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Morphology and expression of genes related to skeletal muscle growth in juveniles of pirarucu (*Arapaima gigas,* Arapaimatidae, Teleostei)

Fernanda Regina Carani*, Bruno Oliveira Da Silva Duran, Tassiana Gutierrez De Paula, Warlen Pereira Piedade and Maeli Dal-Pai-Silva

*Departamento de Morfologia, Instituto de Biociências, Universidade Estadual Paulista, Distrito de Rubião Junior, s/n, 18618-970, Botucatu, São Paulo, Brazil. *Author for correspondence. E-mail: fcarani@gmail.com*

ABSTRACT. Skeletal muscle growth in the pirarucu (*Arapaima gigas*) is highly interesting to fish farmers because it provides information about how the mechanism in muscle mass increase, characteristic of the species, is regulated. Pirarucu has specific muscle growth that highlights the species's significance and commercial value. Current research evaluates the morphology and the growth-related gene expression in the red and white skeletal muscles of the pirarucu. Muscle samples were collected from the lateral anterior region and frozen in liquid nitrogen. Histological sections were performed and stained by HE for morphological analysis. Red and white muscle samples were used to determine MyoD, myogenin, and myostatin genes expression by Real-time Polymerase Chain Reaction. Although MyoD and myogenin were not statistically different in the two types of muscles, myostatin was significantly higher in the white rather than in the red muscle. Results show the muscle growth characteristics of the species and may be helpful for improving aquaculture management programs.

Keywords: muscle fibre morphology, myogenic regulatory factors, myostatin, skeletal muscle growth, *Arapaima gigas*, real time PCR.

Morfologia e expressão de genes relacionados ao crescimento do músculo esquelético em juvenis de pirarucu (*Arapaima gigas,* Arapaimatidae, Teleostei)

RESUMO. A caracterização do crescimento muscular no pirarucu (*Arapaima gigas*) é de elevado interesse para os piscicultores, pois fornece informações de extrema importância sobre como esse mecanismo é regulado e permite o rápido aumento na massa muscular característico da espécie. O pirarucu possui um crescimento muscular típico, o que destaca sua importância e seu valor comercial. O objetivo do presente trabalho foi avaliar a morfologia e a expressão de genes relacionados ao crescimento da musculatura esquelética vermelha e branca do pirarucu. As amostras de músculo vermelho e branco foram obtidas da região lateral anterior superficial e profunda, respectivamente, e estas foram congeladas em nitrogênio líquido. Para análise morfológica, cortes histológicos obtidos em criostato foram submetidos à coloração com HE. A expressão dos genes MyoD, miogenina e miostatina foi feita por PCR em tempo real após transcrição reversa (RT-qPCR). Em relação à expressão de MyoD e miogenina, não houve diferença estatística na comparação entre os músculos; por outro lado, a expressão da miostatina foi significativamente maior no músculo branco, em comparação com o músculo vermelho. Estes resultados refletem as características de crescimento muscular do pirarucu e podem ser úteis na tentativa de melhorar as condições de criação e a sobrevivência da espécie.

Palavras-chave: morfologia das fibras musculares, fatores reguladores miogênicos, miostatina, crescimento muscular, *Arapaima gigas*, PCR em tempo real.

Introduction

Muscle growth in fish depends on the proliferation and differentiation of myogenic precursor cells (termed satellite cells or myoblasts) (JOHNSTON et al., 2000; ROWLERSON; VEGGETTI, 2001). When these cells are activated, they proliferate and differentiate and their nuclei are internalized by existing fibres, which characterize hypertrophic muscle growth (ROWLERSON; VEGGETTI, 2001). Proliferating myoblasts are aggregated to the fibre surface and generate new myotubes. When they separate, they give rise to new muscle fibres in a process called hyperplasia (DAL PAI-SILVA et al., 2003a and b; JOHNSTON et al., 2000).

