MICRORNAS E SEU PAPEL NO DESENVOLVIMENTO E DIFERENCIAÇÃO SEXUAL DA TILÁPIA DO NILO (*OREOCHROMIS NILOTICUS*)

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BOTUCATU - SP

2015

UNIVERSIDADE ESTADUAL PAULISTA

"Júlio de Mesquita Filho"

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> Tese apresentada ao Instituto de Biociências, Câmpus de Botucatu, UNESP, para obtenção do título de Doutor no Programa de Pós-Graduação em Ciências Biológicas (Genética).

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Agradecimentos

A Deus por me guiar em todas as minhas decisões e por me proteger sempre.

Aos meus pais que sempre primaram pela minha educação, e o fizeram da forma mais doce que poderiam. Se cheguei até aqui, foi porque sempre tive quem me incentivasse, com a certeza de que caso precisasse recuar, seria acolhida com amor.

Ao meu irmão Eduardo que é meu mais leal amigo e companheiro de todas as horas! Te amo, truta!

Ao meu marido Thiago que com seu amor e companheirismo sempre esteve comigo, mesmo longe. Obrigada por andar comigo nessa longa e tortuosa jornada, mas que ao seu lado se faz muito prazerosa.

Ao meu orientador Cesar Martins, agradeço por receber-me em seu laboratório e por todas as oportunidades únicas que tive durante o período em que aqui estive. Obrigada pela paciência e pelos ensinamentos.

Aos meus colegas de laboratório Adauto, Érica, Bruno, Bianca, Natália, Diego, Diogo, Rafael, Farhan, Maryam e Spike, muito obrigada por me ajudarem em meu crescimento profissional. Obrigada pelas risadas, pela companhia para ir comer o "Bolo de Leite Ninho" mais incrível do mundo e por todo companheirismo!

Aos meus amigos brasileiros e brasileiro-ingleses por todo companheirismo, risadas, "foras", conselhos... enfim, obrigada por estarem comigo sempre que precisei! Vocês são minha família do coração!

A pós-graduação em genética, por possibilitar condições muito boas para o desenvolvimento dos pós-graduandos.

Ao departamento de Morfologia, ao instituto de Biociência e a UNESP de Botucatu, pela infraestrutura fornecida para o desenvolvimento desse trabalho.

Aos professores membros da banca de qualificação e de defesa, por terem dispendido um precioso tempo contribuindo para essa tese. Assim, considerem-se agentes ativos na minha formação profissional.

A Dra. Andrea Münsterberg, por receber-me de braços abertos em seu laboratório e por ser sempre tão gentil e solícita todas as vezes que precisei dela.

Aos meus colegas ingleses de laboratório que me receberam com carinho e respeito e que me ajudaram a enxergar a ciência de uma forma eficiênte a aplicada.

A University of East Anglia, pela estrutura e profissionais maravilhos que ajudaram muito na condução dessa tese.

A FAPESP e CAPES pelo auxílio financeiro, imprescindível para o desenvolvimento desse trabalho.

Muito obrigada!

"Com as perda, só há um jeito: Perdê-las. Com os ganhos, o proveito é saborear cada um como

uma fruta boa da estação"

Lia Luft

Índice

Resumo

A Tilápia do Nilo é uma espécie de grande relevância na aqüicultura mundial. Espécimes do sexo masculino têm maior crescimento quando comparado as fêmeas, uma característica importante em sistemas de produção de peixe. Infelizmente, os mecanismos envolvidos no desenvolvimento que levam à diferenciação sexual em peixes ainda são pouco compreendidos. Vários genes e miRNAs tem sido propostos como influênciadores desses processos, porém seu efetivo papel, permanece desconhecido. Considerando a importância biológica do desenvolvimento embrionário e determinação do sexo, microRNomas de tilápia do Nilo, expressos durante o desenvolvimento e em indivíduos machos e fêmeas adultos, foram analisados. Bibliotecas de pequenos RNAs foram geradas a partir de embriões de 3 e 5 dias pós fertilização (dpf), e cérebro e gônadas de indivíduos adultos de ambos os sexos, e seqüenciados via plataforma Illumina. Foram identificados 194 miRNAs já conhecidos com base em bancos de dados referência de zebrafish, sendo que destes 120 apresentaram expressão diferencial entre macho e fêmea de *O. niloticus*. Foram identificados ainda 74 novos miRNAs não presentes no banco de dados referência. Vinte e dois miRNAs foram testados por qPCR e cinco tiveram seus genes-alvo candidatos testados pelo ensaio de gene repórter da luciferase. Os resultados de qPCR mostraram que a expressão da família do miR-10 foi alta durante os períodos de 3 e 5dpf. *Softwares* de predição sugerem que essa família de miRNAs regula a expressão de membros dos genes Hox. Sendo assim, foram testados por qPCR e pelo ensaio do gene réporter da luciferase três genes dessa família: HoxA3a, HoxB3a e HoxD10a. Os resultados mostraram que miR-10b tem como alvos Hoxb3a e HoxD10a. Já na diferenciação do sexo, os resultados de qPCR apontaram uma alta expressão do miR-145-5p durante o desenvolvimento de embriões de fêmea, enquanto seu possível alvo, Sox9a, teve expressão reduzida. Por outro lado, a expressão do miR-181a diminuiu e do seu gene alvo, Cyp191a, aumentou. Os ensaios do gene repórter da luciferase validaram o Cyp19a1 como um alvo direto do miR-181a, e o Sox9 como alvo do miR-145-5p. Nossos dados sugerem que, assim como nos demais vertebrados, os genes Hox são de extrema importância no desenvolvimento da tilápia do Nilo e que são regulados por membros da família do miR-10. Quanto a diferenciação sexual, os miR-145-5p e miR-181a são importantes no gatilho de diferenciação do sexo masculino e feminino, respectivamente, regulando os genes Sox9 e Cyp19a1.

Abstract

The Nile tilapia is a species of great importance in world aquaculture. Male specimens have increased growth compared with females, an important feature in fish production systems. Unfortunately, the mechanisms involved in the development and sexual differentiation in fish are still poorly understood. Several genes and miRNAs have been reported as influencing these processes, but its effective role remains unknown. Given the biological importance of miRNAs to embryonic development and sex determination, microRNomes were analyzed during development and in adult male and female of *O. niloticus*. Small RNAs libraries were generated from 3 and 5 dpf embryos, and brain and gonads of adult specimens, and sequenced using Illumina platform. Out of 194 miRNAs identified based on zebrafish database, 120 showed differential expression between male and female of *O. niloticus*. Additionally, 74 new miRNAs not detected in zefrafish database were found. Twenty-two miRNAs were tested by qPCR and five had their target candidate genes tested by luciferase reporter gene assay. The qPCR results showed that miR-10 family expression was high during the periods of 3 and 5dpf. Prediction software suggested that this family of miRNAs regulate the Hox genes family members. Therefore, were tested by qPCR and luciferase gene reporter assay, three Hox genes: Hoxa3a, HoxB3a and HoxD10a. The results showed that miR-10b have Hoxb3a and HoxD10a as target. In sex differentiation, the results of qPCR showed a high expression of miR-145-5p during the development of female embryos, while its possible target, Sox9, decreased. Moreover, the miR-181a expression decreased and his target gene, Cyp191a increased. The luciferase gene reporter assay validated the Cyp19a1 as a direct target of miR-181a, and Sox9 as a target of miR-145-5p. Our data suggest that, as in other vertebrates, Hox genes are extremely important in the development of Nile tilapia and are regulated by miR-10 members family. Considering sexual differentiation, miR-145-5p and miR-181a are important to trigger differentiation of male and female respectively, and act in the regulation of Sox9 and Cyp19a1 genes.

1. Introdução

A presente tese é focada no papel biológico dos microRNAs no desenvolvimento e determinação/diferenciação sexual de peixes, utilizando a tilápia (*Oreochromis niloticus*) como modelo experimental. Esse tema foi abordado utilizando análises de seqüenciamento em larga escala de bibliotecas de pequenos RNAs, qPCR e análise do gene repórter da luciferase. Nesse contexto, a introdução dessa pré-tese está dividida em quatro tópicos principais: 1.1 –MicroRNAs e a Regulação da Expressão Gênica; 1.2 - Papel dos microRNAs durante o desenvolvimento; 1.3 - Sistema de determinação e diferenciação sexual em vertebrados; 1.4 - Papel dos microRNAs na determinação do sexo.

1.1 MicroRNAs e a Regulação da Expressão Gênica

Micro-RNAs (miRNAs) são pequenos RNAs reguladores não codificantes com tamanho variando de 17 a 25 nucleotídeos (miRBase, http://microrna.sanger.ac.uk/). A definição de miRNA é baseada na sua formação pela ação da enzima RNase III (Dicer), uma RNase que processa precursores com estrutura de hairpin (conhecidos como pré-miRNA) originando o miRNA maduro (Ambros, 2003). Os miRNAs reprimem pós-transcricionalmente a expressão gênica pelo reconhecimento de locais complementares na região 3' não traduzida (3' UTR) de seus RNAs mensageiros alvos.

Atualmente, mais de 10000 miRNAs de 115 espécies estão registrados no banco de dado miRBase (miRBase v. 14.0); são conhecidos 721 miRNAs humanos, mas esse número pode aumentar, pois os estudos predizem a existência de aproximadamente 1000 miRNAs (Bentwich et al., 2005; Berezikov et al., 2005). Os miRNAs são nomeados como miR- mais números (ex: mir-133), entretanto, existem algumas exceções. Os miRNAs de seqüências similares são geralmente distinguidos por uma letra adicional seguida do número do miRNA (ex: miR-133a). Um miRNA com uma seqüência madura idêntica pode aparecer em diferentes loci genômicos com diferentes seqüências precursoras. Nesses casos, os diferentes genes de miRNA são usualmente distinguidos por outro número adicional no final da seqüência (ex: mir-133a-1).

A maioria dos genes de miRNAs possuem múltiplas isoformas (parálogos), as quais são provavelmente o resultado da duplicação de genes. Por exemplo, o genoma humano possui 12 loci para os miRNAs da família let-7. Parálogos geralmente possuem seqüências idênticas de nucleotídeos nas posições 2-7 relativa à extremidade 5' do miRNA. Como esses seis nucleotídeos, chamados de semente (*seed*, do inglês), são fundamentais no pareamento

das bases com o mRNA alvo, acredita-se que os parálogos atuem de forma redundante. Entretanto, como as seqüências 3' dos miRNA também contribuem para a ligação ao alvo e como o padrão de expressão desses miRNAs são geralmente diferentes entre si, membros da mesma família podem apresentar diferentes papéis *in vivo* (Ventura et al., 2008).

Aproximadamente 50% dos loci de miRNA de mamíferos são encontrados muito próximos de outros miRNAs. Esses aglomerados de miRNAs são transcritos a partir de uma única unidade de transcrição policistrônica (UT) (Lee et al., 2002), embora existam casos excepcionais nos quais miRNAs individuais são originados a partir de promotores de genes distintos. Alguns miRNAs são gerados a partir de UT não codificantes, enquanto outros são codificados por UT que codificam proteínas (Kim et al., 2009). Aproximadamente 40% dos loci de miRNA estão localizados na região intrônica de transcritos não codificantes, enquanto aproximadamente 10% estão localizados na região exônica de UT não codificantes. Os miRNAs em UTs que codificam proteínas são geralmente encontrados em regiões intrônicas, as quais correspondem à aproximadamente 40% de todos os loci de miRNA. Alguns genes de miRNAs podem estar entre os grupos de miRNA intrônicos ou exônicos, dependendo dos padrões de *splicing* alternativo.

A biogênese de um miRNA começa com a síntese de um longo transcrito primário conhecido como pri-miRNA. Os pri-miRNAs são preferencialmente transcritos pela RNA polimerase II e mantém características tais como estrutura de cap na sua região 5' e cauda de poli (A) na sua região 3' (Lee et al., 2004; Kim et al., 2005). Entretanto, outras vias geram um conjunto menor de miRNAs, especialmente a partir de repetições genômicas. Por exemplo, a RNA polimerase III é responsável pela transcrição de miRNAs em repetições Alu (Borchert et al., 2006).

No núcleo, o pri-miRNA é processado para pré-miRNA pela enzima RNAse III (Drosha), a qual requer um cofator, a proteína DGCR8 (*DiGeorge Syndrome critical region gene 8*) em humanos (Pasha em *D. Melagonaster* e *C. Elegans*) (Landthaler et al., 2004). A DGCR8 forma com a Drosha um grande complexo conhecido como complexo microprocessador, que em humanos possui ~ 650 kDa (Gregory et al., 2004). Esse complexo microprocessador reconhece e cliva o pri-miRNA, originando uma molécula com estrutura de hairpin de aproximadamente 70 pb, o pré-miRNA (Han et al., 2006). Um subconjunto de miRNAs, conhecidos como miRtrons utiliza uma via alternativa, na qual os pré-miRNA são derivados como produto secundário de um evento de *splicing*, sem a necessidade de processamento pela Drosha (Ruby et al., 2007). Após o processamento nuclear, cada pré-miRNAs é exportado para o citoplasma pela exportina-5 (EXP5), membro da família de

receptores de transporte nuclear (Lund et al., 2004) sendo convertido para miRNAs maduro e funcional pela Dicer (Hutvagner et al., 2001). Após a clivagem pela Dicer, a molécula de dupla fita de RNA com aproximadamente 22 nucleotídeos associa-se à proteína Argonauta para formar o complexo de silenciamento induzido por RNA (RISC) (Kim et al., 2009). Uma das fitas de aproximadamente 22 nucleotídeos do RNA dupla fita permanece na proteína Argonauta como o miRNA maduro (fita guia ou miRNA), enquanto a outra fita (fita passageiro ou miRNA*) é degradada (Rand et al., 2005). Da mesma maneira como ocorre a seleção de um siRNA (Salzman et al., 2007), a fita que possui sua extremidade 5' formando o duplex mais instável com sua fita parceira parece preferencialmente sobreviver como o miRNA no RISC (Han et al., 2006 en Schwarz et al., 2003). O complexo miRNA-RISC interage com sítios ligantes da região 3' UTR do RNA mensageiro alvo inibindo a expressão ou degradando o RNA mensageiro alvo (Lim et al., 2005; Lee et al., 2009). A interação entre o RNA mensageiro alvo e o complexo miRNA-RISC ocorre devido à complementaridade total ou parcial de uma seqüência de 5-7 nucleotídeos do miRNA e do RNA mensageiro alvo (Sen et al., 2005).

Estima-se que cada miRNA possa se ligar a muitos RNAs mensageiros, e que os RNAs mensageiros podem ter sua estabilidade ou tradução regulada por mais de um miRNA (Doench et al., 2004). Os alvos de miRNAs reguladores parecem trabalhar de forma orquestrada para controlar uma via ou função biológica comum (Esau et al., 2006; Leung et al., 2007); essa característica única dos miRNAs os tornam ferramentas eficientes para determinação de vias específicas envolvidas em processos biológicos.

1.2 Papel dos microRNAs durante o desenvolvimento

Estudos vem demonstrando o papel dos microRNAs durante o desenvolvimento embrionário em vertebrados, sendo alguns destes também conduzidos em peixes teleósteos. Embriões e larvas de zebrafish mutantes, sem a presença da enzima Dicer1, tiveram lenta taxa de crescimento e sobrevida de apenas 2 semanas (Wienholds et al., 2005). Da mesma forma, embriões com Dicer materna e zigótica mutantes tiveram defeitos morfogenéticos durante a gastrulação, formação do cérebro, somitogênese e no desenvolvimento do coração (Giraldez et al., 2005). Neste último estudo, injeções de miR-430 em embriões deficientes em Dicer, tiveram o processo de gastrulação parcialmente resgatado e os defeitos de formação do ventrículo cerebral reparados, indicando que os miRNAs são fundamentais nesse processo.

Além disso, alguns microRNAs como o miR-430 só são transcritos, após a ativação do genoma zigótico, realizado por fatores de transcrição maternos (Lee et al., 2012).

Os fatores de transcrição são componentes importantes durante toda a vida animal, e em especial, durante o desenvolvimento. Algumas dessas moléculas são codificadas por uma família gênica muito relevante no processo de desenvolvimento, os genes Hox. Esses genes têm conservado papel na padronização do eixo ântero-posterior durante a embriogênese em animais bilateriais (Alexander et al., 2009). Os mecanismos de regulação da transcrição destas moléculas são ditadas pela organização dos genes em *clusters* (Duboule et al., 2007). Este tipo de organização genômica permite o compartilhamento de espaço nuclear, estrutura da cromatina, elementos reguladores comuns e até mesmo os promotores. Como resultado, o tempo e local de expressão de genes Hox são em grande parte determinada pela posição relativa de cada gene no seu conjunto (Duboule et al., 2007). Sendo assim, sítios formadores de microRNAs que regulam a expressão dos genes Hox, geralmente, estão nos mesmos *clusters*, ou em *clusters* próximos, aos de seus alvos, como o miR-10c e seu alvo HoxB4a em zebrafish (Woltering & Durston., 2008).

1.3 Determinação sexual em vertebrados

A existência de dois sexos é quase universal no reino animal e a diferença morfogênica das gônadas é similar entre os vertebrados, porém os mecanismos de determinação do sexo, são consideravelmente variáveis (Bronwyn & Sinclair, 2002). Embora o sexo possa ser determinado por vários mecanismos em vertebrados, as gônadas são estrutural e funcionalmente muito semelhantes. Sendo assim, é possível que mecanismos genéticos de determinação do sexo sejam parecidos nos diferentes organismos.

Nos peixe, a gama de tipos de diferenciação gonadal já foram descritas e incluem espécies de um único sexo, que possuem tecidos puramente ovarianos ou testiculares, bem como espécies hermafroditas. A determinação do sexo em peixes é um processo muito flexível com relação a padrões evolutivos observados entre os gêneros e famílias, e dentro dos indivíduos está sujeita a alteração por fatores externos (Devlin et al., 2002). Estas influências podem afetar o destino de ambas as células, somáticas e germinativas primordiais no desenvolvimento das gônadas. Esses fatores incluem: a ação de uma base genética, ação ambiental (temperatura, salinidade e fotoperíodo, por exemplo), comportamental e de fatores fisiológicos (Baroiller et al., 1999; Devlin et al., 2002).

A manipulação de alguns desses fatores tem orientado a diferenciação do sexo em peixes. O mais usual tem sido o tratamento com esteróides sexuais em peixes, répteis, anfíbios e, em certa medida, nas aves. Durante as últimas décadas, grande parte das pesquisas envolvendo peixes tem incidido sobre a implicação desses hormônios na diferenciação do sexo (Guiguen et al., 2000).

Outro fator que vem sendo amplamente explorado é a determinação genética do sexo. Nos peixes teleósteos, existem dois sistemas genéticos determinantes que envolvem controle poligênico, fatores dominantes combinados com controles autossômicos, ou um controle cromossomo sexual, com machos heterogaméticos (sistema genético XX/XY) ou fêmeas heterogaméticos (ZZ/ZW sistema genético) (Devlin et al., 2002). A tilápia do Nilo possue o sistema cromossômico XX-XY, o mesmo de muitos vertebrados, porém o gatilho da determinação do sexo na espécie ainda é desconhecido.

O início da diferenciação sexual em *O. niloticus* acontece de 5-6 dias após a fertilização (dpf) dos ovos (Ijiri et al., 2008), estendendo-se até o 15° dpf. Porém, a observação das gônadas morfologicamente diferenciadas, só é possível entre 23-26 dpf (formação da cavidade ovariana na gônada XX ou os ductos eferentes na gônada XY).

