

Expression of Growth-Related Factors in Skeletal Muscle of Pirarucu (*Arapaima Gigas*) during Growth

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Abstract

The skeletal muscle of the species *Arapaima gigas* (pirarucu) constitutes the major edible part of the fish and is, therefore, an important protein source for human consumption. Post-natal muscle growth is regulated by the expression of myostatin as well as the Myogenic Regulatory Factors (MRFs) MyoD and myogenin. Once pirarucu reaches large size, we assume that the control of muscle growth by MRFs and myostatin occurs differentially at the initial life stages. In the present work we evaluated the morphological aspects and expression of MRF genes in skeletal muscle of pirarucu during early juvenile stage (Group A, up to 50 g, n=7), and post juvenile stages (Groups B, from 50 to 400 g, n=7; C, from 400 g to 5 kg, n=7, and D, from 5 to 9 kg, n=7). Transverse sections of red and white muscles were obtained to evaluate muscle fiber morphological and morphometric characteristics. MyoD, myogenin and myostatin genes and protein expressions were determined after quantitative real-time polymerase chain reaction and western blotting, respectively. Pirarucu skeletal muscle exhibited similar morphologies at different life stages. It was possible to conclude that both hyperplasia and hypertrophy occur during muscle growth in early and post-juvenile stages. As regard as expression, both mRNA and protein levels were similar among all groups for MyoD and myogenin. Myostatin presented lower mRNA levels and higher protein levels in early-juvenile stage, compared with the other groups. The levels of MRFs and myostatin might be involved in a balance that controls hyperplasia and hypertrophy occurring during post-natal muscle growth. Myostatin does not appear to play a crucial role during pirarucu early-juvenile stages. Since this species represents an interesting model for aquaculture programs due to high growing rates, our data suggest that the best phase to improve muscle growth in pirarucu is at post-juvenile stage and this will turn the employment of pirarucu farming economically better.

Keywords: Fish skeletal muscle; Muscle growth; 46 Myogenic regulatory factors; Myostatin; RT-qPCR; Western blotting; Pirarucu *arapaima gigas*

Introduction

Pirarucu (*Arapaima gigas*) is a member of the largest freshwater fish group in the world (the Teleosts) and is considered one of the most important species of the Amazonian ichthyofauna. The pirarucu fast-growing characteristic allows it to reach up to 3 m length and 250 kg weight, when the 4 years-old pirarucu is called adult [1,2]. This species exhibits some intrinsic characteristics, such as rapid growth rates and rusticity, which enable it to be included in intensive rearing programs with excellent performance results. For this reason, it is important to investigate muscle growth mechanisms in this species. Skeletal muscle comprises the entire fillet in this fish species and lacks spines, one of the reasons it is widely appreciated as a food source for human [3,4]. Teleost fish skeletal muscle comprises around 60% of total body mass and is composed of different muscle fiber types organized in distinct areas [5]. The myotomes of adult fish contain a superficial lateral zone with red aerobic fibers, while the bulk of the musculature consists of white anaerobic fibers as described in [6]. Between the two layers, there is an intermediate layer of fibers with mixed characteristics related to metabolic and contractile activities [5,7-9]. During slow swimming speeds, as fish migrates, red muscle layers are the major active portion of muscle mass, whereas the white muscle is mostly recruited for bursts of rapid, vigorous activity [8,10-12]. The amount and arrangement of red and white muscle fibers, as well the red/white muscle ratio, can vary according to the fish species and swimming requirements, being an interesting characteristic regarding environmental adaptations [13].

