

Postprandial molecular responses in the liver of the barramundi, *Lates calcarifer*.

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Abstract

The regulation of gene expression by nutrients is an important mechanism governing energy storage and growth in most animals, including fish. Understanding the timing and intensity of these responses is the first critical step in defining the molecular effects of different dietary nutrient sources. In this study, changes in key metabolic regulators of nutritional pathways were investigated in barramundi fed a meal of a diet formulated with 500, 150 and 110 g kg⁻¹ dry matter of protein, lipid and carbohydrate, respectively. Plasma glucose levels showed a postprandial peak two hours after feeding, and had returned to basal levels within four hours. Significant activation of genes that regulate glycolytic and lipogenic pathways immediately post feeding were observed, in combination with down-regulation of genes that control gluconeogenesis and activation of the Akt-mTOR pathway. Strong correlations were identified between a number of different metabolic genes, and the coordinated co-regulation of these genes may underlie the ability of this fish to effectively regulate circulating plasma glucose levels. Overall, post-prandial responses in barramundi were observed to be extremely rapid compared with other fish species, and there was no prolonged hyperglycaemia with a diet low in carbohydrates. These data clearly demonstrate, for the first time, the molecular changes that control intermediary metabolism in barramundi in response to feeding a single meal of a diet formulated within optimal specifications for this species.

Keywords

Fish, Liver, Metabolism, Nutrigenomics

Introduction

The basic goal of fish nutrition in aquaculture is to supply adequate amounts of energy to promote optimal growth. Understanding the molecular pathways that regulate the utilization of dietary energy and macronutrients is a critical element to understanding the feeding and growth response in fish. In order to use molecular responses to understand fish nutrition, the first step is to define the molecular responses that occur each time a fish receives food. The changes in gene expression after feeding have been investigated for various metabolic genes in a range of fish species, although there is considerable variation in the molecular responses of different fish to nutrients, particularly in response to carbohydrates (reviewed in Panserat and Kaushik 2010; Polakof et al. 2012). In barramundi, there is no understanding of the changes in expression of any genes that regulate key energy partitioning pathways or the induction of signalling cascades after feeding.

Barramundi, like salmon and trout, are an economically important, carnivorous fish species. Traditionally, aquaculture production of these species has relied on diets containing high levels of protein and lipid, based on wild-harvest fishmeal and fish oil. The need to reduce the reliance on wild fisheries resources has resulted in the increased use of plant based raw materials such as soybean meal, lupin meals (Gatlin et al. 2007; Glencross et al. 2007). However, this introduces a range of complex carbohydrates, such as cellulose, polysaccharides and starches (Gatlin et al., 2007; Glencross et al. 2007). Whilst these complex carbohydrates do not form part of the natural diet for carnivorous fish, most fish species have the capacity to digest various types of grains, particularly after extrusion and gelatinisation (Bergot and Breque 1983; Glencross et al. 2011a, 2011b; Jeong et al. 1991; Wilson 1994). This is also the case for barramundi (Allan et al. 2003; Catacutan and Coloso 1997; Glencross et al. 2011c, 2012).

Past studies have shown that barramundi growth performance and feed conversion ratio (FCR) is maximised when feeds contain 450-550 g kg⁻¹ dry matter (DM) of protein, 140-160 g kg⁻¹ DM of lipid and between 18-22 MJ kg⁻¹

gross energy (reviewed in Glencross et al. 2006). Barramundi, like most fish, have no specific nutritional requirement for carbohydrates, and have been shown to be intolerant of high levels of simple carbohydrates compared with omnivorous and herbivorous fish (Allan et al. 2003). In addition, the digestibility of the carbohydrate component of different plant based raw ingredients has been shown to be extremely variable (Allan et al. 2003; Glencross et al. 2012). As a consequence, levels of carbohydrates in commercial diets for barramundi are generally limited to under 20%, with formulations focussing on achieving high protein content. Carbohydrate is included primarily as a binding agent and secondarily as a minor source of energy, and at levels of 10-20% there has been shown to be little effect on the growth performance or protein digestibility (Allan et al. 2003; Glencross et al. 2012).

In this study, a diet was formulated within optimal protein and lipid specifications for this species, while carbohydrate levels were kept to minimum levels required for pellet binding and extrusion. Changes in barramundi plasma glucose and free amino acid levels were investigated over time after feeding. In addition, the molecular responses of key genetic regulators of energy partitioning pathways in the liver, including carbohydrate, fatty acid, amino acid and glycogen metabolism were examined, and the activation of signalling cascades that regulate growth and metabolism assessed. This study seeks to define the post-prandial changes in liver gene expression that regulate a range of metabolic pathways in barramundi, and assess the timing of signalling cascade activation that regulate downstream growth pathways over time after a fish receives food.

Methods

Fish and Experimental Protocol

All experiments were performed in accordance with the Australian code of practice for the care and use of animals for scientific purposes and were approved by the CSIRO Animal Ethics Committee (approval number: A8/2010). Barramundi (*Lates calcarifer*) were held indoors under artificial lighting in 200 L tanks receiving $\sim 2 \text{ Lmin}^{-1}$ flow of continuously aerated marine water at 30°C and salinity at 35 gL^{-1} . Two tanks, each containing 24 fish ($99.3 \pm 7.8 \text{ g}$ and $99.4 \pm 11.8 \text{ g}$, respectively), were fed to apparent satiety twice daily (0830 and 1600) using the diet outlined in Table 1. This diet was designed using optimal protein, lipid and energy requirements for barramundi of this size (Glencross et al. 2006). Nutritional composition and digestibility of individual ingredients had been calculated from other studies (Glencross et al. 2012).

Fish were fed for five days, then once on Day six (0830) and starved for 24 hr to ensure that no effects were present from previous feeding events. On Day seven, fish weighed $113.2 \pm 11.8 \text{ g}$ and $109.7 \pm 16.5 \text{ g}$, respectively. Three fish were sampled from each of the two tanks as a pre-feeding control one hour before a 100 g ration of the experimentally prepared diet was fed to each of the two tanks over a 10 min feeding event. Three fish were then sampled from each of the two tanks at 30 min, 1 hr, 2 hr, 4 hr, 8 hr, 12 hr and 24 hr post feeding. For sampling, fish were euthanized by placing them in seawater containing 0.2 ml L^{-1} AQUI-S (AQUI-S New Zealand Ltd). Stomach contents recorded over the first four hours showed that sampled fish ate an average of $3.1 \pm 1.31 \text{ g}$ of feed. Blood was collected by caudal vein puncture using a syringe pre-treated with a solution containing 0.2M EDTA, then centrifuged and plasma kept frozen at -80°C until analysis. Liver samples were collected, snap frozen on dry ice then stored at -80°C prior to further RNA and protein analyses.

