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Tesi Doctoral

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Tracing metabolic routes of dietary carbohydrate and protein in rainbow trout (*Oncorhynchus mykiss*) using stable isotopes ([^{13}C]starch and [^{15}N]protein): effects of gelatinisation of starches and sustained swimming

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Abstract

Here we examined the use of stable isotopes, [^{13}C]starch and [^{15}N]protein, as dietary tracers to study carbohydrate assimilation and distribution and protein utilisation, respectively, by rainbow trout (*Oncorhynchus mykiss*). The capacity of glucose uptake and use by tissues was studied, first, by varying the digestibility of carbohydrate-rich diets (30 % carbohydrate), using raw starch and gelatinised starch (GS) and, second, by observing the effects of two regimens of activity (voluntary swimming, control; sustained swimming at 1·3 body lengths/s, exercise) on the GS diet. Isotopic ratio enrichment (^{13}C and ^{15}N) of the various tissue components (protein, lipid and glycogen) was measured in the liver, muscles, viscera and the rest of the fish at 11 and 24 h after a forced meal. A level of 30 % of digestible carbohydrates in the food exceeded the capacity of rainbow trout to use this nutrient, causing long-lasting hyperglycaemia that raises glucose uptake by tissues, and the synthesis of glycogen and lipid in liver. Total ^{13}C recovered 24 h post-feeding in the GS group was lower than at 11 h, indicating a proportional increase in glucose oxidation, although the deposition of lipids in white muscle (WM) increased. Prolonged hyperglycaemia was prevented by exercise, since sustained swimming enhances the use of dietary carbohydrates, mainly through conversion to lipids in liver and oxidation in muscles, especially in red muscle (RM). Higher recoveries of total ^{15}N for exercised fish at 24 h, mainly into the protein fraction of both RM and WM, provide evidence that sustained swimming improves protein deposition, resulting in an enhancement of the protein-sparing effect.

Key words: δ^{13}C and δ^{15}N: Carbohydrate: Protein: Sustained swimming

The capacity of rainbow trout (*Oncorhynchus mykiss*) to use dietary carbohydrates is controversial[1–3], although several authors have claimed that diets with a high content of digestible starch (20–30 %) can stimulate growth[4,5]. However, while a protein-sparing effect from lipids has been reported in this species[6–8], there is no clear evidence that carbohydrates also have a protein-sparing effect. The replacement of fish-meal by plant ingredients is a common practice in aquaculture, even in carnivorous fish. Since plant sources contain large amounts of carbohydrates, the use of this alternative energy source is of interest. The physical state of the animal, the molecular complexity and the amount of starch in the diet influence carbohydrate digestibility and tolerance, and also the efficiency of fish growth. If the protein content of the diet is adequate, low levels of gelatinised starches promote growth in carnivorous fish, such as European eel (*Anguilla anguilla*)[9], cod (*Gadus morhua*)[10], sturgeon (*Acipenser transmontanus*)[11], Atlantic salmon (*Salmo salar*)[12,13] and turbot (*Scophthalmus maximus*)[14]. As we have shown in brown trout, uptake and use of glucose by tissues depends on plasma glucose concentration[15] and after an aortic glucose overload, almost all tissues increase glucose uptake. This is particularly true for skeletal muscle, which is the main target of the glucose load[16]. This effect may also be exerted by high levels of dietary carbohydrates, although, to our knowledge, this has not been measured experimentally.

Fish generally swim aerobically at submaximal velocities[17–19]. Several fish species, when made to swim at about 1·3 body lengths/s (BL/s), show improved growth rate and food conversion efficiency[20] through the increase of aerobic potential of red muscle (RM) and white muscle (WM). There is no general agreement on how metabolic fuels support aerobic

Abbreviations: at %, atom percentage; BL/s, body lengths/s; GS, gelatinised starch; RM, red muscle; RS, raw starch; WM, white muscle.

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swimming in fish[21]. Protein and lipids were traditionally believed to be the main source of energy during sustained swimming in teleost fish, and carbohydrate utilisation was considered to be minimal[22–26]. However, Alsop & Wood[27] found that in satiation-fed rainbow trout, protein did not become more important as a fuel source during exercise. Further, Lauff & Wood[21], using respirometric analyses, demonstrated that the most oxidised substrates during moderate swimming (55–85% U_{crit}; critical swimming speed) were lipids, followed by carbohydrates, and then protein. Kieffer *et al.*[28] also found in rainbow trout that during swimming at 1 or 3 BL/s, protein use decreased to 15% while the relative contribution of both lipid and carbohydrates increased. On the other hand, Shanghavi & Weber[29] noted that sustained swimming for a period of 3 h causes a 33% decline in hepatic glucose production, but plasma glucose levels are maintained stable by closely matching peripheral glucose utilisation. However, glucose disposal can also be conditioned by the source or type of diet, carbohydrate content, feeding regimen, gelatinisation process, etc. (reviewed by Hemre *et al.*[2]). Traditionally, studies on fish metabolism have used radioactive isotopes, but labelling feed ingredients with radioactive markers can be harmful to users and the aquatic environment. Stable isotopes are now used to study protein metabolism in fish. Thus, ^{15}N has been administered orally to measure protein synthesis in species such as rainbow trout (*O. mykiss*)[30], flounder (*Pleuronectes flesus*)[31] or carp (*Cyprinus carpio*)[32]. In the present study, we used stable isotopes as dietary tracers ([^{13}C]starch and [^{15}N]protein), as a preferred method for tracing dietary nutrient allocation in fish[33], with the following two aims: (1) to measure the effects of gelatinisation of starch on [^{13}C]glucose uptake and use by tissues in rainbow trout fed a carbohydrate-rich diet; (2) to determine the effects of sustained swimming on the efficiency of carbohydrate use in this species. To our knowledge, there are no studies on routing both carbohydrates and protein from [^{13}C]starch and [^{15}N]protein added to the diet. This alternative method has allowed us to show improved assimilation and distribution of dietary carbohydrates, and their protein-sparing effect in rainbow trout under sustained swimming.

Experimental methods

Experimental design and sampling

Expt 1: effects of raw and gelatinised starch on glucose uptake by tissues of rainbow trout fed with carbohydrate-rich diets. Rainbow trout from a local fish farm (Truchas del Segre, Lleida, Spain) were held in the facilities of the Faculty of Biology (University of Barcelona, Barcelona, Spain) in 1000 litre tanks with fresh water within a semi-closed system (10% of water renovation daily) with physical and biological filters, ozone skimmers and continuous aeration at 15°C and a 12 h light–12 h dark photoperiod. Fish with an average body weight of 180 g were randomly distributed into two experimental groups (twenty-five fish/tank), which were fed with two experimental diets with a high level of raw starch (RS) or gelatinised starch (GS) (see diet compositions given in Table 1). After 1 month, fourteen fish from each group were lightly anaesthetised and then force-fed a bolus, equivalent to 1% of body weight, with a gastric cannula. Fish were held in separated tanks for only a few minutes to check the acceptance of the forced meal. Any fish showing some degree of regurgitation was disqualified, and another one was used in its place. Fish were returned to their respective tanks and maintained for 11 or 24 h post-feeding. These two periods were chosen as they represent the post-absorption maximum (11 h) and nutrient use completion (24 h) time points. Diets were labelled with 3% [^{13}C]starch ([^{13}C]algal starch; Martek Biosciences Corporation, Columbia, MD, USA). Another four animals from each group received the same dietary ration containing non-labelled starch, and they were used to measure the background level of ^{13}C, to establish the natural abundance (i.e. blank value of each sample). At 11 h after the oral administration of the diets, half of the fish (seven fish fed the diets labelled with stable isotope plus two fish as blanks) were anaesthetised, killed by sectioning the spinal cord and sampled. Blood samples from the caudal vessel were centrifuged (12 000 ***g***, 5 min at 4°C) to obtain plasma. Portions of the liver, and WM and RM and viscera (gut plus perivisceral fat) were excised, frozen in liquid N_2 and stored at −80°C until analysis, as were the rest of the fish and plasma samples. The entire sampling procedure took less than 3 min from the death of the fish, and the tissues with high glycogen hydrolytic capacity, such as muscle, were frozen first. After 24 h of forced-feeding, the remaining animals were sampled using the same protocol.

Table 1. Ingredients and chemical composition of the experimental diets

	RS	GS
Ingredients (% DM)		
Fishmeal CP70	59·10	59·10
CPSP G	5·00	5·00
Crude wheat starch	30·00	0·00
Gelatinised wheat starch	0·00	30·00
Fish oil	2·90	2·90
Binder	1·00	1·00
Mineral premix*	1·00	1·00
Vitamin premix†	1·00	1·00
Proximate composition		
Crude protein (% DM)	44·39	44·39
Crude fat (% DM)	10·00	10·00
Digestible protein (% DM)	39·16	39·16
Digestible energy (kJ/g DM)	14·67	16·82
P (% DM)	1·77	1·77

RS, raw starch; GS, gelatinised starch; CPSP G, fish soluble concentrate protein with high-fat level.

* Supplied the following (mg/kg diet, except as noted): calcium carbonate (40% Ca) 2·15 g, magnesium hydroxide (60% Mg) 1·24 g, potassium chloride 0·9 g, ferric citrate 0·2 g, potassium iodide 4 mg, NaCl 0·4 g, calcium hydrogen phosphate 50 g, copper sulphate 0·3, zinc sulphate 40, cobalt sulphate 2, manganese sulphate 30, sodium selenite 0·3.

† Supplied the following (mg/kg diet): retinyl acetate 2·58, DL-cholecalciferol 0·037, DL-α-tocopheryl acetate 30, menadione sodium bisulphite 2·5, thiamin 7·5, riboflavin 15, pyridoxine 7·5, nicotinic acid 87·5, folic acid 2·5, calcium pantothenate 2·5, vitamin B_{12} 0·025, ascorbic acid 250, inositol 500, biotin 1·25, choline chloride 500.

Expt 2: effects of sustained swimming on the use of carbohydrates by rainbow trout. Juvenile rainbow trout (with an average weight of 60 g) from the same fish farm were acclimatised indoors as in the previous experiment. For individual monitoring, sixty fish were identified with a passive integrated transponder (PIT) tag (Trovan Electronic Identification Systems, Madrid, Spain) near the dorsal fin, and were randomly distributed into four 200 litre circular tanks (fifteen fish/tank) at a density of 4 kg/m^3. Of these four tanks, two were kept on standard rearing conditions, with a water flow of 350 litres/h and vertical water inflow. Fish in these conditions presented only spontaneous movements (voluntary swimming) and were used as the control group. The other two tanks (exercise group) were kept in a circular, uniformly distributed flow of 700 litres/h, induced by the perpendicular water entrance at the surface and a submerged water pump at the bottom of the tank, isolated from the free-living area. The shape of the tank prevented the fish from entering a central area of lower velocity, thus guaranteeing similar swimming velocities throughout the experiment. Consequently, water volume and fish density were the same as in the control group. This design and water flow resulted in a swimming velocity of 1·3 BL/s, measured and adjusted at three different tank depths (surface, mid-tank and near the bottom) using a low-speed mechanical flow meter (General Oceanics, Inc., Miami, FL, USA). All fish were kept in the same semi-closed circuit, guaranteeing that physico-chemical water parameters were the same for both groups, and they were fed twice a day to apparent satiety with the diet rich in digestible carbohydrates (GS) for 1 month (see diet composition given in Table 1). Feed intake was recorded daily for each tank and the specific growth rate ($100 \times$ (ln final weight − ln initial weight)/d) and food conversion ratio (feed intake:wet bodyweight gain) were also calculated for each tank at the end of the experimental period. After 1 month, eighteen fish from the exercise group and twelve from the control group were lightly anaesthetised and force-fed with a gastric cannula a ration of 1 % of diet labelled with 1 % [^{15}N]*Spirulina* protein and 3 % [^{13}C]algal starch. From each group, two other fish received the same dietary ration containing similar proportions of non-labelled *Spirulina* protein and algal starch. These four fish were used to measure natural abundances of ^{15}N and ^{13}C in samples (blank values). After force-feeding, fish were held for a few minutes in separate tanks as indicated for the first experiment. Then, fish were returned to their respective tanks and maintained for 11 or 24 h post-feeding (exercise group swam until the moment they were sampled). At 11 h after feeding, nine fish from the sustained swimming group and six from the control group were anaesthetised and killed by sectioning the spinal cord. Samples of blood were extracted from caudal vessels, and then, samples of liver, WM and RM, viscera (gut plus perivisceral fat) and the rest of the fish were rapidly excised, frozen in liquid N_2 and stored at −80°C until analysis. As in the first experiment, the entire sampling procedure took less than 3 min from the death of the fish, and the tissues with high glycogen hydrolytic capacity, such as muscle, were frozen first. The same procedure was repeated at 24 h post-feeding with the other nine and six fish from the sustained swimming and control groups, respectively. Although the initial body weight of rainbow trout differed between the two experiments, all fish can be considered as juvenile fish in a linear phase of growth. For the exercise trial, we used fish of 60 g constrained by the size and number of the tanks available for implementing sustained swimming.

Before conducting the animal trials, prior approval of the Comitè Ètic d'Experimentació Animal de la Universitat de Barcelona (CEEA-UB, Ethics Committee) was obtained. The specific ethics approval number for the protocol was CEEA-96/09.

Plasma analysis and proximal composition of tissue samples

Plasma was used to determine glucose concentration (Commercial Kit Glucofix, Menarini, Italy) based on the enzymatic method of glucose oxidase described by Werner *et al.*[34]. Tissue samples (liver, muscles and viscera) were homogenised in liquid N_2 using a pestle and mortar to obtain a fine powder. The rest of the fish was homogenised at −20°C using a food homogeniser (Pacojet AG, Zug, Switzerland). Samples were apportioned for the various analyses: percentage of lipids, proteins, glycogen and water determination, and one part of the sample was used for isotopic analysis. Tissue water content was determined gravimetrically after drying the samples at 100°C for more than 24 h. Lipids were extracted as described by Folch *et al.*[35]. The washed lipid extracts were dried under a N_2 atmosphere and the lipid content was determined gravimetrically. Protein was purified from defatted samples via precipitation with 10 % (v/v) trifluoroacetic acid. Protein extracts were dried by a vacuum system (Speed Vac Plus, AR, Savant Speed Vac Systems, South San Francisco, CA, USA) and protein content was calculated from N obtained by elemental analysis (Elemental Analyser Flash 1112, ThermoFinnigan, Bremen, Germany), assuming that N content is 1 g for every 6·25 g of protein. Glycogen was extracted and purified by alcoholic precipitation after alkaline tissue hydrolysis with 30 % KOH in heat[36]. Glycogen content was analysed using the anthrone colorimetric method described by Fraga[37].

δ^{15}N and δ^{13}C determination in tissues and expression of results

The enrichments in ^{13}C were determined in both experiments, and the enrichments in ^{15}N were measured in the second experiment. Dried samples of diets and tissues, as well as the purified lipid, glycogen and protein fractions of each tissue, were lyophilised and ground in a mortar to a homogeneous powder for isotope-ratio mass spectrometry analysis. Aliquots ranging from 0·3 to 0·6 mg were accurately weighed in small tin capsules (3·3 × 5 mm; Cromlab, Barcelona, Spain). Samples were analysed for C and N isotope composition using a Mat Delta C isotope-ratio mass spectrometry (Finnigan MAT, Bremen, Germany) coupled to an elemental analyser (Flash 1112) at Barcelona University 'Serveis Cientifico-Tècnics'. Isotope ratios (^{15}N/^{14}N, ^{13}C/^{12}C) are expressed

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on a relative scale as deviation, referred as δ units with the notation ‰, parts per thousand, relative to the isotope ratio content of international standards: Pee Dee Belemnite (a calcium carbonate) for C and air for N.

