Postprandial changes in plasma free amino acids and hepatic gene expression in response to variable methionine inclusion in the diet of barramundi (*Lates calcarifer*).

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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 of this species for dietary Met/TSAA, three treatments were selected (with deficient (DEF; 8.6g/kg), adequate (ADQ; 14.9g/kg) and excessive (EXC; 21.4g/kg)) levels of dietary Met, based on their respective growth responses. Amino acid analysis indicated a peak in plasma free Met at 2h post-feeding in fish fed the DEF and ADQ diets and at 4h post-feeding in fish in the EXC 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 the Met turnover (CGL, MAT1) and taurine biosynthetic pathways (CSAD, ADO, CDO), target of rapamycin signalling (Redd-1), the somatotropic axis (GHR-II, IGF-1, IGF-II) and protein turnover (Mul-1, ZFAND-5) pathways. Sulphur amino acid turnover was 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, rather than by the composition. Further, metabolised Met appeared likely to have been directed through SAM-dependent pathways, rather than converted to Cys, which may have contributed to the observed growth response. Both genes influencing MAT appear to be active at this lifestage in barramundi. The importance of Tau synthesis to this species was highlighted by longer-term differences in biosynthetic enzyme gene expression responses between treatments. 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 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 isolated for the first time in this species and shown to be nutritionally regulated.

Accepted refereed manuscript of: Poppi DA, Moore SS, Wade NM & Glencross BD (2019) 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. Aquaculture, 501, pp. 345-358. DOI: https://doi.org/10.1016/j.aquaculture.2018.11.044

1 Introduction

Consideration of essential amino acid inclusion has become increasingly important in the formulation of diets for carnivorous fish species 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 a number of important roles in *fish metabolism*. It, and its metabolite cysteine (Cys) are two of the 20 canonical 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.

Dietary sulphur amino acid supply has been linked to growth modulation through a number of 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 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 and cellular protein synthesis (Clemmons and Underwood, 1991) and has been shown by Rolland *et al.* (2015) to be affected, directly or indirectly, by Met availability in diets for rainbow trout.

The availability of amino acids for these processes can be directly influenced by the availability of the precursor amino acids required for their synthesis, when limiting, and by the requirement for synthesis of product amino acids. These amino acid biosynthetic/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 these amino acid biosynthetic enzymes (Fafournoux *et al.*, 2000) but few studies have investigated the relationship between the levels of these compounds and the expression of the genes responsible for the production of these enzymes 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, therefore, was to investigate the mechanisms by which barramundi metabolise Met and what effect the level of its supply in the diet had on some important growth-related metabolic pathways.

2 Materials and Methods

2.1 Diets

Three diets (Table 1) were selected from a previously published TSAA requirement growth trial (Poppi et al., 2017?): those that elicited the minimum and maximum growth responses, as well as one with a clearly excessive level of Met/TSAA (which had apparently approached a toxic level). These diets were designated as either Met deficient (DEF; 8.6g/kg), adequate (ADQ; 14.9g/kg) or excessive (EXC; 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 for the requirement study on a laboratory-scale twin-screw extruder (APV MFP19:25; APV-Baker, Peterborough, UK), with intermeshing, co-rotating screws following the protocol of Glencross *et al.* (2016), incorporating a 3mm diameter die in order to produce pellets of a 4mm diameter.

2.2 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).

2.2.1 Preconditioning

Twenty one juvenile barramundi (*Lates calcarifer*) (mean initial weight: 116g) were housed in each of three 1000L tanks per treatment, having been randomly assigned to these tanks at the commencement of a 49 day growth trial preceding the current experiment. 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.

2.2.2 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 fish consumed the same average ration to negate any impact of feed intake on the postprandial response.

2.3 Sampling

On the day of sampling, mean weights of the fish in each treatment were as follows: DEF (132g), ADQ (135g), EXC (132.4g). Three fish were randomly selected from each tank 24 hours after their last meal and euthanased by overdose of anaesthetic (AQUI-S) (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 for 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.

2.4 Chemical analyses

Plasma samples were deproteinised by addition of 380µl acetonitrile to 60µl each of plasma and 500µM internal standard (α-Aminobutyric acid) according to the protocol of Davey and Ersser (1990). Samples were centrifuged at 2200g for four minutes at 4^0C and the supernatant extracted for analysis. 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 quaternary gradient module, coupled with a Shimadzu RF-20A XS prominence fluorescence detector using the Waters AccQ·tag system (Waters Corporation, Milford, MA).

2.5 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). 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).

2.6 Quantitative real-time RT-PCR

The differential expression of several genes associated with the somatotropic index and sulphur amino acid and protein turnover pathways was assessed by real-time PCR 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, showing a high level of homology with published sequences of known genes from related species. 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 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.

2.7 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. Statistical analyses were conducted in the R-project statistical environment (R Core Team, 2014).

