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# Moderate and sustained exercise modulates muscle proteolytic and myogenic markers in gilthead sea bream (*Sparus aurata*)

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**Running title:** Proteolytic and myogenic markers modulation by exercise

**Authors' contributions:** JFB, JB and JG conceived the study. EJV, ShA, EL and EC performed the *in vivo* experiments and laboratory analyses. All authors analyzed the data, contributed to the writing and discussion and approved the final manuscript.

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**Abstract**

Swimming activity primarily accelerates growth in fish by increasing protein synthesis and energy efficiency. The role of muscle in this process is remarkable and especially important in teleosts, where muscle represents a high percentage of body weight, and by the continuous growth that many fish species present. The aim of this work was to characterize the effects of five weeks of moderate and sustained swimming in gene and protein expression of myogenic regulatory factors, proliferation markers and proteolytic molecules in two muscle regions (anterior and caudal) of gilthead sea bream fingerlings. Western blot results showed an increase in the proliferation marker PCNA, proteolytic systems' members CAPN1 and CTSD, as well as vascular-endothelial growth factor protein expression. Moreover, quantitative real-time PCR data showed that exercise increased the gene expression of proteases: calpains, cathepsins and members of the ubiquitin-proteasome system in the anterior muscle region; and the gene expression of the proliferation marker PCNA and the myogenic factor MyoD in the caudal area, compared to control fish. Overall, these data suggest a differential response of the two muscle regions during swimming adaptation, with tissue remodeling and new vessels formation occurring in the anterior muscle and enhanced cell proliferation and differentiation in the caudal area. In summary, the present study contributes to improve the knowledge on the role of proteolytic molecules and other myogenic factors in the adaptation of muscle to moderate sustained swimming in gilthead sea bream.

**Keywords:** Growth, MRFs, proteolytic molecules, IGFs, VEGF, growth potential, swimming, *Sparus aurata*

## 1. Introduction

In fish, growth depends on the accretion of muscle, principally white skeletal muscle (48, 63), and compared with other vertebrates, differences in regulation exist among species. Whereas some fish species such as zebrafish, *Danio rerio* (6) follow a determinate-growth pattern similarly to mammals, most of the teleost species can grow in weight and length in an indeterminate way throughout their life (49, 92). Moreover, continuous fish muscle growth consists in hypertrophy (increase in fiber size) and hyperplasia (new muscle fibers formation) (90). These processes are the result of increased myogenesis, as well as down-regulation of growth inhibitors, as is the case of some members of the transforming growth factor beta (Tgf-ß) family (i.e. myostatin, MSTN) (62, 88). During myogenesis, muscle satellite cells, which can be recognized for the expression of Pax3/7 (34), turn into myoblasts that after proliferation and differentiation, fuse together or with existing fibers to form multinucleated myofibers (14, 48). In mammals, this process is modulated by key transcription factors called myogenic regulatory factors (MRFs), which are expressed in a sequential manner, being either essential for muscle lineage determination and cell proliferation (Myf5 and MyoD), or responsible for the initiation of the differentiation program from myoblast to myotubes (Mrf4 and Myogenin) (18, 103). In fish, these transcription factors have been also identified and a similar function to that in mammals has been demonstrated (9, 10, 16, 34, 36, 48, 56). In the last part of the myogenic process, and concomitant with the multinucleated myofibers formation, it is well known the necessity of structural and contractile proteins (e.g. actin or myosin), as it has been demonstrated in fish by means of both *in vivo* and *in vitro* experiments (36, 66).

Furthermore, muscle growth is the result of the positive balance between protein synthesis and degradation. The main endogenous proteolytic systems involved (ubiquitin-proteasome, calpains, cathepsins and caspases) are well known in mammals (5); and in the last years, different authors have identified and characterized some of these molecules also in fish (85-87). A few studies have shown in different species that these molecules are regulated by nutritional status (57, 75, 81). In this line, Salmerón and coauthors (82) have identified in gilthead sea bream (*Sparus aurata*), one of the most important species in Mediterranean aquaculture (31), few calpain members and demonstrated that diet composition and fasting/refeeding modulate their expression, being the changes well correlated with flesh texture. Moreover, it is commonly known

that protein turnover has a greater relevance during higher growth periods (i.e. fingerlings *versus* juvenile or adult fish) (72, 73, 83), and thus the proteolytic systems, should be considered to completely understand growth in fish. In this sense, an *in vitro* study with rainbow trout (*Oncorhynchus mykiss*) myocytes has demonstrated that, in comparison with mammals, the protein degradation in fish is mainly due to the autophagy-lysosomal system (including cathepsins), at the expense of a minor important role of the ubiquitin-proteasome system (85).

In addition to muscle growth, white muscle cellularity depends on various factors such as exercise (49, 50). In previous publications from this same experimental trial, Blasco et al., (8) and Vélez et al., (98) have demonstrated that five weeks of moderate sustained swimming stimulates growth in fingerlings of gilthead sea bream without increasing food intake. Furthermore, this exercise-related growth was modulated by an increase in the aerobic capacity of the muscle and the plasma insulin-like growth factor (IGF-I)/growth hormone (GH) ratio, variations on the mRNA levels of GH-IGFs axis-related genes in liver and muscle, and activation of the target of rapamycin (TOR) signaling pathway (98). Overall, these changes accelerate growth by improving both muscle protein synthesis and energy efficiency (8); however, complementary information on myogenic regulators or proteolytic systems to better picture muscle development and protein turnover was not reported, becoming the main objective of the present study.

