

UNIVERSIDADE ESTADUAL PAULISTA – UNESP
CENTRO DE AQUICULTURA DA UNESP

**CONTROLE ENDÓCRINO E MOLECULAR
ENVOLVIDOS NA ESPERMATOGÊNESE DE
ZEBRAFISH (*Danio rerio*): EFEITOS DOS
HORMÔNIOS CORTICOIDES E
TIREOIDIANOS**

Aldo Tovo Neto

Botucatu, São Paulo
2018

UNIVERSIDADE ESTADUAL PAULISTA - UNESP

CENTRO DE AQUICULTURA DA UNESP

**CONTROLE ENDÓCRINO E MOLECULAR
ENVOLVIDOS NA ESPERMATOGÊNESE DE
ZEBRAFISH (*Danio rerio*): EFEITOS DOS
HORMÔNIOS CORTICOIDES E
TIREOIDIANOS**

Aldo Tovo Neto

Orientador: Dr. Rafael Henrique Nóbrega

Tese apresentada ao Programa de Pós-Graduação em Aquicultura do Centro de Aquicultura da Unesp, *campus* de Jaboticabal, como parte das exigências para a obtenção do título de Doutor em Aquicultura.

Botucatu, São Paulo

2018

T736c Tovo Neto, Aldo
Controle endócrino e molecular envolvidos na espermatogênese de zebrafish (*Danio rerio*) : efeitos dos hormônios corticoides e tireoidianos / Aldo Tovo Neto. – – Jaboticabal, 2018
v, 87 p : il. ; 29 cm

Tese (doutorado) - Universidade Estadual Paulista, Centro de Aquicultura, 2018
Orientador: Rafael Henrique Nóbrega
Banca examinadora: Maeli Dal Pai, Renata Guimarães Moreira Whitton, Hamid Habibi, Gustavo Manuel Somoza

Bibliografia

1. Zebrafish. 2. Espermatogênese. 3. Expressão gênica. 4. Fsh. 5. Hipotireoidismo. I. Título. II. Jaboticabal-Centro de Aquicultura.

CDU 639.3

Ficha catalográfica elaborada pela Seção Técnica de Aquisição e Tratamento da Informação – Diretoria Técnica de Biblioteca e Documentação - UNESP, Câmpus de Jaboticabal.

CERTIFICADO DE APROVAÇÃO

TÍTULO DA TESE: **CONTROLE ENDÓCRINO E MOLECULAR ENVOLVIDOS NA
ESPERMATOGÊNESE DE ZEBRAFISH (Danio rerio): EFEITOS DOS HORMÔNIOS
CORTICOIDES E TIREOIDIANOS**

AUTOR: ALDO TOVO NETO

ORIENTADOR: RAFAEL HENRIQUE NÓBREGA

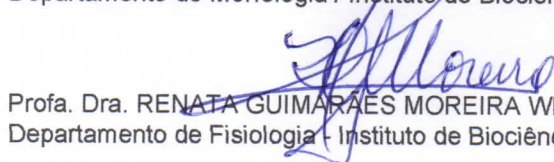
Aprovado como parte das exigências para obtenção do Título de Doutor em AQUICULTURA, pela
Comissão Examinadora:



Prof. Dr. RAFAEL HENRIQUE NÓBREGA
Departamento de Morfologia / Instituto de Biociências de Botucatu - UNESP



Profa. Dra. MAELI DAL PAI
Departamento de Morfologia / Instituto de Biociências de Botucatu - UNESP

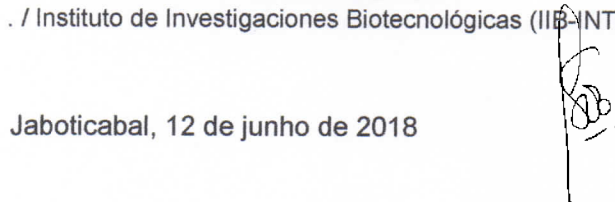


Profa. Dra. RENATA GUIMARÃES MOREIRA WHITTON
Departamento de Fisiologia - Instituto de Biociências / Universidade de São Paulo - SP

Prof. Dr. HAMID HABIBI
. / Universidade de Calgary, Canadá



Prof. Dr. GUSTAVO MANUEL SOMOZA
. / Instituto de Investigaciones Biotecnológicas (IIB-INTTECH)-Argentina



Jaboticabal, 12 de junho de 2018

SUMÁRIO

CAPÍTULO I - INTRODUÇÃO GERAL	8
1. Hormônio cortisol e definição de agentes estressores	15
2. Hormônios tireoidianos (HTs)	21
3. O zebrafish (<i>D. rerio</i>) como modelo experimental	25
Objetivo Geral	26
Objetivos específicos	26
Referências	27
CHAPTER II - CORTISOL PROTECTS TYPE A UNDIFFERENTIATED SPERMATOGONIA AGAINST DIFFERENTIATION AND STIMULATES ZEBRAFISH SPERMATOGENESIS <i>IN VITRO</i>	38
Introduction	41
Material and Methods	42
Results	48
Discussion	54
Conclusion	57
References	58
CAPÍTULO III - THYROID HORMONES ON ZEBRAFISH (<i>Danio rerio</i>) SPERMATOGENESIS	65
Introduction	67
Material and Methods	68
Results and Discussion	71
Conclusion	81
References	82
GENERAL CONCLUSION	85
APPENDIX	87

DEDICATÓRIA

“Aos meus pais, Suely Sandra S. Tovo e Paulo Reinaldo Tovo (in memoriam), por não pouparem esforços para minha educação, os quais me fizeram chegar até aqui, DEDICO”.

AGRADECIMENTOS

Ao Programa de Pós-Graduação em Aquicultura da Unesp (CAUNESP), pela oportunidade de cursar Doutorado nesta Instituição.

Ao Departamento de Morfologia da Unesp, *campus* de Botucatu.

Ao prof. Dr. Rafael Henrique Nóbrega (Orientador), pela confiança depositada e orientação durante esses 4 anos de curso, assim como pelo conhecimento compartilhado, que muito me fizeram crescer profissionalmente.

Ao Dr. Emanuel Martinez (Coorientador), pelos ensinamentos práticos e teóricos, que foram decisivos para o desenvolvimento desta tese.

Ao Dr. Hamid Habibi (University of Calgary), por me receber em seu laboratório durante o estágio no exterior.

Aos colegas de laboratório, Reproductive and Molecular Biology Group, pelo companheirismo, troca de conhecimentos e ajuda nos experimentos.

Aos colegas, Aline Melo, Giovana Branco e Arno Butzge, pela ajuda com os experimentos de cultura com o cortisol.

À amiga Maira Rodrigues, pela ajuda com as análises histológicas e figuras desta Tese.

Aos colegas de laboratório, HRH Lab, pelo companheirismo, troca de conhecimentos e ajuda nos experimentos.

Ao técnico José Eduardo Bozzano, pela disponibilidade em ajudar sempre.

À Prof. Dra. Renata Guimarães, por disponibilizar seu laboratório para os ensaios de ELISA. Gostaria de agradecer também ao Renato Honji, pela ajuda nas análises de ELISA.

À minha noiva, Tamara Galvão, pelo companheirismo e apoio durante essa longa caminhada do Doutorado.

APOIO FINANCEIRO

O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Código de Financiamento 001.

O presente trabalho também foi realizado com apoio da FAPESP, através do convênio FAPESP/CAPES, PROCESSO nº 2014/16280-5, Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), pela concessão da bolsa no país; e PROCESSO nº 2017/09218-0, Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), pela concessão da bolsa de estágio no exterior (BEPE).

O presente trabalho também foi realizado com apoio do Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), através do Programa “Ciência Sem Fronteiras”, pela concessão da bolsa no exterior (Processo 234548/2014-2).

RESUMO

Diversas pesquisas deixam claro que os hormônios do metabolismo como o cortisol e hormônios tireoidianos (HTs, T3 e T4) podem influenciar a reprodução de peixes diretamente afetando hormônios do eixo hipotalâmico-hipofisário-gonadal, ou indiretamente por diferentes mecanismos. Embora os efeitos do cortisol na reprodução tenham sido estudados extensivamente, os efeitos diretos à nível gonadal receberam menos atenção nesse processo. Em relação aos HTs, estudos mostram uma correlação positiva entre HTs e o status reprodutivo, onde os HTs são frequentemente associados com o desenvolvimento testicular, crescimento e maturação. O objetivo desta tese foi estudar os efeitos do cortisol e HTs na espermatogênese de zebrafish utilizando cultura de órgãos *ex vivo*. Foram avaliados a proliferação de células germinativas, quantificação de cistos germinativos, liberação de andrógenos (ELISA) e expressão de genes testiculares. O cortisol tem um efeito positivo na espermatogênese estimulando a diferenciação de células germinativas e aumentando a quantidade de espermatozoides no lúmen, de forma independente de andrógeno. O T3 afetou a expressão de genes expressos em células germinativas, enquanto o T4 não alterou a expressão destes genes. Além disso, os estudos *in vivo* mostraram que o metimazol aumentou a área ocupada pelas espermatogônias do tipo A e tipo B, enquanto que a área do lúmen e a quantidade de espermatozoides reduziram significativamente, sugerindo que o hipotireoidismo induzido prejudica a meiose, além de modular a expressão de genes nas células de Sertoli e Leydig.

PALAVRAS-CHAVE

zebrafish, espermatogênese, expressão gênica, Fsh, hipotireoidismo.

ABSTRACT

It has been increasingly clear that metabolic hormones such as cortisol and thyroid hormones (THs, T3 and T4) can also influence fish reproduction either directly by affecting hormones of the hypothalamic-pituitary-gonadal axis or indirectly by different mechanisms. Although the effects of cortisol in reproduction were extensively studied, direct effects at gonadal level have received less attention in this process. Regarding THs, it has been shown a positive correlation between THs and fish reproductive status, where THs are frequently associated with testicular development, growth and maturation. The aim of this thesis was to study the effects of cortisol and THs on zebrafish spermatogenesis using an *ex vivo* organs culture. We evaluated germ cell proliferation activity, quantification of germ cysts, androgen release by ELISA and the mRNA expression of testicular genes. Cortisol has a positive role in zebrafish spermatogenesis by stimulating germ cells differentiation and increase of free spermatozoa in the lumen, in an androgen independent manner. T3 affected germ cells related genes, while T4 did not alter these evaluated genes. Moreover, *in vivo* studies showed that methimazole increased the area occupied by the undifferentiated type A spermatogonia and type B spermatogonia, while spermatozoa and the lumen area were reduced significantly, which suggests that hypothyroidism-induced impairs meiosis and modulates the mRNA expression of Sertoli and Leydig cells genes.

KEYWORDS

zebrafish, spermatogenesis, gene expression, Fsh, hypothyroidism.

Capítulo I

Introdução Geral

Este capítulo foi parcialmente publicado como revisão na revista “General and Comparative Endocrinology” (anexo)

TOVO-NETO, A.; RODRIGUES, M.S.; HABIBI, H.R.; NÓBREGA, R.H. 2018. Thyroid hormones action on male reproductive system of teleost fish. Gen. Comp. Endocrinol. <https://doi.org/10.1016/j.ygcen.2018.04.023>

Processo reprodutivo em peixes

O processo reprodutivo em peixes, como nos demais vertebrados, é dependente do eixo hipotalâmico-hipofisário-gonadal (HPG). Este eixo se inicia com a produção hormônio liberador de gonadotrofina (*gonadotropin releasing hormone*, GnRH). O GnRH é um decapeptídeo, conhecido por regular a síntese e liberação dos hormônios gonadotróficos (GtHs), hormônio folículo estimulante (FSH) e hormônio luteinizante (LH), que por sua vez, estimulam a função gonadal nos vertebrados via indução da síntese de esteroides sexuais (ANDREU-VIEYRA e HABIBI, 2007).

Os vertebrados (incluindo os peixes) expressam duas ou mais formas de GnRHs, sendo que até o momento, oito formas já foram reportadas entre os peixes teleósteos (ZOHAR et al., 2010). Os GnRHs são expressos em inúmeros tecidos, mas a forma produzida no hipotálamo parece ter um papel primário na ativação do eixo HPG via estimulação sináptica das células produtoras de gonadotrofinas (gonadotrófos) (PANKHURST, 2016).

Em algumas espécies de peixes, a produção destas gonadotrofinas (FSH e LH) está sob a ação inibitória de neurônios produtores de dopamina (Figura 1). Desta forma, o balanço entre a ação inibitória da dopamina e a estimulatória do GnRH, modulam a síntese e liberação de gonadotrofinas (DUFOUR et al., 2010). O efeito inibitório da dopamina foi demonstrado em algumas ordens de teleósteos, tais como Cypriniformes, Salmoniformes, Siluriformes, Cichliformes e Mugiliformes (veja DUFOUR et al., 2010).

Além disso, em um estudo mais recente foi descrito, outro mecanismo que modula a síntese e liberação de GnRH que ocorre através do sistema kisspeptina, o qual parece ser responsável por transformar estímulos ambientais exógenos e endógenos em efeitos no sistema reprodutivo (Figura 1) (ZOHAR et al., 2010; SHAHJAHAN et al., 2013). Por fim, estudos recentes descobriram outro neuropeptídeo hipotalâmico, conhecido como hormônio inibidor das gonadotrofinas (GnIH), que inibe a síntese e a liberação de gonadotrofinas (Figura 1) (TSUTSUI et al., 2018).

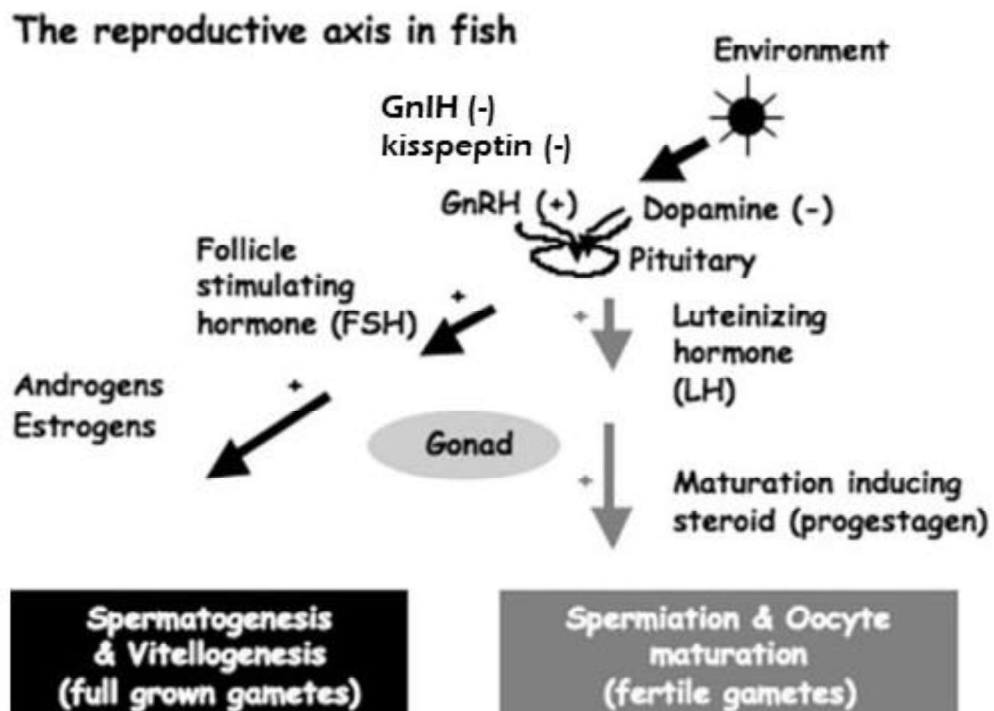


Figura 1. Visão esquemática do eixo reprodutivo de peixes. A partir dos estímulos ambientais, estimula a síntese e secreção do GnRH, hormônio liberador de gonadotrofina. Entretanto, a liberação do GnRH é regulada pelo hormônio inibidor de gonadotrofina (GnIH), kisspeptinas e Dopamina. O GnRH estimula a produção das gonadotrofinas FSH (hormônio folículo estimulante) e LH (hormônio luteinizante), as quais vão agir localmente nas gônadas e irão estimular a produção de andrógenos, estrógenos e progestágenos. Modificado de MYLONAS et al., 2010.

As gonadotrofinas (Fsh e 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 gonadotróficos possuem papel crucial na regulação da espermatogênese (PIERCE e PARSONS, 1981). Quando liberadas na corrente sanguínea, as gonadotrofinas exercem sua atividade biológica na via receptores de proteínas G. Com exceção da classe dos Agnathas (lampréias e hagfishes) que possuem somente uma glicoproteína (SOWER et al., 2009), a presença de ambos FSH e LH e respectivos receptores é bem documentada nos vertebrados em geral (SCHULZ et al., 2010), e ambas gonadotrofinas desempenham diferentes papéis nas gônadas.

Em mamíferos, ambas as gonadotrofinas são claramente definidas, dadas as interações altamente específicas entre cada hormônio e seu respectivo

receptor (GÁRCIA-LOPEZ et al., 2010). O Lh (hormônio luteinizante) atua na esteroidogênese das células de Leydig e o Fsh (hormônio folículo estimulante) regula a função das células de Sertoli.

Em peixes teleósteos, o Fsh também atua nas células de Leydig pois tais células apresentam receptores de Fsh como descrito em *Clarias gariepinus* (GARCIA-LOPEZ et al., 2009) e *Danio rerio* (GARCIA-LOPEZ et al., 2010). As células de Leydig estão no compartimento intertubular dos testículos geralmente localizadas ao redor de vasos sanguíneos (LEAL et al., 2009). As células de Leydig são responsáveis pela produção de andrógenos e fatores de crescimento (SCHULZ et al., 2010).

Processo de espermatogênese em mamíferos e peixes

A espermatogênese é um processo altamente conservado entre os cordados e compreende uma série de eventos altamente precisos e coordenados, nos quais uma única espermatogônia tronco se diferencia para originar milhares de espermatozóides. Este processo é dividido em três grandes fases (RUSSELL et al., 1990; SHARPE, 1994; FRANÇA e CHIARINI-GARCIA, 2005; NÓBREGA et al., 2009; SCHULZ et al., 2010): (1) fase espermatogonial ou proliferativa, caracterizada por sucessivas divisões mitóticas das espermatogônias; (2) fase espermatocitária ou meiótica, em que o material genético dos espermatócitos é duplicado, recombinado e segregado, formando células haplóides denominadas de espermátides; e (3) fase espermiogênica ou de diferenciação, na qual as espermátides passam por modificações estruturais e funcionais altamente complexas para originar os espermatozóides, que estão aptos para a fecundação.

Embora altamente conservada, a espermatogênese apresenta certas peculiaridades em termos de sua unidade morfofuncional dependendo do grupo de estudo. Em peixes teleósteos, por exemplo, a espermatogênese ocorre no interior de estruturas denominadas espermatocistos, ou cistos, que se formam quando uma única espermatogônia primária ou do tipo A é completamente envolvida pelos prolongamentos das células de Sertoli (Figura 2) (GRIER, 1993; PUDNEY, 1993, 1995; SCHULZ et al., 2010). As células germinativas derivadas desta espermatogônia dividem-se sincronicamente para constituir um clone de

células germinativas que é envolvido por um número variado de células de Sertoli, dependendo do tipo de cisto (VILELA et al., 2003).

Diferente dos mamíferos, onde as células de Sertoli estão em contato com várias gerações de células germinativas (Figura 2A) (RUSSELL et al., 1990), na espermatogênese cística as células de Sertoli normalmente estão em contato com apenas um tipo específico de célula germinativa durante a evolução do processo espermatogênico (Figura 2B) (NÓBREGA et al., 2009; SCHULZ et al., 2010). Estes cistos encontram-se apoiados na túnica própria dos túbulos seminíferos, que é formada por uma camada acelular denominada de membrana basal e pelas células peritubulares mióides (Figura 2B) (KOULISH et al., 2002).

Nos vertebrados em geral, o testículo é composto de dois compartimentos principais, o compartimento intertubular (ou intersticial), e o compartimento tubular (SCHULZ et al., 2010). O compartimento intertubular abriga as células de Leydig, vasos sanguíneo/linfáticos, macrófagos e mastócitos, células de tecido conectivo e neural envolvidos pela túnica albugínea (KOULISH et al., 2002). Já o compartimento tubular é delineado pela membrana basal e as células mióides peritubulares, abrigando também o epitélio germinativo (SCHULZ et al., 2010). O epitélio germinativo é composto por dois tipos celulares, as células somáticas (Sertoli) e as células germinativas, estas últimas sendo encontradas em diferentes estágios de desenvolvimento (SCHULZ et al., 2010). *In vivo*, as células germinativas somente podem sobreviver em contato e contínua interação com as células de Sertoli (SCHULZ et al., 2010). Dessa forma, o número de células de Sertoli determina a capacidade espermatogênica do testículo, o que é um importante alvo na sinalização regulando a espermatogênese (MATTA et al., 2002).

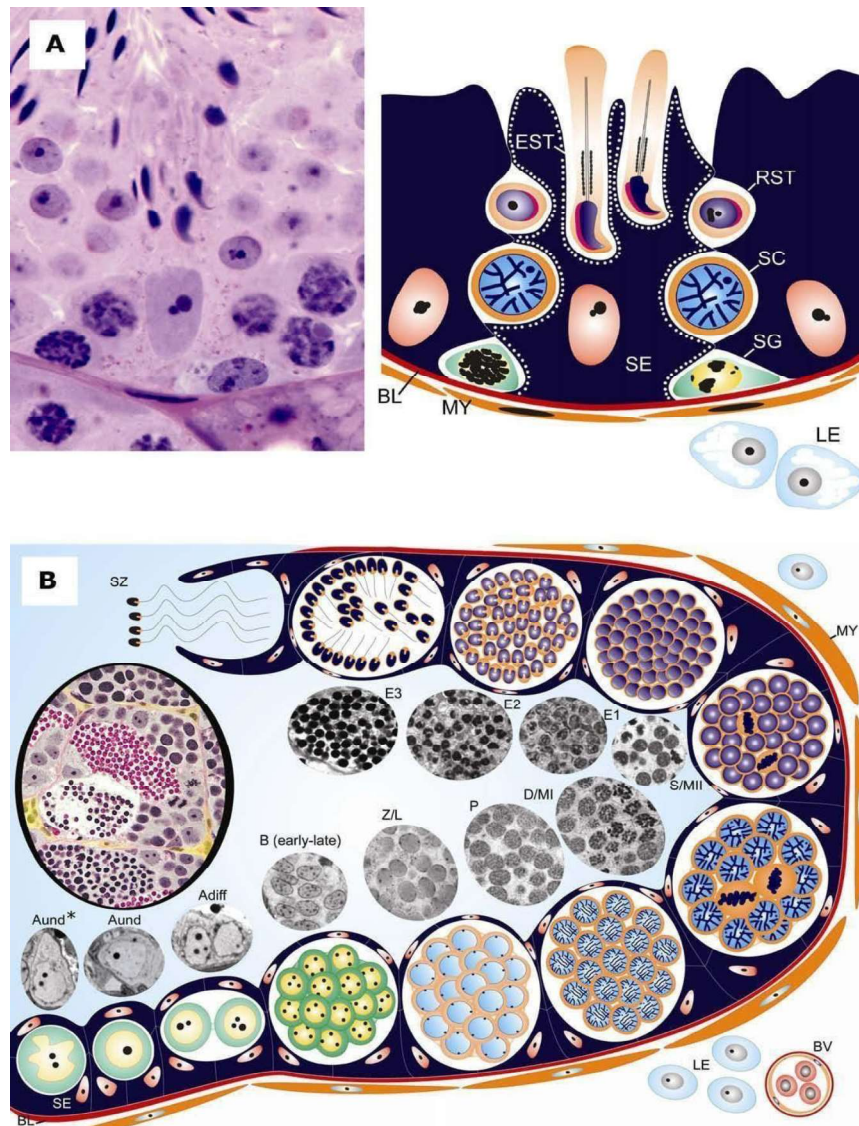


Figura 2. Comparação entre a espermatogênese não-cística de amniotas (répteis, aves, mamíferos) (A) e anamniotas (peixes, anfíbios) (B). A figura ilustra as diferenças entre a relação célula de Sertoli/célula germinativa na espermatogênese não-cística e cística. Em A, a célula de Sertoli suporta ao mesmo tempo diferentes clones de células germinativas em diferentes fases de desenvolvimento. Enquanto que em B, a célula de Sertoli suporta apenas um clone em uma mesma fase de desenvolvimento por vez. Legendas: células de Sertoli (SE); lâmina basal (BL); células peritubulares mióides (MY), células de Leydig (LE), espermatogônia (SG); espermatócito (SC); espermatíde arredondada (RST); espermatíde alongada (EST); espermatogônia do tipo A indiferenciada* (Aund*) (célula tronco?); espermatogônia do tipo A indiferenciada (Aund); espermatogônia do tipo A diferenciada (Adiff); 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); espermatídes iniciais (E1); intermediárias (E2); finais (E3); espermatozóides (SZ); e vasos sanguíneos (BV). Retirado de SCHULZ et al., 2010.

Em pássaros e mamíferos, as células de Sertoli proliferam até a puberdade de modo que no testículo adulto, um determinado número de células de Sertoli residentes pós-mitóticas, podem suportar sucessivas ondas de espermatogênese (FRANÇA et al., 2015). Durante essas ondas de espermatogênese, um certo

número de células de Sertoli suportam vários tipos de células germinativas ao mesmo tempo (Figura 2A) (SCHULZ et al., 2010). Já nos vertebrados anamniotas (peixes e anfíbios), por outro lado, a unidade funcional do epitélio germinativo é o cisto espermatogênico (Figura 2B) (FRANÇA et al., 2015). Esses cistos consistem inicialmente de uma espermatogônia envolta pelas extensões citoplasmáticas das células de Sertoli (FRANÇA et al., 2015). Quando esses cistos entram no processo espermatogênico, ambas as células de Sertoli e células germinativas entram em processo de diferenciação (SCHULZ et al., 2010). Um número específico de células de Sertoli é associado com um número em determinado estágio da espermatogênese, estabelecendo uma razão previsível entre células de Sertoli/germinativas para o desenvolvimento de determinado número de células germinativas (FRANÇA et al., 2015).

Hormônios do metabolismo e sua relação com a reprodução

A reprodução em vertebrados é controlada pelo eixo HPG, tendo alguns elementos chaves neste processo como o GnRH e as gonadotrofinas (Fsh e Lh). Entretanto, sabe-se que os hormônios relacionados com o metabolismo (como o cortisol e hormônios tireoidianos, por exemplo) também exercem papéis no processo reprodutivo numa atuação orquestrada entre os eixos hipotalâmico-pituitário-tireoide (HPT), hipotalâmico-hipofisário-interrenal (HPI) e o HPG.

Os níveis elevados de cortisol plasmático desencadeiam inúmeras respostas no organismo, incluindo impactos no processo reprodutivo, quando em condições de estresse. De forma geral, os trabalhos na literatura têm reportado tanto efeitos inibitórios como estimulatórios do cortisol na reprodução (CARRAGHER et al., 1989; CARRAGHER e SUMPTER, 1990; CAMPBELL et al., 1992; CAMPBELL et al., 1994). Vale mencionar que os peixes estão expostos à situações de estresse frequentemente, seja por problemas com a baixa qualidade de água (poluição), alta densidade, manejo dos animais e o próprio confinamento. Dessa forma, estudos para elucidar os efeitos do estresse/cortisol, sejam no metabolismo e/ou processo reprodutivo, são de extrema importância, pois ajudam a entender como funciona a ação conjunta dos eixos.

Além disso, inúmeros estudos têm mostrado que os hormônios tireoidianos também exercem um importante papel no sistema reprodutivo dos vertebrados, incluindo os peixes (CYR e EALES, 1996, BLANTON e SPECKER, 2007), HABIBI et al. (2012) e FLOOD et al. (2013). Sendo assim, é importante direcionar as pesquisas no sentido de entender também o papel dos HTs na reprodução dos peixes e a interação entre os eixos do metabolismo e o da reprodução.

1. Hormônio cortisol e definição de agentes estressores

O cortisol é o principal glicocorticoide em peixes teleósteos, sendo sintetizado e liberado em situações de estresse. Deve-se mencionar que os peixes adultos estão expostos diariamente a situações de estresse seja na natureza, ou sob condição de criação ou confinamento (SCHRECK et al., 2001). Brevemente será abordada a definição de situação de estresse para contextualizar a importância e ação do cortisol para os peixes.

Agentes estressores podem variar desde uma situação muito rápida (aguda), por exemplo, uma situação em que o peixe está sendo pego na rede ou escapando de um predador; até aquelas situações que são mais prolongadas e permanentes (crônico), como exemplo quando os animais estão em um tanque com alta densidade ou quando os animais ocupam a classe mais baixa na hierarquia de comunidade (SCHRECK e TORT, 2016). Os termos “agudo” e “crônico” são dependentes do contexto e difíceis de definir.

Existem três estágios principais da resposta ao estresse: alarme, resistência e compensação ou exaustão (morte) (SELYE, 1950; SCHRECK, 2000). A natureza (magnitude e duração) da resposta ao estresse é dependente da severidade e duração do agente estressor. Essencialmente a fase de alarme (ou resposta primária) consiste no estímulo do sistema locomotor (envolvidos na fuga, voo, luta, ou mesmo para simplesmente sobreviver à essa condição, por exemplo, é fase de ativação do eixo interrenal) (Figura 3). A resposta secundária inclui respostas do sistema cardiovascular e respiratório (RODNICK e PLANAS, 2016), as quais aumentam a distribuição de oxigênio e disponibilizam substrato de energia na circulação como resultado da resposta ao estresse (Figura 3). Outras respostas (secundárias) são a disfunção hidromineral, pois a adrenalina altera o

fluxo de sangue para as brânquias e permeabilidade destas, impactando no gradiente osmótico, assim, deixando fluir a água para dentro ou fora do animal de acordo com a salinidade do ambiente (Figura 3) (TAKEI e HWANG, 2016). A Resposta terciária seria mais relacionada aos efeitos em longo prazo, como comportamento, crescimento do animal e reprodução (Figura 3).

