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Caracterização molecular de híbridos heteróticos das linhagens Red Stirling e Chitralada da tilápia do Nilo (*Oreochromis niloticus*)

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Botucatu, SP, Brasil.

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Caracterização molecular de híbridos heteróticos das linhagens Red Stirling e Chitralada da tilápia do Nilo (*Oreochromis niloticus*)

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Resumo

A tilápia do Nilo (*Oreochromis niloticus*) possui importância econômica na piscicultura mundial. Com a finalidade de atender à demanda do mercado consumidor, foram desenvolvidas várias linhagens com maior viabilidade econômica, dentre elas a Chitralada, que possui rápido crescimento, e a Red Stirling, com filé de cor rosado, mais apreciado pelo consumidor. Com o objetivo de combinar estas características, foi desenvolvido um híbrido, que apresentou heterose. A pesquisa genética em peixes mostrou a interferência direta e indireta de vários genes no desempenho animal, principalmente os genes relacionados ao eixo GH/IGF e a miostatina (MSTN). Além disso, uma classe de RNAs não-codificadores, os microRNAs (miRNAs), possuem papel fundamental na regulação de vários pontos em vias biológicas conhecidas, principalmente na modulação de genes codificadores de proteínas relacionadas ao crescimento. Portanto, o objetivo deste trabalho foi avaliar a expressão gênica dos genes relacionados ao eixo GH/IGF e MSTN; determinar os miRNAs envolvidos e seus alvos; e avaliar o conjunto de proteínas relacionando-as aos respectivos fenótipos. Amostras biológicas foram coletadas das linhagens Chitralada e Red Stirling e do híbrido (7/8 Chitralada). Para a análise de expressão gênica por RT-qPCR, foram coletadas amostras de cérebro, fígado e músculo branco. Para a análise de miRNAs por RNA-seq, foram utilizados *pools* das amostras de músculo branco, assim como para a análise por ESI-q-TOF e *shotgun*. Os genes relacionados ao eixo GH/IGF foram super expressos e a MSTN sub expressa no híbrido em relação aos seus parentais. Os dados de expressão dos miRNAs let-7, miR-122, miR-194 e miR-219 corroboram com os dados de expressão dos genes alvos.. Na comparação entre os perfis de expressão de miRNAs e proteínas, verificou-se que proteínas oriundas de genes alvos de miRNAs sub expressos estavam altamente expressas sugerindo atividade regulatória. Conclui-se que existe uma ação dos miRNAs em proteínas associadas às vias metabólicas relacionadas à heterose na tilápia do Nilo.

Abstract

Nile tilapia (*Oreochromis niloticus*) has great economic importance in world fish farming. To attend the commercial demand, several lines were developed, among them: Chitralada, with fast growth, and Red Stirling, with red color fillet of pink, which is more appreciated by the consumer. With the aim to combine the main characteristic of these lines, a crossbreed was developed, which also showed heterosis. Although crossbreeding between lines, or species, is a common breeding strategy for producing animals with heterosis, the molecular mechanisms that affect gene expression and biological pathways to create this phenotype still understood. Genetic research on fish showed the direct and indirect interference of several genes in animal performance, especially genes related to the GH/IGF axis and myostatin (MSTN). In addition, a class of non-coding RNAs, microRNAs (miRNAs), play a key role in regulating many biologically known pathways, mainly growth-related. Therefore, this study aimed to evaluate the GH/IGF axis genes expression and MSTN; determine the miRNAs involved and their targets; and the protein involved in the heterosis fenotype. Morphometric parameters, such as weight and length, were collected from the 4th to the 6th month of life. Biological samples were also collected from the Chitralada and Red Stirling, and their crossbreed (7/8 Chitralada). For the analysis of gene expression by RT-qPCR, brain, liver and white muscle samples were collected, ; for miRNA analysis, samples of white muscle were used, as well as ESI-q-TOF and shotgun GH/IGF axis genes were sup-regulated and the MSTN down-regulated in crossbreed in relation to their parents. Analysis of the miRNAs showed that let-7, miR-122, miR-194 and miR-219 in the crossbreed are associated with the qPCR data. Regarding the comparison of the miRNAs and with the proteins, several miRNAs showed to be sub expressed and consequently there was increase in the expression of the proteins that have targets of these miRNAs in their genes. We conclude that there is an action of miRNAs on proteins associated with the metabolic pathways and may be responsible for heterosis in Nile tilapia.

Lista de abreviações

CEVAP - Centro de Estudos de Venenos e Animais Peçonhentos;

CHIT - Chitralada;

ESI – eletrospray;

GH - hormônio de crescimento (growth hormone);

GHR - receptores do GH;

GIFT - Genetic Improvement of Farmed Tilapia;

IBP – indústria brasileira de peixe;

IGF - fator de crescimento semelhante à insulina (insuline-like growth fator);

mRNA - RNA mensageiro (messenger RNA);

miRNA - microRNA;

MREs - elementos de reconhecimento de miRNAs (miRNAs recognition elements);

MRF - fatores reguladores de miogênese (myogenic regulatory factors);

MS – espectrometria de massas (mass spectrometry);

MSTN - miostatina;

NCBI - National Center for Biotechnology Information;

ncRNAs - RNAs não-codificadores (non-coding RNA);

REDS - Red Stirling;

RIN – número de integridade do RNA (RNA integrity number);

RISC - complexo de indução de silenciamento de RNA (RNA-induced silencing complex);

RNAi - RNA de interferência;

SL – somatolactina;

SNP - polimorfismo de nucleotídeo único (single nucleotide polymorphism);

THW - Technische Hochschule Wildau;

TOF - tempo de flutuação (time of flight);

1. Introdução

1.1. Tilápia do Nilo: caracterização das linhagens investigadas

A tilápia do Nilo (*Oreochromis niloticus*) e suas variedades pertencentes ao grupo dos ciclídeos africanos são considerados excelentes modelos experimentais para estudos em diversas áreas da genética (Wang et al., 2007; Cnaani et al., 2008; Poletto et al., 2010). A facilidade adaptativa da *O. niloticus* permite sua manipulação por diversas técnicas, como a inversão sexual através da administração de esteróides sexuais e a produção de linhagens transgênicas (Maclean et al., 2002). Outras características como fertilização externa e alta fecundidade tornam essa espécie particularmente adequada como organismo modelo para análises experimentais comparativas com diversos tipos de animais.

A tilápia do Nilo possui grande importância econômica para a aquicultura (Meurer et al., 2000; Boscolo et al., 2001), sua produção alcançou 4,85 milhões de toneladas no mundo em 2014 (FAO, 2015). É a espécie de peixe mais cultivada no Brasil, com a produção de 198,49 mil toneladas em 2014, correspondendo à 41,9% do total de pescados. Além disso, a espécie registrou um aumento de 17,3% em relação à produção obtida em 2013 (IBGE, 2014).

Esta espécie foi introduzida ao Brasil na década de 70 (Lovshin et al., 1976) e está muito bem adaptada às condições climáticas brasileiras, apresentando diversas características zootécnicas que a tornam adequada ao cultivo, tais como resistência ao manejo e a doenças, tolerância a baixos teores de oxigênio, hábito alimentar diversificado, rápido crescimento e alto rendimento de filé (Hilsdorf, 1995; Popma e Lovshin, 1995; Freitas et al., 2009).

Com o objetivo de aumentar a produtividade, variedades de tilápia foram desenvolvidas, e dentre elas, destacam-se as variedades GIFT (*Genetic Improvement of Farmed Tilapia*) (Eknath e Acosta, 1997), a Chitralada (ou Tailandesa) (Zimmermann, 2000) e as variedades vermelhas. Uma das variedades vermelhas atualmente utilizada com êxito é a Red Stirling

(REDS). Essa variedade foi originalmente obtida de uma população de *O. niloticus* selvagem do Lago Manzala, no Egito, em 1979, e desde então, tem sido mantida pelo Instituto de Aquicultura da Universidade de Stirling, Escócia (McAndrew e Majumdar, 1983). Embora as variedades vermelhas sejam mais apreciadas pelos consumidores, a REDS possui índices de produção inferiores às linhagens de tilápia não vermelhas (Moreira et al., 2005). Isso levou aquicultores e melhoristas a realizarem o cruzamento desta linhagem com uma outra de maior produtividade: a linhagem Chitralada (CHIT).

Juvenis da linhagem CHIT foram introduzidos no Brasil em 1996 em Londrina no Paraná. Essa linhagem foi domesticada desde a década de 40 inicialmente no Japão e depois na Tailândia (Zimmermann, 1999). Sua introdução concomitante à técnica de incubação artificial melhorou o desempenho e resolveu os problemas de baixa eficiência da técnica de reversão sexual tradicional.

Nos últimos anos o cruzamento das linhagens CHIT e REDS levou à produção de animais com heterose (Moreira et al., 2007). A heterose, ou também vigor híbrido, é um termo utilizado para caracterizar a superioridade média da prole em relação à média dos progenitores (Pereira, 2012). No caso das linhagens utilizadas nesse programa, a variedade REDS apresenta menor crescimento e menor ganho de peso em relação à CHIT, no entanto, possui a coloração vermelha apreciada pelo mercado consumidor. Em função dessas particularidades, estudos de variabilidade genética e de crescimento têm sido realizados comparando-se as variedades REDS, CHIT e híbridos vermelhos (Moreira et al., 2005). A análise do germoplasma parental dessas variedades gerou animais com baixos índices de endogamia e variabilidade genética suficiente para o desenvolvimento de processos de melhoramento por cruzamentos e seleção (Moreira et al., 2007).

Cruzamentos entre estas duas variedades de tilápia foram realizados objetivando maiores ganhos com a heterose , maior velocidade de ganho de peso, aumento da eficiência

produtiva, entre outros atributos desejáveis. Híbridos dessas linhagens (e.g., genótipo 7/8 Chitralada: 1/8 Red Stirling) têm sido produzidos por melhoramento clássico e analisados quanto a parâmetros zootécnicos diversos (Lago, 2014). No entanto, os mecanismos genéticos envolvidos no vigor desses híbridos ainda são pouco conhecidos.

Dentre os fatores que influenciam diretamente o crescimento dos peixes (um dos atributos da heterose), destaca-se o papel dos hormônios, principalmente os componentes do eixo GH (*growth hormone*)/IGF (*insuline-like growth factor*). O eixo GH/IGF corresponde à via pela qual a maioria dos fatores que atuam no processo de crescimento exercem sua ação.

1.2. Regulação hormonal do crescimento em peixes

Investigações genéticas em peixes demonstraram a interferência direta e indireta de vários genes nos índices produtivos. Particularmente se destacam genes relacionados ao eixo GH/IGF, assim como os receptores do GH (GHR) (Grobet et al., 1997; Kambadur et al., 1997; Bellinge et al., 2005), e os IGF1 e IGF2 (Martinelli et al., 2008

Estes genes atuam no crescimento animal, modulando as maiores vias endócrinas anabólicas do corpo através do aumento da síntese proteica e diminuição da proteólise (Reinecke et al., 1997; Butler e LeRoith, 2001; Rodgers et al., 2001; Jiao et al., 2006; Chen et al., 2007; Ma et al., 2007; Wang et al., 2008; Biga e Meyer, 2009), tornando-se alvos no estudo do crescimento e no melhoramento genético de animais de produção.

Nos peixes, os peptídeos IGF-1 e IGF-2 são produzidos principalmente no fígado, que é a principal fonte endócrina de IGFs, sob a influência do GH. Após serem liberadas na corrente sanguínea, estas moléculas atuam em diversos tipos de tecido. O IGF-1 desempenha um papel central em um sistema complexo que regula o crescimento, diferenciação e reprodução. Ele, seletivamente, promove mitogênese e diferenciação celular e também inibe a apoptose (Jones e Clemmons, 1995; Reinecke e Collet, 1998).

Embora os papéis fisiológicos do IGF-1 estejam claros, os do IGF-2 estão em discussão (Berishvili et al., 2010). A expressão desta molécula foi identificada no fígado, cérebro, brânquias, coração, trato gastrointestinal, pâncreas, rim, músculo esquelético, baço e nas gônadas masculina e feminina (Ayson et al.,2002; Caelers et al., 2004; Vong et al., 2003), porém algumas de suas funções necessitam ser testadas.

1.3. Miostatina (MSTN)

Em geral, o músculo esquelético é a principal parte comestível dos animais. Nos peixes, o processo que envolve a formação muscular inclui o crescimento por hiperplasia e hipertrofia em estágios pós-juvenis (Mommsen, 2001). A taxa de crescimento hipertrófico varia de acordo com a taxa de crescimento somático (hiperplásico) em diferentes fases de desenvolvimento (Braun e Gautel, 2011).

Em cada etapa da miogênese, processo responsável pelo crescimento hiperplásico do tecido muscular, proteínas diferentes realizam papéis cruciais para os processos de proliferação e diferenciação celular. Dentre estes, destaca-se um grupo de genes conhecidos como fatores de regulação miogênica (*myogenic regulatory factors* - MRFs) e que são indispensáveis e responsáveis pelo processo de transformação de células não-musculares em células musculares (Bentzinger et al., 2012; Rudnicki e Jaenisch, 1995; Weintraub et al., 1991). A principal função dos MRFs (MyoD, Miogenina, Myf5 e o MRF4) é realizar a ativação e inibição dos demais genes da via de diferenciação muscular, agindo de forma orquestrada (Bentzinger et al., 2012).

O processo de miogênese possui como etapa inicial o recrutamento de células do mesoderma, através da ação do gene Pax3 (**Figura 1**). A proteína PAX 3 é um fator de transcrição que inicia a preparação das células do mesoderma para que sejam transformadas

em células musculares, e promovem a expressão de dois outros MRFs (MyoD e Myf5) (Bentzinger et al., 2012).

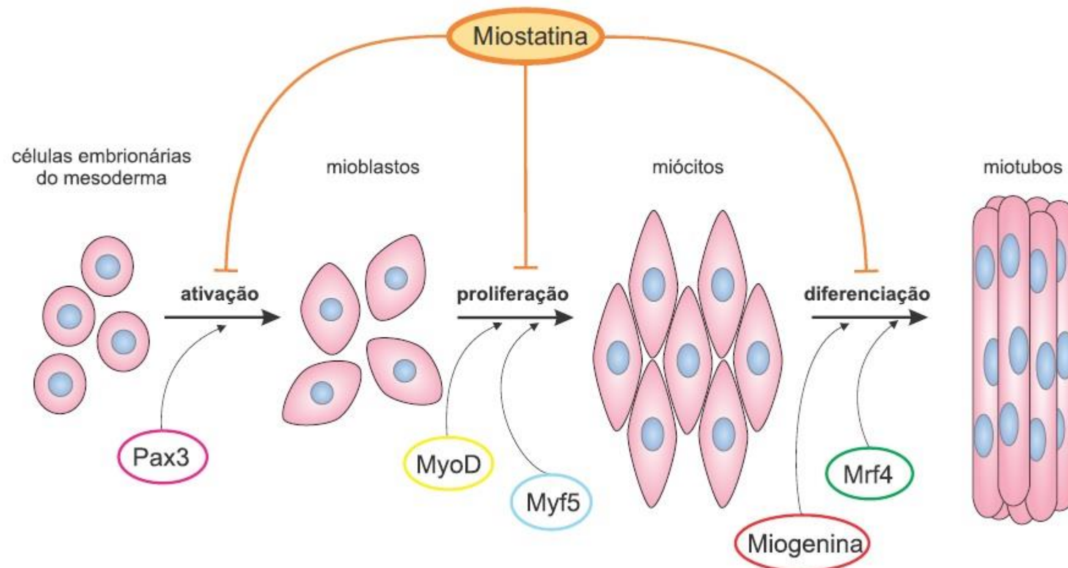

Figura 1. Na miogênese as células-tronco embrionárias do mesoderma são ativadas e recrutadas pela ação de Pax3, diferenciando-se nos mioblastos. Estes, por sua vez, proliferam e se diferenciam pela ação dos fatores MyoD e Myof5, transformando-se em miócitos. Durante a diferenciação por ação da miogenina e de MRF4, ocorre a organização dos miócitos em miotubos, formando o tecido muscular. A miostatina inibe a proliferação de células-tronco embrionárias do mesoderma impedindo a ligação de Pax3, que é a proteína recrutadora das células precursoras bloqueando a miogênese.

Quando um animal atinge a vida adulta, a miogênese tem sua atividade reduzida por uma série de fatores, dentre eles, a miostatina (MSTN) (Bentzinger et al., 2010; Kuang et al., 2008). A MSTN é membro da família TGF-β, que desempenha papel chave na regulação do crescimento do músculo esquelético (Lee, 2004). Esta proteína desempenha função inibitória sobre a proliferação celular, ou seja, inibe o crescimento hiperplásico se ligando ao receptor IIB da activina (Lee et al., 2016). O processo de bloqueio da miogênese promovido pela MSTN ocorre pela regulação negativa na expressão do fator de transcrição Pax3, o que acarreta na diminuição da expressão do gene MyoD (**Figura 1**) (Bentzinger et al., 2010; Kuang et al., 2008).

Ratos portadores de mutação no gene da MSTN apresentaram musculatura duas vezes maior do que aquela observada nos animais portadores do alelo selvagem, sugerindo que o aumento de massa seria o resultado da combinação de hiperplasia e hipertrofia muscular (McPherron et al., 1997). Estas mutações, que ocorrem de forma natural, foram identificadas também nas raças bovinas de dupla musculatura (Grobet et al., 1997; Kambadur et al, 1997; McPherron e Lee, 1997), em cães (Mosher et al., 2007) e até mesmo em seres humanos (Schuelke et al., 2004).

Acosta et al. (2005) realizaram estudo em zebrafish, onde a expressão da MSTN foi inibida por RNA de interferência (RNAi), resultando em fenótipo cujo tamanho corpóreo fora bastante superior em relação ao controle. No pirarucu, Carani et al. (2013) mostraram que a expressão deste gene é maior no músculo branco quando comparado ao músculo vermelho. Na truta, houve o desenvolvimento de uma musculatura dupla em um estudo utilizando animais transgênicos para o gene da folistatina, devido à sua ação na inibição da MSTN (Medeiros et al., 2009).

No estudo de Huang et al. (2012) houve um diferencial de expressão de MSTN entre duas linhagens de tilápia, com até 50% de redução na linhagem com maior crescimento. Portanto, este gene é um alvo importante para o melhoramento animal dos peixes em geral (Forabosco et al, 2013).

1.4. Regulação gênica: o papel central dos microRNAs

Na última década, diversos estudos mostram que virtualmente todos os genes têm sua expressão regulada pré ou pós-transcricionalmente por RNAs não-codificadores (ncRNAs; Mattick e Makunin, 2006). Para o completo entendimento das funções desempenhadas por diferentes produtos gênicos (e.g. IGFs e MSTN), torna-se necessária a análise de seus respectivos reguladores. Dentre os ncRNAs reguladores conhecidos, uma classe de pequenos

RNAs, os microRNAs (miRNAs), tem se destacado como moléculas chave em diversos processos biológicos.

Os miRNAs são transcritos formados por ~17-22 nucleotídeos encontrados no genoma de animais, plantas e vírus (Xia et al., 2011). Estas moléculas regulam pós-transcricionalmente a expressão gênica por pareamento com sequências complementares em seus RNAs mensageiros (mRNA) alvo (Bartel, 2004), atuando desde a formação de heterocromatina até a regulação traducional (Chu e Rana, 2007; Filipowicz et al., 2008); sendo fundamentais em vários processos biológicos que permitem o controle do desenvolvimento, diferenciação, proliferação e morte celular (Ambros, 2004; Flynt et al., 2007, 2009; Shkumatava et al., 2009; Liu e Olson, 2010; Takacs e Giraldez, 2011).

A via canônica de ação dos miRNAs ocorre por meio da interação do miRNA ao RISC (RNA-induced silencing complex), e destes com sítios ligantes ou MREs (*miRNAs Recognition Elements*) na região 3' UTR do RNA mensageiro (mRNA) alvo inibindo a sua expressão (Lee e Dutta, 2009). Entretanto estudos recentes verificaram ainda a ação de miRNAs a partir de interações com outras porções dos mRNAs, incluindo éxons e região 5'UTR dos mRNAs (Rigoutsos, 2009).

A interação melhor descrita entre o mRNA alvo e o complexo miRNA-RISC ocorre devido à complementaridade - total em plantas e parcial em animais - de uma sequência de 7 nucleotídos da região 5' do miRNA (nucleotídeos 2 a 8), chamada de sequência *seed*, com o mRNA (Lee e Dutta, 2009). Entretanto importância cada vez maior tem sido atribuída ao pareamento complementar da porção 3' do miRNA nesse processo (Broughton et al., 2016)., Estima-se que cada miRNA possa se ligar a até centenas de RNAs mensageiros e um único RNA mensageiro possa ter sua estabilidade ou tradução regulada por diversos miRNAs (Doench e Sharp, 2004; Brenneck et al., 2005; Lim et al., 2005).

De modo geral, os miRNAs são abundantes e altamente conservados entre os vertebrados estudados, o que sugere sua participação em processos celulares e ontogenéticos vitais comuns a esses organismos (Heimberg et al., 2010). Por outro lado, diversos miRNAs não conservados (i.e., "linhagem-específicos") foram também detectados em peixes, aves, répteis e mamíferos. Acredita-se que esses miRNAs evolutivamente divergentes poderiam desempenhar um papel no estabelecimento e manutenção da diversidade fenotípica entre diferentes organismos (Plasterk, 2006; Sempere et al., 2006; Xia et al., 2011).

Essas características inerentes aos miRNAs os tornam elementos chave para a determinação de vias específicas envolvidas em diversos processos biológicos (Esau et al., 2006).

1.4.1. MicroRNAs e o melhoramento animal

Por exercerem participação fundamental na regulação gênica, os miRNAs tornaram-se também alvos de investigações direcionadas ao melhoramento animal de animais de produção. Desta forma, estudos foram realizados em bovinos (Coutinho et al., 2007; Gu et al., 2007; Strozzi et al., 2009), suínos (Sharbati-Tehrani et al., 2008; Wernersson et al., 2005), em espécie de aves (Darnell et al., 2006, 2007; Glazov et al., 2008; Hicks et al., 2008, 2009) e inclusive na Tilápia do Nilo (Huang et al., 2012; Yan et al., 2012; Yan et al., 2013a, 2013b).

O estudo de Huang e et al. (2012) associou miRNAs e polimorfismos (SNPs) em alguns genes alvo, porém, seu não utilizou híbridos heteróticos como modelo experimental. A combinação de diferentes sequências dos miRNAs dos parentais podem ter ocasionado a heterose, pelo fato do fenótipo ser o resultado de mecanismos regulatórios na expressão gênica (Sun et al., 2004)

Considerando-se o grande número de miRNAs comumente detectados em vertebrados - grande parte tecido-específicos e de expressão restrita às etapas de desenvolvimento – um

amplo conjunto de miRNAs ainda estão por ser identificados na tilápia do Nilo. O uso da tilápia do Nilo como modelo biológico também contribui para estudos de evolução e comparação de genomas entre espécies, visto que genes reguladores do desenvolvimento, incluindo miRNAs, são altamente conservados entre peixes e humanos (Loh et al., 2011).

1.4.2. Descoberta de miRNAs utilizando sequenciamento de alta performance (RNA-Seq)

Evidências sugerem que o repertório completo de miRNAs de qualquer espécie - animal ou vegetal - compreende um conjunto de miRNAs conservados de origem evolutiva antiga, assim como muitos miRNAs espécie-específicos de origem recente (Plasterk, 2006; Rajagopalan et al., 2006; Zhao e Srivastava, 2007; Yan et al., 2012).

Existem duas abordagens utilizadas para a descoberta de miRNAs: análises de bioinformática e métodos experimentais, ambas com as suas limitações (Berezikov, 2011).

Tecnologias de sequenciamento de alta performance surgiram como uma poderosa abordagem para identificar e quantificar miRNAs. Por permitirem análises em larga escala, facilitaram a descoberta de miRNAs novos, espécie-específicos e de reduzida expressão em diversos organismos, pertencentes aos mais diversos táxons (Berezikov, 2011). Nesta abordagem, as ferramentas de bioinformática são utilizadas somente após a geração de dados experimentais.

Mesmo com a elevada capacidade de geração de sequências inerentes a essa tecnologia, estudos mostram que esse tipo de dado está longe da saturação, vide o grande número de novos miRNAs descobertos e representados apenas uma única vez no miRBase. Além disso, muitas das bibliotecas, de pequenos RNAs não codificadores analisados, foram geradas a partir de um número limitado de tecidos, e por isso uma ampla gama de miRNAs permanece desconhecida (Kloosterman et al., 2006; Reddy et al., 2009).

Portanto, estas tecnologias de sequenciamento em larga escala trazem inúmeras possibilidades para descoberta e análise de miRNAs e contribuem para estudos mais detalhados sobre a funcionalidade do genoma, particularmente quanto às interações genes-reguladores.

1.5. Proteômica

Uma proteína é o produto final da síntese de um mRNA e a sua tradução em uma sequência de aminoácidos. Essas moléculas contribuem decisivamente para determinar alterações fenotípicas, principalmente em decorrência de variações qualitativas e quantitativas nos vários tipos de tecidos orgânicos. Assim, sua produção está condicionada à expressão dos genes codificadores e respectivos ncRNAs regulatórios, como os miRNAs, atuantes nos diferentes tecidos.

O termo proteômica refere-se ao estudo do conjunto de proteínas responsáveis, direta ou indiretamente, pelo controle de todos ou quase todos os processos biológicos (Barbosa et al., 2012). Portanto, analisar o proteoma é conhecer o conjunto de proteínas resultantes da codificação do genoma e da interação com outras moléculas (Wilkins et al., 1996), inclusive outras proteínas e miRNAs. Portanto, um proteoma não é apenas o resultado dos produtos traduzidos a partir das sequências dos genomas, mas também o resultado de processos pós-transcricionais e pós-traducionais, assim como complexos formados por essas biomoléculas (Ahrens et al., 2010).

Além da complexidade, um proteoma é dinâmico e seu perfil se altera de acordo com o estado fisiológico, devido a condições ambientais e as fases da diferenciação celular. Estimativas sugerem que mais de um milhão de diferentes tipos de proteínas estão presentes nas células em momentos distintos (Jensen, 2004). A proteômica estuda de forma descritiva e quantitativa o conjunto de proteínas de uma de uma célula, ou tecido, suas variações na

população, mudanças em resposta a um ambiente ou decorrentes do desenvolvimento normal ou alterado (Valledor e Jorrin, 2011). As abordagens proteômicas têm permitido estudos em larga escala da expressão proteica nos mais variados tecidos e condições experimentais (Barbosa et al., 2012). Atualmente há várias técnicas disponíveis para a análise de proteínas, no entanto, destaca-se a utilização da espectrometria de massa (*mass spectrometry*, MS), que tem sido aplicada de forma abrangente na investigação de sistemas biológicos. (Domon e Aebersold, 2006; Sparkman, 2000).

A MS é a técnica instrumental mais apropriada para a investigação da estrutura e reatividade de íons. O alto vácuo produzido pelo espectrômetro é ideal para se estudar as propriedades iônicas das moléculas em fase gasosa, possibilitando estabelecer uma correlação com a fase condensada. Muitos íons difíceis de serem isolados podem ser facilmente gerados na fase gasosa por MS (Cabrini, 2007).

Muitas das técnicas empregadas em proteômica têm como foco a identificação de biomarcadores, mas são limitadas. Outras têm potencial para automatização e até utilização na rotina clínica. De maneira geral, as metodologias empregadas podem ser classificadas nos tipos *bottom-up* ou *top-down* (Barbosa et al., 2012).

As metodologias por *bottom-up*, mais conhecidas como *shotgun* (Ahrens et al., 2010), inclui separação por cromatografia líquida dos peptídeos obtidos após digestão tríptica de soluções proteicas complexas, seguida de análise por MS. Já as metodologias por *top-down*, ao contrário, são processos em que as proteínas intactas são submetidas à MS (Barbosa et al., 2012).

O *shotgun* possui muitas vantagens, como sensibilidade e reprodutibilidade, mesmo para proteomas complexos. Embora as respostas obtidas são fragmentos de um todo, é possível a identificação de uma proteína com base em alguns peptídeos (Barbosa et al., 2012).

Associado a isso, o surgimento de técnicas de ionização, principalmente a ionização por Electrospray (ESI) expandiu a gama de moléculas que podem ser analisadas por espectrometria de massas (Diniz, 2011). Para auxiliar o método de ionização, analisadores são utilizados após os íons passarem pela câmara à vácuo. Os tipos mais comuns de analisadores são o TOF (Time Of Flight), o quadrupolo e o *ion trap* (May et al., 2011). Nos analisadores TOF, os íons são acelerados por um potencial entre dois eletrodos e atravessam um tubo de vácuo com velocidade inversamente proporcional à sua massa. Este método é utilizado para derivar o valor m/z, o tempo decorrido entre a ionização e a detecção dos íons (Barbosa et al., 2012).

O potencial de aplicação das técnicas de proteoma para a produção animal é muito grande. Estudos têm sido realizados utilizando outros modelos biológicos de interesse econômico, como bovinos, como objetivo, por exemplo, analisar as alterações de proteomas em M. Músculos semitendinosos durante o armazenamento pós-mortem (Yu et al., 2017). Um dos estudos mais notáveis, mostrou que alimentação com restrição alimentar em bovinos não só leva à índices de produção diferenciadas como também na alternação da composição proteína da carne (Almeida et al., 2017).Em peixes, técnicas de proteoma foram utilizadas com objetivo avaliar resistência à infecção bacteriana na tilápia do Nilo (Chang et al., 2017; Li et al., 2017), na criopreservação de sêmen de truta arco-íris (*Oncorhynchus mykiss*) (Nynca et al., 2015), na qualidade e textura muscular da carne de salmão (*Salmo salar*) (Ørnholt-Johansson et al., 2017) e toxicologia hepática também em salmão (*S. salar*) (Hampel et al., 2015). No entanto, este é o primeiro estudo buscando informações sobre as diferenças no conjunto de proteínas no híbrido em questão, e também o primeiro estudo com o foco na heterose.

2. Justificativa

Há alguns anos, a aquicultura vem se tornando uma das atividades que mais cresce na agropecuária do Brasil e do mundo. Além de ser uma espécie comumente cultivada no mundo, a tilápia é a espécie mais cultivada no Brasil.

Para atender à demanda do mercado consumidor, várias linhagens foram desenvolvidas. Com o objetivo de combinar as características de duas linhagens potenciais, foi desenvolvido um híbrido 7/8 de Chitralada e 1/8 Red Stirling, o qual apresenta heterose.

Considerando-se o fato de que pouco é conhecido a respeito dos mecanismos moleculares envolvidos na heterose de animais, e que esse conhecimento pode direcionar programas de melhoramento animal prevendo, por exemplo, quais linhagens quando cruzadas, produzirão um híbrido com heterose, torna-se desejável a investigação de mecanismos genéticos envolvidos na heterose. Para que desta forma, seja mais fácil e também mais assertivo prever quais cruzamentos entre diferentes linhagens, ou até mesmo espécies, que resultem na geração de animais com vigor híbrido.

