UNIVERSIDADE FEDERAL DO RIO GRANDE
INSTITUTO DE OCEANOGRAFIA
PROGRAMA DE PÓS-GRADUAÇÃO EM AQUICULTURA

EFEITOS DA DUPLA TRANSGENIA PARA O EIXO SOMATOTRÓFICO SOBRE O CRESCIMENTO E REPRODUÇÃO DO ZEBRAFISH (*Danio rerio*)

ANA CECILIA GOMES SILVA

RIO GRANDE
Abril 2016

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Ana Cecilia Gomes Silva

Tese apresentada como parte dos requisitos para a obtenção do grau de doutor em Aquicultura no Programa de Pós-Graduação em Aquicultura da Universidade Federal do Rio Grande

Orientador: Dr. Luis Fernando Marins
Co-orientadora: Dra. Daniela Volcan Almeida

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Pós Graduação em
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DE DEFESA DA 40ª TESE DE DOUTORADO EM AQUICULTURA

No dia vinte e nove de abril de dois mil e dezesseis, às quatorze horas, no Auditório da Estação Marinha de Aquacultura da FURG, reuniu-se a Banca Examinadora de Tese de Doutorado em Aquicultura, da **ANA CECILIA GOMES SILVA**, orientada pelo Professor. Dr. Luis Fernando Fernandes Marins, composta pelos seguintes membros: Prof. Dr. Luis Fernando Fernandes Marins (Orientador – IO/FURG), Profa. Dra. Daniela Volcan Almeida (Co-orientadora – FURG), Prof. Dr. Marcelo Borges Tesser (IO/FURG), Prof. Dr. Antonio Sérgio Varela (ICB/FURG), Prof. Dr. Ricardo Berteaux Robaldo (UFPel) e Prof. Dr. Carlos Frederico Ceccon Lanes (UNIPAMPA). Título da Tese: "Efeitos da dupla transgenia para o eixo somatotrófico sobre o crescimento e reprodução do zebrafish (*Danio rerio*)". Dando início à defesa, o Coordenador do PPGAq, Prof. Dr. Marcelo Borges Tesser passou a presidência da sessão ao Prof. Dr. Luis Fernando Fernandes Marins, que na qualidade de orientador, passou a palavra para a candidata apresentar a Tese. Após ampla discussão entre os membros da Banca e a candidata, a Banca se reuniu sob a presidência do Coordenador. Durante esse encontro ficou estabelecido que as sugestões dos membros da Banca Examinadora devem ser incorporadas na versão final, ficando a cargo do Orientador o cumprimento desta decisão. A candidata **ANA CECILIA GOMES SILVA** foi considerada **APROVADA**, devendo a versão definitiva da Tese ser entregue na Secretaria do PPGAq, no prazo estabelecido nas Normas Complementares do Programa. Nada mais havendo a tratar, foi lavrada a presente ata, que após lida e aprovada, será assinada pela Banca Examinadora, pela candidata e pelo Coordenador do PPGAq.

PROF. DR. LUIS FERNANDO FERNANDES MARINS (IO/FURG) ORIENTADOR

PROFA. DRA. DANIELA VOLCAN ALMEIDA (FURG) CO-ORIENTADORA

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SUMÁRIO

Dê ao mundo o melhor de você,
Mas isso pode nunca ser o bastante,
Dê o melhor de você assim mesmo!
E veja você que no final das contas é entre você e Deus,
Nunca foi entre você e eles!

Madre Teresa de Calcutá

Dedido esta tese à minha família, em especial aos meus pais e ao meu marido, que pacientemente me incentivaram e ajudaram de forma incondicional.

MUITO OBRIGADO!!!

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Agradeço ao meu marido Evandro Malanski pelo amor e dedicação. Que sempre me incentivou a querer ser uma pessoa melhor a cada dia. Te amo muitão!!

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Às amigas Ana Luzia, Liane Artico, Maíra, Tati, Lupe, Isabel e Camilão meu muito obrigada vocês são à família que ganhei aqui. Obrigado por todos esses anos de amizade!!! Não podia esquecer as esquiletes Renatinha, Lo e Priscila Lange amo vocês!

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RESUMO GERAL

A aquicultura está assumindo um papel importante no atendimento da crescente demanda por pescado em nível mundial. Atualmente, existe uma interação benéfica entre a aquicultura e a biotecnologia que favorece a otimização da produção através da aplicação de ferramentas biotecnológicas. A transgênese é uma das ferramentas mais promissoras, onde o hormônio do crescimento (GH) tem sido o alvo mais importante na busca de linhagens de peixes geneticamente melhorados. Entretanto, está estabelecido que o excesso de GH ocasiona uma série de efeitos adversos devido à sua ação pleiotrópica. Neste sentido, o objetivo desta tese foi avaliar o efeito da dupla transgenia para o eixo somatotrófico sobre o crescimento e a reprodução do zebrafish (*Danio rerio*). Desta forma, o primeiro trabalho relata a produção de um duplo-transgênico (GH/GHR) através do cruzamento da linhagem F0104 (que superexpressa GH) com a linhagem Myo-GHR (que superexpressa o gene do receptor do hormônio do crescimento de maneira músculo-específica). No segundo estudo foi avaliado o efeito da dupla transgenia sobre o crescimento e estrutura muscular esquelética. O duplo-transgênico apresentou um crescimento comparável ao da linhagem F0104, embora com fator de condição mais baixo. Adicionalmente, houve diminuição na expressão de um gene relacionado com a sinalização intracelular em paralelo com uma forte diminuição na quantidade de fibras musculares grossas. O terceiro trabalho teve como objetivo avaliar os parâmetros reprodutivos de machos de zebrafish duplo-transgênicos. Os resultados mostraram uma melhora significativa em quase todos os parâmetros analisados, evidenciando uma redução dos efeitos negativos do GH sobre a função reprodutiva. Em conclusão, fica claro que o duplo-transgênico é um modelo de estudo interessante que pode gerar novos conhecimentos sobre os efeitos do GH em vários aspectos biológicos e fisiológicos. É necessário observar que a utilização deste hormônio deve respeitar os níveis máximos suportados pelo organismo a ser manipulado de forma que a transgenia do GH possa ser viável como ferramenta biotecnológica na aquicultura.

Palavras-chave: eixo somatotrófico; hormônio do crescimento; reprodução; zebrafish.

GENERAL ABSTRACT

Aquaculture is assuming an important role in meeting the growing demand for seafood worldwide. Currently there is a beneficial interaction between aquaculture and biotechnology, allowing production to be optimized through the application of biotechnological tools. Transgenesis is one of the most promising tools, where the growth hormone (GH) has been the most important target in the search for genetically improved fish lines. However, it is established that the GH excess causes a series of side effects due to its pleiotropic action. In this sense, the aim of this thesis was to evaluate the effect of double transgenesis for the somatotrophic axis on growth and reproduction of zebrafish (*Danio rerio*). Thus, the first paper describes the production of a double transgenic (GH/GHR) by crossing the F0104 line (which overexpresses GH) with Myo-GHR line (which overexpresses the growth hormone receptor gene (GHR) in a muscle-specific manner). In the second study, we evaluated the effect of double transgenesis on growth and skeletal muscle structure. The double transgenic showed a growth comparable to that of the F0104 line, although with lower condition factor. Additionally, there was a decrease in the expression of a gene related to intracellular signaling in parallel to a strong decrease in the amount of thick muscle fibers. The third study aimed to evaluate the reproductive parameters of double transgenic zebrafish males. The results showed a significant improvement in almost all parameters, showing a reduction of the negative effects of GH on reproductive function. In conclusion, it is clear that the double transgenic is an interesting study model that can generate new knowledge about the effects of GH in several biological and physiological aspects. It should be noted that the use of this hormone should comply with the maximum levels supported by the organism to be manipulated so that GH-transgenesis can be viable as a biotechnological tool in aquaculture.

Keywords: growth hormone; reproduction; somatotrophic axis; zebrafish.

INTRODUÇÃO GERAL

Ao longo dos últimos 30 anos, a produção global de alimentos aquáticos cultivados aumentou rapidamente e tem impulsionado a aquicultura para ser um dos setores de mais rápido crescimento (Allison 2011; UN 2011). Atualmente a aquicultura é responsável por quase metade do peixe consumido no mundo e estima-se que este setor será responsável por mais de 60% da produção mundial de pescado para consumo humano em 2030 (FAO 2014). A produção mundial de pescado atingiu o recorde de 158,5 milhões de toneladas em 2012, sendo que 40% deste total referem-se à aquicultura. É o setor da produção de alimentos de origem animal que mais cresce no mundo (FAO 2014). Assim, vemos claramente que a tendência dos últimos anos deve continuar nas próximas décadas, com a aquicultura sendo a maior responsável por atender a crescente demanda de pescado em nível mundial. Entretanto, para que a aquicultura cresça em um ritmo mais acelerado, uma série de obstáculos precisa ser superada. Além dos fatores referentes à economia mundial e ao preço do pescado, existe a necessidade do desenvolvimento de novos pacotes tecnológicos.

Os avanços na biotecnologia nas últimas décadas têm fornecido ferramentas importantes para intensificar a produção, contribuindo de várias formas para o desenvolvimento da aquicultura, como o desenvolvimento de vacinas, a incorporação de probióticos e enzimas nas rações e melhorando o desempenho nutricional de muitas espécies (Freitas et al. 2012). Além disso, uma das técnicas da biotecnologia que se destaca é a transgenia, devido ao potencial de melhoramento na produção que esta tecnologia pode oferecer (Zbikowska 2003; Dunham 2004). Desta forma, características novas, estáveis e determinadas geneticamente poderão ser incorporadas ao organismo receptor, com a possibilidade de serem transmitidas para a progênie. Entretanto, é importante salientar que não obstante o desenvolvimento desta tecnologia seja estratégico para qualquer país, mais estudos devem ser desenvolvidos sobre o bem-estar dos peixes manipulados (Hallerman et al. 2007).

Embora haja controvérsias na aplicação da transgenia, recentemente a agência de controle de alimentos e medicamentos dos Estados Unidos (Food & Drug Administration - FDA) aprovou a produção do salmão do Atlântico (*Salmo salar*) transgênico para o GH conforme notícia veiculada na *Nature News* em 19 de dezembro de 2015. Este é o primeiro animal transgênico aprovado para consumo humano nos Estados Unidos. Este peixe consegue crescer satisfatoriamente durante todas as estações

do ano, através da utilização de um promotor de um gene de uma proteína anticongelante derivado de uma enguia (*Zoarces americanus*), permitindo que o gene do GH do salmão chinook fosse expresso durante os meses de inverno, onde os peixes desaceleram sua taxa de crescimento. A FDA ressalta que não há diferenças biológicas significativas na composição nutricional do animal transgênico quando comparado a outros salmões criados em cativeiro.

O crescimento tem sido um dos alvos mais frequentes da manipulação genética, com o intuito de desenvolver espécies melhoradas para aquicultura, e para utilização como modelos para examinar a fisiologia do crescimento (Leggatt et al. 2012). O hormônio do crescimento (GH) tem como principal efeito o crescimento somático dos vertebrados e tem sido manipulado em mais de trinta e cinco espécies, sendo os melhores resultados com salmão coho (*Oncorhynchus kisutch*) e com *Misgurnus mizolepis* que tiveram um aumento considerável nas taxas de crescimento (Devlin et al. 1994; Nam et al. 2001). O processo de crescimento se dá, principalmente, através da síntese e secreção do GH pela hipófise que é liberado na corrente sanguínea, onde irá atuar em determinados órgãos através da sua associação com receptores específicos (GHR) presentes na membrana das células alvo (Figura 1). A ligação do GH com o GHR induz a fosforilação e consequente ativação de membros de uma família de enzimas conhecidas como Janus Kinases (JAKs) comumente associadas à parte intracelular do receptor (Lanning & Carter-Su 2006). Esta ligação induz a fosforilação e consequente ativação das Janus Kinase 2 (JAK2), que estão associadas à porção intracelular do receptor (Argetsinger et al. 1993; VanderKuur et al. 1995). O complexo JAK2 ativado por GH fosforila os sinais de tradução e ativadores de transcrição (STATs), levando à sua dimerização, translocação para o núcleo, ligação ao DNA e ativação da transcrição de genes relacionados ao crescimento (Herrington & Carter-Su 2001). As respostas biológicas do GH são desenvolvidas e controladas principalmente pelo denominado “eixo somatotrófico”, onde os fatores de crescimento tipo insulina (IGFs) são o principal mediador da ação fisiológica do GH (Moriyama et al. 2000; Butler & Roith 2001; Herrington & Carter-Su 2001). Os IGFs são pequenas cadeias polipeptídicas produzidas principalmente no fígado, que exercem uma influência direta nos processos de crescimento e desenvolvimento animal (Yakar et al. 1999). O crescimento, o dimorfismo sexual e a reprodução são mecanismos que são

principalmente regulados pela ação dos IGFs, também chamados de somatomedinas (Daughaday 2000; Le Roith et al. 2001).

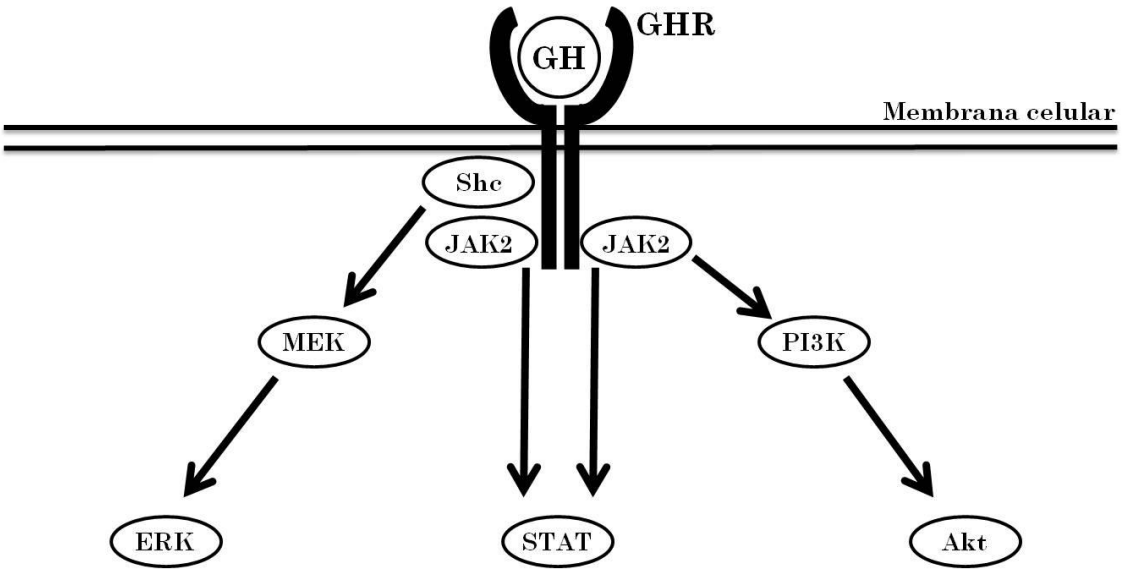

Fig. 1: Vias de sinalização intracelular do hormônio do crescimento (GH). Extraída de Figueiredo (2012).

O IGF-I tem sido reconhecido como um agente hipertrófico importante (Glass 2003; Clemmons 2009). IGF-I induz aumento de massa muscular pelo estímulo da via PI3K/Akt (fosfatidilinositol-3 quinase/proteína quinase B), resultando em ativação da síntese protéica (Rommel et al. 2001), associada à hipertrofia (Glass 2005; Clemmons 2009). Sabe-se que, além da via de sinalização intracelular PI3K/Akt, que está mais relacionada ao processo de diferenciação e síntese protéica (Coolican et al. 1997; Clemmons 2009), o IGF-I ativa também a via MAP-quinase, relacionada ao processo proliferativo (Clemmons 2009). Desta forma, genes que codificam para o IGF-I e seu receptor (IGF-IR), assim como genes que codificam para outras proteínas intermediárias da via PI3K/Akt surgem como alvos interessantes para a produção de linhagens transgênicas com o objetivo de aumentar o processo de hipertrofia muscular.

O crescimento da musculatura esquelética em peixes se faz pela proliferação e diferenciação das células progenitoras miogênicas também conhecidas como mioblastos adultos ou células miossatélites, responsáveis pelo crescimento hiperplásico e hipertrófico das fibras musculares (Koumans & Akster 1995, Johnston 1999, Johansen

& Overturf 2005). O crescimento hiperplásico do músculo se refere ao aumento no número de fibras musculares devido à formação de novas fibras (Johnston, 2004). Na hiperplasia, a fusão entre células satélites ativadas resultam na formação de novos miotubos na superfície das fibras existentes, com posterior diferenciação em fibras musculares (Stellabotte & Devoto, 2007). Durante o crescimento hipertrófico, as células satélites ativadas fundem-se com fibras musculares existentes, aumentando o número de mionúcleos e a síntese de miofibrilas, levando ao aumento no diâmetro ou área da fibra muscular (Johnston, 1999) (Figura 2). A contribuição relativa da hiperplasia e hipertrofia para o crescimento muscular nas espécies de peixes são variáveis, dependendo da espécie, fase de crescimento e tipo de músculo (Aguiar et al. 2005; Dal Pai-Silva et al. 2003). Em geral, nos peixes com crescimento indefinido ocorrem os dois tipos de crescimento muscular (hiperplasia e hipertrofia), durante todo o período de crescimento. No entanto, a hiperplasia é um processo de crescimento mais evidente durante a fase juvenil e o crescimento hipertrófico mais intenso e mais evidente durante a fase adulta do animal (Almeida et al. 2010).

