Title

Growth-promoting effects of sustained swimming in fingerlings of gilthead sea bream (*Sparus aurata* L.)

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ABSTRACT

Fish growth is strongly influenced by environmental and nutritional factors and changing culture conditions can help optimize it. The importance of early-life experience on the muscle phenotype later in life is well known. Here we study the effects of five weeks of moderate and sustained swimming activity (5BL·s^{-1}) in gilthead sea bream during early development. We analysed growth and body indexes, plasma IGF-I and GH levels, feed conversion, composition (proximate and isotopic ($^{15}N/^{13}C$)) and metabolic key enzymes (COX, CS, LDH, HOAD, HK, ALAT, ASAT) of white muscle. Moderate and continuous exercise in fingerlings of gilthead sea bream increased plasma IGF-I, whereas it reduced plasma GH. Under these conditions, growth rate improved without any modification to feed intake through an increase in muscle mass and a reduction in mesenteric fat deposits. There were no changes in the content and turnover of muscle proteins and lipid reserves. Glycogen stores were maintained, but glycogen turnover was higher in white muscle of exercised fish. A lower LDH/CS ratio demonstrated an improvement in the aerobic capacity of white muscle, while a reduction in the COX/CS ratio possibly indicated a functional adaptation of mitochondria to adjust to the tissue-specific energy demand and metabolic fuel availability in exercised fish. We discuss the synergistic effects of dietary nutrients and sustained exercise on the different mitochondrial responses.

Key words: exercise, white muscle, $\delta^{13}C/\delta^{15}N$, IGF-I/GH, RNA/DNA, COX/CS

Introduction

For several species of fish, especially salmonids, moderate exercise (i.e. training) induced by a slow water current enhances growth rate, being often accompanied by improved food conversion (Davison 1997). Previous studies by our group observed similar effects of sustained swimming in juvenile gilthead sea bream (Ibarz et al. 2011; Martín-Pérez et al. 2012), a fish species with rapidly increasing significance in the aquaculture of the Mediterranean region (Apromar 2014). Moreover, there is evidence that fish exposed to moderate water currents exhibit less aggressive behaviour (Christiansen et al. 1992). Lower levels of circulating stress hormones would act lowering the metabolic rate and exerting an energy-saving effect. In line with this theory, gilthead sea bream expend 2.5-fold more energy under spontaneous (i.e. voluntary) activity than when they are forced to swim at a speed of 0.5 body length per second ($BL \cdot s^{-1}$) (Steinhausen et al. 2010). It is clear that spontaneous swimming costs may be considerably higher than those of a sustained swimming speed. In this regard, fish seem to adapt their metabolism to nutritional regime and environmental conditions and depend on their ability to match fuel supply to energy use in order to grow (Magnoni et al. 2013). Thus, sustained swimming can enhance the utilization of dietary carbohydrates on a low-protein, high-carbohydrate regime, and spare the use of dietary proteins for muscle growth in both rainbow trout, *Oncorhynchus mykiss* (Felip et al. 2012) and gilthead sea bream (Martín-Pérez et al., 2012; Felip al. 2013).

In general, the response of fish to chronic submaximal swimming is qualitatively similar to endurance exercise training in mammals, and it results in the emergence of a more aerobic phenotype (Johnston and Moon 1980; McClelland et al. 2006; Le Moine et al. 2010; and reviewed by McClelland and Scott 2013). This is consistent with results observed in juvenile gilthead sea bream (Martín-Pérez et al. 2012). The selection of farmed fish on the basis of growth performance, coupled with a sedentary life-style and high-energy diet, has resulted in suboptimal production efficiency (Tørud and Hillestand 2004), given that these are symptoms of a reduced ability to perform aerobic exercise (Claireaux et al. 2006). Enders et al. (2004) indicated that domestication could lead to lower swimming capacity of fish. Since a hatchery provides a regular food supply and protection from predators, it leads to a sustainable growing environment and allows individuals with low fitness levels to survive (Anttila et al. 2008). Rearing units adjust water flow to maintain water quality and avoid pathologies. As a consequence, they create an environment where fish experience few physical challenges in terms of their swimming capacity.

The importance of early-life experience on the muscle phenotype later in life has long been recognized, but only recently has the influence of developmental exposure on muscle plasticity and swimming performance in adult fish been explicitly demonstrated (Scott and Johnston 2012). Whether the beneficial effects of exercise on growth performance can be induced during the early development of gilthead sea bream is not known. Nevertheless, we observed that certain rearing conditions in three different hatcheries affected the growth rate of fingerlings of this species and, more interestingly, that some of these characteristics were maintained for a long time, even under new feeding regimes and culture conditions (Martin-Pérez et al. 2011). In that study we used, for the first time, the potential of stable isotopes (SIA) of tissue deposits combined with metabolic and growth parameters to discriminate fish seeds with a higher growth capacity and to relate it to their previous history.

The aim of the present work was to analyse the effects of sustained and moderate exercise in the early growth period of gilthead sea bream. To achieve this, fingerlings were maintained under optimal farming conditions. One group was forced to swim for five weeks and the other was maintained without induced water current, showing only spontaneous swimming, acting as a control. Specifically, we analysed growth rate, food conversion rate, white

muscle composition and metabolism (isotopic fingerprint of energy reserves, RNA/DNA content and key metabolic enzymes activity). Plasma growth hormone (GH) and insulin growth factor (IGF-I) levels were also analysed. Although there is no general consensus on the positive correlation between specific growth rate (SGR) and plasma GH levels, it is well known that IGF-I mediates the growth-promoting effects of GH in teleost fish (*reviewed* by Jönsson and Björnsson 2002). However, the effects of exercise on the GH/IGF-I axis are not well known, and we related all these variables to the increased growth promotion observed in the exercised group.

