Dietary starch promotes hepatic lipogenesis in barramundi (*Lates calcarifer*)

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Abstract

Barramundi (*Lates calcarifer*) are a highly valued aquaculture species, and, as obligate carnivores, they have a demonstrated preference for dietary protein over lipid or starch to fuel energetic growth demands. In order to investigate how carnivorous fish regulate nutritional cues, we examined the metabolic effects of feeding two isoenergetic diets that contained different proportions of digestible protein or starch energy. Fish fed a high proportion of dietary starch energy had a higher proportion of liver SFA, but showed no change in plasma glucose levels, and few changes in the expression of genes regulating key hepatic metabolic pathways. Decreased activation of the mammalian target of rapamycin growth signalling cascade was consistent with decreased growth performance values. The fractional synthetic rate (lipogenesis), measured by TAG ^{2}H-enrichment using ^{2}H NMR, was significantly higher in barramundi fed with the starch diet compared with the protein diet (0·6 (SE 0·1) *v.* 0·4 (SE 0·1) % per d, respectively). Hepatic TAG-bound glycerol synthetic rates were much higher than other closely related fish such as sea bass, but were not significantly different (starch, 2·8 (SE 0·3) *v.* protein, 3·4 (SE 0·3) % per d), highlighting the role of glycerol as a metabolic intermediary and high TAG-FA cycling in barramundi. Overall, dietary starch significantly increased hepatic TAG through increased lipogenesis. Compared with other fish, barramundi possess a unique mechanism to metabolise dietary carbohydrates and this knowledge may define ways to improve performance of advanced formulated feeds.

Key words: Liver: Fish: Nutrigenomics: ^{2}H: Lipogenic flux: NMR

Dietary energy is derived from three broad macronutrient classes: protein, lipid and carbohydrates (CHO) (such as starch). Metabolism of these macronutrient classes is known to occur with different levels of efficiency in terrestrial animals[1,2] and has been examined in several fish species[3,4–8]. In past studies, varying the dietary macronutrient composition while maintaining the dietary digestible energy content resulted in significant changes in growth performance, feed utilisation and body composition[5,6,9]. These studies across three different fish species showed that increasing dietary CHO composition reduced feed intake, growth and the efficiency of dietary energy utilisation, with a preference for protein-derived energy. This clearly indicated that dietary macronutrients were not utilised equally, despite the prevailing theory that only total dietary energy was important, but did not reveal how these effects might be occurring. Many studies have sought to understand the metabolic utilisation of glucose and other CHO in carnivorous fish[10,11]. Despite possessing shared metabolic pathways,

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fundamental differences in the hepatic and extra-hepatic regulation of dietary starch metabolism are beginning to be discovered in fish[12–15].

Barramundi (*Lates calcarifer*) are an obligate carnivorous fish species at trophic level 3·8[16] and are the basis of a significant aquaculture industry in Southeast Asia and Australia[17]. A series of factorial bioenergetic nutritional models have been developed to provide estimations of feed demand and idealised feed compositions to support growth performance[18–21]. Although the aim in finfish aquaculture is to reduce dependence on protein (for costs and sustainability), there has been a resulting shift in dietary energy proportion towards formulations with higher levels of lipids and CHO, with a range of implications for farmed fish[13]. As expected for a carnivorous fish, growth performance and feed conversion in barramundi were more efficient when a high proportion of dietary energy was supplied as protein, followed by lipid energy[9]. However, a high proportion of dietary starch was also shown to restrict protein utilisation, suggesting a limited capacity for barramundi to utilise starch-derived energy over protein or lipid energy[22]. The types of physiological adaptations or underlying molecular mechanisms responsible for such differential macronutrient utilisation and subsequent growth performance are yet to be defined.

Molecular tools such as quantitative gene expression analysis have been successfully applied to understanding the effects of dietary nutrients on intermediary metabolism[23–26]. This includes key genes regulating glycolysis or gluconeogenesis (glucokinase (*gck*), pyruvate kinase (*pk*), glucose-6-phosphatase (*g6pca*) and fructose-1,6-bisphosphatase (*fbp1*)), lipogenesis or lipid oxidation (ATP citrate lyase (*acyl*), stearoyl CoA desaturase (*scd*), fatty acid synthase (*fasn*), carnitine palmitoyl transferase 1a (*cpt1a*), glucose-6-phosphate dehydrogenase (*g6pd*) and sterol responsive element binding protein (*srebf1*)). In addition, the activation of signalling cascades (serine/threonine specific protein kinase B (Akt), mammalian target of rapamycin (mTOR), ribosomal S6 kinase (S6), ribosomal protein S6 kinase beta-1 (S6K1), forkhead homebox protein O1 (FoxO1) and FoxO1-3) has been defined as primary regulators of growth and metabolism in fish[25,27]. When sampled at peak times after feeding, these studies provide a snapshot of the molecular regulation underlying dietary metabolic changes[24]. As a complementary technique, the use of stable isotopes is further able to define metabolite flux and re-routing of major macronutrient metabolic pathways within tissues of fish fed different diets. Deuterated water (^2H_2O) has been particularly effective for assessing contributions of gluconeogenesis to circulating glucose[28], direct or indirect pathways of hepatic glycogen synthesis[29,30] or lipogenesis of hepatic TAG[31] in European sea bass (*Dicentrarchus labrax*).