Dentre os mecanismos potencialmente envolvidos na formação de um fenótipo de crescimento superior ou acelerado, destacam-se tanto a expressão diferencial dos genes envolvidos no eixo GH/IGF e da miostatina, quanto a regulação destes mediada pela atividade de miRNAs. Além disso, como a presença e o acúmulo de proteínas são o produto final da expressão regulada de genes codificadores, a investigação do conjunto de proteínas relacionadas à formação do fenótipo heterótico é um aspecto biológico central na busca pelo entendimento dos mecanismos moleculares do vigor híbrido.

Partindo destas informações, a hipótese deste trabalho é que o fenótipo do híbrido heterótico decorre da existência de expressão proteica diferencial, como produto final da regulação da expressão de genes chave e consequente modulação de vias metabólicas por miRNAs.

3. Objetivos

3.1. Objetivo geral:

O presente estudo tem como objetivo elucidar alguns dos possíveis mecanismos moleculares relacionados à heterose nos híbridos da Tilápia do Nilo (*Oreochromis niloticus*).

3.2. Objetivos específicos:

- Comparar os perfis de expressão dos seguintes genes: *gh*; *ghr1*; *ghr2*; *sl*; *igf1*; *igf2* e *mstn* entre o híbrido e seus parentais;

- Identificar os miRNAs potenciais associados às características fenotípicas diferenciais entre o híbrido e os parentais, por RNA-seq, e com seus alvos preditos, por ferramentas de bioinformática.

- Caracterizar a composição do proteoma e detectar potenciais variações entre o híbrido e as duas linagens parentais pelas técnicas de ESI-q-TOF e *shotgun*.

4. Material e métodos

4.1. Animais e condições experimentais

Os animais utilizados foram provenientes da Indústria Brasileira do Peixe Ltda. (IBP) localizada na Rodovia Dom Gabriel Bueno Paulino Couto, km 73,5 Serra do Japi, Jundiaí, SP (latitude 23°20' S, longitude 47°02' O, altitude média 695 m). Nas Fazendas Santa Ignês e Rio das Pedras, propriedades da IBP, estão alocados os plantéis de tilápias das variedades Chitralada e Red Stirling, e seu híbrido intraespecífico (7/8 Chitralada: 1/8 Red Stirling) com melhores índices produtivos (Lago, 2014). Estes animais foram mantidos sob condições controladas de temperatura (25 +/- 1ºC), ciclo claro-escuro (12h/12h) e alimentados *ad libitum* (Hilsdorf et al., 2002).

Para a coleta dos tecidos, foram utilizados 5 animais, entre machos e fêmeas, com 4, 5 e 6 meses de idade – totalizando 45 animais. A curva de crescimento típica de todos os animais durante a vida apresentou uma forma sigmoidal, de modo que o crescimento durante o primeiro estágio de vida foi lento, seguido por um período de aceleração até atingir a taxa de pico do crescimento, em torno da puberdade, e em seguida ocorreu uma fase de desaceleração (Berg e Butterfield, 1976). Esta fase de inflexão da reta varia de 121,2 a 156,2 dias para as fêmeas, e 134,1 a 166,6 dias para os machos, o que corresponde a cerca de 4 a 5 meses (Oliveira, 2013), justificando a coleta de animais com 4, 5 e 6 meses de vida.

Após a coleta, os animais foram eutanasiados utilizando o anestésico tricainametilsulfonato tamponado (MS-222, Sigma Chemical, St. Louis, MO). Em seguida foram coletadas amostras de fígado, cérebro e músculo branco, imediatamente preservadas em nitrogênio líquido e posteriormente acondicionadas à -80ºC. O cérebro é o principal órgão de liberação do GH sistêmico e o fígado é o órgão de liberação sistêmica de IGFs (Martinelli et al., 2008). Todos os experimentos com os animais foram aprovados pela Comissão de Ética no Uso de Animais local (protocolos CEUA 788/2015 e 352/2011).

4.2. Extração de RNA

O RNA foi extraído de amostras de fígado, cérebro e músculo branco com Trizol (Life Technologies, EUA) seguindo as recomendações do fabricante. O tecido foi triturado na solução de Trizol, tomando o cuidado para não aquecer as amostras, na proporção de 1ml:1mg de tecido. Após trituradas, as amostras foram acondicionadas por 5 minutos no gelo.

A solução foi transferida para tubos contendo 400µl de clorofórmio, os quais foram agitados por 15 segundos, mantidos por 3 minutos no gelo e em seguida centrifugados por 15 minutos a 12.000g a 4ºC. A fase aquosa foi transferida em outro tubo contendo 500µl de álcool isopropanol, o qual foi agitado por 30 segundo, mantido em gelo por 10 minutos e em seguida centrifugado por 10 minutos a 12.000g a 4ºC. Foi descartado o sobrenadante e adicionado 1ml de etanol 75% e em seguida foi centrifugado a 7500g por 5 minutos a 4ºC. Após a centrifugação, foi descartado o sobrenadante e adicionado 1ml de etanol 100% e centrifugado mais uma vez a 7500g a por 5 minutos a 4ºC. Em seguida, foi descartado o sobrenadante, e após secarem foi adicionado 20µl de água livre de RNAses. O RNA total foi quantificado por espectrofotometria utilizando o equipamento com absorbância a 260 mm (Thermo Fisher Scientifir, EUA). A qualidade do RNA foi obtida pelo número da integridade do RNA (*RNA Integrity Number - RIN*), a partir da análise dos RNAs ribossomais baseadas em microfluídos, utilizando-se o sistema 2100 Bioanalyzer (Agilent, EUA) (Fleige e Pfaffl, 2006; Becker et al., 2010).

4.3. Análise de expressão gênica por PCR em tempo real após transcrição reversa (RT-qPCR)

O RNA extraído das amostras de cérebro, fígado e músculo branco foi submetido à reação de transcriptase reversa utilizando-se o kit *High Capacity RNA-to-cDNA Master Mix*

(*Life Technologies*, EUA), segundo as orientações do fabricante. A qPCR do cDNA gerado a partir dos RNAs mensageiros foi realizada com ensaios *SYBR® Green* (Life Technologies, EUA), contendo oligos para os mRNAs alvo em estudo. Alguns genes constitutivos foram testados e o de melhor expressão no tecido muscular foi selecionado para normalização dos dados. As reações foram realizadas no Sistema de PCR em Tempo Real *ABI StepOne Plus*™ (*Life Technologies,* EUA) utilizando o *SYBR® Green Real-Time PCR Master* (*Life Technologies*, EUA). Os valores de Cq (ciclo de quantificação) foram calculados utilizando o software SDS 1.4, utilizando a configuração automática de baseline e um threshold de 0,2. O Cq é ponto de encontro entre a curva de amplificação corrigida pelo baseline e o threshold (de acordo com o RDML, HTTP://rdml.org). A quantificação relativa da expressão gênica foi realizada pelo método do Cq comparativo com análise de variância e teste Tukey (5% de significância). Os oligos utilizados, suas sequências e suas referências estão listados na **Tabela 1**.

Tabela 1.Sequências dos oligonucleotídeos utilizados para análise de expressão dos genes relacionados ao crescimento. GH (growth hormone), SL (Somatolactina) GHR (growth hormone receptor;),IGF (insulin-like growth factor) e MSTN (miostatina).

Gene alvo	Acesso no GenBank	Sequencia do oligo (F = Forward; R = Reverse)	Referência
GH	M26916	F - 5′-CTGTCTGTCTGTCTGTCAGTCGT-3′ R- 5′-AGAGGAGACGCCCAAACAC-3′	Rentier-Delrue et al. (1989)
SL	AB442015	F – 5’- CCCTTTGCGACTTCAGAGTAA-3’ R – 5’- ACAGAGTGGAGCAACCATTT-3’	Uchida et al. (2008)
GHR1	EF052861	F - 5′-TCTCAGCAGAACCGATTAATGA-3′ R - 5′-TTTGATTTTGGGTGCAGGA-3′	Ma et al. (2007)
GHR2	EF052862	F - 5′-CGACCCAGAACCATCACC-3′ R - 5′-GTCTCCTGACTGAGGGCAAG-3′	Ma et al. (2007)
IGF-1	EU272149	F - 5′-CCCGAACTTCCTCGACTTGA-3′ R - 5′-CCTCAGCCAGACAAGACAAAAA-3′	Wang et al. (2008)
IGF-2	EU272150	F - 5′-CCCCTGATCAGCCTTCCTA-3′ R - 5′-GACAAAGTTGTCCGTGGTGA-3′	Wang et al. (2008)
MSTN	AF197193	F - 5′-ACCAGCCCCACCTGAACT-3′ R - 5′-ATCTGGGACGTGGCTCTCT-3′	Rodgers et al. (2001)
Beta-actin	EU887951	F - 5′- ACCCACACAGTGCCCATC -3′ R - 5′- CAGGTCCAGACGCAGGAT -3′	Monteiro et al. (2009)
18S ribosomal RNA	DQ397879	F – 5’- CCTGAGAAACGGCTACCACAT-3’ R – 5’ – CAGACTTGCCCTCCAATGGAT-3’	Pang et al. (2013)

4.4. Construção de bibliotecas enriquecidas e sequenciamento de alta performance (RNA-Seq) de miRNAs

Para análise de miRNAs foi utilizado o RNA isolado a partir de amostras de músculo branco da tilápia do Nilo. Na construção das bibliotecas de miRNAs, foi utilizado o kit TrueSeq Small RNA Sample Prep (Illumina, EUA), segundo protocolo informado pelo fabricante. O RNA foi fracionado em fragmentos de diferentes tamanhos a partir da eletroforese em gel de poliacrilamida (SDS-PAGE). A porção do gel com fragmentos entre 15 a 30 nucleotídeos foi excisada. Os fragmentos foram recuperados por resfriamento *overnight* com solução salina, seguido por extração, precipitação e filtração em *micropore*.

Após a eluição em água DEPC, adaptadores foram ligados às extremidades 3' e 5' dos fragmentos de pequenos RNA isolados. Com a ligação dos adaptadores à ambas extremidades, oligos com complementariedade à sequência dos adaptadores (fornecidos no kit) foram então utilizados para a transcrição reversa e amplificação seletiva de fragmentos de pequenos RNAs por PCR (RT-PCR). Os fragmentos de cDNA construídos foram purificados e se constituiram na biblioteca utilizada para a subsequente geração de clusters e sequenciamento. Cada biblioteca gerada foi validada a partir da análise da pureza, concentração e do tamanho dos fragmentos de pequenos RNAs no sistema *2100 Bioanalyzer*, utilizando o chip *DNA-1000* (Agilent, EUA).

As amostras de cDNA seguiram para o sequenciamento por RNA-seq a partir de serviços contratados da empresa (LC Sciences, EUA). A plataforma utilizada foi a Illumina/Solexa capaz de gerar milhões de pequenas sequências ou *reads* (50-100 pares de bases) com alta qualidade.

4.5.Análise de miRNAs *in silico*

A identificação de miRNAs foi realizada com o software miRDEEP (Friedländer et al., 2008). O algoritmo utilizado por esse software opera pela da atribuição de um "score" baseado na probabilidade de dados de sequenciamento para locus de miRNAs candidatos e ajuste ao modelo de biogênese de miRNAs e, em seguida, esse software avalia estatisticamente os resultados. Diversos estudos utilizaram este programa, o qual se encontra plenamente validado, apresentando acurácia e robustez nas predições geradas (Wienholds et al., 2005; Kloosterman et al., 2006; Thatcher et al., 2008; Wei et al., 2012).

A predição *in silico* de alvos dos miRNAs que apresentaram expressão diferencial entre o híbrido e suas linhagens parentais (foldchange >2) foi realizada utilizando-se o algoritmo TargetScan que realiza buscas de genes alvo no genoma de peixes, além de outros vertebrados (http://www.targetscan.org). Para tal, primeiramente foi feito o download da região 3'UTR do genoma da tilápia (v 1.0) do banco de dados do Ensembl (http://www.ensembl.org). Uma vez que a anotação do genoma desta espécie ainda está incompleta para os genes em que não havia anotação de 3'UTR utilizou-se os primeiros 500 nucleotídeos posteriores ao *stop codon*.

Para predição de alvos de melhor qualidade, os resultados provenientes da ferramenta foram filtrados utilizando-se apenas valores de *Context+Score*<= -0.2. Análises prévias realizadas em nosso laboratório (Oliveira et al., 2017) demonstraram que este filtro, dentre todos os outros, proporciona resultados de maior confiabilidade para a versão 6.2 (utilizada em nosso experimento). Adicionalmente, a fim de verificar um efeito tecido-específico, para cada tecido analisado foram utilizados apenas genes alvo que eram expressos naquele tecido (FPKM > 10).

Os resultados gerados a partir da predição de alvos foram submetidos à análise de enriquecimento funcional utilizando o algorítmo EmpiricalGO. O enriquecimento funcional visa caracterizar os principais processos biológicos em que os genes alvo dos miRNAs

analisados estão atuando, fornecendo valiosas informações sobre a possível contribuição destes miRNAs para o crescimento muscular do híbrido.

4.6. Análise de proteínas

4.6.1. Extração de proteínas de eletroforese

Esta etapa do trabalho foi realizada em parceria com o grupo de pesquisa da *Technische Hochschule Wildau* (THW) em Wildau na Alemanha e também com o Centro de Estudos de Venenos e Animais Peçonhentos (CEVAP) em Botucatu-SP. Foram extraídas amostras de músculo branco decinco indivíduos com 6 meses de vida por grupo (CHIT, REDS e híbrido), totalizando 15 amostras. Na extração de proteínas, amostras refrigeradas de músculo branco à -20ºC foram processadas e homogeneizadas. A preparação das amostras ocorreu conforme o protocolo utilizado por Bouley et al. (2004).

As amostras de músculo branco (50-100mg) foram homogeneizadas em uma solução de lise contendo 8,3 M de uréia, 2 M de tiouréia, 1% de DTT, 2% de CHAPS e 2% de tampão IPG pH 3-10 com politron, e centrifugadas à 10.000g por 30 minutos. Foi recolhido o sobrenadante. A concentração de proteínas foi determinada pela reação de Bradford.

As amostras foram separadas em um gel de duas dimensões (2D) de acordo com o protocolo descrito por Eravci e et al. (2007). O primeiro passo foi a separação por um gradiente de pH 3-10 e uma fita. Esta etapa separa as proteínas pelo seu ponto isoelétrico. A segunda etapa consiste em uma separação por 90º, separando as proteínas pelo seu peso molecular.

4.6.2. Aquisição e análises das imagens de géis

Para obtenção das imagens, os géis 2D foram digitalizados utilizando-se o scanner ImageScanner III (GE Healthcare Life Sciences) no modo de transmissão calibrado. As

imagens dos géis bidimensionais foram analisadas pelo software Image Master 2D Platinum v7.05 (Ge Healthcare). A autenticidade de cada spot foi validada por inspeção visual e editada quando necessário.

4.6.3. Digestão Enzimática

As manchas de proteínas, coradas com CBB, foram recortadas do gel, descoloridas com solução Destain (etanol 50% [v/v] e ácido acético 2,5% [v/v]), desidratadas com acetonitrila 100% (v/v), sendo então os pedaços de gel secos por centrifugação à vácuo. Em seguida, os pedaços de géis desidratados foram digeridos com tripsina, seguindo-se o protocolo: foram dissolvidos 20 g de tripsina não autolítica em 1600 µL de bicarbonato de amônio 50 mM, pH 8,0. Cada pedaço de gel seco foi incubado com 30 µL da solução de tripsina, durante 30 minutos em banho de gelo; a digestão foi interrompida removendo o excesso de solução de tripsina e adicionando-se 50 µL de bicarbonato de amônio 50 mM e mantendo-se a suspensão de gel overnight em banho-maria à 37°C; em seguida, foram adicionados na suspensão contendo o gel, 10 µL de solução de ácido fórmico 5% (v/v), incubados por 10 minutos à temperatura ambiente, o sobrenadante foi coletado e colocado em novos microtubos (marca Eppenorf, modelo Lobind – volume 0,5 mL); nos tubos contendo os géis foram adicionados 12 µL de solução de ácido fórmico 5% (v/v) e acetonitrila 50% (v/v), incubados por 10 minutos à temperatura ambiente; o sobrenadante foi coletado e adicionado ao microtudo da etapa anterior. Os sobrenadantes coletados foram secos por centrifugação a vácuo, sendo então mantidos a – 20ºC até serem analisados por espectrometria de massas (Santos et al., 2011).

4.6.4. Sequenciamento peptídico por espectrometria de massas e processamento dos dados

As análises de espectrometria de massas dos digestos trípticos oriundos do processamento de géis de eletroforese bidimensional foram realizadas em um equipamento do tipo electrospray (ESI) quadropolo, modelo MicrQ-TOF III (marca Bruker Daltonics), acoplado à um cromatógrafo líquido LC-20AT (marca Shimadzu). O cromátografo liquido foi equipado com um sistema binário de bombas e um aplicador de amostras automático. A fase móvel consistiu de água (A) e acetonitrila (B), contendo ácido fórmico 0,1% (v/v). Em adição, a separação cromatográfica foi realizada por meio de uma coluna de fase reversa C18 (4,5 mm x 100 mm, 1,8 um). As condições de eluição foi otimizada num gradiente linear de 0 a 85% do solvente B por 60 minutos, num fluxo de 0,2 mL/min. A coluna e o aplicador automático de amostras foram mantidos a 25°C e 10°C, respectivamente. O volume de injeção dos compostos de referência e das amostras foi de 10 uL. O espectrômetro de massas utilizoi os seguintes parâmetros: voltagem do capilar 4.5 kV, temperatura de secagem de 180°C, fluxo de nitrogênio de 6 L/min e pressão de 0.8 bar. A calibração externa foi realizada utilizando-se o kit Tunning MIX ESI (Agilent Tecnologies) antes das análises individuais. Os espectros de massas foram adquiridos no modo de ionização electrospray positiva (ESI) em um intervalo de ionização entre 400 m/z a 2500 m/z. Após a espectrometria de massas, os dados foram exportados e submetidos à análise automática com a ferramenta de bioinformática MASCOT v.2.1 (www.matrixscience.com), utilizando os seguintes parâmetros: enzima tripsina; taxonomia *Oreochromis niloticus* (banco de dados NCBI; modificação fixa carbamidometilação; modificação variável oxidação da metionina; uma clivagem perdida pela enzima; massa molecular do tipo monoisotópica; erro de tolerância de peptídeos (MS) ± 0,2 Da e erro de tolerância (MS/MS) ± 0,2 Da; protonação + 1 e tipo de instrumento ESI-q-TOF.

A análise dos peptídeos trípticos das amostras de mistura complexa (Shotgun) foram realizadas em um sistema nano ACQUITY UPLC (Waters, Milliford, USA) acoplado ao espectrômetro de massas Xevo Q-TOF G2 (Waters, Milliford, USA). Para tanto, o sistema

UPLC nanoACQUITY foi equipado com uma coluna do tipo HSS T3 (Acquity UPLC HSS T3 column 75 mm x 150 mm; 1,8 µm, Waters), previamente equilibrada com 7% da fase móvel B (100% ACN + 0,1 % ácido fórmico). Os peptídeos foram separados através de um gradiente linear de 7-85 % de fase móvel B durante 20 min com fluxo de 0,35 µL/min e a temperatura da coluna mantida a 45°C. O MS foi operado em modo íon positivo, com o tempo de aquisição de dados de 20 min. Os dados obtidos foram processados através do software ProteinLynx GlobalServer (PLGS) versão 3.0 (Waters, Milliford, USA). A identificação das proteínas foi obtida através do algoritmo de contagem de íons incorporado ao software. Os dados obtidos foram comparados com o banco de dados *Oreochromis niloticus* baixado do catálogo do NCBI (National Center for Biotechnology Information), utilizando os seguintes parâmetros: modificação fixa carbamidometilação; modificação variável oxidação da metionina; uma clivagem perdida pela enzima; massa molecular do tipo monoisotópica; erro de tolerância de peptídeos (MS) ± 0,2 Da e erro de tolerância (MS/MS) ± 0,2 Da; protonação + 2,+3+4, tipo de instrumento ESI-Tof.

O enriquecimento funcional foi executados utilizando-se a ferramenta do g:Profiler (http://biit.cs.ut.ee/gprofiler/) na base de dados da tilápia do Nilo, adotando-se g:SCS threshold como parâmetro estatístico.

5. Resultados

5.1. Expressão gênica

5.1.1. Expressão do *gh*

A expressão relativa do *gh* no cérebro e no músculo branco apresentou a mesma tendência nos três meses avaliados, estando super expresso no híbrido em comparação às linhagens CHIT e REDS (**Figura 2**). Se comparada à expressão relativa do *gh* nos 5º e 6º meses, utilizando o mês quatro como referência, a linhagem CHIT apresentou tendência de aumento da expressão do gene no cérebro, enquanto que a linhagem RED e o híbrido super expresso no 5º mês e sub expresso no 6º mês s (**Figura 3**). Já no músculo branco, a expressão relativa do *gh* foi super expresso na linhagem REDS e no híbrido, enquanto que não variou entre os meses quatro e cinco, estando super expresso no mês seis.

5.1.2. Expressão de *sl*

A expressão relativa do *sl* no cérebro seguiu uma tendência de estar super expresso no híbrido se comparado com as linhagens REDS e CHIT (**Figura 2**). Avaliando os meses cinco e seis, tomando o 4º mês como controle, a *sl*se mostrousub expressa na linhagem CHIT e no híbrido, enquanto que na linhagem REDS foi super expresso (**Figura 3**).

5.1.3. Expressão do *ghr1* e *ghr2*

A expressão relativa do *ghr1* no músculo branco se mostrou muito mais expresso na REDS no mês quatro, enquanto que no mês cinco e seis foi super expresso no híbrido em comparação às linhagens, e a linhagem REDS foi sub expresso em relação à CHIT (**Figura 2**). Se comparada à expressão relativa do *ghr1* nos meses cinco e seis utilizando o mês quatro como referência, ele foi subexpresso no mês cinco e super expresso no mês seis (**Figura 3**).

O *ghr2* no músculo branco teve sua expressão relativa super expresso na CHIT comparado as duas linhagens no mês quatro, enquanto que no mês cinco e seis foi super expresso em relação no híbrido em relação aos seus parentais (**Figura 2**) Ao longos dos meses, o *ghr2* mostrou um padrão semelhante de expressão entre as três linhagens **(Figura 3**).

5.1.4. Expressão do *igf1* e *igf2*

Durante o 4º mês - considerando o cérebro, fígado e músculo branco - a expressão relativa do *igf2* esteve super expresso no híbrido em comparação as duas linhagens avaliadas (**Figura 2**). No 5º mês, esse gene foi regulado de maneira similar no cérebro, no músculo branco e no fígado, sua expressão não apresentou diferença entre as linhagens e o híbrido (**Figura 2**). No 6º mês, a expressão desse gene mostrou padrões diferenciados (**Figura 2**).

O *igf1* mostrou também padrões variáveis ao longo dos meses em todos os tecidos, no entanto, em sua maioria se mostrou mais expresso no híbrido em comparação com os seus parentais (**Figura 2**).

Se comparada à expressão relativa do *igf1* nos meses cinco e seis, utilizando o mês quatro como referência, a linhagem CHIT e o híbrido estiveram sub expresso no quinto mês e super expresso no sexto mês, e super expresso nos dois meses na linhagem REDS, ambos avaliados no cérebro. No fígado e no músculo branco, o *igf2* foi super expresso nos meses cinco e seis, exceto a linhagem REDS, apresentando sub expressão no mês cinco e seis no músculo branco (**Figura 3**).

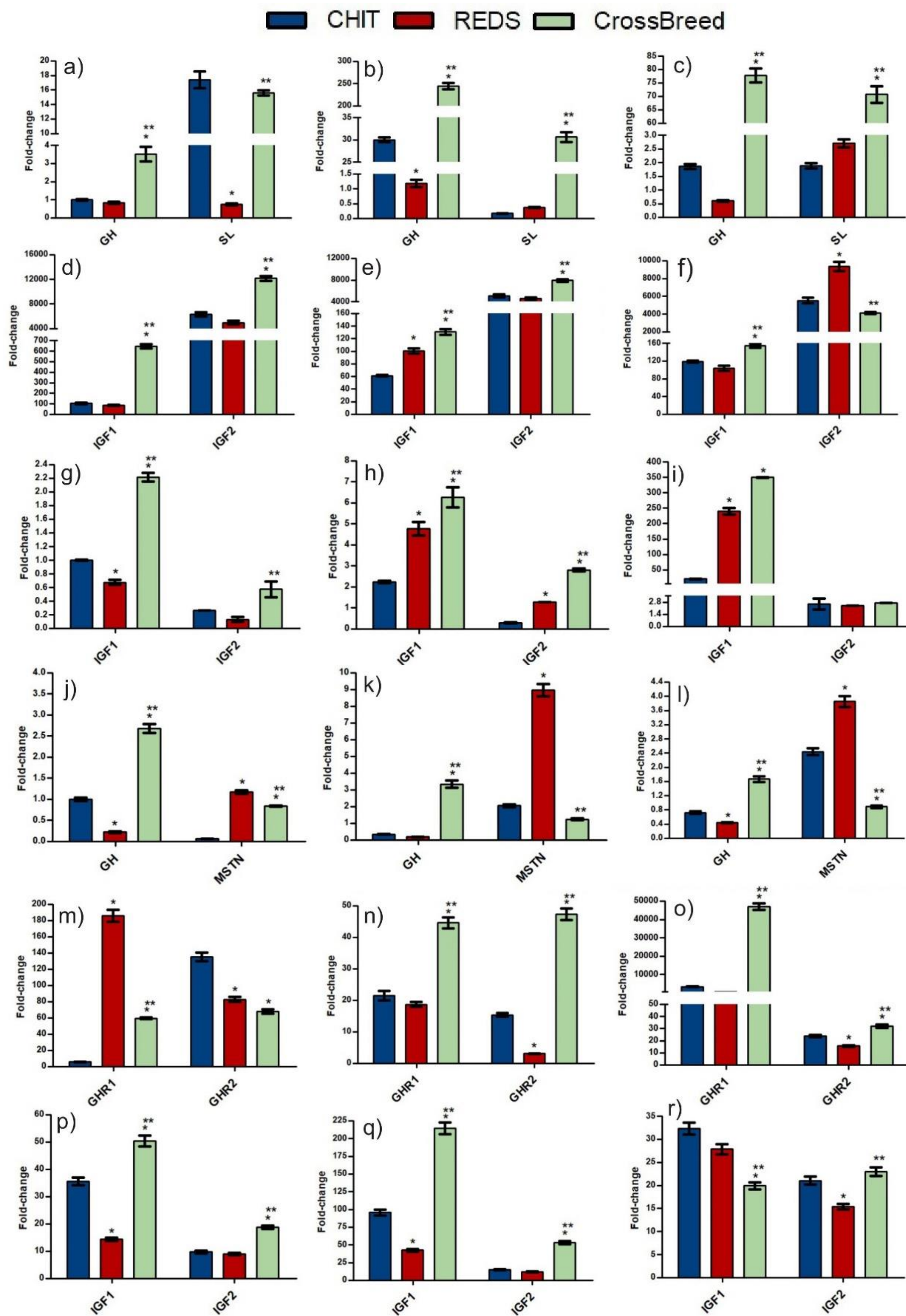

Figura 2 Expressão relativa dos genes do eixo GH/IGF e MSTN no cérebro, músculo branco e fígado. Na legenda CHIT = Chitralada; RED = Red Stirling; e CrossBreed = híbrido. * (P<0,05 comparando com CHIT) ** (P<0,05 comparando com RED). a) e d) expressão relativa dos genes durante o 4º mês no cérebro; b) e e) expressão relativa dos genes durante o 5º mês no cérebro; c) e f) expressão relativa dos genes durante o 6º mês no cérebro; g) expressão relativa dos genes durante o 4º mês no fígado; h) expressão relativa dos genes durantes o 5º mês no fígado; i) expressão relativa dos genes durante o 6º mês no fígado; j), m) e p) expressão relativa dos

genes durante o 4º mês no músculo branco; k), n) e q) expressão relativa dos genes durante o 5º mês no músculo branco; e l), o) e r)expressão relativa durante o 6º mês no músculo branco.

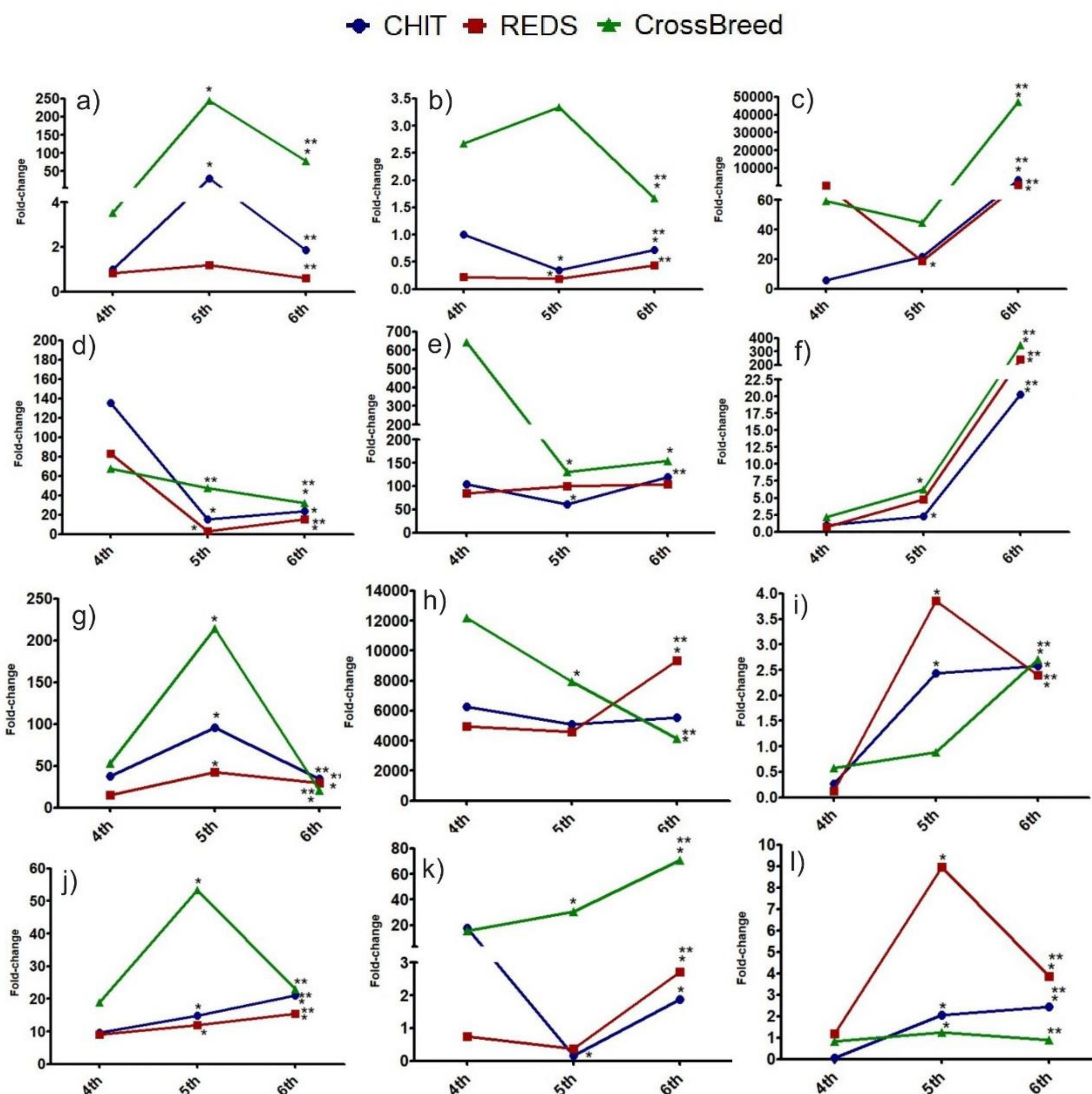

Figura 3 Expressão relativa dos genes ao longo dos 3 meses comparando a expressão inicial de cada grupo. Na legenda CHIT = Chitralada; RED = Red Stirling; e CrossBreed = híbrido. * super expresso em relação ao 4º mês em cada grupo (P<0,05) ** sub expresso em relação ao 4º mês em cada grupo (P<0,05). a) expressão relativa do *gh* no cérebro; b) expressão relativa do *gh* no músculo branco; c) expressão relativa do *ghr1* no músculo branco; d) expressão relativa do *ghr2* no músculo branco; e) expressão relativa do *igf1* no cérebro; f) expressão relativa do *igf1* no fígado; g) expressão relativa do *igf1* no músculo branco; h) expressão relativa do *igf2* no cérebro; i) expressão relativa do *igf2* no fígado; j) expressão relativa do *igf2* no músculo branco; k) expressão relativa da *sl* no cérebro; e l) expressão relativa da *mstn* no músculo branco.