Metabolite assays

Plasma and liver glucose levels ($n=6$) were measured using a commercially available glucose assay kit (BioVision #K606-100). Plasma free amino acids (FAA) were determined using a fluorometric detection method (Fisher et al. 2000) and using Amino Acid Standard H (Pierce #20088) as a reference.

Quantitative real-time RT-PCR.

Total RNA was extracted using Trizol reagent (Invitrogen) according to the manufacturers instructions, and precipitated by adding 0.5 volumes of isopropyl alcohol and 0.5 volumes of RNA precipitation solution (1.2 M sodium chloride, 0.8 M disodium citrate) for purity improvement (Green and Sambrook 2012). Total RNA was DNase digested with the Turbo DNA-free kit (Applied Biosystems). RNA quantity was assessed on a NanoDrop spectrophotometer (NanoDrop Technologies) and RNA quality was assessed using a Bioanalyser (Agilent Technologies) and RNA nanochips (Agilent #5067-1511). All RNA samples were diluted to 200 ng μ l⁻¹. Reverse transcription was performed on 1 μ g of total RNA using Superscript III (Invitrogen) with 25 μ M oligo(dT)₂₀ and 25 μ M random hexamers (Resuehr and Spiess, 2003). Expression of a range of genes that regulate various metabolic pathways in all samples was analyzed by real-time PCR as described below.

Real-time PCR primers specific to each gene of interest (Table 2) were designed with PerlPrimer v1.1.17 (Marshall, 2004). Verification that there was no genomic DNA contamination was carried out by PCR amplification of a pool of DNase-treated RNA samples using gene-specific primers. In all cases, PCR efficiency (E) was optimized to be between 95 % and 105 % using the slope of a standard curve over a five-fold serial dilution of a pooled cDNA sample containing all samples. Real-time PCR amplification reactions were carried out using 1X SYBR Green PCR Master Mix (Applied Biosystems), 0.2 μ mol⁻¹ of each primer and the equivalent of 7.5 ng of reverse-transcribed RNA. Amplification cycle conditions were 2 min at 50°C, 10 min at 95°C followed by 40 cycles of 15 sec at 95°C and 40 sec at 60°C. Amplification specificity was verified by cloning and sequencing and a dissociation melt curve analysis was routinely performed to assess amplified transcripts. Reactions were set up using the epMotion 5070 robot (Eppendorf) and run in triplicate on a Viiia7 real-time PCR system (Applied Biosystems). Normalization was performed using the Δ C_q method (where C_q is the quantification cycle), as it was considered the least biased approach (de Santis et al., 2011). Expression levels of each gene relative to one another were determined by normalizing the cycle threshold values for each gene to Ef1 α , then to the average cycle threshold of all genes at each time point to express relative transcript abundance. Changes in expression levels of each gene over time were

determined by normalizing the cycle threshold values for each gene to Ef1 α , then to the cycle threshold of each gene at time zero. The variation in amplification of Ef1 α across all samples was 0.68 cycles and did not significantly change over time (data not presented). The gene Ef1 α has been routinely used as a reference gene for gene expression analysis in barramundi (de Santis et al. 2011) and for postprandial metabolic gene expression analysis other species (Craig and Moon 2011; Enes et al. 2012; Menningen et al. 2012; Olsvik et al. 2004; Skiba-Cassy et al. 2009).

Protein Extraction and Western Blotting

Frozen liver samples (~100 mg) were homogenized on ice with an Ultraturrax homogenizer in a buffer containing 150 mmol l⁻¹NaCl, 10 mmol l⁻¹Tris, 1 mmol l⁻¹ EGTA, 1 mmol l⁻¹ EDTA (pH 7.4), 100 mmol⁻¹ sodium fluoride, 4 mmol l⁻¹ sodium pyrophosphate, 2 mmol l⁻¹sodium orthovanadate, 1% (v/v) Triton X-100, 0.5% (v/v) NP40-IGEPAL and a protease inhibitor cocktail (Roche). Homogenates were centrifuged at 1000 x g for 15 min at 4°C and supernatants were then centrifuged for 30 min at 15 000 x g. The resulting supernatants (n = 6 for each time point) were stored at -80°C. Protein concentrations were determined using the Bio-Rad Protein assay kit. Quantities of 20 μ g protein per sample were separated by SDS-PAGE and analysed for the presence of specific proteins by western blotting and using the appropriate antibodies. Primary antibodies for the analysis of the insulin signaling pathway were obtained from Cell Signaling Technologies (Akt-p #9272; Akt #9271; mTOR-p #2972; mTOR#2971S; S6-p #4856S; S6 #2217S). All were used at a dilution of 1:1000 and have previously been shown to cross-react with rainbow trout proteins of interest (Seiliez et al. 2008). After incubation with a goat anti-rabbit IRDye infrared secondary antibody (LI-COR Inc. Biotechnology), bands were visualized and quantified by Infrared fluorescence using the OdysseyH Imaging System (LI-COR Inc. Biotechnology).

Statistical Analyses

Where comparison between individual measurements was required, statistical significance was assessed by single factor analysis of variance (ANOVA), followed by Tukey's HSD test allowing 5% error. A Pearson correlation was used to determine linear dependence between measurements, using a 5% confidence

interval and a Student's t-test to attribute statistical significance. All statistical analyses were performed using StatPlus:Mac 2009 (AnalystSoft Inc, 2009).

Results

Metabolites

Plasma glucose levels peaked two hours post-feeding, and then returned to initial levels by 4 hours (Table 3). The levels at 2 hours after a meal were significantly different from those recorded at 8, 12 and 24 hours after a meal. Liver glucose levels remained stable throughout the various time points (Table 3). Plasma free amino acids were significantly elevated 2 and 4 hours after feeding, and had returned to pre-feeding levels by 8 hours (Table 3).

Relative Differences in Liver Transcript Levels During Starvation

Transcript levels of each gene were calculated relative to one another using raw cycle threshold values for each gene, normalised to *Ef1 α* , relative to the average Ct value for all the genes at either before feeding or 2 hours after feeding. Among the 18 genes analysed in this study, the gluconeogenic gene glucose-6-phosphatase (EC 3.1.3.9; *Lc G6Pase*) showed the highest expression in the liver of barramundi that had been starved for 24 hours (Figure 1). The next most abundant transcripts were fructose-1,6-bisphosphatase (EC 3.1.3.11; *Lc F1,6BPase*) and glutamate dehydrogenase (EC 1.4.1.2; *Lc GLUD1*) which had very similar total expression levels.