3 Results

3.1 Plasma amino acid contents

As was expected, plasma amino acid levels fluctuated significantly over time after feeding (Figure 1, Figure 2 andFigure 3), peaking between one and four hours post-feeding and returning 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).

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) (Figure 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 (Figure 2) was significantly higher ($p<0.001$) in fish fed the EXC diet than those in the DEF treatment at the pre-feeding timepoint and further diverged at 1h post-feeding, being significantly higher in fish fed the EXC diet than those fed the ADQ diet, which, in turn had significantly greater levels than those in the DEF treatment. This significant separation was maintained until 8h after the meal. Similarly, plasma Gly (Figure 2) levels were significantly lower in fish fed the EXC diet than those fed the DEF diet at 1h post-feeding.

3.2 Gene expression

3.2.1 Met and Cys metabolism

The differential expression of three genes (MAT-1, MAT-2b and 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 Figure 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 diet was observed.

3.2.2 Taurine metabolism

The differential expression of two genes (CDO and CSAD) involved in the synthesis of Tau from precursor amino acids in barramundi fed the three experimental diets was assessed and is presented in Figure 5. A significant effect of time on expression of both genes was observed

($p<0.001$; 0h=4h; 0h<2h>4h). Additionally, CDO expression was significantly affected by diet ($p<0.05$; DEF=ADQ; ADQ=EXC; DEF<EXC), with a significant diet:time interaction effect ($p<0.05$) also observed ($p<0.05$;EXC>DEF at 0h timepoint).

3.2.3 Somatotropic index

The effect of dietary Met intake on expression of selected genes of the somatotropic index (IGF-I, IGF-II and GHR-II) over time was observed (Figure 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 ($p<0.001$; DEF>ADQ>EXC) was observed. Expression of IGF-II was additionally significantly affected by diet ($p<0.05$; DEF=ADQ; ADQ=EXC; DEF<EXC). A significant diet:time interactive effect on the expression of IGF-I was also observed ($p>0.05$; ADQ>DEF at 0h timepoint).

3.2.4 Protein degradation

The differential gene expression of two indicators of protein degradation (MUL1 and ZFAND-5) in the hepatic tissue of fish fed the three experimental diets was assessed and is presented in Figure 7. A significant effect of time ($p<0.001$; 0h=2h; 0h>4h; 2h>4h) and a significant diet:time interaction effect ($p<0.05$; 2h>4h for DEF;0h>4h for ADQ) on expression of MUL1 was observed. ZFAND-5 expression was significantly affected by both time ($p<0.001$; 0h>4h; 0h>2h>4h) and diet ($p<0.01$; DEF=ADQ; DEF=EXC;ADQ>EXC).

3.2.5 TOR activity signalling (protein synthesis).

Redd1 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 gene fragments in a partial barramundi transcriptome. The effect of the dietary treatments on expression of this gene in barramundi hepatic tissue is presented in Figure 8. Whilst large variation in expression level can be seen within the ADQ 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 diet:time interaction ($p<0.05$; ADQ>DEF at 0h timepoint).

4 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” of 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). This has led to a renewed need to properly characterize the true requirement of the animal for individual amino acids in order to optimise the use of these more sustainable sources of protein. The re-evaluation of the requirement of barramundi for Met/Total Sulphur Amino Acids (TSAA) conducted prior to the current experiment concluded the requirement to be between 10.5 and 13.6g kg^{-1} of diet dry matter (1.8-2.3% CP). While it's important to know this requirement figure, it's equally as important to define why the animal requires this dietary component. Does it have direct effects on metabolic processes or can other dietary components, which may be more abundant in available ingredients fulfil the same roles? Cys, for example, which is relatively abundant in Met-deficient soybean meal, was 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, in Poppi et al. (2017). In the present study, we investigated the effect of variable dietary Met on the metabolism of circulating amino acids and the differential expression of several genes associated with markers of growth and sulphur amino acid and protein turnover.

In this study, dietary Met supply appeared to have no effect on the metabolism of other essential amino acids, nor non-essential amino acids. It might have been expected that production of direct Met metabolites, such as Cys or Tau might have been increased in fish fed the ADQ and EXC diets once the metabolic requirement for Met had been fulfilled and circulating Met became increasingly available for other processes, including protein synthesis which, in turn, would for example require additional Cys. 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. It is not clear why excessive dietary Met lead to a prolonged peak in plasma Thr.

