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

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26   **Abbreviation list**
27   ^2H deuterium; ^2H_2O deuterated water; ACLY, ATP citrate lyase; ACO, acyl-CoA
28   oxidase; CHO carbohydrates; CPT1, carnitine palmitoyl transferase I; DHAP,
29   dihydroxyacetone phosphate; DNL, *de novo* lipogenesis; FA, fatty acids; FAS, fatty
30   acid synthase; G3P, glyceraldehyde 3-phosphate; G6PDH, glucose-6-phosphate
31   dehydrogenase; DNL, *de novo* lipogenesis; HOAD, 3-hydroxyacyl-CoA
32   dehydrogenase; LPL, lipoprotein lipase; NMR, nuclear magnetic resonance; PFK, 6-
33   phosphofructokinase; PK, pyruvate kinase; TAG, triacylglycerol; VAT, visceral
34   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 ^2H_2O. Incorporation of ^2H-labelling follows well-defined metabolic steps, and analysis of triacylglycerols (TAG) ^2H-enrichment by ^2H-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 ^2H-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 ^2H-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 ^2H_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$_2$O) 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.

129 **Material and Methods**

130 *Diet formulation*

131 For this study two diets were formulated to fulfill the energetic requirements of each

132 species: one low- (LS) and one high-starch (HS) diet (Table 1). For seabass, HS was

133 formulated by replacing an inert filler of cellulose without nutritional value for 17.8%

134 starch from gelatinized pea. For barramundi, HS was formulated by replacing protein

135 and fat for 32.5% starch from gelatinized wheat. Further details on the formulations

136 may be found in (Viegas et al., 2015) and (Glencross et al., 2014) for seabass and

137 barramundi, respectively. Fish of both experiments were acclimated to the diets and

138 experimental conditions for one week.

139

140 *Fish handling*

141 For European seabass, experimental protocol was the same as described previously

142 (Viegas et al., 2015). Briefly, European seabass (*D. labrax*) from Tinamenor

143 (Cantabria, Spain) were transported to the lab (Coimbra, Portugal) and randomly

144 assigned to 2 different 200 L tanks (32 fish per tank; initial mean body weight of

145 21.9±0.3 g) of a recirculated system supplied with well-aerated seawater (20±1ºC;

146 30±1‰ salinity). Fish were fed twice daily to satiety for more than 21 days the

147 correspondent diet. Experimental procedures complied with the Guidelines of the

148 European Union Council (86/609/EU). Similarly, barramundi (*L. calcarifer*) from

149 Betta Barra fish hatchery (Atherton, QLD, Australia) were transported to the lab

150 (Bribie Island, QLD, Australia) and randomly assigned to 2 different 200 L tanks (30

151 fish per tank; initial mean body weight of 51.4±0.5 g) of a recirculated system

152 supplied with well-aerated seawater (28±1ºC; 35±1‰ salinity). Fish were fed once

153 daily to satiety for 21 days the correspondent diet. All experiments were performed in

154 accordance with the Australian code of practice for the care and use of animals for

155 scientific purposes and were approval by the CSIRO Animal Ethics Committee

156 (approval numbers: A8-2010 and A8-2016). No mortality was registered.

157

158 *Fish residence in ^2H_2O and sampling*

159 Following the feeding period each group of fish from each species was transferred to

160 a separate tank of ^2H-enriched seawater for 6 days. Approximate 3.5-5.0% ^2H-

161 enrichment was achieved by the addition of 99%-enriched ^2H_2O (seabass: Eurisotop,

162 France; barramundi: Sigma cat. #151882) and tank water ^2H enrichment was assessed

163   at the beginning and end of the trials (Viegas et al., 2011). This tank was maintained

164   with an independent closed filtering system but with similar characteristics to the

165   tanks used during the feeding period in terms of size, volume of water (200 L),

166   opacity, filtering material and water parameters. During the 6-day residence in ^{2}H-

167   enriched saltwater, fish were fed once a day (*ad libitum*) and sacrificed 24 h after last

