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ORIGINAL ARTICLE

Response to dietary carbohydrates in European seabass (*Dicentrarchus labrax*) muscle tissue as revealed by NMR-based metabolomics

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Abstract

Introduction Feed optimization is a key step to the environmental and economic sustainability of aquaculture, especially for carnivorous species. Plant-derived ingredients can contribute to reduce costs and nitrogenous effluents while sparing wild fish stocks. However, the metabolic use of carbohydrates from vegetable sources by carnivorous fish is still not completely understood.

Objectives We aimed to study the effects of diets with carbohydrates of different digestibilities, gelatinized starch (DS) and raw starch (RS), in the muscle metabolome of European seabass (*Dicentrarchus labrax*).

Methods We followed an NMR-metabolomics approach, using two sample preparation procedures, the intact muscle (HRMAS) and the aqueous muscle extracts (^{1}H NMR), to compare the variations in muscle metabolome between the two diets.

Results In muscle, multivariate analysis revealed similar metabolome shifts for DS and RS diets, when compared with the control diet. HRMAS of intact muscle, which included both hydrophobic and hydrophilic metabolites, showed increased lipid in DS-fed fish by univariate analysis. Regardless of the nature of the starch, increased glycine and phenylalanine, and decreased proline were observed when compared to the Ctr diet. Combined univariate analysis of intact muscle and aqueous extracts indicated specific diet related changes in lipid and amino acid metabolism, consistent with increased dietary carbohydrate supplementation.

Conclusions Due to differential sample processing, outputs differ in detail but provide complementary information. After tracing nutritional alterations by profiling fillet components, DS seems to be the most promising alternative to fishmeal-based diets in aquaculture. This approach should be reproducible for other farmed fish species and provide valuable information on nutritional and organoleptic properties of the final product.

Keywords Aquaculture · Diet optimization · Digestible starch · Fish · Tissue extracts · Metabolism

Ivana Jarak and Ludgero Tavares have contributed equally to the manuscript.

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1 Introduction

Efficient feeding formulations and protocols are one of the most critical and demanding steps in fish farming. Optimal diets should ensure all the fish nutritional needs and promote maximal growth, without increasing production costs and nitrogen input into the system. In this context, plant-derived diets could be an interesting alternative to the standard high-protein diets (Gatlin et al. 2007). The effective replacement of proteins with carbohydrates in carnivorous fish diet entails that the sparing of dietary amino acids does not compromise muscular growth and its nutritional composition while reducing ammonia production (Amirkolaie 2011). Moreover, diets with higher carbohydrate percentages are less expensive by depending

less on marine-derived ingredients thus increasing production income margins. In European seabass (*Dicentrarchus labrax*), the reduction of dietary protein by starch incorporation (raw and gelatinized), did not affect overall growth performance nor protein and energy retention (Peres and Oliva-Teles 2002). Due to its higher digestibility coefficient, gelatinized starch has improved metabolic utilization (Enes et al. 2011). However, it has been shown that such alterations in diet composition can directly affect metabolic pathways (Castro et al. 2015a, b; Enes et al. 2011; Kamalam et al. 2017; Viegas et al. 2016), and impact the organoleptic properties of the fillet (Adamidou et al. 2009; Grigorakis 2017; Messina et al. 2013). Understanding how these nutritional regimes affect the muscle metabolome is an essential step to optimize new diets and assure the quality of the end product for farmed fish species.

Intrinsic attributes of nuclear magnetic resonance (NMR) spectroscopy make it ideal for metabolic studies (Markley et al. 2017). This potential has been explored to assess the effects of plant-derived carbohydrates in Atlantic salmon (*Salmo salar*) diets (Wagner et al. 2014) and to profile metabolic alterations in several other farmable aquatic animals, such as mussels (*Dreissena polymorpha*) (Watanabe et al. 2015), red abalone (*Haliotis rufescens*) (Rosenblum et al. 2005), common carp (*Cyprinus carpio*) (Lardon et al. 2013), channel catfish (*Ictalurus punctatus*) (Allen et al. 2015), gilthead seabream (*Sparus aurata*) (Savorani et al. 2010a), and European seabass (*Dicentrarchus labrax*) (Heude et al. 2015; Mannina et al. 2008; Vidal et al. 2012). Moreover, NMR-based metabolic studies can be paired with mass spectrometry (Karakach et al. 2009) or with isotopic labeled compounds (Marques et al. 2016; Rito et al. 2018), which enable a detailed analysis of metabolic pathways affected directly by a specific dietary substrate.

