

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

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Abstract

A trial was conducted to investigate the effect of dietary taurine (Tau) supply on the plasma amino acid composition and hepatic expression of several genes in juvenile barramundi (*Lates calcarifer*) after feeding. Triplicate tanks of fish (average weight: 89.3g) were fed diets containing either a deficient (1g/kg), adequate (8g/kg) or excessive (19g/kg) level of dietary Tau. Liver tissue collected before feeding, and at two hour and four hours post-feeding, were analysed for expression of genes involved in pathways of sulphur amino acid turnover, Tau biosynthesis and transport, target of rapamycin (TOR) signalling, the somatotropic axis and protein turnover.

The treatment had no significant effect on the profiles of any amino acid in plasma collected over time after feeding, other than Tau and glycine. The expression profile of cystine and Tau synthetic genes suggested an effect of Tau excess on the metabolism of cystine. Markers of two pathways of Tau biosynthesis appear to be active in this species, providing proof that this species possesses the ability to synthesise Tau from SAA precursors. A marker for the regulation of Tau transport and homeostasis was shown to be directly regulated by Tau availability, while a link between adequate supply of Tau and TOR pathway-mediated growth stimulation was also apparent. An observed depression in expression of genes of the somatotropic axis, coupled with upregulation of the proteolytic and TOR-suppressing genes, in response to excessive Tau supply in the diet, signalled that excessive Tau may not be conducive to optimal growth in this species.

Keywords: Barramundi, *Lates calcarifer*, Taurine, Postprandial, Protein turnover.

1 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, important in responding to stress events (Huxtable, 1992; Asha and Devadasan, 2013). 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), as well as benefitting glucose homeostasis in juvenile turbot (*Scophthalmus maximus*) (Zhang *et al.*, 2019). 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). Taurine has been shown to be important throughout the lifecycle of several fish species, positively affecting markers of egg quality in Nile tilapia (*Oreochromis niloticus*) (Al-Feky *et al.*, 2016b), as well as growth and development in red sea bream (*Pagrus major*) larvae (Kim *et al.*, 2016). While many fish species possess the ability to synthesise Tau from precursor sulphur amino acids (El-Sayed, 2014), supplementation, particularly to high plant protein diets, has been shown to improve growth, indicating that the synthetic capacity of the animal alone may be insufficient to meet the entire requirement (Takagi *et al.*, 2008, 2010; Yun *et al.*, 2012, Martins *et al.*, 2018). 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 those fish possessing the capacity for synthesis. 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 its supplementation required for optimal growth. Tau supplementation of non-fishmeal based diets has been linked to improved growth in a number of fish species (Salze *et al.*, 2012, Velasquez *et al.*, 2015, Li *et al.*, 2016), 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 the autophagy-lysosomal and ubiquitin-proteasome protein turnover pathways in rainbow trout (*Oncorhynchus mykiss*). The regulation of components of the somatotrophic 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 somatotrophic 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 by-product of improved overall health and metabolic functioning would be an significant step in defining the importance of this amino acid.

A Tau dose response trial conducted prior to the current experiment (Poppi *et al.*, 2018) 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. That study utilised a restricted pair-feeding approach, whereby all tanks of fish were fed an identical ration at each meal, irrespective of fish size. It was conceded by the authors that this approach may have also restricted the growth of any fish responding to increases in dietary Tau by reducing the rate of feed intake of those fish, as a percentage of body weight, limiting the divergence of the growth rates. The aim of the current experiment, then, was to determine whether any markers of growth are positively affected by dietary Tau content, which may indicate whether Tau does, in fact, impact growth pathways in this species more subtly than observed in Poppi *et al.* (2018), independent of the limitations of the restricted pair-feeding approach used 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.

2 Materials and Methods

2.1 Diets

Three diets (Table 1), containing either a deficient (DEF; 1g/kg), adequate (ADQ; 8g/kg) or excessive (EXC; 15g/kg) dietary Tau content, were selected based on performance of fish fed these diets in a 42-day growth trial preceding this experiment (Poppi *et al.*, 2018), where the weight of the fish increased from an average of 27g to an average of 72g (2.7 x initial weight). The diets chosen elicited a poor growth response (DEF), the maximum response (ADQ) or contained the highest inclusion of dietary Tau (EXC), 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).

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.

2.2 Fish Trial

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).

Following the conclusion of a Tau dose response growth trial, described in Poppi *et al.* (2019), fish fed diets with deficient, adequate and excessive levels of dietary Tau (as described above) remained in triplicated 1000L tanks per treatment (nine tanks in total) and continued consumption of the same diets during an acclimation period of six days. Twenty four juvenile barramundi (*Lates calcarifer*) (mean initial weight: 72.1g) were housed in each tank. Diets were fed to satiety once daily for the six day period prior to the experimental feed ration determination process described below.

All feed was refrigerated (< 4°C), 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.