δ values were determined as follows:

$$\delta = ((R_{sa}/R_{st}) - 1) \times 1000,$$

where $R_{sa} = {}^{15}N/{}^{14}N$ or ${}^{13}C/{}^{12}C$ of samples and $R_{st} = {}^{15}N/{}^{14}N$ or ${}^{13}C/{}^{12}C$ of standards. The same reference material analysed over the analysis period was measured with about 0·2‰ precision for natural materials and about 0·4‰ precision for enriched materials. The δ values are expressed as atom percentage (at%) as follows:

$$^{13}C\,at\,\% = 100 \times ({}^{13}C/({}^{13}C + {}^{12}C)),$$
$$^{15}N\,at\,\% = 100 \times ({}^{15}N/({}^{15}N + {}^{14}N)).$$

The net enrichments (atom percentage excess) in ^{13}C and ^{15}N of glycogen, lipid, protein and whole tissue were calculated by the difference between the atom percentage of samples and their corresponding blank values:

$$\text{Atom percentage excess} = \text{at\% sample} - \text{at\% blank}.$$

Finally, using the values of atom percentage excess, molecular weight and Avogadro's number, the results are expressed as the percentage of marker in relation to the ingested dose (g/100 g ^{13}C or ^{15}N ingested) in each tissue fraction (glycogen, lipid and protein), which was calculated as follows:

$$100 \times ((\text{g } {}^{13}C \text{ or } {}^{15}N/\text{g t. fr.}) \times (\text{g t. fr./g tissue})$$
$$\times (\text{g tissue/g b.w.})/(\text{g ingested } {}^{13}C \text{ or } {}^{15}N/\text{g b.w.})) \quad (1)$$

where t. fr. is the tissue fraction and b.w. is the body weight. The free pool of each tissue was calculated as the difference between isotope levels in the entire organ or tissue and the sum of the three tissue fractions. So, the measure in an entire organ, or tissue, represents the sum of all fractions (Eq. (1) + free pool) for ^{13}C or ^{15}N. In whole fish, it was calculated as the sum of all tissues (liver, WM and RM, viscera and the rest of the fish) for ^{13}C or ^{15}N.

For liver and viscera, the exact mass of the total tissue sample was measured by weighing the entire individual organs from the experimental fish. However, in order to estimate the total mass of WM and RM, we made accurate dissections of another ten fish under the same conditions as indicated earlier. The muscle-somatic index (g muscle/100 g body weight) obtained presented the values of 40% for WM and 4% for RM.

Statistics

Results are presented as means with their standard errors. Unpaired *t* tests were used to compare the two experimental groups at 11 and 24 h, respectively, and in the two periods of the same condition. All statistical analyses were performed using SPSS version 14 (SPSS, Inc., Chicago, IL, USA).

Results

Expt 1: effects of raw and gelatinised starch on glucose uptake by tissues of rainbow trout fed with carbohydrate-rich diets

Plasma glucose levels and tissue proximal composition. The two forms of starch, RS and GS, in the diets are digested and absorbed at different rates, as reflected in the postprandial plasma glucose levels shown in Fig. 1. At 11 h after the meal, rainbow trout in the GS group presented plasma glucose levels 3-fold higher than the RS group (17 and 5·6 mM, respectively). Although at 24 h after a meal, plasma glucose levels in the GS group decreased by 30%, the hyperglycaemic situation was maintained (10·3 mM; $P<0·05$). Higher amounts of assimilated carbohydrates also entailed 5-fold higher liver glycogen content in the GS group than in the RS group (GS, 10·28 (SEM 1·58)% wet weight; RS, 2·02 (SEM 0·29)% wet weight; $P<0·05$), causing hypertrophy of the organ (hepatosomatic index: GS, 1·82 (SEM 0·09)% wet weight; RS, 1·29 (SEM 0·03)% wet weight; $P<0·05$) and a concomitant reduction of the other tissue components (lipid: GS, 2·7 (SEM 0·2)% wet weight; RS, 4·3 (SEM 0·12)% wet weight; $P<0·05$; protein: GS, 11·2 (SEM 0·3)% wet weight; RS, 16·2 (SEM 0·4)% wet weight; $P<0·05$).

δ^{13}C taken up by tissues. The total recovery of ^{13}C from whole fish and entire organs is shown in Fig. 2. Changes in this variable paralleled those observed in plasma glucose. Thus, while there were no differences in the RS group in the ^{13}C recovered between 11 and 24 h (18 and 22%, respectively), higher levels were recovered in the GS group at 11 h (28%), with a marked decrease at 24 h (18%). The uptake of ^{13}C in RM and WM increased from 11 to 24 h post-feeding in both groups. Thus, in the RS group, ^{13}C increased by 60% in RM and 78% in WM, and in the GS group, it increased

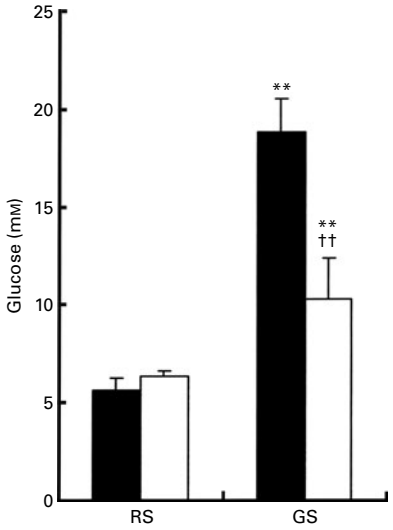

Fig. 1. Plasma glucose concentration (mM) in rainbow trout fed with the raw starch (RS) and gelatinised starch (GS) diets, 11 (■) and 24 h (□) after force-feeding. Values are means, with their standard errors represented by vertical bars (n 9). ** Mean values were significantly different between the RS and GS groups ($P<0·01$). †† Mean values were significantly different between 11 and 24 h ($P<0·01$).

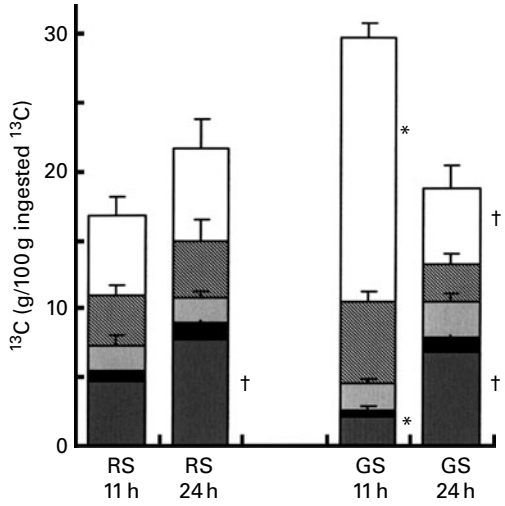

Fig. 2. Recovery of ^{13}C (as a percentage of ingested isotope) from entire organs or tissues (WM (■), white muscle; RM (■), red muscle; L (▨), liver; V (▧), viscera; R (□), the rest of the fish) of rainbow trout fed with the raw starch (RS) and gelatinised starch (GS) diets, 11 and 24 h after force-feeding. The sum of the stacked bar represents the total recovery from whole fish (see the Experimental methods section for details of the calculations). Values are means, with their standard errors represented by vertical bars (*n* 7). * Mean values were significantly different between the RS and GS groups ($P<0·05$). † Mean values were significantly different between 11 and 24 h ($P<0·05$).

by 175% in RM and 100% in WM. Taking into account the total muscle mass of rainbow trout, more than 40% of body weight, the ^{13}C taken up by muscles was the main allocation site of the dietary [^{13}C]starch (43% of the ^{13}C ingested was recovered in the RS group and 41% in the GS group).

The comparison of the values of ^{13}C recovered in each tissue fraction (protein, lipid and glycogen) in liver and WM is shown in Fig. 3. There were clear differences between the two diets in the fate of nutrients. The highest amount of ^{13}C in the liver of the RS group was found in protein (36% of total) and in glycogen (32%), with only 8% in lipids. In the GS group, the highest labelled fraction in liver was lipids (27% of total), then glycogen (19%) and protein (12%) (Fig. 3(a)). In WM, the levels of ^{13}C recovered in protein and lipid components of the GS group increased significantly between 11 and 24 h post-feeding (Fig. 3(b)). The incorporation of ^{13}C from dietary starch to muscle glycogen is shown in Fig. 4. No differences were found between the diets, although there was a significant correlation between ^{13}C levels in glycogen and the amount of glycogen present in RM (r 0·84, $P<0·01$) and WM (r 0·94, $P<0·001$), where ^{13}C deposition in glycogen in RM was 9-fold higher than that in WM.

Expt 2: effects of sustained swimming on the use of carbohydrates by trout

Food intake, fish growth, plasma glucose levels and tissue proximal composition. Sustained swimming caused a significant increase in food intake (control, 2·54 (SEM 0·14)% body weight; exercise, 3·09 (SEM 0·15)% body weight; $P<0·05$), reflecting higher metabolic costs but without impairing growth (specific growth rate: control, 2·24 (SEM 0·34)% body weight/d; exercise, 2·63 (SEM 0·03)% body weight). The food conversion ratio did not change significantly (control, 1·32 (SEM 0·37); exercise, 1·36 (SEM 0·11)). Under exercise, plasma glucose levels increased at 11 h after feeding ($P<0·05$), but decreased significantly with respect to the control group at 24 h (Fig. 5).

Proximate composition of liver, RM and WM, at both 11 and 24 h post-feeding, is shown in Table 2. Exercise modified liver composition due to a transient increase in glycogen content at 11 h and lipid mobilisation at 24 h post-feeding. Fish under exercise increased the glycogen content in RM and the lipid content in WM.

$\delta^{13}C$ and $\delta^{15}N$ taken up by tissues. The recoveries of ^{13}C in glycogen, lipid, protein and free pool in liver, and WM and RM are shown in Fig. 6. In liver, sustained swimming induced higher total recoveries of ^{13}C (2-fold) with respect to the control group at both 11 and 24 h, also in parallel with plasma glucose changes. The higher recovery in the liver of the exercise group was due, in part, to significant depositions of ^{13}C in protein and lipid fractions at 11 h (4- and 5-fold higher, respectively) and in the free pool at 24 h (7-fold increase). In WM, sustained swimming induced a significantly higher recovery of ^{13}C ($P<0·05$), especially due to the recovery in the free pool at 11 h post-feeding. The ^{13}C recovered in

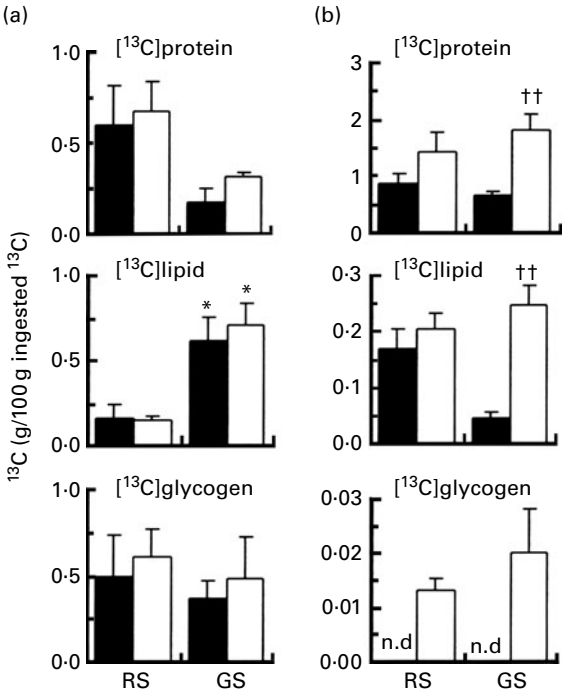

Fig. 3. Recovery of ^{13}C (as a percentage of ingested isotope) from (a) liver and (b) white muscle fractions (protein, lipid and glycogen) of rainbow trout fed with the raw starch (RS) and gelatinised starch (GS) diets, 11 (■) and 24 h (□) after force-feeding (see the Experimental methods section for details of the calculations). Recovery of ^{13}C from the glycogen fraction of white muscle was below the limit of detection (n.d., not detected). Values are means, with their standard errors represented by vertical bars (*n* 7). * Mean values were significantly different between the RS and GS groups ($P<0·05$). †† Mean values were significantly different between 11 and 24 h ($P<0·01$).

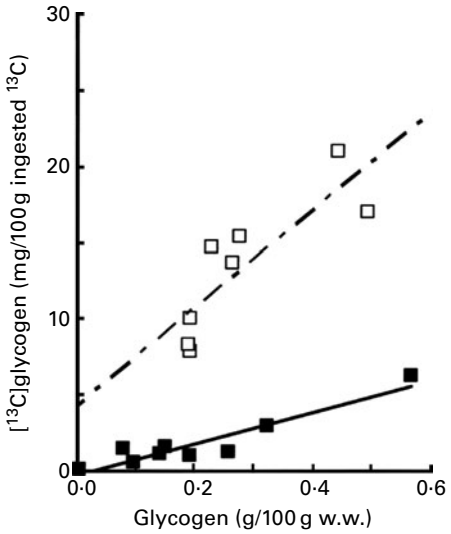

Fig. 4. Relationship between the glycogen content (g/100 g wet weight (w.w.)) and the percentage of [^{13}C]glycogen recovered from dietary starch in white muscle (■; $y = 10{\cdot}315x - 0{\cdot}2796$, $R^2 = 0{\cdot}8829$; $P < 0{\cdot}001$) and red muscle (□; $y = 32{\cdot}18x + 4{\cdot}316$, $R^2 = 0{\cdot}7049$; $P < 0{\cdot}01$) of rainbow trout.

the protein fraction increased at 24 h ($P < 0{\cdot}05$) in both groups, but the incorporation of ^{13}C in the other tissue stores (lipid and glycogen) differed in the two groups. Thus, the control group significantly increased ^{13}C in glycogen (control, 95 (SEM 29) *v.* exercise, 2·2 (SEM 0·3) mg [^{13}C]glycogen/100 g of ^{13}C ingested; $P < 0{\cdot}05$), whereas in the exercise group higher deposition of ^{13}C in the lipid fraction was observed (control, 29 (SEM 12) *v.* exercise, 92 (SEM 16) mg [^{13}C]lipid/100 g of ^{13}C ingested; $P < 0{\cdot}05$). In RM, more than 85 % of ^{13}C labelling was in the free pool, whereas depositions in protein and glycogen reserves were lower and no deposition was observed in lipids. However, the exercise group presented lower deposition of ^{13}C in the glycogen fraction than that of the control group.

The recoveries of ^{15}N in protein and the free pool fractions of liver, and WM and RM are shown in Fig. 7. Sustained swimming did not modify the ^{15}N recovery in liver, and the fate of ^{15}N from dietary protein revealed the same pattern in WM and RM. The greatest recovery of total ^{15}N, due to the higher recovery in the protein fraction, occurred at 24 h in both muscles of the exercise group ($P < 0{\cdot}05$).

As a summary of all results, Fig. 8(a) and (b) presents the total recoveries of ^{13}C and ^{15}N, respectively, after a single forced meal, including all tissues and fractions. The high ^{13}C recovery in whole fish under exercise, although not significantly different than that of the control group at 11 h, decreased significantly between 11 and 24 h. At 24 h, the total ^{15}N recovered in whole fish of the exercise group was significantly higher.

