FERNANDA ANTUNES ALVES DA COSTA

Isolamento e caracterização de DETs (*Differentially Expressed Transcripts*) em espécies de peixes: *Leporinus macrocephalus* (Characiformes) e *Danio rerio* (Cypriniformes)

Tese apresentada ao Instituto de Biociências da Universidade Estadual Paulista "Júlio de Mesquita Filho", Campus de Botucatu, para obtenção do título de Doutor em Ciências Biológicas - Genética

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... Quando paramos de aprender e de progredir, começamos a morrer realmente...

C. Torres Pastorino

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SUMÁRIO

RESUMO

O presente estudo teve o intuito de isolar e caracterizar transcritos diferencialmente expressos em espécies de peixes, visando contribuir com novos dados genéticos acerca deste grupo basal de vertebrados. Desta forma, diferentes análises foram realizadas em *Leporinus macrocephalus* (piauçu) (Characiformes, Anostomidae), espécie de peixe que representa um importante recurso alimentar que tem sido intensamente explorado na aqüicultura subtropical e que apresenta um sistema de determinação sexual ZZ/ZW, e também em *Danio rerio* (*zebrafish*) (Cypriniformes, Cyprinidae), um organismo modelo já bem estabelecido em diferentes áreas da pesquisa científica. Os resultados indicaram que: (1) As α-actinas esqueléticas de tecido muscular branco e vermelho parecem ser genética e funcionalmente distintas não somente em *L. macrocephalus* como também em outras espécies de peixes, característica que, até o momento, não foi encontrada em outro grupo de vertebrados. Além disso, as características de cada tipo de actina podem ser relacionadas às demandas energéticas dos tecidos musculares branco e vermelho; (2) Um expressão dimórfica entre machos e fêmeas ocorre em tecido cerebral de *L. macrocephalus*, relacionada ao gene que codifica a proteína APBA2 (*amyloid beta (A4) precursor protein-binding, family A, member 2*), que está associada à produção da proteína β-amilóide a qual, por sua vez, é relacionada à doença de Alzheimer; (3) A estrutura do gene *fabp6* (*fatty acid-binding protein 6*) de *D. rerio* é similar à maioria dos outros membros da família multigênica *fabp* de outros vertebrados, e sua distribuição tecidual em embriões, larvas e adultos da espécie também é similar à encontrada em outros organismos, especialmente mamíferos; (4) Os genes que codificam a proteína p300 (*E1A binding protein p300*) e a proteína ADCY2 (adenilato ciclase 2) representam dois genes candidatos diferencialmente expressos entre amostras não-transgêncas e homozigotas e hemizigotas transgênicas para o gene do hormônio do crescimento (GH), reforçando dados prévios que evidenciam que estas proteínas estão envolvidas na formação/crescimento muscular e liberação de GH. Desta forma, os dados obtidos contribuem para a compreensão da evolução, função e organização molecular de diversos genes e podem servir de base a futuros estudos. Além disso, os resultados obtidos confirmam a utilidade de *Danio rerio* como espécie modelo em estudos genéticos e indicam que

Leporinus macrocephalus representa um potencial modelo experimental em análises direcionadas a genes envolvidos no desenvolvimento de doenças humanas, como alterações neurodegenerativas, distrofias musculares e câncer.

ABSTRACT

The present study intent to isolate and characterize differentially expressed transcripts in fish species in order to contribute to new genetic data on this basal vertebrate group. Therefore, different analyses were performed in *Leporinus macrocephalus* (piauçu) (Characiformes, Anostomidae), a fish species that represents an important food resource that has been intensively exploited in the subtropical aquaculture and that presents a ZZ/ZW sex determination system, and also in *Danio rerio* (zebrafish) (Cypriniformes, Cyprinidae), a well-established model organism in different areas of scientific research. The results indicated that: (1) The α-skeletal actins from white and red muscle tissues appear to be somewhat genetically and functionally distinguishable not only in *L. macrocephalus* but also in other fish species, a feature that was not found in other vertebrate groups so far. Moreover, the characteristics of each actin type could be related to the energetic demands of white and red muscle tissues; (2) A sexually dimorphic gene expression occurs in brain tissue of males and females of *L. macrocephalus* related to the gene that codes for the protein APBA2 (amyloid beta (A4) precursor protein-binding, family A, member 2), that is associated to the production of the β-amyloid protein which, in turns, is related to the Alzheimer disease; (3) The *fabp6* (fatty acid-binding protein 6) gene structure of *D. rerio* is similar to most of the other members of the multigenic *fabp* family of other vertebrates, and its tissue distribution in embryos, larvae, and adults of the species is also similar to the one found in other organisms, specially mammals; (4) The genes that code for the protein p300 (E1A binding protein p300) and for the ADCY2 protein (adenylate cyclase 2) represent two candidate differentially expressed genes among non-transgenic and growth hormone (GH)-transgenic homozygote and hemizygote samples of *Danio rerio*, reinforcing previous data that evidenced that these proteins are involved in muscle formation/growth and in GH release. Therefore, the obtained data contributes to the comprehension of the evolution, function, and molecular organization of several genes and can be used as basal information on future studies. Moreover, the results confirm the utility of *Danio rerio* as a model species in genetic analyses and indicate that *Leporinus macrocephalus* represents a potential experimental model in analyses of genes involved in the development

of human diseases, as neurodegenerative alterations, muscle dystrophies and cancer.

Considerações Iniciais e Objetivos

Nos últimos anos, vários estudos genéticos têm dedicado especial importância a análises de expressão gênica diferencial. Estes estudos permitem a caracterização de genes e identificação e avaliação do nível de expressão de determinados transcritos em relação a diversas variáveis. Desta forma, é possível analisar e comparar, por exemplo, amostras relacionadas a diferentes períodos de desenvolvimento ou a tecidos distintos, submetidas a diferentes tratamentos, presentes em distintos ambientes, ou mesmo amostras de diferentes sexos e de organismos transgênicos e não transgênicos.

Entre as técnicas mais utilizadas para isolamento e identificação de transcritos diferencialmente expressos (DETs - *Differentially Expressed Transcripts*), destacam-se: RT-PCR (*Reverse Transcription - Polymerase Chain Reaction*), DD (*Differential Display),* SH (*Suppression Hybridization*), SSH (*Suppression Subtractive Hybridization*), RAP-PCR (*RNA Arbitrarily Primed PCR*), RDA (*Representational Difference Analysis*), SAGE (*Serial Analysis of Gene Expression*), *microarrays* de DNA, PCR em tempo real, hibridação *in situ* e *Northern blotting*. Das metodologias citadas acima, RT-PCR, DD, PCR em tempo real e hibridação *in situ* foram utilizadas no presente trabalho. Os métodos de RT-PCR e DD, além de exibirem considerável eficiência e ampla aplicação em estudos de expressão diferencial, caracterizam-se pela simplicidade, custo relativamente reduzido e, em especial para DD, não requerem um conhecimento prévio do genoma que se deseja estudar.

Os trabalhos relacionados à identificação e caracterização de DETs abrangem grande diversidade de organismos e incluem análises em vários modelos experimentais como bactérias, fungos, plantas, invertebrados e

vertebrados. Entretanto, esta área de pesquisa é ainda recente e, entre os vertebrados, embora os peixes representem um dos grupos mais diversificados, até o momento, poucas espécies apresentam seus genomas caracterizados.

Entre os peixes mais amplamente utilizados em estudos genômicos, destaca-se a espécie *Danio rerio*, popularmente conhecida como *zebrafish* ou paulistinha e de grande importância econômica para a área de aquariofilia. Apesar de nativa do continente asiático, apresenta ampla distribuição geográfica, podendo ser encontrada em quase todos os países do mundo e representa um dos mais importantes modelos animais utilizados em estudos de desenvolvimento embrionário, comportamentais, fisiológicos e genéticos. A facilidade de manipulação genética nesta espécie permitiu o desenvolvimento de várias linhagens transgênicas, o que tem contribuído para o desenvolvimento de novos estudos relacionados à expressão gênica. Atualmente, o genoma de *Danio rerio* apresenta-se quase totalmente caracterizado (~ 75%) devido ao projeto "*Danio rerio Sequencing*", iniciado em 2001 pelo *Wellcome Trust Sanger Institute* para pesquisas genômicas. Atualmente, inúmeras pesquisas, em diferentes áreas, vêm sendo desenvolvidas em *D. rerio*, especialmente direcionadas à criação de novas linhagens transgênicas e à compreensão do surgimento e desenvolvimento de doenças humanas, como câncer e processos degenerativos.

Por outro lado, trabalhos genéticos direcionados para diversos outros peixes são extremamente escassos e, para um grande número de espécies, são ainda inexistentes. Nesta perspectiva, o território brasileiro representa uma fonte imensa de recursos ainda inexplorados, especialmente levando-se em consideração que várias espécies apresentam estimável valor comercial e ecológico, sendo muitas delas endêmicas. Entre estas espécies, pode-se citar

Leporinus macrocephalus, popularmente conhecida como piauçu ou piavuçu, nativa do Pantanal e que representa uma importante fonte alimentar que, nos últimos anos, tem sido intensivamente explorada como recurso natural e de piscicultura no Brasil. Apesar de sua importância econômica, os dados genéticos acerca desta espécie são ainda extremamente reduzidos.

Dada a importância de *Danio rerio* e *Leporinus macrocephalus*, o presente trabalho teve o intuito de isolar e caracterizar transcritos diferencialmente expressos nestas duas espécies, visando ampliar os dados genéticos em peixes, grupo que ocupa uma posição basal na história evolutiva dos vertebrados. Desta forma, a aquisição de um maior número de informações genéticas em espécies de peixes pode contribuir à compreensão da evolução, função e organização molecular de diversos genes. Além deste objetivo geral, foram traçados objetivos específicos, relacionados a diferentes sub-propostas de pesquisa, detalhados abaixo.

- Identificar e caracterizar genes de actina de tecido muscular esquelético branco e vermelho de *Leporinus macrocephalus*.

- Identificar e caracterizar transcritos diferencialmente expressos em tecido cerebral de machos e fêmeas de *Leporinus macrocephalus*.

- Determinar a estrutura, a organização e a distribuição espaço-temporal do gene *fabp6* (*fatty acid binding protein type 6*) em *Danio rerio*.

- Identificar e caracterizar transcritos diferencialmente expressos em tecido cerebral de linhagens de *Danio rerio* transgênicas e não-transgênicas para o gene do hormônio do crescimento (GH).

Introdução Geral

Transcritos diferencialmente expressos

Nas últimas décadas, pôde-se presenciar um considerável crescimento da genômica, área que se refere à aquisição de dados do genoma, especialmente à obtenção da seqüência completa do material genético dos organismos. Embora a obtenção desta informação não seja uma tarefa simples, a ampliação desta área de pesquisa está intimamente relacionada aos conhecimentos genéticos gerados com o advento dos denominados Projetos Genomas. Atualmente, vários projetos que visam desvendar o código genético vêm sendo desenvolvidos em organismos diversos como bactérias, fungos, plantas, invertebrados e vertebrados e os resultados gerados podem representar dados básicos para o desenvolvimento de trabalhos posteriores que visem compreender os mecanismos de expressão de diversos genes (Carneiro *et al.* 2006). Além dos dados de projetos de seqüenciamento total do genoma de várias espécies, resultados de projetos que objetivam a construção de bibliotecas de ESTs (*Expressed Sequence Tags*) são diretamente utilizados em pesquisas voltadas à elucidação dos mecanismos biológicos envolvidos na expressão gênica e em sua regulação (Carneiro *et al.* 2006).

Embora abordagens mais amplas de análise de expressão gênica em nível genômico (*Genome-Wide Expression Profiling*) constituam o maior desafio atual para identificação e análise simultânea de um grande número de genes envolvidos nos mais diversos processos biológicos - desde o desenvolvimento dos organismos até suas interações com fatores ambientais (Donson *et al.* 2002) - projetos pontuais, direcionados à identificação e caracterização de genes

específicos, também vêm sendo realizados em diversas espécies. Através da aplicação de diferentes tecnologias, análises deste tipo podem gerar dados primordiais para futuros projetos genômicos de larga escala (Matz & Lukyanov 1998, Ponsuksili *et al.* 2001).

Nas últimas décadas, inúmeras técnicas em biologia molecular foram desenvolvidas visando facilitar o isolamento e a caracterização de transcritos diferencialmente expressos (DETs - *Differentially Expressed Transcripts*), ou seja, genes expressos como RNAs mensageiros (RNAm) para síntese de proteínas que diferem em abundância entre amostras de tipos celulares ou tecidos específicos e cuja regulação pode estar relacionada a mecanismos ambientais, fisiológicos e/ou químicos. Entre estas diversas metodologias, podem ser citadas RT-PCR (*Reverse Transcription-Polymerase Chain Reaction*) (Kawasaki 1990), hibridação subtrativa (Duguid *et al.* 1988), DD (*Differential Display*) (Liang & Pardee 1992), RAP-PCR (*RNA Arbitrarily Primed PCR*) (Welsh *et al.* 1992), RDA (*Representational Difference Analysis*) (Lisitsyn *et al.* 1993), SAGE (*Serial Analysis of Gene Expression*) (Velculescu *et al.* 1995), *microarrays* de DNA (Shena *et al.* 1995), SSH (*Suppression Subtractive Hybridization*) (Diatchenko *et al.* 1996), PCR em tempo real (Freeman *et al.* 1999), hibridação *in situ* (Wilkinson 1998) e *Northern blotting* (Alwine *et al.* 1979).

Embora esta vasta gama de técnicas para isolamento e caracterização de transcritos diferencialmente expressos permita sua aplicação em estudos de diversas áreas, a escolha de uma destas metodologias deve considerar as características dos experimentos, os objetivos propostos e o organismo em estudo (Matz & Lukyanov 1998). Além disso, apesar de suas inúmeras vantagens, normalmente, a aplicação de metodologias experimentais para prospecção de

transcritos diferencialmente expressos nem sempre representa uma tarefa fácil, pois, na maioria das vezes, estes apresentam níveis de expressão extremamente baixos. Entre os milhares de genes que compõem o genoma dos organismos vivos, somente cerca de 10-15% são expressos em um determinado tipo de tecido durante um dado período, o que torna relativamente difícil sua identificação e caracterização (Liao & Zhang 2006). Além disso, as tecnologias disponíveis para o isolamento de DETs são geralmente laboriosas e de alto custo.

O crescente interesse por estudos de transcritos diferencialmente expressos pode ser comprovado pela publicação de mais de 30.000 trabalhos nas mais diversas áreas de aplicação e pelas inúmeras revisões sobre o tema (Medline, Julho de 2008 - PubMed, NCBI Web site http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=PubMed). Grande parte das pesquisas baseadas em genes que apresentam expressão diferencial entre amostras distintas vem sendo direcionada à biologia do desenvolvimento (Kanamori 2000, Xie *et al*. 2001, Ando & Sakai 2002, Anway *et al*. 2003, Dann *et al*. 2003, Kim *et al*. 2004, Sunagawa & Magae 2005, Jianzhen *et al*. 2007, Williams *et al*. 2007, Chu *et al*. 2008, Elis *et al*. 2008, Sugimoto & Endoh 2008), biologia celular e fisiologia de tecidos (Eriksson *et al*. 1999, Ahmed *et al*. 2000, Boag *et al*. 2000, Kim *et al.* 2001, Blásquez & Piferrer 2004, Saunders & Magor 2004, Azumi *et al.* 2005, Venkatesh *et al.* 2005, Jin *et al.* 2006, Koury *et al.* 2007, Bevan *et al.* 2008, Cervigni *et al.* 2008, Kim *et al.* 2008, Smartt & Erickson 2008, Wong *et al.* 2008, Zhang *et al.* 2008) e a processos tumorais e diagnóstico de diversas doenças (An *et al*. 2000, Miller *et al.* 2001, Lee & Cicila 2002, Hoei-Hansen *et al.* 2004, Li & Chen 2004, Clinton *et al.* 2005, Kim *et al.* 2005, Poulin & Labelle 2005, Sharma *et al.* 2005, Zhang *et al.* 2005, Blaes *et al.* 2007, Collins *et*

al. 2007, Nagai *et al.* 2007, Kamalian *et al.* 2008, Shin *et al.* 2008), visando gerar informações sobre a função gênica e suas relações com processos metabólicos e fisiológicos.

Embora análises nas áreas de biologia do desenvolvimento, fisiologia e carcinogênese representem a maior gama de dados relativos a transcritos diferencialmente expressos, outras áreas de pesquisa também têm merecido destaque. Uma destas áreas refere-se à caracterização de DETs em relação a alterações ambientais - como contaminação, variação drástica de temperatura e/ou disponibilidade de água (Llado *et al.* 1998, Casagrande *et al.* 2001, D'Cotta *et al.* 2001, Carginale *et al.* 2002, Chang *et al.* 2004, Basile *et al.* 2005, Meyer *et al.* 2005, Markovskaya *et al.* 2007, Mishra *et al.* 2007, Li *et al.* 2008, Smith-Keune & Dove 2008, Xu *et al.* 2008) - e a tratamentos químicos (Denslow *et al.* 2001a, Denslow *et al.* 2001b, Miller *et al.* 2001, Wong *et al.* 2002, Akbar *et al.* 2004, Hosoi-Tanabe *et al.* 2005, Badonel *et al.* 2007, Caamal-Velázquez *et al.* 2007, Zhang *et al.* 2007, Anderson *et al.* 2008, Karakurt & Huber 2008, Kavar *et al* 2008, Torelli *et al.* 2008). Estudos com este enfoque vêm também sendo, ao longo dos anos, ampliados, especialmente devido ao crescente aumento dos problemas gerados por alterações ambientais antrópicas.

Apesar do grande número de trabalhos direcionados a análises de expressão gênica diferencial, que abrangem uma grande diversidade de organismos, esta área de pesquisa é ainda recente. Particularmente em peixes, até o momento, existem poucos dados, especialmente se considerarmos que estes representam um dos grupos de vertebrados mais diversificados (Nelson 1994). Grande parte dos trabalhos de expressão gênica diferencial restringe-se a espécies nativas da América do Norte e Europa, especialmente de peixes de interesse comercial, como

Salmoniformes, visando gerar benefícios para a piscicultura (Parrington & Coward 2002). Estes estudos incluem o isolamento e a caracterização de genes relacionados à determinação sexual (D'Cotta *et al.* 2001, Denslow *et al.* 2001a, Denslow *et al.* 2001b), ao crescimento e desenvolvimento (Kanamori 2000, Xie *et al.* 2001, Dann *et al.* 2003), à resistência a patógenos (Collins *et al.* 2007) e a condições ambientais adversas (Carginale *et al.* 2002, Larkin *et al.* 2003, Meyer *et al.* 2005). Além disso, espécies de peixes consideradas modelos animais também têm sido alvo de estudos em expressão gênica diferencial. Devido ao genoma compacto (390 Mb) e ao conteúdo de genes similar ao de humanos, *Fugu rubripes* (baiacu ou *pufferfish*) tem sido explorado como um modelo do genoma de vertebrados para identificação de genes e outros elementos funcionais nos genomas de outros animais (Brenner *et al.* 1993).

Desta forma, a ampliação de análises genéticas em peixes, que compreendem um grupo vertebrado basal, poderá levar à identificação e caracterização de diversos genes, assim como fornecer maiores informações sobre sua organização, função e evolução.

A espécie *Leporinus macrocephalus*

A família Anostomidae, grupo de peixes da ordem Characiformes relativamente rico em espécies distribuídas por toda a América do Sul e América Central, compreende 12 gêneros: *Abramites, Anostomus, Gnathodolus, Laemolyta, Leporellus, Leporinus, Rhytiodus, Schizodon, Synaptolaemus, Anostomoides, Pseudanos e Sator*. Entre estes, o gênero *Leporinus* é o mais diverso, possuindo 87 espécies válidas (Garavello & Britski 2003).

Popularmente conhecida como piauçu ou piavuçu e nativa da região do Pantanal, a espécie *Leporinus macrocephalus* (Figura 1) representa um dos principais peixes de pesca de subsistência e de pesca esportiva no Brasil (Garavello & Britski 1988, Martins & Yoshitoshi 2003). Além disso, seu cultivo em estações de piscicultura tem se mostrado um empreendimento promissor, devido a seu hábito alimentar omnívoro, facilidade de adaptação às condições de cativeiro, bom desempenho reprodutivo e excelente taxa de crescimento (Corrêa Filho 2000, Takahashi *et al.* 2004). A espécie é gonocorística, ou seja, os indivíduos se desenvolvem somente como machos ou somente como fêmeas e permanecem, assim, durante todo seu ciclo de vida. Embora machos e fêmeas da espécie atinjam, na maturidade, cerca de 60 cm de comprimento, diferenças de peso e tamanho podem ser observadas entre os sexos em indivíduos adultos - machos da espécie apresentam um maior tamanho corporal (J. Senhorini - CEPTA/IBAMA, comunicação pessoal).

Dada a importância de *L. macrocephalus* como recurso alimentar, diversos estudos voltados à criação da espécie em cativeiro vêm sendo realizados, especialmente análises de crescimento, alimentação e reprodução (Soares *et al.* 2000, Pinto *et al.* 2001, Nagae *et al.* 2002, Ribeiro & Godinho 2003, Minucci *et al.* 2005, Normandes *et al.* 2006, Godinho 2007, Albinati *et al.* 2007). Além disso, *L. macrocephalus* tem sido, nos últimos anos, uma das espécies de destaque para pesca esportiva, especialmente nos estados do sul e sudeste do Brasil (Queiroz *et al.* 2002).

Figura 1: Exemplar adulto de *Leporinus macrocephalus* (piauçu ou piavuçu).

Por outro lado, os estudos genéticos referentes a *Leporinus macrocephalus* são escassos e a maioria das análises refere-se a dados citogenéticos. Embora grande parte das espécies que compõem o gênero *Leporinus* não apresente cromossomos sexuais morfologicamente diferenciados entre machos e fêmeas (Galetti *et al.* 1995, Venere *et al.* 2004), *L. macrocephalus* é caracterizado por apresentar um cariótipo composto por 54 cromossomos e, em especial, por apresentar um sistema de determinação sexual do tipo ZZ/ZW, o que caracteriza uma heterogametia feminina. Assim, as fêmeas possuem um par cromossômico heteromórfico consistindo de um cromossomo submetacêntrico (cromossomo Z), equivalente ao cromossomo n^{o} 2 dos machos, e um cromossomo subtelocêntrico (cromossomo W), o maior do complemento cariotípico e ausente nos machos (Galetti & Foresti 1987). Tal característica torna a espécie extremamente interessante para estudos relacionados à determinação e diferenciação sexual e para estudos sobre a evolução dos caracteres envolvidos nestes processos.

As demais análises genéticas em *L. macrocephalus* referem-se à caracterização do DNA ribossomal 5S para análises filogenéticas (Ferreira *et al.* 2007) e isolamento e caracterização de locos microssatélites para utilização em

estudos de diversidade genética populacional (Morelli *et al.* 2007). Embora os dados genéticos em *L. macrocephalus* sejam ainda extremamente restritos, este cenário tende a mudar nos próximos anos. O crescente desenvolvimento do setor de piscicultura no Brasil - devido ao grande potencial hídrico, à enorme riqueza de espécies nativas e às áreas de clima propício à atividade encontradas no país -, associado à disponibilidade de diversas técnicas de biologia molecular, deverão levar à implementação de novas análises genéticas em espécies de peixes de interesse comercial, incluindo *Leporinus macrocephalus*.

A espécie *Danio rerio*

A espécie *Danio rerio* (Figura 2) (Ordem Cypriniformes, Família Cyprinidae), popularmente conhecida como paulistinha ou *zebrafish*, embora nativa do Sul e Sudeste da Ásia, tornou-se amplamente distribuída, podendo ser encontrada em todos continentes. Características como a fácil adaptação ao ambiente de cativeiro, o espaço relativamente pequeno necessário para sua manutenção, a prole numerosa, a produção contínua de ovos e o período curto de desenvolvimento embrionário e larval representam aspectos interessantes para os produtores, especialmente da área de aquariofilia (Westerfield 2000). Além destas características, *Danio rerio* é considerada uma espécie modelo em estudos de desenvolvimento embrionário, comportamentais, fisiológicos e genéticos de vertebrados, especialmente devido à transparência de estruturas como ovos e larvas, que permitem análises diretas dos estágios embrionários e da expressão espaço-temporal de determinados genes de interesse, através da aplicação de métodos de hibridação *in situ* (Detrich *et al.* 1998, Dahm & Geisler 2006, Dahm *et al.* 2007, Bass & Gerlai 2008, Fadool & Dowling 2008).

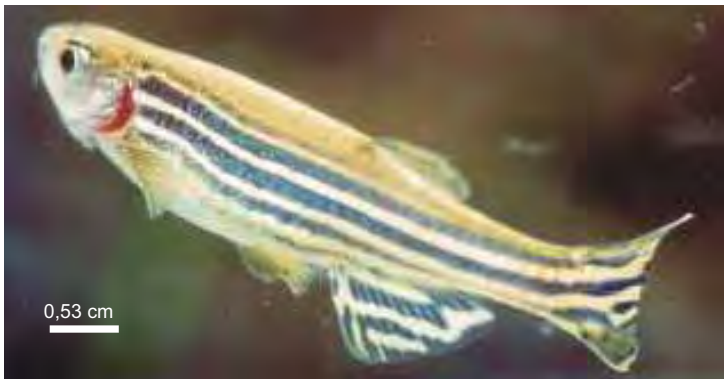

Figura 2: Exemplar adulto de *Danio rerio* (paulistinha ou *zebrafish*).

Ao contrário de diversas outras espécies de peixes, entre estas *Leporinus macrocephalus*, *D. rerio* apresenta inúmeros dados referentes à caracterização de seu genoma. A espécie apresenta um cariótipo composto por 50 cromossomos e não apresenta cromossomos sexuais morfologicamente diferenciados, sendo seu mecanismo de determinação sexual desconhecido e, até o momento, nenhum gene relacionado à determinação sexual foi identificado (Amores & Postlethwaite 1999, Sola & Gornung 2001). Acredita-se que fatores ambientais e ecológicos, como temperatura e densidade populacional, podem influenciar na determinação do sexo de *zebrafish* (Lawrence *et al.* 2008).

