UNIVERSIDADE ESTADUAL DE CAMPINAS

INSTITUTO DE BIOLOGIA

DANILO HENRIQUE AGUIAR

EXPRESSÃO DOS FATORES DE REGULAÇÃO MIOGÊNICA E DA CADEIA PESADA DA MIOSINA NO MÚSCULO ESTRIADO ESQUELÉTICO DA TILÁPIA DO NILO (*OREOCHROMIS NILOTICUS*) DURANTE O CRESCIMENTO

Tese apresentada ao Instituto de Biologia para obtenção do Título de Doutor em Biologia Celular e Estrutural, na área de Histologia.

Orientadora: Profa. Dra. Maeli Dal Pai Silva

Campinas, 2008

FICHA CATALOGRÁFICA ELABORADA PELA
BIBLIOTECA DO INSTITUTO DE BIOLOGIA – UNICAMP

Ag93e

Aguiar, Danilo Henrique
Expressão dos fatores de regulação miogênica e da cadeia pesada da miosina no músculo estriado esquelético da tilápia do Nilo (*Oreochroms niloticus*) durante o crescimento / Danilo Henrique Aguiar. – Campinas, SP: [s.n.], 2008.

Orientador: Maeli Dal Pai Silva.
Tese (doutorado) – Universidade Estadual de Campinas, Instituto de Biologia.

1. Crescimento muscular. 2. Fatores de regulação miogênica. 3. Cadeias pesadas de miosina. 4. Tilápia do Nilo. I. Silva, Maeli Dal Pai. II. Universidade Estadual de Campinas. Instituto de Biologia. III. Título.

(scs/ib)

Título em inglês: Miogenic regulatory factors and myosin heavy chain expression in the striated skeletal muscle of the Nile tilapia (*Oreochroms niloticus*) during growth.
Palavras-chave em inglês: Muscle growth; Miogenic regulatory factors; Myosin heavy chain; Nile tilapia.
Área de concentração: Histologia.
Titulação: Doutorado em Biologia Celular e Estrutural.
Banca examinadora: Maeli Dal Pai Silva, Maria Alice da Cruz Hoffling, Humberto Santo Neto, Selma Maria Michelin Matheus, Maria Célia Portella.
Data da defesa: 07/03/2008.
Programa de Pós-Graduação: Biologia Celular e Estrutural.

Campinas, 07 de março de 2008.

BANCA EXAMINADORA

Profa. Dra. Maeli Dal Pai Silva (Orientadora)

Assinatura

Profa. Dra. Maria Alice da Cruz Höfling

Assinatura

Prof. Dr. Humberto Santo Neto

Assinatura

Profa. Dra. Selma Maria Michelin Matheus

Assinatura

Profa. Dra. Maria Célia Portella

Assinatura

Profa. Dra. Margarida Maria Barros

Assinatura

Prof. Dr. Sérgio Luís Felisbino

Assinatura

Profa. Dra. Valéria Helena Alves Cagnon Quitete

Assinatura

Dedico este trabalho…

Aos meus pais Laura de Fátima Montanha Aguiar e José Fernando Aguiar que sempre estão somando todos os seus esforços e dedicação para proporcionar que minha jornada seja contemplada de luz, amor e paz.

Agradecimentos

À DEUS pelo dia-a-dia de crescimento com muita luz, amor e paz.

Aos meus pais, Fernando e Laura, que estão sempre ao meu lado em todos os momentos.

À minha noiva, Clarianna Martins Baicere Silva, uma benção de Deus na minha vida.

Aos meus irmãos, Rodrigo e Andreo, pelo apoio e amizade.

À minha orientadora, Profa. Dra. Maeli Dal Pai Silva, pelo respeito, pelos ensinamentos, pela dedicação e pelo auxílio transmitido às minhas atividades de pós-graduação.

Ao amigo Cláudio Bock, através do CEPTA, pelo grande auxílio durante a parte experimental.

À Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) pelo apoio financeiro. Proc. 04/13286-0.

Aos docentes do programa de pós-graduação em Biologia Celular e Estrutural da Unicamp e aos professores do Departamento de Morfologia da Unesp, por contribuírem para a minha formação acadêmico-científica.

Ao professor Vitalino Dal Pai (*in memoriam*) por me conceder a oportunidade de realizar estágios com a professora Maeli.

Aos colegas de pós-graduação da Unicamp e da Unesp.

À todos os funcionários do Departamento de Morfologia da Unesp.

Às secretárias Luciana, do departamento de Morfologia da Unesp e Liliam da Unicamp, pela atenção e ótimo desempenho profissional.

À todos que colaboraram direta ou indiretamente para a realização deste trabalho.

Sumário

I. RESUMO

Nos peixes, o conhecimento dos fatores que controlam o crescimento muscular e a análise das proteínas miofibrilares, é importante para entender a dinâmica do crescimento, a plasticidade e as adaptações musculares, principalmente, em espécies com grande valor comercial como a tilápia do Nilo (*Oreochromis niloticus*). No presente estudo, utilizou-se a tilápia do Nilo em quatro estágios: alevinos de 35 dias (0.65g ± 0.08); juvenis de 60 dias (13.67g ± 1.35); adultos de 90 dias (73.18g ± 4.70) e adultos de 190 dias (349.76g ± 34.62). Em cada estágio, fragmentos musculares foram coletados e submetidos às seguintes análises: morfométrica, para caracterizar o crescimento muscular hiperplásico e hipertrófico no músculo branco; imunohistoquímica, para analisar a expressão dos fatores de regulação miogênica MyoD e miogenina e a expressão da proteína PCNA no músculo branco; histoquímica da ATPase miofibrilar (mATPase) e à eletroforese em gel de poliacrilamida – duodecil sulfato de sódio (SDS-PAGE) para observar as características da mATPase e da cadeia pesada da miosina nos músculos branco e vermelho, respectivamente. Os resultados indicaram que a expressão de MyoD e miogenina foi similar em alevinos, juvenis e adultos de 90 dias, porém, em adultos de 190 dias a expressão de miogenina foi maior do que a de MyoD. A expressão do PCNA, em cada estágio, foi mais acentuada do que MyoD e miogenina com picos no estágio de alevinos e adultos de 90 dias. A expressão de MyoD e miogenina nos estágios de alevinos, juvenis e adultos de 90 dias, mostrou que a hiperplasia e a hipertrofia ocorreram como resultado da proliferação e da diferenciação dos mioblastos. O aumento da expressão de miogenina em adultos de 190 dias, indicou que a diferenciação celular e a hipertrofia foi mais significativa nesse estágio. A análise da mATPase indicou, além da presença de fibras musculares vermelhas e brancas, fibras híbridas tanto no músculo vermelho como no músculo branco, ao longo do crescimento muscular da tilápia. A partir de alevinos, o músculo vermelho da região superficial mostrou a presença de cadeia pesada da miosina *slow* e o músculo branco, que forma a maior parte da massa muscular, cadeia pesada da miosina *fast.* Essas isoformas apresentaram massa molecular semelhante à cadeia

pesada da miosina do tipo I do músculo sóleo de rato. No músculo branco, a partir dos alevinos, foi observada outra isoforma de miosina de massa molecular superior à cadeia pesada da miosina do tipo I do músculo sóleo de rato. No músculo vermelho a partir dos adultos, observou-se outra isoforma de miosina de massa molecular semelhante à cadeia pesada da miosina do tipo II do músculo sóleo de rato. A expressão das isoformas de cadeias pesadas da miosina no músculo estriado esquelético da tilápia do Nilo durante o crescimento, pode estar relacionada com a plasticidade fenotípica que ocorre durante o crescimento muscular e reflete na capacidade desses peixes de se adaptar às variações ambientais, importantes para a sobrevivência.

II. ABSTRACT

In fish, the knowledge of factors that control the muscle growth and the myofibrillar proteins analyze is important to understand the dynamic of growth, the plasticity and the muscle adaptations, mainly, in species with high commercial valuable, as the Nile tilapia (*Oreochromis niloticus*). In the present study, Nile tilapia into four age stages were used: 35 day alevins (0.65g ± 0.08); 60 day juveniles (13.67g ± 1.35); 90 day adults (73.18g ± 4.70) and 190 day adults (349.76g ± 34.62). In each stage, muscle fragments were collected and submitted to the following analyzes: morphometric, to characterize the hyperplastic and hypertrophyc growth in the white muscle; immunohistochemical, to analize the myogenic regulatory factors MyoD and myogenin expression, and the PCNA protein expression in white muscle; histochemical of the myofibrillar ATPase (mATPase) and electrophoresis by sodium duodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in the red and white muscle to observed mATPase and myosin heavy chain characteristics, respectively. The results indicate that MyoD and myogenin expression was similar in alevins, juveniles and 90 day adults, however, in 190 day adults the myogenin was higher than the MyoD expression. The PCNA expression, in each stage, was higher than MyoD and myogenin with peaks in alevins and 90 day adults. The MyoD expression in alevins, juveniles and 90 day adults, showed that the hyperplasia and hypertrophy occurred due to the results of myoblasts proliferation and differentiation. The increased of myogenin expression in 190 day adults indicated that cellular differentiation and the hypertrophy was more expressive in this stage. The mATPase showed, beyond red and white muscle fibers, hybrid fibers in both red and white muscle during growth. From alevins, the red muscle showed slow myosin heavy chain (MHCs) and the white muscle, fast myosin heavy chain (MHCf). These isoforms had a molecular mass similar to the type I myosin heavy chain (MHCI) of soleus rat muscle. In the white muscle, from alevins was observed other myosin isoform with molecular mass superior to the MHCI of soleus rat muscle. In the red muscle, in adults, was observed other myosin isoform with molecular mass similar to the type II myosin heavy chain (MHC II) of soleus rat muscle. The expression of myosin

isoforms in the skeletal muscle of Nile tilapia during growth, can be related to the phenotypic plasticity that occur during muscle growth and reflects this fish capacity to adapt to changes in environmental conditions which are important for its survival.

III. INTRODUÇÃO

III.1. Tilápia do Nilo (Oreochromis niloticus)

Nos últimos anos, as tilápias tiveram sua distribuição expandida para todos os continentes. Isto ocorreu em função de seu potencial para a aqüicultura intensiva e para a piscicultura de subsistência em países em desenvolvimento, por apresentar carne de excelente sabor, com boas características organolépticas e com boa aceitação no mercado consumidor (Hilsdorf, 1995; Lovshin, 1997). A demanda tem aumentado com o crescimento paralelo da pecuária e da avicultura, onde esse peixe é utilizado como fonte de proteínas, além de contribuir como alimento em potencial e alternativo para a população (Tacon, 1993; 1997).

III.2. Músculo estriado esquelético em peixes

O tecido muscular estriado esquelético dos peixes é dividido em miômeros constituídos de fibras musculares arranjadas em trajetórias helicoidais que se inserem, via tendões, em camadas de fibras colágenas chamadas miosseptos (Alexander, 1969).

Esse tecido está distribuído em camadas referidas como, vermelha, intermediária e branca. A camada muscular vermelha ou superficial está localizada abaixo da derme, como uma camada uniforme ao longo de todo o corpo do animal ou distribuída mais localmente, na região da linha lateral. Essa camada aumenta em proporção na região da nadadeira caudal e raramente excede em 10% da área de secção transversal total do músculo (Johnston, 1981; 1999). A camada muscular branca ou profunda representa a maior parte da massa muscular (Alexander, 1969; Dal Pai-

Silva *et al*., 1995a e b). Entre as camadas de músculo vermelho e branco, está a camada intermediária, em menor proporção, apresentando características intermediárias (Rowlerson *et al*., 1985; Kilarski, 1990; Johnston, 1999). As fibras do músculo vermelho são utilizadas na realização de movimentos lentos e contínuos como na natação constante enquanto as fibras do músculo branco são utilizadas na realização de atividades bruscas de locomoção, como na fuga de predadores e captura de presas (Bond, 1979; Akster *et al*., 1995; Johnston, 1999). As fibras da camada intermediária de músculo apresentam características intermediárias em relação às fibras do músculo vermelho e branco, podendo ser recrutadas para atividades de sustentação e em movimentos rápidos (Akster *et al*., 1995, Johnston *et al*., 1997, Johnston, 1999).

III.3. Hiperplasia e hipertrofia das fibras musculares estriadas em peixes

O crescimento pós-embrionário do músculo estriado esquelético em peixes, que corresponde à fase tardia da miogênese, se inicia antes da absorção do saco vitelínico e continua até a maturação sexual. Esse crescimento depende da proliferação e da diferenciação de uma população de células progenitoras miogênicas ou mioblastos, fonte de núcleos para o recrutamento de novas fibras musculares (hiperplasia) e para o aumento em espessura das fibras musculares (hipertrofia) (Koumans *et al*., 1991; Koumans & Akster, 1995; Johnston, 1999). Esses mioblastos estão localizados entre a lâmina basal e a membrana plasmática da fibra muscular (Veggetti *et al*., 1990), sendo equivalentes às células satélites descritas nos mamíferos (Mauro, 1961).

Uma característica importante dos peixes é que tanto a hiperplasia quanto a hipertrofia contribuem para o crescimento muscular pós-larval, enquanto que na maioria dos outros vertebrados, o crescimento muscular ocorre predominantemente pela hipertrofia (Weatherley *et al*., 1979; Carpene & Veggetti, 1981; Egginton & Johnston, 1982; Rowlerson *et al*., 1985).