5.1.5. Expressão da *mstn*

Em relação a expressão da *mstn* no músculo branco, comparando a CHIT com a REDS e o híbrido, e de super expressão se comparada a linhagem REDS com o híbrido. No mês 5 e 6, se mostrou sub expresso no híbrido em relação aos seus parentais (**Figura 2**). Comparando

ao longos dos meses, houve um aumento acentuado na expressão da *mstn* na REDS (**Figura 3**).

5.2.Análise de miRNAs por RNA-Seq

Para a construção das bibliotecas de cDNA foram utilizados os indivíduos do 5º e 6º mês (período de maior crescimento nas tilápias-do-nilo) das linhagens parentais, sendo estas os grupos controles, e os híbridos totalizando seis bibliotecas. A qualidade e a integridade das amostras foram avaliadas pelo BioAnalyzer antes de enviadas à empresa LC Sciences (EUA) para serem sequenciadas. As amostras que não possuíam um RNA Integrity Number (RIN) superior a 7, não foram utilizadas para construção das bibliotecas de cDNA.

Os resultados de RNA-Seq foram submetidos à análise pelo software miRDEEP (Friedländer et al., 2008). No total o sequenciamento de alta performance Illumina gerou de 3.173.730 a 5.362.730 (**Tabela 2**) *reads* dependendo da biblioteca. O número de *reads* mapeáveis variou de 1.949.529 a 3.182.923 sequências menores que 15nt, maiores que 26nt, com baixa complexidade e correspondências com sequências de tRNA e rRNA foram descartadas.

Tabela 2 Sumário dos dados brutos de RNA-seq gerados a partir das diferentes bibliotecas de miRNAs.

Sequenciamento	Amostras					
	C5	C6	R5	R6	H5	H6
Reads geradas	3.386.678	4.388.364	3.173.118	5.362.730	3.007.943	5.529.611
Reads descartadas	19.467	10.404	24.936	9.878	8.295	10.997
Reads mapeáveis	2.084.755	2.568.552	2.036.241	3.182.923	1.949.529	3.453.399

As bibliotecas foram separadas de acordo com a linhagem e o período.
* C5 indica a Chitralada no 5º mês, C6 a Chitralada no 6º mês, R5 a Red Stirling no 5º mês, e R6 a Red Stirling no 6º mês, H5 o híbrido no 5º mês e H6 o híbrido no 6º mês.

Dentre os 264 miRNAs identificados houve expressão diferencial entre os grupos controle e o híbrido. Entre o híbrido a linhagem REDS, os miRNAs: let-7e-3p, mir-16-3p, mir-24-5p, mir-122-5p, mir-124-3p, mir-135a-5p, mir-153-3p, mir-192-5p, mir-194-5p, mir-

216b-3p, mir-219-5p, mir-301-5p e mir-458-5p (**Tabela 3**) se mostraram menos expressos no híbrido quando comparada à REDS. Os miRNAs das famílias mir-124-3p, mir-196-3p, mir-301-3p e mir-7641-3p se mostram menos expressas no híbrido do que na CHIT (**Tabela 4**).

Tabela 3. Número de "reads" dos miRNAs que foram menos expressos no híbrido comparado com a Red Stirling.

miRNA	Amostras					
	H5	**H6**	**R5**	**R6**	**R5 x H5**	**R6 x H6**
let-7e-3p	1,50	0,50	2,50	2,00	0,67	2,11*
mir-16-3p	1,03	0,58	0,98	4,08	-0,25	1,25
mir-24-5p	1,03	2,32	5,40	6,44	2,39*	1,47
mir-122-5p	40,01	2356,23	609,95	2317,05	3,93*	-0,02
mir-124-3p	0,51	1,88	0,57	13,67	0,15	2,86*
mir-135a-5p	0,77	0,43	0,25	3,30	-1,64	2,92*
mir-153-3p	0,51	4,15	2,29	8,48	2,15*	1,03
mir-192-5p	17,44	1008,28	215,10	1171,56	3,62*	0,21
mir-194-5p	1,54	23,74	7,86	44,61	2,35*	0,90
mir-216b-3p	0,00	0,58	0,98	3,14	0,00	2,43*
mir-219-5p	0,00	0,14	0,00	0,63	0,00	2,11*
mir-301-5p	0,51	6,37	3,44	5,03	2,74*	0,34
mir-458-5p	0,51	6,66	3,93	5,66	2,93*	0,23

H5 indica os "reads" do híbrido no 5º mês, H6 do híbrido no 6º mês, R5 da Red Stirling no 5º mês e R6 da Red Stirling no 6º mês. R5 vs H5 compara os dados do híbrido com a Red Stirling no 5º mês e R6 vs H6 compara o híbrido com a Red Stirling no 6º mês. O valor mínimo quando comparada a diferença de expressão entre miRNA é de dois, devendo estar duas vezes mais expresso para ser considerado estatisticamente diferente *(P<0,05).

Tabela4. Número de "reads" dos miRNAs que foram mais expressos no híbrido comparado com a Chitralada.

miRNA	Amostra					
	H5	**H6**	**C5**	**C6**	**C5 x H5**	**C6 x H6**
mir-124-3p	0,51	1,88	0,32	0,26	-0,68	-2,85*
mir-219-5p	9,23	24,03	1,44	24,92	-2,68*	0,05
mir-301-3p	1,03	0,87	0,24	0,32	-2,09*	-1,42
mir-458-5p	0,51	0,14	0,12	0,19	-2,09*	0,42

H5 indica os "reads" do híbrido no 5º mês, H6 do híbrido no 6º mês, C5 da Chitralada no 5º mês e C6 da Chitralada no 6º mês. A penúltima coluna compara os dados do híbrido com a Chitralada no 5º mês e a última coluna compara o híbrido com a Chitralada no 6º mês. O valor mínimo quando comparada a diferença de expressão entre miRNA é e dois, devendo estar duas vezes mais expresso para ser considerado estatisticamente diferente *(P<0,05).

5.3. Proteínas identificadas por ESI-q-TOF e shotgun

Após a extração quantificação e das proteínas, foi feito um pool com cinco amostras ou cada grupo (CHIT, REDS e híbrido). Para a análise de *shotgun*, foram utilizadas um pool por

cada grupo e para a análise de ESI-q-TOF foram feitos géis em triplicata utilizando para cada pool.

Pela análise de *shotgun*, após a exclusão de proteínas não caraterizadas, foram encontradas 64 proteínas diferentemente expressas e/ou únicas de um grupo comparando CHIT com REDS; 64 comparando CHIT com híbrido; e 40 REDS com o híbrido. Após o enriquecimento funcional, foram verificadas a atuação de proteínas exclusivas da CHIT – comparada com a REDS – em atividades oxiduredutases e processos metabólicos de nucleosídeo monofosfato e nucleosídeos trifosfato (**Tabela 5**). Comparando REDS com CHIT, foram encontradas duas proteínas exclusivas da REDS que atuam participando de processos metabólicos de nucleosídeo monofosfato, e comparado ao híbrido, possui várias proteínas mais expressas que atuam em reações catalíticas. Comparando a CHIT com o híbrido, foram encontradas proteínas exclusivas da CHIT ou mais expressas atuando em atividade catalítica.

Tabela 5. Análise comparativa de expressão relativa das proteínas identificadas por *shotgu.*

Grupos comparados	Função	Acesso	Proteína	Score	Expressão
CHIT x híbrido	Atividade catalítica	I3IY11	Fator de alongamento 1-alfa	99,17	CHIT
	Atividade catalítica	I3IYA4	Malato desidrogenase	373	CHIT
	Atividade catalítica	I3IZU4	L-lactato desidrogenase	155,38	1,36
	Atividade catalítica	I3J2Y9	Isocitrato desidrogenase	167,16	CHIT
	Atividade catalítica	I3J820	Fosfoglicerato mutase	7709,17	1,05
	Atividade catalítica	I3JB48	Frutose-bifosfato aldolase	925,28	1,35
	Atividade catalítica	I3JB82	Frutose-bifosfato aldolase	906,55	1,35
	Atividade catalítica	I3JBJ3	Tubulina cadeia beta	77,14	CHIT
	Atividade catalítica	I3JFX6	Fator de alongamento 1-alfa	102,68	CHIT
	Atividade catalítica	I3JIG9	Glicerol-3-fosfato desidrogenase	420,05	CHIT
	Atividade catalítica	I3JIV9	Triosefosfato isomerase	6052,29	1,04
	Atividade catalítica	I3JIY6	Gliceraldeído-3-fosfato desidrogenase	10041,98	1,26
	Atividade catalítica	I3JKV4	ATP sintase subunidade alfa	321,13	CHIT
	Atividade catalítica	I3JPS3	L-lactato desidrogenase	135,98	1,31
	Atividade catalítica	I3JQZ4	Fator de alongamento 1-alfa	99,17	CHIT
	Atividade catalítica	I3JTH2	Tubulina cadeia beta	77,14	CHIT
	Atividade catalítica	I3JTS7	Histona-lisina N-metiltransferase	76,21	CHIT

	Atividade catalítica	I3K1F7	ATP-dependente 6-fosfofrutoquinase	76,04	CHIT
	Atividade catalítica	I3K556	Gliceraldeído-3-fosfato desidrogenase	29411,03	1,23
	Atividade catalítica	I3K5M6	Tubulina cadeia beta	77,14	CHIT
	Atividade catalítica	I3K606	Frutose-bifosfato aldolase	934,10	1,36
	Atividade catalítica	I3KB11	L-lactato desidrogenase	2324,30	1,12
	Atividade catalítica	I3KDA6	Isocitrato desidrogenase	218,920	CHIT
	Atividade catalítica	I3KDJ7	ATP sintase subunidade alfa	330,24	CHIT
	Atividade catalítica	I3KME8	Fosfoglicerato mutase	771,93	1,90
	Atividade catalítica	I3KMP5	Fator de alongamento 1-alfa	82,80	CHIT
	Atividade catalítica	I3KTF2	Glicogênio sintase	219,78	CHIT
	Atividade catalítica	I3KTW7	Tubulina cadeia beta	168,40	CHIT
	Atividade catalítica	I3KW15	Frutose-bifosfato aldolase	20950,48	1,16
CHIT x REDS	Atividade oxidoredutase (atuando nos grupos doadores de CH-OH)	I3IYA4	Malato desidrogenase	373,00	CHIT
	Atividade oxidoredutase (atuando nos grupos doadores de CH-OH)	I3J2Y9	Isocitrato desidrogenase	167,16	CHIT
	Atividade oxidoredutase (atuando nos grupos doadores de CH-OH)	I3JIG9	Glicerol-3-fosfato desidrogenase	420,05	CHIT
	Atividade oxidoredutase (atuando nos grupos doadores de CH-OH)	I3KDA6	Isocitrato desidrogenase	218,92	CHIT
	Atividade oxidoredutase (atuando nos grupos doadores de CH-OH)	I3KY60	Malato desidrogenase	565,26	CHIT
	Atividade oxidoredutase (atuando nos grupos doadores de CH-OH, NAD ou NADP como receptores)	I3IYA4	Malato desidrogenase	373,00	CHIT
	Atividade oxidoredutase (atuando nos grupos doadores de CH-OH, NAD ou NADP como receptores)	I3J2Y9	Isocitrato desidrogenase	167,16	CHIT
	Atividade oxidoredutase (atuando nos grupos doadores de CH-OH, NAD ou NADP como receptores)	I3JIG9	Glicerol-3-fosfato desidrogenase	420,05	CHIT
	Atividade oxidoredutase (atuando nos grupos doadores de CH-OH, NAD ou NADP	I3KDA6	Isocitrato desidrogenase	218,92	CHIT

como receptores)				
Atividade oxidoredutase (atuando nos grupos doadores de CH-OH, NAD ou NADP como receptores)	I3KY60	Malato desidrogenase	565,26	CHIT
Transporte transmembranar acoplado à hidrólise ATP	I3J7E7	Cálcio transporte ATPase	1719,79	0,28
Transporte transmembranar acoplado à hidrólise ATP	I3JQC4	ATP sintase subunidade beta	350,84	0,35
Transporte transmembranar acoplado à hidrólise ATP	I3K022	Cálcio transporte ATPase	7609,72	0,31
Transporte transmembranar acoplado à hidrólise ATP	I3K1D1	ATP sintase subunidade beta	371,56	0,33
Transporte transmembranar acoplado à hidrólise ATP	I3KG39	Cálcio transporte ATPase	1896,69	0,29
Transporte transmembranar acoplado à hidrólise ATP	I3KLR1	Cálcio transporte ATPase	1356,76	0,27
Transporte transmembranar acoplado à hidrólise ATP	I3KNX2	Cálcio transporte ATPase	1427,54	0,29
Transporte transmembranar acoplado à hidrólise ATP	I3KTJ3	Cálcio transporte ATPase	1917,07	0,28
Processo metabólico	I3IVP0	Alfa-1 4 glucan fosforilase	748,96	0,33
Processo metabólico	I3IZU4	L-lactato desidrogenase	155,38	0,77
Processo metabólico	I3J3H6	Piruvato quinase	753,33	0,43
Processo metabólico	I3J409	Glucose-6-phosphate isomerase	1398,68	0,26
Processo metabólico	I3J820	Fosfoglicerato mutase	7709,17	0,45
Processo metabólico	I3JB48	Frutose-bifosfato aldolase	925,28	0,50
Processo metabólico	I3JB82	Frutose-bifosfato aldolase	906,55	0,48
Processo metabólico	I3JBN0	Alfa-1 4 glucano fosforilase	7411,72	0,29
Processo metabólico	I3JIY6	Gliceraldeído-3-fosfato desidrogenase	10041,98	0,66
Processo metabólico	I3JJ47	Alfa-1 4 glucano fosforilase	909,24	0,31
Processo metabólico	G3LHF4	Gliceraldeído-3-fosfato desidrogenase	476,04	0,50
Processo metabólico	I3JNZ0	Piruvato quinase	8041,23	0,36
Processo metabólico	I3JPS3	L-lactato desidrogenase	135,98	0,73
Processo metabólico	I3JQC4	ATP sintase subunidade beta	350,84	0,35
Processo metabólico	I3JS50	AMP desaminase	353,70	0,47

	Processo metabólico	I3K1D1	ATP sintase subunidade beta	371,56	0,33
	Processo metabólico	I3K556	Gliceraldeído-3-fosfato desidrogenase	29411,03	0,62
	Processo metabólico	I3K5C3	Triosefosfato isomerase	32638,06	0,49
	Processo metabólico	I3K606	Frutose-bifosfato aldolase	934,10	0,49
	Processo metabólico	I3KB11	L-lactato desidrogenase	2324,30	0,39
	Processo metabólico	I3KCM4	Adenilato quinase isoenzima 1	4607,64	0,61
	Processo metabólico	I3KF87	Alfa-1 4 glucan fosforilase	1256,05	0,31
	Processo metabólico	I3KL67	Fosfoglicerata quinase	5468,08	0,45
	Processo metabólico	I3KSF9	ATP-dependente 6-fosfofrutoquinase	588,18	0,48
	Processo metabólico	I3KW15	Frutose-bifosfato aldolase	20950,48	0,58
	Nucleosídeo monofosfato processo metabólico	I3JKV4	ATP sintase subunidade alfa	321,13	CHIT
	Nucleosídeo monofosfato processo metabólico	I3K1F7	ATP-dependente 6-fosfofrutoquinase	76,04	CHIT
	Nucleosídeo monofosfato processo metabólico	I3KDJ7	ATP sintase subunidade alfa	330,24	CHIT
	Nucleosídeo monofosfato processo metabólico	I3KPD3	Glucose-6-phosphate isomerase	280,43	CHIT
	Nucleosídeo trifosfato processo metabólico	I3JKV4	ATP sintase subunidade alfa	321,13	CHIT
	Nucleosídeo trifosfato processo metabólico	I3K1F7	ATP-dependente 6-fosfofrutoquinase	76,04	CHIT
	Nucleosídeo trifosfato processo metabólico	I3KDJ7	ATP sintase subunidade alfa	330,24	CHIT
	Nucleosídeo trifosfato processo metabólico	I3KPD3	Glucose-6-phosphate isomerase	280,43	CHIT
	Nucleosídeo monofosfato processo metabólico	I3JFG4	AMP desaminase	44,33	REDS
	Nucleosídeo monofosfato processo metabólico	I3KEH5	AMP desaminase	44,33	REDS
REDS x híbrido	Atividade catalítica	I3IVP0	Alfa-1 4 glucan fosforilase	334,79	1,63
	Atividade catalítica	I3IZU4	L-lactato desidrogenase	268,60	1,86
	Atividade catalítica	I3J3H6	Piruvato quinase	198,33	2,05
	Atividade catalítica	I3J409	Glucose-6-phosphate isomerase	277,69	2,51
	Atividade catalítica	I3J7E7	Cálcio transporte ATPase	355,60	1,46
	Atividade catalítica	I3J820	Fosfoglicerato mutase	4464,83	2,44
	Atividade catalítica	I3JB48	Frutose-bifosfato aldolase	1016,65	2,89
	Atividade catalítica	I3JB82	Frutose-bifosfato aldolase	1016,65	2,83
	Atividade catalítica	I3JBN0	Alfa-1 4 glucan fosforilase	2624,60	1,54
	Atividade catalítica	I3JIV9	Triosefosfato isomerase	3046,24	1,75
	Atividade catalítica	I3JIY6	Gliceraldeído-3-fosfato desidrogenase	9367,36	1,99

Atividade catalítica	I3JJ47	Alfa-1 4 glucan fosforilase	242,88	1,77
Atividade catalítica	G3LHF4	Gliceraldeído-3-fosfato desidrogenase	721,53	1,88
Atividade catalítica	I3JNZ0	Piruvato quinase	3188,85	2,36
Atividade catalítica	I3JPS3	L-lactato desidrogenase	275,44	1,79
Atividade catalítica	I3JS50	AMP desaminase	158,51	1,58
Atividade catalítica	I3K022	Cálcio transporte ATPase	1766,68	1,86
Atividade catalítica	I3K556	Gliceraldeído-3-fosfato desidrogenase	20028,87	1,99
Atividade catalítica	I3K5C3	Triosefosfato isomerase	20126,63	1,73
Atividade catalítica	I3K606	Frutose-bifosfato aldolase	1016,65	2,83
Atividade catalítica	I3KB11	L-lactato desidrogenase	794,85	2,05
Atividade catalítica	I3KCM4	Adenilato quinase isoenzima 1	4756,79	1,75
Atividade catalítica	I3KF87	Alfa-1 4 glucan fosforilase	457,43	1,92
Atividade catalítica	I3KG39	Cálcio transporte ATPase	450,58	1,22
Atividade catalítica	I3KL67	Fosfoglicerata quinase	3819,14	1,84
Atividade catalítica	I3KLR1	Cálcio transporte ATPase	175,07	1,31
Atividade catalítica	I3KME8	Fosfoglicerato mutase	190,69	2,12
Atividade catalítica	I3KNX2	Cálcio transporte ATPase	238,29	1,45
Atividade catalítica	I3KPS8	Fosfoglicerato mutase	213,21	2,14
Atividade catalítica	I3KSF9	ATP-dependente 6-fosfofrutoquinase	156,05	1,77
Atividade catalítica	I3KTJ3	Cálcio transporte ATPase	423,46	1,57
Atividade catalítica	I3KW15	Frutose-bifosfato aldolase	20193,59	2,03

CHIT – indica que houve apenas expressão na Chitralada, e não houve expressão no grupo comparado; REDS – indica que houve apenas expressão na Red Stirling, e não houve expressão no grupo comparado;

Na técnica de ESI-q-TOF, após a eletroforese em 2D, os géis (**Figura 2**) foram escaneados e o software Platinum encontrou 401 spots diferentemente expressos ou exclusivos entre os três grupos. Os spots foram excisados dos géis, digeridos e identificados pela técnica ESI-q-TOF. Pela ferramenta Mascot, foram identificadas 115 proteínas com escore superior a 36 (**Tabela 6**). Sendo que várias destas proteínas, validam as proteínas encontradas pela técnica de *shotgun*.

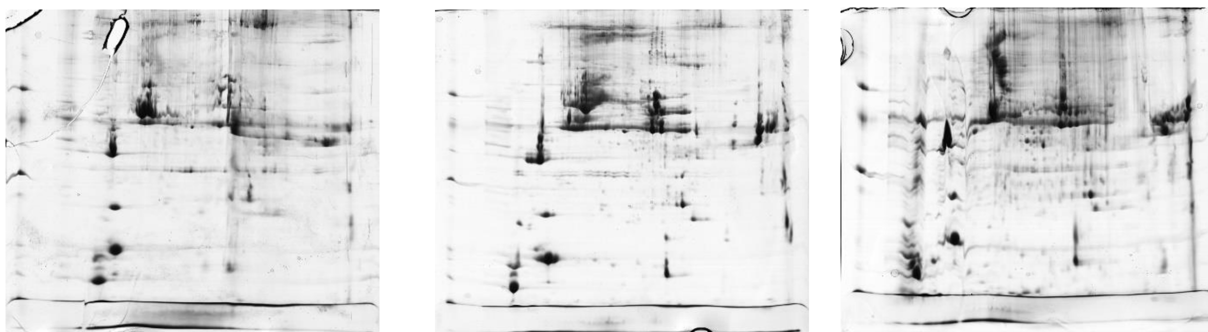

Figura 2 Exemplares dos géis 2D. Da esquerda para a direita: um gel de CHIT, REDS e um gel do híbrido.

Tabela6. Análise de ESI-q-TOF: comparação de proteínas encontradas nos três grupos estudados (CHIT, REDS e híbrido).

Grupo	Comparado à	Nº do spot	Nº acesso (NCBI)	Proteína	Score	Sequências
CHIT	Híbrido	10	ABN58890.1	Alfa actina	285	14 (10)
CHIT	Híbrido	22	XP_003450633.1	Triosefosfatase isomerase B	552	11 (8)
CHIT	Híbrido	27	XP_003444406.1	Fosfoglicerato mutase 2	132	11 (6)
CHIT	Híbrido	46	XP_003449849.1	Creatina quinase tipo M	72	5 (4)
CHIT	Híbrido	47	XP_003449849.1	Creatina quinase tipo M	88	6 (4)
CHIT	Híbrido	48	XP_003438531.1	Frutose-bifosfato aldolase A	309	9 (6)
CHIT	Híbrido	49	XP_003449849.1	Creatina quinase tipo M	595	15 (11)
CHIT	Híbrido	54	XP_003441591.1	Filamento intermediário da proteína ON3	66	1 (1)
CHIT	Híbrido	55	XP_003447595.1	Alfa actinina 3	50	2 (2)
CHIT	Híbrido	56	ABN58888.1	Alfa actina	33	4 (2)
CHIT	Híbrido	57	ABN58890.1	Alfa actina	159	6 (5)
CHIT	Híbrido	58	XP_019213388.1	Miosina cadeia pesada	36	7(0)
CHIT	Híbrido	60	XP_019213374.1	Miosina cadeia pesada	324	39 (20)
Híbrido	CHIT	74	XP_003442598.1	Nucleosídeo difosfato quinase B	156	8(0)
Híbrido	CHIT e REDS	75	XP_003449849.1	Creatina quinase tipo M	41	3(0)
Híbrido	CHIT e REDS	78	XP_003442598.1	Nucleosídeo difosfato quinase B	62	5(0)
Híbrido	CHIT	79	XP_003455014.1	Adenilato quinase isoenzima 1 isoforma X2	220	12 (7)
Híbrido	CHIT e REDS	80	XP_003455014.1	Adenilato quinase isoenzima 1isoforma X2	136	7 (3)
Híbrido	CHIT e REDS	84	XP_003450633.1	Triosefosfato isomerase B	299	10(2)
Híbrido	CHIT	85	XP_003450633.1	Triosefosfato isomerase B	260	3 (3)
Híbrido	CHIT e REDS	89	XP_003450633.1	Triosefosfato isomerase B	497	13 (10)
Híbrido	CHIT e REDS	90	XP_003449849.1	Creatina quinase tipo M	76	8 (5)
Híbrido	CHIT e REDS	91	XP_003455014.1	Adenilato quinase isoenzima 1 isoforma X2	220	9 (5)
Híbrido	CHIT	92	XP_003452738.1	Gliceraldeído-3-fosfato desidrogenase	111	5 (5)
Híbrido	CHIT e REDS	93	XP_003450633.1	Triosefosfato isomerase B	175	3 (3)
Híbrido	CHIT e REDS	94	XP_003445346.1	Miosina cadeia leve 1	39	3 (2)

Híbrido	CHIT e REDS	95	XP_003452738.1	Gliceraldeído-3-fosfato desidrogenase	82	4 (2)
Híbrido	CHIT e REDS	96	XP_003456429.1	Creatina quinase tipo M	192	6 (4)
Híbrido	CHIT e REDS	98	ABN58888.1	Alfa actina	169	6 (4)
Híbrido	CHIT e REDS	99	ABN58898.1	Beta actina	107	4 (2)
Híbrido	CHIT	100	XP_003442453.1	Miosina regulatória cadeia leve 2	380	5 (5)
Híbrido	CHIT e REDS	101	XP_003442453.1	Miosina regulatória cadeia leve 2	317	6 (5)
Híbrido	CHIT e REDS	102	XP_003442453.1	Miosina regulatória cadeia leve 2	262	5(2)
Híbrido	CHIT e REDS	103	XP_003442453.1	Miosina regulatória cadeia leve 2	280	6 (5)
Híbrido	CHIT e REDS	105	XP_003450633.1	Triosefosfato isomerase B	118	3(2)
Híbrido	CHIT e REDS	106	ABN58888.1	Alfa actina	46	2(0)
Híbrido	CHIT	108	XP_003458789.1	Miosina regulatória cadeia leve 2	132	5(2)
Híbrido	CHIT	111	XP_003445346.1	Miosina cadeia leve 1	1009	12 (10)
Híbrido	CHIT e REDS	112	XP_003458204.1	Cofilina-2	256	5(2)
Híbrido	CHIT	113	XP_003453231.1	Miosina cadeia leve 3	448	10 (10)
Híbrido	CHIT e REDS	114	XP_003439438.2	Troponina I	493	8 (3)
Híbrido	CHIT e REDS	116	XP_003449849.1	Creatina quinase tipo M	92	6 (3)
Híbrido	CHIT e REDS	117	XP_003449849.1	Creatina quinase tipo M	390	8 (7)
Híbrido	CHIT e REDS	119	XP_003442598.1	Nucleosídeo difosfato quinase B	39	4(0)
Híbrido	CHIT e REDS	121	XP_003447786.1	Actina	259	11 (8)
Híbrido	CHIT e REDS	127	XP_003447595.1	Alfa actinina 3	48	4 (1)
Híbrido	CHIT e REDS	129	XP_003440474.1	Tropomiosina cadeia alfa-1 isoforma X1	324	14 (6)
Híbrido	CHIT e REDS	132	ABN58894.1	Alfa actina	216	7 (6)
Híbrido	CHIT e REDS	133	XP_003440474.1	Tropomiosina cadeia alfa-1 isoforma X1	324	9 (7)
Híbrido	CHIT	134	XP_003453231.1	Miosina cadeia leve 3	448	10 (10)
Híbrido	CHIT e REDS	147	XP_019213372.1	Miosina cadeia pesada	303	13(4)
Híbrido	CHIT e REDS	151	XP_003437612.1	Tropomiosina cadeia alfa-1 isoforma X5	99	5 (4)
Híbrido	CHIT e REDS	153	XP_003456429.1	Creatina quinase tipo M	120	9(2)
Híbrido	CHIT	176	XP_003456429.1	Creatina quinase tipo M	232	12 (10)