Discussion

For the first time, two stable isotopes ([^{13}C]starch and [^{15}N]protein) have been incorporated into fish diets as labels to study the fate of both nutrients in a species of reference, the rainbow trout. The use of [^{13}C]starch allowed us to analyse the distribution of dietary carbohydrates depending on the degree of gelatinisation. However, we should point out two limitations with this method that reduces its value for the assessment of the use of [^{13}C]starch: the amount of label lost with the undigested food and the small amount of glucose lost via the urine in fish with the highest glycaemia[15,38]. The long-lasting hyperglycaemia observed in the group fed the GS diet indicates higher absorption of carbohydrates in this group than in those fed RS. The digestibility of the two diets was not analysed in the present study, but we assume that the theoretical values of 58 % for RS and 90 % for GS are correct (see Brauge *et al.*[39] and Kaushik *et al.*[40]). This assumption is consistent with the postprandial glucose levels measured. Long-lasting hyperglycaemia in trout was previously seen as a result of feeding a high-carbohydrate diet[41] or of a high glucose dose administrated orally[16,42]. Many fish species correct hyperglycaemia following a high-carbohydrate meal less rapidly than endotherms[1,43,44]. Apart from the differences in body temperature and metabolic rate, another reason, at least in rainbow trout, is a persistent, high level of endogenous glucose production from the liver[45]. Key enzymes of gluconeogenesis in this species are always highly expressed, independently of nutritional status[46]. In the present study, fish fed GS took up much more glucose into tissues, as shown by their higher plasma glucose levels and higher total recovery of ^{13}C in tissues at 11 h. These higher uptake rates could contribute to the marked reduction of plasma glucose levels at 24 h in this group. These results are consistent with our previous study showing that brown trout (*Salmo trutta*) after an aortic glucose load labelled with ^{14}C presented higher glucose uptake rates into tissues, in proportion to the hyperglycaemia[16]. In

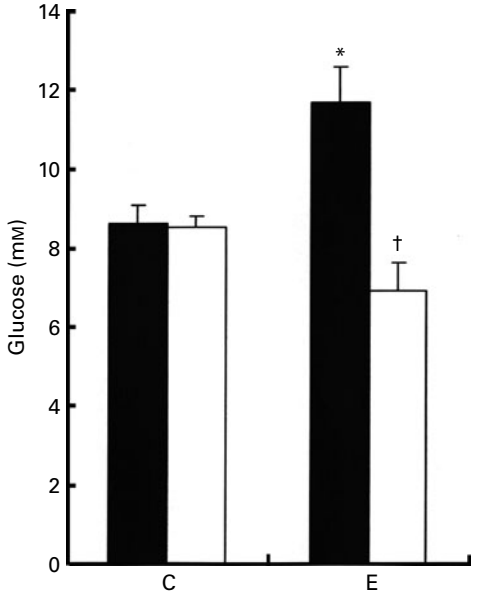

Fig. 5. Plasma glucose concentration (mM) in rainbow trout subjected to sustained swimming (exercise, E) or to voluntary swimming (control, C), 11 (■) and 24 h (□) after force-feeding. Values are means, with their standard errors represented by vertical bars (*n* 6, C) and (*n* 9, E). * Mean values were significantly different between the two experimental groups ($P < 0{\cdot}05$). † Mean values were significantly different between 11 and 24 h ($P < 0{\cdot}05$).

Table 2. Proximal composition of liver and muscle in rainbow trout subjected to sustained swimming (Mean values with their standard errors, *n* 6)

	11 h				24 h			
	Control		Exercise		Control		Exercise	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Liver								
HSI	1·8	0·11	2·1	0·25	1·5	0·09	1·8*	0·08
Liver glycogen (% wet weight)	5·6	0·54	10·3*	1·24	5·2	0·90	7·3	0·58
Liver lipid (% wet weight)	4·2	0·09	4·0	0·11	4·4	0·18	3·4*†	0·13
Liver protein (% wet weight)	12·8	0·33	11·3	0·76	13·8	0·60	12·4	0·48
RM								
RM glycogen (% wet weight)	0·68	0·09	1·00	0·11	0·52	0·04	0·77*	0·06
RM lipid (% wet weight)	7·2	0·93	8·1	0·94	7·2	0·37	6·6	0·60
RM protein (% wet weight)	13·4	0·49	16·5	0·53	13·3	0·87	14·4	0·30
WM								
WM glycogen (% wet weight)	0·13	0·03	0·08	0·01	0·28	0·10	0·07	0·01
WM lipid (% wet weight)	2·04	0·30	2·88	0·39	1·03†	0·3	3·49*	0·43
WM protein (% wet weight)	17·7	0·42	17·2	0·32	18·2	0·22	17·5	0·23

HSI, hepatosomatic index; RM, red muscle; WM, white muscle.
* Mean values were significantly different between the two experimental groups ($P<0·05$).
† Mean values were significantly different between 11 and 24 h ($P<0·05$).

the present study, the [^{13}C]glucose dilution was different in the RS and GS groups due to the similar amount of tracer delivered and the different glycaemia observed. Since the percentage of total ^{13}C recovered in muscle (WM plus RM) was similar in the two groups (nearly 40 % of the total ^{13}C recovered), the total amount of glucose taken up by the GS group must have been higher than that by the RS group. These results also reinforce the idea that skeletal muscle of rainbow trout is the main peripheral site of glucose disposal, similarly to what was observed in cod[47], Atlantic salmon[48] and brown trout[16]. The ^{13}C deposition rate into glycogen depots in RM was nine times higher than in WM, in agreement with the different capacity of glycogen repletion of each kind of muscle in exercised rainbow trout[49]. So, the importance of WM as the main site of glucose disposal cannot be overlooked, based on the large relative mass of the tissue. Higher carbohydrate uptake by the muscles of the GS group caused a proportional increase in ^{13}C recovered in all muscle reserves, especially in protein and lipid, in agreement with the results observed in brown trout after an aortic glucose load[16]. In rainbow trout, high dietary levels of digestible carbohydrates increased hepatic lipid content[50], suggesting hepatic lipogenesis from carbohydrates[51]. In accordance with these studies, the present results show that higher glucose uptake in the liver of the GS group produced an increase of the glycogen depots (observed after 1 month of the GS diet) and the *de novo* synthesis of lipids in the liver. In the present study, nearly 20 % of the total ^{13}C ingested was recovered at 24 h, whereas in Atlantic salmon, the ^{14}C recovered ranged between 13 and 15 %[48]. Differences in species and in methodological conditions (tracer, starch or glucose) might explain these discrepancies. Glucose oxidation can be calculated from the difference between ^{13}C ingested and ^{13}C recovered from the whole fish after 24 h post-feeding,

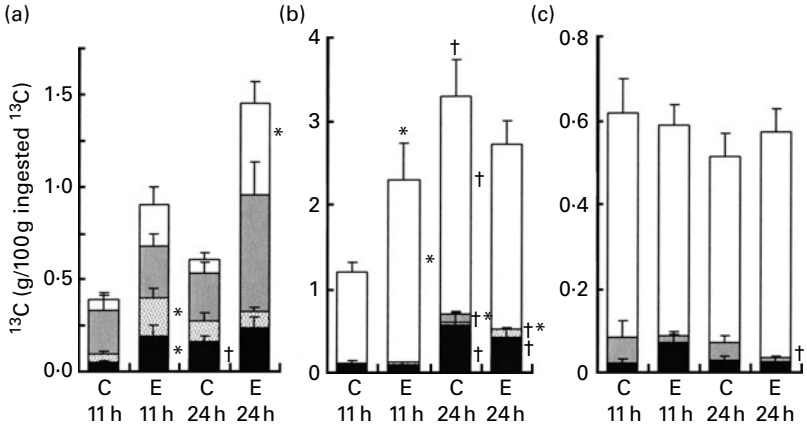

Fig. 6. Recovery of ^{13}C (as a percentage of ingested isotope) from (a) liver, (b) white muscle and (c) red muscle fractions (protein (■), lipid (▨), glycogen (▩) and free pool (□)) of rainbow trout subjected to sustained swimming (exercise, E) or to voluntary swimming (control, C), 11 and 24 h after force-feeding (see the Experimental methods section for details of the calculations). Values are means, with their standard errors represented by vertical bars (*n* 6, C) and (*n* 9, E). * Mean values were significantly different between the two experimental groups ($P<0·05$). † Mean values were significantly different between 11 and 24 h ($P<0·05$).

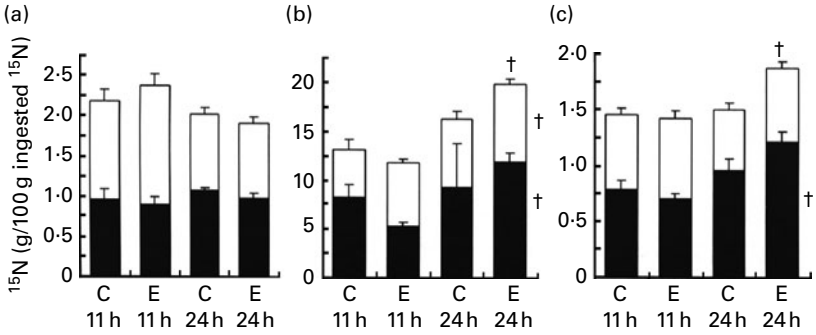

Fig. 7. Recovery of ^{15}N (as a percentage of ingested isotope) from the protein (■) and free pool (□) fractions of the (a) liver, (b) white muscle and (c) red muscle of rainbow trout subjected to sustained swimming (exercise, E) or to voluntary swimming (control, C), 11 and 24 h after force-feeding (see the Experimental methods section for details of the calculations). Values are means, with their standard errors represented by vertical bars (n 6, C) and (n 9, E). * Mean values were significantly different between the two experimental groups ($P<0{\cdot}05$). † Mean values were significantly different between 11 and 24 h ($P<0{\cdot}05$).

but the values of 82 % of glucose oxidised in the GS group and 77 % in the RS group can be overestimates of the actual values due to the losses indicated before. The main issue, however, is that the measurement of ^{13}C in tissue stores confirms that excess glucose promotes lipogenesis, leading to significantly higher depots of newly formed, saturated fat in liver and muscle, even in trout accustomed to this diet.

Summarising all the results of the first experiment, gelatinisation of starch improved the absorption of dietary carbohydrates, causing a long-lasting hyperglycaemia, and glucose uptake by tissues rose in proportion to the plasma glucose concentrations in the post-absorptive period. This higher rate of glucose uptake by tissues favoured the synthesis of lipid in the liver. A level of 30 % digestible carbohydrates fed daily, however, exceeds the capacities of rainbow trout to use this nutrient, as reflected in the hyperglycaemia maintained after 24 h and the deposition of lipids in WM. Consequently, if these lipids are not consumed as energy fuel, they will be deposited as saturated fat, which in excess can affect the fish fillet quality. This can be prevented by increasing energy expenditure through the induction of exercise. In the second experiment, only the GS diet was used to determine the effects of moderate, sustained swimming on the use of nutrients as energy fuels. The swimming regimen of 1·3 BL/s for 1 month was used as a 'metabolic promoter'. In this situation of induced activity, rainbow trout increased feed intake to compensate for the higher energy costs due to the exercise. Although Davison & Goldspink[52] found an increase of food conversion ratio in exercised brown trout fed chopped liver, no significant differences were observed between the groups in the present study, perhaps due to the dietary differences. Rainbow trout growth rate showed a tendency to increase in exercise, in agreement with the results reported by Houlihan & Laurent[53] and Farrell *et al.*[54].

A carbohydrate-rich diet fed under exercise also induced transitory hyperglycaemia 11 h post-feeding, but glycaemia returned to control values at 24 h. This transitory hyperglycaemia in the swimming group is attributed to higher food ingestion. As indicated before, the maintenance of hepatic gluconeogenesis in rainbow trout feeding carbohydrate-rich diets[45,55] can contribute to hyperglycaemia. As observed in the first experiment, hyperglycaemia raised glucose uptake rates in liver and WM. Thus, in liver, the total recovery of ^{13}C was 2-fold higher in the exercise group, indicating greater

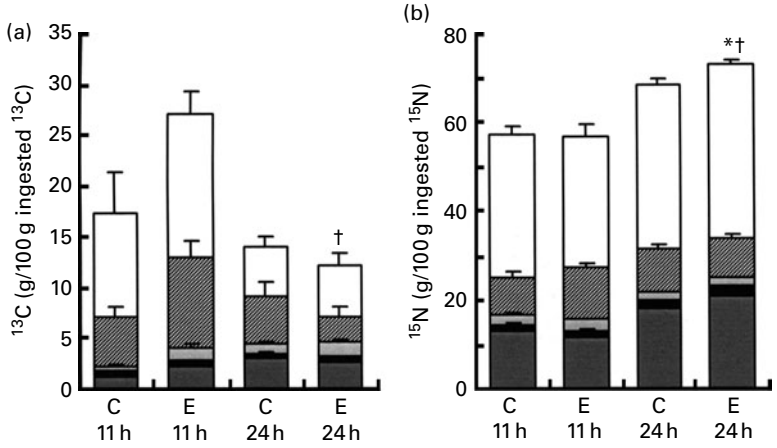

Fig. 8. Recovery of (a) ^{13}C and (b) ^{15}N (as a percentage of ingested isotope) from entire organs or tissues (WM (■), white muscle; RM (■), red muscle; L (■), liver; V (▨), viscera; R (□), the rest of the fish) of rainbow trout subjected to sustained swimming (exercise, E) or to voluntary swimming (control, C), 11 and 24 h after force-feeding. The sum of the stacked bar represents the total recovery from whole fish (see the Experimental methods section for details of the calculations). Values are means, with their standard errors represented by vertical bars (n 6, C) and (n 9, E). * Mean values were significantly different between the E and C groups ($P<0{\cdot}05$). † Mean values were significantly different between 11 and 24 h ($P<0{\cdot}05$).

uptake and deposition into the various tissue reserves. The postprandial variation of glycogen content, related to glycaemia, was evidence of higher carbohydrate uptake and use in exercised fish than in controls. Moreover, hepatic lipogenesis from dietary carbohydrates was also enhanced and, combined with the lower level of liver fat in the exercise group at the end of the experiment, this observation supports the idea of higher mobilisation of hepatic lipids to extra-hepatic tissues. The increase in muscle lipid content in the exercised rainbow trout, in agreement with the increase in muscle lipid content found in several fish species under exercise conditions[52,56,57], should in part be caused by such mobilisation. Fish rely mainly on fatty acids to fuel submaximal exercise[28], mediated by an enhancement of lipoprotein lipase activity in RM[58]. However, the origin of these lipids has not been traced previously. The addition of [^{13}C]algal starch to the diet enabled us to ensure that lipid synthesised *de novo* in the liver is mobilised and transported to skeletal muscles and oxidised or used to replenish stores. The present results on the lack of ^{13}C in the lipid fraction of RM support the notion that lipids are highly used as aerobic energy fuel by this tissue, as reported by Magnoni & Weber[58]. On the other hand, the balance in WM results in a net lipid deposition. Additional information can be drawn from high levels of ^{13}C recovered in the free pool of RM. This labelling corresponds to several kinds of molecules of the intermediary metabolism and is indicative of the higher metabolic activity of RM. During moderate and sustained swimming, RM burns not only fatty acids provided by the liver, but also glycogen. The present results support that dietary carbohydrates play a key role in muscle metabolism during exercise. Moreover, West *et al.*[59] reported a 30-fold rise in glucose use by RM in rainbow trout under a steady swimming speed at 80 % U_{crit}.

Non-protein energy sources spare dietary protein from oxidation as fuel, thus releasing it for growth. Thus, high protein efficiency ratios in rainbow trout fed diets containing about 30 % of digestible carbohydrates have been reported[4,60]. In the present study, the use of [^{15}N]protein as a dietary tracer allowed us to measure the amount of protein allocated to the main tissues following a single meal. Houlihan & Laurent[53] reported that both protein synthesis and protein degradation increased in exercised rainbow trout, leading to increased growth rate. In the present study, the greatest recovery of total ^{15}N for exercised fish at 24 h, mainly in the protein fraction of RM and WM, is evidence that exercise improves protein deposition. Exercise may also reduce N wastes. In conclusion, one forced-feeding with labelled nutrients, [^{13}C]starch and [^{15}N]protein, has allowed us to show that sustained swimming in rainbow trout improves the use of digestible carbohydrates and of lipid and glycogen depots, resulting in an enhancement of the protein-sparing effect.

