Effects of dietary carbohydrate on hepatic de novo lipogenesis in European seabass (*Dicentrarchus labrax* L.)

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Abstract **Farmed seabass have higher adiposity than their wild counterparts and this is often attributed to carbohydrate (CHO) feeding. Whether this reflects a reduction in fat oxidation, increased de novo lipogenesis (DNL), or both, is not known. To study the effects of high CHO diets on hepatic TG biosynthesis, hepatic TG deuterium (^{2}H) enrichment was determined following 6 days in ^{2}H-enriched tank water for fish fed with a no-CHO control diet (CTRL), and diets with digestible starch (DS) and raw starch (RS). Hepatic fractional synthetic rates (FSRs, percent per day^{-1}) were calculated for hepatic TG-glyceryl and FA moieties through ^{2}H NMR analysis. Glyceryl FSRs exceeded FA FSRs in all cases, indicating active cycling. DS fish did not show increased lipogenic potential compared to CTRL. RS fish had lower glyceryl FSRs compared with the other diets and negligible levels of FA FSRs despite similar hepatic TG levels to CTRL. DS-fed fish showed higher activity for enzymes that can provide NADPH for lipogenesis, relative to CTRL in the case of glucose-6-phosphate dehydrogenase (G6PDH) and relative to RS for both G6PDH and 6-phosphogluconate dehydrogenase. This approach indicated that elevated hepatic adiposity from DS feeding was not attributable to increased DNL.—**Viegas, I., I. Jarak, J. Rito, R. A. Carvalho, I. Metón, M. A. Pardal, I. V. Baanante, and J. G. Jones. **Effects of dietary carbohydrate on hepatic de novo lipogenesis in European seabass (*Dicentrarchus labrax* L.). *J. Lipid Res.* 2016.** 57: **1264–1272.**

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In aquaculture, there is high interest in substituting fishmeal protein with carbohydrate (CHO)-based substrates such as vegetable starch. Procurement of fishmeal protein remains highly dependent on overexploited wild fisheries (1); hence any reduction in its consumption by farmed fish would reduce the ecological burden and improve the sustainability of aquaculture (2). Furthermore, to the extent that dietary CHO replaces protein for systemic glucose and energy demands (3), it decreases waste ammonia generation from protein catabolism, thereby reducing nitrogenous effluents. For carnivorous fish such as the European seabass (*Dicentrarchus labrax* L.), the efficacy of this approach depends on the capacity of the fish to adapt from their natural diet that is high in both protein and fat, but lacking in CHO, to a regime where the proportion of dietary CHO to total caloric content is increased (4). The capacity to digest complex CHO varies widely between different fish species (5). Generally, carnivorous fish are poorly able to digest raw starch (RS). Cooking or gelatinizing the starch significantly improves its digestibility

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Abbreviations: CHO, carbohydrate; CTRL, no-carbohydrate control diet; DNL, de novo lipogenesis; DS, digestible starch; FSR, fractional synthetic rate; G6PDH, glucose-6-phosphate dehydrogenase; ^2H_2O, deuterated water; 6PGDH, 6-phosphogluconate dehydrogenase; RS, raw starch.

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(6, 7), in part through stimulating α-amylase expression and activity (8). Seabass can tolerate a 15–30% substitution of protein by gelatinized starch with preservation of growth rates, nutrient utilization, and apparent digestibility (4). This is accompanied by a significant drop in ammonia production consistent with the reduction in protein catabolism without affecting protein or energy retention and even increasing feed efficiency and digestive utilization (7).

Farmed fish in general (9) and farmed seabass in particular (10, 11) have higher overall lipid levels than their wild counterparts. Dietary CHO supplementation can further augment adiposity (12), which is generally detrimental to flesh quality and market value (13). CHO intake in seabass also increases lipid retention leading to lipid accumulation in the liver and viscera (14). Given that high CHO intake promotes hepatic de novo lipogenesis (DNL) in mammals, it was hypothesized that, for farmed seabass, a diet with a high digestible starch (DS) content would also promote hepatic DNL activity and might, in part, explain the increased adiposity associated with dietary CHO supplementation. To test this hypothesis, we estimated DNL by measuring incorporation of deuterated water (^2H_2O) into hepatic TG using ^2H NMR as previously described for mammals (15–17). For fish metabolic studies, ^2H_2O can be conveniently incorporated into tank water without disturbing feeding and behavior (18, 19). We applied this method to a group of fish fed on a control CHO-free diet and a second group fed a diet supplemented with gelatinized starch. To determine whether starch digestibility had an independent effect on DNL activity, a third group of fish fed a diet supplemented with RS was also studied.

MATERIALS AND METHODS

Fish handling and sampling

European seabass (*D. labrax* L.) provided by Tinamenor (Cantabria, Spain) were transported to the laboratory and randomly assigned to three different tanks (n = 20–32 per tank; initial mean length of 10.8 ± 0.5 cm and initial mean body weight of 21.9 ± 0.3 g). Fish were acclimated at 20°C and 30‰ salinity in 200 l tanks supplied with well-aerated filtered seawater in a recirculation system equipped with a central filtering unit and UV unit. Tank water temperature, salinity, pH, and dissolved oxygen were continuously monitored and NH_4^+, NO_3^-, and NO_2^- were assessed every 7 days and maintained within optimal ranges.