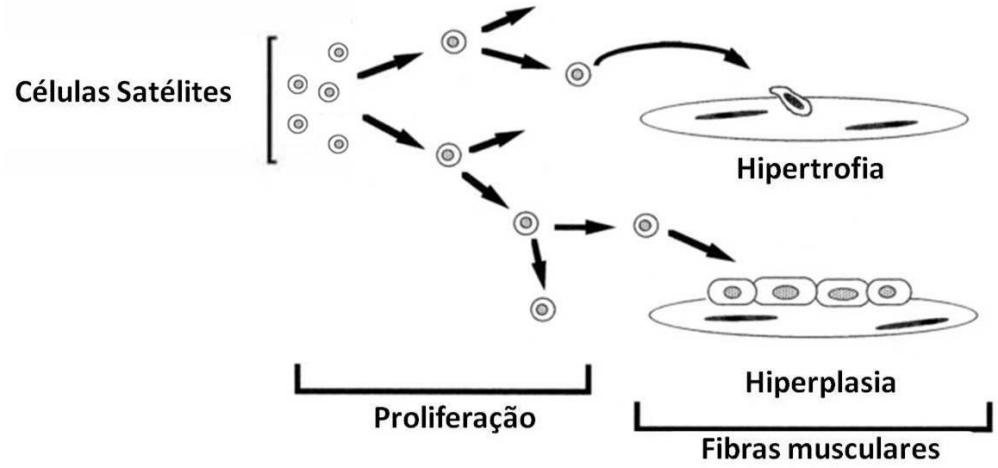

Fig. 2: Esquema demonstrativo dos principais mecanismos de crescimento muscular nos peixes: hipertrofia e hiperplasia. Adaptado de Johnston (1999).

A miogênese envolve uma série de processos que permitem o desenvolvimento e o crescimento do tecido muscular. Todos os eventos da miogênese são iniciados e controlados pela expressão diferencial de fatores transcricionais conhecidos como fatores de regulacão miogênica (*myogenic regulatory factors* ou MRFs), dos quais fazem parte a MyoD, Myf5, miogenina e MRF4 (Watabe 1999, 2001; Weintraub, 1993). Quando ativadas, MyoD e Myf5 induzem os mioblastos a expressar outras

proteínas miogênicas como a Miogenina e MFR4 (Watabe 1999, 2001). A MyoD e o Myf5 são conhecidos como fatores primários, sendo expressos na fase de proliferação dos mioblastos, enquanto os fatores secundários, miogenina e MRF4, são expressos em mioblastos nas fases de fusão e diferenciação em fibras musculares maduras (Watabe, 1999).

O crescimento e a reprodução estão intimamente relacionados em vertebrados. Há uma relação entre os eixos neuroendócrinos que controlam o crescimento e a reprodução (Trudeau, 1997; Hull and Harvey. 2002; Klausen et al. 2002). Além do seu papel na promoção do crescimento, GH atua diretamente sobre as gônadas estimulando a espermatogênese e síntese de hormônios ovarianos (Miura et al. 2011; Van Der Kraak et al. 1990), ou indiretamente no desenvolvimento gonadal (Berishvili et al. 2006). Como o GH participa da síntese de hormônios, ovulação, crescimento e renovação dos folículos, na maturação do oócito, na espermatogénese, na motilidade do espermatozóide, e outros aspectos do desenvolvimento reprodutivo, pode ser considerado um co-gonadotropina (Hull & Harvey. 2002). Estudos *in vitro* com carpa capim (*Ctenopharyngodon idellus*) utilizando células da pituitária revelou a regulação parácrina da secreção do hormônio luteinizante (LH) pelo GH (Zhou et al. 2004). Ao contrário de liberação do hormônio do crescimento em mamíferos, que é regulada principalmente pela secreção do hormônio liberador do GH e somatostatina secretado pelo hipotálamo, a liberação de GH em peixes também é modulada por outros fatores neuroendócrinos, incluindo hormônio liberador da gonadotrofina, o estradiol, e testosterona (Klausen et al. 2002; Lin et al. 1993; Trudeau et al. 1992; Wong et al. 2006; Xu et al. 2011). A superexpressão do GH provoca inúmeros efeitos biológicos podendo também afetar a reprodução. Tilápias transgênicas para o GH apresentaram uma baixa produção de espermatozóides e menor índice de gonadosomático ocasionando uma redução da capacidade reprodutiva deste animais e isto pode ser causado pelo direcionamento da energia para o crescimento somático, em vez de desenvolvimento gonadal (Rahman et al. 1998, 2001).

O desenvolvimento gonadal é cíclico na maioria dos teleósteos. A renovação das células germinativas, sua diferenciação, desenvolvimento, maturação e liberação ocorrem ao longo de cada ciclo reprodutivo e resultam em alterações gonadais que caracterizam diferentes fases reprodutivas (Lowerre-Barbieri et al. 2011). Segundo Ross et al. (1993), a espermatogênese é o processo no qual ocorre a produção de

espermatozóides e se inicia pela produção dos cistos espermatogênicos. Os principais hormônios hipofisários que regulam a espermatogênese são as gonadotrofinas folículo estimulante (FSH) e o hormônio luteinizante (LH) (Schult et al. 2010). Os cistos são formados quando as células de Sertoli envolvem as espermatogônias primárias. As células de Sertoli são células de sustentação e secretam o hormônio antimülleriano (*antimüllerian hormone* – AMH), uma glicoproteína que suprime o desenvolvimento dos ductos de Müller, precursores do trato reprodutor feminino. O AMH está envolvido na esteroidogênese e no desenvolvimento folicular (Josso et al. 2006). Em testículos de zebrafish foi demonstrado que o AMH tem um efeito inibitório sobre a espermatogênese. Em primeiro lugar, o AMH reduz ou bloqueia completamente a FSH ou estimula a libertação de 11-KT através da regulação de genes relacionados com a produção de andrógeno (Figura 3) (Skaar et al. 2011).

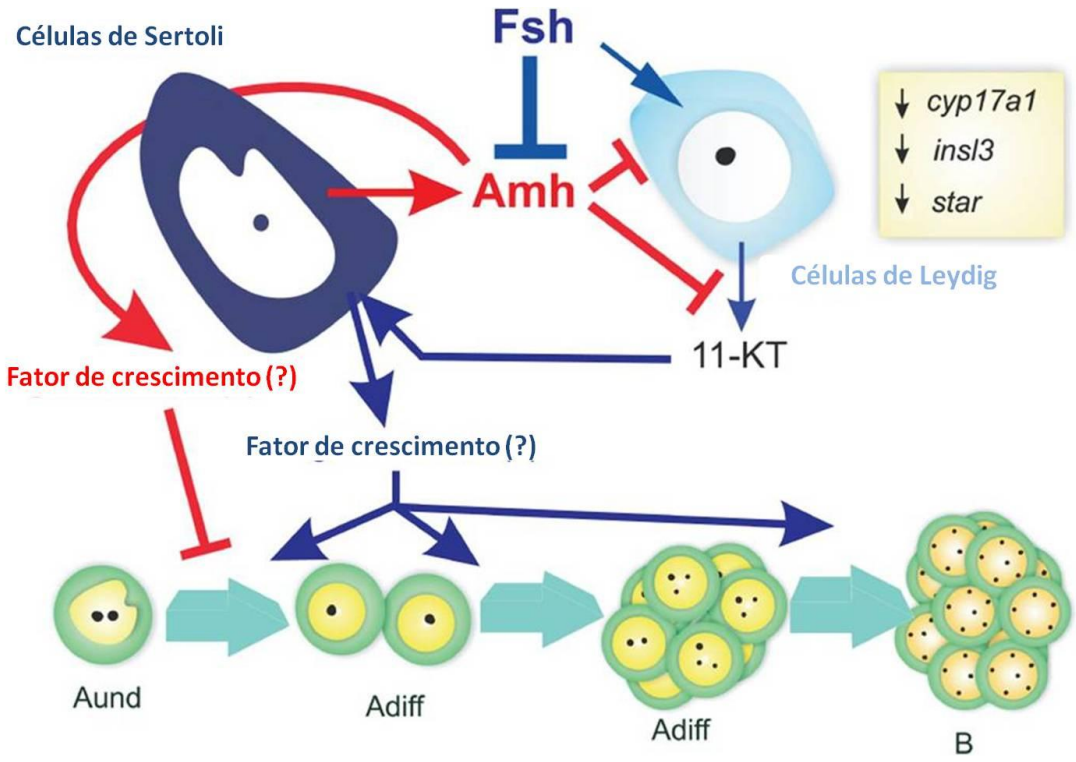

Fig. 3: Efeitos AMH sobre as funções do testículo de zebrafish. Adaptado de Skaar et al. (2011).

A Figura 4 mostra um esquema para a espermatogênese. As espermatogônias (2n), derivadas de células germinativas primordiais, sofrem uma série de divisões mitóticas e diferenciação morfológica, ao final das quais são produzidos espermatócitos (2n), os quais se dividem meioticamente e produzem as espermátides (n). Estas células haplóides sofrem diferenciação morfológica acentuada, conhecida como espermiogênese, até derivarem em espermatozóides (Ross et al. 1993). Finalmente, é na espermiação que ocorre a liberação do espermatozóide nos túbulos eferentes. A divisão meiótica de um espermatócito primário resulta na formação de quatro espermátides haplóides estruturalmente idênticas, mas geneticamente distintas cada uma com a capacidade de se diferenciar em um espermatozóide.

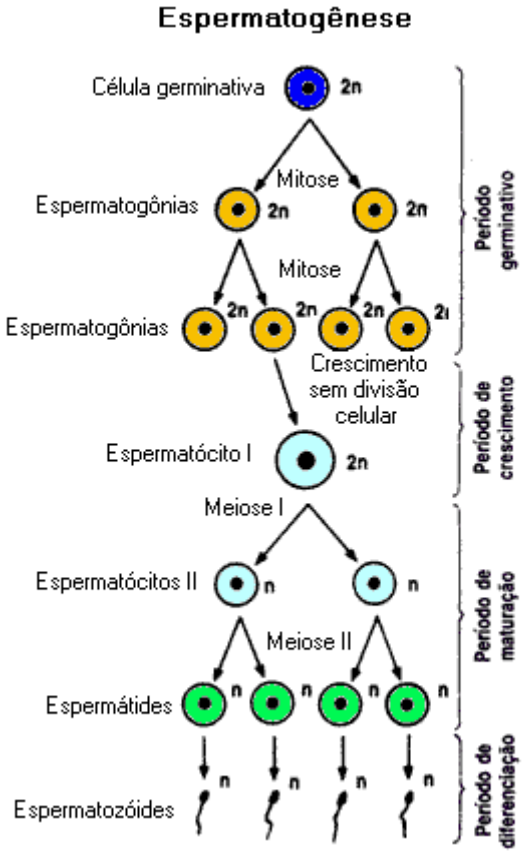

Fig. 4: Esquema da espermatogênese. Figura extraída da internet (https://fabiomizote.wordpress.com/espermatogenese-maculina).

O grupo de pesquisa do Laboratório de Biologia Molecular do Instituto de Ciências Biológicas (ICB/FURG) desenvolveu um modelo de peixe transgênico (zebrafish - *Danio rerio*) que foi denominada F0104. Esta linhagem se caracteriza por

superexpressar o GH do peixe rei marinho (*Odonthestes argentinensis*) (Figueiredo et al. 2007). Embora a manipulação do gene do GH tenha mostrado resultados promissores com relação ao crescimento em peixes, é claro que o GH atua em vários processos além do crescimento, produzindo efeitos pleiotrópicos sobre a morfologia, fisiologia, metabolismo, imunologia e comportamento (Devlin et al. 2006). Estudos com diferentes genótipos da linhagem F0104 reportam um aumento significativo na taxa de crescimento de indivíduos hemizigotos e também no nível de expressão dos genes do GHR e do IGF-I no fígado. Entretanto, os homozigotos expressam o dobro do GH em relação aos hemizigotos, porém o crescimento não foi diferente dos controles não transgênicos (Figueiredo et al. 2007), o que sugere um nível ótimo do hormônio em condições de alimentação controlada (Figueiredo et al. 2007). Ao analisar o sistema de defesa anti-oxidante da linhagem F0104, foi observado um aumento na geração de espécies reativas de oxigênio (ROS), provavelmente relacionado ao efeito anabólico do GH, e como consequência do aumento do consumo de oxigênio e da taxa metabólica (Rosa et al. 2008, 2010). Em outros estudos com esta mesma linhagem, Rosa et al. (2010) observaram envelhecimento acelerado provavelmente pela diminuição das defesas antioxidantes, alterações na estrutura muscular (Kuradomi et al. 2011) decréscimo na maturação do sistema imunológico (Batista et al. 2014) e diminuição significativa da qualidade espermática (Figueiredo et al. 2013).

O problema da qualidade espermática da linhagem F0104 tem implicações sérias no que diz respeito à aplicação desta tecnologia na aquacultura. Se reprodutores não puderem produzir grandes quantidades de larvas e juvenis, não haverá possibilidade de sustentar a produção em larga escala. Visando contornar este e os outros problemas gerados pelo excesso de GH na linhagem F0104, nosso grupo desenvolveu uma nova linhgem de zebrafish transgênico que superexpressa o GHR no tecido muscular esquelético, com o intuito de obter um aumento da massa muscular a partir de uma hipersensibilização deste tecido ao GH (Figueiredo et al. 2012). Entretanto, esta linhagem (denominada Myo-GHR) não apresentou o esperado aumento da massa muscular. Uma possível explicação para esta observação está baseada na redução significativa da expressão muscular dos genes relacionados ao eixo somatotrófico por bloqueio da sinalização na porção intracelular do receptor (Figueiredo et al. 2012).

A regulação entre o crescimento desejado e a viabilidade reprodutiva é um ponto extremamente importante a ser considerado no planejamento de um peixe

geneticamente modificado. No caso dos modelos transgênicos correntemente utilizados, o excesso de GH circulante não pode ser regulado pelo organismo, o que implica um crescimento obrigatório mesmo em condições desfavoráveis, acarretando efeitos metabólicos indesejados, especialmente na capacidade reprodutiva como já demonstrado para o zebrafish (Figueiredo et al. 2013). Estudos com outros vertebrados já comprovaram que a superexpressão do GH provoca muitos efeitos colaterais e a redução no desempenho reprodutivo é uma das consequências. O retardo no desenvolvimento gonadal ou a ausência total de gametas são sintomas reportados em camundongos (Naar et al. 1991), suínos (Pinkert et al. 1991), salmão (Devlin et al. 1994), tilápia (Rahman et al. 1998) e carpa (Yu et al. 2011).

Desta forma, as duas linhagens transgênicas descritas acima parecem ter características complementares. A linhagem F0104 tem excesso de GH circulante, enquanto que a linhagem Myo-GHR tem excesso de GHR no tecido muscular esquelético. O cruzamento destas duas linhagens poderia resultar em um animal duplo-transgênico com menos efeitos adversos decorrentes do excesso de GH circulante, pois a musculatura com mais GHR poderia diminuir os níveis de GH circulante e este hormônio em maior quantidade poderia ainda romper o bloqueio da sinalização intracelular do eixo somatotrófico no músculo causado pelo excesso de GHR. Espera-se um consequente aumento de massa muscular associado a uma melhora na capacidade reprodutiva desta nova linhagem duplo transgênica.

REFERÊNCIAS BIBLIOGRÁFICAS

Aguiar DH, Barros MM, Padovani CR, Pezzato LE, Dal Pai-Silva M (2005) Growth characteristics of skeletal muscle tissue in *Oreochromis niloticus* larvae fed on a lysine supplemented diet. Journal of Fish Biology 675:12877–1298. doi: 10.1111/j.1095-8649.2005.00823.x

Allison EH (2011) Aquaculture, Fisheries, Poverty and Food Security. Security 61. doi: Working Paper 2011–65.

Almeida FLA, Pessotti NS, Pinhal D, Padovani CR, Leitao NJ, et al. (2010) Quantitative expression of myogenic regulatory factors MyoD and myogenin in pacu (Piaractus mesopotamicus) skeletal muscle during growth. Micron, 41:997–1004. doi:10.1016/j.micron.2010.06.012

Argetsinger LS, Campbell GS, Yang X, et al (1993) Identification of JAK2 as a growth

hormone receptor-associated tyrosine kinase. Cell 74:237–44. doi:http://dx.doi.org/10.1016/0092-8674(93)90415-M

Batista CR, Figueiredo MA, Almeida DV, et al (2014) Impairment of the immune system in GH-overexpressing transgenic zebrafish (*Danio rerio*). Fish Shellfish Immunol 36:519–524. doi: 10.1016/j.fsi.2013.12.022

Berishvili G, D'Cotta H, Baroiller JF, Segner H, Reinecke M (2006) Differential expression of IGF-I mRNA and peptide in the male and female gonad during early development of a bony fish, the tilapia Oreochromis niloticus. Gen. Comp. Endocrinol 146:204–210

Butler AA, Roith D Le (2001) Control of growth by the somatropic axis: growth hormone and the insulin-like growth factors have related and independent roles. Annu. Rev. Physiol. 63:141–164. doi: 10.1146/annurev.physiol.63.1.141

Clemmons DR (2009) Role of IGF-I in skeletal muscle mass maintenance. Trends Endocrinol Metab 20:349–356. doi: 10.1016/j.tem.2009.04.002

Coolican SA, Samuel DS, Ewton DZ, et al (1997) The Mitogenic and Myogenic Actions of Insulin-like Growth Factors Utilize Distinct Signaling Pathways. J Biol Chem 272:6653–6662. doi: 10.1074/jbc.272.10.6653

Dal pai-silva M; Carvalho RF; Pellizzon CH; Pai VDal (2003) Muscle growth in Nile tilapia (Oreochromis niloticus): histochemical, ultrastructural and morphometric study. Tissue and Cell, 35:179–187. doi:10.1016/S0040-8166(03)00019-3

Daughaday WH (2000) Growth hormone axis overview--somatomedin hypothesis. Pediatr Nephrol 14:537–40.

Devlin RH, Yesaki TY, Biagi CA, et al (1994) Extraordinary salmon growth. Nature 371:209–210. doi: 10.1038/371209a0

Devlin RH, Sundstrom LF, Muir WM (2006) Interface of biotechnology and ecology for environmental risk assessments of transgenic fish. Trends Biotechnol 24:89–97. doi: 10.1016/j.tibtech.2005.12.008

Dunham R. (2004) Aquaculture and fisheries biotechnology: genetic approaches. Cambridge, Mass. CABI Publishing p 372.

FAO (2014) The state of the world fisheries and aquaculture. Rome: Food and Agriculture Organization of the United Nations. ISBN 978-92-5-108276-8.