Additionally, Egginton and Cordiner (28) have shown that the swimming performance is directly related with the capillary density in rainbow trout, being angiogenesis an integrated response to optimize aerobic performance. These authors also suggested that this process could be stimulated by different mechanical factors in muscle. Similarly, in zebrafish different authors have studied the increased capillarity, mitochondrial biogenesis and the muscle remodeling induced by swimming adaptation (43, 52, 61, 70). In gilthead sea bream juveniles forced to sustained and moderate swimming, a differential response/adaptation to exercise between the epaxial-anterior and the epaxial-caudal region of white muscle has been also reported (44). In this sense, Vélez and colleagues (98) found a differential regulation between regions of several members of the GH-IGFs axis in fingerlings of this species as an effect of swimming, highlighting an increase in IGF-II gene expression in the caudal region and that of IGFBP-5 in the anterior one. Moreover in another study, while in the anterior region the number of capillaries increased, in both anterior and caudal muscles, the fiber size was also enlarged with exercise (96), illustrating the different work of both muscle regions

in the *Sparidae* family during swimming. Regarding this, the classical theories proposed that most of the power for fish swimming is generated by the anterior muscle (95, 99), although in later studies it seems clear that this depends on the type of muscle considered in each region. Thereby, on red muscle the power comes mainly from the posterior region (23, 40, 47, 80, 91), whereas in the case of white muscle, the power is generated mostly by the anterior part (2, 29, 95). In any case, the anterior muscle has generally faster contractile properties than the posterior one for both red and white muscle types (22), and the recruitment of the different fiber types across the axis length of the muscle depends on the swimming speed and it is different among species (2, 21, 30).

The aim of this work was to characterize the gene and protein expression of MRFs, proliferation and angiogenic markers and proteolytic molecules in the anterior and caudal skeletal muscle regions of gilthead sea bream fingerlings after five weeks of moderate and sustained activity, completing the previous metabolic and endocrine studies reported from these fish (8, 98).

## 2. Material and methods

### 2.1. Animals and experimental trial

Five hundred and forty gilthead sea bream (*S. aurata* L.) fingerlings [~5 g body weight and 7 cm body length (BL)] were obtained from a hatchery located in the north of Spain, randomly distributed into eight 0.2 $m^3$ tanks, and maintained within a temperature-controlled seawater recirculation system at 23±1ºC at the facilities of the School of Biology at the University of Barcelona (Spain). The exchange rate of water was 35% per week and it was done through the filtering system without altering the inflow to the tanks; oxygen level was over 90% of saturation and the photoperiod was 15 h light/9 h dark. After the acclimation period, four tanks were kept with a vertical water inflow of 350 l/h (standard rearing conditions), where the fish presented only spontaneous and voluntary movements (control group); and in the other four tanks a circular uniformly distributed flow of 700 l/h was set up, where the fish were forced to undertake moderate and sustained swimming (5 BL/s) (exercise group), as reported before (8, 98). Fish were fed with a commercial diet (Gemma Diamond, Skretting, Burgos, Spain) three times a day, representing a 5% ration (until apparent satiety).

Biometric parameters (weight and length) were determined at the beginning of the experiment, and at the last time point (5 weeks) to monitor fish growth. Fish were fasted for 24 h before any manipulation and sampling. As previously reported (98), in the final sampling at week 5, twelve fish of each group (4 fish per tank) were sacrificed and samples of anterior (from 25% total length behind the tip of the snout to 40% total length) and caudal (from 60% to 75% total length) epaxial white muscle regions were collected, frozen in liquid nitrogen and stored at -80ºC until further analyses.

All animal handling procedures were conducted following the European Union Council (86/609/EU), Spanish and Catalan Government-established norms and procedures and with the Ethics and Animal Care Committee of the University of Barcelona approval (permit number DAAM 7644).

## 2.2. Gene expression

### 2.2.1 RNA extraction and cDNA synthesis

Total RNA was extracted from 100 mg of tissue with 1 mL of TRI Reagent Solution (Applied Biosystems, Alcobendas, Spain) as has been described before (98). Briefly, total RNA concentration and purity were determined using a NanoDrop 2000 (Thermo Scientific, Alcobendas, Spain). After verifying the samples integrity in a 1% agarose gel (w/v) stained with SYBR-Safe DNA Gel Stain (Life Technologies, Alcobendas, Spain), 1 µg of total RNA was treated with DNase I (Life Technologies, Alcobendas, Spain) to remove all genomic DNA following the manufacturer's recommendations. Finally, RNA was reverse transcribed with the Affinity Script$^{TM}$ QPCR cDNA Synthesis Kit (Agilent Technologies, Las Rozas, Spain).

### 2.2.2. Quantitative real-time PCR (qPCR)

The mRNA transcript levels of the following groups of interest (Table 1): proliferation marker, growth inhibitors, MRFs, structural molecules, proteolytic markers, ubiquitin-proteasome system, plus three reference genes, were examined in a CFX384$^{TM}$ Real-Time System (Bio-Rad, El Prat de Llobregat, Spain) according to the requirements of the MIQE guidelines (13). The analyses were performed in triplicate in a final volume of 5 µL including 2.5 µL of iTaq $^{TM}$ Universal SYBR Green Supermix (Bio-Rad), 250

nM of forward and reverse primers (Table 1) and 1 µL of cDNA for each sample. As described before (82), the reactions consisted on: initial activation step of 3 min at 95ºC, 40 cycles of 10 s at 95ºC, 30 s at 55-68ºC (primer dependent, Table 1) followed by an amplicon dissociation analysis from 55 to 95ºC at 0.5ºC increase each 30 s. Before the analyses, the appropriate cDNA dilution for each assay was determined, as well as the specificity of the reaction and the absence of primer-dimers confirmed by running a dilution curve with a pool of samples. The expression level of each gene analyzed was calculated relative to the geometric mean of the reference genes RPS18 and RPL27a, the two most stable of the genes analyzed, using the Pfaffl method (74).