A hiperplasia pode ocorrer de forma estratificada ou de mosaico. A hiperplasia estratificada ocorre em todas as espécies de peixes a partir de 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. A hiperplasia em mosaico resulta em um grande aumento no número de fibras musculares, principalmente na fase juvenil, sendo observada na musculatura das espécies que atingem um tamanho maior e de grande valor comercial (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).

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). Há evidências de que a hiperplasia diminui com o aumento da idade dos peixes (Alfei *et al*., 1994), e sua contribuição nunca excede 50% do crescimento muscular (Mommsen, 2001).

Considerando as adaptações às mudanças funcionais, os mioblastos podem ser influenciadas por vários fatores intrínsecos e extrínsecos durante o desenvolvimento muscular, crescimento, e em respostas a doenças ou traumas e regeneração (Johnston *et al*., 2000; Parry, 2001; Camargo *et al*., 2004).

A temperatura é um dos fatores ambientais que podem influenciar consideravelmente o crescimento muscular devido à redução nas taxas de proliferação celular, ou seja, na ativação dos mioblastos (Mommsen, 2001).

Estudos de Brodeur *et al*., 2003 indicaram que a duração do ciclo celular em mioblastos aumenta com a diminuição da temperatura, reduzindo o crescimento muscular. Por outro lado, a taxa de proliferação celular também pode ser influenciada pela dieta como descrito por Aguiar *et al*., 2005, que observaram um aumento da proliferação celular no músculo estriado em peixes tratados com suplementação do aminoácido lisina.

III.4. Fatores de regulação miogênica

Durante o crescimento embrionário e pós-embrionário, a proliferação e a diferenciação dos mioblastos, é regulada pelos fatores de regulação miogênica conhecidos como MyoD, miogenina, myf-5 e MRF-4 (Watabe, 2001; Rescan, 2001). Esses fatores transcricionais músculo-específicos regulam a ativação, proliferação e a diferenciação dos mioblastos durante o desenvolvimento e crescimento muscular. A

MyoD e Myf5 são conhecidos como fatores primários, sendo expressos em mioblastos na fase de proliferação, que antecede a diferenciação, enquanto que, a miogenina e o MRF4 são expressos em células na fase de fusão e diferenciação em fibras musculares imaturas (Megeney & Rudnicki, 1995; Grobet *et al*., 1997). Esses fatores ligam-se a seqüências de DNA (5´-CANNTG-3´), conhecidas como *Ebox,* presentes na região promotora de vários genes músculo-específicos, levando à expressão dos mesmos. Além disso, podem iniciar a transcrição de seus próprios genes durante o crescimento (Murre *et al*., 1989; Sassoon, 1993).

Embora os níveis de MyoD e miogenina apareçam aumentados durante os primeiros meses após o nascimento, ambos são detectados no músculo de mamíferos adultos com o aumento da idade (Duclert *et al*., 1991; Alway *et al*., 2001) e, estão relacionados com a plasticidade e adaptação muscular (Sabourin & Rudnicki, 2000). Além disso, a expressão dos fatores de regulação miogência em músculo esquelético de animais adultos pode auxiliar a síntese e a degradação de proteínas, responsáveis pela manutenção do fenótipo do músculo (Mozdziak *et al*., 1998).

A MyoD e a miogenina são proteínas altamente conservadas entre os vertebrados, com seqüência de aminoácidos muito similar entre as espécies animais, incluindo os peixes (Kobiyama *et al*., 1998). Nestes últimos, a expressão dessas proteínas ocorre tanto no músculo estriado esquelético embrionário quanto no adulto (Rescan *et al*., 1994; Rescan & Gauvry, 1996).

A proliferação dos mioblastos e a hiperplasia celular nos peixes, podem ser inferidos pela maior expressão dos MRFs, MyoD e Myf5, nas fases iniciais de crescimento. Já, a expressão de Miogenina e MRF4 está relacionada com os processos

de diferenciação dos mioblastos e hipertrofia das fibras musculares, mais intensos na fase adulta (Johansen & Overturf, 2005).

A expressão dos fatores de regulação miogênica pelos mioblastos está diretamente relacionada com a modulação do crescimento muscular, e pode influenciar o tamanho dos peixes no estágio adulto. Fatores extrínsecos, tais como, alimentação, temperatura, pH e óxigênio também podem influenciar a expressão dos fatores de regulação miogênica durante o crescimento, podendo alterar o fenótipo das fibras de acordo com as necessidades do animal (Heap *et al*., 1985).

III.5. Cadeia pesada da miosina

A molécula de miosina é um hexâmero constituído de 6 polipeptídeos: duas cadeias pesadas e quatro cadeias leves. A região N terminal de cada cadeia pesada consiste de uma porção globular onde a força é gerada por “pontes cruzadas” que promovem o deslizamento dos filamentos durante a contração. Além disso, a porção globular contém o domínio no qual se liga a actina e um sítio de atividade da ATPase miofibrilar, que promove a hidrólise do ATP (Weeds & Lowey, 1971; Johnston, 2001).

As fibras musculares podem ser classificadas em lentas e rápidas, conforme a velocidade de hidrólise do ATP que está relacionada com o tipo de isoforma de cadeia pesada da miosina (Schiaffino & Reggiani, 1994). Em mamíferos, as fibras musculares lentas expressam a isoforma de cadeia pesada da miosina do tipo I, enquanto as fibras musculares rápidas expressam as isoformas do tipo IIa, IId e IIb, ditas. No entanto, podem existir fibras que expressam duas ou mais isoformas de cadeia pesada da miosina, sendo denominadas de fibras híbridas (Staron *et al*., 1999, Pette & Staron,

2000). Estas diferentes isoformas permitem a adaptação do músculo estriado esquelético conforme a demanda funcional (Pette & Staron, 2000).

Nos peixes, o músculo estriado esquelético é o tecido mais abundante, constituindo a maior parte da massa muscular e dentre as proteínas musculares, a miosina da classe II é a mais abundante. As miosinas dessa classe não são apenas proteínas estruturais, estando diretamente relacionadas com o processo de contração muscular. A locomoção e a manutenção dos processos vitais dos organismos, como alimentação e respiração, são dependentes da constituição morfofisiológica dos músculos e dos tipos de fibras musculares envolvidas, incluindo o tipo de miosina existente nas fibras (Goldspink *et al*., 2001).

O tecido muscular dos peixes é dividido em regiões discretas, compostas de diferentes tipos de fibras: o músculo vermelho, constituído de fibras oxidativas lentas, o músculo branco, constituído de fibras glicolíticas rápidas e o músculo intermediário, constituído de fibras glicolíticas oxidativas rápidas (Bond, 1979). A especialização muscular nestes animais envolve a adaptação associada com fatores físicos do ambiente e às mudanças do comportamento dos mesmos (Johnston, 1999)**.** Estudos de Johnston *et al*. (1977) mostraram que o treinamento de natação sustentada leva a um aumento na proporção das fibras musculares do músculo vermelho e a um aumento sutil na proporção das fibras musculares do músculo branco, embora estas sejam passivas nesse tipo de movimento muscular.

Em comparação com outros vertebrados, o conhecimento sobre os tipos de cadeias pesadas da miosina no músculo estriado esquelético em peixes é limitado (Gauthier *et al*., 2000). Nesses animais, ocorre uma variação na expressão das

isoformas da cadeia pesada da miosina durante o desenvolvimento e crescimento das fibras musculares (Narusawa *et al*., 1987), que podem expressar as isoformas embrionária, larval e outras, na dependência da espécie estudada (Scapolo *et al*., 1988; Johnston, 2001). Sabe-se que, o músculo vermelho e o músculo branco dos peixes expressam, respectivamente, cadeia pesada da miosina *slow* e cadeia pesada da miosina *fast*. Porém, diferentes espécies exibem variações no tipo de atividade da mATPase e nos mecanismos de crescimento muscular e, por isso, as fibras musculares podem co-expressar diferentes cadeias pesadas da miosina, sendo denominadas fibras híbridas, similares às descritas em músculos de mamíferos (Karasinski & Kilarski, 1989; Radice, 1995; Martinez *et al*., 1991).

Há diferenças estruturais nas cadeias pesadas da miosina do músculo branco e vermelho dos peixes (Karasinski, 1993). Por outro lado, de acordo com Weaver *et al*. (2001) as cadeias pesadas da miosina do músculo dos peixes são quase idênticas àquelas encontradas em aves e anfíbios. Os tipos de fibras musculares dos peixes não são permanentes, ou seja, são capazes de mudar o seu fenótipo e, consequentemente, diferentes isoformas de cadeias pesadas da miosina são expressas sequencialmente durante o desenvolvimento (Martinez *et al*., 1993).

As fibras musculares também são muito dinâmicas, isto é, as mudanças nas isoformas da miosina ocorrem como resultado do desenvolvimento e da demanda ambiental (Coughlin *et al*., 2001). Por exemplo, estudos em goldfish aclimatados a temperaturas baixas exibem maior atividade da mATPase do que em peixes da mesma espécie aclimatados a altas temperaturas (Johnston *et al*, 1975). Além disso, estudos em carpa demonstraram que a flutuação da temperatura pode aterar a expressão das

isoformas de cadeias pesadas da miosina no músculo estriado (Hirayama & Watabe, 1997; Watabe *et al*., 1998).

Desta forma, a análise das características das proteínas miofibrilares, como a cadeia pesada da miosina, e o conhecimento sobre a expressão dos fatores que controlam os mecanismos de crescimento muscular hiperplásico e hipertrófico possuem uma importância prática para as pesquisas em aqüicultura, e podem ser utilizados para a discussão das possíveis oscilações que ocorrem nas respostas biológicas, principalmente, em espécies cultivadas em grande escala, como, a tilápia do Nilo (*Oreochromis niloticus*).

IV. OBJETIVOS

O presente trabalho estudou o tecido muscular estriado esquelético da tilápia do Nilo (*Oreochromis niloticus*) durante o crescimento e teve como objetivos:

- Caracterizar os mecanismos de crescimento hipertrófico e hiperplásico;
- Analisar a expressão protéica dos fatores de regulação miogênica MyoD e miogenina e a proliferação celular através do PCNA;
- Analisar as características da mATPase e da cadeia pesada da miosina.

V. REFERÊNCIAS BIBLIOGRÁFICAS

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, 1287-1298.

Alami-Durante, H., Fauconneau, B., Rouel, M., Escaffre, A. M., 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.

Akster, H.A., Verreth, J.A.J., Spierts, I.L.Y., Berbner, T., Schmidbauer, M. & Osse, J.M.W. (1995). Muscle growth and swimming in larvae of *Clarias gariepinus* (Burchell). *ICES Marine Science Symposia* 201, 45-50.

Alexander, R. (1969). The orientation of muscle fibres in the myomeres of fish. *J. Mar. Biol. Assoc*. UK 49, 263-290.

Alfei, L., Onali, A., Spano, L., Columbari, P.T., Altavista, P.L. & De Vita, R. (1994). PCNA/cyclin expression and BrdU uptake define proliferating myosatellite cells during hyperplastic muscle growth of fish (*Cyprinus carpio* L.). *Eur. J. Histochem.* 38, 151-162.

Alway, S.E., Lowe, D.A. & Chen, K.D. (2001). The effects of age and hindlimb suspension on the levels of expression of the myogenic regulatory factors MyoD and myogenin in rat fast and slow skeletal muscles. *Exp. Physiol*. 86, 509-517.

Bond, C.E. (1979). Endoskeleton and musculature. In Biology of Fishes (Bond, C.E. ed), pp 61-81. Philadelphia PA: Saunders College Publishing.

Brodeur, J.C., Calvo, J., Clarke, A. & Johnston, I.A. (2003). Myogenic cell cycle duration in Harpagifer species with sub-Antarctic and Antarctic distributions: evidence for cold compensation. *J. Exp. Biol.* 206, 1011-1016.

Camargo, A.A.P., Carvalho, R. F., Pellizzon, C. H. & Maeli, Dal Pai-SILVA (2004). Morphological aspects of muscle regeneration in the Nile tilapia (*Oreochromis niloticus*). *J. Subm. Cytology and Pathology*, 319-326.

Carpene E. & Veggetti, A. (1981). Increase in muscle fibres in the lateralis muscle (white portion of Mugilidae) (Pisces, Teleostei). *Experientia* 37, 191-193.

Coughlin, D.J., Burdick, J., Staufer, K.A. & Weaver, F.E. (2001). Rainbow trout display a development shift in red muscle kinetcs, swimming kinematics and myosin heavy chain isoform. *J. Fish Biol.* 58, 701-715.

Dal Pai-Silva, M., Dal Pai, V. & Mota, D. L. (1995a). Histochemical study of muscle fibre types in *Synbranchus marmoratus*. *Ann. 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. Biologia* 55, 685-691.

Duclert, A., Piette, J. & Changeux, J-P. (1991). Influence of innervation of myogenic factors and acetylcholine receptor α- subunit mRNAs. *Neuro Report.* 2, 25-28.

Egginton, S. & Johnston, I.A. (1982). A morphometric analysis of regional differences in myotomal muscle ultrastructure in the juvenile eel. *Cell Tissue Res*. 222, 579-596.

Gauthier, F.V., Qadir, M.A., Merrifield, P.A. & Atkinson, B.G. (2000). Postembryonic expression of the myosin heavy chain genes in the limb, tail and heart muscles of metamorphosing amphibian tadpoles. *Microsc. Res. Tech*. 50, 458-472.