Figueiredo MA, Lanes CFC, Almeida DV, et al (2007) The effect of GH overexpression on GHR and IGF-I gene regulation in different genotypes of GH-transgenic

zebrafish. Comp Biochem Physiol Part D Genomics Proteomics 2:228–33. doi: 10.1016/j.cbd.2007.04.004

Figueiredo MA, Mareco EA, Silva MDP, Marins LF (2012) Muscle-specific growth hormone receptor (GHR) overexpression induces hyperplasia but not hypertrophy in transgenic zebrafish. Transgenic Res 21:457–69. doi: 10.1007/s11248-011-9546-2

Figueiredo MA, Fernandes RV, Studzinski AL, et al (2013) GH overexpression decreases spermatic parameters and reproductive success in two-years-old transgenic zebrafish males. Anim Reprod Sci 139:162–7. doi: 10.1016/j.anireprosci.2013.03.012

Freitas AC, Rodrigues D, Rocha-santos TAP, et al (2012) Marine biotechnology advances towards applications in new functional foods ☆. Biotechnol Adv 30:1506–1515. doi: 10.1016/j.biotechadv.2012.03.006

Glass DJ (2003) Signalling pathways that mediate skeletal muscle hypertrophy and atrophy. Nature Cell Biology 5:87–90. doi:10.1038/ncb0203-87

Glass DJ (2005) Skeletal muscle hypertrophy and atrophy signaling pathways. Int J Biochem Cell Biol 37:1974–1984. doi: 10.1016/j.biocel.2005.04.018

Hallerman EM, McLean E, Fleming IA. (2007) Effects of growth hormone transgenes on the behavior and welfare of aquacultured fishes: A review identifying research needs. Appl Anim Behav Sci 104:265–294. doi: 10.1016/j.applanim.2006.09.008

Herrington J, Carter-Su C (2001) Signaling pathways activated by the growth hormone receptor. Trends Endocrinol Metab 12:252–7. doi: http://dx.doi.org/10.1016/S1043-2760(01)00423-4

Hull KL, Harvey S (2002) GH as a co-gonadotropin: the relevance of correlative changes in GH secretion and reproductive state. J. Endocrinol. 172:1–19. doi: 10.1677/joe.0.1720001

Johansen KA, Overturf K (2005) Quantitative expression analysis of genes affecting muscle growth during development of rainbow trout (*Oncorhynchus mykiss*). Mar Biotech 7:576–587.

Johnston IA (1999) Muscle development and growth: potential implication for flesh quality in fish. Aquaculture, 177:99–115.

Johnston IA (2004) Mechanisms of muscle development and responses to temperature change in fish larvae. American Fisheries Society Symposium 85–116.

Josso N, Picard JY, Rey R , di Clemente N (2006) Testicular anti-müllerian hormone: history, genetics, regulation and clinical applications. Pediatric endocrinology 3:347–358.

Klausen C, Chang JP, Habibi HR (2002) Time and dose related effects of gonadotropin releasing hormone on growth hormone and gonadotropin subunit gene expression in the goldfish pituitary. Can. J. Physiol. Pharmacol 80:915–924.

Koumans JTM, Akster HA (1995). Myogenic cells in development and growth of fish. Comp. Bioch. Physiol. A 110:3–20. doi:10.1016/0300-9629(94)00150-R

Kuradomi RY, Figueiredo MA, Lanes CFC, et al (2011) GH overexpression causes muscle hypertrophy independent from local IGF-I in a zebrafish transgenic model. Transgenic Res 20:513–21. doi: 10.1007/s11248-010-9429-y

Lanning NJ, Carter-Su C (2006) Recent advances in growth hormone signaling. Rev Endocr Metab Disord 7:225–35. doi: 10.1007/s11154-007-9025-5

Le Roith D, Bondy C, Yakar S, et al (2001) The somatomedin hypothesis: 2001. Endocr Rev 22:53–74. doi: 10.1210/er.22.1.53

Leggatt RA, Biagi CA, Smith JL, Devlin RH (2012) Growth of growth hormone transgenic coho salmon *Oncorhynchus kisutch* is influenced by construct promoter type and family line. Aquaculture 356-357:193–199. doi: 10.1016/j.aquaculture.2012.05.016

Lin X, Lin H, Peter RE (1993) Growth hormone and gonadotropin secretion in the common carp (*Cyprinus carpio L.*): in vitro interactions of gonadotropin- releasing hormone, somatostatin, and the dopamine agonist apomorphine. Gen. Comp. Endocrinol. 89:62–71. doi:10.1006/gcen.1993.1009

Lowerre-Barbieri SK, Brown-Peterson NJ, Murua H, et al (2011) Emerging Issues and Methodological Advances in Fisheries Reproductive Biology. Mar Coast Fish 3:32–51. doi: 10.1080/19425120.2011.555725

Miura C, Shimizu Y, Uehara M, Ozaki Y, Young G, Miura T (2011) Gh is produced by the testis of Japanese eel and stimulates proliferation of spermatogonia. Reproduction 142:869–877. doi: 10.1530/REP-11-0203

Moriyama S, Ayson FG, Kawauchi H (2000) Growth Regulation by Insulin-like Growth Factor-I in Fish. Biosci Biotechnol Biochem 64:1553–1562. doi: 10.1271/bbb.64.1553

Naar EM, Bartke A, Majumdar SS, et al (1991) Fertility of transgenic female mice

expressing bovine growth hormone or human growth hormone variant genes. Biol Reprod 45:178–87.

Nam YK, Noh JK, Cho YS, et al (2001) Dramatically accelerated growth and extraordinary gigantism of transgenic mud loach *Misgurnus mizolepis*. Transgenic Res. 10:353–362.

Pinkert CA, Kooyman DL, Dyer TJ (1991) Enhanced growth performance in transgenic swine. Biotechnology 16:251–258.

Rahman MA, Mak R, Ayad H, Smith A, Maclean N (1998) Expression of a novel piscine growth hormone gene results in growth enhancement in transgenic tilápia (*Oreochromis niloticus*). Transgenic Res 7:357–369.

Rahman MA, Ronyai A, Engidaw BZ, Jauncey K, et al (2001) Growth and nutritional trials on transgenic Nile tilapia containing an exogenous fish growth hormone gene. J. Fish Biol. 59:62–78. doi: 10.1111/j.1095-8649.2001.tb02338.x

Rommel C, Bodine SC, Clarke BA, et al (2001) Mediation of IGF-1-induced skeletal myotube hypertrophy by PI(3)K/Akt/mTOR and PI(3)K/Akt/GSK3 pathways. Nat Cell Biol 3:1009–13. doi: 10.1038/ncb1101-1009

Rosa CE, Figueiredo MA, Lanes CFC, et al (2008) Metabolic rate and reactive oxygen species production in different genotypes of GH-transgenic zebrafish. Comp Biochem Physiol B Biochem Mol Biol 149:209–14. doi: 10.1016/j.cbpb.2007.09.010

Rosa CE, Kuradomi RY, Almeida DV, et al (2010) GH overexpression modifies muscle expression of anti-oxidant enzymes and increases spinal curvature of old zebrafish. Exp Gerontol 45:449–456. doi: 10.1016/j.exger.2010.03.012

Ross MH; Reith EJ; Romrell LJ (1993) Histologia: texto e atlas. Panamericana, 779. São Paulo.

Skaar KS, Nobrega RH, Magaraki A, Olsen LC, et al (2011) Proteolytically activated, recombinant anti-Müllerian hormone inhibits androgen secretion, proliferation, and differentiation of spermatogonia in adult zebrafish testis organ cultures. Endocrinology 152:225–234.

Skaar KS, Nóbrega RH, Magaraki A, et al (2011) Proteolytically activated, recombinant anti-müllerian hormone inhibits androgen secretion, proliferation, and differentiation of spermatogonia in adult zebrafish testis organ cultures. Endocrinology 152:3527–3540. doi: 10.1210/en.2010-1469

Stellabotte F, Devoto SH (2007) The teleost dermomyotome. Developmental dynamics: an official publication of the American Association of Anatomists,236:2432–43.

Schulz RW, de Franca LR, Lareyre JJ, et al. (2010) Spermatogenesis in fish. General and comparative endocrinology 165:390–41. doi: 10.1016/j.ygcen.2009.02.013

Trudeau VL (1997). Neuroendocrine regulation of gonadotrophin II release and gonadal growth in the goldfish, Carassius auratus. Rev. Reprod 2:55–68

UN (2011) Aquaculture Has Potential to Cut Poverty, Combat Food Security. United Nations News Cent. URL. http://www.un.org/apps/news/story.asp?NewsID=40343#.Vxo3IvkrK00.

VanderKuur JA, Wang X, Zhang L, et al (1995) Growth Hormone-dependent Phosphorylation of Tyrosine 333 and/or 338 of the Growth Hormone Receptor. J Biol Chem 270:21738–21744. doi: 10.1074/jbc.270.37.21738

VanderKuur G, Rosenblum PM, Peter RE (1990) Growth homone dependent potentiation of gonadotropin stimulated steroid production by ovarian follicles of the goldfish. Gen. Comp. Endocrinol. 79:233–239. doi:10.1016/0016-6480(90)90108-X

Watabe S (1999) Myogenic regulatory factors and muscle differentiation during ontogeny in fish. Journal of Fish Biology 55:1–18.

Watabe S (2001) Myogenic regulatory factors. In I.A. Johnston (Ed.), Muscle development and growth. San Diego - California: Academic press inc. (1st ed., Vol. 156:19–39)

Weintraub, Harold. 1993. The MyoD family and myogenesis: Redundancy, networks,and thresholds. Cell, 75:1241–1244

Wong AOL, Zhou H, Jiang Y, Ko WKW (2006) Feedback regulation of growth hormone synthesis and secretion in fish and the emerging concept of intrapituitary feedback loop. Comp. Biochem. Physiol. A: Mol. Integr. Physiol. 144:284–305. doi:10.1016/j.cbpa.2005.11.021

Xu J, Huang W, Zhong C, Luo D, et al (2011) Defining global gene expression changes of the hypothalamic-pituitary-gonadal axis in female sGnRH-antisense transgenic common carp (*Cyprinus carpio*). PLoS One 6, e21057. doi: org/10.1371/journal.pone.0021057

Yakar S, Liu JL, Stannard B, et al (1999) Normal growth and development in the

absence of hepatic insulin-like growth factor I. Proc Natl Acad Sci U S A 96:7324–9.

Yu F, Xiao J, Liang X, et al (2011) Rapid growth and sterility of growth hormone gene transgenic triploid carp. Chinese Sci Bull 56:1679–1684. doi: 10.1007/s11434-011-4446-7

Zbikowska HM (2003) Fish can be first – advances in fish transgenesis for commercial. 379–389.

Zhou H, Wang X, Ko WK, Wong AO (2004) Evidence for a novel intrapituitary autocrine/paracrine feedback loop regulating growth hormone synthesis and secretion in grass carp pituitary cells by functional interactions between gonadotrophs and somatotrophs. Endocrinology 145:5548–5559 doi: http://dx.doi.org/10.1210/en.2004-0362

OBJETIVOS

Objetivo Geral

Produzir um zebrafish duplo-transgênico que se caracteriza por uma superexpressão músculo-específica do gene do receptor do hormônio do crescimento (GHR) associada à superexpressão de forma constitutiva do gene do hormônio do crescimento (GH), e avaliar os efeitos desta manipulação genética sobre o crescimento e a reprodução.

Objetivos Específicos

1. Cruzar as linhagens transgênicas F0104 e Myo-GHR para obtenção dos duplo transgênicos (GH/GHR);
2. Analisar o crescimento, morfometria e histologia do músculo do duplo transgênico comparando com as demais linhagens transgênicas (F0104 e Myo-*GHR*) e com peixes não-transgênicos;
3. Analisar a expressão dos genes do eixo somatotrófico no tecido muscular, assim como dos genes que codificam para os principais fatores miogênicos e proteínas musculares;
4. Verificar os parâmetros reprodutivos de machos de zebrafish duplo transgênicos através de citometria de fluxo, histologia e expressão de genes relacionados com a maturação espermática.
5. Comparar a capacidade reprodutiva do machos duplo transgênicos com machos não transgênicos quanto aos percentuais de fertilização e eclosão.

CAPÍTULO 1

Double Transgenic Zebrafish for Somatotrophic Axis: A Tool for Muscle Development and Growth Studies

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Double Transgenic Zebrafish for Somatotrophic Axis: A Tool for Muscle Development and Growth Studies

Ana C. Silva, Daniela V. Almeida, Marcio A. Figueiredo, and Luis F. Marins

Transgenic zebrafish lines overexpressing growth-related genes are rare. Our research group has developed two of them expressing growth hormone (GH) ubiquitously (actb1 promoter) or its receptor (GHR) specifically in the skeletal muscle (mylz2 promoter). Besides GH, the former line also expresses GFP under control of the actb1 promoter. Similarly, the GHR line expresses DsRED in the skeletal muscle. Breeding the two lines generated Mendelian proportions of four genotypes (1:1:1:1): nontransgenic, GH transgenic, GHR transgenic, and a double GH/GHR transgenic identified by both green and red fluorescence (Fig. 1). Double (GH/GHR) transgenics can be used to address not only the effects of circulating GH excess but also increased GH signaling in skeletal muscle.

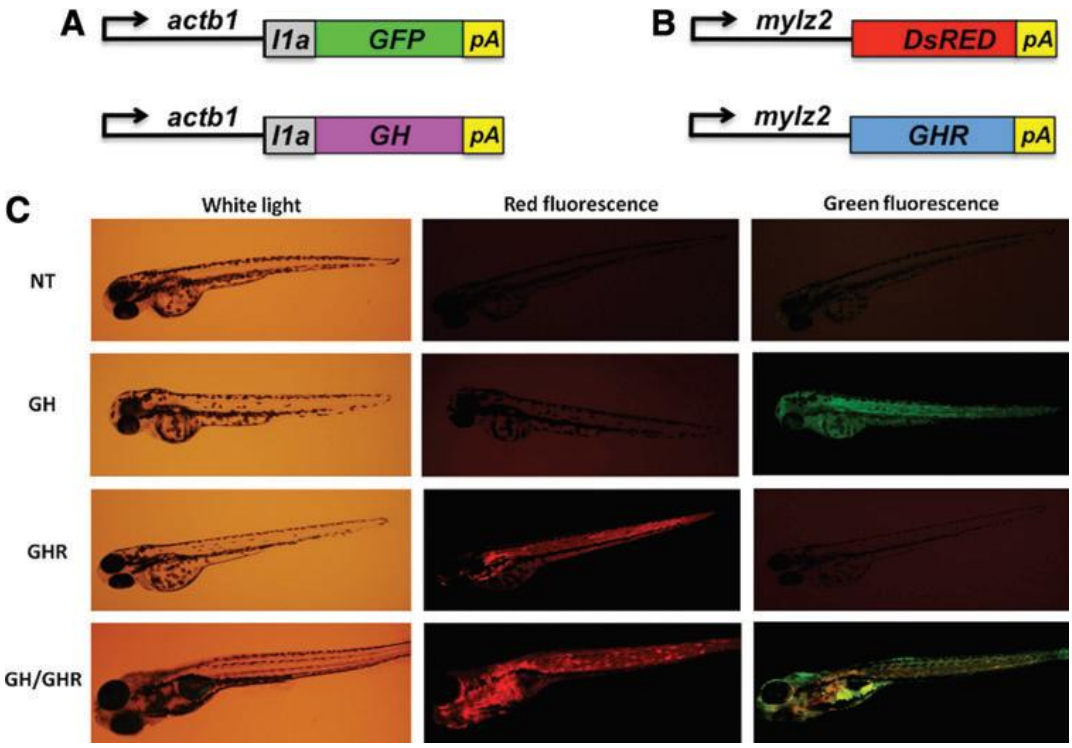

FIG. 1. (A) The GH line [Tg(actb1:GH/actb1:GFP)] expresses both GH and GFP from a ubiquitous actb1 promoter that includes the first intron (I1a). (B) The GHR line [Tg(mylz2:GHR/mylz2:DsRED)] expresses both GHR and DsRED from a muscle-

specific mylz2 promoter. (C) Side view of zebrafish larvae from different genotypes under white light and red and green fluorescence. Nontransgenic (NT) shows no fluorescence, the GH line shows only GFP expression, the GHR line shows only DsRED expression, while double (GH/GHR) transgenic embryos show expression of both GFP and DsRED. GH, growth hormone; GHR, growth hormone receptor.

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References

1. Figueiredo MA, Lanes CFC, Almeida DV, Marins LF. Improving the production of transgenic fish germlines: *in vivo* evaluation of mosaicism in zebrafish (*Danio rerio*) using a green fluorescent protein (GFP) and growth hormone cDNA transgene coinjection strategy. Genet Mol Biol 2007;30:31–36.

2. Figueiredo MA, Mareco EA, Silva MDP, Marins LF. Muscle-specific growth hormone receptor (GHR) overexpression induces hyperplasia but not hypertrophy in transgenic zebrafish. Transgenic Res 2012;21:457–469.

CAPÍTULO 2

Effects of double transgenesis of somatotrophic axis (GH/GHR) on skeletal muscle growth of zebrafish (*Danio rerio*)

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Effects of double transgenesis of somatotrophic axis (GH/GHR) on skeletal muscle growth of zebrafish (*Danio rerio*)

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Abstract

Transgenic fish for growth hormone (GH) has been considered as a potential technological improvement in aquaculture. In this study, a double-transgenic zebrafish was used to evaluate the effect of GH and its receptor (GHR) on muscle growth. Double-transgenics reached the same length of GH-transgenic, but with significantly less weight, featuring an unbalanced growth. The condition factor of GH/GHR transgenic fish was lower than the other genotypes. Histological analysis showed a decrease in the percentage of thick muscle fibers in GH/GHR genotype of approximately 80% in comparison to GH-transgenic line. The analysis of gene expression showed a significant decrease in genes related to muscle growth in GH/GHR genotype. It seems that concomitant overexpression of GH and GHR resulted in a strong decrease of the somatotrophic axis intracellular signaling by diminishing its principal transcription factor STAT5.1.

Keywords: Growth hormone; growth hormone receptor; transgenic zebrafish; muscle hypertrophy.