Fish were fed to apparent satiety twice a day (6 days per week) except for the 7 days prior to euthanization when they were fed only once. Three diets were formulated for this experiment (Sparos Lda., Loulé, Portugal; **Table 1**): a no-CHO control diet (CTRL) with no CHO (except for an inert filler of cellulose of no nutritional value to maintain pellet integrity in water); and two experimental diets, one with 33% DS, and another with 33% (RS). All diets were formulated to fulfill the known nutritional requirements of the species. Following the feeding period, each group was transferred to a ^2H_2O-enriched seawater tank for 6 days. This 200 l tank was maintained with an independent closed filtering system, but had similar characteristics as the other tanks used during the rearing phase in terms of size, opacity, filtering material, and water parameters. Seawater was enriched with ^2H_2O to 5% with the addition of 99% enriched ^2H_2O (Eurisotop, France), as previously described by Viegas et al. (19), and ^2H-enrichment was quantified after each set of experiments, as described by Jones, Merritt, and Malloy (20). During this 6 day period in ^2H_2O-enriched saltwater, fish were fed once a day and provided with the last meal 24 h before euthanization. Fish were anesthetized in a 30 l tank of 5% ^2H-enriched saltwater containing 0.1 g l^{-1} of MS-222, measured, weighed, and sampled for blood from the caudal vein with heparinized syringes. Fish were then euthanized by cervical section; livers and samples of skeletal muscle and perivisceral fat were excised, weighed, freeze-clamped in liquid N_2, and stored at −80°C until further analysis. All experimental procedures complied with the Guidelines of the European Union Council (86/609/EU).

Sample preparation and enzyme activities

A portion of liver was stored separately (n = 6 per diet) at −80°C for glycogen quantification, as described by Keppler and Decker (21). Blood glucose, TG, and hepatic TG levels were quantified by using commercial kits (Cromatest; Linear Chemicals, Spain). Glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (6PGDH; EC 1.1.1.43) liver activities were assayed as previously described (22). All enzyme activity assays were carried out at 30°C and followed at 340 nm. Total protein content was determined by the Bradford method (Bio-Rad, Spain) at 30°C in liver crude extracts using BSA as a standard and followed at 600 nm. All assays for metabolites, enzyme activities, and total protein were adapted for automated measurement using a Cobas Mira S spectrophotometric analyzer (Hoffman-La Roche, Switzerland). Enzyme activities were expressed per milligram of soluble protein (specific activity). One unit of enzyme activity was defined as the amount of enzyme necessary to transform 1 μmol of substrate per minute.

Hepatic lipids from the remaining pulverized livers were extracted by the method of Folch, Lees, and Stanley (23). The TG fraction was separated from the rest of the lipids by column chromatography according to the modified procedure described by Hamilton and Comai (24). Briefly, extracted lipids were dissolved in hexane/methyl-*t*-butyl-ether mixture (200:3, v/v) and applied to a silica gel prepacked column (Sigma prepacked 2 g Discovery DSC-Si SPE tubes). The column was eluted with the hexane/methyl-*t*-butyl-ether mixture (200:3, v/v) and the fractions containing TG evaporated to dryness. To determine the identity and the purity of collected lipid fractions, thin-layer chromatography was carried out on silica gel plates. A petroleum ether/diethyl ether/acetic acid (7:1:0.1, v/v/v) system was used as the mobile phase (TG R_f = 0.55). Lipid fractions were visualized by iodine vapors.

^2H NMR analysis

Body water ^2H-enrichments were determined from 10 μl aliquots of fish plasma by ^2H NMR, as described by Jones, Merritt, and Malloy (20). Water content was assumed to be 92% of total plasma. Fully relaxed ^1H NMR spectra of TG samples were obtained at 25°C with a 14.1 T Agilent 600 system equipped with a 3 mm broadband probe. Spectra were acquired with a 90° pulse and 8 s of recycling time (3 s of acquisition time and 5 s pulse delay). Proton-decoupled ^2H NMR spectra were obtained under the same conditions with the observe coil tuned to ^2H. Up to 5,400 scans were collected per sample, corresponding to a maximum of 12 h collection time. TG ^2H-enrichments were quantified from the ^1H and ^2H NMR spectra by measuring the ^1H and ^2H intensities of selected signals relative to the ^1H and ^2H intensities

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TABLE 1. Ingredients and proximate composition of the experimental diets provided to seabass (*D. labrax*)

	CTRL	DS	RS
Ingredients (%)			
Fishmeal[a]	65.5	54.6	54.6
Gelatinized pea starch[b]	—	33.4	—
Raw pea starch[c]	—	—	33.4
Fish oil[d]	9.8	10.5	10.5
Vitamin and mineral premix[e]	1.0	1.0	1.0
Binder (diatomaceous earth)[f]	0.5	0.5	0.5
Cellulose (inert filler)[g]	23.2	—	—
Proximate composition (% dry weight)			
Dry matter	96.0	95.6	97.4
Crude protein	50.2	50.2	50.2
Crude fat	16.1	16.1	16.1
Starch	0.2	17.8	17.8
Ash	11.5	9.3	11.1
Gross energy (kJ g^{-1} dry weight)	22.66	22.03	22.03