European seabass (*Dicentrarchus labrax* L.) is a carnivorous fish and one of the most representative aquaculture marine species in Mediterranean region. The effect of dietary carbohydrates (digestible and raw starch) on hepatic de novo lipogenesis and systemic carbohydrate fluxes (Viegas et al. 2016, 2015) was already investigated. Results were indicative of a decrease in the gluconeogenic pathway, without increasing the lipogenic potential in diets with digestible starch.

The aim of this work was to study the effects of two diets with different carbohydrate digestibilities on the muscle metabolome of European seabass. To address this, an NMR-metabolomics based approach was applied using two different sample preparation procedures (aqueous extracts and intact muscle). Besides contributing to a comprehensive insight into the metabolic response of seabass muscle to dietary manipulation, this approach should generate an output capable of informing optimization strategies for other farmed fish species.

2 Materials and methods

2.1 Animal handling and sampling

Juvenile European seabass (*Dicentrarchus labrax* L.) with an initial mean body weight of 21.9 ± 0.3 g, were randomly assigned to three different tanks and reared under the conditions described in detail by Viegas et al. (2016). Briefly, fish were fed with three experimental diets (Sparos Lda., Loulé, Portugal): a control carbohydrate-free diet (Ctr), a diet with 30% digestible starch from gelatinized pea meal (DS), and a raw starch diet from crude pea meal (RS) (Supplementary material, Table S1). After this period, each group was transferred to a 5% ^{2}H-enriched seawater tank for 6 days to perform hepatic glucose and lipid metabolic studies (Viegas et al. 2016, 2015). As this setup was used for different parallel studies, significant enrichment of metabolites such as lipids, glucose and amino acids with deuterium (^{2}H) could lead to decreased signal intensities in the ^{1}H spectra and thus influence the observed relative concentrations. However, relatively low ^{2}H-enrichments were observed: 0.1–0.5% in the case of hepatic alanine (Marques et al. 2016); below 0.1% in hepatic (Viegas et al. 2016), and even lower in muscular triglyceride-bound fatty acids (unpublished results). Twenty-four hours after last meal, fish were anesthetized in seawater with MS-222 (0.1 g L^{-1}), weighed and sacrificed by cervical section. Muscle fillet samples from the epaxial quadrant were dissected. A biopsy core was punched into each sample, immediately dipped in N_2 and stored while the remaining fillet was freeze-clamped in N_2. Both samples were stored at − 80 °C until further processing. All experimental procedures complied with the Guidelines of the European Union Council (86/609/EU).

2.2 Sample preparation for NMR analysis

Free amino acids (FAA) were extracted following Matyash et al. (2008) (Ctr, n = 10; DS, n = 9; RS, n = 8). Fish muscle samples were ground in N_2, and to each gram of frozen tissue 15.4 mL of methyl tert-butyl ether (MTBE) and 4.6 mL of methanol were added. The mixture was incubated for 1 h at room temperature under agitation and centrifuged for 10 min at 13,000×*g*. The liquid fraction was separated and extracted with 4 mL of distilled water for 10 min at room temperature and centrifuged for 10 min at 1000×*g*. The aqueous fraction, containing among other FAA, was collected and evaporated to dryness. Samples were reconstituted in 200 µL of 150 mM deuterated phosphate buffer, pD = 7.4, and transferred to 3 mm diameter NMR tubes for ^{1}H NMR analysis.