Introduction

According to FAO[1], aquaculture is currently one of the fastest growing production sectors in the world, with a series of tools being developed for perfecting production techniques. In this context, modern biotechnology presents great potential for meeting production demands and improving the phenotypical characteristics of aquatic organisms. Transgenesis is an important genetic manipulation tool used for modifying the traits of commercially important species such as fish. For aquaculture purposes, this manipulation has focused mainly on increasing growth rates of lines.[2] After the first transgenic fish was produced in China by Zhu et al.[3], this technology has been successfully applied to dozens of fish species including some with high commercial interest such as carps, tilapias, salmonids and catfish.[4]

The growth process in fish begins with growth hormone (GH) synthesis in the hypophysis, followed by its release into the bloodstream where it acts on certain organs through association with specific receptors (GHR) present in the membranes of target cells. Activation of intracellular signaling pathways culminates with the transcription of genes involved in the development of biological responses to GH. In this manner, GH effects are produced and mostly controlled by the somatotrophic axis, with insulin-like growth factor (IGF-I) as the main physiological mediator. [5,6]

Although manipulation of the GH gene has shown promising results for increasing fish growth [7–9], it is known that GH also acts in other processes producing pleiotropic effects on morphology, physiology, metabolism and behavior.[10] Figueiredo et al. [11] developed a transgenic zebrafish (*Danio rerio*) model overexpressing silverside (*Odontesthes argentinensis*) GH, and reported a significant increase in transgenic fish growth rates. More recently, Figueiredo et al. [12] created a new transgenic line overexpressing GHR in muscle tissue, in order to increase skeletal muscle intracellular signaling independently of supraphysiological levels of circulating GH and its resulting pleiotropic effects. However, this line did not present the expected muscle mass, seeming that hypertrophy is independent from GHR overexpression alone. Here the main objective was to evaluate if double transgenesis with increased circulating GH and a higher number of GH receptors on muscle membranes results in increased muscle growth. For achieving this, analyses of growth, gene expression and muscle hypertrophy of double-transgenic (GH/GHR) zebrafish were performed.

Material and Methods

Zebrafish genotypes

All analyzed genotypes were obtained by breeding males from F0104 line [11] with females from MYO-GHR line. [12] Besides GH, the F0104 line also expresses the green fluorescent protein (GFP) under transcriptional control of the β-actin promoter of carp *Cyprinus carpio*. On the other hand, the MYO-GHR line expresses, apart from GHRa gene, the red fluorescent protein (DsRED) in skeletal muscle tissue (zebrafish myosin light chain promoter). Breeding the two lines generated mendelian proportions of four genotypes (1:1:1:1), identified by epifluorescence microscopy: non-transgenic (NT) = no fluorescence; F0104 (GH) line = green fluorescence; MYO-GHR (GHR) line = red fluorescence; and double-transgenic (GH/GHR) = green and red fluorescence (Fig. 1).

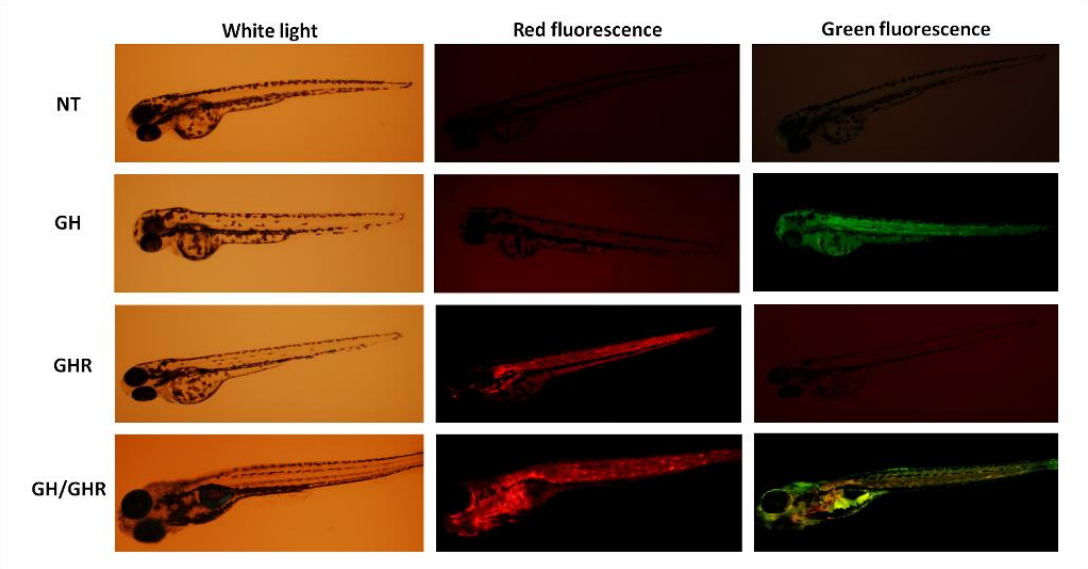

Fig. 1 Side view of zebrafish (*Danio rerio*) larvae from different genotypes under white light, red and green fluorescence. Non-transgenic fish (NT) no shows expression fluorescent, GH lineage shows the expression of green fluorescent protein GFP (excitation = 485 nm, emission = 520 nm), GHR lineage shows the expression of red fluorescent protein DsRed (excitation = 557 nm, emission = 579 nm), and GH/GHR (double) transgenic shows the expression of both red and green fluorescent protein.

Growth analysis

Fish were separated by genotypes and cultivated for 165 days in 15 L aquariums (N = 10 per aquarium, in triplicate). Culture conditions were maintained according to zebrafish requirements.[13] Fish were fed twice daily *ad libitum* with commercial feed (47.5% crude protein). For final biometric analysis, fish were anesthetized with tricaine (0.1 mg/mL), weighed, and photographed in lateral decubitus for standard length measurements (L_s), obtained through ImageJ (Image processing and analysis in Java, http://rsb.info.nih.gov/ij/). Condition factor (K) was calculated using the equation $K = (W. L^{-3}) \times 10^3$, where W is mass (mg) and L the total length (mm).

Gene expression analysis

At the end of the growth experiment, five individuals of each group were randomly sampled for gene expression analysis. Genes related to the muscle growth such as insulin-like growth factor 1 (*igf1*), myogenic regulatory factors (*myod* and *myf5*) and muscle structural proteins (*mylz2* and *myhc4*) were chosen. In order to evaluate intracellular signaling, we analyzed the Signal Transducer and Activator of Transcription 5.1 (*stat5.1*) gene. Fish were sacrificed with tricaine (0.5 mg/mL) for removal of skeletal muscle, total RNA extraction using Trizol reagent (Invitrogen, Brazil), and cDNA synthesis through High Capacity cDNA Reverse Transcription kits (Applied Biosystems, Brazil). All procedures were conducted as suggested by manufacturers.

Expression was analyzed through quantitative Real Time PCR (qPCR), with each sample analyzed in triplicate. Table 1 shows primer sequences used for each gene, designed using Primer Express 3.0 software (Applied Biosystems, Brazil) based on sequences available in GenBank (http://www.ncbi.nlm.nih.gov). qPCR reactions were performed using Platinum SYBR Green qPCR SuperMix-UDG kits (Invitrogen, Brazil). Serial dilutions were done for all primers in order to determine the reaction's efficiency (data not shown). PCR conditions were 50 °C/2 min, 95 °C/2 min, followed by 40 cycles of 95 °C/15 s and 60 °C/30 s. Expression of target genes was normalized by the constitutive genes elongation factor 1 alpha (*ef1α*) and beta-actin (*βactin*), which did not vary between experimental groups (data not shown).

Table 1. Gene-specific primers for qPCR analyses.

Gene	Sequence	GenBank	Efficiency
igf1	F: 5'-CAGGCAAATCTCCACGATCTC-3'	NM131825	86%
	R: 5'-TTTGGTGTCCTGGAATATCTGT-3'		
myf5	F: 5'-TCCAATGGGCCTGCAAA-3'	AF270789	105%
	R: 5'-CGGCGGTCCACCGTACT-3'		
myod	F: 5'-GGAGCGAATTTCCACAGAGACT-3'	BC114261	104%
	R: 5'-GTGCCCCTCCGGTACTGA-3'		
mylz2	F: 5'-TGGAGGCCATGATCAAGGAA-3'	BC045520	90%
	R: 5'-TGGTGAGGAAAACGGTGAAGT-3'		
myhc4	F: 5'-GCGCGCTGACATTTCTGA-3'	AY921650	85%
	R: 5'-CAGCGTCACGGCTTTTGG-3'		
stat5.1	F: 5'-AAATTGGCGGCATCACTATAGC-3'	NM194387	90,7%
	R: 5'-CCTTTCCCCTGCTTTGTTAGG-3'		
ef1α	F: 5'-GGGCAAGGGCTCCTTCAA-3'	NM131263	101%
	R: 5'-CGCTCGGCCTTCAGTTTG-3'		
ßactin	F: 5'-CTGTCCACCTTCCAGCAGAT-3'	AF180887	100%
	R: 5'-GATGGACCTGCCTCGTCGTA-3		

qPCR, quantitative real-time polymerase chain reaction.

Histological analysis

Eight fish of each group were sacrificed with tricaine (0.5 mg/mL) for skeletal muscle removal. Muscle tissue was positioned according to methods described by Lillie [14], fixed in Bouin's solution for 4 hours, and preserved in 70% ethanol. Histological samples were prepared using classic methods and cut into 4 µm sections, stained with hematoxylin/eosin, and analyzed following methods described in Weibel et al. [15] modified by Romano et al. [16] Muscle fibers were classified according to their diameter: thin (≤ 40 µm) and thick (> 40 µm) fibers.

Statistical analyses

Growth data was submitted to one-way ANOVA followed by Tukey's post hoc test. Relative gene expression was calculated through $2^{-\Delta\Delta CT}$. [17] Muscle fiber variance was analyzed through an r x c table with a 5% significance level. When significant difference was encountered, Marascuillo multiple comparison test was applied (National Institute of Statistics, http://www.itl.nist.gov/div898/handbook).

Results

Growth

The evaluated genotypes presented final weight (mg ± SE) of: NT = 254 ± 15, GH = 734 ± 37, GHR = 309 ± 39 and GH/GHR = 476 ± 35. Statistical analysis showed that the GH and GH/GHR groups weighed significantly more than the NT and GHR groups (Fig. 2A). No differences were observed when comparing NT versus GHR, but the GH genotype presented significant weight increase in relation to GH/GHR. In terms of standard length (mm ± SE), all transgenic fish (GH = 33 ± 0.5; GHR = 26 ± 0.9; GH/GHR = 31 ± 0.7) were significantly larger than the NT (23 ± 0.4) ones. However, the GHR was significantly smaller than the GH and GH/GHR groups (Fig. 2B). No significant difference in condition factor (± SE) was observed between NT (2 ± 0.04), GH (2.1 ± 0.04) and GHR (1.8 ± 0.09) fish. On the other hand, the GH/GHR group (1.6 ± 0.08) presented lower condition factor when compared to the NT and GH groups (Fig. 2C). The survival of the animals at the end of the growth experiment was 100%.

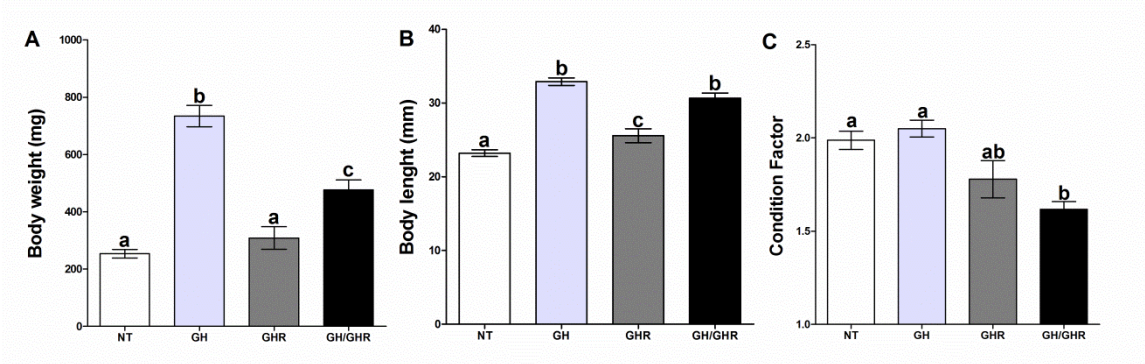

FIG. 2. Biometry of zebrafish (*Danio rerio*). GH=GH transgenics; GHR=GHR transgenics; GH/GHR = double transgenics. Zebrafish (165 days old) were measured on the following parameters: body weight (A), body length (B), and condition factor (C). Different letters indicate significant differences ($p < 0.05$).

Gene expression

GH/GHR genotype presented, in general, significant decrease in skeletal muscle expression of *stat5.1*, *igf1*, *myod*, *myf5* and *myhc4* when compared to the other groups (Fig. 3A, B, C, D and E). Expression of the *mylz2* was significantly decreased in all transgenics in relation to NT group (Fig. 3F).

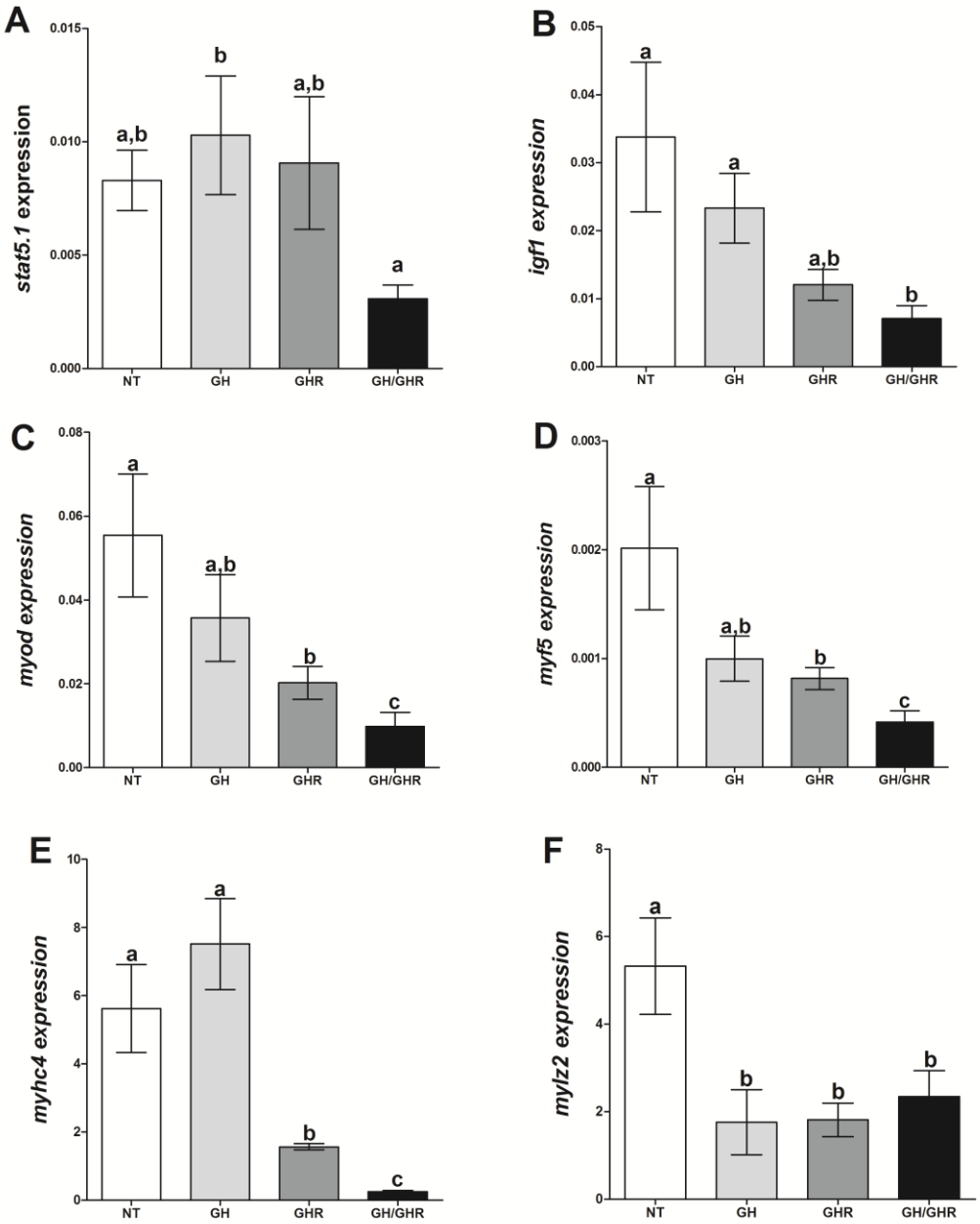

FIG. 3. Expression of growth-related genes in skeletal muscle of 165-day-old zebrafish (*Danio rerio*). Analyzed genes: (A) *stat5.1*, (B) *igf1*, (C) *myod*, (D) *myf5*, (E) *myhc4*,

(F) *mylz2*. The gene expression was normalized by expression of the constitutive genes: elongation factor 1 alpha (*ef1α*) and beta-actin (*bactin*). Data are expressed as mean – SEM of $2^{-\Delta\Delta CT}$ (n = 5). Different letters represent significant differences ($p < 0.05$). The groups represented by ‘‘a’’ and ‘‘b’’ are significantly different between them, but the group represented by ‘‘a,b’’ was not significantly different either from ‘‘a’’ or ‘‘b.’’

Muscle histology

Analysis of muscle fibers revealed significantly different profiles between study groups (Fig. 4). Highest percentage of thick fibers was observed in the GH (36%), followed by NT (14%), GH/GHR (7%) and GHR (2%) groups. The fibers showed different sizes, separated by loose connective tissue the endomysium, and organized into fascicles by a thicker connective tissue septum, forming a structure similar to the perimysium. The same cell types (myoblasts and satellite cells) were found in all genotypes.