Estudos tem relatado ainda, alguns genes importantes nesse processo. Em therianos, o sexo tem sido atribuido ao gene SRY (região sexo-determinante no cromossomo Y) onde encontra-se o gene da proteína HMG-box 9-relacionada a Sry (Sox9), que desempenha um papel importante na cascata do desenvolvimento dos machos em vertebrados. Em peixes, em especial, influenciam a formação de túbulos testiculares (Ijiri et al., 2008). Em ratos, o Sox9 medeia o início da expressão do gene do hormônio anti-Mülleriano (Amh) em células de Sertoli (Yao & Capel., 2005). O gene Amh tem sido apontado como responsável pela regressão dos Ductos de Müller em machos. Em tilápia, o *amh* parece ser expresso antes do Sox9, o mesmo observado em frango e em répteis (Smith e Sinclair., 2004; Shoemaker et al., 2007).

O estrogênio, por sua vez, age nas gônadas induzindo o desenvolvimento ovariano de vertebrados (Yamamoto, 1969; Nakamura et al., 1998). A enzima aromatase (gene Cyp19) catalisa a conversão de androgênios em 17β-estradiol (Baroiller et al., 1999) e se inibida, bloqueia a produção de estrogênio, causando a reversão de uma fêmea em macho (Guiguen et al., 1997; Kwon et al., 2000). No desenvolvimento dos ovários em peixes XX o Cyp19a1 tem função importante (Ijiri et al., 2008), apresentando níveis mais elevados nos futuros ovários a partir do 9 dpf e aumentando rapidamente até 19 dpf. O promotor de Cyp19a tem ligação com regiões para SF-1/Ad4 BP, WT1-KTS e SRY, genes importantes na determinação do sexo em

mamíferos (Baroiller et al., 2008). A diminuição na expressão de Cyp19a pode ser vinculada a formação do testículo, pois diminui os níveis de 17β-estradiol, levando a diferenciação do macho (Chang et al., 2005).

Outro gene importante na diferenciação do ovário é Foxl2 (Forkhead box L2), um fator de transcrição envolvido na formação do ovário em mamíferos, aves e truta arco-íris (Loffler et al., 2003; Baron et al., 2004). Na tilápia, o Foxl2 é expresso em gônadas XX e XY logo no início do desenvolvimento (9dpf) e aumenta linearmente seus níveis de expressão até a completa formação dos ovários (Ijiri et al., 2008). Foi observado em vertebrados, que padrões de expressão desse gene foram altamente correlacionados com a expressão de Cyp19a (Ijiri et al., 2008; Baroiller et al., 2008).

Muitos estudos tem comparando a expressão desses genes entre espécies e vem demostrando, que apesar de padrões conservados durante o desenvolvimento das gônadas, existem diferenças espaciais e temporais, e isso pode indicar diferentes funções em cada espécie (Baroiller et al., 2008).

1.4 Micro-RNAs na diferenciação sexual

É sabido que os genes sofrem regulação pós-transcricional. Esse processo é realizado por pequenos RNAs não codificadores, que na maioria das vezes, ligam-se a região 3'UTR dos mRNAs impedindo-os de serem traduzidos. Sendo assim, os genes que norteiam a diferenciação sexual, provavelmente também passem por essa regulação e sejam modulados pela expressão dessas pequenas moléculas de controle.

Estudos têm sugerido que os miRNAs estejam envolvidos nos processos de desenvolvimento embrionário e organizacional dos tecidos (Aboobaker et al., 2005). E por esse motivo, surge a hipótese de que diferenciação sexual na tilápia do Nilo poderia estar relacionada à presença de miRNAs distintos ou diferencialmente expressos entre os sexos durante o período de desenvolvimento.

A expressão diferencial de miRNAs relacionada ao sexo foi recentemente detectada no genoma de vários organismos, desde urocordados até diversos grupos de vertebrados como aves e mamíferos (Bannister et al., 2009; Ciaudo et al., 2009). No genoma de peixes, a existência de miRNAs diferencialmente expressos entre machos e fêmeas necessita ainda ser elucidada.

Com a recente conclusão do seqüênciamento do genoma de *O. niloticus* (Brawan et al., 2014), acessível pelo banco de dados BouillaBase (bouillabase.org), valiosas informações

estão disponíveis para análise estrutural e funcional do genoma desta espécie. O uso da tilápia do Nilo como um modelo dinâmico de desenvolvimento traz muitas vantagens para o estudo de miRNAs, sendo de considerável relevância a possibilidade de análise comparativa da presença e abundância de transcritos de RNA durante o desenvolvimento por seqüenciamento de nova geração. Esse tipo de análise foi realizada no genoma completo de *zebrafish* e possibilitou o mapeamento cromossômico e a determinação da organização estrutural dos genes de miRNAs (Thatcher et al., 2008). No caso da tilápia do Nilo, existem poucos miRNAs descritos (26 segundo o banco de dados miRNEST), sendo assim, a exploração de dados do genoma completo atrelada à análise da composição e expressão global dos miRNAs pode trazer contribuições tanto teórico-evolutivas quanto prático-aplicadas. Em trabalho recente utilizando ciclídeos (Brawand et al., 2014) foram identificados 1.344 locos de miRNA em dados de sequenciamento em larga escala de embriões. Comparando esses locos com miRNAs conhecidos em teleosteos foram descobertos 40 casos miRNA e nove casos de perda de miRNA; quatro miRNAs maduros distintos com mutação(s) na seqüência sementes (*seed*); um caso de região semente inconstante e 92 miRNAs distintos com mutação(s) fora da sequência semente (Brawand et al., 2014).

Dessa forma, estes trabalhos iniciais de miRNAs em *O. niloticus* abrem a perspectiva de que tais moléculas de fato apresentam papéis importantes na biologia da espécie e em que momentos do desenvolvimento tem sua ação, contribuindo para o avanço do conhecimento e norteando futuros estudos.

2. Hipótese e objetivos

2.1 Justificativa e hipótese

Trata-se de senso comum que mecanismos relacionados ao desenvolvimento dos organismos possuam uma base muito conservada, já que são indispensáveis para a manutenção da vida do indivíduo e consequentemente a manutenção da espécie. Nesse contexto, temos processos biológicos importantes ao longo do desenvolvimento, como formação de tecido, órgãos e sistemas. Na produção animal, o processo de diferenciação sexual é de grande interesse. Embora a existência de dois sexos seja quase universal no reino animal, sendo a diferença morfogênica das gônadas similar entre os vertebrados, os mecanismos de determinação destas, são consideravelmente variáveis (Bronwyn & Sinclair, 2002). De fato, apesar da grande variedade de mecanismos de determinação sexual nos vertebrados, é observado uma conservação na determinação e desenvolvimento das gônadas

tanto em nível histológico quanto molecular. Em nível molecular, é compreendido que um mesmo conjunto de genes atuem de forma semelhante nos diversos mecanismos existentes, podendo diferir principalmente no seu padrão temporal de expressão (revisado por, Graves & Peichel, 2010) e que miRNAs atuem modulando a expressão desses genes. Nesse contexto, usando a tilápia do Nilo como modelo, o presente trabalho baseia-se na hipótese de que as interações entre genes e microRNAs que agem no início do desenvolvimento embrionário e consequentemente, no início das vias de determinação sexual em vertebrados são conservadas, porém os níveis de expressão podem ser diferentes.

2.2 Objetivos

-Identificar miRNAs importantes ao longo do desenvolvimento e na diferenciação sexual de *O. niloticus*, a partir de bibliotecas de miRNAs de diferentes períodos de desenvolvimento;

-Avaliar a expressão dos miRNAs selecionados, em diferentes períodos de desenvolvimento e em tecidos adultos (gônada e cérebro);

-Identificar genes-alvo dos miRNAs selecionados.

3. Material e Métodos

3.1 Material biológico

Todos os procedimentos envolvendo animais foram realizados de acordo com os princípios do Comitê de Ética em Experimentação Animal - Instituto de Biociências de Botucatu - UNESP (protocolo 34/08). Todos os peixes foram anestesiados com benzocaína, antes de serem sacrificados.

Embriões de sexo desconhecido e adultos de *O. niloticus* foram coletados na Royal Piscicultura, Jundiaí, São Paulo, Brasil. Os embriões foram retirados da boca de fêmeas e selecionados com base na morfologia (Fujimura e Okada., 2007), utilizando-se lupa estereoscópica. Embriões de cruzamentos selecionados foram obtidos no Intitute of Aquaculture, da Universidade de Stirling, Stirling, Escócia.

As bibliotecas de pequenos RNAs foram contruídas utilizando embriões de 3 e 5 dias após a fertilização (dpf) (*pools* de cinco embriões inteiros em cada período). Estas fases foram baseadas em estágio de diferenciação celular do ciclo de desenvolvimento embrionário nesta espécie (Fujimura & Okada, 2007; Ijiri et al., 2008). Tecidos adultos de gônada e cérebro

(*Pool* de dois animais por tecido) de macho e fêmea também foram utilizados para construção de bibliotecas.

Para a validação dos dados encontrados nas bibliotecas, foram utilizados embriões de sexo conhecido (5dpf), provenientes de cruzamentos entre fêmea XX e macho YY e fêmea XX e macho XX, e embriões de sexo desconhecido (1, 3, 5, 16 e 30 dpf). Além disso, foram utilizados ainda tecidos adultos: cérebro e gônada de macho (n=5) e fêmea (n=5). Os embriões foram colocados numa solução de benzocaína, congelados em nitrogênio líquido e armazenado em -80 °C até o isolamento de RNA. Os tecidos provenientes dos indivíduos adultos foram coletados após sacrifício dos mesmos, seguindo os mesmos procedimentos utilizados em embriões.

Preparação de bibliotecas

3.2 Extração de RNA total

A extração do RNA total dos diferentes tecidos foi realizada utilizando o protocolo de TRIzol (Life Technologies). O RNA total foi ressuspendido em água destilada e autoclavada (tratada com Dietilpirocarbonato-Sigma® - DEPC, a 0,01%), e armazenado a -80 ºC.

A qualidade e quantidade do RNA total extraído foi mensurada utilizando-se o espectrofotômetro NanoVue (GE Healthcare Life Sciences). Com base na densidade óptica (OD) 260 nm (específica para ácidos nucléicos), foi verificada a quantidade do RNA. Além disso, as razões das absorbâncias 260/280nm e 260/230nm, superiores a 2,0 e 1,8 respectivamente, forneceram uma estimativa da qualidade da extração.

3.3 Análise da integridade do RNA total

Após a quantificação do RNA extraído, foi realizada a avaliação da integridade do material. O processo é realizado pela identificação das bandas correspondentes aos RNAs ribossomais 18S e 28S, após eletroforese capilar (2100 bioanalyzer, Agilent Technologies). A integridade do RNA foi verificada pelo cálculo do número de integridade de RNA (RIN), encontrando-se uma média dos valores de todas as amostras (embriões e tecido adulto) igual a 8,0± 0,3 (escala 1–10), indicando um RNA de alta qualidade e com mínimo de degradação.

3.4 Construção de Bibliotecas e seqüenciamento de pequenos RNAs

Pequenos RNAs de 16-32 nt de comprimento foram isolados a partir do RNA total através do fracionamento por tamanho num gel de TBE e poliacrilamida a 15% e a ligação desses fragmentos a adaptadores. Usando a transcrição reversa, os RNAs foram convertidos em cDNA. Subsequentemente, a reação de PCR foi realizada utilizando os iniciadores complementares para as duas direções. Após a purificação das construções de cDNA amplificados, os produtos foram sequenciados utilizando-se o equipamento Hiseq2000 Illumina Genome Analyzer (Illumina).

3.5 Análise de bioinformática

As sequências (*reads*) obtidas foram submetidas à remoções dos adaptadores, remoção de sequências de baixa qualidade, resultando em *reads* de boa qualidade de 18-32 nt de comprimento, que foram selecionados para as análises de bioinformática. Os *reads* de alta qualidade foram mapeados no genoma da tilápia do Nilo (bouillabase.org) com tolerância de incompatibilidade na seqüência semente (*seed)* utilizando o *software* Patman (https://bioinf.eva.mpg.de/patman/). Posteriormente, RNAs como rRNA, tRNA, snRNA, ncRNA e outras seqüências como snoRNA foram removidos utilizando-se o *software* Workbench -pequenos RNAs (http://srna-workbench.cmp.uea.ac.uk/). Este pacote executa uma análise completa de pequenos conjuntos de dados de RNAs em uma ou várias amostras, e identifica as características estruturais dos pequenos RNAs (Stocks et al., 2012). Além de identificar as seqüências restantes como miRNAs conservados em tilápia do Nilo, utilizando BLAST contra o banco de dados de miRNA, miRBase (version18.0, http://www.mirbase.org/).

Para comparar os miRNAs diferencialmente expressos nas bibliotecas, cada *read* de miRNA identificado foi normalizado pelo número total de *reads* encontrado em cada biblioteca e multiplicado por um milhão. A partir dos *reads* normalizados, foi calculada a relação entre as bibliotecas (fold-change e p-value). Foram considerados mais expressos (*up-regulated*) miRNAs que apresentaram 5 fold-change para cima ou para baixo.

Predição de mRNA alvo

Foram preditos potenciais genes-alvo para alguns dos miRNAs identificados. Foram utilizados três bases de dados: TargetScan (Grimson et al., 2007), PicTar (Krek et al., 2005) e

miRanda (Enright et al., 2003). Estes *softwares* utilizam algoritmos para a identificação de alvos de microRNAs, com base no alinhamento da seqüência semente (*seed sequence*) do miRNA com a região 3 'UTR do mRNA alvo.

Preparação das amostras de RT-qPCR

3.6 Tratamento do RNA com DNase

O RNA total utilizado nas análises de expressão de RNA mensageiro, foi tratado com a enzima DNase para remoção de possível DNA genômico contaminante, conforme as instruções do protocolo da enzima DNase I (Sigma). O RNA total destinado à reação de transcrição reversa foi transferido para microtubo estéril, onde foram acrescentados 1μL de tampão DNase, 1μL de DNase I (1 unidade/μL) e água Milli-Q tratada com dietilpirocarbonato (DEPC) e autoclavada na quantidade suficiente para um volume final de 10μL. Essa solução permaneceu à temperatura ambiente (25°C) durante 15 minutos e, em seguida, foi acrescida de 1μL de EDTA (25mM) e incubada a 65ºC por 5 minutos.

3.7 Reação de Transcrição Reversa (RT)
-qPCR dos RNAs mensageiros

A reação de transcrição reversa (RT) foi realizada utilizando-se o High-Capacity RNA-to-cDNA™ Kit (Life Technologies), com 360ng de RNA total, ao qual foram adicionados 10μL tampão de transcriptase reversa (10x RT buffer), 1μL de enzima de transcriptase reversa (50 unidades/μL) (Life Technologies) e o volume final da reação ajustado para 20μL com água livre de RNases. Cada amostra foi incubada a 37 °C por 60 minutos e em seguida 95 °C por 5 minutos. As amostras permaneceram estocadas em freezer a -20 °C.

-qPCR de microRNAs

O cDNA para miRNA foi feito utilizando o TaqMan MicroRNA kit de transcrição reversa (Life Technologies) seguindo as instruções do fabricante. Foram utilizados 10ng de RNA total acrescidos de 0,15μL de 100nM dNTPs, 0,19μL de Rnase inhibitor, 1μL de enzima de transcrição reversa (50U/μL), 3μL de *primer* (específico para cada ensaio de miRNA a 5μM/ μL) e o volume final da reação ajustado para 15μL com água livre de RNases.

3.8 Reação em cadeia da polimerase em tempo real após transcrição reversa (RT-qPCR)

Foram selecionados 22 miRNAs diferencialmente expressos entre as bibliotecas, para serem validados por qPCR (Tabela 1) em diferentes períodos embrionários (1, 3, 5, 16 e 30 dpf) por RT-qPCR.

Tabela 1. Ensaios de miRNA utilizados em análises de qPCR

miRNA ID	Sequências de miRNA maturas
dre-miR-460-3p	CACAGCGCAUACAAUGUGGAUG
cfa-miR-10	ACCCUGUAGAUCCGAAUUUGU
hsa-miR-100	AACCCGUAGAUCCGAACUUGUG
hsa-miR-10b	UACCCUGUAGAACCGAAUUUGU
dre-miR-10d	UACCCUGUAGAACCGAAUGUGUG
hsa-miR-99a	AACCCGUAGAUCCGAUCUUGUG
dre-miR-192	AUGACCUAUGAAUUGACAGCC
dre-miR-429a	UAAUACUGUCUGGUAAUGCCGU
dre-miR-200a	UAACACUGUCUGGUAACGAUGU
dre-miR-222-3p	AGCUACAUCUGGCUACUGGGUCUC
dre-miR-132-3p	UAACAGUCUACAGCCAUGGUCG
dre-miR-27a	UUCACAGUGGCUAAGUUCCGCU
hsa-miR-181a	AACAUUCAACGCUGUCGGUGAGU
dre-miR-145	GUCCAGUUUUCCCAGGAAUCCC
dre-mir-122-5p	UGGAGUGUGACAAUGGUGUUUG
mmu-mir-182	UUUGGCAAUGGUAGAACUCACACCG
dre-miR-142a-5p	CAUAAAGUAGAAAGCACUACU
dre-miR-125b	UCCCUGAGACCCUAACUUGUGA
dre-miR-455a	UAUGUGCCCUUGGACUACAUCG
dre-miR-19b*	AGUUUUGCUGGUUUUGCAUUCAG
dre-miR-143	UGAGAUGAAGCACUGUAGCUC
dre-mir-29b	UAGCACCAUUUGAAAUCAGUGU
RNU6B	CGCAAGGAUGACACGCAAAUUCGUGAAGCGUUCCAUAUUUUU

- Escolha dos controles endógenos

Para as análises de miRNAs, o gene RNU6B (controle U6) foi utilizado como controle endógeno.

Para os RNAs mensageiros, foram testados dois genes de referência usualmente utilizados em mamíferos, segundo a literatura: 18S rRNA e hipoxantina-guanina fospforribosiltransferase (HPRT).

Foi utilizado o *software* Data Assist v2.0 (Life Technologies) para a realização das análises de expressão e escolha dos genes de referência. O programa analisou a estabilidade dos genes propostos e o HPRT foi apontado como mais estável neste conjuto de amostras.

As reações de RT-qPCR foram realizadas na plataforma *Step-one Plus* de acordo com o protocolo da Life Technologies.

Foram utilizados conjuntos de *primers* e sondas de hidrólise (ensaios TaqMan®) para os miRNAs (Tabela 1), expressos em *Danio rerio* e *Homo sapiens* (Life Technologies).

Para os mRNAs foram desenhados *primers* específicos baseados nas seqüências disponíveis no banco de dados GenBank do *National Center for Biotechnology Information*

(*NCBI*) (Tabela 2), utilizando-se o *software Primer Express®* 3.0 (Life Technologies). Para as análises de mRNA foi utilisado o sistema *Syber Green* de detecção.

Tabela 2. Sequencia de *primers* para RNA mensageiro utilizados em análises de qPCR

mRNA	Sequência *primers*
HoxA3	Forward: TAACCGAACGGCAGGTGAAA
	Reverse: TGTCGCTGGATTCATGGCTT
HoxB3a	Forward: TCTGGAAGCCGTTTTCCTCC
	Reverse: ACGTGACGGTGTCTTTCCAA
HoxD10	Forward: CTGAATCGTGTCCGGTCGAT
	Reverse:TGCTTCCCGTTCGCATAA
Cyp19a1	Forward:GCACCGAGTTTTTCCTCAAA
	Reverse:TCTACAGGCTGCTGGGAAAG
Sox9a	Forward:TACCAAGAATAAGCCCCA
	Reverse: TGATCCTTCTTGTGCTGCA
Foxl2	Forward:CACGACCAAGGAGAAAGAGC
	Reverse:TGGCAATGAGAGCGACATAG
Amh	Forward:CAAAGAACTGAGTGCGTTAC
	Reverse:ACTGGGGTCTGCTCTGGTGG
HPRT	Forward: GACATCATGGATGACATGGGGG
	Reverse: GTAGTCGAGCAGGTCTGCAAAAA

-qPCR

Para os ensaios de mRNA (*Syber Green*) foram realizados os seguintes procedimentos:

a) Descongelamento das amostras de cDNA e dos demais reagentes no gelo, seguindo-se de agitação, ressuspensão e breve centrifugação.

b) O reagente master mix foi misturado suavemente, evitando dano ao material. Foi calculada a quantidade de cada reagente de acordo com o número de reações em triplicata a serem feitas por dia.

