



UNIVERSIDADE ESTADUAL PAULISTA
“JÚLIO DE MESQUITA FILHO”
Campus de Botucatu



PG-BGA

“Hormônios tireoidianos e sua relação com o eixo reprodutivo-somatotrópico e seu possível papel no desenvolvimento embrionário em resposta ao estresse materno em zebrafish (*Danio rerio*)”

MAIRA DA SILVA RODRIGUES

TESE APRESENTADA AO INSTITUTO DE
BIOCIÊNCIAS, CAMPUS DE BOTUCATU, UNESP,
PARA OBTENÇÃO DO TÍTULO DE DOUTORA NO
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA
GERAL E APLICADA, ÁREA DE CONCENTRAÇÃO
BIOLOGIA CELULAR, ESTRUTURAL E FUNCIONAL

DR. RAFAEL HENRIQUE NÓBREGA

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Tese apresentada ao Instituto de Biociências, Campus de Botucatu, UNESP, para obtenção do título de Doutora no Programa de Pós-Graduação em Biologia Geral e Aplicada, Área de concentração em Biologia Celular, Estrutural e Funcional

Orientador: *Dr. Rafael Henrique Nóbrega*

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Hormônios tireoidianos e sua relação com o eixo reprodutivo-somatotrópico e seu possível papel no desenvolvimento embrionário em resposta ao estresse materno em zebrafish (*Danio rerio*) / Maira da Silva Rodrigues. -- Botucatu, 2025
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Impacto potencial desta pesquisa

A pesquisa intitulada “Hormônios tireoidianos e sua relação com o eixo reprodutivo-somatotrófico e seu possível papel no desenvolvimento embrionário em resposta ao estresse materno em zebrafish (*Danio rerio*)” apresenta um impacto social significativo, especialmente nas áreas da saúde, reprodução, biologia do desenvolvimento e toxicologia. O estudo aborda um tema central na compreensão de como distúrbios nos níveis dos hormônios tireoidianos (HTs) podem afetar o eixo reprodutivo de machos adultos e implicar na progressão da espermatogênese, assim como flutuações dos níveis hormonais maternos podem influenciar o desenvolvimento embrionário, com implicações tanto para a saúde humana quanto para a preservação das espécies. O zebrafish tem sido amplamente utilizado como modelo experimental na medicina translacional devido à sua similaridade genética com os seres humanos, especialmente no que se refere à regulação hormonal e ao desenvolvimento embrionário. Seu ciclo de vida rápido, embriões transparentes e facilidade de manipulação tornam esse organismo ideal para investigações sobre a influência dos hormônios tireoidianos no desenvolvimento e na reprodução. Dessa forma, o uso do zebrafish permite que os achados dessa pesquisa sejam extrapolados para a compreensão de desordens endócrinas e reprodutivas humanas que afetam a fertilidade e o crescimento e desenvolvimento fetal. Primeiramente, a compreensão dos mecanismos pelos quais os HTs afetam a espermatogênese em zebrafish pode fornecer *insights* relevantes sobre a interação entre o sistema endócrino e reprodutivo. Isso pode contribuir para a identificação precoce de desordens endócrinas que afetam a fertilidade e a saúde masculina em geral. A conscientização sobre a importância da homeostase hormonal no funcionamento reprodutivo pode resultar em maior atenção às doenças endócrinas, como hipo e hipertireoidismo e seus efeitos na gametogênese. Além disso, a pesquisa sobre a relação entre os eixos tireoidiano-reprodutivo em zebrafish pode inspirar avanços em biotecnologias voltadas para a reprodução, como técnicas avançadas de microscopia para investigação da dinâmica das células germinativas nos testículos, e ainda, no aprimoramento de técnicas de criopreservação destas células e no desenvolvimento de protocolos de tratamentos para infertilidade masculina, tanto em seres humanos quanto em animais de interesse econômico e ecológico. Em fêmeas, a identificação de complicações no desenvolvimento embrionário induzidas por alterações hormonais maternas pode contribuir para a compreensão de implicações na gravidez, como restrições de crescimento fetal e distúrbios endócrinos, além de potencialmente auxiliar no desenvolvimento de estratégias para prevenir tais problemas em populações vulneráveis. Nossa pesquisa também pode ter implicações no campo da ecotoxicologia, uma vez que o *Danio rerio*



é um organismo modelo para estudos ambientais. Alterações hormonais maternas podem ser exacerbadas por poluentes ambientais que afetam os níveis hormonais e consequentemente, o desenvolvimento e a sobrevivência da prole. O desequilíbrio hormonal causado por contaminantes ambientais, como disruptores endócrinos, pode comprometer a reprodução de populações de peixes e outros organismos aquáticos, ameaçando a biodiversidade. Investigar essas relações é crucial para a formulação de políticas ambientais mais eficazes para controlar poluentes que interferem no sistema endócrino, e consequentemente, no sucesso reprodutivo de espécies aquáticas. Além disso, a proposição de políticas públicas para conservação de espécies e proteção de ecossistemas é urgente diante dos desafios atuais e futuros relacionados a contaminação ambiental, e emergência climática, principalmente relacionados com o aumento da temperatura da água, na qual pode impactar a gametogênese. O impacto social da pesquisa também se estende a educação, promovendo maior entendimento sobre a biologia reprodutiva masculina e a importância dos hormônios maternos no desenvolvimento dos organismos. Esse impacto contribui para a formação de uma base científica que apoie discussões sobre saúde materna, bem-estar nas fases infanto-juvenil e sustentabilidade ambiental, promovendo maior conscientização sobre a interconexão entre biologia, ecologia, e saúde pública.

Potential impact of this research

The study entitled “Thyroid hormones and their relationship with the reproductive-somatotropic axis and their potential role in embryonic development in response to maternal stress in zebrafish (*Danio rerio*)” presents significant societal impacts, particularly in the fields of health, reproduction, developmental biology, and toxicology. This research addresses a central topic in understanding how disruptions in thyroid hormone (THs) levels can affect the reproductive axis of adult males and influence the progression of spermatogenesis, as well as how fluctuations in maternal hormone levels can impact embryonic development, with implications for both human health and species conservation. The zebrafish has been widely used as an experimental model in translational medicine due to its genetic similarity to humans, particularly in hormonal regulation and embryonic development. Its rapid life cycle, transparent embryos, and ease of manipulation make this organism ideal for investigating the influence of thyroid hormones on growth and reproduction. Consequently, the use of zebrafish enables the extrapolation of research findings to the understanding of human endocrine and reproductive disorders that affect fertility, as well as fetal growth and development. First, understanding the mechanisms by which THs affect spermatogenesis in zebrafish may provide relevant insights



into the interaction between the endocrine and reproductive systems. This could contribute to the early identification of endocrine disorders that affect fertility and overall male health. Awareness of the importance of hormonal homeostasis in reproductive function may lead to increased attention to endocrine diseases, such as hypo and hyperthyroidism, and their effects on gametogenesis. Additionally, research on the relationship between the thyroid-reproductive axes in zebrafish could inspire advances in reproductive biotechnologies, such as advanced microscopy techniques for investigating germ cell dynamics in the testis, and improvements in cryopreservation techniques for these cells, as well as the development or treatment protocols for male infertility in both humans and economically and ecologically important animals. In females, identifying complications in embryonic development induced by maternal hormonal alterations may contribute to the understanding of pregnancy-related issues, such as fetal growth restriction and endocrine disorders, while also potentially aiding in the development of strategies to prevent such problems in vulnerable populations. Our research may also have implications in ecotoxicology, as *Danio rerio* is a model species for environmental studies in aquatic ecosystems. Maternal hormonal alterations may be exacerbated by environmental pollutants that affect hormonal levels and offspring development and survival. Hormonal imbalances caused by environmental contaminants, such as endocrine disruptors, could compromise the reproduction of fish populations and other aquatic organisms, threatening biodiversity. Investigating these relationships is crucial for the formulation of more effective environmental policies to control pollutants that interfere with the endocrine system and, subsequently, the reproductive success of aquatic species. Furthermore, the proposal of public policies for species conservation and ecosystem protection is urgent in light of current and future challenges related to environmental contamination and climate change, especially with the increase in water temperatures, which could impact gametogenesis. The social impact of the research also extends to education, promoting a greater understanding of male reproductive biology and the importance of maternal hormones in organism development. This impact contributes to building a scientific foundation that supports discussions on maternal health, well-being during childhood and adolescence, and environmental sustainability, raising awareness about the connection between biology, ecology, and public health.

ATA DA DEFESA PÚBLICA DA TESE DE DOUTORADO DE MAIRA DA SILVA RODRIGUES, DISCENTE DO PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA GERAL E APLICADA, DO INSTITUTO DE BIOCIÊNCIAS - CÂMPUS DE BOTUCATU.

Aos 31 dias do mês de janeiro do ano de 2025, às 9h, no(a) Sala 7 da Central Nova, realizou-se a defesa de TESE DE DOUTORADO de MAIRA DA SILVA RODRIGUES, intitulada **Hormônios tireoidianos e sua relação com o eixo reprodutivo-somatotrópico e seu possível papel no desenvolvimento embrionário em resposta ao estresse materno em zebrafish (Danio rerio)**. A Comissão Examinadora foi constituída pelos seguintes membros: Prof. Dr. RAFAEL HENRIQUE NÓBREGA (Orientador(a) - Participação Presencial) do(a) Departamento de Biologia Estrutural e Funcional / Instituto de Biociências de Botucatu UNESP, Profa. Dra. RENATA GUIMARÃES MOREIRA WHITTON (Participação Virtual) do(a) Departamento de Fisiologia Geral / Universidade de São Paulo, Instituto de Biociências (IB/USP), Dra. GIOVANA SOUZA BRANCO (Participação Virtual) do(a) Departamento de Fisiologia / Universidade de São Paulo - USP, Prof. Dr. BERNARDO BALDISSEROTTO (Participação Virtual) do(a) Departamento de Fisiologia e Farmacologia / Universidade Federal de Santa Maria - UFSM, Prof. Dr. LÁZARO WENDER OLIVEIRA DE JESUS (Participação Presencial) do(a) Departamento de Biologia Estrutural e Funcional / Instituto de Biociências de Botucatu - UNESP. Após a exposição pela doutoranda e arguição pelos membros da Comissão Examinadora que participaram do ato, de forma presencial e/ou virtual, a discente recebeu o conceito final: APROVADA. Nada mais havendo, foi lavrada a presente ata, que após lida e aprovada, foi assinada pelo(a) Presidente(a) da Comissão Examinadora.

Prof. Dr. RAFAEL HENRIQUE NÓBREGA

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Dedico

Aos meus amados pais, *Marlene* e *Luiz Sérgio* que,
com muito carinho e apoio enxergaram comigo
que a educação poderia ser transformadora em nossas vidas.
E à minha irmã de coração, *Luiza (In memoriam)*, por me mostrar na dor
que a vida pode ser bela!



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ser meu par na jornada da vida. Sou grata por compartilhá-la com você. Tudo fica mais leve, prazeroso e próspero ao seu lado. Eu te amo, meu esposo!

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Epígrafe

“Ah, comigo o mundo vai modificar-se. Não gosto do mundo como ele é”

Carolina Maria de Jesus

(Escritora, poetisa, cantora e compositora brasileira)



Resumo

Em vertebrados, os hormônios tireoidianos (HTs) estão envolvidos na regulação de vários processos fisiológicos, incluindo o metabolismo, comportamento, estresse, desenvolvimento embrionário, e sistema reprodutivo de machos e fêmeas. Com base nisso, para aprofundar nosso conhecimento sobre a importância dos HTs na biologia reprodutiva de peixes, o presente estudo teve como objetivos: 1) investigar a interação entre os hormônios tireoidianos e o eixo hipotalâmico-hipofisário-tireoide e Gonadal (HHG) em machos adultos de zebrafish, e 2) investigar os efeitos dos hormônios maternos no desenvolvimento embrionário e larval de zebrafish, com foco no eixo somatotrópico. No primeiro objetivo, foi investigado os efeitos do hipotireoidismo induzido por metimazol na função testicular dos machos de zebrafish. Metimazol é um medicamento antitireoideano conhecido por atuar na supressão da atividade da peroxidase tireoideana (TPO), responsável por catalisar a conversão de iodeto em iodo para produção de T3 e T4. O tratamento dos peixes com metimazol, *in vivo*, alterou significativamente a espermatogênese, inibiu a diferenciação celular e a meiose, além de diminuir o número relativo de espermatozoides. O comprometimento da espermatogênese observado devido ao hipotireoidismo induzido pelo metimazol foi correlacionado com mudanças significativas nos níveis de transcritos de vários genes implicados no controle da reprodução. Na abordagem *ex vivo*, foi demonstrado que, além de afetar os componentes do eixo hipotalâmico-hipofisário, o T3 (triiodotironina) exerceu ação direta sobre os testículos. Neste tópico destaca-se a hipótese de que os HTs são um elemento essencial do controle multifatorial da reprodução e da função testicular em zebrafish. Para atender ao segundo objetivo, fêmeas adultas de zebrafish foram alimentadas com ração comercial com diferentes concentrações de T4 (tetraiodotironina) (12.5 e 25 µg) por grama de massa corporal por 5 dias. Um grupo controle foi alimentado com ração comercial contendo o veículo (etanol 100 %). No sexto dia, o sangue de cinco fêmeas foi coletado de cada grupo, e o plasma amostrado para mensuração dos níveis de T3. As fêmeas restantes foram transferidas para tanques de reprodução com machos não tratados. Em seguida, os embriões e larvas (F1) de cada grupo foram coletados nos períodos de 0 a 144 horas pós fertilização (hpf) para análises de sobrevivência, frequência cardíaca e presença de anomalias morfológicas. Além disso, a progênie de 0 a 72 -hpf foi coletada para mensuração dos níveis de T3 e expressão gênica. Os níveis relativos de mRNA dos seguintes genes foram avaliados: *crf*, *tsh*, *thra*, *thrβ*, *dio2*, *dio3*, *mct8*, *gh*, *igf1*, *mtor*, *foxO3a*, *myoG* e *myf5*. Os resultados do nosso estudo demonstraram que os níveis plasmáticos de T3 estavam aumentados em fêmeas alimentadas com uma alta



concentração de T4 (25 µg), o que, consequentemente, afetou o conteúdo de T3 nos embriões de zebrafish. Além disso, a progênie descendente apresentou crescimento reduzido, bem como anomalias morfológicas e fisiológicas, incluindo edema do saco vitelínico e pericárdio, bexiga natatória não inflada, deformação da coluna seguida de escoliose, aumento da frequência cardíaca e alto índice de mortalidade. Neste mesmo grupo, a análise da expressão gênica mostrou alterações nos transcritos relacionados ao eixo tireoidiano, assim como, em genes associados ao crescimento e desenvolvimento responderam as alterações nas concentrações materna de HTs. Foi demonstrado neste grupo que os níveis de mRNA de *gh* e *igf1* tenderam a diminuir. Por outro lado, os transcritos de *mtor* foram diminuídos e aumentados nas proles com 48 e 72 -hpf, respectivamente. No entanto, esses mesmos níveis tenderam a aumentar na prole obtida de fêmeas alimentadas com uma concentração baixa de T4. Nesta abordagem, nós demonstramos que flutuações nos níveis dos HTs materno apresentam consequências significantes no desenvolvimento da progênie de zebrafish pós-fertilização. Por fim, fêmeas adultas de zebrafish foram alimentadas com cortisol (25 µg por grama de massa corporal) por cinco dias. No terceiro dia após o tratamento, essas fêmeas foram acasaladas com machos não tratados. Embriões, larvas e juvenis foram coletados e analisados em diferentes períodos para avaliar a sobrevivência, o crescimento, a presença de anormalidades morfológicas, como edema de pericárdio, e a expressão de genes relacionados ao eixo somatotrópico. Os resultados indicaram que a suplementação materna com cortisol influenciou significativamente o desenvolvimento da prole, resultando em alta taxa de mortalidade, e alterações cardíacas. Além disso, foram observadas alterações na expressão de genes associados à síntese proteica, sugerindo um crescimento acelerado nas primeiras horas pós-fertilização. Em juvenis com 30 dias pós-fertilização, também foram identificadas alterações morfológicas no tecido muscular. De modo geral, este estudo destacou a importância dos hormônios materno no início do desenvolvimento embrionário e larval, e seu papel no eixo reprodutivo de machos adultos de zebrafish.

Palavras-chave: Hormônios tireoidianos (HTs), espermatogênese, hipotireoidismo, hormônios maternos, programação fetal, efeitos intergeracionais



Abstract

In vertebrates, thyroid hormones (THs) are involved in the regulation of various physiological processes, including metabolism, behavior, stress, embryonic development, and the reproductive system of males and females. To deepen our knowledge of the importance of THs in the reproductive biology of fish, the present study aimed to 1) investigate the crosstalk between thyroid hormones and the Hypothalamic-Pituitary-Thyroid and -Gonadal (HPG) axes in adult male zebrafish, and 2) investigate the effects of maternal hormones on the embryonic and larval development of zebrafish, with a focus on the somatotrophic axis. The first objective was to investigate the effects of methimazole-induced hypothyroidism on the testicular function in zebrafish. Methimazole acts by suppressing the thyroid peroxidase (TPO) activity, which is responsible for catalyzing the conversion of iodide to iodine for the production of T3 and T4. The treatment of fish with methimazole, *in vivo*, significantly altered zebrafish spermatogenesis by inhibiting cell differentiation and meiosis, as well as decreasing the relative number of spermatozoa. The observed impairment of spermatogenesis by methimazole was correlated with significant changes in transcript levels for several genes implicated in the control of reproduction. Using an *in vitro* approach, we also demonstrated that T3 (triiodothyronine) also exerts direct action on the testis in addition to affecting the components of the brain-pituitary axis. This topic highlights the hypothesis that THs play a crucial role in the multifactorial control of reproduction and testicular function in zebrafish. To attend to the second objective, zebrafish females were fed commercial food with either a low or high concentration of T4 (tetraiodothyronine) (12.5 or 25 μg T4) per gram of body mass for 5 days. A control group was fed commercial food containing the vehicle used to dissolve the hormone (100% ethanol). Blood was collected from five females in each group on the sixth day, and plasma was sampled to extract and measure T3 levels. The remaining females were transferred to a breeding tank with untreated males. Subsequently, embryos/larvae (F1) from each group were evaluated from 0 hours post-fertilization (hpf) to 144 -hpf based on parameters such as survival rate, heartbeat rate, and morphological abnormalities. Additionally, embryos/larvae were collected from 0 to 72 -hpf to measure T3 levels and examine gene expression. The relative mRNA levels of the following genes were evaluated: *crf*, *tsh*, *thra*, *thr β* , *dio2*, *dio3*, *mct8*, *gh*, *igf1*, *mtor*, *foxO3a*, *myoG* and *myf5*. Our study demonstrated that T3 plasma levels were significantly increased in females fed a high concentration of T4 (25 μg), which consequently affected the T3 content in zebrafish embryos. Moreover, this progeny exhibited reduced growth, as well as morphological and physiological abnormalities, including yolk sac and pericardial edema, uninflated swim



bladder, spine deformation, increased heartbeat rate, and higher mortality. Analysis of gene expression revealed alterations in the transcripts related to the THs axis in the progeny obtained from females fed a high concentration of T4. Additionally, the expression of genes associated with growth and development responded to changes in maternal TH concentrations. In the high T4 concentration group, mRNA levels of *gh* and *igf1* tended to decrease. Conversely, in the same group, *mtor* levels were down- and up-regulated in the progeny with 48 and 72 hpf, respectively. However, these levels tended to increase in the progeny obtained from females fed a low concentration of T4. In this approach, we suggest that fluctuations in maternal THs had significant consequences for the zebrafish offspring post-fertilization. Adult female zebrafish were fed with cortisol (25 µg per gram of body mass) for five days. On the third day after treatment, these females were mated with untreated males. Embryos, larvae, and juveniles were collected and analyzed at different time points to assess survival, growth, the presence of morphological abnormalities such as pericardial edema, and the expression of genes related to the somatotropic axis. The results indicated that maternal cortisol supplementation significantly affected offspring development, leading to high mortality rates and cardiac alterations. Additionally, changes in the expression of genes associated with protein synthesis were observed, suggesting accelerated growth during the early hours post-fertilization. In juveniles, at 30 days post-fertilization, morphological alterations in muscle tissue were also observed. Overall, this work demonstrated the significance of maternal hormones in embryonic and larval development and their role in the reproductive axis of adult male zebrafish.

Keywords: Thyroid hormones (THs), spermatogenesis, hypothyroidism, maternal hormones transfer, fetal programming, intergenerational effects



SUMMARY

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*Development of Testicular Organoids as a Model for Studying Fish Spermatogenesis and
Developing Innovative Aquaculture Applications*

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1. General introduction

1.1 Spermatogenesis in teleosts fish

Fishes are the most diverse and abundant category of vertebrates, accounting for around 32.000 species, or more than half of all vertebrates (Nelson et al., 2006, 2016). This group's success is attributed to a series of adaptations developed throughout evolution that reflect in morphological, behavioral, and physiological diversity, as well as in reproductive strategies, which are defined as the set of characteristics that a species develops to succeed in reproduction and thus ensure the species survival (Reis et al., 2003; Nelson et al., 2006, 2016). These reproductive strategies are directly related to the morphology of the gonads of different species. Externally fertilized teleosts typically have elongated and paired testes (Billard et al., 1982; Vazzoler, 1996; Grier & Aranzábal, 2009), which are positioned in the celomic cavity and surrounded by connective tissue known as the albuginea tunica. This tissue extends into the organ-forming lobules or tubules, which support the germinal epithelial tissue (Grier, 1980). Microscopically, the testis present two distinct compartments. The first is the interstitial compartment composed of connective tissue, which contains Leydig cells, responsible for the production of steroids, as well as peritubular myoid cells, blood vessels, macrophages, granulocytes, and other cellular types. The second is the germinal compartment, which contains germ and Sertoli cells. Together, these compartments constitute the germinal epithelium (Schulz & Nóbrega, 2011; Siqueira-Silva et al., 2018). Spermatogenesis is dependent on the interactions of cells from both compartments (Grier et al., 1980) (Figure 1).

The spermatogenic process is thought to be highly coordinated, with the first diploid cells known as spermatogonial stem cells, which not only self-renew following reproductive events but also undergo mitotic proliferation and differentiation to generate the spermatozoa (Schulz et al., 2010). At the end of several species-specific mitotic divisions, spermatocytes initiate the meiotic process and ultimately undergo cellular differentiation or spermiogenesis, which will result in the formation of haploid cells, the spermatozoa (Grier et al., 1980; Nóbrega et al., 2009; Schulz et al., 2010; Siqueira-Silva et al., 2012). In contrast to amniotes, (Figure 1A), in fish and other anamniote vertebrates, spermatogenesis occurs within structures known as spermatocytes, or cysts, which form when Sertoli cells surround developing germ cells (Figure 1B) (Vilela et al., 2003; Nóbrega et al., 2009; Schulz et al., 2010). Sertoli cells provide the mechanical, nutritional, and factors required for germ cell survival, proliferation, and



differentiation, as well as hormonal mediation and phagocytosis of cellular remains and residual spermatozoa (Grier et al., 1980; Nóbrega et al., 2009; Schulz et al., 2010; Siqueira-Silva et al., 2012; França et al., 2016).

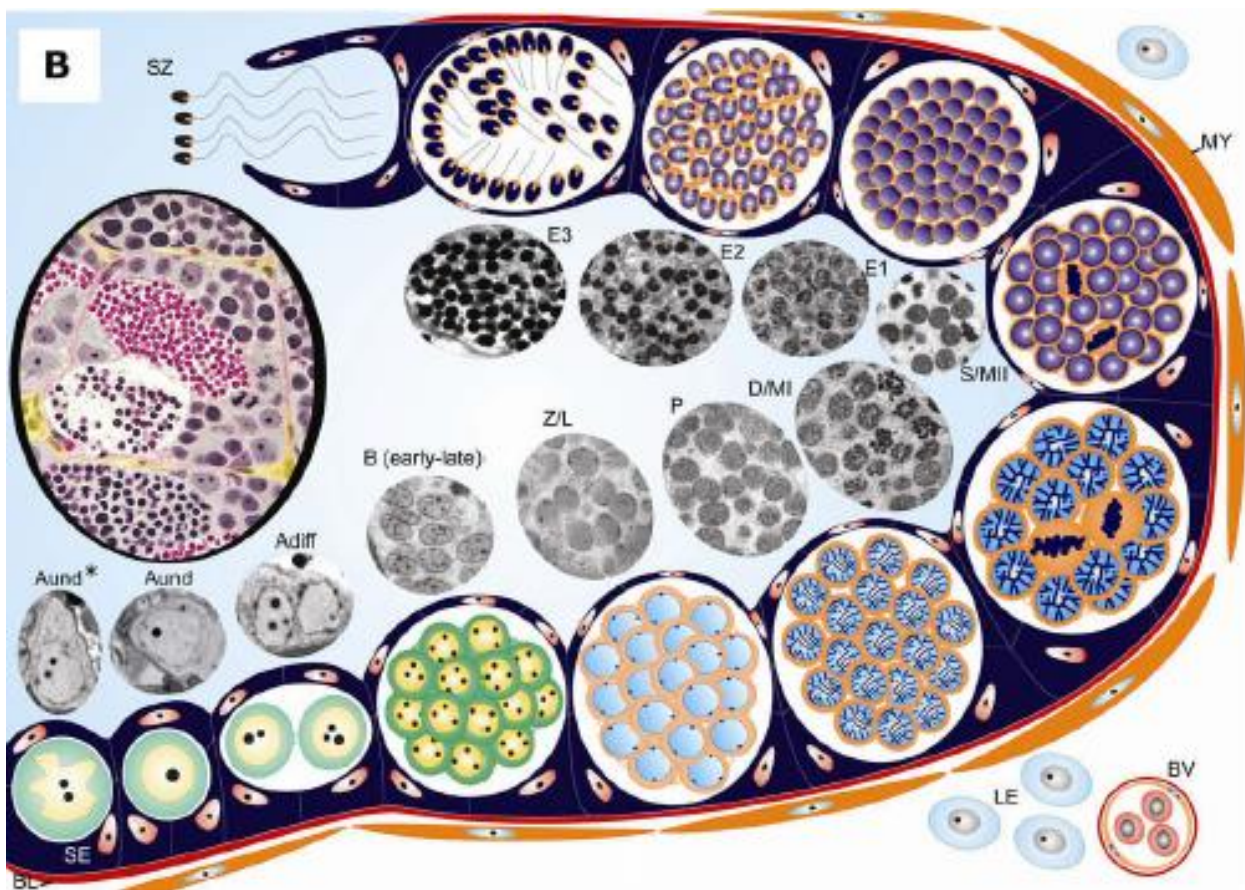
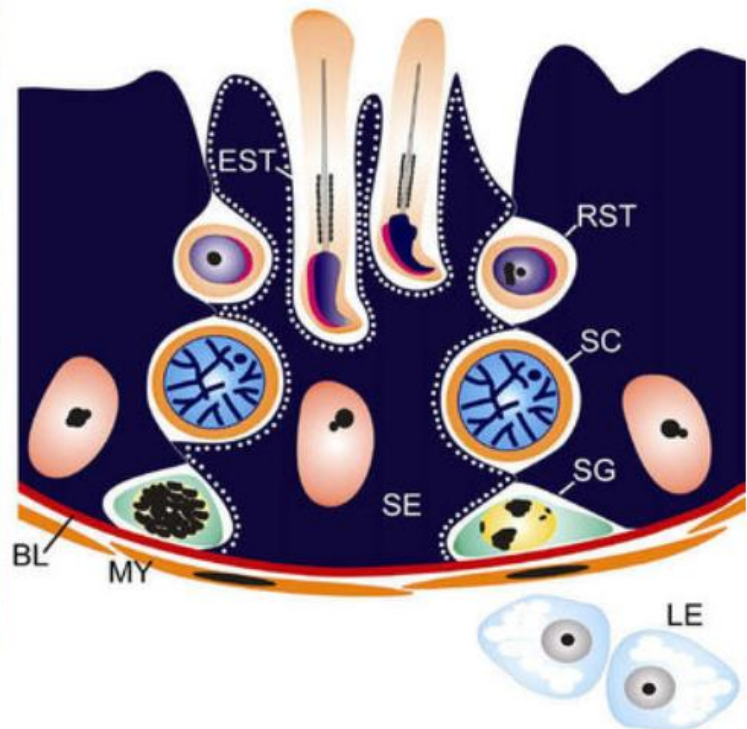
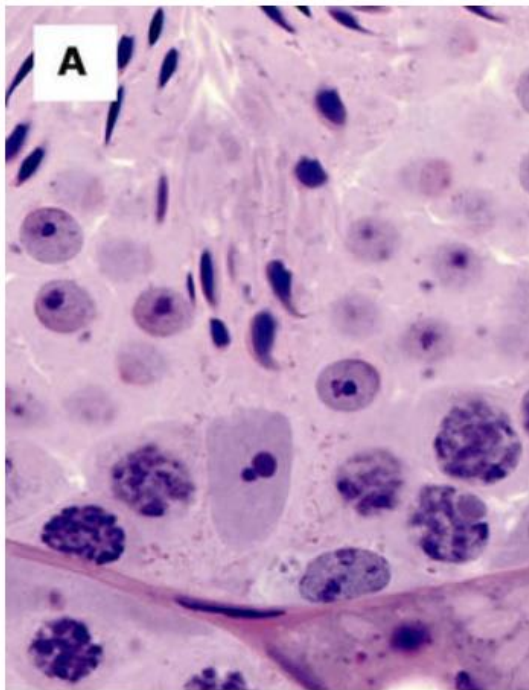


Figure 1. Comparison between spermatogenesis in mouse **(A)** and zebrafish **(B)**. Sertoli cells (SE), basal lamina (BL), and peritubular myoid cells (MY); interstitial Leydig cells (LE) and blood vessels (BV) are shown. **(A)** - Spermatogonia (SG), spermatocyte (SC), round spermatid (EST), and elongated spermatid (EST). **(B)** – Type A undifferentiated spermatogonia (Aund* and Aund), type A differentiated spermatogonia (Adiff), spermatogonia type B (early and late), leptotene/zygotene primary spermatocytes (L/Z), pachytene primary spermatocyte (P), diplotene spermatocyte/metaphase I (D/MI), secondary spermatocytes/metaphase II (S/MII), early, intermediate and final spermatids (E1, E2 and E3, respectively), and spermatozoa (SZ). The figure presented is derived from the study conducted by Schulz and colleagues (2010).

1.2 Spermatogenesis and characteristics of germ cells in zebrafish – 2D approach

The morpho-functional properties of different phases of germ cell development during zebrafish spermatogenesis have been characterized using a two-dimensional method (Leal et al., 2009; Schulz et al., 2010). These cells are classified into three phases based on their nuclear features, such as chromatin condensation, presence of nucleoli, nuclear diameter, and cell size and number per cyst. The three phases are: (1) mitotic or spermatogonial phase, characterized by successive mitotic divisions of undifferentiated spermatogonia (Aund* and Aund), differentiated spermatogonia (Adiff), and type B spermatogonia (SpgB); (2) meiotic or spermatocyte phase, in which the genetic material of spermatocyte is duplicated, recombined, and segregated, forming haploid cells, the spermatids; (3) and the phase called spermiogenesis, in which spermatids undergo highly complex structural and functional modifications to become spermatozoa capable of fertilization (Nóbrega et al., 2009; Schulz et al., 2010; Nóbrega & Lareyre, 2024) (Figures 2 and 3).

Spermatogonial or proliferative phase

Type A undifferentiated spermatogonia (Aund* and Aund) are found isolated in the germinal epithelium, surrounded by Sertoli cells (Nóbrega, Batlouni & França, 2009; Schulz et al., 2010). These cells are the biggest cells of the germinal lineage, with nuclear diameter and cellular volume of around 8.6 μm and $\sim 667 \mu\text{m}^3$, respectively (Leal et al., 2009). The chromatin is not completely condensed, and one or two compact nucleoli are present (Leal et al., 2009). Type Aund* spermatogonia are distinguishable from other spermatogonia by the irregularity of



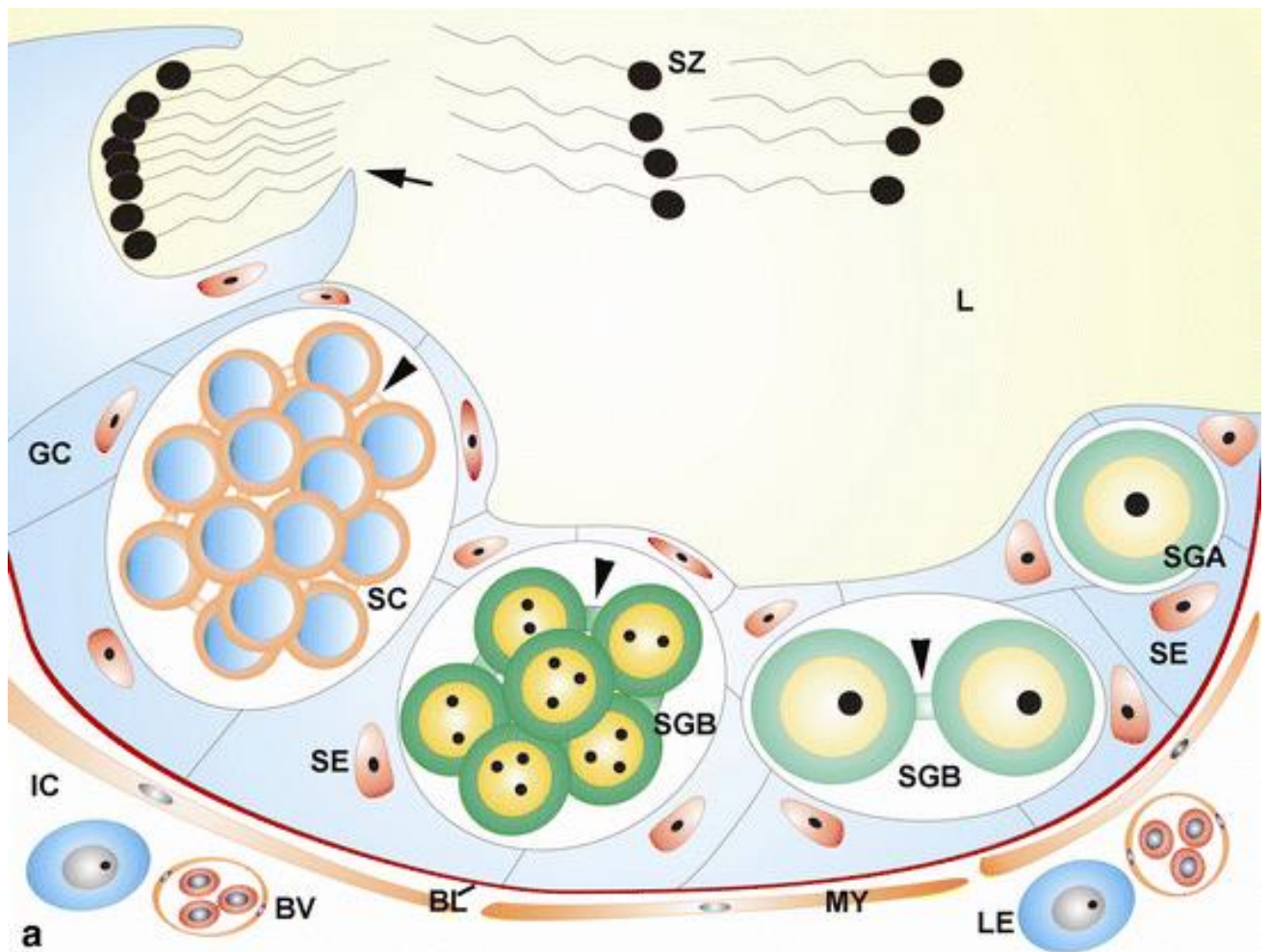
their nuclear envelope and greater nucleus-cytoplasm ratio. Additionally, by incorporation of BrdU (5'-bromo-2'-deoxyuridine), a synthetic nucleotide analogous to thymidine, during the synthesis phase (S phase), it is feasible to determine the presence of putative stem spermatogonia, known as "labeling-retaining cells", that retained BrdU for a long-term. These purported cells are among the undifferentiated spermatogonia (Nóbrega et al., 2010). Differentiated spermatogonia (Adiff) arises from mitosis with incomplete cytokinesis. These cells form cysts comprising two to eight cells. Type Adiff spermatogonia are smaller than the preceding categories, with an average nuclear diameter of 6 μm . They have a regular, rounded form, with one, two, or more nucleoli. Heterochromatin has concentrated spots along the nucleus (Leal et al., 2009). The type B early spermatogonia are more compact, with an elongated nucleus and one or two nucleoli, and the amount of heterochromatin increases. The type B late spermatogonia includes a greater number of cells within the cyst. The nucleus is spherical, dense, and with condensed chromatin as compared to the previous generation of spermatogonia (Leal et al., 2009) (Figures 2 and 3).

Meiotic phase

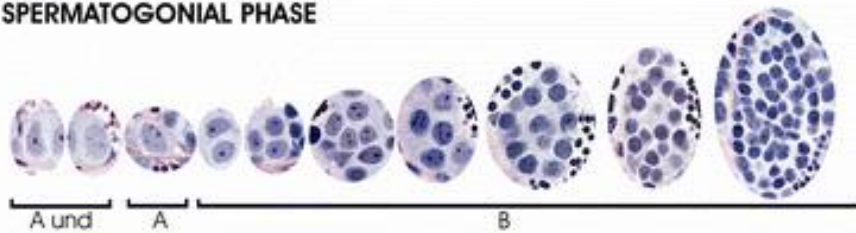
Spermatocytes have an average nuclear diameter of 5 μm and are at various stages of meiosis. They may be identified and distinguished by nuclear size, chromatin condensation, and meiotic figures. Leptotene/zygotene cells are more spherical than type B spermatogonia, and chromatin contains tiny spots near the nuclear envelope. Spermatocytes in pachytene are bigger, with a denser nucleus and homologous chromosomes synapse and chromatin organized in a tight loop-axis array. Spermatocytes are observed in diplotene together with metaphase I figures. In this cell type, the chromosomes reach their highest level of condensation. Finally, secondary spermatocytes are rare because they quickly enter meiosis II. This cell type has a rounded nucleus and dense chromatin (Leal et al., 2009) (Figures 2 and 3).

Spermiogenic phase

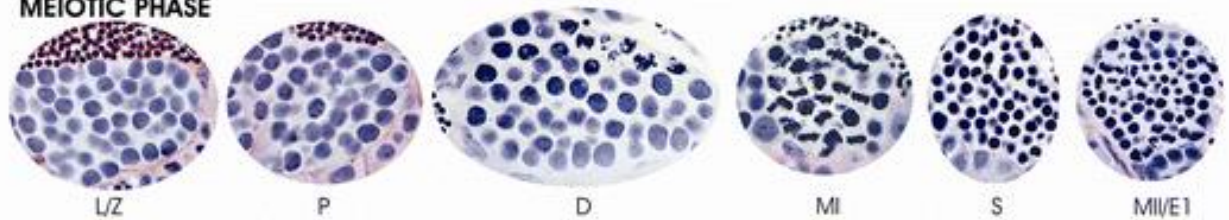
During spermiogenesis, the nuclear volume of the cells is reduced, and the classification of three types of spermatids: initial (E1), intermediate (E2), and final (E3) is based on the increase in nuclear compaction, decrease in cell volume, and spacing between cells due to the formation of flagella (Leal et al., 2009) (Figures 2 and 3).



SPERMATOGONIAL PHASE



MEIOTIC PHASE



SPERMIOGENIC PHASE

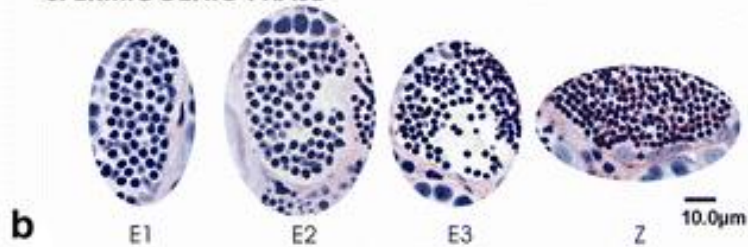


Figure 2. The basic organization of the fish testis **(a)** here was also observed in Figure 1B, and zebrafish testis showed the three phases of spermatogenesis in fish **(b)**. **(a)** – Tubular or germinal compartment (GC) and the intertubular or interstitial compartment (IC). Leydig cells (LE), blood vessels (BV); myoid cells (MY), basal lamina (BL), primary or type A spermatogonia (SGA), Sertoli cells (SE); cytoplasmatic bridge (arrowheads), type B spermatogonia (SGB), spermatocytes (SC), spermiation (arrow), spermatozoa (SZ), lumen (L). **(b)** – Cellular progression of germ cells during spermatogenesis: Primitive/type A undifferentiated spermatogonia (A und), primary/type A differentiated spermatogonia (A), secondary/type B spermatogonia (B), primary spermatocytes at leptotene/zygotene (L/Z), pachytene (P), diplotene (D), metaphase I (MI), secondary spermatocytes (S), metaphase II/early spermatids (MII/E1), early spermatids (E1), intermediate spermatids (E2), late spermatids (E3), and spermatozoa (Z). The figure presented is derived from the study conducted by Nóbrega, Batlouni & França (2009).

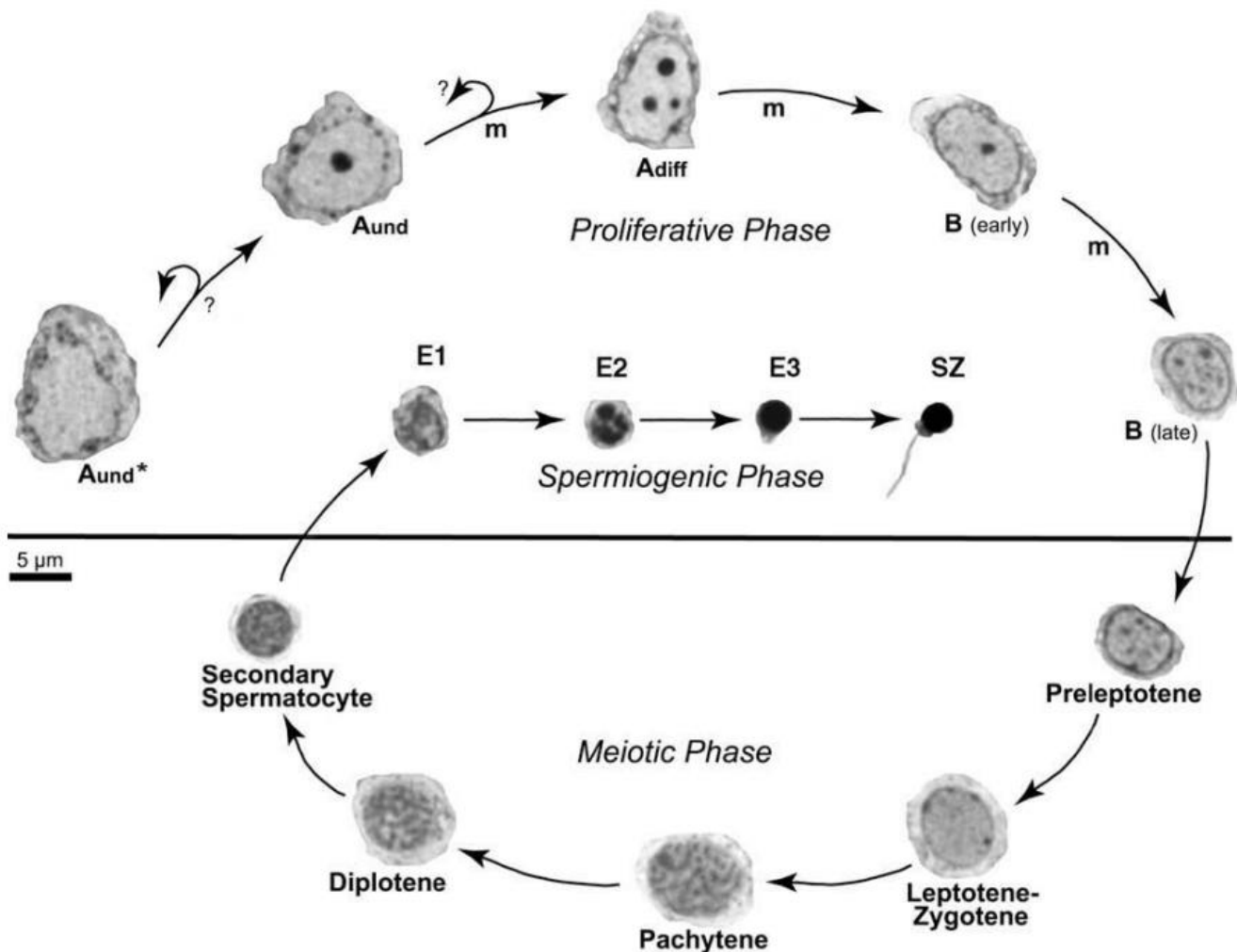


Figure 3. Detailed zebrafish testis showing the three phases of spermatogenesis in fish. Type A undifferentiated spermatogonia (Aund* and Aund), type A differentiated spermatogonia (Adiff), type B spermatogonia (B), self-renew (curved arrows), mitosis (m), early spermatids (E1), intermediate spermatids (E2), late spermatids (E3), and spermatozoa (SZ). The figure presented is derived from the study conducted by Schulz and colleagues (2010).

1.3 Endocrine control of spermatogenesis: hypothalamic-pituitary-gonadal (HPG) axis

Reproduction is essential for the survival and evolution of species (Osugi et al., 2014). It requires the coordination of the nervous and endocrine systems, as well as various external and internal clues (Meethal et al., 2005; Habibi & Andreu-Vieyra, 2007). In fish and other vertebrates, the reproductive process is controlled by the hypothalamic-pituitary-gonadal (HPG) axis, which involves a series of hormonal signals and cascades. The axis begins with the release of gonadotropin-releasing hormone (GnRH) from the hypothalamus (Meethal et al., 2005; Habibi & Andreu-Vieyra, 2007) and is stimulated by kisspeptin (Kiss). GnRH was first identified and isolated in pigs (Baba et al., 1971). It is a decapeptide with several isoforms made up of 10 amino acids. This neuropeptide is essential for the activation of the HPG axis because it stimulates pituitary cells that produce and release gonadotropins (Fsh and Lh) (Pankhurst, 2016), which then act on the gonads to regulate gametogenesis and steroidogenesis (Figure 4). Interestingly, the mechanism by which GnRH reaches gonadotropin cells differs between teleosts and other vertebrates. GnRH is released in the median eminence, and a portal-hypophyseal vascular system is responsible for transporting the neurohormones to the gonadotrophic cells through the bloodstream (Fink, 1988). In teleosts, GnRH is secreted near and directly into gonadotrophic cells. The terminal of neuron fibers in the pituitary connects with pituitary cells, and together these fibers form the neurohypophysis (Yamamoto et al., 1995).

Teleost fish have three distinct forms of GnRH, which are located in different regions of the brain. In contrast, most vertebrates produce only two types of GnRH (Kawai et al., 2009). GnRH3 has specifically been detected in terminal nerve ganglia in fish, and it is postulated to function as a neuromodulator. GnRH2 is conserved across all species studied thus far and is thought to modulate reproductive processes. Conversely, GnRH1, the hypothalamic variant, is synthesized in the preoptic region and stimulates the pituitary gland (Kuo et al., 2005; Kawai et al., 2009). Additionally, another neuropeptide involved in the regulation of gonadotropin

synthesis and release, known as gonadotropin-inhibitory hormone (GnIH), was initially discovered in birds and later identified in fish (Tsutsui et al., 2010; Zohar et al., 2010). GnIH has direct effects on the pituitary gland but also exerts modulatory effects on GnRH. These neuropeptides collectively play a significant role in the control of reproduction.

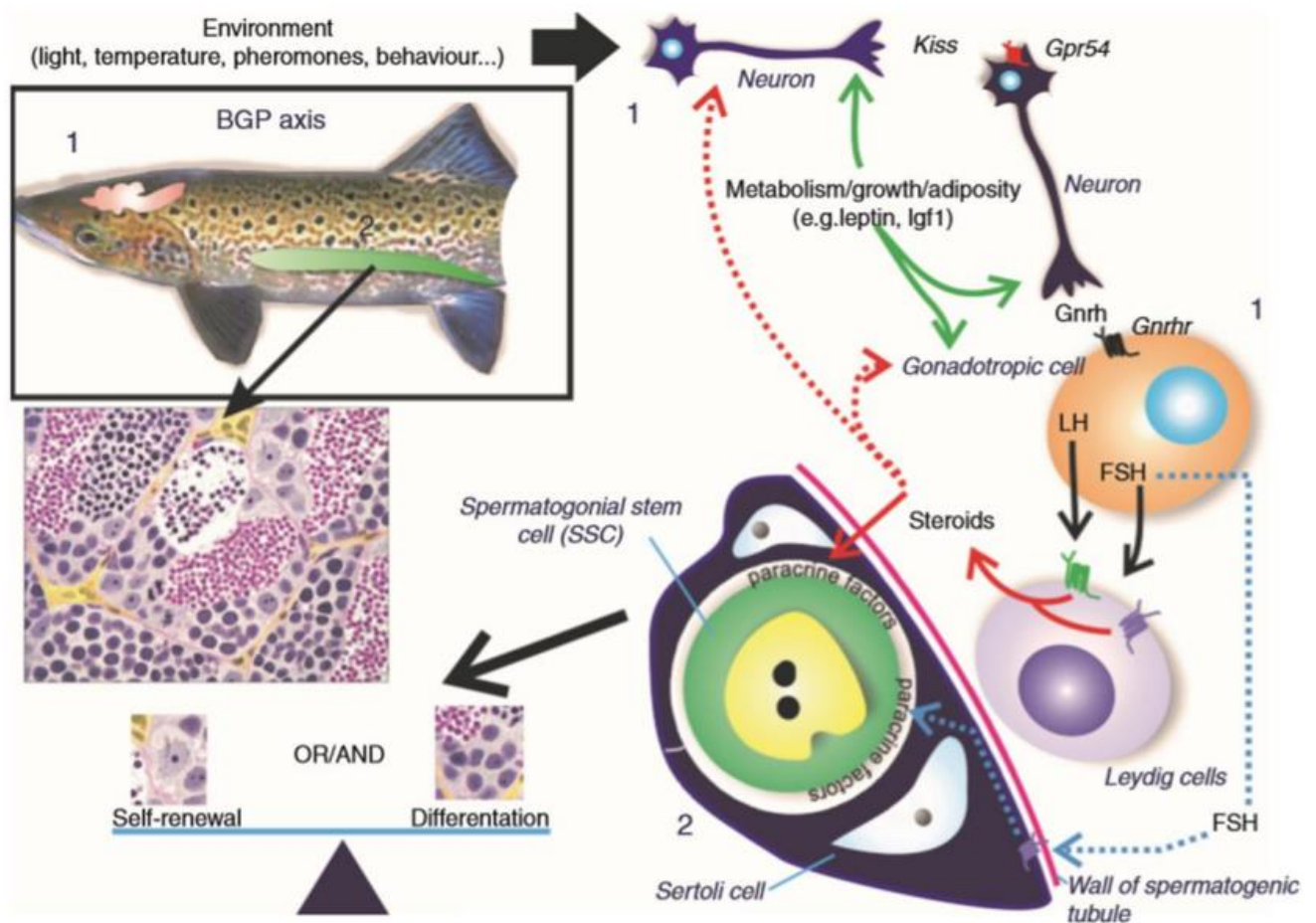


Figure 4. Illustration of the reproductive axis in fish. The initiation of the process is determined by environmental stimuli, such as water temperature and photoperiod, followed by a cascade of hormones along the hypothalamic-pituitary-gonadal (HPG) axis. This cascade begins with Kiss neurons in the hypothalamus, which stimulate the production of gonadotropin-releasing hormone (GnRH) through their receptors (Gpr54). GnRH then acts on the pituitary cells, stimulating the production and release of gonadotropins (follicle-stimulating hormone [Fsh] and luteinizing hormone [Lh]). Both Fsh and Lh are transported through the bloodstream and act on their target cells in the testes. In turn, Fsh and Lh stimulate the production and release of steroids. These steroids have a negative feedback effect on the hypothalamus and pituitary



(represented by the dashed line). Fsh also regulates the development of spermatogenesis stem cells through the production of paracrine factors produced by Sertoli cells. (1) represents the nervous system, composed of the hypothalamus and pituitary, while (2) indicates the testes. Additionally, the figure depicts the influence of internal factors, such as metabolism, growth, and adiposity, on the Kiss and GnRH-producing neurons. The figure presented is derived from the study conducted by Schulz & Nóbrega (2011).

1.4 Follicle-stimulating hormone (Fsh) and its role in fish spermatogenesis

GnRH stimulates the pituitary gland to produce and release gonadotropins (Fsh and Lh) (Pankhurst, 2016; Nóbrega & Lareyre, 2024), which then act on the gonads to regulate the production of gametes and hormones. The gonadotropins, follicle-stimulating hormone (Fsh), and luteinizing hormone (Lh) are glycoproteins composed of two different subunits, the α subunit, which is common to both hormones, and the β subunit, which is specific to the biological activity of each hormone (Schulz et al., 2001; Nóbrega & Lareyre, 2024). These two gonadotropic hormones play a crucial role in the regulation of spermatogenesis (Pierce & Parsons, 1981).

Both gonadotropins are precisely defined in mammals due to the highly particular interactions between each hormone and its receptor (McLachlan et al., 1996). Lh acts on Leydig cells, promoting androgen synthesis and release. While Fsh modulates Sertoli cell function, providing structural, nutritional, and regulatory support for germ cell development (Huhtaniemi & Themmen, 2005). However, in fish, gonadotropin-receptor interactions do not appear to be specific (Sambroni et al., 2007). Studies on salmon show that, while the Fsh receptor prefers Fsh, it also has an affinity for Lh (Miwa et al., 1994). Furthermore, in fish, Fsh is regarded more effective than Lh in promoting androgen production, due to the existence of the receptor Fsh in Leydig cells (Planas et al., 1993), as described in *Clarias gariepinus* (García-Lopez et al., 2009) and *Danio rerio* (García-Lopez et al., 2010). Nóbrega and colleagues (2015) found that Fsh in Sertoli cells in fish promotes spermatogonial proliferation and differentiation through the production of insulin-like growth factor 3 (Igf3). At the same time, Fsh reduced the expression of the antimüllerian hormone (Amh), which is seen in Sertoli cells associated with type A undifferentiated spermatogonia (Skaar et al., 2011). Amh is known to block the differentiation of type A undifferentiated spermatogonia and the synthesis of steroids mediated by Fsh (Skaar et al., 2011; Adolphi et al., 2018). As a result, Fsh serves as a crucial regulator of



the spermatogenic process stimulating and inhibiting components required for its development. Crespo and colleagues (2016) provided another example of the relevance of Fsh in the spermatogenic process in fish. The authors demonstrated that Fsh enhanced spermatogonial proliferation while modulating multiple signaling networks. Additionally, this gonadotropin had a considerable impact on metabolic genes such as lactate and fatty acid metabolism, as well as Sertoli cell barrier components (Crespo et al., 2016). Furthermore, research with salmonids demonstrated that higher plasma Fsh levels are associated with higher levels of androgens as well as increased spermatogonia proliferation and differentiation activity (Campbell et al., 2003; Melo et al., 2014). These findings support that Fsh is significant in the early phases of spermatogenesis, particularly in regulating the spermatogonial phase.

1.5 Mechanism of action of thyroid hormones (THs)

The proper functioning of the hypothalamic-pituitary-thyroid (HPT) axis relies on the release of thyrotropin-releasing hormone (TRH) by the hypothalamus. TRH, in turn, triggers the release of thyroid-stimulating hormone (TSH) (Larsen et al., 1998; Flik et al., 2006). In fish and reptiles, for example, the release of Tsh is facilitated by the corticotropin-releasing hormone (Crh or Crf) (Flik et al., 2006; Tovo-Neto et al., 2018). Additionally, Crf is also responsible for the release of adrenocorticotrophic hormone (Acth), which stimulates the synthesis and secretion of cortisol by the interrenal gland in fish (Larsen et al., 1998; Flik et al., 2006). Cortisol is the primary glucocorticoid present in the circulatory system of both humans and teleost fish, playing a key role in mediating the physiological stress response (Faught et al., 2016). Tsh stimulates the thyroid follicles, facilitating the synthesis of tetraiodothyronine (T4), which serves as the principal thyroid hormone (Flik et al., 2006). T4 undergoes metabolism and conversion into triiodothyronine (T3), the biologically active form that plays a crucial role in different physiological processes, including growth and reproduction (Basset et al., 2003; Wagner et al., 2008; Habibi et al., 2012; Flood et al., 2013; Rodrigues et al., 2022) (Figure 5).

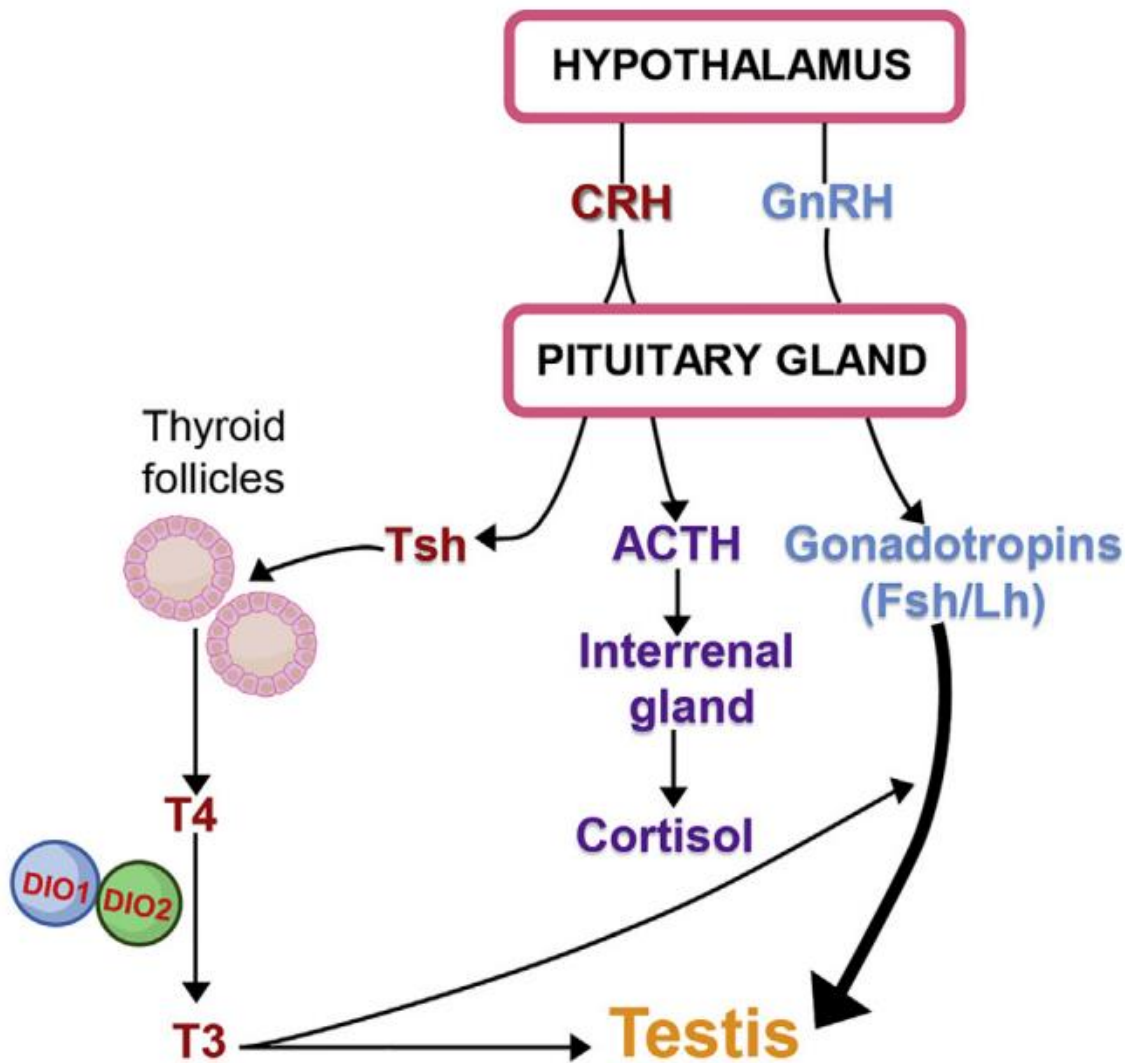


Figure 5. hypothalamic-pituitary-thyroid (HPT) axis in fish. The pituitary gland releases the thyroid-stimulating hormone (Tsh), which is crucial in regulating the thyroid gland. Additionally, the corticotropin-releasing hormone (Crh) stimulates adrenocorticotrophic hormone (Acth) production. Acth, in turn, facilitates the adrenal gland synthesis and release of cortisol. In fish, Tsh stimulates the secretion of thyroxine (T4) within the thyroid follicles. T4 is then converted to triiodothyronine (T3) by the deiodinase enzymes, DIO1 and DIO2. The resulting T3 exerts its influence on the reproductive axis. The figure presented is derived from the study conducted by Tovo-Neto and colleagues (2018).

The production and metabolism of THs occur through the action of three enzymes known as iodothyronine deiodinases type I, II, and III (Figure 6) (Duarte-Guterman et al., 2014).

