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Impact of dietary starch on extrahepatic tissue lipid metabolism in farmed European (*Dicentrarchus labrax*) and Asian seabass (*Lates calcarifer*)

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Abbreviation list

^{2}H deuterium; $^{2}H_2O$ deuterated water; ACLY, ATP citrate lyase; ACO, acyl-CoA oxidase; CHO carbohydrates; CPT1, carnitine palmitoyl transferase I; DHAP, dihydroxyacetone phosphate; DNL, *de novo* lipogenesis; FA, fatty acids; FAS, fatty acid synthase; G3P, glyceraldehyde 3-phosphate; G6PDH, glucose-6-phosphate dehydrogenase; DNL, *de novo* lipogenesis; HOAD, 3-hydroxyacyl-CoA dehydrogenase; LPL, lipoprotein lipase; NMR, nuclear magnetic resonance; PFK, 6-phosphofructokinase; PK, pyruvate kinase; TAG, triacylglycerol; VAT, visceral adipose tissue

Abstract

In aquaculture, there is high interest in substituting marine-derived with vegetable-based ingredients as energy source. Farmed carnivorous fish under high carbohydrate diets tend to increase adiposity but it remains unclear if this happens by increased lipid retention/accumulation, promotion of lipogenic pathways, or both. In order to determine the response of extrahepatic tissue to dietary starch, European (*Dicentrarchus labrax*) and Asian (*Lates calcarifer*) seabass were fed a control (low starch; LS) or experimental (high starch; HS) diet, for at least 21 days and then transferred for 6 days to saltwater enriched with deuterated water $^{2}H_2O$. Incorporation of ^{2}H-labelling follows well-defined metabolic steps, and analysis of triacylglycerols (TAG) ^{2}H-enrichment by ^{2}H-NMR allowed evaluation of *de novo* lipogenesis (DNL) in muscle and visceral adipose tissue (VAT). Fractional synthetic rates for TAG-bound fatty acids and glycerol were quantified separately providing a detailed lipogenic profile. The FA profile differed substantially between muscle and VAT in both species, but their lipogenic fluxes revealed even greater differences. In European seabass, HS promoted DNL of TAG-bound FA, in muscle and VAT. High ^{2}H-enrichment also found in muscle TAG-bound glycerol was indicative of its role on lipid cycling. In Asian seabass, HS had no effect on muscle FA composition and lipogenic flux, with no ^{2}H-enriched TAG being detected. VAT on the other hand revealed a strong enhancement of DNL in HS-fed fish along with high TAG-bound glycerol cycling. This study consolidated the use of $^{2}H_2O$ as tracer for fish lipid metabolism in different tissues, under different dietary conditions and suitable to use in different fish models.

Key words: seabass, barramundi, muscle, visceral adipose tissue, NMR, triacylglycerol, fatty acids, glycerol.

Introduction

As the farming of carnivorous fish species such as seabass continues to grow worldwide, the industry struggles to find affordable source ingredients that are able to provide a balanced fatty acid (FA) profile. This should preferably occur while reducing overall dependence on fishmeal with plant-based ingredients, and as consequence, increasing dietary carbohydrates (CHO) such as starch. This has been the case for European seabass *Dicentrarchus labrax* (Messina et al., 2013; Moreira et al., 2008; Viegas et al., 2016) and Asian seabass (also known as, and hereafter referred to as barramundi) *Lates calcarifer* (Glencross et al., 2016; Glencross et al., 2012; Salini et al., 2016). In this context, balanced formulations should be able to maintain growth performances and yields on the one hand, and provide a final product that delivers optimal levels of critical nutrients for human consumption on the other.

From tissue composition, somatic indices and circulating lipids, feed formulations with higher CHO content have been considered responsible for an overall increase in adiposity (Dias et al., 1998; Glencross et al., 2014; Nankervis et al., 2000; Peres and Oliva-Teles, 2002). However, based on studies addressing the regulation and transcription of hepatic enzymes (Castro et al., 2015a; Castro et al., 2015b; Dias et al., 1998; Enes et al., 2006; Glencross et al., 2016), it remains unclear if this happens by increased lipid retention/accumulation, promotion of lipogenic pathways, or by their combined effects. Moreover, muscle and visceral adipose tissue (VAT) metabolism, regarded as preferential lipid storage sites in teleost fish (Weil et al., 2013), are often overlooked in relation to the liver, as are the factors that control lipid deposition in these locations. The whole-body fatty acid balance method (Turchini et al., 2008), already applied in barramundi (Salini et al., 2015) but, to our knowledge not under high CHO dietary treatments, may be considered as a more holistic analysis. Nonetheless, this method is still blind to lipid compartmentalization in tissues, both in terms of content (Castro et al., 2015a; Messina et al., 2013) but also distribution (Wu et al., 2015).