[a]Peruvian fishmeal LT: 67% crude protein, 9% crude fat (EXALMAR, Peru).
[b]Aquatex 8071, gelatinized dehulled microground pea meal: 23% crude protein, 50% starch (SOTEXPRO, France).
[c]Aquatex G2000 (bran), crude dehulled microground pea meal: 23% crude protein, 50% starch (SOTEXPRO, France).
[d]Marine oil omega 3 (Henry Lamotte Oils GmbH, Germany).
[e]PVO 40.01 premix for marine fish (PREMIX Lda, Portugal).
[f]Kielseguhr (LIGRANA GmbH, Germany).
[g]Microcrystalline cellulose (Blanver, Brazil).

of a pyrazine standard, as described previously by Duarte et al. (16). From this methodology, the fraction of hepatic TG-bound FAs (or fatty acyls) derived from DNL and the fraction of newly synthesized TG-bound glycerol (or glyceryl) over the ^2H_2O administration were estimated. Excess enrichments were calculated after systematic subtraction of the values with 0.012%, the mean background ^{2}H-enrichment value reported for FAs isolated from salmon muscle (25). If the values were below zero, these were considered as 0.0 for fractional synthetic rate (FSR) calculation purposes. Spectra were processed by applying exponential line broadening (^{1}H, 0.1 Hz; ^{2}H. 1.0 Hz) and analyzed using the curve-fitting routine supplied with ACDLabs 1D NMR processor software 2.4.

Statistical analysis

Data are presented as mean ± SEM. ANOVA was used to test significant differences between the three dietary treatments. A posteriori Tukey's multiple comparisons test was performed when significant differences were found. Differences were considered statistically significant at $P < 0.05$.

RESULTS

The daily growth index, somatic indexes, and biochemical parameters determined are summarized in **Table 2**. CTRL and DS fish had similar growth rates and somatic indexes, while RS fish had significantly lower values for these parameters. The perivisceral fat index was significantly higher for DS compared with RS, but neither group was significantly different from CTRL. Surprisingly, neither DS nor RS feeding resulted in significant increases of glycemia and tissue glycogen levels compared with the CTRL diet. Indeed, plasma glucose levels for RS were significantly lower compared with CTRL, while DS fish had intermediate values that were not significantly different from either RS or CTRL. In contrast to glycemic status, the effect of the diets on plasma and hepatic TG levels were more clear-cut with DS showing a distinctly more lipidic profile compared with the other two diets. Plasma TG levels were significantly elevated in DS compared with both

TABLE 2. Growth and physiological parameters for seabass (*D. labrax*) fed with a CTRL diet, a 30% DS diet, and a 30% RS diet

	CTRL	DS	RS
Growth parameters			
Daily growth index[a]	0.79 ± 0.03^{b}	0.80 ± 0.02^{b}	0.63 ± 0.04^{a}
Hepatosomatic index[b]	1.56 ± 0.03^{b}	1.50 ± 0.04^{b}	1.01 ± 0.05^{a}
Perivisceral fat index[c]	$4.93 \pm 0,24^{ab}$	5.11 ± 0.18^{b}	4.22 ± 0.21^{a}
Plasma			
Glucose (mM)	10.0 ± 1.5^{b}	8.2 ± 0.5^{ab}	6.0 ± 0.7^{a}
TGs (mM)	11.1 ± 1.6^{a}	18.2 ± 1.4^{b}	9.9 ± 1.6^{a}
Liver			
Glycogen (g/100 g^{-1} liver)	9.9 ± 0.3^{b}	11.6 ± 0.6^{b}	4.1 ± 1.1^{a}
TGs (g/100 g^{-1} liver)	11.1 ± 0.2^{a}	35.4 ± 0.7^{b}	8.7 ± 0.1^{a}

Mean values ± SEM are presented. Significant differences between dietary treatments are indicated by different letters (one-way ANOVA followed by Tukey's test).
[a]Daily growth index = [(final bodyweight$^{1/3}$ − initial body weight$^{1/3}$)/days] × 100.
[b]Hepatosomatic index = (liver weight/body weight) × 100.
[c]Perivisceral fat index = (perivisceral fat weight/body weight) × 100.

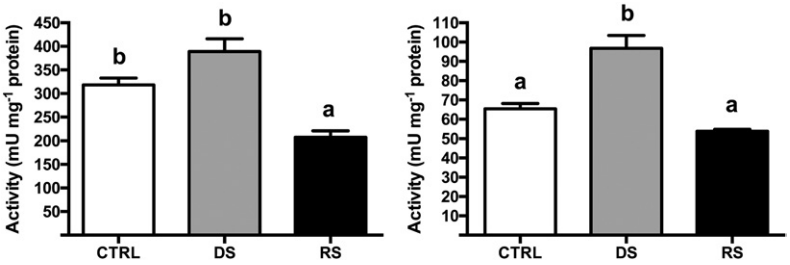

Fig. 1. Specific activity (in mU mg^{-1} protein) of G6PDH (left) and 6PGDH (right) in liver of seabass (*D. labrax*) fed with a CTRL diet, a 33% DS diet, and a 33% RS diet. Mean values ± SEM are presented (n = 6). Significant differences between dietary conditions are indicated by different letters (one-way ANOVA followed by Tukey's test; $P < 0.05$).