This study sought to evaluate the underlying mechanisms that regulate nutrient utilisation that potentially drive fish performance. Two experiments were used to investigate gene expression, signalling pathways and metabolic labelling changes that define metabolic nutrient preference and metabolic flux re-routing. The first used liver samples from barramundi fed either a high-protein or high-starch diet in a 12-week growth performance study[9]. The second used liver samples from a complementary metabolic labelling experiment that fed fish the same two diets in the presence of ^2H_2O to track the fate of dietary

starch using ^2H NMR[12]. These experiments defined several potential mechanisms that underlie the utilisation of dietary starch in barramundi. It also highlighted the diversity of mechanisms that regulate growth, metabolism and nutrient utilisation in fish as dietary protein is replaced by CHO.

Methods

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 numbers: A8-2010 and A8-2016).

Fish Expt 1 – macronutrient source

Samples from the protein and starch treatments from a previous experiment were used in this study[9], where fish were fed diets formulated to the same digestible energy specifications but were biased to increase the relative contributions from protein or starch (Table 1). Twenty juvenile barramundi (81·2 (SE 1·48) g) were allocated to each of the six 300-litre tanks, maintained at 27·8 (SE 0·45)°C, dissolved O_2 5·6 (SE 0·18) mg/l, at flow rates of approximately 3 litres/min and under a 12 h light–12 h dark photoperiod. Three replicate tanks were hand fed one of the two experimental diets for a period of 12 weeks. Diets were fed twice daily (09.00–09.30 and 16.30–17.00 hours) to slight excess based on the loss of observed feeding behaviour. All feed fed and all uneaten feed were accounted for and correction

Table 1. Formulation, proximate composition and digestible protein and energy parameters of the diets*

	Protein diet	Starch diet
Formulation		
Fishmeal†	640	560
Gluten‡	100	100
Casein§	100	50
Fish oil†	40	0
Pregelled starch‡	0	240
Yttrium oxide	2	2
Vitamins and minerals‖	5	5
Cellulose§	113	43
Composition		
DM	930	890
Crude protein	633	502
Digestible protein	575	448
Total lipid	117	66
Ash	90	115
Total carbohydrates	161	317
Total starch	16	325
Gross energy (kJ/g DM)	21·3	20·8
Digestible energy (kJ/g DM)	15·9	15·2
Protein energy (%)	78	66
Lipid energy (%)	19	11
Starch energy (%)	0	20

* All values are g/kg DM basis unless otherwise shown.
† *Peruvian anchoveta* fishmeal and fish oil: Skretting Australia.
‡ Wheat gluten and pre-gelatinised wheat starch: Manildra.
§ Cellulose and vitamin-free casein: Sigma.
‖ Vitamin and mineral premix includes (g/kg of premix): vitamin A, 0·75 g; vitamin D3, 6·3 mg; vitamin E, 16·7 g; vitamin K, 3, 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; Cu, 2·5 g; ferrous Fe, 10·0 g; Mg, 16·6 g; Mn, 15·0 g; Zn, 25·0 g.

factors applied to obtain an accurate estimate of feed intake. At the end of the 12-week trial, four random fish from each of the three tank replicates for each treatment were sedated in anaesthetic Aqui-S® (0·02 ml/l) 2 h after their final meal, the time by which key gene regulatory pathways of intermediary metabolism in barramundi liver tend to peak[24]. Blood was collected by caudal vein puncture using a syringe pre-treated with a solution containing 0·2M EDTA, then centrifuged at 3000 **g** for 5 min and the plasma transferred to a new tube and kept frozen at –80°C until analysis. Fish were euthanised by overdose in anaesthetic Aqui-S® (0·2 ml/l) before liver samples were collected, snap frozen on dry ice and then stored at –80°C prior to further RNA and protein analyses.

Fish Expt 2 – metabolic labelling with ^2H_2O

The greatest differences in growth performance were observed in fish fed a high proportion of dietary energy in the form of protein or starch; therefore, these two diets were further investigated through a metabolic tracer experiment, meaning that fish were fed their respective diets in the presence of deuterated (heavy) water (^2H_2O). Initially, two treatment groups of thirty fish each (initial 51·3 (SE 0·5) g) were housed in two independent 200 litre recirculated seawater systems maintained at 29·7 (SE 0·7) or 29·8 (SE 0·8)°C, respectively, and dissolved O_2 of 6·4 (SE 1·0) or 6·8 (SE 0·8) mg/l, respectively. Each group was assigned to one of the two diets protein or starch (Table 1) and fed once daily (09.00 hours) to apparent satiety for 21 d. The fish were sequentially transferred into a separate 200 litre tank enriched with about 3·5 % ^2H_2O and fed to apparent satiety once per d for 5 d and sampled on day 6, 24 h after their last meal. This ^2H_2O tank was maintained with an independent closed filtering system but had similar characteristics to each of the holding tanks used during the feeding period in terms of size, volume of water (200 litres), opacity, filtering material and water parameters (28·0 (SE 0·6)°C and dissolved O_2 of 6·6 (SE 1·6) mg/l). Seawater was enriched by the addition of 99·9 % ^2H_2O (Sigma catalogue no. 151882) as described previously[28]. Fish were sedated using 0·02 ml/l of anaesthetic Aqui-S®, then measured, weighed and sampled for blood from the caudal vein with heparinised syringes. Approximately 100 µl aliquot was centrifuged (3000 **g**, 10 min), and plasma was stored for quantification of body water (BW) ^2H-enrichments. Fish were then euthanised by an overdose in anaesthetic Aqui-S® (0·2 ml/l) before livers were excised, weighed and stored at –80°C until further analysis.

Metabolite assays

Plasma glucose levels (n 12) were measured using an AccuCheckPerforma glucose meter (Roche). Plasma TAG levels (n 12) were determined using a colorimetric commercial kit adapted to microplates (Biomerieux). Plasma free amino acid levels (n 12) were determined using a fluorometric detection method[32] and using Amino Acid Standard H (Pierce no. 20088) as a reference.