In aquaculture, excess fat deposition in the muscle has been mainly attributed to environmental and/or nutritional factors that promote lipid retention, such as temperature (Cordier et al., 2002; Ibarz et al., 2007; Moreira et al., 2008), unbalanced FA profiles in fish feed (Izquierdo et al., 2003), and starch (Alvarez et al., 1999; Castro et al., 2015b; Dias et al., 1998; Lanari et al., 1999; Messina et al., 2013), respectively. As the edible fraction of the fish, this tissue is also under permanent

scrutiny by the consumer who associates visible fat depots with fish of "inferior" quality, especially if compared with wild fish (Grigorakis, 2007; Manthey-Karl et al., 2016; Vidal et al., 2012). Excess fat deposition around the viscera decreases yields for producers and appeal for consumers. However, VAT is no longer regarded as a metabolically inert sink for excess (dietary or synthesized) fat after being associated in mammalian models with endocrine action, energy homeostasis and metabolic interplay in several conditions such as obesity and cardiovascular disease (Ronti et al., 2006). This has spurred recent efforts to study fish adipocyte metabolism and its response to dietary CHO (Bou et al., 2016) and hormonal treatments, particularly those closely related to high CHO intake like insulin (Caruso and Sheridan, 2011).

Overall, the capacity of fish to endogenously utilize, accumulate or modify FA, and then compartmentalize excess fat is still far from being understood. This has been mostly assessed by tissue composition and indirect interpretation from mRNA abundance of the enzymatic machinery and transporters involved. In this context the use of isotopes, both stable and radioactive, has been a useful tool to directly follow the location and metabolic transformation of dietary substrates. Interesting data on how extrahepatic tissues handle high CHO levels and its consequences on lipid metabolism by *in vivo* studies: injection (Brauge et al., 1995) or forced feeding (Hemre and Storebakken, 2000) of ^{14}C-glucose; dietary incorporation of ^{13}C-starch and subsequent recovery in different tissues (Felip et al., 2012); whole body conversion to lipids (Ekmann et al., 2013). *In vitro* studies, namely the infusion of adipocytes with ^{14}C-glucose (Bou et al., 2016) have also contributed to a better understanding this subject. Instead of following the metabolic fate of a single labeled substrate, the stable isotope deuterium (^{2}H), presented as deuterated water ($^{2}H_2O$) in fish tanks, rapidly equilibrates with fish body water (Viegas et al., 2011) and gets widely incorporated in newly synthesized metabolites such as alanine (Marques et al., 2016), glucose or glycogen (Viegas et al., 2015). Similarly, ^{2}H gets incorporated into different sites of the triacylglycerol (TAG) molecule following well-defined metabolic steps from which estimations for *de novo* lipogenesis (DNL) and glycerol turnover can be derived (Viegas et al., 2016). In order to determine the response of extrahepatic tissues to dietary starch in terms of lipid metabolism we measured incorporation of ^{2}H into muscle and VAT TAG, in seabass and barramundi farmed in similar settings and fed a control (low starch) or an experimental (high starch) diet.

Material and Methods

Diet formulation

For this study two diets were formulated to fulfill the energetic requirements of each species: one low- (LS) and one high-starch (HS) diet (Table 1). For seabass, HS was formulated by replacing an inert filler of cellulose without nutritional value for 17.8% starch from gelatinized pea. For barramundi, HS was formulated by replacing protein and fat for 32.5% starch from gelatinized wheat. Further details on the formulations may be found in (Viegas et al., 2015) and (Glencross et al., 2014) for seabass and barramundi, respectively. Fish of both experiments were acclimated to the diets and experimental conditions for one week.

Fish handling

For European seabass, experimental protocol was the same as described previously (Viegas et al., 2015). Briefly, European seabass (*D. labrax*) from Tinamenor (Cantabria, Spain) were transported to the lab (Coimbra, Portugal) and randomly assigned to 2 different 200 L tanks (32 fish per tank; initial mean body weight of 21.9±0.3 g) of a recirculated system supplied with well-aerated seawater (20±1ºC; 30±1‰ salinity). Fish were fed twice daily to satiety for more than 21 days the correspondent diet. Experimental procedures complied with the Guidelines of the European Union Council (86/609/EU). Similarly, barramundi (*L. calcarifer*) from Betta Barra fish hatchery (Atherton, QLD, Australia) were transported to the lab (Bribie Island, QLD, Australia) and randomly assigned to 2 different 200 L tanks (30 fish per tank; initial mean body weight of 51.4±0.5 g) of a recirculated system supplied with well-aerated seawater (28±1ºC; 35±1‰ salinity). Fish were fed once daily to satiety for 21 days the correspondent diet. All experiments were performed in accordance with the Australian code of practice for the care and use of animals for scientific purposes and were approval by the CSIRO Animal Ethics Committee (approval numbers: A8-2010 and A8-2016). No mortality was registered.