For the high resolution magic angle spinning (HRMAS) analysis of muscle biopsies (0.05 ± 0.01 g of intact tissue; Ctr, n = 15; DS, n = 6; RS, n = 7), intact frozen tissues were washed with ^2H_2O saline to remove excess blood and packed immediately into 50 µL HRMAS 4 mm zirconia rotors containing 10 µL of ^2H_2O saline with 0.25% 3-(trimethylsilyl)propionate sodium salt (TSP)-d_4.

2.3 NMR spectroscopy

1D proton spectra of aqueous extracts were acquired on a Varian VNMR 600 MHz (Varian Inc., Palo Alto, CA, USA) spectrometer equipped with a 3 mm ^{1}H(X)-PFG inverse configuration probe. ^{1}H *presat* pulse sequence was used with the following acquisition parameters: spectral width of 6 kHz, 45° pulse angle, 4 s of acquisition time, 55 s interpulse delay and 16 transients at 298 K. All ^{1}H-HRMAS spectra of intact muscle biopsies were acquired on an 800 MHz Bruker Avance II+ (Rheinstetten, Germany) spectrometer using a 4 mm HRMAS probe. Spectra were recorded at 277 K with a spinning rate of 4 kHz, employing the Carr–Purcell–Meiboom–Gill (CPMG) spin-echo pulse sequence with water presaturation and a total spin echo time of 120 ms ($n = 444$, $\tau = 270$ µs). Typically, 256 transients were acquired with 72 k points, spectral width of 12 kHz, relaxation delay of 2 s, and acquisition time of 3 s. Spectral assignments were based on matching the recorded spectra to the reference data available in public databases such as Human Metabolome Database (HMDM) and Biological Magnetic Resonance Data Bank (BMRB), and further supported by statistical total correlation spectroscopy (STOCSY) (Cloarec et al. 2005). Metabolites have been identified at Metabolomics Standards Initiative (MSI) level 2 according to the guidelines for metabolite identification (Sumner et al. 2007).

2.4 NMR spectral data analysis

All spectra were processed using Topspin 3.2 software (Bruker, Ettlingen, Germany) applying: zero-filling to 64 k points, line broadening of 0.3 Hz, phasing and baseline correction. The chemical shifts were referenced internally to the alanine signal at δ 1.48 ppm. Spectral regions between 0.7 and 8.85 ppm (for aqueous extracts) and 0.67 and 5.6 ppm (for biopsies) were used to build data matrices for multivariate analysis, using one-point bucketing (Amix V3.9.15 software, Bruker Biospin, Germany). Water signal region was excluded (4.40–5.25 and 4.75–5.20 ppm for aqueous extracts and biopsies, respectively). Chemical shift variations in recorded spectra were minimized by using icoshift (Savorani et al. 2010b) alignment, followed by recursive segment-wise peak alignment (Veselkov et al. 2009) where needed, using R statistical package (R Core Team 2014). Probabilistic quotient normalization (PQN) (Dieterle et al. 2006) was used to account for different sample sizes and dilutions. Multivariate analysis: principal component analysis (PCA) and partial least square discriminant analysis (PLS-DA) were performed in SIMCA-P14.1 (Umetrics AB, Umeå, Sweden) after unit variance scaling has been applied. PLS models were validated by permutation testing, following a sevenfold cross-validation test (Triba et al. 2015). To compare the magnitude of the response of the metabolome between the tested diets, the peak area of metabolites of interest were integrated using Amix V3.9.15 (Bruker BioSpin, Germany) and normalized by the quotients retrieved from PQN. For each metabolite, the difference between the groups (control and experimental diets) was assessed using parametric ANOVA or nonparametric Kruskal–Wallis test, followed by Tukey's or Dunn's multiple comparisons test, respectively. The metabolomics data generated during the current study were submitted to the EMBL-EBI MetaboLights database and are publicly available with the identifier MTBLS619 (https://www.ebi.ac.uk/metabolights/MTBLS619).