168   meal on day 5. Fish were anesthetized in a 30 L tank of ^{2}H-enriched seawater

169   (seabass: 0.1 g L^{-1} of MS-222; barramundi: 0.02 mL L^{-1} of Aqui-S$^®$), measured,

170   weighed and blood was drawn from the caudal vein with heparinized syringes. A

171   ~100 µL aliquot was centrifuged (3000xg, 10 min), and plasma was stored at -20ºC

172   for quantification of body water ^{2}H enrichments. Fish were sacrificed (seabass: by

173   cervical section; barramundi: overdose of Aqui-S$^®$); muscle tissue was dissected from

174   the epaxial quadrant (seabass: n=8, 1.6±0.1 g; barramundi: n=5, 1.1±0.1 g) and

175   visceral adipose tissue (VAT) (seabass: n=8, 0.5±0.1 g; barramundi: n=6, 0.5±0.1 g)

176   was carefully stripped from the peritoneal cavity. Both tissues were weighed, freeze-

177   clamped with aluminum tongs cooled in liquid nitrogen, pulverized with chilled pestle

178   and mortar and stored at -80ºC until further analysis.

179

180   *Sample treatment*

181   Muscle and VAT lipids were extracted from pulverized tissue according to Matyash et

182   al. (2008) using methyl tert-butyl ether, methanol and water mixture

183   (MTBE:MeOH:H$_2$O, 10:3:2.5; 20 mL g^{-1} of tissue) transferred to glass amber vials,

184   dried under N$_2$ stream and stored at −20°C. Triacylglycerols (TAG) were purified by

185   solid phase extraction with prepacked 2 g cartridges (Discovery$^®$ DSC-NH$_2$ 52641-U,

186   Supelco) according to Ruiz et al. (2004). Muscle TAG quantifications were performed

187   in a fully-automated analyzer Miura 200 (I.S.E. S.r.l.; Guidonia, Italy) using its

188   dedicated reagent kit (ref. A-R0100000901; seabass: n=7, barramundi: n=5).

189

190   *NMR analysis*

191   Tank water (TW) and fish body water (BW) ^{2}H enrichments were determined by ^{2}H

192   NMR using calibrated acetone and analyzed in duplicates as previously described

193   (Jones et al., 2001). Tank and plasma water content was assumed to be 96,5% and

194   92% of total sample, respectively. NMR spectra of TAG samples were obtained at

195   25°C with a Bruker Avance III HD system with an UltraShield Plus magnet (11.7 T,

196   ^{1}H operating frequency 500 MHz) equipped with a 5-mm ^{2}H-selective probe with ^{19}F

197 lock and ^{1}H-decoupling coil. TAG were reconstituted in chloroform containing a

198 pyrazine standard as previously described (Viegas et al., 2016) - representative TAG

199 ^{1}H and ^{2}H NMR spectra are provided as supplementary material (Figure S1). As ^{1}H

200 and ^{2}H signals are essentially isochronous, the identity of the ^{2}H signals can be

201 confirmed by matching their chemical shifts with their ^{1}H counterparts, meaning that

202 the respective signal assignments apply to both nuclei (Table S1). As control for the

203 TAG extraction, a FA/glycerol ratio (should be ~3) was calculated from the area of all

204 α protons times 2, divided by TAG-bound glycerol sn1,3 protons (Duarte et al., 2014).

205 The FA profile (in percentage) for saturated (SFA) and unsaturated fatty acids (UFA),

206 both poly- (PUFA) and monounsaturated fatty acids (MUFA) were estimated from ^{1}H

207 NMR spectra according to Viegas et al., (2016). Positional ^{2}H-enrichments were

208 quantified from the ^{1}H and ^{2}H NMR spectra by measuring the ^{1}H and ^{2}H intensities

209 (areas) of selected signals relative to the ^{1}H and ^{2}H intensities of a pyrazine standard,

210 after correction for linoleic acid contribution according to Duarte et al. (2014).