### 2.3. Western blot analysis

Protein homogenates from muscle tissue were obtained as described by García de la serrana et al. (37). The amount of protein from each sample was measured (11) and 10-20 µg of protein were separated by electrophoresis (SDS-PAGE) on 10% polyacrylamide gel (125 V for 1 h 30 min) following the procedure previously reported (97) with slight modifications. After SDS-PAGE, samples were transferred to a PVDF membrane. Then, all the membranes were stained with a Ponceau S solution (Sigma-Aldrich, Tres Cantos, Spain) and scanned for posterior bands quantification. Later, the membranes were washed to eliminate the Ponceau staining, and then blocked in non-fat milk 5% buffer and incubated with the respective primary antibodies in a 1:200 dilution. The primary antibodies used were as follows: rabbit polyclonal anti-PCNA (Cat. No. sc-7907), rabbit polyclonal anti-MAFbx (Cat. No. sc-33782), rabbit polyclonal anti-VEGF (vascular endothelial growth factor, Cat. No. sc-152), goat polyclonal anti-CAPN1 (Cat. No. sc-7530) and goat polyclonal anti-CTSD (Cat. No. sc-6486), all from Santa Cruz Biotechnology (Inc. Santa Cruz CA., USA). All these antibodies has been previously demonstrated that cross-react successfully with the proteins of interest in gilthead sea bream (3, Azizi et al., unpublished data); with the exception of anti-VEGF, which specificity has been tested in a pooled sample by blocking overnight with an excess (5-fold) of the immunization peptide (Cat. No. sc-152 P). After washing, the membranes were incubated with the correspondent peroxidase-conjugated secondary antibody: goat anti-rabbit (Cat. No. 31460. Thermo Scientific, Alcobendas, Spain), or donkey anti-goat (Cat. No. sc-2020, Santa Cruz Biotechnology, Inc. Santa Cruz CA., USA). The membranes were rewashed and the different immunoreactive bands were developed by

using an enhanced chemiluminescence kit (Pierce ECL WB Substrate, Thermo Scientific, Alcobendas, Spain). Finally, the bands were quantified by densitometry using ImageJ software (National Institutes of Health, Bethesda, MD, USA). Hence, results from the densitometry analysis of each specific bands are presented normalized by the densitometry values of the Ponceau, since its staining showed similar amounts of loaded and transferred protein. In the case of CTSD, the densitometric analysis was performed with the sum of the bands corresponding to the immature and mature forms.

### 2.4. Statistical analyses

Data was analyzed using IBM SPSS Statistics v.21 and presented as mean ± SEM. Normal distribution was first analyzed using the Shapiro-Wilk test followed by Levene's to test homogeneity of variances. Statistical differences were analyzed using Student's T test and considered significant when P value $<0.05$ (*) or $<0.001$ (**).

## 3. Results

### 3.1. Proliferation marker and growth inhibitor expression

Fig. 1A shows the effects of five weeks of sustained swimming in fingerlings of gilthead sea bream on the gene and protein expression of the proliferation marker PCNA. PCNA gene expression was significantly increased in the caudal muscle region of exercised fish; nonetheless, the protein levels of PCNA were significantly higher in the anterior region in comparison with control fish.

Fig. 1B presents the mRNA levels of two MSTN paralogues in both muscle regions. While the expression of MSTN1 was not modified by effect of exercise, the transcript levels of MSTN2 were down-regulated in the anterior muscle of exercised fish.

### 3.2. Myogenic regulatory factors and structural markers expression

The analysis of MRFs gene expression showed that while none of these factors (MyoD1, MyoD2, Myf5, Myogenin and Mrf4) were affected by swimming activity in the anterior muscle (Fig. 2A), the expression of MyoD1 was significantly increased on the caudal region (Fig. 2B).

Regarding the structural markers (MHC, MLC2A, and MLC2B), in the anterior muscle of exercised fish higher mRNA levels of MLC2A were found (Fig. 3A). However, no differences were observed for any of these genes in the caudal muscle region (Fig. 3B).

### 3.3. Proteolytic and angiogenesis markers

Swimming activity significantly increased the expression of several of the most important proteolytic-related genes in the anterior muscle region of the fish, such as CAPN1, CAPN2, CAPNs1a and CAPNs1b, CTSDb, MuRF1, MAFbx and N3 (Fig. 4A). Nevertheless, in the caudal region the expression of all these molecules remained stable, being only the expression of CAPN2 down-regulated in exercised fish (Fig. 4B). These results are also supported by Western blot analysis. Thus, the protein expression of CAPN1 and CTSD was significantly up-regulated in the anterior region of exercised fish in comparison with control fish (Fig. 5A and 5B), meanwhile no differences were found for any of these molecules in the caudal muscle region. On the other hand, the protein levels of the ubiquitin ligase MAFbx were not modified in none of both muscle regions (Fig. 5C).

After validation of the specificity of the VEGF antibody using the immunization peptide to block the signal (Fig. 6A), Western blot results showed that swimming activity significantly increased the protein expression of VEGF in the anterior muscle, whereas no differences were found in the caudal region (Fig. 6B).

## 4. Discussion

The effects of exercise have been investigated in different fish species (7, 12, 25, 43, 58, 68-71, 94). Our group has demonstrated that moderate and sustained swimming activity increases body weight in rainbow trout and gilthead sea bream with clear changes in metabolism and muscle structure (8, 32, 33, 44, 59). Thus in these studies, exercise triggered an increase in muscle aerobic capacity with rise in muscle glycogen turnover and muscle mass. Although changes in muscle protein content were not found, a significant decrease on mesenteric fat was observed in exercised gilthead sea bream fingerlings (8). Moreover, muscle structure was also adapted to exercise, with increases in hypertrophic condition and capilarization in the anterior but not the caudal muscle region (44). In addition, we have also shown that swimming increases the plasma IGF-I

levels (8, 84), and modulates the gene expression of several GH-IGF axis molecules, including hepatic IGF-I, and GHR-I in muscle (98). Furthermore, under these conditions the TOR signaling pathway is activated at both gene and protein levels, thus stimulating protein synthesis. All these changes resulted in enhanced growth in comparison to control fish.

