Upregulation of aspartate aminotransferase 2 (which catalyses the 'aspartate + α-ketoglutarate ↔ oxaloacetate + glutamate' interconversion reaction, an alternative pathway for metabolism of excess glutamate) and asparagine synthetase (which converts aspartate to asparagine) were also reported. While these specific genes were not mentioned, a more comprehensive array of genes were analysed in a study by Panserat *et al.* (2009), who observed 176 hepatic genes to be differentially expressed at 8h after feeding in response to complete replacement of fishmeal and fish

oil with plant-derived alternatives in a diet for rainbow trout, 32 of which were for factors involved in protein metabolism. A comparison of longer-term changes to protein metabolism with this post-prandial response may have been interesting. It is clear, however, that, even in diets with similar crude compositions, the source of dietary protein and lipid can have a significant effect on nutrient metabolism.

1.7.3 Novel gene networks/metabolic pathways

One of the major advantages of the molecular approach to nutrient metabolism studies is the ability to simultaneously map the response to a specific nutrient across a large number of genes and their associated metabolic pathways, including those which are not necessarily known to be affected by variations in supply of that nutrient. The complex nature of metabolism, whereby individual nutrients, amino acids in particular, can have numerous seemingly unrelated functions, gives rise to the possibility of discovering novel pathways in which certain nutrients can exert previously undocumented effects. For example, most nutrigenomic-based studies investigate the effects of varying the nutrient source or inclusion level on factors directly related to metabolism of that nutrient (e.g. the effect of protein source on expression of genes for enzymes known to regulate amino acid catabolism and synthesis), however, recent studies have used these techniques to investigate the role of individual nutrients as signals for the activation of metabolic pathways indirectly involved in nutrient metabolism.

The TOR pathway, for example, has gained significant recent attention due to its role as a regulator of energy homeostasis and protein translation. It is known to be activated by amino acids (although the exact mechanism of this action remains unknown). In an in-vitro study with rainbow trout hepatocytes by Lansard *et al.* (2010), it was observed that cells incubated in a medium containing a concentrated amino acid solution (the content of which was not disclosed), in comparison to an amino acid-free solution, exhibited significant changes in expression of several genes (including glucose-6-phosphatase, phosphoenolpyruvate carboxykinase, pyruvate kinase, 6-phospho-fructo-1-kinase and serine dehydratase) as a result of an unknown molecular pathway. When cells were incubated in a concentrated amino acid solution with added insulin, however, a dramatic upregulation of lipogenic and glycolytic genes in a TOR-dependent manner was observed (as evidenced by comparison with rapamycin-inhibited treatments). This implies that the combined action of amino acids and insulin can activate the TOR transduction pathway and in this way have a modulating effect on both lipid and energy metabolism pathways. In another study by Lansard *et al.* (2011), the amino acid responsible for TOR activation was suggested to be leucine (in combination with insulin), which resulted in a reduction in the expression of G6Pase 90% and a 4-

fold increase in expression of fatty acid synthetase (FAS) in cells incubated in a medium containing these two products. Wacyk *et al.* (2012) also observed a TOR-related response in rainbow trout in that the expression of *Redd-1*, a gene known to repress TOR function, was significantly upregulated in the livers of fish fed diets containing SPI compared to those on the control diet, possibly contributing to the reduced growth seen in these animals.

1.7.4 Nutrigenomic research in barramundi

A number of studies have been undertaken in characterising non-nutrition related gene expression in barramundi (Xu *et al.*, 2006; He *et al.*, 2012; Lee *et al.*, 2012; Sinthusamran *et al.*, 2013), as well as one using gene expression to characterise the development of digestive enzymes during larval development of this species (Srichanun *et al.*, 2013). The few studies which have investigated the influence of nutritional manipulation on gene expression in barramundi have focussed on fatty acid metabolism (Mohd-Yusof *et al.*, 2010; Alhazzaa *et al.*, 2011; Tu *et al.*, 2012b; Tu *et al.*, 2012a; Salini *et al.*, 2016). One study, however, looked at the expression of genes involved in the regulation of a number of intermediary metabolism pathways in this species (Wade *et al.*, 2014). In this study, the authors first assembled a partial liver transcriptome, due to the paucity of genes for nutrient metabolism which had been identified in barramundi, and used this to investigate changes in expression of 24 genes known to be key regulators of glycolytic and lipogenic pathways in response to feeding. This study provided important information in moving forward with barramundi nutrition research in that it identified a number of markers for use, or avoidance, in future studies of this type. For example, the authors concluded that the use of farnesoid X receptor (*Lc* FXR), pyruvate dehydrogenase kinase (*Lc PDK*) and citrate synthase (*Lc CS*) was effective in indicating the time-course of TCA cycle utilisation of metabolic intermediates. As well, it was suggested that genes controlling glycogen turnover were not reliable markers of glycogen regulation. In addition, the molecular analysis revealed metabolic responses to feeding which are apparently unique to this species compared to other fish, for example the repression of the final step of gluconeogenesis and a response in liver X receptor (*Lc LXR*) expression normally seen in mammals. A number of important areas not investigated in the study were identified by the authors, providing direction for future studies (the response of hepatic glucose regulating genes to high levels of starch to quantify the ability of barramundi to metabolise starch and simple sugars, investigation of the presence of the *cALAT*2 isoform of ALAT and correspondence of enzyme activity with the gene expression responses). Further investigation should also include the role of individual amino acids in these pathways.

1.8 Conclusion

Sulphur amino acids have been proven to be important components in the diet of a number of fish species, including barramundi, with dietary deficiencies reported to cause pathologies ranging from muscular degeneration to the formation of cataracts. While a great deal of research on this topic has been undertaken in other animals, the specific roles of these nutrients in fish metabolism, particularly in barramundi, are not fully known. In order to optimise nutritional models used in the formulation of manufactured diets for these animals, it is necessary to more fully quantify the contribution of these nutrients to the various metabolic pathways which promote the growth of the fish (primarily the deposition of protein). This will allow more accurate determination of their optimal required inclusion in the diet. The use of molecular techniques as a complementary tool to phenomic (growth, feed utilisation, composition) studies, can provide a more complete picture of the animal's response. Isolation of the specific mechanism(s) of action of a nutrient on the growth of an animal allows for determination of the optimal levels/acceptable limits of the supply of each specific component, as well as providing evidence of the efficacy of use of alternative components which share the same metabolic roles and may have a sparing effect. In addition, the response to manipulation of the dietary supply of these components may be measurable on a molecular scale after a shorter experimental period than might be required for manifestation on a phenomic level in the animal. While they has been used previously to examine lipid and energy metabolic pathways in barramundi, these techniques have not been applied to the investigation of amino acid metabolism in this species.

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1.10 Outline of the thesis

Methionine (Met) is commonly the first limiting amino acid in diets formulated to contain high levels of plant proteins. Its metabolite cysteine (Cys), however, is often relatively abundant in these ingredients and has been shown to be able to replace a certain proportion of dietary Met in various fish species, resulting in an assertion that fish have a requirement for total sulphur amino acids (TSAA; Met+Cys). As such, it is becoming increasingly important, with the growing use of plant proteins in aquafeeds, that the requirement for Met is properly defined for each species and that the replacement value of dietary Cys be assessed in order to optimise the inclusion of these more environmentally and economically sustainable sources of protein. Similarly, the inclusion of taurine (Tau) in the diet of carnivorous fish species, such as barramundi (*Lates calcarifer*) has become an important consideration due to its abundance in fishmeal and absence in plant meals. As a metabolite of the sulphur amino acids, the requirement for Tau may be significantly affected by the dietary inclusion of Met and Cys. For these reasons, the aim of the first experiment was to confirm a previously published estimate of Met and TSAA requirement in barramundi and to investigate the degree to which Cys can replace Met within this requirement. These results, presented herein in Chapter Two, enabled the appropriate formulation of diets/choice of treatments for the subsequent experiments.

Following on from Chapter Two, the second study, outlined in Chapter Three, aimed to elucidate some of the mechanisms underpinning the observed growth responses seen by comparing the expression of several genes isolated from barramundi liver tissue of fish fed either a deficient, adequate or excessive level of dietary TSAA. While establishing the requirement for an amino acid is an important step in optimising the inclusion of these dietary components, elucidation of their metabolic roles, particularly those relating directly to growth such as protein turnover, underlines their importance to the animal. Additionally, this information may allow the supplementation or substitution of other compounds which fulfil the same roles, including those which may be more abundant in available ingredients.

Chapter Four describes the results of an experiment designed to establish the essentiality of dietary Tau provision to juvenile barramundi through observation of the effect of graded dietary Tau supplementation to a diet already sufficient in sulphur amino acids (SAA). A secondary aim of this experiment was to assess the effect of variable dietary Met supply on the response to varying Tau, in an effort to determine whether the demand for Tau is dependent on supply of precursors. The results of this experiment may suggest the extent to which this species can synthesise Tau, given sufficient precursors and, in the event that this capacity is limited, could be used to establish a baseline inclusion level of this amino acid in diets for barramundi.

This capacity of carnivorous fish to synthesise Tau from precursor amino acids has been reported to vary widely, which can have a significant impact on the requirement to supplement aquafeeds with this amino acid. Additionally, while Tau has been observed to positively affect growth in a number of species, the exact mechanism of this stimulatory effect is poorly understood. The experiment outlined in Chapter Five aimed to investigate the impact dietary Tau supply has on the activity of some established protein turnover and growth-related pathways known to be affected by its precursor SAA in other species. As well, the capacity of barramundi to synthesise Tau, the mechanisms by which it does this and the effect of Tau supplementation on the activity of these pathways was investigated.

The final experimental chapter, Chapter Six, investigates the inevitable losses of the proteinaceous amino acids from barramundi over a period of starvation. It also provides an alternative way to examine the demand for certain essential nutrients (e.g. Met), as derived from endogenous reserves, and the rate at which these are used, relative to animal size, to assist in deriving estimates of basal and maintenance demands for such nutrients. This approach allows us to establish allometric scaling exponents for use in examining nutrient utilisation and growth models on a size independent basis. The scaling exponents derived for Met were then applied to the assessment of marginal utilisation efficiencies of this amino acid in two previous experiments (including that discussed in Chapter Two). These figures were further applied in the calculation of maintenance, growth and total requirements for Met. The utility of using the derived Met exponent over the protein loss exponent traditionally applied to the derivation of both marginal utilisation and the prediction of requirements is also assessed.

Chapter Seven, the General Discussion, is a summary of the major findings of the experimental chapters and how they relate to the aims of the project. The relationships between the growth, body composition and molecular responses reported in those chapters are examined and conclusions are made regarding their implications for feed formulations and future amino acid nutrition studies in this species. As well, any shortcomings in the techniques used in this thesis are identified and recommendations made for ameliorating them in future studies.

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

2.1 Abstract

This study was designed to confirm a previous estimate of the methionine (Met) and total sulphur 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.

2.2 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 sulphur 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).

2.3 Materials and Methods

2.3.1 Diets

2.3.1.1 Formulation

Experiment One

A series of five isonitrogenous and isoenergetic diets were formulated (Table 2.1) 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.

Table 2.1. 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 2.2. 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.

Experiment Two

Five isonitrogenous and isoenergetic diets were produced (Table 2.2) 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).

2.3.1.2 Diet manufacture

Diets were prepared 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. Briefly, a dry mash (without water or oil) of each of the diets (with the exception of the commercial control) was mixed thoroughly using an upright Hobart mixer (Hobart, Sydney, NSW, Australia). This mash was extruded using a laboratory-scale twin-screw extruder (MPF24; Baker Perkins, Peterborough, UK), with intermeshing, co-rotating screws. On extrusion, pellets of approximately 4mm diameter were cut into 4-5mm lengths using a four-bladed variable speed cutter before being dried at 65°C for 24 hours.

Once dry, the pellets were transferred to a stainless steel mixing bowl and mixed on a Hobart mixer, during which time heated (65°C) fish oil was added according to the dietary formulation. After pellet oil coverage was observed to be uniform, mixing ceased and a clear perspex lid with rubber seal was fitted to the bowl before application of a vacuum. Air was slowly evacuated from the bowl until visible signs of air escaping from the pellets was observed to have ceased, at which time the air pressure was allowed to re-equilibrate, drawing the oil into the pellets.

2.3.2 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.

2.3.3 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.

2.3.4 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.

2.3.5 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 $p<0.05$.

Table 2.3. Nutrient response models fitted to the data from Experiment Two.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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 2.3) 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.

2.4 Results

2.4.1 Experiment One

2.4.1.1 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 2.4 and Fig. 2.1).

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.

Table 2.4. 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

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

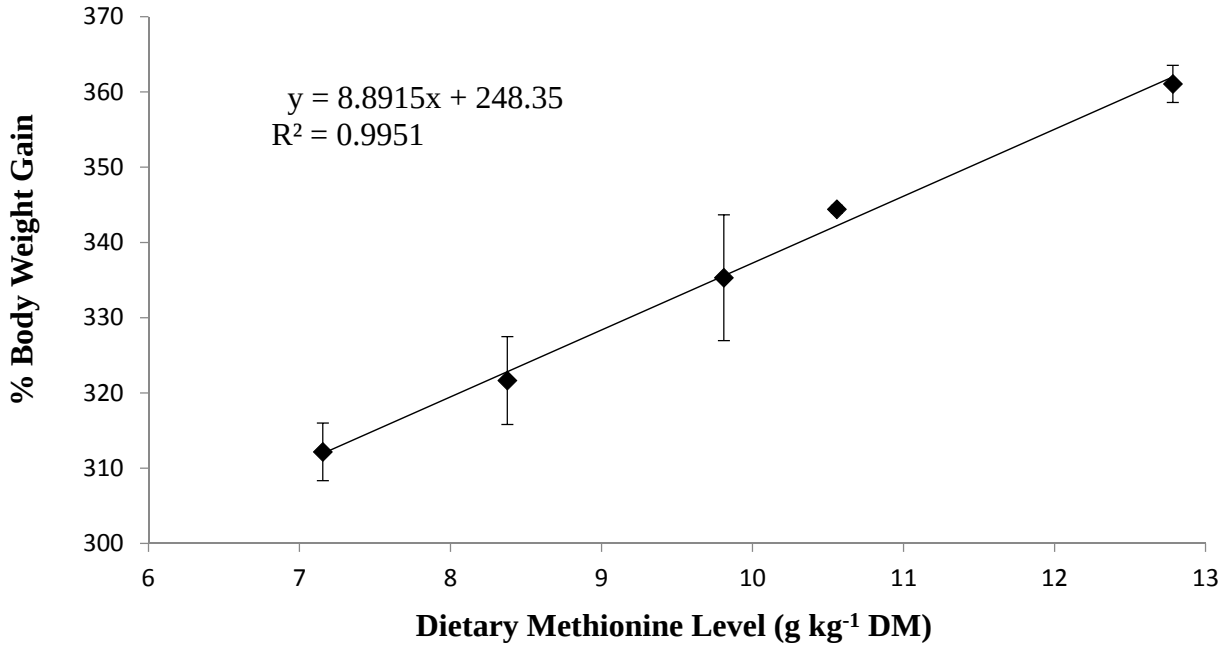

Figure 2.1. Percent Weight Gain (± S.E.M.) of fish fed diets with variable methionine content in Experiment One (mean initial weight = 18.1g).

2.4.2 Experiment Two.

1.1.1.1 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 2.5). 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 as well as its consideration of the small variations in initial body weight, which may be especially relevant given the relatively small numerical variation in final body weights. Nine models (Table 2.3) 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.

Table 2.5. 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

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

All models fit the data well but, based on maximum R^2 and lowest SSE, the Compartmental Model (Fig. 2.2) was deemed the most appropriate model, explaining 71% of the variation in percent body weight gain (Table 2.6) 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.

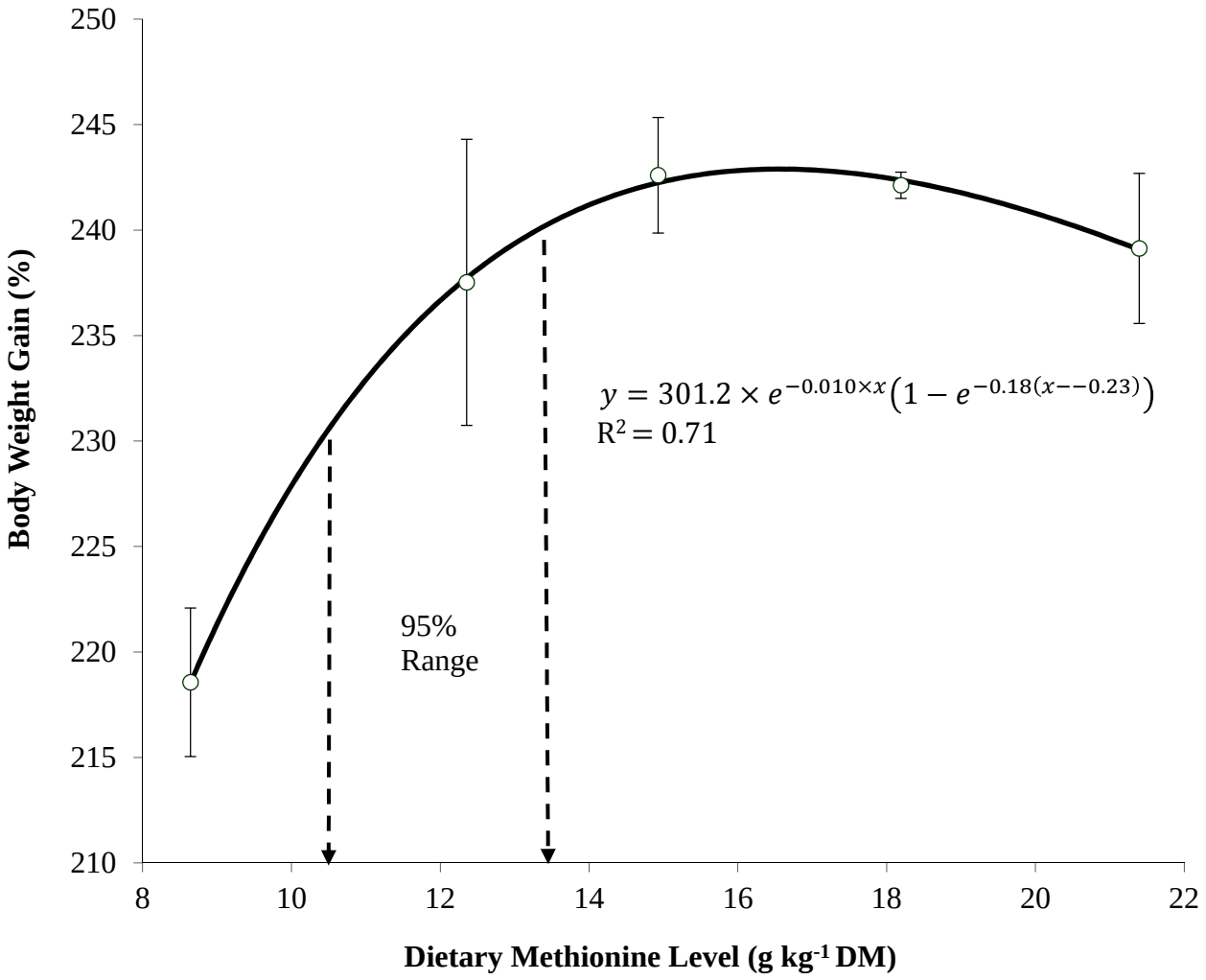

Figure 2.2. Percent Weight Gain (±S.E.M.) (mean initial weight = 35.0g) of fish in Experiment Two with Met requirement as predicted by the Compartmental model (arrows indicate 95% and 99% of the asymptote).

Table 2.6. Comparison of goodness of fit and dietary methionine requirements predicted by nine nutrient response models based on %BW Gain data from Experiment Two (data are 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

2.4.2.1 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 2.9). 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.

2.4.3 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 2.7) 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}).

Table 2.7. Comparison of goodness of fit and dietary methionine requirements predicted by nine nutrient response models based on the %BW Gain data of Coloso et al. (1999) (data are 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	492.8	98.2	10.3	8.9
Broken-Line (Quadratic Ascending)	528.2	98.0	11.8[2]	N/A
Compartmental	590.7	97.8	10.6	8.9
Broken-Line (Linear Ascending)	642.0	97.6	10.1[2]	N/A
Logistic, 4 Parameter	728.4	97.3	11.7	9.1
Sigmoidal	728.5	97.3	11.7	9.1
4-SKM	765.4	97.1	12.8	9.4
Logistic, 3 Parameter	776.3	97.1	13.1	9.6
Exponential	812.7	97.0	14.0	9.8

[1] SSE: Sum of Squared Errors
[2] requirement predicted by the abscissa of the breakpoint of the curve

2.4.4 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 2.8 for comparison with that of the diets.

Table 2.8. 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

Table 2.9. 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

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

2.5 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 2.7, 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=0.71), 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) Met 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 sulphur 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)).

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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Chapter 3 - Postprandial plasma free amino acid profile and hepatic gene expression in juvenile barramundi (*Lates calcarifer*) is more responsive to feed consumption than to dietary methionine inclusion.

3.1 Abstract

The effects of dietary methionine (Met) supply on the postprandial pattern of plasma free amino acids and the differential expression of several genes associated with a number of sulphur amino acid and protein turnover pathways in the liver of juvenile barramundi (*Lates calcarifer*) was investigated. At the conclusion of a 49-day growth trial assessing the requirement for dietary Met, three treatments were selected (with deficient (MetDEF; 8.6g/kg), adequate (MetADQ; 14.9g/kg) and excessive (MetEXC; 21.4g/kg) levels of dietary Met, based on their respective growth responses. A peak occurred in plasma free Met at 2h post-feeding in fish fed the MetDEF and MetADQ diets and at 4h post-feeding in fish in the MetEXC treatment. Liver samples collected at these timepoints, as well as those taken as a pre-feeding control, were analysed for expression of genes involved in Met turnover (CGL, MAT-1, MAT-2a) and taurine biosynthetic pathways (CSAD, ADO, CDO), target of rapamycin inhibition (Redd-1), the somatotropic axis (GHR-II, IGF-I, IGF-II) and protein turnover pathways (MUL-1, ZFAND-5). Markers of sulphur amino acid turnover were more significantly affected by time after feeding than by dietary Met level, suggesting production of these enzymes may be primarily regulated by the consumption of feed or protein, rather than by the composition. Further, metabolised Met appeared likely to have been directed through S-Adenosylmethionine (SAM) dependent pathways, rather than converted to Cys, which may have contributed to the observed growth response. Both genes influencing the conversion of Met to SAM appear to be active at this lifestage in barramundi. Elevated expression of the Tau biosynthetic pathway genes CDO and CSAD in the livers of fish in the MetEXC, compared to the MetDEF, treatment after 24 hours starvation suggested Tau biosynthesis may be important in this species. Pre-feeding IGF-I expression in response to Met supplementation reflected the long term growth responses seen, however, these differences were less pronounced after feeding. Previously described markers of proteolytic pathways appear to be conserved in this species and we have confirmed that ZFAND-5 is a reliable biomarker of this process in barramundi. A number of important genes were investigated for the first time in this species and shown to be nutritionally regulated.

3.2 Introduction

Consideration of essential amino acid inclusion in the formulation of diets for carnivorous fish species has become progressively important in recent years with the increased replacement of fishmeal with alternative sources of protein such as terrestrial plant meals. This is especially important for methionine (Met) given that it is often the first limiting amino acid in diets containing high levels of non-cereal plant meals such as soybean meal (Takagi *et al.*, 2001; Hansen *et al.*, 2007).

Methionine plays several important roles in fish metabolism. It, and its metabolite cysteine (Cys) are two of the 20 proteinogenic amino acids which make up the many proteins in the body and, as such, are essential for muscle synthesis and, thus, growth of the animal. In addition, it is a precursor for a number of key metabolic compounds, including S-Adenosylmethionine (SAM), which is considered the most important biological methyl donor (Garcia-Trevijano *et al.*, 2000) and, as a sulphur amino acid, may contribute to the production of taurine, another metabolically important amino acid which is low or absent in terrestrial plant meals. As such, the requirements for, and metabolism of, methionine is substantially more complex than that of those amino acids, such as lysine, which are primarily proteinogenic.

Dietary sulphur amino acid (SAA) supply has been linked to growth modulation through several mechanisms. One key component of growth is the deposition of body protein, the sum result of the balance of protein degradation and synthesis. These processes are under the control of a number of systems, some of which may be affected by dietary Met supply. Expression of markers of the ubiquitin-proteosome and autophagy-lysosomal proteolytic pathways in the white muscle of rainbow trout (*Oncorhynchus mykiss*), for example, have previously been suggested to be influenced by the level of dietary Met by Belghit *et al.* (2014). The mechanism of this response is not fully understood but it may be related to either the induction of hormonal changes or the role of Met as a signal for a range of metabolic processes, including protein synthesis, a role which has gained increasing attention in recent years (Kimball and Jefferson, 2006; Hevrøy *et al.*, 2007; Lansard *et al.*, 2010).

The target of rapamycin (TOR) pathway, too, is thought to be a major contributor to the regulation of growth in animals through its role in stimulating protein synthesis (Mennigen *et al.*, 2012). It has been studied extensively in terrestrial animals (see review by Hay and Sonenberg, 2004) but has only recently been investigated in aquatic species (Seiliez *et al.*, 2008; Seiliez *et al.*, 2011; Liang *et al.*, 2016). This pathway, in turn, is linked to another driver of growth in animals, the somatotropic axis, or GH/IGF system. This system of factors, incorporating a series of binding proteins, growth factors and growth hormone receptors, stimulates muscle growth through the

stimulation of amino acid transport as well as RNA transcription and cellular protein synthesis (Clemmons and Underwood, 1991) and has been shown by Rolland *et al.* (2015b) to be affected, directly or indirectly, by Met availability in diets for rainbow trout.

The availability of amino acids for roles supplementary to acting as substrates for protein synthesis can be directly influenced by both the supply of the precursor amino acids required for their synthesis, when limiting (for the non-essential amino acids), and by the demand for synthesis of their product amino acids. Amino acid biosynthetic and degradation pathways involve a series of complex reactions catalysed by substrate-specific enzymes. Substrate and product availability has been shown to directly influence the activity of the amino acid biosynthetic enzymes (Fafournoux *et al.*, 2000) but few studies have investigated the relationship between the levels of specific amino acids and the expression of the genes responsible for the control of these pathways in fish. With the increasing use of molecular techniques in the study of the nutritional regulation of growth in fish, it is important to characterise the relationship between gene expression and phenotypic responses such as enzyme activity and substrate/product turnover.

The purpose of this study was to investigate the mechanisms by which barramundi metabolise Met and to examine what effect the level of its supply in the diet had on some important growth-related metabolic/gene expression pathways.

3.3 Materials and Methods

3.3.1 Diets

Three diets (Table 3.1) were selected from a previously published TSAA requirement growth trial (Poppi *et al.*, 2017): those diets that elicited the minimum and maximum growth responses, as well as one with an apparently excessive level of Met/TSAA (causing a depression in growth in that study). These diets were designated as either Met deficient (MetDEF; 8.6g/kg), adequate (MetADQ; 14.9g/kg) or excessive (MetEXC; 21.4g/kg) with a constant Cys content of 6.6g kg^{-1} DM.

All diets were otherwise formulated to be equal in all other dietary components (with the exception of glycine which was used to replace Met) and to exceed the requirements of this species for all amino acids not under investigation, based on the ideal protein concept with reference to the amino acid profile reported by Glencross *et al.* (2013).

Diets were produced on a laboratory-scale twin-screw extruder (MPF24; Baker Perkins, Peterborough, UK), with intermeshing, co-rotating screws following the protocol of Glencross *et al.* (2016), using a 3mm diameter die in order to produce pellets of ~ 4mm diameter. After drying, the pellets were vacuum coated with their oil allocation to produce a sinking feed.

Table 3.1. Formulations and analysed compositions of experimental diets.

	MetDEF	MetADQ	MetEXC
Ingredients (g kg^{-1})			
Fishmeal[1]	150	150	150
SPC	490	490	490
Fish oil[2]	100	100	100
Cellulose	76	76	76
Pregel Starch	53	53	53
$CaHPO_4$	20	20	20
Vit. and Min. Premix[3]	6	6	6
Choline Cl$^-$ [4]	1	1	1
Marker (Y_2O_3)	1	1	1
DL-Met	-	6.5	13.0
L-Tau	5.0	5.0	5.0
L-Gly	13.0	6.5	-
EAA Premix 1[5]	85	85	85
Composition as determined (g kg^{-1}DM unless otherwise stated)			
DM (g kg^{-1} as is)	960	954	957
CP	600	582	589
DP	530	519	520
Lipid	116	117	114
Ash	67	67	67
GE (MJ kg^{-1} DM)	22.5	22.5	22.2
DE(MJ kg^{-1} DM)	14.9	15.7	13.9
Arg	44.3	44.3	44.0
His	17.3	16.7	16.4
Ile	28.6	29.0	28.7
Leu	46.1	29.2	44.7
Lys	39.4	40.7	40.0
Met	8.6	14.9	21.4
Cys	6.6	6.8	6.6
Phe	34.9	34.4	34.8
Thr	29.7	31.1	29.7
Val	35.4	35.6	35.2
Tau	6.4	6.5	6.3

[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.

3.3.2 Fish management

This experiment was 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 number: A6/2014) and The University of Queensland Animal Ethics Committee (approval number: CSIRO/QAAFI/391/14).

3.3.2.1 Preconditioning

Twenty one juvenile barramundi (*Lates calcarifer*) (mean initial weight: 116g) were housed in each of three replicate 1000L tanks per treatment, having been randomly assigned to these tanks at the commencement of a 49 day growth trial preceding the current experiment (Poppi *et al.*, 2017). These fish were fed their respective diets (the same as was fed throughout the growth trial) to satiety once daily for a further two weeks before sampling commenced.

All feed was refrigerated (< 4ºC) except during periods of feeding and ration preparation.

The experimental system was set up such that each tank was supplied with ~ 3 L/min flow of continuously aerated marine water (~35PSU) of 29.5°C ± 0.2°C for the duration of the interim and experimental periods. Photoperiod manipulation was achieved using artificial lighting set at 12:12 (light:dark), supplemented with a similar photoperiod of natural light provided by evenly distributed opaque roof panels.

3.3.2.2 Feed Ration Determination

For three days prior to the sampling day, feed intake over a five minute period (the allotted time for feeding on sampling day) was assessed and the average intake of the poorest feeding tank was assigned as the feed ration (1.3g $fish^{-1}$) in order to ensure all tanks consumed the same average ration to negate any impact of large-scale variation in feed intake on the postprandial response.

3.3.3 Sampling

On the day of sampling, the mean weights of the fish in each treatment were as follows: MetDEF (132g), MetADQ (135g), MetEXC (132g). Three fish were randomly selected from each tank 24 hours after their last meal and euthanized by an overdose of anaesthetic (AQUI-S^{TM}) (zero hour control). All fish were individually weighed and checked for stomach contents. A volume of blood greater than 1ml was removed from the caudal vein by heparinised syringe, centrifuged at 6000 rpm for 2 min, and the plasma separated from the clotted material. At this time, samples of liver tissue were also collected and immediately placed with the plasma samples on dry ice before storage at -80ºC until required for analysis. The remaining fish were allowed to recover for a

further two hours before being fed the assigned ration. Feeding was undertaken over a period of five minutes and in such a fashion as to ensure all following sample points were completed at precise intervals after feeding of each tank. All rations were consumed within this feeding period.

One, two, four, eight, 12 and 24 hours after feeding, three fish were again randomly selected from each tank and processed as previously described. Stomach contents were assessed for the first four hours after feeding. Seven fish out of the 108 sampled during this period were observed not to have consumed any feed and were excluded from analyses. All other fish were determined to have consumed a similar amount of feed.

3.3.4 Chemical analyses

Plasma samples were deproteinised by addition of 380µl acetonitrile to 60µl each of plasma sample and a 500µM internal standard (α-Aminobutyric acid) according to the protocol of Davey and Ersser (1990). Samples were centrifuged at 2200 x *g* for four minutes at 4^0C and the supernatant extracted for analysis. The amino acid composition of this supernatant was determined by reverse-phase ultra high-performance liquid chromatography after pre-column derivatisation with 6-aminoquinolyl-N-hydroxysuccinimidyl (AQC). Analyses were undertaken on a Shimadzu Nexera X2 series UHPLC (Shimadzu Corporation, Kyoto, Japan) with a quaternary gradient module, coupled with a Shimadzu RF-20A XS prominence fluorescence detector using the Waters AccQ·tag system (Waters Corporation, Milford, MA).

3.3.5 Molecular analyses

3.3.5.1 RNA extraction and cDNA synthesis

Total RNA was isolated from frozen hepatic tissue (two fish per tank per timepoint; n=6 per treatment per timepoint) using the Trizol (Invitrogen) method according to the manufacturer's instructions and precipitated in equal volumes of isopropyl alcohol and precipitation solution (0.8M disodium citrate with 1.2M sodium chloride in ultrapure distilled water) (Green and Sambrook, 2012). The extracted RNA was DNase digested using the Turbo DNA-free kit (Applied Biosystems) before assessment of RNA quantity on a NanoDrop spectrophotometer (NanoDrop Technologies) and quality using RNA nanochips (Agilent #5067-1511) in a bioanalyser (Agilent Technologies). All RNA samples were normalised to 200ng/µl.

Total RNA (1µg) was reverse-transcribed using the Superscript III first strand synthesis system (Invitrogen) with 25µM oligo$(dT)_{20}$ and 25µM random hexamers (Resuehr and Spiess, 2003).

Table 3.2. Target genes of sulphur amino acid and protein turnover; and growth in barramundi, and the primer sequences used in the qPCR assays of their expression.

Target Gene[1]	Accession Number	Primer Name	Sequence	Length
Met and Cys metabolism				
MAT-1	XM_018678413	MAT1 qPCR F1	TGTCAATCTCCTTGTTCACCT	21
		MAT1 qPCR R1	GCCTCTTCAGATTCAGTTCC	20
MAT-2a	XM_018669469	MAT2a- qPCR F2	GAGACCGATGAGTGTATGCCT	21
		MAT2a- qPCR R2	ACCGTAACCTGTGTCTTTGAG	21
CGL	XM_018673132	CGL qPCR F2	CACAAGACGAGCAGAACGAC	20
		CGL qPCR R2	CACCACAGCCATTGACTTCC	20
Tau metabolism				
CDO	XM_018674402	CDO- qPCR F2	GTTGCCTACATAAATGACTCCA	22
		CDO- qPCR R2	CTGTCCTCTGGTCAAAGGTC	20
CSAD	XM_018666199	CSAD qPCR F1	GTACATTCCACCAAGTCTGAG	21
		CSAD qPCR R1	CCCAGGTTGTGTATCTCATCC	21
Somatotropic axis				
IGF-I	XM_018697285	IGF-1 qPCR F2	CTGTATCTCCTGTAGCCACAC	21
		IGF-1 qPCR R2	AGCCATAGCCTGGTTTACTG	20
IGF-II	XM_018664155	IGF-II qPCR F1	AGTATTCCAAATACGAGGTGTG	22
		IGF-II qPCR R1	GAAGATAACCTGCTCCTGTG	20
GHR-II	XM_018702499	GHR-2 qPCR F2	CGTCCATATCCCATCTAAAGTGTC	24
		GHR-2 qPCR R2	GTCATTCTGCTCCTCAATGTC	21
Proteolysis				
MUL-1	XM_018686248	Mul1 qPCR F1	GGCTTCCGTTATTTCCTCAC	20
		Mul1 qPCR R1	TGCTCTCCTCTATGTTAAGTTCAC	24
ZFAND-5	XM_018669382	ZFAND5 qPCR F1	CTAGAGCCTGTTGTAAGCCA	20
		ZFAND5 qPCR R1	CTCGGCCTTGTAATCATAGGG	21
TOR activity signaling				
Redd-1	XM_018699192	Redd1 qPCR F2	TTTCAGCACATCCACTAACGG	21
		Redd1 qPCR R2	CCACTACTTCTTTCAGGATTGTC	23
Control genes				
Luc	NA	Luc qPCR F	GGTGTTGGGCGCGTTATTTA	20
		Luc qPCR R	CGGTAGGCTGCGAAATGC	18
EF1α	NA	Lcal EF1α F	AAATTGGCGGTATTGGAAC	19
		Lcal EF1α R	GGGAGCAAAGGTGACGAC	18

[1]*MAT-1*, methionine adenosyltransferase-1*; MAT-2a*, methionine adenosyltransferase-2a; *CGL*, cystathionine-γ-lyase; *CDO*, cysteine dioxygenase; *CSAD*, cysteine sulphinic acid decarboxylase; *IGF-I*, insulin-like growth factor-I; *IGF-II*, insulin-like growth factor-II; *GHR-II*, growth hormone receptor-II; *MUL-1*, mitochondrial ubiquitin ligase activator of NF-κβ-1; *ZFAND-5*, zinc finger AN1-type domain-5; *Redd-1*, regulated in development and DNA damage response-1; *Luc*, luciferase; *EF1α*, elongation factor 1α.

3.3.5.2 Quantitative real-time RT-PCR

The differential expression of several genes associated with the somatotropic axis and sulphur amino acid and protein turnover pathways was assessed by real-time quantitative polymerase chain reaction (rt-qPCR) as follows.

Real-time PCR primers were designed for each of the target genes using PerlPrimer version 1.1.21 (Marshall, 2004), using sequences of gene fragments, or whole genes, in a partial, unannotated barramundi transcriptome (Hook *et al.*, 2017), showing a high level of homology with published sequences of known genes from related species. Sequences of genes described in an annotated barramundi transcriptome published after the completion of this experiment were identical to those derived for this study. GenBank accession numbers and primer sequences for all genes are presented in Table 3.2.

A five-fold serial dilution of pooled cDNA from all samples to be analysed was PCR-amplified and the slope of the standard curve used to optimise the efficiency of each primer to between 95 and 105%. A pool of DNase-digested RNA was also PCR-amplified at this time to verify the absence of genomic DNA contamination. Real-time PCR amplification of the equivalent of 7.5ng of reverse-transcribed RNA was conducted using 2X SYBR Green PCR Master Mix (Applied Biosystems) and 0.5μM RT-PCR gene-specific primers. Reaction components were allocated to each well of a MicroAmp Optical 384-well reaction plate (Applied Biosystems) using an epMotion 5070 robot (Eppendorf). Each reaction was run in triplicate on a Viia7 real-time PCR system (Applied Biosystems). Amplification cycle conditions were: 2 minutes at 50^0C and 10 minutes at 95^0C, followed by 40 cycles of 15 seconds at 95^0C and 40 seconds at 60^0C. Dissociation melt curves for each gene were assessed after amplification to confirm the specificity of the primers. Variation in gene expression magnitude between treatments over time was assessed by normalisation of the cycle threshold values for each gene to that of the elongation factor 1α (EF1α), as used in several other gene expression studies with this species (De Santis *et al.*, 2011; Wade *et al.*, 2014; Salini *et al.*, 2015), and Luciferase reference genes. Amplification variation of these genes was 1.35 cycles and 2.58 cycles respectively, which did not change significantly over time.

3.3.6 Statistical analysis

Plasma amino acid contents (n=9 per treatment per timepoint) and gene expression (n=6 per treatment per timepoint) values are expressed as means with their standard errors. All data was log-transformed to obtain homoscedasticity prior to analysis. The effects of dietary treatment and time after feeding on both plasma amino acid composition and gene expression were determined by two-way ANOVA. Differences between treatments group means within timepoints and between

timepoint means within treatment were measured by Tukey's Honestly Significant Difference *post-hoc* test. Any differences were considered significant at an alpha level of 0.05. Correlations between Met intake and either peak plasma Met levels or the area under the plasma Met curve were examined using Pearson's correlation test. All statistical analyses were conducted in the R-project statistical environment (R Core Team, 2014).

