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Fisiologia Animal Comparada

Efeitos do hormônio do crescimento sobre o transporte de glicose cerebral e o controle do apetite em um modelo de *zebrafish* (*Danio rerio*) transgênico

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Sumário

Resumo geral

As ações biológicas do hormônio do crescimento (GH) são pleiotrópicas, incluindo promoção do crescimento, mobilização de energia, desenvolvimento gonadal, apetite e comportamento. Para exercer estas diferentes funções, muitos fatores endócrinos e ambientais estão envolvidos. Em peixes, o controle neuroendócrino do GH é multifatorial, com fatores inibitórios e estimulatórios agindo sobre sua produção e liberação na hipófise. Peixes geneticamente modificados para o GH, onde ocorre a superexpressão do hormônio, possuem aceleração do crescimento, aumento da quantia de alimento ingerida e, consequentemente, alteração da taxa metabólica e da utilização de substratos energéticos. Entretanto, a maneira como a superexpressão do GH em peixes altera diferentemente estes fatores ainda foi pouco explorada. No presente trabalho, avaliamos o efeito do GH sobre os transportadores de glicose (*gluts*) no cérebro em uma situação de exposição aguda ou crônica ao hormônio, sobre diferentes estados de alimentação, e os possíveis efeitos diferenciados entre os sexos. Além disto, foi avaliado o efeito do excesso de GH sobre a regulação do apetite. Os resultados obtidos demonstraram que: (1) a exposição crônica (animais transgênicos) tem efeito anti-insulínico (diabetogênico), fazendo com que os animais mantenham glicemia alta por mais tempo e reduzam a expressão de *gluts* no cérebro. O efeito agudo do GH (não-transgênicos injetados com GH) resultou em aumento na expressão dos *gluts* no cérebro; (2) animais transgênicos alimentados ou em jejum apresentaram queda na expressão de *gluts* e de transportadores de corpos cetônicos (*mcts*) no cérebro, sendo estes efeitos mais pronunciados em machos; (3) o excesso de GH levou a uma maior ingestão de alimento, além de ter alterado a forma

como machos e fêmeas controlam a fome e a saciedade antes da alimentação, após a alimentação e em estado de jejum.

1. Introdução

Fisiologia animal é o ramo da biologia que estuda as múltiplas funções mecânicas, físicas e bioquímicas nos seres vivos. De uma forma mais geral a fisiologia preocupa-se em compreender, integrativamente, o funcionamento dos vários níveis de organização: tecidos, órgãos e sistemas de órgãos dos animais multicelulares. Nestes diferentes níveis de organização ocorre diferenciação e divisão de trabalho celular e, apesar da grande variedade de formas, eles partilham algumas propriedades essenciais. Obtenção e processamento da energia proveniente do meio, excreção dos produtos finais do metabolismo, reparo de tecidos e reprodução estão entre estas propriedades (Randall *et al*., 2006). Cada uma destas tarefas está relacionada a órgãos, tecidos ou células especializadas, os quais têm necessidades energéticas e padrões metabólicos característicos em cada tipo de organismo (Nelson & Cox, 2008). A escolha do substrato energético por tecidos particulares também obedece a um complexo sistema de controle e diversos fatores podem estar envolvidos (Chinetti *et al*., 2001).

Os processos fisiológicos de um organismo são coordenados, principalmente, pelos sistemas nervoso e endócrino, agindo juntamente com os efetores, músculos e glândulas, para gerar respostas (Randall *et al.*, 2006). Em vertebrados, o hipotálamo é o centro coordenador do sistema neuroendócrino: ele recebe e integra sinais vindos do Sistema Nervoso Central, respondendo através da produção de hormônios que vão diretamente até a adenohipófise. Na adenohipófise, os hormônios estimulam a produção de hormônios específicos, que irão atuar em todo o organismo (Nelson & Cox, 2008). No cérebro, em especial no eixo somatotrófico, um dos principais hormônios que

está relacionado ao controle destas atividades é o hormônio do crescimento (GH) (Hallerman *et al.*, 2007).

1.1 O hormônio do crescimento (GH)

O GH é um hormônio pluripotente produzido na adenohipófise, e que realiza suas funções através da sua ligação ao receptor transmembrana específico, o receptor do hormônio do crescimento (GHR), nos tecidos alvo (Pérez-Sánchez *et al*., 2002). A secreção do GH, que ocorre de forma pulsátil, está sob o controle de neurohormônios hipotalâmicos, sendo que o estímulo de sua produção e liberação está a encargo do hormônio liberador do hormônio do crescimento (GHRH), e o efeito contrário, a inibição, ocorre pela ação do hormônio inibidor do hormônio do crescimento (GHIH) ou somatostatina (Randall *et al.*, 2006). Além do controle exercido pelo GHRH e GHIH, o próprio GH pode regular sua secreção através de retroalimentação negativa sobre suas células produtoras e sobre a produção de GHRH (Wallenius *et al*., 2001). Em alguns grupos, o padrão de secreção do GH tem influência do sexo do indivíduo: em humanos, a secreção de GH em machos é em forma de picos, com períodos praticamente livres de GH circulante, ao passo que em fêmeas a secreção se mantém relativamente constante (Jaffe *et al*., 1998). Além dos fatores citados, a grelina, produzida pelo trato gastrointestinal, pode estimular a secreção de GH (Dimaraki & Jaffe, 2006). Na circulação, o GH pode ser encontrado livre ou ligado a uma forma truncada do seu receptor, a proteína ligante GHPB, sendo a predominância de cada forma dependente do padrão de secreção pulsátil do hormônio (Vijayakumar *et al*., 2010).

Afirmações generalizadas tendo como base o sistema hipofisário de mamíferos, onde o eixo GH hipofisário - fator de crescimento tipo insulina I hepático (IGF-I) era visto como a via pela qual o IGF-I mediava as ações fisiológicas do GH, indicavam que praticamente todos os efeitos do GH eram indiretos (Reinecke *et al*., 2005). Em mamíferos, o IGF-I circulante pode realizar o controle de sua concentração através de retroalimentação negativa, agindo sobre a produção de GHRH no hipotálamo e sobre a produção de GH na adenohipófise, reduzindo a produção de GH (Wallenius *et al*., 2001). No entanto, apesar de todas estas informações, a ampla distribuição tecidual de células produtoras de IGF-I (Reinecke *et al.*, 1997) e seus receptores (IGF-R) (Radaelli *et al*., 2003), juntamente com a ampla distribuição de receptores de GH nos tecidos (Pérez-Sánchez *et al*., 2002) torna difícil identificar como o GH exerce suas funções.

Inúmeras funções são atribuídas ao GH, sendo que este hormônio atua sobre muitos tecidos do organismo. Ele promove o crescimento de quase todos os tecidos do organismo que têm capacidade de crescer, tendo ação tanto hipertrófica quanto hiperplásica. Além disso, está envolvido na diferenciação de certos tipos de células, como as células ósseas e musculares no estágio inicial da maturação (Guyton, 2008). Efeitos metabólicos também podem ser vistos sob a influência do GH: ele está envolvido na incorporação de aminoácidos e na redução da excreção de compostos nitrogenados, tendo assim um efeito anabólico sobre o metabolismo proteico (Norris, 2007); promove aumento da mobilização de ácidos graxos do tecido adiposo, elevando o nível sanguíneo destas substâncias e o seu uso como fonte energética; e reduz a utilização de glicose pelas células, deixando-a disponível no sangue (Mauras & Haymond,

2005). Além deste efeito direto sobre o metabolismo energético, o GH aumenta a motivação para o forrageamento e a quantia ingerida (Le Bail & Boeuf, 1997). Estas inúmeras funções exercidas pelo GH podem se dever a especificidade de resposta entre os tecidos, às diferentes vias de sinalização intracelular ativadas por ele, ou também pelo "*cross-talk*" entre estas vias.

A ligação hormônio-receptor induz a fosforilação e ativação de membros de uma família conhecida como Janus Kinases (JAKs), as quais estão normalmente ligadas à porção intracelular do receptor (Carter-Su *et al*., 1996; Croker *et al*., 2008). A JAKs ativas fosforilam resíduos de tirosina da porção intracelular do receptor, os quais servem de sítios de ancoragem para outra família, esta de Transdutores de Sinal e Ativadores de Transcrição, as STATs. As STATs fosforiladas se dimerizam e translocam-se para o núcleo para ativar a transcrição de genes relacionados principalmente com o crescimento (Lanning & Carter-Su, 2006).

Além da via das STATs, as vias MEK/ERK e PI3K/Akt também podem ser ativadas pelo GH. A cascata MEK/ERK participa do controle da proliferação, diferenciação e migração celular. O GH ativa esta via através do recrutamento de uma proteína adaptadora de sinalização chamada Shc (proteína semelhante ao colágeno com homologia src) junto ao complexo GHR-JAK2 ativo, a qual é fosforilada e desencadeia a ativação de outras moléculas como Ras, Raf, MEK (MAPK ERK kinase), e finalmente a kinase regulada extracelularmente ERK1/2 (González *et al*., 2011).

O GH também pode ativar a via PI3K, tanto através do recrutamento de substratos do receptor de insulina (IRS), quanto através da interação da proteína adaptadora CrkII-IRS1 (González *et al*., 2011), ou pela interação direta

da PI3K com resíduos fosforilados de tirosina do GHR (Lanning & Carter-Su, 2006). Este braço de sinalização do GH leva a múltiplas respostas, entre elas alterações no metabolismo de carboidratos e proteínas. A via IRSs/PI3K é a principal via ativada pela insulina e pode, portanto, contribuir ao efeito tipo insulina do hormônio do crescimento (Dominici *et al*., 2002). Além do IRS, outro efetor da via PI3K é a proteína kinase B (PKB), também chamada de Akt. A Akt, por sua vez, ativa outra proteína kinase, a mTOR (proteína alvo da rapamicina em mamíferos), a qual regula a tradução de proteínas no ribossomo, levando à síntese de proteínas. Além disto, a Akt regula a captação de glicose, promovendo o recrutamento dos transportadores de glicose para a superfície celular e regulando a síntese de glicogênio (White, 1998). As principais vias de sinalização do GH estão esquematizadas na Figura 1.

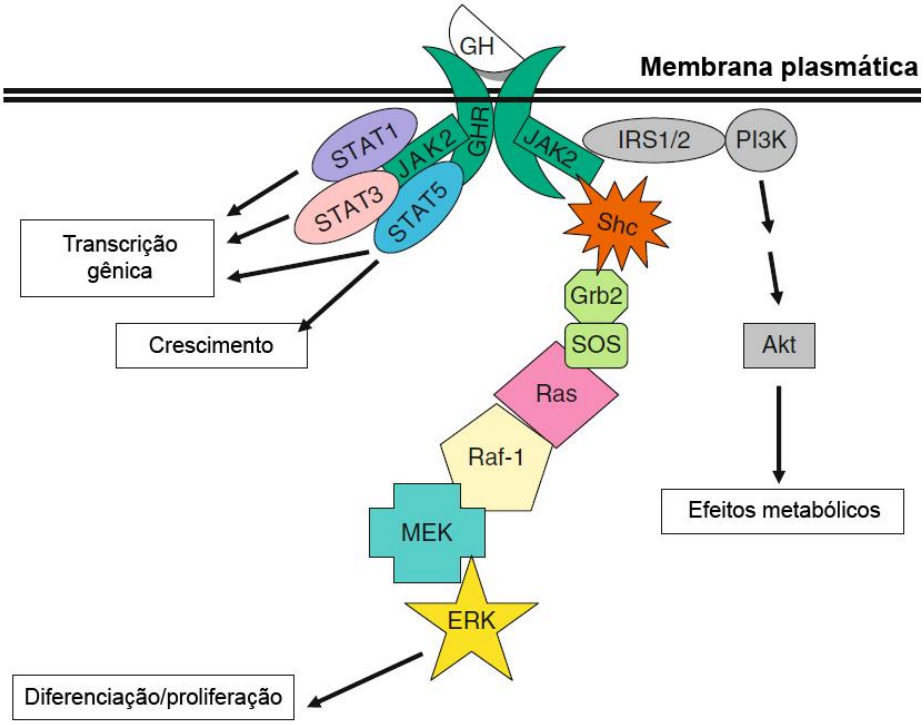

Figura 1: Principais vias de sinalização ativadas pelo GH. As maiores vias de sinalização utilizadas pela ativação do complexo GHR/JAK2 incluem STATs, MEK/ERK e PI3K.

Dentre os genes que têm a transcrição ativada pelas vias de sinalização do GH, encontram-se alguns que atuam regulando negativamente estas vias, como as SOCSs (Supressores de Sinalização de Citoquinas). Estas proteínas atuam inativando as JAKs ou bloqueando o recrutamento das STATs, criando eixo de retroalimentação negativa na sinalização do GH (Flores-Morales *et al.*, 2006). Experimentos realizados com *zebrafish* transgênicos superexpressando GH, demonstraram que as principais SOCSs envolvidas na regulação negativa da sinalização do GH eram SOCS1 e SOCS3 (Studzinski *et al.*, 2009). Além disto, foi demonstrado que as SOCS podem mediar resistência à insulina induzida pelo GH, interagindo com o receptor de insulina e inibindo a fosforilação dos IRSs, consequentemente inativando as vias ERK1/2 e Akt. Outra forma que as SOCS podem usar para inativar a via de sinalização da insulina é através da promoção da ubiquitinação dos IRSs e sua consequente degradação (Rui *et al.*, 2002).

Apesar do vasto conhecimento existente sobre a ação do GH em mamíferos, a ação deste hormônio em peixes continua em discussão. Nestes organismos, além do crescimento somático, ele participa de quase todos os principais processos fisiológicos, incluindo a regulação do equilíbrio iônico e osmótico, metabolismo de lipídios, proteínas e carboidratos, crescimento esquelético e dos tecidos moles, reprodução e função imune (Yousefian & Shirzad, 2011). Estudos recentes têm indicado que o GH atua, também, sobre diversos aspectos do comportamento, incluindo fome, comportamento alimentar, agressividade e fuga do predador (Canosa *et al*., 2007).

Muitos aspectos da ação do GH têm sido objeto de investigação em peixes como salmonídeos, ciprinídeos e sparídeos (Reinecke *et al*., 2005). O

GH já foi sequenciado e/ou sua proteína isolada em diversas famílias de teleósteos e linhagens de peixes transgênicos para o GH já foram estabelecidas (Chen & Powers, 1990), sendo estes modelos de grande importância para o estudo deste hormônio e suas possíveis interações com outros hormônios e fatores regulatórios do organismo como um todo.

Muitos experimentos realizados com animais transgênicos superexpressando GH já foram realizados. Estes trabalhos pontuaram diferenças morfológicas e comportamentais entre a linhagem selvagem e a geneticamente modificada. Os espécimes transgênicos apresentaram taxa de crescimento superior à dos animais selvagens e também maior consumo e conversão alimentar. Outro fato relacionado aos animais transgênicos é que estes podem apresentar anormalidades morfológicas principalmente no crânio e opérculo, as quais podem estar relacionadas à sobrevivência reduzida destes animais em comparação com os selvagens (Chen & Powers, 1990; Devlin *et al*., 2004).

Além disto, trabalhos realizados com salmões e *zebrafish* transgênicos superexpressando o GH, mostraram que estes animais têm seu crescimento e chegada à idade adulta acelerados (Figueiredo *et al*., 2007a; Raven *et al*., 2008). Apesar do crescimento elevado destes animais ser resultado da elevada produção de GH resultante da expressão do transgene em todo o corpo (Devlin *et al*., 2004), a indicação exata dos mecanismos de controle hormonais, neuroendócrinos e metabólicos associados a estes eventos ainda não foram identificados com precisão (Raven *et al*., 2008).

1.2 O hormônio do crescimento e a utilização de glicose

Como citado anteriormente, o GH promove o crescimento em todos os vertebrados, além de ter efeito sobre o metabolismo de diversas moléculas do organismo. Entende-se como metabolismo a soma das transformações químicas que ocorrem em uma célula ou organismo, através de vias metabólicas finamente reguladas. As reações que ocorrem no metabolismo podem ser divididas em dois tipos distintos: o catabolismo e o anabolismo. Como catabolismo, se compreende a fase degradativa do metabolismo, onde moléculas energéticas (carboidratos, lipídeos e proteínas) são quebradas a produtos menores e mais simples, liberando energia para ser conservada em ATP ou na forma de coenzimas reduzidas (NADH, NADPH, and $FADH_2$). Já o anabolismo é o caminho contrário do catabolismo, onde ocorre, a partir de pequenos precursores, a biossíntese de moléculas maiores e mais complexas como carboidratos (mono e polissacarídeos), lipídeos (fosfolipídeos, triglicerídeos), proteínas e ácidos nucleicos, utilizando energia armazenada nas moléculas energéticas citadas anteriormente (Nelson & Cox, 2008). Dentre estas moléculas, os carboidratos têm papel chave no metabolismo energético dos vertebrados (Moon & Foster, 1995). Neste sentido, a glicose é o carboidrato mais importante, sendo absorvida do trato gastrointestinal para a corrente sanguínea ou produzida no fígado a partir de outras moléculas, como aminoácidos e triacilgliceróis (Nelson & Cox, 2008).

A glicose, por sua natureza hidrofílica, não tem a capacidade de atravessar a membrana das células livremente. Devido a isto a presença de carreadores transmembrana específicos, os quais são responsáveis pela passagem de glicose da circulação para o interior das células, é necessária

para que essa assimilação ocorra. Estes transportadores responsáveis pela captação de glicose pertencem à família de proteínas SLC2A ou *glut*. Muitas isoformas de *gluts* já foram descritas, sendo que cada uma destas possui diferentes distribuições teciduais, além de parâmetros cinéticos específicos. De forma geral, tecidos como o muscular e adiposo têm captação de glicose dependente de insulina, pois a presença deste hormônio estimula a translocação dos transportares das vesículas onde são armazenados para a membrana da célula. Já outros locais como cérebro, fígado, rins, eritrócitos e ilhotas de Langerhans são considerados predominantemente insulino-independentes, sendo a entrada de glicose nestas células desvinculada da presença do hormônio (Alquier *et al*., 2006).