Fish residence in $^{2}H_2O$ and sampling

Following the feeding period each group of fish from each species was transferred to a separate tank of ^{2}H-enriched seawater for 6 days. Approximate 3.5-5.0% ^{2}H-enrichement was achieved by the addition of 99%-enriched $^{2}H_2O$ (seabass: Eurisotop, France; barramundi: Sigma cat. #151882) and tank water ^{2}H enrichment was assessed

at the beginning and end of the trials (Viegas et al., 2011). This tank was maintained with an independent closed filtering system but with similar characteristics to the tanks used during the feeding period in terms of size, volume of water (200 L), opacity, filtering material and water parameters. During the 6-day residence in ^{2}H-enriched saltwater, fish were fed once a day (*ad libitum*) and sacrificed 24 h after last meal on day 5. Fish were anesthetized in a 30 L tank of ^{2}H-enriched seawater (seabass: 0.1 g L^{-1} of MS-222; barramundi: 0.02 mL L^{-1} of Aqui-S®), measured, weighed and blood was drawn from the caudal vein with heparinized syringes. A ~100 µL aliquot was centrifuged (3000xg, 10 min), and plasma was stored at -20ºC for quantification of body water ^{2}H enrichments. Fish were sacrificed (seabass: by cervical section; barramundi: overdose of Aqui-S®); muscle tissue was dissected from the epaxial quadrant (seabass: n=8, 1.6±0.1 g; barramundi: n=5, 1.1±0.1 g) and visceral adipose tissue (VAT) (seabass: n=8, 0.5±0.1 g; barramundi: n=6, 0.5±0.1 g) was carefully stripped from the peritoneal cavity. Both tissues were weighed, freeze-clamped with aluminum tongs cooled in liquid nitrogen, pulverized with chilled pestle and mortar and stored at -80ºC until further analysis.

Sample treatment

Muscle and VAT lipids were extracted from pulverized tissue according to Matyash et al. (2008) using methyl tert-butyl ether, methanol and water mixture (MTBE:MeOH:H_2O, 10:3:2.5; 20 mL g^{-1} of tissue) transferred to glass amber vials, dried under N_2 stream and stored at −20°C. Triacylglycerols (TAG) were purified by solid phase extraction with prepacked 2 g cartridges (Discovery® DSC-NH_2 52641-U, Supelco) according to Ruiz et al. (2004). Muscle TAG quantifications were performed in a fully-automated analyzer Miura 200 (I.S.E. S.r.l.; Guidonia, Italy) using its dedicated reagent kit (ref. A-R0100000901; seabass: n=7, barramundi: n=5).

NMR analysis

Tank water (TW) and fish body water (BW) ^{2}H enrichments were determined by ^{2}H NMR using calibrated acetone and analyzed in duplicates as previously described (Jones et al., 2001). Tank and plasma water content was assumed to be 96,5% and 92% of total sample, respectively. NMR spectra of TAG samples were obtained at 25°C with a Bruker Avance III HD system with an UltraShield Plus magnet (11.7 T, ^{1}H operating frequency 500 MHz) equipped with a 5-mm ^{2}H-selective probe with ^{19}F

lock and ^{1}H-decoupling coil. TAG were reconstituted in chloroform containing a pyrazine standard as previously described (Viegas et al., 2016) - representative TAG ^{1}H and ^{2}H NMR spectra are provided as supplementary material (Figure S1). As ^{1}H and ^{2}H signals are essentially isochronous, the identity of the ^{2}H signals can be confirmed by matching their chemical shifts with their ^{1}H counterparts, meaning that the respective signal assignments apply to both nuclei (Table S1). As control for the TAG extraction, a FA/glycerol ratio (should be ~3) was calculated from the area of all α protons times 2, divided by TAG-bound glycerol sn1,3 protons (Duarte et al., 2014). The FA profile (in percentage) for saturated (SFA) and unsaturated fatty acids (UFA), both poly- (PUFA) and monounsaturated fatty acids (MUFA) were estimated from ^{1}H NMR spectra according to Viegas et al., (2016). Positional ^{2}H-enrichments were quantified from the ^{1}H and ^{2}H NMR spectra by measuring the ^{1}H and ^{2}H intensities (areas) 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. (2014). Briefly, during their residence in ^{2}H-enriched seawater, newly synthesized FA from *de novo* lipogenesis incorporate ^{2}H-enrichment in the FA terminal methyl group (signal at 0.80 ppm). The same principle applies to for newly synthesized glycerol, incorporating ^{2}H-enrichment in the sn-1,3 glyceryl site (signals at 4.15 ppm). Fractional synthetic rates (FSR; in % d^{-1}) of newly synthesized TAG-bound FA and TAG-bound glycerol were estimated by dividing the respective positional ^{2}H-enrichment by the BW. ^{2}H-enrichments were calculated after systematic subtraction of 0.015%, taken as the mean background ^{2}H-enrichment. If the values were below zero, these were considered as 0.0 for FSR calculation purposes. Spectra were processed by applying exponential multiplication to the free-induction decay (^{1}H: 0.1 Hz; ^{2}H: 1.0 Hz). Spectra processing and peak integration was performed using ACD/NMR Processor Academic Edition from ACD\Labs 12.0 software (Advanced Chemistry Development, Inc.).

