UNIVERSIDADE ESTADUAL PAULISTA – UNESP

CENTRO DE AQUICULTURA DA UNESP

EFEITOS DO HIPOTIREOIDISMO NA FUNÇÃO TESTICULAR DE *Danio rerio* E RELAÇÃO DOS HORMÔNIOS TIREOIDIANOS COM O EIXO HIPOTALÂMICO-HIPOFISÁRIO-GONADAL

Maira da Silva Rodrigues

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Jaboticabal, 05 de julho de 2019.

SUMÁRIO

DEDICATÓRIA

Dedico este trabalho aos meus pais,
Luiz Sergio *e* ***Marlene*** *que,*
com muito carinho e humildade,
me ensinaram amor, gratidão pela vida e respeito
ao próximo.
Vocês são os meus heróis!

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A vaidade e a fortuna são os que governam essa farsa da vida; cada um se põe no teatro com a pompa com que a fortuna e a vaidade o põem; ninguém escolhe o papel; cada um recebe o que lhe dão. Aquele que sai sem fausto, nem cortejo, e que logo no rosto indica que é sujeito à dor, à aflição e à miséria, esse é o que representa o papel de homem. A morte está de sentinela, em uma mão tem o relógio do tempo, na outra tem a foice, e com esta, de um golpe certo e inevitável, dá fim à tragédia, corre a cortina e desaparece (...) assim acaba o homem, assim acabam as suas glórias, e só assim acaba sua vaidade.

Matias Aires Ramos da Silva de Eça- filósofo e escritor português nascido em São Paulo- Brasil

RESUMO

Dentre os aspectos hormonais que influenciam o desenvolvimento dos vertebrados, pode-se destacar o papel dos hormônios tireoidianos (Hts). Esses hormônios (T4/T3) mediam funções imprescindíveis para a homeostase dos organismos, modulando processos fisiológicos como a regulação do crescimento, metabolismo e reprodução. Pensando nisso o presente trabalho teve como objetivo avaliar os efeitos do hipotireoidismo *in vivo* e *in vitro* na função testicular de machos adultos de *D. rerio* e a relação dos Hts no eixo hipotalâmico-hipofisário. No presente trabalho nós mostramos que *in vivo* o hipotireoidismo induzido pelo methimazole afetou a espermatogênese de zebrafish levando ao acúmulo de células pré-meióticas, atraso na diferenciação e meiose, reduzindo a quantidade de espermatozoides formados. *in vitro* foi observado que os efeitos biológicos do hormônio folículo estimulante (Fsh) na função testicular são dependentes dos hormônios tireoidianos, assim como os efeitos estimulatórios da gonadotropina coriônica humana (hCG) e inibitório do hormônio inibidor de gonadotropina (GnIH) necessitam dos Hts. Além disso, a expressão de genes hipotalâmicos e hipofisários foram afetados de modo antagônico quando na ausência ou presença dos Hts. Portanto nossos resultados indicam que os hormônios tireoidianos são essenciais na manutenção da espermatogênese e do eixo hipotalâmico-hipofisário em adultos de zebrafish.

Palavras-chave: hipotireoidismo, hormônios tireoidianos, espermatogênese, GnIH, zebrafish

ABSTRACT

Among the hormonal aspects that influence the development of vertebrates, the role of thyroid hormones (Ths) are crucial. These hormones (T4/T3) have a key role on the organism's homeostasis by modulating physiological processes, including regulation of growth, metabolism and reproduction. This study assessed the effects of *in vivo* and *in vitro* hypothyroidism on the testicular function of *D. rerio* adult males and the interaction between thyroid hormones in the brain-pituitary axis. Here we show that methimazole-induced hypothyroidism *in vivo* affected the progression of zebrafish spermatogenesis by inducing the accumulation of pre-meiotic cells, delaying cell-differentiation and meiosis as well reducing the number of spermatozoa. It was established *in vitro* that the effects of the follicle-stimulating hormone (Fsh) on testis function are dependent of thyroid hormones, likewise the stimulatory effects of human chorionic gonadotropin (hCG) and the inhibitory effects of gonadotropin- inhibitory hormone (GnIH) are also subject to Ths. In addition to that, gene expression of brain-pituitary was antagonistically affected both in the absence and in the presence of Ths. These results point to how essential the thyroid hormones are in regulating spermatogenesis and maintaining the brain-pituitary axis in adult zebrafish.

Key-words: hypothyroidism, thyroid hormones, spermatogenesis, GnIH, zebrafish

1. INTRODUÇÃO GERAL

1.1. Espermatogênese em peixes teleósteos

Dentre aproximadamente 60.000 espécies de vertebrados conhecidas, descritas e vivas do mundo, os peixes ocupam o grupo com maior diversidade e abundância, constituindo cerca de 32.000 espécies, o que representa mais de 50% dos vertebrados (Nelson et al., 2006 e 2016). O sucesso desse grupo é atribuído a uma série de adaptações desenvolvidas ao longo da evolução que refletem na diversidade morfológica, comportamental, fisiológica e principalmente nas estratégias reprodutivas, que são definidas como o conjunto de características que uma espécie desenvolve para ter sucesso na reprodução e assim garantir a sobrevivência da espécie (Reis et al., 2003; Nelson et al., 2006 e 2016).

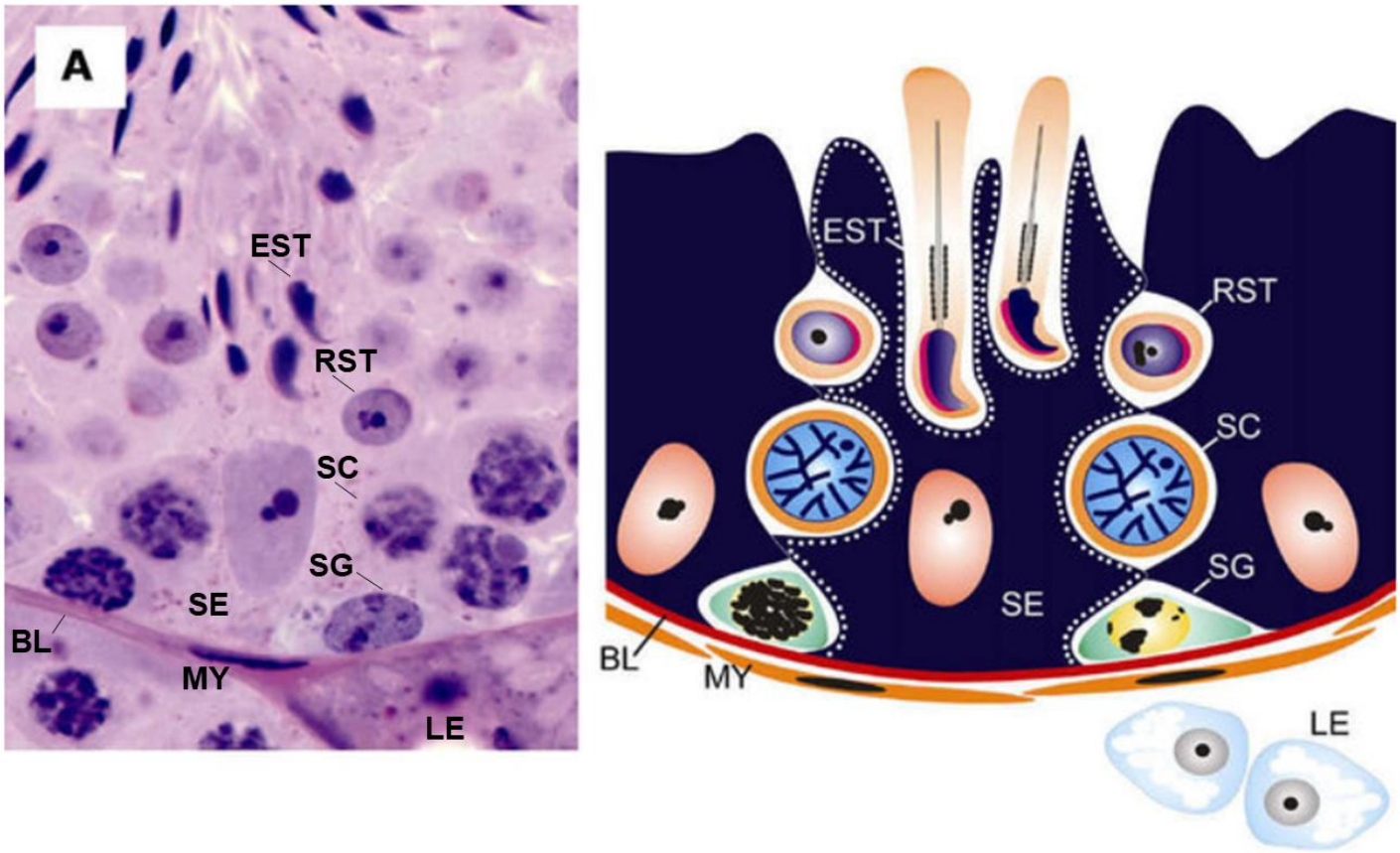
Estas estratégias reprodutivas estão diretamente relacionadas com a morfologia das gônadas das diferentes espécies. Nos teleósteos com fertilização externa, os testículos geralmente são pares e alongados (Billard et al., 1982; Vazzoler, 1996; Grier e Aranzábal, 2009), localizados na cavidade celomática e envolvidos pelo tecido conjuntivo, denominado de túnica albugínea. Esse tecido emite projeções para o interior do órgão formando lóbulos ou túbulos seminíferos que sustentam o tecido epitelial germinativo (Grier, 1980).

Microscopicamente os testículos apresentam dois compartimentos distintos: (1) intersticial formado por um tecido conjuntivo, contendo células de Leydig (produtoras de esteroides), vasos sanguíneos, macrófagos, granulócitos e outros; (2) compartimento germinativo, que contém as células germinativas e as células de Sertoli que em conjunto formam o epitélio germinativo (Schulz e Nóbrega, 2011; Siqueira-Silva et al., 2018) (Fig. 1). A espermatogênese depende

da interação dos componentes celulares presentes nestes dois compartimentos (Grier et al., 1980).

O processo espermatogênico é considerado altamente coordenado, no qual as células diploides iniciais conhecidas como espermatogônias tronco, que além de se auto-renovarem após os eventos reprodutivos, passam pelos processos de proliferação mitótica e diferenciação para formar os espermatozoides (Schulz et al., 2010). Ao final de diversas divisões mitóticas espécie-específica, os espermatócitos iniciam o processo meiótico e por fim passam pela diferenciação celular ou espermiogênese, que resultará na formação das células haploides, os espermatozoides (Grier et al., 1980; Nóbrega et al., 2009; Schulz et al., 2010; Siqueira-Silva et al., 2012).

Diferente dos amniotas, considerados os vertebrados derivados (Fig. 1A), em peixes e outros vertebrados anamniotas, a espermatogênese desenvolve-se no interior de estruturas denominadas de espermatocistos, ou cistos, que são formados quando as células de Sertoli envolvem as células germinativas em desenvolvimento (Fig.1B) (Vilela et al., 2003; Nóbrega et al., 2009; Schulz et al., 2010). As células de Sertoli fornecem suporte mecânico, nutricional e fatores necessários para a sobrevivência, proliferação e diferenciação das células germinativas, assim como intermediação hormonal e fagocitose de restos celulares e espermatozoides residuais (Grier et al., 1980; Nóbrega et al., 2009; Schulz et al., 2010; Siqueira-Silva et al., 2012; França et al., 2016).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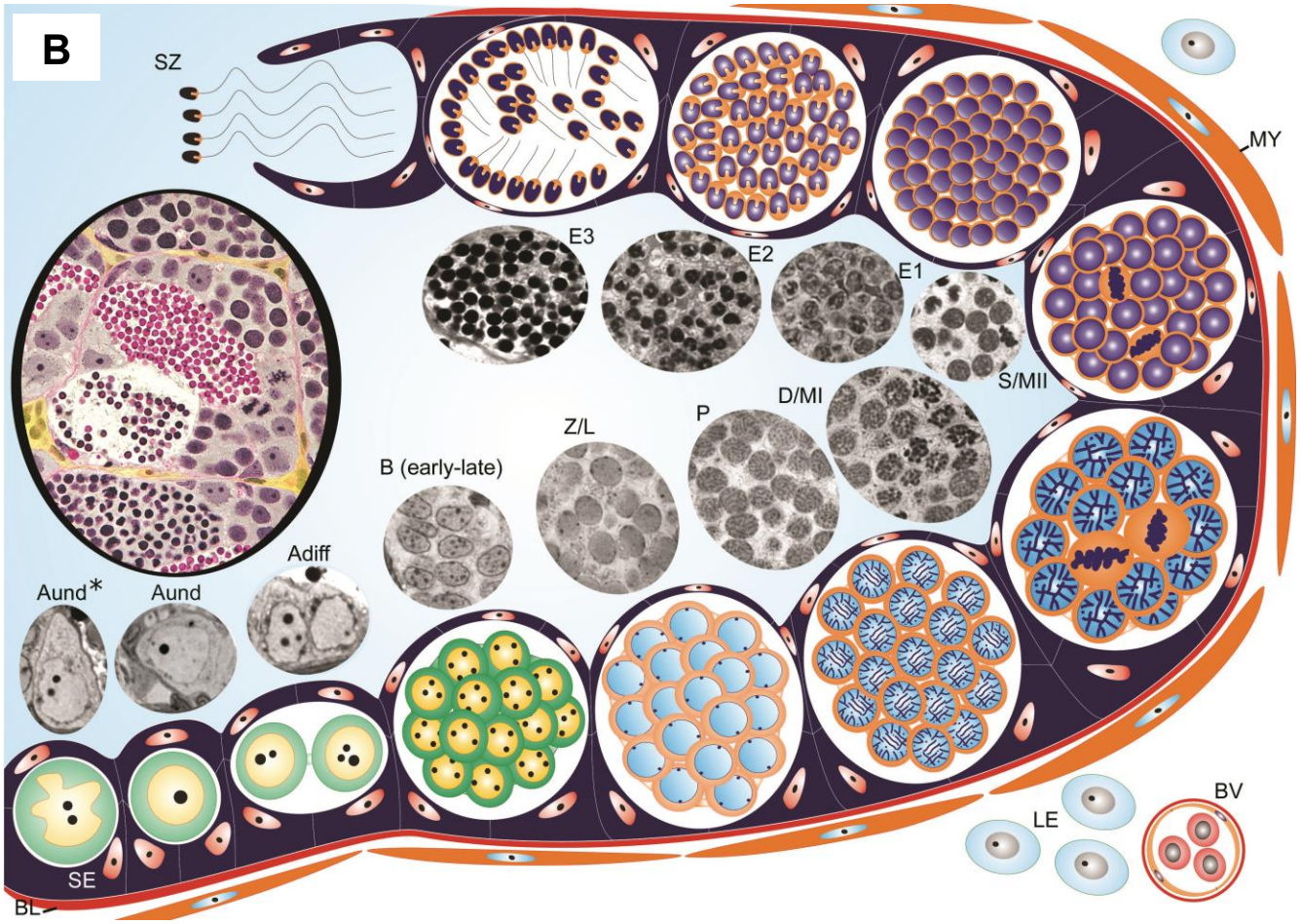
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Figura 1 A) Espermatogênese em mamíferos. **B)** Espermatogênese em zebrafish. Legenda: células de Sertoli (SE); lâmina basal (BL); células peritubulares mióides (MY), células de Leydig (LE), espematogônia (SG); espermatócito (SC); espermátide arredondada (RST); espermátide alongada (EST); espermatogônia do tipo A indiferenciada* (A_{und*}) (célula tronco?); espermatogônia do tipo A indiferenciada (A_{und}); espermatogônia do tipo A diferenciada (A_{diff}); espermatogônia do tipo B (B early-late); espermatócitos primários em leptóteno/zigóteno (L/Z), paquíteno (P), diplóteno/metáfase I (D/MI); espermatócitos secundários/metáfase II (S/MII); espermátides iniciais (E1); intermediárias (E2); finais (E3); espermatozoides (SZ); e vasos sanguíneo (BV). Adaptado de Schulz e colaboradores (2010).

1.2. Espermatogênese e características das células germinativas em zebrafish

As características morfofuncionais dos diferentes estágios de desenvolvimento das células germinativas durante a espermatogênese em zebrafish foram descritas (Leal et al., 2009; Schulz et al., 2010). Estas células são identificadas de acordo com o formato do núcleo, condensação da cromatina e tamanho da célula, sendo divididos em três fases: (1) fase mitótica ou espermatogonial, caracterizada por sucessivas divisões mitóticas das espermatogônias indiferenciadas (A_{und*} e A_{und}), espermatogônia diferenciada (A_{diff}) e do tipo B; (2) a fase espermatocitária ou meiótica na qual o material genético dos espermatócitos é duplicado, recombinado e segregado formando as células haploides, as espermátides; (3) e a fase denominada espermiogênese, na qual as espermátides passam por modificações estruturais e funcionais altamente complexas para originar os espermatozoides aptos a fecundação (Nóbrega et al., 2009; Schulz et al., 2010) (Fig. 2).

Fase mitótica ou espermatogonial

As espermatogônias indiferenciadas do tipo A (A_{und*} e A_{und}) são encontradas isoladas no epitélio germinativo e estão envoltas por um conjunto de células de Sertoli (Schulz et al., 2010). Além disso são as maiores células da

linhagem germinativa, com diâmetro nuclear e volume celular medindo cerca de 8.6 µm e ~ 667 μm^3, respectivamente (Leal et al., 2009). A cromatina não está completamente condensada e pode ser encontrado um ou dois nucléolos compactos (Leal et al., 2009). As espermatogônias A_{und*} são distinguidas das demais espermatogônias pela irregularidade do seu envoltório nuclear e pela razão entre núcleo e citoplasma serem maior. Além disso, por meio da técnica de BrdU (5'-bromo-2'-deoxiuridina), um nucleotídeo sintético e análogo à timidina, o que confere a capacidade de poder ser incorporado e retido no DNA durante a fase de síntese (fase S), foi possível identificar a existência de possíveis espermatogônias tronco, as chamadas "labeling-retaining cells" que retiveram BrdU a longo prazo, estas supostas células estão entre as espermatogônias indiferenciadas (A_{und*} e A_{und}) (Nóbrega et al., 2010).

As espermatogônias diferenciadas (A_{diff}) são resultantes da mitose com citocinese incompleta. Estas células estão agrupadas em pequenos cistos contendo de duas a oito células. Além de serem menores, apresentando diâmetro nuclear médio de 6 µm, comparada aos tipos anteriores, possuem um formato regular e arredondado, com um, dois ou mais pequenos nucléolos. A heterocromatina possui pontos densos ao longo do núcleo (Leal et al., 2009).

As espermatogônias iniciais do tipo B (B early) são mais condensadas e possuem um núcleo alongado com um ou dois nucléolos pequenos. A quantidade de heterocromatina aumenta. As células do tipo tardia (B late) possuem um número maior de células dentro do cisto. O núcleo é arredondado, denso e heterocromático (Leal et al., 2009) (Fig.2).

Fase espermatocitária ou meiótica

Os espermatócitos apresentam um diâmetro nuclear médio de 5 µm e estão em diferentes fases da meiose. Podem ser identificados e diferenciados pelo tamanho do núcleo, condensação da cromatina e pelas figuras meióticas. Em leptóteno/zigóteno as células são mais arredondadas em relação as espermatogônias do tipo B e a cromatina apresenta pequenos pontos próximos ao envoltório nuclear. Espermatócitos em paquíteno são maiores, o núcleo é denso e os cromossomos estão mais condensados. Já os espermatócitos em diplóteno são encontrados em conjunto com as figuras da metáfase I. Nesse tipo celular os cromossomos atingem o grau máximo de condensação. Por fim, os espermatócitos secundários são considerados raros pois entram rapidamente na meiose II. Este tipo celular possui um núcleo arredondado e cromatina densa (Leal et al., 2009) (Fig. 2).

Espermiogênese

Durante a espermiogênese, o volume nuclear das células é reduzido e a classificação de três tipos de espermátides: iniciais, intermediárias e finais (E1, E2 e E3) são baseadas no aumento da compactação nuclear, diminuição do volume celular e espaçamento entre as células devido a formação dos flagelos (Leal et al., 2009) (Fig. 2).

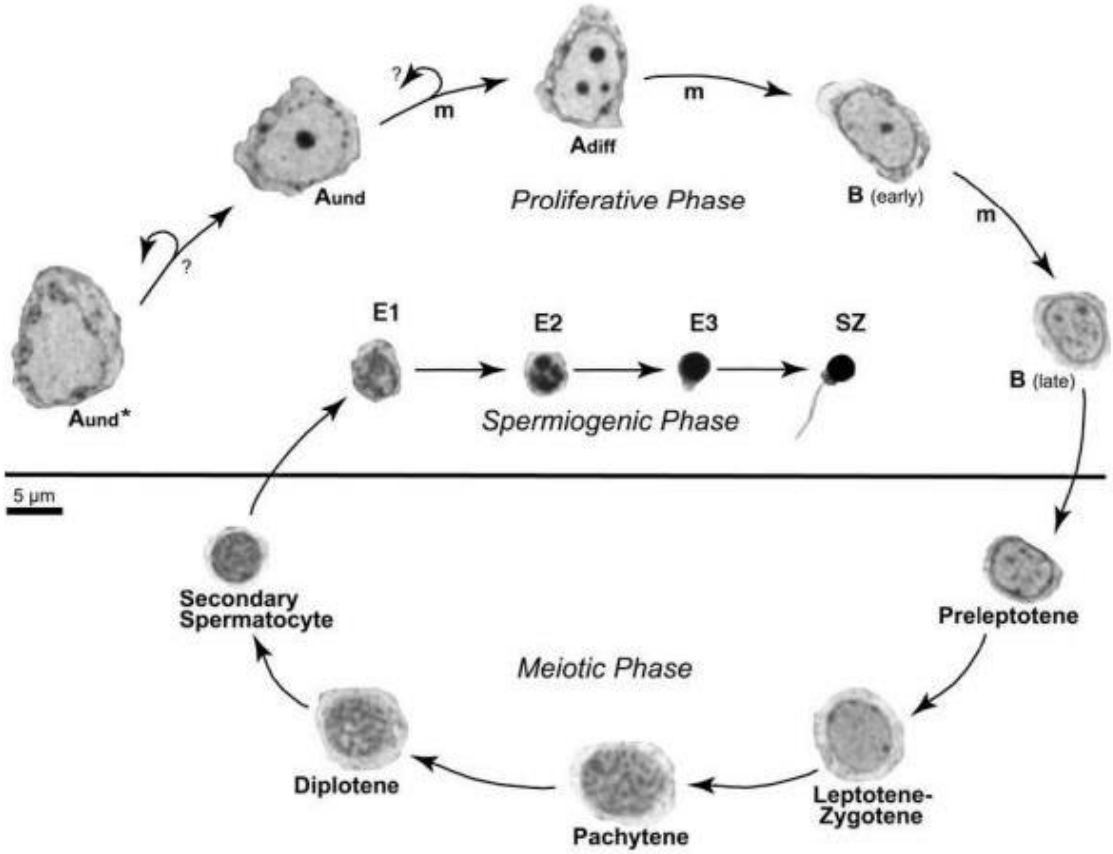

Figura 2: Representação da espermatogênese em zebrafish. **Fase espermatogonial:** espermatogônia indiferenciada do tipo A (A_{und*} e A_{und}), espermatogônia diferenciada (A_{diff}), espermatogônia do tipo B inicial e final (B early-late). **Fase meiótica:** pre-leptóteno, leptóteno/zigóteno, paquíteno, diplóteno e espermatócito secundário. **Fase espermiogênica:** espermátides iniciais, intermediárias e finais (E1,E2,E3) e espermatozoide (SZ). Retirado de Schulz e colaboradores (2010).

1.3. Controle endócrino da gametogênese: Eixo Hipotalâmico-Hipofisário-Gonadal (HHG)

A reprodução é um dos mecanismos mais importantes para a perpetuação das espécies e evolução (Osugi et al., 2014). Para que ocorra o sucesso reprodutivo é necessário a combinação do sistema nervoso com o endócrino, integrado à fatores externos como o fotoperíodo, alimentação e predação e a fatores internos, como ação hormonal. Nos peixes e demais vertebrados, a reprodução é regulada por uma cascata hormonal ao longo do eixo hipotalâmico-

hipofisário-gonadal (HHG) (Fig. 3). Esse sistema é iniciado com a produção do hormônio liberador de gonadotropina (GnRH) proveniente do hipotálamo (Meethal et al., 2005; Habibi e Andreu-Vieyra, 2007) e estimulado pela kisspeptina (Kiss).

O GnRH foi inicialmente descoberto e isolado em porcos (Baba et al., 1971). Estruturalmente é composto por dez aminoácidos, sendo um decapeptídeo e com diferentes isoformas. Este neuropeptídeo tem papel fundamental na ativação do eixo HHG pela estimulação das células hipofisárias produtoras e secretoras das gonadotropinas (Fsh e Lh) (Pankhurst, 2016), que, por sua vez, atuam nas gônadas regulando a gametogênese e esteroidogênese. Interessante que o modo de ação do GnRH para alcançar as células gonadotrópicas é diferente entre teleósteos e os demais vertebrados. Neste último, o GnRH é liberado na eminência média e um sistema vascular porta-hipofisário para o transporte de neuro-hormônios, que o leva até as células gonadotrópicas por meio da corrente sanguínea (Fink, 1988). Em teleósteos, o GnRH é liberado nas proximidades das células gonadotrópicas. Os terminais das fibras dos neurônios na hipófise conectam-se com as células da hipófise e juntos estas fibras formam a neuro-hipófise (Yamamoto et al., 1995).

Peixes teleósteos têm três diferentes formas de GnRH localizadas em diferentes regiões do encéfalo, enquanto a maioria dos vertebrados expressa apenas duas formas de GnRH (Kawai et al., 2009). O GnRH3 foi identificado nos gânglios nervosos terminais apenas nos peixes e acredita-se ser um neuromodulador. O GnRH2 é idêntico nas espécies até hoje examinadas e têm papel na modulação das vias reprodutivas. Já o GnRH1 é a forma hipofisiotrópica

produzidas na área pré-óptica no hipotâlamo que estimula a glândula hipofisária (Kuo et al., 2005; Kawai et al., 2009).

Adicionalmente, foi identificado primeiramente em aves e depois em peixes outro neuropeptídeo com ação regulatória na síntese e liberação de gonadotropinas, o hormônio inibidor de gonadotropinas (GnIH) (Tsutsui et al., 2010; Zohar et al., 2010). O GnIH atua diretamente na hipófise, no entanto como observado por Zohar e colaboradores (2010), pode exercer ação modulatória nos neurônios de GnRH devido as fibras de GnIH serem emitidas na direção do GnRH. Juntos estes neuropeptídeos estão envolvidos no controle da reprodução.