É de amplo conhecimento a relação entre o metabolismo de glicose e o GH circulante (Gerich, 2000; Jørgensen *et al*., 2004). De forma geral, este hormônio exerce quatro efeitos principais sobre o metabolismo de glicose:

(1) redução do uso de glicose como fonte energética: isto ocorre, em parte, devido a maior mobilização e utilização de ácidos graxos para produção de energia, os quais formam grandes quantidades de acetil-CoA e provocam um efeito de retroalimentação, bloqueando a degradação da glicose e do glicogênio.

(2) aumento da deposição de glicogênio nas células: uma vez que a glicose e o glicogênio não podem ser facilmente utilizados para fornecimento de energia, a glicose que penetra nas células é rapidamente convertida a glicogênio e armazenada.

(3) redução da captação de glicose pelas células (aumento da glicemia): de forma geral, o primeiro efeito da administração GH a um animal é o aumento

da captação celular de glicose, reduzindo a glicemia. Entretanto, certo tempo depois esta ação é revertida, havendo redução no transporte de glicose para o interior da célula.

(4) efeito diabetogênico do GH: o aumento da glicemia ocasionado pelo hormônio do crescimento estimula a secreção de quantidades adicionais de insulina pelas células beta das ilhotas de Langerhans e, além disto, o GH também exerce efeito estimulante direto sobre as células beta. Estas duas ações do GH promovem, em longo prazo, um “esgotamento” nas células β, desenvolvendo um quadro de *Diabetes mellitus*.

1.3. O controle da ingestão de alimento e o GH

Em vertebrados o controle da ingestão alimentar envolve uma extensa rede de fatores produzidos pelo sistema nervoso central ou mesmo pelos tecidos periféricos do organismo. Sob condições ótimas, a ingestão nutricional é adequada para a manutenção do metabolismo basal, crescimento, desenvolvimento, reprodução e, havendo algum excedente, deposição de estoques energéticos (Volkoff *et al*., 2009).

Em todo o grupo de vertebrados, o forrageamento é regulado por fatores endócrinos chave, os quais têm a capacidade de estimular (orexígenos) ou inibir (anorexígenos) a fome, agindo em centros controladores da alimentação no cérebro e regulando este aspecto da fisiologia do animal tanto a curto quanto a longo prazo (Valassi *et al*., 2008) (Figura 2). Tais fatores endócrinos podem ser produzidos no hipotálamo (fatores de origem central), ou no trato gastrointestinal, pâncreas, fígado e tecido adiposo (fatores de origem periférica). A ação dos fatores periféricos se faz no sentido de transmitir as

informações aos centros de alimentação do cérebro, atuando diretamente em receptores centrais (Volkoff *et al*., 2009). Dentre os fatores orexígenos produzidos centralmente podemos citar o neuropeptídeo Y (NPY) (Silverstein & Plisetskaya, 2000) e as orexinas (Volkoff *et al*., 2009), e como anorexígenos o sistema proopiomelanocortina/ hormônio estimulador dos melanócitos (POMC/MSH) (Cerdá-Reverter *et al*., 2003). Como sinais periféricos estão incluídos os hormônios de saciedade colecistocinina (CCK), de origem gastrointestinal (Silverstein & Plisetskaya, 2000), e a leptina, produzida principalmente pelos adipócitos (Rousseau & Dufour, 2007); e estimulando a fome de forma periférica, há a grelina, um hormônio produzido pelo estômago (Unniappan *et al*., 2002).

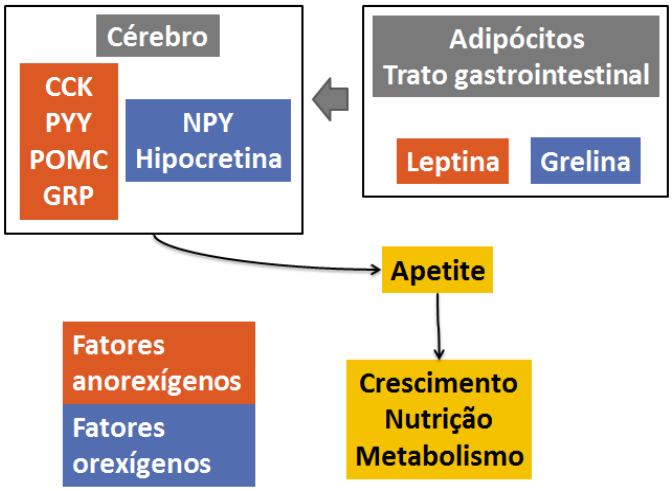

Figura 2: Resumo de alguns fatores anorexígenos e orexígenos produzidos central ou perifericamente, relacionados ao controle do apetite.

Nos últimos anos, um crescente número de fatores reguladores da fome homólogos aos de mamíferos foram caracterizados em peixes (Volkoff *et al*., 2005), sugerindo que a regulação da ingestão alimentar é relativamente bem conservada ao longo da escala evolutiva dos vertebrados (Volkoff *et al*., 2009).

De forma semelhante a outros vertebrados, algumas espécies de peixes estão submetidas a variações ambientais, as quais exigem diversas adaptações na alimentação. Além disto, mudanças sazonais no forrageamento muitas vezes coincidem com migrações de desova e reprodução, sugerindo uma ligação entre o estado nutricional do animal e o eixo reprodutivo. No entanto, devido à vastidão do grupo filogenético ao qual os peixes pertencem, sugere-se que este grupo tenha o controle endócrino da alimentação também diverso, envolvendo diferentes moléculas e/ou mecanismos específicos de regulação (Volkoff *et al*., 2009). Apesar do aumento de informações disponíveis acerca do papel destes neuropeptídeos no controle da ingestão alimentar em peixes, o conhecimento dos mecanismos envolvidos nestes processos ainda é muito limitado.

Um dos eixos fortemente influenciado pela disponibilidade de alimento é o eixo somatotrófico: reprodução, crescimento, sobrevivência, entre outros processos, estão intimamente relacionados às condições nutricionais do animal. Durante o jejum ocorre um paradoxo, onde o crescimento cessa e a energia mobilizada dos tecidos é utilizada para a manutenção do metabolismo, apesar da elevação do GH e redução do IGF no plasma. Este fato ocorre devido ao desenvolvimento de resistência ao GH no fígado, o qual parece uma medida adaptativa de proteção, já que o baixo nível de IGF circulante limita o crescimento, enquanto que o elevado nível de GH estimula o uso de ácidos graxos como fonte energética (Pierce *et al*., 2005). Na maioria dos casos, o cessamento do jejum irá restaurar os níveis de IGF-I e o crescimento (Metón *et al*., 2000). Também se sabe que a elevação dos níveis de GH durante o jejum e a administração de GH, além de estarem relacionadas à estimulação da

lipólise, estimulam a retenção do uso de proteínas e glicose como substrato energético, gerando um estado hiperglicêmico (Møller & Nørrelund, 2003), e este estado hiperglicêmico gerado pelo GH pode ter efeito inibitório sobre a ingestão alimentar (Le Bail & Boeuf, 1997).

Experimentos com trutas arco-íris (*Oncorhynchus mykiss*) que receberam injeções de GH mostraram aumento da atividade dopaminérgica no hipotálamo, sugerindo que o GH pode estar atuando diretamente nos centros de controle da fome, modulando o comportamento alimentar (Johnsson & Bjornsson, 1994). O GH atua também sobre o controle da ingestão alimentar de forma indireta, através dos fatores reguladores da fome, como o NPY e a CCK. O NPY é um orexígeno (Silverstein & Plisetskaya, 2000) que responde positivamente ao GH, tendo seus níveis de mRNA aumentados em resposta ao jejum e tem efeito contrário ao da CCK, um sinal de saciedade produzido pelo intestino e o cérebro (Volkoff *et al*., 2003).

Além do NPY e da CCK, vários outros fatores tem ação conhecida sobre o balanço energético e a fome e podem sofrer influência do GH, como a leptina (Rousseau & Dufour, 2007), grelina (Unniappan *et al*., 2002), MSH (Volkoff *et al*., 2005), POMC (Cerdá-Reverter *et al*., 2003), orexinas (OXs), peptídeo liberador da gastrina (GRP), transcrito regulado pela cocaína e anfetamina (CART) (Volkoff *et al*., 2009), entre outros.

1.4. Fatores reguladores da fome

Orexígenos

Neuropeptídeo Y (NPY)

Em mamíferos, o NPY é um dos mais potentes orexígenos conhecido (Valassi *et al*., 2008). A composição de aminoácidos do peptídeo maturo é relativamente bem conservada entre os vertebrados, incluindo peixes (Sundström *et al*., 2004), sugerindo a existência de semelhanças para as funções fisiológicas deste fator. Estudos mostraram que injeções centrais de NPY em peixes como peixinho-dourado (*Carassius auratus*) e truta arco-íris (*Oncorhynchus mykiss*) causaram aumento na ingestão alimentar de forma dose-dependente, sugerindo que o NPY atua na regulação da alimentação dos peixes. Tanto em peixinho-dourado (Narnaware & Peter, 2001) como no bacalhau do Atlântico (*Gadus morhua*) (Kehoe & Volkoff, 2007), a expressão de mRNA de NPY sofre variações peri-prandiais, sendo que níveis mais elevados são encontrados pouco antes do horário em que o animal é normalmente alimentado, sugerindo que o NPY atua como um fator que estimula a fome a curto prazo nestas duas espécies. Em peixinho-dourado, um período de jejum de 1 a 4 semanas induz aumento na expressão de mRNA de NPY no hipotálamo, sugerindo que o NPY também poderia atuar como um regulador de longo prazo da fome em peixes (Narnaware & Peter, 2001).

Estudos mostraram que o NPY é um potente estimulador da liberação de GH em peixinho-dourado (Barb & Barrett, 2005; Raven *et al*., 2008), sendo que este estímulo pode ser influenciado de forma direta, onde o NPY atua nas células da hipófise produtoras de GH, ou indireta, através do estímulo para liberação de GHRH (Hao-Ran, 1997).

Grelina

A grelina é um peptídeo secretado predominantemente pelo estômago, mas que também tem sua síntese ocorrendo no cérebro. Receptores de grelina já foram identificados nos neurônios aferentes vagais de ratos, sugerindo que os sinais deste fatore são transmitidos do estômago para o cérebro indiretamente via nervo vago (Sakata *et al*., 2003). A sequência do peptídeo já foi isolada e identificada em várias espécies de peixes (Volkoff *et al*., 2009).

Estudos relatam que injeções centrais ou periféricas de grelina humana ou de peixe estimulam o consumo alimentar em peixinho-dourado (Miura *et al*., 2007) ao passo que, em trutas arco-íris, apenas injeções periféricas de grelina não afetam o consumo alimentar (Jönsson *et al*., 2007). Experimentos realizados com o bacalhau do Atlântico (*Gadus morhua*) mostraram que os maiores níveis de grelina são encontrados no horário em que o animal é alimentado, sendo que nos períodos peri-prandiais (2h antes ou depois do horário de alimentação) a expressão de mRNA do peptídeo é menor. No mesmo trabalho, os autores mostraram que animais cultivados em regime de restrição na quantia do alimento não tiveram afetada a expressão de grelina no estômago, sugerindo que este peptídeo não atua como um sinal para fome em longo prazo nesta espécie. Já em outros trabalhos, o jejum induzido provocou redução nos níveis de grelina.

Em humanos, a grelina atua como um forte estímulo para a liberação de GH, sendo este fato mediado através do eixo estômago-hipófise (grelina-GH). O contrário também ocorre, sendo que alterações nos níveis de GH podem modificar a homeostase da grelina no estômago. Quando ocorre redução nos

níveis de GH, ocorre aumento na produção e liberação de grelina, e no caso contrário, quando ocorre aumento no GH, há redução na produção e liberação deste fator (Qi *et al*., 2003).

Hipocretina (Orexinas - OXs)

As hipocretinas são compostas por dois peptídeos orexígenos, hipocretina A e B, originadas da clivagem de um único precursor, a pré-pro-hipocretina (Valassi *et al*., 2008). Em peixes, mRNAs que codificam pré-pro-hipocretinas foram identificados no cérebro de *zebrafish* (Kaslin *et al*., 2004), bacalhau do atlântico (Xu & Volkoff, 2007) e peixinho-dourado (Hoskins *et al*., 2008). Assim como em mamíferos, as orexinas parecem ter papel na regulação da ingestão alimentar de peixes (Korczynski *et al*., 2006).

Em peixinho-dourado, injeções centrais de hipocretina causam aumento significativo na fome (Volkoff *et al*., 2009). No bacalhau, quando ocorre jejum de longo prazo ou restrição alimentar, há aumento no mRNA para pré-pro-hipocretina no cérebro, sugerindo que este peptídeo possa ter ação sobre a regulação da ingestão alimentar a longo prazo (Xu & Volkoff, 2007). Também no bacalhau, a expressão de hipocretina no cérebro sofre variações peri-prandiais, ocorrendo elevação desta expressão no horário em que o animal é normalmente alimentado. Apesar disto, estas mudanças são somente observadas quando o animal é alimentado de forma restrita (Xu & Volkoff, 2007). Estes dados sugerem que, ao menos no bacalhau, as hipocretinas podem ser mais importantes como reguladores da fome de longo prazo do que de curto prazo (Volkoff *et al*., 2009).

Relatos de experimentos realizados em ratos mostraram que injeções centrais de hipocretina tem capacidade de reduzir o nível de GH endógeno . Em peixes, apesar de não haver relato da existência de receptores de hipocretina na hipófise, a presença deste fator neste local sugere a existência de receptores, podendo haver sinalização autócrina ou parácrina, como por exemplo, sobre a liberação do GH (Suzuki *et al*., 2007).

Anorexígenos

Leptina

A leptina é uma proteína codificada pelo gene *obese* (OB), produzida e secretada principalmente pelos adipócitos, mas também por outros tecidos, como o cérebro e o epitélio gástrico (Farrell, 2011). Grandes concentrações de receptores de leptina foram identificadas em núcleos hipotalâmicos contendo neurônios que expressam importantes agentes orexígenos (como NPY e hipocretina) e também fatores anorexígenos (como CART, CCK e peptídeos derivados do POMC), sugerindo que a leptina afeta a ingestão alimentar através da inibição hipotalâmica dos agentes orexígenos e estimulando os anorexígenos (Harvey & Ashford, 2003).

O peptídeo leptina já foi isolado em mamíferos e aves (Doyon *et al.*, 2001), no entanto evidências sugerem que também é expressa em outros animais, como peixes. Nesta classe, tecidos imunorreativos à leptina foram detectados em várias espécies (Yaghoubian *et al*., 2001; Nieminen *et al*., 2003). Em estudos realizados com salmão (*Oncorhynchus kisutch*) (Baker *et al*., 2000) e sunfish (*Lepomis cyanellus*) (Londraville & Duvall, 2002) não foram relatados efeitos do tratamento com leptina sobre a ingestão alimentar ou peso

corporal. No peixinho-dourado, contudo, injeções tanto periféricas quanto centrais de leptina de roedor culminaram com diminuição da ingestão de alimento (Volkoff *et al*., 2003). Em peixinho-dourado, maiores doses periféricas de leptina são necessárias para provocar redução na ingestão alimentar, em comparação com doses administradas centralmente, o que sugere que em peixes, assim como em mamíferos, a leptina atua principalmente no cérebro para controlar a enantiostase energética (Volkoff *et al*., 2003). Também no peixinho-dourado, injeções centrais de leptina alteram a expressão de mRNA de alguns fatores relacionados ao apetite, acentuando os efeitos de alguns anorexígenos como CART e CCK, e inibindo os orexígenos NPY e hipocretina (Volkoff *et al*., 2003).

Em humanos a relação entre a leptina e o GH é bem estudada e interessante, pois estes dois fatores exercem ação sobre a composição corporal. A deficiência de GH em adultos é associada ao aumento da gordura corporal e diminuição da massa magra, levando a um aumento nas concentrações séricas de leptina, sendo que o tratamento do indivíduo com GH proporciona redução da gordura corporal e aumento da massa magra (Lissett *et al*., 2001). Outro aspecto da relação entre GH e leptina é a obesidade, a qual está associada com redução do GH em humanos (Company *et al*., 2001).

Colecistocinina (CCK)

Em mamíferos e aves, CCK é sintetizada no cérebro, intestino e células endócrinas do intestino delgado. Substâncias tipo CCK foram identificados também no cérebro e intestino de várias espécies de peixe. Dois tipos de

receptores (A e B) foram detectados, mas somente o tipo A parece estar relacionado à via de regulação da saciedade (Le Bail & Boeuf, 1997).

Várias observações apoiam a influência da CCK no controle da ingestão alimentar, sendo seu principal efeito o de inibir o apetite. Injeções periféricas ou intraventriculares de CCK provocam redução ou atraso no consumo alimentar em mamíferos (Figlewica *et al*., 1996) e peixes, sendo que neste ultimo grupo esta inibição ocorre logo após injeção do peptídeo (Himick & Peter, 1994). Em mamíferos, injeções de antagonistas de CCK restauram a ingestão alimentar provocada pela saciedade pós-prandial. Assim, este peptídeo parece ter ação de curto prazo sobre a regulação da ingestão alimentar (Le Bail & Boeuf, 1997).

Estudos realizados em peixes mostraram imunorreatividade de CCK no cérebro e no intestino de espécies como truta arco-íris (*Onchorynchus mykiss)* e robalo (*Dicentrarchus labrax)* (Silverstein & Plisetskaya, 2000). Estes trabalhos, em conjunto com experimentos *in vitro* mostrando os efeitos da CCK sobre a contração do músculo liso (Aldman & Holmgren, 1987), e o de Himick & Peter (1994) onde foi analisado o efeito inibitório de injeções periféricas de CCK sobre a alimentação, sugerem que a CCK pode atuar tanto centralmente quanto perifericamente sobre a regulação do consumo alimentar (Silverstein & Plisetskaya, 2000).

Assim como o NPY, a CCK também tem ação estimulatória sobre a liberação de GH nas células da hipófise (Hao-Ran, 1997; Raven *et al*., 2008). Resultados de experimentos realizados com peixes transgênicos superexpressando o GH mostram que, apesar destes dois fatores terem efeitos antagônicos (CCK anorexígeno; GH orexígeno), a regulação da sazonalidade na alimentação dos animais é determinada primeiramente pelo GH, sendo a

ação da CCK dependente da concentração do primeiro peptídeo (Lõhmus *et al*., 2008).