RS and CTRL, while hepatic TG was ~3-fold higher for fish fed DS diet compared with both CTRL and RS. Activities of enzymes that can provide NADPH for lipogenesis were affected by the inclusion and type of CHO used (**Fig. 1**). DS-fed fish showed higher activity relative to CTRL in both enzymes, even if in the case of G6PDH, these differences were not statistically significant. RS-fed fish on the other hand, revealed lower activity compared with DS-fed fish for both enzymes.

Following extraction and subsequent isolation from other lipid classes by solid phase extraction, hepatic TG gave well-characterized ^{1}H NMR spectra (**Fig. 2A**, **Table 3**). The FA/glyceryl ratio was consistent and was ~3 in all the diets, as would be expected from a successful TG separation (**Table 4**). Signals from saturated FA and MUFA moieties dominated the spectrum, while contributions from PUFAs were relatively minor, as seen by the resolved PUFA methyl resonance downfield of the main methyl signal. This was also reflected in the composition and structure of FAs calculated by ^{1}H NMR (Table 4). Despite no significant differences observed in saturated FAs and unsaturated FAs between dietary treatments, the percentage of PUFAs increased from CTRL to RS. MUFAs revealed the inverse pattern with DS-fed fish presenting intermediate values in both cases. Consequently, the estimated FA chemical structure varied accordingly in terms of the average number of protons and methylene units.

Because ^{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. The observed ^{2}H NMR signals (Fig. 2B, Table 3) represent the background TG ^{2}H-enrichment of ~0.012% (25) plus excess enrichment from the metabolic incorporation of $^{2}H_2O$ into FAs, and glyceryl hydrogens for TG synthesized and/or elongated during the 6 day period the fish remained in ^{2}H-enriched tank water. Besides the well-resolved multiplet observed by ^{1}H NMR from the four hydrogens of glyceryl positions 1 and 3, no other signals in the 3–5 ppm region of the spectrum were detectable. Therefore, the broad ^{2}H NMR signal centered at 4.2 ppm could be confidently attributed to ^{2}H enrichment of the *sn*-1,3 glyceryl sites. The TG enrichment pattern observed by ^{2}H NMR revealed the activities of de novo synthesis of FA chains from acetyl CoA, chain elongation of existing and newly-synthesized FAs, FA desaturation, and synthesis of the TG glyceryl moiety (16), as shown in **Fig. 3**.

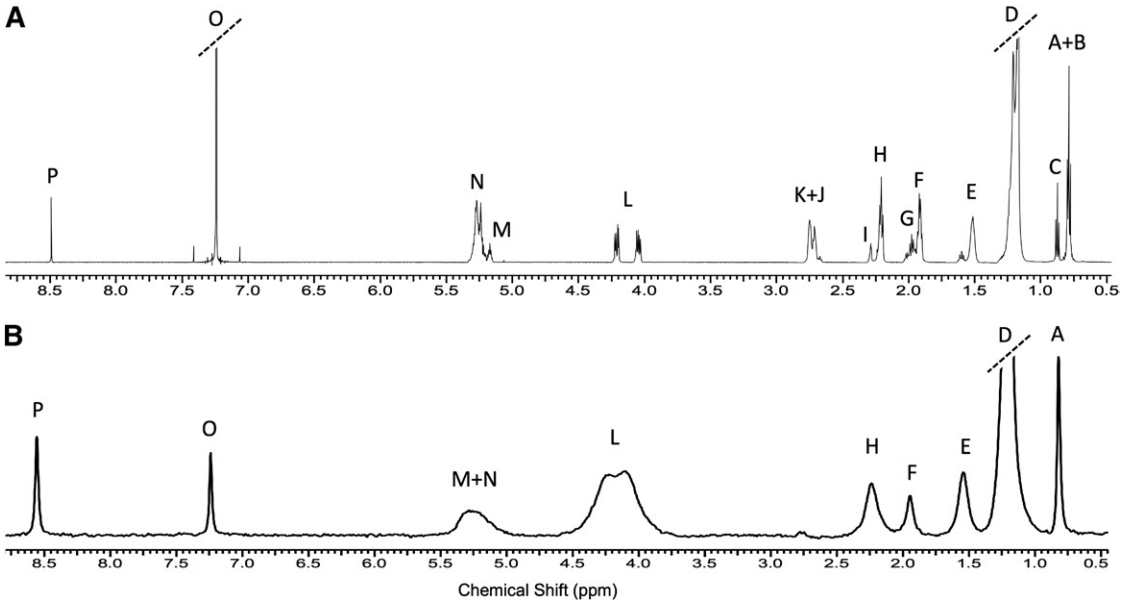

Fig. 2. Representative ^{1}H (A) and ^{2}H (B) NMR spectra of hepatic TG of seabass (*D. labrax*) fed with a CTRL diet, a 33% DS diet, and a 33% RS diet after a 6 day residence in a tank with 5% ^{2}H-enriched water. Letters on peaks indicate: A, non n-3 methyls; B, partial n-6 methyls; C, n-3 methyls; D, aliphatic chain methylenes; E, β methylenes; F, monounsaturated allylic hydrogens; G, polyunsaturated allylic hydrogens; H, α methylenes; I, DHA α and β methylenes; J, linoleic acid bisallylic hydrogens; K, other bisallylic hydrogens; L, *sn*-1,3 of TG-bound glycerol; M, *sn*-2 of TG-bound glycerol; N, olefinic hydrogens; O, chloroform (solvent); P, pyrazine standard. Assignments are listed in detail in Table 4.