Postnatal muscle growth in teleost fish involves the activation and proliferation of a set of quiescent myogenic precursor cells,

termed satellite cells, located at the periphery of the muscle fibers [14,15]. Their nuclei are absorbed by existing fibers as they expand in size (hypertrophy) or fuse together to form multinucleated myotubes and new fibers (hyperplasia). In fish, muscle grows continuously via hyperplastic and hypertrophic mechanisms throughout life [16,17]. These mechanisms are regulated by several types of transcriptional factors, which are muscle-specific, such as the Myogenic Regulatory Factors (MRFs), and by growth factors produced locally by muscle cells or neighbor tissues [18-21]. There are four MRFs described in the literature; MyoD, Myf5, myogenin and MRF4. MyoD and Myf5 are the first MRFs to be expressed in active satellite cells during post-natal period, regulating muscle fiber proliferation and, therefore, controlling hyperplasia. Myogenin and MRF4 roles are cell differentiation. They are also involved in hypertrophy, causing fiber maturation [21-25].

Another important regulator of skeletal muscle growth is Growth and Differentiation Factor 8 (GDF-8), widely known as myostatin. Myostatin is a member of the TGF- β superfamily of proteins which

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negatively regulates muscle growth, inhibiting both myoblast proliferation and differentiation [26-29]. It has been shown that the blockade of myostatin produces larger specimens of zebrafish (*Danio rerio*) and rainbow trout (*Oncorhynchus mykiss*), due to elevation of muscle hypertrophy and hyperplasia processes [30-33]. Myostatin has been isolated and characterized in several fish species [29,34-40]. In the present work, we have investigated the morphological characteristics and the expression of growth-related factors in skeletal muscle of pirarucu during growth. The results shown in the present study indicate that post-juvenile phase of pirarucu represents an interesting developmental stage for understanding 96 molecular mechanisms involved with muscle growth and our findings may help to improve the intensive rearing programs and production in fish farms.

Materials and Methods

Sample collection

This study was approved by the Biosciences Institute Ethics Committee of UNESP, Botucatu, SP, Brazil (Protocol N° 72/07-CEEA). Pirarucu (*Arapaima gigas*) juvenile specimens were obtained from the Liberdade fish farm located in Uirapuru, GO, Brazil, and grouped according to their average body weight as follows: early juvenile stage (Group A, up to 50 g, n=7), and post juvenile stages (Groups B, from 50 to 400 g, n=7; C, from 400 g to 5 kg, n=7, and D, from 5 to 9 kg, n=7). The animals were euthanized in a tank containing ice. Red and white muscle samples were obtained from superficial and deep lateral line regions of the fish, respectively; white muscle was also collected from the dorsal region. Both sampling areas were located close to the cranial region. The collected muscle samples were immediately frozen in liquid nitrogen and stored at -80°C.

Morphological and morphometric analysis

To evaluate morphological characteristics, transverse histological sections of red and white muscle samples (10 µm thick) were obtained with a -20°C cryostat microtome and stained with hematoxylin-eosin (HE) [41]. The smallest diameter of 200 white muscle fibers per animal were measured using a computerized image analyzer (DIGITAL IMAGE ANALYSIS SYSTEM QWIN V.3 FOR WINDOWS/LEICA, WETZLAR, GERMANY). We examined white muscle because it constitutes the prevailing part of the fish fillet. The muscle fibers were grouped into the following classes according to their diameter (adapted from [16] and [42]): class 10 (<10 µm), class 20 (≥10 µm, <20 µm), class 30 (≥20 µm, <30 µm), class 50 (≥30 µm, <50 µm), class 80 (≥50 µm, <80 µm), and class >80 (≥80 µm) to evaluate the hyperplastic and hypertrophic white muscle growth rates.

