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ANÁLISE GENÉTICA DE CARACTERÍSTICAS DE CRESCIMENTO DO *Colossoma macropomum* E IDENTIFICAÇÃO DE NOVOS PROMOTORES DE CRESCIMENTO MUSCULAR UTILIZANDO COMO ESPÉCIE MODELO *Oncorhynchus mykiss*

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“À minha família, pelo apoio incondicional em todos os momentos, principalmente nos de incerteza, muito comuns para quem tenta trilhar novos caminhos”.

Dedico

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“Nós somos aquilo que fazemos repetidamente. Excelência, então,
não é um modo de agir, mas um hábito”.
(*Aristóteles*)

[1]IDENTIFICAÇÃO DE NOVOS REGULADORES DO CRESCIMENTO MUSCULAR UTILIZANDO A ESPÉCIE MODELO *Oncorhynchus mykiss* APLICADO AO MELHORAMENTO DO DESEMPENHO ZOOTÉCNICO EM *Colossoma macropomum*

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Orientador: Danilo Pedro Streit Júnior
Co-orientador: Ricardo Pereira Ribeiro
Orientador no exterior: Jean-Charles Gabillard

RESUMO
Objetivou-se com este trabalho (1) estimar os parâmetros genéticos das características de desempenho e morfométricas em duas fases de cultivo, 12 e 24 meses de idade; (2) estimar o padrão de crescimento entre fêmeas e machos em tambaqui utilizando o modelo de regressão não linear de Gompertz; (3) determinar o crescimento muscular em tambaqui na fase juvenil por hiperplasia e/ou hipertrofia das fibras musculares; (4) caracterizar a expressão de genes envolvidos com o crescimento muscular em peixes na espécie modelo *Oncorhynchus mykiss* utilizando sequências nucleotídicas e proteicas específicas; e (5) caracterizar a regulação dos promotores de crescimento na miogênese *in vitro* e *in vivo* na espécie modelo *O. mykiss* e como estes são regulados pelos fatores de crescimento conhecidos, visando uma transferência de tecnologia para o melhoramento do tambaqui. As herdabilidades (h^2) estimadas para os caracteres morfométricos e de desempenho foram valores de moderados a alta magnitude, variando 0,17-0,49 aos 12 e 24 meses. A análise do crescimento corporal indicou diferenças no crescimento entre sexos, sendo ajustadas curvas diferentes para machos e fêmeas para peso corporal (BW), altura (BH) e comprimento da cabeça (LH). No entanto, não foram observadas diferenças entre machos e fêmeas no peso de 2 kg, idade comercial de abate (516 dias), o que indica que ambos os sexos podem ser utilizado para fins de piscicultura. Neste estudo, foram identificadas 13 novas sequências de genes envolvidos com o crescimento muscular em peixes específicas para *O. mykiss* depositadas no banco de dados Genbank. As novas sequências sao conservadas, mas o padrão de expressão é diferente, sugerindo funções não redundantes na miogênese *in vivo*. Os resultados aqui encontrados demonstram que é possível obter um melhor desempenho dos animais através da seleção para as características morfométricas e de desempenho, que apresentam a vantagem de fácil mensuração.

Palavras-chave: características morfométricas, dimorfismo sexual, ganho em peso, hipertrofia, miogênese, parâmetros genéticos

[1]Tese de doutorado em Zootecnia- Produção Animal, Faculdade de Agronomia, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brasil. Dezembro, 2013.

[1]IDENTIFICATION OF NEW MUSCLE GROWTH REGULATORS BY THE MODEL SPECIES *Oncorhynchus mykiss* APPLIED TO GENETIC IMPROVEMENT OF TRAITS PERFORMANCES IN *Colossoma macropomum*

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ABSTRACT

The objective of this study was (1) to estimate genetic parameters of performance traits and morphometric in two cultivation phases, 12 and 24 months of age, (2) to estimate the growth curves between males and females in tambaqui using the model Gompertz nonlinear regression, (3) to determine muscle growth in the juvenile stage tambaqui by hyperplasia and / or hypertrophy of muscle fibers, (4) to characterize the expression of genes involved in muscle growth in model species *Oncorhynchus mykiss* using sequences nucleotide and protein specific , and (5) to characterize the regulation of growth promoters on myogenesis *in vitro* and *in vivo* in model species *O. mykiss* and how these are regulated by known growth factors, aiming at technology transfer to improve the tambaqui. The heritability (h^2) estimates for the morphometric traits and performance values were moderate to high magnitude, ranging from 0.17 to 0.49 at 12 and 24 months. The analysis of body growth indicated differences in growth between sexes, adjusted curves different for males and females for body weight (BW), height (BH) and head length (LH). However, no differences between males and females were observed in the 2 kg weight, age commercial slaughter (516 days) , indicating that both sexes can be used for fish farming. In this study, 13 new sequences were identified genes involved with muscle growth in fish specific to *O. mikyss* deposited in the GenBank database. The new sequences are conserved, but the expression pattern is different, suggesting non-redundant roles in myogenesis *in vivo*. The results showed that it is possible to obtain a better performance of animals by selection for morphometric and performance traits, which have the advantage of easy measurement.

Keywords: hypertrophy, genetic parameters, morphometric traits, myogenesis, sexual dimorphism, weight gain

[1]Doctoral Thesis in Animal Science, Faculdade de Agronomia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil. December, 2013.

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h^2	Herdabilidade
BW	Body weight Peso corpóreo
DWG	Daily weight gain Ganho de peso diário
BH	Body height Altura do corpo
BWi	Body width Largura do corpo
BL	Body legth Comprimento do corpo
HL	Head leght Comprimento de cabeça
TGF- β	Transforming growth factor-β
Inh βA	Inhibin subunit βA
Fst	Fosllistatin

CAPÍTULO I

INTRODUÇÃO

O tambaqui (*Colossoma macropomum*) é um peixe de grande porte, nativo da bacia amazônica que apresentou um crescimento exponencial na sua produção nos últimos cinco anos. As possibilidades de aumento na produtividade desta espécie são grandes, principalmente porque a produção comercial desta espécie utiliza animais selvagens. A partir da iniciativa de implantação recente do Programa de Melhoramento Genético do Tambaqui (PMGT) dentro do projeto Aquabrasil - Bases tecnológicas para o desenvolvimento sustentável da aquicultura no Brasil as perspectivas de ganhos na produção desta espécie aumentaram.

O crescimento é caracterizado pelo aumento de peso, comprimento, altura e circunferência do corpo, dentre outras características, em função da idade. No entanto, não estão disponíveis na literatura informações sobre estimações do crescimento corporal, diferença de desempenho entre machos e fêmeas ou estimações de parâmetros genéticos para as características de interesse econômico. A falta destas informações é uma barreira a ser ultrapassada para se atingir o melhoramento genético para esta espécie.

Abordagens a nível molecular sobre as características de interesse econômico também são difíceis de serem realizadas devido a falta de dados de tal natureza. Neste sentido, o uso de espécies modelos para desenvolvimento de tecnologias de ponta aplicáveis ao melhoramento genético do tambaqui é justificável devido a possibilidade de transferência de tecnologia.

Assim, a geração de informações a cerca do desenvolvimento corpóreo, deposição de tecido muscular e ganho em peso, bem como o conhecimento da porção herdável destas características, são imprescindíveis para o sucesso do programa de melhoramento do tambaqui. Todas estas variáveis do desenvolvimento implicam em mudanças na conformação corporal e das funções fisiológicas regidas por diferentes vias metabólicas que envolvem diferentes grupos de genes. A compreensão destas mudanças de forma ampla assume posição estratégica em uma produção mais eficiente.

O músculo esquelético constitui a parte comestível do peixe. Estudos de crescimento muscular são, por conseguinte, importante para o desenvolvimento da criação de peixe. Programas de melhoramento genético e avanços na compreensão das necessidades nutricionais conseguiram aumentar drasticamente as taxas de crescimento muscular em peixes. Em tilápia do Nilo (*Oreochromis niloticus*), por exemplo, o programa de melhoramento genético alcançou um crescimento de 18% sobre a geração anterior em quatro anos de acasalamento. Houve uma redução de 21 dias no cultivo representando uma economia de 11% no custo total de produção (Santos et al., 2007). Em sistemas de produção da carne, características de desempenho, como por exemplo, peso corporal e ganho de peso diário são importantes critérios de seleção a ser utilizados para melhorar as características de interesse econômico.

O crescimento muscular é dependente da divisão de uma população de células estaminais, embora a origem e natureza destas células são ainda mal compreendidas. Crescimento do músculo pós-embrionário envolve a

hipertrofia das fibras embrionárias e a produção de novas fibras. Assim, ao contrário de aves e mamíferos, o número de fibras continua a aumentar na maior parte da vida em peixes. Neste sentido, o conhecimento dos fatores que determinam o crescimento e o desenvolvimento dos tecidos e do organismo como um todo é fundamental para a adequação de programas de melhoramento, de manejo nutricional, ambiente, definição da idade de abate, entre outros para alterar a quantidade e a qualidade da carne produzida. O conhecimento da complexa sinalização da proliferação e diferenciação celular é muito importante para a manipulação do crescimento, para se obter melhores índices produtivos e maior quantidade de carne de qualidade na carcaça.

Este trabalho teve por objetivos estudar os aspectos relacionados ao crescimento corpóreo e muscular em *Colossoma macropomum* por meio da estimação dos parâmetros genéticos das características morfométricas e de desempenho; do estudo de curva de crescimento; e análise de expressão de genes envolvidos no desenvolvimento muscular, proliferação e diferenciação celular, em outros vertebrados por meio da espécie modelo *Oncorhynchus mykiss*.

REVISÃO BIBLIOGRÁFICA

Produção aquícola

O melhoramento genético animal vem sendo impulsionado pela crescente competitividade das atividades de produção e pela importância, cada vez maior, da qualidade do produto e da eficiência da produção nas mais diferentes espécies exploradas comercialmente. A aplicação da tecnologia de melhoramento genético em espécies de animais aquáticos tem sido menor em comparação às espécies terrestres, mas existe um grande potencial para sua expansão vinculado ao aumento da demanda mundial por alimentos. Desta forma, justificam-se o planejamento, desenho e implantação de pesquisa, desenvolvimento e de programas de melhoramento em espécies aquáticas.

Considerando-se o potencial de cultivo de espécies nativas, o tambaqui (*Colossoma macropomum*) é a espécie nativa mais cultivada no Brasil com uma produção de 54.313,1 toneladas em 2010 e um crescimento de 39% de 2008 a 2010 (Ministério da Pesca e Aquicultura, 2013). Sua produção é realizada principalmente em viveiros escavados no sistema semi-intensivo. Esta espécie é nativa da bacia Amazônica e Orinoco, apresenta um hábito alimentar onívoro (Goulding and Carvalho, 1982), alimentando-se de zooplâncton, sementes e frutos. Sua criação tem sido impulsionada principalmente pelo fato desta espécie apresentar alto valor comercial, crescimento rápido principalmente durante a fase jovem (Melo et al., 2001), adaptação fisiológicas e anatômicas aos ambientes com baixa concentração de oxigênio e pode ser cultivado em altas densidades (Chagas et al., 2007).

Entretanto, a produção de tambaqui atual trabalha com o potencial genético silvestre, sem nenhum melhoramento. Desta forma, a espécie está incluida no projeto “Melhoramento de espécies aqüícolas no Brasil”, componente da Rede Aquabrasil, pertencente à programação atual de pesquisa da Embrapa. Este projeto visa o desenvolvimento um programa nacional de reprodução seletiva para quatro espécies aquáticas: camarão marinho do Pacífico (*Litopenaeus vannamei*), a tilápia do Nilo (*Oreochromis niloticus*), o tambaqui (*Colossoma macropomum*) e a cachara (*Pseudoplatystoma reticulatum*); buscando implementar estratégias para a disseminação dos indivíduos de alto desempenho a aqüicultores.

Melhoramento Genético do Tambaqui (*Colossoma macropomum*)

O tambaqui é a espécie endêmica mais produzida no Brasil (Ministério da Pesca e Aquicultura, 2013) (Figura 1). Originária de águas tropicais apresenta comportamento reprodutivo migratório com alta prolificidade (Viveiros and Godinho, 2009). O excelente sabor da carne, ótima aceitação no mercado e o hábito alimentar onívoro fazem com que a demanda atual desta espécie seja exponencial. Em decorrência, os estoques naturais de tambaqui encontram-se praticamente esgotados devido à pesca extrativista não planejada e o impacto ambiental gerado pela destruição da mata ciliar e dos locais de recria natural (berçários).

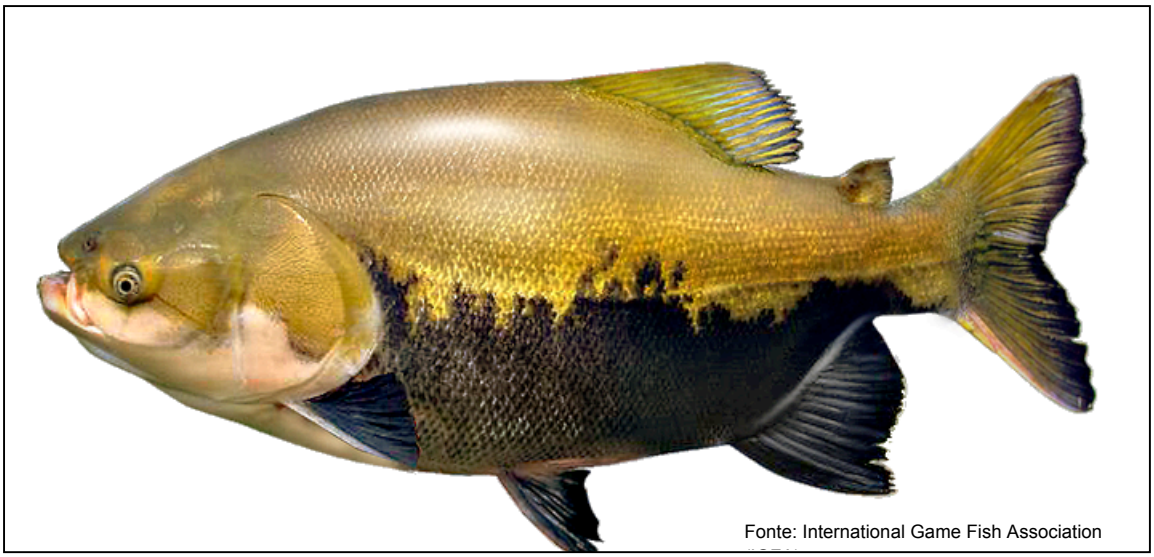

Figura 1. Tambaqui (*Colossoma macropomum*).

Atualmente, o sistema de seleção realizado no sistema de cultivo de tambaqui é, quase exclusivamente, por meio de seleção fenotípica, como por exemplo, o ganho em peso à despesca. O processo de seleção desconsiderando características correlacionadas e grau de parentesco entre os animais pode comprometer a produtividade do plantel (Croquet et al., 2006; Falconer and Mackay, 1996). Assim, selecionar animais sem controle de pedigree e avaliação genética caracteriza um sistema de produção baseado no uso de espécies e linhagens não-melhoradas, ou melhoradas por seleção massal (por meio do fenótipo). A não discriminação de acasalamentos endogâmico pode levar ao uso de animais com potencial produtivo menor ou igual aos animais disponíveis no ambiente natural (Lind et al., 2012).

Neste cenário atual, a aquicultura tem posição estratégica para suprir um mercado consumidor mais exigente e crescente na demanda pela produção desta espécie. Entretanto, a produção em larga escala de qualquer organismo para consumo humano somente será eficiente se o recurso for facilmente renovável. Desta forma, um programa de melhoramento genético vem de encontro às necessidades do mercado consumidor, uniformizando a produção da espécie em cativeiro, bem como possibilita o reestabelecimento das populações selvagens.

Critérios de seleção

A implantação e desenvolvimento de programas de seleção que conduzam a ganhos genéticos expressivos e duradouros devem atender a critérios específicos para determinar quais são os objetivos de seleção e a forma como estes objetivos serão alcançados.

A definição dos critérios de seleção, ou seja, eleger as características que serão usadas para alcançar o objetivo de seleção, é essencial para o delineamento do programa de melhoramento genético (Thompson, 2008). Estas características devem ser de fácil mensuração, apresentar resposta à seleção e estar relacionada com o objetivo de seleção, o qual foi estabelecido com base na relevância econômica. Entretanto, até o

momento, não existem estimativas de componentes de (co)variância e parâmetros genéticos disponíveis para as características de crescimento e desempenho em tambaqui. Desta forma, não é possível inferir sobre a resposta a seleção ou correlações entre as principais características nesta espécie, sendo difícil o delineamento do sistema de avaliação genética ou estimação do mérito genético dos animais.

Uma das ferramentas para promover o melhoramento genético é a seleção genética e o cruzamento de indivíduos geneticamente superiores para determinadas características, causando alterações nas frequências de alelos envolvidos na expressão das características, aumentando a frequência dos alelos favoráveis e reduzindo a participação dos alelos desfavoráveis. A identificação dos animais que devem ter prioridade de acasalamento, contribuindo de forma desproporcional com descendentes para as próximas gerações, depende da determinação correta dos critérios de seleção.

A realização de medições de características morfométricas, a estimação dos componentes de (co)variância e parâmetros genéticos destas características podem fornecer informações que permitam a identificação de critérios de seleção adequados para selecionar animais com superioridade genética.

Melhoramento genético da velocidade para ganho em peso e aumento da massa muscular

À medida que se intensificam os sistemas de produção e que aumenta a demanda por eficiência, maior é a necessidade de se ter programas de melhoramento genéticos bem estruturados. Como uma das principais premissas para alcançar sucesso, o programa de melhoramento genético de qualquer espécie animal deve ter objetivos e metas bem definidos, que sejam coerentes com a estrutura de mercado vigente e, certamente, condizentes com as condições ambientais existentes.

O objetivo do Programa de Melhoramento Genético do Tambaqui (PMGT) é melhorar a taxa de crescimento corporal, ou seja, animais com uma velocidade de ganho de peso e da massa muscular maior. O crescimento muscular em peixes ocorre de forma ininterrupta, durante toda a sua vida, sendo que músculo esquelético constitui a maior parte comestível do peixe (Silva et al., 2009). Estudos de crescimento muscular são, por conseguinte, importantes para o desenvolvimento do melhoramento de espécies de peixes.

A seleção genética visando aumentar a velocidade de ganho em peso proporciona um aumento no número e no tamanho das fibras musculares. Esta seleção tem influência no processo de proliferação dos mioblastos e na taxa de proliferação das células satélites musculares. Assim, é importe compreender os processos pelos quais um animal apresenta maior quantidade de músculo esquelético. Para isso, é necessário entender, de forma mais aprofundada, o processo de formação das fibras musculares, bem como o processo de formação de novas fibras, que em peixes é indeterminado. A via complexa de sinalizações celulares que estimulam as *stem cells* musculares, denominadas células satélites, a se proliferarem e diferenciarem-se para a formação de novas fibras permanece pouco entendido (Gabillard et al., 2013, 2010).

Precursores musculares

O desenvolvimento embrionário nos vertebrados é o conjunto de transformações que decorrem desde a fecundação, a partir de um rearranjo das células em simultâneo com a divisão celular do período de gastrulação. O músculo é formado a partir da diferenciação da mesoderme (segundo folheto embrionário) em mesoderme paraxial, intermediária e placa lateral (Figura 2).

Somito é uma estrutura epitelial transitória que se formam nas primeiras etapas do desenvolvimento embrionário dos vertebrados. A sua formação é regulada no espaço e no tempo, pois é fundamental para a correta formação da coluna vertebral, dos músculos esqueléticos do corpo e da organização segmentar do sistema nervoso periférico (Moore and Persaud, 2000). O somito é formado pela divisão do mesoderma paraxial formado pela transição epitélio mesênquimal realizada pelo epiblasto (posterior ectoderme).

Em peixes, como em outros vertebrados, o músculo esquelético do tronco e cauda deriva de um compartimento embrionário específico, o miótomo. Somitos se condensam a partir mesoderme imediatamente adjacente ao eixo central do corpo, a chamada mesoderme paraxial, e num segmento rostral estereotípica à progressão caudal. Estudos em zebrafish demonstraram que mesoderme paraxial deriva de uma região específica do embrião identificável apenas antes da gastrulação (Kimmel et al., 1990). Estas células sofrem uma série complexa de movimentos celulares antes de se iniciar a programação miogênica que é sinalizada pelo inicio da expressão de famílias miogênicas de fatores de transcrição.

O processo no qual as células, espalhadas por todo o hemisfério do embrião de zebrafish, estão no mesoderme paraxial e da placa lateral, envolve um conjunto de movimentos celulares denominados convergência e extensão. Este processo é essencial para a geração do limite entre populações axiais paraxiais e, consequentemente, a especificação apropriada das células musculares. Uma vez que as células foram atribuídas a mesoderme paraxial ou placa lateral, a próxima fase de especificação de músculo derivado da placa lateral se inicia. As células tornam-se comprometida com o destino miogênese notavelmente precoce nos embriões de peixe, o início da expressão do fator de transcrição de regulação miogênica indica a restrição das linhagens musculares no final da gastrulação.

O processo de miogênese

A miogênese é o processo de formação do tecido muscular em vertebrados e é um mecanismo plástico que envolve um conjunto de células precursoras miogênicas chamadas de mioblastos (Figura 3) (Johnston, 1999). Os mioblastos são células indiferenciadas presentes no miótomo que vão se especificar em miócitos e dar origem às fibras musculares. Durante o desenvolvimento muscular, uma população de mioblastos não se diferencia, permanecendo associada à periferia da fibra muscular, entre a lâmina basal e o sarcolema, como células satélites musculares quiescentes (Schultz and McCormick, 1994). As células satélites possuem grande atividade mitogênica e a sua progênie pode se fundir formando novas fibras musculares, ou mesmo se unir às fibras musculares já existentes. Dessa forma, elas contribuem para o crescimento, reparo e manutenção do músculo esquelético (Hawke and Garry,

2001). A forma ativa das células satélites, assim como as células precursoras musculares durante as fases iniciais de desenvolvimento é denominada de mioblasto (Rantanen et al., 1995).

Em um segundo estágio do desenvolvimento, os mioblastos fundem-se entre si formando os miotubos. Aparentemente, após os mioblastos serem incorporados pelos miotubos, os núcleos perdem a capacidade de se dividir. Os fatores regulatórios da miogênese são proteínas que funcionam primariamente como ativadoras da transcrição, se ligando ao DNA através de sítios específicos conhecidos como E-box, onde controlam os eventos da miogênese.

Diferentes fatores de regulação miogênica (myogenic regulatory factors (MRFs)) foram identificados em peixes até o momento, dentre eles, existem quatro fatores mais claramente entendidos: MyoD (Rudnicki and Jaenisch, 1995), Miogenina (Hasty et al., 1993), Myf-5 e a MRF4 (Kassar-Duchossoy et al., 2004), que compreende um sub-grupo dentro da hélice-volta-hélice (bHLH) superfamília de proteínas básicas. Entretanto, existem muitos outros fatores envolvidos na miogênese já identificados, mas que necessitam ser mais bem avaliados para entendermos a participação dos mesmos neste processo de sinalização celular complexo.

Os fatores de transcrição *paired-box* 3 (PAX3) e PAX7 têm papéis essenciais e sobreposição na miogênese. PAX3 age para especificar precursores musculares embrionárias, enquanto PAX7 reforça a programação miogênica das células satélites, mantendo o estado indiferenciado. Experiências recentes têm sugerido que PAX7 é dispensável em células satélite em animais adultos (Figure 3) (Wang and Rudnicki, 2012). O entendimento do modo de ação destes fatores pode levar a manipulação do processo de miogênese.

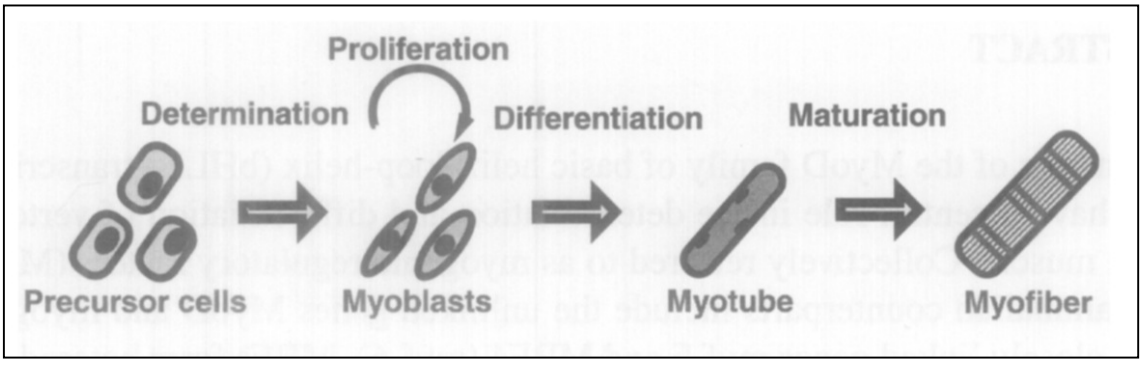

Figura 2. Determinação da linhagem miogênica e diferenciação celular até a maturação múscular (Adaptado de Johnston, 1999).