2.2.1 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 the experimental day of the present study. 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 fish¹) was assigned as the ration for all tanks. This ration was offered and consumed in its entirety on the day preceding the experimental day.

2.2.2 Sampling

Mean weights of the fish in each treatment on the day of sampling were: DEF (86.7g), ADQ (88.7g), EXC (91.0g). Twenty four hours after the last meal, three fish were randomly selected from each tank and euthanised by an overdose of anaesthetic (0.2 ppt AQUI-S™). Fish were individually weighed and approximately 2 mL 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, transferred to 2 mL screw capped microcentrifuge tubes and placed immediately on dry ice, along with hepatic tissue samples measuring 1cm x 1 cm, before storage at -80°C (zero hour control). The assigned ration was then fed after a further two hours of recovery following the zero hour sampling. Hand 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.

2.3 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 in an Eppendorf 5417R refrigerated centrifuge (Eppendorf AG, Hamburg, Germany) at 2200g for four minutes at 4°C. 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.

2.4 Molecular analyses

2.4.1 RNA extraction and normalisation

Total RNA was extracted from the frozen liver tissue taken from two fish from each of the three replicate tanks at three selected timepoints (0h, 2h and 4h; based on observed peaks in the post-prandial plasma amino acid response) (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). RNA quality was then assessed using RNA nanochips (Agilent #5067-1511) in a bioanalyser (Agilent Technologies), with visual assessment of the band integrity in the generated densitometry plots and achievement of an RNA integrity number greater than 4, used as measures of sufficient quality for RT-qPCR. All samples were normalised to a concentration of 200ng/µl before cDNA synthesis.

2.4.2 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), incorporating 400 pg of an exogenous Luciferase (Luc) RNA template (Promega L4561) as an internal control (De Santis *et al.*, 2011). 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, including identity with an annotated barramundi transcriptome published after these analyses were undertaken (NCBI BioProject PRJNA345597) were used to generate a series of primer pairs. Pairs with similar amplicon size (100-300bp), GC content between 50 and 60%, melting temperatures close to 60°C, and never more than 3°C different between primers, were chosen for the assessment. Pairs with the least chance of formation of homodimer or heterodimer complexes were selected based on assessment by PerlPrimer of the energy required to break any potential duplexes. 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 2.

The differential expression of selected genes of the somatotrophic 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°C and 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C and 40 seconds at 60°C. 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 endogenous reference gene elongation factor 1α (EF1α), as previously used in several other gene expression studies with barramundi (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. Luciferase control RNA (Promega L4561) was added as an exogenous reference to correct for differences in cDNA amplification across all samples. Amplification variation of this genes was 2.73 cycles, which did not change significantly over time.

2.5 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).

3 Results

3.1 Plasma amino acid contents

Plasma amino acid contents varied significantly over time after feeding as digestion of the feed released amino acids into circulation. Peak concentrations were seen at between one (for arginine in fish fed the EXC diet – Fig. 1) and 12 hours post-feeding (for glutamic acid in fish fed the EXC diet – Fig. 2 - although fluctuations were small in this amino acid), gradually returning to pre-feeding levels by 24 hours post-feeding for most amino acids. Most EAAs (Fig. 1) fluctuated significantly over time. Histidine (His), however, was the only EAA to be also be significantly affected by dietary treatment (EXC > ADQ; $p < 0.05$), 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. 2). 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 DEF diet having the highest concentration of Ser in their plasma, followed by those fed the ADQ diet, then those in the EXC 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 (DEF) 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.

3.2 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.

3.2.1 Met and Cys metabolism

The differential expression of two genes involved in the turnover of Met (methionine adenosyltransferase 1, *Lc-MAT-1*) and synthesis of Cys (cystathionine gamma-lyase, *Lc-CGL*) in response to variation in dietary Tau was investigated and is presented in Fig.3 (A and B). Expression of both *Lc-MAT-1* and *Lc-CGL* was highly significantly affected by time ($p < 0.001$; 0h<2h; 0h<4h; 2h=4h). No effect of treatment was observed.

3.2.2 Hypotaurine biosynthesis

The effect of dietary Tau on the expression of genes of two hypotaurine biosynthetic pathways (cysteine dioxygenase, *Lc-CDO*; cysteamine dioxygenase, *Lc-ADO*; and cysteine sulfinic acid decarboxylase, *Lc-CSAD*) was observed (Fig. 3, C, D, E). *Lc-CSAD* expression was significantly affected by treatment ($p < 0.05$; DEF>ADQ; DEF>EXC; ADQ=EXC) and highly significantly affected by time ($p < 0.001$; 0h<2h<4h; 0h<4h) with a significant interactive effect between the two ($p < 0.05$; EXC<DEF at the zero hour timepoint). *Lc-ADO* and *Lc-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 *Lc-CDO* expression.