Statistical analysis

Data are presented as mean ± S.E.M. Whenever possible, two-way ANOVA was used to test main effects of, and interactions between, diet and tissue. In the case of statistically significant interactions, or in the impossibility to perform a two-way ANOVA, differences between diets were tested using a Student's two-tailed unpaired

t-test. Analyses were performed in GraphPad Prism® software (GraphPad Software, Inc.). Differences were considered statistically significant at $P < 0.05$.

Results

Following extraction and subsequent isolation from other lipid classes by solid phase extraction, TAG gave well-resolved ^{1}H NMR spectra. The FA/glycerol ratio was not affected by diet for any tissue of both species, and was overall consistent with a successful TAG separation (seabass: n=32, 2.96±0.03; barramundi: n=22, 3.03±0.04; t-test, $P > 0.05$). Signals from PUFA and MUFA moieties dominated the spectra, while contributions from SFA were relatively minor. In seabass, TAG-bound FA composition as categorized by ^{1}H NMR spectra was tested for effects of diet (LS vs. HS) and tissue distribution (muscle vs. VAT) (Table 2). All were significant for the latter, symptomatic of a differential profile where SFA were stored to a higher degree in VAT than in muscle while the opposite trend was observed regarding UFA. Within UFA, more PUFA, and consequently also more n-3 were found in muscle than in VAT. In the case of PUFA and MUFA effects were also observed due to dietary treatment, with PUFA decreasing, and MUFA increasing with elevated dietary starch. No interactions between dietary treatment and the analyzed tissues were observed, as summarized in Table 2. For barramundi, the same lipid composition analysis from ^{1}H NMR spectra was performed as shown in Table 3. Due to poor signal-to-noise ratio, probably resulting from technical problems associated with the lipid extraction and not to low lipid concentration *per se*, two muscle samples (one per diet) were considered unsuited for spectral analysis. As in seabass, barramundi lipid composition revealed differential tissue distribution, with the exception of PUFA. As previously observed, SFA were stored to a higher degree in VAT, while UFA, mainly driven by MUFA, were preferentially stored in muscle. None of the lipid species were affected by the dietary treatment even if PUFA mean values seemed to indicate otherwise. Again, no interactions between variables were observed (Table 3).

As equivalent ^{1}H and ^{2}H signals resonate at approximately the same chemical shift, the observed ^{2}H NMR signals correspond to specific enriched sites in the TAG molecule. This enrichment is derived from ~0.015% ^{2}H natural abundance (mean background ^{2}H-enrichment) plus excess enrichment from the metabolic incorporation of ^{2}H from the 6-day residence in $^{2}H_2O$-enriched seawater. Specifically, this incorporation resulted in peaks corresponding to FA terminal methyl group site (non

n-3) (signal at 0.80 ppm) revealing TAG-bound FA synthesized *de novo*, and to the sn1,3 glyceryl site, revealing newly synthesized or cycled TAG-bound glycerol. Labeling was not detected in glycerol of seabass VAT (Fig. 1), and in both FA and glycerol of muscle of barramundi (Fig. 2). For both species, glyceryl FSR were substantially higher than for FA; ~20-80 times higher in the case of seabass muscle, and ~2-20 times higher in the case of barramundi VAT.

In seabass (Fig. 1), differences in FA FSR were attributable to both tissue distribution and diet, being significantly higher in muscle in regard to VAT, and HS in regard to LS. Glycerol revealed higher FSR when compared to FA FSR and was also statistically lower in the HS when compared with LS. In barramundi VAT, FA FSR was significantly higher in fish fed the HS diet while glyceryl FSR on the other hand was unaffected by diet. Muscle TAG levels did not differ between diets for both species (seabass: LS 8.4±1.2 vs. HS 7.4±0.6; barramundi LS 1.0±0.2 vs. HS 0.7±0.1, in g $100g^{-1}$ of tissue; t-test $P > 0.05$).