Crescimento muscular

A massa muscular produzida por um animal e a velocidade de crescimento depende do número de fibras musculares (hiperplasia) que compõe um músculo e do diâmetro de cada fibras (hipertrofia).A hipertrofia das fibras musculares necessita de uma fonte adicional de núcleos que são proporcionados por um conjunto de células indiferenciadas localizadas abaixo da lâmina basal, as células satélites (Moss and Leblond, 1971). O crescimento

muscular em peixes envolve a formação de novas fibras musculares além da hiperplasia das fibras (Johnston et al., 1998; Koumans et al., 1993; Stickland, 1983). A expressão continuada de membros de fatores de regulação miogênicos em juvenis de carpa pode refletir na ativação de células miogênicas. Essas células constituem uma proporção decrescente do número total de núcleos muscular durante as fases larval e juvenil como o crescimento prossegue (Johnston et al., 1998; Koumans et al., 1993).

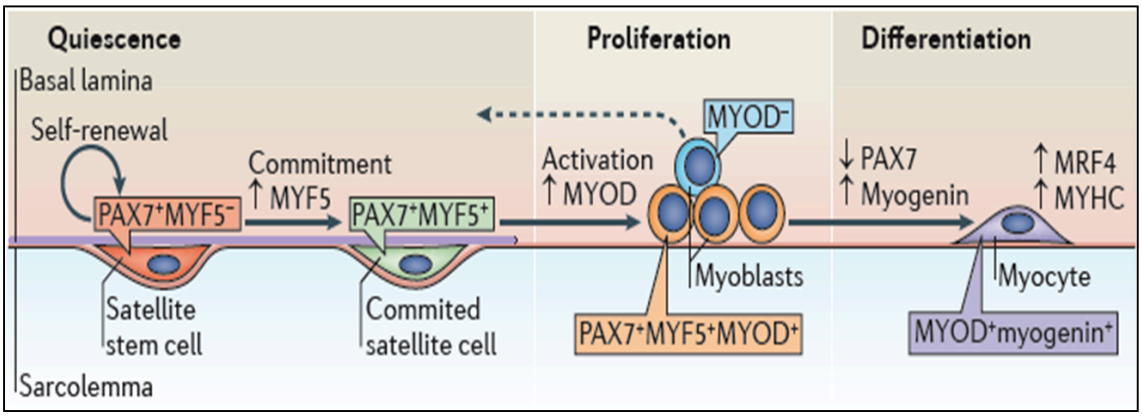

Figura 3. Esquema simplificado contendo fatores de regulação miogênica identificados até o momento (Adaptado de Wang and Rudnicki, 2012).

O músculo é um tecido pós-mitótico onde o crescimento pós-embrionário envolve uma população de mioblastos indiferenciadas (Figura 4) (Johnston, 1999). Quando as fibras se expandem, elas absorvem núcleos, a fim de manter uma relativamente constante proporção nuclear : citoplasma (Koumans et al., 1994).

Em peixes, novas fibras se formam sobre a superfície de fibras existentes através da fusão de mioblastos em miotubos multinucleados. A decisão de mioblastos para segurar no ciclo celular ou a diferenciação é determinada através de sinais de rejeição à proliferação e diferenciação, que não são completamente compreendidos em mamíferos e pouco estudados em tudo em peixes. Os membros da família do gene MyoD são considerados ativadores de diferenciação muscular e inibem a proliferação das células produtoras de mioblastos (Rescan and Gauvry, 1996). No entanto, estes fatores de transcrição são componentes de um sistema de regulação altamente redundantes e mal compreendidos na modulação do crescimento muscular.

O uso da truta arco-íris (*Oncorhynchus mykiss*) como espécie modelo

A truta arco-íris (*Oncorhynchus mykiss*) que deve o seu nome às numerosas manchas coloridas da sua pele, é uma das principais espécies cultivadas em água doce no mundo (Figura 5). É um salmonídeo nativo das águas frias da costa do Pacífico dos Estados Unidos, desde o rio Kuskokwim no Alasca até o rio Santa Domingo no México (Page et al., 1991). Entretanto, esta espécie foi amplamente introduzida nas águas frias da América do Norte e no resto do mundo, sendo diversos os relatos o impacto ecológico negativo

após a introdução (Froese, R. and D. Pauly., 2013). A truta arco-íris é um peixe muito resistente, que tolera ambientes muito diversos, bem como o manuseamento. O crescimento e a maturação são igualmente influenciados pela temperatura da água, bem como pela alimentação. Em condições normais, a truta arco-íris atinge a maturidade aos 3-4 anos. As trutas arco-íris são carnívoras, pelo que necessitam de uma dieta alimentar rica em proteínas.

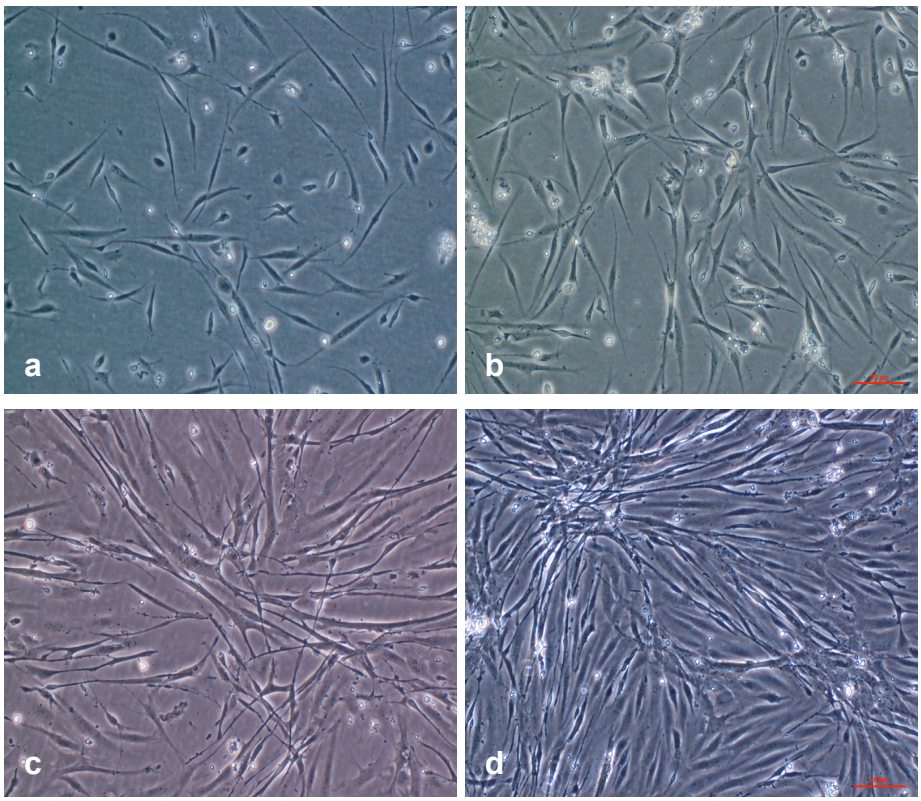

Figura 4. A diferenciação de células satélites extraídos de músculo esquelético branco de *Oncorhynchus mykiss* juvenis (20-30 g) e cultivadas em substratos de laminina em meio de cultura F10 10% / DMEM 2% a 15 ° C durante (**a**) 1 e (**b**) 3 dia de proliferação, (**c**) 4 e (**d**) 6 dia de diferenciação celular.

Produção e comércio

Em 2009, os principais países produtores a nível mundial são a União Europeia (UE), Estados Unidos, Chile, Noruega e Turquia (Figura 6). Atualmente, a quase totalidade das trutas arco-íris comercializadas na União Européia provêm da aquicultura, sendo, na sua maior parte, produzidas localmente. Os principais países produtores na UE são a Itália, França, Dinamarca, Alemanha e Espanha. No Brasil, os principais produtores estão na Serra da Mantiqueira, englobando os estados de São Paulo, Rio de Janeiro e Espírito Santo, e Santa Catarina. A produção de truta é pouco expressiva, sendo produzidos 500 toneladas/ano (Ministério da Pesca e Aquicultura, 2013).

Devido ao grande mercado consumidor e, consequentemente, a grande importância econômica desta espécie, um grande aporte para o desenvolvimento de biotecnologias para uma produção mais eficiente tem sido fortemente financiado por países como Canadá, Estados Unidos, Chile e

França. Desta forma, a truta arco-íris tornou-se um importante modelo experimental para estudos em reprodução, fisiologia, genética e criobiologia.

Muitos estudos têm sido realizados para entender a complexa sinalização e regulação celular do crescimento muscular a fim de se encontrar meios para uma manipulação do mesmo. Recentemente, uma grande oportunidade de avanço científico foi obtida a partir da corrida para o sequenciamento do genoma da espécie entre França, Chile e o consórcio Canadá-Estados Unidos. Novas sequências nucleotídicas e proteicas foram disponibilizadas permitindo inferência espécie-específica, aumentando ainda mais o potencial desta espécie como um modelo para as diferentes espécies de peixes economicamente importantes.

Figura 5. Truta arco-íris (*Oncorhynchus mykiss*). Apresenta o corpo acastanhado ou amarelado, com pintas pretas na zona do dorso, também presentes nas barbatanas dorsal e caudal. Como característica distintiva tem uma risca rosada que se prolonga das guelras à barbatana caudal.

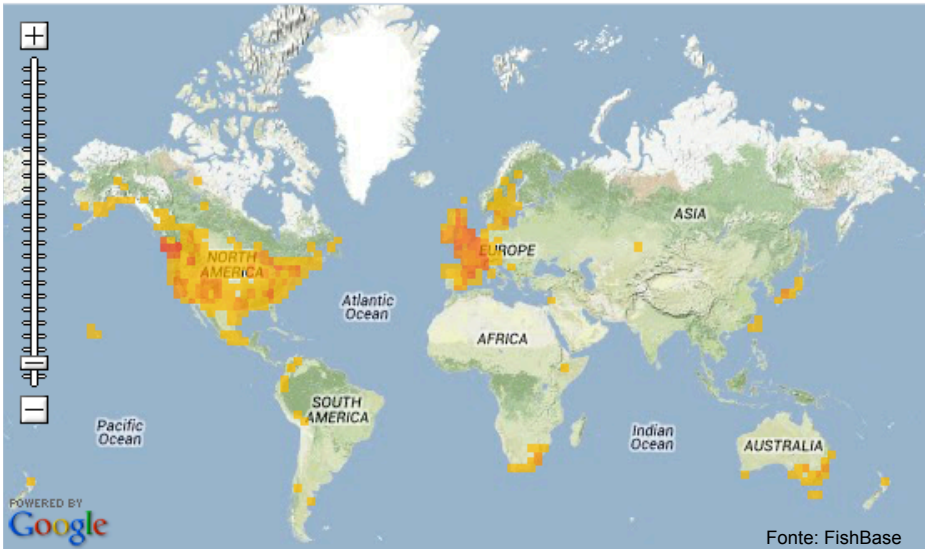

Figura 6. Principais produtores de truta arco-íris (*Oncorhynchus mykiss*) no mundo (Cores laranja e amarelo).

HIPÓTESES E OBJETIVOS

Objetivou-se com o presente trabalho avaliar o crescimento corpóreo e a performance da espécie *Colossoma macropomum*. Para isso, avaliou-se a porção herdável das características e a existência de diferenças no crescimentoentre machos e fêmeas. Buscou-se compreender a sinalização do crescimento muscular por meio análises de expressão de genes promotores de crescimento no contexto fisiológico do crescimento muscular *in vivo* e *in vitro* utilizando a espécie modelo *Oncorhynchus mykiss,* visando a transferência de tecnologia para o programa de melhoramento do tambaqui.

Hipóteses

a)Grande parte da performance observada nos animais é de origem genética, sendo possível o melhoramento da velocidade do ganho em peso por meio da seleção utilizando as características morfométricas, peso do corpo e ganho em peso diário como critérios de seleção;

b)Existem diferenças de crescimento corporal e na performance entre machos e fêmeas em tambaqui, podendo ser mais efetivo o uso de um dos sexos no cultivo comercial;

c)O crescimento corpóreo e o aumento do peso observado nos animais é o resultado do aumento no número de fibras musculares (hipertrofia) e\ou no diâmetro das fibras musculares existentes (hiperplasia);

d)Existe diferença no perfil de expressão dos genes envolvidos no crescimento muscular, proliferação e diferenciação celular, nos diferentes tecidos do corpo, permitindo identificar\validar promotores do crescimento em *Oncorhynchus mykiss*;

e) Os genes com expressão diferencial durante a miogênese participam da complexa via de sinalização do crescimento muscular, ou seja, regulam a profileraçao e\ou diferenciação e são regulados pelos fatores de crescimento.

Objetivos

a)Estimar os parâmetros genéticos para peso do corpo, ganho de peso diário e para as características morfométricas;

b)Estimar o padrão de crescimento corpóreo entre fêmeas e machos de tambaqui utilizando o modelo de regressão não linear de Gompertz;

c)Determinar o crescimento muscular em tambaqui na fase juvenil por meio da avaliação de hiperplasia e/ou hipertrofia das fibras musculares;

d)Caracterizar a expressão de genes envolvidos com o crescimento muscular em mamíferos e em peixes na espécie modelo *Oncorhynchus mykiss* utilizando sequências nucleotídicas e proteicas específicas*;*

e)Caracterizar a regulação dos promotores de crescimento na miogênese *in vitro* e *in vivo* na espécie modelo *Oncorhynchus mykiss* e como estes são regulados pelos fatores de crescimento conhecidos.

[1]CAPÍTULO II

[1] Artigo redigido de acordo com as normas do periódico *Journal of Fish Biology*

[2] Artigo redigido de acordo com as normas do periódico *Aquaculture*

Estimation of the genetic parameters for body weight and morphometric characteristics in tambaqui Colossoma macropomum

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Abstract

Heritability estimated for the morphometric and performance characters in *Colossoma macropomum* were moderate to high values, ranging from 0.17-0.49 at 12 and 24 months. The highest values of heritability were for the characteristics daily weight gain and body weight, 0.49 and 0.44 respectively, indicating that the majority of the observed variations have a genetic origin, which largely will be passed to their progeny. These findings demonstrate that can be possible to achieve improved animal performance through the use these characteristics as selection criteria in the population, such as body weight, of obvious benefit to farmers.

Keywords: Aquaculture; Bayesian inference; common family environment effect, genetic improvement; performance characteristic; heritability

To satisfy increasing demands for fish as food is essential to develop strategies that maximize productivity per area, reducing cost and increasing profitability (Lind et al., 2012). The genetic improvement comes to meet this need, increasing the productive efficiency by identification and selection of the animals with high performance in the population. For this, it is necessary to know the genetic parameters of economic important characteristics to guide the actions to improvement. However, few estimates of additive genetic variance and heritability wild populations are available, particularly for fish (Serbezov et al., 2010). Aquatic animals have undergone a limited amount of genetic improvement or domestication, with the exception of a few fish species, and most aquaculture stocks in current use in developing countries are genetically similar or inferior to wild, undomesticated stocks (Brummett et al., 2004; Gjedrem, 2000, 2010). Genetic progress with classical breeding approaches has been achieved based at accurate estimates. Possible sources of variation are included in statistical models to increase the accuracy of estimated breeding values (EBV), such as the maternal genetic effect has been considered an incorporated effect on an individual's performance for different characteristics (Bijma, 2006; Chen et al., 2007).

In this sense, Bayesian inference has offered a major advance for genetic parameters estimation (Waldmann, 2009). The Bayesian approach has some advantages as flexibility in choosing distributions for sample data and unknown parameters (Sorensen, 2009). This seems very suitable for populations at the domestication process, as the case of tambaqui *C. macropomum*, where no prior information on the estimation of genetic parameters is available. Thus, the aim of this study is to estimate the genetic parameters for morphometric and performance characteristics measured in two growth stages in tambaqui *C. macropomum* using Bayesian inference.

The data set was analyzed comprised of eleven families of maternal half-sib sexed. The offspring of each family remained in the same environment until it reached a body weight of 20g (more or less 1 month) when it was possible to mark the animals by Passive Integrated Transponder Tags (PIT) in the dorsal muscle. After, the animals were transferred to earth ponds tank remained during the whole period with alimentation two-fold per day. The biometrics data were collected at 12 and 24 months of cultivation, corresponding to an average of 475.5 and 854.5 days of the age, respectively; a total of 96 and 99 males to 12

and 24 months of cultivation, respectively and 101 females for the same period. Were measured body weight (BW) (grams), head length (HL) (cm) (measured between the front end of the head and the caudal edge of the operculum), body width (BWi) (cm) (measured in front of the first dorsal fin ray), body height (BH) (cm) (measured in front of the first dorsal fin ray), body length (BL) (cm) and daily weight gain (DWG) (grams). The DWG was measured as the ratio between the measured weight and the initial weight during cultivation period in days.

Were performed preliminary analyses by PROC GLM (Generalized Linear Model) of SAS to determine the model and the sex and age effects was included. Estimates of genetic parameter and (co)variance components were performed to 12 months of age by animal model: $y=XB+Z_1a+Z_2m+e$; where B, a, m, and *e* are identifiable environmental effects vectors, genetic values, common family environment effects, and residue, respectively. X, Z_1 and Z_2 are incidence matrices of the identifiable environmental effects (B), genetic additives (a) and common family environment (m). The estimation to 24 months of age was performed with the same animal model described above, but not considering the common family environment effect.

The (co)variance and genetic parameters were carried out by MTGSAM (Multiple Trait Gibbs Sampler for Animal Models) system (Van Tassel and Van Vleck, 1995). The *a posteriori* distributions were obtained from 400.000 cycles, using disposal intervals and 40.000 burned at 50 cycles, totaling 7.200 samples of the (co)variance components. Convergence monitoring of chains generated were carried out by Gibbs sampler via graphic analysis; the credibility intervals estimation at 95% were carried out via Heidelberger and Welch test (1983), available at the CODA library (Convergence Diagnosis and Output Analysis), implemented in the program R (version 2.8.1).

Heritability values estimates were high values for morphometric characteristics, body weight and daily weight gain, ranging from 0.25 to 0.49 at 12 months and from 0.17 to 0.42 at 24 months of age (Table I). Heritability values estimates for body weight (BW) and daily weight gain (DWG) were higher than that 0.40, indicating great genetic participation at the variance observed. Body length (BL), body height (BH) and body width (BWi) estimates were 0.31, 0.29, and 0.35, respectively, for 12 months. For 24 months, there was 5, 6.8 and 15 percentage points reduction for BH, LH and BWi characteristics, respectively, compared at 12 months. However, for the body length (BL), there was an increase for heritability values from 0.31 at 12 months to 0.46 at 24 months. Body weight (BW) and daily weight gain (DWG) showed the highest heritability at 12 months, which were maintained at 24 months for BW. The phenotypic variance was lowest for the trait body weight (BW) (0.13), which indicates a lower relative participation of the environment in the animal's performance.

Here, were first estimate the heritability and phenotypic variance for the population for tambaqui *C. macropomum*, native species of South America. Estimate of genetic parameters for importance economic characteristics measured in two stages in tambaqui *C. macropomum* showed heritability values of high magnitudes (Table I).

Estimates for the all morphometric characteristics showed heritability

values high with exception of the head length (LH); which showed the moderate heritability value (0.17). Likewise to that observed for body weight (BW) and daily weight gain (DWG), there is a greater genetic influence on growth performance in 12 months. This hypothesis is corroborated by greater heritability estimates for the 12 months that decrease with time of cultivation, with the lowest estimates at 24 months. Similarly, the estimates of phenotypic variance at 24 months were higher, in general, coinciding with the decrease of heritability estimates.

This study is consistent with the heritability estimates for body weight at other important species. For rainbow trout (Gjerde and Schaeffer, 1989) and atlantic salmon (Fjalestad et al., 1995), with estimations of 0.21 and 0.35, respectively, similar the tambaqui *C. macropomum* heritability values. In fact, the estimation of heritability estimates showed similar variation such as other fish species, which may vary according with development stage. Body length (BL) heritability in trout *Salmo trutta* ranged from 0.16 and 0.31, according to the age (Serbezov et al., 2010). Similarly, it was observed in sea bass *Dicentrarchus labrax* L., the heritability values ranged from 0.21~ 0.56 at 341 and 818 days, respectively (Saillant et al., 2006). In masu salmon *Oncorhynchus masou masou*, the heritability values for body weight and fork length were 0.41~0.51 and 0.46~0.54, for 12 and 24 months, respectively, suggesting that the heritability for the growth characteristics is related to the cultivation stages (Zhang et al., 2013). Likewise, the heritability for body mass, body standard length and body height using microsatellite data in yellow croaker *Larimichthys crocea* were very close to those estimated here. The heritability estimates at 13 month-old were 0.30 for M, 0.25 for LS and 0.36 for HB (Liu et al., 2013).

In general, the effect of common family environment was lower than 11%, except for body width (BWi) that had a 20% interest relative at 12 months. Were consider common family environment effect only for the first year of cultivation because the importance of the effects related to early stages of life tends to reduce and lose importance as the animal becomes older. In tambaqui *C. macropomum* is not observed parental care, being expected a low relative contribution of common effect of family. In fish species where the progeny depends more maternal care, such as tilapia *Oreochromis niloticus*, being incubation of embryos at the female mouth a very important strategy for the viability of offspring. Thus, is observed to decrease the effect of common environment with time, from 0.30 to 0.12 in five months of cultivation (Santos, 2009).

The available database for this first analysis was reduced, because we had to edit them to get a higher consistency. The credible intervals estimated were large for heritability, common family environment effect and phenotypic variances for all characteristics. It is desirable that the estimates have narrower credibility intervals, because indicate a more accurate estimation (Waldmann and Ericsson, 2006). However, Bayesian inference was methodology with better estimation, more accurate, for data structure available. On the other hand, the phenotypic variance showed credibility intervals with lower amplitude, indicating that the data structure had low influence on the accuracy for this estimates.

In conclusion, the estimated genetic parameters for body weight (BW),

daily weight gain (DWG), body height (BH), body width (BWi), body length (BL), and head length (HL) indicate that can be improved by selection in both stages of cultivation analyzed. This allows to predict the response to selection on performance and morphometric characteristics, assisting to design an effective breeding strategy. The characteristics with high heritability values are promising characteristics for selection to improve the performance of native fish species.

Table 1. *A posteriori* estimation of the means and credibility intervals (in parentheses) for the heritability (h^2), common family environment (m^2), and phenotypic variance (σ^2_p) of morphometric and performance* characteristics in tambaqui *Colossoma macropomun* measured at 12 and 24 months.

	12 months			24 months	
	h^2	m^2	σ^2_p	h^2	σ^2_p
DWG	0.49 (0.15-0.86)	0.11 (0.03-0.30)	0.64 (0.46-0.90)	0.40 (0.12-0.84)	0.76 (0.58-1.05)
BL	0.31 (0.05-0.79)	0.09 (0.02-0.27)	6.37 (4.84-9.16)	0.46 (0.13-0.89)	7.40 (5.52-10.41)
LH	0.25 (0.05-0.73)	0.08 (0.02-0.23)	0.32 (0.25-0.46)	0.17 (0.04-0.56)	0.91 (0.73-1.17)
BH	0.29 (0.05-0.82)	0.07 (0.01-0.23)	0.12 (0.10-0.18)	0.22 (0.05-0.65)	0.27 (0.21-0.36)
BWi	0.35 (0.14-0.62)	0.20 (0.07-0.44)	1.24 (0.93-1.79)	0.20 (0.05-0.57)	1.96 (1.57-2.54)
BW	0.44 (0.09-0.85)	0.06 (0.01-0.24)	0.13 (0.10-0.19)	0.42 (0.14-0.85)	0.55 (0.42-0.77)

*body weight (BW); daily weight gain (DWG); body width (BWi); body height (BH); length of head (LH), and body length (BL).

References

Bijma, P. (2006). Estimating maternal genetic effects in livestock. J. Anim. Sci. *84*, 800–806.

Brummett, R.E., Angoni, D.E., and Pouomogne, V. (2004). On-farm and on-station comparison of wild and domesticated Cameroonian populations of Oreochromis niloticus. Aquaculture *242*, 157–164.

Chen, C.Y., Johnson, R.K., Newman, S., and Van Vleck, L.D. (2007). A general review of competition genetic effects with an emphasis on swine breeding. Genet. Mol. Res. GMR *6*, 594–606.

Fjalestad, K.T., Gjedrem, T., and Refstie, T. (1995). Selection for enhanced disease resistance in Atlantic salmon. Aquaculture *137*, 275.

Gjedrem, T. (2000). Genetic improvement of cold-water fish species. Aquac. Res. *31*, 25–33.

Gjedrem, T. (2010). The first family-based breeding program in aquaculture. Rev.

Aquac. *2*, 2–15.

Gjerde, B., and Schaeffer, L.R. (1989). Body traits in rainbow trout: II. Estimates of heritabilities and of phenotypic and genetic correlations. Aquaculture *80*, 25–44.

Lind, C.E., Ponzoni, R.W., Nguyen, N.H., and Khaw, H.L. (2012). Selective breeding in fish and conservation of genetic resources for aquaculture. Reprod. Domest. Anim. Zuchthyg. *47 Suppl 4*, 255–263.

Liu, X.D., Zhao, G.T., Cai, M.Y., and Wang, Z.Y. (2013). Estimated genetic parameters for growth-related traits in large yellow croaker Larimichthys crocea using microsatellites to assign parentage. J. Fish Biol. *82*, 34–41.