211 Briefly, during their residence in ^{2}H-enriched seawater, newly synthesized FA from

212 *de novo* lipogenesis incorporate ^{2}H-enrichment in the FA terminal methyl group

213 (signal at 0.80 ppm). The same principle applies to for newly synthesized glycerol,

214 incorporating ^{2}H-enrichment in the sn-1,3 glyceryl site (signals at 4.15 ppm).

215 Fractional synthetic rates (FSR; in % d^{-1}) of newly synthesized TAG-bound FA and

216 TAG-bound glycerol were estimated by dividing the respective positional ^{2}H-

217 enrichment by the BW. ^{2}H-enrichments were calculated after systematic subtraction

218 of 0.015%, taken as the mean background ^{2}H-enrichment. If the values were below

219 zero, these were considered as 0.0 for FSR calculation purposes. Spectra were

220 processed by applying exponential multiplication to the free-induction decay (^{1}H: 0.1

221 Hz; ^{2}H: 1.0 Hz). Spectra processing and peak integration was performed using

222 ACD/NMR Processor Academic Edition from ACD\Labs 12.0 software (Advanced

223 Chemistry Development, Inc.).

224

225 *Statistical analysis*

226 Data are presented as mean ± S.E.M. Whenever possible, two-way ANOVA was used

227 to test main effects of, and interactions between, diet and tissue. In the case of

228 statistically significant interactions, or in the impossibility to perform a two-way

229 ANOVA, differences between diets were tested using a Student's two-tailed unpaired

230   t-test. Analyses were performed in GraphPad Prism® software (GraphPad Software,

231   Inc.). Differences were considered statistically significant at $P < 0.05$.

232

233   **Results**

234   Following extraction and subsequent isolation from other lipid classes by solid phase

235   extraction, TAG gave well-resolved ^{1}H NMR spectra. The FA/glycerol ratio was not

236   affected by diet for any tissue of both species, and was overall consistent with a

237   successful TAG separation (seabass: n=32, 2.96±0.03; barramundi: n=22, 3.03±0.04;

238   t-test, $P > 0.05$). Signals from PUFA and MUFA moieties dominated the spectra,

239   while contributions from SFA were relatively minor. In seabass, TAG-bound FA

240   composition as categorized by ^{1}H NMR spectra was tested for effects of diet (LS vs.

241   HS) and tissue distribution (muscle vs. VAT) (Table 2). All were significant for the

242   latter, symptomatic of a differential profile where SFA were stored to a higher degree

243   in VAT than in muscle while the opposite trend was observed regarding UFA. Within

244   UFA, more PUFA, and consequently also more n-3 were found in muscle than in

245   VAT. In the case of PUFA and MUFA effects were also observed due to dietary

246   treatment, with PUFA decreasing, and MUFA increasing with elevated dietary starch.

247   No interactions between dietary treatment and the analyzed tissues were observed, as

248   summarized in Table 2. For barramundi, the same lipid composition analysis from [1]H

249   NMR spectra was performed as shown in Table 3. Due to poor signal-to-noise ratio,

250   probably resulting from technical problems associated with the lipid extraction and

251   not to low lipid concentration *per se*, two muscle samples (one per diet) were

252   considered unsuited for spectral analysis. As in seabass, barramundi lipid composition

253   revealed differential tissue distribution, with the exception of PUFA. As previously

254   observed, SFA were stored to a higher degree in VAT, while UFA, mainly driven by

255   MUFA, were preferentially stored in muscle. None of the lipid species were affected

256   by the dietary treatment even if PUFA mean values seemed to indicate otherwise.

257   Again, no interactions between variables were observed (Table 3).

258   As equivalent ^{1}H and ^{2}H signals resonate at approximately the same chemical shift,

259   the observed ^{2}H NMR signals correspond to specific enriched sites in the TAG

260   molecule. This enrichment is derived from ~0.015% [2]H natural abundance (mean

261   background [2]H-enrichment) plus excess enrichment from the metabolic incorporation

262   of ^{2}H from the 6-day residence in ^2H_2O-enriched seawater. Specifically, this

263   incorporation resulted in peaks corresponding to FA terminal methyl group site (non

264   n-3) (signal at 0.80 ppm) revealing TAG-bound FA synthesized *de novo*, and to the

265   sn1,3 glyceryl site, revealing newly synthesized or cycled TAG-bound glycerol.