3 Results and discussion

3.1 General NMR profiling and multivariate analysis

Average 1D ^{1}H NMR spectra of intact muscle biopsies and aqueous extracts are presented in Fig. 1. Some major metabolites are highlighted while the full spectral assignment is available as supplementary material (Table S2). Spectra of intact muscle were dominated by the signals of creatine and lactate, with taurine, glycine and trimethylamine oxide (TMAO) also observed in large quantities. Some other amino acids, namely proline, valine, glutamine, alanine and lysine were also present in smaller amounts. The CPMG pulse sequence was applied to filter out the broad peaks belonging to the metabolites with shorter T_2 relaxation times, such as lipids and proteins. While the majority of the broad signals responsible for the distortion of the baseline were successfully filtered out, broad lipid signals were not completely suppressed under the experimental conditions suggesting the presence of highly mobile lipids (Griffin et al. 2001). Unlike the CPMG spectra of intact muscle, the predominant metabolites in muscle extracts were lactate and TMAO. Furthermore, acetate and betaine, which were not detected in the biopsy spectra, were clearly observed in the extracts while lysine multiplets were not visible. The upfield signals of aromatic metabolites were more prominent in the spectra of extracts and revealed a number of amino acid and nucleic acid derivatives. Low levels of glucose were observed in both types of sample preparations, as expected from muscle composition described for seabass

