UNIVERSIDADE ESTADUAL DE CAMPINAS

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EXPRESSÃO DO FATOR DE REGULAÇÃO MIOGÊNICA MyoD, NA MUSCULATURA ESTRIADA ESQUELÉTICA DO PACU (*Piaractus mesopotamicus*), DURANTE O CRESCIMENTO

Este exemplar corresponde à redação final da tese defendida pelo(a) candidato (a) Fernanda Losi Alves de Almeida e aprovada pela Comissão Julgadora.

Maeli Dal Pai Silva

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Orientadora: Profa. Dra. Maeli Dal Pai Silva

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ÍNDICE

1. Resumo..... 1

2. Abstract..... 3

3. Introdução..... 5

Organização da musculatura estriada esquelética nos peixes..... 5

Desenvolvimento embrionário do músculo estriado esquelético em peixes..... 8

Fatores de regulação miogênica..... 10

Crescimento pós-embrionário da musculatura estriada esquelética em peixes..... 13

Piaractus mesopotamicus..... 16

4. Objetivos..... 18

Artigo

Differential expression of Muscle Regulatory Factor MyoD from pacu (*Piaractus mesopotamicus*), during juvenile and adult growth phases. 19

5. Conclusões Gerais..... 46

6. Referências Bibliográficas Gerais..... 47

1. RESUMO

Nos peixes, o crescimento do tecido muscular ocorre por hipertrofia e/ou hiperplasia a partir da proliferação e diferenciação de mioblastos adultos ou células miossatélites, processos regulados pela expressão diferencial dos fatores de regulação miogênica (MRFs). O objetivo desse trabalho foi avaliar os mecanismos de crescimento muscular hiperplásico e hipertrófico e a expressão do MRF MyoD, na musculatura branca do pacu (*Piaractus mesopotamicus*), durante o crescimento. Exemplares juvenis (n=5) e adultos (n=5) de pacu foram anestesiados, sacrificados e determinados o peso corporal (g) e o comprimento total (cm). Fragmentos musculares brancos da região dorsal de cada exemplar, em cada fase estudada, foram congelados e imersos em n-hexano congelado em nitrogênio líquido. Cortes histológicos (10 μm), obtidos em criostato, foram submetidos à coloração hematoxilina-eosina para avaliação da morfologia e morfometria das fibras musculares brancas. Foi calculado o menor diâmetro de 100 fibras musculares brancas em cada animal de cada fase estudada. As fibras musculares foram distribuídas em classes, na dependência do seu diâmetro (<20, 20-50, >50μm), para avaliar o grau de crescimento hipertrófico e hiperplásico da musculatura. A expressão do MRF MyoD na musculatura branca foi analisada por Reação em Cadeia da Polimerase após Transcrição Reversa (RT - PCR). Todos os produtos visualizados em gel de agarose a 1% foram clonados e seqüenciados. A morfologia da musculatura dos exemplares juvenis e adultos foi semelhante, apresentando um padrão em mosaico caracterizado por fibras de diferentes diâmetros. Nos exemplares juvenis, foi observado um predomínio de fibras com diâmetro menor que 20 μm, caracterizando intensa hiperplasia. Nos exemplares adultos, houve o

predomínio de fibras musculares com diâmetro maior que 50 μm, caracterizando intensa hipertrofia da musculatura. A expressão do RNAm para o gene MyoD foi significativamente maior na fase juvenil, se comparada com a fase adulta. Foi obtida a seqüência consenso parcial do gene MyoD (338 pares de bases) expresso na musculatura branca do pacu. Essa seqüência apresentou similaridade com as seqüências de MyoD de várias espécies de vertebrados, incluindo peixes teleósteos. A expressão diferencial do MRF MyoD, observada nas fases de crescimento juvenil e adulta do pacu, possivelmente seja responsável pelas diferenças observadas no padrão de crescimento, com a hiperplasia predominando nos juvenis e a hipertrofia, nos adultos.

2. ABSTRACT

Skeletal muscle growth in fish occurs by hypertrophy and hyperplasia and is dependent of the proliferation and differentiation of myogenic progenitor cells, events regulated by the diferential expression of the myogenic regulatory factors (MRFs). The aim of this study was to analyze the hyperplasia and hypertrophy processes and the MRF MyoD expression in the white muscle in pacu (*Piaractus mesopotamicus*) during growth. Juvenile (n=5) and adult (n=5) fishes were anaesthetized, sacrificed and the weight (g) and the total length (cm) were determined. White muscle samples from dorsal region of each sample, in each growth phase, were collected and and immersed in n-Hexane cooled in liquid nitrogen. Transverse sections (10 µm thick), obtained in a cryostat, were stained with Haematoxilin-Eosin to morphological and morphometric analysis. We calculated the smallest diameter from 100 white muscle fibres per animal in each group. White muscle fibers were grouped in three classes: <20, 20-50 and >50µm to evaluate hypertrophy and hyperplasia in pacu white skeletal muscle. MyoD gene expression was determined by using RT-PCR. All PCR products visualized in 1% agarose gels were cloned and sequenced. Juvenile and adult pacu fish skeletal muscle showed similar morphology, with mosaic pattern characterized by fibers with different diameters. The great number of muscle fibers with diameter inferior 20µm observed in juvenile fish confirms the active hyperplasic process. In adult fish, most fibers were over 50µm diameter and denote the more intense muscle fiber hypertrophy. MyoD mRNA level in the juvenile fish was higher compared to adult fish. A consensus partial sequence for MyoD gene (338 bases pairs) was obtained. This sequence showed similarity with various vertebrate species, including teleost fishes. Differential expression

of MyoD gene observed in white muscle of pacu possibly is related to differences in growth patterns during the phases analysed, with predominance of hyperplasia in juveniles and hypertrophy in adult fish.

3. INTRODUÇÃO

3.1. Organização da musculatura estriada esquelética nos peixes

Nos peixes, a maior parte da massa corporal é representada pelo tecido muscular estriado esquelético que constitui de 40 a 60% do peso total do animal. Essa abundante massa muscular não representa somente um mecanismo específico para a adaptação desses animais no meio aquático, mas serve como importante fonte de proteínas utilizadas na alimentação humana (Weatherley & Gill, 1985).

Na maioria das espécies de peixes, a musculatura estriada esquelética (musculatura miotomal) está organizada em unidades morfofuncionais, os miômeros, que se repetem ao longo do corpo do animal e são separados por bainhas de tecido conjuntivo, os miosseptos (Alexander, 1969) (Figura 1). Na região onde se encontra o nervo da linha lateral, um septo de tecido conjuntivo, o septo transverso, separa a massa muscular em regiões epiaxial e hipoaxial (Alexander, 1969; Grizzle & Rogers, 1979).

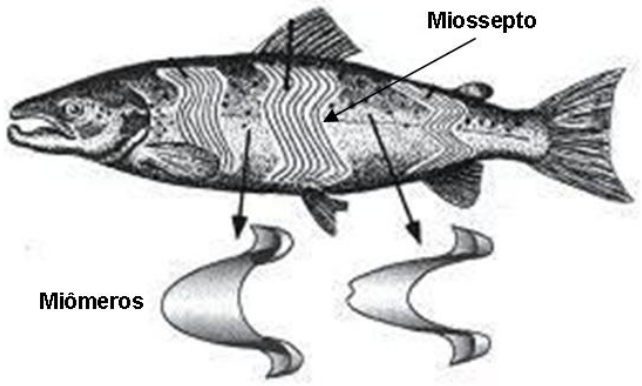

Figura 1. Organização anatômica da musculatura estriada esquelética (miotomal) em miômeros e miosseptos (adaptado de Johnston, 2001).

As análises morfológica e histoquímica do tecido muscular esquelético mostram três tipos básicos de fibras musculares: vermelhas, intermediárias e brancas (Johnston, 1981). Na maioria das espécies de peixes, as fibras estão distribuídas em compartimentos: vermelho, intermediário e branco, com as fibras brancas correspondendo a mais de 90% do volume total do tecido muscular (Weatherley & Gill, 1989; Kilarski, 1990) (Figura 2).

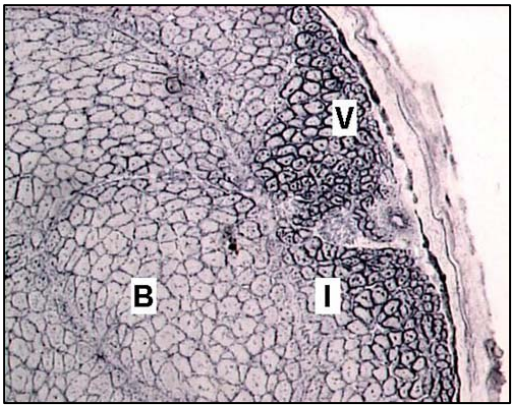

Figura 2. Corte transversal da musculatura estriada esquelética da tilápia do Nilo (*Oreochromis niloticus*). Compartimento vermelho superficial (V), compartimento intermediário (I) e compartimento branco profundo (B). Reação NADH-TR (adaptado de Aguiar *et al*., 2005).

A distribuição dos tipos de fibras em compartimentos nos peixes difere do padrão encontrado nos demais vertebrados, nos quais os tipos de fibras estão distribuídos em um padrão em mosaico. Nos mamíferos, de um modo geral, a maioria dos músculos é formada por uma população heterogênea de fibras, distribuídas de maneira a formar um mosaico ou pequenos grupos de fibras de um mesmo tipo (Armstrong *et al*., 1982; Armstrong & Pheleps, 1984).

A distribuição das fibras musculares nos compartimentos é variável entre as espécies de peixes, como também nos diferentes estágios de desenvolvimento desses

animais. Assim, o compartimento de músculo vermelho pode aparecer na região subdermal como uma camada fina e uniforme ao longo de todo o corpo do animal (Egginton & Johnston, 1982; Dal Pai-Silva *et al*., 1995) ou pode apresentar uma distribuição mais localizada, aparecendo somente na região do nervo da linha lateral, onde assume um aspecto triangular (Hoyle *et al*., 1986; Sanger & Stoiber, 2001).

O compartimento vermelho é formado por fibras musculares de contração lenta e metabolismo oxidativo. As fibras vermelhas normalmente apresentam pequeno diâmetro (25-45 μm), alta concentração de mioglobina, muitas mitocôndrias, lipídios e um excelente suprimento sanguíneo (Bone, 1978; Johnston, 1981; Sänger & Stoiber, 2001), sendo utilizadas na realização de movimentos lentos e de sustentação, como a migração (Bone, 1966; Johnston *et al.,* 1977).

A maior parte da massa muscular é formada pelo compartimento branco, constituído por fibras de contração rápida e metabolismo glicolítico (Driedzic & Hochachka, 1976). As fibras musculares brancas apresentam diâmetros que variam de 50 a 100μm, baixa concentração de mioglobina, poucas mitocôndrias, lipídios e miofibrilas ocupando entre 75 e 95% do volume total da fibra (Sänger & Stoiber, 2001). Esse tipo de musculatura é utilizado nos movimentos bruscos de natação, como a captura de alimento e fuga de predadores (Altringham & Johnston, 1988).