Lipid quantification

The determination of the fatty acid (FA) profile of diets and liver utilised an adapted protocol described by Coutteau &

Sorgeloos[33]. Lipids were esterified by an acid-catalysed methylation and to each sample was added 0·3 mg of an internal standard (21 : 0 Supelco). The FA methyl esters were separated by GC using an Agilent Technologies 6890N GC system (Agilent Technologies) fitted with a DB-23 capillary column. The carrier gas used was H_2 at a flow rate of 40 ml/min. The GC was programmed with the following temperature, 50–175°C at 25°C min then 175–230°C at 2·5°C min. FA methyl esters were detected by a flame ionization detector with the injector and detector temperatures being set at 250 and 320°C, respectively. The FA methyl esters were detected by comparing peak retention times to known standards (37 Comp. FAME mix, Supelco).

Quantitative real-time RT-PCR

Total RNA was extracted using Trizol reagent (Invitrogen), according to the manufacturer's instructions, and precipitated by adding 0·5 volumes of isopropyl alcohol and 0·5 volumes of RNA precipitation solution for purity improvement[34]. 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 no. 5067-1511). All RNA samples were diluted to 200 ng/µl. Reverse transcription was performed on 1 µg of total RNA using Superscript III (Invitrogen) with 25 µM oligo(dT)$_{20}$, 25 µM random hexamers and 400 pg of luciferase RNA (Promega L4561) as an exogenous control gene.

Real-time PCR amplification using primers specific to each gene of interest (online Supplementary Table S1) was performed as previously described[24]. Real-time PCR amplification reactions were carried out using 1X SYBR Green PCR Master Mix (Applied Biosystems); 0·2 µM 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 forty cycles of 15 s at 95°C and 40 s at 60°C. 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. Normalisation was performed using the ΔCq method (where Cq is the quantification cycle), and expression levels of each gene relative to one another were determined by normalising the cycle threshold values for each gene to the endogenous control gene *Ef1α* and the exogenous Luciferase control, then to the average cycle threshold of each gene relative to the control diet. The variation in amplification of *eef1a1* or luciferase across all samples was 0·63 and 0·10 cycles, respectively (data not presented).

Protein extraction and Western blotting

Frozen liver samples (about 100 mg) were extracted as previously described[24], and the resulting supernatants (n 9 for each treatment) were stored at –80°C until required. Protein concentrations were determined using the Bio-Rad Protein assay kit. Quantities of 20 µg 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 signalling pathways were obtained from Cell Signaling Technologies (Akt-p no. 9272; Akt

no. 9271; mTOR-p no. 2972; mTOR no. 2971; S6-p no. 4856; S6 no. 2217S; S6K1-p no. 9205; S6K1 no. 9202; FoxO1-p no. 9461; FoxO1-3-p no. 9464 and Tubulin no. 2775) or Epitomics (Labome FKHR (Fox-O1) no. 1874-1) and used at a dilution of 1:1000 as described previously[35]. After incubation with a goat anti-rabbit IRDye infrared secondary antibody (LI-COR Inc. Biotechnology), bands were visualised and quantified by Infrared fluorescence using the Odyssey Imaging System (LI-COR Inc. Biotechnology).

Metabolite preparation

To obtain a sufficient amount of analytes for generating ^{2}H NMR spectra with a high signal:noise ratio, the livers of five fish were pooled into six replicate groups (pooled analyses: n 6 per diet). Lipids were extracted from homogenised livers according to Matyash *et al.*[36] using a mixture of methyl tert-butyl ether (Sigma) and methanol (Sigma). Briefly, homogenised livers were added to a mixture of methyl tert-butyl ether:methanol, and after phase separation, the upper lipid phase was carefully separated. The lipid extract was further fractionated into TAG and NEFA using solid-phase extraction (SPE) with prepacked 2 g cartridges (Discovery® DSC-NH$_2$ 52641-U, Supelco) according to Ruiz *et al.*[37]. Hepatic TAG and NEFA extracts were analysed separately by ^{2}H NMR. Hepatic TAG quantifications were performed in a fully-automated analyzer Miura 200 (I.S.E. S.r.l.) using a dedicated TAG reagent kit (ref. A-R0100000901; n 5).

^{1}H and ^{2}H NMR analysis of lipids

Tank water and fish BW ^{2}H-enrichments were analysed in duplicate from 10 µl samples of water and plasma by ^{2}H NMR as described previously[38]. Tank and plasma water content was assumed to be 96·5 % (35‰ salinity) and 92·0 %[39] of total sample, respectively. NMR spectra of TAG and NEFA samples were obtained at 25°C with a Bruker Avance III HD system with an UltraShield Plus magnet (11·7 T, ^{2}H operating frequency 500 MHz) equipped with a 5-mm ^{2}H-selective probe with 19F lock and ^{1}H-decoupling coil. Lipids were reconstituted in chloroform containing a pyrazine standard as previously described[40] generating well-resolved spectra (online Supplementary Fig. S1). As control for the TAG extraction, a FA:glycerol ratio was calculated from the area of all FA α protons (online Supplementary Fig. S1(a)); A) times 2, divided by TAG-glycerol sn-1,3 protons (online Supplementary Fig. S1(a)); L). If successful, a TAG-only extraction theoretical FA:glycerol ratio should be approximately 3[41]. As control for the NEFA extraction, all spectra were confirmed for absent glycerol sn-1,3 proton signals. The FA profile (in percentage) for SFA and unsaturated FA, both PUFA and MUFA were estimated by ^{2}H NMR according to Viegas *et al.*[31].