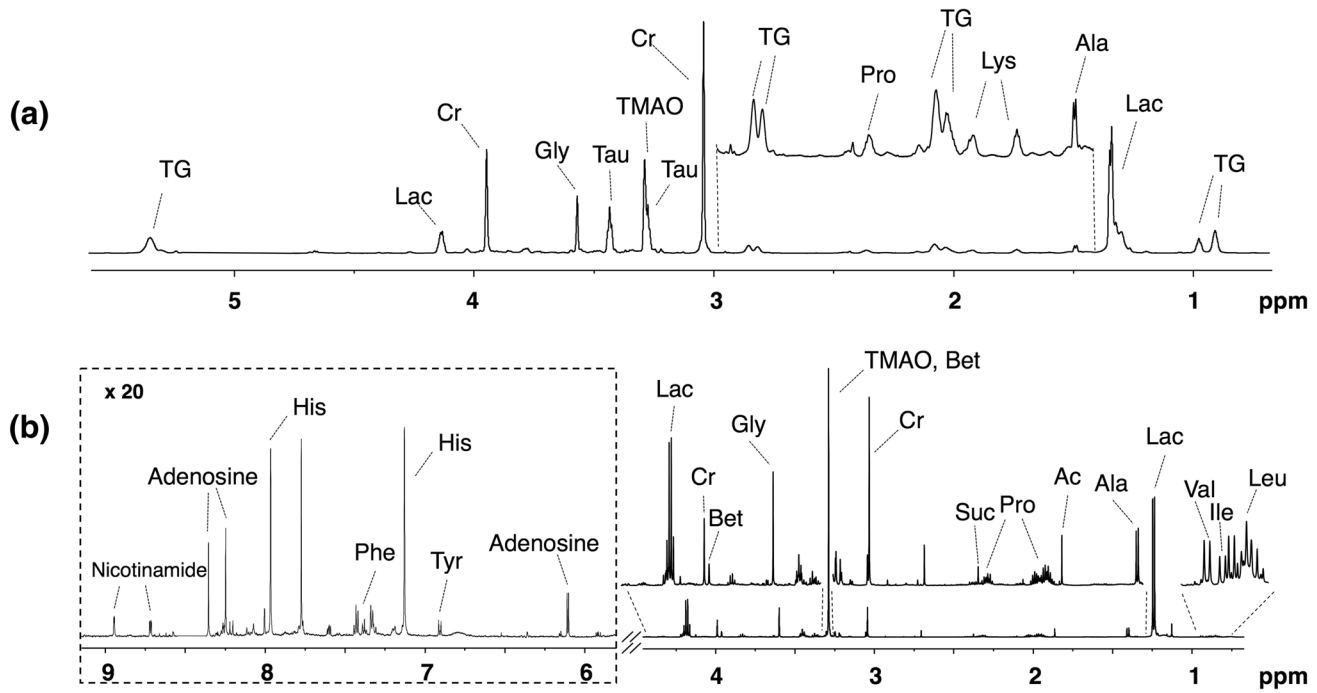

Fig. 1 Average 1D ^{1}H NMR spectra of the control group fish muscle: **a** CPMG spectrum of intact muscle; **b** spectrum of aqueous muscle extracts. Some major metabolites are assigned. *Ac* acetate, *Bet* betaine, *Cr* creatine, *His* histidine, *Lac* lactate, *Suc* succinate, *Tau* taurine, *TG* triglyceride, *TMAO* trimethylamine N-oxide, three letter codes for amino acids

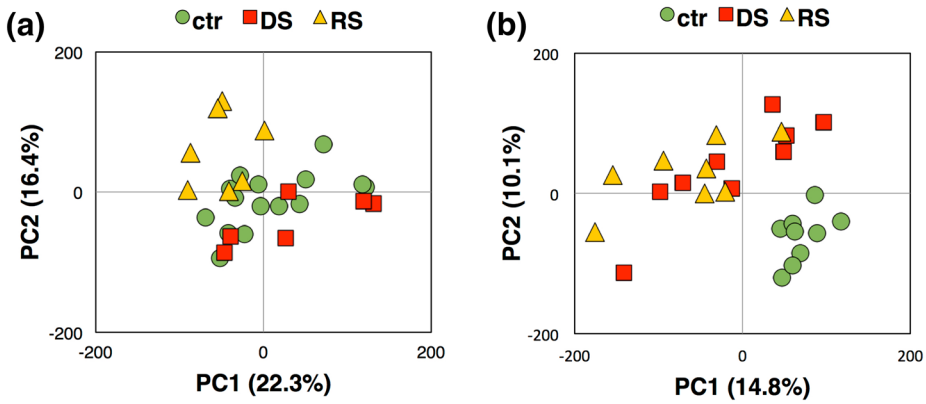

Fig. 2 PCA scatter scores plots obtained by multivariate analysis of 1D ^{1}H NMR spectra of muscle of seabass fed a control carbohydrate-free diet (Ctr), and two experimental diets containing gelatinized starch (DS), or raw starch (RS); **a** intact muscle (n(PC) = 5) and **b** aqueous extract (n(PC) = 6)

(Adamidou et al. 2009; Messina et al. 2013). The PCA scores plot of intact muscle (Fig. 2a) revealed a certain clustering between dietary treatments, however, overlap was observed between groups fed with control and experimental diets, indicating similarities of their respective metabolomes. On the contrary, the PCA scores plot of the first two principal components of aqueous extracts (Fig. 2b) showed complete overlap of experimental diets while a cluster of the control diet was clearly separated. PLS-DA was also applied to both intact muscle and aqueous extract groups (Supplementary material, Fig. S1) however, permutation tests did not validate the models.

3.2 Univariate analysis of muscle metabolome response to experimental diets: RS vs. DS

When RS and DS diets were compared following univariate analysis (Fig. 3), the most significant differences between them were observed in lipid content and amino acids. The muscle of DS-fed fish was found to be significantly richer in lipids than the RS-fed fish, an output only illustrated in intact muscle analysis since the preparation of aqueous extracts involved the discarding of the organic phase. As previously reported, the nature of starch used to substitute a portion of normal protein-based diets significantly impacts the lipogenic capacity of carnivorous fish, displaying overall