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TABLE 3. Functional groups (with NMR signal assignments in bold), chemical shifts, and positional excess of ^{2}H-enrichments in hepatic TG of seabass (*D. labrax*) fed with a CTRL diet, a 30% DS diet, and a 30% RS diet after a 6 day residence in a tank with 5% ^{2}H-enriched water

Signal	Functional Group	Chemical Shift (ppm)	Assignment	^{2}H-enrichments		
				CTRL	DS	RS
A+B	Non n-3 + partial n-6 methyls	0.80	C**H$_3$**-CH$_2$-	0.043 ± 0.010^b	0.030 ± 0.007^b	−0.004 ± 0.004^a
C	n-3 Methyls	0.90	C**H$_3$**-CH$_2$-CH=	—	—	—
D	Aliphatic chain methylenes	1.20	CH$_3$-(C**H$_2$**)$_n$-	0.050 ± 0.011^b	0.023 ± 0.005^{ab}	−0.001 ± 0.002^a
E	β Methylenes	1.50	-C**H$_2$**-CH$_2$-COO-	0.093 ± 0.013^b	0.033 ± 0.003^a	0.008 ± 0.006^a
F	MU allylic hydrogens	1.90	-C**H$_2$**-CH=CH-	0.023 ± 0.006	0.030 ± 0.006	0.009 ± 0.008
G	PU allylic hydrogens	2.00	-C**H$_2$**-CH=CH-	—	—	—
H	α Methylenes	2.20	-CH$_2$-C**H$_2$**-COO-	0.094 ± 0.014^b	0.032 ± 0.005^a	0.010 ± 0.011^a
I	DHA α and β methylenes	2.30	-C**H$_2$**-C**H$_2$**-COO-	—	—	—
J+K	Bisallylic methylenes	2.70	-CH=CH-C**H$_2$**-CH=CH-	—	—	—
L	*sn*-1, *sn*-3 of TG-glycerol	4.15	HOC**H$_2$**-CHOH-C**H$_2$**OH	0.661 ± 0.037^b	0.312 ± 0.020^b	0.306 ± 0.057^b
M	*sn*-2 of TG-glycerol	5.15	HOCH$_2$-C**H**OH-CH$_2$OH	—	—	—
N	Olefinic hydrogens	5.25	-C**H**=C**H**-	—	—	—
O	Chloroform	7.25	Solvent	—	—	—
P	Pyrazine	8.60	Standard	—	—	—

Mean values ± SEM are presented (n = 6). Significant differences between dietary treatments are indicated by different letters (one-way ANOVA followed by Tukey's test; $P < 0.05$). Enrichments adjusted for tank water at 5.0% ^{2}H$_2$O. MU, monounsaturated; PU: polyunsaturated.

Analysis of the ^{2}H NMR signals corresponding to *sn*-1,3 glyceryl enrichment (L in Figs. 2, 3 and Table 3) indicated a 7- to 30-fold higher incorporation of ^{2}H in comparison to the FA enrichment, as indicated by the terminal methyl site (A in Fig. 3, Table 3). This translated into much higher glyceryl FSR rates (**Fig. 4**) compared with those of FAs (**Fig. 5**). Glyceryl enrichments and FSRs varied according to diet, with CTRL fish showing significantly higher glyceryl enrichments and FSRs compared with both DS and RS. Significant differences in TG FA enrichment distributions were also found between the three diets. RS fish had essentially no excess enrichment of FA hydrogens (Table 3), translating to negligible FA FSRs (Fig. 5). Both CTRL and DS groups had significantly higher TG FA chain enrichments reflecting measurable DNL contributions to hepatic TG FAs. Enrichments of the FA methylenes nearest to the carboxyl terminal (H and E in Fig. 3, Table 3) were significantly higher in CTRL fish compared with the other two diets. Because these sites are enriched by both de novo synthesis and chain elongation, whereas the terminal methyl site is only enriched by de novo synthesis, differences in enrichment between either E or H sites and that of site A indicated chain elongation (Fig. 3) (16). The fraction of FAs that underwent elongation was pronouncedly higher in CTRL fish compared with the other two groups (Fig. 5), but the average number of FA carbons was not significantly changed. Differences in the incorporation of ^{2}H into the monounsaturated allylic hydrogens (F in Figs. 2, 3 and Table 3) are an indication of FA desaturation activity. This had a tendency to be lower than average in RS-fed fish compared with the other two groups, but with no statistical relevance (**Fig. 6**).