3.2.3 Taurine transport/homeostasis

The impact of dietary Tau on the expression of the taurine transporter gene (*Lc-TauT*), a regulator of the absorption and homeostasis of Tau was examined and is presented in Figure 3F. Expression of this gene was significantly influenced by the dietary treatment ($p < 0.05$; EXC<DEF) and highly significantly affected by time after feeding ($p < 0.001$; 0h<2h; 0h<4h; 2h=4h).

3.2.4 Somatotropic axis

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

3.2.5 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. 4D and E)). The expression of mitochondrial ubiquitin ligase activator of NF- κ B-1 (*Lc-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 (*Lc-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 EXC treatment and those in the DEF and ADQ 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 (EXC > ADQ; EXC > DEF; DEF = ADQ).

3.2.6 TOR (protein synthesis) suppression signalling.

The expression of the gene *Lc-Redd-1* (regulated in development and DNA damage response-1), a suppressor of 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 Figure 4F. Highly significant effects of both treatment ($p < 0.001$; DEF > ADQ; DEF > EXC; ADQ < EXC) and time after feeding ($p < 0.001$; $0h < 2h > 4h$; $0h < 4h$) were observed.

4 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 many of these observations focusing primarily on its influence on growth rate. Consideration of the underlying mechanisms behind any effect on growth is central to our understanding of *why* it is required, if at all, in the diet. 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. An experiment aimed at assessing the impact of dietary Tau supply on growth of barramundi (Poppi *et al.*, 2018), observed a positive effect on growth at between 6 and 8 g 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 of a previous study with this species (Poppi *et al.*, 2019), 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 EXC diet, before levels declined, then rose to a secondary peak at four hours post-feeding. Similar fluctuations were also apparent in the plasma levels of the EAAs arginine, lysine and, to a lesser extent, leucine. 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 DEF and ADQ 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 DEF 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 twenty four 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 DEF 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 DEF treatment and that this would be reflected in an upregulation of *Lc*-MAT-1 (Met → SAM) and *Lc*-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 *Lc*-MAT-1 appeared to be more highly expressed in the livers of fish in the ADQ and EXC treatments than those fed the DEF 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 EXC (and, to a lesser degree, ADQ) 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 gene for the Cys producing enzyme *Lc-CGL* at two hours post-feeding in fish fed the EXC diet and at four hours post-feeding in fish in the ADQ 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 *Lc-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 *Lc-MAT-1* in the livers of fish fed the DEF diet points to the fate of this Met likely being Tau synthesis. While patterns in the data are discussed here, no significant differences were seen which may support the assertion in Poppi *et al.* (2019) that expression of these genes may be 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. It is also acknowledged that the number of replicate samples taken at each time point for molecular analyses is relatively low, compared with similar studies (Kwasek *et al.*, 2014; Liang *et al.*, 2016) and that this may have contributed to the observed variation and resulting lack of significant differences in relative expression of some genes.

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 components of the first taurine biosynthetic pathway (Cys → cysteine sulfinic acid → hypotaurine) showed significant upregulation at two hours post-feeding, presumably in response to increased availability of substrate for these enzymes. Expression of *Lc-CDO*, catalysing production of cysteine sulfinic acid from Cys then plateaued, while expression of *Lc-CSAD*, an indicator of conversion of cysteine sulfinic acid to hypotaurine, continued to increase significantly. Interestingly, the patterns of response of these two genes, as well as the gene for *CGL*, which is involved in the production of the Cys substrate for *CDO*, are quite similar, indicating the flow of metabolites along this pathway. *Lc-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 *Lc-ADO* and *Lc-CSAD* expression, the genes for the enzymes which catalyse competing pathways for hypotaurine synthesis. The *CSAD*-catalysed 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. There is evidence, however, that the cysteamine pathway, incorporating *ADO*, could also be significant in other animals (Ueki and Stipanuk, 2008). In the present study, both pathways appear to be

stimulated by feed intake. *Lc*-ADO appeared to respond much faster than *Lc*-CSAD to feeding but remained at a lower level of expression, with *Lc*-ADO expression levels peaking at two hours and *Lc*-CSAD at four hours post-feeding. The pattern of response in the present study provides further proof that this species does indeed possess the ability to synthesise Tau from SAA precursors and seems to support the CSAD-mediated pathway as being the more active. The sensitivity, rather than the magnitude, of enzyme gene expression to the presence of precursors, however, has previously been linked to the capacity of fish species to synthesise Tau by Wang *et al.* (2016), who observed that CDO expression was more responsive to the presence of Cys in rainbow trout (which have a high taurine biosynthetic capacity) compared with Japanese flounder (with a reduced capacity for taurine biosynthesis), despite a lower overall expression of this gene in the rainbow trout. It could be argued, then, that both Tau synthetic pathways investigated in the present study may play a role in barramundi, 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 Tau supply. *Lc*-TauT was most highly expressed in the livers of fish fed the DEF 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 *Lc*-TauT expression is directly regulated by Tau availability in this species, as suggested in turbot (*Psetta maxima*) by Wang *et al.* (2017), 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 Poppi *et al.* (2018) 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 *Lc*-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 barramundi in the study reported in Poppi *et al.* (2019) and seems 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 EXC treatment, and was significantly more highly expressed in fish in this treatment, compared to those fish fed the DEF and ADQ diets, at two hours post-feeding. 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 *Lc-MUL-1* did not change significantly over time. This may be a reflection of the continuous nature of protein turnover (Kaushik and Seiliez, 2010). While both this gene and *Lc-ZFAND-5* are indicative of proteolysis, perhaps their respective pathways are differentially active and are stimulated or suppressed by separate stimuli. Dietary Tau level was, however, observed by de Moura *et al.* (2019) to have no effect on total protease activity in meagre (*Argyrosomus regius*). This may be indicative of the complexity of the post-transcriptional processes occurring between mRNA production (and, hence, expression of the gene of interest) and translation to associated enzymes (Panserat and Kaushik 2010), confounding interpretation of the relationship between proteolytic pathway gene expression and measurable protein degradation. In other words, Tau may stimulate expression of particular genes but not activity of the enzyme/s.