A primeira resposta do sistema endócrino frente ao agente estressor é a rápida elevação do nível de epinefrina no plasma (a catecolamina predominante) assim como os níveis de cortisol, principal glicocorticoide em peixes (Figura 3) (REID et al., 1998; MOMMSEN et al., 1999). Em outras palavras, o agente estressor induz a cascata neuroendócrina envolvendo a liberação imediata de catecolaminas e ativação do eixo HPI (SCHRECK et al., 2001).

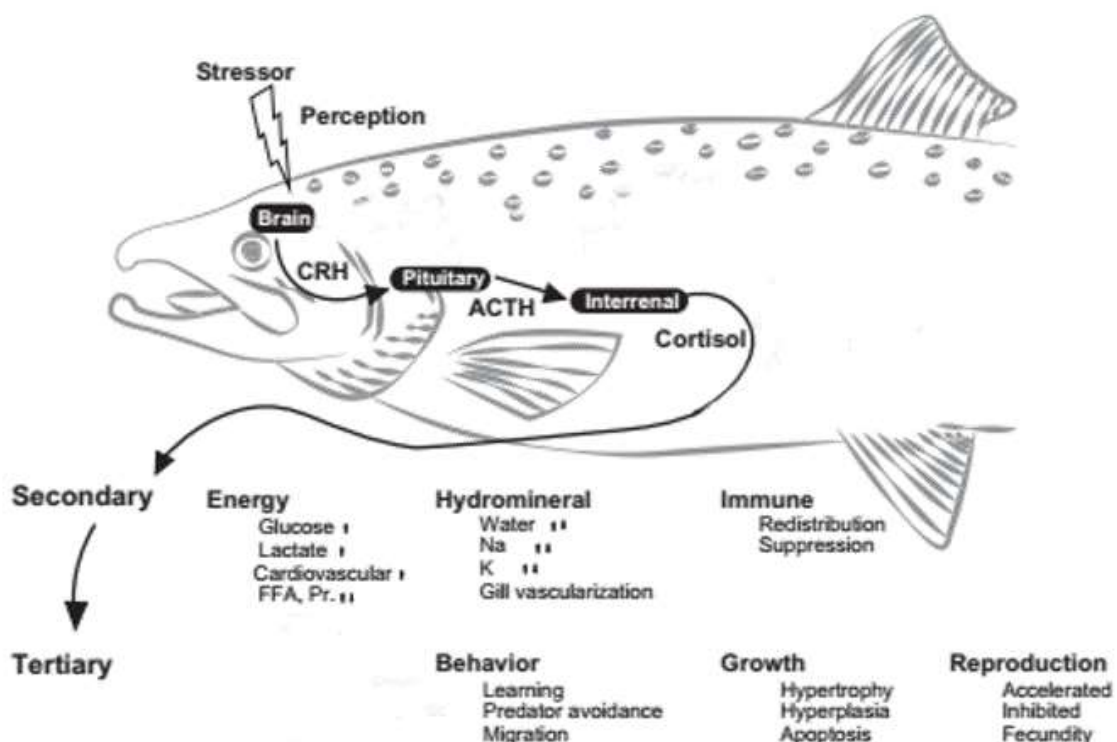


Figura 3. Visão esquemática dos efeitos causados pelo agente estressor em peixes. A primeira resposta é à nível do eixo hipotalâmico-hipofisário-interrenal e eventos cascata a baixo que culminam na liberação de catecolaminas e cortisol. A resposta secundária interfere em processos como: balanço de energia, hidromineral e resposta imune. A resposta terciária está relacionada a alterações no comportamento, crescimento e reprodução. Retirado de SCHRECK e TORT (2016).

Outra particularidade, o eixo hipotalâmico-hipofisário-interrenal (HPI) presente nos teleósteos é diferente daquele encontrado em mamíferos (eixo

hipotalâmico-hipofisário-adrenal (HPA) (WENDERLAAR BONGA, 1997). De maneira diferente, o tecido interrenal nos peixes teleósteos se localiza dentro da parte anterior dos rins e é homólogo ao córtex adrenal em mamíferos (MILLA et al., 2009).

Eixo hipotalâmico-hipofisário-interrenal-gonadal e a produção de cortisol

A secreção de cortisol está sob o controle do eixo HPI em peixes (WENDERLAR BONGA, 1997; MOMMENSEN et al., 1999). O fator liberador de cortisol (CRF) é secretado a partir de neurônios hipotalâmicos e estimulam a síntese do hormônio corticotrófico (ACTH) (ALURU e VIJAHAN, 2009). O ACTH é produzido na hipófise, e esse peptídeo pituitário se liga ao receptor de melanocortina 2 (MCR2) nas células esteroidogênicas no tecido interrenal levando a corticoesteroidogênese (ALURU e VIJAHAN, 2008).

A via de sinalização do cortisol em tecidos-alvo envolve a ativação de receptores corticoesteroides, da família de receptores nucleares (ALURU e VIJAHAN, 2009). Entretanto, os receptores de mineralcorticoides também podem atuar como ligantes fisiológicos para o cortisol (STURM et al., 2005). Na espécie de peixe *Haplochromis burtoni*, a sensibilidade do receptor de mineralcorticoide parece ser similar àquela encontrada em mamíferos, sendo mais sensível para ambos - cortisol e aldosterona (mineralcorticoide) - quando comparado aos receptores de glicocorticoides (GREENWOOD et al., 2003). A expressão gênica dos receptores de corticoesteroides (glico e mineralcorticoides) foi encontrada nas gônadas masculina e feminina (MILLA et al., 2009). Ambos receptores foram também identificados em testículo de truta arco-íris e outras espécies de peixes (COLOMBE et al., 2000; PARK et al., 2007; FILBY e TYLER, 2007; MILLA et al., 2008). No testículo de peixes, o receptor de mineralcorticoide é estrategicamente localizado ao longo do eixo reprodutivo e consequentemente em posição de regular a função reprodutiva (MILLA et al., 2008).

O papel do cortisol na reprodução de peixes

Os dados de efeitos do estresse ou administração do cortisol nos níveis plasmáticos dos esteroides gonadais são, de forma geral, controversos (CASTRANOVA et al., 2005). O estresse mostrou reduzir os níveis de testosterona (T) em truta arco-íris (CARRAGHER et al., 1989) e 11 ketotestosterona (11-KT) em truta marrom (PICKERING et al., 1987; CAMPBELL et al., 1994), e similarmente em *Acanthopagrus butcheri* (HADDY e PANKHURST, 1999). Por outro lado, em outro estudo, implantes de cortisol aplicados em machos de truta arco-íris e marrom em maturação não alteraram os níveis de 11-KT comparados ao grupo controle (CARRAGHER et al., 1989).

Em fêmeas, o estresse pode resultar na redução dos níveis de T e estradiol (E₂) (CAMPBELL et al., 1994; HADDY e PANKHURST, 1999; CLEARY et al., 2000). Esse efeito do estresse nos esteroides gonadais tem sido atribuído ao efeito do cortisol inibindo esteroidogênese ovariana em folículos de truta arco-íris (CARRAGHER e SUMPTER, 1990). O estresse teve efeito negativo também nos níveis de vitelogenina em truta brook (*Salvelinus fontinalis*) (ROY et al., 1990), assim como o cortisol retardou o crescimento do oócito acompanhado de redução nos níveis plasmáticos de testosterona e 17 β -oestradiol em tilápia (*Oreochromis niloticus*) (FOO e LAM, 1993). Entretanto, a adição de cortisol *in vitro* não inibiu a produção ovariana de T e E₂ em goldfish, carpa comum e *Pagrus auratus* (PANKHURST et al., 1995).

Inúmeros trabalhos reportam a correlação entre os níveis de cortisol no plasma e a fase reprodutiva do animal, demonstrando picos de cortisol durante a fase reprodutiva (CARRAGHER et al., 1989; CARRAGHER e SUMPTER, 1990; 1990; CAMPBELL et al., 1992; CAMPBELL et al., 1994). Além disso, o cortisol também é importante para a mobilização de reservas de energia (e.g. catabolismo proteico, lipólise, gliconeogênese) durante a maturação gonadal final em peixes (DICKHOFF et al., 1989).

Experimentalmente, a adição de cortisol na ração afetou o desenvolvimento testicular de carpas, retardando a maturação gonadal nesses animais (CONSTEN et al., 2001). O tratamento com acetato de hidrocortisona em machos de *Labeo gonius* causou redução no volume e comprimento dos testículos, e consequente redução do IGS (índice gonadossomático) (JOSHI, 1982). Similarmente, quando o

cortisol foi administrado na forma de implantes durante a fase de maturação da truta marrom, este reduziu o tamanho dos testículos dos animais (CARRAGHER et al., 1989).

Outra evidência da importância do cortisol na reprodução de peixes é o fato dele participar também do processo de reversão sexual. YAMAGUCHI e colaboradores (2010) demonstraram que o cortisol está envolvido na reversão sexual de fêmeas para machos de linguado japonês (*Paralichthys olivaceus*), devido a inibição da aromatase *cyp19a1*. Além disso, o inibidor de síntese do cortisol (*metirapone*) impede a masculinização de fêmeas de linguado japonês em temperaturas elevadas, sugerindo que o cortisol induz a reversão sexual nesta espécie. A administração exógena de cortisol induz à apoptose das células germinativas na gônada, aumento da expressão do *amh* e diminuição da aromatase gonadal *cyp19a1* nas larvas de pejerrey (HATTORI et al., 2009).

Uma possível via regulada pelo cortisol é a enzima 11 β -hidroxiesteroide desidrogenase (11 β HSD). Em peixes, somente uma enzima 11 β -Hsd foi descrita até o momento, e ela é considerada homóloga à 11 β -HSD2 encontrada em mamíferos (BAKER, 2004). Entretanto, a importância da 11 β -Hsd2 para o metabolismo dos glicocorticóides em peixes ainda não está clara, mas certamente está envolvida no processo final da biossíntese de 11-Ketotestosterona (11-KT) (Figura 4), o qual é o principal andrógeno nos peixes (FERNANDINO et al., 2012).

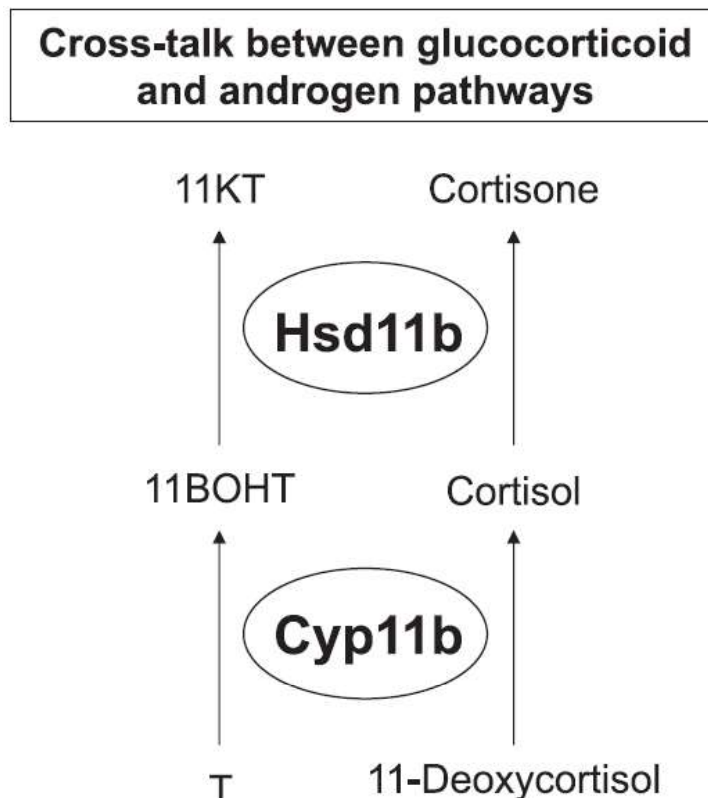


Figura 4 – Representação esquemática mostrando a relação entre as vias androgênica e metabolismo do cortisol. Dois tipos da enzima 11 β -Hydroxysteroid dehydrogenase - HSD11B1 e HSD11B2 – foram identificadas e caracterizadas em mamíferos (Albiston et al., 1994). Em peixes, entretanto, somente um tipo desta enzima foi identificado (Hsd11b2) em algumas espécies. Esta enzima está envolvida em tanto no metabolismo do Cortisol em Cortisona, quanto no metabolismo do 11B-hidroxitestosterona (11B-OHT) em 11 cetotestosterona (11-KT). T, testosterona; Cyp11b, 11- β hidroxilase. Retirado de GOIKOETXEA et al. (2017).

Efeitos do estresse e cortisol na espermatogênese de peixes

Em mamíferos, o cortisol parece ter um efeito inibitório durante todo o processo de espermatogênese (WEBER et al., 2000). Os glicocorticoides inibem a produção de testosterona pelas células de Leydig por uma via mediada pelos receptores de glicocorticoides (GE et al., 2005). Os glicocorticoides podem também induzir a apoptose de espermatogônias e espermatócitos, assim como diminuir o rendimento espermático (GAO et al., 2003; WAGNER e CLAUS, 2004). A atividade da enzima 11 β HSD2 nas células de Leydig e Sertoli protegeria o testículo de efeitos adversos do cortisol convertendo esses hormônios em cortisona ou 11-dihidrocorticosterona, respectivamente (Figura 4) (NACHARAJU et al., 1997; GE et al., 2005).

Em peixes, geralmente o nível de cortisol no plasma apresenta baixos níveis durante a fase de espermatogênese (PICKERING e CHRISTIE, 1981; HOU et al., 2001), que pode ser devido à uma adaptação fisiológica para proteger os testículos de possíveis efeitos adversos do cortisol (POTTINGER et al., 1995). De fato, existem relatos de que o cortisol retardou o desenvolvimento testicular atrasando a puberdade em carpas, efeito que parece ser independente de LH (hormônio luteinizante) (CONSTEN et al., 2001). Interessantemente, em outro estudo com carpa comum (*Cyprinus carpio*), a administração do RU-486 (potente antagonista do receptor de glicocorticoide) preveniu o efeito da diminuição do crescimento gonadal e atraso do início da espermatogênese (induzidos pelo cortisol), mostrando que os efeitos do cortisol são mediados via receptor de glicocorticoide (GOOS e CONSTEN, 2002).

Não obstante, outro estudo *in vitro* incubando testículos de enguia japonesa (*Anguilla japonica*) na presença do cortisol, reporta que o cortisol aumentou a replicação de DNA e a mitose das espermatogônias em doses de 0.1 a 100 ng/mL (OKAZAKI et al., 2006), estimulando também a liberação de 11-KT no nível de 100 ng/mL. Em outro estudo com adição de cortisol na ração (20, 40 e 60µg/ peixe), mostram que o cortisol promoveu a espermatogênese e aumentou o índice gonado-somático (IGS) em *Notopterus notopterus* (SHANKAR E KULKARNI, 2000), novamente um efeito positivo do cortisol na espermatogênese.

O cortisol inibiu *in vivo* a secreção dos esteroides durante a fase de espermatogênese. Em trabalhos com agentes estressores ou aplicação de cortisol durante a espermatogênese em peixes reduziram os níveis de andrógenos nos peixes tratados, quando comparados ao controle (PICKERING et al., 1987; CARRAGHER et al., 1989; POTTINGER, 1999; GOOS e CONSTEN, 2002; CONSTEN et al., 2002; LISTER et al., 2008). Essas observações sugerem que o efeito do cortisol no retardamento da espermatogênese *in vivo* pode ser parcialmente pela inibição da produção de andrógenos (CONSTEN et al., 2002; PICKERING et al., 1987; SCOTT e BAYNES, 1982).

2. Hormônios tireoidianos (HTs)

Os hormônios tireoidianos (HTs) são conhecidos pelo seu envolvimento em inúmeros processos biológicos como crescimento, morfogênese, pigmentação da pele, osmorregulação e reprodução (BLANTON e SPECKER, 2007). Em relação à reprodução, há um número crescente de estudos mostram que os HTs exercem um importante papel no sistema reprodutivo masculino em vertebrados.

O eixo hipotalâmico-hipofisário-tireoide em peixes

O funcionamento da tireoide é ativado pelo HPT, o qual foi revisado em vertebrados incluindo peixes (BROWN e CAI, 2007; BLANTON e SPECKER, 2007; CARR e PATIÑO, 2011). Uma característica particular do eixo HPT em peixes é a ausência do hormônio liberador de tireotrofinas (TRH) (Figura 5), como ocorre em mamíferos (LARSEN et al., 1998). Por outro lado, a secreção do hormônio estimulante da tireoide (TSH) em peixes parece ser dependente do hormônio liberador de corticotrofinas (CRH) (Figura 5) (LARSEN et al., 1998), semelhante ao encontrado em anfíbios, répteis e aves (De GROEF et al., 2003; CASTAÑEDA CORTÉS et al., 2014).

O TSH secretado, por sua vez, estimula a síntese e liberação dos hormônios tireoidianos pelos folículos tireoidianos (Figura 5). Nos peixes, os folículos tireoidianos não são encapsulados em um órgão, mas sim dispersos entre as arteríolas branquiais aferentes da região ventral da faringe (CYR e EALES, 1996; CARR e PATIÑO, 2011). A L-tiroxina (T_4) é o principal hormônio tireoidiano secretado sob condições normais, enquanto que o T_3 , a forma ativa do hormônio, é produzida em sua maior parte por tecidos periféricos a partir da conversão do T_4 (Figuras 5 e 6) (BASSET et al., 2003).

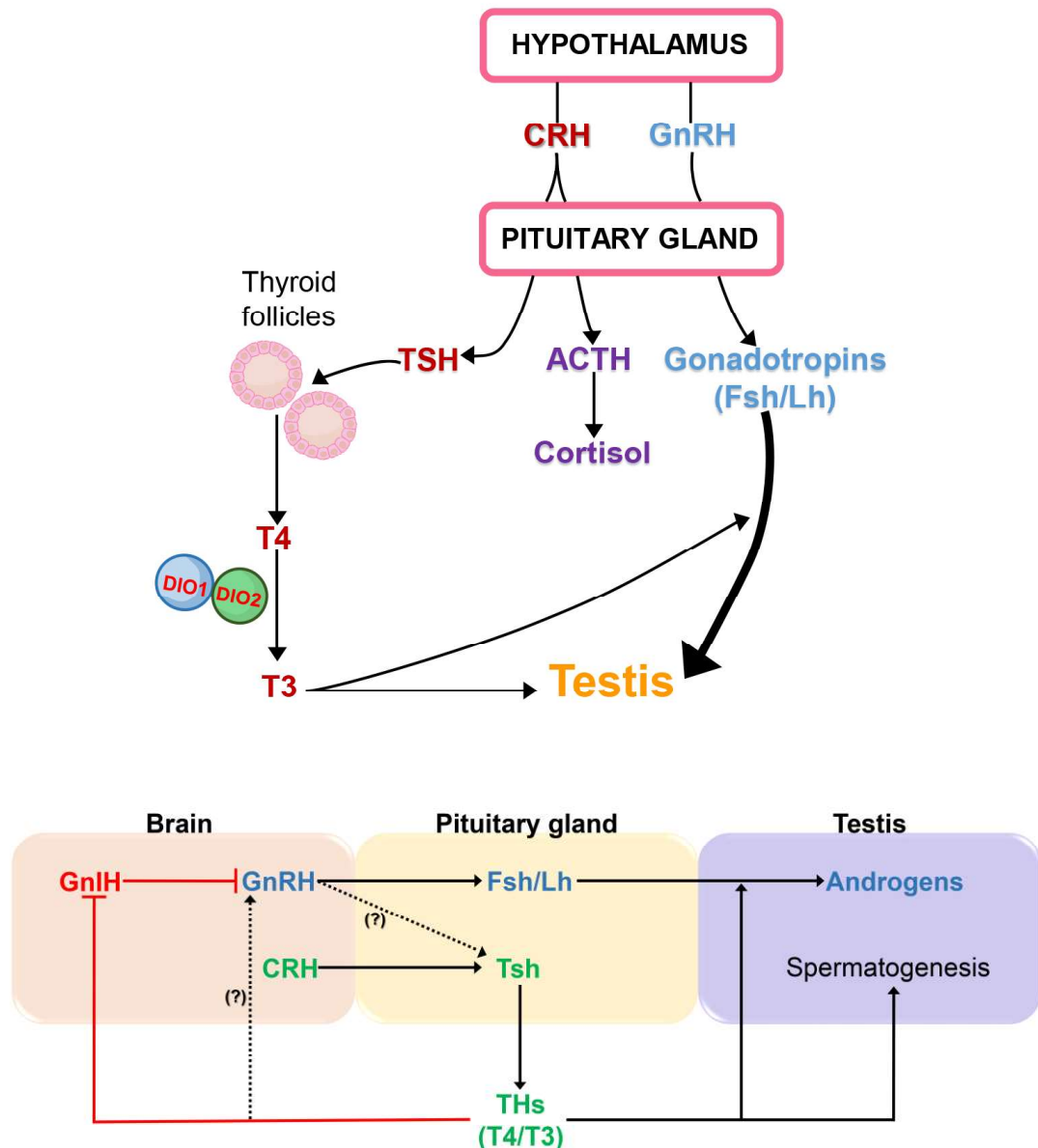


Figura 5. A. Eixos: hipotalâmico-hipofisário-tireoide, -interrenal e -testicular. A secreção hipofisária do Tsh (hormônio estimulador da tireoide) parece ser dependente do hormônio liberador de corticotropinas (CRH), de modo similar ao observado em anfíbios, répteis e aves. O CRH é o precursor também do hormônio corticotrófico (ACTH), que estimula a síntese e secreção do cortisol pela glândula interrenal. Nos folículos tireoidianos, os quais são dispersos em peixes, o Tsh estimula a secreção do T4. Dois tipos de desidases (DIO1 e DIO2) convertem o T4 em T3 via extra tireoide. T3 é a forma ativa do HT e dentre suas funções no testículo, está associado com o desenvolvimento testicular, crescimento e maturação. Estudos recentes mostraram que o T3 aumentou a função do Fsh e Lh no testículo (seta larga). B. Principais efeitos dos HTs no eixo hipotalâmico-pituitário-testicular. Em mamíferos, os HT inibem a síntese de GnIH (TSUTSUI et al., 2018). Outros estudos mostraram que o GnRH aumenta o nível de Tsh e a função da tireoide em algumas espécies de peixes e anfíbios (JACOBS et al., 1988a,b; ROY et al., 2000; CHIBA et al., 2004). O ponto de interrogação (?) indica os dados controversos na literatura a respeito da relação entre GnRH e tireoide. Retirado de TOVO-NETO et al. (2018).

O metabolismo dos HTs é regulado por três tipos de enzimas iodotironinas deiodinases (tipo I, II e III), como mostra a Figura 2 (DUARTE-GUTERMAN et al.,

2014). A deiodinase tipo I (D1) catalisa a desiodação do anel externo para produzir T3 a partir do T4 e também faz a desiodação no anel interno para produzir o T3 reverso (T3r, inativo) a partir do T4. O tipo II (D2) atua exclusivamente na ativação dos HTs pela catálise da conversão de T4 para T3 (DUARTE-GUTERMAN e TRUDEAU, 2010) (Figura 6). O tipo III (D3) catalisa unicamente a desiodação do anel interno das moléculas de T4 e T3, o que resulta na formação de metabólitos inativos rT3 e T2, respectivamente (MEYER et al., 2007) (Figura 6). A coordenação da expressão e atividade das enzimas desiodases em tecidos individuais regulam a concentração dos HTs ativos, de acordo com as necessidades específicas do tecido (FLOOD et al., 2013).

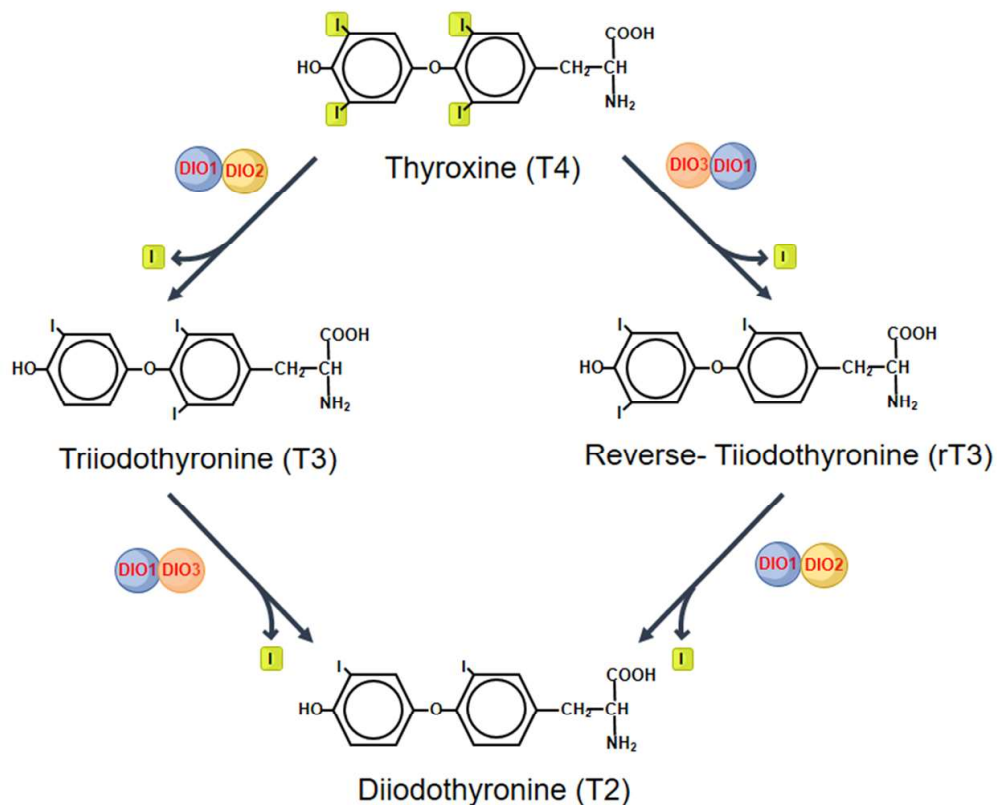


Figura 6. Metabolismo do hormônio tireoidiano (T4) a partir das desiodases. O T4 é a principal forma produzida sob circunstância normal. O T4 pode ser tanto metabolizado em T3 (que é a forma ativa dos HTs geralmente) pela ação das DIO1e2; como pode também ser metabolizado em uma forma inativa conhecida como rT3 (reverso) pela ação das DIO1e3. O T3 pode ser metabolizado em T2 pelas DIO1e3, assim como o rT3 pode ser convertido em T2 pela ação das DIO1e2. Retirado de TOVO-NETO et al. (2018).

A forma ativa dos HTs (T3) atua por meio de receptores nucleares (TR, *Thyroid hormone receptor*), que possuem duas isoformas, o TR α e TR β . Os receptores nucleares dos HTs são codificados pelos genes *THRA* e *THRB*, os quais são transcritos em múltiplas isoformas de RNAm. As isoformas Tr α 1, Tr α 2, Tr β 1 e Tr β 3 são expressas amplamente ao passo que a expressão do Tr β 2 é restrita ao eixo hipotalâmico-hipofisário, onde atua negativamente regulando a transcrição do TSH em mamíferos (para revisão veja BASSET et al., 2003). Os TRs atuam como fatores de transcrição ligando-se à regiões regulatórias do gene alvo, ativando ou reprimindo sua transcrição (BASSET et al., 2003).

O papel dos HTs na reprodução de peixes (machos) foi recentemente revisto por TOVO-NETO et al. (2018) e não será discutido neste capítulo (veja anexo). TOVO-NETO et al. (2018) reporta dados de que os HTs são importantes em todas as etapas da formação da gônada em peixes, seja na fase de reversão sexual, na fase de desenvolvimento testicular ou na espermatogênese em animais adultos.

3. O zebrafish (*D. rerio*) como modelo experimental

O *D. rerio*, originário da Ásia (principalmente Índia), é também conhecido como peixe zebra (zebrafish), paulistinha ou peixe-bandeira, devido às suas listras horizontais que lembram a bandeira do Estado de São Paulo. Este pequeno teleósteo de água doce (~5cm de comprimento) pertence à família Cyprinidae e à ordem Cypriniformes.

Em função de seu fácil manejo, tolerância à altas densidades, reprodução padronizada em laboratório, assim como transparência ótica do desenvolvimento dos embriões, fazem do zebrafish um excelente modelo experimental para pesquisas científicas em vertebrados (MCGONNELL e FOWKES, 2006). Este teleósteo possui vida média em torno de três anos e sua primeira maturação ocorre com três meses de vida. Normalmente, a temperatura de conforto da espécie é em média de 27-28°C. A fêmea desta espécie pode desovar cerca de 100-200 ovos semanalmente.

O sequenciamento recente do genoma do zebrafish, disponibilizado no site ZFIN (The zebrafish Information Network), abriu enormes possibilidades para que

estudos na área de genética sejam desenvolvidos neste teleósteo, propiciando inclusive a geração de excelentes modelos transgênicos na área da biologia da reprodução.

Além da aplicabilidade do zebrafish em pesquisas básicas como no estudo das funções gênicas e da biologia do desenvolvimento, a espécie também é utilizada em pesquisas aplicadas no estudo da base genética de inúmeras doenças em humanos (arritmia cardíaca, doenças neurodegenerativas, endocrinologia, doenças hematológicas, e até mesmo no estudo do câncer) (HEILESEN, 2017). Outro uso importante do zebrafish é na área de toxicologia. Tais características fazem do zebrafish um modelo bem interessante para pesquisa tanto básica quanto aplicada.

Objetivo Geral

Estudar os efeitos do cortisol e hormônios tireoidianos na espermatogênese de zebrafish (*Danio rerio*).

Objetivos Específicos

1. Estudar os efeitos do cortisol na espermatogênese do zebrafish utilizando o sistema *ex vivo* de cultura de explantes.
2. Estudar os efeitos dos hormônios tireoidianos (T3 e T4) na espermatogênese do zebrafish utilizando o sistema *ex vivo* de cultura de explantes.
3. Estudar os efeitos do T3 combinado com a gonadotrofina Fsh na espermatogênese do zebrafish utilizando o sistema *ex vivo* de cultura de explantes.
4. Estudar os efeitos do hipotireoidismo induzido quimicamente pelo metimazol na espermatogênese do zebrafish *in vivo*.