Following this line of research, in the present work we have demonstrated that the gene and protein expression of the cellular proliferation marker PCNA, increases in the caudal and anterior muscle regions respectively with exercise. PCNA is considered a good marker of proliferation processes (1), and we have observed in gilthead sea bream cultured myocytes, that the highest levels of PCNA expression coincide with the stage of maximum proliferation (36). Also, we have demonstrated that both IGF-I and IGF-II stimulate the increase of PCNA positive cells using the same cell model system (78). This concurs with the increase of IGF-II gene expression (98) parallel to that of PCNA in the caudal muscle of exercised fish. The fact that in the anterior muscle, PCNA increased in terms of protein, but not at the mRNA level, while in the caudal muscle the response was opposite, supports the distinct response to exercise throughout the muscle already observed in our previous studies (44, 98). Moreover, this is in agreement with the longitudinal shifts in contractile properties caused by the different combination and coordination of a great variety of proteins in the white muscle of several fish species (19, 20, 26, 100), which provide faster contractile properties to the anterior muscle region (22). Nevertheless, with only these data, we cannot conclude a different proliferative activity between both muscle regions and it becomes necessary to analyze other growth-regulating molecules such as inhibitors, transcription factors or structural muscle proteins. Furthermore, differences between gene and protein expression along the myogenic process have already been reported (3), and should be taken into account to understand this differential PCNA response.

In terms of growth repression, MSTN is the main inhibitor of muscle development in vertebrates (51, 79). In fish, it has been observed that fasting increases MSTN gene expression while refeeding decreases it (36, 64). In gilthead sea bream, as in other fish species, two main paralogues are expressed in muscle (MSTN1 and MSTN2), which can exert their function depending on physiological conditions (35, 54, 55). This is the case in this study, where only MSTN2 gene expression decreased in the anterior muscle of exercised fish, but no response of MSTNs gene expression was observed in the

caudal region, supporting the different effect of exercise all through the muscle in fish. The significant decrease in the anterior muscle of MSTN2 would suggest the possibility that in this region, the muscle is in a state of remodeling because of exercise, as we previously suggested due to the differential increase of GHR-I, IGF-I and IGFBP-5 observed in this region (98).

In mammals, exercise determines diverse effects on myogenesis depending on its intensity and duration, which can modify the expression of MRFs, structural molecules and IGFs (41, 102). Palstra and coworkers (70, 71) showed that zebrafish trained to perform intense exercise up-regulates in muscle the expression of several MRFs, and Hasumura and Meguro (43) in the same species demonstrated very recently that a short but intense exercise is able to induce the expression of MyoD and Myogenin. Contrarily in this study, 5 weeks of sustained and moderate exercise in gilthead sea bream did not change importantly MRFs transcripts levels. Only the increase of MyoD1 gene expression in the caudal muscle area was significant, which could indicate the initiation of the determination stage of myogenesis in this part of the muscle that would later extend to the anterior muscle region. We should not forget that this is a dynamic process and our results only reflect a snapshot from the time of sampling. In any case, the up-regulation of MyoD1 agrees with the increase in IGF-II gene expression previously observed in these fish (98), corroborating the stimulatory effect of this growth factor on MyoD expression previously demonstrated in gilthead sea bream myocytes (46).

With regards to the structural myofribrillar molecules, little effects were observed in the gene expression of the different myosins during exercise, with the exception of MLC2A, which expression was increased in the anterior muscle. This coincides with the up-regulation of MLC gene expression observed in the white muscle of rainbow trout (58) and zebrafish (70) under sustained swimming. Very recently, this molecule has been related with the intensive stratified formation of new fibers during muscle growth in larvae of gilthead sea bream (38). Moreover, in concordance with previous studies, it has been suggested that MLC2A is more involved in larval growth (mainly hyperplasic growth), whereas MLC2B is more related to the hypertrophic growth that takes places in the adult stages (39, 67). Similarly, Silva et al., (89) also suggested previously that MLC could be a good marker of hyperplasia in blackspot sea bream. Overall, this is in agreement with the increase in PCNA protein expression and the down-regulation of MSTN2 mRNA observed in this study, and with the up-regulation

of IGFs and GHR-I described before in anterior muscle (98), suggesting that this part of the muscle might be in a proliferative state condition.

The proteolytic systems play a key role in muscle, and in mammals, it is known that they are necessary for normal day-to-day function. During exercise, proteolysis is increased, being a requirement for the remodeling and regeneration processes that occur in response to activity (24, 53, 76, 77, 101). Thus, the calpain, cathepsin and proteasome systems, including the genes MuRF or MAFbx among others, will be activated during exercise (4, 24, 27, 77, 101). In gilthead sea bream, we have recently characterized several members of the calpains, cathepsins and ubiquitin-proteasome families and their response to nutritional changes (82, 83). However, information available on fish proteolytic systems in relation to swimming is scarce, especially in this species. Besides, as introduced before it has been shown that in contrast to mammals, the autophagy-lysosomal system (in which cathepsins are included) has a more important contribution than the ubiquitin-proteasome on the total protein degradation in rainbow trout myotubes (85). In the present study, the anterior muscle showed a clear activation of all proteolytic systems, with the significant up-regulation of most of the molecules studied. Such a uniform response also observed in the protein expression of CAPN1 and CTSD, is a good indication of the proteolytic process that may be taking place in the anterior muscle, but not the caudal one, where the unique change observed was the decrease of CAPN2. In fact, muscle expression of calpains and cathepsins, increased during the mobilization accompanying fasting in gilthead sea bream (82, 83). The up-regulation of the calpain system observed in the present study can thus be related with two different situations known to occur in mammals; first, prior to myoblast fusion it is necessary to remodel the membrane of myogenic cells at the fusion point through breaking the cytoskeletal/plasma membrane linkages (4, 42, 65), and in this is involved CAPN2. Secondly, CAPN1 is more associated with the cleavage of different cytoskeletal proteins to smaller fragments (4, 101), that can be later degraded by the ubiquitin-proteasome system or cathepsins, as it would be expected in fish (85) and confirmed in this study by the increased protein levels of CTSD. Parallel to these molecules, exercise determined in the anterior muscle the increase of MuRF1 and MAFbx, two muscle-specific ubiquitin ligases that participate in the last step of ubiquitination, normally induced in muscle atrophy (17). Both enzymes are frequently used as markers of muscle proteolysis in fish (9, 15, 83, 86). However, the interpretation

of these results should be cautious, since at least in mammals MAFbx is thought to exert specific control regulating MRFs such as MyoD, while MuRF regulates myofibrillar and sarcomeric stability (93, 101). In this sense, these results are consistent with the increased expression observed for these two molecules together with MyoD and Myogenin in exercised zebrafish (43). Similarly, the beta-type proteasome subunit N3 resulted also elevated in the anterior muscle of exercised gilthead sea bream in the present study. In this species, it has been recently demonstrated that during the greater-growth stage along ontogeny (i.e. fingerlings), the fish have higher proteolysis rates than during juvenile and adult stages and, in parallel, have also higher rates of protein synthesis, producing in combination enhanced growth (83). Altogether, these data support the observation that the anterior muscle, differently from the caudal muscle, was subjected during swimming to an important remodeling.