3.4 Results

3.4.1 Plasma amino acid contents

Plasma amino acid levels fluctuated significantly over time after feeding (Figs. 3.1, 3.2 and 3.3; and Tables A1, A2 and A3 in Appendix A). Levels peaked between one and four hours post-feeding and returned to pre-feeding levels by 12 hours after the meal in most cases. A secondary peak was also seen at 12h post-feeding for aspartic acid (Asp), glycine (Gly), arginine (Arg) and alanine (Ala). For brevity, only those amino acids which showed significant or relevant patterns of response are addressed in the discussion section.

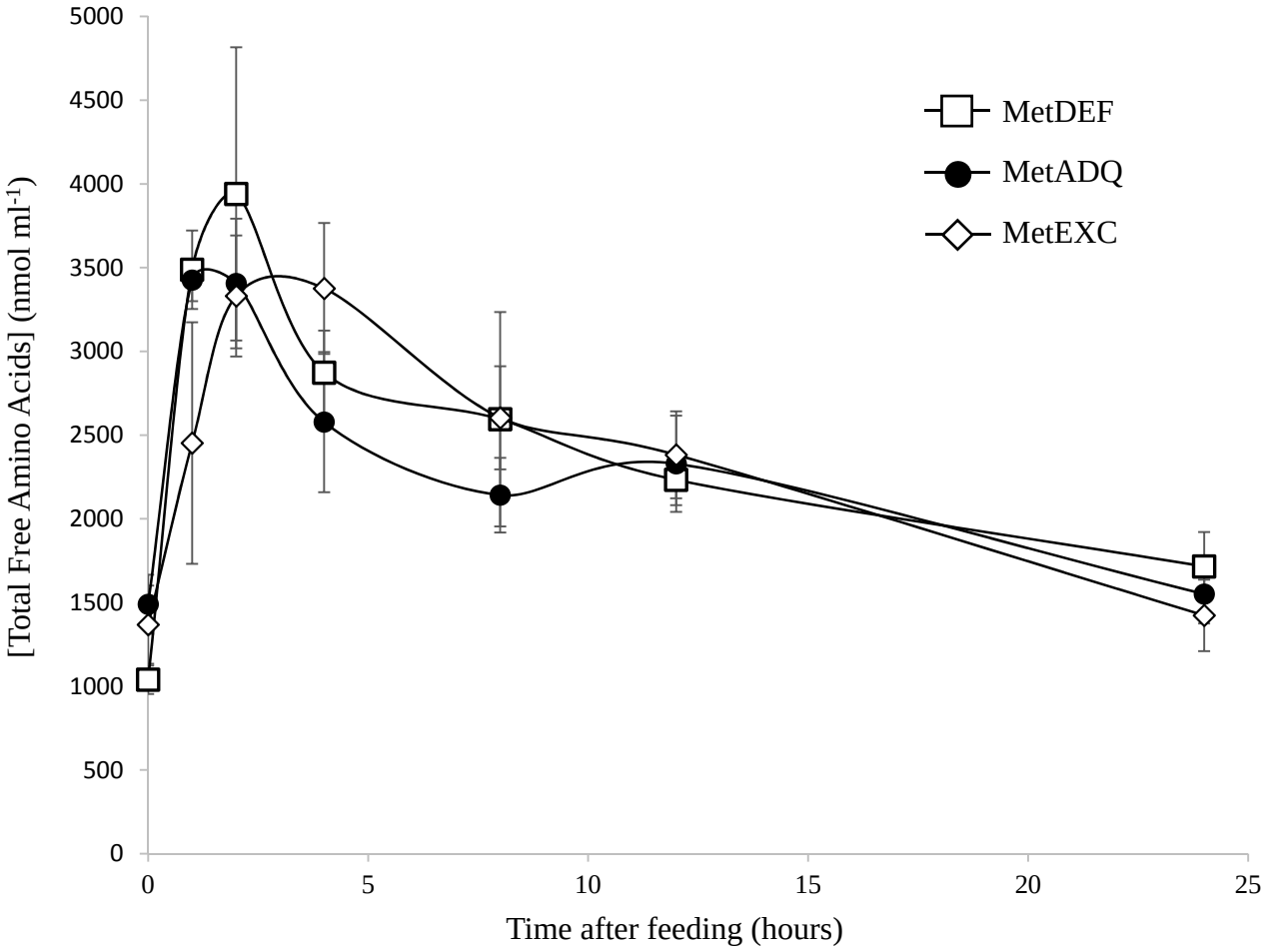

Figure 3.1. Concentrations of total free amino acids (TFAA) present in the plasma of juvenile barramundi over a 24 hour period following consumption of a single meal containing either a deficient (MetDEF), adequate (MetADQ) or excessive (MetEXC) level of dietary methionine.

A significant effect of time ($p<0.05$) on total and individual plasma amino acid contents was observed for all essential and non-essential amino acids measured, with the exceptions of glutamic acid (Glu) and tyrosine (Tyr) (Fig. 3.3) which remained elevated throughout the sampling period. Tyr levels were subject to large variation within treatments, possibly contributing to the lack of significance of the response, the average of which did vary over time.

Significant dietary effects were seen only for threonine (Thr), which remained elevated for a longer period in the plasma of fish fed the Met excess diet, and for Gly and Met, the only two amino acids which varied in the dietary formulation, with responses reflecting their level in the diet. Plasma Met (Fig. 3.2) was significantly higher ($p<0.001$) in fish fed the MetEXC diet than those in the MetDEF treatment at the pre-feeding timepoint and further diverged at 1h post-feeding, being significantly higher in fish fed the MetEXC diet than those fed the MetADQ diet, which, in turn had significantly greater levels than those in the MetDEF treatment. This significant separation was maintained until 8h after the meal. Met intake was highly significantly positively correlated to both the peak plasma Met content ($r = 0.939$, $p<0.001$) and the area under the plasma Met curve (i.e. the longevity of the elevation in plasma Met) ($r = 0.986$, $p<0.001$). Plasma Gly (Fig. 3.2) levels were significantly lower in fish fed the MetEXC diet than those fed the MetDEF diet at 1h post-feeding.

3.4.2 Gene expression

Expression of the target genes was assessed in liver tissue of those fish sampled after 24 hours starvation (the pre-feeding or zero-hour timepoint), as well as those taken at two and four hours post-feeding, where the peaks in plasma Met and total free amino acids were seen.

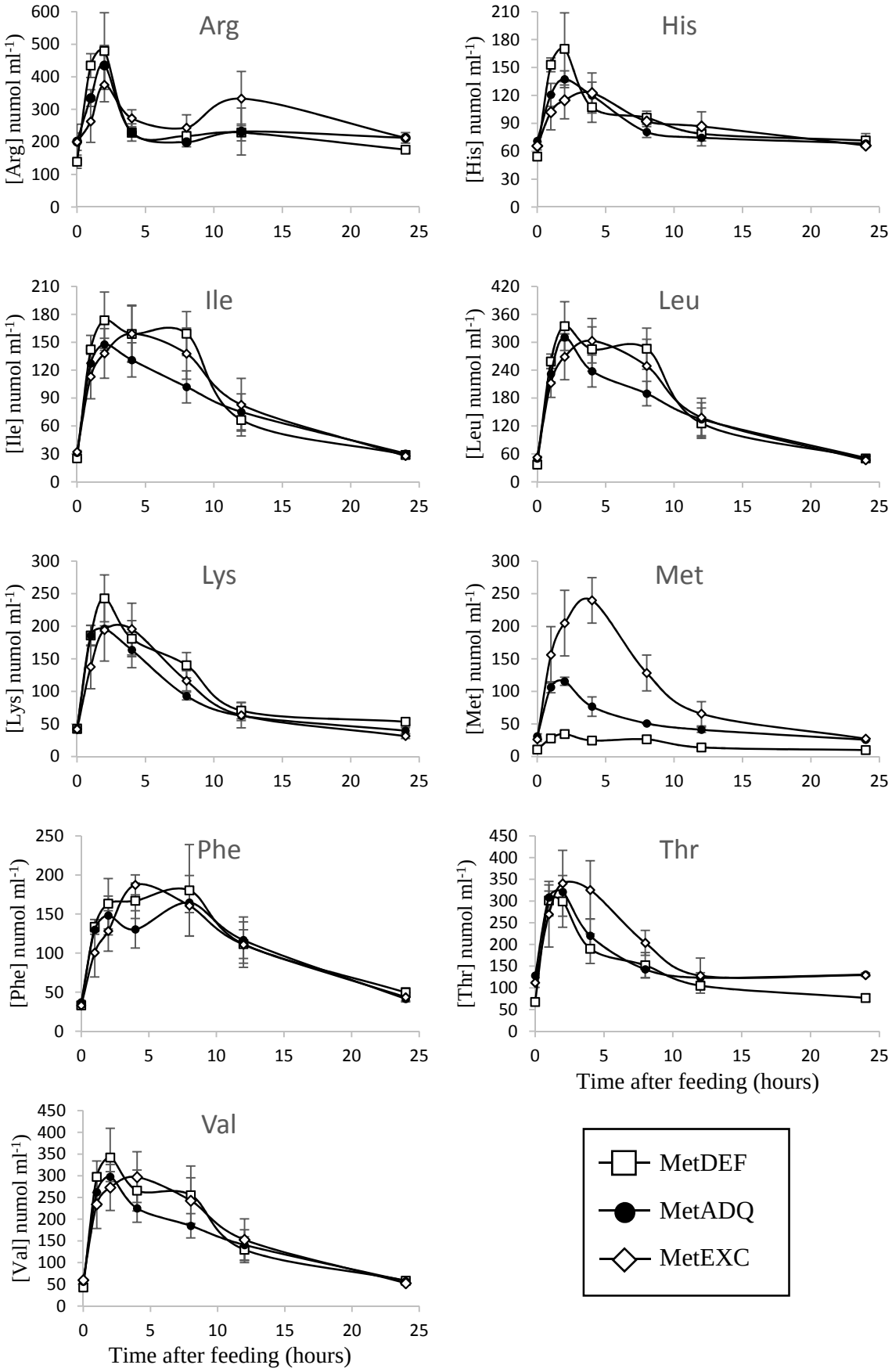

Figure 3.1. Concentrations of individual essential amino acids present in the plasma of juvenile barramundi over a 24 hour period following consumption of a single meal containing either a deficient (MetDEF), adequate (MetADQ) or excessive (MetEXC) level of dietary methionine.

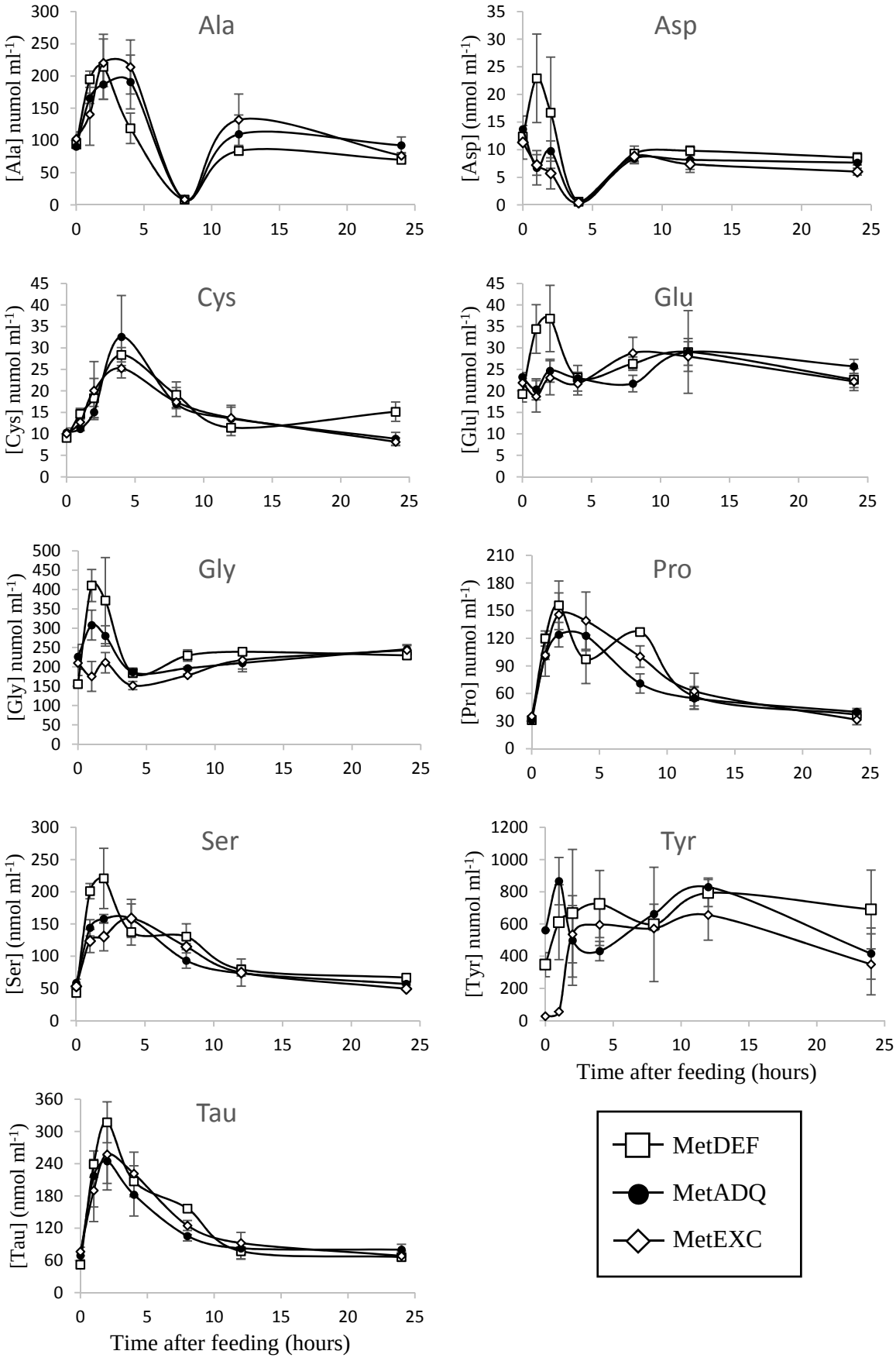

Figure 3.2. Concentrations of individual non-essential amino acids present in the plasma of juvenile barramundi over a 24 hour period following consumption of a single meal containing either a deficient (MetDEF), adequate (MetADQ) or excessive (MetEXC) level of dietary methionine.

3.4.2.1 Met and Cys metabolism

The differential expression of three genes (methionine adenosyltransferases 1 and 2a (MAT-1, MAT-2a) and cystathionine gamma-lyase (CGL)) involved in the turnover of Met and Cys in the liver tissue of juvenile barramundi in response to variable dietary Met over time after a meal is presented in Fig. 3.4. Expression of MAT-1 and MAT-2a was significantly affected by time ($p<0.001$; 0h=4h; 0h<2h>4h). CGL expression levels were also significantly affected by time ($p<0.001$; 0h<4h; 0h<2h>4h). No effect of treatment was observed.

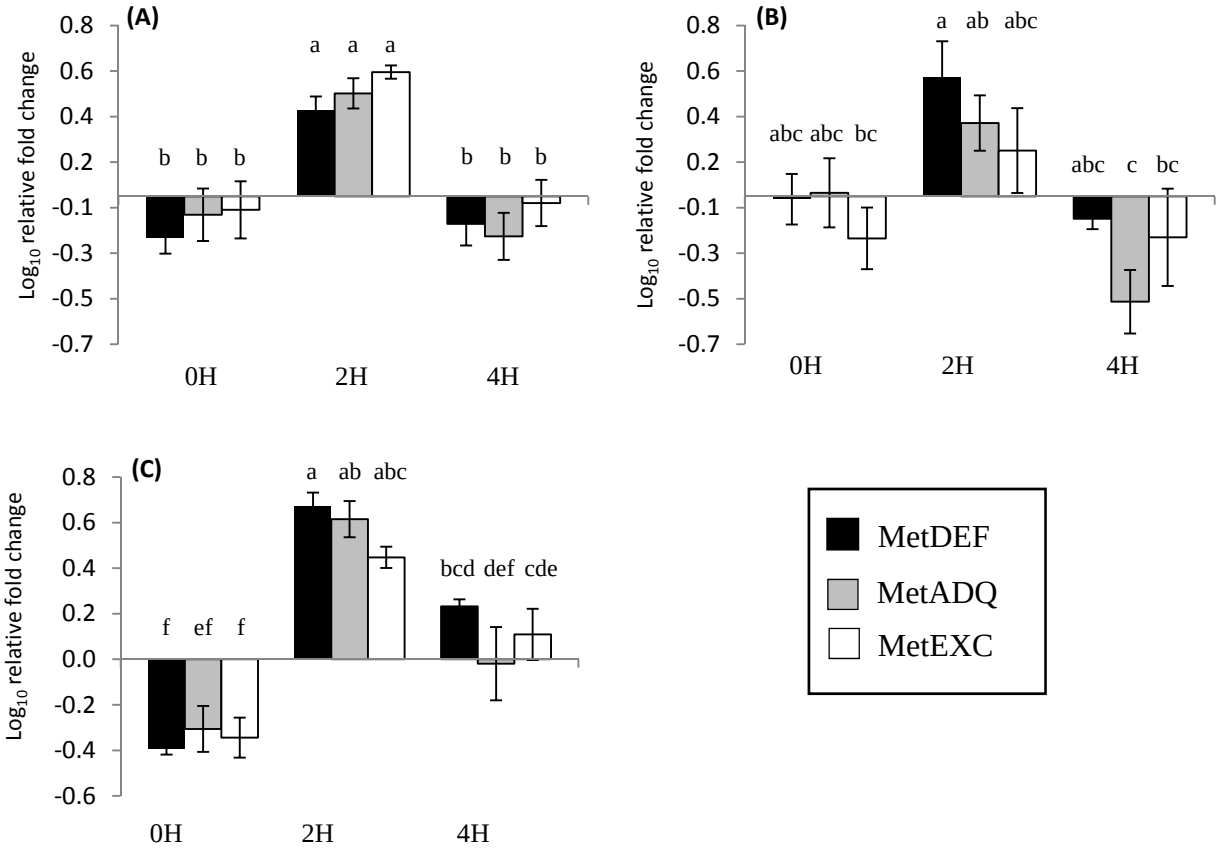

Figure 3.3. Transcript levels of selected genes (A, MAT-1; B, MAT-2a; C, CGL) of methionine and cysteine metabolic pathways in the liver tissue of juvenile barramundi sampled prior to (0H), and two (2H) and four (4H) hours after, consumption of a single meal containing either a deficient (MetDEF), adequate (MetADQ) or excessive (MetEXC) level of dietary methionine. Values were normalised to those of elongation factor 1α (Ef1α) and log10 transformed. Values presented are means (n=6) ±S.E. (represented by vertical bars). Significance analyses were performed by Two-Way ANOVA with *post-hoc* analysis by way of Tukey's honestly significant difference test. Columns with the same superscript letter are not significantly different. Letters are presented in order of magnitude from largest to smallest RQ. Gene abbreviations can be found in Table 3.2.

3.4.2.2 Taurine metabolism

The differential expression of two genes (cysteine dioxygenase (CDO) and cysteine sulphinic acid decarboxylase (CSAD)) involved in the synthesis of Tau from precursor amino acids in barramundi fed the three experimental diets was assessed and is presented in Fig. 3.5. A significant effect of time on the expression of both genes was observed ($p<0.001$; 0h=4h; 0h<2h>4h). Additionally, CDO expression was significantly affected by treatment ($p<0.05$; MetDEF=MetADQ; MetADQ=MetEXC; MetDEF<MetEXC), with a significant treatment:time interaction effect ($p<0.05$) also observed ($p<0.05$; MetEXC>MetDEF at 0h timepoint).

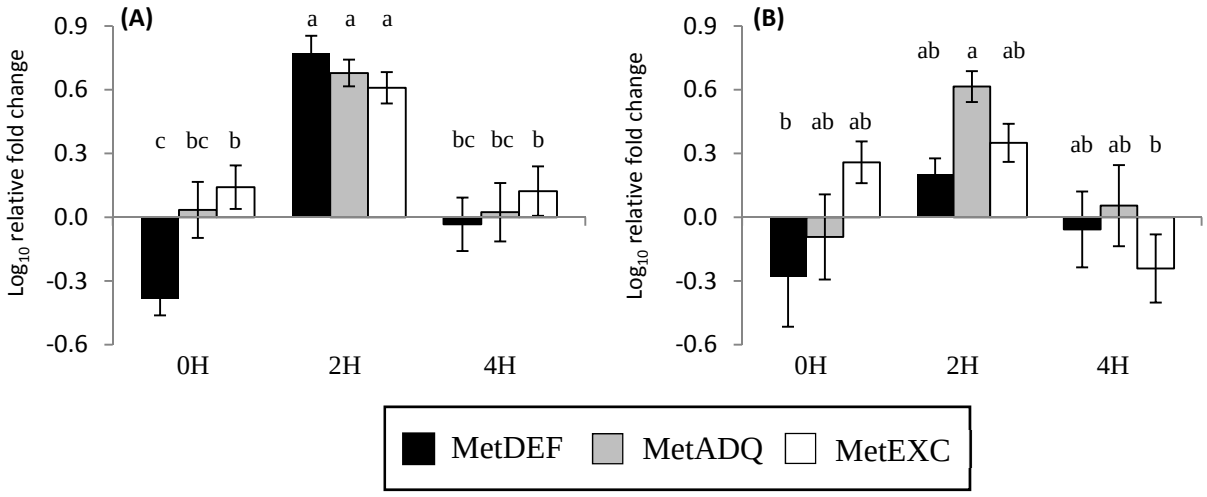

Figure 3.5. Transcript levels of selected genes (A, CDO; B, CSAD) of taurine biosynthetic pathways in the liver tissue of juvenile barramundi sampled prior to (0H), and two (2H) and four (4H) hours after, consumption of a single meal containing either a deficient (MetDEF), adequate (MetADQ) or excessive (MetEXC) level of dietary methionine. Values were normalised to those of elongation factor 1α (Ef1α) and log10 transformed. Values presented are means (n=6) ±S.E. (represented by vertical bars). Significance analyses were performed by Two-Way ANOVA with *post-hoc* analysis by way of Tukey's honestly significant difference test. Columns with the same superscript letter are not significantly different. Letters are presented in order of magnitude from largest to smallest RQ. Gene abbreviations can be found in Table 3.2.

3.4.2.3 Somatotropic axis

The effect of dietary Met intake on the expression of selected genes of the somatotropic axis (insulin-like growth factors I and II (IGF-I, IGF-II) and growth hormone receptor II (GHR-II)) over time was observed (Fig. 3.6). A significant effect of time on expression of IGF-I ($p<0.001$; 0h=2h; 0h>4h; 2h>4h), IGF-II ($p<0.001$; 0h>4h; 0h<2h>4h) and GHR-II ($p<0.001$; 0h>2h>4h) was observed. Expression of IGF-II was additionally significantly affected by treatment ($p<0.05$; MetDEF=MetADQ; MetADQ=MetEXC; MetDEF>MetEXC). A significant treatment:time interactive effect on the expression of IGF-I was also observed ($p>0.05$; MetADQ and MetEXC>MetDEF at 0h timepoint).

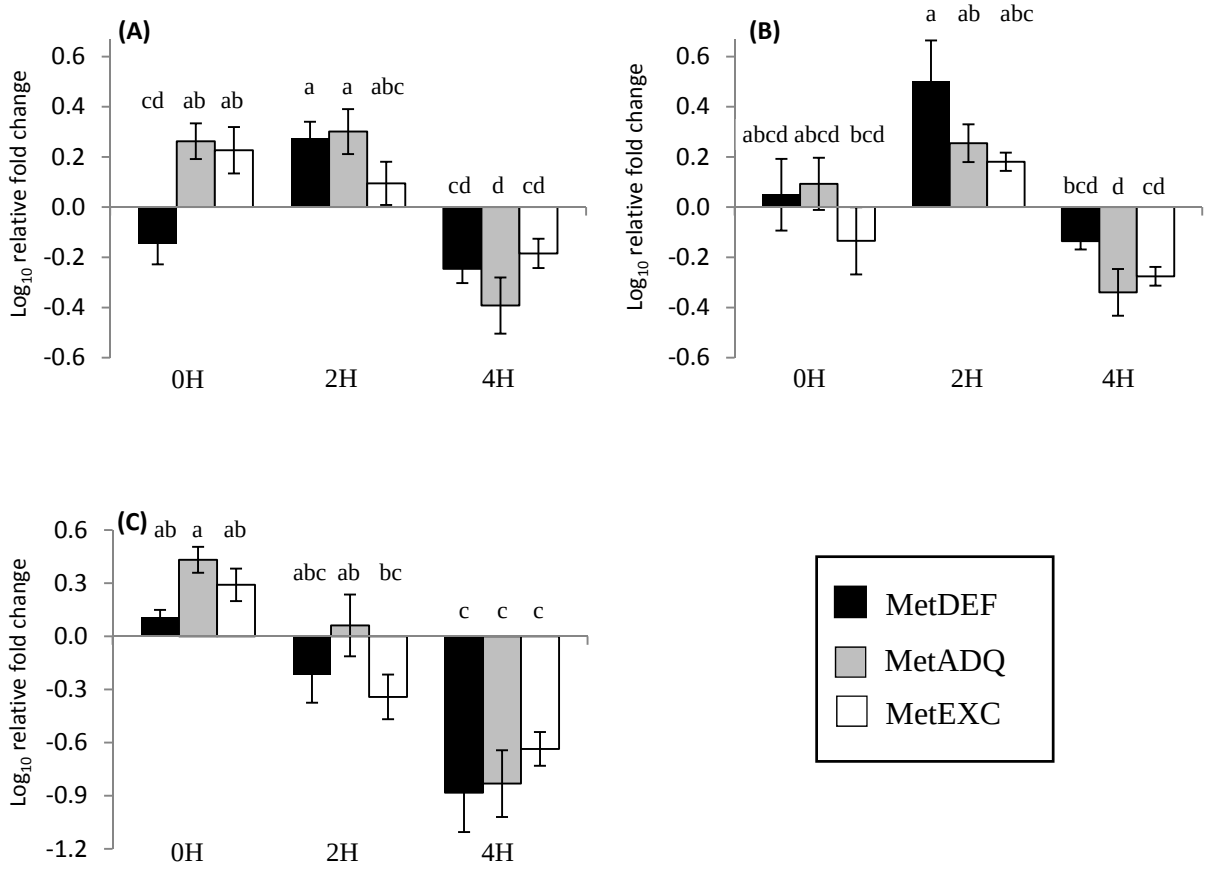

Figure 3.4. Transcript levels of selected genes (A, IGF-I; B, IGF-II; C, GHR-II) of the somatotropic axis in the liver tissue of juvenile barramundi sampled prior to (0H), and two (2H) and four (4H) hours after, consumption of a single meal containing either a deficient (MetDEF), adequate (MetADQ) or excessive (MetEXC) level of dietary methionine. Values were normalised to those of elongation factor 1α (Ef1α) and log10 transformed. Values presented are means (n=6) ±S.E. (represented by vertical bars). Significance analyses were performed by Two-Way ANOVA with *post-hoc* analysis by way of Tukey's honestly significant difference test. Columns with the same superscript letter are not significantly different. Letters are presented in order of magnitude from largest to smallest RQ. Gene abbreviations can be found in Table 3.2.

3.4.2.4 Protein degradation

The differential gene expression of two indicators of protein degradation (mitochondrial ubiquitin ligase activator of NF-κβ-1 (MUL1) and zinc finger AN1-type domain-5 (ZFAND-5)) in the hepatic tissue of fish fed the three experimental diets was assessed and is presented in Fig. 3.7. A significant effect of time ($p<0.001$; 0h=2h; 0h>4h; 2h>4h) and a significant treatment:time interaction effect ($p<0.05$; 2h>4h for MetDEF;0h>4h for MetADQ) on the expression of MUL1 was observed. ZFAND-5 expression was significantly affected by both time ($p<0.001$; 0h>4h; 0h>2h>4h) and treatment ($p<0.01$; MetDEF=MetADQ; MetDEF=MetEXC; MetADQ>MetEXC).

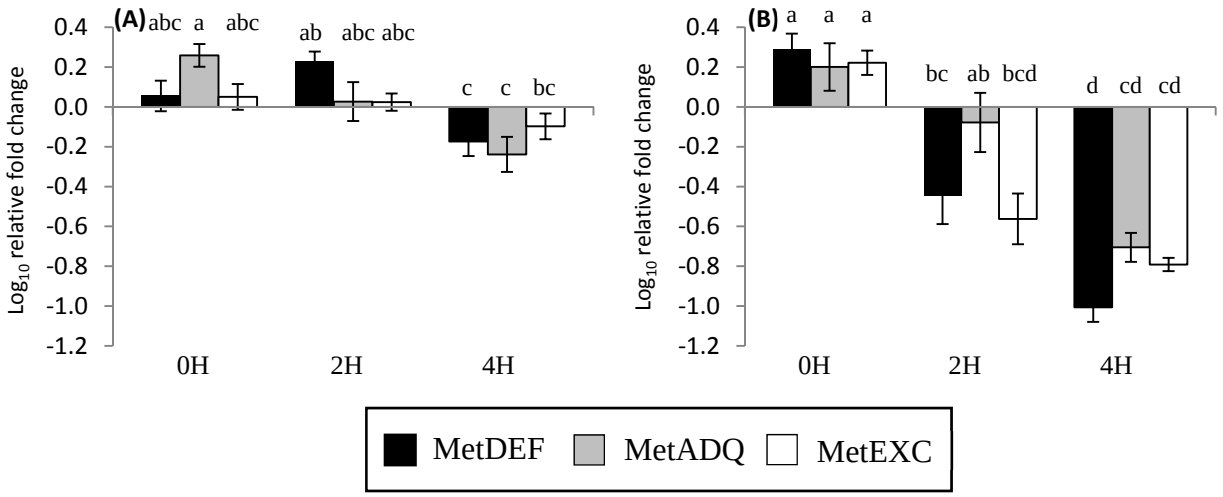

Figure 3.7. Transcript levels of selected genes (A, MUL-1; B, ZFAND-5) of proteolytic pathways in the liver tissue of juvenile barramundi sampled prior to (0H), and two (2H) and four (4H) hours after, consumption of a single meal containing either a deficient (MetDEF), adequate (MetADQ) or excessive (MetEXC) level of dietary methionine. Values were normalised to those of elongation factor 1α (Ef1α) and log10 transformed. Values presented are means (n=6) ±S.E. (represented by vertical bars). Significance analyses were performed by Two-Way ANOVA with *post-hoc* analysis by way of Tukey's honestly significant difference test. Columns with the same superscript letter are not significantly different. Letters are presented in order of magnitude from largest to smallest RQ. Gene abbreviations can be found in Table 3.2.

3.4.2.5 TOR activity signalling (protein synthesis).

Redd1 (regulated in development and DNA damage response-1) was used as an indicator of TOR pathway inhibition due to a lack of sufficient homogeneity between published piscine TOR gene sequences and those of the gene fragments in our partial barramundi transcriptome. The effect of the dietary treatments on expression of this gene in barramundi hepatic tissue is presented in Fig. 3.8. Whilst large variation in expression level can be seen within the MetADQ treatment at the pre-feeding timepoint, a significant effect of time on gene expression was seen ($p<0.001$; 0h>4h; 0h<2h>4h) as well as a significant treatment:time interaction ($p<0.05$; MetADQ>MetDEF at 0h timepoint).

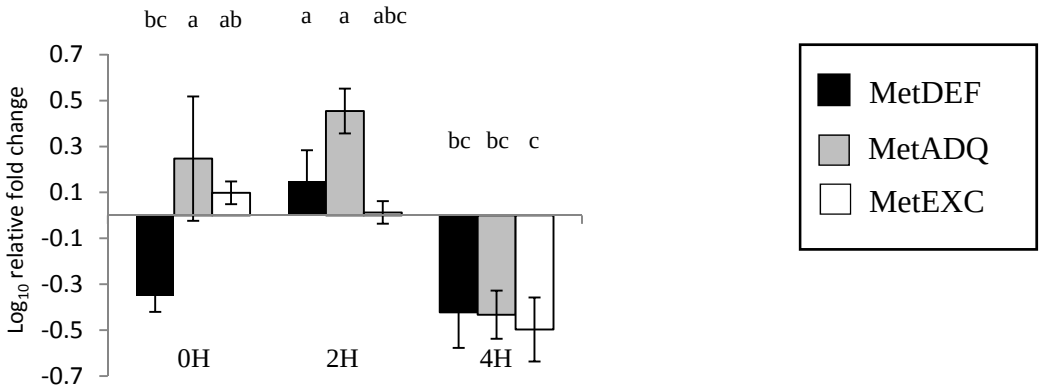

Figure 3.5. Transcript levels of Redd-1 (signifying inhibition of the TOR pathway) in the liver tissue of juvenile barramundi sampled prior to (0H), and two (2H) and four (4H) hours after, consumption of a single meal containing either a deficient (MetDEF), adequate (MetADQ) or excessive (MetEXC) level of dietary methionine. Values were normalised to those of elongation factor 1α (Ef1α) and log10 transformed. Values presented are means (n=6) ±S.E. (represented by vertical bars). Significance analyses were performed by Two-Way ANOVA with *post-hoc* analysis by way of Tukey's honestly significant difference test. Columns with the same superscript letter are not significantly different. Letters are presented in order of magnitude from largest to smallest RQ. Gene abbreviations can be found in Table 3.2.

3.5 Discussion

Traditionally, feed formulations have included amino acids at levels well in excess of their reported requirements, however, the use of plant proteins with amino acid profiles considered "imbalanced" to that required by the animal has necessitated the increased use of supplementary amino acids which are both expensive and, if not bound properly within the feed matrix, less efficiently used (Peres and Oliva-Teles, 2005; Ambardekar *et al.*, 2009). The re-evaluation of the requirement of barramundi for Met/Total Sulphur Amino Acids (TSAA) reported in Chapter Two, concluded the requirement to be between 10.5 and 13.6g kg^{-1} of diet dry matter (1.8-2.3% CP) (also published as Poppi *et al.*, 2017). While it is important to know this requirement figure, it's equally as important to define why the animal requires the nutrient. Does it have direct effects on metabolic processes or can other dietary nutrients, which may be more abundant in available ingredients fulfil the same roles? Cysteine, for example, which is relatively abundant in Met-deficient soybean meal, has been shown able to constitute 40% and possibly up to 54% of the TSAA content in the diet of barramundi without significantly compromising growth, sparing Met in the process (Poppi *et al.*, 2017). In the present study, we investigated the effect of variable dietary Met on the metabolism of plasma amino acids and the differential expression of several genes associated with markers of growth and sulphur amino acid metabolism and protein turnover.

In this study, dietary Met supply appeared to have no effect on the presence of other essential amino acids, nor any non-essential amino acids, in the plasma, contrary to the findings of Rolland *et al.* (2015a), who found that dietary Met significantly affected the plasma concentrations of all EAAs, in particular the branched chain amino acids. It might have been expected that Cys or Tau might have been more abundant in the plasma of fish in the MetADQ and MetEXC treatments once the metabolic/protein synthetic requirement for Met had been fulfilled and circulating Met became increasingly available for other processes, such as synthesis of these metabolites. This wasn't apparent, however, possibly due to these amino acids having been already supplied in the diet above adequate levels. The significant responses to dietary treatment seen for Met and Gly are almost certainly an artefact of their supply in the diet, given that plasma levels were highest in fish fed the diets with the greatest inclusion of the respective amino acid and lowest in fish in the low inclusion treatment. The postprandial peak in plasma Met of 115 nmol mL^{-1} at four hours post-feeding in fish fed a diet with an adequate inclusion of dietary Met compares well with a peak of around 130 nmol mL^{-1} at seven hours post-feeding in rainbow trout (*Oncorhynchus mykiss*) fed a fishmeal-based diet (where dietary methionine was also provided to meet EAA requirements) by Larsen *et al.* (2012). It is not clear why excessive dietary Met lead to a prolonged peak in plasma Thr, nor why substantial reduction in circulating levels of alanine (Ala) and aspartic acid (Asp) at

eight and four hours post-feeding respectively, followed by immediate return to a secondary peak at the following timepoint was observed. While analytical error seems evident, similar rapid fluctuations in plasma amino acid levels were also observed by Larsen *et al.* (2012).

Another important aim of this study was to investigate differences in the mechanisms by which barramundi metabolise Met when it is nutritionally limiting, as opposed to supplied in excess. The main hypothesis being tested was that the genes responsible for Met turnover, and that of subsequent metabolites, would respond in a step-wise fashion to increasing dietary Met supply, with more Met in the diet resulting in a greater level of gene expression to produce more of each specific enzyme for processing of this additional level of Met. This, however, wasn't what the gene expression data revealed. Despite significant differences in plasma Met contents between treatments at two and four hours post-feeding, we observed very little, if any, effect of the dietary levels of Met on the expression of the genes of these enzymes.

Methionine adenosyltransferase (MAT) catalyses the formation of SAM from Met and ATP and is the product of two genes (MAT1 and MAT2)(Garcia-Trevijano *et al.*, 2000). These genes are differentially expressed at different lifestages in other animals, with MAT2 in the foetal rat liver being replaced by MAT1 during maturation (Torres *et al.,* 2000). This balance during the ontogenesis of marine fish has not yet been reported to our knowledge so it was decided to investigate both forms in an attempt to capture whichever response was most substantial at this lifestage. Expression levels of both genes were relatively similar, suggesting that both are active in the liver in barramundi of this size. Numerically higher average expression of MAT-1 observed in the livers of fish in the MetADQ and MetEXC treatments, compared to that of fish fed the MetDEF diet, at two hours post-feeding, indicated that this gene may have been more active with increased Met. This response, however, wasn't reflected in the expression of MAT-2a, despite the fact that it is thought to have an even higher affinity for L-Met (Kotb and Geller, 1993) so should have been more sensitive to supplementation. The lack of significant difference between treatments may indicate a high requirement for Met metabolites for metabolic processes further downstream of Met. One theory may be that the system is flooded with Met catabolic enzymes in an effort to shunt the available Met through to SAM or Tau, which may have, for example, health implications, as a priority over protein synthesis or somatic growth. In that situation, only the remaining Met would then be available for protein synthesis. If this notion were true and a proportion of the dietary Met in the deficient diets was utilised for processes other than protein deposition, a poorer growth response may be seen in fish fed a Met deficient diet as was reported in Poppi *et al.* (2017). The partitioning of dietary Met between protein synthesis, metabolite production and other metabolic roles, however, was not measured so it cannot be said if this were truly a contributing factor.

Quantification of these roles through the measurement of fluxes of labelled Met may be valuable in elucidating this further. The lack of a significant effect of dietary Met content on expression of CGL, which is responsible for the conversion of cystathionine into Cys, may suggest that the Met processed by the MATs is not preferentially routed to Cys production but, rather may be temporarily retained as SAM, S-Adenosyl homocysteine (SAH) or another intermediate metabolite. SAM is a major donor of methyl groups for transmethylation reactions involving nucleic acids and proteins (Lieber and Packer, 2002) as well as aminopropyl groups for the synthesis of polyamines (Espe *et al.*, 2014) which are critical for a range of metabolic processes such as cell proliferation and differentiation (Li *et al.*, 2009). Increased levels of circulating SAM, is likely to provide an advantage in maintaining a rapid rate of growth as seen in those fish in the MetADQ treatment relative to the MetDEF treatment fish in Poppi *et al.* (2017). Accumulation of superfluous SAM in the liver has been linked to the apparent toxicity of excessive dietary Met levels in rats (Regina *et al.*, 1993) which may also explain the depression in weight gain seen in those fish fed excessive Met in Poppi *et al.* (2017). In the present study it might have been beneficial to measure the plasma content of these amino acids for confirmation of this, however it has been reported that SAM, in particular, is subject to degradation when stored for as little as two months, even at -80^{0}C, (Krijt *et al.*, 2009), requiring acidification of plasma with citric acid immediately after sampling to obtain accurate measurements (Kirsch *et al.*, 2009), a fact not taken into consideration in the design of the present experiment. The observation that expression of these genes changed significantly over time after the meal, but were not significantly affected by dietary Met supply, also suggests that production of these enzymes may be regulated by the consumption of feed, regardless of the amino acid composition. It should be noted, however, that the link between gene expression and enzyme activity may not be a direct relationship and can be confounded by a range of other factors (Panserat and Kaushik, 2010), resulting in these observations being indicative of activity, rather than measurably comparable. The use of enzyme assays and comparison between amino acid turnover, gene expression and enzyme activation, may shed more light on this subject and would be an interesting avenue of further investigation if we are to use these genes as indicators of enzyme activity.