Atualmente, aproximadamente 75% do genoma de *Danio rerio* já se encontra caracterizado, devido ao projeto de seqüenciamento iniciado em 2001 pelo *Wellcome Trust Sanger Institute* (Cambridge, Inglaterra) para pesquisas genômicas, e uma rede de dados (*Zebrafish Information Network* - ZFIN: http://www.zfin.org) foi criada com o intuito de integrar diversos dados referentes ao genoma desta espécie de peixe (Sprague et al. 2003). Além disso, nos últimos cinco anos, com o advento de novas técnicas biotecnológicas e experimentais de análises genômicas, o número de estudos genéticos neste modelo animal cresceu

significativamente - mais de 640 mil ESTs (*Expressed Sequence Tags*) que representam transcritos de *zebrafish* foram seqüenciados e mais de 6.800 RNAs mensageiros foram identificados e caracterizados (Zon & Peterson 2005, Dahm & Geisler 2006). A facilidade de manipulação genética nesta espécie ainda levou ao desenvolvimento de várias linhagens transgênicas, o que tem contribuído para o desenvolvimento de novos estudos relacionados à expressão gênica e já permitiu um grande avanço na elucidação das relações existentes entre diversos genes de *zebrafish* e de outros vertebrados (Sprague *et al.* 2003).

Atualmente, diversas pesquisas, em diferentes áreas, vêm sendo desenvolvidas em *D. rerio*, especialmente direcionadas à criação de novas linhagens transgênicas (Linney & Udvadia 2004, Ashworth & Brennan 2005, Huang *et al.* 2005, Burket *et al.* 2008, Wu *et al.* 2008) e à compreensão do surgimento e desenvolvimento de doenças humanas, como câncer e processos degenerativos (Patton & Zon 2005, Lam & Gong 2006, Panulo *et al.* 2006, Goessling *et al*. 2007, Merlino & Khanna 2007, Beckman 2007, Feitsma & Cuppen 2008). Como perspectiva, pode-se prever a continuação e ampliação das análises genéticas em *Danio rerio*, tanto na área biológica como na área de medicina.

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Resultados e Discussão

Os resultados e a discussão dos dados obtidos encontram-se apresentados na forma de capítulos, referentes a trabalhos científicos. Visando facilitar a apresentação e compreensão de cada um destes capítulos, as figuras e tabelas foram numeradas em relação a cada trabalho específico. Da mesma forma, as referências bibliográficas citadas em cada capítulo não se encontram ao final do item "Introdução Geral" da tese e sim discriminadas no final de cada trabalho.

Capítulo 1: Molecular organization and expression analyses of skeletal actin genes from white and red fish muscle tissues: insights into the musculature energetic demands;

Capítulo 2: Identification of sexually dimorphic gene expression in brain tissue of the fish *Leporinus macrocephalus* through mRNA differential display and real time PCR analyses;

Capítulo 3: Spatio-temporal distribution of fatty acid-binding protein 6 (*fabp6)* gene transcripts in the developing and adult zebrafish (*Danio rerio*);

Capítulo 4: A comparative expression analysis of gene transcripts in brain tissue of wild-type and GH-transgenic zebrafish (*Danio rerio*) using a DDRT-PCR approach.

CAPÍTULO 1

Organização molecular e análises de expressão de genes de actina esquelética de tecidos musculares branco e vermelho de peixes: compreensão sobre a demanda energética muscular

Molecular organization and expression analyses of skeletal actin genes from white and red fish muscle tissues: insights into the musculature energetic demands

Abstract

Two α-skeletal actin genes were isolated from the neotropical fish *Leporinus macrocephalus*, referring to white and red muscle tissues. Similar actin isoforms were also identified in other fish species, mainly differing by a Ser/Ala^{155} substitution, which can have a functional significance related to actin-ATP interaction. A Ser^{155}, identified in the α-skeletal white muscle actin, seems to lead to a higher affinity to ATP molecules which, in turns, could be related to the fast contraction fibers of this tissue. However, an Ala^{155}, as observed in the α-skeletal actin from red muscle might result in a decrease in actin's affinity for ATP, which could also be associated to its tissue slow contractile performance. FISH mapped the skeletal muscle actin genes to a pair of submetacentric chromosomes. Comparisons on the predicted secondary protein structure evidenced no differences between the two analyzed actin isoforms, although a Phe/Ile^{262} substitution at the red muscle actin leads to a hydrophobicity variation at the D-plug of the protein which could alter its stability. Data on qRT-PCR evidenced a significant gene expression variation between white and red muscle actin genes (T=105 Mann Whitney; $p<0.001$). Moreover, although no significant variation was observed between adult males and females (white muscle: females 0.01175±0.00247 vs. males 0.01082±0.004447; red muscle: females 0.000847±0.000613 vs. males 0.000754±0.000374), there was a great variation on gene expression within individuals of each sex. Available data on muscle actins

lead to the proposal that white and red α-skeletal actins are genetically and functionally distinguishable in fish species, a feature that is not found in other vertebrate groups.

Introduction

Muscles are a major source of energy consumption and heat generation and there has been a longstanding interest in the mechanisms that underlie muscle energetic properties. Skeletal muscle is found throughout the body and, in higher vertebrates, form an integrated network with a prominent skeletal system via tendons, resisting gravity and facilitating mobility. Lower vertebrates, particularly those that are aquatic, have a proportionally greater muscle mass (e.g. Sambasivan & Tajbakhsh 2007). In fish, body mass is mainly composed by skeletal musculature that constitutes 40-75% of the total weight of the animal. In most fish species, this tissue is composed by different muscle fibers that occupy distinct axial regions - a large portion of a deeper layer of white muscle (with fast contraction fibers and glycolytic metabolism, for energy supply), a superficial thin layer of red muscle (with slow contraction fibers and oxidative metabolism), and an intermediate layer between the red and white musculatures (with fibers of fast contraction and oxidative/glycolytic metabolism). While white muscle fibers are associated to fast swimming in fish, as predation and escape behaviors, the red muscle fibers are associated to slow movements, as migration and foraging habits (Driedzic & Hochachka 1976, Luther *et al.* 1995, Sänger & Stoiber 2001).

One of the major components of muscle tissues is actin that, together with myosin, tropomyosin, and troponin, represents a protein that composes the parallel

fibrils (miofibrils) of the striate musculature. Actin plays a crucial role in maintaining cytoskeletal structure, contractile processes, cell motility and division, and intracellular movements, and is essential to eukaryotic cells (Clarke & Spudich 1977, Lazarides & Revel 1979). More recently, it was evidenced that actin is also involved in diverse nuclear functions, including transcription, ribonucleoprotein packaging and transport, chromatin remodeling, and the formation of karyoskeletal elements (Pederson & Aebi 2002, Bettinger *et al.* 2004, Kiseleva *et al.* 2004). Reflecting the importance of its biological functions, the actin structure is highly conserved among different organisms.

Although the genes that code for actin proteins can occur in single copies, as observed in *Sacharomyces cerevisae*, some protozoans, and several green algae, actin proteins are encoded by a multigene family in animals, plants, and in many protozoans examined to date (Vandeckerckhove & Weber 1984, Hightower & Meagher 1986, Reece *et al*. 1992, Bhattacharya *et al.* 2000). Mammals have four muscle actin isoforms - two in striated muscles (α-skeletal and α-cardiac) and two in smooth muscles (γ-enteric and α-vascular) - and two non-muscle or cytoplasmic types, the β- and γ-isoforms (e.g. Vandekerckhove & Weber 1984, Kusakabe *et al.* 1997, Mounier & Sparrow 1997). However, teleost fish seems to contain a higher number of distinct actin isoforms, as revealed by the most extent research on actin's diversity and tissue expression profile in fish genome performed to date on *Takifugu rubripes,* revealing nine isoforms (two α-skeletal, three α-cardiac, an α-anomalous testis type actin, two β-cytoplasmic, and one β-cytoplasmic vascular type-actin) (Venkatesh *et al.* 1996). Similarly, insects have at least six actin genes (Fyrberg *et al*. 1980). The actin gene family of plants is much larger, comprising 8-44 genes, depending on the taxa (Reece *et al*. 1992).

Thus, the actin multigene family provides an excellent model for the study of the molecular mechanisms of gene regulation and expression and for insights into its evolution and function.

The multiple actin isoforms are expressed in different stages and/or tissues and seem to be encoded by a set of structurally related genes that were originated by gene duplication followed by functional divergence (Hightower & Meagher 1986, Miwa *et al.* 1991, Venkatesh *et al.* 1996). The controversy between the actin high structure conservation and several biophysical properties suggests that its several isoforms result from a very few amino acid substitutions in key functional positions. Small differences related to nucleotide or amino acid substitutions can constitute the structural base for changes in the conformation of the actin protein which, in turns, could modify its affinity to numerous bindings found in distinct cellular types (Ooi & Soematsu 2007).

Although there are several data on mammalian actins, the evolutionary origin, pattern of organization, and their diversity in lower vertebrates, especially fishes, remain to be better investigated. Moreover, data on the organization and expression of the main components of the muscle tissues can give new insights on the molecular events underlying muscle contraction and/or growth. Therefore, the purpose of the present study was the isolation and characterization of skeletal muscle actin genes of *Leporinus macrocephalus*, a fish species that represents an important food resource that has been intensively exploited in the subtropical aquaculture (Takahashi *et al.* 2004). The analyses resulted in the first description of white and red skeletal muscle actin genes that may be genetically and functionally distinguishable in fish species.

Material and Methods

Animal samples

Ten adult specimens (five males and five females) of *Leporinus macrocephalus* (piauçu) (Characiformes, Anostomidae) were obtained from a private fishery station (Piscicultura Água Milagrosa, Itajobi, São Paulo State, Brazil), where they were maintained under the same conditions. Animals with at least 40 days were selected since they already present gonadal differentiation (Toriyama 2001) which permits the sex identification under stereomicroscopy. White and red muscle samples from the dorsal region of the animals were collected and immediately stored at -80^{o}C until RNA extraction. It was not possible to obtain muscle samples from the intermediate layer between the red and white musculature due to the difficulty to correctly isolate this tissue. Kidney tissue was also collected and immediately used for chromosome preparations.

RNA isolation

White and red muscle frozen samples were mechanically homogenized with TRizol Reagent (Invitrogen) and total RNA extraction followed the manufacturer's protocol. RNA samples were eluted in RNase-free water and quantified (NanoDrop 1000 Spectrophotometer) by measuring the optical density (OD) at 260nm. RNA purity was ensured by obtaining a 260/280nm OD ratio ≥1.80.

RT-PCR and cDNA amplification

Each RNA sample was reverse transcribed with the commercial kit SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen) using an oligonucleotide $(dT)_{12-18}$ as an anchor primer, according to the manufacturer's instructions. cDNA (2μL) was amplified using forward AactF (5'-AGGCCAACAGGGAGAAGATT-3') and reverse AactR (5'-TCCATACCGATGAAGGAAGG-3') primers, designed based on alpha-actin gene sequence GenBank databases of the fish *Takifugu rubripes* (http://www.ncbi.nlm.nih.gov), in order to amplify a segment of this gene. Each PCR consisted of 2μL of cDNA, 0.2mM of each primer, 1x 25mM $MgCl_2$ PCR buffer, 0.2mM of dNTPs, and 1 unit of Platinum *Taq* DNA polimerase (Invitrogen), in a final volume of 25μL. PCR amplifications were carried out with an initial denaturation step at 95°C for 2 minutes, followed by 34 cycles at 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute, with an additional extension step at 72°C for 5 minutes. PCR products were fractionated on 1% agarose gel, stained with ethidium bromide, and visualized under UV light using Eagle Eye II image documentation system (Strategene).

Semi quantitative RT-PCR and statistical analysis

Four pools of RNA samples were prepared in order to minimize the detection of simple variations among *Leporinus macrocephalus* individuals (pool of RNA samples from white muscles of males; pool of RNA samples from white muscles of females; pool of RNA samples from red muscles of males; pool of RNA samples from red muscles of females). Each RNA pool was reverse transcribed individually as described above. cDNA amplification followed the methodology

described in Marone *et al.* (2001) using 2μL of the obtained cDNA, and PCR was carried out at 95°C for 2 minutes, followed by 30 cycles with denaturation at 95°C (30 seconds), annealing at 55°C (30 seconds), and extension at 72°C (1 minute and 15 seconds), followed by an additional 5 minute extension step at 72°C. The PCR signals were normalized to the housekeeping 18S rRNA gene. A 245 bp segment of the 18S rRNA gene was amplified using the four RNA pools individually and the primers 18S1 (5'-TACCACATCCAAAGAAGGCAG-3') and 18S2 (5'-TCGATCCCGAGATCCAACTAC-3'), designed based on consensus sequences of this gene described for several fish species. Preliminary PCR experiments, using 28-30-32-34 cycles, were conducted to determine the number of cycles that represented the linear amplification range for analysis (Tom *et al.* 2004). PCR products were fractionated on 1% agarose gel, stained with ethidium bromide, and visualized under UV light using Eagle Eye II image documentation system (Strategene). The bands corresponding to each gene were quantified by densitometry as Integrated Optical Density (IOD) using Imaging Master VDS Software version 3.2 (GE Life Sciences).

Quantitative PCR and statistical analysis

Quantitative RT-PCR was performed using Power SYBER Green PCR Master Mix Kit (Applied Biosystems), according to the manufacturer's instructions. Standard reactions (25μL) were assembled using 12.5μL of Power SYBER Green PCR Master Mix 2x, 2μL of forward primer AactF2 (5μM) (5'-CCATCTATGAGGGTTACGCTCTTC-3'), 2μL of reverse primer AactR2 (5μM), (5'-CGGTTGTGACGAAAGAGTAGCC-3'), 2μL of template (10ng/μL), and 6.5μL of ultrapure water. Primers were designed with the software Primer Express v.2.0

(Applied Biosystems), based on the actin gene sequences previously obtained from white and red muscles of *L. macrocephalus*. Templates cDNA were 1:10 diluted, and cDNA samples were replaced by DEPC water in the negative controls. All real time assays were carried out in duplicate using an Applied Biosystem 7300 HT Real-Time PCR system (Applied Biosystems). Forty amplification cycles were performed and each cycle consisted of 94^{o}C for 15 seconds followed by 60^{o}C for 1 minute. Amplification and dissociation curves generated by the 7300 System/Sequence Detection Software version 4.0 (Applied Biosystems) were used for gene expression analysis. As performed for the semi-quantitative analysis, the qRT-PCR signals were normalized to a segment of the 18S rRNA housekeeping gene using the primers 18S3 (5'-CGGAATGAGCGTATCCTAAACC-3') and 18S4 (5'-GCTGCTGGCACC AGACTTG-3'), also designed based on consensus sequences of this gene described for several fish species. Following the removal of outliers, raw fluorescence data were exported to the online program QPCR Standard Curve Slope to Efficiency Calculator (http://www.stratagene.com/techtoolbox/calc/qpcr_slope_eff.aspx) to determine the PCR amplification efficiency. The Ct values were used to calculate a relative gene expression value for each transcript, according to $2^{-\Delta Ct}$ method (Livak & Schimittgen 2001). The relative gene expression values were submitted to statistical analysis using Student's unpaired t-test ($p<0.05$) to normal distribution, or Mann-Whitney T value (Mann-Wh) ($p<0.05$) to data which did not display a normal distribution (Zar 1999). Differences were considered significant when $p<0.05$ and a 95% confidence level of the difference was used.

Cloning, sequencing, and sequence analysis

PCR products were cloned into pGEM-T (Promega) vector and used to transform competent cells of the *E. coli* strain DH5α (Invitrogen), following the manufacturers' instructions. Clones were submitted to automated sequencing on an ABI 377 Automated DNA Sequencer (Applied Biosystems) with a DYEnamic ET Terminator Cycle Sequencing kit (GE Healthcare Life Sciences), following the manufacturer's instructions, and using primers complementary to vector arms. Nucleic acid and amino acid sequence database searches were performed using BLAST/N (Altschul *et al.* 1990) at the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/blast). Sequence alignments were obtained by Clustal-W function (Thompson *et al.* 1994) and the consensus sequences were manually determined. The deduced amino acid sequences were subjected to the bioinformatics computer program CLC Main Workbench 4.0.1 to generate protein secondary structure data and hydropathy plots using the Kyte-Doolittle scale with a window size default value of 9 (Kyte & Doolitle 1982).

Chromosome analyses

Mitotic chromosomes were obtained from a suspension of anterior kidney cells using direct preparations (Bertollo *et al*. 1978). A cDNA actin product (300-1.000ng), obtained using primers AactF and AactR, was isolated from agarose gels using a commercial kit (Sephaglas^{TM} BandPrep Kit, GE Healthcare Life Sciences). A SCOMP (single cell comparative genomic hybridization) methodology for DNA amplification was performed. Briefly, the cDNA was digested using 24U of *Mse*I (New England Biolabs) at 37^{o}C for 3 hours. In a separate tube, the adaptor-oligonucleotides LIB1 (5'-AGTGGGATTCCTGCTGTCAGT-3') and ddMseI (5'-

TAACTGACAGCdd-3') were incubated at 65ºC to 15ºC, using a slope of 1 min/ºC. Further, 1µL of ATP (10mM) and 1µL of T4 DNA ligase (5U/µL) were added. The digested cDNA was mixed to this solution and incubated at 15ºC overnight. The kit Elongase Amplification System (Invitrogen) was used for the primary DNA amplification, following the manufacturer's instructions. The amplified product was used as a template for a second amplification reaction, using the QIAquick PCR Amplification Kit (QIAGEN), following its instructions. The oligonucleotide LIB1 was removed by a*Tru*I treatment at 65ºC for 3 hours. The DNA probe (8µg) was further precipitated in the presence of the competitor Human Cot-1 DNA (50x) and salmon sperm DNA (50x), and labeled with biotin-11-dUTP by nick translation using the kit Bionick™ Labeling System (Gibco), according to the manufacturer's instructions. After chromosome treatment with 10% pepsine for 5 minutes at 37°C, FISH (Fluorescent *in situ* hybridization) was performed as detailed in Wasko & Galetti (2000). Metaphases were examined with an Olympus BX50 epifluorescence microscope.

Results and Discussion

The alpha-skeletal actin plays a key role in biological muscle movement and represents the major protein in muscles along with myosin. This protein has been intensively studied in mammals and its functions include polymerization in neutral salt and binding to Ca^{2+}, Mg^{2+}, adenine nucleotides, tropomyosin, and myosin (Estes *et al.* 1992, Reisler 1993). These multiple functions make it one of the structurally best-conserved cell proteins. Although muscles of most vertebrates contain one α-skeletal actin, the presence of two isoforms in the muscles of fish species has been reported. Venkatesh *et al.* (1996) identified two α-skeletal actins in the pufferfish *Takifugu rubripes*, which have 98.7% of identity at the amino acid sequence level. Two other fish species - *Coryphaenoides acrolepis* and *C. cinereus* - also present two distinct α-skeletal muscle actin isoforms (Morita 2000). However, the functional difference of these two actins in fish muscle was not elucidated.

Distinct muscle actin isoforms, with tissue and/or stage-specific patterns, could be related to the different structure of the muscle tissues of fish species. As so, the aim of the present study exploits the isolation and characterization of actin genes of white and red skeletal muscles of the neotropical fish species *Leporinus macrocephalus*, through the idea that it should be feasible to also find distinct α-skeketal actin genes, as already identified in other fish species. Moreover, it should be possible to identify the genetic bases that could be beyond the specific functions of different skeletal actin isoforms.

The amplification of the cDNA samples of *L. macrocephalus* from white and red muscle, using the two selected actin primers, revealed fragments of approximate sizes of 450 bp. Cloning and sequencing of the PCR products lead to the characterization of segments composed by 472 bp (Figure 1). Database

DDBJ/EMBL/GenBank searches for nucleotide and amino acid similarity index indicated that the isolated fragments of *L. macrocephalus* correspond to partial α-skeletal muscle actin genes, containing the complete regions of exons 2, 3, and 4, and a fragment of exon 5 (Figure 1). These results were confirmed through comparisons with data on vertebrate skeletal muscle actin genes that are composed by 6 exons, whose proteins present 375 amino acid residues (Venkatesh *et al.* 1996).

The results evidenced no nucleotide differences between the isolated actin genes from white muscles of males and females of *L. macrocephalus*. In the same manner, no differences could be detected between sexes when comparing the actin gene from red muscle. However, several base substitutions were identified when comparing the isolated α-skeletal muscle actin genes from white and red muscle tissues - 7 and 12 nucleotide substitutions were found in the exons 3 and 4, respectively (Figure 1). Despite these differences between the white and red muscle actin gene sequences of *L. macrocephalus*, their deduced amino acid sequences, referring to the residues 113 to 269, are very conserved (Figure 2), since most nucleotide variations correspond to synonymous substitutions and just three nucleotide differences are related to amino acid substitutions.

Available data on fish actins indicate the occurrence of two different skeletal muscle actin genes, firstly denominated *α-Sk1* and *α-Sk2* by Venkatesh *et al.* (1996). Comparisons with amino acid sequences of database banks indicated that the α-skeletal muscle actin gene isolated from white muscle of *L. macrocephalus* presents a higher identity with the actin gene type 2 identified in the fish species *Takifugu rubripes* (Venkatesh *et al.* 1996), *Coryphaenoides acrolepis*, and *C. cinereus* (Morita 2000). On the other hand, the characterized α-skeletal muscle

actin gene from red muscle of *L. macrocephalus* is more similar to the actin gene type 1 also characterized in these same other fish species.

The precursors of vertebrate muscle actins (muscle-like actins) are found only in urochordates and represent a transition from non-muscle like actins to vertebrate skeletal muscle actins (Kovilur *et al*. 1993). Actins have undergone a rapid diversification during the emergence of vertebrates to give rise to several muscle-type actins, as seen in fish species. Distinct α-skeletal muscle actin isoforms were not evidenced in other vertebrate groups. Therefore, it is possible that some of the actins that have been characterized in fish species are either specific to the teleost lineage or are yet to be identified in higher vertebrates.

The actin monomer structure, already characterized in several species, presents two domains, originally termed large and small, although they are now known to be nearly identical in size. The small domain comprehends the subdomain 1 (a.a. residues 1-32, 70-144, and 338-375) and the subdomain 2 (a.a residues 33-69), while the large domain comprises the subdomains 3 (a.a. residues 145-180 and 270-337) and 4 (a.a. residues 181-269) (Kabsch *et al.* 1990) (Figure 3a). The structure and rotations of these domains with respect to one another are important in many biological processes, such as enzyme catalysis, ligand binding, and oligomerization (Page *et al.* 1998). Amino acid substitutions could lead to conformational changes of the protein which in turn would modify chemical affinities for numerous ligands found inside the cells (Mounier & Sparrow 1997). The strong conservation of actin isoforms indicates a great restriction in the rate and nature of amino acid changes in this protein during evolution. Only substitutions compatible with the folded structure and protein function will have

been retained. As so, no extreme change on protein conformation is thus expected, but, rather, small and local modifications (Mounier & Sparrow 1997).

One of the actin ligands is the ATP molecule (Figure 3a), which is hydrolysed to ADP.Pi, playing a role in force generation by the actomyosin complex and accompanies assembly of actin monomers (G-actin) in to filaments (F-actin) (Chen *et al.* 2000). Amino acid residues 12-17 and 154-161 comprehend two protruding loops that form a hydrogen bond with the phosphate of ATP (Kabsch *et al.* 1990, Schüler *et al.* 1999, Morita 2000) since their amine nitrogen can be hydrogen bounded to oxygen atoms of the nucleotide phosphate tail (Schüler *et al.* 1999). This binding bridges the two loops between the small and the large domains, and prevents the unfolding of the protein and stabilizes its structure (Kabsch *et al* 1990, Schüler *et al.* 1999). Any disturbance in the interdomain coupling is expected to result in a decreased stability.

An amino acid difference observed between the α-skeletal muscle actins from white muscle and from red muscle of *L. macrocephalus* corresponds to a substitution of a Serine (white muscle) for an Alanine (red muscle) at the residue 155 (Figure 2) that lies inside one of the ATP-binding site to the actin molecule, found at the subdomain 3. This amino acid substitution can influence the actin's affinity for ATP, a feature that has been described in actin isoforms of some vertebrate species, including fish, and in *Sacharomyces cervisae* (Table 1), an yeast that has one essential actin gene that encodes a protein that shares a high identity degree (87%) to vertebrate skeletal muscle actins (Geeves *et al.* 2005). Similarly, it has been evidenced that a Ser/Ala substitution at the amino acid 14, at the actin subdomain 1, results in a 40 to 60-fold decrease in actin's affinity for ATP (Chen *et al.* 1995) (Table 1), since Alanine has a lower hydrogen bonding

capacity. Ser^{14} hydroxyl forms a polar bridge between the γ-phosphate ATP and the amide nitrogen of Gly^{74}, thus conferring additional stability on the actin small domain (Morita 2000, Ooi & Soematsu 2007). Therefore, the Ser/Ala^{155} substitution found in the α-skeletal actin isoform of red muscle of *L. macrocephalus* could also influence the actin-ATP interaction and, consequently, alter this protein stability, structure, or function.