Entre os compartimentos vermelho e branco, encontra-se o compartimento intermediário (musculatura intermediária), com fibras que apresentam propriedades morfofisiológicas intermediárias entre as das fibras musculares brancas e vermelhas (Romanello *et al*., 1987; Mascarello *et al*., 1995; Zhang *et al*., 1996; Sänger & Stoiber, 2001), como contração rápida e metabolismo oxidativo/glicolítico (Johnston *et al*., 1977).

3.2. Desenvolvimento embrionário do músculo estriado esquelético em peixes

Nos peixes, a formação das primeiras fibras musculares ocorre nas fases iniciais da embriogênese. O tecido muscular estriado esquelético origina-se do mesoderma paraxial, localizado adjacente à notocorda e ao tubo neural em formação. O mesoderma paraxial segmenta-se na direção rostro-caudal do embrião, formando os somitos (Kimmel *et al*., 1990). Cada somito é formado por uma porção ventral, o esclerótomo, que dará origem ao esqueleto e à cartilagem do embrião, e uma dorsal, o dermomiótomo, que formará a derme e as musculaturas do tronco e da cauda (Currie & Ingham, 2001). Nos peixes, o esclerótomo é altamente reduzido, pois, no ambiente aquático, o animal apresenta maior facilidade em sustentar o peso do corpo. Dessa forma, a maior parte do somito é constituída pelo miótomo (Bone, 1966).

Nos somitos, uma população de células mesodérmicas dispõe-se em uma única camada que flanqueia ambos os lados da notocorda. Pela morfologia e padrão de expressão gênica, essas células são denominadas células adaxiais, células musculares não-pioneiras ou mioblastos “slow” (Currie & Ingham, 2001; Johnston & Hall, 2004). Sob o estímulo de glicoproteínas secretadas pela notocorda e pelo tubo neural (Blagden *et al*., 1997), essas células sofrem alongamento e migram em direção à superfície do miótomo, dispondo-se em uma única camada abaixo da epiderme. As células adaxiais fundem-se umas às outras, formando miotubos que se diferenciam em fibras musculares vermelhas, originando a musculatura vermelha do embrião. Uma subpopulação de células adaxiais, denominadas células adaxiais pioneiras, emite processos citoplasmáticos que as mantêm conectadas à notocorda, possivelmente para orientar a migração das demais células adaxiais em direção à superfície do miótomo.

Nessa região medial, forma-se uma estrutura especializada, o miossepto horizontal, que divide o miótomo nas regiões ventral (hipoaxial) e dorsal (epiaxial). As demais células do miótomo (mioblastos “fast”) fundem-se para formar miotubos, dando origem ao compartimento de músculo branco do embrião (Currie & Ingham, 2001; Johnston & Hall, 2004). Assim, nos peixes, as musculaturas vermelha e branca formam-se a partir de populações diferentes de mioblastos – as células adaxiais formarão o compartimento vermelho e as demais células do miótomo darão origem à musculatura branca (Devoto *et al.*, 1996) (Figura 3).

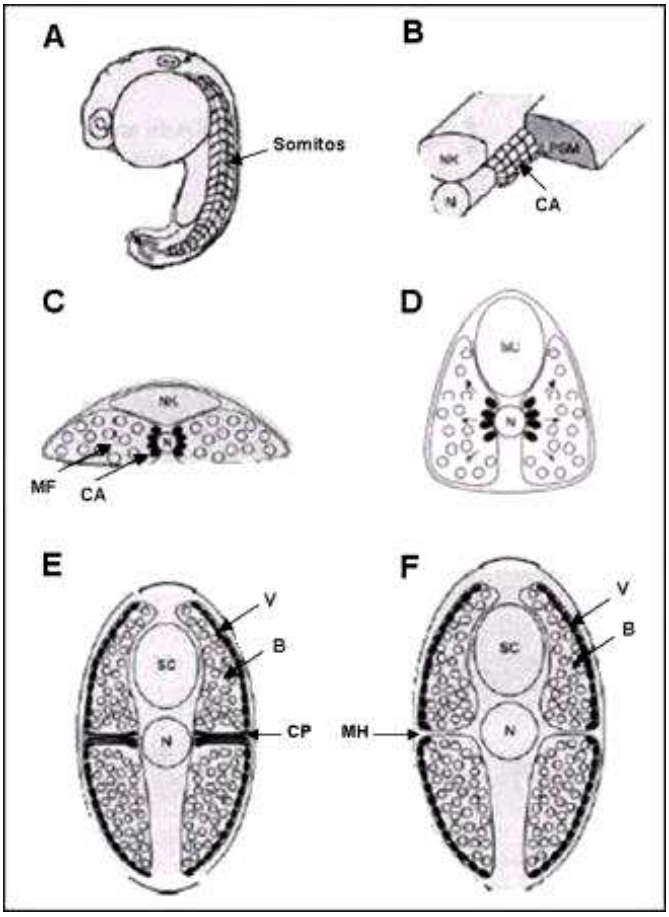

Figura 3. Miogênese no embrião de zebrafish. **A.** Embrião com 21 somitos. **B.** Células adaxiais (CA) adjacentes à notocorda (N). **C.** Corte transversal do embrião ilustrado em (B). MF: mioblastos “fast”. **D.** Células adaxiais alongam-se e migram em direção à superfície do miótomo. **E.** Células adaxiais formando o compartimento de músculo vermelho (V). Células pioneiras (CP) mantêm-se conectadas à notocorda, na região do futuro miossepto horizontal (MH). As demais células do miótomo (MF) se diferenciam em fibras musculares brancas (B). **F.** Final da miogênese, mostrando uma monocamada superficial de fibras vermelhas (V) circundando uma grande quantidade de fibras brancas (B) (adaptado de Johnston & Hall, 2004).

Os mioblastos (mononucleados) fundem-se uns aos outros, formando miotubos. Esses miotubos possuem um ou mais núcleos em posição central, miofibrilas em posição periférica e características morfológicas e fisiológicas próprias (Johnston *et al*., 1995; Johnston, 1999). Nos miotubos, ocorre a organização das proteínas que irão constituir a unidade contrátil, o sarcômero (Huxley, 1969). O sarcômero é constituído, principalmente, pelos filamentos grossos, compostos pelas cadeias de miosina, e pelos filamentos finos, compostos pelas proteínas actina, troponina e tropomiosina. A contração muscular depende do deslizamento dos filamentos finos sobre os filamentos grossos (Huxley, 1969; Huxley, 1971; Huxley, 1983).

Durante a diferenciação dos miotubos, ocorre a organização dos sarcômeros da periferia em direção ao centro do miotubo, enquanto os núcleos migram do centro para a periferia e as miofibrilas passam a ocupar quase todo o sarcoplasma (Sänger *et al*., 1990, Sänger, 1992). Paralelamente, ocorre o desenvolvimento do sistema de membranas constituído por Túbulos T e Retículo Sarcoplasmático, ambos envolvidos no processo de contração muscular (Schiaffino & Margreth, 1969; Kelly, 1971; Flutcher *et al*., 1992). Ao final desses processos, o miotubo passa a ser chamado de fibra muscular adulta.

3.3. Fatores de regulação miogênica

Todos os eventos da miogênese ocorrem sob o controle de fatores transcricionais conhecidos como Fatores de Regulação Miogênica (do inglês *myogenic regulatory factors* ou MRFs), dos quais fazem parte a MyoD, Myf5, Miogenina e MRF4 (revisado em Watabe, 2001). Os MRFs compartilham um domínio altamente conservado, conhecido como *basic helix-loop-helix* (bHLH), que apresenta 80% de

similaridade na sua seqüência de aminoácidos (Edmonson & Olson, 1993). A região *helix-loop-helix* é caracterizada por duas α-hélices separadas por um *loop*. A região básica (*basic*) compreende uma extensão de uma das α-hélices da região HLH (Cole *et al*, 2004) (Figura 4).

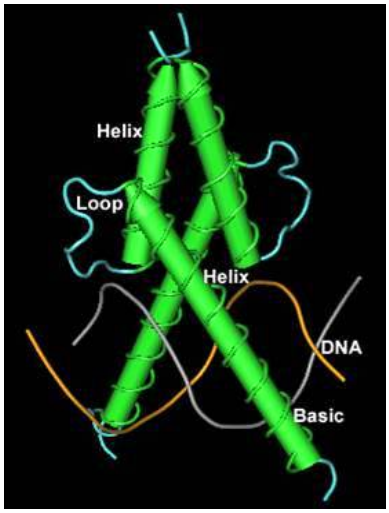

Figura 4. Estrutura cristalográfica do complexo formado pelo dímero do fator transcricional da família "basic Helix-Loop-Helix" (bHLH) MyoD e o DNA (adaptado de Ma *et al*., 1994).

Os MRFs reconhecem, através de seu domínio básico, uma seqüência consenso no DNA conhecida como E-box (5´-CANNTG-3´), presente na região promotora da maioria dos genes músculo-específicos (Lassar *et al*., 1989; Murre *et al*., 1989; Blackwell & Weintraub, 1990). A região *helix-loop-helix* dos MRFs constitui o domínio de ligação dessa molécula com proteínas E, como E12 e E47 (Murre *et al*., 1989). A ligação do heterodímero MRF-proteína E ou de homodímeros dos MRFs à seqüência E-box ativa a transcrição dos genes músculo-específicos, levando à sua expressão (Murre *et al*., 1989, 1994; Lassar *et al*., 1991).

A MyoD e o Myf5 são conhecidos como fatores primários, sendo expressos na fase de proliferação dos mioblastos, enquanto a miogenina e o MRF4 são expressos

em mioblastos nas fases de fusão e diferenciação em fibras musculares imaturas (Menegey and Rudnicki, 1995; Rudnick and Jaenisch, 1995; Watabe, 1999) (Figura 5).

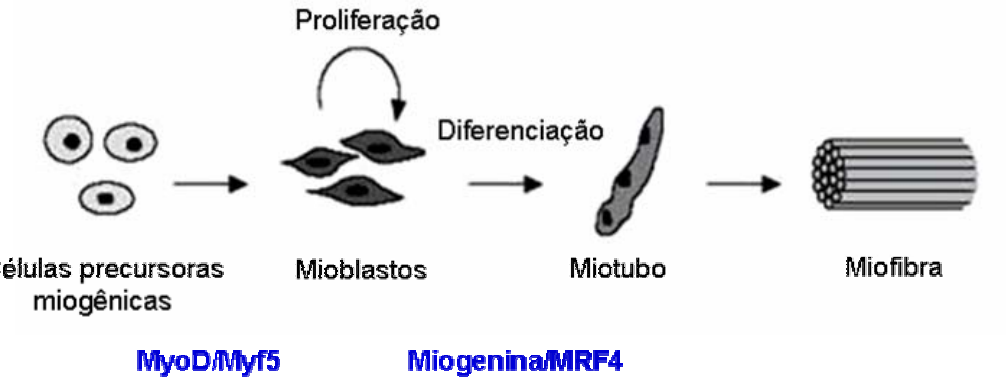

Figura 5. Células precursoras miogênicas, oriundas dos somitos, tornam-se mioblastos, que iniciam a proliferação. Esses eventos são controlados pela expressão dos MRFs MyoD e Myf-5. A expressão de miogenina e MRF4 controla a diferenciação dos mioblastos em miotubos, que posteriormente se diferenciam para formar as miofibras maduras (adaptado de Watabe, 1999).