-mRNA

Amostras de cDNA dos genes HoxA3, HoxB, HoxD, Cyp19a, FOXL2, Amh e Sox9a foram quantificadas através de reações compostas por 1μL GoTaq probe qPCR master mix (2x), 1,8μL de *Forward primer* (10nM), 1,8μL *Reverse primer*, 1,3 μL de cDNA (18 ng/μL) e 5,1μL de água RNase-free, totalizando 20μL de solução. As placas foram lacradas com os adesivos e centrifugadas brevemente.

-miRNA

Os 22 miRNAs (Tabela 1) foram quantificados utilizando reações compostas por 2μL de TaqMan 2x master mix universal, 1 μL de TaqMan miRNA assay mix 20x, 1,33μL de

cDNA (2 ng/µL) e 7,67µL de água RNase-free, totalizando 20µL de solução. As placas foram lacradas com adesivos e centrifugadas brevemente.

- Determinação dos parâmetros da reação

Para a realização das reações foi utilizado o sistema *Real Time Step-one-Plus* (Life Technologies) utilizando-se as seguintes condições: 10 min a 95 °C, e em seguida 40 ciclos de 30 seg à 95 °C e 1 min a 60 °C.

A quantificação da expressão gênica de cada animal foi feita em reações triplicatas, e foi utilizado o método $\Delta\Delta$CT, que gera uma relação entre o Cq do gene-alvo e o fator normalizador (média dos Cqs dos genes de referência de uma amostra calibradora ou controle interno (Livak e Schmittgen, 2001). Todo procedimento matemático foi realizado pelo programa Data Assist v2.0 (Life Technologies).

3.9 Análise da região promotora de genes alvo de microRNAs

Para confirmar o efeito regulador de miRNAs em seus possíveis genes-alvo, foi realizado o ensaio do gene repórter da luciferase. As regiões 3'UTR de cinco genes - HoxA3a, HoxB3a, HoxD10a, Cyp19a1 e Sox9a - da Tilápia do Nilo, foram obtidas no banco de dados Ensembl (http://www.ensembl.org/index.html). *Primers* específicos foram desenhados e foram realizadas amplificações a partir do cDNA de embriões e adultos (gônada) através de PCR semi-quantitativo (Tabela 3).

Tabela 3. *Primers* utilizados na amplificação das regiões 3'UTR dos genes-alvo de miRNAs

| miRNA regulador	Gene -alvo	Sequência dos *primers*	enzima de restrição
miR-99a-5p	HoxA3	Forward: AT*AGATC*TAACCGGCGGATGAAACACAA	BglII
		Reverse: AT*GCTAG*CTGTTGAGGTCTCAGAAGTTG	NheI
miR-10b	HoxB3a	Forward: AT*AGATC*TTTCCACTTCAACAAGTACCTGT	BglII
		Reverse: AT*GCTAG*CTTTCAGTTTGTGTTGAGGT	NheI
miR-10b-5p	HoxD10	Forward: AT*AGATC*T GTTTGATGCTTCGCTGGCAT	BglII
		Reverse: AT*GCTAG*C TGCCTGCTGTTATGTGCAAC	NheI
miR-181a	Cyp19a1	Forward: AT*AGATC*TCCTGCACTTTTGTATACTTAATTTGT	BglII
		Reverse: AT*CTCGA*GTTTCAGTGTTAGCAGGTTTAAATG	XhoI
miR-145-5p	Sox9a	Forward: AT*AGATC*T CCCGTCAATCAGACACTTGA	BglII
		Reverse: AT*GCTAG*CCCTCCCTTCAAGAAGGTTCA	NheI

Grifado: Sítio de restrição enzimática

Foram construídos controles negativos com mutações dirigidas nos dois primeiros nucleotídeos da região semente (*seed*) dos miR-99a-5p, miR-10b, miR-10b-5p, miR-181a e miR-145-5p (Tabela 4).

Tabela 4. Primers utilizados na construção dos plasmídeos com a região semente mutada

Gene - alvo	Sequência dos *primers*	Enzima de restrição
HoxA3	Forward: TTACGGCATCGGGGTGCTCTGAC*CCCGGG*GCGGCTCGTCACTCTCCGCCGGCGTT	
	Reverse:AACGCCGGCGGAGAGTGACGAGCCGC*CCCGGG*GTCAGAGCACCCCGATGCCGTAA	
HoxB3a	Forward: TTACGGCATCGGGGTGCTCTGAC*CCCGGG*GCGGCTCGTCACTCTCCGCCGGCGTT	SmaI
	Reverse:AACGCCGGCGGAGAGTGACGAGCCGC*CCCGGG*GTCAGAGCACCCCGATGCCGTAA	
HoxD10	Forward: GCGTCCTGTATTTGTTTTGTTTCATC*CCCGGG*TAACACTTTGTGGTCTTAAATTATT	
	Reverse:AATAATTTAAGACCACAAAGTGTTA*CCCGGG*GATGAAACAAAACAAATACAGGACGC	
Cyp19a1	Forward: AAAGATTGTATTTGTACTCAAA*CTCGAG*GCATGATGTGAAAT	
	Reverse: ATTTCACATCATGC*CTCGAG*TTTGAGTACAAATACAATCTTT	
Sox9a	Forward: AGAGACTTTGCCTCCCAGTGCCGCGCTGT*CTCGA*TGGCGGGGAGGGAGACGGCGA	XhoI
	Reverse: TCGCCGTCTCCCTCCCCGCCATCGAGACAGCGCGGCACTGGGAGGCAAAGTCTCT	

Os fragmentos foram amplificados por PCR e visualisados em gel de agarose 1%. A banda correspondente ao amplicom foi recortada do gel, purificada utilizando o kit Gel Extraction Qiaex II (Qiagen) e inserida no vetor plasmidial pGEMT-easy vector (Promega). O plasmídeo foi inserido em células DH5alfa que posteriormente foram plaqueadas e cultivadas *over-night.*

Foram selecionadas de 3-5 colônias, cujos DNAs plasmidiais foram extraídos (QIAGEN) e submetidos a digestão com a enzima de restrição (EcoRI), procedimento que permite confirmar a inserção do fragmento de DNA de interesse (Figura 1).
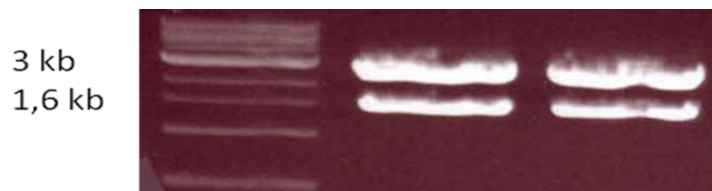
Figura 1. Fragmento do gene Sox9 (1,6 kb) e vetor PGEM-T easy vector (3 kb). Gel de agarose 1%.

Os fragmentos foram sequenciados a fim de confirmar se os insertos eram de fato as regiões 3'UTR dos genes de interesse. Posteriormente, as regiões foram removidas do plasmídeo PGMT-easy vector, utilizando as enzimas de restrição BglII e NheI ou XhoI (dependendo do inserto) e inseridos no vetor PGL-3 luciferase assay (Promega).
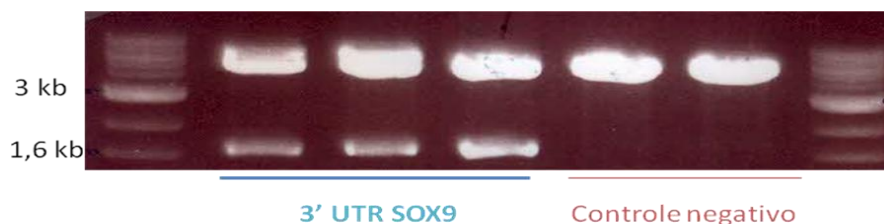
Figura 2. Fragmento da região 3' UTR do gene SOX9 (1,6 kb), vetor PGL-3 (3,5 kb) e contole negativo. Gel de agarose 1%.

-Cultura Celular

A linhagem celular DF1 (fibroblasto de galinha) foi cultivada em meio líquido contendo DMEM Glutamax^{-1}, 10% de soro fetal bovino e 5% de antibiótico (Gibco/Life Technologies). A cultura foi mantida em estufa sob condição de 5% de CO2, a 37 °C.

-Ensaio de Gene Reporter Luciferase

-Transfecção transiente

Dia 1:

Foram plaquedas $6x10^4$ células DF1/poço em 100 uL em placa de 96 poços e incubada em estufa a 5% CO_2 a 37°C *over night,* a fim de fixar as células na placa de transfecção.

Dia 2:

Foram removidos 50 uL de meio/poço e adicionado 50 uL de meio sem antibiótico. Dois tubos foram preparados:

Tubo 1. 50 nM de miRNA mímico, 100 ng de plasmídeo (3' UTR do mRNA + PGL-3 vector), 25 ng de Renilla (20ng/ul), em um volume total de 25 uL de meio livre de antibiótico.

Tubo 2. 25 uL meio livre de antibiótico e 0,2 uL lipofectamina 2000 (100ng/ul)

O conteúdo de ambos os tubos foram misturados e mantidos a temperatura ambiente por 5 minutos. O mix foi adicionado as células e incubadas a 37 °C por 24 horas.

Dia 3:

As células foram lavadas com PBS gelado e acrescidas de 60uL de 1xPLB (Baffer de lise celular). As placas foram mantidas sob agitação a temperatura ambiente por 15 minutos. A quantificação nesse ensaio foi realizada utilizando-se o kit Dual-Luciferase® Reporter Assay System (Promega).

As células foram transferidas para placas de fundo branco e acrescidas de LARII (reagente da luciferase II) e mensuradas pelas primeira vez em um luminômetro (Thermo Fisher Scientific). Posteriormente, foram acrescidas de 50uL de Stop&Glo reagente (solução de parada) e mensuradas novamente. Essa última quantificação serve para medir a fluorescência da Renilla, fator normalizador utilizado no experimento.

3.10 Análise Estatística

Os dados de PCR quantitativo foram expressos como mediana ± desvio padrão. A distribuição dos dados foi não-paramétrica e por isso o teste estatístico utilizado foi o teste de

Wilcoxon-Mann-Whitney. As diferenças significantes foram confirmadas pelo teste de Kruskal-Wallis. Foi adotado um percentual acima de 80 para identificar o potencial de expressão diferencial entre as bibliotecas de miRNA. O ensaio do gene repórter da luciferase foi avaliada por ANOVA one-way, seguido pelo teste de Dunn's de comparação múltipla. A significância estatística foi definida como $p < 0,05$.

4. Resultados e Discussão

O presente tópico será apresentado na forma de manuscritos a serem submetidos a revistas científicas da área de genética e biologia molecular.

CAPÍTULO 1

Mir-10 family involved in the regulation of Hox genes in Nile tilapia

Abstract

Hox gene clusters encode a family of transcription factors that govern the anterior-posterior axis patterning during embryogenesis in all bilaterian animals. The time and place of Hox genes expression are largely determined by the relative position of each gene within its cluster. Furthermore, Hox genes were shown to have their expression fine-tuned by regulatory microRNAs. However, the mechanisms of miRNA mediated-regulation of transcription factors during fish early development remain largely unknown. Here we have profiled miRNomes of Nile tilapia embryos at early developmental stages using Illumina high throughput sequencing in order to identify highly expressed miRNAs or miRNA families. We identified 194 known miRNAs and 74 putative novel miRNAs. Three highly expressed miRNAs and their possible target genes (determined by *in silico* prediction) were evaluated by qPCR, and subsequently had their interaction validated by luciferase reporter gene assays. qPCR results showed that miR-10 family expression increase during developmental stages, whereas HoxA3, HoxB3a and HoxD10 decrease. It was demonstrated using luciferase gene report assays that HoxB3a and HoxD10 are directly targetedbymiR-10b. Overall, our data evidenced that miR-10 family directly regulates Hox genes during Nile tilapia embryogenesis.

Introduction

The development of a multicellular organism from a single cell is a complex process involving many molecules including transcription factors, which must be present in specific cells at the right time (Montavon & Soshnikova, 2010). Some transcription factors are encoded by Hox genes family and have conserved roles in patterning the anterior-posterior axis during embryogenesis in all bilaterian animals (Alexander et al., 2009). The mechanisms of transcriptional regulation of these molecules are dictated by the organization of the genes in clusters (Duboule et al., 2007). This type of genomic organization allows for sharing nuclear space, chromatin structure, common regulatory elements, such as enhancers, and even promoters. As a result, time and place of Hox genes expression are largely determined by the relative position of each gene within its cluster (Duboule et al., 2007).

Hox genes from the same group (transparalogous or paralogues genes) arose from duplication, and share more similarity in protein sequence and expression pattern than other genes within a cluster. An example of duplication role can be looked in mice and other mammals that have 39 Hox genes arranged in four clusters (A, B, C and D) located on four different chromosomes, whereas teleosts have at least 48 Hox genes in eight clusters (Aa, Ab, Ba, Bb, Ca, Cb, Da and Db) resulting from a whole genome duplication (Amores et al., 2004). The Nile tilapia has 51 genes arranged in seven clusters, one less than zebrafish (cluster loss Cb). Several studies have compared the Hox genes organization, and Tilapia Hox seems to be more similar to the pufferfish and the medaka than to the zebrafish (Santini & Bernardi, 2005). The complex diversity of Hox in teleost fish is a consequence of three rounds of genome duplications that took place during vertebrate evolution. Following the first duplication (500 My), the AB cluster lost the Hox12 gene and the CD cluster lost the Hox2 and Hox7 genes. After the second duplication, the cluster A lost the Hox8 gene, the cluster B lost the Hox11 gene, the cluster C lost the Evx gene and the cluster D lost the Hox6 gene. The divergence of the tetrapods lineage followed the second duplication (400 My) and the tetrapods underwent specific gene losses, while the ancient ray-finned fish (teleosts) ancestor underwent the third duplication (350 My) and lost the genes: Hox6 in cluster Aa, Hox1, 3, 4, 5, 6, 7, Evx in cluster Ab, Hox2, 4, 7, 9, 10, 13, Evx in cluster Bb, Hox1, 3, 4, 5, 8, 9, 10 in cluster Cb, Hox1, 5, 8 in cluster Da and Hox1, 3, 5, 8, 9, 12, 13, Evx in cluster Db evolving in the teleosts ancestor. The teleosts underwent further gene losses and in one case (Fugu), a further partial duplication, independently. The organization of the "a" clusters appear to be very conserved, whereas "b", have higher losses. Tilapia completely lacks HoxCb cluster, but this feature is also shared by pufferfish and medaka (Santini & Bernardi, 2005).

The organization of paralogous Hox genes often plays diverse biological roles, as evidenced by their mutant phenotypes, but also shows extensive redundancy and functional overlap. In fruit flies and mice, deletion of a single Hox gene leads to altered axial identities and transformation of specific embryonic structures into more anterior ones (Rijli et al., 1973; Kaufman et al., 1978; Gendron-Maguire et al., 1993). Conversely, ectopic expression of a single Hox gene can also result in a posterior transformation or loss of the body structures (Denell et al., 1981; Van de Ven et al., 2011). Once the development process is extremely complex all events must be extremely accurate thus requiring fine regulatory mechanisms. In this sense, microRNAs (miRNAs) were reported as key regulatory elements for proper organism development based in their ability to modulate gene expression, including the expression of transcriptional factors (Mallo & Alonso, 2013). As members of an abundant class of small noncoding RNAs, miRNAs repress gene expression by preferentially binding to complementary target sequences in the 3'UTRs of mRNAs leading to mRNA degradation and/or translational repression (Bartel, 2009; Lee & Shin, 2012).

In fact Hox genes regulation by miRNAs has been reported in mouse (Mansfield & McGlinn, 2012) and *Drosophila* (Bender, 2008) implying that regulation via miRNAs stand for an extra tier in the complex molecular regulatory circuit controlling Hox genes expression. Notably, most examples available to date show that Hox-regulating miRNAs are encoded within the Hox clusters. This genomic arrangement might provide an effective mechanism to guide the production of miRNAs in the same cells and at the same time as their Hox mRNA targets (Alonso et al., 2013).

In vertebrates, several miRNAs have been shown to regulate Hox gene expression (Bender, 2008; Mansfield & McGlinn, 2012). In mammals, miR-196 was the first miRNA to be experimentally shown to regulate the expression of HoxB8 gene (Yekta et al., 2004). Down regulation of miR-196 in chick (McGlinn et al., 2009) and zebrafish (He et al., 2011) leads to Hox protein de-repression and the generation of homeotic transformations, highlighting the importance of this miRNA. Similarly, down regulation of miR-10 in zebrafish led to over expression of HoxB3a (Woltering & Durston, 2008), this gene is conserved in tilapia. In humans, over expression of HoxA3 in contrast with down regulation of miR-10a, suggested a correlation, either proved by reporter assay (Garzon et al., 2006).

The accumulated data confirm Hox complex miRNAs are important factors that modulate Hox protein expression during vertebrate development. However, the exactly role of miRNAs in Hox genes regulation is still poorly understood. Studies have suggested whatever the biological roles of miRNAs during Hox expression, they might only be detectable in

specific developmental contexts or come about as result of combinatorial interactions with other RNA regulators or when coupled with selective RNA processing patterns of their targets (Mallo & Alonso, 2013). In this paper we investigate the role of miR-10 family members in the regulation of HoxA3a, HoxB3a and HoxD10a genes during the development of Nile tilapia fish, *Oreochromis niloticus*. We obtained miRNA libraries from two development stages (embryos of 3 and 5 days post fertilization) and found high expression of miR-10 members in the results. To validate this data, we used quantitative real-time PCR (RT-qPCR). Target prediction software were used to looking for the possible target genes of selected miRNAs. Additionally, the Hox gene expression was analyzed by qPCR and to validate the interaction miRNA-target, functional analyzes were performed.

Our results confirm that HoxB3a and HoxD10a are regulated by miR-10b-5p during the development in Nile tilapia, providing input for future research in vertebrates and in fish development.

Materials and Methods

Samples and RNA purification

All procedures involving animals were performed according with principles settled by the Ethics Committee for Animal Experimentation – Biosciences Institute –São Paulo State University(protocol 34/08). All fish were anesthetized with benzocaine (100mg/L of water) before being euthanized.

Unsexed embryos (1, 3, 5, 7 and 30 days post fertilization - dpf) and adults of *O. niloticus* were collected in the Royal Fish Farm, Jundiaí, São Paulo, Brazil. The embryos were removed from female mouth and selected based on morphology (Fujimura & Okada, 2007) with a stereomicroscope. These sampling periods were based on cell differentiation stage of embryonic development cycle of *O. niloticus* (Fujimura & Okada, 2007; Ijiri et al., 2008).The embryos were placed in a solution of benzocaine, frozen in liquid nitrogen and stored in -80°C until RNA isolation.

RNA extraction was performed using TRIZOL kit (Ambion) according to the manufacturer's instructions. The RNA samples were quantified by spectrophotometry (NanoVue, GE Healthcare Life Sciences). The quality of RNA was obtained by the RNA integrity number (RIN) analysis. RNA samples were treated with DNA FreeTM Kit (Ambion) to remove genomic DNA contamination. Samples 3 and 5 dpf were used to obtain RNA small libraries, and samples 1, 3, 5, 7 and 30 dpf were used to validate the data by qPCR.