Hypertrophy and hyperplasia are regulated by the sequential expression of Myogenic Regulatory

Factors (MRFs), *myoD*, *myf-5*, *myogenin*, and *mrf4*. They are transcriptional factors that bind to a specific DNA sequence, termed *E-box* (5'-CANNTG-3'), found in the promoter region of many muscle-specific genes (JOHANSEN; OVERTURF, 2005; RESCAN, 2001; SABOURIN; RUDNICKI, 2000; WATABE, 2001). *MyoD* and *myf-5* control the determination of myogenic lineage and regulate myoblast activation and proliferation (GOULDING et al., 1994). *Myogenin* and *mrf4* act in the myoblast differentiation stage in which myotubes fuse to form new myofibres (GROBET et al., 1997).

Myostatin (*mstn*) is a member of the Transforming Growth Factor beta (*TGF-β*) superfamily of growth and differentiation factors. It is known for its potent abilities to negatively regulate mammalian skeletal muscle growth (LEE, 2004). *Myostatin* in mammals is mainly restricted to skeletal muscles (McPHERRON et al., 1997), adipose tissues (GONZALEZ-CADAVID et al., 1998), mammary glands (JI et al., 1998) and cardiac muscles (SHARMA et al., 1999). Due to its wide expression, its function in fish may be more diverse and may influence many other tissues (DELGADO et al., 2008; HELTERLINE et al., 2007; KO et al., 2006; KOCABAS et al., 2002; MACCATROZZO et al., 2001; OSTBYE et al., 2001; RESCAN et al., 2001; ROBERTS; GOETZ, 2001; RODGERS et al., 2001; XU et al., 2003; XUE et al., 2006). Mutations in the *myostatin* gene result in a 'double-muscling' phenotype in many cattle breeds and mice (KAMBADUR et al., 1997; MCPHERRON et al., 1997), whereas a similar muscle increase pattern has also been observed in pigs (JI et al., 1998), chickens (KOCAMIS et al., 1999) and fish (ACOSTA et al., 2005; LEE et al., 2009; MEDEIROS et al., 2009).

The Amazonian pirarucu (*Arapaima gigas*) is an economically important large-size fish for aquaculture projects. It grows fast and the adult reaches up to 3 m in length and 250 kg in weight (SOUZA; VAL, 1990). It has been bred under intensive conditions, with satisfactory production results, reaching 10 kg in just one year (IMBIRIBA, 2001; PEREIRA-FILHO et al., 2003).

Muscle morphology and *myoD*, *myogenin*, and *myostatin* genes expression in red and white muscle of pirarucu were analyzed to understand the cellular and molecular mechanisms involved in regulating fish muscle growth. Current study is a contribution towards fish aquaculture since improved production rates are directly related to muscle growth dynamics of fish species, in particular aquaculture species.

Material and methods

Fish

Experiment was approved by the Ethics Committee of the Bioscience Institute, UNESP, Botucatu, São Paulo State, Brazil (Protocol N. 72/07-CEEA). Specimens of pirarucu (*Arapaima gigas*) (n=7), weighting 47.94 ± 3.64 g and two months old, were euthanized with MS-222 (Tricaine Methanensulfonate-SIGMA®). White and red muscles were isolated from deep and surface layers, respectively, close to the head and near the lateral line (Figure 1). Muscle samples were immediately frozen in liquid nitrogen and stored at -80°C.

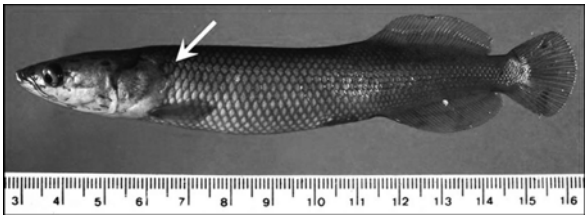

Figure 1. Pirarucu specimen (*Arapaima gigas* C.). Muscle sample area is indicated.

Morphological analysis

Histological transversal sections of red and white muscle fibre (10 μm thick) were obtained in a -20°C cryostat and stained with Haematoxylin-Eosin (BANCROFT; STEVEN, 1990).