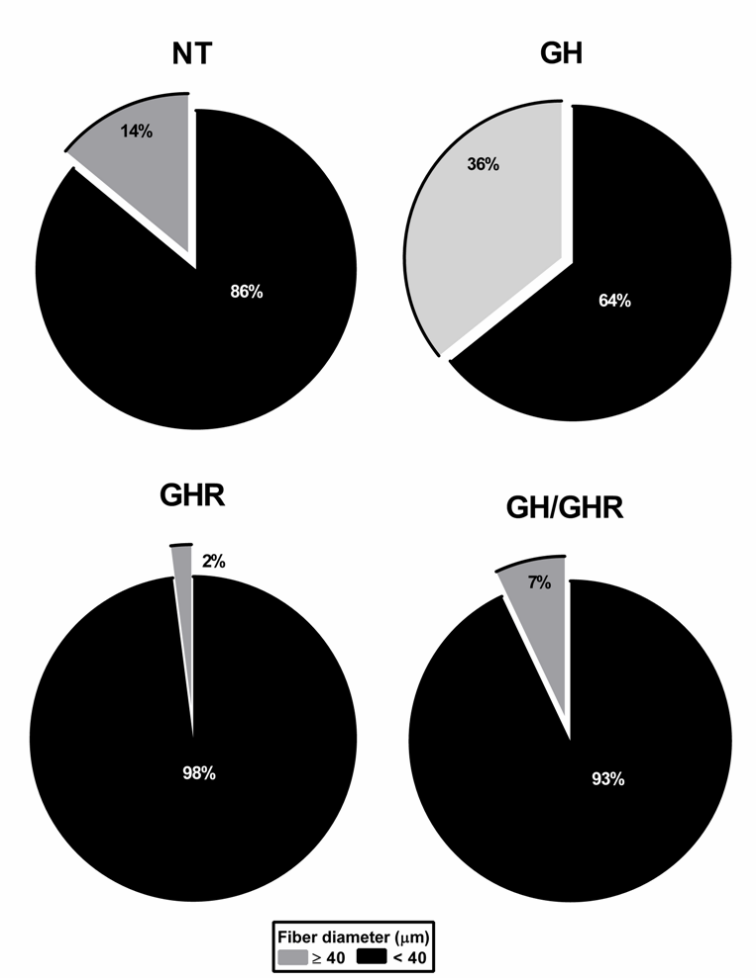

FIG. 4. Proportion of muscle fibers of 165-day-old zebrafish (*Danio rerio*) from different genotypes: NT, GH, GHR, GH/GHR. Muscle fibers were classified as thin (≥ 40 μm) or thick (<40 μm). The differences were significantly different at $p < 0.05$.

Discussion

In this study we used two transgenic lines, one overexpressing GH and the other GHR, for production of a double-transgenic zebrafish (GH/GHR). Both lines express fluorescent proteins for non-invasive genotype identification. The main objective was to evaluate if a breed between the two above-cited lines could increase muscle growth. Results from analyses of growth, muscle structure and gene expression demonstrated that, differently from our initial hypothesis, muscle growth was not enhanced in the GH/GHR double-transgenics when compared to GH genotype.

Condition factor (K), which represents the degree of wellbeing and robustness of an animal,[18] showed significant decrease (21%) in the double-transgenic when

compared to the GH group. This decrease was due to the fact that the GH/GHR group did not present proportional growth, i.e. attained the same length as GH fish, but with significantly lower weights. The result of this morphological alteration in double-transgenics was a more slender fish than the other analyzed genotypes. In addition, muscle structure alterations were also observed in the double-transgenic group, with approximately 80% decrease in thick fibers when compared to the line overexpressing GH. A possible explanation for these observations could be that the excess of circulating GH caused increase in longitudinal growth, but overexpression of GHR in muscle tissue blocked intracellular signaling of this hormone, with consequent decrease of hypertrophy.

Regarding gene expression analyses, it was observed a significant decrease of *igf1* (approximately 70%) in GHR and GH/GHR genotypes. IGFs are present in a wide variety of tissues at relatively high concentrations, displaying hypertrophic and hyperplasic properties and being important regulators of myogenesis. [5,21] This decline in *igf1* expression could be result of a generalized decrease in GH intracellular signaling. Phosphorylation is induced when GH links to GHR, with consequent activation of Janus Kinases (JAKs), a family of enzymes commonly associated with the intracellular portion of the receptor. [19] Activated JAKs phosphorylate specific tyrosine-rich regions of the receptors, which are anchorage sites for molecules related to different signaling pathways, and culminate with the activation of specific genes for GH-mediated biological actions. Transcriptional activation of *igf1* follows the classic route driven by proteins known as Signal Transducer and Activator of Transcription (STATs), which when phosphorylated form dimers for nuclear translocation. [20] In fact, *stat5.1* expression analysis showed a decrease of approximately 75% in GH/GHR transgenic zebrafish when compared to GH group. This result could be interpreted as a consequence of the increased intracellular signalization due to concomitant GH and GHR overexpression. In fact, muscle cells of double transgenics would be producing less STATs as a way to avoid excessive signaling due to the presence of large amounts of membrane GHR and circulating GH. Considering that the STATS are the main transcription factors associated with IGF1, its lower production could explain the observed decline in *igf1* expression.

Apart from the JAK/STAT pathway, GH activates two other intracellular signaling pathways. The PI3K/Akt pathway is related to IGF1-induced muscle mass

increase, through activation of genes associated with protein synthesis increase, hypertrophy, apoptosis inhibition, and decrease in muscle protein degradation.[22–24] The MEK/ERK pathway is related to cell proliferation and differentiation,[24,25] through phosphorylation of tyrosines mediated by serine/threonine kinases[26]. PI3K/Akt and MEK/ERK pathways can activate genes that code not only for myogenic regulatory factors (MRFs), but also for muscle proteins.[27,28] In this study, expression of *myod* and *myf*5 was respectively 73% and 58% lower in the double-transgenics when compared to the GH group. Regarding muscle proteins, the gene that codifies for the *myhc4* presented the largest decrease (97%) when comparing the GH to the GH/GHR group. When analyzed as a whole, gene expression results support the hypothesis that GH/GHR overexpression causes a significant intracellular signaling decrease in the main somatotrophic axis pathways.

To our knowledge, this is the first work to use a double-transgenic zebrafish overexpressing growth hormone in a constitutive way, and its receptor in a muscle-specific manner. The results obtained with this model allowed an *in vivo* analysis of the main responses related to the effects of GH/GHR axis on muscle growth. In the case of the double-transgenic (GH/GHR) fish used in the present study, a scenario of elevated somatotrophic axis signaling in muscle tissue was expected. However, concomitant overexpression of GH and GHR probably leads to intracellular signaling levels not supported by the cells, which respond by decreasing the production of molecules associated with major intracellular signaling pathway of the somatotrophic axis.

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References

1. FAO. The state of the world fisheries and aquaculture. Rome: Food and Agriculture Organization of the United Nations. ISBN 978-92-5-108276-8; 2014.

2. Leggatt RA, Biagi CA, Smith JL, Devlin RH. Growth of growth hormone transgenic coho salmon *Oncorhynchus kisutch* is influenced by construct promoter type and family line. Aquaculture 2012; 356-357: 193–199.

3. Zhu ZG, He L, Chen S. Novel gene transfer into the fertilised eggs of goldfish (*Carassius auratus*). Journal of Applied Ichthyology 1985; 1: 32–34.

4. Rasmussen RS, Morrissey MT. Biotechnology in aquaculture: transgenics and polyploidy. Comprehensive Reviews in Food Science and Food Safety 2007; 6: 2–16.

5. Moriyama S, Ayson FG, Kawauchi H. Growth regulation by insulin-like growth factor-I in fish. Bioscience, Biotechnology and Biochemistry 2000; 64: 1553–1562.

6. Le Roith D, Bondy C, Yakar S, Liu JL, Butler A. The somatomedin hypothesis: 2001. Endocrine Reviews 2001; 22: 53–74.

7. Nam YK, Noh JK, Cho YS. et al. Dramatically accelerated growth and extraordinary gigantism of transgenic mud loach *Misgurnus mizolepis*. Transgenic Research 2001; 10: 353–362.

8. Devlin RH, Biagi CA, Yesaki TY. Growth, viability and genetic characteristics of GH transgenic coho salmon strains. Aquaculture 2004; 236: 607–632.

9. Fletcher GL, Shears MA, Yaskowiak ES, King MJ, Goddard SV. Gene transfer: potential to enhance the genome of Atlantic salmon for aquaculture. Australian Journal of Experimental 2004; 44: 1095–1100.

10. Devlin RH, Sundström LF, Muir WM. Interface of biotechnology and ecology for environmental risk assessments of transgenic fish. Trends in Biotechnology 2006; 24: 89–97.

11. Figueiredo MDA, Lanes CFC, Almeida DV, Marins LF. Improving the production of transgenic fish germlines: in vivo evaluation of mosaicism in zebrafish (*Danio rerio*) using a green fluorescent protein (GFP) and growth hormone cDNA transgene co-injection strategy. Genetics and Molecular Biology 2007; 30: 31–36.

12. Figueiredo MA, Mareco EA, Silva MDP, Marins LF. Muscle-specific growth hormone receptor (GHR) overexpression induces hyperplasia but not hypertrophy in transgenic zebrafish. Transgenic Research 2012; 21: 457–469.

13. Westerfield M. The zebrafish book: a guide for the laboratory use of zebrafish *Danio rerio*. Third ed. University of Oregon Press, Eugene, OR, USA, 1995.

14. Lillie RD. Histopathologic technic and practical histochemistry. 2nd ed. New York, Blakiston, 1947.

15. Weibel R, Scherle F. Practical stereological methods for morphometric cytology. The Journal of Cell Biology 1966; 30: 23–38.

16. Romano LA, Ferder MD, Stella IY et al. High correlation in renal tissue between computed image analysis and classical morphometric analysis. The Journal of Histotechnology 1996; 19: 121–123.

17. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001; 25: 402–408.

18. Williams JE. The Coefficient of Condition of Fish. Chapter 13 In: J.C. Schneider (Ed.) The manual of fisheries survey methods II: with periodic updates. Michigan, 2000.

19. Brooks AJ, Waters MJ. The growth hormone receptor: mechanism of activation and clinical implications. Nature Reviews Endocrinology 2010; 6 :515-525.

20. Ihle JN, Jude S. STATs: Signal transducers and activators of transcription. Cell 1996; 84: 331–334.

21. Mommsen TP. Paradigms of growth in fish. Comparative Biochemistry and Physiology Part B 2001; 129: 207–219.

22. Bodine SC, Stitt TN, Gonzalez M, et al. Akt/mTOR pathway is a crucial regulator of skeletal muscle hypertrophy and can prevent muscle atrophy in vivo. Nature Cell Biology 2001; 3: 1014–1019.

23. Glass DJ. Skeletal muscle hypertrophy and atrophy signaling pathways. The International Journal of Biochemistry & Cell Biology 2005; 37: 1974–1984.

24. Clemmons DR. Role of IGF-I in skeletal muscle mass maintenance. Trends in Endocrinology & Metabolism 2009; 20: 349– 356.

25. Coolican SA, Samuel DS, Ewton DZ, McWade FJ, Florini JR. The mitogenic and myogenic actions of insulin-like growth factors utilize distinct signaling pathways. Journal of Biological Chemistry 1997; 272: 6653–6662.

26. Chiou MJ, Wang YD, Kuo CM, Chen JC, Chen JY. Functional analysis of mitogen-activated protein kinase-3 (MAPK3) and its regulation of the promoter region in zebrafish. DNA and Cell Biology 2007; 26: 781–790.

27. Bryantsev AL, Baker PW, Lovato TL, Jaramillo MS, Cripps RM. Differential requirements for myocyte enhancer factor-2 during adult myogenesis in Drosophila. Developmental Biology 2012; 361: 191–207.

28. Jang YN, Baik EJ. JAK-STAT pathway and myogenic differentiation. Jak-Stat 2 2013; e23282.

CAPÍTULO 3

Reproductive parameters of double transgenic zebrafish (*Danio rerio*) males overexpressing both the growth hormone (GH) and its receptor (GHR)

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Reproductive parameters of double transgenic zebrafish (*Danio rerio*) males overexpressing both the growth hormone (GH) and its receptor (GHR)

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Abstract

Growth hormone (GH) transgenesis has high potential application in aquaculture. However, GH excess can cause serious consequences due to its pleiotropic actions. In order to study these effects in zebrafish (*Danio rerio*), our laboratory has developed two transgenic lines. The first expresses the GH ubiquitous and constitutively (F0104 line), while the second one expresses the GH receptor in a muscle-specific manner (Myo-GHR line). The F0104 line showed accelerated growth but with reproductive difficulties, while Myo-GHR did not show the expected increase in muscle mass. Since the two lines seem to have complementary characteristics, a double transgenic (GH/GHR) was created from crossing between them. Taking into account that the double transgenic also has accelerated growth, the objective of this study was to estimate the reproductive capacity of males of this new line, evaluating sperm parameters, expression of spermatogenesis-related genes, and reproductive tests. Double transgenics showed a strong recovery in almost all sperm parameters analyzed when compared to the F0104 line. Gene expression analyses revealed that Anti-Müllerian Hormone gene (*amh*) appears to be primarily responsible for this recovery. Reproductive tests showed that double transgenic males do not differ from non-transgenics. It is possible that GHR excess in the double transgenic muscle may have contributed to lower circulating GH levels and thus reducing the negative effects of this hormone on reproduction. In general, it is clear that GH-transgenesis technology should take into account the need to obtain adequate levels of circulating hormone in order to achieve maximum growth with minimal unwanted side effects.

Keywords: Somatotrophic axis, GH side effects, double transgenesis, zebrafish, sperm.

Introduction

The stagnation of the fishing industry and a rapid increase in human population have driven aquaculture to become one of the food production sectors with the fastest growth (Béné et al. 2015). Fish is one of the most commercialized food worldwide, and it is the main source of animal protein in several regions (FAO 2014). Aquaculture accounts for almost half of all fish consumed in the world and 67% of the exports of all global fisheries output are intended for human consumption (FAO 2014). With the continuous growth of this demand, new alternatives to optimize production are necessary, and among them is biotechnology.

Improvements in biotechnology in recent decades have provided important tools that contribute in several ways to the enhancement of production in aquaculture, such as the development of vaccines, the use of probiotics and enzymes in the stock feed and other developments that improve the nutritional performance of many species (Freitas et al. 2012). Furthermore, one of main techniques of biotechnology, which has a huge potential of improving the aquaculture production, is transgenesis (Zbikowska 2003; Dunham 2004).

Transgenesis involves a transfer of a characteristic of an organism to another, altering the genome of a species by introducing one or more gene sequences. Thus, new features can be incorporated into the recipient organism and can be passed on to the progeny. A wide variety of genes are being used to produce transgenic fish in order to improve important characteristics for cultivation such as maturation, growth, and disease resistance (Rasmussen and Morrissey 2007).

Improved fish growth has been one of the main targets of genetic engineering (Leggatt et al. 2012). This process takes place mainly through the synthesis and secretion of the growth hormone (GH) by the pituitary gland. This hormone is released into the bloodstream, where it will act in certain organs through its association with specific growth hormone receptors (GHR) in the membrane of target cells. GH biological responses are developed and controlled mainly by the somatotrophic axis, where IGF-I is the main mediator of GH physiological action (Moriyama et al. 2000; Butler and LeRoith 2001).

Although GH-transgenesis has shown promising results regarding fish growth, it is known that GH acts in several processes other than growth, having pleiotropic effects on morphology, physiology, metabolism, immunology and behavior (Devlin et al.

2006). Figueiredo et al. (2007) developed a GH-transgenic zebrafish line (named F0104), which show a significant increase in growth rate. However, other studies using the same fish line showed several problems related to the excess of GH, such as the rise of oxygen consumption and the generation of reactive oxygen species (ROS), premature aging (Rosa et al. 2008; 2010; 2011), changes in the muscle structure (Kuradomi et al. 2011) and decreased maturation of the immune system (Batista et al. 2014). However, one of the main problems observed in the F0104 strain was difficulty in reproduction evidenced by a significant decrease of sperm quality (Figueiredo et al. 2013). This fact raises an important question regarding the application of GH-transgenesis, since genetically modified breeders would have difficulties to sustain a high production of fingerlings to support a commercial fish farming.

In order to reduce the effects of the GH excess previously demonstrated for F0104 fish, a new line of transgenic zebrafish that overexpresses the GH receptor in a muscle-specific manner was developed (Myo-GHR). The aim was to obtain an increase in muscle mass from a sensitization of the tissue to circulating GH due to greater amount of membrane receptors. Thus, the unwanted side effects of the excess circulation hormone would be avoided. However, the Myo-GHR line did not show increased muscle mass as expected probably due to the production of proteins that block the intracellular signaling of GH (Figueiredo et al. 2012). Recently, F0104 and Myo-GHR lines were crossbred to obtain a double transgenic (GH/GHR), since the two transgenic lines appear to have complementary characteristics (Silva et al. 2015a). A study with this double transgenic showed that growth was very similar to the F0104 line (Silva et al. 2015b), raising the hypothesis that the increased amount of receptors in muscle could minimize the side effects of excess GH circulating, even in reproduction. In this context, the aim of this study was to evaluate the reproductive parameters of double transgenic (GH/ GHR) zebrafish males via flow cytometry, testes histology and expression of genes related to sperm maturation.

Materials and Methods

Fish

To analyze the fish reproductive capacity, this study used non-transgenic males; males from the F0104 strain (GH), MYO-GHR strain (GHR) and double-transgenic

strain (GH/GHR), with the latter obtained by a crossbreeding of males of the F0104 strain (Figueiredo et al. 2007) and females of MYO-GHR line (Figueiredo et al. 2012). The fish were separated in groups by genotypes and raised in a closed-circulation water system, composed of 15L tanks at 28°C, 14h of light/10h of dark, and fed with high protein content (47.5%) twice a day, *ad libitum*. The water quality was monitored once a week, and the temperature, pH, nitrogen compounds and photoperiod were maintained in accordance with zebrafish requirements (Westerfield 1995). When the fish reached one year of age, 10 males of each genotype were sacrificed for the removal of the testes, where one of them was used in semen analysis and the other preserved in Trizol Reagent (Invitrogen, Brazil) for gene expression analysis. All procedures were performed as suggested by the Ethics Committee for Animal Use from the Federal University of Rio Grande (FURG, Brazil) under the process number 23116.005574/2013.

Sperm analyses

In all analysis of each genotype (n=10), the sperm were used immediately after the gonads removal (<30min). The collected sperm were activated by mixing 1 μL of sample with 4 μL of distilled water solution (Varela Junior et al. 2015), then placed on a slide under a cover slip and examined by the *Computer Assisted Semen Analysis (CASA)* through *Androvision (Minitube - Germany) software* (Dziewulska et al. 2011). At least 1,000 cells were captured on 10-15 fields, with the captures made 10 seconds after activation. The parameters evaluated were total motility (%), progressive motility (%), distance average path DAP (μm), distance curved line DCL (μm), distance straight line DSL (μm), average path velocity VAP (μm/s), velocity curved line VCL (μm/s), straight-line velocity VSL (μm/s), straightness STR (VSL/VAP, %), linearity LIN (VSL/VCL), balancing (WOB), amplitude of lateral head displacement (ALH) and beat cross frequency (BCF) (Dziewulska et al. 2011). The motility period (MotP) was evaluated from the time of activation until the end of the sperm progressive motility (Varela Junior et al. 2015).