Lc-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 is 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 *Lc-ZFAND-5* expression, significant upregulation of this gene in fish fed the taurine excess diet (EXC), in comparison to those fed the adequate diet (ADQ), at both two and four hours after feeding suggests a depressive effect of taurine on growth at high levels of inclusion, as also seen in juvenile totoaba (*Totoaba macdonaldi*) (Satriyo *et al.*, 2017) and larval Nile tilapia (*Oreochromis niloticus*) (Al-Feky *et al.*, 2016a). 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 the initiation and stimulation of growth processes in fish. Expression of components of the somatotrophic 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 *Lc-IGF-I*, *Lc-IGF-II* and *Lc-GHR-II* were similar within each timepoint. Fish in the ADQ 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 *Lc-Redd-1* expression. Before feed was consumed, the lowest level of expression of these genes was seen in the excessive taurine treatment (EXC). After feeding, however, expression was consistently lowest in those fish fed the DEF diet. This trend is consistent with the pattern of growth observed in Poppi *et al.* (2018), reaffirming the connection between growth and expression of genes of the somatotrophic 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 a 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. While Pierce *et al.* (2012) showed GHR-II to be most sensitive to growth hormone and insulin supplementation *in-vivo* in tilapia (*Oreochromis mossambicus*) hepatocytes, prompting measurement of its expression in the present study, this variant is generally more abundant in the muscle, while GHR-I is the more abundant in hepatic tissue (Gabillard *et al.*, 2006). Selection of GHR-I as an indicator of growth hormone regulation in the liver in the present study, therefore, may have yielded more meaningful results.

5 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 somatotrophic 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 Poppi *et al.* (2018) 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.

Acknowledgements

The authors wish to acknowledge the staff of the CSIRO Bribie Island Aquaculture Centre (BIRC): Mr. Simon Irvin, Ms. Natalie Habily, Mr. Isaak Kadel and Mr. Joel Slinger for their assistance in the collection of samples and Mr. David Blyth in the preparation of the diets. This research did not receive a specific grant but was funded by the Commonwealth Scientific and Industrial Research Organisation (CSIRO) and The Queensland Alliance for Agriculture and Food Innovation. David Poppi received support through an Australian Government Research Training Program Scholarship.

Conflict of Interest: The authors state that they have no conflict of interest.

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Table 1. Formulations and analysed compositions of experimental diets.

	DEF	ADQ	EXC
<i>Ingredients (g kg⁻¹)</i>			
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 ₂ O 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
<i>Composition as determined (g kg⁻¹DM unless otherwise stated)</i>			
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
<i>EAA</i> s			
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