Peptídeo YY (PYY)

O polipeptídio Y (PYY) é produzido e liberado, principalmente, pelas células da mucosa intestinal, e seus níveis plasmáticos se elevam quase que imediatamente após a ingestão alimentar (Le Roux & Bloom, 2007). Estudos têm apontado que o efeito anorexigênico do PYY seja principalmente mediado pelo sistema da melanocortina, através dos receptores Y2 sobre os neurônios produtores de NPY, inibindo seus efeitos orexígenos (Ellacott *et al*., 2006). Experimentos realizados com ratos demonstraram que o PYY pode diminuir, além do apetite, os níveis circulantes de grelina (Barazzoni *et al*., 2003).

Poucos estudos com PYY foram realizados em peixes, mas já foi relatada expressão de PYY em salmão (*Salmo salar*), onde a produção ocorre principalmente no trato gastrointestinal e cérebro (Murashita *et al*., 2009).

Sistema melanocórtico: proopiomelanocortina (POMC)

As melanocortinas são um grupo de fatores hipofisários que incluem a adrenocorticotrofina (ACTH) e os hormônios estimuladores dos melanócitos α, β e γ (MSHs). Todas estas substâncias são derivados de uma única molécula precursora, a proopiomelanocortina (POMC), e ocorrem em tecidos específicos por meio de clivagem pós-traducional (Volkoff *et al*., 2005). Em mamíferos as melanocortinas estão envolvidas no controle da homeostase energética (Ellacott & Cone, 2004), fato demonstrado pela administração de agonistas e

antagonistas para os receptores de MSHs, onde os agonistas inibiram a fome e os antagonistas estimularam (Schiöth *et al*., 2003).

Nos peixes, o gene da POMC codifica para três isoformas de MSH (α, β, γ, δ), sendo que as isoformas α e β foram detectadas tanto em peixes ósseos quanto cartilaginosos (Cerdá-Reverter *et al.*, 2011), a isoforma γ não ocorre em teleósteos e a quarta isoforma, δ, foi identificada em peixes cartilaginosos (Dores *et al.*, 2003). Com relação aos receptores de MSH, seis isoformas foram identificadas em *zebrafish* (Logan *et al*., 2003) e duas em peixinho-dourado (Cerdá-Reverter *et al*., 2003). Em peixinho-dourado, mRNA para estes receptores são amplamente distribuídos no cérebro e também em tecidos periféricos (Cerdá-Reverter *et al*., 2003).

Em peixes, muitos estudos indicam que os MSHs e seus receptores estão envolvidos na regulação do metabolismo energético e ingestão alimentar. Em trutas arco-íris, o α-MSH parece estimular a atividade lipásica no fígado, elevando os níveis plasmáticos de ácidos graxos. Além disso, animais com defeito na síntese de α-MSH são hiperfágicos, têm fígado hipertrófico e acumulação de gordura na cavidade abdominal (Yada *et al*., 2002), sugerindo que o sistema melanocórtico desempenha um papel no controle do equilíbrio energético de peixes, diminuindo a ingestão de alimentos, aumentando a atividade lipolítica, e elevando o gasto energético (Volkoff, 2006).

Estudos realizados por Cerdá-Reverter (2003) mostraram que em peixes o POMC é exclusivamente expresso no núcleo tuberal lateral (homólogo ao núcleo arqueado de mamíferos) e que o jejum não altera os níveis de mRNA desta substância no tecido.

Peptídeo liberador da gastrina (GRP)

O GRP é um peptídeo anorexígeno estruturalmente relacionado à bombesina (BBS), e originalmente isolado da pele de anfíbios (Anastasi *et al*., 1970). Peptídeos tipo BBS/GRP foram detectados no trato gastrointestinal e cérebro de peixes (Merali *et al.*, 1999). A este peptídeo foi atribuída função sobre a regulação das funções gástricas de peixes, como a secreção de ácidos e motilidade intestinal (Aldman & Holmgren, 1987). Em peixinho-dourado, injeções de BBS suprimem a ingestão alimentar, sugerindo que peptídeos tipo BBS/GRP podem atuar como fatores de saciedade (Canosa *et al.*, 2005). No entanto, em trutas arco-íris os níveis plasmáticos de GRP são muito baixos e parecem não influenciar a alimentação, sugerindo que o GRP não tem ação importante sobre a regulação da alimentação a curto prazo (Canosa *et al*., 2005). No bacalhau, experimentos realizados com animais super-alimentados e sub-alimentados mostram que o primeiro grupo têm expressão de mRNA para GRP maior. Além disto, foi observado que o jejum prolongado induz reduções na expressão de mRNA de GRP no estômago, sugerindo que o GRP pode atuar como um regulador na alimentação do bacalhau a longo prazo (Xu & Volkoff, 2009).

No peixinho-dourado, quando peptídeos tipo BBS/GRP são administrados central ou perifericamente, ocorre aumento na liberação de GH (Canosa *et al*., 2005), sendo possível que estes fatores contribuam para a regulação da saciedade e o aumento pós-prandial de GH (Canosa & Peter, 2004).

Levando em consideração que o hormônio do crescimento pode atuar em diversos sistemas, este trabalho objetivou relacionar este fator à alteração

da glicemia, à absorção de glicose no cérebro, e aos fatores que estimulam ou inibem a fome, utilizando como modelo zebrafish selvagens e transgênicos superexpressando GH.

2. Objetivos

2.1. Objetivo Geral

Avaliar os efeitos do GH sobre a expressão dos transportadores de glicose no cérebro, bem como sobre o comportamento alimentar e a expressão de mRNA de fatores que controlam o apetite, levando em consideração o estado alimentar e o sexo, em uma linhagem de *zebrafish* (*Danio rerio*) transgênico.

2.2. Objetivos específicos

- Avaliar as alterações provocadas pela ação crônica ou aguda do GH sobre a expressão dos transportadores de glicose (*gluts*) no cérebro;

- Verificar como os animais transgênicos superexpressando GH respondem em relação à expressão de transportadores de glicose (*gluts*), hexokinases (*hks*) e transportadores de corpos cetônicos (*mcts*) em estado alimentado ou de jejum, entre machos e fêmeas, em comparação aos animais não-transgênicos;

- Determinar diferenças no comportamento alimentar entre animais transgênicos e não-transgênicos;

- Verificar a expressão de genes relacionados ao apetite (agentes orexígenos: hipocretina, NPY, grelina; e anorexígenos: CCK, GRP, POMC,

PYY, leptina) entre animais transgênicos e não-transgênicos, bem como diferenças entre os tecidos analisados;

- Verificar a expressão dos genes que controlam o apetite em situações de jejum curto, pós-prandial e jejum longo, entre animais transgênicos e não-transgênicos;

- Verificar a expressão dos fatores envolvidos no estímulo e inibição do apetite através de genes que regulam negativamente o eixo somatotrófico (SOCS).

3. Justificativa

A maioria dos modelos genéticos atualmente utilizados para o estudo do metabolismo e utilização de glicose, bem como do comportamento alimentar e fome é de mamíferos. Entretanto, uma linhagem de *zebrafish* geneticamente modificada (*Danio rerio* – linhagem F0104) foi produzida superexpressando o gene do GH (Figueiredo *et al*., 2007a). Este modelo animal é de grande importância para estudos nesta área, já que o GH está intimamente envolvido nestes processos.

Em estudos utilizando esta linhagem de peixes transgênicos, Figueiredo *et al*. (2007b) avaliaram o crescimento e a expressão de genes relacionados ao eixo somatotrófico em peixes hemizigóticos (HE) e homozigóticos (HO). Os resultados demonstram que, apesar dos HO terem uma maior expressão do GH exógeno em relação aos HE e aos não transgênicos (NT), o seu crescimento não foi significativamente diferente dos não transgênicos. Já os HE apresentaram um crescimento significativamente superior às outras duas

classes, sugerindo que o excesso de GH não reflete, necessariamente, no aumento direto no crescimento.

Outras observações relacionadas a esta linhagem de peixes transgênicos estão relacionadas à fome e reprodução: os animais geneticamente modificados apresentam fome voraz, são provavelmente diabéticos e sua reprodução é deficiente.

ARTIGO 1

A ser submetido para a revista

Comparative Biochemistry and Physiology B

Chronic and acute effect of GH excess over glucose transporters in zebrafish (*Danio rerio*) brain

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Abstract

The aim of this study was to evaluate the response of glucose transporters (*gluts*) in brain of male *Danio rerio* (zebrafish) under a chronic or acute dose of growth hormone (GH). For this, the chronic treatment was conducted with transgenic zebrafish overexpressing GH, and the acute treatment was performed in wild-type zebrafish injected with rbGH. It is known that GH is a major growth-promoting and metabolic regulatory hormone. Interaction of GH with its cell surface receptor (GHR) causes activation of intracellular cascades that leads to alterations on cell division and growth, metabolism of carbohydrates, proteins and lipids. Due some overlapping functions, GH and insulin can functionally interact with each other, regulating cellular metabolism. Our results showed that GH-transgenic males present a persistent hyperglycemia versus wild-type animals, holding blood glucose levels higher even after 3 days without food. Expression data showed that GH-transgenic animals have strong down-regulation of almost all analyzed *gluts* in brain. In the other hand, acute treatment causes an increment in *gluts* expression. These data demonstrate that chronic high levels of GH might leads to an anti-insulin response (fall on *gluts* expression and rise in blood glucose levels). The opposite, an insulin-like effect of GH, leads to a rise on *gluts* expression and glucose uptake by tissues, decreasing the level of this monosaccharide on blood.

Keywords: GH, glucose, GH-transgenic, *gluts*, diabetes

1. Introduction

The somatotrophic axis is a complex series of carefully controlled physiological processes that regulate key aspects of growth and metabolism. The central hormone acting in this pathway is growth hormone (GH), a pluripotent peptidic compound produced by the pituitary gland of vertebrates (Canosa *et al*., 2007). The secretion of growth hormone is regulated by a dual system, in which hypothalamic releasing hormone (GHRH) stimulates and somatostatin (SS) inhibits its secretion into the hypothalamo-pituitary portal system (Zhou *et al*., 2004). The biological actions of GH might occur directly via binding to GH receptor (GHR) in target tissues, or indirectly by stimulating the production of another hormone of the somatotropic axis, the insulin-like growth factor (IGF-I) in liver (Pérez-Sánchez *et al*., 2002; Mauras & Haymond, 2005). Negative feedback control of GH release is mediated by GH itself and by IGF-I (Vijayakumar *et al*., 2010).

Growth hormone receptor belongs to a type I cytokine receptor superfamily, which does not have intrinsic tyrosine kinase activity, depending on the associated tyrosine Janus kinases (JAKs) for signal transduction (Miqu*et et al.*, 2004). GHR-associated JAKs, activate several signaling pathways, including the STATs, ERK1/2, and PI3K pathways (Bartke *et al*., 1999). This system might be negatively regulated by a family of proteins identified as suppressors of cytokine signaling (SOCS) (Larsen & Ropke, 2002).

Growth hormone, as its name implies, promote growth since early stages of development, but its secretion continues in adults, revealing other functions besides somatic growth (Møller & Jørgensen, 2009). These other actions of GH include adjustments on protein, carbohydrates and lipid metabolism (Rousseau

& Dufour, 2007). It might promote protein synthesis (anabolism), or stimulates the utilization of fat stores, causing fatty acids release from adipose tissue into the bloodstream (catabolism) (Møller & Nørrelund, 2003). It is also known that GH might reduce glucose utilization by the tissues, increasing its levels on blood, leading to the “diabetogenic effect” of the hormone (Le Bail & Boeuf, 1997).

Throughout the last years, many aspects of GH effects have been studied using genetic manipulation, such overexpression of GH, and models in groups like fish (Devlin *et al*., 2004a; Reinecke *et al*., 2005; Figueiredo *et al*., 2007a) and mammals (Costa *et al.*, 1998; Kopchick *et al*., 1999) have been established. In fact, fish become great subjects to study some illness, such diabetes, due the property of GH on augment blood glucose concentration. High blood glucose levels are known to lead to tissue damage depending of the time exposure to the condition (Simpson *et al*., 1999), and tissues like brain, that is highly glucose dependent, might be more susceptible.

Glucose uptake in brain, as in other tissues, is mediated by a passive transport process, in which the family of facilitative glucose transporters SLC2A (or GLUT) plays a key role. Thirteen members of this family have been identified, *glut 1-12* and *hmit* (*glut1*3), plus four pseudogenes (Zhao & Keating, 2007). The 13 isoforms, described to mammals, might be divided in two groups, accordingly to its dependence or independence of insulin. The degree of insulin dependence varies accordingly to the tissue and local energy demand, and even in the same tissue, more than one type of *glut* can be found (Augustin, 2010). For example, in liver *gluts* are highly insulin dependent, and in brain glucose transport is less reliant of insulin (Zhao & Keating, 2007). This partial

insulin independence guarantees continuous supply of glucose to brain, especially in conditions of hyperglycemia, a condition that could stimulate insulin release and provoke a dramatic fall in blood glucose, preventing a possible brain damage (Harik *et al*., 1988). Together to the amount of glucose transporters, the other limiting factor in glucose utilization is the rate glucose phosphorylation by hexokinases (*hks*), which holds glucose into the cells (Soengas & Aldegunde, 2002). Alterations in *gluts* expression has been reported under several glycemic conditions in fish (for review, see Polakof *et al*., 2012), and these alterations seems to be also tissue (Hall *et al*., 2006), and hormone-dependent, such as of insulin (Díaz *et al*., 2009).

Considering that growth hormone is related to control of energetic substrates metabolism, possessing the property of elevate blood glucose level; that *gluts* might be under control of humoral or hormonal effectors; and chronic or acute exposure to this condition may generate different responses on cell signaling, altering its machinery of signalization and action; this study aimed verify alterations on *gluts* expression in GH-injected wild-type zebrafish (acute exposure) and transgenic zebrafish model overexpressing GH (chronic exposure).

2. Materials and methods

2.1. Fish and maintenance conditions

The zebrafish (*Danio rerio*) used in these experiments has two different genotypes: wild-type adult males (WT) and adult transgenic (T) males. Wild-type adult males were obtained from local commercial suppliers. Transgenic males were obtained by crossing hemizygous males from the F0104 lineage

(Figueiredo et al., 2007a) with wild type females, generating hemizygous transgenic and wild-type fish. The F0104 lineage harbor a transgene comprised of carp (*Cyprinus carpio*) *β-actin* promoter driving the expression of the growth hormone cDNA from the marine silverside fish *Odonthestes argentinensis*. As a label to identify the transgenic organisms, the green fluorescent protein (GFP) gene was added on construction under the control of the same promoter.

Both wild-type and transgenic fish were reared until around 3 months of age before the experiments. All fish were maintained in a closed circulation water system composed of 25 L tanks at 28°C, 14 h light/10 h dark photoperiod, fed twice a day (at 10am and 4pm) with high-protein (47.5%) commercial food (Tetra Color Tropical Granules, Spectrum Brands, Inc) *ad libitum*. All experiments were carried out in accordance with international norms of use and animal experimentation.

2.2. Blood glucose measurements

Aiming test the glucose clearance capacity of WT and T zebrafish, 44 animals (22 WT and 22 T) were maintained under conditions described above and submitted to two conditions: 11 WT and 11 T were starved for 24h, followed by a normal meal and blood collection 1h after this meal, and 11 WT and 11 T were starved for 24h, fed, and then starved again for 72h followed by blood collection. To blood collection, both groups were anesthetized in tricaine solution (0.1 mg/mL), and caudal vein were punctured. A strip test were putted on the blood drop and read in Accu-Check Performa Nano® (Roche) apparatus.

2.3. Gluts expression analysis

For testing differences among chronic and acute GH effect over glucose transporters (*gluts*) in brain, two experiments were conducted. The chronic experiment compared wild-type and transgenic males; and acute experiment compared wild-type males injected with saline or saline plus recombinant bovine GH (rbGH; Lactotropin®, Elanco).

2.4. Chronic GH exposure (TGH group)

Aiming test how glucose transporters (*gluts*) express in a situation of high lifetime GH levels, wild-type males (control group, n=6) and transgenic males (tested group, n=6) were used in this test. Fish were maintained in the previous described conditions, fasted for 24h and then sacrificed for total brain tissue collection. Each collected sample was stored, separately, in Trizol® Reagent (Invitrogen, Brazil) at -80ºC for later procedures.

2.5. Acute GH exposure (IGH group)

To test how *gluts* respond to an acute GH stimulus, twelve wild-type males were maintained in the previous described conditions, and then fasted for 24h before saline or GH injections. On the day of experiment, animals were anesthetized in 0.05% tricaine methanesulfonate for 2 minutes. Under anesthetic effect, fish received a Hamilton® syringe intraperitoneal injection 2µL of saline solution (control group, n = 6), or 2 µL of saline plus rbGH (120 µg/g body wt) (injected group, n = 6). rbGH concentration were previously tested by Biga & Goetz (2006). After the procedure, animals returned to their respective tanks. Six hours after injection animals were sacrificed, total brain tissue of each

animal was collected and stored, separately, in Trizol® Reagent (Invitrogen, Brazil) at -80ºC for later procedures.

2.6. Total RNA extraction and qRT-PCR

Total RNA were extracted from collected tissues using Trizol® Reagent (Invitrogen, Brazil), according to manufacturer's instructions. The RNA integrity was evaluated based on the intensity of 28S and 18S rRNA bands in 1% agarose gels. Total extracted RNA was treated with DNA-*free* (Invitrogen, Brazil) in order to remove a possible contamination by DNA. Synthesis of complementary DNA (cDNA) was accomplished with 10µL of total RNA solution, and RT High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Brazil), according to the protocol suggested by the manufacturer. The synthesized cDNA was utilized as template for qPCRs using specific primers designed with basis on specific gene sequences available at GenBank (www.ncbi.nlm.nih.gov). Primer efficiencies qPCRs were performed using a 7500 RealTime PCR System equipment (Applied Biosystems, Brazil), with total volume of 10 µL, containing 5 µL of SYBR® Green PCR Master Mix (Applied Biosystems, Brazil), 0.25 µL of each primer, 3.5 µL of Ultra Pure Water (Invitrogen, Brazil), and 1 µL of cDNA (5x, 10x, 20x, 40x, and 80x diluted). All expression qPCR reactions were performed using the same equipment, with total volume of 10 µL, containing 5 µL of SYBR® Green PCR Master Mix (Applied Biosystems, Brazil), 0.25 µL of each primer, 3.5 µL of Ultra Pure Water (Invitrogen, Brazil), and 1 µL of cDNA (5x diluted). Analyzed *gluts*, forward and reverse primer sequences, and primer efficiencies are described in Table 1. Primer sequences were described by Tseng *et al*. (2009). Dissociation curve

analysis was performed in all series to confirm the specificity of amplified segments. In all gene expression analyses, *β-actin* gene was used as reference gene.