A significant aim of this study was to investigate differences in the mechanisms by which barramundi metabolise Met when it is limiting, as opposed to supplied in excess, in the diet. The main hypothesis we were working under was that the genes responsible for Met turnover would respond in a step-wise fashion to increasing Met supply in the diet, in other words, the more Met in the diet, the more enzyme would be required to process this Met. This, however, wasn't what the gene expression data told us. Despite significant differences in plasma Met contents between treatments at two and four hours post-feeding, we saw very little, if any, effect of the treatment on 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 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 significant at this lifestage. Expression levels of both genes were relatively similar, suggesting both are active in the liver in barramundi of this size. Numerically higher average expression of MAT-1 in the livers of fish fed the DEF and EXC diets, compared to that of fish fed the DEF 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-2b, 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. In other words, 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. Only the remaining Met would then be available for protein synthesis. If the majority of the dietary Met in the deficient diets, then, is utilised for other processes rather than deposited in protein, this would result in the lower long term growth response of fish fed the Met deficient diet reported in Poppi et al. (2017). The lack of a significant effect of dietary Met content on expression of CGL, which is responsible for the conversion of cystathionine into Cys, suggests that the Met processed by the MATs is not preferentially routed to Cys production but, rather may be temporarily retained as SAM 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, then, is likely an advantage in maintaining a rapid rate of growth as seen in those fish fed the ADQ diet. Accumulation of superfluous SAM in the liver, however, 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 fish fed excessive Met in Poppi et al., (2017). 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 this experiment. The observation that expression of these genes changed significantly over time after the meal, but were not significantly affected by dietary Met supply, may also suggest that production of these enzymes

may be regulated by the consumption of feed, regardless of the composition. It should also 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.

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 fed the EXC diet compared to those in the DEF treatment at the pre-feeding timepoint, when plasma Met levels were similarly significantly different, 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 fed the ADQ diet at two and four hours post-feeding, suggesting adequate Met is required for taurine synthesis and, possibly, that excess Met may depress Tau synthesis. Given the non-significance of plasma Tau levels between treatments precluding a negative feedback of sufficient or excess circulating taurine, however, the mechanism for this is not clear. The lack of a significant response in the Cys and Tau biosynthetic genes may also be an artefact of sufficient dietary supply of these aminos negating the requirement for synthesis.

Given the significant differences in growth seen between these treatments in the previous growth trial, it was expected that Met intake would have an equivalent effect on growth related factors such the GH/IGF complex and protein turnover. Similarly to the SAA turnover genes, expression of genes of the somatotropic index (IGF-I, IGF-II and GHR-II) was significantly affected by time after feeding with that of the IGFs increasing two hours after feeding and rapidly returned 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 increases the activity of components of these pathways (Rolland *et al.*, 2015), 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 and its association with improved growth was reflected in the growth response reported in the growth trial, highlighting the role 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 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, with expression of this gene decreasing with increasing dietary Met. As these two growth factors share the same function, it is unclear why they would respond in opposition, however, 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*). 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.*, 2015) and GHR-II (Saera-Vila *et al.*, 2007), however, this was not the case, with GHR-II expression decreasing significantly in a linear fashion after feeding. It was decided to investigate GHR-II as opposed to GHR-I expression as this variant was 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 also be that the upregulation of IGF-I 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.

The proteolytic pathways were shown to be depressed by feed intake, as indicated by significant downregulation of the expression of ZFAND-5 and, to a lesser extent, MUL1 at two hours and again at four hours post-feeding. 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 pathway is more active and may be a good hepatic marker of proteolysis in barramundi. What was unexpected, however, was that proteolysis appeared to be stimulated, significantly so in expression of ZFAND-5, by adequate Met supply. This upregulation seen in both genes at the pre-feeding timepoint 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 deposits in these fish. This may also be a factor in the effect seen at two and four hours postprandially, until circulating amino acids were sufficient for the metabolic needs.

It was expected that increased dietary Met, and the associated observed significant increases in plasma Met, would increase the activity of protein synthetic pathways, given the additional Met

available for those processes. Protein synthesis, as indicated by the differential expression of Redd-1, signalling TOR pathway inhibition, was not significantly affected by dietary Met supply after feeding. Similarly to protein degradation, repression of TOR appeared to be stimulated by adequate Met supply, significantly so at the pre-feeding timepoint and to a lesser extent at two hours post-feeding, 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.*, 2015) or possibly that there may have been some interaction with insulin, as has been reported elsewhere (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 TOR is in fact receptive, but less sensitive, to Met in this species or is not a major determinant of protein synthesis. 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.

5 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 turnover in the plasma. Similarly, few significant differences between treatments were observed in the expression of selected genes of sulphur amino acid protein turnover pathways after feeding, suggesting production of these enzymes may be primarily regulated by the consumption of feed, rather than composition. Met processing by MAT-1, however did appear to be more active with increased Met after feeding and both MATs appear to be active. A lack of concomitant increases in plasma Cys or Cys-related metabolism genes suggested that this 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. IGF-I expression was also positively affected by dietary Met supplementation, reiterating its relationship with growth stimulation. Previously described 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 things 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 showed that they are nutritionally regulated, which will prove useful in further study of the impact of SAA metabolism in this species.