Table 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				
<i>Lc-MAT-1</i>	XM_018678413	MAT1 qPCR F1	TGCAATCTCCTTGTTACCT	21
		MAT1 qPCR R1	GCCTCTCAGATTCAGTTCC	20
<i>Lc-CGL</i>	XM_018673132	CGL qPCR F2	CACAAGACGAGCAGAACGAC	20
		CGL qPCR R2	CACCACAGCCATTGACTTCC	20
Tau metabolism				
<i>Lc-CDO</i>	XM_018674402	CDO- qPCR F2	GTTGCCTACATAAATGACTCCA	22
		CDO- qPCR R2	CTGTCCTCTGGTCAAAGGTC	20
<i>Lc-CSAD</i>	XM_018666199	CSAD qPCR F1	GTACATTCCACCAAGTCTGAG	21
		CSAD qPCR R1	CCCAGGTTGTGTATCTCATCC	21
<i>Lc-ADO</i>	XM_018660792	ADO qPCR F5	AGCCTGTTAGTACTGTGATCC	21
		ADO qPCR R5	AGACATCAATGCTGAAATGGAC	22
Somatotropic axis				
<i>Lc-IGF-I</i>	XM_018697285	IGF-1 qPCR F2	CTGTATCTCCTGTAGCCACAC	21
		IGF-1 qPCR R2	AGCCATAGCCTGGTTTACTG	20
<i>Lc-IGF-II</i>	XM_018664155	IGF-II qPCR F1	AGTATTCCAAATACGAGGTGTG	22
		IGF-II qPCR R1	GAAGATAACCTGCTCCTGTG	20
<i>Lc-GHR-II</i>	XM_018702499	GHR-2 qPCR F2	CGTCCATATCCCATCTAAAGTGTC	24
		GHR-2 qPCR R2	GTCATTCTGCTCCTCAATGTC	21
Proteolysis				
<i>Lc-MUL-1</i>	XM_018686248	Mul1 qPCR F1	GGCTTCCGTTATTTCTCAC	20
		Mul1 qPCR R1	TGCTCTCCTCTATGTTAAGTTCAC	24
<i>Lc-ZFAND-5</i>	XM_018669382	ZFAND5 qPCR F1	CTAGAGCCTGTTGTAAGCCA	20
		ZFAND5 qPCR R1	CTCGGCCTTGTAATCATAGGG	21
TOR suppression signaling				
<i>Lc-Redd-1</i>	XM_018699192	Redd1 qPCR F2	TTTCAGCACATCCACTAACGG	21
		Redd1 qPCR R2	CCACTACTTCTTTCAGGATTGTC	23
Control genes				
<i>Luc</i>	na	Luc qPCR F	GGTGTGGGCGCGTTATTTA	20
		Luc qPCR R	CGGTAGGCTGCGAAATGC	18
<i>EF1α</i>	GQ_507427	Lcal EF1 α F	AAATTGGCGGTATTGGAAC	19
		Lcal EF1 α 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 growth factor-I; *IGF-II*, insulin growth factor-II; *GHR-II*, growth hormone receptor-II; *MUL-1*, mitochondrial ubiquitin ligase activator of NF- κ B-1; *ZFAND-5*, zinc finger AN1-type domain-5; *Redd-1*, regulated in development and DNA damage response-1; *Luc*, luciferase; *EF1 α* , elongation factor 1 α .