Goldspink, G., Wilkes, D. & Ennion, S. (2001). Myosin expression during ontogeny, post-hatching growth, and adaptation. In: Johnston IA. Muscle Development and Growth, San Diego, *Academic Press*, p. 43-72.

Grobet, L., Royo Martin, L.J. & Poncelet, D. (1997). A deletion in the bovine myostatin gene causes the double-muscled phenotype in cattle. *Nat. Genetics* 17, 71-74.

Heap, S.P., Watt, P.W. & Goldspink, G. (1985). Consequences of thermal change on the myofibrillar ATPase of five freshwater teleosts. *J. Fish Biol.* 26, 733-738.

Hildsorf, A.W.S. (1995). Genética e cultivo de tilápias vermelhas, uma revisão. *Boletim do Instituto de Pesca* 22, 73-87.

Hirayama, Y. & Watabe, S. (1997). Structural differences in the crossbridge head of temperature-associated myosin subfragment-1 isoforms from carp fast skeletal muscle. *Eur. J. Biochem.* 246, 380-387.

Johansen K.A. & Overturf K. (2005). Quantitative expression analysis of genes affecting muscle growth during development of rainbow trout (*Oncorhynchus mykiss*). *Mar. Biotechnol.* 7, 576-587.

Johnston, I.A. (1981). Quantitative analysis of muscle breakdown during starvation in the marine flatfish *Pleuronectes platessa*. *Cell Tissue Res*. 214, 369-386.

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

Johnston, I.A. (2001). Myosin expression during ontogeny, post-hatching growth, and adaptation. In: Johnston, I.A. Muscle Development and Growth. Ed. San Diego, Academic Press, p. 43-70.

Johnston, I.A., Cole, N.J., Vieira, V.L.A. & Davison, I. (1997). Temperature and developmental plasticity of muscle phenotype in herring larvae. *J. Exp. Biol.* 200, 849-868.

Johnston, I.A., Davison, W. & Goldspink, G. (1975). Adaptations in Mg^{2+}–activated myofibrillar ATPase activity induced by temperature acclimation. *FEBS Lett*.50, 293-295.

Johnston, I.A., Davison, W. & Goldspink, G. (1977). Energy metabolism of carp swimming muscles. *J. Comp. Physiol*. 114, 203-216.

Johnston, I.A., McLay, H.A., Abercromby, M. & Robbins, D. (2000). Phenotypic plasticity of early myogenesis and satellite cell numbers in Atlantic salmon spawning in upland and lowland tributaries of a river system. *J. Exp. Biol*. 203, 2539-2552.

Karasinski, J. & Kilarski, W. (1989). Polymorphism of myosin isoenzymes and myosin heavy chain in histochemically typed skeletal muscles of the roach. *Comp. Biochem. Physiol.* 92B, 727-731.

Karasinski, J. (1993). Diversity of native myosin and myosin heavy chain in fish skeletal muscles. *Comp. Biochem. Physiol.* 106, 1041-1047.

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

Kobiyama, A., Nihei, Y., Hirayama, Y., Kikuchi, Y., Suetake, H., Johnston, I.A. & Watabe, S. (1998). Molecular cloning and developmental expression patterns of the

MyoD and MEF2 families of muscle transcription factors in the carp. *J. Exp. Biology* 201, 2801-2813.

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

Koumans, J.T.M., Akster, H.A., Booms, G.H.R., Lemmens, C.J.J. & Osse, J.W.M. (1991). Numbers of myosatellite cells in white axial muscle of growing fish: *Cyprinus carpio* L. (Teleostei). *Am. J. Anat.* 192, 418-424.

Lovshin, L.L. (1997). Tilápia farming: A Growing Worldwide Aquaculture Industry. In: Simpósio Sobre Manejo E Nutrição De Peixes. Piracicaba. *Anais*. Piracicaba: CBNA, p. 137-164.

Martinez, I., Bang, B., Hatlen, B. & Blix, P. (1993). Myofibrillar proteins in skeletal muscles of parr, smolt and adult Atlantic salmon (*Salmo salar*) – comparison with another salmonid, the artic charr *salvelinus-alpinus*. *Comp. Biochem. Physiol.* B 106, 1021-1028.

Martinez, I., Christiansen, J.S., Ofstad, R. & Olsen, R.L. (1991). Comparison of myosin isoenzymes present in skeletal and cardiac muscles of the Artic charr Salvelinus alpinus (L). *Eur. J. Biochem.* 195, 743-753.

Mauro, A. (1961). Satellite cells of skeletal muscle fibers. *J. Biophys. Biochem. Cytol*. 9, 493-495.

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

Mommsen, T.P. (2001). Paradigms of growth in fish. *Comp. Biochem. Physiol*. B 129, 207-219.

Mozdziak, P.E., Greaser, M.L. & Schultz, E. (1998). Myogenin, MyoD, and Myosin expression after pharmacologically and surgically induced hypertrophy. *J. Appl. Physiol.* 84(4), 1359-1364.

Murre, C., Mccaw, P.S., Vaessin, H., Caudy, M., Jan, L.Y., Yan, J.N., Cabrera, C.V., Buskin, J.N., Hauschka, S.D., Lassar, A.B., 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.

Narusawa, M., Fitzsimons, R.B., Izumo, S., Nadal-Ginard, B. & Rubistein, N.A. (1987). Slow myosin in developing rat skeletal muscle. *J.Cell Biol*. 104, 447-459.

Parry, D.J. (2001). Myosin heavy chain expression and plasticity: role of myoblast diversity. *Exerc. Sport Sci. Rev*. 29, 175-179.

Pette, D. & Staron, R.S. (2000). Myosin isoforms, muscle fiber types, and transitions. *Microsc. Res.* Tech. 50, 500-509.

Radice, G.P. (1995). Spatial expression of two tadpole stage specific myosin heavy chains in Xenopus laevis. *Acta Anat.* 153, 254-262.

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

Rescan, P.Y. (2001). Regulation and function of myogenic regulatory factors in lower vertebrates. *Comp. Biochem. Physiol*. 130, 1-12.

Rescan, P-Y. & Gauvry, L. (1996). Genome of the rainbow trout (*Oncorhynchus mykiss*) encodes two distinct muscle regulatory factors with homology to MyoD. *Comp. Biochem. Physiol.* 113B, 711-715.

Rowlerson, A., Scapolo, P.A., Mascarello, F., Carpene, E. & Veggetti, A. (1985). Comparative study of myosins present in the lateral muscle of some fish: species variations in myosin isoforms and their distribution in red, pink and white muscle. *J. Muscle Res. Cell Motil*. 6, 601-640.

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, pp. 103-140.

Sabourin, L.A. & Rudnicki, M.A. (2000). The molecular regulation of myogenesis. *Clin. Genet*. 57, 16-25.

Sassoon, D.A. (1993). Myogenic Regulatory Factors: Dissecting Their Role And Regulation During Vertebrate Embryogenesis. *Dev. Biol*. 156, 11-23.

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 *Discentrarchus labrax* (L.) *Anat. Embriol*. 178, 287-195.

Shiaffino, S. & Reggiani, C. (1994). Myosin isoforms in mammalian skeletal muscle. *J.Appl. Physiol*. 77, 493-501.

Staron, R.S., Kraemer, W.J., Hikida, R.S., Fry, A.C., Murray, J.D. & Campos, G.E.R. (1999). Fiber type composition of four hindlimb muscles of adult fisher 344 rats. *Histochem. Cell.Biol*. 111, 117-123.

Tacon, A.G.J. (1993). Feed ingredients for warmwater fish. Fish meal and other processed feedstuffs, FAO Fish. Circ. No.856, FAO, Rome, Italy, 64p.

Tacon, A.G.J. (1997). Global trends in aquaculture and aquafeed production 1984-1995. Ind. *Aquafeed directory and buyers guide,* 1987/1998. pp.5-37.

Veggetti, A., Mascarello, F. & Scapolo, P.A. (1990). Hiperplastic and hypertrophic growth of lateral muscle in *Dicentrarchus labrax*. An ultrastructural and morphometric study. *Anat. Embriol.* 182, 1-10.

Watabe, S. (2001). Myogenic regulatory factor. In: Johnston, I.A. Muscle Development and Growth. Ed. San Diego, Academic Press, p. 19-41.

Watabe, S., Hirayama, Y., Nakaya, M., Kakinuma, M., Kikuchi, K., Guo, X.-F., Kanoh, S., Chaen, S. & Ooi, T. (1998). Carp expression fast skeletal myosin isoforms with altered motor functions and structural stabilities to compensate for changes in environmental temperature. *J. Therm. Biol*. 22, 375-390.

Weatherley, A.H., Gill, H.S. & Rogers, S.C. (1979). Growth dynamics of muscle fibres, dry weight, and condition in relation to somatic growth rate in yearling rainbow trout (*Salmo gairdneri*). *Can. J. Zool*. 57, 2385-2392.

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.

Weaver, F.E., Stauffer, K.A. & Coughlin, D.J. (2001). Myosin heavy chain expression in the red, white, and ventricular muscle of juvenile stages of rainbow trout. *J. Exp. Zool.* 290(7), 751-758.

Weeds, A.G. & Lowey, S. (1971). Substructure of the Myosin Molecule. The Light Chains of Myosin. *J.Mol. Biol*. 61, 701-725.

VI. ARTIGO 1

Submitted to Aquaculture Research, 2007
Manuscript ID:ARE-OA-07-Sep-157

MyoD, Myogenin, and PCNA expression in growing Nile tilapia (*Oreochromis niloticus* L.)

Danilo Henrique Aguiar [a,d], Cláudio Bock [b], Carlos Roberto Padovani [c], Maeli DalPai Silva [d *]

[a] Department of Cellular Biology, Institute of Biology, UNICAMP, Campinas, SP, Brazil

[b] Center of Research and Training in Aquaculture, CEPTA, IBAMA, Pirassununga, SP, Brazil

[c] Department of Biostatistics, Institute of Biosciences, UNESP, Botucatu, SP

[d] Department of Morphology, Institute of Biosciences, UNESP and CAUNESP, Botucatu, SP, Brazil

Correspondence: Maeli Dal Pai-Silva, Department of Morphology, Institute of Biosciences, UNESP, Botucatu, 18618-000, São Paulo, Brazil.

Phone/Fax: +55 (14) 3811-6264.

E-mail: dpsilva@ibb.unesp.br

Running title: MyoD, Myogenin, and PCNA expression in growing Nile tilapia

Key Words: Skeletal muscle growth, Myogenic regulatory factors, Nile tilapia

Abstract

Muscle growth characterization to have proven to be more challenging in teleost fishes compared with their others vertebrates. The study of muscle growth regulation in fish is very important, mainly in the commercial species, like as Nile tilapia (*Oreochromis niloticus*). In the present study, immunohistochemical and morphometric analysis was used to show myogenic regulatory factor expression during fiber recruitment and hypertrophy of muscle growth in Nile tilapia. Fish were classified into four age stages: 35 day alevins (0.65g ± 0.08); 60 day juveniles (13.67g ± 1.35); 90 day adults (73.18g ± 4.70); and 190 day adults (349.76g ± 34.62). Frozen sections (5-7μm thick) of white muscle were cut in a cryostat (–20°C) and mounted on glass slides coated with poly-L-lysine. Sections were air-dried and submitted to the following immunohistochemical reactions: MyoD and myogenin to evaluate the degree of myogenic progenitor cell proliferation and differentiation, respectively, and PCNA to evaluate the degree of cell proliferation. The number of nuclei expressing MyoD and myogenin were similar in alevins, juveniles, and 90 day adults, however more nuclei expressed myogenin than MyoD in 190 day adults. The number of PCNA stained nuclei in each stage was higher than MyoD and myogenin staining with peaks in alevins and 90 day adults. These data suggest that growth per se stimulated cellular proliferation and differentiation, nuclei accretion and hyperplasia of Nile tilapia muscle fibers in alevins, juveniles, and 90 day adults. Muscle fiber differentiation and hypertrophy was more pronounced in 190 day adults.

Introduction

White myotomal muscle makes up at least 60% of body mass in most teleost species and is important in determining overall energy budget. Skeletal muscle growth in fish is both hypertrophic and hyperplasic from undifferentiated myogenic progenitor cells or myoblasts (Johnston 1999; Johnston 2006). In hyperplasia, myoblasts aggregate to the surface of existing fibers forming myotubes which then separate giving rise to new muscle fibers. Therefore in hyperplasia, a mosaic of different diameter fibers (large and small associated fibers) is often seen in white muscle (Johnston 1999, 2001). During hypertrophy, fibers expand and absorb myoblast nuclei with increasing fiber size in order to maintain a relative constant nuclear to cytoplasmatic ratio (Koumans *et al.*, 1994; Johnston 2001). Hypertrophic growth rate varies with somatic growth rate and in different stages of life (Rowlerson, Mascarello, Radaelli & Veggetti 1995). Hypertrophic growth in many fish species continues through life up to adult stages, even after hyperplasic growth has ceased (Weatherley, Gill & Rogers 1980; Stickland, 1983; Alfei, Maggi, Parvopassu, Bertoncello & de Vita 1989; Kiessling, Storebakken & Asgard 1991).