Furthermore, the effects reported on the proteolytic systems are in agreement with the increase in PCNA protein expression and the decrease on MSTN2 gene expression in the anterior muscle. Moreover, this is also coincident with the up-regulation of VEGF only in this muscle region. Although information on VEGF regulation in fish (45) is not as broad as in mammals (76, 102), VEGF is considered a good marker of muscle remodeling regulating the increase of capillary supply, necessary for the new myofibers produced. Thus, the observed VEGF increase in this study is another demonstration of the transformation process that is occurring in the anterior muscle region of exercised fish. Changes in capilarization were already observed in gilthead sea bream under equivalent exercise conditions. Thus, while in control conditions the caudal muscle showed more capilarization than the anterior muscle, in exercised fish the capillaries in the anterior muscle increased to equal the values in caudal muscle (44). Similarly, Palstra et al., (70, 71) reported in zebrafish that swimming promotes an increase in muscle hypertrophy and vascularization accompanied by up-regulation of several genes including calpains and VEGF. Preliminary immunohistochemistry results have indicated that in our trial exercised fish show a general increase also, in muscle hyperplasia and capilarization (Moya et al., unpublished data). Vélez et al., (98) found in anterior muscle a significant increase of both TOR gene and protein expression, which reinforces the existing equilibrium between protein degradation and synthesis; and this protein balance, was further confirmed by the conservation of the protein

content observed in these fish (8). Overall, this is consistent with the expected muscle remodeling as effect of exercise (60).

### 4.1. Perspectives and Significance

In the present study, after 5 weeks of moderate and sustained swimming in gilthead sea bream fingerlings, the significantly increased PCNA and MyoD1 gene expression in the caudal muscle region, indicates the cell proliferation and differentiation condition that correlates well with the higher gene expression levels of IGF-II and IGF-I in this region previously observed in these fish (98). Besides, in the anterior muscle region, a remodeling condition seems to exist that is confirmed by the increased gene expression of most proteolytic markers and VEGF. This over activation of proteolytic systems corresponds with the increase in protein turnover (cathepsins and ubiquitin-proteasome) and structural changes (calpains), that are necessary for the tissue renovation and new vessels formation that take place during exercise adaptation.

Overall, the present study contributes to improve knowledge on the growth-promoting effects of swimming in fish, demonstrating for the first time the role of several proteolytic factors and the growth factor VEGF in gilthead sea bream muscle remodeling, which can help to optimize fish growth and flesh quality in this important aquaculture species, and confirms the use of moderate sustained swimming as an excellent intervention for achieving this goal.

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753 **Table 1. Primers used for qPCR.** Sequences, melting temperatures (Tm) and
754 GenBank accession numbers.

| Gene | Primer sequences (5'–3') | Tm (ºC) | Accession number |
|---|---|---|---|
| *RPS18* | **F**:GGGTGTTGGCAGACGTTAC<br>**R**:CTTCTGCCTGTTGAGGAACCA | 60 | AM490061.1 |
| *RPL27a* | **F**:AAGAGGAACACAACTCACTGCCCCAC<br>**R**:GCTTGCCTTTGCCCAGAACTTTGTAG | 68 | AY188520 |
| *β-Actin* | **F**: TCCTGCGGAATCCATGAGA<br>**R**: GACGTCGCACTTCATGATGCT | 60 | X89920 |
| *PCNA* | **F**:TGTTTGAGGCACGTCTGGTT<br>**R**:TGGCTAGGTTTCTGTCGC | 58 | AY550963.1 |
| *MSTN1* | **F**:GTACGACGTGCTGGGAGACG<br>**R**:CGTACGATTCGATTCGCTTG | 60 | AF258448.1 |
| *MSTN2* | **F**:ACCTGGTGAACAAAGCCAAC<br>**R**:TGCGGTTGAAGTAGAGCATG | 60 | AY046314 |
| *MyoD1* | **F**:TTTGAGGACCTGGACCC<br>**R**:CTTCTGCGTGGTGATGGA | 60 | AF478568.1 |
| *MyoD2* | **F**:CACTACAGCGGGGATTCAGAC<br>**R**:CGTTTGCTTCTCCTGGACTC | 60 | AF478569 |
| *Myf5* | **F**:CTACGAGAGCAGGTGGAGAACT<br>**R**:TGTCTTATCGCCCAAAGTGTC | 64 | JN034420 |
| *Myogenin* | **F**:CAGAGGCTGCCCAAGGTCGAG<br>**R**:CAGGTGCTGCCCGAACTGGGCTCG | 68 | EF462191 |
| *Mrf4* | **F**:CATCCCACAGCTTTAAAGGCA<br>**R**:GAGGACGCCGAAGATTCACT | 60 | JN034421 |
| *MHC* | **F**:AGCAGATCAAGAGGAACAGCC<br>**R**:GACTCAGAAGCCTGGCGATT | 58 | AY550963.1 |
| *MLC2a* | **F**:GCCCCATCAACTTCACCGTCTTT<br>**R**:GGTTGGTCATCTCCTCAGCGG | 60 | AF150904 |
| *MLC2b* | **F**:TCCCTTTGCTATTCTGCCTTC<br>**R**:AAATCAGCCCTATTCCCCATA | 60 | FG618631 |
| *CAPN1* | **F**:CCTACGAGATGAGGATGGCT<br>**R**:AGTTGTCAAAGTCGGCGGT | 56 | KF444899 |
| *CAPN2* | **F**:ACCCACGCTCAGACGGCAAA<br>**R**:CGTTCCCGCTGTCATCCATCA | 61 | KF444900 |
| *CAPN3* | **F**:AGAGGGTTTCAGCCTTGAGA<br>**R**:CGCTTTGATCTTTCTCCACA | 56 | ERP000874 |
| *CAPNs1a* | **F**:CGCAGATACAGCGATGAAAA<br>**R**:GTTTTGAAGGAACGGCACAT | 56 | KF444901 |
| *CAPNs1b* | **F**:ATGGACAGCGACAGCACA<br>**R**:AGAGGTATTTGAACTCGTGGAAG | 56 | ERP000874 |
| *CTSDb* | **F**:AAATTCCGTTCCATCAGACG<br>**R**:CTTCAGGGTTTCTGGAGTGG | 56 | KJ524456 |
| *MuRF1* | **F**:GTGACGGCGAGGATGTGC<br>**R**:CTTCGGCTCCTTGGTGTCTT | 60 | FM145056 |
| *MAFbx* | **F**:GGTCACCTGGAGTGGAAGAA<br>**R**:GGTGCAACTTTCTGGGTTGT | 60 | ERA047531 |
| *N3* | **F**:AGACACACACTGAACCCGA<br>**R**:TTCCTGAAGCGAACCAGA | 54 | KJ524458 |