Material and methods

Experimental design

Five hundred and forty fingerlings of gilthead sea bream (5.05 ± 0.09 g body weight) obtained from a hatchery in Northern Spain were held in the facilities of the Faculty of Biology (University of Barcelona, Spain). Fish were randomly distributed into eight circular tanks equipped with a semi-closed recirculation system and physical and biological filters at 23ºC and with 15L:9D photoperiod at a stocking density of 1.5 kg/m^3 (65-70 fish per tank). Four 200-L tanks were kept in standard rearing conditions, with a water flow of 350 L/h and vertical water inflow. In these conditions fish presented only spontaneous, voluntary movements and were used as a control group. Other four 400-L tanks were equipped with a cylindrical tube in the central area, which resulted in a cylinder-toric living area corresponding to an effective space of 200 L. These were maintained with a water flow of 700 L/h and a circular, uniformly distributed flow induced by a perpendicular water inflow at the surface and an additional lateral tube with holes to maintain the flux in the water column. The resulting flow was adjusted to a swimming velocity of 5 BL·s^{-1}, in line with other studies (Ibarz et al. 2011; Martin-Pérez et al. 2012). The water current was measured at three tank depths (near the surface, mid-tank and near the bottom) using a low-speed mechanical flow meter (General Oceanics Inc., Miami, FL, USA). During the five-weeks experimental period, both groups were fed by hand with a commercial diet (Gemma Diamond, Skretting) three times per day (7 a.m., 2 p.m. and 10 p.m.) until apparent satiety. Feed intake was recorded on a daily basis for each tank and the food conversion ratio (FCR) was calculated as fish biomass gained per feed administered. Body weight, standard length and total length were measured at 21 and 35 days after the start of the trial. Specific growth rate (SGR = 100 · (ln final body weight - ln initial body weight) ·day^{-1}) was calculated for each tank biomass, weighing all fish from the tanks and averaging fish weights at the beginning and the end of the experimental period (n=4 for each condition).

Sample preparation

At the end of the experiment, 12 fish from each treatment were captured (three fish at random per tank, four tanks per experimental condition), anaesthetized, weighed and sized. Samples of blood from caudal vessels were obtained with heparinizing syringes. After centrifugation, plasma aliquots were frozen in liquid nitrogen and kept at -80°C until IGF-I and GH analysis. The animals were then killed by sectioning the spinal cord and eviscerated. The weight of mesenteric fat and liver was obtained and the percentage of the mesenteric-fat index, hepatosomatic index and muscle-somatic index was calculated. Samples of epaxial white skeletal muscle under the dorsal fin were dissected and frozen immediately in liquid nitrogen and kept at -80ºC. The experiments complied with the guidelines of the Council of the European Union (86/609/EU), the Spanish government (RD 1201/2005) and the University of Barcelona (Spain) for the use of laboratory animals (DAAM 7644).

Analytical procedures

Plasma GH and IGF-I analysis and muscle proximal composition

Plasma GH levels were assayed using a homologous gilthead sea bream radioimmunoassay (RIA) in accordance with the procedure described previously (Martínez-Barberá et al. 1995). This assay has a sensitivity of 0.15 ng/mL and a mid-range of 1.8 ng/mL. Plasma IGFs were extracted by acid-ethanol cryoprecipitation (Shimizu et al. 2000), and the IGF-I concentration was measured by means of a generic fish IGF-I RIA validated for Mediterranean perciform fish (Vega-Rubín de Celis et al. 2004). The sensitivity and mid-range of the assay were 0.05 and 0.7-0.8 ng/mL, respectively.

Samples of white muscle were ground in liquid N_2 using a pestle and mortar to obtain a fine powder. Aliquots of each sample were taken for use in isotopic analyses and to assess the lipid, protein, glycogen and water content. Water content was determined gravimetrically after drying the samples at 95°C for 24 h. Lipids were extracted as described by Blight and Dyer (1959), and lipid extracts were dried under a N_2 atmosphere and total lipids determined gravimetrically. Proteins were purified from defatted tissue samples via precipitation with 10% (v/v) trifluoroacetic acid. The extracts were dried using a vacuum system (Speed Vac Plus AR, Savant Speed Vac Systems, South San Francisco, CA, USA) and the protein content was calculated from the total N content obtained by elemental analysis (Elemental Analyser Flash 1112, ThermoFinnigan, Bremen, Germany), assuming 1 g of N for every 6.25 g of protein. Glycogen was extracted and purified from tissues following alkaline hydrolysis by boiling with 30% KOH and an alcoholic precipitation, as described by Good et al. (1933). Glycogen content was then assessed using the anthrone colorimetric method described by Fraga (1956).

Nucleic acid quantification and metabolic enzyme activity

Muscle nucleic acid levels (RNA and DNA) were determined using the UV-based procedures for fish samples described by Buckley and Bulow (1987). RNA and DNA from muscle samples were hydrolysed to nucleotides, and their concentrations were calculated based on their absorbance at 260 nm. Nucleic acid concentrations were expressed as μg of RNA or DNA per mg of wet tissue. An aliquot of supernatant was also used to determine protein content (Bradford 1976).