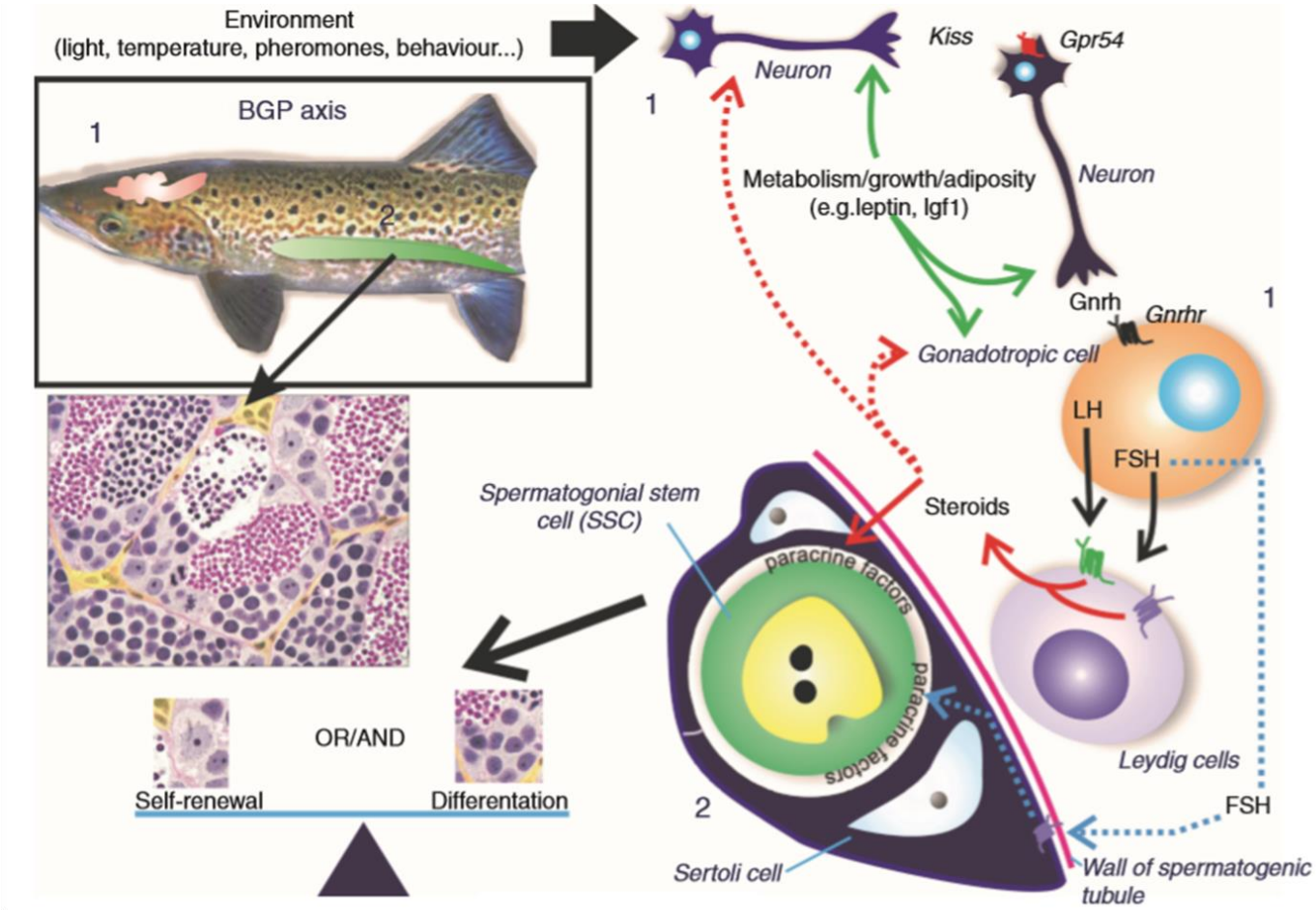

Figura 3: Representação do eixo reprodutivo nos peixes. O início do processo se dá a partir dos estímulos ambientais, como temperatura da água e fotoperíodo seguido pela cascata de hormônios ao longo do eixo hipotalâmico-hipofisário-gonadal, que tem início com os neurônios de kisspeptina (Kiss) no hipotálamo. Por meio de seus receptores (Gpr54), a Kiss estimula a produção do hormônio liberador de gonadotropina (GnRH), que atua sobre as células hipofisárias estimulando a produção e liberação das gonadotropinas (Fsh e Lh). Ambos são levados pela corrente sanguínea atuando em suas células alvo no testículo. Fsh e Lh estimulam a produção e liberação de esteroides, estes tem efeito feedback negativo (linha tracejada) no hipotálamo e hipófise. O Fsh regula o desenvolvimento das espermatogônias tronco (auto-renovação e/ou diferenciação) através da produção de fatores parácrinos produzidos pelas células de Sertoli. (1) representa o sistema nervoso composto pelo hipotálamo e hipófise. (2) indica o testículo. A figura também representa a influência dos fatores internos, como: metabolismo, crescimento, adiposidade, dentre outros) sob os neurônios produtores de Kiss e GnRH. Retirado de Schulz e Nóbrega (2011).

1.4. O papel da gonadotropina Fsh na espermatogênese dos peixes

As gonadotropinas, hormônio folículo estimulante (Fsh) e hormônio luteinizante (Lh) são glicoproteínas formadas por duas subunidades diferentes, a subunidade α, comum aos dois hormônios e a subunidade β, específica de

acordo com a atividade biológica de cada hormônio (Schulz et al., 2001). Estes dois hormônios gonadotrópicos possuem papel crucial na regulação da espermatogênese (Pierce e Parsons, 1981).

Em mamíferos, ambas as gonadotropinas são claramente definidas, dadas as interações altamente específicas entre cada hormônio e seu respectivo receptor (McLachlan et al., 1996). O Lh atua nas células de Leydig estimulando a produção e liberação de andrógeno. Enquanto o Fsh regula a função das células de Sertoli, como suporte estrutural, nutricional e regulatório no desenvolvimento das células germinativas (Huhtaniemi e Themmen, 2005). Porém em peixes, as interações entre as gonadotropinas e seus receptores parecem não serem específicas (Sambroni et al., 2007). Estudos em salmão fornecem evidências de que o receptor de Fsh apesar de ter preferência pelo Fsh também apresenta afinidade pelo Lh (Miwa et al., 1994). Também, em peixes, o Fsh é considerado mais potente que o Lh na estimulação da produção de andrógeno, devido a presença do receptor de Fsh nas células de Leydig (Planas et al., 1993), como descrito em *Clarias gariepinus* (García-Lopez et al., 2009) e *D. rerio* (García-Lopez et al., 2010).

Ainda, segundo Nóbrega e colaboradores (2015) a atuação do Fsh nas células de Sertoli em peixes tem sido associado ao aumento da proliferação e diferenciação de espermatogônias através da produção do fator de crescimento semelhante à insulina 3 (Igf3) e também do peptídeo 3 semelhante a insulina (Insl3) (García-Lopez et al., 2010). Porém, foi demonstrado por Skaar e colaboradores (2011) que o hormônio antimülleriano (Amh), na qual é expresso nas células de Sertoli associadas às espermatogônias indiferenciadas, inibe a ação estimulatória do Fsh sobre os fatores de crescimento. A ação do Amh é

conhecida por bloquear a diferenciação das espermatogônias indiferenciadas e a síntese de esteroides dependentes de Fsh (Skaar et al., 2011; Adolf et al., 2018). Desta forma, pode-se dizer que o Fsh é um importante regulador do processo espermatogênico, atuando na estimulação e inibição de fatores necessários para o desenvolvimento deste processo.

Outro indício que o Fsh está relacionado com a espermatogênese e é um fator chave para esse processo é descrito por Crespo e colaboradores (2016), na qual mostraram que o Fsh estimulou a proliferação espermatogonial acompanhada pela modulação de vários sistemas de sinalização. Além disso essa gonadotropina impactou significativamente nos genes metabólicos, como no metabolismo de lactato e ácidos graxos e nos componentes da barreira das células de Sertoli. Também, estudos realizados com salmonídeos mostraram que o aumento dos níveis de Fsh no plasma estão associados com os elevados níveis de andrógenos e com a maior atividade de proliferação e diferenciação das espermatogônias (Campbell et al., 2003; Melo et al., 2014).

Além disso, há evidências de que o Fsh quando associado aos hormônios tireoidianos (Hts) tem sua ação potencializada, principalmente na liberação de esteroides, como o cyp17a1 nos testículos adultos de zebrafish (Morais et al., 2013).

1.5. O papel dos hormônios tireoidianos (Hts) no desenvolvimento e reprodução dos peixes

O eixo hipotalâmico-hipofisário-tireoide (HHT) é dependente da liberação de TRH (hormônio liberador de tireotrofinas) pelo hipotálamo. O TRH, por sua vez, estimula a secreção do hormônio estimulante da tireoide (TSH) (Larsen et

al., 1998; Flik et al., 2006). Em peixes, a liberação do Tsh é mediado pelo Crh ou Crf (hormônio liberador de corticotrofina) (Flik et al., 2006). O Crf é também o responsável pela liberação do Acth (hormônio corticotrófico) que estimula a síntese e secreção do cortisol pela glândula interrenal, em peixes (Larsen et al., 1998; Flik et al., 2006) (Fig. 4).

O Tsh atua nos folículos tireoidianos estimulando a produção da tetraiodotironina (T4) que é a principal configuração de Ht (Flik et al., 2006). A T4 é metabolizada e convertida em triiodotironina (T3), que corresponde a forma bioativa na maioria dos processos fisiológicos, como crescimento e maturação (Basset et al., 2003; Wagner et al., 2008; Flood et al., 2013).

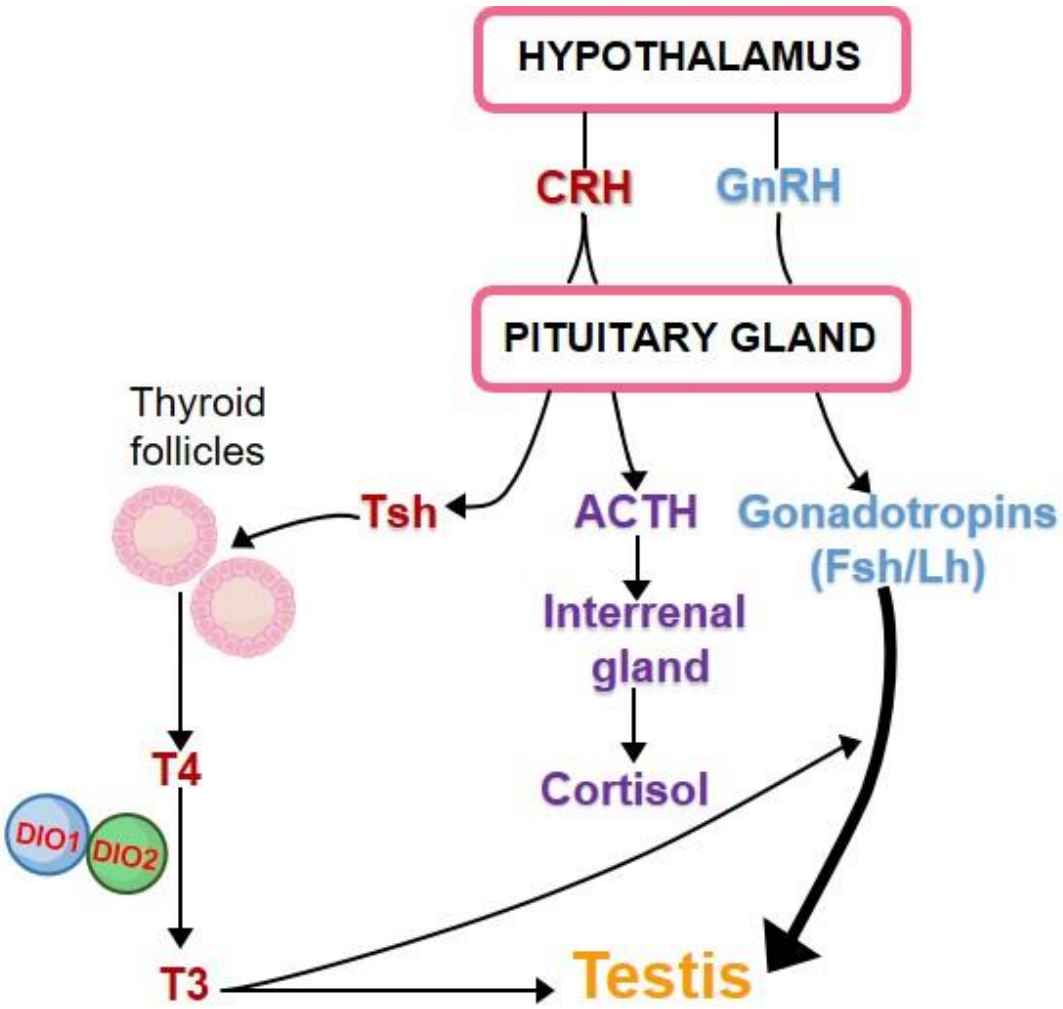

Figura 4: Eixo hipotalâmico-hipofisário-tireoide (HHT). A hipófise secreta Tsh (hormônio estimulador da tireoide). O Crh também estimula o hormônio corticotrófico (Acth), que promove a síntese e secreção do cortisol pela glândula interrenal. Nos folículos tiroidianos, os quais são dispersos em peixes, o Tsh estimula a secreção do T4. Pela ação das (DIO1 e DIO2) convertem o T4 em T3, na qual atuará no eixo reprodutivo. Retirado de Tovo-Neto e colaboradores (2018).

A produção e metabolismo dos Hts ocorrem por meio de três tipos de enzimas denominadas de iodotironinas desiodases, do tipo I, II e III (Fig. 5) (Duarte-Guterman et al., 2014).

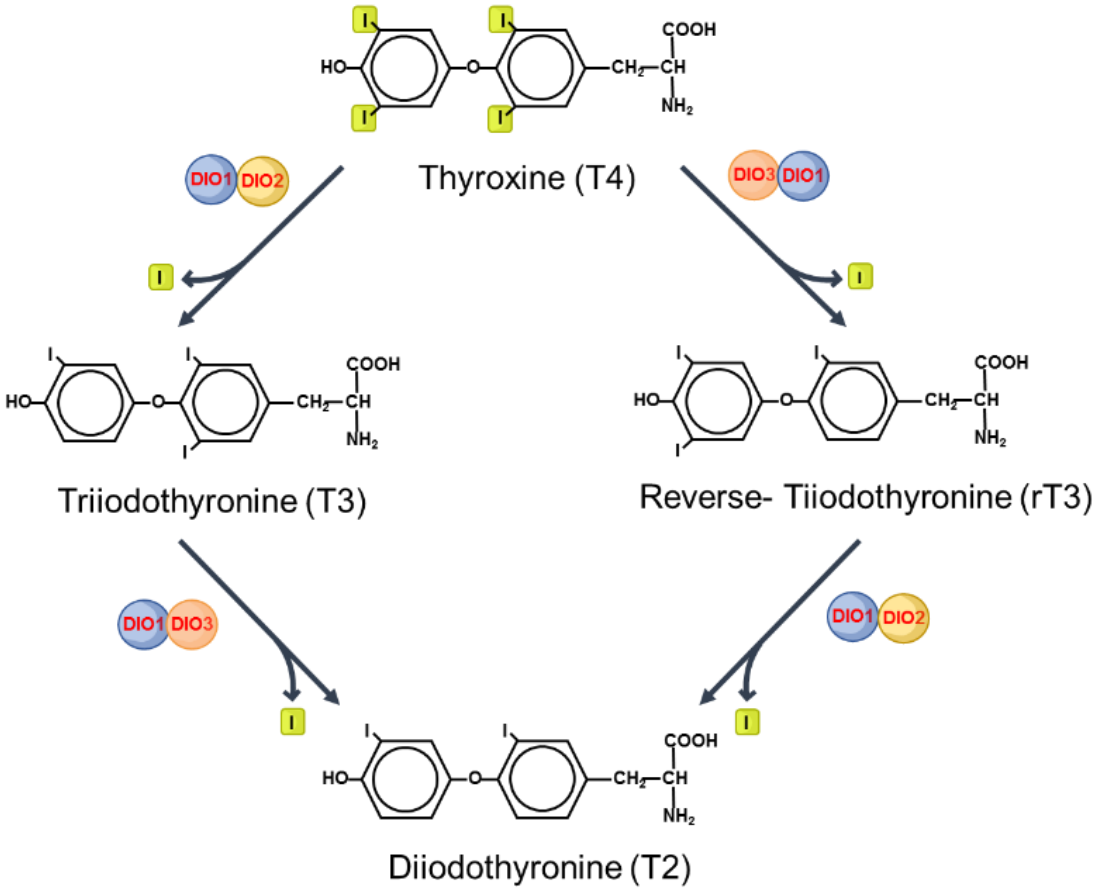

Figura 5: Metabolismo do hormônio tireoidiano (T3) pelas deiodinases. A tiroxina (T4), principal produto secretado pelos folículos tireoidianos, é transformada no hormônio bioativo (T3) pela ação das iodotironinas desiodases tipo I e II (DIO1 e DIO2). A DIO1 também atua na produção do T3r, que é a sua forma inativa por meio da T4. Retirado de Tovo-Neto e colaboradores (2018).

A desiodase tipo I (DIO1) catalisa a desiodação do anel externo da T4 para produzir a forma bioativa, T3. Além disso, a DIO1 também desiodisa o anel interno da T4 para produzir T3 reverso (T3r), que é a sua forma inativa. O tipo II (DIO2) atua exclusivamente na ativação dos Hts pela conversão de T4 em T3

(Duarte-Guterman e Trudeau, 2010). A expressão e atividade das enzimas desiodases nos tecidos individuais regulam a concentração dos Hts ativos dependendo das necessidades específicas do tecido (Flood et al., 2013).

Os receptores dos Hts (TRα e TRβ) atuam principalmente como fatores de transcrição nuclear, os quais ao se ligarem aos Hts formando um complexo hormônio-receptor, e a partir desse momento são capazes de desencadearem respostas celulares pela ligação à região regulatória dos promotores dos genes, estimulando ou inibindo a transcrição (Mendis-Handagama et al., 2005).

Embora os Hts sejam notadamente conhecidos por regularem o metabolismo, sua ação na reprodução vem sendo investigada. Nos roedores machos, o T3 tem a função de regular a maturação e o crescimento dos testículos, controlar a proliferação e diferenciação das células de Sertoli e Leydig durante o desenvolvimento testicular (Cooke et al., 2005).

Em peixes, a estrutura da tireoide é difusa e não encapsulada, embora apresentem uma estrutura folicular semelhante à dos mamíferos (Higgs et al., 1982). A função da tireoide é dependente da captação da iodina, e está envolvida na síntese e transporte dos Hts (Brucker-Davis, 1998). Esses hormônios regulam o crescimento e desenvolvimento (Chang et al., 2012) e suas funções são mediadas por meio de ligações a receptores específicos (Habibi et al., 2012).

Em fêmeas de salmonídeos, os níveis de Hts são mais elevados nos ovos, supostamente de origem materna, devido ao seu acúmulo nos oócitos durante a maturação ovariana (Tagawa et al., 1994). Em outras espécies de peixes teleósteos, tais como o linguado japonês (*Paralichthys olivaceus)*, o zebrafish (*D. rerio*) e a dourada (*Sparus aurata*), os níveis de Hts também permanecem elevados durante o desenvolvimento larval (Power et al., 2001). Na fase adulta,

os níveis de Hts permanecem estáveis (Chang et al., 2012) e estes parecem desempenhar um papel regulatório no eixo reprodutivo.

Neste contexto, Cooke e colaboradores (2005) demonstraram que a alteração da tireoide pode impactar no desenvolvimento da espermatogênese em ratos. A exposição ao 6-propil-2-tiouracil (PTU, um inibidor da produção de Ht) durante o período neonatal de ratos resultou no aumento dos testículos e maior produção de espermatozoides na fase adulta (Cooke 1991). Esse aumento dos testículos no tratamento com PTU é resultante do aumento da área do túbulo seminífero e dos componentes intersticiais (Hess et al., 1993), acompanhado pelo aumento da proliferação das células de Sertoli (Hess et al., 1993). Estudos explicam que esse efeito ocasionado pela ausência dos Hts é resultante do retardamento da transição da fase proliferativa para a fase de maturação das células de Sertoli, resultando no prolongamento da mitose deste tipo celular e consequentemente no amadurecimento destas células (Hess et al., 1993; Cooke et al., 2005). A imaturidade das células de Sertoli torna mais lenta a abertura dos túbulos seminíferos e prejudica a sua capacidade de suportar os estágios mais avançados das células germinativas (França et al., 1995).

Os efeitos do PTU na espermatogênese de peixes também foram avaliados. Matta e colaboradores (2002) avaliaram os efeitos do hipotireoidismo neonatal transitório em juvenis de tilápias do Nilo (*Oreochromis niloticus)*. Nestes animais, o tamanho dos testículos foi duplicado, semelhante ao que foi encontrado em ratos (Joyce et al., 1993). Estes resultados sugerem que o hipotireoidismo neonatal transitório em juvenis de tilápias levou a um aumento na proliferação das células de Sertoli e consequentemente maior proliferação das células germinativas que refletiu no tamanho do testículo.

Outros estudos abordando o papel dos Hts na função reprodutiva foram reportados em goldfish adultos (Habibi et al., 2012). Neste estudo, os animais que receberam doses de T3 (25 e 250 ng/peixe) diminuíram significativamente a expressão dos níveis de RNAm de *lhβ* e *fshβ* (Nelson et al., 2010). Sendo assim, os resultados sugerem que os Hts podem reduzir as funções reprodutivas, considerando que os Hts regulam o metabolismo e estimulam o crescimento na maioria das espécies (Hulbert, 2010), é provável que o aumento dos níveis de Ht possa desviar a energia da reprodução e promover funções somatotrópicas, o que explicaria a redução de Lh e Fsh (Habibi et al., 2012).

Outro resultado interessante é descrito na revisão de Flood e colaboradores (2013) sobre este assunto. Nesta revisão foi demonstrado que Fsh é um importante mediador do hormônio estimulador da tireoide (Tsh) no desenvolvimento sexual em machos e os genes da biossíntese de andrógenos são induzidos pelos Hts (Flood et al., 2013). Desta forma, os Hts induzem respostas na produção e secreção das gonadotropinas e também influenciam a biossíntese dos hormônios esteroides masculinos.

Neste contexto, Morais e colaboradores (2013) mostraram em zebrafish que o hormônio T3 potencializa os efeitos do Fsh nos machos, aumentando a liberação de andrógeno (11KT, principal andrógeno nos peixes) induzido pelo Fsh, bem como a expressão gênica do *ar* (receptor de andrógeno) e da enzima esteroidogênica *cyp17a1*.

1.6. Alteração do estado da tireoide: uma abordagem dos efeitos do hipotireoidismo no desenvolvimento e reprodução dos vertebrados

O uso de goitrogênicos - substâncias que dificultam a absorção de iodo, como PTU, perclorato e methimazole são comuns para entender os mecanismos dos Hts envolvidos no desenvolvimento geral do organismo, incluindo a reprodução (Sharma e Patinõ, 2013; Sharma et al., 2016).

O 6-n-propil-2- tiouracil (PTU) inibe a captação de iodo e a síntese de T4 pela tireoide e também a deiodinização periférica do hormônio T4 em T3 (Aires, 2008). Em roedores, o tratamento com este composto afeta o desenvolvimento da espermatogênese, desde o aumento da população das células de Sertoli no período neonatal (Cooke et al., 1994) até o desenvolvimento normal dos espermatócitos e espermátides (Sakai et al., 2004).

O perclorato bloqueia a captação de iodo no folículo tireoidiano (Sharma e Patinõ, 2013; Sharma et al., 2016). Estudos com peixes mostraram que o hipotireoidismo induzido pelo perclorato induziu a feminização das gônadas durante o desenvolvimento inicial em zebrafish (Mukhi e Patiño, 2007). O methimazole inibe a incorporação de iodo à tireoglobulina, o precursor dos hormônios tireoidianos (T4/T3). Esse composto não induziu a reversão sexual de macho para fêmea em zebrafish, porém promoveu um atraso na transformação do ovário em testículo (Sharma e Patinõ, 2013). O estudo desses compostos em peixes é ainda limitado aos estágios iniciais de desenvolvimento (Vancamp et al., 2018).

1.7. Hormônio Inibidor de Gonadotropina (GnIH)

O hormônio inibidor de gonadotropina (GnIH), neuropeptídeo hipotalâmico descoberto em codornas, está relacionado com a regulação dos processos reprodutivos através da inibição da síntese e secreção das gonadotropinas

(Tsutsui et al., 2010). Esse peptídeo pertence à família LPXRF-amida (X=L ou Q) e foi caracterizado como parte integrante do eixo HHG em muitos vertebrados (Tsutsui et al., 2013). Em mamíferos, por exemplo, foram identificados ortólogos do GnIH que exibem semelhante ação inibitória das gonadotropinas, como em codornas (Tsutsui et al., 2013).

Em peixes, ortólogos de GnIH exercem papel conflitante, pois apresentam ação estimulatória e inibitória na expressão, síntese e liberação das gonadotropinas em diferentes espécies (Ubuka e Parhar, 2018). Em goldfish GnIH foi responsável pela redução dos níveis plasmáticos de Lh (Zhang et al., 2010), porém em *Astyanax altiparanae* (Branco et al., 2018) não foram encontrados efeitos na expressão dos níveis de Fsh e Lh nos explantes hipofisários. Nesse mesmo estudo, o co-tratamento entre GnIH e GnRH mostram que o GnIH promoveu a diminuição das gonadotropinas (Fsh e Lh), exercendo papel regulatório no eixo reprodutivo, modulando as células hipofisárias e a ação do GnRH.

Adicionalmente, ortólogos de GnIH e seus receptores podem ser identificados nos tecidos periféricos, como nas gônadas de diferentes teleósteos, tais como zebrafish e tilápia (Zhang et al., 2010; Muñoz-Cueto et al., 2017; Fallah et al., 2019), sugerindo que esse neuropeptídeo é um potente regulador autócrino e parácrino na função reprodutiva. Em zebrafish, foi demonstrado que *in vitro* o GnIH reduziu a liberação de testosterona (Fallah et al., 2019). O co-tratamento com a gonadotropina coriônica humana (hCG), similar ao hormônio luteinizante (Lh), também foi utilizada nesse estudo para investigar o papel do GnIH na regulação da espermatogênese. Interessante, pois o tratamento com hCG estimulou a produção de testosterona e o aumento da população das

células haploides. Estes resultados demonstram a necessidade das gonadotropinas na regulação da produção de andrógenos para o desenvolvimento da espermiogênese. Diretamente nos testículos, a alta concentração de GnIH (1000 nM) aumentou o número de espermátides e espermatozoides.

Qi e colaboradores (2013), por exemplo, avaliaram *in vitro* os efeitos do GnIH na esteroidogênese de machos e fêmeas de goldfish (*Carassius auratus*). Nas fêmeas não houve mudanças nos níveis de estradiol. No entanto, os machos apresentaram aumento nos níveis de testosterona no plasma, o que indica que o peptídeo pode modular a síntese de andrógeno. Foi observado que o rg GnIH (recombinante de goldfish) estimulou a liberação das gonadotropinas e do hormônio de crescimento (GH) em salmão (*Oncorhynchus nerka*) (Amano et al., 2006). Porém, Zhang e colaboradores (2010) mostraram que rzf GnIH (recombinante de zebrafish) diminuiu os níveis plasmáticos de Lh em goldfish adulto. Segundo Muñoz-Cueto e colaboradores (2017) as diferenças fisiológicas encontradas nestes trabalhos podem ser devido ao uso inespecífico de recombinantes para mostrar os efeitos fisiológicos espécie-específica. Além do GnIH ser considerado um regulador do eixo reprodutivo, há indícios de que esse peptídeo também regula o eixo tireoidiano (HHT) (ver abaixo) (Tsutsui et al., 2018).

1.8. Interação do GnIH nos eixos HHG-HHT na regulação da reprodução

Segundo Tsutsui e colaboradores (2018) a atividade da tireoide está relacionada a alterações do eixo reprodutivo e ao desenvolvimento inicial da puberdade em mamíferos. Na revisão de Tsutsui e colaboradores (2018) foi

mostrado que as fêmeas de camundongos induzidas quimicamente ao hipotireoidismo com PTU apresentaram níveis de GnIH elevado e níveis mais baixos de gonadotropinas e estradiol, refletindo na puberdade tardia observada nestes animais. Isso ocorre porque neurônios hipotalâmicos que produzem GnIH também expressam os receptores dos hormônios tireoidianos (TRα e TRβ) (Tsutsui et al., 2018).

Com base nesses experimentos, Kiyohara e colaboradores (2017) demostraram que fêmeas de camundongos GnIH-KO e também tratadas com PTU não apresentaram atraso na puberdade, enquanto em fêmeas normais o hipotireoidismo retardou o início da puberdade. Dessa forma, o GnIH pode mediar a disfunção tireoidiana no desenvolvimento da puberdade. A hipótese sugerida é que o GnIH é um potente mediador da interação dos eixos HHG-HHT durante o desenvolvimento reprodutivo nos vertebrados, como ilustra a figura abaixo (Fig. 7).