Saillant, E., Dupont-Nivet, M., Haffray, P., and Chatain, B. (2006). Estimates of heritability and genotype-environment interactions for body weight in sea bass (Dicentrarchus labrax L.) raised under communal rearing conditions. Aquaculture *254*, 139–147.

Serbezov, D., Bernatchez, L., Olsen, E.M., and Vollestad, L.A. (2010). Quantitative genetic parameters for wild stream-living brown trout: heritability and parental effects. In J Evol Biol, (Switzerland), pp. 1631–1641.

Sorensen, D. (2009). Developments in statistical analysis in quantitative genetics. Genetica *136*, 319–332.

Van Tassel, C.P., and Van Vleck, D.L. (1995). A manual for use of MTGSAM. A set of FORTRAN programs to apply Gibbs sampling to animal models for variance component estimation (DRAFT). 86.

Waldmann, P. (2009). Easy and flexible Bayesian inference of quantitative genetic parameters. In Evolution, (United States), pp. 1640–1643.

Waldmann, P., and Ericsson, T. (2006). Comparison of REML and Gibbs sampling estimates of multi-trait genetic parameters in Scots pine. Theor Appl Genet *112*, 1441–1451.

Zhang, Y.-Y., Jia, Z.-Y., Bai, Q.-L., Chen, S.-Q., Shi, L.-Y., and Wang, B.-Q. (2013). [Heritability of body weight and fork length for Oncorhynchus masou masou]. Yi Chuan Hered. Zhongguo Yi Chuan Xue Hui Bian Ji *35*, 202–207.

[2] CAPÍTULO III

[2] Artigo redigido de acordo com as normas do periódico *Aquaculture*

Growth curve by Gompertz nonlinear regression model in female and males in tambaqui (*Colossoma macropomum*)

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Abstract
The aim was to evaluate the pattern of growth among females and males in tambaqui by the Gompertz nonlinear regression model. Were measured economic important traits from 145 animals collected during three years totally 981 biometric data. Were adjusted different curves to males and females for traits of body weight, height and head length. For body weight, the asymptotic value (*a*) and relative growth rate to maturity (*k*) showed to be differences between sexes. However, no difference was observed between males and females at commercial slaughter age (451 days), which indicates that both sexes can be used for fish farm purposes currently defined. In general, males have lower for growth rate (8.66 g/day) than females (9.34 g/day), being the body height and body length the traits more important to body weight to the 451 days (P<0.001). Estimates suggest that males should be slaughtered at 476 days and 486 days for females, thereby you can use the maximum potential of growth in animals.
Key words: Gompertz curve, growth patterns, maximum growth rate, slaughtered point.

Introduction

The tambaqui (*Colossoma macropomum*: CUVIER, 1818) is the native species to the Amazon basin of highest production at continental aquaculture in Brazil with a demand is expressive and growing (Ministério da Pesca e Aquicultura, 2013). Due to the large importance of this species to aquaculture, studies have been conducted evaluating mainly diets and metabolism (Corrêa et al., 2007), reproduction (Carneiro et al., 2012; Maria et al., 2012), molecular genetics (Gomes et al., 2012; Hamoy and Santos, 2012; Marcuschi et al., 2010) among others, however, few is known about the pattern of growth in tambaqui (Penna et al., 2005).

Multiple factors affect the efficiency of animal growth in fish farm such as genetics, the sex, nutrition and environment. The knowledge on body growth has strategic importance for genetic improvement of this species, besides providing information on traits of economic interest could be used to predict or fit the optimal age of slaughter (Knizetova et al., 1991). The prior knowledge of these aspects, for example sexual dimorphism, allows adapt the processing techniques of meat in accordance with the sex (Contreras-Guzmán, 1994; Gomiero et al., 2003). Animal growth patterns can be modified by selection. Thus, the knowledge about the speed of growth of different tissues has high value to the meat production industry; however, due the early phase of the fish supply chain, such information is still unknown.

The aim of this study was to estimate body growth curves and evaluate differences between males and females in tambaqui using the Gompertz nonlinear regression model.

Materials and Method

Were analyzed 981 biometric data from 145 animals measured for three years (2009 - 2011) for economic important traits. The offspring of each family remained in the same environment until it reached a body weight of 20g (more or less 1 month) when it was possible to mark the animals by Passive

Integrated Transponder Tags (PIT) in the dorsal muscle. After, the animals were transferred to earth ponds tank (500m^2) remained during the whole period with alimentation two-fold per day. Were measured body weight (BW) (grams), length of head (LH) (cm) (from anterior end of the head and caudal operculum), body width (BWi) (cm) (from 1st dorsal fin ray), body height (BH) (cm) (from 1st dorsal fin ray) and body length (BL) (cm) by pachymeter, ictiometer and measuring tape graduated (mm).

Gompertz nonlinear regression model was used to describe growth curves in tambaqui sorted by sex. This can be described as, $y = a.e^{(-e)^{(-b(t-k)}}$, where, *y* represents the traits of interest, *a* is the maximum value of *y* (asymptotic values), *b* is a constant rate of exponential growth, *e* the base of natural logarithms, *t* is the age in days and *k* is the age at inflection point. The growth rate, g/day, is given by the derivative of the above (Fialho, 1999):

$$\frac{dy}{dt} = a.b.e^{-b(t-k)-e^{-b(t-k)}}$$

Were tested the adequacy of the curve for each trait using eight equations to obtain the best fit of the Gompertz model parameters in the population, i.e., it would be better to set a single curve for the entire population or a curve for each sex. The adequacy was performed using an adaptation of the likelihood ratio test proposed by Regazzi (Regazzi and Silva, 2004). The simple equation, called M8, considered that Gompertz parameters were the same at males and females; and the complete equation, called M1, considered a specific parameter for each sex; other models tested were simplifications of the M1 equation (Table 1). The estimate of the Gompertz model parameters was carried out by NLIN (Nonlinear Regression Models) procedure; the regression analysis was carried out by GLM (Generalized Linear Model) procedures and the principal factor analysis was performed by FACTOR procedure (SAS 9.2 statistical package).

Results and Discussion

The initial hypothesis was that there no difference in growth among males and females. From these results it is possible to corroborate the field observations that females grow faster than males after reproductive maturation. In fact, the growth showed allometric characteristics, i.e., each tissue has different growth rate. The first tissue to be deposited and stop the growth was the bone tissue followed of the muscle tissue. That explains why the animals reached the inflexion point for length and body height (BH) and head length (HL) (Figures 1b, 1d and 1e). Then, and still continuing, was being deposited muscle tissue, explaining the later inflection point for weight and body width (Fig. 1a; 1c).

Growth patterns were similar between males and females for length (BL) and body width (BWi), with a single curve fitted for the all population (Fig. 1a; 1b). For weight and body height, and head length the suitability test revealed different parameters between males and females, adjusting different curves. Were observed differences of the female's asymptotic values (*a*) and age at inflection point (*k*) in comparison to males; fitting curves with different estimates for the parameters at each sex (M3 equation) (Fig. 1c). Females presented higher weight than males after sexual maturation (846 days), however, this difference was not seen in immature animals (451 days) (Fig. 1c). There were

differences at the estimates of the *b* parameter for body height (BH) and head length (HL) (M6 equation) indicating a constant exponential growth different between males and females (Fig. 1d; 1e).

Was carried out the principal component analysis (PCA) until 2 kg, obtained at 516 days. Was observed that animals with greater body length (BL), length head (LH), presenting better results, showed lower body height (BH). Length head (LH) and body length (BL) are traits of the most influence the body weight (BW) and dairy wehgt gain (DWG) until 2 kg ($P<0.001$) (Fig. 2).

According current processing industry the slaughter weight of this species is 2 kg, which was achieved at 516 days of cultivation. In this point, is possible to observe at both sexes the same growth pattern (Fig. 1c); thereby, indicating males and females for fish farming (Table 2). However, the females showed a maximum growth rate for body weight higher than males, 9.34 and 8.66 g/day, respectively, achieved at different days of cultivation, 476 days to males and 486 days to females (Table 3).

Analyze growth curves is a complex problem because the evolution of growth over time is nonlinear, and it is affected by genetic and environmental effects (Ibáñez-Escriche and Blasco, 2011). However, it is possible to estimate the maximum growth rate and, thus, fit the better slaughter age for each sex. The shape of the body, sex and body weight are very important in choosing the processing performed by the industry, as it influences the operations of beheading, gutting and cleaning, besides influencing the final yield of the processing (Carneiro et al., 2004; Contreras-Guzmán, 1994; Faria et al., 2003).

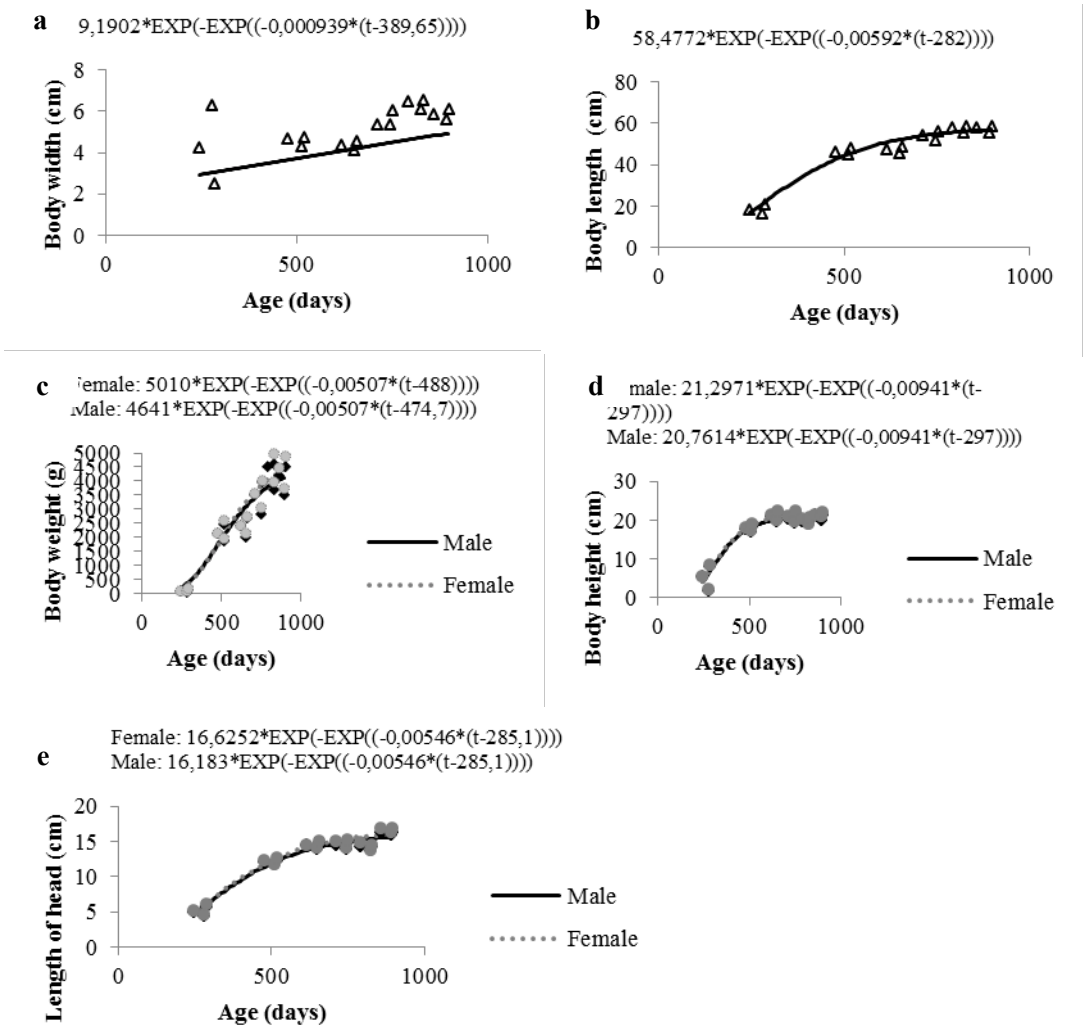

Figure 1. Growth profiles body according to adjusted of the Gompertz nonlinear model at 896 days: body width (a); body length (b); body weight (c); head length (d) and body height (e) at function of age in tambaqui (*Colossoma macropomum*). Gray data represent the females and black the males.

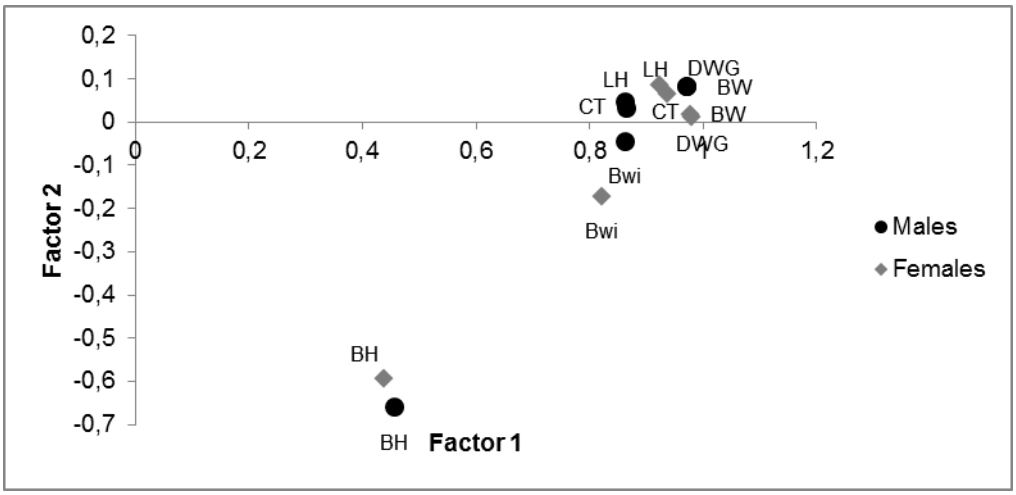

Figure 2. Principal Component Analysis (PCA) in tambaqui (*Colossoma macropomum*) growth traits of the factors 1 and 2 (86% accumulated variance); body weight (BWE), daily weight gain (DWG), body width (BWI), body height (BH), length of head (LH) and body length (BL) for 516 days of age (± 2 Kg).

Table 1. Description of the eight equations tested to obtain the best fit of the parameters in the nonlinear regression of the Gompertz model.

Parameters	M1	M2	M3	M4	M5	M6	M7	M8
a	*ai*	*a*	*ai*	*ai*	*a*	*ai*	*a*	*a*
b	*bi*	*bi*	*b*	*bi*	*b*	*b*	*bi*	*b*
k	*ki*	*ki*	*ki*	*k*	*ki*	*k*	*k*	*k*

i= different parameters tested for males and female

Table 2. Mean ± standard deviation for morphometric traits in age at commercial slaughter for tambaqui (*C. macropomum*) (516 days).

Characteristic	Male	Female
Body weight (grams)	2140.31 ± 387.1	2217.66 ± 388.7
Body height (cm)	17.65 ± 0.99	18.1 ± 1.05
Length of head (cm)	12.02 ± 0.58	12.2 ± 0.56
Body width (cm)	4.54 ± 0.33	4.66 ± 0.32
Body length (cm)	46.23 ± 2.5	46.56 ± 2.53

Table 3. Maximum growth rate and estimated days to inflection point for body weight (BW), body length (BL), length of head (LH), body width (BWi) and body height (BH) in tambaqui (*Colossoma macropomum*).

	Male		**Female**	
Traits		Days		Days
BW (g/day)	8.66	476	9.34	486
BL (cm/day)	0.1274	256	0.1274	256
LH (cm/day)	0.0325	246-236	0.03339	246-236
BWi (cm/day)	0.00317	366-376	0.00317	366-376
BH (cm/day)	0.0718	256-346	0.0737	256-346

Conclusion

There were differences in growth patterns between adult male and female tambaqui evidenced by the different curves fitted, indicating the existence of sexual dimorphism of this specie. Were showed differences at the maximum growth rate between the sexes suggesting the need to adapt the production system to use the maximum growth potential of each sex.

References

Carneiro, P.C.F., Mikos, J.D., Bendhak, F., and Ignacio, S.A. (2004). Processamento do jundiá Rhamdia quelen: rendimento de carcaça. Rev. Acadêmica Ciênc. Agrár. E Ambient. *2*, 11–17.

Carneiro, P.C.F., Azevedo, H.C., Santos, J.P., and Maria, A.N. (2012). Cryopreservation of tambaqui (Colossoma macropomum) semen: extenders, cryoprotectants, dilution ratios and freezing methods. Cryo Letters *33*, 385–393.

Contreras-Guzmán, E. (1994). Bioquímica de Pescados e Derivados (Jaboticabal: FUNEP).

Corrêa, C.F., de Aguiar, L.H., Lundstedt, L.M., and Moraes, G. (2007). Responses of digestive enzymes of tambaqui (Colossoma macropomum) to dietary cornstarch changes and metabolic inferences. Comp. Biochem. Physiol. A. Mol. Integr. Physiol. *147*, 857–862.

Faria, R.H.S., Souza, M.L.R. de, Wagner, P.M., Povh, J.A., and Ribeiro, R.P. (2003). Rendimento do processamento da tilápia do nilo (Oreochromis niloticus Linnaeus, 1757) e do pacu (Piaractus mesopotamicus, Holmberg 1887). Acta Sci. *25*, 21–24.

Gomes, F., Schneider, H., Barros, C., Sampaio, D., Hashimoto, D., Porto-Foresti, F., and Sampaio, I. (2012). Innovative molecular approach to the identification of Colossoma macropomum and its hybrids. An. Acad. Bras. Ciênc. *84*, 517–526.

Gomiero, J.S.G., Ribeiro, P.A.P., Ferreira, M.W., and Logato, P.V.R. (2003). Rendimento de carcaça de peixe matrinxã (Brycon cephalus) nos diferentes cortes da cabeça. Ciênc. Agrotecnológica *27*, 211–216.

Hamoy, I.G., and Santos, S. (2012). Multiplex PCR panel of microsatellite markers for the tambaqui, Colossoma macropomum, developed as a tool for use in conservation and broodstock management. Genet. Mol. Res. *11*, 141–146.

Ibáñez-Escriche, N., and Blasco, A. (2011). Modifying growth curve parameters by multitrait genomic selection. J. Anim. Sci. *89*, 661–668.

Knizetova, H., Hyanek, J., Knize, B., and Roubicek, J. (1991). Analysis os growth-curves of fowl. 1. Chickens. Br. Poult. Sci. *32*, 1027–1038.

Marcuschi, M., Espósito, T.S., Machado, M.F.M., Hirata, I.Y., Machado, M.F.M., Silva, M.V., Carvalho, L.B., Jr, Oliveira, V., and Bezerra, R.S. (2010). Purification, characterization and substrate specificity of a trypsin from the Amazonian fish tambaqui (Colossoma macropomum). Biochem. Biophys. Res. Commun. *396*, 667–673.

Maria, A., Azevedo, H., Santos, J., and Carneiro, P. (2012). Hormonal induction and

semen characteristics of tambaqui Colossoma macropomum. Zygote *20*, 39–43.

Ministério da Pesca e Aquicultura (2013). Boletim estatístico da pesca e aquicultura: Brasil 2011.

Penna, M.A., Villacorta-Correa, M.A., Walter, T., and Petrere-Jr, M. (2005). Growth of the tambaqui Colossoma macropomum (Cuvier) (Characiformes: Characidae): which is the best model? In Braz J Biol, (Brazil), pp. 129–139.

Regazzi, A.J., and Silva, C.H.O. (2004). Teste para verificar a igualdade de parâmetros e a identidade de modelos de regressão não-linear. I. Dados no delineamento inteiramente casualizado. Rev. Matemática E Estat. *22*, 33–45.

[3] CAPÍTULO IV

[3] Artigo redigido de acordo com as normas do periódico *Anatomia, Histologia, Embryologia*

Morphological and morphometric analysis skeletal muscle tissue in tambaqui (*Colossoma macropomum*)

ANÁLISE MORFOLÓGICA E MORFOMÉTRICA DO TECIDO MUSCULAR ESQUELÉTICO EM TAMBAQUI (*Colossoma macropomum*)

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Abstract

The objective of this study was to evaluate the morphological and morphometric characteristics of the growth of skeletal muscle in tambaqui (*Colossoma macropomum*). Young-adults at 300 days of age were used. After euthanasia, gonad and muscle fragments of dorsal regions were collected and fixed in formalin (10%). Histological sections (5 mm) were subjected to HE and Masson's trichrome for morphological analysis and sexing of animals. The pattern of distribution of the fibers observed in tambaqui is characteristic of muscular growth by hyperplasia and hypertrophy. The body weight was accompanied by a continuous increase in fiber diameter. The occurrence of smaller diameter fibers represents a continuation of myogenesis during young-adult life. This muscle growth is histological characterized by a mosaic of fibers of different diameters, with no correlation with the sex of the animals.

Keywords: muscle growth, hypertrophy, hyperplasia, sex correlation.

Introduction

In fish, most of the species have continued growth, implying that the size is never fixed. As in other vertebrates, skeletal muscle derives from the somites that are formed during the rostro-caudal segmentation of the paraxial mesoderm (Chauvigné et al., 2005). Muscle fibers have hyperplastic and hypertrophic capacity where post-larval muscle growth is dependent of the contributions these two mechanisms (Veggetti et al., 1993; Weatherley, 1990; Weatherley and Gill, 1985). In fish species that ultimately reach a large size, muscle fiber formation continues in a long lasting hyperplastic process disseminated throughout the entire myotome giving a typical mosaic appearance to muscle cross-sections (Marschallinger et al., 2009).

Tambaqui (*Colossoma macropomum* Cuvier; Serrasalminae, Characidae, Teleostei) is the largest characin of South America and important aquaculture specie widely distributed in Amazon basin (Araujo-Lima, 1997; Ibama, 2012). This specie presents fast growth, omnivorous habit, easily adapts to artificial feed for possessing great ability to digest plant and animal protein (Nunes et al., 2006). Previous studies on this species have been concerned mostly on metabolism and physiology (Corrêa et al., 2007), reproduction (Carneiro et al., 2012; Maria et al., 2012), nutrition (Chagas and Val, 2003; Guimarães and Storti Filho, 2004) and genotoxicity aspects (Matsuo et al., 2005). However, very little is known about the muscle development and growth in tambaqui (Araujo-Lima, 1994). Thereby, the goal this study was to descript the organization of the skeletal muscle and determine the mechanism of muscle fibers growth in tambaqui.

Material and Methods

Histology and Morphometry Studies

Were analyzed 10 young-adults, full siblings with approximately 300 days of age and weight different from the same fish farm (Table 1).

To collection of muscle the animals were anaesthetized with Benzocaine (75 mg/L), on ice stunned and sacrificed by spinal section. Samples of 1 cm^2, approximately, skeletal muscle were collected and fixed in Bouin solution for 24

hours, dehydrated in ethanol at increasing concentrations, diaphanized in xylol, impregnated and embedded in paraffin. Subsequently, histological sections of 5 *μm* of thickness, transverse and vertical, were obtained and stained with hematoxylin-eosin to evaluate the morphology of muscle fibers. Following the same processing of the samples of muscle, gonads were collected for sexing of animals. Sections were viewed by light microscopy and analyzed with the software Image-Pro Plus.

To estimate fibers diameter (*μm*), totals of from 420 to 615 fibers of each animal were measured using the smallest diameter method (Dubowitz and Brooke, 1984). Based on the fibers diameter, muscle fibers were grouped into four classes: <10 *μm*, <20 *μm*, 20 - 50 *μm* and >50 *μm* (Romanello et al., 1987). The mean diameter and standard deviation for each muscle fiber in each class of fiber were determined. Were estimates the Pearson Correlation between: (i) body weight and muscle fibers diameters correlations and (ii) sex and muscle fibers diameters. The statistical data analyses were carried out by ANOVA one-way and supplemented by test Tukey (P<0.05).

Results

Somatic growth and Morphometry

The striated muscle in tambaqui exhibited a typical morphologic pattern of other fish species, fibers rounded with peripheral nuclei and multinucleated. The fibers presented different diameters; they are individually surrounded by a connective tissue layer and grouped into bundles to form a skeletal muscle (Figure 1). Skeletal muscle is highly vascularized to provide essentials nutrients for muscle function.

Were observed many small fibers between the fibers of larger diameter in all the animals, featuring fibers with a mosaic of different sizes (Figure 2). The numbers of fibers with 20-50 *μm* of diameter was superior in the animals of low weight than the frequency the fibers of other diameters (P=0.05). Among the animals more weight was observed higher frequency fibers of larger diameters, however, there was no difference between the frequencies of fibers of 20-50 *μm* e >50 *μm* (P=0.174).

The correlation between the fibers diameters and body weight was 0.5, being the class of fibers diameter 20-50 *μm* that contributed most to the weight of the animals (P<0.0001) (Figure 2).

Gonadal development

Despite of the initial gonadal development was possible carried out the sexing of the animals; at 300 days of age it can be observed oocytes and spermatozoids maturation (Figure 3). It's possible to observer this stage of gonadal two distinctly lots of oocyte, a stock group that does not participate in next reproduction (Figure3a, dotted arrow) and a maturation group which accumulates yolk and will be released in the next spawning (Vieira et al., 1999) (Figure 3a, solid arrow). An immature stage of the reproductive cycle in males consisted of small seminiferous lobules and much interstitial tissue (Nagaki et al., 2003) (Figure 3b).