Enzyme activity was assayed from crude muscle extracts obtained by homogenizing frozen tissue (250 mg) in 2 mL of detergent solution (1.24 mM TRITON X-100, 1 mM EDTA, and 1 mM $NaHCO_3$) and stabilizing solution (3.7 mM EDTA and 5 mM 2-β-mercaptoethanol), 1:1 v/v. Homogenates were centrifuged at 700 g at 4°C for 10 min. The microtitration assays to obtain maximal enzyme activity were performed at room temperature (20ºC) in 96-well plates, as follows (final volume 200 μL): ASAT (aspartate aminotransferase, EC 2.6.1.1): 50 mM Tris-HCl buffer (pH 7.4), 10 mM α-ketoglutarate, 0.3 mM NADH, 25 mM L-aspartate (substrate); ALAT (alanine aminotransferase, EC 2.6.1.2): 50 mM Tris-HCl buffer (pH 7.4), 10 mM α-ketoglutarate, 0.3 mM NADH, 25 mM L-alanine (substrate); GDH (glutamate dehydrogenase, EC 1.4.1.2): 50 mM imidazole-HCl buffer (pH 8.0), 100 mM ammonium acetate, 0.2 mM NADH, 1 mM ADP, 2IU LDH, 10 mM α-ketoglutarate (substrate); LDH (lactate dehydrogenase, EC 1.1.2.4): 50 mM Tris-HCl buffer (pH 7.4), 0.16 mM NADH, 1 mM pyruvate (substrate). HK (hexokinase, E.C. 2.7.1.1): 71.4 mM imidazole buffer (pH 7.4), 100 mM $MgCl_2$, 50 mM ATP, 8 mM NADP, 200 mM glucose 6-phosphate dehydrogenase (substrate). HOAD (3-hydroxiacyl CoA dehydrogenase, E.C 1.1.1.35): 71.4 mM imidazole buffer (pH 8.0), 2 mM NADH, 2 mM acetoacetyl-CoA (substrate).

CS (citrate synthase, EC 2.3.3.1) activity was determined from absorbance increases at 412 nm of DTNB reagent, using oxaloacetic acid as the substrate, following the method described by Srere (1969). COX (cytochrome-c-oxidase, EC 1.9.3.1) activity was determined by adapting a commercial kit (CYTOC-OX1, Sigma-Aldrich Inc., St. Louis, MO, USA). This colorimetric assay measures the reduction in ferrocytochrome c absorbance caused by oxidation of the latter by COX. Enzymatic activity measurements were performed in duplicate and expressed in milliunits (mUI) per mg of wet tissue, one unit being the amount of enzyme that converts 1 μmol of substrate per min. Another aliquot of the supernatant was used to determine the protein content in accordance with the Bradford method.

Isotopic composition analysis ($\delta^{15}N$ and $\delta^{13}C$)

Samples of diet and white muscle were lyophilized and grounded into a homogenous powder for isotopic analysis. Aliquots of the diet and their purified fractions (lipid and protein), and of white muscle, together with their purified tissue fractions (glycogen, lipid and protein), which ranged from 0.3 mg to 0.6 mg, were weighed in small tin capsules. Samples were analysed to determine the carbon and nitrogen isotope composition using a Mat Delta C Isotope Ratio mass spectrometer (Finnigan MAT, Bremen, Germany) coupled to a Flash 1112 Elemental Analyzer. Isotope ratios (^{15}N /^{14}N, ^{13}C /^{12}C) determined by isotope-ratio mass spectrometry are expressed in delta (δ) units (parts per thousand, ‰), as follows:

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

where R_{sa} is the ^{15}N /^{14}N or ^{13}C /^{12}C ratio of samples and R_{st} is the ^{15}N /^{14}N or ^{13}C /^{12}C ratio of the international standards (Vienna Pee Dee Belemnite for C and air for N). The same reference material analysed over the experimental period was measured with ±0.2‰ precision. Nitrogen and carbon isotopic fractionation values ($\Delta\delta^{15}N$ and $\Delta\delta^{13}C$) were calculated as the difference between the δ value in the tissue and their corresponding δ value in the diet.

Statistics

The results of tissue composition, nucleic acid contents, IGF-I and GH plasma levels, enzymatic activity and isotopic analyses are presented as mean ± standard error of the mean (SEM). A two-way ANOVA was used with activity (exercise (E) or control (C) groups) as a fixed factor and tank as a random factor, after checking for normal distribution of data and equality of variances using the Shapiro-Wilk and Levene's test, respectively. As there were not significant effects of factor tank for any of the variables analysed, activity factor comparing 3 fish per 4 tanks resulted in an n of 12 individuals per condition. Two-way ANOVA (activity and time) and Tukey's post-hoc test were used to analyse fish body weight (n=4). All statistical analyses were performed using SPSS v.16 (SPSS Inc., Chicago, IL, USA).

Results

The effects of sustained swimming on the growth performance and feed intake of gilthead sea bream fingerlings are shown in Figure 1 and Table 1, respectively. Exercised fish significantly ($p<0.05$) increased body weight after 20 days of moderate swimming, being 20% larger than control fish after five weeks of experiment ($p<0.001$). Although SGR showed the same tendency, no significant differences were observed ($p=0.059$). Feed intake did

not change significantly as a result of exercise, and exercised fish showed similar FCR with respect to the control group (Table 1). The hepato-somatic and muscle-somatic indexes did not differ significantly between groups, but the percentage of mesenteric fat significantly decreased in exercised fish compared to control fish (Table 1).

A slight but significant increase ($p<0.01$) in plasma IGF-I levels was found in exercised fish (Figure 2a). At the same time, a significant two-fold decrease was found in GH levels ($p<0.01$) (Figure 2b).

Table 2 shows the changes in nucleic acids contents, proximate composition and isotopic levels of the white muscle of gilthead sea bream fingerlings. White muscle RNA and DNA concentrations did not change significantly in response to exercise. Proximate composition of muscle (protein, lipid and glycogen content) did not differ between groups. $\delta^{13}C$ values of muscle glycogen in exercised group were significantly lower than those of control group, revealing turnover differences of this reserve between both groups. The isotopic composition of the other two energy stores did not change significantly. ^{15}N-fractionation in muscle tissue and in the protein fraction did not vary, which indicates that there was no change in the isotopic fractionation of the protein gained in white muscle in the exercised group.