Library construction and deep sequencing of small RNAs

MicroRNA libraries were obtained for two embryonic stages (3dpf and 5dpf) of unsexed embryos. Small RNAs of 16-32 nt in length were isolated from the total RNA by size fractionation in a 15% TBE urea polyacrylamide gel and ligated to adaptors. Using reverse transcription, the RNAs were converted in cDNA constructs. Subsequently, PCR reaction was performed using primers complementary to the two adaptors. Following the purification of the amplified cDNA constructs, the products were sequenced by Hiseq2000 in Illumina Genome Analyzer (Illumina).

Bioinformatics Analysis

The reads obtained in small RNA libraries were subject to adaptor trimming, removal of low-quality sequences and orphan reads, resulting in good quality reads of 18-32 nt in length that were selected for further bioinformatics analysis. High quality reads were mapped to Nile tilapia genome (bouillabase.org) with a tolerance of one mismatch in the seed sequence using PatMan software (https://bioinf.eva.mpg.de/patman/). Subsequently, RNAs such rRNA, tRNA, snRNA, ncRNA and other snoRNA sequences were depleted using Small RNA Workbench software (http://srna-workbench.cmp.uea.ac.uk/). This package performs a complete analysis of single or multiple-sample small RNA datasets identifying structural characteristics of small RNAs (Stocks et al., 2012). The remaining sequences were identified for the conserved miRNAs in Nile tilapia by a BLAST search against the miRNA database, miRBase (version18.0, http://www.mirbase.org/).

To compare the differentially expressed miRNAs in the development stages, each identified miRNA read was normalized following the formula (miRNA read number/total number read per library)x1,000,000. After normalized miRNA reads count, the $\log_{10}$fold-change and p-value were calculated from the normalized data. It was considered upper expression5 fold-change over and down..

Potential target genes for the newly identified miRNAs were predicted by integrating three databases TargetScan (Grimson et al., 2007), Pictar (Krek et al., 2005) and miRanda (Enright et al., 2003). These software use algorithms for the identification of microRNA targets, based in alignment of miRNA seed sequence with 3' UTR region of mRNA target.

Quantitative RT-PCR validation

miR-10 family members showed high expression in the sequence data and were selected to be quantified (Table 1)in different embryonic periods (1, 3, 5, 7 and 30dpf) by RT-

qPCR. The cDNA for miRNAs was reverse transcript using TaqMan MicroRNA reverse transcription kit (Life Technologies) following the manufacturer's instructions. qPCR was carried out using TaqMan 2x universal master mix 1x, Taqman miRNA assay mix 1x, 2ng/uL cDNA and the volume was completed to20µL with nuclease-free water.

The mRNA of HoxA3, HoxB3a and HoxD10 was quantified by qPCR using n=5 for each period. Reverse transcription of total mRNA was performed using High Capacity kit RNA-to-cDNA Master Mix (Life Technologies) following the manufacturer's guidelines. qPCR was performed using 1xGoTaq® probe qPCR master mix based on SYBR Green chemistry (Promega), 40ng/uL of RT reaction, 900nM of primers (forward and reverse) to 10 mM (Table 1) and the volume was completed to 20µL with nuclease-free water.

Thermocycling was performed on Step-one PCR System (Applied Biosystems) according to the following conditions: 10 min at 95°C, and then 40 cycles of 30 sec of 95°C and 1 min of 60°C. Relative expression was evaluated using the comparative quantification method and Hypoxanthine phosphoribosyltransferase gene (HPRT) was used as an endogenous control.

Action of miRNAs on target genes

To confirm the regulatory effect of miRNAs on their target genes, it was conducted the luciferase reporter gene assay. The 3'UTR regions of three genes - HoxA3, HoxB3a and HoxD10 - from tilapia cDNA were PCR amplified and individually cloned into the pGL3 vector (Promega) by directional cloning. Negative controls were built mutating the seed region of the predicted miR-99a-5p and miR-10b-5pmiRNA sites.

DF1 cells were co-transfected with 0.4 mg of firefly luciferase reporter vector and 0.02 mg of the control vector containing Renilla luciferase (Promega) using lipofectamine 2000 (Invitrogen) in 24-well plates(Costar). Each transfection was performed in four wells. Luciferase assays were carried out 24 hours after transfection using the Dual Luciferase Reporter Assay System (Promega). Firefly luciferase activity was normalized to Renilla luciferase activity.

Statistical Analysis

The sequence data was parametric then T-test was used to calculated the difference between reads number of miR-10 family members. The data of quantitative PCR were expressed as median± standard deviation. The qPCR and Luciferase gene reporter assay data distribution were parametric then the Two-way ANOVA test was used. The significant

differences were confirmed by Bonferroni multiple comparison tests. Statistical significance was defined as $p < 0.05$.

Results

Deep sequence data

In total, 54,508,897 raw reads were obtained from two embryo periods (3 and 5 dpf). After filtering the low-quality sequences, empty adaptors and single-read sequences, 20,638,502 clean reads (average) of each library of 18-32 nt were selected for further analysis. Among the cleaned reads, 4,803,715 sequences mapped perfectly to the tilapia genome. The analysis identified 194 mature miRNA, being 120 known miRNAs and 74 Novel miRNAs, many of them present in both libraries. The Illumina small RNA deep sequencing approach help us to determine the relative abundance of miRNA families by calculating the sequencing frequency. miR-10 family showed large number of read. Each microRNA presented 7,000 reads per library, on average, while the members of the miR-10 family have an average of 40,000 reads, suggesting a large involvement in development stages in Nile tilapia.

Library screening for miR-10 family members and their validation by qPCR

The screening of miRNAs libraries of 3 and 5 dpf formiR-10 family members allowed the identification of miR-10-5p, miR-100-5p, miR-10b-5p, miR-10d-5p and miR-99a-5p with high expression in both miRNA library sequence data, but all these miRNAs were more expressed in 3dpf sequence data (P<0.05) (Figure 1a).

The qPCR results showed that all the miRNAs had an increase expression tendency during the development, but only miR-100-5p and miR-99a-5p presented differential expression, getting to be 8 times more expressed between 1 dpf and other periods (P<0.001).These two miRNAs present very similar expression patterns and the same was observed for the miR-10-5p, miR-10b-5p and miR-5p-10d-5p (Figure 1b).

Target genes expression profiles

The analysis of the target genes of miR-10 members family showed HoxA3a is the most expressed in compares onto remaining Hox genes evaluated (P<0.05) (Figure 2). This gene showed high expression in 1 dpf (p<0.001), followed by decrease during the subsequent development stages and increase in 30 dpf. This result is common for all the Hox genes tested and all showed statistical differences (P<0.01). On the other hand, all the miRNAs showed

increased expression throughout development (Figure 1b), suggesting a strong relationship of control.

Luciferase reporter gene assay results

To checkthe control relationship between miRNAs and Hox genes, we used the Luciferase reporter gene assay. The results showed that HoxA3a is not a direct target to miR-99a-5p (P>0.05) (Figure 3a), although the mRNA expression decreased over time (Figure 2), while the expression of miR-99a-5p increased (Figure 1b). On the other hand HoxB3a and HoxD10a have their expression modulated by miR-10b-5p (P<0.05) (Figures 3b and c). In qPCR data, miR-10b-5p increased his expression over the time (Figure 1b), exactly the opposite observed in HoxB3a and HoxD10a expression (Figure 2).

Discussion

Is known that this miRNA family has an important role in Hox genes expression regulation (Garzon et al., 2006; Woltering & Durston, 2008).The miR-10 family, as well as, Hox genes (HoxA3a, HoxB3a and HoxD10a) showed the same pattern over different stages of development. The miR-10-5p, miR-10b-5p and miR-10d-5p showed down expression in 1dpf, followed by increase at 3, 5 and 7 dpf and down again at 30 dpf. miR-100-5p and miR-99a-5p, on the other hand, started their increase in 3 dpf and kept the high expression until 30 dpf.

In contrast we have the Hox genes expression. These genes showed high expression in 1 and 3 dpf, followed by decrease in 5 and 7 dpf and a short increase in 30 dpf. The time of Hox gene activation is functionally important because experimental conditions has been showed that in premature or delayed Hox gene activation to produce phenotypic alterations, even in cases when the final Hox expression patterns are preserved (Zákány et al., 1997; Kondo & Duboule, 1999). This make sense when we think about the existence of distinct functional activities associated with early and late phases of vertebrate Hox gene expression (Carapuço et al., 2005). It has also been suggested that during early vertebrate development the general repressed state of the Hox cluster keeps the late regulatory elements in a "silent state", and that it is only after global repression is erased that these elements become accessible to transcriptional regulators and, therefore, functional (Tschopp & Duboule, 2011).

The high expression of HoxA3a makes sense when we look to his function. HoxA3a is required in patterning the anterior body plan during embryogenesis process (Chen et al., 2010). Extensive genetic studies in mouse have demonstrated HoxA3a roles in patterning and

development of endodermal, mesodermal, and ectodermal derivatives, and in cell migration, proliferation, apoptosis and differentiation (Chen et al., 2010). These processes are abundant in beginning of development in vertebrates. The target predictions showed this gene could be regulated by miR-99a, but the reporter gene luciferase analysis showed that this miRNA does not directly target HoxA3a. Following the same pattern expression HoxB3a was high expressed in 1 and 3dpf. HoxB3a was described involved in the spinal cord formation and repressed by miR-10 in zebrafish (Woltering & Durston, 2008). The prediction showed HoxB3a might be regulated by miR-10b in Nile tilapia. The luciferase gene assay results confirm miR-10b has HoxB3a as target. The luciferase assay using miR-10b was tested for HoxD10a also, and showed this miRNA have both genes as targets. The activation of two Hox genes belonging to different clusters was described before (Chambeyron & Bickmore, 2004; Morey et al., 2007). In addition, HoxD10a is involved in the specification of the arm and forearm (or leg and lower leg), along the limb axis. This phase displays both temporal and spatial collinearities, such genes located closer to the enhancers are transcribed earlier and in a broader territory within the bud (Andrey et al., 2013). Therefore, HoxD10a is expressed after another Hox genes that are closer 3' regions.

The mechanism wherein a miRNA regulate more than one target gene is known. In zebrafish miR-10 repressesthe nearby HoxB1a and HoxB3a genes and that its overexpressionalso induces the associated loss of function phenotypes for both (Woltering & Durston, 2008).

Conclusion

MicroRNA-10 family are really important in Nile tilapia development and has direct connection in Hox genes regulation. The HoxB3a and HoxA10 seem to be a directly targeted by miR-10b-5p, and both are important to embryogenesis in Nile tilapia, as well as, in most of vertebrates. These results are important in the knowledge of development in teleosts and especially in the Nile tilapia and can be use to understand de pathways regulation in early development.

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CAPÍTULO 2

MicroRNAs involved in sex determination in Nile tilapia

Abstract

The Nile tilapia (*Oreochromis niloticus*) is an important fish species due to its economical relevance. Male specimens have increased growth when compared with females, important factor for feature in fish production systems. Unfortunately the mechanisms that lead to sex differentiation in tilapia and fish as general are still poorly understood. In the past few years, several new master gene regulators of sex determination and other factors involved in sexual development have been discovered in teleost fishes. Some genes were proposed as triggers of differentiation processes, but it is also likely that miRNAs play an important role in this process. Considering the biological importance of sex determination we have analyzed microRNomes of Nile tilapia focusing in which miRNAs or miRNA families are differentially expressed in males and females during development. MicroRNome libraries were generated through Illumina sequencing prepared from two embryo stages (3 and 5 dpf whole embryos), and adult male and female brains and gonads. The results obtained identified 194 miRNAs expressed in all the libraries, being 120 with differential expression between male and female (gonads and brain) and 74 Novel miRNAs. Three miRNAs and selected target genes (determined by *in silico* prediction) were evaluated for expression by qPCR, and subsequently their interaction was validated by luciferase reporter gene assays. qPCR results showed that miR-145-5p expression increase in female embryos during early developmental stages whereas Sox9a decrease. In the other hand, miR-181a decreases at the same developmental stages and its target Cyp191a increase. In the report assays we demonstrated that Cyp19a1 is directly targeted by miR-181a and Sox9a is target of miR-145-5p. This was further supported by checking the presence of miRNAs binding sites in the 3'UTR of selected mRNAs. Additionally, these miRNAs inhibited the expression of a reporter protein when these binding sites replaces the 3'UTR of the luciferase mRNA. Overall, our data evidenced miR-145-5p and miR-181a are important for tilapia male and female sex differentiation, respectively, by regulating Sox9a and Cyp19a1 genes.

Introduction

The Nile tilapia (*Oreochromis niloticus)* belongs to Cichlidae family and is considered an excellent experimental model for studies in genetics and genomics (Kocher, 2004). Nile tilapia allows easy handling, such as androgen and production of gynogenetic individuals, sex reversal by administering sex steroids, production of interspecific hybrids and production of transgenic lines (Maclean et al., 2002). In addition, this species has great value in aquaculture, being the second species of freshwater fish most widespread cultivated (FAO, 2006).

Several studies have reported a higher growth in the Nile tilapia males compared to females (Fryer & Iles, 1972; Schreiber et al., 1998) and such characteristic is therefore profitable in the production system. For this reason, various techniques involving crosses and alteration of reproductive physiology by sex reversal via hormonal dosage have been employed to produce monosex populations (Borges et al., 2005). In the same way, the identification of males and females is of great interest allowing the production of monosex animals.

Sex determination can be affected by several mechanisms: environmental (density, feeding rate, photoperiod, pH, salinity, social factors and temperature); behavioural; physiological and genetic factors.

In most mammals, for example, the sex is determined genetically by the presence of the SRY gene (sex-determining region on the Y chromosome), which encodes the testis-determining factor on the Y chromosome (Sinclair et al., 1990; Koopman et al., 1991). Similarly, avian sex is determined genetically, although unlike mammals, the heterogametic sex in birds is female (ZW sex chromosomes), whereas males are homogametic (ZZ sex chromosomes). Fish species have a high diversity and plasticity of sex determining mechanisms, including the existence of XY and ZW systems, multiple sex chromosome system, X0 and Z0 systems, influence of autosomal genes, environmental determination and sex reversal (protrandrya and protogyny mediated by temperature, pH or social factors) (Devlin & Nagahama, 2002; Mank et al., 2006; Sandra & Norma, 2010). Furthermore, studies on sex determination has shown that genes like Amh, Dmrt1, Sox9, Foxl2, and Cyp19a, known for their involvement in sex differentiation in mammals, also have similar functions in Nile tilapia (Baroiller et al., 2009).

In birds and fish, unlike in mammals (Vidal et al., 2001), expression of Amh in the undifferentiated gonads of both sexes occurs in the absence of Sox9 (Oreal et al., 2002). The expression of Dmrt1 and Sox9 throughout the sex-determining period is testis specific in non mammalian vertebrates (Yao & Capel, 2005).

In teleosts, FOXL2 has been described as a repressor of several genes coding steroidogenic enzymes and is an essential transcription factor in ovarian sex differentiation, follicle development and maintenance (Caburet et al., 2012). It is possible that this gene make a regulation of cytochrome P450 aromatase (Cyp19a1). However, the regulation mechanism is still unclear (Bentsi-Barnes et al., 2010). In mouse (*Mus musculus*) pituitary, has been suggested that FOXL2 have influence in FSH production (Justice et al., 2011). In mammals, FOXL2 is an It also maintains the ovarian phenotype throughout adulthood by repressing Sox9 expression and by regulating granulose cell differentiation, maintenance and function (Schmidt et al., 2004). Sox9 has been implicated in testicular differentiation as one of the immediate gene products after SRY in Nile tilapia (Ijiri et al., 2008). *Amh,* in zebrafish, expression peaked at 20 dpf at a period when the oocytes undergo apoptosis in presumptive males, leading to the development of testis (Uchida et al., 2002).

In addition to genes, other molecules may be involved in the regulation of sex differentiation process. Previous studies show that microRNAs (miRNAs) play an important role in gene pathways regulation that leads to sex differentiation. miRNA are small noncoding RNAs, about 21 nucleotides in length. Many are conserved, and may regulate up to 30% of gene expression by base-pairing to partially complementary mRNAs. The differential expression of miRNAs related to sex has been recently detected in genomes of several organisms, including vertebrates such birds and mammals (Bannister et al., 2009; Ciaudo et al., 2009). The characterization of 55 miRNA signatures in testis and ovary of mouse (Mishima et al., 2008) illustrated their importance for the proliferation of spermatogonia (Hayashi et al., 2008). Additional studies in chicken and zebrafish identified sex-specific pattern of miRNA expression in brain, embryo and gonads (Giraldez et al., 2006, Bannister et al., 2009).

In Nile tilapia, the existence of miRNAs involved in sex differentiation is poorly understood. In this way, the aim of this study was to investigate miRNAs involved in sex differentiation of Nile tilapia and their target genes. For this purpose, six small RNA libraries were constructed from male and female tissues (adult gonads and brain) and two development stages (embryos of 3 and 5 days after hatching) using Solexa-Ilumina deep sequencing. Additionally, we investigated mRNA and miRNA expression using quantitative real-time PCR (RT-qPCR). We found sexually dimorphic expression of many miRNAs, some of which have also been observed to have a sex-dependent expression pattern in other vertebrates. Comparative analysis indicate a wide conservation of miRNAs in teleost and suggest their possible roles in vertebrate gonadal development.

Materials and Methods

Tissue and embryo sampling and RNA purification

All procedures involving animals were performed according with principles settled by the Ethics Committee for Animal Experimentation - Biological Institute of Botucatu - UNESP (protocol 34/08). All fish were anesthetized with benzocaine before being euthanized.

Embryos with unknown sex (1, 3, 5, 16 days post fertilization)and adults of *O. niloticus* were collected in the Royal Fish Farm, Jundiai, São Paulo, Brazil. Additionally, XY and XX embryos (5 dpf) from selected crosses (female XX with male YY, and female XX with male XX) were obtained from Institute of Aquaculture, Stirling University, Stirling, Scotland. The embryos were collected based on cell differentiation stage of embryonic development defined for this species (Fujimura&Okada, 2007; Ijiri et al., 2008). All the animals were sacrified using benzocaine solution. The gonads and brains of male and female adult animals were removed and immediately frozen in liquid nitrogen and stored in -80°C until RNA isolation. The whole embryos were anesthetized, frozen in liquid nitrogen and stored at -80 ° C.

RNA extraction was performed using TRIZOL kit (Ambion) according to the manufacturer's instructions. The RNA samples were quantified by spectrophotometry (NanoVue, GE Healthcare Life Sciences). The quality of RNA was obtained by the RNA integrity number (RNA Integrity number, RIN). RNA samples were treated with DNA FreeTM Kit (Ambion) to remove genomic DNA contamination.

Library construction and deep sequencing of small RNAs

MicroRNA libraries were obtained for adult male and female brain, testis and ovary and for two embryonic stages (3dpf and 5dpf) of unsexed embryos. Small RNAs of 16-32 nt in length were isolated from the total RNA by size fractionation in a 15% TBE urea polyacrylamide gel and ligated to adaptors. Using reverse transcription, the RNAs were converted in cDNA constructs. Subsequently, PCR reaction was performed using primers complementary to the two adaptors. Following the purification of the amplified cDNA constructs, the products were sequenced by Hiseq2000 in Illumina Genome Analyzer (Illumina).

Bioinformatics analysis

The reads obtained were subject to adaptor trimming, removal of low-quality sequences and orphan reads, resulting in good quality reads of 18-32 nt in length that were selected for further bioinformatics analysis. High quality reads were mapped to Nile tilapia genome (bouillabase.org) with a tolerance of one mismatch in the seed sequence using PatMan software (https://bioinf.eva.mpg.de/patman/). Subsequently, RNAs such rRNA, tRNA, snRNA, ncRNA and other snoRNA sequences were depleted using Small RNA Workbench software (http://srna-workbench.cmp.uea.ac.uk/). This package perform a complete analysis of single or multiple-sample small RNA datasets identifying structural characteristics of small RNAs (Stocks et al., 2012). The remaining sequences were identified for the conserved miRNAs in Nile tilapia by a BLAST search against the miRNA database, miRBase (version18.0, http://www.mirbase.org/) (Griffiths-Jones, 2004).