755 ***RPS18***: Ribosomal Protein S18. ***RPL27a***: Ribosomal Protein L27a. ***PCNA***: Proliferating Cell Nuclear Antigen.
756 ***MSTN1***: Myostatin-1. ***MSTN2***: Myostatin-2. ***MyoD1***: Myoblast determination protein 1. ***MyoD2***: Myoblast

757 determination protein 2. ***Myf5***: Myogenic factor 5. ***Mrf4***: Myogenic regulatory factor 4. ***MHC***: Myosin heavy chain.
758 ***MLC2a***: Myosin light chain 2a. ***MLC2b***: Myosin light chain 2b**.** ***CAPN1***: Calpain 1. ***CAPN2***: Calpain 2. ***CAPN3***:
759 Calpain 3. ***CAPNs1a***: Calpain small-subunit 1a. ***CAPNs1b***: Calpain small-subunit 1b. ***CTSDb***: Cathepsin Db.
760 ***MuRF1***: Muscle Ring-Finger protein 1. ***MAFbx***: Muscle Atrophy F-box or Atrogin-1. ***N3***: Proteasome beta type-4
761 subunit or PSMB4.

762 **Figure legends**

763 **Fig. 1. Effects of exercise on the expression of a proliferation marker and a growth**
764 **inhibitor in muscle tissue.** (**A**) On the left, relative mRNA expression of PCNA and in
765 right the PCNA protein expression normalized to total protein staining with Ponceau S,
766 and (**B**) relative mRNA levels of MSTN1 and MSTN2 normalized to RPS18 and
767 RPL27a in both anterior and caudal muscle region of gilthead sea bream after 5 weeks
768 of sustained and moderate exercise (Exercise), or reared in control conditions (Control).
769 Data are shown as mean ± SEM (n=12 for qPCR. n=4 for WB). Significant differences
770 are indicated with * ($P<0.05$).

771 **Fig. 2. Effects of exercise on myogenic regulatory factors gene expression.** Relative
772 mRNA expression normalized to RPS18 and RPL27a of MyoD1, MyoD2, Myf5,
773 Myogenin and Mrf4 in (**A**) the anterior muscle and (**B**) the caudal muscle regions of
774 gilthead sea bream fingerlings after 5 weeks of sustained and moderate exercise, or in
775 control conditions. Data are shown as mean ± SEM (n=12). Significant differences were
776 indicated with * ($P<0.05$).

777 **Fig. 3. Effects of exercise on structural markers gene expression.** Relative mRNA
778 expression normalized to RPS18 and RPL27a of MHC, MLC2a and MLC2b in (**A**) the
779 anterior muscle and (**B**) the caudal muscle regions of gilthead sea bream fingerlings
780 after 5 weeks of sustained and moderate exercise, or in control conditions. Data are
781 shown as mean ± SEM (n=12). Significant differences were indicated with * ($P<0.05$).

782 **Fig. 4. Effects of exercise on proteolytic markers gene expression.** Relative mRNA
783 expression normalized to RPS18 and RPL27a of CAPN1, CAPN2, CAPN3, CAPNs1a,
784 CAPNs1b, CTSDb, MuRF1, MAFbx and N3 in (**A**) the anterior muscle and (**B**) the
785 caudal muscle regions of gilthead sea bream fingerlings after 5 weeks of sustained and
786 moderate exercise, or in control condition. Data are shown as mean ± SEM (n=12).
787 Significant differences were indicated with * ($P<0.05$).

788 **Fig. 5. Effects of exercise on proteolytic markers protein expression.** Protein
789 expression of (**A**) CAPN1, (**B**) CTSD immature (imm.) and mature (m.) forms and (**C**)

MAFbx in the anterior and caudal muscle regions of gilthead sea bream fingerlings after 5 weeks of sustained and moderate exercise (Ex), or in control conditions (C). Data are normalized to total protein staining with Ponceau S and shown as mean ± SEM (n=4). Significant differences were indicated with * ($P<0.05$).

**Fig. 6. Effects of exercise on angiogenesis marker protein expression.** (**A**) Western blot for VEGF specificity validation with muscle protein extract pooled samples and blocking immunization peptide. (**B**) Protein expression of VEGF in the anterior and caudal muscle regions of gilthead sea bream fingerlings after 5 weeks of sustained and moderate exercise (Ex), or in control conditions (C). Data are normalized to total protein staining with Ponceau S and shown as mean ± SEM (n=4). Significant differences were indicated with * ($P<0.05$).