Positional ^{2}H-enrichments were quantified from the ^{1}H and ^{2}H NMR spectra by measuring the ^{1}H and ^{2}H intensities of selected signals relative to the ^{1}H and ^{2}H intensities of a pyrazine standard, after correction for linoleic acid contribution according to Duarte *et al.*[41]. Briefly, this involved, (1) the determination of the ^{2}H-enrichment in the FA terminal methyl group for both TAG-bound FA and NEFA derived from lipogenesis (online Supplementary Fig. S1; A); (2) the determination of the

^{2}H-enrichment in the sn-1,3 glycerol site for newly synthesised or cycled TAG-bound glycerol (online Supplementary Fig. S1; L); (3) the determination of the ^{2}H-enrichment in the MUFA's allylic protons for desaturation of SFA (online Supplementary Fig. S1; F). Moreover, while the terminal methyl group is enriched with ^{2}H during the first round of FA synthesis (thus indicative of DNL), the α protons (online Supplementary Fig. S1; H) incorporate ^{2}H in the last round of elongation. Therefore, if elongation occurs on pre-existing (unlabeled) FA, the α- and methyl protons will be differentially labelled and will inform of the fractional contribution of elongation to lipid synthesis.

Fractional synthetic rates (FSR; in % per d) from (1) lipogenesis; (2) newly synthesised/cycled glycerol; (3) desaturation and (4) elongation rates (% per d) were estimated by dividing the respective positional ^{2}H-enrichments by that of BW. ^{2}H-enrichments were calculated after systematic subtraction of the values with 0·015 %, taken as the mean background ^{2}H-enrichment[41]. If the values were below zero, these were considered as 0·0 for FSR calculation purposes.

Statistical analyses

For comparison of the relationships between blood chemistry, gene expression and signalling pathways, each value was normalised to the average of the entire group and then log$_2$-transformed. Prior to statistical comparison, measured values were assessed for normality using a Kolmogorov–Smirnov test. Where comparison between individual measurements was required, statistical significance was assessed by t test analysis of means allowing 5 % error. Statistical analyses were performed using a combination of StatPlus:Mac 2009 (AnalystSoft Inc.), Statistica (StatSoft) or R-software packages (R-Core Team). For ^{1}H and ^{2}H NMR analysis of lipids, Student's two-tailed unpaired t test was used to compare means between dietary treatments. Analyses were performed in GraphPad Prism software (GraphPad Software). Differences were considered statistically significant at $P < 0·05$.

Results

Plasma metabolites

Levels of plasma metabolites were largely unaffected by the proportion of dietary energy supplied as different macronutrients at the sample time assessed. No significant differences were observed between the diets for plasma glucose, TAG levels or free amino acid (Table 2).

Liver lipids

When the FA content of the liver of animals fed the starch diet was compared with the protein diet, liver TAG levels were significantly elevated (Table 2). After separation of liver FA by GC, there was a significant elevation in the proportion of SFA (38·8–41·3 %) and MUFA (29·5–33·9 %), along with a significant decrease in PUFA (24·9–18·8 %), in particular *n*-3 PUFA (online Supplementary Table S2). Differences were predominantly caused by changes in 16 : 0 and 18 : 1*n*-9 *cis* FA. Decreased proportions were recorded for EPA (7·8–4·2 %), DPA (2·7–1·8 %)

Table 2. Plasma and liver metabolites
(Mean values with their standard errors)

	Protein		Starch	
	Mean	SEM	Mean	SEM
Plasma (n 12)†				
Glucose (mmol/l)	5·4	0·6	8·0	1·2
TAG (g/l)	1·0	0·5	1·4	0·8
Free amino acids (μmol/l)	553	38	452	37
Liver (n 6)‡				
TAG (mg/g tissue)	5·7	1·3	11·9*	1·6

Significant difference between diets (t test; * $P < 0·05$).
† From fish Expt 1 – macronutrient source, 2 h post-feeding.
‡ From fish Expt 2 – metabolic labelling with ^2H_2O.

and DHA (6·3–4·5 %). The n-3:n-6 ratio found in the liver was significantly lower in starch fed fish compared with protein fed fish (2·9–1·6; online Supplementary Table S2). There was also a shift in the proportion of FA in the liver compared with the levels of FA in the diets, dependent upon the dietary treatment. Compared with the levels in the diet, liver SFA significantly increased by 10·8 % in animals fed the starch diet, along with a 7·9 % increase in MUFA. This caused the greatest relative reduction in liver PUFA in the starch diet fed animals, with 24·7 % less PUFA recorded in the liver than in the diet.

The liver lipid analysis as obtained by ^1H NMR for the ^2H_2O experiment corroborated this profile and statistical changes for the protein and starch diets (online Supplementary Table S3). TAG ^1H NMR analysis revealed a consistent FA:glycerol ratio of approximately 3 for protein-fed fish (3·1 (SE 0·1)) and starch-fed fish (3·0 (SE 0·1)), while NEFA ^1H NMR spectra showed no presence of glycerol peaks (online Supplementary Fig. S1(a) (inlet at 4·14 parts per million)). Finally, after the metabolic tracer experiment, hepatic TAG levels were significantly elevated in fish fed with the starch diet (Table 2).

Gene expression

Gene expression changes at 2 h after feeding were generally small, but the expression of several genes was significantly affected by varying the dietary macronutrient source. Compared with the protein diet, barramundi fed the starch diet showed significantly decreased expression of *pk* and *fbp1* but increased expression of *pck2*. None of the genes regulating FA synthesis or breakdown was affected by diet.