Durante a embriogênese das fibras musculares, alguns mioblastos não se fundem, permanecendo como células indiferenciadas no tecido muscular. Esse tipo celular é referido como mioblasto adulto, precursor miogênico ou célula miossatélite. Nos mamíferos e em peixes na fase adulta, as células miossatélites estão localizadas entre a lâmina basal e a membrana plasmática (Mauro, 1961; Campion, 1984; Koumans & Akster, 1995; Johnston *et al*., 1998). No entanto, nas fases larval e juvenil, mioblastos indiferenciados podem ser observados no tecido conjuntivo do endomísio, entre as fibras musculares já diferenciadas (Veggetti *et al*., 1990; Koumans & Akster, 1995; Stoiber & Sänger, 1996; Fauconneau & Paboeuf, 2001).

3.4. Crescimento pós-embrionário da musculatura estriada esquelética em peixes

Nos peixes, o crescimento pós-embrionário da musculatura estriada esquelética é dependente da proliferação e diferenciação das células miossatélites, que são responsáveis pelo crescimento hiperplásico e hipertrófico das fibras musculares. Na hipertrofia, as células miossatélites fundem-se com fibras musculares existentes, aumentando o número de núcleos para maior síntese de miofibrilas, enquanto na hiperplasia, ocorre a formação de novos miotubos na superfície das fibras existentes, com posterior diferenciação em novas fibras musculares (Koumans & Akster, 1995; Johnston, 1999; Rowlerson & Veggetti, 2001) (Figura 6).

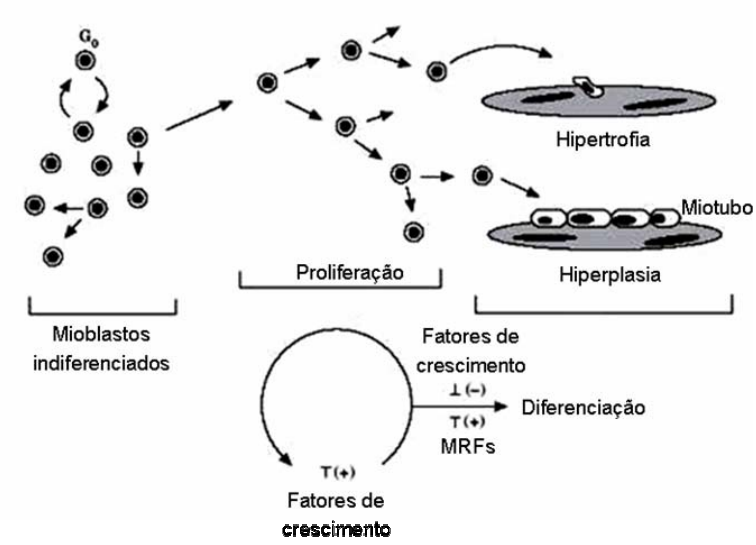

Figura 6. População de mioblastos indiferenciados (mioblastos adultos ou células miossatélites) que contribui para o crescimento hiperplásico e hipertrófico da musculatura estriada esquelética nos peixes. A proliferação e diferenciação dos mioblastos ocorrem sob o controle da expressão dos fatores de regulação miogênica (MRFs) e de fatores de crescimento (adaptado de Johnston, 1999).

A hiperplasia pode ocorrer de forma estratificada ou em mosaico. A hiperplasia estratificada ocorre em todas as espécies de peixes a partir das zonas germinais de

proliferação de mioblastos localizadas nas regiões dorsal e ventral dos miótomos e entre os compartimentos vermelho e branco. Esse tipo de hiperplasia é responsável pelo espessamento das camadas musculares nas fases iniciais do desenvolvimento (Figura 7A). A hiperplasia em mosaico resulta em um grande aumento no número de fibras musculares, principalmente na fase juvenil, sendo observada na musculatura das espécies de grande valor comercial que atingem um tamanho maior (Rowlerson & Veggetti, 2001). Quando a hiperplasia em mosaico está ocorrendo, observam-se fibras pequenas (diâmetro menor que 25μm) entre fibras maiores, formando um mosaico de fibras de diferentes tamanhos e estágios de diferenciação, melhor observado na musculatura branca (Johnston, 1999; Rowlerson & Veggetti, 2001) (Figura 7B).

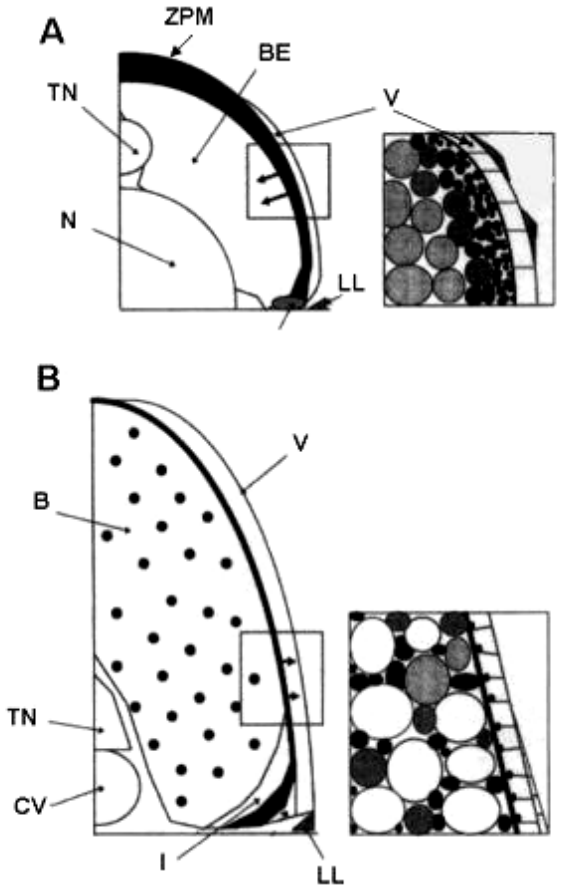

Figura 7. Representação esquemática da hiperplasia estratificada (A) e hiperplasia em mosaico (B) durante o crescimento da musculatura estriada esquelética. TN = Tubo neural, N = notocorda, BE = fibras musculares brancas embrionárias, ZPM = zona de proliferação de mioblastos, V = fibras musculares vermelhas, LL = linha lateral, CV = Coluna vertebral, B = fibras musculares brancas, I = fibras musculares intermediárias (adaptado de Rowlerson & Veggetti, 2001).

Entre todos os vertebrados, os peixes apresentam uma característica peculiar, principalmente quando os padrões de crescimento muscular são considerados. Com poucas exceções, os peixes tendem a apresentar crescimento indeterminado (Mommsen, 2001). Nos mamíferos, a hiperplasia cessa em um curto período após o desenvolvimento embrionário (Goldspink *et al*., 2001). Nos peixes, a hiperplasia e a hipertrofia contribuem por todo o período de crescimento da musculatura estriada esquelética. Nas espécies que atingem tamanho de poucos centímetros, o crescimento muscular envolve principalmente a hipertrofia de fibras formadas nas fases iniciais da embriogênese e o período de crescimento hiperplásico é mais curto. Nas espécies que atingem um tamanho maior, novas fibras musculares são continuamente recrutadas em todas as fases do crescimento (Weatherley *et al*., 1988; Alami-Durante *et al*., 1997; Rowlerson & Veggetti, 2001).

Durante o crescimento hiperplásico e hipertrófico da musculatura, é observada a retomada dos eventos ocorridos durante a miogênese. O crescimento pós-embrionário da musculatura ocorre pela ativação, proliferação e diferenciação dos mioblastos indiferenciados (células miossatélites) em fibras musculares, eventos iniciados e controlados pela expressão diferencial dos MRFs (Watabe, 1999). A proliferação dos mioblastos e a hiperplasia celular podem ser inferidos pela maior expressão dos MRFs MyoD e Myf5, nas fases iniciais de crescimento (Johansen & Overturf, 2005). Já, a expressão de Miogenina e MRF4 está relacionada com os processos de diferenciação dos mioblastos e hipertrofia das fibras musculares, mais intensa na fase adulta (Johansen & Overturf, 2005). Assim, o conhecimento sobre a expressão dos MRFs, que regulam os mecanismos de crescimento hiperplásico e

hipertrófico, pode indicar estratégias que beneficiem o crescimento muscular em espécies de peixes importantes economicamente, propiciando cultivo mais lucrativo e qualidade na produção em larga escala.

O balanço entre os mecanismos de crescimento muscular hipertrófico e hiperplásico pode deteminar a taxa de crescimento e o tamanho da espécie. Pesquisas que visam caracterizar esses processos e a expressão dos fatores de regulação miogênica, nas diferentes fases de crescimento do animal, são de fundamental importância para o melhoramento da aqüicultura, principalmente em espécies cultivadas em grande escala.

3.5. *Piaractus mesopotamicus*

O pacu, *Piaractus mesopotamicus* (Holmberg, 1887), é representante da superordem Ostariophysi, na qual se incluem os peixes de maior valor comercial na pesca e na piscicultura brasileiras (Urbinati & Gonçalves, 2005). Pertence à ordem Characiforme, grupo dominante entre os peixes de água doce da América do Sul (Urbinati & Gonçalves, 2005).

O pacu apresenta grande interesse econômico, sendo extensivamente utilizado nos programas brasileiros de aqüicultura (Hernandez, 1989). É um dos principais peixes dos rios do Pantanal, sendo encontrado nas Bacias dos rios Paraná, Paraguai e Uruguai (Godoy, 1975). Sua maior distribuição ocorre nas planícies alagadas da região Centro-Oeste, principalmente nos estados de Mato Grosso e Mato Grosso do Sul (Petrere, 1989). No entanto, a disponibilidade do pacu oriundo de piscicultura vem aumentando e pode ajudar a popularizar o seu consumo em outras regiões do Brasil.

Sua carne é saborosa e pode apresentar alto teor de gordura dependendo da idade, época de captura ou tipo de alimento utilizado no cultivo (Bernardino & Colares de Melo, 1989). No seu habitat natural, o pacu apresenta hábito onívoro, alimentando-se de folhas, caules, flores, frutos e sementes (Bernardino & Colares de Melo, 1989). Havendo necessidade, ele pode se alimentar de insetos, aracnídeos, moluscos e peixes (Urbinati & Gonçalves, 2005).

O pacu possui crescimento rápido, com um peso que varia de 1,0 a 1,5 kg no primeiro ano de cultivo (Bernardino & Colares de Melo, 1989). Os mecanismos de crescimento por hiperplasia e hipertrofia das fibras ocorrem por um período prolongado (Dal Pai *et al*., 2000).