The enzyme activities of aerobic (CS, COX) and anaerobic metabolism (LDH), protein catabolism (ALAT, ASAT, GDH), β-oxidation (HOAD) and glucose use (HK) are shown in Table 3. Muscle CS activity increased three-fold ($p<0.001$), while COX activity decreased by 30% ($p<0.05$) in exercised fish compared to control fish. No significant changes were observed in ALAT, ASAT, GDH or HOAD activities, but HK activity decreased significantly by 30% ($p<0.05$) in exercised fish compared to control fish. Figure 3a shows the relationship between LDH and CS activity and reveals that the high anaerobic capacity of the white muscle of gilthead sea bream is maintained under exercise, but that the aerobic capacity of this muscle also increases. However, COX and CS did not show any correlation (as shown in Figure 3b). It is worth noting that individual variability in most of the studied variables, including COX, was much higher in the control group, than in the exercised group.

Discussion

Many studies have shown the positive effects of swimming activity on growth rate in adult fish, and McDonald et al. (1998) and Yogata and Oku (2000) also observed the growth-promoting effects of exercise in fingerlings of Atlantic salmon (*Salmo salar*) and yellowtail (*Seriola quinqueradiata*), respectively. In the present work, moderate and sustained exercise improved the growth performance of gilthead sea bream fingerlings, in line with previous findings in juveniles of this fish species (Ibarz et al. 2011; Martin-Pérez et al. 2012). As mentioned above, this is primarily due to the enhanced utilization of nutrients (Ibarz et al. 2011, Martín-Pérez et al. 2012), whereas the growth-promoting effects of aerobic training in salmonids is mostly driven by the increased feed intake (Davidson 1997; Felip et al. 2013).

It is important to note that the energy partitioning of dietary nutrients depends on feeding status and diet composition (Cho et al. 1982). When energy demand changes, as it occurs in exercise, the use of energy fuels depends on the intensity of the activity and the species (*reviewed* by Magnoni et al. 2013). In previous studies on gilthead sea bream, we observed that sugars are preferentially used as metabolic fuels in juveniles subjected to moderate activity and fed a low-protein/high-carbohydrate diet. In this scenario, COX activity increased in white muscle, whereas a parallel reduction in CS activity was reported (Martin-Pérez et al. 2012). This implies that the entry of amino acids into the tricarboxylic acid cycle is reduced and there is an increase in growth through a

protein- sparing effect. In the present study, fingerlings of gilthead sea bream were fed a high-protein diet under sustained swimming. Therefore, in order to obtain energy, amino acid oxidation had to increase in the exercised group, as reflected by the significant increase in mitochondrial CS activity. A meta-analysis of microarray gene expression profiling shows that mitochondria are among the first responders to nutritional and environmental stressors in gilthead sea bream (Calduch-Giner et al. 2014). For instance, the gene expression of CS is up-regulated by thermal stress to cope with the enhanced oxidative capacity (Bermejo-Nogales et al. 2014). In contrast, the same study showed that mitochondrial activity and CS in particular are down-regulated by multiple sensory perception stressors. White muscle CS activity also increases under moderate activity in zebrafish (*Danio rerio*) (McCelland et al. 2006), but a significant reduction has been observed in rainbow trout after four weeks at 1.5 $BL \cdot s^{-1}$ (Morash et al. 2014), which demonstrates a complex and sometimes contradictory regulation. In exercised brown trout (*Salmo trutta*), the COX activity of skeletal muscle also increased at a low velocity (42% of maximal swimming capacity), but decreased when the swimming capacity increased to 83% of maximal capacity (Antilla et al. 2010). In the present study, COX activity also decreased in exercised fingerlings, which emphasizes the fact that the switch of metabolic flux towards aerobic and glycolytic metabolism varies with energy demand and the oxidative capacity of the fish species at a given allostatic load. In that respect, the statistically significant reduction in LDH/CS ratio in exercised fish would be indicative of an enhanced aerobic phenotype, but here it was concurrent with the reduction in the COX/CS ratio in the white muscle of exercised gilthead sea bream. The enhanced aerobic phenotype was also observed in the white muscle of zebrafish through increased CS activity (McClelland et al. 2006; Le Moine et al. 2010) and in the COX increase in the white muscle of juvenile gilthead sea bream (Martín-Pérez et al. 2012). A possible explanation is that sustained exercise reduces energy wastage and decreases mitochondrial respiration uncoupling, which serves as a safety valve when the energy supply exceeds the energy demand or the oxidative capacity of the tissue. This futile cycle is mostly driven by uncoupling proteins that are expressed at a higher level in the glycolytic skeletal muscle of gilthead sea bream than in highly oxidative muscle tissues (e.g. cardiac muscle and red skeletal muscle) (Bermejo-Nogales et al. 2014). At the same time, the low mitochondrial density and poor vascularization of fish white muscle (Johnston and Moon 1981) may limit maximal rates of protein synthesis (Pelletier et al. 1993), and the increase in the mitochondrial enzyme activity of axial muscle serves to meet the enhanced demand for aerobic ATP generation caused by increased growth rates (Goolish and Adelman 1987). All of this demonstrates the great metabolic plasticity of fish to adjust tissue-specific demands in response to exercise and energy availability. Indeed, a recent study on gilthead sea bream indicated that the transcriptional regulation of almost all enzyme subunits of the mitochondrial oxidative-phosphorylation (OXPHOS) pathway are regulated by fasting in a tissue-specific manner according to the different metabolic capabilities of liver, cardiac muscle and glycolytic white skeletal muscle (Bermejo-Nogales et al. 2015).