Signalling pathways

There were very few significant changes in the phosphorylation of various signalling molecules in response to different dietary macronutrients. At 2 h after feeding, there was a significant down-regulation in the level of phosphorylated mTOR in fish fed the starch diet (Fig. 2). A similar trend was observed in the average phosphorylation status of S6, S6K1 and FoxO1-3 in starch-fed fish, although this decrease was not significant (Fig. 2). Phosphorylation of FoxO1 tended to increase in response to a starch diet, but again was not considered significant.

Table 3. Fractional synthetic rate (FSR) for hepatic NEFA and TAG-bound fatty acids (FA) and glycerol (expressed as percentage of newly synthesised FA from lipogenesis per d; FSR in % per d) and modification (elongation and desaturation) rates (expressed as % of FA) in barramundi (*Lates calcarifer*) fed with protein or starch diet, after a 6 d residence in a tank with approximately 3·5 % ^2H-enriched water
(Mean values with their standard errors; n 6 unless indicated)

	Protein		Starch	
FSR (% per d)	Mean	SEM	Mean	SEM
NEFA	0·78	0·29	0·85	0·10
Elongation rate	0·77†	0·35	0·44‡	0·23
Desaturation rate	ND		ND	
TAG-bound FA	0·35	0·09	0·62*	0·07
TAG-bound glycerol	3·40	0·31	2·82	0·29
Elongation rate	0·44	0·08	0·12**	0·04
Desaturation rate	0·30	0·12	0·50	0·06

ND, not detected.
Significant differences between diets (t test; *$P < 0·05$, **$P < 0·01$).
† n 5 (1 ND).
‡ n 4 (2 ND).

Lipogenic flux from ^2H_2O

Equilibration of BW and tank water was confirmed, with no apparent differences being observed on either diet (protein-fed fish: BW 3·25 (SE 0·09) v. tank water 3·46 (SE 0·19); starch-fed fish: BW 3·74 (SE 0·12) v. tank water 3·79 (SE 0·04); ^2H-enrichment in %). Separate estimations for lipogenesis revealed differential fluxes for hepatic lipids. The FSR for TAG-bound FA was significantly increased in the starch-fed fish (starch 0·6 (SE 0·1) % per d; protein 0·4 (SE 0·1) % per d; $P = 0·035$), while the FSR for NEFA was unaffected by diet (starch 0·9 (SE 0·1) % per d; protein 0·8 (SE 0·3) % per d; $P > 0·05$) (Fig. 3; Table 3). The FSR for newly synthesised/cycled TAG-bound glycerol was similarly unaffected by diet (starch-fed fish 2·8 (SE 0·3) % per d; protein-fed fish 3·4 (SE 0·3) % per d; $P > 0·05$), even though the FSR of TAG-bound glycerol was found to be 5–10-fold higher than the FSR for TAG-bound FA (Table 3). Estimations for modifications of FA revealed different dynamics (Table 3). For TAG-bound FA, the rate of elongation was significantly lower in starch-fed fish, while the rate of desaturation showed no statistical difference, even if slightly elevated in starch-fed fish compared with protein-fed fish. For NEFA, elongation rates were calculated in nine out of the twelve samples. These revealed no statistical difference between diets. Peaks for calculating desaturation rates on the other hand were not detected in all samples (online Supplementary Fig. S1(b) inlet). In agreement with the increased FSR for TAG-bound FA, there was a significant increase in total hepatic TAG in starch-fed fish (protein 5·7 (SE 1·3) mg/g tissue; starch 11·9 (SE 1·6) mg/g tissue).

Discussion

Presently, there is no understanding of the molecular mechanisms that regulate intermediary metabolism in barramundi, a highly valued species of global importance, in response to feeding different dietary energy sources. This study combined gene

expression and signalling cascades with a metabolic labelling approach to define the hepatic mechanisms by which barramundi assimilate and store excess dietary CHO energy. These changes underlie the significantly reduced growth performance (protein 3·72 g/d, starch 3·32 g/d)[9], increased lipid deposition efficiency (protein 77·3 %, starch 182·8 %)[9] and reduced protein energy utilisation coefficient (protein $\theta k_{PE} = 0.557$, starch $\theta k_{PE} = 0.412$)[22] observed in barramundi attributed to the replacement of dietary protein with plant-based raw materials that contain high levels of digestible CHO.