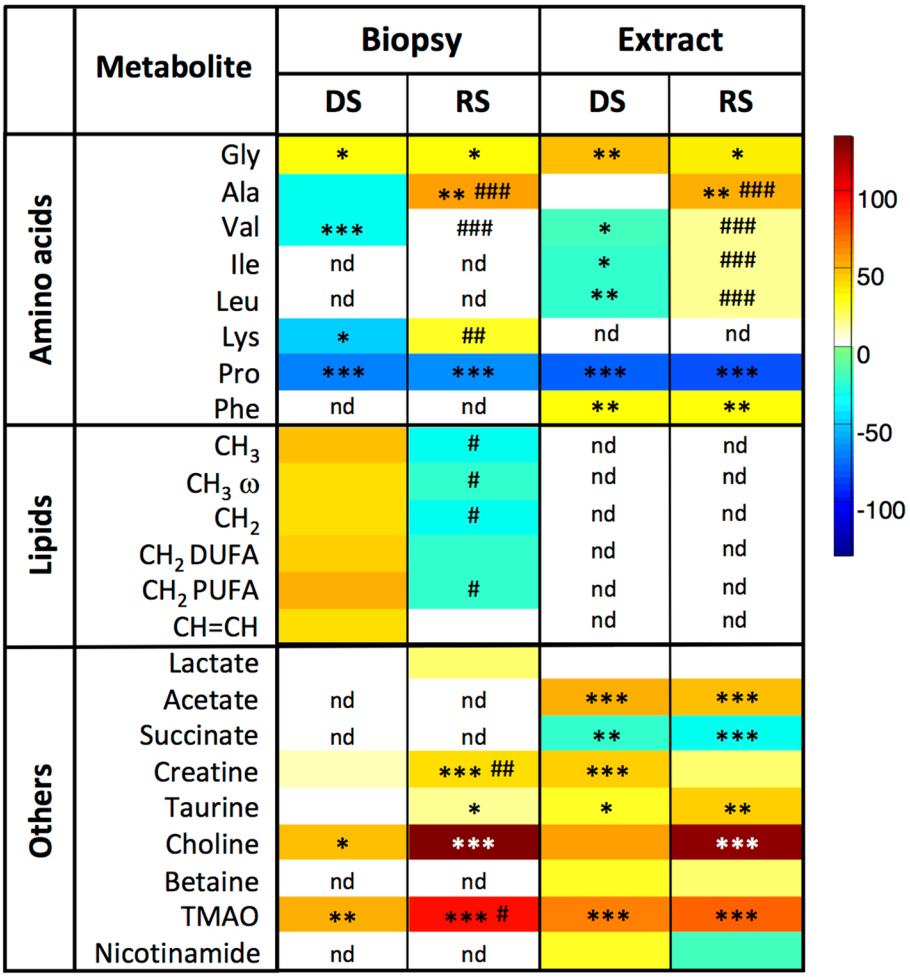

Fig. 3 Heatmap showing the changes in metabolites in experimental diets relative to the Ctr diet in intact muscle (biopsy) and aqueous extracts. The scale is color-coded expressed as a function of the % variation, ranging from maximum decrease (dark blue) to maximum increase (dark red). Statistically significant differences are indicated for comparisons of experimental diets with control diet (*$p<0.05$, **$p<0.01$, ***$p<0.001$) and between experimental diets ($^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$). *DUFA* diunsaturated fatty acids, *PUFA* polyunsaturated fatty acids, three letter code used for amino acids, *nd* not determined

increased adiposity (Enes et al. 2011; Kamalam et al. 2017). For the same experimental setting here described, this was corroborated by increased plasma triglyceride (TG) levels and perivisceral somatic indices of DS-fed fish compared with RS (Viegas et al. 2016).

Muscle of RS-fed fish was also characterized by significant accumulation of alanine and branched-chain amino acids (BCAA) generally evident in both types of sample processing. Opposing trends in lysine and BCAA indicate different metabolic responses to nutritional cues. Several studies have thus far been conducted to search for amino acid supplementation and specific dietary formulations that would promote optimal growth in farmed seabass (Kousoulaki et al. 2015). Lysine is one of the major limiting amino acids in plant-based diets and is primarily used for muscle buildup. However, it can also be oxidized for energy or used for lipid and carnitine synthesis. Carnitine regulates lipid metabolism by transporting long chain fatty acids from cytosol to mitochondrial matrix for β-oxidation thus reducing lipid deposition in fish muscle (Li et al. 2017). Furthermore, lysine (Lansard et al. 2011) and carnitine (Li et al. 2017) positively influence protein synthesis through activation of the mTOR pathway, proposed as a possible mechanism for explaining the increased growth induced by carnitine in juvenile seabass (Santulli and D'Amelio 1986). Leucine is also an mTOR activator (Lansard et al. 2011) thereby consistent with our observations of increased BCAA levels.