Discussion

In vertebrates the liver is the centerpiece for metabolic regulation and in teleost fish it is the main lipogenic organ where key endocrine and nutrient sensing mechanisms intersect (Conde-Sieira and Soengas, 2017). In similar feeding trials, hepatic TAG content and FA composition was sensitive to high CHO diets (Castro et al., 2015a; Lanari et al., 1999; Messina et al., 2013; Peres and Oliva-Teles, 2002; Viegas et al., 2016). In the present study, muscle and VAT from both species presented different FA profiles, but only in seabass these profiles were responsive to HS diet, particularly PUFA and MUFA. CHO-stimulated accumulation of muscle lipids has been associated with transcriptional response of hepatic FA desaturation and elongation enzymes in rainbow trout (*Oncorhynchus mykiss*) (Kamalam et al., 2012), and enhanced metabolic elongation rates in the same seabass analyzed in the present study (Viegas et al., 2016). Interestingly in barramundi muscle, lack of CHO-stimulated FA profile alterations may relate to absolute lack of ^{2}H-enriched TAG, either in the FA or glycerol moieties. This suggests that this pool had very limited turnover, either in terms of net FA replacement, or FA-TAG cycling (this latter process accounting for the higher enrichment of glycerol compared to FA in other tissues as discussed ahead). Muscle FA profile in this species has proven to be sensitive to dietary lipid manipulations (Alhazzaa et al., 2011), but as regard to CHO, further studies should be

conducted. Notwithstanding, potential muscle FA profile alterations were often accompanied by little or no variation in muscle lipid content as verified in seabass (Boujard et al., 2004; Castro et al., 2015a; Dias et al., 1998; Messina et al., 2013; Peres and Oliva-Teles, 2002). The same could be indirectly inferred in barramundi by the fact that head-on-gutted lipid composition, mainly composed by muscle tissue, was unaffected by HS (Glencross et al., 2014). This is most likely associated with the poor capacity of muscular tissue, which represents about 50-60% of the whole-body mass, to effectively clear excess CHO. Rainbow trout is one of the most intensely studied fish species, and under high CHO diets muscle mRNA levels for GLUT4 remained unaltered (Kamalam et al., 2012; Panserat et al., 2009). After a short-term administration of insulin, muscle GLUT4 (Polakof et al., 2010b), along with muscle FAS (Polakof et al., 2010a) mRNA levels decreased. A long-term (chronic) insulin administration reverted these effects, but regardless of the duration of the insulin treatment, muscle glycogen always increased significantly (Polakof et al., 2010b). In the seabass from the present study, incorporation of ^{2}H into glycosyl units revealed that hepatic glycogen synthesis via direct pathway was significantly augmented in HS-fed fish (Viegas et al., 2015), so the same could be expected in muscle tissue. It is worth noting that in rainbow trout fed with ^{13}C-labelled dietary starch, the fraction recovered in muscle tissue was for the most part present in lipids and glycogen. Interestingly, no differences we observed for both parameters between fish fed digestible and raw starch (Felip et al., 2012). Regardless of its lipogenic action in the liver, under normal feeding the effects of insulin in fish muscle seem to ineffectively regulate CHO disposal, particularly in a species where insulin release was poorly stimulated by dietary CHO (Enes et al., 2010) like the seabass. There is however margin for improvement and revision of these metabolic models, particularly in the context of aquaculture, as incredibly versatile trait-selected fish (e.g. Fat and Lean lines of rainbow trout) unveiled the combined effects of i) modulation of lipogenesis in the liver by the mTOR pathway (Skiba-Cassy et al., 2009), ii) modulation of muscle glycolytic (PK and PFK) (Song et al., 2018) and fatty acid oxidation enzymes (CPT1, HOAD and ACO) (Jin et al., 2014a), and finally iii) improved response to insulin (Jin et al., 2014b) to achieve fish able to display higher muscle lipid content. Contrary to barramundi, considerable levels of ^{2}H-enriched TAG were detected in seabass muscle. When compared with the liver (Viegas et al., 2016), CHO-stimulated DNL and/or incorporation of starch into body lipid (muscle and VAT) was lower.

Despite the fact that the labeling was recovered from a single type of lipid, the synthesis, and therefore the ^{2}H-enrichment of the TAG glyceryl backbone and its three esterified FA, happens independently. FA terminal methyls hydrogens become enriched in ^{2}H by DNL (other TAG fatty acyl hydrogens may also become enriched by elongation reactions). TAG glyceryl hydrogens become enriched via *de novo* glycerol-3-phosphate synthesis from glyceraldehyde-3-phosphate (G3P) and exchange between glycerol-3-phosphate and G3P. Their enrichment is suitably discriminated by ^{2}H NMR, a feature that mass-based technologies are unable to do unless additional sample treatment was performed. Regardless of the dietary treatment and species, TAG-bound glycerol revealed considerably higher ^{2}H-enrichment when compared to TAG-bound FA. It has been hypothesized that fish maintain high glycerol rates of appearance by constantly cycling (reesterifying) up to two-thirds of circulating TAG to free FA (Bernard et al., 1999; Magnoni et al., 2008). During exercise, fish muscle augments lipoprotein lipase (LPL), critical for lipid mobilization (Magnoni et al., 2013) but TAG-FA turnover rates were maintained unalterably high. In the absence of glycerol kinase in muscle, this glycerol cannot be recycled to glycerol-3-phosphate and reused in TAG cycling, being instead shuttled to the liver. Alternatively, *de novo* synthesis of glycerol-3-phosphate must occur and as consequence, enrichment of the triglyceride glyceryl moiety is typically much higher than that of the FA, which also include FA from dietary (unlabeled) origin. Glucose can be a contributing precursor to glycerol synthesis (through dihydroxyacetone phosphate; DHAP) via an abbreviated pathway of glycolysis as confirmed by ^{13}C- (Rito et al., 2018) and ^{14}C-glucose (Walter et al., 2006) tracer studies. Higher levels of circulating (unlabeled) glucose may putatively explain why, glyceryl FSR was lower in the muscle of seabass fed with HS diet, as already observed in the liver if these fish (Viegas et al., 2016). This same study also revealed that despite elevated hepatic TAG levels (LS: 11.1±0.2 and HS: 35.4±0.7 g 100 g^{-1} liver; t-test $P < 0.001$), this was not attributable to increased hepatic DNL. So, it was particularly intriguing to find that in muscle, TAG-bound FA from DNL were significantly increased in the HS diet, even if muscle TAG levels remained unaltered (LS: 8.4±1.2 vs. HS: 7.4±0.6 g 100 g^{-1} muscle; t-test $P > 0.05$). This indicates an independent regulation of muscle TAG synthesis activity during starch feeding, previously observed for rainbow trout (Brauge et al., 1995) but whose mechanisms are yet to be addressed.