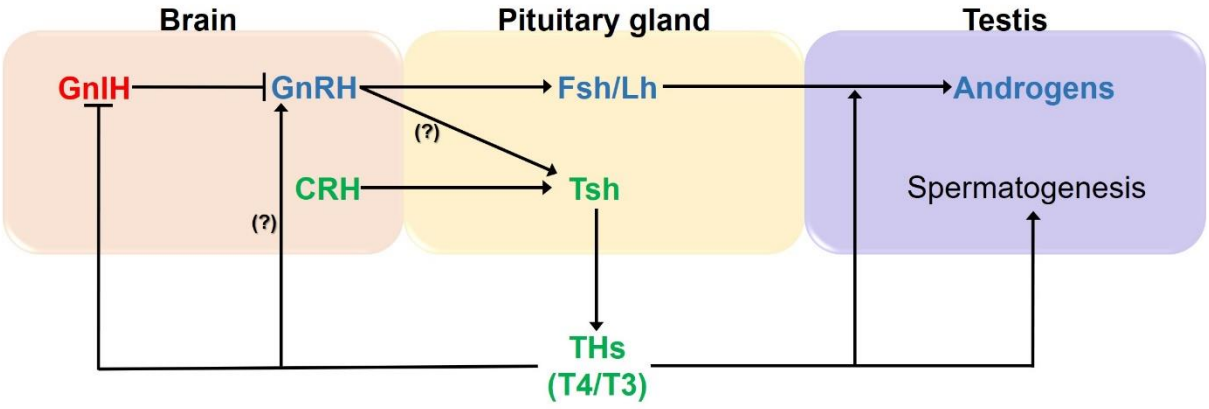

Figura 7: Efeitos dos hormônios tireoidianos (Hts) (T4/T3) no eixo reprodutivo de vertebrados. Altas concentrações de Hts inibem a síntese de GnIH (Hormônio inibidor das gonadotropinas). Na ausência dos Hts a expressão de GnIH aumenta. A interrogação (?) significa que os dados na literatura são inconclusivos sobre o papel regulatório entre GnRH e os Hts. Retirado de Tovo-Neto e colaboradores (2018).

1.9. O zebrafish como modelo biológico

O *Danio rerio* é um pequeno teleósteo de água doce originário da Índia, pertencente à família Cyprinidae e à ordem Cypriniformes, conhecido popularmente como zebrafish. Além do tamanho reduzido, esta espécie se destaca pelo fácil manejo em laboratório, podendo ser mantido em altas densidades populacionais, apresentando fertilização externa com geração de alto número de indivíduos e os embriões são transparentes, facilitando a visualização e monitoramento de seu desenvolvimento (Nasiadka e Clark, 2012). Além disso, este teleósteo possui uma vida média de três anos, com a primeira maturação podendo ocorrer nos primeiros três meses de vida, a temperatura média ideal para se reproduzirem varia de 27ºC a 28ºC, por aproximadamente 18 meses e a fêmea pode desovar em torno de 200-300 ovos semanalmente. Estas características credenciam esta espécie como um excelente modelo biológico para pesquisas científicas experimentais, seja na área da fisiologia, toxicologia ou endocrinologia (McGonnell e Fowkes, 2006).

O sequenciamento do genoma desta espécie, disponibilizado no site ZFIN (The Zebrafish Information Network - https://zfin.org/), despertou ainda mais o interesse da comunidade científica em utilizar este modelo como referência para inúmeras pesquisas, possibilitando que estudos genéticos também sejam aplicados neste teleósteo, inclusive a geração de excelentes modelos transgênicos na área da biologia da reprodução (McGonnell e Fowkes, 2006).

2. JUSTIFICATIVA

Dentre os aspectos hormonais que influenciam o desenvolvimento da espermatogênese, pode-se destacar o papel dos hormônios tireoidianos (Hts). Esses hormônios desempenham funções importantes para a homeostase dos

organismos, modulando processos fisiológicos, tais como a regulação do crescimento, comportamento, estresse e reprodução em vertebrados (Terrien e Prunet, 2013). Segundo Habibi e colaboradores (2012), a relação entre os Hts e a reprodução tem sido investigada, mostrando ser espécie-específica, e importante na regulação dos processos reprodutivos. No entanto, os mecanismos que regulam essa relação ainda é desconhecida. Com base nisso, a presente dissertação fornece informações sobre os efeitos do hipotireoidismo no desenvolvimento da espermatogênese e nos diferentes níveis do eixo hipotalâmico-hipofisário-gonadal de machos adultos de zebrafish.

3. OBJETIVOS

Avaliar os efeitos do hipotireoidismo *in vivo* e *in vitro* na função testicular de machos adultos de *D. rerio* e a relação dos hormônios tireoidianos (Hts) com o hormônio folículo estimulante (Fsh) e com hormônio inibidor de gonadotropina (GnIH) no eixo reprodutivo.

Objetivos específicos:

1. Avaliar os efeitos *in vivo* do hipotireoidismo induzido farmacologicamente (methimazole) na espermatogênese e no eixo hipotalâmico-hipofisário-gonadal de machos adultos de zebrafish;

2. Investigar os efeitos *in vitro* do Fsh na capacidade de liberação de andrógenos e expressão gênica de testículos de zebrafish adultos induzidos farmacologicamente ao hipotireoidismo (methimazole);

3. Investigar os efeitos *in vivo e in vitro* dos Hts na espermatogênese e eixo hipotalâmico-hipofisário-gonadal de machos adultos de zebrafish;

4. Verificar a relação do GnIH com os Hts nos testículos de machos adultos de zebrafish.

4. REFERÊNCIAS

ADOLF, M.C.; NAKAJIMA, R.T.; NÓBREGA, R.H.; SCHARTL, M. Intersex, hermaphroditism, and gonadal plasticity in vertebrates: evolution of the Mullerian duct and Amh/Amhr2 signaling. **Annual review of animal biosciences**. 7:7.1-7.24. 2018.

AIRES, M.M. Fisiologia. 3º ed. Rio de Janeiro, Guanabara Koogan.2008.

AMANO M, MORIYAMA S, IIGO M, KITAMURA S, AMIYA N, YAMAMORI K, UKENA K, TSUTSUI K. Novel fish hypothalamic neuropeptides stimulate the release of gonadotrophins and growth hormone from the pituitary of sockeye salmon. **J Endocrinol**. 188:417–423. 2006.

BABA, Y., MATSUO, H., SCHALLY, A.V. Structure of the porcine LH- and FSH-releasing hormone. II. Confirmation of the proposed structure by conventional sequential analyses. **Biochemical and Biophysical Research Communications** 44, 459-463. 1971.

BASSET, J.H.D, HARVEY, C.B., WILLIAMS, G.R. Mechanisms of thyroid hormone receptor-specific nuclear and extra nuclear actions. **Mol. Cell.** Endocrinol. 213, 1-11. 2003.

BILLARD R. Attempts to inhibit testicular growth in rainbow trout with antiandrogens (cyproterone, cyproterone acetate, oxymetholone) and busulfan given during the period of spermatogenesis. **Gen Comp Endocr.** 48:33-8.1982.

BRANCO, G. S.; MELO, A. G.; RICCI, J. M. B.; DIGMAYER, M.; DE JESUS, L. W. O.; HABIBI, H. R.; NÓBREGA, R. H. Effects of GnRH and the dual regulatory actions of GnIH in the pituitary explants and brain slices of Astyanax altiparanae males. **General and Comparative Endocrinology**. 2018.

BRUCKER-DAVIS F. Effects of environmental synthetic chemicals on thyroid function. **Thyroid**, 8: 827–856. 1998.

CAMPBELL, B.; DICKEY, J.T.; SWANSON, P. Endocrine changes during onset of puberty in male spring Chinook salmon, *Oncorhynchus tshawytscha*. **Biol Reprod**, v. 69, n. 6, p. 2109-2117, 2003.

CHANG, J.; WANG, M.; GUI, W.; ZHAO, Y.; YU, L.; ZHU, G. Changes in Thyroid Hormone Levels during Zebrafish Development. **Zoological Science**, v. 29, n. 3, p. 181-184. 2012.

COOKE, P.S. Thyroid hormones and testis development: a model system for increasing testis growth and sperm production. In: Robaire B, ed. The Male Germ Cell: Spermalogonium to Fertilization**. Ann NYAcadSci**, 1991.

COOKE, P.S., ZHAO, Y.D., BUNICK, D. Triiodothyronine inhibits proliferation and stimulates differentiation of cultured neonatal Sertoli cells: possible mechanism for increased adult testis weight and sperm production induced by neonatal goitrogen treatment. **Biol. Reprod**. 51, 1000–1005. 1994.

COOKE, P.S, HOLSBERGER DR, FRANÇA LR. Thyroid hormone regulation of Sertoli cell development. In*:* Skinner MK, Griswold MD (Ed.). **The Sertoli Cell Biology**. Philadelphia, PA: Elsevier Science. Pp. 217-226. 2005.

CRESPO, D.; ASSIS, L.H.C.; FURMANEK, T.; BOGERD, J.; SCHULZ, R.W. Expression profiling identifies Sertoli and Leydig cell genes as Fsh targets in adult zebrafish testis. **Molecular and Cellular Endocrinology**. vol. 437, p.237-251. 2016.

DUARTE-GUTERMAN, P.; NAVARRO-MARTÍN, L.; TRUDEAU, V.L. Mechanisms of crosstalk between endocrine system: Regulation of sex steroid hormone synthesis and action by thyroid hormones. **General and Comparative Endocrinology**, in press. 2014.

DUARTE-GUTERMAN, P.; TRUDEAU, V.L. Regulation of Thyroid Hormone-, Oestrogen- and Androgen- Related Genes by Triiodothyronine in the Brain of *Silurana tropicalis*. **Journal of Neuroendocrinology**, vol. 22, p. 1023-1031. 2010.

FALLAH, H. P., TOVO-NETO, A., YEUNG, E. C., NÓBREGA, R. H., HABIBI, H. R. Paracrine/autocrine control of spermatogenesis by gonadotropin-inhibitory hormone. Molecular and Cellular Endocrinology. 2019.

FRANÇA, L.R., HESS, C.A., COOKE, P.S., RUSSEL, L.D. Neonatal hypothyroidism causes delayed Sertoli cell maturation in rats treated with propylthiouracil: evidence that the Sertoli cell controls testis growth. T**he Anatomical Record**. 242: 57-69.1995.

FINK, G. Gonadotropin secretion and its control. In: Knobil E, Neil, J. The physiology of reproduction. New York: Raven Press. 1349-1377. 1988.

FLOOD, D.E.K.; FERNANDINO, J.I.; LANGLOIS, V.S. Thyroid hormones in male reproductive development: Evidence for direct crosstalk between the androgen and thyroid hormone axes. **General and Comparative Endocrinology**, vol. 192, p. 2-14. 2013.

FLIK, G.; KLAREN, P.H.M.; BURG, E.V.; METZ, J.; HUISING, M.O. CRF and stress in fish. **General and Comparative Endocrinology**. 146: 36-44.2006.

FRANÇA, L.R.; HESS, R.A; DUFOUR, J.M.; HOFMAN, M.C.; GRISWOLD, M.D. The Sertoli cell: one hundred fifty years of beauty and plasticity. **Andrology**. Vol. 4. 2016.

GARCÍA-LÓPEZ, A.; BOGERD, J.; GRANNEMAN, J.C.M.; et al. Leydig cells express follicle-stimulating hormone receptors in African catfish. **Endocrinology**, v. 150, n. 1, p. 357–365, 2009.

GARCÍA-LÓPEZ, A.; JONGE, H.; NOBREGA, R.H.; WAAL, P.P.; VAN DIJK, W.; HEMRIKA, W.; TARANGER, G.L.; BOGERD, J.; SCHULZ, R.W. Studies in Zebrafish Reveal Unusual Cellular Expression Patterns of Gonadotropin

Receptor Messenger Ribonucleic Acids in the Testis and Unexpected Functional Differentiation of the Gonadotropins. **Endocrinology**. 2010.

GRIER, H.J.; LINTON, J.R.; LEATHERLAND, J.F.; VLAMING, V.L. Structural evidence for two different testicular types in Teleost fishes. **The American Journal of Anatomy**, 331-345. 1980.

GRIER, H. J. & M. C. U. ARANZABAL. The Testis and Spermatogenesis in Teleost. Pp. 119-142. In: Jamieson, B. G. M. (Ed.). **Reproductive Biology and Phylogeny of Fishes (Agnathans and Bony Fishes).**Vol. 8. Part B. Enfield: Science Publishers. 2009.

HABIBI, H.R., ANDREU-VIEYRA, C.V. Hormonal Regulation of Follicular atresia in the teleost fish. In: Babin, P.J., Cerda, J., Lubzens, E. (Eds.), The Fish Oocyte: From Basic Studies to Biotechnological Applications. **Springer Pub Co**., pp. 231–250. 2007.

HABIBI, H.R.; NELSON, E.R.; ALLAN, E.R.O. New insights into thyroid hormone function and modulation of reproduction in goldfish. **General and Comparative Endocrinology**, vol. 175, p. 19-26. 2012.

HIGGS, D.A.; FAGERLUND, U.H.M.; EALES, J.G.; McBRIDE, J.R. Application of thyroid and steroid hormones as anabolic agents in fish culture. **Comparative Biochemistry and Physiology**, vol. 73B, n. 1, p. 143-176. 1982.

HESS, R.A; COOKE, P.S; BUNICK, D.; KIRBY, J.D. Increased Sertoli and germ cell numbers accompany adult testicular enlargement induced by neonatal hypothyroidism. **Endocrinology**, 32:2607-2613. 1993.

HUHTANIEMI, I.T., THEMMEN, A.P. Mutations in human gonadotropin and gonadotropin receptor genes. **Endocrine** 26, 207–217. 2005.

HULBERT, A.J. Thyroid hormones and their effects: a new perspective. **Biol. Rev.** 75. 519–631. 2000.

JOYCE, K.L.; PORCELLI, J.; COOKE, P.S. Neonatal goitrogen treatment increases adult testis size and sperm production in the mouse. **Journal of Andrology**, 14:448–455. 1993.

KAWAI T, OKA Y, EISTHEN H. The role of the terminal nerve and GnRH in olfactory system neuromodulation. **Zoolog Sci.** 26:669–80. doi: 10.2108/zsj.26.669. 2009.

KIYOHARA M, SON YL, TSUTSUI K. Involvement of gonadotropin-inhibitory hormone in pubertal disorders induced by thyroid status. **Sci Rep** 7:1042.2017.

KUO, M.W., LOU, SW., POSTLETHWAIT, J., CHUNG, B.C. Chromossomal organization, evolutionary relationship and expression of zebrafish GnRH family members. **J BiomSci**. 12:629-639. 2005.

LARSEN, D.A., SWANSON, P., DICKEY, J.T., RIVIER, J., DICKHOFF, W.W. In Vitro Thyrotropin-Releasing Activity of Corticotropin-Releasing Hormone-Family Peptides in Coho Salmon, Oncorhynchus kisutch. **Gen. Comp. Endocrinol**. 109, 276-285. 1998.

LEAL, M.C.; CARDOSO, E.R.; NOBREGA, R.H.; BATLOUNI, S.R.; BOGERD, J.; FRANCA, L.R.; SCHULZ, R.W. Histological and stereological evaluation of zebrafish (*Danio rerio*) spermatogenesis with an emphasis on spermatogonial generations. **Biol Reprod**, Vol. 81, n. 1, pp. 177-87. 2009a.

MATTA, S.L; VILELA, D.A; GODINHO, H.P; FRANÇA, L.R**.** The goitrogen 6-n-propyl-2-thiouracil (PTU) given during testis development increases Sertoli and germ cell numbers per cyst in fish: the tilapia (*Oreochromis niloticus*) model. **Endocrinology**, 143: 970 –978. 2002.

McGONNELL, I.M.; FOWKES, R.C. Fishing for gene function – endocrine modelling in the zebrafish. **Journal of Endocrinology**, vol. 189, p. 425-439. 2006.

MCLACHLAN, R.I.; WREFORD, N.G.; O'DONNELL, L.; DE KRETSER, D.M.; ROBERTSON, D.M. The endocrine regulation of spermatogenesis: independent roles for testosterone and FSH. **J Endocrinol** 148:1–9. 1996.

MELO, M.C.; ANDERSSON E.; FJELLDAL, P.G.; BOGERD, J.; FRANÇA, L.R.; TARANGER, G.L.; SCHULZ, R.W. Salinity and photoperiod modulate pubertal development in Atlantic salmon (*Salmo salar*). **J Endocrinol**, 220: 319-332. 2014.

MENDIS-HANDAGAMA, S.M.L.C.; ARIYARATNE, H.B.S. Leydig cells, thyroid hormones and steroidogenesis. **Indian Journal of Experimental Biology**, vol. 43, p. 939-962. 2005.

MEETHAL, S. V.; ATWOOD, C. S. Alzheimers disease: the impact of age-related changes in reproductive hormones. CMLS Cellular and Molecular Life Sciences, 62(3), 257–270.doi:10.1007/s00018-004-4381-3. 2005.

MIWA, S.; YAN, L.; SWANSON, P. Localization of two gonadotropin receptors in the salmon gonad by in vitro ligand autoradiography. **Biol Reprod** 59:629 – 642. 1994.

MORAIS, R.D.; NOBREGA, R.H.; GOMEZ-GONZALEZ, N.E.; SCHMIDT, R.; BOGERD, J.; FRANÇA, L.R.; SCHULZ, R.W. Thyroid hormone stimulates the proliferation of Sertoli cells and single type A spermatogonia in adult zebrafish (*Danio rerio*) testis. **Endocrinology**, vol. 154, p. 4365-4376. 2013.

MUÑOZ-CUETO, J.A., PAULLADA-SALMERÓN, J.A., ALIAGA-GUERRERO, M., COWAN, M.E., PARHAR, I.S., UBUKA, T. A Journey through the Gonadotropin-Inhibitory Hormone System of Fish 2017.

MUKHI, S., PATIÑO, R. Effects of Prolonged Exposure to Perchlorate on Thyroid and Reproductive Function in Zebrafish. **Toxicol. Sci.** 96, 246-254. 2007.

NASIADKA, A.; CLARK, M.D. Zebrafish Breeding in the Laboratory Environment. **ILAR Journal**, v. 53, n. 2, p. 161–168, 2012.

NELSON, J.S. Fishes of the world 50 ed. Wiley – Interscience, New York. 624p. 2006.

NELSON, J.S.; GRANDE, T.; WILSON, M.V.H. Fishes of the world 5th ed. Wiley – Interscience, New Jersey. 752p. 2016.

NELSON, E.R.; ALLAN, E.R.; PANG, F.Y.; HABIBI, H.R. Thyroid hormone and reproduction: regulation of estrogen receptors in goldfish gonads, **Mol Reprod Dev**, 77. 784–794. 2010.

NÓBREGA, R.H.; BATLOUNI, S.R.; FRANÇA, L.R. An overview of functional and stereological evaluation of spermatogenesis and germ cell transplantation in fish. **Fish Physiology Biochemistry,** 35: 197-206. 2009.

NÓBREGA, R.H.; GREEBE, C.D.; VAN DE KANT, H.; BOGERD, J.; DE FRANCA, L.R.; SCHULZ, R.W. Spermatogonial stem cell niche and spermatogonial stem cell transplantation in zebrafish. **PLoS ONE**, vol. 5, n. 9, e12808. 2010.

NÓBREGA, R.H; MORAIS, R.D.V.S.; CRESPO, D.; WAAL, P.P.; FRANÇA, L.R.; SCHULZ, R.W.; BOGERD, J. Fsh Stimulates Spermatogonial Proliferation and Differentiation in Zebrafish via Igf3. **Endocrinology**, 156 (10): 3804–3817. 2015.

OSUGI, T., UBUKA, T., TSUTSUI, K. Review: evolution of GnIH and related peptides structure and function in the chordates. **Frontiers in Neuroscience**, 8.doi:10.3389/fnins.2014.00255. 2014.

PANKHURST, N.W. Reproduction and Development, In: SCHRECK, C.B.; TORT, L.; FARRELL, A.; BRAUNER, C. **Biology of Stress in Fish**. Academic Press. p.295-331. 2016.

PIERCE, J.G.; PARSONS, T.F. Glycoprotein hormones: structure and function. **Annu Rev Biochem**, 50: 465–495. 1981.

PLANAS, J.V., SWANSON, P., DICKHOFF, W.W. Regulation of testicular steroid production in vitro by gonadotropins (GTH I and GTH II) and cyclic AMP in Coho salmon (*Oncorhynchus kisutch*). **Gen. Comp. Endocrinol**. 91, 8–24. 1993.

POWER, D.M.; LLEWELLYN, L.; FAUSTINO, M.; NOWELL, M.A.; BJORNSSON, B.T.; EINARSDOTTIR, I.E.; et al. Thyroid hormones in growth and development of fish. Comparative Biochemistry and Physiology Part C: **Toxicology Pharmacology**, 130(4): 447-59. 2001.

QI, X., ZHOU, W., LU, D., WANG, Q., ZHANG, H., LI, S., LIN, H. Sexual Dimorphism of Steroidogenesis Regulated by GnIH in the Goldfish, Carassius auratus1. **Biology of Reproduction**, 88(4). 2013.

REIS R. E.; KULLANDER S. O.; FERRARIS J.R; C. J. Check list of the freshwater fishes of south and Central America. **Edipucrs**. Porto Alegre. 742p. 2003.

SAMBRONI, E., LEGAC, F., BRETON, B., LAREYRE, J.J. Functional specificity of the rainbow trout (Oncorhynchus mykiss) gonadotropin receptors as assayed in a mammalian cell line. **J. Endocrinol.** 195, 213–228. 2007.

SAKAI, Y., YAMASHINA, S., & FURUDATE, S. Developmental delay and unstable state of the testes in the rdw rat with congenital hypothyroidism. **Development, Growth and Differentiation**. 46(4), 327–334. 2004.

SKAAR,K.S.; NÓBREGA,R.H.; MAGARAKI,A.; OLSEN,L.C.; SCHULZ,R.W. Ma le Proteolytically activated, recombinant anti-mullerian hormone inhibits androgen secretion, proliferation, and differentiation of spermatogonia in adult zebrafish testis organ cultures. **Endocrinology**.152 pp. 3527-3540. 2011.

SCHULZ, R.W.; VISCHER, H.F.; CAVACO, J.E.; SANTOS, E.M.; TYLER, C.R.; GOOS, H.J.; BOGERD, J. Gonadotropins, their receptors, and the regulation of testicular functions in fish. **Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology**, v. 129, p. 407–17, 2001.

SCHULZ, R.W.; FRANÇA, L.R.; LAREYRE, J.J.; LEGAC, F.; CHIARINI-GARCIA, H.; NOBREGA, R.H.; MIURA, T. Spermatogenesis in fish. **General and Comparative Endocrinology**, 165: 390-411. 2010.

SCHULZ R. W. & NÓBREGA R. H. Regulation of Spermatogenesis. In: Farrell A.P., (ed.), **Encyclopedia of Fish Physiology: From Genome to Environment**, San Diego: Academic Press v.1, p. 627–634. 2011.

SHARMA, P., PATIÑO, R. Regulation of gonadal sex ratios and pubertal development by the thyroid endocrine system in zebrafish. Gen. Comp. Endocrinol. 184, 111-119. 2013.

SHARMA, P.; TANG, S.; MAYER, G.D.; PATIÑO, R. Effects of thyroid endocrine manipulation on sex-related gene expression and population sex ratios in Zebrafish. **General and Comparative Endocrinology**, vol. 235, p. 38-47. 2016.

SIQUEIRA-SILVA, D.H.; DA SILVA COSTA, R.; NINHAUS-SILVEIRA, A.; VICENTINI, C.A.; VERÍSSIMO-SILVEIRA, R. Ultrastructural analysis of spermiogenesis in the neotropical cichlid Kullander (Perciformes: Cichlidae). **Journal of Applied Ichthyology**, 28: 878-882. 2012.

SIQUEIRA-SILVA, D.H.; RODRIGUES, M. S.; NOBREGA, R. H. Testis structure, spermatogonial niche and Sertoli cell efficiency in Neotropical fish. **General and comparative endocrinology**. v. x, p. 1, 2018.

TAGAWA, M.; OGASAWARA, T.; SAKAMOTO, T.; MIURA, T.; YAMAUCHI, K.; HIRANO, T. Thyroid hormone concentrations in the gonads of wild chum salmon during maturation. **Fish Physiol Biochem**, 13, 233-240. 1994.

TSUTSUI, K.; BENTLEY, G. E.; BEDECARRATS, G.; OSUGI, T.; UBUKA, T.; AND KRIEGSFELD, L. J. Review: gonadotropin-inhibitory hormone (GnIH) and its control of central and peripheral reproductive function. **Front. Neuroendocrinol**. 31, 284–295. 2010.

TSUTSUI, K.; UBUKA, T. Gonadotropin-inhibitory hormone, in Handbook of Biologically Active Peptides. Section on Brain Peptides, eds A. J. Kastin and H. Vaudry (London: Academic Press). 802–811. 2013.

TSUTSUI, K., SON, Y.S., KIYOHARA, M., MIYATA, I. Discovery of GnIH and Its Role in Hypothyroidism-Induced Delayed Puberty. **Endocrinology** 159, 62-68. 2018.

TERRIEN, X.; PRUNET, P. Cross regulation of the Thyroid Hormone and Corticosteroids in Amphibians and Fish: The Effects of Endocrine Disruption. **Insights from Veterinary Medicine**, 2013.

TOVO-NETO, A., DA SILVA RODRIGUES, M., HABIBI, H. R., NÓBREGA, R. H. Thyroid hormone actions on male reproductive system of teleost fish. **General and Comparative Endocrinology**. 265-230. 2018.

UBUKA, T., PARHAR, I. Dual Actions of Mammalian and Piscine GonadotropinInhibitory Hormones, RFamide-Related Peptides and LPXRFamide Peptides, in the Hypothalamic–Pituitary–Gonadal Axis. Front. Endocrinol. 8:377. doi: 10.3389/fendo.2017.00377. 2018.

VANCAMP, P., HOUBRECHTS, A.M., DARRAS, V.M. Insights from zebrafish deficiency models to understand the impact of local thyroid hormone regulator action on early development. **General and Comparative Endocrinology**. 2018.

VILELA, D.A.R.; SILVA, S.G.B.; PEIXOTO, M.T.D.; GODINHO, H.P.; FRANÇA, L.R. Spermatogenesis in teleost: insights from the Nile tilapia (*Oreochromis niloticus)* model. **Fish Physiology and Biochemistry**, 28: 187-190. 2003.

VAZZOLER A. E. A. DE M. Biologia da reprodução de peixes teleósteos: teoria e prática. Maringá, EDUEM/SBI/CNPq/Nupélia. 1996.

WAGNER, M.S.; WAJNER, S.M.; MAIA, A.L. The Role of Thyroid Hormone in Testicular Development and Function. **J Endocrinol**, 199(3): 351–365. 2008.

YAMAMOTO, N.; OKA, Y.; AMANO, M.; AIDA, K.; HASEGAWA, Y.; KAWASHIMA, S. Multiple gonadotropin-releasing hormone (GnRH)-immunoreactive system in the brain of the dwarf gourami, *Colisa lalia*: immunohistochemistry and radioimmunassay. **J Comp Neurol**. 355: 354-368. 1995.

ZHANG, Y.; LI, S.; LIU, Y.; LU, D.; CHEN, H.; HUANG, X. Structural diversity of the GnIH/GnIH receptor system in teleost: its involvement in early development and the negative control of LH release. **Peptides**. 31.1034–1043. 2010.

ZOHAR, Y., MUNOZ-CUETO, J.A., ELIZUR, A., KAH, O. Neuroendocrinology of reproduction in teleost fish. **Gen Comp Endocrinol**. 165:438-55. 2010.