Pouco é sabido sobre a expressão dos genes que controlam os mecanismos de crescimento muscular nas espécies cultivadas, em especial, nas espécies brasileiras de peixes, como o pacu. Esse estudo descreve pela primeira vez a expressão do fator regulador miogênico MyoD na musculatura estriada do pacu. Os resultados obtidos serão fundamentais para o estudo dos genes envolvidos no controle do crescimento muscular no pacu e em outras espécies brasileiras de peixes, iniciando a compreensão dos processos que regulam o crescimento muscular para propiciar a obtenção de peixes saudáveis com rápido crescimento na piscicultura.

A hipótese desse trabalho é que ocorre uma expressão diferencial do MRF MyoD na musculatura branca do pacu, nas fases de crescimento juvenil e adulta, uma vez que a contribuição dos mecanismos de crescimento muscular hiperplásico e hipertrófico é variável nessas fases de crescimento.

4. OBJETIVOS

Avaliar a expressão do fator de regulação miogênica MyoD, na musculatura branca do pacu (*Piaractus mesopotamicus*), correlacionando-a com os mecanismos de crescimento muscular hiperplásico e hipertrófico, nas fases de crescimento juvenil e adulta.

Differential expression of Muscle Regulatory Factor MyoD from pacu (*Piaractus mesopotamicus*), during juvenile and adult growth phases.

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Abstract

Skeletal muscle growth in fish occurs by hypertrophy and hyperplasia and is dependent of the proliferation and differentiation of myogenic progenitor cells, events regulated by the differential expression of myogenic regulatory factors (MRFs). The aim of this study was to analyze hyperplasia and hypertrophy and MRF MyoD expression in white muscle of pacu (*Piaractus mesopotamicus*) during the juvenile and adult growth stages. Juvenile (n=5) and adult (n=5) fishes were anaesthetized, sacrificed; weight (g) and total length (cm) were determined. White muscle samples from the dorsal region were collected and immersed in n-Hexane cooled in liquid nitrogen. Transverse sections (10μm thick) were cut and stained with Haematoxilin-Eosin for morphological and morphometric analysis. The smallest diameter from 100 white muscle fibres per animal was calculated in each growth phase. These fibres were grouped into three classes: <20, 20-50, and >50μm to evaluate hypertrophy and hyperplasia in white skeletal muscle. MyoD gene expression was determined by RT-PCR. All PCR products visualized in 1% agarose gels were cloned and sequenced. Juvenile and adult pacu fish skeletal muscle had similar morphology, with mosaic pattern characterized by different diameter fibres. The large number of <20μm diameter muscle fibres observed in juvenile fish confirms active hyperplasia. In adult fish, most fibres were over 50μm diameter and denote the more intense muscle fibre hypertrophy. The MyoD mRNA level in juvenile fish was higher than in adult fish (juvenile 0.50±0.04 vs. Adult 0.26±0.05; $p<0.05$). A consensus partial sequence for MyoD gene (338 bases pairs) was obtained. This sequence showed similarity with various vertebrate species, including teleost fishes. MyoD gene differential expression observed in pacu white muscle is possibly related to

differences in growth patterns during the phases analysed, with hyperplasia predominant in juveniles and hypertrophy in adult fish.

Keywords: skeletal muscle growth, Myogenic Regulatory Factors, MyoD, *Piaractus mesopotamicus*.

Introduction

Fish skeletal muscle is mainly composed of white muscle, which never comprises less then 70% of the bulk of myotomal muscle (Zhang *et al*., 1996); this musculature is made up of glycolytic metabolism muscle fibre for energy supply (Driedzic and Hochachka, 1976), fast contractors and is used in fast swimming such as predation and escape behavior (Altringham and Johnston, 1988). Red muscle forms a thin layer in the subdermal region, which is more developed in the lateral line nerve region, but makes up less than 30% of total musculature (Greer-Walker and Pull, 1975; Hoyle *et al*., 1986; Luther *et al*., 1995). Red muscle fibres show aerobic metabolism and slow contraction; they are associated with slow cruise swimming, such as migration and foraging (Bone, 1966; Johnston *et al*., 1977). There is an intermediate layer between the red and white musculature with intermediate characteristics (Sänger and Stoiber, 2001).

Muscle growth in fish is a plastic mechanism involving populations of myogenic precursor cells, also called adult myoblast or myosatellite cells (Johnston, 1999). These cells provide the essential nuclei for new muscle fibre formation (hyperplasia) and hypertrophy (Koumans and Akster, 1995). During hypertrophic growth, as fibres expand they absorb myoblast nuclei in order to maintain a relatively constant nuclear to cytoplasmatic ratio (Koumans *et al*., 1994). In hyperplasic growth, new fibres form on

the surface of existing fibres by myoblasts fusing to form multinucleated myotubes (Johnston, 1999; Rowlerson and Veggetti, 2001). Final body weight depends on both hypertrophy and hyperplasia in muscle growth. In large, fast growing fish, hyperplasia is particularly active during the larval and juvenile stages (Weatherley and Gill, 1984). In small, slow-growing species, its contribution during adult life is low and muscle growth primarily involves hypertrophy of fibres formed in the embryo and during the early larval stage (Weatherley and Gill, 1984; Weatherley *et al*., 1988).

The process of myogenic progenitor cell activation, proliferation and differentiation, is characterized and regulated by the sequential expression of members of the myogenic regulatory factor family (MRFs) that include MyoD, Myf5, Myogenin, and MRF4 (Watabe, 1999; reviewed by Watabe, 2001). MRFs are transcription factors that share a highly conserved central region termed the basic helix-loop-helix (bHLH) domain (Edmonson and Olson, 1993). This basic domain mediates sequence-specific DNA binding, whereas the helix-loop-helix domain regulates dimerization with E12 and E47 proteins (Murre *et al*, 1989). This heterodimer binds to a consensus DNA sequence (5'-CANNTG-3') called E-box, present in the promoters of many skeletal muscle specific genes (Lassar *et al*., 1989; Murre *et al*., 1989; Blackwell and Weintraub, 1990).

The primary MRFs, MyoD and Myf5, direct proliferating myogenic progenitor factors towards a myogenic lineage, whereas the secondary MRFs, Myogenin and MRF4, control the differentiation and fusion of myoblasts to form myofibres (Menegey and Rudnicki, 1995; Rudnicki and Jaenisch, 1995; Watabe, 1999). As per Johansen and Overturf (2005), during the initial growth phases, myoblast proliferation and hyperplasia can be infered by the high expression of MyoD and Myf5; whereas Myogenin and MRF4 expression can be related to myoblast differentiation and hypertrophy, more intense

during adult growth phase. Understanding the molecular control of postembryonic muscle growth in fish is of particular importance in aquaculture.

The neotropical characid pacu (*Piaractus mesopotamicus*) has extensively been used in Brazilian aquaculture programs (Hernandez, 1989). It is one of the most important food species farmed in the Pantanal wetlands area of the Paraná-Paraguai basin (Godoy, 1975). It is an economically important fish in South America and its hyperplastic and hypertrophic muscle growth mechanisms occur over a long period of growth (Dal Pai *et al*., 2000). This investigation was undertaken to examine MyoD mRNA expression in pacu muscle growth during the juvenile and adult growth phases.

Materials and methods

Fish

Specimens of pacu (*Piaractus mesopotamicus*) were obtained from the Aquaculture Center, UNESP, in Jaboticabal, São Paulo State, Brazil. Two development stages, juvenile (n=5) and adult (n=5), were used in this study. Fishes were anaesthetized with MS-222 (Tricaine Methanensulfonate-SIGMA) and sacrificed. Body weight (g) and total length (cm) were determined.

Morphologic and morphometric analysis

White muscle samples from the dorsal region were collected, immersed in n-Hexane cooled in liquid nitrogen (-159ºC) and then stored at -80°C in a freezer until sectioning. Transverse 10µm thick sections were obtained in a -20°C cryostat and stained with Haematoxilin-Eosin (HE) (Bancroft and Steven, 1990). This was used to

evaluate morphology and calculate the diameter of muscle fibres (Dubowitz and Brooke, 1973).

To estimate fibre cross-section diameter (μm), 100 white muscle fibres from each animal per group were measured using a compound microscope attached to a computerized imaging analysis system (Qwin, Leica), based on the smallest diameter method (Dubowitz and Brooke, 1973). The smallest fibre diameter was used to avoid any errors that might have been caused by the cross-sections not being completely true (Dubowitz and Brooke, 1973). White muscle fibres were grouped into three diameter classes: <20μm, 20-50μm and >50μm, based on Valente *et al*. (1999). Muscle fibre frequency was expressed as the number of fibres from each diameter class relative to the total number of fibres measured.

Semi-quantitative RT-PCR analysis of mRNA for MyoD gene

Total RNA was extracted from frozen juvenile and adult white muscle samples from each animal, with TRIzol Reagent (Invitrogen), which is based on the guanidine thiocyanate method. Frozen muscle samples were mechanically homogenized on ice in 1mL of ice-cold TRIzol reagent. Total RNA was solubilized in RNase-free H_2O and quantified by measuring the optical density (OD) at 260nm. RNA purity was ensured by obtaining a 260/280nm OD ratio >1.70. These samples of total RNA were then PCR amplified to ensure no DNA contamination of RNA. Four micrograms of RNA were reverse transcribed with random hexamer primers and First Strand cDNA Synthesis Kit (GE Healthcare – Amersham Biosciences) in a total volume of 33μL, according to standard methods. One microliter of cDNA was then amplified using 0.2mM of each primer (Table 1), 1 X PCR buffer minus Mg, 1.5mM of $MgCl_2$, 0.2mM of

deoxyribonucleotide triphosphates and 1 unit of Platinum Taq DNA Polymerase (Invitrogen) in a final volume of 25µL.

Primer pairs for MyoD were designed with reference to cDNA nucleotide sequence from *Ictalurus furcatus* (GenBank accession nº AY562555) (Table 1). PCR amplifications for a segment of MyoD gene were carried out for 3 minutes at 94ºC, followed by 35 cycles of denaturation for 1 minute at 94°C, 1.5 minute of annealing at 55°C, 2 minutes of extension at 72°C, and an additional 5 minute extension step. A set of primers designed from the 18S ribosomal RNA (rRNA) consensus fish sequences was used to amplify a segment of the 18S rRNA gene (Tom *et al*., 2004) (Table 1). This gene was used as the housekeeping gene in semi-quantitative RT-PCR analysis. PCR amplifications for 18S rRNA gene were carried out for 2 minutes at 94°C, followed by 32 cycles of denaturation for 1 minute at 94°C, 1 minute of annealing at 57°C, 1 minute of extension at 72°C, and an additional 5 minute extension step.

Preliminary experiments were conducted to determine the number of PCR cycles that represented the linear amplification range. All PCR products were verified by cloning and sequencing. The cDNA from each muscle for both juvenile and adult groups were amplified simultaneously by using aliquots from the same PCR mixture. After PCR amplification, 10 µL of each reaction underwent electrophoresis on 1% agarose gels and were stained with Sybr Safe (Invitrogen). The bands were visualized under UV illumination (Hoefer UV-25) and the gel image was retrieved using the EDAS program (Electrophoresis Documentation and Analysis System 120 - Kodak Digital Science 1D). The bands corresponding to each gene were quantified by densitometry as Integrated Optical Density (IOD) using Labworks Analysis Software 3.0. PCR products were run in

duplicate on different gel for each gene and results averaged. The PCR signals were normalized to the housekeeping 18S rRNA.