Myoblast proliferation and differentiation are characterized and regulated by the sequential expression of muscle-specific myogenic regulatory factors (MRFs) (Watabe 1999). Primary MRFs (MyoD and myf5) direct proliferating myogenic progenitors towards a myogenic lineage, and secondary MRFs (myogenin and MRF4) control myoblast differentiation and fusion, forming myofibers (Megeney & Rudnicki, 1995; Rudnicki & Jaenisch, 1995). Other markers, such as the PCNA are used to study cell proliferation, and this protein in association with MyoD in myogenic cells is a marker of

myogenic progenitor cell activation (Yablonka–Reuveni & Rivera, 1994; Bravo *et al*., 1987; Baserga, 1991).

The aim of this paper is to show myogenic regulatory factor expression during fiber recruitment and hypertrophy during muscle growth in Nile tilapia. This should provide information for development biologists and those seeking to refine tilapia musculature for aquaculture.

Materials and Methods

Fish

Newly hatched, 48h old, Nile tilapia larvae (*Oreochromis niloticus*) were purchased from Aquabel Fisheries, Rolândia, PR and transported to the Research Center and Fishery Resource Management Continental, CEPTA – IBAMA, Pirassununga, SP. Larvae were initially distributed into three 250L aquaria, each with a re-circulating freshwater system (25-27ºC, 14/10h light/dark). Two larvae/L were placed in each aquarium and kept for five days adaptation before being fed. Then, when yolk sacs had been reabsorbed, food was administered with hormone α-methyl-testosterone for sexual reversion.

Fish were fed to satiation six times a day (8:00; 10:00; 12:00; 14:00; 16:00, and 18:00) for 30 days. Initial body mass and standard length, L_s were 0.010$\pm$0.001g and 0.93$\pm$0.031cm (mean$\pm$SD, N=50), respectively.

After sex reversal, fish were transferred to 4.0m long, 1.80m wide, 1.0m high outside tanks (n=10) under natural conditions. Alevins (n=100) were put in each tank for 160 days until they reached a commercially interesting weight (350 and 400g). As fish grew, they were checked every 15 days to accompany growth and manage their food needs in each stage. During the experimental period, fish were fed with commercial food pellets four times a day (9:00, 12:00, 14:00, 17:00) until satiation. Water quality was monitored daily. Mean (09:00 am) and (17:00 pm) water temperatures were 26.5ºC and 27.5ºC, respectively; oxygen content was 6.20% and pH around 5.75.

Sacrifice and muscle sample extraction

Fish were classified, as per fishery classification, into four age stages: 35 day alevins (0.65g ± 0.08); 60 day juveniles (13.67g ± 1.35); 90 day adults (73.18g ± 4.70) and 190 day adults (349.76g ± 34.62).

In each study period, 50 fish from each stage were anaesthetised with MS-222 (Tricaine Methanensulfonate) - SIGMA, placed in water and sacrificed. Weight gain (g) and length (cm) were determined. White muscle samples from the epaxial region, at dorsal fin level were withdrawn, immersed in n-Hexane cooled in liquid nitrogen (-159ºC), and then stored in a freezer at –80°C until sectioning. Samples from other specimens were fixed in 4% paraformaldehyde as per Bancroft & Stevens (1990) for later analysis.

Morphometric and immunohistochemical analysis

White muscle cross-sections (5 to 7µm thick) were obtained by –20°C cryostat, and then stained with haematoxylin-eosin (Bancroft & Stevens 1990). These were used to calculate the smaller diameter muscle fibers (Dubowitz & Brooke 1973). The smallest diameters were measured from 100 white muscle fibers from five animals per stage using a Leica Qwin Software 3.1 (Leica-England), image analysis system. Muscle fibers were later distributed into diameter classes based on Veggetti, Rowlerson, Radaelli, Arrighi & Domeneghini (1999), and Rowlerson *et al*. (1995).

Frozen sections (5-7µm thick) from ten animals per stage were cut in a cryostat (–20°C) and mounted on glass slides coated with poly-L-lysine. Sections were air-dried and submitted to the following immunohistochemical reactions: MyoD and myogenin to

evaluate the degree of myogenic progenitor cell proliferation and differentiation, respectively.

First sections were fixed in acetone for 10 min and then washed 3 times for 5 min in PBS. The primary antibody used against MyoD and myogenin (Santa Cruz Biotechnology) was diluted 1:20 in a solution containing 1% (m/v) bovine serum albumin (BSA) in phosphate buffered saline (PBS). Sections were incubated overnight at 4ºC. After three washes in PBS, sections were incubated for one hour with biotinylated goat anti-rat secondary antibody (IgG; Vector) diluted 1:20 in a solution containing 1% (m/v) BSA in PBS. Sections were again washed 3 times for 5 min in PBS and incubated for 45 min in a 1:20 dilution with Avidin-Biotin-Peroxidase (ABC – VECTOR). Peroxidase was located with diamino benzidine (DAB; Sigma). Sections were washed 3 times for 2 min, the last with 1% triton, and were mounted in permount (Hsu, Raine & Fanger 1981).

Specimens fixed in 4% paraformaldehyde were embedded in Paraplast and 5-7μm thick sections from ten animals per stage were submitted to proliferating cell nuclear antigen (PCNA) immunohistochemical reaction, to evaluate the degree of cell proliferation. After deparafinization, primary PCNA antibody (NCL-PCNA – NOVOCASTRA) was diluted 1:100 in a solution containing 1% (m/v) BSA in PBS. Sections were incubated overnight at 4°C. Later, sections were incubated with secondary antibody (Anti-rat IgG – VECTOR), diluted 1:100 in a solution containing 1% BSA in PBS, and finally with ABC (VECTOR), diluted 1:20 in PBS. At each solution change sections were washed 3 times for 5min each in PBS. Peroxidase was located with DAB (SIGMA). Sections were then mounted in permount (Hsu *et al*. 1981)

The number of nuclei stained for MyoD, myogenin, and PCNA were identified and quantified using a Leica Qwin Software 3.1 (Leica-England), image analysis system. Immunoreactive cell counts were made from three different 1.30mm^2 areas at 400X for each fish stage. Only two areas were counted in alevin because of the small myotomal area available in fish at this stage.

Statistical analysis

The following statistical tests were used to compare experimental stages: ANOVA for L_T and mass; Goodman-test for association between stages and diameter distributions (Goodman 1964, 1965); Non-parametric Kruskal-Wallis test for nuclei frequency (Zar, 1999; Norman & Streiner, 1994). Differences were considered significant at $P<0.05$.

Results

Morphology and morphometry

At the end of the experiment fish from all stages showed increased muscle mass and length. HE stain showed white skeletal muscle making up most of the muscle mass in all stages; this muscle was composed of round or polygonal muscle fiber separated by a fine septa of connective tissue, the endomysium. Thicker septa of connective tissue separated the muscle fibers into fascicles and made the perimysium. Muscle fibers were distributed in a mosaic pattern, characterized by fibers of different diameter (Figure 1).

Muscle fiber frequency distribution showed the highest percentage of below 8.0μm diameter fibers in alevins, followed by juveniles; in 90 and 190 day adults, their frequency was lower and similar. The frequency of 8.0 to 16μm fibers was higher in alevins and decreased from alevins to 190 day adults. Frequency of 16 to 24μm fibers was higher in alevins and juveniles, and lower in 90 and 190 day adults. Juveniles had a higher frequency of 24 to 32μm fibers than the other stages. All stages had different frequencies for fibers over 32μm; alevins the smallest and 190 day adults the highest (Figure 2).

Immunohistochemistry

The number of nuclei expressing MyoD and myogenin was similar in alevins, juveniles, and 90 day adults; however in 190 day adults, the number of nuclei expressing myogenin was higher than those expressing MyoD. The number of PCNA

stained nuclei in each stage was higher than MyoD and myogenin stained with peaks in alevins and 90 day adults (Figure 3). MyoD, myogenin and PCNA positive nuclei in white muscle are illustrated in Figure 4.

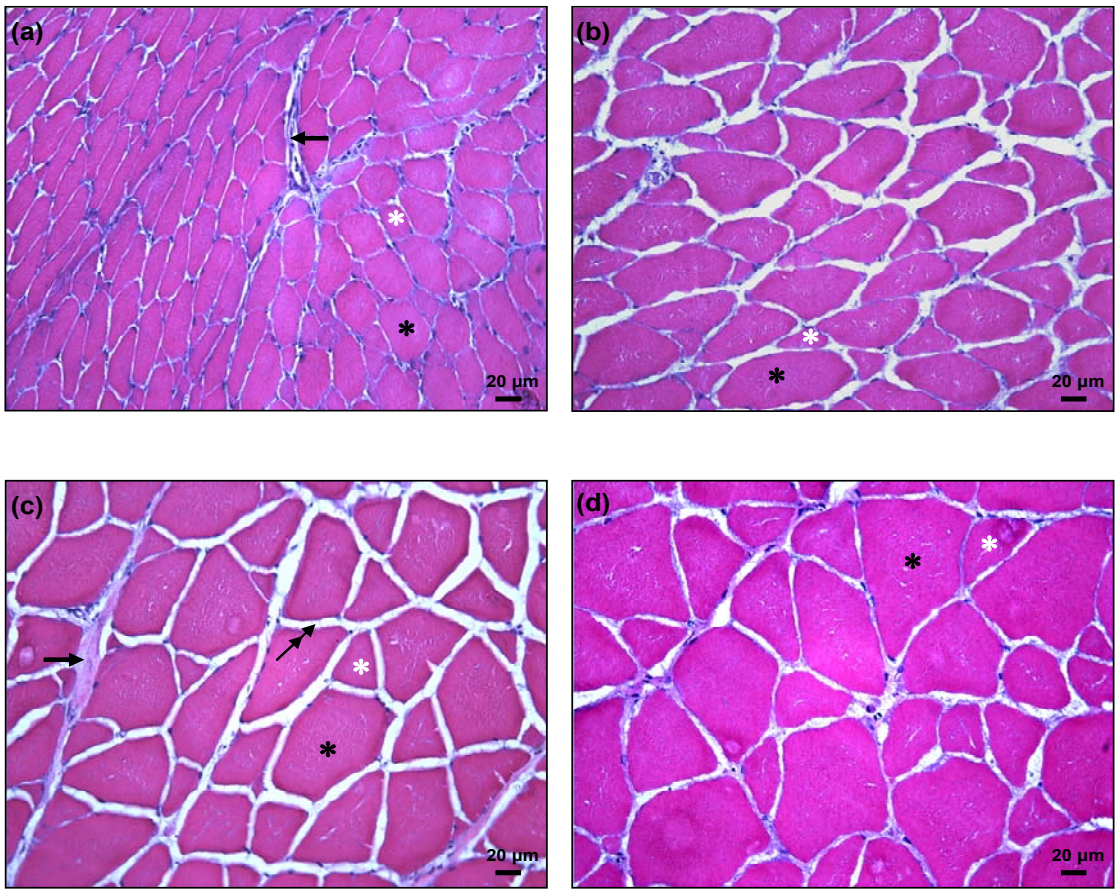

Figure 1. Nile tilapia (*O. niloticus*) skeletal muscle. Transverse section of the white muscle displaying the association between small (white asterisks) and large (black asterisks) fibers. Endomysium (double arrows) and perimysium (single arrows). a. alevin. b. juvenile. c. 90 day adults. d. 190 day adults.

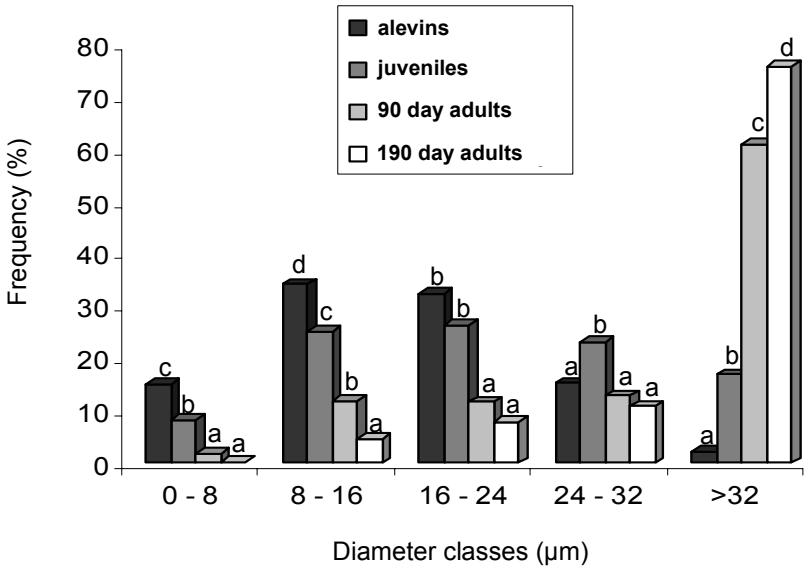

Figure 2. Relative frequency distribution of white muscle fibers smaller diameters in Nile tilapia (*O. niloticus*) during growth. (letters in the column show size classes with significant variation, $p<0.05$).