Gene expression analysis

Total RNA isolation and reverse transcription: Total RNA was extracted from pirarucu white muscle tissue using the TRIZOL® 129 reagent (INVITROGEN LIFE TECHNOLOGIES, CARLSBAD, CA, USA) following the manufacturer's protocol. The RNA samples were re-suspended in nuclease-free water and quantified by measuring the optical density (OD) at 260 and 280 nm using a NANOVue™ PLUS SPECTROPHOTOMETER (GE HEALTHCARE, PISCATAWAY, NJ, USA). A 260/280 nm OD ratio ≥ 1.8 was obtained to ensure high RNA purity, and RNA integrity was confirmed via electrophoresis on agarose gels. Total RNA was incubated with DNASE I AMPLIFICATION GRADE (INVITROGEN LIFE TECHNOLOGIES, CARLSBAD, CA, USA) to remove any residual genomic DNA present in the samples. Two micrograms of RNA were reverse transcribed using the HIGH CAPACITY cDNA ARCHIVE KIT (APPLIED BIOSYSTEMS, FOSTER

CITY, CA, EUA), as per the manufacturer's recommendations, and the final volume was adjusted to 100 µL with RNase-free water.

Reverse Transcription-Polymerase Chain Reaction 143 (RT-PCR), sequencing and sequence analysis: Complementary DNA (cDNA) was amplified using primer pairs designed based on the *Ictalurus furcatus* MyoD (Accession N° AY562555), myogenin (Accession N° AY540993) and myostatin (Accession N° AY540992) gene sequences available in the GenBank database (<http://www.ncbi.nlm.nih.gov>). A set of primers designed based on the consensus fish 18S ribosomal RNA sequence was used to amplify a segment of the 18S rRNA gene in pirarucu muscle samples [43]. Each PCR assay consisted of 0.2 µg of cDNA, 0.2 mM of each primer and 22.5 µL of PLATINUM® PCR SUPERMIX (22 U/mL Platinum®152 Taq DNA Polymerase, 22 mM Tris-HCl pH 8.4, 55 mM KCl, 1.65 mM MgCl₂, 220 µM dNTPs; LIFE TECHNOLOGIES, CARLSBAD, CA, USA) in a final volume of 25 µL. PCR amplifications were carried out with an initial denaturation step at 94°C for 2 minutes, followed by 35 cycles of 94°C for 1 minute, 55°C for 30 seconds and 72°C for 1.5 minutes, with a final extension step at 72°C for 5 minutes. The obtained PCR products were fractionated on 1.5% agarose gels, stained with GELRED® (BIOTIUM, HAYWARD, CA, USA), and visualized under UV light using an image documentation system. The RT-PCR products were subjected to automated sequencing in an ABI 377 AUTOMATED DNA SEQUENCER (APPLIED BIOSYSTEMS, USA) using the BIGDYE® 163 TERMINATOR V.3.1 CYCLE SEQUENCING KIT (APPLIED BIOSYSTEMS, FOSTER CITY, CA, EUA) according to the manufacturer's instructions. Nucleic acid sequence database searches were performed using the BLAST tool [44] on the National Center for Biotechnology Information (NCBI) website (<http://www.ncbi.nlm.nih.gov/blast>). The partial MyoD, myogenin and myostatin mRNA 168 sequences, and the 18S rRNA sequence, obtained from pirarucu skeletal muscle were used to design specific primers for quantitative PCR (real time PCR) analysis.