Taurine (Tau) metabolism, as indicated by the expression of CDO (converting cysteine to sulphinoalanine) and CSAD (producing Tau from cysteate or hypotaurine from sulphinoalanine), was seemingly largely unaffected by the dietary Met content. A significantly higher expression of CDO, the gateway into the primary pathway for taurine biosynthesis, in fish in the MetEXC treatment compared to those in the MetDEF treatment at the pre-feeding timepoint (24 hours after the last meal), when plasma Met levels were at their lowest and were significantly depressed in the

MetDEF treatment, suggests that taurine synthesis may be an important ongoing function of available circulating sulphur amino acids in this species during periods where nutrients are limiting. This is also reflected in the pattern of CSAD expression after 24 hours starvation. This gene was also most highly expressed in the livers of fish in the MetADQ treatment at two and four hours post-feeding, suggesting that adequate Met may contribute to taurine synthesis and that excess Met may depress Tau synthesis in this species. Given the non-significance of plasma Tau levels between treatments precluding a negative feedback of sufficient or excess circulating taurine, the mechanism for this is not clear. However, the lack of a significant response in the Cys and Tau biosynthetic genes may also be an artefact of sufficient dietary supply of these amino acids negating the requirement for synthesis.

Given the significant differences in growth seen between these different dietary Met levels in Poppi *et al.* (2017), it was expected that Met intake would have a similar effect on growth related factors such the GH/IGF complex. It was further hypothesised that genes of the selected protein turnover pathways would be differentially expressed in response to dietary Met supply. Protein deposition, occurring when the rate of protein synthesis exceeds that of degradation (Klasing *et al.*, 1987) has been isometrically linked to body weight gain in fish (Dumas *et al.*, 2007)). It could be reasonably assumed, then, that increased weight gain, as observed in response to the MetADQ-equivalent and MetEXC-equivalent treatments by Poppi *et al.* (2017) (reported in Chapter Two of this thesis) would be reflected in upregulation of genes of the protein synthetic pathways and downregulation of genes of protein degradation pathways (resulting in net accretion of protein). Similarly to the SAA turnover genes, expression of genes of the somatotropic axis (IGF-I, IGF-II and GHR-II) were significantly affected by time after feeding, with that of IGF-II increasing, though not significantly, two hours after feeding before rapidly returning to pre-feeding levels, or below, within a further two hours in a similar fashion to that reported by Ayson *et al.* (2007). It has previously been reported in rainbow trout (*Oncorhynchus mykiss*) that Met supplementation stimulates the activity of components of these pathways (Rolland *et al.*, 2015b), which is thought to be related to an inhibitory effect of Met limitation on the transcriptional response to growth hormone (Stubbs *et al.*, 2002). This stimulatory effect was seen in the pattern of response of IGF-I at the pre-feeding timepoint, with a significantly higher expression of IGF-I observed in fish in the MetADQ and MetEXC treatments, and its association with improved growth was reflected in the enhanced growth response to these diets reported in Poppi *et al.* (2017), highlighting the role that this factor plays in growth stimulation. It is not known, however, whether this response is the result of the direct action of Met on production of IGF-I, or of additional available Met permitting an increased protein deposition which, in turn interacts with other growth related compounds such as

growth hormone (GH) (i.e. IGF-I may be acting as a signal of growth stimulation, rather than a direct driver). IGF-II responded in an opposing fashion to that of IGF-I at two hours post-feeding, with expression of this gene decreasing with increasing dietary Met. A similar lack of homogenous response in hepatic IGF-I and IGF-II expression was observed by Hevrøy *et al.* (2007) in response to variable lysine intake in Atlantic salmon (*Salmo salar*), however, IGF-II was shown to be more active in fast muscle, suggesting a functional differentiation between tissues. It was expected that expression of GHR-II, which has the function of transducing the signal from circulating GH into the cell (Ma *et al.*, 2012) would be positively associated with that of IGF-I, as has been reported elsewhere for both GHR-I (Rolland *et al.*, 2015b) and GHR-II (Saera-Vila *et al.*, 2007), however this was not the case, with GHR-II expression showing an apparent decrease in all treatments at two hours post-feeding, while IGF-I expression increased significantly in fish in the MetDEF treatment and remained constant in those in the MetADQ treatment, decreasing only in those fed an excessive amount of Met. We decided to investigate GHR-II as opposed to GHR-I expression in the present study as this variant has been shown to be more sensitive to growth hormone supplementation in-vivo in tilapia hepatocytes (Pierce *et al.*, 2012). Gabillard *et al.* (2006), however, showed that these two receptors can respond quite differently in rainbow trout, a functional partitioning also observed by Saera-Vila *et al.* (2007). It may be that upregulation of the IGFs at two hours post-feeding, which is known to have a negative feedback effect on GH production (Fruchtman *et al.*, 2002), suppressed the level of circulating GH, decreasing the requirement for GHR transduction.

Markers of the proteolytic pathways were shown to be depressed after the consumption of feed, as indicated by significant downregulation of the expression of ZFAND-5 in fish in the MetDEF and MetEXC treatments between the pre-feeding timepoint and two hours post-feeding and in the MetDEF and MetADQ treatments between two and four hours post-feeding. MUL1 was also observed to be significantly downregulated in fish in the MetADQ treatment between the pre-feeding and four hour post-feeding timepoints. This was not entirely unexpected, given that protein degradation to meet metabolic demands for essential amino acids would be less required when these amino acids are more readily available, however, the magnitude of the response of ZFAND-5 indicates that this gene may be a good hepatic marker of proteolysis in barramundi. What was unexpected, however, was that proteolysis appeared to be stimulated by adequate Met supply at the pre-feeding timepoint, as indicated by the higher expression of MUL-1. This pattern may be an indication that the metabolic EAA requirements (on a g fish^{-1} basis) of fish in this treatment, which were larger than those in either of the other two treatments, was greater. Having been fed identical rations of feed 24 hours prior to the first sampling point, the absence of sufficient circulating amino acids may have required greater turnover of protein in these fish. This may also be a factor in the

increased expression of ZFAND-5 at the two hour post-feeding timepoint, until circulating amino acids were at a level sufficient to meet metabolic needs, although no difference was seen in expression of this gene after starvation.

Methionine should have been the first limiting factor to protein synthesis in fish in the MetDEF and MetADQ treatments. It was expected then that increases in dietary Met, and the associated observed significant increases in plasma Met, should have increased protein synthesis, as was suggested by the significantly improved protein retention efficiencies reported between these treatments in Poppi *et al.* (2017). It was further expected that this may have been reflected in molecular markers of protein synthesis. This process, as indicated by the differential expression of Redd-1, which provides a signal of TOR pathway inhibition, however, was not significantly affected by dietary Met supply after feeding. Similarly to the protein degradation pathways, repression of TOR appeared to be stimulated by adequate Met supply, significantly so at the pre-feeding timepoint, which may suggest that either TOR was suppressed by Met, which is in direct contradiction to what has been reported previously in other species (Rolland *et al.*, 2015b) or possibly that there may have been some interaction with insulin, as has been reported elsewhere in other fish species (Lansard *et al.*, 2010), which masked any effect of Met supply. Excessive Met levels, however, did induce lower expression of Redd1 two hours after the meal, compared with feeding a deficient or adequate Met diet, so perhaps the TOR pathway is in fact receptive, but less sensitive to Met in this species, or alternatively is not a major pathway of protein synthesis in barramundi. Indeed, the effect of Met on TOR pathway modulation has been shown to be highly variable (Lansard *et al.*, 2011; Skiba-Cassy *et al.*, 2016), likely due to the complexity of the processes, and conditions surrounding their regulation, within this cascade. More work is clearly required to properly characterise this pathway in fish and the role individual dietary components play in its activity.

Conclusion

In conclusion, dietary Met supply appeared to have little to no effect on the metabolism of other amino acids in this study, as indicated by the largely uniform patterns of amino acid fluctuation in the plasma postprandially. Similarly, few significant differences were observed in the expression of selected genes of sulphur amino acid and protein turnover pathways after intake of different levels of Met, suggesting that the production of these enzymes may be primarily regulated by the consumption of feed, rather than the amino acid composition of that food. Methionine processing by MAT-1, however did appear to be more active with increased Met levels after feeding and both MAT transcripts appear to be active. A lack of a concomitant increases in plasma

Cys or Cys-related metabolism genes suggested that the metabolised Met was directed through SAM-dependent pathways. Results also indicated that Tau synthesis is an important process in this species and is somewhat sensitive to precursor (Met) supply. Longer term IGF-I expression was positively affected by dietary Met supplementation, reiterating its relationship with growth stimulation. Previously described markers of proteolytic pathways appear to be conserved in this species and we have confirmed that ZFAND-5 is a reliable biomarker of this process in barramundi. The apparent response of mTOR repression seemed to suggest either suppression of TOR by Met or an interaction with insulin, however there was also evidence that this pathway may be active but less sensitive to Met supply than anticipated. One of the other valuable observations to come out of this study was that we were able to isolate a number of important genes for the first time in this species and demonstrated that they are nutritionally regulated. This will prove useful in further study of the impact of SAA metabolism in this species.

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Chapter 4 - The effect of taurine supplementation to a plant-based diet for barramundi (*Lates calcarifer*) with varying total sulphur amino acid contents.

4.1 Abstract

The effect of variable levels of taurine (Tau) in the diet of juvenile barramundi (*Lates calcarifer*) on growth and nutrient utilisation was investigated. Five isonitrogenous and isoenergetic diets were formulated with dietary Tau contents ranging from 1g kg^{-1} DM to 15g kg^{-1} DM in order to determine the dietary inclusion level eliciting the maximum growth response (a "requirement"). Six additional diets were formulated (three with a deficient and three with an excessive level of methionine (Met) with either one, six or 12g kg^{-1} DM Tau) to investigate the effect of dietary Met level on metabolism of Tau. Diets were fed to juvenile barramundi (average starting weight: 26.8g) twice daily under a restricted pair-fed regime for a period of 42 days. No significant effect of variable dietary taurine supply on growth or nutrient utilisation was observed at any level of Met inclusion. Numerical variations suggested there may have been a positive effect of Tau provision at the mid-level of supplementation (6-8g kg^{-1} DM). The best-fit response model (5-SKM), fitted to the percent body weight gain data of fish fed diets with an adequate level of Met, suggested a relatively weak pattern of response ($R^2 = 0.183$) and predicted a Tau requirement of 5.47g kg^{-1} DM (0.96% CP) which is similar to that reported for several other species. Based on findings for other fish species, the lack of a significant response to variable Tau was unexpected. The possible confounding effect of the feeding regime and choice of total sulphur amino acid inclusion level on the magnitude of the response is discussed, as is the applicability of defining a "requirement" for non-essential amino acids. It was concluded that taurine appears to be conditionally essential to barramundi and should be provided in the diet when sulphur amino acid supply is insufficient to meet biosynthetic demands and that the predicted requirement is likely reasonably accurate for use as a minimum level of inclusion. This result may provide further support that barramundi possess the ability to synthesise Tau from precursor sulphur amino acids (SAA). Further, the response to variable dietary Met was significantly more pronounced than to Tau inclusion, highlighting the importance of Met/TSAA in the diet of this species.

4.2 Introduction

Taurine (Tau) is an amino sulphonic acid, possessing a sulphonate group in place of the carboxyl group indicative of the proteinogenic amino acids, nevertheless is often grouped with the sulphur amino acids owing to the fact it is one of the end products of sulphur amino acid metabolism (Hayes, 1976). It is known to be involved in a range of processes important to the health and metabolic functioning of animals including acting as an organic osmolyte, regulating cellular volume and thus osmotic stress and osmoregulation (an important function for marine, and especially euryhaline fish species such as barramundi)(Yancey, 2005). It also plays an important role in nutrient absorption, conjugating with bile acids in the liver to improve absorption of lipids and possibly contributing to cholesterol clearance (Yun *et al.*, 2012). Its antioxidant properties are central in stabilising cellular membranes during periods of disease challenge (Asha and Devadasan, 2013) and it is also thought to regulate mitochondrial protein synthesis (Jong *et al.*, 2012).

Taurine is generally regarded as a "conditionally essential" amino acid in fish, meaning it is required primarily in situations where endogenous production occurs, but at a rate which cannot meet demand. In cases such as inadequate supply of precursors (El-Sayed, 2014) or where enzymes of the biosynthetic pathway are lacking or have a reduced activity, it may need to be supplemented in the diet. Taurine (4.50 g kg^{-1}) and its precursor amino acids methionine (Met, 20.80 g kg^{-1}) and cysteine (Cys, 7.25 g kg^{-1}) (measured in white fish meal by Yamamoto *et al.* (1998)) are relatively abundant in fishmeal, traditionally the major source of protein for formulated diets for these species. Consequently, little attention has been paid in the past to the inclusion of this nutritional component in the diet of carnivorous fish species. The recent trend towards increasing the use of more environmentally and economically sustainable sources of protein such as plant meals, which are largely devoid of Tau and often limiting in Met, has created an impetus for focus on this issue in recent years (Watson *et al.*, 2015). Many of the earlier studies have primarily focused on concluding whether or not Tau is required at all in the diet of the studied species without a quantitative estimation of the level at which maximum gains are made (see review by Salze and Davis, 2015). Estimations of "requirement" for non-essential amino acids can be confounded by the inclusion levels of the amino acid(s) from which they can be synthesised, leading most studies (even those investigating multiple levels of Tau inclusion) to conclude only whether they consider it to be "required" or "not required" in the diet. Of those which have offered an estimate of requirement, estimates have varied between 0.2% of the diet for common dentex (*Dentex dentex*) (Chatzifotis *et al.*, 2008) and sea bass (*Dicentrarchus labrax*) (Brotons Martinez *et al.*, 2004) and 1.7% of the diet for Japanese flounder (*Paralichthys olivaceous*) (Kim *et al.*, 2005).

A recent study concluded the requirement of juvenile barramundi for total sulphur amino acids (TSAA; Methionine+Cystine) to be 20.2g kg^{-1} (99% of maximum response) in a diet with 592g kg^{-1} CP (3.4% CP) (Poppi *et al.*, 2017). Given that most animals have the capacity to synthesise Tau from these sulphur amino acid precursors, we were interested in whether a proportion of the TSAA requirement estimated in that study was due to a requirement for the production of Tau and, by association, whether the Tau level used in that study was indeed adequate. In particular, we were interested in whether variation of dietary Tau at a level marginally below the TSAA level eliciting maximum growth in that study would significantly affect the growth and nutrient utilisation of similar sized barramundi. In addition, just as variations in dietary Cys content can affect the requirement for Met (Twibell *et al.*, 2000), if the animal has an efficient mechanism for synthesizing Tau from Met, then variation in the supply of Met should impact the amount of supplementary Tau required to elicit optimal growth from plant-based diets for carnivorous fish.

The primary aim of the present study was to assess the effect of varying dietary Tau supply on the growth and nutrient utilisation of juvenile barramundi, with a view to determining an optimal level of dietary inclusion in formulated feeds for this species. Additionally, we aimed to define the essentiality of this amino acid and the relationship this has with dietary Met/TSAA supply.

4.3 Materials and Methods

4.3.1 Diets

4.3.1.1 Formulation

A series of five isonitrogenous and isoenergetic diets were formulated (Table 4.1) to assess the quantitative requirement for dietary Tau by juvenile barramundi. These diets contained varying levels of dietary Tau inclusion between one (deficient) and 15g kg^{-1} DM (excessive dietary Tau), based around an assumed requirement of 4g kg^{-1} DM derived from the ideal protein concept, with reference to lysine and according to the barramundi carcass profile of Glencross *et al.* (2013) and Glencross (unpublished carcass Tau content data); and with an adequate level of dietary Met/TSAA (13g kg^{-1} DM Met; 18g kg^{-1} DM TSAA), according to Poppi *et al.* (2017). The non-essential amino acid glycine replaced the varying amino acid(s) (Tau and/or Met) in order to maintain the sum free amino acid, crude protein and energy contents of the diets consistent with that done by other authors investigating sulphur amino acid requirements (Simmons *et al.*, 1999; Liao *et al.*, 2014).

Table 4.1. Formulations and analysed compositions of the experimental diets.

	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7	Diet 8	Diet 9	Diet 10	Diet 11
Ingredients (g kg^{-1})											
Fishmeal[1]	150	150	150	150	150	150	150	150	150	150	150
SPC	490	490	490	490	490	490	490	490	490	490	490
Fish oil[2]	100	100	100	100	100	100	100	100	100	100	100
Cellulose	67	67	67	67	67	67	67	67	67	67	67
Pregel Starch	53	53	53	53	53	53	53	53	53	53	53
$CaHPO_4$	20	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	6
Choline chloride[4]	1	1	1	1	1	1	1	1	1	1	1
Marker (Y_2O_3)	1	1	1	1	1	1	1	1	1	1	1
DL-Met	6.5	6.5	6.5	6.5	6.5	0	0	0	13	13	13
L-Tau	0	3	8	13	19	0	8	19	0	8	19
L-Gly	25.5	22.5	17.5	12.5	6.5	32	24	13	19	11	0
EAA Premix[5]	80	80	80	80	80	80	80	80	80	80	80
Composition as determined (g kg^{-1}DM unless otherwise stated)											
Dry matter (g kg^{-1} as is)	971	972	971	971	968	974	966	968	970	968	976
Crude Protein	582	569	569	558	558	580	567	559	557	556	553
Digestible Protein	484	437	474	448	446	466	480	453	427	465	422
Lipid	116	115	114	118	117	119	120	120	114	120	121
Ash	75.6	74.5	74.5	74.9	74.3	74.5	74.1	74.6	74.9	74.8	74.7
Gross Energy (MJ kg^{-1} DM)	21.9	21.6	21.6	21.7	21.6	21.8	22.0	22.0	22.0	21.8	21.8
DE (MJ kg^{-1} DM)	16.9	16.1	16.6	16.2	16.1	16.0	17.1	16.6	16.1	16.6	15.9
EAAs											
Arg	40	39	39	38	38	37	37	37	42	40	39
His	10	12	11	11	10	11	12	11	11	11	13
Ile	26	28	27	27	27	27	26	27	28	29	28
Leu	42	44	44	45	46	47	47	47	48	49	49
Lys	37	38	38	38	36	33	33	35	36	36	35
Met	13	13	13	13	13	8	8	8	18	18	18
Cys	5	5	5	5	5	5	5	5	5	5	5
Phe	28	30	29	29	28	28	27	28	28	29	29
Thr	28	28	28	28	28	28	28	28	28	29	29
Val	32	33	33	33	33	33	32	33	33	34	34
Tau	1	4	8	12	15	1	6	12	1	6	12

[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, 75.0g; L-Valine, 125.0g; L-Leucine, 187.5g; L-Phenylalanine, 87.5g; L-Threonine, 150.0g; L-Lysine, 187.5g; L-Arginine, 187.5g

Six additional diets were formulated to contain equivalent dietary Tau contents to the deficient (1g kg^{-1} DM), adequate (4g kg^{-1} DM) and excess (15g kg^{-1} DM) diets but with either a deficient (8g kg^{-1} DM) or excessive (18g kg^{-1} DM) level of dietary Met, in order to investigate the effect of dietary Met supply on the response to Tau. Post-experiment analyses determined the dietary Tau contents of these additional diet series to be one, six or 12g kg^{-1} DM.

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, with reference to lysine and according to the barramundi carcass profile of Glencross *et al.* (2013) and Glencross (unpublished carcass Tau content data).

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

4.3.1.2 Diet manufacture

Diets were prepared, manufactured on a laboratory-scale twin-screw extruder (MPF24; Baker Perkins, Peterborough, UK) and vacuum infused with fish oil according to the methods described in Glencross *et al.* (2016) to produce a 6mm diameter pellet.

4.3.2 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 number: A6/2015) and The University of Queensland Animal Ethics Committee (approval number: CSIRO/QAAFI/391/14).

The experiment was undertaken as 11 treatments, with each treatment randomly assigned to triplicate tanks.

In order to obtain a pooled average weight, forty juvenile hatchery-reared barramundi (*Lates calcarifer*) were randomly selected from a pooled population and individually weighed to 0.1 g accuracy. Thirty fish with an average weight of 26.8g ± 6.7g were randomly allocated to each of the thirty three 1000L tanks. Fish were anaesthetised prior to weighing with AQUI-S (~0.02mL/L) (AQUI-S New Zealand Ltd) before recovering in their allocated tank.

Experimental tanks were provided with ~3 L/min flow of continuously aerated marine water (~35PSU) of 28.9°C ± 0.2°C throughout the experiment. Photoperiod was set at 12:12 (light:dark).

A restricted pair-fed feeding strategy, as recommended by Glencross *et al.* (2007), was used to negate any influence of feed intake on the observed response to feed composition variation. For a period of seven days prior to commencing the experiment, consumption of a commercial barramundi diet (6mm Marine Float, Ridley Aquafeed Pty Ltd), fed twice daily to satiety, was monitored in order to establish a satiety feeding rate. Feed intake during this period was observed to be 0.7g $fish^{-1}$ day^{-1} on average.

Based on this, the initial rations were set at 0.6g $fish^{-1}$ day^{-1} for all tanks. Rations were hand 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 0.3g $fish^{-1}$ day^{-1} on Day 7 and increased by the normal amount on two consecutive days (Day 31 and 32) based on enthusiastic feeding response in all tanks).

Feed consumption was recorded daily for assessment of feed conversion and feed efficiency ratios. Uneaten feed was removed and an equivalent amount added to the subsequent ration. Consequently, feed intake did not differ between treatments.

All feed remained in cold storage (< 4ºC) other than during feeding and weighing.

4.3.3 Sample collection

At the commencement of the experiment, five fish were randomly selected and euthanised by overdose of anaesthetic (AQUI-S) and stored at -20° C until required for baseline proximate analysis. After 42 days, all fish were individually weighed for comparison of growth rate between treatments. At this time, a random sample of five fish from each replicate tank were euthanised by overdose of anaesthetic (AQUI-S) and stored at -20°C. Feed was withheld for a period of 24 hours prior to this sampling.

In order to calculate dietary digestible protein and energy contents, faeces were stripped from the fish over a period of six days after a three day rest period subsequent to the conclusion of the growth trial. During this period, all fish were fed their respective diets between 0800 and 1000 with faecal collection undertaken from selected tanks between 1600 and 1800 on the same day. In order to minimise stress and maximise feed intake on the day of collection, fish were stripped on non-consecutive days. Faecal collection was carried out according to the procedures outlined in Glencross (2011). Fish were transferred from their respective tanks by net and placed in a 100L tank containing aerated seawater and a light dose of AQUI-S (~0.02mL/L) and observed until loss of equilibrium occurred, at which point they were removed for stripping. Specific attention was paid to the relaxation of the ventral abdominal muscles of the fish to ensure collection of the faeces occurred before involuntary

evacuation. Once removed from the anesthetic tank, faeces were stripped from the distal intestine using gentle abdominal pressure into a pooled plastic specimen jar and frozen at -20°C. Contamination of the faeces with urine or mucous was minimised by rinsing of the hands between fish.

4.3.4 Chemical and digestibility analyses

Whole animals were minced, freeze dried and ground prior to analysis. Diets and faecal samples were similarly ground and homogenised and all samples were analysed for dry matter, ash, nitrogen, lipid and gross energy content as described below. Amino acid profiles of the diets were also determined as were yttrium contents of diets and faeces.

Sample dry matter contents were calculated by mass change after drying at 105^0C for 16h. Gross ash contents were determined gravimetrically following combustion in a muffle furnace at 550^0C for 16 hours. Crude lipid contents were determined after extraction of sample lipid according to the method of Folch *et al.* (1957). Total nitrogen content was determined by organic elemental analysis (CHNS-O, Flash 2000, Thermo Scientific, USA) and the sample protein content calculated based on *N* x 6.25. Gross energy analysis was undertaken using isoperibolic bomb calorimetry in a Parr 6200 oxygen bomb calorimeter (Par Instrument Company, Moline, IL, USA). Mass detection after reverse-phase ultra high-performance liquid chromatography, using pre-column derivatisation with 6-aminoquinolyl-N-hydroxysuccinimidyl (AQC) was used to determine diet amino acid composition. Analyses were undertaken on a Shimadzu Nexera X2 series UHPLC (Shimadzu Corporation, Kyoto, Japan) coupled with a Shimadzu 8030 Mass Spectrometer. Ground diet samples were prepared and hydrolysed in 6N phenolic HCl in accordance with the protocol for complex feed samples of Waters Corp. (1996). It is known that Cys is destroyed by acid hydrolysis and methionine is inconsistently oxidised to methionine sulfone (Rutherfurd and Gilani, 2009). Consequently, contents of these amino acids were quantified based on the abundance of cysteic acid and methionine sulfone respectively in the samples, following pre-hydrolysis oxidation with performic acid according to a protocol adapted from that of Chavali *et al.* (2013) (with the exception that 11mL glass vials were used and the samples dried by Speedivac vacuum drier).

Feed and faeces were microwave digested in 5mL HNO_3, based on a modification of EPA method 3051 (EPA, 1994), and yttrium concentrations determined by inductively coupled plasma mass spectrometry (ICP-MS).

Individual nutritional parameter (DM, protein and gross energy) apparent digestibilities ($AD_{Parameter}$) were calculated by consideration of the difference in ratios of the parameter of interest to yttrium concentration between the diets and faeces using 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. Digestible protein and energy contents of the diets were then calculated from these values.

4.3.5 Statistical analysis

The trends of the responses (linear, quadratic or cubic) to variable Tau inclusion at an adequate level of dietary Met was analysed by multiple regression analysis. All parameters of interest within each experiment (Initial and Final Body Weight, % Body Weight Gain, FCR, Feed Intake, Protein and Energy Retention Efficiencies and final carcass compositions) were also analysed by a One-Way ANOVA with a *post hoc* comparison of treatment group means by Tukey's honestly significant difference (HSD) multiple range test in order to illustrate the magnitude of the differences.

The effects of both Tau and of Met were analysed by two-way ANOVA. Simultaneous tests for General Linear Hypotheses were undertaken using Tukey's HSD contrasts to elucidate significant differences in response between individual treatments.

Percentage data was arcsin transformed prior to analyses. All statistical tests were conducted in the R-project statistical environment (R Core Team, 2014). Effects were considered significant at $p<0.05$.

The response of the fish, in percent body weight gain, to variable dietary Tau at an adequate Met level was analysed using nine nutrient response models (linear and quadratic ascending broken line, four- and five-parameter Saturation Kinetics, three- and four-parameter logistics, a compartmental, a sigmoidal and an exponential model) as described by Poppi *et al.* (2017). Coefficient of determination (R^2) and sum of squared errors (SSE) terms were calculated for each of the models according to Pesti *et al.* (2009). The "best fit" model was determined to be that which resulted in the lowest SSE and highest R^2.

Table 4.2. Response of juvenile barramundi to variable dietary taurine content at an adequate dietary methionine level (13g kg^{-1})[1].

	Initial Fish	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Pooled SEM	Polynomial Contrasts		
								Linear	Quadratic	Cubic
Dietary Tau (g kg^{-1}DM)		1	4	8	12	15				
Initial Weight (g $fish^{-1}$)		26.7[a]	27.1[a]	26.8[a]	26.9[a]	26.9[a]	0.07	n.s.	n.s.	n.s.
Final Weight (g $fish^{-1}$)		71.3[a]	72.3[ab]	72.6[b]	72.4[ab]	72.5[b]	0.16	<0.01	<0.05	n.s.
BW Gain (%)		166.9[a]	166.6[a]	171.1[a]	169.5[a]	169.8[a]	0.19	n.s.	n.s	n.s
FCR[2]		1.00[a]	0.98[a]	0.97[a]	0.97[a]	0.97[a]	0.00	n.s.	n.s	n.s
Feed Intake (g/fish)		44.4[a]	44.4[a]	44.4[a]	44.4[a]	44.4[a]	0.00	n.s.	n.s	n.s
ERE[3]		36.3[a]	37.4[a]	35.7[a]	36.1[a]	35.3[a]	0.40	n.s.	n.s	n.s
PRE[4]		27.7[a]	30.6[b]	29.5[ab]	30.0[ab]	30.2[ab]	0.36	n.s.	n.s	n.s
Carcass composition as determined (g kg^{-1}as is unless otherwise stated)										
DM	320	294[a]	287[a]	286[a]	291[a]	289[a]	0.16	n.s.	n.s	n.s
CP	209	176[a]	177[a]	177[a]	177[a]	177[a]	0.09	n.s.	n.s	n.s
Lipid	49	85[a]	80[a]	78[a]	82[a]	77[a]	0.14	n.s.	n.s	n.s
Ash	54	36[a]	33[ab]	31[b]	34[ab]	33[ab]	0.04	n.s.	<0.05	n.s
GE (MJ kg^{-1} as is)	6.9	7.4[a]	7.2[a]	7.1[a]	7.2[a]	7.1[a]	0.06	n.s.	n.s	n.s

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

A "requirement" for Tau was also calculated and was considered to be the Tau level eliciting 99% of the maximum response as predicted by the model best fitting the observed percent body weight gain data.

4.4 Results

4.4.1 Response to increasing dietary Tau content at adequate Met level

Increasing dietary Tau content seemed to have little effect on growth or nutrient utilisation in the studied fish when they were fed diets containing adequate levels of Met (Table 4.2). A significant effect of treatment on final weight of the fish was observed with fish fed the diet with the lowest dietary Tau content (Diet 1) being significantly smaller than those in either the assumed adequate (Diet 3) or excessive (Diet 5) Tau treatments. This effect was significantly linear with a less significant quadratic effect. No significant effect of treatment on percent body weight gain was observed, however, which may be a more robust indicator of treatment influence on growth given the very small differences in overall weight gain (1.3g $fish^{-1}$ maximum) between treatments.

Similarly, dietary treatment had little influence on body composition with ash content being the only variable exhibiting any significant response, decreasing significantly between Diet one and Diet three.

Of the nine nutrient response models fitted to the data, the best fitting model, the 5-parameter saturation kinetics model (5-SKM) (Fig. 4.1), explained 18.3% of the variation in the percent body weight gain of all replicate tanks fed with diets containing adequate Met and variable Tau. The Tau requirement predicted by this model was 5.47g kg^{-1} DM (0.96% CP).

4.4.2 Response to variable dietary Tau and Met content

Dietary Met content exerted a greater effect on response than did dietary Tau when the effect of Met intake on the response to increasing dietary Tau was examined (Table 4.3). Both Final Weight and PRE were significantly affected overall by dietary Tau content, however no significant differences were seen within individual dietary Met levels. These responses, however, along with %BW gain and FCR were highly significantly affected by dietary Met content, with Final Weight, %BW gain and PRE being significantly lower, and FCR significantly higher, in fish fed the Met deficient diet than those fed the Met adequate and excess diets at both shared Tau levels. ERE was similarly highly significantly affected by dietary Met content with fish fed the Met and Tau deficient diet (Diet 6) retaining

significantly less energy than those fish in the Met adequate and excess treatments at the taurine deficient level (Diets 1 and 9).

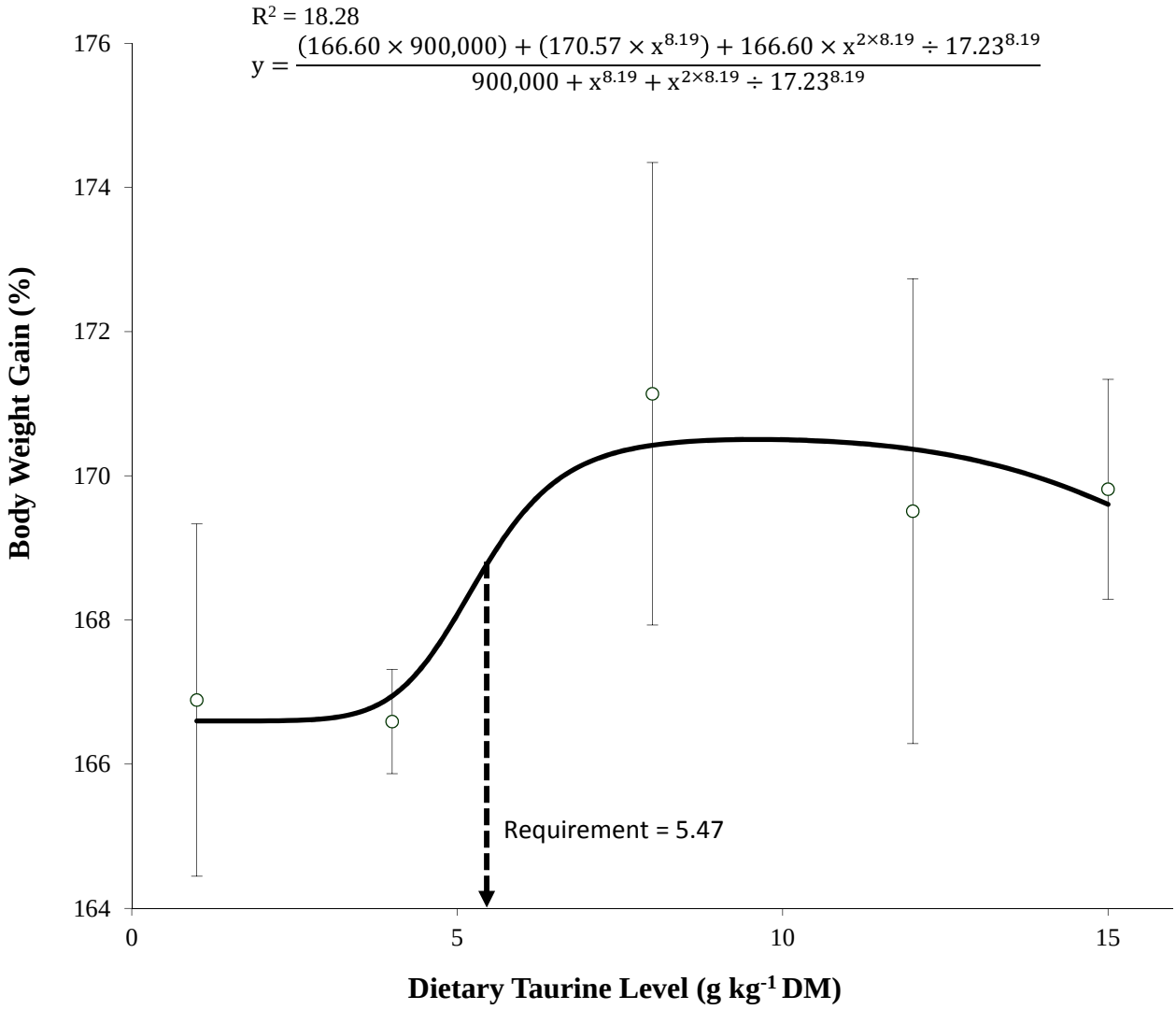

Figure 4.1. Percent body weight gain (±S.E.M.) (mean initial weight = 26.8g) of juvenile barramundi with taurine requirement predicted by the five-parameter saturation kinetics model.

Table 4.3. Response of juvenile barramundi to variable Tau content at three levels of dietary Met

													Main Effects		
	Initial Fish	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7	Diet 8	Diet 9	Diet 10	Diet 11	Pooled SEM	Met	Tau	Met:Tau
Dietary Tau (g kg^{-1}DM)		1	4	8	12	15	1	6	12	1	6	12				
Dietary Met (g kg^{-1}DM)		13	13	13	13	13	8	8	8	18	18	18				
Initial Weight (g $fish^{-1}$)		26.7^{a}	27.1^{a}	26.8^{a}	26.9^{a}	26.9^{a}	26.8^{a}	26.8^{a}	26.9^{a}	26.8^{a}	26.7^{a}	26.9^{a}	0.04	n.s.	n.s.	n.s.
Final Weight (g $fish^{-1}$)		71.3^{b}	72.3^{bc}	72.6^{bc}	72.4^{bc}	72.5^{bc}	65.4^{a}	65.7^{a}	65.8^{a}	72.0^{bc}	73.0^{c}	72.0^{bc}	0.54	<0.001	<0.05	n.s.
BW Gain (%)		166.9^{b}	166.6^{b}	171.1^{b}	169.5^{b}	169.8^{b}	143.8^{a}	145.2^{a}	143.8^{a}	169.0^{b}	173.5^{b}	167.5^{b}	14.67	<0.001	n.s.	n.s.
FCR[2]		1.00^{a}	0.98^{a}	0.97^{a}	0.97^{a}	0.97^{a}	1.15^{b}	1.14^{b}	1.14^{b}	0.98^{a}	0.96^{a}	0.98^{a}	0.09	<0.001	n.s.	n.s.
Feed Intake (g/fish)		44.4^{a}	44.4^{a}	44.4^{a}	44.4^{a}	44.4^{a}	44.4^{a}	44.4^{a}	44.4^{a}	44.4^{a}	44.4^{a}	44.4^{a}	0.00	n.s.	n.s.	n.s.
ERE[3]		36.3^{bd}	37.4^{d}	35.7^{ad}	36.1^{bd}	35.3^{ad}	30.9^{a}	32.3^{abc}	31.3^{abc}	35.9^{bd}	37.2^{cd}	34.9^{ad}	3.07	<0.001	n.s.	n.s.
PRE[4]		27.7^{b}	30.6^{bc}	29.5^{bc}	30.0^{bc}	30.2^{bc}	21.4^{a}	22.1^{a}	24.2^{a}	29.7^{bc}	32.2^{c}	29.4^{bc}	2.45	<0.001	<0.05	<0.05
Carcass composition as determined (g kg^{-1}as is unless otherwise stated)																
DM	320	294^{a}	287^{a}	286^{a}	291^{a}	289^{a}	294^{a}	297^{a}	292^{a}	294^{a}	296^{a}	287^{a}	0.13	n.s.	n.s.	n.s.
CP	209	176^{ab}	177^{b}	177^{ab}	177^{ab}	177^{ab}	168^{a}	167^{a}	174^{ab}	177^{ab}	182^{b}	176^{ab}	0.10	<0.001	n.s.	n.s.
Lipid	49	85^{a}	80^{a}	78^{a}	82^{a}	77^{a}	91^{a}	94^{a}	86^{a}	77^{a}	80^{a}	78^{a}	0.14	<0.01	n.s.	n.s.
Ash	54	36^{a}	33^{a}	31^{a}	34^{a}	33^{a}	34^{a}	34^{a}	36^{a}	33^{a}	34^{a}	32^{a}	0.04	n.s.	n.s.	n.s.
GE (MJ kg^{-1} as is)	6.9	7.4^{a}	7.2^{a}	7.1^{a}	7.2^{a}	7.1^{a}	7.3^{a}	7.4^{a}	7.3^{a}	7.3^{a}	7.3^{a}	7.2^{a}	0.04	n.s.	n.s.	n.s.

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

Carcass crude protein and lipid levels were also deemed to be significantly impacted by dietary Met level only, though no significant differences were seen between diets with the same Tau content. The effect of dietary Tau on carcass ash content was not considered significant by this statistical method.

While not statistically significant, it is notable that at all three levels of dietary Met, %BW gain peaked numerically at the middle level of Tau (at either 6 or 8g kg^{-1}) which may indicate some sort of nutritional adequacy at around this level.