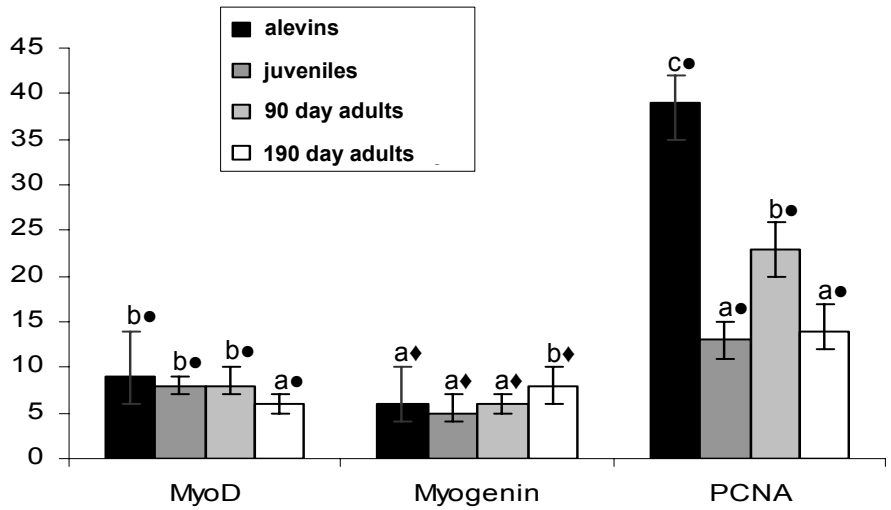

Figure 3. Frequency of MyoD+, Myogenin+ and PCNA+ myonuclei in the white muscle fibers of Nile tilapia (*O. niloticus*). Comparison of the proteins expression among the stages values with the same lower case letter were not significantly different. ●p<0,001; ♦p<0,005

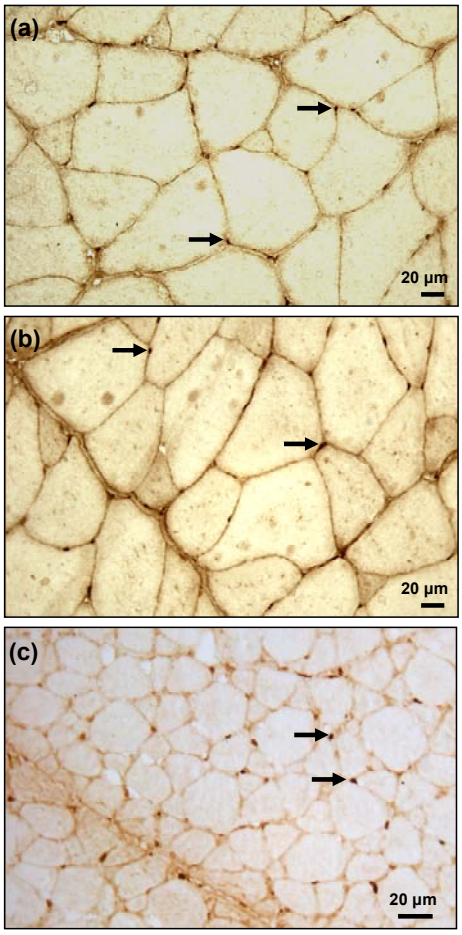

Figure 4. Nile tilapia (*O. niloticus*) skeletal muscle. Transverse section of white muscle. a, b and c: MyoD, myogenin and PCNA positive nuclei (arrow), respectively.

Discussion

In Nile tilapia, most musculature consists of white muscle fibers, the most commercially important (Zhang, Swank & Rome, 1996). We were able to characterize post-larval muscle growth in Nile tilapia making reference to the hypertrophy, hyperplasia and protein expression involved in muscle growth mechanisms during the commercially essential stages.

Comparing stages, we observed a general increase in higher diameter muscle fiber frequency in 90 and 190 day adults associated to a gradual decrease in small diameter fiber frequency during growth. This indicated that muscle fiber recruitment was more evident in early growth (alevins and juveniles), which agrees with Valente, Rocha & Gomes (1999), and Rowlerson & Veggetti (2001) where the presence of small diameter muscle fibers, 20-25μm, indicated hyperplasia.

Hyperplasia began in the germinal zones of cell proliferation near the intermediate layer, mainly consisting of new fibers which are responsible for thickening muscle mass in the early development stages (Rowlerson & Veggetti, 2001; Johnston, Fernandez, Calvo, Vieira, North, Abercromby & Garland, 2003). This growth event is known as stratified hyperplasia and occurs in most fish species (Johnston, 1999). Stratified hyperplasia is followed by mosaic hyperplasia, showing large and small associated muscle fibers (Johnston, 2006).

The proportion of small diameter fibers depends on final fish size and is a relevant factor in comparing hyperplasic growth in large economically beneficient species (Kiessling *et al*., 1991; Fauconneau, André, Chmaitilly, Lê Bail, Krieg & Kaushik,

1997). Our study showed that alevins and juveniles muscle growth was predominantly stratified and mosaic hyperplasia. In 90 and 190 day adults there was a higher frequency of >32μm diameter fibers than in alevins and juveniles, showing that hypertrophy was more intense in 90 and 190 day adults muscle growth.

According to Zimmerman & Lowery (1999), new fiber recruitment during muscle growth stops when fish reach about 56% of their full grown size; after this muscle growth is mainly due to hypertrophy. Although the 190 day adults are not indicative of their final size, our study showed that muscle fiber recruitment in this stage was less than in the others (alevins, juveniles, and 90 day adults).

Skeletal muscle growth in fish is dependent on the proliferation and differentiation of myogenic progenitor cells, processes regulated by the differential expression of myogenic regulatory factors (MRFs), MyoD, Myf5, myogenin, and MRF4. These transcriptional factors control the expression of muscle specific genes and contribute to hyperplasia and hypertrophy (Watabe, 1999).

Nuclei expressing MyoD and myogenin were observed in Nile tilapia throughout growth. The number of nuclei expressing both MyoD and myogenin was similar in alevins, juveniles, and 90 day adults, but in 190 day adults the number of myogenin expressing nuclei was higher than the number expressing MyoD.

During early fish development and growth, intense new muscle fiber recruitment is due to the proliferation of undifferentiated myogenic progenitor cells that express MRFs, MyoD, and Myf5 (Rescan, Gauvry, Paboeuf & Fauconneau, 1994; Watabe, 1999; Megeney & Rudnicki, 1995; Grobet, Royo Martin & Poncelet, 1997). In Nile tilapia

alevins, juveniles, and 90 day adults a great number of MyoD expressing nuclei are related to the intense cell proliferation occurring in these growth periods. These nuclei contributed to both hyperplasia and hypertrophy as observed by morphometric analysis (Brooks & Johnston, 1993).

The presence of equal numbers of MRF myogenin expressing nuclei in alevins, juveniles, and 90 day adults shows that both cell proliferation and differentiation are occurring. During the differentiation process nuclei fuse to each other forming new muscle fibers; this was confirmed by analyzing muscle fibers diameters in these stages. Our findings confirm that during the initial phases of tilapia growth, myogenic progenitor cell proliferation and differentiation is occurring.

The presence of MyoD positive cells in 190 day adults indicates that myoblast proliferation is occurring and this is involved in muscle fiber hypertrophy, the predominant muscle growth mechanism as seen by morphometric analysis. MRF expression in adult skeletal muscle may help protein synthesis and degradation, responsible for maintaining muscle phenotype (Mozdziak, Greaser & Schultz, 1998). Also, myoblast proliferation may be involved in nuclear turnover, as 2% of nuclei are recycled weekly in adult skeletal muscle (Schmalbruch & Lewis, 2000).

Myogenin expression increased in this stage indicating that differentiation was predominant during hypertrophy at the same time as a decreasing in muscle fiber recruitment. As gene targeting experiments suggest that MRF myogenin is involved with myotube fusion and growth (Hasty, Bradley, Morris, Edmondson, Venuti, Olson & Klein, 1993; Black & Olson, 1998), hypertrophy can be inferred by analyzing the expression of these MRFs (Johansen & Overturf, 2005).

According to Fauconneau & Paboeuf (2000) and Brodeur, Peck & Johnston (2002), increased MyoD and myogenin expression during muscle growth should theoretically also be in response to a stimulus, such as forced swimming or feeding status. However, in Nile tilapia with no external interference, muscle growth displayed uniformity with myoblast proliferation and differentiation until the 190th day.

In all studied stages, PCNA expression was higher than both MyoD and myogenin expression. PCNA expression higher than MyoD and myogenin in each stage implies that it regulates myogenic progenitor cell activation and proliferation (Martin & Johnston, 2005; Yablonka-Reuveni & Rivera, 1994).

In conclusion, our results suggest that growth per se stimulated cellular proliferation and differentiation, nuclei accretion and hyperplasia of Nile tilapia muscle fibers in alevins, juveniles, and 90 day adults. Muscle fiber differentiation and hypertrophy was more pronounced in 190 day adults.

Acknowledgments

This work is part of the PhD Thesis that will be presented by DHA to Universidade Estadual de Campinas – UNICAMP. This study was supported by Fapesp, Proc. N° 04/13286-0. Researchers from CEPTA were extremely helpful during planning and execution of this experiment. Colleagues from the Department of Morphology and the Biosciences Institute did valuable work in the laboratory.

References

Alfei L., Maggi F., Parvopassu F., Bertoncello G. & de Vita R. (1989). Post-larval muscle growth in fish: DNA flow cytometric and morphometric analysis. *Basic Applied Histochem*istry **33**, 147-158.

Bancroft J.D. & Stevens A. (1990). Theory and Practice of Histological Techniques. 3th ed. Churchill Livingstone, pp. 81-92.

Baserga R. (1991). Growth regulation of the PCNA gene. *Journal Cell Science* **98**, 433-436.

Black B.L. & Olson E.N. (1998). Transcriptional control of muscle development by myocyte enhancer factor-2 (MEF2) proteins. *Annual Review of Cell and Developmental Biology* **14**, 167-196.

Bravo R., Frank R., Blundell P. A., MacDonald-Bravo H. (1987). Cyclin/PCNA is the auxiliary protein of DNA polymerase-δ. *Nature* **326**, 515-517.

Brodeur J.C., Peck L.S. & Johnston I.A. (2002). Feeding increases MyoD and PCNA expression in myogenic progenitor cells of *Notothenia coriiceps*. *Journal of Fish Biology* **60**, 1475-1485.

Brooks S. & Johnston I.A. (1993). Influence of development and rearing temperature on the distribution, ultrastructure and myosin sub-unit composition of myotomal muscle fiber types in the plaice *Pleuronectes platessa*. *Marine Biology* **117**, 501-513.

Dubowitz V. & Brooke M. (1973). Muscle Biopsy: A Modern Approach. London: Saunders, pp. 475.

Fauconneau B., André S., Chmaitilly J., Lê Bail P.-Y., Krieg F. & Kaushik S.J. (1997). Control of skeletal muscle fibers and adipose cells size in the flesh of rainbow trout. *Journal of Fish Biology* **50**, 296-314.

Fauconneau B. & Paboeuf G. (2000). Effect of fasting and refeeding on in vitro muscle cell proliferation in rainbow trout (*Onchorhynchus mykiss*). *Cell and Tissue Research* **301**, 459-463.

Goodman L.A. (1964). Simultaneous confidence intervals for contrasts among multinomial populations. *Annals of Mathematical Statistics* **35(2)**, 716-725.

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

Grobet L., Royo Martin L.J. & Poncelet D. (1997). A deletion in the bovine myostatin gene causes the double-muscled phenotype in cattle. *Nature Genetics* **17**, 71-74.

Hasty P., Bradley A., Morris J.H., Edmondson D.G., Venuti J.M., Olson E. N. & Klein W.H. (1993). Muscle deficiency and neonatal death in mice with a targeted mutation in myogenin gene. *Nature* **364**, 501-506.

Hsu S.M., Raine L. & Fanger H. (1981). Use of avidin-biotin-peroxidase complex (abc) in immunoperoxidase techniques: a comparison between abc and unlabeled antibody (pap) procedures. *Journal of Histochemistry and Cytochemistry* **29**, 577-580.

Johansen K.A. & Overturf K. (2005). Quantitative expression analysis of genes affecting muscle growth during development of rainbow trout (*Oncorhynchus mykiss*). *Marine Biotechnology* **7**, 576-587.

Johnston I.A., Fernandez D.A., Calvo J., Vieira V.L.A., North A.W., Abercromby M. & Garland T. (2003). Reduction in muscle fiber number during the adaptative radiation of notothenioid fishes: a phylogenetic perspective. *Journal of Experimental Biology* **206**, 2595-2609.

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

Johnston I.A. (2001). Myosin expression during ontogeny, post-hatching growth, and adaptation. In: Muscle Development and Growth. Ed.Academic Press, pp. 43-70.

Johnston I.A. (2006). Environment and plasticity of myogenesis in teleost fish. *Journal of Experimental Biology* **209**, 2249-2264.

Kiessling A., Storebakken T. & Asgard T. (1991). Changes in the structure and function of the epaxial muscle of rainbow trout (O. Mykiss) in relation to ration and age. I. Growth dynamics. *Aquaculture* **93**, 335-356.

Koumans J.T.M., Akster H.A., Witkam A. & Osse J.W.M. (1994). Numbers of muscle nuclei and myosatellite cell nuclei in red and white axial muscle during growth of the carp (*Cyprinus carpio*). *Journal Fish Biology* **44**, 391-408.

Martin C.L. & Johnston I.A. (2005). The role of myostatin and the calcineurin-signalling pathway muscle mass in response to exercise training in the rainbow trout (*Oncorhynchus mykiss*) Walbaum. *Journal of Experimental Biology* **208**, 2083-2090.

Megeney L.A. & Rudnicki M.A. (1995). Determination versus differentiation and the MyoD family of transcription factors. *Biochemistry and Cell Biology* **73**, 723-732.