2.7. Statistical analysis

Differences on blood glucose levels between WT and T 1h after meal or under 72h of starvation were analyzed with Student's t-test. Differences on gene expression between wild-type or transgenics (chronic exposure), and between wild-type injected with saline or saline plus rbGH (acute exposure), were analyzed with the Relative Expression Software Tool (REST, 2009) (Pfaffl *et al*., 2002). REST uses the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001), followed by a randomization test to assess statistical significance of up- or down-regulation of the target genes, taking into account reaction efficiency and reference gene normalization. To generate standard error estimation (SE), Taylor algorithm is used by the software (Pfaffl, 2004). Values for tested groups (transgenics, or saline plus rbGH-injected wild-type) varied upwards or downwards relative to control groups (wild-type, and saline-injected wild-type, =1 on graphics). In all situations statistical differences were assumed when $p<0.05$.

3. Results

Aiming to verify any possible trouble on glucose clearance in transgenics, blood glucose level was measured under two situations: post-prandial and after 3 days of fasting. Figure 1 shows the result of wild-type and transgenic groups. Figure 1A compared blood glucose level 1h after meal, and presented no

differences between groups. Figure 1B shows a marked difference ($p < 0.01$) between groups after 72h of fasting, pointing out the difficulty of transgenics to reduce blood glucose level, even after 3 days.

Chronic exposure to GH promoted down-regulation ($p<0.01$) of *glut1a, glut1b, glut3, glut6, glut10, glut 13*. No differences were observed to *glut2, glut5 and glut12* (Fig. 2).

Acute exposure to rbGH promoted significantly up-regulation of *glut3* and *glut13a*. No differences were observed to *glut1a, glut1b, glut6, glut10* and *glut12. glut2, glut5 and glut11b* were not detected (Fig. 3).

4. Discussion

Growth hormone, secreted by pituitary gland, is strongly known to exert many effects in whole organism, promoting somatic growth and alterations on metabolism (Vijayakumar *et al*., 2010). Among the metabolic alterations, influence on lipid and protein metabolism were described, but its effect on carbohydrates metabolism, notably glucose, has been most extensively studied, mainly due its capacity to promote a diabetogenic situation (Holt *et al.*, 2003).

It is known that glucose is an essential metabolic substrate in the majority of vertebrates, and some tissues use glucose as virtually their only energetic substrate. Amongst the most dependent tissue is brain, which works almost exclusively with glucose from the circulation (Kauppinen *et al*., 1989; Duelli & Kuschinsky, 2001). Although total brain tissue represents approximately 0.1-1% of the body weight, it is responsible for 2.7–3.4% of total body energy consumption (Ginneken *et al*., 1996).

The present work begun with the hypothesis that GH could affect, in some way, blood glucose levels, exerting the called “diabetogenic” effect. Figure 1A shows that, 1h after meal, both wild-type and transgenic presented the same blood glucose level, whereas figure 1B exhibit the high glucose level of transgenic group after 72h of starvation. This result is in agreement with many others, showing that a constant and high level of GH could affect the capacity of glucose clearance, especially in fasting conditions (Holt *et al*., 2003; Jørgensen *et al*., 2004). It is known that growth hormone exerts both insulin-like and anti-insulin effects *in vitro* (Fowelin *et al*., 1991), depending on the exposure time. Chronic effects of GH are characterized by a pattern of insulin resistance in a number of parameters, including hepatic and muscle glycolysis and glycogenolysis, hepatic gluconeogenesis, and lipid metabolism, leading to and increase on glucose production/ availability and reduction in glucose consumption (Valera *et al*., 1993).

Besides glycemic condition, the present work evaluated the expression of ten glucose transporters (*gluts*) in brain of zebrafish subjected to conditions of chronic and acute exposure to a high amount of growth hormone. The animal model used in chronic exposure is originated of F0104 lineage, and presents a constitutive overexpression of GH. Figure 2 shows a strong down-regulation of almost all analyzed *gluts* in chronic exposure. Figure 3 shows the result of *gluts* expression in brain under acute exposure (6h) to rbGH, achieved by a singular bovine growth hormone injection. It was shown an up-regulation to *glut3* and *glut13a*, no differences were observed to *glut1a, glut1b, glut6, glut10* and *glut12*, and no signal detection were observed for *glut2, glut5 and glut11b*.

Since GH discovery many functions have been described to it. Among the metabolic activities of growth hormone, two conflicting actions have been described: its effect in a chronic, late anti-insulin (diabetogenic) way; and acute, early insulin-like activities. The delayed anti-insulin activities of GH include hyperglycemia (Møller & Jørgensen, 2009), hyperinsulinemia (Costa *et al.*, 1998), increased lipolysis (Goodman & Grichting, 1983), decrease in glucose transporters, decreased glucose metabolism (Pelligrino *et al*., 1990), and insulin resistance (Fowelin *et al*., 1991). These anti-insulin activities require relatively long periods of GH treatment in cultured cells or *in vivo*, and are thought to represent a major physiological effect of GH. Acute insulin-like activities include hypoglycemia (Milman & Russell, 1950; Swislocki, 1967), increased glucose through *gluts*, amino acid transport and metabolism (Goodman, 1978), increased glycogenesis (O'Connor *et al*., 1993), and increased lipogenesis (Caruso & Sheridan, 2011). These insulin-like activities are seen primarily *in vitro*, or under special circumstances, such as hypophysectomy. But why this marked and opposite differences between the chronic and acute exposure? What actually happens: an anti-insulin or insulin-like effect? The response might be behind the intracellular mechanisms of GH signaling.

Growth hormone signals a response in cells through the growth hormone receptor (GHR), a member of the cytokine receptor gene family. Activated receptor begun a downstream response, activating signal transducers and activators of transcription (STATs), MEK/ERK, and phosphatidylinositol 3 kinase (PI3K)/Akt pathways (Carter-Su *et al.*, 1996). Each pathway activated by GH is known to exert a different action. STATs pathway classically activates target gene transcription and promotes body growth; MEK-ERK route besides growth,

promotes cell proliferation; and PI3K-Akt generate mainly metabolic effects (White, 1998).

The insulin-like effect of GH might be ensured due the fact that both GH and insulin share some of these intracellular signalization cascades. Via activation of PI3K pathway, GH may stimulate tyrosine phosphorylation of IRSs, exerting effect on downstream signalization to alterations on cellular metabolism (White, 1996). On the other side, insulin signalization cascades begins when insulin binds to cell-surface insulin receptors (IR), which possess intrinsic tyrosine kinase activity. This binding recruits intracellular SH2 domain-containing molecules, such as IRSs, to the activated IR and subsequent tyrosine phosphorylation of IRSs. PI3K pathway plays a crucial role in insulin actions, including stimulation of glucose transport, activation of glycogen synthase, and inhibition of hepatic gluconeogenesis (Dominici *et al.*, 2002; Taniguchi *et al.*, 2006).

Beneath a normal condition of GH, an acute response of GH presence are insulin-like, occurring stimulation of glucose and amino acid transport, lipogenesis, and protein synthesis (Dominici *et al.*, 2002; Jørgensen *et al.*, 2004). On the other hand, chronic GH treatment in normal or elevated doses is also reported to cause diabetes mellitus (Lippe *et al*., 1981). The fact that GH possesses the ability to interfere with insulin signaling may contribute to elucidate why insulin resistance and elevated blood glucose levels occur under chronic GH exposure.

Johansen *et al*. (2005) have shown that prolonged GH treatment in rat liver and muscle increases the association of JAK2 with IRS-1, resulting in reduced amount of IRS-1 available as a substrate for phosphorylation by the

activated IR. Suppressor of cytokine signaling (SOCS) proteins, inducible by GH and other cytokines, has also potential to mediate GH-induced insulin resistance under chronic exposure (Ueki *et al.*, 2004). Experiments demonstrated that SOCS-1 and SOCS-6 interact with the IR and inhibit insulin-induced phosphorylation of IRS-1 *in vitro*, and activation of ERK1/2 and Akt pathways *in vivo* (Mooney *et al*., 2001). Diabetic mice overexpressing SOCS-1 and SOCS-3 in liver presented insulin resistance, while inhibition of the same molecules in diabetic and obese mice raises insulin sensitivity (Ueki *et al*., 2004). Rui *et al*. (2002) demonstrated that SOCS-1 and SOCS-3 have the ability to bind to recombinant and endogenous IRS-1 and IRS-2, leading to their ubiquitination and subsequent degradation. Data from experiments performed with transgenic zebrafish overexpressing GH have shown that, in these animals, SOCS 1 and SOCS3 are up-regulated and probably are down-regulating GH signaling via this negative modulator (Studzinski *et al*., 2009). Other data shows that forced expression of SOCS-1 reduces tyrosine phosphorylation of IRS-1, and the opposite, SOCS-1 deficiency, results in sustained tyrosine phosphorylation of IRS-1 in response to insulin. Moreover, SOCS-1 deficient mice exhibit a significantly low level of blood glucose. Therefore, SOCS proteins might inhibit GH signaling cascades both by binding directly to IRS-1 or by suppressing JAK (Kawazoe *et al*., 2001), interfering with proper insulin signaling at both receptor and post-receptor levels (IRS-1 and JAK), and ultimately provoke a strong down-regulation on glucose transporters.

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Table

Table 1 Gene-specific primers used for qRT-PCR analysis, primer sequences, and primer efficiencies.

Genes	Primer sequences (5' – 3')	Primer efficiencies (%)
***β-actin* F**	GCTGTTTTCCCCTCCATTGTT	100
***β-actin* R**	TCCCATGCCAACCATCACT	
***glut1a* F**	TGACCGGCCCATACGTTTTC	102
***glut1a* R**	ATCATCTCGGTTATATTTATCTGCC	
***glut1b* F**	CCATTTCTCCTGGGCTTTACCTTTA	87
***glut1b* R**	CAGATTTGGCTTTGCTTTCCTCGTT	
***glut2* F**	TTAACAGGCACGCTCGCTCT	107
***glut2* R**	TTCATGCTCTGTGCCATTTCC	
***glut3* F**	TCGTCAATGTCTTGGCTCTG	85
***glut3* R**	CAACATACATTGGCGTGAGG	
***glut5* F**	TCTCTGGTTGCTGGATTTGGT	84
***glut*5 R**	CAAGAGGGTGAGGAGATTGTCC	
***glut6* F**	TTTGGCCTGATTTTGCCGTG	94
***glut6* R**	GTGGTAACGTGGAGAGGTCG	
***glut10* F**	CTGATAGATTAACAGAGGAAACGG	90
***glut10* R**	CACATTTGGTTGACCTGTGAA	
***glut11b* F**	AAGGATGAGTACTGGCCGATCCTC	90
***glut11b* R**	AATGCCGAGAGCGCTGACCCTTTC	
***glut12* F**	GGGACAATCCTGGACCACTA	111
***glut12* R**	ACATCCCAACCAGCATTCTC	
***glut13a* F**	CGTCGCACGCAATAAAGAGG	108
***glut13a* R**	ATCGACGCGATTCCTAACCC	

Figures

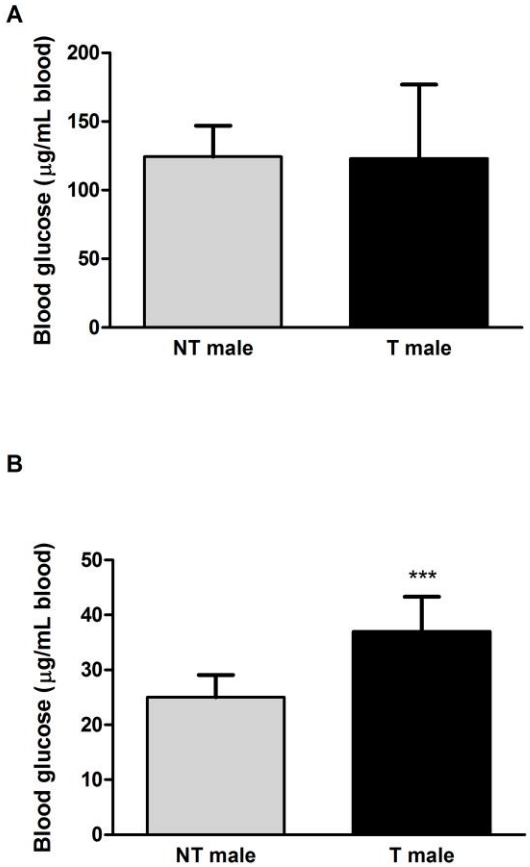

Figure 1 Blood glucose level of wild-type (WT) and transgenic (T) zebrafish (*Danio rerio*) males 1h after meal (A) and after 72h starvation (B). Asterisks (***) indicate significant difference ($p < 0.01$).

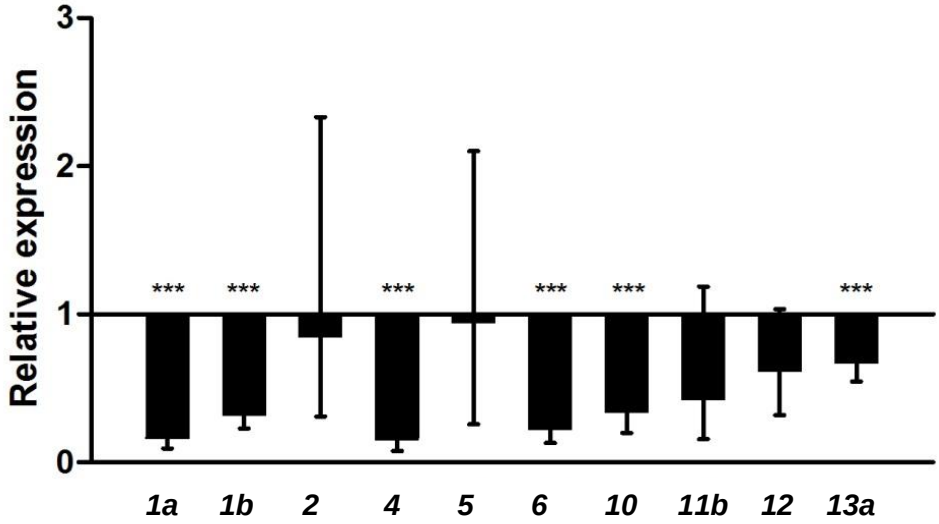

Figure 2 Relative gene expression of glucose transporters (*gluts*) in brain of males comparing wild-type and transgenic zebrafish (*Danio rerio*). Gene expression was normalized by the expression of *β-actin* gene. Wild-type were considered controls, where gene expression = 1. Experimental n = 6. Asterisks (***) indicate significant differences ($p < 0.01$).

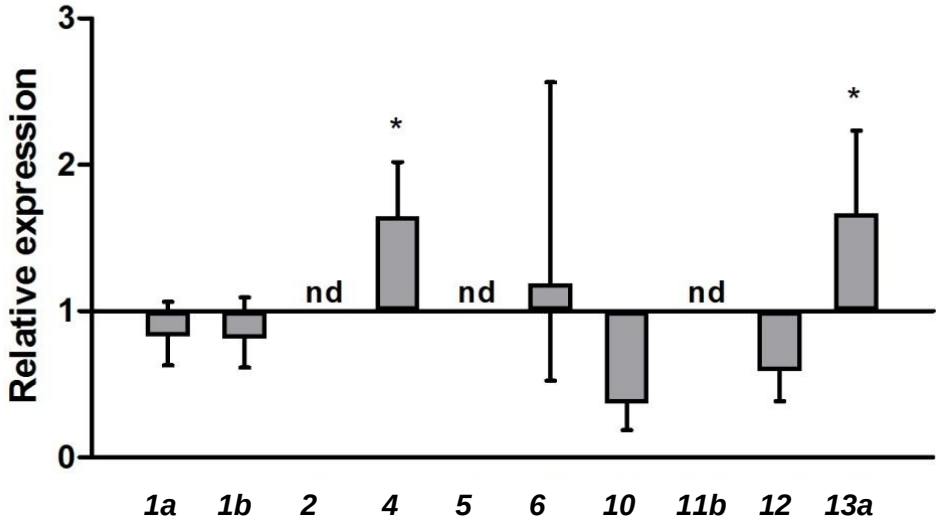

Figure 3 Relative gene expression of glucose transporters (*gluts*) in brain of males comparing saline-injected and bGH-injected zebrafish (*Danio rerio*). Gene expression was normalized by the expression of *β-actin* gene. Saline-injected fish were considered controls, where gene expression = 1. Experimental n = 6. Asterisk (*) indicate significant differences ($p < 0.05$).

ARTIGO 2

A ser submetido para a revista

Transgenic Research

Energy delivery to the brain: effects of genotype and fasting in transgenic zebrafish model overexpressing growth hormone

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Abstract

It is known that the energy requirements of the brain are very high, and tight regulatory mechanisms must operate to ensure adequate delivery of energy substrates to maintain normal neuronal activity. More than any other organ, the brain is entirely dependent on a continuous supply of glucose from the circulation, since glucose is the main substrate for energy metabolism. Besides glucose, but in a lesser extent, brain may also use ketone bodies as additional energetic substrate when plasmatic glucose falls. The present work aimed evaluates the effect of GH-transgenesis upon some molecules involved in energetic metabolism of brain. For this, expression analyses of glucose transporters (*gluts*), hexokinases (*hks*) and monocarboxylate transporters (*mcts*) were performed, seeking for alterations under fed or fasting condition due to transgenesis, and show a possible difference among these key factors between males and females. As an overall, our data demonstrate that chronic GH exposure due to transgenesis impairs the energetic uptake of brain, overlapping even the effect of fasting, through the down-regulation of *gluts* and *mcts*, in both males and females. Moreover, the differential analysis among sexes shows that males seem to be more affected by transgenesis, probably due the strong alteration of the normal pattern of GH secretion in males.