Table 1. Oligonucleotide primers used for PCR amplification of reverse transcribed RNA.

Product	Sequence	T_A, ºC	Cycles
MyoD	Forward: 5'- CTAACCAGAGGCTGCCHAAG - 3' Reverse: 5'- CACGATGCTGGACAGACAGT - 3'	55	35
18S rRNA	Forward: 5'- TACCACATCCAAAGAAGGCAG-3' Reverse: 5'- TCGATCCCGAGATCCAACTAC-3'	57	32

T_A: annealing temperature; bp: base pairs.

cDNA cloning of MyoD and 18S rRNA genes

All amplified cDNA fragments were inserted in the plasmid PGEM-T (Promega) which was used to transform competent cells of *Escherichia coli* strain DH5α (Invitrogen). The positive clones were sequenced on an ABI Prism 377 automatic DNA sequencer (Perking-Elmer) with a DYEnamic ET Terminator Cycle Sequencing kit (GE Healthcare - Amersham Biosciences) following manufacturer instructions.

Sequence comparisions

Nuclei acid sequences obtained from clones were subject to BLASTN (Altschul *et al*., 1997) searches at the National Center for Biotechnology Information (NCBI) web site (http://www.ncbi.nlm.nih.gov/blast) to confirm putative similarity with MyoD and 18S rRNA genes. Sequence aligment was performed using the Molecular Evolutionary Genetics Analysis computer program - MEGA version 3.1 (Kumar *et al*., 2004). MyoD and 18S rRNA consensus sequences were obtained using the Bioedit computer program (Hall, 1999).

Statistical analysis

Body weight data were expressed as median ± total semi-amplitude. The non-parametric Mann Whitney test was used for weight analysis (Norman and Streiner, 1993). Body total length data were expressed as mean ± SD and analysis was performed using the Student's unpaired t-test (Norman and Streiner, 1993).

White muscle fibre diameters and semi-quantitative RT-PCR data were expressed as mean ± SD. White muscle fibre diameters were analyzed using the Goodman test (Goodman, 1964; 1965). In semi-quantitative RT-PCR analysis,

comparisons between groups were performed using the Student's unpaired t-test. Differences were considered significant at $p<0.05$.

Results

Anatomical Data

Median and total semi-amplitude weight was 16.45±9.37g for juvenile and 768.00±238.50g for adult fish ($p<0.001$). Mean and SD of total length was10.29±1.29cm for juvenile and 35.36±2.8cm for adult fish ($p<0.001$).

Morphologic and morphometric analysis

HE stain showed the white skeletal muscle making up most of the muscle mass in both juvenile and adult fish. This muscle was consisted of round or polygonal muscle fibre separated by fine septa of connective tissue, the endomysium. Thicker septa of connective tissue separated the muscle fibres into fascicles and made up the perymisium. Muscle fibres were distributed in a mosaic pattern, characterized by fibres of different diameters (Figure 1).

Frequency distribution of <20μm diameter white muscle fibres in juvenile fish was significantly higher than in adults. The frequency of >20 to <50μm diameter fibres was significantly different between groups, decreasing from juvenile to adult. Adults showed a significantly higher frequency of >50μm diameter fibres than juvenile fish (Figure 2).

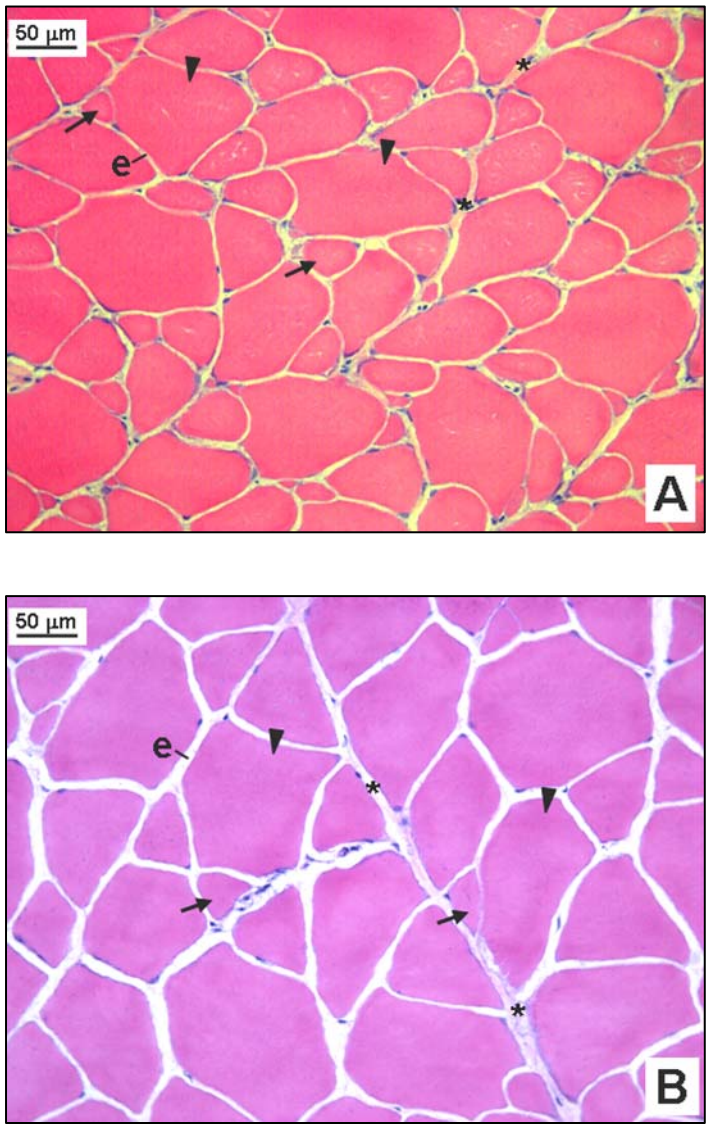

Figure 1. Transverse sections of white skeletal muscle in pacu (*Piaractus mesopotamicus*) (A) Juvenile fish. (B) Adult fish. A mosaic pattern of muscle fibre with different diameters. Small fibres (arrows) between large fibres (arrowhead). Perymisium (*). Endomysium (e). HE. Scale bars: 50 µm.

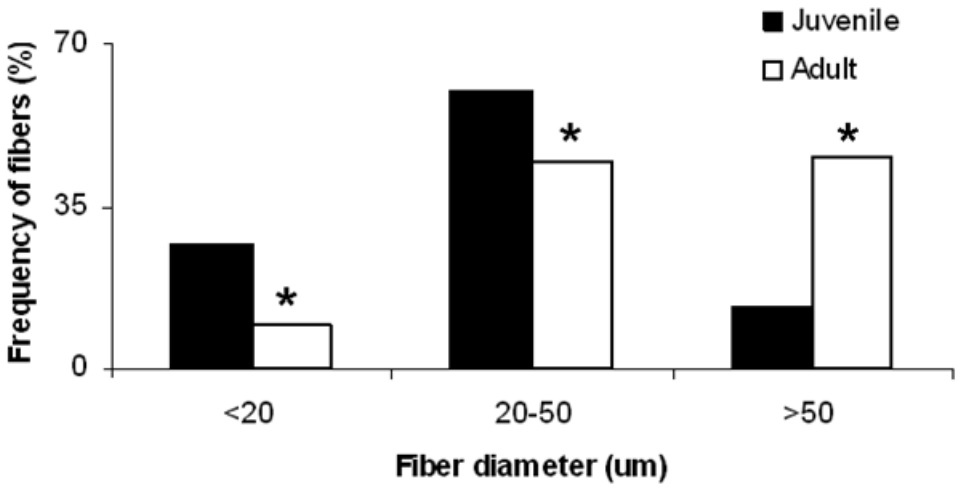

Figure 2. White muscle fibre diameter distribution in juvenile (n=5) and adult (n=5) pacu (*Piaractus mesopotamicus*). Columns represent white fibre frequencies (%) in each group (asterisks in the column show size classes with significant variation; $p<0.05$).

MyoD mRNA levels estimated by semi-quantitative RT-PCR

PCR amplification of pacu cDNA for MyoD gene generated one band of approximately 300 base pairs (bp), and for pacu cDNA with the set of primers for 18S rRNA gene generated one band of approximately 250 bp (Figure 3A). Estimated MyoD mRNA level decreased in the adult group compared to its juvenile counterpart (juvenile 0.50 ± 0.04 vs. adult 0.26 ± 0.05; $p<0.05$) (Figure 3B).

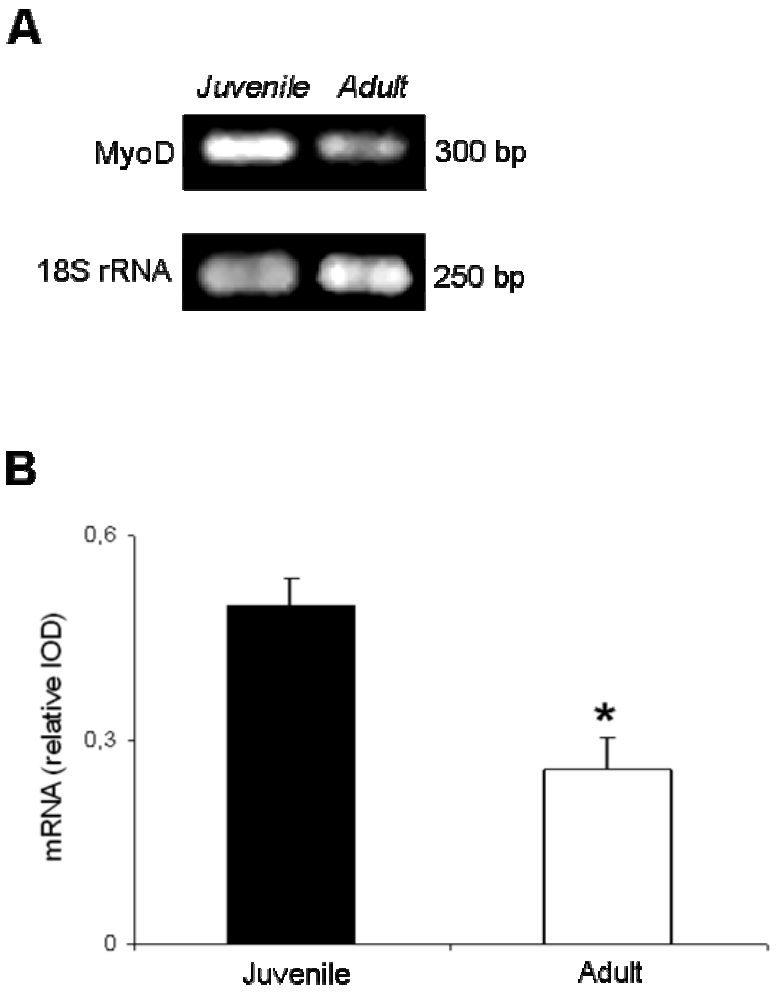

Figure 3. MyoD representative PCR result (A) and content estimated by RT-PCR (B) from white muscles in juvenile (n=5) and adult (n=5) fish. Data were run in duplicate on different gels for each gene and results averaged. PCR products were visualized with SyBR Safe staining. Quantification of PCR signals was obtained by densitometric analysis of the product as Integrated Optical Density (IOD). MyoD gene expression was normalized to the 18S rRNA gene signal from the same RT product. Normalized data are expressed as means ± SE. * $p<0.05$ statistical significance.

cDNA cloning of MyoD and 18S rRNA genes

The PCR products obtained with the set of primers for MyoD were cloned and a total of six clones (three of juvenile muscle samples and three of adult muscle samples) were sequenced. The PCR products obtained with the set of primers for 18S rRNA were cloned and a total of seven clones (three of juvenile muscle and four of adult muscle samples) were sequenced. After sequencing, the exact total length of cDNA fragments, including primer regions, was 338bp for MyoD and 241bp for 18S rRNA.