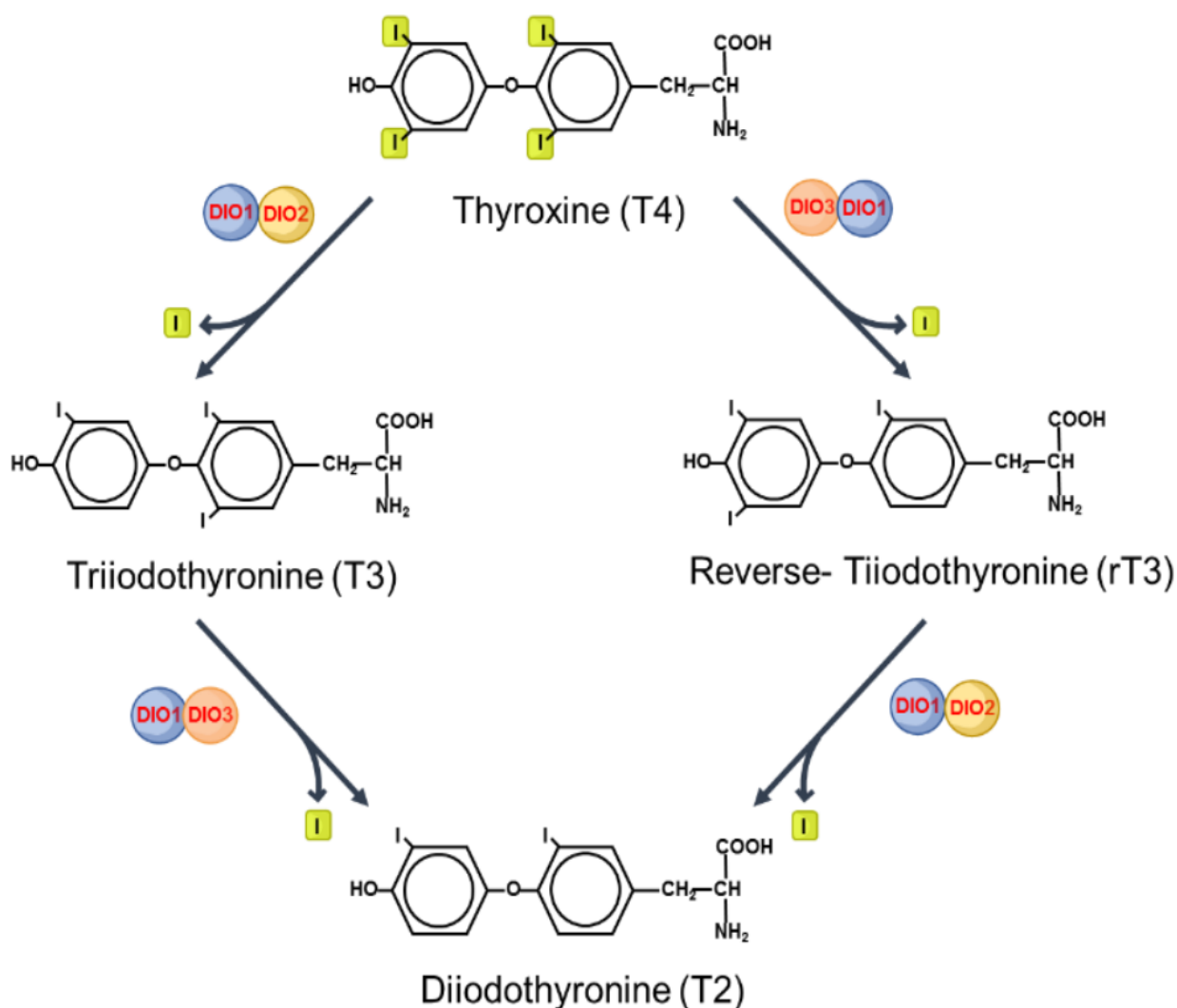


Figure 6. Deiodinases play an important role in the metabolism of the thyroid hormone T3. Type I and type II iodothyronine deiodinases (DIO1 and DIO2) convert thyroxine (T4), the main product released by thyroid follicles, into the bioactive T3. Furthermore, DIO1 also plays a crucial role in the production of rT3, which is the inactive form of the hormone T3. The figure presented is derived from the study conducted by Tovo-Neto and colleagues (2018).

The type I deiodinase (DIO1) catalyzes the deiodination of the outer ring of T4, producing the bioactive form, T3. DIO1 also deiodinates the inner ring of T4, converting it into



reverse T3 (rT3), the inactive form. On the other hand, type II deiodinase (DIO2) acts on the activation of THs by converting T4 into T3 (Duarte-Guterman & Trudeau, 2000). The expression and activity of the deiodinase enzymes, which are responsible for the production of the bioactive forms of THs, are regulated according to the specific needs of each tissue (Flood et al., 2013).

The receptors of THs ($TR\alpha$ and $TR\beta$) are nuclear components that act as nuclear transcription factors. Circulating levels of T4 and T3 are transported to target cells by specific transporters. They form a hormone-receptor complex capable of triggering cellular responses through binding to the regulatory region of promoters known as thyroid hormone response elements (TREs) in target genes. In this way, THs modulate the expression of target genes, both positively and negatively (Mendis-Handagama et al., 2005; Wagner et al., 2009; Habibi et al., 2012).

In mammals, the interaction between T3 and the nuclear receptors TRs (thyroid hormone receptors) is highly specific and occurs with a strong affinity to regulate gene expression (Yen, 2006; Nelson & Habibi, 2008). In the absence of T3, $TR\alpha$ and $TR\beta$ form homodimers or heterodimers and bind to the TREs. They then interact with co-repressor proteins such as nuclear receptor co-repressor (NCoR) or silencing mediator for retinoic acid and thyroid hormone receptor (SMRT), which leads to modifications in the chromatin structure near the promoter region and repression of transcription (Yen, 2006). Conversely, in the presence of T3, the co-repressor proteins dissociate from TRs and instead associate with co-activator proteins, forming a co-activator complex that stimulates gene transcription (Yen, 2006; Walter et al., 2019) (Figure 7). Additionally, T3 can also exert negative regulation on the transcription of TRH and TSH (Friesema et al., 2005; Yen, 2006; Wagner et al., 2009; Habibi et al., 2012), although the mechanisms underlying this action are still not fully understood. According to Nelson and Habibi (2008), there is limited research characterizing TREs and their relationship with THs in fish.

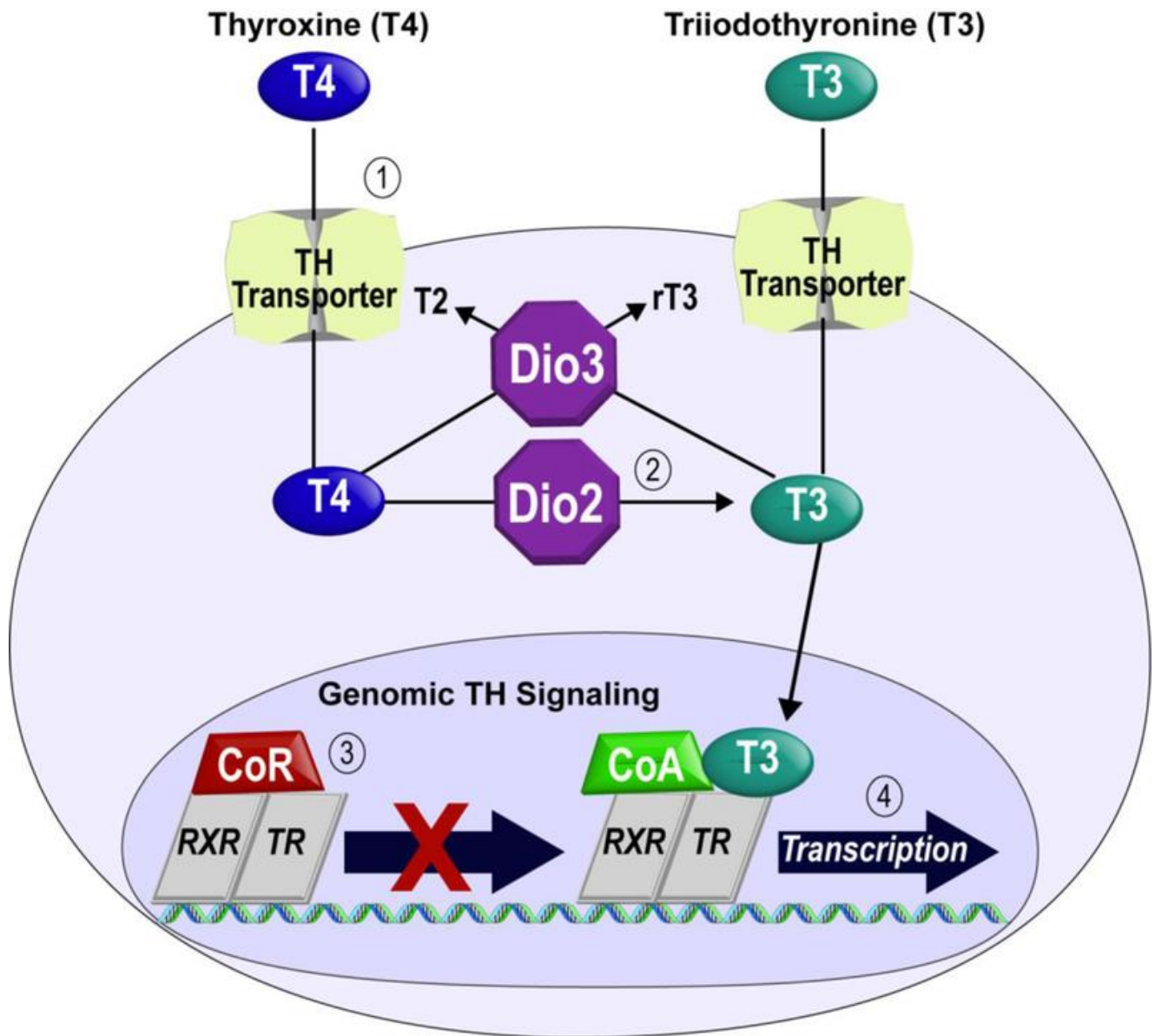


Figure 7. TH signaling. **(1)** Circulating levels of T4 and T3 are transported to target cells by specific transporters. **(2)** T4 is converted to the active T3 by deiodinase type II (DIO2). T4 and T3 can also be converted to rT3 and T2, respectively, by DIO3. **(3)** In the nucleus, the thyroid hormone receptor (TR) is located on DNA, usually as a heterodimer with the retinoic-X-receptor (RXR). Without a T3 ligand, a co-repressor (CoR) prevents transcription. **(4)** When T3 translocates into the nucleus and binds to the TR-RXR heterodimer, the CoR dissociates and a co-activator (CoA) is recruited, inducing transcription of genes responsive to THs. The figure presented is derived from the study conducted by Walter and colleagues (2019).

1.6 Crosstalk between thyroid hormones and reproduction in fishes



THs are vital for regulating metabolism, growth, and development in adult vertebrates (Yen, 2001). They impact several specific homeostasis-related activities, including metabolic rate regulation, glucose mobilization, heat production, heart function, fat metabolism, stress response, nervous system development, and reproduction (Yen, 2001; Yamano, 2005; Marelli & Persani, 2017; Balasch & Tort, 2019; Rodrigues et al., 2022).

In recent decades, there has been increasing interest in the investigation of the role of THs in reproduction, emphasizing its significance in the sexual differentiation, gonadal development, and maturation of vertebrates (Kirby et al., 1996; Tovo-Neto et al., 2018; Houbrechts et al., 2019). In male rodents, T3 plays a vital role in the regulation of testicular growth and maturation, as well as in the control of Sertoli and Leydig cell proliferation and differentiation during testicular development (Cooke et al., 2005; Meroni et al., 2019). Cooke and colleagues (2005) showed that thyroid alterations affect spermatogenesis development in rats. Neonatal animals' exposure to 6-propyl-2-thiouracil (PTU), an inhibitor of THs production, resulted in increased testicular size and greater spermatozoa production in adulthood (Cooke, 1991). These effects are caused by increased seminiferous tubule area and interstitial components (Hess et al., 1993) and Sertoli cell proliferation (Hess et al., 1993). Studies explained that this effect occurs due to the delay in the transition from the proliferative phase to the maturation phase of Sertoli cells, resulting in the prolonged mitosis of these cells and, consequently, delaying their differentiation and maturation (Hess et al., 1993; Cooke et al., 1994). The immaturity of Sertoli cells delays the opening of the seminiferous tubules and impairs their ability to support the more advanced stages of germ cells (França et al., 1995).

The intricate relationship between thyroid hormones and reproduction in teleost fish has been recognized (Tovo-Neto et al., 2018; Deal & Volkoff, 2020; Rodrigues et al., 2021; 2022; Habibi & Raine, 2024) (Figure 8). Therefore, understanding the intricacies of this crosstalk is pivotal in elucidating the underlying mechanisms governing reproductive processes. Previous research has examined the impact of T3 on adult fish reproduction (Habibi et al., 2012). T3 dosages (25 and 250 ng/fish) resulted in a significant decrease of *lh β* and *fsh β* mRNA levels (Nelson et al., 2010). These results suggest that T3 can negatively affect reproductive function. THs regulate metabolism and growth (Hulbert, 2000), which can divert energy from reproduction and promote growth functions, resulting in reduced Lh and Fsh production and levels (Habibi et al., 2012).



Matta and colleagues (2002) investigated the consequences of transitory neonatal hypothyroidism in juvenile Nile tilapia (*Oreochromis niloticus*). The size of the testes in these animals doubled, as observed in rats (Joyce et al., 1993). These results suggest that transitory neonatal hypothyroidism in fish increased Sertoli cell proliferation, which in turn increased germ cell proliferation, affecting testicular size. Houbrechts and colleagues (2019) investigated the impact of the absence of THs in zebrafish, specifically caused by the genetic deletion of type 2 deiodinase (DIO2), on androgen levels in the testis, and subsequently reproductive activity in this species.

1.7 Role of thyroid hormones in the somatotropic axis

Adequate THs (T4 and T3) levels are required to regulate the metabolic process for appropriate growth and development in adult vertebrates (Yen, 2001). The proper levels of THs promote homeostasis of cardiac function, lipid metabolism, and muscle physiological processes such as myogenesis and regeneration (Yen, 2001; Yamano, 2005; Brent, 2012; Mullur et al., 2014; Marelli & Persani, 2017; Bloise et al., 2018; Balasch & Tort, 2019). THs dysregulation affects the somatotropic axis in different levels. In the hyperthyroidism conditions, which are characterized by elevated THs levels, there is a state of hypermetabolism. In this condition, there is an increase in energy expenditure, weight loss, and cholesterol levels are reduced, while lipolysis and gluconeogenesis increase (Brent, 2008). However, hypothyroidism has the opposite effect (Brent, 2012). In this way, THs regulate the primary metabolic pathways that control the balance of energy storage and expenditure. THs regulate metabolism through molecular mechanisms, lipid metabolism regulation, nuclear receptor interaction, and thermogenesis, and are characterized by actions on the brain, fat, liver, and skeletal muscle (Figure 8) (Mullur et al., 2014).

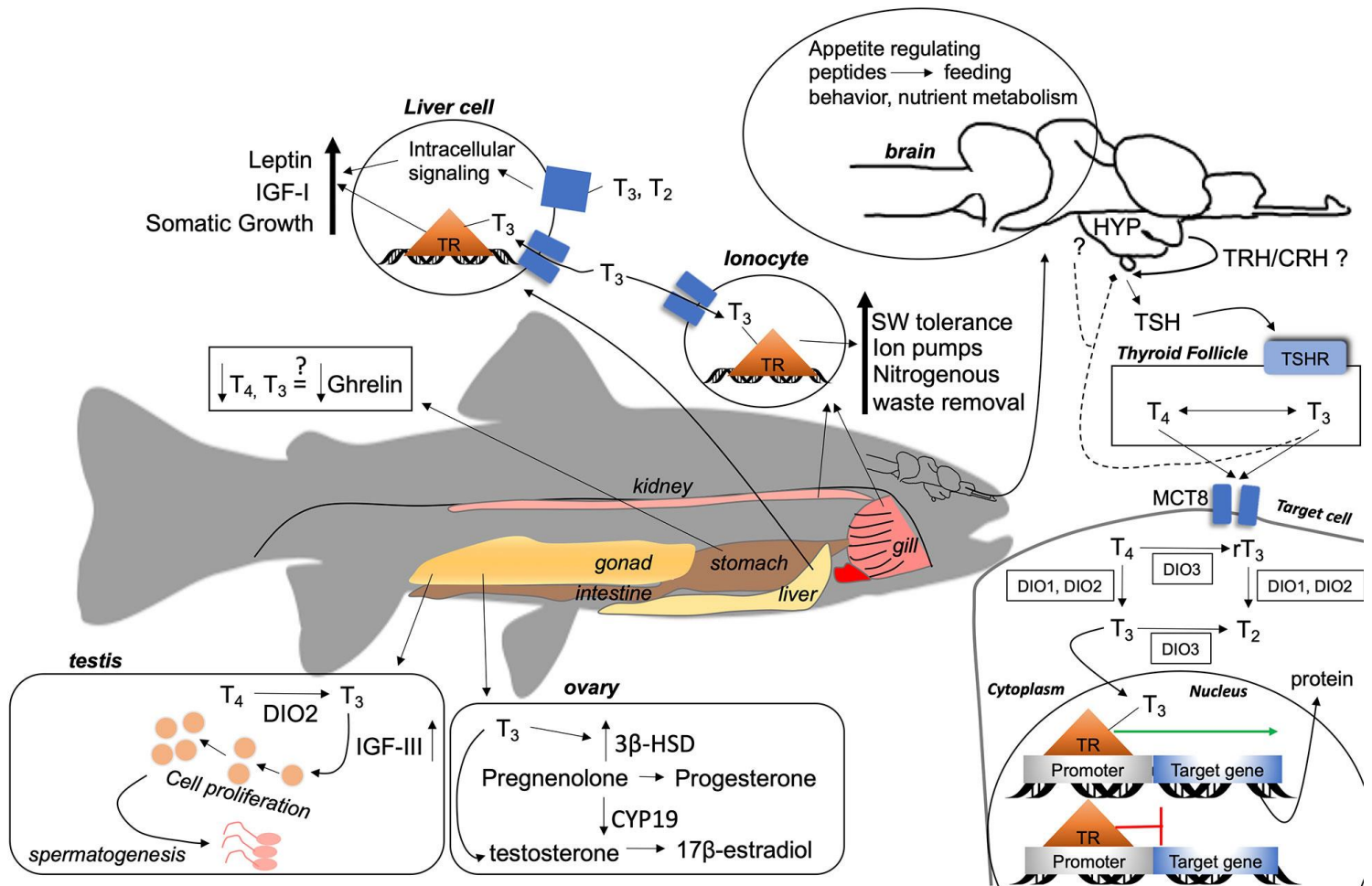


Figure 8. An overview of the general actions of THs in fish. Corticotropin-releasing hormone (CRH) stimulates the pituitary to release TSH, which binds to the TSH receptor (TSHR) on the thyroid follicles. Intracellular processes produce T4 and T3 that enter the circulation to target cells (solid line) or feedback (dashed line) to the hypothalamus-pituitary axis. THs enter target cells through the membrane transporters, MCT8, where bioactivation of T4 to T3 occurs via the action of deiodinases type 1 and type 2 (DIO1 and DIO2), or metabolism of rT3 or T2 through DIO1, DIO2, or DIO3. THs enter the target cell nucleus from the cytoplasm and bind to TRs located on promoter regions of THs response elements (TRE). When T3 is bound, gene transcription occurs (green arrow), in the contrary, transcription is repressed (red line). THs may act on different tissues in fish, as demonstrated by general mechanisms in central and peripheral tissues. Up arrows indicate that THs increase activity, and down arrows indicate repression of synthesis/production. HYP (hypothalamus), TRH (thyrotropin-releasing hormone), CRH (corticotropin-releasing hormone), TSH (thyrotropin), TSHR (thyrotropin



receptor), MCT8 (monocarboxylase transporter 8); T4 (thyroxine), T3 (triiodothyronine), rT3 (reverse triiodothyronine), T2 (diiodothyronine), DIO1, DIO2, DIO3 (deiodinases type I, II and III, respectively), TR (thyroid receptor), IGF-I and IGF-III (insulin-like growth factor I and III, respectively); 3 β -HSD, 3 β -hydroxysteroid dehydrogenase, CYP19 (aromatase). The figure presented is derived from the study conducted by Deal and Volkoff (2020).

Components of the HPT axis also affect the GH/IGF-1 axis in vertebrates (Deal & Volkoff, 2020). Growth hormone (GH) acts on tissue both directly and indirectly by stimulating and releasing insulin-like (IGF-1 and IGF2) from the liver, which stimulates cell proliferation and differentiation (Blanco, 2020). THs enhance the liver's IGF-1 synthesis and release (Blanco, 2020). T3 has been shown to stimulate *igf1* mRNA levels in tilapia (Schmid et al., 2003) and zebrafish (Wang & Zhang, 2011), both *in vivo* and *in vitro*, respectively.

Furthermore, THs play an important role in skeletal muscle metabolic processes, including muscle contraction, metabolism, myogenesis, regeneration, and regulation of protein synthesis and degradation (Simonides & Van Hardeveld, 2008; Salvatore et al., 2014; Bloise et al., 2018; Koganti et al., 2021). The HPT axis must work properly to control T4 and T3 levels ensuring homeostasis in these crucial processes (Ortiga-Carvalho et al., 2016; Koganti et al., 2021). The type 2 deiodinase (DIO2) – an enzyme required for the intracellular uptake of T3 – is crucial in regulating T3 levels in muscle tissue. This regulation enhances the final stages of myogenic differentiation, specifically targeting MyoD and MyoG (Marsili et al., 2011; Dumont et al., 2015). Myogenesis begins early in fish embryogenesis, similar to that observed in birds and mammals (Johnston et al., 2011). This process is initiated by the transcription factor paired-box Pax7, which promotes the expression of myogenic regulatory factors (MRFs) (Dumont et al., 2015). These MRFs, including Myf5 (myogenic factor 5) and MyoD, are crucial for driving myogenesis forward and regulating the expression of MyoG. MRFs and MyoG work in concert with other transcript factors to induce and sustain differentiation and the initiation of muscle-specific gene expression (Hinitz et al., 2009).

1.8 Maternal origin of hormones

The production of fertile eggs with the capacity to develop into larvae and marketable fish is crucial to maintaining the aquaculture industry. An egg is the result of oocyte development and differentiation. Oocyte development and maturation share basic



morphological and physiological aspects between fish species. Detailed descriptions of these processes may be found in different reviews (Selman et al., 1993; Clelland & Peng, 2009; Lubzens et al., 2010; 2017). Briefly, the dynamics of oogenesis include the formation of PGCs and their transformation in oogonia, followed by five stages of follicle development that result in ovulation in zebrafish (Selman et al., 1993), which occurs over 10 days.

According to Selman and colleagues (1993), and Clelland and Peng (2009), Stage I, or the primary growth phase is initiated when oocytes begin to enlarge, and follicles start to form. Oocytes progress through the early stages of the first meiotic division and become arrested in prophase I. In stage II or cortical alveolus, yolk vesicles accumulate within the oocytes. Stage III oocytes undergo vitellogenesis, increasing the size of follicles. In stage IV or oocyte maturation, meiosis is resumed, the germinal vesicle migrates from the center of the oocyte to the periphery and the nuclear membrane breaks down. Finally, in stage V, mature eggs are ovulated and ready for spawning (Figure 9). At the end of this process, the oocytes become competent to undergo fertilization and contain maternal mRNAs, proteins, lipids, carbohydrates, vitamins, and hormones that are vital for proper embryo development and high survival of offspring to juvenile and growth (see Lubzens et al., 2010; 2017).

Studies have shown that maternal contributions to the development of embryos, including transcripts are important to regulate cell division and determine oocyte polarity, pattern development during early and late embryonic stages, and the transition from maternal to zygotic gene expression and translation (Lubzens et al., 2017). After ovulation, fertilization, and egg activation, the embryonic developmental program begins. In zebrafish, Lee and colleagues (2013) demonstrated that maternal factors are crucial for development and trigger zygotic genome activation (ZGA) at the maternal-to-zygotic transition (MZT). ZGA is required for gastrulation and clearance of maternal messenger RNAs. In this study, the investigators showed that *Nanog*, *SoxB1*, and *Pou5f1* are the most highly translated sequence-specific transcription factors in the pre-MTZ transcriptome. These factors are involved in maintaining pluripotency of embryonic stem cells (Onichtchouk, 2012). Also, *Pou5f1* in zebrafish provides control of gene expression and together with *SoxB1* regulates neural development (Okuda et al., 2010), whereas *Nanog* is important for endoderm formation (Xu et al., 2012). Furthermore, Lee and colleagues (2013) also demonstrated that *Nanog*, *SoxB1*, and *Pou5f1* directly regulate the microRNA *miR-430*, which is responsible for the clearance of maternal mRNAs during the MZT of gene expression, and enhances reprogramming efficiency (Subramanyam et al., 2011; Lee et al., 2014; Lubzens et al., 2017). However, data on the roles of miRNAs in oogenesis and



oocyte development are scarce, and further studies are needed to understand their functions in maternal transfer and embryo/larval and juvenile development.

In addition to the factors mentioned above, hormones related to stress and metabolism, such as cortisol and THs, also have a role in early development (Peter, 2011). Studies have demonstrated the role of cortisol on fish offspring development (Nesan & Vijayan, 2013; Faught et al., 2016; Rodrigues et al., 2025). Cortisol is deposited in the embryos by maternal transfer, and gradually decreases during embryogenesis (Nesan & Vijayan, 2016). The maternal cortisol concentration transfer to embryos influences development, including activation of the hypothalamic-pituitary-interrenal (HPI) axis (Nesan & Vijayan, 2016). However, THs also have a vital function for the progenies (Power et al., 2001). In oviparous fish, there is evidence that maternal THs are transferred (Lam, 1994). Fathead minnow and zebrafish eggs display high TH levels and high transcript levels of thyroid-related transcriptions, including thyroid hormone receptors, and deiodinases (Vergauwen et al., 2018) before 72 -hpf, when the endogenous TH synthesis begins. In alligator gas (*Atractosteus spatula*), injecting females with THs or Tsh increased the concentration of T4 and T3 in early embryos (Castillo et al., 2015). Additionally, maternal injections or egg immersion have been shown to enhance the hatching, larval growth rate, muscle development, and metabolic capacity of important commercial fish, *Brycon orbignyanus* (Landines et al., 2010) and *B. amazonicus* (Urbinati et al., 2008). However, the molecular, biochemical, and physiological mechanisms to produce high-quality eggs by maternal transfer remain unclear to our knowledge.

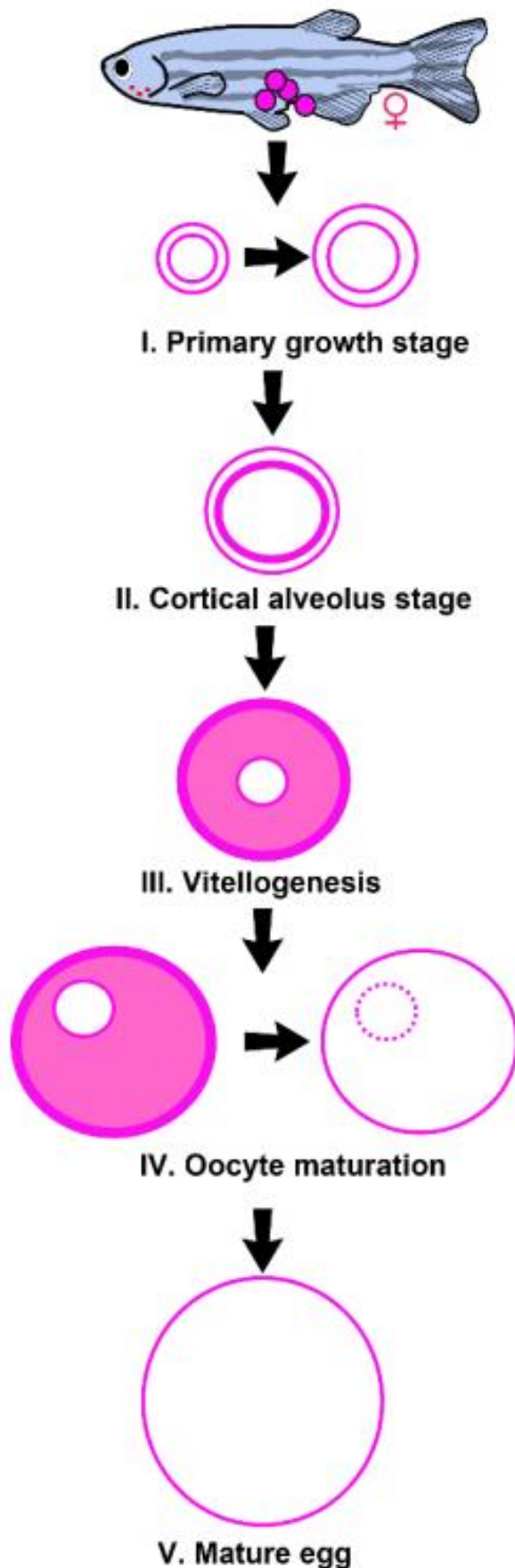


Figure 9. Schematic drawing of stages of follicle development in zebrafish. **Stage I/Primary growth stage:** the oocytes begin to enlarge, and follicles start to form. **Stage II/cortical alveolus stage:** cortical alveolus accumulates within the oocytes. **Stage III/Vitellogenesis:** follicles undergo vitellogenesis, resulting in a significant increase in their sizes. **Stage IV/Oocyte maturation:** germinal vesicle (dashed circle) migrates from the center of the oocyte to the periphery and the nuclear membrane breaks down. **Stage V/Mature eggs:** eggs are ovulated and read for spawning. Adapted from Selman and colleagues (1993), and Clelland & Peng (2009) (Illustration created by the author).



1.9 Zebrafish as a model organism in reproductive biology

D. rerio, commonly known as zebrafish, is a small freshwater teleost native to India, belonging to the family Cyprinidae and the order Cypriniformes. This species is highly valued in laboratory research due to its small size and ease of maintenance in high population densities. Zebrafish exhibit external fertilization, which facilitates the production of large numbers of offspring, and their transparent embryos allow direct observation and monitoring during embryogenesis (Kimmel, 1995; Nasiadka & Clark, 2012) (Figure 10). In terms of life history traits, zebrafish have an average lifespan of approximately three years, with sexual maturation occurring as early as three months of age. Optimal reproductive conditions are achieved at temperatures ranging from 27 to 28 °C, with females capable of producing 200 to 300 eggs per week for up to 18 months. These attributes position *D. rerio* as an ideal model organism for experimental studies in physiology, toxicology, and endocrinology (McGonnell & Fowkes, 2006; Rodrigues et al., 2021; 2022; Doretto et al., 2022; Costa et al., 2023; Souza et al., 2024).

The sequencing of the zebrafish genome is accessible through the ZFIN website (The Zebrafish Information Network – <https://zfin.org/>), which has further increased the scientific community's interest in using this model for a wide range of research. This genomic resource has facilitated the application of genetic studies to this teleost, including the development of robust transgenic models, particularly in the field of reproductive biology (McGonnell & Fowkes, 2006; Rodrigues et al., 2021; 2022; Doretto et al., 2022; Costa et al., 2023; Souza et al., 2024).

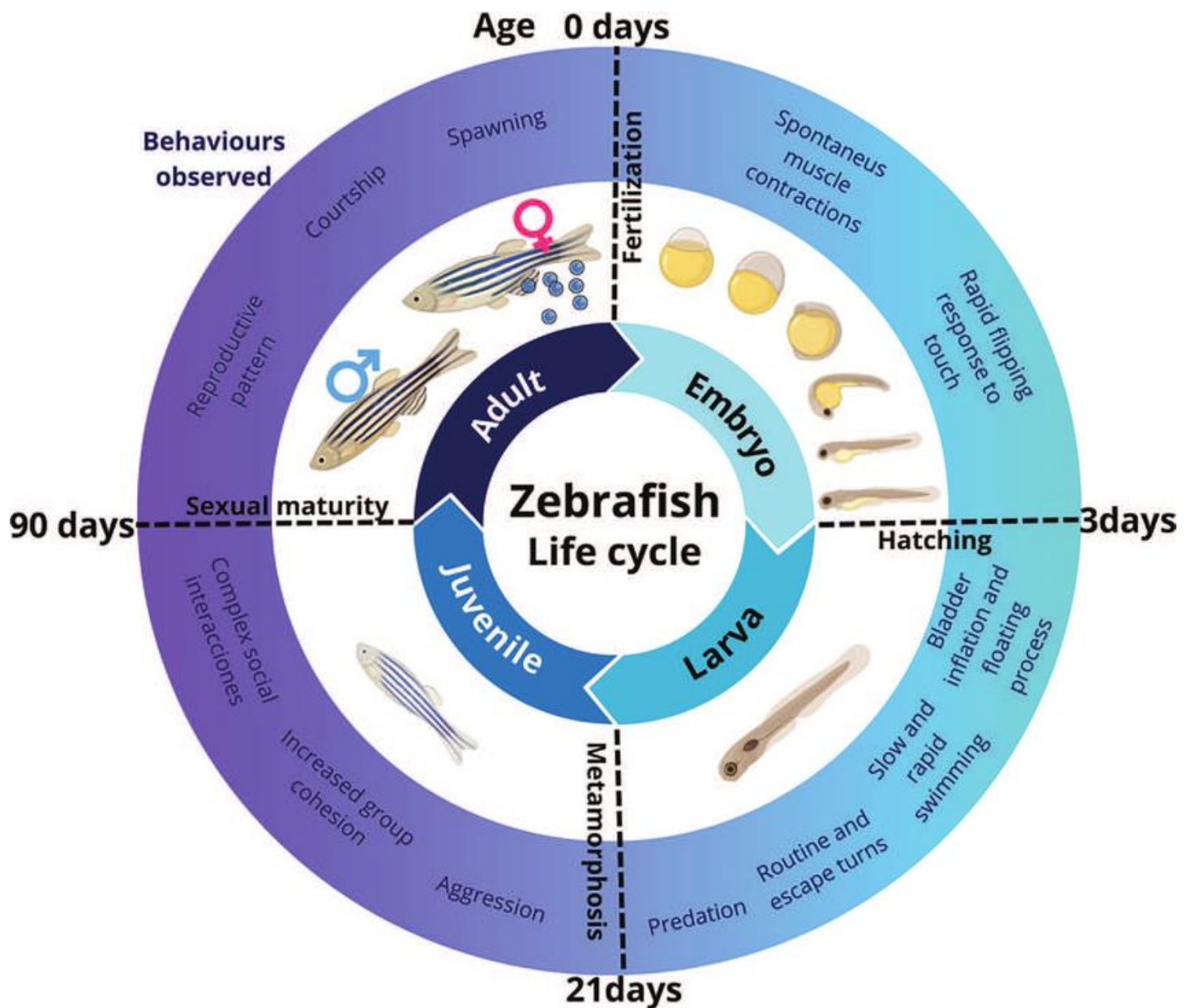


Figure 10. Zebrafish life cycle: Embryo, larvae, juvenile, and adult. The figure presented is derived from the study conducted by Elizalde-Velázquez & Herrera-Vásquez (2023).

2. Importance of the thesis

Thyroid hormones (THs) are pivotal in vertebrate development and essential for maintaining physiological homeostasis (Terrien & Prunet, 2013). Several studies (Power et al., 2001; Habibi et al., 2012; Rodrigues et al., 2021, 2022) have investigated the crosstalk between THs and the reproductive axis in fish, demonstrating that fluctuations in TH levels can directly



affect reproduction, development biology, toxicology, and environmental health. The thesis investigated how THs disruptions affect the reproductive axis of adult males, influencing spermatogenesis and advancing knowledge of endocrine-reproductive system interactions, with implications for diagnosis and managing fertility disorders. The research also examines how maternal THs fluctuations alter embryo development, with implications for both human health and species conservation. Chapter 1 investigated the mechanisms by which THs affect spermatogenesis in zebrafish may provide relevant insights into the interaction between the endocrine and reproductive system. Additionally, the importance of hormonal homeostasis in reproductive function may lead to increased attention to endocrine diseases, such as hypo and hyperthyroidism, and their effects on gametogenesis. On the other hand, thyroid-reproductive axes in zebrafish could inspire advances in reproductive biotechnologies, such as improvements in cryopreservation techniques for germ cells, as well as the development of protocols for male infertility in both humans and economically and ecologically important. Chapter 2 of the thesis showed that identifying complications in embryonic development caused by maternal hormonal alterations can enhance understanding of pregnancy-related issues, including fetal growth restriction and endocrine disorders. Another important aspect addressed in the thesis is the potential implications of maternal THs disruption in the field of ecotoxicology. Hormonal imbalances caused by environmental contaminants, such as endocrine disrupts, could compromise the reproduction of fish populations and other aquatic organisms. Furthermore, the proposal of public policies for species conservation and ecosystem protection is urgent in light of current and future challenges related to environmental contamination and climate change. In general, this thesis aimed to investigate the role of THs in regulating the thyroid-reproductive axis and the impact of maternal hormones transfer on offspring growth and survival, employing molecular and cellular approaches.

3. General objectives

This thesis aims to evaluate the effects of hypothyroidism *in vivo* and *ex vivo* on the testicular function of adult *D. rerio* and to investigate the crosstalk between the hypothalamic-pituitary-thyroid, and -gonadal axes (Chapter 1). Additionally, this research aims to explore the influence of maternal hormones transfer on offspring development (Chapters 2 and 3).

3. 1 Specific objectives



1. To evaluate the *in vivo* effects of pharmacologically induced hypothyroidism on spermatogenesis and in the expression of important key genes involved in the HPG axis of adult male zebrafish;
2. To investigate the *ex vivo* effects of Fsh on the androgen release capacity and testicular gene expression of adult zebrafish, which had been pharmacologically induced to hypothyroidism;
3. To investigate the effects of maternal hormones on zebrafish embryos and larval development, with a focus on the somatotropic axis.

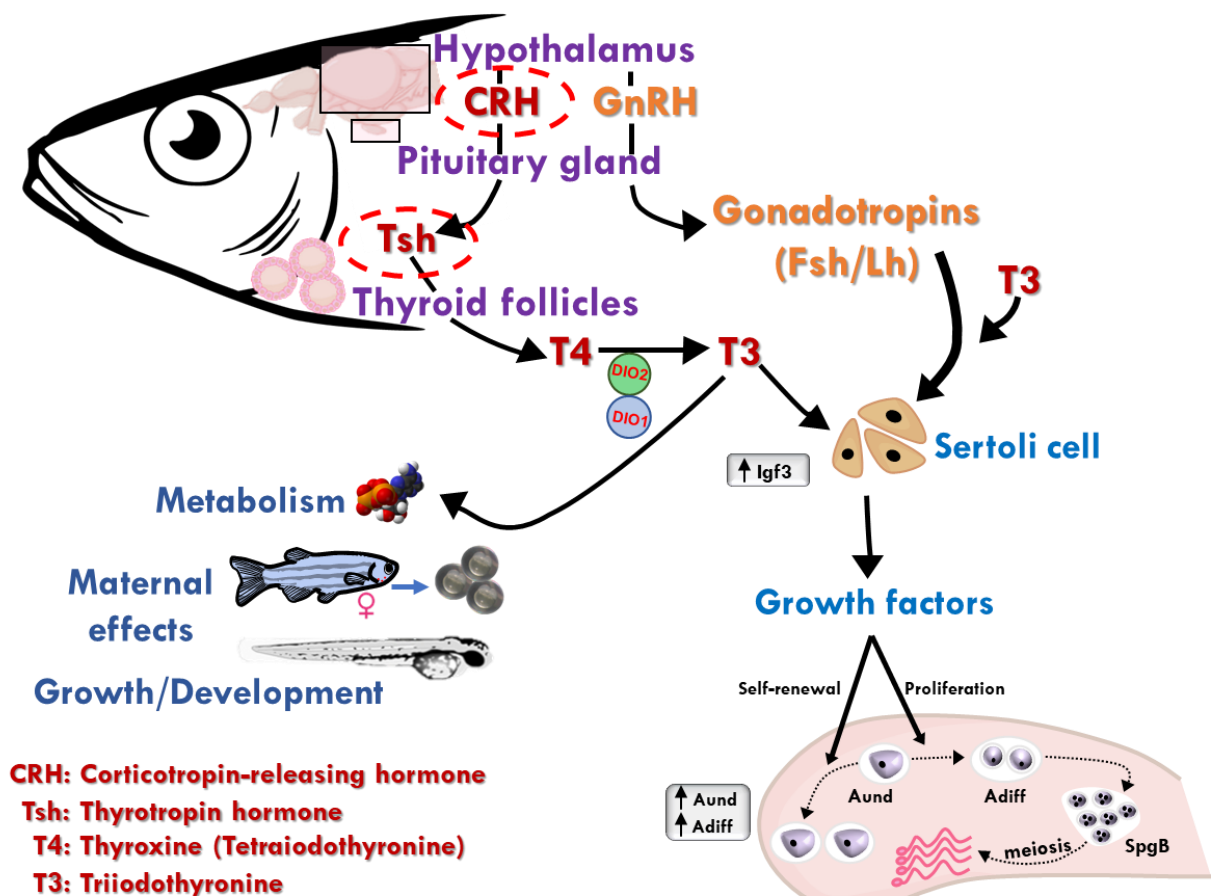
Chapter 1

Thyroid Hormones Deficiency Impairs Male Germ Cell Development: A Cross Talk Between Hypothalamic-Pituitary-Thyroid, and-Gonadal Axes in Zebrafish

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Hypothalamic-Pituitary-Thyroid, and-Gonadal Axes in Teleosts



Graphical abstract
(Figure by the author)



Thyroid hormones deficiency impairs male germ cell development: a cross talk between hypothalamic-pituitary-thyroid, and - gonadal axes in zebrafish

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Keywords: hypothyroidism, methimazole, thyroid hormones, spermatogenesis, zebrafish

Abstract

In vertebrates, thyroid hormones are critical players in controlling different physiological processes such as development, growth, metabolism among others. There is evidence in mammals that thyroid hormones are also an important component of the hormonal system that controls reproduction, although studies in fish remain poorly investigated. Here we tested this hypothesis by investigating the effects of methimazole-induced hypothyroidism on the testicular function in adult zebrafish. Treatment of fish with methimazole, *in vivo*, significantly altered zebrafish spermatogenesis by inhibiting cell differentiation and meiosis, as well as decreasing the relative number of spermatozoa. The observed impairment of spermatogenesis by methimazole was correlated with significant changes in transcript levels for several genes implicated in the control of reproduction. Using an *in vitro* approach, we also demonstrated that in addition to affecting the components of the brain-pituitary-peripheral axis, T3 (triiodothyronine) also exerts direct action on the testis. These results reinforce the hypothesis



that thyroid hormones are an essential element of multifactorial control of reproduction and testicular function in zebrafish and possibly other vertebrate species.

1 Introduction

The production of thyroid hormones in fish and other vertebrates is under the control of the hypothalamic-pituitary-thyroid (HPT) axis (Cooke et al., 1994; Tousson et al., 2011; Duarte-Guterman et al., 2014; Kang et al., 2020). The thyrotropin-releasing factor [thyrotropin-releasing hormone (TRH)/corticotropin-releasing hormone (CRH)] stimulates the pituitary to release the thyrotropic hormone (TSH), which in turn, promotes the synthesis and release of thyroid hormones, thyroxine (T₄) and triiodothyronine (T₃), by thyroid follicles (Larsen et al., 1998). Of the two, T₃ is the more biologically active thyroid hormone owing to its affinity for the nuclear thyroid hormone receptor (Carr and Patiño, 2011). The HPT axis acts parallel to the hypothalamic-pituitary-gonadal (HPG) axis, which involves a large number of hormones, including the gonadotropin-releasing hormone (GnRH) that promotes the secretion of gonadotropin hormones, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (Schulz et al., 2010; Pankhurst, 2016) which are crucial for testis development and spermatogenesis in fish (Schulz et al., 2010; for a review see Xie et al., 2020). There is evidence for interaction between HPT and HPG axes in vertebrates (Teerds et al., 1998; Ariyaratne et al., 2000; Matta et al., 2002; Wagner et al., 2009; Morais et al., 2013; Kang et al., 2020), although this subject remains poorly investigated in fish, particularly, in the male reproductive system (Habibi et al., 2012; Castañeda-Cortés et al., 2014; Tovo-Neto et al., 2018; Ma et al., 2020a, b). In mammals, it has been shown that T₃ regulates the growth and maturation of testis by inhibiting immature Sertoli cell proliferation and stimulating their terminal differentiation (Cooke and Meisami, 1991; Hess et al., 1993; Cooke et al., 1994). Furthermore, in postnatal rat testis, an important action of thyroid hormones is to initiate the onset of Leydig cell differentiation and stimulation of steroidogenesis (Ariyaratne et al., 2000), in part, by stimulating the expression of steroidogenic acute regulatory protein (StAR) (Mendis-Handagama and Ariyaratne, 2005). The presence of thyroid hormone receptors in the mammalian testis, particularly in Leydig cells, suggests both direct and indirect actions of thyroid hormones on testicular function (Hernandez, 2018). The regulatory role of thyroid hormones is complex, species specific, and dependent on developmental stages. Neonatal hypothyroidism was shown to impair testicular growth and sperm production in rats (França et al., 1995), hamsters (*Mesocricetus auratus*) (Jansen et al., 2007), and juvenile teleost fish (*Oreochromis niloticus*) (Matta et al., 2002).

A number of researchers have investigated the role of thyroid hormones in fish embryogenesis, larval development, and growth (Blanton and Specker, 2007; Mukhi et al., 2007; Orozco et al., 2012). With regards to fish male reproduction, there are some evidence that thyroid hormones can affect spermatogenesis (Cyr and Eales, 1996; Wagner et al., 2009; Nelson et al., 2010; Habibi et al., 2012; Morais et al., 2013; Safian et al., 2016; Tovo-Neto et al., 2018). In adult catfish, *Clarias gariepinus*, treatment with thiourea (a thyroid disruptor) decreased 11-ketotestosterone (11-KT) and testosterone (T) production (Swapna et al., 2006), leading to male reproductive system disruption. Morais and collaborators (2013) demonstrated the influence of thyroid hormones on zebrafish spermatogenesis using an *ex vivo* approach. In the same study, the authors revealed that T₃ stimulated the increase in mitotic index of type A undifferentiated spermatogonia (A_{und}) and Sertoli cells through Igf3 (Insulin-like growth factor like 3), a Sertoli cell stimulatory growth factor (Morais et al., 2013). Moreover, the same authors showed that T₃ potentiated FSH actions on steroid release and enhanced Fsh-stimulated *cyp17a1* (17 α -hydroxylase/17,20 lyase) and *ar* mRNA levels in adult zebrafish testis (Morais et al., 2013). There is also evidence that thyroid hormones interact with other reproductive



peptides such as GnRH and gonadotropin-inhibitory hormones (GnIH) *in vivo* (Ma et al., 2020a, b) and *in vitro* in the zebrafish testis (Rodrigues et al., 2021). Other studies have demonstrated that GnRH stimulates thyroid activity in a freshwater murrel, *Channa gachua*, and two carp species, *Catla catla* and *Cirrhinus mrigala* (Roy et al., 2000). GnRH injection increases plasma T4 levels in different species (Jacobs et al., 1988; Roy et al., 2000; Chiba et al., 2004), suggesting an effect of endogenous pituitary gonadotropin release due the heterothyrotropic activities of GnRH on the thyroid (Mackenzie, 1982, 1987). In general, these findings support the hypothesis that normal thyroid hormone action is critical for HPG axis function and normal gonadal function. However, significant gaps remain regarding exact physiological significance of thyroid hormones on fish male reproductive function.

The aim of this research was to further explore the influence of thyroid hormones on male zebrafish reproduction adopting *in vivo* and *ex vivo* approaches. We first evaluated the effect of hypothyroidism induced by methimazole and co-treatment with T4 on zebrafish spermatogenesis by histomorphometrical measurement and measured testicular transcript levels for genes related to reproduction, as well as 11-KT plasma levels and basal and FSH-induced 11-KT release *in vitro*. Subsequently, we investigated the hypothyroidism induced by methimazole treatment and T3 injection on zebrafish brain and pituitary by transcript measurement. Finally, we investigated long-term effects of T3 on zebrafish spermatogenesis by histomorphometrical analysis, and transcript levels of a selected genes. The results provide a framework for understanding of the influence of thyroid hormone in the control of male reproduction in adult zebrafish.

2 Methods

2.1 Animals

Sexually mature male zebrafish (4–5-months-old) were maintained in the aquarium facility of the Department of Structural and Functional Biology, Institute of Biosciences, Botucatu, São Paulo State University (UNESP) in 6-L tanks in the recirculating system under constant temperature conditions (28 °C), and proper photothermal conditions (14-h light/10-h dark). The following water parameters were monitored in all tanks every other day: pH, salinity, dissolved oxygen, and ammonia concentration. The animals were fed twice a day with commercial food (Sera Vipán Flakes®). Handling and experimentation were performed according to the Brazilian legislation regulated by National Council for the Control of Animal Experimental (CONCEA) and Ethical Principles in Animal Research (Protocol n. 1031-CEUA) and University of Calgary animal care committee and in agreement with the procedures of the Canadian Council of Animal Care (Protocol #AC19-0161).

2.2 Treatment solutions: methimazole-induced hypothyroidism and reversal treatment with T4

In this study, we used methimazole (1-methyl-3H-imidazole-2-thione) (CAS 60-56-0; MW, 114.17 g/mol; purity, ≥99%; Sigma-Aldrich) to chemically generate hypothyroidism in adult zebrafish males. Exposure concentration of 1 mM was prepared following the methodology described in Rodrigues et al. (2021). The working concentration of 1 mM methimazole and 100 µg/L T4 were chosen based on previous studies (Sharma and Patiño, 2013; Sharma et al., 2016; Rodrigues et al., 2021). In this study, adult male zebrafish (n = 144) were divided into four replicate tanks per experimental group: control [only filtered water (n = 36)]; group I [filtered water with T4 (100 µg/L) (n = 36)]; group II [filtered water with

methimazole (1 mM) (n = 36)]; group III [1 mM of methimazole followed by addition of T4 (100 µg/L) (n = 36) as reversal treatment group (methimazole + T4)]. In the T4 group, males were exposed to T4 (100 µg/L) from the second week until the end of treatment. In the methimazole group, males were exposed to 1 mM methimazole for 21 days. In addition, zebrafish males were exposed to 1 mM methimazole for 21 days, and T4 (100 µg/L) was added in the water from the second week until the end of treatment. The reversal treatment was performed to assess whether the apparent effects were due to lowering thyroid hormone levels. After euthanasia, the heads (control, methimazole and methimazole + T4) were sampled for histology, while the testes were dissected and immediately used for *in vivo* experiments (histomorphometric analysis and gene expression); androgen plasma levels and *ex vivo* organ culture experiment [short-term (18 h) incubation for androgen release by zebrafish testicular explants] were available on the treatments (control, T4, methimazole and methimazole + T4 groups). The brain and pituitary from the control and methimazole groups were sampled for gene expression.

2.3 Thyroid follicles histology

Thyroid follicles from the different experimental groups were analyzed histologically. As thyroid follicles in fish appear distributed among the afferent branchial arterioles (Patinõ et al., 2003; Van der Ven et al., 2006), head was separated from the trunk and fixed in 2% glutaraldehyde and 4% paraformaldehyde in Sorensen buffer [0.1 M, pH 7.2] for at least 24 h at room temperature. The material was dehydrated, embedded in glycol methacrylate (GMA) resin (Technovit 7100 - Heraeus Kulzer, Wehrheim, Germany), and serial sections (3 µm thickness) were stained with 0.1% toluidine blue in 1% sodium borate and examined and documented using a Leica DMI6000 microscope.

2.4 Histomorphometrical evaluation of zebrafish spermatogenesis

After exposure to methimazole or methimazole + T4, zebrafish testes (n = 8 per treatment) were dissected and immediately fixed in 2% glutaraldehyde and 4% paraformaldehyde in Sorensen buffer [0.1 M, pH 7.2] at 4°C overnight. Subsequently, testes were dehydrated, embedded in GMA resin (Technovit 7100 - Heraeus Kulzer, Wehrheim, Germany), sectioned at 3 µm thickness, and stained with 0.1% toluidine blue. The slides were evaluated, and the proportion of section surface area of spermatogenic cysts containing different germ cell types were determined: type A undifferentiated spermatogonia (A_{und*} and A_{und}), type A differentiated spermatogonia (A_{diff}), type B spermatogonia (SpgB), spermatocytes (Spc), and spermatids (Spt). Intersection points were counted on the histologic fields, for which five fields per slide (n = 8 slides per treatment) were quantified using a grid of 540 (54 × 10) intersections under 100x objective lens. The proportion of section area occupied by spermatogenic cysts containing different germ cell types were represented as fold-change of control value.

For the quantification of the relative number of spermatozoa, twenty different histological fields were captured at 100x objective lens and analyzed by IMAGEJ Software (available at <http://imagej.nih.gov/ij/index.html>) according to Fallah and collaborators (2019, 2020), Tovo-Neto and collaborators (2020) and Rodrigues and collaborators (2021).

2.5 Transcript analysis by quantitative real-time PCR (qPCR)

Total RNA from testes (control, methimazole, and methimazole + T4 groups) was extracted using TRIzol™ (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions, and quantity and purity were checked with a NanoDrop™ One Spectrophotometer (Thermo Scientific, Madison, WI, USA). cDNA synthesis was performed as described previously (Nóbrega et al., 2010). qPCR reactions were conducted using 5 µL of 2x SYBR-Green Universal Master Mix, 1 µL of forward primer (9 mM), 1 µL of reverse primer (9 mM), 0.5 µL of DEPC water, and 2.5 µL of cDNA. The relative mRNA levels of *thra* and *thrβ* (thyroid hormone receptors), *fshr* (follicle-stimulating hormone receptor), *cyp17a1* (17α-hydroxylase/17,20 lyase/17,20 desmolase), *insl3* (insulin-like peptide 3), *cx43* (testicular connexin), *igf3* (insulin-like growth factor 3), *amh* (anti-Müllerian hormone), *gsdf* (gonadal somatic cell derived factor), *nanos2* (marker of undifferentiated spermatogonia), *dazl* (deleted in azoospermia-like), *sycp3l* (synaptonemal complex protein 3) and *odf3a* (outer dense fiber protein 3) were measured in the different treatments.

Brain (n = 8) and pituitary (n = 4 pools of 4 pituitaries for each pool) were collected from control and methimazole groups. Brain of each fish was kept separate. Total RNA was extracted from the brain using TRIzol™ (Invitrogen, Carlsbad, CA, USA) method. At least four pituitary glands were pooled per group (n = 4 pools per treatment), and total RNA was extracted using a commercial kit (PureLink™ RNA Mini Kit, Ambion, Life Technologies, Carlsbad, CA, USA). After RNA extraction, the usual downstream methods were followed according to procedures described above. The relative mRNA levels of *gnrh2* and *gnrh3* (gonadotropin-releasing hormones), *gnih* (gonadotropin-inhibitory hormone), and *crf* (corticotropin-releasing hormone) were analyzed in the brain, and the *lhb* (luteinizing hormone), *fshb* (follicle-stimulating hormone), and *tsh* (thyroid-stimulating hormone) mRNA levels were determined in the pituitary gland. mRNA levels of the targets (Cts) were normalized by the transcript levels of *β-actin* and expressed as relative values of the control group. Primers were designed according to zebrafish sequences (**Table 1**).

2.6 11-KT plasma levels

Blood from adult male zebrafish in different conditions (control, T4, methimazole, and methimazole + T4 groups) were collected (n = 8 per condition). Fish were euthanized, and the caudal peduncle was cut for blood sampling using heparinized syringes and tubes. Subsequently, blood was centrifuged at 4°C for 10 min at 800 ×g (Eppendorf Centrifuge 5424 R), and 11-Ketotestosterone (11-KT) plasma levels were quantified by ELISA (582751, Cayman Chemical), following the manufacturer's instructions. The results were evaluated as nanograms of 11-KT per milliliter of plasma.

2.7 Testis tissue culture

An *ex vivo* testis culture system described previously (Leal et al., 2009) was used to culture zebrafish testes. For short-term incubations (18 h for 11-KT release analysis), testes were submerged in a culture medium, whereas for long-term exposure (7 days for histomorphometrical analysis and gene expression), testes were placed on a nitrocellulose membrane on top of a cylinder of agarose (1.5% w/v, Ringer's solution, pH 7.4) and exposed to 1 mL of medium culture in 24-well flat-bottom plates, as described by Leal and collaborators (2009).

2.8 Short-term (18 h) incubation



Zebrafish testes were collected from eight animals per condition (control, T4, methimazole, methimazole + T4) post-dissection. One testis was cultivated in the Lebovitz medium (L-15), whereas its contra-lateral one in L-15 containing recombinant zebrafish Fsh (rzfFsh; 100 ng/mL). Following incubation, testes were individually weighed, and the medium was collected and stored at -20°C for androgen release (11-KT) assay (see sub-section 2.9).

2.9 *in vitro* 11-KT release by zebrafish testicular explants in short-term incubation

This technique was used to examine whether treatment conditions (T4, methimazole, methimazole + T4) modulated rzfFsh (100 ng/mL)-induced androgen release by zebrafish testis. The androgen (11-Ketotestosterone, 11-KT) release capacity of zebrafish testes into culture medium was measured after 18 h incubation as described previously (García-López et al., 2010). The levels of 11-KT released by zebrafish were quantified by ELISA using a commercial kit (Cayman Chemical) following manufacturer's instructions.

2.10 Long-term (7 days) incubation

To examine the effects of T3 (100 nM) ($n = 8$) on zebrafish spermatogenesis, long-term incubations were performed according to Leal and collaborators (2009). For that, one testis was incubated in the presence of T3 alone and its contralateral one in a basal culture medium. The proportion of section area occupied by different germ cell types were represented as fold-change of basal value. The relative number of spermatozoa per area was quantified as described above (see sub-section 2.4). Also, this technique was used to analyze if different concentrations of T3 and T4 (10, 100 and 1000 nM) modulate expression of selected genes in zebrafish testis. For that, total RNA from testis explants ($n = 8$) was extracted and the relative mRNA levels of *nanos2*, *sycp3l*, *3 β -HSD* (3-beta (β)-hydroxysteroid dehydrogenase), and *cyp17a1* were evaluated as described above (see sub-section 2.5) (**Table 1**).

2.11 T3 injections

Adult zebrafish were intracelomically injected with 0 and 250 ng of T3 per fish ($n = 16$). The dose was selected according to its ability to stimulate deiodinase type 3 mRNA levels as described previously (Nelson and Habibi, 2008; Nelson et al., 2010). A stock solution of T3 was dissolved in sodium hydroxide (0.02 M) and further diluted in physiologic saline. The control group was injected only with the physiologic saline solution. After twelve hours, the animals were euthanized, and brain and pituitary glands were sampled. Brain of each fish ($n = 8$) was kept separate. The pituitaries were pooled ($n = 4$ pools of 4 pituitaries per condition) for RNA extraction. mRNA levels of *gnrh2* and *gnrh3*, *gnih*, and *crf* were quantified in the brain, and *lhb*, *fshb*, and *tsh* were quantified from pituitary glands (**Table 1**). RNA extraction and downstream procedures were followed as described in sub-section 2.5.

2.12 Statistical analysis

All data were subjected to normality Shapiro-Wilk test followed by the Bartlett homogeneity variance test. Significant differences between two groups were identified using unpaired or paired t-tests, while for three or more groups, one-way ANOVA followed by the Student–Newman–Keuls or Dunnett's tests was used. Significance level (p) was considered $\leq$

0.05 in both cases. Data are represented as mean $\pm$ SEM (Standard Error of Mean). All statistical analysis was performed using Graph Pad Prism software 7.04 (Graph Pad Software, Inc., San Diego, CA, USA, <http://www.graphpad.com>).

3 Results

3.1 Thyroid follicles analysis

Thyroid gland follicles were examined histologically in the control group and following treatments with methimazole and methimazole + T4 (**Fig. 1(A)-(D)**). The control group thyroid follicles consisted of squamous or cuboidal epithelial cells filled with colloid (**Fig. 1(B)**). The results demonstrate a significant modification in the histological condition of thyroid follicles in fish treated with methimazole. Three-week exposure to 1 mM methimazole resulted in thyroid gland follicles with colloid depletion, columnar epithelium and follicle cell hypertrophy (**Fig. 1(C)**). These morphological changes were consistent with previous studies in which adult male zebrafish were exposed to methimazole (Rodrigues et al., 2021), and other goitrogens, such as perchlorate (Patiño et al., 2003) and 6-n-propyl-2-thiouracil (PTU) (Van der Ven et al., 2006). The observed effect of methimazole was reversed by co-treatment with T4, in which the thyroid follicles were found to be morphologically similar to the control group (**Fig. 1(D)**). The results demonstrate that methimazole-induced hypothyroidism in zebrafish had altered thyroid function following treatment with the goitrogen. Also, the results demonstrate that co-treatment with T4 restored the zebrafish thyroid follicular structure.

3.2 Methimazole-induced hypothyroidism and reversal treatment with T4: histomorphometrical analysis of the zebrafish testis

Methimazole-induced hypothyroidism promoted histomorphometrical alterations in the proportion of germ cell cysts compared to the control (**Fig. 2(A)-(D)**). There was a significant increase in the proportion of the area occupied by type A undifferentiated spermatogonia (A_{und*}), type A differentiated (A_{diff}) and spermatogenic cysts containing type B spermatogonia (SpgB) in the methimazole group as compared to control (**Fig. 2 (D)**). The number of meiotic cells (Spc) and post-meiotic haploid cell population (Spt) did not change between control and methimazole-induced hypothyroidism group (**Fig. 2 (D)**). However, the relative number of spermatozoa reduced drastically when compared to control as clearly evidenced in the photomicrographs and analysis of spermatozoa number by field (**Fig. 2(A), (B) and (E)**). Co-treatment with T4 rescued the proportion of A_{und*} , A_{diff} and SpgB types returned to its basal values, while the proportion area occupied by Spc and Spt significantly increased (**Fig. 2 (D)**). Remarkably, the production of spermatozoa was recovered in the co-treatment with T4 (as viewed in the fields of **Fig. 2(A), (C) and (E)**).