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ORIGINAL PAPER

Beneficial effects of sustained activity on the use of dietary protein and carbohydrate traced with stable isotopes ^{15}N and ^{13}C in gilthead sea bream (*Sparus aurata*)

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Abstract To determine the effects of sustained swimming on the use and fate of dietary nutrients in gilthead sea bream, a group of fish were forced to undertake moderate and sustained swimming (1.5 BL s^{-1}) for 3 weeks and compared with a control group undertaking voluntary activity. The exercise group showed a significant increase in specific growth rate (C: 1.13 ± 0.05; E: 1.32 ± 0.06 % day^{-1}, $P < 0.05$) with no significant change in food intake (C: 3.56 ± 0.20; E: 3.84 ± 0.03 % of body weight). The addition of ^{13}C-starch and ^{15}N-protein to a single meal of 1 % ration allowed analysis of the fate of both nutrients in several tissues and in their components, 6 and 24 h after force-feeding. In exercised fish improved redistribution of dietary components increased the use of carbohydrates and lipid as fuels. Gilthead sea bream have a considerable capacity for carbohydrate absorption irrespective of swimming conditions, but in trained fish ^{13}C rose in all liver fractions with no changes in store contents. This implies higher nutrient turnover with exercise. Higher retention of dietary protein (higher ^{15}N uptake into white muscle during the entire post-prandial period) was found under sustained exercise, highlighting the protein-sparing effect. The combined effects of a carbohydrate-rich, low-protein diet plus sustained swimming enhanced amino acid retention and also prevented excessive lipid deposition in gilthead sea bream.

Keywords Exercise · Carbohydrate-rich diet · Liver · White muscle

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The experiments complied with the Guidelines of the European Union Council (86/609/EU), the Spanish Government (RD 1201/2005) and the University of Barcelona (specific ethics approval number for the protocol was CEEA-96/09) for the use of laboratory animals.

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Introduction

In recent years, scientific advances in our knowledge of the physiology and metabolism of several fish species have improved aquaculture management. The increasing demand for fish meal protein makes that alternative sources of energy should be a requirement for the sustainable development of this industry (Watanabe 2002; Gatlin et al. 2007; Tacon et al. 2010; Kaushik and Seiliez 2010). One approach would be to find fish species naturally adjusted to obtain energy from non-protein sources. Saving dietary amino acids for growth instead of using them for oxidative purposes (known as the protein-sparing effect) implies that the total amount of carbohydrates or lipids has to be increased in fish diets. Several studies have assayed different proportions of dietary non-protein energy sources (lipid and carbohydrate) in different species (rainbow trout, Brauge et al. 1995; brown trout, Arzel et al. 1998; dentex, gilthead sea bream and sea bass, Company et al. 1999; gilthead sea bream, Vergara et al. 1999). The use of dietary

lipids instead of protein is well established in fish; even in some cases very high-lipid inclusion in the diet have caused some problems as hepatomegalia and excessive lipid accumulation in the white muscle. However, the effect of carbohydrates saving protein for growth (i.e., the entry of high amounts of carbohydrates in the energy production pathways) remains controversial, particularly in marine fish species (Peres et al. 1999; Enes et al. 2011). Classically, carnivorous fish have been described as diabetics or at least with a limited ability to metabolize glucose (Wilson 1994; Moon 2001; Hemre et al. 2002; Panserat and Kaushik 2002; Stone et al. 2003). High intake of digestible carbohydrate results in prolonged post-prandial hyperglycaemia that persists for many hours in rainbow trout (Bergot 1979; Kaushik and Oliva-Teles 1985; Brauge et al. 1994). However, carbohydrates can promote growth in rainbow trout, because glucose is the preferred oxidative substrate for nervous tissue and blood cells. As carbohydrate digestibility has been increased in modern fish commercial diets through starch extrusion, it has re-opened the discussion about how these carbohydrates affect growth, feed utilization and nutrient deposition. Also carbohydrates in the diet can depress the high rate of amino acids use in gluconeogenic pathways (Cowey et al. 1977; Sanchez-Muros et al. 1996).

Gilthead sea bream is one of the most important cultured fish in the Mediterranean areas, explaining the high numbers of studies about the physiology of this species. Surprisingly, only few studies have focused on the metabolism of carbohydrates and their effects promoting growth (Metón et al. 1999; Couto et al. 2008; Enes et al. 2008). Previous studies from our group suggest that gilthead sea bream can use carbohydrate more efficiently as energy fuel than other teleosts, especially salmonids. Enes et al. (2010) found that dietary carbohydrate complexity did not affect growth performance but that feed utilization was more efficient for complex carbohydrate than for glucose.

The activity of α-glucosidase in gilthead sea bream whole intestine was almost twice as much in European sea bass (Papoutsoglou and Lyndon 2005), indicating that gilthead sea bream have a higher capacity to digest carbohydrates than European sea bass. High-carbohydrate, low-protein diets stimulated 6-phosphofructokinase, pyruvate kinase, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activity in the liver of gilthead sea bream but decreased alanine aminotransferase activity (Metón et al. 1999). High-carbohydrate, low-protein diets increased glucokinase (GK) expression (Caseras et al. 2002) and thus allowed metabolic adaptation favouring glycolysis over gluconeogenesis. Gut transit times and enzyme activities indicate differences that could affect the nutritional value of the carbohydrate sources evaluated (Venou et al. 2003). Although it has been suggested that diets containing around 20 % digestible carbohydrates ensure high digestibility, growth and feed utilization in this species (reviewed by Enes et al. 2011), other studies did not find any big adverse effects on fish growth rate with higher inclusion levels (Metón et al. 1999; Venou et al. 2003).

Such studies on fish nutrition have paid more attention to nutrient substitution than their assimilation, deposition and use. Discrepancies in the results are related not only to differences in dietary composition, but also to the feeding strategies (Enes et al. 2011 review) and maintenance conditions used in the different studies. There is a delicate equilibrium in the interchange of molecules in the different pathways of intermediary metabolism, because none of the compounds involved can be modified without affecting the function of the others. So, it is necessary to provide the most efficient supply of nutrients to use as energy providing fuels, as growth-promoting structures and as tissue depots between meals. Obviously, the nutrients required also depend on energy requirements. Exercise is closely related to energy consumption and feeding, and it has been associated with animal welfare and improved growth in most animals including various fish species. Husbandry can reduce physical activity and animal performance, and it is well known that wild fish show superior swimming performance to fish kept in captivity, including the brook trout (Vincent 1960), coho salmon (Brauner et al. 1994), Atlantic salmon (McDonald et al. 1998) and gilthead sea bream (Basaran et al. 2007). Pelagic fish can easily be exercised in artificial rearing conditions by forcing them to swim against a current, because this is a reflex. Swimming activity changes the amount and proportion of nutrients used by fish, especially in their muscles, and several reviews have been dedicated to this topic (Davison 1997; Palstra and Planas 2011). From a practical point of view, moderate and sustained activity has also been used to improve growth rate and food conversion efficiencies in fish (Jorgensen and Jobling 1993; Davison 1997). Indeed, swimming speeds of up to 1.5 body lengths per second (BL s^{-1}) are acceptable for training fish without adverse effects on growth rates (reviewed by Davison 1997). Although gilthead sea bream is now the main species cultivated in the Mediterranean area, there has only been one study of the costs of swimming at different speeds in this species (Steinhausen et al. 2010). These authors found that gilthead sea bream swimming at 1.5 BL s^{-1} accounted for 30–40 % of critical velocity (*Ucrit*). Thus, animals were swimming at moderate, but sustained, velocity for a long period.

The aim of the present study was to analyse the effects of sustained swimming on the use and fate of dietary protein and carbohydrates labelled with ^{15}N and ^{13}C to trace the intermediary metabolism of gilthead sea bream,

nutrient uptake by the main organs and deposition into stores. Prior to the current study, we conducted a pilot experiment to validate labelling in rainbow trout and gilthead sea bream (Beltrán et al. 2009) and another one to demonstrate that sustained swimming in rainbow trout improves growth, feed intake and carbohydrate use (Felip et al. 2012). We should note that there are no previous studies of the relationship between nutrition and swimming in gilthead sea bream. Our results show that, as exercise improves fish growth and the use of carbohydrates, feed efficiency should increase because feed intake does not change significantly.

Materials and methods

Experimental design and sampling procedures

Juvenile gilthead sea bream (*Sparus aurata L.*) were purchased from a local commercial supplier (Cripesa, Tarragona, Spain) and were held in indoor facilities at the Faculty of Biology (University of Barcelona, Barcelona, Spain) in 400-l seawater tanks equipped with a semi-closed recirculation system with physical and biological filters, ozone skimmers and continuous aeration at 22 °C and photoperiod 12L/12D, with a 35 % weekly seawater renewal rate. Temperature, flow, oxygen concentration and water characteristics such as pH, nitrite and nitrate parameters were recorded daily. Fish were acclimated to an experimental carbohydrate-rich diet (see Experimental diet composition in Table 1), and the amount supplied was adapted to the daily level of ingestion. After this acclimatization period, all fish were lightly anaesthetized and fitted with a passive integrated transponder (PIT) tag (Trovan Electronic Identification Systems, Madrid, Spain) near the dorsal fin to allow subsequent identification and individual monitoring. Fish were weighed and randomly distributed in four trial tanks (initial weight 50–60 g). In two of the experimental tanks (sustained swimming group, E) the water supply was modified to produce a circular, uniformly distributed flow of 700 litres/h, induced by the perpendicular entrance of water at the surface and a submerged water pump at the bottom of the tank, isolated from the free-living area. The shape of the tank prevented the fish from entering a central area of lower velocity, thus guaranteeing similar swimming velocities throughout the experiment. Both water volume and fish density were the same as in the control group. This design and water flow resulted in a sustained swimming velocity of 1.5 BL s^{-1}, measured and adjusted at three different tank depths (surface, mid-tank and near the bottom) using a low-speed mechanical flow meter (General Oceanics, Inc., Miami, FL, USA). In the other two tanks fish were held in standard rearing conditions, with a water flow of 350 l/h and vertical water inflow. Fish in these conditions (control group, C) showed only spontaneous movements (voluntary swimming). During the experimental period of 3 weeks both groups were fed twice a day (9:30 a.m. and 5:30 p.m.) until apparent satiety with an experimental diet rich in digestible carbohydrates. Food intake was recorded on a daily basis for each tank and the specific growth rate (SGR = 100 × (ln final weight − ln initial weight)/day) was calculated for each individual of each tank at the end of the experimental period.

Table 1 Ingredients and chemical composition of the experimental diet

Ingredients and additives (g/kg)	
Fish meal	450
Gelatinized wheat starch	470
Fish oil	70
Vitamins	10
Proximate composition (% DM)	
Crude protein	37.15
Crude fat	12.51
Crude carbohydrates	40.04
Crude fibre	1.77
Total ashes	8.52
Gross energy	19.3 MJ/kg

After 3 weeks, 20 fish were randomly sampled from each group (E and C), lightly anaesthetized and force-fed a bolus, equivalent to a ration of 1 % of diet labelled with stable isotopes (3 % ^{13}C algal starch and 1 % ^{15}N *Spirulina* protein; Martek Biosciences Corporation, Columbia, MD, USA) using a gastric cannula. Two other fish from each group received the same dietary ration containing similar proportions of non-labelled *Spirulina* protein and algal starch. These four fish were used to determine the natural abundance of ^{15}N and ^{13}C in the samples (blank values). After force-feeding, fish were held for a few minutes in separate tanks to check the acceptance of the forced meal. Any individual showing a degree of regurgitation was disqualified and another fish was used in its place. The food ingested by each animal was recorded to calculate the percentage of label ingested. Fish were then returned to their respective tanks and maintained for 6 or 24 h post-feeding (the exercise group were swimming until the point of sampling). These two periods were chosen as they represent the times of maximum post-absorption (6 h) and nutrient use completion (24 h). Six hours after force-feeding, ten fish from each group plus two more fish from each group as blanks were anaesthetized and killed by sectioning the spinal cord. The final body weight, body length and body indices (hepatosomatic, muscle-somatic

and perivisceral fat content) were recorded and blood samples were extracted from the caudal vessels using EDTA-Li as an anticoagulant. Plasma was obtained by centrifuging the blood at 13,000×*g* for 5 min at 4 °C and then kept at −80 °C until analysis. Samples of liver, white muscle, viscera (gut plus perivisceral fat) and the rest of the fish were rapidly excised, frozen in liquid N_2 and stored at −80 °C until analysis. The entire sampling procedure took less than 3 min from the death of the fish, and tissues with high glycogen hydrolytic capacity, such as muscle, were frozen first. The same procedure was repeated at 24 h post-feeding with the other ten fish from each group plus two more fish as blanks.

Analytical procedures

A commercial kit (Commercial Kit Glucofix, Menarini, Italy) based on the enzymatic method of glucose-oxidase described by Werner et al. (1970) was used to determine the plasma glucose concentration. Tissue samples (liver, muscle and viscera) were homogenized in liquid N_2 using a pestle and mortar to obtain a fine powder, and the rest of the fish was homogenized at −20 °C with a food homogenizer (Pacojet AG, Zug Switzerland). Samples were shared out for the analysis of percentage lipids, proteins, glycogen and water content, and one part of the sample was used for isotopic analysis. Tissue water content was determined gravimetrically after drying the samples at 100 °C for more than 24 h. Lipids were extracted according to the method described by Folch et al. (1957). The washed lipid extracts were dried under a N_2 atmosphere and the lipid content was determined gravimetrically. Protein purification was carried out using defatted samples via precipitation with 10 % (v/v) trifluoroacetic acid. The proteins were dried using a vacuum system (Speed Vac Plus, AR, Savant Speed Vac Systems, South San Francisco, CA, USA), and protein content was calculated according to the nitrogen obtained by elemental analysis (Elemental Analyser Flash 1112, ThermoFinnigan, Bremen, Germany) assuming the following conversion factor: 1 g of nitrogen corresponds to 6.25 g of protein. Glycogen extraction and purification were carried out via alcoholic precipitation after tissue hydrolysis using 30 % KOH (Good et al. 1933). Glycogen content was analysed using the anthrone colorimetric method described by Fraga (1956).

δ^{15}N and δ^{13}C determination in tissue

The ^{13}C enrichment in the whole tissue and in the main components (glycogen, lipids and proteins) of each sampled tissue and the ^{15}N enrichment in the whole tissue and in the protein fraction of each tissue were analysed using an elemental analysis isotope ratio mass spectrometer (IRMS). Dried samples of diets and whole tissues, as well as the purified lipid, glycogen and protein from each tissue, were lyophilized and ground in a mortar to a homogeneous powder for isotopic analysis. Aliquots ranging from 0.3 to 0.6 mg were accurately weighed in small tin capsules (3.3 × 5 mm, *Cromlab,* Barcelona, Spain). Samples were analysed for C and N isotope composition using a Mat Delta C isotope-ratio mass spectrometer (Finnigan MAT, Bremen, Germany) coupled to an Elemental Analyser (Flash 1112) (at Barcelona University SCT). The EA-IRMS burned the samples and converted them into gas (N_2 and CO_2). That gas was transported in a continuous helium (He) flux to determine the percentage carbon and nitrogen in the sample. The isotope ratios ($^{13}C/^{12}C$, $^{15}N/^{14}N$) in the samples were compared with reference gases (N_2 and CO_2) and were expressed on a relative scale as deviation, referred to as delta (δ) units using the notation ‰, parts per thousand, relative to the isotope ratio content of international standards: PDB (Pee Dee Belemnite, a calcium carbonate) for C and air for N.