266   Labeling was not detected in glycerol of seabass VAT (Fig. 1), and in both FA and

267   glycerol of muscle of barramundi (Fig. 2). For both species, glyceryl FSR were

268   substantially higher than for FA; ~20-80 times higher in the case of seabass muscle,

269   and ~2-20 times higher in the case of barramundi VAT.

270   In seabass (Fig. 1), differences in FA FSR were attributable to both tissue distribution

271   and diet, being significantly higher in muscle in regard to VAT, and HS in regard to

272   LS. Glycerol revealed higher FSR when compared to FA FSR and was also

273   statistically lower in the HS when compared with LS. In barramundi VAT, FA FSR

274   was significantly higher in fish fed the HS diet while glyceryl FSR on the other hand

275   was unaffected by diet. Muscle TAG levels did not differ between diets for both

276   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,

277   in g $100g^{-1}$ of tissue; t-test $P > 0.05$).

278

279   **Discussion**

280   In vertebrates the liver is the centerpiece for metabolic regulation and in teleost fish it

281   is the main lipogenic organ where key endocrine and nutrient sensing mechanisms

282   intersect (Conde-Sieira and Soengas, 2017). In similar feeding trials, hepatic TAG

283   content and FA composition was sensitive to high CHO diets (Castro et al., 2015a;

284   Lanari et al., 1999; Messina et al., 2013; Peres and Oliva-Teles, 2002; Viegas et al.,

285   2016). In the present study, muscle and VAT from both species presented different

286   FA profiles, but only in seabass these profiles were responsive to HS diet, particularly

287   PUFA and MUFA. CHO-stimulated accumulation of muscle lipids has been

288   associated with transcriptional response of hepatic FA desaturation and elongation

289   enzymes in rainbow trout (*Oncorhynchus mykiss*) (Kamalam et al., 2012), and

290   enhanced metabolic elongation rates in the same seabass analyzed in the present study

291   (Viegas et al., 2016). Interestingly in barramundi muscle, lack of CHO-stimulated FA

292   profile alterations may relate to absolute lack of ^{2}H-enriched TAG, either in the FA or

293   glycerol moieties. This suggests that this pool had very limited turnover, either in

294   terms of net FA replacement, or FA-TAG cycling (this latter process accounting for

295   the higher enrichment of glycerol compared to FA in other tissues as discussed

296   ahead). Muscle FA profile in this species has proven to be sensitive to dietary lipid

297   manipulations (Alhazzaa et al., 2011), but as regard to CHO, further studies should be