To compare the differentially expressed miRNAs in the sex libraries, each identified miRNA read was normalized following the formule: (miRNA read number/total number read per library)x1,000,000. After normalized miRNA reads count, the log10fold-change and p-value were calculated from the normalized data. It was considered upper or down expression 5 fold-change.

Potential target genes for the newly identified miRNAs were predicted by integrating three databases TargetScan (Grimson et al., 2007), Pictar (Krek et al., 2005) and miRanda (Enright et al., 2003). These software use algorithms for the identification of microRNA targets, based in alignment of miRNA seed sequence with 3' UTR region of mRNA target.

Quantitative RT-PCR validation

The miRNAs that showed different expression levels between sexes based in the whole miRNA libraries (Table 1) had their expression levels quantified in different embryonic periods (1, 3, 5 and 16 dpf) by RT-qPCR.The cDNA for miRNA was reverse transcribed using TaqMan MicroRNA reverse transcription kit (Life Technologies) following the manufacturer's instructions. qPCR was realized using TaqMan 2x universal master mix 1X, Taqman miRNA assay mix 1x, 2ng/ul cDNA and the volume was completed 20μL with nuclease-free water.

Predicted target genes of highly expressed miRNAs detected in the libraries were selected for expression analysis by qPCR using n=5 for each sample: XX and XY 5 dpf embryos, female and male brains, and gonads. Reverse transcription of total mRNA was performed using the High Capacity kit RNA-to-cDNA Master Mix (Life Technologies)

following the manufacturer's guidelines. qPCR was realized using 1x GoTaq ® probe qPCR master mix based on SYBR Green chemistry (Promega), 40ng/ul of RT reaction, 900nM of primers (forward and reverse) to 10 mM and the volume was completed to 20µL with nuclease-free water to mRNA reactions.

Thermocycling was performed on equipment Step-one PCR System (Applied Biosystems) according to the following conditions: 10 min at 95°C, and then 40 cycles of 30 sec. of 95°C and 1 min of 60°C. Relative expression was evaluated using the comparative quantification method. The assay number and primers are described in supplement data (Table S1). The Hypoxanthine phosphoribosyl transferase (HPRT) and U6 spliceosomal RNA (U6) genes were used as endogenous control.

Action of miRNAs on target genes

To confirm the regulatory effect of miRNAs on their target genes, it was conducted the luciferase reporter gene assay. The 3'UTR regions of two genes - Cyp19a1 and Sox9a - from tilapia cDNA were PCR amplified and individually cloned into the pGL3 vector (Promega) by directional cloning. Negative controls were built mutating the seed region of the predicted mir-181a and miR-145-5p miRNA sites.

DF1 cells were co-transfected with 0.4 mg of firefly luciferase reporter vector and 0.02 mg of the control vector containing Renilla luciferase (Promega) using lipofectamine 2000 (Invitrogen) in 24-well plates(Costar). Each transfection was performed in four wells. Luciferase assays were carried out 24 hours after transfection using the Dual Luciferase Reporter Assay System (Promega). Firefly luciferase activity was normalized to Renilla luciferase activity.

Statistical Analysis

The data of quantitative PCR were expressed as median± standard deviation. The data distribution was nonparametric then the Wilcoxon-Mann-Whitney test was used. The significant differences were confirmed by Kruskal-Wallis test. The miRNAs that showed over 5 fold-change up were considered upper regulated and miRNAs lower than 5 fold-change considered down regulated.

Luciferase assay was assessed by one-way ANOVA followed by Bonferroni multiple comparison tests. Statistical significance was defined as $p < 0.05$.

Results

Discovery and characterization of miRNAs in Nile Tilapia

In total, 138,060,300 raw reads were obtained from tissue samples of sexually mature adults and two embryo periods. After filtering the low-quality sequences, empty adaptors and single-read sequences, 6,668,124 clean reads of each library (average for each library) of 18-32 nt were selected for further analysis. Among the clean reads, 5,095,751 sequences from all the libraries mapped perfectly to the tilapia genome.

The results showed 194 mature miRNA, being 120 known miRNAs and 74 Novel miRNAs (Table S4), many of them expressed in all libraries (Table S3). The Illumina small RNA deep sequencing approach help us to determine the relative abundance of various miRNAs families by calculating the sequencing frequency. Highly expressed miRNA had likely a large number of repeated sequences. Several miRNAs showed different read numbers between libraries (Table S3), but only the miRNAs with possible influence in sex differentiation were selected to be tested by qPCR (Table 1). Members of miR-99 family were observed in all libraries, except among ovary sequence data (Table S5).

Differential Expression of miRNAs

The results of deep sequencing showed different expression levels of miRNA between sex and development periods (Figure 1). The sequence data can be found in supplementary material.

It was found 54 miRNAs conserved in embryos and gonads and 70 in embryos and brain (Figure 2). An interesting point is that do not have sex-specific miRNA in brain tissue. All the miRNAs are expressed in brain, is expressed in male and female, but the opposite happens in gonads. In this paper we chose the miRNAs that showed expression in gonads and embryos, since, the aim of this study is understand which miRNAs are involved in sex determination process, and the gonads differentiation occurs during the development stages.

The results showed miR-130, miR-135, miR-139, miR-143, miR-145, miR-181, miR-183, miR-192, miR-20, miR-203, miR-206, miR-212, miR-458, miR-724 and miR-192 being expressed in embryos and testis. The miRNAs, miR-122, miR-132, miR-137, miR-142, mir-182, miR-184, miR-200, mir-222, miR-27, miR-455, miR-722 and miR-19 were expressed in ovary and embryos. Some of these miRNAs were validated by qPCR.

miRNAs validation by qPCR

MicroRNAs that showed a higher difference in expression between the sexes (Table 1) and were already described in the literature as involved in sex differentiation process, were tested using qPCR. We select seventeen miRNAs that showed different read numbers between libraries. All miRNAs were tested in embryos 1, 3, 5 and 16 dpf and in 5dpf XX and XY embryos, to confirm the data found in the libraries (Figure 3a and b).

Some miRNAs expressed in 5dpf showed similar expression in qPCR and in sequence data (Figure 4). However, the correlations between sequence data and qPCR are high (r^2=0.65 P<0.005).

Target prediction and quantification by qPCR analyzes

We used TargetScan, Pictar and miRanda software to predict mRNA that could be regulated by miRNAs detected in libraries (Table S6) and tested by qPCR. mRNA that were detected with more than two software were chosen. Four mRNA were selected (Sox9a, Foxl2, Cyp19a1 and *amh*) and their expression levels quantified by qPCR in 1, 3, 5, 16 dpf and XX and XY 5dpf embryos and adult tissue (brain and gonads) samples (Figure 5a and b).

The results showed that Sox9a increased his expression in 3dpf and was differential expression between XX and XY embryos (p>0.05). Looking to adult tissue, this genes was expressed in testis, ovary, male and female brain, but the hight expression was observed female brain (P>0.05). Foxl2, in the other hand, demonstrated increase expression during the development stages, being more expressed in XX embryos (p>0.05) and in ovary tissue (p>0.05). The Cyp19a1 expression was down in 1 and 5dpf and high in 3 and 16dpf. This genes was expressed in both embryos (XX and XY), but showed high expression in XY embryos (p>0.05). In adult tissue, Cyp19a1 changed and expressed only in ovary (p>0.05). *amh*, like Foxl2, started his expression in 1 dpf and improved during the following stages, but decreased in 16dpf. This genes showed differential expression between XX and XY embryos, being more expressed in XY embryos and adult testis (p>0.05).

Luciferase gene reporter assay

The genes Sox9a and Cyp19a1 are targets of miR-181a and miR-145-5p, respectively and were selected to conduct functional experiments of miRNA action on mRNA control expression (Figures 7a and 7b). The results showed that the selected genes are direct targets of the respective miRNAs (P<0.05).

The wild assay to Sox9a showed decrease in luciferase relative light unit to the Pgl-3 vector with wild 3'UTR Sox9a+miR-145-5p.

In Cyp19a1 assay, the decrease luciferase relative light unit was observed in Pgl-3 vector with wild 3'UTR Cyp19a1+Positive control and Pgl-3 vector with wild 3'UTR Cyp19a1+miR-181a wild. The decrease luciferase relative light unit in mutant 3'UTR region for both assay were not significant.

Discussion

MicroRNAs miR-182, miR-27a, miR-125, miR-200, miR-29, miR-192 and miR-222 were highly expressed ($P<0.05$) in XX 5dpf embryos (Figure 8). The miR-182 plays a key role in cell-surface, structure adhesion, migration, and organization (Li et al., 2014); miR-27 exert roles of silencing self-renewal through directly targeting several important pluripotency-associated cell factors (Ma et al., 2014); in mice miR-125 presented high levels in interstitial Leydig cells (Zhigang et al., 2013), these cells produce testosterone when stimulated by LH, showing important function in the body of males. MicroRNAs 29, 222 and 19b were found in high concentrations in ovarian fluid in humans. These miRNAs seem to be connected to progesterone secretion in mammals (Sang et al., 2013). In rainbow trout, miR-29 is also highly expressed in the mature ovary than in the pre-vitellogenic ovary, increasing its expression during ovary development (Juanchich et al., 2013). In Nile tilapia, all these miRNAs seem to be acting in different developmental processes, but directed to the regulation of genes involved in ovary differentiation.

In mammals and fish, Cyp19a1 and Foxl2 have been identified as important genes in the developmental cascate of female gonads. We quantified their mRNA expression by qPCR and the Cyp19a1 expression was up-regulated in embryos up to 5dpf, changing in embryos 16dpf ($P<0.01$), corroborating the results found by Ijiri and coworkers (2008). The miR-181a was identified as possible post-transcriptional regulator of this mRNA. In porcine female, miR-181a was described as involved in embryo implantation regulation (Su et al., 2014). During the development, this miRNA showed high expression in embryos until 5dpf and decrease in 16dpf embryos ($P<0.05$), indicating miR-181a may has regulatory role in Cyp19a1. The luciferase reporter gene assay demonstrated miR-181a has Cyp19a1 as a target-gene in Nile tilapia.

The Foxl2 is involved in transcriptional regulation of Cyp19a1 and both are co-localizing in the stromal and interstitial cells in gonads of XX, before the morphological sex differentiation (Wang et al., 2007). The mRNA expression of Foxl2 was increased during the

embryonic period in tilapia, with the highest level in 16dpf. In contrast, Ijiri and co-workers (2008) observed high Foxl2 expression from 9dpf. The expression of this gene has been observed in ovary and female brain, but not in male brain and testis.

Some miRNAs tested by qPCR showed high expression in different tissue of sequence data. The miR-145-5p and miR-143 were highly expressed only in testis microRNomes data, but qPCR detected both highly expressed only in ovary tissue only. These miRNAs showed increase expression during the developmental stages in Nile tilapia and prediction software showed the Sox9a gene as a possible target to miR-145-5p. This possible relationship can be tested by comparing the miRNA expression and Sox9a expression. Sox9a was down expressed, whereas miR-145-5p was high expressed in XX embryos. The functional analysis conducted confirm that miR-145-5p have role in Sox9a regulation. MicroRNA miR-145-5p has been suggested as a possible player in apoptosis of germinal epithelium and also in Sertoli cells differentiation process, leading to early male gonad differentiation in mammals (Morrish et al., 2002). In tilapia, this gene is expressed in both sex in early stages, but increase his expression in male. Thus, miR-145-5p is highly expressed in XX enbryos and down-regulate Sox9a expression.

The miR-122, miR-132, miR-455 and miR-19 were expressed in XY embryos differently than expected. All these miRNAs showed high expression in ovary libraries. This unexpected expression can be linked to the regulation of androgen conversion to estrogen (Guiguen et al.,1999; Kwon et al., 2000). Estrogen is essential for the development of females, but should be inhibited so that there is the beginning of the formation of testicles. In mouse, miR-132 have involvement in estrogen conversion (Tripurani et al., 2013) and miR-122 has been implicated as a regulator of luteinizing hormone (LH) receptors ovaries (Menon et al., 2013). Thus, in Nile tilapaia, these miRNAs can be working together to block aromatase enzyme and estrogen production, causing formation testis.

In males, an important gene in sexual differentiation in vertebrates is Amh. Higher expression of Amh than Sox9a in early periods of development (Smith & Sinclair, 2004; Shoemaker et al., 2007) have been observed for vertebrates (including fish) and similar results were found here for *O. niloticus*. The *amh* mRNA level increased during development, being more abundantly expressed in 5dpf and XY embryos, followed by decrease in 16dpf (P<0.05). This gene was expressed in all adult tissue, but highly expressed in testis (P<0.05).

Conclusion

This study provides data on mature miRNAs, their precursor sequences, and target genes of *Oreochromis niloticus*. Seventeen miRNAs were validated by qPCR and two gene targets checked by luciferase gene reporter assay. Our data proves that miRNAs miR-145-5p and miR-181a are important for sex development in Nile tilapia. The miR-145-5p down regulate Sox9a expression, leading to the development of females. The same occur in Cyp19a1 under the action of miR-181a. The results presented here represents promissing to understand the the process of sex differentiation on Nile tilapia.

Acknowledgements

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5. Conclusão

A presente tese relatou o padrão de expressão de microRNAs e genes importantes no processo de desenvolvimento e diferenciação sexual em Oreochromis niloticus. Nossos dados demostraram que, assim como nos demais vertebrados, os genes Hox são de extrema importância no desenvolvimento da tilápia do Nilo e que são regulados por membros da família do microRNA-10. Na segunda bordagem, relacionada à diferenciação sexual, obtemos resultados interessantes, tendo os miR-145-5p e miR-181a como importantes no gatilho de diferenciação do sexo de machos e fêmeas, respectivamente, e como regulandores dos genes SOX9 e Cyp19a1.

Nesse contexto, nossos dados estão em concordância com alguns relatos na literatura e com a hipótese desse trabalho. Visto que os miRNAs e genes descritos, possuem papel importante nos processos de desenvolvimento e diferenciação sexual em outros grupos de vertebrados, porém agem de forma específica em cada espécie.

Além disso, um novo conjunto de microRNAs candidatos foram elencados como sendo importantes nos processos investigados. Os resultados obtidos abrem perspectivas para novas discussões e trabalhos voltados para o estudo do desenvolvimento e da determinação e diferenciação sexual nos vertebrados, assim como apresentam potencial na produção de populações monosexo de grande valor na aquicultura.

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7. Apêndices e material suplementar

-Capítulo 1

Tables

Table 1. MicroRNA assays and Hox genes primers used in qPCR analyzes

miRNA ID	Mature miRNASequence
cfa-miR-10-5p	ACCCUGUAGAUCCGAAUUUGU
hsa-miR-100-5p	AACCCGUAGAUCCGAACUUGUG
hsa-miR-10b-5p	UACCCUGUAGAACCGAAUUUGU
dre-miR-10d-5p	UACCCUGUAGAACCGAAUGUGUG
hsa-miR-99a-5p	AACCCGUAGAUCCGAUCUUGUG
RNU6B	CGCAAGGAUGACACGCAAAUUCGUGAAGCGUUCCAUAUUUUU
mRNA	**Sequência *primers***
HoxA3	Forward:TAACCGAACGGCAGGTGAAA
	Reverse: TGTCGCTGGATTCATGGCTT
HoxB3a	Forward:TCTGGAAGCCGTTTTCCTCC
	Reverse:ACGTGACGGTGTCTTTCCAA
HoxD10	Forward:CTGAATCGTGTCCGGTCGAT
	Reverse:TGCTTCCCGTTCGCATAA
HPRT	Forward:GACATCATGGATGACATGGGGG
	Reverse:GTAGTCGAGCAGGTCTGCAAAAA

Figures
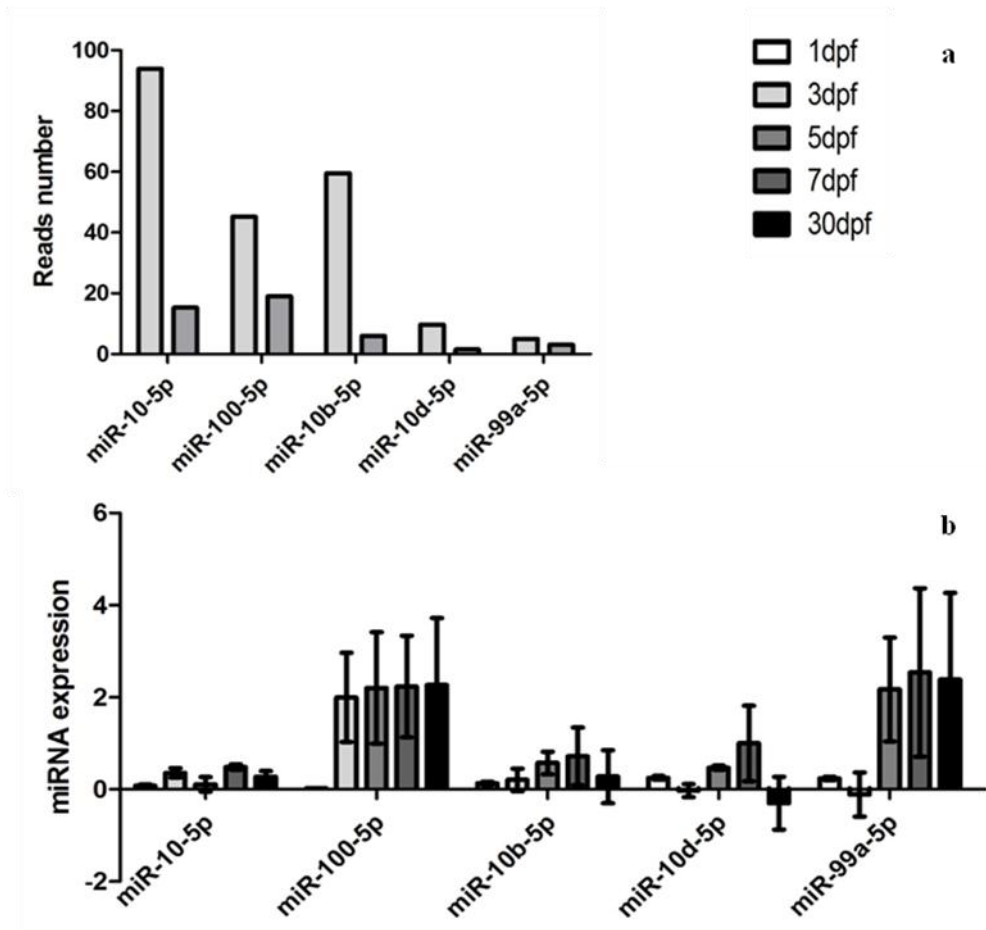
Figure 1. Total reads of miR-10 family member in sequence data (a); relative quantification of microRNA expression during development based on qPCR ($\log_{10}$)(b).
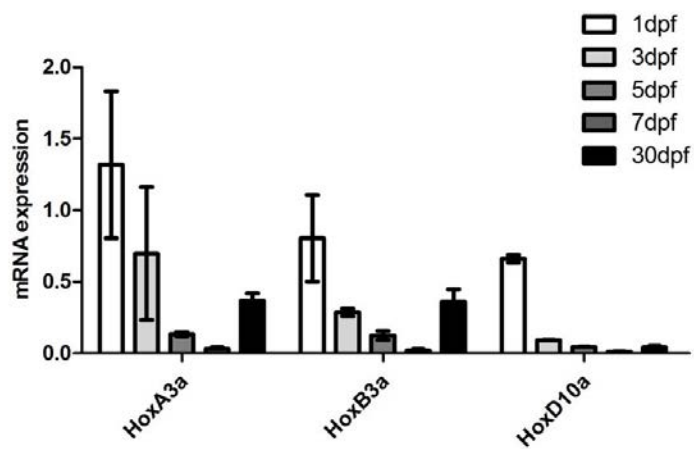
Figure 2. Relative quantification of Hox genes expression during development based on qPCR ($\log_{10}$)
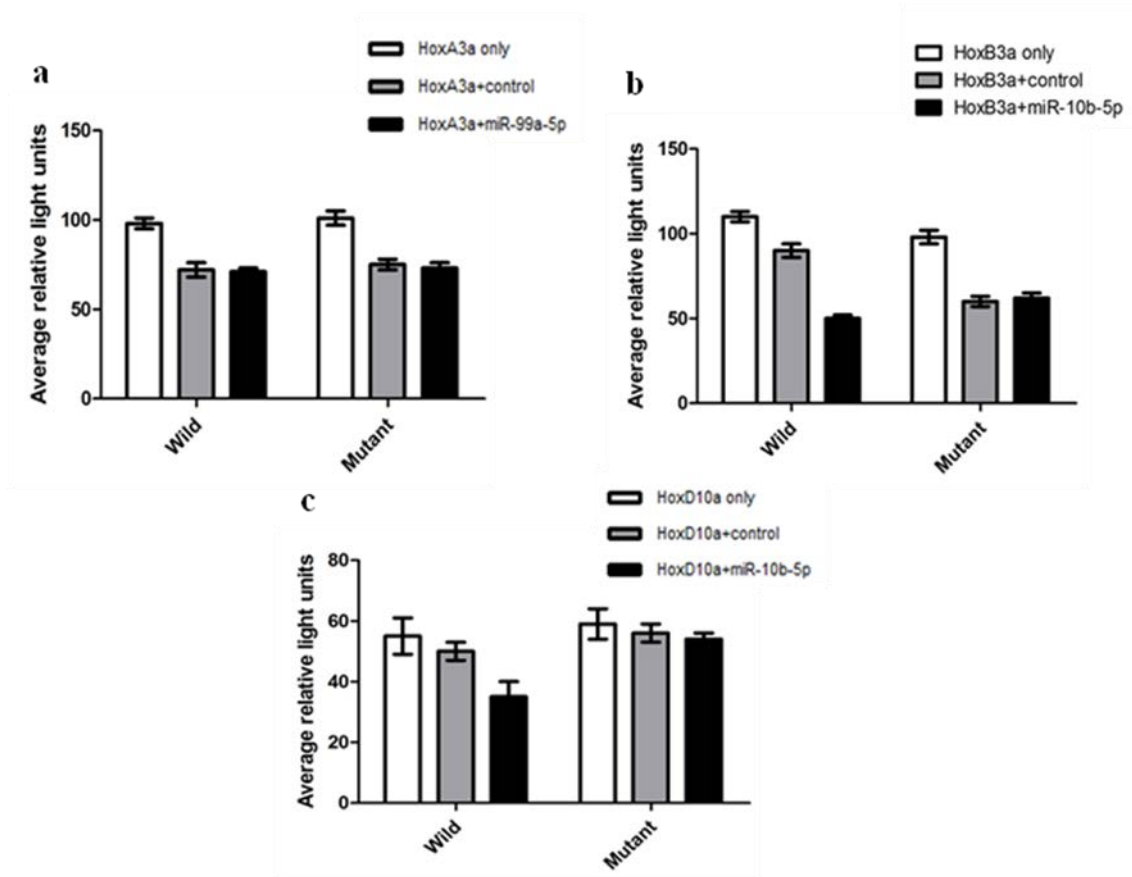
Figure 3. Luciferase assay with Pgl-3 vector (3'UTR ofHoxA3a, HoxB3a and HoxD10a) and miR-99a-5p and miR-10b-5p. The first section demonstrates relative level of luciferase activity after transfection of DF-1 cells with 3'UTR region wild only; 3'UTRwild+positivecontrol and 3'UTR wild+miR mimic. Second section: 3'UTR mutant only; 3'UTRmutant+positive control and 3'UTR mutant+miR mimic. Bars represent the normalized average of relative luciferase units.