For the analysis of cell disruption, membrane fluidity, DNA fragmentation, lipid peroxidation, reactive oxygen species, membrane and mitochondrial integrity, the Attune Acoustic Focusing® flow cytometer (Life Technologies) equipped with blue laser (Argon 488 nm) and laser violet (UV 405 nm) was used. Analyses were performed

by the 2.1 software version (Life Technologies). The violet laser of 405 nm (450/40, VL-1) was used in all tests to determine the population of sperm cells stained with Hoechst 33342 at a concentration of 16.2 mM, except in DNA fragmentation (Martínez-Alborcia et al. 2012). Non-spermatic events were ruled out by scatter graphs FSC x SSC and negative Hoechst 33342. In all parameter readings, fluorophore stained cells were diluted in calcium-free PBS (80 g of NaCl, 11,5 g of KCl, 24 g of Na2HPO4, 2 g of KH2PO4 in 1L of water Mili-Q), using a total of 10,000 sperm per analysis.

The fluorophores Sybr 14 and propidium iodide (PI) (MiniTube, Tiefenbach, Germany) were used to verify the plasma membrane integrity. A semen aliquot was incubated for 5 min in fluorescent probe containing 0.25 mM of Sybr14 and 7.5 μM IP, according to the manufacturer's instructions - Minitube. The sperm were classified as not damaged and with a functional membrane (Sybr + / PI-) and injured and/or with non-functional membrane (Sybr + / PI +; Sybr- / PI +; Sybr- / PI-) (Figueroa et al. 2015).

Membrane fluidity was verified using 2.7 μM hydrophobic merocyanine dye 540 (M540) and 0.1 μM YO PRO-1 (Invitrogen - Eugene, OR, USA) in 10 μL sample for 5 min. Sperm cells with high fluidity (high concentration M540) were evaluated and of those with low fluidity (low concentration M540), only samples with intact sperm (YO-PRO negative) (Fernandez-Gago et al. 2013) were evaluated. The rate of membrane fluidity was calculated as the number of sperm cells with low fluidity / number of sperm with low fluidity + sperm with high fluidity x 100.

The sperm cells were classified by their levels of high mitochondrial functionality (high fluorescence by the accumulation of Rhodamine) and low mitochondrial functionality (low fluorescence, low accumulation of Rhodamine), assessing only intact sperm (negative PI) in the latter (Liu et al. 2015). In the assay, 3.1 mM of Rhodamine 123 (green fluorescence) and IP 7.5 uM were added in 10 uL of sample for 5 min. Mitochondria functionality rate was calculated by the number of sperm with high mitochondrial membrane potential / number of sperm with high mitochondrial membrane potential + sperm with low mitochondrial membrane potential x 100.

DNA integrity was assessed by chromatin structure (SCSA). To test this parameter, 10 μL of sperm was added to 5 μL TNE (0.01 M Tris-HCl; 0.15 M NaCl; 0.001 M EDTA; pH 7.2), and 10 μL of 1X Triton (Triton X-100, 1%) (v/v) with 30

seconds intervals. The orange acridine dye was added and incubated for 30 s to 2 min prior to reading. Sperm was classified as integrated DNA (green) and fragmented DNA (orange/red) (Jenkins et al. 2015). The DNA fragmentation rate was calculated as the number of sperm with fragmented DNA / number of sperm with integrate DNA + sperm with fragmented DNA x 100.

The concentration of ROS in sperm cells was measured using 1.0 μM of the fluorophore 2'7'-dichlorofluorescein diacetate (H2DCFDA) (emits green fluorescence when oxidized) and 7.5 μM IP. The median intensity of green fluorescence was used only to measure live sperm (negative IP) (Domínguez-Rebolledo et al. 2011).

The lipid peroxidation of sperm was assayed by using a final concentration of 1 μM Bodipy C11 (Hagedorn et al. 2012) in 10 μL sample, and incubated for 2 hours at room temperature (20°C), where only live sperm were analyzed. The lipid peroxidation rate was calculated by the median of the green fluorescence intensity (peroxidized lipid) /median of the green fluorescence intensity + median of the red fluorescence intensity (non-peroxidized lipid) x 100.

Gene expression

Testes from five individuals of each group were randomly sampled for gene expression. Total RNA extraction was performed using Trizol reagent (Invitrogen, Brazil), and cDNA synthesis using the High Capacity cDNA Reverse Transcriptiont kit (Applied Biosystems, Brazil). All procedures were performed as suggested by the manufacturers. The gene expression was analyzed by real time quantitative PCR (qPCR), with each sample being analyzed in duplicate. Specific primers for each gene (Table 1) were designed using Primer-Blast tool from GenBank (http://www.ncbi.nlm.nih.gov). The qPCR reactions were performed using the Platinum SYBR Green qPCR kit SuperMix-UDG (Invitrogen, Brazil). Serial dilutions were performed for all primers to determine the efficiency of the reaction (data not shown). The conditions for PCRs were 50°C / 2 min, 95°C / 2 min followed by 40 cycles of 95°C / 15 sec and 60°C / 30 sec. In this study, four reference genes (elongation factor 1 alpha - *ef1α*, beta-2-microglobulin - *b2m*, beta-actin - *actb1*; and ribosomal protein L13 alpha - *rp113a*) were tested using geNorm VBA applet for Microsoft Excel (Vandesompele et al. 2002). Therefore, a normalization factor based on the expression

levels of the reference genes with the best performance was calculated. The reference genes selected were *actb1* and *b2m*.

Table 1: Sequence of primers used in real time PCR in zebrafish (*Danio rerio*).

Gene	Sequence	Efficiency	Amplicon	GenBank
lhcgr	F: 5'-ACAATCACTCACGCTCTCCG-3'	98.0	104 bp	NM_205625
	R: 5'-GGAAGGAGTGCCATCGCTAA-3'			
bdnf	F: 5'- GCCGGACAACCCAGTCTTAC-3'	97.0	70 bp	NM_001308649
	R: 5'- ATAAACCGCCAGCCGATCTT-3'			
bik	F: 5'-TGGCTGTCAGGAGGCTAGAAA-3'	102.0	60 bp	NM_001045038
	R: 5'-CAGTCAGAAACATGCAAGTTGGA-3'			
ar	F: 5'-GGGCGAATGGATGGATGTAA-3'	101.0	59 bp	NM_001083123
	R: 5'-CACTCCTCCCTCCGTCAAAC-3'			
amh	F: 5'- GGCAGGCCGGAAGATGAT -3'	93.0	60 bp	NM_001007779
	R: 5'- GGAGAAAGCGCACTCAGTTCA-3'			
cyp19a1a	F: 5'- ACATTGTGCGTGTCTGGATCA -3'	97.8	58 bp	NM_131154
	R: 5'- GCTGACGACCTGCTCAAGATG -3'			
ef1α	F: 5'-GGGCAAGGGCTCCTTCAA-3'	100.0	54 bp	NM131263
	R: 5'-CGCTCGGCCTTCAGTTTG-3'			
sox9a	F: 5'-CGGGACGCTCGGAGACT-3'	97.8	55bp	NM_131643
	R: 5'-GATGCACACCGGGAACTTG-3'			
sox9b	F: 5'- TCGGTGCGGTGGACATC-3'	102.9	60 bp	NM_131644
	R: 5'- CGAAGGCCTCTATGTTGGAGAT -3'			
actb1	F: 5'-GCTGTTTTCCCCTCCATTGTT-3'	100.5	60 bp	NM_131031
	R: 5'-TCCCATGCCAACCATCACT-3'			
b2m	F: 5'-GCCTTCACCCCAGAGAAAGG-3'	100.5	101 bp	NM131163
	R: 5'-GCGGTTGGGATTTACATGTTG-3'			
rp113a	F: 5'-TCTGGAGGACTGTAAGAGGTATGC-3'	102.9	148 bp	NM212784
	R: 5'-AGACGCACAATCTTGAGAGCAG-3'			

Histology of testes

After the extraction, three gonads were fixed in buffered paraformaldehyde 4% for 24 hours and stored in 70% alcohol. Then, the samples were dehydrated in increasing alcohol solutions, cleared in xylene, and embedded in Paraplast Xtra (Sigma, Brazil), as stated in Carson and Hladik (2009). Subsequently, the material was cut into motorized rotary microtome (Leica RM2255) in a thickness of 6 microns, and then subjected to haematoxylin-eosin (Carson and Hladik 2009). The slides were observed in optical microscope.

Reproduction experiment

To check the reproductive behavior of NT and GH/GHR genotypes, three non-transgenic couples and three couples of NT females with double-transgenic males were formed. A rotation among the wild females was held weekly, distributing them randomly between water tanks, regardless of genotype. During 30 days traps were placed for the collection of spawn. The eggs were collected one hour after spawning and examined in a stereoscope. The fertilization rate was expressed in percentage as the total number of eggs divided by the number of fertilized eggs. Fertilized eggs, characterized by 4-8 blastomere, were counted and incubated at 28°C until hatching. Mortality and hatching rates were then recorded during the trial period. The eggs were considered dead when parts of the content were white. Dead eggs were counted and removed to prevent mold growth. Hatching was defined as rupture of the egg membranes by the tail. The rate of hatching was determined and expressed as a percentage relative to the proportion of the total number of eggs to the number of hatched larvae.

Statistical analysis

The significant differences in sperm analysis and gene expression between treatments were compared using analysis of variance (ANOVA) by Tukey test and Newman-Keuls multiple comparison test, respectively. Data are expressed as mean ± SE. For all analyzes, the significant difference was considered when $P<0.05$. The normality and homogeneity of variances were previously verified.

Results

The first result that draws attention was the macroscopic observation of the testis during fish dissection. GH-transgenesis produced a very significant increase in the testis size of transgenics both to the GH genotype, and for the double transgenic GH/GHR. No differences were found between NT and GHR (Fig. 1).

Fig. 1 Testes of adult zebrafish (*Danio rerio*) from different genotypes (indicated by the black arrow). NT: non-transgenic; GH: GH-transgenic; GHR: GHR-transgenic; GH/GHR: double transgenic. Scale bar 1 cm.

Histological analysis shown in Figure 2 evidences a significant loss in the sperm amount of the GH genotype when compared to NT and GHR. Although GH/GHR testis also seemed macroscopically altered, it was surprising to observe an apparent recovery of normal amounts of sperm in this group.

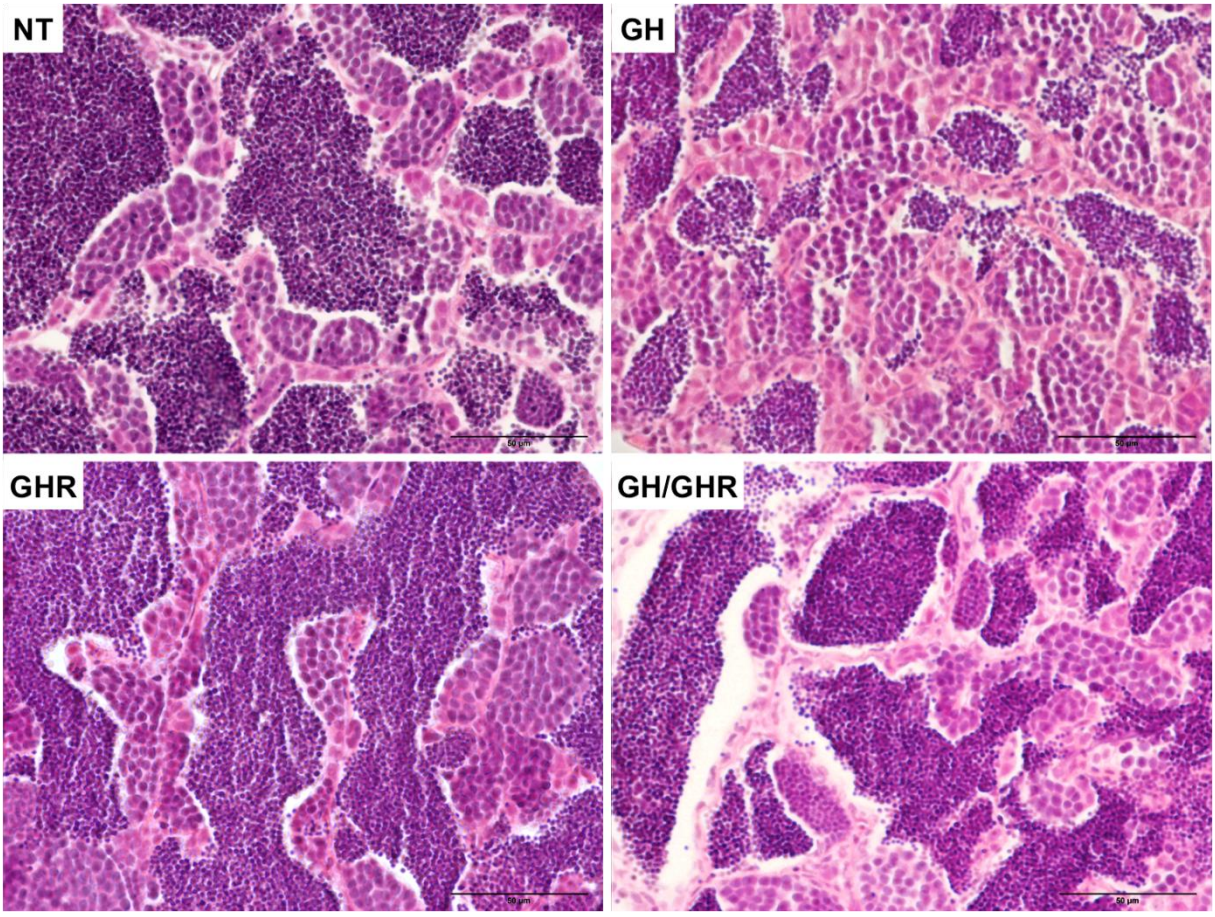

Fig. 2 Photomicrographs of testes from adult zebrafish (*Danio rerio*) males. Sperm cells are colored in dark purple. NT: non-transgenic; GH: GH-transgenic; GHR: GHR-transgenic; GH/GHR: double transgenic. Scale bar 50 µm.

To verify whether this apparent histological recovery is well founded, sperm analyses were performed by flow cytometry. The results of these analyzes are shown in table 2. As noted in the preliminary histology, GH/GHR genotype showed a strong recovery in sperm concentration (CON=452.412±51.294), including no statistical difference when compared to NT (CON=425.552±96.630) and GHR (CON=408.106±58.558) groups. The GH genotype showed a significant decrease in this parameter (CON=254.477±79.674) when compared to the other groups. In fact, this genotype has also lost in sperm quality compared to the NT in the cytoplasmic membrane funcionality (MEM), mitochondrial membrane potential (MIT), cell membrane fluidity (FL) and DNA fragmentation (DNA). On the other hand, the double-transgenic GH/GHR showed a recovery to normal levels of MEM, MIT and DNA parameters. Regarding to the double-transgenic FL (25.90±3.93), it improved even when compared to NT (46.12±2.74). The cellular integrity (CI) and the oxidative stress

parameters (ROS and LPO) also improved significantly in the double transgenic compared to GH.

Table 2: Flow cytometry analyses of zebrafish (*Danio rerio*) sperm. NT: non-transgenic; GH: GH-transgenic; GHR: GHR-transgenic; GH/GHR: double transgenic. Data are expressed as mean and standard error of the mean.

	Genotypes			
	NT	GH	GHR	GH/GHR
CI (%)	76,9 ± 2,74^{ab}	70,0 ± 1,87^{b}	82,6 ± 2,89^{a}	83,0 ± 3,39^{a}
MEM (%)	62,5 ± 2,25^{ab}	43,8 ± 2,78^{c}	58,9 ± 368^{b}	68,6 ± 3,78^{a}
MIT (%)	61,2 ± 2.78^{a}	40,3 ± 2,77^{b}	34,1 ± 3,22^{b}	55,0 ± 4,43^{a}
FL (%)	46,1 ± 2,74^{b}	56,5 ± 2,37^{c}	44,8 ± 3,08^{b}	25,9 ± 3,93^{a}
DNA (%)	2,72 ± 0,33^{a}	4,70 ± 0,26^{b}	2,88 ± 0,21^{a}	3,51 ± 0,37^{a}
ROS	721,1 ± 156,2^{ab}	1204,9 ± 336,0^{bc}	1561,1 ± 289,3^{c}	290,6 ± 35,7^{a}
CON (nºzpt/ml)	425552 ± 96630^{a}	254477 ± 79674^{b}	408106 ± 58558^{a}	452412 ± 51294^{a}
LPO (%)	34,1 ± 4,56^{ab}	39,4 ± 4,04^{b}	24,1 ± 3,44^{a}	23,9 ± 3,14^{a}

*Cellular integrity (CI), cytoplasm membrane integrity (MEM), mitochondrial functionality (MIT), fluidity cytoplasm membrane (FL), DNA fragmentation (DNA), reactive oxygen species (ROS), concentrations spermatic (CON), lipid peroxidation (LPO) of transgenic zebrafish sperm (*D. rerio*). Different letters in the same row have statistical difference test Tukey ($P < 0.05$).

The results of sperm kinetics analysis are shown in Table 3. In agreement with results previously observed on histology and flow cytometry, the GH genotype also showed loss in the majority of parameters, in particular: motility (TM, PM and MotP), travelled distance (DAP, DCL and DSL) and speed (VAP, VCL and VSL). On the other hand, double transgenic showed a significant recovery in practically all kinetic parameters not only in relation to GH but also to NT, particularly regarding sperm motility (TM, PM and MotP). Only STR, LIN, WOB and BCF parameters showed no differences among genotypes.

Table 3: Kinetic analyses of zebrafish (*Danio rerio*) sperm. NT: non-transgenic; GH: GH-transgenic; GHR: GHR-transgenic; GH/GHR: double transgenic. Data are expressed as mean and standard error of the mean.