CAPÍTULO 1

ROLE OF THYROID HORMONES IN THE BRAIN-PITUITARY-TESTES AXIS OF ADULT ZEBRAFISH, *Danio rerio*

Artigo em preparação

Role of thyroid hormones in the brain-pituitary-testes axis of adult zebrafish, *Danio rerio*

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ABSTRACT

Thyroid hormones (Ths) plays a crucial role on the vertebrates homeostasis, including regulating of growth, metabolism and reproduction. An increasing number of studies have been focused on the involvement of Ths in the male reproductive system of vertebrates, including fish. The data in the literature have showed that Ths have effects on different levels of the brain-pituitary-testes axis. However, the role of Ths regulation on male reproductive system in adult fish remains unclear. In this study, we discuss the effects of *in vivo* and *in vitro* the of thyroid hormones on the zebrafish spermatogenesis and the correlation between Ths and zebrafish reproductive system. *in vivo* we show that exposure to methimazole-induced hypothyroidism affected the progression of zebrafish spermatogenesis by inducing the accumulation of pre-meiotic cells, delaying cell-differentiation and meiosis, as well as reducing the number of spermatozoa. This delayed suggest that Ths play a crucial role in the entry of meiosis. Using this approach, we demonstrated that gene expression of brain-pituitary axis was antagonistically affected both in the methimazole-treatment or T3-treatment. It was established *in vitro* that the effects of the follicle-stimulating hormone (Fsh) on testis function are dependent of thyroid hormones. In conclusion, this results demonstrate that Ths exert a modulatory influence on brain-pituitary axis and consequently the spermatogenic function in adult zebrafish.

Key-words: triiodothyronine (T3), hypothyroidism, methimazole, spermatogenesis, zebrafish

INTRODUCTION

The thyroid hormones play an essential role in growth, metabolism and reproduction in vertebrates (Mullur et al., 2014). In rats, tri-iodothyronine (T3) affects Sertoli cell proliferation and differentiation, the onset of adult Leydig cell population formation and their function during neonatal-prepubertal period (Teerds et al., 1998; Ariyaratne et al., 2000). Induced-hypothyroidism in the neonatal rat impairs testicular growth, germ cell maturation, seminiferous tubule formation and other differentiation processes in the testis (França et al., 1995; Maran and Aruldhas, 2002). The effects caused by neonatal induced-hypothyroidism can be rescue with hormonal treatment (T3) which has been demonstrated to be stimulatory for sperm production in the adult rats (Cooke, 1991). On the other hand, hyperthyroidism leads to early cessation of Sertoli cell proliferation, decrease in testis size and decreased sperm production in adults rats (Van Haster et al., 1993; Cooke et al., 1994).

The role of thyroid hormone on testicular development is well described in mammals, however in fish it has received less attention. There are few papers describing the effects of hypothyroid state on testicular development. According to Matta et al. (2002), Nile tilapia juveniles treated with PTU (propylthiouracil, antithyroid drug similar to methimazole) doubled the size of their testis, similar to the effects found in rats. This data suggests a conserved mechanism controlling testis development through thyroid hormones among vertebrates. In this case, Nile tilapia Sertoli cell remained immature and able to proliferate longer, resulting in a higher Sertoli cell capacity and efficiency (Matta et al., 2002).

In adult catfish (early recrudescence/early preparatory phase), *Clarias gariepinus*, treatment with thiourea (another thyroid disrupter) reduced the 11-

ketotestosterone (11-KT) and testosterone (T) plasma and tissue levels after thyroid depletion. After thiourea withdrawal, 11-KT and thyroid hormone plasma levels were partially recovered, indicative an important role of thyroid hormones in adult catfish testis function (Swapna et al., 2006).

More recently, the molecular mechanisms underlying the role of thyroid hormones in spermatogenesis have been elucidated in the zebrafish model. In this model, *ex vivo* studies showed that T3 increases the Sertoli cell production of Igf3, which is a stimulatory growth factor of spermatogenesis (Morais et al., 2013). Moreover, T3 acts through Leydig cells to enhance the gonadotropin-induced synthesis and release of androgens (Morais et al., 2013). Based on the evidences from the literature, it can be concluded that thyroid hormones modulate male reproductive system during the different life stages of fish.

In addition, there are several studies that the hypothalamic-pituitary-thyroid (HPT) axis is interact with the hypothalamic-pituitary-gonadal (HPG) axis, important on controlling reproduction in vertebrates (Tsutsui et al., 2010; Zhang et al., 2010; Kumar et al., 2019). However, the physiological impact of such regulation in fish is still unclear. The aim of this study was to investigate the effects of *in vivo* and *in vitro* of thyroid hormones on the zebrafish spermatogenesis and the correlation between Ths and zebrafish reproductive system.

MATERIAL AND METHODS

Animals

Adult zebrafish male was bred and raised in the aquarium facility of Department of Morphology, Biosciences Institute, São Paulo State University. Handling and experimentation were consistent with the Brazilian National Regulation Guideline for Animal Care and Use in São Paulo State University (CEUA number 1031).

Hypothyroidism-induced by *in vivo* methimazole exposure

A working solution of methimazole (1- methyl-3H-imidazole-2-thione) (CAS 60-56-0, Sigma-Aldrich) was used to induce the hypothyroidism as reported in the literature (Sharma et al., 2013). In this context, adult zebrafish male (n=60) were divided into three groups: control [only filtered water (n=20)]; group I [filtered water adding 1mM of methimazole (n=20)]; and group II [1mM of methimazole following addition of T4 (100 µg/ L) (n=20) from the second week of exposure until the end of experimental period, as rescue treatment group (methimazole+T4)]. Fishes were exposed to the treatments for 21 days according the literature (Sharma and Patino, 2013; Sharma et al., 2016). Methimazole was diluted in destiled water, while T4 was previously diluted in DMSO and then added into the water. To avoid problems adding a new variable, the same amount of DMSO was added in all the tanks. The water was renewed every day and the temperature of the room was set to 28°C, with 14L:10D photoperiod. Fishes were fed twice a day using a commercial food (Zeigler®). After the experimental period fish were euthanized by overdose with benzocaine hydrochloride (250 mg) previously dissolved in ethanol and then mixed with water. The animals were

immersed in the benzocaine solution until death. Then, the testes were collected for morphological, morphometrical and gene expression analysis. Control and hypothyroid-induced males had their plasma collected for ELISA measurements of androgens plasma levels. Moreover, brain and pituitary of these groups were collected for gene expression analysis (see below).

Morphological and morphometrical analysis of spermatogenesis

After the *in vivo* treatment (methimazole and methimazole+T4), zebrafish testes (n=8) were fixed in 4% in Karnovsky at 4°C overnight, dehydrated, embedded in Technovit (7100) historesin, sectioned at 3µm thickness, and stained with toluidine blue, according conventional histological procedures. The slides were evaluated, and the proportion occupied by germ cell cysts (type A spermatogonia undifferentiated, type B spermatogonia, spermatocytes, spermatid, spermatozoa and abnormal cells) was determined. The measuring was performed by counting the intersection point on the histologic fields. For that, 5 fields per slide (n=8 slides per treatment) were quantified using a grid of 540 (54x10) intersections under 100x objective lens. Spermatozoa number was also quantified for each treatment; 20 different fields were taken from 100x objective lens and analyzed by IMAGE J Software (available at http://imagej.nih.gov/ij/index.html) for quantification of spermatozoa number according Fallah et al. (2019). This software allows quantification of several types of cells by limiting the size of the particle. First, it is necessary to defocus the image, and this filter may avoid false positive cells and improve the measurement. Following up, threshold feature is activated, which separates the background of unspecific particles of interest. Then the RGB image is converted into gray scale picture (8 or 16 bytes) and the

threshold allows that lower intensity signals are white and higher intensity signals as black. The algorithm (analyze particles) can be applied for measuring the black particles in that range of pre-fixed size.

Quantification of relative testicular mRNA levels

Total RNA was extracted from the testes (control, methimazole, methimazole+T4) using Trizol™ (Invitrogen, Carlsbad, CA, USA) method, following manufacturer's instructions. To determine the relative mRNA levels of selected genes, qPCR was performed as described by De Waal et al. (2008). The level of *β-actin* mRNA, chosen as endogenous control RNA, remained stably expressed under the different experimental conditions. All qPCRs were performed in 20 µl reaction volume and quantification cycle (Cq) values were determined by Step One Plus (Applied Biosystems) using default settings. Relative mRNA levels were calculated as reported previously (Nóbrega et al., 2015). The mRNA levels of thyroid receptors (*thrα* and *thrβ*), 17α-hydroxylase/17,20 lyase (*cyp17a1*), insulin-like peptide 3 (*insl3*), insulin-like growth factor (*igf3*), testicular connexin (*cx43*), anti-Müllerian hormone (*amh*) and gonadal soma factor (*gsdf*) were measured (Table 1). The relative mRNA levels of germ cell markers, such as *nanos2*, *dazl*, *shippo1* and *sycp3l* were also evaluated among the groups.

Quantification of androgen (11-KT) plasma levels

Blood from control and hypothyroid-induced males (n=8 per condition) were collected. Fish were euthanized by overdose with benzocaine hydrochloride and the caudal peduncle was cut for blood sampling using heparinized syringes and

tubes. Samples were centrifuged at 4°C for ten minutes at 800g (Eppendorf Centrifuge 5424 R) and the 11-KT plasma levels quantified by enzyme-linked immunosorbent assay ELISA (582751, Cayman Chemical), following the manufacturer's instructions.

Brain and pituitary sampling for qPCR

Brain (n=8) and pituitary were collected from control and methimazole-treated. Total mRNA was extracted using Trizol™ (Invitrogen, Carlsbad, CA, USA) method, following manufacturer's instructions. Pituitary gland was pooled four into individual glands per group and used for mRNA extraction using a commercial kit (Pure Mini Kit, Ambion). After RNA extraction, the usual downstream procedures were followed according to methods described above. The relative mRNA levels of gonadotropin-releasing hormone (*gnrh2* and *gnrh3*), gonadotropin-inhibitory hormone (*gnih*) and corticotropin-releasing hormone (*crf*) were evaluated in the brain, while the luteinizing hormone (*lhb*) and follicle-stimulating hormone (*fshb*) mRNA levels were determined in the pituitary gland (Table 2).

***In vitro* studies: steroidogenic release capacity and gene expression**

Testes were collected from eight animals per condition tested (control and methimazole group), and the two testes from each fish were incubated in parallel, one serving as control for the contralateral one incubated with 100 ng/mL rzf Fsh, as previously described (Nóbrega et al., 2015). Incubations lasted 18h at 28°C in 96-well plates using a final volume of 200 µL culture medium. After incubation,

testes were weighed and collected for gene expression analysis, while the medium was processed to evaluate the 11-Ketotestosterone (11-KT) release into the medium by immunoenzymatic assay ELISA (582751, Cayman Chemical), following the manufacturer's instructions and the described methods in García-Lopez et al. (2010). For gene expression, the total RNA was extracted from both groups (control and methimazole) using a commercial Kit (Pure Mini Kit, Ambion). The relative mRNA levels of 17α-hydroxylase/17,20 lyase (*cyp17a1*), steroidogenic acute regulatory protein (*star*), insulin-like peptide 3 (*insl3*), insulin-like growth factor (*igf3*), anti-Müllerian hormone (*amh*) and androgen receptor (*ar*) were evaluated as previously described.

In vitro thyroid hormones effects in combination or not with Fsh

To assess the long term *in vitro* effects of T3, a well-established *ex vivo* system was used (Leal et al., 2009). Three levels of T3 (Sigma Aldrich) (10, 100 and 1000 nM/mL) and three levels of T4 (Sigma Aldrich) (10, 100 and 1000 nM/mL) were tested. To evaluate the crossover between T3 and Fsh in the testes, one level of T3 (100 nM/mL) was selected based on previous experiments (Morais et al., 2013) and then combined with recombinant zebrafish follicle-stimulating hormone (rzf Fsh, 100 ng/mL). Testes were incubated in a 24 well plate, containing 1 mL of culture medium per well at 28 ºC for seven days, changing the medium every 3 days. After the experimental period, testes were weighed and collected for gene expression analysis. Testes incubated with T3 (100 nM/mL were sampled for morphometric analysis, as described above.

In vivo T3 injections

Zebrafish adult males were injected intracoelomatic with 0 and 250ng of tri-iodothyronine (T3) per fish (n=8). Dose were chosen based on their ability to induce deiodinase 3 transcript as described previously (Nelson and Habibi, 2008; Nelson et al., 2010). First, a stock solution of T3 was made in 0.02 M sodium hydroxide and diluited in physiologic saline. The control group were injected with vehicle (only physiologic saline). After twelve hours, the animals were euthanized, and brain and pituitary glands were collected. Brain of each fish (n=8) were kept separate. The pituitaries were pooled in groups of four for RNA extraction. The release mRNA levels of gonadotropin-releasing hormone (*gnrh2* and *gnrh3*), gonadotropin-inhibitory hormone (*gnih*) and corticotropin-releasing hormone (*crf*) were measured from brain, while the luteinizing hormone (*lhb*), follicle-stimulating hormone (f*shb*) and thyroid stimulating hormones (*tsh*) were measured from pituitary glands (Table 2). RNA extraction and downstream procedures were followed as previously described here.

Statistical analysis

Data are presented as mean ± SEM (standard error of mean). Significant differences were identified using unpaired or paired t-tests for two groups or one-way ANOVA followed by the Student–Newman–Keuls or Dunnett's tests for three or more groups. Significance level (*p*) was considered at 0.05 in both cases. All data analysis was performed by Graph Pad Prism software 7.04 (Graph Pad Software, Inc., San Diego, CA, USA, http://www.graphpad.com).

RESULTS

Methimazole and T4 co-treatment: morphological and morphometrical analysis

Methimazole-induced hypothyroidism promoted morphological changes on zebrafish testes (Figs. 1A-D; 2). By morphometric analysis the number of isolated type A undifferentiated spermatogonia (A_{und}) and such as spermatogenic cysts containing type B spermatogonia increased in the methimazole group. The number of meiotic (spermatocytes) and post-meiotic (spermatids) cells did not change between control and methimazole group (Fig. 3A). On the other hand, the number of spermatozoa in the lumen was drastically reduced (Fig. 1B-D; Fig. 3A-B).

Interestingly, the germ cell morphology was altered in the methimazole group (Fig. 2, 3). We named them as abnormal cells (Figs. 2, 3).These cells were found loosen in the lumen and have morphological changes in their cytoplasm and nucleus (Figs. 2, 3). The abnormal cells have condensed pyknotic or fragmented nuclei and some cells have different and irregular nuclear shape (Fig. 2).

The co-treatment with T4 rescued nullified the number of altered germ cells (abnormal cells) induced by methimazole (Fig. 3A). The increased proportion of spermatogonia (A_{und} and B) returned to its basal values (Fig. 3A). However, the proportion of meiotic and post-meiotic spermatogenic cysts (spermatocytes and spermatids) increased (Fig. 3A) with T4 co-treatment.

Interestingly, the production of spermatozoa was recovered in the co-treatment with T4 (Figs. 3A-B).

Methimazole and co-treatment with T4: gene expression

Testicular mRNA levels were clearly affected with the methimazole exposure and co-treatment with T4 *in vivo* (Figs. 4, 5). The expression of thyroid hormones receptors (*thrα* and *thrβ*) increased in the co-treatment with T4 (Fig. 4A-B). Interestingly, *thrα* mRNA levels were up-regulated only in the T4 treatment, while *thrβ* transcriptions were elevated in both groups (Fig. 4A-B). The mRNA levels for Fsh receptor (*fshr*) were also increased only in the T4 treatment (Fig. 4C).

The genes expressed by Leydig cells, *cyp17a1* and *insl3*, had similar expression pattern, showing reduction of mRNA levels in both methimazole and methimazole co-treated with T4 groups (Fig. 5A-B).

The Sertoli cell growth factors, *amh* (anti-Müllerian hormone) and *igf3* (insulin-like growth factor 3), showed no changes in gene expression in both treatments (Fig. 5D-E). However, the gonadal somatic cell derived factor (*gsdf*) transcripts were higher in methimazole and methimazole+T4 (Fig. 5F). Connexin 43 (*cx43*) mRNA levels were affected by methimazole and decreased significantly, while T4 co-treatment nullified this effect (Fig. 5C).

Regarding the germ cell genes, *nanos2* (marker of type A_{und}) mRNA levels were up-regulated in both treatments (methimazole and methimazole+T4). However, *nanos2* levels were higher in methimazole group in comparison with the T4 co-treatment (Fig. 6A)

The *dazl*, expressed by types A_{diff} and B spermatogonia increased in both treatments (Fig. 6B). The expression of *shippo1* (marker of spermatids) increased in methimazole treatment only (Fig. 6C), while *sycp3l* (marker of spermatocytes) did not change in any experimental group (Fig. 6D).

11-KT plasma levels and *in vitro* androgen (11-KT) release capacity by zebrafish testes

11-KT plasma levels were significantly reduced in males exposed to methimazole in comparison to the control group (Fig. 7).

rzf Fsh (100ng/mL) was able to stimulate 11-KT release from control zebrafish testes after 18h of culture (Fig. 8). However, Fsh-induced 11-KT release was abolished on testes from methimazole treated zebrafish (Fig. 8).

Short term effects of Fsh: gene expression

Zebrafish testis explants incubated with 100ng/mL rzf Fsh for 18h had mRNA levels clearly affected by methimazole *in vivo* exposure (Fig. 9). In euthyroid testis, the *igf3* was up-regulated by Fsh, such as steroidogenic enzyme (*star*), but not c*yp17a1* (Fig. 9A). With respect to selected receptors genes, the transcript levels of *ar* was also up-regulated in the presence of Fsh (Fig. 9A). On the other hand, methimazole treatment nullified the capacity of Fsh to stimulate *igf3, ar* and *star* expression in the zebrafish testes (Fig. 9B). Interestingly, mRNA levels of *amh* and c*yp17a1* were highly expressed in the testes of methimazole treated males in the presence of Fsh (Fig. 9B).

Brain and pituitary: expression of selected genes

Expression of neuro hypothalamic peptides, such *gnrh2* and *gnrh3* and *crf* did not change in the methimazole treatment in relation to control fish (Fig. 10A). However, the absence of thyroid hormones significantly stimulated *gnih* mRNA levels (Fig. 10A). In the pituitary *fshb* mRNA levels was up-regulated in the males

treated with methimazole (Fig. 10B). The mRNA levels of *lhb* did not change (Fig. 10B).

After 12h post-injection of 250ng T3 in control fish, *gnrh2, gnih* and *crf* did not change, while the mRNA levels of *gnrh3* were up-regulated with T3 injection (Fig. 10C). In the pituitary T3 injection strongly supressed *lhb* expression (Fig. 10D). The mRNA levels of *fshb* also decreased with T3 injection, but not significantly different from control fish (Fig. 10D).

In vitro long-term effects of T3 on zebrafish function testes

Different concentrations of T3 and T4 (10, 100, 1000 nM) were tested in zebrafish testes during 7 days of culture (long-term) for all selected genes evaluated. T4 did not modulate the expression of any transcript (Figs. 11, 12). On the other hand, T3 (1000 nM) increased the expression of two germ cell markers: *nanos2* (type A_{und}) and *sypc3l* (spermatocytes) (Fig. 11A, C). Higher concentrations of T3 (1000 nM) only increased the *nanos2* mRNA levels (Fig. 11A). Steroidogenic related genes did not change their expression in the presence of T3 (Fig. 12A, C).

To evaluate the combined effects of Fsh and T3, zebrafish testes were incubated with both hormones (100 nM T3+100 ng/mL rzf Fsh) for 7 days of culture (Fig. 13). T3 alone did not affect steroidogenic genes (*3B-HSD* and *cyp17a1*) (Fig. 13A-B), however when combined with Fsh, T3 potentiated the effects of Fsh in zebrafish for *cyp17a1* mRNA levels (Fig. 13B).

Morphometrical analysis revealed that the proportion of type A undifferentiated spermatogonia (A_{und*} and A_{und}) were increased after 7 days of culture with T3 (100 nM), while the spermatogenic cysts containing

spermatocytes and spermatids decreased (Fig. 14A). Interestingly the decreased proportion of meiotic and post-meiotic cysts did not prevent the increased of spermatozoa production in zebrafish testes exposed with T3 (Fig. 14A-B).

DISUSSION

The current work showed that the *in vivo* exposure to 1mM methimazole for three weeks induced a disturbance on the brain-pituitary-testes axis, inducing drastic changes on testis function. The *in vivo* exposure to 1 mM methimazole affected the progression of zebrafish spermatogenesis.

The morphometrical data showed that the number of spermatozoa decreased in the testes of zebrafish exposed to methimazole *in vivo*. Although the proportion of spermatocytes and spermatids were not affected, therefore it is suggested that the germ cell loss occurs during spermiogenesis. The presence of abundant abnormal cells could be related to the germ cell loss during this phase. Interestingly that in the testes of neonatal rats showed the increase number of apoptotic cells post PTU-treatment (Santos-Ahmed et al., 2011). Loosen germ cells in the lumen, condensed, pyknotic and different nuclear phases were recorded as germ cell abnormalities in zebrafish testes treated with methimazole. Moreover, it is suggested that thyroid hormones play a role in the entry of meiosis. This is because methimazole exposed fish exhibited an accumulation of pre-meiotic cells, such as types A_{und*}, A_{diff} and B spermatogonia. The impairment zebrafish spermatogenesis agrees with Swapna et al. (2006) who showed that the absence of thyroid hormones in catfish caused changes in the development of germ cells, such as sperm depletion.

To evaluate whether the effects seen in zebrafish testes were not due to the methimazole, co-treatment with T4 was carried out. Co-treatment rescued zebrafish spermatogenesis, sperm production and abnormal cells were gone. Interestingly, co-treatment with T4 increased the proportion of spermatocytes and spermatids, another evidence that thyroid hormones stimulate meiosis.

Gene expression analysis shared similar results in agreement of the morphometrical data. For example, *nanos2* (marker of type A_{und}) (Aoki et al., 2009) and *dazl* (marker of differentiation) (Chen et al., 2013) showed higher mRNA levels in methimazole treated group supporting accumulation of pre-meiotic cells in these testes. Although the proportion of spermatids was not affected in the morphometrical analysis, *shippo1* (marker of spermatids) (Yano et al., 2008) was up-regulated in the methimazole group. The rescue and stimulating of zebrafish spermatogenesis by T4 co-treatment also reflected in the gene expression by higher *nanos2* and *dazl* mRNA levels in the testes. With respect to somatic gene expression, T4 co-treatment stimulated *thrα* and *fshr* mRNA levels in the testes. This data indicates that thyroid hormones would increase testicular response to thyroid hormones and Fsh signaling. In zebrafish testes, *thrα* is expressed in Sertoli and Leydig cells, while *thrβ* is expressed only by Leydig cells (Morais et al., 2013). Therefore, Sertoli cells co-localize both receptors (*fshr* and *thrα*). Moreover, Morais et al. (2013) showed that T3 potentiated Fsh effects on zebrafish testes *in vitro*. These effects could be explained by thyroid hormones induced *fshr* expression as shown in this work. This might be another evidence of the crossover between thyroid hormones and gonadotropin signaling.

igf3 and *amh* mRNA levels did not change their mRNA levels in methimazole or methimazole+T4. Interestingly, *gsdf* was up-regulated in both

group. The *gsdf* (gonadal soma-derived growth factor) is expressed by Sertoli cells and exert a crucial role on the control of germ cell proliferation and differentiation in males spermatogenesis (Gautier et al., 2011, Yan et al., 2017).

Recent studies suggested that connexin 43 is a potential target for thyroid hormones (Gilleron et al., 2006). T3 has been reported to enhance gap junctional communication and cx43 in rat liver epithelial cells (Poguet et al., 2003), cardiomyocytes (Tribulova et al., 2004) and hepatic cells (Fischer et al., 2005). Gilleron et al. (2006) demonstrated that PTU (anti-thyroid drug) decreased cx43 that is associated with reduced Sertoli cell proliferation and reduced spermatogenic yield. In our work, cx43 also seems to be a potential target for thyroid hormones in zebrafish testes. Methimazole group showed lower testicular *cx43* mRNA levels which could be associated with the reduced sperm formation in this group. In rainbow trout (*Oncorhynchus mykiss*) and brook trout (*Salvelinus fontinalis*) – four different Cxs/*cxs* were identified in the testis (de Montgolfier et al., 2007; de Montgolfier et al., 2009) and cx43 is involved in maturation of trout testis because its expression levels increased during the onset of spermatogenesis (de Montgolfier et al., 2007; de Montgolfier et al., 2009). Although, thyroid hormone levels were not measured in methimazole group, the lower expression of *cx43* is a strong evidence that methimazole induced hypothyroidism in zebrafish. Interestingly, the lower levels of *cx43* were nullified after the T4 co-treatment, indicating the restoration of thyroid hormones levels in the zebrafish testes.

In this study, 11-ketotestosterone (KT) plasma levels were decreased in methimazole-induced hypothyroidism males. Previously studies have shown that PTU-treatment also decreased serum testosterone concentrations in rats (Chiao

et al., 2000). Similar, in African catfish, *Clarias gariepinus* exposed to thiourea (other anti-thyroid drug) also showed reduced androgen levels (Swapna et al., 2006). Interestingly, Houbrechts et al. (2019) showed that the Dio2-knockout (KO) zebrafish (*dio2−/−*) strongly decreased of androgens (11-KT and testosterone) in the testes, also the steroidogenesis and steroid signaling were similarly affected compared to control animals (Houbrechts et al., 2019). This provide that absence of thyroid hormones by DIO2 (KO) (supress thyroid hormones production) or goitrogen treatment may act directly on the testis to repress steroidogenesis. This data indicate that the thyroid hormones levels are crucial to reproduction (Houbrechts et al., 2019). In agreement to the lower androgen levels in zebrafish testes, steroidogenic enzyme *cyp17a1* and androgen-sensitive gene, *insl3*, were down-regulated in the methimazole group. Although, 11-KT plasma levels were not measured after T4 co-treatment, the lower androgen condition remained after T4 co-treatment, *cyp17a1* and *insl3* were still down-regulated. This data indicates that spermatogenesis recovery after T4 treatment was not mediated by androgens.

To explain the reduced levels of androgens, brain-pituitary axis was evaluated. The expression levels of *gnrh2* and *gnrh3* did not change, but *gnih* was highly up-regulated in the brain of methimazole treated. In fish, GnIH ortólogos inhibit pituitary hormones in zebrafish and other fish species (Zhang et a., 2010, Moussavi et al., 2012; Branco et al., 2018). In addiction, Tsutsui et al. (2018) have shown that PTU increased GnIH levels and reduced gonadotropins and steroid plasma levels in mammals. Therefore, it is suggested that the reduction of androgen plasma levels in methimazole group could be associated with the inhibition of the brain-pituitary axis by GnIH. This inhibition might be at

the protein level, because gonadotropin mRNA levels were not reduced in this group. On the other hand, *fshb* transcriptions were up-regulated in methimazole group. The up-regulation of *fshb* could be associated to the positive feedback exerted by the lower 11-KT plasma levels seen in these animals. Similar, Yoshiura et al. (1999) found in goldfish that the thiourea-treatment (2 weeks) increased Fsh levels.

Interestingly, T3 injection in zebrafish post 12h shows opposite effects in the brain-pituitary axis compared to the ones seen in methimazole. *gnrh3* mRNA levels were up-regulated and both gonadotropins mRNA decreased (not significant *fshb*, although it is reduced). Previously studies from Nelson et al. (2010) reported that in goldfish the T3 injection (25 or 250ng/fish) was drastically decreased *lhb* transcript in male pituitary after 36h. The down-regulated of gonadotropins would be decreased synthesis of gonadal steroids, which include estrogen (Nelson et al., 2010). Altogether these data indicate a relation between thyroid hormones and reproductive axes in zebrafish.

However, studies evaluating the cross-talk between thyroid hormones and gonadotropins in male reproduction are few. In this context, Morais et al. (2013) showed that T3 potentiated the *in vitro* effects of Fsh by increasing androgen release (11-KT) and *cyp17a1* mRNA levels. In our study, T3 also potentiated the Fsh induced *cyp17a1* expression. When testing the effects of Fsh on androgen release and gene expression in the methimazole group, this work shows that Fsh-induced androgen release was supressed. This results might be explained by the lower *cyp17a1* mRNA levels seen in the methimazole group. Similar studies showed that PTU decreased the enzymatic activity of STAR in mice (Cooke et al., 1994). In addition, Fsh did not stimulate the expression of *igf3*, *insl3* and *star*

in this work. Previous studies have shown that these genes respond to Fsh in zebrafish testes (Gárcia-Lopez et al., 2010, Nóbrega et al., 2015, Crespo et al., 2016). On the other hand, *cyp17a1* mRNA levels were increased as result of the positive feedback to increase androgen production.