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Table 1. Dietary formulations.

	Diet 1	Diet 3	Diet 5
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
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.

Table 2. Genes investigated and primer sequences (EC numbers to be added)

Target gene	Gene abbreviation	EC number	Primer name	Sequence	Length
Met and Cys metabolism					
Methionine adenosyltransferase-1	MAT-1		MAT1 qPCR F1	TGTCAATCTCCTTGTTCACCT	21
			MAT1 qPCR R1	GCCTCTTCAGATTCAGTTCC	20
Methionine adenosyltransferase-2b	MAT-2b				21
			MAT2b- qPCR F2	GAGACCGATGAGTGTATGCCT	
			MAT2b- qPCR R2	ACCGTAACCTGTGTCTTTGAG	21
Cystathionine-γ-lyase	CGL				20
			CGL qPCR F2	CACAAGACGAGCAGAACGAC	
			CGL qPCR R2	CACCACAGCCATTGACTTCC	20
Tau metabolism					
Cysteine dioxygenase	CDO				22
			CDO- qPCR F2	GTTGCCTACATAAATGACTCCA	
			CDO- qPCR R2	CTGTCCTCTGGTCAAAGGTC	20
Cysteine sulphinic acid decarboxylase	CSAD				21
			CSAD qPCR F1	GTACATTCCACCAAGTCTGAG	
			CSAD qPCR R1	CCCAGGTTGTGTATCTCATCC	21
Somatotropic index					
Insulin growth factor-I	IGF-I				21
			IGF-1 qPCR F2	CTGTATCTCCTGTAGCCACAC	
			IGF-1 qPCR R2	AGCCATAGCCTGGTTTACTG	20
Insulin growth factor-II	IGF-II				22
			IGF-II qPCR F1	AGTATTCCAAATACGAGGTGTG	
			IGF-II qPCR R1	GAAGATAACCTGCTCCTGTG	20
Growth hormone receptor-II	GHR-II				24
			GHR-2 qPCR F2	CGTCCATATCCCATCTAAAGTGTC	
			GHR-2 qPCR R2	GTCATTCTGCTCCTCAATGTC	21
Proteolysis					
Mitochondrial ubiquitin ligase activator of NFKB-1	MUL-1				20
			Mul1 qPCR F1	GGCTTCCGTTATTTCCTCAC	
			Mul1 qPCR R1	TGCTCTCCTCTATGTTAAGTTCAC	24
Zinc finger, AN1-type domain 5	ZFAND-5				20
			ZFAND5 qPCR F1	CTAGAGCCTGTTGTAAGCCA	
			ZFAND5 qPCR R1	CTCGGCCTTGTAATCATAGGG	21
TOR activity signalling					
DNA Damage Inducible Transcript 4	Redd-1				21
			Redd1 qPCR F2	TTTCAGCACATCCACTAACGG	
			Redd1 qPCR R2	CCACTACTTCTTTCAGGATTGTC	23