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

332 Despite the fact that the labeling was recovered from a single type of lipid, the
333 synthesis, and therefore the ^{2}H-enrichment of the TAG glyceryl backbone and its
334 three esterified FA, happens independently. FA terminal methyls hydrogens become
335 enriched in ^{2}H by DNL (other TAG fatty acyl hydrogens may also become enriched
336 by elongation reactions). TAG glyceryl hydrogens become enriched via *de novo*
337 glycerol-3-phosphate synthesis from glyceraldehyde-3-phosphate (G3P) and exchange
338 between glycerol-3-phosphate and G3P. Their enrichment is suitably discriminated by
339 ^{2}H NMR, a feature that mass-based technologies are unable to do unless additional
340 sample treatment was performed. Regardless of the dietary treatment and species,
341 TAG-bound glycerol revealed considerably higher ^{2}H-enrichment when compared to
342 TAG-bound FA. It has been hypothesized that fish maintain high glycerol rates of
343 appearance by constantly cycling (reesterifying) up to two-thirds of circulating TAG
344 to free FA (Bernard et al., 1999; Magnoni et al., 2008). During exercise, fish muscle
345 augments lipoprotein lipase (LPL), critical for lipid mobilization (Magnoni et al.,
346 2013) but TAG-FA turnover rates were maintained unalterably high. In the absence of
347 glycerol kinase in muscle, this glycerol cannot be recycled to glycerol-3-phosphate
348 and reused in TAG cycling, being instead shuttled to the liver. Alternatively, *de novo*
349 synthesis of glycerol-3-phosphate must occur and as consequence, enrichment of the
350 triglyceride glyceryl moiety is typically much higher than that of the FA, which also
351 include FA from dietary (unlabeled) origin. Glucose can be a contributing precursor
352 to glycerol synthesis (through dihydroxyacetone phosphate; DHAP) via an
353 abbreviated pathway of glycolysis as confirmed by ^{13}C- (Rito et al., 2018) and ^{14}C-
354 glucose (Walter et al., 2006) tracer studies. Higher levels of circulating (unlabeled)
355 glucose may putatively explain why, glyceryl FSR was lower in the muscle of seabass
356 fed with HS diet, as already observed in the liver if these fish (Viegas et al., 2016).
357 This same study also revealed that despite elevated hepatic TAG levels (LS: 11.1±0.2
358 and HS: 35.4±0.7 g 100 g^{-1} liver; t-test $P < 0.001$), this was not attributable to
359 increased hepatic DNL. So, it was particularly intriguing to find that in muscle, TAG-
360 bound FA from DNL were significantly increased in the HS diet, even if muscle TAG
361 levels remained unaltered (LS: 8.4±1.2 vs. HS: 7.4±0.6 g 100 g^{-1} muscle; t-test $P >$
362 0.05). This indicates an independent regulation of muscle TAG synthesis activity
363 during starch feeding, previously observed for rainbow trout (Brauge et al., 1995) but
364 whose mechanisms are yet to be addressed.

365 In seabass, CHO-derived overall increased adiposity is driven not only by increased

366 liver lipid content but also by VAT accumulation (Castro et al., 2015a; Dias et al.,

367 1998; Peres and Oliva-Teles, 2002). This was not observed in the present experiment,

368 as perivisceral fat index remained unaltered (LS: 4.9 ± 0.2 vs. HS: 5.1 ± 0.2) (Viegas et

369 al., 2016). In rainbow trout, contrary to documented for muscle, in VAT insulin is

370 regarded as an important hormone for lipid deposition: i) via its anti-lipolytic effects

371 (Albalat et al., 2006); ii) through upregulation of ACLY and FAS and iii) by

372 production of NADPH for lipogenesis (Polakof et al., 2010b). In a species from the

373 same genus, coho salmon (*O. kisutch*), adipocyte GLUT4 transporter was sensitive to

374 insulin notwithstanding a lower affinity for glucose compared to the mammalian

375 homolog (Capilla et al., 2004). In another salmonid species, *in vitro* studies revealed

376 even though the DNL pathway is active in Atlantic salmon (*Salmo salar*) adipocytes,

377 rates of conversion of glucose into lipids were relatively low (Bou et al., 2016). It is

378 worth noting that in salmonids, adipocytes were not nutritionally regulated by CHO

379 (Figueiredo-Silva et al., 2012; Kamalam et al., 2013), but displayed elevated G6PDH

380 activity and NADPH levels even in the absence of CHO (Barroso et al., 2001). This

381 seems not to be the case for seabass and barramundi VAT where TAG-bound FA

382 synthesis via DNL was significantly higher for HS diet. This was also described in

383 gilthead seabream (*Sparus aurata*) adipocytes which under high CHO diets, up-

384 regulated G6PDH expression and FAS also tended to increase (Bou et al., 2014).

385 Besides a stronger response to insulin (Albalat et al., 2007) through LPL activation,

386 CHO utilization in this species' VAT was also favored over FA oxidation by

387 modulation of the PPARs transcription factors (peroxisome proliferator-activated

388 receptors) (Bou et al., 2014).