Mozdziak P.E., Greaser M.L. & Schultz E. (1998). Myogenin, MyoD, and Myosin expression after pharmacologically and surgically induced hypertrophy. *Journal of Applied Physiology* **84(4)**, 1359-1364.

Norman G.R. & Streiner D.I. (1994). Biostatistics. The bare essentials. Mosby-Year Book, St.Louis, pp.260.

Rescan P.Y., Gauvry L., Paboeuf G. & Fauconneau B. (1994). Identification of a muscle factor related to MyoD in fish species. *Biochimica and Biophysica Acta* **1218**, 202-204.

Rowlerson A., Mascarello F., Radaelli G. & Veggetti A. (1995). Differentiation and growth of muscle in the fish *Sparus Aurata* (L): Ii. Hyperplastic and hypertrophic growth of lateral muscle from hatching to adult. *Journal of Muscle Research and Cell Motility* **16**, 223-236.

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, pp. 103-140.

Rudnicki M.A. & Jaenisch R. (1995). The MyoD family of transcription factors and skeletal muscle myogenesis. *Bioessays* **17**, 203-209.

Schmalbruch H. & Lewis D.M. (2000). Dynamics of nuclei of muscle fibers and connective tissue cells in normal and denervated rat muscles. *Muscle Nerve* **23**, 617-626.

Stickland N.C. (1983). Growth and development of muscle fibres in the rainbow trout (*Salmo gairdneri*). *Journal of Anatomy* **137**, 323-333.

Valente L.M.P., Rocha E. & Gomes E.F.S. (1999). Growth dynamics of white and red muscle fibers in fast and slow growing strains of rainbow trout. *Journal of Fish Biology* **55**, 675-691.

Veggetti A., Rowlerson A., Radaelli G., Arrighi S. & Domeneghini, C. (1999). Post-hatching development of the gut and lateral muscle in the sole. *Journal of Fish Biology* 55 A, 44-65.

Watabe S. (1999). Myogenic regulatory factors and muscle differentiation during Ontogeny in fish. *Journal of Fish Biology* **55**, 1-18.

Weatherley A.H., Gill H.S. & Rogers S.C. (1980). The relationship between mosaic muscle fibres and size in rainbow trout (*Salmo gairdneri*). *Journal of Fish Biology* **17**, 603-610.

Yablonka-Reuveni Z. & Rivera A.J. (1994). Temporal expression of regulatory and sctructural muscle proteins during myogenesis of satellite cells on isolated adult rat fibres. *Developmental Biology* **164**, 588-603.

Zar J.H. (1999). Biostatistical analysis, 4th ed. Prentice Hall, New Jersey, 663.

Zhang G., Swank D.M. & Rome L.C. (1996). Quantitative distribution of muscle fiber types in the scup (*Stenotomus chrysops*). *Journal of Morphology* **229**, 71-81.

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

VII. ARTIGO A SER SUBMETIDO PARA PUBLICAÇÃO

Myosin heavy chain (MHC) characteristic in the Nile tilapia (*Oreochromis niloticus*) during growth

Aguiar, D.H.[a,d] ; Bock,C.L.[b]; Carani, F.R. [d]; Padovani,C.R.[c]; Dal-Pai-Silva, M. [d]

[a] *Department of Cellular Biology, Institute of Biology, UNICAMP, Campinas, SP, Brazil*

[b] *Center of Research and Training in Aquaculture, CEPTA – IBAMA, Pirassununga, SP*

[c] *Department of Biostatistics, Institute of Biosciences, UNESP, Botucatu, SP*

[d] *Department of Morphology, Institute of Biosciences, UNESP, Botucatu, SP*

Abstract

The swimming muscle of fishes is divided into discrete regions, which are composed of functionally different fiber types: the red muscle, made up of slow twitch oxidative fibers, are used in steady swimming; the white muscle, made up of fast twitch glycolytic fibers, produce short-term bursts and intermediate muscles with fast oxidative glycolytic fibers with intermediate characteristics. In the present work, we studied the skeletal muscle characteristic in Nile tilapia during growth. Fish were classified into five age stages: 2 day larvae (0.010g ± 0.001), 35 day alevins (0.65g ± 0.08); 60 day juveniles (13.67g ± 1.35); 90 day adults (73.18g ± 4.70) and 190 day adults (349.76g ± 34.62). Samples of red and white skeletal muscle were submitted to myofibrillar ATPase activity (mATPase) and electrophoresis by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) to analyze the mATPase and the Myosin Heavy Chain (MHC) characteristics and to comparing results with a standard of known molecular mass. The standard used was rat soleus muscle with predominant MHC I and II. In all stages red muscle of the superficial compartment showed fibers with strong mATPase reaction and most of the muscle mass showed white fibers which weak reaction. Small fibers were identified between the red and white muscle fibers with moderate mATPase enzyme activity. These were named hybrid fibers. In all stages, red and white muscle

expressed slow and fast MHC isoforms, respectively, which together approximate the molecular mass of rat soleus muscle MHC I. In the white muscle, from alevins was observed other myosin isoform with molecular mass superior to the rat soleus muscle MHCI. In contrast, in the red muscle from adults was observed other myosin isoform of molecular mass similar to rat soleus muscle MHC II. We concluded that the expression of different MHC isoforms in Nile tilapia during growth, can be related to the phenotypic plasticity that occur during muscular growth and reflects this fish capacity to adapt to changes in the environmental conditions which are important for its survival.

Key Words: Fish; Skeletal Muscle growth; Myosin Heavy Chain; *Oreochromis niloticus*

Introduction

The swimming muscle of fishes is divided into discrete regions, which are composed of functionally different fiber types: the red muscle, made up of slow twitch oxidative fiber types, are used in steady swimming, and the white muscles, made up of fast twitch glycolytic fibers, are used to produce short-term bursts swimming (Bond, 1979; Akster *et al*., 1995; Johnston *et al*., 1997). There is an intermediate layer between the red and white muscle with intermediate characteristics (Sanger & Stoiber, 2001).

The myosin is the most abundant protein in muscle tissue and produce the force for muscular contraction. The myosin contain a portion of the myosin heavy chain (MHC) that consists in the region where the actin binding and contain the ATPase sites (Johnston, 1999). During the contraction the main determinant is the type of MHC that is predominantly expressed in the muscle fibers types (Buckingham, 1985).

Althought the types of MHC of red and white muscles fish are, respectively, MHCs (*slow myosin heavy chain*) and MHCf (*fast myosin heavy chain*), there are structural differences dependent on developmental stage and species (Karasinski, 1993; Karasinski & Kilarski, 1989). Moreover, the MHC are capable of changing their phenotype that occur as a changes in protein isoforms (Martinez *et al*., 1993; Weaver *et al*., 2001).

In fish, few studies have looked at characterizing MHC isoforms expressed in muscles during the embryonic and post embryonic periods, and a complex transition and change in myosin isoform phenotypes occur during embryonic development and growth (Focant *et al*., 1992; Johnston *et al*., 1997; Weaver *et al*., 2001).

The different contractile properties on the swimming muscles show in fish a wide diversity in modes of life and habitats and the adaptation of the myosins is particularly interesting (Johnston, 1999). Then, the study and the characterization of the myosin heavy chain in important species, like as Nile tilapia, may provide some very useful pointers in elucidating the contractile mechanism and for understanding fish adaptation to environment.

Material and Methods

Fish

Newly hatched, 48h old, Nile tilapia larvae (*Oreochromis niloticus*) were purchased from Aquabel Fisheries, Rolândia, PR and carried to the Center of Research and Training in Aquaculture, CEPTA – IBAMA, Pirassununga, SP. Larvae were initially distributed into three Bernauer aquariums utilized to sexual reversion with capacity of 250L each and re-circulating freshwater system (25-27ºC, 14/10h light/dark). Fish were placed at 2 larvae/L in each aquarium and kept for five days adaptation before being fed. After this period, when yolk sacs had been reabsorbed, food was administered with hormone α-methyl-testosterone for sexual reversion. In this period were sacrificed ten larvae (2 day larvae stage) and immersed in liquid nitrogen, then stored in a freezer at –80°C until sectioning after of determined to weight and length.

Fish were fed to satiation six times a day (8:00; 10:00; 12:00; 14:00; 16:00 e 18:00) for 30 days. Initial body mass and standard length, L_T were 0.010g $\pm$ 0.001 and 0.93 cm $\pm$ 0.031 (mean$\pm$SD, N=50), respectively.

After sexual reversion, fish were transferred to 4.0m long, 1.80m wide, 1.0m high outside tanks (N=10) under natural conditions. Alevins (n=100) were puts in each tank for 160 days until they reached a commercially interesting weight between 350 and 400g. As they grew, the fish were managed every 15 days to accompany growth and suitable food levels for their need at each stage. During the experimental period, fish were fed with commercial food pellets four times a day (9:00, 12:00, 14:00, 17:00) until satiation. Water quality was monitored daily. Mean am (09:00) and pm (17:00) water temperatures were 26.5ºC and 27.5ºC, respectively; oxygen content was 6.20% and pH around 5.75.

Sacrifice and extract of muscle samples

Fish were classified into five age stages: 2 day larvae (0,01g ± 0,001), 35 day alevins (0.65g ± 0.08); 60 day juveniles (13.67g ± 1.35); 90 day adults (73.18g ± 4.70) and 190 day adults (349.76g ± 34.62).

In each study period, ten fish from each stage were anaesthetised with MS-222 (Tricaine Methanensulfonate) - SIGMA, placed in water and sacrificed. Weight gain (g) and length (cm) were determined. Red and white Muscle samples from the epaxial region, at dorsal fin level were withdrawn, immersed in n-Hexane cooled in liquid nitrogen (-159ºC), and then stored in a freezer at –80°C until sectioning.

Histochemical analysis (mATPase activity)

Histological sections (7µm) from a cryostat at –20ºC were submitted to myofibrillar ATPase reaction (mATPase) in pH 9.4 after preincubation in acid pH (4.6) based on Dubowitz & Brooke (1973) to study muscle fiber types during muscle growth.

Slides were initially immersed in a solution containing sodium acetate (54.3mM), sodium barbiturate (32.6mM) at pH4.6 for 7 minutes. Then slides were immersed at ambient temperature in the following sequence of solutions: sodium chloride 0.18M at pH7.8 for 1 minute; sodium barbiturate 0.1M; calcium chloride 0.18M; 15mg ATP (adenosine triphosphate) at pH 9.4 for 45 minutes; calcium chloride 1% in deionized water for 9 minutes (3X3'); calcium chloride 2% in deionized water for 3 minutes (2X1'30"); sodium barbiturate 0.1M for 2 minutes (6X20") and ammonium sulphate 2% for 45 seconds. Cover slides were then mounted with Permount for analysis.

Electrophoresis by SDS-PAGE

Electrophoresis was performed using complete larvae specimens, white muscle fragments of alevins, and red and white muscle fragments from juveniles, and 90 and 190 day adults.

Muscle fragments were frozen in liquid nitrogen and 12µm thick histological slices, obtained in a microtome cryostat, were placed in 450µl of a solution containing glycerol 10% (w/vol), 2-mercaptoethanol 5 % (vol/vol), SDS 2.3% (w/vol), and Tris HCl 0.9% (w/vol) for 10 minutes at 60°C. Sample methods were standardized so that tissue

sizes and quantities were the same for each method whether taking muscle samples or sections by cryostat.

Electrophoresis was then performed using 7µl of each sample, with a gradient gel (6 and 10%) for protein separation and a binding gel at 4%. This was run for 24 hours at 70V. The gels were then placed in fixing solution (50% methanol, 7% acetic acid) for 10 minutes, stained with Coomassie Blue for 30 minutes, and immersed in discolouring solution (80% methanol, 7% acetic acid).

The band proteins of SDS-PAGE, were photographed and then were analyzed comparing results with a standard of known molecular mass. The standard was rat soleus muscle with predominant myosin heavy chain isoforms (MHC) I and II.

Results

Histochemical analysis (mATPase activity)

In alevins, red muscle fibers formed a red muscle layer at the superficial region, mostly in the lateral line nerve. This red muscle was distributed in a triangular shape making it thinner towards the dorsal and ventral regions; some fibers were distributed in the transverse septum region. The red fibers reaction was strong. Most of the muscle mass, showed fiber which weak reaction, forming white muscle (Figure 1A). Small fibers were identified around the white muscle fibers which showed moderate enzyme activity. These were named hybrid fibers (Figure 1B).

In juveniles, red muscle was more developed and from this stage a moderate number of small fibers, the hybrid fibers with weak reaction were seen between the red fibers which strongly reacted. An intermediate layer was seen between the red and white muscle whose fibers had different reaction intensities. In the white muscle, fibers reacted weakly and between these were small hybrid fibers with moderate enzyme activity (Figure 2A).

In 90 day adults, red muscle showed fibers with high enzymatic activity and small hybrid fibers with moderate intensity. In intermediate muscle between the red and white, fibers presented different reaction intensities. White fibers reacted in a weakly and small hybrid fibers were identified between the white fibers with moderate activity (Figure 2B).

In 190 day adults, the distribution pattern was similar to that in 90 day adults (Figure 3).

Electrophoresis by SDS-PAGE analysis

Under our electrophoresis conditions, larvae showed a lightly band protein stained. This protein was located at the same level as rat soleus muscle MHC I (figure 4).