The expression of *amh* in the zebrafish testes of methimazole group was up-regulated *in vitro* in the presence of Fsh. This effects were the opposite seen by Skaar et al. (2011) and Morais et al. (2017) for the euthyroid condition in zebrafish. These studies showed that *amh* mRNA levels decreased in response to Fsh and this down-regulated requires exposure to Fsh (75 ng/mL) for more than 3 days (Morais et al., 2017). The results found in methimazole treatment suggest that thyroid hormones could act as repressors in the *amh* decreased expression by Fsh. Amh is expressed in Sertoli cells and inhibits androgen production and blocks spermatogonial differentiation in zebrafish testes (Skaar et al., 2011). This condition fits with the effects found in the testes in the absence of thyroid hormones reported here. This is another evidence that thyroid hormones are necessary for Fsh actions in zebrafish testes.

In addition to these data, we evaluated the effects of thyroid hormones on zebrafish spermatogenesis. Expression analysis showed that T3 (100nM and 1000 nM) increased *nanos2* (marker of type A undifferentiated spermatogonia). Morais et al. (2013) using lower dose (50 ng/ mL) reported an increase of type A undifferentiated spermatogonia, which was corroborated by higher BrdU mitotic index of this cell type in T3-treated tissue. Interestingly, T3 also stimulated the *sycp3l*, the meiotic marker expression (100 nM/mL). Levels of *3β-hsd* and *cyp17a1* were also measured and did not change within any level of both T3 and

T4 treatments. Another interestingly result is that T4 does not exert any change, thyroid hormones act via T3 in zebrafish testis.

The morphometrical analysis showed an increase of type A undifferentiated spermatogonia (A_{und*} and A_{und}) proportion in T3 *in vitro* exposure, which may reflect the up-regulation of *nanos2*. The number of spermatozoa and lumen also increased. Previously, Morais et al. (2013) reported that the mitotic indices of A_{und} and Sertoli cells were stimulates after T3 treatment. Also, Safian et al. (2016) demonstrated that T3 enhanced the formation of new cysts of $A_{und,}$ accumulation of differentiated spermatogonia (A_{diff}) and reduction of spermatogonial B. Altogether this data indicates that thyroid hormones (T3) stimulates zebrafish spermatogenesis by proliferation, differentiation and meiosis. These results supply important data about the effects of thyroid hormones on brain-pituitary-testes and support interaction between reproductive and thyroid axes via Fsh signaling, which needs T3 to promote its functions in zebrafish testes.

DECLARATION OF INTEREST

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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REFERENCES

ARIYARATNE, H.B., MILLS, N., MASON, J.I., MENDIS-HANDGMA, S.M. Effects of thyroid hormone on Leydig cell regeneration in the adult rat following ethane dimethane sulphonate treatment. **Biology of Reproduction**. 63:1115-1123. 2000.

AOKI Y, NAKAMURA S, ISHIKAWA Y, TANAKA M. Expression and syntenic analyses of four nanos genes in medaka. **Zool Sci** 26:112–118. 2009.

BRANCO, G. S.; MELO, A. G.; RICCI, J. M. B.; DIGMAYER, M.; DE JESUS, L. W. O.; HABIBI, H. R.; NÓBREGA, R. H. Effects of GnRH and the dual regulatory actions of GnIH in the pituitary explants and brain slices of Astyanax altiparanae males. **General and Comparative Endocrinology**. 2018.

CHEN, S.X., BOGERD, J., SCHOONEN, N.E., MARTIJN, J., DE WAAL, P.P., SCHULZ, R.W. A progestin (17a,20β-dihydroxy-4-pregnen-3-one) stimulates early stages of spermatogenesis in zebrafish. **General and Comparative Endocrinology**. 185 1-9. 2013.

COOKE, P.S. Thyroid hormones and testis development: a model system for increasing testis growth and sperm production. In: Robaire B, ed. The Male Germ Cell: Spermalogonium to Fertilization**. Ann NYAcadSci**, 1991.

COOKE, P.S., ZHAO, Y.D., BUNICK, D. Triiodothyronine inhibits proliferation and stimulates differentiation of cultured neonatal Sertoli cells: possible mechanism for increased adult testis weight and sperm production induced by neonatal goitrogen treatment. **Biol. Reprod**. 51, 1000–1005. 1994.

CRESPO, D.; ASSIS, L.H.C.; FURMANEK, T.; BOGERD, J.; SCHULZ, R.W. Expression profiling identifies Sertoli and Leydig cell genes as Fsh targets in adult zebrafish testis. **Molecular and Cellular Endocrinology**. vol. 437, p.237-251. 2016.

CHIAO, Y.-C., LIN, H., WANG, S.-W., & WANG, P. S. Direct effects of propylthiouracil on testosterone secretion in rat testicular interstitial cells. **British Journal of Pharmacology**. 130: 1477–1482. 2000.

De MONTGOLFIER, B., DUFRESNE, J., LETOURNEAU, M., NAGLER, J.J., FOURNIER, A., AUDET, C., CYR, D.G. The Expression of Multiple Connexins Throughout Spermatogenesis in the Rainbow Trout Testis Suggests a Role for Complex Intercellular Communication. **Biology of Reproduction**. 76: 2-8. 2007.

De MONTGOLFIER, B., FAYE, A., AUDET, C., CYR, D.G. Seasonal variations in testicular connexins levels and their regulation in the brook trout, *Salvelinus fontinalis*. **General and Comparative Endocrinology.** 162: 276-285. 2009.

FALLAH, H. P., TOVO-NETO, A., YEUNG, E. C., NÓBREGA, R. H., HABIBI, H. R. Paracrine/autocrine control of spermatogenesis by gonadotropin-inhibitory hormone. Molecular and Cellular Endocrinology. 2019.

FRANÇA, L.R., HESS, C.A., COOKE, P.S., RUSSEL, L.D. Neonatal hypothyroidism causes delayed Sertoli cell maturation in rats treated with propylthiouracil: evidence that the Sertoli cell controls testis growth. T**he Anatomical Record**. 242: 57-69.1995.

FISCHER R, REINEHR R, LU TP, SCHONICKE A, WARSKULAT U, DIENES HP, HAUSSINGER D. Intercellular communication via gap junctions in activated rat hepatic stellate cells. **Gastroenterology** 128:433–448. 2005.

GARCÍA-LÓPEZ, A.; JONGE, H.; NOBREGA, R.H.; WAAL, P.P.; VAN DIJK, W.; HEMRIKA, W.; TARANGER, G.L.; BOGERD, J.; SCHULZ, R.W. Studies in Zebrafish Reveal Unusual Cellular Expression Patterns of Gonadotropin Receptor Messenger Ribonucleic Acids in the Testis and Unexpected Functional Differentiation of the Gonadotropins. **Endocrinology**. 2010.

GAUTIER, A., SOHM, F., JOLY, J.-S., LE GAC, F., & LAREYRE, J.-J. The Proximal Promoter Region of the Zebrafish gsdf Gene Is Sufficient to Mimic the Spatio-Temporal Expression Pattern of the Endogenous Gene in Sertoli and Granulosa Cells1. **Biology of Reproduction.** 85: 1240–1251. 2011.

GILLERON, J., NEBOUT, M., SCARABELLI, L., SENEGA-BALAS, F., PALMERO, S., SEGRETAIN, D., POINTIS, G. A. Potential Novel Mechanism Involving Connexin 43 Gap Junction for Control of Sertoli Cell Proliferation by Thyroid Hormones. **Journal of Cellular Physiology.** 209: 153-161. 2006.

HOUBRECHTS, A.M., HOUCKE, J.V., VEERLE, M.D. Disruption of deiodinase type 2 in zebrafish disturbs male and female reproduction. Journal of Endocrinology. 241: 111-123. 2019.

KUMAR, P., WISDOM, K. S., BHAT, I. A., PATHAKOTA, G.-B., NAYAK, S. K., REANG, D., SHARMA, R. Molecular characterization of gonadotropin-inhibitory hormone (GnIH) gene and effect of intramuscular injection of GnIH peptide on the reproductive axis in *Catla catla*. **Animal Biotechnology**. 1–15. 2019.

LEAL, M.C.; DE WAAL, P.P.; GARCIA-LOPEZ, A.; CHEN, S.X.; BOGERD, J.; SCHULZ, R.W. Zebrafish primary testis tissue culture: An approach to study testis function ex vivo. **General Comparative Endocrinology**, vol. 162, n. 2, p.134-138. 2009b.

MARAN, R.R., ARULDHAS, M.M. Adverse effects of neonatal hypothyroidism on Wistar rat spermatogenesis. **Endocrinology Research**. 28:141-154. 2002.

MATTA, S.L; VILELA, D.A; GODINHO, H.P; FRANÇA, L.R**.** The goitrogen 6-n-propyl-2-thiouracil (PTU) given during testis development increases Sertoli and germ cell numbers per cyst in fish: the tilapia (*Oreochromis niloticus*) model. **Endocrinology**, 143: 970 –978. 2002.

MORAIS, R.D.; NOBREGA, R.H.; GOMEZ-GONZALEZ, N.E.; SCHMIDT, R.; BOGERD, J.; FRANÇA, L.R.; SCHULZ, R.W. Thyroid hormone stimulates the proliferation of Sertoli cells and single type A spermatogonia in adult zebrafish (*Danio rerio*) testis. **Endocrinology**, vol. 154, p. 4365-4376. 2013.

MORAIS, R. D. V. S., CRESPO, D., NÓBREGA, R. H., LEMOS, M. S., VAN DE KANT, H. J. G., DE FRANÇA, L. R., SCHULZ, R. W. Antagonistic regulation of spermatogonial differentiation in zebrafish (*Danio rerio*) by Igf3 and Amh. **Molecular and Cellular Endocrinology.** 454, 112–124. 2017.

MOUSSAVI, M., WLASICHUK, M., CHANG, J. P., AND HABIBI, H. R. Seasonal effect of GnIH on gonadotrope functions in the pituitary of goldfish. **Mol. Cell. Endocrinol**. 350, 53–60. 2012.

MULLUR, R.; LIU, Y.Y.; BRENT, G.A. Thyroid hormone regulation of metabolism. **Physiol Rev**. 94:355–82. 10.1152. 2014.

NELSON, E.R., HABIBI, H.R. Seasonal-related homologous regulation of goldfish liver Estrogen receptor subtypes. **Cybium.** 32: 248-25. 2008.

NELSON, E.R.; ALLAN, E.R.; PANG, F.Y.; HABIBI, H.R. Thyroid hormone and reproduction: regulation of estrogen receptors in goldfish gonads, **Mol Reprod Dev**, 77. 784–794. 2010.

NÓBREGA, R.H; MORAIS, R.D.V.S.; CRESPO, D.; WAAL, P.P.; FRANÇA, L.R.; SCHULZ, R.W.; BOGERD, J. Fsh Stimulates Spermatogonial Proliferation and Differentiation in Zebrafish via Igf3. **Endocrinology**, 156 (10): 3804–3817. 2015.

POGUET AL, LEGRAND C, FENG X, YEN PM, MELTZER P, SAMARUT J, FLAMANT F. Microarray analysis of knockout mice identifies cyclin D2 as a possible mediator for the action of thyroid hormone during the postnatal development of the cerebellum. **Dev Biol**. 254:188–199. 2003.

SAFIAN, D.; MORAIS, R.D.V.S.; BOGERD, J.; SCHULZ, R.W. Igf Binding Proteins Protect Undifferentiated Spermatogonia in the Zebrafish Testis Against Excessive Differentiation. **Endocrinology**, 157, 4423-4433. 2016.

SHARMA, P.; TANG, S.; MAYER, G.D.; PATIÑO, R. Effects of thyroid endocrine manipulation on sex-related gene expression and population sex ratios in Zebrafish. **General and Comparative Endocrinology**, vol. 235, p. 38-47. 2016.

SANTOS-AHMED, J.; BROWN, C.; SMITH, S. D.; WESTON, P.; RASOULPOUR, T.; GILBERT, M. E.; HIXON, M. L. Akt1 protects against germ cell apoptosis in the postnatal mouse testis following lactational exposure to 6 N-propylthiouracil. **Reproductive Toxicology**. 31(1), 17–25. 2011.

SHARMA, P., PATIÑO, R. Regulation of gonadal sex ratios and pubertal development by the thyroid endocrine system in zebrafish. Gen. Comp. Endocrinol. 184, 111-119. 2013.

SWAPNA, I.; RAJASEKHAR, M.; SUPRIYA, A.; RAGHUVEER, K.; SREENIVASULU, G.; RASHEEDA, M.K.; MAJUMDAR, K.C.; HAGAWA, H.; TANAKA, H.; DUTTA-GUPTA, A.; SENTHILKUMARAN, B. Thiourea-induced thyroid hormone depletion impairs testicular recrudescence in the air-breathing catfish, *Clarias gariepinus*. **Comp Biochem Physiol**, Part A. 144, 1-10. 2006.

SKAAR,K.S.; NÓBREGA,R.H.; MAGARAKI,A.; OLSEN,L.C.; SCHULZ,R.W. Ma le Proteolytically activated, recombinant anti-mullerian hormone inhibits androgen secretion, proliferation, and differentiation of spermatogonia in adult zebrafish testis organ cultures. **Endocrinology**.152 pp. 3527-3540. 2011.

TEERDS, K.J., DE ROOJI, D.G., DE JONG, F.H., VAN HAASTER, L.H. Development of the adult-type Leydig cell population in the rat is affected by neonatal thyroid hormone levels. **Biology of Reproduction**. 59:344-350. 1998.

TRIBULOVA N, SHNEYVAYS V, MAMEDOVA LK, MOSHEL S, ZINMAN T, SHAINBERG A, MANOACH M, WEISMANN P, KOSTIN S. Enhanced connexin-43 and alpha sarcomeric actin expression in cultured heart myocytes exposed to triiodo-L thyronine. **J Mol Histol** 35:463–470. 2004.

TSUTSUI, K.; BENTLEY, G. E.; BEDECARRATS, G.; OSUGI, T.; UBUKA, T.; AND KRIEGSFELD, L. J. Review: gonadotropin-inhibitory hormone (GnIH) and its control of central and peripheral reproductive function. **Front. Neuroendocrinol**. 31, 284–295. 2010.

TSUTSUI, K., SON, Y.S., KIYOHARA, M., MIYATA, I. Discovery of GnIH and Its Role in Hypothyroidism-Induced Delayed Puberty. **Endocrinology** 159, 62-68. 2018.

VAN HAASTER, L.H., DE JONG, F.H., DOCTER, R., DE ROOJI, D.G. High neonatal triiodothyronine levels reduce the period of Sertoli cell proliferation and accelerate tubular lumen formation in the rat testis and increase serum inhibin levels. **Endocrinology**. 133: 755–760. 1993.

YAN, Y.L., DESVIGNES, T., BREMILLER, R., WILSON, C., DILLON, D., HIGH, S., DRAPER, B., BUCK, C.L., POSTLETHWAIT, J. Gonadal soma controls ovarian follicle proliferation through Gsdf in zebrafish. **Developmental dynamics** 246:925-945. 2017.

YANO, A., SUZUKI, K., YOSHIZAKI, G. Flow-cytometric isolation of testicular germ cells from rainbow trout (Oncorhynchus mykiss) carrying the green fluorescent protein gene driven by trout vasa regulatory regions. Biol. Reprod., 78:151-158. 2008.

YOSHIURA, Y., SOHN, Y. C., MUNAKATA, A., KOBAYASHI, M., AIDA, K. Molecular cloning of the cDNA encoding the β subunit of thyrotropin and regulation of its gene expression by thyroid hormones in the goldfish, Carassius auratus. **Fish Physiology and Biochemistry**. 21: 201–210. 1999.

ZHANG, Y.; LI, S.; LIU, Y.; LU, D.; CHEN, H.; HUANG, X. Structural diversity of the GnIH/GnIH receptor system in teleost: its involvement in early development and the negative control of LH release. **Peptides**. 31.1034–1043. 2010.

Table 1 – Primers used for qPCR analysis

Target genes **Testis**	**Primers sequence (5'→3')**	**Access (NCBI)**
β-actin	**FW** AGACATCAGGGAGTGATGGT **RV** CAATACCGTGTCAATGGGG	BC045846
cyp17a1	**FW** GGGAGGCCACGGACTGTTA **RV** CCATGTGGAACTGTAGTCAGCAA	NM_212806.3
star	**FW** CCTGGAATGCCTGAGCAGAA **RV** ATCTGCACTTGGTCGCATGAC	NM_131663.1
amh	**FW** CTCTGACCTTGATGAGCCTCATTT **RV** TGCAAAAGTTCTTAAGGGACATCC	NM_001007779.1
insl3	**FW** TCGCATCGTGTGGGAGTTT **RV** TGCACAACGAGGTCTCTATCCA	NM_001115053.2
ar	**FW** ACGTGCCTGGCGTGAAAA **RV** CAAACCTGCCATCCGTGAAC	NM_001083123.1
igf3	**FW** TGTGCGGAGACAGAGGCTTT **RV** CGCCGCACTTTCTTGGATT	HQ241070.1
thrα	**FW** CTGCTGTGATTGGATGCTGGAT **RV** GCTCTTGTCGGATGTGCTGTT	NM_205625
thrβ	**FW** ATCGACCAGAGCCCACACA **RV** TAGGTGCCGATCCAATGTCTT	NM_131340.1
fshr	**FW** GAGGATTCCCAGTAATGCTTTCCT **RV** TCTATCTCACGAATCCCGTTCTTC	NM_001001812.1
cx43	**FW** CTACAGGGCTCTCCACTCTTTACTTCT **RV** CGCACTCCAGTCACCCATCT	NC_007131.7
nanos2	**FW** AGAGACTGCGCAGATTTACC **RV** AGCATAACGGACACACGTAG	NC_007116.7
dazl	**FW** AGTGCAGACTTTGCTAACCCTTATGTA **RV** GTCCACTGCTCCAAGTTGCTCT	NC_007130.7
shippo1	**FW** GATGCCTGGAGACATGACCAA **RV** CAAAGGAGAAGCTGGGAGCTTT	NC_007129.7
sypc3l	**FW** TGGAGCTTCGGAAGGAAATG **RV** CAGAACAGCATGGACTGAAGA	NC_007118.7
3β-HSD	**FW** GCAACTCTGGTTTTCCACACTG **RV** CAGCAGGAGCCGTGTAGCTT	NC_007114.7
gsdf	**FW** CATCTGCGGGAGTCATTGAAA **RV** CAGAGTCCTCCGGCAAGCT	NC_007132.7

Table 2 – Primers used for qPCR analysis

Target genes **Brain**	**Primers sequence (5'→3')**	**Access (NCBI)**
β-actin	**FW** AGACATCAGGGAGTGATGGT **RV** CAATACCGTGTCAATGGGG	BC045846
gnrh2	**FW** TCAGGATTACCAACACCGGG **RV** CAGACCAGCACCATCACTTCA	NM_181439.4
gnrh3	**FW** AACACAGCAGTTTTAGCATGGAG **RV** TATGACCAGTGCTGGCAAAGA	NM_182887.2
Gnih	**FW** AAGAAAGGACTCAAGTCCACGA **RV** GGGCTTTGTTAGTCTAAAGCTGTG	GU290218.1
Crf	**FW** CACCGCCGTATGAATGTAGA **RV** GAAGTACTCCTCCCCCAAGC	NM_001007379.1
Target genes **Pituitary**	**Primers sequence (5'→3')**	**Access (NCBI)**
β-actin	**FW** AGACATCAGGGAGTGATGGT **RV** CAATACCGTGTCAATGGGG	BC045846
lhb	**FW** GCAGAGACACTTACAACAGCC **RV** AAAACCAAGCTCTGAGCAGCC	AY714132.1
fshb	**FW** CAGATGAGGATGCGTGTGC **RV** ACCCCTGCAGGACAGCC	NM_205624.1
tsh	**FW** GCAGATCCTCACTTCACCTACC **RV** GCACAGGTTTGGAGCATCTCA	NM_001145763.2

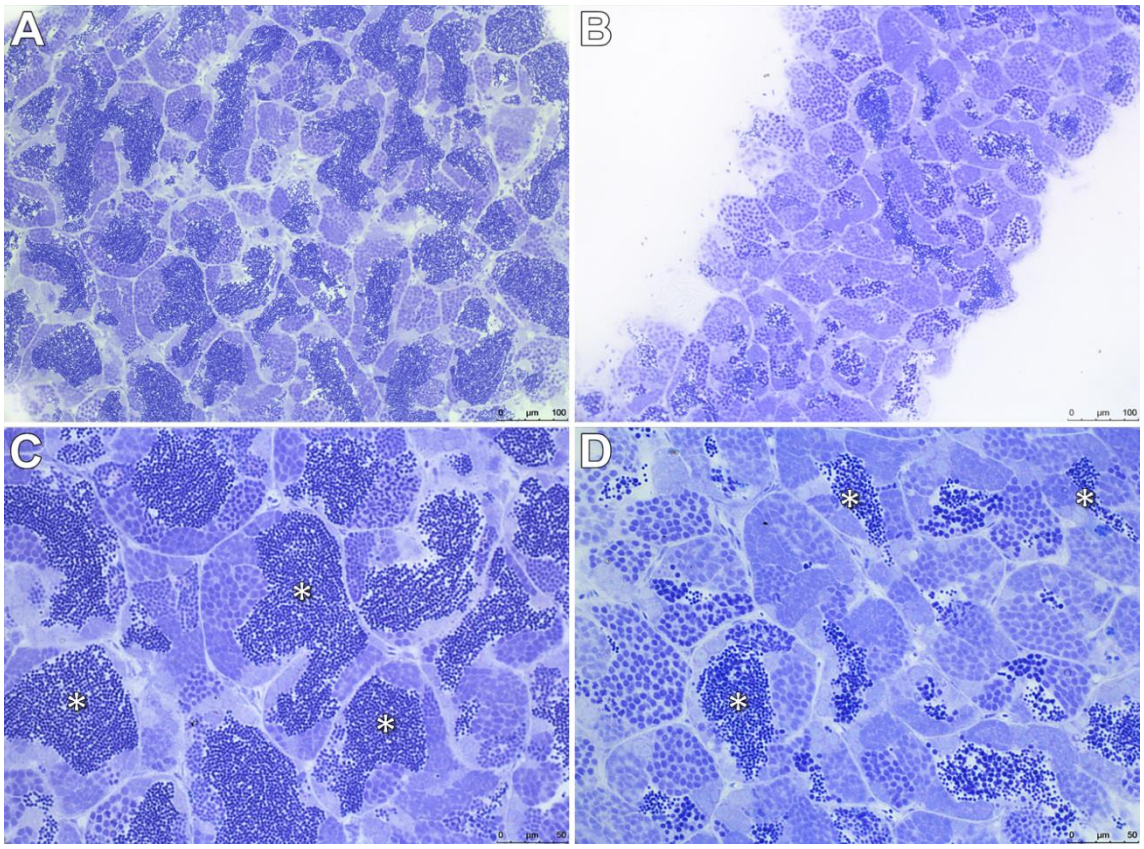

Figure 1 – Histological sections from zebrafish testis after *in vivo* exposure to methimazole for 21 days. **(A and C):** control group **(B and D):** methimazole treated group. * area occupied by the lumen. Staining: Toluidine blue.

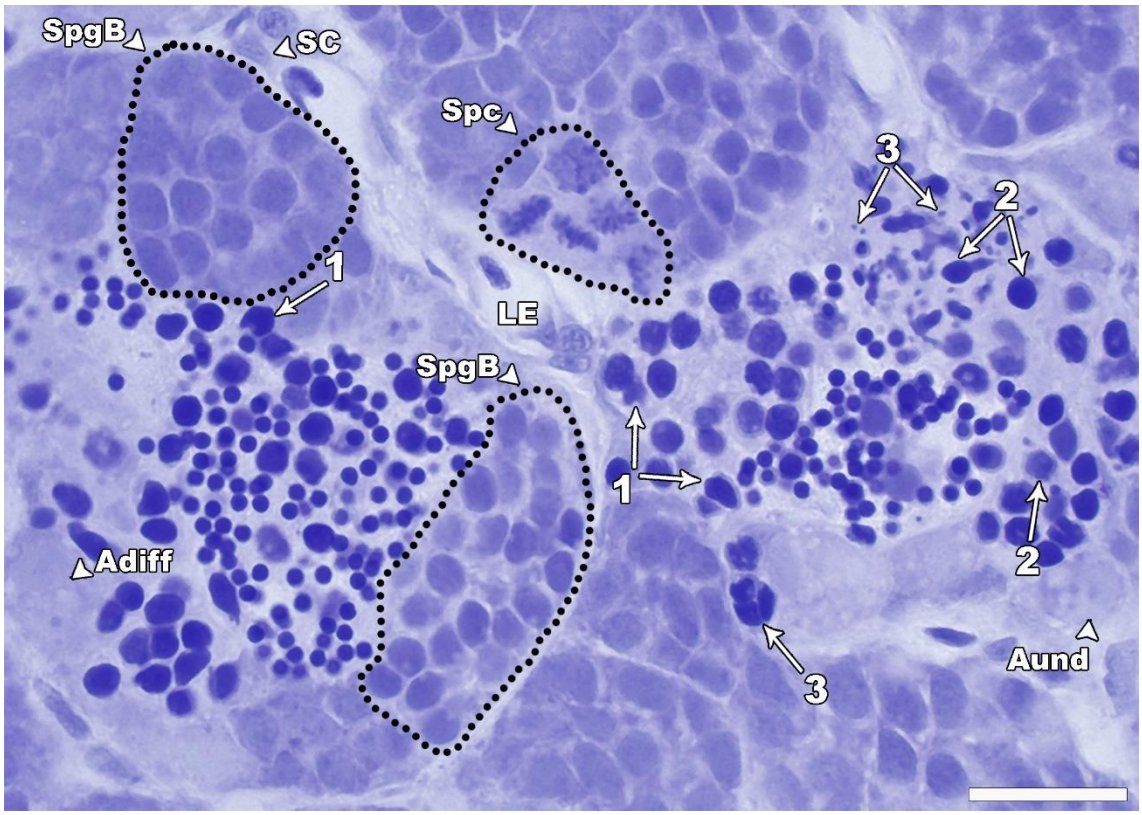

Figure 2 – Abnormal cells in the methimazole testis. **1-** irregularities on germ cell morphology. **2-** condensed nucleus. **3-** pyknotic and fragmented nuclei. Type A undifferentiated spermatogonia (A_{und}), differentiated spermatogonia (A_{diff}), type B spermatogonia (SpgB), spermatocytes (Spc), Sertoli cell (SC), Leydig cell (LE). Staining: Toluidine blue. Scale bar=100µm.