Keywords: glucose transporter, hexokinase, monocarboxylate transporter, GH, *Danio rerio*

1. Introduction

The vertebrate brain is metabolically one of the most active of all organs and notably sensitive to perturbations of energy metabolism. Although total brain tissue represents approximately 0.1-1% of the body weight of non-primate vertebrates, it is responsible for 2.7–3.4% of total body energy consumption (Ginneken *et a*l., 1996). In this tissue, glucose from the circulation is the major energy source (Kauppinen *et al*., 1989; Duelli & Kuschinsky, 2001). However, in cases of higher energy requirement or fasting, other energy sources might be used instead of glucose, such as ketone bodies (β-hydroxybutyrate and acetoacetate), and perhaps lipids and proteins (Gibbs & deRoos, 1991).

Glucose is taken up into cells by facilitated diffusion, which is mediated by members of the GLUT or SLC2A family (Glucose Transporters or Solute Carrier Family 2A) (Zhao & Keating, 2007). Therefore, the main limiting factor in the cerebral utilization of glucose may be the rate of sugar transport, amongst to the rate of glucose phosphorylation by hexokinases (HKs), which 'holds' glucose into the cells (Soengas & Aldegunde, 2002). As well as glucose, ketone bodies also have a specific transport system, the monocarboxylate transporters (MCTs), which are responsible for the transport of monocarboxylates across cellular and sub-cellular membranes (Ngan & Wang, 2009).

It is well established that glucose is an essential metabolic substrate in the majority of mammals (Duelli & Kuschinsky, 2001), but in fish its importance appears limited (Enes *et al*., 2009). In this group, glucose metabolism remains under debate as many fish species are traditionally considered glucose intolerant (Polakof *et al*., 2012). This question aroused since observations have shown a persistent hyperglycemia after a glucose load (Moon, 2001), but until

now, the physiological basis for such apparent glucose intolerance has not been fully understood. Many approaches try to explain why fish present this profile of glucose utilization, but as a general feature of the wide group of fish, the more carnivorous is the species, the long time needed to clear a glucose load (Wilson, 1994; Moon, 2001). An observation of glucose uptake and utilization in peripheral tissues may also contribute to poor glucose clearance: the largest tissue of fish, the white muscle, is responsible for only 10–15% of the glucose utilization (West *et al*., 1994), and smaller ones, such as brain, kidney and gills are so much dependent, using 60–90% of the glucose uptake (Legate *et al*., 2001).

Alterations on glucose availability and utilization due to humoral factors might occur, and unbalanced food intake or hormones related to energetic metabolism are able to affect this status. Under this scenario, the organism must respond to achieve and maintain the homeostasis. Generally, in a state of hyperglycemia insulin is released and stimulate glucose uptake by tissues, and one of the hormones acting in a situation of hypoglycemia is glucagon, working to raise blood glucose levels (Polakof *et al.*, 2011). It has been already demonstrated that growth hormone has the capacity of elevate blood glucose both directly or indirectly. Directly, GH release decrease glucose uptake in muscle; and indirectly stimulating mobilization of free fatty acids from adipose tissue, which themselves inhibit glucose utilization (Mauras & Haymond, 2005; Møller & Jørgensen, 2009). Furthermore, there are evidences that the reduction on glucose uptake provoked by GH may be reached by down-regulation of *gluts* (Pardridge *et al*., 1990; Duelli & Kuschinsky, 2001).

Taking into account that brain is one of the most energetically demanding organ in vertebrates utilizing specially glucose as energy source, and the narrow relationship among glucose levels and GH, this work were conducted with wild-type and GH-transgenic zebrafish (*Danio rerio*). Our lineage (F0104) is, at our knowledge, a unique strain of GH-transgenic zebrafish, and has proved to be useful for GH signaling studies. We have published several papers focusing on the collateral effects of GH overexpression over somatotropic-related genes (Figueiredo *et al*., 2007b), as well as control mechanisms of intracellular signaling in this axis (Studzinski *et al*., 2009), metabolism and reactive oxygen species (Rosa *et al.*, 2008), and others. Summarily, our objectives were to detect the effect of GH-transgenesis upon *gluts*, *hks* and *mcts* expression, seeking for alterations due to transgenesis in the path of response under fed or fasting condition, and show a possible difference among these key factors between males and females in brain.

2. Materials and methods

2.1. Fish and maintenance conditions

Animal experiments and care procedures were reviewed and approved by the Institutional Animal Care and Use Committee. The zebrafish used in these experiments has two different genotypes: wild-type (WT) and transgenic (T) sibling males and females. Adult WT and T animals were obtained by crossing hemizygous males from the F0104 lineage with wild type females, generating wild-type and hemizygous transgenic sibling fish. The transgenic lineage harbor a transgene comprised of carp (*Cyprinus carpio*) *β-actin* promoter driving the expression of the growth hormone cDNA from the marine

silverside fish (*Odonthestes argentinensis*). As a label to identify the transgenic organisms, the green fluorescent protein (GFP) gene was added on construction under the control of the same promoter (Figueiredo *et al*., 2007a).

All experimental animals were reared until 4 months of age in a closed circulation water system composed of 25 L tanks at 28°C, 14 h light/10 h dark photoperiod, fed twice a day with high-protein (47.5%) commercial food (Tetra Color Tropical Granules, Spectrum Brands, Inc) *ad libitum*. All experiments were carried out in accordance with international norms of use and animal experimentation.

2.2. Gluts, mcts and hks expression analysis

Alterations in expression of glucose transporters (*gluts*), monocarboxylate transporters (*mcts*) and hexokinases (*hks*) in brain of males or females, under fed or fasting condition, due transgenesis procedure were targeted in this experiment.

For the analysis, 12 WT males, 12 T males, 12 WT females and 12 T females were kept under the maintenance conditions described above. In the experimental day, one hour after usual morning feeding time (fed animals), six animals of each type were randomly sampled, euthanized, and total brain tissue was collected by dissection. The remaining six animals of each type were kept without food for 72 hours (fasting) and after this period were sacrificed, and total brain tissue was collected by dissection. All collected tissues were stored, separately, in Trizol® Reagent (Invitrogen, Brazil) at -80ºC for later procedures.

2.3. Total RNA extraction and qRT-PCR

Total RNA were extracted from collected tissues using Trizol® Reagent (Invitrogen, Brazil), according to manufacturer's instructions. The RNA integrity was evaluated based on the intensity of 28S and 18S rRNA bands in 1% agarose gels. Total extracted RNA was treated with DNA-*free* (Invitrogen, Brazil) in order to remove a possible contamination by DNA from RNA samples. Synthesis of complementary DNA (cDNA) was accomplished with 10µL of total RNA solution, and RT High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Brazil), according to the protocol suggested by the manufacturer. The synthesized cDNA was utilized as template for qPCRs using specific primers designed with basis on specific gene sequences available at GenBank (www.ncbi.nlm.nih.gov). Primer efficiencies qPCRs were performed using a 7500 RealTime PCR System equipment (Applied Biosystems, Brazil), with total volume of 10 µL, containing 5 µL of SYBR® Green PCR Master Mix (Applied Biosystems, Brazil), 0.25 µL of each primer, 3.5 µL of Ultra Pure Water (Invitrogen, Brazil), and 1 µL of cDNA (5x, 10x, 20x, 40x, and 80x diluted). All expression qPCR reactions were performed using the same equipment, with reactions of total volume of 10 µL, containing 5 µL of SYBR® Green PCR Master Mix (Applied Biosystems, Brazil), 0.25 µL of each primer, 3.5 µL of Ultra Pure Water (Invitrogen, Brazil), and 1 µL of cDNA (5x diluted). Analyzed genes, forward and reverse primer sequences, and primer efficiencies are described in Table 1. Primer sequences were described by Tseng *et al*. (2009) (*gluts*), Ngan & Wang (2009) (*mcts*) and González-Alvarez *et al*. (2009) (*hks*). Dissociation curve analysis was performed in all series to confirm the specificity of amplified segments. In all gene expression analyses, *β-actin* gene was used as reference gene.

2.4. Statistical analysis

Differences on gene expression between WT and T, or fed and fasting groups were analyzed with the Relative Expression Software Tool (REST, 2009) (Pfaffl *et al*., 2002). REST uses the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001), followed by a randomization test to assess statistical significance of up- or down-regulation of the target genes, taking into account reaction efficiency and reference gene normalization. To generate standard error estimation (SE), Taylor algorithm is used by the software (Pfaffl, 2004). Values for tested groups (transgenics, or saline plus rbGH-injected wild-type) varied upwards or downwards relative to control groups (wild-type, and saline-injected wild-type, =1 on graphics). In all situations statistical differences were assumed when $p<0.05$.

3. Results

Figure 1 shows wild-type fasting males up-regulating *gluts* 3, 6, 10 and 12 ($p < 0.01$) in relation to fed ones. Wild-type fasting females presented down-regulation of *gluts* 2 and 6 ($p < 0.01$) and up-regulation of *glut3* ($p < 0.01$) in relation to fed animals. Hexokinases expression (*hk1* and *hk2*) (Figure 2) presented no differences among fed and fasted animals in both sexes. Regarding *mcts* expression (Figure 3), no differences were observed in *mct1* expression in males or females. Wild-type fasting males presented up-regulation ($p < 0.01$) of *mct3* in relation to fed ones.

Figure 4A shows the result of *gluts* expression in fed males (M) and females (F). Transgenic males presented down-regulation of *gluts 1a, 1b, 3, 6,*

10, 11b and *13a* ($p < 0.01$) and up-regulation only for *glut5* ($p < 0.01$). Fed females presented down-regulation of *gluts 12* and *13a*. On fasting period (Figure 4B), males presented down-regulation of *gluts 1a, 1b, 3, 6, 10, 11b, 13a* ($p < 0.01$), and *12* ($p < 0.05$). Also in this period, females presented down-regulation of *gluts 1a, 3, 10, 12*, and *13a* ($p < 0.01$). About hexokinases expression (Figure 5), no differences were observed in transgenic males to *hk1* and *hk2* in fed or fasting periods. Transgenic females, in relation to wild-type ones, presented up-regulation of *hk1* in fed ($p < 0.05$) and fasting ($p < 0.01$) condition, and down-regulation of *hk2* on fasting ($p < 0.05$). Expression analysis of *mcts* between WT and T animals are showed in figure 6. Fed males presented down-regulation of *mct1* and *mct3* ($p < 0.05$) and females only of *mct3* ($p < 0.01$). In fasting period, *mct1* and *mct3* of males are down-regulated ($p < 0.01$), and only *mct3* of female is down-regulated ($p < 0.01$).

4. Discussion

Amongst all metabolic active tissues of vertebrates, brain is one of the most demanding organs (Soengas & Aldegunde, 2002). This organ has specific energy requirements and in this way, glucose is the major fuel in a normal feeding condition (Peters, 2011). Brain lacks fuel stores and hence requires a continuous supply of glucose from circulation to maintain its normal function (Peters *et al*., 2004). Glucose enters into brain cells through glucose transporters (GLUTs) (Maher *et al*., 1994). These transporters has a low value of K_m for glucose, which means that they are saturated under most conditions, and thus usually provide a constant supply of glucose to brain (Gould & Holman, 1993). After glucose enters the cell, it is rapidly phosphorylated to form

glucose-6-phosphate by the enzyme hexokinase (HK), then cannot be exported back to blood (Soengas & Aldegunde, 2002). Many different glucose transporters isoforms have already been described, and their distribution seems to be tissue-specific (Gould & Holman, 1993). Under extreme conditions, such as starvation and diabetes, the brain is also able to use ketone bodies (products of lipid oxidation, such as α-ketoglutatate and acetoacetate) as an alternative source of energy, but it still requires glucose, which in this situation is typically provided by liver gluconeogenesis (Peters *et al*., 2004).

The first purpose of our work was evaluate how *gluts*, *hks* and *mcts* express in wild-type zebrafish under fasting condition, compared to fed animals. We observed the *gluts* tend to up-regulate its expression in males when available glucose falls due the absence of a meal (Figure 1). Many other authors have demonstrated this inverse relationship: in a condition of low blood glucose level, the tendency of *gluts* is overexpress to maintain constant supply of glucose to brain (Koranyi *et al*., 1991; Boado & Pardridge, 1993; Nagamatsu *et al*., 1994; Kumagai *et al.*, 1995). In a condition of low systemic blood glucose concentration, as occurs in hypoglycemia, glucose transport becomes the limiting step of cerebral glucose utilization (Qutub & Hunt, 2005). Instead this positive correlation between our data and published ones, it is know that the rise on *gluts* under fasting is time-dependent: Polakof *et al.* (2011) reported that, in mammalian brain, food deprivation enhances glucose uptake only the first days of fasting, whereas a depression in transport rates occurs over longer periods. Regarding to *hks* expression in fasting animals (Figure 2), compared to feed ones, no differences were observed to both isozymes (*hk1* and *hk2*). This result suggests that hexokinases do not alter their expression under variations

in food intake such as hypoglycemia. Studies have demonstrated that expression of hexokinase might inducible by feeding and available dietary carbohydrates in many fish species, including zebrafish (Robison *et al.*, 2008). The absence of changes in *hk* in fasted fish are consistent with the lack of changes reported in food deprived gilthead sea bream (*Sparus aurata*) (Sangiao-Alvarellos *et al*., 2005), although others describe a fall of *hk* in whole brain of fasted rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*) (Soengas *et al*., 1996, 1998). All differences among these species may relate to specific differences in brain metabolic patterns, or that other enzyme such glucokinase (GK), another isozyme of HK, are more involved in fish brain metabolism instead of HK (Soengas *et al*., 2006). The obtained results of monocarboxylate transporters (*mcts*) expression comparing fed and fasting fish (Figure 3) have shown that only males up-regulate *mct3*. This result is in accordance to others that report up-regulation of *mcts* in situations of starvation, in which glucose availability in the blood is scarce, and an alternative energy source must be awake (Soengas *et al*., 1998; Leino *et al*., 2001) . The observed differences between sexes in wild-type animals demonstrated that males respond more greedily to the depletion on blood glucose available than females and have a greater energetic need, since besides up-regulation of four *gluts* it also up-regulated the alternative energy transporter *mct3*. On the other hand, the almost absence of response of females facing the fasting situation might indicate that they need less energy or that it is using another energy source, being useless up-regulate *gluts* or *mcts*.

In our experiments, as an overall, we observed that transgenesis provoke a down-regulation on analyzed *gluts* mRNA in both fed and fasting males and

females, in comparison to wild-type ones, despite the fall was more pronounced in males (Figure 4). Transgenic fed males had altered 8 of 10 analyzed *gluts*, and 7 of these changes were down-regulations, and in contrast transgenic fed females has altered 2 *gluts*, both down-regulated (Figure 4A). On fasting condition (Figure 4B), males are still down-regulating *gluts*, but now 8 of 10, and females began to down-regulate 5 *gluts*. This overview makes clear that the action of growth hormone is overlapping the feeding status of the animal, and that even in fasting period, which occur a virtual situation of glucose depletion and *gluts* should up-regulate to catch more glucose from circulation, their expression is down-regulated. Another point to take into account in these analyses: unpublished data from our research group have shown that post-prandial glycemia is similar between wild-type and transgenic fish, but after 3 days of starvation transgenic animals presented a higher level of blood glucose than wild-type ones (about 1.5 fold), revealing the diabetogenic effect of GH excess. Thus, transgenic animals after feeding probably present an overload of glucose coming from food and due to the effect of GH excess, and fasting animals do not have the glucose load from food, but its glycemic condition is presumably moderately above a wild-type animal subjected to fasting.

The observed effect of growth hormone over the great fall in *gluts* expression might be due the anti-insulin properties of GH, affecting especially insulin-responsive glucose transporters. Under certain circumstances, GH might alter the recruitment of *gluts* to the plasma membrane (Carter-Su *et al*., 1996), which suggests that GH and insulin may share some signaling molecules. Growth hormone binding to its receptor (GHR) results in the phosphorylation and activation of Janus kinase 2 (JAK2) and GHR (Argetsinger *et al*., 1993;

Carter-Su *et al*., 1996). This active complex leads to activation of three different signaling pathways: the signal transducers and activators of transcription (STATs), the MEK/ERK kinases, and the phosphatidylinositol 3 kinase (PI3K)/Akt pathways (Carter-Su *et al*., 1996; Lanning & Carter-Su, 2006). The last cascade (PI3K/Akt pathway) share some compounds of insulin signaling, such as insulin receptor substrates (IRSs), and this might be one of the points by which GH and insulin crosstalk their signalization cascades. It has been demonstrated that GH treatment in rats leads to reduction of IRS-1 protein levels due association of IRS to JAK2, diminishing the pool of IRS-1 available as a substrate for phosphorylation by the activated insulin receptor (IR) (Johansen *et al*., 2005). Other way by which insulin signaling might be blocked is through ubiquitination followed by degradation of IRSs by the suppressor of cytokine signaling SOCS (Rui *et al*., 2002), an inhibitor of GH signaling that is present when GH levels are raised (Studzinski *et al.*, 2009). In this way, the GH excess may be blocking insulin-like signalization, leading to elevation on blood glucose and affecting negatively insulin-sensitive *gluts*, blocking the entry of glucose into the cell.

Although almost all *gluts* of males and females are down-regulated in fed and fasting condition, males seems to be more affected. This fact might be due the alteration in the normal path of production and secretion of GH between sexes: in a normal situation, males presented a profile of GH secretion in peaks, followed by GH-free intervals, and females present a middle-constant presence of GH on plasma (Jaffe *et al*., 1998). Due to the transgenesis this path of secretion is altered, and the GH-free intervals of males are probably absent, leading to strong consequences in comparison to females.

Regarding hexokinases (*hks*), it has been demonstrated that *hk1* is the main isoform of this enzyme that responds to alterations on energetic condition in brain (Wilson, 2003). In our results (Figure 5), we observed that females seem to be more affected by transgenesis than males, since it up-regulate *hk1* in fed and fasting situations, and have a slightly fall in *hk2* on fasting. Males, in contrast, do not alter any analyzed *hks* isozymes. A study performed by Sebastian & Kenkare (1999) suggest that one of the intermediary compound of GH action, the insulin-like growth factor I (IGF-I), has the ability to stimulate glucose uptake and utilization by brain. In that study, administration of recombinant IGF promoted up-regulation of *hk1*, suggesting that IGF might be one of the regulatory factors of hexokinase gene expression in brain and possibly of brain glucose metabolism.