Delta values were determined as follows:

$$\delta = [(R_{sa}/R_{st}) - 1] \times 1000,$$

where $R_{sa} = {}^{15}N/{}^{14}N$ or $^{13}C/^{12}C$ of samples and $R_{st} = {}^{15}N/{}^{14}N$ or $^{13}C/^{12}C$ of international standards. The same reference material analysed during the analysis period was measured with about 0.2 ‰ precision for natural materials and about 0.4 ‰ precision for enriched materials. The δ values were expressed as atom percentage (at %) as follows:

$$^{13}\text{C at}\,\% = 100 \times (^{13}C/(^{13}C + {}^{12}C))$$

$$^{15}\text{N at}\,\% = 100 \times (^{15}N/(^{15}N + {}^{14}N))$$

The net enrichment (APE (*atom percentage excess*)) of ^{13}C and ^{15}N in glycogen, lipid, protein and whole tissue was calculated by the difference between the atom % of samples and their corresponding blank atom % values:

$$\text{APE} = \text{at}\,\%\,\text{sample} - \text{at}\,\%\,\text{blank}.$$

Finally, using the APE values, molecular weight and Avogadro's number, the results were expressed as the percentage of marker in relation to the ingested dose (g/100 g ^{13}C or ^{15}N ingested) in each tissue fraction (glycogen, lipid and protein), which was calculated as follows:

$$100 \times ((\text{g}\,^{13}\text{C or}\,^{15}\text{N/g t. fr.}) \times (\text{g t. fr./g tissue}) \times (\text{g tissue/g b.w.})/(\text{g ingested}\,^{13}\text{C or}\,^{15}\text{N/g b.w.})) \quad (1)$$

where t. fr. is the tissue fraction and b.w. is the body weight. The free pool of each tissue was calculated as the difference between isotope levels in the entire organ or tissue and the sum of the three tissue fractions. So, the

value in an entire organ, or tissue, is the sum of all fractions (Eq. (1) + free pool) for ^{13}C or ^{15}N. In whole fish, this was calculated as the sum of all tissues (liver, WM, viscera and the rest of the fish) for ^{13}C or ^{15}N.

For liver and viscera, the exact mass of the total tissue sample was calculated by weighing the entire individual organs from the experimental fish. However, to estimate the total mass of the WM, we accurately dissected another ten fish under the conditions indicated earlier. The muscle-somatic index (g muscle/100 g body weight) obtained was 38.04 % ± 0.79 for the WM of exercised fish and 38.68 % ± 0.68 for the WM of control fish ($P < 0.05$).

Statistics

Data for all parameters are presented as mean ± standard error of mean. Differences of body weight, food intake and SGRs were obtained from Two-way ANOVAs, with tank and conditions (control *vs* exercise) as the two factors. For the rest of analysis, unpaired T-tests were used to compare the two experimental groups and the two post-prandial times, 6 and 24 h, within the same condition. Differences were considered statistically significant for $P < 0.05$. All statistical analyses were performed using SPSS Statistics v.17.0 (SPSS Inc., Chicago, Illinois).

Results

Sustained, moderated swimming for 3 weeks led to an increase in final body weight in gilthead sea bream, although this increase was not significant due to individual variability (Control group (C): 70.15 ± 1.76; Exercise group (E): 74.15 ± 1.70 g). Nevertheless, the increase in the specific growth rate in exercised fish was significant (SGR, C: 1.13 ± 0.05; E: 1.32 ± 0.06 % day^{-1}, $P < 0.05$) despite there being no significant change in food intake (C: 3.56 ± 0.20 %; E: 3.84 ± 0.03 % b. w.). The tissue composition of liver and white muscle is presented in Table 2, showing very similar values in both groups, with the exception of a transitory increase in lipid content at 6 h post-feeding in the white muscle of exercised fish.

Six hours after force feeding a carbohydrate-rich diet, a significant, but transitory increase in plasma glucose levels were seen in the C and E groups. These levels were twofold higher than those recorded 24 h post-feeding, and the changes in plasma glucose profiles were also similar in each group (Fig. 1). Thus, irrespective of the exercise regime, gilthead sea bream showed a notable capacity for carbohydrate absorption and distribution.

The labelling of the forced meal with 3 % starch-^{13}C and 1 % protein-^{15}N allowed us to measure the levels of post-prandial nutrient distribution at 6 and 24 h, reflecting the peak in assimilation and the daily total use, respectively. Total ^{13}C recovery in whole fish decreased markedly from 6 to 24 h (percentage of ^{13}C ingested: 24 and 26 % at 6 h, 13 and 15 % at 24 h) with no significant differences between groups (see Fig. 5a below for a more detailed explanation of these results). At 6 h post-feeding, transitory increases in ^{13}C deposition were observed in the liver (up to 5 %) and viscera (4.5 %). In the liver, ^{13}C was found mainly in the tissue fractions of glycogen and free-pool (i.e., ^{13}C soluble intermediary metabolites) (Fig. 2a), whereas in the viscera ^{13}C was found in the protein and free-pool fractions (Fig. 3a) with no significant differences between E and C groups. At 24 h post-feeding, ^{13}C

Table 2 Effect of the activity to the proximal composition of liver and white muscle in gilthead sea bream

	6 h		24 h	
	Control	Exercise	Control	Exercise
Liver				
HSI	1.99 ± 0.11	1.94 ± 0.14	1.80 ± 0.15	2.04 ± 0.06
Liver water (% w.w.)	62.9 ± 1.02	64.0 ± 0.68	61.2 ± 0.57	60.67 ± 0.54^{+}
Liver glycogen (% w.w.)	11.9 ± 0.59	12.9 ± 0.80	10.6 ± 1.21	11.1 ± 0.67
Liver lipid (% w.w.)	13.2 ± 0.66	11.3 ± 0.59	11.7 ± 0.88	11.0 ± 0.71
Liver protein (% w.w.)	7.9 ± 0.39	7.8 ± 0.08	7.7 ± 0.34	7.7 ± 0.33
White muscle				
MSI	38.68 ± 0.68	38.04 ± 0.79	38.68 ± 0.68	38.04 ± 0.79
WM water (% w.w.)	76.0 ± 0.29	77.0 ± 0.22*	76.4 ± 0.29	76.1 ± 0.24^{+}
WM glycogen (% w.w.)	0.15 ± 0.02	0.07 ± 0.01*	0.14 ± 0.04	0.12 ± 0.04
WM lipid (% w.w.)	3.7 ± 0.50	9.5 ± 1.24*	6.9 ± 0.96^{+}	4.7 ± 0.42^{+}
WM protein (% w.w.)	18.2 ± 0.41	18.4 ± 0.17	18.7 ± 0.18	19 ± 0.19^{+}

Values are mean ± SEM. $n = 10$

* Significant differences ($P < 0.05$) between the two experimental groups and + between 6 and 24 h

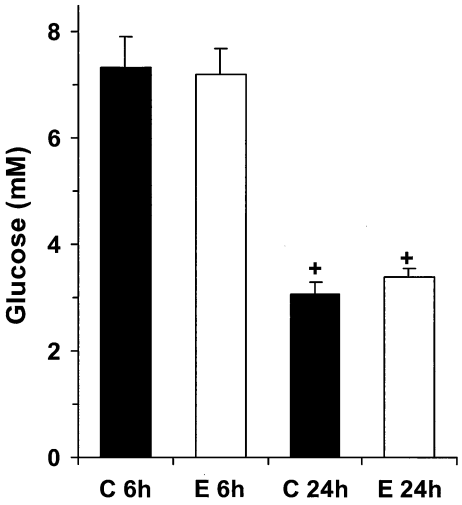

Fig. 1 Plasma glucose concentration (mM) in gilthead sea bream subjected to sustained swimming (E) or to voluntary swimming (C), at 6 and 24 h after force-feeding. Mean values with their standard errors, *n* 10. *Significant differences between the two experimental groups ($P < 0.05$); $^{+}$significant differences between 6 and 24 h ($P < 0.05$)

recovery from the liver and viscera markedly decreased, revealing the transient role of the gut and liver and the plasticity of liver stores. However, although the liver lipid contents of the two groups were similar, the amount of ^{13}C taken up from dietary starch into the hepatic lipid fraction by the exercised group 24 h post-feeding was twofold higher than that of the control group, indicating a significant increase in lipogenesis under exercise. Likewise, ^{15}N recovery from the whole liver showed the same pattern as that of ^{13}C (Fig. 2b). Dietary ^{15}N-protein was rapidly incorporated into the whole liver at 6 h, but its fate at 24 h was dependent on swimming condition (3.5 ± 0.1 C and 4.2 ± 0.3 E g/100 g ingested ^{15}N $P < 0.05$). Recovery of ^{15}N from whole viscera tissue (Fig. 3b) followed the same pattern as that in the liver, showing significantly higher ^{15}N deposition in the protein fraction in the exercised group at the end of the postprandial period. The recovery of ^{13}C and ^{15}N from components of the white muscle of gilthead sea bream is shown in Fig. 4a, b, respectively. At 6 h, labelled carbon was mainly found in the free-pool fraction, with the exercised group showing significantly higher incorporation of carbon from dietary starch into the muscle protein fraction. After 24 h, ^{13}C levels in the total white muscle mass of exercised fish were 15–20 % higher than in the control group. ^{15}N recovery in white muscle of exercised fish was also higher, mainly in the protein fraction, indicating greater protein incorporation into the muscle and dietary protein retention in this group compared with the control.

To summarize the results, Fig. 5a, b show the total ^{13}C and ^{15}N recovery (sum of all organ or tissue recoveries) from a single meal in whole fish. Sustained swimming in the conditions studied led to higher retention of both ^{13}C (via nutrient transformation and isotope retention in protein and lipid fractions) and ^{15}N (by retention), especially in the protein fractions. It should also be noted that all observed differences between exercised and control gilthead sea bream were achieved after only 3 weeks of sustained swimming at 1.5 BL s^{-1}.

Discussion

Gilthead sea bream has become, at least among marine teleosts, a clue species for aquaculture because it has been the subject of many studies of fish physiology during the last three decades. Nevertheless, the effects of spontaneous and forced activity on its physiology, especially energy demands and use, have received little attention. The present work is the first demonstration of the improvement in the assimilation and distribution of dietary nutrients in gilthead sea bream in response to sustained swimming activity of

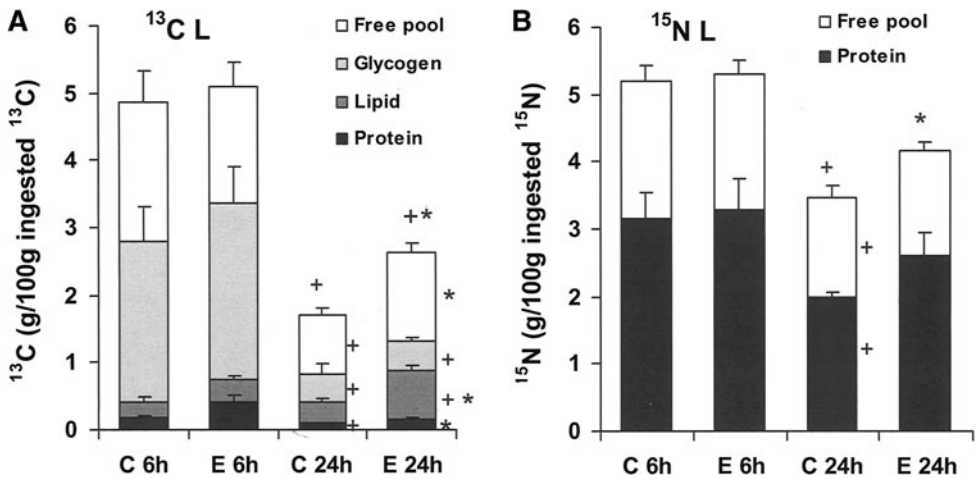

Fig. 2 Recovery of ^{13}C (**a**) and ^{15}N (**b**) (as percentage of ingested isotope) from liver fractions (protein, lipid, glycogen and free pool) of gilthead sea bream subjected to sustained swimming (E) or to voluntary swimming (C), 6 and 24 h after force-feeding. Mean values with their standard errors, *n* 10. *Significant differences between the two experimental groups ($P < 0.05$); $^{+}$significant differences between 6 and 24 h ($P < 0.05$)

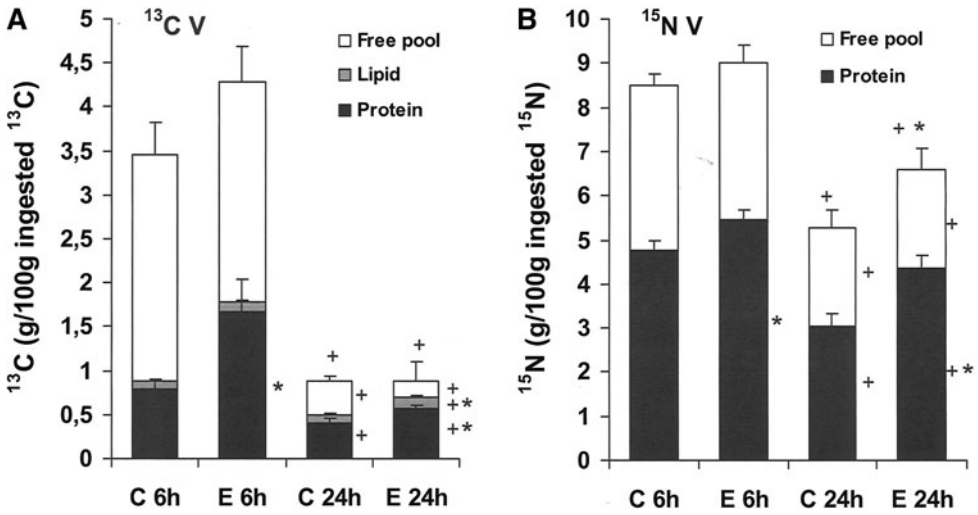

Fig. 3 Recovery of ^{13}C (**a**) and ^{15}N (**b**) (as percentage of ingested isotope) from viscera fractions (protein, lipid and free pool) of gilthead sea bream subjected to sustained swimming (E) or to voluntary swimming (C), 6 and 24 h after force-feeding. Mean values with their standard errors, *n* 10. *Significant differences between the two experimental groups ($P < 0.05$); $^{+}$significant differences between 6 and 24 h ($P < 0.05$)

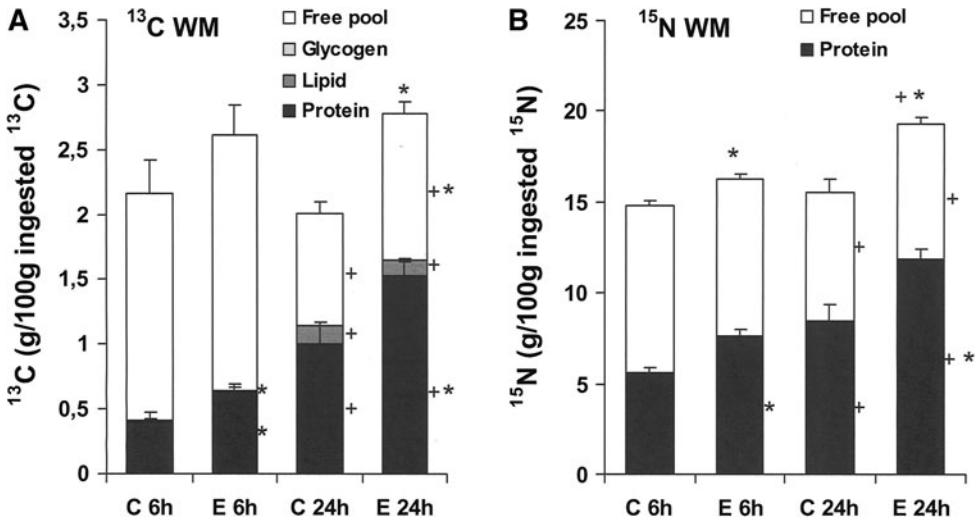

Fig. 4 Recovery of ^{13}C (**a**) and ^{15}N (**b**) (as percentage of ingested isotope) from white muscle fractions (protein, lipid, glycogen and free pool) of gilthead sea bream subjected to sustained swimming (E) or to voluntary swimming (C), 6 and 24 h after force-feeding. Mean values with their standard errors, *n* 10. *Significant differences between the two experimental groups ($P < 0.05$); $^{+}$significant differences between 6 and 24 h ($P < 0.05$)

1.5 BL s^{-1}. After 3 weeks of this regime, the exercised group grew better than the control group (under voluntary activity) without increasing feed intake. The significantly higher growth rate of the exercised gilthead sea bream indicates an improved use of energy sources other than dietary protein, whose ingestion was similar in the two groups. Higher growth rates have also been reported in other fish species subjected to exercise, such as the brown trout (Davison and Goldspink 1977), rainbow trout (Houlihan and Laurent 1987; Farrell et al. 1990), Atlantic salmon (Totland et al. 1987) and Arctic charr (Adams et al. 1995). Our group (Felip et al. 2012) observed in rainbow trout under exercise a tendency to better growth but increasing food ingestion. Nevertheless, Christiansen and Jobling (1990) reported on Arctic charr that the improved weight gain in the exercising fish does not appear to be due primarily to increased food consumption, but is rather the result of better fish feed efficiency. In the present study, the proportion of protein in the experimental diet was low (37.2 % DM), especially in comparison with the levels proposed by the majority of nutritionists (around 45 % DM) for this species, size of fish, and rearing temperature. It should, however, be noted that the animals in our study were fed to apparent satiety, so total food intake was not limited by the experimental procedure.