3.3 Methimazole and co-treatment with T4: testicular transcript levels

Transcript levels of selected genes involved in reproduction were measured by qPCR in the testis from methimazole-induced hypothyroidism and rescued groups (methimazole + T4) (**Fig. 3**). In this study we measured transcript levels of two thyroid hormone receptor subtypes (*thra*, *thrβ*). The *thra* transcript level was higher in the methimazole-treated group than control, but the difference was not statistically significant (**Fig. 3(A)**). The *thra* transcript level was further increased significantly in the methimazole + T4 treated group (**Fig. 3(A)**). Similarly, the

thrβ was increased in the methimazole and methimazole + T4 treated groups, compared to the control (**Fig. 3(B)**).

We also measured mRNA levels of *fshr* which was not altered in the methimazole treated group, but considerably increased in the methimazole + T4 treated fish, compared to the control (**Fig. 3(C)**).

In the present research we quantified transcript levels for *cyp17a1* and insulin-like peptide 3 (*insl3*) genes. Treatment with methimazole significantly reduced the *cyp17a1* and *insl3* transcript levels compared to control (**Fig. 3(D) and (E)**). Co-treatment with T4 did not influence the methimazole induced response on the expression of these transcripts (**Fig. 3(D) and (E)**). We also observed significant reduction in the testicular connexin (*cx43*) mRNA levels in the methimazole treated group, compared to control (**Fig. 3(F)**). Co-treatment with T4 in this case increased the *cx43* mRNA to a level not significantly different from the control (**Fig. 3(F)**).

Among others, *igf3*, *amh*, and *gsdf* genes are known to be expressed in the Sertoli cells. Treatment with methimazole or methimazole + T4 were without significant effects on *igf3* and *amh* transcript levels (**Fig. 3(G) and (H)**). The *gsdf* mRNA level, however, was higher in the methimazole and methimazole + T4 treated groups (**Fig. 3(I)**).

With regard to germ cell marker genes, such as marker for type A undifferentiated spermatogonia, *nanos2* mRNA level was considerably higher in the methimazole-treated group compared to control (**Fig. 3(J)**). Co-treatment with T4, significantly reduced the methimazole-induced response to a level higher than the basal control (**Fig. 3(J)**). The *dazl* (deleted-in azoospermia-like) transcript level expressed by A_{diff} and SpgB, increased in the methimazole treated group compared to the control (**Fig. 3(K)**). Co-treatment with T4 did not influence the methimazole-induced response (**Fig. 3(K)**). In our study, synaptonemal complex protein 3 (*sycp3l*) which is a marker for spermatocytes was not significantly affected by methimazole or methimazole + T4 treatments (**Fig. 3(L)**). However, the transcript level of the outer dense fiber protein 3 (*odf3a*) which is a marker for spermatids was increased following treatment with methimazole (**Fig. 3(M)**). Co-treatment with T4 reduced the methimazole-induced response to a level not significantly different from the basal control (**Fig. 3(M)**).

3.4 Measurement of 11-KT levels

In the present research, we measured the 11-KT concentration to partially assess the effect of methimazole-induced hypothyroidism on steroidogenesis, *in vivo* and *in vitro* (**Fig. 4(A)**). Four treatment groups were studied in this experiment, including control, control + T4, methimazole and co-treatment (methimazole + T4) groups (**Fig. 4(A)**). Animal exposure with T4 (control+T4) significantly reduced the plasma 11-KT level as compared to control levels (**Fig. 4(B)**). Also, treatment with methimazole significantly reduced the plasma 11-KT concentration to almost undetectable level compared to the control level of over 40 ng/mL (**Fig. 4(B)**). However, co-treatment with T4 significantly increased and nullified the methimazole-induced response to a level not significantly different from the control (**Fig. 4(B)**).

We also measured the androgen (11-KT) release capacity of zebrafish testicular tissue using the *ex vivo* culture system (**Fig. 4(A)**). In this experiment, we compared testis taken from control and those exposed to T4 (100 µg/L), methimazole (1 mM) and methimazole co-treated with T4. We tested the 11-KT release response into culture media following *in vitro* treatment for 18 h to recombinant zebrafish Fsh (100 ng/ml) (**Fig. 4(A)**). In the basal medium condition,

the treatments (T4, methimazole and methimazole + T4) did not change the basal androgen (11-KT) release capacity of zebrafish testicular tissue (**Fig. 4(C)**). However, as expected, treatment with Fsh significantly increased the 11-KT level in the control group (**Fig. 4(C)**). Also, treatment with T4 was responsive to Fsh but to a level not significantly different from the control (**Fig. 4(C)**). In comparison, the isolated testis from methimazole-treated zebrafish was completely unresponsive to Fsh (**Fig. 4(C)**). However, co-treatment with T4 restored the Fsh-induced response by increasing the 11-KT concentration to the level observed following treatment of the control group with Fsh alone (**Fig. 4(C)**).

3.5 Transcript levels of selected genes in the brain and pituitary

In this experiment, we measured brain transcript levels of a number of neurohypothalamic peptides, including *gnrh2* and *gnrh3*, *gnih*, and *crf*, as well as pituitary gonadotropin hormone subunits, *fshb*, *lhb*, and *tshb* in the methimazole-induced hypothyroidism in fish (**Fig. 5(A) and (C)**). As shown in **Fig. 5(C)**, we observed a significant increase in the *gnih* transcript level compared to the control shown as the dotted line. Transcript levels of the other neuropeptides measured including *gnrh2*, *gnrh3* and *crf* remained unchanged. In the same group of methimazole-treated fish, the results the pituitary glycoprotein hormone subunits, demonstrate significant increase in *fshb* mRNA and a massive increase in *tshb* transcript levels in the methimazole-induced hypothyroid fish (**Fig. 5(C)**). No change was observed in the *lhb* transcript level (**Fig. 5(C)**).

We also measured the same transcript levels following 12 hours acute treatment with 250 ng of T3/fish *in vivo* (**Fig. (B) and (D)**). In this experiment T3 injection did not significantly alter *gnih*, *gnrh2* and *crf*, but significantly increased the *gnrh3* transcript level (**Fig. 5(D)**). In the same group of fish, acute treatment with 250 ng of T3/fish significantly reduced the pituitary *lhb* but was without effect on the *fshb* and *tshb* transcript levels (**Fig. 5(D)**).

3.6 *in vitro*: effects of T3 on zebrafish spermatogenesis

In this research, we also examined the direct action of T3 at 100 nM on *ex vivo* culture of zebrafish testis for 7 days (**Fig. 6(A)**) and results provide information on the proportion of germ cells in the cultured testis. Histomorphometrical evaluation of zebrafish testis revealed that treatment with T3 significantly stimulated the type A undifferentiated spermatogonia (A_{und}) abundance with no effect on type A differentiated spermatogonia cells as compared to basal medium incubation (**Fig. 6(B)**). In the same tissue, we observed a reduction in type B spermatogonia (SpgB) abundance (**Fig. 6(B)**). As for meiotic and post-meiotic cells, treatment with T3 reduced the proportion of area occupied by Spc and Spt when compared to basal condition (**Fig. 6(B)**). Interestingly, the number of spermatozoa was stimulated in zebrafish testis treated with T3 as clearly shown by the analysis of spermatozoa number by field (**Fig. 6(C)**). In addition to these results, the effect of thyroid hormones on testicular gene expression was analyzed (**Fig. 6 (D)-(G)**) (see **Supplementary Figure**). Several concentrations of T3 and T4 (10, 100 and 1000 nM) were tested in zebrafish testis. T4 did not change the expression of any transcript (*nanos2*, *sycp3l*, *3 β -HSD* and *cyp17a1*) (see **Supplementary Figure**). On the other hand, expression analysis revealed that T3 (100 and 1000 nM) increased *nanos2* (**Fig. 6 (D)**), and up-regulated *sycp3l* transcript levels at 100 nM (**Fig. 6 (E)**). Levels of *3 β -HSD* and *cyp17a1* were also quantified and did not change within any levels of T3 (**Fig. 6(F)-(G)**).

4 Discussion



This study demonstrated the importance of thyroid hormones as a factor controlling zebrafish spermatogenesis, using methimazole-induced hypothyroidism in fish as a model organism. Several other studies have used antithyroid agents to investigate the effects of thyroid hormones on vertebrate reproduction, including fish (Swapna et al., 2006; Weng et al., 2007; Sharma and Patiño, 2013; Sharma et al., 2016; Tovo-Neto et al., 2018; Figueiredo et al., 2019; Rodrigues et al., 2021). In our study, the efficacy of hypothyroidism was considered based on histological conditions of thyroid follicles. Our results demonstrated that thyroid follicles clearly disturbed following 21 days exposure to 1 mM methimazole. However, the normal thyroid follicles were recovered after co-treatment with T4. Also, testicular connexin (cx43) levels as it is the potential target for thyroid hormones (Poguet et al., 2003; Gilleron et al., 2006). Interestingly, Gilleron and collaborators (2006) demonstrated that propylthiouracil (PTU; another thyroid disruptor) decreases cx43 levels in rats. In our study, the lower expression of cx43 in the methimazole group indicates that methimazole treatment led to hypothyroidism in the male zebrafish.

The present study provides clear evidence that hypothyroidism disrupts germ cell development in the zebrafish testis. Plasma concentration of androgen (11-KT) levels was significantly lower in the hypothyroid group when compared to that in the euthyroid group. Moreover, the testicular mRNA levels of selected genes expressed by somatic and germ cells were altered in hypothyroidism, and in addition to decreased 11-KT plasma levels, hypothyroidism induced by methimazole also impaired genes expressed in the brain and pituitary gland. We used co-treatment with T4, which is the primary secretory product of the thyroid gland, to potentially reverse the effects of methimazole treatment. In addition to these *in vivo* experiments, we also examined the acute effects of thyroid hormones in zebrafish testicular explants in the presence or absence of rzfFsh in different treatments. Thus, this research provides a novel and practical approach to better understand the nature of thyroid hormone actions on the HPG axis in male zebrafish.

Histomorphometric evaluation of zebrafish spermatogenesis demonstrated that methimazole-induced hypothyroidism remarkably increased the proportion area occupied by type A_{und}* (the most undifferentiated spermatogonia), type A_{diff} and type B spermatogonia, while the number of spermatozoa was decreased. Furthermore, our results demonstrate that the proportion of Spc and Spt were not affected in hypothyroid testis compared with the control animals. It has been well-established that spermatogenesis development is supported and regulated by gonadotropic hormones, FSH and LH which mainly target actions on somatic testicular cells such as Sertoli and Leydig cells (Planas et al., 1993; Campbell et al., 2003; Huhtaniemi and Themmen, 2005; García-López et al., 2010; Schulz et al., 2010; Flood et al., 2013). Likewise, thyroid hormones have also been shown to play an essential role in testicular physiology (Chiao et al., 2002; Cooke et al., 2004; Holsberger and Cooke, 2005; Wagner et al., 2008; 2009; Morais et al., 2013; Tovo-Neto et al., 2018). In rats, previous studies have reported that hypothyroidism decreases plasma levels of gonadotropins and reduces the number and size of gonadotrophs (Bruni et al., 1975; Ruiz et al., 1988), suggesting that the absence of thyroid hormones is associated with gonadal dysfunctions (Amin and El-Sheikh, 1977; Hernandez, 2018). Further, the level of relevant androgen (11-KT) that stimulates spermatogenesis decreased significantly in the plasma of zebrafish following treatment with methimazole. These results, in part, could explain the observed increase in different populations of spermatogonia (A_{und}*, A_{diff} and B) and decrease in spermatozoa number, demonstrating the impact of thyroid hormone depletion on germ cell development and testicular function. Similar findings have been reported for male Japanese quail, wherein treatment with methimazole decreased body and testes weight as well as plasma levels of LH and testosterone (Weng et al., 2007). In this



same study, the data showed decreased spermatogenesis in seminiferous tubules of the treatment group (Weng et al., 2007). Altogether, these results demonstrate that gonadal function is associated with normal thyroid action. Fluctuations in thyroid hormone levels can directly modulate gonadotropin actions and affect Sertoli and Leydig cell proliferation (Castañeda-Cortés et al., 2014), resulting in the impairment of spermatogenesis, which may be caused by low FSH level and delay of Sertoli cell maturation (Hernandez, 2018).

We also examined the effects of methimazole co-treated with T4. Our results demonstrate that co-treatment with T4 partially restored zebrafish spermatogenesis and spermatozoa production in the methimazole-induced hypothyroid fish. Interestingly, the administration of T4 to the hypothyroid group increased the proportion of meiotic (Spc) and post-meiotic (Spt) cells compared with the control and methimazole groups. These results support the hypothesis that thyroid hormones are involved, directly or indirectly, with meiosis entry in the zebrafish testis.

The observed transcript analysis in zebrafish testis is to some extent in agreement with histomorphometric results. With respect to the germ cell markers genes of zebrafish spermatogenesis, the results indicated that mRNA levels of *nanos2* (marker of type A undifferentiated spermatogonia) (Aoki et al., 2009) and *dazl* (marker of differentiation) (Chen et al., 2013) were greater in the methimazole-treated group, in agreement with the histomorphometry showing an accumulation of pre-meiotic cells (A_{und*} , A_{diff} and B). The observed upregulation of these transcripts in the methimazole treated group suggest that euthyroid condition may be important for normal spermatogonial cell development in zebrafish. However, the level of *odf3a* (marker of spermatids) (Yano et al., 2008) was upregulated in the methimazole group, although the proportion of spermatids was not affected in the present study. The affected testicular functions did not always recover following co-treatment with T4 to counter hypothyroidism.

Other essential components of the thyroid hormones axis are the thyroid receptors (TRs), which are crucial for testis development and function (Valadares et al., 2008; Dittrich et al., 2011; Lema et al., 2009; Wajner et al., 2009). According to Habibi and collaborators (2012), TRs are expressed by some different cell types, and thyroid hormones have pleiotropic effects, including effects on the gonads. In the latter study in goldfish, *in vivo* and *in vitro*, thyroid hormones were shown to exert both direct and indirect actions on gonadal steroid synthesis, and steroid receptor expression in a seasonally dependent manner (Allan and Habibi, 2012; Habibi et al., 2012). In zebrafish testis, *thra* was shown to be expressed in the Sertoli and Leydig cells, whereas *thrβ* expression was only observed in the Leydig cell (Morais et al., 2013). In the present study, methimazole-induced hypothyroidism stimulated *thrβ* transcript level, but increase in *thra* was only significant in presence of T4 compared to control. Other investigators also reported that T3 treatment only elevated *thrβ* in the ovary and testis of *Pimephales promelas* (Lema et al., 2009). Similarly, *fshr* mRNA levels were also upregulated in hypothyroid co-treated with T4. These results suggest that chronic hyperthyroidism may alter thyroid hormone sensitivity and possibly alter other parameters not clear at the present time. Similarly, in rats, higher FSH-R mRNA levels were detected in hypothyroidism. It was suggested that the upregulated FSH-R mRNA level may have resulted from the elevated proportion of Sertoli cells in rats (Rao et al., 2003). Likewise, in zebrafish, the increase of *fshr* transcripts following co-treatment with T4 could be due to Sertoli cell proliferation. Morais and collaborators (2013) revealed that T3 stimulates *in vitro* Sertoli cell proliferation in zebrafish testes. Moreover, the same study showed that Sertoli cells express both *thra* and *fshr*, and treatment with T3 potentiates Fsh *in vitro* actions in the zebrafish testis (Morais et al., 2013). This is also consistent with the observation that *fshr* transcript levels was stimulated by



methimazole-induced hypothyroid fish co-treated with T4. These results support the view that thyroid hormones and gonadotropins stimulate spermatogenesis by stimulating gonadal androgen production, which in turn mediate the start of spermatogenesis.

Interestingly, mRNA expression of other key gonadal growth factors as *igf3* (Wang et al., 2008) and *amh* (Miura et al., 2002) did not change in the methimazole or methimazole co-treated with T4 groups. However, *gsdf* (Gautier et al., 2011) was upregulated in both groups. *gsdf* is expressed by Sertoli cells and exerts an essential role in the control of spermatogenesis, including germ cell proliferation and differentiation (Gautier et al., 2011; Yan et al., 2017).

Decreased thyroid hormones levels by methimazole in male zebrafish are associated with decreased 11-KT plasma levels. Previous reports have demonstrated that PTU treatment also decreases serum testosterone concentrations in other vertebrate species, such as rats (Chiao et al., 2000). In another study, exposure of *Clarias gariepinus* to thiourea, reduced androgen levels leading to testicular regression (Swapna et al., 2006; Swapna and Senthikumar, 2007). This is consistent with the present study demonstrating lower 11-KT plasma levels in the methimazole group, in addition to lower levels of steroidogenic enzyme (*cyp17a1*) and androgen-sensitive gene (*insl3*) mRNA levels. These transcript levels remained the same even after co-treatment with T4. However, treatment of methimazole-induced hypothyroid fish effectively rescued 11-KT levels in the plasma which was correlated with the revival of spermatozoa number in zebrafish testis. Thus, impairing thyroid action within the physiological limit of restoration is a valid approach to explain the role of thyroid hormones in male zebrafish reproduction. Interestingly, Houbrechts and collaborators (2019) demonstrated the Dio2-knockout (KO) zebrafish (*dio2^{-/-}*) significantly reduced androgen (11-KT and testosterone) levels in the testis, and steroidogenesis and steroid signaling were similarly disturbed when compared with control group. This indicates that absence of thyroid hormones by goitrogen treatment or DIO2 KO, which suppresses thyroid hormone production, may act directly on the testis to repress steroidogenesis. These data also reveal that normal thyroid hormone levels are fundamental to normal reproduction in zebrafish.

The result in this study helps to elucidate the mechanisms underlying the observed reduction in androgen level in male zebrafish following treatment with methimazole. While transcript levels of gonadotropin-releasing hormones, *gnrh2* and *gnrh3* did not change, *gnih* mRNA levels were significantly elevated in the brain of animals after methimazole exposure. In fish, GnIH orthologs have double mechanism, including stimulatory and inhibitory actions on gonadotropin secretion and growth hormone production, depending on the species studied, season, and mode of treatment (Amano et al., 2006; Zhang et al., 2010; Moussavi et al., 2012; 2013; 2014; Branco et al., 2018; Fallah et al., 2019; Ma et al., 2020a, b). In addition, Tsutsui and collaborators (2017) showed that hypothyroidism induced by PTU increases GnIH expression and reduces gonadotropins and plasma steroid levels in female mice. The present results support the hypothesis that decrease in plasma androgen level in methimazole group is involved with the inhibition of the hypothalamic–pituitary–gonadal axis mediated, in part, by increased GnIH. As shown previously in goldfish, GnIH mediated inhibition of reproduction does not always correlate closely by inhibition of gonadotropin transcript levels due to uncoupling of release and synthesis. In this context, it was demonstrated that GnIH can increase gonadotropin subunit mRNA levels while reducing secretion of the hormones (Moussavi et al., 2012; 2013; 2014; Ma et al., 2020a, b). Thus, the inhibition may be at the protein level, since *fshb* transcript level was increased in the pituitary of the methimazole-treated group. Similarly, Yoshiura and collaborators (1999) showed in goldfish that thiourea exposure per two weeks increased Fsh mRNA levels. A contributing factor could be due to feedback exerted by lower plasma 11-KT levels seen in these animals (Rojdmark et al., 1988; Trudeau et al., 1993; Habibi



and Huggard-Nelson, 1998; Huggard-Nelson et al., 2002). There is increasing evidence that the effects of thyroid hormones as well as brain-pituitary-gonadal hormones are diverse, and change with season, mode of action, time-course, and concentration (Nelson and Habibi 2006, 2008, 2009; Nelson et al., 2011; Habibi et al., 2012; Moussavi et al., 2012, 2013, 2014; Nelson and Habibi 2016; Ma et al., 2020a, b). This is reflected in the present study as acute treatment for 12 hours with T3 injection resulting in a different effect than chronic treatments with T4 and methimazole on the hypothalamic-pituitary-gonadal axis. Injection with T3 increased *gnrh3* mRNA levels and either decreased (*lhb*) or had no effects (*fshb*, *tsh*) on the pituitary glycoprotein hormone subunit transcript levels. This is coherent with earlier study in goldfish showing reduction in gonadotropins and synthesis of gonadal steroids following 12 hours injection with T3 (Nelson et al., 2010).

In addition to these data, we examined the actions of thyroid hormones on zebrafish spermatogenesis. Histomorphometrical analysis revealed an increase of type A undifferentiated spermatogonia (A_{und}) proportion in T3 *in vitro* exposure (100 nM), which may reflect the up-regulation of *nanos2* (marker of type A undifferentiated spermatogonia) (100 and 1000 nM of T3). The number of spermatozoa also increased. This data corroborates the histomorphometry of the zebrafish testes from *in vivo* exposures using methimazole and methimazole co-treated with T4, indicating, therefore, a direct action of thyroid hormones in the zebrafish spermatogenesis. Previously, Morais and collaborators (2013) reported that the mitotic indices of A_{und} and Sertoli cells were stimulated after T3 treatment. Likewise, Safian and collaborators (2016) reported that T3 improved the formation of new cysts of A_{und} , accumulation of differentiated spermatogonia (A_{diff}) and reduction of spermatogonia B. Morais and collaborators (2013) using lower dose of T3 (50 ng/mL) also demonstrated an increase of type A undifferentiated spermatogonia, which was corroborated by higher BrdU mitotic index of this cell type in T3-treated tissue. In this study, we also investigated the effects of T3 and T4 on gene expression. Several concentrations of T3 and T4 (10, 100 and 1000 nM) were tested in zebrafish testis. Interestingly, T3 also stimulated *sycp3l* (meiotic marker) mRNA levels at 100 nM. Levels of *3 β -HSD* and *cyp17a1* were also quantified and did not change within any level of T3 exposure. Another interesting result is that T4 does not exert any change on testicular gene expression, indicating that thyroid hormones act via T3 in zebrafish testis. In sum, this data support that thyroid hormone T3 stimulates zebrafish spermatogenesis by proliferation, differentiation, and meiosis. These results provide important data about the roles of thyroid hormones on brain-pituitary-testis and reinforce the interaction between reproductive and thyroid axes.

5 Conclusion

The present study clearly demonstrated that hypothyroidism induced by methimazole significantly impair zebrafish germ cell development by delaying differentiation and meiosis, and decreasing the number of spermatozoa as well as impairing the hypothalamic-pituitary axis. We also provide evidence that testicular function is dependent on thyroid hormones. Taken together, these results provide support for the hypothesis that thyroid hormones are essential for spermatogenesis and maintaining normal function of the hypothalamic-pituitary-gonadal axis in adult zebrafish.



Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

R.H.N., M.S.R., A.T.N., and H.R.H. designed the study; M.S.R., A.T.N., I.F.R., L.B.D., and H.P.F performed the experiments; All authors analyzed the data; R.H.N., M.S.R., A.T.N., and H.R.H. wrote the paper. All authors edited the article.

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Table legend

Table 1. Primers used for gene expression studies (qPCR). (FW = Forward; RV = Reverse).



Target name Testis	Primers sequence (5' - 3')		Access (NCBI)
<i>β-actin</i>	AGACATCAGGGAGTGATGGT	FW	BC045846
	CAATACCGTGTCAATGGGG	RV	
<i>cyp17a1</i>	GGGAGGCCACGGACTGTTA	FW	NM_212806.3
	CCATGTGGAAGTGTAGTCAGCAA	RV	
<i>amh</i>	CTCTGACCTTGATGAGCCTCATTT	FW	NM_001007779.1
	TGCAAAAGTTCTTAAGGGACATCC	RV	
<i>insl3</i>	TCGCATCGTGTGGGAGTTT	FW	NM_001115053.2
	TGCACAACGAGGTCTCTATCCA	RV	
<i>igf3</i>	TGTGCGGAGACAGAGGCTTT	FW	HQ241070.1
	CGCCGCACTTTCTTGATT	RV	
<i>thra</i>	CTGCTGTGATTGGATGCTGGAT	FW	NM_205625
	GCTCTTGTCGGATGTGCTGTT	RV	
<i>thrβ</i>	ATCGACCAGAGCCACACA	FW	NM_131340.1
	TAGGTGCCGATCCAATGTCTT	RV	
<i>fshr</i>	GAGGATTCCCAGTAATGCTTTCCT	FW	NM_001001812.1
	TCTATCTCACGAATCCCGTTCTTC	RV	
<i>cx43</i>	CTACAGGGCTCTCCACTCTTACTTCT	FW	NC_007131.7
	CGCACTCCAGTCACCCATCT	RV	
<i>nanos2</i>	AGAGACTGCGCAGATTACC	FW	NC_007116.7
	AGCATAACGGACACACGTAG	RV	
<i>dazl</i>	AGTGCAGACTTTGCTAACCTTATGTA	FW	NC_007130.7
	GTCCACTGCTCCAAGTTGCTCT	RV	
<i>shippo1</i>	GATGCCTGGAGACATGACCAA	FW	NC_007129.7
	CAAAGGAGAAGCTGGGAGCTTT	RV	
<i>sypc3l</i>	TGGAGCTTCGGAAGGAAATG	FW	NC_007118.7
	CAGAACAGCATGGACTGAAGA	RV	
<i>gsdf</i>	CATCTGCGGGAGTCATTGAAA	FW	NC_007132.7
	CAGAGTCCTCCGGCAAGCT	RV	
<i>gnrh2</i>	TCAGGATTACCAACACCGGG	FW	NM_181439.4
	CAGACCAGCACCATCACTTCA	RV	
<i>gnrh3</i>	AACACAGCAGTTTTAGCATGGAG	FW	NM_182887.2
	TATGACCAGTGCTGGCAAAGA	RV	
<i>gnih</i>	AAGAAAGGACTCAAGTCCACGA	FW	GU290218.1
	GGGCTTTGTTAGTCTAAAGCTGTG	RV	
<i>crf</i>	CACCGCCGTATGAATGTAGA	FW	NM_001007379.1
	GAAGTACTCCTCCCCCAAGC	RV	
<i>lhb</i>	GCAGAGACACTTACAACAGCC	RV	AY714132.1
	AAAACCAAGCTCTGAGCAGCC	FW	
<i>fshb</i>	CAGATGAGGATGCGTGTGC	RV	NM_205624.1
	ACCCCTGCAGGACAGCC	FW	
<i>tsh</i>	GCAGATCCTCACTTCACCTACC	RV	NM_001145763.2
	GCACAGGTTTGGAGCATCTCA	FW	

Figures legends

A. Experimental design

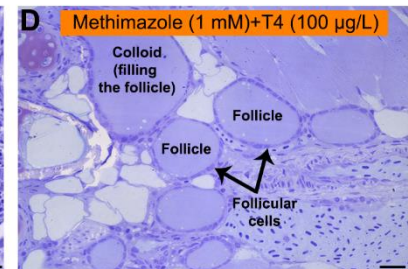
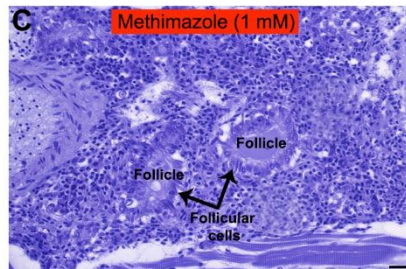
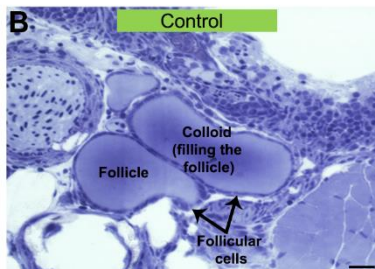
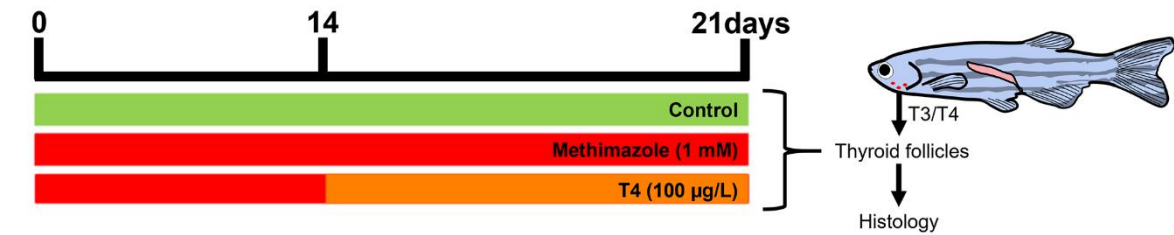


Figure 1: (A) Experimental design representation of treatments: control (non-treated fish), methimazole (1 mM) and methimazole (1 mM) co-treated with T4 (100 µg/L) groups. Zebrafish adult males were exposed to reconstituted water containing 1 mM methimazole (goitrogen) for 21 days or 1 mM methimazole following T4 (100 µg/L) added in the water from the second week until the end of exposure. The control group received the same volume of vehicle solution. After three weeks heads were dissected for histological analysis of thyroid follicles **(B-D)**. Control group **(B)** have thyroid follicles typical of euthyroid animals. These thyroid follicles display squamous or cuboidal epithelial cells and are totally filled with colloid. However, animals treated with methimazole **(C)** revealed disturbed thyroid follicles with columnar epithelium, follicle cell hypertrophy and colloid depletion, while fish co-treated with T4 **(D)** showed follicles similar to the control animals. Staining: Toluidine blue with sodium borate. Scale bar = 20 µm.

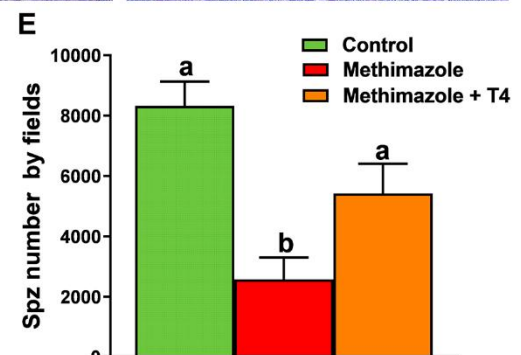
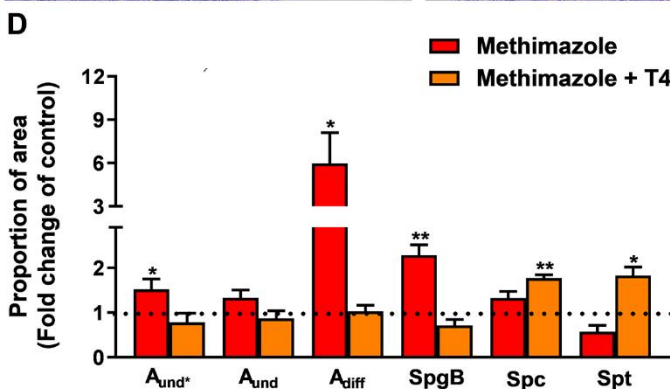
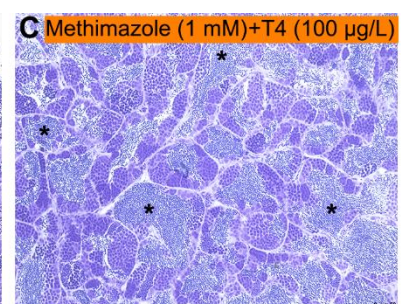
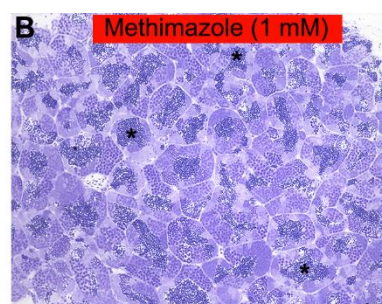
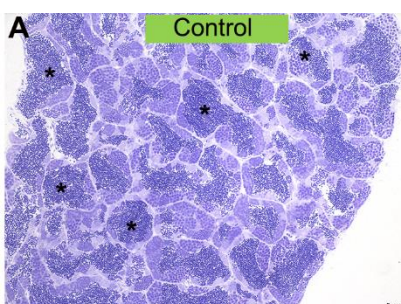


Figure 2: Histomorphometrical evaluation of zebrafish testes after *in vivo* exposure to methimazole and co-treatment with T4 for three weeks. Control group (non-treated fish) **(A)**. Methimazole-treated group **(B)**. Methimazole co-treated with T4 **(C)**. Asterisks in **(A)**, **(B)** and **(C)** indicate the testicular lumen with spermatozoa that appeared reduced in the methimazole group. **(D)** Proportion of section area occupied by different spermatogenic cysts: type A undifferentiated spermatogonia (A_{und*} , A_{und}), type A differentiated spermatogonia (A_{diff}), type B spermatogonia (SpgB), spermatocytes (Spc), and spermatids (Spt). Bars (mean $\pm$ SEM; $n = 8$) are expressed as fold-change relative to the no-treated fish (control group) (dotted black line set at 1). **(E)** Spermatozoa number per field generated by using IMAGEJ Software from control and treatments. ANOVA followed by Dunnett's multiple comparison tests. Distinct letters denote significant differences ($p < 0.05$) between different treatment conditions with the untreated group. Asterisks denote statistical significance differences between control, methimazole and methimazole + T4 groups; * $p < 0.05$; ** $p < 0.01$ (Student unpaired *t*-test; $n = 8$). Staining: Toluidine blue. Scale bar = 50 μ m.

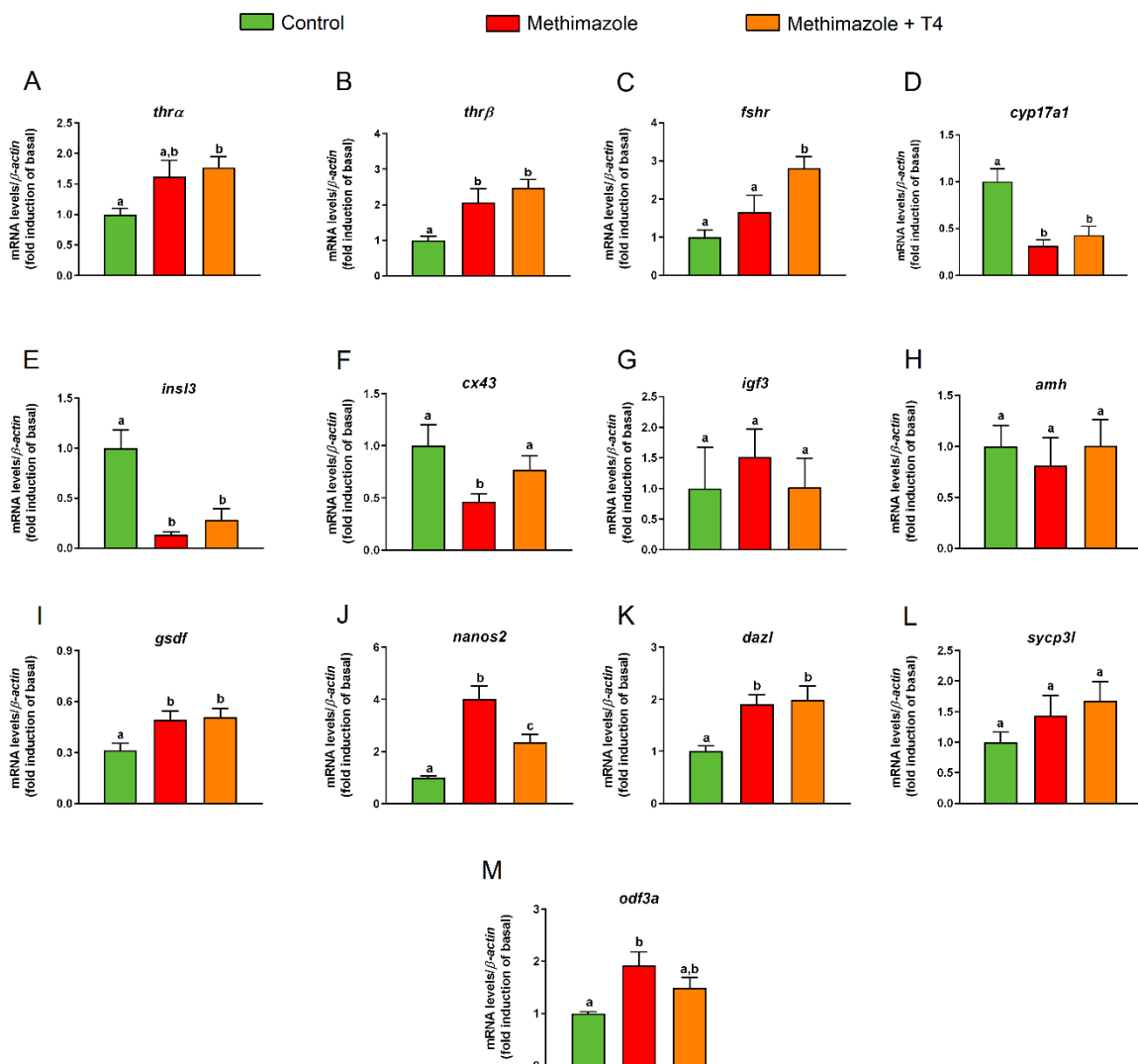
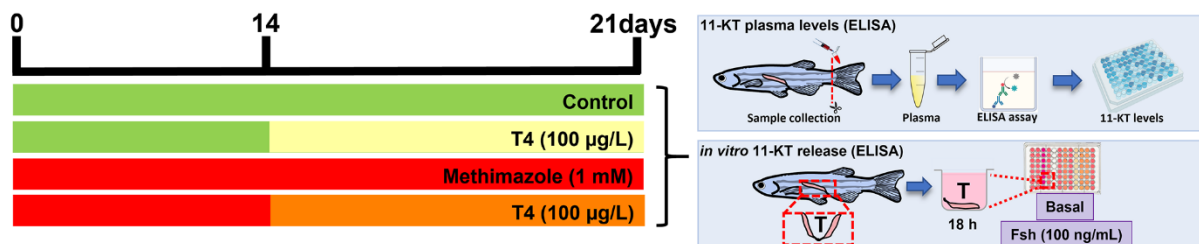
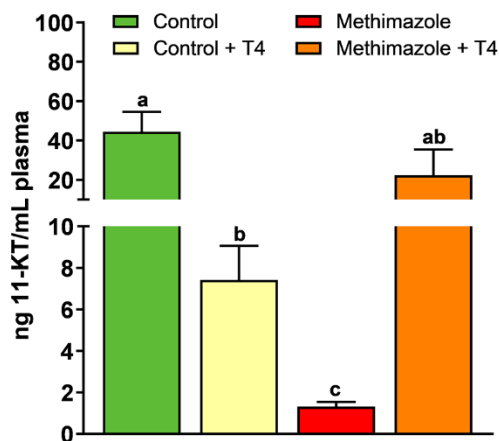


Figure 3: Relative mRNA levels of selected genes expressed in zebrafish testis after *in vivo* exposure to methimazole and methimazole co-treated with T4 for three weeks. The selected genes *thra* and *thrβ* (thyroid hormones receptor) (A-B); *fshr* (follicle-stimulating hormone receptor) (C); genes expressed by somatic cells (Leydig and Sertoli cells) (D-I); *cyp17a1* (17 α -hydroxylase/17,20 lyase) (D); *insl3* (insulin-like peptide 3) (E); *cx43* (testicular connexin) (F); *igf3* (insulin-like growth factor 3) (G); *amh* (anti-Müllerian hormone) (H); *gsdf* (gonadal somatic cell derived factor) (I); and germ cell markers (J-M); *nanos2* (J); *dazl* (deleted-in azoospermia-like) (K); *sycp3l* (synaptonemal complex protein 3) (L); *odf3a* (outer dense fiber of sperm tails 3B) (M) were evaluated. Ct values were normalized with β -actin and expressed as relative values of control levels of expression. Bars represent the mean $\pm$ SEM fold change (n = 8) relative to the control, which is set at 1. Student unpaired *t*-test. Different letters denote significant differences ($p < 0.05$) between different treatment conditions with the control.

A. Experimental design



B



C

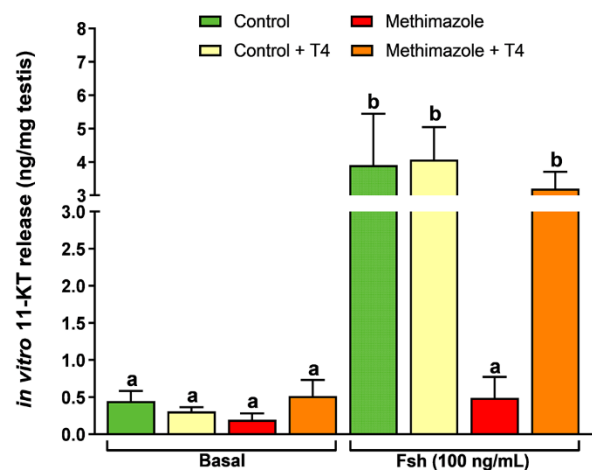


Figure 4: (A) Experimental design. 11-Ketotestosterone (11-KT) plasma levels (ng/mL plasma) was evaluated in zebrafish males exposed to different treatments: Control (non-treated fish), T4 (100 μ g/L), methimazole (1 mM) and methimazole (1 mM) + T4 (100 μ g/L). Amounts of 11-KT (ng/mg of testis weight) released by zebrafish testes were measured in the incubation media after 18h (short-term exposure) in the presence or absence of Fsh (100 ng/mL) from control, T4, methimazole and methimazole + T4 groups. (B) Effect of T4, methimazole and combination of methimazole and T4 on 11-KT plasma levels. (C) Androgen (11-KT) release from zebrafish testicular explants previously treated with T4, methimazole or methimazole co-treated with T4. Bars represent the mean $\pm$ SEM (n = 8). ANOVA followed by Tukey's test. Different letters denote significant difference ($p < 0.05$) between different treatments compared to the respective control group.

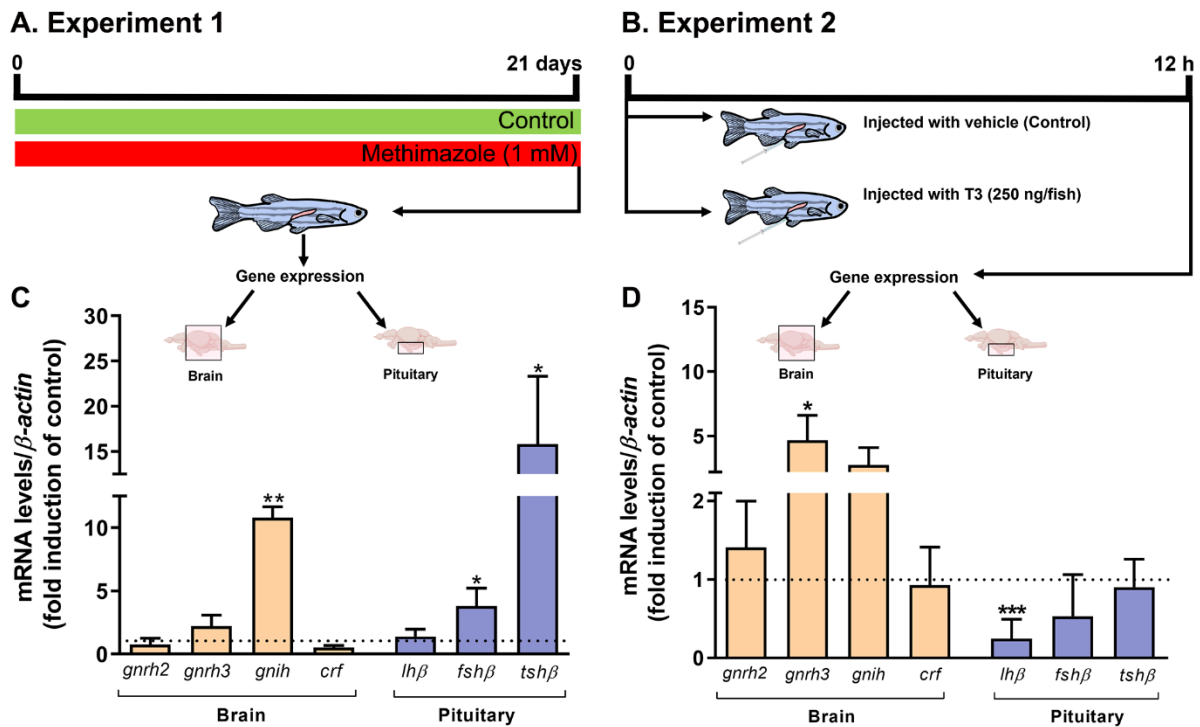


Figure 5: (A) Experiment 1: zebrafish adult males were exposed to methimazole-induced hypothyroidism for three weeks. (B) Experiment 2: zebrafish adult males were injected with 0 or 250 ng of T3/fish and tissue were collected 12 h post-injection. Relative mRNA levels of selected genes, including *gnrh2* and *gnrh3* (gonadotropin-releasing hormones), *gnih* (gonadotropin-inhibitory hormone), and *crf* (corticotropin-releasing hormone), expressed in the brain ($n = 8$), and *lh β* (luteinizing hormone), *fsh β* (follicle-stimulating hormone), and *tsh β* (thyroid-stimulating hormone) expressed in the pituitary ($n = 4$ pools of 4 pituitaries for each pool) from the methimazole group (C) and 12 h post-injection with 0 and 250 ng of T3/fish (D). Ct values were normalized with β -actin and expressed as relative values of control (no-treated fish) levels of expression. Bars represent mean $\pm$ SEM fold-change relative to the control, which is set at 1. Student unpaired t -test, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

A. Experimental design

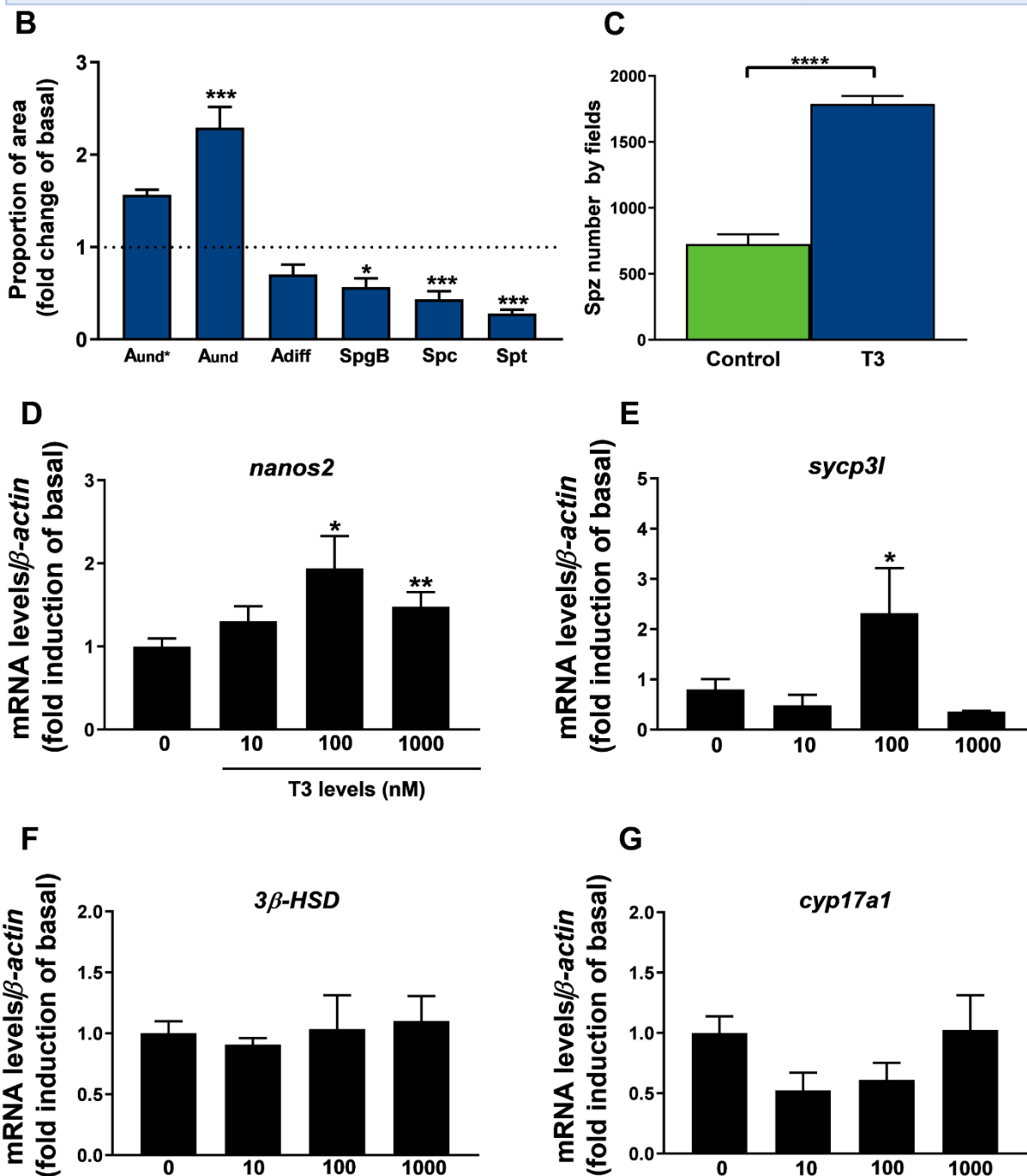
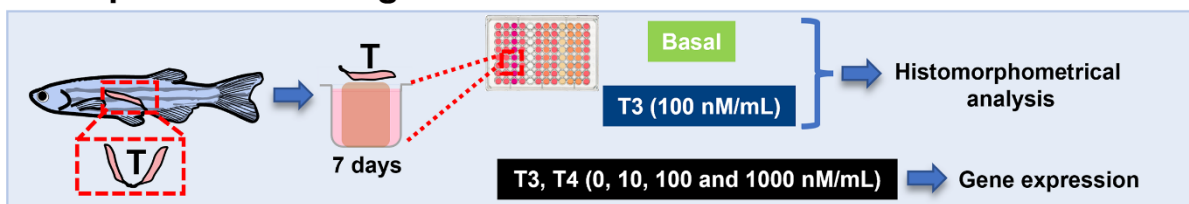
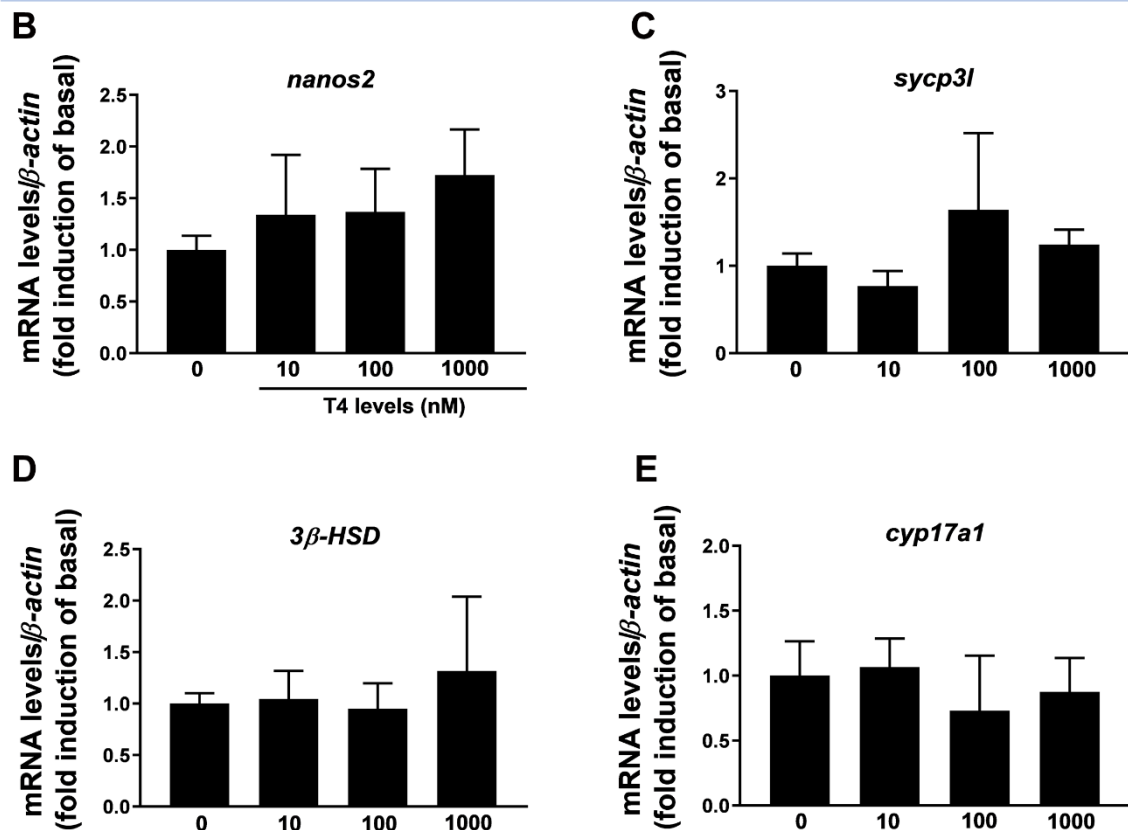
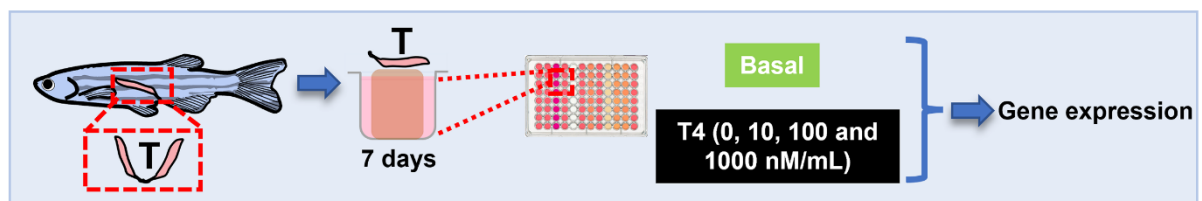


Figure 6: (A) Experimental design of histomorphometrical analysis of zebrafish testicular explants incubated for 7 days (long-term exposure) with T3 (100 nM) compared to the control (basal) and gene expression of relative mRNA levels of several selected genes in zebrafish testis incubated to different concentrations of T3 (0, 10, 100 e 1000 nM) for 7 days. **(B)** Histomorphometrical analysis of testicular explants containing types A undifferentiated

spermatogonia (A_{und}^* , A_{und}), type A differentiated spermatogonia (A_{diff}), type B spermatogonia (SpgB), spermatocytes (Spc), and spermatids (Spt). Bars (mean $\pm$ SEM; $n = 8$) are expressed as fold-change relative to the untreated group (control) (dotted black line set at 1). (C) Spermatozoa number per field generated by using IMAGEJ Software from zebrafish explants incubated for 7 days with basal (L-15) and T3 (100 nM). Bars (mean $\pm$ SEM; $n = 8$). Student paired t -test, * $p < 0.05$ and *** $p < 0.001$ denote significant differences between control and treated fish. The selected genes *nanos2* (D), *sycp3l* (synaptonemal complex protein 3) (E), *3 β -HSD* (3-beta (β)-hydroxysteroid dehydrogenase) (F), and *cyp17a1* (17 α -hydroxylase/17,20 lyase/17,20 desmolase) (G) were evaluated. Ct values were normalized with β -actin and expressed as relative values of basal levels of expression. Bars represent the mean $\pm$ SEM fold change ($n = 8$), relative to the control (basal), which is set at 1. Paired t -test, * $p < 0.05$, ** $p < 0.01$.

A. Experimental design



Supplementary Figure: (A) Relative mRNA levels of selected genes expressed in zebrafish testis after incubated for 7 days to different concentrations of T4 (0, 10, 100 e 1000 nM). The following genes were evaluated: *nanos2* (B), *sycp3l* (synaptonemal complex protein 3) (C), *3 β -HSD* (3-beta (β)-hydroxysteroid dehydrogenase) (D), and *cyp17a1* (17 α -hydroxylase/17,20 lyase/17,20 desmolase) (E). Ct values were normalized with β -actin and expressed as relative



values of basal levels of expression. Bars represent the mean $\pm$ SEM fold change ($n = 8$), relative to the control (basal), which is set at 1. Paired t -test.

References

- Allan, E.R.O., Habibi, H.R. (2012). Direct effects of triiodothyronine on production of anterior pituitary hormones and gonadal steroids in goldfish. *Mol Reprod Dev.* 79: 592-602. doi:10.1002/mrd.22066.
- Amano, M., Moriyama, S., Iigo, M., Kitamura, S., Amiya, N., Yamamori, K., Ukena, K., Tsutsui, K. (2006). Novel fish hypothalamic neuropeptides stimulate the release of gonadotrophins and growth hormone from the pituitary of sockeye salmon. *J Endocrinol.* 188:417–423. doi: 10.1677/joe.1.06494.
- Amin, S.O., El-Sheikh, A.S. (1977). Pituitary-testicular function changes in hypo- and hyperthyroid male rats. *Acta Anatomica.* 98:121-129. doi:10.1159/000144788.
- Ariyaratne, H.B.S., Mason, J.I., Mendis-Handagama, S.M. (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. doi:10.1095/biolreprod63.3.898.
- Aoki, Y., Nakamura, S., Ishikawa, Y., Tanaka, M. (2009). Expression and syntenic analyses of four nanos genes in medaka. *Zool Sci.* 26:112–118. doi:10.2108/zsj.26.112.
- Blanton, M.L., Specker, J.L. The hypothalamic-pituitary-thyroid (HPT) axis in fish and its role in fish development and reproduction. (2007). *Crit Rev Toxicol.* 37:97–115. doi:10.1080/10408440601123529.
- Branco, G. S., Melo, A. G., Ricci, J. M. B., Digmayer, M., De Jesus, L. W. O., Habibi, H. R., Nóbrega, R. H. (2018). Effects of GnRH and the dual regulatory actions of GnIH in the pituitary explants and brain slices of *Astyanax altiparanae* males. *Gen Comp Endocrinol.* 273:209-217. doi: 10.1016/j.ygcen.2018.08.006.
- Bruni, J.F., Marshall, S., Dibbet, J.A., Meites, J. (1975). Effects of hyper- and hypothyroidism on serum LH and FSH levels in intact and gonadectomized male and female rats. *Endocrinol.* 97:558-563. doi:10.1210/endo-97-3-558.
- Campbell, B., Dickey, J.T., Swanson, P. (2003). Endocrine changes during onset of puberty in male spring Chinook salmon, *Oncorhynchus tshawytscha*. *Biol Reprod.* 69:2109-2117. doi: 10.1095/biolreprod.103.020560.
- 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. doi:10.1016/j.ygcen.2010.06.001.
- Castañeda-Cortés, D.C., Langlois, V.S., Fernandino, J.I. (2014). Crossover of the hypothalamic pituitary-adrenal/interrenal, -thyroid, and -gonadal axes in testicular development. *Front. Endocrinol.* 139:1–11. doi: doi.org/10.3389/fendo.2014.00139.



Chen, S.X., Bogerd, J., Schoonen, N.E., Martijn, J., De Waal, P.P., Schulz, R.W. (2013). A progestin (17 α ,20 β -dihydroxy-4-pregnen-3-one) stimulates early stages of spermatogenesis in zebrafish. *Gen Comp Endocrinol.* 185:1-9. doi:10.1016/j.ygcen.2013.01.005.

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. doi:10.1007/s10695-005-8676-y.

Cyr, D.G., Eales, J.G. (1996). Interrelationships between thyroidal and reproductive endocrine systems in fish. *Rev Fish Biol Fisheries.* 6:165–200. doi:10.1007/BF00182342.

Cooke, P.S., Meisami, E. (1991). Early postnatal hypothyroidism causes increased adult size of testis and other reproductive organs but does not increase testosterone levels. *Endocrinol.* 129:237–243. doi:10.1210/endo-129-1-237.

Cooke, P.S., Zhao, Y-D., Bunick, D. (1994). 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. doi: 10.1095/biolreprod51.5.1000.

Cooke, P.S., Holsberger, D.R., Witorsch, R.J., Sylvester, P.W., Meredith, J.M., Treinen, K.A. Chapin, R.E. (2004). Thyroid hormone, glucocorticoids, and prolactin at the nexus of physiology, reproduction, and toxicology. *Toxicol Appl Pharmacol.* 14:309–35. doi: 10.1016/j.taap.2003.09.016.

Chiao, Y.C., Lin, H., Wang, S.-W., Wang, P. S. (2000). Direct effects of propylthiouracil on testosterone secretion in rat testicular interstitial cells. *Br J Pharmacol.* 130:1477–1482. doi:10.1038/sj.bjp.0703444.

Chiao, Y.C., Cho, W.L., Wang, P.S. (2002). Inhibition of testosterone production by propylthiouracil in rat Leydig cells. *Biol Reprod.* 67:416–22. doi:10.1095/biolreprod67.2.416.

Dittrich, R., Beckmann, M.W., Oppelt, P.G., Hoffmann, I., Lotz, L., Kuwert, T. (2011). Thyroid hormone receptors and reproduction. *J Reprod Immunol.* 90:58–66. doi:10.1016/j.jri.2011.02.009.

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. doi:10.1016/j.ygcen.2014.03.015.

Fallah, H. P., Tovo-Neto, A., Yeung, E. C., Nóbrega, R. H., Habibi, H. R. (2019). Paracrine/autocrine control of spermatogenesis by gonadotropin-inhibitory hormone. *Mol Cell Endocrinol.* 492:110440. doi:10.1016/j.mce.2019.04.020.

Fallah, H.P., Rodrigues, M.S., Corchuelo, S., Nóbrega, R.H., Habibi, H.R. (2020). Role of GnRH isoforms in paracrine/autocrine control of zebrafish (*Danio rerio*) spermatogenesis. *Endocrinol.* 161:1-16. doi:10.1210/endocr/bqaa004.



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. doi:10.1002/ar.1092420108.