Quantitative PCR: Analyses of the expression of the MyoD, myogenin and myostatin genes were performed using the ABI 7300 REAL TIME PCR SYSTEM (APPLIED BIOSYSTEMS, FOSTER CITY, CA, EUA). Primers were designed with PRIMER EXPRESS® 176 software (APPLIED BIOSYSTEMS, FOSTER CITY, CA, EUA) from pirarucu sequences obtained via RT-PCR. The primer sequences employed in these analyses were as follows: MyoD (forward) 5' CCA GCC CCA GGT CCA ACT, (reverse) 5' ACA CGT TGG GCC ATT GAA A; Myogenin (forward) 5' AGG CTA CCC AAG GTG GAG ATC, (reverse) 5'TGC AGC CGC TCG ATG TAC T; Myostatin (forward) 5' CGA AGT ACA TGC ACC TGC AGA A, (reverse) 5' CGT GGG TTG GCC TTG TTT AC; and 18S rRNA (forward) 5' TAC CAC ATC CAA AGA AGG CAG, (reverse) 5' TCG ATC CCG AGA TCC AAC TAC. The 18S rRNA gene was used as the reference gene in this study because it exhibits a similar level at each developmental stage analyzed. The PCR efficiencies for target and reference genes were evaluated based on seven serial dilutions (1:10) of sample cDNAs and showed an acceptable slope value of 188-3.32 [45]. Each qPCR amplification mixture contained 12.5 µL of 2.5X POWER SYBR® GREEN PCR MASTER MIX (APPLIED BIOSYSTEMS, FOSTER CITY, CA, EUA), 40 ng of cDNA (20 ng/µL), 500 nM of each primer and RNase-free water to a final volume of 25 µL. Amplifications were performed in duplicate with the following thermal cycling conditions: initial activation at 95°C for 10 minutes, followed by 40 cycles of 15s at 95°C and 1 minute at 60°C. The control reactions included a no template control (NTC) and a no reverse transcriptase control (-RT). Dissociation analysis of the PCR products was performed by running a gradient from 60°C to

95°C to confirm the presence of a single PCR product. The products were also sequenced to confirm their identity. The fluorescence signal baseline and threshold were set manually for each detector (for MyoD, myogenin, myostatin, and 18S rRNA), generating a threshold cycle (Ct) for each sample. Sample quantification was accomplished via the comparative Ct method ($\Delta\Delta Ct$), and the data are expressed as the fold-change in gene expression normalized to the reference gene and relative to the calibrator sample [46].

Protein expression analysis: The levels of the MyoD, myogenin and myostatin proteins in pirarucu white muscle were determined by western blotting using β -actin protein for normalization. Muscle samples were homogenized with IKA UltraTurrax/T-25 in lysis buffer (1% Triton X-100, 10 mM sodium pyrophosphate, 100 mM NaF, 10 μ g/mL aprotinin, 1 mM phenylmethylsulfonylfluoride (PMSF), 0.25 mM Na_3VO_4 , 150 mM NaCl, and 50 mM Tris-HCl, pH 7.5). The samples were centrifuged at 11,000 \times g for 20 min, and 50 μ L of the homogenate fraction was re-suspended in 25 μ L of Laemmli loading buffer (2% SDS, 20% glycerol, 0.04 mg/mL bromophenol blue, 0.12 M Tris-HCl, pH 6.8, and 0.28 M β -mercaptoethanol). Total protein was quantified based on Bradford method [46a] and 70 μ g was fractionated via one-dimensional SDS-PAGE (12%). The proteins were then transferred from the gel to a nitrocellulose membrane. The membrane nonspecific binding sites were blocked using skim milk in Tris-HCl buffered saline containing Tween (TBS-T: 10 mM Tris-HCl pH 8.0, 150 mM NaCl, and 0.05% Tween-20), followed by primary antibody incubation 218 overnight at 4°C (MyoD M-318 sc-760; Myogenin M-225 sc-576; Myostatin C-20 sc-6884 and β -actin R-22 sc-130657, SANTA CRUZ BIOTECHNOLOGY, CA, USA). After three steps of 10-minute washing with TBS-T buffer, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (goat anti-rabbit IgG-HRP sc-2004 and donkey anti-goat IgG-HRP sc-2020, SANTA CRUZ BIOTECHNOLOGY, CA, USA). Variations in the blocking solution concentration, blocking time duration, and primary and secondary antibody dilutions were determined according to each analyzed protein. Immunoreactive protein signals were detected using the SUPERSIGNAL WEST PICO CHEMILUMINESCENT SUBSTRATE KIT (THERMO FISHER SCIENTIFIC, ROCKFORD, IL, USA), according to the manufacturer's recommendations. The signals were captured on film, and the band intensities were quantified using densitometry analysis software (IMAGE J SOFTWARE FOR WINDOWS, VERSION 1.71, 2006, AUSTRIA).