Plasma metabolites and hepatic gene expression

Most carnivorous fish display an elevation of blood glucose levels and prolonged periods of hyperglycaemia after consuming a CHO-rich diet, often associated with increased glycolytic and lipogenic enzyme activity and gene expression[10,11,42]. Similar to most carnivorous fish, certain forms of starch are highly digested by barramundi[43,44], including >85 % digestibility of the pre-gelatinised starch in this study[9]. However, 2 h after consuming a high-starch meal, barramundi showed no significant elevation of plasma glucose, TAG or free amino acid compared with protein-fed fish (Table 2). The expression of genes regulating key metabolic pathways was also largely unaffected, with the exception of *pk*, *fbp1* and *pck2* (Fig. 1). The lack of increased *acyl* or *fas* expression do not suggest that lipogenesis was affected, although strong evidence demonstrating this role was defined in the metabolic labelling experiment, as is further discussed below. This is despite evidence that many barramundi
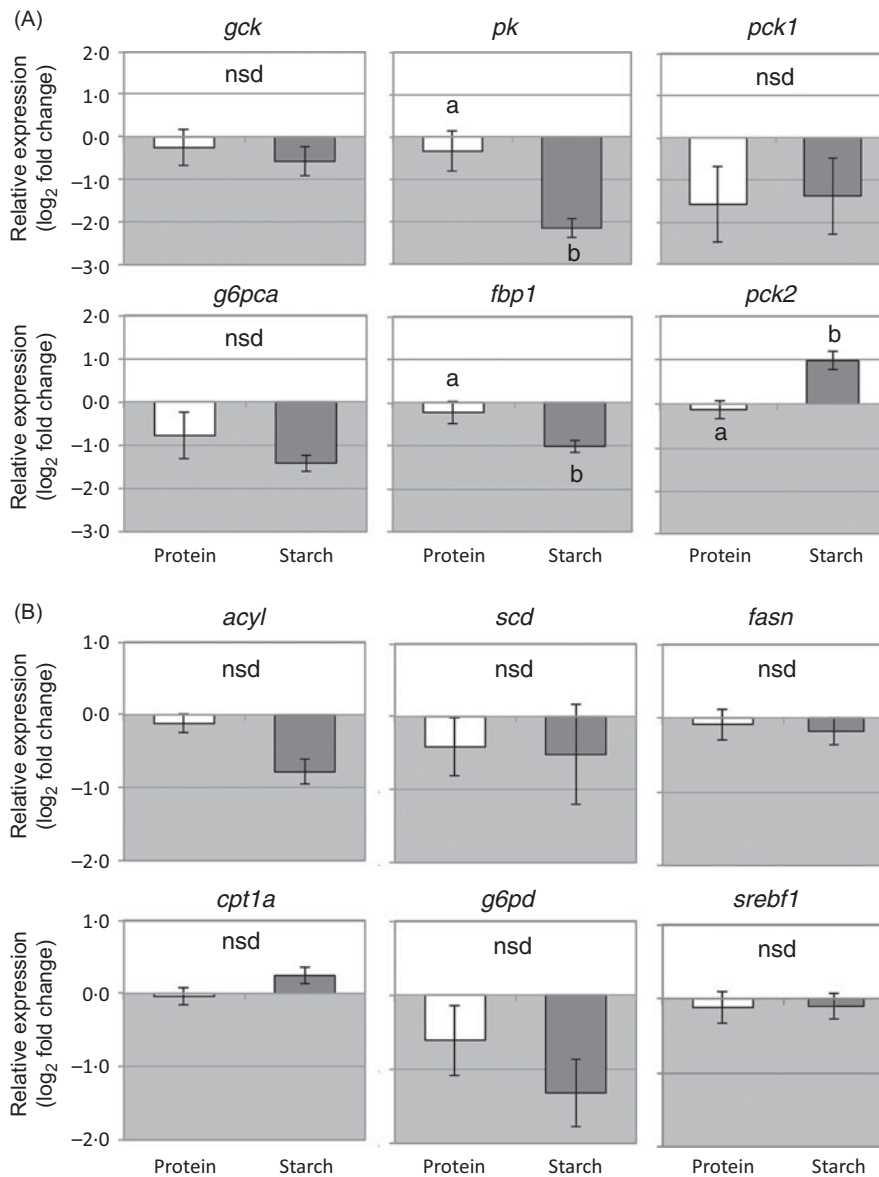

Fig. 1. Change in liver expression of genes regulating glucose metabolism (A) or fatty acid metabolism (B) in fish fed a diet that differed in protein or starch macronutrient composition. Expression values are shown as $\log_2$-fold change of each gene relative to the expression in the protein diet. [a,b] Unlike letters indicate significant ($P < 0.05$) differences between the different diets; nsd denotes no significant difference.
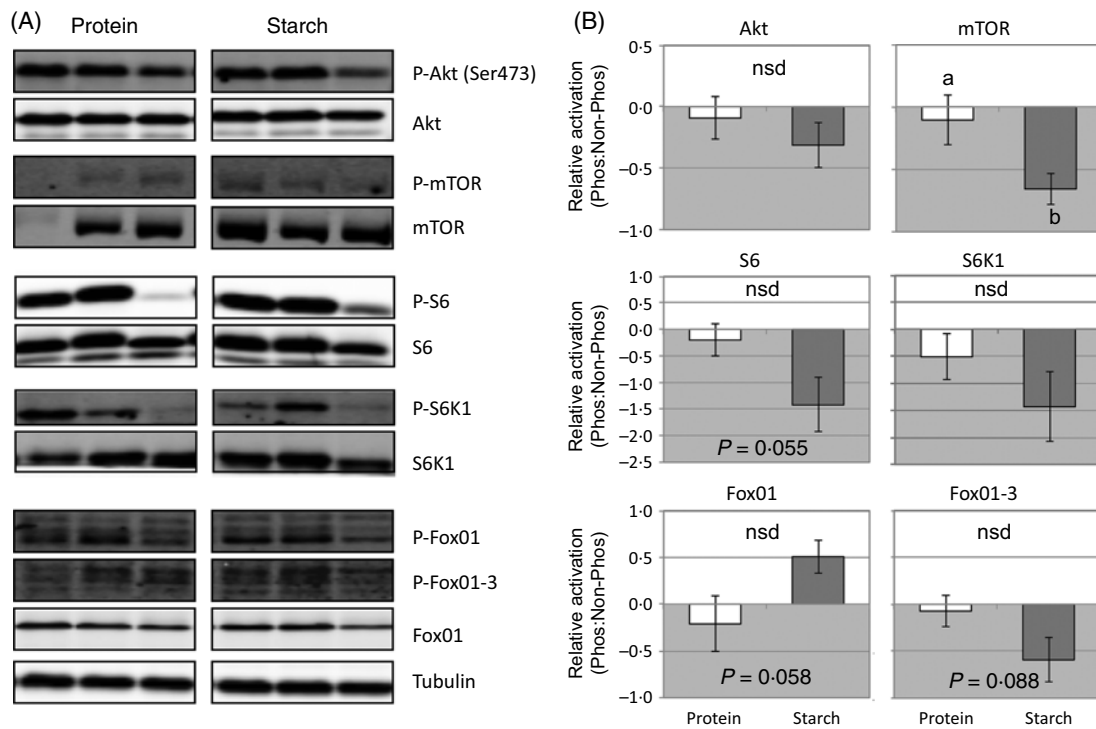
Fig. 2. Change in liver activation of signalling cascades that regulate growth and metabolism in fish fed a diet that differed in protein or starch macronutrient composition. (A) Raw gel images used for quantification of band intensity of active (phosphorylated) to total (phosphorylated and unphosphorylated forms) of each signalling protein, with tubulin used to demonstrate equal protein loading. (B) Mean values with their standard errors (log$_2$-fold change) of the proportion of active to total forms of each signalling protein relative to the activation in the protein diet. [a,b] Unlike letters indicate significant ($P < 0.05$) differences between the different diets; nsd denotes no significant difference.
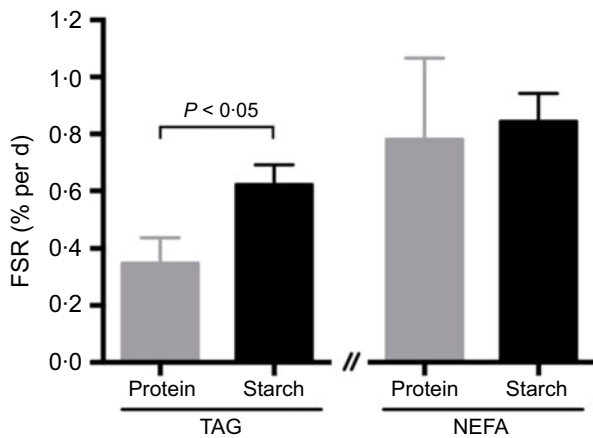