Following the observed reduction on *gluts* expression in brain, and probably on glucose uptake, it would be expected that other sources of energy were activated in transgenic animals, such as the second preferential fuel of brain, the ketone bodies transported into the brain by the *mcts* (Peters *et al.*, 2004). In the present study we observed, instead of a rise, a fall on *mct1* and *mct3* in both fed and fasting states of males (Figure 6), but as overall, fasting males presented lower level of *mcts* than fed group, and only *mct3* is down-regulated in fed and fasting transgenic females. Data from mammals has demonstrated that starving animals cannot maintain sufficient circulating glucose to the efficient functioning of the central nervous system, and then must use ketone bodies produced by liver to compensate the lack of glucose (Soengas *et al*., 1996). Then, in situations of hyperglycemia or high ketotic diet, *mcts* might be up-regulated in brain (Mason *et al*., 2006; Pierre *et al*., 2007;

Canis *et al*., 2009). In fish, Zammit & Newsholme (1979) suggested a very low importance of ketone bodies on energetic metabolism of teleost brain during fasting, in contrast to elasmobranchs in which these fuels have great importance (Speers-Roesch & Treberg, 2010). As an overall, the expression pattern of *mcts* was not different between the two analyzed feeding status, suggesting that the effect over the decrease of *mcts* between wild-type and transgenic fish is due to the presence of GH, rather than the glycemic condition of the animal.

Although additional experiments will be required to clearly these issues, our data demonstrate that chronic GH exposure might impairs the energetic uptake of brain, overlapping even the effect of fasting, through the down-regulation of *gluts* and *mcts*, in both males and females.

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Table

Table 1: Gene-specific primers used for qRT-PCR analysis, primer sequences, and primer efficiencies.

Genes	Primer sequences (5' – 3')	Primer efficiencies (%)
***β-actin* F**	GCTGTTTTCCCCTCCATTGTT	100
***β-actin* R**	TCCCATGCCAACCATCACT	
***glut1a* F**	TGACCGGCCCATACGTTTTC	102
***glut1a* R**	ATCATCTCGGTTATATTTATCTGCC	
***glut1b* F**	CCATTTCTCCTGGGCTTTACCTTTA	87
***glut1b* R**	CAGATTTGGCTTTGCTTTCCTCGTT	
***glut2* F**	TTAACAGGCACGCTCGCTCT	107
***glut2* R**	TTCATGCTCTGTGCCATTTCC	
***glut3* F**	TCGTCAATGTCTTGGCTCTG	85
***glut3* R**	CAACATACATTGGCGTGAGG	
***glut5* F**	TCTCTGGTTGCTGGATTTGGT	84
***glut5* R**	CAAGAGGGTGAGGAGATTGTCC	
***glut6* F**	TTTGGCCTGATTTTGCCGTG	94
***glut6* R**	GTGGTAACGTGGAGAGGTCG	
***glut10* F**	CTGATAGATTAACAGAGGAAACGG	90
***glut10* R**	CACATTTGGTTGACCTGTGAA	
***glut11b* F**	AAGGATGAGTACTGGCCGATCCTC	90
***glut11b* R**	AATGCCGAGAGCGCTGACCCTTTC	
***glut12* F**	GGGACAATCCTGGACCACTA	111
***glut12* R**	ACATCCCAACCAGCATTCTC	
***glut13a* F**	CGTCGCACGCAATAAAGAGG	108
***glut13a* R**	ATCGACGCGATTCCTAACCC	
***hk1* F**	GGTGAATTGGACGAAGGGCTTTAA	86
***hk1* R**	CCTCTTGATCCCCTCTCTCAGAAG	
***hk2* F**	AAAACTCGCGGGATCTTCGA	89
***hk2* R**	CTCAATGCCAAACGATCACTTTCA	
***mct1* F**	AGCCAGGTGTCATGGATCTCC	100
***mct1* R**	CAACTAATCCCGTGCCTGACA	
***mct3* F**	GACACGGCTTGGATCTCCTCTA	91
***mct3* R**	TGCCAAGACCATACCCAATGA	

Figures

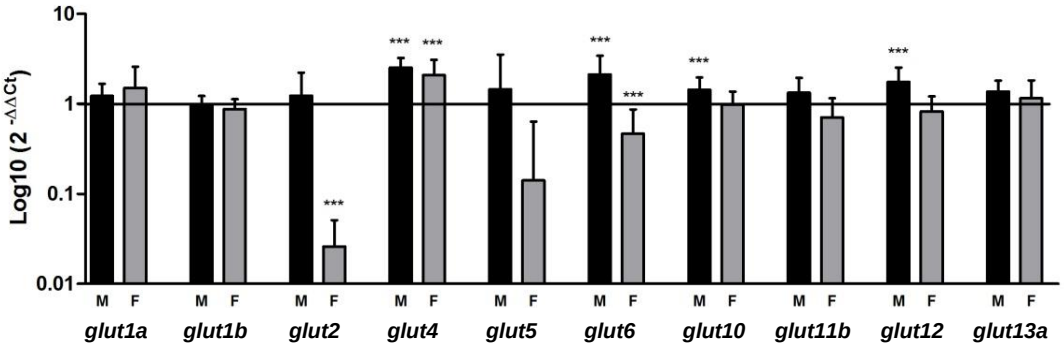

Fig 1 Relative gene expression of brain *gluts*, comparing fed and fasting wild-type zebrafish (*Danio rerio*) males (M) and females (F). Gene expression was normalized by the expression of *β-actin* gene. Fed males and females were used as control groups, which gene expression = 1. Data are expressed as Log10 ($2^{-\Delta\Delta Ct}$) ± SE (n = 6). Asterisk (***) indicate significant differences ($p < 0.01$).

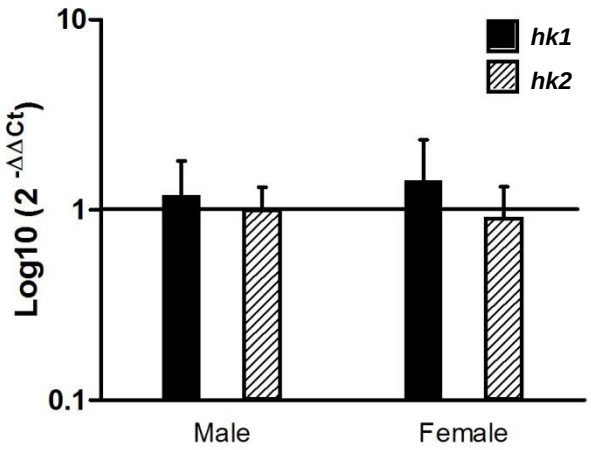

Fig 2 Relative gene expression of brain *hk1* and *hk2*, comparing fed and fasting wild-type males and females of zebrafish (*Danio rerio*). Gene expression was normalized by the expression of *β-actin* gene. Fed animals were considered control, which gene expression = 1. Data are expressed as Log10 (2-ΔΔCt) ± SE (n = 6).

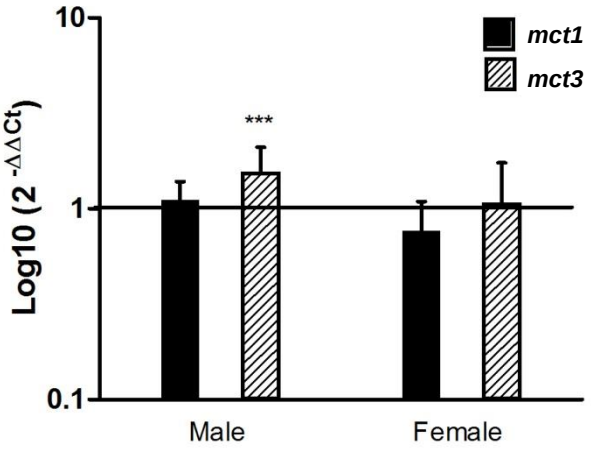

Fig 3 Relative gene expression of brain *mct1* and *mct3*, comparing fed and fasting wild-type males and females of zebrafish (*Danio rerio*). Gene expression was normalized by the expression of *β-actin* gene. Fed animals period were considered control, which gene expression = 1. Data are expressed as Log10 (2-ΔΔCt) ± SE (n = 6). Asterisk (***) indicate significant differences ($p < 0.01$).

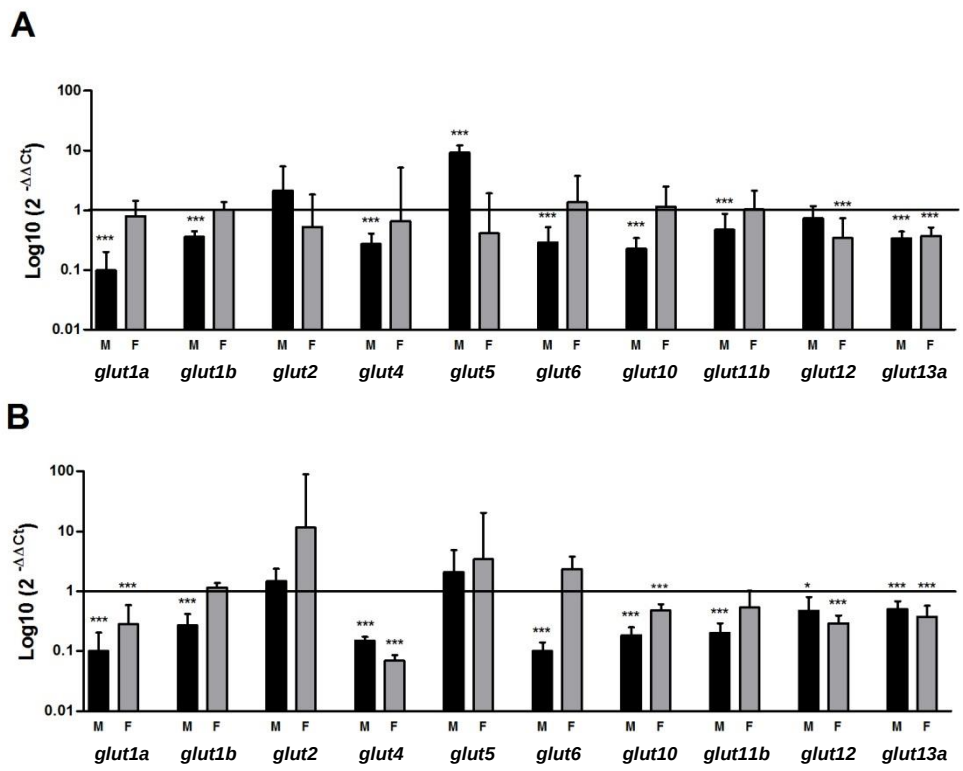

Fig 4 Relative gene expression of brain *gluts* of fed (A) and fasting (B) transgenic males (M) or females (F) zebrafish (*Danio rerio*) versus wild-type ones. Gene expression was normalized by the expression of *β-actin* gene. wild-type animals were considered controls, which gene expression = 1. Data are expressed as Log10 (2-ΔΔCt) ± SE (n = 6). Asterisk (*) or (***) indicate significant differences ($p < 0.05$) and ($p < 0.01$), respectively.

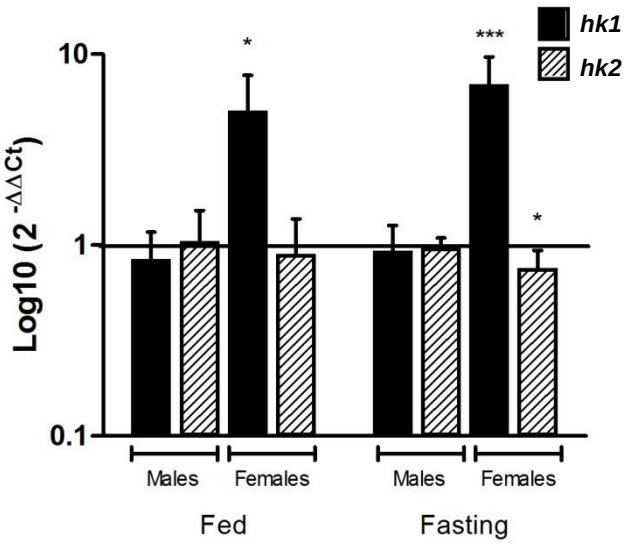

Fig 5 Relative gene expression of brain *hk1* and *hk2* in fed or fasting states, and males or females, between wild-type and transgenic zebrafish (*Danio rerio*). Gene expression was normalized by the expression of *β-actin* gene. Wild-type animals were considered controls, which gene expression = 1. Data are expressed as Log10 (2-ΔΔCt) ± SE (n = 6). Asterisk (*) or (***) indicate significant differences ($p < 0.05$) and ($p < 0.01$), respectively.

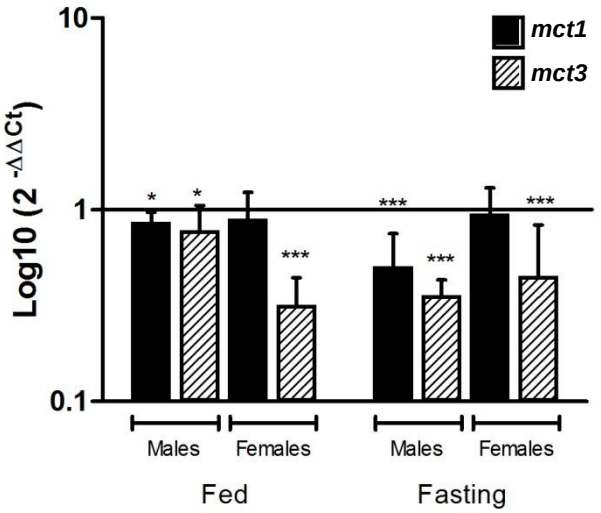

Fig 6 Relative gene expression of brain *mct1* and *mct3* in fed or fasting states, and males or females, between wild-type and transgenic zebrafish (*Danio rerio*). Gene expression was normalized by the expression of *β-actin* gene. Wild-type animals were considered controls, which gene expression = 1. Data are expressed as Log10 (2-ΔΔCt) ± SE (n = 6). Asterisk (***) indicate significant differences ($p < 0.01$).

ARTIGO 3

A ser submetido para a revista

General and Comparative Endocrinology

GH-transgenesis induces different mechanisms of appetite control in a sex-dependent manner in zebrafish

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Abstract

The present study aimed to investigate the effect of growth hormone (GH) overexpression on appetite control mechanisms in a transgenic zebrafish model. We analyzed feeding behavior and the expression of the main appetite-related genes at three different feeding periods: pre-prandial, post-prandial, and fasting. In addition, we tested the hypothesis that GH overexpression can affect appetite control in a sex-specific way, since the natural sex profile of GH secretion is modified by transgenesis. Results showed that transgenic fish eat significantly more and faster than wild-type. Expression analyses of anorexigenic/orexigenic genes pointed out that the peripheral system contributes, especially through ghrelin, to the food intake behavior. Brain genes revealed that GH-transgenic males and females present different mechanisms of appetite control. Pre-prandial appetite is kept by a down-regulation of anorexigenic genes in males, while up-regulation of orexigenic genes is observed in females. Concerning post-prandial appetite, neither males nor females presented a satiation state, probably due to a permanent down-regulation of Proopiomelanocortin (POMC). In fact, none of the anorexigenic genes were induced after meal. In fasting, both sexes used a strong up-regulation of hypocretin to maintain appetite. These differences between sexes seem to be dependent of a protein family that negatively regulates GH signaling, called SOCS (suppressors of cytokine signaling). The present study suggests, for the first time, a sex-dependent mechanism of appetite control in GH-transgenic fish.

Keywords: appetite control, growth hormone, suppressors of cytokine signaling, transgenic, zebrafish

1. Introduction

As a general feature, growth in fish is under the influence of many cellular, hormonal and environmental factors (Raven *et al*., 2008) but it is controlled mainly by the neuroendocrine brain - growth hormone (GH) - insulin-like growth factor I (IGF-I) axis (Mauras & Haymond, 2005). GH binds to receptors located at the membranes of target cells, promoting an intracellular signaling constituted by a series of cascading phosphorylation reactions that culminate with the transcription of specific genes involved in its biological responses. Negative control of GH intracellular signaling has been attributed to proteins of the suppressors of cytokine signaling (SOCS) family (Croker *et al*., 2008).

Alterations in growth rate can be reached by manipulation of the regulators of GH production, occurring only when food intake is adequate (Arendt, 1997; Devlin *et al*., 2004). Among techniques and target genes chosen to produce fish models for growth and development studies, the overexpression of GH is one of the most applied (Nam *et al*., 2001; Devlin *et al*., 2004), and these transgenic fish are considered interesting models for the study of physiological effects of GH *in vivo.*

Both transgenesis (Raven *et al*., 2008) or GH-chronic administration (Silverstein *et al*., 2000) in fish result in increased growth, probably due to a higher food intake and improved food conversion efficiency. In addition, GH-induced hyperphagia might be a result of hormone action on hypothalamic appetite control center (Hallerman *et al*., 2007). The relationship between GH and nutritional status occurs through substances that inhibit or stimulate appetite, with several neuropeptides and amides also regulating the GH

secretion in fish (Lin *et al*., 2000). In goldfish (*Carassius auratus*), administration of CCK-8s (sulfated cholecystokinin) suppress feeding and increase GH circulating levels after 30 min (Himick & Peter, 1994), and this rise happens via direct action of CCK on the pituitary (Lin *et al*., 2000). Also in goldfish, neuropeptide Y (NPY) stimulates GH secretion *in vivo* and *in vitro* (Peng *et al*., 1993). Moreover, experiments performed by Minami *et al*. (1998) showed that GH acts on NPY neurons in the hypothalamus inhibiting their secretion. Regarding ghrelin secretion, it was showed that might exist a pituitary GH – ghrelin – stomach axis, where an elevation of circulating GH levels causes a reduction in stomach ghrelin production and secretion (Qi *et al*., 2003). Other substances involved on appetite regulation that might be related to GH secretion are hypocretin (orexin) (Volkoff, 2006), peptide YY (Murashita *et al*., 2009), melanocyte-stimulating hormone (MSH) (Volkoff *et al*., 2005), proopiomelanocortin (POMC) (Cerdá-Reverter, 2003), gastrin-releasing peptide (GRP), cocaine- and amphetamine-regulated transcript (CART) (Volkoff *et al*., 2009), melatonin (Piccinetti *et al*., 2010), and leptin (Company *et al*., 2001).

It has been well demonstrated that GH secretion, in mammals, occurs in a sex-dependent fashion: males secrete GH in peaks followed by moments without GH in plasma, while in females the secretion of this hormone is virtually constant (Jaffe *et al*., 1998). In fish, there is evidence from carp (Zhang *et al*., 1994) that GH secretion follows the same pattern as mammals. Thus, GH-transgenesis might alter not only the amount of plasmatic hormone levels, but also the male/female secretion profile.