REFERÊNCIAS

- ALURU, N.; VIJAYAN, M.M. 2008. Molecular characterization, tissue specific expression and regulation of melanocortin 2 receptor in rainbow trout. *Endocrinology* 149, 4577–4588.
- ALURU, N.; VIJAYAN, M.M. 2009. Stress transcriptomics in fish: A role for genomic cortisol signaling. *Gen. Comp. Endocrinol.* 164, 142-150.
- ANDREU-VIEYRA, C.V.; HABIBI, H.R., 2007. Hormonal regulation of follicular atresia in teleost fish, In: BABIN, P.J.; CERDÀ, J.; LUBZENS, E. *The Fish Oocyte: From Basics Studies to Biothechnological Applications*. Springer Netherlands, p.235-253.
- BAKER, M.E. 2004. Evolutionary analysis of 11-beta-hydroxysteroid dehydrogenase-type 1, -type 2, -type3 and 17 β -hydroxysteroid dehydrogenase-type 2 in fish. *FEBS Lett.* 574, 167-170.
- BASSET, J.H.D.; HARVEY, C.B.; WILLIAMS, G.R. 2003. Mechanisms of thyroid hormone receptor-specific nuclear and extra nuclear actions. *Mol. Cell. Endocrinol.* 213, 1-11.
- BLANTON, M.L.; SPECKER, J.L. 2007. The Hypothalamic-Pituitary-Thyroid (HPT) Axis in Fish and Its Role in Fish Development and Reproduction. *Crit. Rev. Toxicol.* 37, 97-115.
- BROWN, D.D.; CAI, L.Q. 2007. Amphibian metamorphosis. *Dev. Biol.* 306, 20-33.
- CAMPBELL, P.M.; POTTINGER, T.G.; SUMPTER, J.P. 1992. Stress reduces the quality of gametes produced by rainbow trout. *Gen. Bio. Reprod.* 47, 1140-1150.
- CAMPBELL, P.M.; POTTINGER, T.G.; SUMPTER, J.P. 1994. Preliminary evidence that chronic confinement stress reduces the quality of gametes produced by brown and rainbow trout. *Aquaculture* 120, 151-169.
- CARR, J.A.; PATIÑO, R. 2011. The hypothalamus-pituitary-thyroid axis in teleosts and amphibians: Endocrine disruption and its consequences to natural populations. *Gen. Comp. Endocrinol.* 170, 299-312.

- CARRAGHER, J.F.; SUMPTER, J.P.; POTTINGER, T.G.; PICKERING, A.D., 1989. The deleterious effects of cortisol implantation on reproductive function in two species of trout, *Salmo trutta* L., & *Salmo gairdneri* Richardson. Gen. Comp. Endocrinol. 76, 310.
- CARRAGHER, J.F.; SUMPTER, J.P. 1990. The effect of cortisol on the secretion of sex steroids from cultured ovarian follicles of rainbow trout. Gen. Comp. Endocrinol. 77, 403.
- CASTAÑEDA CORTÉS, D.C.; LANGLOIS, V.S.; FERNANDINO, J.I. 2014. Crossover of the hypothalamic pituitary-adrenal/interrenal, -thyroid, and –gonadal axes in axes in testicular development. Front. Endocrinol. 139, 1-11.
- CASTRANOVA, D.A.; KING, W.; WOODS, L.C. 2005. The effects of stress on androgen production, spermiation response and sperm quality in high and low cortisol responsive domesticated male striped bass. Aquaculture 246, 413–422.
- CHIBA, H.; AMANO, M.; YAMADA, H.; FUJIMOTO Y.; OJIMA, D.; OKUZAWA, K.; YAMANOME, T.; YAMAMORI, K.; IWATA, M. 2004. 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.
- CLEARY, J.J.; PANKHURST, N.W. 2000. The effect of capture and handling stress on plasma steroid levels and gonadal condition in wild and farmed snapper *Pagrus auratus* (Sparidae). J. World Aquacult. Soc. 31, 558–567.
- COLOMBE, L.; FOSTIER, A.; BURY, N.R.; PAKDEL, F.; GUIGUEN, Y. 2000. A mineralocorticoid-like receptor in the rainbow trout, *Oncorhynchus mykiss*: cloning and characterization of its steroid binding domain. Steroids 65, 319–328.
- CONSTEN, D.; LAMBERT, J.G.D.; GOOS, H.J.Th. 2001. Cortisol affects testicular development in male common carp, *Cyprinus carpio* L., but not via an effect on LH secretion. Comp. Biochem. Physiol. B 129, 671-677.

- CONSTEN, D.; TERLOU, M.; LAMBERT, J.G.D.; GOOS, H.J.Th. 2002. Corticosteroids affect the testicular androgen production in male common carp (*Cyprinus carpio* L.). Biol. Reprod. 66, 106–111.
- CYR, D.G.; EALES, J.G. 1996. Interrelationships between thyroidal and reproductive endocrine systems in fish. Rev. Fish Biol. Fish. 6, 165-200.
- De GROEF, B.; GORIS, N.; ARCKENS, L.; KÜHN, E.R.; DARRAS, V.M. 2003. Corticotropin-Releasing Hormone (CRH)-Induced Thyrotropin Release Is Directly Mediated through CRH Receptor Type 2 on Thyrotropes. Endocrinology 144, 5537-5544.
- DICKHOFF, W.W.; YAN, L.; PLISETSKAYA, E.M.; SULLIVAN, C.V.; SWANSON, P.; HARA, A.; BERNARD, M.G. 1989. Relationship between metabolic and reproductive hormones in salmonid fish. Fish Physiology and Biochemistry 7, 147-155.
- DUARTE-GUTERMAN, P.; TRUDEAU, V.L. 2010. Regulation of Thyroid Hormone-, Oestrogen- and Androgen- Related Genes by Triiodothyronine in the Brain of *Silurana tropicalis*. J. Neuroendocrinol. 22, 1023-1031.
- DUARTE-GUTERMAN, P.; NAVARRO-MARTÍN, L.; TRUDEAU, V.L. 2014. Mechanisms of crosstalk between endocrine system: Regulation of sex steroid hormone synthesis and action by thyroid hormones. Gen. Comp. Endocrinol. 203, 69-85.
- DUFOUR, S.; SEBERT, M.E.; WELTZIEN, F.A.; ROUSSEAU, K.; PASQUALINI, C. 2010. Neuroendocrine control by 44 dopamine of teleost reproduction. J. Fish Biol. 76, 129-160.
- FERNANDINO, J.I.; HATTORI, R.S.; KISHII, A.; STRÜSSMANN, C.A.; SOMOZA, G.M. 2012. The Cortisol and Androgen Pathways Cross Talk in High Temperature-Induced Masculinization: The 11 β -Hydroxysteroid Dehydrogenase as a Key Enzyme. Endocrinology 153, 6003-6011.
- FILBY, A.L.; TYLER, C.R. 2007. Cloning and characterization of cDNAs for hormones and/or receptors of growth hormone, insulin-like growth factor-I, thyroid hormone, and corticosteroid and the gender-, tissue-, and

- developmental-specific expression of their mRNA transcripts in fathead minnow (*Pimephales promelas*). Gen. Comp. Endocrinol. 41, 563–565.
- FLOOD, D.E.K.; FERNANDINO, J.I.; LANGLOIS, V.S. 2013. Thyroid hormones in male reproductive development: Evidence for direct crosstalk between the androgen and thyroid hormone axes. Gen. Comp. Endocrinol. 192, 2-14.
- FOO, J.T.W.; LAM, T.J. 1993. Serum cortisol response to handling stress and the effect of cortisol implantation on testosterone level in the tilapia, *Oreochromis mossambicus*. Aquaculture 115, 145-158.
- FRANÇA, L.R.; CHIARINI-GARCIA, H. Célula de Sertoli. In: CARVALHO, H.F.; COLLARES-BUZATO, C.B. A Célula: Uma abordagem multidisciplinar. São Paulo: Ed. Manole, 2005. p.302-324.
- FRANÇA, L.R.; NÓBREGA, R.H.; MORAIS, R.D.V.S.; ASSIS, L.H.C.; SCHULZ, R.W. Sertoli cell structure and function in anamniote vertebrates, in: GRISWOLD, M.D. Sertoli cell biology. Amsterdam: Elsevier, 2015. p.385–407.
- GAO, H-B.; TONG, M-H.; HU, Y-Q.; YOU, H-Y.; GUO, Q-S.; GE., R-N.; HARDY, M.P. 2003. Mechanisms of glucocorticoid-induced Leydig cell apoptosis. Mol. Cell. Endocrinol. 199, 153-163.
- GARCÍA-LÓPEZ, A.; BOGERD, J.; GRANNEMAN, J.C.; Van DIJK, W., TRANT, J.M., TARANGER, G.L., SCHULZ, R.W. 2009. Leydig cells express follicle-stimulating hormone receptors in African catfish. Endocrinology 150, 357–365.
- GARCÍA-LÓPEZ, A.; De JONGE, H.; NÓBREGA, R.H.; DE WAAL, P.P.; VAN DIJK, W.; HEMRIKA, W.; TARANGER, G.L.; BOGERD, J.; SCHULZ, R.W., 2010. Studies in Zebrafish Reveal Unusual Cellular Expression Patterns of Gonadotropin Receptor Messenger Ribonucleic Acids in the Testis and Unexpected Functional Differentiation of the Gonadotropins. Reprod. Dev. 151, 2349-2360.
- GE, R.S.; DONG, Q.; NIU, E.M.; SOTTAS, C.M.; HARDY, D.O.; CATTERALL, J.F.; LATIF, S.A.; MORRIS, D.J.; HARDY, M.P. 2005. 11 β -hydroxysteroid dehydrogenase 2 in rat Leydig cells: its role in blunting glucocorticoid action at physiological levels of substrate. Endocrinology 146, 2657–2664.

- GOIKOETXEA, A.; TODD, E.V.; GEMMELL, N.J. 2017. Stress and sex: does cortisol mediate sex change in fish? *Reproduction* 154, R149-160.
- GOOS, H.J.TH.; CONSTEN, D. 2002. Stress adaptation, cortisol and pubertal development in the male common carp, *Cyprinus carpio*. *Mol. Cell. Endocrinol.* 197, 105-116.
- GREENWOOD, A.K.; BUTLER, P.C.; WHITE, R.B.; DEMARCO, U.; PEARCE, D.; FERNALD, R.D. 2003. Multiple corticosteroid receptors in a teleost fish: distinct sequences, expression patterns, and transcriptional activities. *Endocrinology* 144, 4226–4236.
- GRIER, H.J. 1993. Comparative organization of Sertoli cells including the Sertoli cell barrier. In: RUSSELL, L.D.; GRISWOLD, M.D. *The Sertoli cell*. Clearwater: Cache River Press, p. 703–739.
- HABIBI, H.R.; NELSON, E.R.; ALLAN, E.R.O. 2012. New insights into thyroid hormone function and modulation of reproduction in goldfish. *Gen. Comp. Endocrinol.* 175, 19-26.
- HADDY, J.A.; PANKHURST, N.W. 1999. Stress-induced changes in concentrations of plasma sex steroids in black bream. *J. Fish Biol.* 55, 1304–1316.
- HATTORI, R.S.; FERNANDINO, J.I.; KISHII, A.; KIMURA, H.; KINNO, T.; OURA, M.; SOMOZA, G.M.; YOKOTA, M.; STRÜSSMANN, C.A.; WATANABE, S. 2009. Cortisol-Induced Mascunilization: Does Thermal Stress Affect Gonadal Fate in Pejerrey, A Teleost Fish with Temperature-Dependent Sex Determination? *PLoS One* 4, e6548.
- HEILESEN, E. 2017. The zebrafish is an important animal model. Disponível em < <http://mbg.au.dk/en/news-and-events/news-item/artikel/the-zebrafish-is-an-important-animal-model/>>. Acesso em: jun. 2017.
- HOU, Y.Y.; HAN, X.D.; YUZURI, S. 2001. Annual changes in plasma levels of cortisol and sex steroid hormones in male rainbow trout, *Oncorhynchus mykiss*. *Chin. J. Ocean. Limnol.* 19, 217–221.

- JACOBS, G.F.M.; GOYVAERTS, M.P.; VANDORPE, G.; QUAGHEBEUR, A.M.L.; KUHN, E.R. 1988a. Luteinizing hormone-releasing hormone as a potent stimulator of the thyroïdal axis in ranid frogs. *Gen. Comp. Endocrinol.* 70, 274-283.
- JACOBS, G.F.M.; MICHELSEN, R.P.; KUHN, E.R. 1988b. 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.
- JOSHI, S.N. 1982. Effect of hydrocortisone acetate on the maturity of male *Labeo gonius* (ham.). *Endocrinology* 80, 299-303.
- KOULISH, S.; KRAMER, C.R.; GRIER, H.J. 2002. Organization of the male gonad in a protogynous fish, *Thalassoma bifasciatum* (Teleostei: Labridae). *J. Morphol.* 254, 292-311.
- LARSEN, D.A.; SWANSON, P.; DICKEY, J.T.; RIVIER, J.; DICKHOFF, W.W. 1998. *In Vitro* Thyrotropin-Releasing Activity of Corticotropin-Releasing Hormone-Family Peptides in Coho Salmon, *Oncorhynchus kisutch*. *Gen. Comp. Endocrinol.* 109, 276-285.
- LEAL, M.C.; De WAAL, P.P.; GARCÍA-LÓPEZ, A.; CHEN, S.X.; BOGERD, J.; SCHULZ, R.W. 2009. Zebrafish primary testis tissue culture: an approach to study testis function ex vivo. *Gen. Comp. Endocrinol.* 162, 134–138.
- LISTER, A.; NERO, V.; FARWELL, A.; DIXON, D.G.; Van Der KRAAK, G. 2008. Reproductive and stress hormone levels in goldfish (*Carassius auratus*) exposed to oil and sands process-affected water. *Aquat. Toxicol.* 87, 170–177.
- MATTA, S.L.P.; VILELA, D.A.R.; GODINHO, H.P. FRANÇA, L.R., 2002. 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.
- MCGONNELL, I.M.; FOWKES, R.C. 2006. Fishing for gene function – endocrine modelling in the zebrafish. *J. Endocrinol.* 189, 425-439.

- MEYER, E.L.S.; WAGNER, M.S.; MAIA, A.L. 2007. Expressão das iodotironinas Desiodases nas Neoplasias Tireoidianas. Arq. Bras. Endocrinol. Metab. 51, 690-700.
- MILLA, S.; TERRIEN, X.; STURM, A.; IBRAHIM, F.; GITON, F.; FIET, J., PRUNET, P., LE GAC, F., 2008. Plasma 11-deoxycorticosterone (DOC) and mineralocorticoid receptor testicular expression during rainbow trout *Oncorhynchus mykiss* spermiation: implication with 17alpha, 20beta-dihydroxyprogesterone on the milt fluidity? Reprod. Biol. Endocrinol. 6, 19.
- MILLA, S.; WANG, N.; MANDIKI, S.N.M.; KESTEMONT, P. 2009. Corticosteroids: Friends or foes of teleost fish reproduction? Comp. Biochem. Physiol. Part A. 153, 242-251.
- MOMMSEN, T.P.; VIJAYAN, M.M.; MOON, T.W. 1999. Cortisol in teleosts: dynamics, mechanisms of action, and metabolic regulation. Rev. Fish Biol. Fish. 9, 211-268.
- MYLONAS, C.C.; FOSTIER, A.; ZANUY, S. 2010. Broodstock management and hormonal manipulations of fish reproduction. Gen. Comp. Endocrinol. 165, 516-534.
- NACHARAJU, V.L.; MUNEEYIRCI, O.; KAHN, N.; 1997. Presence of 11 beta-hydroxysteroid dehydrogenase in human semen: evidence of correlation with semen characteristics. Steroids 62, 311–314.
- NÓBREGA, R.H.; BATLOUNI, S.R.; FRANÇA, L.R.; 2009. An overview of functional and stereological evaluation of spermatogenesis and germ cell transplantation in fish. Fish. Physiol. Biochem. 35, 197-206.
- OZAKI, Y.; HIGUCHI, M.; MIURA, C.; YAMAGUCHI, S.; TOZAWA, Y.; MIURA, T. 2006. Roles of 11 β -Hydroxysteroid Dehydrogenase in Fish Spermatogenesis. Endocrinology 147, 5139-5146.
- PANKHURST, N.W.; VAN DER KRAAK, G.; PETER, R.E. 1995. Evidence that the inhibitory effects of stress on reproduction in teleost fish are not mediated by the action of cortisol on ovarian steroidogenesis. Gen. Comp. Endocrinol. 99, 249-257.

- PANKHURST, N.W. Reproduction and Development, In: SCHRECK, C.B.; TORT, L.; FARRELL, A.; BRAUNER, C. Biology of Stress in Fish. Academic Press, 2016. p.295-331.
- PARK, C.B.; TAKEMURA, A.; ALURU, N.; PARK, Y.J.; KIM, B.H.; LEE, C.H.; LEE, Y.D.; MOON, T.W.; VIJAYAN, M.M. 2007. Tissue-specific suppression of estrogen, androgen and glucocorticoid receptor gene expression in feral vitellogenic male Mozambique tilapia. *Chemosphere* 69, 32–40.
- PICKERING, A.D.; CHRISTIE, P. 1981. Changes in the concentrations of plasma cortisol and thyroxine during sexual maturation of the hatchery-reared brown trout, *Salmo trutta*. *Gen. Comp. Endocrinol.* 44, 488–496.
- PICKERING, A.D.; POTTINGER, T.G.; CARRAGHER, J.; SUMPTER, J.P.; 1987. The effects of acute and chronic stress on the levels of reproductive hormones in the plasma of mature male brown trout, *Salmo trutta* L. *Gen. Comp. Endocrinol.* 68, 249–259.
- PIERCE, J.G.; PARSONS, T.F. 1981. Glycoprotein hormones. Structure and function. *Ann. Rev. Biochem.* 50, 465–495.
- POTTINGER, T.G.; BALM, P.H.M.; PICKERING, A.D. 1995. Sexual maturity modifies the responsiveness of the pituitary–interrenal axis to stress in male rainbow trout. *Gen. Comp. Endocrinol.* 98, 311–320.
- POTTINGER, T.G. The impact of stress on animal reproductive activities. In: BALM, P.H.M. *Stress Physiology in Animals*. Sheffield Academic Press, 1999, p.130–177.
- PUDNEY, J. Comparative cytology of the non-mammalian vertebrate Sertoli cell. In: Russell, L.D.; Griswold, M.D. *The Sertoli Cell*. Clearwater: Cache River Press, 1993. p.611–657.
- PUDNEY, J., 1995. Spermatogenesis in nonmammalian vertebrates. *Microsc. Res. Tech.* 32, 459–497.
- REID, S.G.; BERNIER, N.J.; PERRY, S.F. 1998. The adrenergic stress response in fish: control of catecholamine storage and release. *Comp. Biochem. Physiol. Part C* 120, 1–27.

- RODNICK, K.J.; PLANAS, J.V. The Stress and Stress Mitigation Effects of Exercise: Cardiovascular, Metabolic, and Skeletal Muscle Adjustments. In: SCHRECK, C.B.; TORT, L.; FARRELL, A.P.; BRAUNER, C.J. Biology of Stress in Fish. Amsterdam: Elsevier, 2016. p.251-294.
- ROY, P.; DATTA, M.; DASGUPTA, S.; BHATTACHARYA, S. 1990. Gonadotropin-Releasing Hormone Stimulates Thyroid Activity in a Freshwater Murrel, *Channa gachua* (Ham.), and Carps, *Catla catla* (Ham.) and *Cirrhinus mrigala* (Ham.). General and Comparative Endocrinology 117, 456-463.
- RUSSELL, L.D.; ETHLIN, R.A.; SINHA HIKIM, A.P.; CLEGG, E.D. Histological and Histopathological Evaluation of the Testis. Clearwater: Cache River Press, 1990.
- SCHRECK, C.B.; CONTRERAS-SANCHEZ, W.; FITZPATRICK, M.S. 2001. Effects of stress on fish reproduction, gamete quality, and progeny. Aquaculture 197, 3-24.
- SCHRECK, C.B. Accumulation and long-term effects of stress in fish. In: The Biology of Animal Stress: Basic Principles and Implications for Animal Welfare. MOBERG, G.P.; MENCH, J. A. Wallingford: CAB International, 2000. p.147–158.
- SCHRECK, C.B.; TORT, L. 2016. The Concept of Stress in Fish. In: SCHRECK, C.B.; TORT, L.; FARREL, A.P.; BRAUNER, C.J. Fish Physiology. Amsterdam: Elsevier, p.1-34.
- SCHULZ, R.W.; FRANÇA, L.R.; LAREYE, J.J.; LE GAC F.; CHIARINI-GARCIA H.; NÓBREGA R.H.; MIURA, T. 2010. Spermatogenesis in fish. Gen. Comp. Endocrinol. 165, 390-411.
- SCHULZ, R.W.; VISCHER, H.; CAVACO, J.E.B.; SANTOS, E.M.; TYLER, C.R.; GOOS, H.J.Th.; BOGERD, J. 2001. Gonadotropins, their receptors, and the regulation of testicular functions in fish. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 129, 407-417.
- SCOTT, A.P.; BAYNES, S.M. 1982. Plasma levels of sex steroids in relation to ovulation and spermiation in rainbow trout (*Salmo gairdneri*). In: RICHTER, C.J.J., GOOS, H.J. Proceedings of the 2nd International Symposium on

- Reproductive Physiology of Fish: 2–6 august 1982. Pudoc, Wageningen, p. 103–106.
- SELYE, H., 1950. Stress and the general adaptation syndrome. Br. Med. J. 1, 1383–1392.
- SHAHJAHAN, M.; KITAHASHI, T.; OGAWA, S.; PARHAR, I.S. 2013. Temperature differentially regulates the two kisspeptin systems in the brain of zebrafish. Gen. Comp. Endocrinol. 193, 79-85.
- SHANKAR, D.S.; KULKARNI, R.S. 2000. Effect of cortisol on testis of freshwater fish *Notopterus notopterus* (Pallas). Indian J. Exp. Biol. 38, 1227-1230.
- SHARPE, R.M. Regulation of spermatogenesis, In: KNOBIL E.; NEILL, J.D. 1994. The Physiology of Reproduction. Raven Press. p.1363-1434.
- SOWER, S.A.; FREMAT, M.; KAVANAUGH, S.I. 2009. The origins of the vertebrate hypothalamic–pituitary–gonadal (HPG) and hypothalamic–pituitary–thyroid (HPT) endocrine systems: New insights from lampreys. Gen. Comp. Endocrinol. 161, 20-29.
- STURM, A.; BURY, N.; DENGREVILLE, L.; FAGART, J.; FLOURIOT, G.; RAFESTIN-OBLIN, M.E.; PRUNET, P. 2005. 11-Deoxycorticosterone is a potent agonist of the rainbow trout (*Oncorhynchus mykiss*) mineralocorticoid receptor. Endocrinology 146, 47–55.
- TAKEI, Y.; HWANG, P-P. 2016. Homeostatic Responses to Osmotic Stress. In: SCHRECK, C.B; TORT, L.; FARRELL, A.P.; BRAUNER, C.J. Biology of Stress in Fish. Elsevier. p.207-249.
- TOVO-NETO, A.; RODRIGUES, M.S.; HABIBI, H.R.; NÓBREGA, R.H. 2018. Thyroid hormones action on male reproductive system of teleost fish. Gen. Comp. Endocrinol. In press.
- TSUTSUI, K.; SON, Y.S.; KIYOHARA, M.; MIYATA, I. 2018. Discovery of GnIH and Its Role in Hypothyroidism-Induced Delayed Puberty. Endocrinology 159, 62-68.

- VILELA, D.A.R.; SILVA, S.G.B.; PEIXOTO, M.T.D.; GODINHO, H.P.; FRANÇA, L.R. 2003. Spermatogenesis in teleost: insights from the Nile tilapia (*Oreochromis niloticus*) model. *Fish Physiol. Biochem.* 28, 187-190.
- WAGNER, A.; CLAUS, R. 2004. Involvement of glucocorticoids in testicular involution after active immunization of boars against GnRH. *Reproduction* 127, 275–283.
- WENDELAAR BONGA, S.E, 1997. The stress response in fish. *Physiol. Revs.* 77, 591–625.
- WEBER, M.A.; GROOS, S.; HÖPFL, U.; SPIELMANN, M.; AUMÜLLER, G.; KONRAD, L. 2000. Glucocorticoid receptor distribution in rat testis during postnatal development and effects of dexamethasone on immature peritubular cells *in vitro*. *Andrologia* 32, 23-30.
- YAMAGUCHI, T.; YOSHINAGA, N.; YAZAWA, T.; GEN, K.; KITANO T. 2010. Cortisol is involved in temperature-dependent sex determination in the Japanese flounder. *Endocrinology* 151, 3900-3908.
- ZOHAR, Y.; MUÑOZ-CUETO, J.A.; ELIZUR, A.; KAH, O. 2010. Neuroendocrinology of reproduction in teleost fish. *Gen. Comp. Endocrinol.* 165, 438-455.

Chapter II

Cortisol Protects Type A undifferentiated Spermatogonia Against Differentiation And Stimulates Zebrafish Spermatogenesis *In Vitro*

Cortisol protects type A undifferentiated spermatogonia against differentiation and stimulates zebrafish spermatogenesis *in vitro*

Aldo Tovo-Neto^{1,2,4}, Aline G. Melo⁴, Emanuel R.M. Martinez⁴, Arno J. Butzge⁴, Maira S. Rodrigues^{1,4}, Lucas B. Doretto⁴, Hamid R. Habibi^{2,3}, Rafael H. Nóbrega^{1,4*}

¹Aquaculture Program, São Paulo State University (CAUNESP), Jaboticabal, São Paulo, Brazil.

²Departments of Biological Sciences and ³Physiology and Pharmacology, University of Calgary, Calgary, Alberta, Canada.

⁴Departament of Morphology, Reproductive and Molecular Biology Group, São Paulo State University (UNESP), Botucatu, São Paulo, Brazil.

*corresponding author

Correspondence should be addressed to RH Nóbrega

Email address: biorhn@yahoo.com.br

Address: Departament of Morphology, Reproductive and Molecular Biology Goup, São Paulo State University (UNESP), Botucatu, São Paulo, Brazil.

Contact: +55 (14) 3880-0482

ABSTRACT

Corticosteroids correspond to the class of steroid hormones produced in the adrenal cortex of vertebrates, and within corticosteroids, cortisol is the main glucocorticoid produced in fish. In male fish, inhibitory and stimulatory effects of cortisol are reported on literature, such as decrease of androgen plasma levels and delayed testicular development. On the other hand, the *in vitro* approach is less explored and seems to have stimulatory effects. The aim of this study was to investigate the *in vitro* effects of cortisol in zebrafish spermatogenesis using an *ex vivo* organ culture system. We evaluated the germ cell proliferation activity by quantifying BrdU incorporation and cysts frequency were also determined in the presence of cortisol and cortisol + inhibitor (RU486) (Supplemental data). The transcript levels of selected testicular genes were quantified after 18h (short-term) and 7 days (long-term) of incubation in response to different levels of cortisol by qPCR. In addition, the androgen release (11-Ketotestosterone, 11-KT) in the culture medium was evaluated in the 18h incubation by enzyme-linked immunosorbent assay (ELISA). Localization of primary transcripts of *gr α* and differential plating were also used to determine sites of *gr* mRNA expression. Cortisol did not affect androgen release (11-KT), however stimulated steroidogenic genes in short-term culture (*ar*, *star*, *cyp17a1* and *hsd11b2*), as well *gr α* and *gr β* receptors. In the long-term culture, cortisol stimulated *sypc3l* and *shippo1* mRNA expression, but the inhibitor did not change any gene evaluated. Morphological analysis showed that cortisol stimulated zebrafish spermatogenesis, as well increase BrdU-mitotic index, even when in the treatment with RU486. In situ hybridization shows expression sites of *gr α* in both somatic cells, which was corroborated by differential plating. Cortisol has a positive role in zebrafish spermatogenesis *in vitro* by stimulating Sertoli and Leydig cell genes, as well stimulating differentiation process of germ cells.

Key-words

cortisol, spermatogenesis, spermatogonia, germ cell differentiation, zebrafish.

INTRODUCTION

The corticosteroids correspond to the class of steroid hormones produced by the adrenal cortex of vertebrates, or interrenal gland, as seen in fish. The corticosteroids are divided into glucocorticoids and mineralocorticoids, which are involved in a wide range of physiological responses, such as stress, immune and inflammatory, energetic metabolism and osmoregulation (Milla et al., 2009), as well reproduction (Consten et al., 2002). In general, the corticosteroids exert their physiological actions through nuclear receptors which act as transcription factors regulating the expression of target genes (Milla et al., 2009). Among the corticosteroids, cortisol is the main hormone involved in the stress response of vertebrates (Arterbery et al., 2011). In fish, for example, higher cortisol plasma levels are normally associated with stressful conditions, such as handling, toxicants in the water, and in situations where the fish can perceive threat (Barton, 2002).

All the levels of brain-pituitary-gonadal axis (BPG-axis) have been shown to be affected by stress in vertebrates (Rivier and Rivest, 1991; Wolfenson et al., 2000). For example, a decrease in the hypothalamic GnRH and LH plasma levels in male rats after chronic restraint stress has been reported (López-Calderón et al., 1990), similarly as observed in rams and ewes (Tilbrook et al., 1999).

Within fish species, cortisol affects both female and male reproduction. In female, depending of length of treatment and intensity of stressor, it may cause follicular atresia, advanced or delayed oocyte maturation, affecting egg size, fertilization success (Clearwater and Pankhurst, 1997; Coward et al., 1998; Schreck et al., 2001), and vitellogenesis process (Campbell et al., 1992; Roy et al., 1990).

In male fish, inhibitory and stimulatory effects of cortisol are reported on literature. *In vivo* studies have shown deleterious effects of cortisol such as lower androgen plasma levels during spermatogenesis phase of brown trout (Pickering et al., 1981), and delayed testicular development in common carp (Consten et al., 2001, 2002). These responses should be understood as consequences of cortisol actions on the BPG axis. On the other hand, when cortisol was tested *in vitro* by using immature Japanese eel (*Anguilla japonica*) testis explant, Okazaki et al. (2006) pointed out “positive effects” of cortisol by enhancing DNA replication and

mitosis of spermatogonia when added at moderate doses (0.1-100 ng/mL). Moreover, cortisol administration increased gonadosomatic index (GSI) and activated spermatogenesis in immature knifefish (*Notopterus notopterus*) (Shankar and Kulkarni, 2000).