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. doi:10.1016/j.ygcen.2013.02.038.

Figueiredo, A.F.A., Wnuk, N.T., Tavares, A.O., Miranda, J.R., Hess, R.A., França, L.R., Costa, G.M.J. (2019). Prepubertal PTU treatment in rat increases Sertoli cell number and sperm production. *Reprod*. 158:199-209. doi: 10.1530/REP-19-0127.

García-López, A., Jonge, H., Nóbrega, R.H., 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. *Endocrinology*. 151-2349-60. doi:10.1210/en.2009-1227.

Gautier, A., Sohm, F., Joly, J.-S., Le Gac, F., Lareyre, J.-J. (2011). The proximal promoter region of the zebrafish *gsdf* gene is sufficient to mimic the spatio-temporal expression pattern of the endogenous gene in sertoli and granulosa cells. *Biology of Reproduction*. 85:1240–1251. doi: 10.1095/biolreprod.111.091892.

Gilleron, J., Nebout, M., Scarabelli, L., Senega-Balas, F., Palmero, S., Segretain, D., Pointis, G. A. (2006). Potential novel mechanism involving connexin 43 gap junction for control of sertoli cell proliferation by thyroid hormones. *Journal of Cellular Physiology*. 209:153-161. doi: 10.1002/jcp.20716.

Habibi, H.R., Huggard-Nelson, D. L. (1998). Testosterone regulation of gonadotropin production in goldfish. *Comp Biochem Physiol C Pharmacol Toxicol Endocrinol*. 119:339-44. doi: 10.1016/s0742-8413(98)00022-x.

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. doi: 10.1016/j.ygcen.2011.11.003.

Hernandez, A. (2018). Thyroid hormone role and economy in the developing testis. *Vitam Horm*. 106:473–500. doi:10.1016/bs.vh.2017.06.005.

Hess, R.A., Cooke, P.S., Bunick, D., Kirby, J.D. (1993). Adult testicular enlargement induced by neonatal hypothyroidism is accompanied by increased Sertoli and germ cell numbers. *Endocrinol*. 132:2607-2613. doi:10.1210/endo.132.6.8504761.

Houbrechts, A.M., Houcke, J.V., Veerle, M.D. (2019). Disruption of deiodinase type 2 in zebrafish disturbs male and female reproduction. *J Endocrinol*. 241:111-123. doi:10.1530/JOE-18-0549.



Holsberger, D.R., Cooke, P.S. (2005). Understanding the role of thyroid hormone in Sertoli cell development: a mechanistic hypothesis. *Cell Tissue Res.* 322:133–40. doi: 10.1007/s00441-005-1082-z.

Huggard-Nelson, D.L., Nathwani, P.S., Kermouni, A., Habibi, H.R. (2002). Molecular characterization of LH-beta and FSH-beta subunits and their regulation by estrogen in the goldfish pituitary. *Mol Cell Endocrinol.* 188:171–193. doi:10.1016/s0303-7207(01)00716-x.

Huhtaniemi, I.T., Themmen, A.P. (2005). Mutations in human gonadotropin and gonadotropin receptor genes. *Endocrine.* 26:207–217. doi: 10.1385/ENDO:26:3:207.

Jacobs, G.F.M., Michielsen, R.P., Kuhn, E.R. (1988). Thyroxine and triiodothyronine in plasma and thyroids of the neonetic and metamorphosed axolotl *Ambystoma mexicanum*: influence of TRH injections. *Gen Comp Endocrinol.* 70:145–151. doi:10.1016/0016-6480(88)90103-7.

Jansen, H.T., Kirby, J.D., Cooke, P.S., Arambepola, N., Iwamoto, G.A. (2007). Impact of neonatal hypothyroidism on reproduction in the male hamster, *Mesocricetus auratus*. *Physiol Behav.* 90:771–781. doi:10.1016/j.physbeh.2006.12.017.

Kang, H., Kenealy, T. M., Cohen, R. E. (2020). The hypothalamic-pituitary-gonadal axis and thyroid hormone regulation interact to influence seasonal breeding in green anole lizards (*Anolis carolinensis*). *Gen Comp Endocrinol.* 292:113446. doi:10.1016/j.ygcen.2020.113446.

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. doi:10.1006/gcen.1997.7031.

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. doi:10.1016/j.ygcen.2009.03.003.

Lema, S., Dickey, J., Schultz, I.R., Swanson, P. (2009). Thyroid hormone regulation of mRNAs encoding thyrotropin beta-subunit, glycoprotein alpha-subunit, and thyroid hormone receptors alpha and beta in brain, pituitary gland, liver, and gonads of an adult teleost, *Pimephales promelas*. *J Endocrinol.* 202:43–54. doi:10.1677/JOE-08-0472.

Ma, Y., Ladisa, C., Chang, J. P., Habibi, H. R. (2020a). Multifactorial control of reproductive and growth axis in male goldfish: Influences of GnRH, GnIH and thyroid hormone. *Mol Cell Endocrinol.* 500:110629. doi:10.1016/j.mce.2019.110629.

Ma, Y., Ladisa, C., Chang, J. P., Habibi, H.R. (2020b). Seasonal related multifactorial control of pituitary gonadotropin and growth hormone in female goldfish: Influences of neuropeptides and thyroid hormone. *Front Endocrinol.* 11:175. doi:10.3389/fendo.2020.00175.

Mackenzie, D. S. (1982). Stimulation of the thyroid gland of a teleost fish, *Gill ichthys mirabilis*, by tetrapod pituitary glycoprotein hormones. *Comp Biochem Physiol A Comp Physiol.* 72:477–482. doi: 10.1016/0300-9629(82)90111-6.



Mackenzie, D.S., Sokolowska, M., Peter, R.E., Breton, B. (1987). Increased gonadotropina levels in goldfish do not result in alterations in circulating thyroid hormone levels. *Gen Comp Endocrinol.* 67:202–213. doi:10.1016/0016-6480(87)90149-3.

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. *Endocrinol.* 143:970–978. doi:10.1210/endo.143.3.8666.

Mendis-Handagama, S.M.L.C, Siril Ariyaratne, H.B. (2005). Leydig cells, thyroid hormones and steroidogenesis. *Indian J Exp Biol.* 43:939-62.

Miura, T., Miura, C., Konda, Y., Yamauchi, K. (2002). Spermatogenesis-preventing substance in *Japanese eel*. *Development.* 129:2689-2697.

Morais, R.D., Nóbrega, R.H., Gomez-Gonzalez, 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. *Endocrinol.* 154:4365-4376. doi:10.1210/en.2013-1308.

Moussavi, M., Wlasichuk, M., Chang, J. P., Habibi, H. R. (2012). Seasonal effect of GnIH on gonadotrope functions in the pituitary of goldfish. *Mol Cell Endocrinol.* 350:53–60. doi:10.1016/j.mce.2011.11.020.

Moussavi, M., Wlasichuk, M., Chang, J.P., Habibi, H.R. (2013). Seasonal effect of gonadotrophin inhibitory hormone on gonadotrophin-releasing hormone-induced gonadotroph functions in the goldfish pituitary. *J Neuroendocrinol.* 25:506–516. doi:10.1111/jne.12024.

Moussavi, M., Wlasichuk, M., Chang, J.P., Habibi, H.R. (2014). Seasonal effects of GnIH on basal and GnRH-induced goldfish somatotrope functions. *J Endocrinol.* 223:191–202. doi:10.1530/joe-14-0441.

Mukhi, S., Torres, L., Patiño, R. (2007). Effects of larval–juvenile treatment with perchlorate and co-treatment with thyroxine on zebrafish sex ratios. *Gen Comp Endocrinol.* 150:486–494. doi: 10.1016/j.ygcen.2006.11.013.

Nelson, E.R., Habibi, H.R. (2006). Molecular characterization and sex-related seasonal expression of thyroid receptor subtypes in goldfish. *Mol Cell Endocrinol.* 253:83–95. doi: 10.1016/j.mce.2006.05.003.

Nelson, E.R., Habibi, H.R. (2008). Seasonal-related homologous regulation of goldfish liver Estrogen receptor subtypes. *Cybiurn.* 32:248-249. doi:10.26028/cybiurn/2008-322SP-124.

Nelson, E.R., Habibi, H.R. (2009). Thyroid receptor subtypes: Structure and function in fish. *Gen Comp Endocrinol.* 161:90–96. doi: 10.1016/j.ygcen.2008.09.006.

Nelson, E.R.; Allan, E.R.; Pang, F.Y.; Habibi, H.R. (2010). Thyroid hormone and reproduction: regulation of estrogen receptors in goldfish gonads. *Mol Reprod Dev.* 77:784–794. doi: 10.1002/mrd.21219.



Nelson, E.R., Allan, E.R., Pang, F.Y., Habibi, H.R. (2011). Auto-regulation of thyroid hormone receptors in the goldfish ovary and testis. *Gen Comp Endocrinol.* 172:50–55. doi: 10.1016/j.ygcen.2010.12.017.

Nelson, E.R., Habibi, H.R. (2016). Thyroid hormone regulates vitellogenin by inducing estrogen receptor alpha in the goldfish liver. *Mol Cell Endocrinol.* 436:259-267. doi: 10.1016/j.mce.2016.08.045.

Nóbrega, R.H., Greebe, C.D., Van De Kant, H., Bogerd, J., França, L.R., Schulz, R.W. (2010). Spermatogonial stem cell niche ad spermatogonial stem cell transplantation in zebrafish. *Plos One.* 5:e12808. doi:10.1371/journal.pone.0012808.

Orozco, A., Valverde, C.R., Olvera, A., García, C.G. (2012). Iodothyronine deiodinases: a functional and evolutionary perspective. *J Endocrinol.* 215:207–219. doi:10.1530/JOE-12-0258.

Pankhurst, N.W. (2016). Reproduction and development. In *Fish Physiology – Biology of Stress in Fish.* (eds. Schreck, C.B., Tort, L., Farrell, A., Brauner, C. San Diego, CA: Academic Press. 295-331.

Patiño, R., Waincott, M.R., Cruzli, E.I., Balakrishnan, S., Mcmurry, C., Blazer, V.S., Anderson, T.A. (2003). Effects of ammonium perchlorate on the reproductive performance and thyroid follicle histology of zebrafish. *Environ Toxicol Chem.* 22:1115-21.

Planas, J.V., Swanson, P., Dickhoff, W.W. (1993). Regulation of testicular steroid production in vitro by gonadotropins (GTH I and GTH II) and cyclic AMP in coho salmon (*Oncorhynchus kisutch*). *Gen Comp Endocrinol.* 91:8-24. doi:10.1006/gcen.1993.1099.

Poguet, A.L., Legrand, C., Feng, X., Yen, P.M., Meltzer, P., Samarut, J., Flamant, F. (2003). Microarray analysis of knockout mice identifies cyclin D2 as a possible mediator for the action of thyroid hormone during the postnatal development of the cerebellum. *Dev Biol.* 254:188–199. doi: 10.1016/s0012-1606(02)00039-8.

Rao, J.N., Liang, J.Y., Chakraborti, P., Feng, P. (2003). Effect of thyroid hormone on the development and gene expression of hormone receptors in rat testes in vivo. *J Endocrinol Invest.* 26:435–43. doi:10.1007/BF03345199.

Rodrigues, M.S., Fallah, P.H., Zanardini, M., Malafaia, G., Habibi, H.R., Nóbrega, R.H. (2021). Interaction between thyroid hormones and gonadotropin inhibitory hormone in ex vivo culture of zebrafish testis: An approach to study multifactorial control of spermatogenesis. *Mol Cell Endocrinol.* 532:111331. doi:10.1016/j.mce.2021.111331.

Rojdmark, S., Berg, A., Kallner, G. (1988). Hypothalamic-pituitary-testicular axis in patients with hyperthyroidism. *Horm Res.* 29:185-90. doi:10.1159/000181000.

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. doi: 10.1006/gcen.1999.7432.



Ruiz De Oña, C., Obregón, M. J., Escobar Del Rey, F., Morreale De Escobar, G. (1988). Developmental changes in rat brain 5h-deiodinase and thyroid hormones during the fetal period: The effects of fetal hypothyroidism and maternal thyroid hormones. *Pediatric Research*. 24:588–594. doi: 10.1203/00006450-198811000-00010.

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. *Endocrinol*. 157:4423-4433. doi:10.1210/en.2016-1315.

Schulz, R.W., França, L.R., Lareyre, J.J., Legac, F., Chiarini-Garcia, H., Nóbrega, R.H., Miura, T. (2010). Spermatogenesis in fish. *Gen Comp Endocrinol*. 165:390-411. doi: 10.1016/j.ygcen.2009.02.013.

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. doi:10.1016/j.ygcen.2016.05.028.

Sharma, P., Patiño, R. (2013). Regulation of gonadal sex ratios and pubertal development by the thyroid endocrine system in zebrafish. *Gen Comp Endocrinol*. 184:11-119. doi:10.1016/j.ygcen.2012.12.018.

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 A Mol Integr Physiol*. 144:1-10. doi: 10.1016/j.cbpa.2006.01.017.

Swapna, I., Senthilkumaran, B. (2007). Thyroid hormones modulate the hypothalamo–hypophyseal–gonadal axis in teleosts: molecular insights. *Fish Physiol Biochem*. 33:335–45. doi:10.1007/s10695-007-9165-2.

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. *Biol Reprod*. 59:344-350. doi:10.1095/biolreprod59.2.344.

Tovo-Neto, A., Rodrigues, M.S., Habibi, H. R., Nóbrega, R. H. (2018). Thyroid hormone actions on male reproductive system of teleost fish. *Gen Comp Endocrinol*. 265:230-236. doi: 10.1016/j.ygcen.2018.04.023.

Tovo-Neto, A., Martinez, E. R. M., Melo, A. G., Doretto, L. B., Butzge, A. J., Rodrigues, M. S., Nakajima, R. T., Habibi, H. R., Nóbrega, R. H. (2020). Cortisol directly stimulates spermatogonial differentiation, meiosis, and spermiogenesis in zebrafish (*Danio rerio*) testicular explants. *Biomolecules*. 10:429. doi:10.3390/biom10030429.

Tousson, E., Ali, E.M., Ibrahim, W., Mansour, M.A. (2011). Proliferating cell nuclear antigen as a molecular biomarker for spermatogenesis in PTU- induced hypothyroidism of rats. *Reprod Sci*. 18:679-86. doi:10.1177/1933719110395401.



Trudeau, V.L., Murthy, C.K., Habibi, H. R., Sloley, B. D., Peter, R .E. (1993). Effects of sex steroid treatments on gonadotropin-releasing hormone-stimulated gonadotropin secretion from the goldfish pituitary. *Biol Reprod.* 48:300-7. doi:10.1095/biolreprod48.2.300.

Tsutsui, K., Son, Y. L., Kiyohara, M., Miyata, I. (2017). Discovery of GnIH and its role in hypothyroidism-induced delayed puberty. *Endocrinol.* 159:62–68. doi:10.1210/en.2017-00300.

ValadareS, N.F., Polikarpov, I., Garratt, R.C. (2008). Ligand induced interaction of thyroid hormone receptor beta with its coregulators. *J Steroid Biochem Mol Biol.* 112:205–12. doi:10.1016/j.jsbmb.2008.10.006.

Van Der Ven, L.T.M., Van Den Brandhof, E.-J., Vos, J.H., Power, D.M., Wester, P.W. (2006). Effects of the antithyroid agent propylthiouracil in a partial life cycle assay with zebrafish. *Environ Sci Technol.* 40:74-81. doi:10.1021/es050972c.

Xie, X., Nóbrega, R., Pšenička, M. (2020). Spermatogonial stem cells in fish: characterization, isolation, enrichment, and recent advances of in vitro culture systems. *Biomolecules.*10:644. doi: 10.3390/biom10040644.

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. doi:10.1677/JOE-08-0218.

Wagner, M.S., Wajner, S.M., Maia, A.L. (2009). Is there a role for thyroid hormone on spermatogenesis? *Microsc Res Tech.* 72:796–808. doi:10.1002/jemt.20759.

Wajner, S.M., Wagner, M.S., Maia, A.L. (2009). Clinical implications of altered thyroid status in male testicular function. *Arq Bras de Endocrinol Metab.* 53:976–982. doi: 10.1590/S0004-27302009000800011.

Wang, D.S., Baowei, J., Chongjiang, H., Xigui, H., Zhihao, L., Christopher, H.K.C. (2008). Discovery of a gonad-specific IGF subtype in teleost. *Biochemical and Biophysical Research Communications.* 367:336–341. doi:10.1016/j.bbrc.2007.12.136.

Weng, Q., Saita, E., Watanabe, G., Takahashi, S., Sedqyar, M., Suzuki, A. K., Taneda, S., Taya, K. (2007). Effect of methimazole-induced hypothyroidism on adrenal and gonadal functions in male Japanese quail (*Coturnix japonica*). *J Reprod Dev.* 53:1335-41. doi:10.1262/jrd.19081.

Yan, Y.L., Desvignes, T., Bremiller, R., Wilson, C., Dillon, D., High, S., Draper, B., Buck, C.L., Postlethwait, J. (2017). Gonadal soma controls ovarian follicle proliferation through Gsdf in zebrafish. *Dev Dyn.* 246:925-945. doi:10.1002/dvdy.24579.

Yano, A., Suzuki, K., Yoshizaki, G. (2008). Flow-cytometric isolation of testicular germ cells from rainbow trout (*Oncorhynchus mykiss*) carrying the green fluorescent protein gene driven by trout vasa regulatory regions. *Biol Reprod.* 78:151-158. doi:10.1095/biolreprod.107.064667.

Yoshiura, Y., Sohn, Y. C., Munakata, A., Kobayashi, M., Aida, K. (1999). Molecular cloning of the cDNA encoding the β subunit of thyrotropin and regulation of its gene expression by



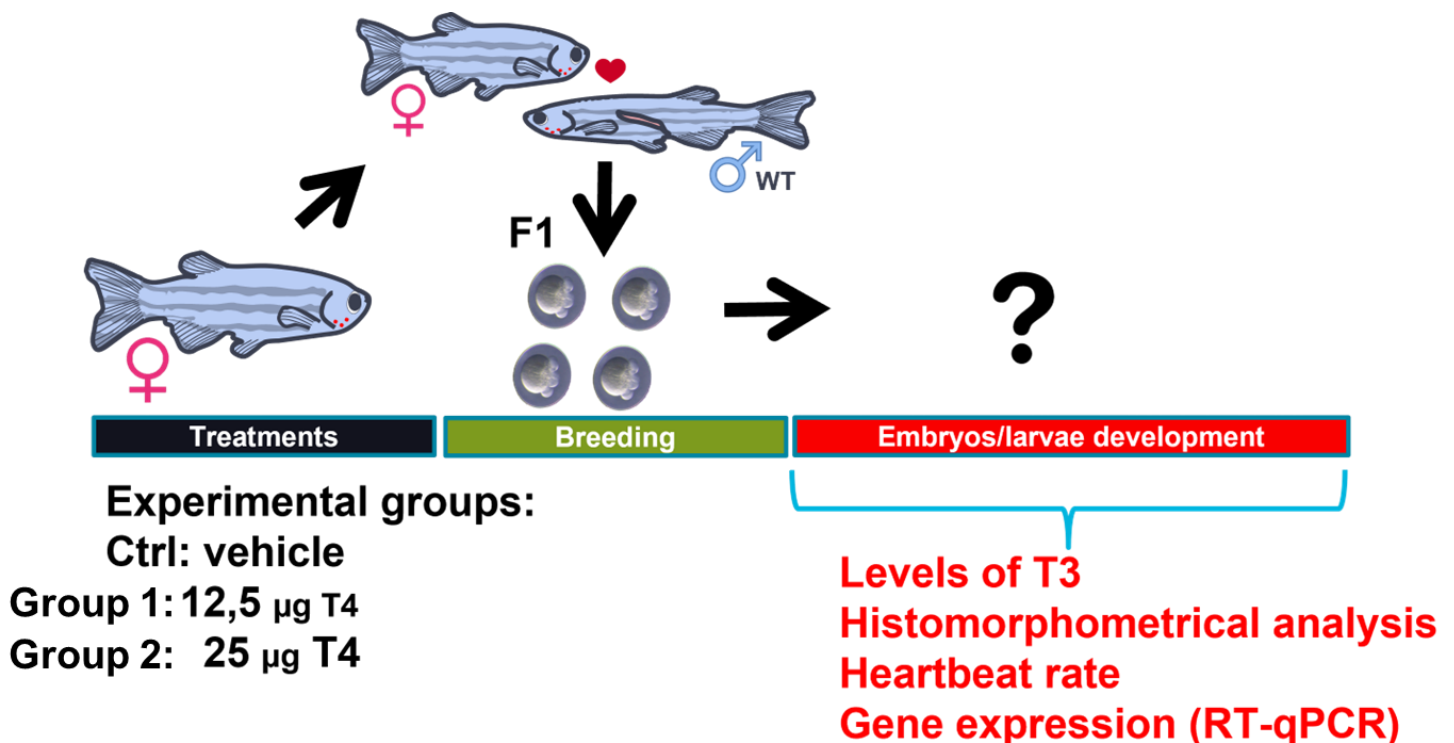
thyroid hormones in the goldfish, *Carassius auratus*. *Fish Physiology and Biochemistry*. 21:201–210. doi: 10.1016/s0303-7207(01)00392-6.

Zhang, Y., Li, S., Liu, Y., Lu, D., Chen, H., Huang, X., Liu, X., Meng, Z. Lin, H., Cheng, C.H.R. (2010). Structural diversity of the gnih/gnih receptor system in teleost: Its involvement in early development and the negative control of LH release. *Peptides*. 31:1034–1043. doi: 10.1016/j.peptides.2010.03.003.

Chapter 2

Maternal Thyroid Hormones Affect Zebrafish Embryo Development

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Graphical abstract
(Figure by the author)



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Maternal thyroid hormones affect zebrafish embryo development

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Abstract

In vertebrates, thyroid hormones (THs), specifically thyroxine (T4) and triiodothyronine (T3) play regulatory roles in various biological processes, including embryo and larval development. The initial stages of embryo and larval development rely on the supply of THs from the mother. In fish, the transfer of maternal THs to eggs and embryos and their regulatory roles in development have not been well studied. Therefore, this study aimed to investigate the effects of maternal THs on zebrafish embryos and larval development, with a focus on the somatotrophic axis. To accomplish this, zebrafish females were fed commercial food with either a low or high concentration of T4 (12.5 or 25 µg T4) per gram of body mass for 5 days. A control group was fed commercial food containing the vehicle used to dissolve the hormone (100% ethanol). On the sixth day, blood was collected from five females in each group, and plasma was sampled to extract and measure TH levels. The remaining females were transferred to a breeding tank with untreated males. Subsequently, embryos/larvae (F1) from each group were evaluated from 0 hours post-fertilization (hpf) to 144 -hpf based on parameters such as survival rate, heartbeat rate, and the presence of morphological abnormalities. Additionally, embryos/larvae were collected from 0 to 72 -hpf to measure T3 levels and examine gene expression. The relative mRNA levels of the following genes were evaluated: *crf*, *tsh*, *thra*, *thrβ*, *dio2*, *dio3*, *mct8*, *gh*, *igf1*, *mtor*, *foxO3a*, *myoG* and *myf5*. The results of our study demonstrated that T3 plasma levels were significantly increased in females fed a high concentration of T4, which consequently affected the T3 content in zebrafish embryos. Moreover, this progeny exhibited reduced growth, as well as morphological and physiological abnormalities, including yolk sac and pericardial edema, uninflated swim bladder, spine deformation, increased heartbeat rate, and higher mortality. Analysis of gene expression revealed alterations in the transcripts related to the THs axis in the progeny obtained from females fed a high concentration of T4. Additionally, the expression of genes associated with growth and development responded to changes in maternal TH concentrations. In the high T4 concentration group, mRNA levels of *gh* and *igf1* tended to decrease. Conversely, in the same group, *mtor* levels were down- and up-regulated in the progeny with 48 and 72 hpf, respectively. However, these levels tended to increase in the progeny obtained from females fed a low concentration of T4. Overall, this study highlights the critical role of maternal THs in coordinating embryo and larval developmental programs related to the somatotrophic axis. Alterations in maternal THs can have severe effects on development and the phenotype of the offspring.

Keywords: Maternal THs transfer, fetal programming, somatotrophic axis



1. Introduction

Thyroid hormones, specifically T4 (L-thyroxine = 3,5,3',5'-tetraiodo-L-thyronine) and T3 (3,3,3'-triiodo-L-thyronine) play a crucial role in the regulation of homeostasis in vertebrates, such as controlling metabolic rate, thermogenesis, cardiac function, stress response, and development (Yen, 2001; Yamano, 2005; Peter, 2011; Marelli & Persani, 2017; Balasch & Tort, 2019). Concerning development, there is evidence that maternal provision of thyroid hormones (THs) is vital for both embryonic and adult development (Power et al., 2001; Deal & Volkoff, 2020). In humans, elevated levels of maternal T4 are essential for optimal embryonic differentiation (Burrow et al., 1994). While placental transfer ensures TH supply in mammalian embryos (Stepien & Huttner, 2019), oviparous fish rely on yolk-deposited THs during embryonic development. This maternal endowment is essential for endogenous TH synthesis and facilitating proper tissue differentiation (Ayson & Lam, 1993; Lam, 1994; Brown, 1997; McComb et al., 2005). Studies conducted on zebrafish and fathead minnow show significant maternal transfer of TH-related transcripts (TR α , TR β , deiodinases, thyroid peroxidase (TPO), sodium-iodine symporter, TRH-receptors, TSH-receptor, thyroglobulin (TG), and transthyretin (TTR)) within the first 48-72 hours post-fertilization (hpf), which coincides with the onset of endogenous TH synthesis (Lazcano et al., 2023). This intergenerational transfer underscores the crucial role of THs in ensuring the fitness and viability of the offspring (Walpita et al., 2007; Campinho et al., 2014; Vergauwen et al., 2018). As a result, fluctuations in maternal THs may have a profound impact on the growth and development of offspring.

In teleost fish, numerous studies have shown a positive relationship between exogenous THs and accelerated developmental processes (Brown et al., 2014). For example, injecting T4 into pre-spawning females of *Siganus guttatus* increases circulating and oocyte THs levels, leading to improved offspring survival (Ayson & Lam, 1993). Brown (1997) observed enhanced zebrafish pigmentation, while goldfish displayed growth promotion and pathogen resistance following TH stimulation (Swain & Sahoo, 2003). In aquaculture species like *Brycon amazonicus* and *B. orbignyanus*, which experience high mortality rates due to cannibalism during early developmental stages in captivity (Senhorini et al., 1998), T3 supplementation through maternal injection or egg immersion has been found to reduce cannibalism, enhance survival, and stimulate early growth (Urbinati et al., 2008; Leonardo et al., 2013). Similarly, Alinezhad and colleagues (2019) reported that immersing sterlet sturgeon eggs in T4 treatment improved embryonic and larval survival, as well as hatching success. These findings emphasize



the crucial role of THs during key stages of growth and development in commercially important fish species. On the contrary, insufficient maternal THs levels can lead to delayed development, decreased metabolic rates, and impaired organ function, underscoring the need to maintain a precise hormonal balance for normal development (Ortiga-Carvalho et al., 2016; Koganti et al., 2021). The dual nature of THs effects highlights the importance of carefully regulating these hormones to optimize developmental outcomes in fish.

Furthermore, THs play a critical role in regulating organogenesis and growth in vertebrates during both embryonic and postnatal development. They primarily interact with the growth hormone-insulin-like growth factor I (GH-IGF-I) axis to exert their effects (Wang & Zhang, 2011; Segers et al., 2012). The anabolic effects of GH are mediated by IGF, and THs status can impact the expression of both GH and IGF-1. Disorders that disrupt THs, such as hyperthyroidism and hypothyroidism, can have severe systemic effects on vertebrates, interfering with normal growth and development (Devdhar et al., 2007; De Leo et al., 2016). For example, in hypothyroid rats, serum IGF-1 levels are reduced *in vitro*, which may be due to a direct influence of THs on *Igf1* expression or an indirect effect through GH secretion (Miell et al., 1993). THs have also been found to induce *gh* mRNA expression in fish species like rainbow trout and carp (Moav & McKeown, 1992; Farchi-Pisanty et al., 1995). Similarly, in *Oreochromis mossambicus*, a bony fish, THs stimulate hepatic *igf1* mRNA transcription in both *in vivo* and *in vitro* conditions (Schmid et al., 2003). In zebrafish larvae, exogenous T3 administration increases *igf1* transcript levels, leading to accelerated development, swim bladder inflation, and eye formation (Molla et al., 2019). Since THs play a crucial role in regulating growth, any inhibition of their activity can result in significant developmental impairments affecting various organs, including the heart (Vargas-Uricoechea et al., 2014), eyes (Houbrechts et al., 2016), skeleton (Keer et al., 2019), and brain (Fariás-Serratos et al., 2021). Understanding the mechanisms underlying the interactions between THs and the somatotrophic axis is essential for comprehending the complex regulatory network that governs fish growth and development. This knowledge has significant implications for aquaculture, where optimizing growth rates and feed efficiency is paramount. Additionally, insights into maternal THs are particularly relevant in environmental management, as controlling or predicting the impact of maternal THs can provide valuable information for conserving wild fish populations facing ecological challenges (Sun et al., 2024).

This study aimed to investigate how maternal THs influence the somatic development, growth, and phenotype of zebrafish offspring. Using zebrafish as a model organism, we



manipulated maternal THs levels by supplementing diets with varying concentrations of T4 and examined their effects on offspring at different developmental stages. By measuring plasma T3 levels, analyzing the expression of key genes related to thyroid and somatotrophic axes, and assessing survival, growth, heart rate, and phenotypic traits, the study provides critical insights into the regulatory role of maternal THs in embryonic and larval development. These findings highlight the profound impact of altered maternal TH levels on somatic development, gene expression, and offspring phenotype, contributing to a deeper understanding of maternal hormonal influence on early developmental processes.

2. Methods

2.1 *Animal husbandry*

Sexually mature male and female zebrafish (outbred) (4-12 months old) were maintained in the aquarium facility of the Department of Structural and Functional Biology, Institute of Bioscience, Botucatu, São Paulo State University (UNESP) in 6-L tanks in the recirculating system under constant temperature conditions (28 °C), and proper photothermal conditions (14h light/10h dark). The following water parameters were monitored in all tanks every other day (pH: 6.8 – 7.2, ammonia $\leq$ 0.05 ppm, and nitrite $\leq$ 0.5 ppm). The animals were fed twice a day with commercial food (Tropical Poytara Flakes, Araraquara, Brasil). Handling and experimentation were performed according to the Brazilian legislation regulated by the National Council for the Control of Animal Experimental (CONCEA) and were approved by the Ethics Committee on Animals Experiments of São Paulo State University (Protocol n. 8520250320 – CEUA).

2.2 *Thyroid hormone treatment – Preparation of spiked food*

Figure 1 illustrates an overview of the experimental workflow used in this study. Three groups of adult female zebrafish (n = 15 per group; 5 per replicate) were distributed into three replicate 6-L tanks per experimental condition as follows: One group (control) was fed with a zebrafish diet without any treatment, while the other group (group 1) was fed with a diet supplemented with thyroxine (L-Thyroxine, T4) (CAS 51-48-9; MW, 776.87 g/mol; purity $\geq$ 98%; Sigma-Aldrich, St. Louis, MO, United States) at a concentration of 12.5 $\mu\text{g T4 g}^{-1}$ of the body weight per day, while group 2 was fed diet laced with 25 $\mu\text{g T4 g}^{-1}$ of the body weight per



day. To date, no studies have specifically employed dietary T4 supplementation to elevate endogenous T3 levels in zebrafish, although similar approaches have been widely utilized to mitigate stress-induced cortisol elevation (Faught et al., 2016). Dietary thyroid hormone supplementation is an established method to modulate endocrine responses, particularly in aquaculture and experimental settings, where it is used to influence growth, metabolism, and stress response. For instance, dietary T4 has been shown to increase circulating T3 levels by deiodination *in vivo*, as T4 serves as a prohormone for the active form, T3 (Power et al., 2001). The concentrations chosen for this study, while arbitrary, were informed by previous studies employing similar methods to manipulate maternal cortisol levels and endocrine signaling, ensuring alignment with established protocols in teleost research. The zebrafish diets were prepared by soaking food pellets of commercial food in 100 % ethanol either alone (control group) or with T4 (0.25 mg or 0.5 mg of T4 per g feed) and allowing ethanol to evaporate as previously reported (Faught et al., 2016). Post complete ethanol evaporation, the pellets were stored in the refrigerator and used to feed the females. Fish weighing an average of 0.8 g were maintained in 6-L tanks and were fed 2.05% (control), 1.31% (group 1), or 1.95% (group 2) of their body weight twice per day (Figure 1, step 1). The fish received an equivalent of 12.5 μg T4 (g body mass)⁻¹ d⁻¹ (group 1) and 25 μg T4 (g body mass)⁻¹ d⁻¹ (group 2). Fish were held under these conditions for 5 days (Faught et al., 2016), and then 5 females from each experimental group (control and treatments) were collected for fish sampling and measuring maternal plasma thyroid hormone levels. Given that initial growth and larval development are dependent on the maternal supply of THs (Figure 2), the remaining females were transferred to breeding tanks on day 6 for reproduction and subsequently offspring analysis, similar to previous studies (Faught et al., 2016).

2.3 Fish sampling

On day 6, females (n = 5) from each group (control, group 1, and group 2) were euthanized with an overdose of benzocaine (250 mg/L). The body mass (BM), and ovary weight (OW) were recorded to calculate the gonadosomatic index ($\text{GSI} = \text{OW}/\text{BM} \times 100$). The blood from each animal per experimental condition was collected, and the plasma was sampled for thyroid hormone extraction and measurement levels (see Figure 1, step 3, and section 2.5).

2.4 Breeding



For a detailed schematic representation of the experimental workflow, refer to Figure 1, specifically steps 2 and 4. On day 6, female zebrafish from each experimental group (control, group 1, and group 2; $n = 5$ per group/replicate) were designated for reproduction. Females were individually placed in separate tanks ($n = 1$ female per tank), each paired with two unexposed males, following standardized zebrafish breeding protocols (Tsang et al., 2017). Breeding was initiated daily at 18:00, with fish transferred to breeding traps within the same tank. Fertilized eggs were collected the following morning at first light (09:00) post-mating. Collected eggs were transferred to Petri dishes containing embryo medium (Hank's medium, as described in ZFIN) and sampled at predefined intervals: 0, 24, 48, 72, 96, 120, and 144 hours post-fertilization (hpf). From 0 to 72 -hpf, eggs, embryos, and early larvae were counted, and pools of 20 individuals ($n = 5$ pools per group) were snap-frozen in liquid nitrogen and stored at -80°C for downstream gene expression analysis. Additionally, three pools of 50 individuals were collected for thyroid hormone extraction and quantification. Furthermore, eggs, embryos, and larvae from 0 to 144 -hpf were systematically sampled for phenotypic characterization.

2.5 Thyroid hormone extraction and measurement

Blood samples were collected from adult female zebrafish under different experimental conditions (control, group 1, and group 2; $n = 5$ per condition/replicate) to assess plasma T3 levels. Fish were euthanized, and blood was obtained by severing the caudal peduncle, using heparinized syringes and tubes to prevent coagulation. Plasma was isolated by centrifugation at 4°C for 10 minutes at $800 \times g$ (Eppendorf Centrifuge 5424 R). Following the manufacturer's protocol, plasma T3 levels were quantified using an Enzyme-Linked Immunosorbent Assay (ELISA) kit (EIAT3C, Thermo Fisher Scientific). Plasma samples were prepared by extracting T3 with ethyl acetate at a solvent-to-sample ratio of 5:1 (v/v). Samples were snap-frozen, and the solvent phase was collected after extraction. The extraction process was repeated to maximize recovery and pooled solvent extracts were dried in a vacuum concentration (SpeedVac) for 2-3 hours. The dried extracts were reconstituted in 1X Assay Buffer at room temperature. A high-binding 96-well plate was coated with donkey anti-sheep IgG, and $100\ \mu\text{l}$ of either standards or prepared samples were added to the wells in duplicate. Each well-received $25\ \mu\text{l}$ of T3 conjugate and T3 antibody. The plate was incubated for 2 hours at room temperature with gentle shaking, followed by washing with 1X Wash Buffer. Detection was achieved by



adding 100 μ l of Tetramethylbenzidine (TMB) substrate solution, incubating for 30 minutes, and stopping the reaction with 50 μ l of 1 M HCL. Absorbance was measured at 450 nm using a microplate reader (Epoch, Agilent, Santa Clara, CA, USA). T3 concentrations were determined using a standard curve and expressed as nanograms of T3 per mL of plasma.

Embryos and larvae from 0 to 72 -hpf were collected from each maternal group (control, group 1, and group 2), snap-frozen in liquid nitrogen, and stored at -80 °C. Three pools of 50 specimens per maternal group (n = 3 pools of 50 specimens each) were homogenized in 0.4 mL ELISA Buffer using an Ultra-TurraxT8 basic homogenizer (IKA, Staufen, Germany). Samples were disrupted by intermittent sonification for 5 minutes on ice, followed by vigorous vortexing for 10 minutes and centrifugation at 4 °C for 10 minutes at 5000 x g (Eppendorf Centrifuge 5424 R). The resulting supernatant was collected and stored at -80 °C until analysis. T3 levels in embryos and larvae were measured using the same ELISA protocol described above, with 100 μ l of each sample analyzed in duplicate. Hormonal levels were quantified using a standard curve and expressed as picograms of T3 per embryo or larvae. For a schematic overview of the experimental workflow, see Figure 1, steps 3-5.

2.6 Transcript levels of thyroid and somatotropic axes

To find mechanistic linkages between the disrupted levels of maternal thyroid hormones and embryo development, mRNA abundance of key genes involved in thyroid and somatotropic axes was measured by qPCR in 0 to 72 -hpf embryos (Figure 1, step 5). Embryos were pooled into 20 (n = 5 pools of 20 individuals for each pool) per experimental group, then snap-frozen on liquid nitrogen and stored at -80°C for gene expression. Total RNA was extracted from the pools using a commercial kit (PureLink™ RNA mini kit, Ambion, Life Technologies, Carlsbad, CA, United States). After RNA extraction, the usual downstream methods were followed according to the manufacturer's instructions, and quality and purity were checked with a NanoDrop™ One Spectrophotometer (Thermo Scientific, Madison, WI, United States). cDNA synthesis was performed as described by Nóbrega and colleagues (2010). qPCR reactions were conducted using 5 μ l of 2X SYBR-Green Universal Master Mix, 1 μ l of forward primer (1.125 nM), 1 μ l of reverse primer (1.125 nM), 0.5 μ l of DEPC water, and 2.5 μ l of cDNA (Figure 1, step 5). The relative mRNA levels of *crf* (corticotropin-releasing hormone), *tsh* (thyroid-stimulating hormone), *thra* and *thr β* (thyroid hormone receptors), *mct8* (monocarboxylate transporter 8), *dio2* and *dio3* (deiodinases type 2 and 3), *gh* (growth

hormone), *igf1* (insulin-like growth factor 1), *mtor* (mammalian target of rapamycin), *foxo3a* (forkhead box O3), *myf5* (myogenic factor 5), and *myoG* (myogenin). mRNA target expressions (CTs) were normalized using the β -actin gene as a reference, considering the stable expression levels of this reference gene among the experimental groups (García-Lopez et al., 2010; Costa et al., 2024). Results were expressed as relative values to the control group (as fold induction), using the $2^{-(\Delta\Delta CT)}$ method. Primers were designed based on zebrafish genetic sequences available in GenBank (NCBI, <https://www.ncbi.nlm.nih.gov/genbank/>) and are listed in Table 1.

Table 1: Primers used for gene expression studies (qPCR) (FW = Forward; RV = Reverse).

Target name	Primers sequence (5' - 3')		Reference
<i>β-actin</i>	AGACATCAGGAGTGATGGT	FW	BC045846
	CAATACCGTGTCATGGGG	RV	
<i>crf</i>	CACCGCCGTATGAATGTAGA	FW	NM_001007379.1
	GAAGTACTCCTCCCCCAAGC	RV	
<i>tsh</i>	GCAGATCCTCACTTCACCTACC	FW	NM_001145763.2
	GCACAGGTTTGAGCATCTCA	RV	
<i>thra</i>	CTGCTGTGATTGGATGCTGGAT	FW	NM_205625
	GCTCTTGTCGGATGTGCTGTT	RV	
<i>thrβ</i>	ATCGACCAGAGCCCACACA	FW	NM_131340.1
	TAGGTGCCGATCCAATGTCTT	RV	
<i>mct8</i>	GAAGGCTTTTCCCTTTGCTCG	FW	This work
	AGCTCTGCGTTCAGTTTCAGA	RV	
<i>dio2</i>	TGGATGCCTACAAACAGGTGA	FW	This work
	GTCTTACCGCTGATGCTCCA	RV	
<i>dio3</i>	AGCAATGCTTCTGGGTCTCA	FW	This work
	GGCTGTGTTCAAAACCTCCT	RV	
<i>gh</i>	TGACCATCGGAAACCCCAAC	FW	This work
	GTCCATCGAGACATCCCTTG	RV	
<i>igf1</i>	CCCAGGACACCAAAGAAACCTA	FW	This work
	CGGCTCGAGTTCTTCTGATGA	RV	
<i>mtor</i>	GGAATGCAACAGCAAGCACA	FW	This work
	GCTTCAGAAAACACCGTGCC	RV	
<i>foxo3a</i>	GCTCTGCAGGATGGAAGAACT	FW	This work
	TCCACCATGAACTCTTTCCTGT	RV	
<i>myf5</i>	TGGAGCAATACTACAGCCTGC	FW	This work
	TGTTGCAGTCAACCATGCTCT	RV	
<i>myoG</i>	GGTCAGTTCACTCAACCAGC	FW	This work
	AGAGGACGACACCCCAAGTAT	RV	



2.7 Characterization of embryo and larvae phenotypes

Embryos and larvae from the control and treatment groups (groups 1 and 2) were collected from spawning tanks and systematically evaluated for morphological changes throughout development. At 48 - 144 -hpf, 30 embryos, and larvae were randomly selected from each group. Bright-field images of each specimen ($n = 30$ per group/replicate) were captured using an Olympus MVX10 MacroView stereomicroscope. Morphometric parameters were measured using the annotation capabilities of the Olympus cellSens dimensions software (<https://www.olympuslifescience.com/pt/software/cellsens/>) and further analyzed with ImageJ software (available at <http://imagej.nih.gov/ij/index.html>). Morphometric analyses included measurements of total larval length, as well as assessments of developmental anomalies, including pericardial and yolk sac edema, swim bladder inflation, and the frequency of deformities such as abnormal body curvature were analyzed, based on the OECD FET test.

Survival rates of zebrafish embryos and larvae from 0 to 144 -hpf were evaluated using the Kaplan-Meier method. Survival was calculated as the percentage of viable embryos and larvae relative to the normal number of specimens collected at 0 -hpf. Non-viable embryos and larvae, identified as those settling at the bottom of the Petri dish with an opaque appearance, abnormal coloration, and hyporesponsiveness to mechanical stimuli, were classified as dead (Figure 1, step 5). All morphological and morphometrical evaluations were conducted by a single observer to ensure consistency and minimize variability in the assessments. This analysis provided a detailed comparison of developmental progression and survival rates between the control and treatment groups.

2.8 Heart rate measurement

Videos from randomly zebrafish embryos at 48 -hpf from control and treatments ($n = 20$ /condition) were taken to calculate the heart rate per minute. The non-anesthetized zebrafish embryos were immobilized using excavated slide blanks containing sufficient embryonic medium for survival during filming. Our basic setup consists of an Olympus MVX10 MacroView stereomicroscope linkage and an Olympus cellSens dimensions software (<https://www.olympuslifescience.com/pt/software/cellsens/>) to obtain the videos. The video files (.AVI) are obtained at 20 frames per second (fps) totaling 50 seconds of recording, and a resolution of 4080 x 3072 pixels. For quantification, we used a Python script named CardiOT2[®]



software that uses the Fast Fourier (FFT) methodology to calculate the heart rate contraction cycles (Figure 1, step 5).

2.9 Statistical analysis

All data were first assessed for normality using the Shapiro-Wilk test and for homogeneity of variance using Bartlett's test. Data are presented as mean $\pm$ standard error of the mean (SEM). Significant differences between groups were determined using unpaired Student's t-tests (non-parametric) to compare treated versus control groups, or a one-way analysis of variance (ANOVA) to analyze the difference between the means of more than two groups. A significant level of $p \leq 0.05$ was considered for all tests. The normality of the data for all genes in this study was further verified using the Kolmogorov-Smirnov test. Statistical analyses were performed using Graph Pad Prism software 8.0.1 (Graph Pad Software, Inc., San Diego, CA, USA, <http://www.graphpad.com>).

3. Results

3.1 Thyroid hormones (T4) supplementation alters embryo yield without affecting somatic or gonadal parameters in female zebrafish

The whole-body and ovary weights did not differ significantly between the control and treated groups of female zebrafish (Figure 3A-B). Similarly, the gonadosomatic index (IGS) was comparable across the experimental groups (Figure 3C). After breeding females from the control and treated groups, we collected and quantified the embryos. The treatment with 25 μ g of T4 (group 2) significantly reduced the mean embryo yield compared to the control group (Figure 3D). In contrast, the treatment with 12.5 μ g of T4 (group 1) increased the mean embryo yield relative to the control group, but this difference was not statistically significant (Figure 3D).

3.2 High-dose thyroid hormone supplementation elevates plasma T3 levels and modulates embryonic T3 content in zebrafish

Plasma samples were collected from females of control, groups 1 and 2 to evaluate potential alterations in T3 plasma levels. The analysis revealed a significant increase in plasma T3 levels in females subjected to group 2 (approximately 972 ng/ml) compared to the control group (approximately 298 ng/ml) (Figure 4A). In contrast, T3 levels in females from group 1 (approximately 152 ng/ml) were statistically similar to those in the control group (Figure 4A).

In the present study, the administration of elevated thyroid hormone concentrations ($25 \mu\text{g T4 (g body mass)}^{-1} \text{d}^{-1}$, group 2) to female zebrafish for 5 days modulated the T3 content in their embryos. T3 concentrations were quantified in pooled samples of 50 embryos at various developmental stages from 0 to 72 -hpf. At 0 -hpf, the T3 concentration in whole embryos from group 2 was significantly lower than that in the control group (vehicle, 100 % alcohol) (Figure 4B). No significant differences in T3 levels were observed between the control and experimental groups 1 [$12.5 \mu\text{g T4 (g body mass)}^{-1} \text{d}^{-1}$] and 2 at 24 -hpf (Figure 4B). In contrast, at 48 -hpf and 72 -hpf, T3 content in embryos from group 2 was significantly elevated compared to the control group (Figure 4B). Embryos from group 1 exhibited T3 concentrations that were comparable to those of the control group across the evaluated time points (Figure 4B).

3.3 Maternal THs status affects relative mRNA levels of genes involved in the thyroid and somatotrophic axes in zebrafish offspring

Samples were collected at 0, 24, 48, and 72 -hpf for analysis of gene expression by qPCR. Of the genes evaluated in embryos, the expression of essential components of the thyroid hormones axis such as *crf*, *tsh*, *thra*, *thrβ*, *mct8*, *dio2*, and *dio3* were significantly altered in the zebrafish progeny from females fed with different concentrations of thyroid hormones (T4) when compared to control (Figure 5). The analysis revealed that the highest maternal T3 concentration ($25 \mu\text{g T4 (g body mass)}^{-1} \text{d}^{-1}$, group 2) significantly up-regulated *crf* and *tsh* mRNA levels in embryos between 24 and 72 -hpf (Figure 5A-B). In contrast, embryos from group 1 [$12.5 \mu\text{g T4 (g body mass)}^{-1} \text{d}^{-1}$] exhibited an initial upregulation of *crf* mRNA at 0 -hpf, followed by a marked decline at 48 and 72 -hpf (Figure 5A), while *tsh* expression also decreased at 72 -hpf (Figure 5B).

The study further examined the response of thyroid receptor subtypes (*thra*, *thrβ*) during embryonic development following maternal treatment. Both receptor mRNA levels increased during embryonic development in both treatments (Figure 5C-D). Specifically, *thra* was up-regulated at 24 -hpf in embryos from both treatments (Figure 5C). Although, *thra* levels

decreased at 48 -hpf in both groups, a significant increase was observed at 72 -hpf in embryos from group 1 (Figure 5C). In contrast, *thrβ* levels were elevated in embryos at 24-48 -hpf in both treatments (Figure 5D) and remained significantly increased at 72 -hpf in treatment 2 (Figure 5D).

The expression of thyroid hormone (TH) transporters (*mct8*) and deiodinases (*dio2* and *dio3*) was significantly altered in embryos/larvae obtained from females of both treatment groups (Figure 5E-G). *mct8* expression increased at 0 and 24 -hpf in embryos derived from females treated with 25 $\mu\text{g T4 (g body mass)}^{-1} \text{d}^{-1}$ (Figure 5E). The expression of *dio2* and *dio3* were significantly reduced in both treatments when compared to the control group (Figure 5F-G). Specifically, mRNA levels of *dio2* were down-regulated in group 2 (25 $\mu\text{g T4 (g body mass)}^{-1} \text{d}^{-1}$) at 24 -hpf, while *dio3* expression decreased at this time in both treatment groups as compared to control (Figure 5F-G). At 48 -hpf, the levels of *dio2* (48 -hpf, group 1) and *dio3* (48 -hpf, both groups) remained low, although no statistical differences were observed. However, at 72 -hpf, *dio2* expression was drastically decreased in embryos from both treatments (Figure 5F-G).

To further explore the role of maternal thyroid hormones in zebrafish embryonic development, the expression of genes related to the somatotropic axis, including *gh*, *igf1*, *mtor*, *foxo3a*, *myf5*, and *myoG*, was assessed in embryos at various developmental stages (0 to 72 -hpf) under different treatments (control, groups 1 and 2). At 48 -hpf, *gh* mRNA levels were down-regulated in embryos derived from group 2 (25 $\mu\text{g T4 (g body mass)}^{-1} \text{d}^{-1}$) and remained low at 72 -hpf compared to the control group (Figure 6A), while *igf1* expression was up-regulated at 0 and 72 -hpf (Figure 6B). For the embryos originated from group 1 (12.5 $\mu\text{g T4 (g body mass)}^{-1} \text{d}^{-1}$), the expression patterns of *gh* and *igf1* contrasted with those observed in group 2. At 48 -hpf, *gh* mRNA levels increased when compared to control levels (Figure 6A), while *igf1* levels decreased at 24 -hpf (Figure 6B). After 24 -hpf, *igf1* expression tended to increase, but no statistical differences were found between group 1 and the control (Figure 6B).

Next, the expression of *mtor* and *foxo3a*, genes involved in muscle protein synthesis and degradation, respectively, were evaluated. The regulation of these genes is crucial for maintaining growth. *foxo3a* expression remained unchanged across treatments and time points evaluated (Figure 6D). In contrast, maternal thyroid hormone alterations affected *mtor* expression, leading to disturbances in embryonic development. *mtor* mRNA levels were elevated at 24 -hpf in embryos from group 1 (Figure 6C), while for group 2, *mtor* levels were decreased at 48 -hpf and increased at 72 -hpf (Figure 6C).

Finally, the expression of myogenesis regulatory factors was assessed in the offspring of females treated with T4. The expression of *myf5* and *myoG* were modulated as follows: 12.5 $\mu\text{g T4 (g body mass)}^{-1} \text{d}^{-1}$ maternal treatment did not affect *myf5* expression during the evaluated period (Figure 6E), while *myoG* expression decreased only at 24 -hpf in this group (Figure 6F). Both *myf5* and *myoG* embryonic mRNA levels increased following 25 $\mu\text{g T4 (g body mass)}^{-1} \text{d}^{-1}$ maternal treatment at 0 -hpf (Figure 6E-F), and *myf5* expression showed elevated levels at 72 -hpf in this group as compared to control (Figure 6E).

3.4 Disruption of maternal thyroid hormone levels impairs zebrafish embryonic survival, growth, and development

The impact of maternal THs on embryonic and larval survival and phenotypes was evaluated by analyzing developmental stages from 0 to 144 -hpf across experimental groups (control, groups 1 and 2). The findings revealed the cumulative effects of altered maternal and embryonic TH levels in the different groups when compared to the control (Figures 7-9). Survival rates, assessed using the Kaplan-Meier method, remained consistently high (~100 %) in control and group 1 (12.5 $\mu\text{g T4 (g body mass)}^{-1} \text{d}^{-1}$) throughout the evaluation period (Figure 7A). Progressive and accelerated growth was observed between 48 and 144 -hpf in the control and group 1. At 144 -hpf, larvae derived from group 1 reached an average total body length of approximately 4.1 mm, closely aligning with the 4.06 mm reported for the control group, which is consistent with observations by Kimmel et al. (1995) (Figure 7B). In contrast, embryos originated from 25 $\mu\text{g T4 (g body mass)}^{-1} \text{d}^{-1}$ maternal treatment exhibited a significant decline in survival rates, dropping to 70 % at 120 -hpf and further plummeting to 20 % at 144 -hpf (Figure 7A). Larvae from this group also showed a marked reduction in body length, measuring 3.5 mm at 144 -hpf (Figure 7B). While embryonic and larval development remained unaffected in control and group 1 (Figures 7-9), 25 $\mu\text{g T4 (g body mass)}^{-1} \text{d}^{-1}$ maternal treatment induced multiple developmental abnormalities (Figures 7-9). These abnormalities included pericardial and yolk sac edema (Figures 7C-D, and 9) observed between 48 and 144 -hpf, an increased frequency of uninflated swim bladders post 72 -hpf (Figures 7E and 9), and a higher incidence of scoliosis between 120 and 144 -hpf (Figures 7F and 9).

3.5 Elevated maternal thyroid hormones disrupt cardiac function in zebrafish embryos



The normal embryonic heart rate in zebrafish closely aligns with that of humans, ranging from 120 to 180 beats per minute (bpm) (Baker et al., 1997). In the present study, embryos originated from $25 \mu\text{g T4 (g body mass)}^{-1} \text{d}^{-1}$ maternal treatment exhibited a significant increase in cardiac rate as compared to control (Figure 10A-D). The average heart rate, measured across 20 zebrafish embryos, reached approximately 300 bpm, as confirmed by statistical analysis (Figure 10D; Supplementary V1). In contrast, maternal treatment with $12.5 \mu\text{g T4 (g body mass)}^{-1} \text{d}^{-1}$ resulted in embryonic heart rates comparable to those observed in the control group (Figure 10A-D).

4. Discussion

This study highlights the significant impact of elevated maternal TH levels on zebrafish embryonic and larval development. Notably, elevated maternal plasma T3 levels disrupted offspring homeostasis, as insufficient T3 at 0 -hpf impaired its regulation at subsequent stages (48 and 72 -hpf). This study showed that increased maternal TH levels stimulated the expression of genes associated with the HPT axis in the offspring at different stages of embryonic development disrupting TH synthesis and inactivation during critical developmental stages. Consequently, these cumulative hormonal imbalances led to adverse outcomes, including reduced survival rates, growth inhibition, morphological abnormalities, and irregular heart rates. These developmental disturbances were closely linked to elevated maternal T4 levels and dysregulated gene expression in the somatotropic axis, underscoring the interconnected effects of maternal TH alterations on offspring development.

Thyroid hormones (THs) are well-documented components of both fertilized and unfertilized mature eggs of maternal origin. These hormones are transferred or accumulated in the developing oocyte during vitellogenesis, as extensively reported in various species (Kobuke et al., 1987; Greenblatt et al., 1989; Tagawa & Hirano, 1990; Lam, 1994; Power et al., 2001; Yamano, 2005; Szisch et al., 2005; Ruuskanen & Hsu, 2018). The deposition of maternal THs into the yolk facilitates endocrine communication during early development in oviparous fishes, providing a critical regulatory mechanism before the formation of embryonic secretory follicles and the HPT axis (Brown et al., 2014). Maternal THs, primarily thyroxine (T4), are instrumental in this process (Tagawa & Hirano, 1989).

In *Siganus guttatus* (rabbitfish), maternal THs have been shown to enhance larval performance, growth, and survival (Ayson & Lam, 1993). Similarly, in freshwater species such



as *Carassius auratus* (goldfish) and *D. rerio* (zebrafish), exogenous THs improve pathogen resistance and promote growth (Brown, 1997; Swain & Sahoo, 2003). These findings highlight the potential of maternal TH supplementation to positively influence larval development and survival. However, disruptions in maternal TH supply have been linked to adverse reproductive outcomes, including reduced egg-laying in avian species such as *Gallus gallus domesticus* (domestic chicken) (Chen et al., 2008; Wilson, 2018; Darras et al., 2019).

In the present study, maternal whole-body weight, ovary weight, and gonadosomatic index (IGS) were unaffected by thyroid supplementation. However, higher concentrations of T4 significantly impaired egg fertilization and hatching in zebrafish. These abnormalities are likely attributed to dysregulated maternal TH levels, highlighting the critical role of balanced TH homeostasis in ensuring successful reproductive and developmental outcomes.

Our findings revealed that exposure of zebrafish females to exogenous T4 disrupts maternal plasma triiodothyronine (T3) levels and alters TH content in F1 eggs, resulting in impaired TH homeostasis across generations. This disruption manifests as abnormal plasma T3 levels during embryonic and larval development. Elevated maternal circulating T3 levels are likely due to the partial enzymatic conversion of T4 into T3, catalyzed by type 1 and type 2 deiodinases (DIO1 and DIO2, respectively). These enzymes are critical for regulating the bioavailability of THs, as T3 represents the active form required for physiological processes (Brent, 2012).

Transport of T3 into target tissues is facilitated by monocarboxylate Mct8, which demonstrates a high affinity for T3 and likely mediates TH exchange between tissues and circulation (Jansen et al., 2005; Vancamp et al., 2018). Excessive T3 can also be inactivated via conversion to reverse T3 by type 3 deiodinase (DIO3), highlighting the importance of a tightly regulated TH metabolic network (Basset et al., 2003; Darras et al., 2006; Gereben et al., 2008; Orozco et al., 2012; Tovo-Neto et al., 2018). Similar enzymatic activities have been observed in other teleosts, including *Oncorhynchus mykiss* (rainbow trout) and *S. guttatus* (rabbitfish), following exogenous T4 administration (Fok & Eales, 1984; Ayson & Lam, 1993).

The significantly elevated maternal plasma T3 levels observed in this study suggest the activation of a negative feedback mechanism within the hypothalamic-pituitary-thyroid (HPT) axis in response to high T4 doses. This feedback loop likely leads to altered TH transfer to F1 embryos, as evidenced by the reduced levels of T3 in the fertilized eggs (0 -hpf) and its further accumulation at 48 and 72 -hpf. Impaired deiodinase activity may exacerbate this accumulation, disrupting the synthesis and regulation of THs during critical developmental windows. These



findings emphasize the necessity of optimal maternal TH levels for ensuring normal embryonic development.

In humans, maternal hypothyroidism or hyperthyroidism similarly affects fetal growth and development, emphasizing the conserved role of THs across species (Darras, 2019). During pregnancy, elevated maternal T4 levels support embryonic differentiation before the onset of fetal TH production, with placental deiodinases modulating fetal exposure to THs and protecting against excess (Burrow et al., 1994). In avian models, such as *G. gallus domesticus* (domestic chicken), excessive maternal goitrogens, such as methimazole, have been shown to inhibit embryonic thyroid gland function, leading to delayed or failed hatching (Van Herck et al., 2013; Darras, 2019). Environmental contaminants, including endocrine-disrupting chemicals, further complicate maternal TH regulation. Studies have demonstrated that exposure to bisphenol S (BPS) in zebrafish altered maternal plasma TH levels and F1 egg content, resulting in delayed embryonic development (Wei et al., 2018). Additionally, exposure to decabromodiphenyl ethane (DBDPE) induced transgenerational epigenetic modifications in the thyroid endocrine system, highlighting sex-specific responses within the HPT axis (Sun et al., 2024). These studies collectively emphasize the sensitivity of maternal TH regulation to external stressors and the implications for offspring health.

This research demonstrates the critical role of maternal TH homeostasis in supporting normal embryonic and larval development in zebrafish. The findings highlight the necessity for further investigation into the mechanisms of maternal TH transfer, the impact of environmental stressors, and the intergenerational effects of endocrine disruption on TH-mediated developmental processes.

To elucidate the impact of maternal thyroid hormones (THs) on the offspring of zebrafish, our study analyzed the expression of key genes associated with the thyroid and somatotrophic axes during early development. The findings revealed significant stage-specific alterations in thyroid-related gene expression, emphasizing the critical role of maternal THs in shaping developmental processes. Elevated maternal TH levels were linked to up-regulation of *crf*, *tsh*, *thra*, and *thrβ* in offspring from 24 to 72 -hpf. This result suggests that the observed response may be associated with reduced embryonic T3 levels following maternal treatment with 25 $\mu\text{g T4 (g body mass)}^{-1} \text{ d}^{-1}$, potentially representing a compensatory mechanism aimed at stimulating the hypothalamic-pituitary-thyroid (HPT) axis to counteract the low T3 levels. Moreover, maternal T4 significantly up-regulated *mct8* mRNA levels during the 0-24 -hpf window, while suppressing *dio2* and *dio3* transcripts at later stages (24 and 72 -hpf). These



findings align with previous research showing maternal transfer of *dio1* and *dio2* transcripts to zebrafish embryos at the 2-cell stage (Dong et al., 2013).