Híbrido	CHIT	186	XP_003451499.1	Fosfoglicerato quinase 1	80	4 (2)
Híbrido	CHIT	187	ABN58890.1	Alfa actina	120	6 (4)
Híbrido	CHIT	188	XP_003456429.1	Creatina quinase tipo M	269	8 (6)
Híbrido	CHIT e REDS	211	XP_003438531.1	Frutose-bifosfato aldolase A	368	15 (7)
Híbrido	CHIT e REDS	216	XP_003451163.1	Beta enolase	301	9 (6)
Híbrido	CHIT e REDS	217	XP_003438531.1	Frutose-bifosfato aldolase A	283	12 (10)
Híbrido	CHIT e REDS	218	XP_003438531.1	Frutose-bifosfato aldolase A	482	9 (6)
Híbrido	CHIT e REDS	221	XP_003438531.2	Frutose-bifosfato aldolase A	319	7 (4)
Híbrido	CHIT	222	XP_003440474.1	Tropomiosina cadeia alfa-1 isoforma X1	88	6 (3)
Híbrido	CHIT e REDS	223	XP_003440474.1	Tropomiosina cadeia alfa-1 isoforma X1	202	7(3)
Híbrido	CHIT e REDS	224	XP_003438531.1	Frutose-bifosfato aldolase A	371	10(3)
Híbrido	CHIT e REDS	225	XP_005455495.1	Gliceraldeído-3-fosfato desidrogenase	83	12 (7)
Híbrido	CHIT e REDS	226	XP_003438531.1	Frutose-bifosfato aldolase A	121	5 (3)
Híbrido	CHIT	227	XP_003453016.1	Creatina quinase tipo S, mitocondrial	127	7 (3)
Híbrido	CHIT e REDS	234	XP_019213375.1	Miosina cadeia pesada	129	39 (13)
Híbrido	CHIT e REDS	235	ABN58890.1	Alfa actina	156	7 (5)
Híbrido	CHIT	237	XP_019213375.1	Miosina cadeia pesada	453	28 (18)
Híbrido	CHIT e REDS	241	XP_003447786.1	Actina	313	13 (11)
Híbrido	CHIT e REDS	242	XP_003447786.1	Actina	103	14 (6)
Híbrido	CHIT e REDS	243	XP_003442598.1	Nucleosídeo difosfato quinase B	61	6 (5)
Híbrido	CHIT e REDS	244	XP_019213387.1	Miosina cadeia pesada	39	5 (2)
Híbrido	CHIT e REDS	248	XP_019213375.1	Miosina cadeia pesada	249	30 (13)
Híbrido	CHIT e REDS	252	XP_019213375.1	Miosina cadeia pesada	462	45 (28)
Híbrido	CHIT e REDS	253	XP_019213375.1	Miosina cadeia pesada	318	35 (15)
Híbrido	CHIT e REDS	256	XP_003447786.1	Actina	305	13 (7)
Híbrido	REDS	263	XP_003449849.1	Creatina quinase tipo M	78	5(0)
Híbrido	CHIT e REDS	265	XP_003456429.1	Creatina quinase tipo M	36	3(0)
Híbrido	CHIT e REDS	274	XP_003438531.1	Frutose-bifosfato aldolase A	41	3(1)

Híbrido	REDS	276	XP_003449849.1	Creatina quinase tipo M	58	7(0)
Híbrido	CHIT e REDS	277	XP_003449849.1	Creatina quinase tipo M	103	9 (4)
Híbrido	CHIT e REDS	293	XP_003440474.1	Tropomiosina cadeia alfa-1 isoforma X1	123	5(3)
Híbrido	CHIT e REDS	294	XP_003440474.1	Tropomiosina cadeia alfa-1 isoforma X1	419	12 (8)
Híbrido	CHIT e REDS	297	XP_003449849.1	Creatina quinase tipo M	74	3(2)
Híbrido	REDS	299	XP_003438531.1	Frutose-bifosfato aldolase A	162	3 (2)
REDS	Híbrido	600	XP_003442453.1	Miosina regulatória cadeia leve 2	651	15 (13)
REDS	Híbrido	606	XP_003450753.1	Aldose reductase	94	10(2)
REDS	Híbrido	609	XP_003453231.1	Miosina cadeia leve 3	487	9 (8)
REDS	Híbrido	611	XP_003457006.1	Troponinaa C	688	13 (11)
REDS	Híbrido	613	XP_003440474.1	Tropomiosina cadeia alfa-1 isoforma X1	222	8 (6)
REDS	Híbrido	618	XP_003440474.1	Tropomiosina cadeia alfa-1 isoforma X1	124	11(2)
REDS	Híbrido	620	XP_003440474.1	Tropomiosina cadeia alfa-1 isoforma X1	333	12(5)
REDS	Híbrido	622	XP_003440474.1	Tropomiosina cadeia alfa-1 isoforma X1	266	11 (7)
REDS	Híbrido	623	XP_003440474.1	Tropomiosina cadeia alfa-1 isoforma X1	202	7(3)
REDS	Híbrido	629	XP_003447786.1	Actina	241	13 (9)
REDS	Híbrido	638	XP_003456429.1	Creatina quinase tipo M	262	15 (10)
REDS	Híbrido	650	ABN58888.1	Alfa actina	36	2(0)
REDS	Híbrido	667	XP_019213372.1	Miosina cadeia pesada	134	14 (8)
REDS	Híbrido	672	XP_003446809.1	Glicogênio fosforilase	73	10(2)
REDS	Híbrido	690	XP_003452738.1	Gliceraldeído-3-fosfato desidrogenase	76	4(1)
REDS	Híbrido	698	XP_003444406.1	Fosfoglicerato mutase 2	215	9(2)
REDS	Híbrido	700	XP_005455495.1	Gliceraldeído-3-fosfato desidrogenase	119	10 (3)

REDS	Híbrido	701	XP_005455495.1	Gliceraldeído-3-fosfato desidrogenase	102	10 (5)
REDS	Híbrido	702	XP_005455495.1	Gliceraldeído-3-fosfato desidrogenase	114	11 (6)
REDS	Híbrido	706	XP_003438531.1	Frutose-bifosfato aldolase A	368	9 (8)
REDS	Híbrido	708	XP_003438531.1	Frutose-bifosfato aldolase A	432	17 (12)
REDS	Híbrido	711	XP_003439438.2	Troponina I	283	3 (2)

5.4. Interação miRNA e proteína

Com a finalidade de comparar os dados da proteoma e do miRnoma, os genes de suas respectivas proteínas, identificadas pela técnica de shotgun, foram acessados nas bases de dados e obtidos seus números de acesso. Com estes números de acesso, foi possível a identificação, por TargetScan, dos miRNAs reguladores destes genes. Foram separados, então, os miRNAs encontrados com expressões diferenciadas no presente trabalho (**Tabela 3 e 4**). Desta forma, foi possível encontrar uma relação dos miRNAs com as proteínas e separar os miRNAs e as proteínas de interesse (**Tabela 7 e 8**).

Tabela 7 Relação miRNAs e as proteínas de seus genes alvos diferentemente expressos no híbrido em relação à CHIT (vermelho indica sub expressão no híbrido; e azul indica super expressão no híbrido).

MiRNA	Proteína	Gene (acesso)	Proteína (acesso)	Função
let-7e-3p	Frutose bifosfato aldolase	ENSONIG00000020092	I3KW15	processo metabólico; atividade catalítica
miR-16-3p	Frutose bifosfato aldolase	ENSONIG00000013159	I3K606	processo metabólico; atividade catalítica
miR-16-3p	L-lactato desidrogenase	ENSONIG00000001705	I3IZU4	processo metabólico; atividade catalítica
miR-216b-3p	L-lactato desidrogenase	ENSONIG00000001705	I3IZU4	processo metabólico; atividade catalítica
miR-153-3p	Frutose bifosfato aldolase	ENSONIG00000004866	I3JB82	processo metabólico; atividade catalítica
miR-153-3p	Tubulina cadeia beta	ENSONIG00000019514	I3KTW7	atividade catalítica
miR-204a-3p	L-lactato desidrogenase	ENSONIG00000014546	I3KB11	processo metabólico; atividade catalítica
miR-205-3p	L-lactato desidrogenase	ENSONIG00000001705	I3IZU4	processo metabólico; atividade catalítica
miR-205-3p	Frutose bifosfato aldolase	ENSONIG00000004866	I3JB82	processo metabólico; atividade catalítica
miR-205-3p	L-lactato desidrogenase	ENSONIG00000014546	I3KB11	processo metabólico; atividade catalítica

Tabela 8 Relação miRNAs e as proteínas de seus genes alvos diferentemente expressos no híbrido em relação à REDS (vermelho indica sub expressão no híbrido; e azul indica super expressão no híbrido).

MiRNA	Proteína	Gene (acesso)	Proteína (acesso)	Função
miR-7641-3p	Piruvato quinase	ENSONIG00000002725	I3J3H6	atividade catalítica; processo metabólico
miR-7641-3p	L-lactato desidrogenase	ENSONIG00000001705	I3IZU4	atividade catalítica; processo metabólico
miR-7641-3p	Gliceraldeído-3-fosfato desidrogenase	ENSONIG00000007262	G3LHF4	atividade catalítica; processo metabólico
miR-7641-3p	Alpha-1_4 glucan phosphorylase	ENSONIG00000015719	I3KF87	atividade catalítica; processo metabólico
miR-7641-3p	Cálcio-transporte ATPase	ENSONIG00000019415	I3KTJ3	ATP hidrólise acoplado transporte transmembranar; atividade catalítica
miR-219-5p	Cálcio transporte ATPase	ENSONIG00000004866	I3KNX2	ATP hidrólise acoplado transporte transmembranar; atividade catalítica
let-7e-3p	Gliceraldeído-3-fosfato desidrogenase	ENSONIG00000007262	G3LHF4	ATP hidrólise acoplado transporte transmembranar; atividade catalítica
let-7e-3p	Frutose bifosfato aldolase	ENSONIG00000020092	I3KW15	atividade catalítica; processo metabólico
miR-16-3p	Frutose bifosfato aldolase	ENSONIG00000013159	I3K606	atividade catalítica; processo metabólico

miR-16-3p	Piruvato quinase	ENSONIG00000002725	I3J3H6	atividade catalítica; processo metabólico
miR-16-3p	L-lactato desidrogenase	ENSONIG00000001705	I3IZU4	atividade catalítica; processo metabólico
miR-216b-3p	Piruvato quinase	ENSONIG00000002725	I3J3H6	atividade catalítica; processo metabólico
miR-216b-3p	L-lactato desidrogenase	ENSONIG00000001705	I3IZU4	atividade catalítica; processo metabólico
miR-216b-3p	Cálcio transporte ATPase	ENSONIG00000018111	I3KNX2	ATP hidrólise acoplado transporte transmembranar; atividade catalítica

6. Discussão

6.1. A relação dos genes do eixo GH/IGF e da MSTN com a heterose

A causa da heterose sempre foi muito discutida. A teoria da dominância e a teoria da sobredominância, sempre foram utilizadas na tentativa de explicar a heterose. Atualmente, é aceito que estas duas teorias se completam, e não se contradizem. Adicionalmente, a interação entre genes normais com genes de ganho de função *(gain-of-function)* pode ter influência (Huyen, 2016) e a identificação de variabilidade genética de SNPs pode ser utilizada para a seleção de animais que terão heterose em gado (Kelleher et al., 2017).

Híbridos de tilápia geneticamente melhorados por meio de seleção combinada de linhagens comerciais vêm sendo obtidos visando taxa de crescimento rápido, como é o caso do híbrido apresentado neste estudo. O aumento da expressão do GH no híbrido em comparação às linhagens tanto no cérebro quanto no músculo branco corrobora com o observado por Huang et al. (2012), confirmando a maior aptidão ao crescimento do híbrido com relação às linhagens. Huang e et al. (2012) realizaram um estudo de expressão gênica comparando uma linhagem de tilápia do Nilo de rápido crescimento. Assim como os dados apresentados aqui no híbrido, a expressão de *gh* foi muito maior na linhagem de rápido crescimento (Huang et al., 2012). Devido à atuação do GHR1 e GHR2 como receptores do GH, a regulação inadequada dessas moléculas pode levar a diferentes quadros de retardo de crescimento. Diante disso, observa-se estreita relação entre os perfis de expressão destes genes que, de maneira geral, se mostraram super expressos no híbrido em comparação às linhagens.

Assim como no caso do *gh, ghr1* e *ghr2*, aumentos similares na expressão no*igf1* no fígado foram observados no híbrido e na linhagem de rápido crescimento (Huang et al., 2012). A linhagem de rápido crescimento, como o híbrido também, mostrou declínio na expressão de *mstn*. Visto que a MSTN é reguladora negativo de crescimento muscular, ou

seja, sua presença inibe o crescimento muscular, a sua sub expressão é muito positiva para a produção animal.

Analisando um transcriptoma de um híbrido heterótico entre duas espécies do gênero *Epinephelus* comparando com os seus parentais, foi encontrado vários genes diferencialmente expressos no híbrido associados ao crescimento, como *gnrh1*, *gnrh3* e *gh* no cérebro e *igf1*, *igf2b, igffbp1, igfb2a, igfbp4, igfbp5a* e *igfbp5b* no fígado (Sun et al., 2016). Entre eles, os níveis de expressão de *igf1* e *igf2b* no fígado do híbrido foram significativamente maiores do que os de seus pais, indicando IGF-1 e IGF-2b pode desempenhar um papel crítico no crescimento deste híbrido (Sun et al., 2016).

Em um híbrido de uma espécie de tartaruga (*Pelodiscus sinensis*) de interesse econômico na aquicultura da China, foram encontrados 220 transcritos expressos por RNA-Seq apenas no híbrido, dentre eles, o *ghrh* (gene do hormônio de liberação do GH - *growth hormone releasing hormone*), que possui papel no eixo GH/IGF (Zhang et al., 2017).

Os dados apresentados no híbrido (CHIT 7/8 e REDS 1/8) corroboram com os dados apresentados em outros estudos envolvendo a expressão dos genes do eixo GH/IGF. O músculo é um tecido altamente organizado, sendo que as implicações resultantes da alteração do eixo GH/IGF está amplamente descrito, e seu papel se mostra fundamental na regulação do crescimento modulando a síntese protéica e a proteólise (Biga e Meyer, 2009). A maior expressão do *gh* no músculo da linhagem CHIT em comparação à linhagem REDS está de acordo com o obtido por Lago (2014) em teste de desempenho zootécnico das mesmas linhagens avaliadas nesse estudo, onde o crescimento foi cerca de 70% maior na CHIT, comprovando a relação do GH com o desenvolvimento muscular na espécie, fato de suma importância uma vez que se refere à porção de maior interesse

econômico. Embora seja evidente a maior expressão destes genes nos híbridos heteróticos, não se sabe se o aumento nessas expressões é devido à heterose ou se é a sua causa.

Estes dados, assim como os dados apresentados neste trabalho, mostram evidências da atuação dos genes do eixo GH/IGF com a heterose. No entanto, a maioria das evidências em outros trabalhos sugere que a causa da heterose seja oriunda de vários fatores genéticos combinados.

6.2. A relação dos miRNAs, seus alvos e a expressão dos genes do eixo GH/IGF e MSTN

Dentre os miRNAs que se mostraram menos expressos no híbrido em relação à REDS (**Tabela 3**), destacam-se o let-7 (Barozai, 2012; De Santis et al., 2008; Lee, 2004) e o miR-122 (Chen et al., 2012). Existem estudos que mostram correlação de duas destas famílias, let-7 e miR-122, com características que podem favorecer a produção animal, principalmente na aquicultura.

De acordo com Barozai (2012), que utilizou o software RNAhybrid na predição de alvos, o let-7a-3p, pode ter alguma relação com o *gh2* no salmão do atlântico. Em barramundi, verificou-se que os miRNAs da família let-7 interagem com a região 3'UTR da *mstn* (De Santis et al., 2008). A MSTN atua de forma fundamental no crescimento do músculo esquelético (Lee, 2004), principal parte comestível dos animais domésticos. Desta forma esta interação pode favorecer o crescimento. Já o miR-122 foi associado com deposição de gordura em suínos (Chen et al., 2012) e é especifico do fígado pertencendo a uma família que atua de forma específica no metabolismo de ácidos graxos e do colesterol (Elmén et al., 2008; Esau et al., 2006), sugerindo um forte envolvimento desses dois miRNAs com o crescimento.

Os miRNAs das famílias mir-124-3p, mir-196-3p, mir-301-3p e mir-7641-3p se mostraram sub expressas no híbrido comparado à CHIT (**Tabela 4**). Até o momento, nenhum miRNA de nenhuma destas famílias foi associado com alguma característica favorável à produção animal.

Na predição de alvos de miRNAs isolados por TargetScan, destacam-se os alvos do miR-194, miR-122, miR-124, miR-216b e miR-219. O miR-194, assim como o miR-122, tem como alvo a *mstn* e apresenta uma expressão maior comparando a REDS com o híbrido. Estudos em camundongos mostraram que a supressão do miR-122 resultado em aumento da oxidação e queda nos índices de síntese de ácidos graxos (Krutzfeldt et al., 2005; Esau et al., 2006; Elmen et al., 2008).

O miR-194, mais expresso no híbrido do que na REDS, também possui como alvo a *mstn*, corroborando com os dados de expressão. Outro miRNA mais expresso no híbrido, tendo como alvo a *mstn*, é o miR-219. O miR-122, miR-194 e o miR-219 podem ser responsáveis pela baixa expressão da *mstn* e consequentemente no maior crescimento do híbrido.

Além do miR-219, outros miRNAs mais expressos mostraram como alvos alguns dos genes com as expressões avaliadas. Dentre eles, o miR-124 tendo como alvo o *gh* e o *ghr1*; e o miR-216b o *igf1*, *igf2* e *ghr1*. O miR-124 se mostrou sub expresso e o miR-216b super expresso em relação à CHIT.

6.3. A relação dos miRNAs e as proteínas

A regulação negativa das transcrições de mRNA por miRNAs específicos tem papel importante em processos biológicos. Apesar do mecanismo simples de ação repressiva compartilhado por todos os miRNAs, os processos biológicos utilizam os miRNAs para realizar uma série de diferentes resultados regulatórios.

No híbrido em comparação aos seus parentais, encontrou-se diversos tipos de interação. Comparando com a CHIT, onde só a sub expressão de miRNAs mostrou alguma interação significativa com as proteínas, pode-se observas as duas situações: sub expressão de miRNAs com sub expressão de proteínas; e sub expressão de miRNAs com super expressão de proteínas. A segunda interação, é uma interação clássica, onde a expressão do miRNA é inversamente proporcional ao seu alvo. A via canônica de ação dos miRNAs ocorre através da interação do complexo miRNA-RISC com sítios ligantes do mRNA alvo inibindo a sua expressão (Lee e Dutta, 2009). Desta forma, a sub expressão dos miRNAs justifica a super expressão de seus alvos, uma vez que a inibição dos alvos é reduzida as proteínas tendem a se expressar mais. É o caso dos seguintes miRNAs e seus alvos: miR-216b com L-lactato desidrogenase; miR-153 com frutose bisfosfato aldolase e tubulina cadeia beta; miR-204a com L-lacatato desidrogenase; miR-205 com L-lacatato desidrogenase e frutose bifosfato aldolase (**Tabela 7**). Já a sub expressão dos miRNAs nos casos em que também foram observados sub expressão nas proteínas, pode ser oriunda de uma resposta biológica a baixa expressão de proteínas e desta forma seus miRNAs reguladores também tiveram sua expressão reduzida.

No caso da comparação com a REDS, o híbrido mostrou dois tipos de padrões de expressão miRNAs e proteínas também: super expressão de miRNA e super expressão de proteínas; e sub expressão de miRNAs e super expressão de proteínas. O primeiro caso, assim como sub expressão de miRNAs e sub expressão comparando com a CHIT, pode ser oriunda de uma resposta biológica, ao contrário do caso da comparação com a CHIT, à super expressão das proteínas e, consequentemente, resultou em um aumento da expressão de seus miRNAs reguladores como uma tentativa de trazer as expressões de volta à normalidade. Já a sub expressão de determinados miRNAs em comparação a REDS resultou no aumento das proteínas que tem os seus genes como alvos dos mesmos

miRNAs, no caso: let-7e e frutose bifosfato aldolase; miR-16 e frutose bifosfato aldolase e L-lactato desidrogenase; e miR-216b e L-lactato desidrogenase (**Tabela 8**).

Dentre estes miRNAs, o let-7e - sub expresso no híbrido com a proteína super expressa em comparação com a REDS – já mostrou relação com ganho crescimento em estudos anteriores (Barozai, 2012; De Santis et al., 2008; Lee, 2004). Zhang et al. (2014), utilizando *Megalobrama amblycephala* como modelo experimental, mostrou que miR-16 é pouco expresso em animais com uma dieta alta em gordura. Assim como no trabalho de Zhang et al. (2014), ele se mostrou sub expresso no híbrido em comparação com REDS, e consequente, L-lactato desidrogenase e frutose bifosfato aldolase, que tem os seus genes como alvo do miR-16, apresentaram super expressão.

7. Conclusões

Os dados de expressão gênica demonstraram que os genes do eixo GH/IGF e a MSTN podem contribuir para o crescimento superior do híbrido heterótico devido ao aumento na síntese de proteínas e metabolismo. De uma maneira geral, a maioria dos genes relacionados ao eixo GH/IGF mostraram uma expressão maior no híbrido e a MSTN uma expressão menor em relação aos seus parentais. Esses genes atuam na heterose, embora não se sabe se são oriundos ou responsáveis por ela.

A análise dos miRNAs mostrou que o let-7 está mais expresso no híbrido em relação aos seu parentais, podendo desempenhar alguma função na heterose. Os dados das predições de alvos de miRNAs corroboram com alguns dos dados de expressão. As expressões aumentadas do miR-122, miR-194 e miR-219 no híbrido estão diretamente relacionados a baixa expressão de *mstn* no mesmo. Desta forma, assim como a *mstn, sl, gh e ghr1*, a expressão destes miRNAs também está relacionado ao ganho de peso e tamanho do híbrido em relação aos seus parentais.

Em relação a comparação dos miRNAs e com as proteínas, vários miRNAs se mostraram sub expressos e consequentemente houve aumento na expressão das proteínas que possuem alvos destes miRNAs em seus genes. No caso, o miR-216, miR-153, miR-204 e miR205 comparando o híbrido com a CHIT; e o let-7e, miR-16 e miR-216 comparando o híbrido com a REDS. Esses dados mostram que existe uma ação dos miRNAs em proteínas associadas às vias metabólicas, relacionadas à heterose nas tilápias do Nilo.

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Anexo I: Artigo

Expression of GH/IGF axis and myostatin genes in a heterotic Nile tilapia (*Oreochromis niloticus*) crossbreed

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Abstract

The Nile tilapia (*Oreochromis niloticus*) is an economically important farmed fish in different countries and particularly in Brazil. Crossbreeding between two strains of Nile tilapia has been carried out Red color Stirling and wild type Chitralada strains, combining the superior performance of Chitralada strain with the red dominant coloration of Red Stirling strain. Crossbreeding is a breeding strategy to produce crossbred fish showing heterosis, and therefore expected better performance. The molecular mechanisms affecting gene expression and the biological pathways behind heterosis phenotypic effects are still unknown. Genetic studies have shown direct and indirect interference of growth hormone - GH, insuline-like growth factor-IGF, somatolactin (SL) and myostatin – MSTN genes in farmed animals. Studies showed that the expressions of these gene related proteins are different among strains. Thus, the aim of this study was to assess the expression of these

genes comparing the crossbreed (7/8 Chitralada and 1/8 Red Stirling) with Chitralada and Red Stirling strains as control groups. Expression assessments were carried out on brain, liver and white muscle tissues. Extracted RNA was subjected to RT-qPCR using REST 2009 software. The crossbreed fish showed best growth performance. Gene expression of these proteins showed mostly up-regulated, with exception of MSTN which was down-regulated almost all the time when comparing the crossbreed with Red Stirling and Chitralada. These results suggest that growth hormone and somatolactin genes may be involved in heterosis phenotype and can be target of breeding programs.

Key words: growth; GH/IGF axis; myostatin (MSTN); Nile tilapia; RT-qPCR.

1.Introduction

Nile tilapia (*Oreochromis niloticus)* is the fifth the most commonly cultivated aquaculture species with world production of 3,670,260 MT in 2014 (FAO, 2016). In Brazil, Nile tilapia remains the most cultivated aquaculture species with an annual production of 219,329 t in 2015, corresponding to 45.4% of the total aquaculture production (IBGE, 2016).

Genetically improved nile tilapia strains have been developed by breeding programs, such as Genetic Improvement of Farmed Tilapia-GIFT (Bentsen et al., 1998; Eknath and Acosta, 1998), and other GIFT derived strains, Akosombo strain (Ansah et al., 2014), Chitralada (Chinnabut et al., 2007) and red phenotype strains (Castillo-Campos, 2001; Ramírez-Paredes et al., 2012; Thodesen et al., 2013.). Red Stirling strain originated from the University of Stirling - Scotland (McAndrew and Majumdar, 1983; Hussain, 1994). Crossbreeding program using Red Stirling and Chitralada strains has been carried

out to yield red phenotypes with superior farming performance, which also performed a heterosis (Lago, et al., 2016).

Heterosis genetics mechanisms have been debated for a long time. Dominance and the over dominance were allways used to explain heterosis phenomen. Nowadays, there is accepted that these two theories do not contradict but complement each other to explain. Additionally, regular genes together with gain-of-function genes contribute to heterosis (Huyen, 2016). However, understanding the molecular mechanism underlying crossbreeding heterosis are still unknown so far.

In teleosts, GH/IGF axis hormones play a key role in the regulation of somatic growth (Duan, 1997, 1998). GH is a pituitary hormone that regulates several physiological processes, such as somatic growth, lipid and protein metabolism, osmotic regulation and feeding behavior in fish (Albalat et al., 2005; Kawauchi and Sower, 2006). In addition, it is widely known that GH stimulates the release of circulating IGFs through the liver (Vong et al., 2003; Pierce et al., 2004). Among the IGFs, IGF-1 is directly related to growth rates and serum levels, and is used as indicators of growth in fish (Adams et al., 1991; Reinecke, 2010). This has been observed in Nile tilapia (Oreochromis niloticus) (Vera Cruz et al., 2006), Mozambique tilapia (Oreochromis mossambicus) (Kajimura et al., 2001), and in carp (Cirrhinus molitorella) (Zhang et al., 2006). There another hormone known as somatolactin (SL), which paly role in alternative GH/IGF axis and its function still unknown.

Considering the importance of GH/IGF axis hormones in the growth rate particularly in farmed fishes, the present study was designed to evaluate and compare the expression of GH levels, SL, GHR1, GHR2, IGF1, IGF2 and MSTN genes levels in two strains of *O. niloticus* and their crossbred in an intensive cage production system.

2. Materials and methods

2.1. Experimental animals

The two strains used in the present study were Thai Chitralada strain (CHIT) from the Asian Institute of Technology and the Red-Stirling strain (REDS) from the University of Stirling. Both broodstock strains has been kept by Brazilian fish farming company (Indústria Brasileira do Peixe Ltd.) as part of a breeding program to produce better lines of red strains. Contemporary fingerling of REDS and CHIT as well as their crossbred (7/8 CHIT: 1/8 REDS) were produced in reproduction hapas where they were kept to the minimum size to be marked with electronic microchips (Passive Integrated Transponder (PIT) tags). Tagged fingerlings were transferred to net cages according to the study carried out by Lago, et al., (2016). Twenty-seven fish were randomly collected during the six month grow-out cycle, three from each genetic group in the fourth, fifth and sixth months. During each sampling, morphological parameters were taken and the animals were sacrificed using tricaine methanesulfonate (MS-222, Sigma Chemical, St. Louis, MO). After euthanasia has been performed, samples of liver, brain and white muscle were collected and stored at -80ºC. The experiment was approved by the local animal ethics committed with the protocol number (CEUA, protocol 792/2015).

2.2. RNA extraction

Total RNA was extracted from the tissues using Trizol (Life Technologies, EUA) following the manufacture recommendations. The tissue was mixed, titrated and homogenized in Trizol solution. Then, 400 µL of chloroform was added to the sample, and the mixture was manually homogenized for 15 seconds and kept in ice for 3 min. The

samples were then centrifuged for 15 min at 12,000 rpm and 4 °C. The aqueous phase was collected and transferred to a clean tube containing 500 μL of isopropanol per tube, then again homogenized and centrifuged for 10 min at 12,000 rpm and 4 °C. The supernatant was discarded, and the precipitate was washed in 1000μl of 75% ethanol. The material was once again centrifuged at 12,000 rpm for 5 min and 4 ºC, and the supernatant was discarded. The precipitate was washed in 1000μl of 100% ethanol and centrifuged at 12,000 rpm for 5 min and 4 ºC. After discard the supernatant, the pellet was dried at room temperature (25ºC) until complete dry and then resuspended in ultrapure RNase-free water.

The RNA concentration was measured using Nanodrop (Thermo Fisher Scientific, USA) at a wavelength of 260 nm and RNA integrity was analyzed using 1% agarose gel stained with GelRed and visualized under ultraviolet light. The RNA samples were treated with DNase I (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions in order to remove possible genomic DNA contamination.

2.3. cDNA synthesis and qPCR

High Capacity RNA-to-cDNA Master Mix (Life Technologies, EUA) were used in the cDNA reaction according with the manufacture. For this reaction we used 10 μL of total RNA, 0.8 μL of oligo dNTPs (100 mM), 2 μL random primers (10x), 2 μL buffer, 1 μL RT(U/μL), 0.5 μL RNAse inhibitor and 3.7 μL nuclease free water.The solution was incubated for 10 min at 50 °C, 120 min at 37 ºC and 5 min at 85ºC for the synthesis of complementary DNA. Next, the samples were stored at −20 °C until further use.

The qPCR of the cDNA was performed using SYBR® Green assays (Life Technologies, USA), containing primers for the target messenger RNAs study (Table 1). Some housekeeping genes were tested and the best expression was selected for data

normalization. The qPCR reactions were performed in the ABI real-time system StepOne PlusTM (Life Technologies, USA).

Table 1.Sequences of the primers used for expression analysis of genes related to growth. GH (growth hormone), SL (SomatolactinaGHR (growth hormone receptor), IGF (insulin-like growth factor) e MSTN (myostatin). * were used for data normalization.

Taget gene	GenBank	Sequences (F = Forward; R = Reverse)	Reference
GH	M26916	F - 5′-CTGTCTGTCTGTCTGTCAGTCGT-3′	Rentier-Delrue et al. (1989)
		R- 5′-AGAGGAGACGCCCAAACAC-3′	
SL	AB442015	F – 5'- CCCTTTGCGACTTCAGAGTAA-3'	Uchida et al. (2008)
		R – 5'- ACAGAGTGGAGCAACCATTT-3'	
GHR1	EF052861	F -5′ TCTCAGCAGAACCGATTAATGA-3′	Ma et al. (2007)
		R -5′-TTTGATTTTGGGTGCAGGA-3′	
GHR2	EF052862	F - 5′-CGACCCAGAACCATCACC-3′	Ma et al. (2007)
		R - 5′-GTCTCCTGACTGAGGGCAAG-3′	
IGF-1	EU272149	F - 5′-CCCGAACTTCCTCGACTTGA-3′	Wang et al. (2008)
		R - 5′-CCTCAGCCAGACAAGACAAAAA-3′	
IGF-2	EU272150	F - 5′-CCCCTGATCAGCCTTCCTA-3′	Wang et al. (2008)
		R - 5′-GACAAAGTTGTCCGTGGTGA-3′	
MSTN	AF197193	F - 5′-ACCAGCCCCACCTGAACT-3′	Rodgers et al. (2001)
		R - 5′-ATCTGGGACGTGGCTCTCT-3′	
*18S	DQ397879	F- 5′- GGCAACCAACGGTAAAACAA -3′	Pang et al. (2013)
		R - 5′- AGCTAGCTGCGTTCTTCATTG -3′	
*Beta-actin	EU887951	F - 5′- ACCCACACAGTGCCCATC -3	Monteiro et al. (2009)
		R - 5′- CAGGTCCAGACGCAGGAT -3′	

Cq values (cycle quantification) were calculated using the SDS 1.4 software by automatic configuration baseline and threshold of 0.2. The Cq is the meeting point between the amplification curve corrected for baseline and threshold (according to RDML, HTTP://rdml.org). The relative quantification of gene expression was carried out by the comparative method Cq (Livak and Schmittgen, 2001).