Transcript levels of genes that regulate the glycolysis (glucokinase: EC 2.7.1.1; *Lc GK*, and pyruvate kinase: EC 2.7.1.40; *Lc PK*), pentose phosphate pathway (glucose-6-phosphate dehydrogenase: EC 1.1.1.49; *Lc G6PDH*) and lipogenesis (fatty acid synthase: EC 2.3.1.85; *Lc FAS*, stearoyl-CoA desaturase: EC 1.14.19.1; *Lc SCD*) and ATP citrate lyase (EC 2.3.3.8; *Lc acyl*) were between 5 and 11 fold less abundant than transcripts regulating glucose production. Transcript levels of *Lc GLUD1* were approximately 4-fold higher than those of the other amino acid aminotransferase genes (alanine aminotransferase: EC 2.6.1.2; *Lc cALAT*, and aspartate aminotransferase: EC 2.6.1.1; *Lc ASAT*). The changes in gene expression were rapid, and the expression of the majority of the genes peaked 2 hours after a meal (Figure 2). Interestingly, this coincided with the peak of plasma glucose and plasma free amino acids at 2 hours after a meal. Also at this time, the glycolytic and lipogenic genes *Lc GK* and *Lc SCD* were the most highly expressed, respectively, and exceeded the relative levels of expression of *Lc G6Pase* (Figure 1).

Changes in Gene Expression Induced by Feeding

The relative response of each gene to a meal was calculated for each gene independently using raw cycle threshold values for each gene, normalised to *Ef1 α* , relative to the average Ct value for that gene prior to feeding. The glycolytic genes *Lc GK* and *Lc PK* were significantly up-regulated from 2 to 12 hours after a meal (Fig. 2). Meanwhile, the expression of gluconeogenic genes *Lc G6Pase* and *Lc F1,6BPase* were significantly repressed after feeding, although the change in *Lc FBPase* expression was very small (Figure 2). The *Lc G6Pase* gene was significantly down-regulated from 2 to 12 hours after feeding, resulting in a significant negative correlation between *Lc G6Pase* and the glycolytic and lipid metabolism genes, as well as plasma free amino acid levels (Figure 3). None of the genes analysed in this study were correlated with either plasma glucose or liver glucose (Figure 3).

As an alternative to glycolysis, the expression of *Lc G6PDH* regulates the production of metabolic intermediates through the pentose phosphate pathway. After feeding, the expression of the *Lc G6PDH* gene was significantly elevated from 2 to 12 hours after feeding (Figure 2). This was matched by the significant up-regulation of the fatty acid synthesis genes *Lc acyl*, *Lc FAS* and *Lc SCD* over the same period (Figure 2), and resulted in a significant positive correlation between all four of these genes (Figure 3). One known regulator of lipogenesis is the transcription factor sterol regulatory element binding protein 1c (*SREBP-1c*). Expression of the *Lc SREBP-1c* gene was significantly elevated immediately post feeding, and was positively correlated with *Lc G6PDH*, *Lc acyl*, *Lc SCD*, *Lc PPAR α* , *Lc cALAT*, *Lc ASAT*, *Lc CS* and *Lc pygl* expression, as well as TOR and S6 activation (Fig 3).

The opposing pathway to lipogenesis is the breakdown of fatty acids through β -oxidation, and known to regulate key steps in this pathway in the liver are genes such as carnitine palmitoyltransferase 1A (EC 2.3.1.21; *CPT1A*) and peroxisome proliferator-activated receptor alpha (*PPAR α*). Although significantly downregulated at 0.5 and 1 hours after a meal, expression of the *Lc CPT1A* gene was significantly elevated at 4, 8 and 12 hours after feeding and had returned to pre-feeding levels by 24 hours (Figure 2). This expression pattern showed a significant positive correlation with the expression of *Lc GK*, *Lc PK*, *Lc G6PDH* and

Lc FAS and significant negative correlation with the expression of *Lc G6Pase* and *Lc gys2* (Figure 3). Similarly, *Lc PPARα* expression was significantly elevated at 1 and 2 hours after feeding and significantly repressed at 24 hours (Fig 2), which resulted in significant positive correlations with *Lc G6PDH*, *Lc acyl*, *Lc SREBP-1c*, *Lc ASAT*, *Lc CS* and *Lc pygl* (Fig 3).

In this study we have focussed on some of the genes involved in amino acid inter-conversion and entry into the TCA cycle for energy production. These included the alanine and aspartate aminotransferases (EC 2.6.1.2; *Lc cALAT* and EC 2.6.1.1; *Lc ASAT*), glutamate dehydrogenase (EC 1.4.1.2; *Lc GLUD1*), as well as citrate synthase (EC 2.3.3.1; *Lc CS*) which is the pace making enzyme of the TCA cycle. Expression of *Lc ALAT* was significantly up-regulated within 0.5 hours of feeding and had returned to normal levels by 8 hours. The change in expression of the other genes *Lc ASAT* and *Lc gluc* was only very small after a feeding event, and in most cases this change was not significant. However, the significant up-regulation of *Lc ASAT* at 2 and 4 hours after a meal resulted in a positive correlation with *Lc G6PDH*, *Lc acyl* and *Lc SREBP-1c*. In addition, expression levels of pyruvate dehydrogenase kinase (EC 2.7.11.2; *Lc PDK*) were analysed, and showed a significant decrease between 4 and 12 hours after feeding, that produced strong negative correlations with glycolytic and lipogenic genes.

The expression of the hepatic isoform of the glycogen synthase gene (EC 2.4.1.11; *Lc gsy2*) was progressively down regulated to a low point 12 hours after feeding (Figure 3). This caused *Lc gys2* expression to be significantly negatively correlated with *Lc PK* and *Lc CPT1A*. The glycogen phosphorylase gene (EC 2.4.1.1; *Lc pygl*) was significantly up-regulated after feeding, and peaked 12 hours after feeding. The *Lc pygl* expression showed a significant positive correlation with the expression of lipid synthesis genes (*Lc acyl*, *Lc FAS* and *Lc SREBP-1c*) as well as the expression of *Lc PPARα*, *Lc G6PDH* and *Lc ASAT*. Although they appeared complementary in their post feeding expression pattern, there was no significant negative correlation between *Lc gys2* and *Lc pygl*.