4.5 Discussion

Taurine is not considered an essential amino acid in the diet of most fish species as it can generally be synthesised from precursor sulphur amino acids. It has, however, been reported that this biosynthetic capacity is not shared by all species (Yokoyama *et al.*, 2001; Salze *et al.*, 2016). In these situations, and where sufficient precursors are not available (such as in unsupplemented plant meal-based diets), Tau may be considered to be conditionally essential and must be provided in the diet (Watson *et al.*, 2014). The capacity of fish to synthesise Tau can be dependent on a number of factors, including feeding habit, dietary protein source, species and size, among other things (El-Sayed, 2014). Many marine fish species, for example, have been shown to have a reduced activity of key enzymes involved in the taurine biosynthetic pathways (Goto *et al.*, 2001; Kim *et al.*, 2008), while this activity is generally not limiting in freshwater species (Yokoyama *et al.*, 2001; Wang *et al.*, 2015). However, no biological reason for this separation between environments has been put forward. As barramundi are euryhaline, determination of their biosynthetic capacity may add to clarification of this issue. Often, initial dietary formulations for novel species use amino acid profiles based on that of the animal itself. Indeed, the diets for the current experiment were formulated using the ideal protein concept, with reference to lysine, based on the barramundi carcass amino acid composition published by Glencross *et al.* (2013) and Glencross (unpublished carcass Tau content data). While this concept often provides reasonably accurate estimates of essential amino acid (EAA) requirements (Mambrini and Kaushik, 1995), it does not hold true for all amino acids. Certain amino acids may be utilised at different rates, depending on their metabolic roles (NRC, 2011), leading some to be under- or overestimated by ideal protein (Rollin *et al.*, 2003). This could be especially true for Tau given its role in, for example, conjugating with bile acids in the liver, the requirement for which may be affected by the dietary composition. As well, Tau is not a proteinogenic amino acid, and thus its level in the body is likely both temporally variable and not genetically

controlled (as protein content is), body composition at a specific point in time may not be the ideal basis on which to base dietary Tau requirement. It is necessary, then, to determine this figure experimentally. A previous observation in our lab suggested that Tau supplementation may have positively impacted growth in juvenile barramundi fed a diet deficient in TSAA (unpublished data). The purpose of this experiment, then, was to investigate the impact of variable dietary Tau content on the growth and nutrient utilisation of juvenile barramundi when fed a diet with a sufficient TSAA content, with the intention of estimating the level at which growth is optimised (a "requirement" level). Additionally, we were interested to know whether varying the dietary TSAA content affected the response to variable taurine which may have suggested whether the animal was in fact synthesising Tau from these precursors.

At an adequate level of TSAA, changes in dietary Tau from one to 15g kg^{-1} had no significant effect on growth performance of the fish. This would seem to suggest that Tau is not required to be supplemented in the diet of juvenile barramundi at all when this level of dietary TSAA is supplied and may be an indication that most, if not all, of the necessary Tau may have been provided through synthesis from precursor sulphur amino acids (SAA). This seems unlikely, however, as it would suggest that the response to a diet containing approximately 18g kg^{-1} TSAA (as in the current experiment) with or without Tau supplementation should be no different to one containing less TSAA and with Tau making up the deficiency. This, however, was not what was reported in Poppi *et al.* (2017), with significant growth effects observed between Diet one (13.8g kg^{-1} TSAA + 6.4g kg^{-1} Tau) and Diet two (19g kg^{-1} TSAA + 6.6g kg^{-1} Tau) in that study, so it seems some other factor may have affected the response.

The relatively small differences in weight gain between treatments made it difficult to detect significance, especially when natural variations in growth and feeding behavior between fish are taken into account. This lack of divergence in growth may be attributable, in part, to the restricted feeding pair-feeding regime applied. It was decided to follow this method, similar to that used by Glencross *et al.* (2003) and Enes *et al.* (2008), in order to negate the impact of feed intake (which can be influenced by several factors unrelated to nutritional adequacy) on physiological response, which is often seen as a confounding factor in satiety fed studies (Glencross *et al.*, 2007) and isolate the effect of variable taurine supply. A negative aspect of this approach, however, is that for the faster growing animals (presumably being those fed at least an "adequate" amount of Tau, assuming Tau positively impacts growth), the feed intake becomes increasingly restrictive with respect to the requirement of the animal for protein and energy, which may have resulted in a greater

proportion of the ingested amino acids being catabolised for energetic needs and not deposited in the body. It is possible then that feeding to satiety (as is most common in the fish nutrition literature) might have yielded more significant results. Whether the results using that method would have been more accurate, however, is debatable given that variations in feed intake were identified by El-Sayed (2014) as possibly a major factor in the growth stimulation seen in response to taurine supplementation in several studies. Perhaps feeding in proportion to animal body weight would negate this confounding aspect, although the regular weighing required would be impractical and may have its own impacts on feed intake. Ultimately, any improvements in the response presented herein, while not as divergent as might otherwise have been seen with a different feeding regime, can be said to be primarily due to the dietary composition.

Nutrient utilisation was similarly unaffected by taurine supplementation as also reported by Gaylord *et al.* (2007). Neither were significant differences seen in the final composition of the carcass other than ash content, however supplementation of taurine effected a downward trend in carcass lipid content between the lowest and moderate levels of supplementation at the adequate Met level while carcass crude protein remained relatively unchanged. This observation was also reported by Espe *et al.* (2012) and was attributed to a known interaction between lipid deposition and polyamines, the production of which S-Adenosylmethionine (SAM) is a donor of aminoisopropyl groups (Brosnan and Brosnan, 2006). Those authors suggested that additional Tau may have spared hepatic Met for SAM synthesis which, in turn, increased production of polyamines.

The response of fish to variable Tau was investigated at a level of TSAA close to the optimal requirement reported in Poppi *et al.* (2017). The diets were formulated to contain a level of TSAA marginally below the upper end of the requirement range, however, analysed values for both Met and Cys, while consistent across diets, were lower than expected, most likely due to variation in ingredient compositions between that used in our formulation model (based on a database of compositions of previous batches of ingredients used in our lab) and that of the batch used to produce the diets in this study. Nevertheless, the levels were still well within the requirement range and remain relevant to the original objectives. It was decided to use a level close to the asymptotic response, rather than closer to the minimum requirement, in order to assess the contribution of dietary Tau level to the response seen in that previous study. It could have been reasonably assumed that, if a proportion of the TSAA eliciting the maximum response in Poppi *et al.* (2017) was being used to synthesise Tau, reducing the dietary Tau content at that same Met level should have impacted growth (i.e.

indicating the reported TSAA requirement is a Met+Cys+Tau requirement). While this would have been an interesting observation, it was perhaps not the best approach for estimating the true Tau requirement independent of the effect of Met. It may have been more accurate to use a dietary Met level marginally limiting or at the lower end of the requirement range as suggested by Salini *et al.* (2015) for assessing the impact of fatty acid deficiency in this species (barramundi). In this way, the response to dietary treatment could have been separated between that due to the supplementary Tau and Tau being synthesised from excess SAA.

What was clear was that varying the dietary Met content had a much more significant effect on growth and nutrient utilisation than did Tau inclusion level. The purpose of varying the dietary Met inclusion at common Tau levels was to investigate whether the response to Tau was dependent on the level of Met. This may also serve as an indication of whether Tau is being synthesised at all from Met. It was expected that, if Tau biosynthesis was active in this species, those fish fed the Met deficient diet series would exhibit a more significant response to increasing Tau than those fed diets with adequate or excessive levels of Met. There was, however, no significant effect of Tau supplementation on growth between those fish fed the deficient and assumed adequate and excessive Tau diets. In this case, as all other essential amino acids (EAA) were provided in the diet well in excess of their requirements, Met would have been, by far, the most limiting amino acid for protein synthesis. It appears, then, that any sparing effect of Tau, even at excessive levels of inclusion, was not enough to make up for this deficiency. It should also be noted that the highest level of Tau inclusion was well in excess of the assumed requirement (4g kg^{-1}). Perhaps, even when precursors are limiting, supplemental Tau is only advantageous up to the point where the requirement is met and does not impact the metabolism of other essential amino acids. The lack of response may also indicate that Tau synthesis is a high priority function of Met and that, even at this low level of Met, a significant proportion of the Tau requirement was already being met (i.e. Tau synthesis may have been prioritised over other Met roles). Indeed, it was shown in Chapter Three that the gene for cysteine sulfinic acid decarboxylase (CSAD), an enzyme catalysing the rate limiting step in taurine biosynthesis, was more highly expressed in the livers of fish fed an adequate or excessive amount of dietary Met after 24 hours starvation, suggesting circulating SAAs were being used for Tau synthesis, even when circulating SAA levels were considerably reduced. In the present study, at the excessive Met level, where Met would clearly not be limiting protein synthesis, the lack of response to increasing Tau may confirm

that barramundi can indeed efficiently synthesise Tau from Met, with synthesis possibly fulfilling the requirement for Tau, without the need for supplementation.

The available evidence seems to suggest that Tau is not required by barramundi, at least at this level of dietary TSAA inclusion. Nevertheless, the best fitting of nine nutrient response models applied to the replicate *tank* average percent body weight gain data, the five-parameter saturation kinetics model (5-SKM), revealed a relatively weak pattern of response ($R^2 = 0.18$) and predicted a Tau requirement of 5.47g kg^{-1} DM (0.96% CP). When applied to the average percent body weight gain of each *treatment*, however, the 5-SKM explained 90.3% of the variation in the data, emphasising the importance of data selection when applying statistical models. Using the treatment averages ignores the variation in the data and is, therefore, less accurate, giving false-confidence in prediction outputs of that model (although the two predicted similar Tau requirements) so that based on the *tank* averages must be considered to be the more reliable representation. This figure is similar to the recommended beneficial level of supplementation of 5g kg^{-1} reported for several marine species, including cobia (*Rachycentron canadum*) (Lunger *et al.*, 2007) and red sea bream (*Pagrus major*) (Matsunari *et al.*, 2008; Takagi *et al.*, 2010), as well as the freshwater fish rainbow trout (*Oncorhynchus mykiss*) (Gaylord *et al.*, 2007), suggesting it may be valid. As well, this figure equates to 14.6% of the lysine (Lys) content of the diet, compared with the assumed requirement of 16.7% of Lys used in the formulation of the diets, based on the ideal protein concept with respect to Lys and the carcass profile of Glencross *et al.* (2013) and Glencross (unpublished carcass Tau content data). This similarity seems to suggest that Tau requirements can be reasonably predicted by the ideal protein concept. Other analyses of barramundi carcass amino acid profiles, however, showed carcass Tau contents to be 34.8% and 36.1% of carcass Lys in two separate experiments (unpublished data from Poppi *et al.*, 2017), highlighting the variability of this amino acid and the limitations of applying the ideal protein concept to requirement for a non-proteinaceous amino acid.

Defining a requirement for a non-essential amino acid is difficult as the level of supplementation required to elicit the maximum response would be dependent on the availability of the precursors required for their synthesis. Tau requirement, in particular, may be highly variable due to the range of processes in which it is involved. Fluctuations in salinity, for example, may necessitate the modulation of cellular Tau content, which has been shown to improve the osmotic tolerance of erythrocytes in yellowtail (*Seriola quinqueradiata*) (Takagi *et al.*, 2006). In addition, it may be problematic to draw conclusions from a model fitted to data which has been shown to not differ significantly between

treatments. It is perhaps more accurate to conclude that Tau appears to positively affect growth in this species but that it is not clear the level at which this effect is optimised and that it may not be required to be supplemented in the diet at all if sufficient precursors are provided. In this way, it can be considered to be "conditionally essential" as suggested by various authors (Salze *et al.*, 2011; Espe *et al.*, 2012). Numerical peaks in percent body weight gain at the middle level of Tau supplementation (6-8g kg^{-1}) at all levels of dietary Met, coupled with a significantly positive effect of Tau supplementation on protein retention, when varying Met level was taken into account, supports this assertion and, at least, confirms this level is a good starting point from which to further investigate this topic.

In order to get a more accurate estimate of the relationship between precursor supply and Tau requirement, it may be useful to conduct a more thorough investigation of the effectiveness of TSAA variation, in particular that of Cys, the major amino acid precursor for Tau synthesis and one which is relatively abundant in terrestrial plant meals, in sparing Tau. In addition, the present experiment was conducted in seawater. As this species is euryhaline and is cultured in freshwater in some countries (Ayson *et al.*, 2013), acclimation of the fish to fresh water would be possible and a re-assessment of the response to Tau supplementation under these conditions may be an interesting avenue of future exploration. Comparison of these results may suggest the contribution of osmoregulatory demands to the overall requirement for Tau supplementation.

Conclusion

The results of the present study demonstrate that Tau supplementation to plant-based diets may have a positive effect on the growth of juvenile barramundi, however, this effect is diminished by adequate supply of precursor sulphur amino acids. This further supports that this species likely has an efficient mechanism for synthesising Tau. Additionally, adequate or excessive supply of Tau cannot ameliorate the negative impact of Met deficiency. Further work is required to define the sparing effect of dietary Cys on the Tau requirement and the impact salinity has on this requirement for barramundi.

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Chapter 5 - Adequate supply of dietary taurine stimulates expression of molecular markers of growth and protein turnover in juvenile barramundi (*Lates calcarifer*).

5.1 Abstract

A trial was conducted to investigate the effect of variable dietary supply of taurine (Tau) on postprandial changes in plasma amino acid composition and hepatic expression of several genes in juvenile barramundi (*Lates calcarifer*). Triplicate tanks of 24 fish (average weight: 89.3g) were fed one meal of 2.5g/fish of a diet containing either a deficient (TauDEF; 1g/kg), adequate (TauADQ; 8g/kg) or excessive (TauEXC; 19g/kg) level of dietary taurine. Plasma and liver samples were then taken 1h, 2h, 4h, 8h, 12h and 24h post-prandially. Liver tissue collected at the two hour and four hour post-feeding timepoints, in addition to samples taken as a pre-feeding control, were analysed for expression of genes involved in pathways of sulphur amino acid turnover (CGL, MAT-1), Tau biosynthesis and transport (CSAD, CDO, ADO, TauT), target of rapamycin signalling (Redd-1), the somatotropic axis (GHR-II, IGF-I, IGF-II) and protein turnover (Mul1, ZFAND-5).

Dietary Tau content had no significant effect on the postprandial plasma profiles of any amino acid in this study other than Tau and glycine. The expression profile of CGL and the Tau synthetic genes suggested an effect of Tau excess on the metabolism of Cys. A lack of significant difference in the expression of the sulphur amino acid turnover genes, however, may provide further evidence that their expression is regulated primarily by feed intake, rather than the dietary amino acid profile. Markers of two pathways of Tau biosynthesis (ADO and CSAD) appear to be active in this species, however, CSAD appeared more responsive to feeding. The gene expression pattern observed in this study provides further proof that this species does indeed possess the ability to synthesise Tau from SAA precursors. Taurine transport and homeostasis by TauT was shown to be directly regulated by Tau availability in this species. A link between adequate supply of taurine and TOR pathway-mediated growth stimulation was also apparent. Signalling of growth stimulation in response to Tau supplementation was evident in the expression of genes of the somatotropic axis which were all more highly expressed in the hepatic tissue of fish fed the TauADQ and TauEXC diets. Interestingly, an observed depression in expression of these genes, coupled with upregulation of the proteolytic and TOR-suppressing genes in response to excessive Tau supply in the diet possibly signals that excessive Tau is not conducive to optimal growth.

5.2 Introduction

Taurine (2-aminoethane sulfonic acid) is an end-product of sulphur amino acid (SAA) metabolism and, while not incorporated into protein, is the most abundant free amino acid in the tissues of numerous animal species (Schuller-Levis and Park, 2003; Ripps and Shen, 2011). Accordingly, it plays a number of important functions in fish health and metabolism. Among its many roles, it is known to act as both an organic osmolyte and an antioxidant, both important roles in responding to stress events (Huxtable, 1992; Asha and Devadasan, 2013). In addition, by conjugating with bile acids in the liver, resulting in the formation of taurocholic acid, taurine (Tau) has been shown to improve absorption of lipids (Kim *et al.*, 2008) which may, in turn, spare other amino acids, particularly crystalline amino acids which are absorbed more rapidly than those bound in protein (Ambardekar *et al.*, 2009), from catabolism for energetic demands. It also possesses the characteristics of a feeding stimulant for fish (Chatzifotis *et al.*, 2008) which may help to overcome some of the palatability issues commonly associated with the use of plant feed ingredients in diets for carnivorous fish (McGoogan and Gatlin, 1997). While most fish species possess the ability to synthesise Tau from precursor sulphur amino acids, this capacity is often insufficient to meet the entire requirement without supplementation (Yun *et al.*, 2012). Given the importance of Tau in fish health and metabolism, therefore, it is often considered to be "conditionally essential" rather than a non-essential amino acid (NEAA). In certain species, such as yellowtail (*Seriola quinqueradiata*), bluefin tuna (*Thunnus thynnus*) and skipjack (*Katsuwonus pelamis*), however, this capacity has been reported to be reduced, or even absent, primarily due to variations in activity of cysteine sulfinic acid decarboxylase (CSAD) (Yokoyama *et al.*, 2001). In these cases, Tau may be considered to be an essential component of the diet.

Consideration of the dietary Tau supply has become increasingly important in recent years given the negligible Tau content of terrestrial plant meals. If the use of these more sustainable ingredients is to be increased, accurate characterization of the dietary and metabolic requirements of Tau in the species of interest is necessary to determine its importance and the level of supplementation required for optimal growth. Tau supplementation of non-fishmeal based diets has been linked to improved growth in a number of fish species (Takagi *et al.*, 2010; Salze *et al.*, 2012), however the exact mechanism of this stimulatory effect is so far unclear. One theory is that Tau may be sparing methionine (Met), a precursor and essential amino acid (EAA), for protein synthesis and other important physiological processes such as production of S-Adenosylmethionine (SAM) (Pinto *et al.*, 2013).

Markers of protein turnover and other growth-related pathways have been used in the past to link amino acid supplementation-related growth stimulation to the mechanisms underpinning the

observed response. Belghit *et al.* (2014), for example, reported that Met directly affected the expression level of several genes of autophagy-lysosomal and ubiquitin-proteosome protein turnover pathways in rainbow trout (*Oncorhynchus mykiss*). The regulation of components of the somatotropic axis: a series of growth factors (insulin-like growth factors one and two, IGF-I and IGF-II) and related growth hormone receptors (GHRs) and binding proteins (IGFBPs) which are considered signals of growth modulation (Clemmons and Underwood, 1991); as well as elements of the target of rapamycin (TOR) pathway, have also been linked to amino acid supplementation in fish (Gómez-Requeni *et al.*, 2003; Vélez *et al.*, 2014). Rolland *et al.* (2015) found that Met affects these pathways "directly or indirectly" in rainbow trout and Hevrøy *et al.* (2007) observed a significant upregulation of several genes of the somatotropic axis in response to lysine supplementation in Atlantic salmon (*Salmo salar*). The effect of Tau supplementation on these pathways, however, has yet to be investigated in fish. Clarifying whether the reported apparent positive influence of Tau on growth is due to direct effects, such as signalling the initiation of specific pathways or sparing of precursor SAA, or is simply a byproduct of improved overall health and metabolic functioning would be an important step in defining the importance of this amino acid.

The Tau dose response trial reported in Chapter Four suggested that Tau may positively affect growth in this species, although no significant differences were seen in that study in response to variable dietary Tau. The aim of the current experiment, then, was to determine whether any markers of growth were positively affected by dietary Tau content which may indicate whether Tau does, in fact, impact growth pathways in this species more subtly than we had previously observed, independent of the limitations of the restricted pair-fed growth trial identified in that study. In addition, the present study aimed to investigate the impacts of variable dietary Tau supply on sulphur amino acid and Tau turnover, indicating the role of Tau in sparing SAA as well as further elucidating the pathways used by this species to synthesise it.

5.3 Materials and Methods

5.3.1 Diets

Three diets (Table 5.1), containing either a deficient (TauDEF; 1g/kg), adequate (TauADQ; 8g/kg) or excessive (TauEXC; 15g/kg) dietary Tau content, were selected based on performance of the fish in those treatments in a 42-day growth trial preceding this experiment. The diets chosen elicited a poor growth response (TauDEF), the maximum response (TauADQ) or contained the highest inclusion of dietary Tau (TauEXC), deemed excessive according to a formulation model based on the ideal protein concept with reference to lysine and the amino acid profile reported by Glencross *et al.* (2013).

Table 5.1. Formulations and analysed compositions of the experimental diets.

	TauDEF	TauADQ	TauEXC
Ingredients (g kg^{-1})			
Fishmeal[1]	150	150	150
SPC	490	490	490
Fish oil[2]	100	100	100
Cellulose	67	67	67
Pregel Starch	53	53	53
$CaHPO_4$	20	20	20
Vit. and Min. Premix[3]	6	6	6
Choline chloride[4]	1	1	1
Marker (Y_2O_3)	1	1	1
DL-Met	6.5	6.5	6.5
L-Taurine	0	8	19
L-Glycine	25.5	17.5	6.5
EAA Premix[5]	80	80	80
Composition as determined (g kg^{-1}DM unless otherwise stated)			
Dry matter (g kg^{-1} as is)	971	971	968
Crude Protein	582	569	558
Digestible Protein	484	474	446
Lipid	116	114	117
Ash	75.6	74.5	74.3
Gross Energy (MJ kg^{-1} DM)	21.9	21.6	21.6
DE (MJ kg^{-1} DM)	16.9	16.6	16.2
EAAs			
Arg	40	39	38
His	10	11	10
Ile	26	27	27
Leu	42	44	46
Lys	37	38	36
Met	13	13	13
Cys	5	5	5
Phe	28	29	28
Thr	28	28	28
Val	32	33	33
Tau	1	8	15

[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, 75.0g; L-Valine, 125.0g; L-Leucine, 187.5g; L-Phenylalanine, 87.5g; L-Threonine, 150.0g; L-Lysine, 187.5g; L-Arginine, 187.5g

Dietary formulations were designed to be isonitrogenous and isoenergetic. Amino acid contents were similarly intended to be equal and to exceed the amino acid requirements of barramundi according to the aforementioned formulation model, excluding glycine (used to balance the total crystalline amino acid content of the diets), total sulphur amino acids (included at an adequate level according to Poppi *et al.* (2017)) and Tau, the amino acid of interest.

Diets were manufactured for the growth trial on a laboratory-scale twin-screw extruder (MPF24; Baker Perkins, Peterborough, UK), with intermeshing, co-rotating screws according to an adaptation of the protocol of Glencross *et al.* (2016), to produce pellets ~ 6mm in diameter.

5.3.2 Fish management

This experiment was 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 number: A6/2015) and The University of Queensland Animal Ethics Committee (approval number: CSIRO/QAAFI/391/14).

5.3.2.1 Preconditioning

Twenty four juvenile barramundi (*Lates calcarifer*) (mean initial weight: 72.2g) from each of the triplicated 1000L tanks of the three selected treatments from the growth trial remained in their respective tanks. The relevant diets were fed to satiety once daily for a further six days before feeding of the experimental ration commenced.

All feed was refrigerated (< 4ºC) except during feeding and ration allocation.

Continuously aerated marine water (~35PSU) of 29.5°C ± 0.2°C was supplied to each tank at a rate of ~ 3 L/min. Tank illumination was achieved using a combination of artificial lighting set at 12:12 (light:dark) and natural light of a similar photoperiod.

5.3.2.2 Feed Ration Determination

Feed intake over a five minute period (that allocated for feeding on the sampling day) was assessed for three days prior to commencing the experiment. In order to exclude any influence of feed intake on the postprandial response, the average intake over this period in the poorest feeding tank (2.5g $fish^{-1}$) was assigned as the ration for all tanks in order to negate any large-scale variation in feed intake confounding the response to the treatments. This ration was offered and consumed in its entirety on the day preceding the experimental day.

5.3.3 Sampling

Mean weights of the fish in each treatment on the day of sampling were: TauDEF (86.7g), TauADQ (88.7g), TauEXC (91.0g). Twenty four hours after the last meal, three fish were randomly selected from each tank and euthanised by an overdose of anaesthetic (AQUI-S™). Fish were individually weighed and approximately 2mL of blood was removed from the caudal vein by heparinised syringe before centrifugation at 6000 rpm for 2 min. Plasma was separated from the clotted material and placed, along with hepatic tissue collected concurrently, immediately on dry ice before storage at -80ºC (zero hour control). The assigned ration was then fed after a further two hours of recovery following the zero hour sampling. Feeding of the experimental diets was carried out over a period of five minutes and staggered according to a plan designed to ensure the precision of sampling intervals of each tank. All feed was observed to have been consumed within the allotted period.

Three fish were again randomly selected from each tank one, two, four, eight, 12 and 24 hours after this meal and processed as described above. During the first three sampling points (until 4 hours post-feeding), the stomach contents of each fish was assessed. During this period, one fish out of the 81 sampled was observed to have consumed a negligible amount of feed and was replaced by an additional fish sampled at the same time for the purposes of analyses. All remaining fish were deemed to have consumed an equivalent amount of feed.

5.3.4 Chemical analyses

Plasma samples were deproteinised according to the protocol of Davey and Ersser (1990). Briefly, equal volumes (60µl) of plasma and 500µM internal standard (α-Aminobutyric acid) were combined with 380µl of acetonitrile before centrifugation at 2200 x *g* for four minutes at 4^0C. The supernatant was extracted and derivatised with 6-aminoquinolyl-N-hydroxysuccinimidyl (AQC) using the Waters AccQ·tag system (Waters Corporation, Milford, MA). Derivatised samples were analysed for amino acid composition using mass detection after reverse-phase ultra high-performance liquid chromatography on a Shimadzu Nexera X2 series UHPLC (Shimadzu Corporation, Kyoto, Japan) coupled with a Shimadzu 8030 Mass Spectrometer.

Table 5.2. Target genes of sulphur amino acid and protein turnover; and growth in barramundi, and the primer sequences used in the qPCR assays of their expression.

Target Gene[1]	Accession Number	Primer Name	Sequence	Length
Met and Cys metabolism				
MAT-1	XM_018678413	MAT1 qPCR F1	TGTCAATCTCCTTGTTCACCT	21
		MAT1 qPCR R1	GCCTCTTCAGATTCAGTTCC	20
CGL	XM_018673132	CGL qPCR F2	CACAAGACGAGCAGAACGAC	20
		CGL qPCR R2	CACCACAGCCATTGACTTCC	20
Tau metabolism				
CDO	XM_018674402	CDO- qPCR F2	GTTGCCTACATAAATGACTCCA	22
		CDO- qPCR R2	CTGTCCTCTGGTCAAAGGTC	20
CSAD	XM_018666199	CSAD qPCR F1	GTACATTCCACCAAGTCTGAG	21
		CSAD qPCR R1	CCCAGGTTGTGTATCTCATCC	21
ADO	XM_018660792	ADO qPCR F5	AGCCTGTTAGTACTGTGATCC	21
		ADO qPCR R5	AGACATCAATGCTGAAATGGAC	22
Somatotropic axis				
IGF-I	XM_018697285	IGF-1 qPCR F2	CTGTATCTCCTGTAGCCACAC	21
		IGF-1 qPCR R2	AGCCATAGCCTGGTTTACTG	20
IGF-II	XM_018664155	IGF-II qPCR F1	AGTATTCCAAATACGAGGTGTG	22
		IGF-II qPCR R1	GAAGATAACCTGCTCCTGTG	20
GHR-II	XM_018702499	GHR-2 qPCR F2	CGTCCATATCCCATCTAAAGTGTC	24
		GHR-2 qPCR R2	GTCATTCTGCTCCTCAATGTC	21
Proteolysis				
MUL-1	XM_018686248	Mul1 qPCR F1	GGCTTCCGTTATTTCCTCAC	20
		Mul1 qPCR R1	TGCTCTCCTCTATGTTAAGTTCAC	24
ZFAND-5	XM_018669382	ZFAND5 qPCR F1	CTAGAGCCTGTTGTAAGCCA	20
		ZFAND5 qPCR R1	CTCGGCCTTGTAATCATAGGG	21
TOR activity signaling				
Redd-1	XM_018699192	Redd1 qPCR F2	TTTCAGCACATCCACTAACGG	21
		Redd1 qPCR R2	CCACTACTTCTTTCAGGATTGTC	23
Control genes				
Luc	NA	Luc qPCR F	GGTGTTGGGCGCGTTATTTA	20
		Luc qPCR R	CGGTAGGCTGCGAAATGC	18
EF1α	NA	Lcal EF1α F	AAATTGGCGGTATTGGAAC	19
		Lcal EF1α R	GGGAGCAAAGGTGACGAC	18

[1]*MAT-1*, methionine adenosyltransferase-1; *CGL*, cystathionine-γ-lyase; *CDO*, cysteine dioxygenase; *CSAD*, cysteine sulphinic acid decarboxylase; *ADO*, cysteamine dioxygenase; *IGF-I*, insulin-like growth factor-I; *IGF-II*, insulin-like growth factor-II; *GHR-II*, growth hormone receptor-II; *MUL-1*, mitochondrial ubiquitin ligase activator of NF-κβ-1; *ZFAND-5*, zinc finger AN1-type domain-5; *Redd-1*, regulated in development and DNA damage response-1; *Luc*, luciferase; *EF1α*, elongation factor 1α.

5.3.5 Molecular analyses

5.3.5.1 RNA extraction and normalisation

Total RNA was extracted from frozen liver tissue (two fish per tank per timepoint; n=6 per treatment per timepoint) using Trizol reagent (Invitrogen) in accordance with the manufacturer's instructions. Extracted RNA was then precipitated using equal volumes of isopropyl alcohol and precipitation solution (0.8M disodium citrate with 1.2M sodium chloride in ultrapure distilled water) (Green and Sambrook, 2012) before DNase digestion with the Turbo DNA-free kit (Applied Biosystems) to remove any remaining DNA. RNA quantity was assessed on a NanoDrop spectrophotometer (NanoDrop Technologies) and quality assessed using RNA nanochips (Agilent #5067-1511) in a bioanalyser (Agilent Technologies) prior to normalisation to a concentration of 200ng/µl for cDNA synthesis.

5.3.5.2 Quantitative real-time RT-PCR

Reverse transcription was undertaken on 1µg of total RNA using the Superscript III first strand synthesis system (Invitrogen) with 25µM oligo(dT)$_{20}$ and 25µM random hexamers (Resuehr and Spiess, 2003). Real-time PCR primers for those genes not previously isolated in this species (ADO and TauT) were designed using PerlPrimer version 1.1.21 (Marshall, 2004). Sequences of gene fragments, or whole genes, within a partial, unannotated barramundi transcriptome (Hook *et al.*, 2017), which showed significant homology with published sequences from related species were used to generate a series of primer pairs. Those which showed the greatest specificity to the target gene were selected for use in the analyses. Barramundi gene sequences published by a third party following the completion of this experiment were identical to those derived for the present study by the process outlined above. The efficiency of each primer was optimised to between 95 and 105% using the slope of the standard curve of a PCR-amplified five-fold serial dilution of pooled cDNA. Genomic DNA contamination was excluded at this time by concurrent PCR-amplification of a pool of DNase-digested RNA. GenBank accession numbers and primer sequences for all genes are presented in Table 5.2.

The differential expression of selected genes of the somatotropic axis as well as Tau, sulphur amino acid and protein turnover pathways in response to the dietary treatments over time was evaluated by real-time PCR in the following fashion. The equivalent of 7.5ng of reverse-transcribed RNA was PCR-amplified following addition of 2X SYBR Green PCR Master Mix (Applied Biosystems) and 0.5µM RT-PCR gene-specific primers. Allocation of reaction components was carried out in triplicate by an epMotion 5070 robot (Eppendorf) before being run on a Viia7 real-time PCR system (Applied Biosystems). Amplification cycle conditions were: 2

minutes at 50^0C and 10 minutes at 95^0C, followed by 40 cycles of 15 seconds at 95^0C and 40 seconds at 60^0C. The specificity of each primer was confirmed on completion of the reaction by assessment of dissociation melt curves for each gene. Normalisation of the cycle threshold values for each gene to that of the elongation factor 1α (EF1α) and luciferase reference genes, as used in several other gene expression studies with this species (De Santis *et al.*, 2011; Wade *et al.*, 2014; Salini *et al.*, 2015) was used to assess the variation in gene expression magnitude between treatments over time. Amplification variation of these genes was 2.73 cycles and 2.79 cycles respectively, which did not change significantly over time.

5.3.6 Statistical analysis

All values are expressed as means with their standard errors. Prior to statistical analysis, plasma amino acid contents (n=9 per treatment per timepoint) and gene expression (n=6 per treatment per timepoint) data were log-transformed to obtain homoscedasticity. The impact of dietary treatment and time after feeding on plasma amino acid composition and the differential expression of the target genes was assessed by two-way ANOVA with Tukey's honestly significant difference *a posteriori* test to identify differences between treatments group means within timepoints and between timepoint means within treatment. Differences between groups were considered significant at $p<0.05$. Correlations between Tau intake and both the area under the plasma Tau curve and peak plasma Tau levels were examined using Pearson's correlation test. Statistical analyses were conducted in the R-project statistical environment, version 3.1.0 (R Core Team, 2014).

5.4 Results

5.4.1 Plasma amino acid contents

Plasma amino acid contents varied significantly over time after feeding as digestion of the feed released amino acids into circulation (Figs. 5.1, 5.2, and 5.3; and Tables B1, B2 and B3 in Appendix B). Peak concentrations were seen at between one (for arginine in fish fed the TauEXC diet) and 12 hours post-feeding (for glutamic acid in fish fed the TauEXC diet – although fluctuations were small in this amino acid), gradually returning to pre-feeding levels between 12 and 24 hours post-feeding for most amino acids. Levels of the sum of the standard proteinogenic amino acids (minus Aspartic acid, Glutamine and Tryptophan and plus Tau) (Fig. 5.1) was highly significantly affected by time ($p<0.001$) after feeding but did not respond to dietary treatment. Similarly, most EAAs (Fig. 5.2) fluctuated significantly over time. Histidine (His), however, was the only EAA to be also be significantly affected by dietary treatment (TauEXC>TauADQ;

p<0.05), with a significant treatment:time interaction (p<0.05) also observed, although changes in the presence of this amino acid were small in comparison to others. The NEAAs, too, were predominantly affected only by time after the meal (Fig. 5.3). Glycine (Gly) and Tau (the two amino acids which varied in the dietary formulation) were additionally highly significantly affected by treatment (p<0.001), with the response of Tau also displaying a highly significant treatment:time interactive effect (p<0.001). Tau intake was highly significantly positively correlated with both the area under the plasma Tau curve (i.e. the longevity of the plasma Tau elevation) (r = 0.959, p<0.001) and the peak plasma Met content (r = 0.952, p<0.001). Additionally, plasma serine (Ser) content was highly significantly affected by treatment (p<0.001). Levels of this amino acid diverged significantly two hours post-feeding, with those fish fed the TauDEF diet having the highest concentration of Ser in their plasma, followed by those fed the TauADQ diet, then those in the TauEXC treatment. This pattern of response remained for the duration of the experiment. Most amino acids gradually rose to a peak and declined thereafter, as might be expected after digestion. Plasma levels of Gly in the diet containing the highest level of this amino acid (TauDEF) declined between the pre-feeding timepoint and the 12 hour post-feeding timepoint, returning to the pre-feeding level after a further 12 hours. The Tau plasma content followed a similar pattern, declining between the pre-feeding and four hour post-feeding timepoints before gradually rising to return to pre-feeding levels 24 hours post-feeding.

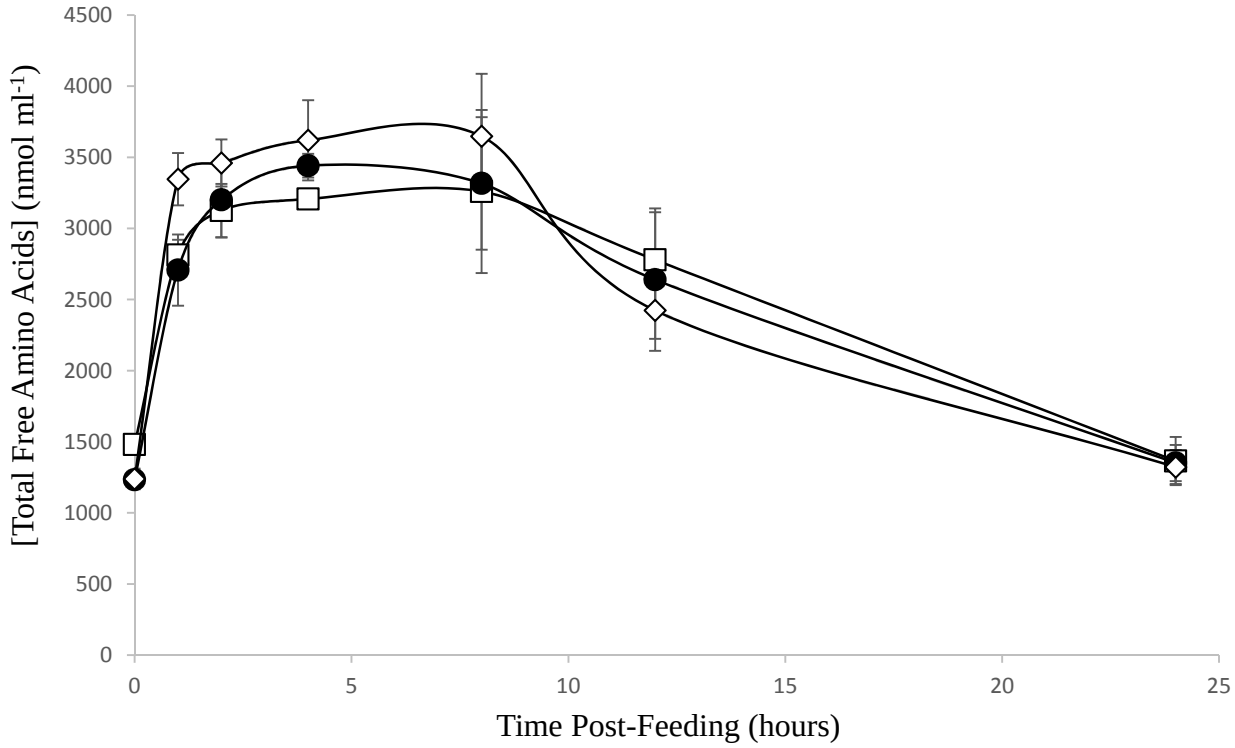

Figure 5.1. Concentrations of total free amino acids (TFAA) present in the plasma of juvenile barramundi over a 24 hour period following ingestion of a single meal containing either a deficient (TauDEF), adequate (TauADQ) or excessive (TauEXC) dietary taurine content.