IGF-1 and IGF-2 expression assessment was carried out using brain, liver and white muscle tissues; GH expression used white muscle and brain tissues; GHR1, GHR2 and MSTN expression used white muscle; and SL expression used only brain tissue.

The gene expression was evaluated comparing the three groups in the 3 months and comparing over the months in each one individually using the analysis of variance (ANOVA) and the Tukey test, with a level of 5% of significance.

3. Results

3.1 *gh* expression

Gh expression in brain and white muscle showed the same pattern in the three evaluated months, being up-regualted in crossbreed compared to both lines (**Figure 2**). Using the 4th month as control in each group, CHIT lshowed a tendency to increase the expression of the gene in the brain, while the RED line and the up-regulated in crossbreed in 5th and 6th month (**Figure 3**). In the white muscle, *gh* was up-regulated in the REDS line and in crossbreed, whereas it did not vary between 5th and 6th months, being up-regulated in 6th month.

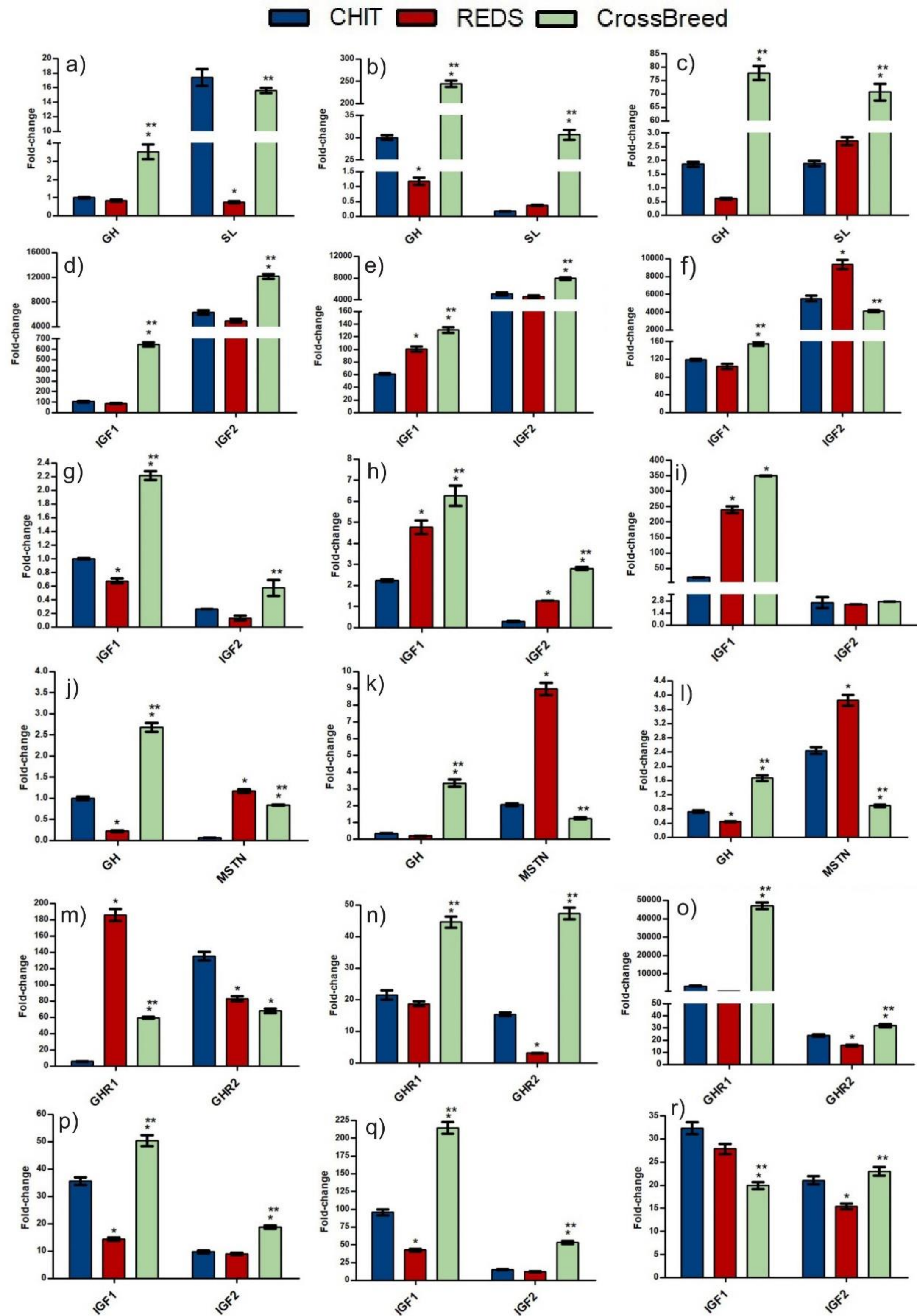

Figure 1 Relative expression of GH/IGF and MSTN axis genes in brain, white muscle and liver. In the legend CHIT = Chitralada; RED = Red Stirling; And CROSS = hybrid. * (P <0.05 compared to CHIT) ** (P <0.05 compared to RED). a) and d) relative expression of the genes during the 4th month in the brain; b) and e) relative expression of the genes during the 5th month in the brain; c) and f) relative expression of the genes during the 6th month in the brain; g) relative expression of the genes during the 4th month in the liver; h) relative expression of the genes during the fifth month in the liver; i) relative expression of the genes during the 6th month in the liver; j), m) and p) relative expression of the genes during the 4th month in the white

muscle; k), n) and q) relative expression of the genes during the 5th month in the white muscle; And l), o) and r) relative expression during the 6th month in the white muscle.

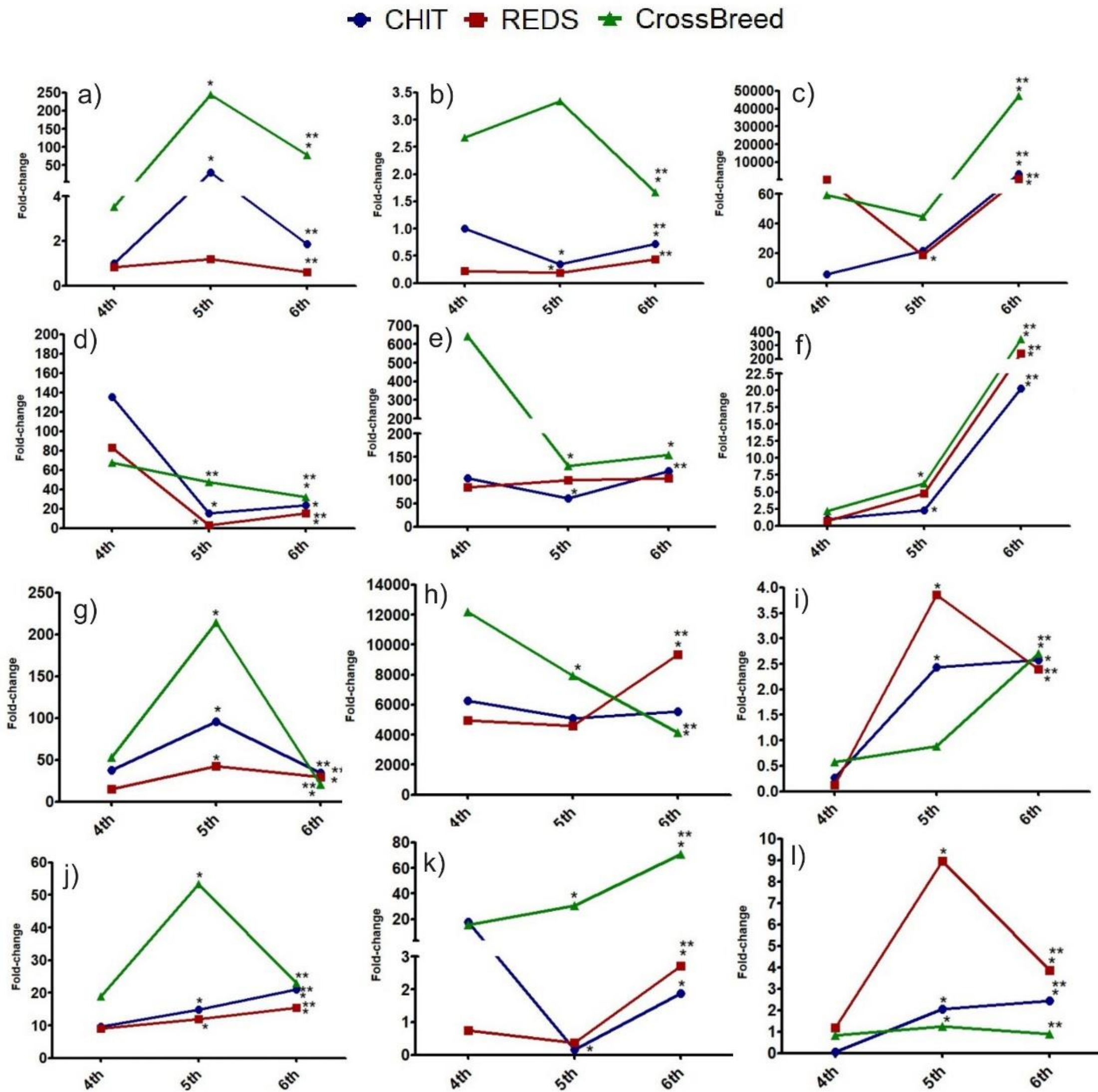

Figure 2 Relative expression of the genes over the 3 months comparing the initial expression of each group. In the caption CHIT = Chitralada; RED = Red Stirling; And CROSS = hybrid. * Super expressed in relation to the 4th month in each group (P <0.05) ** sub expressed in relation to the 4th month in each group (P <0.05). a) gh relative expression in brain; b) gh relative expression in white muscle; c) ghr1 relative expression in white muscle; d) ghr2 relative expression in white muscle; e) igf1 relative expression in brain; f) igf1 relative expression in liver; g) igf1 relative expression in white muscle; h) igf2 relative expression in brain; i) igf2 relative expression in liver; j) igf2 relative expression in white muscle; k) sl relative expression in brain; And l) mstn relative expression in white muscle.

3.2 *ghr1* and *ghr2* expression

Ghr1 was up-regulated in white muscle during 4th month in REDS, while was up-regulated in crossbreed during 5th and 6th month, and REDS *ghr1* was down-regulated in

both months comparing to CHIT (**Figure 2**). Using 4th as control in each group, *ghr1* was down-regulated during the 5th month and up-regulated during the 6th month in the three groups (**Figure 3**).

CHIT *ghr2* was up-regulated during 4th month, while crossbreed *ghr2* was up-regulated during 5th and 6th month (**Figure 2**) G*hr2* show the same pattern among the groups along the months **(Figura 3**).

3.3 *igf1* and *igf2* expression

During the 4th month – considering brain, liver and white muscle – *igf2* was up-regulated in crossbreed (**Figure 2**). In 5th month, *igf2* expressiondid not show any difference among the groups. In the 6th month, *igf2* show different expression pattern (**Figure 2**).

Igf1 showed also diffent expression patterns along the month, however, it was monstly up-regulated in crossbreed (**Figure 2**). Along the months using the 4th month as control in each group, CHIT and crossbreed *igf1* was down-regulated during 5th month and up-regulated during 6th month, and REDS *igf1* up-regulated during 5th and 6th months, both cases in brain. In liver and white muscle, *igf1* was up-regulated during 5th and 6th month, except in REDS, which was down-regulated during 5th and 6th month (**Figure 3**).

3.4 SL mRNA expression

Sl expression in the brain followed a tendency to be up-regulated in crossbreed compared to the REDS and CHIT (**Figure 2**). Evaluating the 5th and 6th months, using the fourth month as control, *sl* was down-regulated in CHIT and in crossbreed, while in the REDS was up-regulated (**Figure 3**).

3.5 *mstn* expression

CHIT *mstn* was down-regulated during 4th month. Comparing REDS with crossbreed, *mstn* was up-regulated during 4th month. During 5th and 6th months, crossbreed *mstn* was upr-regulated (**Figure 2**). Along the months, REDS *mstn* expression showed progressive increase (**Figure 3**).

4. Discussion

Heterosis causes has always been discused. Dominance and overdominance theories were always used attempting to explain heterosis. Nowadays, its accepted that both theories complete itselfs, and would not contradict.

In addition, the interaction between normal genes and gain-of-function genes may have influence (Huyen, 2016).

Genetically improved tilapia crossbreeds by means of the combined selection of commercial lines have been obtained aiming at fast growth rate, as is the case of the crossbreed presented in this study. Increased *gh* expression in crossbreed compared to both lines, in brain and white muscle, corroborates with that observed by Huang et al. (2012), confirming the greater ability to grow the hybrid in relation to the lines.

Huang et al. (2012) performed a gene expression study comparing a fast growing nile tilapia line. As with the data presented here in crossbreed, *gh* expression was much higher in the fast-growing lineage (Huang et al., 2012). Due to the performance of GHR1 and GHR2 as GH receptors, inadequate regulation of these molecules may lead to different growth retardation conditions. In view of this, a close relationship is observed between the expression profiles of these genes, which in general have been superexpressed in the hybrid in comparison to the lineages.

As in the case of *gh*, *ghr1* and *ghr2*, similar increases in igf1 expression in the liver were observed in the hybrid and in the fast growing lineage (Huang et al., 2012). The fast growing line as well as the hybrid showed a decline in *mstn* expression. Since MSTN is a negative regulator of muscle growth, that is, its presence inhibits muscle growth, its sub expression is very positive for animal production.

Analyzing a transcriptome of a crossbreed with heterosis between two species of the genus *Epinephelus* comparing with its parents, it was found several differentially expressed genes in the hybrid associated to the growth, like *gnrh1, gnrh3* and *gh* in the brain and *igf1, igf2b, igffbp1, igfb2a, igfbp4, Igfbp5a* and *igfbp5b* in the liver (Sun et al., 2016). Among them, *Igf1* and *Igf2b* expression levels in the liver of the crossbreed were significantly higher than those of their parents, showing that IGF1 and IGF2b may play a critical role in the growth of this hybrid (Sun et al., 2016).

In a crossbreed of a species of tortoise (Pelodiscus sinensis) of economic interest in aquaculture in China, 220 transcripts expressed by RNA-Seq were only found in the crossbreed, among them, *ghrh* (growth hormone releasing hormone Hormone), which play role in the GH/IGF axis (Zhang et al., 2017).

The data presented in crossbreed (CHIT 7/8 and REDS 1/8) corroborate with the data presented in other studies involving GH / IGF axis expression. Muscle is a highly organized tissue, and the implications resulting from the alteration of the GH/IGF axis are widely described, and their role is fundamental in regulating growth by modulating protein synthesis and proteolysis (Biga and Meyer, 2009).

The highest expression of *gh* in the muscle of the CHIT line in comparison to the REDS is in agreement with the data obtained by Lago (2014) in test of zootechnical performance of the same lineages evaluated in this study, where the growth was about 70%

greater in CHIT. Proving the relation of the GH with the muscular development in the species, fact of great importance since it refers to the portion of greater economic interest. Although the increased expression of these genes in crossbreeds with heterosis is evident, it is not known whether the increase in these expressions is due to heterosis or whether it is the cause.

These data, as well as the data presented in this study, show evidence of the performance of GH/IGF axis genes with heterosis. However, most of the evidence in other papers suggests that the cause of heterosis comes from several genetic factors combined.

5. Conclusion

Gene expression data demonstrated that GH/IGF axis and MSTN genes may contribute to superior crossbreed with heterosis growth due to increased protein synthesis and metabolism. In general, most genes related to the GH / IGF axis and the MSTN showed a greater expression in the hybrid with respect to their parents. These genes act on heterosis, although it is not known whether they originate or are responsible for it.

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Anexo II: Artigo

MicroRNAs expression protein profile of crossbreed between Chitralada and Red Stirling lines of Nile Tilapia (*Oreochromis niloticus*).

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Abstract

MicroRNAs (miRNAs) are a class of small RNAs that control gene expression post-transcriptionally by pairing at the 3' untranslated region (UTR) of their target mRNA. Since miRNAs are key elements in gene regulation, they have also became focus of investigations regarding breeding of farming animals. MiRNAs participate actively in several biological pathways relevant in aquaculture (e.g. growth, metabolism, muscle development and fat deposition). However, the protein is the final product of the expression of a gene and its interaction with other factors such as miRNAs and other processes that can't be detected by expression analysis like posttranslational modifications,

phosphorylation or proteolysis. Data from the white muscle proteome, which is the edible part of fish, can help us set new strategies that could help to determine the molecular mechanism behind the hybrid vigor. Nile tilapia (*Oreochromis niloticus*) is the most important farmed fish species in Brazil. Based on this assumption, the present work aims to assessing the expression of the miRNAs and protein profiles in the crossbred animals and its relationships to the molecular regulation of crossbreed vigor. We collected muscle samples and morphometric parameters such as weight and length from individual animals (parental and crossbred) from 5th and 6th month of life. We evaluated the miRNA expression of the 5th and 6th months from the three genetic groups (Chitralada, Red-Stirling as control groups and crossbred individuals) and protein profiles. To do this, we performed RNA-seq to detect all miRNAs and protein analysis by ESI-q-TOF and shotgun expressed in skeletal muscle among the lineages. Regarding the comparison of the miRNAs and with the proteins, several miRNAs were sub-expressed and consequently there was an increase in the expression of the proteins that have targets of these miRNAs in their genes. In this case, miR-216, miR-153, miR-204 and miR205 comparing the crossbreed with CHIT; And let-7e, miR-16 and miR-216 comparing the crossbreed with REDS. These data show, as shown in other studies, that there is an action of miRNAs on heterosis.

Key words: growth; RNA-Seq; miRNA; ESI-q-TOF; shotgun; Nile tilapia;

1. Introduction

Nile tilapia (*Oreochromis niloticus*) is economically important for aquaculture (Meurer et al., 2000; Boscolo et al., 2001), due to several zootechnical characteristics such

as easy management, disease resistance, low oxygen content tolerance, diversified dietary habits, rapid growth, and high yield of fillet (Hilsdorf et al., 1995; Popma and Lovshin, 1995; Freitas et al., 2009).

With the aim to improve productivity, some varieties of tilapia were produced by genetic selection processes and crossbreeding. Among them, the GIFT varieties (Eknath and Acosta, 1997), Chitralada or Thai (Zimmermann, 2000) and red varieties stand out. A red variety currently used is Red Stirling, originally obtained from a wild *O. niloticus* population from Lake Manzala in Egypt in 1979. This variety has been maintained by the Aquaculture Institute of the University of Stirling, Scotland (McAndrew and Majumdar, 1983). Crossbreeding between Chitralada and Red Stirling lines led to the production of animals with high heterosis (Moreira et al., 2007). Red Stirling variety has slower growth compared to Chitralada, but a red colored meat that is attractive to the consumer. On the other hand, the Chitralada variety has rapid growth. Currently, a crossbreed between these lines (e.g., genotype 7/8 Chitralada: 1/8 Red Stirling) has been produced by successive crosses and has shown high quality heterosis (Lago, 2014).

MicroRNAs (miRNAs) are a large class of non-coding RNA with around 22 nucleotides found in the genome of animals, plants and viruses (Bartel, 2004; Kim et al., 2009; Lee et al., 1993; Xia et al., 2011).By acting on gene regulation, they have also become targets for research in animal breeding. In this way, studies were carried out on bovines (Coutinho et al., 2007; Gu et al., 2007; Strozzi et al., 2009), pigs (Sharbati-Tehrani et al., 2008; Wernersson et al 2005), poultries (Darnell et al., 2006, 2007; Glazov et al., 2008; Hicks et al., 2008, 2009), and in Nile tilapia (Huang et al., 2012; Yan et al., 2012; Yan et al., 2013a, 2013b) in order to identify potential miRNAs that could help improve the productivity in aquaculture.

Additionally, a protein is the final product of the synthesis of an mRNA and its translation into amino acid sequence. These molecules contribute decisively to determine phenotypic changes, mainly due to qualitative and quantitative changes in various body tissues. Thus, their production is subject to the expression of genes encoding and their regulatory ncRNAs such as miRNAs, active in different tissues and also by Posttranslational modifications that cannot be seen by RNA measurements and the different functions of proteins (e.g. enzymes in metabolism, signaling, structural and mechanical functions in muscels).

Currently there are several techniques available for the analysis of proteins. The most important step in the proteome analysis is separation of proteins and the detection of group differences by two-dimensional gel electrophoresis (2DE) (O'Farrell, 1974). After the 2D gel some techniques are then used to identify the interesting protein spots in the gel. One of these techniques is the mass spectrometry (MS), which has been applied comprehensively in the investigation of biological systems (Domon e Aebersold, 2006; Sparkman, 2000).

Similar to plants, the combination of different parental miRNA sequences may also cause heterosis in animals (Sun et al., 2004). Thus, this study aims to identify miRNAs potentially associated with differential phenotype traits between the crossbreed and the parental lines by using RNA-seq and bioinformatics to identify putative targets of miRNAs associated with aquaculture advantages.

2. Materials and methods

2.1. Experimental animals

The two strains used in the present study were Thai Chitralada strain (CHIT) from the Asian Institute of Technology and the Red-Stirling strain (REDS) from the University of Stirling. Both broodstock strains has been kept by Brazilian fish farming company (Indústria Brasileira do Peixe Ltd.) as part of a breeding program to produce better lines of red strains. Contemporary fingerling of REDS and CHIT as well as their crossbred (7/8 CHIT: 1/8 REDS) were produced in reproduction hapas where they were kept to the minimum size to be marked with electronic microchips (Passive Integrated Transponder (PIT) tags). Tagged fingerlings were transferred to net cages according to the study carried out by Lago, et al., (2016). Twenty-seven fish were randomly collected during the six month grow-out cycle, three from each genetic group in the fourth, fifth and sixth months. During each sampling, morphological parameters were taken and the animals were sacrificed using tricaine methanesulfonate (MS-222, Sigma Chemical, St. Louis, MO). After euthanasia has been performed, samples of liver, brain and white muscle were collected and stored at -80ºC. The experiment was approved by the local animal ethics committed with the protocol number (CEUA, protocol 792/2015).

2.2. RNA isolation and small RNA sequencing

Total RNA was extracted by using TRIzol (Life Technologies, EUA), according to manufacturer's instruction. RNA integrity was assessed by electrophoresis on 1.0% agarose gel stained with GelRed (Uniscience, USA) and RIN (RNA Integrity Number) by using the 2100 Bioanalyzer system (Agilent, USA) (Fleige and Pfaffl, 2006; Becker et al., 2010). For subsequent analysis, we only used samples with RIN > 7.0. The RNA samples were also quantified by using a NanoDrop 1000 (Thermo Scientific, USA). RNA purity was

measured by the ratio of A260/280 and A260/230, and only samples with ratio > 1.8 were used.

Small RNA cloning was performed using the TrueSeq Small RNA Sample Prep kit (Illumina, EUA), according to manufacturer's instruction. The previously isolated RNA was fractionated into fragments of different sizes using polyacrylamide gel electrophoresis (SDS-PAGE). The fragments ranging from 15 to 30 nucleotides, were excised from the gel and recovered by cooling overnight in saline solution, followed by extraction, precipitation and filtration by micropore. The adapters were bounded to the 3' and 5' of the isolated small RNA fragments. Primers (provided by the kit) were then bind to the sequence of the adapters and were used for reverse transcription and selective amplification of small fragments of RNA by RT-PCR. The cDNA fragments were purified and used to build the libraries used to generate clusters and subsequent sequencing. The libraries were sequenced using Illumina/Solexa RNA-Seq by LC Sciences enterprise (USA).

2.3. Bioinformatics analysis

The raw reads were checked for quality inspection with FastQC (www.bioinformatics.babraham.ac.uk/projects/fastqc/) being the reads with Phred Score < 30 removed from the analysis. The adapters were trimmed by using FASTX-toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). The trimmed sequences between 18 and 25 nt were used for miRNAs identification. The reads were mapped to genome by using Bowtie2.0. Conserved known and novel miRNAs were identified using miRDeep2 (Friedländer et al., 2008) and normalized with miRProf from the UEA sRNA workbench v3.2 (Stocks et al., 2012). Expression values of these miRNAs were obtained using the weight, which is the number of total reads divided by the number of the loci that matched

with that read. The weight count was used to known the RPM (reads per million) count, which is the weight count divided by the total reads mapped by the sample sequenced multiplied for 1 million. FOLD CHANGE (log_2 of the ration of two samples (one sample divided by the other sample) was used to compare the RPM among the libraries.

Target prediction was performed using TargetScan (v 6.2) with a cutoff of Context+Score <= -0.2. The 3'UTR sequences of Nile tilapia (v 1.0) were downloaded at BioMart in Ensembl database. For genes with no annotated 3'UTR sequence, we used the 500 first nucleotides after the stop codon.

2.4. Protein extraction

White muscle samples were extracted from five individuals with 6 months of life per group (CHIT, REDS and crossbreed), totaling 15 samples. In protein extraction, refrigerated samples of white muscle at -20ºC were processed and homogenized. Samples were prepared according to the protocol used by Bouley et al. (2004).

The white muscle samples (50-100mg) were homogenized in a lysis solution containing 8.3 M urea, 2 M thiourea, 1% DTT, 2% CHAPS and 2% IPG pH 3-10 buffer with Politron, and centrifuged at 10,000g for 30 minutes. The supernatant was collected. Protein concentration was determined by the Bradford reaction.

The samples were separated on a two-dimensional (2D) gel according to the protocol described by Eravci et al. (2007). The first step was the separation by a gradient of pH 3-10. This step separates proteins by their isoelectric point. The second step consists of a separation by 90 °, separating the proteins by their molecular weight.

2.5. Acquisition and analysis of gel images

The 2D gels were scanned using the ImageScanner III (GE Healthcare Life Sciences) scanner in the calibrated transmission mode. Images of the two-dimensional gels were analyzed by Image Master 2D Platinun v7.05 (Ge Healthcare) software. The authenticity of each spot was validated by visual inspection and edited when necessary.

2.6. Enzyme Digestion

CBB-stained protein spots were cut from the gel, decolorized with Destain solution (50% ethanol [v / v] and 2.5% acetic acid [v / v]) dehydrated with 100% acetonitrile (v / v), The pieces of gel being then dried by vacuum centrifugation. Then the dehydrated gels pieces were digested with trypsin, following the protocol: 20 g of non-autolytic trypsin was dissolved in 1600 µl of 50 mM ammonium bicarbonate, pH 8.0. Each piece of dry gel was incubated with 30 µL of trypsin solution for 30 minutes in an ice bath; The digestion was stopped by removing the excess trypsin solution and adding 50 µl of 50 mM ammonium bicarbonate and maintaining the overnight gel suspension in a water bath at 37 ° C; (V / v), incubated for 10 minutes at room temperature, the supernatant was collected and placed in new microtubes (Eppenorf, model Lobind - Volume 0.5 mL); In the tubes containing the gels were added 12 µl of 5% (v / v) formic acid solution and 50% (v / v) acetonitrile, incubated for 10 minutes at room temperature; The supernatant was collected and added to the microwell from the previous step. The collected supernatants were dried by vacuum centrifugation and then kept at -20 ° C until analyzed by mass spectrometry (Santos et al., 2011).

2.7. Peptide sequencing by mass spectrometry and data processing

The mass spectrometric analysis of tryptic digesting from the processing of two-dimensional electrophoresis gels was carried out in an electrospray (ESI) quarter pole type

equipment, model MicrQ-TOF III (Bruker Daltonics brand) coupled to an LC-20AT liquid chromatograph (Shimadzu). The liquid chromate was equipped with a binary pump system and an automatic sample applicator. The mobile phase consisted of water (A) and acetonitrile (B), containing 0.1% (v / v) formic acid. In addition, the chromatographic separation was performed by means of a C18 reverse phase column (4.5 mm x 100 mm, 1.8 μm). The elution conditions were optimized in a linear gradient from 0 to 85% of solvent B for 60 minutes at a flow rate of 0.2 mL / min. The column and automatic sample applicator were maintained at 25 ° C and 10 ° C, respectively. The injection volume of the reference compounds and the samples was 10 μl. The mass spectrometer used the following parameters: capillary voltage 4.5 kV, drying temperature 180 ° C, nitrogen flow 6 L / min and pressure 0.8 bar. The external calibration was performed using the Tunning MIX ESI kit (Agilent Technologies) before the individual analyzes. Mass spectra were acquired in the electrospray positive ionization (ESI) mode at an ionisation range between 400 m/z and 2500 m/z. After mass spectrometry, the data were exported and submitted to automatic analysis with the MASCOT v.2.1 bioinformatics tool (www.matrixscience.com), using the following parameters: trypsin enzyme; Taxonomy *Oreochromis niloticus* (database NCBI, fixed modification carbamidomethylation, modified modification oxidation of methionine, cleavage lost by the enzyme, molecular weight of the monoisotope type, peptide tolerance error (MS) ± 0.2 Da and tolerance error (MS / MS) ± 0.2 Da, protonation + 1 and type of instrument ESI-q-TOF.

The tryptic peptides of the complex mixture samples (Shotgun) were run in a nano ACQUITY UPLC system (Waters, Milliford, USA) coupled to the Xevo Q-TOF G2 mass spectrometer (Waters, Milliford, USA). The nano ACQUITY UPLC system was equipped with a HSS T3 (Acquity UPLC HSS T3 column 75 mm x 150 mm, 1.8 μm, Waters) column, previously equilibrated with 7% of mobile phase B (100% ACN + 0.1% formic

acid). The peptides were separated by a linear gradient of 7-85% mobile phase B for 20 min at a flow rate of 0.35 µL/min and the column temperature maintained at 45 ° C. The MS was operated in positive ion mode, with the data acquisition time of 20 min. The data obtained were processed using ProteinLynx GlobalServer software (PLGS) version 3.0 (Waters, Milliford, USA). Protein identification was obtained through the ion count algorithm incorporated into the software. The data obtained were compared with the Oreochromis niloticus database downloaded from the NCBI catalog, using the following parameters: fixed modification carbamidomethylation; Variable modification oxidation of methionine; Cleavage lost by the enzyme; Molecular mass of the monoisotope type; Peptide tolerance error (MS) ± 0.2 Da and tolerance error (MS / MS) ± 0.2 Da; Protonation + 2, + 3 + 4, type of ESI-Tof instrument.

The functional enrichment was done using the g:Profiler tool (http://biit.cs.ut.ee/gprofiler/) using the nile tilapia database and g: SCS threshold as a statistical parameter.

3. Results

3.1. miRNAs libraries

Six miRNAs libraries were obtained from the three groups in the 5th and 6th month of life. Chitralada and Red Stirling were used as control groups. The libraries from 5th and 6th of Chitralada had 2,084,755 and 2,568,552 reads, respectively; 5th and 6th of Red Stirling 2,036,241 and 3,182,923 reads; and 5th and 6th of crossbreed 1,949,529 and 3,453,399 reads

3.2. Expression and miRNAs identification

The miRNAs miR-122-5p, miR-192-5p, miR-194-5p, miR-301b-5p, miR-458-5p and miR-2188-5p were up-regulated and miR-125a-3p was down-regulated in crossbreed during the 5th month, and let-7e-3p, miR-16-3p, miR-124-3p, , , miR-135a-5p, miR-216b-3p, and miR-219-5p were up-regulated and miR-7641-3p was down-regulated in crossbred during the 6th month when compared to the same periods of Red Stirling.