We previously observed that sustained and moderate swimming reduced the lipid content of white muscle in gilthead sea bream juveniles (Ibarz et al. 2011). After four weeks of swimming, fingerlings of this species presented no change in white muscle lipid content, HOAD activity (a marker of β-oxidation pathway) or $\delta^{13}C$ values of lipid deposits. All these results indicate that the turnover of lipid deposits did not change in the white muscle of the exercised group compared to the control group, which is consistent with the results obtained for brown trout submitted to five weeks of submaximal and continuous exercise (Antilla et al. 2010). Although aerobic enzyme activity increases in the white muscle of zebrafish (McClelland et al. 2006) and brown trout (Antilla et al. 2010), HOAD levels remained unchanged, which shows that lipids are not the preferred fuel during any type of

exercise. However, there are some conflicting results relating to muscle HOAD activity after training; endurance training in the brook trout increases HOAD activity in red and white muscles, which would indicate an increase in fatty acid oxidation (Johnston and Moon 1980), but the HOAD activity of zebrafish submitted to a progressive training program from 2 to 5 BL/s remains unchanged (McClelland et al. 2006). With respect to the use of lipids while swimming, some authors have observed a reduction in lipid content after training when the whole fish is taken into account (red sea bream: Forster and Ogata 1996; rainbow trout: Lauff and Wood 1996). According to these findings, we have observed a reduction in the mesenteric fat content in fingerlings of gilthead sea bream under exercise. Therefore, the maintenance of muscle lipid content would indicate a good balance between dietary lipid supply and its use as fuel for energy and growth, without the fat deposition presented by the control group.

The total glycogen store in white muscle was maintained in exercised fingerlings of gilthead sea bream, but the $\delta^{13}C$ levels of this reserve decreased approaching the $\delta^{13}C$ values of the dietary protein. This is a good indicator of a higher rate of *de novo* synthesis of glucose (and then of glycogen) with significant incorporation of carbon skeletons from dietary proteins, which presented those lower $\delta^{13}C$ values.

Although gluconeogenesis from dietary amino acids should be increased in liver and thus glucose exported to peripheral tissues, including muscle, plasma glucose levels were not modified by exercise (control: 2.7 mM ± 0.13; exercise: 2.9 ± 0.19). At the same time, HK activity in white muscle decreased compared to the control group. Sánchez-Gurmaches et al. (2013) found lower insulin and higher IGF-I levels in the plasma of juveniles of gilthead sea bream submitted to sustained swimming. The increase in plasma IGF-I concentration was also observed in the present study, but insulin was not measured. The lower levels of muscle HK activity, possibly associated with lower plasma insulin, may indicate that the use of dietary carbohydrates was not enhanced under the swimming conditions of the present study, but it merits further study.

Regarding proteins, N fractionation (a good marker of protein turnover, Martín-Pérez et al. 2011) was unchanged in exercised fingerlings compared to control fish. A similar observation was found in exercised juveniles (Martín-Pérez et al. 2012). Moreover, exercise did not modify ASAT or ALAT activity. Therefore, a positive balance between protein synthesis and degradation would explain the higher growth rate observed in exercised fingerlings of gilthead sea bream, and may be promoted by the high levels of IGF-I. IGF-I and GH are the main regulatory hormones of fish growth (Reindl and Sheridan 2012), and gilthead sea bream in particular (Mingarro et al. 2002), although the role of these hormones in exercise is not fully understood. Thus, an increase in circulating levels of plasma GH has been reported in coho salmon (*Oncorhynchus kisutch*) (Barrett and McKeown 1988a) and rainbow trout (Barrett and McKeown 1988b; Nielsen et al. 1994) submitted to submaximal swimming, but a close association between exercise-induced growth and circulating levels of GH and IGF-I has not been reported in masu salmon (*Oncorhynchus masou masou)* (Azuma et al. 2002). This contrasts with the observations made in gilthead sea bream, in which moderate exercise increased plasma IGF-I levels, both in this study and previous studies on juvenile fish (Sánchez-Gurmaches et al. 2013). Moreover, we found that this endocrine feature was related to a pronounced reduction in plasma GH levels, which was probably due to the negative feedback inhibition of systemic IGF-I upon pituitary GH synthesis and release. Experimental evidence for this has previously been reported for primary cultures of rainbow trout pituitary cells (Pérez-Sánchez et al. 1992). Therefore, the best growth performance, in both salmonids and gilthead sea bream, is achieved with high levels of IGF-I and low levels of GH, regardless of age and nutritional condition (Pérez-Sánchez and Le Bail 1999; Gómez-Requeni et al. 2004; Benedito-Palos et al. 2007). In our experimental model, this was the case for the exercised fish. In this regard, the

significant increase in body growth and the tendency towards higher muscle somatic index without changes in DNA concentration might indicate that hypertrophic processes are produced in white muscle, which correspond to a protein gain in white muscle, as observed in exercised rainbow trout (Houlihan and Laurent 1987). Moreover, a tendency for the RNA content of exercised fingerlings to increase compared to control fish would indicate that the synthetic capacity of white muscle is enhanced in the former. Both hypertrophy and a higher RNA content were also observed in the white muscle of juveniles (Ibarz et al. 2011; Martin-Pérez et al. 2012), but further histological studies are required to confirm this.