RNA extraction and RT-PCR

Total RNA was extracted from muscle samples using *TRIzol*® Reagent (Invitrogen, Carlsbad CA USA) protocol. Purity and yield were determined by measuring absorbance of aliquots at 260 and 280 nm and total RNA integrity was confirmed by inspecting the electrophoresis pattern of 28S and 18S ribosomal RNA. Total RNA was incubated with DNase I - Amplification Grade (Invitrogen Life Technologies, Carlsbad CA USA) to remove any genomic DNA present and two micrograms of RNA were reverse transcribed using High Capacity cDNA Archive Kit (Applied Biosystems, Foster City CA USA). These samples were PCR amplified with specific primers pairs to *myoD*, *myogenin* and *myostatin* genes, designed from *Ictalurus furcatus* sequences (Accession n. AY562555, AY540993 and AY540992, respectively), available in GenBank database (http://www.ncbi.nlm.nih. gov). A set of primers designed from the 18S ribosomal RNA consensus fish sequences was used to amplify a segment of 18S rRNA gene in pirarucu muscle samples (TOM et al., 2004), used as reference gene to normalize the relative quantification results.

All PCR products were sequenced and used to design primer pairs for the qPCR analysis with *Primer Express®* software (Applied Biosystems, Foster City CA USA): *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; *18S rRNA* (forward) 5' TAC CAC ATC CAA AGA AGG CAG, (reverse) 5' TCG ATC CCG AGA TCC AAC TAC.

Quantitative PCR (qPCR)

MyoD, *myogenin*, and *myostatin* genes expression analyses were performed in an *ABI 7300 Real Time PCR System* (Applied Biosystems, Foster City CA USA). Data from *Power SYBR® Green PCR* amplicons were collected with *ABI 7300 System SDS software v. 1.4* (Applied Biosystems, Foster City CA USA). Fluorescence signal baseline and threshold were set manually for each detector (*myoD*, *myogenin*, *myostatin* and *18S* rRNA), generating a threshold cycle (Ct) for each sample. Relative quantification was obtained by the Comparative Ct Method (ΔΔCt Method), which required an amplification validation, showing that efficiencies of target and reference genes were similar (LIVAK; SCHMITTGEN, 2001). All PCR efficiencies in current study were measured and found adequate (slope < 0.1). Differences between Ct values were calculated for each mRNA by taking mean Ct of duplicate reactions and subtracting mean Ct of duplicate reactions for the reference gene mRNA ($\Delta Ct = Ct_{target\ gene} - Ct_{reference\ gene}$). All samples were then normalized to ΔCt rate of a calibrator sample to obtain a ΔΔCt rate ($\Delta Ct_{target} - \Delta Ct_{calibrator}$). Red muscle (which had the lowest relative expression, i.e., highest Ct value, for all genes) was chosen as calibrator sample to evaluate putative target gene differential mRNA expression between the two muscle types. The Comparative Ct method (ΔΔCt) calculated relative quantifications in relation to calibrator sample concentrations ($2^{-\Delta\Delta Ct}$), expressed in arbitrary units and normalized for the endogenous reference gene (18S rRNA). Therefore, by using the $2^{-\Delta\Delta Ct}$ method, data were recorded as the fold-change in gene expression normalized with the reference gene and relative to the calibrator sample (LIVAK; SCHMITTGEN, 2001). Dissociation melting curves confirmed the specific amplification of the cDNA target and the absence of nonspecific amplification products.

Statistical Analysis

Unpaired t-Test compared differences in *myoD* and *myogenin* mRNA levels between the calibrator sample (red muscle) and the white muscle samples. Welch's corrected unpaired t-Test compared differences in *myostatin* mRNA expression levels for the two groups. The statistical significance level was set at 5% ($p < 0.05$) for all analyses. *GraphPad InStat v. 3.01 software for Windows* for the two tests (1998, GraphPad Software, San Diego CA USA) were employed.