Signalling Pathways

The activity of members of the mTOR signalling cascade were assessed by quantifying the ratio between the phosphorylated and unphosphorylated form of each enzyme. There was a significant activation of the insulin signalling cascade,

where the phosphorylated form of each protein was significantly more abundant after feeding (Figure 4). Phosphorylation of barramundi Akt, TOR and S6 significantly increased at least 30 min after the meal and remained elevated until 4 hours after feeding. Positive correlations were observed between Akt, mTOR and S6, as well as the other genes *Lc SREBP-1c*, *Lc cALAT* and, in the case of S6, *Lc CS* (Figure 3).

Discussion

The barramundi in this study showed the ability to effectively regulate blood glucose levels, since plasma glucose reached a maximum value 2h after refeeding but returned to basal level at the most 2h after that. This result was as expected given the diet was only composed of 11% total carbohydrates, of which was approximately 30% digestible to barramundi (Glencross et al. 2012). The result of this study mimicked the peak in plasma glucose levels at 2 hours post-feeding recorded previously in this species when using a diet containing 30% pre-gelatinised starch that is known to be poorly utilised in this species (Allan et al. 2003). However, in the same study, including high levels of highly digestible glucose in the diet (up to 30%) or intraperitoneal injection of over 1g kg⁻¹ of glucose caused pronounced elevation of plasma glucose levels for at least 8 hours (Allan et al. 2003). The present study demonstrated that there was no persistent post-feeding hyperglycaemia using a diet that is composed of 50% digestible protein energy, 15% lipid energy and approximately 3%, carbohydrate energy. The response to feeding this nutritionally optimised diet enabled the analysis of how these fish are able to metabolise dietary energy and how these processes are regulated at the molecular level.

Although provided at low levels in the diet in this study, the mechanism by which barramundi utilise dietary carbohydrates appears to be very different to other fish species, despite its carnivorous status. Glucokinase (GK) and pyruvate kinase (PK) are the two first enzymes involved in hepatic glucose utilization, along with the entry of glycolytic pyruvate to the TCA cycle via the pyruvate dehydrogenase complex (PDC). The ability of barramundi to rapidly and simultaneously up-regulate *Lc GK*, *Lc PK* and down-regulate *Lc G6Pase* and *Lc PDK* may underlie the metabolism of dietary carbohydrates in this species. The rapid induction of *Lc GK* and *Lc PK* gene expression during the first 2 hours following the meal is consistent with the ability of barramundi to metabolise simple carbohydrates, although the response of these genes at higher carbohydrate levels has not been investigated to date. Induction of glucokinase after feeding has been reported in a number of fish species (Panserat et al. 2001; Méton et al. 2004), as well as in response to increased levels of dietary carbohydrates (Panserat et al. 2000). However, the regulation of *Lc PK* gene expression in barramundi after a meal

seems to be closer to that observed in mammalian species, rather than in other fish. In mammals, regulation of PK occurs at both transcriptional and post-transcriptional levels (Assimacopoulos-Jeannet and Jeanrenaud 1990), whereas *PK* gene expression has been shown to be poorly regulated in trout (Panserat et al. 2001; Skiba-Cassy et al. 2009).

In some fish species, such as trout, the lack of post-prandial *G6Pase* repression has also been proposed to be a contributing factor towards the prolonged elevation of plasma glucose levels (Panserat et al., 2001). Some fish, such as trout and sea bream, have shown the ability to reduce *G6Pase* expression in response to dietary post-feeding (Caseras et al. 2002; Méton et al. 2004; Saldago et al. 2004; Meningen et al., 2012), but not until 4-8 hours after the feeding event. Barramundi in this study showed the ability to down-regulate *Lc G6Pase* expression within 2 hours after a meal, a result consistent with the low metabolic requirement for endogenous glucose production while it is available at low levels in the diet. Where both gene expression and enzyme activity have been studied, *G6pase* expression is often linked to G6Pase enzyme activity (Caseras et al. 2002; Méton et al. 2004; Saldago et al. 2004). However, down regulation of *G6Pase* gene expression does not automatically mean inhibition of gluconeogenesis, as a recent study performed in trout showed a post-prandial inhibition of *G6Pase* gene expression was not linked to the modification of G6Pase enzyme activity (Skiba-Cassy et al., 2012).

Expression of *Lc cALAT* was observed to significantly increase immediately after feeding. This enzyme product of this gene functions to link carbohydrate and amino acid metabolism, and increased ALAT activity is connected with enhanced gluconeogenesis and fasting (Méton et al. 1999; Fernandez et al. 2007; Pérez-Jiménez et al. 2012). In fish studied to date, including trout and seabream, one mitochondrial form (*mALAT*) and two cytosolic isoforms (*cALAT1* and *cALAT2*) have been identified (Méton et al. 2004; Fernandez et al. 2007; Anaemet et al. 2008). In addition, *cALAT1* was shown to be the predominant isoform in liver tissue, and the two cytosolic isoforms were independently regulated in response to nutrients, whereby starvation decreased *cALAT1* expression, and refeeding increased *cALAT1* expression and decreased *cALAT2* expression (Anaemet et al. 2008). Unfortunately, it is not known if barramundi possess these two *cALAT*

isoforms, as the sequence of the *Lc cALAT* gene analysed in this study is shared by both isoforms. However, if the mechanism of *cALAT* regulation was conserved between barramundi and sea bream, it is likely that the barramundi sequence corresponds to the *cALAT1* isoform. Whether two *cALAT* isoforms exist in barramundi requires clarification, with important implications for the use of analysing gene expression in response to nutrients.

Pyruvate dehydrogenase kinase (PDK) phosphorylates and inactivates the pyruvate dehydrogenase complex (PDC), a group of enzymes that converts pyruvate to acetyl-CoA as an entry point into the TCA cycle. The expression of *Lc PDK* was lowest 4 hours after a meal, suggesting that there was also an increase in both the activity of the PDC and potentially the entry of metabolic intermediates into the TCA cycle. Since the PDC is active during metabolism of metabolic intermediates generated through glycolysis, as well as gluconeogenic amino acids such as alanine, serine and glycine, it is not possible to distinguish the proportion of energy metabolism derived from glycolysis or amino acid metabolism. **Something about CS here.....** The results of this study demonstrate that measuring *Lc PDK* gene expression may be used to assess the activation of the PDC and hence the entry of metabolic intermediates to the TCA cycle. Similarly, *Lc CS* expression may reflect the timing of energy production through the TCA cycle after a meal is consumed.