DISCUSSION

The effect of CHO feeding on metabolic regulation has been far less studied in fish compared with humans and mammalian animal models, namely rodents. The paradigms developed from mammalian studies have only been

TABLE 4. Percentage of lipid species and chemical structure as determined from ^{1}H NMR spectra of hepatic TG of seabass (*D. labrax*) fed with a CTRL diet, a 30% DS diet, and a 30% RS diet

	CTRL	DS	RS
Lipid species (%)			
Non n-3	82.0 ± 2.0	78.6 ± 1.8	80,9 ± 6.9
SFA	26.1 ± 1.5	25.7 ± 2.5	23.8 ± 0.6
UFA	73.9 ± 1.5	74.3 ± 2.5	76.2 ± 0.6
PUFA	27.4 ± 1.7^a	33.9 ± 2.0^b	41.2 ± 0.6^c
MUFA	46.5 ± 0.9^c	40.8 ± 0.8^b	31.8 ± 1.0^a
Chemical structure			
ANC	18.8 ± 0.4	18.5 ± 0.4	17.1 ± 1.3
ANP	31.3 ± 0.5^b	29.9 ± 0.4^{ab}	28.1 ± 1.0^a
Olefinic (HC=CH)	2.6 ± 0.3	3.0 ± 0.3	2.6 ± 0.9
Methylenic (CH_2)	11.5 ± 0.3^b	10.1 ± 0.2^a	9.9 ± 0.4^a
FA/glyceryl	2.9 ± 0.1	2.8 ± 0.1	2.7 ± 0.0
AMW	289.0 ± 5.6	283.8 ± 5.4	265.9 ± 16.9

Mean values ± SEM are presented (n = 6). Significant differences between dietary treatments are indicated by different letters (one-way ANOVA followed by Tukey's test; $P < 0.05$). Chemical structure: FAs were considered polymers of olefinic (HC=CH) and methylenic (CH_2) subunits [i.e., $^-OOC-(CH_2)_x-(HC=CH)_y-CH_3$] calculated as in Duarte et al. (16) except for the olefinic hydrogens that were calculated directly as equal to the correspondent area in the spectra. ANC, average number of carbons; ANP, average number of protons; AMW, average molecular weight. SFA, saturated FA; UFA, unsaturated FA.

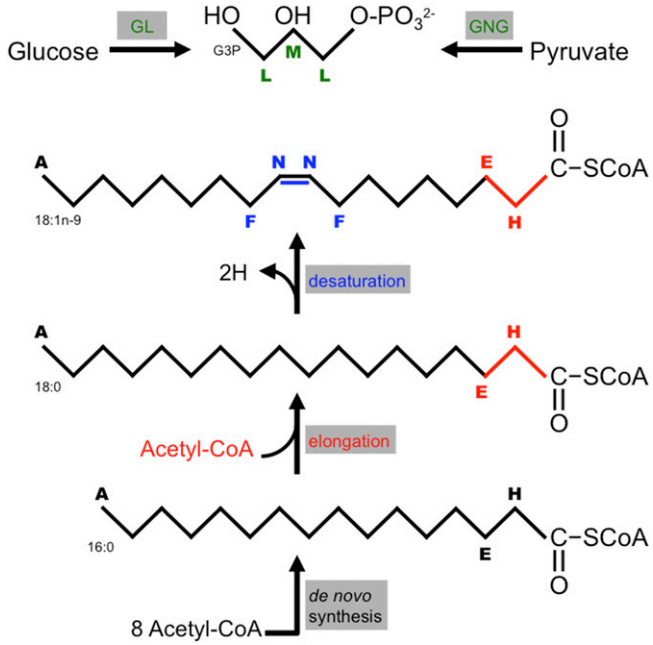

Fig. 3. Summary of the component pathways of TG biosynthesis and relationship with the ^{2}H NMR signals from $^{2}H_2O$ incorporation shown in Fig. 2. For the fatty acyl-CoA precursors, these include de novo synthesis of palmitoyl-CoA (16:0) from acetyl-CoA; elongation to stearoyl-CoA (18:0), and desaturation to the monounsaturated oleoyl-CoA (18:1n-9). For simplicity, the synthesis, chain elongation, and desaturation of other less common fatty acyl-CoA precursors (i.e., myristoyl-CoA, palmitoleoyl-CoA, etc.) are not shown. Also, FA elongation and desaturation can involve unlabeled dietary or endogenous preexisting FAs, as well as the newly synthesized labeled species. The glycerol-3-phosphate (G3P) precursor can be synthesized from glucose via glycolysis (GL) and from pyruvate via glyceroneogenesis (GNG). In the presence of $^{2}H_2O$, both pathways result in enrichment of the glyceryl *sn*-1- and *sn*-3-methylene hydrogens, as well as the carbon 2 hydrogen. The TG positions corresponding to the ^{2}H NMR signals shown in Fig. 2 are indicated by the bold lettering (**A**, **E**, **F**, **H**, **L**, **M**, **N**). For simplicity, the remaining FA aliphatic chain methylenes (D in Fig. 2) are not indicated.

partially successful in explaining the metabolic response to CHO supplementation of fish feeds, particularly for carnivorous fish (26, 27). This has been evolving, in part, by the development of more effective gene expression methods for assessing transcriptional regulation, as well as novel tracer methodologies for identifying carbon fluxes through the pathways that interconnect CHO and lipid metabolism. The fishfeed industry shifts their attention to the use of different types of CHO, especially in balance with vegetable oils (2) to spare the use of fishmeal. In mammals, high CHO feeding results in a significant upregulation of DNL activity and fractional contribution to hepatic and systemic TG (28, 29). Thus, there is high interest in determining the metabolic basis of lipid accumulation in fish, either by enhanced DNL and/or by restricted FA oxidation.