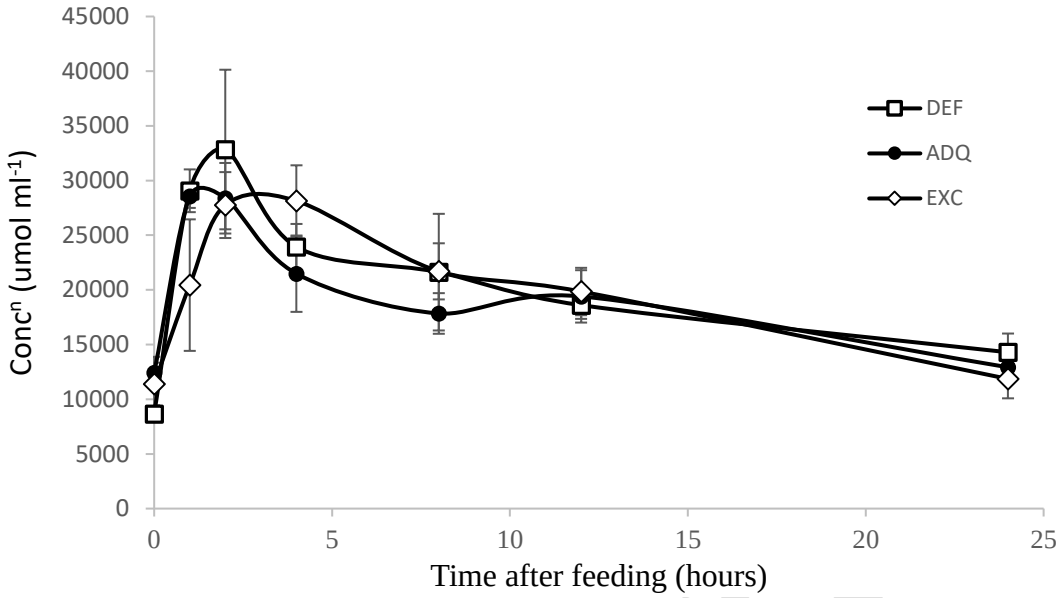

Figure 1. Total Free Amino Acids

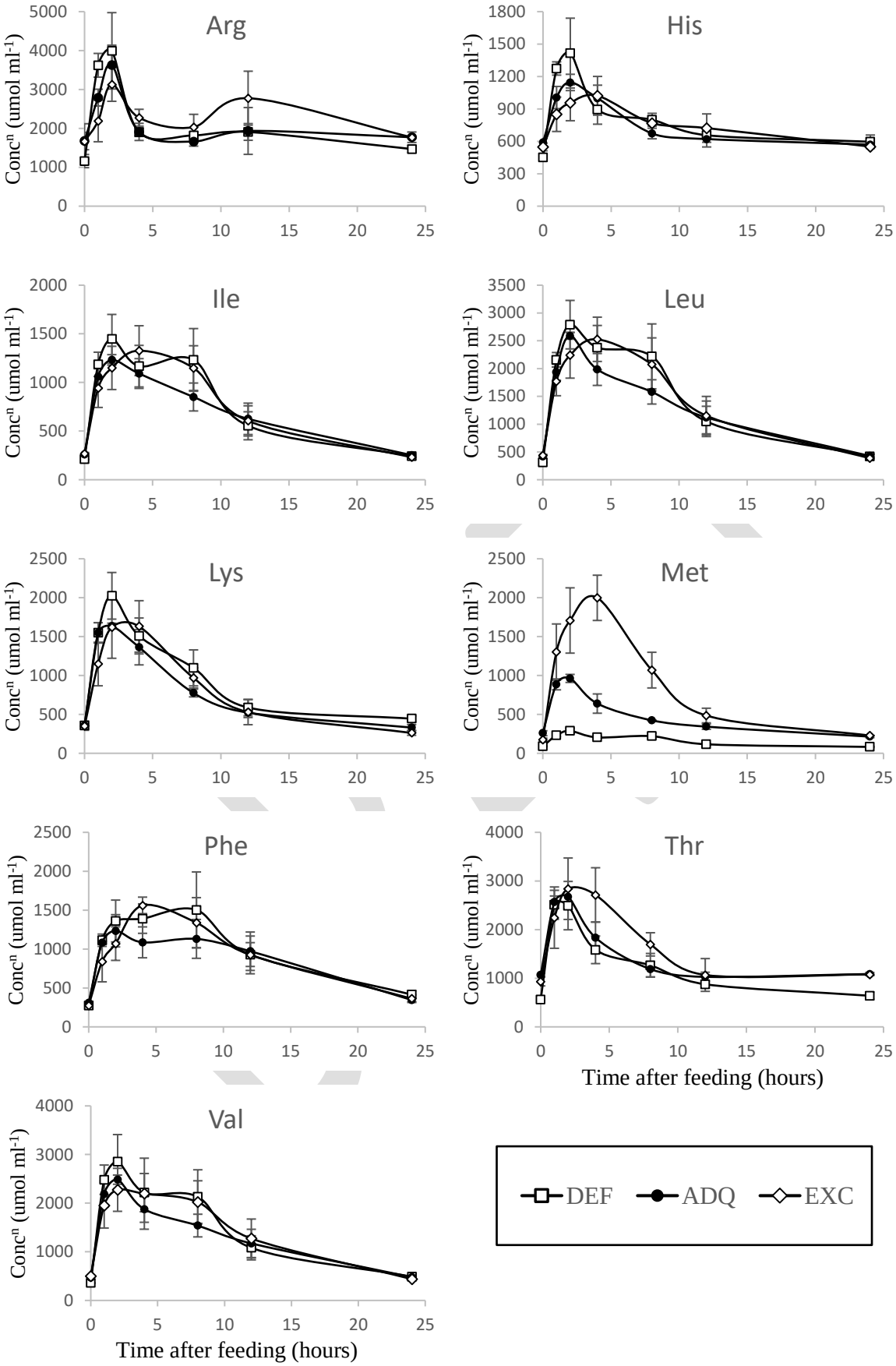