In fish, the presence of a $Serine^{155}$ has been also identified in an α-skeletal muscle actin type of some species, as *Takifugu rubripes* (Venkatesh *et al.* 1996), *Coryphaenoides acrolepis* and *C. cinereus* (Morita 2000), and in α-cardiac muscle actins, as identified in *T. rubripes* (Venkatesh *et al.* 1996), *Oreochromis niloticus* and *O. mossambicus* (Wasko *et al.* 2007), and *Salmo trutta* (Mudalige *et al.* 2007). On the other hand, Alanine was also identified as the 155^{th} amino acid in a second α-skeletal muscle actin isoform in the fish *Takifugu rubripes* (Venkatesh *et al.* 1996), *Coryphaenoides acrelepis*, *C. cinereus*, and *Cyprinus carpio* (Morita 2000). Therefore, it is reasonable to suppose that the occurrence of a Serine or an Alanine in the vicinity of the actin ATP-binding site, at the amino acid residue 155, could be related to the type of contraction of the muscle fibers (fast vs. slow contraction), since force generation during muscle contraction can be understood in terms of cyclical length changes in segments of actin thin filaments moving trough the tree-dimensional lattice of myosin filaments (Schutt & Lindberg 1998, Sieck & Regnier 2001) and any disturbance in these proteins could alter the chemomechanical transduction (work, force, and shortening) in muscle.

Therefore, the residue Ser^{155}, that seems to lead to a higher affinity to ATP molecules, could be related to the contractile performance of white skeletal and cardiac muscle tissues (fast contraction fibers). On the other hand, the presence of

an Ala^{155} might result in a decrease in actin's affinity for ATP, which could also be related to the slow contraction fibers of the red muscle tissue. Moreover, it is possible that the genetic base differences between white and red skeletal muscle actins of fish species may have been hidden since previous analyses probably have used both muscle tissues together and not individually, as performed in the present study. Therefore, some of the α-skeletal muscle actin isoforms already described for other fish species, as the denominated *Sk1* actin of *Takifugu rubripes* (Venkatesh *et al.* 1996), actin-1 of *Coryphaenoides acrolepis* and *C. cinereus* (Morita 2000), and the actin-2b of *Coryphaenoides yaquinae* and *C. armatus* (Morita 2003), that also present an Ala^{155} may represent a red skeletal muscle actin type, while the other skeletal actin isoforms with a Ser^{155} identified in these same fish species may be related to a white muscle type. As so, the amino acid 155 might be a diagnostic position in α-skeletal actins of red and white muscle tissues of fish species. In contrast, other vertebrates, as rabbit, chicken, mouse, human, and frog, synthesize a single α-skeletal actin isoform with Ser^{155}. This different pattern may reflect that fish skeletal musculature is layered organized and largely confined to locomotion demands rather than bundled as in the other vertebrates (Luther *et al.* 1995).

Comparisons between the two distinct α-skeletal muscle actin isoforms of *L. macrocephalus* also evidenced another amino acid substitution (Phe/Ile^{262}) (Figure 2). Although there is no available data regarding to actin amino acid substitutions at this residue, analyses of the protein structure show that residues Phe^{262} to Gly^{273} form an outer loop which buttresses both subdomains 3 and 4, and stabilizes the extensive intersubdomains interface (Page *et al.* 1998). Therefore, the identified Phe/Ile^{262} amino acid variation could also influence the F-actin

formation, nucleotide binding and exchange, and interactions with various actin-binding proteins.

The proposal that the different α-skeletal actin isoforms already described in some fish species may be correlated to white and red muscle tissues led us to predict their protein secondary structure, since amino acid differences can lead to a weak aggregation of these residues which could strongly influence the chain's motifs - as the alpha helices (coiled conformation in which every backbone N-H group donates a hydrogen bond to the backbone C=O group of the amino acid four residues earlier) and the beta strands (typically 5-10 amino acid long whose peptide backbones are almost fully extended) - and its folding (Kurgan 2008). As the present study characterized a partial region of two different α-skeletal actins of *Leporinus macrocephalus*, a protein diagram representation (Figure 3b) was achieved based on the complete amino acid sequence of the skeletal actin-1 and skeletal actin-2 described for *Coryphaenoides acrolepis* and *C. cinereus* (Morita 2000), since they present a identity level of 99.6% and 100% with the partial deduced actin amino acid sequences (residues 113-269) of the actin isoforms from red and white muscles of *L. macrocephalus*, respectively. Comparisons between the predicted secondary protein structures evidenced no differences between the two analyzed actin isoforms, since their alpha helices and beta strands configurations were identical, even at the 155th and 262th amino acid residues that evidence a Ser/Ala and a Phe/Ile substitution, respectively.

However, a comparison of the amino acids hydropathy of the two actin isoforms evidenced that a Serine instead of an Alanine as the 155th residue results in a small decrease in the protein hydrophobicity. Similarly, the presence of an Isoleucine instead of a Phenylalanine as the 262th amino acid also leads to a

reduction in the actin hydrophobicity. Although the Ser^{155} found in the white muscle actin isoform of *L. macrocephalus* probably not alter the protein stability or function, the Ile^{262} identified in the red muscle actin isoform of this fish species can alter the protein since amino acids 262 to 274 form an actin hydrophobic plug (H-plug) that is important for the globular actin monomer to polymerize reversibly into double-stranded actin filaments and also important for the protein stability (Shvetsov *et al.* 2008). Actin stability problems due to hydrophobicity variations have been shown to be related to amino acid substitutions along the 262-274 residues, even when these substitutions caused no significant changes in overall secondary structure (Kuang & Rubenstein 1997).

Genetic analyses of actins in fish species are mainly restricted to nucleotide and amino acid sequences descriptions and there is no data concerning the chromosome localization of these genes throughout *in situ* hybridization, probably due to the difficulty in obtaining a good resolution level, specially using PCR products directly or in plasmid vectors as hybridization probes. However, FISH mapping of actin genes could be a reasonably efficient and straightforward way of improving the resolution of comparative maps. A cDNA amplification product of *L. macrocephalus*, related to actin genes, was used as a probe in chromosome fluorescence in situ hybridization analyses. Preliminary results evidenced that the characterized α-skeletal muscle actin genes were localized at the pericentromeric region of the short arm of a large submetacentric chromosome pair (Figure 4). No additional fluorescent signals were detected and generaly two signals were observed in interphase cells.

Data on vertebrate chromosome in situ localization of muscle actin genes were mostly obtained for human cells, indicating that the α-cardiac, α-skeletal, α-vascular, β-cytoplasmic, and γ-cytoplasmic genes are located on chromosomes

15q14, 1q42.1, 10q23.3, 7p22, and 17q25, respectively (Ueyama *et al.* 1995, 1996). This distribution pattern of each actin gene to a particular chromosome pair seems to be common in other mammal species. However, actin loci in the genus *Drosophila* are dispersed over all chromosomal elements (Bondinas *et al.* 2002). Linkage mapping data on fish species, as *Danio rerio* (http://www.ncbi.nlm.nih.gov/genome/guide/zebrafish/index.html), also suggest that in this vertebrate group actin loci can be spread through several chromosomes. Therefore, even though the two different isolated α-skeletal actin genes of *L. macrocephalus* seems to be localized in a major group along a chromosome pair, other small actin cistrons might not be identified due to the FISH technical procedure.

Although no variations were identified in the nucleotide sequences of the actin genes isolated from males and females of *L. macrocephalus*, quantitative PCR analyses were performed in the α-skeletal actin genes of white and red muscle tissues from each sex in order to verify the eventual possibility of finding a gender-associated gene expression in this fish species. The occurrence of actin genes associated to a sex was already observed in some eukaryotes, as *Aedes aegypti* (Muñoz *et al*. 2004, Vyazunova & Lan 2004), *Schistosoma mansoni* (Davis *et al.* 1986), and *Schistosoma japonicum* (Fitzpatrick *et al*. 2004), a feature that seems to be associated to muscles that are required to sustain specific activities in each sex, as feeding, reproduction, or particular movements (Fitzpatrick *et al.* 2004).

The semi-quantitative analysis performed using RNA sample pools from white and red muscles of males and females of *L. macrocephalus* separately, evidenced an estimated actin mRNA level decrease in females compared to males (IOD ratio values of white muscle: males 0.675 vs. females 0.492; IOD ratio values

of red muscle: males 0.873 vs. females 0.642) (Figure 5). Further gene expression analyses were also performed, throughout Real-Time PCR, using RNA samples of white and red muscles of each male and each female separately, in order to confirm the previous semi-quantitative gene expression results. Surprisingly, individual results indicated that the characterized α-skeletal muscle actin genes had a higher relative expression in most females than in males (white muscle: females 0.01175±0.00247 vs. males 0.01082±0.004447; red muscle: females 0.000847±0.000613 vs. males 0.000754±0.000374) (Figure 6a, b). However, statistical analysis revealed no significant difference in gene expression between sexes, as values of t=-0.479, p=0.641, and t=-0.343, p=0.738 were obtained when comparing the α-skeletal actin genes from white and red muscles of males and females, respectively.

The apparently different results obtained through semi-quantitative and quantitative gene expression analyses could be related to the use of a mixture of RNA samples and the use of individual RNA samples in each of these two analyses, respectively. Moreover, although there was no significant difference when comparing all males and all females together, there was a relevant variation on gene expression when comparing individuals within each sex (Figure 6a, b). Although the biological significance of these differences is unknown, it may be related to the analysis of animals in different developmental stages, even these individuals were in adult phase and presented gonadal differentiation. As so, future gene expression analyses should be done in order to verify the occurrence of a variation in α-skeletal muscle actin gene expression throughout the developmental stages of *L. macrocephalus*.

Gene expression comparisons between the two characterized α-skeletal muscle actin genes of *L. macrocephalus*, through Real-Time PCR, also revealed a significant higher actin mRNA level in white muscle when compared to red muscle (T=105 Mann-Wh; $p<0.001$) (Figure 6c). This finding could be related to the energetic demands of the white muscle tissue, with fast contraction fibers and glycolytic metabolism for energy supply (Driedzic & Hochachka 1976, Luther *et al.* 1995, Sänger & Stoiber 2001).

Due to its conserved and ubiquitous nature, actin genes are often used as internal controls in gene expression analyses based on data that they are expressed at a constant level across all samples. However, the present results and also data on β-actin genes of fish species (e.g. Tang *et al.* 2007) indicate that these constitutive genes can exhibit variable expression and should be used with careful as internal standards in order to normalize each particular set of experimental RNA samples.

Taken together, the present data propose that α-skeletal actins from white and red muscle tissues appear to be somewhat genetically and functionally distinguishable in fish species and should be under different evolutionary selective pressures, a feature that was not found in other vertebrate groups so far. Further analyses comparing the organization and expression pattern of distinct actin isoforms in several vertebrate species would be useful to better understand the molecular evolution and the function of these genes and to verify if the variable muscle actin isoforms of fish that are not found in other animal groups are either specific to this lineage or are yet to be identified in higher vertebrates.

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Table, Figures and Legends

Table 1: Actin amino acid residues as adenosine triphosphate binding sites and actin's affinity for ATP related to some actin isoforms of distinct organisms. The vertebrate data were based on comparisons with the chicken α-skeletal muscle actin, and the yeast data were based on *Saccharomyces cerevisae* wild type actin, which were used as controls.

Actin isoform	Amino acid residue	ATP binding	Reference
carp (*Cyprinus carpio*) α-skeletal muscle actin *	a.a. 155 Ser → Ala	weak	Ooi & Soematsu (2007)
brown trout (*Salmo trutta*) α-skeletal muscle actin	a.a. 155 Ser → Ala	weak	Mudalige *et al.* (2007)
Atlantic salmon (*Salmo salar*) α-skeletal muscle actin	a.a. 155 Ser	same as control	Mudalige *et al.* (2007)
Pacific grenadier (*Coryphaenoides acrolepis*) α-skeletal muscle actin-1	a.a. 155 Ser → Ala	weak	Morita (2003)
Pacific grenadier (*Coryphaenoides. acrolepis*) α-skeletal muscle actin-2a	a.a. 155 Ser	same as control	Morita (2003)
Loosescale grenadier (*Coryphaenoides yaquinae*) α-skeletal muscle actin-2b	a.a. 155 Ser → Ala	weak	Morita (2003)
Abyssal grenadier (*Coryphaenoides armatus*) α-skeletal muscle actin-2b	a.a. 155 Ser → Ala	weak	Morita (2003)
Saccharomyces cerevisae actin *	a.a. 14 Ser → Ala	weak	Chen *et al.* (1995); Chen & Rubenstein (1995)
Saccharomyces cerevisae actin *	a.a. 157 Asp → Ala	same as control	Schüler *et al.* (1999)
Saccharomyces cerevisae actin *	a.a 177 Arg → Asp	same as control	Schüler *et al.* (2000)
Saccharomyces cerevisae actin *†	a.a. 177 Arg → His	same as control	Wen & Rubenstein (2003)
Saccharomyces cerevisae actin *	a.a. 38 Pro → Ala	same as control	Aspenström *et al.* (1993)
Saccharomyces cerevisae actin *	a.a. 374 Cys → Ser	same as control	Aspenström *et al.* (1993)
chicken smooth actin	a.a. 17 Val → Cys a.a. 89 Thr → Ser	weak	Strezelecka-Gotlaszewska *et al.* (1985)
bovine smooth actin	a.a. 17 Val → Cys a.a. 89 Thr → Ser	weak	Strezelecka-Gotlaszewska *et al.* (1985)

* refer to induced actin amino acid substitutions

† equivalent to the not induced zebrafish (*Danio rerio*) cardiofunk actin mutation and to the not induced human cardiomyopathy mutation that cause abnormal heart development (Wong *et al.* 2001)

Figure 1: Consensus nucleotide sequences of partial á-skeletal muscle actin genes isolated from white muscle (WM) and red muscle (RM) of *Leporinus macrocephalus*. Red letters indicate nucleotide variations. Regions of beginning and ending of the exons are indicated by arrows.

```
        113                                                                155                 167
WM  KANREKMTQIMFETFNVPAMYVAIQAVLSLYASGRTTGIVLDSGDGVTHNVPIYE
RM  ..........................................A.............

                                        222
WM  GYALPHAIMRLDLAGRDLTDYLMKILTERGYSFVTTAEREIVRDIKEKLCYVALD
RM  ....................................................

                                  262   269
WM  FENEMATAASSSSLEKSYELPDGQVITIGNERFRCPETLFQPSFIGM
RM  ......................................I.......
```

Figure 2: Deduced amino acid sequences (residues 113-269) of the partial α-actin skeletal muscle genes isolated from white muscle (WM) and red muscle (RM) of *Leporinus macrocephalus*. Dots indicate identical amino acid residues. The 155th and the 262th residues are indicated by bold letters.

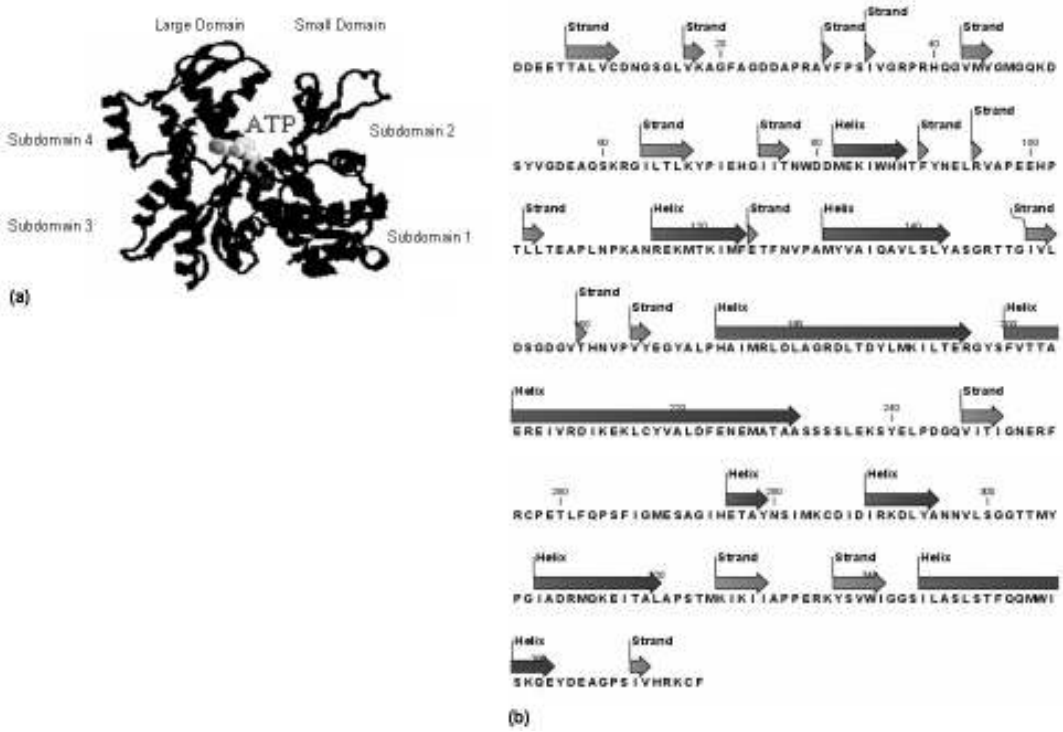

Figure 3: Actin monomer (G-actin) tridimensional structure. **(a)** the small subdomain (composed of subdomains 1 and 2) and the large subdomain (composed of subdomains 3 and 4), and the ATP-binding site at the subdomain 3. The N and C termini are located in subdomain 1. **(b)** predicted secondary structure of the α-skeletal muscle actin-2 of *Coryphaenoides acrolepis* and *C. cinereus* (based on amino acid data described by Morita 2000), evidencing the alpha helices and the beta strands configurations.

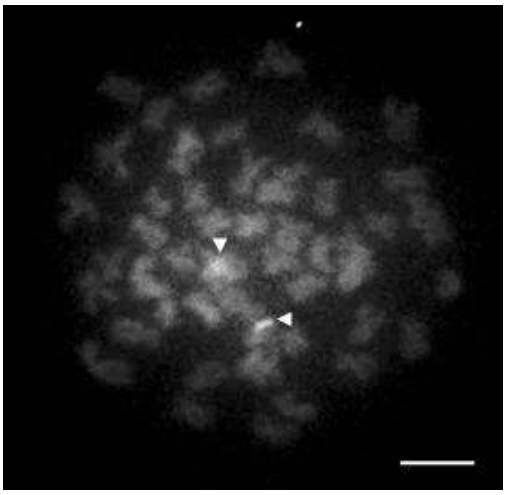

Figure 4: *In situ* hybridization results of the actin genes in metaphase chromosomes of *Leporinus macrocephalus*. Arrows indicate the actin loci. Scale bar = 3μm.

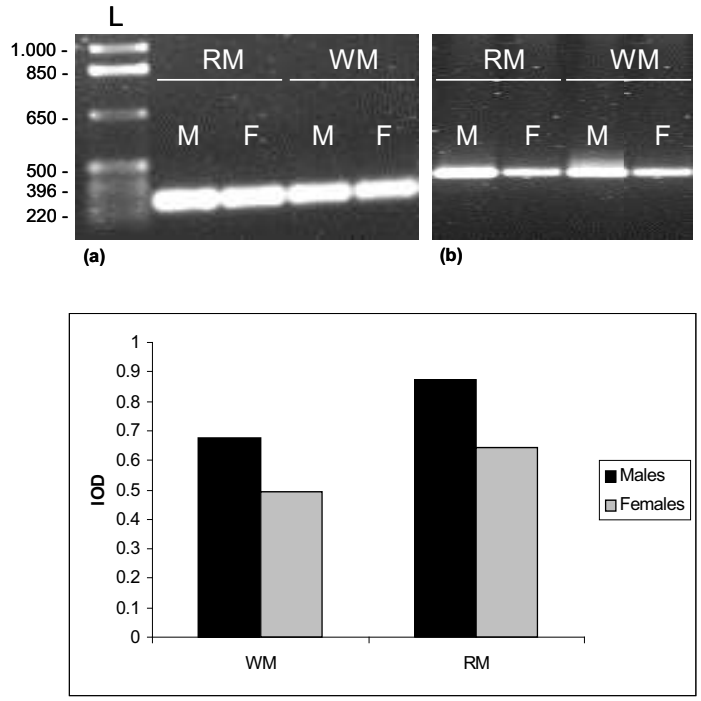

Figure 5: Alpha-skeletal muscle actin representative semi-quantitative RT-PCR results from white muscle (WM) and red muscle (RM) of males (M) and females (F) of *Leporinus macrocephalus*. 1% agarose gel evidencing the expression of the constitutive 18S rRNA gene used as control **(a)**, and the expression of the α-skeletal muscle actin genes of white and red muscle tissues **(b)**, statistical data on gene expression levels of the α-skeletal muscle actin genes of white and red muscle tissues **(c)**. Quantification of PCR signals was obtained by densitometric analysis of the product as Integrated Optical Density (IOD). **L**, 1Kb DNA molecular marker.

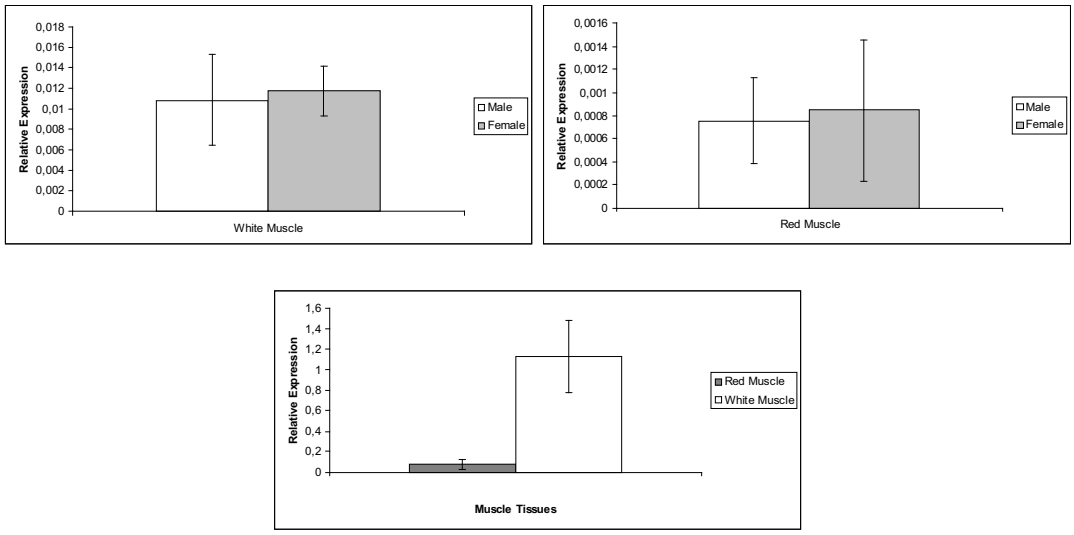

Figure 6: Relative gene expression analysis (Real-Time PCR) of the α-skeletal muscle actin genes of white and red **(c)** muscle tissues of males and females **(a, b)** of *Leporinus macrocephalus* relative to the constitutive 18S rRNA gene (18S) used as control. Data were run in duplicate. Normalized data were expressed as means ± SD.

CAPÍTULO 2:

Identificação de expressão gênica dimórfica relacionada ao sexo em tecido cerebral da espécie de peixe *Leporinus macrocephalus* através de análises de *display* diferencial e PCR em tempo real

Identification of sexually dimorphic gene expression in brain tissue of the fish *Leporinus macrocephalus* through mRNA differential display and real time PCR analyses

Abstract

Differentially expressed genes in males and females of vertebrate species generally have been investigated in gonads and, to a lesser extent, in other tissues. Therefore, we attempted to identify sexually dimorphic gene expression in the brains of adult males and females of *Leporinus macrocephalus*, a gonochoristic fish species that presents a ZZ/ZW sex determination system, throughout a comparative analysis using differential display reverse transcriptase-PCR and real-time PCR. Four cDNA fragments were characterized, representing candidate genes with differential expression between the samples. Two of these fragments presented no significant identity with previously reported gene sequences. The other two fragments, isolated from male specimens, were associated to the gene that codes for the protein APBA2 (amyloid beta (A4) precursor protein-binding, family A, member 2) and to the Rab 37 gene, a member of the Ras oncogene family. The overexpression of these genes has been associated to a greater production of the β-amyloid protein which, in turns, is the major factor that leads to Alzheimer's disease, and to the development of brain-tumors, respectively. Quantitative RT-PCR analyses revealed a higher Apba2 gene expression in males, thus validating the previous data on differential display. *L. macrocephalus* may represent an interesting animal model to the understanding of the function of several vertebrate genes, including those involved in neurodegenerative and cancer diseases.

Introduction

The development of the sexual phenotype in several vertebrates involves a complex cascade of events, initiated at conception by the fusion of two gametes and involving the action of regulatory sex determination genes - often in a response to a karyotype signal - and ending in the production of sex-specific molecules, anatomy, and behaviors (Marin & Baker 1999, Eriksson *et al.* 1999). Sex determination pathways are highly variable among different vertebrate groups, although they often include the presence or absence of a testis determining factor (SRY), leading to the formation of ovaries or testis, which in turns determine several sex-specific expression of gene networks in somatic tissues (Zarkower 2001, Wilhelm *et al.* 2007). This lack of conservation is intriguing itself, but means that the pathways of gender-specific genes must be dissected at a species level.

Mammals and birds present a relative stability in the sexual determination process, due to the presence of a XX/XY or a ZZ/ZW sex chromosome system, respectively. However, the presence and role of sex chromosomes in sex determination and differentiation might not be always clear in all vertebrates, particularly in fish species. Teleost fish can have different sex determination systems, including chromosomes that are not morphologically differentiated between males and females and sex chromosomes that are completely different between the two sexes. Moreover, males (XY) or females (ZW) can represent the heterogametic sex in different species. It has been also reported that some teleost fish have sex determination systems composed by multiple chromosomes (Tave 1993, Moreira-Filho *et al.* 1993, Almeida-Toledo & Foresti 2001). Despite this, the sexual differentiation in fish can also be determined by environmental factors and,

depending on the species, different hermaphroditic and gonochoristic modes of reproduction can occur (Yamamoto 1969, Conover *et al.* 1992).

The chromosomal, gonads, and behavioral differences observed between males and females of vertebrate species represent the major factors that lead to several efforts in the isolation and characterization of genes that are differentially expressed, especially in gonads. In fish, sex-specific genes have been isolated from male and females gonads generally in order to understand the sexual determination, and also sexual reversion, hermaphroditism, and unisexuality processes (Liu *et al.* 1996, Kanamori 2000, Guan *et al.* 2000, D´Cotta *et al.* 2001, Denslow *et al.* 2001a, b, Xie *et al.* 2001, Matsuda *et al.* 2002, Kondo *et al.* 2003, Lutfalla *et al.* 2003).