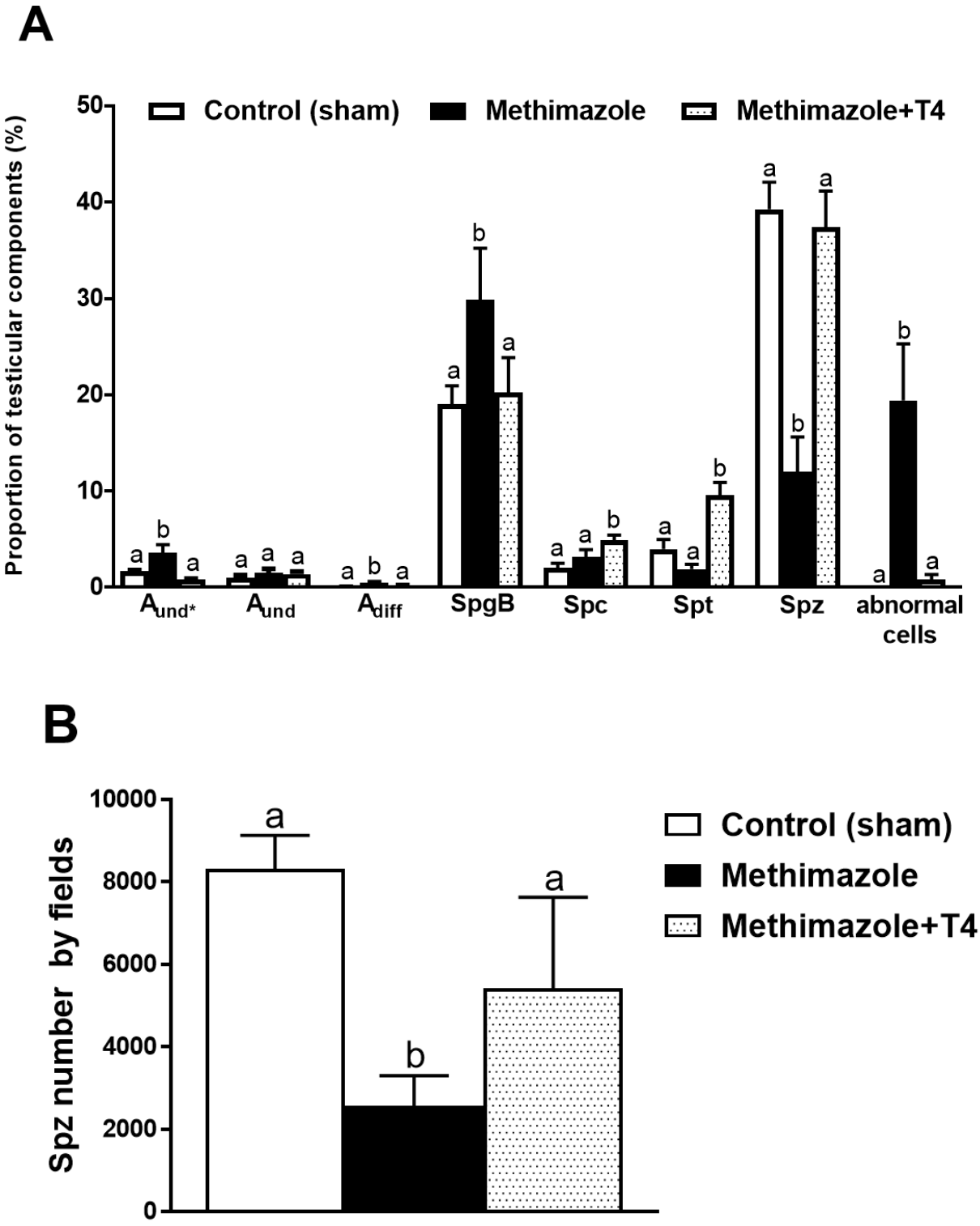

Figure 3 – **(A)** Morphometrical evaluation of germ cells (type A undifferentiated spermatogonia A_{und*} and A_{und}), differentiated spermatogonia (A_{diff}), type B spermatogonia (SpgB), spermatocytes

(Spc), spermatids (Spt), spermatozoa (Spz) and abnormal cells. **(B)** Spermatozoa number by fields. Bars represent the mean±SE. Different letters in A and B denote statistic differences between the different groups with the control ($p < 0,05$; Dunnett's Multiple Comparison Test; n=8).

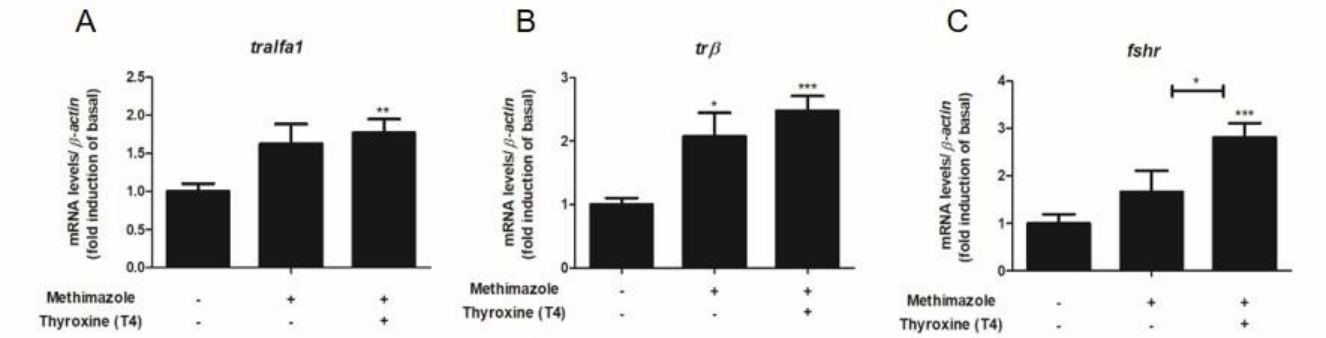

Figure 4 – Relative mRNA levels of thyroid hormones and Fsh receptors in zebrafish testes after *in vivo* exposure to methimazole and methimazole+T4 for 21 days. Bars represent the mean±SE. Gene expression levels were normalized to *β-actin* mRNA levels; * ($p<0.05$) ** ($p<0.01$) and *** ($p<0.001$) denote statistical significance between treatment and control among the groups (Student unpaired t-test; n=8).

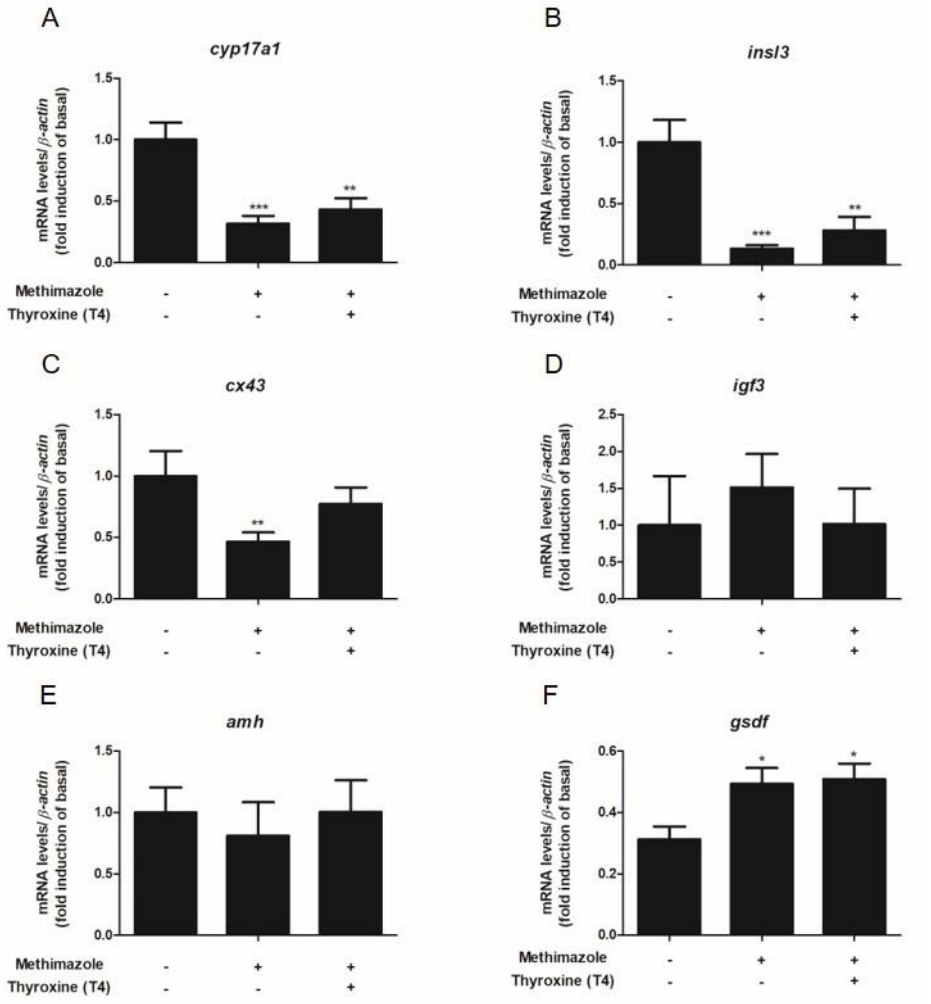

Figure 5 – Relative mRNA levels of selected testicular genes expressed by somatic cells in zebrafish testes after *in vivo* exposure to methimazole and methimazole+T4 for 21 days. Bars represent the mean±SE. Gene expression levels were normalized to *β-actin* mRNA levels; * (p<0.05) ** (p<0.01) and *** (p<0.001) denote statistical significance between treatment and control among the groups. (Student unpaired t-test; n=8).

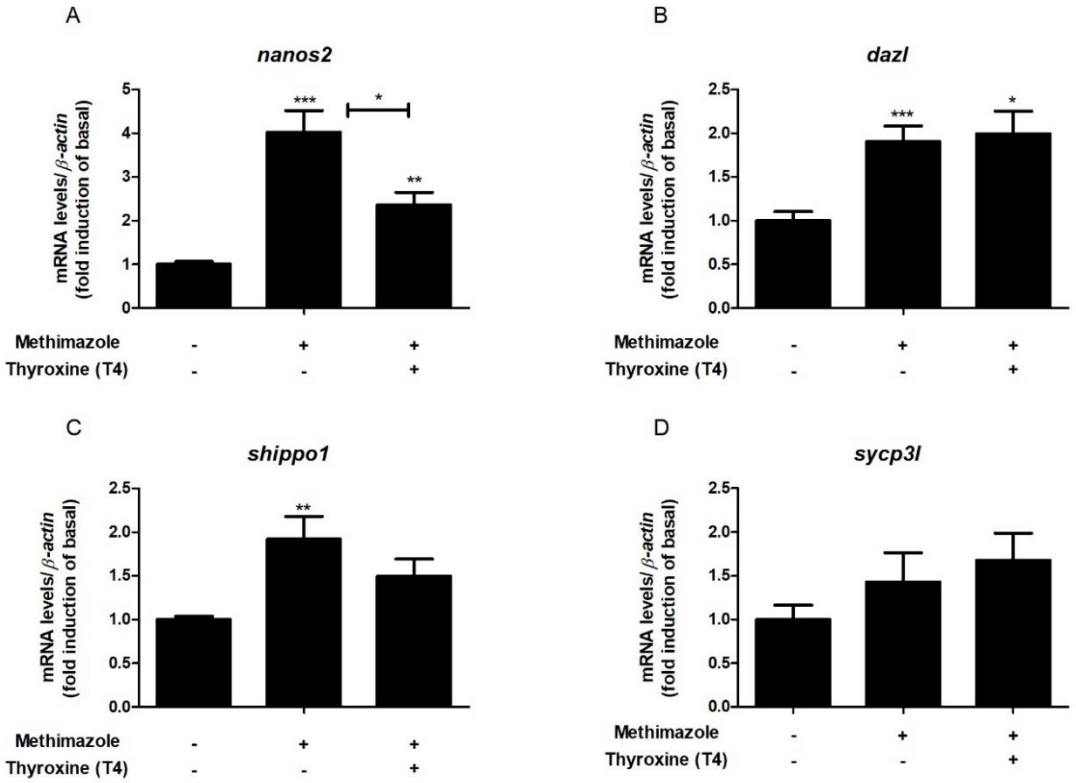

Figure 6 – Relative mRNA levels of selected germ cell markers in zebrafish testes after *in vivo* exposure to methimazole and methimazole+T4 for 21 days. Bars represent the mean±SE. Gene expression levels were normalized to *β-actin* mRNA levels.* (p<0.05) ** (p<0.01) and *** (p<0.001) denote statistical significance between treatment and control among the groups. (Student unpaired t-test; n=8).

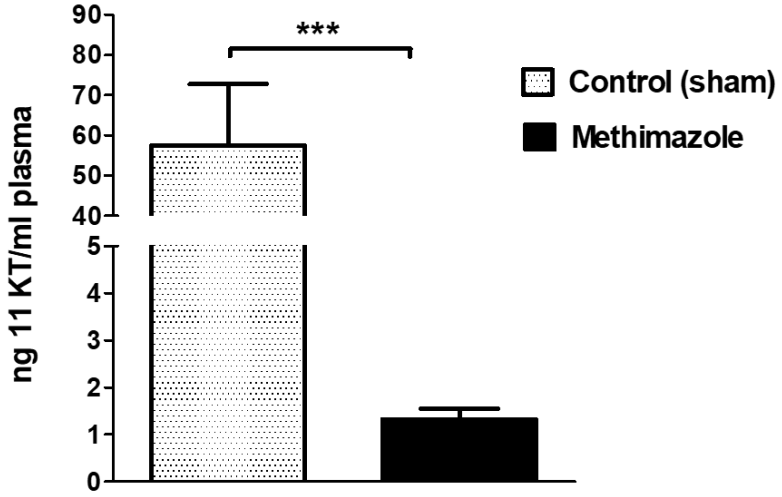

Figure 7 – 11-KT plasma levels (ng/ml plasma) from zebrafish males: control and exposure to methimazole for 21 days. Bars represent the mean±SE. *** ($p<0.001$) indicate statistical significance between control and methimazole (Student unpaired t-test; n=8).

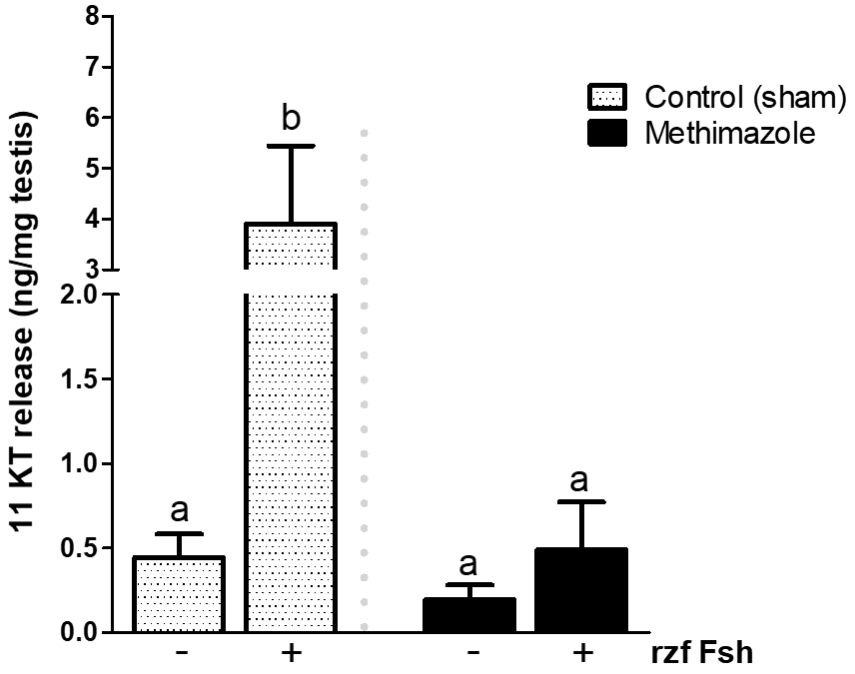

Figure 8 – *in vitro* quantification of 11-KT (ng/mg testis) release from control and methimazole treatment in the absence or presence of 100 ng/mL rzf Fsh after 18h of culture. Bars represent the mean±SE. Different letters ($p<0.005$) denote significance differences between basal and Fsh treatment group (Student paired t-test; n=8).

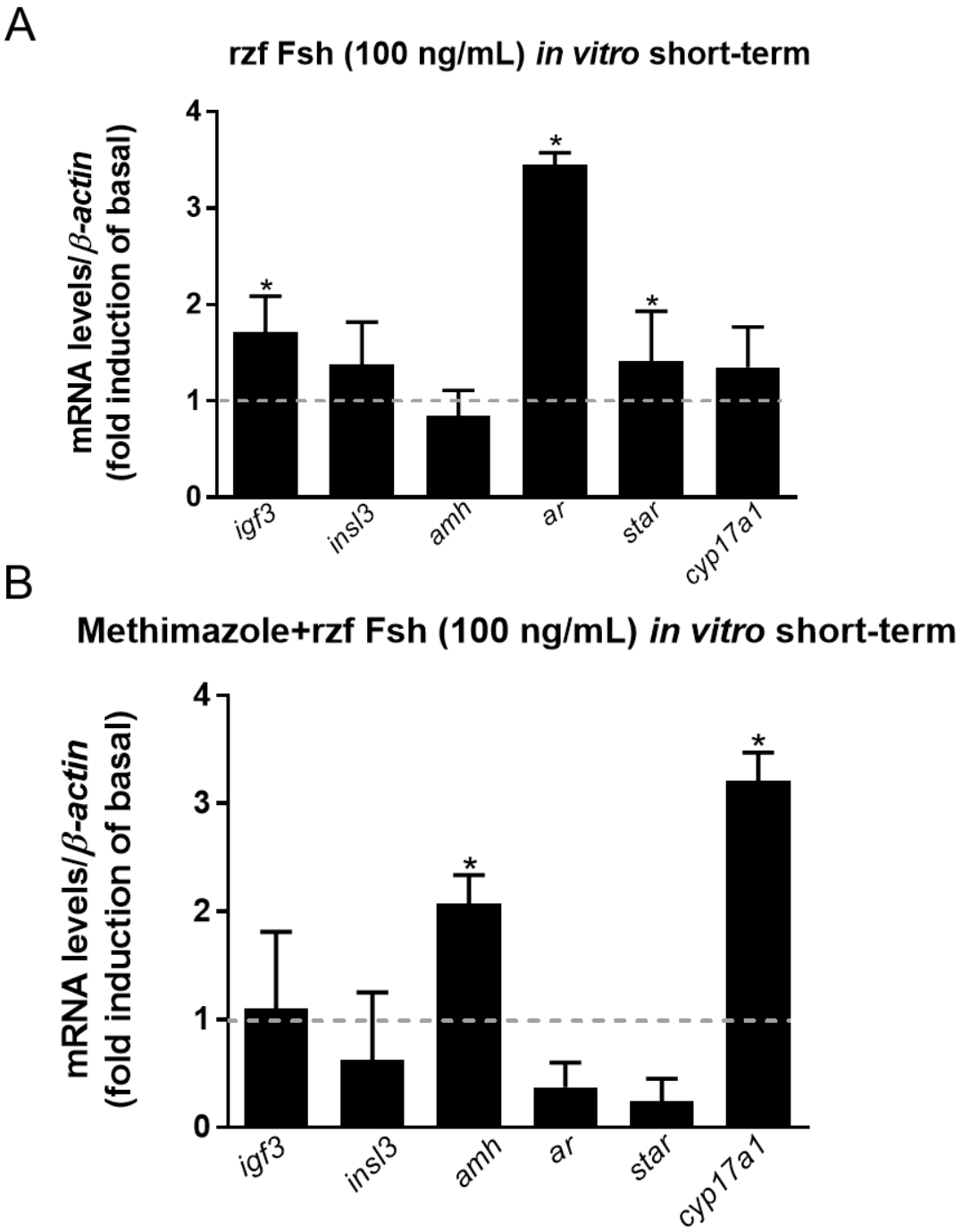

Figure 9 – (A and B) Relative mRNA levels of several testicular genes (*igf3*, i*nsl3*, *amh*, *ar*, *star*, *cyp17a1*) from control and methimazole treatment after incubation with 100 ng/mL rzf Fsh *in vitro* for 18h. Bars represent the mean±SE. Gene expression levels were normalized to *β-actin* mRNA levels. *($p<0.05$) ** ($p<0.01$) and *** ($p<0.001$) denote statistical differences between control/ basal condition and Fsh exposure. (Student unpaired t-test; n=5).

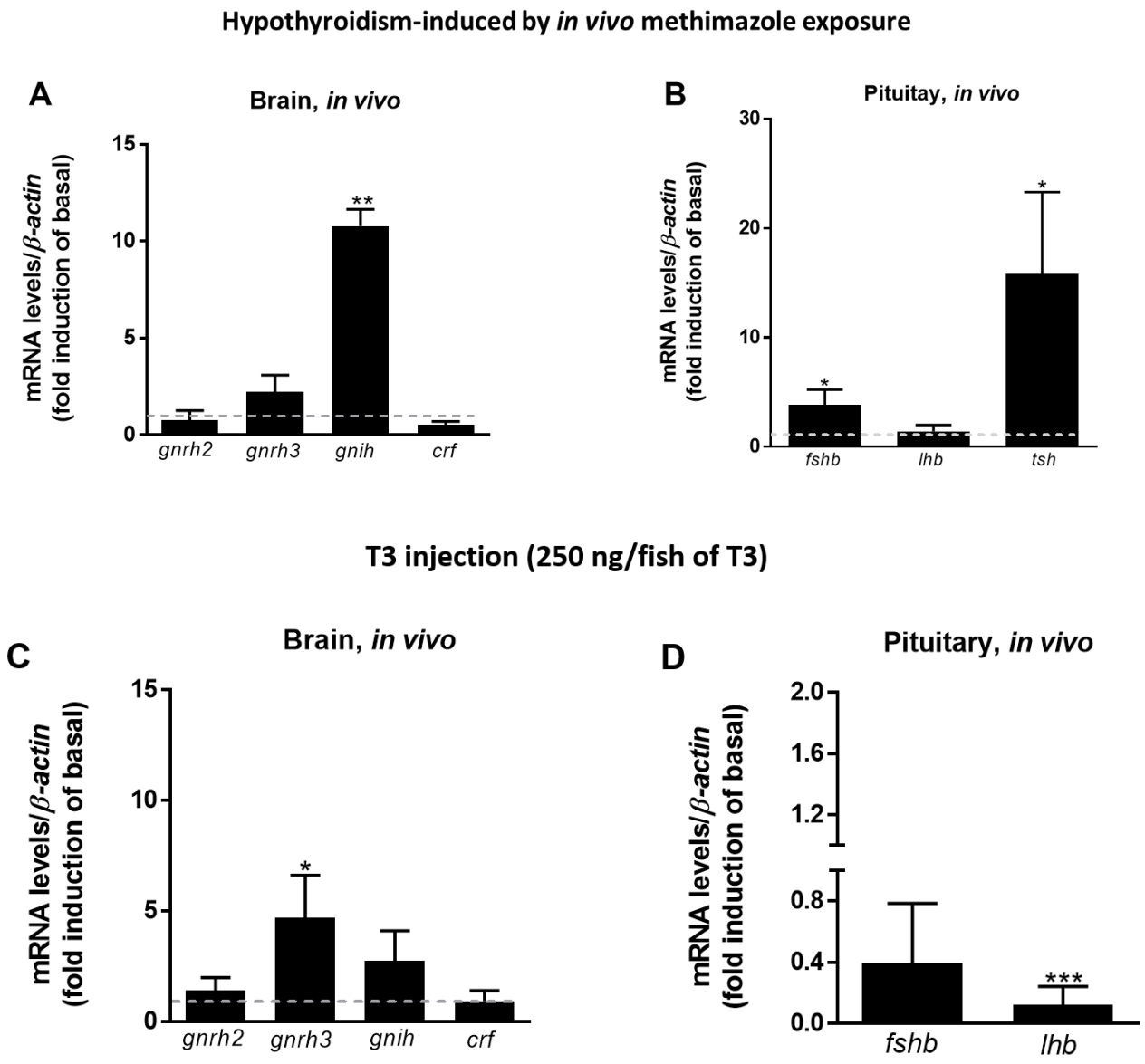

Figure 10 – Relative mRNA levels of selected genes expressed in brain (n=8) (*gnrh2*, *gnrh3*, *gnih* and *crf*) and pituitary (n=4, pool=4) (*lhb*, *fshb* and *tsh*) after methimazole-induced hypothyroidism **(A-B)** and after 12h pos-injection with 0 and 250ng T3 per fish **(C-D).** Bars represent the mean±SE. Gene expression levels were normalized to *β-actin* mRNA levels. * ($p<0.05$) ** ($p<0.01$) and *** ($p<0.001$) denote significant differences between control and treated fish. (Student unpaired t-test).

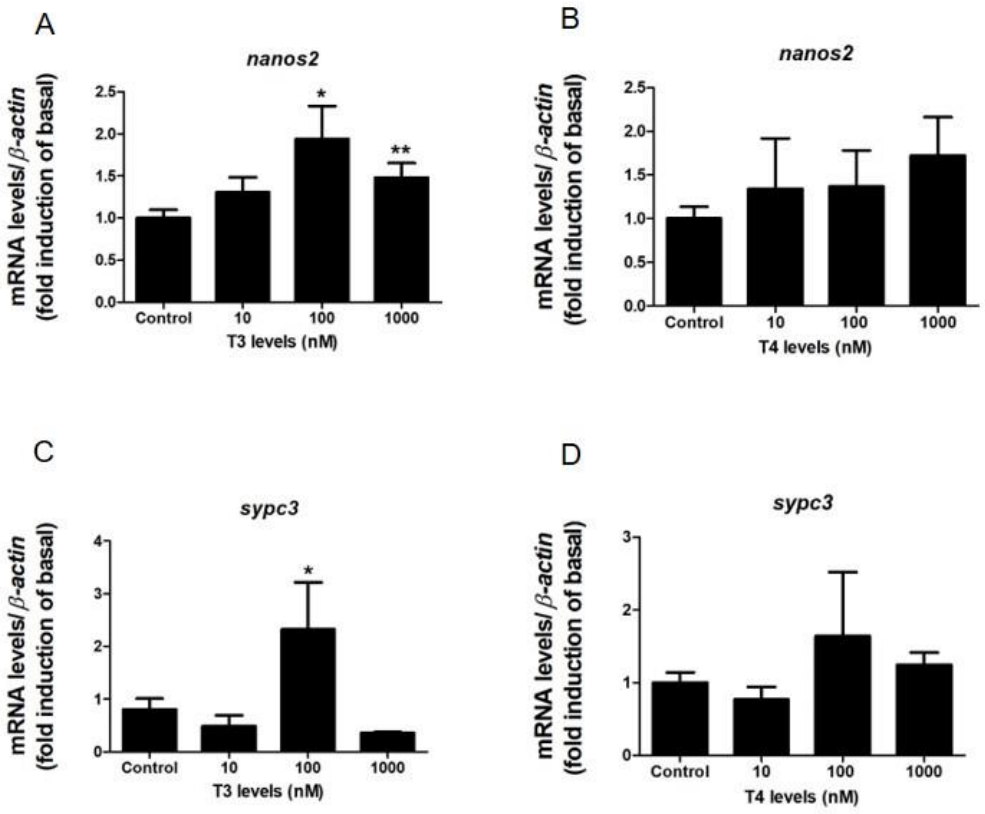

Figure 11 – Relative mRNA levels of germ cell markers (*nanos2*, *sycp3l*) from zebrafish testes incubated with different T3 and T4 concentrations during 7 days. Bars represent the mean±SE. Gene expression levels were normalized to *β-actin* mRNA levels. * ($p<0.05$) ** ($p<0.01$) and *** ($p<0.001$) denote significant differences between basal and hormonal treatment. (Student unpaired t-test; n=8).

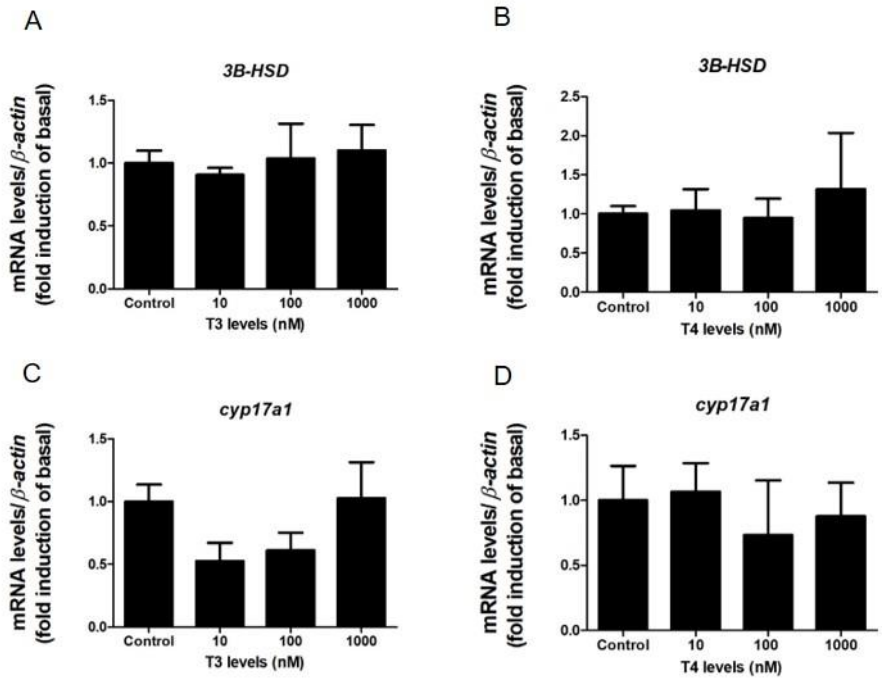

Figure 12 – Relative mRNA levels of steroidogenic related genes (*3B-HSD* and *cyp17a1*) in zebrafish testes incubated with different T3 and T4 concentration after 7 days. Bars represent the mean±SE. Gene expression levels were normalized to ***β-actin*** mRNA levels. * ($p<0.05$) ** ($p<0.01$) and *** ($p<0.001$) denote significant differences between control and hormonal treatment (Student unpaired t-test; n=8).