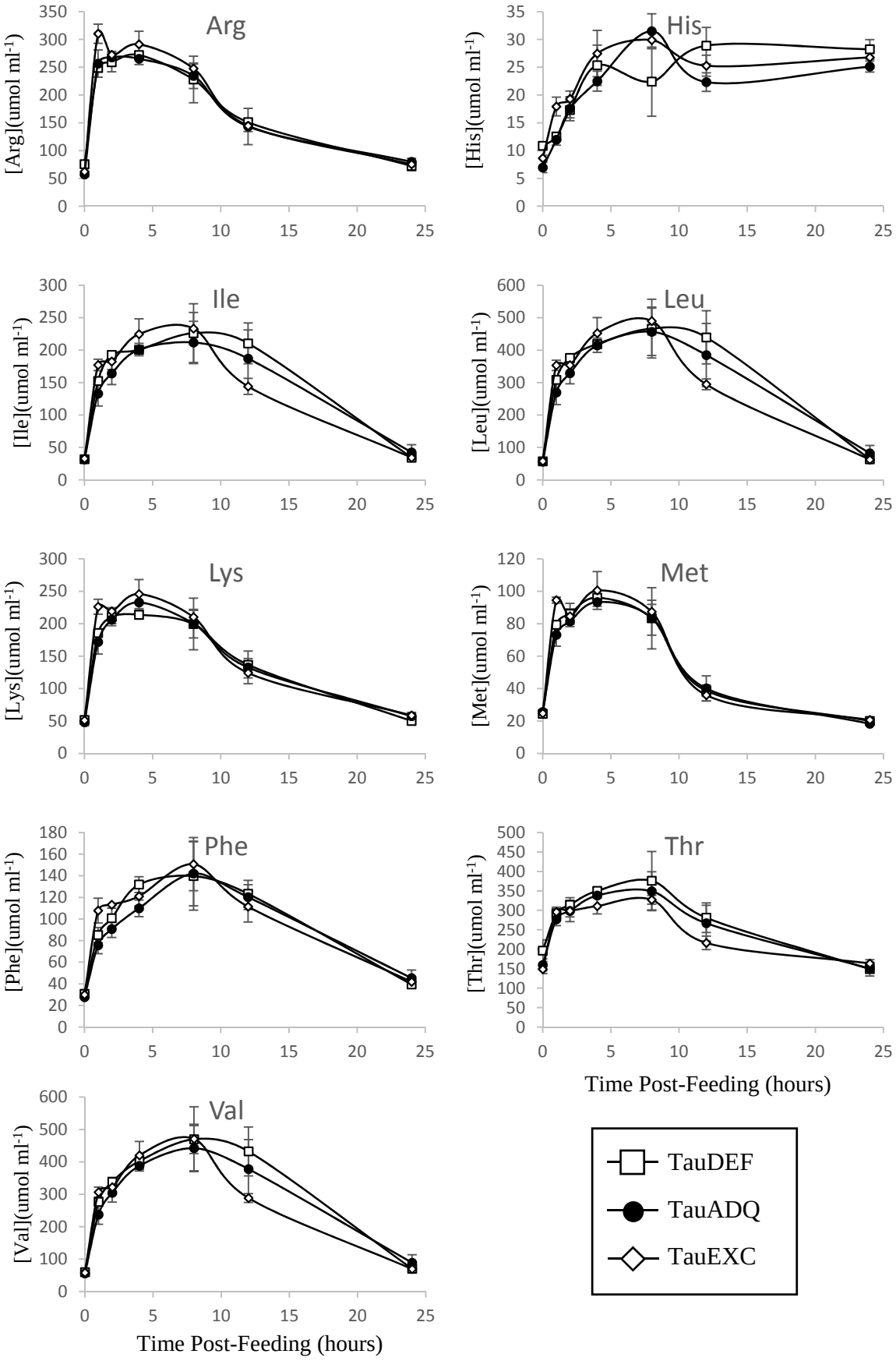

Figure 5.2. Concentrations of individual essential amino acids present in the plasma of juvenile barramundi over a 24 hour period following ingestion of a single meal containing either a deficient (TauDEF), adequate (TauADQ) or excessive (TauEXC) dietary taurine content.

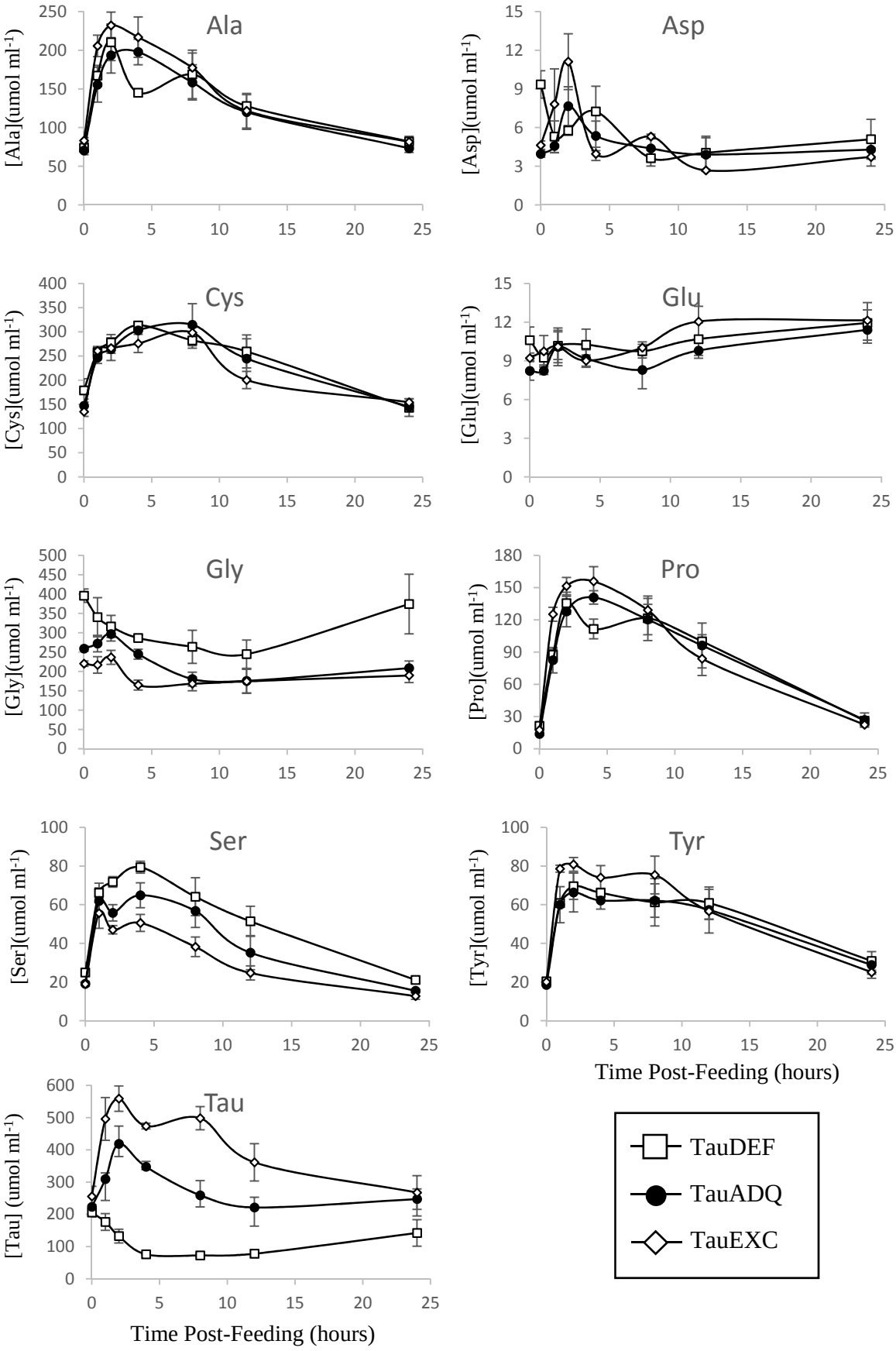

Figure 5.3. Concentrations of individual non-essential amino acids present in the plasma of juvenile barramundi over a 24 hour period following ingestion of a single meal containing either a deficient (TauDEF), adequate (TauADQ) or excessive (TauEXC) dietary taurine content.

5.4.2 Gene expression

The differential expression of the target genes was assessed in the hepatic tissue of fish sampled at two and four hours post-feeding (where peaks in plasma Tau; and a depression in plasma Tau and peak in plasma Met, respectively, were observed), as well as those taken as a pre-feeding control.

5.4.2.1 Met and Cys metabolism

The differential expression of two genes involved in the turnover of Met (methionine adenosyltransferase 1, MAT-1) and synthesis of Cys (cystathionine gamma-lyase, CGL) in response to variation in dietary Tau was investigated and is presented in Fig. 5.4. Expression of both MAT-1 and CGL was highly significantly affected by time ($p<0.001$; 0h<2h; 0h<4h; 2h=4h). No effect of treatment was observed.

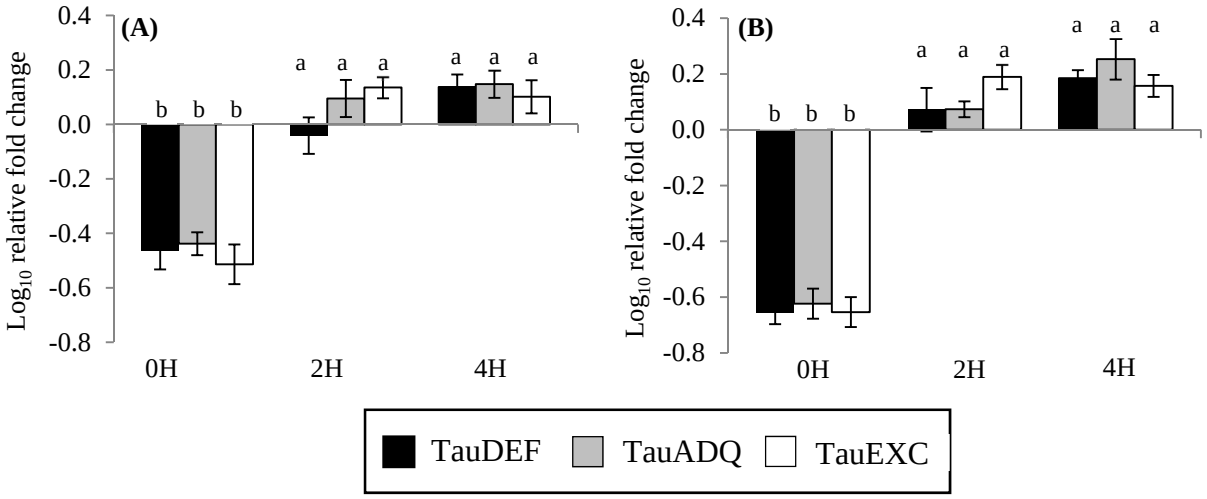

Figure 5.4. Transcript levels of selected genes (A, MAT-1; B, CGL) of methionine and cysteine metabolic pathways in the liver tissue of juvenile barramundi sampled after 24 hours starvation (0H); and two (2H) and four (4H) hours after ingestion of a single meal containing either a deficient (TauDEF), adequate (TauADQ) or excessive (TauEXC) dietary taurine content. Values were normalised to those of elongation factor 1α (Ef1α) before log10 transformation. Values presented are means (n=6) ±S.E. (represented by vertical bars). Significance analyses were performed by Two-Way ANOVA followed by *post-hoc* analysis of means by Tukey's honestly significant difference test. Columns with the same superscript letter are not significantly different. Letters are presented in order of magnitude from largest to smallest relative RQ. Gene abbreviations can be found in Table 5.2.

5.4.2.2 Hypotaurine biosynthesis

The effect of dietary Tau on the expression of genes of two hypotaurine biosynthetic pathways (cysteine dioxygenase, CDO; cysteamine dioxygenase, ADO; and cysteine sulphinic acid decarboxylase, CSAD) was observed (Fig. 5.5). CSAD expression was significantly affected by treatment ($p<0.05$; TauDEF>TauADQ; TauDEF>TauEXC; TauADQ=TauEXC) and highly significantly affected by time ($p<0.001$; 0h<2h<4h; 0h<4h) with a significant interactive effect between the two ($p<0.05$; TauEXC<TauDEF at the zero hour timepoint). ADO and CDO expression was similarly highly significantly affected by time ($p<0.001$; 0h<2h; 0h<4h; 2h=4h). Neither responded significantly to treatment, however, a significant treatment:time interactive effect ($p<0.05$) was observed for CDO expression.

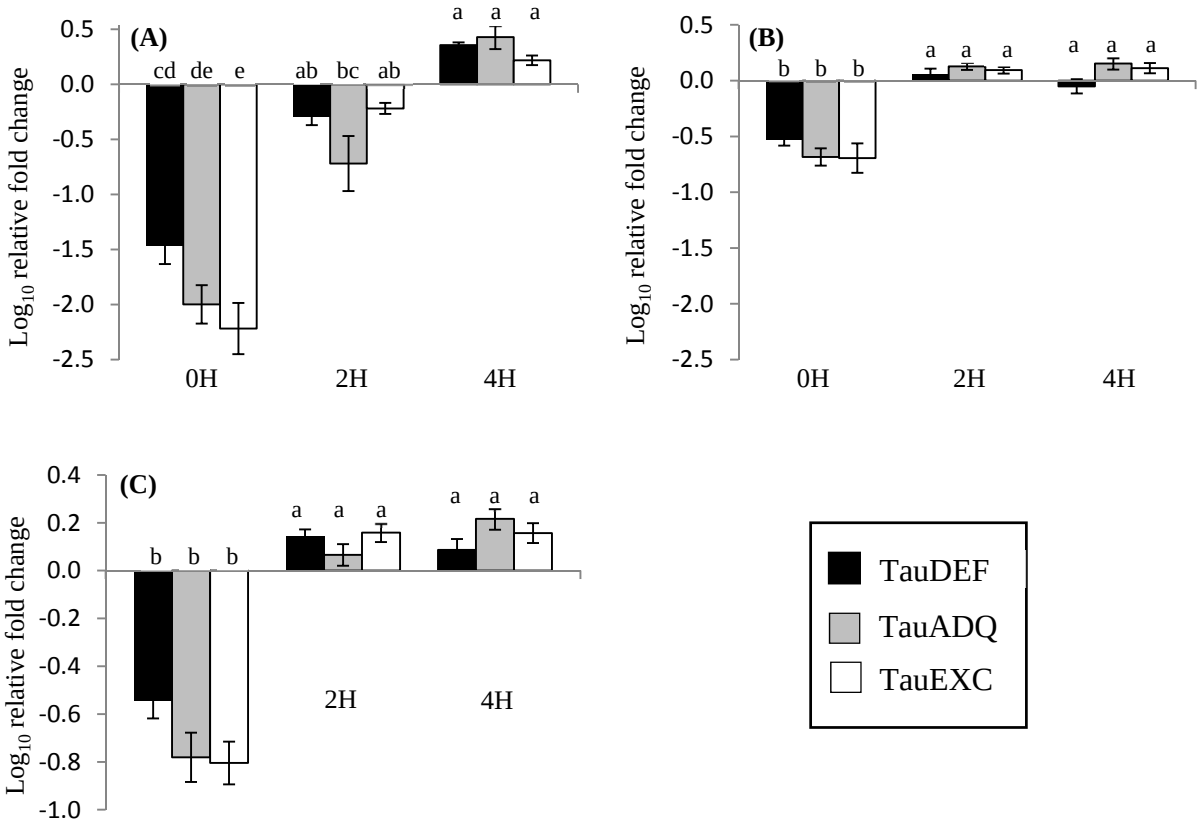

Figure 5.5. Transcript levels of selected genes (A, CSAD; B, ADO; C, CDO) of taurine biosynthetic pathways in the liver tissue of juvenile barramundi sampled after 24 hour starvation (0H); and two (2H) and four (4H) hours after ingestion of a single meal containing either a deficient (TauDEF), adequate (TauADQ) or excessive (TauEXC) dietary taurine content. Values were normalised to those of elongation factor 1α (Ef1α) before log10 transformation. Values presented are means (n=6) ±S.E. (represented by vertical bars). Significance analyses were performed by Two-Way ANOVA followed by *post-hoc* analysis of means by Tukey's honestly significant difference test. Columns with the same superscript letter are not significantly different. Letters are presented in order of magnitude from largest to smallest RQ. Gene abbreviations can be found in Table 5.2.

5.4.2.3 Taurine transport/homeostasis

The impact of dietary Tau on the expression of the taurine transporter gene (TauT), a regulator of the absorption and homeostasis of Tau was examined and is presented in Fig. 5.6. Expression of this gene was significantly influenced by the dietary treatment ($p<0.05$; TauEXC<TauDEF) and highly significantly affected by time after feeding ($p<0.001$; 0h<2h; 0h<4h; 2h=4h).

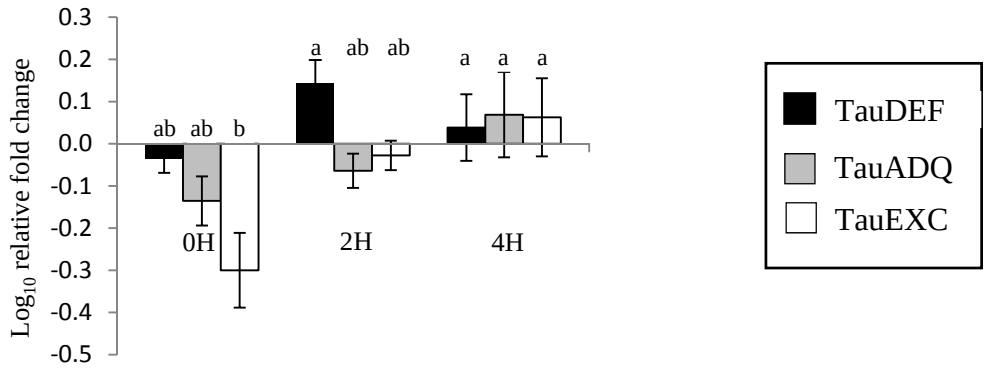

Figure 5.6. Transcript levels of the Taurine Transporter (TauT) gene in the liver tissue of juvenile barramundi sampled after 24 hour starvation (0H); and two (2H) and four (4H) hours after ingestion of a single meal containing either a deficient (TauDEF), adequate (TauADQ) or excessive (TauEXC) dietary taurine content. Values were normalised to those of elongation factor 1α (Ef1α) before log10 transformation. Values presented are means (n=6) ±S.E. (represented by vertical bars). Significance analyses were performed by Two-Way ANOVA followed by *post-hoc* analysis of means by Tukey's honestly significant difference test. Columns with the same superscript letter are not significantly different. Letters are presented in order of magnitude from largest to smallest RQ. Gene abbreviations can be found in Table 5.2.

5.4.2.4 Somatotropic axis

The expression of selected genes of the somatotropic axis (insulin growth factors I and II, IGF-I, IGF-II; and growth hormone receptor II, GHR-II) in response to variable dietary Tau over time after feeding is presented in Fig. 5.7. All examined genes of this group were highly significantly affected by diet ($p<0.001$; TauDEF<TauADQ>TauEXC; TauDEF=TauEXC). IGF-I and IGF-II expression were also highly significantly affected by time ($p<0.001$; 2h<0h>4h; 2h=4h). GHR-II was similarly highly affected by time after feeding but between all three timepoints ($p<0.001$; 0h>2h>4h; 0h>4h).

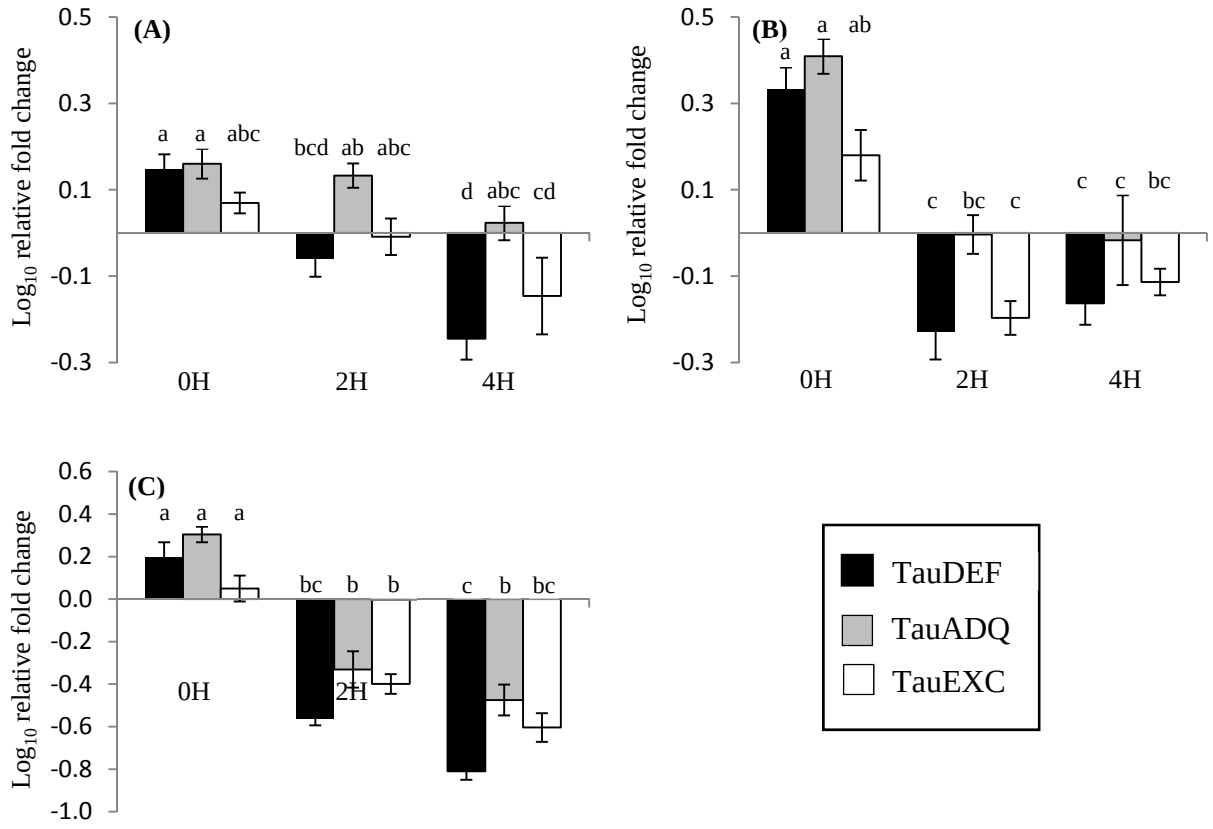

Figure 5.7. Transcript levels of selected genes (A, IGF-I; B, IGF-II; C, GHR-II) of the somatotropic axis in the liver tissue of juvenile barramundi sampled after 24 hours starvation (0H); and two (2H) and four (4H) hours after ingestion of a single meal containing either a deficient (TauDEF), adequate (TauADQ) or excessive (TauEXC) dietary taurine content. Values were normalised to those of elongation factor 1α (Ef1α) before log10 transformation. Values presented are means (n=6) ±S.E. (represented by vertical bars). Significance analyses were performed by Two-Way ANOVA followed by *post-hoc* analysis of means by Tukey's honestly significant difference test. Columns with the same superscript letter are not significantly different. Letters are presented in order of magnitude from largest to smallest RQ. Gene abbreviations can be found in Table 5.2.

5.4.2.5 Protein degradation

The effect of varying dietary Tau supply on the expression of two genes involved in the turnover of body protein was assessed (Fig. 5.8). The expression of mitochondrial ubiquitin ligase activator of NF-κβ-1 (Mul1) remained constant throughout the analysed period, with no significant differences in expression seen in the liver tissue of fish in the different treatments. Zinc finger AN1-type domain-5 (ZFAND-5) expression, however, was highly significantly affected by time ($p<0.001$; 0h>2h>4h; 0h>4h). No significant main effect of treatment was seen on expression of this gene ($p=0.0522$), however, the *post-hoc* analysis revealed a significant difference ($p<0.05$) between fish in the TauEXC treatment and those in the TauDEF and TauADQ treatments. This effect, highlighted by the highly significant treatment:time interactive effect ($p<0.001$) was observed to be only at the two hours post-feeding timepoint (TauEXC>TauADQ; TauEXC>TauDEF; TauDEF=TauADQ).

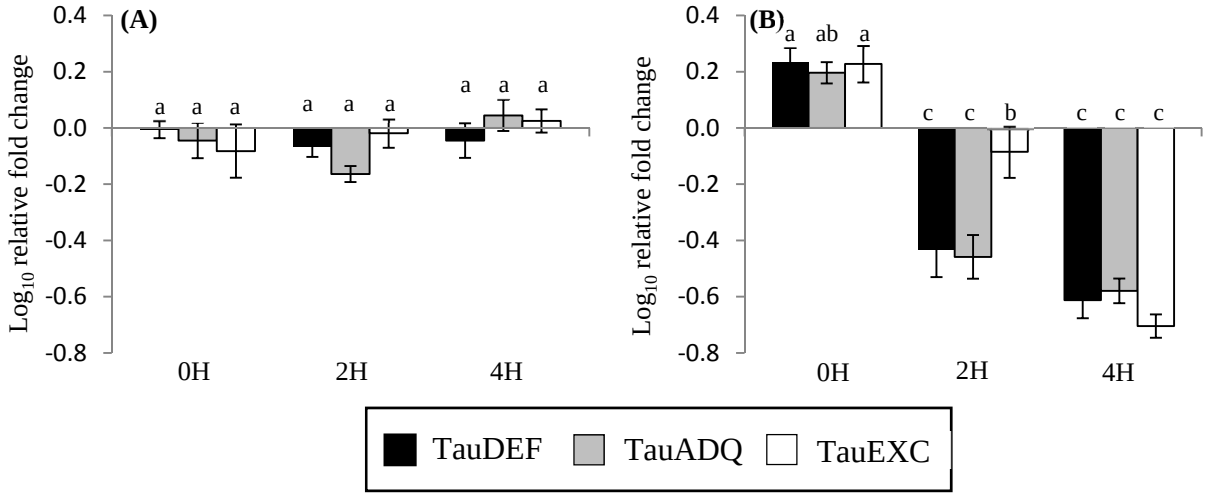

Figure 5.8. Transcript levels of selected genes (A, MUL-1; B, ZFAND-5) of proteolytic pathways in the liver tissue of juvenile barramundi sampled after 24 hours starvation (0H); and two (2H) and four (4H) hours after ingestion of a single meal containing either a deficient (TauDEF), adequate (TauADQ) or excessive (TauEXC) dietary taurine content. Values were normalised to those of elongation factor 1α (Ef1α) before log10 transformation. Values presented are means (n=6) ±S.E. (represented by vertical bars). Significance analyses were performed by Two-Way ANOVA followed by *post-hoc* analysis of means by Tukey's honestly significant difference test. Columns with the same superscript letter are not significantly different. Letters are presented in order of magnitude from largest to smallest RQ. Gene abbreviations can be found in Table 5.2.

5.4.2.6 TOR activity signalling (protein synthesis).

The expression of the gene Redd-1 (regulated in development and DNA damage response-1), a suppressor the target of rapamycin (TOR) pathway, thought to be a major driver of growth in fish, was used as an indicator of activity of this important pathway as suggested by Wacyk *et al.* (2012). The effect of Tau supply on the expression of this gene is presented in Fig. 5.9. Highly significant effects of both treatment ($p<0.001$; TauDEF>TauADQ; TauDEF>TauEXC; TauADQ<TauEXC) and time after feeding ($p<0.001$; 0h<2h>4h; 0h<4h) were observed.

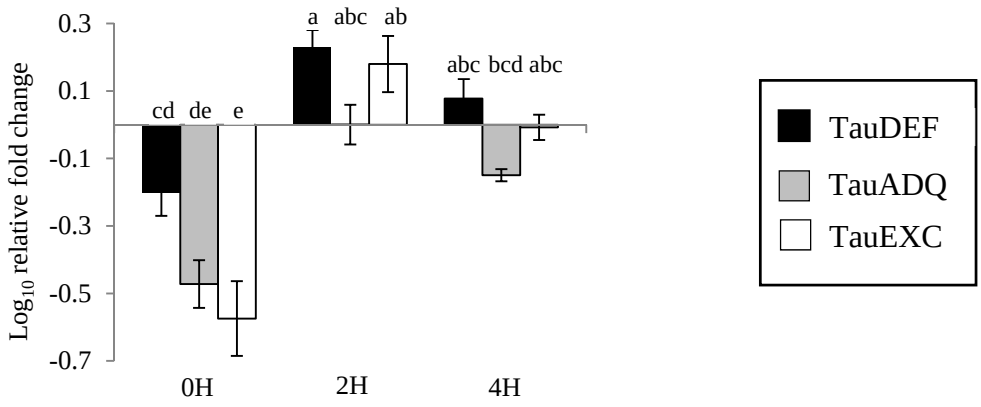

Figure 5.9. Transcript levels of Redd-1 (signifying inhibition of the TOR pathway) in the liver tissue of juvenile barramundi sampled after 24 hours starvation (0H); and two (2H) and four (4H) hours after ingestion of a single meal containing either a deficient (TauDEF), adequate (TauADQ) or excessive (TauEXC) dietary taurine content. Values were normalised to those of elongation factor 1α (Ef1α) before log10 transformation. Values presented are means (n=6) ±S.E. (represented by vertical bars). Significance analyses were performed by Two-Way ANOVA followed by *post-hoc* analysis of means by Tukey's honestly significant difference test. Columns with the same superscript letter are not significantly different. Letters are presented in order of magnitude from largest to smallest RQ. Gene abbreviations can be found in Table 5.2.

5.5 Discussion

Consideration of the inclusion of Tau in the diet of carnivorous fish species has become increasingly important in recent years with the increased use of Tau deficient plant meals to replace fishmeal as the primary source of protein (Watson *et al.*, 2015). A number of studies have been conducted to investigate the essentiality of this ingredient to various fish species (see review by Salze and Davis, 2015), with most observations limited to its influence on growth rate. Few studies, however, have endeavored to elucidate the underlying mechanisms behind this effect. Effects on the metabolism of Met, a precursor for Tau and the amino acid most commonly first limiting for protein synthesis in diets with high inclusion levels of non-cereal plant proteins (Jia *et al.*, 2013), for example, may have implications for supplementation of this amino acid. In addition, understanding the pathways through which Tau impacts growth (if at all), may allow substitution of alternative dietary components (i.e. other amino acids which may be more abundant in available ingredients) which exert the same effect on these pathways. The experiment assessing the impact of dietary Tau supply on growth of barramundi, reported in Chapter Four, observed a positive effect on growth at between 6 and 8g kg^{-1} inclusion. The aim of the present study was to investigate whether this effect was reflected in selected protein turnover and growth-related pathways previously linked to dietary amino acid supplementation. A secondary aim was to confirm that this species does possess the ability to synthesise Tau and to elucidate the mechanism by which it does this (if at all).

Whilst Tau is not a precursor for synthesis of other amino acids, the multitude of metabolic processes in which it has been reported to be involved in other animals suggests that its absence or excess in the diet could have an impact on the utilisation of other amino acids with which it interacts to effect these processes. Dietary Tau content, however, was observed to have little effect on the metabolism of other amino acids in the present study, consistent with the observations in Chapter Three, with reference to varying dietary Met content. It was thought that increased Tau supply in the diet may have had a sparing effect on Met, a proportion of which would otherwise have been processed for synthesis of Tau, and that this would be reflected in an increased presence, or more prolonged peak, of Met in the plasma. This, however, was not the case with no significant differences observed in Met levels at any timepoint. A primary peak in Met was observed at one hour post feeding in fish fed the TauEXC diet, before levels declined, then rose to a secondary peak at four hours post-feeding. Similar fluctuations were also apparent in the plasma EAAs arginine, lysine and, to a lesser extent, leucine levels. These amino acids were included at the highest concentrations in the EAA premix so this small initial peak may be a reflection of rapid uptake of free amino acids from the feed rather than an effect of Tau on metabolism of specific amino acids.

Alam *et al.* (2004) suggested that binding of crystalline amino acids within a matrix using casein-gelatin could assist in negating this differential absorption and this may have been advisable in this case. Interestingly, this response was also seen in the plasma levels of the NEAA serine (Ser) which was not supplemented in the diet, so the reason for this fluctuation is not known. Levels of Ser were also observed to decrease significantly in the plasma in response to increasing dietary Tau. As a precursor for Gly synthesis, it may be that increasing Gly supplementation (which was used to replace Tau in the TauDEF and TauADQ diets) spared Ser catabolism, increasing the circulating levels. The declining levels of Gly for the first 12 hours after feed consumption in the plasma of fish fed the TauDEF diet (which had the highest inclusion of Gly) was unexpected and there appears to be no logical explanation for it. The levels returned to pre-feeding concentrations by 24 hours post-feeding which seems to suggest that this response is cyclical, though there seems no reason for excess Gly or a deficiency in Tau to precipitate this response.

One of the fates of dietary Met may be the synthesis of Tau when it is limiting in the diet (as would be expected in the TauDEF treatment). It was anticipated that more sulphur amino acids (SAA) would be required to be turned over for biosynthesis of Tau in fish in the TauDEF treatment and that this would be reflected in an upregulation of MAT-1 (Met → SAM) and CGL (cystathionine → Cys) in those fish. These genes were significantly upregulated in the livers of fish in all treatments two hours after the meal and remained elevated after a further two hours, reflecting the changes in plasma Met and Cys level. What was unexpected was that MAT-1 appeared to be more highly expressed in the livers of fish in the TauADQ and TauEXC treatments than those fed the TauDEF diet at two hours post feeding. It has been suggested previously that excess circulating Tau, as seen in the plasma profiles of fish fed the TauEXC (and, to a lesser degree, TauADQ) diets reported in the present study, may spare dietary Met for other processes (Espe *et al.*, 2012), possibly the most important of which is the production of S-Adenosylmethionine (SAM). The increased expression of the Cys producing enzyme CGL at two hours post-feeding in fish fed the TauEXC diet and at four hours post-feeding in fish in the TauADQ treatment group, however, suggests the priority may be for a cysteine metabolite further along the Met metabolic pathway, possibly glutathione or pyruvate, which play important roles in protein and energy metabolism (Wang *et al.*, 1997; Lahnsteiner and Caberlotto, 2012). The synthesis of glutathione, specifically, has been suggested to be an important metabolic fate of Met (Li *et al.*, 2009). Even if this were the case in the present experiment, it would be expected that MAT-1 expression would remain constant, as any redirection of metabolites would be occurring further downstream. There is no evidence, therefore, that Tau had any sparing effect on Met requirement in the present study. Taurine has been shown to induce gastric acid secretion in rats (Huang *et al.*, 2011) so the resulting rapid digestion may have

hastened the availability of Met, although the similarity in plasma Met levels does not support this. The continual increase over time of expression of MAT-1 in the livers of fish fed the TauDEF diet points to the fate of this Met to likely be Tau synthesis. While the patterns in the data are discussed here, no significant differences were seen which may support the assertion in Chapter Three that expression of these genes is regulated primarily by feed intake, rather than by the dietary amino acid profile. MAT-1, for example, may be produced in such a volume as to convert a predetermined amount of Met to SAM. Whether this SAM is destined for Tau synthesis or production of some other metabolite may be dependent on other factors.

Similarly, it was expected that genes directly associated with the biosynthesis of Tau would be significantly affected by supplementation of Tau, in response to a reduced requirement for synthesis. Genes of the first taurine biosynthetic pathway showed significant upregulation at two hours post-feeding, presumably in response to increased availability of substrate for these enzymes. Expression of CDO, catalysing production of cysteine sulphinic acid from cysteine then plateaued while that of CSAD, possibly suggesting conversion of this compound to hypotaurine continued to increase significantly. Interestingly, the patterns of response of these two genes, as well as that of CGL, which is involved in the production of the Cys substrate for CDO, are quite similar, indicating the flow of metabolites along this pathway. CSAD expression appeared to be depressed by excessive Tau intake at four hours post-feeding, indicating a negative feedback on the regulation of expression of this gene by excess circulating Tau. An interesting pattern emerged in the comparison of ADO and CSAD expression, the enzymes which catalyse competing pathways for hypotaurine synthesis. The CSAD-catalaysed sulphinoalanine pathway is considered the primary pathway used by mammals (Schuller-Levis and Park, 2003) and has been assumed to be as such for teleost fish as well, although there has been some discussion that the cysteamine pathway incorporating ADO could also be significant (Salze and Davis, 2015). In the present study, both pathways appear to be stimulated by feed intake. ADO appeared to respond much faster than CSAD to feeding but remained at a lower level of expression with ADO expression levels peaking at two hours and CSAD at four hours post-feeding. The pattern of response provides further proof that this species does indeed possess the ability to synthesis Tau from SAA precursors and seems to support the CSAD-mediated pathway as being the more active. It could be argued, however, that both pathways play a role, depending on substrate availability. Quantification of the contribution of each enzyme/pathway to overall Tau biosynthesis through a combination of enzyme activity assays and more comprehensive metabolite profiling may add considerably to this assertion.

As might have been expected, taurine homeostasis was significantly affected by dietary taurine supply. TauT was most highly expressed in the livers of fish fed the TauDEF diet at the pre-

feeding and two-hour post-feeding timepoints, suggesting that the available taurine was in high demand in these animals, requiring dynamic transport. This difference, however, was not reflected in the plasma Tau contents which were not significantly different at the pre-feeding timepoint. It may be that *cellular* Tau, rather than *circulating* Tau, was limiting at this time, signalling production of TauT to transport Tau to areas in most demand. The significant downregulation of this gene in these fish at four hours post-feeding may be in response to the depletion of circulating Tau below a threshold for transport. This may be an indication that TauT expression is directly regulated by Tau availability in this species, as suggested in turbot (*Psetta maxima*) by Wang *et al.* (2016), and also illustrates that hepatic Tau is rapidly used up when dietary Tau is limiting, reflecting the patterns seen in the plasma profile.

Methionine, a precursor of Tau, has been shown in the past to significantly affect factors signalling the turnover of protein in other fish species (Belghit *et al.*, 2014), which directly or indirectly may impact overall growth stimulation. In this study, we were interested to know whether the apparent stimulatory effect of Tau inclusion on growth in this species reported in Chapter Four was related to interactions with these same pathways, whether directly through signalling activation of the pathway or cascade, or indirectly through sparing of Met for involvement as previously outlined. Expression of the proteolytic pathway gene ZFAND-5 was downregulated over time after the meal, being significantly higher pre-feeding than at two hours post-feeding and decreasing significantly again after a further two hours. This same pattern of response after feeding was also seen in the study reported in Chapter Three and is logical considering proteolysis would be less required as circulating amino acids become increasingly available. Expression of this gene declined over time in a slower fashion in the livers of fish in the TauEXC treatment, and was significantly more highly expressed in fish in this treatment, compared to those fish fed the TauDEF and TauADQ diets, at two hours post-feeding. This is unlikely to be related to fish size, as theorised Chapter Three, as fish in this treatment were slightly smaller than those in the TauADQ treatment. It may suggest a stimulatory effect of excessive taurine on protein degradation during the brief period where circulating Tau was at its peak, although no biological reason for this is apparent. Unexpectedly, expression of MUL-1 did not change significantly over time, in contrast to what was reported in Chapter Three. This may be a reflection of the continuous nature of protein turnover (Kaushik and Seiliez, 2010). While both this gene and ZFAND-5 are indicative of proteolysis, perhaps their respective pathways are differentially active and are stimulated or suppressed by separate stimuli.

Redd-1 expression was significantly lower in fish in the taurine adequate treatment than those in the deficient treatment at all sampling points, signalling a reduction in the suppression of

TOR, and possibly suggesting a link between adequate supply of taurine and TOR pathway-mediated growth stimulation. It's not clear why expression of this gene in all treatments increased after feeding when it might be expected that nutrient availability would stimulate growth-related pathways such as mTOR. Similarly to ZFAND-5 expression, significant upregulation of this gene in fish fed the taurine excess diet (TauEXC), in comparison to those fed the adequate diet (TauADQ), at both two and four hours after feeding suggests a depressive effect of taurine on growth at high levels of inclusion. Alternatively, any effect of Tau supplementation on this pathway may have been concealed by an interaction with circulating insulin, a factor not measured in this study. This relationship between amino acid supply and insulin level on the activation of the TOR cascade has been documented in other species (Lansard *et al.*, 2010).

Variations in the dietary inclusion of other amino acids have similarly been shown to affect the expression of genes associated with growth stimulation and initiation in fish. Expression of components of the somatotropic axis, for example, were reported to be impacted by dietary Met inclusion by Rolland *et al.* (2015) and by dietary Lys inclusion in the study of Hevrøy *et al.* (2007). In the present study, the patterns of response of IGF-I, IGF-II and GHR-II were similar within each timepoint. Fish in the TauADQ treatment exhibited the highest relative expression of each gene at all sampling points, confirming the positive effect of adequate Tau supply on growth processes in this species, as also supported by the reduced Redd-1 expression. Before feed was consumed, the lowest level of expression of these genes was seen in the excessive taurine treatment (TauEXC). After feeding, however, expression was consistently lowest in those fish fed the TauDEF diet. This trend is consistent with the pattern of growth observed in the experiment reported in Chapter Four, reaffirming the connection between growth and expression of genes of the somatotropic axis. The depression in expression of these growth-related genes in response to excessive Tau supply in the diet reflects the upregulation of proteolytic and TOR-suppression markers described above, possibly further signalling that excessive Tau is not conducive to optimal growth. Whether this effect is due to direct action of Tau on stimulating or suppressing expression of these genes or is simply a reflection of differential growth regulated through alternative pathways remains unclear.