The correlation between fiber diameters and sex was positive, despite low, 0.2. However, there was no correlation between the body weight and sex (P=0.8).

Discussion

The pattern of the fibers distribution observed in tambaqui is characteristic of muscular growth by hyperplasia and hypertrophy (Johnston, 1999; Veggetti et al., 1993). However, it was observed frequencies significantly different of different fiber diameters in animals with different body weights, although at the same age. This differential enlargement of body weight and fiber diameter differs significantly from the situation found in mammals. For example, in adults rabbits, hypertrophy of white fibers was still intense, and the gain in weight was very low (Pai and Curi, 1992). In contrast, in the juvenile and young-adult stages of large, continuously growing fish, the increase in muscle mass is accomplished mainly by the hyperplastic process (Johnston, 1999; Stickland, 1983). The large contribution of hyperplasia to muscle growth in tambaqui described here is consistent with the results the other morphometric studies in different species (Alves-Costa et al., 2013; Carani et al., 2008; Dal Pai et al., 2000).

In this study, both body weight and sex showed a positive correlation with muscle fiber diameter from young-adult. However, the study of growth curves indicates that there is no difference in performance between males and females weight tambaqui until the first reproduction, around 400 days (de Mello et al, 2014).

The body weight was accompanied by a continuous increase in fiber diameter (hypertrophy), as was confirmed when these parameters are plotted together. The occurrence of smaller diameter fibers represents a continuation of myogenesis during young-adult life. The absence of such small fibers in the caudal region of adult rainbow trout (>39 - 40 cm) was associated with the end of hyperplastic growth (Stickland, 1983; Weatherley, 1990).

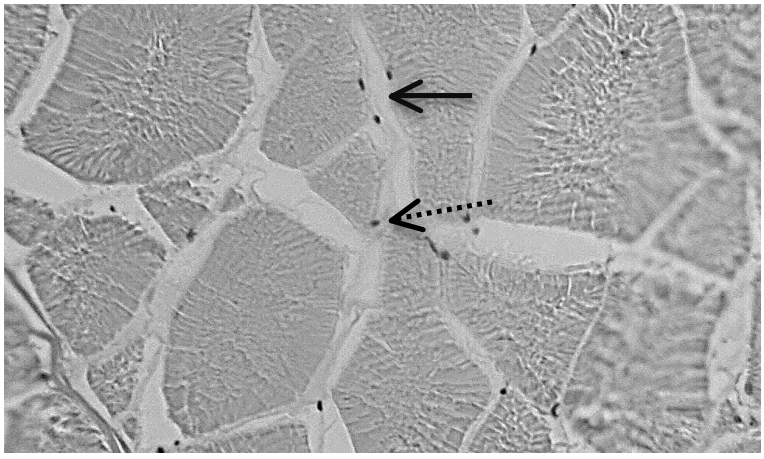

Figure 1. Morphological description of skeletal muscle young-adult of tambaqui; myofibers grouped in bundles within the perimysium. The myofibers are multinucleated (solid arrow) with nuclei located at the periphery (dotted arrow). Muscle cross-section stained with hematoxylin and eosin. 40X.

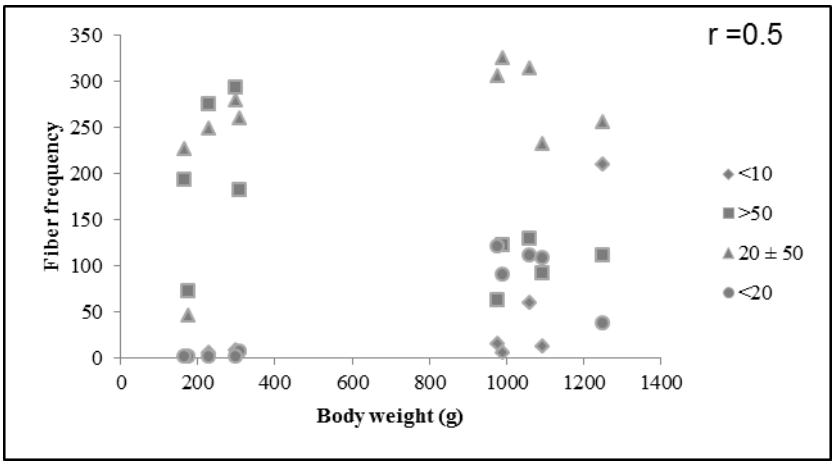

Figure 2. Pearson correlation for body weight (grams) and muscle fiber frequency of the diameters different (*µm*) in tambaqui (*Colossoma macropomum*).

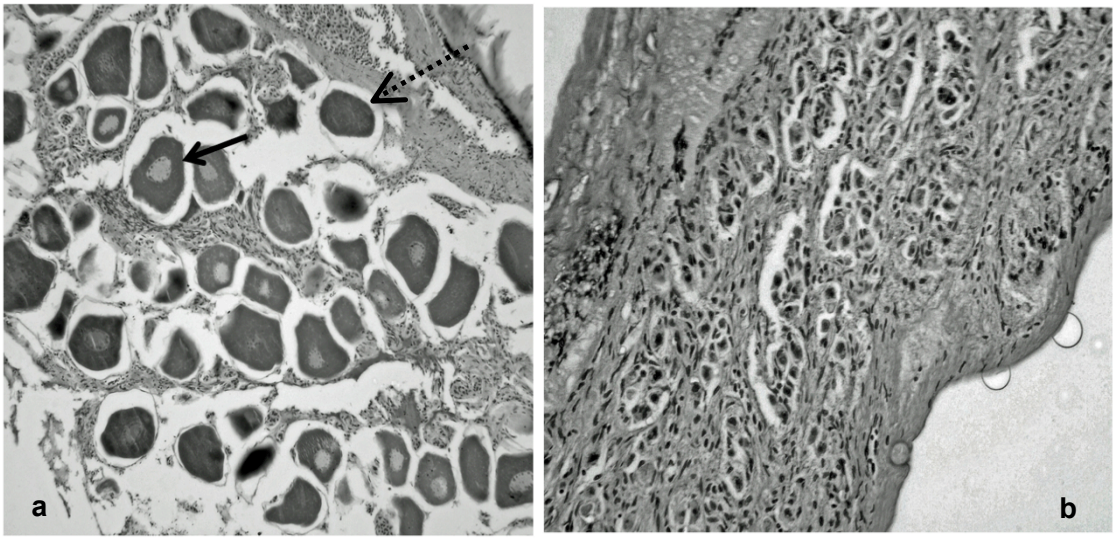

Figure 3. Gonadal tissue of young-adult at 300 days of age of the tambaqui (*Colossoma macropomum*); (a) ovary at maturation and (b) testicle immature showing primary spermatogonia throughout the lobules. Solid arrow: oocyte in vitellogenesis incipient, dotted arrow: oocyte reserve stock.

Table 1. Body weight, sex (1=male and 2=female), number of fibers (n) sampled and means (X) and standard deviation (±SD) fibers diameters of sampled animals.

Fish	Weight (g)	Sex	n	X ± SE
1	308	2	615	153.75 ± 16.1
2	300	1	545	136.25 ± 7.5
3	178	1	505	126.25 ± 16
4	165	*	444	111 ±
5	228	2	615	153.75 ±
6	1250	2	455	113.75 ±
7	990	2	583	145.75 ±
8	976	1	119	119 ±
9	1093	1	423	105.75 ±
10	1060	1	531	132.75 ±

* There was no material for analysis.

Conclusion

Muscle growth at 300 days of age in tambaqui occurs by hyperplastic and hypertrophic mechanism. This muscle growth is histological characterized by a mosaic of the fibers of different diameters, with no correlation with the sex of the animals.

References

Alves-Costa, F.A., Barbosa, C.M., Aguiar, R.C.M., Mareco, E.A., and Dal-Pai-Silva, M. (2013). Morphometry and expression of MyoD and Myogenin in white and red skeletal muscles of juvenile fish *Colossoma macropomum* (Cuvier 1818). Acta Zool. n/a–n/a.

Araujo-Lima, C. (1997). So fruitful fish: ecology, conservation, and aquaculture of the Amazon's tambaqui. 157.

Araujo-Lima, C. a. R.M. (1994). Egg size and larval development in Central Amazonian fish. J. Fish Biol. *44*, 371–389.

Carani, F.R., Aguiar, D.H., Almeida, F.L.A. de, Gonçalves, H.S., Padovani, C.R., and Silva, M.D.P. (2008). Morfologia e crescimento do músculo estriado esquelético no pirarucu Arapaima gigas Cuvier, 1817 (Teleostei, Arapaimidae). Acta Sci. Biol. Sci. *30*.

Carneiro, P.C.F., Azevedo, H.C., Santos, J.P., and Maria, A.N. (2012). Cryopreservation of tambaqui (Colossoma macropomum) semen: extenders, cryoprotectants, dilution ratios and freezing methods. Cryo Letters *33*, 385–393.

Chagas, E.C., and Val, A.. (2003). Efeito da vitamina C no ganho de peso em parâmetros hematológicos de tambaqui. *38*, 397–402.

Chauvigné, F., Cauty, C., Rallière, C., and Rescan, P.Y. (2005). Muscle fiber differentiation in fish embryos as shown by in situ hybridization of a large repertoire of muscle-specific transcripts. Dev. Dyn. Off. Publ. Am. Assoc. Anat. *233*, 659–666.

Corrêa, C.F., de Aguiar, L.H., Lundstedt, L.M., and Moraes, G. (2007). Responses of digestive enzymes of tambaqui (Colossoma macropomum) to dietary cornstarch changes and metabolic inferences. Comp. Biochem. Physiol. A. Mol. Integr. Physiol. *147*, 857–862.

de Mello F, Oliveira CAL, Ribeiro RP, Resende EK, Povh JA, Fornari DC, Barreto RV, McManus C, Streit Jr D (2014). Growth curve by Gompertz nonlinear regression model in female and males in tambaqui (Colossoma macropomum).

Guimarães, S., and Storti Filho, A. (2004). Produtos agrícolas e florestais como alimento suplementar de tambaqui em policultivo com jaraqui/ Agricultural and forest products as supplementary feeding for tambaqui in polyculture with jaraqui. Pesqui. Agropecu. Bras. *39*, 293–296.

Ibama (2012). Boletim estatístico da pesca e aquicultura: Brasil 2010.

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

Maria, A., Azevedo, H., Santos, J., and Carneiro, P. (2012). Hormonal induction and semen characteristics of tambaqui Colossoma macropomum. Zygote *20*, 39–43.

Marschallinger, J., Obermayer, A., Sanger, A.M., Stoiber, W., and Steinbacher, P. (2009). Postembryonic Fast Muscle Growth of Teleost Fish Depends Upon a Nonuniformly Distributed Population of Mitotically Active Pax7+ Precursor Cells. Dev. Dyn. Off. Publ. Am. Assoc. Anat. *238*, 2442–2448.

Matsuo, A., Wood, C., and Val, A. (2005). Effects of copper and cadmium on ion transport and gill metal binding in the Amazonian teleost tambaqui (Colossoma macropomum) in extremely soft water. Aquat. Toxicol. *74*, 351–364.

Nunes, E.S., Cavero, B.A.S., Pereira-Filho, M., and Roubach, R. (2006). Enzimas digestivas exógenas na alimentação de juvenis de tambaqui. *41*, 139–143.

Pai, V.D., and Curi, P.R. (1992). Crescimento pós-natal do coelho Norfolk: correlação entre parâmetros somáticos e área dos tipos de fibras musculares. Pesqui. Agropecuária Bras. *27*, 1623–1633.

Dal Pai, V., Dal Pai-Silva, M., Carvalho, E.D., Fujihara, C.Y., Gregorio, E.A., and Curi, P.R. (2000). Morphological, Histochemical and Morphometric Study of the Myotomal Muscle Tissue of the Pacu (Piaractus mesopotamicus Holmberg 1887: Serrasalminae, Characidae, Teleostei). Anat. Histol. Embryol. J. Vet. Med. Ser. C *29*, 283–289.

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

Veggetti, A., Mascarello, F., Scapolo, P.A., Rowlerson, A., and Carnevali, C. (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. (Berl.) *187*, 353–361.

Vieira, E.F., Isaac, V.J., and Fabré, N.N. (1999). Biologia reprodutiva do tambaqui, Colossoma macropomum Cuvier, 1818 (Teleostei, Serrasalmidae), no baixo Amazonas, Brasil. *29*, 625–638.

Weatherley, A.H. (1990). Approaches to Understanding Fish Growth. Trans. Am. Fish. Soc. *119*, 662–672.

Weatherley, A.H., and Gill, H.S. (1985). Dynamics of increase in muscle fibers in fishes in relation to size and growth. Experientia *41*, 353–354.

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Gene expression profile of the new genes sequences to TGF- β superfamily and follistatin genes in *Oncorhynchus mykiss*

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ABSTRACT

Since its initial discovery, TGF-β superfamily has been considered are multifunctional growth and differentiation factors in many cell types. Different studies have demonstrated clearly the pathway to muscle growth through the TGF-β superfamily genes; as TGF-β isoforms, myostatin and inhibin involving the follistatin. Follistatin is an inhibin-binding protein that prevents inhibin from binding to its receptors and neutralizes its activity. Among the proteins that contain follistatin domains, only follistatins are reported to bind TGF-b family members. In this study, we report the identification of sequences novel of three isoforms and four paralogs to TGF-β, four sequences paralogs to inhibin βA and two sequences paralogs of follistatin from rainbow trout. Alignments and phylogenetic analysis showed that sequence of the mature protein and nucleotídica are extremely well conserved between the genes of TGF-β, inhibin βA and follistatin (>80%) whereas their regulation and expression pattern are different. Follistatin b2 e inhibin βA showed most abundant expression in gonads, mainly in the testis; with except inhibin βA 2 that showed more expression in liver. However, recent investigations suggest that follistatin exerts its functions mainly in a paracrine manner, despite this activity in gonadal development. *TGF-β2* and *–β3* had a most abundant expression in muscle and are the main TGF-β members expressed in muscle. Our findings of multiple members of TGF-β superfamily and follistatin had different expression patterns suggests non-redundant functions in rainbow trout. This is not only shed new light on the molecular diversity of genes involved in muscle growth, but also suggest that the regulatory mechanisms biological signaling of the muscle growth are more dynamic and complex than thought previously.

INTRODUCTION

Muscle growth in animals is controlled by numerous growth factors and muscle-specific gene, which act in development of the myotome. In particular, genes of the transforming growth factor-β (TGF-β) superfamily have provided definitive evidence about its role in muscle growth (Kollias and McDermott 2008). Different studies have demonstrated clearly the pathway to muscle growth through the TGF-β superfamily genes; as *TGF-β* isoforms, *myostatin* and *inhibin* involving the *follistatin* (Matzuk et al. 1995; McPherron, Lawler, and Lee 1997; Amthor et al. 2004). Interaction between inhibin and follistatin lead a control of negative regulation of the muscle growth. Transgenic animals overexpressing or deletion/inhibition to genes as *myostatin* (S. J. Lee and McPherron 2001), *inhibin βA* (S.-J. Lee et al. 2012; Phelps, Jaffe, and Bradley 2013) or *follistatin* (Medeiros et al. 2009; S.-J. Lee 2007) present a modification the cell signaling cascade resulting at phenotypes known in mammals as double muscling and “six pack” in fish.

To date, the study of genes *follistatin* and *inhibin βA* in trout evaluated the role in muscle growth based on the premise of only one copy of the gene. However, the salmonids underwent two round of whole genome duplication (WGD) that create extra copies of the genes in early vertebrate lineage evolution (Guyomard et al. 2012). Thus, studies to date may have been overestimated gene expression by considering complementary or similar functions to those paralogs. Throughout evolution some copies can remain, get

new atemporal role or develop a novel function (Zhang 2003).

From the project of trout genome sequencing is possible to analyze the expression profile of the different copies of a gene and infer more indeed on relative participation on muscle growth. Nevertheless, the challenge post sequencing is to characterize the huge number of gene products, because DNA sequencing alone does not provide enough information to determine the molecular pathways (Janitz 2007). Therefore, study of gene expression has strategic importance in the description of the new sequences expression profile potentially involved on muscle growth.

To report of the expression profile of potential involved genes of the muscle growth in rainbow trout we analyze the new gene sequences to *TGF-β* (three isoforms and four paralogs), *growth differentiation factor 11*, *inhibin βA* (four paralogs) and *follistatin* (two paralogs). We located the exons sequence in each new gene sequences corresponding the protein sequences not publisher or partially available in public databases.

MATERIAL & METHODS

Animal to tissues analysis

Rainbow trout were maintained at the *Laboratoire de Physiologie et Génomique des Poissons* (LPGP-INRA) in 0.6 m^3 tanks in a re-circulated system at 18°C. All experiments were carried out in accordance with legislation governing the ethical treatment of animals (Decreet No. 2001-464, May 29, 2001). Investigators were certified by the French Government to carry out animal experiments (No. agreement 35–47). All animal work was approved by the *Ministère de l'Enseignement Supérieur et de le Recherche* (Authorisation No. A352386). The animals were initially anaesthetized with phenoxyethanol (3 mL/L) and sacrificed by spinal section. Tissues were collected from three imature fish weighing ±400g, analyzed in triplicate and immediately stored in liquid nitrogen after collected to RNA extraction.

RNA extraction, cDNA synthesis and quantitative PCR analyses

Total RNA was extracted from 100 mg using TRI Reagent (Sigma-Aldrich T9424) of white muscle, red muscle, skin, heart, brain, adipose, liver, spleen, hypophysis, kidney, ovary, gill, testicle and intestine tissues. The total RNA was resuspended in 200 µL MiliQ water and quantified in a spectrophotometer based on absorbance at 260 nm (NanoDrop ND-1000 spectrophotometer). The cDNA was obtained from 1 µg total RNA by reverse transcriptase (Applied Biosystems kit #N808-0234; Applied Biosystems, Foster City, CA,USA) in triplicate, following the manufacturer's recommendations. After, the reaction was diluted 10x and stocked at -80 ° C.

Quantitative PCR analyses were carried out with 5 µL cDNA using a SYBR® Green PCR Master Mix (Eurogentec, Seraing, Belgium), according to the manufacturer's instructions with end-concentration 300 nM of each primer. The amplification was performed by StepOnePlus® Real Time PCR System (Applied Biosystems®) using the following cycle: 95°C, 20 s; 62°C, 1 min, for 40 times. The realtive abundance of target cDNA within the sample set was calculated from a serially diluted cDNA pool (1:1–1:256) (standard curve) using StepOne™ Software V2.0.2 (Applied Biosystems®). Subsequently, real-time

PCR data were normalized using 18S rRNA by dividing the raw data by the reference gene expression.

Multiple alignments and phylogenetic analysis

All sequences analyzed were available from genome project rainbow trout (*ANR GENOTROUT*, INRA, France). We analyzed the nucleotide sequences retrieved from EST (Expressed Sequence Tag) of the interest genes duly identified. Were identified 13 new sequences of the mRNA (messenger RNA) for rainbow trout by MEGA5 software and BLAST (http://www.ncbi.nlm.nih.gov) (Table 1). Gene sequences with predicted exons and intro, and scaffolds were analyzed for the correct prediction of mRNA based translated protein by sequence manipulation suite tools (http://bioinformatics.org/sms2/).

The amino acid sequences of 30 protein-coding genes were combined and aligned by using Clustal X (Thompson et al. 1997) software with the default settings, followed by minor manual adjustment. Phylogenetic tree were performed using sequences of TGF-β occurring in fish available on database GenBank and Ensembl (http://www.ensembl.org/index.html) (Annex 1). Phylogenetic tree was inferred by neighbor-joining method by MEGA 5.2 tools. The substitution model was obtained by Protest 2.4 server and the robustness of the nodes of phylogenetic trees was tested by bootstrapping methods (Felsenstein, 1985).

The sequences of trout *follistatin* and *inhibin βA* were aligned with sequences of frog (BC080943.1/ ENSXETG00000001861), zebrafish (NM_131037.3; ENSDARG00000057992/ ENSDART00000004241; ENSDART00000047468), mouse (NM_008046.2/ ENSMUST00000164993) and chicken (NM_205200.1/ ENSGALT00000037439) available in databases GenBank and Ensembl by BioEdit and Mega 5. Regulatory sequences were identified based on the information available in Ensembl. The new sequences of the complementary DNA (cDNA) and protein to *TGF- β1a*, *TGF- β1*b, *TGF- β1c*, *TGF-β2*, *TGF-β3*, *TGF-β6*, *Inhibin βA 1*, *Inhibin βA 2*, *Inhibin βA 3*, *Inhibin βA 4*, *follistatin b1*, *follistatin b2* and *GDF11* genes for rainbow trout were deposited in GenBank database (Table1).

Primers design

The design of gene-specific primers was performed based on cDNA sequences obtained and sequences deposited in the public database by MEGA5.2 (Table 1). The fragment amplified was tested to secondary structures formation by mFOLD (SantaLucia Jr., 1998; Zuker M., 2003) and the alignments of the forward and reverse primers sequences were assembled by BLAST to verify the specificity primers. The amplifications condition were optimized before the analysis of expression.

Statistical analysis

The differential expression profile in different tissue was analyzed with a one-way ANOVA using the non-parametric Wilcoxon/Kruskal–Wallis rank test adjusted using the tukey method. The results are presented as a ratio of gene expression and 18S rRNA expression. All analyzes were performed using of the SAS 9.2 statistical package.

Table 1. Access number, primers forward and reverse, and fragment amplified size (bp) for *transforming growth factor beta* (*TGF-β*), *folistatin* (*Fst*), *Inhibin βA subunit* (*Inh*) and *growth differentiation factor 11* (*GDF11*) new gene sequences (*sequence published).

Gene	Access number	Forward primer (5' - 3')	Reverse primer (5' - 3')	Size
TGF-β1a	KF870471	ACCAATGGAGGAATTCCGCTTCA	GCTTTGATGGCATTTTCATGGCT	90
TGF-β1b	KF870472	CTGGATGAAGAGGAGGAGTACTT	CCTCATCTCATTCAGGTTAAACA	108
TGF-β1c	KF870473	AGCAATGGAAGAATTCCTCTTTGA	AGGCTTTGGCATGTTCACGAAG	88
TGF-β2	KF870474	TGAATGGTTGCTTCACAGAGACA	GTTTCCAGCTCCTCACTCTTATT	120
TGF-β3	KF870475	ACCAACCTCTTCAGAGCAGAGTT	GATGTAGCGCTGCTTAGCTATGT	129
TGF-β6	KF870476	CTGCCCATACCTCTGGAGCG	GCAGCACGGAGTTGCCGACG	95
Inh βA 1	KF870467	TAGGCGACACTACATCCGCTG	CCTCGTGTCCACCATCTTCTCA	167
Inh βA 2	KF870468	CTAGGAAACTCTCCATCTGCCA	CCGTGTGTCCACCATCTTCTCT	204
Inh βA 3	KF870469	ATTACCTTTGCAGAGGCTGGCG	GGAGGAAGATCCATGCATTAGC	106
Inh βA 4	KF870470	CTTACCTTCGCAGAGGCCGGT	GGAAGATCCATACATTAGCCTG	106
*Fst a1**	NM_001160483.1	TGTAAAGTCCATCTGGACCTAGAA	GATCCGATTACACATCACACAATAT	138
*Fst a2**	NM_001160488	GTAAAGTCCATCTGGACCTGGAT	TCCGATTACAGGTCACACAGTAC	135
Fst b1	KF870464	TTTACGCCAGCTCCTGCCAC	CCCGACACACAATGTCCTCG	120
Fst b2	KF870465	CTGCTCCAAAGTCACAAGGAGG	GGGCAGCGAACTCCATGACAC	159
GDF11	KF870466	GAGGACGAATATCACGCCACTAC	CTGAACTTGAAGAAGCAGCAGTTA	107

RESULTS

Phylogeny

This study was made possible by the sequences available from the rainbow trout genome project. We analyzed new sequences for TGF-β family presents in fish in a phylogenetic analysis of *TGF-β* other vertebrates: *TGF-β1*, *TGF-β2*, *TGF-β3* and *TGF-β6*. Were identified three *TGF-β1* isoforms in rainbow trout genome, referred to as *TGF-β1**a***, *TGF-β1**b*** and *TGF-β1**c***. All new sequences analyzed are active as disulphide-linked homo-or heterodimers, sequences motifs characteristics of the TGF-β family members. Sequences motifs to TGF-β family were identified cross of alignments with *TGF-β* sequences of different vertebrate groups. Cluster analyzes were performed using know and new sequences of *TGF-β* (data no presented), but for analyze phylogenetic were utilized only *TGF-β1* isoforms, *TGF-β2*, *-β3* and *-β6* because these are presents in fish (Figure 1).

We inferred the neighbor-joining tree followed by bootstrapping (1000

replicates) to examine the statistical reliability. The tree has shown clearly the existence of four monophyletic groups, *TGF-β1*, *-β2*, *-β3* and *-β6*. *TGF-β2*, *-β3* and *-β6* are single copy in the trout genome, and these are separated of the mammals and birds group. Probably *TGF-β2*, *-β3* and *-β6* were originated in 2nd WGD deriving from a common ancestor of mammals and birds. Perhaps this is the reason why mammals and birds form a group clearly separated from the other species of fish. *TGF-β1* exists in more than one copy in the trout genome, in effect, there are three isoforms. *TGF-β1a* and *-β1c* new sequences in rainbow trout are closest and appear to have been derived after of *TGF-β1b* in different fish species.