Notably, thyroid receptor isoforms have been identified in unfertilized eggs of zebrafish and fathead minnow (*P. promelas*), highlighting their conserved role during early development (Takayama; 2008; Vergauwen 2018). The maternal contributions of deiodinases and thyroid receptors are essential for organogenesis, including the development of the brain, eyes, ears, and swim bladder (Walpita et al., 2009; Houbrechts et al., 2016; Van Dingenen et al., 2023). Inhibition of deiodinases in zebrafish embryos has been shown to impair swim bladder inflation, further highlighting the importance of maternal TH regulation (Van Dingenen et al., 2023). In our study, embryos from T4-treated females displayed a higher incidence of uninflated swim bladders between 96 and 144 -hpf. This observation may be attributed to dysregulation of TH levels and the inhibition of deiodinases, as evidenced by the suppression of *dio2* and *dio3* transcripts at later developmental stages (24 and 72 -hpf).

Maternal TH dysregulation also influenced the somatotrophic axis, as evidenced by changes in the expression of *gh* and *igf1* transcripts. Offspring of females fed a diet supplemented with high concentrations of T4 [$25 \mu\text{g T4 (g body mass)}^{-1} \text{d}^{-1}$] exhibited reduced *gh* mRNA levels and fluctuations in *igf1* expression, correlating with observed delays in somatic growth and morphological abnormalities, including scoliosis. Conversely, offspring from females receiving a diet with lower T4 supplementation [$12.5 \mu\text{g T4 (g body mass)}^{-1} \text{d}^{-1}$] showed increased *gh* expression, suggesting a dose-dependent antagonist effect of maternal thyroid hormones (THs). These findings highlight the critical role of the growth hormone/insulin-like growth factor-1 (Gh/Igf-1) axis in mediating the detrimental effects of excessive maternal THs on offspring growth and development.

Thyroid hormones play a pivotal role in metabolic regulation, particularly in skeletal muscle. Our study examined the expression of genes involved in protein synthesis (*mtor*) and degradation (*foxo3a*). Embryos originated from females fed with $25 \mu\text{g T4 (g body mass)}^{-1} \text{d}^{-1}$ showed decreased *mtor* expression at 48 -hpf, followed by an increase at 72 -hpf, while *foxo3a* levels remained unchanged. These alterations in protein synthesis pathways suggest disrupted muscle metabolism and impaired myogenesis. Supporting this, unpublished data from our laboratory showed that adult zebrafish males treated with methimazole exhibited reduced *mtor* levels in skeletal muscle, which did not normalize after T4 rescue.

Myogenesis factors (*myoG* and *myf5*) were up-regulated immediately post-fertilization, with *myf5* levels remaining elevated at 72 -hpf. This sustained increase likely reflects the onset

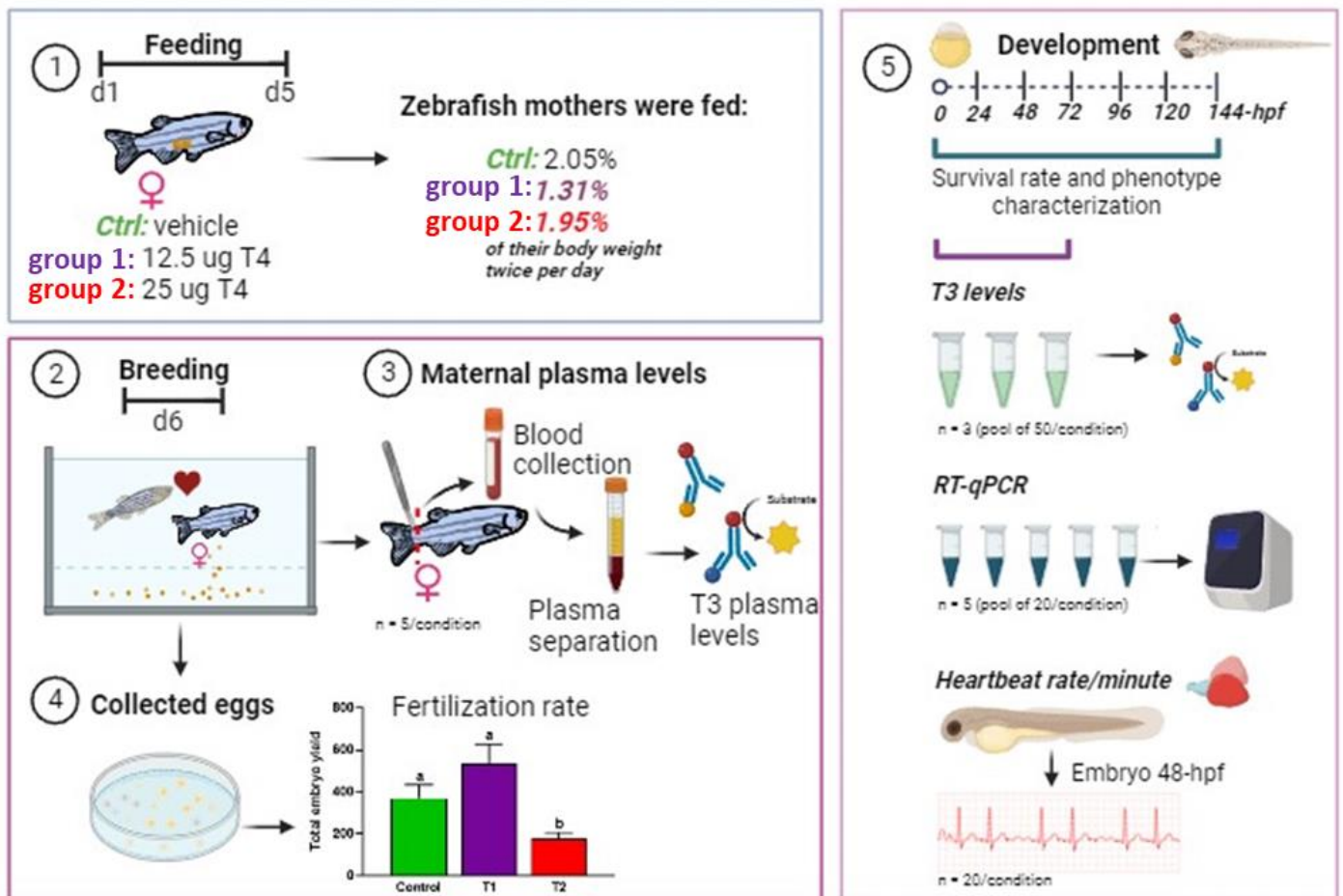


of endogenous TH production, as T3 signaling is known to promote satellite cell differentiation and enhance myogenesis (Dentice et al., 2014; Salvatore et al., 2014; Dumont et al., 2015). Persistent imbalances in myogenic factor expression may result from maternal TH manipulation, leading to long-term developmental consequences.

Elevated maternal THs also affected cardiac function in zebrafish embryos. Increased heart rates (tachycardia) and pericardial edema were observed at 48 -hpf, followed by a decline in heart rate and subsequent mortality by 144 -hpf. These findings suggest that fluctuating TH levels disrupt normal cardiac development and function. Consistent with previous studies, hyperthyroidism increases heart rate and myocardial contractility, whereas hypothyroidism weakens cardiac function (Vargas-Uricoechea et al., 2014).

In conclusion, this study highlights the critical role of maternal thyroid hormones in zebrafish development, demonstrating their profound influence on growth, metabolism, muscle development, and cardiac function. Disruptions in TH homeostasis, whether due to endogenous factors or environmental pollutants, can have far-reaching consequences for offspring survival and aquatic ecosystem health. A comprehensive understanding of these processes will inform strategies to address environmental challenges and protect aquatic biodiversity.

Figures



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Figure 1. Experimental design. **1)** Three groups of female zebrafish ($n = 15$ per group; 5 per replicate) were assigned into three replicates per experimental group. One control group was fed commercial food, while the other groups were fed commercial food containing $12.5 \text{ ug T4 (g body mass)}^{-1} \text{ d}^{-1}$ (group 1) or $25 \text{ ug T4 (g body mass)}^{-1} \text{ d}^{-1}$ (group 2) for 5 days. **2-4)** On day 6, females ($n = 5$) from each group were placed for reproduction with unexposed males **(2)**. The blood was collected and processed ($n = 5$ /per group), and the maternal plasma was sampled for thyroid hormone (THs, T3) extraction and measurement levels **(3)**. **(4)** Eggs were collected for fertilization rate. **(5)** Eggs, embryos, and larvae were collected at 0 to 144 -hpf for survival rate, and phenotype characterization such as length, pericardial and yolk sac edema, uninflated swim bladder, and scoliosis. The eggs, embryo, and early larvae from 0 to 72 -hpf were counted and pools were collected for T3 levels ($n = 3$; pool of 50/condition) or pools ($n = 5$; pool of 20/condition) were snap frozen on liquid nitrogen and stored at $-80 \text{ }^{\circ}\text{C}$ for gene expression analysis. Embryos at 48 -hpf from control and treatments ($n = 20$ /condition) were taken to calculate the heart rate per minute.

Maternal THs supply

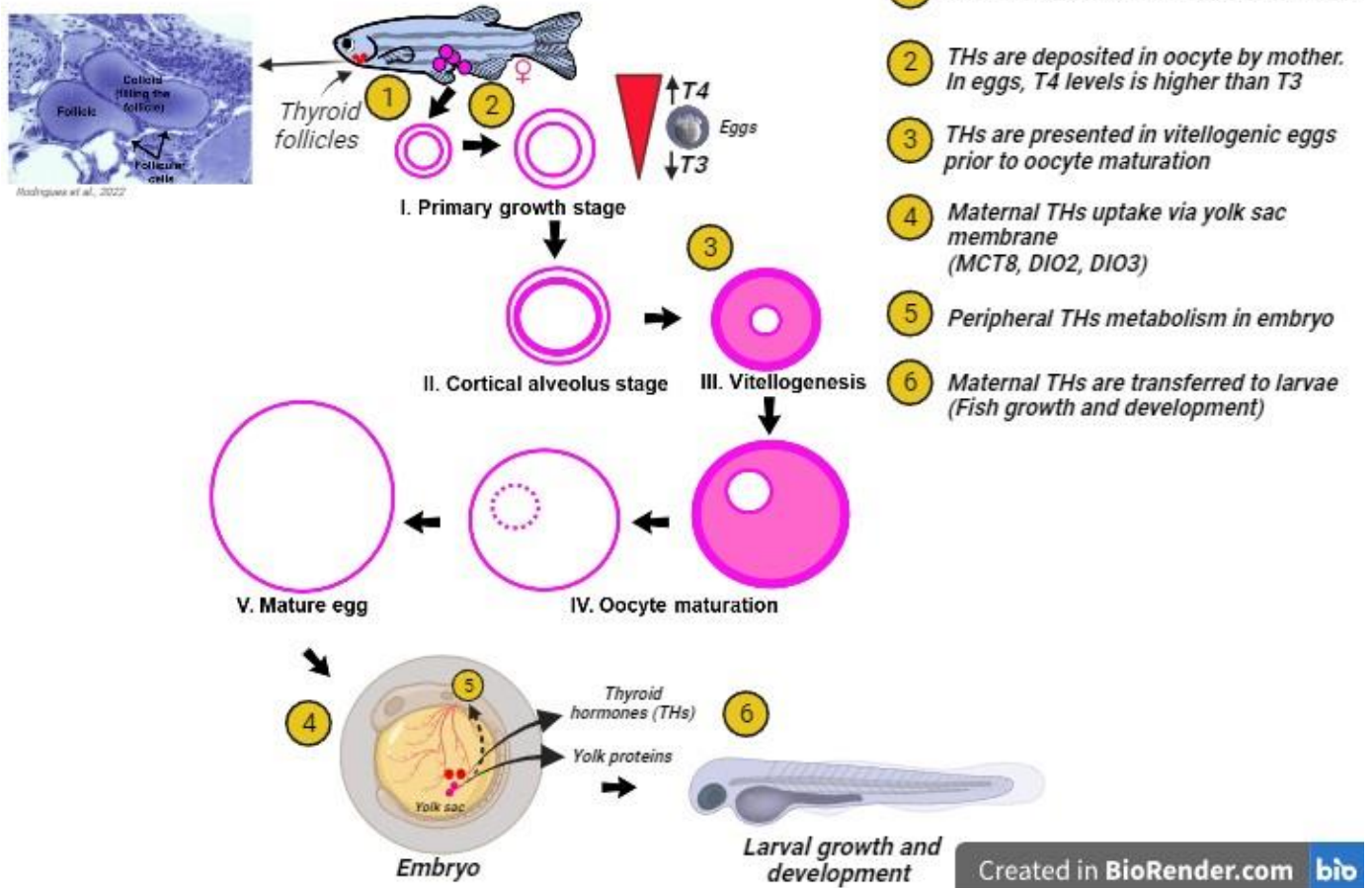


Figure 2. A schematic diagram illustrates the five stages of follicle development in zebrafish during the oogenesis process (adapted from Clelland & Peng, 2009). The process begins with Stage I, known as the primary growth phase when follicles start to form and enlarge. Stage II referred to as the cortical alveolus stage, is characterized by the accumulation of cortical alveoli within the oocyte. Moving on to Stage III, follicles enter vitellogenesis, where the oocytes accumulate vital nutrients. Stage IV corresponds to oocyte maturation, involving germinal vesicle migration from the center of the oocyte to the periphery, followed by the breakdown of the nuclear membrane. Finally, Stage V represents mature eggs ready for ovulation and spawning. THs maternal circulation provided by thyroid follicles (1) (for review see Tovo-Neto et al., 2018) are deposited into the oocyte during follicle development (2). The hormones are present in both fertilized and unfertilized mature eggs, as well as in vitellogenic eggs before oocyte maturation (3). The stock of maternal THs in fish egg yolk is substantial and is used by the embryo until hatching even if the embryonic thyroid follicles become active. The factors controlling THs transporter and metabolism (MCT8 and deiodinases) are shown to be present (4 and 5). The initial growth and larval development are dependent on the maternal supply of THs (6).

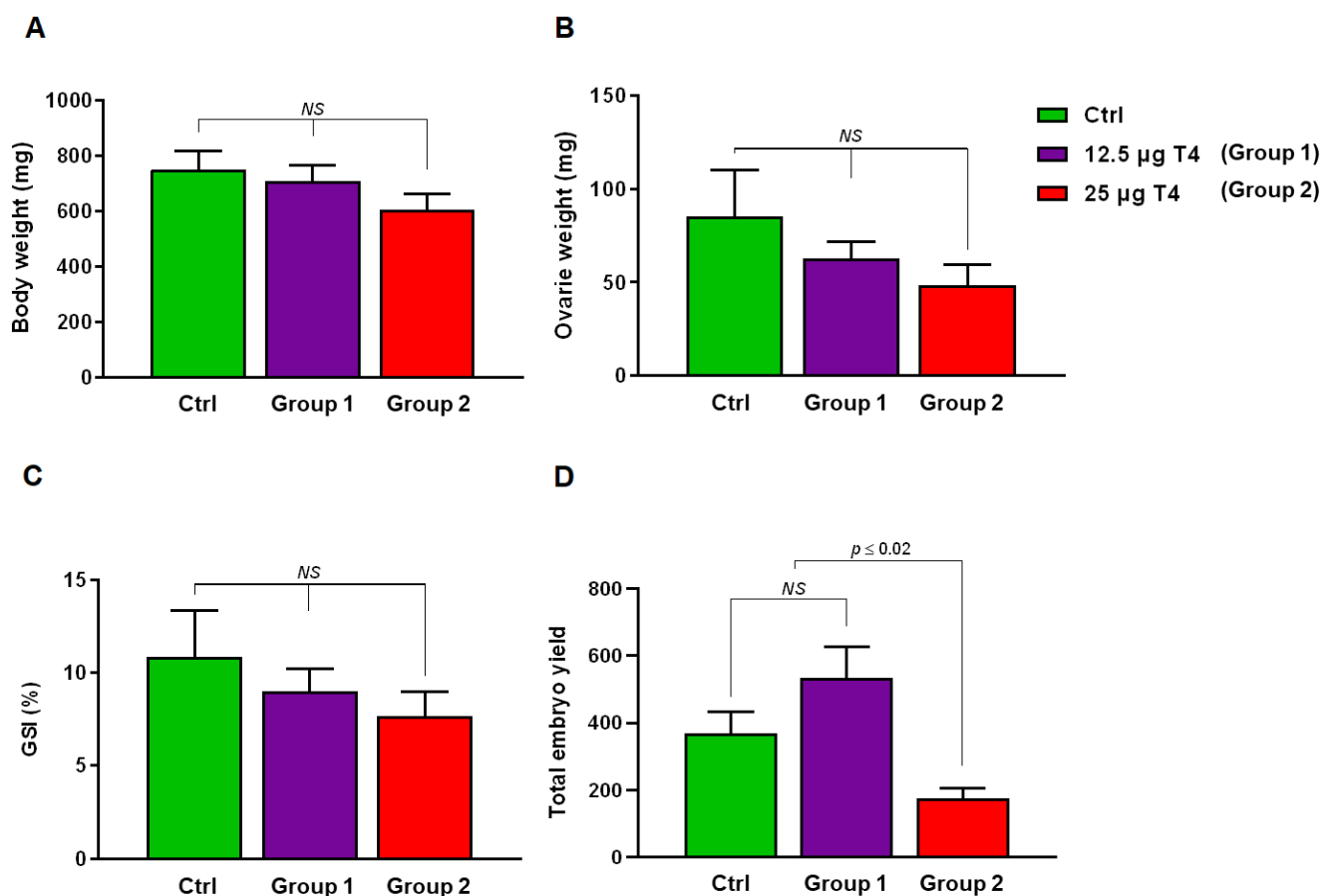


Figure 3. The body weight (**A**), ovary weight (**B**), GSI (**C**), and total embryo yield (**D**) from zebrafish females following 5 days of feeding with commercial food containing vehicle (control group), low or high dose of T4: 12.5 µg T4 (g body mass)⁻¹d⁻¹ (group 1) or 25 µg T4 (g body mass)⁻¹d⁻¹ (group 2), respectively. Values represented the mean ± SEM. One-way ANOVA followed by Tukey's multiple comparison tests; $p \leq 0.05$; n = 5/group). NS not significant.

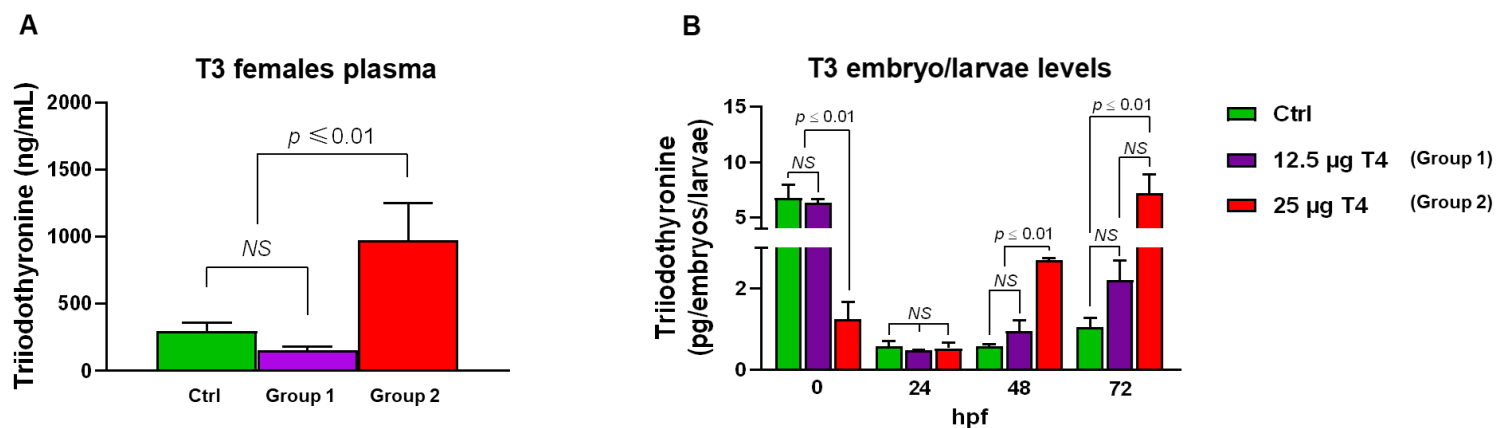


Figure 4. Quantification of T3 levels in females' plasma **(A)** under different conditions including control and T4 treatment at dosages of 12.5 (g body mass)⁻¹ d⁻¹ (group 1) and 25 μg T4 (g body mass)⁻¹ d⁻¹ (group 2), as well as in embryos at 0 to 72 -hpf **(B)** from each females group. ANOVA followed by Tukey's multiple comparison tests; $p \leq 0.05$. NS not significant.

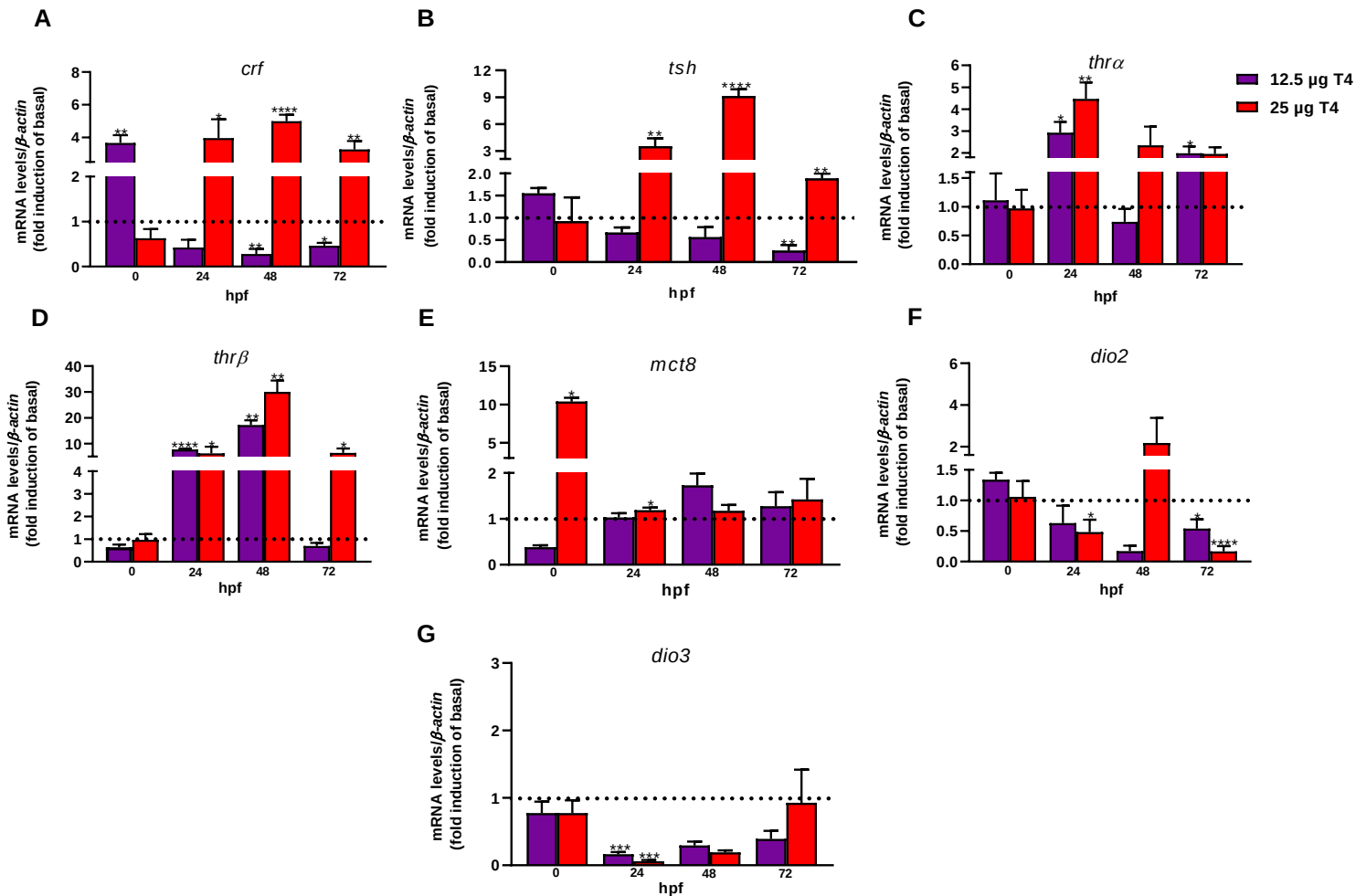


Figure 5. Thyroid-related genes in embryos and early larvae at 0 to 72-hpf from mothers fed with T4 enriched in the diet (0.25 mg in purple and 0.5 mg T4 in red) compared to the untreated group (control) (dotted black line set at 1). The selected genes *crf* (corticotropin-releasing factor) (A); *tsh* (thyroid-stimulating hormone) (B); *thra* and *thrβ* (thyroid hormones receptor) (C, D); *mct8* (monocarboxylate transporter 8) (E); and *dio2* and *dio3* (deiodinases type 2 and 3) (F, G) were evaluated. Ct values were normalized with β -actin and expressed as relative values of control (no treated fish) expression levels. Bars represent the mean $\pm$ SEM fold change (n = 5 pools of 20 individuals for each pool) relative to the control. Student unpaired *t*-test, **p* < 0.05, ***p* < 0.01, ****p* < 0.001, and *****p* < 0.0001.

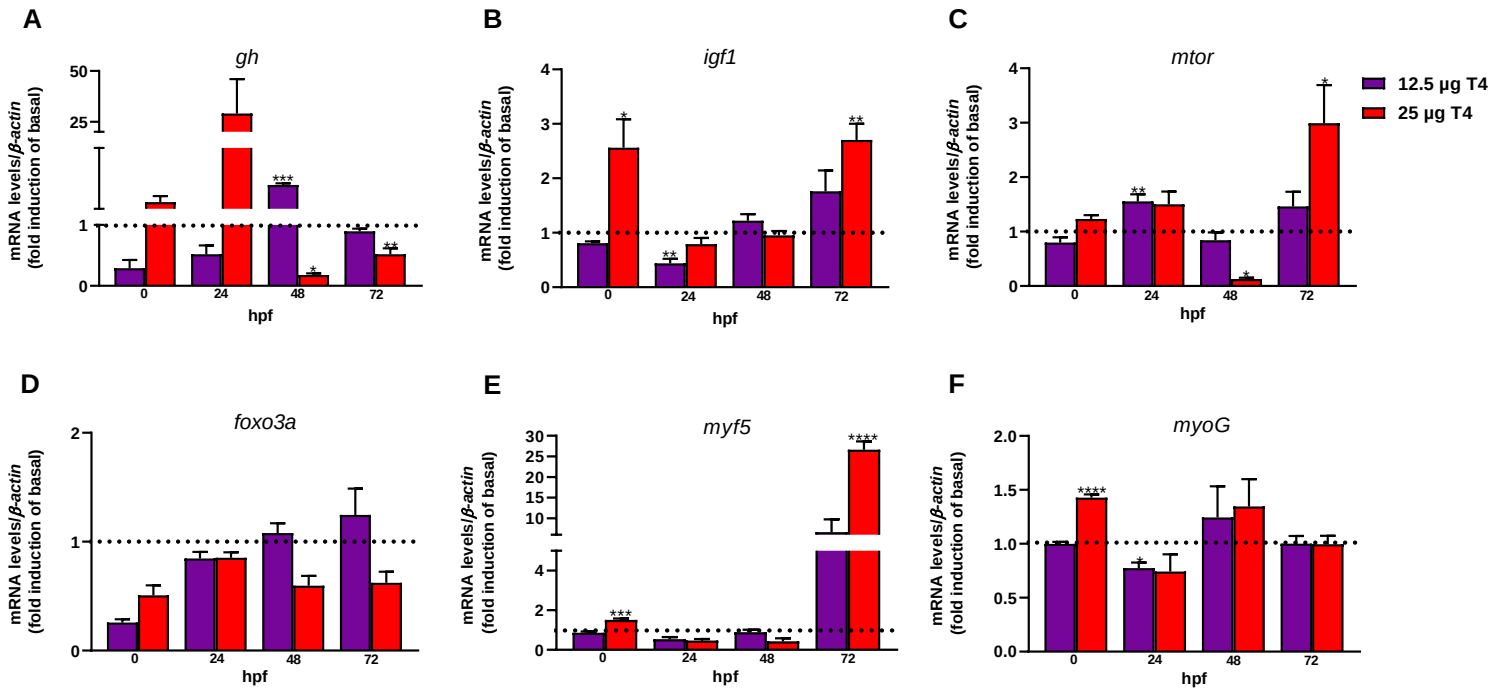


Figure 6. Comparison of somatotropic-related gene expression in embryos and early larvae at 0 to 72-hpf between mothers that were fed with diets enriched with T4 (0.25 mg in purple and 0.5 mg T4 in red) and the untreated group (control) (dotted black line set at 1). The selected genes *gh* (growth hormone) (A); *igf1* (insulin-like growth factor 1) (B); *mtor* (mammalian target of rapamycin) (C); *foxo3a* (forkhead box O3) (D); *myf5* (myogenic factor 5) (E); and *myoG* (myogenin) (F) were evaluated. Ct values were normalized with β -actin and expressed as relative values of control (no treated fish) levels of expression. Bars represent the mean $\pm$ SEM fold change (n = 5 pools of 20 individuals for each pool) relative to the control. Student unpaired *t*-test, **p* < 0.05, ** *p* < 0.01, *** *p* < 0.001, and **** *p* < 0.0001.

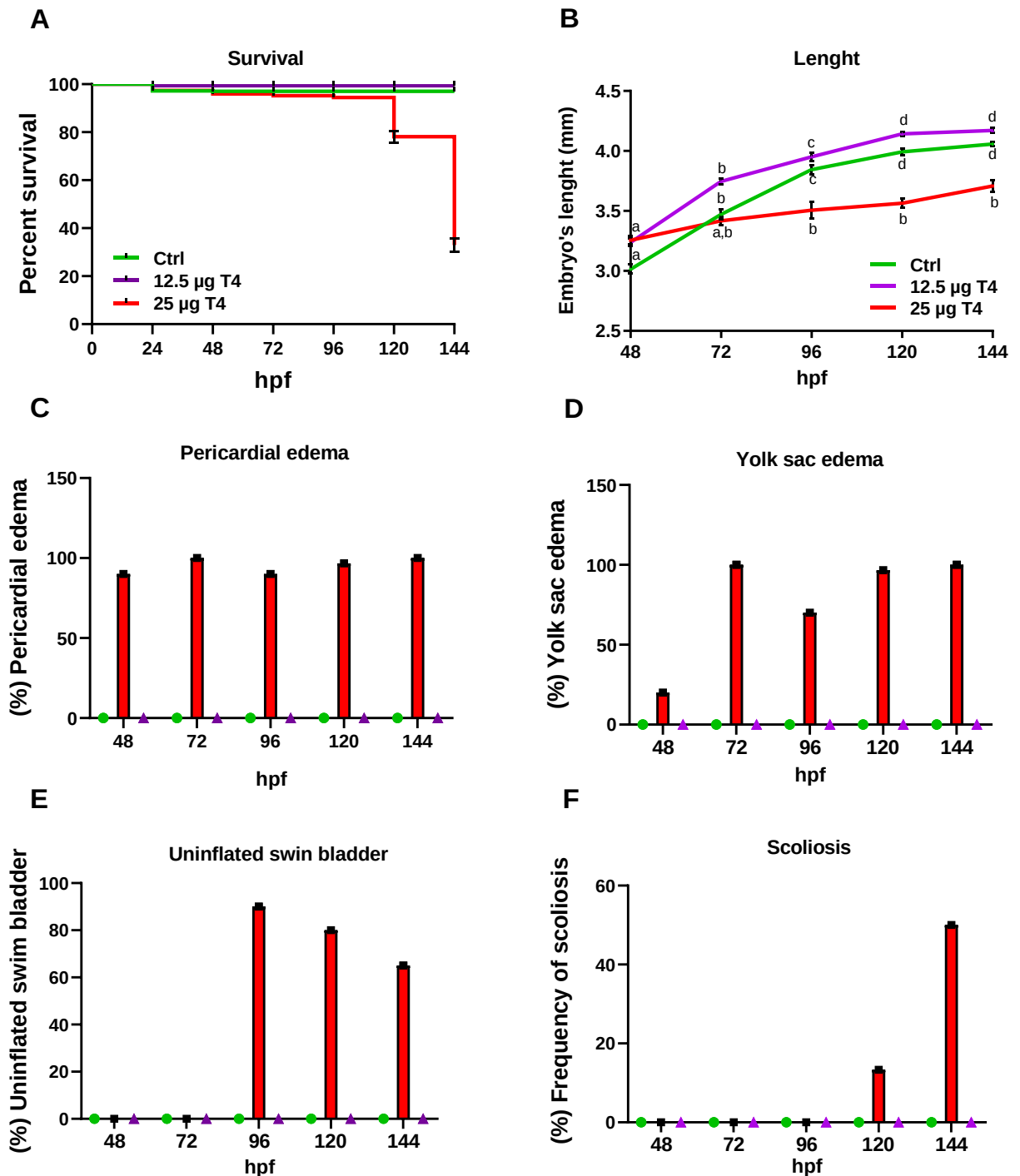


Figure 7. The survival rate of embryos and larvae at 0 to 144 -hpf (**A**) was demonstrated (Log-rank (Mantel-Cox) test; chi-square = 68.71; $p < 0.0001$). Embryos and larvae length (**B**) (Bars represent the mean $\pm$ SEM; ANOVA followed by Tukey's multiple comparison tests. Distinct letters denote significant differences; $p \leq 0.05$), percentage of pericardial (**C**), and yolk sac (**D**) edema, percentage of uninflated swim bladder (**E**), and frequency of scoliosis (**F**) in embryos and larvae at 48 to 144 -hpf were showed ($n = 30$, vehicle or treatments).

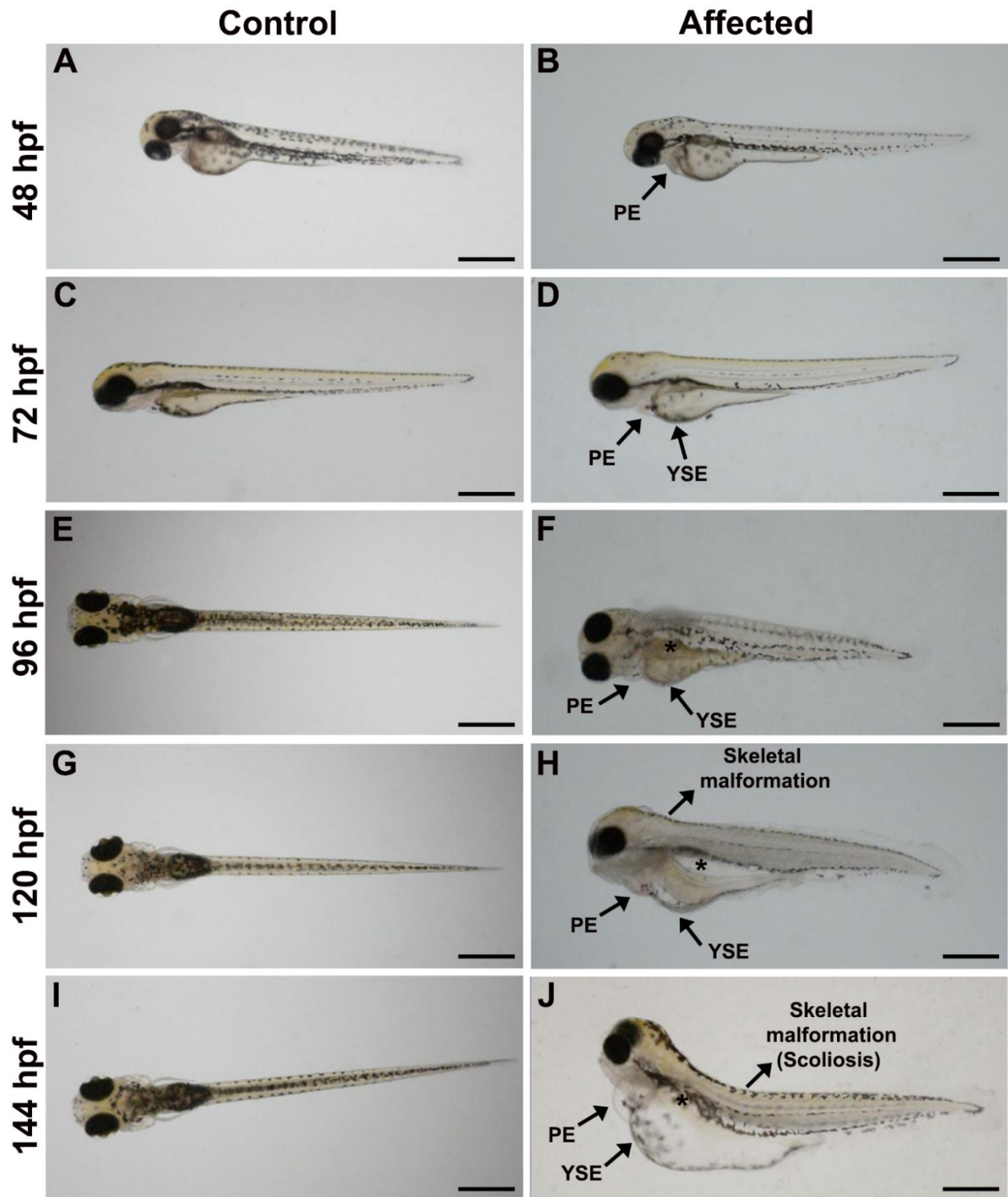


Figure 8. The phenotype of zebrafish embryos and larvae from control females (fed a commercial diet) (A, C, E, G, and I) was compared with those of progeny impacted by maternal transfer of THs (offspring from females fed a diet enriched with $25 \mu\text{g T4 (g body mass)}^{-1} \text{d}^{-1}$ (group 2) (B, D, F, H, and J) at 48 to 144 -hpf. Arrows highlight the presence of pericardial edema (PE), yolk sac edema (YSE), and skeletal malformations, following scoliosis. Asterisk (*) denotes the absence of swim bladder inflation. Scale bar = 500 μm .

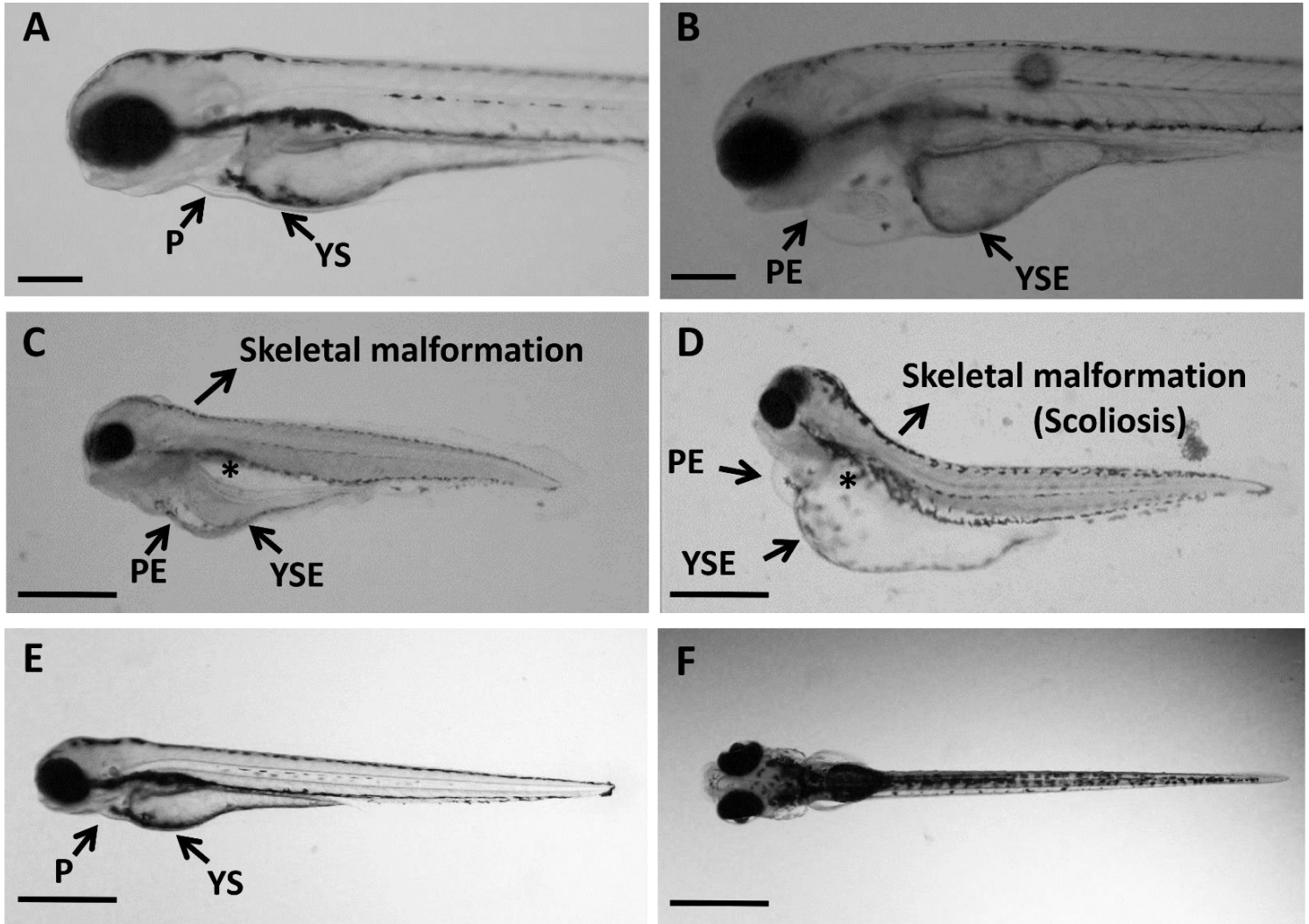


Figure 9. Detailed photomicrographs representing the morphological alterations of *D. rerio* embryos and larvae after mothers' exposure to higher concentrations of T4 for 5 days. Control (no treated females) (A) and affected progeny after mothers were fed a diet laced with 25 µg T4 (g body mass)⁻¹ d⁻¹ (group 2) (B-D). Arrow indicates pericardial (PE) and yolk sac (YSE) edema, followed by skeletal malformation (scoliosis). Asterisk (*) indicates that the swim bladder has not been inflated (C-D). The progeny of mothers from group 1 (females fed with 12.5 µg T4 (g body mass)⁻¹ d⁻¹) did not show morphological change or malformation during embryogenesis (E-F). P = pericardium; YS = Yolk sac. Scale bar = 500 µm (A-B) and 200 µm (C-F).

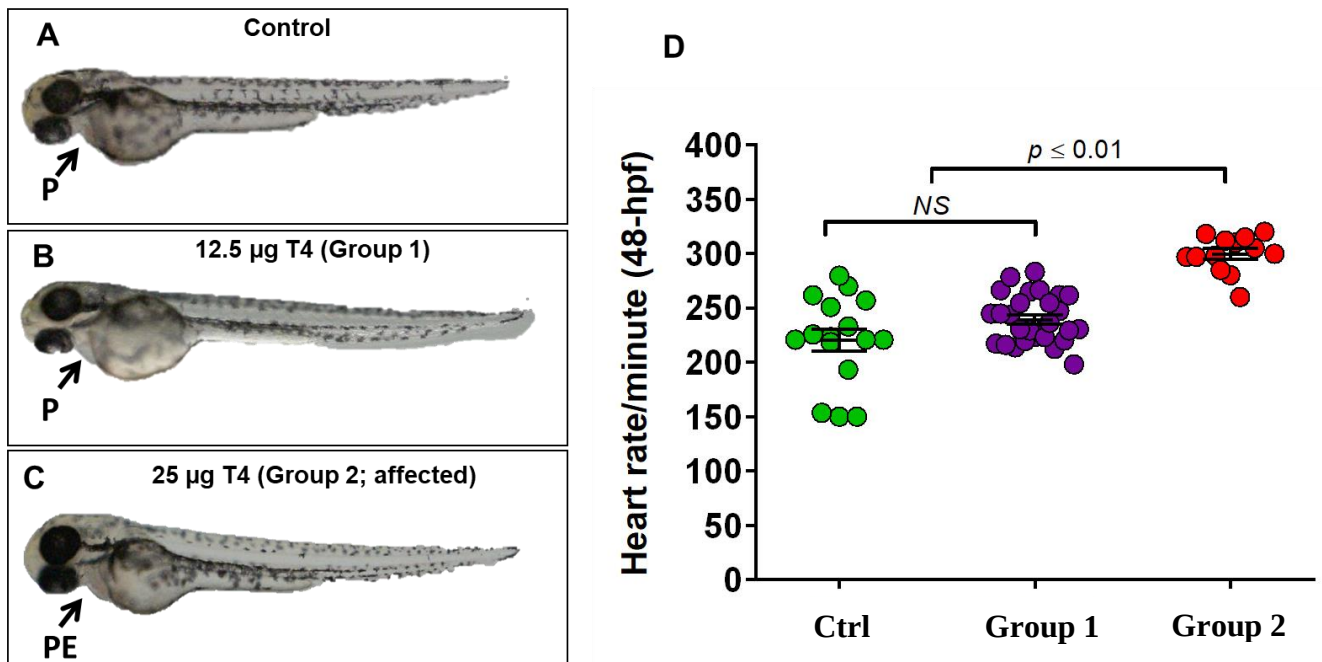
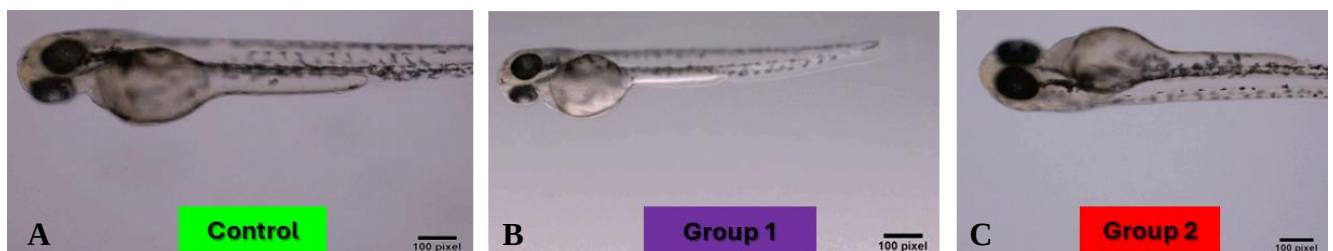


Figure 10. The phenotype of zebrafish embryos with 48 -hpf from control (**A**) or maternal treatment with low (**B**) or higher concentration of T4 (12.5 or 25 µg T4, respectively) (**C**). Control and group 1 (12.5 µg T4 (g body mass)⁻¹ d⁻¹) showed normal morphology of pericardium (P) (**A-B**). However, embryos from group 2 (25 µg T4 (g body mass)⁻¹ d⁻¹) were affected with a higher concentration of maternal T4 (**C**). Pericardial edema (PE), and tachycardia were observed (**C-D**). Scale bar = 500 µm. ANOVA followed by Tukey's multiple comparison tests; $p \leq 0.05$. NS not significant (n = 20 per group).

Supplementary Material

Supplementary Video V1: Heartbeat of embryos with 48 -hpf from the control group (**A**) and maternal treatment with 12.5 µg T4 (g body mass)⁻¹ d⁻¹ (group 1) (**B**), and 25 µg T4 (g body mass)⁻¹ d⁻¹ (group 2) (**C**).



Author Contributions

Conception and design of experiments, R.H.N., M.S.R.; performance of experiments, M.S.R., B.M.S., I.F.R., L.B.D; data analysis, M.S.R., B.M.S., I.F.R., L.B.D and R.H.N.; contribution of



reagents/materials/analysis tools, R.H.N.; writing of the manuscript, M.S.R. and R.H.N. All authors have read and agreed to the published version of the manuscript.

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The National Council approved the Animal Study Protocol for the Control of Animal Experimental (CONCEA) and Ethical Principles in Animal Research (Protocol n. 8520250320-CEUA).

Informed Consent Statement

Not applicable.

Data Availability Statement

Data is contained within the article or supplementary material. The data presented in this study are available in this manuscript and supplemental material.

Conflicts of Interest

The authors declare no conflict of interest.

5. References

- ALINEZHAD, S., ABDOLLAHPOUR, H., JAFARI, N., FALAHATKAR, B. Effects of thyroxine immersion on Sterlet sturgeon (*Acipenser ruthenus*) embryos and larvae: variations in thyroid hormone levels during development. **Aquaculture**. 2019.
- AYSON, F. D., AND T. J. LAM. Thyroxine injection of female rabbitfish *Siganus guttatus* broodstock: Changes in thyroid hormone levels in plasma, eggs, and yolk-sac larvae, and its effect on larval growth and survival. **Aquaculture**, 109: 83–93. 1993.
- BALASCH, J.C.; TORT, L. Netting the Stress Responses in Fish. **Front Endocrinol (Lausanne)**.10: 62. 2019.
- BASSET, J.H.D, HARVEY, C.B., WILLIAMS, G.R. Mechanisms of thyroid hormone receptor-specific nuclear and extranuclear actions. **Mol. Cell. Endocrinol.** 213, 1-11. 2003.



BAKER, K.; WARREN, K.S.; YELLEN, G.; FISHMAN, M.C. Defective “pacemaker” current (I_h) in a zebrafish mutant with a slow heart rate. **Proc Natl Acad Sci USA**. 94:4554–4559. 1997.

BLOISE F. F., CORDEIRO A., ORTIGA-CARVALHO T. M. Role of thyroid hormone in skeletal muscle physiology. **J. Endocrinol**. 236 R57–R68. 2018.

BRENT, G. A. Mechanisms of thyroid hormone action. **J. Clin. Invest**. 122, 3035– 3043. 2012.

BROWN, D. D. The role of thyroid hormone in zebrafish and axolotl development. **Proc. Natl. Acad. Sci.**, 94: 13011–13016. 1997.

BROWN, C.L.; URBINATI, E.C.; ZHANG, W.; BROWN, S.B.; McCOMB-KOBZA, M. Maternal thyroid and glucocorticoid hormone interactions in larval fish development, and their applications in aquaculture. **Reviews in Fisheries Science & Aquaculture**, 22:207:220. 2014.

BURROW, G.N.; FISHER, D.A.; LARSEN, P.R. Maternal and fetal thyroid function. **The New England journal of medicine**. 331:1072–1078. 1994.

CAMPINHO, M.A.; SARAIVA, J.; FLORINDO, C.; POWER, D. Maternal thyroid hormones are essential for neural development in zebrafish. **Mol. Endocrinol**. 28, 1136-1149. 2014.

CHAN, S.Y.; VASILOPOULOU, E. & KILBY, M.D. The role of the placenta in thyroid hormone delivery to the fetus. **Nature Clinical Practice. Endocrinology & Metabolism** 5 45–54. 2009.

CHEN, Y.; SIBLE, J.C.; MCNABB, F.M. Effects of maternal exposure to ammonium perchlorate on thyroid function and the expression of thyroid-responsive genes in Japanese quail embryos. **Gen Comp Endocrinol**. 159:196–207. 2008.

CLELLAND, E.; PENG, C. Endocrine/paracrine control of zebrafish ovarian development. **Molecular and Cellular Endocrinology**. 312:42-52. 2009.

DARRAS, V.M.; VERHOELST, C.H.; REYNS, G.E.; KUHN, E.R.; VAN DER GEYTEN, S. Thyroid hormone deiodination in birds. **Thyroid**. 16:25–35. 2006.

DARRAS, V. M. The Role of Maternal Thyroid Hormones in Avian Embryonic Development. **Frontiers in Endocrinology**. 2019.

DEAL CK, VOLKOFF H. The Role of the Thyroid Axis in Fish. **Front Endocrinol (Lausanne)**. 6;11:596585. 2020.

DE LEO, S.; LEE, S.Y.; BRAVERMAN, L.E. Hyperthyroidism. **Lancet**. 388:906–918. 2016.

DENTICE M., AMBROSIO R., DAMIANO, V., SIBILIO, A., LUONGO, C., GUARDIOLA O, YENNEK S, ZORDAN P, MINCHIOTTI G & COLAO A. Intracellular inactivation of thyroid hormone is a survival mechanism for muscle stem cell proliferation and lineage progression. **Cell Metabolism**. 20 1038–1048. 2014.



DEVDHAR, M.; OUSMAN, Y.H.; BURMAN, K.D. Hypothyroidism. **Endocrinol Metab Clin North Am.** 36:595–615. 2007.

DONG, W.; MACAULAY, L.; KWOK, K.W.H.; HINTON, D.E.; STAPLETON, H.M. Using whole-mount in situ hybridization to examine thyroid hormone deiodinase expression in embryonic and larval zebrafish: A tool for examining OH-BDE toxicity to early life stages. **Aquat. Toxicol.** 132–133, 190–199. 2013.

DUMONT NA, BENTZINGER CF, SINCENNES MC & RUDNICKI MA. Satellite cells and skeletal muscle regeneration. **Comprehensive Physiology** .5 1027–1059. 2015.

FARCHI-PISANTY, O.; HACKETT, P.B.; JR MOAV, B. Regulation of fish growth hormone transcription. **Mol Marine Biol Biotechnol.** 1995.

FARÍAS-SERRATOS, B.M.; LAZCANO, I.; VILLALOBOS, P.; DARRAS, V.M.; OROZCO, A. Thyroid hormone deficiency during zebrafish development impairs central nervous system myelination. **PLoS One.** 2021.

FAUGHT, E., BEST, C.; VIJAYAN, M. M. Maternal stress-associated cortisol stimulation may protect embryos from cortisol excess in zebrafish. **R. Soc. Open Sci.** 3, 160032. 2016.

FORHEAD, A.; FOWDEN, L. Role of thyroid hormones in the control of fetal growth and maturation. 2006.

FOK, P.; EALES, J.G. Regulation of plasma T3 levels in T4-challenged rainbow trout, *Salmo gairdneri*. *Gen Comp Endocrinol.* 53:197-202. 1984.

GEREBEN, B.; ZEÖLD, A.; DENTICE, M.; SALVATORE, D.; BIANCO, A.C. Activation and inactivation of thyroid hormone by deiodinases: Local action with general consequences. **Cell. Mol. Life Sci.** 65, 570–590. 2008.

GREENBLATT, M.; BROWN, C. L.; LEE, M.; DAUDER, S.; BERN, H. A. Changes in thyroid hormone levels in eggs and larvae and iodide uptake by eggs of coho and chinook salmon, *Oncorhynchus kisutch* and *O. tshawytscha*. **Fish Physiol. Biochem.** 6(5): 261–278. 1989.

HOUBRECHTS, A.M.; VERGAUWEN, L.; BAGCI, E., VAN HOUCKE, J.; HEIJLEN, M.; KULEMAKA, B.; HYDE, R.D.; KNAPEN, D.; DARRAS, V.M. Deiodinase knockdown affects zebrafish eye development at the level of gene expression, morphology, and function. **Molecular and Cellular Endocrinology**, vol. 424, 81-93. 2016.

JANSEN, J.; FRIESEMA, E.C.H.; MILICI, C.; VISSER, T.J. Thyroid hormone transporters in health and disease. **Thyroid.** 15 757–768. 2005.

KEER, S.; COHEN, K.; MAY, C.; HU, Y.; MCMENAMIN, S.; HERNANDEZ, L.P. Anatomical assessment of the adult skeleton of zebrafish reared under different thyroid hormone profiles. **Anatomical Record.** 302(10):1754–69. 2019.



KIMMEL, C.B.; BALLARD, W.W.; KIMMEL, S.R.; ULLMANN, B.; SCHILLING, T.F. Stages of embryonic development of the zebrafish. **Dev Dyn.** 203(3):253-310. 1995.

KOBUKE, L.; SPECKER, J.L.; BERN, H. A. Thyroxine content of eggs and larvae of coho salmon, *Oncorhynchus kisutch*. **J. Exp. Zool.**, 242, 89-94. 1987.

KOGANTI, P.; YAO, J.; CLEVELAND, B.M. Molecular Mechanisms Regulating Muscle Plasticity in Fish. **Animals**.11, 61. 2021.

LAM, T.J. Hormones and egg/larval quality in fish. **J. World Aquac. Soc.** 25: 2-12. 1994.

LAZCANO, I., PECH-POOL, S.M., OLVERA, A., GARCÍA-MARTÍNEZ, I., PALACIOS-PÉREZ, S., OROZCO, A. The importance of thyroid hormone signaling during early development: lessons from the zebrafish model. **Gen. Comp. Endocrinol.** 334, 114225. 2023.

LEONARDO, A. F. G., M. A. MARCIO AQUINO HOSHIBA, E. C. URBINATI, AND J. A. Jose Augusto Senhorini. Improvement of matrinxã, *Brycon amazonicus*, and larviculture by exposing eggs to triiodothyronine. **J. World Aquacult. Soc.** 44(1): 141–147. 2013.

MARELLI F, PERSANI, L. How zebrafish research has helped in understanding thyroid diseases. **Research**. 2017.

MCCOMB, D. M., J. GELSLEICHTER, C. A. MANIRE, R. BRINN, C. L. BROWN. Comparative thyroid hormone concentration in maternal serum and yolk of the bonnethead shark (*Sphyrna tiburo*) from two sites along the coast of Florida. **Gen. Comp. Endocrinol.** 144: 167–173. 2005.

MIELL, J.P.; TAYLOR, A.M.; ZINI, M.; MAHESHWARI, H.G.; ROSS, R.J.M.; VALCAVI, R. Effects of hypothyroidism and hyperthyroidism on insulin-like growth factors (IGFs) and growth hormone and IGF binding proteins. **Journal of Clinical Endocrinology and Metabolism**. 76 950–953. 1993.

MOAV, B.; MCKEOWN, B.A. Thyroid hormone increases transcription of growth hormone mRNA in rainbow trout pituitary. **Hormone Metab Res.** 1992.

MOLLA, M.H.R.; HASAN, M.D.T.; JANG, W.J.; SORIA, D.C.D.; APPENTENG, P.; MARUFCHONI, H.; JAHAN, B.; BROWN, C.L. Thyroid hormone-induced swim bladder and eye maturation are transduced by IGF-1 in zebrafish embryos. **Aquaculture Research**, 50(11), 3462–3470. 2019.

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

OROZCO A., VALVERDE-R C., OLVERA A., GARCÍA-G C. Iodothyronine Deiodinases: a Functional and Evolutionary Perspective. **J. Endocrinol.** 215, 207–219. 10.1530/JOE-12-0258. 2012.



ORTIGA-CARVALHO, T.M, CHIAMOLERA, M.I, PAZOS-MOURA, C.C & WONDISFORD, F.E Hypothalamus-pituitary-thyroid axis. **Comprehensive Physiology**. 6 1387–1428. 2016.

PETER, M.C.S. The role of thyroid hormones in stress response of fish. **Gen Comp Endocrinol**. 172:198–210. 10.1016/j.ygcen.2011.02.023. 2011.

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

RUUSKANEN, S.; HSU, B.-Y. Maternal thyroid hormones: an unexplored mechanism underlying maternal effects in an ecological framework. **Physiological and biochemical zoology**, 91, 904–916. 10.1086/697380. 2018.

SALVATORE, D, SIMONIDES, WS, DENTICE, M, ZAVACKI, A.M, LARSEN PR. Thyroid hormones and skeletal muscle--new insights and potential implications. **Nat Rev Endocrinol**. Apr;10(4):206-14. 2014.

SCHMID, A.C., REINECKE, M., LUTZ, I., KLOAS, W. Thyroid hormone stimulates hepatic IGF-I mRNA expression in a bony fish, the tilapia *Oreochromis mossambicus*, *in vitro* and *in vivo*. **Gen. Com. Endocrinol**. 130, 129–134. 2003.

SEGRS, F.H.; BERISHVILI, G.; TABORSKY, B. Egg size-dependent expression of growth hormone receptor accompanies compensatory growth in fish. **Proc Biol Sci**.279 (1728):592-600. 2012.

SENHORINI, J. A., F. L. M. MANTELATTO, AND S. M. C. CASANOVA. Growth and survival of larvae of Amazon species “matrinxã,” *Brycon cephalus* (Pisces, Characidae), in larviculture ponds. **Boletim Técnico CEPTA** , 11: 13–28. 1998.

SIMONIDES, W. S. & VAN HARDEVELD, C. Thyroid hormone as a determinant of metabolic and contractile phenotype of skeletal muscle. **Thyroid** 18, 205–216. 2008.

STAPIEN, B. K., HUTTNER, W. B. Transport, Metabolism, and Function of Thyroid Hormones in the Developing Mammalian Brain. **Frontiers in Endocrinology**. 2019.

SUN, Y.; WNAG, X.; GUO, W.; LI, F.; HUA, J.; ZHU, B.; GUO. Y.; YANG, L.; ZHOU, B. Life-time exposure to decabromodiphenyl ethane (DBDPE) caused transgenerational epigenetic alterations of thyroid endocrine system in zebrafish. **Science of the Total Environment**. 2024.

SWAIN, S. K., AND P. K. SAHOO. Effects of feeding triiodothyronine on growth, food conversion and disease resistance of goldfish, *Carassius auratus* (Linn.). **Asian Fisheries Science**, 16: 291–298 2003.



SZISCH, V., N. PAPANDROULAKIS, E. FANOURLAKI, AND M. PAVLIDIS. Ontogeny of the thyroid hormones and cortisol in the gilthead sea bream, *Sparus aurata*. **Gen. Comp. Endocrinol.**, 142: 186–192. 2005.

TAGAWA, M., HIRANO, T. Changes in tissue and blood concentrations of thyroid hormones in developing *chum salmon*. **Gen. Comp. Endocrinol.** 76, 437–443. 1989.

TAGAWA, M., AND T. HIRANO. Effects of thyroid-hormone deficiency in eggs on early development of the medaka, *Oryzias latipes*. **J. Exp. Zool.**, 257: 360–366. 1990.

TAKAYAMA, S., HOSTICK, U.; HAENDEL, M.; EISEN, J.; DARIMONT, B. An F-domain introduced by alternative splicing regulates activity of the zebrafish thyroid hormone receptor α : role of zebrafish TR α F-domain. **Gen. Comp. Endocrinol.** 155, 176–189. 2008.