The present study indicates that, quantitatively, DNL fluxes did not explain the 3-fold increase in hepatic TG levels of CHO-fed fish, regardless of CHO type (raw or gelatinized), compared with fish fed with CHO-free diets.

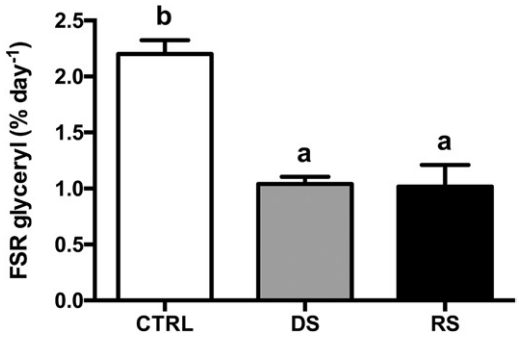

Fig. 4. TG-bound glycerol FSR expressed as percent of newly synthesized glyceryl per day in hepatic TG of seabass (*D. labrax*) fed with a CTRL diet, a 33% DS diet, and a 33% RS diet after a 6 day residence in a tank with 5% ^{2}H-enriched water. Mean values ± SEM are presented (n = 6). Significant differences between dietary treatments are indicated by different letters (one-way ANOVA followed by Tukey's test; $P < 0.05$).

Overall, the data obtained from studies in fish species have been equivocal on the extent of DNL stimulation in this setting. In rainbow trout (*Oncorhynchus mykiss*), one of the most intensely studied fish species, high CHO intake, either by diet (30, 31) or by bolus injection (32), consistently failed to increase expression of hepatic DNL enzymes; whereas high levels of dietary protein were found to be effective (33). On the other hand, selective breeding studies have revealed some degree of plasticity in hepatic CHO assimilation for this species. In two divergent breeding lines selected for lean or fat muscle, the fat muscle phenotype demonstrated higher expression of hepatic DNL enzymes and higher plasma TG levels, as well as increased hepatic glycogen (34). It is worth noting that line selection, a process that has taken centuries for terrestrial farmed animals, is just taking its first steps in fish farming. The influence of dietary CHO on DNL in seabass has been, in most cases, derived from combining analyses of FA profiles in different tissues (6, 13, 35, 36) with determination of the activities and/or expression levels of metabolic pathway enzymes directly involved in DNL as well as NADPH generation (6, 12, 36–42). Recently, these

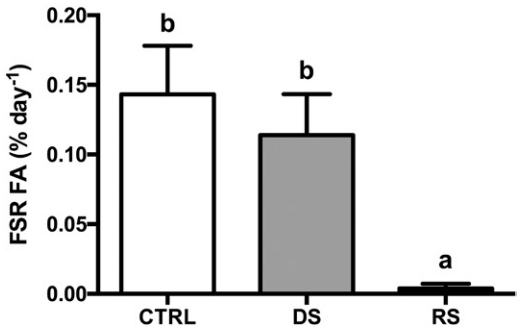

Fig. 5. FA FSR expressed as percent of newly synthesized FAs from DNL per day in hepatic TG of seabass (*D. labrax*) fed with a CTRL diet, a 33% DS diet, and a 33% RS diet after a 6 day residence in a tank with 5% ^{2}H-enriched water. Mean values ± SEM are presented (n = 6). Significant differences between dietary conditions are indicated by different letters (one-way ANOVA followed by Tukey''s test; $P < 0.05$).

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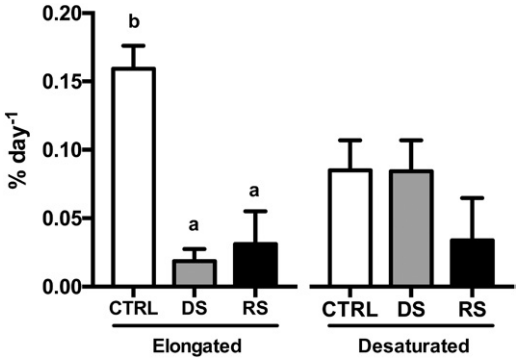

Fig. 6. Fractional elongation and desaturation rate of TG-bound FA expressed as percent of FAs per day in hepatic TG of seabass (*D. labrax*) fed with a CTRL diet, a 33% DS diet, and a 33% diet after a 6 day residence in a tank with 5% ^{2}H-enriched water. Mean values ± SEM are presented (n = 6). Significant differences between dietary conditions are indicated by different letters (one-way ANOVA followed by Tukey's test; $P < 0.05$).