Interestingly, this study also showed that moderate exercise reduces the individual variability for most of the variables analysed, especially in terms of body weight and enzyme activities (LDH, COX). Jobling et al. (1993) found that groups of salmonids exposed to water currents were less heterogeneous than groups held under static water conditions. Thus, normal sedentary rearing conditions, as found in the control group, produce more interactions between individual fish (Christiansen and Jobling 1990). The sudden changes in spontaneous activity are associated with substantially higher energy costs compared with fish under sustained swimming at a constant velocity (Steinhausen et al. 2010). The different levels of efficiency in the use of energy between the two groups of fish affected their growth rates.

In conclusion, moderate and continuous aerobic exercise improves body growth and nutrient use in gilthead sea bream fingerlings, expands the aerobic capacity of white muscle and reduces mesenteric fat deposits. These changes in muscle growth and metabolic signatures may occur at later stages if the training is maintained, although further studies are required to fully explore the potential benefits of exercise in fish farming. In this sense, considering that fingerlings and juveniles present similar response to swimming activity, it seems that the sooner this is applied the better the benefits for sustainable production.

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Figure captions

Fig. 1 Effect of moderate sustained swimming on body weight of gilthead sea bream fingerlings. Values are mean ± SEM (n= 4). Significant differences were considered at $p<0.05$ (*), $p<0.001$ (***)

Fig. 2 Effect of moderate sustained swimming on plasma IGF-I (a) and GH (b) levels in gilthead sea bream fingerlings. Values are mean ± SEM (n=12). Significant differences were considered at $p<0.01$ (**)

Fig. 3 Effect of moderate sustained swimming on LDH/CS (a) and COX/CS (b) relationships in muscle gilthead sea bream fingerlings. Values are mean ± SEM (n=12). Pearson's correlation coefficients and p values: a) r= -0.194, p=0.37; b) r=-0.285, p=0.19

Table 1. Effect of moderate swimming activity on growth performance, feed intake and somatic indexes of gilthead sea bream fingerlings.

	Control	Exercise	
Initial body weight (g)	**4.97** ± 0.04	**5.14** ± 0.20	
Final body weight (g)	**17.54** ± 0.46	**20.28** ± 0.38	***
[a]SGR	**3.41** ± 0.08	**3.72** ± 0.13	
Feed intake (g/fish·day)	**0.37** ± 0.01	**0.36** ± 0.01	
[b]FCR	**1.17** ± 0.16	**0.93** ± 0.06	
[c]HSI	**1.12** ± 0.07	**1.27** ± 0.08	
[d]MSI	**34.89** ± 1.00	**37.27** ± 0.99	
[e]MFI	**1.37** ± 0.16	**0.81** ± 0.15	*

Values are mean ± SEM (n=4 for initial and final body weight, SGR, feed intake and FCR) (n=12 for HSI, MSI and MFI).

[a] Specific growth rate % =

= [100 (ln final body weight - ln initial fish weight)] $days^{-1}$.

[b] Food conversion ratio = (Feed intake·daily weight $gain^{-1}$)

[c] Hepato-somatic index (g liver·100g body $weight^{-1}$).

[d] Muscle-somatic index (g muscle·100g body $weight^{-1}$).

[e] Mesenteric fat index (g fat·100g body $weight^{-1}$)

Significant differences were considered at $p<0.05$ (*), $p<0.001$(***).

Table 2. Effect of moderate swimming activity on nucleic acid, proximate and isotopic composition of muscle of gilthead sea bream fingerlings.

	Control	**Exercise**
Protein (% w.w)	**19.47** ± 1.34	**19.02** ± 1.47
Lipids (% w.w)	**2.69** ± 0.43	**2.66** ± 0.41
Glycogen (% w.w)	**0.26** ± 0.02	**0.30** ± 0.03
RNA (µg/mg prot)	**4.88** ± 0.50	**5.84** ± 0.47
DNA (µg/mg prot)	**1.56** ± 0.20	**1.26** ± 0.13
RNA/DNA	**4.59** ± 0.43	**4.02** ± 0.37
δ^{13} C-muscle	**-19.80** ± 0.07	**-19.90** ± 0.09
δ^{13} C-protein	**-20.93** ± 0.33	**-20.51** ± 0.05
δ^{13} C-lipid	**-24.84** ± 0.11	**-24.84** ± 0.13
δ^{13} C-glycogen	**-19.14** ± 0.36	**-21.05** ± 0.36 *
δ^{15} N-muscle	**11.87** ± 0.03	**11.85** ± 0.02
δ^{15} N-protein	**12.84** ± 0.04	**12.77** ± 0.07
[a]Δ^{15} N-muscle	**2.33** ± 0.02	**2.33** ± 0.04

Values are mean ± SEM (n =12).

[a] Isotopic fractionation (δ^{15}N-muscle- δ^{15}N-diet).

δ^{15}N-diet: 9.50 ± 0.02; δ^{13}C-diet: -21.82 ± 0.04

Significant differences were considered at $p<0.05$ (*).

Table 3. Effect of moderate sustained swimming on the enzyme activities of muscle of gilthead sea bream fingerlings

	Control	Exercise	
Cox (UI/g w.w)	**0.34** ± 0.03	**0.25** ± 0.01	*
CS (UI/g w.w)	**0.36** ± 0.04	**1.07** ± 0.08	***
Cox / CS	***0.84*** *± 0.07*	***0.24*** *± 0.02*	*
LDH (UI/g w.w)	**475** ± 25.7	**467** ± 13.0	
LDH / (CS*1000)	***1.18*** *± 0.26*	***0.37*** *± 0.03*	*
ASAT (UI/g w.w)	**15.23** ± 0.70	**16.69** ± 0.7	
ALAT (UI/g w.w)	**1.38** ± 0.06	**1.35** ± 0.08	
GDH (UI/g w.w)	**51.85** ± 2.80	**52.76** ± 2.50	
HOAD (UI/g w.w)	**0.14** ± 0.01	**0.13** ± 0.01	
HK (mUI/g w.w)	**91.8** ± 10.0	**65.2** ± 5.0	*

Values are mean ± SEM (n =12).