Conclusion

Tau appears to have a positive influence on several markers of growth or growth stimulation in barramundi. Specifically, adequate Tau may reduce the expression of the TOR suppressing gene Redd-1 and increase the expression of genes of the somatotropic axis. Quantification of the capacity of a species to synthesise Tau from precursor SAA is important in defining the essentiality of Tau supplementation in the diet. This study has confirmed that barramundi possess this

capability and showed that they may be able to effect this through at least two different biosynthetic pathways, with both CSAD and ADO expression responding to feed consumption. Transport of taurine within the animal was also suggested to be directly regulated by Tau availability. It appears, then, that the growth stimulating effects of Tau which were proposed, though not significant, in Chapter Four may have been more pronounced in the absence of the restricted pair-feeding regime, identified as a possible confounding factor in limiting the magnitude of growth divergence of fish in that study. While Tau may not be required in the diet *per se*, supplementation may be advantageous where sufficient SAA precursor supply is limiting.

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Chapter 6 - Allometric scaling exponents for individual amino acids differ significantly from that of protein in juvenile barramundi (*Lates calcarifer*).

6.1 Abstract

An experiment was conducted to assess the effect of varying fish size on the losses of protein and individual amino acids during a period of starvation in barramundi (*Lates calcarifer*). Fifteen fish within each of six size classes (Group A, 10.5 ± 0.13g; Group B, 19.2 ± 0.11g; Group C, 28.3 ± 0.05g; Group D, 122.4 ± 0.10g; Group E, 217.6 ± 0.36g; Group F, 443.7 ± 1.48g; mean ± SD) were housed in triplicate tanks and deprived of food for a period of 21 days. Losses in protein and individual amino acids over this period, relative to the geometric mean weight of the animals, were used to calculate the allometric scaling exponents for each of these parameters. The exponent value derived for loss of protein (0.693 ±0.0029) compared well with previous estimates in various species. Live weight exponents for the majority of the proteinaceous amino acids differed significantly from that of protein, with only those calculated for the branched chain amino acids and cysteine being lower. No significant difference was seen in the exponent values for losses of the sum of essential, compared with non-essential, amino acids, however the exponent value for losses of the sum of all proteinaceous amino acids was significantly higher than that of protein, suggesting an influence of fish size on utilisation of non-protein nitrogen. Metabolic requirements for methionine for maintenance and growth in barramundi were calculated based on data from two previous dose response experiments. The impact on these predictions of using the methionine weight exponent derived in the present study, compared to the traditionally used protein weight exponent, in these calculations was assessed. A requirement for weight gain was predicted to be the major driver of utilisation of this amino acid in smaller fish, while proportionally more methionine may be used for maintenance in larger fish. It was concluded that the use of a methionine specific weight exponent may correct for changes in Met requirements in barramundi more accurately than use of the protein weight exponent commonly used in feed utilisation modelling.

6.2 Introduction

Developing reliable models to describe nutrient demands and utilization by fish species and extrapolating these for prediction of growth in response to variable nutrient supply is an important step in allowing feed formulators to optimise nutrient supply. This may have impacts both economically, in terms of reducing wastage at the manufacturing and feeding level, and environmentally, through the reduction of waste outputs from aquaculture operations, an important consideration in maintaining the social licence of the aquaculture industry to continue its expansion.

A number of such models of varying complexity have been proposed for various fish species over the years (Machiels and Henken, 1987; Cho and Bureau, 1998; Dumas *et al.*, 2007; Pirozzi *et al.*, 2010). Some of these have been iteratively developed to incorporate additional functions or adapted for various species (e.g. Lupatsch *et al.*, 1998; Lupatsch and Kissil, 2005; Glencross, 2008; Glencross and Bermudes, 2012). These models have primarily focused on the demands for the macronutrients protein and lipid as well as energy. In order to optimise these models further, it may be necessary to characterize the impact individual components of these macronutrients have on the overall requirements of the animal. This may be especially pertinent for the amino acids given their roles in numerous metabolic processes and the impact the balance of their profile can have on protein metabolism (Aragão, 2004) and, thus the growth and development of the animal. Rollin *et al.* (2003) suggested that fish may utilise dietary amino acids at differing efficiencies. Considering individual dietary amino acid utilisation to be proportional to that of crude protein, therefore, may be problematic when the amino acid profile of the protein used differs from that used to establish the model components relating to protein utilisation. A recent focus on the amino acid requirements of fish, particularly carnivorous species, has come about due to a need for the aquafeed industry to reduce its reliance on fishmeal and concomitant increases in the use of plant proteins, which often have amino acid profiles imbalanced with that required by the animal (Espe *et al.*, 2012). It has become necessary, therefore, to properly characterise the true requirements for these dietary components, particularly those such as Met which are abundant in fishmeal but limiting in many plant meals, in order to ensure dietary formulations containing high levels of these ingredients meet or exceed minimum requirements. As a result, there is an increasing body of information relating to the amino acid requirements of a range of fish species (see summary in NRC, 2011). What is less understood, however, is how these demands change with animal size. While essential amino acid requirements have been defined for certain species separately at different lifestages (the requirement of rainbow trout (*Oncorhynchus mykiss*) for lysine, for example, has been studied in fish ranging from 5g (Walton *et al.*, 1984) to 51g (Rodehutscord *et al.*, 1997) starting weight) only one attempt at modelling this mathematically has been published to our knowledge, published in

NRC (2011) utilizing the "hybrid nutrient-flow bioenergetics model" of Bureau and Hua (2006). Further work is clearly needed to properly characterise this relationship.

Relative protein demands are known to decrease with increasing fish size (Wilson, 2002), likely due to a decreased rate of protein synthesis and turnover as the specific rate of growth of the animal decreases (Houlihan *et al.*, 1986). This phenomenon has been accounted for in several fish feed utilisation models (Lupatsch *et al.*, 2003; Glencross, 2008; Booth *et al.*, 2010; Van Tien *et al.*, 2016) through the use of a body weight exponent for protein. While experimentally-derived values for this exponent can vary marginally (Beck and Gropp, 1995; Van Tien *et al.*, 2016), the generally accepted value of 0.70 is widely used (Lupatsch *et al.*, 1998; Helland *et al.*, 2010; Glencross and Bermudes, 2012).

This exponent, describing the relationship between protein loss over a period of starvation and the live weight of the animal, is then used to scale protein requirements for maintenance and maximum growth at any given weight for incorporation into equations underpinning feed utilisation models such as that of Lupatsch and Kissil (2005) :

$$\mathrm{DP_{maint}}\ (\mathrm{g\ fish^{-1}\ day^{-1}}) = a * \mathrm{BW(kg)^{\mathbf{0.70}}}$$

$$\mathrm{DP_{req}}\ (\mathrm{g\ fish^{-1}\ day^{-1}}) = \mathrm{a} * \mathrm{BW(kg)^{\mathbf{0.70}}} + b * \mathrm{protein\ gain}$$

Where DP_{maint} is the digestible protein required for maintenance and DP_{req} is the total digestible protein required for maintenance and growth. *a* is a temperature-dependent coefficient and *b* is the cost in units of DP to deposit one unit of protein as growth (Glencross and Bermudes, 2010; Glencross and Bermudes, 2011).

Whether this can be extended to that of individual amino acids remains unknown. Accounting for the effect of growth on the basal, maintenance and growth requirements for individual amino acids and incorporation of this information into current nutritional models would improve the accuracy of predictions made when formulating diets containing novel sources of protein.

One model already developed for barramundi (*Lates calcarifer*) is that of Glencross (2008), which was further refined to incorporate the effects of water temperature by Glencross and Bermudes (2012). This model, like many before it, assessed mainly crude protein, lipid and energy utilisation by this species. A recent study by Salini *et al.* (2016) examined the allometric scaling effect of specific fatty acids in this species, allowing application of the Glencross (2008) model to the determination of metabolic demands of these fatty acids for maintenance and growth. The aim of the present study, therefore, was to extend this further and provide estimates of the relationship

between fish size and the inevitable losses of individual amino acids in this species in order to allow further optimisation of that model.

6.3 Materials and Methods

6.3.1 Fish Management

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 number: A3/2015) and The University of Queensland Animal Ethics Committee (approval number: QAAFI/422/15/CSIRO).

Juvenile barramundi (*Lates calcarifer*) were procured from the BettaBarra fish hatchery (Atherton, QLD, Australia) and on-grown at the Commonwealth Scientific and Industrial Research Organisation's (CSIRO) Bribie Island Aquaculture Research Facility for a period of approximately four months. During this period, fish were fed to satiety with commercial barramundi feed (Ridley Marine Float; Ridley Aquafeeds) and graded periodically in order to reduce cannibalism and to separate broad size classes. A second batch of juvenile fish was sourced from the same hatchery two weeks prior to the commencement of the experiment and treated as per the previous batch. These fish were graded regularly over the following two weeks to isolate the smaller size classes.

On commencement of the experiment, forty fish from each pool tank were individually weighed to obtain a size group average, from which six specific weight classes were established. These groups (± S.E.M.) were as follows: Group A, 10.5 (± 0.13)g; Group B, 19.2 (± 0.11)g; Group C, 28.3 (± 0.05)g; Group D, 122.4 (± 0.10)g; Group E, 217.6 (± 0.36)g and Group F, 443.7 (± 1.48)g. Fifteen fish within each weight group were then randomly allocated to each of three replicate tanks per group. All fish were anaesthetised with a light dose of AQUI-S (~0.02mL/L) (AQUI-S New Zealand Ltd) and individually weighed before being allowed to recover in their allocated tanks.

The experimental tanks (600L capacity) were supplied with seawater at a rate of 3L min^{-1}. Water temperature was maintained at 30.0 (±0.2) ^{0}C with a dissolved oxygen content of 6.6 (±0.3) mg L^{-1}, and the system maintained under a photoperiod of 12h light:12h dark using artificial lighting. During the experimental period (21 days), the fish remained unfed.

6.3.2 Chemical analyses

At the commencement of the experimental period, five fish from each weight group, having remained unfed for the preceding 24 hours, were euthanised by overdose of anaesthetic and stored at -20^{0}C until required for proximate analyses. Additionally, five fish from each experimental tank

were sampled at the conclusion of the experiment and were treated in the same manner. Whole fish within each weight group were pooled and homogenised by commercial meat mincer (MGT-012, Taiwan). Samples of this mince were then taken for whole-fish dry matter analysis while a random proportion of the remaining homogenate was freeze-dried (Alpha 1-4, Martin Christ, Germany) for further analyses.

Carcass dry matter contents were determined by mass change after 24 hours of drying at 105^0C. Similarly, gross ash contents were determined gravimetrically following combustion in a muffle furnace for 24 hours at 550^0C. Total lipid contents were determined by extraction of the lipid portion of the samples according the method of Folch *et al.* (1957). Measurement of total nitrogen content was undertaken using organic elemental analysis (CHNS-O Flash 2000, Thermo Scientific, USA) and sample protein content calculated based on *N* x 6.25. Determination of gross energy by isoperibolic bomb calorimetry was performed on 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 on a Shimadzu Nexera X2 series UHPLC (Shimadzu Corporation, Kyoto, Japan) coupled with a Shimadzu 8030 Mass Spectrometer following pre-column derivatisation with 6-aminoquinolyl-N-hydroxysuccinimidyl (AQC) using the Waters AccQ·tag system (Waters Corporation, Milford, MA). Proteins were hydrolysed and prepared for analysis according to the protocol for complex feed samples of Waters Corp. (1996) following hydrochloric acid hydrolysis. Cysteine (Cys) and methionine (Met) contents were determined independently as described by Poppi *et al.* (2017).

6.3.3 Assessment of protein and amino acid loss

Losses of crude protein across all size groups after the starvation period were assessed according the formula reported by Glencross and Bermudes (2011):

$$\text{Protein loss (mg fish}^{-1}\text{ day}^{-1}) = \frac{\text{Wi} * \text{Pi} - \text{Wf} * \text{Pf}}{\text{t}}$$

Where the Wi and Wf are the average initial and final weights, respectively, of fish in each replicate tank. Pi represents the protein content of the pooled initial (pre-starvation) carcass samples of each weight group and Pf the replicate tank average final protein content of the whole fish on a live-weight basis. The observation period is denoted as t. Individual essential and non-essential amino

acid losses were calculated in the same fashion by substitution of the applicable Pi and Pf values with the equivalent values for the respective amino acids.

6.3.4 Methionine utilisation efficiency assessment

Marginal efficiencies of Met utilisation (EU) were calculated for a soy protein concentrate-based diet in a published barramundi total sulphur amino acid (TSAA) requirement study (Poppi *et al.*, 2017; Chapter Two). Only data from Experiment One of that study was evaluated as insufficient Met intake data points preceding the observed growth plateau in Experiment Two were available for application of the linear relationship required for calculation of the marginal efficiency. Regression of the mass-independent (marginal) Met gain as a function of the mass-independent (marginal) digestible Met intake was used to determine the marginal efficiency constant (K_{Met}), taken as the slope of the linear relationship (Rodehutscord *et al.*, 2000). The marginal Met gain was calculated using the following formula:

$$\text{Marginal gain} = \text{Gain}_{\text{Met}} / \text{GMW}^{x} / \text{t}$$

Where: Gain_{Met} is the Met gained (g $fish^{-1}$), GMW is the geometric mean weight of the fish ($(\text{Weight}_{initial} - \text{Weight}_{final})^{0.5}$), *x* is either the Met loss (0.81) or protein loss (0.70) exponent derived in this chapter and t is the study period duration (Glencross and Bermudes, 2011). Calculations of marginal intake were undertaken in a similar fashion with intake of Met replacing gain of Met in the above equation.

A comparison was made between the calculations using either the Met (MetBW) or protein (PBW) exponent-transformed GMW to examine the effect of the exponent value on estimates of Met EU. Met utilisation efficiencies calculated in a published barramundi Met utilisation study (Poppi and Glencross, 2014) on a PBW basis were also recalculated using MetBW.

6.3.5 Iterative determination of metabolic demands for methionine

The marginal efficiency constant (K_{Met}) was applied to the calculation of maintenance, growth and total Met requirements of barramundi of varying size, based on data from two earlier studies (Poppi and Glencross, 2014; Poppi *et al.*, 2017). Predicted losses of Met from barramundi of 50, 100, 500, 1000 and 2000g were calculated based on the power functions derived from Met loss (the components of which are presented in Table 6.4). Extrapolation of the marginal efficiency slope from these predicted losses (the *y* intercept) to the point of zero Met loss or

retention (the *x* intercept), yielded estimates of maintenance requirements for Met for each dietary formulation.

Methionine gain was estimated as a function of its quantity in the whole body with reference to the daily growth of the fish predicted by the barramundi growth equation of Glencross (2008):

$$\text{Gain (g/fish/d)} = (\text{K} + x\text{T} + y\text{T}^2 + z\text{T}^3) * (\text{weight})^{ax+b}$$

Where: K and *b* are constants and *x*, *y*, *z* and *a* are defined coefficients of the growth response model. *T* is the temperature within an operating range of 16 to 39°C and weight is the GMW of the fish (g $fish^{-1}$). The requirement for Met for growth was determined as Met gained in relation to its utilisation efficiency (Salini *et al.*, 2015).

The total Met demand, then was considered to be the sum of the requirements for maintenance and growth.

6.3.6 Statistical analysis

All values are presented as mean ± standard error of the mean (SEM). Losses of protein and individual amino acids (mg $fish^{-1}$ day^{-1}), relative to the geometric mean weight (g $fish^{-1}$), within each replicate tank were examined using power functions ($y=aX^b$) in Microsoft Excel (Microsoft Office 2013). Plotting of these relationships yielded R^2 values used to assess the goodness of fit of the model. A bootstrapping approach was further employed to generate replicate coefficient (slope) and exponent values for each relationship. This approach, involving manually removing each replicate individually and assessing the effect on the parameters of interest has previously been employed in studies of this type (Glencross and Bermudes, 2011; Salini *et al.*, 2016) and was assessed, in a cross-validation of selected parameters (data not presented), to produce standard errors identical to those derived by an alternate method used by Lupatsch *et al.* (1998). Protein and individual amino acid coefficients were analysed by One-Way ANOVA with *post-hoc* comparison of group means by Tukey's HSD multiple range test. All statistical analyses were performed in the R-project statistical environment (R Core Team, 2014) with effects considered significant at an alpha level of 0.05.

6.4 Results

6.4.1 Carcass composition

Gross compositions of fish at the commencement and conclusion of the starvation period and indicators of fish performance are presented in Table 6.1. Weight losses as a percentage of initial weight decreased with increasing fish size, ranging from 12.9% to 5.3%. Protein, energy and lipid density were decreased in starved fish whilst carcass ash contents were observed to have increased. Condition factor decreased across all groups after starvation. Survival was 100% in all groups.

Initial and final carcass protein contents were best described by power functions (R^2 = 0.941 and 0.911 respectively; Fig. 6.1).

Similarly, the relationships between fish size and individual amino acid density in both the pre- and post-starvation fish were best fitted to power functions, with increases in all proteinogenic amino acids observed in response to increasing fish size (Table 6.2 and Table 6.3). Carcass taurine content was highly variable but followed the same general pattern in relation to fish weight before and after starvation.

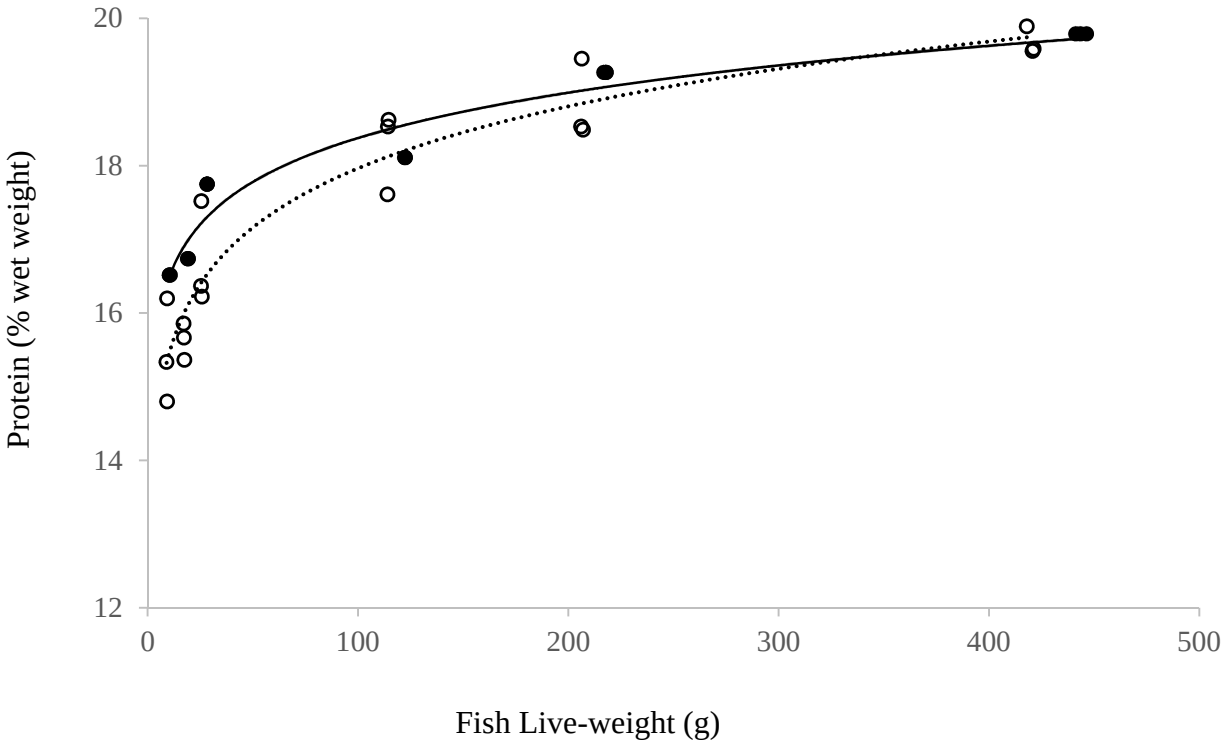

Figure 6.1. Protein density of barramundi of varying live-weight pre- (● = $14.761x^{0.0476}$, R^2 = 0.941) and post- (○ = $13.257x^{0.066}$, R^2 = 0.911) feed deprivation for 21 days.

Table 6.1. Performance indicators and proximate composition of barramundi within each size class before and after feed deprivation for 21 days. Figures are presented as ±SEM where appropriate.

	Group A	Group B	Group C	Group D	Group E	Group F
Initial composition (g kg^{-1} as is unless otherwise indicated)						
Dry matter	244.1	243.9	272.4	271.9	296.7	308.5
Protein	165.2	167.4	177.5	181.1	192.6	197.9
Ash	39.4	39.2	37.5	31.5	41.4	49.2
Lipid	39.2	40.7	61.4	64.4	73.3	64.8
Gross energy (MJ kg^{-1})	5.2	5.3	6.3	6.6	7.1	7.0
SumAA[a]	120.6	124.3	130.0	138.4	144.2	156.0
NPN (%)[b]	27.0	25.7	26.7	23.6	25.1	21.2
Final composition (g kg^{-1} as is unless otherwise indicated)						
Dry matter	210.4 ±0.4	220.2 ±0.1	255.6 ±0.3	273.6 ±0.3	281.0 ±0.3	295.8 ±0.2
Protein	149.7 ±0.2	156.3 ±0.1	167.0 ±0.4	182.5 ±0.3	188.2 ±0.3	196.8 ±0.1
Ash	45.0 ±0.0	45.4 ±0.3	42.2 ±0.1	37.6 ±0.1	42.4 ±0.1	55.0 ±0.1
Lipid	15.9 ±0.2	19.6 ±0.1	43.7 ±0.1	54.2 ±0.2	53.1 ±0.3	51.8 ±0.1
Gross energy (MJ kg^{-1})	4.2 ±0.1	4.5 ±0.1	5.5 ±0.1	6.4 ±0.1	6.5 ±0.1	6.5 ±0.1
SumAA[a]	111.2±1.5	117.0±2.9	124.8±0.3	137.4±1.6	140.2±2.0	146.0±0.3
NPN (%)[b]	27.8±0.0	25.1±0.0	25.2±0.0	24.7±0.0	25.5±0.0	25.8±0.0
Performance indicators(g fish^{-1} as is unless otherwise indicated)						
Initial weight	10.5 ±0.1	19.2 ±0.1	28.3 ±0.1	122.4 ±0.1	217.6 ±0.4	443.7 ±1.5
Final weight	9.2 ±0.1	17.3 ±0.1	25.6 ±0.1	114.3 ±0.1	206.5 ±0.3	420.0 ±1.0
Weight loss	1.4 ±0.1	1.9 ±0.0	2.7 ±0.2	8.1 ±0.2	11.1 ±0.2	23.7 ±1.6
Weight loss (%)	12.9 ±0.7	9.9 ±0.1	9.6 ±0.5	6.6 ±0.2	5.1 ±0.1	5.3 ±0.3
Condition factor initial[c]	1.2 ±0.0	1.2 ±0.0	1.3 ±0.1	1.2 ±0.0	1.2 ±0.1	1.2 ±0.0
Condition factor final[c]	1.1 ±0.0	1.0 ±0.0	1.1 ±0.1	1.1 ±0.0	1.1 ±0.1	1.1 ±0.0
Survival (%)	100	100	100	100	100	100

[a] Sum of all proteinaceous amino acids (minus Trp)

[b] Proportion of total nitrogen comprised of non-protein nitrogen (derived from difference between SumAA and Protein).

[c] Condition factor = (average fish weight (g fish^{-1}) /average fish length(cm)^3)*100

Table 6.2. Whole body proteinaceous amino acid (+Tau and minus Trp) composition (g kg^{-1} live weight) of barramundi at the commencement of the experiment. Data across size classes were fitted to power functions ($y = a * X^b$) with function components presented as means ± SEM.

	Group A	Group B	Group C	Group D	Group E	Group F	Equation	R^2
EAAs								
His	2.46	2.55	2.46	2.59	2.84	3.20	$y = 2.068(\pm0.0040)x^{0.062(\pm0.0005)}$	0.777
Arg	9.06	9.28	9.89	10.57	11.47	13.14	$y = 7.161(\pm0.0096)x^{0.092(\pm0.0004)}$	0.938
Thr	5.55	5.73	5.96	6.29	6.41	6.92	$y = 4.907(\pm0.0027)x^{0.054(\pm0.0002)}$	0.966
Val	5.66	5.75	6.02	6.30	6.36	6.54	$y = 5.203(\pm0.0024)x^{0.038(\pm0.0001)}$	0.962
Met	3.60	3.68	3.95	4.27	4.32	4.65	$y = 3.092(\pm0.0021)x^{0.066(\pm0.0001)}$	0.969
Lys	9.90	10.34	11.18	12.49	12.83	13.64	$y = 8.179(\pm0.0058)x^{0.085(\pm0.0001)}$	0.982
Ile	8.94	9.23	9.51	10.16	10.14	10.41	$y = 8.219(\pm0.0039)x^{0.040(\pm0.0001)}$	0.961
Leu	5.08	5.25	5.43	5.82	5.75	5.84	$y = 4.723(\pm0.0032)x^{0.037(\pm0.0002)}$	0.908
Phe	5.23	5.43	5.70	6.02	5.99	6.26	$y = 4.790(\pm0.0036)x^{0.044(\pm0.0001)}$	0.935
NEAAs								
Asp	11.72	12.24	13.04	13.22	13.74	14.03	$y = 10.810(\pm0.0111)x^{0.044(\pm0.0002)}$	0.901
Ser	5.75	5.86	6.17	6.54	6.55	7.07	$y = 5.106(\pm0.0031)x^{0.051(\pm0.0002)}$	0.954
Glu	14.63	15.30	16.32	17.40	18.15	19.82	$y = 12.356(\pm0.0088)x^{0.075(\pm0.0002)}$	0.972
Gly	12.48	12.78	12.87	14.25	15.59	18.45	$y = 9.558(\pm0.0205)x^{0.096(\pm0.0006)}$	0.984
Ala	7.77	7.90	8.32	8.67	9.38	10.04	$y = 6.526(\pm0.0557)x^{0.066(\pm0.0002)}$	0.948
Pro	7.41	7.62	7.78	8.08	9.16	10.13	$y = 6.037(\pm0.0083)x^{0.077(\pm0.0004)}$	0.877
Cys	1.11	1.08	1.16	1.15	1.13	1.16	$y = 1.080(\pm0.0010)x^{0.011(\pm0.0002)}$	0.380
Tyr	4.21	4.24	4.27	4.64	4.41	4.73	$y = 3.901(\pm0.0021)x^{0.030(\pm0.0002)}$	0.792
Tau	1.06	0.98	1.09	0.61	0.59	0.82	$y = 1.465(\pm0.0094)x^{-0.135(\pm0.0019)}$	0.559

Table 6.3. Whole body proteinaceous amino acid (+Tau and minus Trp) composition (g kg^{-1} live weight) of barramundi after 21 days feed deprivation. Data across size classes were fitted to power functions ($y = a * X^b$) with function components presented as means ± SEM.

	Group A	Group B	Group C	Group D	Group E	Group F	Equation	R^2
EAAs								
His	1.90 ±0.03	2.06 ±0.08	2.21 ±0.09	2.48 ±0.06	2.57 ±0.03	2.65 ±0.13	$y = 1.618(\pm0.0043)x^{0.085(\pm0.0007)}$	0.834
Arg	7.40 ±0.12	8.11 ±0.28	8.96 ±0.45	9.68 ±0.53	9.99 ±0.45	10.78 ±0.60	$y = 6.320(\pm0.0189)x^{0.089(\pm0.0008)}$	0.752
Thr	5.07 ±0.08	5.37 ±0.19	5.63 ±0.07	6.25 ±0.07	6.36 ±0.09	6.54 ±0.07	$y = 4.453(\pm0.0070)x^{0.067(\pm0.0003)}$	0.900
Val	5.42 ±0.07	5.76 ±0.20	6.07 ±0.17	6.59 ±0.10	6.69 ±0.15	6.89 ±0.14	$y = 4.846(\pm0.0087)x^{0.061(\pm0.0004)}$	0.837
Met	3.34 ±0.08	3.46 ±0.04	3.76 ±0.10	4.26 ±0.02	4.42 ±0.11	4.39 ±0.05	$y = 2.826(\pm0.0053)x^{0.080(\pm0.0005)}$	0.889
Lys	8.33 ±0.07	8.93 ±0.21	9.74 ±0.20	10.89 ±0.23	11.08 ±0.08	11.31 ±0.25	$y = 7.192(\pm0.0134)x^{0.080(\pm0.0004)}$	0.889
Ile	8.23 ±0.07	8.72 ±0.23	9.16 ±0.19	10.26 ±0.12	10.40 ±0.21	10.39 ±0.19	$y = 7.290(\pm0.0119)x^{0.065(\pm0.0004)}$	0.862
Leu	4.68 ±0.02	4.97 ±0.11	5.28 ±0.11	5.88 ±0.04	5.96 ±0.13	5.95 ±0.11	$y = 4.157(\pm0.0069)x^{0.066(\pm0.0004)}$	0.862
Phe	4.61 ±0.06	4.83 ±0.15	5.21 ±0.01	5.69 ±0.02	5.79 ±0.08	5.84 ±0.07	$y = 4.084(\pm0.0068)x^{0.064(\pm0.0004)}$	0.879
NEAAs								
Asp	10.73 ±0.14	11.10 ±0.17	12.06 ±0.10	13.34 ±0.10	13.56 ±0.17	13.58 ±0.19	$y = 9.407(\pm0.0141)x^{0.066(\pm0.0004)}$	0.892
Ser	5.32 ±0.04	5.48 ±0.06	5.78 ±0.02	6.43 ±0.06	6.49 ±0.10	6.69 ±0.04	$y = 4.656(\pm0.0036)x^{0.063(\pm0.0002)}$	0.949
Glu	14.19 ±0.55	15.07 ±0.69	15.77 ±0.22	17.76 ±0.38	17.73 ±0.34	18.55 ±0.29	$y = 12.477(\pm0.1273)x^{0.070(\pm0.0005)}$	0.830
Gly	11.93 ±0.19	12.40 ±0.23	13.42 ±0.41	14.18 ±0.56	14.88 ±0.55	16.69 ±0.62	$y = 10.011(\pm0.0280)x^{0.079(\pm0.0006)}$	0.822
Ala	7.94 ±0.24	8.33 ±0.28	8.53 ±0.18	9.30 ±0.16	9.52 ±0.10	10.06 ±0.15	$y = 7.001(\pm0.0129)x^{0.059(\pm0.0004)}$	0.863
Pro	7.41 ±0.15	7.70 ±0.16	8.12 ±0.05	8.73 ±0.16	9.18 ±0.12	10.23 ±0.06	$y = 6.212(\pm0.0088)x^{0.077(\pm0.0003)}$	0.927
Cys	1.02 ±0.02	1.03 ±0.07	1.05 ±0.04	1.16 ±0.03	1.19 ±0.05	1.13 ±0.00	$y = 0.934(\pm0.0024)x^{0.039(\pm0.0006)}$	0.456
Tyr	3.73 ±0.08	3.72 ±0.38	4.06 ±0.13	4.55 ±0.08	4.44 ±0.20	4.31 ±0.13	$y = 3.357(\pm0.0133)x^{0.050(\pm0.0008)}$	0.414
Tau	1.23 ±0.03	1.12 ±0.08	1.16 ±0.06	0.64 ±0.03	0.65 ±0.05	0.69 ±0.03	$y = 1.922(\pm0.0093)x^{-0.194(\pm0.0014)}$	0.799

6.4.2 Metabolic live-weight exponent determination

The relationships between geometric mean weight and losses of protein and individual amino acids were well fitted to power functions ($y = a * X^b$) with R^2 values of at least 0.895. The coefficients (slope) and exponents derived from these functions are presented in Table 6.4.

A protein loss live weight exponent of 0.693 at 30^0C was derived based on protein loss after starvation across variable body size, the relationship of which is represented by the following equation:

Protein $loss_{30°C}$ (mg $fish^{-1}$ day^{-1}) = 0.065 (±0.0007) * (live weight)$^{0.693(\pm 0.0029)}$, R^2 = 0.8981

Table 6.4. Coefficient and exponent values for protein and amino acid losses derived from the power function ($y = a * X^b$). Exponent values with the same superscript letter, number of asterisks or plus signs are not significantly different.

	Coefficient (a)	Exponent (b)	R^2	Weighted Exponent[1]
Protein	0.065(±0.0007)	0.693(±0.0029)$^{a+}$	0.898	NA
EAAs				
His	0.826(±0.0161)	0.889(±0.0051)^{b}	0.903	0.012(±0.0007)
Arg	2.404(±0.0342)	0.970(±0.0036)^{c}	0.957	0.047(±0.0041)
Thr	1.724(±0.0268)	0.792(±0.0045)^{di}	0.915	0.025(±0.0015)
Val	3.965(±0.0484)	0.429(±0.0037)^{e}	0.926	0.011(±0.0016)
Met	1.015(±0.0156)	0.811(±0.0045)^{d}	0.925	0.017(±0.0010)
Lys	2.503(±0.0166)	1.007(±0.0017)^{f}	0.991	0.062(±0.0006)
Ile	3.763(±0.0639)	0.662(±0.0050)^{g}	0.895	0.032(±0.0028)
Leu	2.572(±0.0356)	0.594(±0.0039)^{h}	0.920	0.016(±0.0014)
Phe	2.016(±0.0172)	0.795(±0.0026)^{di}	0.965	0.025(±0.0002)
		Av. = 0.772*		
NEAAs				
Asp	5.127(±0.0578)	0.702(±0.0035)^{a}	0.910	0.047(±0.0028)
Ser	1.835(±0.0214)	0.784(±0.0037)^{i}	0.928	0.027(±0.0002)
Glu	2.865(±0.0494)	0.902(±0.0047)^{b}	0.936	0.075(±0.0065)
Gly	1.602(±0.0319)	1.024(±0.0038)^{f}	0.954	0.063(±0.0082)
Ala	1.488(±0.0288)	0.737(±0.0051)^{j}	0.922	0.028(±0.0037)
Pro	1.179(±0.0209)	0.809(±0.0042)^{d}	0.914	0.034(±0.0030)
Cys	0.571(±0.0082)	0.648(±0.0043)^{g}	0.902	0.003(±0.0004)
Tyr	1.439(±0.0283)	0.805(±0.0050)^{di}	0.895	0.017(±0.0015)
		Av. = 0.801*		
Tau	0.007(±0.0008)	1.512(±0.0177)^{k}	0.917	NA
SumAA[2]	1.487(±0.0118)	0.858(±0.0020)$^{++}$	0.9766	

[1] Weighted exponent calculated as: the geometric mean percent of crude protein of each amino acid x the exponent (b).
[2] Sum of all proteinaceous amino acids (minus Trp)

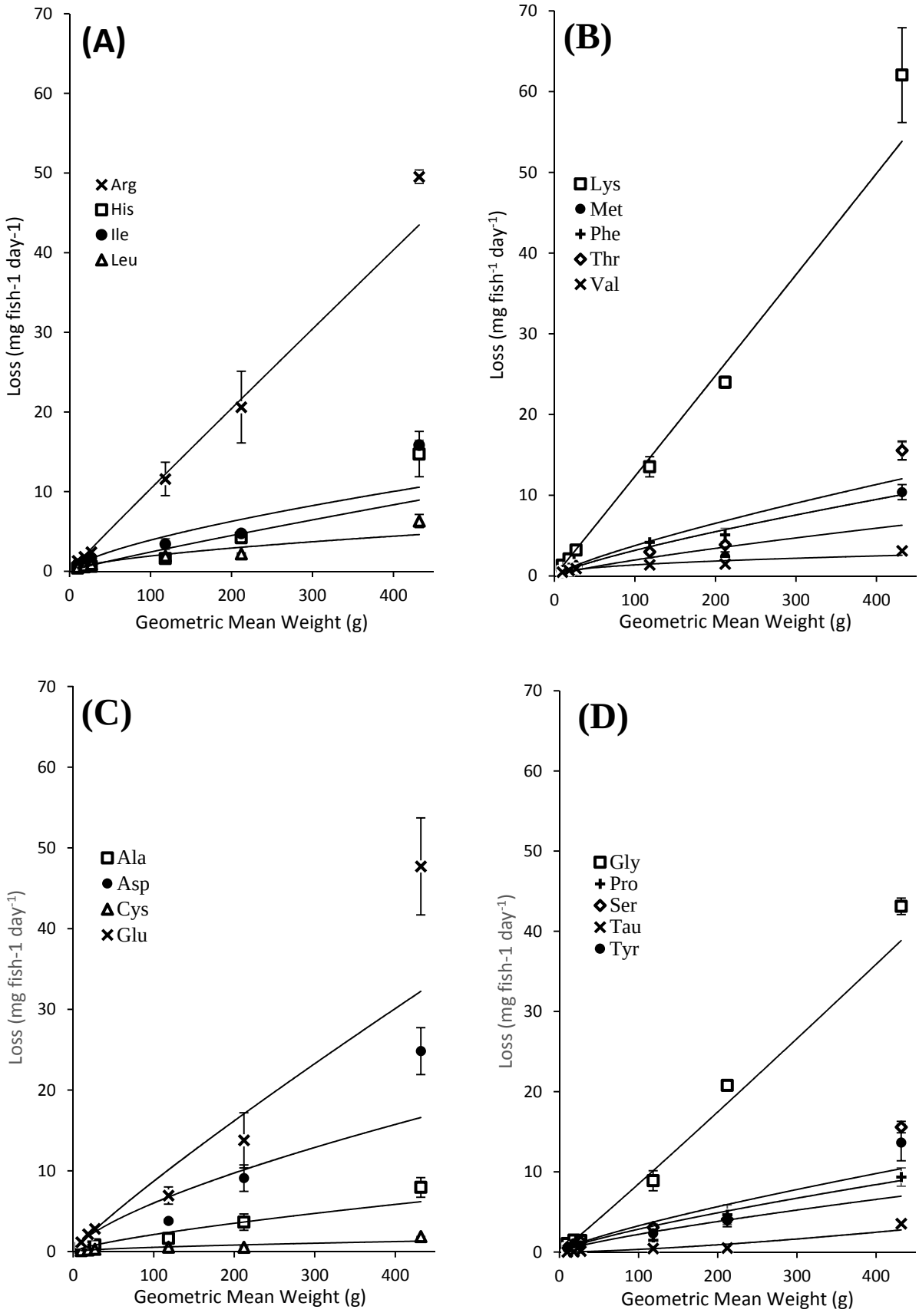

Figure 6.2. Essential (A and B) and non-essential (C and D) amino acid losses in barramundi of varying size after 21 days feed deprivation.

Amino acid live weight exponents showed substantial variation and, with the exception of aspartic acid, were significantly different ($p<0.001$) from that of protein (Table 6.4). Valine (Val), isoleucine (Ile), leucine (Leu) and cysteine (Cys) exponents were significantly lower than that of protein, while exponents for the remaining amino acids were significantly higher. Similarly, most coefficients were highly significantly different ($p<0.001$) from one another with some exceptions. Threonine (Thr), phenylalanine (Phe) and tyrosine (Tyr) were not significantly different from each other, proline (Pro) or serine (Ser). Additionally, Histidine (His) and glutamic acid (Glu); lysine (Lys) and glycine (Gly); methionine (Met) and proline (Pro); and Ile and Cys live weight exponents were not significantly different. The mean exponent values for the essential amino acids was not significantly different from that of the non-essential amino acids. The exponent for the relationship between the loss of the sum of the proteinaceous amino acids and geometric mean body weight was significantly ($p<0.05$) higher than that relating to loss of protein. Weighted exponent values for individual proteinaceous amino acids were also derived (Table 6.4) and summed to 0.543 (±0.0402), representing 78.4% of the exponent for protein (sum and proportion not presented).