Figure 2. Essential Amino Acid Response

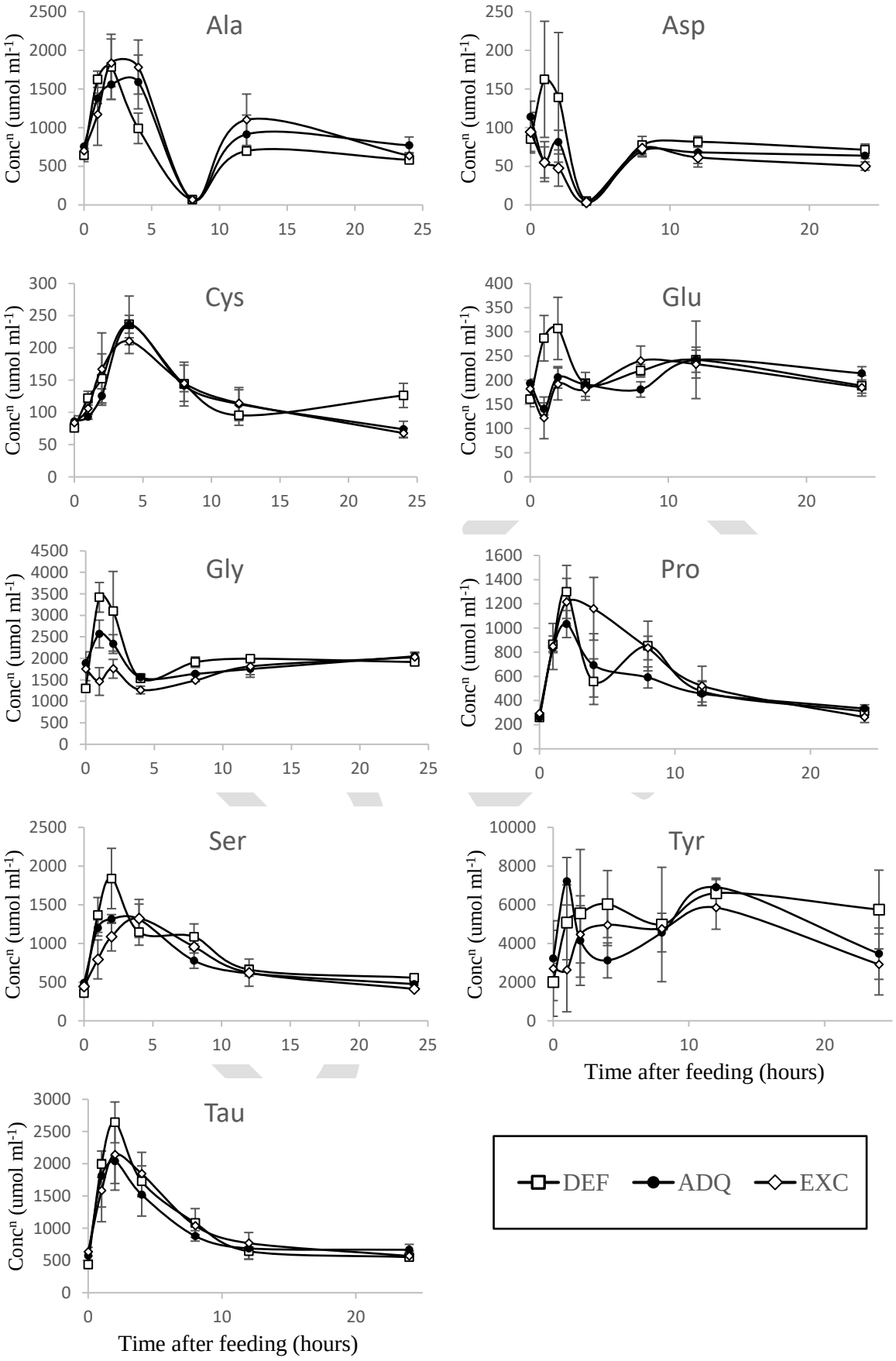

Figure 3. Non-Essential Amino Acid Response

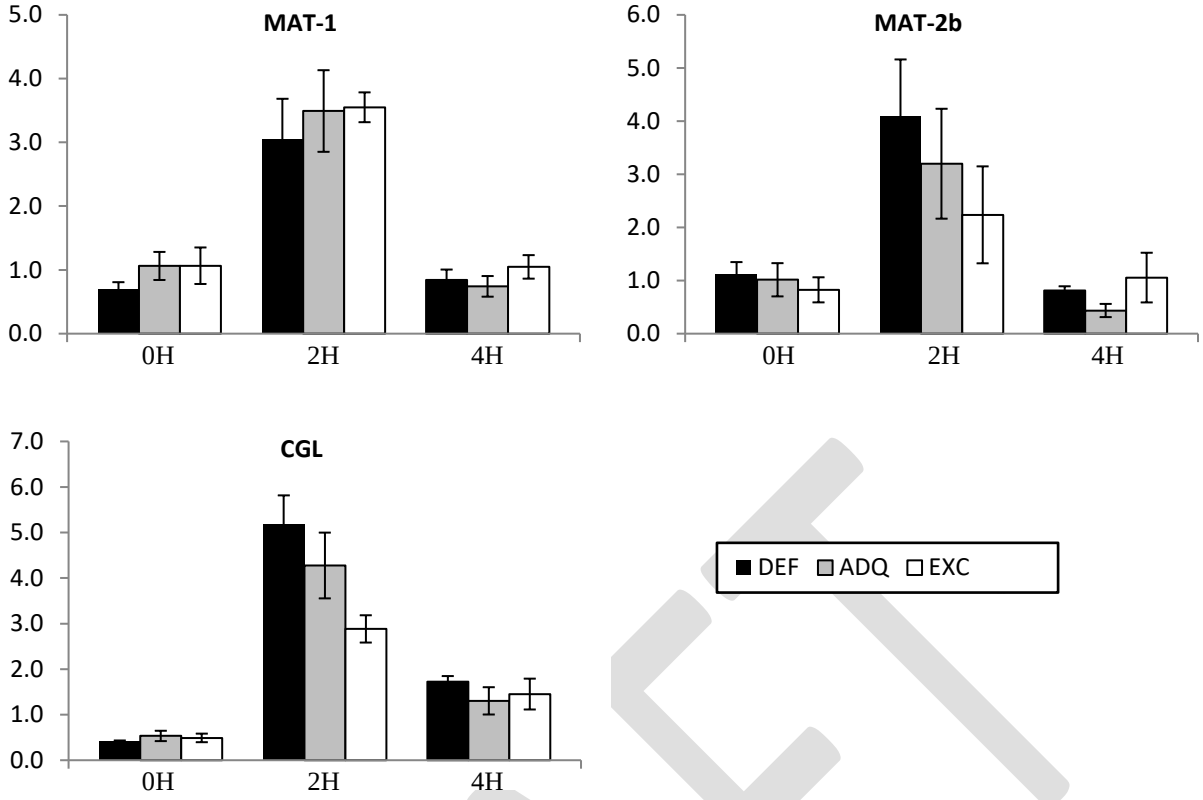

Figure 4. Methionine and cysteine metabolic gene expression - see belghit for more detail on format for this caption)