Statistical analysis: The Goodman statistical test for multinomial proportions was used for analysis of the muscle fiber class distribution [47,48]. For gene and protein expression analysis, Kruskal-Wallis test was employed followed by Dunn multiple comparisons post-test to detect statistical significance. For all analysis, the results were considered significant at 95% of confidence interval ($p < 0.05$). GraphPad InStat v. 3.01 software for Windows (1998, GRAPHPAD SOFTWARE, SAN DIEGO, CA, USA) was used.

Results

Morphological analysis

In all studied groups, red and white lateral muscles exhibited a similar morphological pattern. Muscle fibers presented normal features and were polygonal or round in shape, and multinucleated; nuclei were located at the periphery of the fibers. Fibers of different sizes were separated by a thin layer of connective tissue, endomysium. Groups of fibers were distributed in clusters separated by connective tissue, perimysium (Figure 1).

Morphometric analysis

Small-diameter fibers occurred between large ones, resulting in a variety of fibers with different diameters in white muscle (Figures 1B and 1D). This pattern was observed both in dorsal and lateral musculatures. The distribution of muscle fibers followed an increase in diameter according to body weight, considering the predominant size. Early juvenile pirarucu (Group A) had most fibers in classes 20 and 30; post juveniles (Groups B, C and D) had fibers mostly between classes 50 and 80 (Figure 2).

Gene expression analysis

No statistical difference among groups were found for 18S rRNA gene expression and it was used as reference to the other genes. MyoD, myogenin and myostatin transcript levels were quantitatively evaluated (RT-qPCR) and normalized based on 18S rRNA transcript levels (Figure 3). No differences were found between dorsal and deep lateral white muscles regarding to gene expression and both are referred in the text as white muscle.

Relative amounts of MyoD, myogenin, and myostatin transcripts in pirarucu white muscle are shown in Figure 3. No differences were found for the amplification of MyoD and myogenin mRNA among the

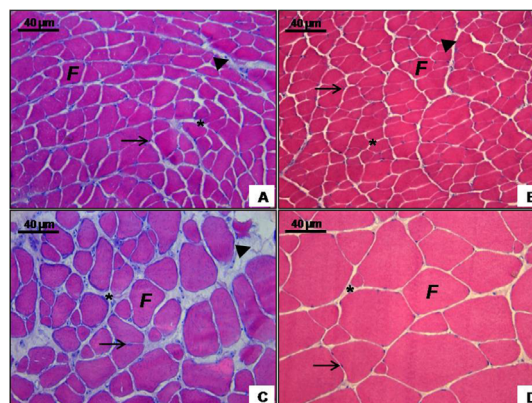


Figure 1: Histological sections of pirarucu (*A. gigas*) lateral skeletal muscle from group A (A and B) and group C (C and D). In A and C red muscle and in B and D, white muscle. Polygonal or round muscle fibers (F) surrounded by a thin connective tissue, the endomysium (*). Note the perimysium (arrowhead) grouping muscle fibers into fascicles. Nuclei (arrow) are in the peripheral location. HE stain (40X).

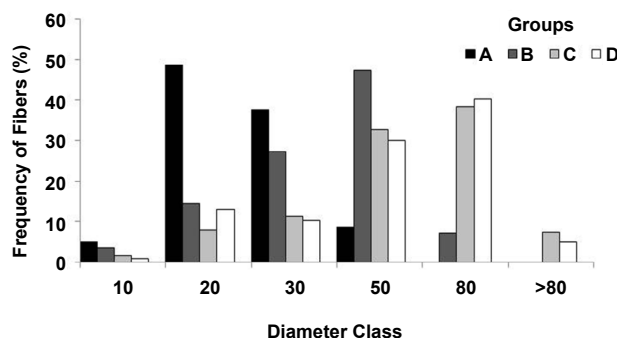


Figure 2: Frequency distribution of white muscle fibers in pirarucu (*A. gigas*), according to their diameter classes. Group A: up to 50 g weight, Group B: from 50 to 400 g weight, Group C: from 400 g to 5 kg weight, Group D: from 5 to 9 kg weight.