Fig. 3. Fractional synthetic rate (FSR) for TAG-bound fatty acid (FA) and NEFA, expressed as percentage of newly synthesised FA from lipogenesis per d in hepatic lipids of barramundi (*Lates calcarifer*) fed with a protein or starch diet, after a 6-d residence in a tank with approximately 3·5 % ^{2}H-enriched water. Mean values with their standard errors are presented (*n* 6). A significant difference between diets is indicated (*t* test; $P < 0.05$).

genes displayed significant postprandial regulation, including *gk*, *g6pca*, *acyl* and *fas*[24], which implies the expression of these genes is regulated in a coordinated way after feeding.

In rainbow trout *Oncorhynchus mykiss*, elevated dietary starch levels of more than 20 % have been shown to significantly elevate both plasma glucose and hepatic *gk* gene expression and enzyme activity[45–47]. Barramundi are most closely related to

European sea bass *Dicentrarchus labrax*, common dentex *Dentex dentex* and gilthead sea bream *Sparus aurata*, yet the response to dietary starch of these species was similar to that reported in rainbow trout[48–50], through stimulation of glycolysis rather than inhibition of gluconeogenesis. The inability to down-regulate gluconeogenesis has been suggested as a cause of the deficiencies in post-prandial glucose regulation in fish[10,42,51]. Gluconeogenesis was down-regulated in carp *Cyprinus carpio* and sea bream fed 20 % starch[46], through reduced *gck1* and *g6pca* expression, respectively, suggesting these fish repress glucose production when not required. The expression of *g6pca* was down-regulated after feeding in both barramundi[24] and sea bream[52]. However, sea bass and sea bream fed with high levels of gelatinised starch showed no effect on *g6pca* mRNA abundance and/or enzyme activity[29,49,52], with a similar result observed in the present study (Fig. 2). These data suggest that glucose metabolism is not directly regulated by dietary starch/glucose levels in these fish, and the underlying metabolic regulators have not been identified.

The phosphofructokinase/fructose-1,6-bisphosphatase substrate cycle is another important regulatory locus for glucose metabolism and has been hypothesised as a potential mechanism for the poor regulation of blood glucose levels in fish. The expected shift towards glycolysis over gluconeogenesis in response to the more abundant dietary CHO, through reduction of *fbp1* expression or activity, has been observed in sea bass[53], sea bream[46,54–56] and trout[23,57]. The present study in barramundi showed a similar significant down-regulation of *fbp1*

expression 2 h after feeding as part of their response to dietary CHO (Fig. 2). However, although endogenous gluconeogenesis was also significantly repressed in sea bass fed 30 % dietary starch[29], half the circulating glucose in starch-fed fish was still derived from gluconeogenic pathways. Gluconeogenesis remains a major contributor of hepatic glucose-6-phosphate synthesis in carnivorous fish[58], yet the precise contribution of gluconeogenesis in barramundi glucose utilisation remains to be determined. Combined, molecular data suggest that there is at least one component of the gluconeogenic pathway strongly decreased (phosphoenolpyruvate carboxykinase, cytosolic form (*pck1*), *g6pca* or *fbp1*), and/or at least one component of the glycolytic pathway strongly increased (*gk*, *pfkl* or *pk*). These responses form part of a variety of species-specific mechanisms involved in fish metabolism of dietary CHO, although that utilisation remains universally poor in most carnivorous fish[51].