Multiple alignments

Follistatin

We analyzed four protein sequences of the *follistatin* present in rainbow trout, two sequences already published, referred to as *Fst a1* and *Fst a2*, and two new sequences identified from the genome sequencing of rainbow trout, referred to as *Fst b1* and *Fst b2*. Were performed alignments between sequences of follistatin of rainbow trout and publish sequences in amphibian (*Xenopus tropicalis*), mammal (*Mus musculus*), bird (*Gallus gallus*) and zebrafish (*Danio rerio*). The sequence of *follistatin* is conserved among species, and can display a high degree of similarity at 80% between different phylogenetic groups (Figure 2).

The nucleotide sequences of *follistatin* identified in rainbow trout are smaller than the sequences of *M. muscles*, *X .tropicalis*, *G. gallus* and *D. rerio*. However, resemble the sequences already published *follistatin*: *Fst a1*, *Fst a2*, *Fst b1* and *Fs tb2*, with 969, 582, 981 and 984 bp, respectively.

Inhibin βA

We analyzed four nucleotide sequences of *inhibin βA* present in rainbow trout. All four sequences presents are first described and here referred to as *Inh βA1*, *Inh βA2*, *Inh βA3* and *Inh βA4*. The sequences were very similar to each other and the primer design to gene expression profile analysis was possible only after exhaustive analysis of sequence-specific. The alignments analysis between *inhibin βA* of rainbow trout and *inhibin βA* sequences from other species reveals the great variability present in this gene. However, conserved regions are observed with 80% similarity, indicating that inhibin gene sequences are important in evolution course of the different vertebrates (Figure 3).

Gene expression profile

Not all new genes have introns and exons sequences well defined, corresponding to functional protein sequenced, because sequencing of rainbow trout genome is not finished. All DNA sequences were evaluated to the correspondence with the protein sequence. The beginning and end of each exon was extensively tested through the alignments from protein and nucleotide sequenced, between the nucleotide sequence identified for trout and other species; which correspond to the conserved domains of each family. After refined sequence analysis, it was possible to design primers specific for each gene and carry the gene expression profile.

Transforming growth factor beta (TGF-β)

All genes have different gene expression patterns in different tissues analyzed (P<0.005), confirming the function non-redundancy of the isoforms

and paralogs present in the genome of rainbow trout. We cannot define a clear gene expression pattern that could be associated with muscle growth in rainbow trout for *TGF-β1a*, *-β1b*, *-β1c* and *TGF-β6*. However, for *TGF-β2* and *-β3* genes was observed a major gene expression in red muscle followed by expression in gonads, male and female (P<0.001) (Figure 4). Surprisingly, the isoforms *TGF-β1a* and *-β1b* have more gene expression in tissues related to immune system and osmoregulation; as spleen, gill and kidney (P<0.001) (Figure 4A and 4B). Whereas *TGF-β1c* gene expression was detected in all tissues, including red and white muscles; confirming the importance of *TGF-β* family in different tissues, derived of embryonic annexes different. *TGF-β6* almost did not detect in the tissues analyzed, except gill and skin (*P*=0.001) (Figure 4F).

Inhibin βA

Were analyzed the gene expression for *Inh βA1*, *Inh βA2*, *Inh βA3* and *Inh βA4. Inh βA1*and *Inh βA2* showed expression profiles different in various tissues analyzed, unlike *Inh βA3* and *Inh βA4* that showed an expression pattern very similar (Figure 4I and 4J). Despite this similarity, sequencing of the amplified product confirmed the specificity of the primers utilized for this analysis. This is reinforced by the different temperatures of melting curve analysis (data not shown). *Inh βA3* and *Inh βA4* appear to have a major involvement in the gonads, especially in the testicle (*P*=0.008 and *P*=0.004, respectively). However, the expression level in intestine and gill was superior to other tissues, where almost no expression was detected. *Inh βA1* and *Inh βA2* showed different gene expression profile. *Inh βA1* showed higher expression level in testis and at lower levels in the ovaries, but still could be detected clearly (*P*=0.009) (Figure 4G). *Inh βA2* showed higher expression level in the liver, followed by adipose tissue (*P*<0.0001). Inversely to what was expected, *Inh βA2* showed a lower level of expression in testicle and a barely detectable expression in ovary (Figure 4H). In all others tissue analyzed, the level of *Inh βA1* and *Inh βA2* expression was quite low.

Follistatin

Among all the paralogs sequences analyzed, the *Fst a2* and *Fst b1* showed a wide expression in different tissues of rainbow trout (*P*<0.001 and *P*=0.007, respectively) (Figure 4L and 4M). *Fst a1* and *Fst a2* showed higher level of expression in red muscle indicating a possible participation in the myogenic, although the expression in white muscle was lower than in other tissues (*Fst a2*). However, *Fst a1* and *Fst a2* were more strongly expressed in the gonads; *Fst a1* in ovary and *Fst a2* in testis (*P*=0.008 and *P*=0.001, respectively) (Figure 4K and 4L).

DISCUSSION

Phylogeny

We believe that the observed tree topology is consistent with the evolutionary events described for salmonids, result of 4 events of whole genome duplication (WGD). Probably *TGF-β2*, *-β3* and *-β6* were originated in 2nd WGD deriving from a common ancestor of mammals and birds. The same hypothesis is not so easy to explain to *TGF-β6* because this gene occurs specifically in fish group (Funkenstein, Olekh, and Jakowlew 2010). However, the presence of

TGF-β6 in *Latimeria chalumnae* allows strengthens this hypothesis. *L. chalumnae* belonging to the Coelacanthimorpha group, which is the group of fish closest to terrestrial vertebrates that, like all other Actinopterygii not passed the 3rd WGD. Hypothesize that *TGF-β* are the result of the 2nd WGD event implies that the presence of only one copy of the gene after the 3rd and 4th WGD is the result of the evolutionary process.

TGF-β1 is present in more than one copy in the rainbow trout genome; in effect there are three isoforms. Conversely, *TGF-β1* has only one copy in mammals (Derynck et al. 1985). This fact probably indicates that after the second duplication event. This topology indicates that the derivation of *TGF-β1b* is preceding 4ht WGD, but the derivation of the sequences *TGF-β1a* and *-β1c* are postdating 4ht WGD.

The conservation of gene sequence *TGF-β* after these 4ht WGD and long evolutionary process is to confirm their importance in physiology and development, reinforcing the importance of studies to characterize this group of genes.

Gene expression profile

In this report, we hypothesized that the new gene sequences and isoforms of the *TGF-β*, *inhibin βA* and *follistatin* discoveries from the genome sequencing of rainbow trout are involvement in the course of myogenesis but do not have redundant functions. This hypothesis is strengthened because these new gene sequences and isoforms have a different pattern of gene expression in different tissues of rainbow trout as a result of gene and protein sequence different. All new sequences identified are well conserved among different vertebrate groups and also between different species analyzed, indicating the evolutionary importance of these sequences.

In fact, the analysis of gene expression reveals different expression patterns in different tissues evaluated for both new gene sequences (*TGF-β*, *inhibin βA* and *follistatin*) as for *TGF-β1* isoforms (Figure 4), which also supports the hypothesis of different roles for different gene sequences analyzed. This work, only the *TGF-β*2 and *-β*3 expression suggest an involvement in the course of myogenesis in rainbow trout due the highest expression level of transcripts observed in red muscle following of the white muscle (Figure 4D and E). However, *in vitro* studies are necessary to more clearly understand the relationship between *TGF-β2* and *-β3* with the regulation of muscle growth. *TGF-β1b* and *-β1c* isoforms, *Fst a1*, *a2*, *b1* and *b2* also showed expression in white and red muscle, although in a much less magnitude than in other tissues. However this is not sufficient to discard the involvement of the genes and isoforms in myogenesis. It is known that gene expression is temporal and would be needed analysis of expression in different moments of myogenesis to eliminate the involvement these genes on muscle growth (Hutcheson and Kardon 2009).

Since its initial discovery, TGF-β superfamily has been considered a multifunctional peptide in many cell types. The *TGF-β* are expressed and secreted near their biological target, acting as paracrine or autocrine factors (Bilezikjian et al. 2006). This fact becomes more complex the interpretation of the expression profile of *TGF-β* and other genes involved in muscle growth

pathway and its relationship with the different physiological events. In mammals and fish, *TGF-β* and *inhibin βA* are key regulators involved in the migration (Richards, Enders, and Resnick 1999), survival and proliferation of the primordial germ cells (PGC) during the fetal life (Itman et al. 2006; Itman et al. 2009) and a important role in germ cell differentiation (Neumann et al. 2011). The high expression of the *TGF-β* and *inhibin βA* in gonads, ovary and/or testicle, may be the result of actuation of this genes on gonadal development in rainbow trout. It is possible that the expression of *Fst a1*, *a2* and *b1* (even that *Fst b1* has been detected at lower level) detected in the gonads may be a response to expression of *inhibin βA*, considering that the activity of inhibin is regulated by follistatin, its binding protein (Phillips and de Kretser 1998; Wang et al. 1999). The follistatin to regulate by sequestration of inhibin allows the cell signaling cascade (Matzuk et al. 1995; Sidis et al. 2006; Schneyer et al. 2008).

Actually, despite the evidenced importance gonadal development reported in the literature we believe that the levels of gene expression detected here for *TGF- β*2 and *-β*3 are related to muscle growth. *TGF-β1*, *-β*2 and *-β*3 also are associated as regulators of embryonic skeletal muscle development (Sanders, Hu, and Wride 1994; Kocamis, H., and J. Killefer. 2003). However, these factor stimulates the growth of normal cells in culture but can acting like an inhibitor or a stimulator depending on the culture conditions (Roberts et al. 1985; Shipley et al. 1986). *TGF-β2* and *-β3* were associated with muscle growth in mammals, was observed increasing the gene expression after differentiation of the myoblast cell line C2C12, delaying differentiation while increasing proliferation (Lafyatis et al. 1991; Schabort et al. 2009).

Only two sequence showed the same pattern of gene expression in the different tissues analyzed, *Inh βA3* and *Inh βA4*, and is difficult to support the hypothesis of non-complementary function in this case (Figure 4I and J). Although sequencing has confirmed the specificity of the primers used in the analysis, the sequences of these genes are very close, suggesting the same or complementary function (Figure 3). The *TGF-β6*, specific to fish group, was associated with muscle growth in *Sparatus aurata* with a high number of transcripts detected in muscle (Funkenstein, Olekh, and Jakowlew 2010). Surprisingly, in the analysis performed on rainbow trout was not observed the same tendency of expression, being observed actually an expression almost undetectable (Figure 4F). However, the sampling conditions are different, which explains the difference of results.

Annex 1. TGF-β and myostatin protein gene sequences, species and access number of database used for construction of phylogenetic tree.

Gene	Species	Access number
Transforming growth factor beta 1 isoform a (TGF-β1a)	*Oncorhynchus mykiss*	KF870471
Transforming growth factor beta 1 isoform a (TGF-β1a)	*Danio rerio*	ENSDARG00000041502
Transforming growth factor beta 1 isoform b (TGF-β1b)	*Oncorhynchus mykiss*	KF870472
Transforming growth factor beta 1 isoform b (TGF-β1b)	*Danio rerio*	ENSDARG00000034895
Transforming growth factor beta 1 isoform c (TGF-β1c)	*Oncorhynchus mykiss*	KF870473
Transforming growth factor beta 1 (TGF-β1)	*Sparus aurata*	AAN03842.1
Transforming growth factor beta 1 (TGF-β1)	*Tetraodon nigroviridis*	CAG12751.1
Transforming growth factor beta 1 (TGF-β1)	*Mus musculus*	CAA08900.1
Transforming growth factor beta 1 (TGF-β1)	*Homo sapiens*	NP_000651.3
Transforming growth factor beta 1 (TGF-β1)	*Gallus gallus*	P09531.2
Transforming growth factor beta 2 (TGF-β2)	*Oncorhynchus mykiss*	KF870474
Transforming growth factor beta 2 (TGF-β2)	*Danio rerio*	AAQ18012.1
Transforming growth factor beta 2 (TGF-β2)	*Atlantic code*	ENSGMOT00000019121
Transforming growth factor beta 2 (TGF-β2)	*Mus musculus*	AAH11170.1
Transforming growth factor beta 2 (TGF-β2)	*Homo sapiens*	AAA50405.1
Transforming growth factor beta 2 (TGF-β2)	*Gallus gallus*	NP_001026216.1
Transforming growth factor beta 3 (TGF-β3)	*Oncorhynchus mykiss*	KF870475
Transforming growth factor beta 3 (TGF-β3)	*Danio rerio*	AAU14139.1
Transforming growth factor beta 3 (TGF-β3)	*Tetraodon nigroviridis*	CAG09133.1
Transforming growth factor beta 3 (TGF-β3)	*Mus musculus*	AAI08427.1
Transforming growth factor beta 3 (TGF-β3)	*Homo sapiens*	CAA33024.1
Transforming growth factor beta 3 (TGF-β3)	*Gallus gallus*	NP_990785.1
Transforming growth factor beta 6 (TGF-β6)	*Oncorhynchus mykiss*	KF870476
Transforming growth factor beta 6 (TGF-β6)	*Sparus aurata*	ACY78405.1
Transforming growth factor beta 6 (TGF-β6)	*Gasterosteus aculeatus*	ENSGACG00000016971
Transforming growth factor beta 6 (TGF-β6)	*Takifugu rubripes*	ENSTRUG00000013167
Transforming growth factor beta 2 LIKE (TGF-β2)	*Atlantic code*	ENSGMOT00000009706
Transforming growth factor beta 2 LIKE (TGF-β2)	*Latimeria chalumnae*	ENSLACG00000017213
Myostatin 1a	*Oncorhynchus mykiss*	AAZ85121.1
Myostatin 1b	*Oncorhynchus mykiss*	ABA42586.1

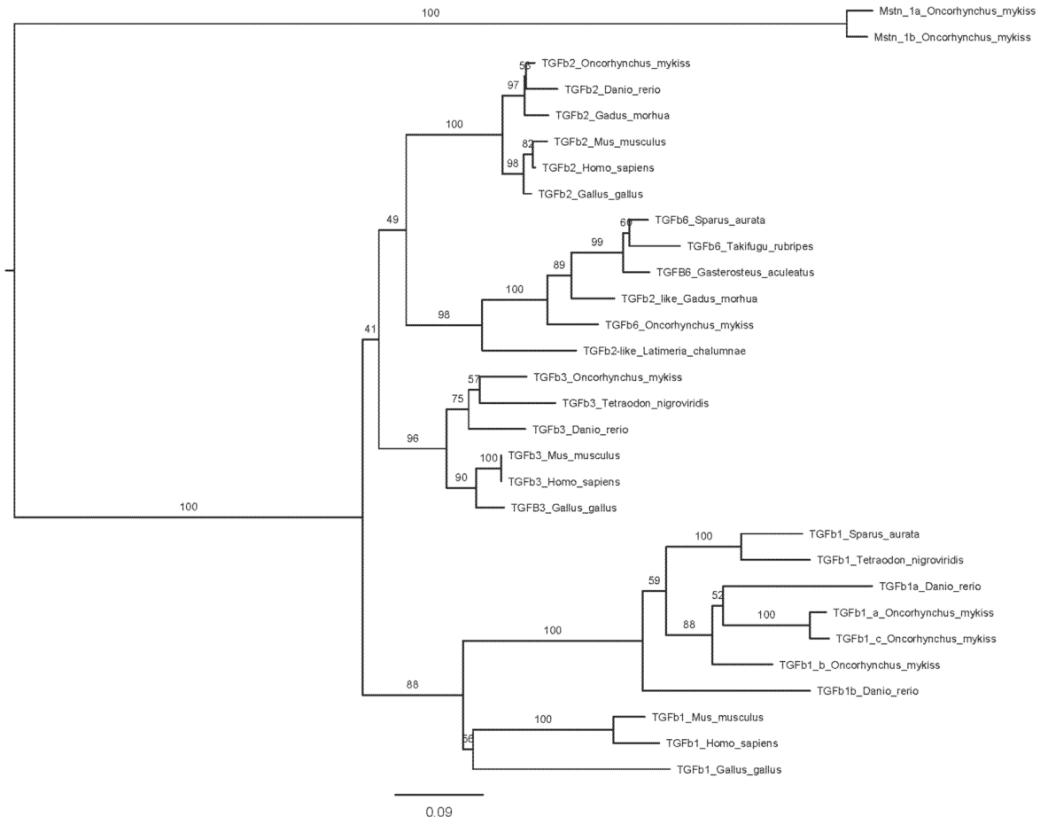


Figure 1. Phylogenetic tree obtained by amino acid sequence comparison for the TGF-β isoforms known in fish. The phylogenitic tree was constructed from a single multiple alignment of the complete protein sequences using neighbor-joining method by MEGA 5.2 tools. The substitution model was obtained by ProtTest 2.4 Server and the numbers at the tree nodes represent percentage bootstrap values after 1000 replicates. Full scientific names of species and respective accession numbers are detailed in Additional file.

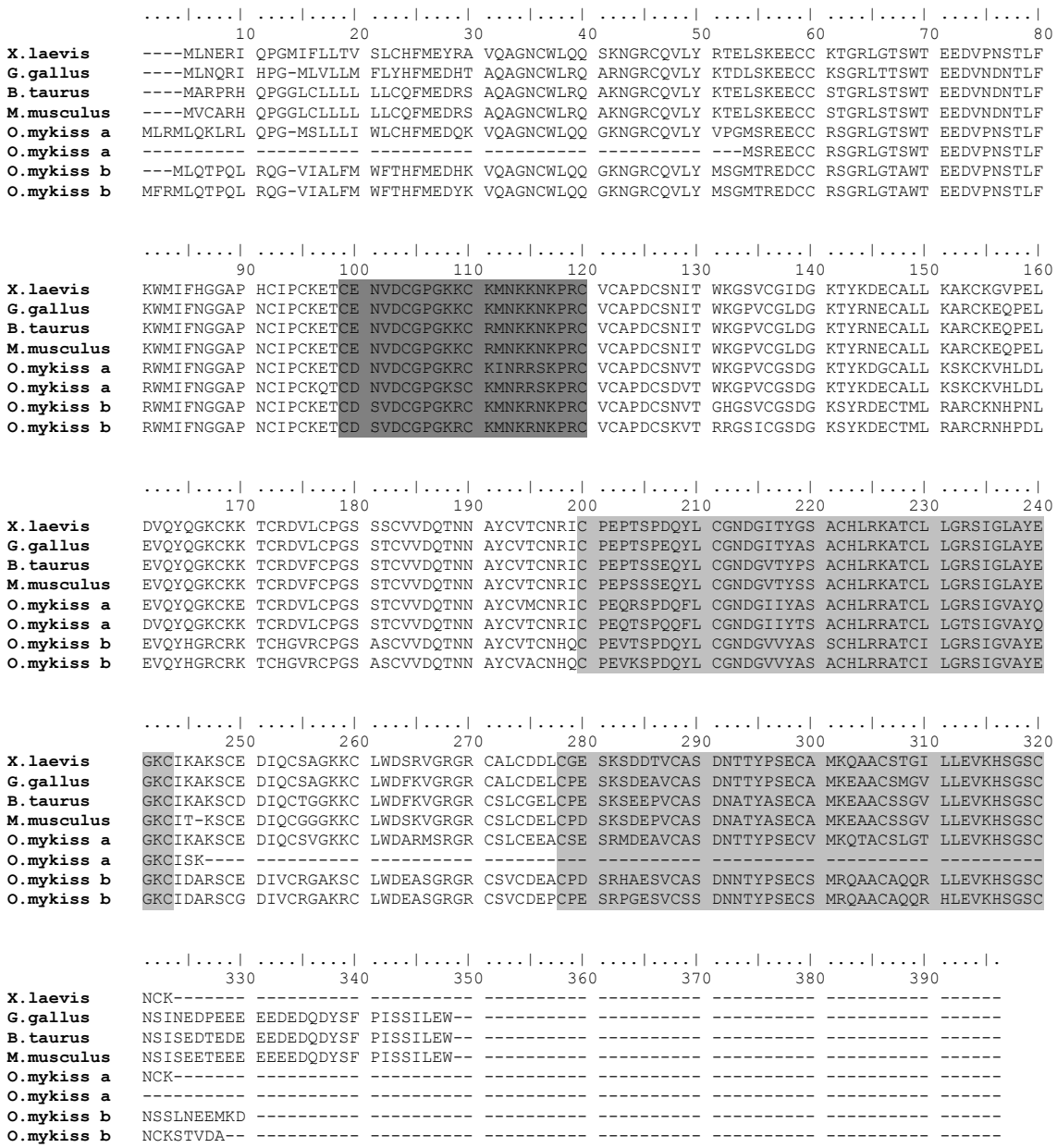


Figure 2. Multiple alignments for new protein sequences of the follistatin gene in *Oncorhynchus mykiss*. The abbreviation were used for species with GenBank ID and Ensembl as follows: *Danio rerio*, NM_131037.3; *Danio rerio* b, ENSDARG00000057992; *Mus musculus*, NM_008046.2; *Gallus gallus*, NM_205200.1; *Xenopus tropicalis*, BC080943.1. The sequences marked in dark gray represent Follistatin/Osteonectin-like EGF domain; the sequences marked in light gray represent FS domain consists of an N-terminal beta hairpin (FOLN/EGF-like domain) and a Kazal-like domain and has five disulfide bonds. The alignment was made from complete sequences using ClustalW Multiple alignment (Thompson et al., 1994) by BioEdit version 7.1.