Results

Muscle morphology

Skeletal muscle fibres in the pirarucu are organized in distinct compartments: the surface red layer, immediately under the skin, composed of slow-twitch oxidative fibres, and the deep white layer, which predominates in the myotome, is formed by fast-twitch glycolytic fibres. Morphological analysis of skeletal muscle in the pirarucu showed a similar muscle fibres pattern within the compartments; fibres were polygonal or round in shape, multinucleate, and with peripheral nuclei. The muscles had fibres of different sizes separated by a connective tissue, usually called endomysium, and arranged in fascicules by a thick connective septum (Figure 2). Further, the surface red muscle in the pirarucu appeared as a large layer in the lateral line region across the whole body (not shown).

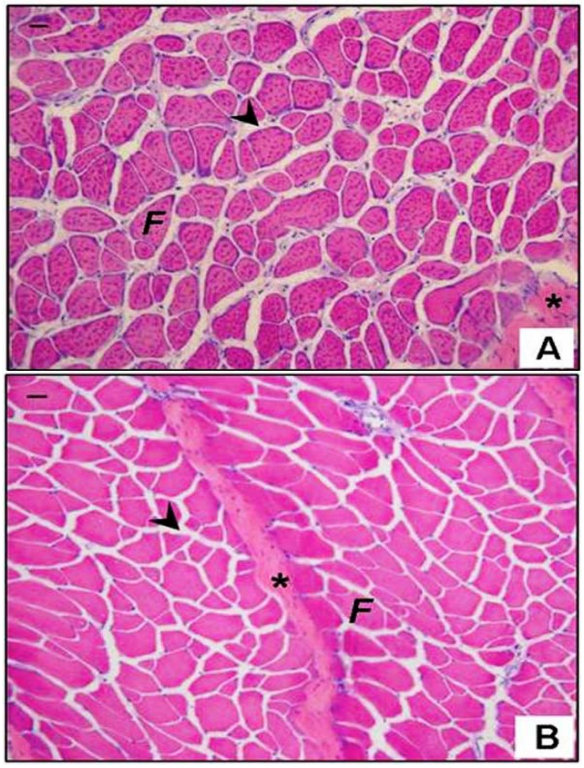

Figure 2. Transversal sections of red (*A*) and white (*B*) muscles from juvenile pirarucu (*Arapaima gigas*); Polygonal fibres (F); Endomysium (arrowheads); Connective septum (*); Scale: 20 μm. (HE).

MyoD, myogenin and myostatin mRNA expression

Quantitative analysis showed that estimated *myoD* mRNA levels were not statistically different ($p > 0.05$) in the pirarucu's red and white muscles (Figure 3). Similarly, estimated *myogenin* mRNA levels were not different ($p > 0.05$) between the two muscle types (Figure 4). Contrastingly, estimated *myostatin* mRNA levels showed a striking difference ($p < 0.01$) between muscle types (Figure 5). Data are expressed as mean ± SEM, represented as arbitrary units.

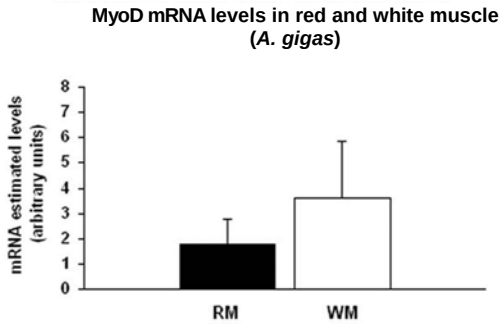

Figure 3. Relative MyoD mRNA expression in red (*RM*) and white (*WM*) muscles of pirarucu (*Arapaima gigas*) by Real-time PCR. No significant differences between groups were reported by unpaired t-Test ($p > 0.05$).