In seabass, CHO-derived overall increased adiposity is driven not only by increased liver lipid content but also by VAT accumulation (Castro et al., 2015a; Dias et al., 1998; Peres and Oliva-Teles, 2002). This was not observed in the present experiment, as perivisceral fat index remained unaltered (LS: 4.9±0.2 vs. HS: 5.1±0.2) (Viegas et al., 2016). In rainbow trout, contrary to documented for muscle, in VAT insulin is regarded as an important hormone for lipid deposition: i) via its anti-lipolytic effects (Albalat et al., 2006); ii) through upregulation of ACLY and FAS and iii) by production of NADPH for lipogenesis (Polakof et al., 2010b). In a species from the same genus, coho salmon (*O. kisutch*), adipocyte GLUT4 transporter was sensitive to insulin notwithstanding a lower affinity for glucose compared to the mammalian homolog (Capilla et al., 2004). In another salmonid species, *in vitro* studies revealed even though the DNL pathway is active in Atlantic salmon (*Salmo salar*) adipocytes, rates of conversion of glucose into lipids were relatively low (Bou et al., 2016). It is worth noting that in salmonids, adipocytes were not nutritionally regulated by CHO (Figueiredo-Silva et al., 2012; Kamalam et al., 2013), but displayed elevated G6PDH activity and NADPH levels even in the absence of CHO (Barroso et al., 2001). This seems not to be the case for seabass and barramundi VAT where TAG-bound FA synthesis via DNL was significantly higher for HS diet. This was also described in gilthead seabream (*Sparus aurata*) adipocytes which under high CHO diets, up-regulated G6PDH expression and FAS also tended to increase (Bou et al., 2014). Besides a stronger response to insulin (Albalat et al., 2007) through LPL activation, CHO utilization in this species' VAT was also favored over FA oxidation by modulation of the PPARs transcription factors (peroxisome proliferator-activated receptors) (Bou et al., 2014).

In seabass, FA FSR was significantly lower in VAT than in muscle but was nevertheless influenced by diet. Contrary to observed in muscle, TAG-FA cycling did not occur in VAT as interpreted by the lack of ^{2}H-enrichment in TAG-bound glycerol. In barramundi the opposite seemed to take place; on the one hand, no ^{2}H-enrichment was detected in muscle TAG, and on the other, not only VAT FA were extremely stimulated by the HS diet, but also high FSR for glycerol were estimated. Similar findings were obtained from Atlantic salmon (*Salmo salar*) adipocytes incubated with ^{14}C-glucose. Analysis of TAG revealed 16-fold more ^{14}C incorporation into glycerol than FA (Bou et al., 2016). This is well within the range observed in the present study for barramundi VAT TAG with 19-fold excess ^{2}H-enrichment of glycerol over FA

during HS feeding. Bou and colleagues proposed that the way glucose stimulates lipogenesis was by stimulating the pentose phosphate pathway, which in turn generates G3P precursors for TAG glycerol as well as providing NADPH for sustaining FA synthesis. Our study provides further evidence for this mechanism, not only in barramundi adipose tissue but also seabass muscle. It should be noted that despite our best efforts to provide a framework for comparing both species by using isoenergetic diets, certain parameters differed. Particularly, the diets for barramundi were not isoproteic but none was protein limiting as it was ensured that the DP:DE ratio exceeded the established requirements for this species (Glencross, 2008). The fact that these were not isolipidic may have alone interfered with overall lipid metabolism, perhaps with stronger effects in the liver rather than in muscle or VAT. Nonetheless, the present study has provided further insight into the impact and fate of dietary starch in extrahepatic tissues of two carnivorous seabass species. The FA profile differed substantially between muscle and VAT, but their lipogenic fluxes revealed even greater differences. In seabass, HS promoted DNL of TAG-bound FA, in muscle and VAT. High ^{2}H-enrichment found in muscle TAG-bound glycerol was indicative of its role on lipid cycling. In barramundi, HS had no effect on muscle FA composition and lipogenic flux, with no ^{2}H-enriched TAG being detected. VAT on the other hand revealed a strong enhancement of DNL in HS-fed fish along with high TAG-bound glycerol cycling.