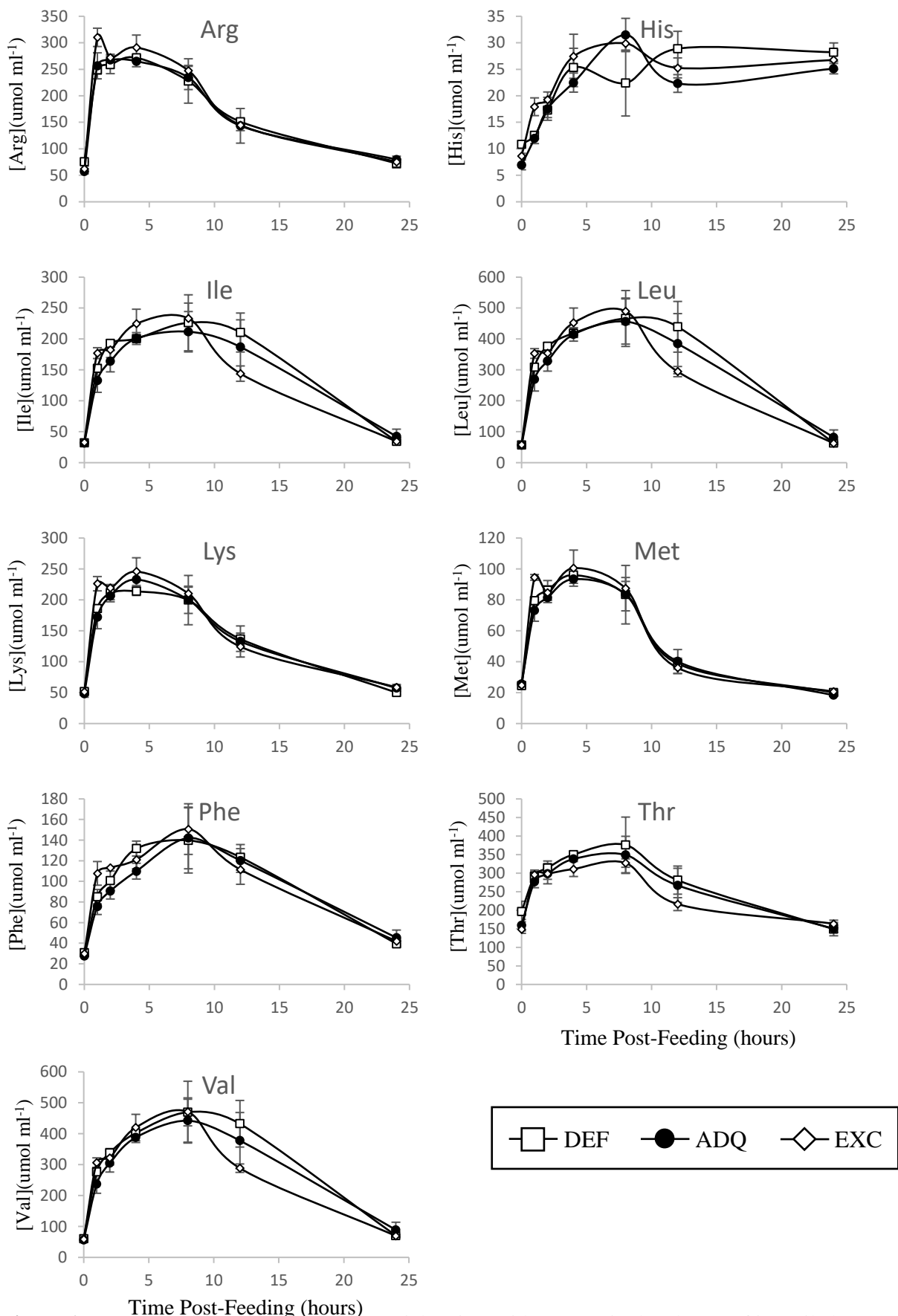


Figure 1. Concentrations of individual essential amino acids present in the plasma of juvenile barramundi over a 24 hour period following ingestion of a single meal containing either a deficient (DEF), adequate (ADQ) or excessive (EXC) dietary taurine content.

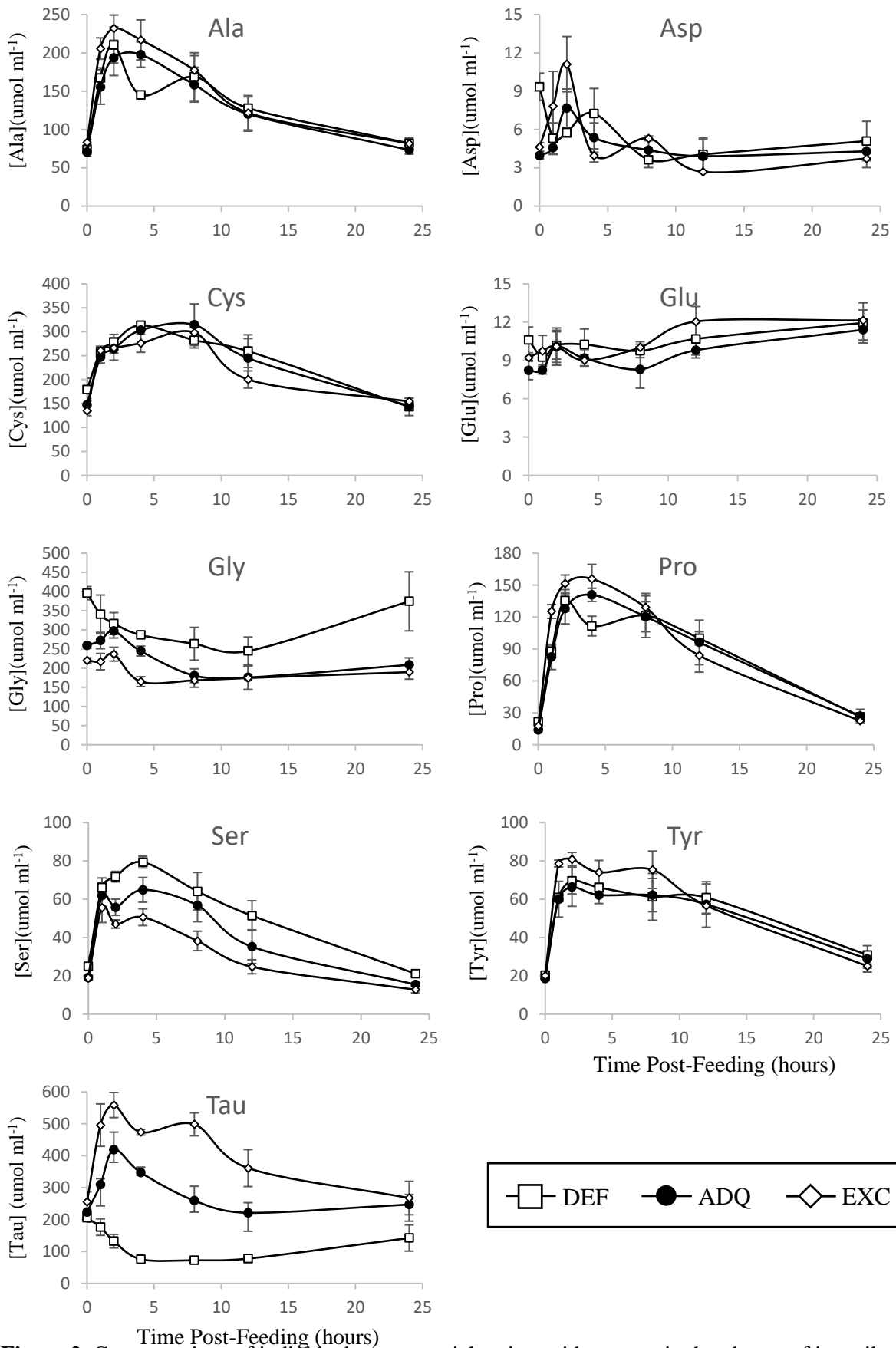


Figure 2. Concentrations of individual non-essential amino acids present in the plasma of juvenile barramundi over a 24 hour period following ingestion of a single meal containing either a deficient (DEF), adequate (ADQ) or excessive (EXC) dietary taurine content.