Figure 1

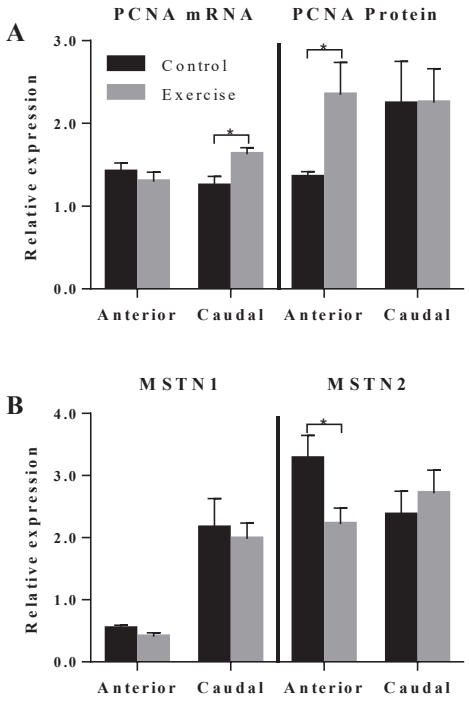

A
PCNA mRNA
PCNA Protein
Control
Exercise
Relative expression
3.0
2.0
1.0
0.0
*
*
Anterior
Caudal
Anterior
Caudal
B
MSTN1
MSTN2
Relative expression
4.0
3.0
2.0
1.0
0.0
*
Anterior
Caudal
Anterior
Caudal

Figure 2

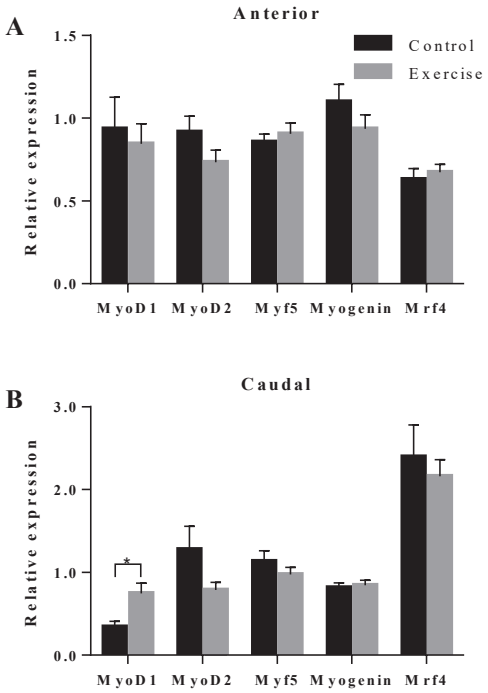

A
Anterior
Control
Exercise
Relative expression
1.5
1.0
0.5
0.0
MyoD1
MyoD2
Myf5
Myogenin
Mrf4
B
Caudal
Relative expression
3.0
2.0
1.0
0.0
*
MyoD1
MyoD2
Myf5
Myogenin
Mrf4

Figure 3

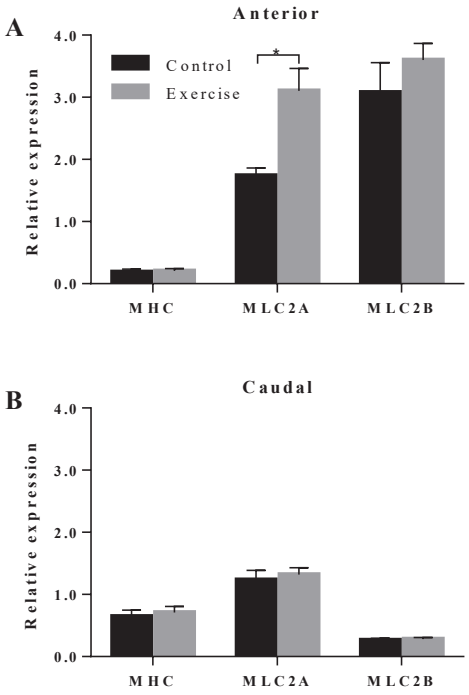

A
Anterior
Relative expression
4.0
3.0
2.0
1.0
0.0
Control
Exercise
*
MHC
MLC2A
MLC2B
B
Caudal
Relative expression
4.0
3.0
2.0
1.0
0.0
MHC
MLC2A
MLC2B

Figure 4

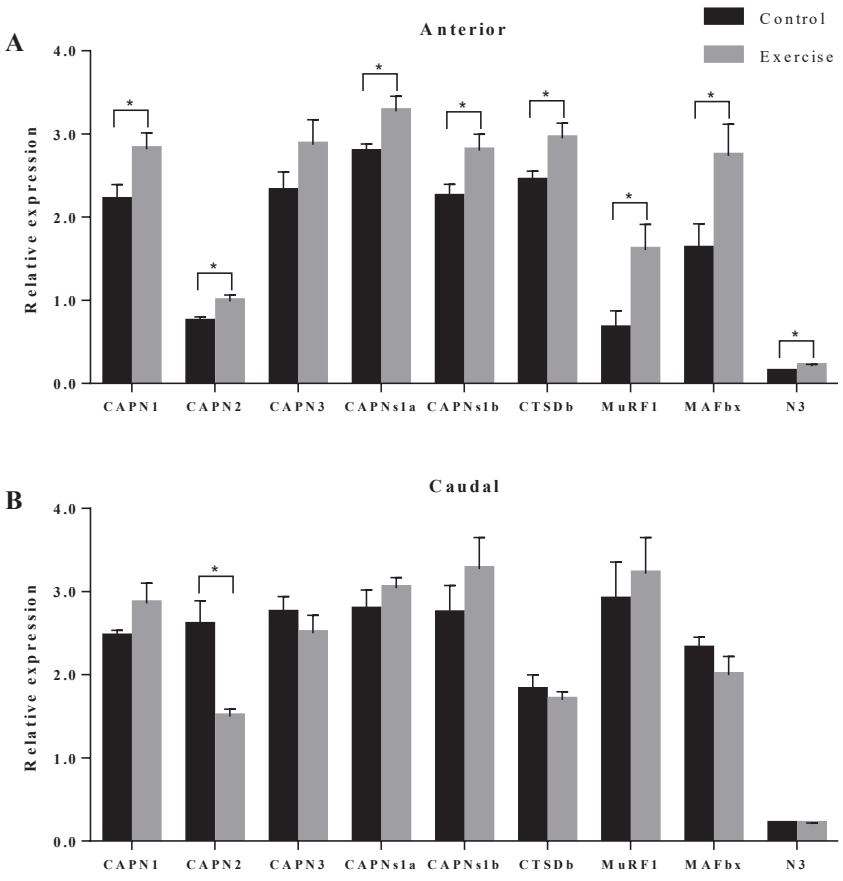

A
Anterior
Control
Exercise
Relative expression
4.0
3.0
2.0
1.0
0.0
CAPN1
CAPN2
CAPN3
CAPNs1a
CAPNs1b
CTSDb
MuRF1
MAFbx
N3
B
Caudal
Relative expression
4.0
3.0
2.0
1.0
0.0
CAPN1
CAPN2
CAPN3
CAPNs1a
CAPNs1b
CTSDb
MuRF1
MAFbx
N3