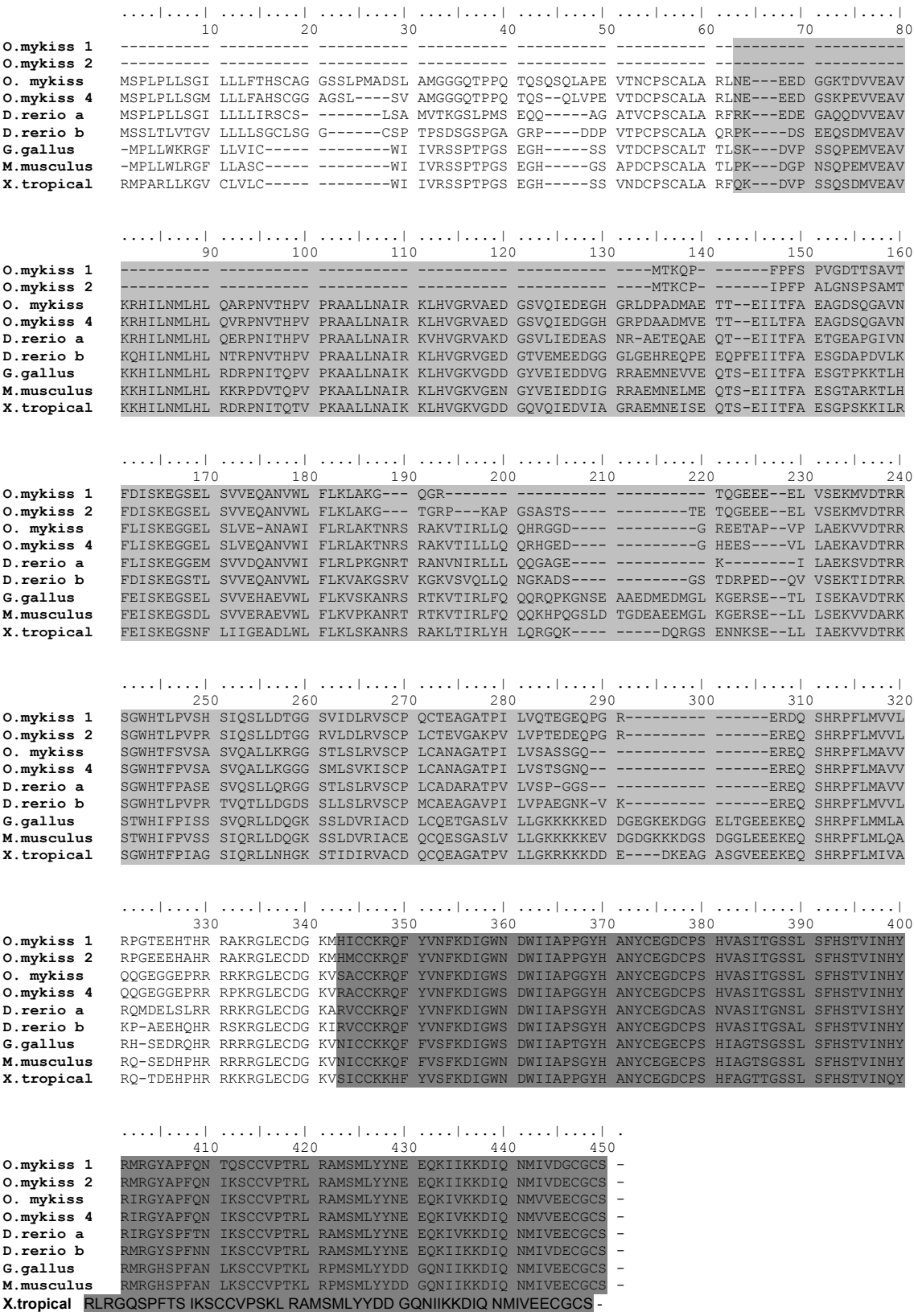
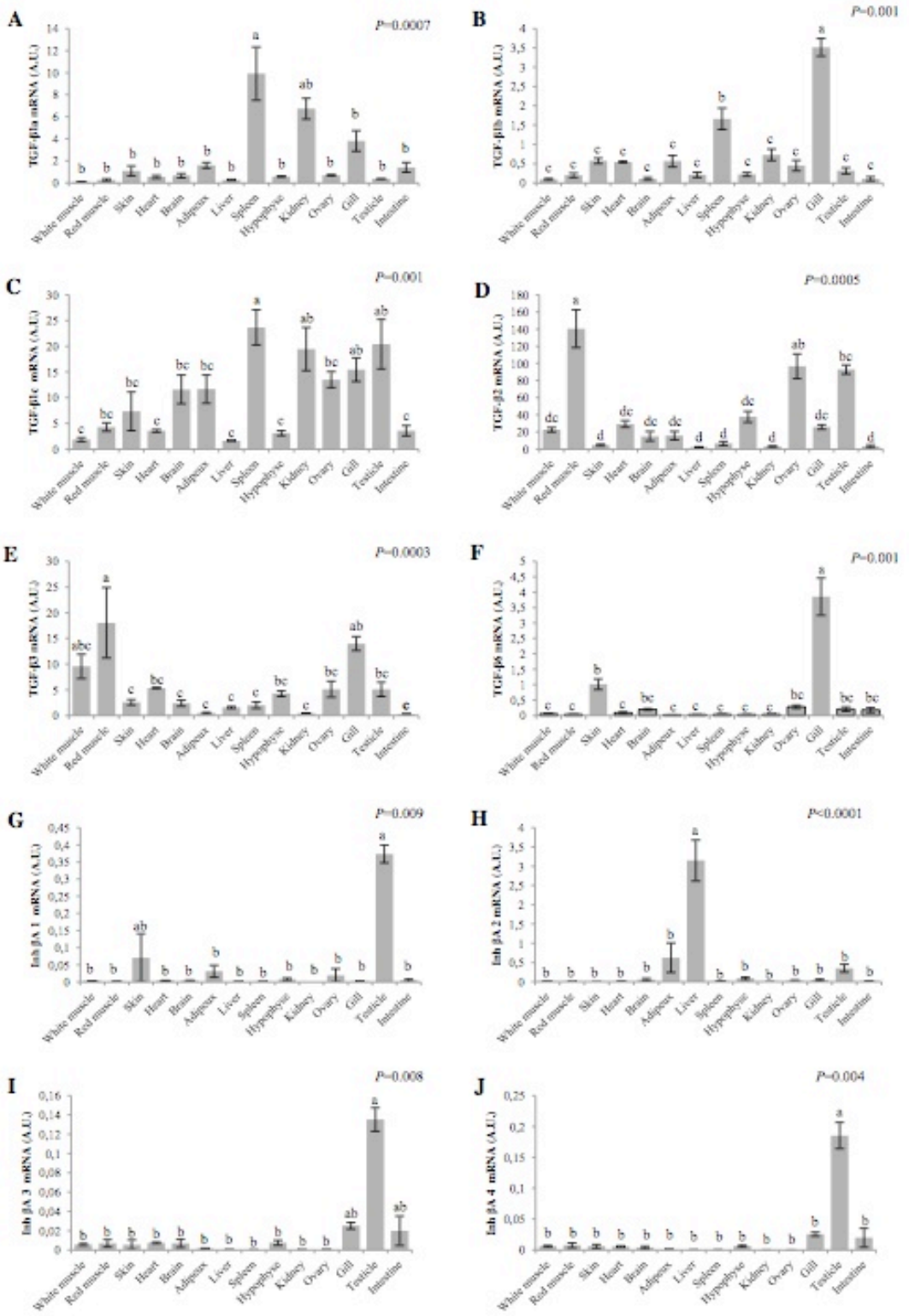


Figure 3. Multiple alignments between new protein sequences of the inhibin βA gene in *Oncorhynchus mykiss* and protein sequences of other vertebrate. The abbreviations were used for species with GenBank ID and Ensembl as follows: *Danio rerio a*, ENSDARP00000006400; *Danio rerio b*, ENSDARP00000047467; *Xenopus tropicalis*,

ENSXETP00000003924; *Gallus gallus*, ENSGALP00000036647; *Mus musculus*, ENSMUSP00000132085. The sequences marked in light gray represent the propeptide is known as latency associated peptide (LAP) in TGF-beta (LAP is a homodimer which is disulfide linked to TGF-beta binding protein); the sequences marked in dark gray represent disulphide-linked homo- or heterodimers and transforming growth factor beta like domain. The alignment was made from complete sequences using ClustalW Multiple alignment (Thompson et al., 1994) by BioEdit version 7.1.


A
P=0.0007
TGF-β1a mRNA (A.U.)
B
P=0.001
TGF-β1b mRNA (A.U.)
C
P=0.001
TGF-β1c mRNA (A.U.)
D
P=0.0005
TGF-β2 mRNA (A.U.)
E
P=0.0003
TGF-β3 mRNA (A.U.)
F
P=0.001
TGF-β5 mRNA (A.U.)
G
P=0.009
Inh βA 1 mRNA (A.U.)
H
P<0.0001
Inh βA 2 mRNA (A.U.)
I
P=0.008
Inh βA 3 mRNA (A.U.)
J
P=0.004
Inh βA 4 mRNA (A.U.)
White muscle
Red muscle
Skin
Heart
Brain
Adipose
Liver
Spleen
Hypophyse
Kidney
Ovary
Gill
Testicle
Intestine

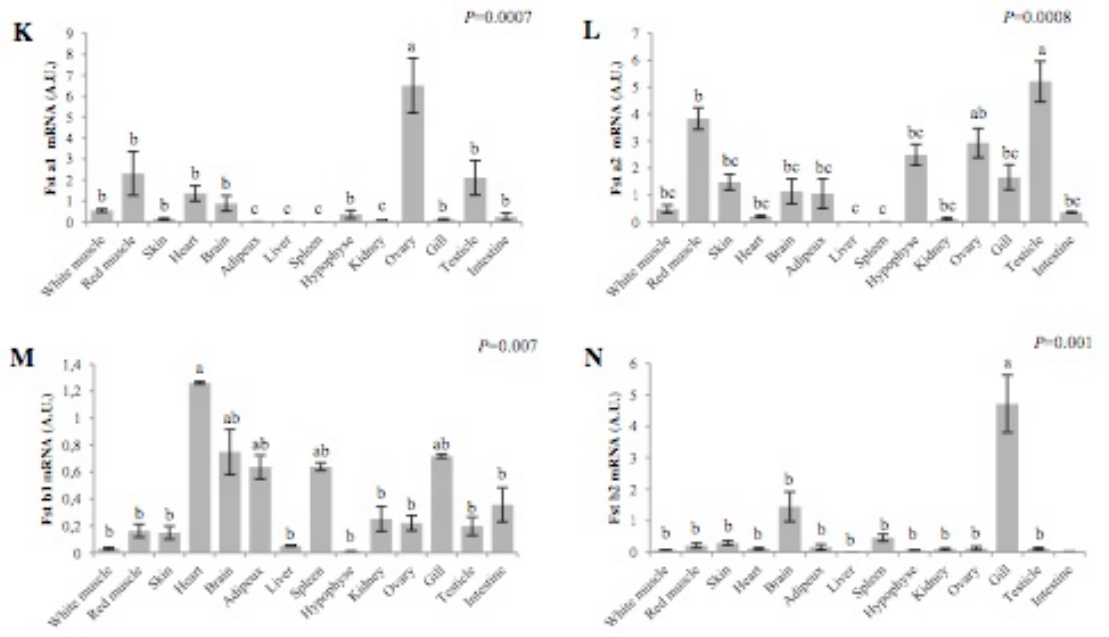


Figure 4. Gene expression of *TGF-β1a* (A), *TGF-β1b* (B), *TGF-β1c* (C), *TGF-β2* (D), *TGF-β3* (E), *TGF-β6* (F), *inhibin βA 1* (G), *inhibin βA 2* (H), *inhibin βA 3* (I), *inhibin βA 4* (J), *follistatin a1* (K), *follistati a2* (L), *follistati b1* (M) and *follistati b2* (N) in rainbow trout tissues. Total RNA was extract from three imature fish (±200 g). The results are presented as a ratio of gene expression and 18S rRNA expression. Letters reveal the significant differences between means in rainbow trout tissues (Wilcoxon/Kruskal–Wallis rank test; $P<0.05$).

CONCLUSION

We showed that the sequence of the mature protein and nucleotídica are extremely well conserved between the genes different of *TGF-β*, *inhibin βA* and *follistatin*, whereas their regulation and expression pattern are different. *TGF-β2* and *–β3* are the main TGF-β members expressed in muscle, white and red.

REFERENCES

Amthor H, Nicholas G, McKinnell I, Kemp CF, Sharma M, Kambadur R & Patel K 2004 Follistatin complexes Myostatin and antagonises Myostatin-mediated inhibition of myogenesis. *Developmental Biology* **270** 19–30. (doi:10.1016/j.ydbio.2004.01.046)

Bilezikjian LM, Blount AL, Donaldson CJ & Vale WW 2006 Pituitary actions of ligands of the TGF-β family: activins and inhibins. *Reproduction* **132** 207–215. (doi:10.1530/rep.1.01073)

Derynck R, Jarrett JA, Chen EY, Eaton DH, Bell JR, Assoian RK, Roberts AB, Sporn MB & Goeddel DV 1985 Human transforming growth factor-beta complementary DNA sequence and expression in normal and transformed cells. *Nature* **316** 701–705.

Funkenstein B, Olekh E & Jakowlew SB 2010 Identification of a novel transforming growth factor-? (TGF-?6) gene in fish: regulation in skeletal muscle by

nutritional state. *BMC Molecular Biology* **11** 37. (doi:10.1186/1471-2199-11-37)

Guyomard R, Boussaha M, Krieg F, Hervet C & Quillet E 2012 A synthetic rainbow trout linkage map provides new insights into the salmonid whole genome duplication and the conservation of synteny among teleosts. *BMC Genetics* **13** 15. (doi:10.1186/1471-2156-13-15)

Hutcheson DA & Kardon G 2009 Genetic manipulations reveal dynamic cell and gene functions: Cre-ating a new view of myogenesis. *Cell Cycle (Georgetown, Tex.)* **8** 3675–3678.

Itman C, Mendis S, Barakat B & Loveland KL 2006 All in the family: TGF-beta family action in testis development. *Reproduction (Cambridge, England)* **132** 233–246. (doi:10.1530/rep.1.01075)

Itman C, Whiley PAF, Zhou W, Meistrich M, Sahin Z & Loveland KL 2009 Regulated production of SnoN2 is a feature of testicular differentiation. *Microscopy Research and Technique* **72** 833–844. (doi:10.1002/jemt.20739)

Janitz M 2007 Assigning functions to genes--the main challenge of the post-genomics era. *Reviews of Physiology, Biochemistry and Pharmacology* **159** 115–129. (doi:10.1007/112_2007_0703)

Lafyatis R, Lechleider R, Roberts AB & Sporn MB 1991 Secretion and transcriptional regulation of transforming growth factor-beta 3 during myogenesis. *Molecular and Cellular Biology* **11** 3795–3803.

Lee SJ & McPherron AC 2001 Regulation of myostatin activity and muscle growth. *Proceedings of the National Academy of Sciences of the United States of America* **98** 9306–9311. (doi:10.1073/pnas.151270098)

Lee S-J, Huynh TV, Lee Y-S, Sebald SM, Wilcox-Adelman SA, Iwamori N, Lepper C, Matzuk MM & Fan C-M 2012 Role of satellite cells versus myofibers in muscle hypertrophy induced by inhibition of the myostatin/activin signaling pathway. *Proceedings of the National Academy of Sciences of the United States of America* **109** E2353–2360. (doi:10.1073/pnas.1206410109)

Matzuk MM, Lu N, Vogel H, Sellheyer K, Roop DR & Bradley A 1995 Multiple defects and perinatal death in mice deficient in follistatin. *Nature* **374** 360–363. (doi:10.1038/374360a0)

McPherron AC, Lawler AM & Lee SJ 1997 Regulation of skeletal muscle mass in mice by a new TGF-beta superfamily member. *Nature* **387** 83–90. (doi:10.1038/387083a0)

Neumann JC, Chandler GL, Damoulis VA, Fustino NJ, Lillard K, Looijenga L, Margraf L, Rakheja D & Amatruda JF 2011 Mutation in the type IB bone morphogenetic protein receptor Alk6b impairs germ-cell differentiation and causes germ-cell tumors in zebrafish. *Proceedings of the National Academy of Sciences of the United States of America* **108** 13153–13158. (doi:10.1073/pnas.1102311108)

Phelps MP, Jaffe IM & Bradley TM 2013 Muscle growth in teleost fish is regulated by factors utilizing the activin II B receptor. *The Journal of Experimental Biology*.

(doi:10.1242/jeb.086660)

Phillips DJ & de Kretser DM 1998 Follistatin: a multifunctional regulatory protein. *Frontiers in Neuroendocrinology* **19** 287–322. (doi:10.1006/frne.1998.0169)

Richards AJ, Enders GC & Resnick JL 1999 Activin and TGFbeta limit murine primordial germ cell proliferation. *Developmental Biology* **207** 470–475. (doi:10.1006/dbio.1998.9174)

Roberts AB, Anzano MA, Wakefield LM, Roche NS, Stern DF & Sporn MB 1985 Type beta transforming growth factor: a bifunctional regulator of cellular growth. *Proceedings of the National Academy of Sciences of the United States of America* **82** 119–123.

Sanders EJ, Hu N & Wride MA 1994 Expression of TGFβ1/β3 during early chick embryo development. *The Anatomical Record* **238** 397–406. (doi:10.1002/ar.1092380314)

Schabort EJ, van der Merwe M, Loos B, Moore FP & Niesler CU 2009 TGF-beta's delay skeletal muscle progenitor cell differentiation in an isoform-independent manner. *Experimental Cell Research* **315** 373–384. (doi:10.1016/j.yexcr.2008.10.037)

Schneyer AL, Sidis Y, Gulati A, Sun JL, Keutmann H & Krasney PA 2008 Differential antagonism of activin, myostatin and growth and differentiation factor 11 by wild-type and mutant follistatin. *Endocrinology* **149** 4589–4595. (doi:10.1210/en.2008-0259)

Shipley GD, Pittelkow MR, Wille JJ Jr, Scott RE & Moses HL 1986 Reversible inhibition of normal human prokeratinocyte proliferation by type beta transforming growth factor-growth inhibitor in serum-free medium. *Cancer Research* **46** 2068–2071.

Sidis Y, Mukherjee A, Keutmann H, Delbaere A, Sadatsuki M & Schneyer A 2006 Biological activity of follistatin isoforms and follistatin-like-3 is dependent on differential cell surface binding and specificity for activin, myostatin, and bone morphogenetic proteins. *Endocrinology* **147** 3586–3597. (doi:10.1210/en.2006-0089)

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F & Higgins DG 1997 The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* **25** 4876–4882.

Wang EY, Draper LB, Lee E, Polak A, Sluss P, Weiss J & Woodruff TK 1999 Identification of naturally occurring follistatin complexes in human biological fluids. *Biology of Reproduction* **60** 8–13.

Zhang J 2003 Evolution by gene duplication: an update. *Trends in Ecology & Evolution* **18** 292–298. (doi:10.1016/S0169-5347(03)00033-8)

[5]CAPÍTULO VI

[5] Artigo redigido de acordo com as normas do periódico *Journal of Molecular Endocrinology*

TGF-B2, TGF- B3 and Inhibin βA are regulated during myogenesis in vivo and in vitro in Oncorhynchus mykiss

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INTRODUCTION

Muscle growth in animals is controlled by numerous of growth factors that interacting of complex form in pathways of the cell signaling. Whereas the muscle stem cells, also called the satellite cells, are quiescent in mammals, in fish that exhibit continuous growth, these cells have a strong activity. In particular, transforming growth factor-β (TGF-β) superfamily exerts a strongly control over proliferation, migration and adhesion on the satellite cells, which are essential in growth processes (Kollias and McDermott, 2008). The TGF-β superfamily constitutes of a large number of structurally related, extracellular polypeptide growth factors that regulate a diverse spectrum of biological processes. Major advance has been made in understanding the role of TGF-β and its closely related family member, myostatin (MSTN), in skeletal muscle ontogeny and postnatal physiology. However, research on TGF-β in fish has been limited to its involvement in the immune system and in reproduction, but very few information is available to date on the possible involvement of TGF-β in fish muscle growth (Chim et al., 2011; Harms et al., 2003; Yang and Zhou, 2008). Transforming growth factor-β1 (TGF-β1), a multifunctional regulator of cell growth and differentiation (Roberts and Sporn, 1985), has been shown to inhibit both myoblast proliferation and differentiation (Allen and Boxhorn, 1987). TGF-β2 and TGF-β3 in mammals, increasing the gene expression after differentiation of the myoblast cell line C2C12, delaying cell differentiation while increasing cell proliferation (Lafyatis et al., 1991; Schabort et al., 2009).

Different secreted proteins have common domains able to bind to growth factors and thereby regulate their activity (Maurer et al., 1995; Tanaka et al., 1998; Zwijsen et al., 1994). Interestingly, only follistatins are reported to bind TGF-b family members, in particular activins/inhibin and bone morphogenetic protein (BMP) family members (Bickel et al., 2008; Nakamura et al., 1990; Roberts-Galbraith and Newmark, 2013). Several studies have revealed a role of extreme importance of follistatin in muscle growth in both mammals and fish (Amthor et al., 2004; Lee and McPherron, 2001; Matzuk et al., 1995; Medeiros et al., 2009). Initially, it was assumed that follistatin interacted with myostatin by binding and blocking it, resulting in a phenotype of muscle growth. However, transgenic mice overexpressing of follistatin and lacking myostatin can also cause substantial muscle growth. Indeed, a quadrupling of muscle mass was observed, higher than the phenotypes observed when the follistatin was overexpressed or when the myostatin has been blocked. These results suggesting that other TGF-β related ligands normally cooperate with myostatin to suppress muscle growth (Lee, 2007).

Extensive effort directed at understanding the mechanisms of muscle growth and role of the genes involved has been made. However, investigate the regulation in rainbow trout is complicated due the two whole genome duplication occurred in early vertebrate lineage evolution (Guyomard et al., 2012). Those duplication events create extra copies of the genes that can remain expressed and disappear or either duplicated copies can get new atemporal role developing a novel function (Zhang, 2003). In this regard, a considerable

progress was obtained recently by genome sequencing of the rainbow trout allowing us here characterize the specific genes expression profile involved with muscle growth. To evaluate the muscle growth regulation were measured the gene expression profile of the *TGF-β2*, *TGF-β3*, and four new sequences for genes to both *inhibin* β_A (*inhβA*) and *follistatin* (*fst*) during the myogenesis in rainbow trout. We analyzed the effect of growth factors such as Growth Hormone, Insulin-like Growth Factor 1 and Myostatin on mRNA levels in satellites cells culture.

METHODOLOGY

Experimental design

Refeeding

Were evaluated two groups of female immature trout with a mean weight of 136 ± 10 g. Three experimental groups (S1, S2 and S3) and control group were cultured in triplicate (50 fish/tank) and were starved for 1 month and refed for 1 month with food available *ad libitum*. Before starvation (day 30) and at days 0, 1, 2, 4, 7, 15 and 29 following refeeding, 7 fish were sampled to each day alternating tank collected to minimize the animal stress. For the control group, 7 fish were sampled to each day: 0 and 29. The control group had food available *ad libitum* throughout the experiment. Fish were cultured in fish freshwater tanks (PEIMA-INRA, Sizun, France) under a natural photoperiod and fed with a commercial diet (BioMar, Nersac, France). The animals were anaesthetized with Eugenol (10 mL/L), on ice stunned and sacrificed by spinal section to collection of muscle.

Cell culture

Satellites cell culture was performing from immature rainbow trout extracted from 40g of white muscle according with Gabillard *et al.* (Gabillard et al., 2010). A first cell culture was carried out to evaluate the kinetics of cell proliferation and differentiation. This procedure yields suspension cells of the density of the 160.000.000 cells/cm^2. The cell suspension was divided equally among four boxes with six wells. To stimulate of cell proliferation was used F10 medium (Nutrient mixture Ham's F10, Sigma, ref: N6635 Sigma) with 10% of the fetal bovine serum (Sigma ref: F-7524) and to stimulate of cell differentiation was used DMEM (Dulbecco's Modified Eagle's Medium, Sigma, ref: D7777) with 2% of the fetal bovine serum. The cells were collected to RNA extraction on the third day of proliferation, following the first, second and third day of cell differentiation.

The second cell culture was carried out to evaluate of cell proliferation and differentiation with the growth factors. Was used the same protocol to cell proliferation and differentiation, as described above. This procedure yields suspension cells of the density of the 160.000.000 cells/cm^2. The cells were cultivated for seven days and hormones were added to the culture medium for 24 h: 0.5 nM of Growth Hormone (Rainbow Trout Recombinant GH) (Le Bail et al., 1991); 50 nM of Myostatin (Recombinant Human Myostatin, ProSpec CYT-418) and 4 nM Insulin-like growth factor 1 (Salmon/Trout IGF-1 *Oncorhynchus* Receptor Grade, GroPep WU100). The cell suspension was divided equally among five boxes with six wells. The cells were collected to RNA extraction on the third day of proliferation (proliferation control), first day of cell differentiation

(differentiation control) and after 24 h to each hormone. Each time sampled has been cultivated in six replicates.

RNA extraction and cDNA synthesis

Total RNA was extracted from satellite cells recovered from the substrate (cell culture experiment) and from 100 mg of muscle (refeeding experiment) using TRI Reagent (Sigma-Aldrich T9424). Extracted RNA was quantified based on absorbance at 260 nm (NanoDrop ND-1000 spectrophotometer), and 1 µg total RNA were used to perform the reverse transcription reaction (Applied Biosystems kit #N808-0234; Applied Biosystems, Foster City, CA,USA), stocked in -80°C.

Primers design mRNA quantification by Real-Time PCR

Was analyzed the DNA and protein sequences previously available in the private database of the *ANR GENOTROUT* project (INRA, France). Were analyzed four isoforms of the *follistatin* (*fst*), two previously published to trout, four new sequences of the *inhibin βA* subunit (*inhβA*) and complete sequences of the *TGF-β2* and *TGF-β3* genes (Table 1).

The design was performed based on the full-length cDNA sequences obtained from *ANR GENOTROUT* database (INRA, France) and sequences deposited in the public database. Different genes sequences were aligned by MEGA5 software to gene-specific primers design. Amplicon sequences were tested to secondary structures formation by mFOLD (SantaLucia Jr., 1998; Zuker M., 2003) and forward and reverse primers sequences alignments were assembled by BLAST (http://www.ncbi.nlm.nih.gov) to verify the specificity primers. The amplifications condition were optimized before the analysis of expression.

Quantitative PCR analyses were carried out with 5 µL cDNA using a real-time PCR kit provided with an SYBR® Green fluorophore (Eurogentec, Seraing, Belgium), according to the manufacturer's instructions with end-concentration 300 nM of each primer. The amplification was performed using the following cycle: 95°C, 20 s; 62°C, 1 min, for 40 times. The realtive abundance of target cDNA within the sample set was calculated from a serially diluted (1:1–1:256) (standard curve) cDNA pool using StepOneTM Software V2.0.2 (Applied Biosystems). Subsequently, real-time PCR data were normalized using elongation factor-1 alpha (eF1α) gene expression by dividing the raw data by the reference gene expression.

Statistical analysis

The refeeding and cell culture effect on gene expression profile and gene expression profile in different tissue was analyzed with a one-way ANOVA using the non-parametric Wilcoxon/Kruskal–Wallis rank test adjusted using the tukey method. All the data are presented as the mean$\pm_{S.E.M}$. All analyzes were performed using of the SAS 9.2 statistical package.

RESULTS

Refeeding

Compared to fasted animals, the analysis *TGF-β2* mRNA levels showed a significant decrease of expression mainly on days 1, 2 and 4 post-refeeding.

This down-regulation of *TGF-β2* gene expression was no longer observed 4 days after ingestion of food, returning to the same level of control expression from the day 7 (Fig. 1a). Differently, the animals maintained the same mRNA levels to *TGF-β3* throughout the fasting period, but seven days after ingestion of food was possible observer a increased significantly of the mRNA levels (P<0.001). The increases observed were 2-fold and 3.5-fold at 2 and 7 days, respectively, indicating a dramatic elevation of *TGF-β3* mRNA levels followed by a decline as refeeding proceeded (Fig. 1b).