Although *in vivo* studies provide a powerful and robust response of cortisol in the BPG axis, the *in vitro* experiments are more suitable to unravel the role specific role of cortisol. Therefore, the aim of this study was to investigate the *in vitro* effects of cortisol in zebrafish spermatogenesis using an *ex vivo* organ culture system well established for zebrafish (*Danio rerio*) testis (Leal et al., 2009a).

MATERIAL AND METHODS

Animals

Adult male of zebrafish (outbred) were bred and raised in the aquarium facility of Department of Morphology, Biosciences Institute, São Paulo State University. Handling and experimentation were consistent with Brazilian legislation regulated by National Council for the Control of Animal Experimental (CONCEA) and Ethical Principles in Animal Research (Protocol n. 671).

Testis tissue culture

Testes were dissected out and cultured using an *ex vivo* organ culture system as previously described (Leal et al., 2009a), for a short term (18 hours for steroid releasing and gene expression, in some cases) or a long term (07 days in all other cases) incubations.

Short term (18 h) incubation

For the short-term culture (18 h), testes were cultured in Leibovitz medium (L-15) and distributed in the follow scheme: one testis in L-15 and their contralateral one in L-15 containing increasing levels of cortisol (0.1; 1; 10; 100 and 1000 ng/mL) (Figure 1A). The number of animals used for each treatment was 6. Testes were incubated in 96 well plates in 200 µl of solution in each well at 28°C.

Following the period, the testes were individually weighted and stored at -80°C, and the medium was collected and stored at -20°C. The androgen release (11-Ketotestosterone, 11-KT) and mRNA levels of *ar* (androgen receptor), *cyp17a1* (17 α -hydroxylase/17,20 lyase/17,20 desmolase), *star* (steroidogenic acute regulatory), *gr α* (glucocorticoid receptor type alfa), *gr β* (glucocorticoid receptor type beta) and 11 β -hsd or *hsd11b2* (11-beta hydroxysteroid dehydrogenase) were quantified by quantitative PCR as described below.

In vitro 11-Ketotestosterone (11-KT) release

The androgen (11-KT) release capacity of zebrafish testicular tissue was measured after 18 h of *ex vivo* culture. The experiment evaluated the cortisol modulation on basal androgen releasing by zebrafish testis. In this context, zebrafish testes were immersed in a culture medium (basal medium) and their contra-lateral ones in medium containing different concentrations of cortisol (0.1; 1; 10; 100 and 1000 ng/mL). The amount of testicular 11-KT released in the culture medium was quantified using a steroid release bioassay previously established for zebrafish testis (Leal et al., 2009a).

Gene expression analysis by real-time, quantitative PCR (qPCR)

Total RNA from the testes was extracted by using a commercial kit RNAqueous-Micro kit (Ambion, TX, USA), according to manufacturer's instructions. The cDNA synthesis was performed as usual procedures (Nóbrega et al., 2010). qPCR reactions were conducted using 10 μ l 2X SYBR-Green Universal Master Mix, 2 μ l of forward primer (9mM), 2 μ l of reverse primer (9mM), 1 μ l of DEPC water and 5 μ l of cDNA. mRNA levels of the targets (Cts) were normalized by reference genes *ef1* (elongation factor 1) (in the case of *gr α* , *gr β* , *hsd11b2*) and *β -actin* (*ar*, *star*, *cyp17a1*), and expressed as relative values of Cts means of control group (ddCt). Primers were designed based on zebrafish sequences available at Genbank-pubmed plataform (NCBI) (Table 1).

Table 1 - Primers Used for Gene Expression Studies and to Generate DNA Templates for DIG-labeled cRNA Probe Syntheses for In Situ Hybridization

Target name	Primers sequence (5' - 3')		Access (NCBI)	Technical
<i>ef1α</i>	GCCGTCCCACCGACAAG CCACACGACCCACAGGTACAG	FW RV	FJ915061.1	qPCR
<i>b-actin</i>	AGACATCAGGGAGTGATGGT CAATACCGTGCTCAATGGGG	FW RV	NM_131031.1	qPCR
<i>ar</i>	ACGTGCCTGGCGTGAAAA CAAACCTGCCATCCGTGAAC	FW RV	EU708622.1	qPCR
<i>star</i>	CCTGGAATGCCTGAGCAGAA ATCTGCACTTGGTCGCATGAC	FW RV	NM_131663.1	qPCR
<i>cyp17a1</i>	GGGAGGCCACGGACTGTTA CCATGTGGAACTGTAGTCAGCAA	FW RV	NM_212806.3	qPCR
<i>hsd11b2</i>	GGGGGTCAAAGTTTCCACTA TGGAAGAGCTCCTTGGTCTC	FW RV	NM_212720.2	qPCR
<i>grα</i>	ACTCCATGCACGACTTGGTG GCATTTCGGGAACTCCACG	FW RV	EF436284.1	qPCR
<i>grβ</i>	GATGAACACGAATGTCTTA GCAACAGACAGCCAGACGCTCACT	FW RV	EF436285.1	qPCR
<i>sycp3l</i>	AGAAGCTGACCCAAGATCATTCC AGCTTCAGTTGCTGGCGAAA	FW RV	BC115343.1	qPCR
<i>dazl</i>	AGTGCAGACTTTGCTAACCTTATGTA GTCCACTGCTCCAAGTTGCTCT	FW RV	NM_131524.1	qPCR
<i>nanog</i>	TGTCCTACAACAAGACTGAGCC CAGGAATCTGGCGTGTGGG	FW RV	JN809237.1	qPCR
<i>shippo1</i>	GATGCCTGGAGACATGACCAA CAAAGGAGAAGCTGGGAGCTTT	FW RV	NM_199958.1	qPCR
<i>grα</i>	GGGCGGGTGTATTAAACCTCACTAAAGACTCCATGCACGACTTGGTG CCGGGGGGTGAATACGACTCACTATAGGCATTCGGGAACTCCACG	T3 T7	EF436284.1	ISH

*ISH, *in situ* hybridization.

Long term (07 days) incubation

In order to evaluate the effects of cortisol on spermatogenesis, the long-term culture was adopted. After dissecting out the testes, each tissue was placed on a piece of nitrocellulose membrane measuring 0.25cm² (25µm of thickness and 0.22µm of porosity) on top of cylinder of agarose with 750µl of volume (1.5% w/v, Ringer's solution – pH 7.4) into a 24 well plate. One testis was incubated with cortisol (100 ng/mL) and the contra-lateral one in a basal culture medium (only containing L-15), in a total of 24 samples (Figure 1). The medium was changed every 3 days of culture. This concentration (100 ng/mL) was selected based on the testicular mRNA expression of *ar* of short term culture (Figure 4A), avoiding extreme levels of 0.1 and 1000 ng/mL, as well as based on Okazaki et al. (2006) who showed stimulatory effects of cortisol in doses up to 100 ng/mL. For all experiments, total RNA was extracted from testis explants (n = 8) and mRNA levels of *star*, *ar*, *cyp17a1*, *hsd11b2*, *grα*, *grβ*, *sycp3l*, *dazl*, *nanog* and *shippo1*

were evaluated as described above (qPCR). In addition, some testes were also processed for morphological and histomorphometrical analysis (n = 8), and for BrdU incorporation (n = 8) (see below).

Morphological analysis

After 7 days of culture, zebrafish testis explants (n=8) from all culture experiments described above were fixed in 4% buffered glutaraldehyde at 4°C overnight, dehydrated, embedded in Technovit (7100 - Heraeus Kulzer, Wehrheim, Germany), sectioned at 4µm thickness, and stained with toluidine blue, according conventional histological procedures. Following the procedure, the slides were evaluated and the cysts frequency were determined, from 80 different fields per treatment. Morphological analysis was done by using conventional light microscopy and objective lens of 100x.

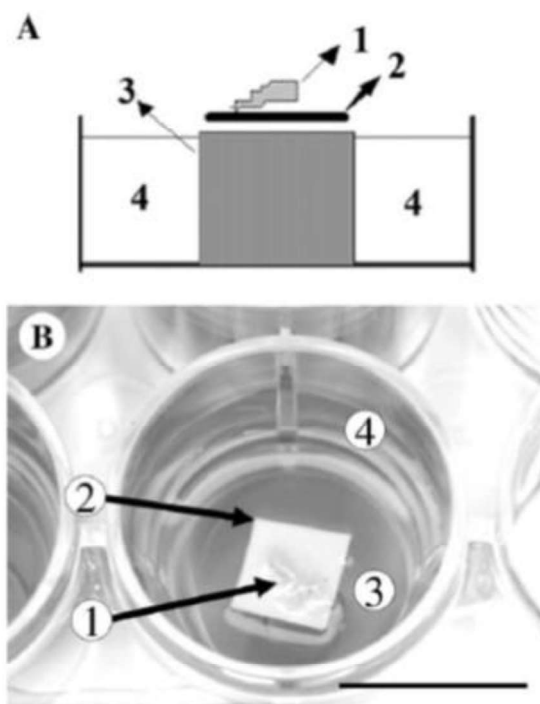


Figure 1 – A, schematic view and B, photograph of the zebrafish testis organ culture. 1- testis tissue, 2- nitrocellulose membrane, 3- agarose cylinder, 4- culture medium (L-15) surrounding the cylinder (1mL). Adapted from Leal et al. (2009a).

Germ cell proliferation - BrdU incorporation

To evaluate the effects of cortisol on germ cell proliferation, 100µg/mL bromodeoxyuridine (BrdU) was added to the culture medium during the last 6 hours of incubation of all culture experiments described above. After incubation, zebrafish testes (n = 8) were fixed at 4°C overnight in freshly prepared methacarn (60% [v/v] absolute ethanol, 30% chloroform, and 10% acetic acid) for 4 hours. Following up, tissues were dehydrated, embedded in Technovit 7100, sectioned at 4µm thickness, and submitted to BrdU immunodetection, as described before by Leal et al. (2009b).

In situ hybridization

For *in situ* hybridization, specific PCR products from glucocorticoid sequence was generated with primers (Table 1). The specific PCR product was gel purified and served as a template for digoxigenin (DIG)-labelled cRNA probe synthesis using the RNA labeling (Roche) kit. Zebrafish testes were dissected out and fixed in 4% paraformaldehyde (PFA) in PBS at 4°C overnight in RNase free conditions. The protocol used for *in situ* hybridization were performed with adaptations, as described in (Thisse e Thisse, 2008). Detection of hybridization was done with fluorescence HNPP (Roche).

Differential plating

Testis tissue was digested with 0.2% collagenase and 0.25% trypsin as described previously (Hinfray et al., 2013), and the cell suspension resulting was submitted to a differential plating (Luo et al., 2006). By using this method, somatic cells adhere to the bottom of the plate while germ cells either remain in suspension after 2-3 days of culture or only weakly associated with the firmly adhering cells somatic cells (Figure 2). Following up, the floating fraction was collected and somatic fraction (2C) was removed by scraping the bottom of the wells and washing several times with PBS solution.

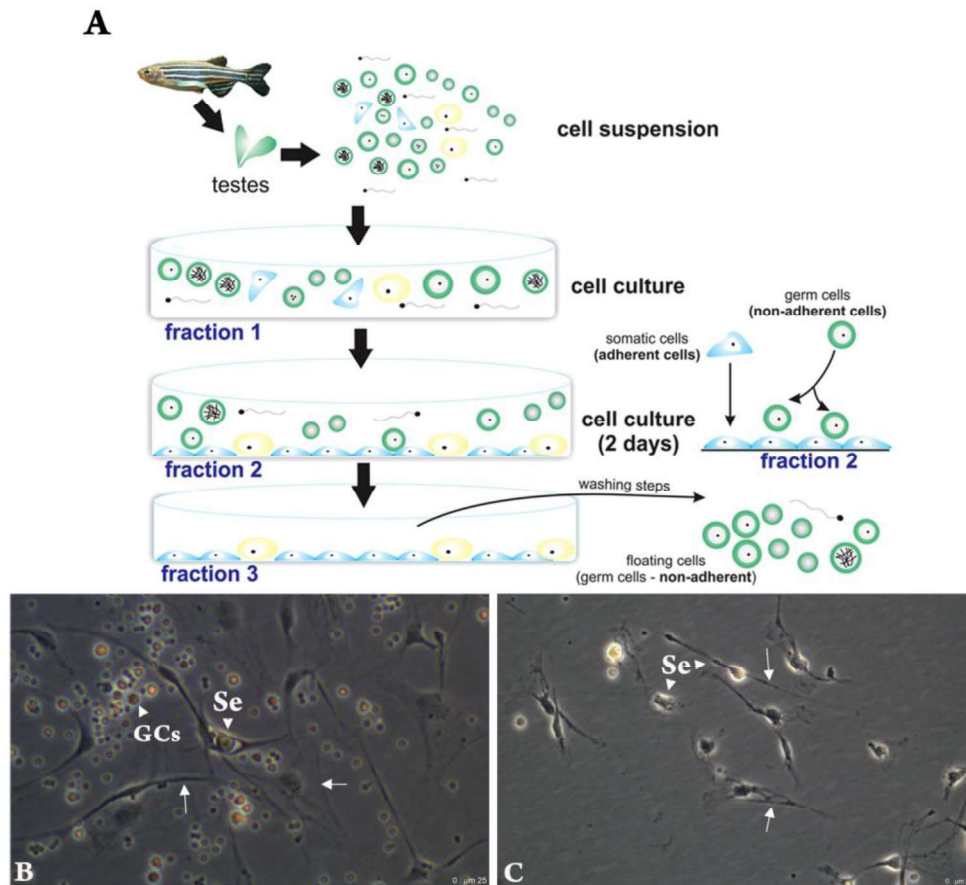


Figure 2 - Differential plating method. (A) Scheme showing the differential plating method adapted for zebrafish testicular cell suspension from Luo et al. (2006). A total cell suspension (fraction 1, 2B) was obtained from zebrafish testes and harvested in cell culture medium (L-15). After 2 days of culture, only somatic cells with adhesive properties (Sertoli cells, blue triangular symbols; Leydig cells, yellow oval symbols) adhere to the bottom of the plate (fraction 2), while germ cells remain floating or attach loosely to the firmly attached Sertoli cells. After the culture, floating germ cells were removed. After an extensive washing step, it is possible to remove those germ cells weakly attached to the somatic cells, leaving the adherent somatic cells (Sertoli cells, Leydig cells, fibroblast and others – fraction 3, 2C) at the bottom of the plate. From Hinfray et al. (2013).

Statistical analysis

Data are presented as mean $\pm$ SEM (Standard Error of Mean). Significant differences were identified using paired t-tests for two groups 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 were performed by Graph Pad Prism software 4.0 (Graph Pad Software, Inc., San Diego, CA, USA, <http://www.graphpad.com>).

RESULTS

In vitro 11-Ketotestosterone (11-KT) release

As known in Japanese eel (*A. japonica*) cortisol modulates the action of the 11 β HSD enzyme, which can both metabolize cortisol into cortisone and into androgen 11-oxygenated, thereby increasing 11-KT release by adult testis (Okazaki et al., 2016). To test whether cortisol increased 11-KT release by zebrafish testis, we measured the levels of 11-KT released by the testis in the culture medium. The graph represents the amount of 11-KT (ng) released by milligram of testis in culture medium (L-15) after 18h of incubation. The results showed that cortisol at different concentrations (from 0.1 ng/mL to 1000 ng/mL) has no effect on basal androgen release in zebrafish testicular explants (Figure 3).

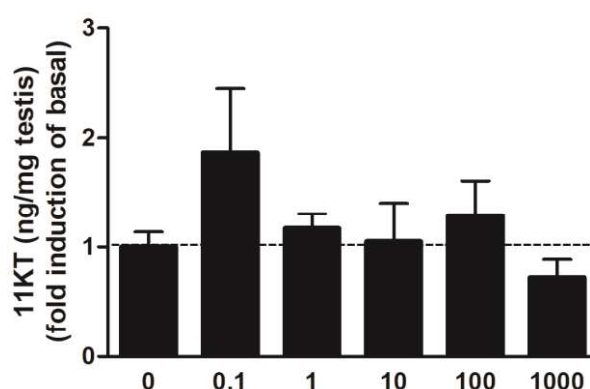


Figure 3 – *in vitro* 11-KT release by zebrafish testes cultured for 18h in different concentrations of cortisol (0; 0.1; 1; 10; 100 and 1000 ng/mL) ($n=8$). Data were normalized by milligrams of testis and expressed as fold induction of basal release.

In vitro short-term actions of cortisol on testicular gene expression

The lowest concentration of cortisol (0.1 ng/mL) was able to increase the androgen receptor (*ar*) mRNA levels (~8 fold induction of control) (Figure 4A). On the other hand, *ar* mRNA expression level was significantly down-regulated at the highest concentration of cortisol (1000 ng/mL). Intermediary concentrations (1, 10 and 100 ng/mL) have no effect on *ar* expression (Figure 4A).

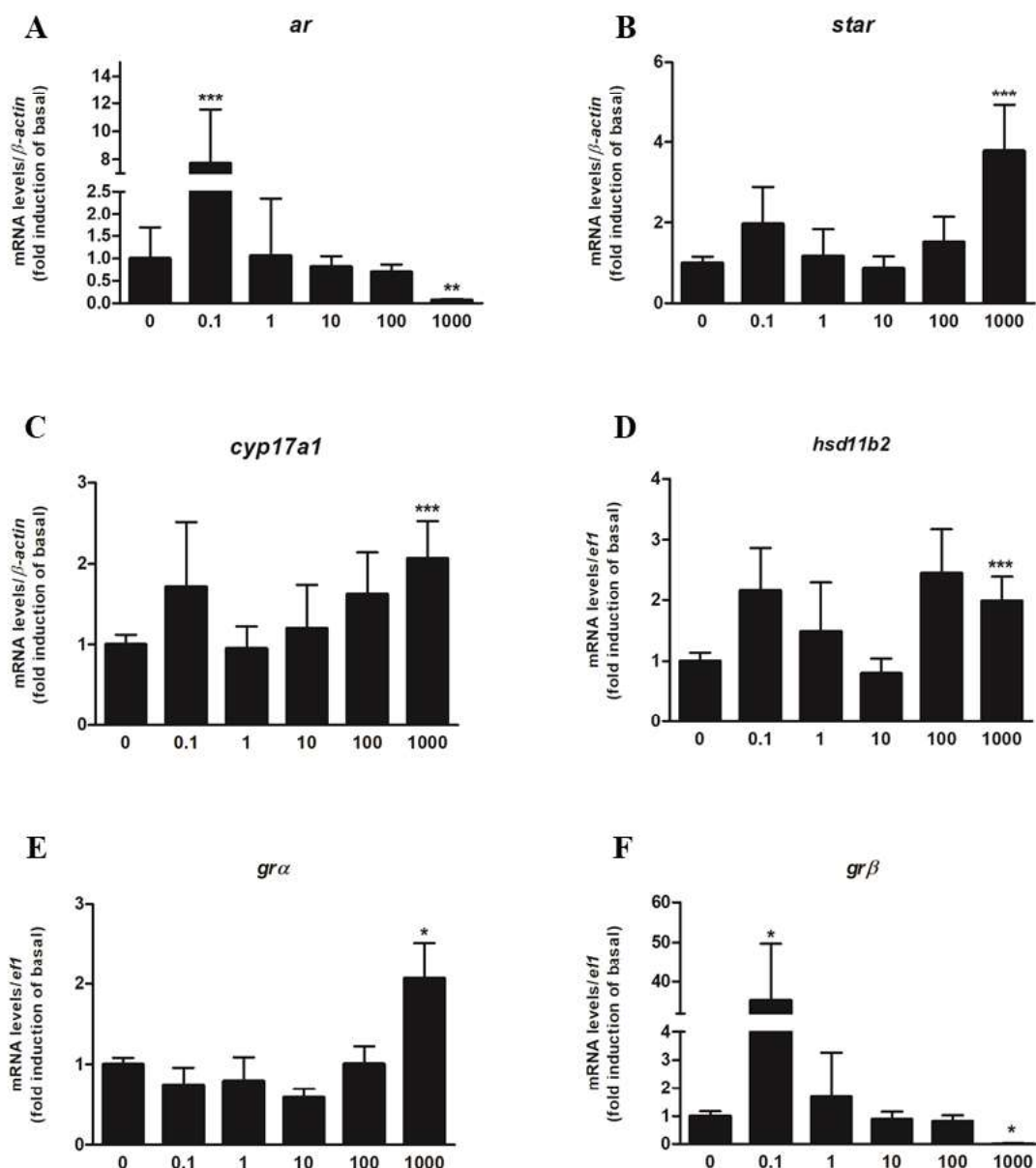


Figure 4 – Relative mRNA levels of steroidogenic related genes A-D (*ar*, *star*, *cyp17a1*, *hsd11b2*), glucocorticoid receptors E-F (*gr α* and *gr β*) and of zebrafish testes incubated with different concentrations of cortisol (0; 0.1; 1; 10; 100 and 1000 ng/mL) after 18h of culture. Bars represent the mean $\pm$ standard error of mean. Ct values were normalized with β -actin A-C (*ar*, *star*, *cyp17a1*) and *ef1* D-F (*hsd11b2*, *gr α* and *gr β*) and expressed as relative values of the basal (0 ng/mL) levels of expression. (*, when $p < 0.05$ compared to the control group; **, $p < 0.01$ compared to the control group; and ***, $p < 0.001$ compared to the control group).

With regards to the steroidogenic enzymes (*star* and *cyp17a1*), the highest concentration of cortisol (1000 ng/mL) increased significantly the transcript levels of both enzymes (~4 and ~2 folds for *star* and *cyp17a1* compared to control group,

respectively) (Figures 4B and C). Similar pattern was observed for *hsd11b2* mRNA levels, gene involved on steroidogenesis pathway and cortisol metabolism (Figure 4D). Although the expression of these genes were up-regulated, there was no 11-KT release associated (Figure 3).

Glucocorticoid receptor alfa (*gr α*) transcripts were significantly elevated at the highest concentration of cortisol (1000 ng/mL), while *gr β* mRNA levels were significantly down-regulated (Figures 4E and F). Interestingly, the lowest concentration of cortisol (0.1 ng/mL) stimulated *gr β* expression (~35 fold compared to control group) in relation to the control (Figure 4F).

In vitro long-term actions of cortisol on testis function

Morphological analysis showed that long-term effects of cortisol stimulated zebrafish spermatogenesis, exhibiting cysts at different stages of development (Figure 5) and quantitatively larged amount of spermatozoa inside the testicular lumen of the seminiferous tubules (Figure 6). Moreover cortisol significantly decreased the area occupied by type A undifferentiated spermatogonia (*A_{und}** and *A_{und}*) (Figure 5), while increased the one by type B spermatogonia. Interestingly, the meiotic and post-meiotic cysts became more frequent in the zebrafish testis treated with cortisol (100 ng/mL) after 7 days of culture (Figure 5).

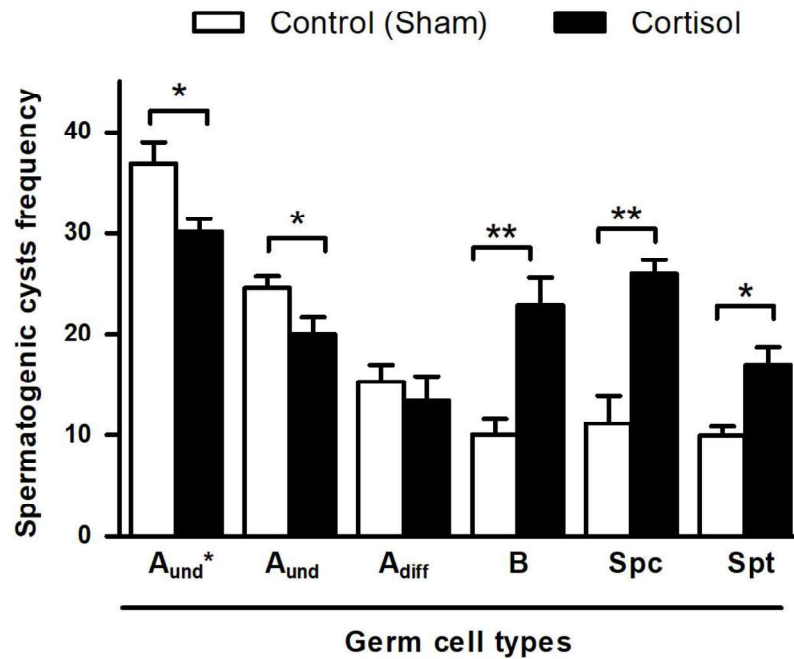


Figure 5 – Morphometric quantification (percentage of the total number of cysts at different stages of germ cells development; mean $\pm$ SEM; n=6) of the different stages of germ cell cyts in zebrafish testis. White bars mean control and black bars the treatment with 100 ng/mL of cortisol. A_{und}*, type undifferentiated spermatogonia; A_{und}, type undifferentiated spermatogonia; A_{diff}, differentiated spermatogonia; B, type B spermatogonia; Spc, spermatocyte; Spt, spermatid. *, when $p < 0.05$; **, when $p < 0.01$, compared to control group.

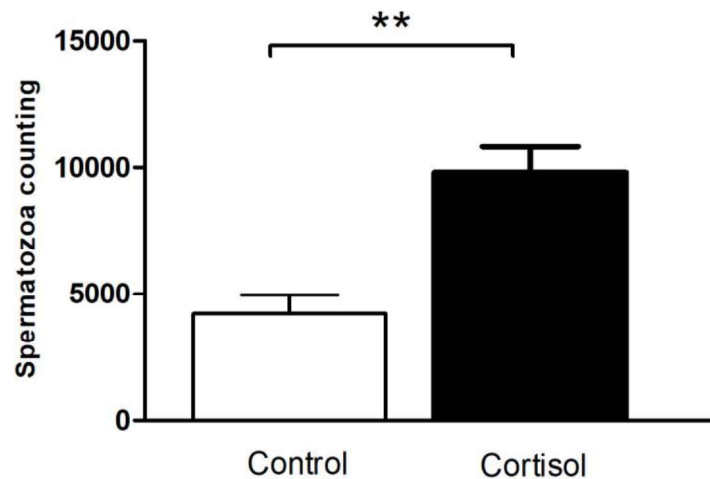


Figure 6 – Spermatozoa counting per field by using IMAGE J Software. **, when $p < 0.01$, compared to control group.

Evaluation of the BrdU-mitotic index showed that cortisol increased mitosis of type A undifferentiated spermatogonia (A_{und}), type A differentiated

spermatogonia (A_{diff}) and type B spermatogonia (Figure 7C). Interestingly, localization of BrdU-incorporated showed that fewer amount cysts are labeled in the control (Figure 7A), whereas in the presence of cortisol (100ng/mL) there is an increase of type B spermatogonia labeled (Figure 7B). These results are in agreement with BrdU labeling index (7C).

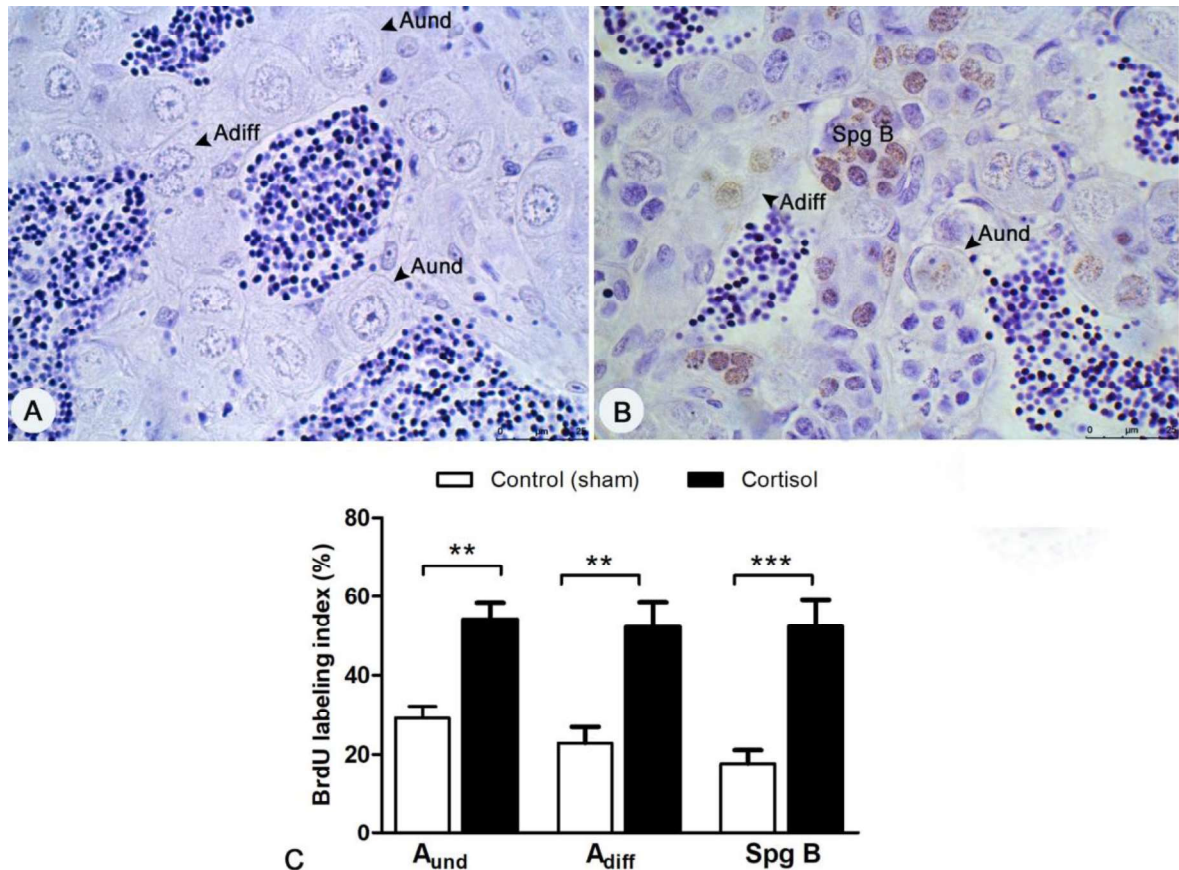


Figure 7 – Localization and quantification of BrdU-incorporated during the last 6 hours of incubation into zebrafish testis tissue explants after 7 days in culture. Transversal section showing BrdU immunodetection in tissue incubated in a culture medium (without treatment) (A), in the presence of 100 ng/mL of cortisol (B). BrdU positive single type A undifferentiated spermatogonia (A_{und} , black arrowheads); unlabeled A_{und} (white arrowheads). **, $p < 0.01$ and ***, $p < 0.001$ compared to the control group.