-Capítulo 2

Table

Table 1. MiRNA assays used in qPCR analyzes

miRNA ID	Mature miRNA Sequences
dre-miR-460-3p	CACAGCGCAUACAAUGUGGAUG
dre-miR-192	AUGACCUAUGAAUUGACAGCC
dre-miR-429a	UAAUACUGUCUGGUAAUGCCGU
dre-miR-200a	UAACACUGUCUGGUAACGAUGU
dre-miR-222-3p	AGCUACAUCUGGCUACUGGGUCUC
dre-miR-132-3p	UAACAGUCUACAGCCAUGGUCG
dre-miR-27a	UUCACAGUGGCUAAGUUCCGCU
hsa-miR-181a	AACAUUCAACGCUGUCGGUGAGU
dre-miR-145	GUCCAGUUUUCCCAGGAAUCCC
dre-mir-122-5p	UGGAGUGUGACAAUGGUGUUUG
mmu-mir-182	UUUGGCAAUGGUAGAACUCACACCG
dre-miR-142a-5p	CAUAAAGUAGAAAGCACUACU
dre-miR-125b	UCCCUGAGACCCUAACUUGUGA
dre-miR-455a	UAUGUGCCCUUGGACUACAUCG
dre-miR-19b	AGUUUUGCUGGUUUGCAUUCAG
dre-miR-143	UGAGAUGAAGCACUGUAGCUC
dre-mir-29b	UAGCACCAUUUGAAAUCAGUGU

Figures
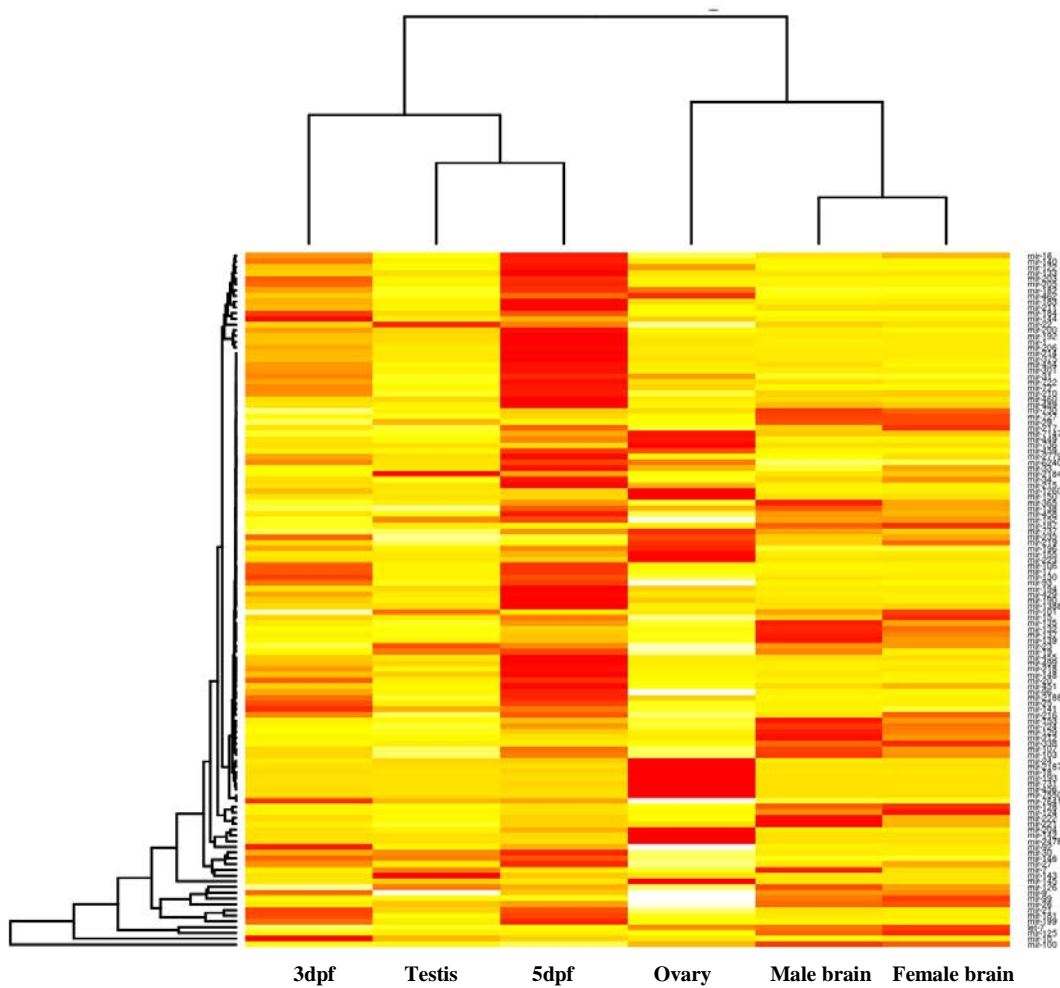
Figure 1. Hierarchical clustering of miRNAs differentially expressed between 3 dpf, 5dpf, female and male brain, ovary and testis. The heat map was drawn with $\log_{10}$ normalized data of each miRNA. White, yellow and red indicate low, middle and high frequency miRNA in the library, respectively.
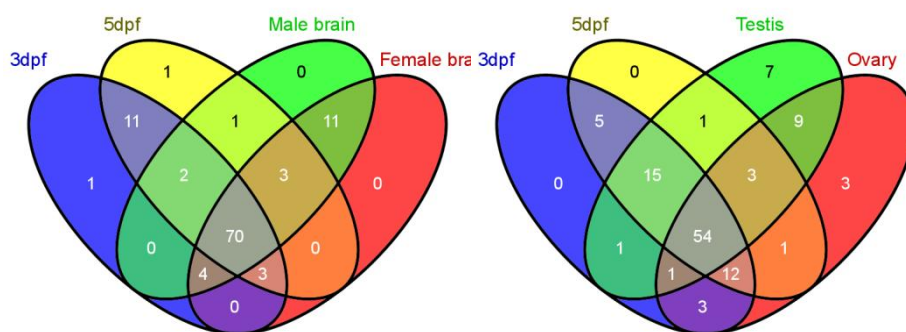
Figure 2. Venn diagram comparing the expression distribution of miRNAs in 3 dpf, 5dpf, female and male brain, ovary and testis
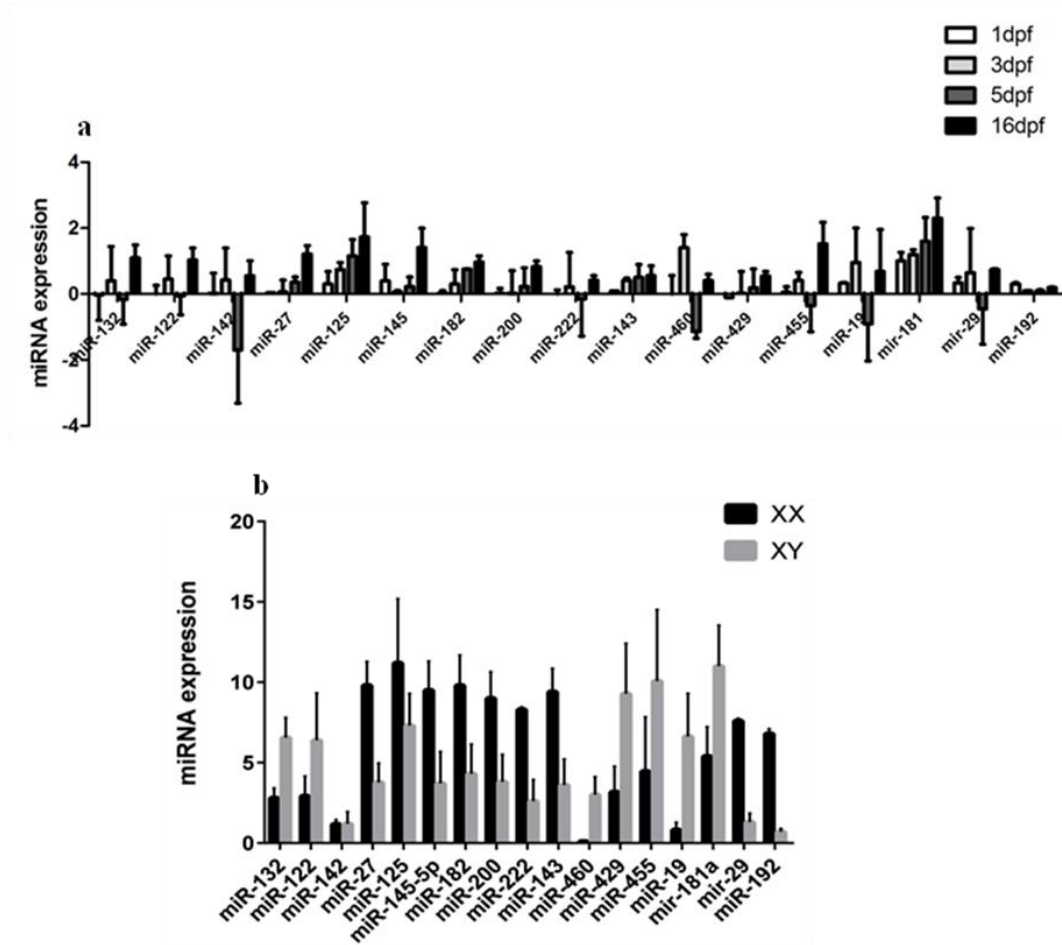
Figure 3. (a) miRNAs expression levels during the *O. niloticus* development by qPCR $(\log_{10})$ (b) miRNA differential expression in XX and XY embryos (5dpf)
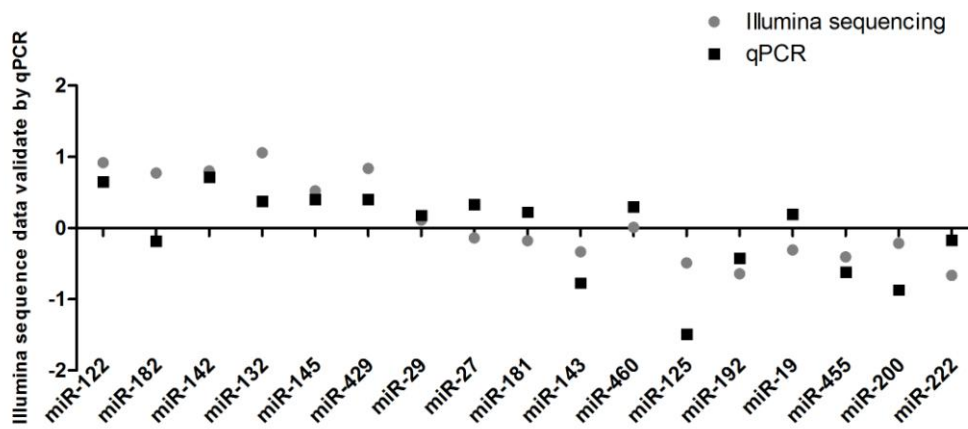
Figure 4. Correlation between Illumina sequence data and qPCR data (log10).
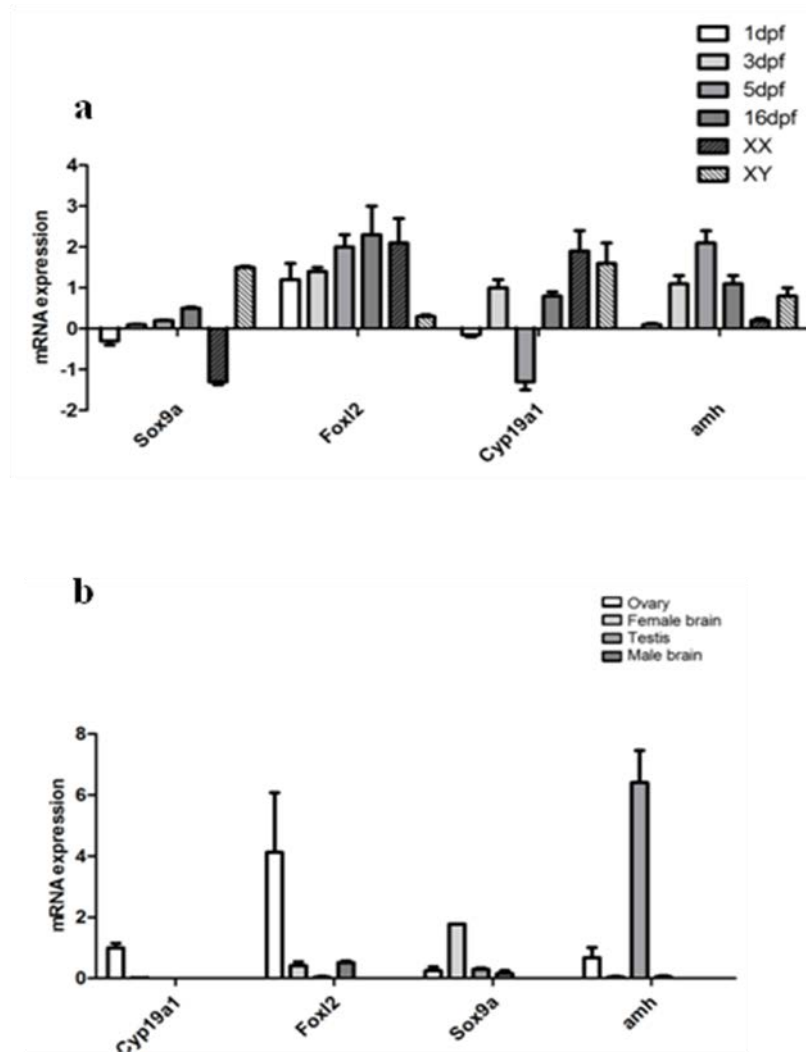
Figure 5. Expression levels of mRNA during development (a) and in adult tissues (b).
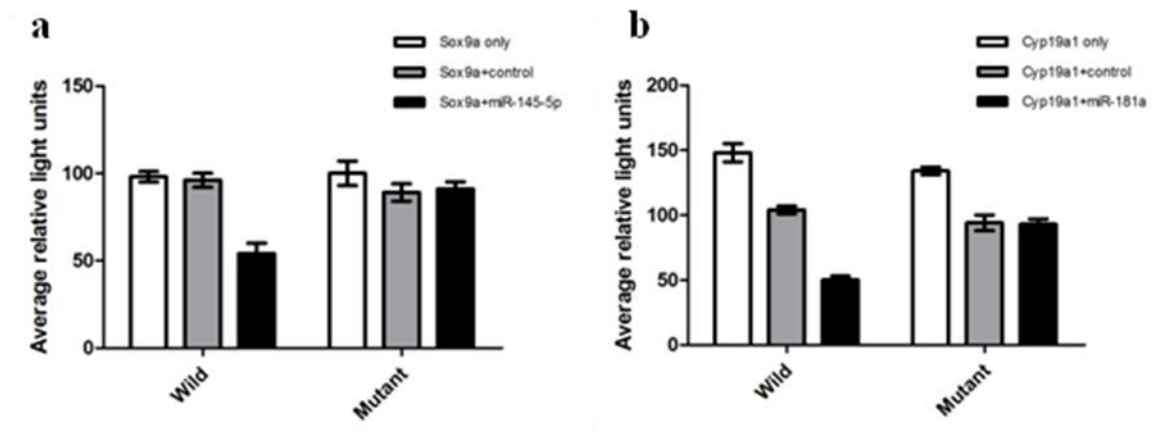
Figure 7. a) Luciferase Assay with Pgl-3 vector (3'UTR Sox9a) and miR-145-5p. The first section demonstrates relative level of luciferase activity after transfection of DF-1 cells with Sox9 wild only; Sox9 wild+positive control and Sox9 wild+miR-145-5p mimic. Second section: Sox9 mutant only; Sox9 mutant+positive control and Sox9 mutant+miR-145-5p mimic. Y-axis represents the normalized average of relative luciferase units.**b)**Luciferase Assay with Pgl-3 vector (3'UTR Cyp19a1) and miR-181a. The first section demonstrates relative level of luciferase activity after transfection of DF-1 cells with Cyp19a1 wild only; Cyp19a1 wild+positive control and Cyp19a1 wild+miR-181a mimic. Second section: Cyp19a1 mutant only; Cyp19a1 mutant+positive control and Cyp19a1 mutant+miR-181a mimic. Y-axis represents the normalized average of relative luciferase units.
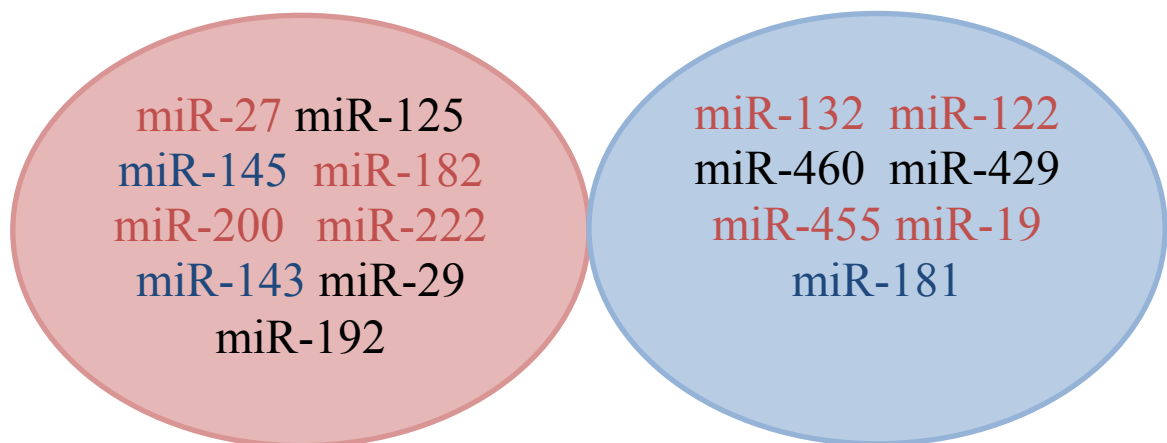
Figure 8. Comparison between results of sequence data and qPCR using XX and XY embryos. Pink and blue circles represents the qPCR result in ovary and testis, respectively. Word color (pink, blue and black) represent miRNAs that were more expressed in ovary, testis and embryos, respectively, in the sequence data.