Sequence comparisions

A search in BLASTN of MyoD and 18S rRNA genes expressed in pacu white muscle showed sequence similarity with several vertebrates, including fish. Partial consensus sequences for MyoD (Figure 4A) and 18S rRNA genes (Figure 4B) were obtained from alignment sequence of their respectively clones.

Sequence analysis of MyoD partial consensus sequence showed a high similarity with various vertebrate species, including teleost fishes, such as *Ictalurus furcatus, Sternopygus macrurus, Cyprinus carpio, Danio rerio, Tetraodon nigroviridis, Sparus aurata, Oreochromis aureus, Takifugu rubripes, Paralichthys olivaceus, Hippoglossus hippoglossus, Ictalurus punctatus,* and *Salmo salar*.

Sequence analysis of 18S rRNA partial consensus sequence also showed a high similarity with various 18S rRNA vertebrate species, including teleost fish, such as *Ictalurus punctatus, Rutilus rutilus, Clupea harengus, Notropis hudsonius, Cyprinus carpio and Prochilodus marggravii, Salmo salar, Sparus aurata, Oncorhynchus masou, Sebastiscus marmoratus,* and *Tetraodon nigroviridis.*

(A)

CTAACCAGAGGCTGCCHAAGGTGGAGATCCTGAGGARCGCCATCAGCTACATCGAGTCCCTGCAG
GCTCTGCTGCGCAGCCAGGAGGACTCCTACTACCCCGTCCTGGATCAGTACAGCGGGGACTCGGA
CGCGTCCAGCCCCAGATCCAACTGCTCCGATGGCATGGTGAGGACACTGAAGCGGCTCTGACGCG
TCCTTGATTGACCAGTAGATAGATAGTGTAGTGGGTGAATCCGTCGCCTGGCTGTTTTTATTGTG
TTTTGATTTCCTCCAGGTGACGTGCGGAGCAGCAAGTCCACAGTGGTGTCCAGTCTGGACTGTCT
GTCCAGCATCGTG

(B)

TACCACATCCAAAGAAGGCAGGCGCGCAAATTACCCATTACCGACACGGTGAGGTAGTGACGAAA
AATAACGATACAGGTCTCTTTCGAGGCCCTGTAATCGGAATGAGCGTATCCTAAACCCATGGGTG
AGGACCCATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATAT
TAAAGTTGCTGCAGTTAAAAAGCTYGTAGTTGGATCTCGGGATCGA

Figure 4. Consensus sequences (5'→ 3') for MyoD (A) and 18S rRNA (B) genes obtained from alignment sequences of their clones, expressed in pacu white skeletal muscle. Primer annealing regions are shadowed. H: adenine, timine, or cytosine. R: adenine or guanine.

Discussion

This study describes, for the first time, the differential expression of myogenic regulatory factor MyoD in skeletal muscle of *Piaractus mesopotamicus* during the juvenile and adult growth phases. MyoD mRNA level was significantly higher in juvenile than in adult fish.

Morphological examination of skeletal muscle in pacu (*Piaractus mesopotamicus*) showed the majority of musculature composed of deep white compartment, in both phases studied. This musculature comprises muscle mass with considerable economic significance (Zhang *et al*., 1996). In both stages, the white muscle morphology was similar to other fish species (Fernandez *et al*., 2000; Dai Pai-Silva *et al*., 2003a and b; Aguiar *et al*., 2005). Althought muscle fibre distribution in compartments is a common characteristic in fish (Scapolo *et al*., 1988; Veggetti *et al*., 1993; Galloway *et al*., 1999; Johnston, 1999), the fibre distribution pattern can vary according to species (Te Kronnie *et al*., 1983; Dal Pai Silva *et al*., 1995a and b) and growth stage (Dal Pai Silva *et al*., 2003a and b).

As previously described by Dal Pai *et al*. (2000), juvenile and adult phases pacu muscle fibres are distributed in mosaic pattern, characterized by different diameter fibres, as observed in others fish species (Rowlerson and Veggetti, 2001). Frequency distribution of <20μm diameter muscle fibres was significantly higher in juvenile fish and the frequency of >50μm diameter fibres was significantly higher in adult fish.

The large number of <20μm diameter muscle fibres observed in juvenile fish confirm an active hyperplasic growth process in skeletal muscle during this developmental stage (Valente *et al*., 1999; Rowlerson and Veggetti, 2001). Hyperplastic growth occurs mainly in two waves in teleost fishes (Rowlerson and Veggetti, 2001). The

first, a continuation of embryonic myogenesis, takes place during at least part of larval life and generates new fibres along a germinal or proliferative zone (Usher *et al.*, 1994), and is responsible for thickening muscle mass in early development stages (Rowlerson and Veggetti, 2001; Johnston *et al.*, 2003). This event is known as stratified hyperplasia and occurs in most of fish species (Johnston, 1999). The second, a mosaic hyperplasia, occur in fish which grow to large sizes, such as pacu, and new fibre production is disseminated across the whole myotome. This results in a mosaic pattern of fibre diameters, as observed in the morphological analysis of pacu skeletal muscle. Mosaic hyperplasia results in a large increase in fibre number during juvenile growth and is very important for commercial aquaculture species; this characteristic is lacking in species that remain small (Rowlerson and Veggetti, 2001). In juvenile pacu, the contribution of hyperplasia, mainly mosaic, was higher than hypertrophy in skeletal muscle growth.

In adult pacu, a majority of >50μm diameter fibres denotes muscle fibre hypertrophy (Valente *et al.*, 1999; Rowlerson and Veggetti, 2001). According to Zimmerman and Lowery (1999), the recruitment of new fibres during muscle growth stops when the fish reach about 44% of their final size; after this muscle growth is mainly by hypertrophy. Although the commercially interesting size of the pacu is not fixed, our study showed that muscle fibre recruitment in the adult phase was less than in the juvenile phase.

Hyperplasia and hypertrophy in muscle fish growth is dependent on the activation, proliferation, and differentiation of myogenic precursor cells, also called adult myoblast or myosatellite cells (Koumans and Akster, 1995; Johnston, 1999). These processes are regulated by the sequential expression of myogenic regulatory factors (MRFs) (Watabe, 1999; reviewed by Watabe, 2001). MRFs are transcription factors that

bind to a consensus DNA sequence called E-box (CANNTG), present in the promoters of many skeletal muscle specific genes (Lassar *et al*., 1989; Murre *et al*., 1989; Blackwell and Weintraub, 1990).

MRF expression levels play an essential role during myogenesis and are related to myoblast specification and differentiation, and regulating muscle development and growth in the growing fish (Zhang *et al*., 2006). In Flounder (*Paralichthys olivaceus*) MyoD expression was detected in precursor muscle cells from the initial phases of embryogenesis (Zhang *et al*., 2006). Johansen and Overturf (2005) showed a continuous and differential MRF (MyoD, Myf5, Myogenin and MRF4) expression in skeletal muscle of rainbow trout (*Oncorhyncus mykiss*) during different growth phases. These authors inferred that differential MRF expression may be related to muscle growth mechanisms.

In our study MyoD mRNA level was significantly higher in juvenile than adult pacu. During early development and the juvenile stage, muscle growth occurs by intense recruitment of new muscle fibres from the proliferation of undifferentiated myogenic progenitor cells that express primary MRF, MyoD and Myf5 (Rescan *et al*., 1994; Megeney and Rudnicki, 1995; Watabe, 2001). Myoblast proliferation is directly related to the hyperplasia process and both can be inferred by analyzing the expression levels of MyoD and Myf5 (Johansen and Overturf, 2005). In our study, the high expression levels of MyoD in juvenile fish can be associated with a predominance of the hyperplasia mechanism in muscle growth.

In adult *Piaractus mesopotamicus*, muscle growth was mainly by hypertrophy. In this stage, myoblast proliferation and hyperplasia do not occur at a significant level, with MyoD expression being smaller than in juvenile fish (Johansen and Overturf, 2005).

This can explain the low MyoD expression in adult pacu compared to their juvenile counterparts.

The expression of genes that control muscle growth is still unknown in Brazilian fish. The results obtained in our study should provide a foundation for understanding the molecular control of skeletal muscle growth in economically important species, with a view to improving production quality.

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References

Aguiar, D.H., Barros, M.M., Padovani, C.R., Pezzato, L.E., Dal Pai-Silva, M., 2005. Growth characteristics of skeletal muscle tissue in *Oreochromis niloticus* larvae fed on a lysine supplemented diet. J. Fish Biol. 67, 1-12.

Altringham, J.D., Johnston, I.A., 1988. The mechanical properties of pollyneuronally myotomal muscle fibres isolated from a teleost fish (*Myoxocephalus scorpius*). Pflügers Arch. 412, 524-529.

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389-3402.

Bancroft, J.D., Steven, A., 1990. Theory and Practice of Histological Techniques. Churchill Livingstone, New York, 704 pp.

Blackwell, T., Weintraub, H., 1990. Differences and similarities in DNA-binding preferences of MyoD and E2A protein complexes revealed by binding site selection. Science 250, 1104-1110.

Bone, Q., 1966. On the function of the two types of myotomal muscle fibre in elasmobranch fish. J. Mar. Biol. Ass. U.K. 46, 321-349.

Dal Pai-Silva, M., Dal Pai, V., Mota, D.L., 1995a. Histochemical study of muscle fibre types in *Synbranchus marmoratus*. Annals Anat. 177,65-70.

Dal Pai-Silva, M., Dal Pai, V., Mota, D.L., 1995b. Características morfológicas e histoquímicas do tecido muscular do *Synbranchus marmoratus*, com fenótipo I e II. Rev. Bras. Biol. 55, 685-691.

Dal Pai, V., Dal Pai-Silva, M., Carvalho, E.D., Fujihara, C.Y., Gregório, E.A., Curi, P.R., 2000. Morphological, histochemical and morphometric study of the myotomal muscle

tissue of pacu (*Piaractus mesopotamicus*, Holmberg, 1887: Serrasalminae, Characidae, Teleostei). Anat. Histol. Embriol. 29(5), 283-289.