Despite the relative extensive data on genes with differential expression in vertebrate's testis and ovaries, data on sexually differential expressed genes in other organs are not so frequent, probably because these transcripts seem to be less common in somatic tissues (Santos *et al.* 2008). Although gender-associated genes have been described in heart, liver, kidney, adipose tissue, and muscles of some vertebrate species (Hutchison 1997, Mayer *et al.* 1998, Eriksson *et al.* 1999, Grossmann *et al.* 2004, Rinn & Snyder 2005, Yang *et al.* 2006, Isensee *et al.* 2008), data on sexually dimorphic genes in brain are still scarce - a well characterization of these transcripts was only performed in mouse (Eriksson *et al.* 1999, Xu *et al.* 2001, Yang *et al.* 2006, Yuge *et al.* 2007), specially due to the brain structure dimorphism between males and females. However, brain represents an extremely interesting tissue in order to analyze sexually dimorphic gene expression. Hundreds of sex differences have been identified in human and animal brains, ranging from structure and behaviors to molecules. Many of these differences have been

proven to be due to hormones and its metabolites, acting in the developing brain and permanently wiring it in a sex specific fashion (McCarthy & Konkle 2005, Becker *et al*. 2005, Morris *et al*. 2004, Xu & Disteche 2006).

Gene expression differences in brain associated to males and females have only been described in few fish species, as *Onchorhynchos nerka* (Jadhao *et al.* 2001), *Danio rerio* (Goto-Kazeto *et al.* 2004, Kallivretaki *et al.* 2007, Santos *et al.* 2008), *Fundulus heteroclitus* (Lauer *et al.* 2006), *Pimephales promelas* (Filby & Tyler 2007), *Oreochromis niloticus* (Davis *et al.* 2008), and *Dicentrarchus labrax* (Moles *et al.* 2007). There is no data on differentially expressed genes on brain tissues of neotropical fish species. Analyses in the brain of lower vertebrates represent an important source for the identification and characterization of target genes that could be related to differences in neurophysiology between males and females and for the understanding of the molecular pathways that are responsible for these differences.

In an attempt to isolate sexually dimorphic genes expressed in adult fish brain we used a DDRT-PCR approach for mRNA screening in *Leporinus macrocephalus* (piauçu), a gonochoristic fish species that represents an important food resource that has been intensively exploited in the subtropical aquaculture (Takahashi *et al.* 2004) and that presents a ZZ/ZW sex determination system (Galetti & Foresti 1987). Further quantitative PCR analysis, through real-time PCR, permitted to identify the expression levels of the isolated transcripts.

Material and Methods

Animal samples

Adult specimens of *Leporinus macrocephalus* (Characiformes, Anostomidae) were obtained from a private fishery station in São Paulo State, Brazil (Piscicultura Kabeya - municipality of Glicério), where they were maintained under the same conditions. Animals with at least 40 days were selected since they already present gonadal differentiation (Toriyama 2001) which permits the sex identification under stereomicroscopy. Brain tissue samples (including the medulla, cerebellum, optic lobes, pineal gland, pituitary gland, cerebral hemispheres, and olfactory lobes) of the animals were collected and immediately stored at -80^{o}C until RNA extraction.

RNA isolation

Approximately 100mg of the brain samples were mechanically homogenized with 1mL of TRizol Reagent (Invitrogen) and total RNA extraction followed the manufacturer's protocol. RNA samples were eluted in RNase-free water and quantified (NanoDrop 1000 Spectrophotometer) by measuring the optical density (OD) at 260nm. RNA purity was ensured by obtaining a 260/280nm OD ratio ≥1.80.

DDRT-PCR

Approximately 2μg of five RNA samples from males and females were used to prepare RNA pools of each sex which were reverse transcribed (RT-PCR), separately, with the commercial kit SuperScript First-Strand Synthesis System for

RT-PCR (Invitrogen) using an oligonucleotide $(dT)_{12-18}$ as an anchor primer, according to the manufacturer's instructions. The cDNA samples were amplified using as single primers some oligonucleotides with 15-24 bp that were designed based on VNTR core sequences (Table 1), and also RAPD (Randomly Amplified Polymorphic DNA) decamer primers (OPP series - Operon Technologies Inc.) (Table 2). Each cDNA amplification reaction consisted of 2μL of cDNA (10%), 0.2mM of primer, 1x 25mM $MgCl_2$ PCR buffer, 0.2mM of dNTPs, and 0.2 unit of Platinum *Taq* DNA polymerase (Invitrogen), in a final volume of 50μL. Reactions using the 15-24 bp primers were carried out with an initial denaturation step at 94°C for 2 minutes, followed by 40 cycles at 94°C for 50 seconds, 55°C for 2 minutes, and 72°C for 50 seconds, with an additional extension step at 72°C for 5 minutes. Reactions using the 10-mer primers were carried out with an initial denaturation step at 95°C for 5 minutes, followed by 45 cycles at 94°C for 1 minute, 36°C for 1 minute, and 72°C for 2 minutes, following the manufacturer's instructions of the Kit Ready-To-Go RAPD Analysis Beads (GE Healthcare Biosciences). DDRT-PCR products (10μL) were fractionated on 6% polyacrilamide gel, stained with 0.17% silver nitrate ($AgNO_3$) (Sambrook & Russel 2001), and visualized under white light. The molecular weigh of the amplified fragments was assigned through comparison with 1Kb DNA ladder (Invitrogen).

DNA isolation and reamplification

Selected DNA fragments that seemed to correspond to genes with differential expression between the analyzed samples were reamplified in order to obtain a higher DNA content to be used on cloning and nucleotide sequencing. The fragments were cut from the polyacrilamide matrix and purified using a

QIAquick® Gel Extraction Kit (Qiagen), following the manufacturer's instructions. The purified fragments were used on reamplification reactions, as described in Dakis & Kouretas (2002). The amplification reactions followed the conditions described above. PCR products were analyzed through 6% polyacrilamide gel electrophoresis.

Cloning, sequencing, and sequence analysis

Products of reamplification were cloned into pGEM-T (Promega) vector and used to transform competent cells of the *E. coli* strain DH5α (Invitrogen). Clones were submitted to automated sequencing on an ABI 377 Automated DNA Sequencer (Applied Biosystems) with a DYEnamic ET Terminator Cycle Sequencing kit (GE Healthcare Life Sciences), following the manufacturer's instructions, and using primers complementary to vector arms. Nucleic acid sequence database searches were performed using BLAST/N (Altschul *et al.* 1990) at the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/blast). Sequence alignments were obtained by Clustal-W function (Thompson *et al.* 1994) and the consensus sequences were manually determined.

Real-time PCR and statistical analysis

Quantitative RT-PCR was performed using Power SYBER Green PCR Master Mix Kit (Applied Biosystems), according to the manufacturer's instructions. Standard reactions (25μL) were assembled using 12.5μL of Power SYBER Green PCR Master Mix 2x, 2μL of each primer (5μM), 2μL of template cDNA treated with

DNase I (Invitrogen) (10ng/μL), and 6.5μL of ultrapure water. Primers were designed with the software Primer Express v.2.0 (Applied Biosystems), based on the DNA sequences previously obtained (Table 3). Templates cDNA were 1:10 diluted, and cDNA samples were replaced by DEPC water in the negative controls. All real time assays were carried out in duplicate using an Applied Biosystem 7300 HT Real-Time PCR system (Applied Biosystems). Forty amplification cycles were performed and each cycle consisted of 94^{o}C for 15 seconds followed by 60^{o}C for 1 minute. Amplification and dissociation curves generated by the 7300 System/Sequence Detection Software version 4.0 (Applied Biosystems) were used for gene expression analysis. The qRT-PCR signals were normalized to a segment of the 18S rRNA housekeeping gene using the primers 18S3 (5'-CGGAATGAGCGTATCCTAAACC-3') and 18S4 (5'-GCTGCTGGCACCAGACTTG-3'), also designed based on consensus sequences of this gene described for several fish species. Following the removal of outliers, raw fluorescence data were exported to the online program QPCR Standard Curve Slope to Efficiency Calculator (http://www.stratagene.com/techtoolbox/calc/qpcr_slope_eff.aspx) to determine the PCR amplification efficiency. The Ct values were used to calculate a relative gene expression value for each transcript, according to $2^{-\Delta Ct}$ method (Livak & Schimittgen 2001). The relative gene expression values were submitted to statistical analysis using Student's unpaired t-test ($p<0.05$) to normal distribution, or Mann-Whitney T value (Mann-Wh) ($p<0.05$) to data which did not display a normal distribution (Zar 1999). Differences were considered significant when $p<0.05$ and a 95% confidence level of the difference was used.

Results and Discussion

Polymerase chain reaction (PCR)-based mRNA differential display has been widely used for identifying differentially expressed transcripts in a variety of species (e.g. Liang 2002). The choice of applying a DDRT-PCR approach in *Leporinus macrocephalus* in order to identify genes with different expression levels in males and females was mainly due to the principle that this methodology does not need a previous knowledge on the mRNA sequences of the target biological samples. Moreover, the differential display (DD) technique can simultaneously visualize increased or decreased expression of numerous mRNAs from many samples, and requires relatively small amounts of starting material (Pardee & McClelland 1999). Although DD also has some disadvantages, as the possibility of isolation “false-positive” transcripts (PCR products that appear to be differentially expressed on the gel, but that cannot be verified when subsequent expression analyses are performed) (McClelland *et al.* 1995, Liang 1998, Pardee & McClelland 1999), technical improvements have been increasing its successful application. Modifications which have been proposed to improve differential display include the use of primers tailored to amplify members of a particular gene family (Jurecic 1996), the optimization of annealing temperature (Malhotra *et al.* 1998), and optimization of primer design (Graf *et al.* 1997).

In order to improve the isolation of differentially expressed genes in *L. macrocephalus*, total RNA samples were incubated with DNase I to remove DNA contamination which represents a further source of false-positive bands that can be observed on differential display gels. After DNase treatment, small aliquots of each sample were loaded onto an agarose gel to check for the RNA integrity. Subsequently, good quality RNA samples were used to prepare RNA pools of

males and females, separately, in order to avoid the detection of interindividual variations not related to differences between sexes.

Further, preliminary analyses were performed to determine primers that give good and reproducible amplification results. As so, cDNA amplification was achieved using decamer oligonucleotides commonly used in RAPD analysis, and also oligonucleotides that were designed based on VNTR (Variable Number of Tandem Repeats) core sequences that correspond to minisatellite short and highly conserved regions (Jeffreys *et al.* 1985). The use of the 10-mer primers usually resulted on several faint and diffuse bands, and the results were generally not reproducible (data not shown). These faint bands often make interpretation of the data difficult. Moreover, just a reduced number of candidate differentially expressed transcripts could be identified when comparing males and females of *L. macrocephalus* through the use of these RAPD primers. The use of longer primers on differential display strategies can also lead to detection of DNA polymorphism through RAPD-like results. Some studies evidenced that primers with 13 bases or longer have better effects in the DD efficiency (Zhao *et al.* 1995, Liang 1998, Motlik *et al.* 1998, Huang *et al.* 2001). As the oligonucleotides of VNTR core sequences (14-24 bp), used as single primers to amplify the cDNA samples of *L. macrocephalus*, are longer than RAPD primers, the methodology could be effectively carried out at a relatively high stringency, thus yielding better results – the generated bands were more distinct and the results of cDNA amplification were consistently repeatable.

The DD methodology generally leads to the visualization of 5 to 100 amplification products on polyacrilamide gel (McClelland *et al.* 1995). The obtained results indicated that, even using longer primers, it was not possible to achieve

complex amplification patterns with a high number of bands, a fact that could be due to the high stringency of the PCR conditions, trough an annealing temperature of 55^{o}C, and due to the use of RNA sample pools that can avoid the detection of individual variations. Despite the relative reduced number of amplified fragments (around 5 to 15 bands) visualized on polyacrilamide gel, some of them were identified only in males or females, which indicate that they correspond to presumptive differentially expressed transcripts between the two samples. Thirty one cDNAs appeared to be differentially expressed between males and females of *L. macrocephalus*.

During the course of the experiments, we experienced a failure to reconfirm differences in mRNA expression, due to difficulties in re-amplifying and/or cloning the selected transcripts, a problem that is relatively common in differential display strategies (Liang *et al.* 1993, Pardee & McClelland 1999). Therefore, just three of the detected candidate fragments (Figure 1) were re-amplified and cloned into a plasmid vector. The primer INS leaded to the identification of a conspicuous fragment of approximately 400 bp only in male samples of *L. macrocephalus* (Figure 1a). A fragment of around 300 bp was also identified only in male samples through the use of the primer HBV5 (Figure 1b). The primer OPP-11 permitted the visualization of a band of approximately 400 bp, unique to females of the species (Figure 1c). Since different cDNA species can be contained theoretically within one differentially expressed fragment (Liang & Pardee 1995), at least five white colonies were selected and analyzed from each cloned fragment.

Real-time PCR was used to validate the differential display results. The indication that some of the isolated genes do not present a sexually dimorphic expression between males and females of *L. macrocephalus*, as previously

deduced through the differential display data, can be associated to several factors that can affect results. Not only the experimental designs and the amplification reaction conditions, but also the systems being compared can alter the obtained data. Comparisons between the same type of cells, as performed in the present study, leads to a higher rate of false-positives simply due to the fewer differences in gene expression (Liang 1998).

DDRT-PCR results using primer INS

Cloning and sequencing of the selected band from males of *L. macrocephalus*, obtained with the primer INS, permitted the characterization of two distinct fragments with 265 bp and 231 bp (Figure 2a, b).

Database searches for nucleotide similarity evidenced that the 265 bp fragment has a high identity level with a partial region of the gene that codes for the protein APBA2 or amyloid beta (A4) precursor protein-binding, family A, member 2, of several vertebrates. A high identity level was evidenced with the *apba2* gene described for the species *Danio rerio* (85%) and *Xenopus tropicalis* (77%), in relation to a 95% query coverage of the analyzed sequence. Gene expression analyses were performed throughout Real-Time PCR using primers denominated Bami (F) and Bami (R) (Table 3), leading to the amplification of a fragment of 126 bp. Further cloning and sequencing of this fragment confirmed that it represents a segment of the *apba2* gene. The qRT-PCR analysis (reaction efficiency above 99%; R2 correlation coefficients ≥0.97) revealed a significant difference between males and females, as a higher gene expression was detected in males of *L. macrocephalus* (Figure 3a, d; Table 4) thus validating the isolated

fragment as a differentially expressed transcript. The mRNA level of males was almost three-fold higher than in females.

The APBA2 corresponds to a transmembrane cell surface glycoprotein that is mainly expressed in brain (Sutcliffe *et al.* 2003, Taru & Suzuki 2004, Sano *et al*. 2006) and that can modulate the AβPP (amyloid β-protein precursor) expression, leading to its decrease or increase. Consecutive cleavages of the AβPP by secretases generate beta-amyloid proteins (βA) (Taru & Suzuki 2004, Sano *et al*. 2006) that, at normal physiological levels, appear to be involved in neurotrophic events, i.e. the growth and survival of developing neurons and the maintenance of mature neurons (Deister & Schimidt 2006). However, an overexpression of the *aβpp* gene can lead to an increased production of the beta-amyloid protein (e.g. Mattson *et al.* 1993), a feature that has been related to Alzheimer's disease, a frequent neurodegenerative disorder of the central nervous system that causes mental deterioration and progressive dementia due to neuropathologic lesions including the presence of senile plaques mainly composed by the beta-amyloid protein (e.g. Selkoe 1994). Furthermore, it also seems that mutations along the *aβpp* gene or mutations in the enzymes that cleave the AβPP into beta-amyloid proteins can also lead to the development of Alzheimer's disease (Harrison *et al.* 1991, Hendriks & VanBroeckhoven 1996, Hartmann *et al.* 1996, Panegyres 1997, Cole *et al.* 2001, Yamamoto & Behl 2001, Xie *et al.* 2003).

Data on Alzheimer's disease indicate a prevalence of this neurodegenerative disorder in females of some human populations, a result that seem to be correlated not only to hormones, as estrogen and gonadotropins (Casadesus *et al.* 2008), but also to age and education level (Letenneur *et al.* 2000). The identification of a higher *apba2* gene expression in males of *L.*

macrocephalus can be correlated to an increase in amyloid β-protein precursor which, in turns, would lead to a higher content of beta-amyloid protein in the brain of animals of this sex. Even though the functional significance of the increased *apba2* expression in males than in females of *L. macrocephalus* is unclear, this fish species, similarly to other vertebrates as mouse (Fisher *et al.* 1991), *Xenopus laevis* (Van den Hurk *et al.* 2001) and chicken (Carrodeguas *et al*. 2005), can also be used as a model in order to studying Alzheimer's disease development or even disease therapeutic agents.

The second characterized 231 bp fragment of *L. macrocephalus*, isolated through the use of the primer INS, evidenced a high similarity with a partial region of the *Rab 37* gene, a member of the *Ras* oncogene family. This isolated transcript of *L. macrocephalus* has 87% and 85% identity, at nucleotide level, to the *Rab 37* gene described for *Danio rerio* and with the variant transcript 3 of this gene of *Homo sapiens*, respectively. The quantitative gene expression analysis (reaction efficiency above 99%; R2 correlation coefficients ≥0.97) was performed using designed primers denominated Rab37 (F) and Rab37 (R) (Table 3), leading to the amplification of a fragment of 170 bp. Further cloning and sequencing of this fragment confirmed that it represents a segment of the *Rab37* gene. Although the statistical analyses revealed a higher expression level in males than in females of *L. macrocephalus*, the differences were not statistically significant (Figure 3b, d; Table 4).

The *Rab37* gene is a member of the most common oncogenes related to human tumors - the *Ras* oncogene family. The Ras proteins correspond to small GTP-binding proteins that are involved in signal transduction pathway of several normal cellular functions, as cell proliferation, differentiation, adhesion, apoptosis,

and migration (Masuda *et al.* 2000, Stenmark & Olkkonen 2001). In brain, the *Rab37* gene is mainly related to the differentiation of neuronal cells (Bar-Sagi & Feramisco 1985, Noda *et al.* 1985, Hagag *et al.* 1986). Cancer induction seems to be related to overexpression of *Ras* genes or alterations in their proteins, which lead to an abnormal cellular proliferation (Capon *et al.* 1983, Brown *et al.* 1984, Feramisco *et al.* 1984, Stacey & Kung 1984).

Higher expression levels of *Ras* oncogenes have been described in human brain-tumors (Zimmer *et al.* 1987, Lee *et al.* 2008). However, there is no conclusive data on a sexually differential expression of these genes, since some genes of the *Ras* family present a higher expression in women while others are highly expressed in men, a feature that can also be related to the type of the cancer and age (Stenmark & Olkkonen 2001). Therefore, the obtained results that suggest a higher expression of the *Rab37* gene in males of *L. macrocepalus* remain to be elucidated. Comprehending the ways in which the regulatory actions of Rab proteins intertwine with cell-signaling cascades and developmental processes remains to be clarified.

DDRT-PCR results using primer HBV5

After cloning and sequencing the cDNA fragment that was amplified through the use of primer HBV5, observed in male samples of *L. macrocephalus*, it was possible to characterize a 194 bp segment (Figure 2c). Data base searches revealed no significant similarity with any known gene, despite a ~30 bp region that showed an identity level of 93% with a region of the gene encoding protein C (PROC) of *Gallus gallus* that functions as an anti-inflammatory and also as an inhibitor of the coagulation factors Va and VIIIa, subsequently blocking the

generation of thrombin (Jijo *et al*. 2007). Although the isolated transcript did not reveal a significant similarity to the *proc* gene (database searches evidenced a 15% query coverage of the analyzed sequence), it is also possible that the characterized segment represents an untranslated region of the mRNA which is commonly less conserved than the coding regions between homologous genes in different organisms (Colona-Romano *et al.* 1998).

A new set of primers - HBV5 (F) and HBV5 (R) - was designed in order to be used on qRT-PCR (reaction efficiency above 99%; R2 correlation coefficients ≥0.96), leading to the amplification of a fragment of 108 bp. Cloning and sequencing of this fragment confirmed a high identity with the previous isolated fragment trough the differential display strategy. Although most males presented a higher expression level of this transcript, the observed difference between both sexes was not statistically significant (Figure 3c, d; Table 4).

DDRT-PCR results using primer OPP-11

The cDNA amplification using primer OPP-11 generated a female-specific band of approximately 400 bp (Figure 1c). Cloning and sequencing of this fragment permitted the characterization of a 414 bp segment that revealed no significant identity level with previously reported gene sequences. An 89% identity level could be observed between a 36 bp of the isolated fragment of *L. macrocephalus* and the BAC clone CH251-315P9 of *Pan troglodytes* and the BAC clone RP11-54F22 of *Homo sapiens* that were mapped to the X chromosome of these species. Despite no information on the sequences contained in these BACs, the database searches evidenced extremely reduced query coverage (9%) of the analyzed sequence.

The characterized nucleotide sequence of the isolated transcript was used to design a set of primers – primers 11P(F) and 11P (R) (Table 3)– to be used on the real time PCR assay. However, there was no success in obtaining a single amplification product. Primer design and PCR conditions could have lead to the observed results, a feature that seem to be common in quantitative RT-PCR analyses (Roling *et al*. 2004). Although the obtained data do not provide relevant information about the nature of the isolated fragment, its putative dimorphic expression in males and females of *L. macrocephalus* indicates that this transcript should also be better investigated.

Final considerations

Sexually dimorphic genes have been described for different vertebrates in somatic tissues, a feature that has been proven to be due to sexual hormones, specially estrogens and androgens and its metabolites (Dewing *et al.* 2003, Morris *et al.* 2004, McCarthy & Konkle 2005, Becker *et al.* 2005, Tomassini & Pozzilli 2006, Verma & Shapiro 2006). Recent evidences indicate that differences in the expression of some genes in brain also arise under the influence of the sex chromosome complement, independent of gonadal sex steroids levels (Arnold 2004). However, there is still several lacks in the comprehension of the regulation of these genes in brain, especially in lower vertebrates. Improving data on the isolation and characterization of these transcripts in fish species, that comprise the basal vertebrate group, could give new insights into genes structure, function and evolution.

To get better insights into several genes that can present a different expression level depending on the sex, the availability of a proper model system may be crucial. The zebrafish *Danio rerio* is actually one of the few lower

vertebrate models in which genome-wide methods can be employed effectively. However, the fundamental process controlling sex assignment in this species has yet to be clarified, since no heteromorphic sex chromosomes have been detected (Sola & Gornung 2001). *Leporinus macrocephalus* is a gonochoristic species that presents a ZZ/ZW sex determination system (Galetti & Foresti 1987). Moreover, it has been intensively exploited in the subtropical aquaculture due to characteristics as the facility to adaptation to captive breeding conditions, good reproductive performance and excellent growth rate (Takahashi *et al.* 2004). Therefore, it may represent an interesting fish model to the understanding of the function of several vertebrate genes associated to sex determination and differentiation. In addition, as evidenced by the present results on the *apba2* and *Rab37* genes, it could also be used as a natural model for the assay of drugs that can regulate several processes related to genes involved in neurodegenerative and cancer diseases.

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Tables, Figures and Legends

Table 1: Oligonucleotides that were designed based on VNTR core sequences and used as single primers for the amplification of the cDNA samples of *Leporinus macrocephalus*.

Primer	Sequence (5' - 3')	Reference
INS	ACAGGGGTGTGGGG	Nakamura *et al.* (1987)
YNZ22	CTCTGGGTGTCGTGC	Nakamura *et al.* (1987)
HBV5	GGTGTAGAGAGGGGT	Nakamura *et al.* (1987)
HBV3	GGTGAAGCACAGGTG	Nakamura *et al.* (1987)
FvIIex8	ATGCACACACACAGG	Murray *et al.* (1988)
EMBL	AGAGCTTCAGGCTGGGCAGCTAAG	Harris & Wright (1995)

Table 2: Decamer oligonucleotides used as single primers for the amplification of the cDNA samples of *Leporinus macrocephalus*.

Primer	Sequence (5' - 3')
OPP-1	GTAGCACTCC
OPP-2	TCGGCACGCA
OPP-3	CTGATACGCC
OPP-4	GTGTCTCAGG
OPP-5	CCCCGGTAAC
OPP-6	GTGGGCTGAC
OPP-7	GTCCATGCCA
OPP-8	ACATCGCCCA
OPP-9	GTGGTCCGCA
OPP-10	TCCCGCCTAC
OPP-11	AACGCGTCGG
OPP-12	AAGGGCGAGT
OPP-13	GGAGTGCCTC
OPP-14	CCAGCCGAAC
OPP-15	GGAAGCCAAC
OPP-16	CCAAGCTGCC
OPP-17	TGACCCGCCT
OPP-18	GGCTTGGCCT
OPP-19	GGGAAGGACA

Table 3: Set of primers that were designed based on the nucleotide sequences of the isolated DNA fragments from *Leporinus macrocephalus,* in order to be used on Real-Time PCR assays.

Primer	(5' - 3')
Bami (F)	CATTCTTACGCTCTGCCATG
Bami (R)	TAGTAGCCCGTGATAAGGAC
Rab37 (F)	GTGGGAGTTCAAATGCTGGG
Rab37 (R)	GGAAATTCATCCCTGGCTCC
HBV5 (F)	CCCTGTTACTGCGCATGAG
HBV5 (R)	CACGCGGTATCAGGATCA
P11 (F)	GCTACATTTCAGAGTATAATAG
P11 (R)	CATATGAAAGCTTAGAATCTCT

F = forward; R = reverse

Table 4: Data on the relative expression of the isolated genes from brain tissue of *Leporinus macrocephalus*.