	Genotypes			
	NT	GH	GHR	GH/GHR
TM(%)	35,6 ± 2,31^{b}	28,1 ± 1,88^{c}	30,4 ± 3,27^{bc}	47,9 ± 2,75^{a}
PM(%)	29,2 ± 2,46^{b}	20,9 ± 1,73^{c}	25,3 ± 3,21^{bc}	38,7 ± 2,70^{a}
MotP (s)	114,3 ± 23,6^{ab}	38,2 ± 8,41^{c}	72,2 ± 11,6^{bc}	147,6 ± 14,5^{a}
DAP (µm)	27,7 ± 1,37^{ab}	20,6 ± 1,06^{c}	29,8 ± 1,51^{a}	24,8 ± 1,11^{b}
DCL (%)	31,2 ± 1,42^{ab}	23,9 ± 1,17^{c}	39,4 ± 1,60^{a}	28,1 ± 1,17^{b}
DSL	24,5 ± 1,28^{a}	18,2 ± 1,02^{b}	26,1 ± 1,45^{a}	21,1 ± 1,00^{b}
VAP (µm/s)	59,5 ± 2,95^{ab}	44,2 ± 1,24^{c}	64,9 ± 3,27^{a}	54,1 ± 2,46^{b}
VCL (µm/s)	67,1 ± 3,05^{ab}	51,3 ± 2,49^{c}	72,9 ± 3,45^{a}	61,2 ± 2,59^{b}
VSL (µm/s)	52,8 ± 2,77^{ab}	39,0 ± 2,17^{c}	56,8 ± 3,18^{a}	46,0 ± 2,20^{b}
STR	0,87 ± 8,81^{a}	0,86 ± 0,01^{a}	0,85 ± 0,01^{a}	0,83 ± 7,52^{a}
LIN	0,76 ± 0,01^{a}	0,74 ± 0,01^{a}	0,76 ± 0,01^{a}	0,73 ± 9,89^{a}
WOB	0,87 ± 0,01^{a}	0,85 ± 0,01^{a}	0,87 ± 8,93^{a}	0,86 ± 7,42^{a}
ALH	1,37 ± 0,08^{ab}	1,05 ± 0,05^{c}	1,47 ± 0,08^{a}	1,19 ± 0,04^{ab}
BCF	29,4 ± 1,07^{a}	26,2 ± 0,98^{a}	27,8 ± 0,79^{a}	26,2 ± 0,69^{a}

* Total motility (TM), progressive motility (PM), motility period (MotP) traveled an average distance (DAP), curvilinear distance (DCL), straight away (DSL), route medium speed (VAP), straight line velocity (VSL), curvilinear velocity (VCL), straightness (STR), linearity (LIN), oscillation (WOB), lateral displacement of the head (ALH), cross flagellar beat frequency (BCF). Different letters in the same row have statistical difference test Tukey ($P < 0.05$).

Figure 3 shows the results of expression analysis of genes related to the differentiation and sperm maturation. Basically, transgenics to GH significantly affected the expression of seven of the eight genes analyzed. Unlike what was observed in the sperm analysis, the double-transgeny GH/GHR did not recover most of the genes affected by GH (*bdnf*, *lhcgr*, c*yp19a1a*, s*ox9a* and s*ox9b*). However, in the double-transgenic, genes *amh* and *bik* had their expressions returned to the levels observed for the NT genotype (Fig. 3a and 3b). The only gene that showed no difference between genotypes was *ar* (Fig. 3h).

Fertilization and hatching rates did not differ between the GH/GHR genotypes (74,3±4,3 and 55,7±5) and NT (73,1±5,8 and 56,3 ±4), respectively.

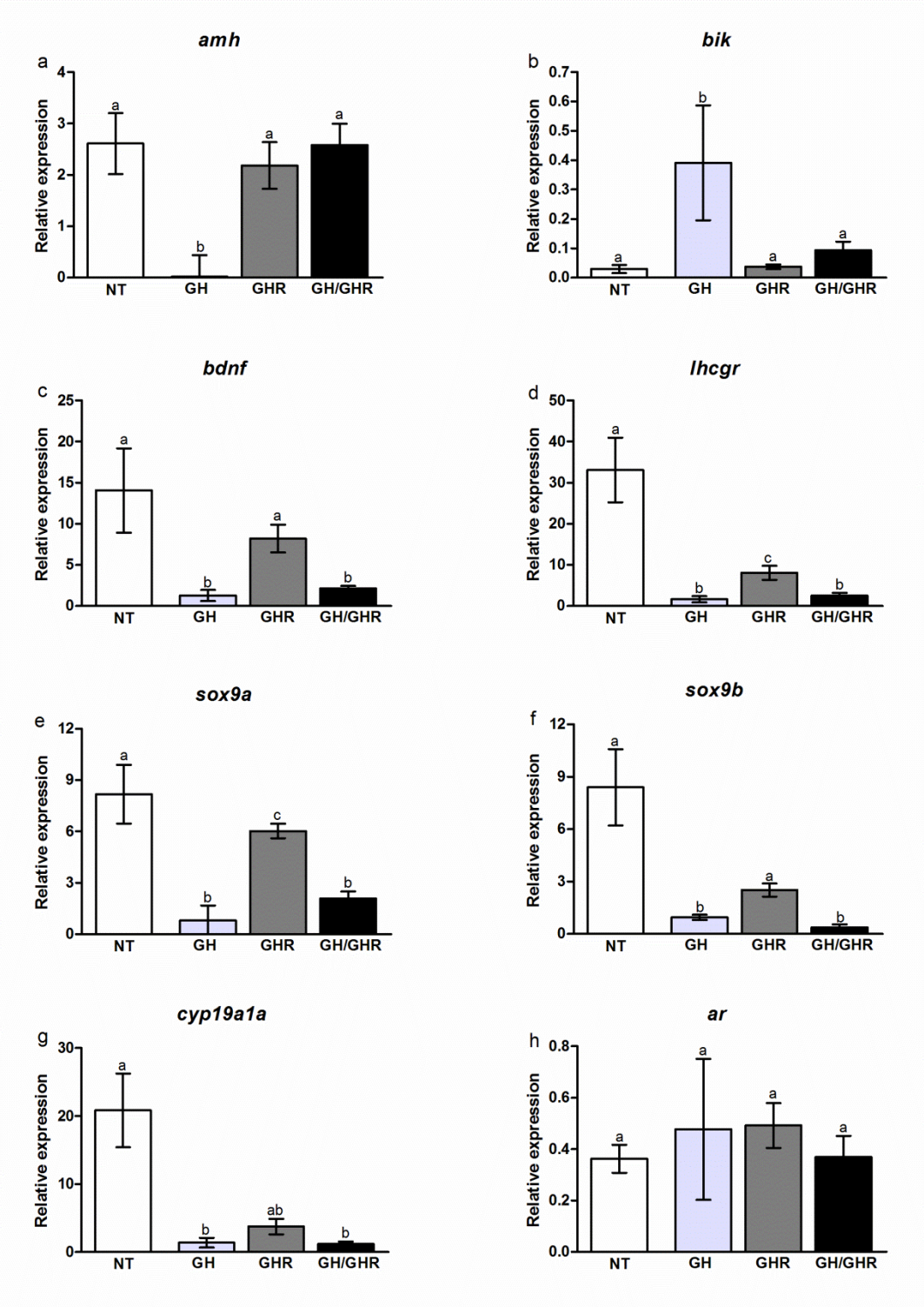

Fig. 3 Relative expression of sperm maturation-related genes in zebrafish (*Danio rerio*) testes. (a) *amh*: anti-müllerian hormone; (b) *bik*: BCL2-interacting killer (apoptosis-inducing); (c) *bdnf*: Brain-derived neurotrophic factor; (d) *lhcgr*: Luteinizing hormone/choriogonadotrophin receptor; (e) *sox9a*: SRY (sex determining region Y)-box 9a; (f) *sox9b*: SRY (sex determining region Y)-box 9b; (g) *cyp19a1a*: cytochrome P450; (h) *ar*: androgen receptor. NT: non-transgenic; GH: GH-transgenic; GHR: GHR-transgenic; GH/GHR: double transgenic. Different letters represent statistical differences ($P<0.05$).

Discussion

It is undoubted that biotechnological tools can represent a major breakthrough for the development of aquaculture. Among these, transgenesis has shown a great application potential, especially in the manipulation of somatotropic axis for growth. However, the main gene that has been manipulated for this purpose has shown undesirable side effects. The GH overexpression in fish has led to meaningful structural and/or functional changes related to various commercial such as salmon (Raven et al. 2008), tilapia (Shved et al. 2011) and carp (Cao et al. 2014), which bring concerns about the viability of their application in aquaculture. One of the major causes of these changes may be supraphysiological GH levels due to transgenesis from genetic constructs composed by gene promoters of strong and ubiquitous expression. Recently, a study of transgenic mice showed that GH excess leads to serious morphological alterations in the liver, kidney and pituitary gland (Blutke et al. 2014). Previous studies had already shown changes in the same organs (Stefaneanu et al. 1993; Wanke et al. 1991; Wolf et al. 1993; Wanke et al. 1991, 2001; Wolf et al. 1993) and also in the skin (Wanke et al. 1999). Furthermore, Ohyama et al. (1999) showed that the GH treatment increased growth and differentiation of the rat testis.

Information regarding changes in organs and tissues caused by excess of GH in fish are rare. Recently, a study with GH-transgenic salmon showed a proportional decrease of the eyes and brain, while the heart, spleen and liver seem to have been affected (Devlin et al. 2012). However, to our knowledge, there are no data regarding the GH action on testis of fish. In this study, a significant increase in testicular volume was observed in both transgenic fish lines overexpressing GH used herein. Although this increase could be related to a decrease in sperm quality as previously described for the GH genotype (Figueiredo et al. 2013), histological results showed that the double transgenic (GH/GHR) has an apparent recovery in sperm count. Additional analysis by flow cytometry confirmed this recovery, since the concentration of double-transgenic sperm has matched the NT genotype levels.

Regarding sperm quality, the result that draws the most attention is the significant ROS production decrease in the double transgenic. This reduction has important implications for all other parameters. For example, a lesser quantity of reactive oxygen species reduces the possibility of the occurrence of lipid peroxidation (LPO) and damage to the plasma membrane (MEM), with consequent improvement in

the membrane fluidity (FL). Yet, it reduces the possibility of DNA fragmentation and increases the mitochondrial functionality (MIT). All these parameters were significantly improved in the double transgenic. Likewise, the double transgenic also showed a significant recovery in virtually all sperm kinetic parameters analyzed for GH genotype. There was improvement in motility parameters (TM, PM and MotP), as well as distance (DAP and DCL) and speed (VAP, VCL and VSL). All these results indicate that the double transgenesis recovered the reproductive capacity of transgenic males overexpressing GH and GHR at the same time. The question is why this double transgenesis had a positive effect on sperm quality when compared to GH genotype?

As previously reported by Figueiredo et al. (2013) and confirmed in this study, the GH genotype has a high ROS production with consequent increase of LPO and DNA damage. These parameters are strong apoptosis inducers. Indeed, pro-apoptotic *bik* gene showed an increase of about 20 times in its expression in GH genotype compared to NT, which may be related to the low concentration of sperm in this genotype. Guerra et al. (2013) demonstrated that *bik* expression can be considered as an indicator of sperm quality in fish. These authors showed that expression of such gene is strongly increased in zebrafish selected as poor breeders. Another gene that shows an important result in this study was *amh*. This gene codes for anti-müllerian hormone (AMH), which is a glycoprotein produced and secreted by Sertoli cells responsible for the regression of the müllerian ducts during testis differentiation in embryos of vertebrate tetrapods (Josso 1986). Although teleost do not have müllerian ducts, in zebrafish, for example, AMH is important in the negative control of spermatogenesis and steroidogenesis (Skaar et al. 2011). Thereby, the drop observed in *amh* expression on GH genotype in approximately 99% probably represents a compensatory attempt of these fish to increase sperm production. All other analyzed genes (*bdnf*, *lhcgr*, c*yp19a1a*, s*ox9a*, sox9b and *ar*) do not seem to be related to the sperm quality recovery observed in the double transgenics. Complementing these remarks are the results of breeding tests comparing males from NT and GH/GHR genotypes. The percentages of hatching and fertilization resulting from breeding with NT females showed no difference between the evaluated males. Thus, it is clear that double transgenics recovered the reproductive capacity lost by GH genotype males, as previously shown by Figueiredo et al. (2013).

What sets apart the double transgenics from GH genotype is the increased expression of GHR receptor in muscle tissue, which apparently can reduce the amounts of circulating GH. Although this decrease has not been measured in the double transgenic blood, this hypothesis can be taken into account since it is probable that all downsides in relation to poor breeding of GH genotype individuals must be related to the excess of circulating hormone (Figueiredo et al. 2013). If this is the case, it is clear that the double transgenic muscle worked as a regulator of the GH circulating quantity, enabling a reduction of the negative side effects on breeding without losing the ability to grow as recently shown by Silva et al. (2015b). Thus, this study raises an important issue in the use of GH as an agent to induce growth by transgenesis. It is noteworthy to point out that the use of this hormone should comply with the maximum levels supported by the organism to be manipulated. GH strongly induces growth through the somatotrophic axis, which involves the action of other hormones such as IGFs. This entire system in action implies a high-energy demand, which can drain large amounts of ATP, damaging other systems such as the reproductive. Which would be the alternative? It is likely that a first answer to this question would be the use of controllable promoters for the expression of the hormone, in order to obtain a circulating GH level that could stimulate maximum growth without any prejudice to other important physiological systems. Another possibility would be the use of genetic constructs allowing expression of constitutively activated GH or IGF receptors. In this case, the design of new molecules that combine different protein domains could promote intracellular growth signaling in a "hormone-free" manner. A pioneering study in this regard has shown that a chimeric molecule of GHR can activate the signaling for growth in a GH-independent manner (Ahmed et al. 2011). In this study, the authors have produced a novel receptor from a signal peptide derived from a pig GHR linked to the amino acid sequence coding for the leucine zipper found in the mouse transcription factor c-jun, all this fused to pig GHR transmembrane and cytoplasmatic domains. This genetic construct used the strong CMV promoter and induced the growth of zebrafish approximately 86% higher than non-transgenic controls. Although the authors have not assessed the side effects of this transgenic line, it is likely that these fish have fewer problems once growth was obtained without dependence on the excess of circulating GH. Overall, this paper presents results that allow to re-evaluate the potential of GH-transgenesis in aquaculture and suggest new research lines in this area that seek the best

way to induce growth with fewer negative side effects for genetically modified organisms.

References

Ahmed ASI, Xiong F, Pang SC, et al (2011) Activation of GH signaling and GH-independent stimulation of growth in zebrafish by introduction of a constitutively activated GHR construct. Transgenic Res 20:557–567. doi: 10.1007/s11248-010-9439-9

Batista CR, Figueiredo MA, Almeida DV, et al (2014) Impairment of the immune system in GH-overexpressing transgenic zebrafish (*Danio rerio*). Fish Shellfish Immunol 36:519–524. doi: 10.1016/j.fsi.2013.12.022

Béné C, Barange M, Subasinghe R, et al (2015) Feeding 9 billion by 2050 – Putting fish back on the menu. Food Secur 7:261–274. doi: 10.1007/s12571-015-0427-z

Butler AA and LeRoith, DL (2001) Control of growth by the somatotrophic axis: Growth hormone and the insulin–like growth factors have related and independent roles. Annual Review of Physiology 63:141–164. doi: 10.1146/annurev.physiol.63.1.141

Blutke A, Schneider MR, Renner-Muller I, et al (2014) Genetic dissection of IGF1-dependent and -independent effects of permanent GH excess on postnatal growth and organ pathology of mice. Mol Cell Endocrinol 394:88–98. doi: 10.1016/j.mce.2014.07.002

Carson FL, Hladik C, 2009. Histotechnology—A Self–instructional Text, 3rd ed. ASCP Press, Chicago

Cao M, Chen J, Peng W, et al (2014) Effects of growth hormone over-expression on reproduction in the common carp Cyprinus carpio L. Gen Comp Endocrinol 195:47–57. doi: 10.1016/j.ygcen.2013.10.011

Devlin RH, Sundström LF, Muir WM (2006) Interface of biotechnology and ecology for environmental risk assessments of transgenic fish. Trends Biotechnol 24:89–97. doi: 10.1016/j.tibtech.2005.12.008

Dziewulska K, Rzemieniecki A, Czerniawski R, Domagała J (2011) Post-thawed motility and fertility from Atlantic salmon (Salmosalar L.) sperm frozen with four cryodiluents in straws or pellets. Theriogenology 76: 300 –311. doi: 10.1016/j.theriogenology.2011.02.007

Raven P A, Uh M, Sakhrani D, et al (2008) Endocrine effects of growth hormone overexpression in transgenic coho salmon. Gen Comp Endocrinol 159:26–37. doi: 10.1016/j.ygcen.2008.07.011

FAO (2014) The state of the world fisheries and aquaculture. Rome: Food and Agriculture Organization of the United Nations. ISBN 978-92-5-108276-8.