Dal Pai-Silva, M., Carvalho, R.F., Pellizzon, C.H., Dal Pai, V., 2003a. Muscle fibre types in tilapia do Nilo (*Oreochromis niloticus*) from larval to adult: histochemical, ultrastructural and morphometric study. Tissue and Cell 35, 179-187.

Dal Pai-Silva, M., Freitas, E.M.S., Dal Pai, V., Rodrigues, A.C., 2003b. Morphological and histochemical study of myotomal muscle in pacu (*Piaractus mesopotamicus*) during the initial growth phases. Arch. Fish Mar. Res. 50, 149-160.

Dubowitz, V., Brooke, M.H., 1973. Muscle biopsy: A modern approach. WB Saunders Company, London, 472 pp.

Driedzic, W.R., Hochachka, P.W., 1976. Control of energy metabolism in fish white muscle. Am. J. Physiol. 230, 579-582

Edmonson, D.G., Olson, E.N., 1993. Helix loop helix proteins as regulators of muscle-specific transcription. J. Bio. Chem. 268, 755-758.

Fernandez, D.A., Calvo, J., Franklin, C.E., Johnston, I.A., 2000. Muscle fibre types and size distribuition in sub-antartic notothenoid fishes. J. Fish Biol. 56, 1295-1311.

Galloway, T.F., Kjorsvik, E., Kryvi, H., 1999. Muscle growth and development in Atlantic cod larvae (*Gadus morhua* L.) related to different somatic growth rates. J. Exp. Biol. 202, 2111-2120.

Godoy, M.P., 1975. Peixes do Brasil: subordem Characoidei: bacia do rio Mogi-Guassu. Franciscana, Piracicaba, 216 pp.

Goodman, L.A., 1964. Simultaneous confidence intervals for contrasts among multinomial populations. Annals Math. Stat. 35(2), 716-725.

Goodman, L.A., 1965. On simultaneous confidence intervals for multinomial proportions. Technometrics 7(2), 247-254.

Greer-Walker, M.G., Pull, G.A., 1975. A survey of red and white muscle in marine fish. J. Fish Biol. 7, 295-300.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis. Nucl. Acids Symp. Ser. 41, 95-98.

Hernandez, R.A., 1989. Cultivo de *Colossoma*. Guadalupe, Bogotá, 475 pp.

Hoyle, J., Gill, H.S., Weatherley, A.H., 1986. Histochemical characterization of myotomal muscle in the grass pickrel, *Esox americanus vermiculatus* (LeSueur) and the muske kellunge, *E. masquinongy* (Mitchill). J. Fish Biol. 28, 393-401.

Johansen, K.A., Overturf, K., 2005. Quantitative expression analysis of genes affecting muscle growth during development of rainbow trout (*Oncorhynchus mykiss*). Mar. Biotechnol. 7(6), 576-87.

Jonhston, I.A., Davison, W., Goldspink, G., 1977. Energy metabolism of carp swimming muscles. J. Comp. Physiol. 114, 203-216.

Johnston, I.A., 1999. Muscle development and growth: potential implication for flesh quality in fish. Aquaculture 177, 99-115.

Johnston, I.A., Manthri, S., Alderson, R., Smart, A., Campbell, P., Nickel, D., Robertson, B., Paxton, C.G.M., Burt, M.L., 2003. Freshwater environment affects growth rate and muscle fibre recruitment in seawater stages of Atlantic salmon (*Salmo salar* L.). J. Exp. Biol. 203, 2539-2552.

Koumans, J.T.M., Akster, H.A., Brooms, G.H.R., Lemmens, C.J.J., Osse, J.W.M., 1994. Numbers of myonuclei and of myosatellite cells in red and white axial muscle during growth of carp *Cyprinus carpio* L. (Teleostei). J. Fish Biol. 44, 391-408.

Koumans, J.T.M., Akster, H.A., 1995. Myogenic cells in development and growth of fish. Comp. Biochem. Physiol. 110(A), 3-20.

Kumar, S., Tamura, K., Nei, M., 2004. MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. Briefings in Bioinformatics 5,150-163.

Lassar, A., Buskin, J.N., Lockshon, D., Davis, R.L., Apone, S., Hauschka, S.D., Weintraub, H., 1989. MyoD is a sequence-specific DNA binding protein requiring a region of myc homology to bind to the muscle creatine kinase enhancer. Cell 58, 823-831.

Luther, P.K., Munro, P.M.G., Squire, J.M., 1995. Muscle ultrastructure in the teleost fish. Micron 26, 431-459.

Menegey, L.A., Rudnicki, M.A., 1995. Determination versus differentiation and the MyoD family of transcription factors. Biochem. Cell Biol. 73, 723-732.

Murre, C., McCaw, P.S., Vaessin, H., Caudy, M., Jan, L.Y., Jan, Y.N., Cabrera, C.V., Buskin, J.N., Hauschka, S.D., Lassar, A.B., 1989. Interactions between heterologous helix-loop-helix proteins generate complexes that bind specifically to a common DNA sequence. Cell 58(3), 537-544.

Norman, G.R., Streiner, D.L., 1993. Biostatistics – The Bare Essencials. Mosby Year Book, St. Louis, 260 pp.

Rescan, P.Y., Gauvry, L., Paboeuf, G., Fauconneau, B., 1994. Identification of a muscle factor related to MyoD in fish species. Biochim. Biophys. Acta 1218, 202-204.

Rowlerson, A., Veggetti, A., 2001. Cellular Mechanisms of Post-Embryonic Muscle Growth in Aquaculture Species. In: Johnston, I.A., Muscle Development and Growth. Academic Press, London, pp. 103-140.

Rudnicki, M.A., Jaenish, R., 1995. The MyoD family of transcription factors and skeletal muscle myogenesis. Bioessays 17, 203-209.

Sänger, A.M., Stoiber, W., 2001. Muscle fiber diversity and plasticity. In: Johnston, I.A., Muscle Development and Growth. Academic Press, London, pp. 187-250.

Scapolo, P.A., Veggetti, A., Mascarello, F., Romanello, M.G., 1988. Developmental transitions of myosin isoforms and organization of the lateral muscle in the teleost *Dicentrarchus labrax* (L.). Anat. Embriol. 178, 287-295.

Te Kronnie, G., Tatarczuch, L., Van Raamsdonk, W., Kilarski, W., 1983. Muscle fibre types in the myotome of stickleback *Gasterosteus aculeatus* L.: a histochemical, immunohistochemical and ultrastructural study. J. Fish Biol. 22, 303-316.

Tom, M., Chen, N., Segev, M., Herut, B., Rinkevich, B., 2004. Quantifying fish metallothionein transcript by real time PCR for its utilization as an environmental biomarkers. Marine Pollution Bulletin 48, 705-710.

Usher, M.L., Stickland, N.C., Thorpe, J.E., 1994. Muscle development in Atlantic salmon (*Salmo salar*) embryos and the effect of temperature on muscle cellularity. J. Fish Biol. 44, 953-964.

Valente, L.M.P., Rocha, E., Gomes, E.F.S., Silva, M.W., Oliveira, M.H., Monteiro, R.A.F., Fauconneau, B., 1999. Growth dynamics of white and red muscle fibres in fast and slow-growing strains of rainbow trout. J. Fish Biol. 55, 675-691.

Veggetti, A., Mascarello, F., Scapolo, P.A., Rowlerson, A., Candia, C.M.D., 1993. Muscle growth and myosin isoform transitions during development of a small teleost fish, *Poecilia reticulata* (Peters) (Atheriniformes, Poeciliidae): a histochemical, immunohistochemical, ultrastructural and morphometric study. Anat. Embryol. 187, 353-361.

Watabe, S., 1999. Myogenic regulatory factors and muscle differentiation during ontogeny in fish. J. Fish Biol. 55(A), 1-18.

Watabe, S., 2001. Myogenic regulatory factors. In: Johnston, I.A., Muscle Development and Growth. Academic Press, London, pp. 19-41.

Weatherley, A.H., Gill, H.S., 1984. Growth dynamics of white myotomal muscle fibres in the bluntnose minor, *Pimaphales notatus* (Rafinesque) and comparison with rainbow trout *Salmo gairdneri* (Richardson). J. Fish Biol. 25, 13-24.

Weatherley, A., Gill, H., Lobo, A.F., 1988. Recruitment and maximal diameter of axial muscle fibers in the teleosts and their relationship to somatic growth and ultimate size. J. Fish Biol. 33, 851-859.

Zhang, G., Swank, D.M., Rome, L.C., 1996. Quantitative distribuition of muscle fiber types in the scup *Stenoteomus chrysops*. J. Morphol. 229, 71-81.

Zhang, Y., Tan, X., Zhang, P.J., Xu, Y., 2006. Characterization of muscle-regulatory gene, MyoD, from flounder (*Paralichthys olivaceus*) and analysis of its expression patterns during embryogenesis. Mar. Biotechnol. 8(2):139-48.

Zimmerman, A.M., Lowery, M.S., 1999. Hyperplastic development and hypertrophic growth of muscle fibers in the white seabass (*Atractoscion nobilis*). J. Exp. Zool. 284, 299-308.

5. CONCLUSÕES GERAIS

O mRNA do fator regulador miogênico MyoD é expresso de forma diferencial na musculatura branca do pacu (*Piaractus mesopotamicus*) nos períodos de crescimento juvenil e adulto.

Essa expressão diferencial pode estar relacionada com as diferenças no padrão de crescimento muscular nas fases analisadas, com a hiperplasia predominante na fase juvenil e a hipertrofia, na fase adulta.

6. REFERÊNCIAS BIBLIOGRÁFICAS GERAIS

Aguiar DH, Barros MM, Padovani CR, Pezzato LE, Dal Pai-Silva M (2005) Growth characteristics of skeletal muscle tissue in *Oreochromis niloticus* larvae fed on a lysine supplemented diet. J Fish Biol 67:1-12.

Alami-Durante H, Fauconneau B, Rouel M, Escaffre AM, Bergot P (1997) Growth and multiplication of white skeletal muscle fibres in carp larvae in relation to growth raye. J Fish Biol 50:1285-1302.

Alexander R (1969) The orientation of muscle in the myomers of fishes. J Mar Biol Assoc UK 49:263-290.

Altringham JD, Johnston IA (1988) The mechanical properties of pollyneuronally myotomal muscle fibres isolated from a teleost fish (*Myoxocephalus scorpius*). Pflügers Arch 412:524-529.

Armstrong RB, Saubert CW, Seeherman HJ, Taylor CR (1982) Distribuition of fiber types in locomotory muscles of dogs. Am J Anat 163: 87-98.

Armstrong RB, Pheleps RO (1984) Muscle fiber type composition on the rat hindlimb. Am J Anat 171:259-272.

Bernardino G, Colares de Melo JS (1989) Estimativa do tamanho mínimo da amostra de pacu (*Piaractus mesopotamicus*, Holmberg, 1887) em monocultura, em viveiros experimentais. Boletim Técnico do CEPTA 2:75-89.

Blackwell T, Weintraub H (1990) Differences and similarities in DNA-binding preferences of MyoD and E2A protein complexes revealed by binding site selection. Science 250:1104-1110.