Alanine is an important source of energy and a glucogenic amino acid through the activity of alanine transaminase (ALT; EC 2.6.1.2). In fact, for the same experimental setting here described, hepatic cytosolic ALT activity was significantly increased in RS group compared to both DS and control (Viegas et al. 2015; Fig. S2). The buildup of alanine in RS-fed fish could indicate a decreased utilization of pyruvate through the TCA cycle, resulting in less available energy for sustaining growth. Decreased daily growth index (Viegas et al. 2016) and muscular alanine fractional synthetic rate (Marques et al. 2016) observed in RS fish seem to corroborate the obtained results. The analysis of the intact muscle spectra revealed that the predominant osmolyte in seabass muscle was TMAO, with higher values in RS-fed

fish compared to DS-fed fish, which was not statistically significant in aqueous extracts.

3.3 Univariate analysis of muscle metabolome response to carbohydrate supplementation: Ctr vs. starch

As revealed in the muscle biopsy ^{1}H-HRMAS spectra, neither RS nor DS showed statistical differences when compared to Ctr regarding lipid composition. This reinforces the assumption that carbohydrate supplementation in diets for carnivorous fish is a feasible option with limited impact on fillet overall lipid composition. However, as discussed previously based on Fig. 3, experimental diets showed opposing trends, with lipid levels higher in DS and lower in RS. Since raw starch has low digestibility and assimilation rates, the RS-fed fish presented impaired growth parameters when compared to Ctr and DS (Viegas et al. 2016).

Regardless of the nature of the starch, some common trends were observed when compared to the Ctr diet, namely an increase in glycine and phenylalanine, and decrease in proline. Proline is a substrate for the synthesis of polyamines, which are involved in cell proliferation and differentiation. The decreased levels of proline observed in both DS- and RS-fed fish muscle could be explained by the reduced proline bioavailability in the starch-supplemented diets. It is interesting to point out that no free proline was observed in the ^{1}H NMR spectra of muscle extracts of adult seabass (Mannina et al. 2008) indicating a possible role for free proline in juvenile fish.

Significant changes were also noticed in the group of metabolites with osmolytic properties. The analysis of the intact muscle spectra revealed that the predominant osmolyte in juvenile seabass muscle was taurine, followed by glycine. As hypo-osmoregulators, marine teleost fish regulate osmolality using specialized organs (gills); but they also accumulate organic osmolytes in the muscle cells in order to regulate cell size and ensure normal physiological functioning (Tseng and Hwang 2008). Apart from being an osmolyte, taurine has many other important roles in physiological processes in fish, such as regulation of bile acid composition (Kim et al. 2007). Although plants contain $<1\%$ of the taurine found in animals, increased taurine levels were observed in the muscle of fish fed both digestible and raw starch diets. However, taurine tissue levels are determined not only by dietary intake, but also by the presence and activity of specific intestinal epithelium and renal transporters (TauT) that regulate taurine absorption. In addition, fish are capable of endogenous taurine biosynthesis (Liu et al. 2017) from sulfur-containing amino acids. Differing biosynthetic capacities encountered across fish species make taurine an essential nutrient being that taurine-deficient formulations have been associated with reduced growth and survival, and increased susceptibility to diseases. On the other hand, direct beneficial effects deriving from taurine supplementation on fish growth are still under debate since taurine function and response vary greatly between fish species, and in some cases, within species depending on life stage (Salze and Davis 2015). In seabass fry taurine supplementation improved growth rates (Martinez et al. 2004), while in juvenile seabass taurine supplementation alone (Feidantsis et al. 2014), or paired with methionine (Coutinho et al. 2017) seemed to have no effect on growth, feed utilization or whole-body composition.