Comparing the crossbred with Chitralada, miR-734-3p was up-regulated, while miR-196-3p, miR-301a-3p, miR-301b-3p and miR-7641-3p were down-regulated during the 5th month and let-7e-3p, miR-16-3p, miR-153a-3p, miR-153-3p, miR-204a-3p, miR-205-3p and miR-216b-3p were up-regulated, while miR-124-3p was down-regulated during the 6th month.

3.3. Target prediction

The differentially expressed miRNAs were subject to target prediction. Being predicted 794 targets for miR-124-3p; 455 targets for let-7e-3p; 606 targets for miR-216b-3p; 504 targets for miR-153a-3p; 342 targets for miR-204a-3p; 1525 targets for miR-205-3p; 650 targets for miR-125a-3p; 437 targets for miR-219-5p; 614 targets for miR-122-5p; 414 targets for miR-192-5p; 306 targets for miR-2188-5p; 695 targets for miR-194-5p; and 356 targets for miR-196-3p.

Among these differentially expressed miRNAs, we highlight miR-194-5p, miR-122-5p, miR-124-3p, miR-216b-3p and miR-219-5p due to the potential regulation of the GH/IGF axis genes predicted by our analysis.

3.4. Proteins identified by ESI-q-TOF and shotgun

After quantification and protein extraction, a pool was made with five samples or each group (CHIT, REDS and crossbreed). For the shotgun analysis, a pool was used for each group and for ESI-q-TOF analysis gels were made in triplicate using for each pool.

By the shotgun analysis, after the exclusion of uncharacterized proteins, we found 64 differently expressed and / or unique proteins from a group comparing CHIT with REDS; 64 comparing CHIT with crossbreed; And 40 REDS with the crossbreed. After functional enrichment, the performance of CHIT-exclusive proteins compared to REDS was verified in oxidized and nucleoside monophosphate and nucleoside triphosphate metabolic processes (**Table 1**). Comparing REDS with CHIT, two REDS-exclusive proteins that participate in nucleoside monophosphate metabolic processes, and compared to the crossbreed, have several proteins that are more expressive that act on catalytic reactions. Comparing CHIT with the crossbreed, CHIT-exclusive proteins or more expressed acting in catalytic activity were found.

Table 1. Comparative analysis of relative expression of the proteins identified by shotgun.

Groups	Function	Acess	Protein	Score	Expression
CHIT x crossbreed	Catalytic activity	I3IY11	Elongation factor 1-alpha	99,17	CHIT
	Catalytic activity	I3IYA4	Malate dehydrogenase	373	CHIT
	Catalytic activity	I3IZU4	L-lactate dehydrogenase	155,38	1,36
	Catalytic activity	I3J2Y9	Isocitrate dehydrogenase	167,16	CHIT
	Catalytic activity	I3J820	Phosphoglycerate mutase	7709,17	1,05
	Catalytic activity	I3JB48	Fructose-bisphosphate aldolase	925,28	1,35
	Catalytic activity	I3JB82	Fructose-bisphosphate aldolase	906,55	1,35
	Catalytic activity	I3JBJ3	Tubulin beta chain	77,14	CHIT
	Catalytic activity	I3JFX6	Elongation factor 1-alpha	102,68	CHIT
	Catalytic activity	I3JIG9	Glycerol-3-phosphate dehydrogenase	420,05	CHIT
	Catalytic activity	I3JIV9	Triosephosphate isomerase	6052,29	1,04
	Catalytic activity	I3JIY6	Glyceraldehyde-3-phosphate dehydrogenase	10041,98	1,26
	Catalytic activity	I3JKV4	ATP synthase subunit alpha	321,13	CHIT
	Catalytic activity	I3JPS3	L-lactate dehydrogenase	135,98	1,31
	Catalytic activity	I3JQZ4	Elongation factor 1-alpha	99,17	CHIT
	Catalytic activity	I3JTH2	Tubulin beta chain	77,14	CHIT
	Catalytic activity	I3JTS7	Histone-lysine N-methyltransferase	76,21	CHIT
	Catalytic activity	I3K1F7	ATP-dependent 6-phosphofructokinase	76,04	CHIT
	Catalytic activity	I3K556	Glyceraldehyde-3-phosphate dehydrogenase	29411,03	1,23
	Catalytic activity	I3K5M6	Tubulin beta chain	77,14	CHIT
	Catalytic activity	I3K606	Fructose-bisphosphate aldolase	934,10	1,36
	Catalytic activity	I3KB11	L-lactate dehydrogenase	2324,30	1,12
	Catalytic activity	I3KDA6	Isocitrate dehydrogenase	218,920	CHIT
	Catalytic activity	I3KDJ7	ATP synthase subunit alpha	330,24	CHIT
	Catalytic activity	I3KME8	Phosphoglycerate	771,93	1,90

			mutase		
	Catalytic activity	I3KMP5	Elongation factor 1-alpha	82,80	CHIT
	Catalytic activity	I3KTF2	Glycogen synthase	219,78	CHIT
	Catalytic activity	I3KTW7	Tubulin beta chain	168,40	CHIT
	Catalytic activity	I3KW15	Fructose-bisphosphate aldolase	20950,48	1,16
CHIT x REDS	Oxidoreductase activity (acting in the CH-OH donor groups)	I3IYA4	Malate dehydrogenase	373,00	CHIT
	Oxidoreductase activity (acting in the CH-OH donor groups)	I3J2Y9	Isocitrate dehydrogenase	167,16	CHIT
	Oxidoreductase activity (acting in the CH-OH donor groups)	I3JIG9	Glycerol-3-phosphate dehydrogenase	420,05	CHIT
	Oxidoreductase activity (acting in the CH-OH donor groups)	I3KDA6	Isocitrate dehydrogenase	218,92	CHIT
	Oxidoreductase activity (acting in the CH-OH donor groups)	I3KY60	Malate dehydrogenase	565,26	CHIT
	Oxidoreductase activity (acting on the donor groups of CH-OH, NAD or NADP as receptors)	I3IYA4	Malate dehydrogenase	373,00	CHIT
	Oxidoreductase activity (acting on the donor groups of CH-OH, NAD or NADP as receptors)	I3J2Y9	Isocitrate dehydrogenase	167,16	CHIT
	Oxidoreductase activity (acting on the donor groups of CH-OH, NAD or NADP as receptors)	I3JIG9	Glycerol-3-phosphate dehydrogenase	420,05	CHIT
	Oxidoreductase activity (acting on the donor groups of CH-OH, NAD or NADP as receptors)	I3KDA6	Isocitrate dehydrogenase	218,92	CHIT
	Oxidoreductase activity (acting on the donor groups of CH-OH, NAD or NADP as receptors)	I3KY60	Malate dehydrogenase	565,26	CHIT
	Transmembrane transport coupled to ATP hydrolysis	I3J7E7	Calcium-transporting ATPase	1719,79	0,28

Transmembrane transport coupled to ATP hydrolysis	I3JQC4	ATP synthase subunit beta	350,84	0,35
Transmembrane transport coupled to ATP hydrolysis	I3K022	Calcium-transporting ATPase	7609,72	0,31
Transmembrane transport coupled to ATP hydrolysis	I3K1D1	ATP synthase subunit beta	371,56	0,33
Transmembrane transport coupled to ATP hydrolysis	I3KG39	Calcium-transporting ATPase	1896,69	0,29
Transmembrane transport coupled to ATP hydrolysis	I3KLR1	Calcium-transporting ATPase	1356,76	0,27
Transmembrane transport coupled to ATP hydrolysis	I3KNX2	Calcium-transporting ATPase	1427,54	0,29
Transmembrane transport coupled to ATP hydrolysis	I3KTJ3	Calcium-transporting ATPase	1917,07	0,28
Metabolic process	I3IVP0	Alpha-1 4 glucan phosphorylase	748,96	0,33
Metabolic process	I3IZU4	L-lactate dehydrogenase	155,38	0,77
Metabolic process	I3J3H6	Piruvato quinase	753,33	0,43
Metabolic process	I3J409	Glucose-6-phosphate isomerase	1398,68	0,26
Metabolic process	I3J820	Phosphoglycerate mutase	7709,17	0,45
Metabolic process	I3JB48	Fructose-bisphosphate aldolase	925,28	0,50
Metabolic process	I3JB82	Fructose-bisphosphate aldolase	906,55	0,48
Metabolic process	I3JBN0	Alpha-1 4 glucan phosphorylase	7411,72	0,29
Metabolic process	I3JIY6	Glyceraldehyde-3-phosphate dehydrogenase	10041,98	0,66
Metabolic process	I3JJ47	Alpha-1 4 glucan phosphorylase	909,24	0,31
Metabolic process	G3LHF4	Glyceraldehyde-3-phosphate dehydrogenase	476,04	0,50
Metabolic process	I3JNZ0	Piruvato quinase	8041,23	0,36
Metabolic process	I3JPS3	L-lactate dehydrogenase	135,98	0,73
Metabolic process	I3JQC4	ATP synthase subunit beta	350,84	0,35
Metabolic process	I3JS50	AMP desaminase	353,70	0,47
Metabolic process	I3K1D1	ATP synthase subunit beta	371,56	0,33
Metabolic process	I3K556	Glyceraldehyde-3-	29411,03	0,62

			phosphate dehydrogenase		
	Metabolic process	I3K5C3	Triosephosphate isomerase	32638,06	0,49
	Metabolic process	I3K606	Fructose-bisphosphate aldolase	934,10	0,49
	Metabolic process	I3KB11	L-lactate dehydrogenase	2324,30	0,39
	Metabolic process	I3KCM4	Adenilato quinase isoenzima 1	4607,64	0,61
	Metabolic process	I3KF87	Alpha-1 4 glucan phosphorylase	1256,05	0,31
	Metabolic process	I3KL67	Phosphoglycerate kinase	5468,08	0,45
	Metabolic process	I3KSF9	ATP-dependent 6-phosphofructokinase	588,18	0,48
	Metabolic process	I3KW15	Fructose-bisphosphate aldolase	20950,48	0,58
	Nucleoside monophosphate metabolic process	I3JKV4	ATP synthase subunit alpha	321,13	CHIT
	Nucleoside monophosphate metabolic process	I3K1F7	ATP-dependent 6-phosphofructokinase	76,04	CHIT
	Nucleoside monophosphate metabolic process	I3KDJ7	ATP synthase subunit alpha	330,24	CHIT
	Nucleoside monophosphate metabolic process	I3KPD3	Glucose-6-phosphate isomerase	280,43	CHIT
	Nucleoside triphosphate metabolic process	I3JKV4	ATP synthase subunit alpha	321,13	CHIT
	Nucleoside triphosphate metabolic process	I3K1F7	ATP-dependent 6-phosphofructokinase	76,04	CHIT
	Nucleoside triphosphate metabolic process	I3KDJ7	ATP synthase subunit alpha	330,24	CHIT
	Nucleoside triphosphate metabolic process	I3KPD3	Glucose-6-phosphate isomerase	280,43	CHIT
	Nucleoside monophosphate metabolic process	I3JFG4	AMP desaminase	44,33	REDS
	Nucleoside monophosphate metabolic process	I3KEH5	AMP desaminase	44,33	REDS
REDS x crossbreed	Catalytic activity	I3IVP0	Alpha-1 4 glucan phosphorylase	334,79	1,63
	Catalytic activity	I3IZU4	L-lactate dehydrogenase	268,60	1,86
	Catalytic activity	I3J3H6	Piruvato quinase	198,33	2,05
	Catalytic activity	I3J409	Glucose-6-phosphate	277,69	2,51

		isomerase		
Catalytic activity	I3J7E7	Calcium-transporting ATPase	355,60	1,46
Catalytic activity	I3J820	Fosfoglicerato mutase	4464,83	2,44
Catalytic activity	I3JB48	Fructose-bisphosphate aldolase	1016,65	2,89
Catalytic activity	I3JB82	Fructose-bisphosphate aldolase	1016,65	2,83
Catalytic activity	I3JBN0	Alpha-1 4 glucan phosphorylase	2624,60	1,54
Catalytic activity	I3JIV9	Triosephosphate isomerase	3046,24	1,75
Catalytic activity	I3JIY6	Glyceraldehyde-3-phosphate dehydrogenase	9367,36	1,99
Catalytic activity	I3JJ47	Alpha-1 4 glucan phosphorylase	242,88	1,77
Catalytic activity	G3LHF4	Glyceraldehyde-3-phosphate dehydrogenase	721,53	1,88
Catalytic activity	I3JNZ0	Piruvato quinase	3188,85	2,36
Catalytic activity	I3JPS3	L-lactate dehydrogenase	275,44	1,79
Catalytic activity	I3JS50	AMP desaminase	158,51	1,58
Catalytic activity	I3K022	Calcium-transporting ATPase	1766,68	1,86
Catalytic activity	I3K556	Glyceraldehyde-3-phosphate dehydrogenase	20028,87	1,99
Catalytic activity	I3K5C3	Triosephosphate isomerase	20126,63	1,73
Catalytic activity	I3K606	Fructose-bisphosphate aldolase	1016,65	2,83
Catalytic activity	I3KB11	L-lactato desidrogenase	794,85	2,05
Catalytic activity	I3KCM4	Adenilato quinase isoenzima 1	4756,79	1,75
Catalytic activity	I3KF87	Alpha-1 4 glucan phosphorylase	457,43	1,92
Catalytic activity	I3KG39	Calcium-transporting ATPase	450,58	1,22
Catalytic activity	I3KL67	Fosfoglicerata quinase	3819,14	1,84
Catalytic activity	I3KLR1	Calcium-transporting ATPase	175,07	1,31
Catalytic activity	I3KME8	Fosfoglicerato mutase	190,69	2,12
Catalytic activity	I3KNX2	Calcium-transporting ATPase	238,29	1,45
Catalytic activity	I3KPS8	Fosfoglicerato mutase	213,21	2,14
Catalytic activity	I3KSF9	ATP-dependent 6-	156,05	1,77

			phosphofructokinase		
	Catalytic activity	I3KTJ3	Calcium-transporting ATPase	423,46	1,57
	Catalytic activity	I3KW15	Fructose-bisphosphate aldolase	20193,59	2,03

CHIT - indicates that there was only expression in Chitralada, and there was no expression in the compared group; REDS - indicates that there was only Red Stirling expression, and there was no expression in the compared group;

In the ESI-q-TOF technique, after 2D electrophoresis, the gels (Figure 1) were scanned and the Platinum software found 401 spots differently expressed or exclusive among the three groups. The spots were excised from the gels, digested and identified by the ESI-q-TOF technique. From the Mascot tool, 115 proteins with a score greater than 36 were identified. Several of these proteins validate the proteins found by the shotgun technique.

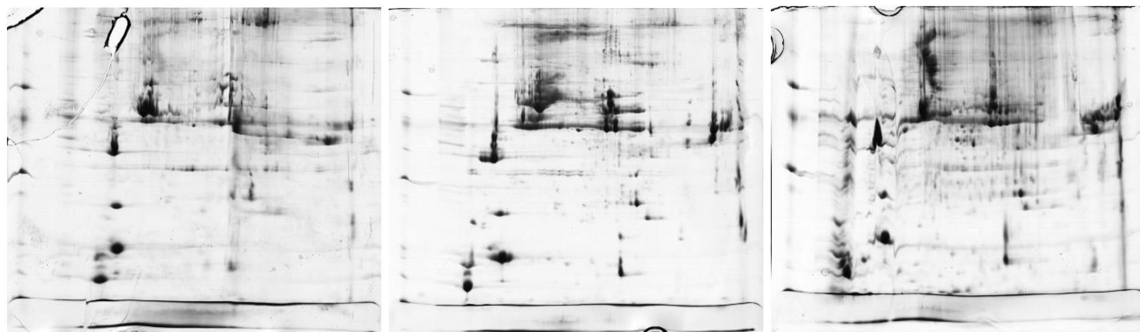

Figure 1 Specimens of the 2D gels. From left to right: a CHIT gel, REDS and a crossbreed gel.

3.5. MiRNA and protein interaction

In order to compare the proteome and miRnoma data, the genes of their respective proteins, identified by the shotgun technique, were accessed in the databases and obtained their access numbers. With these access numbers, it was possible to identify, by TargetScan, the regulatory miRNAs of these genes. The miRNAs were then separated with differentiated expressions in the present work. In this way, it was possible to find a relation of the miRNAs with the proteins and to separate the miRNAs and the proteins of interest (**Table 2 and 3**).

Table 2 Relationship miRNAs and the proteins of their target genes differently expressed in the crossbreed relative to CHIT (red indicates down-regulated in the crossbreed, and blue indicates up-regulated in the crossbreed).

MiRNA	Protein	Gene (acess)	Protein (acess)	Function

let-7e-3p	Frutose bifosfato aldolase	ENSONIG00000020092	I3KW15	processo metabólico; atividade catalítica
miR-16-3p	Frutose bifosfato aldolase	ENSONIG00000013159	I3K606	processo metabólico; atividade catalítica
miR-16-3p	L-lactato desidrogenase	ENSONIG00000001705	I3IZU4	processo metabólico; atividade catalítica
miR-216b-3p	L-lactato desidrogenase	ENSONIG00000001705	I3IZU4	processo metabólico; atividade catalítica
miR-153-3p	Frutose bifosfato aldolase	ENSONIG00000004866	I3JB82	processo metabólico; atividade catalítica
miR-153-3p	Tubulina cadeia beta	ENSONIG00000019514	I3KTW7	atividade catalítica
miR-204a-3p	L-lactato desidrogenase	ENSONIG00000014546	I3KB11	processo metabólico; atividade catalítica
miR-205-3p	L-lactato desidrogenase	ENSONIG00000001705	I3IZU4	processo metabólico; atividade catalítica
miR-205-3p	Frutose bifosfato aldolase	ENSONIG00000004866	I3JB82	processo metabólico; atividade catalítica
miR-205-3p	L-lactato desidrogenase	ENSONIG00000014546	I3KB11	processo metabólico; atividade catalítica

Table 3 Relationship miRNAs and the proteins of their target genes differently expressed in the crossbreed relative to REDS (red indicates down-regulated in the crossbreed, and blue indicates up-regulated in the crossbreed).

MiRNA	Proteína	Gene (acesso)	Proteína (acesso)	Função
miR-7641-3p	Piruvato quinase	ENSONIG00000002725	I3J3H6	atividade catalítica; processo metabólico
miR-7641-3p	L-lactato desidrogenase	ENSONIG00000001705	I3IZU4	atividade catalítica; processo metabólico
miR-7641-3p	Gliceraldeído-3-fosfato desidrogenase	ENSONIG00000007262	G3LHF4	atividade catalítica; processo metabólico
miR-7641-3p	Alpha-1_4 glucan phosphorylase	ENSONIG00000015719	I3KF87	atividade catalítica; processo metabólico
miR-7641-3p	Cálcio-transporte ATPase	ENSONIG00000019415	I3KTJ3	ATP hidrólise acoplado transporte transmembranar; atividade catalítica
miR-219-5p	Cálcio transporte ATPase	ENSONIG00000004866	I3KNX2	ATP hidrólise acoplado transporte transmembranar; atividade catalítica
let-7e-3p	Gliceraldeído-3-fosfato desidrogenase	ENSONIG00000007262	G3LHF4	ATP hidrólise acoplado transporte transmembranar; atividade catalítica
let-7e-3p	Frutose bifosfato aldolase	ENSONIG00000020092	I3KW15	atividade catalítica; processo metabólico
miR-16-3p	Frutose bifosfato aldolase	ENSONIG00000013159	I3K606	atividade catalítica; processo metabólico
miR-16-3p	Piruvato quinase	ENSONIG00000002725	I3J3H6	atividade catalítica; processo metabólico
miR-16-3p	L-lactato desidrogenase	ENSONIG00000001705	I3IZU4	atividade catalítica; processo metabólico
miR-216b-3p	Piruvato quinase	ENSONIG00000002725	I3J3H6	atividade catalítica; processo metabólico
miR-216b-3p	L-lactato desidrogenase	ENSONIG00000001705	I3IZU4	atividade catalítica; processo metabólico
miR-216b-3p	Cálcio transporte ATPase	ENSONIG00000018111	I3KNX2	ATP hidrólise acoplado transporte transmembranar; atividade catalítica

4. Discussion

Negative regulation of mRNA transcripts by specific miRNAs plays an important role in biological processes. Despite the simple mechanism of repressive action shared by all miRNAs, biological processes utilize the miRNAs to perform a range of different regulatory outcomes.

In the crossbreed compared to their parents, several types of interaction were found. Comparing with the CHIT, where only the miRNAs sub-expression showed some significant interaction with the proteins, one can observe the two situations: sub-expression of miRNAs with sub-expression of proteins; And sub-expression of miRNAs with super-expression of proteins. The second interaction, is a classical interaction, where miRNA expression is inversely proportional to its target. The canonical pathway of the miRNAs occurs through the interaction of the miRNA-RISC complex with binding sites of the target mRNA inhibiting its expression (Lee and Dutta, 2009). In this way, the sub expression of the miRNAs justifies the super expression of their targets, once the inhibition of the targets is reduced the proteins tend to express themselves more. This is the case of the following miRNAs and their targets: miR-216b with L-lactate dehydrogenase; MiR-153 with fructose bisphosphate aldolase and tubulin beta chain; MiR-204a with L-lactate dehydrogenase; MiR-205 with L-lactate dehydrogenase and fructose bisphosphate aldolase. On the other hand, the sub-expression of the miRNAs in cases where sub-expression was also observed in the proteins, may be due to a biological response to the low expression of proteins and in this way their regulatory miRNAs also had their expression reduced.

In the case of comparison with REDS, the crossbreed showed two types of expression patterns miRNAs and proteins also: super miRNA expression and super protein expression; And sub-expression of miRNAs and super-expression of proteins. The first case, as well as sub-expression of miRNAs and sub-expression compared to CHIT, may be the result of a biological response, unlike the case of comparison with CHIT, to super-expression of proteins and, consequently, resulted in an increase in Expression of its regulatory miRNAs as an attempt to bring the expressions back to normality. Already the sub-expression of certain miRNAs in comparison to REDS resulted in the increase of the

proteins that have their genes as targets of the same miRNAs, in the case: let-7e and fructose bisphosphate aldolase; MiR-16 and fructose bisphosphate aldolase and L-lactate dehydrogenase; And miR-216b and L-lactate dehydrogenase (Table 8).

Among these miRNAs, the let-7e-sub expressed in the crossbreed with super expressed protein compared to REDS - has already shown a relationship with growth gain in previous studies (Barozai, 2012; De Santis et al., 2008; Lee, 2004) . Zhang et al. (2014), using Megalobrama amblycephala as an experimental model, showed that miR-16 is poorly expressed in animals with a high fat diet. As in the work of Zhang et al. (2014), it was shown to be sub-expressed in the crossbreed compared to REDS, and consequently, L-lactate dehydrogenase and fructose bisphosphate aldolase, which has its genes as the target of miR-16, showed over expression.

5. Conclusion

Regarding the comparison of the miRNAs and with the proteins, several miRNAs were sub-expressed and consequently there was an increase in the expression of the proteins that have targets of these miRNAs in their genes. In this case, miR-216, miR-153, miR-204 and miR205 comparing the crossbreed with CHIT; And let-7e, miR-16 and miR-216 comparing the crossbreed with REDS. These data show, as shown in other studies, that there is an action of miRNAs on heterosis.

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Anexo III: Artigo de revisão

MiRNAs in farmed fishes: tiny molecules with great impact on aquaculture

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Abstract

In the last decade, several studies have been focused onrevealing the microRNA (miRNA) repertoire and determining their functions in farm animals such as poultry, pigs, cattle, and fish. These minute non-protein coding RNA molecules (18–25 nucleotides) are capable of controlling gene expression by binding to messenger RNA (mRNA) targets, thus interfering in the final protein output. MiRNAs have been recognized as the main regulators of biological features of economic interest, including body growth, muscle development, fat deposition, and immunology, among other highly valuable traits, in aquatic livestock. Currently, the miRNAs repertoire of a limited number of farmed fish species has been identified and characterized, in order to determinethe functions of miRNAs. In this review, we summarize the current advances in miRNA diversity and functional implications for production by examining available data on 15 key species exploited by fisheries and in aquaculture worldwide. We also discuss strategies of miRNA manipulation for forthcoming research.

Keywords: aquaculture, farm animals, teleost fish, gene expression, microRNAs

1. Introduction

MicroRNAs (miRNAs) are a class of small (17–22 nucleotides), single-stranded, non-coding RNAs that inhibit gene expression post-transcriptionally by pairing with complementary sequences in their target mRNA (Box 1). These minute regulatory molecules are present in the genome of animals, plants, and even in some viruses (Lee *et al.*, 1993; Bartel, 2004; Kim *et al.*, 2009; Xia *et al.*, 2011). The first miRNAs, lin-4 and let-7, were both discovered in *Caenorhabditis elegans* (Lee *et al.*, 1993), and have subsequently been found to correspond to a novel and extensive class of small non-coding RNAs (Lagos-Quintana *et al.*, 2001; Lau *et al.*, 2001; Lee & Ambros, 2001).

Since then, numerous miRNAs have been identified and quantified in several organisms, mainly owing to frequent enhancements in high throughput sequencing technologies, bioinformatics programs, and experimental methods (Guerra-Assumpção & Enright, 2012). Together, these approaches have allowed for large-scale analysis, thereby facilitating the discovery of species-specific and novel miRNAs, even those with reduced expression, in many organisms belonging to diverse taxa (Berezikov, 2011).

Owing to the key involvement of miRNAs in the regulation of growth, metabolism, and homeostasis, among hundreds of other functions, diverse studies in the last decade have sought to uncover genuine miRNA-to-target interactions in farm animals such as poultry, pigs, cattle (reviewed by Wang *et al.*, 2013),and fish (Rasal *et al.*, 2016), to maximize production (Box 2). In addition, several patents have been developed in various countries on the commercial exploration of miRNAs, e.g., a US patent (US 2006/0246491 A1) using miRNAs to regulate muscle cell growth.

Here, we review the available data on miRNA identification and functional characterization in the most important farmed fish species globally and discuss the current challenges and future directions for the use of miRNAs in aquaculture.

2. MicroRNAs in animal breeding

Since miRNAs are the key elements in gene regulation, they have been the focus of studies on improving the health and productivity of farm animal species. Diverse studies have been conducted in cattle (Townley-Tilson *et al.*, 2010; Kozomara and Griffiths-Jones, 2011; Li *et al.*, 2011b; Miretti *et al.*, 2011), pigs (Cho *et al.*, 2010; Cirera *et al.*, 2010; Xie *et al.*, 2010; Li *et al.*, 2011a; Chen *et al.*, 2012), and birds(Hicks *et al.*, 2008, 2009; Li *et al.*, 2011c; Yao *et al.*, 2011; Wang *et al.*, 2012b;). These studies have reported that miRNAs play an important role part in a wide range of biological pathways, such as growth, metabolism, immunology, muscle development, and fat deposition. Therefore, these small RNAs are of great value in animal breeding, steering research towards the development of new solutions to current and emerging threats to the health and welfare of farm animal species.

Studies on miRNAs in fish have increasedrecently and miRNAs have been characterized for a few but very important farmed species in the aquaculture industry worldwide (Figure 1 and Table 1).

3. A systematic review of miRNAs in farmed fishes

3.1. Nile tilapia (*Oreochromis niloticus,* Cichlidae)

Nile tilapia is one of the most commonly farmed species in freshwater aquaculture worldwide. Fast growth rate and adaptation to a wide range of culture conditions are the attributes responsible for successful fish farming (Yue *et al.*, 2016).

Using bioinformatics, Loh *et al.* (2011) analyzed the evolution of miRNAs and their pairing sites in target genes in cichlid fishes from Lake Malawi (East Africa) and detected 100 cichlid miRNAs, including those from tilapia, that are highly conserved in metazoan's genomes. Brawand *et al.* (2014) also exploring cichlid miRNAs, described diverse loci in tilapia, comprising 7 novel miRNAs.

Yan *et al.* (2012a), using small RNA cloning and Sanger sequencing, discovered 25 conserved miRNAs in tilapia skeletal muscle. Huang *et al.* (2012) employed next-generation sequencing (NGS) in muscle tissue to explore the complete miRNA transcriptome, and identified the expression of 184 known mature miRNA sequences.

Functional roles of miRNAs were also evaluated under variable contexts. Several studies have investigated muscle development because muscle constitutes the major edible part of fishes, and is therefore an economically important trait. Many miRNAs have been proven to assist in the regulation of muscle growth by controlling genes involved in hyperplasia and hypertrophy (Lagos-Quintana *et al.*, 2002).

In tilapia, Yan *et al.* (2012a) and Nachtigall *et al.* (2015) showed that miR-1, miR-133a, and miR-206 have similar expression patterns in adult males and females, and may assist each other to accurately control the development of skeletal muscles, although performing distinct biological functions. MiR-1 is responsible for repressing the expression of histone deacetylase 4 (HDAC4), which is a negative regulator of cellular differentiation, and thus promotes myocyte differentiation. MiR-1 also blocks a repressor of the MEF2 (myocyte enhancer factor-2) transcription factor. MiR-133a promotes, in part, myocyte proliferation by repressing the serum response factor (SRF) (Chen *et al.*, 2006), whereas miR-206 plays an important role in regulating the differentiation of C2C12 myoblasts *in vitro* (Kim *et al.*, 2006). In addition, miR-206 loss-of-function *in vivo* was shown to significantly improve tilapia growth performance by targeting insulin-like growth factor-1

(IGF-1) (Yan *et al.*, 2013c). IGF-1 is known to play a central role in a complex system that regulates growth, differentiation, and reproduction by selectively promoting mitogenesis and cell differentiation, and inhibiting apoptosis (Jones and Clemmons, 1995; Reinecke and Collet, 1998). In many fish species, IGF-1 blood or tissue levels positively correlate with dietary protein levels and body growth rate (Beckman *et al.*, 2004; Carnevali *et al.*, 2006).Therefore, miR-206 could affect tilapia growth by modulating IGF-1 gene expression levels (Yan *et al.*, 2014). Similarly, miR-203b was shown to promote myogenesis (hyperplastic growth) by targeting MyoD (Yan *et al.*, 2013a), a key protein that initiates the cascade of regulatory events during muscle differentiation. These authors have shown that blocking miR-203b results in a significant increase in MyoD expression.