TSANG, B.; ZAHID, H.; ANSARI, R.; LEE, R.C. PARTAP, A.; GERLAI, R. Breeding Zebrafish: A Review of Different Methods and a Discussion on Standardization. **Zebrafish**. 2017.

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

URBINATI, E. C., L. H. VASQUES, J. A. SENHORINI, V. L. SOUZA, AND F. D. GONCALVES. Larval performance of matrinxã, *Brycon amazonicus* (Spix & Agawssiz 1829), after maternal triiodothyronine injection or egg immersion. **Aquacult. Res.** 39: 1355–1359. 2008.

VAN DINGENEN, I.; VERGAUWEN, L.; HAIGIS, A-C.; BLACKWELL, B.R.; STACY, E.; VILLENEUVE, D.L.; KNAPEN, D. Deiodinase inhibition impairs the formation of the three posterior swim bladder tissue layers during early embryonic development in zebrafish. **Aquatic Toxicology**. 106632. 2023.

VAN HERCK, S.L.; GEYSSENS, S.; BALD, E.; CHWATKO, G.; DELEZIE, E.; DIANATI, E.; AHMED, R.G.; DARRAS, V.M. Maternal transfer of methimazole and effects on thyroid hormone availability in embryonic tissues. **J Endocrinol.** 218:105–15. 2013.

VANCAMP, P.; BOURGEOIS, N.M.A.; HOUBRECHTS, A.M.; DARRAS, V.M. Knockdown of the thyroid hormone transporter MCT8 in chicken retinal precursor cells hampers early retinal development and results in a shift towards more UV/blue cones at the expense of green/red cones. **Exp Eye Res.** 178:135–47. 2018.

VARGAS-URICOECHEA, H.; BONELO-PERDOMO, A.; SIERRA-TORRES, C.H. Effects of thyroid hormones on the heart. 26(6):296–309. **Arteriosclerosis**. 2014.

VERGAUWEN L, CAVALLIN JE, ANKLEY GT, BARS C, GABRIËLS IJ, MICHIELS EDG, FITZPATRICK KR, PERIZ-STANACEV J, RANDOLPH EC, ROBINSON SL, SAARI TW, SCHROEDER AL, STINCKENS E, SWINTEK J, VAN CRUCHTEN SJ, VERBUEKEN E, VILLENEUVE DL, KNAPEN D. Gene transcription ontogeny of hypothalamic-pituitary-



thyroid axis development in early-life stage fathead minnow and zebrafish. **Gen Comp Endocrinol.** 2018.

WALPITA, C.N., VAN DER GEYTEN, S., RURANGWA, E., DARRAS, V.M. The effect of 3, 5, 3'- triiodothyronine supplementation on zebrafish (*Danio rerio*) embryonic development and expression of iodothyronine deiodinases and thyroid hormone receptors. **Gen. Comp. Endocrinol.** 152, 206-214. 2007.

WALPITA, C.N., CRAWFORD, A.D.; JANSSENS, E.D.R.; VAN DER GEYTEN, S.; DARRAS, V.M. Type 2 iodothyronine deiodinase is essential for thyroid hormonedependent embryonic development and pigmentation in zebrafish. **Endocrinol.** 150(1), 530-539. 2009.

WANG, Y.; S. ZHANG. Expression and regulation by thyroid hormone (TH) of zebrafish IGF-I gene and amphioxus IGF-I gene with implication of the origin of TH/IGF signaling pathway. **Comp. Biochem. Physiol. A Mol. Integr. Physiol.** 160: 474–479. 2011.

YAMANO, K. The Role of Thyroid Hormone in Fish Development with Reference to Aquaculture. **Japan Agricultural Research Quarterly.** 39(3):161-168. 2005.

YEN, P.M. Physiological and molecular basis of thyroid hormone action. **Physiol Rev** 81:1097–1142. 2001.

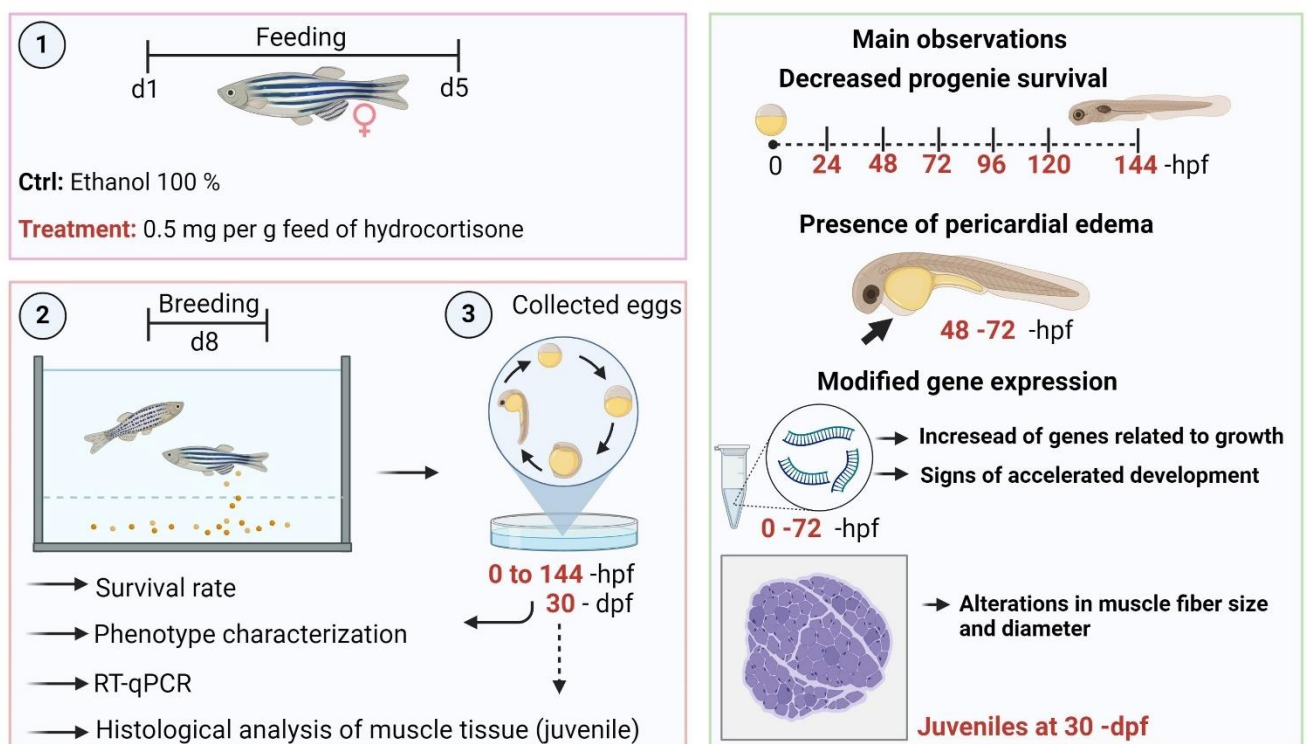
ZHANG, C.; LI, L.; ALAVA, J.J.; YAN, Z.; CHEN, P.; GUL, Y.; WANG, L.; XIONG, D. Female zebrafish (*Danio rerio*) exposure to polystyrene nanoplastics induces reproductive toxicity in mother and their offspring. *Aquatic Toxicology*. 2024.

Chapter 3

Effects of Maternal Stress on the Development of the Somatotrophic Axis During the Larval and Juvenile Stages of Zebrafish (*Danio rerio*)

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Graphical abstract

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Article

Effects of maternal stress on the development of the somatotrophic axis during the larval and juvenile stages in zebrafish (*Danio rerio*)

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Abstract: Stress is recognized as an adaptive response to potentially harmful environmental stimuli. The primary physiological adaptation to stress is an increase in circulating cortisol levels, which, in excess, can be transferred and incorporated into the oocytes of maturing females, affecting the embryonic developmental program. Additionally, maternal energy availability is an essential environmental factor that modulates this program. Based on this background, we investigated the effects of maternal cortisol on the development of the somatotrophic axis in the zebrafish offspring and juveniles. Zebrafish mothers were divided into two groups based on diet: Group 1 received a cortisol-enriched diet, to mimic maternal stress, while Group 2 (control) received a standard diet, for five days, followed by breeding on day three. Offspring were assessed at 0, 24, 48, 72-, 96-, 120, and 144 -hours post-fertilization (hpf). Morphological analyses were performed during embryonic development, including survival rate, body length, presence of pericardial edema, and heartbeat. We examined the gene expression of key somatotrophic axis components, including *mtor*, *foxO3a*, *mafbx*, *murfl*, *mstna*, *gh*, *igf1*, *igf2a*, *igf2b*, *11hsdb2*, and *fkbp5*. Our finding offers critical insights for aquaculture by elucidating the complex interaction between maternal factors and somatotrophic axis development in offspring. The study demonstrated that cortisol-treated females significantly affected offspring survival, morphology, and cardiac structures, in addition to altering gene expression related to protein synthesis, and showed signs of accelerated development. At 30 days post-fertilization, juveniles exhibited a notable increase in muscle bundle size and cross-sectional diameter. This evidence underscores the importance of aquaculture and animal research regarding how maternal environments influence somatotrophic axis development in fish, providing a foundation for improved breeding protocols and management practices.

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Keywords: cortisol; maternal stress; stress response; zebrafish; somatotrophic axis

Key Contribution: This study suggests that maternal treatment and environmental conditions can modulate the Hypothalamic-Pituitary-Interrenal (HPI) axis function, a key endocrine pathway, thereby impacting the growth and survival of offspring. These findings underscore the need for standardized breeding and reproductive methods in aquaculture to ensure optimal offspring growth.



1. Introduction

Developmental programming is a concept originating from maternal environment stimuli or early environmental conditions, which can lead to significant consequences in embryonic development and result in physiological changes that persist throughout life. Epigenetic mechanisms are central in mediating the effects of adverse environmental factors during early life, influencing long-term metabolic health by inducing enduring alterations in gene expression and organ function [1]. The exploration of early programmed changes dates back several decades. Notably, research conducted in 1957 on rats revealed modifications in the Hypothalamic-Pituitary-Adrenal (HPA) axis response to parental stress [2]. This study demonstrated that early life experiences could alter adults' nervous system reactivity to stress. This subject is being extensively explored across different species, including humans [3]. Exposure to a stressful environment during fetal development has been associated with behavioral dysfunction and disruptions in the HPA axis [4-6], along with metabolic [7], neurological [8-10], immunological [11,12], cardiovascular [13], and aging-related dysfunctions [14]. Studies on reptiles and birds have shown that maternal stress can lead to abnormal corticosterone deposition in embryos, the primary hormone involved in stress response in vertebrates. This abnormal corticosterone deposition has been found to cause developmental alterations after hatching [15,16]. In fish, cortisol manipulations in embryos or parents have been shown to result in significant alterations in the development of the somatotrophic axis [17,18].

The environmental conditions that fish are cultured can subject them to a diverse array of stressors, both physical and chemical. Consequently, understanding the physiological alterations induced by stress is significant in aquaculture [19]. Stress is an adaptive response that triggers the activation of the central nervous system and endocrine pathways to restore homeostasis [11]. The primary response involves the release of catecholamines and corticosteroids via the Hypothalamic-Pituitary-Interrenal (HPI) axis. This is followed by secondary responses, which include the mobilization of energy substrates, enhancement of oxygen transport capacity, and modifications to the immune system. Finally, tertiary effects manifest as behavior, reproduction, development, and growth changes. Thus, stress responses extend beyond simple glucose mobilization, reflecting a complex metabolic adaptation that impacts multiple systems within the organism [20, 21].

The HPI axis plays a central role in the primary stress response and releases glucocorticoids in fish. The HPI axis is functionally like the HPA axis in mammals, sharing key similarities. Upon exposure to stress, the hypothalamus releases corticotropin-releasing hormone (CRH), which in turn stimulates the synthesis of adrenocorticotrophic hormone (ACTH). ACTH then targets the interrenal tissue, which is comparable to the adrenal gland in mammals, leading to the release of cortisol. Cortisol is the major glucocorticoid in the circulatory system of humans and teleost fish, mediating the physiological stress response [17].

In zebrafish, cortisol during the embryonic phase originates from the mother, as *de novo* synthesis of this steroid occurs only post-hatching [22]. Early cortisol is crucial for modulating developmental programming, including the activating of the HPI axis [23]. During maternal stress, cortisol is transferred into developing oocytes and subsequently deposited into fertilized embryos. In the embryos, cortisol binds to glucocorticoid receptors (GRs). These GRs when bound to cortisol act as transcription factors that regulate the expression of various target genes in the embryo. Maternal cortisol is deposited in the developing oocyte and is present in decreasing quantities throughout embryogenesis. The enzyme 11 β -hydroxysteroid dehydrogenase (11 β -HSD) converts cortisol into its inactive form, cortisone, thereby protecting oocytes and embryos from excessive cortisol during maternal stress [17, 23]. The interplay between maternal stress and cortisol in oocytes is significant and complex. Therefore, research into the effects of maternal cortisol during early embryonic development is essential for understanding how these effects influence early developmental processes and contribute to phenotypic changes in offspring.

Furthermore, the development of muscle tissue or myogenesis, which initiates in the early stages of fish development can be affected by stress and cortisol. Embryonic muscle formation originates from cellular compartments known as somites, which are derived from mesodermal tissues. The dorsal region of the somites gives rise to myogenic precursor cells (MPCs) that express transcription factors Pax3 and Pax7, which are specific to muscle fiber types. This region also produces a population of



reserve progenitor cells, referred to as satellite cells, which are crucial for adult muscle growth [24]. Muscle tissue growth needs a balance between self-renewal and differentiation of precursor stem cells. This balance is regulated by different factors that influence the cell cycle of precursor stem cells, including Meox1, a critical factor for their activity. Zebrafish's muscular system maintains a continuous population of self-renewing precursor cells, facilitating secondary myogenesis throughout life. Secondary myogenesis occurs through hyperplasia and hypertrophy [25]. The activation of precursor stem cells, proliferation of myoblasts, differentiation, formation of myotubules, and maturation of myofibrils are regulated by myogenic regulatory factors (MRFs) such as MyoD, Myf5, myogenin, and MRF4, each with specific roles [26].

In teleost fish and other vertebrates, skeletal muscle represents a significant portion of body mass, and its growth is regulated by a balance between anabolic and catabolic processes influenced by stress and cortisol. The absence of glucocorticoid signaling has been shown to disrupt the expression of myogenic regulatory factors and muscle-specific genes, as evidenced by studies on zebrafish lacking glucocorticoid receptors (GRs) [13, 27]. Additionally, stress and cortisol can affect the somatotrophic axis. The somatotrophic axis comprises the hypothalamus, the pituitary gland, which releases growth hormone, GH-, and the liver, the primary source of insulin-like growth factors (IGFs). Studies in salmonids have demonstrated that acute stress and intraperitoneal cortisol administration lead to significant GH levels decrease in plasma [20]. Therefore, it is plausible that stress and cortisol also influence IGF1 production, as IGF1 synthesis in the liver is stimulated by GH. IGF1 is a key hormone in promoting the proliferation, differentiation, and hypertrophy of muscle precursor cells.

The importance of the somatotrophic axis in the growth of fish has been elucidated. On the other hand, perturbations in the growth axis involving environmental factors, including energy availability, stress, photoperiod, temperature, and others, can influence the development of this axis. Recent studies suggest that excessive maternal cortisol can impact embryonic developmental programming. However, the molecular mechanisms underlying these processes remain unclear. This study aimed to explore how the maternal environment affects the development of the somatotrophic axis in offspring, using zebrafish as a model organism. Zebrafish were chosen due to their rapid development, ease of manipulation, well-characterized genome, and the transparency of embryos and larvae, which facilitate structural and morphological observations. Their genomic similarity to humans also makes zebrafish a valuable model. The knowledge generated from this study will contribute to research in mammals, including humans, and optimize aquaculture techniques to ensure proper growth and development of offspring.

2. Materials and Methods

2.1. Animal husbandry

Sexually mature males and females (outbred) (4-5 months old) were maintained in the aquarium facility of the Department of Structural and Functional Biology, Institute of Biosciences, Botucatu, São Paulo State University (UNESP) in 6-L tanks on a recirculating system with a 14:10 light: dark cycle, and constant temperature conditions (28 °C). The following water parameters were monitored in all tanks every other day: pH, dissolved oxygen, and ammonia concentration. Males were fed twice a day with commercial food (Pentair Aquatic Habitats, Florida, USA), while females were fed twice a day with a diet prepared with hydrocortisone to assess the effects of maternal stress-associated cortisol on offspring development, as described below. Handling and experimentation were performed according to the Brazilian legislation regulated by the National Council for the Control of Animal Experimental (CONCEA) and Ethical Principles in Animal Research (Protocol n. 8520250320 -CEUA).

2.2. Maternal stress-associated cortisol

Ten sexually mature female zebrafish were assigned to each of the two 6-L tanks. One group (n = 5) was fed a control zebrafish diet, while the other group (n = 5) was fed a diet laced with cortisol, according to methods previously described [17]. For that, the zebrafish diet was prepared by soaking food pellets in 100 % ethanol either alone (control group) or with 0.5 mg per g feed (hydrocortisone group) (Sigma-Aldrich, St Louis, MO, USA) and allowing ethanol to evaporate [17, 28]. This



concentration was chosen based on previous studies with maternal cortisol [17]. Zebrafish mothers received thereabout of 25 µg cortisol/ g body mass. Fish were fed twice per day for 5 days. On day 3 post-treatment, five females from each group were transferred to breeding tanks (Figure 1).

2.3. Breeding

On day 3, female fish from each experimental group (control and cortisol) (n = 5/group) were placed for reproduction. The females were divided among five tanks (n = 1 per tank) along with unexposed males in each tank, following the usual methods for zebrafish reproduction [29]. The daily breeding set-up at 18 h, fish were transferred into breeding traps in the same tank, and eggs were collected within the first light (at 9 h) after mating the following morning. Fertilized eggs were collected in Petri dishes containing embryo medium (Hank's medium, https://zfin.org/zf_info/zfbook/chapt10.html#wptotml16). The eggs, embryos, and larvae from each group were collected and evaluated from 0-, 24-, 48- 72-, 96-, 120-, and 144 -hours post-fertilization (hpf). In the period from 0 to 72 -hpf, the eggs, embryos, and early larvae were counted and pools of 20 (n = 5 pools of 20 individuals for each pool) were snap-frozen on liquid nitrogen and stored at - 80 °C for gene expression analysis. Furthermore, eggs, embryos, and larvae from 0 to 144 -hpf were sampled for phenotype characterization and larval morphology. Juveniles at 30 -days post fertilization (dpf) were also collected for muscle fiber analysis in both groups, as described above (Figure 1).

2.4. Transcript levels of the somatotropic axis by quantitative real-time PCR (qPCR)

mRNA abundance of key genes involved in the somatotropic axis was measured by qPCR in embryos from 0 to 72 -hpf. Embryos were pooled into 20 (n = 5 pools of 20 individuals for each pool) per experimental group (control and cortisol treatment). Total RNA was extracted from the pools using a commercial kit (PureLink™ RNA mini kit, Ambion, Life Technologies, Carlsbad, CA, United States). After RNA extraction, the usual downstream methods were followed according to the manufacturer's instructions, and quality and purity were checked with a NanoDrop™ One Spectrophotometer (Thermo Scientific, Madison, WI, United States). cDNA synthesis was performed as described by Nóbrega et al. (2010) [30]. qPCR reactions were conducted using 5 µl of 2X SYBR-Green Universal Master Mix, 1 µl of forward primer (1.125 nM), 1 µl of reverse primer (1.125 nM), 0.5 µl of DEPC water, and 2.5 µl of cDNA. The relative mRNA levels of intracellular genes related to growth regulation through syntheses, such as *mtor* (mammalian target of rapamycin), and protein degradation, including *foxo3a*, *mafbx*, and *murfl* (forkhead box O3, muscle atrophy F-box, and muscle ring-finger protein-1, respectively) were quantified. Additionally, genes involved in the systemic or hormonal control of anabolic pathways, such as *gh*, *igf1*, *igf2a*, and *igf2b* (growth hormone, insulin-like growth factor 1, insulin-like growth factor 2a and 2b, respectively), as well as *mstna* (myostatin), which is related to the regulation of catabolism, were also measured. Furthermore, the relative mRNA levels of *hsd11b2* (hydroxysteroid 11-beta dehydrogenase 2) and *fkbp5* (glucocorticoid receptor chaperone protein) were evaluated to denote changes in the Hypothalamic-Pituitary-Interrenal (HPI) axis [17, 31]. mRNA levels of the targets (Cts) were normalized by the transcript levels of β -actin and expressed as relative values of the control group (as fold induction), according to the $2^{-(\Delta\Delta CT)}$ methods. Primers were designed according to zebrafish sequences (Table 1). Each primer was validated using a melting curve (Figure S1).

2.5. Embryo and larvae phenotypes

Embryos and larvae at 48 to 72 -hpf from both control and cortisol-treated groups were collected from spawning tanks and evaluated for morphological changes. The selected period corresponds to the post-hatching stage, during which larvae are presumed to experience the most significant influence of maternal cortisol deposition, as *de novo* synthesis of this steroid begins only after hatching. A total of 30 larvae were randomly chosen from each group, and 30 bright-field images from each specimen were captured using an Olympus MVX10 MacroView stereomicroscope. These images were subsequently utilized to measure morphometric parameters in developing larvae with annotation performed using

the Olympus cellSens dimensions software (<https://www.olympuslifescience.com/pt/software/cellsens/>), and analysis conducted via ImageJ software (available at <http://imagej.nih.gov/ij/index.html>). The developmental differences between the control and treatment groups were assessed through morphometric evaluations. The total length of larvae was measured, and abnormal developments were identified, including pericardial and yolk sac edema, inflated swim bladder, and frequency of body deformation and/or abnormal curvature. The survival rate of zebrafish embryos and larvae in the range of 0 to 144 -hpf was evaluated for each group using the Kaplan-Meier method. Survival was assessed based on the percentage of viable larvae relative to the total number of specimens collected post-fertilization (0 hpf). Embryos and larvae found at the bottom of the Petri dish with opaque appearance and abnormal coloration, along with hyporesponsiveness to mechanical stress were considered dead.

2.6. Heart rate measurement in embryos at 48 -hpf

Videos from zebrafish embryos at 48 -hpf from both maternal control and cortisol-treated groups ($n = 30/\text{condition}$) were recorded to calculate heart rate per minute. The non-anesthetized embryos were immobilized using excavated slide blanks containing sufficient embryonic medium to ensure survival during filming. The setup consists of an Olympus MVX10 MacroView stereomicroscope linkage to Olympus cellSens dimensions software (<https://www.olympuslifescience.com/pt/software/cellsens/>) for video acquisition. The video files (.AVI) were obtained at 30 frames per second (fps) totaling 60 seconds of recording, with a resolution of 4080 x 3072 pixels. Heart rate quantification was performed using a Python script named CardiOT2[®] software, which employs the Fast Fourier (FFT) methodology to calculate heart rate contraction cycles.

2.7. Histological analysis of muscle tissue

Five fish at 30 days post-fertilization (dpf) were collected from each experimental group (control and cortisol) for cross-sectional analysis, while three larvae per group were used for longitudinal analysis. The specimens were anesthetized with 0.1% benzocaine and sacrificed for skeletal muscle tissue collection. Samples were immediately fixed in Karnovsky solution (glutaraldehyde 2.5 %, paraformaldehyde 2 %, 0.1 M phosphate buffer, pH 7.2) and preserved in 70 % alcohol. Samples were dehydrated using graded ethanol concentrations (80, 95, and 95 %) and embedded in Technovit 7100 resin (Heraeus Kulzer, Wehrheim, Germany), according to the manufacturer's protocol. Longitudinal and cross-section histological areas (4 μm) of muscle fibers were prepared through a microtome equipped with a glass knife. Sections were stained with 0.1 % toluidine blue in 1 % sodium borate. Fiber diameter and frequency were determined using a Leica DMI6000 microscope (Leica, Heidelberg, Germany) coupled with an image analyzer. Muscle fibers were classified based on the criteria previously described [32], with fibers smaller than 9 μm categorized as thin fibers, and those larger than 9 μm as thick fibers.

2.8. Statistical analysis

The data were subjected to the mean $\pm$ SEM (Standard Error of Mean). Significant differences between the two groups were identified using Student's t-tests non-parametric (unpaired) to compare two groups, the treated group versus the control group. The significance level (p) was considered ≤ 0.05 in both cases. The normal distribution of the samples was assessed using the Kolmogorov-Smirnov test, which was performed for all genes used in this study. All analysis was carried out using Graph Pad Prism software 8.0.1 (Graph Pad Software, Inc., San Diego, CA, United States).

3. Results

3.1. Maternal cortisol influences the mRNA expression levels of genes associated with the somatotrophic and HPI axes in zebrafish offspring

Samples were collected at 0, 24, 48, and 72 -hpf of both the control and cortisol-treated groups for gene expression analysis by qPCR (Figure 1). The expression levels of essential components involved

in the somatotrophic and HPI axes, such as *mtor*, *foxo3a*, *mafbx*, *murf1*, *mstna*, *gh*, *igf1*, *igf2a*, *igf2b*, *fbp5*, and *11hsdb2*, were significantly altered in the zebrafish progeny from females fed with cortisol-enriched diet when compared to the control (Figure 2). Our analysis revealed that maternal cortisol treatment up-regulated *mtor* mRNA levels in embryos at 24 -hpf (Figure 2A). We also evaluated the genes involved in muscle protein degradation. Embryos and larvae from the maternal cortisol-treated group exhibited increased levels of *foxo3a* at 24, 48, and 72 -hpf compared to the control (Figure 2B). The mRNA expression levels of *mafbx* e *murf1*, which are genes involved in the catabolic pathway, were significantly altered in the treated group. While *mafbx* levels were significantly elevated at 48 and 72- hpf (Figure 2C), *murf1* increased expression at 0 and 48 -hpf (Figure 2D). In this regard, the expression of *mstna*, a protein that inhibits myogenesis, a significant decrease in its expression was observed in the treated group at 0 -hpf, followed by a significant increase at 24 -hpf (Figure 2E). Maternal cortisol treatment also significantly increased the expression of *gh* in embryos at 0 -hpf (Figure 2F). However, a substantial decrease in primary *gh* transcripts was observed in early larvae at 72 -hpf from females fed with a cortisol-treated diet (Figure 2F). The expression levels of *igf1*, *igf2a*, and *igf2b* were measured. *igf1* was up-regulated from 0 to 48 -hpf in the cortisol-treated group (Figure 2G), while *igf2b* expression increased only at 0 -hpf (Figure 2I). On the other hand, the mRNA levels of *igf2a* remained unchanged across the experimental groups at the sampled time points (Figure 2H). Finally, we evaluated the expression of genes responsive to the HPI axis. A significant decrease in *fbp5* expression levels was observed exclusively at 0 -hpf (Figure 2J). However, the mRNA levels of the enzyme 11 β -HSD2 (*11hsdb2*), responsible for converting cortisol into its inactive form, cortisone, showed no significant differences between the experimental groups (Figure 2K).

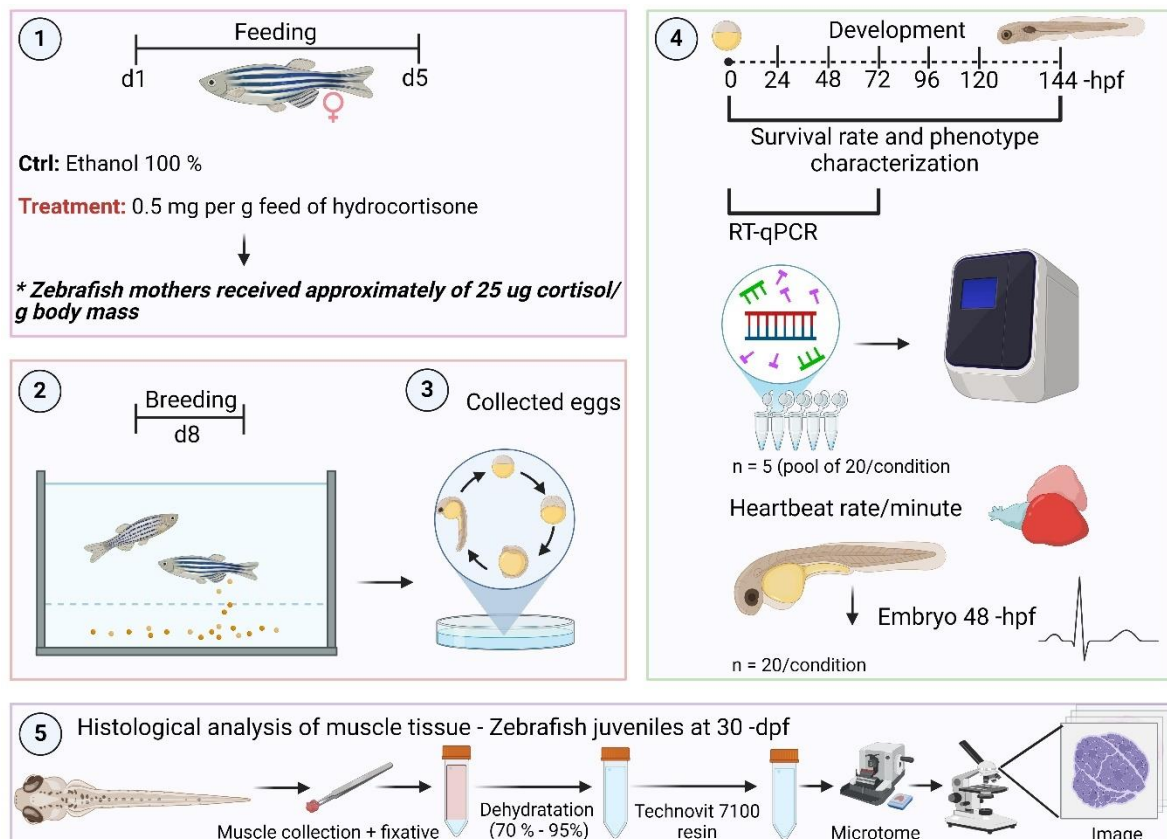


Figure 1. Experimental design. **(1)** Two groups of female zebrafish (n = 30) were assigned into three replicates per experimental group. One group was fed commercial food, while the other one was fed commercial food containing 0.5 mg per feed of hydrocortisone. Zebrafish mothers received thereabout of 25 μ g cortisol/ g body mass. Fish were fed twice per day for 5 days. **(2)** On day 3 post-treatment, five females from each group were transferred to breeding tanks with males untreated. **(3)** Fertilized eggs were collected in Petri dishes containing embryo medium. **(4)** Eggs, embryos, and larvae were collected at 0 to 144 -hpf for survival rate, and phenotype characterization such as length,

and pericardial edema. The eggs, embryos, and early larvae from 0 to 72 -hpf were counted and pools were collected for gene expression analysis. Embryos at 48 -hpf from the control and cortisol groups were taken to calculate the heartbeat rate per minute. (5) Juveniles at 30 -dpf were also collected for histological analysis of muscle fiber in both groups. Illustration created in <https://BioRender.com>.

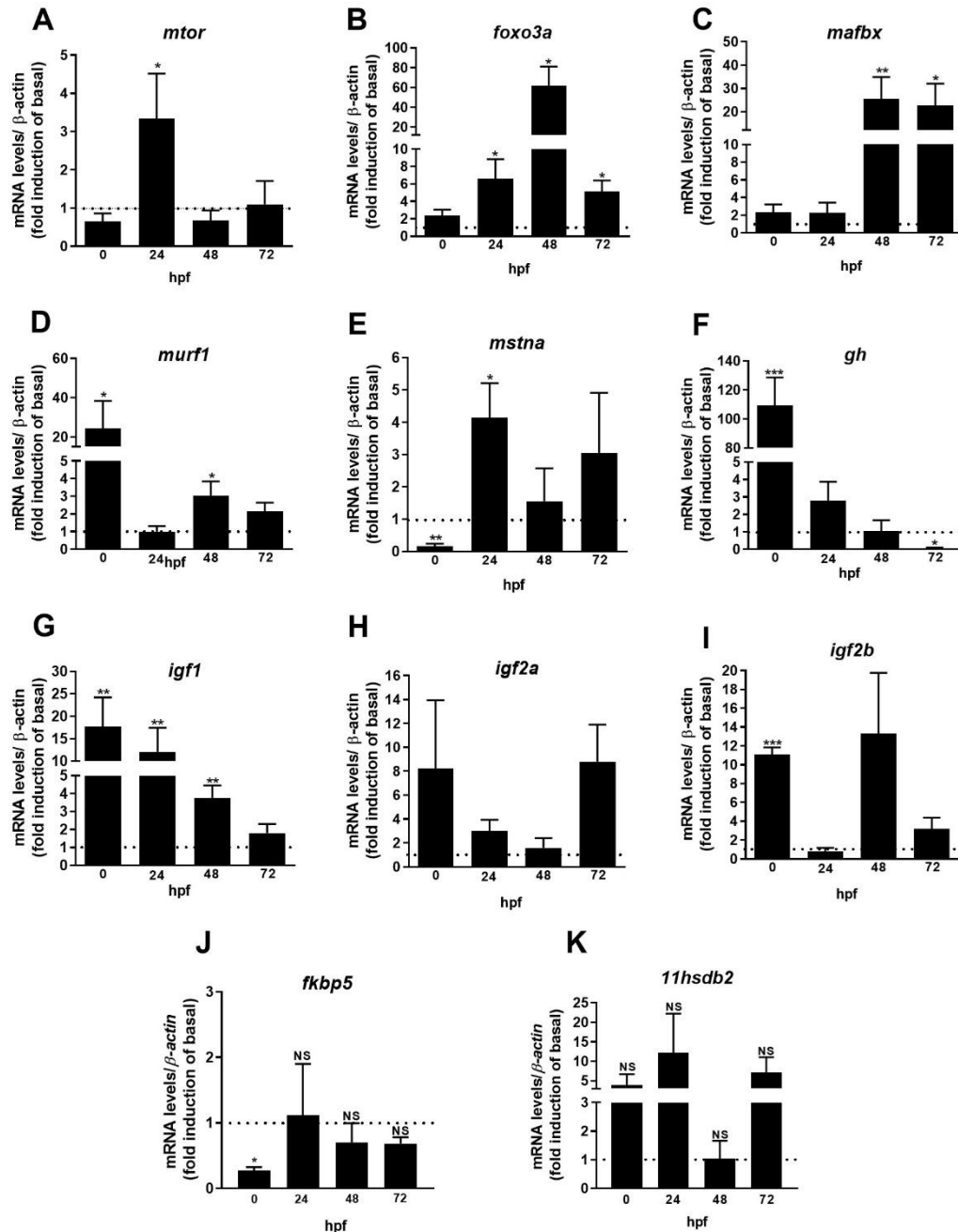


Figure 2. Somatotrophic and HPI-related genes in embryos and early larvae at 0 to 72 -hpf from mothers fed with hydrocortisone enriched in the diet (0.5 mg per g feed) compared to the untreated group (control) (dotted black line set at 1). The selected genes *mtor* (mammalian target of rapamycin) (A); *foxo3a* (forkhead box O3) (B); *mafbx* (muscle atrophy F-box) (C); *murf1* (muscle ring-finger protein-1) (D); *mstna* (myostatin) (E); *gh* (growth hormone) (F); *igf1* (insulin-like growth factor 1) (G); *igf2a* (insulin-like growth factor 2a) (H); *igf2b* (insulin-like growth factor 2b) (I); *fkbp5* (glucocorticoid receptor chaperone protein) (J); and *11hsdb2* (hydroxysteroid 11-beta dehydrogenase 2) (K) were evaluated. Ct values were normalized with β -actin and expressed as relative values of control (no treated fish) levels of expression. Bars represent the mean $\pm$ SEM fold change (n = 5 pools of 20 individuals for each pool) relative to the control. Student unpaired *t*-test, **p* < 0.05, ***p* < 0.01, ****p* < 0.001. NS not significant.



3.2. Maternal cortisol treatment affected different stages of offspring development in different parameters of evaluation

We investigated the impact of maternal cortisol on embryos and larvae survival and phenotype. Embryos and larvae were collected at intervals from 0 to 144 -hpf in both the control and cortisol-treated groups for survival analysis. The survival rate was conducted using the Kaplan-Meier method. The results showed a higher mortality rate among offspring from females fed a cortisol-enriched diet compared to the control group (Figure 3A). The Log-rank test (Mantel-Cox) indicated a significant relative risk of death between the groups during the study period (chi-square = 64; $p < 0.0001$). The control group maintained a survival rate of 90.5 % at 24 -hpf, with no decline observed up to 144 -hpf. However, the offspring from the cortisol group showed survival rates of 79.6 %, 78.6 %, 73.5 %, and 70.9 % at 24, 48, 72, and 144 -hpf, respectively.

Body length measurements of larvae at 48 and 72 -hpf were measured. Maternal cortisol exposure significantly increased larval length at 48 -hpf compared to the control group (Figure 3B). The morphological evaluation indicated that offspring from females fed a cortisol-enriched diet exhibited moderate cardiac edema, as classified previously [13]. Specifically, 63.3 % and 36.6 % of larvae displayed moderate pericardial edema at 48 and 72 -hpf, respectively (Figure 3C-D). No morphological abnormalities were noted in the control group (Figure 3E-F).

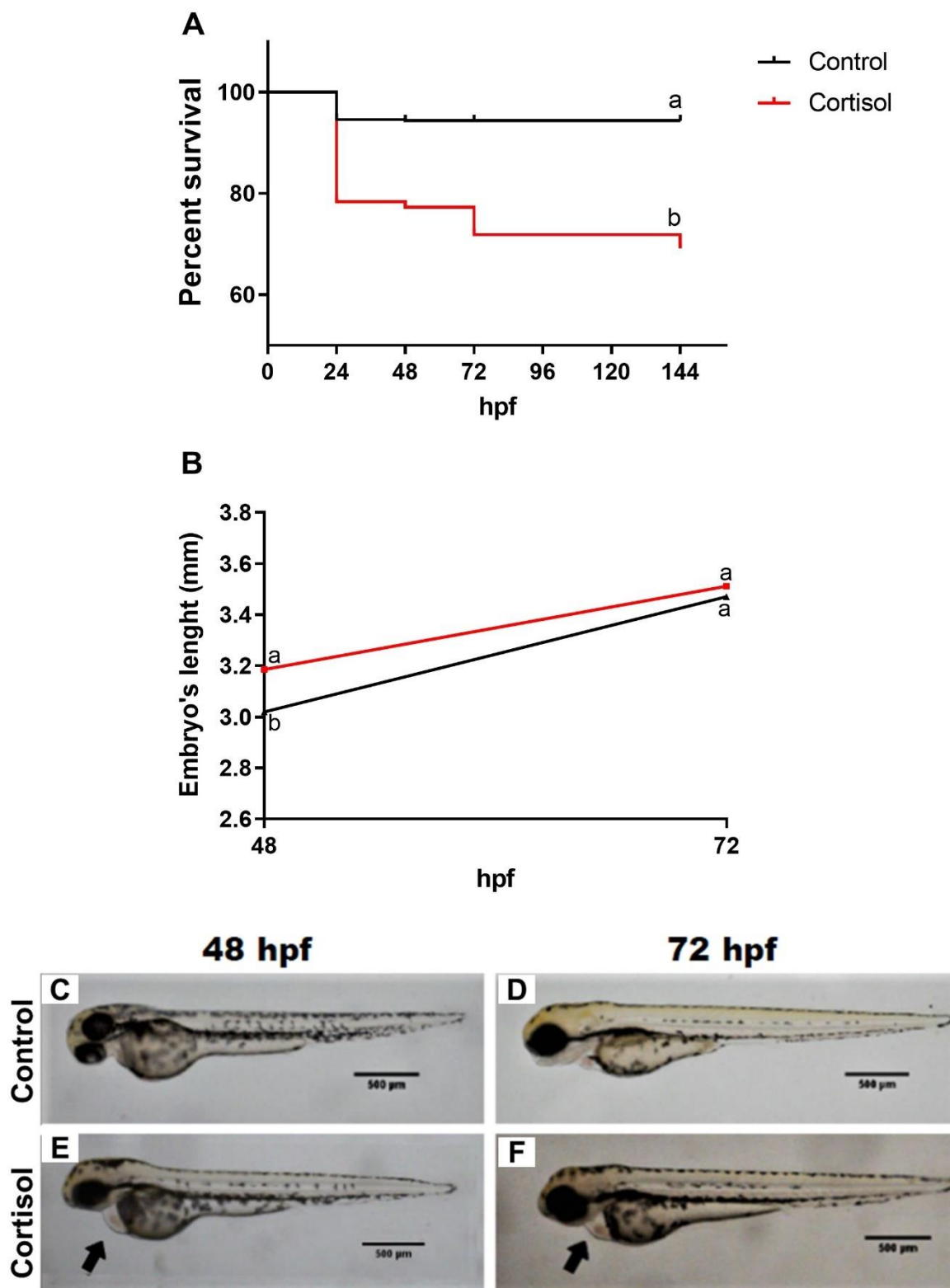


Figure 3. The survival rate of embryos and larvae at 0 to 144 -hpf (**A**) was demonstrated in both control and treated animals. Embryos and larvae length at 48 to 72 -hpf (**B**) were measured (Bars represent the mean $\pm$ SEM; ANOVA followed by Tukey's multiple comparison tests or Student unpaired *t*-test. Distinct letters denote significant differences; $p \leq 0.05$). The phenotype of embryos and early larvae from control females (**C-D**) compared with those

of progeny impacted by maternal transfer of corticosteroid (E-F). Control animals showed normal morphology of pericardium (C-D). However, embryos from maternal cortisol-treated were affected. Arrows highlight the presence of pericardial edema (E-F). Scale bar = 500 μ m (n = 30, basal and treatment).

Additionally, heart rate measurements at 48 -hpf showed no significant difference between offspring from cortisol-treated and control females (Figure 4).

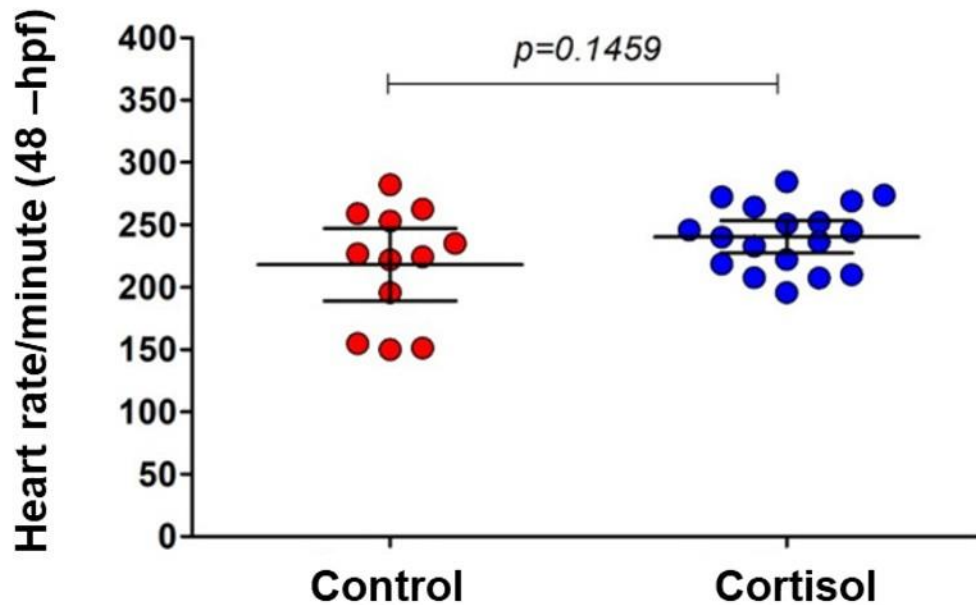


Figure 4. The heartbeat of embryos with 48 -hpf from control (red circle) and maternal cortisol treatment (blue circle) was demonstrated. No significant heartbeat difference was found between control and treatment embryos. Student unpaired *t*-test, $p = 0.1459$.

At 30 -dpf, histological analysis of muscle fibers in the progeny of cortisol-treated females revealed distinct changes. Longitudinal sections revealed that juveniles from the maternal cortisol-treated group had significantly longer muscle bundles compared to the control group (mean $387.6 \pm 6.71 \mu$ m, and $287.6 \pm 24.81 \mu$ m, respectively) (Figure 5A-C).

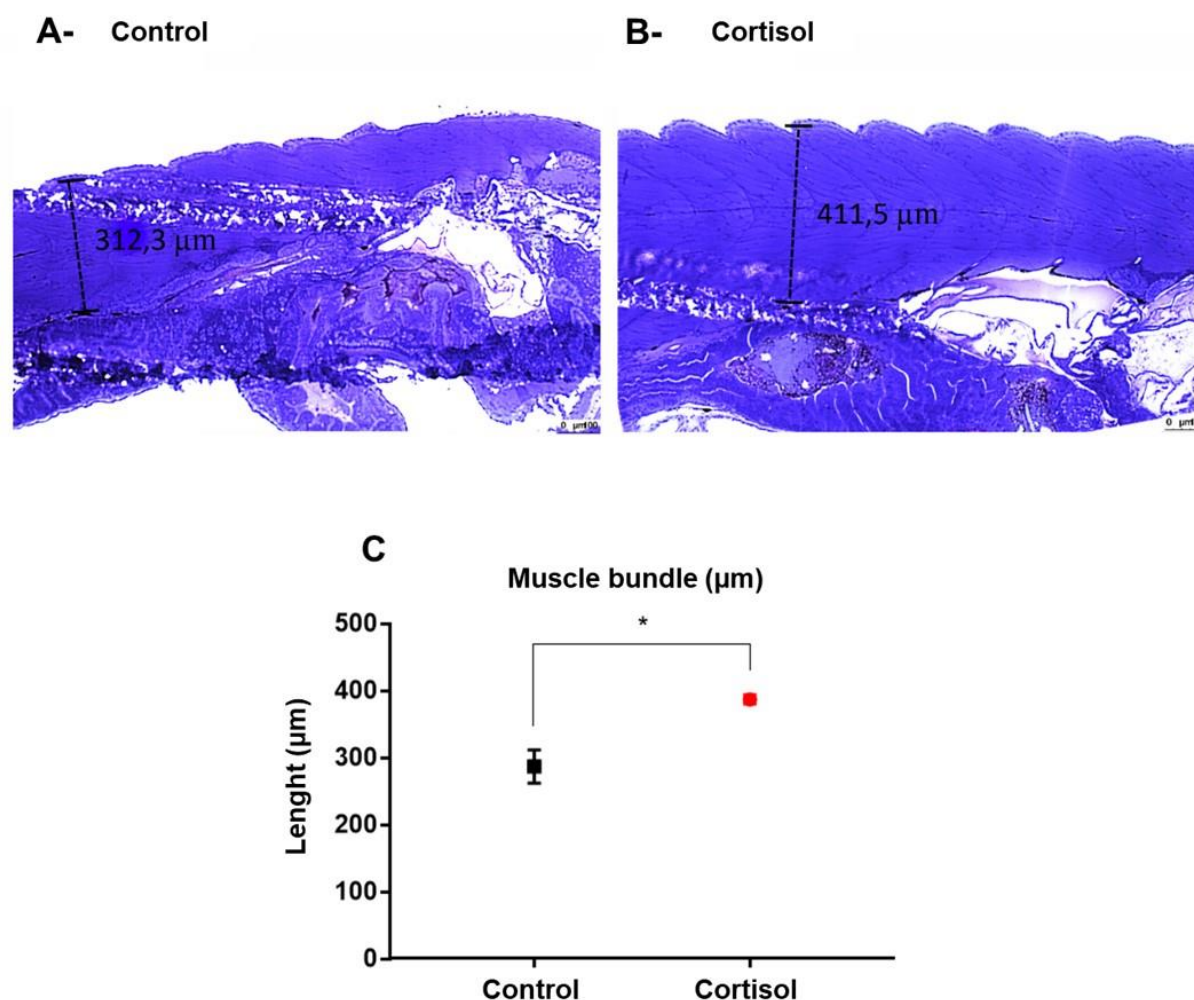


Figure 5. Longitudinal histological section of muscle from 30 -dpf zebrafish juvenile from females fed with a commercial diet (A) or a diet supplemented with cortisol (B). Muscle bundle length in 30 -dpf from juvenile from control (black) and cortisol-treated (red) females (C). (Bars sampled in each group represent the mean $\pm$ SEM; Student unpaired *t*-test, $*p < 0.05$). Scale bar = 100 μm . Staining: Toluidine blue ($n = 3$, basal and treatment).

Furthermore, cross-section analysis indicated that the total muscle diameter was larger in juveniles from mothers treated with cortisol ($254 \pm 9.27 \mu\text{m}$) compared to those from control females ($187.7 \pm 6.44 \mu\text{m}$) (Figure 6A, B, E). Furthermore, cross-sections showed a higher percentage of thick muscle fiber ($> 9 \mu\text{m}$) in the juvenile from maternal cortisol-treated, averaging 72.5 %, compared to 35.7 % in the control group. However, the progeny from the control group exhibited a higher proportion of thin muscle fibers ($< 9 \mu\text{m}$), averaging 63.7 % compared to 27.5 % in the progeny from the maternal cortisol group (Figure 6C, D, F).

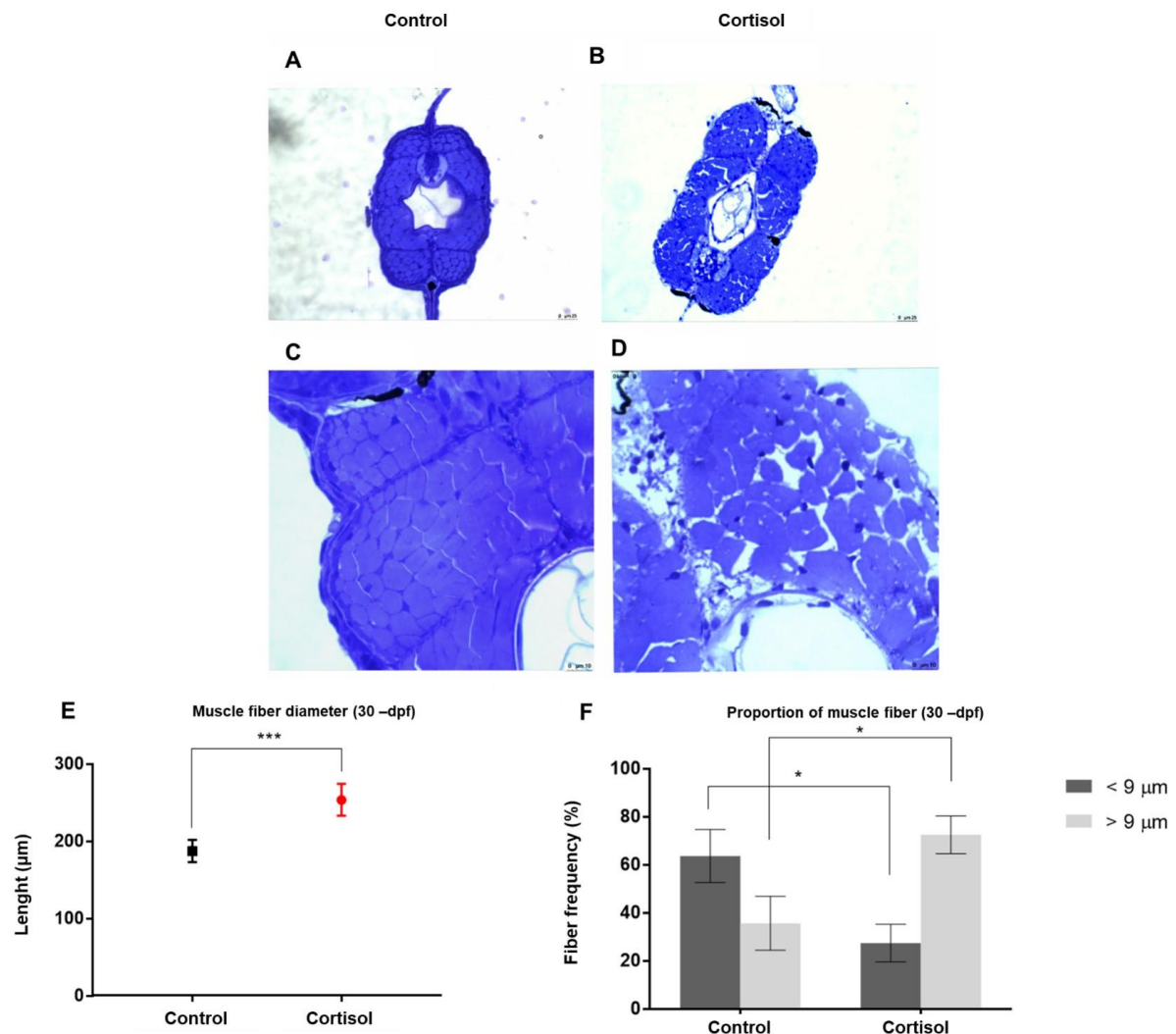


Figure 6. Histological cross-section of zebrafish muscle juvenile at 30 -dpf from females fed a commercial diet (control) (A, C) or a diet supplemented with cortisol (B, D). The cross-sectional diameter of muscle fiber in both progeny from non-treated and cortisol-treated females (E). The proportion of thin (< 9 μm) and thick (> 9 μm) muscle fiber in zebrafish juveniles at 30 -dpf from control and cortisol-treated groups. (Symbols or bars represent the mean ± SEM; Student unpaired *t*-test, **p* < 0.05; ****p* < 0.001). Scale bar = 25 μm (A-B); 10 μm (C-D). Staining: Toluidine blue (n = 5; 75 muscle fiber per animal, basal and treatment).

4. Discussion

Zebrafish (*Danio rerio*) is an ideal model to investigate the early developmental effects of cortisol signaling, as *de novo* synthesis of cortisol begins only after hatching (48 – 72 hours post-fertilization, hpf). Therefore, maternal cortisol is a major influence on early developmental programming [22]. Previous research has highlighted the critical role of maternal cortisol in embryogenesis [13, 27]. Studies employing morpholino oligonucleotides to reduce glucocorticoid receptor expression in zebrafish embryos showed mesodermal malformations, including bent tails and deformed somites, as well as reduced growth and decreased survival rates [13, 27].

On the other hand, to investigate the influence of increased maternal cortisol deposition in embryos, several experiments have been conducted in fish to assess phenotypic changes in offspring, particularly concerning the somatotrophic axis [33]. In zebrafish, Nesan and Vijayan (2016) [23] demonstrated that microinjection of 32 pg pf cortisol into newly fertilized embryos – a technique used to mimic maternal cortisol transfer and deposition – resulted in increased embryo size at 48 and 72 -hpf.



Similarly, our study found that adding cortisol to the maternal diet resulted in maternal transfer of cortisol, which increased offspring size at 48 -hpf. Interestingly, the same researchers [23] demonstrated that injection of cortisol-specific antibodies resulted in decreased embryo length and abnormal morphologies, including moderate tail twist and deformed body curvature. These findings demonstrated the crucial role of cortisol in mesoderm formation and muscle development [13], prompting further investigation into whether a positive adjustment of cortisol levels could be beneficial for embryogenesis and myogenesis.

Another critical aspect to consider is the cardiac structure. Offspring from females fed a cortisol-enriched diet exhibit pericardial edema. This finding is particularly relevant to our study, as the heart is composed of muscle tissue and originates from the mesodermal layer during embryogenesis [34]. Interestingly, Nesan and Vijayan (2012) [13] demonstrated that increasing cortisol levels in newly fertilized zygotes via microinjection also disrupted cardiogenesis, leading to pericardial edema, and cardiac chamber malformations. Similar results support the hypothesis that maternal cortisol programs embryogenesis, though elevated levels of this steroid in the embryo can have adverse effects. Moreover, the morphometric data from our study align with the literature confirming the effectiveness of using a cortisol-enriched diet to simulate maternal stress and subsequent cortisol transfer to the offspring [17]. Nesan and Vijayan (2012) [13] also observed a reduction in heart rate in 72 -hpf larvae that were exposed to elevated cortisol levels during pre-hatch. On the other hand, our study showed that maternal cortisol treatment did not alter the heart rate of larvae at 48 -hpf, despite observed pericardial edema. We hypothesize that the discrepancy between the results may be attributed to differences in the treatment methods – such as cortisol microinjection into embryos versus maternal cortisol diet supplementation – or to a specific time when heart rate was measured.

To gain a deeper understanding of how cortisol influences zebrafish embryonic development, we focused on gene expression analysis. We examined key developmental genes involved in the somatotrophic axis, including *gh*, *igf1*, *igf2a*, and *igf2b*. In vertebrates, GH functions directly by binding to receptors on the muscle sarcolemma and indirectly by stimulating the production and release of insulin-like growth factors (Igf) in the liver and peripheral tissues [35]. In zebrafish, the Igf system includes *igf1*, *igf2*, and *igf3*, which *igf3* being specific to gonadal tissue [36]. *igf2* and *igf3* are involved in midline development. The particular relevance to our study is *igf1*, which acts through the PI3K-Akt-TOR anabolic pathways by binding to its receptor, thereby being essential for normal embryonic development and myogenesis [24]. Indeed, reduced *igf1* signaling in zebrafish has been associated with decreased synthesis of major muscle proteins, leading to a reduction in muscle fiber diameter [32]. The gene expression data reveal a significant increase in *gh* mRNA levels after fertilization, suggesting that this transcript is maternally inherited and positively regulated by maternal cortisol. As development progresses, relative *gh* levels normalize at 24 and 48 -hpf. However, there was a marked decline of *gh* levels at 72 -hpf in the offspring of cortisol-treated females. Consistent with this, *igf1* mRNA levels increased at 0, 24, and 48 -hpf in embryos and larvae from cortisol-treated females. Notably, the increase in larval size at 48 -hpf brings into line with the elevated levels of the growth factor. In our study, the elevated transfer of maternal cortisol resulted in a coincidence increase in *gh* and *igf1* levels in the first hour post-fertilization, followed by a subsequent downregulation during embryogenesis. This suggests a direct relationship between the HPI and somatotrophic axes. Our findings are consistent with the literature, which emphasizes the crucial role of cortisol in the proper morphological development of zebrafish [21, 37].

Additionally, we analyzed the mRNA levels of genes related to skeletal muscle development, including *mtor*, *foxo3a*, and *myf1*. The regulation of muscle fiber volume and quantity involves a complex interplay of anabolic and catabolic proteins, which respectively promote protein synthesis and degradation [24]. The IGF-Akt pathway plays a pivotal role in this process, functioning through two key mechanisms in fish. In the anabolic process, *mtor* protein is phosphorylated and activated, playing a central role in cell growth and proliferation, which drives hypertrophy and hyperplasia. On the other hand, muscle atrophy is prevented by the phosphorylation and inactivation of the *foxo3a* protein,



resulting in the downregulation of the ubiquitin ligases *murfl* and *mafbx*, which mark proteins for degradation by the proteasome complex [38]. In this study, we observed an upregulation of *mtor* and *foxo3a* expression in embryos at 24 -hpf in the maternal cortisol-treated group, suggesting an increase in muscle protein turnover. This phenomenon may explain the increased embryo length observed in larvae at 48 -hpf in the treated group. Subsequently, we observed the upregulation in the mRNA expression of protein degradation markers, such as *foxo3a*, *murfl*, and *mafbx* in embryos at 48 and 72 -hpf along with stabilization of *mtor* expression, which may have prevented the exacerbated growth post-fertilization. Consequently, there was no significant difference in embryo length at 72 -hpf between maternal cortisol-treated and control groups.

On the other hand, myostatin, a member of the tumor growth factor- β (TGF- β) superfamily, is a well-established negative regulator of myogenesis in vertebrates. In zebrafish, inhibition of myostatin expression leads to a significant increase in muscle mass [39, 40]. Furthermore, two myostatin gene variants, *mstna* and *mstnb*, can be found in zebrafish. *mstna* mRNA being maternally inherited and highly expressed in embryos at 48 -hpf [41-43]. Our study demonstrated that *mstna* mRNA levels were inhibited in embryos after fertilization in the maternal cortisol-treated group. This suggests that the increase in larval length at 48 -hpf is related to the inhibition of *mstna* levels, leading to enhanced muscle growth, as indicated by elevated *mtor* expression. However, this interpretation was complex when we observed the suppression of mRNA transcripts of genes involved in protein degradation, *murfl* and *mafbx*. Additionally, there was a significant increase in *mstna* expression in embryos at 24 -hpf corresponding to the treated group, which could be directly related to the elevated expression of *foxo3a*, *murfl*, and *mafbx* in the period of 48 and 72 -hpf. These findings suggest that this process's regulatory mechanisms are still poorly understood, particularly during the post-fertilization period.

Finally, we also evaluated the expression of cortisol-responsive genes: *hsd11b2* e *fkbp5* [17, 31, 44]. FK506-binding protein 5 (Fkbp5) is a glucocorticoid receptor chaperone protein that is responsive to elevated glucocorticoid levels in zebrafish. This observation was confirmed in Fdx1b-deficient zebrafish males, in which its expression was decreased in response to decreased plasma cortisol levels [45]. In the present study, *fkbp5* expression was reduced in 0 -hpf embryos from cortisol-treated females, indicating either that maternal cortisol transfer did not occur as expected, or that there are counter-regulatory mechanisms in response to excess glucocorticoids in the offspring. The low response to cortisol receptors activating during the early stages of embryogenesis could have influenced the increased larval size observed at 48 -hpf in the treated group, which indicates the crosstalk between the HPI and somatotropic axes.