In alevins, in the white muscle, there was a very evident band protein at the same level as the larva, in the region corresponding to soleus muscle MHC I. Immediately below this strongly stained band protein, a other band protein with weaker stained was observed in the following stages (figure 4).

In juveniles, in the white muscle, was observed the same bands proteins as in alevins. However, in the red muscle, only a band protein strongly stained was observed.

In 90 and 190 day adults, considering white muscle, there was a very evident strong band protein at the same level as in larva, alevins and juveniles muscle corresponding to soleus muscle MHC I. There was also a thin slightly stained band protein below this main band. In relation to red muscle, as well as the evident band protein at the corresponding level to soleus muscle MHC I, there was also a thin band at the same level as a soleus muscle MHC II. This was more evident in 190 day adults (figure 4).

The most evident band protein, in all stages, was at the same level as soleus muscle MHC I. White muscle bands proteins were more strongly stained in all stages studied (figure 4).

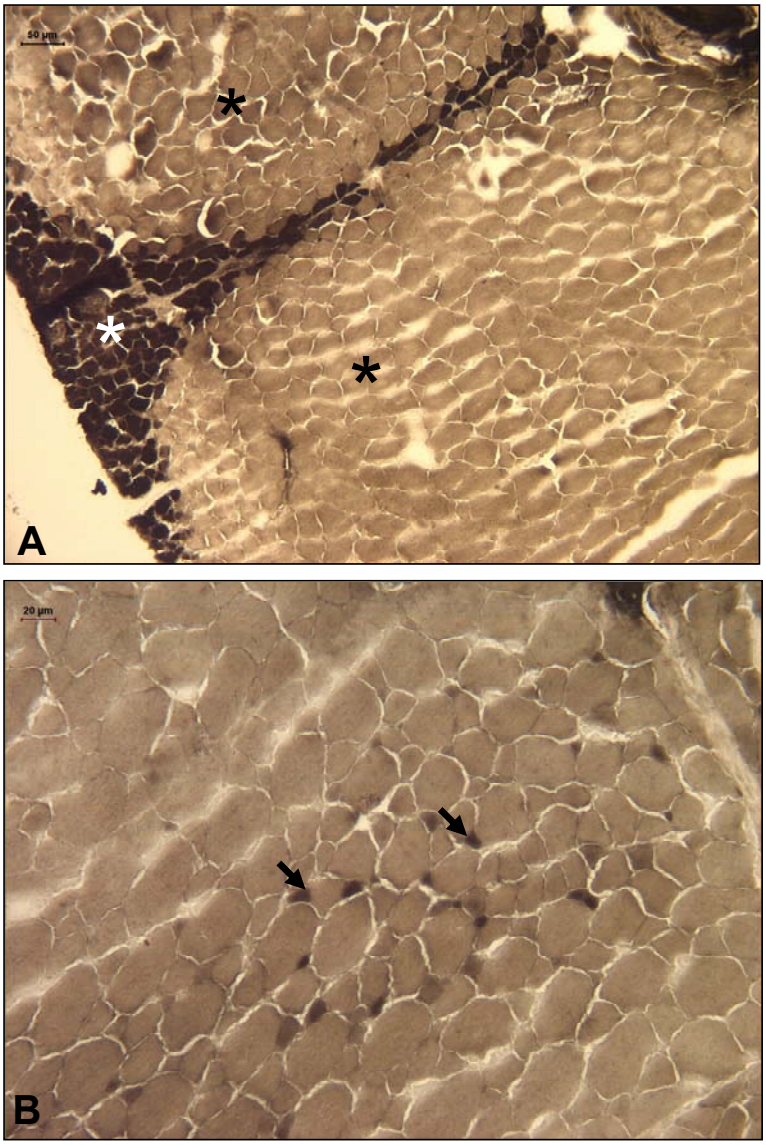

Fig. 1. **Transverse section of myotomal muscle in 35 day alevins**. **A**. Red muscle with red muscle fibers (white asterisk). White muscle with white muscle fibers (black asterisks). **B**. Detail of white muscle. Small fibers (hybrid) with moderate enzyme activity in deep white muscle (arrows). **mATPase, pH 4,6.**

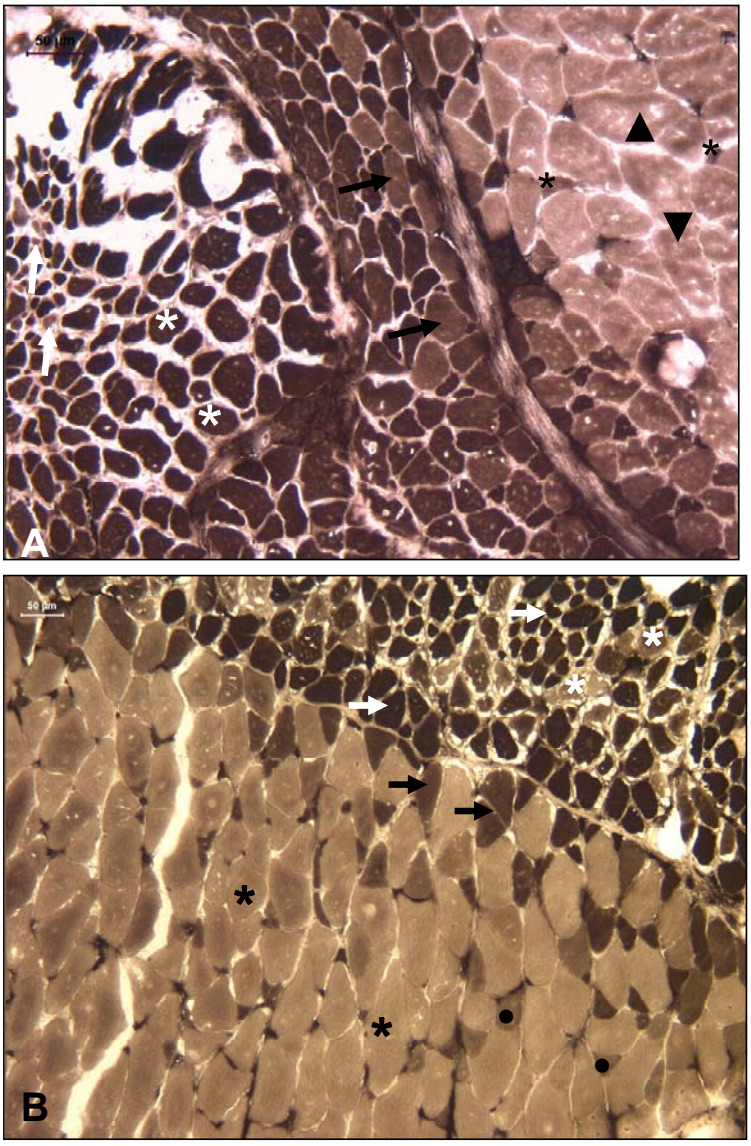

Fig. 2. **A: Tranverse section of myotomal muscle in 60 day juveniles**. Red muscle fibers (white asterisks) with several enzimatic activity and hybrid fibers (white arrows); muscle fibers of the enzimatic activit between red and white fibers that to form the intermediate muscle (black arrows); white fibers in white muscle (arrows-heads); hybrid fibers in white muscle (black asterisks). **B: Transverse section of myotomal muscle in 90 day adults**. Red muscle fibers (white arrows) around hybrid fibers (white asterisks); fibers of the intermediate muscle (black arrows); White fibers (black asterisks) and hybrid fibers (circles). **mATPase, pH 4,6.**

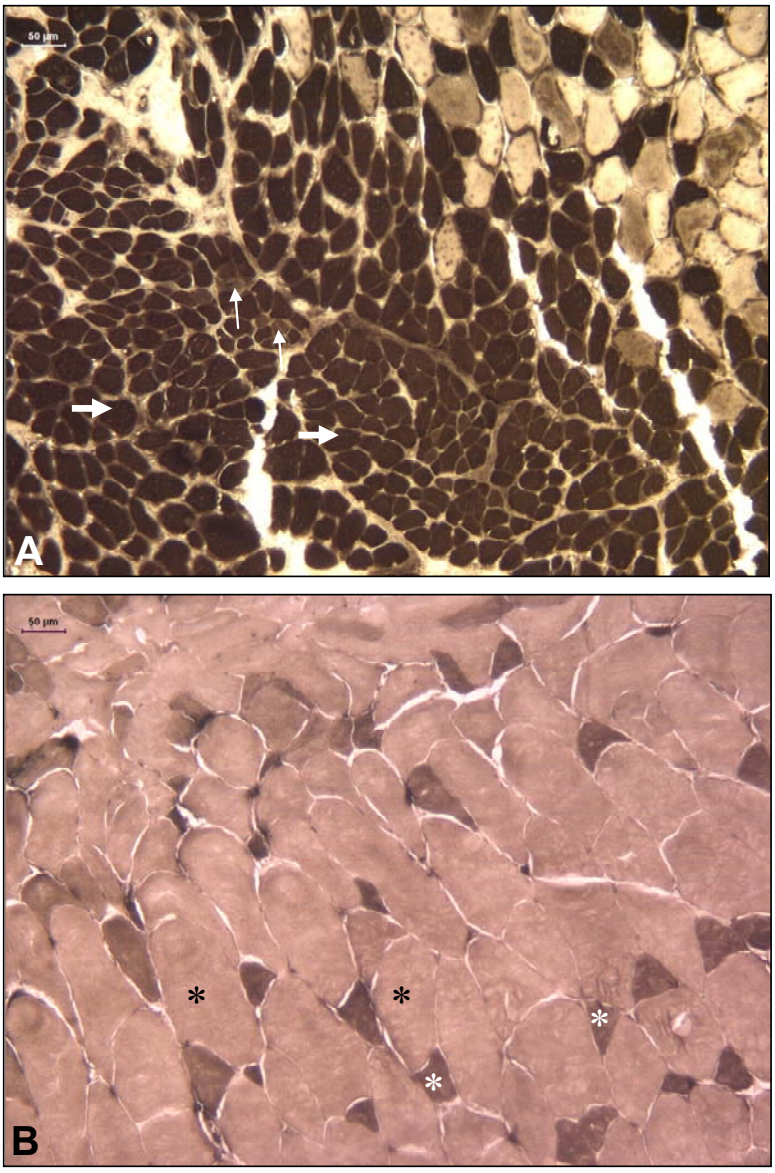

Fig. 3. **Transverse section of myotomal muscle in 190 day adults**. **A:** Red muscle fibers (thick arrows) and hybrid fibers (thin arrows). **B**. White muscle fibers (white asterisks) and hybrid fibers (black asterisks). **mATPase, pH 4,6.**

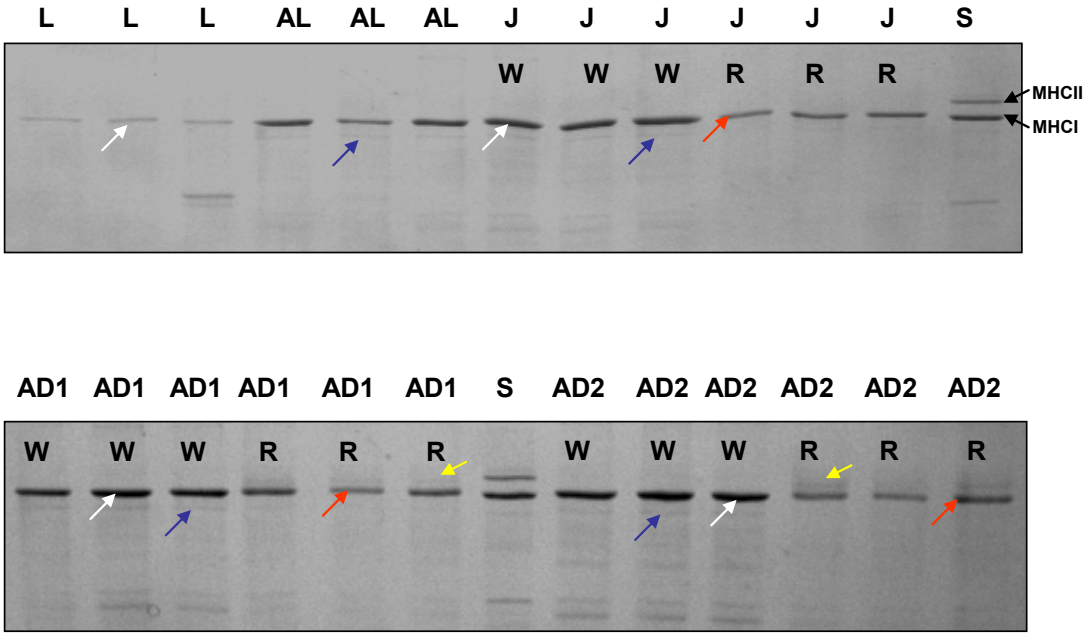

Fig. 4. **Electrophoretic separating of the myosin heavy chain (MHC) in the Nile tilapia**. L, 2 day larvae; AL, 35 day alevins; J, 60 day juveniles; AD1, 90 day adults; AD2, 190 day adults; R, red muscle; W, white muscle; MHC fast (white arrows); MHC slow (red arrows); MHC of hybrid red muscle fibers (yellow arrows); MHC of hybrid white muscle fibers (blue arrows); S, standard: rat soleus muscle with MHC I and MHC II. **SDS-PAGE, gradient of 6 - 10%.**

Discussion

In Nile tilapia, in all stages studied, mATPase reaction showed skeletal muscle divided into superficial compartment with red fibers, and the deep layer with white fibers making up the majority of muscle mass as observed for Stickland (1983).