Compared with mammals, food intake control is relatively unknown in fish. Fish eat not only to satisfy their energy requirements, suggesting a possible role of energy or nutrient utilization, and thus of nutrient source, in food intake regulation in fish (Saravanan et al. 2012). We hypothesize that the correct combination of dietary composition and exercise may improve growth, and we have done it because gilthead sea bream grew more after 3 weeks of sustained swimming without increasing food intake. Nahhas et al. (1982) and Bagatto et al. (2001) reported that long-term exercise improved swimming efficiency in rainbow trout and zebrafish by reducing the metabolic costs of aerobic swimming. Likewise,

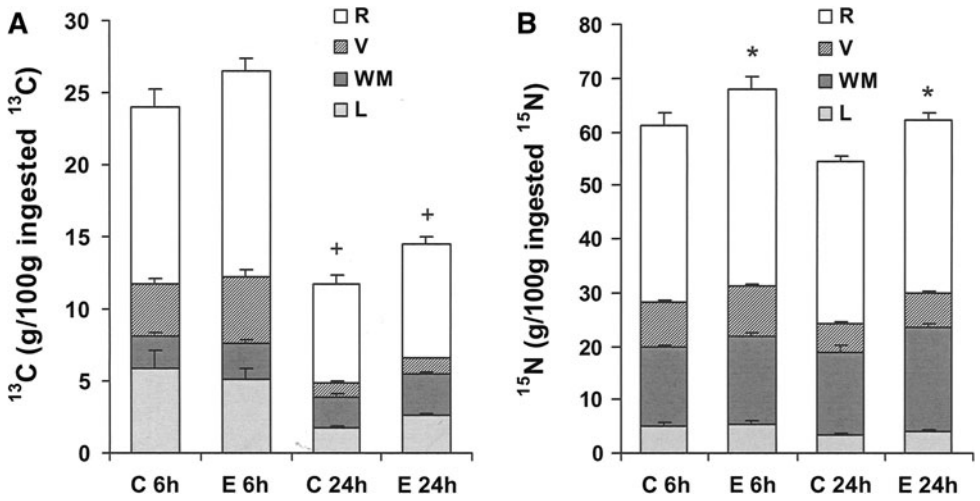

Fig. 5 Recovery of ^{13}C (**a**) and ^{15}N (**b**) (as percentage of ingested isotope) from entire organs or tissues (*L* liver, *WM* white muscle, *V* viscera, *R* the rest of the fish) of gilthead sea bream subjected to sustained swimming (E) or to voluntary swimming (C), 6 and 24 h after force feeding. The sum of the stacked bar represents the total recovery from the whole fish (see "Materials and methods" for details of calculations). Mean values with their standard errors, *n* 10. *Significant differences between E and C group ($P < 0.05$); $^{+}$significant differences between 6 and 24 h ($P < 0.05$)

Steinhausen et al. (2010) found that the oxygen consumption of gilthead sea bream under sustained swimming at 0.5–1.5 BL s^{-1} was not higher than that of fish swimming voluntarily with spontaneous activity. These authors demonstrate that the costs of spontaneous activity may be high and even comparable to those of a fixed, moderate swimming speed. In this sense, sustained swimming at optimal speeds also reduces aggressive interactions in Arctic charr, and such interactions are associated with anaerobic peaks of energy consumption (Adams et al. 1995). Lowered plasma cortisol levels have been reported for Atlantic salmon that was subjected to moderate exercise of between 0.5 and 1.5 BL s^{-1} (Boesgaard et al. 1993; Herbert et al. 2011), which improve animal welfare. Thus, some fish species can swim and grow at the same time suggesting either that multiple metabolic costs are easily balanced or that some form of behavioural or physiological efficiency, including changes in digestive and assimilation capacities, arise in response to long-term exercise as it has been proposed by Christiansen et al. (1991) and Adams et al. (1995) in Arctic charr, and by Brown et al. (2011) in New Zealand yellowtail kingfish.

The use of carbohydrate as nutrient varies according to species, size of fish, levels of dietary carbohydrates, and the kind of feed ingredient and digestibility (reviewed by Hemre et al. 2002). The inclusion of carbohydrates in the diet at appropriate levels can improve growth performance, even in carnivorous fish species (Degani et al. 1986; Hemre et al. 1989, 1995; Hung et al. 1989; Stephan et al. 1996; Hemre and Hansen 1998). However, the controversy is open because other studies have found no improvement in growth rate, despite its not being detrimental to growth at certain amounts or with certain kinds of carbohydrate (Peres and Oliva-Teles 2002; Enes et al. 2006, 2008). The transitory increase observed in this study in plasma glucose levels at 6 h and the similar decline at 24 h post-feeding in both groups revealed that gilthead sea bream have the capacity to incorporate and use a high proportion of carbohydrates from the diet. For many years it has been considered that the use of dietary carbohydrates for energy purposes in carnivorous fish species is limited or at least slow (see reviews by Wilson 1994 and Hemre et al. 2002), so anything that would promote the use of dietary carbohydrates as energy fuel, i.e., sustained swimming, should have beneficial effects. Thus, the long-lasting post-prandial hyperglycaemia produced by the ingestion of a carbohydrate-rich diet in rainbow trout (Bergot 1979; Wilson 1994; Hemre et al. 2002; Stone 2003) becomes transitory hyperglycaemia when the animals are forced to exercise (Felip et al. 2012). In the present study, gilthead sea bream fed with a carbohydrate-rich diet showed only short-transient hyperglycaemias. This was also seen in the group under voluntary activity. Thus, we expect that the beneficial effects of sustained exercise on the use of this diet may be greater in gilthead sea bream than in trout. The observed improvement of fish growth due to moderate, sustained swimming can be explained by tracing the use of two main nutrients (carbohydrate and protein) with ^{13}C and ^{15}N, respectively. Regardless of activity and in parallel with the transient hyperglycaemia, the highest amount of ^{13}C recovered from gilthead sea bream liver at 6 h was in the glycogen fraction, showing that in both groups ingested carbohydrates were initially converted into glycogen, and also found in the free–pool fraction (intermediary

metabolites). Both the transient hyperglycaemia and the increase in labelled compounds of intermediary metabolism in the liver should respond to the high GK hepatic activity induced by dietary carbohydrates, as reported by Panserat et al. (2000), Caseras et al. (2002) and Metón et al. (2004) in gilthead sea bream. Nevertheless, in fish subjected to sustained exercise, higher levels of ^{13}C were recovered in all tissue fractions of the liver at 24 h post-feeding without modifying the proximal composition of that tissue (i.e., the amounts of protein, lipid and glycogen in percentages of liver fresh weight). These results demonstrate that exercise induced more metabolic transformations in the liver of gilthead sea bream. Moreover, higher incorporation and deposition of ^{13}C into the white muscle of the exercise group throughout all the postprandial period, with significant differences at 24 h post-feeding, reveals that exercise increases the transformation of carbohydrates and their use in extrahepatic tissues. Our results demonstrated that gilthead sea bream had the capacity to incorporate and distribute high amounts of carbohydrates (as seen in the control group) and that their use improved in exercise (sustained swimming group).

Carnivorous fish such as rainbow trout, Atlantic salmon, European sea bass and gilthead sea bream do not modify liver gene expression and/or the activity of enzymes involved in the gluconeogenic pathways (PEPCK, FBase and G6Pase) in response to high dietary carbohydrate levels (reviewed by Enes et al. 2009); instead, they seem to regulate the glycolysis/gluconeogenesis ratio through the proportion of carbohydrate and protein in their diets. Thus, high carbohydrate intake increases the rate of glycolysis (gilthead sea bream, Metón et al. 2000), whereas low protein intake depresses the rate of gluconeogenesis (rainbow trout, Kirchner et al. 2003). Some differences in the responses among species could explain the more rapid restoration of plasma glucose levels in gilthead sea bream.

When the percentage of digestible carbohydrates consumed is high, the rate of glucose uptake by tissues increases (Felip et al. 2012) favouring the synthesis of lipid in the liver (Panserat et al. 2009). As previously observed in rainbow trout (Felip et al. 2012), in the present study higher ^{13}C recovery in the hepatic lipid fraction under exercise reflected enhanced lipogenesis from dietary carbohydrates. However, the fact that hepatic lipogenesis increased in the exercised group without changing the total liver fat content, already signalled, indicates higher mobilization of lipids in gilthead sea bream to extrahepatic tissues. In fish species such as rainbow trout, brown trout, Atlantic salmon and fingerling yellowtail, exercise increases the muscle lipid content. These lipids may be derived from an excess of dietary carbohydrates, as in the rainbow trout (Felip et al. 2012), or an excess of dietary lipids, as found in the gilthead sea bream (Santinha et al. 1999). In the present swimming conditions, gilthead sea bream did not accumulate lipids in white muscle. Thus, although the exercise regime (sustained swimming at 1.5 BL s^{-1}) promoted the incorporation of circulating lipids into the white muscle of gilthead sea bream, as demonstrated by the transitory increase in lipid content at 6 h post-feeding in the white muscle of exercised fish, it also promoted their use, as reflected by the reduction in lipid content 24 h post-feeding. The plasticity of white muscle lipid stores in exercised gilthead sea bream during the day indicates higher rates of lipid transformation and oxidation in the white muscle of this species, because rainbow trout under similar sustained swimming conditions deposit lipids in white muscle (Felip et al. 2012). Endurance swimming increases muscle lipid levels in the white muscle of brown trout (Davison and Goldspink 1977), in the red muscle of Atlantic salmon (Totland et al. 1987) and in the whole body fat of yellowtail (Yogata and Oku 2000). The fact that gilthead sea bream did not increase lipid deposition in white muscle under sustained exercise is consistent with results reported for the red muscle of rainbow trout subjected to moderate exercise (Magnoni and Weber 2007; Felip et al. 2012). The observation that lipid levels in the white muscle of rainbow trout tend to decrease after exhaustive exercise (Milligan and Girard 1993) supports that the use of lipid reserves is determined by the balance between energy input from the diet and energy outputs. In the present trial conditions, the lower levels of lipid in the white muscle of exercised gilthead sea bream, compared with the levels in the control group, are similar to the levels reported for the flesh of wild gilthead sea bream (Morrison et al. 2007; Lenas et al. 2011). Thus, the introduction of forced exercise could promote animal welfare and determine body compositions similar to those found in wild fish, avoiding hepatomegalia or excessive fat deposition in muscle.

Under sustained exercise, gilthead sea bream increased both ^{13}C and ^{15}N incorporations into the protein fractions of all analysed tissues (liver, white muscle and viscera). It demonstrates, first, a high ^{13}C isotopic routing from dietary starch to non-essential amino acids and then to proteins and, second, the increased efficiency of protein retention in the swimming group fed a low-protein diet. The lower recoveries of ^{13}C and ^{15}N in the voluntary activity group in the present study indicated lower energy efficiency in the use of dietary carbohydrates, due to the already mentioned high costs of spontaneous activity. In another study on gilthead sea bream, under similar exercise and dietary conditions, we observed an increase of the aerobic work in white muscle and a reduction in red muscle (Martin-Perez et al. 2012), being the enzymatic machinery of white muscle modified (increased activity of cytochrome-c oxydase and decreased of citrate synthase) in a form that

indicates a marked reduction in the entry of all amino acids into the Krebs cycle and their preservation for protein synthesis. It is of interest to note that following just one forced-feeding with 1 % ration, ^{15}N deposition in the whole fish of exercised group increased approximately 34 % more than that in the control group. Higher recoveries of total ^{15}N from the protein fraction of both red and white muscles was previously observed in exercised rainbow trout (Felip et al. 2012), but in gilthead sea bream the higher growth rate occurred without increasing feed intake significantly, thus leading to a conclusion that a protein-sparing effect occurred in the conditions of the study (low protein, high carbohydrate and exercise). That sparing effect associated with enhanced growth may be derived from shifts in fuel use (Ozório 2008; Kaushik and Seiliez 2010) combined with the increased use of dietary carbohydrates under exercise conditions (present study).

In conclusion, the total recovery of dietary ^{13}C-starch and ^{15}N-protein demonstrated that gilthead sea bream, regardless of exercise regime, has a good capacity to incorporate dietary carbohydrates and to use them in energy production and lipogenesis. Swimming at 1.5 BL s^{-1} did not increase the catabolic use of dietary protein in gilthead sea bream, but instead increased the efficiency of protein retention and the use of other energy reserves (carbohydrates and lipids), caused a protein-sparing effect and avoided excessive lipid deposition in white muscle.

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ORIGINAL PAPER

Sustained swimming improves muscle growth and cellularity in gilthead sea bream

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Abstract Two groups of juvenile gilthead sea bream were kept on two different swimming regimes (Exercise, E: 1.5 body length s^{-1} or Control, C: voluntary activity) for 1 month. All fish were first adapted to an experimental diet low in protein and rich in digestible carbohydrates (37.2% protein, 40.4% carbohydrates, 12.5% lipid). The cellularity and capillarisation of white muscle from two selected areas (cranial (Cr), below the dorsal fin, and caudal (Ca), behind the anal fin) were compared. The body weight and specific growth rate (SGR) of group E rose significantly without an increment in feed intake, pointing to higher nutrient-use efficiency. The white muscle fibre cross-sectional area and the perimeter of cranial samples increased after sustained activity, evidencing that sustained exercise enhances hypertrophic muscle development. However, we cannot conclude or rule out the possibility of fibre recruitment because the experimental period was too short. In the control group, capillarisation, which is extremely low in gilthead sea bream white muscle, showed a significantly higher number of fibres with no surrounding capillaries (F0) in the cranial area than in the caudal area, unlike the exercise group. Sustained swimming improved muscle machinery even in tissue normally associated with short bouts of very rapid anaerobic activity. So, through its effect on the use of tissue reserves and nutrients, exercise contributes to improvements in fish growth what can contribute to reducing nitrogen losses.