7.1 Material suplementar

CAPÍTULO 2

Table S1. Sequence of messenger RNA primers used in qPCR analysis

mRNA	Sequência *primers*
Cyp19a1	Forward:GCACCGAGTTTTTCCTCAAA
	Reverse:TCTACAGGCTGCTGGGAAAG
Sox9a	Forward:TACCAAGAATAAGCCCCA
	Reverse: TGATCCTTCTTGTGCTGCA
Foxl2	Forward:CACGACCAAGGAGAAAGAGC
	Reverse:TGGCAATGAGAGCGACATAG
Amh	Forward:CAAAGAACTGAGTGCGTTAC
	Reverse:ACTGGGGTCTGCTCTGGTGG
HPRT	Forward:GACATCATGGATGACATGGGGG
	Reverse:GTAGTCGAGCAGGTCTGCAAAAA

Table S2. Number of total reads per library and adaptor percentage

	3dpf	5dpf	Female brain	Ovary	Male brain	Testis	Media
Total reads	26472081	28036816	23322873	2585083	28774743	28868704	23010050
Matching adaptor	6573969	6657923	6589729	1532301	8984543	4534455	5812153
% Matching adaptor	25	24	28	59	31	16	31
Matching adaptor & genome	5245970	4361460	5187743	468491	7169340	3514242	4324541
% Matching adaptor and genome	20	16	22	18	25	12	19

Table S3. Normalized counts of known miRNAs

miRNA	3 dpf	5dpf	Female brain	Ovary	Male brain	Testis
let-7	14200.00	38900.00	149000.00	107000.00	118000.00	30000.00
mir-1	3402.72	18100.00	455.42	1480.13	220.90	1583.56
mir-10	264000.00	50000.00	24500.00	26800.00	1620.67	79900.00
mir-100	59600.00	30200.00	253000.00	143000.00	296000.00	66300.00
mir-101	77.53	346.24	894.11	349.15	583.68	752.76
mir-103	991.11	2034.11	1813.89	180.77	2845.07	136.29
mir-106	617.54	751.39	69.65	60.04	47.53	57.84
mir-107	1071.78	2389.69	1864.47	191.22	2938.00	165.21
mir-122	1237.03	5207.03	7.78	2022.45	10.36	4.75
mir-124	389.44	2299.90	26200.00	13.05	11900.00	8.72
mir-125	6734.76	11000.00	150000.00	308.69	110000.00	10800.00
mir-126	20000.00	35500.00	37500.00	22500.00	47300.00	41300.00
mir-1260	40.92	26.58	3.89	179.47	1.84	10.30
mir-128	1148.14	3056.79	20500.00	5.22	16600.00	204.43
mir-129	93.20	663.27	1508.66	224.50	2634.67	3.57
mir-130	690.76	635.97	140.26	0.65	159.04	108.56
mir-132	27.41	393.65	1283.57	56.78	1857.04	0.79
mir-133	518.07	5313.83	44.74	30.02	25.11	209.98
mir-135	274.50	902.00	794.31	3.26	1829.09	34.47
mir-137	137.45	432.44	863.17	2.61	1556.25	15.85
mir-138	34.07	240.41	158.16	140.31	242.04	6.34
mir-1388	65.00	685.78	83.46	5.87	44.41	52.30
mir-139	100.64	365.64	583.62	43.73	1267.96	107.76
mir-140	3145.84	5354.29	105.05	251.91	71.51	142.23
mir-141	2135.73	1428.31	6.61	48.95	253.25	895.39
mir-142	204.80	1060.27	432.27	39100.00	159.90	293.97
mir-143	1623.92	6822.35	1463.52	1794.69	881.06	45700.00
mir-144	16800.00	4639.30	248.04	2474.06	140.18	141.04
mir-145	527.67	1043.03	861.42	107000.00	1905.13	12600.00
mir-146	25000.00	26800.00	2682.32	327.61	3498.13	21400.00

mir-148	427.22	1415.62	173.92	150.10	225.59	364.49
mir-15	317.97	644.11	918.23	0.65	336.68	233.75
mir-150	0.00	12.45	6.61	177.51	4.97	1.58
mir-152	117.67	428.13	298.62	9.14	286.31	324.08
mir-153	501.43	1279.61	1939.56	56.12	3945.90	326.06
mir-155	9.20	132.65	7.39	511.00	4.26	11.89
mir-16	2682.20	5242.71	2014.66	41.77	1107.35	574.87
mir-17	632.61	751.39	66.53	9.79	48.24	57.45
mir-18	158.59	296.44	11.09	3488.87	10.36	8.32
mir-181	56800.00	59700.00	7419.56	698.30	8098.51	2743.21
mir-182	4259.32	8608.15	118.86	5719.50	58.60	27.34
mir-183	4331.57	14800.00	506.97	1649.81	235.23	58.24
mir-184	12900.00	11600.00	15.56	0.65	9.79	2477.36
mir-187	3.52	44.78	411.84	49.60	319.37	59.43
mir-19	328.54	1299.00	387.33	12.40	977.96	1042.37
mir-190	122.76	606.28	30.74	124.00	58.45	19.41
mir-192	3953.49	23700.00	3.31	33.94	0.28	1439.75
mir-193	0.78	2.87	0.58	3441.88	0.00	1.19
mir-194	35.44	1013.34	0.19	112.25	0.00	70.13
mir-196	244.55	311.76	0.78	547.54	0.28	55.86
mir-199	65300.00	99200.00	1794.44	10.44	1761.13	6840.98
mir-20	872.85	1093.32	114.97	77.66	113.08	130.74
mir-200	8130.58	21700.00	27.24	11.75	700.31	3502.30
mir-203	5112.40	6742.37	7.00	56.78	10.22	5.15
mir-204	2996.05	11400.00	1456.33	41800.00	1324.57	179.47
mir-205	4122.46	6157.40	2.72	5.87	3.55	1.98
mir-206	5693.33	19400.00	1.75	77.66	6.24	2.77
mir-21	48000.00	45300.00	12400.00	10.44	14600.00	17700.00
mir-210	109.45	193.23	66.92	16.32	62.28	21.00
mir-211	2995.66	11400.00	1456.33	2.61	1324.57	179.47
mir-212	77.34	370.67	1862.14	263.66	4274.91	11.89
mir-214	88.30	420.23	2.14	2.61	1.84	5.15
mir-215	6.66	85.96	0.00	24.80	0.00	5.94
mir-216	1496.46	1900.02	1845.21	5.22	588.79	76.86
mir-217	51.30	178.87	228.00	14.36	72.78	7.92
mir-218	554.10	1394.06	98.24	25.45	55.33	9.11
mir-2184	0.78	18.20	10.51	0.65	5.11	51.11
mir-2187	0.78	13.17	11.09	5892.45	42.85	53.49
mir-2188	1430.28	2001.78	318.07	587.35	251.97	92.31
mir-219	177.98	77.58	501.33	651.96	216.36	0.00
mir-22	5021.75	9512.54	4463.13	18.93	5224.78	14600.00
mir-221	1550.30	2970.11	4408.47	2489.72	12700.00	299.52
mir-222	2634.42	3393.45	5412.49	0.65	18500.00	282.88
mir-223	19.78	83.81	21.40	836.00	17.88	5.15
mir-23	284.88	1271.71	1153.82	8.48	1143.11	1662.80
mir-235	292.32	48.13	200.77	331.53	139.04	0.00
mir-24	273.33	882.84	464.37	5326.63	277.51	694.12
mir-2478	113.37	54.35	7.59	27400.00	6.95	41.20
mir-25	2438.04	2622.19	304.45	3.92	328.59	291.99
mir-26	30900.00	54300.00	79500.00	14.36	72300.00	43600.00
mir-27	17100.00	26000.00	14800.00	3496.70	9616.60	9110.74
mir-2779	2.55	6.47	1.17	0.65	0.43	1.19
mir-29	6.27	35.44	149.99	29.37	148.26	76.07
mir-30	18400.00	39800.00	8241.68	11.09	8810.17	23700.00
mir-301	184.44	452.08	11.48	1.31	8.09	3.57
mir-31	189.73	318.47	0.97	153.36	0.99	3.96
mir-33	1.37	7.66	3.70	3.26	0.99	1.98
mir-338	53.45	234.18	3524.48	3.92	2465.26	195.72
mir-34	22.71	121.16	60.50	2.61	27.38	1.98
mir-365	31.13	196.11	180.92	28.06	377.39	73.69
mir-375	71.07	337.86	0.00	20.23	1.14	9.51

mir-429	266.67	859.38	0.19	33.94	13.90	24.56
mir-449	3.92	17.00	2.33	31.98	2.41	1.19
mir-451	417.24	1960.12	601.32	42.42	337.95	119.65
mir-454	151.94	353.90	13.03	26.10	48.66	8.32
mir-455	252.38	1552.58	114.97	0.65	112.08	63.79
mir-456	59.52	58.43	22.96	1428.57	26.25	6.74
mir-458	84.78	436.75	167.11	1.96	216.93	39.62
mir-459	1.17	36.87	0.00	31.98	0.00	0.00
mir-460	13.90	267.46	10.31	0.65	18.73	21.39
mir-462	1751.19	4228.89	673.50	5890.49	483.66	316.95
mir-489	14.88	208.08	34.43	0.65	51.93	3.57
mir-499	256.69	1285.12	4.67	20.23	3.97	146.19
mir-6240	2.94	4.31	0.19	3.26	0.00	1.19
mir-7	3318.92	3687.97	5680.37	18.27	34200.00	19400.00
mir-7147	2.94	14.61	11.09	32.63	11.07	3.96
mir-72	102.99	205.21	0.78	38.50	0.71	3.57
mir-722	104.16	255.01	33.85	44.38	48.52	9.51
mir-724	333.63	1786.28	2367.75	3.92	3249.42	2.77
mir-727	8.42	27.30	118.09	4.57	121.31	0.00
mir-730	6.07	53.40	106.22	45.68	128.40	35.26
mir-731	12.34	132.41	15.37	1965.02	11.63	16.64
mir-736	0.00	0.48	0.00	49.60	0.00	0.00
mir-737	160.36	249.98	212.05	330.22	244.88	157.68
mir-7550	2.55	6.23	1.17	12900.00	0.43	1.19
mir-7641	8.03	3.83	0.39		0.14	3.17
mir-9	40200.00	23600.00	33600.00		32200.00	19.41
mir-92	36400.00	11900.00	2913.62		2172.00	1608.92
mir-93	393.16	532.29	87.35		113.93	69.33
mir-96	997.97	2490.02	127.62		170.25	118.46
mir-99	10500.00	7456.16	68700.00		49700.00	11400.00

Table S4. Normalized counts of Novel microRNAs

Sequence	3 dpf	5 dpf	Female brain	Ovary	Male brain	Testis
AAACAAAGGTGTGTACTCTCC	5.1	1.9	0.2	0.0	0.0	0.8
AAACTTGACATGTTGGTACTGC	2.9	4.8	1.6	0.0	1.7	0.0
AAAGCATTGCTCTACCTGCAC	0.4	0.5	1.9	0.7	3.0	0.4
AAAGTACTCTAGCGTCACT	30.0	3.1	0.0	26.8	0.0	0.0
AAAGTGTTATCCCCATCCACTT	8.2	2.4	0.0	1.3	0.0	0.0
AAATGTAGTAGACTATAAGTATAC	0.8	9.3	4.5	2.6	12.5	0.0
AAATGTAGTAGACTTTAAGTATAC	2.2	7.9	1.8	0.0	4.1	0.4
AACATGAGAGCGGAAACTTTGC	0.0	0.2	0.0	0.0	0.0	0.8
AACCTATGAACTTCAAGCACAT	0.0	0.0	0.0	0.0	0.0	0.0
AACGCTACGTCCAGATGAAC	11.7	2.4	1.2	1.3	0.6	0.4
AACGTCCAGATGAACAATCAAC	1144.8	388.4	18.9	28.1	16.6	25.4
AAGATAGAATGAGATGAGGACT	171.9	97.2	2.3	15.0	1.7	2.4
AAGGATAACTACAACTGTACT	0.0	0.0	0.0	0.0	0.1	0.4
AAGGGAATATCGTGAGTGTAGCT	10.2	2.9	0.0	1.3	1.6	0.0
AATGATCCAAGTGTCCTGAGCCATT	0.2	0.0	0.0	0.0	0.0	12.7
ACCTCTCTCCCCCTGCCTTTGT	1.0	3.8	1.0	1.3	0.0	0.4
ACTAGCACCACTTAATGAACAA	0.6	0.7	0.0	0.7	0.0	0.8
ACTCCAGACTCTGTGATTATGT	12.3	5.3	0.2	0.7	0.1	1.6
AGAATAATGCCAGCAGTCGGCC	4.9	30.6	16.3	0.0	29.7	2.0
AGAATAATGCCAGCAGTCGGTC	26.4	49.6	89.5	0.0	95.2	2.0
AGACTATTTGGAAACCACTGGA	7.4	4.5	0.0	0.7	0.0	0.0
AGCCGAGTTTGTAAGCTGAACT	0.0	0.7	0.0	22.8	0.3	2.4
AGGAAATCTGCTTCTGTTGGTTT	1.4	11.3	0.0	0.0	0.0	0.0
AGTGGCTTTGGTCAGCTTTGGTA	0.0	0.0	0.0	44.4	0.0	0.0
AGTGGTTTCCAAATAGTCTGAT	4.5	5.7	0.0	0.0	0.1	0.0

ATCCAGACTGACATCTCCTCAC	3.1	0.5	0.0	0.7	0.1	0.0
ATCCGGGACATGCAGCTCTCC	3.3	0.0	0.6	1.3	0.3	0.0
ATGAGAATGCTGACAGGGAGCT	0.4	0.0	0.4	0.0	0.3	0.0
ATGAGTTTGGACTGTGCCGAGT	3.3	1.4	2.1	0.0	1.3	1.2
ATGTGGAAAAACAACGGACTGA	0.4	0.7	0.4	0.0	0.3	0.8
ATTCTGATTCACATGAACTCGT	46.2	57.5	6.2	7.2	4.8	7.9
CAATGTCAACGCGTGATTGGTC	0.0	1.0	1.4	0.0	2.6	0.0
CAGCTCACACTGCTCTGCTACT	4.7	8.9	4.9	0.7	6.0	0.8
CATACCGTGTCCTGTGCTTGAAC	4.9	0.2	0.2	3.9	0.0	0.0
CATCATTCACTCTAAGCTGACT	12.1	6.7	0.2	0.7	0.0	4.8
CCACCTCCCCTGCAAACGTCC	0.2	0.5	0.6	0.0	0.3	0.0
CCTCAAGCTGCTGTTGCTCGTCT	5.7	2.9	0.4	0.0	0.6	0.0
CGAGTCCAGCTGAAGTTGTAGT	4.1	2.2	0.0	5.9	0.6	0.0
CGTCTCGTCCTGGATGTGAGCT	0.8	6.2	0.0	0.0	0.3	0.0
CGTGATTGCGGATGCTGTGCAGG	5.1	2.6	0.0	0.7	0.0	0.0
CTCGAAGAATTCCTTGTTTTAG	13.7	3.1	0.8	60.7	1.6	1.2
CTGATGAAATCACTCTATGTTCCT	5.5	4.5	0.4	0.0	3.1	0.8
CTGATTTCATTTGGTGACGTAGA	46.6	32.1	1.6	72.4	2.1	13.5
CTGCTCCACAGTTCCAAGCCAG	0.8	0.5	0.0	0.7	0.0	0.0
CTGGGAAAACGTGTAATAGACA	0.0	0.2	0.2	0.0	1.6	0.0
GACACCAATTTGCTCTGTAAGC	8.8	10.8	3.9	17.0	5.0	11.9
GACAGACTCGTCTGATATGGTGA	4.5	1.9	0.0	0.0	0.0	0.0
GAGGAAACTCTGTAGCCGAACACTA	7.6	18.4	0.0	1.3	1.1	6.3
GATGTTGAGTATCAAACTGTAT	0.6	13.9	1.2	0.7	1.7	0.8
GGAATTCCGCTAGTTCTGAACT	22.7	50.0	0.2	0.7	0.0	4.4
GTGCCATATCTGACTGTACGCAAT	1447.7	136.2	1.8	2.6	5.1	4.4
TAAAAGAGGATCCAGCAAGAGA	2.5	1.9	1.9	0.0	2.0	0.0
TAAGTCTCACACCAGTGCAAAAC	7.4	22.5	14.0	1.3	38.7	19.0
TAAGTGCTCATTGTTGTTGTGT	227.7	3.6	0.4	3.9	0.0	0.0
TACAGTCAGGCAAGTATGGCT	0.0	7.9	0.2	0.0	0.0	0.0
TACATGCAGAGGTGGAGCAAGA	4.5	1.4	3.7	2.0	1.7	0.4
TACGCCCAGATGAACAGAATCA	6.9	8.9	1.4	0.0	3.8	5.5
TAGGCGTGTCACTGCGTGTCACA	0.6	10.1	2.3	0.0	8.8	0.0
TATATGGTCTTTTTGTGGGAAA	2.2	0.0	0.0	984.1	0.0	0.0
TCAAGTGCTAATTGTTGTTGT	43.5	1.9	0.0	0.7	0.0	0.0
TCAGTAACTGGAATCTGTCCCT	150.0	305.8	0.0	506.4	0.0	2.0
TCCAGGGACTGCCTGCATACT	4.9	2.9	0.0	0.0	0.9	0.4
TCCCCAAGATGTCAAACTAGT	3.7	1.0	0.2	0.0	0.1	0.0
TCCGGGTGGAGCACTGCTGACT	1.2	2.4	2.5	2.0	2.4	0.4
TCGGCTTTCTCACTCGACGGC	2.7	1.0	0.0	0.0	1.0	11.1
TCTTCCATGCACTTTGATGACT	17.2	52.0	81.5	8.5	74.5	3.2
TCTTGGAGGGTTTGCTACAGTGC	0.6	0.7	0.0	0.0	0.3	0.0
TGAACCGGACACGACGCTCGCT	2.5	0.5	0.0	0.0	0.1	0.0
TGACCCCACCTCCACAGAGGGA	2.9	4.5	0.6	0.0	0.1	5.9
TGCAAATCAGAACGCGGCACGA	0.4	1.7	1.2	0.0	0.4	0.4
TGCACAGTGTGGATTAGTTGACT	8.8	1.7	1.4	2.6	0.1	1.6
TGCAGATGGTGTTTTTTCTGA	0.6	3.6	0.2	0.0	2.0	0.8
TGCAGTGAGTCAGTCTGAAGCT	5.3	8.9	0.4	0.0	1.6	0.0
TGCCATCGGGTGAGAAAGACGA	129.4	18.2	1.0	1.3	0.0	2.0
TGCTTGGGCTCAGTCAAACTGT	5.7	4.1	1.4	0.0	2.7	0.0
TGGAGTTATGGCTCAGAGGCAC	1.0	3.1	2.1	0.7	5.0	0.0
TGGCATTAGTGCTTGAGCAGCT	1.2	5.5	0.2	0.0	0.6	0.0
TGGGTTCAGTGACTACAGACC	13.7	5.5	3.1	11.7	0.4	3.2
TGTAGAGCAGACTGGATTCTCT	17.8	13.6	2.5	4.6	9.1	2.4
TGTAGAGTCTGTGTGTGGAGCTT	1.0	0.5	0.0	0.0	0.9	0.4
TGTGACCCAGTATACCTCCACACC	1.8	0.5	0.4	0.0	2.0	0.0
TGTGGATTCTATAATGTCTTGT	4.9	3.6	0.6	0.0	0.9	0.8
TGTGTTCAGGCTCTGGCTGACA	0.4	1.2	2.3	0.0	0.6	0.4
TTAACATGAATGCACGGCTCA	0.0	0.0	0.0	0.0	0.0	0.0
TTACAATTAAAGGATATTTCT	0.4	21.1	112.4	0.0	459.1	0.8
TTACAATTAAAGGATATTTCTT	4.7	124.8	197.8	0.0	711.5	0.8