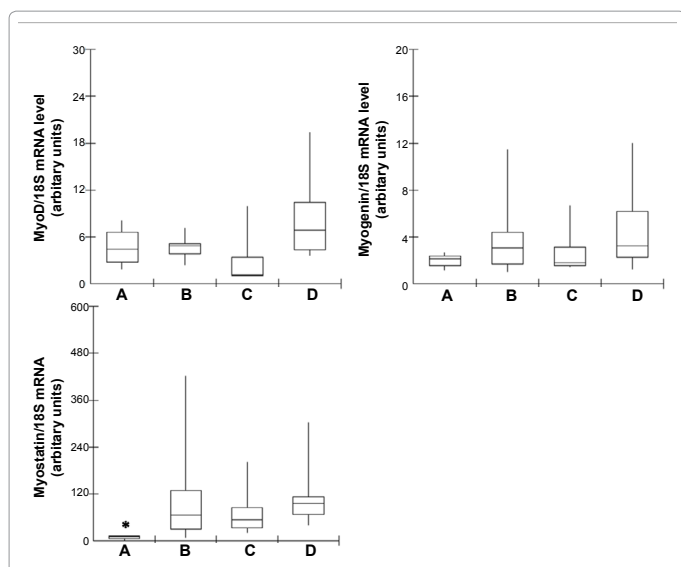


Figure 3: MyoD, myogenin and myostatin mRNA estimated levels in white skeletal muscle of pirarucu (*A. gigas*) during growth. Data were normalized to 18S ribosomal RNA expression values. Data are presented as minimum, 1st quartile, median, 3rd quartile and maximum values. Group A: up to 50 g weight, Group B: from 50 to 400 g weight, Group C: from 400 g to 5 kg weight, Group D: from 5 to 9 kg weight. (*) Statistical difference at 5% significance (Group A compared to B, C and D).

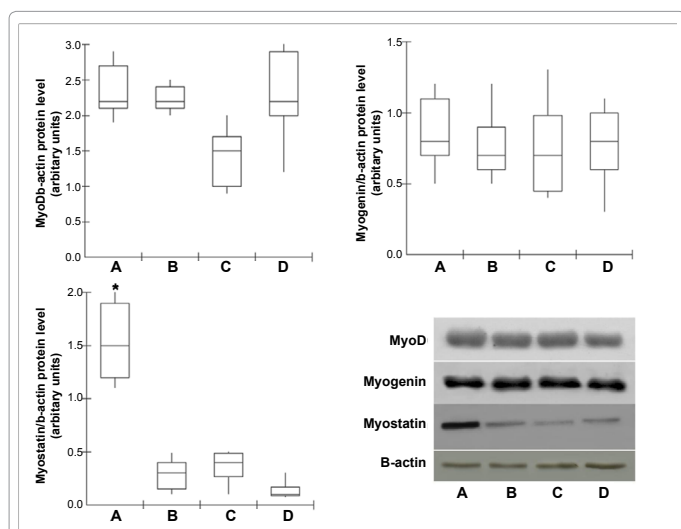


Figure 4: MyoD, Myogenin and Myostatin protein levels in white skeletal muscle of pirarucu (*A. gigas*) during growth. Data were normalized to the β -actin protein levels. Data are expressed as minimum, 1st quartile, median, 3rd quartile and maximum values. Group A: up to 50 g weight, Group B: from 50 to 400 g weight, Group C: from 400 g to 5 kg weight, Group D: from 5 to 9 kg weight. (*) Statistical difference at 5% significance (Group A compared to B, C and D). Figure shows protein bands identified by chemiluminescent detection system.

studied groups (p values: 0.2063 and 0.4533, respectively), indicating that these genes contributed similarly during the whole period of growth from young to juveniles. In contrast, myostatin levels exhibited significant variation. The youngest group showed lower myostatin mRNA expression compared to groups B, C and D (p value: 0.0047), which levels were statistically similar.