Overall, while the aquaculture industry seeks to further stretch the optimization of fish feed production, the administration of isotopes will provide deeper insights into the regulation of lipid storage and oxidation in fish species in response to different combination of macronutrients. This may be particularly interesting in the procurement of new species to farm or in the optimization and selection of existing ones, currently happening by trait preference (Jin et al., 2014b; Song et al., 2018) or by recurring to the wide spectrum of metabolic responses occurring in nature (Betancor et al., 2016; Marandel et al., 2018).

Disclosures

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Figure 1

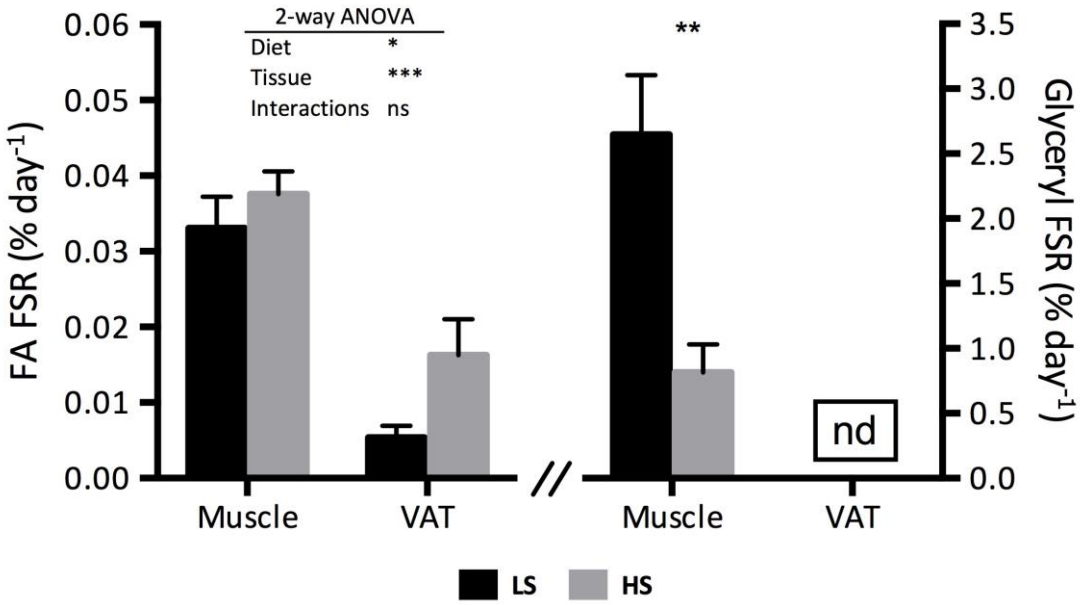
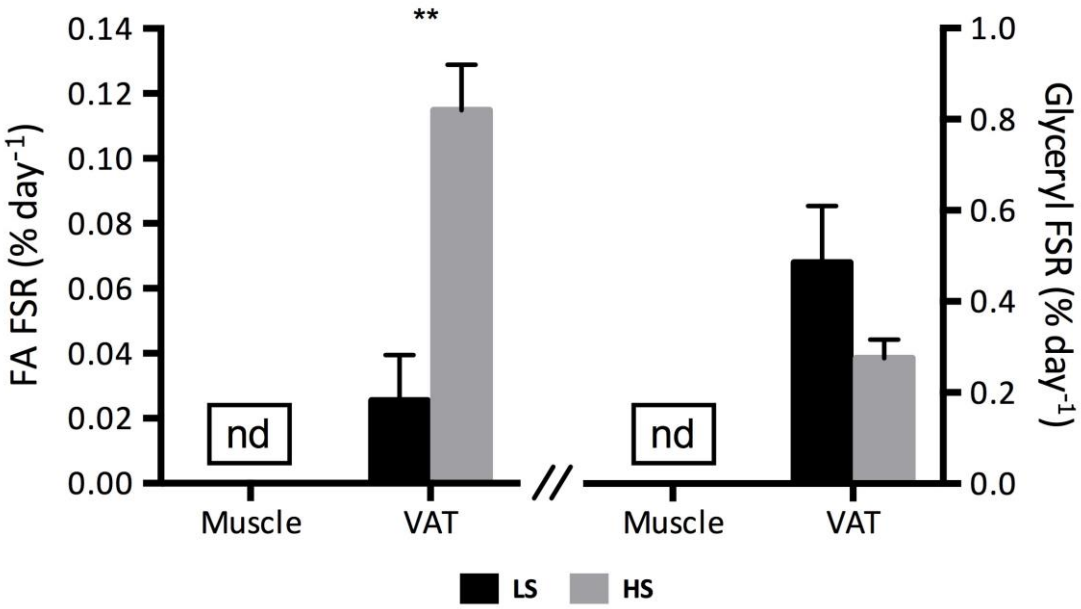