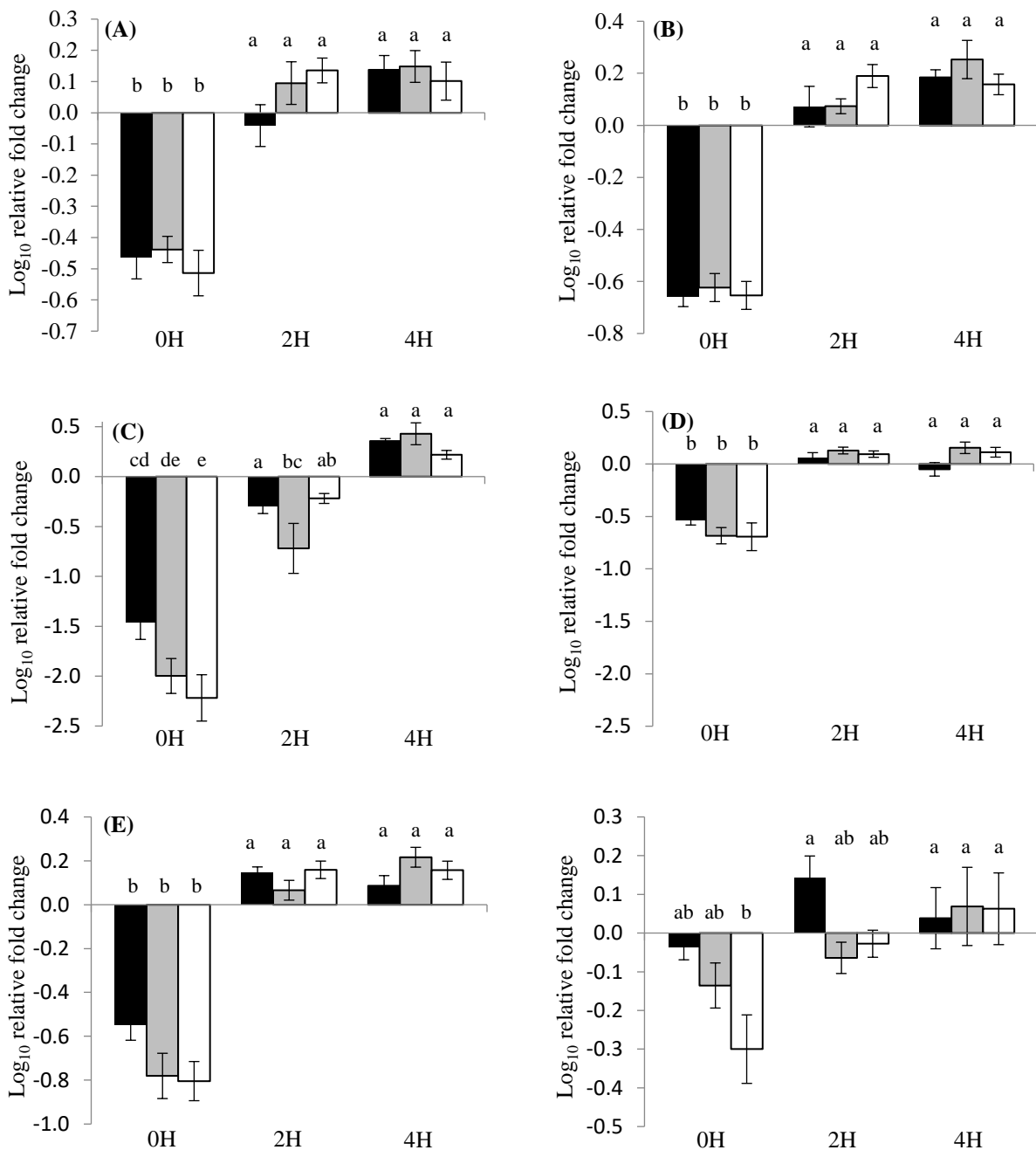


Figure 3. Transcript levels of selected genes of methionine (A, *Lc-MAT-1*) and cysteine (B, *Lc-CGL*) metabolic; taurine biosynthetic (C, *Lc-CSAD*; D, *Lc-ADO*; E, *Lc-CDO*); and taurine transport (E, *Lc-TauT*) pathways in the liver tissue of juvenile barramundi sampled after 24 hours starvation (0H); and two (2H) and four (4H) hours after ingestion of a single meal containing either a deficient (DEF), adequate (ADQ) or excessive (EXC) dietary taurine content. Values were normalised to those of elongation factor 1 α (Efl α) before log10 transformation. Values presented are means (n=6) \pm 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 log10 transformed relative fold change. Gene abbreviations can be found in Table 2.