When evaluating the long-term effects of cortisol on testicular gene expression, the results showed that most of the evaluated genes did not change their expression, such as the steroidogenic-related genes (*star*, *cyp17a1*, *hsd11b2*, *ar*) and the glucocorticoid receptors (*gr α* and *gr β*) (Figure 8). However, analysis of germ cell transcripts, such as *sycp3l* (synaptonemal complex) and *shippo1* (spermatid) shown a significant increase in zebrafish testis cultivated with

100 ng/mL cortisol after 7 days of culture (Figure 8). On the other hand, the expression of other germ cell markers were not affected, such as *dazl* and *nanog* (Figure 8).

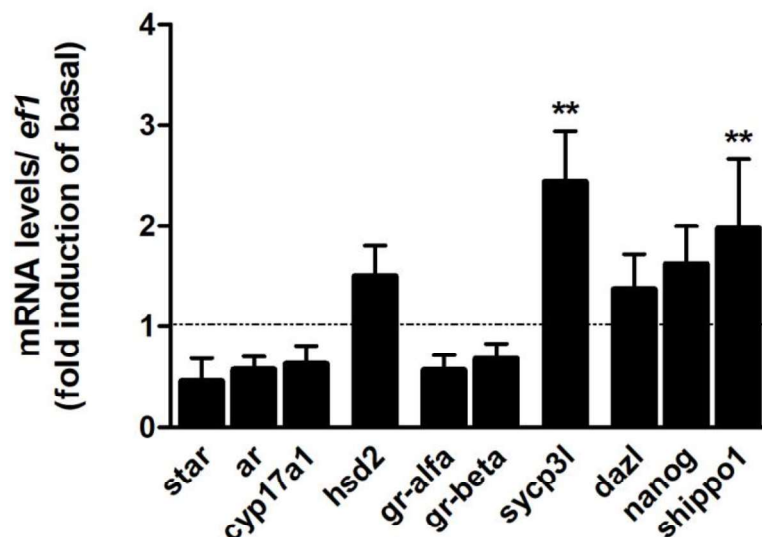


Figure 8 – Relative mRNA levels of selected genes (fold induction of control) from zebrafish testes incubated *in vitro* using 100 ng/mL of Cortisol and cultured for 7 days. Bars represent the mean $\pm$ standard error of relative mRNA values normalized using *ef1*. ** when $p < 0.01$ compared to control group.

Localization of primary transcripts of $gr\alpha$

Identification of the cell types expressing the mRNA coding for glucocorticoid receptor (GR) was accomplished by ISH. Based on the shape and localization of the stained cells in the tubular and intertubular compartments, the cells specifically labeled were identified as Sertoli and Leydig cells (Figures 9B and 9C), however, we also found expression in germ cells (Figure 9C). No specific signal was obtained when sections were incubated with sense probe (9A).

Differential plating: $gr\alpha$ and $gr\beta$ gene expression

Evaluating transcripts of *gr α* showed mRNA expression in both somatic and germ cells (flotating cells), however the germ cells showed higher expression compared to the control (9D). Interestingly, transcripts of *gr β* showed higher expression in germ cells comparing to both control and somatic cells (9E).

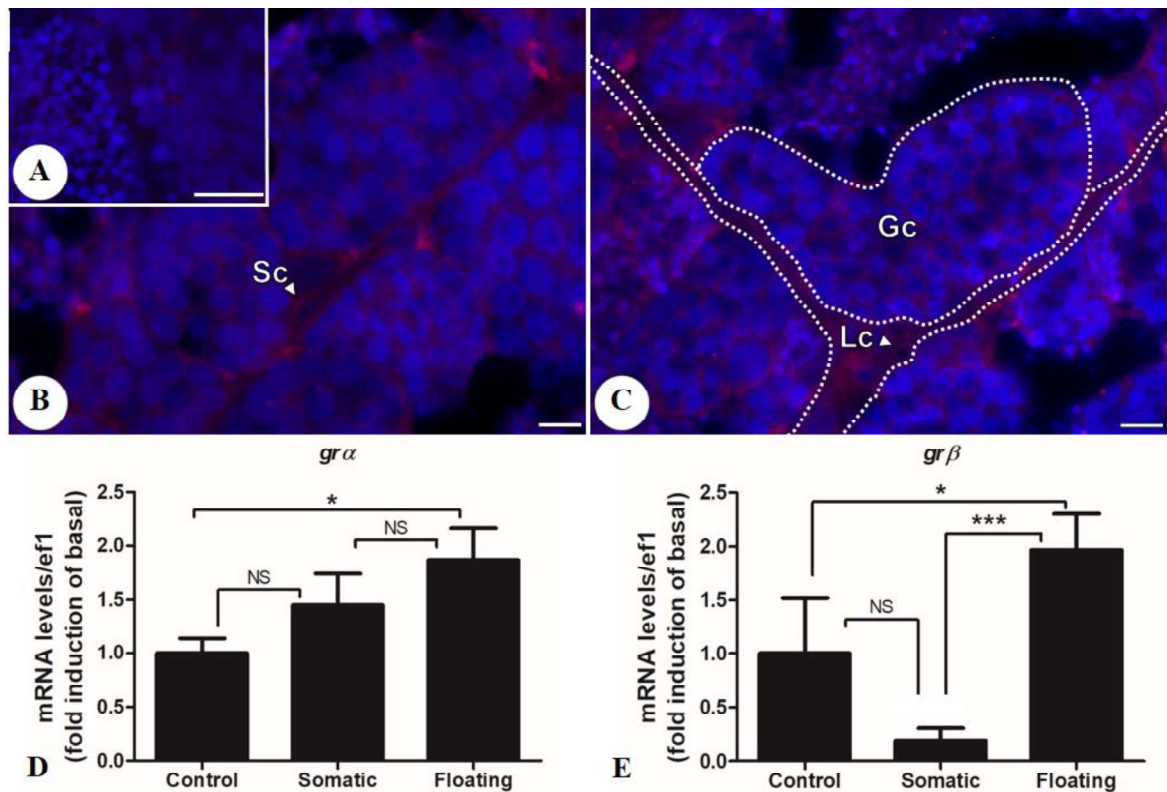


Figure 9 - Localization of GR mRNA in zebrafish testis using whole-mount ISH and post hybridization embedding and sectioning. No specific staining was obtained with the sense cRNA probes (A). Staining representing *gr* mRNA (B and C) was found in Sertoli cells (Sc), Leydig cells (Lc) and germ cells (gc). Dashed line indicates a germ cell cyst containing stained gc. No specific signal was obtained when sections were incubated with sense probe. Relative mRNA levels of *grα* (9D) and *grβ* (9E) from differential plating. *, $p < 0.05$ and ***, $p < 0.001$ compared to the control group.

DISCUSSION

The inhibitory effect of cortisol on fish spermatogenesis was demonstrated systematically through *in vivo* experiments incorporating cortisol in the fish feed (Consten et al. 2001) and through hormone implants in fish (Consten et al., 2002). In the same line, it was also demonstrated that cortisol is capable to interfere on gonadal differentiation of fish larvae (Hattori et al., 2009, Hayashi et al., 2010, Fernandino et al., 2010, Yamaguchi et al., 2010, Fernandino et al., 2012, Mankiewicz et al., 2013), masculinizing the larvae. However, the direct effects of

cortisol on fish gonads still is less explored (see Fernandino et al., 2012, Ozaki et al., 2006, Yamaguchi et al., 2010), and provide interesting results because can decrease the interference in crossover reactions.

Here we present some biological effects of direct action of cortisol on zebrafish testes. In japanese eel (*A. japonica*), cortisol direct stimulated *in vitro* 11-KT production (doses of 10 and 100 ng/mL) (Ozaki et al., 2006). In the same line, in pejerrey (*Odontesthes bonariensis*), Fernandino et al., (2012) has shown that 0.1 ng/mL of cortisol induces 11-KT releasing on testicular explants of that species, following by the 1 and 10 ng/mL which also stimulated the androgen release when compared to control group. These results could be explained by activation of 11- β -hydroxysteroid dehydrogenase (11 β HSD) (Fernandino et al., 2013).

Our findings show that in the zebrafish case, cortisol does not modulate androgen (11-KT) releasing in the culture medium (Figure 3). It seems that the androgen levels did not alter because cortisol does not stimulate the expression of 11 β HSD enzyme (*hsd11b2*) in the zebrafish testes, except in the highest level of 1000 ng/mL. This could imply that is this enzyme is trying to metabolize the over amount of cortisol into the inactive form (cortisone), once the androgen releasing is not showing any changes.

In vitro studies with affinity and transactivation of androgen receptor (*ar*) (by the MMTV promoter related to luciferase – see de Waal et al., 2008), showed that in zebrafish cortisol presents low binding affinity to *ar* (K_i of cortisol = ~352nM versus K_i of methyltestosterone = ~0.73nM), which means that ~1855nM (~672ng/mL) of cortisol is required to obtain 50% of maximum activation of androgen receptor (de Waal et al., 2008). Interestingly, dosage of 100 nM (~0.3ng/mL) had any effect on receptor activation (de Waal et al., 2008). Despite of cortisol has low affinity with *ar* receptor (de Waal et al., 2008), here we demonstrate that cortisol regulates the expression of this receptor on zebrafish testes *in vitro* culture. Lower dosage of cortisol (0.1ng/mL) up-regulated its expression in the short-term culture experiments, whereas higher dosage downregulated its expression. This stimulation in lower dosage suggest that sensibility is higher in this dosage, while the highest dosage has an inhibitory

effect on androgenic sensitivity. This is also reported on mammalian, which the glucocorticoids suppress the mRNA *ar* expression (Burnstein et al., 1995). Thus, we conclude that higher dosage of cortisol would have an inhibitory effect on androgen stimulation of zebrafish testes. It should be noted that on 1000ng/mL, it is possible that the *ar* reached its maximum activity (see de Waal et al., 2008, EC50=~672ng/mL).

In our study cortisol stimulated the *cyp17a1* expression at the highest level (1000ng/mL) upregulated the expression of this gene and also the *star* expression. It could be associated with the relief caused by the androgen insensitivity in the highest level of cortisol. The inhibitory effect of *cyp17a1* and *star* expression mediated by androgen was reported in zebrafish by García-López et al. (2010). On this work, they have been used a competitive inhibitor (trilostane) of the enzyme involved on androgen production. So, when the testes were incubated with trilostane in absence or presence of Fsh (potent steroidogenesis inducer), even with androgen pathway blocked, upregulated the levels of *cyp17a1*, *star* and *hsd11b2* mRNA levels. This finding is similar to these data reported here at the level of 1000 ng/mL of cortisol. The inhibitory effect of androgen pathway could be explained by the negative feedback loop in order to prevent an overstimulation of steroidogenesis (García-López et al., 2010). The high levels of *cyp17a1*, *star* and *hsd11b2* reported here may be explained by the androgen insensitivity at the level of 1000ng/mL. This high level could be an attempt to stimulate the steroidogenesis, increasing the androgen levels.

Despite the reports on literature brings inhibitory effects of cortisol, it should be mentioned that the majority of the studies addressed indirect effects of cortisol in the gonads. Cortisol in intermediary dosage of 100 ng/mL stimulates the spermatogonia differentiation from undifferentiated type A to type B spermatogonia, as well protects type Aund* from exhaustive differentiation. This pattern is also observed in another stem cell types, as the case of neural stem cells (NSCs). Interestingly, although HPA axis activation has been strongly associated with suppression of neurogenesis, there are some stressors that consistently increase the proliferation rate and enhance the survival of NSCs (reviewed by Koutmani and Karalis (2015)). In addition, “stressful” experiences e.g running and physical exercise (van Praag et al., 1999, 2002; Droste et al., 2003),

psychological challenge such as housing in enriched environment (van Praag et al., 1999; Kempermann et al., 2002), sexual experience and learning (Bonilla-Jaime et al., 2006; Leuner et al., 2010) allow not only protection of NSCs from negative effects of glucocorticoid, but even more, they exert positive effects on NSCs (Koutmani and Karalis, 2015).

CONCLUSION

Cortisol has a positive effect in zebrafish spermatogenesis by stimulating types Aund* and Aund differentiation and increasing type B spermatogonia population, in an androgen independent manner. When the Gr inhibitor is added, differentiation still is stimulated suggesting that cortisol also acts protecting type Aund* spermatogonia from an excessive differentiation. Cortisol also stimulated zebrafish spermatogenesis *in vitro* by increasing spermatocitary-meiosis and free spermatozoa in the lumen.

FUNDING

This work was supported by National Council for Scientific and Technological Development (CNPq) – Science without Borders (234548/2014-2) (awarded to Aldo Tovo-Neto) and FAPESP (2014/16280-5 and 2017/09218-0 – granted to Aldo Tovo-Neto; 2014/07620-7 – granted to Dr. Rafael Henrique Nóbrega).

REFERENCES

- Arterbery AS, Fergus DJ, Fogarty EA, Mayberry J, Deitcher DL, Kraus WL & Bass AH 2011 Evolution of ligand specificity in vertebrate corticosteroid receptors. *BMC Evolutionary Biology* **11** 1-14.
- Barton BA Stress in Fishes: A Diversity of Responses with Particular Reference to Changes in Circulating Corticosteroids. *Integrative and Comparative Biology* **42** 517-525.
- Bonilla-Jaime H, Vazquez-Palacios G, Arteaga-Silva M & Retana-Marquez S 2006 Hormonal responses to different sexually related conditions in male rats. *Hormones and Behavior* **49** 376–382.
- Burnstein KL, Maorino CA, Dai JL & Cameron DJ 1995 Androgen and glucocorticoid regulation of androgen receptor cDNA expression. *Molecular and Cellular Endocrinology* **115** 177-186.
- Campbell PM, Pottinger TG & Sumpter JP 1992. Stress reduces the quality of gametes produced by rainbow trout. *Biology of Reproduction* **47** 1140-1150.
- Clearwater SJ & Pankhurst NW, 1997. The response to capture and confinement stress of plasma cortisol, plasma sex steroids and vitellogenic oocytes in the marine teleost, red gurnard. *Journal of Fish Biology* **50** 429-441.
- Consten D, Lambert JGD & Goos HJTh 2001 Cortisol affects testicular development in male common carp, *Cyprinus carpio* L., but not via an effect on LH secretion. *Comparative Biochemistry and Physiology - Part B* **129** 671-677.
- Consten D, Keuning ED, Terlouw M, Lambert JGD & Goos HJTh 2002 Cortisol effects on the testicular androgen synthesis capacity in common carp, *Cyprinus carpio* L. *Fish Journal of Physiology and Biochemistry* **25** 91-98.
- Coward MP, Daltaban TS & Johnson H. (eds) 1998. Structural geology in reservoir characterization. Geological Society, London, Special Publications, **127**.
- Droste SK, Gesing A, Ulbricht S, Muller MB, Linthorst AC & Reul JM 2003 Effects of long-term voluntary exercise on the mouse hypothalamic-pituitary-adrenocortical axis. *Endocrinology* **144** 3012–3023.

Fernandino JI, Popescu JT, Paul-Prasanth B, Xiong H, Hattori RS, Oura M, Strüssmann CA, Somoza GM, Matsuda M, Nagahama Y & Trudeau VL 2010 Analysis of Sexually Dimorphic Expression of Genes at Early Gonadogenesis of Pejerrey *Odontesthes bonariensis* Using a Heterologous Microarray. *Sexual Development* **15** 1-13.

Fernandino JI, Hattori RS, Kishii A, Strüssmann CA & Somoza GM 2012 The Cortisol and Androgen Pathways Cross Talk in High Temperature-Induced Masculinization: The 11 β -Hydroxysteroid Dehydrogenase as a Key Enzyme. *Endocrinology* **153** 6003-6011.

Fernandino JI, Hattori RS, Acosta ODM, Strüssmann CA & Somoza GM 2013 Environmental stress-induced testis differentiation: Androgen as a by-product of cortisol inactivation. *Endocrinology* **192** 36-44.

García-López Á, de Jonge H, Nóbrega RH, de Waal PP, van Dijk W, Hemrika W, Taranger GL, Bogerd J & Schulz RW 2010 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* **151** 2349-2360.

Hattori RS, Fernandino JI, Kishii A, Kimura H, Kinno T, Oura M, Somoza GM, Yokota M, Strüssmann CA & Watanabe S 2009 Cortisol-Induced Masculinization: Does Thermal Stress Affect Gonadal Fate in Pejerrey, A Teleost Fish with Temperature-Dependent Sex Determination? *PLoS One* **4** e6548.

Hayashi Y, Kobira H, Yamaguchi T, Shiraishi E, Yazawa T, Hirai T, Kamei Y & Kitano T 2010 High Temperature Causes Masculinization of Genetically Female Medaka by Elevation of Cortisol. *Molecular Reproduction and Development* **77** 679-686.

Hinfray N, Nóbrega RH, Caulier M, Baudiffier D, Maillot-Maréchal E, Edith Chadili E, Palluel O, Porcher J-M, Rüdiger Schulz RW & Brion F 2013 Cyp17a1 and Cyp19a1 in the zebrafish testis are differentially affected by oestradiol. *Journal of Endocrinology* **216** 375-388.

Kempermann G, Gast D & Gage FH 2002. Neuroplasticity in old age: sustained fivefold induction of hippocampal neurogenesis by long-term environmental enrichment. *Annals of Neurology* **52** 135–143.

Koutmani Y & Karalis KP 2015 Neural stem cells respond to stress hormones: distinguishing beneficial from detrimental stress. *Frontiers in physiology* **6** 1-9.

Leal MC, de Waal PP, García-López Á, Chen SX, Bogerd J & Schulz RW 2009a Zebrafish primary testis tissue culture: An approach to study testis function *ex vivo*. *General and Comparative Endocrinology* **162** 134-138.

Leal MC, Cardoso ER, Nóbrega RH, Batlouni SR, Bogerd J, França LR & Schulz RW 2009b Histological and stereological evaluation of zebrafish (*Danio rerio*) spermatogenesis with an emphasis on spermatogonial generations. *Biology of Reproduction* **153** 6003-6011.

Leuner B, Glasper ER & Gould E 2010 Sexual experience promotes adult neurogenesis in the hippocampus despite an initial elevation in stress hormones. *PLoS ONE* **5** e11597.

López-Calderón A, González-Quijano M, Tresguerres J & Ariznavarreta C 1990 Role of LH-RH in the gonadotrophin response to restraint stress in intact male rats. *Journal of Endocrinology* **124** 241-246.

Luo J, Megee S, Rathi R & Dobrinski I 2006 Protein gene product 9.5 is a spermatogonia-specific marker in the pig testis: Application to enrichment and culture of porcine spermatogonia. *Cell Biology and Biochemistry* **73** 1531-1540.

Mankiewicz JL, Godwin J, Holler BL, Turner PM, Murashige R, Shamey R, Daniels HD & Borski RJ 2013 Masculinizing Effect of Background Color and Cortisol in a Flatfish with Environmental Sex-Determination. *Integrative and Comparative Biology* **1** 1-11.

Milla S, Wang N, Mandiki SNM & Kestemont P 2009 Corticosteroids: Friends or foes of teleost fish reproduction? *Comparative Biochemistry and Physiology - Part A* **153** 242-251.

Nóbrega RH, Greebe CD, de Kant H, Bogerd J, França LR & Schulz RW 2010 Spermatogonial Stem Cell Niche and Spermatogonial Stem Cell Transplantation in Zebrafish. *PLoS One* **5** e12808.

Ozaki Y, Higuchi M, Miura C, Yamaguchi S, Tozawa Y & Miura T 2006 Roles of 11 β -Hydroxysteroid Dehydrogenase in Fish Spermatogenesis. *Endocrinology* **147** 5139-5146.

Pickering AD & Christie P 1981 Changes in the concentrations of plasma cortisol and thyroxine during sexual maturation of the hatchery-reared brown trout, *Salmo trutta*. *General and Comparative Endocrinology* **44** 488–496.

Rivier C & Rivest S 1991 Effects of stress on the activity of the hypothalamic-pituitary-gonadal axis: peripheral and central mechanisms. *Biology of Reproduction* **45** 523-532.

Roy P, Datta M, Dasgupta S & Bhattacharya S 1990. Gonadotropin-Releasing Hormone Stimulates Thyroid Activity in a Freshwater Murrel, *Channa gachua* (Ham.), and Carps, *Catla catla* (Ham.) and *Cirrhinus mrigala* (Ham.). *General and Comparative Endocrinology* **117** 456-463.

Schreck CB, Contreras-Sanchez W & Fitzpatrick MS, 2001. Effects of stress on fish reproduction, gamete quality, and progeny. *Aquaculture* **197**, 3-24.

Shankar DS & Kulkarni RS 2000 Effect of cortisol on testis of freshwater fish *Notopterus notopterus* (Pallas). *Indian Journal of Experimental Biology* **38** 1227-1230.

Thisse C & Thisse B 2008. High-resolution in situ hybridization to whole-mount zebrafish embryos. *Nature Protocols* **3** 59-69.

Tilbrook AJ, Canny BJ, Serapiglia MD, Ambrose TJ & Clarke IJ 1999. Suppression of the secretion of luteinizing hormone due to isolation/restraint stress in gonadectomized rams and ewes is influenced by sex steroids *Journal of Endocrinology* **160** 469–481.

van Praag H, Kempermann G & Gage FH 1999 Running increases cell proliferation and neurogenesis in the adult mouse dentate gyrus. *Nature Neuroscience* **2** 266–270.

vanPraag H, Schinder AF, Christie BR, Toni N, Palmer TD & Gage FH 2002 Functional neurogenesis in the adult hippocampus. *Nature* **415** 1030-1034.

de Waal PP, Wang DS, Nijenhuis WA, Schulz RW & Bogerd J 2008 Functional characterization and expression analysis of the androgen receptor in zebrafish (*Danio rerio*) testis. *Reproduction Research* **136** 225-234.

Wolfenson D, Roth Z & Meidan R 2000 Impaired reproduction in heat-stressed cattle: basic and applied aspects. *Animal Reproduction Science* **60-61** 535-547.

Yamaguchi T, Yoshinaga N, Yazawa T, Gen K & Kitano T 2010 Cortisol Is Involved in Temperature-Dependent Sex Determination in the Japanese Flounder. *Endocrinology* **151** 3900-3908.

SUPPLEMENTAL DATA

In vitro effects of a glucocorticoid receptor antagonist (RU-486) in zebrafish testis treated with cortisol

To evaluate whether the cortisol effects were specific, a glucocorticoid receptor antagonist (RU-486) was used in a long-term culture of zebrafish testis. For this purpose, zebrafish testis were incubated with cortisol in the presence or absence of RU-486 (1 μ g/mL) for 7 days of culture. Regarding gene expression, when the inhibitor was added, all the genes evaluated were not affected by the treatment (Figure 3). Spermatozoa quantification showed no difference within cortisol and cortisol + inhibitor (Figure 2). On the other hand, evaluation of the BrdU-mitotic index showed that even in the presence of the inhibitor, types A_{und} and A_{diff} spermatogonia still increased mitotic index of these types (Figure 1).

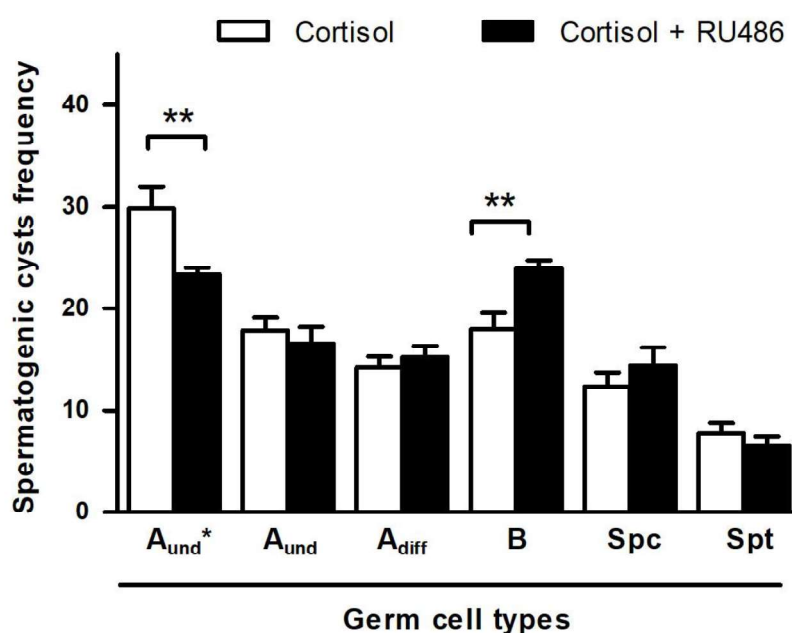


Figure 1 – Morphometric quantification (percentage of the total number of cysts at different stages of germ cells development; mean $\pm$ SEM; n=6) of the different stages of germ cell cyts in zebrafish testis. White bars mean control (100ng of cortisol) and black bars treated with 100 ng/mL of cortisol+inhibitor. A_{und}^* , type undifferentiated spermatogonia; A_{und} , type undifferentiated spermatogonia; A_{diff} , differentiated spermatogonia; B, type B spermatogonia; Spc, spermatocyte; Spt, spermatid. **, when $p < 0.01$ compared to control group.

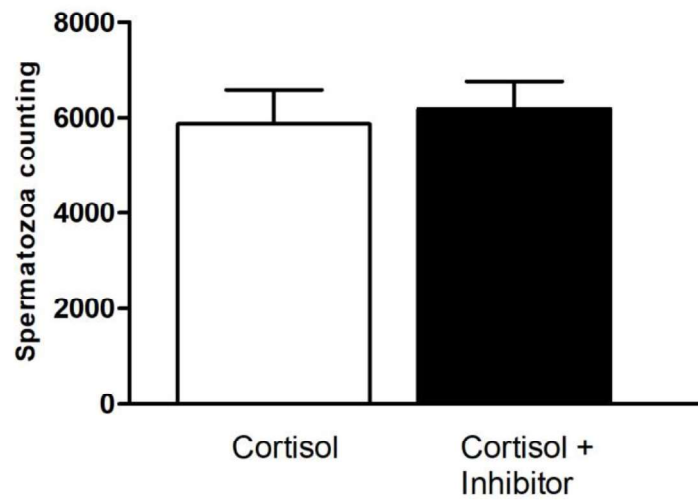


Figure 2 – Spermatozoa counting per field by using IMAGE J Software.

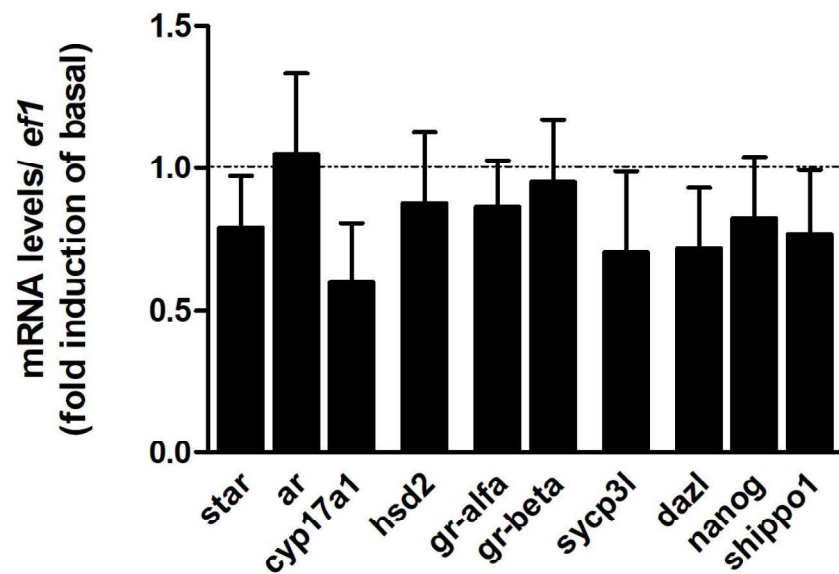


Figure 3 – Relative mRNA levels of selected genes (fold induction of control (100 ng/mL of cortisol)) from zebrafish testes incubated *in vitro* using 100 ng/mL of Cortisol and 1µg/mL of inhibitor and cultured for 7 days. Bars represent the mean $\pm$ standard error of relative mRNA values normalized using *ef1*. ** when $p < 0.01$).

Chapter III

Thyroid Hormones On Zebrafish
***(Danio Rerio)* Spermatogenesis**

Manuscript in preparation

ABSTRACT

Thyroid hormones (THs) play important roles in the regulation of many biological processes of vertebrates, such as growth, metabolism, morphogenesis and reproduction. An increasing number of studies have been focused on the involvement of THs in the male reproductive system of vertebrates, in particular of fish. Considering that in mammals most of the experimental studies to date are focused on thyroid hormone effects on the developing testes, only limited data are available on adult testis and spermatogenesis. Therefore, the aim of this study was to investigate TH effects on zebrafish (*Danio rerio*) spermatogenesis by using methimazole, as well *in vitro* studies. For that, *in vitro* studies were conducted testing different dosages of T3 and T4 (0, 10, 100 and 1000 nM/mL), as well the co-treatment of T3 (100 nM/mL) with Fsh (100ng/mL). Following up, *in vivo* experiments were conducted with three groups (control, methimazole, methimazole + T4) for 21 days. Morphological quantification (area occupied) and mRNA levels of thyroid receptors (*thra* and *thrβ*), *cyp17a1*, *insl3*, *igf3*, testicular connexin (cx43), *amh*, *gsdf* and germ cell markers (*nanos2*, *dazl*, *shippo1* and *sycp3*) were determined, as well the caspase activity in the testis. *In vitro*, T3 stimulated *nanos2* and *sycp3* expression, however did not change *igf3*, *amh*, *3β-HSD* and *cyp17a1*. Intriguingly, when in the presence of T4, all genes did not differ. When T3 was cultured co-treated with Fsh, T3 potentiated gonadotropin action and up-regulated *cyp17a1* expression. Interestingly, *in vivo* treatment with methimazole affected mRNA expression of genes that control Sertoli and Leydig cells function, whereas when T4 was co-treated with methimazole, still the effect of methimazole persisted. Measurement of the area occupied by the germ cells showed increase of undifferentiated type A spermatogonia and type B, while spermatozoa and the lumen area were reduced significantly, associated with apoptosis. Overall our data shows that thyroid absence induced by methimazole impairs zebrafish spermatogenesis by modulating Sertoli and Leydig cells functions.