Individual amino acid losses as a function of the geometric mean body weight are presented in Fig. 6.2 (A to D).

6.4.3 Methionine metabolic demands

Evaluation of the marginal efficiencies of Met use in a previously published Met/total sulphur amino acid (TSAA) requirement study (Poppi *et al.*, 2017), as well as an as-yet unpublished study (Poppi and Glencross, 2014), are presented in Table 6.5. A comparison of the components of the equations describing the relationships between mass-independent digestible Met intake and mass-independent Met gain derived using either the protein or Met exponent-adjusted GMW (PBW and MetBW respectively) is presented for comparison. Marginal efficiencies of Met utilisation (EU) were markedly different between diets, however, the basis for this was not the focus of this study. Application of the MetBW to the calculation of the mass-independent Met intakes and gains resulted in marginally reduced EUs in comparison to those derived using the protein exponent body weight in both the LPCM diet of Poppi and Glencross (2014) and the data from Poppi *et al.* (2017), while it remained constant in the LPC diet and increased slightly in the FML diet of Poppi and Glencross (2014).

Extrapolation of the EU relationships was used to identify the predicted losses of Met based on the *x* intercept generated estimates of Met maintenance demands (Table 6.6). The effect of using MetBW or PBW on these estimates was dependent on the size of the animal, with higher maintenance demands predicted for smaller and medium-sized fish across all treatments when the

PBW was used in calculations, whilst higher demands were predicted for the larger fish using the MetBW.

Methionine requirements for growth calculated using the MetBW adjustment were predicted to be marginally higher across all fish sizes when applied to the Poppi *et al.* (2017) data and the LPCM, but not FML and LPC, data of Poppi and Glencross (2014).

A similar trend was seen in the predictions of total Met requirement.

Table 6.5. Comparison of the marginal efficiencies (slope) of Met utilisation by barramundi in two previous studies, calculated using either the Met (MetBW) or protein (PBW) transformed live weight exponent values derived in the current experiment. Also indicated is the estimated maintenance demands for Met (intercept) from each transformation and diet.

		Slope	Intercept	R^2	1/k[a]
Poppi and Glencross (2014)					
Diet FML[b]					
	PBW	0.894	0.001	0.90	1.119
	MetBW	0.900	0.001	0.89	1.111
Diet LPCM[c]					
	PBW	0.256	0.004	0.86	3.906
	MetBW	0.255	0.006	0.85	3.922
Diet LPC[d]					
	PBW	0.318	-0.017	0.52	3.145
	MetBW	0.318	-0.029	0.51	3.145
Poppi et al. (2017)[e]					
	PBW	0.266	0.058	0.88	3.759
	MetBW	0.260	0.085	0.87	3.846

[a] Intake to gain ratio.
[b] Diet FML: a fishmeal-based diet
[c] Diet LPCM: a lupin protein concentrate-based diet with supplemental DL-Met.
[d] Diet LPC: a lupin protein concentrate-based diet.
[e] Experiment 1 data only

Maintenance Met demands as a proportion of the total Met requirements were consistent across all datasets, with maintenance constituting a higher proportion of total demands when calculated using the PBW for all fish sizes except the largest two sizes. Figures derived from the Poppi and Glencross (2014) data were almost identical across the treatments. The proportion of the total Met requirement occupied by the requirement for Met maintenance increased with increasing

fish size, ranging from 0.8% of the total requirement for 50g fish in the FML treatment of Poppi and Glencross (2014), up to 43.2% for 2000g fish in the Poppi *et al.* (2017) data.

6.5 Discussion

Several authors have argued that the amino acid requirements of an animal can be derived from the use of an "ideal protein", one which contains each of the essential amino acids (EAA) in a specified ratio relative to a key amino acid such as lysine, and usually with reference to the proportions present in the whole body or muscular tissue of the animal (Boisen *et al.*, 2000; Furuya *et al.*, 2004; Gaylord and Barrows, 2009). The merits and limitations of this school of thought have been discussed extensively (Mambrini and Kaushik, 1995; Hart *et al.*, 2009; NRC, 2011), however, there is some consensus that the whole body amino acid profile reflects the minimum requirements for most EAA. Consequently, consideration of dietary digestible protein supply should suffice as a component of modelling growth in fish, if this concept is considered accurate. Digestible protein requirements could be calculated over a range of parameters, using a "balanced" amino acid profile, and the magnitude of individual EAA inclusion in dietary formulations could be based on this "ideal protein" supplied to meet the crude protein requirement, as is the assumption when applying current factorial growth models for fish. It has been asserted, however, that fish do not have a requirement for protein *per se* but for a balance of individual essential and non-essential amino acids (Lim *et al.*, 2002), which together make up the reported protein requirement (or the majority thereof). One of the major limitations of the ideal protein concept is that it does not account for the differences in utilisation or rate of turnover of individual amino acids identified by authors such as Rollin *et al.* (2003) and Adeola (1998). These differences may be a result of variations in the roles of these amino acids, possibly overestimating the requirement for some and underestimating others, such as those substantially involved in processes other than protein synthesis. Additionally, these roles may change with the lifestage of the fish (Rollin *et al.*, 2003). Incorporating these differences into current models, such as by incorporation of exponent values which allow the effect of the size of the animal on nutrient utilisation to be taken into consideration, may improve the accuracy of predictions, allowing optimisation of the use of less expensive or more sustainable sources of protein.

If the ideal protein is indeed representative of the requirements for individual amino acids, it might be expected that the utilisation of whole protein would be representative of that of individual amino acids. In the present study, a protein loss exponent of 0.693 (±0.0029) was derived based on protein (nitrogen x 6.25) loss after feed deprivation in fish of variable size.

Table 6.6. Methionine demands in growing barramundi maintained at 30^0C as predicted by published growth models for this species and based on responses to variable methionine in two published dose response studies, in addition to utilisation efficiencies derived in the present study. Comparison between the use of carcass methionine and protein loss exponent-adjusted body weights derived in the present study (MetBW and PBW respectively) are also presented.

	Live Wt. (g fish^{-1})	Expected Growth (g day^{-1})[a]	Met maint (mg fish^{-1} d^{-1})[b]	Met gain (mg fish^{-1} d^{-1})[c]	Met growth (mg fish^{-1} d^{-1})[d]	Total (mg fish^{-1} d^{-1})[e]	Maint:Total (%)[f]
Poppi and Glencross (2014)							
Diet FML[g]							
PBW	50	2.13	0.16	11.74	13.13	13.29	1.2
MetBW	50	2.13	0.11	11.74	13.05	13.17	0.8
PBW	100	2.88	0.45	15.87	17.75	18.20	2.5
MetBW	100	2.88	0.35	15.87	17.65	17.99	1.9
PBW	500	5.81	5.15	31.97	35.74	40.89	12.6
MetBW	500	5.81	4.74	31.97	35.54	40.28	11.8
PBW	1000	7.85	14.68	43.22	48.32	63.00	23.3
MetBW	1000	7.85	14.59	43.22	48.04	62.63	23.3
PBW	2000	10.61	41.85	58.43	65.33	107.18	39.0
MetBW	2000	10.61	44.93	58.43	64.95	109.88	40.9
Diet LPCM[h]							
PBW	50	2.13	0.55	11.74	45.94	46.49	1.2
MetBW	50	2.13	0.40	11.74	46.14	46.54	0.9
PBW	100	2.88	1.58	15.87	62.10	63.68	2.5
MetBW	100	2.88	1.23	15.87	62.37	63.60	1.9
PBW	500	5.81	18.01	31.97	125.08	143.09	12.6
MetBW	500	5.81	16.75	31.97	125.62	142.36	11.8
PBW	1000	7.85	51.36	43.22	169.09	220.45	23.3
MetBW	1000	7.85	51.58	43.22	169.82	221.40	23.3
PBW	2000	10.61	146.44	58.43	228.60	375.03	39.0
MetBW	2000	10.61	158.83	58.43	229.58	388.42	40.9
Diet LPC[i]							
PBW	50	2.13	0.45	11.74	36.98	37.43	1.2
MetBW	50	2.13	0.32	11.74	36.92	37.24	0.9
PBW	100	2.88	1.27	15.87	50.00	51.27	2.5
MetBW	100	2.88	0.98	15.87	49.92	50.90	1.9
PBW	500	5.81	14.50	31.97	100.69	115.19	12.6
MetBW	500	5.81	13.40	31.97	100.53	113.94	11.8
PBW	1000	7.85	41.34	43.22	136.12	177.47	23.3
MetBW	1000	7.85	41.28	43.22	135.91	177.19	23.3
PBW	2000	10.61	117.89	58.43	184.03	301.92	39.0
MetBW	2000	10.61	127.12	58.43	183.74	310.86	40.9
Poppi et al. (2017)[j]							
PBW	50	2.13	0.54	10.68	40.66	41.20	1.3
MetBW	50	2.13	0.40	10.68	41.66	42.05	1.0
PBW	100	2.88	1.54	14.44	54.96	56.50	2.7
MetBW	100	2.88	1.22	14.44	56.31	57.54	2.1
PBW	500	5.81	17.52	29.08	110.70	128.22	13.7
MetBW	500	5.81	16.62	29.08	113.42	130.04	12.8
PBW	1000	7.85	49.97	39.31	149.65	199.62	25.0
MetBW	1000	7.85	51.20	39.31	153.33	204.52	25.0
PBW	2000	10.61	142.48	53.15	202.32	344.80	41.3
MetBW	2000	10.61	157.66	53.15	207.29	364.94	43.2

[a] Modelled daily growth based on 30°C water temperature (Glencross, 2008; Glencross and Bermudes, 2012).
[b] Maintenance digestible Met requirement, per exponent-transformed Met (MetBW) or protein (ProBW) body weight (as per exponents presented in Table 6.4).
[c] Met content of the modelled live-weight gain.
[d] Digestible Met demand for gain derived by gain of Met predicted by the model divided by the utilisation efficiency of Met.
[e] Combined digestible Met demand for both maintenance and growth.
[f] Proportion of maintenance Met demand in total Met demand.
[g] Diet FML: a fishmeal-based diet
[h] Diet LPCM: a lupin protein concentrate-based diet with supplemental DL-Met.
[i] Diet LPC: a lupin protein concentrate-based diet.
j Experiment 1 data only

This exponent does not differ greatly from the generally accepted protein loss exponent of 0.70 reported by Lupatsch *et al.* (1998) from 1-250g gilthead seabream (*Sparus aurata*) and of 0.67 - 0.72 derived by Glencross and Bermudes (2010) from barramundi of a similar size range at water temperatures consistent with that used in this study (~30^0C). Unusually, the crude protein content of the fish at the start of the experiment increased with increasing fish size, which is in disagreement with the data presented by Glencross and Bermudes (2012) and had a flow-on effect of fluctuating initial amino acid profiles on a whole-body basis. Nevertheless, the pattern of relative losses of protein from those fish with an apparently higher carcass content remained consistent with what has been seen in other fish species. The homology between the derived exponent for protein in the present study and those reported previously in this species suggests that the derived individual amino acid exponents should also be representative of turnover of these amino acids by this species. Contrary to the assumptions of the ideal protein concept, live weight exponents for the majority of the proteinaceous amino acids differed significantly from that of whole protein. Most amino acids, with the exceptions of Val, Ile, Leu and Cys, were significantly higher than that of protein as a whole. Exponents ranged from 0.429 (±0.0037) for Val up to 1.024 (±0.0038) for Gly and were generally significantly different between amino acids. The amino acid compositions of individual tissue proteins can vary between organs, however they, and that of the carcass as a whole, are thought to be tightly controlled in larger fish (Shearer, 1994; Wilson, 2003) and, therefore, unable to change substantially. The assumption in any variation of whole body amino acid composition across fish size during starvation, then, is that it is the result of either variation in the composition of the free amino acid pool or in the rate of turnover or catabolism within individual organs. While it would seem logical that the free amino acid pool would fluctuate as individual amino acids are selectively removed at differing rates for the synthesis of proteins, there has been no in-vivo proof that this is impacted significantly by the size of the animal, despite the fact that the relative size of the pool has been shown to vary with lifestage (Houlihan *et al.*, 1995; Srivastava *et al.*, 1995). Relative organ sizes have, however, been observed to scale to fish size (most often isometrically, except in the case of white muscle, which exhibited some allometry in rainbow trout (*Oncorhynchus mykiss*) in a study by Houlihan *et al.* (1986)). The relative area of the gill tissue, with its high rate of protein turnover (synthesis and degradation) and low rate of protein accretion, for example, declines with increasing fish size (Houlihan *et al.*, 1986), whereas muscle tissue continues to accumulate. The rate of turnover of these organs, with their differing amino acid profiles, could have a significant impact on the requirement for individual amino acids.

The exponent values of around 1.00 calculated for Lys and Gly indicate that the relative rate of loss of these amino acids is in direct proportion to the size of the fish, seemingly unrelated to the

relative rate of standard metabolism of the animal which is known to decrease proportionally with increasing fish size (Wootton, 1990). This may indicate that the demand for these amino acids is greater for biological processes other than retention of protein/tissue. Glycine, for example, plays an important role in responding to environmental stressors through acting as an organic osmolyte, regulating cellular volume (Fiess *et al.*, 2007) which can have a range of health implications, while the lysine derivatives hydroxylysine and allysine are essential in stabilizing the structure of collagen (Ricard-Blum and Ville, 1989), a key constituent of connective tissues as well as skin and scales of fish, which, in itself, may have osmoregulatory consequences. It may also indicate that these amino acids were preferentially catabolized for energy production rather than retained during protein turnover or that their rate of inevitable catabolism is higher. While the use of amino acids for energetic purposes is significant in fish, little is known about the selection of individual amino acids for this function. The NRC (2011) asserts that this preferential catabolism may be difficult to separate from the inevitable catabolism of amino acids through other catabolic pathways, the magnitude of which has been suggested to vary between amino acids (based on variations in utilisation efficiencies of individual amino acids) (Fuller, 1994). Glycine has been observed in rats to have an additional energetic role through action on gluconeogenesis (Nadkarni *et al.*, 1960), a pathway shown to be active in this species (Wade *et al.*, 2014). This function, however, is also shared by serine which exhibited a much lower weight loss exponent in the present study, as might be more expected given a decreasing metabolic rate implies a lower relative demand for energy in the larger fish.

A significant observation in this study was that those amino acids with live weight exponent values below that of protein, indicating a greater biological demand in smaller fish, were the branched chain amino acids (BCAAs) and Cys. The BCAAs are known to have a number of important roles, including determining the structure of globular proteins such as myoglobin and hemoglobin (Brosnan and Brosnan, 2006), responsible for oxygen transport in the muscle and blood respectively, the relative demand for which might be expected to be greater in smaller fish given their higher metabolic rate (Brett and Glass, 1973). Catabolism of this group of amino acids is thought to occur primarily in the muscle tissue, the rate of which increases rapidly during stressful events such as exhaustive exercise and seawater acclimation (Li *et al.*, 2009) when energy may be limiting as would also be the case during periods of starvation. Muscle tissue has been observed to be the major site of protein degradation during starvation in Atlantic salmon, for example, (Einen *et al.*, 1998) which may explain the higher relative catabolism of the BCAAs by smaller fish, whose rate of protein turnover is higher (Houlihan *et al.*, 1986). It is not immediately clear why the body weight exponent for loss of Cys is also below that of protein but, similarly to the BCAAs, may be

related to its role in protein structure or its regulation of transcription factor NFκβ, an important function in influencing cell survival and proliferation (Métayer *et al.*, 2008). Cysteine also acts as a precursor for the aminosulfonic acid taurine (Tau), an important compound in osmoregulation and protection from oxidative stress (Ripps and Shen, 2011) which may be especially important during starvation when energy reserves are depleted, reducing the capacity of the animal to respond to external stressors.

Although not a proteinogenic amino acid, taurine contents were variable in the body before and after feed deprivation but were proportionally lowest in fish in the larger size classes. This may suggest either that Tau is less required in larger fish, as has been suggested by Qi *et al.* (2012), or that it was somehow limiting in these fish, requiring synthesis from Cys, to maintain a constant body pool. The body weight exponent for the loss of Tau, however, was the highest calculated at 1.5117(±0.0177) which seems to suggest larger fish have a higher requirement per unit of body weight, supporting the latter theory. This high exponent appears to be driven by the high losses of Tau in fish in the largest size group which increased noticeably from the preceding size group. It might have been interesting to see the response of fish of an intermediate size (e.g. ~300 g/fish), or perhaps larger still, to elucidate whether this was an anomaly or a true exponential response.

One limitation of drawing conclusions on amino acid utilisation from the results of this study, and extrapolating to deduce amino acid requirements, is that these figures were derived based on the assumption that the rates of amino acid losses observed in the starved fish are constant and will remain so in fed fish. As mentioned in relation to the losses of the BCAAs, some of the observations made may be associated with a response to amino acid or energy limitation, rather than inevitable catabolic processes. Indeed, reduced protein synthesis has been associated with feed deprivation in several fish species, a condition reversed with feeding, and may, therefore, have a significant impact on the standard metabolic rate of the animal (O'Connor *et al.*, 2000).

It might have been expected that the EAAs would be lost at a greater rate than the NEAAs, given their additional role in the synthesis of deficient NEAAs during protein turnover further to fulfilling their various metabolic roles and as components of proteins themselves. This, however, was not observed, with the average exponent for the EAAs not differing significantly from that of the NEAAs. Perhaps the need to maintain a specific ratio of EAA:NEAA within the proteins and free amino acid pools, as proposed is necessary in dietary formulations for optimal fish health (Oliva-Teles, 2012), is the major driver in selection of amino acids for catabolism.

An additional caveat of the present study is that losses in protein are assumed to be representative of catabolism of muscle and/or organ proteins, however, this may not necessarily be the case. Current methods of estimating animal protein composition (and, consequently, protein

accretion and loss), including that used in the present study, assume a protein content of 6.25 times the measured amount of nitrogen in the sample (i.e. that nitrogen comprises 16% of an intact protein). While convenient, these methods ignore both the fact that not all proteins have the same amino acid profile and, thus, will contain variable levels of nitrogen, as well as the contribution of non-protein nitrogen (NPN) to the overall nitrogenous composition of the animal. NPN may be present in the fish in the form of volatile bases (ammonia and various amines), nucleic acids, phospholipids and purine derivatives, among other compounds (Mariotti *et al.*, 2008) and has been shown to constitute up to 18% of total nitrogen in teleost fish (Niimi, 1972). In the present study, for example, the sum of the measured amino acids accounted for between only 73 and 79% of the total nitrogen composition of the fish before starvation and between 72 and 75% after starvation, suggesting the majority of the remaining 21 to 28% (excluding Trp which was not measured) was made up of NPN. A similar result was seen when the weighted exponents for the proteinaceous amino acids were calculated, where the sum of these weighted exponents represented only 78.4% of the protein loss exponent. Helland *et al.* (2010) reported differences in the calculated requirements of Atlantic salmon post-smolts for digestible protein (DP) and sum amino acids (SumAA), surmising that the rate of NPN recycling was much higher than that of the body AA. Estimates of losses and, by extension, utilisation of protein as a whole, then, may be confounded by the turnover of this proportion of the nitrogenous content of the animal. The body weight to loss relationships for the majority of amino acids, and of the sum of all proteinaceous amino acids, in the present study exhibited exponents higher than that of protein as a whole, indicating a greater effect of fish size on the proportional losses of those amino acids than that seen for protein (nitrogen). This seems to suggest that the losses of NPN, as a proportion of the total CP losses, was greater in the smaller fish than in the larger fish. It is unclear why smaller fish would utilise NPN at a greater rate than their larger counterparts, however it may be due to increased excretion of nitrogen (via ammonia) as a consequence of their greater metabolic rate, as opposed to cycling of amino acids, a proportion of which may end up as NPN in the larger fish, resulting in losses of individual aminos but lower overall losses of nitrogen (protein). Alternatively, NPN may be preferentially catabolised over amino acids for energetic purposes, the relative demand for which is greater in the smaller fish, although no direct evidence of this has been reported to our knowledge. It is also assumed in this case that complete hydrolysis of the proteins was achieved. It is acknowledged that any incomplete hydrolysis could contribute to disparities between total nitrogen (protein) and SumAA.

The utility of the amino acid-specific metabolic weight exponents is dependent on the assumption that the allometric scaling of individual amino acid loss is a better indicator of utilisation of that amino acid than is the scaling of protein losses. The significantly different loss

exponents derived in this study suggested utilisation of Met was considerably different to that of protein and that consideration of this should improve the accuracy of predictive models of Met utilisation. However, when considered on the basis of a Met loss exponent-adjusted geometric mean weight, the goodness of fit (R^2) of the linear model in all cases was consistently lower, suggesting that Met deposition was better predicted on a unit of PBW basis. The complexity of the amino acid analysis may introduce slightly more error than that observed in nitrogen analyses, allowing for the possibility that the marginal differences in observed R^2 values are actually just artefacts of this error. Either way, no noticeable improvement in linear model fit was observed when MetBW was used in the calculations. At nutrient intake levels below the requirement, as was the case in the studies analysed here, the relationship between nutrient intake and gain should be well described by this linear model (Sawadogo *et al.*, 1997). While the Poppi *et al.* (2017) data may have approached the minimum requirement, based on further analysis in that study, and was thus better described by a quadratic model when considered on a mass-independent exponent-adjusted basis ($R^2 = 0.99$ for relationships on both MetBW and PBW bases; figures not presented), no improvement in model fit was seen with the use of the MetBW correction. The use of MetBW also had an inconsistent effect on the derivation of the utilisation efficiencies, with both increases and decreases observed in response to the changed exponent, casting further doubt on the advantage of its use over PBW.

When applied to the derivation of Met maintenance demands, however, a clear pattern was observed, whereby lower demands in smaller fish were predicted by the use of the MetBW adjustment, whilst it increased predictions of maintenance requirements in larger fish, in comparison to those derived using the PBW adjustment. This may suggest that the use of the PBW transformation may overestimate the Met maintenance demand in smaller fish and underestimate that of larger fish. From a feed formulation perspective, however, the more important figure is ultimately the total Met demand which in most cases was calculated to be higher in small to medium-sized fish when the PBW was used in the calculations but lower in the largest fish, supporting the assertion that PBW may overestimate requirements in smaller fish. This, however, is predicated on the assumption that MetBW provides a more accurate, rather than simply a different, estimate. It is not immediately clear why the MetBW adjustment resulted in higher total Met requirement predictions across the entire size-range in calculations based on the data of Poppi *et al.* (2017).

The choice of BW exponent affects the calculation of amino acid requirements in two ways. The weight-independent BW (MetBW vs. PBW) of which the exponent is a component, affects the derivation of the utilisation efficiency which, in turn, is used for derivation of maintenance

requirements and, along with the BW exponent itself, is applied directly in the calculation of the requirements. Any variation, therefore, can have a marked impact on these calculations. In the response to the Met-supplemented LPC diet (LPCM) of Poppi and Glencross (2014) and the data of Poppi *et al.* (2017), application of the Met, rather than protein, loss exponent resulted in increased calculated requirements for growth. This requirement derived from the response of fish fed the FML diet in Poppi and Glencross (2014), however was reduced when the Met loss exponent was used. This is largely a function of the much higher Met utilisation efficiency by fish fed this diet which is not overly surprising given the high nutritional value placed on fishmeal as a protein source for carnivorous fish (Espe *et al.*, 2012). This may also be a factor, to a lesser extent, in the reduction in Met requirement for growth seen in the LPC data of Poppi and Glencross (2014), which also had a higher utilisation efficiency.

Overall, Met demands in small barramundi (50g) appear to be driven primarily by a requirement for growth, with maintenance requirements representing a small proportion of the total Met requirement (around 1%). Larger fish (2000g), however, were predicted to expend considerably more (41-43%) of this ingested Met for maintenance, leaving less for somatic growth. This is consistent with the observation of several authors that specific growth rate decreases with increasing fish size (Brett and Shelbourn, 1975; Austreng *et al.*, 1987) and that the relative contribution of maintenance to overall demands may be greater in these slower growing animals (NRC, 2011). Amino acid maintenance demands in relation to their demand for protein deposition has previously been reported in Rainbow trout (*Oncorhynchus mykiss*) by Rodehutscord *et al.* (1997). While these authors did not investigate the requirement for Met, they did report variations between amino acids in the proportion of the total demand occupied by the maintenance requirement of between 4% (for lysine) and 32% (for leucine). Assessment of the compartmentalisation of requirements for other essential amino acids may be an interesting extension of the work presented herein

Conclusion

Contrary to previous assumptions, the present study demonstrated that the scaling of individual amino acid losses in juvenile barramundi during starvation differ significantly from that of protein (nitrogen) as a whole and from each other. While the R^2 values for the assessments of marginal efficiency of Met utilisation remained relatively unchanged when the metabolic body weight of the fish in those calculations was adjusted using the Met loss exponent, compared with that derived from loss of protein, estimates of maintenance, growth and total requirements for Met were affected. It was suggested that the protein loss adjustment typically used in these calculations

may overestimate these requirements for smaller fish and underestimate those of larger fish. This may be, in part, due an apparent effect of fish size on the utilisation of non-protein nitrogen.

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Chapter 7 - General Discussion

Consideration of the dietary amino acid profile in formulated feeds for carnivorous fish species, such as barramundi (*Lates calcarifer*), is becoming increasingly important with the growing use of plant ingredients over the traditionally used fishmeal as the primary source of dietary protein. This is especially the case for methionine (Met) and taurine (Tau), two sulphur amino acids (SAA) known to be limiting or absent in many popular plant protein meals. Determination of their requirement for optimal growth then is paramount to further development of more productive and sustainable aquafeeds. Traditionally, this has been achieved through repeated iterations of dose response studies, however, the development of models describing nutrient utilisation in fish, including barramundi, in conjunction with the principles of the ideal protein concept, has provided an alternative means by which to estimate these requirements. Application of these models to the derivation of successful feed formulations, however, has relied on the assumption that the utilisation of protein is representative of that of individual amino acids across all lifestages, a notion which has been suggested not to be completely accurate. Understanding the mechanisms which drive the importance of individual amino acids to an animal allows consideration of the substitution or supplementation of dietary components (e.g. precursors or metabolites of the component of interest or compounds with similar metabolic roles) which may negate deficiency and shed some light on the reasons behind their changing essentiality.

The experiments described in this thesis endeavoured to address these issues. The overall aim of this series of experiments was to assess the requirements for Met and Tau and to elucidate their influence on the expression of selected genes of some important metabolic pathways. Assessment of the extent to which the main SAAs influence the utilisation of one another and how this utilisation changes with body size were also explored. The requirements for Met, total sulphur amino acids (TSAA) and, to a lesser extent, Tau were established and the roles that these amino acids play in protein turnover, growth signalling and the pathways through which they are metabolised (catabolised and synthesised) were identified. Additionally, allometric scaling exponents were derived for the proteinaceous amino acids and the utility of applying the exponent for Met loss, rather than that of protein, to predictions of Met utilisation efficiency and metabolic requirements was assessed. Several limitations in the methodology used in these experiments were identified and will be addressed in this chapter. This information, along with the results of the experiments reported in this thesis will prove useful in further research on the amino acid requirements of barramundi and/or in optimizing future feed formulations for this species.

7.1 Summary of key findings

7.1.1 Chapter 2 - Redefining the requirement for total sulphur amino acids in the diet of barramundi (*Lates calcarifer*) including assessment of the cystine replacement value.

A previous estimate of the requirement for Met and TSAA in this species was made by Coloso *et al.* (1999). The series of experiments described in Chapter Two concluded this figure may have been an underestimate, re-evaluating the requirement for TSAA to be between 17.1 (95% of maximum response) and 20.2g kg^{-1} (99% of maximum response) in a diet with 592g kg^{-1} CP (10.5-13.6g kg^{-1} Met + 6.6g kg^{-1} Cys; 1.8-2.3% CP Met + 1.1% CP Cys). The compartmental model of Pesti *et al.* (2009) provided the best fit to the data, based on a low sum of squared error and high R^2 value, and was used to derive this figure. The choice of the nutrient response model used for establishing requirement figures, and the mode of expression of these requirements, were highlighted as significant considerations in assessing the results of nutrient dose response studies. Nine nutrient response models were applied to the percent body weight gain data in order to estimate the requirement for Met and TSAA. All models fit the data to a similar degree, however, markedly different estimates of requirement were predicted. Discussion was made in this chapter as to the greater biological relevance of using quadratic over linear-based models in dose-response studies. Expressing amino acid requirements as a percentage of protein has been debated in the past, however, in this case, it was shown that there was more homology between the previous and current estimates of Met requirement when presented in this basis. The TSAA requirement was considerably higher regardless of the mode of expression. Investigation of the capacity of cystine (Cys) to replace Met in the diet suggested at least 40%, and possibly up to 54%, of the Met in the diet of juvenile barramundi could be replaced by Cys without significantly affecting growth.

7.1.2 Chapter 3 - Postprandial plasma free amino acid profile and hepatic gene expression in juvenile barramundi (*Lates calcarifer*) is more responsive to feed consumption than to dietary methionine inclusion.

While defining the dietary inclusion at which growth is maximised is important, the next logical step is to understand why these nutritional components are required by the animal and how they interact with the metabolism of other nutrients. The experiment described in Chapter Three investigated this through the observation of changes, at seven timepoints over a 24 hour period, in the circulating levels of the proteinogenic amino acids (as well as taurine) in the plasma of juvenile barramundi fed a meal containing a deficient, adequate or excessive amount of dietary Met. Levels of all free amino acids in the plasma fluctuated significantly over time, however, no significant effect of dietary Met on the timing or magnitude of peak levels of these amino acids, other than Met

and glycine, was observed. Peaks in plasma free Met were observed at two hours post-feeding in fish fed the Met deficient and adequate diets and at four hours post-feeding in fish in the excessive Met treatment. The differential expression of markers of selected SAA and protein turnover pathways was then assessed in hepatic tissue collected at these timepoints, as well as in that taken as a pre-feeding control. Methionine availability has previously been linked to modulation of the expression of genes associated with protein turnover and markers of growth stimulation in other fish species (Belghit *et al.*, 2014; Rolland *et al.*, 2015). In the present study, little effect of Met availability after the meal was observed on the expression of genes associated with these pathways in barramundi. Insulin-like growth factor I (IGF-I) expression did reflect the observed growth response at the pre-feeding timepoint but it was unclear whether Met acted directly on expression of this gene or if IGF-I was simply a signal of growth stimulation through another pathway. Protein degradation pathways were depressed after consumption of feed, as might have been expected, but this was not directly affected by dietary Met level. Unexpectedly, it appeared to be stimulated by adequate Met supply. It was theorised, however, that the larger size of the fish in this treatment (resulting from an improved growth response in the 49-day growth trial preceding this experiment) may have increased the daily Met requirement (on a unit of body weight basis) affecting the utilisation of the available Met. Similarly, expression of genes associated with sulphur amino acid turnover were significantly upregulated only by ingestion of feed/Met. Within this group of genes, a capacity to synthesise taurine was suggested by significant upregulation in the expression levels of cysteamine dioxygenase (CDO) and cysteine sulphinic acid decarboxylase (CSAD) (two enzymes in the pathway for conversion of cysteine to hypotaurine). Two forms of methionine adenosyltransferase (MAT-1 and MAT-2a) were also observed to be differentially expressed after feeding, suggesting that both forms are active in barramundi of this size, with MAT-1 appearing to be more responsive to Met supply. Notably, these genes were isolated and assessed in barramundi for the first time and proven to be nutritionally regulated, adding to a small but growing number of genes identified in this species to be markers of response to nutritional inputs.

7.1.3 Chapter 4 - The effect of taurine supplementation to a plant-based diet for barramundi (*Lates calcarifer*) with varying total sulphur amino acid contents.

The study of taurine (Tau) requirements of carnivorous fish have received considerable attention recently (see Salze and Davis, 2015), however it has not previously been investigated in barramundi. Depending on the capacity of the fish to synthesise it from other sulphur amino acids, Tau is considered to be either a conditionally essential (i.e. required in certain circumstances such as when sufficient precursors are not available) or essential component of the diet. Results of the

present study indicated that adequate supply of Tau may have had a positive effect on growth with the model of best fit predicting a requirement of 5.47g kg^{-1} DM (0.96% CP). While this figure is similar to those estimated for several other fish species (Lunger *et al.*, 2007; Matsunari *et al.*, 2008), the relatively poor fit of this model to the data suggests it should be considered with some caution. It was postulated that the lack of significant differences between treatments may be an indication of synthesis of Tau from an oversupply of TSAA. The importance of Met to this species was also highlighted with variable dietary Met having a greater influence on growth than supply of Tau, suggesting synthesis of Tau is not the primary fate of dietary Met. Taken together, it was concluded that Tau is likely a conditionally essential amino acid for barramundi but that the requirement figure derived may provide a safe minimum inclusion level.

7.1.4 Chapter 5 - Adequate supply of dietary taurine stimulates expression of molecular markers of growth and protein turnover in juvenile barramundi (*Lates calcarifer*).

While the investigation of the impact of taurine on growth in fish species has gained recent momentum (Salze *et al.*, 2012; Watson *et al.*, 2014), relatively few studies have considered its effect on the expression of genes involved in the metabolic pathways contributing to that improved growth. Those studies which have examined gene expression have mostly been limited to assessing the activity of the direct biosynthetic (Yokoyama *et al.*, 2001; Haga *et al.*, 2015) and taurine transporter genes (Wang *et al.*, 2016). This latter gene was shown in the present study to be directly influenced by Tau availability in barramundi. Similarly, a redirection of Cys utilisation in response to Tau supplementation was suggested by the expression profile of cystathionine-γ-lyase (CGL) and the Tau biosynthetic genes. Notably, markers of two alternative pathways of taurine synthesis were identified as being active with CSAD, mediating the sulphinoalanine pathway, suggested to be the more responsive to feeding. As was seen previously in response to Met supply, expression of the SAA metabolism genes were more significantly affected by time after feeding than supply of Tau. Similarly, no effect of Tau supply on the circulating levels of the proteinogenic amino acids in the plasma was observed. A link between adequate supply of Tau and stimulation/signalling of growth through both the TOR pathway and somatotropic index was established, while excessive Tau was suggested to negatively impact growth. Together, the evidence seems to support the pattern of growth response seen in Chapter Four and confirms that barramundi do possess the capacity to synthesise Tau from precursor SAA.

7.1.5 Chapter 6 - Allometric scaling exponents for individual amino acids differ significantly from that of protein in juvenile barramundi (*Lates calcarifer*).

Current factorial growth and feed utilisation models (e.g. Lupatsch *et al.*, 1998; Glencross, 2008) typically consider only the gross energy, lipid and protein compositions of the feed. Feed formulations derived separately from these models then assume equivalencies of utilisation between, for example, protein and individual amino acids across fish sizes, with amino acid inclusion scaling parallel to that of dietary protein. The results of the present study, however, showed that this assumption was not accurate. Live-weight exponents derived from the whole body losses of individual amino acids from barramundi of varying size over 21 days of starvation were shown, for the first time, to differ significantly from that of protein and from one another. The branched chain amino acids (valine, isoleucine and leucine) and Cys exhibited lower exponent values than protein, suggesting a greater demand in smaller fish, while losses of lysine and glycine appeared to scale directly with fish weight. Significantly, the exponent for loss of the sum of all measured amino acids was significantly higher than that of protein, suggesting non-protein nitrogen utilisation may be affected by fish size. Unexpectedly, when the revised exponent for Met was applied in the assessment of Met utilisation efficiency, marginally reduced goodness of the fit of the linear model to the adjusted retention responses suggested it does not improve the accuracy of utilisation prediction for this amino acid over the protein body weight exponent typically used. Further work is required to assess the utility of the derived exponent values for other amino acids. The relationships observed between starvation losses and the body weight of the animals also allow the calculation of basal demands for each of the amino acids at any fish size and, in conjunction with the utilisation efficiency, maintenance demands for incorporation into the Glencross (2008) barramundi growth model for calculation of total demands for Met. Notably, using this technique a requirement for gain was determined to be the primary driver of Met requirement for smaller barramundi, while maintenance requirements represented a large proportion of the total requirements in larger fish.

7.2 Discussion

The series of experiments described in this thesis have advanced our knowledge of sulphur amino acid (SAA) metabolism in barramundi (*Lates calcarifer*) in several ways. The previously published study focussed only on growth of the animals and may have underestimated the requirement for TSAA. The studies presented in this thesis represent a more comprehensive reassessment of this requirement, as well as that of taurine, and provided corroborating molecular evidence of the effect of these amino acids on growth in this species.

As indicated in Chapter Three, a link between improved growth and expression of components of the somatotropic index was observed. A significant improvement in percent body weight (%BW) gain was observed between fish fed diets containing 8.6g kg^{-1} and 14.9g kg^{-1} Met in the experiment outlined in Chapter Two. This response was mirrored in the long term expression of insulin-like growth factor one (IGF-I), a regulator of cellular growth (Clemmons and Underwood, 1991), in Chapter Three. Prior to feed consumption (i.e. 24 hours after the last meal), IGF-I was observed to be significantly more highly expressed in the liver tissue of fish fed the adequate Met diet (MetADQ; 14.9g kg^{-1} Met) than in that of fish in the deficient Met treatment (MetDEF; 14.9g kg^{-1} Met). An increase in IGF-I expression in response to increased dietary Met supply was also observed 24 hours post-feeding by Rolland *et al.* (2015) and has previously been linked with improved growth in fish (Dyer *et al.*, 2004).

Comparable results were seen in response to variable dietary Tau. Similarly to the response to Met, an improvement in growth (though not significant in this case) was suggested between fish fed a deficient (1g kg^{-1}) and adequate (8g kg^{-1}) amount of dietary Tau, at all levels of dietary Met, in Chapter Four. It was proposed that, rather than a lack of influence of Tau on growth stimulation, the absence of a significant growth response in that study may have been related more so to the restricted pair-feeding regime limiting the magnitude of the growth response, or to the choice of a higher level of dietary TSAA supply masking the effect of Tau supplementation. The molecular response supported this assertion with expression of all measured genes of the somatotropic index showing significant upregulation in response to adequate Tau supply in the diet, suggesting, albeit on a minor scale, that Tau does affect growth, or at least markers of it, and that the response may have been more pronounced in the absence of the potentially confounding factors identified. As discussed in Chapter Three, it is unclear whether these genes were directly stimulated by Met or Tau or were simply acting as signals of growth. In addition to its role in initiating protein synthesis, increasing supply of dietary Met would provide additional substrate for protein synthesis and thus growth of the animal, provided it remains the first limiting amino acid in the diet. It may be that growth was improved in the TSAA requirement experiment as a result of this increased substrate which, in turn, stimulated the somatotropic axis cascade. Unlike Met, however, Tau is not a proteinogenic amino acid and, as such, does not contribute directly to protein synthesis. Whether the positive effect of Tau supplementation on the somatotropic axis is the result of direct action on these genes or through sparing of Met for the processes described above remains to be determined, although no evidence of Met sparing by Tau was observed in the plasma amino acid analyses undertaken. Overall, it appears that supplementation of both Met and Tau can improve growth in this species. This seems to be, at least in part, related to their stimulating action on components of

the somatotropic index. As well, it was confirmed that expression of IGF-I and growth hormone receptor two (GHR-II) are good indicators of growth stimulation in this species.