Growth signalling cascades

Previous work has shown that the starch-fed fish used in this study had approximately 11 % reduced growth than the protein-fed fish[9] and that this was potentially caused by a reduction in the utilisation of total energy (k_E protein $= 0.715$, starch $= 0.481$) mostly due to reduced protein energy utilisation (θk_{PE} protein $= 0.557$, starch $= 0.412$)[22]. Therefore, analysis of the activation of the mTOR signalling cascade in barramundi provides a potential mechanism to explain the poor growth performance observed in fish-fed high-starch diets. Changes in phosphorylation status were subtle, and only significant in mTOR and S6, but consistent with the peak of activation of these signalling proteins at the time of sampling[24] and their relative position within the signalling cascade[25,27]. The role of mTOR as a regulator of growth and whole-body metabolism[59] is consistent with evidence in barramundi that suggests that as starch levels increase, there is a direct negative effect on growth and energy utilisation, particularly that of protein[22]. However, past work in trout has shown that dietary fishmeal replacement with plant ingredients (maize and wheat gluten) did not induce differences in the Akt-mTOR signalling pathway[27]. In addition, trout Akt-mTOR activation has been shown to be a key regulator of hepatic lipogenesis through stimulation of *srebf1*, *fas* and *gk* expression[25], which was not evident in barramundi. Meanwhile, 2 h after a meal of a high-lipid diet, Senegalese sole (*Solea senegalensis*) displayed prolonged hyperglycaemia and down-regulated the Akt-mTOR signalling pathway, while a high-CHO diet had no effect[60]. The results of this study combined with past work favours the notion that species-specific mechanisms are central to glucose homoeostasis in carnivorous fish, but may provide a potential basis for the ineffective utilisation of this macronutrient as a direct energy source for growth in barramundi.

Hepatic lipids and lipogenic flux

Tracer methods provide direct measurements of lipogenic fluxes[61–66], but have still not been widely applied in fish. ^2H_2O in particular rapidly equilibrates with fish BW[28] and gets incorporated into newly synthesised or exchanged metabolites

as described for glucose and glycogen synthesis in sea bass[28–30], lipogenesis of hepatic[31] and extrahepatic[12] TAG, hepatic alanine metabolism in gilthead sea bream[67], and muscle protein synthesis in catfish (*Ictalurus punctatus*)[68]. In barramundi, hepatic lipogenesis was significantly increased (Fig. 3), which provides direct support for the accumulation of hepatic TAG (Table 2) and FA (online Supplementary Table S2) and increased lipid retention and deposition from high-starch diets[9,22]. Dietary lipid levels were reduced in the starch diet, leading to significantly reduced gross lipid intake (protein 31·0; starch 19·6 g per fish) in starch-fed fish despite a slight increase in feed intake[9]. This reduced intake may partially explain the approximately 25 % improved lipid retention but cannot account for the 182 % lipid deposition that is demonstrated here to be a direct result of increased lipogenesis.

Metabolic flux for NEFA revealed no lipogenic increase, no elongation rate alterations, and no evidence for any desaturation. In a similar feeding and tracer administration setting, sea bass revealed that TAG levels were significantly augmented in the liver in response to high-starch diets[29], but this feature could not be attributed to an increase in hepatic TAG-bound FA from lipogenesis. Meanwhile, muscle TAG levels remained unaltered, but TAG-bound FA from lipogenesis significantly increased in starch-fed fish, indicative of TAG-FA recycling in response to diet[12]. In barramundi, no enrichment of muscle TAG could be detected, but in visceral fat, TAG-bound FA and glycerol synthesis/cycling was elevated by starch diets[12], although the rate of lipogenesis was approximately 6-fold higher rates in liver tissue. Changes in endogenous lipids and TAG consisted of increased SFA and MUFA at the expense of PUFA, with SFA accumulation mainly driven by an increase in palmitate (16 : 0), the end product of lipogenesis. Combined, the strong enhancement of visceral fat lipogenesis and high TAG-glycerol cycling in starch-fed fish[12] further supports the accumulation of large amounts of visceral fat and modifications to whole-body composition observed in barramundi as a result of consuming high-starch diets[22].

Observations from other carnivorous species further substantiate that the unaltered expression of lipid synthesis-related gene expression or enzymes 2 h after a meal should not be interpreted as lack of lipogenic potential from dietary starch *per se*[42]. In gilthead sea bream, dietary starch affected lipid absorption and transport, probably due to a delay in lipid absorption[69], which would delay any potential response from FAS for several hours. As outlined above, studies in rainbow trout demonstrated that amino acids, and not CHO, are potent stimulators of lipogenesis[70]. While mRNA abundance and enzymatic rates involved in the metabolism of dietary CHO provide valuable insights, the ^2H_2O experiment captured metabolic changes of free-swimming fish fed over 6 d. Changes in endogenous TAG and glycerol synthesis rates provide a holistic lipogenic flux profile that is independent of short-term post-prandial, circadian or stress-induced enzymatic or gene expression fluctuations.

Conclusion

This study demonstrates that barramundi utilise a unique series of specific hepatic regulatory mechanisms to assimilate and store

excess dietary CHO energy in the form of lipids, to the detriment of protein utilisation and growth. This study also highlights the potential advantages of using metabolic tracers to track dietary nutrient assimilation over several days of feeding, as this method overcomes many of the limitations of molecular-based studies that select a single time point (often 2, 6 or 24 h post-feeding) to draw conclusions. Although metabolic pathways are shared among carnivorous fish, results from hepatic enzyme assays, gene expression and signalling cascades, as well as lipogenic and metabolic flux analysis, support the notion that there is significant diversity in the underlying regulation of dietary nutrient assimilation in different fish species. This study provides a deeper understanding of the metabolic utilisation of feed ingredients in barramundi and therefore the ability to formulate advanced species-specific feeds.

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The authors declare that there are no conflicts of interest.

Supplementary material

For supplementary materials referred to in this article, please visit https://doi.org/10.1017/S0007114520001051

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