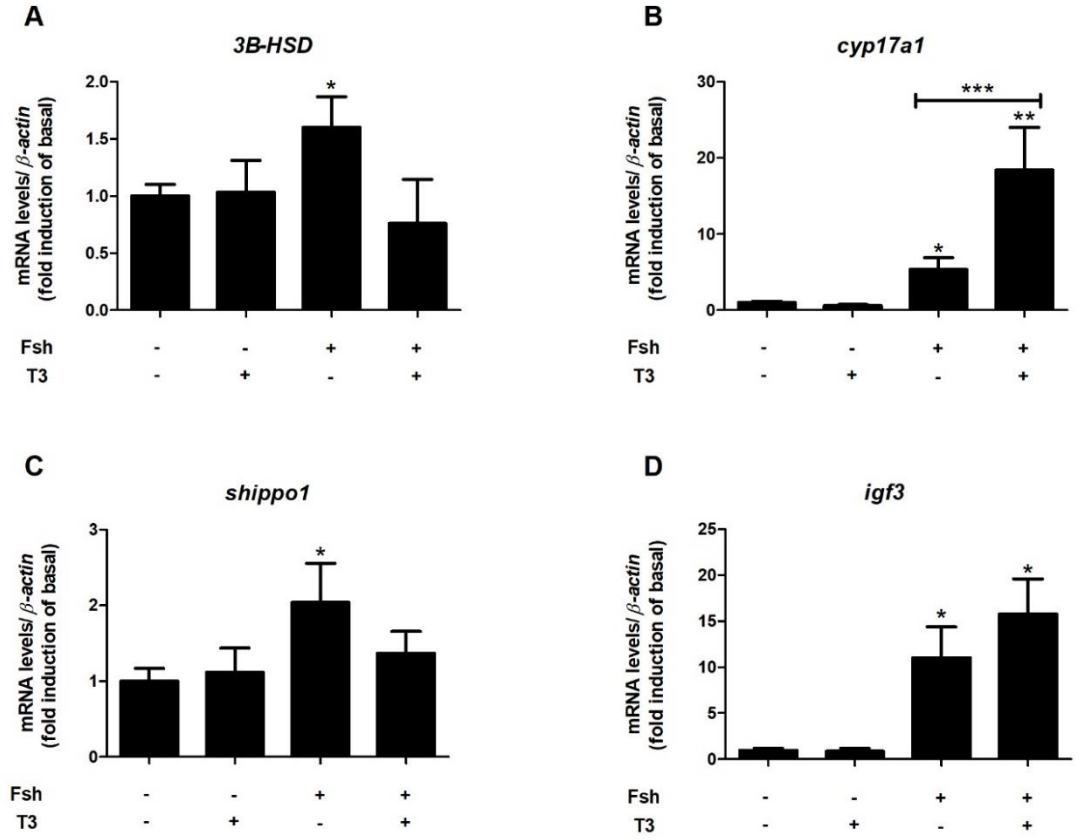

Figure 13 – Relative mRNA levels of selected genes: *3β-HSD*, *cyp17a1*, *shippo1* and *igf3* in zebrafish testes incubated with T3 (100nM/mL) and rzf Fsh (100ng/mL) for 7 days. Bars represent the mean±SE. Gene expression levels were normalized to *β-actin* mRNA levels. * ($p<0.05$) ** ($p<0.01$) and *** ($p<0.001$) indicate significant differences between control and hormonal treatment (Student unpaired t-test; n=8).

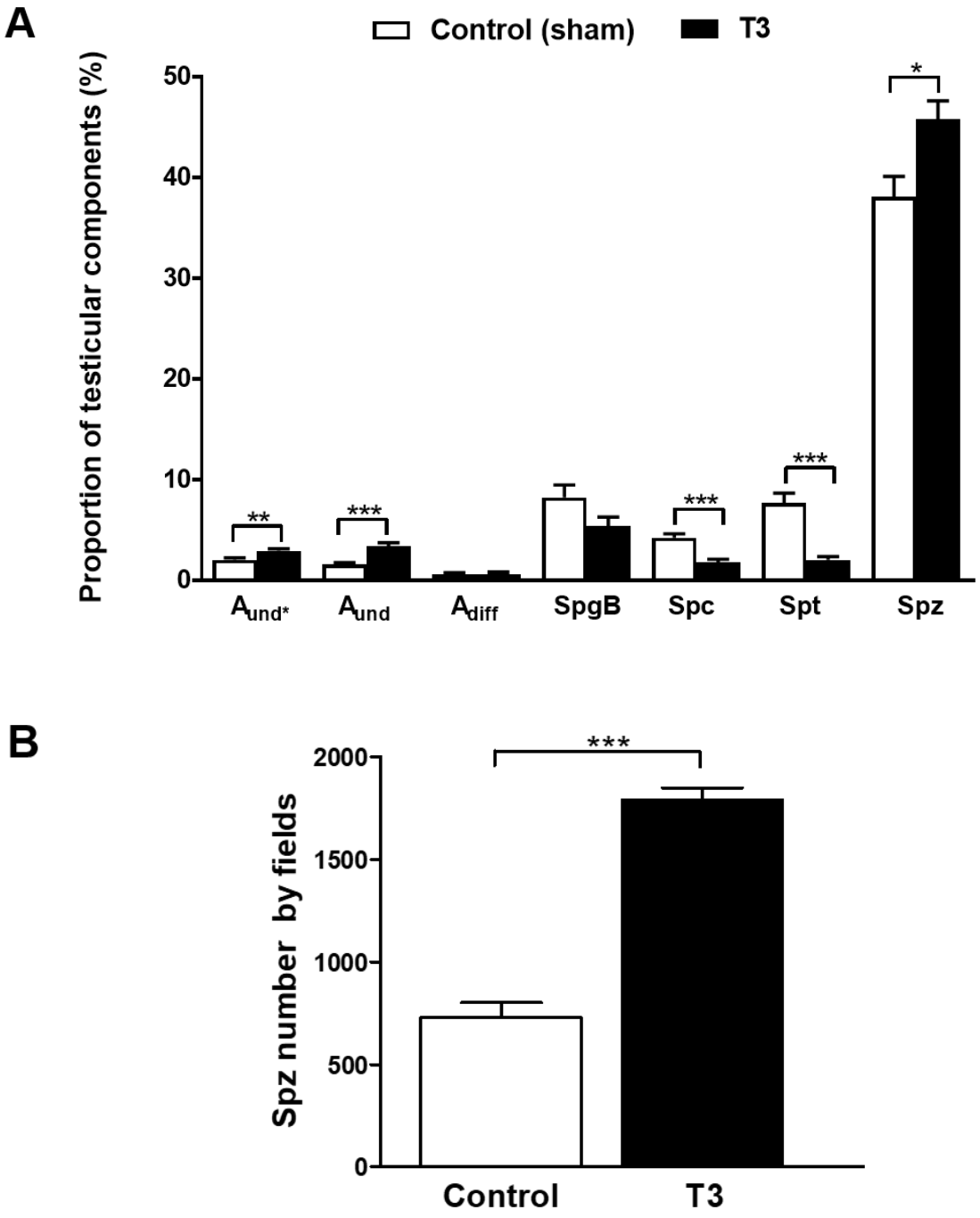

Figure 14 - (A) Morphometric evaluation of germ cells (type A undifferentiated spermatogonia (A_{und*} and A_{und}), differentiated spermatogonia (A_{diff}), type B spermatogonia (SpgB), spermatocytes (Spc), spermatids (Spt), spermatozoa (Spz), lumen (L), interstitium (I) **(B)** Spermatozoa number by fields of control and testes incubated *in vitro* with T3 (100 nM). Bars represent the mean±SE. * ($p<0.05$) ** ($p<0.01$) and *** ($p<0.001$) denote statistical differences between basal and T3 exposed group. (Student paired t-test; n=8).

CAPÍTULO 2

ROLE OF GONADOTROPIN INHIBITORY HORMONE (GnIH) AND THYROID HORMONES (Ths) IN MULTIFACTORIAL CONTROL OF ZEBRAFISH (*Danio rerio*) SPERMATOGENESIS

Artigo em preparação

Role of gonadotropin inhibitory hormone (GnIH) and thyroid hormones (Ths) in multifactorial control of zebrafish (*Danio rerio*) spermatogenesis

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ABSTRACT

Thyroid hormones (Ths) (T4/T3) exert several roles on regulating vertebrate homeostasis acting on several physiological processes such as growth, behavior, stress and on the reproductive system. In mammals, the role of thyroid hormones in reproduction has been well investigated, however the interaction between reproduction and thyroid axes is still unknown. For that, we evaluate the role of thyroid hormones and Gonadotropin-inhibitory hormone (GnIH), an important neuropeptide that control the secretion of pituitary hormones, on zebrafish spermatogenesis. Then, a thyroid hormone disruptor, methimazole, was used to generate a model of thyroid-induced insufficiency. Fishes were exposed to 1 mM of methimazole dissolved into the water for 21 days, after exposure they were dissected, and their testes was collected for flow citometry, using FACScan cell cycle analysis and morphometric histological analysis after cultivation with 100ng/mL zGnih and 5 IU hCG alone and the combination with 100ng/mL zGnih & 5 IU hCG. Here we showed that the stimulatory effects of human chorionic gonadotropin (hCG) and the inhibitory effects of gonadotropin-inhibitory hormone (GnIH) on germ cell development of zebrafish testes are nullified in the absence of thyroid hormones. In conclusion, these data indicate that Ths are necessary for both hCG or GnIH effects in the zebrafish spermatogenesis.

Key-words: GnIH, hypothyroidism, spermatogenesis, thyroid hormones

INTRODUCTION

Hypothalamic-Pituitary-Thyroid and Gonadal axes

Thyroid function is activated by the hypothalamic-pituitary-thyroid axis (HPT), which has been reviewed in many vertebrates, including fish (Brown and Cai, 2007; Blanton and Specker 2007; and Carr and Patiño, 2011). In fish, a particular characteristic of the HPT is the non-involvement of thyrotropin releasing hormone (TRH) in the activation of pituitary-thyroid axis, as occurs in mammals (Larsen et al., 1998). On the other hand, the pituitary secretion of thyrotropin stimulating hormone (TSH) seems to be dependent of corticotropin-releasing hormone (CRH or known as corticotropin releasing factor – CRF (Larsen et al., 1998), similarly as observed in amphibians, reptiles and birds (De Groef et al., 2003, Castañeda Cortés et al., 2014). The secreted TSH stimulates the synthesis and release of THs by thyroid follicles, which are dispersed among the afferent branchial arterioles of the ventral region of pharynx (Cyr and Eales, 1996; Carr and Patiño, 2011). The L-Thyroxine (T4) is the main TH secreted under normal circumstances, while T3, known as the active hormone, is mostly produced by extrathyroidal sources from T4 conversion (Basset et al., 2003). The conversion of T4 into T3 is catalyzed by two distinct selenoenzymes, type 1 and type 2 deiodinases (DIO1 and DIO2, respectively) (Orozco et al., 2012).

Another interesting aspect to be mentioned is the crossover of the HPT and hypothalamic-pituitary-gonadal (HPG) axes (Castañeda Cortés et al., 2014). Next to the well-known function of GnRH (Gonadotropin-releasing hormone) in regulating gonadotropins, GnRH has been shown to induce TSH secretion in amphibians (Denver, 1988; Okada et al., 2004). Other studies have demonstrated

that GnRH exposure increased T4 plasma levels in freshwater murrel (*Channa gachua*) and in two species of carp (Roy et al., 2000), as well in barfin flounder (*Verasper moseri*), masu salmon (*Oncorhynchus masou*), goldfish (*Carassius auratus*) (Chiba et al., 2004), and amphibians (Jacobs et al., 1988a; Jacobs et al., 1988b). However, no changes in T3 plasma levels were observed after injections of an analogue of GnRH in goldfish (MacKenzie et al., 1987). When evaluating the effects of THs on GnRH, studies on tilapia, *Oreochromis niloticus* showed that T3 suppressed the concentration of terminal nerve salmon GnRH mRNA in sexually immature males (Parhar et al., 2000). This fact is consistent with the hypothesis that TH, by suppressing terminal nerve GnRH expression, promotes inhibition of sexual maturation in tilapia males (Parhar et al., 2000).

Recent studies have been focused is a newly discovered hypothalamic neuropeptide, GnIH (Gonadotropin-inhibitory hormone) that inhibits the pituitary gonadotropin synthesis and release (Tsutsui et al., 2018). Interestingly, it has been shown that GnIH neurons express thyroid hormone receptors (TRα and TRβ) in mammals (Tsutsui et al., 2018). Recent study showed that female mice with thyroid dysfunction showed higher levels of GnIH, and presented lower circulating levels of gonadotropins and estradiol, which might be associated with the delayed puberty seen in the animals (Tsutsui et al., 2018). This data suggests a possible role of THs on regulating the GnIH synthesis and release in vertebrates, although no studies have been reported in fish at the moment.

Thyroid hormone actions in spermatogenesis

Although there is a general agreement that THs are important regulators of

the testicular development prior to and during puberty in mammals, the role of these hormones is not very clear in the adult testis (Wagner et al., 2009). Considering that most of the experimental studies to date are focused on TH effects on the developing testes, only limited data are available on its role in vertebrate spermatogenesis (Wagner et al., 2009; Morais et al., 2013; Safian et al., 2016).

Altered thyroid status, natural or experimentally induced, is frequently associated with some sort of sexual dysfunction and/or morphological testicular alteration in mammals (Faraone-Menella et al., 2008). Similar studies in reptiles showed that thyroidectomy impaired spermatogenesis of adult garden lizards (*Calotes versicolor*), while T4 treatment nullified the thyroidectomy-induced testicular atrophy (Haldar-Misra and Tapliyal, 1981). In adult catfish (at early recrudescence/early preparatory phase), treatment with thiourea (a thyroid disrupter) reduced androgen plasma and tissue levels after thyroid depletion, leading to a male reproductive system disruption (Swapna et al., 2006). Interestingly, thiourea treatment withdrawal for 21 days induced a partial recovery of androgen levels, which indicates that thyroid plays a role in maintaining testis functions via androgen stimulation (Swapna et al., 2006). In this context, it has been observed that androgens could also regulate thyroid function. Testosterone propionate injected intramuscularly in immature rainbow trout (*Salmo gairdneri* Richardson) stimulates the thyroid function, increasing T3 plasma levels (Hunt and Eales, 1979).

More recently, Morais et al. (2013) studied the effects of THs on zebrafish spermatogenesis using an *ex vivo* approach. These authors showed that T3 increased both mitotic index of type A undifferentiated spermatogonia (A_{und}) and

Sertoli cell in zebrafish testis. In the follow up experiments, Safian et al. (2016) demonstrated that T3 not only promoted the formation of new cysts with type Aund spermatogonia, but also stimulated the accumulation of A_{diff} (type A differentiated spermatogonia) by increasing their proliferation while reducing development into type B spermatogonia in zebrafish.

GnIH actions in spermatogenesis

Different studies with GnIH in vertebrates have demonstrated that GnIH is also present in the gonads, being considered as a peripheral regulator of gonadal functions (Muñoz-Cueto et al., 2017). In fish, GnIH and GnIH (receptors) have been detected in the gonads of different teleost species such as zebrafish, tilapia and sea bass. However, few studies have been focused on GnIH actions in gonadal gametogenesis and steroidogenesis of fish (Muñoz-Cueto et al., 2017). For example, Paullada-Salmerón and collaborators (2016) studied the effects of GnIH on steroidogenesis of male European sea bass, the peripheral sbGnIH-1 and sbGnIH-2 implants caused decreased of testosterone (T) and 11-ketotestosterone (11-KT) plasma levels at particular reproductive stages (early- and mid-spermatogenesis). Interestingly, intramuscular implant of both GnIH peptides delayed the development of testis, with increase of type A spermatogonia showing a spermatogenesis development.

Qi and collaborators (2013) studied the *in vivo* the effects of GnIH on female and male steroidogenesis in goldfish. In females, implantation of goldfish GnIH peptides did not affect the estradiol serum levels. Interestingly, GnIH peptides increased testosterone serum levels in males. This result supported the

proposal that GnIH peptides affect the synthesis and secretion of sexual steroid hormones. In addition, the action of GnIH on gonadotropin activity is complex and not always inhibitory, presenting conflicting results in fish reproduction. For example, Zhang and collaborators (2010) reported that zebrafish GnIH reduced gonadotropin Lh serum levels in adult of goldfish (*Carassius auratus*), whereas Amano and collaborators (2006) related that goldfish GnIH elevated gonadotropins (Lh/Fsh) release from salmon pituitary cell cultures and possibly these effects may also be directly related to spermatogenesis.

Altogether data suggesting the crossover between thyroid hormones and reproductive axes. The objective this work was analyze the interaction between thyroid hormones and GnIH on germ cell development of zebrafish testis chemically-induced to hypothyroidism by methimazole, using morphological, morphometrical analysis, and flow cytometry.

MATERIAL AND METHODS

Animals

Adult male of zebrafish was bred and raised in the aquarium facility of Department of Biological Sciences, University of Calgary, Calgary, Alberta, Canada. Handling and experimentation were consistent with the Guideline for Animal Care and Use.

Hypothyroidism-induced by *in vivo* methimazole exposure

A working solution of methimazole (1- methyl-3H-imidazole-2-thione) (CAS 60-56-0, Sigma-Aldrich) was used to induce the hypothyroidism. In this context, male adult zebrafish (n=40) were divided into two groups: group I, control (only filtered

water) (n=20); group II, filtered water adding 1mM of methimazole (n=20). Fishes were exposed to the treatments for 21 days according the literature (Sharma and Patino, 2013; Sharma et al., 2016). Methimazole was diluted in destiled water. The water was renewed every day and the temperature of the room was set to maintain 28°C and fishes were fed twice a day using a commercial food (Zeigler®). The fish were euthanized by overdose with benzocaine hydrochloride (250 mg) previously dissolved in ethanol and then mixed with water. The animals were immersed in the benzocaine solution until death. After, testes were dissected out and one testis was incubated in Leibovitz culture medium (L-15) while its contralateral ones in different treatments with hormones: gonadotropin-inhibitory hormone (GnIH) and human chorionic gonadotropin (hCG) (similar to luteinizing hormone (Lh)) alone or combination for morphometric histological analysis and flow citometry using FACScan cell cycle analysis.

Long-term *in vitro* effects of hCG and zGnih on the hypothyroidism testis

The endocrine regulation was evaluated by using a well-established *in vitro* culture system previously described by Leal et al. (2009). For this, zebrafish testes (n=8 for control and methimazole group) were dissected out and one testis was incubated in Leibovitz culture medium (L-15) while its contra-lateral ones in different treatments. We used 100ng/mL zGnih and 5 IU hCG. To evaluate the crossover between GnIH and hCG in the euthyroidism and hypothyroidism testis, the hormones were combined at the tissue culture, 100ng/mL zGnih plus 5 IU hCG. Doses were selected based on previous experiments. Testes were incubated in a 24 well plate containing 1 mL of culture medium per well in a humidified air atmosphere at 28 ºC for seven days, changing the medium every

3 days. The medium was collected and kept at the refrigerator at -20ºC for 11-Ketotestosterone (11-KT) measurement by immunoenzymatic assay ELISA (582751, Cayman Chemical), following the manufacturer's instructions. The testis was collected for morphometrical histological analysis and flow citometry using FACScan cell cycle analysis.

Morphological and morphometrical analysis of spermatogenesis

After the *in vivo* treatment (methimazole), zebrafish testes (n=8) were fixed in 4% in Karnovsky at 4°C overnight, dehydrated, embedded in Technovit (7100) historesin, sectioned at 3µm thickness, and stained with toluidine blue, according conventional histological procedures. The slides were evaluated, and the proportion occupied by germ cell cysts (type A spermatogonia undifferentiated, type A spermatogonia differentiated, type B spermatogonia, spermatocytes, spermatid and spermatozoa) was determined. The measuring was performed by counting the intersection point on the histological fields. For that, 5 fields per slide (n=8 slides per treatment) were quantified using a grid of 540 (54x10) intersections under 100x objective lens. Spermatozoa number was also quantified for each treatment; 20 different fields were taken from 100x objective lens and analyzed by IMAGE J Software (available at http://imagej.nih.gov/ij/index.html) for quantification of spermatozoa number according Fallah et al. (2019). This software allows quantification of several types of cells by limiting the size of the particle. First, it is necessary to defocus the image, and this filter may avoid false positive cells and improve the measurement. Following up, threshold feature is activated, which separates the background of unspecific particles of interest. Then the RGB image is converted into gray scale

picture (8 or 16 bytes) and the threshold allows that lower intensity signals are white and higher intensity signals as black. The algorithm (analyze particles) can be applied for measuring the black particles in that range of pre-fixed size.

Isolation of testicular cells by flow citometry using Fluorescence Activated Cell Sorting (FACScan) cell cycle analysis

FCM is an automated approach to measure the number of cells. This procedure offer precision, sensitivity, accuracy and multiparametric analysis (Cordelli et al., 2005). Flow cytometer was calibrated with three-millimeter rainbow calibration particles (Spherotech, Libertyville, IL). Then, the number of cells in different phases of the cell cycle were counted from the testes previously cultured (7 days). The testes were dissociated, fixed and stained with propidium iodide (PI) (Thermo Fisher Scientific, Gibco®, Canada). The first step the tissue was minced in small fragments and dissociated using 5 mM ethylenediaminetetraacetic acid (EDTA) in phosphate-buffered saline (PBS) and trypsin-EDTA on the proportion 0.05% trypsin and 0.02% EDTA. The cell in suspension was centrifuged at 3500 rpm during 15 minutes at 4°C. In the next step the cells were filtered with a 40 mm nylon mesh with medium culture (L-15) to obtain a suspension of single cells. After centrifugation, the dissociated cells were washed in PBS and fixed in 70% ethanol per 30 minutes. After that, PBS (1X) combined with 20 mg/mL RNase A form (Sigma-Aldrich (Oakville, ON, Canada) was added to the cells to excluded RNA. The cells were stained with 12 µl PI from 1mg/mL stock solution. Finally, the samples were transferred to FACScan tubes. The analysis was performed using a FACSCalibur system. The fluorescence signals of 10000 cells were counted at 65 nm. The PBS with PI (blank solution) and unstained cells were

used as controls. The proportion of haploid cells was detected using FlowJo (FlowJo LLC. Ashland, Oregon), Cyflogic V 1.2.1 (CyFlo Ltd. Turku, Finland) and BD CellQuest Pro (BD Biosciences. San Jose, 170 CA, USA) analysis software (Nóbrega et al., 2015, Fallah et al., 2019).

Statistical analysis

Data are presented as mean ± SEM (standard error of mean). Significant differences were identified using paired t-tests for two groups or one-way ANOVA followed by the Student–Newman–Keuls test for three or more groups. Significance level (*p*) was considered at 0.05 in both cases. All data analysis was performed by Graph Pad Prism software 7.04 (Graph Pad Software, Inc., San Diego, CA, USA, http://www.graphpad.com).

RESULTS

Effects of hCG on zebrafish spermatogenesis

We evaluated the *in vitro* effects of 5 IU hCG on normal and hypothyroid testis on zebrafish spermatogenesis using morphological, morphometrical and FACScan analysis. Morphological and morphometrical analysis showed that the treatment with hCG on normal testis stimulates the increased the number of spermatozoa (Figs. 2A, C and 3A-B), while the proportion of number of type A undifferentiated spermatogonia (A_{und*} and A_{und}), differentiated spermatogonia (A_{diff}), type B spermatogonia (SpgB), spermatocytes (Spc) and spermatids (Spt) did not change. Similar to morphometrical analysis, hCG treatment increased the

number of spermatozoa when compared to the basal condition in the FCM (Fig. 4C).

The number of spermatids and spermatozoa reduced in the testes of methimazole group after 7 days of culture, when compared to the basal condition and hCG-treatment (Figs. 1, 2 and 3). On the other hand, the number of spermatids and spermatozoa were similar to the control and when testes from methimazole group were incubated with hCG exposure (Fig. 3A). The FCM analysis showed the number of haploid cell populations (Spt and Spz) decreased in the methimazole group (Fig. 4C), but the number of these cell populations in the presence of hCG did not change in relation to the control (Fig. 4C). We observed that hCG induced the increase of haploid cell populations o the normal testis, demonstrating that hCG (5 IU/mL) induced zebrafish spermatogenesis.

Effects of zGnih on zebrafish spermatogenesis

We investigated *in vitro* effects of zGnih (100 nM/mL) in the testes of normal and hypothyroid group. Based on the morphological and morphometrical data, the number of type A undifferentiated spermatogonia (A_{und}) decreased in the normal and methimazole group incubation with zGnih (Figs. 2A, E and 3A). On the other hand, the proportion type B spermatogonia was increased in zebrafish in both group by zGnih treatment (Figs. 2A, E and 3A). No significant changes occurred for the spermatozoa when zGnih was added in both groups (Figs. 2A, E and 3A). In line with the morphometrical analysis, FCM data showed that zGnih (Fig. 4) did not affected the basal number of cell populations in control group. However, haploid cells were decreased in the methimazole group by zGnih (Fig. 4). The spermatid proportion decreased in the presence of zGnih in

control but did not change in methimazole group (Fig. 3A). The results obtained from FCM did not show significant differences for diploid cell population among the groups (Fig. 4A), but for the meiotic (Fig. 4B) and haploid cells (Fig. 4C) the results where be consistent with the morphometrical analysis.

Effects of combined treatments (5 IU hCG+100nM zGnih)

The concomitant treatment of zGnih inhibited the stimulatory effect of 5 IU hCG on spermatid and spermatozoa in the normal testis (Figs. 2A-H and 3A-B). interestingly, no changes were seen for methimazole. The FCM analysis showed an increased percentage of undifferentiated and differentiated spermatogonia cell population in the co-treatment for both control and methimazole group (Fig. 4A). The hCG induced increase of haploid cells was decreased in the presence of zGnih for control but this effects of zGnih disappeared for methimazole group (Fig. 4). The morphometrical data showed similar effects for type B spermatogonia and spermatids. The effects in these germ cells from the interaction between zGnih and hCG disappeared in methimazole animals.

DISCUSSION

The current work characterized the direct effects of zGnih and hCG-induced spermatogenesis in methimazole-treated males. We used morphological and morphometrical analysis to quantify the proportion of different germ cell types. Also, the results were evaluated by cell cycle analysis (Nóbrega et al., 2015; Fallah et al., 2019) to quantify diploid and haploid cell populations.

Recently it has been shown that *zgnih* mRNA levels is expressed in germ cells and the interstitial compartment of zebrafish (Fallah et al., 2019), as well as in goldfish (Qi et al., 2013). *in vitro* the concentrations of 100 nM zGnih did not affect basal zebrafish spermatogenesis and gene expression (Fallah et al., 2019). While the co-treatment with 5 IU hCG caused inhibitory effects on haploid population (Fallah et al., 2019).

In additional to the actions of GnIH in vertebrates reproduction, recent studies have demonstrated that GnIH is involved in reproductive dysfunction induced by abnormal thyroid status (Tsutsui et al., 2017). The hypothesis is that a crosstalk between GnIH and thyroid hormones may exist in the zebrafish testes. Our morphometrical and FCM data showed that the 100 nM zGnih did not change the number of germ cells, but GnIH nullified the hCG-induced haploid cells (spermatids and spermatozoa) in control testes. These data are supported by Fallah et al. (2019).

For the methimazole group, hCG increased the proportion of spermatozoa but not stimulate to the control group. zGnih alone did not change the number of spermatozoa. However, interestingly, the negative effects of zGnih in the hCG-induced spermatozoa formation is nullified in the methimazole treatment. The same patter is seen for type B spermatogonia and spermatids. The effects of zGnih and hCG interaction on these germ cells were disappeared in the methimazole. These data indicate that thyroid hormones are necessary for either hCG or GnIH effects in the zebrafish testes.