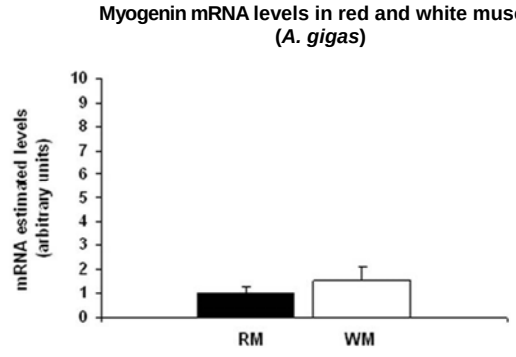

Figure 4. Relative myogenin mRNA expression in red (*RM*) and white (*WM*) muscles of pirarucu (*Arapaima gigas*). No significant differences between groups were reported by unpaired t-Test ($p > 0.05$).

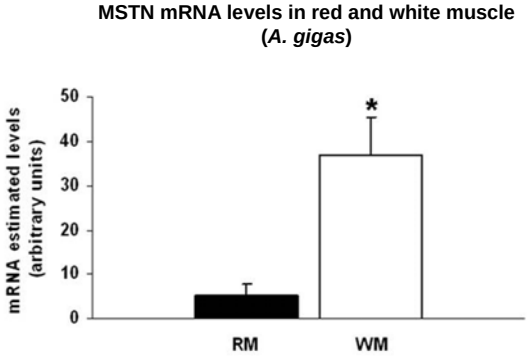

Figure 5. Relative myostatin mRNA expression in red (*RM*) and white (*WM*) muscles of pirarucu (*Arapaima gigas*) by Real-time PCR. Welch's corrected unpaired t-Test shows significant differences between groups (*$p < 0.01$).

Discussion

Morphological analysis of red and white muscles showed a typical muscle fibre pattern – polygonal or round, multinucleated, and with peripheral nuclei. The two muscles had fibres of different sizes separated by connective tissue, usually called endomysium, and arranged in fascicules by a thick connective septum (Figure 2). The fibre arrangement is similar to that of other teleost fish and depends on fish environment requirements (AGUIAR et al., 2005; DAL PAI et al., 2000; FERNANDEZ et al., 2000), fish species, developmental growth stage and swimming behaviour movement (ANDO et al., 1992; DAL PAI-SILVA et al., 2003a and b). The presence of a thicker connective tissue of perimysium in the pirarucu's skeletal muscle may provide a support structure for the maintenance of the whole body and allows the transmission of contraction force from muscle fibres to the axial skeleton and caudal fin. These integrated and simultaneous events generate the body wave motion and propulsion movement, extremely important during swimming behaviour of large-size fish (SÄNGER; STOIBER, 2001) such as the pirarucu.

Muscle fibres in the pirarucu are distributed into distinct layers, surface red and deep white, similar to those of most fish species (AGUIAR et al., 2005; DAL PAI-SILVA et al., 1996; SÄNGER; STOIBER, 2001). White muscle is a widely studied compartment in fish because it comprises most of the fillet and is related to the fast-twitch function and to the greater power outputs required for escape responses and predation behaviour (JOHNSTON, 2006). Although the red muscle in the pirarucu is less important for aquaculture production, a thick layer of red muscle fibres along the whole body lateral line may be directly involved with the breathing behaviour. In fact, it acts as the main source of slow-oxidative fibres for the continuous and sustained swimming movements during aerial respiratory behaviour (CARANI et al., 2008).

During postnatal muscle growth in fish, hyperplasia is mainly regulated by the Myogenic Regulatory Factor *myoD*, which stimulates myoblast proliferation and posterior myotube formation (GOULDING et al., 1994; RESCAN, 2001). Current study failed to detect any difference in *myoD* gene expression between red and white muscles. The above results are similar to *myoD* gene expression studies in other fish species at different development stages, such as those found in the rainbow trout (*Oncorhynchus mykiss*) (JOHANSEN; OVERTURF, 2005). However, many other studies

compare *myoD* expression in fish muscles with no similarity between results (DELALANDE; RESCAN, 1999; TAN; DU, 2002; ZHANG et al., 2006). Discordant results, corroborated by those in current analysis, presume that *myoD* gene expression in fast and slow muscles may reflect differences in regulating the growth mechanisms of these two types of muscle fibre depending on fish species. In the pirarucu, the similar *myoD* gene expression in fast and slow muscles seems to be related to the intense satellite cell proliferation involved with intense hyperplasia and hypertrophy mechanisms occurring in both muscles during this growth stage. These events may contribute towards the great muscle mass increase that occurs in the species.