Figure 1: Fractional synthetic rate for TAG-bound fatty acids (fatty acyls; FA FSR) and TG-bound glycerol (Glyceryl FSR) expressed as percent of newly synthesized TAG from *de novo* lipogenesis per day in muscle and visceral adipose tissue (VAT) of seabass (*D. labrax*) fed with low starch (LS) and high starch (HS) diets, after a 6-day residence in ^{2}H-enriched seawater. Mean values ± S.E.M. (n=8) are presented. When not applicable a two-way ANOVA, significant differences between diets are tested (t-test) and indicated by asterisks ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

683 **Figure 2**



684

685 **Figure 2:** Fractional synthetic rate for TAG-bound fatty acids (fatty acyls; FA FSR)
686 and TG-bound glycerol (Glyceryl FSR) expressed as percent of newly synthesized
687 TAG from *de novo* lipogenesis per day in muscle and visceral adipose tissue (VAT)
688 of barramundi (*L. calcarifer*) fed with low starch (LS) and high starch (HS) diets,
689 after a 6-day residence ^{2}H-enriched seawater. Mean values ± S.E.M. (n=5 for muscle;
690 n=6 for VAT) are presented. Significant differences between diets are tested (t-test)
691 and indicated by asterisks ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

692 **Table 1.** Ingredients and proximate composition of the experimental diets provided to
693 European seabass (*D. labrax*) and barramundi (*L. calcarifer*)
694

	Seabass		Barramundi	
Proximate composition (% dry weight)	LS	HS	LS	HS
Dry matter	96.0	95.6	93.0	89.0
Crude protein	50.2	50.2	63.3	50.2
Crude fat	16.1	16.1	11.7	6.6
Starch	0.2	17.8	1.6	32.5
Ash	11.5	9.3	9.0	11.5
Gross Energy (kJ g^{-1} dry weight)	22.66	22.03	21.3	20.8

695

696 **Table 2.** Percentage of lipid species in triacylglycerol of muscle and visceral adipose
697 tissue (VAT) of European seabass (*D. labrax*) fed with a low starch (LS) and high
698 starch (HS) diet as determined from ^{1}H NMR spectra.
699

		n-3	PUFA	MUFA	UFA	SFA
Muscle (n=8)	LS	27.6±0.3	46.7±0.9	36.5±1.0	82.3±1.7	17.7±1.7
	HS	27.2±0.3	45.1±0.4	37.3±0.6	82.5±0.6	17.5±0.6
VAT (n=8)	LS	25.5±0.2	40.6±1.1	38.4±0.6	79.1±1.1	21.0±1.1
	HS	24.6±0.5	35.5±2.4	41.8±0.4	77.3±2.2	22.7±2.2
2-way ANOVA	Diet	ns	*	**	ns	ns
	Tissue	***	***	***	*	*
	Interactions	ns	ns	ns	ns	ns

700 Mean values±S.E.M are presented. Two-way ANOVA; sources of variations: diet (LS *vs.* HS) and
701 tissue (muscle *vs.* visceral adipose tissue) ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; ns: not significant).
702

703 **Table 3.** Percentage of lipid species in triacylglycerol of muscle and visceral adipose
704 tissue (VAT) of Asian seabass (barramundi *L. calcarifer*) fed with a low starch (LS)
705 and high starch (HS) diet as determined from ^{1}H NMR spectra.
706

		n-3	PUFA	MUFA	UFA	SFA
Muscle (n=5)	LS	7.8±0.9	26.4±3.7	54.2±3.9	80.7±7.0	19.4±7.0
	HS	10.8±1.8	29.4±3.4	51.5±4.1	73.6±5.8	26.4±5.8
VAT (n=6)	LS	15.1±1.2	26.5±2.5	37.4±0.9	64.0±2.1	36.1±2.1
	HS	16.6±0.2	30.2±0.8	34.4±0.9	63.3±1.1	36.7±1.1
2-way ANOVA	Diet	ns	ns	ns	ns	ns
	Tissue	***	ns	***	**	**
	Interactions	ns	ns	ns	ns	ns

707 Mean values±S.E.M are presented. Two-way ANOVA; sources of variations: diet (LS *vs.* HS) and
708 tissue (muscle *vs.* visceral adipose tissue) ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; ns: not significant).