The mammalian target of rapamycin (mTOR) is a central controller of the growth in response to nutrients, growth factors and cellular energy status (Polak et al. 2009; Wullschleger et al. 2006). Recent studies performed in mammals and fish indicate that TOR signalling pathway controls *de novo* lipogenesis by regulating the expression and processing of sterol regulatory element-binding protein 1c (SREBP1c), a master regulator of sterol- and lipogenic gene transcription (Dentin et al 2005; Duvel et al. 2010; Eberle et al. 2004; Lansard et al., 2010; Seilliez et al. 2011). Genes that code the enzymes glucokinase (GK), ATP-citrate lyase (acyl), acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) have been thus identified as targets of the transcription factor SREBP-1c (Girard et al. 1997; Kim 2004; Portsmann et al. 2008; Sato 2000; Skiba-Cassy et al., 2009). In this study, we observed strong correlations between the activation of the mTOR signaling pathway, *Lc SREBP-1c* expression and glycolysis (*Lc GK*

and *Lc PK*), the pentose phosphate pathway (*Lc G6PDH*) and lipogenic gene expression (*Lc acyl*, *Lc FAS* and *Lc SCD*). As such, our data confirm the link between the mTOR pathway and *SREBP-1c* as the master regulator of fatty acid and triglyceride synthesis in barramundi, resulting in increased expression of glycolytic and lipogenic genes. Whereas Akt phosphorylation is mainly controlled by insulin in trout, *in vivo* activation of the mTOR pathway seems to require both insulin and amino acids (Avruch et al., 2009; Lansard et al 2010 and 2011; Seiliez et al. 2011). We observed a small increase in circulating free amino acids in the plasma that coincided with the activation of the mTOR pathway, but this was not significantly correlated. Although likely to be the same as other species, further studies are required to confirm the role of amino acids in the regulation of the TOR signalling pathway in barramundi.

Expression of genes regulating β -oxidation in barramundi were in stark contrast to the response seen in fish, mammalian and avian species. Fasting has been shown to be a potent activator of β -oxidation in the liver (Escher et al. 2001; Leaver et al. 2005). As such, refeeding has been shown to significantly down regulate *CPT1A* expression, as well as the expression of the master regulator of hepatic lipid catabolism, *PPAR α* (Leaver et al. 2005; Meningen et al., 2012). However, in barramundi both *Lc CPT1A* and *Lc PPAR α* were elevated over a prolonged period after refeeding. These results imply that the lipolytic pathway, along with the glycolytic and lipogenic pathways, are for some reason active at the same time, or that one of these pathways is perhaps constitutively active or poorly regulated. One potential explanation is that certain classes of polyunsaturated fatty acids (PUFAs), particularly n-3 and n-6, are known to act as direct ligands to activate *PPAR α* and hence other fatty acid oxidation genes (Keller et al., 1993; Clarke 2000). In a similar way, supply of dietary n-3 and n-6 PUFAs may also be triggering fatty acid catabolism through *PPAR α* in barramundi, although this requires further confirmation through other genes targets such as acyl Co-A oxidase. What remains clear is that the pathways that modulate fatty acid oxidation in barramundi are nutritionally regulated, and that *PPAR α* and downstream targets of *PPAR α* are involved in fatty acid catabolism in barramundi.

The results of the glycogen gene expression data were also counter intuitive, in that glycogen breakdown appeared to be occurring at times when glucose and energy were abundant, since the expression of the glycogen synthase gene underwent a continuous decrease during the 12 hours following the meal. In general, an increase in liver glycogen has been observed once fish are refed after periods of starvation, or in response to elevated dietary carbohydrates (Enes et al. 2011, 2012; Figueiredo-Garutti et al. 2002; Leung and Woo 2011; Panserat and Kaushik 2010; Viegas et al. 2012), including barramundi (Allan et al. 2003). Glycogen is not thought to be a major contributor to the storage of excess energy in fish, and glycogen metabolism is known to be rapidly modified by allosteric effects and phosphoregulation (NRC 2011). At the very least, the data from the present study indicate that in barramundi the expression of genes controlling glycogen turnover is regulated by nutritional status, but may not be suitable markers of glycogen regulation. Genes that regulate phosphorylation events controlling glycogenesis, such as glycogen synthase kinase, may be more suitable molecular targets.

Although these types of functional genetic correlations performed in this study do not indicate that these genes are directly co-regulated, they do provide an indication of the trends that may be occurring in the regulation of various nutritional pathways. A higher degree of confidence in biological function could be attributed to some of the gene expression responses if a corresponding change in enzyme activity was also confirmed. Most often, the focus of current studies of fish intermediary metabolism is directed towards the modification of gene expression in response to modifying dietary energy sources, particularly protein and carbohydrate levels. It is paramount that this type of nutritional regulation be interpreted in conjunction with knowledge of how different species genes that regulate intermediary metabolism are regulated in the hours after feeding. Differences in the timing of activation of, or the ability to regulate, specific metabolic pathways may play a central role in the metabolic consequences of dietary nutrient utilisation for each different fish species.

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Table 1 The ingredient formulation and nutritional composition of experimental diet. All values are g kg⁻¹ dry matter (DM) unless otherwise detailed.

<i>Ingredient Formulation (g kg⁻¹)</i>	
Yttrium oxide	1.0
Pre-mix vitamins*	5.0
Fish oil	94.0
Wheat gluten	100.0
Wheat flour	145.0
Fish meal	655.0
<i>Diet nutritional composition</i>	
Dry matter	909
Crude protein	547
Digestible protein	500
Lipid	150
Carbohydrate	110
Phosphorus	17
Ash	102
Gross energy (MJ kg ⁻¹ DM)	20.51
Digestible energy (MJ kg ⁻¹ DM)	18.68
Lysine	36.04
Threonine	19.71
Methionine	13.20
Isoleucine	22.66
Leucine	38.08
Tryptophan	5.51
Valine	25.53
Phenylalanine	20.90
Histidine	10.84
Arginine	35.71

*Vitamin and mineral premix includes (IU kg⁻¹ or g kg⁻¹ of premix): Vitamin A, 2.5MIU; Vitamin D3, 0.25 MIU; Vitamin E, 16.7 g; Vitamin K₃, 1.7 g; Vitamin B1, 2.5 g; Vitamin B2, 4.2 g; Vitamin B3, 25 g; Vitamin B5, 8.3; Vitamin B6, 2.0 g; Vitamin B9, 0.8; Vitamin B12, 0.005 g; Biotin, 0.17 g; Vitamin C, 75 g; Choline, 166.7 g; Inositol, 58.3 g; Ethoxyquin, 20.8 g; Copper, 2.5 g; Ferrous iron, 10.0 g; Magnesium, 16.6 g; Manganese, 15.0 g; Zinc, 25.0 g.