Fernández–Gago R, Domínguez JC, Martínez–Pastor F (2013) Seminal plasma applied post–thawing affects boar sperm physiology: A flow cytometry study. Theriogenology 80:400–10. doi: 10.1016/j.theriogenology.2013.05.003

Figueiredo MA, Fernandes RV, Studzinski AL, et al (2013) GH overexpression decreases spermatic parameters and reproductive success in two-years-old transgenic zebrafish males. Anim Reprod Sci 139:162–7. doi: 10.1016/j.anireprosci.2013.03.012

Figueiredo MA, Mareco EA, Silva MDP, Marins LF (2012) Muscle-specific growth hormone receptor (GHR) overexpression induces hyperplasia but not hypertrophy in transgenic zebrafish. Transgenic Res 21:457–69. doi: 10.1007/s11248-011-9546-2

Figueiredo MDA, Lanes CFC, Almeida DV, Marins LF (2007) Improving the production of transgenic fish germlines: in vivo evaluation of mosaicism in zebrafish (*Danio rerio*) using a green fluorescent protein (GFP) and growth hormone cDNA transgene co-injection strategy. Genet Mol Biol 30:31–36. doi: 10.1590/S1415-47572007000100008

Freitas AC, Rodrigues D, Rocha-Santos TP, et al (2012) Marine biotechnology advances towards applications in new functional foods. Biotechnol Adv 30:1506–15. doi: 10.1016/j.biotechadv.2012.03.006

Guerra SM, Valcarce DG, Cabrita E, Robles V (2013) An/alysis of transcripts in gilthead seabream sperm and zebrafish testicular cells: MRNA profile as a predictor of gamete quality. Aquaculture 406-407:28–33. doi: 10.1016/j.aquaculture.2013.04.032

Hagedorn M, McCarthy M, Carter VL, Meyers SA (2012) Oxidative Stress in Zebrafish (*Danio rerio*) Sperm. PLoS One 7:e39397. doi: 10.1371/journal.pone.0039397

Jenkins JA, Draugelis–Dale RO, Pinkney AE, Iwanowicz LR, Blazer VS (2015) Flow cytometric method for measuring chromatin fragmentation in fixed sperm from

yellow perch (Percaflavescens) Theriogenology 83:920–931. doi: 10.1016/j.theriogenology.2014.11.028

Josso N (1986) Anti–müllerian hormone. Clin Endocrinol 25:331–345 doi: 10.1111/j.1365-2265.1986.tb01699.x

Kuradomi RY, Figueiredo MA, Lanes CFC, et al (2011) GH overexpression causes muscle hypertrophy independent from local IGF-I in a zebrafish transgenic model. Transgenic Res 20:513–21. doi: 10.1007/s11248-010-9429-y

Leggatt RA, Biagi CA., Smith JL, Devlin RH (2012) Growth of growth hormone transgenic coho salmon Oncorhynchus kisutch is influenced by construct promoter type and family line. Aquaculture 356-357:193–199. doi: 10.1016/j.aquaculture.2012.05.016

Liu Q, Wang X, Wang W, Zhang X, et al (2015) Effect of the addition of six antioxidants on sperm motility, membrane integrity and mitochondrial function in red seabream (Pagrus major) sperm cryopreservation. Fish PhysiolBiochem 41:413–422 doi: 10.1007/s10695-014-9993-9

Raven P a, Uh M, Sakhrani D, et al (2008) Endocrine effects of growth hormone overexpression in transgenic coho salmon. Gen Comp Endocrinol 159:26–37. doi: 10.1016/j.ygcen.2008.07.011

Moriyama S, Ayson FG, Kawauchi H (2000) Growth Regulation by Insulin-like Growth Factor-I in Fish. Biosci Biotechnol Biochem 64:1553–1562. doi: 10.1271/bbb.64.1553

Ohyama K (1999) Growth Rats Agonist Hormone Treated with Advances Spermatogenesis in Premature Hormone. 46:555–562

Rasmussen RS and Morrissey MT (2007) Biotechnology in Aquaculture : Transgenics and Polyploidy. Compr Rev Food Sci Food Saf 6:2–16

Raven PA, Uh M, Sakhrani D, et al (2008) Endocrine effects of growth hormone overexpression in transgenic coho salmon. Gen Comp Endocrinol 159:26–37. doi: 10.1016/j.ygcen.2008.07.011

Rosa CE, Figueiredo MA, Lanes CFC, et al (2008) Metabolic rate and reactive oxygen species production in different genotypes of GH-transgenic zebrafish. Comp Biochem Physiol B Biochem Mol Biol 149:209–14. doi: 10.1016/j.cbpb.2007.09.010

Rosa CE Da, Kuradomi RY, Almeida DV, et al (2010) GH overexpression modifies

muscle expression of anti-oxidant enzymes and increases spinal curvature of old zebrafish. Exp Gerontol 45:449–56. doi: 10.1016/j.exger.2010.03.012

Rosa CE, Figueiredo M a, Lanes CFC, et al (2011) Genotype-dependent gene expression profile of the antioxidant defense system (ADS) in the liver of a GH-transgenic zebrafish model. Transgenic Res 20:85–89. doi: 10.1007/s11248-010-9395-4

Shved N, Berishvili G, Mazel P, et al (2011) Growth hormone (GH) treatment acts on the endocrine and autocrine/paracrine GH/IGF-axis and on TNF-α expression in bony fish pituitary and immune organs. Fish Shellfish Immunol 31:944–952. doi: 10.1016/j.fsi.2011.08.012

Silva ACG, Almeida DV, Figueiredo MA, Marins LF (2015a) Double Transgenic Zebrafish and Growth Studies. 12:268–269. doi: 10.1089/zeb.2015.1090

Silva ACG, Almeida DV, Nornberg BF, et al (2015b) Effects of Double Transgenesis of Somatotrophic Axis (GH/GHR) on Skeletal Muscle Growth of Zebrafish (*Danio rerio*). Zebrafish 12:408–413. doi: 10.1089/zeb.2015.29001.sil

Skaar KS, Nóbrega RH, Magaraki A, et al (2011) Proteolytically Activated, Recombinant Anti-Müllerian Hormone Inhibits Androgen Secretion, Proliferation, and Differentiation of Spermatogonia in Adult Zebrafish Testis Organ Cultures. Endocrinology 152:3527–3540. doi: 10.1210/en.2010-1469

Stefaneanu L, Kovacs K, Bartke A, Mayerhofer A, Wagner TE (1993) Pituitary morphology of transgenic mice expressing bovine growth hormone. Lab. Invest 68: 584–591

Wanke R, Hermanns W, Folger S, Wolf E, Brem G (1991) Accelerated growth and visceral lesions in transgenic mice expressing foreign genes of the growth hormone family: an overview. Pediatr. Nephrol 5:513–521

Wanke R, Milz S, Rieger N, Ogiolda L, Renner–Muller I, et al (1999) Overgrowth of skin in growth hormone transgenic mice depends on the presence of male gonads. J. Invest Dermato 113:967–971

Wanke, R, Wolf E, Brem G, Hermanns W (2001) Role of podocyte damage in the pathogenesis of glomerulosclerosis and tubulointerstitial lesions: findings in the growth hormone transgenic mouse model of progressive nephropathy. Verh. Dtsch. Ges. Pathol 85:250–256

Wolf E, Kahnt E, Ehrlein J, Hermanns W, Brem G, et al (1993) Effects of longterm elevated serum levels of growth hormone on life expectancy of mice: lessons from transgenic animal models. Mech. Ageing Dev 68:71–87

Raven P a, Uh M, Sakhrani D, et al (2008) Endocrine effects of growth hormone overexpression in transgenic coho salmon. Gen Comp Endocrinol 159:26–37. doi: 10.1016/j.ygcen.2008.07.011

Varela Junior AS, Goularte KL, Alves JP, Pereira FA, et al (2015) Methods of cryopreservation of Tambaqui semen, Colossoma macropomum. Animal Reproduction Science 157:71–77 doi: 10.1016/j.anireprosci.2015.03.017

Zbikowska HM (2003) Fish can be first – advances in fish transgenesis for commercial. 379–389

DISCUSSÃO GERAL

O crescimento acelerado da polulação mundial e a estagnação da pesca têm impulsionado a aquicultura para ser um dos setores de mais rápido crescimento (FAO 2014). Ferramentas biotecnológicas estão sendo exploradas por seu potencial para aumentar a eficiência da produção aquícola (Rasmussen e Morrissey 2007; Freitas et al. 2012). A transgenia se destaca dentre as várias técnicas da biotecnologia e tem mostrado um grande potencial de aplicação, especialmente pela manipulação do homônio do crescimento (GH). A transgenia para o GH além de seus efeitos sobre o crescimento também é conhecido por muitas vezes vir acompanhada por efeitos pleiotrópicos (Devlin et al. 1994; Pinkert et al. 1991; Vasudevan et al. 2010; Pursel et al. 1997; Rahman et al. 1998; Nam et al. 2001). Neste contexto, no primeiro capítulo desta tese relatamos a produção de um peixe duplo-transgênico (GH/GHR) que foi obtido através do cruzamento das linhagens F0104 (superexpressa o GH) e Myo-RED (superexpressa o gene do receptor do hormônio do crescimento - GHR). A produção do GH/GHR é viável, e este pode ser usado como um modelo de estudo sobre os efeitos que a superexpressão do GH pode gerar. Com a produção do GH/GHR é possível resolver o problema de manutenção destas linhagens no laboratório, já que quando cruzamos o duplo-transgênico com fêmeas selvagens obtemos uma proporção mendeliana (1:1:1:1) de não-transgênicos; GH: linhagem F0104; GHR: linhagem Myo-GHR e o GH/GHR: duplo transgênico. Este resultado indica que os transgenes estão integrados em cromossomos diferentes. As linhagens F0104 e Myo-GHR parecem ter características complementares e hipotetizamos se a expressão concomitante destas duas características proporcionaria um aumento da sinalização intracelular do eixo somatotrófico no músculo e consequente incremento da hipertrofia deste modelo.

No segundo capítulo desta tese avaliamos o efeito da dupla transgenia sobre o crescimento e estrutura muscular esquelética do duplo-transgênico que apresentou um crescimento comparável ao da linhagem F0104, embora com fator de condição mais baixo, resultando em um peixe comprido e delgado. Já foi previamente observado que a superexpressão do GH em peixes tem levado a alterações estruturais e/ou funcionais importantes relatadas para diversas espécies (Devlin et al. 2012; Blutke et al. 2014). O tratamento com GH exógeno efetivamente estimula o crescimento somático e linear (Holloway e Leatherland 1997) a maturação sexual, gametogênese e esteroidogênese em peixes (Reindl e Sheridan 2012). Figueiredo et al. (2013) relataram uma diminuição

na qualidade espermática para o genótipo GH. Neste sentido, o terceiro capítulo desta tese avaliou os parâmetros espermáticos do duplo-transgênico. O GH/GHR, mesmo apresentando uma alteração macroscópica no tamanho do testículo, teve uma melhora significativa em vários parâmetros espermáticos. Os resultados mostraram uma diminuição significativa na produção de ROS no duplo-transgênico e esta diminuição tem implicações importantes sobre os todos os demais parâmetros. Estes compostos quando presentes em grandes quantidades danificam o seu DNA e membranas, o que pode levar a um funcionamento anormal destas células ou até mesmo à morte celular (De Lamirande e O'Flaherty 2008). Um aumento de ROS foi reportado para o genótipo GH por Figueiredo et al. (2013) e neste trabalho onde observamos um aumento dos níveis de transcrição do gene pró-apoptótico *bik* para o genótipo GH, o que pode estar relacionado com a baixa concentração de espermatozóides neste genótipo. Guerra et al. (2013) demonstraram que *bik* pode ser considerado com um bom indicador de qualidade espermática em peixes. O hormônio anti-mülleriano (AMH) é uma glicoproteína produzida e secretada pelas células de Sertoli, responsável pela regressão dos ductos müllerianos (Josso 1986) e é importante no controle negativo da esteroidogênese e da espermatogênese (Skaar et al. 2011).

No presente estudo foi observado que *amh* está praticamente inativado na linhagem F0104 representando, provavelmente, uma tentativa do organismo de estimular a espermatogênese. Contudo, a causa maior da diminuição na concentração de espermatozóides na linhagem que superexpressa o GH é a alta concentração de ROS no tecido testicular. O excesso de GH demanda mais energia e uma consequência da produção aeróbica de ATP é a produção de ROS, assim, o cenário provável é que o GH estimula a produção de ATP, que causa o aumento de ROS. que danifica o DNA e induz apoptose. Uma diminuição de GH circulante no duplo transgênico pode ter ocorrido devido à maior captação do hormônio circulante pelos receptores em excesso no músculo. Isto deve ter diminuído a concentração de hormônio circulante e, consequentemente, seus efeitos colaterais negativos.

CONCLUSÕES

Baseado nos resultados desta Tese conclui-se:

1. O cruzamento das linhagens F0104 e Myo-GHR origina um peixe duplo-transgênico que superexpressa simultaneamente o GH e o GHR;
2. A proporção mendeliana nos cruzamentos entre o duplo transgênico e não transgênicos resulta numa proporção de 1:1:1:1 para os quatro genótipos, indicando que os transgenes para o GH e GHR estão integrados em cromossomos diferentes.
3. Foi constatado que a dupla-transgenia levou a um aumento de crescimento corporal do GH/GHR, porém com menor fator de condição;
4. A dupla-transgenia melhorou a qualidade espermática e a capacidade reprodutiva contornando um dos efeitos negativos do excesso de GH.

PERSPECTIVAS

Uma vez que fica claro que níveis adequados de GH podem levar a um crescimento "sustentável" do organismo, uma das perspectivas que surge desta Tese é a necessidade de se ampliar a busca de promotores gênicos controláveis. Construções genéticas que pudessem ser ativadas por algum agente físico ou químico poderia permitir um ajuste refinado nos níveis de expressão e secreção do hormônio. Desta forma, os reprodutores poderiam ser criados com os promotores "desligados", enquanto que os peixes destinados à engorda seriam cultivados em um ambiente que levasse à ativação do transgene. Neste cenário, os reprodutores estariam protegidos dos efeitos colaterais negativos do excesso de hormônio.

Outra perspectiva que surge é que, mesmo utilizando promotores controláveis, o ideal seria o crescimento independente de excesso de hormônio. Neste caso, o desenvolvimento de receptores quiméricos que possam se auto-ativar parece uma alternativa bastante plausível, pois não teríamos em nenhuma hipótese excesso de hormônio prejudicando o organismo geneticamente melhorado e, também, nenhum risco de que o excesso de hormônio possa vir a causar algum efeito no consumidor final.

REFERÊNCIAS BIBLIOGRÁFICAS

Blutke A, Schneider MR, Renner-Müller I, et al (2014) Genetic dissection of IGF1-dependent and -independent effects of permanent GH excess on postnatal growth and organ pathology of mice. Mol Cell Endocrinol 394:88–98. doi: 10.1016/j.mce.2014.07.002

De Lamirande E, O'Flaherty C (2008) Sperm activation: Role of reactive oxygen species and kinases. Biochim Biophys Acta.;1784:106–15

Devlin RH, Yesaki TY, Biagi CA, et al (1994) Extraordinary salmon growth. Nature 371:209–210. doi: 10.1038/371209a0

Devlin R, Vandersteen W, Uh M, Stevens ED (2012) Genetically modified growth affects allometry of eye and brain in salmonids. Can J Zool 90:193–202. doi: 10.1139/z11-126

FAO (2014) The state of the world fisheries and aquaculture. Rome: Food and Agriculture Organization of the United Nations. ISBN 978-92-5-108276-8

Figueiredo MA, Fernandes RV, Studzinski AL, et al (2013) GH overexpression decreases spermatic parameters and reproductive success in two-years-old transgenic zebrafish males. Anim Reprod Sci 139:162–7. doi: 10.1016/j.anireprosci.2013.03.012

Freitas AC, Rodrigues D, Rocha-santos TAP, et al (2012) Marine biotechnology advances towards applications in new functional foods ☆. Biotechnol Adv 30:1506–1515. doi: 10.1016/j.biotechadv.2012.03.006

Guerra SM, Valcarce DG, Cabrita E, Robles V (2013) Analysis of transcripts in gilthead seabream sperm and zebrafish testicular cells: MRNA profile as a predictor of gamete quality. Aquaculture 406-407:28–33. doi: 10.1016/j.aquaculture.2013.04.032

Holloway AC, Leatherland JF (1997) The effects of N-methyl-D,L-aspartate and gonadotropin-releasing hormone on in vitro growth hormone release in steroid-primed immature rainbow trout, Oncorhynchus mykiss. Gen Comp Endocrinol 107:32–43. doi: 10.1006/gcen.1997.6907

Josso N (1986) Anti–müllerian hormone. Clin Endocrinol 25:331–345 doi: 10.1111/j.1365-2265.1986.tb01699.x

Nam YK, Noh JK, Cho YS, et al (2001) Dramatically accelerated growth and extraordinary gigantism of transgenic mud loach Misgurnus mizolepis. Transgenic Res 10:353–362. doi: 10.1023/A:1016696104185

Pinkert CA, Kooyman DL, Dyer TJ (1991). Enhanced growth performance in transgenic swine. Biotechnology 16, 251–258.

Pursel VG, Wall RJ, Solomon MB, Bolt DJ, Murray JE, Ward KA (1997) Transfer of an ovine metallothionein–ovine growth hormone fusion gene into swine. J. Anim. Sci. 75, 2208–2214.

Rahman MA, Mak R, Ayad H, Smith A, Maclean N (1998) Expression of a novel piscine growth hormone gene results in growth enhancement in transgenic tilápia (Oreochromis niloticus). Transgenic Res 7:357–369.

Rasmussen RS, Morrissey MT (2007) Biotechnology in Aquaculture : Transgenics and Polyploidy. Compr Rev Food Sci Food Saf 6:2–16.

Reindl KM, Sheridan MA (2012) Peripheral regulation of the growth hormone-insulin-like growth factor system in fish and other vertebrates. Comp Biochem Physiol A Mol Integr Physiol 163:231–45. doi: 10.1016/j.cbpa.2012.08.003

Skaar KS, Nóbrega RH, Magaraki A, et al (2011) Proteolytically Activated, Recombinant Anti-Müllerian Hormone Inhibits Androgen Secretion, Proliferation, and Differentiation of Spermatogonia in Adult Zebrafish Testis Organ Cultures. Endocrinology 152:3527–3540. doi: 10.1210/en.2010-1469

Vasudevan K, Raber J, Sztein J (2010) Fertility comparison between wild type and transgenic mice by in vitro fertilization. Transgenic Res 19:587–594.

ANEXOS

COMISSÃO DE ÉTICA EM USO ANIMAL
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CEUA

AUTORIZAÇÃO DE ALTERAÇÃO DE VIGÊNCIA Nº 005/2015

PROCESSO Nº	23116.005574/2013-03
CEUA Nº	Pq015/2013
UNIDADE	ICB
TÍTULO DO PROJETO	Efeitos da dupla transgenia para o eixo somatotrófico, sobre aspectos estruturais, genéticos e produtivos em *zebrafish*
NÚMERO DE ANIMAIS E VIGÊNCIA	747 (*Danio rerio* - adultos) – 31/07/2016
ENVIO DO RELATÓRIO FINAL	Agosto de 2016
PROFESSOR RESPONSÁVEL	Luis Fernando Marins

PARECER DA CEUA:

Após a análise de sua solicitação de alteração de vigência, enviada a essa comissão em 22 de julho de 2015, a nova data para conclusão dos protocolos experimentais estabelecidos em seu projeto foi alterada para 31 de julho de 2016.

Rio Grande, 27/08/2015.

Med. Vet. Alice T. Meirelles Leite
Coordenadora da CEUA-FURG

Controle da CEUA - Parecer(es) prévio(s) relacionado(s) ao processo: P042/2013, P049/2013
Página 1/1