Significant differences considered at $p<0.05$ (*), $p<0.001$ (***).

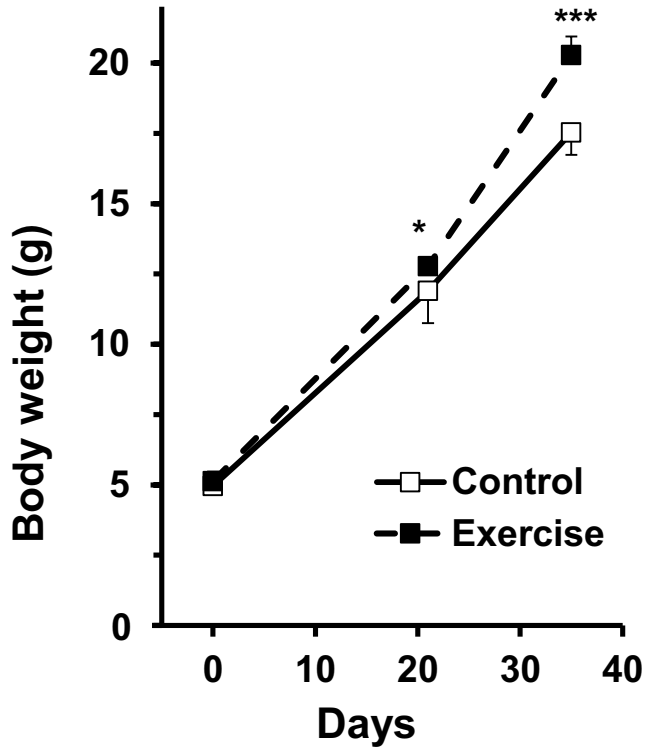


Figure 1

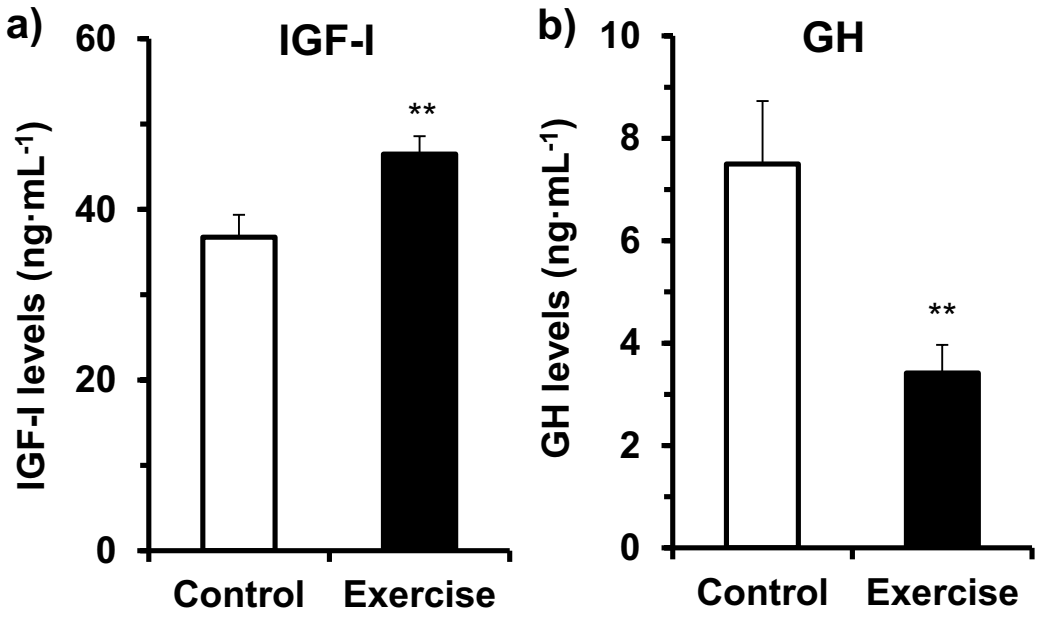
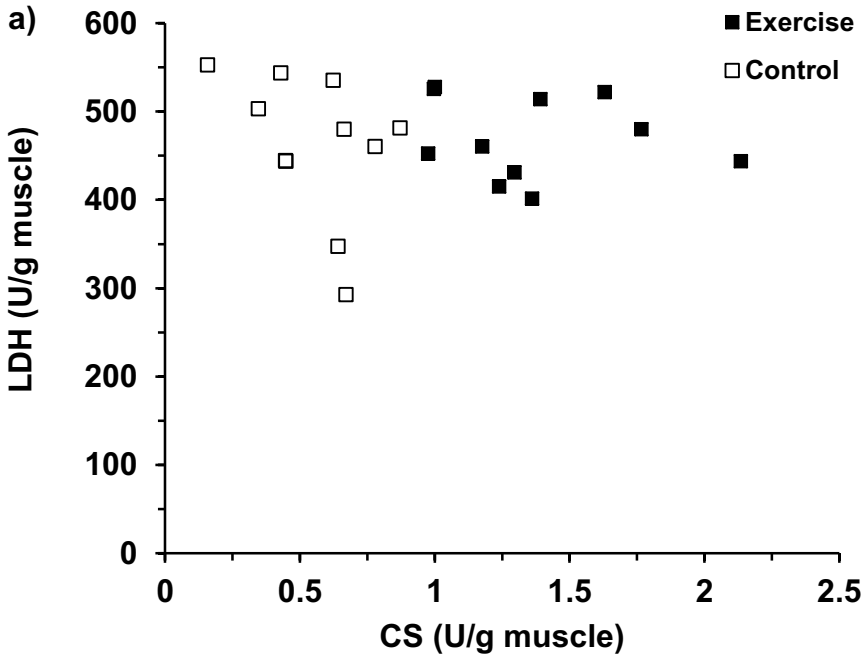

a)
IGF-I
IGF-I levels (ng·mL-1)
60
40
20
0
**
Control
Exercise
b)
GH
GH levels (ng·mL-1)
10
8
6
4
2
0
**
Control
Exercise