Table 2. Target genes of intermediary metabolism in barramundi, and the primer sequences used to analyse their expression.

Target Gene	Gene Abbreviation	EC Number	Genbank Accession	Primer Name	Sequence	Length
Glucose Metabolism						
Glucokinase (Hexokinase IV)	<i>Lc GK</i>	EC 2.7.1.1		GK qPCR.For 2	CTGGTGTGATCAACCGAATG	20
				GK pPCR.Rev 2	ACCACTCCCCTCCTCTGACT	20
Glucose-6-Phosphatase	<i>Lc G6Pase</i>	EC 3.1.3.9		G6Pase qPCR.For 1	TTGCTGGTGTGCATCACAGGT	20
				G6Pase qPCR.Rev 1	CACCTTCTGGGCTTTCTCCAG	20
Fructose-1,6-Bisphosphatase	<i>Lc F1,6BPase</i>	EC 3.1.3.11		F1,6BPaseqPCR.For 1	GATATGTTGGTTCAATGGTGGCT	23
				F1,6BPaseqPCR.Rev 1	GGACTCGTTGGTGGATTGTG	20
Pyruvate Kinase	<i>Lc PK</i>	EC 2.7.1.40		PK qPCR.For 2	TCACGCCTCTCTCCTCTGAT	20
				PK qPCR.Rev 2	TTCTGGTGACCGCAATGATA	20
Glucose-6-Phosphate Dehydrogenase	<i>Lc G6PDH</i>	EC 1.1.1.49		G6PDH qPCR F1	CAAACCTCAACACACACATCC	20
				G6PDH qPCR R1	CTTCTCTACAATCACCCCTGTTC	22
Fatty Acid Metabolism						
Fatty Acid Synthase	<i>Lc FAS</i>	EC 2.3.1.85		FAS qPCR.For 1	TGAATCTCACCACGCTTCAG	20
Stearoyl CoA Desaturase	<i>Lc SCD</i>	EC 1.14.19.1		FAS qPCR.Rev 1	AGGCAGCAATAGAACCCTCA	20
				SCD qPCR.For 1	CCTGGTACTTCTGGGGTGAA	20
CarnitinePalmitoyltransferase	<i>Lc CPT1A</i>	EC 2.3.1.21		SCD qPCR.Rev 1	AAGGGGAATGTGTGGTGGTA	20
				CPT1A qPCR.For 1	TGATGGTTATGGGGTGTCTCCT	20
ATP Citrate Lyase	<i>Lcacyl</i>	EC 2.3.3.8		CPT1A qPCR.Rev 1	CGGCTCTCTTCAACTTTGCT	20
				Lcal acyl F1	CAACACCATTGTCTGTGCTC	20
Sterol Regulatory Element Binding Protein	<i>Lc SREBP-1c</i>	na		Lcal acyl R1	GAAATGCTGCTTAACAAAGTCC	21
				Lcal SREBP F2	GTTCTGGGTGTGCATATTGC	20
				Lcal SREBP R2	GGATCAGAACTCCAGACACC	20
Amino Acid Metabolism						
Alanine Aminotransferase, cytosolic form	<i>Lc cALAT</i>	EC 2.6.1.2		cALATqPCR.For 2	GATGAACCCTCCTACACCAC	20
				cALATqPCR.Rev 2	TTTGAGGTAGAGTGATGCGG	20
Aspartate Aminotransferase	<i>Lc ASAT</i>	EC 2.6.1.1		ASAT qPCR.For 1	CATTCGCTTCTTCGTCTCTG	20
				ASAT qPCR.Rev 1	TCCTGACAATCTTCTCCATCTG	22
Glutamate Dehydrogenase	<i>Lc GLUD1</i>	EC 1.4.1.2		Glud qPCR.For 1	GGATATTGTTCACTCTGGGTTGG	23
				Glud qPCR.Rev 1	GAAGTGTGGGTCTGTGATTGG	20
Glycogen Metabolism						
Glycogen Synthase, liver	<i>Lc gys2</i>	EC 2.4.1.11		Lcal gys2 F2	CAAGATTCCCATGGCAACAG	20

isoform			Lcal gys2 R2	AGACTGTGGTGAAGACATGAG	21
Glycogen Phosphorylase, liver	<i>Lc pygl</i>	EC 2.4.1.1	Lcal pygl F2	CACACTCCAAGACATCATCC	20
isoform			Lcal pygl R2	GTCCACAAAGATTCTCATCAG	21
Control Genes					
Luciferase	<i>Luc</i>	na	Luc qPCR For	GGTGTTGGGCGCGTTATTTA	20
			Luc qPCR Rev	CGGTAGGCTGCGAAATGC	18
Elongation Factor 1 alpha	<i>Lc Ef1α</i>	na	Lcal Ef1 α F	AAATTGGCGGTATTGGAAC	19
			Lcal Ef1 α R	GGGAGCAAAGGTGACGAC	18

Table 3. Plasma and tissue chemistry parameters

Parameter	Units	T0	T0.5	T1	T2	T4	T8	T12	T24
Plasma		5.1 ^{ab}	6.1 ^{ab}	6.4 ^{ab}	7.5 ^b	5.8 ^{ab}	4.4 ^a	3.4 ^a	4.8 ^a
Glucose	mmolL ⁻¹	±1.0	±1.0	±1.1	±0.4	±0.5	±0.6	±0.3	±1.0
Liver		7.4	6.8	7.9	8.4	7.4	8.2	7.6	8.4
Glucose	μmolL ⁻¹	±0.4	±0.4	±0.3	±0.3	±0.6	±0.5	±0.4	±0.5
Plasma Free		867 ^a	914 ^{ab}	827 ^a	1179 ^{bc}	1259 ^c	932 ^{ab}	1090 ^{ab}	902 ^a
Amino Acids	μmolL ⁻¹	±100	±66	±47	±39	±147	±26	±67	±44

Superscripts denote significant (P<0.05) differences among dietary treatments within a parameter. Lack of any superscripts within a row indicate that there were no significant differences among any of those treatments for that parameter.

Fig 1 The relative expression of each gene analysed in this study at both before feeding 0 hrs and 2 hrs post feeding. Before feeding, the *Lc G6Pase* gene was the most highly expressed of those investigated, along with the *Lc GLUD1* gene. Two hours after feeding, relative levels of the *Lc GK*, *Lc FAS* and *Lc SCD* genes had increased above those of *Lc G6Pase*, which had undergone mild repression.