Keywords: triiodothyronine (T3), hypothyroidism, spermatogenesis, zebrafish.

INTRODUCTION

The thyroid gland plays an essential role in the growth and regulation of metabolism and reproduction in vertebrates (Mullur et al., 2014). In the reproductive field, the role of thyroid hormones on ovarian function is well known in mammals (Stradtman, 1993), while studies on male reproductive system presented several conflicting observations in fish (Swapna et al., 2006). In the last two decades, the function of thyroid hormone in testicular development has received much attention due to initial observations of functional thyroid hormone receptors present in neonatal rats Sertoli cells (Francavilla et al., 1991; Krassas et al., 2008).

Different from mammalian thyroid gland which is an encapsulated organ, the thyroid follicles in fish are diffusely dispersed within the connective tissue on the surface of the ventral aorta and sub-pharyngeal and parapharyngeal areas. This particular condition constitutes a bias against thyroidectomy-induced hypothyroidism. Hence, thyroid hormone suppression in fish is usually achieved by chemical compounds, such as methimazole (Swapna et al., 2006).

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 (França et al., 1995; Maran and Aruldas, 2002). The impairments caused by neonatal induced-hypothyroidism can be rescued with T3 which has been demonstrated to be stimulatory to sperm production in the adult (Cooke, 1991). On the other hand, hyperthyroidism leads to early cessation of Sertoli cell proliferation, decrease in testis size, and decreased sperm production (van Haaster et al., 1993; Cooke et al., 1994a,b).

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 larvae treated with PTU (propylthiouracil, antithyroid drug similar to methimazole) doubled the size of their testis, similar to the effects found in rats, suggesting conserved mechanism controlling testis development

through thyroid hormones among vertebrate evolution. In this case of hypothyroidism, 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 reflecting in male reproductive system disruption (Swapna et al., 2006). Interestingly, partial recovery of 11-KT and T levels after thiourea treatment withdrawal for 21 days clearly indicated that thyroid plays a role in maintaining testis functions (Swapna et al., 2006).

More recently, the molecular mechanisms underlying the role of THs in spermatogenesis have been elucidated in the zebrafish testis model (Tovo-Neto et al., 2018). 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. Next to their roles in the adult testis, THs also participate in the gonadal sex differentiation by regulating the expression of sex-related genes, and during testicular development through modulating Sertoli cell proliferation (Matta et al., 2002). Based on the evidences from the literature, it can be concluded that THs modulate the male reproductive system during the different life stages of fish (Tovo-Neto et al., 2018). The aim of this study was to investigate TH effects on zebrafish spermatogenesis by using methimazole, as well *in vitro* studies.

MATERIAL AND METHODS

Thyroid hormones effects in vitro culture of testes

The endocrine regulation was evaluated by using a well established *in vitro* culture system previously described by Leal et al. (2009). For this end, zebrafish testes (n = 8 for each treatment) were dissected out and one testis was incubated in Leibovitz culture medium (L-15) while its contra-lateral ones in different treatments with thyroid hormones. 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 gonads, one level of T3 (100 nM/mL) was selected based on previous experiments (Morais et al., 2013) and then combined with zebrafish recombinant of follicle stimulating hormone (Fsh, 100 ng/mL). Testes were incubated in a 24 well plate, containing 1 mL of culture medium per well at 28 °C for seven days, changing the medium every 3 days. After the experimental period, testes were weighed and stored at -80 °C, until gene expression analysis (see below).

Hypothyroidism-induced by in vivo methimazole exposure

In order to mimic the effects of thyroid inhibition, a thyroid inhibitor, methimazole (MZ), was used to induce the hypothyroidism as previously reported in the literature (Sharma et al., 2013). In this context, male adult zebrafish (n = 60) were divided into three groups: group I, control (only filtered water) (n = 20); group II, filtered water adding 1mM of methimazole (MZ) (n = 20); and group III, 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 (MZ+T4). Fishes were exposed to the treatments for 21 days according previous experiments and available 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, we added the same amount of DMSO in all the tanks. In order to avoid problems with water quality, water was renewed every day. Temperature of the room was set to maintain 28°C and fishes were fed twice a day using a commercial food (Zeigler®).

Sampling

Fishes were dissected out and the liver and some testes (n = 6 for each treatment) were snapfrozen at -80°C for gene expression analysis (see below). A fraction of testes was also fixed in a glutaraldehyde solution for morphology evaluation (n = 6), while another fraction was kept in L-15 medium to proceed with caspase activity measurement (n = 6).

Morphological analysis

After the *in vivo* exposure, zebrafish testes (n=8) were fixed in 4% in Karnovsky at 4°C overnight, dehydrated, embedded in Technovit (7100) historesin, sectioned at 4µm thickness, and stained with toluidine blue, according conventional histological procedures. Following the procedure, the slides were evaluated and the area occupied by germ cell cysts (undifferentiated type A spermatogonia, type B spermatogonia, spermatocytes, spermatid and spermatozoa, lumen and apoptosis) was determined. The measuring was performed by counting the point located on histologic field. For that, 5 fields per slide (n = 8 slides in each treatment) will be quantified using immersion objective from reticulum of 540 points (54x10).

Spermatozoa number was also quantifying and 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>) and for quantification of spermatozoa number. 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 your 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.

Gene expression analysis by real-time quantitative PCR (qPCR)

Total RNA was extracted from the testes using Trizol method, following manufacturer's instructions. To estimate 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 will be determined in 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 (*thra* 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. We also evaluated germ cell markers, such as *nanos2*, *dazl*, *shippo1* and *sycp3*.

Caspase-3 assay

Apoptosis measurement followed procedure adopted by Soverchia et al. (2007). Samples were rinsed with PBS and homogenized in 250 µl extraction/substrate caspase buffer (50 mM Pipes-KOH pH 7.4, 220 mM mannitol, 68 mM sucrose, 50 mM KCl, 5 mM EGTA, 1 mM MgCl₂, 1 mM DTT, 10 µg/mL proteinase inhibitor phenylmethylsulfonyl fluoride) for 30 min on ice. Samples were frozen at -80°C for 3-4h at least. After thawing, samples were spun at 14,000 g at 4°C for 15 min and the supernatants were assessed for caspase-3 activity. Total protein content in tissue samples was determined by the Bradford assay (Bio-Rad Laboratories, Milan, Italy) at 595 nm, using BSA as standard. Fifty-microliter lysates or 20-µg protein tissue samples were used per duplicate in 96-well plates. Caspase-3 colorimetric substrate (final concentration 2 mM) and extraction/substrate buffer was added to a final volume of 100 µl/well and incubated at 37°C for 2h, reading immediately in a plate reader at 405 nm. Results were expressed as caspase-3 activity (mg of product/mg total protein/h for tissue samples) after correction for protein content in the sample and incubation time.

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 were performed by Graph Pad Prism software 4.0 (Graph Pad Software, Inc., San Diego, CA, USA, <http://www.graphpad.com>).

RESULTS AND DISCUSSION

In vitro long-term effects of thyroid hormones on zebrafish testicular gene expression

When analyzing the germ cell markers, *nanos2* mRNA expression was significantly increased at the level of 100 nM/mL for T3, and interestingly, reduced in the level of 1000 nM/mL (Figure 1A). *sycp3* (Synaptonemal complex) expression is high at the level of 100 nM (Figure 1C), while *piwil1* and *shippo1* remained unaltered in all treatments (data not shown). From these graphs it can be inferred that TH regulates mRNA expression of type A undifferentiated spermatogonia (*nanos2*), as well is stimulates the transcription of synaptonemal complex gene (*sycp3*), a meiosis-related gene. T4 treatments (0, 10, 100 and 1000 nM/mL) were similar in all genes evaluated (Figure 1A-D).

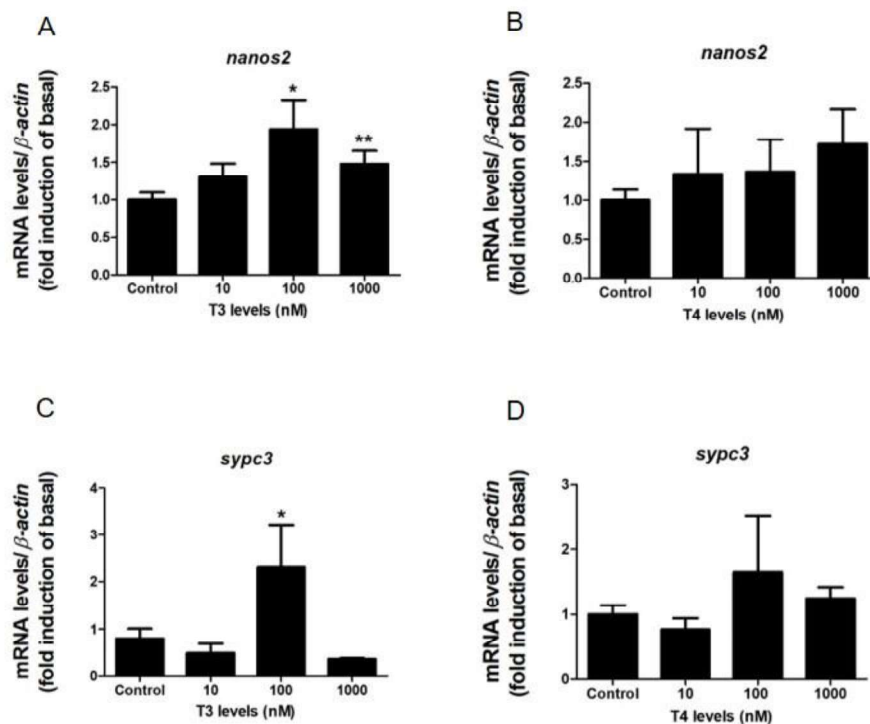


Figure 1 – Relative mRNA levels of germ cell markers (*nanos2*, *sycp3*) from zebrafish testes incubated *in vitro* using different T3 and T4 levels, cultured for 7 days. Bars represent the mean $\pm$ standard error of relative mRNA values normalized using β -actin. (*, when $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$).

In vitro effects shown that T3 stimulated mRNA expression of *nanos2*, which is a germ cell marker for type A undifferentiated of spermatogonia (Figure 1A). Morais et al. (2013) using similar dosage (50 ng/mL) reports increase of type A undifferentiated spermatogonia, which was corroborated by higher BrdU mitotic index of this cell type in T3-treated tissue. Intriguingly, T3 also stimulated *sycp3* expression (100 nM/mL). On other hand, this is different from the data presented by Morais et al. (2013), which reports a decrease of spermatid population.

mRNA levels of 3 β -HSD, *cyp17a1*, were also measured and did not change within any level of both T3 and T4 treatments (Figure 2A-D), as well as *amh* and *igf3* (data not shown). This could imply that *in vitro* effects of TH are not mediated by Sertoli and Leydig cells in zebrafish.

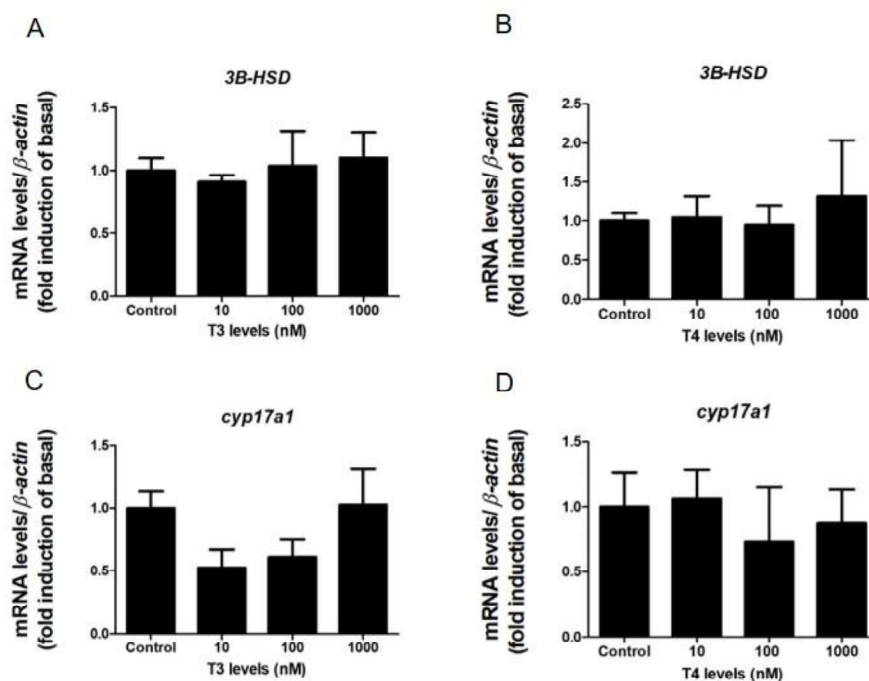


Figure 2 – Relative mRNA levels of steroidogenic genes (*3B-HSD* and *cyp17a1*) in zebrafish testes incubated *in vitro* using different T3 and T4 levels, cultured for seven days. Bars represent the mean $\pm$ standard error of relative mRNA values normalized using β -actin.

In vitro long-term effects of co-treatment of thyroid hormone and Fsh on zebrafish testicular gene expression

When the recombinant zebrafish gonadotropin Fsh is co-treated with T3, TH has potentiated the Fsh-induced gene expression of *cyp17a1* level (Figure 3B). Fsh is a potent steroidogenic gonadotropin in zebrafish testis (Nóbrega et al., 2015). TH alone does not affect steroidogenic genes (*3 β -HSD* and *cyp17a1*) (Figure 3A and B), however when combined with Fsh, TH potentiates the effects of Fsh in zebrafish testis, as seen in *cyp17a1* gene expression (Figure 3B). Our results are in agreement with previous study reported by Morais et al. (2013), when T3 also potentiated FSH action in zebrafish testis, by stimulating *cyp17a1* and *igf3* expression (Figure 3B and D). Interestingly, another finding showing the crossover between TH and FSH is the up-regulation of FSH receptor in the animals treated with methimazole and T4 (see below, Figure 7C).

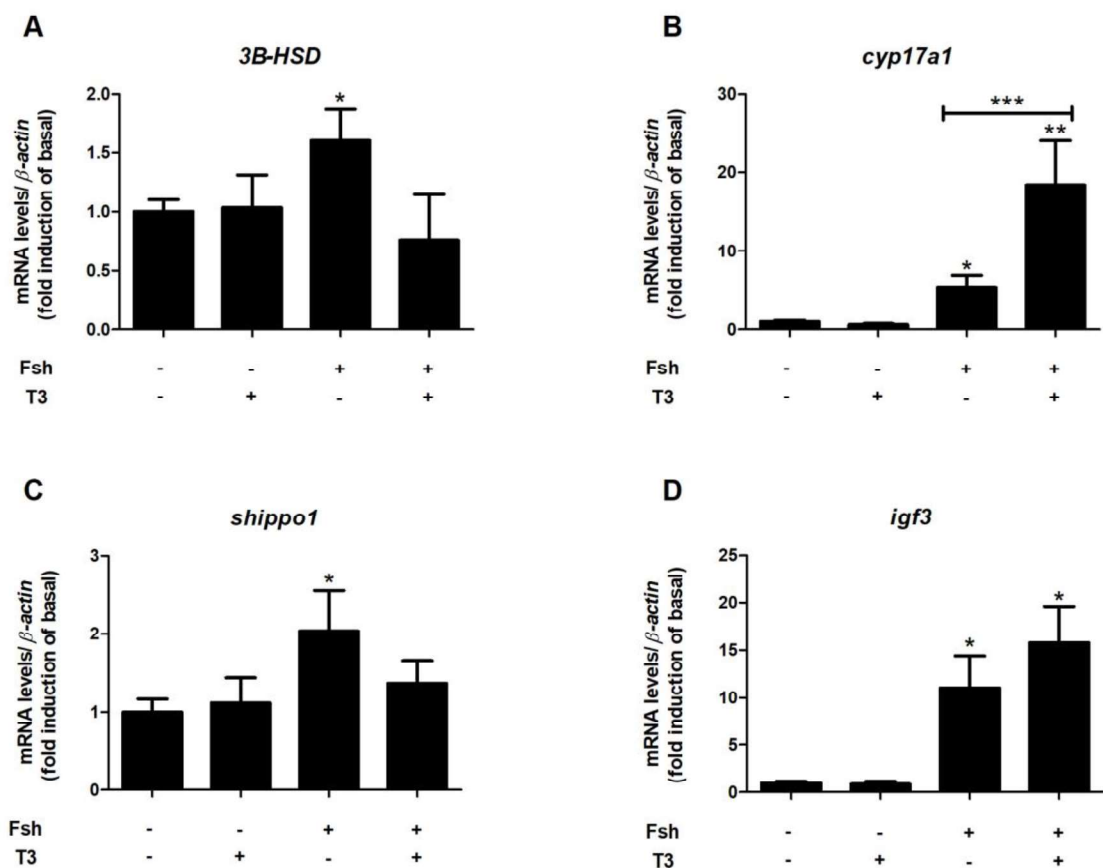


Figure 3 – Relative mRNA levels of steroidogenic genes (*3 β -HSD* and *cyp17a1*) and *shippo1* and *igf3* from zebrafish testes incubated in vitro using T3 (100ng/mL) and Fsh (100ng/mL) cultured for seven days. Bars represent the mean $\pm$ standard error of relative mRNA values normalized using β -actin. (*, when $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$).

In vivo effects of methimazole-induced hypothyroidism: morphological quantification

In vivo exposure to methimazole impaired zebrafish spermatogenesis (Figures 4, 5 and 6), which reduced significantly spermatozoa number. Methimazole-induced increased area occupied by types A_{und}* and B spermatogonias (Figure 4), whereas spermatozoa and lumen area decreased significantly (Figure 4). Interestingly, the area occupied by apoptosis were also measured and it increased significantly (Figure 4). This could indicate that spermiogenesis process was compromised, and as consequence the differentiation of type B spermatogonia into meiotic cells can be reduced, which could explain the large area occupied by type B spermatogonia in the methimazole group (Figure 4).

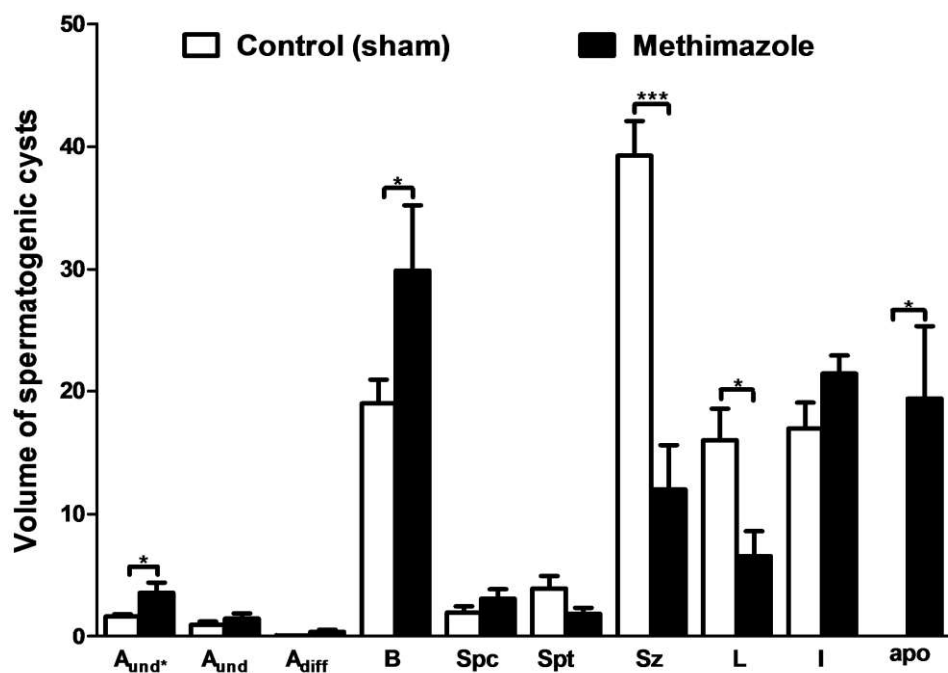


Figure 4 – Measurement of the area occupied by different germ cell types (type A undifferentiated spermatogonia, type A differentiated, type B spermatogonia, spermatocytes, spermatid and spermatozoa, lumen, interstice and apoptosis) from zebrafish testes after *in vivo* exposure to methimazole for 21 days. The frequency was determined by quantification of 50 different fields. (*, when $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$).

Spermatozoa quantification shows, in agreement with morphological evaluation of area, that spermatozoa population decreased significantly after methimazole exposure (Figures 5 and 6). As consequence of spermatozoa reduction, the testicular lumen is clearly decreased, as shown by morphological data (Figura 6).

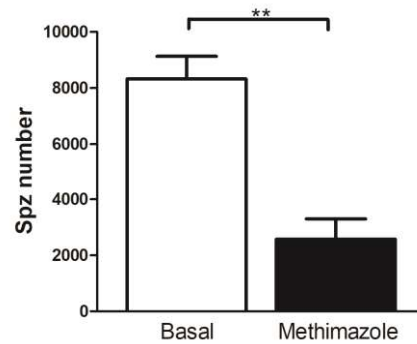


Figure 5 – Spermatozoa quantification by using IMAGEJ Software. Picture were taken from objective of 100x screening throughout the tissue quantifying 20 fields in every testis.

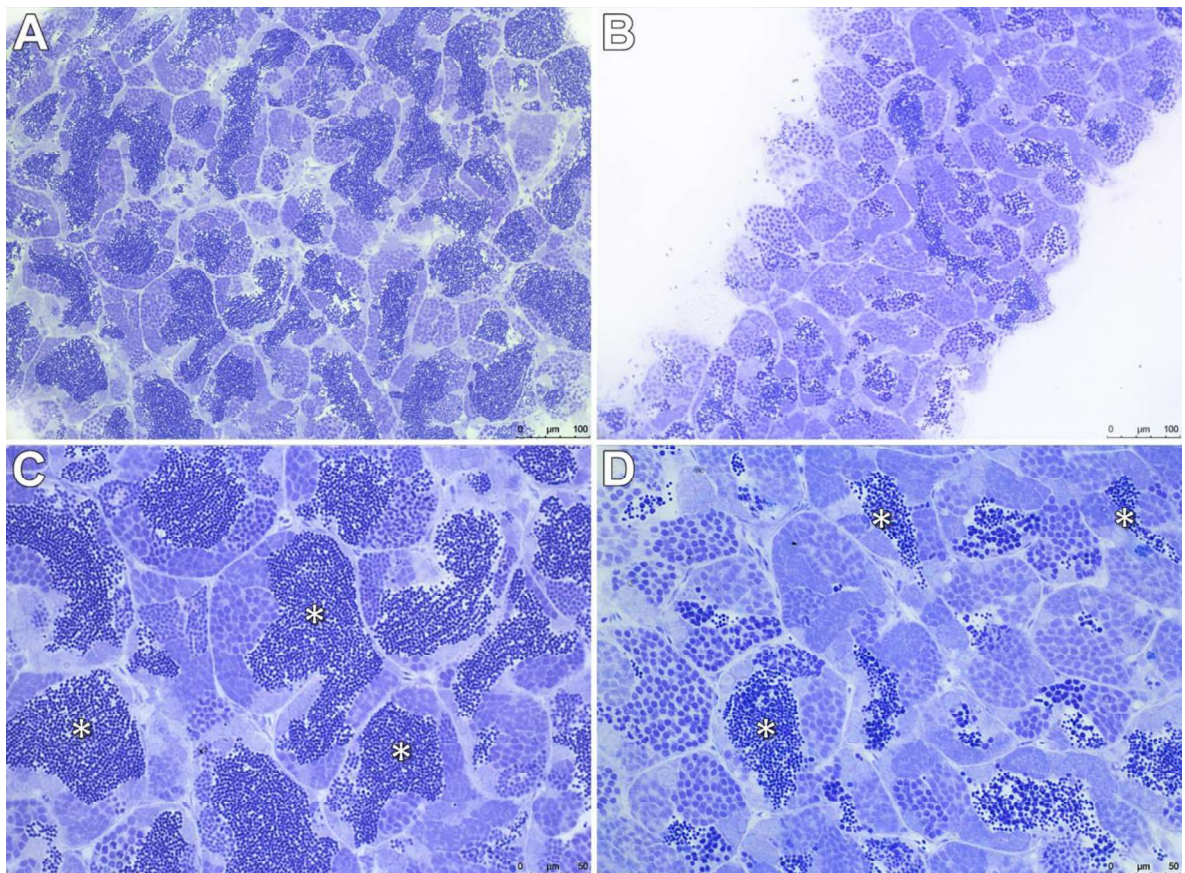


Figure 6 – Morphological sections from zebrafish testis after *in vivo* exposure to methimazole for 21 days. A and C: control group in the lens of 40 and 100x, respectively; B and D: methimazole treated group (MZ). * means the area occupied by the lumen.

In vivo effects of methimazole-induced hypothyroidism: gene expression analysis

Thyroid receptors (*thra* and *thrβ*) had their mRNA levels increased when T4 was added to methimazole treatment (MZ+T4) (Figure 7A and B). However the T4-induced thyroid hormone receptor expression was specific only for *thra* (Figure 7A); *thrβ* transcripts were elevated in both groups MZ and MZ+T4 (Figure 7B). In zebrafish, *thra* is expressed by Sertoli and Leydig cells, while *thrβ* is expressed only by Leydig cells (Morais et al., 2013).

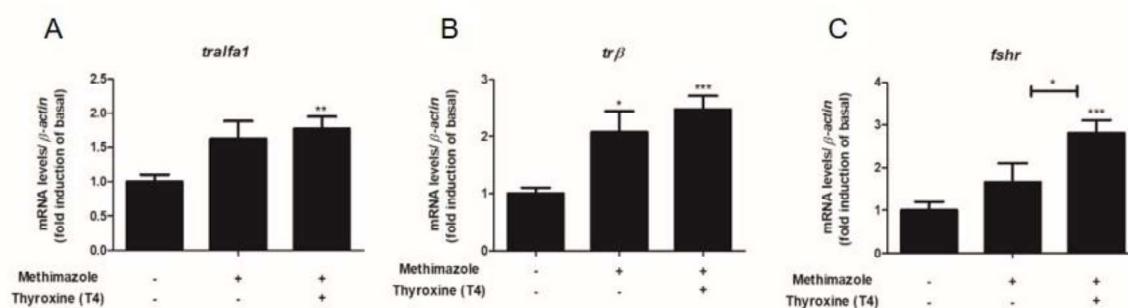


Figure 7 – Relative mRNA levels thyroid receptors and Fsh receptor in *thr* zebrafish testes after *in vivo* exposure to methimazole (MZ) and methimazole + T4 (MZ+T4) for 21 days. Bars represent the mean $\pm$ standard error of relative mRNA values normalized using β -actin. (*, when $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$).

The Fsh receptor (*fshr*) gene expression was higher in the treatment of MZ+T4 (Figure 7C, $p \leq 0.001$). This is an interesting data because it shows the crosstalk between the reproductive and thyroid axis; the addition of T4 in a hypothyroid-induced condition increased the sensibility to Fsh hormone in the testis. Morais et al. (2013) reported interaction between reproductive and thyroid axis, where T3 potentiated the effects of Fsh on androgen release in the culture medium by zebrafish testis explants. Therefore, it can be suggested that thyroid hormones potentiate the Fsh effects by increasing Fsh receptors in the testis.

The genes expressed by Leydig cells, *cyp17a1* and *insl3*, had similar expression trend, showing reduction of mRNA levels in methimazole-treated ($p \leq 0.001$) and methimazole co-treated with T4 (MZ+T4) ($p \leq 0.01$) groups (Figures 8A and B). In the thyroid hormones absence (methimazole-treated), it seems that the

tissue reduces the steroidogenic functions, which could be correlated to the histomorphometrical analysis, where spermatozoa has reduced in the hypothyroid-treated (Figure 6B and 6D) when compared to the basal group (Figure 6A and 6C).

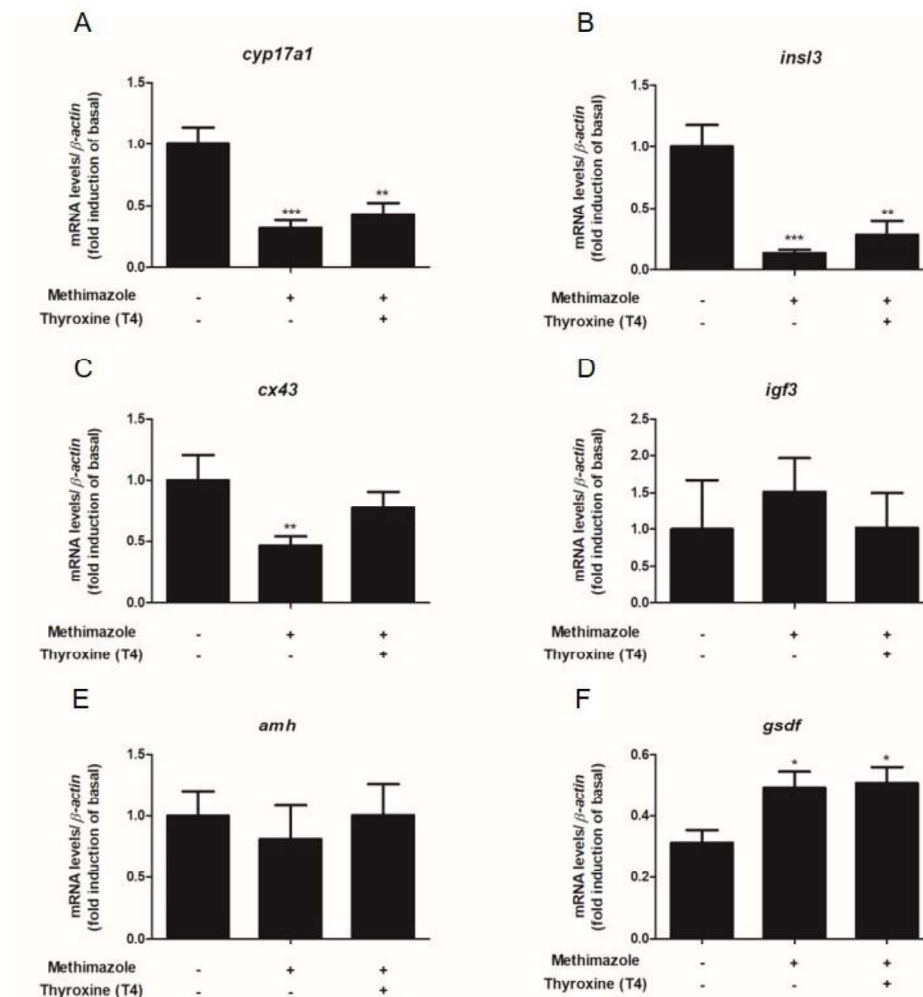


Figure 8 – Relative mRNA levels of selected genes expressed by somatic cells in zebrafish testes after *in vivo* exposure to methimazole and methimazole + T4 for 21 days. Bars represent the mean $\pm$ standard error of relative mRNA values normalized using β -actin. (*, when $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$).