Fsta1 mRNA levels followed the same profile expression of the control group, however, showed significant differences of expression between days -30 and the first day post-refeeding (P<0.01) (Fig. 1c). In contrast, *fsta2* expression increased throughout the period of fasting, but the mRNA levels abruptly decreasing in the first 24 hours after ingestion of food being maintained low throughout the refeeding period. *Fsta2* high expression was at day 0, significantly different from the control and other times analyzed (P<0.001) (Fig. 1d). *Fstb1* expression showed a tendency to decrease during the refeeding experiment. This significant decrease was observed in the fasting period and continued post-refeeding, however not significant (Fig. 1e). Inversely, *fstb2* expression presented the peak on day 2 and 4 after refeeding (P<0.01) (Fig. 1f).

No significant increase in mean *inhβA4* mRNA level was evidenced between fasted and refed trout (Fig. 1h). However, *inhβA1* mRNA level showing a large decrease in expression on day 2 after ingestion of food (P<0.001), and remained below the value in fasted fish throughout the refeeding period (Fig. 1g). It was not possible detect the expression of *inhβA2* and *inhβA3* in the fasted/ refeed experiment, so, these genes were not measured in the other conditions.

Cells culture

Kinetic of cell proliferation and differentiation

TGF-β2 and *TGF-β3* showed different mRNA level *in vitro*. *TGF-β2* mRNA level showed a significant decrease during the course of cell differentiation (P<0.05) (Fig. 2A). Indeed, *TGF-β2* mRNA levels were high only during the cell proliferation, after cell differentiation was observed a dramatic decrease in the mRNA levels and remained below by the end of the experiment. This level decreased was by 200% between third day of proliferation (P) and third days of cell differentiation (DM3) (P< 0.05). Differently, *TGF-β3* mRNA levels observed was lower in the period of cell proliferation which increased during the period of cell differentiation (DM). *TGF-β3* showed an increasing mRNA levels in 50% on the first day of cell differentiation (DM1) (P<0.05) (Fig. 2B).

No significant increase in mean *fsta1* and *inhβA4* mRNA levels were evidenced in kinetic of cell proliferation and differentiation (Fig. 2C and H). *Fstb1* showed a significant decrease of 84% in the mRNA levels in cell differentiation, with lower expression measured in third day (DM3) (P<0.05). Similarly, *fsta2, fstb2* and *inhβA1* showed a higher mRNA levels during cellular proliferation (PM) which have decreased during cell differentiation (DM); P=0.05, P<0.001 and P=0.001, respectively.

Growth factors in cell culture

We investigated the hypothesis of that transcription modulation by growth factors could inhibit or stimulate the *TGF-β2*, *TGF-β3*, *follistatin* and *inhibin βA* mRNA levels. We tested Growth hormone (GH), Insulin-like Growth Factor 1 (IGF-1) and Myostatin (MSTN) growth factors during 24h on satellites cell culture from rainbow trout. Effect was not observed on *TGF-β2* mRNA levels when applied GH, IGF-1 and MSTN growth hormones (Fig. 3A). IGF-1 stimulated the *TGF-β3* mRNA levels (P=0.01), however, was not observed the same trend by GH and MSTN, where the level of *TGF-β3* transcription was equal to the control (Fig. 3B).

The *fstb1* mRNA level was upregulated by GH (P=0.0008) and MSTN (P<0.001) growth factors. The increases observed were 2-fold compared to the control group, though stimulation was 3.4% less by GH (Fig. 3C). *Fstb2* responded to growth factors of very similar form, the mRNA levels were stimulated by GH and MSTN in the same ratio observed in *fstb1* (Fig. 3D). Increased *fstb2* mRNA level stimulated by MSTN was observed, but this was not significant. Differently, the mRNA levels for the *inhβA1* and *inhβA4* paralogs were modulated in different ways front of growth factors (Fig. 3E and F). GH and IGF-1 upregulated the *inhβA1* mRNA levels (P=0.001), compared to the control the transcripts expression were 2-fold and 3-fold at GH and IGF-1, respectively. Compared to control, MSTN and IGF-1 increased at 2-fold and 3-fold in mean *inhβA4* mRNA levels, respectively (P=0.005). This up-regulation of *inhβA4* gene expression was no longer observed by GH (P> 0.05) (Fig. 3F).

DISCUSSION

In this report, we hypothesized that the new sequences of the *TGF-β*, *inhibin βA* and *follistatin* available from genome sequencing of rainbow trout are involved in the muscle growth control. For this propose we analyzed the differential expression of these different genes in fasting and refeeding period; satellite cells cultured in the kinetics of proliferation and differentiation; and cell satellites supplemented with growth factors reported in the literature with key roles in myogenesis. These three experiences were performed in order to identify genes whose expression may indicate an involvement with muscle growth induced by different physiological contexts analyzed.

Indeed, *TGF-β2* and *TGF-β3* showed a differential expression post-refeeding, although in opposite directions, may indicate opposing functions in the regulation of muscle growth. *TGF-β2* may be more closely related to growth regulation due to the dramatic decrease in mRNA levels in the resumption of myogenesis post-refeeding of the animals (Fig. 1A). This is a pattern compatible with a function of negative regulator in muscle growth of rainbow trout. However, the *TGF-β3* expression may indicates an importance role in the resumption of myogenesis post-refeeding; which may involve activation/proliferation of the myogenic precursor. Here, we show that the hypothesized role to *TGF-β2* and *TGF-β3* in myogenesis are supported by consistence of the results obtained *in vivo* and *in vitro* (Fig. 1A and 2A; Fig. 1B and 2B; respectively). The results observed *in vivo* corroborate the results obtained *in vitro*. *TGF-β2* mRNA level was down-regulated soon after the resumption of myogenesis, post-refeeding (Fig. 1A), the same drastic decrease was observed in the period between cell proliferation (PM) and differentiation

(DM3) (Fig. 2A). *TGF-β3* mRNA level was up-regulated post-refeeding, reaching maximum expression level of the day 7 (Fig. 1B). The same is observed in the kinetics of cell proliferation and differentiation; the *TGF-β3* mRNA level was significantly lower during the days of cell proliferation (PM), increasing the its expression from the day 1 of differentiation (DM1) (Fig. 2B). Likewise as *TGF-β2*; the *fsta2* and *inhβA1* mRNA levels also showed a differential expression pattern which are consistents with expression of a negative regulator in myogenesis. *Fsta2* and *inhβA1* were down-regulated in the resumption of myogenesis (Fig. 1D and G). Throughout in the fasting period the mRNA levels were increased, post-refeeding was observed a sharp decrease in expression levels for both, *fsta2* and *inhβA1*. These high mRNA levels also were observed during the cell proliferation (PM) and decreased half from the second day of differentiation (DM2) (Fig. 2D and G).

During myoblast proliferation and differentiation, the expression of the myogenic regulatory factors are increased and stimulate a set of muscle-specific genes, resulting in myoblast proliferation and fusion to form multinucleated myotubes (Florini et al., 1991a). Previous studies have shown that TGF-β act in the regulation of differentiation and proliferation of different ways. For example, TGF-β1 is a negative regulator of muscle cell proliferation and differentiation (Vaidya et al., 1989; Martin et al., 1992). In the mouse C2 muscle cell line and in human myoblasts in primary culture, TGF-β1 inhibited expression of muscle-specific mRNAs and proteins, and impaired myotube formation (Gardner et al., 2011). In birds, *TGF-β1* mRNA level is increased during the 1 day posthatch and decreased after one week and remained low until the sixth week, there is a significant weight reduction of the 1 day for a 6 week. (Li and Velleman, 2009). In this report, was observed that the expression profile of *TGF-β2* mRNA during cell proliferation and differentiation in rainbow trout is very similar the *TGF-β1* expression in birds; an increase in the proliferation phase and decreased during cell differentiation. Moreover, *TGF-β2* considered an important in regulatation of muscle repair in mammals because levels of TGF-β2 immunoreactivity are found elevated in skeletal muscle disorders (Murakami et al., 1999). This pattern expression identified in *TGF-β2 in vivo* and *in vitro* may indicate a negative regulatory feedback to TGF-β2-induced signaling pathway during skeletal muscle growth and development.

Differently, the myogenin compose a regulatory pathway, which during myogenesis is up-regulated leading to terminal differentiation (Molkentin and Olson, 1996). The importance of myogenin actions in muscle is supported by many experimental observations. In rainbow trout, the myogenin expression post-refeeding has been an expression increase from cell differentiation period (Gabillard et al., 2006), likewise as the expression observed in *TGF-β3.* Similarly, the *TGF-β3* has its peak expression at day 7 post-refeeding, decreasing along the cell differentiation. The mRNA expression profile provides evidence that *TGF-β3* is involved in the regulation of myogenesis. This is corroborated by *in vitro* response that was up-regulated by IGF-1 which are potent stimulators of differentiation in myoblasts act by inducing expression of the myogenin gene (Florini et al., 1991b).

Fsta1 and *inhβA4* showed no difference in the mRNA levels in the resumption of myogenesis, post-refeeding, and also showed no differences in

expression between the period of cell proliferation and differentiation (Fig. 1C and 2C; Fig. 1H and 2H; respectively). These results were unexpected, because is reported in the literature the involvement of *follistatin* and *inhibin βA* in the regulation of muscle growth in mammals (Amthor et al., 2004; Lee, 2007; Lee and McPherron, 2001) and fish (Medeiros et al., 2009; Phelps et al., 2013). Indeed, mice homozygous for a targeted mutation in *follistatin*$^{+/-}$ have reduced muscle mass at birth, and the fact, *follistatin* $^{-/-}$ mice die immediately after birth (Matzuk et al., 1995). *Inhibin* β_A $^{-/-}$ was showed lead to embryonic lethality in mice, suggesting that perhaps this is the binder that cooperates with myostatin in muscle growth controlling (Lee, 2007). However, other paralogs of *follistatin* and *inhibin βA* discussed here showed expression differential; this confirms that the paralogs has no function redundant and that can act on different pathways. *Fstb1* showed no significant changes during fasting/refeeding (Fig. 1C), however, was observed a decrease in mRNA levels during cell differentiation (Figure 2C). *Fstb2* showed a pattern of expression similar to *TGF-β3* the resumption of myogenesis, an increase was observed in mRNA levels after refeeding (Fig. 1F). However, during the cell kinetics was observed a decrease of 8-fold between proliferation day (PM) and third day of differentiation (DM3) (Fig. 2E). We believe this is not consistent between experiments to *Fstb1* and *Fstb2* indicate the need for further studies before any conclusion, although the kinetic results have been confirmed in two experiments (data not shown).

Growth factors in cell culture

In order to verify the involvement of genes here analyzed in cell proliferation and differentiation, we hypothesized that the abundance of transcripts (mRNA levels) in muscle would be modulated by growth factors. Were tested three growth factors; growth hormone (GH), insulin-like growth factor 1 (IGF-1) and myostatin (MSTN); which are reported as major regulators of muscle growth in different farmed species.

It has been known for several decades that GH exerts a major impact on muscle fiber development, however, it is now recognized that the growth promoting effect of GH is mediated by IGF-1 and IGF-2 (Wang et al., 2004). Here, we find that IGF-1stimuladed *TGF-β3*, *inhβA1* and *inhβA4* mRNA levels in differentiated myoblasts (Fig. 3). Stimulation of myogenic differentiation by IGFs has been established for many years. Previous studies have shown that IGF-1 and IGF-2 stimulate myotube formation by interactions with IGF binding proteins (IGFBPs). TGF-β has been found to promote the expression of one or more of the six high-affinity IGFBPs; for example IGFBP-2, IGFBP-3, IGFBP-4, and IGFBP-5 (Guo et al., 1995; Hwa et al., 1997; Rousse et al., 2001; Verrecchia et al., 2001). Furthermore, in satellite cultured cells the addition of transforming growth factor1 (TGF-β1) in pigs increases IGFBP-3 mRNA by 200% and IGFBP-5 mRNA by 50% (Yi et al., 2001). We hypothesize that *TGF-β3*, *inhβA1* and *inhβA4* may be cooperating with protein ligands and other genes in the signaling cascade produced of the liver, the muscles and various other tissues by multiple mechanisms. The up-regulated by IGF-1 (by the same way that IGF 1 induces expression of the myogenin gene); the gene expression profile in cell proliferation and differentiation kinetic and other evidences discussed here corroborate this hypothesis. However, even with the response against IGF-1,

cannot support this hypothesis for *inhβA4* due the no consistence with other experiments presented here.

The MSTN also known as growth differentiation factor-8 (GDF-8), is a TGF-β family member that acts as a negative regulator of skeletal muscle mass (Gabillard et al., 2013). Myostatin activity itself is regulated by keeping it in its inactive form or by inactivating it through other proteins such as follistatin, which is capable of acting as a potent myostatin antagonist (Abe et al., 2009). As discussed above, follistatin regulating not only myostatin but also other TGF-β family members that cooperate with mysotatin to limit muscle growth. Genetic evidences indicates that *inhibin βA* may be one of these key cooperating ligands due only deletion in *inhβA* resulted increases in muscle mass in mice heterozygous; the deletions in others genes encoding the inhibin β-subunits not showed the same phenotype (Lee et al., 2010; Souza et al., 2008; Trendelenburg et al., 2009). We found that myostatin up-regulated *follistatins* and *inhibin βA* mRNA levels: *fstb1*, *fstb2* and *inhβA4* (Fig. 3C, D and F). In fact, our findings were not surprising, this expression pattern was expected because corroborate previously reported. We hypothesized that increased of mRNA levels was a negative response to myostatin present in the medium. Myostatin exogenous receive negative feedback by endogenous signaling for growth, increasing levels expreção their ligands.

Table 1. Access number, primers forward and reverse, and fragment amplified size (bp) for *transforming growth factor beta 2* (*TGF-β2*), *transforming growth factor beta 3* (*TGF-β3*), *folistatin* (*Fst*), *Inhibin βA subunit* (*InhβA*) and *elongation factor-1 alpha* (*eF1α*) new gene sequences (*sequence published).

Gene	Access number	Forward primer (5' - 3')	Reverse primer (5' - 3')	Size (bp)
TGF-β2	KF870474	TGAATGGTTGCTTCACAGAGACA	GTTTCCAGCTCCTCACTCTTATT	120
TGF-β3	KF870475	ACCAACCTCTTCAGAGCAGAGTT	GATGTAGCGCTGCTTAGCTATGT	129
InhβA1	KF870467	TAGGCGACACTACATCCGCTG	CCTCGTGTCCACCATCTTCTCA	167
InhβA2	KF870468	CTAGGAAACTCTCCATCTGCCA	CCGTGTGTCCACCATCTTCTCT	204
InhβA3	KF870469	ATTACCTTTGCAGAGGCTGGCG	GGAGGAAGATCCATGCATTAGC	106
InhβA4	KF870470	CTTACCTTCGCAGAGGCCGGT	GGAAGATCCATACATTAGCCTG	106
*Fst a1**	NM_001160 483.1	TGTAAAGTCCATCTGGACCTAGAA	GATCCGATTACACATCACACAATAT	138
*Fst a2**	NM_001160 488	GTAAAGTCCATCTGGACCTGGAT	TCCGATTACAGGTCACACAGTAC	135
Fst b1	KF870464	TTTACGCCAGCTCCTGCCAC	CCCGACACACAATGTCCTCG	120
Fst b2	KF870465	CTGCTCCAAAGTCACAAGGAGG	GGGCAGCGAACTCCATGACAC	159
eF1α		CATTGACAAGAGAACCATTGA	CCTTCAGCTTGTCCAGCAC	107

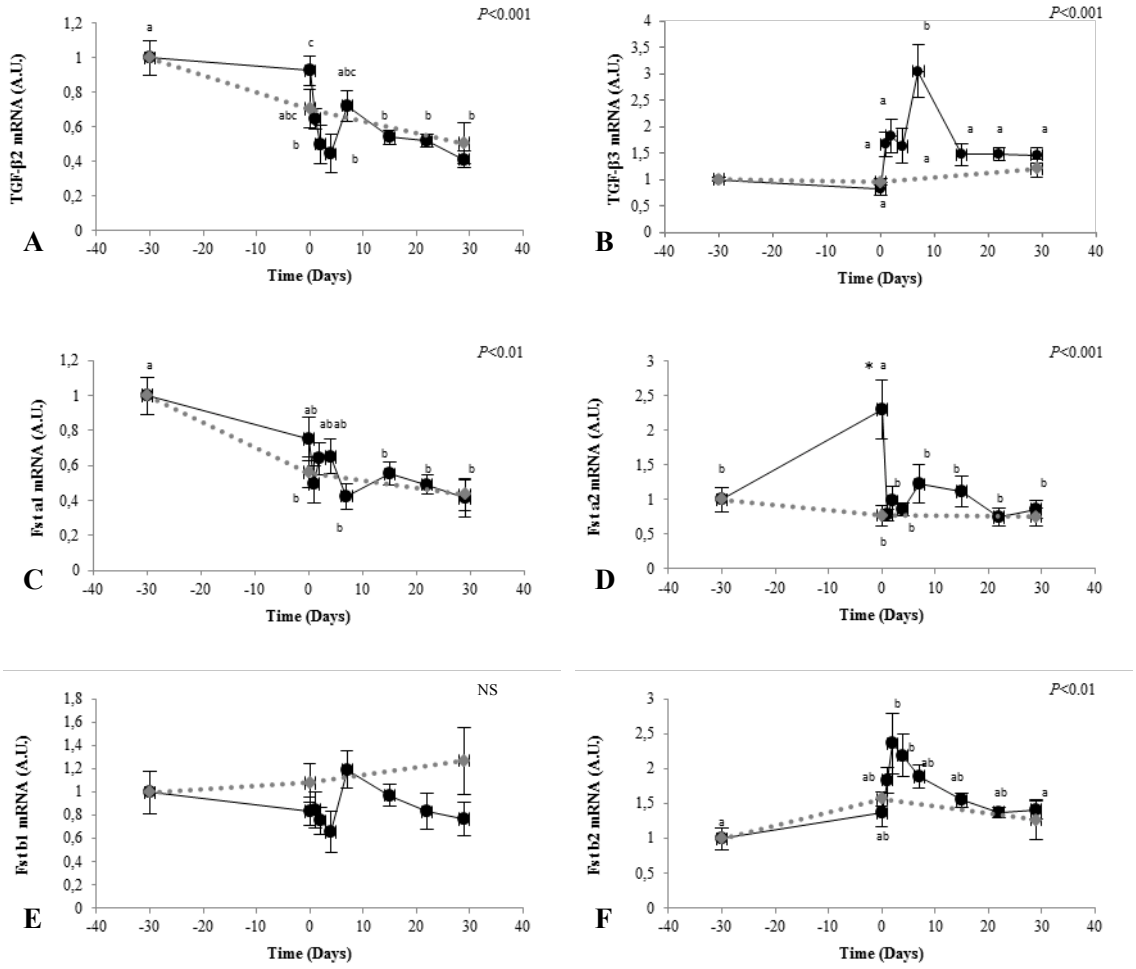

Figure 1. Expression profiles of transcripts to *TGF-β2* (**A**), *TGF-β3* (**B**), *follistatin a1* (**C**), *follistatin a2* (**D**), *follistatin b1* (**E**), *follistatin b2* (**F**), *inhibin βA 1* (**G**) and *inhibin βA 4* (**H**) in muscle. Fish were fasted for 1 month (days -30 to 0) and refed (days 1 to 29). The *P-value* corresponds the results of the one-way analysis of variance Kruskal-Wallis rank test, adjusted using tukey method ($P<0.05$). Bars indicate standard error of the mean. Different letters indicate a significant difference between groups. Stars indicates a significant difference between control (gray) and starved/refed (black) groups.

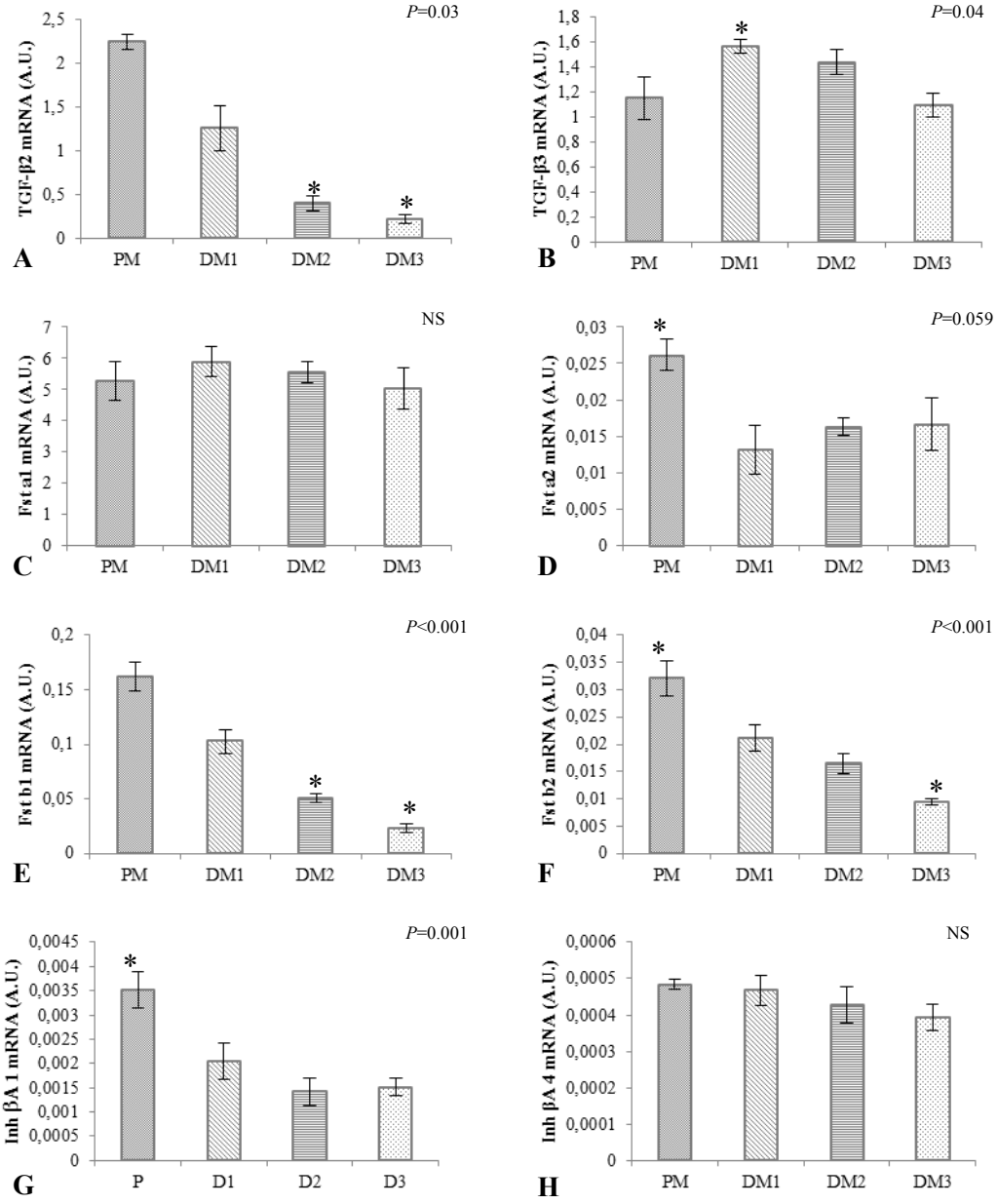

Figure 2. Expression profiles of transcripts to *TGF-β2* (**A**), *TGF-β3* (**B**), *follistatin b1* (**C**), *follistatin b2* (**D**), *inhibin βA 1* (**E**) and *inhibin βA 4* (**F**) in cell culture days: Proliferation (PM); 1st differentiation day (DM1), 2nd differentiation day (DM2) and 3rd differentiation day (DM3). The means of six repetitions in each group are show. The *P-value* corresponds the results of the one-way analysis of variance Kruskal-Wallis rank test, adjusted using tukey method ($P<0.05$). Bars indicate standard error of the mean. Stars indicate significantly different means between groups.