Taking in consideration that GH may interfere in the appetite control process and that transgenesis probably alters the natural sex-dependent

expression profile of this hormone, the present study aimed to evaluate the effects of GH overexpression on food ingestion. In order to test this hypothesis, we used males and females from a GH-transgenic zebrafish model and analyzed feeding behavior and expression of genes coding for anorexigenic (*cck, grp, pomc, pyy*) and orexigenic (*hyp, npy, ghre*) peptides, as well as the main negative modulators of the GH intracellular signaling: the SOCS proteins (Croker *et al*., 2008).

2. Materials and methods

2.1. Obtainment of GH-transgenic zebrafish and maintenance conditions

GH-transgenic zebrafish (*Danio rerio*) used in this work were from the F0104 lineage (Figueiredo et al., 2007a). This lineage harbor a transgene comprised of carp (*Cyprinus carpio*) *β-actin* promoter driving the expression of the growth hormone cDNA from the marine silverside fish (*Odonthestes argentinensis*). As a label to identify the transgenic organisms, the green fluorescent protein (GFP) gene was added on construction under the control of the same promoter.

The experimental fish was obtained by crossing hemizygous males from the F0104 lineage with wild type females, generating hemizygous transgenic and wild-type sibling fish. Transgenic (T; n = 58) and wild-type (WT; n = 64) fish were reared until 4 months of age in a closed circulation water system composed of 25 L tanks at 28°C, 14 h light/10 h dark photoperiod, fed twice a day with high-protein (47.5%) commercial food (Tetra Color Tropical Granules, Spectrum Brands, Brazil) *ad libitum*. All experiments were carried out in accordance with international norms of use and animal experimentation.

2.2. Food intake measurement

In order to determine the food intake by each animal and the time expend on this, the four-month-old reared fish were divided in four groups and housed in tanks as follows: WT males (n = 14), T males (n = 11), WT females (n = 14), and T females (n = 11). Until the day of the experiment, all fish were kept under the same conditions of the rearing period. Fish were starved for 24 h before the beginning of the test. One fish at a time was removed from a housing tank and released in a 25 L tank at 28°C previously prepared. At this time, 1 min was counted as habituation time. After habituation, food pellets of known weight were delivered in the water. The time expended until the fish ate the first pellet was registered as “latency time”, and the number of pellets eaten until satiation was counted to later calculation of % body weight ingested per fish. All remaining pellets were removed from the tank and the next fish were exposed to the same procedure. Throughout the procedure, disturbances were avoided in the behavioral test room. After tests, all fish were euthanized to gene expression analysis.

2.3 Gene expression of appetite regulators

2.3.1. Fish

For gene expression analysis, 18 four-month-old fish of each group (WT males, T males, WT females, and T females) were reared for two months at the same conditions as before, but the animals were fed 5% of body weight/ day of commercial food (47.5% protein) twice a day at 10 am and 4 pm.

2.3.2. Tissue dissection

At the end of 2 months, one hour before the usual feeding time (pre-prandial period), six animals of each group (WT males, T males, WT females, T females) were randomly sampled and euthanized. Total brain tissue, gastrointestinal tract and adipose tissue were collected by dissection. The remaining animals were fed as usual. One hour after feeding (post-prandial period), six animals of each group were randomly sampled, euthanized, and total brain tissue, gastrointestinal tract and adipose tissue were collected by dissection. All peri-prandial euthanasia were performed around the morning feeding. The remaining animals (six animals each group) were kept without food for 2 days (fasting) and after this period were euthanized for total brain tissue, gastrointestinal tract and adipose tissue collection. All collected tissues were stored, separately, in Trizol reagent (Invitrogen, Brazil) at -80ºC.

2.3.3. Total RNA extraction and qRT-PCR

Total RNA was extracted from the tissues using Trizol reagent (Invitrogen, Brazil), according to manufacturer's instructions. The RNA integrity of samples was evaluated based on the intensity of 28S and 18S rRNA bands in 1% agarose gels. Total extracted RNA was treated with DNA-*free* (Invitrogen, Brazil) in order to remove a possible contamination by DNA from RNA samples. Synthesis of complementary DNA (cDNA) was accomplished with 10µL of total RNA solution, and RT High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Brazil), according to the protocol suggested by the manufacturer. The synthesized cDNA was utilized as template for qPCRs using specific primers designed with basis on specific gene sequences available at GenBank (www.ncbi.nlm.nih.gov). Primer efficiencies qPCRs were performed using a

7500 RealTime PCR System equipment (Applied Biosystems, Brazil), with total volume of 10 µL, containing 5 µL of SYBR® Green PCR Master Mix (Applied Biosystems, Brazil), 0.25 µL of each primer, 3.5 µL of Ultra Pure Water (Invitrogen, Brazil), and 1 µL of cDNA (5x, 10x, 20x, 40x, and 80x diluted). All expression qPCR reactions were performed using the same equipment, with reactions of total volume of 10 µL, containing 5 µL of SYBR® Green PCR Master Mix (Applied Biosystems, Brazil), 0.25 µL of each primer, 3.5 µL of Ultra Pure Water (Invitrogen, Brazil), and 1 µL of cDNA (5x diluted). Analyzed genes, forward and reverse primer sequences, GenBank accession number, and primer efficiencies are described in Table 1. Dissociation curve analysis was performed in all series to confirm the specificity of amplified segments. In all gene expression analyses, *ef1α* gene was used as reference gene.

2.4. Statistical analysis

Student's *t* test was performed for testing differences in the food intake experiment between WT and T males, WT and T females. Differences on gene expression between WT and T fish were tested using the Relative Expression Software Tool (REST, 2009) (Pfaffl *et al*., 2002). REST uses the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001), followed by a randomization test to assess statistical significance of up- or down-regulation of the target genes, taking into account reaction efficiency and reference gene normalization. To generate standard error estimation (SE), Taylor algorithm is used by the software. Values for T animals varied upwards or downwards relative to WT control group (=1 on graphics). In all situations statistical differences were assumed when $p<0.05$.

Normality and variance homogeneity of data were previously tested when necessary (Pfaffl, 2004).

3. Results

3.1. Food intake experiments

Table 2 shows that transgenic males eat 58% faster and ingest 42% more food than wild-type males. Regarding females, transgenics eat 60% faster and ingest 128% more food than wild-type.

3.2. Gene expression experiments

In order to determine which regulating factor might be involved with the differences of food intake, qPCRs of appetite-related genes were performed. The results obtained from these analyses (*cck, grp, pomc, pyy, lep* – anorexigenic, *hyp, npy, ghre* – orexigenic, *socs1, socs3*, and *ef1α* – internal control) in different tissues (brain, gastrointestinal tract, or adipose tissue), comparing WT males and T males, or WT females and T females (4 months of age), subjected to three different feeding periods (pre-prandial, post-prandial, and fasting), are described below.

In brain, many differences were observed along the three analyzed periods. Before meal (Fig. 1), T males presented a down-regulation ($p<0.05$) for *cck, grp, pomc, pyy*. No differences were observed for hypocretin (Fig. 1a). In females, no differences were observed for *cck, grp, pomc, pyy*, and *npy*, but hypocretin was up-regulated ($p<0.05$) (Fig. 1b). After meal (Fig. 2), males presented a down-regulation for *pomc, pyy* ($p<0.01$) (Fig. 2a) and *npy* ($p<0.05$) (Fig. 2b). No differences were observed for *cck, grp, leptin, hypocretin* and

ghrelin. Females after meal presented a down-regulation ($p<0.05$) of *cck, pomc* (Fig. 2a), *hypocretin* and *npy*, and up-regulation of *ghrelin* (Fig. 2b). No differences were observed for *grp* and *pyy*. Regarding fasting fish (Fig. 3), males presented an up-regulation ($p<0.01$) for *hypocretin* (Fig. 3b). No differences were observed for *cck, gpr, pomc, pyy* and *npy*. Fasting females presented a down-regulation ($p<0.05$) for *pomc* (Fig. 3a), and an up-regulation ($p<0.01$) for *hypocretin* (Fig. 3b). No differences were observed for *cck, grp, pyy* and *npy*. Results from brain *socs1* and *socs3* genes expression in males showed up-regulation before meal ($p<0.05$) and in fasting ($p<0.01$), while no differences were observed after meal. In females, *socs1* and *socs3* presented up-regulation after meal ($p<0.05$ and $p<0.01$, respectively), while no differences were observed before meal and in fasting.

Gastrointestinal tract of both males and females, in pre-prandial period, presented no differences for *cck* and *ghrelin* (Fig. 1). *ghrelin* up-regulation was observed in females after meal (Fig. 2b) and fasting (Fig. 3b) periods ($p<0.01$).

Another analyzed tissue was the adipose tissue, and the only difference found was a down-regulation of *leptin* in males before meal (Fig. 1a).

4. Discussion

Control of food intake involves many interactions between brain and peripheral signs, conveyed via blood or nervous system. In the brain, hypothalamus is the main region responsible for the food intake control, and two distinct populations of neurons might be identified: one that stimulates food intake (orexigenic), and another one that inhibits it (anorexigenic). Acting jointly, signals from peripheral tissues reach these populations of neurons and also

exert actions on the control of food intake (Volkoff *et al*., 2005). Studies have proposed a relationship between this control system and the GH secretion due to an overlapping of the hormone functions: GH is a key piece on energy metabolism and is positively related to feeding time (Straus, 1994). The animal model used in our experiments was a GH-transgenic zebrafish, and the effects of GH overexpression on quickness and amount of food ingested, as well as the central and peripheral appetite control, were evaluated.

Results from table 2 demonstrated a marked difference between transgenic (T) and wild-type (WT) fish. Transgenic individuals begin meal faster and ate more than wild-type siblings. This might be explained by changes in the metabolic state of GH-transgenics, such as the inducement of anabolism comprised by protein synthesis, which generates high metabolic costs (Rousseau & Dufour, 2007). These anabolic effects of GH were studied in rainbow trout (*Oncorhynchus mykiss*) administered with ovine GH, which enhanced growth rates as a result of the stimulatory effect on protein synthesis rates and little action on protein degradation (Møller & Nørrelund, 2003). In the same sense, other studies showed that administration of GH to teleosts results in an increased food intake (Johnsson & Bjornsson, 1994; Lin *et al*., 2000), and GH-transgenic fish display high feeding levels as well (Farrell, 2011).

In order to assess the mechanisms involved in food intake and appetite control of GH-transgenic zebrafish, we performed experiments in three different situations of appetite control (one hour before meal – pre-prandial period, one hour after meal – post-prandial period, and after 2 days without food – fasting period) and analyzed the expression of the main genes coding for anorexigenic

and orexigenic hormones in three different tissues: brain, gastrointestinal tract (GT) and adipose tissue (AT).

The first gene expression analysis was carried out with transgenic and wild-type fish just one hour before mealtime. Results showed in figure 1a evidence that, in brain, all anorexigenic genes are significantly down-regulated in transgenic males, while no difference was observed in GT. On the other side, transgenic females did not show any variation for the same genes in both brain and GT. In adipose tissue of males, *leptin* presented a down-regulation. *Npy* (Figure 1b) was down-regulated in brain of transgenic males, while transgenic females presented a significant 2.8 fold induction of *hypocretin* in the same tissue. No variations were observed in GT genes of both sexes. *Cck, grp, pomc, pyy* in brain, and *leptin* in adipose tissue are known anorexigenic peptides and their down-regulation before meal is a result that corroborates with the avoidance of satiety (Volkoff *et al*., 2005; Volkoff & Peter, 2006). Hypocretin, as an orexigenic peptide, stimulates food intake and its rise before meal are in accordance with previous reports (Korczynski *et al*., 2006; Volkoff *et al*., 2009). These results indicate that pre-prandial appetite in transgenic fish is maintained by two different gender-dependent mechanisms. Transgenic males remain hungry because transcription of anorexigenic genes is severely down-regulated, while transgenic females up-regulate *hypocretin*. At our knowledge, this is the first report to show that pre-prandial appetite is regulated in a gender-dependent manner in GH-transgenic fish.

The second gene expression analysis comprised fish sampled one hour after meal. In this case, our objective was to assess the satiation mechanism. Figure 2a shows the results for expression of anorexigenic genes in brain, GT

and AT. Transgenic males and females showed a significant down-regulation of *pomc*, with transgenic females also presenting a down-regulation of *cck*, and males for *pyy*. Regarding expression of orexigenic genes (Fig. 2b), both transgenics were down-regulated for *npy*, with transgenic females also down-regulated for *hyp*. In GT, females presented an up-regulation, of around 3 times, of *ghrelin*. These results clearly indicate that satiation expected by down-regulation of orexigenic genes such as *hyp* and *npy* might not be achieved, as the decreased expression of *pomc* in both sexes could keep the hungry of transgenic fish even after meal. It seems that GH overexpression is affecting *pomc* transcription, resulting in an eternal hungry fish. The *pomc* gene is a precursor of other molecules (α-, β-, γ-MSH and ACTH, the melanocortin system) and is expressed in many cell types and tissues, but the main site of production is the pituitary (Metz *et al*., 2006). In fish, POMC has been identified in a number of species (Alrubaian *et al*., 2003; Dores *et al*., 2003), where it is related to the control of food intake. A study performed with *Salmo salar* showed that, after meal, *pomc* mRNA increased, acting as a satiety signal (Valen *et al*., 2011). However, in the present work, GH-transgenic fish did not reach a satiety state after meal due to the absence of up-regulation of any satiety signal, and at our knowledge, this is the first report that suggests a down-regulation of *pomc* as a mechanism for the continuous hunger in transgenic fish. Moreover, the strong up-regulation of *ghrelin*, an important orexigenic factor produced mainly by stomach and gut (Volkoff & Peter, 2006; Volkoff *et al*., 2009), may corroborate to the hypothesis that transgenic fish are still hungry even after meal, in this case due to a peripheral sign. Experiments by Canosa *et al*. (2005) presented a down-regulation of *ghrelin* 1 hour after

meal in GT of goldfish. On the other hand, Hevrøy *et al*. (2011) observed an elevation of this peptide in starving situations. Our results showed a rise in *ghrelin* expression during the hypothetical satiety situation (after meal), increasing the hungry state of fish.

The third gene expression analysis was done with fish after two days of starvation. This experiment had the objective of verifying how GH-transgenic fish appetite genes behave during a period of fasting. Regarding anorexigenic genes, *pomc* in brain of transgenic females and *cck* in GT of males were down-regulated (Fig. 3a). There was a significant induction of *hypocretin* in brain of both transgenic males (2-fold) and females (4.2-fold), and *ghrelin* in GT of females (2.4-fold) (Figure 3b). These results indicate the involvement of *hypocretin* and *ghrelin* in appetite maintenance of fasting GH-transgenic fish. Corroborating our finds, previous studies reported that prolonged fasting significantly increases brain *hypocretin* mRNA in goldfish (Nakamachi *et al*., 2006) and zebrafish (Novak *et al*., 2005). *Hypocretin* is a neuropeptide involved, in fish and mammals, in the regulation of energy balance and sleep-wake cycle. Besides this bidirectional effect, a regulation in locomotor activity seems to exist as well. Intraperitoneal injections of mammalian hypocretin significantly increased feeding and locomotion in the fish *Thalassoma pavo* (Facciolo *et al*., 2009). The relation between hypocretin and locomotion may justify our observations that GH-transgenic fish expended less time than wild-type siblings to start the food intake (Table 2). *Ghrelin* up-regulation may be supported by the results of Amole & Unniappan (2009), where they reported that fasted zebrafish presented an up-regulation of *ghrelin*. Moreover, this result of *ghrelin* might be

linked to its action stimulating orexigenic hypothalamic nuclei, increasing the expression of other orexigenic factors like *hypocretin* (Dimaraki & Jaffe, 2006).

Taking all results reported here into account, it seems that GH overexpression affected the transcription of food intake and appetite-related genes. In transgenic males, pre-prandial appetite was maintained by down-regulation of anorexigenic genes, while transgenic females up-regulate *hypocretin*. Satiation was not reached in GH-transgenics mainly because of *pomc* down-regulation, even after meal. Hunger in fasting GH-transgenic fish was caused by high levels of *hypocretin* transcription. These mechanisms are in agreement with the high-energy demand provoked by GH overexpression. Thus, an interesting question arises: how does GH excess affect anorexigenic/orexigenic genes transcription? This hormone binds to receptors present in the membranes of target cells, and its signaling is negatively controlled by proteins of the SOCS family. SOCS1 and SOCS3 have been described as the main negative modulators of the somatotropic axis in liver of homozygous GH-transgenic zebrafish from the F0104 lineage (Studzinski *et al*., 2009). Within this scenario, it is reasonable to think that the down-regulation of several anorexigenic genes observed in brain of GH-transgenic fish could be related to SOCS genes expression.

In order to test the hypothesis that *socs* genes could be induced in brain of transgenics because of GH excess, we analyzed *socs1* and *socs3* expression in brain of both transgenic males and females. *socs1* and *soc3* expression profiles indicate significant differences among all analyzed appetite situations (pre-prandial, post-prandial and fasting) and between transgenic males and females. In transgenic males, both *socs1* and *socs3* presented the

same expression profile: up-regulation in hungry states (pre-prandial and fasting) and no variation after meal. In transgenic females, both *socs* have changed the expression pattern: no variation during hungry states and up-regulation after meal. It is clear that GH overexpression affects *socs* expression differently in males and females. Probably, the up-regulation of *socs* in males is related to down-regulation of anorexigenic genes, while the increased transcription of the same suppressors in females after meal is related to satiation via *hypocretin* down-regulation.

The way that transgenic males and females control *socs* transcription might be a consequence of how GH excess affects each sex. It is known that there is a sexual dimorphism on GH secretion by pituitary in rats, mice and humans, where males have a highly pulsatile secretion and females a more constant level (Waxman *et al*., 1991; Jaffe *et al*., 1998). In adult rats, the sex differences are more dramatic with males presenting GH plasma peaks about every 3.5 h, followed by GH-free intervals lasting about 2 h. In contrast, adult females have more frequent GH secretion and almost a continuous presence of it in plasma (Waxman & O'Connor, 2006). The animal model used in the present work is a transgenic fish, which have an overexpression of GH. Males of this genotype might be suffering a flatness of pulses on GH secretion with concomitant lack of GH-free intervals, while females are less affected because their GH secretion is already relatively constant. Distortions on the usual GH secretion pattern were already described, where rats or mice treated with exogenous GH for days showed a decrease on the expression of male-specific liver genes and an induction of female-specific genes (Thangavel *et al*., 2004). Considering that the changes in the pattern of GH release modify the individual

sex profile, this deviation may also influence the metabolism and, consequently, the energy consumption and expenditure.