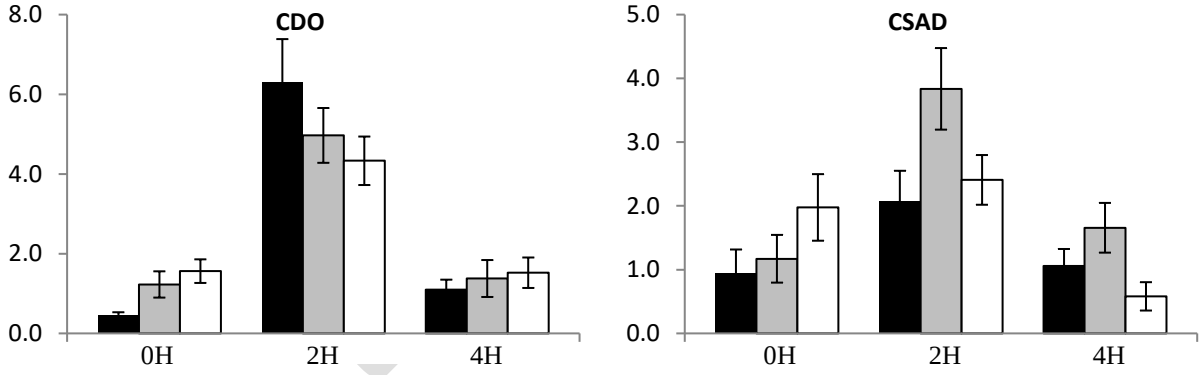

Figure 5. Taurine metabolic gene expression

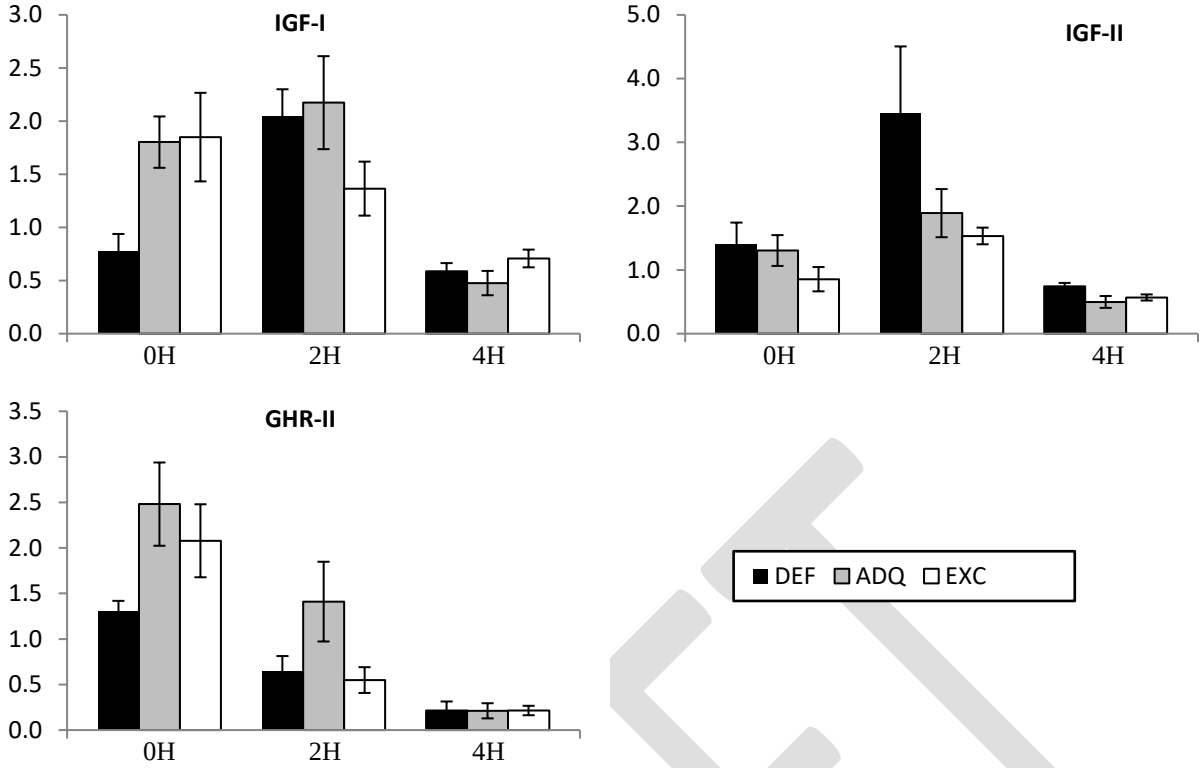

Figure 6. Somatotropic index gene expression

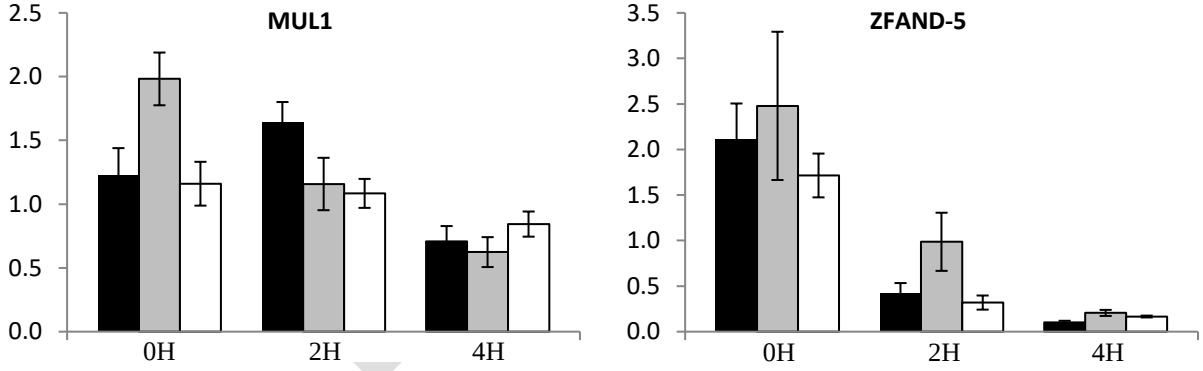

Figure 7. Proteolytic gene expression

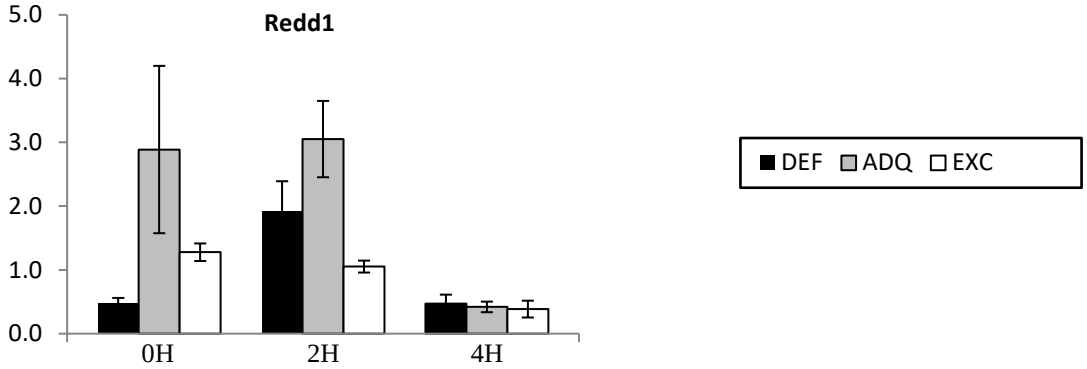

Figure 8. Redd1 expression (TOR activity signalling)