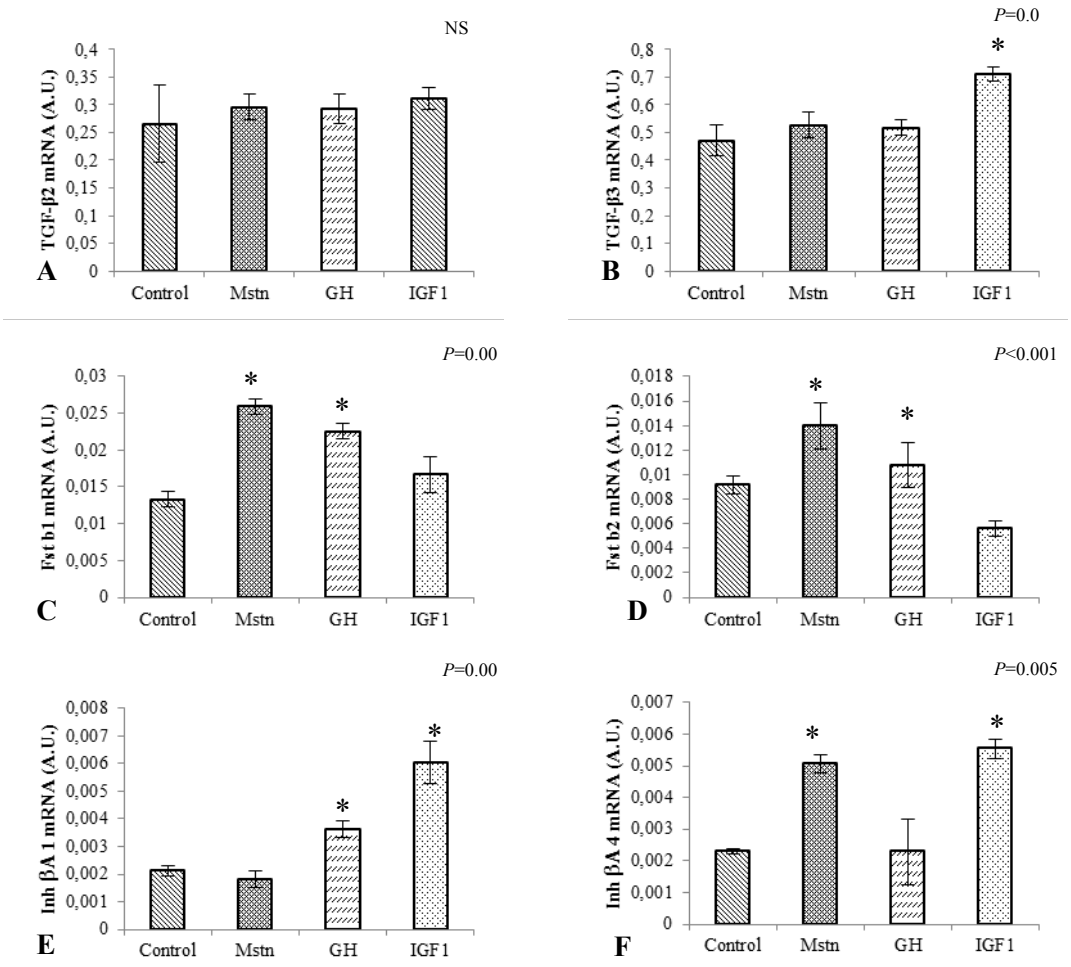

Figure 3. Expression profiles of transcripts to *TGF-β2* (**A**), *TGF-β3* (**B**), *follistatin b1* (**C**), *follistatin b2* (**D**), *inhibin βA 1* (**E**) and *inhibin βA 4* (**F**) in satellites cell culture with added hormones for 24h: Myostatin (Mstn) (50 nM); Growth hormone (GH) (0.5 nM) and Insulin-like growth factor 1 (IGF1) (4 nM). The results are presented as the means of six repetitions in each group. The *P-value* corresponds the results of the one-way analysis of variance Kruskal-Wallis rank test, adjusted using tukey method ($P<0.05$). Bars indicate standard error of the mean. Stars indicate significantly different means between hormone group and control group.

CONCLUSSION

In conclusion, we showed that among the new sequences described and analyzed here for the first time *TGF-β3* mRNA levels displays a differential pattern of expression compatible with a positive regulator in myogenesis (*in vivo* and *in vitro*), such as myogenin, what suggests that it may promote myogenesis. Our data also showed that *inhβA1* and to a lesser extend *TGF-β2*, exhibits a pattern of regulation compatible with a function as negative regulator of rainbow trout muscle growth.

REFERENCES

Abe S, Soejima M, Iwanuma O, Saka H, Matsunaga S, Sakiyama K & Ide Y 2009 Expression of myostatin and follistatin in Mdx mice, an animal model for muscular dystrophy. *Zoological Science* **26** 315–320. (doi:10.2108/zsj.26.315)

Amthor H, Nicholas G, McKinnell I, Kemp CF, Sharma M, Kambadur R & Patel K 2004 Follistatin complexes Myostatin and antagonises Myostatin-mediated inhibition of myogenesis. *Developmental Biology* **270** 19–30. (doi:10.1016/j.ydbio.2004.01.046)

Le Bail PY, Sumpter JP, Carragher JF, Mourot B, Niu PD & Weil C 1991 Development and validation of a highly sensitive radioimmunoassay for chinook salmon (Oncorhynchus tshawytscha) growth hormone. *General and Comparative Endocrinology* **83** 75–85.

Bickel D, Shah R, Gesualdi SC & Haerry TE 2008 Drosophila Follistatin exhibits unique structural modifications and interacts with several TGF-beta family members. *Mechanisms of Development* **125** 117–129. (doi:10.1016/j.mod.2007.09.013)

Chim H, Manjila S, Cohen AR & Gosain AK 2011 Molecular signaling in pathogenesis of craniosynostosis: the role of fibroblast growth factor and transforming growth factor-β. *Neurosurgical Focus* **31** E7. (doi:10.3171/2011.5.FOCUS1197)

Florini JR, Ewton DZ & Magri KA 1991a Hormones, growth factors, and myogenic differentiation. *Annual Review of Physiology* **53** 201–216. (doi:10.1146/annurev.ph.53.030191.001221)

Florini JR, Magri KA, Ewton DZ, James PL, Grindstaff K & Rotwein PS 1991b 'Spontaneous' differentiation of skeletal myoblasts is dependent upon autocrine secretion of insulin-like growth factor-II. *The Journal of Biological Chemistry* **266** 15917–15923.

Gabillard J-C, Kamangar BB & Montserrat N 2006 Coordinated regulation of the GH/IGF system genes during refeeding in rainbow trout (Oncorhynchus mykiss). *The Journal of Endocrinology* **191** 15–24. (doi:10.1677/joe.1.06869)

Gabillard JC, Sabin N & Paboeuf G 2010 In vitro characterization of proliferation and differentiation of trout satellite cells. *Cell and Tissue Research* **342** 471–477. (doi:10.1007/s00441-010-1071-8)

Gabillard J-C, Biga PR, Rescan P-Y & Seiliez I 2013 Revisiting the paradigm of myostatin in vertebrates: Insights from fishes. *General and Comparative Endocrinology* **194** 45–54. (doi:10.1016/j.ygcen.2013.08.012)

Gardner S, Alzhanov D, Knollman P, Kuninger D & Rotwein P 2011 TGF-? Inhibits Muscle Differentiation by Blocking Autocrine Signaling Pathways Initiated by IGF-II. *Molecular Endocrinology* **25** 128–137. (doi:10.1210/me.2010-0292)

Guo YS, Townsend CM Jr, Jin GF, Beauchamp RD & Thompson JC 1995 Differential regulation by TGF-beta 1 and insulin of insulin-like growth factor binding protein-2 in IEC-6 cells. *The American Journal of Physiology* **268** E1199–1204.

Guyomard R, Boussaha M, Krieg F, Hervet C & Quillet E 2012 A synthetic rainbow trout linkage map provides new insights into the salmonid whole genome duplication and the conservation of synteny among teleosts. *BMC Genetics* **13** 15. (doi:10.1186/1471-2156-13-15)

Harms CA, Howard KE, Wolf JC, Smith SA & Kennedy-Stoskopf S 2003 Transforming

growth factor-beta response to mycobacterial infection in striped bass Morone saxatilis and hybrid tilapia Oreochromis spp. *Veterinary Immunology and Immunopathology* **95** 155–163.

Hwa V, Oh Y & Rosenfeld RG 1997 Insulin-like growth factor binding protein-3 and -5 are regulated by transforming growth factor-beta and retinoic acid in the human prostate adenocarcinoma cell line PC-3. *Endocrine* **6** 235–242. (doi:10.1007/BF02820498)

Kollias HD & McDermott JC 2008 Transforming growth factor-beta and myostatin signaling in skeletal muscle. *Journal of Applied Physiology (Bethesda, Md.: 1985)* **104** 579–587. (doi:10.1152/japplphysiol.01091.2007)

Lafyatis R, Lechleider R, Roberts AB & Sporn MB 1991 Secretion and transcriptional regulation of transforming growth factor-beta 3 during myogenesis. *Molecular and Cellular Biology* **11** 3795–3803.

Lee S-J 2007 Quadrupling muscle mass in mice by targeting TGF-beta signaling pathways. *PloS One* **2** e789. (doi:10.1371/journal.pone.0000789)

Lee SJ & McPherron AC 2001 Regulation of myostatin activity and muscle growth. *Proceedings of the National Academy of Sciences of the United States of America* **98** 9306–9311. (doi:10.1073/pnas.151270098)

Lee S-J, Lee Y-S, Zimmers TA, Soleimani A, Matzuk MM, Tsuchida K, Cohn RD & Barton ER 2010 Regulation of Muscle Mass by Follistatin and Activins. *Molecular Endocrinology* **24** 1998–2008. (doi:10.1210/me.2010-0127)

Li X & Velleman SG 2009 Effect of transforming growth factor-β1 on embryonic and posthatch muscle growth and development in normal and low score normal chicken. *Poultry Science* **88** 265–275. (doi:10.3382/ps.2008-00234)

Matzuk MM, Lu N, Vogel H, Sellheyer K, Roop DR & Bradley A 1995 Multiple defects and perinatal death in mice deficient in follistatin. *Nature* **374** 360–363. (doi:10.1038/374360a0)

Maurer P, Hohenadl C, Hohenester E, Göhring W, Timpl R & Engel J 1995 The C-terminal portion of BM-40 (SPARC/osteonectin) is an autonomously folding and crystallisable domain that binds calcium and collagen IV. *Journal of Molecular Biology* **253** 347–357. (doi:10.1006/jmbi.1995.0557)

Medeiros EF, Phelps MP, Fuentes FD & Bradley TM 2009 Overexpression of follistatin in trout stimulates increased muscling. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology* **297** R235–242. (doi:10.1152/ajpregu.91020.2008)

Murakami N, McLennan IS, Nonaka I, Koishi K, Baker C & Hammond-Tooke G 1999 Transforming growth factor-beta2 is elevated in skeletal muscle disorders. *Muscle & Nerve* **22** 889–898.

Nakamura T, Takio K, Eto Y, Shibai H, Titani K & Sugino H 1990 Activin-binding protein from rat ovary is follistatin. *Science* **247** 836–838. (doi:10.1126/science.2106159)

Phelps MP, Jaffe IM & Bradley TM 2013 Muscle growth in teleost fish is regulated by factors utilizing the activin II B receptor. *The Journal of Experimental Biology*. (doi:10.1242/jeb.086660)

Roberts-Galbraith RH & Newmark PA 2013 Follistatin antagonizes Activin signaling and acts with Notum to direct planarian head regeneration. *Proceedings of the National Academy of Sciences* **110** 1363–1368. (doi:10.1073/pnas.1214053110)

Rousse S, Lallemand F, Montarras D, Pinset C, Mazars A, Prunier C, Atfi A & Dubois C 2001 Transforming growth factor-beta inhibition of insulin-like growth factor-binding protein-5 synthesis in skeletal muscle cells involves a c-Jun N-terminal kinase-dependent pathway. *The Journal of Biological Chemistry* **276** 46961–46967. (doi:10.1074/jbc.M104440200)

Schabort EJ, van der Merwe M, Loos B, Moore FP & Niesler CU 2009 TGF-beta's delay skeletal muscle progenitor cell differentiation in an isoform-independent manner. *Experimental Cell Research* **315** 373–384. (doi:10.1016/j.yexcr.2008.10.037)

Souza TA, Chen X, Guo Y, Sava P, Zhang J, Hill JJ, Yaworsky PJ & Qiu Y 2008 Proteomic identification and functional validation of activins and bone morphogenetic protein 11 as candidate novel muscle mass regulators. *Molecular Endocrinology (Baltimore, Md.)* **22** 2689–2702. (doi:10.1210/me.2008-0290)

Tanaka M, Ozaki S, Osakada F, Mori K, Okubo M & Nakao K 1998 Cloning of follistatin-related protein as a novel autoantigen in systemic rheumatic diseases. *International Immunology* **10** 1305–1314.

Trendelenburg AU, Meyer A, Rohner D, Boyle J, Hatakeyama S & Glass DJ 2009 Myostatin reduces Akt/TORC1/p70S6K signaling, inhibiting myoblast differentiation and myotube size. *American Journal of Physiology. Cell Physiology* **296** C1258–1270. (doi:10.1152/ajpcell.00105.2009)

Verrecchia F, Chu M-L & Mauviel A 2001 Identification of Novel TGF-β/Smad Gene Targets in Dermal Fibroblasts using a Combined cDNA Microarray/Promoter Transactivation Approach. *Journal of Biological Chemistry* **276** 17058–17062. (doi:10.1074/jbc.M100754200)

Wang J, Zhou J, Cheng CM, Kopchick JJ & Bondy CA 2004 Evidence supporting dual, IGF-I-independent and IGF-I-dependent, roles for GH in promoting longitudinal bone growth. *The Journal of Endocrinology* **180** 247–255.

Yang M & Zhou H 2008 Grass carp transforming growth factor-beta 1 (TGF-beta 1): molecular cloning, tissue distribution and immunobiological activity in teleost peripheral blood lymphocytes. *Molecular Immunology* **45** 1792–1798. (doi:10.1016/j.molimm.2007.09.027)

Yi Z, Hathaway MR, Dayton WR & White ME 2001 Effects of growth factors on insulin-like growth factor binding protein (IGFBP) secretion by primary porcine satellite cell cultures. *Journal of Animal Science* **79** 2820–2826.

Zhang J 2003 Evolution by gene duplication: an update. *Trends in Ecology & Evolution* **18** 292–298. (doi:10.1016/S0169-5347(03)00033-8)

Zwijsen A, Blockx H, Van Arnhem W, Willems J, Fransen L, Devos K, Raymackers J, Van de Voorde A & Slegers H 1994 Characterization of a rat C6 glioma-secreted follistatin-related protein (FRP). Cloning and sequence of the human homologue. *European Journal of Biochemistry / FEBS* **225** 937–946.

CONSIDERAÇÕES FINAIS

Existe um grande potencial para a expansão da aquicultura no Brasil, principalmente impulsionada pelo aumento na demanda mundial por alimentos. Neste sentido, o melhoramento genético vem ao encontro desta necessidade, possibilitando o aumento na produtividade e, assim, aumentando a eficiência da produção.

O objetivo inicial do melhoramento genético do tambaqui é o aumento do peso corporal em menor tempo de cultivo. Os valores de herdabilidade aqui estimados de média magnitude para as características morfométricas e de desempenho indicam que é possível obter progresso genético. Tendo em vista que o progresso genético é diretamente dependente da magnitude da herdabilidade da característica a ser melhorada.

O estudo de curva do crescimento em tambaqui revelou uma diferença de performance entre os sexos após a maturação sexual, entretanto, devido ao abate comercial ser ainda na fase juvenil, os dois sexos são indicados para o produção. Foi demostrado que os animais em idade de abate tem seu crescimento e aumento de peso do corpo obtidos por meio do incremento no numero de fibras musculares, bem como no aumento do seu calibre. Isso indica que o aumento da produtividade está diretamente relacionada com a hipertrofia muscular, ou seja, ao maior recrutamento de células satélites para a formação de novas fibras musculares, tendo como resultado o progresso genético da população selecionada.

Neste sentido, os resultados de expressão gênica diferencial observados no tecido muscular de animais juvenis *in vitro* e *in vivo* em *O. mykiss* tem grande relevância no contexto do melhoramento genético de peixes. Estes resultados indicam que os diferentes TGF-β e inibinas apresentam funções não redundantes em *O. mykiss* e que, assim como em outros vertebrados, promovem e regulam a proliferação e a diferenciação das células musculares de peixes. TGF-β3 tem expressão compatível com a de um regulador positivo da miogênese e inhβA e TGF-β2 apresentam padrão de expressão compatíveis com regulador negativo. Isto não só representa um novo insight sobre a diversidade molecular dos genes envolvidos no crescimento muscular, mas também sugere que os mecanismos de regulação de sinalização biológica do crescimento muscular são mais dinâmicos e complexos do que se pensava anteriormente.

Estes resultados não representam apenas a certeza do sucesso no melhoramento do tambaqui por meio da seleção tradicional e do cultivo de ambos os sexos ate idade de abate comercial, como também abre novos horizontes pela transferência das tecnologias desenvolvidas a partir dos resultados obtidos para *O. mykiss*.

REFERÊNCIAS BIBLIOGRÁFICAS

CHAGAS, E. C. et al. Tambaqui productivity reared in cages with different feeding rations. **Ciência Rural**, Santa Maria, v. 37, n. 4, p. 1109–1115, ago. 2007.

CROQUET, C. et al. Inbreeding depression for global and partial economic indexes, production, type, and functional traits. **Journal of dairy science**, Champaign, v. 89, n. 6, p. 2257–2267, jun. 2006.

FALCONER, D. S.; MACKAY, T. F. C. **Introduction to quantitative genetics**. Essex, England: Longman, 1996.

FROESE, R.; D. PAULY. **FishBase**. Disponível em: <http://www.fishbase.us/summary/Oncorhynchus-mykiss.html>. Acesso em: 3 jan. 2014.

GABILLARD, J. C.; SABIN, N.; PABOEUF, G. In vitro characterization of proliferation and differentiation of trout satellite cells. **Cell and tissue research**, Freiburg, v. 342, n. 3, p. 471–477, dez. 2010.

GABILLARD, J.-C. et al. Revisiting the paradigm of myostatin in vertebrates: Insights from fishes. **General and Comparative Endocrinology**, Amsterdam, v. 194, n. 1, p. 45–54, 1 dez. 2013.

GOULDING, M.; CARVALHO, M. L. Life history and management of the tambaqui (Colossoma macropomum, Characidae): an important Amazonian food fish. **Revista Brasileira de Zoologia**, Curitiba, v. 1, n. 2, p. 107–133, jan. 1982.

HASTY, P. et al. Muscle deficiency and neonatal death in mice with a targeted mutation in the myogenin gene. **Nature**, London, v. 364, n. 6437, p. 501–506, 5 ago. 1993.

HAWKE, T. J.; GARRY, D. J. Myogenic satellite cells: physiology to molecular biology. **Journal of applied physiology,** Bethesda**,** v. 91, n. 2, p. 534–551, ago. 2001.

IBAMA. Boletim estatístico da pesca e aquicultura: Brasil 2010. 2012.

JOHNSTON, I. I.; COLE, M. Embryonic temperature modulates muscle growth characteristics in larval and juvenile herring. **The Journal of experimental biology**, Cambridge, v. 201 (Pt 12), p. 623–646, jun. 1998.

JOHNSTON, I. A. Muscle development and growth: potential implications for flesh quality in fish. **Aquaculture**, Philadelphia, v. 177, n. 1–4, p. 99–115, 1 jul. 1999.

KASSAR-DUCHOSSOY, L. et al. Mrf4 determines skeletal muscle identity in Myf5:Myod double-mutant mice. **Nature**, London, v. 431, n. 7007, p. 466–471, 23 set. 2004.

KIMMEL, C. B.; WARGA, R. M.; SCHILLING, T. F. Origin and organization of the zebrafish fate map. **Development, Cambridge, England**, v. 108, n. 4, p. 581–594, abr. 1990.

KOUMANS, J. T. M. et al. Growth of carp (Cyprinus carpio) white axial muscle;

hyperplasia and hypertrophy in relation to the myonucleus/sarcoplasm ratio and the occurrence of different subclasses of myogenic cells. **Journal of Fish Biology**, Malden, v. 43, n. 1, p. 69–80, jul. 1993.

KOUMANS, J. T. M. et al. Numbers of muscle nuclei and myosatellite cell nuclei in red and white axial muscle during growth of the carp (Cyprinus carpio). **Journal of Fish Biology**, Malden, v. 44, n. 3, p. 391–408, mar. 1994.

LIND, C. E. et al. Selective breeding in fish and conservation of genetic resources for aquaculture. **Reproduction in domestic animals = Zuchthygiene**, Ohio, v. 47, , n. 1, p. 255–263, ago. 2012. Suplemento 4.

MELO, L. A. S.; IZEL, A. C. U.; RODRIGUES, F. M. Criação de tambaqui (Colossoma macropomum) em viveiros de argila/barragens no Estado do Amazonas. **Embrapa Amazônia Ocidental. Documentos**, 2001.

MINISTÉRIO DA PESCA E AQUICULTURA. Boletim estatístico da pesca e aquicultura: Brasil 2011. 2013.

MOORE, K. L.; PERSAUD, T. V. N. **Embriologia clínica**. Rio de Janeiro: Guanabara Koogan, 2000.

MOSS, F. P.; LEBLOND, C. P. Satellite cells as the source of nuclei in muscles of growing rats. **The Anatomical record**, Salt Lake City, v. 170, n. 4, p. 421–435, ago. 1971.

PAGE, L. M. et al. **A field guide to freshwater fishes:** North America north of Mexico. Boston: Houghton Mifflin, 1991.

RANTANEN, J. et al. Satellite cell proliferation and the expression of myogenin and desmin in regenerating skeletal muscle: evidence for two different populations of satellite cells. **Laboratory investigation;** a journal of technical methods and pathology, London, v. 72, n. 3, p. 341–347, mar. 1995.

RESCAN, P. Y.; GAUVRY, L. Genome of the rainbow trout (Oncorhynchus mykiss) encodes two distinct muscle regulatory factors with homology to myoD. **Comparative biochemistry and physiology. Part B, Biochemistry & molecular biology**, Philadelphia, v. 113, n. 4, p. 711–715, abr. 1996.

RUDNICKI, M. A.; JAENISCH, R. The MyoD family of transcription factors and skeletal myogenesis. **BioEssays:** news and reviews in molecular, cellular and developmental biology, Weinheim, v. 17, n. 3, p. 203–209, mar. 1995.

SCHULTZ, E.; MCCORMICK, K. M. Skeletal muscle satellite cells. **Reviews of physiology, biochemistry and pharmacology**, Heidelberg, v. 123, p. 213–257, 1994.

SILVA, P. et al. Hyperplastic and hypertrophic growth of lateral muscle in blackspot seabream Pagellus bogaraveo from hatching to juvenile. **Journal of fish biology**, Malden, v. 74, n. 1, p. 37–53, jan. 2009.

STICKLAND, N. C. Growth and development of muscle fibres in the rainbow trout (Salmo gairdneri). **Journal of Anatomy**, Malden, v. 137, n. Pt 2, p. 323–333, set. 1983.

THOMPSON, R. Estimation of quantitative genetic parameters. **Proceedings. Biological sciences / The Royal Society**, London, v. 275, n. 1635, p. 679–686, 22 mar. 2008.

VIVEIROS, A. T. M.; GODINHO, H. P. Sperm quality and cryopreservation of Brazilian freshwater fish species: a review. **Fish physiology and biochemistry**, New York, v. 35, n. 1, p. 137–150, mar. 2009.

WANG, Y. X.; RUDNICKI, M. A. Satellite cells, the engines of muscle repair. **Nature reviews. Molecular cell biology**, London, v. 13, n. 2, p. 127–133, fev. 2012.

VITA

Fernanda de Mello, filha de Vera Lúcia de Mello e Jonas Antônio Rosendo de Mello, nasceu aos 23 dias do mês de julho de 1982 na cidade de Canoas, estado do Rio Grande do Sul.

Iniciou o ensino fundamental na instituição confessional católica Colégio Espírito Santo e concluiu o ensino médio na Escola Paula Soares, em 1999. Em 2001, ingressou no curso de Ciências Biológicas na Universidade Luterana do Brasil (ULBRA), iniciando como estagiária no Laboratório da Biodiversidade vinculado ao PPG de Genética e Toxicologia Aplicada/ULBRA. Desde então, a paixão por genética e biologia molecular norteou o seu envolvimento em pesquisa como aluna de Iniciação Científica nos anos que seguiram, graduando-se ao final do ano de 2006.

Em 2007, estagiou no laboratório de Imunogenética, vinculado ao PPG em Genética e Biologia Molecular/UFRGS, onde adquiriu experiência em doenças auto-imune na área humana. Ainda em 2007, foi aprovada no processo seletivo do Programa de Pós-Graduação em Zootecnia da Universidade Federal do Rio Grande do Sul (UFRGS) sob a orientação do Prof. Jaime Araújo Cobuci. Iniciou o curso de mestrado em março de 2008, desenvolvendo sua pesquisa na área de Melhoramento Genético Animal em bovinos leiteiros, o qual finalizou em 2010.

Em 2010 iniciou o curso de Doutorado em Zootecnia na Universidade Federal do Rio Grande do Sul (UFRGS) sob a orientação do Prof. Danilo P. Streit Jr, onde passa a desenvolver pesquisas envolvendo Melhoramento Genético Animal em peixes, conjuntamente com o grupo de pesquisas Peixegen, liderado pelo Prof. Ricardo Pereira Ribeiro, seu co-orientador no doutorado.

Em 2011, é estabelecido o primeiro contato com o renomado grupo de pesquisas em crescimento muscular de peixes, liderado pelo Dr. Jean-Charles Gabillard. Este contato levou ao estabelecimento de uma parceria internacional em pesquisa entre a UFRGS e o INSTITUT NATIONAL DE LA RECHERCHE AGRONOMIQUE (INRA) na França. No ano seguinte é contemplada com a bolsa de doutorado sanduiche no exterior por pelos órgãos de fomento nacional CAPES e CNPq. Permaneceu pelo período de 11 meses junto da *Équipe Croissance et Qualité de la Chair des Poissons* do *Laboratoire de Physiologie et Génomique des Poissons* (LPGP) INRA/Rennes, onde desenvolveu experimentos envolvendo expressão gênica e fisiologia celular do crescimento muscular.

Em agosto de 2013 retorna ao Brasil para finalizar os trabalhos e submeter-se à avaliação de defesa da Tese de doutorado no Programa de Pós-Graduação em Zootecnia, área de concentração Produção Animal (UFRGS).