Designed set of primers	Mean* ± SD	"t" value	"P" values
Bami	M = $2,53x10^{-5}$±$3,47x10^{-6}$ F = $8,16x10^{-6}$±$1,53x10^{-6}$	-14,27	<0,001
Rab37	M = $8,44x10^{-7}$±$4,89x10^{-7}$ F = $7,03x10^{-7}$±$4,33x10^{-7}$	98**	0,623**
HBV5	M = $1,92x10^{-7}$±$8,52x10^{-8}$ F = $1,4x10^{-7}$±$5,85x10^{-8}$	-1,568	0,134

*Mean value refering to the values obtained for $2^{-\Delta Ct}$
** Refers to "T" value obtained for these samples through the Mann-Whitney statistical test
M, male samples; F, female samples

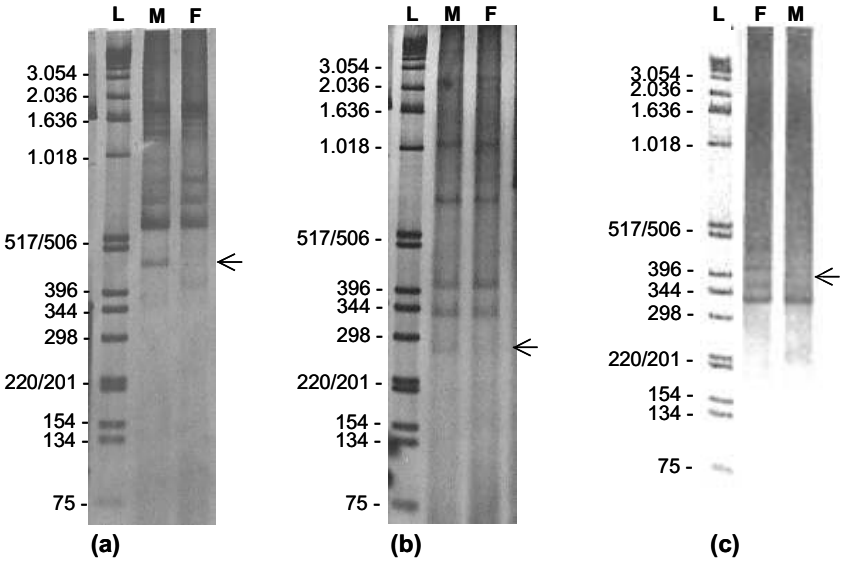

Figure 1: cDNA amplification patterns of *Leporinus macrocephalus*, visualized in 6% polyacrilamide gel, obtained through the use of primers INS **(a)**, HBV5 **(b)**, and OPP-11 **(c)**. Sex-associated fragments are indicated by arrows. L, 1Kb DNA ladder; M, males; F, females.

```
5′ ACAGGGGTGTGGGGAACTCTGCTTGCAGTGGGACGTCGAGTCCTGCGTG.....49
GCTCCTGTCCTGCAGCGATCCTCCAGCCATAGCTTTGGCATTCTTACGCTCT....101
GCCATGATCCGAGAGCAGATCTCATGCAGACTAGTGGCGATGGCTTTGGCAG....153
GCGTGTCACAGCGGAAAACGTGACATTTTAATATCCGAGTGTCCTTATCACG....205
GGCTACATACGCAAAATCCCTCTCTCTGCCGTTGTCCCGGCCTACGCCCCAC....257
ACCCCTGT 3′.........................................265
```

(a)

```
5’ ACAGGGGTGTGGGGGGGTGGGAGTTCAAATGCTGGGTACTATAGTGAAA.....49
GATCAATCATGGAAAGGCAAAAAAAGATGAACTGTGTATTCTGCTCTACGCA....101
ACGCTGCAAATAACATATTAAATGCTTGTTTTATTCGTGAAGCCGATGCCCA....153
CAGTAGCAGAAAAGGAGCCAGGGATGAATTTCCCCTGATCAAACTGGACGAG....205
CAGGGACGTTTTCCCCACACCCCTGT 3’.........................231
```

(b)

```
5’GGTGTAGAGAGGGGTGCTGGAGCTGTGATACCACACGCGGTATCAGGATC.....50
AGAATTCTGGTATCACGACAGACGCACAGCTGCAGTAGCGACCCTGCTGATA....102
ACGGGTACTCGGTTACAGCACTCATGCGCAGTAACAGGGTCTCCGGGCTTTC....154
TGGTTCTCCTGCTGACCCGATGATGACCCCTCTCTACACC 3’.............194
```

(c)

```
5’AACGCGTCGGGCCAGGAATCAAAAATAAATATATTTAAGGATATATCTGA....50
AGATGTNCAGTACCACCAAAGACAATCAAAGTACCATATGAAAGCTTAGAAT...102
CTCTACTTTTCGGTCGTTTTGCCATTTTTTCTTTATTTTCATTCAGAAAATA...154
AATATTTTGTTCAAAAACTAGCGTTAGTATGACAAAATATTAGGTACACTAC...206
GAACATTTTTATTTTTCGTTAAGCCATCTTTTGGCCAATCTGGACATATTAC...258
ACATCAAACGAATGGGGAGACCCTCAGGATTACGATGGTGTAAGCTATTATA...310
CTCTGAAATGTAGCAGTATTTAAATAAAAAATAAAGAATGGCTTTTCCAAAC...362
AGTAACACGCCTCCTAGCGCCGCCACTACTTCTGCGTTTCTCCGACGCGTT 3’.414
```

(d)

Figure 2: Consensus nucleotide sequences of cDNA fragments of *Leporinus macrocephalus,* corresponding to candidate differentially expressed genes between males and females, isolated from brain tissue using primers INS **(a** and **b)**, HBV5 **(c)**, and OPP-11 **(d)**. The underlined regions are homologous to the primer sequences.

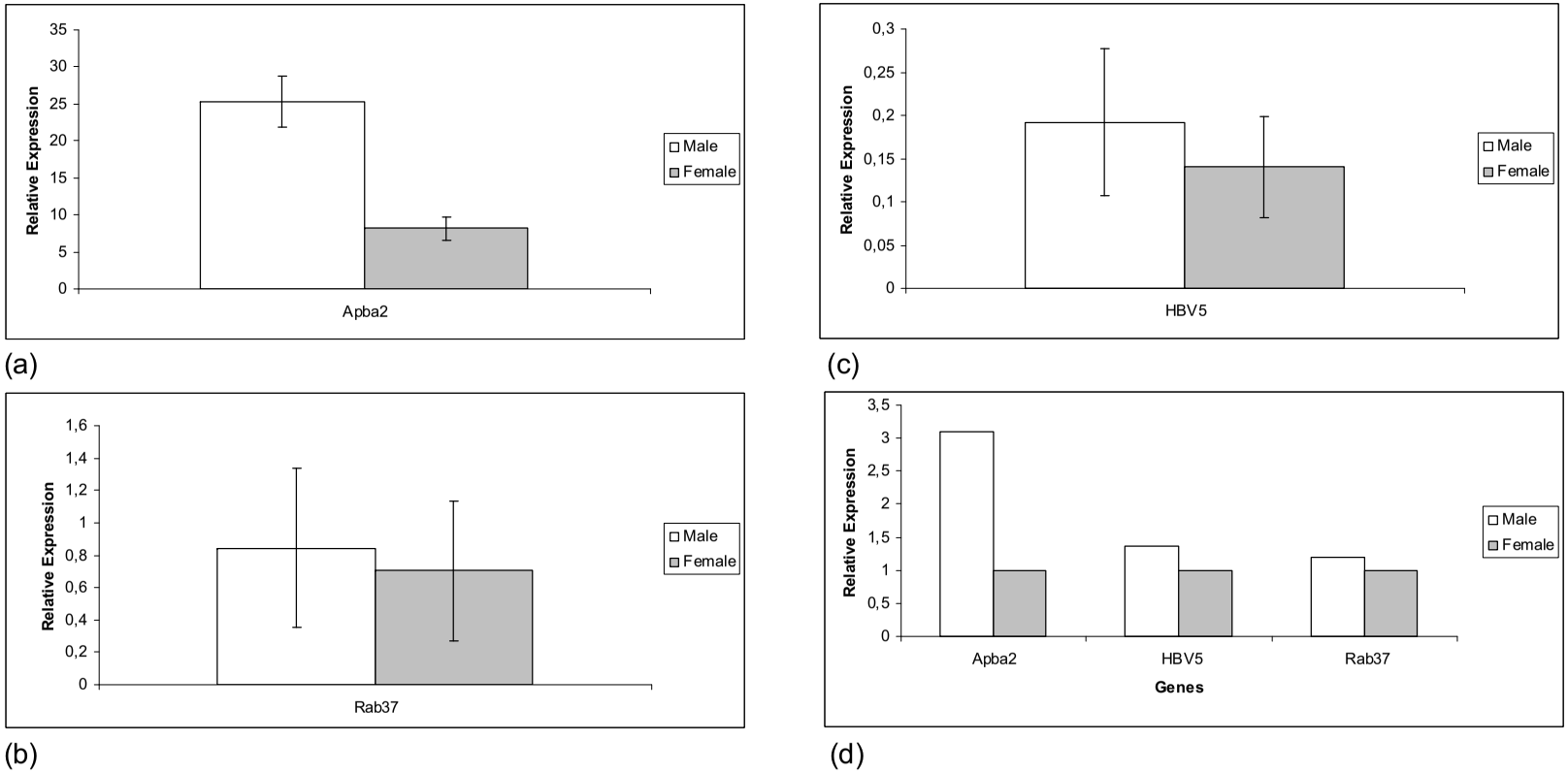

Figure 3: Relative gene expression analysis (Real-Time PCR) of the candidate sexually dimorphic expressed genes isolated through DDRT-PCR from brain tissue of *Leporinus macrocephalus*, relative to the constitutive 18S rRNA gene used as control. **(a)**, **(b)**, and **(c)** represent data on males and females, separately, using set of primers (denominated as Bami, Rab37, and HBV5) designed based on the previous nucleotide sequences of the isolated transcripts; **(d)** represents a comparative analysis of the relative expression of the three isolated genes – the females were assigned by a reference value = 1, and the males were represented by a ratio of their values in relation to the reference female value. Data were run in duplicate. Normalized data were expressed as means ± SD.

CAPÍTULO 3*

Distribuição espaço temporal de transcritos do gene de *fatty acid-binding protein 6* (*fabp6*) em *zebrafish* (*Danio rerio*) adulto e em desenvolvimento

* Artigo publicado: *FEBS Journal* 275: 3325-3334 (2008).

Spatio-temporal distribution of fatty acid-binding protein 6 (*fabp6*) gene transcripts in the developing and adult zebrafish (*Danio rerio*)

Abstract

We have determined the structure of the fatty acid-binding protein 6 (*fabp6*) gene and the tissue-specific distribution of its transcripts in embryos, larvae and adult zebrafish (*Danio rerio*). Like most members of the vertebrate *FABP* multigene family, the zebrafish *fabp6* gene contains four exons separated by three introns. The coding region of the gene and expressed sequence tags code for a polypeptide of 131 amino acids (14 kDa, pI of 6.59). The putative zebrafish Fabp6 protein shared greatest sequence identity with human FABP6 (55.3%) compared to other orthologous mammalian FABPs and paralogous zebrafish Fabps. Phylogenetic analysis showed that the zebrafish Fabp6 formed a distinct clade with the mammalian FABP6s. The zebrafish *fabp6* gene was assigned to linkage group (chromosome) 21 by radiation hybrid mapping. Conserved gene synteny was evident between the zebrafish *fabp6* gene on chromosome 21 and the *FABP6/Fabp6* genes on human chromosome 5, rat chromosome 10 and mouse chromosome 11. Zebrafish *fabp6* transcripts were first detected in the distal region of the intestine of embryos at 72 h postfertilization. This spatial distribution remained constant to 7-day-old larvae, the last stage assayed during larval development. In adult zebrafish, *fabp6* transcripts were detected by RT-PCR in RNA extracted from liver, heart, intestine, ovary and kidney (most likely adrenal tissue), but not in RNA from skin, brain, gill, eye or muscle. *In situ* hybridization of a *fabp6* riboprobe to adult zebrafish sections revealed intense hybridization signals in the adrenal homolog of the kidney and distal region of the intestine, and to a

lesser extent in the ovary and liver, a transcript distribution that is similar, but not identical, to that seen for the mammalian *FABP6/Fabp6* gene.

Introduction

Intracellular lipid-binding proteins (iLBPs) are encoded by a highly conserved multigene family, and include fatty acid-binding proteins (FABP/Fabps), cellular retinol-binding proteins (CRBPs) and the cellular retinoic acid-binding proteins (CRABPs) [1, 2]. Currently, 16 paralogous iLBP genes have been identified in animals, but no member of this multigene family has thus far been identified in plants and fungi. Schaap *et al.* [2] have therefore suggested that the first iLBP gene emerged after the divergence of animals from plants and fungi approximately 930 million years ago. This ancestral iLBP gene presumably then underwent a series of duplication events followed by sequence divergence, giving rise to the extant iLBP multigene family.

To date, 11 isoforms of FABP/Fabps or their genes, or both, have been identified in vertebrate species [3]. Originally, these proteins were named according to the tissue from which they were initially isolated, *e.g.,* liver-type fatty acid-binding protein (L-FABP), brain-type fatty acid-binding protein (B-FABP), intestinal-type fatty acid-binding protein (I-FABP), *etc.* However, this nomenclature has become confusing because different types of FABPs have been isolated from the same tissue, and some orthologous FABPs from different species exhibit distinctly different tissue-specific patterns of distribution [4, 5]. Furthermore, two so-called liver-type *fabp* genes, *fabp1a* and *fabp1b* (based on phylogenetic analysis and conserved gene synteny), are not expressed in the liver of teleost

fishes [6]. In this paper, we have used an alternative nomenclature proposed by Hertzel and Bernlohr [4], which uses numerals to distinguish the FABP proteins and genes corresponding to the chronological order of their discovery, *e.g.,* FABP1 (liver-type FABP), FABP2 (intestinal-type FABP), FABP3 (heart-type FABP), *etc.* We have also followed the gene and protein designations for mammalian and teleost fish genes and proteins according to the recommendations of the Zebrafish Model Organism Database (http://zfin.org), in which zebrafish genes and proteins are represented as *fabp6* and Fabp6, respectively, human genes and proteins are given in upper-case letters, e.g., *FABP6* and FABP6, respectively, and the mouse gene is designated *Fabp6* and its protein FABP6.

Phylogenetic studies have identified three main groups for the FABPs: group 1 includes FABP1, FABP6 and Fabp10 (Fabp10 has only been found in non-mammalian vertebrates), group 2 consists of a single protein, FABP2, and group 3 consists of FABP4, FABP5, FABP8, FABP9 and Fabp11 (Fabp11 may be unique to teleost fishes [3]). Schaap *et al.* [2] estimate that the FABPs from group 1 diverged from the last common ancestral FABP gene approximately 679 million years ago.

Although the first FABP, FABP1, was described almost four decades ago [7], and extensive studies have focused on the tissue distribution and binding activities and regulation of *FABP* genes, including FABP gene knock-out experiments [8], our understanding of the physiological function(s) of these proteins remains limited or, in many cases, unknown. However, sufficient evidence exists to strongly suggest the following roles for FABPs: (a) uptake of fatty acids across the plasma membrane and transport to various subcellular organelles; (b) modulation of activity of enzymes involved in fatty acid metabolism; (c) protection

of enzymes and membranes from the detergent effects of excess fatty acids by sequestering them, and (d) modulation of cell growth and differentiation by transport of fatty acids to the nucleus where they activate specific gene transcription [4, 8, 9].

Here we report studies on the *fabp6* gene from zebrafish, the first *fabp6* gene described for non-mammalian vertebrates. Previous work has reported the cloning and sequencing of mammalian *FABP6/Fabp6* genes and cDNAs, and their expression in mammalian species including human [10], rat [11-13], mouse [14, 15] and pig [16]. Over the years, FABP6 has been given a variety of names, such as the ileal lipid-binding protein, intestinal 15-kDa protein, ileal bile acid-binding protein and gastrotropin, reflecting the speculations of authors on its intracellular function(s). *In vitro* binding assays revealed a surprisingly low affinity of recombinant-derived human FABP6 and rat Fabp6 for long chain fatty acids, such as palmitate and oleate, despite these proteins having a common three-dimensional structural motif with other FABP/Fabps known to bind long chain fatty acids [10, 17]. Work by Gong *et al.* [12] suggests that the ligands of FABP6 are bile salts and that FABP6 is involved in their uptake from the ileal epithelium. However, other studies have detected mammalian *FABP6/Fabp6* gene transcripts and encoded protein in the ovary and steroid endocrine cells of the adrenal gland, leading to speculation that FABP6 may also function in steroid metabolism [11]. Comparative studies of *FABP6/Fabp6/fabp6* gene expression in mammals and teleost fishes may provide additional evidence for the role of this protein in cellular physiology. In this paper, we describe the structure of the zebrafish *fabp6* gene, its linkage group (chromosome) assignment, conserved gene synteny with

mammalian orthologs, and the tissue-specific distribution of the *fabp6* gene transcripts in embryos, larvae and adults.

Material and Methods

Husbandry of zebrafish

The AB strain of zebrafish was used throughout this work and maintained according to established procedures [27]. Experimental protocols were reviewed by the Animal Care Committee of Dalhousie University in accordance with guidelines set down by the Canadian Committee on Animal Care.

Nucleotide sequence of zebrafish fabp6 cDNA and gene

We retrieved a previously uncharacterized Ensembl gene (ENSDAR00000044566) by a BLASTn search of the zebrafish genome sequence database at the Wellcome Trust Sanger Institute (version Zv7, scaffold 296.3, http://www.ensembl.org/Danio_rerio/index.html) using NM_001002076 (GenBank accession number) as the query sequence. This sequence was also used in BLASTn searches for other ESTs coding for zebrafish Fabp6. Based on the NM_001002076 sequence, primers were designed for RT-PCR amplification of this transcript from total RNA extracted from a whole adult zebrafish of strain AB (forward primer, 5'-CTC TTC TTC TCC GCT CAA-3'; reverse primer, 5'-ATC AGT TTA GCT CGT ACA-3'). The resulting product of expected size as estimated by agarose gel electrophoresis was cloned into the pGEM-T vector (Promega, Madison, WI, USA) and six clones sequenced. To identify SNPs, the cDNA sequences obtained by us were compared to the coding sequence of zebrafish *fabp6* gene retrieved from the Zebrafish Genomic Sequence Database at the Wellcome Trust Sanger Institute by alignment using CLUSTALW [28]. The

molecular mass and isoelectric point of the Fabp6 polypeptide encoded by clone NM_001002076 was determined using the program at http://ca.expasy.org/tools/pi_tool.html.

Phylogenetic analysis

Sequence alignment and determination of percentage amino acid sequence identity of FABP/Fabp sequences from zebrafish and other vertebrates was performed using BIOEDIT (v.7.0.9) [29]. Phylogenetic analysis was performed using CLUSTALW [28] to generate a neighbour-joining tree. Bootstrap values were based on 100 replicates.

Linkage group (chromosome) assignment by radiation hybrid mapping of the zebrafish fabp6 gene

Radiation hybrids of the LN54 panel were used to assign the *fabp6* gene to a specific zebrafish linkage group according to the protocol described by Hukriede *et al.* [21]. Two primers were designed (forward primer, 5'-TAG GCA AAG AGA GCC ACA TGC AGA-3'; reverse primer, 5'-TGC TCA AAT CCT GAC ACC ATG GAC-3') to PCR-amplify a portion of the zebrafish *fabp6* gene from genomic DNA samples isolated from the LN54 hybrid panel using Platinum PCR Super Mix (Invitrogen, Burlington, Ontario, Canada).

Whole-mount in situ hybridization to zebrafish embryos and larvae

Whole-mount *in situ* hybridization using a cloned *fabp6* cDNA to generate an antisense riboprobe was performed according to the methods described previously [30].

Detection of fabp6 transcripts in adult zebrafish tissues by RT-PCR

RT-PCR was used to determine the tissue distribution of *fabp6* transcripts in RNA extracted from tissues of adult zebrafish. RNA was extracted from tissue using Trizol reagent (Invitrogen). Following synthesis of cDNA using the Omnicript RT kit (Qiagen, Mississauga, Ontario, Canada), the zebrafish *fabp6* transcripts were amplified by PCR from total RNA extracted from various tissues using the forward primer, 5'-TAG GCA AAG AGA GCC ACA TGG AGA-3', and the reverse primer, 5'-GCG GTT AAA CCT TCT TGC TTG TGC-3', according to the protocol described by Liu *et al.* [23]. The constitutively expressed gene for elongation factor 1α (*ef1α*) was used as a positive control to assay the integrity of RNA extracted from each tissue. The primers and RT-PCR conditions employed have been described previously [31].

Detection of fabp6 transcript in adult sections of zebrafish by in situ hybridization

A synthetic antisense probe, 5'-GTA CTG GGT CCA TGT GAA GTC ATC TCC GTT C-3', was used for *in situ* hybridizations to detect *fabp6* transcripts in sections of adult zebrafish according to the method described by Denovan-Wright *et al.* [32].

Results and Discussion

Identification of zebrafish cDNA and genomic fabp6 sequences

Following BLAST searches of GenBank at the National Center for Biotechnology Information (NCBI), we identified an expressed sequence tag (EST)

(GenBank accession number: NM_001002076) [3] for which the deduced amino acid sequence showed highest percentage sequence identity to the amino acid sequences of mammalian FABP6/Fabp6s (see below). Using this EST sequence as a query, we retrieved numerous other ESTs coding for zebrafish Fabp6 from NCBI and the sequence for the zebrafish *fabp6* gene from the genomic DNA assembly Zv7, scaffold 296.3 (ENSDARG00000044566), at the Wellcome Trust Sanger Institute (http://www.ensembl.org/Danio_rerio/index.html). In order to generate a hybridization probe for further study of the tissue-specific distribution of *fabp6* transcripts in zebrafish embryos, we amplified the *fabp6* transcript by RT-PCR of total RNA extracted from a whole adult zebrafish. The resulting DNA of the expected size was cloned, and six independent clones were sequenced and found to be identical (Genbank accession numbers EU665309 – EU665314). Five single nucleotide polymorphisms (SNP) were seen between the sequence of the *fabp6* transcripts cloned by us and the coding sequence of the *fabp6* gene (Fig. 1). Only one SNP in the coding sequence, located at nucleotide (nt) position +1633 in Fig. 1, changed the deduced amino acid sequence of Fabp6, a change of valine (GTC) to isoleucine (ATC). We attribute the five SNPs to differences between established strains in zebrafish. The coding sequence derived from the *fabp6* gene at the Wellcome Trust Sanger Institute was derived from the Tuebingen strain of zebrafish, while the sequences for the *fabp6* cDNA generated in this study were derived from the AB strain of zebrafish (see http://zfin.org for strain details).

The coding sequence of the *fabp6* gene contained an open reading frame of 393 bp (not including the stop codon), with 5' and 3' untranslated regions of 50 and 74 bp, respectively (Fig. 1). The open reading frame codes for a polypeptide of 131 amino acids with a molecular mass of 14 406 Da and an isoelectric point of

6.59. With the exception of some Fabp10s, which have an isoelectric point of 8.8-9.0, all other FABPs have isoelectric points of approximately 6 [18].

The zebrafish *fabp6* gene consists of four exons of 112, 174, 89 and 141 bp, coding for 22, 59, 30 and 20 amino acids, respectively. The intron/exon structure of the *fabp6* gene from zebrafish is consistent with that of all other *fabp* genes studied to date [1] with the exception of the muscle-type FABP (M-FABP) genes from desert locust [19], which lacks intron II, and the *fabp1b* gene from zebrafish, which contains an additional intron in the 5'-untranslated region [6]. Each of the intron/exon splice junctions in the zebrafish *fabp6* gene conform to the GT/AG rule proposed by Breathnach and Chambon [20].

Alignment of the zebrafish Fabp6 sequence with mammalian FABP6 sequences (human, rat, mouse and pig) and to paralogs of other zebrafish Fabps and human FABPs showed that zebrafish Fabp6 shared greatest sequence identity with human (55.3%), mouse (50%), rat (50%) and pig (49.2%) FABP6 sequences (Fig. 2). Sequence identity of the zebrafish Fabp6 with paralogous zebrafish Fabps and human FABPs varied from 42.1% to 21.8%. Phylogenetic analysis revealed the inclusion of zebrafish Fabp6 with human, rat, mouse and pig FABP6s in a distinct clade with a robust bootstrap value of 99/100 (Fig. 3). The phylogenetic tree indicates a closer evolutionary relationship between FABP6s and FABP1s, and a more distant relationship between FABP6s and FABP7s, a finding consistent with early phylogenetic studies of rat and human FABP6 and FABP1 [10, 12]. The sequence alignment (Fig. 2) and phylogenetic analysis (Fig. 3) strongly suggests that the ESTs and genomic sequence retrieved from DNA assembly Zv7, scaffold 296.3 (Wellcome Trust Sanger Institute zebrafish genome sequence) described above, code for Fabp6 in zebrafish.

Linkage group assignment of the zebrafish fabp6 gene by radiation hybrid mapping and its conserved gene synteny with mammalian FABP6/Fabp6 genes

To provide additional evidence that the gene located on the DNA assembly Zv.7, scaffold 296.3, indeed codes for zebrafish Fabp6, we determined the linkage group (chromosome) assignment of the zebrafish *fabp6* gene and examined its conserved gene synteny with the human, rat and mouse *FABP6/Fabp6* genes. Using the LN54 panel of radiation hybrids [21] and specific primers to exon 2 and introns 2, respectively (see Fig. 1 and experimental procedures), the zebrafish *fabp6* gene was mapped to linkage group (chromosome) 21 at a distance of 26.79 cR from the marker fc08c06 with a LOD (logarithm of the odds [to the base 10]) of 10.8 (mapping data available at http://dir.nichd.nih.gov/Img/devb.htm). This result is consistent with the chromosomal location of *fabp6* on Zv6 at the Wellcome Trust Sanger Institute database, but not with the latest version, Zv7, which places the zebrafish *fabp6* gene on chromosome 3. We have previously observed incompatibilities between radiation hybrid mapping data for other zebrafish *fabp* genes and their chromosomal assignment in the Wellcome Trust Sanger Institute genome database for the zebrafish. Later, versions of the zebrafish genome sequence have been corrected in agreement with the chromosomal assignment of *fabp* genes by radiation hybrid mapping.