Keywords Capillarity · Cellularity · Growth · Fish · Hypertrophy · Muscle · *Sparus aurata* · Swimming

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Introduction

The locomotor strategies of animals play an important role in their response to life challenges, conditioning their nourishment, predator–prey interactions and reproduction, and determining migration capacity. Farmed fish lead a more sedentary life than their counterparts in the wild. Mechanical loads are involved in the development and maintenance of vertebrate tissue (Buchanan and Marsh 2002; Davison 1997). Comparative studies of farmed and wild fish reveal useful data about fish domestication processes and provide an insight into fish nutrition, physiology and production. Several studies have shown that wild fish have superior swimming performances than farmed fish. This is the case for brook trout (Vincent 1960), coho salmon (Brauner et al. 1994), Atlantic salmon (McDonald et al. 1998) and gilthead sea bream (Basaran et al. 2007). Consequently, wild fish show superior aerobic and anaerobic capacities. Moreover, fish-rearing conditions also affect fish quality, with cultured fish presenting a higher fat content than their wild counterparts (Vincent 1960; Thorstad et al. 1997; Grigorakis et al. 2002).

Two basic responses to exercise training can be described in humans and in some other mammals. Activities

such as running, swimming and cycling increase the aerobic capacity of muscle, which improves endurance (Holloszy and Booth 1976; Hoppeler et al. 1985), whereas isometric exercise (for example, weightlifting) results in fibre hypertrophy and an increase in muscle strength (Goldspink et al. 1976; Hoppeler 1986). Fewer studies have been carried out on exercise in lower tetrapods, such as amphibians and reptiles, due to the difficulties involved in forcing them to exercise at repeatable work rates and the highly anaerobic nature of any exercise (Bennett 1978). But by their nature, fish can easily be exercised by forcing them to swim against the stream. Swimming speeds of two body lengths per second (b.l.s^{-1}) or less are acceptable for training fish without any notable consequences for fish welfare or stress (Sänger and Stoiber 2001). Fish offer several advantages over mammalian models in exercise training studies. Many species of fish control buoyancy via their swim bladder, which limits the effects of gravitational forces compared with terrestrial animals. The locomotory musculature of fish is relatively simple: it is located around the axial skeleton, distributed in discrete myotomes, and the slow and fast muscle fibres are spatially segregated. Moreover, in contrast to mammals in which the recruitment of new skeletal muscle fibres ends soon after birth (Goldspink 1972), limiting muscle development and growth to hypertrophy, fish have the capacity to recruit new skeletal muscle fibres not only throughout larval life but also throughout juvenile and adult stages (Weatherley et al. 1988; Rowlerson and Veggetti 2001).

Fish myotomal muscles are arranged to provide the power for different swimming styles. Red muscle, composed of slow oxidative fibres, produces the force required for slower, routine and sustainable movements, including migration over long distances. White muscle is composed of fast glycolytic fibres, which are recruited to produce the force required for rapid movements, such as sprint swimming and escape responses (Gibb and Dickson 2002). In many fish species, an intermediate pink muscle is usually present between the two types of muscle. The white muscle usually represents 35%-50% of body weight, although in male salmon and tuna it can be nearly 70%, whereas red fibres comprise approximately 0.5–13% of body mass (Goolish 1989; reviewed by Dickson 1996). The pattern of fibre number and fibre size distribution and the pattern of capillaries surrounding each fibre in a particular muscle section are commonly referred to as muscle cellularity and capillarisation, respectively (Johnston 1999; Stoiber et al. 2002). Moreover, muscle cellularity is also the main determinant of both muscle growth and flesh quality (Johnston 1999).

Exercise is a powerful factor in improving growth rate and food conversion efficiency in many species (reviewed in Davison 1997). Increases in cell diameter and fibre numbers in aerobic muscle seem to be general features in teleost fish when exercise is moderate—swimming speeds below 1.5 b.l.s^{-1}—but the effects on white muscle are controversial. Some studies have reported that swimming has no effect on white muscle cellularity (Davie et al. 1986; Sänger 1992), but others show increases in white muscle fibre diameter (Hinterleitner et al. 1992; Davison 1994). It has also been demonstrated that the aerobic capacity of the swimming muscles increases due to exercise (Farrell et al. 1991), which is also linked to the changes in the contractile machinery. The reported data regarding the effects of training upon capillarisation are also controversial; no changes were observed in salmonids (Johnston and Moon 1980; Davison 1983), whereas studies on cyprinids (Sänger 1992) and rainbow trout (Davie et al. 1986) reported that training led to higher capillarisation, mainly in red muscle.

Here we examine the effect of mechanical load on gilthead sea bream white muscle by submitting fish to a 1-month training period. During this period fish were fed on an experimental diet (40.4% CHO, 37.2% protein, 12.5% lipid). Two zones of the fish body (cranial and caudal) were selected to evaluate the effects of exercise on the reared fish. Since these variables are of crucial importance in commercial terms and are usually used as growth markers, our study focuses on white muscle cellularity and capillarisation.

Materials and methods

Fish rearing

Juvenile sea bream (*Sparus aurata* L.) were purchased from a commercial supplier (Cripesa, Tarragona, Spain) who reared the fish in marine cages. One hundred fish were transferred and maintained indoors in the facilities of the Faculty of Biology (University of Barcelona, Barcelona, Spain), in six 200-L seawater tanks equipped with a semi-closed recirculation system with physical and biological filters, ozone skimmers and continuous aeration at 20°C and 12L:12D, with a 35% weekly seawater renewal rate. Fish were first acclimated to a carbohydrate-rich diet (composition: 37.15% protein; 12.51% lipid; 40.04% carbohydrate; 1.77% fibre; and 8.52% ash) and fed to satiation for 2 weeks. After this period, fish were slightly anaesthetised, weighed and randomly distributed in trial tanks (initial body weight and length data are shown in Table 1). Control groups (C: 12 fish per tank, in triplicate) were kept under normal rearing conditions in 200-L circular tanks with a water flow of 350 L/h (vertical water entrance). Exercise groups (E: 12 fish per tank, in triplicate) were kept in 400-L circular tanks in the same semi-closed circuit. To obtain sustained activity, the fish were prevented from entering the

Table 1 Growth, body indices and muscle composition of gilthead sea bream submitted to two regimes of swimming activity

	Control	Exercise
Growth[a]		
Initial weight (g)	88.4 ± 0.98	90.5 ± 1.17
Final weight (g)	98.2 ± 2.3	107 ± 2.2*
SGR	0.61 ± 0.15	0.76 ± 0.06*
Body indices[b]		
CF	1.50 ± 0.03	1.41 ± 0.06
HSI	2.06 ± 0.16	2.09 ± 0.15
Periv. fat index	2.15 ± 0.13	2.01 ± 0.13
MSI	40.9 ± 0.78	40.6 ± 0.65
Muscle composition[b]		
% protein	20.5 ± 0.18	20.6 ± 0.18
% lipid	2.17 ± 0.14	1.68 ± 0.15*
µg DNA/g	214 ± 8	201 ± 3*

SGR specific growth rate in % per day = 100 × [ln (final weight) – ln (initial weight)]/21 days; *CF* condition factor = body weight × 100 × total length^{-3}; *HSI* hepatosomatic index = liver weight × 100 × body weight^{-1}; *Periv. fat index* perivisceral fat index = fat weight × 100 × body weight^{-1}; *MSI* musculosomatic index = total muscle weight × 100 × body weight^{-1}

* Significant difference by Student's *t* test ($p < 0.05$)

[a] Values are mean ± standard error of the mean of triplicate tanks

[b] Values are mean ± standard error of the mean of 10 fish for each condition

central area of lower velocity by a cylindrical tube. This results in a living area corresponding to an effective space of 200 L with the same fish density as in the control tanks. Water flow was 700 L/h with a circular and uniformly distributed flow induced by a perpendicular water entrance and one additional submerged water pump (at the bottom of the tank and isolated from the fish living area). This design resulted in a swimming velocity of 1.5 body lengths per second measured at three different tank depths (at the surface, mid-tank and near the bottom). During the experimental period all groups were fed until apparent satiety twice a day (9:30 a.m. and 5:30 p.m.) and intake was recorded daily.

Sampling and histochemical procedures

After 4 weeks, 10 fish randomly sampled from both C and E groups were sacrificed by severing their spinal cord and the final body weight and body indices (hepatosomatic, muscle-somatic and perivisceral fat content) were recorded. For muscle composition, epaxial white muscle samples were obtained and immediately frozen in liquid nitrogen and then kept at –80°C until the main components were analysed. Muscle composition in water, glycogen, lipid, protein and DNA were analysed as explained elsewhere (Ibarz et al. 2007a, b).

White muscles samples for histochemical examination were dissected from the cranial (Cr) and caudal (Ca) regions. Small strips of white muscle with an approximate length of 1 cm, a width of 1 cm and a thickness of 0.5 cm were obtained from each region. Each sample was immediately soaked in 3-methyl-butane pre-cooled to −160°C and stored in liquid nitrogen until subsequent sectioning (Dubowitz 1985). Serial transverse sections from each sample were cut at a thickness of 16–20 µm in a cryostat (Frigocut, Reichart-Jung, Heidelberg, Germany) at −22°C. Sections were mounted on gelatinised slides and incubated for 5 min in a buffered fixative (Viscor et al. 1992) in order to prevent shrinkage or wrinkling. After rinsing the slides thoroughly, we used the ATPase method developed by Fouces et al. (1993) in order to reveal muscle capillaries and a histochemical assay for succinate dehydrogenase to demonstrate the aerobic or anaerobic characteristics of muscle fibres (Nachlas et al. 1957).

Morpho-functional measurements

Images of the stained sections were obtained using a light microscope (BX40, Olympus, Tokyo, Japan) connected to a digital camera (KP-C550, Hitachi, Tokyo, Japan). To ensure accurate calibration of all measurements, an image of a stage micrometer was obtained each time images of samples were taken. All the parameters listed below were empirically determined from 2×10^5 µm^2 windows of tissue from two different zones or muscle fields in each sample. After testing for the absence of differences between the two muscle fields from each sample (see "Statistics"), the data obtained from both fields were considered together so that the sample size was large enough. The following parameters were counted or calculated: capillary density (CD), fibre density (FD), the number of capillaries in contact with each fibre (NCF), the percentage of fibres having no capillaries in contact with them (F0) and the percentage of fibres in contact with at least one capillary (F+). Capillary and fibre counts were calculated to be expressed as capillaries and fibres per mm^2. The fibre cross-sectional area (FCSA) and fibre perimeter (FPER) were determined directly using a personal computer connected to a digitiser tablet and SigmaScan software (Systat Software Inc., San Jose, CA, USA) from digital images. The total number of fibres analysed in each sample muscle ranged from 200 to 300. Two indices expressing the relationship between NCF and the FCSA: CCA = NCF × 10^3/FCSA or FPER: CCP = NCF × 10^2/FPER, were also calculated. These indices are considered a measure of the number of capillaries per 1,000 µm^2 of muscle FCSA and the number of capillaries per 100 µm of muscle FPER. The maximal diffusion

distance (MDD) between the capillary and the central region of the fibre was also calculated for every capillary of the region analysed. A shape factor (SF), circularity, was measured as a function of the FPER and the FCSA following the formula: $SF = (4\pi FCSA)/FPER^2$. Circularity indicates the degree of adjustment of the fibre transverse section to a circular shape (SF = 1 for a perfect circle).

Statistics

Data for growth, body indices and white muscle composition under each condition, initially analysed by one-way ANOVA, did not show any differences between the three tanks. The data were then grouped and Student's *t* tests were performed using $n = 3$ tanks for the growth study, and $n = 10$ animals per condition for the remaining parameters. To test for the absence of differences between both muscle fields from each muscle sample, the non-parametric Wilcoxon rank-sum test was performed. For the percentage of fibre types, the arcsine transformation was applied as a previous step. The normality of the data was tested by the Kolmogorov–Smirnov test (with Lilliefors' correction) and the comparisons between the control and exercise groups were analysed by the Student's *t* tests. Data for all the parameters are expressed as sample means ± standard error of the mean.

For FCSA histograms a dynamic fitting by nonlinear regression was performed for each section (caudal and cranial). The approximation was done by a log-normal (four parameters) equation with a dynamic fit option of 200 for both total number of fits and maximum number of iterations. Log-normal equations have been reported with *R* value and coefficients and standard errors for each parameter (a, b, x_0 and y_0). Individual data were linearised by an ln $(x + 1)$ transformation, thus avoiding negative values and possible ln (0). With this linearisation, a linear regression was obtained to test for the difference in regression slope. All statistical analyses were performed using SigmaStat 4.0 (in SigmaPlot 11.0 Software, Systat Software Inc., San Jose, CA, USA) with significance at $p < 0.05$.

Results

Table 1 shows the body weight and organ indices, growth rate and muscle composition of gilthead sea bream submitted to two swimming-activity regimes. Noticeably, the fish that maintained a swimming speed of 1.5 b.l.s^{-1} for 1 month presented a significantly higher body weight without any differences in food intake (C: 2.8 ± 0.1 vs. E: 2.6 ± 0.1 g of feed per 100 g body weight). Other body indices, such as the condition factor (CF), hepatosomatic index (HSI), body percentage of perivisceral fat and muscle-somatic index (MSI), did not alter significantly (Table 1). Epaxial white muscle samples of the exercising fish reduced lipid content by 30% compared to the control group values, but protein percentage did not change. Therefore, the increment in total body weight with similar muscle-somatic index and protein percentage implies increased muscle mass with a net gain of total protein content in white muscle. A slight decrement in the DNA content (7%) was observed in fish under sustained activity, but there was no difference in the DNA content of total white muscle mass (calculated by multiplying body weight per MSI per DNA levels) between the C and E groups (total muscle DNA ranged from 8.6 to 8.7 mg).

Muscle cellularity and capillarisation were studied in two different zones: cranial (Cr) and caudal (Ca). For both zones and conditions the histochemical assay for succinate dehydrogenase (SDH) revealed a lack of staining, indicating the absence of SDH activity and thus the anaerobic character of those muscle sections. Illustrative images from muscle section are shown in Fig. 1. Morphometrical fibre parameters and fibre density (cellularity) from both muscle samples are shown in Table 2, and capillarisation parameters in Table 3. Fish under the sustained swimming regime presented significant increases in the parameters related to muscle fibre size in the cranial zone (Table 2). Likewise, the fibre perimeter was 11% higher and the fibre area was 17% higher than the control ones. These increases took place without any change in the shape factor of the fibres. Fibre cross-sectional area (FCSA) distributions, along with adjust equations, in both sections of the gilthead sea bream white muscle are shown in Fig. 2, and linearised regression parameters of those equations are reported in Table 3. The slope for the cranial area of control group was higher (in absolute value) than those of the other groups. The number of small fibres (FCSA < 2,500 μm^2) was significantly higher for the cranial area of control group, whereas white muscle fibres with FCSA > 9,000 μm^2 were lower (Table 3), indicating that, in the short 1-month period of sustained activity, white muscle fibres achieved higher perimeters and areas.