The literature suggests that ovarian follicles, eggs, and embryos are not passive recipients of maternal steroids [33]; they possess mechanisms to either transport cortisol out of the embryo or metabolize it into its inactive form. Consequently, the transfer of maternal cortisol to the offspring and its subsequent effects involves passive and counter-regulatory mechanisms. Faught et al. (2016) [17] demonstrated an increase in the *hsd11b2* transcript in zebrafish ovarian follicles following *ex vivo* cortisol incubation, indicating that maternal stress may lead to the activating of ovarian enzymes that mitigate cortisol levels in oocytes. However, our findings suggest that cortisol deposition is transient due to the asynchronous type of zebrafish reproductive, in which vitellogenic oocytes might be susceptible to excessive steroid uptake during a specific window before the upregulation of *hsd11b2* activity in ovarian follicles in response to increased cortisol levels. Notably, no significant differences in *hsd11b2* expression were observed between experimental groups up to 72 -hpf, supporting our hypothesis. Although *hsd11b2* mRNA levels were unchanged, the expression of *fkbp5* was reduced post-fertilization. Therefore, the mechanism behind the increased cortisol levels in embryos remains unclear, and further research is required to better understand the mechanism of maternal cortisol transfer in fish. We propose that maternal cortisol transfer occurred without a counter-regulatory effect on *fkbp5* mRNA levels, as previously demonstrated by Tovo-Neto et al. (2020) [31], who showed that high cortisol concentrations increase *fkbp5* expression. The results obtained from morphological and histological



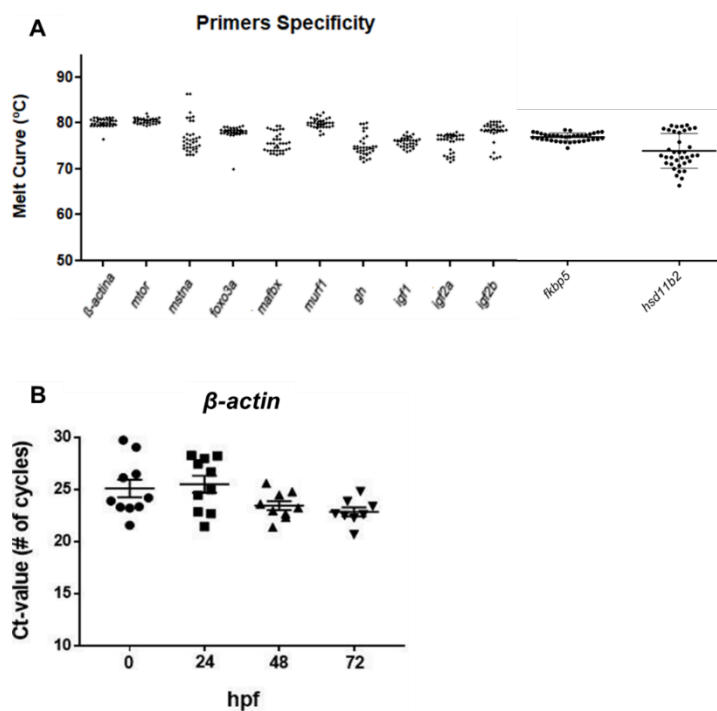
analysis in this study are consistent. We believe that feeding females with cortisol-enriched feed may lead to the transfer of cortisol to their offspring.

Histological analysis of muscle fiber in larvae at 30 -dpf from a mother-fed cortisol-enriched diet revealed a significantly larger muscle bundle size compared to the larvae from the control group. Moreover, larvae from the treated group exhibited an increased cross-section diameter. These findings are consistent with previous studies. Wilson et al. (2016) [44] showed that treating fertilized embryos with the corticosteroid dexamethasone for 5 days resulted in increased larval size at 84 -dpf. This suggests that changes in corticosteroid levels in ovarian follicles or directly in the embryos can induce long-term morphological changes. The same study [44] investigated the acute and chronic effects of the synthetic glucocorticoid receptor (GR) agonist dexamethasone. At 120 -hpf, larvae treated exhibited reduced cortisol levels compared to control larvae. Additionally, Nesan and Vijayan (2016) [23] demonstrated that alterations in maternal cortisol availability affect the cortisol response in zebrafish embryos post-hatching. Specifically, reducing cortisol levels in embryos via microinjection of specific antibodies resulted in an enhanced cortisol response. In contrast, larvae from embryos microinjected with cortisol did not show a cortisol response following an acute physical stressor, indicating potential disruption of the HPI axis activity. In vertebrates, muscle growth occurs through a combination of two main processes: hypertrophy, which is the increase in the size and length of existing muscle fibers, and hyperplasia, which involves the formation of new muscle fibers from the differentiation of muscle stem cells. Research in zebrafish has demonstrated that the cross-section area (CSA) of muscle fibers correlates directly with the fish's age [46]. Additionally, newly formed muscle fibers through hyperplasia in older zebrafish exhibit increased CSA and length compared to those in younger fish [47]. Our results showed that juveniles at 30 -dpf from cortisol-fed females had a higher percentage of thick fibers compared to the control group. These findings suggest that maternal cortisol treatment may have accelerated the metabolic rate and developmental progression of embryos, as evidenced by changes in length during larval development. Another hypothesis is that the treatment induced greater hypertrophy than muscle hyperplasia.

5. Conclusions

In this study, we showed that maternal stress linked to cortisol deposition significantly impacts the morphological and cardiac structures of offspring development. Embryos from cortisol-treated females exhibited modified expression of genes associated with protein synthesis and showed signs of accelerated development. In juveniles, alterations in muscle fiber size and diameter were observed. Overall, our study suggests that parental treatment and environmental conditions can influence the function of the hypothalamic-pituitary-interrenal (HPI) axis in offspring, thereby impacting the growth and viability of offspring. This highlights the importance of standardizing breeding and reproductive methods in the aquaculture system.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure S1: **(A)** Primer specificity. The primer specificity was verified according to the melt curve temperature (dissociation temperature). The dissociation temperature in all qPCR reactions is demonstrated for each primer. Each dot represents the melt curve temperature for one sample. **(B)** A scatter plot was generated to assess the stability of *β-actin* as a reference gene in samples of embryo/early larvae collected at 0 to 72 -hpf from females fed only with commercial food (control group) and fed with a diet laced with 0.5 mg hydrocortisone per gram of feed. No significant differences ($p > 0.05$; ANOVA following Tukey's multiple comparison test) were observed in *β-actin* Ct values between control and treated samples across the experimental groups. **Table S1:** Primers used for gene expression studies (qPCR) (FW = Forward; RV = Reverse).



Target name	Primers sequence (5' - 3')	Reference
<i>B-actin</i>	AGACATCAGGGAGTGATGGT (Fw)	BC045846
	CAATACCGTGCTCAATGGGG (Rv)	
<i>igf1</i>	CCCAGGACACCAAGAAACCTA(Fw)	This work
	CGGCTCGAGTTCTCTGATGA(Rv)	
<i>igf2a</i>	GGCTTCTATTTCAGTCGACCAACTAG(Fw)	This work
	ACTAAACAACACTCTCCACAATCC(Rv)	
<i>igf2b</i>	CTGCCATGGATGATTACCATGTATT(Fw)	This work
	CATGGACAATGACAGAACGAAGAC(Rv)	
<i>mtor</i>	GGAATGCAACAGCAAGCACA(Fw)	This work
	GCTTCAGAAAACACCGTGCC(Rv)	
<i>foxo3a</i>	GCTCTGCAGGATGGAAGAACT(Fw)	This work
	TCCACCATGAACCTTTCTGT(Rv)	
<i>mstna</i>	CAGTATGACCAAAACGGAGGC(Fw)	This work
	TGGGTGATGGCTTGAGGTTT(Rv)	
<i>gh</i>	TGACCATCGGAACCCCAAC(Fw)	This work
	GTCCATCGAGACATCCCTTG(Rv)	
<i>mafbx</i>	GGAGCACCAAGAGCGTCAT(Fw)	This work
	ATCGCCTGGTGCTTGATG(Rv)	
<i>murf1</i>	TCCCGTGCCAACACAATCTC(Rv)	This work
	CTCGAAGCGACAAGTAGGGC(Fw)	
<i>fbp5</i>	TTCCACTCGTGTCGAGA (Fw)	Griffin et al. 2016
	ACGATCCCACCATCTTCTGT (Rv)	
<i>hsd11b2</i>	GGGGTCAAAGTTTCCACTA (Fw)	Tokarz et. al., 2013
	TGGAAGAGCTCCTGGTCTC (Rv)	



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Data Availability Statement: The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher. The data are available from the author, M.S.R., upon request.

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References

1. Tobi, E. W.; Slieker, R. C.; Luijk, R.; Dekkers, K. F.; Stein, A. D.; Xu, K. M. DNA methylation as a mediator of the association between prenatal adversity and risk factors for metabolic disease in adulthood. *Sci Adv.* **2018**, *4*, eaao4364, 1-10.
2. Levine, S. Infantile experience and resistance to physiological stress. *Science.* **1957**, *126* (3270), 405-405.
3. Joseph, D.; Whirledge, S. Stress and the HPA axis: Balancing homeostasis and fertility. *Int J Mol Sci.* **2017**, *18*, 2224.
4. Brunton, P. J.; Russell, J. A. Prenatal social stress in the rat programs neuroendocrine and behavioral responses to stress in the adult offspring: Sex-specific effects. *J Neuroendocrinol.* **2010**, *22*, 258-71.
5. Dreiling, M.; Schiffner, R.; Bischoff, S.; Rupprecht, S.; Kroegel, N.; Schubert, H.; Rakers, F. Impact of chronic maternal stress during early gestation on maternal-fetal stress transfer and fetal stress sensitivity in sheep. *Stress.* **2017**, *21*, 1-10.
6. D'Agostino, S.; Testa, M.; Aliperti, V.; Venditti, M.; Minucci, S.; Aniello, F.; Donizetti, A. Expression pattern dysregulation of stress- and neuronal activity-related genes in response to prenatal stress paradigm in zebrafish larvae. *Cell Stress Chaperones.* **2019**, *24*, 1005-1012.
7. Entringer, S. Impact of stress and stress physiology during pregnancy on child metabolic function and obesity risk. *Curr Opin Clin Nutr Metab Care.* **2013**, *16*, 320-327.
8. Coulon, M.; Wellman, C. L.; Marjara, I. S.; Janczak, A. M.; Zanella, A. J. Early adverse experience alters dendritic spine density and gene expression in prefrontal cortex and hippocampus in lambs. *Psychoneuroendocrinology.* **2013**, *38*, 1112-1121.
9. Babenko, O.; Kovalchuk, I.; Metz, G. A. S. Stress-induced perinatal and transgenerational epigenetic programming of brain development and mental health. *Neurosci Biobehav Rev.* **2015**, *48*, 70-91.
10. Hirst, J. J.; Cumberland, A. L.; Shaw, J. C.; Bennett, G. A.; Kelleher, M. A.; Walker, D. W.; Palliser, H. K. Loss of neurosteroid-mediated protection following stress during fetal life. *J Steroid Biochem Mol Biol.* **2016**, *160*, 181-8.
11. Veru, F.; Laplante, D. P.; Luheshi, G.; King, S. Prenatal maternal stress exposure and immune function in the offspring. *Stress.* **2014**, *17*, 133-148.
12. Marques, A. H.; Bjørke-Monsen, A.-L.; Teixeira, A. L.; Silverman, M. N. Maternal stress, nutrition and physical activity: Impact on immune function, CNS development, and psychopathology. *Brain Res.* **2015**, *1617*, 28-46.
13. Nesan, D.; Vijayan, M.M. Embryo exposure to elevated cortisol level leads to cardiac performance dysfunction in zebrafish. *Mol Cell Endocrinol.* **2012**, *363*, 85-91.
14. Haussmann, M. F.; Longenecker, A. S.; Marchetto, N. M.; Juliano, S. A.; Bowden, R. M. Embryonic exposure to corticosterone modifies the juvenile stress response, oxidative stress and telomere length. *Proc Biol Sci.* **2011**, *279*, 1447-1456.
15. De Fraipont, M.; Clobert, J.; John-Alder, H.; Meylan, S. Increased pre-natal maternal corticosterone promotes philopatry of offspring in common lizards *Lacerta vivipara*. *Journal of Animal Ecology.* **2000**, *69*, 404-4013.



16. Tissier, M. L.; Williams, T. D.; Criscuolo, F. Maternal effects underlie ageing costs of growth in the zebra finch (*Taeniopygia guttata*). *PLoS ONE*. **2014**, 9, e97705.
17. Faught, E.; Best, C.; Vijayan, M. M. Maternal stress-associated cortisol stimulation may protect embryos from cortisol excess in zebrafish. *R Soc Open Sci*. **2016**, 24, 160032.
18. Faught, E.; Vijayan, M. M. The mineralocorticoid receptor is essential for stress axis regulation in zebrafish larvae. *Sci Rep*. **2018**, 8, 18081.
19. Yoshioka, E. T.O.; Mariano, W.S.; Santos, L.R.B. Estresse em peixes cultivados: Agravantes e atenuantes para o manejo rentável. In *Manejo e sanidade de peixes em cultivo*, 1st ed.; Tavares-Dias, M; Macapá: Embrapa Amapá, Brasil, 2009; pp. 226-247.
20. Leatherland, J. F.; Li, M.; BARKATAKI, S. Stressors, glucocorticoids and ovarian function in teleosts. *J of Fish Biol*. **2010**, 76, 86-111.
21. Nesan, D.; Vijayan, M. M. Role of glucocorticoid in developmental programming: Evidence from zebrafish. *Gen Comp Endocrinol*. **2013**, 181, 35-44.
22. Alsop, D.; Vijayan, M. M. Development of the corticosteroid stress axis and receptor expression in zebrafish. *Am J Physiol Regul Integr Comp Physiol*. **2008**, 294, R711-9.
23. Nesan, D.; Vijayan, M. M. Maternal cortisol mediates hypothalamus-pituitary-interrenal axis development in zebrafish. *Sci Rep*. **2016**, 6, 22582.
24. Johnston, I. A.; Bower, N. I.; Macqueen, D. J. Growth and the regulation of myotome muscle mass in teleost fish. *J Exp Biol*. **2011**, 214, 1617-28.
25. Keenan, S.; Currie, P. The developmental phases of zebrafish myogenesis. *J Dev Biol*. **2019**, 7, 12.
26. Hinitz, Y.; Osborn, D. P. S.; Hughes, S. M. Differential requirements for myogenic regulatory factors distinguish medial and lateral somitic, cranial and fin muscle fibre populations. *Development*. **2009**, 136, 403-14.
27. Pikulkaew, S.; Benato, F.; Celeghin, A.; Zucal, C.; Skobo, T.; Colombo, L.; Valle, L. D. The knockdown of maternal glucocorticoid receptor mRNA alters embryo development in zebrafish. *Dev Dyn*. **2011**, 240, 874-89.
28. Alderman, S.L.; McGuire, A.; Bernier, N.J.; Vijayan, M.M. Central and peripheral glucocorticoid receptors are involved in the plasma cortisol response to an acute stressor in rainbow trout. *Gen Comp Endocrinol*. **2012**, 176, 79-85.
29. Tsang, B.; Zahid, H.; Ansari, R.; Lee, R.C. Partap, A.; Gerlai, R. Breeding zebrafish: A review of different methods and a discussion on standardization. *Zebrafish*. **2017**, 14, 561-573.
30. Nóbrega R.H.; Greebe C.D.; Van De Kant H.; Bogerd J.; França L.R.; Schulz R.W. Spermatogonial stem cell niche and spermatogonial stem cell transplantation in zebrafish. *PLoS One*. **2010**, 5, e12808.
31. Tovo-Neto, A.; Martinez, Emanuel R. M.; Melo, A.G.; Doretto, L. B.; Butzge, A. J.; Rodrigues, M. S.; Nakajima, R.T.; Habibi, H. R.; Nóbrega, R.H. Cortisol directly stimulates spermatogonial differentiation, meiosis, and spermiogenesis in zebrafish (*Danio rerio*) testicular explants. *Biomolecules*. **2020**, 10, 429.
32. Figueiredo, M.A.; Mareco, E.A.; Silva, M.D.P.; Marins, L.F. Muscle-specific growth hormone receptor (GHR) overexpression induces hyperplasia but not hypertrophy in transgenic zebrafish. *Transgenic Res*. **2012**, 21, 457-69.
33. Sopinka, N. M.; Capelle, P. M.; Semeniuk, C. A. D.; Love, O. P. Glucocorticoids in fish eggs: variation, interactions with the environment, and the potential to shape offspring fitness. *Physiol Biochem Zool*. **2017**, 90, 15-33.
34. Glickman, N.; Yelon, D. Cardiac development in zebrafish: coordination of form and function. *Semin Cell Dev Biol*. **2002**, 13, 507-13.
35. Wood, A. W.; Duan, C.; Bern, H.A. Insulin-like growth factor signaling in fish. *Int Rev Cytol*. **2005**, 243, 215-85.
36. Nóbrega, R.H.; Morais, R.D.V.S.; Crespo, D.; De Waal, P.P.; França, L.R.; Schulz, R.W.; Bogerd, J. Fsh stimulates spermatogonial proliferation and differentiation in zebrafish via Igf3. *Endocrinology*. **2015**, 156, 3804-17.
37. Wilson, K. S.; Matrone, G.; Livingstone, D. E. W.; Al-Dujaili, E. A. S.; Mullins, J. J.; Tucker, C.S.; Hadoke, P. W. F.; Kenyon, C. J.; Denvir, M. A. Physiological roles of glucocorticoids during early embryonic development of the zebrafish (*Danio rerio*). *J Physiol*. **2013**, 591, 6209-20.
38. Cleveland, B.M.; Weber, G.M.; Blemings, K.P.; Silverstein, J.T. Insulin-like growth factor-I and genetic effects on indexes of protein degradation in response to feed deprivation in rainbow trout (*Oncorhynchus mykiss*). *Am J Physiol Regul Integr Comp Physiol*. **2009**, 297, R1332-42.
39. Acosta J.; Carpio Y.; Borroto I.; Gonzálezo; Estradam.P. Myostatin gene silenced by RNAi show a zebrafish giant phenotype. *J Biotechnol*. **2005**, 119, 324-31.
40. Lee, C.; Hu, S.; Gong, H.; Chen, M.; Lu, J.; Wu, J. Suppression of myostatin with vector-based RNA interference causes a double-muscle effect in transgenic zebrafish. *Biochem Biophys Res Commun*. **2009**, 387, 766-71.
41. Maccatrozzo, L.; Bargelloni, L. ; Cardazzo, B.; Rizzo, G.; Patarnello, T. A novel second myostatin gene is present in teleost fish. *FEBS Lett*. **2001**, 509, 36-40.



42. Amali, A.A.; Lin, C. J.-F.; Chen, Y.-H.; Wang, W.-L.; Gong, H.-Y.; Lee, C.-Y.; Wu, J.-L. Up-regulation of muscle-specific transcription factors during embryonic somitogenesis of zebrafish (*Danio rerio*) by knock-down of myostatin-1. *Dev Dyn.* **2004**, 229, 847-56.
43. Gao, Y.; Dai, Z.; Shi, C.; Zhai, G.; Jin, X.; He, J.; Lou, Q.; Yin Z. Depletion of myostatin b promotes somatic growth and lipid metabolism in zebrafish. *Front Endocrinol.* **2016**, 10, 332.
44. Wilson, K. S.; Matrone, G.; Livingstone, D. E. W.; Al-Dujaili, E. A. S.; Mullins, J. J.; Tucker, C.S.; Hadoke, P. W. F.; Kenyon, C. J.; Denvir, M. A. Physiological roles of glucocorticoids during early embryonic development of the zebrafish (*Danio rerio*). *J Physiol.* **2013**, 591, 6209-20.
45. Oakes, J.A.; Li, N.; Wistow, B.R.C.; Griffin, A.; Barnard, L.; Storbeck, K.-H.; Cunliffe, V.T.; Krone, N.P. Ferredoxin 1b deficiency leads to testis disorganization, impaired spermatogenesis, and feminization in zebrafish. *Endocrinology.* **2019**, 160, 2401-2416.
46. Keenan, S.; Currie, P. The developmental phases of zebrafish myogenesis. *J Dev Biol.* **2019**, 7, 12.
47. Nguyen, P.D.; Gurevich, D.B.; Sonntag, C.; Hersey, L.; Alaei, S.; Nim, H.T.; Siegel, A.; Hall, T.E.; Rossello, F.J.; Boyd, S.E. Muscle stem cells undergo extensive clonal drift during tissue growth via meox1-mediated induction of G2 cell-cycle arrest. *Cell Stem Cell.* **2017**, 21, 107-119.



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General discussion



The role of thyroid hormones (THs) in testicular development is well-documented in mammals; however, their function in fish remains less explored. There are a few papers describing the impact of THs status on the male reproductive system (Matta et al., 2002; Swapna et al., 2006; Morais et al., 2013), and its influence on the hypothalamic-pituitary-gonadal (HPG) axis (Matta et al., 2002; Morais et al., 2013; Rodrigues et al., 2022).

Here, we investigated the role of THs in male zebrafish reproduction adopting *in vivo* and *ex vivo* approaches. Initially, a hypothyroid zebrafish model was generated using methimazole (Chapter 1). This compound inhibits thyroid peroxidase (TPO) activity, thereby preventing the conversion of iodide to iodine necessary for synthesizing T4 and T3 (Heidari et al., 2015). Although it was not possible to completely rule out or monitor potential off-target effects caused by the methimazole drug. However, the findings demonstrated that the observed alterations in zebrafish spermatogenesis were primarily attributable to THs deficiency.

After 21 days of treatment, the altered THs status induced histomorphometrical alterations in the proportion of germ cell cysts compared to euthyroid animals. There was a significant accumulation of pre-meiotic cells (type A undifferentiated and differentiated spermatogonia), meiotic arrest, and a dramatic reduction in spermatozoa number. Interestingly, these effects were reversed when animals were co-treated with T4 in the last week of methimazole exposure. Here, we demonstrated effectively that fluctuations in THs levels in adult male zebrafish disrupt the progression of spermatogenesis. Our findings highlight the critical role of THs in regulating spermatogenesis in fish. However, whether these effects are mediated directly by THs or indirectly through interactions with other pathways remains to be determined. Further research is needed to elucidate the interplay between THs and other components of the endocrine system that governs fish reproduction.

The transcripts analysis of zebrafish testis aligns, to some extent, with histomorphometrical findings. The results indicated that the methimazole-treated group exhibited elevated mRNA levels of *nanos2*, a marker of type A undifferentiated spermatogonia (Aoki et al., 2009). This upregulation corresponds with the histomorphometric data, which revealed an increase in type A undifferentiated spermatogonia. These findings suggest that the euthyroid state is crucial for the normal development of spermatogonial cells in zebrafish.

Reduced THs levels induced by methimazole in male zebrafish were associated with a significant decrease in plasma 11-ketotestosterone (11-KT) levels. Similarly, Chiao and colleagues (2000) reported reduced serum testosterone concentrations in rats following propylthiouracil (PTU) treatment. In *Clarias gariepinus*, exposure to thiourea reduced



androgen levels and subsequently testicular regression (Swapna et al., 2006; Swapna & Senthikumar, 2007). Notably, in our study, methimazole-induced hypothyroid zebrafish co-treated with T4 effectively rescued plasma 11-KT levels, correlating with the recovery of spermatozoa number in the testis. These findings suggest that transient disruption of thyroid function, within physiological limits that allow for recovery, provides a useful model to investigate the role of THs in male zebrafish reproduction.

Interestingly, *fshr* mRNA levels were upregulated in hypothyroid zebrafish co-treated with T4, indicating a potential alteration in THs sensitivity under thyroid status. The increase in *fshr* transcripts following T4 co-treatment could be attributed to the proliferation of Sertoli cells, as demonstrated by Moraes and colleagues (2013), who showed that T3 stimulates Sertoli cell proliferation *ex vivo* and enhances follicle-stimulating hormone (Fsh) actions in zebrafish testis. These observations support the hypothesis that THs and gonadotropins synergistically stimulate spermatogenesis by promoting gonadal androgen production, which is essential for initiating and maintaining spermatogenesis. Further studies are warranted to elucidate the precise mechanisms underlying the interactions between THs and gonadotropins in zebrafish reproduction by exploring molecular approaches, such as the roles of non-coding RNAs (lncRNAs) and microRNAs, which may provide insights into the regulatory network involved in this complex endocrine interplay.

To further investigate the impact of THs on the regulatory components of the HPG axis, we analyzed two experimental scenarios: long-term hypothyroid treatment, where animals were exposed to methimazole for three weeks, and an acute treatment involving a single intracelomic injection of T3 in euthyroid animals, assessed 12 hours post-injection. In hypothyroid animals, *gnih* transcript levels were significantly elevated in the brain. Gonadotropin-inhibitory hormone (GnIH) is known to suppress gonadotropin secretion, leading to reduced plasma levels and impaired reproductive function (Tsutsui et al., 2017). In fish, GnIH orthologs have been reported to exhibit both stimulatory and inhibitory effects on gonadotropin secretion (Amano et al., 2006; Branco et al., 2018; Ma et al., 2020). Specially, in cyprinids, zebrafish GnIH has been shown to decrease plasma gonadotropin levels in goldfish (Zhang et al., 2010). Similarly, in female mice, hypothyroidism induced by PTU increased GnIH expression while reducing gonadotropin and plasma steroid levels (Tsutsui et al., 2017). This evidence supports the hypothesis that the reduction in plasma androgen levels observed in methimazole-treated zebrafish may be mediated by elevated GnIH expression.



Interestingly, while GnIH appears to inhibit reproduction at the protein level, transcript levels of *fshb* were elevated in the pituitary of the methimazole-treated fish, likely as part of a compensatory feedback mechanism. This compensatory effect was further reflected in the acute T3 treatment, where *gnrh3* mRNA levels were upregulated, stimulating the HPG axis. However, this activation was followed by a significant reduction in *lh* mRNA levels, indicative of negative feedback regulation. Similar findings have been reported in goldfish, where T3 injection after 12 hours reduced gonadotropin levels and gonadal steroid synthesis (Nelson et al., 2010).

These results demonstrate that fluctuations in TH levels, whether through long-term hypothyroidism or acute hyperthyroidism conditions, profoundly affect the HPG axis. Collectively, these findings highlight the intricate interactions between the thyroid and reproductive axes, though the distinct regulatory mechanisms underlying these interactions remain to be fully elucidated. Further studies are required to clarify the molecular and functional interplay between these endocrine systems.

Maternal reproduction health was also adversely affected in animals exposed to elevated concentrations of T4. To investigate the impact of maternal THs on the development of the somatotrophic axis in offspring, female zebrafish were fed commercial food containing 0.5 mg of T4 per feed, equivalent to approximately 25 µg of T4 per gram of body weight, for five days, followed by breeding on day six (Chapter 2). The offspring were evaluated at different developmental stages from 0 to 144 -hpf. This study provides critical insights into aquaculture by unraveling the complex interplay between maternal THs and the somatotrophic axis in zebrafish offspring. Our findings reveal that maternal exposure to exogenous T4 disrupts T4 homeostasis, leading to altered maternal plasma T3 levels and changes in TH content in F1 eggs. These disruptions were associated with abnormal plasma T3 levels during offspring development. The elevated maternal circulating T3 levels are probably due to the partial conversion of T4 to T3, catalyzed by deiodinase enzymes DIO1 and DIO2. These suggest that maternal hyperthyroidism negatively impacts the role of THs in regulating offspring development, with potential consequences for growth and somatic regulation. Such findings highlight the importance of maintaining TH homeostasis in broodstock to optimize reproductive and developmental outcomes in aquaculture systems.

To investigate the effects of maternal THs on zebrafish offspring, our study examined the expression of key genes involved in the thyroid and somatotrophic axes during early development. Our findings revealed significant changes in thyroid-related gene expression.



Maternal THs stimulated the offspring's transcript levels of *crf*, *tsh*, *thra*, and *thrβ*. However, at the highest maternal T4 concentrations, the activation of the HPT axis was abnormal, likely due to overstimulation by maternal THs. Additionally, low T3 levels at 0 -hpf in offspring from mothers with elevated THs may have up-regulated the expression of the TH transporter, *mct8*. Alterations were also observed in the expression of type 2 and type 3 deiodinases, which play critical roles in the activation and inactivation of THs. These maternal contributions are crucial for the development of the brain, eyes, ears, and swim bladder (Walpita et al., 2009; Houbrechts et al., 2016; Van Dingenen et al., 2023). Inhibition of deiodinase activity in zebrafish embryos has been shown to impair swim bladder development (Van Dingenen et al., 2023). Consistent with these findings, our results showed a higher percentage of uninflated swim bladders in embryos from mothers exposed to elevated THs. Taken together, this evidence indicates that adequate levels of THs in both parents and embryos are necessary for successful embryogenesis and developmental outcomes.

Disruptions in the somatotrophic axis within muscle tissue can significantly contribute to growth retardation in offspring (Forhead & Fowden, 2006). In zebrafish offspring from females exposed to elevated T4 levels, noticeable delays in growth and development were observed compared to the control group. These developmental delays were accompanied by altered gene expression and morphological abnormalities, including scoliosis, a condition characterized by spinal curvature. Our results revealed that offspring from females exposed to high concentrations of T4 exhibited reduced expression of *gh* mRNA, which correlated with their smaller size. Interestingly, *igf1* transcript levels were up-regulated, likely as a compensatory response to the decreased *gh* expression. Overall, the expression profile of key genes in the somatotrophic axis was significantly altered, indicating that excessive maternal T4 exposure disrupts normal growth regulation and musculoskeletal development in zebrafish offspring.

Manipulation of maternal THs can lead to a persistent imbalance in myogenic regulatory factors in offspring post-fertilization. In addition to the clear changes in external morphology induced by elevated maternal THs, other tissues, such as the heart, were highly responsive to TH treatment. Embryos at 48 -hpf exhibited increased sensitivity, with elevated maternal THs leading to tachycardia and pericardial edema. However, preliminary observations revealed a decline in heart rate beyond 48 -hpf, culminating in mortality, with embryos failing to survive past 144 -hpf. This temporal pattern suggests that fluctuations in THs levels during development may disrupt physiological processes.



The observed effects could be attributed to imbalances in TH-regulated pathways, including deficiencies or excess in deiodinase enzymes, TH receptors, and transporters, which are critical for endogenous hormone production during normal development. Vargas-Uricoechea and colleagues (2014) emphasized the significant role of THs in cardiac function, supporting our findings of impaired heart performance in embryos exposed to abnormal maternal TH levels. Our findings also highlight that the effects of THs on eggs and larvae are likely dose- and species-dependent. Further research is required to elucidate the interplay between maternal THs and the somatotrophic axis in offspring, as well as the potential transgenerational impacts of altered maternal THs in the long term. The role of microRNAs in oogenesis, fertility, and early development in fish offspring remains poorly understood. Investigating non-coding RNAs, such as long non-coding RNAs and microRNAs offers a promising field to uncover the molecular pathways underlying these processes. This approach could provide valuable insights into the maternal transfer of molecules and hormones, as well as the intergenerational and transgenerational roles of THs in fish development.

Another important subject to be mentioned is that maternal hormonal alterations can be further aggravated by environmental pollutants that disrupt endocrine function, potentially impacting hormonal regulation, offspring development, and survival. Endocrine-disrupting chemicals (EDCs) are among the key environmental contaminants known to interfere with hormonal homeostasis. These disruptions may lead to reproductive impairments in fish populations, posing a significant threat to their sustainability. Therefore, this understanding will provide critical insights into how THs and other hormonal factors influence fish embryogenesis and development in different environmental conditions.

Finally, in chapter 3, we investigated the role of maternal cortisol in the regulation of the somatotrophic axis during the larval and juvenile stages of zebrafish. Excess cortisol can be transferred and deposited in the oocytes of maturing females and affect the embryonic developmental program (Faught et al., 2016). Similar to chapter 2, females were fed a cortisol-enriched diet for five days to mimic maternal stress, and on the third day, they were bred with untreated males. Offspring were collected at multiple developmental stages (0 to 144 -hpf) and analyzed for phenotype traits, survival rate, and gene expression. Our results showed that cortisol exposure influenced offspring development, leading to increased mortality rates, and higher morphological abnormalities, particularly pericardial edema. Additionally, gene expression analysis revealed alterations in transcripts related to the somatotrophic axis, including genes involved in protein synthesis, and early growth acceleration. By 30 days post-



fertilization, juveniles from cortisol-treated females exhibited a significant increase in muscle bundle size and cross-sectional diameter compared to the control group. These findings provide valuable insights into the complex interaction between maternal stress factors and the regulation of the somatotrophic axis in offspring development.

In general, our study elucidated insights into the role of THs in spermatogenesis and regulating the reproductive axis in males and highlighted the first observations about their impacts on offspring development through maternal exposure to exogenous THs. Also, although THs and cortisol share corticotropin-releasing factor/hormone (Crh) as a common hypothalamic regulator stimulating the release of thyroid-stimulating hormone (Tsh) and adrenocorticotrophic hormone (Acth) from the pituitary to promote THs and cortisol synthesis, respectively, we observed opposing effects of maternal hormones on the somatotrophic axis in zebrafish. These findings contribute significantly to the discussion on reproductive health in males and females, providing a foundation for a broader exploration of hormonal regulation across generations in fish and other vertebrates.



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Conclusions



The role of THs has been investigated *in vivo* and *ex vivo* approaches in the reproductive axis. The data shown in this study indicate the crosstalk between the HPT-HPG axes in adult male zebrafish. Additionally, we demonstrated that fluctuations in maternal THs levels as well as maternal stress can significantly impact embryonic development, particularly affecting growth and gene expression associated with the somatotrophic axis. Our findings underscore the importance of endocrine hormones in regulating both adult male reproductive and embryonic development in zebrafish. That way:

- 1) Methimazole-induced hypothyroidism significantly impairs zebrafish germ cell development by delaying differentiation, and meiosis, decreasing the number of spermatozoa as well as impairing the Hypothalamic-Pituitary axis;
- 2) Androgen plasma levels and testicular steroidogenic capacity are reduced, which might be due to gonadotropin inhibition by GnIH;
- 3) Altogether, these results reinforce the hypothesis that THs are an essential element of multifactorial control of reproduction and testicular function in zebrafish and possibly other vertebrate species;
- 4) Hyperthyroidism-mediated by high maternal doses of THs severely impaired the offspring, compromising their growth, survival, phenotype, organogenesis, and gene expression of genes related to the thyroid and somatotrophic axis;
- 5) The preliminary results may help explain the mechanisms by which chemicals interfere with the thyroid endocrine system in fish through parental exposure, especially mothers;
- 6) Altogether, maternal THs are crucial to coordinating embryo and larvae development programs related to the somatotrophic axis;
- 7) Maternal stress associated with cortisol deposition significantly impacts the survival, morphological development, and gene expression of the genes involved in protein synthesis, suggesting accelerated growth, as well as muscle tissue in juveniles;



8) Altogether, the results underscore the critical influence of maternal treatment on the regulation of the hypothalamic-pituitary axis in zebrafish.



Literature cited

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

AMANO M., MORIYAMA S., IIGO M., KITAMURA S., AMIYA N., YAMAMORI K. Novel Fish Hypothalamic Neuropeptides Stimulate the Release of Gonadotrophins and Growth Hormone from the Pituitary of Sockeye Salmon. **J. Endocrinol.** 188, 417–423. 2006.

AOKI Y., NAKAMURA S., ISHIKAWA Y., TANAKA M. Expression and Syntenic Analyses of Fournanos Genes in Medaka. **Zoological Sci.** 26, 112–118. 2009.

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

BAI, L., WU, Y., LI, G., ZHANG, W., ZHANG, H., SU, J. AI-enabled organoids : construction, analysis, and application. **Bioactive Materials**. 2014.

BALASCH, J.C.; TORT, L. Netting the Stress Responses in Fish. **Front Endocrinol (Lausanne)**. 2019.

BRANCO G. S., MELO A. G., RICCI J. M. B., DIGMAYER M., DE JESUS L. W. O., HABIBI H. R. Effects of GnRH and the Dual Regulatory Actions of GnIH in the Pituitary Explants and Brain Slices of *Astyanax Altiparanae* Males. **General Comp. Endocrinol.** 273, 209–217. 2019.

BLANCO AM. Hypothalamic– and pituitary–derived growth and reproductive hormones and the control of energy balance in fish. **Gen Comp Endocrinol.** 287:113322. 2020.

BLOISE F. F., CORDEIRO A., ORTIGA-CARVALHO T. M. Role of thyroid hormone in skeletal muscle physiology. **J. Endocrinol.** 236 R57–R68. 10.1530/JOE-16-0611. 2018.

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

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

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

CASTILLO S, BOLLFRASS K, MENDOZA R, FONTENOT Q, LAZO JP, AGUILERA C, ET Al. Stimulatory effect of thyroid hormones improves larval development and reproductive performance in alligator gar (*Atractosteus spatula*) and spotted gar (*Lepisosteus osculates*). **Aquaculture Res.** 2015.



CHAM, T-C., IBTISHAM, F., AL-DISSI, A., HONARAMOOZ, A. An in vitro testicular organoid model for the study of testis morphogenesis, somatic cell maturation, endocrine function, and toxicological assessment of endocrine disruptors. **Reproductive toxicology**. 2024.

CHIAO Y.-C., LIN H., WANG S.-W., WANG P. S. Direct Effects of Propylthiouracil on Testosterone Secretion in Rat Testicular Interstitial Cells. **Br. J. Pharmacol.** 130, 1477–1482. 2000.

CHIARADIA, I., LANCASTER, M.A. Brain organoids for the study of human neurobiology at the interface of *in vitro* and *in vivo*. **Nat Neurosci** 23, 1496–1508. 2020.

CLELLAND, E.; PENG, C. Endocrine/paracrine control of zebrafish ovarian development. **Molecular and Cellular Endocrinology**. 312:42-52. 2009.

CLEVERS, H. Modeling development and disease with organoids. **Cell** 165. 2016.

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

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

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

COSTA, D.F.; OLIVEIRA, A.R.; RICCI, J.M.B.; RODRIGUES, M.S.; OLIVEIRA, M.A.; ROSA, IVANA, F.; DORETTO, L.B.; TAKAHIRO, R.N.; NÓBREGA, R.H. A83-01 and DMH1 effects in the zebrafish spermatogonial niche: unraveling the roles of TGF- β and BMP signaling in the Fsh-mediated spermatogonial fate. **GENE**, v. X, p. 148082, 2023.

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

de ROOIJ, D.G. Proliferation and differentiation of spermatogonial stem cells. **Reproduction-Cambridge**, 121, 347–354. 2001.

de ROOIJ, D.G. Organization of the seminiferous epithelium and the cycle, and morphometric description of spermatogonial subtypes (rodents and primates) J.M. Oatley, M.D. Griswold (Eds.). **The Biology of Mammalian Spermatogonia**. 2017.

DEAL CK, VOLKOFF H. The Role of the Thyroid Axis in Fish. **Front Endocrinol (Lausanne)**. Nov 6;11:596585. 2020.



DORETTO, L. B.; BUTZGE, A. J.; NAKAJIMA, R. T.; MARTINEZ, E. R. M.; SOUZA, B. M.; RODRIGUES, M. S.; ROSA, I.F.; RICCI, J. M. B.; TOVO-NETO, A.; COSTA, D.F.; MALAFAIA, G.; SHAO, C.; NÓBREGA, R. H. Gdnf Acts as a Germ Cell-Derived Growth Factor and Regulates the Zebrafish Germ Stem Cell Niche in Autocrine- and Paracrine-Dependent Manners. **Cells**, v. 11, p. 1295, 2022.

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

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

DUMONT NA, BENTZINGER CF, SINCENNES MC & RUDNICKI MA. Satellite cells and skeletal muscle regeneration. **Comprehensive Physiology** 5 1027– 1059. 2015.

ELIZALDE-VELÁZQUEZ, G.A., HERRERA-VÁSQUEZ, S.E. Zebrafish as model organism in aquatic ecotoxicology: current trends and future perspectives. In book: Zebrafish research – An ever-Expanding Experimental Model. 2023.

FATEHULLAH, A., TAN, S.H., BARKER, N. Organoids as an in vitro model of human development and disease. **Nat Cell Biol.**18(3):246-254. 2016.

FAUGHT, E., BEST, C.; VIJAYAN, M. M. Maternal stress-associated cortisol stimulation may protect embryos from cortisol excess in zebrafish. **R. Soc. Open Sci.** 3, 160032. 2016.

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

FORHEAD, A.; FOWDEN, L. Role of thyroid hormones in the control of fetal growth and maturation. 2006.

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

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

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

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



FRANÇA, L.R., NÓBREGA, R.H., MORAIS, R.D.V.S., ASSIS, L.H.D.C., SCHULZ, R.W. Sertoli cell structure and function in anamniote vertebrates. **In Sertoli Cell Biology, Academic Press: Oxford, UK**, pp. 385–407. 2015.

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

FRETAUD, M., RIVIERE, L., JOB, E., GAY, S., LAREYRE, J-J., JOLY, J-S., AFFATICATI, P., THERMES, V. High-resolution 3D imaging of whole organ after clearing: taking a new look at the zebrafish testis. **Scientific Reports**. 7: 43012. 2017.

FRIESEMA, E.C.H; VISSER, J.J. Thyroid hormone transporters. **Biochemical society transactions**. 2005.

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

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

GÓMEZ-BOUDREAU. Organoids and Their Use in Modeling Gut Epithelial Cell Lineage Differentiation and Barrier Properties During Intestinal Diseases. *Front. Cell Dev. Biol.*, 13 August. 2021.

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

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

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

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

HABIBI, H.E., RAINE, J.C. Thyroid hormones and reproduction in fish. Chapter 5. In book: *Hormones and reproduction of vertebrates*. **Fishes**. Volume 1. 2024.



HARDY, R.W., GATLIN III, D.M. Nutritional strategies to reduce nutrient losses in intensive aquaculture. J.R. Tomasso (Ed.), *Aquaculture and the Environment in the United States*, World **Aquaculture Society**, Baton Rouge, LA, 155-165. 2002.

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

HEIDARI R., NIKNAHAD H., JAMSHIDZADEH A., EGHBAL M. A., ABDOLI N. An Overview on the Proposed Mechanisms of Antithyroid Drugs-Induced Liver Injury. **Adv. Pharm. Bull.** 2015.

HINITIS, Y., OSBORN, D. P. S., HUGHES, S. M. Differential requirements for myogenic regulatory factors distinguish medial and lateral somitic, cranial and fin muscle fibre populations. **Development**. 136(3), 403–414. 2009.

HO, B.X., PEK, N.M.Q., SOH, B.S. Disease Modeling Using 3D Organoids Derived from Human Induced Pluripotent Stem Cells. **Int J Mol Sci**. 2018.

HOUBRECHTS, A.M.; VERGAUWEN, L.; BAGCI, E., VAN HOUCKE, J.; HEIJLEN, M.; KULEMAKA, B.; HYDE, R.D.; KNAPEN, D.; DARRAS, V.M. Deiodinase knockdown affects zebrafish eye development at the level of gene expression, morphology, and function. **Molecular and Cellular Endocrinology**, vol. 424, 81-93. 2016.

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

HUA-MING, X., YI-JIE., R., FA, R., YU, L., TIAN-YU, F., ZHI, W., YE-QING, Y., LI-KUN, Z., JIAN-HONG, H. Recent advances in isolation, identification, and culture of mammalian spermatogonial stem cells. **Asian Journal of Andrology**. 2022.

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

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

IBTISHAM, F., HONARAMOOZ, A. Spermatogonial Stem Cells for In Vitro Spermatogenesis and In Vivo Restoration of Fertility. **Cells**. 2020.

JI X., LIU H., CHEN S., JIANG Y. & TIAN Y. Growth differences and dimorphic expression of growth hormone (GH) in female and male *Cynoglossus semilaevis* after male sexual maturation. **Marine Genomics**, 4, 9–16. 2011.

JOHNSTON, I. A., BOWER, N. I., MACQUEEN, D. J. Growth and the regulation of myotomal muscle mass in teleost fish. **Journal of Experimental Biology**, 214(10), 1617–1628. 2011.

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



KANATSU-SHINOHARA, M., OGONUKI, N., INOUE, K., MIKI, H., OGURA, A., TOYOKUNI, S., SHINOHARA, T. Long-term proliferation in culture and germline transmission of mouse male germline stem cells. **Biol Reprod** 69(2):612–616. 2003.

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

KIMMEL, C.B.; BALLARD, W.W.; KIMMEL, S.R.; ULLMANN, B.; SCHILLING, T.F. Stages of embryonic development of the zebrafish. **Dev Dyn.** 203(3):253-310. 1995.

KIRBY JD, MANKAR MV, HARDESTY D, KREIDER DL. Effects of transient prepubertal 6-N-propyl-2-thiouracil treatment on testis development and functional in the domestic fowl. **Biology of Reproduction**; 55(4):910-916. 1996.

KOGANTI, P.; YAO, J.; CLEVELAND, B.M. Molecular Mechanisms Regulating Muscle Plasticity in Fish. **Animals**.11, 61. 2021.

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

LAM, T.J. Hormones and egg/larval quality in fish. **J. World Aquac. Soc.** 25: 2-12. 1994.

LANDINES MA, SANABRIA AI, SENHORINI JA, URBINATI EC. The influence of triiodothyronine (T3) on the early development of piracanjuba (*Brycon orbignyanus*). **Fish Physiol Biochem.** 2010.

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

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

LEE, M.T., BONNEAU, A.R., TAKACS, C.M., BAZZINI, A.A., DIVITO, K.R., FLEMING, E.S., GIRALDEZ, A.J. Nanog, Pou5f1 and SoxB1 activate zygotic gene expression during maternal-to-zygotic transition. **Nature.** 2013.

LEE, M.T., BONNEAU, A.R., GIRALDEZ, A.J. Zygotic genome activation during the maternal-to-zygotic transition. **Ann. Rev. Cell Dev. Biol.** 30, 581–613. 2014.

LUBZENS, E., BOBE, J., YOUNG, G., CERDÀ, J. Oogenesis in teleosts: How fish eggs are formed. **General and Comparative Endocrinology.** 2010.



LUBZENS, E., BOBE, J., YOUNG, G., SULLIVAN, C.V. Maternal investment in fish oocytes and eggs: The molecular cargo and its contributions to fertility and early development. **Aquaculture**. 2017.

MA Y., LADISA C., CHANG J. P., HABIBI H. R. Multifactorial Control of Reproductive and Growth axis in Male Goldfish: Influences of GnRH, GnIH and Thyroid Hormone. **Mol. Cell. Endocrinol.** 500, 110629. 2020.

MACLATCHY DL, EALES JG. Intra- and extra-cellular sources of T3 binding to putative thyroid hormone receptors in liver, kidney, and gill nuclei of immature rainbow trout, *Oncorhynchus mykiss*. **J Exp Zool.** 1992.

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

MARELLI F, PERSANI, L. How zebrafish research has helped in understanding thyroid diseases. **Research**. 2017.

MAOUCHE, A., CURRAN, E., GOUPIL, AS., SAMBRONI, E., BELLAICHE, J., LE GAC, F., LAREYRE, J.J. New insights into the evolution, hormonal regulation, and spatiotemporal expression profiles of genes involved in the Gfra1/Gdnf and Kit/Kitlg regulatory pathways in rainbow trout testis. **Fish Physiol Biochem** 44, 1599–1616. 2018.

MARSILI, A., AGUAYO-MAZZUCATO, C., CHEN, T., KUMAR, A., CHUNG, M., LUNSFORD, E.P., HARNEY, J.W., VAN-TRAN, T., GIANETTI, E., RAMADAN, W., CHOU, C., BONNER-WEIR, S., LARSEN, P.R., SILVA, J.E., ZAVACKI, A.M. Mice with a targeted deletion of the type 2 deiodinase are insulin resistant and susceptible to diet-induced obesity. **PLoS One** 6, e20832. 2011.

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

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

MERONI, S.B; GALARDO, M.N.; RINDONE, G.; GORGA, A.; RIERA, M.F.; CIGORRAGA, S.B. Molecular Mechanisms and signaling pathways involved in Sertoli cell proliferation. **Frontiers in endocrinology**. 2019.

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

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



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

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

MORAIS R. D. V. S., NÓBREGA R. H., GÓMEZ-GONZÁLEZ N. E., SCHMIDT R., BOGERD J., FRANÇA L. R. Thyroid Hormone Stimulates the Proliferation of Sertoli Cells and Single Type A Spermatogonia in Adult Zebrafish (*Danio rerio*) Testis. **Endocrinology** 154, 4365–4376.2013.

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

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

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

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

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

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

NESAN, D., VIJAYAN M. M. Role of glucocorticoid in developmental programming: evidence from zebrafish. **Gen. Comp. Endocrinol**.181, 35–44. 2013.

NESAN, D., VIJAYAN, M. M. Maternal cortisol mediates hypothalamus-pituitary- interrenal axis development in zebrafish. **Scientific Reports**, 6, 22582. 2016.

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

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

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



NÓBREGA, R.H; LAREYRE, J-J. Testicular function and hormonal regulation in fishes. In Hormones and reproduction of vertebrates. **Fishes**. Volume 1. Second edition. Chapter 3. 2024.

ONICHTCHOUK, D. Pou5f1/oct4 in pluripotency control: insights from zebrafish. **Genesis**. 2012.

ORTIGA-CARVALHO, T.M, CHIAMOLERA, M.I, PAZOS-MOURA, C.C & WONDISFORD, F.E Hypothalamus-pituitary-thyroid axis. **Comprehensive Physiology** 6 1387–1428. 2016.

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

OKUDA, Y., OGURA, E., KONDOH, H. & KAMACHI, Y. B1 SOX coordinate cell specification with patterning and morphogenesis in the early zebrafish embryo. **PLoS Genet**. 2010.

OKUZAWA, K. Puberty in teleost. **Fish Physiology and Biochemistry** 26, 31–41. 2002.

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

PANDERGRAFT, S.S., SADRI-ARDEKANI, H., ATALA, A., BISHOP, C.E. Three-dimensional testicular organoid: a novel tool for the study of human spermatogenesis and gonadotoxicity *in vitro*. **Biol Reprod**. 96(3):720-732. 2017.

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

PIERCE, A. L., SHIMIZU, M., BECKMAN, B. R., BAKER, D. M., & DICKHOFF, W. W. Time course of the GH/IGF axis response to fasting and increased ration in chinook salmon (*Oncorhynchus tshawytscha*). **General and Comparative Endocrinology**, 140(3), 192–202. doi:10.1016/j.ygcen.2004.10.017. 2005.

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

PETER, M.C.S. The role of thyroid hormones in stress response of fish. **Gen Comp Endocrinol**. 172:198–210. 10.1016/j.ygcen.2011.02.023.2011.

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

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



RODRIGUES, M. S., HAMIDEH P. FALLAH, MAYA ZANARDINI, GUILHERME MALAFAIA, HAMID R. HABIBI, RAFAEL H. NÓBREGA. Interaction between thyroid hormones and gonadotropin inhibitory hormones in ex vivo culture of zebrafish testis: An approach to study multifactorial control of spermatogenesis. **Molecular and Cellular Endocrinology**, Volume 532, 111331, ISSN 0303-7207. doi: 10.1016/j.mce.2021.111331. 2021.

RODRIGUES, M.S., TOVO-NETO, A., ROSA, I.F., DORETTO, L. B., FALLAH, H.P., HABIBI, H.R., NÓBREGA, R.H. Thyroid Hormones Deficiency Impairs Male Germ Cell Development: A Cross Talk Between Hypothalamic-Pituitary-Thyroid, and—Gonadal Axes in Zebrafish. **Front. Cell Dev. Biol. Sec. Molecular and Cellular Pathology**. 2022.

ROMERO-GUEVARA, R., LOANNIDES, A., XINARIS, C. Kidney Organoids as Disease Models: Strengths, Weaknesses, and Perspectives. **Frontiers**. 2020.

SALVATORE, D, SIMONIDES, WS, DENTICE, M, ZAVACKI, A.M, LARSEN PR. Thyroid hormones and skeletal muscle--new insights and potential implications. **Nat Rev Endocrinol**. Apr;10(4):206-14. 2014.

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

SAKIB, S., GOLDSMITH, T., VOIGT, A., & DOBRINSKI, I. Testicular organoids to study cell-cell interactions in the mammalian testis. **Andrology**. 2019.

SAKIB, S., LARA, N.L.M., HUYNH, B.C., DOBRINSKI, I. Organotypic Rat Testicular Organoids for the Study of Testicular Maturation and Toxicology. **Front. Endocrinol. Reproduction**. 2022.

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

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

SCHMID, A.C., REINECKE, M., LUTZ, I., KLOAS, W. Thyroid hormone stimulates hepatic IGF-I mRNA expression in a bony fish, the tilapia *Oreochromis mossambicus*, *in vitro* and *in vivo*. **Gen. Com. Endocrinol**. 130, 129–134. 2003.

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



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

SIMONIDES, W. S. & VAN HARDEVELD, C. Thyroid hormone as a determinant of metabolic and contractile phenotype of skeletal muscle. **Thyroid** 18, 205–216. 2008.

SELMAN, K., WALLACE, R.A., SARKA, A., QI, X., Stages of oocyte development in the zebrafish, *Brachydanio rerio*. **J. Morphol.** 218, 203–224. 1993.

SOUZA, V. V.; MOREIRA, D. P.; BRAZ-MOTA, S.; SANTOS, W. V.; COTTA, G. C.; RODRIGUES, M.S.; NÓBREGA, R. H.; CORRÊA, R. D. S.; MELO HOYOS, D. C.; SANCHE, E.A.; VAL, A. L.; SANTOS NASSIF LACERDA, S. M. Simulated climate change and atrazine contamination can synergistically impair zebrafish testicular function. **Science of the Total Environment**, v. 1, p. 174173, 2024.

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

STRANGE, D.P., ZARANDI, N.P., TRIVEDI, G., ATALA, A., BISHOP, C.E., SADRI-ARDKANI, H. & VERMA, S. Human testicular organoid system as a novel tool to study Zika virus pathogenesis. **Emerging Microbes and Infections** 7 82. 2018.

SUBRAMANYAM, D. ET AL. Multiple targets of miR-302 and miR-372 promote reprogramming of human fibroblasts to induced pluripotent stem cells. **Nature Biotechnol.** 2011.

SWAPNA I., RAJASEKHAR M., SUPRIYA A., RAGHUVeer K., SREENIVASULU G., RASHEEDA M. K. Thiourea-induced Thyroid Hormone Depletion Impairs Testicular Recrudescence in the Air-Breathing Catfish, *Clarias gariepinus*. **Comp. Biochem. Physiology Part A Mol. Integr. Physiology** 144, 1–10. 2006.

SWAPNA I., SENTHILKUMARAN B. Thyroid Hormones Modulate the Hypothalamo-Hypophyseal-Gonadal axis in Teleosts: Molecular Insights. **Fish. Physiol. Biochem.** 33, 335–345. 2007.

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

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

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



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

URBINATI, E. C., L. H. VASQUES, J. A. SENHORINI, V. L. SOUZA, AND F. D. GONCALVES. Larval performance of matrinxã, *Brycon amazonicus* (Spix & Agawssiz 1829), after maternal triiodothyronine injection or egg immersion. **Aquacult. Res.**, 39: 1355–1359. 2008.

VAN DER VAART, J., BOSMANS, L., SIJBESMA, S.F., KNOOPS, K., VAN DE WETERING, W. J., OTTEN, H. G., BEGTHEL, H., RINKES, I. H. M. B., KORVING, J., LENTJES, E. G. W. M., LOPEZ-IGLESIAS, C., PETERS, P. J., VAN SANTEN, H.M., VRIENS, M.R., CLEVERS, H. Adult mouse and human organoids derived from thyroid follicular cells and modeling of Graves' hyperthyroidism. **PNAS**. 2021.

VAN DINGENEN, I.; VERGAUWEN, L.; HAIGIS, A-C.; BLACKWELL, B.R.; STACY, E.; VILLENEUVE, D.L.; KNAPEN, D. Deiodinase inhibition impairs the formation of the three posterior swim bladder tissue layers during early embryonic development in zebrafish. **Aquatic Toxicology**. 106632. 2023.

VARGAS-URICOECHEA, H.; BONELO-PERDOMO, A.; SIERRA-TORRES, C.H. Effects of thyroid hormones on the heart. 26(6):296-309. **Arteriosclerosis**. 2014.

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

VERGAUWEN L, CAVALLIN JE, ANKLEY GT, BARS C, GABRIËLS IJ, MICHIELS EDG, FITZPATRICK KR, PERIZ-STANACEV J, RANDOLPH EC, ROBINSON SL, SAARI TW, SCHROEDER AL, STINCKENS E, SWINTEK J, VAN CRUCHTEN SJ, VERBUEKEN E, VILLENEUVE DL, KNAPEN D. Gene transcription ontogeny of hypothalamic-pituitary-thyroid axis development in early-life stage fathead minnow and zebrafish. **Gen Comp Endocrinol**. 2018.

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

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

WALTER, K. M.; MILLER, G.W.; CHEN, X.; YAGHOobi, B.; PUSCHNER, B.; LEIN, P.J. Effects of thyroid hormone disruption on the ontogenetic expression of thyroid hormone signaling genes in developing zebrafish (*Danio rerio*). **General and Comparative Endocrinology**. 272: 20-32. 2019.

WALPITA, C.N., CRAWFORD, A.D.; JANSSENS, E.D.R.; VAN DER GEYTEN, S.; DARRAS, V.M. Type 2 iodothyronine deiodinase is essential for thyroid hormonedependent embryonic development and pigmentation in zebrafish. **Endocrinol**. 150(1), 530-539. 2009.



WANG, Y.; ZHANG, S. Expression and regulation by thyroid hormone (TH) of zebrafish IGF-I gene and amphioxus IGF-1 gene with implication of the origin of TH/IGF signaling pathway. 160(4), 474–479. 2011.

XU, C. ET al. Nanog-like regulates endoderm formation through the Mxtx2-Nodal pathway. **Dev. Cell.** 2012.

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

YAMANO, K. The Role of Thyroid Hormone in Fish Development with Reference to Aquaculture. **Japan Agricultural Research Quarterly** 39(3):161-168.2005.

YEN, P.M. Physiological and molecular basis of thyroid hormone action. **Physiol Rev** 81:1097–1142. 2001.

YEN, P.M. Thyroid Hormones and 3,5-Diiodothyropropionic Acid: New Keys for New Locks. **Endocrinology.** 147(4):1598-1601. 2006.

ZHAO X, WAN W, ZHANG X, WU Z, YANG H. Spermatogonial Stem Cell Transplantation in Large Animals. **Animals.** 11:918. 2021.

ZHANG Y., LI S., LIU Y., LU D., CHEN H., HUANG X. Structural Diversity of the GnRH/gnRH Receptor System in Teleost: Its Involvement in Early Development and the Negative Control of LH Release. **Peptides** 31, 1034–1043. 2010.

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



Attachments

Attachment 1: High-resolution three-dimensional imaging and reconstruction of the whole zebrafish testis using Light-Sheet Fluorescence Microscopy (LSFM): a powerful approach to investigate the spermatogonial niche in fish

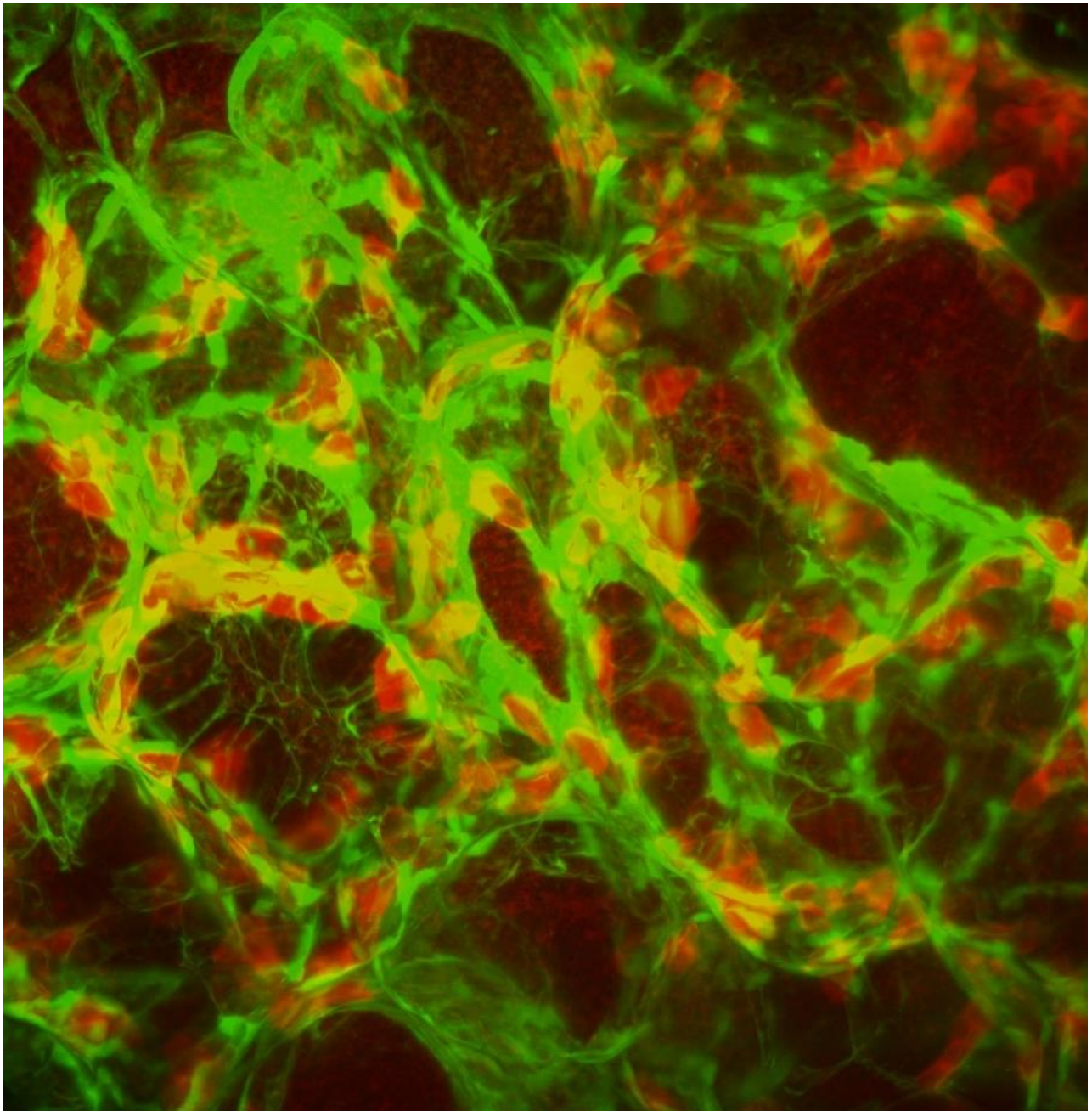
Manuscript in preparation

Attachment 2: Development of testicular organoids as a model for studying fish spermatogenesis and developing innovative aquaculture applications

Manuscript in preparation

Attachment 1: High-resolution three-dimensional imaging and reconstruction of the whole zebrafish testis using Light-Sheet Fluorescence Microscopy (LSFM): a powerful approach to investigate the spermatogonial niche in fish

Manuscript in preparation



3D projection of transgenic zebrafish testis: Green fluorescent generated by the GFP was detected in Sertoli cells, delineating the tubular walls and cysts lining. Red fluorescence generated by DsRed2 in different spermatogonial generations was detected (Figure by the author).