Muscle of starch-fed fish was also characterized by significant increase of choline and TMAO in both biopsy and aqueous extracts. Choline is an important biosynthetic precursor of cell membrane phospholipids and a main source of osmolytes such as TMAO and betaine. In fish gut, choline is converted to trimethylamine by bacteria, which is then oxidized to TMAO in the liver by the enzyme trimethylamine oxidase and later transported to organs or excreted. Retarded growth, decreased survival, low feed efficiency and hepatic lipid accumulation have been documented as a result of choline deficiency in fish (Khosravi et al. 2015). Moreover, in relatively less carnivorous fish like the Jian carp (*Cyprinus carpio* var. Jian), choline deficiency induced oxidative damage in liver and intestines, resulting in increased levels of oxidative stress markers and reduced activities of antioxidant enzymes (Wu et al. 2017).

Finally, energy related metabolism has been altered in both DS and RS groups, as revealed by decreased levels of the TCA metabolite succinate. This metabolite is considered as a reliable proxy for energetic status since succinate dehydrogenase (Complex II) participates in the oxidative phosphorylation. Creatine serves as an energy buffer in its phosphorylated form (phosphocreatine), and participates in ATP regeneration by providing a source of inorganic phosphate. Due to its labile nature, creatine showed unclear results between sample processing methods, however, carbohydrate supplementation seemed to reveal heightened levels of creatine. Sustained swimming in rainbow trout fed a similar DS (high digestible wheat starch) diet upregulated the expression of creatine kinase in muscle (Magnoni et al. 2013). These mechanisms should be finely tuned in aquatic organisms since burst swimming is a crucial adaptive response for feeding and survival and very sensitive to dietary cues such as starch (Felip et al. 2012).

Interestingly, both DS and RS diets exhibited increased acetate levels. This could result from increased gut microbiota fermentation as already verified in seabass also fed with carbohydrate-rich diets (Gatesoupe et al. 2014). Acetyl-CoA is a major hub for connecting metabolic pathways, and acetate and acetyl-CoA pools can be interconverted. Additionally, RS-fed fish may direct metabolism towards β-oxidation of dietary lipids as fuel source. This generates large amounts of acetyl-CoA that can be

converted to acetate, however, rates of endogenous acetate production and utilization are still to be determined for fish species. This finding is further supported by the high lysine levels present in the RS fish intact muscle, suggesting higher carnitine synthesis and increased transport of fatty acids towards β-oxidation ultimately confirming the almost negligible de novo lipogenic rate described in this setting (Viegas et al. 2016). On the contrary, lipid accumulation observed in DS muscle could, at least partially, be explained by the insufficient availability of lysine as biosynthetic precursor of carnitine, resulting in lower lipid transport for β-oxidation.

4 Conclusions

Many similarities were observed in the muscle response of the experimental diets when compared to the Ctr diet as revealed by multivariate analysis, especially in the aqueous extract where the DS and RS groups were clustered aside. After metabolite profiling, the univariate analysis allowed to detect individual variations that, if clustered by functionality or class, elucidate the main metabolic events occurring under the respective experimental diet. This clustering was highlighted using an intuitive color scheme assisting on the interpretation of the direction and intensity of the observed variations as well as evidencing differences between diets and sample processing.

Regarding the feed formulations in the aquaculture context, the apparent decrease in muscle lipids observed in RS-fed fish may be detrimental to the overall nutritional quality of the fillet. DS could therefore be a plausible substitute even if heightened muscle fat content may cause organoleptic changes. The chosen methodologies were able to characterize seabass muscle alterations, where biopsies and extracts provided distinct but complementary and concordant information, which allowed sustaining deeper and complex metabolic considerations. Biopsies in particular, paired with HRMAS analysis, could be a useful application for the industry as pre-harvesting fillet quality control since it is non-lethal and requires minimal sample processing.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest, financial or otherwise.

Ethical approval To fulfill the purpose of this study, the full animal model was required using European seabass (*Dicentrarchus labrax*). All experimental procedures complied with the Guidelines of the European Union Council (86/609/EU).

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