389 In seabass, FA FSR was significantly lower in VAT than in muscle but was

390 nevertheless influenced by diet. Contrary to observed in muscle, TAG-FA cycling did

391 not occur in VAT as interpreted by the lack of [2]H-enrichment in TAG-bound glycerol.

392 In barramundi the opposite seemed to take place; on the one hand, no [2]H-enrichment

393 was detected in muscle TAG, and on the other, not only VAT FA were extremely

394 stimulated by the HS diet, but also high FSR for glycerol were estimated. Similar

395 findings were obtained from Atlantic salmon (*Salmo salar*) adipocytes incubated with

396 ^{14}C-glucose. Analysis of TAG revealed 16-fold more ^{14}C incorporation into glycerol

397 than FA (Bou et al., 2016). This is well within the range observed in the present study

398 for barramundi VAT TAG with 19-fold excess [2]H-enrichment of glycerol over FA

399 during HS feeding. Bou and colleagues proposed that the way glucose stimulates
400 lipogenesis was by stimulating the pentose phosphate pathway, which in turn
401 generates G3P precursors for TAG glycerol as well as providing NADPH for
402 sustaining FA synthesis. Our study provides further evidence for this mechanism, not
403 only in barramundi adipose tissue but also seabass muscle. It should be noted that
404 despite our best efforts to provide a framework for comparing both species by using
405 isoenergetic diets, certain parameters differed. Particularly, the diets for barramundi
406 were not isoproteic but none was protein limiting as it was ensured that the DP:DE
407 ratio exceeded the established requirements for this species (Glencross, 2008). The
408 fact that these were not isolipidic may have alone interfered with overall lipid
409 metabolism, perhaps with stronger effects in the liver rather than in muscle or VAT.
410 Nonetheless, the present study has provided further insight into the impact and fate of
411 dietary starch in extrahepatic tissues of two carnivorous seabass species. The FA
412 profile differed substantially between muscle and VAT, but their lipogenic fluxes
413 revealed even greater differences. In seabass, HS promoted DNL of TAG-bound FA,
414 in muscle and VAT. High ^{2}H-enrichment found in muscle TAG-bound glycerol was
415 indicative of its role on lipid cycling. In barramundi, HS had no effect on muscle FA
416 composition and lipogenic flux, with no ^{2}H-enriched TAG being detected. VAT on
417 the other hand revealed a strong enhancement of DNL in HS-fed fish along with high
418 TAG-bound glycerol cycling.
419 Overall, while the aquaculture industry seeks to further stretch the optimization of fish
420 feed production, the administration of isotopes will provide deeper insights into the
421 regulation of lipid storage and oxidation in fish species in response to different
422 combination of macronutrients. This may be particularly interesting in the
423 procurement of new species to farm or in the optimization and selection of existing
424 ones, currently happening by trait preference (Jin et al., 2014b; Song et al., 2018) or
425 by recurring to the wide spectrum of metabolic responses occurring in nature
426 (Betancor et al., 2016; Marandel et al., 2018).
427
428 **Disclosures**
429 No conflicts of interest, financial or otherwise, are declared by the authors.
430
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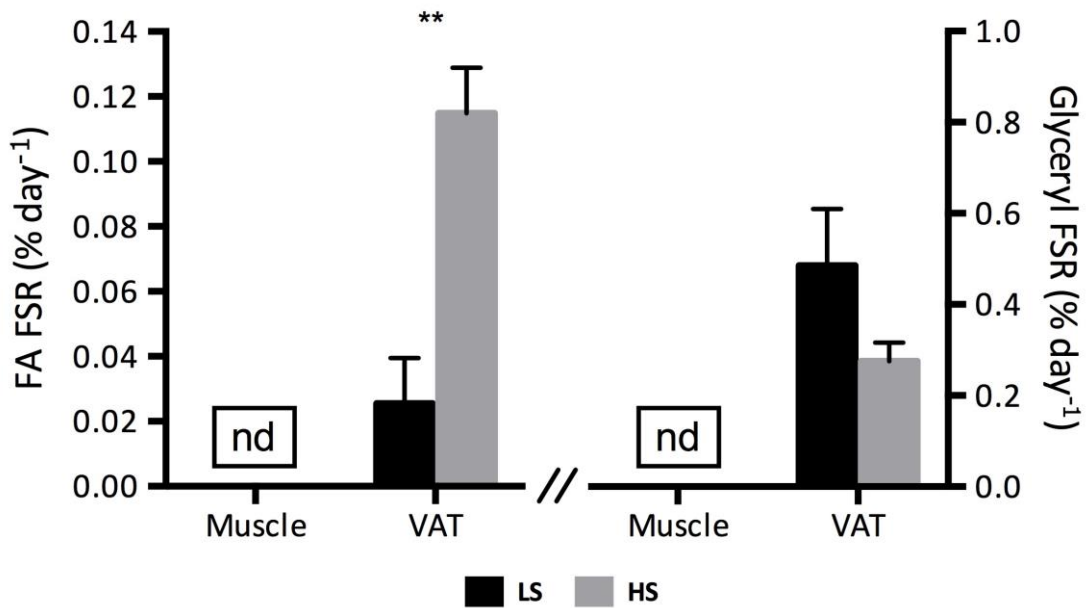673   magnetic resonance imaging (MRI). Aquac 448, 112-122.