Other studies have revealed the function of miRNA in the regulation of metabolic routes that could indirectly enhance production. For instance, there is growing concern about the genetic improvement of salt tolerance in Nile tilapia, which may provide advantages such as adaptation to certain environmental conditions and higher rusticity. Tilapias are euryhaline fish, and most species can live in a wide range of salinity from freshwater to seawater, and therefore are a suitable model organism for studies on ionic and osmotic acclimation in euryhaline teleosts (Deane & Woo, 2004; Wang *et al.*, 2009). For example, miR-30c, a kidney-enriched miRNA, was shown to regulate salt tolerance, since its loss-of-function caused fish to be unable to respond to osmotic stress (Yan *et al.*, 2012b, Yan *et al.*, 2013c). Moreover, osmotic stress transcription factor 1 (OSTF1), was shown to be potentially regulated by miR-429 (Yan *et al.*, 2012c). Recently, Zhao *et al.* (2016b) showed that miR-21 is abundantly expressed and its action modulates alkalinity stress by up-regulating VEGFB and VEGFC expression *in vivo* and *in vitro*, which are responsible for regulating alkalinity tolerance. Therefore, miR-21, miR-30c, and miR-429 may be important markers for tilapia and also for other commercial species.

Another relevant topic for livestock production corresponds to miRNA roles in sex determination and differentiation. This is particularly motivating for Nile tilapia because males grow faster and more than the female. In this way, several studies have sought to uncover the molecular mechanisms of sex-determination regulated by miRNAs (Eshel *et al.*, 2014; Xiao *et al.*, 2014).

In the study by Xiao *et al.* (2014), gonads (testes and ovaries) were screened using high throughput sequencing and recovered data showed distinct miRNA expression signatures. In common, Nile tilapia testes and ovaries displayed miR-181a, miR-181a-5p, miR-143, and miR-143-3p as the most abundant miRNAs. In contrast, the miR-29 and miR-129 family showed a significantly increased expression in ovaries than in testes (Xiao *et al.*, 2014). In humans, the high expression of miR-129 in ovaries is associated with the control of cell growth and differentiation in the final process of ovary maturation through the downregulation of its target mRNAs, whereas miR-29 expression levels that progressively increase throughout oogenesis may also be important (Sirotkin *et al.*, 2009). In tilapia testes, the most abundantly expressed miRNAs families are miR-33a, miR-132, miR-135b, and miR-212 (Xiao *et al.*, 2014). In mammals, miR-212/132 expression is necessary for the development and function of neurons. Furthermore, both miRNAs have been associated with mammary gland development by downregulating the matrix metalloproteinase 9 (MMP-9), which is an activator of TGFβand is involved in cell proliferation, differentiation, and apoptosis (Kubiczkova *et al.*, 2012). miR-33a control fatty acid regulation in mammals by repressing the insulin receptor substrate 2 (Dávalos *et al.*, 2011), and its high expression in tilapia testes may contribute to testes maturation via the regulation of the insulin signaling pathway (Xiao *et al.*, 2014).

Eshel *et al.* (2014) compared miRNA expression in tilapia gonads and found nine sexually dimorphic expressed miRNAs; theydemonstrated a single upregulated miRNA in

male embryos (miR-4585), with a perfect inverse correlation in expression pattern with its six target genes: cr/20β-hsd, psmb8, rtn4ip1, casp8, atp5g3, and an non-annotated gene (down-regulated in males). cr/20β-hsd is known to be part of the oxidoreductase pathway for oocyte maturation preceding the enzymatic activity of cyp19 (cytochrome P450 aromatase) (Senthilkumaran *et al.*, 2004) and cyp19a1a was proposed to be the major gene for female determination in zebrafish (*Danio rerio*) (Rodríguez-Marí *et al.*, 2010). MiR-4585 was upregulated in male embryos at 2 and 5 days post fertilization (dpf) and decreased at 9 dpf, which indicates the significance of this miRNA in males soon after fertilization (Eshel *et al.*, 2014). Therefore, miR-4585 could possibly be manipulated for sex reversal in tilapia. Similarly, the control of cyp19a1a expression may be relevant for sex reversal of females into males, given the aforementioned differential growth between sexes. Recently, Wang *et al.* (2016) using ovaries and testes of young Nile tilapia showed that miR-17-5p and miR-20a were highly expressed in the ovaries and negatively regulated DMRT1 expression, suggesting that these miRNAs could induce estrogen production by inhibiting DMRT1 expression and promoting cyp19a1a expression in Nile tilapia. They found miR-138, miR-338, and miR-200a negatively regulated cyp17a2 (Wang *et al.*, 2016), which is involved 20b-dihydroxy-4-pregnen-3-one biosynthesis, and thus might be essential for spermatogonial cell proliferation and spermatogenesis (Eshel *et al.*, 2014). Wang *et al.* (2016) also showed that miRNAs miR-456 and miR-138 negatively regulates AMH and that a lower expression of these miRNAs promotes testis differentiation by allowing AMH to be expressed in testis.

3.2. Atlantic salmon (*Salmo salar,* Salmonidae)

Atlantic salmon is a domesticated fish of notable economic interest for wild fisheries and aquaculture production. Since 1970, Atlantic salmon has been intensively selected for

genetic traits to improve growth performance, considerably benefiting aquaculture (Bentsen & Thodesen, 2005). The increased growth of this species is estimated at approximately 14% per generation (Gjedrem, 2010). Genome knowledge of salmonids is advanced in comparison to other farmed fish species (Bekaert *et al.*, 2013); however, there are limited studies describing salmonid miRNAs.

The first screening for salmonid miRNAs was performed by Barozai (2012a), using an *in silico* approach to predict miRNAs in salmon genomes. They detected let-7a-3p as a regulator of zonadhesin-like and growth hormone 2 gene, miR-142-5p as a regulator of heparin-binding growth factor 1, and miR-144 as a regulator of the growth factor receptor-bound protein 2. They also found that miR-430 regulates the transforming growth factor-beta-induced protein ig-h3, miR-451 blocks the anti-dorsalizing morphogenic protein, and miR-1594 activates both titin-cap (telethonin)-like mRNA and growth hormone receptor isoform 2. All these miRNAs and targets are associated with growth and developmental processes.

Andreassen *et al.* (2013) characterized miRNA genes in Atlantic salmon by deep sequencing analysis of small RNA libraries from nine different tissues and revealed a total of 180 evolutionary conserved mature miRNAs and 13 distinct novel mature miRNAs. Later, Bekaert *et al.* (2013) deep sequenced miRNA libraries of young juveniles (four months old), and identified 547 miRNA transcripts that mapped to 88 miRNA distinct genes.

Johansen and Andreassen (2014) validated miR-25-3p and miR-455-5p as the best performing two-reference gene combination suitable for quantitative expression analysis in Atlantic salmon. Such findings are relevant for aquaculture because these two miRNA reference genes are now valuable for appropriate diagnostics tests, as those performed for the detection of infection by viral RNA through expression. In addition, Kure *et al.* (2013)

identified the differential expression of miRNAs subjected to an acid environment through RNA-Seq. They found 4 down- and 14 up-regulated miRNAs between exposed groups and the control, suggesting alterations in a number of physiological responses that ultimately may interfere in animal growth performance.

The modulatory effect of miRNAs in collagen formation in salmon muscle has also been investigated (Mitchie, 2001). Muscle firmness is appreciated by consumers and inadequate processing of salmon meat reduces firmness in selected adult salmons (Moreno *et al.*, 2016). The miR-29a, highly conserved between *Salmo salar* and *Danio rerio* (Andreassen *et al.*, 2013), is the major factor in collagen formation and showed low expression in human fibroblasts with systemic sclerosis (Maurer *et al.*, 2010). Thus, miR-29a may be a future target in expression studies to improve fillet quality, with a high potential for application in aquaculture.

Overall, the collection of experimentally identified Atlantic salmon miRNAs provide an important resource for functional genome research in salmonids and, in particular, helps to determine the actual contribution of miRNAs to phenotypic variation in economic and biologically fundamental characters.

3.3. Rainbow trout (*Oncorhynchus mykiss,* Salmonidae)

Rainbow trout, the most widely cultivated cold freshwater fish in the world, has been improved reagarding growth and development by animal breeding programs globally (Sae-Lim *et al.*, 2013). This species is also a model organism for genome-related research on comparative immunology, carcinogenesis, toxicology, disease ecology, physiology, evolutionary genetics, and nutrition (Thorgaard *et al.* 2002).

Ramachandra *et al.* (2008) discovered 14 miRNAs in early embryos (5dpf), among these miR-21, miR-30d, miR-92a, miR-200, and miR-26 are associated with differentiation

and development. Salem *et al.* (2010) showed that miR-133, known to be muscle enriched in mammals (Shingara *et al.*, 2005; Ason *et al.*, 2006; Chen *et al.*, 2006), is also high expressed in trout skeletal muscle, being of interest for animal breeding.

Other miRNAs involved in metabolic pathways related to growth and nutritional support have also been reported. A pioneer study by Meningen *et al.* (2012) describes the postprandial regulation of lipid and glucose metabolism by miRNAs. Among these miRNAs, they predicted conserved targets in fish and humans for miR-103, miR-107, and miR-143 homologs. Authors have also shown miR-33 and miR-122b were upregulated, whereas miR-122a was downregulated. MiR-33 and miR-122b act on the hepatic insulin pathway to stimulate lipogenesis and inhibit lipolysis (Fernandez-Hernando *et al.*, 2011). MiR-33 and miR-122b may promote lipogenesis and simultaneously inhibit lipolysis. The inhibition of miR-33 expression in mice (*Mus musculus*) resulted in a low level of VLDL triglyceride (Fernandez-Hernando *et al.*, 2011). Similarly, inhibition of miR-122 resulted in increased fatty acid oxidation and decrease fatty acid synthesis rates (Krutzfeldt *et al.*, 2005; Esau *et al.*, 2006; Elmen *et al.*, 2008). MiR-122b was inhibited when plasma triglycerides increased, a final product of the lipogenic pathway (Li *et al.*, 2009; Mennigen *et al.*, 2012). Mennigen *et al.* (2013) analyzed the expression of miRNAs by quantitative real-time RT-PCR in rainbow trout fingerlings, switching from endogenous to exogenous feeding, and showed a decrease in miRNA-33 and miRNA-122a/b isomiRs. On the basis of these studies, it is possible that down-regulation of the above miRNAs could contribute to weight gain in rainbow trout.Therefore, this miRNAcould possibly be experimentally manipulated in live animals to improve the production indices in rainbow trout.

3.4. Arctic charr (*Salvelinus alpinus*, Salmonidae)

Arctic charr displays resistance to lower temperatures, and could also provide a different niche in the market, as an alternative to rainbow trout. Although, in Iceland, Arctic charr originated from a single Atlantic lineage (Brunner *et al.*, 2001), it shows an extremely high phenotypic variation between populations and many examples of polymorphism (i.e., sympatric morphs) have been documented (Cresko *et al.*, 2004; Snorrason & Skúlason, 2004; Woods *et al.*, 2012). The Arctic charr morphs of Lake Thingvallavatn constitute an extreme example of local phenotypic diversity. Four morphs grouped into two morphotypes have been described in the lake (Snorrason *et al.*, 1989).

Kapralova *et al.* (2014) studied miRNA expression during embryonic development from two contrasting morphs: a small benthic morph from Lake Thingvallavatn (SB) and an aquaculture stock (AC).The results indicated morphs differed extensively in morphology and adult body size. These authors found a total of 326 conserved and 427 novel miRNA candidates, and from this total, 51 known and six novel miRNA candidates were differentially expressed among the developmental stages studied. Among the novel miRNAs, six miRNAs were found as differentially expressed in developmental time points. Three of these (sal-nov-1, sal-nov-5, and sal-nov-18) were differentially expressed between morphs. These authors have also reported 53 known miRNAs and 19 novel miRNA candidates differentially expressed between AC and SB embryos. MiR-130, miR-133, miR-153, miR-17, miR-30, miR-451, miR-219, miR-26, miR-199a, and miR-145 were highly expressed in AC, whereas miR-206, miR-133, miR-122, miR-181a, miR-192, miR-196a, and miR-223 in SB. The two most abundant miRNAs found, miR-1 and miR-206, exerted functions in skeletogenesis and myogenesis and have been previously studied in more detail (Liu *et al.*, 2007; Sweetman *et al.*, 2008; Inose *et al.*, 2009; Dey *et al.*, 2011).

Several of these miRNAs, including miR-1, miR-206, and let-7, have been associated with development. Kapralova *et al.* (2014) reinforced their importance in the Artic charr because miRNAs act in both osteogenesis and muscle development, being promising molecules to be examined for breeding and production purposes.

3.5. Common carp (*Cyprinus carpio*, Cyprinidae)

Originally from Eurasia, the common carp occupies a prominent global position in freshwater aquaculture. This species has been cultured for thousands of years and a many strains are distributed worldwide (Zhou *et al.* 2003). However, despite the millenary livestock and technological progress in molecular biology, little is known about its genome,Although there are some studies involving miRNAs.

Yan *et al.* (2012d) studied the expression of miRNAs in the skeletal muscle of common carp and compared their conservation to homologs in other vertebrates. Among these, miR-1, miR-133a-3p, and miR-206 were found to be expressed in heart, skeletal muscle, gut, eye, and skin tissue (Yan *et al.*, 2012d). These miRNAs and miR-21 were also upregulated in the skeletal muscle of 30-day post-hatching (dph) to 2-years-old individuals and miR-26a was up-regulated in 2-year-old individuals, suggesting that miR-1, miR-133a-3p, miR-21, miR-26, and miR-206 play roles in the growth and functional maintenance of carp skeletal muscles (Yan *et al.*, 2014d). The miRNAs, miR-27a, miR-214, and miR-222 were expressed at relatively high levels at 30 dph and then decreased in the 1- and 2-yr olds in skeletal muscle, suggesting they are important for muscular proliferation and differentiation (Yan *et al.*, 2014). Using miRNA identification, target prediction and single nucleotide polymorphism(SNP) analysis, Zhu *et al.* (2012b) identified two SNPs in potential miRNA target sites that could be important candidates for the causal variants of common carp phenotypes.

Common carp also has great ornamental value owing to the skin colors, such as red, white, orange, or black (Wang *et al.*, 2009). Yan *et al.* (2013b) identified 14 distinct miRNAs that are differentially expressed between white and red skin. MiR-25, miR-15a-3p, miR-146b, miR-184, miR-429, and miR-141 are abundantly expressed in red skin, whereas miR-18a, miR-137, miR-17a, miR-203a, miR-9-3p, miR-9-5p, miR-129-5p, and miR-204 are highly expressed in white skin. In addition, they examined the miRNA expression profile at five distinct developmental stages (zygote, blastula, gastrula, segmentation, and larvae) in common carp. Their analysis revealed that miR-429 is a positive regulator of skin color, since its loss affects the pigment content in the skin and these effects are mainly mediated by its interaction with Foxd3 (Yan *et al.*, 2013b). The data acquired provided insights into the role of miRNA in the regulation of melanogenesis, implying that miRNAs can be used as a molecular tool to obtain pure lines and ornamental carps with high commercial value.

Recently, two studies focused on the role of miRNAs in the immune system of common carp. First, Li *et al.* (2014) characterized the miRNAs expressed in the spleen tissue of common carp. They identified that let-7 and miR-21 are highly expressed and can be related to immune response, based on previous analyses in mammals (Mondol & Pasquinelli, 2012; Wang *et al.*, 2012a). Second, sequenced miRNAs from the liver tissue of common carp with *Flavobacterium columnare* (FC) infection (Zhao *et al.*, 2016a) were analyzed. They described 30 miRNAs differentially expressed between infected and non-infected animals, with miR-196b, miR-365, and miR-184 considered the most important pathogen-responsive miRNAs owing to functions previously described in humans (Maru *et al.*, 2009; Guo *et al.*, 2013a; Wu *et al.*, 2014a). These studies improved the background knowledge of miRNAs in immune system response and defense mechanisms against pathogens in common carp considerably.

3.6. Bighead (*Hypophthalmichthys nobilis,* Cyprinidae) and silver (*Hypophthalmichthys molitrix*, Cyprinidae)carp

The closely related filter-feeding fish species, bighead and silver carp, are endemic to EastAsia (Ping, 1999, 2000); however, in cage culture, silver carp grow faster (Cremer & Smitherman, 1980) because the growth and development conditions are ideal.

Chi *et al.* (2011) sequenced the miRNAs of two libraries, each with a pool of tissues (heart, liver, brain, spleen, and kidney) for the two carps, and found 108 miRNA families. Among these, 167 miRNAs in bighead and 166 miRNAs in silver carp were identified (Chi *et al.*, 2011). MiR-122, the most abundant in both carps and associated with fat deposition in pigs (Chen *et al.*, 2012), was expressed three times higher in bighead than silver carp (Chi *et al.*, 2011), implying that the control of this miRNA may be a future target for production purposes.

3.7. Grass carp (*Ctenopharyngodon idella*, Cyprinidae)

Grass carp is extensively cultivated in eastern Asia for food, being one of the most important freshwater fish globally (Liu *et al.*, 2009). Grass carp was introduced to 115 countries, and in at least 58 of these (~50%), it appears to have self-sustaining populations (FishBase, 2015a) and high performance. However, there are limited studies on its genetics.

Zhu *et al.* (2014) reported that several miRNAs are likely involved in fast skeletal muscle growth in grass carp, by analyzingthe response to quick refeeding following fasting. They recorded changes in the expression levels of eight miRNAs (miR-1a, miR-181a, miR-133a, miR-214, miR-133b, miR-206, miR-146, and miR-26a) shown to be involved in a strong resumption of myogenesis (Zhu *et al.* 2014).

Xu *et al.* (2014) studied miRNA expression stability in embryos at distinct developmental stages as well as in several tissues from adults to establish the best reference genes for quantitative expression analysis. Seven miRNAs (miR-126-3p, miR-101a, miR-451, miR-22a, miR-146, miR-142a-5p, and miR-192) were found to have optimal stability and should be individually prioritized according to the stage and tissue of interest.

Analyzing two grass carp lineages, *Aeromonas hydrophila*-susceptible (SGC) and resistant (RGC), Xu *et al.* (2015) found miRNAs related to the immune system. MiR-118 and let-7i are differentially expressed and target both tlr4 and nfil3-6 genes. Let-7i is predominantly expressed in the spleen during the bacterial infection and displays a noticeable difference in expression between SGC and RGC (Xu *et al.*, 2015). The let-7 family is known to regulate multiple genes related to the cell cycle and proliferation (Yang *et al.*, 2008), and let-7i was shown to influence innate immunity (Chen *et al.*, 2007). Recently, Xu *et al.* (2016) studied these two carps using two kidney miRNA transcriptomes from SGC or RGC infected with the highly pathogenic *A. hydrophila*, and identified nine miRNAs differentially expressed between these groups. Furthermore, the spatial and temporal expression ofa novel miRNA (cid-miRn-115) and miR-142a-3p, suggests that they are potential regulators of anti-bacterial activity (Xu *et al.*, 2016). Overexpression of these miRNAs resulted in a visible change in the immune effector activity in *C. idella* kidney cells and bioinformatics analysis shows that they directly regulate tlr5 expression (Xu *et al.*, 2016), which is associated with innate immune response (Yoon *et al.*, 2012).

Therefore, all these miRNAs interfere with grass carp health and should be further investigated for improving fish resistance to diseases that account for great economic losses in aquaculture.

3.8. Wuchang bream (*Megalobrama amblycephala*, Cyprinidae)

Wuchang bream (also known as blunt snout bream) is restrictively distributed in the middle and lower reaches of the Yangtze River in Central China and has a high economic value (Li *et al.*, 1991). The species has been farmed in freshwater polyculture systems in China since the 1960s (Ke, 1965; Wan *et al.*, 2015b). Owing to its economic value, the production of Wuchang bream has increased substantially (Wang, 2009), even with a lack of molecular resources.

Yi *et al.* (2013) described 347 mature miRNAs belonging to 123 miRNA families in tissues related to growth (brain, pituitary gland, liver, and muscle). Twenty-seven miRNAs (8 novels) were differentially expressed between big- and small-sized animals. They found miR-462, miR-92, miR-92a, and miR-23b might play roles in the immune system and diseases. In contrast, miR-462 was upregulated with a viral hemorrhagic septicemia virus (VHSV) infection in zebrafish (Cohen & Smith, 2013) and rainbow trout (Bela-Ong *et al.*, 2013);whereas miR-92a and miR-23b have been associated with myeloid cell proliferation and interleukin biosynthesis in mammals (Manni *et al.*, 2009; Zhu *et al.*, 2012a). They predicted that IGF-2a (insulin growth factor 2a) can be targeted by miR-1, miR-122, and miR-462, which indicates functions for these miRNAs in growth control mechanisms of Wuchang bream.

Zhang *et al.* (2014b) also evaluated miRNA profiling in the liver of the species using two treatment groups: NFD (5% fat diet) and HFD (15% fat diet). In animal models, the exposure to an HFD induced hepatic steatosis (Ahn *et al.*, 2011), Let-7, miR-101b, miR-103, miR-107a, miR-122, miR-140-3p, miR-192, miR-21, miR-22a, and miR-221 were the most expressed in both groups, and five (let-7, miR-122, miR-192, miR- 21, and miR-22a)

were also expressed at high levels in the human liver tissue (Girard *et al.*, 2008). Three miRNAs (miR-30c, miR-30e-3p, and miR-31) were upregulated and 9 miRNAs (miR-142b-5p, miR-145, miR-15a-5p, miR-16a, miR-18a, miR-193a, miR-19d, miR-203b-3p, miR-34a) downregulated in the HFD group (Zhang *et al.*, 2014b). In mammals, miR-30c inhibition provoked hyperlipidemia and atherosclerosis (Soh *et al.*, 2013). In the human subcutaneous adipose tissue, miR-30e may be involved in the development of obesity-related disorders (Kloting *et al.*, 2009), whereas miR-15a could promote adipogenesis by inhibiting delta-like 1 homolog (Mitterberger *et al.*, 2012). In bream, miR-30e-3p, highly expressed in the HFD, potentially targeted Fetuin-B and Cyp7a1 (cytochrome P450, family 7, subfamily A, polypeptide 1a). In addition, miR-30c is high expressed in the HFD and targets both stearoyl-CoA desaturase (SCD) and fatty acid synthase (Fas) (Zhang *et al.*, 2014b). Fetuin-B is associated with fatty acid metabolism in liver cells (Choi *et al.*, 2012) and inflammation (Olivier *et al.*, 2000); Cyp7a1 with the pathway of cholesterol removal (Pandak *et al.*, 2001); Fas with hepatic lipogenesis (Radenne *et al.*, 2008); and SCD with complex lipids synthesis, such as triglycerides and cholesterol esters (Roongta *et al.*, 2011). Fetuin-B, Cyp7a1, SCD, and Fas expression were significantly lower in the HFD than NFD group (Zhang *et al.*, 2014b). MiR-15a-5p and miR-145, were expressed less in HFD, target NADH dehydrogenase 1 beta subcomplex subunit 2 (NDUFB2) and 3-oxoacid CoA transferase 1b (Oxct1b), respectively (Zhang *et al.*, 2014b). Oxct1 is essential for ketone body oxidation (Cotter *et al.*, 2011) and NDUFB2, overexpressed in a diet-induced obesity, is involved in oxidative phosphorylation (Buchner *et al.*, 2011). Oxct1b and NDUFB2 were significantly higher in the HFD than NFD group. Zhang *et al.* (2014b) concluded that these miRNAs and their targets may be future potential pharmaceutical and nutritional targets; however, further*in vivo* and *in vitro* investigations are required.

Intramuscular bones (IB) ossified myoseptal tendons inserted into the fish skeletal muscle of mostly early ostariophysi (Ma *et al.*, 2012; Perazza *et al.*, 2016). The undesirable presence of these spicule-like bones in farmed fishes poses drawbacks for processing and consumers (Perazza *et al.*, 2016). Wan *et al.* (2015a; 2016) identified miRNAs in both tissues (IBs and CT) of six-month-old individuals, when the intermuscular bone is actively growing (Wan *et al.*, 2014).The 10 most abundant miRNAs present in the mioceptal tissue were miR-1, miR-206, let-7b, let-7a, let-7c, miR-199-3p, miR-21, let-7f, let-7d, and miR-199a-3p; however, eight of these miRNAs were also among the ten most abundant miRNAs in the CT library (miR-1, miR-206, let-7a, let-7b, let-7c, miR-199-3p, miR-21, let-7f, and miR-22a and let-7g were two remaining CT-enriched miRNAs. Wan *et al.* (2015a) speculated that the high expression of miR-206 in IB and CT could be related to the development and differentiation of IBs since miR-206, was demonstrated as a key regulator of osteoblast differentiation(Kim *et al.*, 2006; Sweetman *et al.*, 2008). Additionally, miR-125a showed lower expression in IB than in CT in mice was modulated the expression of genes involved in the mitogen-activated protein kinases (MAPKs) signaling pathway (Herrera *et al.*, 2009) and the p38 MAPK pathway, required for normal skeletogenesis (Greenblatt *et al.*, 2010).

Another study showed that miR-125b, miR-138, miR-148, miR-152, miR-181c, and miR-217 are involved in the innate and adaptive immune system of this species in response to lipopolysaccharide (LPS) treatment (Yuhong *et al.*, 2016). This study provides an important foundation for aquaculture by producing novel data on miRNA-mediated regulation in host-pathogen interactions.

3.9. Barramundi (*Lates calcarifer*, Latidae)

Barramundi, or Asian sea bass, is a high-value aquaculture fish endemic and widely farmed in Southeast Asia and Australia, and in the last decade, the species has gained increasing aquacultural interest in Europe and North America (Tucker *et al.*, 2002; O'Sullivan, 2004).

Xia *et al.* (2011) identified and characterized 63 miRNAs in the Asian sea bass, using a combination of bioinformatics and sequencing analysis . Expression profiles analyzed by qRT-PCR revealed that a group of miRNAs strongly and ubiquitously expressed in eight different organs and other miRNAs with conserved tissue-specific expression patterns (Xia *et al.* 2011).

De Santis *et al.* (2008) detected miRNAs interacting with myostatin (MSTN) in this species. MSTN is a member of the TGF-β family, which plays a key role by negatively regulating skeletal muscle growth (Lee, 2004). In rainbow trout, transgenic animals for the gene follistatin that inhibit the action of MSTN developed a double muscle phenotype (Medeiros *et al.*, 2009). De Santis *et al.* (2008) verified that miRNAs from the let-7 family interact with the 3′ UTR region from MSTN, thus suggesting a possible new direction to be undertaken for the improvement of fish productivity. However, studies are needed to determine whether there is any actual effect on the interaction of let-7 with MSTN. A previous study described polymorphism in the 3′ UTR region of the sheep MSTN gene that generates an illegitimate new target site for two miRNAs, being this point mutation associated with an increased hypertrophy of muscle fibers (Clop *et al.*, 2006). In mammals,major MSTN loss-of-function natural mutations have been identified, causing giant phenotypes without any other detectable side effects (McPherron & Lee, 1997; Sharma *et al.*, 1999; Schuelke *et al.*, 2004). In barramundi, MSTN transcripts were more highly expressed in skeletal muscle than in other tissues (De Santis *et al.*, 2008). Thus,

studies on loss-of-function of MSTN are needed in fish, wherein the use of the let-7 family may be promising.

Barramundi has reproductive traits that are disadvantageous to its production. This species reaches sexual maturity at the age of 2–3 years and are protandrous, where the fish mature first as male and become female when they grow older and larger (Carrey & Mather, 1999). Studies linking miRNAs in sexual differentiation are extremely important to improve the production of barramundi with the aim to reduce the gap from one generation to another and make the breeding and generation of commercial strains with high performance more efficient.

3.10. European sea bass (*Dicentrarchus labrax L.*, Moronidae)

European sea bass is one of the most appreciated species in Europe for aquaculture owing to its high commercial and ecological value (FAO, 2012b; Cucchi *et al.*, 2012). During its embryonic and larval phases, European sea bass lives in a marine environment,whereas juveniles migrate to coastal zones, estuaries, and lagoons.

Genome information in this species has been significantly enriched in recent years, enhancing gene and miRNAs identification and characterization (Chistiakov *et al.*, 2004; Boutet *et al.*, 2006; Guyon *et al.*, 2010; Tine *et al.*, 2014).

Kaitetzidou *et al.* (2015) investigated the transcriptome of the early developmental stages of the European sea bass, where it has been shown that some miRNAs are not critical in the earliest stage of development, as previously reported for zebrafish (Wienholds *et al.*, 2003; Wienholds & Plasterk, 2005). They found to be true for European sea bass that among 42 miRNAs, some considered classical, such as lin-4, let-7, lsy-6, and miR-273 (Wienholds & Plasterk, 2005), were not all expressed in *D. labrax*. These miRNAs control basic processes in invertebrates, including developmental timing (Lee *et*

al., 1991; Wightman *et al.*, 1991) and body asymmetry (Chang *et al.*, 2004). Kaitetzidou *et al.* (2015) also found a total of 14 miRNAs as nodes linked to modules based on expression patterns. MiR-1, which is one of the nodes, regulates transcripts involved in muscle development (Chen *et al.*, 2005; Gagan *et al.*, 2012; Yan *et al.*, 2012d). MiR-30a was determined to link to several modules, including one module with the putative direct regulation of miR-196a.

Together these miRNAs are involved in early muscle development (Ketley *et al.*, 2013), and are very important for the cultivation of this species because they could reduce theproduction timeand improve fillet quality.

3.11. Atlantic halibut (*Hippoglossus hippoglossus*, Pleuronectidae)

Atlantic halibut is the largest flatfish of the Atlantic Ocean and its early developmental stages are prolonged and morphologically defined, developing from symmetrical larvae to unsymmetrical mature fish (Pittman *et al.*, 1990; Sæle *et al.*, 2004).

The first study characterizing miRNAs in Atlantic halibut identified 199 conserved and one species-specific miRNAs during early developmental stages (Bizuayehu *et al.*, 2012b). A subsequent article described sex-biased expression of miRNAs in brain and gonads tissues (Bizuayehu *et al.*, 2012a). Furthermore, they showed up-regulation of miR-202 in gonads of mature males and in individuals within androgen treatment, indicating a feasible role of this miRNA in testis differentiation and spermatogenesis.

Bizuayehu *et al.* (2013) predicted that halibut miR-103 and miR-107 target growth hormone receptor (GHR) and follicle-stimulating hormone receptor (FSHR), whereas the luteinizing hormone receptor (LHR) is targeted by miR-138 and miR-211. Knowing that GHR, with GH cooperation, acts in the growth process, miR-103 and miR-107 may be associated with growth. These miRNAs and also miR-138 and miR-211 can also play a

role in animal breeding because they regulate FSHR and LHR, which are hormones associated with sexual maturation of females. Moreover, a luciferase assay validated the interaction between miR-24 and kiss1r-2 (Bizuayehu *et al.*, 2013), which plays a crucial role in the control of puberty in vertebrates (Gorodilov, 1996). Interestingly, miR-24 showed high expression in the testis of Atlantic halibut.All these findings indicate the regulatory mechanism of miRNAs in sex differentiation and maturation of Atlantic halibut, which can be helpful for application in fish species with regard to growth and reproduction.

3.12. Olive flounder (*Paralichthys olivaceus*, Paralichthyidae)

Olive flounder, or Japanese flounder,similar to other flatfish, has a peculiar anatomy owing to the metamorphosis that changes its body form from larvae to juvenile. This metamorphosis is characterized by the morphological transformation from a bilaterally symmetrical to an asymmetrical body shape accompanied by extensive morphological and physiological remodeling of tissues and organs (Inui *et al.*, 1995; Schreiber, 2006).