In conclusion, GH-transgenic zebrafish ate more and faster than wild-type siblings. This fact was confirmed by the food intake experiment, where the amount of food eaten was high and the time spent to start meal was low, revealing a strong hungry state. Gene expression results presented a down-regulation of *leptin* in AT, in males before meal. Expression of *ghrelin* in GT revealed a substantial participation of this hormone on maintenance of hungry after meal and on fasting, especially in females. Analyzes of brain genes showed that females have a quite different outcome in comparison to males. While in males, the majority of the differences reported are concentrated on anorexigenic factors, especially *pomc* and *pyy*, in females the regulation of hungry-satiety state is focused on orexigenic pathway, with *hypocretin* in the main place. This anorexigenic/orexigenic expression system seems to be SOCS-dependent, with the transcription of these suppressors affected differently in males and females. Since males and females have considerably differences of GH secretion, transgenesis procedure may have changed this pattern, making its secretion high and constant in both sexes. The results presented in this study suggest that transgenic fish appetite is regulated even under the influence of the transgenesis procedure, and that appetite control systems can adjust the food intake to compensate for the new energy demand set point.

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Tables

Table 1 Gene-specific primers used for qRT-PCR analysis and GenBank accession number of the source sequence.

Genes	Primer Sequences (5' – 3')	GenBank accession number	Primer efficiencies
***cck* F**	AAAGGCTCATACCGCAGAAGTC	XM001346104	93
***cck* R**	TCTGTGAGATGCACCCATGGT		
***ef1α* F**	GGGCAAGGGCTCCTTCAA	BC098530	100
***ef1α* R**	CGCTCGGCCTTCAGTTTG		
***gre* F**	GTGGCACCAGCTTCCTCAGT	AM055940	92
***gre* R**	CACTCTTGGTGGCCTTCGA		
***grp* F**	CATCTGCCTCTCCGGCTGTA	BX248399	98
***grp* R**	GCGGGTTCTCCTGTGTCATT		
***hypocretin* F**	CCGCTGTCGGGATTCTCA	DQ831346	98
***hypocretin* R**	GTGGACGCGGCTTTCG		
***leptin* F**	GGTTACCTGGAAGGCATGGA	BN000830	100
***leptin* R**	CCAGCGCTTTCCCATTTGT		
***npy* F**	CCAAACATGAAGATGTGGATGAG	BC162071	75
***npy* R**	CCAAGCAGACGAACAAGAGAAA		
***pomc* F**	CGCAGACCCATCAAGGTGTA	NM181438	97
***pomc* R**	CGTTTCGGCGGATTCCT		
***pyy* F**	GACCGTCGTCGCCACTGT	CT574577	86
***pyy* R**	TCCACAAATGTCCCGAGACA		
***socs1* F**	CTCCGTTTTAGGATGCAGGAAT	BC077158	92
***socs1* R**	CATTGTGCAGTGTTCAAGTCTG		
***socs3* F**	CTGGTACGATCGCTGATCCA	BC049326	100
***socs3* R**	GGCAAGAATGGCGCTTCA		

Table 2 Average (± SD) of latency time and percentage of food ingested by WT males, T males, WT females and T females. Comparisons were made by sex (WT males x T males; WT females x T females) using *t test*.

Animal	**Latency time (s)**	**% body weight ingested**
Wild-type Male	43.7 (± 5.06)	0.91 (± 0.10)
Transgenic Male	18.4 (± 6.7)	1.28 (± 0.14)
p	<0.01	<0.05
Wild-type Female	54.7 (± 8.94)	0.85 (± 0.11)
Transgenic Female	21.7 (± 7.22)	1.94 (± 0.28)
p	<0.01	<0.01

Figures

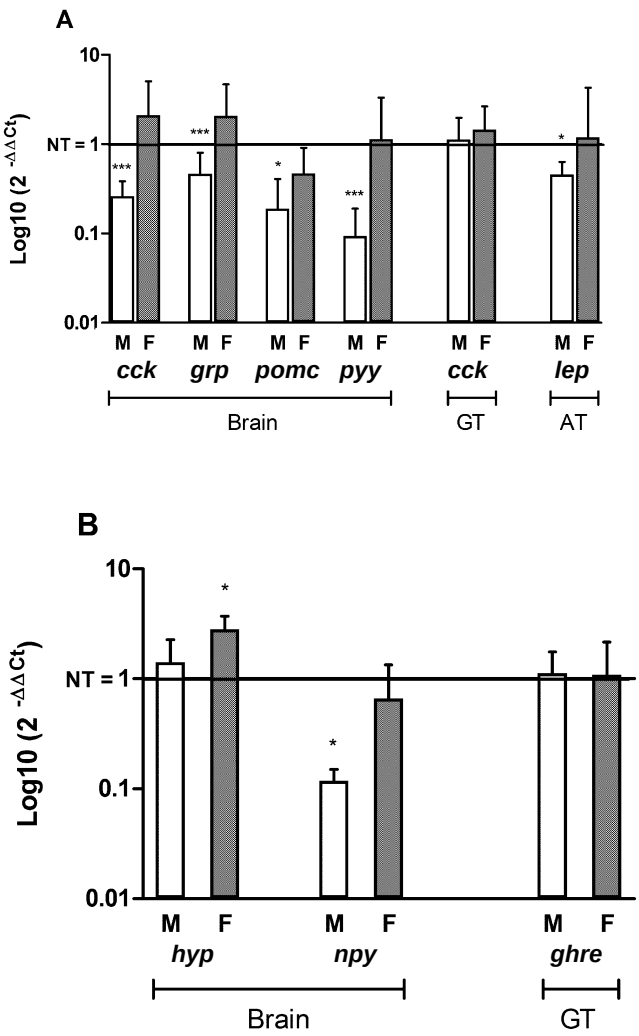

Figure 1 Relative gene expression comparing wild-type (WT) and transgenic (T) zebrafish (*Danio rerio*) in brain, gastrointestinal tract (GT) and adipose tissue (AT) of males (M) and females (F) of anorexigenic (A) and orexigenic (B) genes in pre-prandial period. Gene expression was normalized by the expression of elongation factor 1α (ef1α) gene. WT pre-prandial males and females were considered controls, where gene expression = 1. Data are expressed as Log10 ($2^{-\Delta\Delta Ct}$) ± SE (n = 6). Asterisk (*) and (***) indicate significant differences ($p < 0.05$) and ($p < 0.01$), respectively).

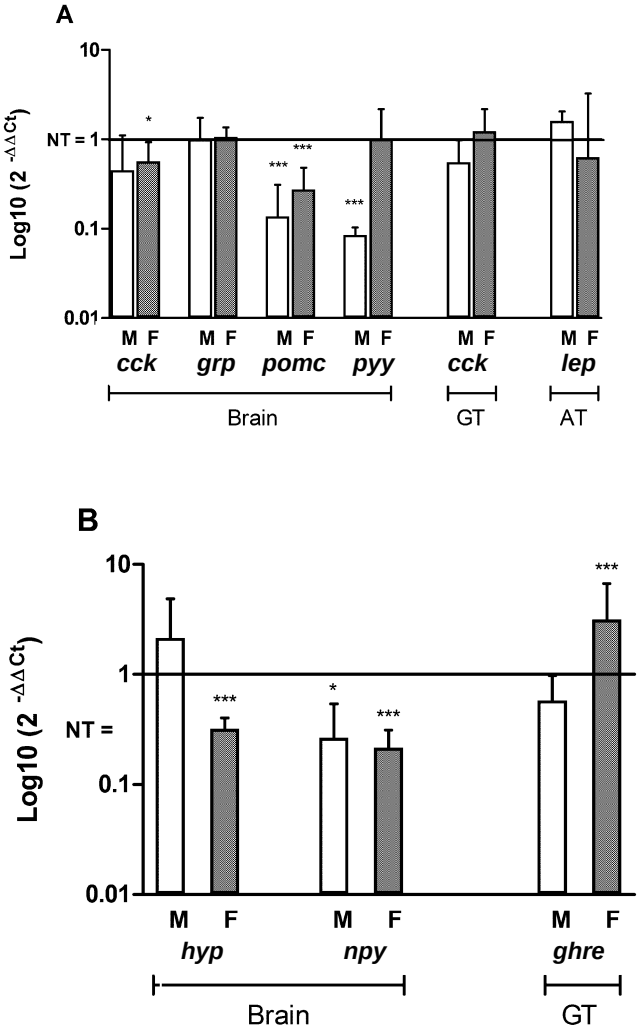

Figure 2 Relative gene expression comparing wild-type (WT) and transgenic (T) zebrafish (*Danio rerio*) in brain, gastrointestinal tract (GT) and adipose tissue (AT) of males (M) and females (F) of anorexigenic (A) and orexigenic (B) genes in post-prandial period. Gene expression was normalized by the expression of elongation factor 1α (ef1α) gene. WT post-prandial males and females were considered controls, where gene expression = 1. Data are expressed as Log10 ($2^{-\Delta\Delta Ct}$) ± SE (n = 6). Asterisk (*) and (***) indicate significant differences ($p < 0.05$) and ($p < 0.01$), respectively).

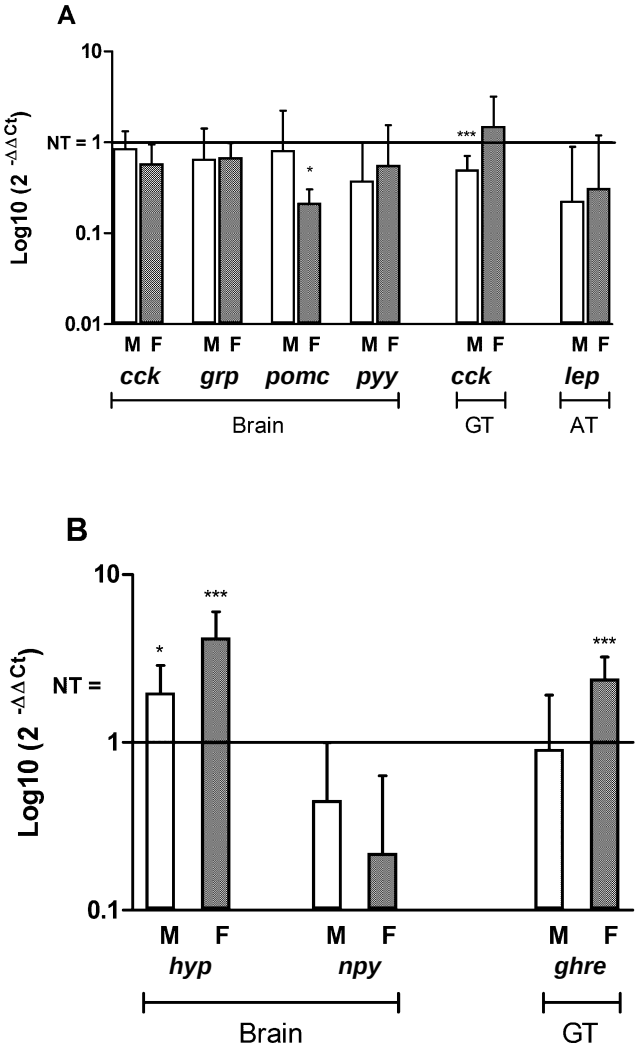

Figure 3 Relative gene expression comparing wild-type (WT) and transgenic (T) zebrafish (*Danio rerio*) in brain, gastrointestinal tract (GT) and adipose tissue (AT) of males (M) and females (F) of anorexigenic (A) and orexigenic (B) genes in fasting period. Gene expression was normalized by the expression of elongation factor 1α (ef1α) gene. WT fasting males and females were considered controls, where gene expression = 1. Data are expressed as Log10 ($2^{-\Delta\Delta Ct}$) ± SE (n = 6). Asterisk (*) and (***) indicate significant differences ($p < 0.05$) and ($p < 0.01$), respectively).

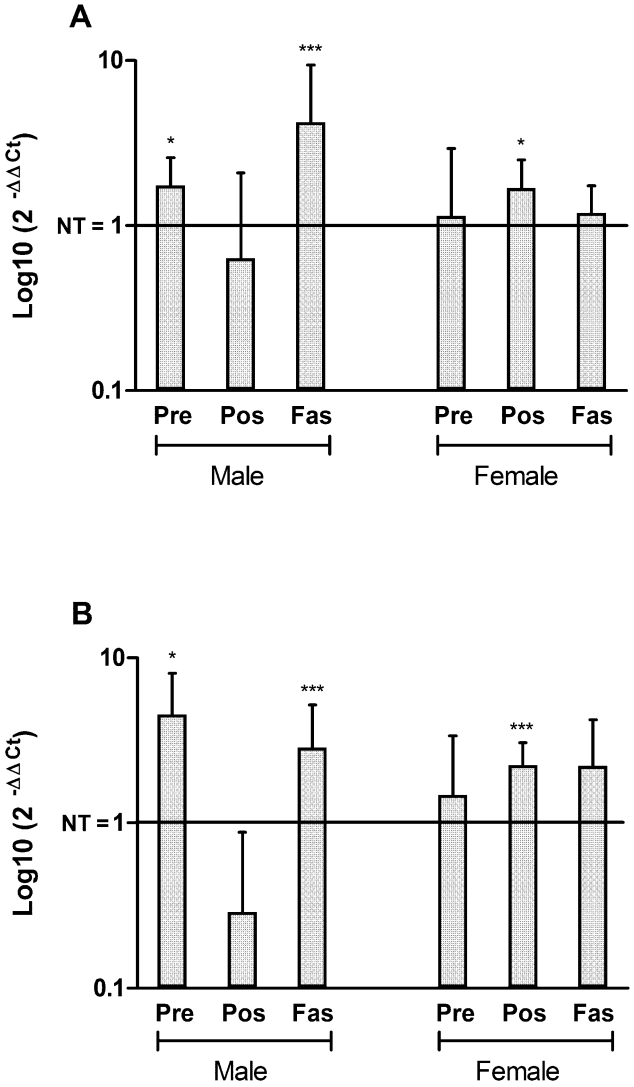

Figure 4 Expression of (A) SOCS1 and (B) SOCS3 genes comparing n wild-type (WT) and transgenic (T) zebrafish (*Danio rerio*) in brain of males and females, in three different periods: pre = pre-prandial, pos = post-prandial and fas = fasting. Gene expression was normalized by the expression of elongation factor 1α (ef1α) gene. WT were considered controls, where gene expression = 1. Data are expressed as Log10 ($2^{-\Delta\Delta Ct}$) ± SE (n = 6). Asterisks indicate significant differences (* = $p<0.05$; *** = $p<0.01$).

4. Conclusões gerais

Os resultados e conclusões desta tese, que teve como objetivo avaliar as ações do hormônio do crescimento (GH) sobre aspectos do metabolismo energético e apetite, utilizando exemplares de zebrafish (*Danio rerio*) selvagens e transgênicos superexpressando o GH, foram:

- a exposição aguda a uma sobredose de GH (via intraperitoneal) levou a um aumento dos transportadores de glicose (*gluts*) no cérebro;

- a exposição crônica a uma sobredose de GH (via superexpressão do GH) levou a um efeito anti-insulínico, com queda na expressão de *gluts* no cérebro e manutenção de hiperglicemia mesmo em uma situação de jejum;

- animais não-transgênicos expostos a uma condição de hipoglicemia devido ao jejum respondem elevando a expressão de *gluts* no cérebro, especialmente em indivíduos machos, os quais ainda elevaram a expressão de *mcts* para compensar a redução na glicose sanguínea;

- animais transgênicos, em relação aos não-transgênicos, respondem de forma parecida às condições de recém alimentados ou jejum, demonstrando que o efeito da transgenia se sobrepõe ao efeito da condição alimentar;

- fêmeas transgênicas recém alimentadas e em jejum mostraram tendência de elevar a expressão da enzima hexoquinase 1;

- machos e fêmeas transgênicos recém alimentados ou em jejum tiveram queda na expressão dos *mcts* analisados em relação aos não-transgênicos;

- com relação ao comportamento alimentar, animais transgênicos comem mais e mais rápido que os não-transgênicos;

- machos transgênicos apresentaram regulação do estado fome-saciedade através de hormônios anorexígenos, especialmente POMC e PYY;

- fêmeas transgênicas apresentaram regulação do estado fome-saciedade principalmente através da via orexígena, com a hipocretina em destaque;

- a regulação anorexígena/orexígena parece ser dependente das SOCS, sendo a expressão destes supressores afetada diferentemente entre os sexos;

- as diferenças em diversos parâmetros encontradas entre os sexos em resposta à transgenia pode se dever, em parte, ao diferente padrão de secreção do GH entre machos e fêmeas.

5. Perspectivas

Desde o surgimento dos primeiros peixes geneticamente modificados, estes têm se mostrado bons modelos para pesquisas básicas e aplicadas em diferentes áreas, como fisiologia e aquicultura. Com a geração de um modelo animal em que o gene do hormônio do crescimento foi alterado e se apresenta com maior expressão, o campo de possibilidades para testar os efeitos deste sobre os diversos aspectos fisiológicos se amplia, sendo seu uso benéfico para elucidar os princípios endócrinos, bioquímicos e moleculares da ação do GH.

No presente estudo observamos que a alteração do padrão de expressão do GH pode ter efeito sobre diferentes sistemas, sendo abordados aqui alguns aspectos do transporte de glicose e apetite. Os resultados apresentados mostram que este hormônio tem a capacidade de interferir de forma diferenciada sobre os transportadores de glicose (*gluts*) no cérebro e sobre hormônios que controlam o apetite, sendo estas alterações dependentes do tempo de exposição ao hormônio, do sexo e do estado alimentar.

Neste contexto, abre-se um leque de possibilidades para avaliar, a nível molecular, a interação do GH sobre os diferentes sistemas. Qual a relação direta do GH com os hormônios sexuais? Qual seu efeito sobre memória e comportamento, já que ele altera a captação de glicose no cérebro? Existe algum mecanismo de compensação?

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