Figure 2



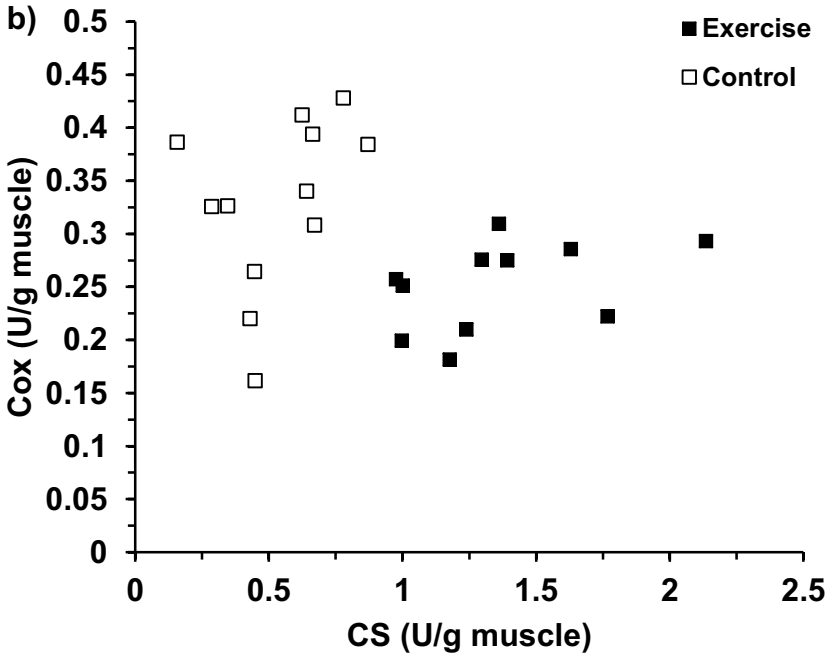


Figure 3a and 3b

Table 1. Effect of moderate swimming activity on growth performance, feed intake and somatic indexes of gilthead sea bream fingerlings.

	Control	Exercise	
Initial body weight (g)	**4.97** ± 0.04	**5.14** ± 0.20	
Final body weight (g)	**17.54** ± 0.46	**20.28** ± 0.38	***
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[e]MFI	**1.37** ± 0.16	**0.81** ± 0.15	*

Values are mean ± SEM (n =4 for initial ande final body weight, SGR, feed intake and FCR) (n=12 for HIS, MSI and MFI).

[a] Specific growth rate % =

= [100 · (ln final body weight - ln initial fish weight)] ·days^{-1}.

[b] Food conversion ratio = (Feed intake·daily weight gain^{-1})

[c] Hepato-somatic index (g liver·100g body weight^{-1}).

[d] Muscle-somatic index (g muscle·100g body weight^{-1}).

[e] Mesenteric fat index (g fat·100g body weight^{-1})

Significant differences were considered at $p<0.05$ (*), $p<0.001$(***).

Table 2. Effect of moderate swimming activity on nucleic acid, proximate and isotopic composition of muscle of gilthead sea bream fingerlings.

	Control	**Exercise**
Protein (% w.w)	**19.47** ± 1.34	**19.02** ± 1.47
Lipids (% w.w)	**2.69** ± 0.43	**2.66** ± 0.41
Glycogen (% w.w)	**0.26** ± 0.02	**0.30** ± 0.03
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δ^{13} C-lipid	**-24.84** ± 0.11	**-24.84** ± 0.13
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δ^{15} N-muscle	**11.87** ± 0.03	**11.85** ± 0.02
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[a]Δ^{15} N-muscle	**2.33** ± 0.02	**2.33** ± 0.04

Values are mean ± SEM (n =12).

[a] Isotopic fractionation (δ^{15}N-muscle- δ^{15}N-diet).

δ^{15}N-diet: 9.50 ± 0.02; δ^{13}C-diet: -21.82 ± 0.04

Significant differences were considered at $p<0.05$ (*).

Table 3

Table 3. Effect of moderate sustained swimming on the enzyme activities of muscle of gilthead sea bream fingerlings

	Control	Exercise	
Cox (UI/g w.w)	**0.34** ± 0.03	**0.25** ± 0.01	*
CS (UI/g w.w)	**0.36** ± 0.04	**1.07** ± 0.08	***
Cox / CS	***0.84*** *± 0.07*	***0.24*** *± 0.02*	*
LDH (UI/g w.w)	**475** ± 25.7	**467** ± 13.0	
LDH / (CS*1000)	***1.18*** *± 0.26*	***0.37*** *± 0.03*	*
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ALAT (UI/g w.w)	**1.38** ± 0.06	**1.35** ± 0.08	
GDH (UI/g w.w)	**51.85** ± 2.80	**52.76** ± 2.50	
HOAD (UI/g w.w)	**0.14** ± 0.01	**0.13** ± 0.01	
HK (mUI/g w.w)	**91.8** ± 10.0	**65.2** ± 5.0	*

Values are mean ± SEM (n =12).

Significant differences considered at $p<0.05$ (*), $p<0.001$ (***).