Myogenin is characterized by regulating the later stages of muscle fibre formation and growth, which culminate in myoblast fusion, myotube formation, and finally, adult muscle fibre differentiation (GROBET et al., 1997; SCHMALBRUCH; LEWIS, 2000). Current investigation showed that *myogenin* gene expression had a similar pattern to *myoD* expression: there was no difference between red and white muscles. This may have been related to the intense process of muscle fibre differentiation occurring during the pirarucu's growth stage. The fibres, primarily similar to undifferentiated myoblasts, which are at first stimulated by *myoD* expression to proliferate, now have to fuse and form multinucleated myotubes that differentiate into muscle fibres. In fact, these later processes are mainly regulated by *myogenin*. Similar *myogenin* expression levels in red and white muscles may be attributed to the intense muscle fibre formation rate and to the differentiation that occur in a highly activated state in both muscles in the pirarucu's initial stage.

Few studies are extant that analyze *myostatin* expression in slow and fast muscles separately. According to Roberts and Goetz (2001), *myostatin* mRNA is higher in the red muscle from the adult brook trout, king mackerel, and yellow perch. It is also predominantly expressed in the white muscle from the little tunny and equally expressed in both muscle types in mahi-mahi (ROBERTS; GOETZ, 2001). Østbye et al. (2001) demonstrated that *myostatin* mRNA in salmon is detectable in the red muscle only. Furthermore, Patruno et al. (2008) have shown high levels of *myostatin* in red and low levels in white muscle of adult sea bass. Nonetheless, there were no significant differences in juveniles. Current study shows a wide difference in *myostatin* expression between the pirarucu's slow and fast muscles. Red muscle exhibited low levels of *myostatin* mRNA content, whereas *myostatin* expression was extremely high in white muscle. The *myostatin* expression pattern is analogous to that observed in adult mammals, where *myostatin* mRNA levels are higher in fast-glycolytic fibres than in slow ones (CARLSON et al., 1999; MATSAKAS et al., 2006). Based on these results and on the observation that satellite cells express *myostatin* and are quiescent (MCCROSKERY et al., 2003), it has been suggested that the normal function of *myostatin* in postnatal muscle is to maintain satellite cells in a quiescent, undifferentiated state (MANCEAU et al., 2008).

Current results show that since the pirarucu's red muscle has low *myostatin* mRNA levels, intense satellite cell proliferation and hyperplasia are indicated. In contrast, white muscles have high *myostatin* mRNA levels and may indicate that it is acting on the white muscle fibre inhibiting satellite cell proliferation. This event disagrees with the high myoblast proliferation rate and fibre hyperplasia that occur at this development stage, regardless of the slow/fast phenotype. Although satellite cells are highly activated, a large population of myotubes has already been formed and their next step is to differentiate into new myofibres. The differentiation process is well characterized and is controlled by the Myogenic Regulatory Factor *myogenin* (GROBET et al., 1997). The idea that *myostatin* is regulating the differentiation process by controlling *myogenin* action is well supported by studies that show *myogenin* as a probable major target of endogenous *myostatin* (JOULIA et al., 2003).

Conclusion

Current results suggest that *myoD*, *myogenin*, and *myostatin* modulate the equilibrium between satellite cells proliferation and differentiation during postnatal muscle growth in the initial growth phase of the pirarucu. Future research within the study of muscle growth-related genes throughout the growth phases are of great interest within the context of the fast body development that occurs in this large freshwater fish during such a short period.

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