The conserved gene synteny between the zebrafish *fabp6* gene on chromosome 21 and human *FABP6* gene on chromosome 5 is extensive (Table 1). Conserved gene synteny was also evident between the zebrafish *fabp6* gene and the *Fabp6* genes on rat chromosome 10 and mouse chromosome 11. Not all the genes that show conserved gene synteny between zebrafish chromosome 21

and human chromosome 5 are located on rat chromosome 10 and mouse chromosome 11. Other genes are located on rat chromosomes 2, 17, 18 and 20, and mouse chromosome, 13, 15 and 18, suggesting chromosomal rearrangements or translocations in these regions after divergence of the human and rodent lineages. Despite these chromosomal rearrangements, the conserved gene synteny shown in Table 1 strongly indicates that a common linkage group containing the *FABP6/Fabp6/fabp6* gene was inherited from a common ancestor of fishes and mammals. The conserved gene synteny (Table 1), sequence identity (Fig. 2) and phylogenetic analysis (Fig. 3) provide compelling evidence that the putative zebrafish *fabp6* gene described here and the mammalian *FABP6/Fabp6* genes are orthologs.

Distribution of fabp6 gene transcripts in zebrafish embryos and larvae

To determine the spatio-temporal distribution of *fabp6* transcripts during zebrafish embryonic and larval development, we performed whole-mount *in situ* hybridization to zebrafish embryos and larvae at various developmental stages (Fig. 4). *fabp6* transcripts were not detected in embryos at 48 h postfertilization (hpf), but a very strong hybridization signal was detected in the distal region of the zebrafish intestine at 72 hpf (Fig. 4A) indicating that initiation of *fapb6* gene transcription occurred between 48 and 72 hfp. The distribution of *fabp6* transcripts remained constant in the distal region of the intestine of zebrafish larvae from 3 to 7 days postfertilization (Fig. 4A-C). A transverse section of a 4-day-old larva showed the presence of *fabp6* transcripts located predominately in epithelial cells of the intestine (Fig. 4B).

To our knowledge, only two studies have investigated the tissue-specific distribution of *FABP6/Fabp6* transcripts during embryogenesis [14,15]. In the mouse, Sacchettini *et al.* [14] showed by dot-blot hybridization that no *Fabp6* transcripts were detected in any tissues during fetal life, or throughout the suckling period of 1-12 postnatal days. Mouse *Fabp6* transcripts were first detected and restricted to the ileum at the beginning of the suckling/weaning transition at postnatal days 12-14. In contrast, Crossman *et al.* [15] did detect *Fabp6* transcripts in mouse embryos. They used Northern blot analysis and quantified mRNA steady-state levels by scanning autoradiograms of RNA extracted from total intestine and sections along the entire length of the intestine (*i.e.*, from the gastroduodenal junction to the rectum). *Fabp6* transcripts were first detected in RNA from total intestine at E18 which is the stage at which the 'proximal-to-distal wave of cytodifferentiation of the pseudo-stratified gut epithelium to a monolayer had reached the ileum' [14]. During postnatal development, *Fabp6* transcripts were restricted to the distal third of the small intestine and cecum. No *Fabp6* transcripts were detected in the duodenum, jejunum or 12 other extraintestinal tissues (the latter tissues were not specified). The transcriptional initiation of the zebrafish *fabp6* gene in the distal region of the intestine at around 72 hpf , prior to hatching (Fig. 5), occurs at approximately the same developmental stage as the transcription initiation of the mouse *Fabp6* gene in the ileum at E18 [14].

Tissue-specific distribution of fabp6 gene transcript in adult zebrafish

We explored the tissue-specific distribution of *fabp6* transcripts in adult zebrafish by RT-PCR amplification from total RNA extracted from various tissues and by *in situ* hybridization of a *fabp6*-specific antisense oligonucleotide probe to

sections of adult zebrafish. A *fabp6*-specific RT-PCR product of expected size was amplified from total RNA extracted from liver, heart, intestine, ovary and kidney (Fig. 5, top panel). No *fabp6*-specific RT-PCR product was amplified from total RNA extracted from skin, brain, gill, eye or muscle. As a positive control to determine the integrity of the RNA samples used in these assays, transcripts for the constitutively expressed elongation factor 1α (*ef1*α) gene were amplified by RT-PCR. A product of the expected size was generated from RNA extracted from all tissues assayed (Fig. 5, bottom panel).

In situ hybridization of an antisense *fabp6* oligonucleotide probe to adult zebrafish sections revealed an intense hybridization signal in the distal region of the intestine (Fig. 6, 1A) and in the adrenal homolog of the kidney (Fig. 6, 1D). Less-intense hybridization signals were observed in the liver (Fig. 6, 1B) and the ovary (Fig. 6, 1C). Despite the difference in sensitivity of the two methods employed, the tissue distribution of *fabp6* transcripts in adult zebrafish assayed by RT-PCR and by *in situ* hybridization was identical.

In adult mammals, the reported tissue distributions of *FABP6/Fabp6* gene transcripts and its protein have varied, probably due to the assay techniques used. For example, Fujita *et al.* [10] used Northern blot analysis to detect a single-sized *FABP6* transcript in RNA extracted from the terminal region of the human ileum, whereas RT-PCR generated an abundant *FABP6*-specific product from total RNA extracted from the ileum, and to a much lesser extent from RNA extracted from the human ovary and placenta. Unfortunately, the authors do not state whether other tissues were assayed by RT-PCR in which *FABP6* transcripts were not detected. Rat *Fabp6* transcripts were detected by Northern blot analysis in RNA extracted from the ileum and ovary, but not in RNA extracted from the stomach, jejunum,

colon, adrenal, brain, heart, or liver [12]. Iseki *et al.* [11] used immunocytochemistry to localize the rat Fabp6 protein and *in situ* hybridization to localize *Fabp6* transcripts to the enterocytes of the ileum, luteal cells of the ovary and a subpopulation of steroid endocrine cells of the adrenal gland. Sato *et al.* [13] also detected rat FABP6 in the adrenal gland and ovary. In adult mouse, *Fabp6* transcripts were only detected by blot hybridization in the intestine, and not in the liver, stomach, pancreas, kidney, spleen, testis, skeletal muscle, heart or lung [14].

With the exception of one report [12], these studies consistently show that the *FABP6/Fabp6* gene transcripts are expressed at high levels in the ileum and to a lesser extent in the ovary and adrenal gland of adult mammals. In zebrafish, we showed by RT-PCR and by *in situ* hybridization that *fabp6* transcripts were detected at high levels in the distal region of the intestine, the tissue homologous to the mammalian ileum. The presence of *fabp6* transcripts suggests that Fabp6 may well play a role in the uptake of lipids from the distal region of the zebrafish intestine, which is similar to the suggested role for FABP6 in the uptake of bile salts from the mammalian ileum [11, 12]. Zebrafish *fabp6* transcripts were shown by RT-PCR assay (Fig. 5) and by *in situ* hybridization (Fig. 6) to be abundant in the ovary and kidney of adult zebrafish, similar to the distribution of mammalian *FABP6/Fabp6* transcripts, which are also generally found in the ovary and adrenal gland. In fishes, the adrenal homolog is not as compact as the adrenal gland found in mammals. In fishes, adrenal tissue exists as aminergic chromaffin and inter-regnal cells mostly inside the head kidney with the two tissues being either mixed, adjacent, or completely separated [22]. The distribution of the hybridization signal for zebrafish *fabp6* transcripts in the adrenal homolog of the kidney (Fig. 6, 1D) is consistent with the structure of the adrenal homolog in teleost fishes. With the

exception of the zebrafish liver and heart, the overall pattern of adult tissue distribution of zebrafish *fapb6* and mammalian *FABP6/Fabp6* gene transcripts, and the transcriptional initiation of these genes at similar embryonic stages of development, is surprisingly concordant, in contrast to some other members of the multigene family of iLBP genes (*e.g., fabp1a/b*, *fabp10, fabp11, rbp2*) [3,6,16,23,24]. As FABP6 has been implicated in human colorectal cancer [25] and type 2 diabetes [26], zebrafish may serve as a useful model experimental system to investigate the role of FABP6 in these disease states.

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Table, Figures and Legends

Table 1: Conserved gene synteny between zebrafish linkage group (chromosome) 21 and human chromosome 5, rat chromosome 10 and mouse chromosome 11.

Genes	Chromosomal Position									
	Zebrafish	Human	Rat				Mouse			
	LG	5	2	10	17 /20	18	11	13	15	18
c6	21	5p13	2q16						15 3.0cM	
c7	21	5p13	2q16						15 3.0cM	
rpl37	21	5p13	2q16						15 A1	
fgf10	21	5p13-p12	2q15-q16					13 75.0cM		
taf9	21	5q11.2-q13.1	2q12					13 D1		
f2rl1	21	5q13	2q12					13 75.0cM		
thbs4	21	5q13	2q12					13 46.99cM		
bhmt	21	5q13.1-q15	2q12					13 D1		
glrx	21	5q14	2q11					13 44.0cM		
ell2	21	5q15	2q11					13C1		
pcsk1	21	5q15-q21	2q11-q12					13 44.0cM		
rnf14	21	5q23.3-q31				18p11				18 17.0cM
cdc23	21	5q31				18p12				18 17.0cM
pou4f3	21	5q31				18p11				18 24.0cM
sept8	21	5q31		10q22			11 28.5cM			
skp1a	21	5q31		10q22			11 31.0cM			
vdac1	21	5q31		10q22			11 29.0cM			
cnot8	21	5q31-q33		10q22			11 B1.3			
ddx46	21	5q31.1			17p14			13 B2		
pdlim4	21	5q31.1	-	-	-	-	11 28.5cM			
rapgef6	21	5q31.1		10q22			11 B1.3			
sara2	21	5q31.1		10q22			11 B1.3			
tcf7	21	5q31.1		10q22			11 28.0cM			
zcchc10	21	5q31.1		10q22			11 28.5cM			
spry4	21	5q31.3				18p11				18 18.0cM
zmat2	21	5q31.3				18p11				18 B2
rbm22	21	5q33.1				18q12.1				18 D2
larp1	21	5q33.2		10q22			11 B2			
sap30l	21	5q33.2		10q22			11 B2			
rnf145	21	5q33.3	-	-	-	-	11 B1.1			
fabp6	**21**	**5q33.3-q34**		**10q21**			**11 24.0cM**			
sgcd	21	5q33.3-q34		10q21			11 B1.2			
mat2b	21	5q34-q35		10q12			11 A5			
drd1	21	5q35.1			20p12			13 32.0cM		
fgfr4	21	5q35.1			17p14			13 33.0cM		
rars	21	5q35.1		10q12			11 A4			
ubtd2	21	5q35.1	-	-	-	-	11 A4			
cnot6	21	5q35.3		10q22			11 B1.2			
nola2	21	5q35.3		10q22			11 28.5cM			

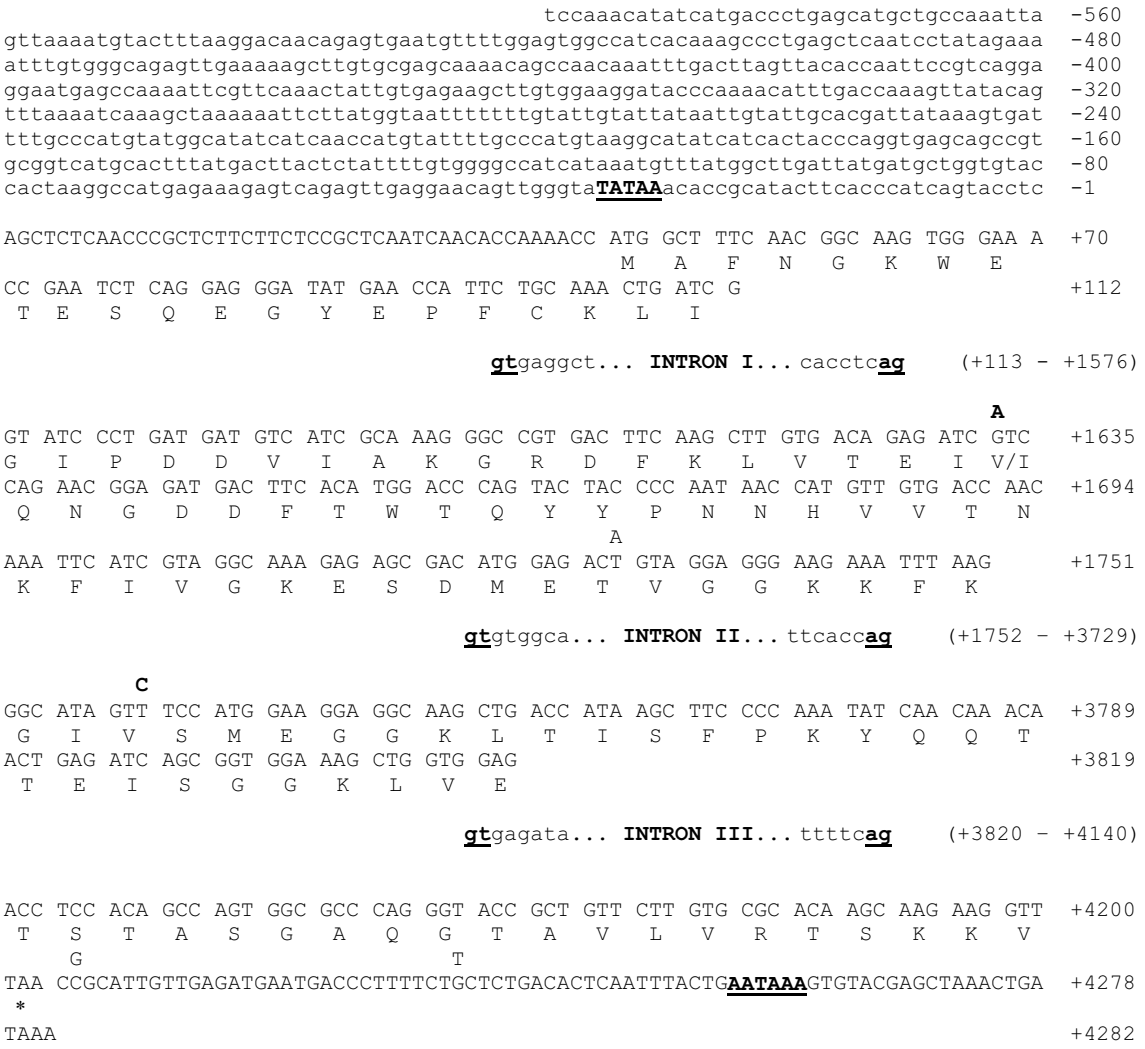

Figure 1. The nucleotide sequence of the zebrafish *fabp6* gene. The coding sequence is shown in uppercase letters with the deduced amino acid sequence indicated below. The size of each intron is shown with each exon/intron splice junctions (gt-ag) highlighted in bold font and underlined and the 5' upstream sequence of the *fabp6* gene is presented by lowercase letters with a putative TATA box in uppercase letters, underlined and in bold font. Single nucleotide polymorphisms (SNPs) based on differences between cDNA sequences from the Tuebingen and AB strains of zebrafish are positioned above the genomic sequence and in bold font. A polyadenylation signal sequence, “AATAAA”, is underlined and in bold font.

```
ZF FABP6     M--AFNGKWETESQEGYEPFCKLIGIPDDVIAKGRDFKLVTEIVQNGDDFTWTQYYPNNHVVTNKFIVGK
HU FABP6     .--..T..F.M..EKN.DE.M..L..SS...E.A.N..I...VQ.D.Q....S.H.SGG.TM....T...
MO FABP6     .--..S..Y.F..EKN.DE.M.RL.L.G...ER..N..II..VQ.D.Q....S.S.SGGNIMS...TI..
RA FABP6     .--..T..Y.F..EKN.DE.M.RL.L.E...ER..N..II..VQ.D.EN...S.S.SGGNIMS...TI..
PI FABP6     .--..T..Y.I..EKN.DE.M.RLAL.S.A.D.A.NL.IIS.VK.D.QN...S.Q..GG.SI..T.TI..
ZF FABP1B    .--S.T..YQL.....F.E.M.AV.L...M.E..K.I.S.S..EE..NQ.KV.VT-TGSK.L..S.TI.Q
HU FABP1     .--S.S..YQLQ...NF.A.M.A..L.EEL.Q..K.I.G.S......KH.KF.IT-AGSK.IQ.E.T..E
ZF FABP1A    .--..T..YQL..H.NF.A.M.AV.V...EVE..K.I.SIS..H.D.K..KV.VT-AGTK.ILYS.T..E
HU FABP3     .VD..L.T.KLVDSKNFDDYM.SL.VGFATRQVASMT.PT.I.EK...IL.LKTH-STFKNTEIS.KL.V
ZF FABP7A    .VD..CAT.KLVDSQNFDEYM.SL.VGFATRQV.NVT.PTIV.SHE..KVVIKTL-STFKNTEIS.KL.E
HU FABP2     .--..DST.KVDRS.N.DK.MEKM.VNIVKRKLAAHDN.KLT.T.E.NK..VKES-SAFRNIEVV.EL.V
HU FABP7     .VE..CAT.KLTNSQNFDEYM.AL.VGFATRQV.NVT.PTVI.S.E..KVVIRTL-STFKNTEIS.QL.E
ZF FABP3     .AD..I.T.NLKESKNFDEYM.G..VGFATRQVANMT.PT.I.SKE..V..LKTV-STFKSTEIN.KL.E
HU FABP4     .CD..V.T.KLV.S.NFDDYM.EV.VGFATRKVAGMA.PNMI.SV...VI.IKSE-STFKNTEIS..L.Q
ZF FABP2     .--T...T.KVDRN.N..K.MEQM.VNMVKRKLAAHDN.KITLE.T..K.NVKEV-STFRTLEIN.TL.V
ZF FABP7B    .VD..C.T.KLV.SDNFDEYM.SL..GFATRQV.NVT.PTLV.SKE.EKVVIKTQ-STFKNTEIS.TL.E

ZF FABP6    ESDMETVGGKKFKGIVSMEG-GKLTISFPK------YQQTTEISG-GKLVETSTASGAQGTAVLVRTSKKV-    100.0%
HU FABP6    ..NIQ.M...T..AT.Q...-...VVN..N------.H..S..V.-D....V..IG.VT----YE.V..RLA    55.3%
MO FABP6    .CE.Q.M......AT.K...-..VVAE..N------.H..S.VV.-D....I..IGDVT----YE.V..RLA    50.0%
RA FABP6    .CE.Q.M......AT.K...-..VVAD..N------.H..S.VV.-D....I..IGDVT----YE.V..R.A    50.0%
PI FABP6    .C.I..I......AT.Q...-..VVVNS.N------.HH.A..VD-.....V..VG.VT----YE.V...LA    49.2%
ZF FABP1B   .A.I..LT.E.V.TT.NR..-N..KVVLNR------ITSI..LVDTNT..N.L.LG.LV----YK.I..R.A    42.1%
HU FABP1    .CEL..MT.E.V.TV.QL..DN..VTT.KN------IKSV..LN.-DIITN.M.LGDIV----FK.I..RI-    39.3%
ZF FABP1A   .CEL..FT.DRA.TV.Q.D.-N...AFVKG------IESV..LD.-DTISN.LSFN.IV----YK.I.RRIS    36.3%
HU FABP3    .F.ET.ADDR.V.S..TLD.-...VHLQKWD--GQETTLVR.LID-...IL.L.HGT.V----CT..YE.EA    26.8%
ZF FABP7A   .F.ET.ADDRHV.ST..L..-DN.VQVQRWD--GKETKFVR..KD-..M.M.L.FE.V.----A...YE.A-    25.5%
HU FABP2    TFNYNLAD.TELR.TW.L..-N..IGK.KRTDNGNELNTVR..I.-DE..Q.YVYE.VE----AK.IF..D-    24.8%
HU FABP7    .F.ET.ADDRNC.SV..LD.-D..VHIQKWD--GKETNFVR..KD-..M.M.L.FGDVV----A..HYE.A-    24.0%
ZF FABP3    .F.ET.ADDR.V.SVITLD.-...LHVQKWD--GKETTLLR.V.D-NN.TL.L.LGDIV----ST.HYV.AE    23.9%
HU FABP4    .F.EV.ADDR.V.STITLD.-.V.VHVQKWD--GKSTTIKRKRED-D...VECVMK.VT----ST.VYERA-    22.6%
ZF FABP2    TF.YSLAD.TELT.SWVI..-DT.KGT.TRKDNGKVLTTVRT.VN-.E..QSYSYD.VE----AK.IF.RA-    22.6%
ZF FABP7B   .FEET.ADDRHC.ST.LLK.-NQ.VHVQKWD--GKETTFIR..KD-..M.MKL.FGDVE----AL..YE.A-    21.8%
```

Figure 2. Sequence alignment and amino acid sequence identity of zebrafish Fabp6 and FABP6 from different species, and paralogues of other zebrafish Fabps and human FABPs. The deduced amino acids sequence of the zebrafish Fabp6 (Ensembl Peptide ID: ENSDARP00000065447) was compared to sequences of FABP6s from human (HU FABP6; U19869), mouse (MO FABP6; CAI24826), rat (RA FABP6; NP_058794), pig (PI FABP6; P10289), zebrafish Fabp paralogues, FABP1A (ZF FABP1A; DQ062095), FABP1B (ZF FABP1B; DQ062096), FABP2 (ZF FABP2; AAH75970), FABP3 (ZF FABP3; NP_694493), FABP7A (ZF FABP7A; NP_571680), FABP7B (ZF FABP7B; AAQ92970), and human FABPs, FABP1 (Hu FABP1; M10617) , FABP2 (HU FABP2; M18079), FABP3 (HU FABP3; X56549), FABP4 (HU FABP4; NP_00133) and FABP7 (HU FABP7; CAI15449). Dots indicate amino acids identity. Dashes have been introduced to maximize alignment. The percentage amino acid sequence similarities between the zebrafish Fabp6 and other FABPs are shown at the end of each sequence.

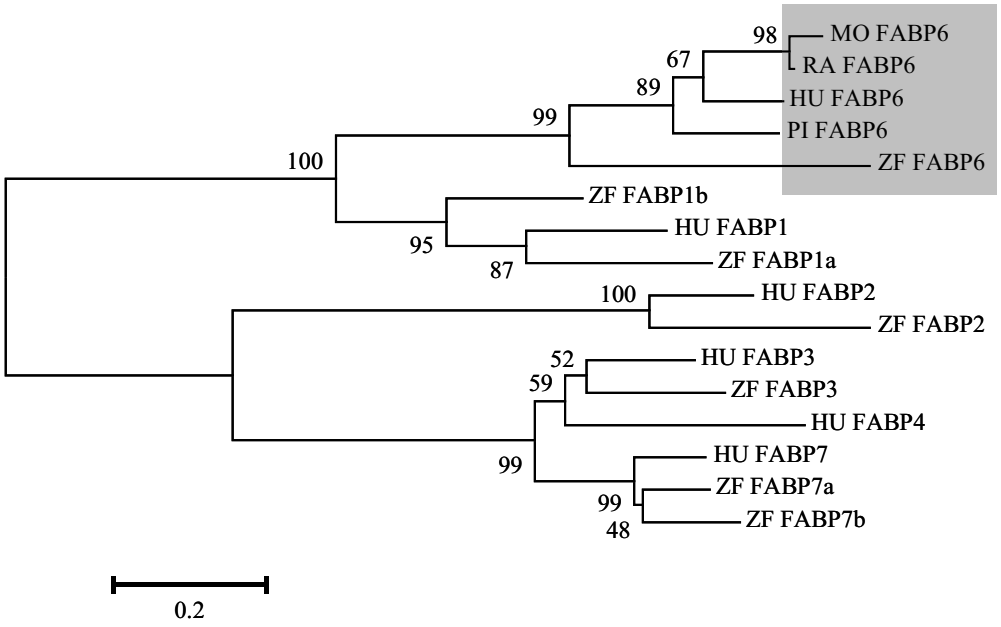

Figure 3. A neighbour-joining tree showing the phylogenetic relationship of zebrafish fabp6 with selected paralogous and orthologous FABP/Fabps from zebrafish and mammals. The bootstrap values (based 100 duplicates) are indicated at the nodes. The sequences used were: zebrafish FABP6 (Ensembl Peptide ID: ENSDARP00000065447); mammalian sequences for FABP6 from human (HU FABP6, U19869), mouse (MO FABP6, CAI24826), rat (RA FABP6, NP_058794) and pig (PI FABP6, P10289); zebrafish FABP1A (ZF Fabp1A, DQ062095), FABP1B (ZF Fabp1B, DQ062096), FABP2 (ZF Fabp2, AAH75970), FABP3 (ZF Fabp3, NP_694493), FABP7A (ZF Fabp7A, NP_571680) and FABP7B (ZF Fabp7B, AAQ92970); human FABP1 (Hu FABP1, M10617) , FABP2 (HU FABP2, M18079), FABP3 (HU FABP3, X56549), FABP4 (HU FABP4, NP_00133) and FABP7 (HU FABP7, CAI15449). The distinct clade of FABP6/Fabp6s is boxed in grey. Scale bar = 0.2 substitutions per site.