Protein expression analysis

MyoD, myogenin and myostatin protein levels were normalized

to β -actin. Muscle growth-related protein in the white muscle of pirarucu is shown in Figure 4. Similarly, MyoD and myogenin proteins contributed equally to all groups (p values: 0.999 and 0.392, respectively). In contrast, myostatin exhibited higher levels in group A, compared to groups B, C and D (p value: 0.038).

Discussion

The present work is the first to describe the mechanisms regulating skeletal muscle growth in pirarucu (*Arapaima gigas*), which is a very important fish regarding economy of Amazon people. The organization of muscle fibers in pirarucu is quite similar to other fish species; red and white muscle fibers showed similar morphological patterns, presenting polygonal or round shapes with peripheral nuclei. Connective tissues of extracellular matrix are distributed in the endomysium, surrounding each muscle fiber, and in the perimysium, where muscle fibers are clustered [7,49-55]. The thick connective tissue found in dorsal muscle can be seen as a support structure for the whole body, allowing the transmission of contraction forces from muscle fibers to the axial skeleton and caudal fin. In all studied groups, analysis of white muscle fiber morphometry showed many small-diameter fibers surrounding larger fibers. This pattern is observed in most fish species during muscle growth and is characteristic of hypertrophy and hyperplasia [12,17,55,56]. In the majority of fish species, these mechanisms are employed throughout their life cycle and have been well documented in a variety of species, particularly in species with a large potential for aquaculture [15,17,55,57]. In the present work, we observed that early juvenile stage pirarucu (Group A) had many immature fibers, demonstrating that hyperplasia is probably the main muscle growth mechanism at that stage. This mechanism is responsible for the new fibers formation in fish species, once proliferative satellite cells aggregate to the mature fibers surface and generate new myotubes [15,58]. Post-juvenile fishes (groups B, C and D) also presented hyperplasia, identified by the occurrence of mosaic groups of fibers. However, they presented predominately larger fibers, what suggests hypertrophy as the main mechanism of muscle growth in these life stages. During this process, satellite cells fuse to pre-existing 313 fibers, increasing protein synthesis and resulting in larger fiber sizes [16,17]. Hypertrophy is, in fact, the main post-natal muscle growth mechanism during the juvenile and adult phases of most fish species [59,60]. Moreover, in larger fish species, it is well documented that hyperplasia predominates in early developmental stages but persists for a prolonged period during growth [16,17,52,61,62]. Pirarucu has the combination of both mechanisms (hyperplasia and hypertrophy) balanced during the different developmental stages, what explain its rapid growth rates observed in nature and aquaculture ponds (approximately 10 Kg per year) [63-65]. Pirarucu grows up to 250 Kg, reaching 2 m long in nature. This is a remarkable growth for a scale freshwater fish, and it is associated with robust and continuous muscle development, which is under powerful machinery that contributes to the great increase in muscle mass during the early growth stages. In most fish, muscle thickening occurs via the apposition of muscle layers, a process known as stratified hyperplasia. The relative timing of this process in relation to the life cycle varies among fish species [16,42,66-68]. In large fish, it may predominate after hatching and persists during the early stages development. Therefore, based on our results, we suggest that stratified hyperplasia also contributes to a great increase in pirarucu's muscle mass. The expression of the MyoD and myogenin genes, as well as their related proteins in white muscle were not different among the several analyzed stages, which is consistent with many other studies [15-17,69,70] and may be attributed to the large requirement of fibers for

muscle compartment thickening. This is a process that occurs rapidly in pirarucu, as it reaches a large size in a short period of time [63-65].