The Sertoli cell related genes (*amh* and *igf3*) showed no alteration in both treatments (Figures 8D and E). However, the *gsdf* mRNA level increased in both treatments, MZ and MZ+T4 ($p \leq 0.05$) (Figure 8F). Connexin level (*cx43*) was affected by methimazole and decreased significantly, while when T4 was co-treated with methimazole this effect disappeared (Figure 8C).

Another marker for Sertoli cells differentiation/ maturation in mammals is the expression of connexins (CXs) (Wagner et al., 2008). Gilleron et al. (2006) demonstrated that T3 increased mRNA levels of CX43, an important gap junctional protein that participates in the control of cell proliferation. In the case of fishes - 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 may be involved in maturation of trout testis because its expression levels increased during the onset of spermatogenesis (Morais et al., 2013). Here we have identified a decreasing of mRNA levels of *cx43* in the hypothyroidism-induced condition (Figure 8C).

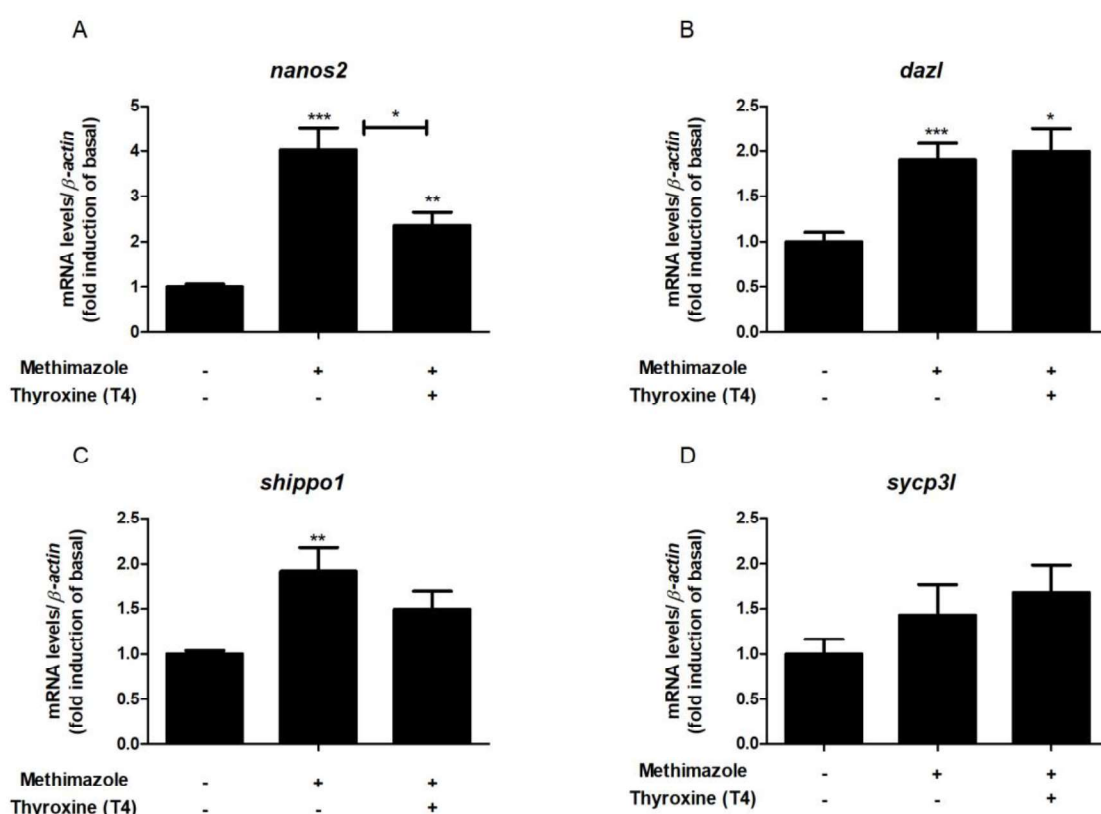


Figure 9 – Relative mRNA levels of selected germ cell markers in zebrafish testes after *in vivo* exposure to methimazole and methimazole + T4 for 21 days. Bars represent the mean $\pm$ standard error of relative mRNA values normalized using β -actin. (*, when $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$).

Regarding the germ cell genes, *nanos2* was increased by MZ treatment ($p \leq 0.001$), while MZ + T4 also showed increased mRNA levels of *nanos2* which was significantly different from control and from MZ treatments (Figure 9A). Interestingly, the increased *nanos2* (marker of type A undifferentiated spermatogonia, A_{und}) in MZ group is similar with the histomorphometrical analysis which showed a large area occupied by A_{und} (Figures 6B and D). This result may be as an accumulation of A_{und} in consequence of the impairment of germ cell differentiation (from B into meiotic). The *dazl* marker (Figure 9B), which is expressed by type B of spermatogonia, also increased in both treatments, MZ and MZ+T4 ($p \leq 0.01$ and $p \leq 0.001$, respectively compared to control group). This might indicate that initial cell types (A and B of spermatogonia) ratios is increased as a result of the impairment of late spermatogenic phases, which is consistent with *shippo1* increase in MZ treatment (Figure 9C).

In vivo effects of methimazole-induced hypothyroidism: caspase activity

As a result of this possible block of late phases of spermatogenesis, we suspected of germ cell apoptosis associated with this impairment of spermatogenesis. For this purpose, apoptosis was determined by caspase 3 activity measurement by fluorescence. The results showed similar levels of caspase 3 activity in all groups (Figure 10).

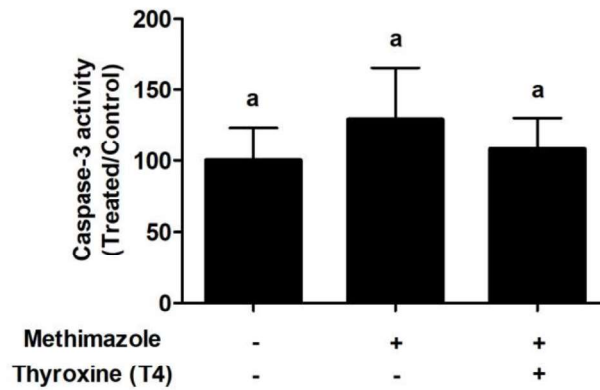


Figure 10 – Caspase-3 activity after *in vivo* exposure to methimazole and methimazole + T4 for 21 days.

CONCLUSION

T3 affects directly zebrafish testis by increasing *nanos2* expression and *sycpl3* *in vitro*, while T4 did not alter gene expression of any evaluated gene. Moreover, methimazole increased the area occupied by the undifferentiated type A spermatogonia and type B spermatogonia, while spermatozoa and the lumen area were reduced significantly, associated with apoptosis. Overall our data shows that thyroid absence induced by methimazole impairs zebrafish spermatogenesis by modulating Sertoli and Leydig cells functions.

REFERENCES

- Ariyaratne, H.B., Mills, N., Mason, J.I., Mendis-Handgma, S.M., 2000. Effects of thyroid hormone on Leydig cell regeneration in the adult rat following ethane dimethane sulphonate treatment. *Biology of Reproduction* 63, 1115-1123.
- Cooke, P.S., 1991. Thyroid hormones and testis development: a model system for increasing testis growth and sperm production. *Annals of the New York Academy of Sciences* 637, 122–132.
- Cooke, P.S., Hess, R.A., Kirby, J.D., 1994a. A model system for increasing testis size and sperm production: potential application to animal science. *Journal of Animal Science* 72, 43-54.
- Cooke, P.S., Zhao, Y.D., Bunick, D., 1994b. 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. *Biology of Reproduction* 51, 1000–1005.
- França, L.R., Hess, C.A., Cooke, P.S., Russel, L.D., 1995. Neonatal hypothyroidism causes delayed Sertoli cell maturation in rats treated with propylthiouracil: evidence that the Sertoli cell controls testis growth. *The Anatomical Record* 242, 57-69
- Francavilla, S., Cordeschi, G., Properzi, G., Di Cicco, L., Jannini, E.A., Palmero, S., Fugassa, E., Loras, B., D'Armiento, M., 1991. Effect of thyroid hormone on the pre- and post-natal development of the rat testis. *Journal of Endocrinology* 129, 35-42.
- Gilleron, J., Nebout, M., Scarabelli, L., Senega-Balas, F., Palmero, S., Segretain, D., Pointis, G., 2006. 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.
- van Haaster, L.H., de Jong, F.H., Docter, R., de Rooji, D.G., 1993. 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
- Krassas, G.E., Papadopoulou, F., Tziomalos, K., Zeginiadou, T., Pontikides, N., 2008. Hypothyroidism Has an Adverse Effect on Human Spermatogenesis: A Prospective, Controlled Study. *Thyroid* 18, 1255-1259.

Leal, M.C., De Waal, P.P., García-López, A., Chen, S.X., Bogerd, J., Schulz, R.W., 2009. Zebrafish primary testis tissue culture: An approach to study testis function *ex vivo*. *General and Comparative Endocrinology* 162, 134-138.

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

Matta, S.L.P., Vilela, D.A.R., Godinho, H.P., França, L.R., 2002. The Goitrogen 6-*n*-Propyl-2-Thiouracil (PTU) Given during Testis Development Increases Sertoli and Germ Cell Number per Cyst in Fish: The tilapia (*Oreochromis niloticus*) Model. *Endocrinology* 143, 970-978.

de Montgolfier, B., Dufresne, J., Letourneau, M., Nagler, J.J., Fournier, A., Audet, C., Cyr, D.G., 2007. 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.

de Montgolfier, B., Faye, A., Audet, C., Cyr, D.G., 2009. Seasonal variations in testicular connexins levels and their regulation in the brook trout, *Salvelinus fontinalis*. *General and Comparative Endocrinology* 162, 276-285.

Morais, R.D.V.S., Nóbrega, R.H., Gómez-González, N.E., Schmidt, R., Bogerd, J., França, L.R., Schulz, R.W., 2013. Thyroid Hormone Stimulates the Proliferation of Sertoli Cells and Single Type A Spermatogonia in Adult Zebrafish (*Danio rerio*). *Endocrinology* 154, 4365-4376.

Mullur, R., Liu, Y.Y., BRENT, G.A. 2014. Thyroid hormone regulation of metabolism. *Physiological Reviews* 94, 355-82.

Nóbrega, R.H., Morais, R.D.V.S., Crespo, D., De Waal, P.P., França, L.R., Schulz, R.W., Bogerd, J., 2015. Fsh Stimulates Spermatogonial Proliferation and Differentiation in Zebrafish via Igf3. *Endocrinology* 156, 3804-3817.

Sharma, P., Patiño, R., 2013. Regulation of gonadal sex ratios and pubertal development by the thyroid endocrine system in zebrafish. *General and Comparative Endocrinology* 184, 111–119.

Sharma, P., Tang, S., Mayer, G.D., Patiño, R., 2016. Effects of thyroid endocrine manipulation on sex-related gene expression and population sex ratios in Zebrafish. *General and Comparative Endocrinology* 235, 38-47.

Soverchia, L., Carotti, M., Andreu-Vieyra, C., Mosconi, G., Cannella, N., Habibi, H., Polzonetti-Magni, A.M., 2007. Role of gonadotropin-releasing hormone

(GnRH) in the regulation of gonadal differentiation in the gilthead seabream (*Sparus aurata*). *Molecular Reproduction and Development* 74, 57-67.

Stradtman, E.W., 1993. Thyroid dysfunction and ovulatory disorders. *In*: Carr, B.R., Blackwell, R.E. (Ed), *Textbook of Reproductive Medicine*. CT Appleton, Norwalk, pp.297-321.

Swapna, I., Rajasekhar, M., Supriya, A., Raghuvver, K., Sreenivasulu, G., Rasheeda, M.K., Majumdar, K.C., Kagawa, H., Tanaka, H., Dutta-Gupta, A., Senthilkumaran, B., 2006. Thiourea-induced thyroid hormone depletion impairs testicular recrudescence in the air-breathing catfish, *Clarias gariepinus*. *Comparative Biochemistry and Physiology* 144, 1-10.

Teerds, K.J., De Rooji, D.G., De Jong, F.H., Van Haaster, L.H., 1998. Development of the adult-type Leydig cell population in the rat is affected by neonatal thyroid hormone levels. *Biology of Reproduction* 59, 344-350.

Tovo-Neto, A., Rodrigues, M.S., Habibi, H.R., Nóbrega, R.H., 2018. Thyroid hormone actions on male reproductive system of teleost fish. *General Comparative Endocrinology*, In Press

Wagner, M.S., Wajner, S.M., Maia, A.L., 2008. The role of thyroid hormone in testicular development and function. *Journal of Endocrinology* 199, 351–365.

de Waal, P.P., Wang, D.S., Nijenhuis, W.A., Schulz, R.W., Bogerd, J., 2008. Functional characterization and expression analysis of the androgen receptor in zebrafish (*Danio rerio*) testis. *Reproduction* 136, 225-234.

General Conclusion

The role of cortisol and THs in zebrafish spermatogenesis was investigated through *in vitro* and *in vivo* studies. It has been clear that cortisol has a positive role in zebrafish spermatogenesis by stimulating germ cells differentiation and increased the number of free spermatozoa in the lumen, in an androgen independent manner.

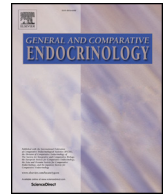
Regarding the THs, *in vitro* studies showed that T3 affected germ cells related genes, while T4 did not alter any gene evaluated. Moreover, *in vivo* studies showed that methimazole increased the area occupied by the type A undifferentiated spermatogonia and type B spermatogonia, while spermatozoa and the lumen area were reduced significantly, which suggests that hypothyroidism-induced impairs meiosis, and modulates the mRNA expression of Sertoli and Leydig cells genes.

Appendix



Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

Thyroid hormone actions on male reproductive system of teleost fish

Aldo Tovo-Neto^{a,c,d}, Maira da Silva Rodrigues^{c,d}, Hamid R. Habibi^{a,b,*},
Rafael Henrique Nóbrega^{d,*}

^a Department of Biological Sciences, University of Calgary, Calgary, Alberta, Canada^b Physiology and Pharmacology, University of Calgary, Calgary, Alberta, Canada^c Aquaculture Program, São Paulo State University (UNESP), Jaboticabal, São Paulo, Brazil^d Department of Morphology, Reproductive and Molecular Biology Group, São Paulo State University (UNESP), Botucatu, São Paulo, Brazil

ARTICLE INFO

Keywords:

Thyroid hormones
Sex differentiation
Testicular development
Adult testis
Reproduction
Spermatogenesis
Teleost fish

ABSTRACT

Thyroid hormones (THs) play important roles in the regulation of many biological processes of vertebrates, such as growth, metabolism, morphogenesis and reproduction. An increasing number of studies have been focused on the involvement of THs in the male reproductive system of vertebrates, in particular of fish. Therefore, this mini-review aims to summarize the main findings on THs role in male reproductive system of fish, focusing on sex differentiation, testicular development and spermatogenesis. The existing data in the literature have demonstrated that THs exert their roles at the different levels of the hypothalamic-pituitary-gonadal (HPG) axis. In general a positive correlation has been shown between THs and fish reproductive status; where THs are associated with testicular development, growth and maturation. Recently, the molecular mechanisms underlying the role of THs in spermatogenesis have been unraveled in zebrafish testis. THs promote germ cell proliferation and differentiation by increasing a stimulatory growth factor of spermatogenesis produced by Sertoli cells. In addition, THs enhanced the gonadotropin-induced androgen release in zebrafish testis. Next to their functions in the adult testis, THs are involved in the gonadal sex differentiation through modulating sex-related gene expression, and testicular development via regulation of Sertoli cell proliferation. In conclusion, this mini-review showed that THs modulate the male reproductive system during the different life stages of fish. The physiological and molecular mechanisms showed a link between the thyroid and reproduction, suggesting a possibly co-evolution and interdependence of these two systems.

1. Introduction

Thyroid hormones (THs) have been known to be involved in many biological processes, such as growth, morphogenesis, skin pigmentation, osmoregulation, and reproduction (Blanton and Specker, 2007). An increasing number of studies has shown that THs exert an important role in male reproductive system of vertebrates. In the past two decades, experimental and clinical studies have shown the involvement of THs in the testicular development and function in mammals (Wagner et al., 2008). Similar functions were reported in other vertebrates, such

as fish, which is the focus of this mini-review. The interaction between THs and fish reproductive system was reviewed before by Cyr and Eales (1996), Blanton and Specker (2007), Habibi et al. (2012) and Flood et al. (2013). While there are significant variations in the nature of interaction between THs and reproduction in different fish species, it would appear that THs may exert stimulatory and/or inhibitory actions by different mechanisms (Habibi et al., 2012). In this context, this mini-review will address some of the direct and indirect actions of THs in male reproductive system of teleost fish, highlighting the main findings on sex differentiation, testicular development and adult testis.

Abbreviations: TH, Thyroid hormone; THs, Thyroid hormones; T₃, 3,5,3'-L-triiodothyronine; HPT, Hypothalamic-pituitary-thyroid axis; HPG, Hypothalamic-pituitary-gonadal axis; CRH, Corticotropin-releasing hormone; CRF, corticotropin-releasing factor; GnIH, Gonadotropin inhibitory hormone; GnRH, Gonadotropin releasing hormone; DIOs, Deiodinases; T₄, Thyroxine; rT₃, Reverse T₃; TRs, Thyroid hormone receptors; TREs, Thyroid response elements; *thr*, Thyroid hormone receptor; Tsh, Thyroid-stimulating hormone; Fsh, Follicle-stimulating hormone; Lh, Luteinizing hormone; GPCR, G-protein coupled receptor; Fshr, Follicle-stimulating hormone receptor; Lhr, Luteinizing hormone receptor; ERbeta, Estrogen receptor beta-isoform; *ar*, Androgen receptor; *srd5alpha1*, Steroid 5α-reductase 1; *srd5alpha2*, Steroid 5α-reductase 2; TR, Thyroid hormone receptor; TRA/α, Thyroid hormone receptor alpha; TRB/β, Thyroid hormone receptor beta; *cyp19a1*, Cytochrome P450 aromatase; *amh*, Anti-Müllerian hormone; PTU, Propylthiouracil or 6-n-propylthiouracil; 11-KT, 11-Ketotestosterone; T, Testosterone; A_{und}, Type A undifferentiated spermatogonia; A_{diff}, Type A differentiated spermatogonia; *igf3*, Insulin-like growth factor 3; IGF, Insulin-like growth factor; *igfbp1a*, Insulin-like growth factor binding protein 1; IGFBP, Insulin-like growth factor-binding protein; *cyp17a1*, 17α-hydroxylase; *cx43*, Connexin 43; *erb*, Estrogen receptor beta

* Corresponding authors at: Department of Morphology, Reproductive and Molecular Biology Group, São Paulo State University (UNESP), Botucatu, São Paulo, Brazil; and Department of Biological Sciences, University of Calgary, Calgary, Alberta, Canada.

E-mail address: nobregarh@ibb.unesp.br (R.H. Nóbrega).

<https://doi.org/10.1016/j.ygcen.2018.04.023>

Received 1 December 2017; Received in revised form 16 April 2018; Accepted 16 April 2018
0016-6480/ © 2018 Elsevier Inc. All rights reserved.

Please cite this article as: Tovo-Neto, A., General and Comparative Endocrinology (2018), <https://doi.org/10.1016/j.ygcen.2018.04.023>

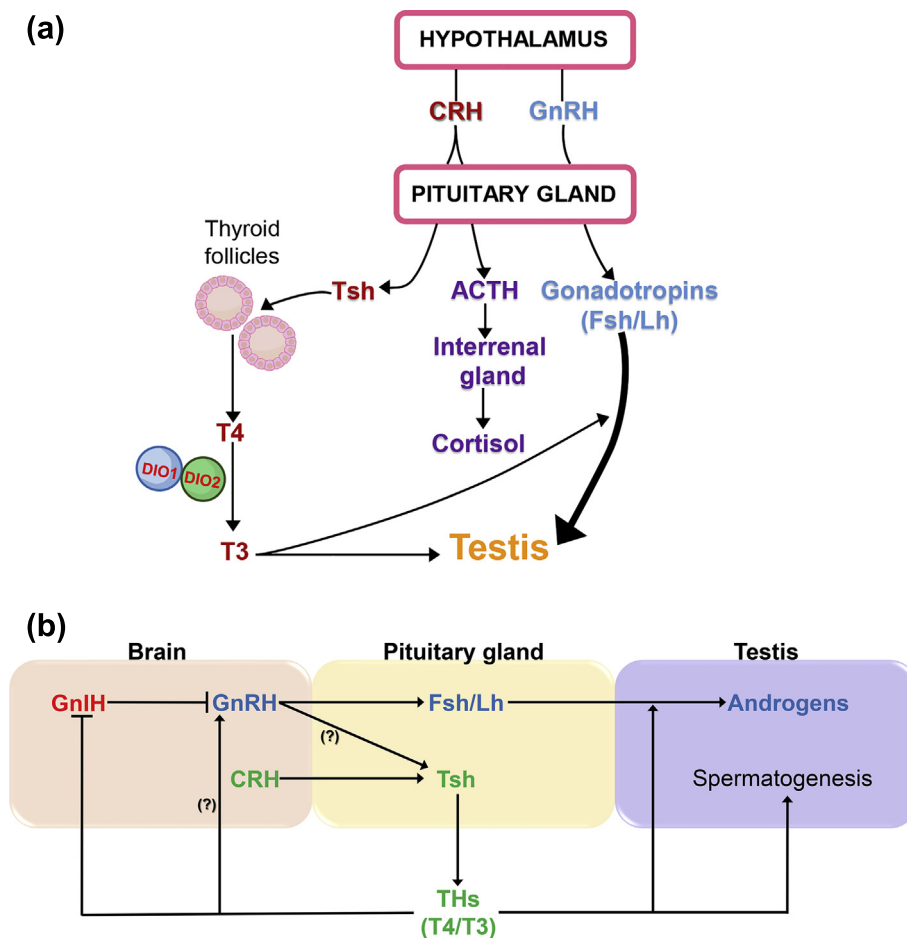


Fig. 1. A The hypothalamic-pituitary-thyroid, -interrenal, and -testicular axes in fish. The pituitary secretion of Tsh (thyroid-stimulating hormone) seems to be dependent on corticotropin-releasing hormone (CRH), similarly as observed in amphibians, reptiles and birds. CRH is also involved in the pituitary secretion of ACTH (adrenocorticotropic hormone), which stimulates the synthesis and secretion of cortisol by the interrenal gland. In the thyroid follicles, which are dispersed in fish, Tsh stimulates the production of T4, which is the main TH secreted. Two types of deiodinase (DIO1 and DIO2) convert T4 into T3 in the extrathyroidal tissues. T3, the active TH form, exerts its role in the testis, which are generally associated with testicular development, growth and maturation. Recent studies have shown that T3 enhanced the gonadotropins (Fsh, follicle-stimulating hormone; Lh, luteinizing hormone) function in the testis (enlarged arrow). B. The main effects of thyroid hormones (THs) in the hypothalamic-pituitary-testicular axis. In mammals, THs inhibit the synthesis of GnRH (Gonadotropin-releasing hormone) (Tsutsui et al., 2018). In tilapia, T3 suppressed the concentration of terminal nerve salmon GnRH mRNA in sexually immature males (Parhar et al., 2000). Other studies have demonstrated that GnRH increased Tsh (thyroid-stimulating hormone) and thyroid function in some species of fish and amphibians (Jacobs et al., 1988a,b; Roy et al., 2000; Chiba et al., 2004). In the testis, T3 stimulates spermatogenesis and enhances the gonadotropin-induced androgens synthesis and release. The question mark (?) indicates controversial data in the literature with respect to the reciprocal regulatory role between GnRH and thyroid function. CRH, corticotropin-releasing hormone; Fsh, follicle stimulating hormone; Lh, Luteinizing hormone.

2. Hypothalamic-pituitary-thyroid axis

Thyroid function is activated by the hypothalamic-pituitary-thyroid axis (HPT), which has been reviewed in many vertebrates, including fish (Fig. 1A,B) (Brown and Cai, 2007; Blanton and Specker 2007; and Carr and Patiño, 2011). A particular characteristic of the HPT in fish is the non-involvement of thyrotropin releasing hormone (TRH) as occurs in mammals (Larsen et al., 1998). On the other hand, the pituitary secretion of TSH (Thyroid-stimulating hormone) seems to be dependent on corticotropin-releasing hormone (CRH, or known as corticotropin-releasing factor – CRF) (Fig. 1A,B) (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. In fish, the thyroid follicles are not encapsulated in an organ, but they 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) (Fig. 1A; Supplemental Fig. 1) (for review see Orozco et al., 2012).

The active form (T3) regulates nuclear gene expression by binding to the TH receptors (TRs), TR α and TR β . The TRs are encoded by *THRA* and *THRB* genes, which are transcribed as multiple mRNA isoforms. TR α 1, TR α 2, TR β 1 and TR β 3 are expressed widely whereas TR β 2 is predominantly restricted to the hypothalamic/pituitary axis, where it acts negatively to regulate TSH transcription (for a review see Basset et al., 2003). TRs act as transcription factors binding to TH response

elements (TREs) in the regulatory regions of target genes, activating or repressing their transcription (Basset et al., 2003). In anamniotes (fish and amphibians), TH receptor (*thr*) is differentially expressed between testes and ovaries; higher *thr* mRNA levels were found in testes than in ovaries of striped parrotfish *Scarus iseri* (Johnson and Lema, 2011) and tropical clawed frog *Silurana tropicalis* (Duarte-Guterman and Trudeau, 2011). In zebrafish testes, *thra* mRNA was detected in Sertoli cells, while *thr β* was detected in both Sertoli and Leydig cells (Morais et al., 2013). Interestingly, testicular *thra* and *thr β* mRNA levels in seasonally reproducing brook trout *Salvelinus fontinalis* are constant during the spermatogenesis, increasing their expression levels after the spawning season (de Montgolfier et al., 2009). The action of sex steroids on TRs is variable in different fish species. In goldfish, acute treatment with gonadal steroids had no effects on liver and gonadal *thra* and *thr β* mRNA in male and female fish, although developmentally-related changes in TRs expression occur in other fish species (Nelson and Habibi, 2009).

Another interesting aspect to be mentioned is the crossover of the HPT and the hypothalamic-pituitary-gonadal (HPG) axes (Castañeda Cortés et al., 2014) (Fig. 1B). 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,b). 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 (Fig. 1B) (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).

More recently, studies have been focused on GnIH (Gonadotropin inhibitory hormone), which is a newly discovered hypothalamic neuropeptide that inhibits the pituitary gonadotropin synthesis and release (Tsutsui et al., 2018). Interestingly, it has been shown that mammalian GnIH neurons express TR α and TR β (Tsutsui et al., 2018). In addition, 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 (Tsutsui et al., 2018). These data suggest a possible role of THs on regulating the GnIH synthesis and release in vertebrates (Fig. 1B).

3. Fish reproduction

The relationship between thyroid function and reproduction has been explored for many years in different species. In fish, it is established that reproductive strategies vary greatly among the species (Eales, 2006), and it has become clear that the nature of interaction between TH and reproduction is highly species-specific (Habibi et al., 2012). Thyroidal function usually increases during early gonadal development in fish, and it is maintained or enhanced during the period of reproduction, and commonly decreased during or after spawning (Cyr and Eales, 1996). Similar function is seen in reptiles, birds and mammals, where TH administration induced testicular growth in thyroidectomized or short photoperiod-induced males (Haldar-Misra and Thapliyal, 1981; Yoshimura et al., 2003; Freeman et al., 2007).

Temporal relationship between thyroidal and reproductive status has been examined in some seasonal species of fish, in particular, non-salmonids and salmonids (for review see Cyr and Eales, 1996). In non-salmonids, a general positive correlation between thyroid activity and reproductive status has been seen among the species. On the other hand, thyroid function in Salmonidae is high in the early stages of gonadal maturation but decreases as vitellogenesis and testis development proceed. However, T3 plasma levels are higher in males than

females just prior to spawning and may even increase (for review see Cyr and Eales, 1996). Table 1 summarizes selected data from the literature about the relationship between thyroid function and reproduction in non-mammalian and mammalian vertebrates. Therefore, the summarized data in the literature suggest that the basic function for THs is associated with gonadal maturation in fish as well as in other vertebrates.