Xie *et al.* (2011) were the first to identify miRNAs related to the asymmetric development of this species. They found 23 unique miRNAs at the metamorphic stage of olive flounder indicating that miRNAs play an important role in larvae to juvenile metamorphosis. Later, Fu *et al.* (2011) identified other 140 conserved miRNAs expressed in flounder cells.

Among the miRNAs identified by Xie *et al.* (2011), stand out miR-1, miR-133 and miR-206, which are muscle enriched and may play a role in the myoblasts proliferation and differentiation (McCarthy & Esser, 2007; Townley-Tilson *et al.*, 2010). They also detected the expression of 10 members of the let-7 family, showing that this miRNAs can be regulating metamorphosis in this species (Fu *et al.*, 2011).

Fu *et al.* (2012) examined the expression of miRNAs and target genes during flounder larval development and metamorphosis. They also evaluated the effects of thyroid hormone (TH) or thiourea (TU) (regulators of metamorphosis; Schreiber & Specker, 1998) on the expression of three miRNAs and three MRFs (MyoD, Myf5 and myogenin). The expression levels of miR-1, miR-133a, and miR-206a were significantly changed by TH or TU treatment during metamorphosis from 17 to 42 dph (Fu *et al.*, 2012). At 14 dph, the pre-metamorphosis stage, the expression of MyoD and Myf5 mRNAs markedly increased compared to that of metamorphic stages. Interestingly, these expressions are remarkably higher than the myogenin expression during larval development. The results suggest that these miRNAs, together with MyoD and Myf5, might be involved in the signaling pathway of TH or TU (Fu *et al.*, 2012).

Su *et al.* (2015) found that serum response factor (SRF) is a potential target gene of miR-133a. Li *et al.* (2005) using a loss-of-function experiment revealed that SRF is essential for the control of gene expression in muscle during embryogenesis. In skeletal muscle development, the expression of a dominant negative SRF mutant blocks myoblast fusion and differentiation (Vandromme *et al.*, 1992; Croissant *et al.*, 1996; Wei *et al.*, 1998).

Fu *et al.* (2013) sought to explore how the let-7 family is distributed within the genome and their expression pattern. These miRNAs, detected from multiple genomic sites, were mainly expressed in metamorphosis and their levels changed with the addition of exogenous TH or TU. The let-7 miRNAs expression was significantly upregulated at 23dph in TH-treated larvae than that of the control, whereas the levels of let-7 miRNAs were significantly up- or downregulated at 29or 36 dph in the TH-treated larvae than that of the control. Thus, let-7 miRNAs are regulated by TH during metamorphosis (Fu *et al.*, 2013).

In addition to the mechanisms involved in the asymmetric formation, others related to cell differentiation were also investigated. Olive flounder females grow faster than males, and the sex differentiation mechanism is extremely important for fish production. Gu *et al.* (2014) identified many miRNAs associated with the sex-biased expression of miRNAs in males or females. Among these, miR-200b and miR-26a/b are distinctive In female mice, double knockout of miR-200b and miR-429 inhibited luteinizing hormone synthesis and caused anovulation and infertility (Hasuwa *et al.*, 2013).Additionally,miR-26a and miR-26b regulated gonad differentiation in olive flounder by targeting EMX2(Yin *et al.*, 2015). EMX2 is highly expressed in bipotential/indifferential gonads in mice (Pellegrini *et al.*, 1997; Miyamoto *et al.*, 1997) and were expressed at higher levels in ovary than testis tissue in olive flounder (Yin *et al.*, 2015), whereas miR-26a/b is highly expressed in testes than that of ovaries.

Further studies investigated the mechanisms mediated by miRNAs supporting disease development in flounder, using some viruses of the Iridoviridae family, whichare serious pathogens of olive flounder (Ariel and Jensen, 2009; Drennan *et al.*, 2005; Shi *et al.*, 2010). MiRNAs play role in the regulation of host-pathogen interactions (Hussain *et al.*, 2012) and virus replication (Jopling *et al.*, 2005; Singh *et al.*, 2012; Song *et al.*, 2012 Guo *et al.*, 2013b). Zhang *et al.* (2014a) experimentally observed viral and host miRNAs associated with megalocytivirus infection in olive flounder. They found 9 novel megalocytivirus and 381 flounder miRNAs differently expressed. Among these, the miR-19a regulates genes involved in several diseasepathways and is downregulated after the infection, which helps to repress the viral invasion. In contrast, miR-146 and miR-221 are upregulated with the infection and target genes that promote megalocytivirus increase, thereby preventing the viral spread.

Zhang *et al.* (2016a) also described a megalocytivirus mechanism for suppressing the host response to viral invasion by upregulating the host teleost specific miR-731. The authors described that miR-731 regulated several responses to viral invasion, and its upregulation by the virus repressed the immune response.

3.13. Channel catfish (*Ictalurus punctatus*, Ictaluridae)

Channel catfish has a prominent position in the United States aquaculture (FAO, 2012a),accounting for over 60% of the domestic aquaculture output in this country (Xu *et al.*, 2012).

Regarding catfish miRNAs, using computational search, Barozai (2012b) found a total 60 novel precursor miRNAs. Xu *et al.* (2012) found 16 miRNAs in ESTs and GSS databases by comparing the genome with precursor sequences of known conserved miRNAs. Xu *et al.* (2013) identified conserved miRNAs in channel catfish and 45 novel miRNAs using sequencing and bioinformatics tools. Most of the novel miRNAs identified were relatively less expressed than conserved miRNAs, a tendency also observed for novel miRNAs from other species (Chi *et al.*, 2011; Fu *et al.*, 2011; Yan *et al.*, 2014d), suggesting that novel miRNAs are usually weakly expressed, whereas conserved miRNAs are highly expressed. Xu *et al.* (2013) employed stem-loop qRT-PCR to validate and profile the expression of novel miRNAs in different tissues. MiR-7147 and miR-203c were highly expressed in heart and weakly in other tissues. MiR-7575 was predominantly expressed in the stomach tissue. These tissue-specific differences in miRNA expression might represent polymorphisms among individuals (Whitehead and Crawford, 2005).

Although there are limited studies on miRNAs in catfish, the results obtainedcould be applied to animal breeding. Xu *et al.* (2012) identified miRNAs that are applicable in aquaculture; Barozai (2012a,b) identified 341 proteins targeted by miRNAs whose targets

genes are involved in the immune system (32%), metabolism (12%), transportation (8%), growth and development (5%), and structural (5%) processes; and Xu *et al.* (2013) identified novel tissue-specific miRNAs that could be applied to animal breeding programs. However, further studies are required to validate the identity and function of these miRNAs, particularly those novel miRNAs.

3.14. Amur sturgeon (*Acipenser schrenckii*, Acipenseridae)

Sturgeons are referred to as living fossils and possess a key phylogenetic position for evolutionary studies (Bemis *et al.*, 1997). They have high economic and ecological value, which has resulted in increased production in several countries (Williot *et al.*, 2001; Wei *et al.*, 2004). Among the species of economic value, Amur or Japanese sturgeon, stands out, which is distributed in the Amur River (also known as the Heilongjiang River). This species is one of the most commonly cultured species in China, accounting for 50% of the total sturgeon production in this country (Zhuang *et al.*, 2002). However, it is not possible to distinguish morphologically between males and females. Therefore, studies on sturgeon miRNAs and interactions with their targets will provide a better understanding of the physiological processes of growth and assist in efficient animal breeding.

Yuan *et al.* (2014) conducted the first deep sequencing analysis on miRNAs in Amur sturgeon. These authors identified 87 high confidence miRNAs in all five tissues analyzed (liver, spleen, muscle, heart, and brain). From this total, 21 miRNAs were identified as being co-expressed in all tissues, suggesting crucial roles on sturgeon general physiology, and, 16 with tissue-specific expression, indicating that these miRNAs could be used to improve particular issues in their related tissues in sturgeon. Furthermore, functional annotation through comparison to other vertebrate species predicted that: (i) eight miRNAs (miR- 21, miR-30c, miR-126-3p, let-7c, let-7e, miR-128, miR-20b, and miR-181b) were

associated with gametogenesis (Niu *et al.*, 2011; Wu *et al.*, 2014b); (ii) miR-144, which is expressed specifically in the spleen, and miR-451, which is expressed in all the five tissues, have functional roles in the sturgeon immune system (Yuan *et al.*, 2014); (iii) miR-133a is highly expressed in muscle tissue and hasa crucial role in proliferation and differentiation of muscle and cardiomyocytes (Chiba & Misawa, 2009; Rao *et al.*, 2010; He *et al.*, 2011); and (iv) sturgeon brain-specific miRNA have crucial roles in neuronal physiology and diseases.

Another study detected 148 miRNAs differentially expressed between the ovary and testis of adult sturgeons (Zhang *et al.*, 2016b). Among these, 83 were upregulated in the ovary and 65 were upregulated in the testis. Through a target prediction putative roles of the targets were identified in gametogenesis, gonad development, meiosis, and germ cell migration and development.

The knowledge regarding miRNAs and their targets in Amur sturgeon could improve fish breeding and reproduction.

3.15. Gilthead seabream (*Sparus aurata*, Sparidae)

Gilthead seabream is produced in Europe and found in the Mediterranean Sea and the eastern coastal regions of the North Atlantic Ocean (FishBase, 2015b). Although economically important, studies involving miRNAs in this species were not directly aimed for animal breeding.

Tiago *et al.* (2014) investigated the role of miR-20a in skeletal development. MiR-20a targets BMP2, which is involved in skeletal tissue development (Yamaguchi *et al.*, 2000; Rafael *et al.*, 2006). Its upregulation also increases MGP expression, which has anti-mineralogical properties. Therefore, Tiago *et al.* (2014) suggests a role of miR-20a in skeletal calcification.

MiR-29a also plays a role in skeletal development regulating osteoblast differentiation by SPARC repression (Kapinas *et al.*, 2009). Roberto *et al.* (2014) investigated the biological effects of miR-29a in gilthead sea bream by gain-of-function, which resulted in increased extracellular matrix mineralization, possibly owing to accelerated differentiation. Although the skeleton is not an edible part of the animal, studies related to its development may support animal breeding through body conformation control, and skeletal deformities cause damages in aquaculture, tthereby reducing performance, increasing mortality rates, and downgrading fillet quality.

4. Conclusions, and Perspectives

In fish, numerous miRNAs have been continuously identified, although only a small fraction has known functions (Figure 1). Considering that miRNAs have an important function in shaping both morphological and physiological phenotypes, the analysis of miRNA profiles is potentially useful for wide applications in fish breeding.

Although limited functional studies on farm fish miRNAs have been conducted, they have provided sufficient evidence for linking miRNA activity to economic aspects, such as muscle development and hypertrophy, oocyte and testis maturation, sex differentiation, and physiological adaptation to environmental changes.

Based on the fact that interactions between miRNAs and their targets can be strongly conserved among species, several interactions verified in previous studies could be extrapolated to other fish species, thereby remarkably improving fish aquaculture. For instance, the interaction between miRNAs of the let-7 family and myostatin,which interferes in the hypertrophic growth of barramundi, may potentially be a widely conserved process in fish. Moreover, previously validated experiments carried out in one species can be easily tested in another. For example, effect of the knockdown of the miR-206 on increased body growth as in Nile tilapia could potentially be applied to other species.

Though a horizontal comparison of features revealed by the aforementioned miRNA studies, we verified that in the muscle, miR-1, miR-133a, and miR-206 have conserved expression patterns in all farmed fish species, which make these miRNAs crucial to muscle development. Some have their targets well defined, as miR-206 target IGF-1; miR-1, miR-122, and miR-462 target IGF-2a; let-7 family target MSTN; miR-103 and miR-107 target GHR and FSHR; miR-138 and miR-211 target LHR. Furthermore, some miRNAs have well-defined functions in humans and mice, and could be applicable in fishes. For example, miR-29a acts in collagen formation and many studies using this miRNA are applicable in Atlantic salmon or Nile tilapia to improve fillet quality.

Other aspects that demand attention are diseases that can affect fishes, mostly when confined, e.g., in cages, owing to various stress factors, such as water quality, density, and diets. The megalocytivirus, from the Iridoviridaefamily, for example, has received focus in research since it results inconsiderable mortality in a wide range of hosts in aquaculture, leading to great economic losses (Subramaniam *et al.*, 2012). For instance, Nile tilapia (Subramaniam *et al.*, 2016), Atlantic salmon (Crane *et al.*, 2011), rainbow trout (Ariel *et al.*, 2009; Crane *et al.*, 2011), grass carp (Subramaniam *et al.*, 2012), barramundi (Wang *et al.*, 2009), and olive flounder (Zang *et al.*, 2016) are confirmed hosts of megalocytivirus. The miRNAs miR-19a, miR-146, miR-221, and even miR-731 described by Zhang *et al.* (2014, 2016) had their expression changed during megalocytivirus infection in olive flounder; therefore they could be further studied as potential regulatory elements of immunity against megalocytivirus in all these species. Moreover, grass carp is a potential carrier of megalocytivirus, being infected without resultingin mortality or clinical changes (Subramaniam *et al.*, 2012). Thus, it can be investigated whether miRNAs differentially expressed in grass carp help the species to overcome megalocytivirus symptoms.

Control of sexual differentiation and maturation is very important in fish production.Experiments in Nile tilapia have shown that miR-456 and miR-138 inhibit testis differentiation by targeting Amh (Wang *et al.*, 2016) and the high expression of miR-4585 in males interfere in the sexual reversal mechanism. Experiments in olive flounder detected both miR-26a and miR-26b acting on testis differentiation by targeting Emx2 (Yin *et al.*,

2015). These studies indicate that these miRNAs can be applied to other species when sex is considered as an important parameter for efficient production. A study on Atlantic halibut showed that miR-138 and miR-211 plays roles in the sexual maturation of females (Bizuayehu *et al.*, 2012b). These findings could be applied to species that require faster sexual maturation of females, such as barramundi. Although some results have been informative, additional studies focusing on miRNA pathways related to sexual development in fishes will be of great value to improve fish breeding.

Environmental temperature plays a key role in maintaining the life cycle of any fish species. Understanding the molecular mechanisms involved in acclimatization at different temperatures fundamental in the current context because of global warming. In addition, these molecular mechanisms can be used to genetically select fish in aquaculture programs. For example, the cultivation of tilapias is restricted to tropical and subtropical areas, since they have a thermal comfort between 27 and 32°C. Handling and transport at low temperatures (<22°C), especially after winter, lead to drastic reduction of appetite and increased risk of disease. Furthermore, the culture of these fish at a temperature below 14°C is generally lethal. As a result, tilapia breeding companies still cannot expand their activities to low-temperature locations. Therefore, miRNAs have been proposed as molecular markers to select cold tolerant fish. In zebrafish, 25 differentially expressed miRNAs were identified in individuals cultured for 10 days at a temperature of 10°C, being related to a cellular response of adaptability to the environment (Yang *et al.*, 2011). Recently, the effect of temperature change on the expression profile of different miRNAs (e.g., miR-206) on the gonads, pituitary, and liver during the early stages of embryonic development of Atlantic cod has been demonstrated. Such alterations reflect changes, especially in the ontogenetic development of individuals, including the adult phase (Bizuayehu *et al.*, 2015). Thus, miRNAs could be studied as epigenetic markers for the selection of cold tolerant tilapias, allowing the production of tilapia in subtropical and temperate regions.

Regarding general metabolism, miRNAs that regulate lipid and glucose metabolismare highly conserved between humans and fishes. For instance, miR-103, miR-

107, miR-122, and miR-143 have constrained metabolic functions in vertebrates and can be targeted in future genetic therapies. Similarly, salt tolerance is an interesting trait and Nile tilapia has strong tolerance associated with the expression of miR-30c and miR-429, which can be broadly investigated and modulated in less tolerant species to increase their productivity. Furthermore, other desirable ornamental characteristics, such as coloration in common carp, are also mediated by the differential expression of miRNAs affecting a subset of genes, through a mechanism that can be deeply exploited in other species of economic interest.

Actually, experiments detecting the quantitative trait locus (QTL) related to important breeding traits have uncovered diverse molecular markers useful for fish production (Sonesson, 2007; Canario *et al.*, 2008). Recently, the relationship between miRNAs and QTLs has been found to be related to sexual differentiation and determination in Nile tilapia (Eshel, 2014) and to defense against viral infection in Atlantic salmon (Lowe *et al.*, 2014). Therefore, miRNAs could be markers for improving breeding in fish speciesused in aquaculture. Similarly, global identification and characterization of miRNAs and QTLs could be important to further understand how coding and non-coding DNA work together to generate attractive traits for selection programs (Cerdà & Manchado, 2013).

Another recent approach based on thecolligated assessmentof miRNAs signatures and single nucleotide polymorphisms (SNP) within QTLs has contributed to the recognition of several miRNA regulatory loci of interest.Such analyses, referred to as miR-eQTLs, possibly is being used to determine phenotypic information through the integration of QTLs polymorphism with transcriptome data, including genomic loci of miRNA that contribute to the variation in mRNAs expression levels. This analysis in European seabass identified 20.779 SNPs over 1,469 gene loci and intergenic spacers (Kuhl *et al.*, 2011), showing they can be extensively used as genetic markers in population and molecular studies. The integration of QTL and miRNA allowed the key identification of regulatory pathways involved in human liver diseases (Gamazon *et al.*, 2013). This approach could be used in fishes to assess susceptibility to diseases and other functional aspects of variable

tissues such as muscle development. Indeed miR-eQTLs are promising molecular tools for enhancing aquaculture productivity and/or species conservation.

Although microRNAs have the potential to be used as molecular markers in fish genetic breeding programs, evaluation of expression levels of these miRNAs are usually performed in specific tissues, necessitating euthanasia of the fish studied, which would make it impossible to use the animal for selection programs. The presence of miRNAs in biofluids, such as blood and urine, has been demonstrated in several studies (Harrill *et al.*, 2016) and results from leakage owing to cell injury, cell death, or active secretion. As an alternative to euthanasia for tissue collection, evaluation of circulating miRNAs markers can be used to avoid euthanasia, allowing fish to be used in the future for genetic selection. In this way, miRNAs could be evaluated in fish blood, since circulating miRNAs are already being proposed as epigenetic markers in mammals and humans (Harrill *et al.*, 2016; Kasimanickam & Kastelic, 2016).

In addition, to evaluate whether miRNAs with described functions in one species can change the phenotype of other organisms, genetic reverse techniques could be performed. This approach changes gene expression to verify its function, helping to determine the potentially beneficial aspects of different farmed fishes. Additionally, when these effects are confirmed in several species, genome editing analysis can be performed to either knock-out or knock-in a relevant miRNA in other species. Genome editing techniques have been recently improved with the CRISPR-Cas system and could positively improve aquaculture. Several papers have already described that CRISPR-Cas studies could be applied in fish, using zebrafish as a model (Bassett *et al.*, 2014).

Future research on farmed fishes should focus on understanding the evolution of fish species through the comparative analysis of transcriptomes, and also develop practical applications to maximize animal breeding programs. Studies on farmed fishes to identify interactions between SNPs and miRNAs are another potential direction. SNPs in miRNA binding sites or in the miRNA precursor sequences may change the final phenotype. This feature could provide an immediate tool for selecting farmed fish with superior production, characteristics of economic interest (e.g., color or flavor of meat), health traits or

immunity, and for improving genetics in fishes, and consequently the profits. Further studies are required on the use of miRNAs in this context and such molecular-based strategies could be useful tools in future animal breeding programs.

In conclusion, this review showed that there are a considerable number of studies focusing on the role of miRNAs in fish farming. These studies have enhanced aquaculture development. In fact, there are several conserved miRNAs involved in animal breeding, which may lead to the application of miRNAs from one fish species to other fishes or even other vertebrates. Finally, there are also some specific characteristics mediated by miRNAs transferable to other species using genome editing. Genome editing is the best technique to increase or reduce miRNAs expression, which could contribute to improving important characteristics to enhance global aquaculture production.

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Tables

Table 1. Methods for validation of miRNA targets. This table lists all techniques and tissues that were used in all species reviewed in this article.

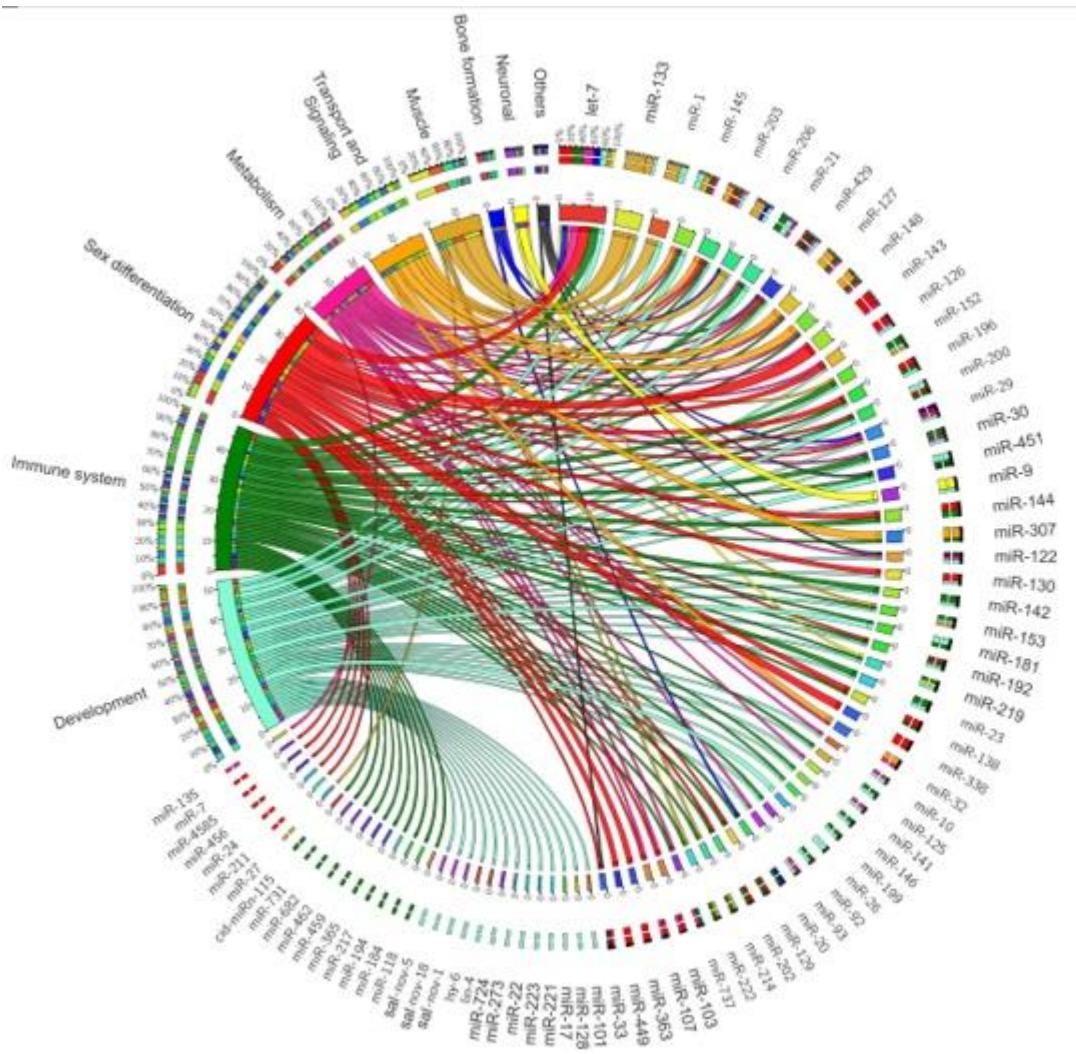
Species	Tissue	Methodology	Reference
Amur sturgeon	brain, muscle, ovary, testis and spleen	RNA-seq high-throughput sequencing with expression assay of microarray and real-time PCR	Yuan *et al.*, 2014
Atlantic halibut	brain and testis	SOLiD deep sequencing technology with expression assay of real-time PCR	Bizuayehu *et al.*, 2012b
	not described and testis	SOLiD deep sequencing technology with expression assay of real-time PCR	Bizuayehu *et al.*, 2013
Atlantic Salmon	blood	RNA-seq high-throughput sequencing	Kure *et al.*, 2013
Artic charr	embryo	RNA-seq high-throughput sequencing	Kapralova *et al.*, 2014
Barramundi	muscle	Target prediction using the miRanda software package	De Santis *et al.*, 2008
Bighead and silver carp	heart, liver, brain, spleen and kidney pool	RNA-seq high-throughput sequencing	Chi *et al.*, 2011
Channel catfish	leukocytes, liver, muscle and spleen	Computational search	Barozai, 2012
Common carp	liver	RNA-seq high-throughput sequencing with real-time PCR expression	Zhao *et al.*, 2016a
	muscle	Solexa sequencing technology	Yan *et al.*, 2012d
	skin	Solexa sequencing-by-synthesis method, expression assay of real-time PCR and Luciferase reporter assay	Yan *et al.*, 2013b
	spleen	Solexa sequencing with real-time PCR expression	Li *et al.*, 2014
European seabass	embryo	RNA-seq high-throughput sequencing	Kaitetzidou *et al.*, 2015
Gilthead sea bream	skeletal tissue	Real-time PCR, Luciferase reporter assay and western-blot analysis	Tiago *et al.*, 2014
		Real-time PCR, nothern-blot and western-blot	Kapinas *et al.*, 2009
		Real-time PCR, Luciferase reporter assay and western-blot analysis	Roberto *et al.*, 2014
Grass carp	blood	Solexa sequencing technology, real-time PCR, directional cloning and induction of expression of three immune factors in vitro.	Xu *et al.*, 2016
	embryo	Real-time PCR	Xu *et al.*, 2014
	spleen	Solexa sequencing technology, real-time PCR, target prediction and integration with mRNA expression and luciferase report system	Xu *et al.*, 2015
Nile tilapia	embryo	RNA-seq high-throughput sequencing and miRNA target prediction analysis	Eshel *et al.*, 2014
	gonads	Solexa sequencing with real-time PCR expression, miRNA target prediction analysis and miRNA pathways analysis	Wang *et al.*, 2016
		Solexa sequencing with real-time PCR expression	Xiao *et al.*, 2014
	kidney	Real-time PCR and luciferase reporter assay	Zhao *et al.*, 2016bYan *et al.*, 2012b; Yan *et al.*, 2012c and

	skeletal muscle	Prediction of MyoD-binding miRNAs, real-time PCR and luciferase reporter assay	Yan *et al.*, 2013c
		Microarray and luciferase reporter assay	Kim *et al.*, 2006
		Silencing of miR-206 in vivo using the antagomir method	Yan *et al.*, 2013b
Olive flounder	larvae/juvenille metamorphosis	Solexa sequencing technology with Real-time PCR and microarray	Fu *et al.*, 2011
	larvae/juvenille metamorphosis	Real-time PCR	Fu *et al.*, 2012
	larvae/juvenille metamorphosis	miRNA target prediction and luciferase reporter assay	Su *et al.*, 2015
	whole organism	Real-time PCR and prediction and analysis of the target genes of miRNAs	Zhang *et al.*, 2014a
		Solexa sequencing method and luciferase report assay	Zhang *et al.*, 2015
Rainbow trout	larvae	Real-time PCR	Ramachandra *et al.*, 2008
	liver	Prediction of miRNA targer gene and real-time PCR	Meningen *et al.*, 2012
	muscle	RNA-seq high-throughput sequencing and target prediction	Salem *et al.*, 2010
Wuchang bream	intermuscular bone	Illumina sequencing technology, real-time PCR and prediction of miRNA target genes	Wan *et al.*, 2015a
	liver	Illumina sequencing technology, real-time PCR and prediction of miRNA target genes	Zhang *et al.*, 2014b
	brain, pituitary, liver and muscle	Solexa sequencing technology, Real-time PCR and target prediction of differently expressed miRNAs and KEGG pathway	Yi *et al.*, 2013
	not described	Illumina sequencing technology, real-time PCR and prediction of miRNA target genes	Yuhong *et al.*, 2016

FIGURE LEGENDS

Figure 1.

MiRNAs and their pathways linked to the economic traits of farmed fishes.In this figure we present the main miRNAs associated with the main functions that reflect the direct production of farmed fish.



BOXES

Box 1. MiRNA biogenesis and action

The miRNA genes are transcribed in the nucleus by RNA polymerase II to generate primary miRNAs (pri-miRNAs). Then, the pri-miRNAs bend into hairpins and are processed by the RNase III Drosha to form precursor miRNAs (pre-miRNAs) with around 70 nt, characterized by the stem-loop structure (Lee *et al.*, 2003; Kim, 2005). The pre-miRNA is transported from the nucleus to cytoplasm by Exportin-5 and is recognized and cleaved by the RNase III Dicer to form a double-stranded RNA with 22 nt (Hutvagner *et al.*, 2001; Lund *et al.*, 2004). The double strands unbind and one of the strands enters in the RNA-induced silencing complex (RISC), becoming a mature miRNA that can exercise its gene silencing function, whereas the other strand is generally degraded or, in some cases, can also form an RISC (Khvorova *et al.*, 2003). The canonical pathway of miRNAs action occurs through miRNA-RISC complex binding to the 3′ UTR region of target mRNA, inhibiting the expression or degrading the mRNA (Lee and Dutta, 2009). It has been estimated that each miRNA may bind to hundreds of messenger RNAs and a single messenger RNA may have its stability or translation controlled by several miRNAs (Doench and Sharp, 2004; Brenneck *et al.*, 2005; Lim *et al.*, 2005).

Box 2. Finding miRNA putative targets

Searching for a true miRNA-mRNA interaction is complicated. Despite the availability of several computational target prediction tools, their results are generally inconsistent and often return variable sets of possible miRNA targets. This happens mainly because the rules governing miRNA target recognition are not completely understood and may vary for each miRNA-target interaction (Ritchie and Rasko, 2014). Moreover, several popular tools such as DIANNA-microT (Maragkakis *et al.*, 2009), miRanda-miRSVR (Betel *et al.*, 2010), and miRWalk (Dweep *et al.*, 2011) were designed exclusively for predicting miRNAs in mammals. Other tools, such as TargetScan (Garcia *et al.*, 2011), miRanda (Enright *et al.*, 2003), and PITA (Kertesz *et al.*, 2007) algorithms are frequently used as target prediction tools for animal miRNAs (Witkos *et al.*, 2011; Reyes-Herrera and Ficarra, 2012; Dweep *et al.*, 2013; Peterson *et al.*, 2014).

Since the target prediction tools lack consistency, once putative targets have been selected, validation experiments must be performed to confirm if the interaction is genuine. The majority of strategies for miRNA-to-target validation rely on the overexpression and/or knockdown of the miRNA of interest and corresponding surveillance of the effect at the mRNA/protein levels of predicted target genes. To evaluate the mRNA and/or protein expression profiles associated with the manipulation of a specific miRNA, several functional analyses have been widely applied (Table 1).