It is well known that hyperplasia depends on fish physiology 337 and lifestyle, and is mainly regulated by Myogenic Regulatory Factor MyoD, which is responsible for stimulating myoblast proliferation with subsequent formation of myotubes [19,22,23]. In this study, no difference was found in MyoD gene expression and protein concentration in differently sized fish. In this work, all groups showed intensive growth rates, as many of their fibers were newly formed. These findings suggest an atypical behavior for pirarucu muscle growth compared with other fish species [71-74], since all groups had similar characteristics of fish at initial stages, i.e., fast muscle growth [3,4,70]. As pirarucu achieves large size in the adult stage, the creation of many fibers during the first developmental stages is the best strategy to improve muscle growth. Myogenin regulates the late stages of muscle fiber formation and growth, which culminates in myoblast fusion, myotube formation, and adult muscle fiber differentiation [24,75-77]. In the present study, neither the myogenin gene nor protein had different expression among the groups. These findings could be related to the intensive differentiation of muscle fibers, which occurs during all investigated growth stages. These fibers must fuse and form multinucleated myotubes that differentiate into muscle fibers, what is mainly regulated by myogenin [21,24,25]. Therefore, such similar myogenin expression levels found in pirarucu fibers may be attributed to the intensive rates of muscle fiber differentiation that occur regardless of fish size. One of the most important factors controlling skeletal muscle growth is myostatin. In the present work we described lower levels of myostatin transcripts in group A, compared to the higher levels in other groups. However, myostatin protein contents were higher in group A compared to other groups. Although there are few studies addressing the function of myostatin in fish, it is known that myostatin negatively regulates myoblast proliferation and differentiation [28,29]. The higher levels of myostatin mRNA in groups B, 362 C and D indicate an intensive rate of gene transcription and this state may have been stimulated by low levels of myostatin protein in these groups, as demonstrated by protein analysis. Once myostatin was no more needed in higher groups, we suppose that the lack of a correlation between transcript and protein levels may be due to a negative feedback mechanism or post-transcriptional down regulation preventing myostatin protein formation. Once satellite cells are activated in these stages, it could be related to the intense muscle fiber formation. As myostatin pauses muscle growth when it appears at a low level, muscle fiber formation and differentiation are fully activated. During the posterior growth stages in pirarucu, myostatin exhibited low levels, indicating that the muscle differentiation rates had increased. Similar to myostatin results, the expression of MyoD and myogenin in pirarucu were associated with high proliferation and differentiation rates in bigger fish groups (B, C and D), confirming the intense muscle differentiation in these groups. Several studies have attempted to describe the structure of the myostatin gene or simply determine its differential expression among distinct tissues [34,35,37,39,40,78]. There are few studies reporting myostatin levels during different fish growth phases [79]. Observed a low level of myostatin during the larval stage of zebrafish (*Danio380 rerio*), when hyperplasia is accentuated; in juvenile and adult phases, where muscle hyperplastic growth is less intense, myostatin expression was higher. These results do not agree with our findings, as this work has demonstrated intense muscle fiber recruitment during all the studied stages, although hypertrophy also occurred. During the early juvenile stages in pirarucu, although the level of myostatin mRNA was low, there was a high level of protein.

It was expected myostatin to be expressed at low levels throughout all evaluated growth stages. In this case, because the protein level was high during this period, it is possible that myostatin 387 may not be directly involved in negatively regulating muscle growth in pirarucu during the early juvenile growth stage. Similar to our results, showed that myostatin plays a dual role in regulating muscle growth at different developmental stages in medaka (*Oryzias latipes*) and regulates the number and size of fibers in a temporally controlled manner during post-hatch growth. Once myostatin effects on muscle growth in fish are not as well understood as in mammals, our results provided interesting insights to develop future experiments to better understanding the biological function of myostatin in skeletal muscle growth dynamics during early stage in fish.

In summary, our data suggest that MyoD, myogenin, and myostatin act to modulate the hyperplastic and hypertrophic muscle growth in pirarucu, which could be the major responsible for the outstanding growth performance observed in this species.

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