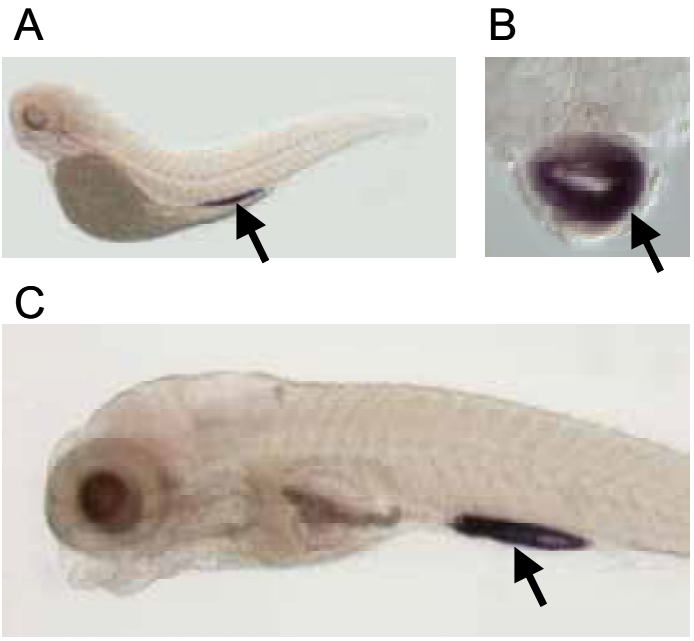

Figure 4. Spatio-temporal distribution of *fabp6* transcripts during zebrafish embryonic and larval development was determined by whole-mount *in situ* hybridization. *fabp6* transcripts were first detected at 72 hours postfertilization (hpf) in the distal region of the intestine (Fig. 4A) and remained confined to this region of the intestine up to 7 day postfertilization (Fig. 4C), the last time point assayed. Fig. 4B shows the distribution of *fabp6* transcripts throughout the enterocytes of the intestine in a transverse section at 4 days postfertilization.

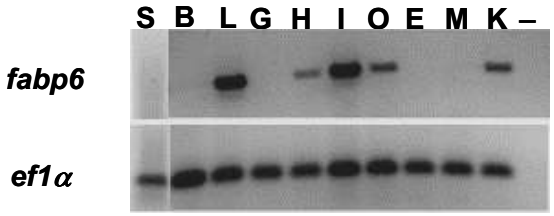

Figure 5. RT-PCR detection of *fabp6* transcripts in RNA extracted from tissues of adult zebrafish. RT-PCR generated a *fabp*6 mRNA-specific product from RNA extracted from adult zebrafish liver (L), heart (H), intestine (I), ovary (O) and kidney (K). No *fabp*6 mRNA-specific product was generated by RT-PCR of RNA extracted from adult zebrafish skin (S), brain (B), gills (G), eyes (E), muscle (M), or the negative control (-) lacking total RNA derived from a whole fish. As a positive control for the integrity of each RNA template, an *ef1á* mRNA-specific product was generated from all the adult zebrafish tissues analyzed.

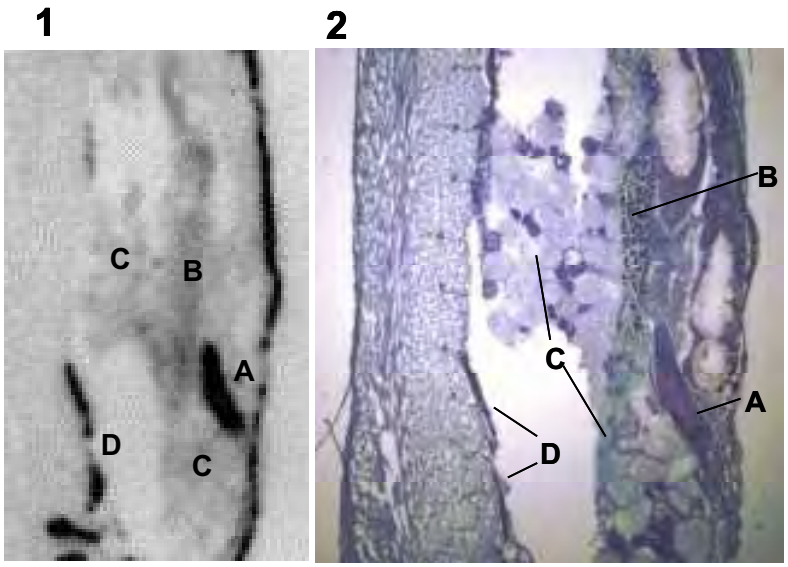

Figure 6. Tissue-specific detection of *fabp6* gene transcripts by *in situ* hybridization of an antisense riboprobe to sections of adult zebrafish. Panel **1** shows the distribution of the *fapb6* transcript in the distal region of the intestine (A), liver (B), ovary (C) and the adrenal homolog in the fish kidney (D). Panel **2** shows the location of distal region of the intestine (A), liver (B), ovary (C) and the adrenal homolog in the fish kidney (D) in an adjacent tissue section stained with cresyl violet.

CAPÍTULO 4

Análise comparativa de expressão de transcritos gênicos em tecido cerebral de *zebrafish* (*Danio rerio*) selvagem e transgênico para o gene do hormônio do crescimento (GH), utilizando uma abordagem de DDRT-PCR

A comparative expression analysis of gene transcripts in brain tissue of wild-type and GH-transgenic zebrafish (*Danio rerio*) using a DDRT-PCR approach

Abstract

Previous data on homozygote individuals of the GH-transgenic zebrafish F0104 line indicated that, despite expressing a higher amount of exogenous growth hormone in relation to hemizygote animals, they presented a significant reduced growth rate (similar to non-transgenic individuals). In the present study, hemizygote (HE) and homozygote (HO) samples of this GH-transgenic fish line were compared to non-transgenic (NT) samples of the species through a DDRT-PCR (*Differential Display Reverse Transcriptase - Polymerase Chain Reaction*) approach, with the goal of identifying candidate differentially expressed transcripts in brain tissue that could be involved in growth process. Two isolated fragments evidenced a high identity level with the genes that code for the proteins p300 (E1A binding protein p300) and ADCY2 (adenylate cyclase 2). Densitometric analyses of the p300 amplification product pointed to a significant lower gene expression in the transgenic genotypes, specially in HE (17.41±5.32) than in HO (32.25±3.21) samples, when compared to NT samples (49.35±12.32). Inferences on the ADCY2 transcript suggested a higher expression level in NT animals (22.73±3.80) than in HE (14.05±4.95) and HO individuals (10.63±0.67). These results corroborate previous data that evidenced that p300 and adenylate cyclase are involved in muscle formation/growth and in GH release, respectively. The obtained data can give support to further analyses into the

elucidation of the mechanisms beyond vertebrate growth and GH expression gene control.

Introduction

Zebrafish (*Danio rerio*) is a well-established model organism in scientific research (Kimmel 1989, Westerfield 1995), especially in behavioral, physiologic, and developmental analyses due to several characteristics of the species. The large, robust and transparent embryos and eggs, and the rapid early development are traits that facilitate experimental manipulation and observation. Moreover, the continuous reproduction, external development, relatively short generation time, large number of progeny, and small size adults, represent important features that allow for large-scale experiments, even in reduced spaces (Kimmel 1989, Westerfield 1995, Higashijima *et al.* 1997, Detrich *et al.* 1998, Dahm *et al.* 2007, Bass & Gerlai 2008, Fadool & Dowling 2008).

The facility to manipulate this species permitted gene transfer experiments, leading to the development of several transgenic lines, which contribute to new research on vertebrate gene expression (Sprague *et al.* 2003). Growth hormone (GH) transgenic zebrafish represents one of the most explored lines of the species with an exogenous gene. There is a great interest in GH gene overexpression in fish species, since generally it can be directly related to a high growth performance of the animals in a short period of time, an important trait in aquaculture (Fletcher & Davies 1991, Hew *et al.*

1995, Zbikowska 2003, Devlin *et al.* 2004, Devlin *et al.* 2006, Figueiredo *et al*. 2007a, Rosa *et al.* 2008).

Even though some studies have shown that only a 20% increase in growth rate can be achieved by an exogenous GH application in zebrafish (Simpson *et al.* 2000, Morales *et al.* 2001, Biga & Goetz 2006), contrasting with other growth hormone-transgenic fish, as coho salmon, that are extremely fast growing (Devlin *et al.* 1994), GH-transgenic *Danio rerio* can be used as an experimental model on basic research analyses to understand vertebrate growth mechanisms and GH expression gene control. Moreover, the growth hormone is also involved in other physiological processes, since it can regulate the metabolism of several molecules, as lipids, proteins, and carbohydrates (Moller & Norrelund 2003), improve the function and maintenance of all major immune cell types maintenance (Jeay *et al.* 2002), and influence the stress response and behavior (Yoshizato *et al*. 1998).

A growth hormone-transgenic line of *Danio rerio*, denominated F0104 lineage, is composed by genetically modified animals with an exogenous GH gene from the marine fish *Odonthestes argentinensis* (Figueiredo *et al.* 2007a). A significant high growth performance was evidenced in hemizygote GH-transgenic animals of this line, although individuals of the homozygote genotype did not present a differential growth rate when compared to wild-type zebrafish. However, homozygote animals presented a higher GH expression than hemizygote and non-transgenic animals, leading to the assumption that a GH excess do not correspond to a higher growth performance in zebrafish (Figueiredo *et al.* 2007b).

Due to growth differences among non-transgenic, and homozygote and hemizygote zebrafish of the F0104 line, it is expected that genes involved in growth process are differentially expressed in these animals. Therefore, the present study intended to identify candidate DETs (Differentially Expressed Transcripts) in these three different samples using a DDRT-PCR (Differential Display Reverse Transcription - Polymerase Chain Reaction) approach. The results can give new insights into the elucidation of the mechanisms beyond vertebrate growth and of the GH expression gene control.

Material and Methods

Animal samples

Adult individuals of zebrafish (*Danio rerio*) were obtained from Fundação Universidade Federal do Rio Grande (Rio Grande, RS), using the transgenic line F0104 that has the carp (*Cyprinus carpio)* β-actin promoter driving the expression of two exogenous genes - the marine silverside fish *Odonthestes argentinensis* growth hormone (msGH) gene and the jellyfish (*Aequorea victoria*) GFP (Green Fluorescent Protein) as a reporter gene to monitor for transformed zygotes (Figueiredo *et al.* 2007a). Different genotypes were obtained through crossing GH-transgenic hemizygote individuals leading to a progeny with a proportion of 25% homozygote (HO), 50% hemizygote (HE), and 25% non-transgenic (NT) animals. Genotypes were determined through UV fluorescence analysis, due to the GFP expression, with an epifluorescence microscope (excitation = 485 nm; emission = 520 nm). HO individuals were identified by presenting a higher fluorescence level.

RNA isolation

Brain tissue was collected from two animals of each genotype (HO, HE, and NT) and immediately stored at -80^{o}C until RNA extraction. Approximately 100mg of the brain samples were mechanically homogenized with 1mL of TRizol Reagent (Invitrogen) and total RNA extraction followed the manufacturer's protocol. RNA samples were eluted in RNase-free water and quantified (NanoDrop 1000 Spectrophotometer) by measuring the optical density (OD) at 260nm. RNA purity was ensured by obtaining a 260/280nm OD ratio ≥1.80.

RT-PCR and cDNA amplification

Each RNA sample (5μg) was reverse transcribed (RT-PCR) with the commercial kit SuperScript III RT (Invitrogen) using the oligonucleotide AP (5´-GGCCACGCGTCGACTAGTAC$(T)_{17}$-3´), according to the manufacturer's instructions. A DDRT-PCR approach was used in order to amplify the cDNA samples. Oligonucleotides with 15-24 bp were designed based on VNTR core sequences (Table 1) and used as single primers for cDNA amplification. Each cDNA amplification reaction consisted of 1μL of cDNA, 0.2mM of primer, 1x 25mM $MgCl_2$ PCR buffer, 0.2mM of dNTPs, and 0.2 unit of Platinum *Taq* DNA polymerase (Invitrogen), in a final volume of 25μL. Reactions were carried out with an initial denaturation step at 95^{o}C for 2 minutes, followed by 40 cycles at 94^{o}C for 50 seconds, 55^{o}C for 2 minutes, and 72^{o}C for 50 seconds, with an additional extension step at 72^{o}C for 5 minutes. DDRT-PCR products (10μL)

were fractionated on 6% polyacrilamide gel, stained with 0.17% silver nitrate ($AgNO_3$) (Sambrook & Russel 2001), and visualized under white light. The molecular weight of the amplified fragments were assigned through comparison with 1Kb DNA ladder (Invitrogen).

Densitometric analysis

The amplified fragments corresponding to putative differentially expressed transcripts were quantified by densitometry as Integrated Optical Density (IOD) using Imaging Master VDS Software version 3.2 (GE Healthcare Life Sciences). Mean and standard deviations (SD) were determined using Macrosoft Excel Software.

DNA isolation and reamplification

Selected DNA fragments that seemed to correspond to genes with differential expression among the analyzed zebrafish samples were reamplified in order to obtain a higher DNA content to be used on cloning and nucleotide sequencing. The fragments were cut from the polyacrilamide matrix and used directly (without any DNA purification) on reamplification reactions, as described in and Dakis & Kouretas (2002). The amplification reactions followed the conditions described above. PCR products were analyzed through 6% polyacrilamide gel electrophoresis.

Cloning, sequencing, and sequence analysis

PCR products were cloned into pGEM-T (Promega) vector and used to transform competent cells of the *E. coli* strain DH5α (Invitrogen), following the manufacturers' instructions. Clones were submitted to automated sequencing on an ABI 377 Automated DNA Sequencer (Applied Biosystems) with a DYEnamic ET Terminator Cycle Sequencing kit (GE Healthcare Life Sciences), following the manufacturer's instructions, and using primers complementary to vector arms. Nucleic acid sequence database searches were performed using BLAST/N (Altschul *et al.* 1990) at the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/blast). Sequence alignments were obtained by Clustal-W function (Thompson *et al.* 1994) and the consensus sequences were manually determined.

Results and Discussion

Muscle development and growth are dynamic processes controlled by a number of factors including growth hormone, insulin-like growth factors (IGFs), anabolic steroids, thyroid hormone, cytokines and cyclins. The GH, that plays a major role in somatic growth, is mainly produced at the anterior pituitary gland, under the control of hypothalamic factors as GHRH (GH release hormone) and somatostatin (GHIH - growth hormone inhibiting hormone) and is further released in circulation blood, acting in specific organs through its association with different receptors present at the surface of target cells (Florini *et al.* 1996, Le Roith *et al.* 2001). Nowadays, several analyses in

disorders that result from under or overproduction of GH, and in transgenic animals that overexpress this hormone have been conducted in order to understand these hormone's interactions with other proteins.

Regardless the mechanisms responsible for the actions of GH, the resulting effect of its overexpression is generally an increase in muscle mass, as observed for some fish species (Nam *et al.* 2001, Devlin *et al.* 2006). However, a higher GH transgene expression level is not related to an enhancement of growth rate in other fish, as observed in the tilapia *Oreochromis niloticus* and *O. hornorum* (Rahman *et al.* 1998, Martínez *et al.* 1999). Homozygotes of the GH-transgenic *Danio rerio* F0104 line also demonstrated a reduced growth, similar to non-transgenic animals, even though they presented a higher GH expression (Figueiredo *et al.* 2007b). The different growth performance between hemizygote (HE) and homozygote (HO) GH-transgenic zebrafish of this line lead to analyses on genes that could be involved in growth process, including IGF-I (insulin-like growth factor I), GHG (GH receptor), and SOCS (suppressor of cytokine signaling). The results permitted to identify distinct expression levels of these genes among the different GH-transgenic genotypes and the non-transgenic (NT) animals (Figueiredo *et al.* 2007b, Studzinski 2008).

In order to implement data on differentially expressed genes in NT, HE, and HO zebrafish, total RNA of brain tissue of these three different samples were obtained and used on a DDRT-PCR approach. cDNA amplification was achieved using oligonucleotides that were designed based on VNTR (Variable Number of Tandem Repeats) core sequences that correspond to minisatellite short and highly conserved regions (Jeffreys *et al.* 1985). The use of longer

primers (14-24pb) on differential display strategies can lead to detection of DNA polymorphism through RAPD-like results. However, since these oligonucleotides are longer than RAPD primers, this methodology can also be effectively carried out at relatively high stringencies, thus yielding more reproducible results (Wasko & Alves-Costa*). Two of the primers (INS and HBV3) used to amplify the cDNA templates lead to the identification of presumptive differentially expressed transcripts among the analyzed samples.

DDRT-PCR using primer INS

cDNA amplifications using primer INS lead to the identification of an intense stained fragment of approximately 1.000 bp, present in NT, and in GH-transgenic HE and HO individuals of zebrafish (Figure 1a), that seemed to correspond to a transcript with differential gene expression among the three samples - the polyacrilamide electrophoresis results permitted the visualization of a fragment with distinctive stain intensities among the different samples (Figure 1a). Densitometric analyses of this amplified product, through IOD values, suggested a significant lower gene expression level in the transgenic genotypes, specially in HE (17.41±5.32) than in HO (32.25±3.21) samples, when compared to NT samples (49.35±12.32) (Figure 2a). The suggested expression levels of the analyzed transcript in HE and HO samples represent approximately 35% and 65% of the IOD value obtained for the non-transgenic samples, respectively.

Cloning and sequencing of the transcript obtained with the primer INS permitted the characterization of a 941 bp DNA fragment (Figure 3). Database searches for nucleotide similarity indicated a 95% identity level with a partial region of the gene that codes for the p300 protein of *Danio rerio* (accession

number XM_001332682, NM_001510744) and also a high identity with the p300 gene of several other vertebrates, as *Ctenopharyngodon idella* (81%), *Gallus gallus* (81%), and *Mus musculus* (78%). The p300, also known as E1A binding protein p300, is involved in several processes, as cellular differentiation and proliferation, cycle cell regulation, growth, apoptosis, and histone acetylation (Huret 2000, Vleugel *et al.* 2006). It has been described as a transcriptional coactivator that interacts with other proteins, leading to the activation of the transcriptional process of several genes (Huret 2000, Tu & Luo 2007), including those related to cancer (Vachtenheim *et al*. 2006, Vleugel *et al*. 2006, Zhao *et al*. 2006) and neural pathologies development (Renoult *et al*. 2007, Francis *et al*. 2007), and to immune responses (Zhang *et al*. 2005).

Recently, it was proposed that the p300 is also involved in muscle tissue formation and growth. The p300 protein can regulate the transcription of the myogenic regulatory factor MyoD (Ji *et al.* 2003) and of the gene *Cugbp1* (Huichalaf *et al.* 2007). The CUGBP1 protein (CUG triplet repeat, RNA binding protein 1) is essential for the muscle differentiation process and any disturbance in the *Cugbp* gene expression leads to a delay in the myogenesis process or to a muscle dystrophy (Bhagwati *et al.* 1996).

Moreover, the interaction between p300 and GH was already evidenced for some genes, as the *RhoA* gene (*Ras* homolog gene family member A) and the early response *c-fos* gene, whose proteins regulate the actin cytoskeleton in the formation of fibers and the cell proliferation and differentiation, respectively (Ling & Lobie 2004, Cui *et al.* 2005). It seems that the growth hormone acts in the regulation of these genes, since GH can

stimulate the p300 protein to occupy the promoter of these genes leading to their activation (Cui *et al.* 2005).

The present results indicate that the p300 gene, isolated from the brain of zebrafish, may have different expression levels in non-transgenic and GH-transgenic animals - HE individuals seem to present a lower expression, while an intermediary and a higher expression levels could be associated to HO and NT animals, respectively. These data reinforce the p300 role in muscle growth and its interaction with the growth hormone.

DDRT-PCR using primer HBV3

The use of the primer HBV3 to amplify the cDNA samples resulted on several faint and diffuse bands (Figure 1b). However, a fragment of approximately 900 bp was clearly visible in the NT, and in the GH-transgenic HE and HO samples of *Danio rerio* (Figure 1b). Although the difference in the stain intensity of this fragment among the three analyzed samples was not so evident, it was possible to notice a darker staining in the NT samples, which led us to infer that this fragment could also correspond to a differentially expressed transcript. The densitometric analyses of this transcript suggested a higher expression level in NT animals (22.73±3.80) than in HE (14.05±4.95) and HO samples (10.63±0.67) (Figure 2b). In the HE and HO samples, the expression level of the analyzed transcript represented approximately 62% and 47% of the IOD value obtained for the NT animals, respectively. Therefore, the IOD value decrease observed in HE and HO individuals can be directly related to the increased exogenous GH level in these two samples of zebrafish.

Nucleotide sequencing analysis of the transcript obtained with the primer HBV3 evidenced a DNA fragment with 831 bp (Figure 4). BLAST

database searches for nucleotide similarity indicated a high identity level (94%) with the 3' end of the gene that codes for the protein adenylate cyclase 2 (ADCY2) found in brain tissue of *Danio rerio* (accession numbers CR759914 and GI6270118). The Adenylate cyclase (AC) catalyses the conversion of ATP into cAMP that then acts to regulate a wide variety of cellular processes. The AC activity can be regulated by hormones, neurotransmitters interactions with their receptors, and calmodulin (Aikawa *et al.* 2000, Niewiadomski *et al.* 2002, Zawilska *et al.* 2003).

Recently, it was evidenced that the neuropeptide PACAP (pituitary adenylate cyclase-activating polypeptide) can also modulate the AC activity, leading not only to the formation of cAMP, but also playing a role in mediating growth hormone release in the pituitary gland of vertebrates (Matsuda *et al.* 2008, Mitchell *et al.* 2008), including fish species, as grass carp (Wong *et al.* 2005), common carp (Xiao *et al.* 2002), sockey salmon (Parker *et al.* 1997), and European eel (Montero *et al.* 1998). Wong *et al.* (2005) verified that increased concentrations of PACAP and/or higher concentrations of cAMP were effective in inducing GH release. Therefore, the performed densitometric data suggest that the lower adenylate cyclase expression level in HO zebrafish could result in a decrease in GH release thus leading to the reduced growth performance of these animals. Since Figueiredo *et al.* (2007b) evidenced that these HO animals, despite presenting a higher level of exogenous GH, were expressing approximately only one third of the endogenous GH observed in non-transgenic animals, the p300 should be involved in the endogenous growth hormone release control.

Final considerations

The growth hormone has physiological actions in several organs, since growth hormone receptors (GHR) have been identified in different tissues (Hughes & Friesen 1985, Kelly *et al.* 1991). As the GH is a multiple-function hormone, its excess can cause several physiological and behavioral collateral effects in the organism, as decrease in fertility (Cecim *et al.* 1995a, b), increase in oxygen consumption (Cook *et al.* 2000, Mackenzie *et al.* 2003), premature aging (Wolf *et al.* 1993, Bartke *et al.* 1998), decrease in learning and memory (Meliska *et al.* 1997), and oxidative stress (Rollo *et al.* 1996, Brown-Borg *et al.* 2001, Rosa *et al.* 2008). Therefore, the presence of a higher level of exogenous GH in transgenic fish could also leads to metabolic alterations that could influence not only the animal growth but also several other traits.

Previous analyses evidenced that HE GH-transgenic zebrafish reached a final average mass higher than HO and NT animals and that, surprisingly, HO fish presented a growth performance similar to NT animals (Figueiredo *et al.* 2007b). Taking into account the amount of endogenous and exogenous growth hormone present in HO and HE animals, both transgenic samples produce higher levels of biological active GH than NT individuals. Therefore, the hormone excess could be inducing a negative feedback system over the endogenous GH expression (Figueiredo *et al.* 2007b). These data could be related to regulation systems of intracellular GH that involves IGF-I (insulin-like growth factor I), GHR (GH receptor), and SOCS proteins (suppressor of cytokine signaling) (Figueiredo *et al.* 2007b, Studzinski 2008). The present data also indicate that the p300 and ADCY2 are also involved in a regulation

system for GH in zebrafish in the case of high circulating hormone levels. Therefore, it seem that the reduced growth performance observed not only in homozygote GH-transgenic *Danio rerio* but also in other vertebrates with an exogenous GH gene is a result of the biological action of several proteins and their interaction. Further research is needed to characterize the significance of these proteins in controlling vertebrate muscle growth.

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Table, Figures and Legends

Table 1: Oligonucleotides that were designed based on VNTR core sequences and used as single primers for the amplification of the cDNA samples of *Danio rerio*.

Primer	Sequence (5' - 3')	Reference
INS	ACAGGGGTGTGGGG	Nakamura *et al.* (1987)
YNZ22	CTCTGGGTGTCGTGC	Nakamura *et al.* (1987)
HBV5	GGTGTAGAGAGGGGT	Nakamura *et al.* (1987)
HBV3	GGTGAAGCACAGGTG	Nakamura *et al.* (1987)
FvIIex8	ATGCACACACACAGG	Murray *et al.* (1988)
EMBL	AGAGCTTCAGGCTGGGCAGCTAAG	Harris & Wright (1995)

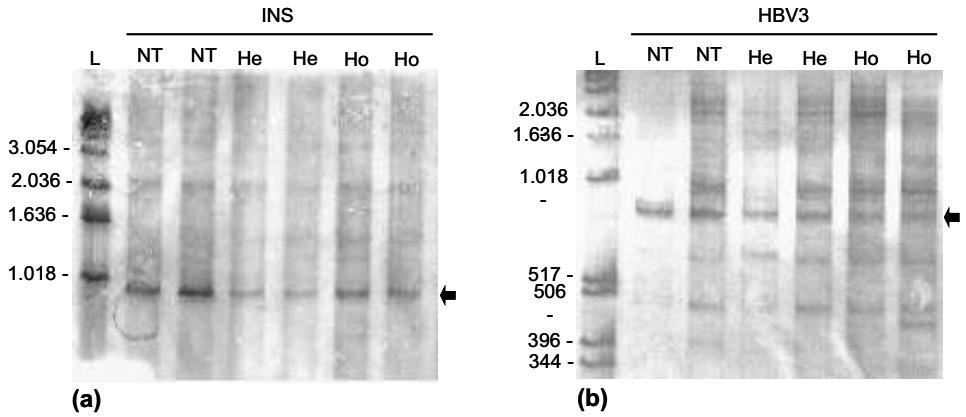

Figure 1: DDRT-PCR patterns of non-transgenic (NT) and of GH-transgenic hemizygote (HE) and homozygote (HO) zebrafish of the F0104 line, visualized trough 6% polyacrilamide gel electrophoresis. **(a)** brain cDNA amplification using primer INS; **(b)** brain cDNA amplification using primer HBV3. L, 1 Kb DNA molecular marker. Arrows indicate putative differentially expressed transcripts among the three analyzed samples.