Figure 5

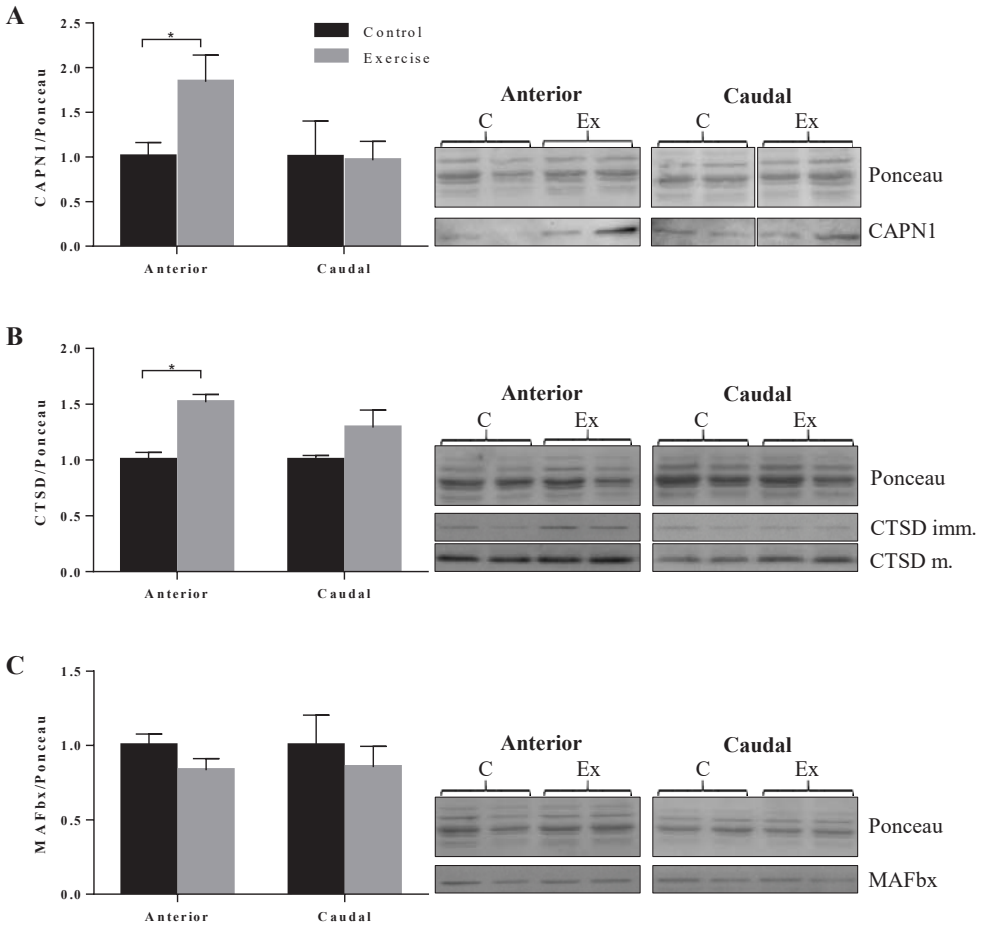
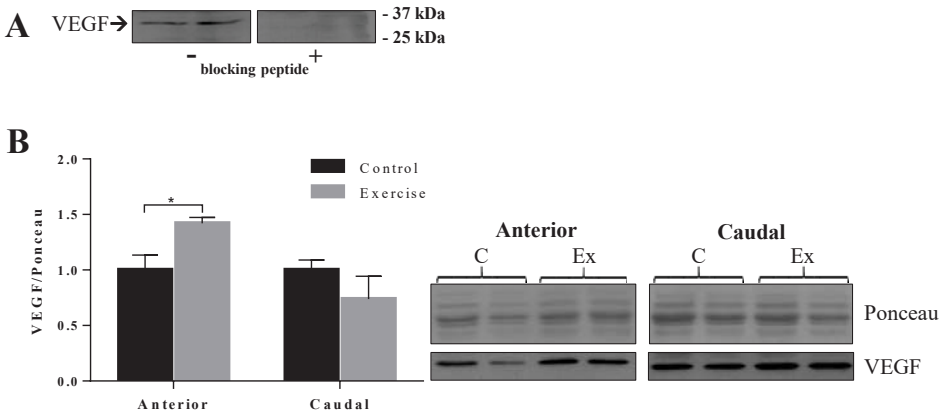

A
Control
Exercise
CAPN1/Ponceau
2.5
2.0
1.5
1.0
0.5
0.0
*
Anterior
Caudal
Anterior
Caudal
C
Ex
C
Ex
Ponceau
CAPN1
B
CTSD/Ponceau
2.0
1.5
1.0
0.5
0.0
*
Anterior
Caudal
Anterior
Caudal
C
Ex
C
Ex
Ponceau
CTSD imm.
CTSD m.
C
MAFbx/Ponceau
1.5
1.0
0.5
0.0
Anterior
Caudal
Anterior
Caudal
C
Ex
C
Ex
Ponceau
MAFbx

Figure 6


A
VEGF→
- 37 kDa
- 25 kDa
-
blocking peptide
+
B
Control
Exercise
VEGF/Ponceau
2.0
1.5
1.0
0.5
0.0
*
Anterior
Caudal
Anterior
Caudal
C
Ex
C
Ex
Ponceau
VEGF