Blagden CS, Currie PD, Inghan PW, Hughes SM (1997) Notochord induction of zebrafish slow muscle mediated by Sonic hedgehog. Gen and Dev 11:2163-2175.

Bone Q (1966) On the function of the two types of myotomal muscle fibre in elasmobranch fish. J Mar Biol Assoc United Kingdom 46:321-349.

Bone Q (1978) Locomotor muscle. In: Hoar WS, Randall DJ, Fish Physiology, Academic Press, New York, 361-424.

Campion DR (1984) The muscle satellite cells. Int Ver Cytol 87:225-251.

Cole NJ, Hall TE, Chrsitopher IM, Chapman MA, Kobiyama A, Nihei Y, Watabe S, Johnston IA (2004) Temperature and the expression of myogenic regulatory factors (MRFs) and myosin heavy chain isoforms during embryogenesis in the common carp *Cyprinus carpio* L. J Exp Biol 207:4239-4248.

Currie PD, Ingham PW (2001) Induction and patterning of embryonic skeletal muscle cells in the zebrafish. In: Johnston IA, Muscle Development and Growth, Academic Press. London, pp. 1-17.

Dal Pai-Silva M, Dal Pai V, Mota DI, Rodrigues AC (1995) Histochemical study of muscle fiber types in *Synbranchus marmoratus* (Boch, 1795). Ann Anat 177:65-70.

Dal Pai V, Dal Pai-Silva M, Carvalho ED, Fujihara CY, Gregório EA, Curi PR (2000) Morphological, histochemical and morphometric study of the myotomal muscle tissue of pacu (*Piaractus mesopotamicus,* Holmberg, 1887: Serrasalminae, Characidae, Teleostei). Anat Histol Embriol 29(5):283-289.

Devoto SH, Melançon EJS, Westerfield M (1996) Identification of separate slow and fast muscle precursor cells in vivo, prior to somite formation. Development 122:3371-3380.

Driedzic WR, Hochachka PW (1976) Control of energy metabolism in fish white muscle. Am J Physiol 230:579-582.

Edmonson DG, Olson EN (1993) Helix loop helix proteins as regulators of muscle-specific transcription. J Bio Chem 268:755-758.

Eggington S, Johnston IA (1982) Muscle fiber differentiation and vascularisation in the juvenile European eel (*Anguilla anguilla* L). Cell Tissue Res 222:563-577.

Fauconneau B, Paboeuf G (2001) Muscle satellite cells in fish. In: Johnston IA, Muscle Development and Growth, Academic Press, London, pp. 73-98.

Flutcher BE, Phillips JL, Powell JA, Andrews SB, Daniels MP (1992) Coordinated development of myofibrils sarcoplasmic reticulum and transverse tubules in normal and dysgenic mouse skeletal muscle, in vivo and in vitro. Dev Biol 155:266-280.

Godoy MP (1975) Peixes do Brasil: subordem Characoidei: bacia do rio Mogi-Guassu. Franciscana Piracicaba, 216 pp.

Goldspink G, Wilkes D, Steven E (2001) Myosin expression during ontogeny, post-hatching growth and adaptation. In: Johnston IA, Muscle Development and Growth, Academic Press, London, pp. 43-72.

Grizzle JM, Rogers WA (1979) Anatomy and histology of the channel cat fish. University Agricultural Experimental Station, Opelika, 94 pp.

Hernandez RA (1989) Cultivo de *Colossoma.* Ed Guadalupe, Bogotá, 475 pp.

Holmberg EL (1897) Sobre algunos peces nuevos o poco conocidos en la República Argentina. Revista Argentina de História Natural, Buenos Aires, 180-193.

Hoyle J, Gill HS, Weatherley AH (1986) Histochemical characterization of myotomal muscle in the grass pickrel, *Esox americanus vermiculatus* (LeSueur) and the muscle kellunge, *E masquinongy* (Mitchill). J Fish Biol 28:393-401.

Huxley HE (1969) The mechanism of muscular contraction. Science 164:1356-1365.

Huxley HE (1971) The structural basis of muscular contraction. Proc R Soc Lond B Biol Sci 178:131-149.

Huxley HE (1983) Molecular basis of contraction in cross-striated muscles and relevance to motile mechanisms in other cells. In: Stracher A, Muscle and Nonmuscle Motility, Academic Press, London.

Johansen KA, Overturf K (2005) Quantitative expression analysis of genes affecting muscle growth during development of rainbow trout (*Oncorhynchus mykiss*). Mar Biotechnol 7(6):576-87.

Jonhston IA, Davison W, Goldspink G (1977) Energy metabolism of carp swimming muscles. J Comp Physiol 114:203-216.

Johnston IA (1981) Quantitative analysis of muscle breakdown during starvation in the marine flat fish *Pleuronectes platessa*. Cell Tissue Res 214:369-379.

Johnston IA, Vieira VLA, Abercromby M (1995) Temperature and myogenesis in embryos of the Atlantic herring, *Clupea harengus.* J Exp Biol 198:1389-1403.

Johnston IA, Cole NJ, Abercromby M, Vieira VLA (1998) Embryonic temperature modulates muscle growth characteristics in larval and juvenile herring (*Clupea harehgus).* J Exp Biol 201:623-646.

Johnston IA (1999) Muscle development and growth: potential implication for flesh quality in fish. Aquaculture 177:99-115.

Johnston IA (2001) Genetic and environmental determinants of muscle growth patterns In: Johnston IA, Muscle Development and Growth, Academic Press, London, pp. 141-186.

Johnston IA, Hall TE (2004) Mechanisms of muscle development and responses to temperature change in fish larvae. Am Fish Soc Symposium 40:85-116.

Kelly AM (1971) Sarcoplasmatic reticulum and T tubules in differentiating rat skeletal muscle. J Cell Biol 49:335-344.

Kilarski W (1990) Histochemical characterization of myotomal muscle in the roach, *Rutilis rutilis* (L). J Fish Biol 36:353-362.

Kimmel CB, Warga RM, Schiling TF (1990) Origin and organization of the zebrafish fate map. Development 108:581-584.

Koumans JTM, Akster HA (1995) Myogenic cells in development and growth of fish. Comp Biochem Physiol 110(A):3-20.

Lassar A, Buskin JN, Lockshon D, Davis RL, Apone S, Hauschka SD, Weintraub H (1989) MyoD is a sequence-specific DNA binding protein requiring a region of myc homology to bind to the muscle creatine kinase enhancer. Cell 58:823-831.

Lassar AB, Davis RL, Wright WE, Kadesch T, Murre C, Voronova A, Baltimore D, Weintraub H (1991) Functional activity of myogenic HLH proteins requires hetero-oligomerization with E12/E-47- like proteins *in vivo.* Cell 66:305-315.

Ma PC, Rould MA, Weintraub H, Pabo CO (1994) Crystal structure of MyoD bHLH domain-DNA complex: perspectives on DNA recognition and implications for transcriptional activation. Cell 77(3):451-459.

Mascarello F, Rowlerson A, Radaelli G, Scapolo PA, Veggetti A (1995) Differentiation and growth of muscle fibers in the fish *Sparus aurata* (L): I Myosin expression and organization of fibers types in lateral muscle from hatching to adult. J Muscle Res Cell Motil 16:213-222.

Mauro A (1961) Satellite cell of skeletal muscle fibers. J Bioph Biochem Cytol 9:493-494.

Mommsen TP (2001) Paradigms of growth in fish. Comp Bioch Physiol 129(B):207-219.

Menegey LA, Rudnicki MA (1995) Determination versus differentiation and the MyoD family of transcription factors. Biochem Cell Biol 73:723-732.

Murre C, Mccaw PS, Vaessin H, Caudy M, Jan LY, Yan JN, Cabrera CV, Buskin JN, Hauschka SD, Lassar AB, Weintraub H, Baltimore D (1989) Interactions between heterologous helix-loop-helix proteins generate complexes that bind specifically to a common DNA sequence. Cell 58:537-544.

Murre C, Bain G, Van Dijk MA, Engel I, Furnari BA, Massari ME, Matthews JR, Quong MW, Rivera RR, Stuiver MH (1994) Structure and function of helix-loop-helix proteins. Biochim Byophys Acta1218:129-135.

Petrere Jr M (1989) River fisheries in Brazil: a review. Regulated Rivers: Research and Management, 4:1-16.

Romanello MG, Scapolo PA, Luprano S, Mascarello F (1987) Post-larval growth in the lateral white muscle of the eel, *Anguilla anguilla.* J Fish Biol 30:161-172.

Rowlerson A, Veggetti A (2001) Cellular mechanisms of post-embryonic muscle growth in aquaculture species In: Johnston IA, Muscle Development and Growth, Academic Press, London, pp. 103-139.

Rudnick MA, Jaenish R (1995) The MyoD family of transcription factors and skeletal muscle myogenesis. Bioessays 17:203-209.

Sänger AM, Kim ZS, Adam H (1990) The fine structure of muscle fibers of roach, *Rutilus rutilus* (L.), and chub, *Leusciscus ceplhalus* (L.), Cyprinidae, Teleostei: interspecific differences and effects of habitat and season. J Fish Biol 36:205-213.

Sänger AM (1992) Quantitative fine structural diversification of red and white muscle fibres in cyprinids. Environ Biol Fishes 33:97-104.

Sänger AM, Stoiber W (2001) Muscle fiber diversity and plasticity In: Johnston IA, Muscle Development and Growth, Academic Press London, pp. 187-250.

Schiaffino S, Margreth A (1969) Coordinated development of the sarcoplasmatic reticulum and T system during postnatal differentiation of rat skeletal muscle. J Cell Biol 41:855-875.

Stoiber W, Sänger AM (1996) An electron microscopic investigation into the possible source of new muscle fibre in teleost fish. Anat Embryol 194:569-579.

Urbinati CU, Gonçalves FD (2005) Pacu (*Piaractus mesopotamicus)*. In: Espécies nativas para a psicultura no Brasil. Baldisserotto B & Gomes LC. Editora UFSM, Santa Maria, 470 pp.

Veggetti A, Mascarello F, Scapollo PA (1990) Hyperplastic and hypertophic growth of lateral muscle in *Dicentrarchus labrax:* an ultrastructural and morphometric study. Anat and Embriol 182:1-10.

Watabe S (1999) Myogenic regulatory factors and muscle differentiation during ontogeny in fish. J Fish Biol 55(A):1-18.

Watabe S (2001) Myogenic regulatory factors In: Johnston IA, Muscle Development and Growth, Academic Press London, pp. 19-41.

Weatherley A, Gill HS (1985) Dynamics of increase in muscle fibres in fishes in relation to size and growth. Experientia 41:353-354.

Weatherley A, Gill H, Lobo AF (1988) Recruitment and maximal diameter of axial muscle fibers in the teleosts and their relationship to somatic growth and ultimate size. J Fish Biol 33:851-859.

Weatherley A, Gill H (1989) The role of muscle in determination growth and size in teleost fish. Experientia 45:875-878.

Zhang G, Swank DM, Rome LC (1996) Quantitative distribution of muscle fiber types in the scup *Stenoteomus chrysops.* J Morphol 229:71-81.