674 **Figure 1**
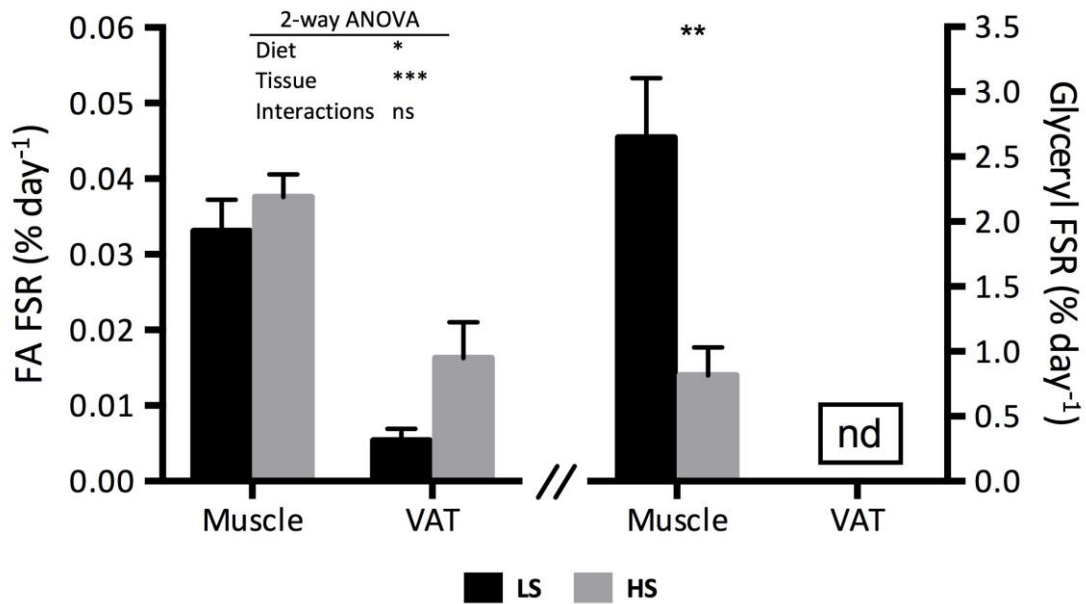
Figure 1: Fractional synthetic rate for TAG-bound fatty acids (fatty acyls; FA FSR) and TG-bound glycerol (Glyceryl FSR) expressed as percent of newly synthesized TAG from *de novo* lipogenesis per day in muscle and visceral adipose tissue (VAT) of seabass (*D. labrax*) fed with low starch (LS) and high 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).