Fig 2 Relative change in gene expression over time after a single feeding event. The expression of many genes was under nutritional regulation, around 2-4 hours after feeding. Expression was significantly elevated in genes that regulate glucose utilisation (*Lc GK*, *Lc PK*, *Lc G6PDH*, *Lc PDK*), and significantly lower in those that regulate glucose synthesis (*Lc G6Pase*). Lipid production genes were significantly elevated (*Lc FAS*, *Lc SCD* and *Lc acyl*), along with those regulating amino acid metabolism (*Lc cALAT*) and energy production (*Lc CS*). However, genes that regulate lipid oxidation (*Lc CPT1A* and *Lc PPAR α*) and glycogen breakdown (*Lc Pygl*) were also active at the same time. Superscripts denote significant ($P < 0.05$) differences between time points.

Fig 3 Pearson correlation coefficient matrix. The colour of each box shows the relationship between the different parameters analysed in this study, with dark red highlighting positive relationships and dark green highlighting negative relationships. Asterisks denote significant ($P < 0.05$) correlations between two parameters.

Fig 4 Activation of signalling cascades in response to feeding. A. Specific antibodies were used to quantify levels of phosphorylated (P-Akt; P-mTOR; P-S6) relative to non-phosphorylated forms (Akt; mTOR; S6). B. The ratio of the active (phosphorylated) form of each member of the signalling cascade demonstrated that there was a rapid phosphorylation and activation of the mTOR signalling pathway. Superscripts denote significant ($P < 0.05$) differences between time points.

Figure 1.

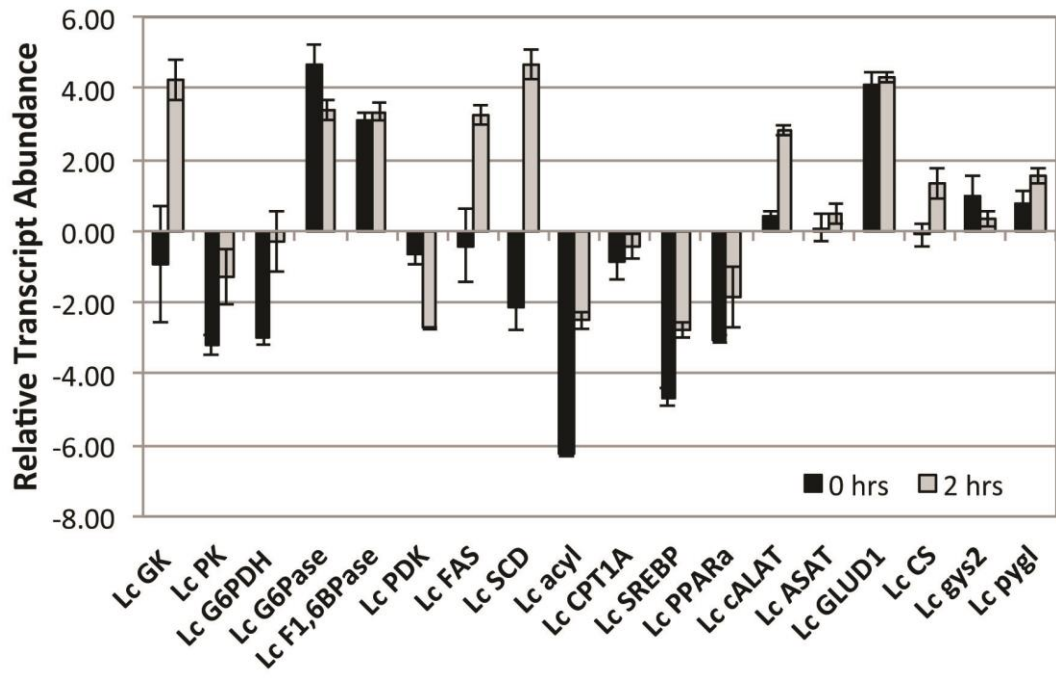


Figure 2. Superscripts denote significant ($P < 0.05$) differences among time points.