Red fibers are normally small in diameter, relatively homogeneous in size, present oxidative metabolism and slow contraction activity (Johnston *et al*., 1972, 1974, 1975; Carpene *et al*., 1982). The white fibers are heterogeneous in size and larger than the red fibers with glycolytic metabolism and fast contraction activity (Carpene *et al*., 1982; Johnston, 1982).

The mATPase staining intensities showed variability in muscle fiber type in Nile tilapia, and several histochemical studies have demonstrated that this variation can be related to the change or transition in the myosin heavy chain (MHC) isoform composition that occur during skeletal muscle growth (Staron & Pette, 1986; Scapolo *et al*., 1988).

Fish red and white muscle fibers express MHCs (*slow myosin heavy chain*) and MHCf (*fast myosin heavy chain*) respectively. However different species display variations in activity type and muscle growth mechanisms, in this way, slow and fast muscle fibers can co-express different MHC isoforms (Karasinski & Kilarski, 1989; Radice, 1995; Martinez *et al*., 1991); these can be named hybrid fibers, similar to that described in mammalian (Karasinski & Kilarski, 1989).

In this study, tilapia larvae skeletal muscle showed that MHC proteins in this growth phase had a similar molecular mass to MHC I in rat soleus muscle. As in Nile tilapia larvae white muscle predominates (Dal Pai Silva *et al*., 2003) and in this and in

embryonic/neonatal phases the fibers are expressing fast MHC (Martinez *et al*., 1991; 1993), it shows that larval phase MHC proteins of Nile tilapia has a similar molecular mass to rat MHC I.

Muscle analysis in alevins of Nile tilapia clearly shows red and white muscles in layers. Fiber hypertrophy occurs in this period and stratified hyperplasia is responsible for muscle thickening; the presence of small fibers between large fibers in white muscle, showing that mosaic hyperplasia is also occurring. The mATPase reaction shows that these small fibers have different activity to the large fibers suggesting the expression of a different myosin isoforms, as demonstrated by Rowlerson *et al*., (1985), in skeletal muscle of carp. As these small fibers are young immature fibers, they are probably expressing an embryonic or neonatal myosin (Martinez *et al*., 1991; 1993), and can be called hybrid fibers.

In Nile tilapia, the two populations of fibers, white and hybrid, seen in white muscle using mATPase reaction, could be related to a very strong fast MHC isoform at the same level as the larvae, and immediately below, a weakly colored MHC could correspond to a MHC of hybrid fibers.

In juveniles, 90 and 190-day adult Nile tilapia, histochemical ATPase reaction demonstrated the presence of both pure and hybrid fiber populations in red, intermediate, and white muscle. In this growth stage, electrophoresis in white muscle demonstrates fast MHC isoforms corresponding to the soleus muscle MHC I and below this MHC, a more weakly coloured MHC isoform suggesting the presence of other MHC isoform only in the white muscle. In red muscle, two bans proteins were seen: a very

strong slow MHC isoform at same level of soleus muscle MHC I, and a weakly colored MHC above, corresponding to soleus muscle MHC II. This MHC isoform was not present in the white muscle of the others stages studied and was more evident at 90 and 190 days, as well as, the number of hybrid fibers was higher in these two growth stages, as confirmed by mATPase reaction.

The amount of protein in white muscle was higher in all analyzed periods, this could be related to the structural differences in extracellular matrix morphology. In red muscle, fibers are smaller and the extracellular matrix is more developed with a higher proportion of collagen (Michelin *et al*., 2007). In white muscle, fibers are larger and the quantity of collagen in the extracellular matrix is lower. This supports our results.

Results from this study show that the molecular mass of fast and slow MHC isoforms, in Nile tilapia, is similar to those of rat soleus muscle MHC I isoforms. Hybrid red fibers in red muscle showed MHC with similar molecular mass to rat soleus muscle MHC II and hybrid fibers in white muscle showed MHC that do not correspond to the isoforms described in rat soleus muscle.

The expression of the MHC isoforms in Nile tilapia during growth can be related to the phenotypic plasticity in muscle during growth and can reflects this fish capacity to adapt to changes in environmental conditions which are important for its survival.

Acknowledgments

This work is part of the PhD Thesis that will be presented by DHA to Universidade Estadual de Campinas – UNICAMP. This study was supported by Fapesp, Proc. N° 04/13286-0. Researchers from CEPTA were extremely helpful during planning and execution of this experiment. Colleagues from the Department of Morphology and the Biosciences Institute did valuable work in the laboratory.

References

Akster, H.A., Verreth, J.A.J., Spierts, I.L.Y., Berbner, T., Schmidbauer, M. & Osse, J.M.W. (1995). Muscle growth and swimming in larvae of *Clarias gariepinus* (Burchell). *ICES Marine Science Symposia* 201, 45-50.

Bond, C.E. (1979). Endoskeleton and musculature. In Biology of Fishes (Bond CE ed), pp 61-81. Philadelphia PA: Saunders College Publishing.

Buckingham, M.E. (1985). Actin and myosin multigene families: their expression during the formation of skeletal muscle. *Essays in Biochemistry* 20, 77-109.

Carpene, E., Veggetti, A. & Mascarello, F. (1982). Histochemical fiber types in the lateral muscle of fishes in fresh, brackish and salt water. *J Fish Biol.* 20, 379-396.

Dal Pai Silva, M., Carvalho, R.F. & Dal Pai, V. (2003). Muscle fiber types in tilapia do Nilo (*Oreochromis niloticus*) from larval to adult: histochemical, ultrastructural and morphometric study.; *Tissue and Cell* 291, 1-9.

Dubowitz, V. & Brooke, M. (1973). Muscle Biopsy: A Modern Approach. London: Saunders, pp. 475

Focant, B., Huriaux, F., Vanderwalle, P., Castelli, M. & Goessens, G. (1992). Myosin, parvalbumin and myofibril expression in barbell (*Barbus barbus* L) lateral white during development. *Fish Physiol Biochem.* 10, 133-143.

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

Johnston, I.A., Frearson, N. & Goldspink, G. (1972). Myofibrillar ATPase activities of red and white myotomal muscles of marine fish. *Experientia* 28, 713-714.

Johnston, I.A., Cole, N.J., Vieira, V.L.A. & Davison, I. (1997). Temperature and developmental plasticity of muscle phenotype in herring larvae. J *Exp Biol.* 200, 849-868.

Johnston, I.A., Patterson, S., Ward, P. & Goldspink, G. (1974). The histochemical demonstration of myofibrillar adenosine triphosphate activity in fish muscle. *Canadian Journal of Zoology* 52, 871-887.

Johnston, I.A., Ward, P. & Goldspink, G. (1975). Studies on the swimming musculature of the rainbow trout. I. Fiber types. *J Fish Biol.* 7, 451-458.

Karasinski, J. (1993). Diversity of native myosin and myosin heavy chain in fish skeletal muscles. *Comp. Biochem. Physiol*, 106, 1041-1047.

Karasinski, J. & Kilarski, W. (1989). Polymorphism of myosin isoenzymes and myosin heavy chain in histochemically typed skeletal muscles of the roach. *Comp. Biochem. Physiol.* 92B, 727-731.

Martinez, I., Christiansen, J.S., Ofstad, R. & Olsen, R.L. (1991). Comparison of myosin isoenzymes present in skeletal and cardiac muscles of the Artic charr *Salvelinus alpinus* (L). *Eur J Biochem.* 195, 743-753.

Martinez, I., Bang, B., Hatlen, B. & Blix, P. (1993). Myofibrillar proteins in skeletal muscles of parr, smolt and adult Atlantic salmon (*salmo salar*) – comparison with another salmonid, the artic charr *salvelinus-alpinus*. *Comp Biochem Physiol.* B 106, 1021-1028.

Michelin, A.C., Justulin, L.A., Padovani, C.R., Felisbino, S.L. & Dal Pai Silva, M. (2007) Differential MMP-2 and MMP-9 activity and collagen composition in skeletal muscle in pacu *(Piaractus mesopotamicus)* during juvenile and adult growth phase. *Cell Biol Int* , 2007. uploaded.

Radice, G.P. (1995). Spatial expression of two tadpole stage specific myosin heavy chains in Xenopus laevis. *Acta Anat.* 153, 254-262.

Rowlerson, A., Scapolo, P.A., Mascarello, F., Carpene, E. & Veggetti, A. (1985). Comparative study of myosins present in the lateral muscle of some fish:species variations in myosin isoforms and their distribution in red, pink and white muscle. *J. Muscle Res. Cell Motil*. 6, 601-640.

Sänger, A.M. & Stoiber, W. (2001). Muscle fiber diversity and plasticity. In: Johnston IA. Muscle Development and Growth, San Diego*, Academic Press*,. p.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.

Staron, R.S. & Pette, D., 1986. Correlation between myofibrillar ATPase activity and myosin heavy chain composition in rabbit muscle fibres. *Histochemistry* 86, 19-23.

Stickland, N.C. (1983). Growth and development of muscle fibers in the rainbow trout (*Salmo gairdneri*). *J. Ana.t* 137, 323-333.

Weaver, F.E., Stauffer, K.A. & Coughlin, D.J. (2001). Myosin heavy chain expression in the red, white, and ventricular muscle of juvenile stages of rainbow trout. *J Exp Zool.* 290(7), 751-758.

VIII. CONCLUSÕES

- A expressão de MyoD e migenina nos estágios de alevinos, juvenis e adultos de 90 dias, mostrou que a hiperplasia e a hipertrofia ocorreram como resultado da proliferação e diferenciação dos mioblastos; em contraste, o aumento da expressão de miogenina no estágio de adultos de 190 dias, indicou que a diferenciação celular e a hipertrofia foi mais significativa nesse estágio;

- O músculo vermelho e branco da tilápia do Nilo apresentam fibras puras e híbridas;

- A cadeia pesada da miosina das fibras puras do músculo branco na larva e branco e vermelho nos estágios de juvenis, adultos de 90 dias e adultos de 190 dias, apresentaram massa molecular semelhante à da cadeia pesada da miosina do tipo I do músculo sóleo de rato;

- As fibras híbridas do músculo vermelho, nos estágios de adultos de 90 dias e adultos de 190 dias, mostraram uma isoforma de cadeia pesada da miosina com massa molecular próxima à cadeia pesada da miosina do tipo II do músculo sóleo de rato;

- As fibras híbridas do músculo branco, nos estágios de juvenis, adultos de 90 e adultos de 190 dias mostraram uma isoforma de cadeia pesada da miosina com massa molecular superior à cadeia pesada da miosina do tipo I do músculo sóleo de rato;

- A expressão das diferentes isoformas de cadeias pesadas da miosina no músculo estriado da tilápia durante o crescimento pode estar relacionada com a plasticidade fenotípica que ocorre durante o crescimento muscular e reflete na capacidade desses peixes de se adaptar às variações ambientais, importantes para a sobrevivência.

DECLARAÇÃO

Declaro para os devidos fins que o conteúdo de minha **Tese de Doutorado** intitulada **"EXPRESSÃO DOS FATORES DE REGULAÇÃO MIOGÊNICA E DA CADEIA PESADA DA MIOSINA NO MÚSCULO ESTRIADO ESQUELÉTICO DA TILÁPIA DO NILO (*OREOCHROMIS NILOTICUS*) DURANTE O CRESCIMENTO"**.

( ) não se enquadra no Artigo 1°, § 3° da Informação CCPG 002/06, referente a bioética e biossegurança.

( ) está inserido no Projeto CIBio (Protocolo nº _____), intitulado _____

(X) tem autorização da Comissão de Ética em Experimentação Animal (Protocolo nº 02/08-CEEA).

( ) tem autorização do Comitê de Ética para Pesquisa com Seres Humanos (?) (Protocolo nº _____).

Danilo Henrique Aguiar
- Aluno -

Profa. Dra. Maeli Dal Pai Silva
- Orientadora -

Para uso da Comissão ou Comitê pertinente:

(X) Deferido ( ) Indeferido

Nome:
Função: Profa. Dra. ANA MARIA A. GUARALDO
Presidente
Comissão de Ética na Experimentação Animal
CEEA/IB - UNICAMP

unesp UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Campus de Botucatu

CERTIFICADO

Certificamos que o Protocolo nº **02/08-CEEA**, sobre "Expressão dos Fatores de Regulação Miogênica (MRF) e da Miosina de Cadeia Pesada (MHC) no músculo estriado esquelético da tilápia do Nilo (*Oreochromis niloticus*) durante o crescimento", sob a responsabilidade de **MAELI DAL PAI SILVA**, está de acordo com os Princípios Éticos na Experimentação Animal adotado pelo Colégio Brasileiro de Experimentação Animal (COBEA) e foi aprovado pela ***COMISSÃO DE ÉTICA NA EXPERIMENTAÇÃO ANIMAL*** (CEEA), em reunião de **04/03/2008**.

Botucatu, 4 de março de 2008.

Prof. Dr. MARCELO RAZERA BARUFFI
Presidente - CEEA

NADIA JOVÊNCIO COTRIM
Secretária – CEEA

Instituto de Biociências – Diretoria Técnica Acadêmica
Distrito de Rubião Júnior s/n CEP 18618-000 Botucatu SP Brasil
Tel 14 3811 6013/6014 fax 14 3815 3744 e-mail: dta@ibb.unesp.br