Another means by which dietary amino acids can modulate growth is through influencing protein turnover. In general, the protein content of fish has been shown to remain relatively constant throughout the lifecycle (Portz and Cyrino, 2003) (although a significantly quadratic increase in protein with increasing fish size was reported in Chapter Six in this thesis). Growth of the animal, then, is driven by the deposition of this protein (Dumas *et al.*, 2007), the product of the balance between synthesis and degradation, processes effected through a number of metabolic pathways. The influence of dietary Met on some of these pathways has previously been investigated in rainbow trout (Lansard *et al.*, 2011; Belghit *et al.*, 2014; Rolland *et al.*, 2015) but not in barramundi. Taurine supplementation has not previously been experimentally linked to protein turnover in any fish species. In the present study, the expression of proteolytic (protein degradation) pathway marker genes was seen to reduce significantly post-prandially in both the Tau and Met response studies (Chapters Three and Five). This response makes biological sense, given that the increased availability of substrates for protein synthesis may reduce the requirement for degradation to meet amino acid demands. This was conserved across experiments, leading to the conclusion that these markers, ZFAND-5 in particular, should be reliable indicators of proteolysis in this species. Confirmation of this with measurements of protein degradation rates over the same timeframe might have been useful. Assuming that ZFAND-5 expression is indeed indicative of protein degradation, it was surprising that this gene was significantly more highly expressed in fish fed an adequate level of Met at all timepoints in Chapter Three, especially as protein retention efficiency was observed to be significantly increased in fish in this treatment. It was theorised that this may have been related to the significantly greater size of fish in this treatment. These larger fish may have had a concomitantly greater maintenance requirement for Met, negating the feedback effect of amino acid sufficiency on protein degradation. A similar result, however, was seen in response to excessive Tau consumption where fish size was not a factor. This latter trend in response to dietary Tau was also reflected in a significant upregulation of the TOR inhibiting gene Redd-1 between those fish fed an adequate and excessive level of dietary Tau. Combined, these observations were interpreted to suggest that excessive Tau may have a negative impact on protein retention and thus growth (with low synthesis and high degradation suggested), however this was not reflected in the measured protein retention efficiencies, suggesting further cross-validation of transcriptomic and phenomic responses may be required. Significant downregulation of Redd1 in the liver tissue of fish fed the Tau adequate diet suggests that adequate Tau does have a positive effect on protein synthesis in barramundi and, as with the somatotropic axis response described

above, may positively affect growth under normal conditions. Direct comparison of the physical amount of protein being synthesised and catabolised through these pathways was not made, however, so while patterns of stimulation or suppression of these pathways in response to amino acid supply have been reported here, the possibility remains that the actual rate of protein synthesis negated the differences in proteolysis seen. Alternatively, perhaps there are protein synthesis pathways, not considered in this study, that are more sensitive to Met supplementation. Markers of a number of such pathways were examined in the hepatic tissue of rainbow trout (*Oncorhynchus mykiss*) by Rolland *et al.* (2015) and were found to be affected by dietary Met level.

Understanding the pathways through which amino acids are catabolised and synthesised allows investigation of the sparing effect of one amino acid on its precursors or metabolites. Excess Met, for example, may spare Cys and Cys has been shown to spare Met (Twibell *et al.*, 2000; Goff and Gatlin, 2004), leading to these requirements being commonly expressed as a combined total sulphur amino acid (TSAA) requirement. In the present study, expression of genes of both the Met metabolising enzymes MAT-1 and MAT-2a and the Cys producing enzyme CGL were significantly upregulated two hours after feeding in both experiments, confirming that barramundi do utilise the pathway for conversion of Met to Cys and suggesting that inadequate supply of Cys may be ameliorated by supplemental Met. The expression profile of MAT-1 before feeding and two hours after feeding suggested that Met may have some influence on modulating this pathway, however no significant differences were seen, leading to theorisation that these genes may be regulated by the consumption of feed (protein), rather than simply Met supply itself. The reverse of this sparing effect, Cys sparing Met, however, is the more important consideration for plant based diets and was proven to also be active in this species, with no significant effect on growth of inclusion of up to 54% of the TSAA content of the diet as Cys observed. The observation that several genes of the metabolic pathway from Met to Cys are active supported this phenotypic response, suggesting that, when Cys is provided in excess, the proportion of Met which otherwise might have been used for Cys synthesis can be directed elsewhere, reducing its apparent requirement for inclusion in the diet.

A topic of great interest in the Tau nutrition of fish is the determination of the Tau biosynthetic capacity of the animal, which has been shown to be absent in some marine fish species (Yokoyama *et al.*, 2001). In the present study, this ability was suggested in the molecular response outlined in Chapter Three, where significant upregulation of selected taurine biosynthetic pathway genes (cysteine dioxygenase (CDO) and CSAD) was seen after feeding and in response to adequate supply of Met. This capacity was confirmed in Chapter 5 with, significantly, two alternative pathways of biosynthesis identified as being active. CSAD is the most widely studied indicator of Tau biosynthesis in fish (Goto *et al.*, 2001; Haga *et al.*, 2015) as it has traditionally been accepted as

the catalyst for the primary synthetic pathway. Salze and Davis (2015) suggested that the alternative pathway catalysed by cysteamine dioxygenase (ADO) could also be biologically significant in fish, although it has previously been observed to be comparatively low in activity (Haga *et al.*, 2015). The evidence presented here suggests that, while CSAD appears confirmed to be the major enzyme involved in this process, responding more vigorously to feed consumption, ADO may be more active when substrate is limiting.

Further refinement of current factorial growth and nutrient utilisation models requires consideration of the contribution of individual amino acids to the overall growth of the animal, rather than relying on assumed equivalencies of utilisation with protein. The significant differences in the allometric (nutrient loss) exponents for the individual amino acids seen in this study suggest that the utilisation of these dietary components changes with the size of the fish, possibly suggesting that requirements may also change. Incorporating this information into factorial models may have implications in the development or refinement of dietary formulations for different lifestages of the fish. A significant finding in this study was that the utilisation of non-protein nitrogen appears to be affected by fish size in this species, an observation not previously published in the fish nutrition literature to my knowledge. This represents further evidence that formulating diets based on a protein (i.e. nitrogen) requirement and establishing amino acid compositions in that diet from a fixed profile may not be optimal.

7.3 Limitations of the study

While several novel observations were made in this thesis relating to amino acid utilisation and the mechanisms underlying the responses seen, particularly as they relate to barramundi, some aspects of the experimental design may have impacted on these results. Interpretation of the results presented should, therefore, be considered in light of these constraints. Future iterations of experiments of this type may benefit from reflection on the limitations presented within.

The primary limitation of this series of studies may be the choice to utilise a restricted pair feeding regime. As outlined in Chapter Four, this may have had a major impact on the lack of significant response to variable levels of dietary Tau in that experiment. The molecular response suggested that Tau does, indeed, affect protein synthesis. This, however, was not reflected significantly in the PRE or the overall growth of the animals, suggesting some other factor was restricting protein deposition. Restricted pair feeding, whereby all treatments are fed an identical ration of feed assumed to be marginally below the satiety ration of the poorest feeding treatment, was used in an effort to separate the effects of feed intake from that of the treatment effects. Unfortunately, as fish size diverges in response to the treatment effects, the larger fish may require

more nutrients (including that being studied) to meet maintenance requirements, leaving less available for deposition and reducing the magnitude of the response to the nutrient of interest. Whether it is preferable to trade this restriction for the confounding effect of feed intake in a satiety fed trial is debatable. However, use of this approach did not greatly affect the responseof barramundi to variable Met levels (although, some greater separation in the response to Met above the apparent requirement may have changed the fit or choice of the model) but the lack of response to Tau detracts from the gravity of the results which may have been more pronounced under a different protocol. A viable alternative might be feeding to a proportion of body weight or the use of a satiety fed control for each treatment to deduce any effect of feed restriction.

An additional confounding factor in the response to variable Tau was the level of Met/TSAA used in the diets. For operational reasons, this experiment was designed and implemented before analysis and interpretation of the results of the TSAA requirement experiment was completed. Consequently, a level of TSAA only marginally below that at which growth was maximised in that study was used in the formulation of the Tau requirement study diets. Subsequent analysis of the data from that study predicted a minimum requirement well below this level. When studying the impact of the dietary provision of amino acids, it is advisable to take into consideration the supply of precursors for that amino acid. As this study has shown, barramundi are capable of synthesising Tau from Met and Cys. It is possible, then, that this level of TSAA was sufficient to meet a proportion of the Tau demands of the fish, masking a proportion of the effect of Tau supplementation. In hindsight, use of a TSAA inclusion level marginally below the minimum requirement (95% of the percent weight gain asymptote), rather than the maximum (99% of the asymptote) as used in the present study, may have yielded more significant results, reducing doubt surrounding the sparing effect of Met and Cys. While these precursors may have had some impact on the growth response, some effect remained apparent suggesting supply of precursors probably cannot completely fulfil the requirement for Tau.

While a specific confounding effect was not identified, it is questionable whether the choice of glycine (Gly) as the amino acid used for replacement of the test amino acid in the experiments described in Chapters Two and Four was ideal. This amino acid was chosen as it is a non-essential amino acid and has been used extensively in experiments of this type, including other studies defining TSAA requirements (Ruchimat *et al.*, 1997; Khan, 2014; Liao *et al.*, 2014). It is, however, a precursor for serine (Ser) which feeds directly into the sulphur amino acid metabolic pathway described in this thesis by combining with homocysteine and water to produce cystathionine. In effect, as inclusion of one precursor for cystathionine decreased, inclusion of the other increased (arriving at homocysteine via different pathways). It seems likely, due to the differing lengths of

each pathway and associated losses along the way, that Gly could contribute more significantly to this reaction, meaning that Met remains the first limiting amino acid as was intended. However, the efficiency of the Gly to Ser conversion was not measured so it cannot be proven what contribution this actually had. It might be advisable, nonetheless, to consider an alternative for future assessments, allowing this effect to be ruled out.

One of the main assumptions on which the interpretation of the differential gene expression measurements were based is that the expression of these genes is directly related to the activity of the pathway or enzyme of interest. In other words, that increased expression of a gene for a certain enzyme directly translates to increased production of that enzyme which, in turn, is assumed to result in increased activity and conversion of one compound to the next in the relevant pathway. As was discussed in Chapter Three, however, this may not necessarily be the case. The multitude of post-transcriptional mechanisms in the steps between mRNA production and the final protein often leads to variable correlation between protein and mRNA expression (Greenbaum *et al.*, 2003). It may be that the production of the enzymes we investigated is controlled at the translational level or later and, as such, the reported gene expression differences may only be indicative of differences in the activity of these enzymes (the amino acid may increase expression of the gene which *might* increase the incidence of the action of the enzyme). As a result, Panserat and Kaushik (2010), suggested that analysis of corresponding phenotypic parameters in conjunction with any molecular analyses was necessary for proper interpretation of these results. These may include assays quantifying the presence of the enzyme and/or post-translational modifications involved in activating the enzyme (e.g. SDS-PAGE/Western blot for detection of phosphorylation). It might have been advisable in the present study, then, to have performed these complementary enzyme assays for those enzymes for which gene expression was quantified as well as a more comprehensive metabolite profile. This might have given a clearer picture of the flow of compounds through the pathways and allowed exploration of the relationship between gene expression, enzyme production, activity and the resulting products. Certainly, future studies should consider this in their design. With the increasing use of molecular tools in the investigation of nutrient modulation of growth and metabolism in fish, and the associated challenges in properly interpreting the outputs, it is important that these relationships are properly characterised.

Finally, it is recognised that a major limitation in the interpretation of the allometric scaling exponent results is the assumption that the utilisation (i.e. losses) of the individual amino acids during starvation is directly representative of their utilisation in fed fish. A review by Young and el-Khoury (1995) proposes that the pattern of total daily obligatory amino acid losses in humans fed a protein free diet can be used to approximate essential amino acid requirements for negating this

loss (i.e. maintenance). This, however, is based on a further assumption that the standard metabolic rate and associated rate of protein synthesis remains unchanged, regardless of nutritional state. The rate of protein synthesis has been observed in several fish species to be reduced during feed deprivation (O'Connor *et al.*, 2000) which will, in turn affect the demand for those amino acids required for protein synthesis, possibly leading to underestimation of requirements in fed fish. In addition, protein synthesis in fish has been said to account for a significant proportion of the metabolic rate of the animal (Houlihan *et al.*, 1988). The reported increased rate of protein synthesis with feeding, then, will also translate to an increased metabolic rate, leading to concomitant increases in demands for those amino acids involved in metabolic processes besides protein turnover. As well, a greater proportion of the maintenance and total requirement for those amino acids may be independent of the rate of metabolism or protein turnover if they are involved in ongoing basal metabolic processes such as osmoregulation. This may be supported by the significant differences reported in this thesis between the protein and individual amino acid allometric exponents which suggest that, even within the proteinaceous amino acids, the utilisation of individual amino acids do not respond in the same manner to the changing protein synthetic rate with increasing fish size. As discussed in Chapter Six, this is most likely a function of their differing metabolic roles and how these may change with fish size. The branched chain amino acids, for example, were all observed to have exponents below that of protein, indicating a higher relative demand in smaller fish, an observation proposed to be linked in part to their role in oxygen transport (Brosnan and Brosnan, 2006) and the fact that they are rapidly catabolised in the muscle (Li *et al.*, 2009), two things which are likely to occur at a greater relative rate in smaller fish given their higher metabolic rate (Brett and Glass, 1973). Further investigation into changes in amino acid utilisation between fed and unfed fish may be necessary to assess the utility of the use of the individual amino acid scaling exponents.

7.4 Conclusions and future directions

The experiments described in this thesis aimed to define the dietary requirements of juvenile barramundi for Met and Tau, to elucidate the mechanisms by which these amino acids affect growth and nutrient turnover and to define the allometric scaling of individual amino acid loss during starvation in this species. These aims were largely met, to varying degrees of certainty and with caveats identified to the interpretation of the results. Several limitations were recognised and their impact on the observed responses acknowledged. Nonetheless, I believe that the results presented in this thesis, and interpretation of these results, represent a significant progression in our understanding of sulphur amino acid metabolism in fish in general, and barramundi specifically.

This new knowledge provides valuable information for the further development of more productive and sustainable dietary formulations, as well as advancement of growth and nutrient utilisation modelling, for this and other fish species.

It was clear that Met is an amino acid essential to the proper growth and metabolic functioning of this species. A significant response to the variable dietary supply of this amino acid was observed and a range of requirements defined based on the observed responses. This assessment allows interested parties to choose the value most relevant to their purposes, be it that which maximises growth or optimises the cost vs. benefit relationship. Significantly for feed formulators, the inclusion of up to 54% of TSAA as Cys, within the total requirement for TSAA, is possible without significantly affecting growth. This may be especially relevant when considering the inclusion of ingredients such as soybean meal which, while low in Met, is relatively abundant in Cys. A limit of 40% of the total TSAA content, however, is suggested as optimal for protein retention efficiency. Results of the present study failed to identify the underlying mechanism for the observed positive effect of Met on growth. A longer-term effect on IGF-I expression was seen but it was unclear if this was a direct effect of Met on this hormone. Significantly, however, the observation that two forms of MAT were suggested to be simultaneously active in these fish has not previously been made in any fish species and may have implications for future research attempting to detect molecular evidence of Met turnover. The expression profiles of several genes in this study, particularly those of the SAA and protein turnover pathways, did not correlate well with the phenotypic observations. There are several reasons this may have occurred, however it is clear that much more work is needed to be done on the relationship between substrate availability; enzyme production, activation and activity; and expression of these genes.

Defining a requirement for Tau was less straightforward than for Met, due to the less pronounced differences in weight gain between treatments, likely due in part to an oversupply of TSAA in the “optimal TSAA” treatments designed to establish the requirement as well as the possible confounding effect of the restricted pair-feeding regime discussed previously. It might have been more suitable to use a TSAA inclusion marginally deficient of the lowest recommended requirement, rather than of the level maximising growth. Consideration of the impact of precursor supply on the estimation of requirements for non-essential nutrients is clearly important. The molecular evidence seemed to show an effect of Tau on growth and protein deposition in this species, suggesting that the magnitude of the effect may have been more pronounced in the absence of the confounding factors. The synthesis of Tau from precursors has previously been reported in a number of fish species, however, the observation that barramundi use two alternative pathways, which appear to be active at different times, is relatively novel. As with the MAT observation,

consideration of both enzyme markers then may be necessary for comprehensive assessment of Tau biosynthesis in this species. Confirmation of the Tau biosynthetic capacity of barramundi is an important observation in itself, given the variability seen even in the marine carnivorous fishes. It was on the basis of this that Tau was declared conditionally essential to this species, dependent on the supply of dietary TSAA. Nevertheless, the homology of the derived requirement with those published for other fish species suggests it might be advisable for feed formulators to consider the requirement presented herein as an optimal level to aim for, regardless of the supply of TSAA. Surprisingly, Tau was not proven to have any sparing effect on Met, suggesting these requirements should be considered separately in feed formulations.

The derivation of allometric scaling exponents for the individual amino acids is, to my knowledge, the first of its kind in fish. While it has been suggested previously that the turnover of individual amino acids may differ in other animals (Baker, 1991), the results presented in this thesis are the first to show that their relative demands also change with fish size at a different rate to each other, as well as to that of protein, contrary to the assumptions of the ideal protein concept often used in conjunction with current factorial fish growth and nutrient utilisation models. Consideration of this changing demand through the incorporation of the derived exponents in place of the protein metabolic weight exponent in these models may improve the accuracy of their outputs over attention to the protein requirements alone. The absence of improvement in the utilisation efficiency model fit when the Met body weight exponent was used in place of the protein exponent was surprising given the significantly different exponent values, however, the case may be different for other amino acids or when all are considered together. Despite the small differences in utilisation efficiencies calculated, differences were seen in the predictions of maintenance demands for this amino acid over varying fish size, suggesting the utility of this exponent may be dependent on the size of the fish.

Perhaps the most significant finding of this experiment was the apparent effect of fish size on the utilisation of NPN, with smaller fish appearing to lose relatively more nitrogen as NPN than larger fish, suggesting a greater utilisation of this source of nitrogen. Defining the contribution of individual sources of NPN (e.g. biogenic amines, nucleic acids and non-proteinogenic amino acids) to overall nitrogen demands and quantifying their presence in feed ingredients may be an important consideration. The use of NPN as a source of nitrogen in fish has not been studied extensively but could be a valuable avenue of further investigation and may add significantly to nutrient modelling. Further, this result highlights a limitation of current methods of “protein” analysis where measured nitrogen contents are assumed to convert directly to crude protein. It may be useful in future studies

investigating protein and/or amino acid nutrition of fish to quantify the NPN proportion of the diets and/or body “protein”.

In summary, the outcomes of this thesis have identified several topics which require further consideration moving forward:

- Continued consideration of the use of quadratic-based regression modelling for the accurate assessment of nutrient requirements in aquatic species.
- Promotion of the need to consider the *total* sulphur amino acid (Met+Cys) requirements of the animal, rather than that of Met alone.
- More consensus on the most appropriate mode of expression of amino acid requirements.
- More comprehensive assessments of the link between the production, activation and activity of individual enzymes and the expression of their associated genes; as well as between molecular and confirmed biochemical markers of other important processes, such as protein turnover. This work is central to advancing the use of molecular techniques in aquaculture nutrition, an approach which represents an opportunity for rapid simultaneous assessment of the influence of individual nutrients on a multitude of metabolic pathways and their interaction with each other and other factors of interest such as environmental conditions and nutritional or health status of the animal.
- Further assessment of the most appropriate method by which to fix feeding rations in nutritional studies in order to reduce the impact of feed intake (whether that be an effect of intentional restriction or of ingredient palatability independent of the studied nutrient).
- Development of factorial growth models that incorporate the variable utilisation and allometric effects of individual amino acids, in addition to that of non-protein nitrogen.
- Elucidation of the sources of non-protein nitrogen in the carcass of the fish species under study and assessment of the utility of their supplementation in future feed formulations. This may be especially important if these formulations are based primarily on individual amino acid requirements, rather than that of crude protein.

7.5 References

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Appendix A

Postprandial free amino acid fluctuations in response to variable dietary methionine supply (Chapter Three supplemental data).

Table A1. Concentrations of individual free amino acids present in the plasma of juvenile barramundi over a 24 hour period following consumption of a single meal containing a deficient (MetDEF), level of dietary methionine.

Time after feeding (hours)	0h	1h	2h	4h	8h	12h	24h
EAA concentrations (nmol ml^{-1})							
Asp	12 (±0.2)	23 (±8.0)	17 (±10.1)	1 (±0.1)	9 (±1.4)	10 (±0.9)	9 (±0.9)
Ser	43 (±1.6)	201 (±11.8)	221 (±46.8)	138 (±20.3)	130 (±19.9)	79 (±7.0)	67 (±3.9)
Glu	19 (±1.9)	34 (±5.7)	37 (±7.7)	23 (±0.8)	26 (±1.6)	29 (±3.1)	23 (±2.6)
Gly	155 (±8.4)	410 (±41.5)	371 (±111.2)	184 (±10.6)	229 (±14.7)	239 (±7.3)	230 (±8.0)
His	54 (±2.9)	153 (±7.4)	170 (±38.7)	107 (±16.3)	96 (±7.1)	79 (±3.4)	72 (±7.4)
Tau	52 (±3.9)	239 (±24.5)	317 (±38.0)	207 (±28.7)	157 (±0.3)	77 (±14.9)	66 (±4.8)
Arg	139 (±20.3)	435 (±36.6)	479 (±117.8)	228 (±13.3)	218 (±8.5)	229 (±25.8)	176 (±11.5)
Thr	68 (±2.2)	301 (±35.8)	299 (±59.6)	190 (±34.1)	152 (±29.2)	105 (±9.1)	77 (±9.2)
Ala	94 (±7.2)	195 (±12.7)	214 (±50.4)	119 (±23.6)	8 (±0.5)	84 (±6.6)	70 (±3.3)
Pro	31 (±0.6)	120 (±8.2)	156 (±26.4)	97 (±26.4)	127 (±1.6)	57 (±10.6)	37 (±3.0)
Cys	9 (±0.5)	15 (±1.3)	18 (±4.6)	28 (±1.7)	19 (±3.0)	11 (±1.9)	15 (±2.3)
Tyr	348 (±75.2)	611 (±232.2)	667 (±396.2)	724 (±208.1)	597 (±354.8)	792 (±83.8)	690 (±244.2)
Val	43 (±1.2)	298 (±36.3)	342 (±67.0)	266 (±47.2)	255 (±67.0)	130 (±29.8)	59 (±4.8)
Met	11 (±0.7)	28 (±1.9)	34 (±4.3)	25 (±1.8)	26 (±4.5)	14 (±1.6)	10 (±0.9)
Lys	43 (±0.4)	186 (±15.4)	243 (±36.0)	181 (±27.7)	140 (±19.3)	71 (±12.7)	53 (±4.2)
Ile	25 (±0.6)	142 (±15.0)	174 (±30.2)	159 (±29.9)	159 (±23.6)	67 (±17.3)	29 (±1.4)
Leu	37 (±1.2)	259 (±15.8)	335 (±52.3)	285 (±47.7)	286 (±44.2)	126 (±32.7)	51 (±4.5)
Phe	33 (±0.9)	134 (±9.5)	163 (±32.3)	167 (±22.7)	180 (±58.6)	112 (±18.4)	50 (±6.0)
Total Free EAA	1045 (±98.6)	3544 (±263)	3940 (±875.7)	2869 (±306.2)	2470 (±456.1)	2233 (±150.7)	1716 (±205.5)

Table A2. Concentrations of individual free amino acids present in the plasma of juvenile barramundi over a 24 hour period following consumption of a single meal containing an adequate (MetADQ), level of dietary methionine.

Time after feeding (hours)	0h	1h	2h	4h	8h	12h	24h
EAA concentrations (nmol ml^{-1})							
Asp	14 (±2.4)	7 (±3.1)	10 (±1.8)	1 (±0.2)	8 (±0.9)	8 (±2.3)	8 (±0.4)
Ser	59 (±5.8)	144 (±12.6)	158 (±6.7)	157 (±23.5)	93 (±11.9)	73 (±4.3)	57 (±1.7)
Glu	23 (±0.4)	20 (±2.5)	25 (±2.7)	23 (±3.0)	22 (±1.9)	29 (±9.6)	26 (±1.6)
Gly	226 (±15.4)	308 (±35.6)	280 (±47.6)	186 (±10.1)	196 (±14.3)	210 (±13.8)	246 (±20.1)
His	71 (±4.2)	121 (±12.3)	137 (±9.0)	120 (±14.4)	81 (±5.9)	75 (±8.8)	68 (±2.8)
Tau	69 (±7.3)	217 (±15.9)	245 (±10.2)	182 (±30.5)	105 (±2.5)	83 (±10.6)	80 (±10.1)
Arg	201 (±26.9)	335 (±25.9)	435 (±61.4)	229 (±26.5)	199 (±14.1)	232 (±72.2)	213 (±16.2)
Thr	128 (±4.5)	308 (±14.9)	321 (±19.5)	220 (±38.9)	142 (±19)	123 (±11.4)	130 (±3.9)
Ala	90 (±3.2)	165 (±7.9)	187 (±23.2)	191 (±41.7)	8 (±0.1)	110 (±29.8)	92 (±13.0)
Pro	31 (±1.5)	100 (±3.3)	124 (±13.4)	123 (±16.3)	71 (±10.5)	55 (±11.9)	40 (±3.7)
Cys	10 (±1.1)	11 (±0.5)	15 (±1.3)	33 (±9.6)	17 (±1.1)	14 (±3.1)	9 (±1.5)
Tyr	561 (±3.1)	866 (±147.3)	498 (±277.9)	432 (±60.3)	662 (±60.8)	829 (±55.9)	416 (±159.1)
Val	56 (±5.1)	262 (±32.2)	298 (±11.3)	225 (±32.3)	185 (±28.0)	141 (±34.9)	57 (±4.2)
Met	31 (±3.8)	106 (±8.4)	115 (±6.2)	77 (±14.9)	51 (±0.6)	41 (±4.0)	26 (±1.5)
Lys	43 (±7.1)	186 (±15.8)	196 (±4.4)	164 (±27.3)	93 (±5.9)	63 (±7.5)	40 (±4.6)
Ile	31 (±2.4)	127 (±14.6)	148 (±6.5)	131 (±18.4)	102 (±17.2)	75 (±19.4)	30 (±1.9)
Leu	50 (±4.3)	232 (±17.4)	311 (±7.2)	238 (±34.4)	190 (±26.2)	135 (±35.4)	51 (±3.8)
Phe	37 (±2.3)	130 (±2.5)	148 (±24.9)	130 (±23.8)	165 (±12.8)	117 (±29.6)	41 (±2.5)
Total Free EAA	1476 (±188.5)	3429 (±125.6)	3406 (±387.2)	2679 (±351.1)	2009 (±345.2)	2329 (±287.3)	1551 (±175.5)

Table A3. Concentrations of individual free amino acids present in the plasma of juvenile barramundi over a 24 hour period following consumption of a single meal containing an excessive (MetEXC), level of dietary methionine.

Time after feeding (hours)	0h	1h	2h	4h	8h	12h	24h
EAA concentrations (nmol ml^{-1})							
Asp	11 (±3.0)	7 (±1.9)	6 (±2.8)	0 (±0.2)	9 (±1.2)	7 (±0.9)	6 (±0.6)
Ser	53 (±3.1)	124 (±17.9)	131 (±22.2)	159 (±29.5)	115 (±4.4)	75 (±21.1)	49 (±1.8)
Glu	22 (±2.4)	19 (±3.6)	23 (±4.0)	22 (±2.6)	29 (±3.7)	28 (±3.5)	22 (±1.4)
Gly	210 (±32.3)	175 (±38.6)	211 (±26.2)	152 (±10.6)	178 (±3.3)	217 (±22.9)	243 (±11.6)
His	66 (±4.2)	102 (±19.1)	115 (±19.9)	123 (±21.5)	92 (±2.9)	87 (±15.7)	66 (±0.5)
Tau	76 (±8.0)	190 (±58.0)	257 (±53.9)	222 (±39.8)	125 (±9.4)	92 (±19.8)	68 (±2.6)
Arg	200 (±54.5)	263 (±64.2)	375 (±51.9)	273 (±26.0)	244 (±39.3)	333 (±83.2)	212 (±4.0)
Thr	112 (±10.9)	270 (±75.6)	341 (±75.8)	326 (±67.1)	204 (±28.7)	128 (±40.4)	129 (±1.6)
Ala	103 (±10.9)	141 (±48.0)	220 (±37.0)	214 (±42.0)	8 (±0.4)	132 (±39.8)	76 (±4.8)
Pro	35 (±0.4)	102 (±22.9)	146 (±23.6)	139 (±31.1)	100 (±11.7)	63 (±19.4)	31 (±5.3)
Cys	10 (±0.4)	13 (±1.0)	20 (±6.7)	25 (±0.6)	17 (±3.4)	14 (±2.5)	8 (±0.9)
Tyr	28 (±0.2)	56 (±0.2)	536 (±177.8)	596 (±129.0)	572 (±12.8)	656 (±156.7)	350 (±189.4)
Val	60 (±1.5)	234 (±55.7)	273 (±53.0)	297 (±58.5)	243 (±52.5)	153 (±48.1)	53 (±4.0)
Met	26 (±3.0)	156 (±43.3)	205 (±50.3)	240 (±34.9)	128 (±27.5)	66 (±18.7)	27 (±0.2)
Lys	42 (±4.2)	138 (±33.5)	194 (±47.6)	196 (±39.7)	116 (±15.5)	63 (±19.3)	31 (±3.6)
Ile	32 (±0.7)	113 (±23.9)	138 (±26.7)	159 (±30.8)	138 (±27.6)	83 (±28.4)	28 (±2.5)
Leu	53 (±1.2)	212 (±31.1)	269 (±49.5)	303 (±47.8)	249 (±57.2)	138 (±41.6)	46 (±3.6)
Phe	33 (±1.2)	101 (±31.1)	128 (±25.9)	187 (±12.7)	161 (±38.6)	111 (±29.1)	44 (±6.0)
Total Free EAA	1086 (±111.4)	2165 (±478.2)	3331 (±361.8)	3409 (±361)	2603 (±308)	2354 (±231.6)	1423 (±213.1)

Appendix B

Postprandial free amino acid fluctuations in response to variable dietary taurine supply (Chapter Five supplemental data).

Table B1. Concentrations of individual free amino acids present in the plasma of juvenile barramundi over a 24 hour period following consumption of a single meal containing a deficient (TauDEF), level of dietary taurine.

Time after feeding (hours)	0h	1h	2h	4h	8h	12h	24h
	EAA concentrations (nmol ml^{-1})						
Asp	9 (±1.1)	5 (±1.2)	6 (±0.4)	7 (±2)	4 (±0.6)	4 (±1.3)	5 (±1.5)
Ser	25 (±5.2)	66 (±4.9)	72 (±2.7)	79 (±3.1)	64 (±9.8)	51 (±7.8)	21 (±1.3)
Glu	11 (±1)	9 (±0.6)	10 (±1.1)	10 (±1.2)	10 (±0.5)	11 (±1.3)	12 (±1.6)
Gly	396 (±17.2)	340 (±50.6)	316 (±28.6)	286 (±7.6)	264 (±42.8)	245 (±36.6)	374 (±77.1)
His	11 (±0.3)	13 (±0.7)	17 (±1.4)	25 (±3.6)	22 (±6.2)	29 (±3.3)	28 (±1.8)
Tau	206 (±11.9)	176 (±25.9)	132 (±21.2)	76 (±1.7)	73 (±5)	78 (±12)	142 (±41)
Arg	76 (±1.1)	249 (±7.7)	259 (±17.1)	272 (±4.1)	228 (±41.9)	151 (±3.8)	72 (±8.6)
Thr	196 (±27.7)	292 (±12.6)	314 (±18.7)	350 (±7.7)	376 (±75)	281 (±37.9)	149 (±17.9)
Ala	74 (±6.5)	167 (±13.3)	210 (±23.6)	145 (±5)	169 (±31.4)	128 (±2.5)	82 (±2.7)
Pro	21 (±0.6)	88 (±5.2)	136 (±10.3)	112 (±9.2)	121 (±20.7)	100 (±6.2)	26 (±2.2)
Cys	179 (±24.1)	258 (±11)	278 (±16.3)	314 (±6.5)	282 (±16.2)	259 (±34.2)	143 (±18)
Tyr	20 (±0.2)	61 (±2.1)	70 (±6.8)	66 (±2.4)	61 (±12.2)	61 (±8.3)	31 (±0.8)
Val	60 (±1.3)	277 (±5.9)	338 (±13.8)	402 (±5.9)	470 (±99.9)	432 (±75.4)	70 (±3.6)
Met	24 (±1.1)	79 (±2.4)	86 (±6.4)	96 (±1.1)	83 (±18.9)	39 (±3.5)	20 (±0.4)
Lys	52 (±2.4)	186 (±4.8)	211 (±13.8)	214 (±6.7)	200 (±39.9)	137 (±9.5)	50 (±2.9)
Ile	32 (±0.8)	153 (±3.6)	193 (±6)	201 (±2.9)	226 (±45.3)	210 (±31.5)	34 (±2.5)
Leu	57 (±1.6)	309 (±9.3)	376 (±10.6)	419 (±7.1)	466 (±90.4)	440 (±82)	64 (±3.8)
Phe	31 (±0.6)	85 (±7)	101 (±9.3)	132 (±7.3)	140 (±31.6)	123 (±12.4)	39 (±2.8)
Total Free EAA	1480 (±26)	2812 (±108)	3125 (±187.5)	3206 (±16.7)	3259 (±573.1)	2779 (±334)	1364 (±169.3)

Table B2. Concentrations of individual free amino acids present in the plasma of juvenile barramundi over a 24 hour period following consumption of a single meal containing an adequate (TauADQ), level of dietary taurine.

Time after feeding (hours)	0h	1h	2h	4h	8h	12h	24h
EAA concentrations (nmol ml^{-1})							
Asp	4 (±0.3)	5 (±0.5)	8 (±1.5)	5 (±1.1)	4 (±0.7)	4 (±1.3)	4 (±0.7)
Ser	19 (±1.3)	62 (±6.7)	56 (±4.2)	65 (±6.4)	57 (±8.6)	35 (±8.8)	15 (±0.6)
Glu	8 (±0.7)	8 (±0.3)	10 (±1.5)	9 (±0.7)	8 (±1.5)	10 (±0.6)	11 (±0.8)
Gly	259 (±9.8)	272 (±14.8)	297 (±35.3)	245 (±10.5)	180 (±20.5)	176 (±19.4)	209 (±16.8)
His	7 (±0.9)	12 (±1)	18 (±2.2)	22 (±1.8)	31 (±3.1)	22 (±1.7)	25 (±1)
Tau	223 (±6.4)	309 (±19)	419 (±55.4)	347 (±17)	259 (±45.3)	221 (±31.8)	247 (±31.4)
Arg	57 (±4.5)	257 (±24.5)	268 (±5.8)	265 (±10.4)	235 (±22.8)	143 (±32.7)	80 (±6.1)
Thr	160 (±15.5)	277 (±16.3)	298 (±27.2)	338 (±1.7)	349 (±50)	267 (±46)	150 (±9.9)
Ala	70 (±5.5)	155 (±22.5)	193 (±23)	198 (±16.4)	158 (±22.7)	120 (±22.4)	73 (±5.5)
Pro	14 (±1.3)	82 (±11.8)	128 (±14.2)	141 (±6.2)	120 (±14.1)	96 (±20.9)	27 (±6.5)
Cys	147 (±13.3)	247 (±12.1)	264 (±23.4)	303 (±0.7)	315 (±43.5)	245 (±40.9)	145 (±8.8)
Tyr	19 (±1.1)	60 (±9.3)	66 (±10.1)	62 (±4.5)	62 (±8.7)	57 (±5)	29 (±6.9)
Val	56 (±3.1)	237 (±30.1)	305 (±28.5)	388 (±16.2)	442 (±69.5)	378 (±90.7)	89 (±24.8)
Met	26 (±1.6)	73 (±6.9)	81 (±3.2)	93 (±2.6)	84 (±10.8)	40 (±7.8)	18 (±0.4)
Lys	48 (±5.3)	172 (±18.5)	206 (±6.2)	233 (±12)	200 (±22.1)	133 (±25.2)	57 (±5.8)
Ile	32 (±2.8)	133 (±19.1)	164 (±17.2)	201 (±9.6)	212 (±32.6)	187 (±44)	42 (±11.8)
Leu	56 (±4.2)	269 (±37.6)	329 (±32.8)	415 (±21.8)	457 (±73.1)	385 (±97.3)	82 (±23.9)
Phe	28 (±1)	76 (±7.9)	91 (±7.7)	110 (±7.7)	142 (±29.9)	120 (±11.5)	45 (±7.5)
Total Free EAA	1233 (±47.3)	2707 (±250.4)	3200 (±264.8)	3441 (±83.7)	3316 (±465.4)	2640 (±501)	1351 (±127)

Table B3. Concentrations of individual free amino acids present in the plasma of juvenile barramundi over a 24 hour period following consumption of a single meal containing an excessive (TauEXC), level of dietary taurine.

Time after feeding (hours)	0h	1h	2h	4h	8h	12h	24h
EAA concentrations (nmol ml^{-1})							
Asp	5 (±0.3)	8 (±2.7)	11 (±2.2)	4 (±0.5)	5 (±0.2)	3 (±0.1)	4 (±0.7)
Ser	19 (±1.1)	56 (±7.8)	47 (±2.1)	51 (±4.4)	38 (±5.1)	25 (±3.7)	13 (±1.7)
Glu	9 (±0.2)	10 (±1.2)	10 (±1.3)	9 (±0.4)	10 (±0.4)	12 (±1.2)	12 (±0.8)
Gly	220 (±6.8)	217 (±21.4)	236 (±18.1)	165 (±12.8)	168 (±17.9)	174 (±31.1)	190 (±18.3)
His	9 (±0.5)	18 (±1.7)	19 (±1.4)	27 (±4.2)	30 (±1.4)	25 (±1.9)	27 (±1.8)
Tau	256 (±31.3)	496 (±66.4)	559 (±39.4)	474 (±8.9)	498 (±35.9)	361 (±57.9)	268 (±52.4)
Arg	62 (±5.2)	310 (±17.3)	272 (±6.1)	291 (±23.4)	248 (±8)	145 (±10.5)	75 (±4.4)
Thr	149 (±10.9)	296 (±12.5)	299 (±15.3)	311 (±20.2)	327 (±11.3)	216 (±17.4)	164 (±9.6)
Ala	83 (±3.8)	206 (±13.9)	232 (±17.4)	217 (±26.1)	177 (±19)	122 (±22.5)	82 (±7.1)
Pro	17 (±1.5)	125 (±6.5)	151 (±8.1)	156 (±13.7)	129 (±10.9)	84 (±15.7)	22 (±2.2)
Cys	135 (±10.1)	261 (±8.6)	266 (±10.9)	276 (±18.9)	298 (±10.6)	200 (±17.9)	154 (±7.3)
Tyr	20 (±0.7)	79 (±1.8)	81 (±3.6)	74 (±6.2)	75 (±9.8)	57 (±11.3)	25 (±1)
Val	58 (±2.9)	307 (±15.4)	322 (±22.7)	420 (±42.7)	471 (±45.3)	289 (±13.6)	69 (±3.8)
Met	25 (±0.6)	95 (±1.8)	85 (±4.6)	101 (±11.7)	87 (±4.6)	36 (±3.6)	21 (±0.8)
Lys	51 (±5.3)	226 (±11.6)	219 (±5.6)	246 (±22.4)	210 (±10.5)	124 (±7.6)	59 (±2.8)
Ile	33 (±1.7)	177 (±8.8)	183 (±12.6)	225 (±23.5)	233 (±24.8)	144 (±12.4)	34 (±2.5)
Leu	58 (±2.9)	353 (±15.7)	354 (±26.9)	453 (±47.7)	490 (±42.6)	295 (±16.5)	63 (±3.6)
Phe	30 (±1.7)	108 (±11.5)	113 (±0.4)	121 (±8.8)	151 (±24.6)	111 (±14.3)	42 (±2.5)
Total Free EAA	1238 (±72.3)	3346 (±184)	3460 (±165.3)	3619 (±281.7)	3647 (±439.6)	2423 (±199.3)	1322 (±119.1)