approaches have been complemented with analyses at the transcriptional level (14) and revealed no regulation of PUFA biosynthesis with increased CHO. In seabass fed a high CHO/low fat diet, activities of ATP-citrate lyase and FAS were significantly higher compared with fish fed low CHO/high fat (6), but acetyl-CoA carboxylase activity showed opposite changes to the other DNL enzymes. Lipid retention in seabass is significantly enhanced by dietary CHO (6, 14, 43), significantly increasing the hepatic and muscular lipid content. This lipid deposition may negatively feedback on the activity of hepatic enzymes like FAS (6, 14). In the Senegalese sole, *Solea senegalensis*, a species that has a very low tolerance for dietary lipid and limited tissue adiposity (44), supplementation of a low fat diet with CHO paradoxically enhanced FAS activity (45, 46), while not being affected by type of starch (46). Because DNL is directly dependent on NADPH, the rate of synthesis of this cofactor via the committing oxidative pentose phosphate pathway enzyme, G6PDH, can also, in principle, exert a strong degree of control on DNL activity. A positive correlation between dietary CHO level and G6PDH activity has been reported for seabass (6, 12, 14, 40) as well as the gilthead seabream (*Sparus aurata*) (22, 43, 47). Others report that increased levels of CHO failed to stimulate G6PDH activity in seabass (48, 49). In blackspot seabream (*Pagellus bogaraveo*), starch supplementation had little effect on glycolytic and lipogenic enzyme activities, while a reduction in dietary protein markedly depressed both G6PDH and FAS activities (50), suggesting that dietary protein was more important in DNL regulation compared with CHO. In summary, many, but not all, studies have found an association between high CHO feeding and increased expression and/or activities of some DNL-related enzymes in carnivorous farmed fish. However, to our knowledge, none have demonstrated the coherent upregulation of all enzymes involved in DNL and NADPH generation, presumably a fundamental requirement for increased DNL fluxes.

Tracer methods provide more direct measurements of lipogenic fluxes (51–57), but due to methodological and cost constraints, are still not widely used for fish studies. $^{2}H_2O$ overcomes the difficulties of performing conventional intravenous infusions and the stressful impact of forced-fed diets. It has been applied so far to study glucose and glycogen synthesis in seabass (58, 59), alanine metabolism in gilthead seabream (60), and muscle protein synthesis in catfish (*Ictalurus punctatus*) (18). In the present study, FA synthesis was affected by the type of starch (raw vs. gelatinized), as already demonstrated in rainbow trout force-fed with ^{13}C-labeled starch (53). However, data from this and other tracer studies in fish suggest a preference for protein over CHO as the carbon source for lipid synthesis. In common carp (*Cyprinus carpio*), labeled glutamate was more effectively incorporated into lipids compared with labeled glucose (57). In gilthead seabream administered with a diet containing ^{13}C-enriched protein, analysis of endogenous lipid ^{13}C-enrichment levels indicated that dietary protein was a major contributor of lipid synthesis. Moreover, this was unaffected when the level of dietary starch was increased (51). Consistent with the poor lipogenic utilization of CHO by fish, studies using ^{14}C-glucose showed low amounts of ^{14}C recovered in liver lipids, suggesting very low de novo synthesis of hepatic lipid from glucose in carp (57), Atlantic salmon (*Salmo salar*) (55), and Atlantic cod (*Gadus morrhua*) (54). With the exception of the latter, labeling of lipid FA chains and glyceryl moieties were not resolved, hence it was not established to what extent the lipids were labeled via DNL or via glucose conversion to glycerol-3-phosphate and incorporation into TG. In Atlantic cod injected with [U-^{14}C]glucose, it was found that the majority of ^{14}C-label in the hepatic lipid fraction was present in TG, while free FAs had much less ^{14}C-labeling (54), suggesting that the lipids were labeled largely via ^{14}C-glucose to ^{14}C-glycerol-3-phosphate conversion followed by esterification into TG. In a study of carp injected with [U-^{14}C]glucose, the extent of labeling of hepatic neutral lipids was assessed by analysis of enzymatically liberated glycerol, but labeling of the FA moieties was not analyzed (57). The present study clearly indicates that the fractional rate of hepatic TG-glyceryl synthesis was far in excess of TG-FA synthesis, a process that is explained by futile cycling between TG and FAs that requires de novo synthesis of glycerol-3-phosphate per turn of the cycle. To the extent that the glycerol-3-phosphate used for esterification is derived from glucose, labeled glucose tracers could result in TG labeling independently of DNL activity. TG-FA cycling is well described in fasting mammals where whole-body TG lipolysis rates are far in excess of FA oxidation, with the majority of liberated FA re-esterified by the liver at a later stage. Although studies have been far fewer, there is evidence of similar activity in fish. In resting as well as exercising rainbow trout, comparison of glycerol and FA appearance rates using tracer methods indicated that more than two-thirds of FAs released during lipolysis underwent TG-FA cycling (56, 61).

In conclusion, it was demonstrated that ^{2}H NMR analysis of tissue lipid fractions of fish that were placed in ^{2}H-enriched tank water provided information on DNL, FA elongation and desaturation, and TG-FA futile cycling.

Also, supplementation of a fishmeal-based diet with RS or DS significantly altered the activity of enzymes that can provide NADPH for lipogenesis with each diet yielding a unique profile of lipid biosynthesis. This approach revealed that elevated hepatic adiposity from DS feeding was not attributable to increased DNL. This approach can usefully complement lipidomic and gene-expression studies of tissue lipid origins and modifications in fish.

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