4. Sex differentiation

THs have considerable influence on the sexual ontogeny of male vertebrates, through direct interactions with genes involved in sex determination and gonadal development along the HPG axis (Flood et al., 2013). The role of THs in the establishment of gonadal sex phenotype has been previously examined in amphibians and reptiles. For example, chronic treatment with TH synthesis inhibitors (perchlorate or thiourea) during larval development resulted in a feminization effect in *Xenopus laevis* (Goleman et al., 2002; Hayes, 1997). In another study, premetamorphic tadpoles (Nieuwkoop and Faber stage 52–54) were exposed to exogenous T3 for 48 h and resulted in a concentration-dependent decrease of estrogen receptor beta isoform (ERbeta) expression at concentrations of 5 and 50 nM T3. The expression of androgen-related genes also changed and significantly increased following treatment with T3 in relation to the control. Treatment with T3 also led to changes in *ar* (androgen receptor, 1.4–1.8 fold), *srd5alpha1* (steroid 5 α -reductase 1; 1.5–1.9 fold) and *srd5alpha2* (steroid 5 α -reductase 2; 1.8–3.9 fold) in tropical clawed frogs (Duarte-Guterman and Trudeau, 2011). In reptiles, hyperthyroid treatment decreased *cyp19a1* transcripts (gonadal aromatase), and consequently estradiol levels, inducing therefore more male offspring in a TSD (Temperature-dependent sex determination) turtle (*Trachemys scripta*) (Sun et al., 2016).

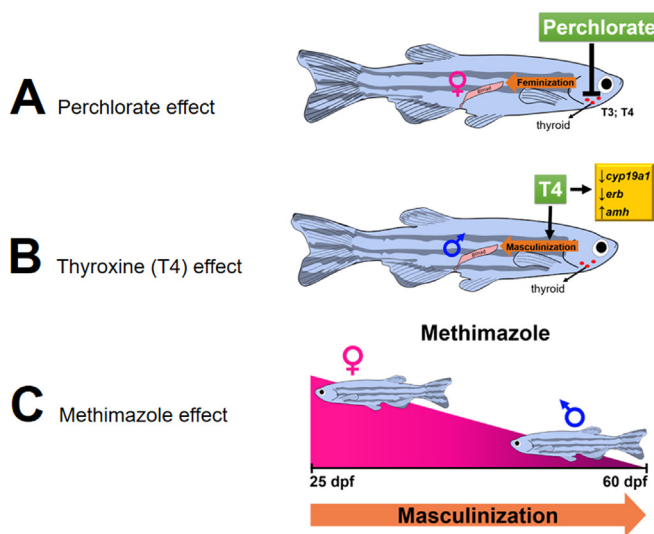
In fish and amphibians, an evidence that THs are involved in gonadal sex differentiation is the different expression of deiodinases and *thr* between males and females, as reported in striped parrotfish, *Scarus iseri* (Johnson and Lema, 2011) and tropical clawed frog *Silurana tropicalis* (Duarte-Guterman and Trudeau, 2011). In addition, An et al. (2010) observed a decrease in gonadal *thra* expression in protandrous

Table 1
Selected experimental results correlating thyroid status and reproduction in vertebrates.

Species	Treatment	Effects
Teleosts	Stimulated thyroid function	Coincides with sexual maturation
	Elevated TH plasma levels	Occurs during gonadal maturation
	Decreased TH plasma levels	Observed at spawning
	Goitrogen exposure	Gonadal atrophy in both sexes
	Presence of goiters in salmon from Great Lakes	Associated with low egg production; low gonadal steroid levels in blood
Amphibians	Exogenous TH*	No effect or mild enhancement of gonadal maturation
	Surgical or chemical thyroidectomy of frogs	Prevents ovulation
	Treatment with thyroxine of thyroidectomized frogs	Permits ovulation
	Treatment of anuran liver with TH	Enhances action of estrogen on vitellogenesis
	Thyroid function	Not associated with reproductive activities
Reptiles	Goitrogen treatment of tadpoles (anurans)	Prevents testicular development
	Elevated thyroxine in the blood of salamanders	Coincides with gonadal growth in males and females
	Thyroxine treatment	Atrophy of steroid secreting cells in testes
	Decreased plasma levels and thyroid function	Associated with peak reproductive and high androgen levels
	Elevated thyroxine levels in blood	Decreased androgen levels in males
Birds	TH treatment	Induced testicular growth of thyroidectomized
	Thyroidectomized males	Testicular regression
Mammals	Exogenous T3	Induced gonadal growth in short-day hamsters
	Exogenous T3	Delayed testicular regression in short photoperiod-induced males

Adapted from Norris (2006).

* TH, Thyroid Hormones.



Sex ratio



Fig. 2. Effects of thyroid hormones during sex differentiation of fish. (A) Treatment with perchlorate induces feminization in early gonadal development, skewing the sex ratio toward females. (B) Treatment with T4 induces masculinization in early gonadal development by reducing the expression of *cyp19a1* (aromatase) and *erb* (estrogen receptor beta) and increasing *amh* (anti-Müllerian hormone). T4 increases the ratio of females. (C) Methimazole treatment delays masculinization from females during the early developmental stage of zebrafish. Sex ratio is 1:1.

black porgy (*Acanthopagrus schlegelii*) following male-to-female sex reversal. During zebrafish metamorphosis and early gonadal development, treatment with perchlorate (a well-known thyroid synthesis inhibitor), significantly skewed the sex ratio toward females (Fig. 2A) (Mukhi et al., 2007). However, the co-treatment with exogenous T4 not only blocked the feminizing effect of perchlorate but also over-compensated by skewing the ratio toward males. In another study, Sharma and Patiño (2013) showed that either perchlorate or methimazole (an antithyroid drug) induced feminization in zebrafish larvae at 45 dpf (days post-fertilization). Interestingly, the feminizing effect was still present in the perchlorate treatment, but not anymore in methimazole treatment at 60 dpf, suggesting that the effect disappeared as fish regained the euthyroid conditions. The conclusion is that methimazole does not cause male-to-female sex reversal but simply delays the ovary-to-testis transformation in presumptive males of this species (Fig. 2C). It is worth emphasizing that studies using antithyroid drugs can provide a powerful tool for understanding TH function but only with an appropriate control due to the extrathyroidal effects these drugs can have. A rigorous study requires a follow up to determine if those effects can be nullified or reversed by simultaneous administration of sufficient TH to regain euthyroid condition.

Interestingly, and more recently, Sharma et al. (2016) evaluated the molecular mechanisms involved in T4-induced masculinization in zebrafish larvae. These authors demonstrated that T4 treatment decreases *cyp19a1* (aromatase) and estrogen receptor expression, while increasing expression of masculinizing transcripts, such as *amh* (anti-Müllerian hormone) and *ar* (androgen receptor) (Sharma et al., 2016) (Fig. 2B).

5. Testicular development

In rodents, THs affect Sertoli cell proliferation and differentiation (Fig. 3A), as well as the onset of adult Leydig cell formation and function during neonatal-prepubertal period (Teerds et al., 1998; Ariyaratne et al., 2000). Induced-hypothyroidism in the neonatal rat impaired testicular growth, germ cell maturation, seminiferous tubule formation and other differentiation processes in the testis (França et al., 1995; Maran et al., 2002). Induced-neonatal hypothyroidism has also been shown to increase Sertoli cell proliferative window, which consequently increased Sertoli number and the Sertoli cell support capacity for germ cells, reflecting directly on the elevated spermatogenic yield and testis size (Fig. 3A) (Cooke et al., 1991). Interestingly, the impairment caused by neonatal induced-hypothyroidism in rats was rescued with T3 (Cooke et al., 1991). On the other hand, hyperthyroidism led to

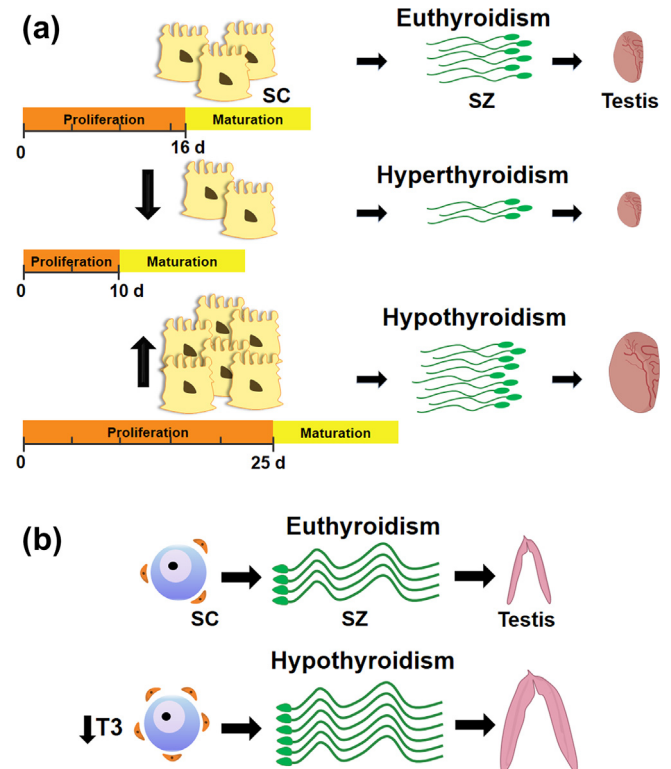


Fig. 3. (A) Effects of hypothyroidism and hyperthyroidism on testicular development in rodents. The induced-hyperthyroidism decreased the Sertoli cell proliferative window, reflecting directly on the final number of adult Sertoli cell, and consequently in the lower spermatogenic yield and testis size/weight. On the other hand, Sertoli cell differentiation is delayed in the hypothyroidism model, increasing their proliferative window. In consequence, there is an elevated number of adult Sertoli cell which leads to higher sperm production and testis size/weight. SC: Sertoli cell; SZ: spermatozoa. (B) Effects of hypothyroidism on testicular development in fish. PTU-induced hypothyroidism increased Sertoli cell proliferation and Sertoli cell capacity reflecting directly on higher number of germ cells per cyst. In this model, higher sperm production and testis weight are seen. SC: Sertoli cell; SZ: spermatozoa.

early cessation of Sertoli cell proliferation, and a decrease in testis size and sperm production in rats (Fig. 3A) (van Haaster et al., 1993; Cooke et al., 1994a,b; Mendis-Handagama and Ariyaratne, 2005).

There are few papers describing the effects of hypothyroid state on

testicular development in fish (see review in Schulz et al., 2010; França et al., 2015). According to Matta et al. (2002), Nile tilapia (*Oreochromis niloticus*) juveniles (~0.95 g body weight and 3.5 cm of length) treated with PTU (propylthiouracil, anti-thyroid drug similar to methimazole) doubled the size of testis which is similar to the effects observed in rats, suggesting that mechanisms controlling testicular development by THs may be conserved among vertebrates. In hypothyroid Nile tilapia, Sertoli cells remained immature and were able to proliferate for longer period during testis development, resulting in a higher number of Sertoli cell per cyst reflected positively on Sertoli cell capacity and efficiency and consequently in the germ cell number in adult tilapia (Fig. 3B) (Matta et al., 2002; França et al., 2015). Sharma and Patiño (2013) reported that goitrogen (e.g. perchlorate) exposure inhibited pubertal development in male zebrafish, as clearly seen at 60 dpf. In the same work, all control and T4-treated males were mature (> 75% of them showed sperm in their testes), while those that were goitrogen-treated were still juvenile and less than 50% had sperm. In contrast, Timmermans et al. (1997) observed no significant increase in body weight and testis diameter or number of spermatogonia following treatment with T4 in two strains of juvenile common carp (*Cyprinus carpio*).

6. Adult testis

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 Thapliyal, 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, directly or indirectly, the thyroid function, increasing T3 plasma levels (Hunt and Eales, 1979).

To study the effects of THs on fish spermatogenesis, a different experimental setting was employed, where African catfish (*Clarias gariepinus*) received T4 (100 ppm) for 21 days (Jacob et al., 2005). In this study, T4 depleted the number of spermatozoa/spermatids during the preparatory and pre-spawning phases (Jacob et al., 2005). Interestingly, the T4 overdose effects were more pronounced during the preparatory phase rather than pre-spawning, suggesting that altered TH status might have significant stage-dependent effects on the testis.

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 A_{und} spermatogonia, but also stimulated the accumulation of A_{diff} (type A differentiated spermatogonia) by increasing their proliferation while reducing

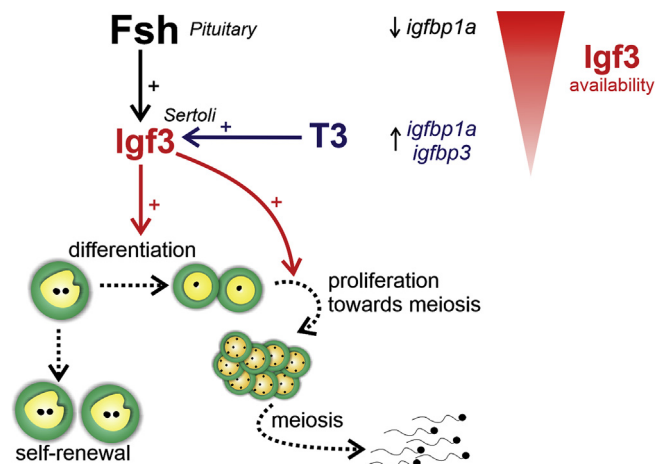


Fig. 4. Endocrine and paracrine regulation of spermatogonial proliferation in zebrafish. Fsh (follicle-stimulating hormone) and T3 stimulate the production of Igf3 (Insulin-like growth factor 3), which in turn promotes spermatogonial differentiation and proliferation towards meiosis. Igf3 is produced by Sertoli cells. T3 increases the transcript levels of two *igfbps* (insulin-like growth factor binding protein): *igfbp1a* and *igfbp3*. Fsh decreases RNAm levels of *igfbp1a*. The differential expression of the *igfbps* reflects directly on the Igf3 availability. Elevating Igf3 bioactivity by blocking Igfbps induced by T3 results in an excessive spermatogonial differentiation, leading to a depletion of type A_{und} spermatogonia in zebrafish.

development into type B spermatogonia in zebrafish (Fig. 4).

To unravel the molecular mechanisms of THs on zebrafish spermatogenesis, Morais et al. (2013) first showed the localization of *thra* and *thrβ* in Sertoli and Leydig cells, respectively. Similar data have been found in mammals, where TRs are localized in Sertoli cells, although these receptors were also found in mammalian germ cells (Buzzard et al., 2000; Singh et al., 2011). Moreover, it has been demonstrated that T3 increased a stimulatory growth factor of spermatogenesis, named Igf3 (Insulin-like growth factor 3) (Fig. 4) (Morais et al., 2013). Igf3 is a Sertoli cell growth factor, exclusively expressed in zebrafish gonads, and Fsh (Follicle-stimulating hormone) responsive (Fig. 4) (Nóbrega et al., 2015). It has been shown that Fsh promotes spermatogonial proliferation and differentiation via Igf3 in zebrafish testis (Nóbrega et al., 2015), which is consistent with T3 stimulation of spermatogonial proliferation and differentiation in this species (Fig. 4) (Morais et al., 2013). Interestingly, co-treatment with T3 and an IGF receptor inhibitor abolished the observed T3 effects on zebrafish spermatogenesis. Altogether, these results suggest that T3 stimulates zebrafish spermatogonial proliferation and differentiation via Sertoli cell production of Igf3 (Fig. 4) (Morais et al., 2013).

More recently, another study in zebrafish testis showed that T3 not only stimulates Igf3, but also increased the transcript levels of *igfbp1a* (Insulin-like growth factor binding protein 1) and *igfbp3* (Insulin-like growth factor binding protein 3) (Fig. 4) (Safian et al., 2016). In this study, Safian et al. (2016) showed a depletion of type A_{und} spermatogonia in zebrafish testis treated with T3 when neutralizing the Igfbps, suggesting that Igfbps protect undifferentiated spermatogonia against excessive differentiation (Fig. 4).

Next to their roles in Sertoli cells, THs also regulate Leydig cell functions in zebrafish testis (Fig. 4) (Morais et al., 2013). It is well known that Leydig cells express Fsh receptor, and Fsh is a potent steroidogenic hormone in zebrafish testis (Nóbrega et al., 2015). In this context, the Fsh steroidogenic capacity is increased in the presence of T3 in zebrafish testis (Morais et al., 2013). The observed increase in androgen secretion by zebrafish testis was accompanied by higher levels of *ar* and *cyp17a1* (17 α -hydroxylase/1720 lyase/1720 desmolase) mRNA levels, suggesting that Fsh effects are enhanced in the presence of T3 (Figs. 1 and 4).

In another study evaluating the action of THs in adult testis, de Montgolfier et al. (2009) examined testicular connexin 43 (cx43) mRNA in brook trout (*Salvelinus fontinalis*) and demonstrated a clear dose-response relationship between T3 and cx43, suggesting a possible regulation of this connexin by T3. These authors also investigated seasonal changes in the basal expression of several connexins and demonstrated higher levels of testicular cx43 in maturing fish, suggesting that changes in the connexins expression may precede the onset of spermatogenesis in this species.

7. Summary

In summary, this mini-review highlighted the main findings on THs role in male reproductive system of fish, focusing on sex differentiation, testicular development and spermatogenesis. The existing data in the literature have been demonstrated that THs exert their roles at the different levels of the HPG axis. In general, it has been shown a positive correlation between THs and fish reproductive status, where THs are frequently associated with testicular development, growth and maturation. More recently, the molecular mechanisms underlying the role of THs in spermatogenesis have been elucidated in the zebrafish testis 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. Moreover, T3 acts through Leydig cells to enhance the gonadotropin-induced synthesis and release of androgens. Next to their roles in the adult testis, THs also participate in the gonadal sex differentiation by regulating the expression of sex-related genes, and during testicular development through modulating Sertoli cell proliferation. Based on the evidences from the literature, it can be concluded that THs modulate the male reproductive system during the different life stages of fish. The physiological and molecular mechanisms showed a link between the thyroid and reproduction, suggesting a possibly co-evolution of these two systems. However, how crucial these systems are to each other remains a topic for future investigation.

Acknowledgements

The authors would like to thank Mr. Rodrigo Augusto Paixão Brasileiro for drawing the scientific illustrations of this mini-review. This work was supported by National Council for Scientific and Technological Development (CNPq) – Science without Borders (234548/2014-2) (awarded to Aldo Tovo-Neto) and FAPESP (2014/16280-5 and 2017/09218-0 – granted to Aldo Tovo-Neto; 2014/07620-7 – granted to Dr. Rafael Henrique Nóbrega; 2017/15793-7 granted to Maira da Silva Rodrigues).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.ygcen.2018.04.023>.

References

An, K.W., An, M.I., Nelson, E.R., Habibi, H.R., Choi, C.Y., 2010. Gender-related expression of TR alpha and TR beta in the protandrous black porgy, *Acanthopagrus schlegelii*, during sex change processes. *Gen. Comp. Endocrinol.* 165, 11–18.

Ariyaratne, H.B.S., Mason, J.I., Mendis-Handagama, S.M.L.C., 2000. Effects of thyroid and luteinizing hormones on the onset of precursor cell differentiation into leydig progenitor cells in the prepubertal rat testis. *Biol. Reprod.* 63, 898–904.

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

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

Brown, D.D., Cai, L.Q., 2007. Amphibian metamorphosis. *Dev. Biol.* 306, 20–33.

Buzzard, J.J., Morrison, J.R., O'Bryan, M.K., Song, Q., Wreford, N.G., 2000. Developmental expression of thyroid hormone receptors in the rat testis. *Biol. Reprod.* 62, 664–669.

Carr, J.A., Patiño, R., 2011. The hypothalamus-pituitary-thyroid axis in teleosts and amphibians: endocrine disruption and its consequences to natural populations. *Gen.*

Comp. Endocrinol. 170, 299–312.

Castañeda Cortés, D.C., Langlois, V.S., Fernandino, J.I., 2014. Crossover of the hypothalamic-pituitary-adrenal/interrenal, -thyroid, and -gonadal axes in axes in testicular development. *Front. Endocrinol.* 139, 1–11.

Chiba, H., Amano, M., Yamada, H., Fujimoto, Y., Ojima, D., Okuzawa, K., Yamanome, T., Yamamori, K., Iwata, M., 2004. 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.

Cooke, P.S., 1991. Thyroid hormones and testis development: a model system for increasing testis growth and sperm production. *Ann. N. Y. Acad. Sci.* 637, 122–132.

Cooke, P.S., Hess, R.A., Kirby, J.D., 1994a. A model system for increasing testis size and sperm production: potential application to animal science. *J. Animal Sci.* 72, 43–54.

Cooke, P.S., Zhao, Y.D., Bunick, D., 1994b. 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.

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

Denver, R.J., 1988. Several hypothalamic peptides stimulate in vitro thyrotropin secretion by pituitaries of anuran amphibians. *Gen. Comp. Endocrinol.* 72, 383–393.

Duarte-Guterman, P., Trudeau, V.L., 2011. Transcript profiles and triiodothyronine regulation of sex steroid- and thyroid hormone-related genes in the gonad-mesonephros complex of *Silurana tropicalis*. *Mol. Cell. Endocrinol.* 331, 143–149.

Eales, J.G., 2006. Modes of action and physiological effects of thyroid hormones in fish, in: Reinecke, M., Zaccane, G., Kapoor, B.G. (Eds). *Fish Endocrinology*. 2, pp. 767–808.

Faraone-Mennella, M.R., Ferone, A., Marino, L., Cardone, A., Comitato, R., Venditti, P., Di Meo, S., Farina, B., 2008. Poly(ADP-ribosyl)ation of proteins and germ cell development in hyperthyroid rat testes. *Mol. Cell. Biochem.* 323, 119–129.

Flood, D.E.K., Fernandino, J.I., Langlois, V.S., 2013. Thyroid hormones in male reproductive development: Evidence for direct crosstalk between the androgen and thyroid hormone axes. *Gen. Comp. Endocrinol.* 192, 2–14.

França, L.H., Hess, R.A., Cooke, P.S., Russell, L.D., 1995. Neonatal hypothyroidism causes delayed sertoli cell maturation in rats treated with propylthiouracil: evidence that the Sertoli cell controls testis growth. *Anat. Rec.* 242, 57–69.

França, L.R., Nóbrega, R.H., Morais, R.D.V.S., Assis, L.H.C., Schulz, R.W., 2015. Sertoli cell structure and function in anamniote vertebrates. In: Griswold, M.D. (Ed.), *Sertoli Cell Biology*. Elsevier, Amsterdam, pp. 385–407.

Freeman, D.A., Teubner, B.J., Smith, C.D., Prendergast, B.J., 2007. Exogenous T3 mimics long day lengths in Siberian hamsters. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 292, R2368–R2372.

Goleman, W.L., Carr, J.A., Anderson, T.A., 2002. Environmentally relevant concentrations of ammonium perchlorate inhibit thyroid function and alter sex ratios in developing *Xenopus laevis*. *Environ. Toxicol. Chem.* 21, 590–597.

De Groef, B., Goris, N., Arckens, L., Kühn, E.R., Darras, V.M., 2003. Corticotropin-releasing hormone (CRH)-induced thyrotropin release is directly mediated through CRH receptor type 2 on thyrotropes. *Endocrinology* 144, 5537–5544.

van Haaster, L.H., de Jong, F.H., Docter, R., de Rooij, D.G., 1993. 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.

Habibi, H.R., Nelson, E.R., Allan, E.R.O., 2012. New insights into thyroid hormone function and modulation of reproduction in goldfish. *Gen. Comp. Endocrinol.* 175, 19–26.

Haldar-Misra, C., Thapliyal, J.P., 1981. Thyroid in reproduction of reptiles. *Gen. Comp. Endocrinol.* 43, 537–542.

Hayes, T.B., 1997. Steroids as potential modulators of thyroid hormone activity in anuran metamorphosis. *Am. Zool.* 37, 185–194.

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

Jacob, T.N., Pandey, J.P., Raghuvver, K., Sreenivasulu, G., Gupta, A.D., Yoshikuni, M., Jagota, A., Senthilkumaran, B., 2005. Thyroxine-induced alterations in the testis and seminal vesicles of air-breathing catfish *Clarias gariepinus*. *Fish. Physiol. Biochem.* 31, 271–274.

Jacobs, G.F.M., Goyvaerts, M.P., Vanderpe, G., Quaghebeur, A.M.L., Kuhn, E.R., 1988a. Luteinizing hormone-releasing hormone as a potent stimulator of the thyroidal axis in ranid frogs. *Gen. Comp. Endocrinol.* 70, 274–283.

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

Johnson, K.M., Lema, S.C., 2011. Tissue-specific thyroid hormone regulation of gene transcripts encoding iodothyronine deiodinases and thyroid hormone receptors in striped parrotfish (*Scarus iserti*). *Gen. Comp. Endocrinol.* 172, 505–517.

Larsen, D.A., Swanson, P., Dickey, J.T., Rivier, J., Dickhoff, W.W., 1998. In Vitro thyrotropin-releasing activity of corticotropin-releasing hormone-family peptides in coho salmon, *Oncorhynchus kisutch*. *Gen. Comp. Endocrinol.* 109, 276–285.

MacKenzie, D.S., Sokolowska, M., Peter, R.E., Breton, B., 1987. Increased gonadotropin levels in goldfish do not result in alterations in circulating thyroid hormone levels. *Gen. Comp. Endocrinol.* 67, 202–213.

Maran, R.R.M., Aruldas, M.M., 2002. Adverse effects of neonatal hypothyroidism on Wistar rat spermatogenesis. *Endocr. Res.* 28, 141–154.

Matta, S.L.P., Vilela, D.A.R., Godinho, H.P., França, L.R., 2002. 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.

- Mendis-Handagama, S.M.L.C., Ariyaratne, H.B.S., 2005. Leydig cells, thyroid hormones and steroidogenesis. *Indian J. Exp. Biol.* 43, 939–962.
- de Montgolfier, B., Faye, A., Audet, C., Cyr, D.G., 2009. Seasonal variations in testicular connexin levels and their regulation in the brook trout, *Salvelinus fontinalis*. *Gen. Comp. Endocrinol.* 162, 276–285.
- Morais, R.D.V.S., Nóbrega, R.H., Gómez-González, N.E., Schmidt, R., Bogerd, J., França, L.R., Schulz, R.W., 2013. Thyroid hormone stimulates the proliferation of sertoli cells and single type a spermatogonia in adult zebrafish (*Danio rerio*) Testis. *Endocrinology* 154, 4635–4676.
- Mukhi, S., Patiño, R., 2007. Effects of prolonged exposure to perchlorate on thyroid and reproductive function in zebrafish. *Toxicol. Sci.* 96, 246–254.
- Nelson, E.R., Habibi, H.R., 2009. Thyroid receptor subtypes: structure and function in fish. *Gen. Comp. Endocrinol.* 161, 90–96.
- Nóbrega, R.H., Morais, R.D.V.S., Crespo, D., de Waal, P.P., França, L.R., Schulz, R.W., Bogerd, J., 2015. Fsh stimulates spermatogonial proliferation and differentiation in zebrafish via Igf3. *Endocrinology* 156, 3804–3817.
- Norris, D.O., 2006. The Hypothalamus-Pituitary-Thyroid (HPT) Axis of Non-mammalian Vertebrates. In: Norris, D.O. (Ed.), *Vertebrate Endocrinology*. Elsevier, Amsterdam, pp. 244–270.
- Okada, R., Yamamoto, K., Koda, A., Ito, Y., Hayashi, H., Tanaka, S., Hanaoka, Y., Kikuyama, S., 2004. 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.
- Orozco, A., Valverde, C.R., Olvera, A., García, C.G., 2012. Iodothyronine deiodinases: a functional and evolutionary perspective. *J. Endocrinol.* 215, 207–219.
- Parhar, I.S., Soga, T., Sakuma, T., 2000. 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.
- Roy, P., Datta, M., Dasgupta, S., Bhattacharya, S., 2000. 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.
- Safian, D., Morais, R.D.V.S., Bogerd, J., Schulz, R.W., 2016. Igf Binding proteins protect undifferentiated spermatogonia in the zebrafish testis against excessive differentiation. *Endocrinology* 157, 4423–4433.
- Sharma, P., Patiño, R., 2013. Regulation of gonadal sex ratios and pubertal development by the thyroid endocrine system in zebrafish. *Gen. Comp. Endocrinol.* 184, 111–119.
- Sharma, P., Tang, S., Mayer, G.D., Patiño, R., 2016. Effects of thyroid endocrine manipulation on sex-related gene expression and population sex ratios in Zebrafish. *Gen. Comp. Endocrinol.* 235, 38–47.
- Schulz, R.W., França, L.R., Lareye, J.J., Le Gac, F., Chiarini-garcia, H., Nóbrega, R.H., Miura, T., 2010. Spermatogenesis in fish. *Gen. Comp. Endocrinol.* 165, 390–411.
- Singh, R., Hamada, A.J., Agarwal, A., 2011. Thyroid hormones in male reproduction and fertility. *Open Reprod. Sci. J.* 3, 98–104.
- Sun, B.J., Li, T., Mu, Y., McGlashan, J.K., Georges, A., Shine, R., Du, W.G., 2016. Thyroid hormone modulates offspring sex ratio in a turtle with temperature dependent sex determination. *Proc. R. Soc. B* 283, 1–7.
- 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., 2006. Thiourea-induced thyroid hormone depletion impairs testicular recrudescence in the air-breathing catfish *Clarias gariepinus*. *Comp. Biochem. Physiol. Part A* 144, 1–10.
- Teerds, K.J., de Rooij, D.G., de Jong, F.H., van Haaster, L.H., 1998. Development of the adult-type leydig cell population in the rats is affected by neonatal thyroid hormone levels. *Biol. Reprod.* 59, 344–350.
- Timmermans, L.P.M., Chmielevsky, D.A., Komen, H., Schipper, H., 1997. Precocious onset of spermatogenesis in juvenile carp (*Cyprinus carpio* L., Teleostei) following treatment with low doses of L-thyroxine. *Eur. J. Morphol.* 35, 344–353.
- Tsutsui, K., Son, Y.S., Kiyohara, M., Miyata, I., 2018. Discovery of GnIH and its role in hypothyroidism-induced delayed puberty. *Endocrinology* 159, 62–68.
- Wagner, M.S., Wajner, S.M., Maia, A.L., 2008. The role of thyroid hormone in testicular development and function. *J. Endocrinol.* 199, 351–365.
- Wagner, M.S., Wajner, S.M., Maia, A.N., 2009. Is there a role for thyroid hormone on spermatogenesis. *Microsc. Res. Tech.* 72, 796–808.
- Yoshimura, T., Yasuo, S., Watanabe, M., Iigo, M., Yamamura, T., Hirunagi, K., Ebihara, S., 2003. Light-induced hormone conversion of T4 to T3 regulates photoperiodic response of gonads in birds. *Nature* 426, 178–181.