DECLARATION OF INTEREST

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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REFERENCES

AMANO M, MORIYAMA S, IIGO M, KITAMURA S, AMIYA N, YAMAMORI K, UKENA K, TSUTSUI K. Novel fish hypothalamic neuropeptides stimulate the release of gonadotrophins and growth hormone from the pituitary of sockeye salmon. **J Endocrinol**. 188:417–423. 2006.

BASSET, J.H.D., HARVEY, C.B., WILLIAMS, G.R. Mechanisms of thyroid hormone receptor specific nuclear and extra nuclear actions. **Mol. Cell. Endocrinol**. 213, 1–11. 2003.

BLANTON, M.L., SPECKER, J.L. The hypothalamic-pituitary-thyroid (HPT) axis in fish and its role in fish development and reproduction. **Crit. Rev. Toxicol**. 37: 97–115. 2007.

BROWN, D.D., CAI, L.Q. Amphibian metamorphosis. Dev. Biol. 306, 20**–**33. 2007.

CARR, J.A., PATIÑO, R. The hypothalamus-pituitary-thyroid axis in teleosts and amphibians: endocrine disruption and its consequences to natural populations. Gen. Comp. Endocrinol. 170: 299**–**312. 2011.

CORDELLI, E., ELEUTERI, P., LETER, G., RESCIA, M., & SPANÒ, M. Flow cytometry applications in the evaluation of sperm quality: semen analysis, sperm function and DNA integrity. **Contraception**. 72: 273–279. 2005.

CASTAÑEDA CORTÉS, D.C., LANGLOIS, V.S., FERNANDINO, J.I. Crossover of the hypothalamic pituitary-adrenal/interrenal, -thyroid, and –gonadal axes in axes in testicular development. **Front. Endocrinol**. 139: 1–11. 2014.

CHIBA, H., AMANO, M., YAMADA, H., FUJIMOTO, Y., OJIMA, D., OKUZAWA, K., YAMANOME, T., YAMAMORI, K., IWATA, M. Involvement of gonadotropin-releasing hormone in thyroxine release in three different forms of teleost fish: barfin flounder, masu salmon and goldfish**. Fish Physiol. Biochem**. 30: 267–273. 2004.

CYR, D.G., EALES, J.G. Interrelationships between thyroidal and reproductive endocrine systems in fish. Rev. **Fish Biol. Fish**. 6: 165–200. 1996.

DE GROEF, B., GORIS, N., ARCKENS, L., KÜHN, E.R., DARRAS, V.M. Corticotropin-releasing hormone (CRH)-induced thyrotropin release in directly mediated through CRH receptor type 2 on thyrotropes. **Endocrinology** 144: 5537–5544.2003.

DENVER, R.J. Several hypothalamic peptides stimulate in vitro thyrotropin secretion by pituitaries of anuran amphibians. **Gen. Comp. Endocrinol**. 72: 383–393.1988.

FALLAH, H. P., TOVO-NETO, A., YEUNG, E. C., NÓBREGA, R. H., HABIBI, H. R. Paracrine/autocrine control of spermatogenesis by gonadotropin-inhibitory hormone. **Molecular and Cellular Endocrinology**. 2019.

FARAONE-MENNELLA, M.R., FERONE, A., MARINO, L., CARDONE, A., COMITATO, R., VENDITTI, P., DI MEO, S., FARINA, B. Poly(ADP-ribosyl)ation of proteins and germ cell development in hyperthyroid rat testes. **Mol. Cell. Biochem**. 323: 119–129. 2008.

HUNT, D.W.C., EALES, J.G.The influence of testosterone propionate on thyroid function of immature rainbow trout, Salmo gairdneri richardson. **Gen. Comp. Endocrinol.** 37: 115–121. 1979.

JACOBS, G.F.M., GOYVAERTS, M.P., VANDORPE, G., QUAGHEBEUR, A.M.L., KUHN, E.R. Luteinizing hormone-releasing hormone as a potent stimulator of the thyroidal axis in ranid frogs. **Gen. Comp. Endocrinol**. 70: 274–283. 1988a.

JACOBS, G.F.M., MICHIELSEN, R.P., KUHN, E.R. Thyroxine and triiodothyronine in plasma and thyroids of the neotenic and metamorphosed axolotl Ambystoma mexicanum: influence of TRH injections. **Gen. Comp. Endocrinol.** 70: 145–151. 1988b.

LARSEN, D.A., SWANSON, P., DICKEY, J.T., RIVIER, J., DICKHOFF, W.W. In Vitro thyrotropin-releasing activity of corticotropin-releasing hormone-family peptides in Coho salmon, Oncorhynchus kisutch. **Gen. Comp. Endocrinol**. 109: 276–285. 1998.

LEAL, M.C.; DE WAAL, P.P.; GARCIA-LOPEZ, A.; CHEN, S.X.; BOGERD, J.; SCHULZ, R.W. Zebrafish primary testis tissue culture: An approach to study testis function ex vivo. **General Comparative Endocrinology**, vol. 162, n. 2, p.134-138. 2009.

MACKENZIE, D.S., SOKOLOWSKA, M., PETER, R.E., BRETON, B. Increased gonadotropina levels in goldfish do not result in alterations in circulating thyroid hormone levels. Gen. Comp. **Endocrinol.** 67: 202–213. 1987.

MORAIS, R.D.V.S., NÓBREGA, R.H., GÓMEZ-GONZÁLES, N.E., SCHMIDT, R., BOGERD, J., FRANÇA, L.R., SCHULZ, R.W. Thyroid hormone stimulates the proliferation of sertoli cells and single type a spermatogonia in adult zebrafish (Danio rerio) Testis**. Endocrinology** 154: 4635–4676. 2013.

MUÑOZ-CUETO, J.A., PAULLADA-SALMERÓN, J.A., ALIAGA-GUERRERO, M., COWAN, M.E., PARHAR, I.S., UBUKA, T. A Journey through the Gonadotropin-Inhibitory Hormone System of Fish. 2017.

NÓBREGA, R.H; MORAIS, R.D.V.S.; CRESPO, D.; WAAL, P.P.; FRANÇA, L.R.; SCHULZ, R.W.; BOGERD, J. Fsh Stimulates Spermatogonial Proliferation and Differentiation in Zebrafish via Igf3. **Endocrinology**. 156: 3804–3817. 2015.

OKADA, R., YAMAMOTO, K., KODA, A., ITO, Y., HAYASHI, H., TANAKA, S., HANAOKA, Y., KIKUYAMA, S. Development of radioimmunoassay for bullfrog thyroid-stimulating hormone (TSH): effects of hypothalamic releasing hormones on the release of TSH from the pituitary in vitro**. Gen. Comp. Endocrinol**. 135: 42–50. 2004.

OROZCO, A., VALVERDE, C.R., OLVERA, A., GARCÍA, C.G. Iodothyronine deiodinases: a functional and evolutionary perspective. J**. Endocrinol**. 215: 207–219. 2012.

PARHAR, I.S., SOGA, T., SAKUMA. Thyroid hormone and estrogen regulate brain region specific messenger ribonucleic acids encoding three gonadotropin-releasing hormone genes in sexually immature male fish, Oreochromis niloticus. **Endocrinology** 141: 1618–1626. 2000.

PAULLADA-SALMERÓN JA, COWAN M, ALIAGA-GUERRERO M, LÓPEZ-OLMEDA JF, MAÑANÓS EL, ZANUY S, et al. Testicular steroidogenesis and locomotor activity are regulated by gonadotropina inhibitory hormone in male European sea bass. PLoS One. 2016.

QI, X., ZHOU, W., LU, D., WANG, Q., ZHANG, H., LI, S., LIN, H. Sexual Dimorphism of Steroidogenesis Regulated by GnIH in the Goldfish, Carassius auratus1. **Biology of Reproduction**. 2013.

ROY, P., DATTA, M., DASGUPTA, S., BHATTACHARYA, S. Gonadotropin-releasing hormone stimulates thyroid activity in a freshwater murrel, Channa gachua (Ham.), and Carps, Catla catla (Ham.) and Cirrhinus mrigala (Ham.). **Gen. Comp. Endocrinol**. 117: 456–463.2000.

SAFIAN, D., MORAIS, R.D.V.S., BOGERD, J., SCHULZ, R.W. Igf Binding proteins protect undifferentiated spermatogonia in the zebrafish testis against excessive differentiation. **Endocrinology** 157: 4423–4433. 2016.

SWAPNA, I., RAJASEKHAR, M., SUPRIYA, A., RAGHUVEER, K., SREENIVASULU, G., RASHEEDA, M.K., MAJUMDAR, K.C., HAGAWA, H., TANAKA, H., DUTTA-GUPTA, A., SENTHILKUMARAN, B. Thiourea-induced thyroid hormone depletion impairs testicular recrudescence in the air-breathing catfish Clarias gariepinus. **Comp. Biochem. Physiol**. 144: 1–10. 2006.

TSUTSUI, K., SON, Y. L., KIYOHARA, M., & MIYATA, I. Discovery of GnIH and Its Role in Hypothyroidism-Induced Delayed Puberty. **Endocrinology**. 159: 62–68. 2017.

TSUTSUI, K., SON, Y.S., KIYOHARA, M., MIYATA, I. Discovery of GnIH and its role in hypothyroidism-induced delayed puberty. **Endocrinology** 159: 62–68. 2018.

WAGNER, M.S., WAJNER, S.M., MAIA, A.N. Is there a role for thyroid hormone on spermatogenesis. **Microsc. Res. Tech**. 72: 796–808.2009.

ZHANG, Y.; LI, S.; LIU, Y.; LU, D.; CHEN, H.; HUANG, X. Structural diversity of the GnIH/GnIH receptor system in teleost: its involvement in early development and the negative control of LH release. **Peptides**. 31.1034–1043. 2010.

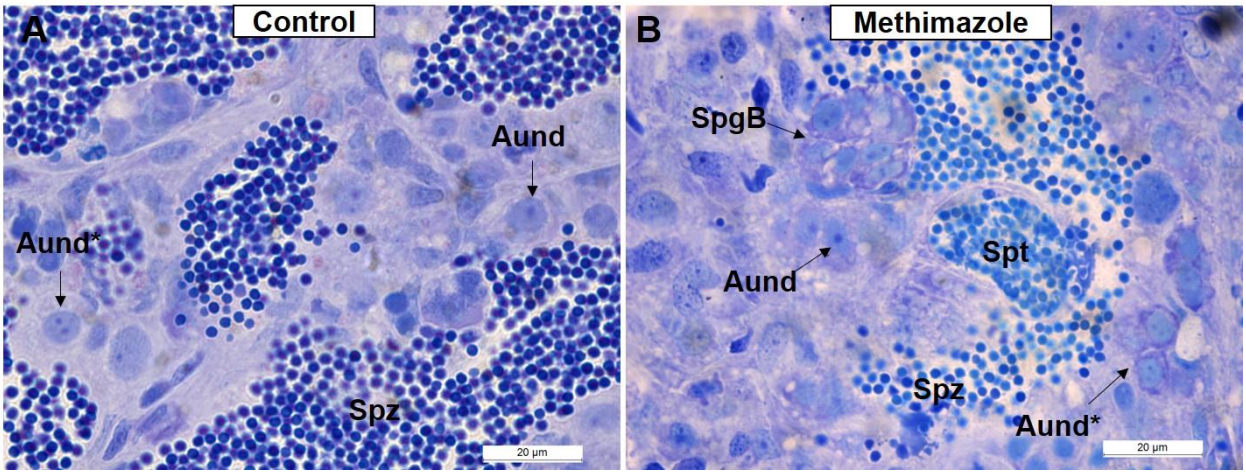
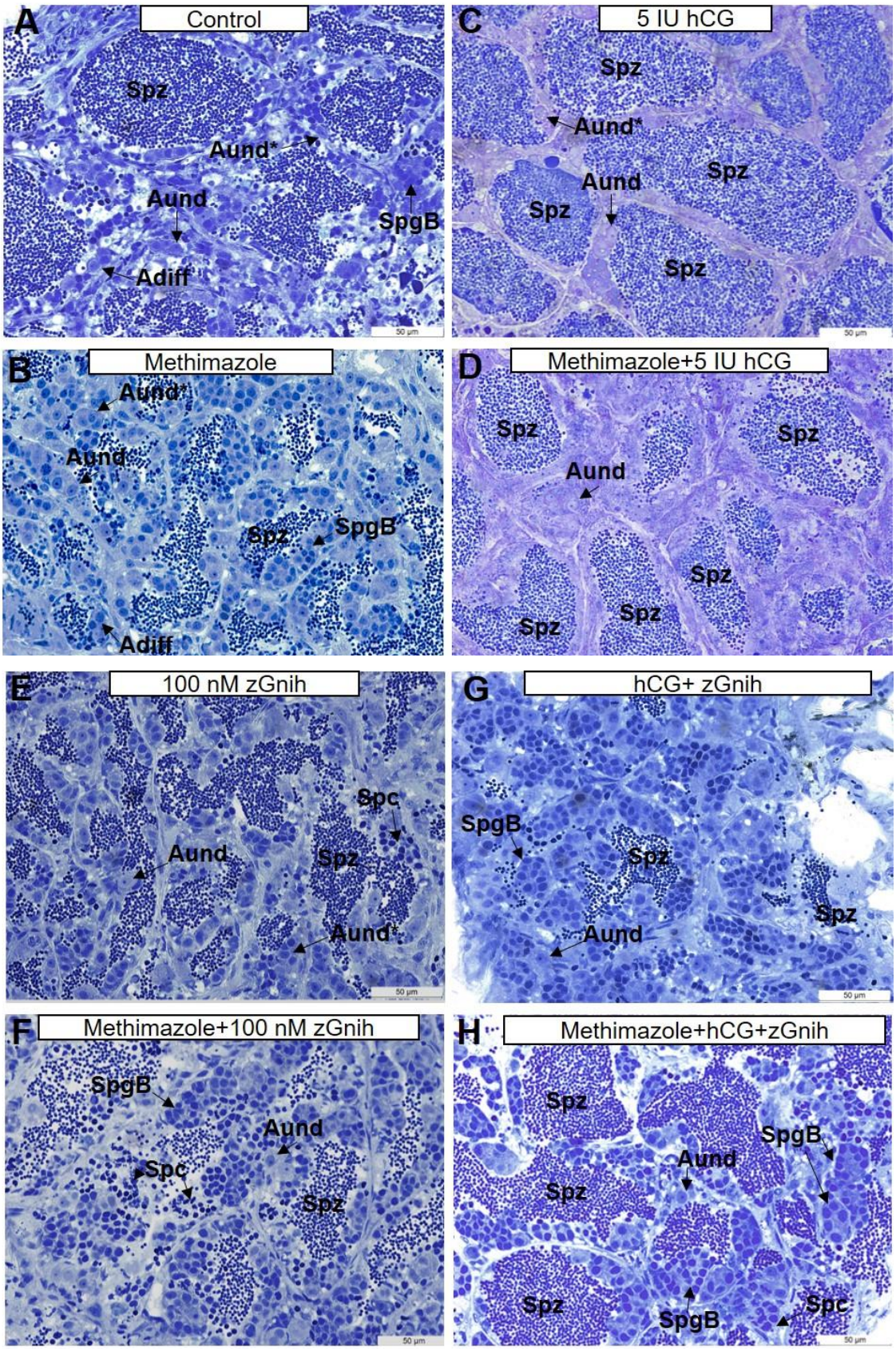


Figure 1 - Morphological sections from zebrafish testis after 7 days culture *in vitro* post methimazole-treatment for 21 days. **(A):** control group **(B):** methimazole treated group. type A undifferentiated spermatogonia (A_{und*} and A_{und}), type B spermatogonia (SpgB), spermatids (Spt), spermatozoa (Spz). Staining: Toluidine blue. 100µm.


A
Control
Spz
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Aund*
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hCG+ zGnih
SpgB
Spz
Spz
Aund
F
Methimazole+100 nM zGnih
SpgB
Aund
Spc
Spz
H
Methimazole+hCG+zGnih
Spz
SpgB
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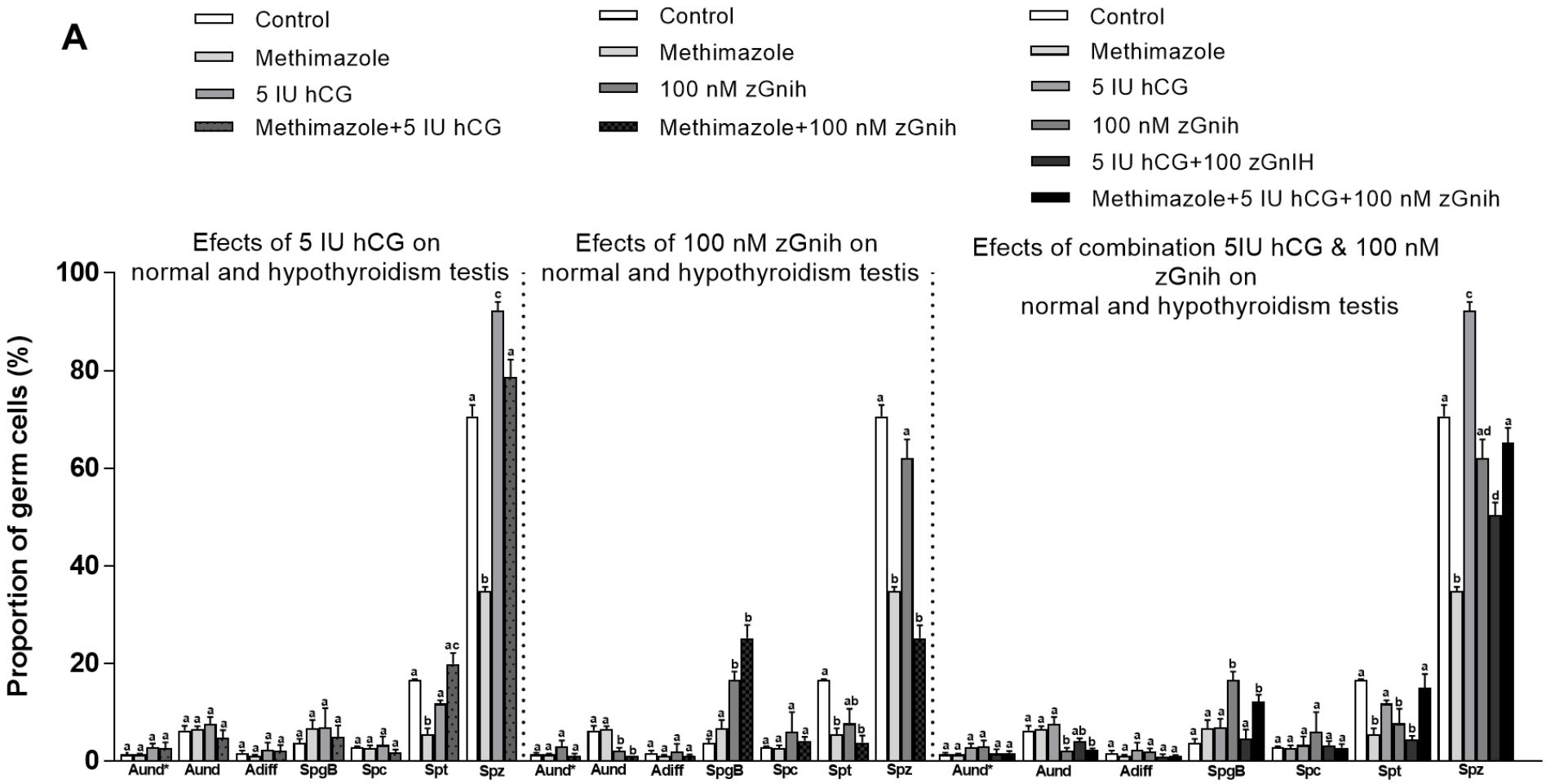
SpgB
Spc

Figure 2 - Morphological sections from zebrafish testis after 7 days culture *in vitro* post methimazole-treatment for 21 days. **(A):** control group **(B):** methimazole treated group. **(C):** 5 IU hCG alone. **(D):** methimazole plus 5 IU hCG. **(E):** 100 nM zGnih alone. **(F):** methimazole plus 100 nM zGnih. **(G):** 5 IU hCG plus 100 nM zGnih. **(H):** methimazole plus 5 IU hCG & 100 nM zGnih. Type A undifferentiated spermatogonia (A_{und*} and A_{und}), differentiated spermatogonia (A_{diff}), type B spermatogonia (SpgB), spermatocytes (Spc), spermatids (Spt), spermatozoa (Spz). Staining: Toluidine blue. 40µm.


A
Control
Methimazole
5 IU hCG
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Control
Methimazole
5 IU hCG
100 nM zGnih
5 IU hCG+100 zGnIH
Methimazole+5 IU hCG+100 nM zGnih
Efects of 5 IU hCG on normal and hypothyroidism testis
Efects of 100 nM zGnih on normal and hypothyroidism testis
Efects of combination 5IU hCG & 100 nM zGnih on normal and hypothyroidism testis
Proportion of germ cells (%)
0
20
40
60
80
100
Aund*
Aund
Adiff
SpgB
Spc
Spt
Spz

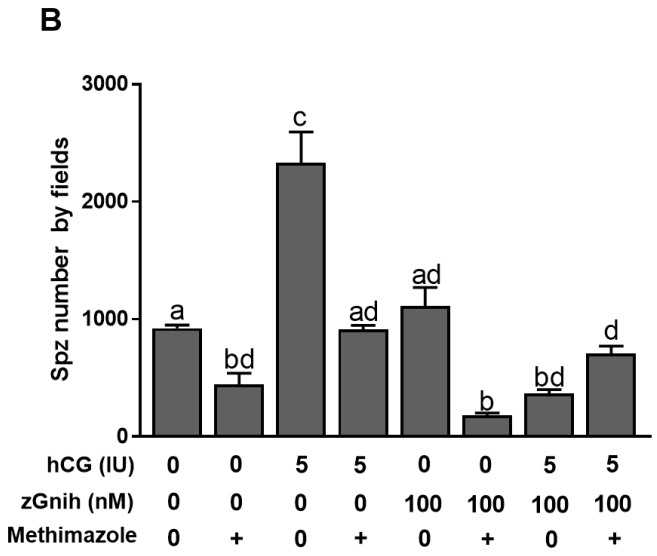

Figure 3 – **(A)** *In vitro* effects of 5 IU/mL hCG and zGnih (100nM) alone or in combination on population of germ cells on normal and hypothyroid zebrafish testis by morphometric analysis. Type A undifferentiated spermatogonia (A_{und*} and A_{und}), differentiated spermatogonia (A_{diff}), type B spermatogonia (SpgB), spermatocytes (Spc), spermatids (Spt), spermatozoa (Spz) on histological points. **(B)** spermatozoa (Spz) number by fields of control and different treatments. Bars represent the mean±SEM. Different letters denote statistically different from the control ANOVA followed by Tukey's multiple comparison test, $P \leq 0.05$).

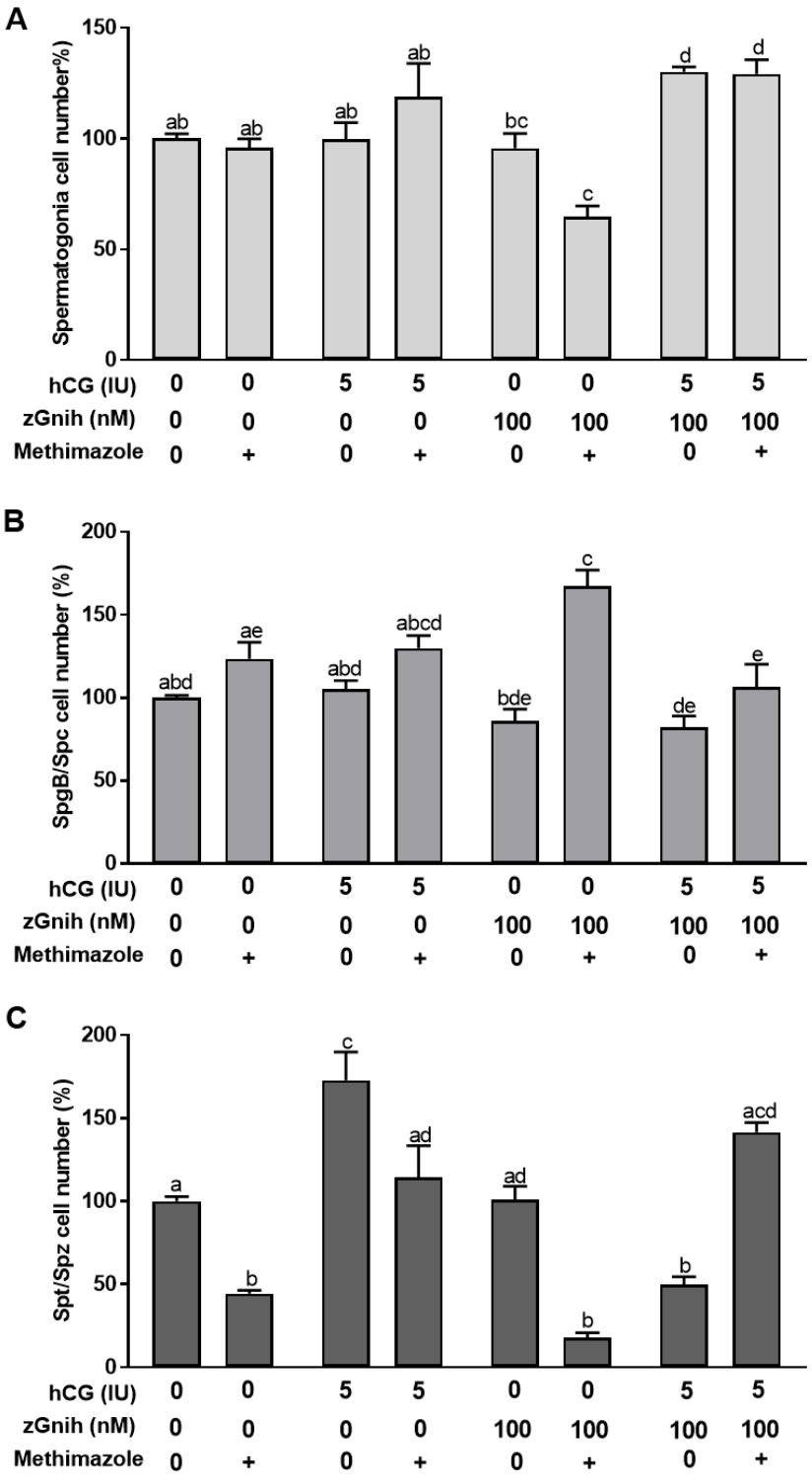

Figure 4 – A comparison of effects of hCG (5 IU/mL) and zGnih (100nM) alone or in combination on euthyroid and hypothyroid testis induced by methimazole on spermatogonia (undifferentiated and differentiated) **(A)**, B spermatogonia (SpgB) and spermatocytes (Spc) **(B)**, spermatids (Spt) and spermatozoa (Spz) **(C)** in zebrafish testis. Following 7 days treatment *in vitro*, zebrafish testes were dissociated and assayed for cell cycle analysis. The percentage of different cell population were determined by FACScan analysis. Counts were normalized to 100%, and treatment groups were normalized against control (mean ± SEM; n = 5) Different letters represent statistical significance between groups (ANOVA followed by Tukey's multiple comparison test, $P \leq 0.05$).

7. CONCLUSÕES GERAIS

O papel dos hormônios tireoidianos (Hts) foram investigados *in vivo* e *in vitro* no eixo reprodutivo. Os dados mostrados na presente dissertação indicam a relação entre os eixos hipotalâmico-hipofisário-gonadal e tireoide (HHP-HHT) em machos adultos de zebrafish. Dessa forma:

1. Os hormônios tireoidianos são essenciais para a função testicular de adultos de zebrafish;

2. O hipotireoidismo induzido pelo methimazole afetou a espermatogênese de zebrafish levando ao acúmulo de células pré-meióticas, atraso na diferenciação e meiose, reduzindo a quantidade de espermatozoides formados;

3. O methimazole afeta o eixo hipotalâmico-hipofisário;

4. Os efeitos biológicos do Fsh na função testicular são dependentes dos hormônios tireoidianos;

5. Os hormônios tireoidianos são cruciais para os efeitos estimulatórios do hCG e inibitório do GnIH na regulação da espermatogênese de adultos de zebrafish.