TTGACACACTGCCACTCCTGTAGG	6.3	0.5	0.0	0.7	0.3	0.0
TTGGCTCCGGTATGTTTCAGCGA	0.8	3.4	9.1	0.0	18.6	0.4
TTGTTGTTGCTGCTGTGATGCT	3.7	2.6	1.2	0.0	1.0	0.0
TTTCCCTTTTTCACACCTTTCC	0.8	3.4	0.4	0.0	0.6	0.0
TTTTCCTCTGATCTGCCTCCTCT	3.9	2.4	0.6	0.0	0.7	0.0

Table S5. Absence of miR-9 family in ovary tissue

miRNA	Ovary	Tests
miR-9	0	19.41
miR-92	0	1608.92
miR-93	0	69.33
miR-96	0	118.46
miR-99	0	11400

Table S6. Predicted targets to miR-145-5p and miR-181a

| ID	Representative transcript ID	Gene
TPM3	NM_001043351	tropomyosin 3
FSCN1	NM_003088	fascin homolog 1, actin-bundling protein (Strongylocentrotus purpuratus)
SRGAP2	NM_001170637	SLIT-ROBO Rho GTPase activating protein 2
FAM108C1	NM_021214	family with sequence similarity 108, member C1
NAV3	NM_014903	neuron navigator 3
ABCE1	NM_001040876	ATP-binding cassette, sub-family E (OABP), member 1
KCNA4	NM_002233	potassium voltage-gated channel, shaker-related subfamily, member 4
PLDN	NM_012388	pallidin homolog (mouse)
SOX9	NM_000346	SRY (sex determining region Y)-box 9
FLI1	NM_001167681	Friend leukemia virus integration 1
SEMA3A	NM_006080	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3A
CSRNP2	NM_030809	cysteine-serine-rich nuclear protein 2
YTHDF2	NM_001172828	YTH domain family, member 2
DAB2	NM_001343	disabled homolog 2, mitogen-responsive phosphoprotein (Drosophila)
TMEM9B	NM_020644	TMEM9 domain family, member B
MPZL2	NM_005797	myelin protein zero-like 2
ADD3	NM_001121	adducin 3 (gamma)
ST6GALNAC3	NM_152996	ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)-N-acetylgalactosaminide alpha-2,6-sialyltransferase 3
SRGAP1	NM_020762	SLIT-ROBO Rho GTPase activating protein 1
EXOC8	NM_175876	exocyst complex component 8
TRIM2	NM_001130067	tripartite motif containing 2
MYO5A	NM_000259	myosin VA (heavy chain 12, myoxin)
ITGB8	NM_002214	integrin, beta 8
CAMSAP1L1	NM_203459	calmodulin regulated spectrin-associated protein 1-like 1
ATXN2	NM_002973	ataxin 2
HHEX	NM_002729	hematopoietically expressed homeobox
ORC4	NM_001190879	origin recognition complex, subunit 4
HNRNPH2	NM_001032393	protein-coding
RPL36A-HNRNPH2	NM_001199973	RPL36A-HNRNPH2 readthrough
SMCHD1	NM_015295	structural maintenance of chromosomes flexible hinge domain containing 1
NEGR1	NM_173808	neuronal growth regulator 1
ZNF41	NM_007130	zinc finger protein 41
EPB41L5	NM_001184937	erythrocyte membrane protein band 4.1 like 5
FLT1	NM_002019	fms-related tyrosine kinase 1 (vascular endothelial growth factor/vascular permeability factor receptor)
RNF170	NM_001160223	ring finger protein 170
FAM135A	NM_001105531	family with sequence similarity 135, member A
MYO6	NM_004999	myosin VI

CASZ1	NM_001079843	castor zinc finger 1
C11orf9	NM_001127392	chromosome 11 open reading frame 9
MBTD1	NM_017643	mbt domain containing 1
UBN2	NM_173569	ubinuclein 2
C13orf35	NM_207440	chromosome 13 open reading frame 35
SLC1A2	NM_001195728	solute carrier family 1 (glial high affinity glutamate transporter), member 2
ARF6	NM_001663	ADP-ribosylation factor 6
HLTF	NM_003071	helicase-like transcription factor
DLGAP1	NM_001003809	discs, large (Drosophila) homolog-associated protein 1
C6orf115	NM_021243	chromosome 6 open reading frame 115
SPSB4	NM_080862	splA/ryanodine receptor domain and SOCS box containing 4
USP31	NM_020718	ubiquitin specific peptidase 31
SLC25A30	NM_001010875	solute carrier family 25, member 30
ZBTB33	NM_001184742	zinc finger and BTB domain containing 33
EXOC2	NM_018303	exocyst complex component 2
OTOR	NM_020157	Otoraplin
REV3L	NM_002912	REV3-like, catalytic subunit of DNA polymerase zeta (yeast)
PALM2	NM_001037293	paralemmin 2
MEST	NM_002402	mesoderm specific transcript homolog (mouse)
SKP1	NM_006930	S-phase kinase-associated protein 1
AGFG1	NM_001135187	ArfGAP with FG repeats 1
NRAS	NM_002524	neuroblastoma RAS viral (v-ras) oncogene homolog
PGM3	NM_001199917	phosphoglucomutase 3
PRKX	NM_005044	protein kinase, X-linked
EIF2AK1	NM_001134335	eukaryotic translation initiation factor 2-alpha kinase 1
KLF5	NM_001730	Kruppel-like factor 5 (intestinal)
PRKD3	NM_005813	protein kinase D3
PTGFR	NM_000959	prostaglandin F receptor (FP)
PLEKHG7	NM_001004330	pleckstrin homology domain containing, family G (with RhoGef domain) member 7
GLIS1	NM_147193	GLIS family zinc finger 1
PLCE1	NM_001165979	phospholipase C, epsilon 1
UNC119B	NM_001080533	unc-119 homolog B (C. elegans)
DOCK9	NM_001130048	dedicator of cytokinesis 9
ZFYVE9	NM_004799	zinc finger, FYVE domain containing 9
ARL6IP5	NM_006407	ADP-ribosylation-like factor 6 interacting protein 5
SPTLC2	NM_004863	serine palmitoyltransferase, long chain base subunit 2
TMEM178	NM_001167959	transmembrane protein 178
KIF21A	NM_001173463	kinesin family member 21A
EBF3	NM_001005463	early B-cell factor 3
RASA2	NM_006506	RAS p21 protein activator 2
NEDD9	NM_001142393	neural precursor cell expressed, developmentally down-regulated 9
CLINT1	NM_001195555	clathrin interactor 1
LRP2	NM_004525	low density lipoprotein receptor-related protein 2
NSUN4	NM_199044	NOP2/Sun domain family, member 4
FAM108B1	NM_001025780	family with sequence similarity 108, member B1
DPH5	NM_001077394	DPH5 homolog (S. cerevisiae)
GFRA1	NM_001145453	GDNF family receptor alpha 1
IGSF5	NM_001080444	immunoglobulin superfamily, member 5
PSIP1	NM_021144	PC4 and SFRS1 interacting protein 1
NTN4	NM_021229	netrin 4
ABCA1	NM_005502	ATP-binding cassette, sub-family A (ABC1), member 1
NUAK1	NM_014840	NUAK family, SNF1-like kinase, 1
SLC27A1	NM_198580	solute carrier family 27 (fatty acid transporter), member 1
AP2B1	NM_001030006	adaptor-related protein complex 2, beta 1 subunit
ASAP2	NM_001135191	ArfGAP with SH3 domain, ankyrin repeat and PH domain 2
CLEC16A	NM_015226	C-type lectin domain family 16, member A
GXYLT1	NM_001099650	glucoside xylosyltransferase 1
NETO1	NM_138966	neuropilin (NRP) and tolloid (TLL)-like 1
YES1	NM_005433	v-yes-1 Yamaguchi sarcoma viral oncogene homolog 1
UXS1	NM_025076	UDP-glucuronate decarboxylase 1
RTKN	NM_001015055	Rhotekin

ID	Representative transcript ID	Gene name
ZNF781	NM_152605	zinc finger protein 781
ZNF439	NM_152262	zinc finger protein 439
ZNF594	NM_032530	zinc finger protein 594
ZNF780B	NM_001005851	zinc finger protein 780B
OSBPL3	NM_015550	oxysterol binding protein-like 3
CYP19A1	NM_019885	cytochrome P450, family 19, subfamily A, polypeptide 1
PRTG	NM_173814	Protogenin
ZNF780A	NM_001010880	zinc finger protein 780A
RAB3IP	NM_001024647	RAB3A interacting protein (rabin3)
KIAA0528	NM_014802	KIAA0528
TMEM87B	NM_032824	transmembrane protein 87B
PPIP5K2	NM_015216	diphosphoinositol pentakisphosphate kinase 2
ATM	NM_000051	ataxia telangiectasia mutated
NUDT12	NM_031438	nudix (nucleoside diphosphate linked moiety X)-type motif 12
FIGN	NM_018086	Fidgetin
IL2	NM_000586	interleukin 2
FBXO34	NM_017943	F-box protein 34
TGFBRAP1	NM_001142621	transforming growth factor, beta receptor associated protein 1
TRDMT1	NM_004412	tRNA aspartic acid methyltransferase 1
TRIM2	NM_001130067	tripartite motif containing 2
TMEM131	NM_015348	transmembrane protein 131
KIAA0195	NM_014738	KIAA0195
PDE5A	NM_001083	phosphodiesterase 5A, cGMP-specific
HOXC8	NM_022658	homeobox C8
ZNF83	NM_001105549	zinc finger protein 83
ZFP36L2	NM_006887	zinc finger protein 36, C3H type-like 2
ZFP14	NM_020917	zinc finger protein 14 homolog (mouse)
MPP5	NM_022474	membrane protein, palmitoylated 5 (MAGUK p55 subfamily member 5)
SPP1	NM_000582	secreted phosphoprotein 1
ACAP2	NM_012287	ArfGAP with coiled-coil, ankyrin repeat and PH domains 2
MARK1	NM_018650	MAP/microtubule affinity-regulating kinase 1
TOM1L1	NM_005486	target of myb1 (chicken)-like 1
TXNDC15	NM_024715	thioredoxin domain containing 15
CLVS1	NM_173519	clavesin 1
MBTPS2	NM_015884	membrane-bound transcription factor peptidase, site 2
C2orf69	NM_153689	chromosome 2 open reading frame 69
BRAP	NM_006768	BRCA1 associated protein
SIPA1L2	NM_020808	signal-induced proliferation-associated 1 like 2
ZNF563	NM_145276	zinc finger protein 563
TMF1	NM_007114	TATA element modulatory factor 1
IPO8	NM_001190995	importin 8
S1PR1	NM_001400	sphingosine-1-phosphate receptor 1
ZNF568	NM_001204838	zinc finger protein 568
FLT1	NM_002019	fms-related tyrosine kinase 1 (vascular endothelial growth factor/vascular permeability factor receptor)
BTBD3	NM_014962	BTB (POZ) domain containing 3
ARHGEF3	NM_001128615	Rho guanine nucleotide exchange factor (GEF) 3
TCERG1	NM_001040006	transcription elongation regulator 1
ZNF468	NM_001008801	zinc finger protein 468
RLF	NM_012421	rearranged L-myc fusion
ITSN1	NM_001001132	intersectin 1 (SH3 domain protein)
KLF6	NM_001160124	Kruppel-like factor 6
ZNF136	NM_003437	zinc finger protein 136
MLL	NM_001197104	myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila)
CTDSPL	NM_001008392	CTD (carboxy-terminal domain, RNA polymerase II, polypeptide A) small phosphatase-like
SLC4A10	NM_001178015	solute carrier family 4, sodium bicarbonate transporter, member 10

SLC25A37	NM_016612	solute carrier family 25, member 37
DDX3Y	NM_001122665	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, Y-linked
MAGOHB	NM_018048	mago-nashi homolog B (Drosophila)
YLPM1	NM_019589	YLP motif containing 1
UBP1	NM_001128160	upstream binding protein 1 (LBP-1a)
RPS6KB1	NM_003161	ribosomal protein S6 kinase, 70kDa, polypeptide 1
YTHDC2	NM_022828	YTH domain containing 2
C15orf29	NM_024713	chromosome 15 open reading frame 29
PGAP1	NM_024989	post-GPI attachment to proteins 1
ZNF673	NM_001129898	zinc finger family member 673
METAP1	NM_015143	methionyl aminopeptidase 1
SYNPR	NM_001130003	Synaptoporin
PITPNB	NM_012399	phosphatidylinositol transfer protein, beta
AFG3L2	NM_006796	AFG3 ATPase family gene 3-like 2 (S. cerevisiae)
FGD4	NM_139241	FYVE, RhoGEF and PH domain containing 4
PBMUCL1	NM_001198815	panbronchiolitis related mucin-like 1
CDON	NM_016952	Cdon homolog (mouse)
LPCAT2	NM_017839	lysophosphatidylcholine acyltransferase 2
DDX3X	NM_001193416	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, X-linked
HOXD1	NM_024501	homeobox D1
ESM1	NM_001135604	endothelial cell-specific molecule 1
ZFP62	NM_001172638	zinc finger protein 62 homolog (mouse)
CLMN	NM_024734	calmin (calponin-like, transmembrane)
TMEM165	NM_018475	transmembrane protein 165
MPP7	NM_173496	membrane protein, palmitoylated 7 (MAGUK p55 subfamily member 7)
PAPD5	NM_001040284	PAP associated domain containing 5
ZFP82	NM_133466	zinc finger protein 82 homolog (mouse)
PI4K2B	NM_018323	phosphatidylinositol 4-kinase type 2 beta
CLIP1	NM_002956	CAP-GLY domain containing linker protein 1
NEK7	NM_133494	NIMA (never in mitosis gene a)-related kinase 7
ZNF655	NM_001009960	zinc finger protein 655
KIAA1239	NM_001144990	KIAA1239
SLC7A2	NM_001008539	solute carrier family 7 (cationic amino acid transporter, y+ system), member 2
ACVR2B	NM_001106	activin A receptor, type IIB
ST8SIA4	NM_005668	ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 4
ZFP36L1	NM_004926	zinc finger protein 36, C3H type-like 1
KRAS	NM_004985	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
PLDN	NM_012388	pallidin homolog (mouse)
RALGAPB	NM_020336	Ral GTPase activating protein, beta subunit (non-catalytic)
PROX1	NM_002763	prospero homeobox 1
GPD2	NM_000408	glycerol-3-phosphate dehydrogenase 2 (mitochondrial)
TBC1D1	NM_015173	TBC1 (tre-2/USP6, BUB2, cdc16) domain family, member 1
TIMP3	NM_000362	TIMP metallopeptidase inhibitor 3
BRWD1	NM_033656	bromodomain and WD repeat domain containing 1
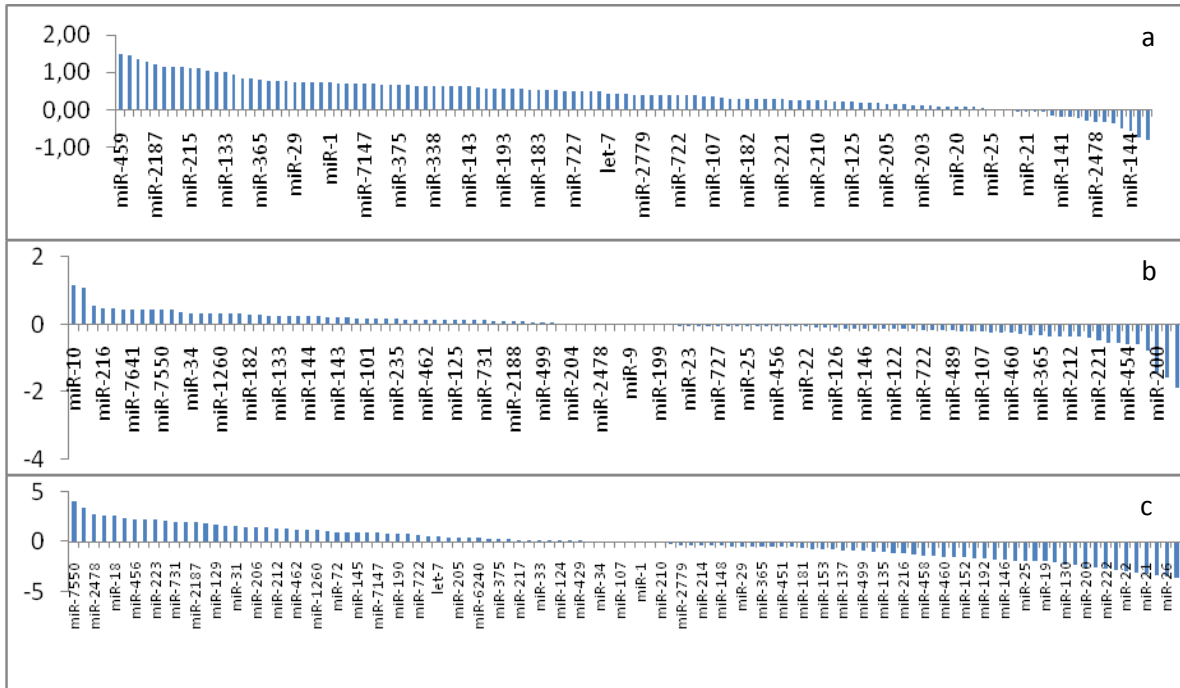
Figure S1. a) miRNA expression based on 5dpf/3dpf; b) brain female/male and c) ovary/testis ratio (log_{10})