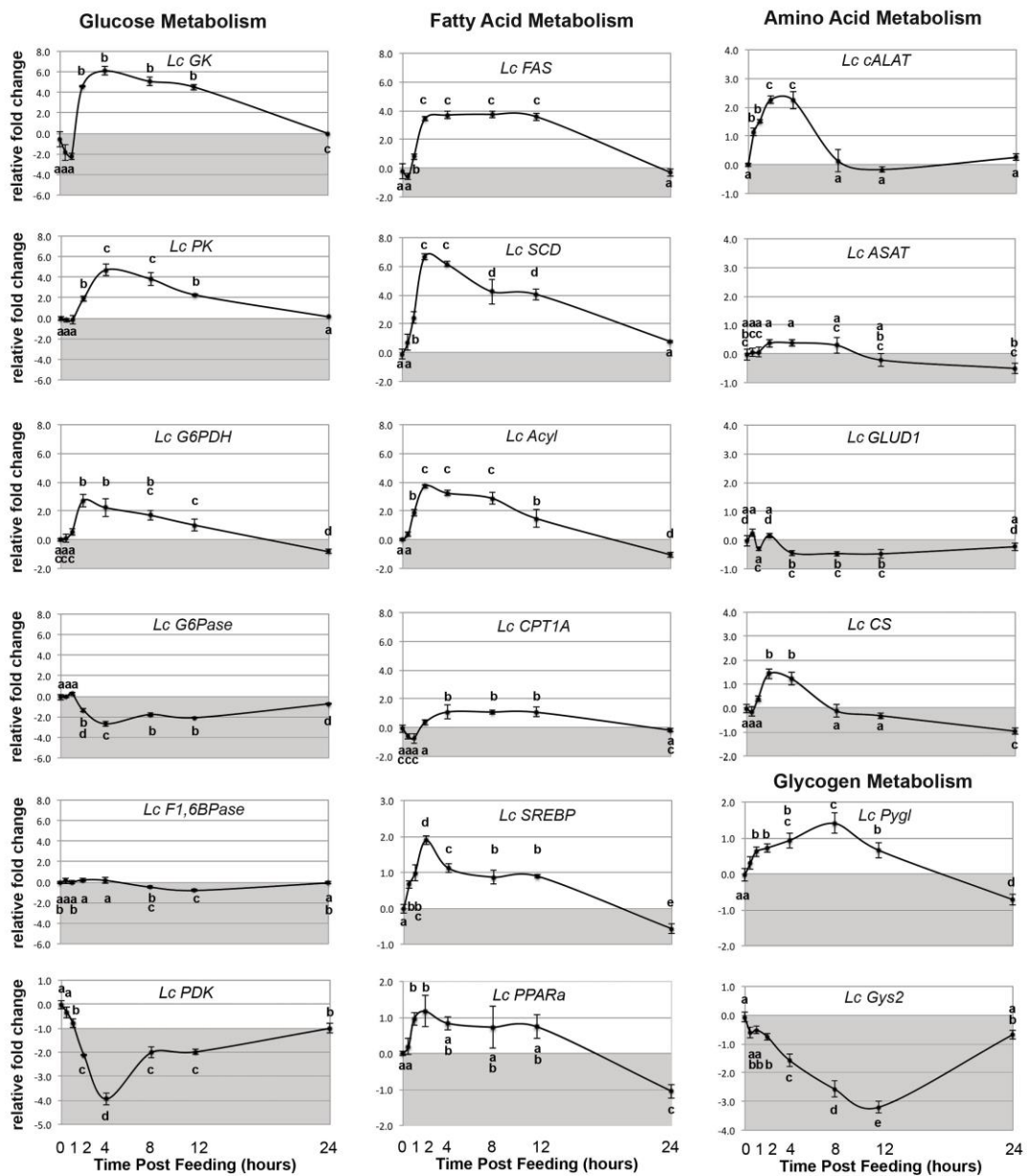


Figure 3. Pearson correlation coefficient matrix.

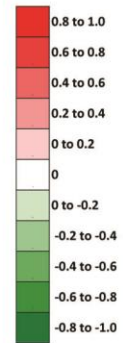
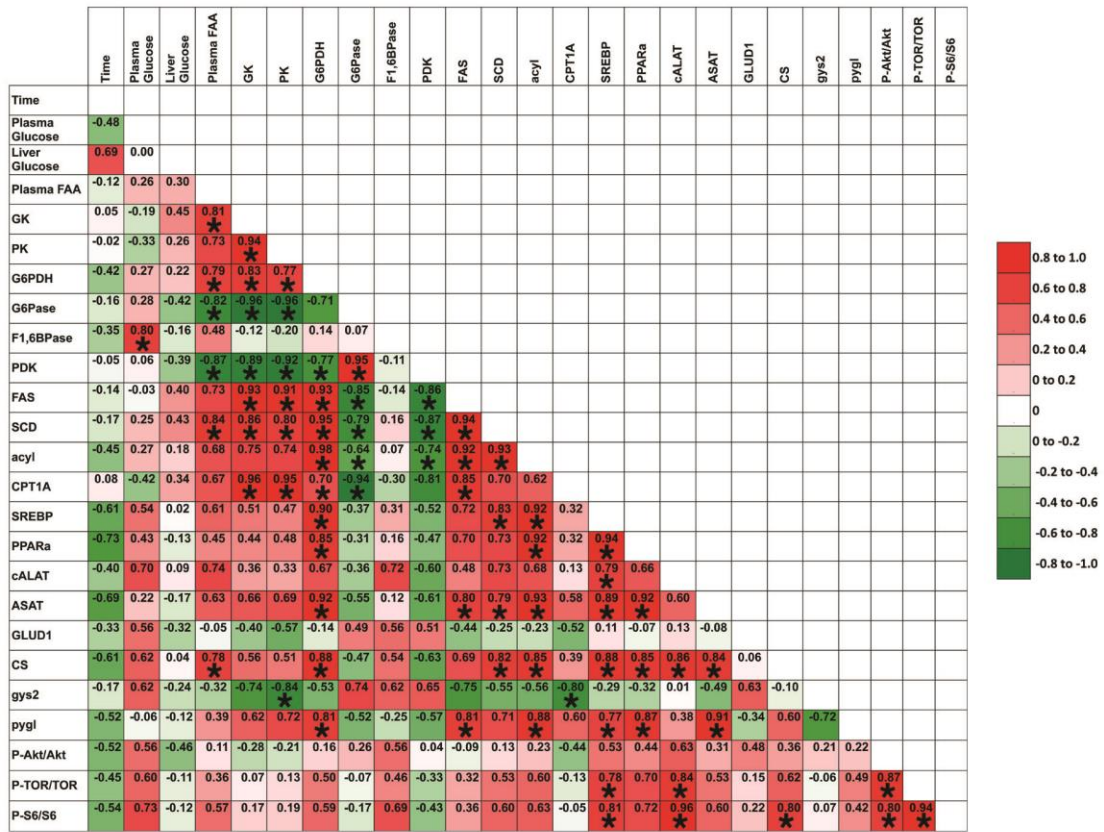
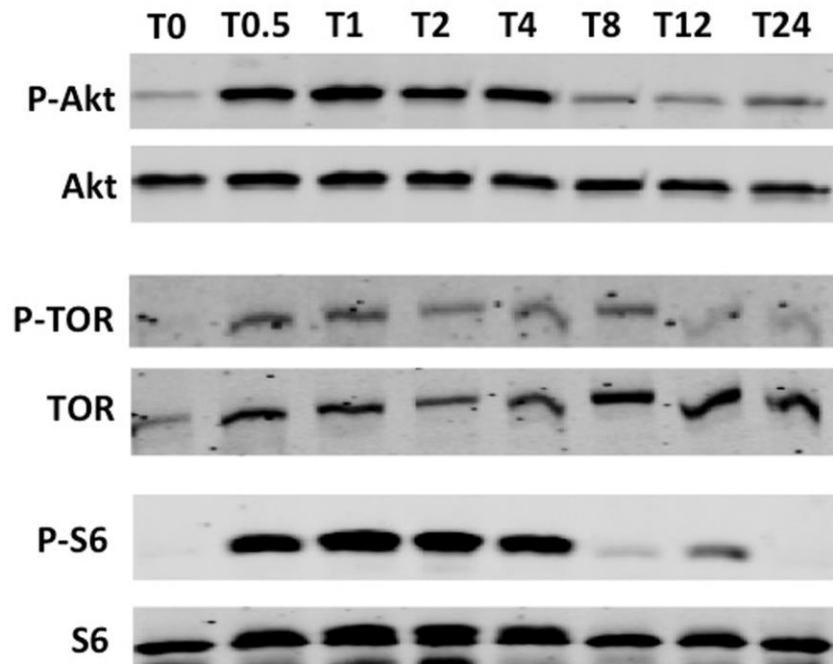


Figure 4. Superscripts denote significant ($P < 0.05$) differences among time points.

A



B