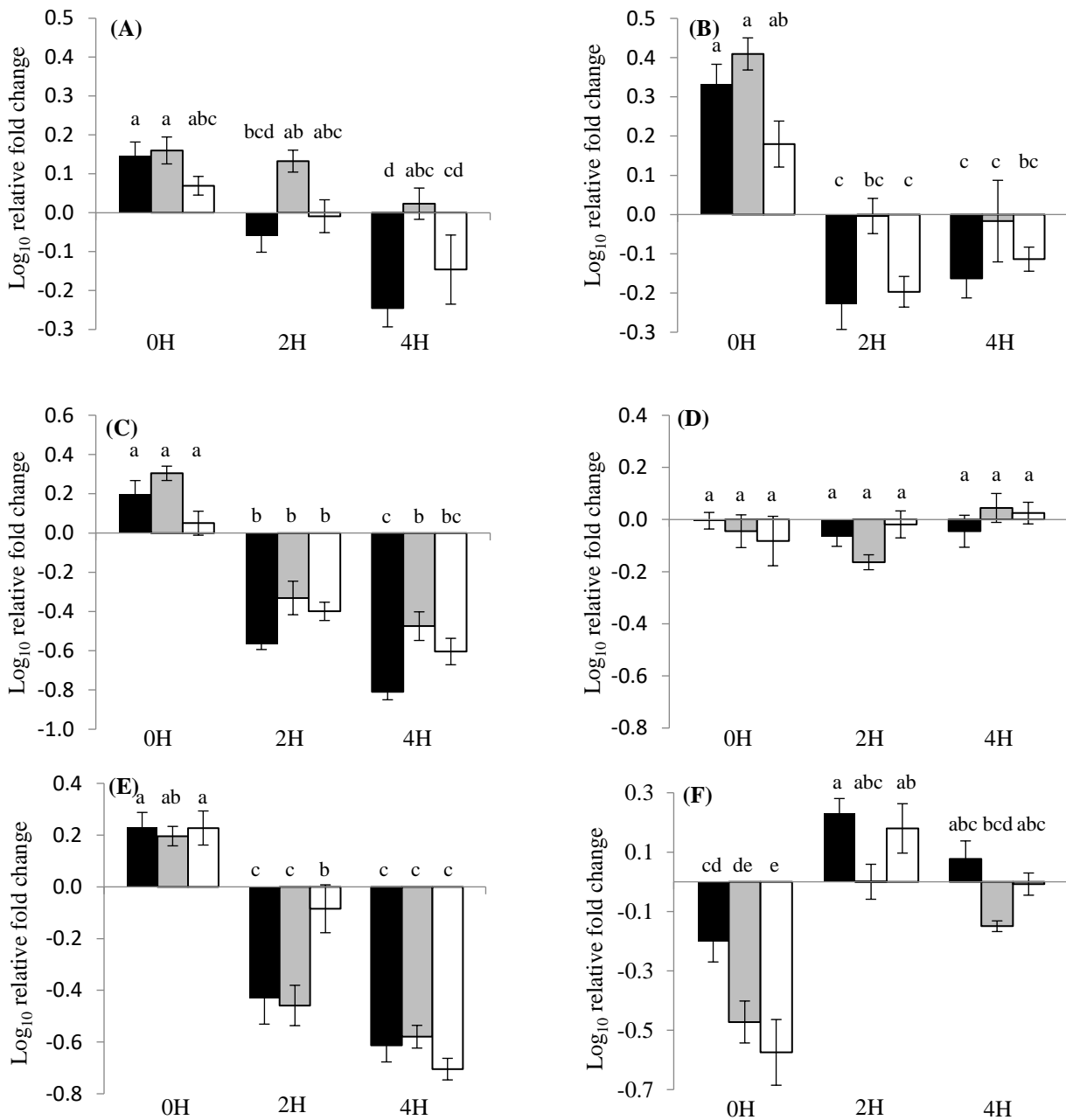


Figure 4. Transcript levels of selected genes of the somatotrophic axis (A, *Lc-IGF-I*; B, *Lc-IGF-II*; C, *Lc-GHR-II*); proteolytic pathways (D, *Lc-MUL-1*; E, *Lc-ZFAND-5*); and inhibition of the TOR pathway (F, *Lc-Redd-1*) in the liver tissue of juvenile barramundi sampled after 24 hours starvation (0H); and two (2H) and four (4H) hours after ingestion of a single meal containing either a deficient (DEF), adequate (ADQ) or excessive (EXC) dietary taurine content. Values were normalised to those of elongation factor 1 α (Efl α) before log10 transformation. Values presented are means (n=6) \pm 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 log10 transformed relative fold change. Gene abbreviations can be found in Table 2.