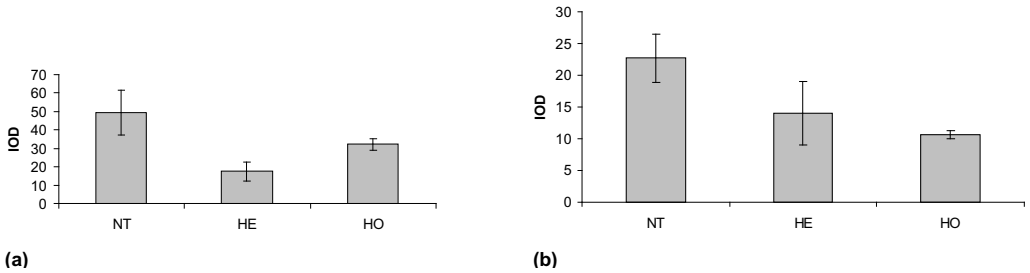

Figure 2: Densitometric analyses of the fragments that correspond to putative differentially expressed transcripts identified through DDRT-PCR in non-transgenic (NT) and in GH-transgenic hemizygote (HE) and homozygote (HO) zebrafish of the F0104 line. **(a)** estimated expression level of the fragment amplified with the primer INS that corresponds to the gene that codes for protein p300 (E1A binding protein p300); **(b)** estimated expression level of the fragment amplified with the primer HBV3 that corresponds to the gene that codes for protein ADCY2 (adenylate cyclase 2).

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5’acaggggtgtcatgangaanatctggcatgangacgtnacccacgatc.....48
tgcgcaaccanctgntgaagaagctagtccaggctatttttcccacacca.....98
gaccctgctgcactgaaagaccgtcggatggagaatctagtggcctatgc....148
acgtaaagttgagggtgacatgtatgagtctgctaacagcagggcggagt....198
attatcactttctggcagagaagatctataaaattcagaaagaactggaa....248
gagaagcgaaggacacggttacagaagcagggtataatgccctcccagga....298
tggcatgaacccctgtggcctgcancaagcgaacgctgggattggtcagc....348
cctggccacccaacaggactgctttataatggcccactattagatccatt....398
cagtagtggtcccaactggcccaaaccagatgatgaacagaatgcaagaa....448
tactgcctgggatgaattcatttgggaatcatatgggaatgcagtccatg....498
ggccagagatcaacaccacctcttaaccagggaagcatggtgcctgggag....548
gatgccacagccgaatgttgcacagatgcaaaatcaatacatgcaaactg....598
gaccgtttcaagcttcaagtcctgttcgtagtgctggtcctgttgaccta....648
gtacacggaggaaacgatggtgctgccactcaacagggacaaatgccgtt....698
atcatctttaccagtcgggagtcctttagcccaacctgggtctgctggcg....748
gggcaggcagcgggtcatctgtgggctccttgggtcccagcagtatgagt....798
gctgttcctccatcatccacccccacccactccatcagcctcagtcattg....848
ccgacctgtacatcagcattcactttcaactgntgatagccggacgcgca....898
gacccatgccaggttaccagacgcctcatccccacacccctgt 3’........941
```

Figure 3: Consensus nucleotide sequence of a partial region of the gene that codes for protein p300, isolated from brain tissue of *Danio rerio* through a DDRT-PCR approach using primer INS. The underlined regions are homologous to the primer sequence.

```
5′ggtgaagcacangtgcaaacagtattatgtgggcaatcctaaaagaga.......48
tgtcaaaaacaaagaggtcggtccaggtggctagcaccagaaaacaataa.......98
acacanatgatctaacatggcagagtaaggcaaggagaaatgctttgtaa......148
aggctgatttatactcctgcgtcaaacactggcgtatgctacggcgctga......198
cgcatagcccttcgccctggccgtcgccgtcactgacgtgcaccactcta......248
aaaattggaactacacatgggaacgacgcgtatcgcaaagtctgtgattg......298
gtcggcttggtagcgttgacgagtcggggcaggaccgagacccgtgcgaa......348
tggtgcaaacccaatgtagcgattgtttacaagtgtggagtcccgtgaag......398
gagcttcagatggaaagttttgttttgtgtttacctcatagttaaagttg......448
ctgcatgtccgccggttgctgcctcaaaatgagcgagtttcagccacttg......498
tacattccggaagtgttcaggaaaagcaaaaaagcagcgaagaaactcaa......548
cacaaaggaacatttacacctcactgccaactagcatttcggaagtgtta......598
atccagaccaacagagacagcgcgcagaagtataaatgcacagccacgtg......648
cgttgcatgcaccgtgggttacgccggtcacttgacgcagaagtattaat......698
caggcttaatgtgtccaggtaacaacaagactcagtaattgcatgtatgt......748
atgtgtctgtgagtgtgtgtgtgtgagcgttgcatgaatagtccatgtaa......798
tgagtgcttaaccagcttcacctgtgcttcacc 3′..................831
```

Figure 4: Consensus nucleotide sequence of a partial region of the gene that codes for protein ADCY2, isolated from brain tissue of *Danio rerio* through a DDRT-PCR approach using primer HBV3. The underlined regions are homologous to the primer sequence.

Considerações Finais e Conclusões

O objetivo principal do presente trabalho, especificado inicialmente, de isolar e caracterizar transcritos diferencialmente expressos em duas espécies de peixes - *Leporinus macrocephalus* e *Danio rerio* - foi alcançado com sucesso, dada a obtenção de resultados referentes às diferentes sub-propostas de pesquisa.

Assim, as análises desenvolvidas no presente estudo evidenciaram:

- Duas isoformas de α-actina esquelética na espécie *Leporinus macrocephalus*, geneticamente e provavelmente funcionalmente distintas, cujas características podem estar relacionadas à demanda energética dos tecidos musculares esqueléticos branco e vermelho.
- Um gene com expressão diferencial em tecido cerebral de machos e fêmeas de *Leporinus macrocephalus*, relacionado à proteína APBA2 (*amyloid beta (A4) precursor protein-binding, family A, member 2*), cuja expressão está associada à produção da proteína β-amilóide a qual, por sua vez, relaciona-se à doença de Alzheimer.
- A estrutura do gene *fabp6* (*fatty acid-binding protein 6*) de *Danio rerio*, similar a grande parte dos demais membros da família multigênica *FABP* de outros vertebrados, e sua distribuição tecidual em embriões, larvas e adultos, também similar à evidenciada em outros organismos, especialmente mamíferos.

- Dois genes candidatos diferencialmente expressos, relacionados à proteína p300 (*E1A binding protein p300*) e à proteína ADCY2 (*adenylate cyclase 2*) envolvidas na formação/crescimento muscular e na liberação de GH, respectivamente, entre exemplares de *Danio rerio* não-transgênicos e transgênicos homozigotos e hemizigotos para gene do hormônio de crescimento.

Dada a importância da obtenção e ampliação de dados genéticos em peixes, que constituem um grupo extremamente diversificado e basal entre os vertebrados, os resultados obtidos contribuem para a melhor compreensão da evolução, função e organização molecular de diversos genes e podem servir de base a futuros estudos. Além disso, os dados obtidos confirmam a utilidade de *Danio rerio* como espécie modelo em estudos genéticos e indicam que *Leporinus macrocephalus* representa um potencial modelo experimental em análises direcionadas a genes envolvidos no desenvolvimento de doenças humanas, como alterações neurodegenerativas, distrofias musculares e câncer.

SÚMULA CURRICULAR

(Outras atividades vinculadas e desenvolvidas no Doutorado)

1. Trabalhos apresentados em congressos:

ALVES, F.A.; FORESTI, F., OLIVEIRA, C., MARTINS, C. 2005. Uso de seqüências de DNAr 5S como marcador molecular em tilápia do Nilo (*Oreochromis niloticus*). **In**: "*XVI Encontro Brasileiro de Ictiologia*", João Pessoa, Paraíba, Brasil.

ALVES, F.A.; MARTINS, C., WASKO, A.P. 2005. Caracterização molecular de genes de actina isolados de músculos branco e vermelho de *Leporinus macrocephalus* (Pisces, Anostomidae). **In**: "*51º. Congresso Brasileiro de Genética",* Águas de Lindóia/ SP, Brasil. Genetics and Molecular Biology (Suppl.) 26, GA123, p.123.

MATOS, F.D.C.; ALVES, F.A.; MARTINS, C.; ALVES, A.L.; FORESTI, F.; OLIVEIRA, C.; WASKO, A.P. 2005. Caracterização do DNAr 5S em espécies da família Sciaenidae (Pisces, Perciformes). **In**: "*51º. Congresso Brasileiro de Genética",* Águas de Lindóia/ SP, Brasil. Genetics and Molecular Biology (Suppl.) 26, GA199, p.199.

WONG, M.S.L.; ALVES, F.A.; WASKO, A.P. 2005. Identificação de um marcador RAPD associado a fêmeas de *Brycon cephalus* (Pisces, Bryconidae). **In**: "*51º Congresso Brasileiro de Genética",* Águas de Lindóia/ SP, Brasil. Genetics and Molecular Biology (Suppl.) 26, GA229, p.229.

ALVES-COSTA, F.A.; MARTINS, C.; WASKO, A.P. 2006. Identificação de transcritos sexo-específicos em *Leporinus macrocephalus* (Pisces, Anostomidae) através da metodologia de DDRT-PCR. **In**: "*XXVI Congresso Brasileiro de Zoologia",* Universidade Estadual de Londrina – UEL, Londrina/ PR, Brasil. Nº. 858_1791.

WONG, M.S.L.; ALVES-COSTA, F.A.; WASKO, A.P. 2006. Prospecção de fragmentos sexo-específicos em *Brycon cephalus* (Pisces, Bryconidae) através da metodologia de RAPD. **In**: "*XXVI Congresso Brasileiro de Zoologia",* Universidade Estadual de Londrina – UEL, Londrina/ PR, Brasil. N°. 2123_1791.

ALVES-COSTA, F.A.; MARTINS, C.; WASKO, A.P. 2006. Identificação e caracterização de transcritos com potencial expressão diferencial entre os sexos de *Leporinus macrocephalus* (Pisces, Anostomidae). **In**: "*52° Congresso Brasileiro de Genética" and "*12°. *Congresso de la Associación Latinoamericana de Genética",* Bourbon Cataratas Resort & Convention Center, Foz do Iguaçu/PR, Brasil. p. 187.

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WONG, M.S.L.; ALVES-COSTA, F.A.; WASKO, A.P. 2006. Identificação e caracterização de marcadores RAPD associados ao sexo em *Brycon cephalus* (Pisces, Bryconidae). **In**: "*52° Congresso Brasileiro de Genética" and "*12° *Congresso de la Associación Latinoamericana de Genética",* Bourbon Cataratas Resort & Convention Center, Foz do Iguaçu/PR, Brasil. p. 235.

GOMES, M.L.; ALVES-COSTA, F.A.; NISHIDA, S.M.; WASKO, A.P. 2006. Análise de locos microssatélites em codorna japonesa *Coturnix japonica* (Galliformes, Phasianidae) para identificação de polimorfismos, visando a elaboração de testes de paternidade. **In**: "*52° Congresso Brasileiro de Genética" and "*12°. *Congresso de la Associación Latinoamericana de Genética",* Bourbon Cataratas Resort & Convention Center, Foz do Iguaçu/PR, Brasil. p. 264.

ALVES-COSTA, F.A.; MARTINS, C.; WASKO, A.P. 2006. Prospecção de DETS (Differentially Expressed Transcripts) em machos e fêmeas de *Leporinus macrocephalus* (Anostomidae). **In**: "*XI Brazilian Symposium on Fish Cytogenetics and Genetics and I International Congress of Fish Genetics"*, Universidade Federal de São Carlos, São Carlos/SP, Brasil. p. 277.

WONG, M.S.L.; ALVES-COSTA, F.A.; WASKO, A.P. 2006. Identificação e caracterização de fragmentos de DNA sexo-específicos em *Brycon cephalus* (Characidae) através da metodologia de RAPD. **In**: "*XI Brazilian Symposium on Fish Cytogenetics and Genetics and I International Congress of Fish Genetics"*, Universidade Federal de São Carlos, São Carlos/SP, Brasil. p. 210.

ALVES-COSTA, F.A.; MARTINS, C.; WASKO, A.P. 2006. Uso de seqüências de DNAr 5S como marcadores moleculares em espécies marinhas da família Sciaenidae (Pisces, Perciformes). **In**: "*VI Jornadas de Ciencias del Mar and XIV Coloquio de Oceanografía",* Puerto Madryn, Chubut, Argentina, p. 158.

TONIATO, J.; MARTINS, C.; ALVES-COSTA, F.A.; ROCHA, K.K.H.R. 2007. Diferenciação molecular da tilápia do Nilo com base em marcadores moleculares de baixo custo. **In**: "*XXII Reunião Anual da FESBE"*, Águas de Lindóia/SP, Brasil. Métodos, 49.011 - Equipamentos e Ensino.

ALVES-COSTA, F.A.; FIGUEIREDO, M.A.; LANES, C.F.C.; ALMEIDA, D.V.; MARINS, L.F.; WASKO, A.P. 2007. Isolamento e caracterização de DETs ("Differentially Expressed Transcripts") em tecido cerebral de uma linhagem de de peixe transgênico (*Danio rerio*) superexpressando o gene do hormônio do crescimento (GH). **In**: "*53º. Congresso Brasileiro de Genética",* Hotel Monte Real Resort, Águas de Lindóia/SP, Brasil. p. 168.

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GOMES, M.L.; ALVES-COSTA, F.A.; NISHIDA, S.M.; WASKO, A.P. 2007. Elaboração de testes de paternidade em codorna japonesa *Coturnix japonica* (Galliformes, Phasianidae). **In**: "*53º. Congresso Brasileiro de Genética",* Hotel Monte Real Resort, Águas de Lindóia/SP, Brasil. p.257.

TONIATO, J.; MARTINS, C.; ALVES-COSTA, F.A. 2007. Aplicação de marcadores RFLP-PCR na identificação de espécies de tilápias (Perciformes: Cichlidae). **In**: "*53º. Congresso Brasileiro de Genética",* Hotel Monte Real Resort, Águas de Lindóia/SP, Brasil. p.91

GOMES, M.L.; ALVES-COSTA, F.A.; NISHIDA, S.M.; WASKO, A.P. 2007 Testes de paternidade para a codorna japonesa *Coturnix japonica* (Galliformes, Phasianidae): ferramenta para evidenciar a competição pós-cópula. **In**: "*XXV Encontro Anual de Etologia",* UNESP/São José do Rio Preto, São José do Rio Preto, SP, Brasil. Oral presentation.

2. Participações em congressos:

XVI Encontro Brasileiro de Ictiologia, realizado em João Pessoa. Paraíba, no período de 24 a 28 de janeiro de 2005.

51º. *Congresso Brasileiro de Genética* realizado em Águas de Lindóia, SP, no período de 7 a 10 de setembro de 2005.

XXVI Congresso Brasileiro de Zoologia, realizado na Universidade Estadual de Londrina e Centro Universitário Filadélfia, Londrina, PR, de 12 a 17 de fevereiro de 2006.

52º. *Congresso Brasileiro de Genética e* 12º. *Congresso de la Associación Latinoamericana de Genética,* realizado no Bourbon Cataratas Resort & Convention Center, Foz do Iguaçu, PR, de 3 a 6 de setembro de 2006.

XI Brazilian Symposium on Fish Cytogenetics and Genetics and I International Congress of Fish Genetics, realizado na Universidade Federal de São Carlos, São Carlos, SP, de 10 a 13 de outubro de 2006.

VI Jornadas de Ciencias del Mar y XIV Coloquio de Oceanografía, realizada na cidade de Puerto Madryn, Chubut, Argentina, de 4 a 8 de dezembro de 2006.

3. Cursos assistidos:

Evolução de proteínas através de embaralhamento de DNA, realizado durante o 51º. *Congresso Brasileiro de Genética,* em Águas de Lindóia, SP, no período de 7 a 10 de setembro de 2005, totalizando 3 horas.

Expressão Gênica: novas formas de análise e abordagens", realizado durante o 52º. Congresso Brasileiro de Genética*,* Bourbon Cataratas Resort & Convention Center, Foz do Iguaçu, PR, no período de 3 a 6 de setembro de 2006, totalizando 3 horas*.*

Melhoramento Genético em Peixes", realizado durante o XI Brazilian Symposium on Fish Cytogenetics and Genetics and I International Congress of Fish Genetics, na Universidade Federal de São Carlos, São Carlos, SP, no período de 10 a 13 de outubro de 2006, totalizando 6 horas.

4. Cursos ministrados:

Curso de extensão Universitária "*Introdução à Biologia Molecular*", realizado no Laboratório de Genética de Peixes, junto ao Departamento de Ciências Biológicas, UNESP, Bauru, SP, durante o período de 16 a 19 de agosto de 2005, com duração de 24 horas.

Curso "*Marcadores Citogenéticos e Moleculares: conceitos e aplicações*", ministrado durante o VI Workshop de Genética, no Instituto de Biociências, UNESP, Botucatu, SP, no período de 20 a 21 de maio de 2006, apresentando carga horária equivalente a 8 horas.

Curso "*Marcadores Moleculares e Sua Aplicação em Teste de Paternidade: uma abordagem sobre conservação genética*", ministrado durante o VII Workshop de Genética, no Instituto de Biociências, UNESP, Botucatu, SP, no período de 20 a 21 de maio de 2007, apresentando carga horária equivalente a 8 horas.

5. Monitoria em cursos de extensão:

Monitoria na disciplina "Métodos de Estudo em Biologia Molecular", sob a responsabilidade do Prof. Cesar Martins, no curso de pós-graduação em Ciências Biológicas (Genética), ministrada de 10 a 19 de outubro de 2005.

Colaboração como monitora durante o curso de extensão universitária (I Curso de Inverno) "Manipulação de Ácidos Nucleicos: PCR, RT-PCR e PCR em tempo real", realizado no Instituto de Biociências, UNESP, Botucatu, SP, no período de 10 a 14 de julho de 2006

Colaboração como monitora da oficina "Experimentando Genética", caracterizada como parte do Prpgrama de Extensão "Difundindo e Popularizando a Ciência", realizada no Instituto de Biociências da UNESP, Botucatu, SP, no período de 15 a 20 de janeiro de 2007, totalizando 48 horas de atividade.

Colaboração como monitora durante curso de extensão universitária (II Curso de Inverno - "Manipulação de Ácidos Nucleicos: PCR, RT-PCR e PCR em tempo real", realizado no Instituto de Biociências, UNESP, Botucatu, SP, no período de 16 a 20 de julho de 2007.

6. Palestras proferidas:

"Expressão de Proteínas Recombinantes", realizada durante curso de extensão universitária "Introdução à Biologia Molecular", Instituto de Biociências de Botucatu, Universidade Estadual Paulista - UNESP (Botucatu, SP) , no dia 13 de julho de 2007.

"Brazilian Conservation Genetics Issues", realizada na Acadia University, Wolfville, Nova Scotia, Canadá, no dia 10 de outubro de 2007.

7. Publicações:

Alves-Costa FA, Wasko AP, Oliveira C, Foresti F, Martins C (2006) Genomic organization and evolution of the 5S ribosomal DNA in Tilapiini fishes. *Genetica* 127:243-252.

Alves-Costa FA, Martins C (2006) DNAr 5S um novo marcador molecular para análise genética de tilápias. *Revista Biotecnologia, KL3 Publicações Ltda* 35: 22-27.

Wasko AP, Bento AP, Ferreira DC, Souza DB, Alves-Costa FA, Rossetto FO, Gatinho IA, Maia IG, Mazzuchelli J, Paiva LRS, Ribolla PEM, Almeida RA, Barbetta SR, Salles VP, Teixeira WG, Martins C (2007) Aliando conceitos e criatividade: proposta de dramatização na área de genética e biologia celular para alunos do ensino médio. *Genética na Escola 4*: 34-38.

Alves-Costa FA, Matos FDC, Foresti F, Oliveira C, Martins C, Wasko AP (2008) 5S rDNA characterization in twelve Sciaenidae fish species (Teleostei: Perciformes): depicting gene diversity and molecular markers. *Genetics and Molecular Biology* 31, 1 (suppl): 303-307.

Alves-Costa FA, Denovan-Wright EM, Thisse C, Thisse B, Wright JM (2008) Spatial-temporal distribution of fatty acid-binding protein 6 (*fabp6)* gene transcripts in the developing and adult zebrafish (*Danio rerio*). *FEBS Journal* 275: 3325-3334.

8. Estágios realizados:

Estágio supervisionado pelo Prof. Dr Luis Fernando Marins, no Laboratório de Biologia Molecular do Departamento de Ciências Fisiológicas, FURG (Fundação Universidade do Rio Grande), realizado de 14 a 26 de outubro de 2006, com participação no desenvolvimento da tecnologia de DDRT-PCR (*Differential Display Transcriptase Reverse – Polymerase Chain Reaction*) dentro do projeto ”Peixes Geneticamente Modificados para o Gene do Hormônio de Crescimento (GH)”, sob a coordenação do mesmo professor.

Estágio supervisionado pelo Prof. Dr. Jonathan M. Wright, Department of Biology, Dalhousie University, Halifax, Nova Scotia, Canada, no período de 30 de julho a 07 de dezembro de 2007, desenvolvendo o trabalho de pesquisa “Caracterização da organização e da expressão gênica da proteína FABP6 (“Fatty Acid-binding Protein 6”) na espécie de peixe *Danio rerio* (zebrafish)”.

9. Participação em projetos de pesquisa:

"Isolamento e caracterização de transcritos sexo-específicos em peixes utilizando a técnica de DDRT-PCR ("Differential Display Reverse Transcriptase"): modelo inicial para implementação de linha de pesquisa em expressão gênica diferencial". Projeto de Jovem Pesquisador Supervisor(a): Dra. Adriane Pinto Wasko) – FAPESP nº. 03/06761-1. Laboratório de Biologia Molecular e do Desenvolvimento, Departamento de Morfologia, Instituto de Biociências, UNESP (Botucatu, SP). Período de Dezembro de 2003 a Novembro de 2006.

"Identificação de marcadores sexo-específicos em *Leporinus macrocephalus* (Pisces, Anostomidae) utilizando a técnica de RAPD". Projeto de Iniciação Científica (Aluna: Marina Seik Lien Wong) – FAPESP nº. 04/10328-4,. Departamento de Morfologia, Instituto de Biociências, UNESP (Botucatu, SP). Dezembro de 2004 à Novembro de 2005.

"Identificação de marcadores sexo-específicos em *Brycon cephalus* (Pisces, Bryconinae) utilizando a técnica de RAPD". Projeto de Iniciação Científica (Aluna: Fernanda Del Campos de Matos) – FAPESP nº. 04/10911-1, Departamento de Morfologia, Instituto de Biociências, UNESP (Botucatu, SP). Período de Dezembro de 2004 a Novembro de 2006.

"Comparação e aperfeiçoamento de técnicas para isolamento de DNA de aves através de amostragem não-invasiva". Projeto de Iniciação Científica (Aluno: Tiago José Benedito Eugênio). Laboratório de Biologia Molecular e do Desenvolvimento, Departamento de Morfologia, Instituto de Biociências, UNESP (Botucatu, SP). Período de Abril de 2005 a Março de 2006.

“Identificação de marcadores RAPD sexo-específicos e DNAs satélites em *Brycon cephalus* (Pisces, Bryconinae): contribuições a caracterização genética da espécie” Projeto de Iniciação Científica (Aluna: Marina Sek Lien Wong) –FAPESP n°. 04/10328-4, Departamento de Genética, Instituto de Biociências, UNESP (Botucatu, SP). Período de Dezembro de 2006 a Novembro de 2007.

“Isolamento e caracterização de transcritos sexo-específicos em *Leporinus macrocephalus* (Pisces, Characiformes), utilizando a técnica de DDRT-PCR (*Differential Display Reverse Transcriptase*)” – Projeto de Doutorado (Aluna de pós-graduação: Fernanda Antunes Alves da Costa) FAPESP n°. 04/10024-5,. Laboratório de Biologia Molecular Animal, Departamento de Genética, Instituto de Biociências, UNESP (Botucatu, SP). Período de Fevereiro de 2005 a Agosto de 2008.

“Determinação das relações entre o comportamento de acasalamento e o sucesso reprodutivo na codorna japonesa (*Coturnix japonica*): uma abordagem multidisciplinar através de análises etológicas e genéticas”. Projeto de Mestrado (Aluna de pós-graduação Magali Lira Gomes) – FAPESP n°.05/57996-4, Laboratório de Biologia Molecular Animal, Departamento de Genética, Instituto de Biociências, UNESP (Botucatu, SP). Período de Abril de 2005 a Agosto de 2008.

10. Disciplinas cursadas:

Disciplinas	Créditos	Conceito
Genética Molecular em Animais de Produção (mamíferos)	03	A
Fundamentos em Genética	06	A
Tópicos especiais em genética: expressão, purificação e caracterização de proteínas recombinantes	03	A
Desenvolvimento embrionário comparado: modelos de estudo	03	A
Sistemática Molecular	03	A
Análises cromossômicas moleculares: técnicas de hibridação *in situ*	03	A
Organização e evolução dos genomas	04	A
Difundindo Genética: popularização da ciência pelos genes	04	A
Mecanismos de Regulaçào e Métodos de Análise da Expressão Gênica	03	A
Conservation Genetics Professores Responsáveis: Adriane Pinto Wasko (Departamento de Genética, Instituto de Biociências, UNESP, Botucatu, SP) e Stephen Mockford (Acadia University, Wolfville, Nova Scotia, Canada)	03	A