The measures of capillarisation shown in Table 3 are the number of capillaries per fibre and distance (NCF and CD), two derived indices relating, respectively, the capillaries that surround a fibre to its cross-sectional area and perimeter (CCA and CCP), and the mean diffusion distances of the capillaries to the centre of the fibre (MDD). Moderate but sustained exercise affected the capillarisation of the caudal area by slightly increasing the surrounding capillarity of the fibre perimeter (CCP data, $p < 0.05$, Table 3). However, white muscle capillarisation was very low in both the cranial and caudal localisations, scarcely reaching a mean of one capillary per fibre. Unlike the exercised fish, the gilthead sea bream in the control group presented a high proportion of fibres with no capillaries (F0) in contact with

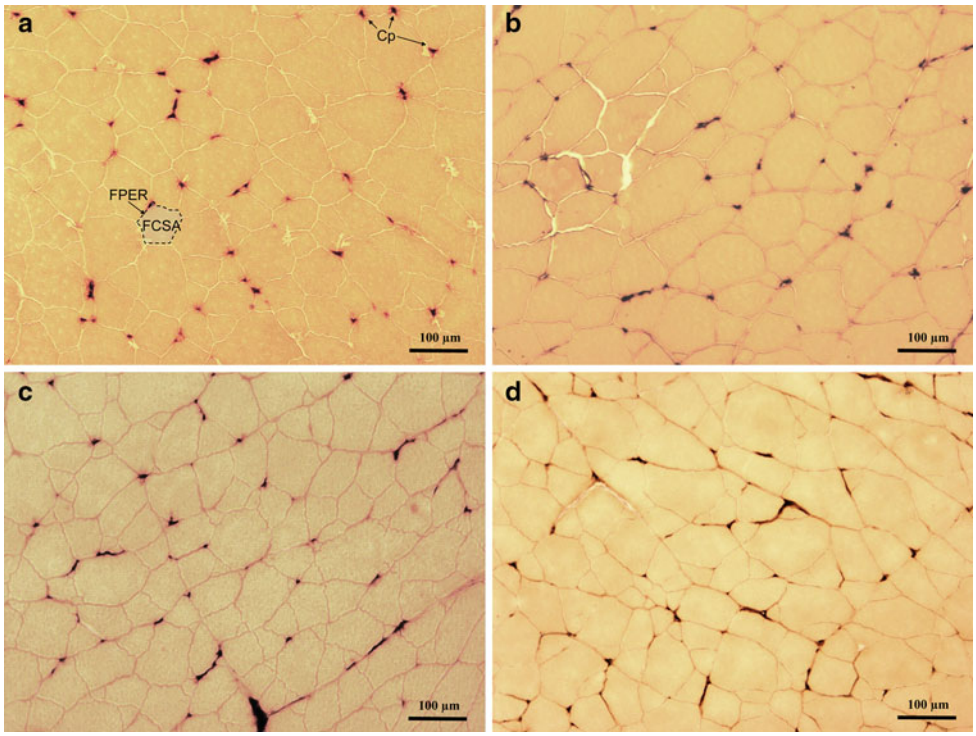

Fig. 1 Illustrative images of cross-sectional muscle of juvenile gilthead sea bream. **a** Cranial section of control fish, **b** cranial section of exercised fish, **c** caudal section of control fish, **d** caudal section of exercised fish. All images correspond to one representative serial transverse section and capillaries revealed by an ATPase detection method (see "Materials and methods" for further information). *Cp* capillaries, *FCSA* fibre cross-sectional area, *FPER* fibre perimeter

Table 2 Morphometrical fibre parameters and fibre density (cellularity) in cranial (Cr) and caudal (Ca) muscles of control (C) and exercising (E) fish

	CrC	CrE	CaC	CaE
FCSA (μm^2)	3749 ± 216	4399 ± 122*	4430 ± 107$^+$	4333 ± 152
FPER (µm)	244 ± 7.6	270 ± 3.1*	273 ± 4.5$^+$	270 ± 4.9
SF	0.68 ± 0.01	0.66 ± 0.01	0.66 ± 0.00	0.65 ± 0.01
FD (fibres/mm^2)	243 ± 18	201 ± 5	198 ± 9	215 ± 17

Values are mean ± standard error of the mean ($n = 5$ animals)

Significant differences ($p < 0.05$) between groups are indicated following the code: *CrC versus CrE; $^+$CrC versus CaC

FCSA fibre cross-sectional area, *FD* fibre density, *FPER* fibre perimeter, *SF* shape factor (circularity)

Table 3 Capillarisation parameters in cranial (Cr) and caudal (Ca) muscles of control (C) and exercising (E) fish

	CrC	CrE	CaC	CaE
CD	56 ± 3.1	56 ± 4.9	65 ± 7.2	71 ± 7.6
NCF	0.73 ± 0.08	0.84 ± 0.13	0.98 ± 0.09	1.03 ± 0.06
CCA	0.58 ± 0.07	0.50 ± 0.04	0.49 ± 0.04	0.58 ± 0.04
CCP	0.58 ± 0.02	0.53 ± 0.02	0.56 ± 0.03	0.60* ± 0.02
MDD (µm)	42 ± 1.8	48 ± 1.8	46 ± 0.9	45 ± 1

Values are mean ± standard error of the mean ($n = 5$ animals)

Significant differences ($p < 0.05$) between groups are indicated following the code: *CrE versus CaE

CD capillary density (capillaries/mm^2), *NCF* number of capillaries in contact with each fibre; CCA = NCF × 10^3/FCSA, relationship between NCF and the FCSA; CCP = NCF × 10^2/FPER, relationship between NCF and the FPER; *MDD* maximal diffusion distance between the capillary and the centre of the fibre

them, these being significantly ($p < 0.05$) more abundant in the cranial zone (38.3 ± 3.4% and 27.2 ± 2.8%, CrC vs. CaC, respectively, Fig. 3). Moreover, those fibres in contact with capillaries (F+) generally only had contact with one or two, with a maximum of four capillaries observed on only a few occasions. This determines high diffusion distances, which confirms the mainly anaerobic character of the fish white muscle.

Discussion

The present work is the first attempt to study the muscle cellularity of gilthead sea bream using sustained activity as a rearing condition to enhance the growth of fish on a rich carbohydrate diet. During recent years, new extrusion feed techniques permit improved availability of highly digestible carbohydrates and recent studies of sea bream have focused on this source to diminish dietary protein levels and waste nitrogen (Georgopoulos and Conides 1999; Venou et al. 2003; Fernández et al. 2007). The aim of replacing dietary protein and lipid by using a carbohydrate source also aims to increase the quality of meat by reducing lipid deposition in the sedentary fish. In the present study,

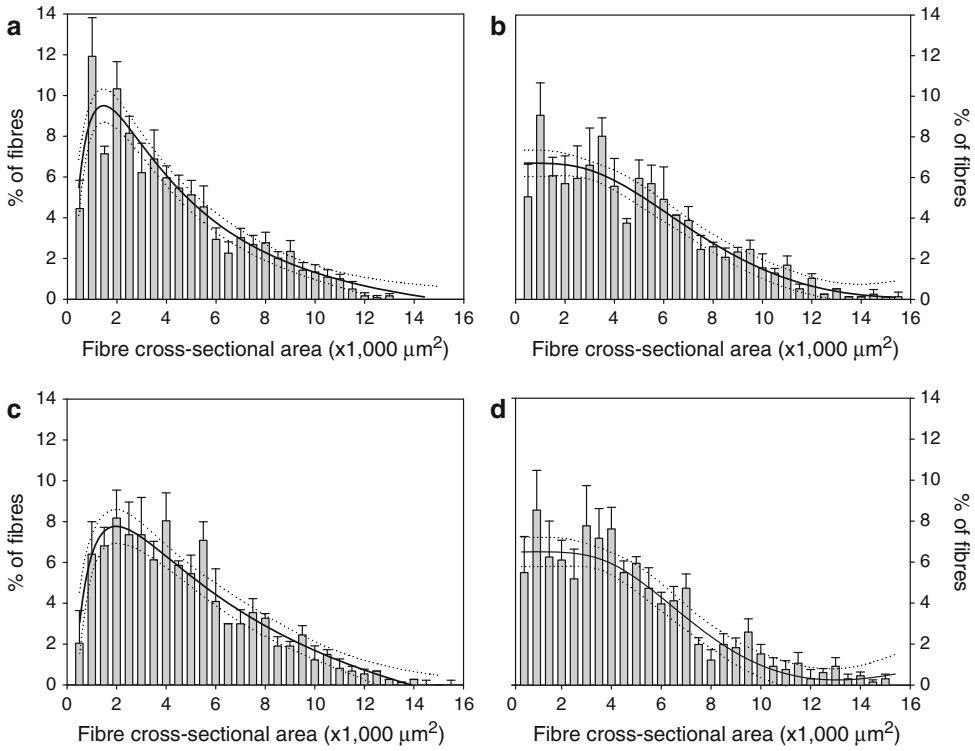

Fig. 2 White muscle cross-sectional area histograms. **a** cranial-control, **b** cranial-exercise; **c** caudal-control, and **d** caudal-exercise. Muscle fibre areas were grouped in 50 × 1,000 μm^2 groups and the data correspond to mean ± SEM frequency of five animals. Regression parameters are shown and analysed in Table 4. See "Materials and methods" ("Statistics") for further information

Table 4 Regression parameters for cranial (Cr) and caudal (Ca) fibre cross-sectional area (FCSA in μm^2) of white muscles of gilthead sea bream submitted to two swimming regimes (C: voluntary activity, and E: 1.5 b.l.s^{-1})

Parameters	CrC	CrE	CaC	CaE
Log-normal regression[a]				
R	0.87	0.84	0.87	0.79
a	10.91 ± 0.84	−6.60 ± 0.64	10.91 ± 0.84	−6.25 ± 0.44
b	1.14 ± 0.13	0.67 ± 0.13	1.14 ± 0.13	0.53 ± 0.09
x_0	1.49 ± 0.10	16.07 ± 2.76	1.49 ± 0.10	13.15 ± 1.15
y_0	−1.52 ± 0.95	6.71 ± 0.33	−1.52 ± 0.95	6.54 ± 0.36
Linear regression[b]				
R	0.91	0.85	0.80	0.80
y_0	2.48 ± 0.06	2.37 ± 0.07	2.32 ± 0.08	2.27 ± 0.08
a	−0.173 ± 0.007 a	−0.151 ± 0.008 b	−0.150 ± 0.009 b	−0.147 ± 0.009 b
N[c]	149	152	149	154
Percentage of muscle fibres with				
FCSA ≤ 2,500	42.0 ± 2.7 a	31.9 ± 1.9 b	30.8 ± 3.3 b	35 ± 4.7 a,b
2,500 < FCSA ≤ 6,000	37.1 ± 0.9	40.5 ± 1.8	44.0 ± 4.5	40.5 ± 4.3
6,000 < FCSA ≤ 9,000	15.1 ± 1.5	17.5 ± 1.5	16.6 ± 2.2	15.1 ± 1.5
FCSA > 9,000	5.8 ± 1.4 a	10.1 ± 1.5 b	8.6 ± 0.9 b	9.4 ± 2.2 b

The R values of both fit regressions (log-normal and linear regression) were significant ($p < 0.0001$) in all groups. For further explanation see "Statistics"

Different letters correspond to significant different groups ($p < 0.05$)

[a] Log-normal regression (four parameters) adjusts the profiles shown in Fig. 1 to the following equation: $F = y_0 + a \times \exp(-0.5 \times (\ln(x/x_0)/b)^2)$

[b] Data was linearised by $\ln(x + 1)$ transformation and follow the equation: $F = y_0 + a \times x$

[c] N corresponds to the number of individual data from five fish for each condition

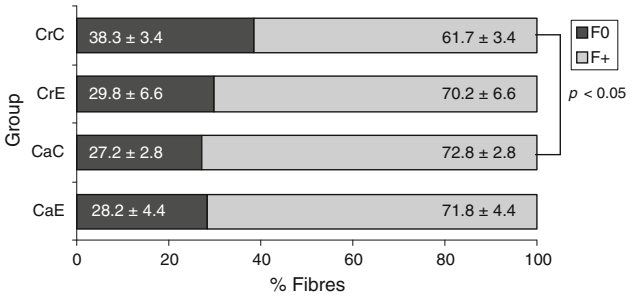

Fig. 3 White muscle capillary surrounding. *Bar chart* showing the percentage of fibres with no surrounding capillaries (F0) and the percentage of fibres contacting at least one capillary (F+). *CrC* cranial-control, *CrE* cranial-exercise, *CaC* caudal-control, and *CaE* caudal-exercise. Data are mean ± SEM of five animals. Significant differences between groups are indicated on the graph

the reduction of dietary protein did not limit energy availability as fish were fed to apparent satiety and thus, the levels of dietary protein proposed (37.2%) should be not considered as a limiting factor for growth. In studies on other species, mainly salmonids, differences in fibre size and number along the length of the body (Stickland 1983; Mascarello et al. 1995) or in ventral and dorsal regions (Kiessling et al. 1991) have been reported. Our data show that untrained gilthead sea bream (with body weight of approximately 100 g) had cranial fibres with lower cross-sectional areas and perimeters than caudal fibres. However, Abdel et al. (2005) did not show significant differences in white muscle size distribution when measuring muscle cellularity in the cranial and caudal areas of sea bass (*D. labrax*) with body weights of over 350 g. Although there are few published data, this disparity could be related to the fish size and age, which would mean that muscle growth potential is not the same in early stages of live as in adults. In fact, fish muscle plasticity has been strongly linked to the existence of seasonal cycles (reviewed by Johnston 1999).

Our results also show that the dynamics of gilthead sea bream growth are highly sensitive to swimming regimes. Thus, induced swimming at 1.5 b.l.s^{-1} affected white muscle fibres at cranial level, with increases in both area and perimeter values, but it did not modify the caudal fibres. Larger fibre sizes led to increased muscle masses and a higher total body weight, supporting the theory that normal sedentary rearing conditions under-exploit the growth potential of fish muscle. Indeed, exercise is a powerful stimulus in muscle hypertrophy, although the effects of muscle recruitment on hyperplasia have not been determined (Johnston 1999). Little is known about the origin of new fibres produced during the post-larval growth phase, and hyperplasic and hypertrophic growth usually occurs simultaneously in fish (reviewed by Johnston 1999). Nevertheless, in fast-growing fish, including cultured species that reach larger sizes, hyperplasia usually continues for longer than in small fish, in which hyperplasia stops earlier and hypertrophy is a more effective growth mechanism (Kiessling et al. 1991; Valente et al. 1999; Rowlerson and Veggetti 2001; Aguiar et al. 2005). In the current trial, the changes observed in the distribution of the muscle fibre cross-sectional area were interpreted as markers of changes in the relative contribution of hyperplasic and hypertrophic growth processes. Muscle protein percentage and total muscle DNA content remained invariable, which indicates that hypertrophy is the main muscle-growth mechanism in this short-term exercise, although hyperplasic effects cannot be ruled out, since the test period was short (1 month).

Few studies exist on muscle capillarity in fish, but all show that fish white muscle is poorly capillarised compared with mammal muscles, reflecting the low O_2 fluxes that fish white muscle needs. Only highly aerobic fish muscle presents high capillary-to-fibre number ratios. In carp, it was 2.2 at 28°C, but increased to 4.8 as the fibre size increased with acclimation to 2°C (Johnston 1982a); in tuna red muscle it was below 2, but with a relatively small fibre size (Mathieu-Costello et al. 1996), and it was 12.9 in the highly aerobic muscle of anchovy (Johnston 1982b). Therefore, the fact that 30–40% of the fibres in the white muscle of gilthead sea bream had no surrounding capillaries (F0) should be not surprising. Apart from escape bouts or prey capture, the low capillarisation of fish white muscle paralleled very low tissue metabolic activity rates and slow oxygen and nutrient supplies, these being lower in the cranial location of untrained fish than in exercised. The induced sustained activity, however, increased the number of capillaries in contact with each fibre (NCF) in the caudal area. Capillarisation in this zone relates to a higher number of pink fibres in the caudal area and, as a consequence, a greater need for oxygen.

In general, fish appetite is stimulated by activity and exercised fish generally consume more food. Nevertheless, a unit mass of growth is achieved when a lower amount of food is consumed, and the mass gain is achieved faster (Davison 1997). Another important aspect of the present study is that for gilthead sea bream under the conditions studied, muscle growth, and consequently whole-body growth, was achieved without significant increases in food consumption. Moreover, the lipid content of white muscle diminished in trained fish, an important aspect that can contribute to increased muscle quality, thus avoiding the excessive fat deposition of many cultured fish species (Cakli et al. 2007; Grigorakis 2007).

Conclusions

All the present results lead to the conclusion that firstly, moderate sustained activity improves whole-body growth

through hypertrophic white muscle growth, and secondly, through its effects on the use of tissue reserves and nutrients, the exercise would contribute to a reduction in water contamination by lowering nitrogen waste from dietary protein. The markedly higher white muscle development was seen in the cranial region.

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