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High-resolution three-dimensional imaging and reconstruction of the whole zebrafish testis using Light-Sheet Fluorescence Microscopy (LSFM): a powerful approach to investigate the spermatogonial niche in fish

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Abstract

The cellular dynamic and the underlying molecular mechanisms allowing the renewal of the germ stem cell stock in adult testes remain poorly understood in vertebrates, particularly in fish. In the absence of a strictly specific marker for adult germ stem cells, the identification and enumeration of the latter into the testis relies mainly on histological analyses using morphological, infrastructural, and topological criteria. Despite the use of serial sections, the difficulty of distinguishing 2D images between single germ stem cells and cells forming doublets of undifferentiated spermatogonial cells is a source of inaccuracy. In addition, such a laborious approach is generally restricted to limited areas of the testis that may not reflect the overall tissue. High-resolution three-dimensional (3D) imaging of whole organs is a promising technique that could circumvent the caveats of the 2D histological approach. In the present study, we tested and improved a modified CUBIC tissue clearing technique to image the testis of adult transgenic zebrafish (*Dr_gsd1:eGFP/vasa: DsRed*) expressing the red fluorescent protein (DsRed) in spermatogonial subpopulations (including single germ stem cells) and the green fluorescent protein (enhanced GFP) in Sertoli cells. Note that the zebrafish reporter line was preferred over other cell labeling techniques to ensure homogenous labeling of the targeted cells throughout the depth of the tissue. Testes were first dissected out and then rapidly fixed in 4% PFA for 1 hour at room temperature. The tissue samples were incubated in modified CUBIC 1A and CUBIC 2 clearing solutions (Clear, Unobstructed Brain Imaging Cocktail) with nuclear DNA labeling in between using Hoechst 33342. The endogenous fluorescence and the nuclear DNA labeling were preserved throughout the testes and were imaged deeply with recordings every 0.5 μm . 3D projections of the image stacks showed that Sertoli cells were present in all regions lining the tubular walls and delineating different cysts, while spermatogonia were either single or organized in clusters containing 2 to 16 cells. The chromatin compaction and the averaged nuclear size decreased in the spermatogonia of 16-cell cysts. The high-resolution 3D imagery described in the present study is a great improvement compared to our anterior achievements (10.1038/srep43012), and it should facilitate and significantly improve the estimation of different spermatogonial subpopulations including single spermatogonial stem cells.

Keywords: Zebrafish testis clearing, light-sheet microscopy, double transgenic zebrafish, stem cells



1. Introduction

Spermatogenesis in teleost fish is a tightly regulated and dynamic process that is essential for species propagation (Schulz et al., 2010). This process is regulated by a complex interplay of endocrine signals, primarily involving gonadotropins and sex steroids, along with local paracrine factors within the testicular microenvironment (Nóbrega et al., 2009; Leal et al., 2009; Schulz et al., 2010). In teleosts, spermatogenesis occurs within cysts formed when the cytoplasmatic extensions of Sertoli cells encase a single spermatogonia (Nóbrega et al., 2009; Leal et al., 2009; Schulz et al., 2010; França et al., 2015). Spermatogonial stem cells (SSCs) are unique in their capacity for both self-renewal and differentiation, ensuring a continuous supply of progenitor spermatogonia that will eventually mature into spermatozoa (de Rooij, 2017; Maouche et al., 2018; Ibtisham & Honaramooz, 2020; Xie et al., 2020; Zhao et al., 2021; Hua-Ming et al., 2022). Proper regulation of this process is essential to prevent stem cell tumors or the depletion of spermatogenesis, which are vital for adult gonadal maturation (de Rooij, 2001; Ehmcke et al., 2006). Despite the importance of understanding the cellular dynamics and molecular mechanisms that regulate germ stem cell renewal in adult testis, these processes remain poorly understood in vertebrates, particularly in fish. The lack of highly specific markers for different germ cell subtypes and the difficulties associated with isolating adult germ stem cells limit current histological analysis for their identification and quantification (Leal et al., 2009; Schulz et al., 2010). This limitation often requires significant data extrapolation, thereby reducing the accuracy of SSCs identification and characterization.

The study of reproductive system morphogenesis and adult homeostasis in vertebrates has been significantly advanced through the development of different techniques (Xie et al., 2020). High-resolution three-dimensional (3D) imaging of whole organs represents a promising approach (Frétaud et al., 2017; Lesage et al., 2020; Folts et al., 2024) that addresses the limitations of traditional 2D methodologies. Whole-organ-level three-dimensional fluorescence imaging techniques, including light-sheet fluorescence microscopy (LSFM), have been developed in conjunction with advanced tissue-clearing protocols to address the challenges associated with imaging depth and resolution (Wan et al., 2019; Zhang et al., 2024). Numerous successful 3D images have been obtained using light-sheet fluorescence microscopy for different whole organs and organoids in mouse (Biswas et al., 2021; Fang et al., 2021; de Medeiros et al., 2022; Spangenberg et al., 2023). Light-sheet fluorescence microscopy, which uses a thin plane of light to optically section transparent tissues or whole organisms labeled



with a fluorophore, has proven particularly effective in this context (Santi, 2011; Wan et al., 2019). Recent advancements in fluorescent labeling technologies have significantly expanded beyond the mere bright and stable labeling of proteins. Live-cell nucleic acid probes have been developed, enabling the monitoring of RNA and DNA dynamics (O'Connell et al., 2014; Qin et al., 2017), with the potential to achieve single-cell, single-molecule sensitivity. In this context, for example, the study of zebrafish embryonic development using transgenic reporter lines that visualize Notch, BMP, and Wnt signaling pathways (Parsons et al., 2009; Collery & Link, 2011; Moro et al., 2012) can be integrated with *in toto* light-sheet imaging. This approach facilitates the investigation of how intrinsic cellular information is integrated with extrinsic signals to regulate cell behaviors, addressing a fundamental question in developmental biology. This technique has also advanced the study of reproductive system morphogenesis in the adult mouse (McKey et al., 2020) and fish (Lesage et al., 2020) ovary. To date, only a few significant achievements have been reported in zebrafish males (Frétaud et al., 2017). Additionally, developing protocols and image analysis pipelines that support high-throughput data analysis will be essential to understand the testis architecture better and to allow accurate cellular characterization in fish models.

On the other hand, over the past decade, significant technical advancements have been made in 3D imaging techniques for large biological samples that are naturally opaque. These include the development of various optical clearing methods designed to enhance tissue transparency and reduce light scattering (Gómez-Gaviro et al., 2020). Numerous protocols have been established to improve tissue clarity, most of which were initially developed for use in mouse neuronal tissue (Hama et al., 2011; Chung et al., 2013; Susaki et al., 2014; Frétaud et al., 2017). In this study, we selected the CUBIC (Clear Unobstructed Brain/Body Imaging Cocktails and Computational Analysis) protocol, chosen based on its demonstrated effectiveness in achieving transparency and preserving GFP fluorescence while avoiding tissue deformation in zebrafish testis, as reported by Frétaud and colleagues (2017). CUBIC enhances tissue transparency through hyperhydration with amino alcohols, urea, and lipid removal using detergents (Susaki et al., 2014, 2015). We applied the CUBIC clearing method to a double transgenic zebrafish line, incorporating minor modifications to the protocol to preserve both the green fluorescence generated by GFP in Sertoli cells lining the lobules, and red fluorescence generated by DsRed2 in germ cells, including single spermatogonial stem cells (Gautier et al., 2011; Lareyre et al., not published).



The next frontier in reproductive biology involves adapting various validated image analysis methods to conduct high-throughput phenotyping and quantitative assessments. Integrating artificial intelligence (AI) with advanced microscopy and clearing techniques will be instrumental in evaluating the quantity and spatial distribution of different spermatogonial populations. This approach will facilitate a deeper understanding of spermatogenesis dynamics, including the proliferation, and differentiation of germ stem cells.

2. Methods

2.1 *Ethics statement*

All housing conditions of the animals used in this study followed the recommendations of the French European Union regulation concerning the protection of experimental animals and were approved by the INRA LPGP, Rennes, France. All the experimental procedures were conducted in agreement with the guidelines for care and use animals at the National Veterinary School of Nantes, France.

2.2 *Animal husbandry*

In the experiments, we utilized adult male zebrafish from the double transgenic line *Dr_gsdf:eGFP/vasa: DsRed* (Gautier et al., 2011; Lareyre et al., not published), aged between 5 to 12 months. The animals were bred and raised in our facilities (INRA LPGP, Rennes) and maintained under oxygen saturation in a recirculating water system at 28°C ±, pH 7.5. Zebrafish were exposed to a photoperiod of 16 hours light/ 8 hours dark. For the dissection procedure, the zebrafish were euthanized by immersion in a lethal dose of Tricaine (MS-222) 300 mg/L. Subsequently, the testes from transgenic zebrafish were carefully excised from the body cavity and promptly used in the experiments below.

2.3 *Double transgenic zebrafish*

To enhance the precision of single germ stem cell detection and to establish reliable automatic cell counting, we devised a high-resolution 3D imaging technique for whole zebrafish testes. To the development of this approach, we took advantage of transgenic lines. At INRA LPGP Rennes, homozygous transgenic reporter lines are maintained. The animals



express red fluorescence in all spermatogonial populations, including single germ stem cells, while green fluorescence is expressed in Sertoli cells. The red fluorescence was generated by the DsRed2 protein whose transcript expression is driven by the zebrafish *vasa* promotor (Fan et al., 2008). Another one, the green fluorescence was generated from the GFP protein, which transcripts were expressed under the control of the *gsdf* promoter (Gautier et al., 2011). Both transgenic reporter lines were crossed to generate zebrafish with red and green fluorescence in the male testis (Figure 1). The use of such double fluorescent reporter fish is preferred to obtain a reproducible fluorescent signal in the cells of interest and to localize the germ stem cell niche.

2.4 Optimization of the transgenic zebrafish testis clearing using CUBIC protocol

In this study, zebrafish ($n = 20$) testis were dissected out. After either immediate dissection, the zebrafish testes were fixed using paraformaldehyde (PFA). The fixation process involved incubating the testes in a solution of 4 % PFA in 0.01 M phosphate buffer saline (PBS) at room temperature for 1 hour. Fixed testes were tested in different protocols of transparization: testes without clearing treatment (control), testes incubated in histodenz (D2158, Sigma-Aldrich) clearing protocol, and CUBIC solution (Frétaud et al., 2017). The best-resolved imaging in transparency and depth was obtained with a CUBIC (Clear, Unobstructed Brain Imaging Cocktails and Computational Analysis) protocol as described previously (Susaki et al., 2014; 2015; Tainaka et al., 2014; Frétaud et al., 2017) with some modifications specifically for the CUBIC 1 solution. Here, we described the CUBIC 1A modified and CUBIC 2 protocol. CUBIC 1A was prepared as a mixture of 10 wt% urea (GE17-1319-01, Sigma-Aldrich), 5 wt% N, N', N'-tetrakis(2-hydroxypropyl)ethylenediamine (122262, Sigma-Aldrich), 10 wt% tritonTM X-100 (9002-93-1, Thermo Fisher Scientific Chemicals, Inc.) in distilled water. CUBIC 2 (IR = 1.45) was prepared as a mixture of 50 wt% sucrose (S-9378, Sigma-Aldrich), 25 wt% urea, 10 wt% triethanolamine (90279, Sigma-Aldrich), and 0.1% (v/v) tritonTM X-100 in distilled water. For whole testis clearing, each fixed testis was immersed in 1 mL of CUBIC 1A 50% (diluted in distilled water) per 3 h at 37°C. Afterward, the solution was exchanged, and samples were immersed in the same volume of fresh CUBIC 1A 100% at 37°C, overnight. The CUBIC 1A 100% solution was renewed once the second morning day. Samples were washed in PBS several times at room temperature. Nuclear staining was performed after clearing with CUBIC 1A 100%. Whole testes were incubated in Hoechst 33342 diluted at 10 µg/mL in PBS at room temperature for 1 hour. Samples were then incubated with CUBIC 2 reagent for 3-4



days. The CUBIC 2 solution was renewed once every day. All samples were agitated on a rotating wheel during the whole protocol. Mounting the samples in agarose was performed at the end of the clearing protocol (Figure 2).

2.5 3D light-sheet imaging processing

Mounting and imaging for light-sheet microscopy were performed at the end of the clearing protocol. The whole testis was embedded in 1% low-melting-point agarose diluted in distilled water. To assemble the sample holder, the agarose drop was positioned at the center of a Petri dish and then drawn into the capillarity system using an insulin syringe. Subsequently, the capillarity was filled with agarose, and the testicular tissue was carefully positioned into the center of the gel. After a few minutes, the capillarity filled up agarose. For image acquisition, the U-shaped capillary was immersed in the CUBIC 2 solution at 20 °C. The samples were then mounted on the light-sheet microscope, sample mounting was imaged directly in CUBIC 2, and full z-stacks were acquired to image the testis using a Zeiss Light-sheet microscope at 20x magnification at 300 μm depth. (Figure 2).

2.6 Data analysis

Brightness and contrast enhancement and measurements were conducted using Fiji software (Schindelin et al., 2012). All images were stacked for a 3D reconstruction of the testis allowing the counting of single germ cells and cell numbers in clustered spermatogonial cells. The quality of the stacks was suitable for the digital segmentation of the fluorescent germ cells using software implementing customizable artificial intelligence (AI) for deep learning. The integration of automatic cell segmentation and counting promises to significantly accelerate the analysis of biological samples. Furthermore, the high quality of 3D reconstruction generated from image stacks is well-suited to precise 3D modeling of the spatial organization within the germinal stem cell niche.

3. Results

3.1 Sample transparency



To evaluate the clearing efficacy of the CUBIC protocol on zebrafish testes, samples were examined at the optical macroscopy level. The apparent transparency of all cleared samples was contrasted with that of non-cleared testes, which were incubated in PBS. Control samples (Figure 3A) appeared whitish and were completely opaque. In contrast, testes tested with CUBIC treatment became completely transparent (Figure 3B).

3.2 Fluorescence preservation

Frétaud and colleagues (2017) demonstrated that the CUBIC protocol was compatible with observing endogenous GFP fluorescence in zebrafish testis. However, while the CUBIC 1 protocol successfully preserved the endogenous GFP fluorescence, it abolished DsRed2 fluorescence. To address this issue, we made modifications to the CUBIC 1 protocol, successfully preserving both types of fluorescence (Figure 4).

3.3 A 3D reconstruction of a whole testis after CUBIC-clearing

The improvement of the CUBIC clearing protocol allowed us to preserve both fluorescence and acquire images up to 1.5 mm in depth with images recorded every 0.5 μm , and it generated approximately 40 GB for a single view of one testis. Both GFP and DsRed2 fluorescence until 300 μm indicated that the CUBIC protocol allows recovery of GFP and DsRed2 signals in the whole testis. Sertoli cells were present in all regions of the organ, lining the tubular walls and the cysts (Figure 5A), and the red fluorescence generated by DsRed2 corresponds to single stem cells, spermatogonial doublets, or 4 to 16 clustered germ cells forming the cysts (Figure 5A). The nuclei were counterstained with Hoechst 33342 to observe the characteristic low chromatin compaction observed in the large nuclei of the undifferentiated spermatogonia cells (Figure 5B). All images were stacked for a 3D reconstruction of the testis allowing the counting of single germ stem cells and cell numbers in clustered spermatogonial cells (Supplementary V1).

3.4 3D CUBIC-imaging of germinal niches: spermatogonial morphology

The types of cells in the zebrafish germinal niches were previously characterized by Leal and colleagues (2009). However, the 3D organization of the male germinal niches in fish



is poorly investigated. In our 3D imaging analysis ($n = 10$ stacks/cyst), we analyzed the composition of germinal niches containing isolated or clustered germ cells by using the 3D reconstruction data. Here, we provide a brief overview of the 3D modeling of spermatogenesis. The morphology and nuclear diameter of the different germ cell types were determined (Figure 6) after treatment with CUBIC protocol and light-sheet microscopy analysis. CUBIC-treatment testes for 3D imaging showed an increased size (Figure 6) of nuclei diameter of germ cells when compared with stereology of the spermatogenic cysts in zebrafish using a 2D approach (Leal et al., 2009). We observed heterogeneity in nuclear diameter and morphology among germ cells within the same cyst (Figure 6). We recognized two types of isolated spermatogonia that can be differentiated in zebrafish based on morphological and nuclear diameter, which vary between 12 to 17 μm (Figure 6A-B). The nuclei are large containing one central and compact nucleoli, and the lower condensation of the chromatin. The nuclear envelope acquires an irregular outline (Figure 6B). Spermatogonial doublets are comprised of two cells arranged within the cyst. The nuclear envelope exhibits a regular/elongated shape, containing poorly condensed chromatin, and one compact nucleolus located in an eccentric position (Figure 6C). The nucleus diameter variation is between 10 to 16 μm (Figure 6A and C). The cysts with 4 to 8 spermatogonial cells (Figure 6D-E) were very similar to the isolated or doublet cells (Figure 6B-C) and arranged in cysts or chains. However, these cysts begin to exhibit a nucleus with dense chromatin (Figure 6D-E), and still with one visible nucleolus. The average nuclear diameter for 4 to 8 cysts was 11 μm and 10 μm , respectively (Figure 6A and D-E). In type B spermatogonia, the number of germ cells inside the cyst increases (Figure 6F-H). Type B early cysts containing 16 cells exhibit a smaller and round nucleus, with no apparent nucleoli (Figure 6F). A notable difference from A spermatogonia is the reduction of nuclear diameter (approximately 8 μm) and an increased amount of heterochromatin (Figure 6F). The nuclear diameter variation between 6 to 15 μm (Figure 6A and F). In type B late spermatogonia, cysts with 32 to 256 cells were observed (Figure 6G-H). The nucleus becomes round and starts to decrease in size, and denser (Figure 6G-H). The nuclear diameter varies between 6 and 7 μm (Figure 6A and G-H). Spermatocytes in the different phases of meiosis were distinguished from the spermatogonial type based on nuclear characteristics, including size, chromosome condensation, and the presence of meiotic figures on the chromosomes (Figure 6I). The nuclear diameter was also measured and ranged from 7 to 15 μm (Figure 6A), with pachytene spermatocytes observed as the largest type among the spermatocytes. Finally, spermiogenesis was characterized by a remarkable reduction in nuclear diameter, along with intense



chromosome condensation as indicated by the bright white color from Hoechst 33342 staining (Figure 6J). In this type of cyst, spermatids were identified with varying nuclear diameters between 3 to 6 μm (Figure 6A). Spermatozoa found in the tubular lumen were smaller, with denser nuclei (Figure 6K), exhibiting an average nuclear diameter ranging from 3 to 5 μm (Figure 6A).

3.5 Characteristic of different germ cell cysts expressing vasa fluorescent protein in transgenic zebrafish testes: a 3D approach

Light-sheet analysis of the expression of *vasa* protein in whole mounts testis showed its presence as a diffuse signal in the cysts (Figure 7). The *vasa* were highly concentrated in doublets and cysts with 4 cells, both classified as undifferentiated spermatogonia (Figure 7A-C). This labeling was either absent or observed at low levels from type B spermatogonia cysts up to spermatozoa (Figure 7).

4. Discussion

The distinct anatomy of the male reproductive organ in fish reflects its multifaceted function, underscored by a complex cell organization. Two-dimensional (2D) light microscopy examinations of the testis structure fall short of adequately representing its complexity and the precise organization of male germinal niches. While recent advances in tissue clearing techniques now enable accurate 3D imaging of various organs in mammals, including the testis (Nakata et al., 2021), the investigation of 3D testis reconstruction and cellular content in fish remains poorly investigated (Frétaud et al., 2017). Here, we tested different tissue-clearing protocols to ascertain their applicability. Among the protocols tested, only the modified CUBIC protocol enabled imaging of the entire transgenic zebrafish testis at a cellular level. To our knowledge, this is the first study that demonstrates the feasibility of an *in vivo* 3D light-sheet investigation in combination with a CUBIC treatment and double transgenic testis was able to explore the cellular organization of clustered undifferentiated spermatogonia within the germinal niches. Previously observed in confocal microscopy that most undifferentiated spermatogonia were not isolated, as proposed previously (Schulz et al., 2010), but instead formed chains of 2, 4, or 6 cells at the periphery of the seminiferous tubules (Frétaud et al., 2017). Here, we observed this organization using the light-sheet technique. Few real isolated



cells were detected throughout the entire zebrafish testis. Despite their isolation, these cells were found in proximity to cysts containing 2 or 4 cells, suggesting a recent division. Light-sheet microscopy offers a greater depth of field across the entire field of view, making it advantageous for precisely observing the spatial distribution of these cells. This allows for determining whether they are truly isolated or situated within cysts, by accessing depth rather than just surface information from a single stack.

Another point to consider in this work is the effect of tissue clearing on nuclear size. Frétaud and colleagues (2017) reported an increase in nuclear volumes with the use of the CUBIC solution. In our study, we observed that CUBIC-clearing testes for 3D imaging showed a greater increase in the nuclear diameter of germ cell cysts compared to 2D histology (Leal et al., 2009). CUBIC urea-based has already been shown to promote volume expansion due to tissue hyperhydration (Susaki et al., 2014; Hama et al., 2015). However, in these studies, the return to the initial size after incubation in CUBIC 2 was observed. We hypothesize that the nuclear diameter of cells in 3D is more pronounced compared to 2D. By assessing cyst depth, we obtained a more accurate dimension of the cells, and selecting cells with large nuclei allowed us to represent the average size of cells more accurately.

The native GFP and DsRed2 fluorescence of the double transgenic line (*Dr_gsdf:eGFP/vasa: DsRed*) was properly preserved with a CUBIC-modified protocol. The fluorescence of GFP and its variants is influenced not only by pH and temperature but also by the protein secondary structure (Tainaka et al., 2016). Detergents, organic solvents, and dehydration processes of some clarifying protocols may lead to an important decay of the GFP fluorescence, likely through denaturation of the GFP proteins (Hama et al., 2015). Frétaud and colleagues (2017) tested and compared various clearing techniques in transgenic zebrafish testis. The comparison of several clearing protocols showed that CUBIC and PACT are the most appropriate among a large number of protocols available. However, the use of one more transgene line, as employed in the present study, proves beneficial for a more realistic evaluation of fluorescence preservation. In our case, the CUBIC protocol showed impairment in preserving DsRed2 fluorescence. This phenomenon might be attributed to the higher concentration of the compounds used in the preparation of the CUBIC 1 solution. To solve this problem, we reduced the organic solvent used in the original protocol to limit the fluorescence decay. Taken together, our data showed that CUBIC 1A modified and CUBIC 2 are suitable methods to preserve both GFP and DsRed2 fluorescence in zebrafish testis for 3D imaging.



Interestingly, both fluorescence signals were preserved for more than one month using the modified CUBIC protocol (personal observations).

The effort to preserve both fluorescence is because the gene *vasa* are expressed in the germ cells of nearly every sexually reproducing animal, including fish (Baat et al., 2000; Gustafson & Wessel, 2010), and the *gsdf* (Gautier et al., 2011) transcripts are expressed in the somatic cells supporting germ cell development. This double transgenic zebrafish testis could be interesting to show in 3D testis reconstruction the spatial organization of the germ stem cell niche and demonstrate the dynamic of *in vivo* spermatogenesis. Here, we observed the characteristics of different germ cell cysts expressing *vasa* fluorescence protein pattern in zebrafish testis. In our 3D imaging analysis of cells expressing *vasa* protein, those in double type exhibit a notably high intensity of DsRed2 expression compared to isolated cells or in other cell cysts. The *vasa* gene is indispensable for germ cell development and is present in both invertebrates and vertebrates. Analysis of these *vasa* homologs revealed a highly conserved role for the *vasa* protein across different organisms (Raz, 2000). In vertebrate oocytes, *vasa* RNA is distributed throughout the cytoplasm, while the protein associates with polar granules and is asymmetrically segregated to the posterior pole (Hay et al., 1988). Then, the *vasa* protein is exclusively localized in the germ cell lineage at later stages of embryogenesis (Fujiwara et al., 1994). In zebrafish, *vasa* RNA is also found throughout oogenesis and embryogenesis (Baat et al., 1999), and *vasa* RNA-positive cells increase in number as migration to the gonads begin, demonstrating their localization in primordial germ cells (PGCs) based on their migration pattern, final location, and morphology (Baat et al., 1999; Weidinger et al., 1999). Furthermore, continuous expression of *vasa* is essential for gametes' maturation and proper function in adult fish.

Our study allowed for the first-time 3D imaging of double zebrafish transgenic testes. The enhancement of the CUBIC clearing solution enabled the preservation of both fluorescence signals and the acquisition of up to 1.5 mm in depth with stacks recorded every 0.5 μ m. GFP and DsRed2 fluorescence signals were observed up to 300 μ m, indicating that the CUBIC protocol modified allows for the recovery of DsRed2 and GFP signals throughout the entire testis. The red fluorescence generated by DsRed2 corresponded to single stem cells, spermatogonial doublets, or clusters of 4 to 16 germ cells forming cysts, while Sertoli cells were found in all regions of the organ, lining the tubular walls and cysts. The combination of 3D light-sheet investigation with CUBIC treatment and double transgenic testis enabled the exploration of the cellular organization of clustered undifferentiated spermatogonia within the



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germinal niches. The application of these protocols will be of utmost interest for understanding the spatial organization of the germ stem cells.

Figures

Zf_*gsdf:eGFP*/*vasa:DsRed2*

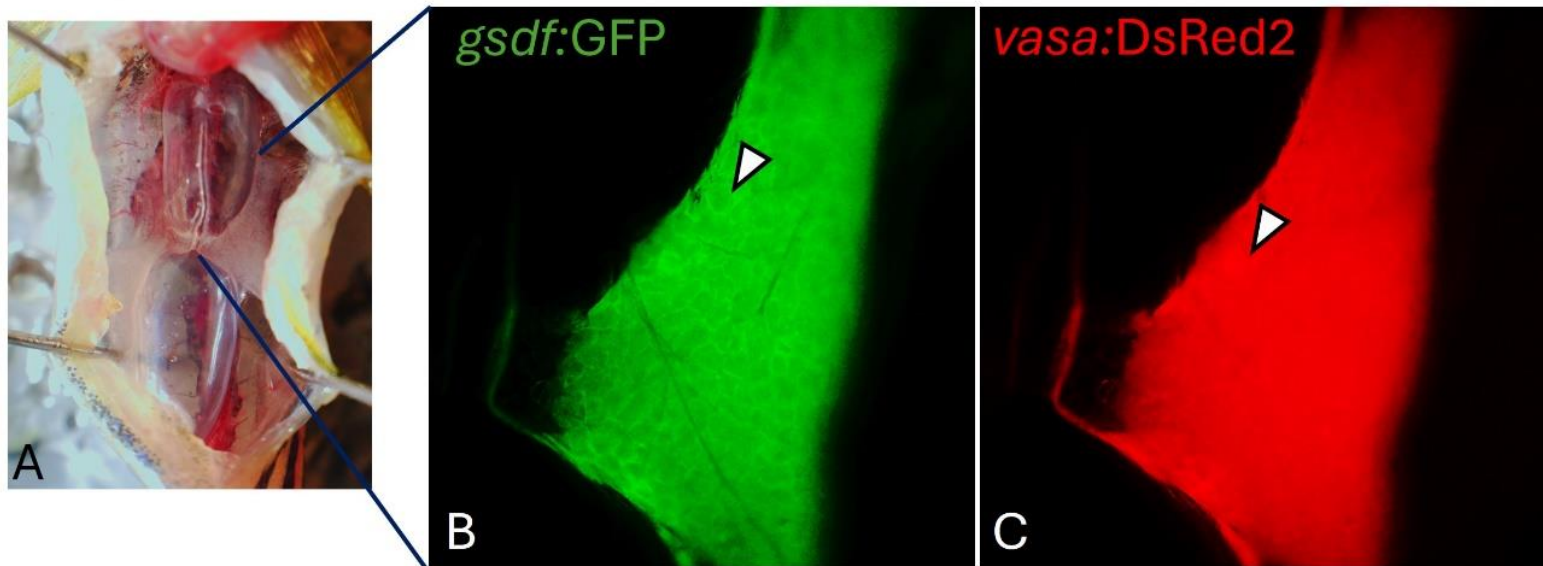


Figure 1: Macroscopic observation of transgenic zebrafish testis (A-C). Green fluorescence generated by the GFP was detected in Sertoli cells lining the lobules (B), and red fluorescence was generated by DsRed2 in germ cells (C). Bars = 250 μ m (A) and 500 μ m (B-C).

Protocol for high-resolution 3D imaging of transgenic zebrafish testis

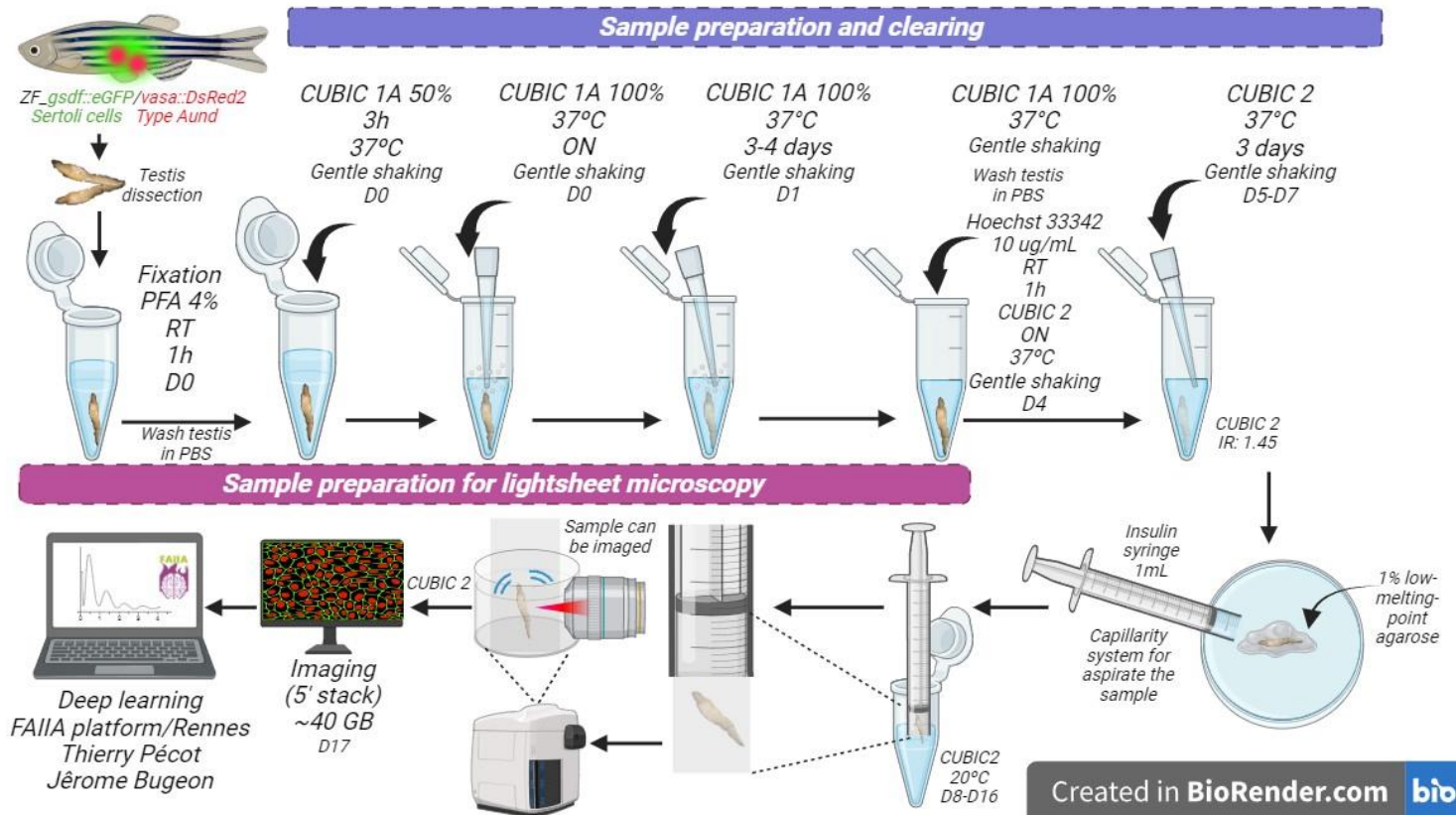


Figure 2: The workflow of tissue preparation for 3D Light-sheet microscopy (LSM) imaging of transgenic zebrafish testis after clearing technique utilizing a modified solution of CUBIC 1A. On day 0 (D0) PAF 4% fixed testes were subjected to clearing following CUBIC protocol (D0-D4). All reporter fluorescence levels were maintained, and nuclei were stained with Hoechst 33342 on day 4 (D4). The samples were then immersed in CUBIC 2 solution for a minimum of 3 days (D5-D7). Subsequently, the whole testis was embedded in 1% low-melting-point. The mounting samples were incubated in CUBIC 2 and stored at 20 °C (D8-D16). Testes were incubated in the refractive index matching solution (CUBIC 2, IR = 1.45) of the last step of the protocol and imaged within 17 days (D17).

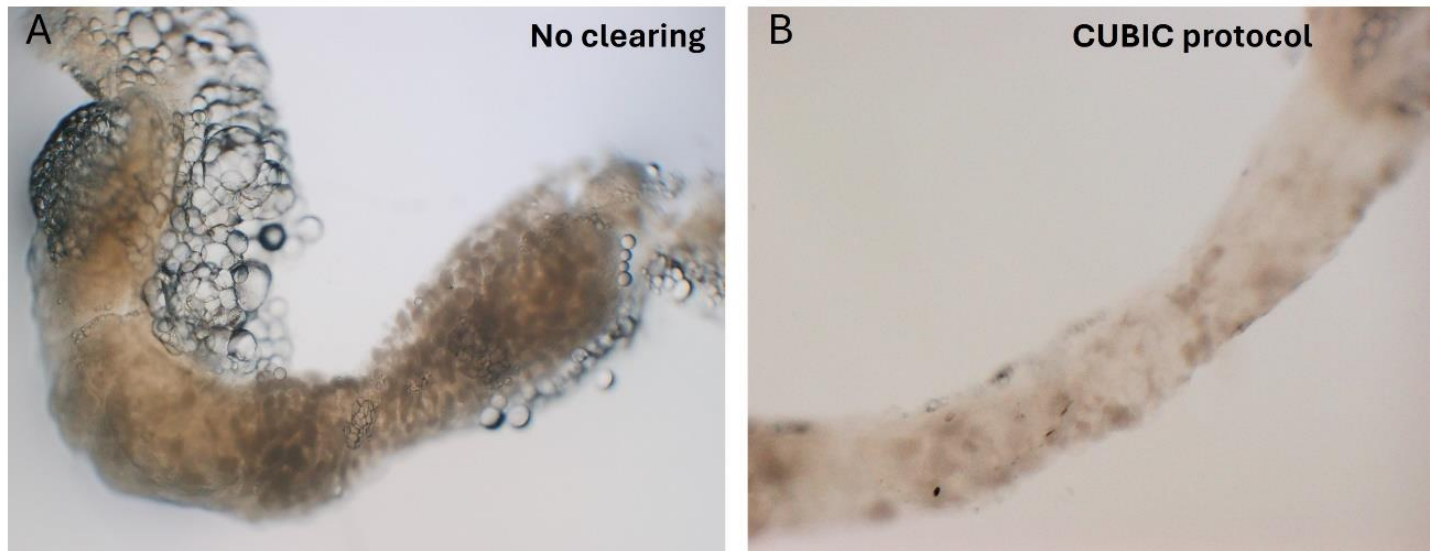


Figure 3: Testes were dissected out from double zebrafish transgenic line *Dr_gsd1:eGFP/vasa:DsRed* and cleared with CUBIC 1A modified. Brightfield images of non-cleared (**A**) and cleared (**B**) testes. Scale bar: 500 μ m.

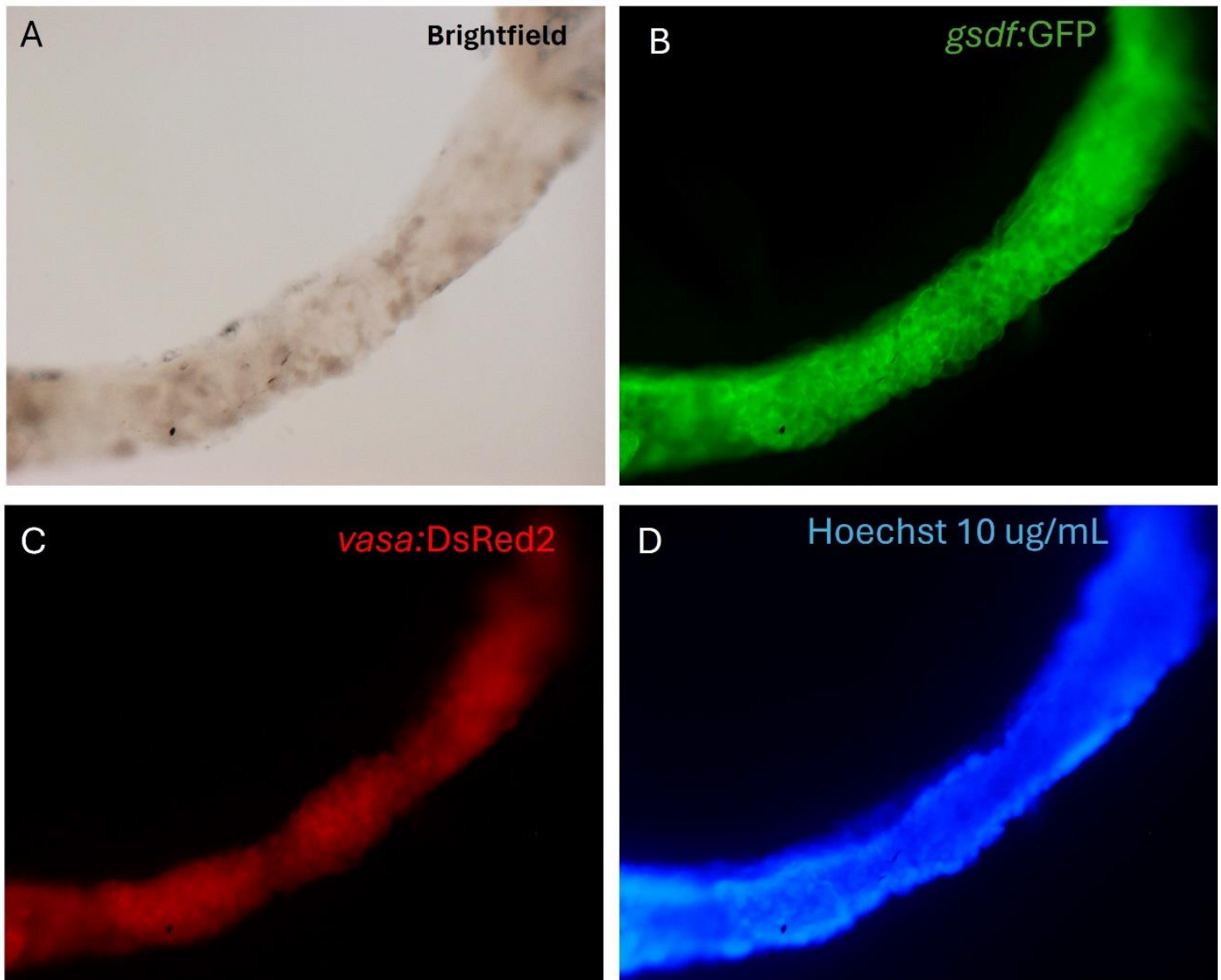


Figure 4: Testes were cleared using CUBIC 1A and CUBIC 2, and the fluorescence was observed afterward with a fluorescence macroscopy. Brightfield images of testis after clearing with CUBIC 1A 100% **(A)**. GFP **(B)** and DsRed2 **(C)** fluorescence of cleared testes. Nuclei were stained with Hoechst 33342 **(D)**. Scale bar: 500 μ m.

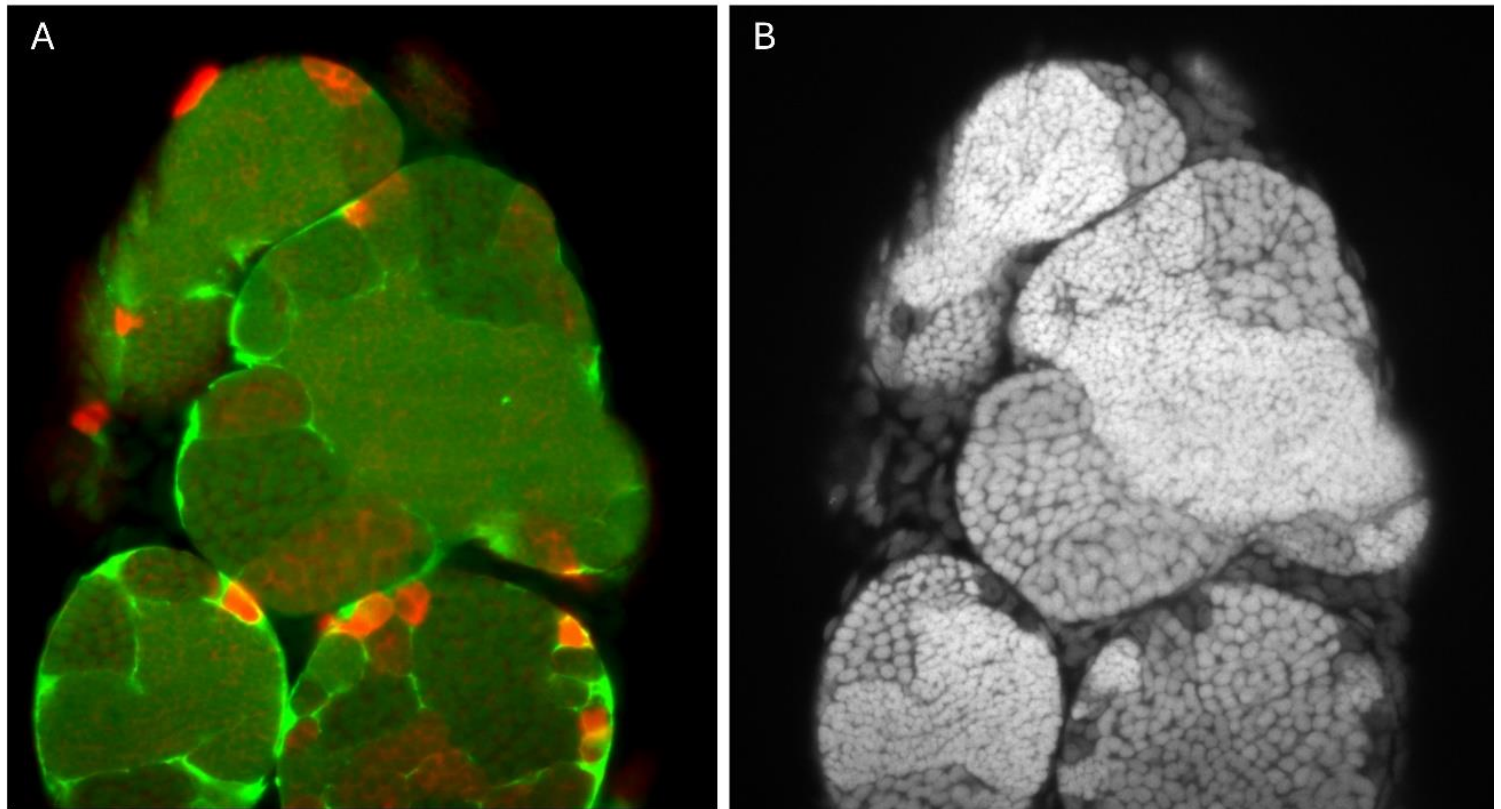
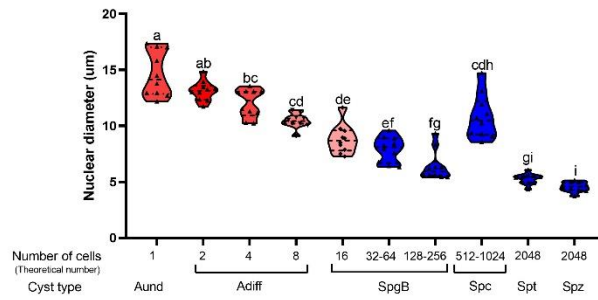
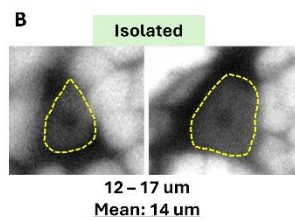


Figure 5: General view of high-resolution of adult zebrafish testis that was acquired using a Zeiss light-sheet microscope at 20x magnification at 300 μm depth. The somatic Sertoli cells are green which corresponds to endogenous GFP fluorescence, and the single stem cell or clustered germ cells are in red, generated by DsRed2 fluorescence **(A)**. The gray-to-white signal corresponds to the Hoechst 33342 observed in cell nuclei **(B)**.

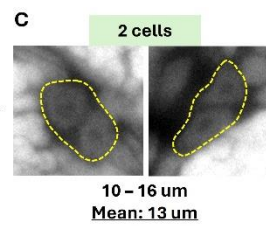
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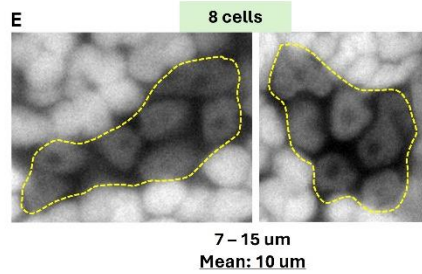
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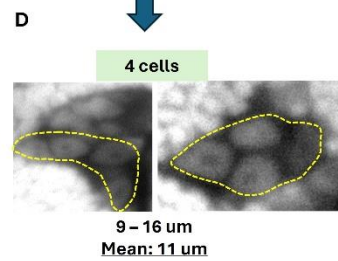
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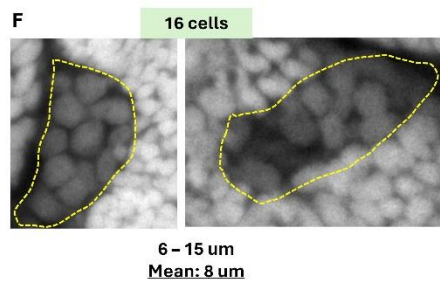
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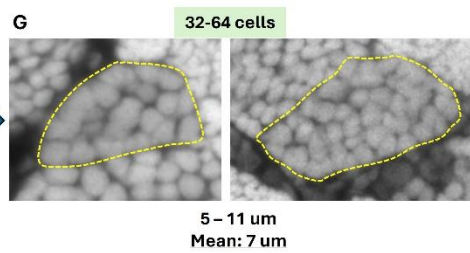
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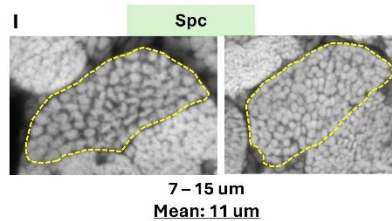
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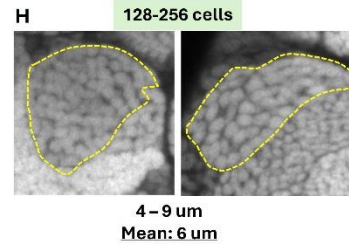
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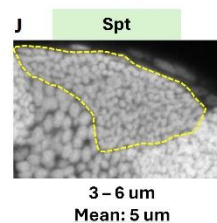
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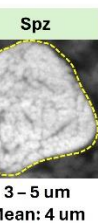
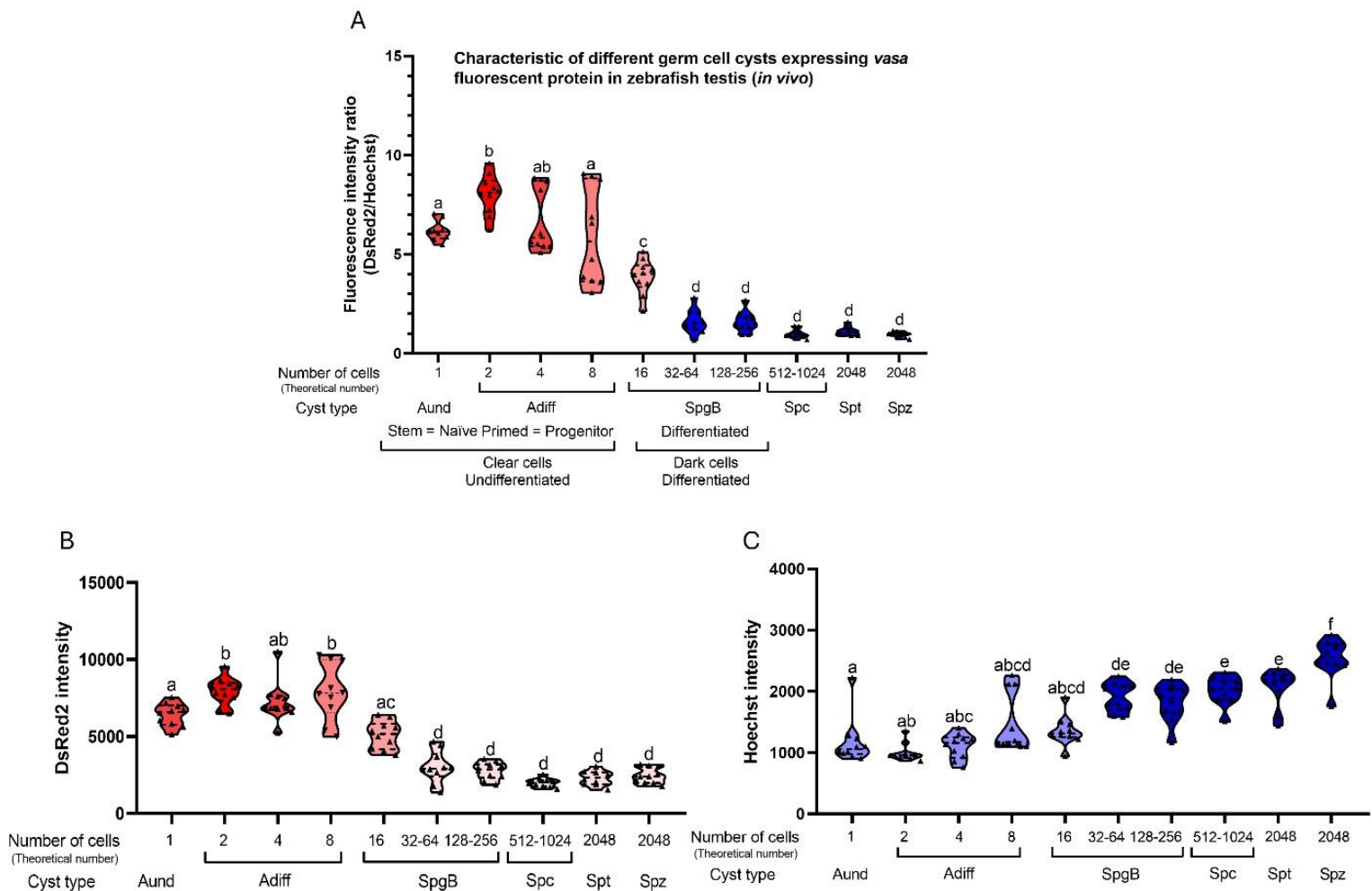
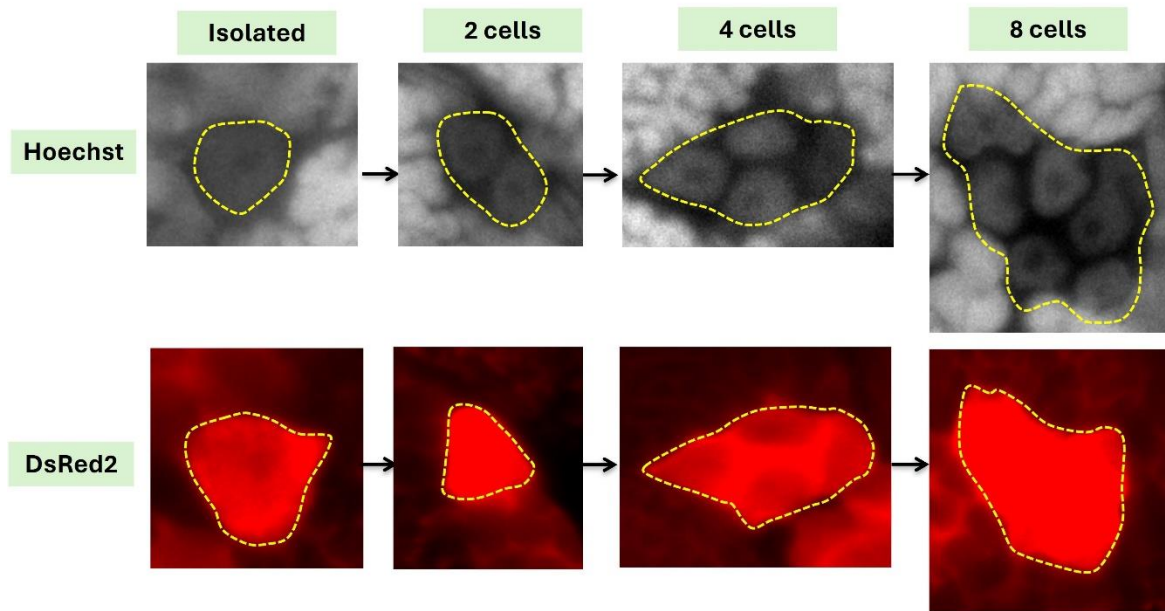


Figure 6: Representative 3D images of male germinal niches in transgenic zebrafish. A tissue section scanned by light-sheet microscopy reveals the composition of germinal niches containing isolated or clustered germ cells by using the 3D reconstruction data. The criteria used to differentiate each generation of the different cell types were the number of cells per cyst and nuclear diameter (**A**). Within the same cyst, the minimum and maximum nuclear diameters of different cell-type cysts were observed, which were plotted on the violin graph (Different letters indicate statistically significant differences; $p < 0.05$). Based on the cell number per cyst, nuclear diameter, and chromatin density, the isolated spermatogonia type (**B**), doublets spermatogonia (**C**), and cysts containing 4 (**D**) or 8 (**E**) cells per cyst were determined. Early and late spermatogonia B type was observed (**F-H**). Spermatocytes in different phases of meiosis were distinguished, and the nuclear diameter varied (**I**). Spermatids (**J**) and spermatozoa (**K**) were differentiated based on cell size, with spermatozoa showing the most condensed nuclear intensity.

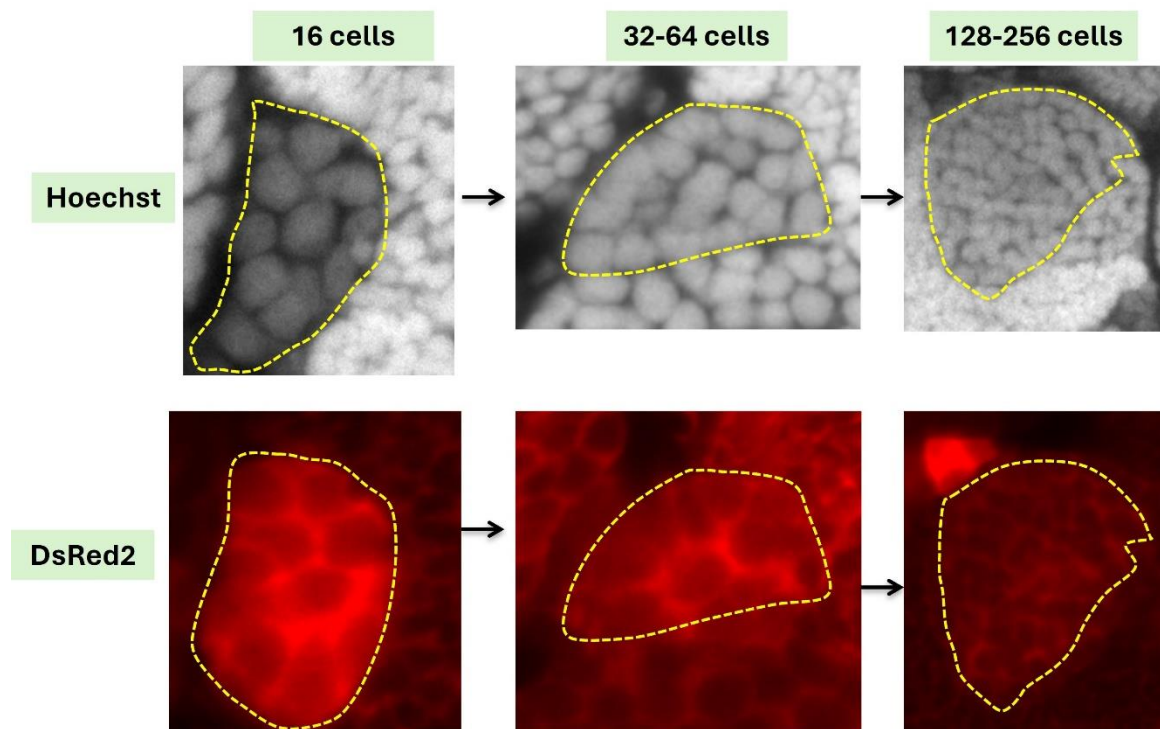


D



Greater heterogeneity of DsRed2/Hoechst ratio fluorescence intensity according to the graph

E



F

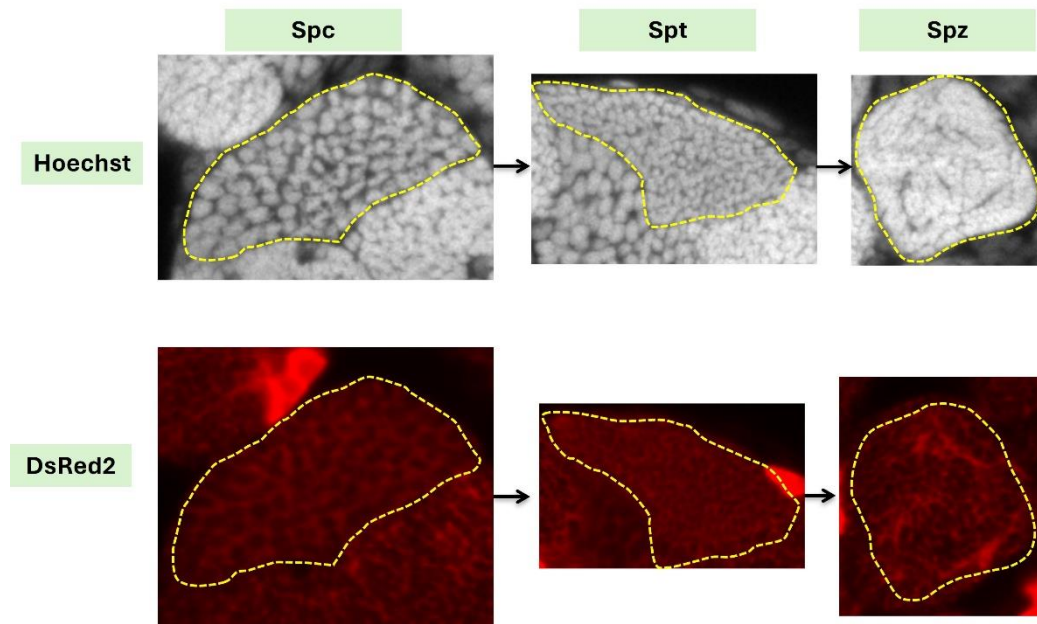


Figure 7: Light-sheet analysis of whole-mount transgenic zebrafish testis. Z-stacks were recorded with a Zeiss light-sheet microscope at 20x magnification at 300 μm depth. Images were processed using Fiji software. The fluorescence ratio between DsRed2 and Hoechst 33342 was measured (**A**). Distinct cysts expressing the *vasa* protein were observed (**B**). While Hoechst 33342 nuclear staining increased in cysts with intense chromosome condensation, indicative of differentiated spermatogonia, as well as in meiotic and post-meiotic cysts (**C**), the expression pattern of *vasa* decreased significantly (**B-C**) (Different letters indicate statistically significant differences ($p < 0.05$)). The more pronounced expression of *vasa* was observed in doublets and 4-cell cysts (**A-D**), these correspond to undifferentiated spermatogonia. The pattern of *vasa* expression has decreased in early and late B spermatogonia type (**A-C and E**), spermatocyte, spermatids, and spermatozoa (**A-C and F**).

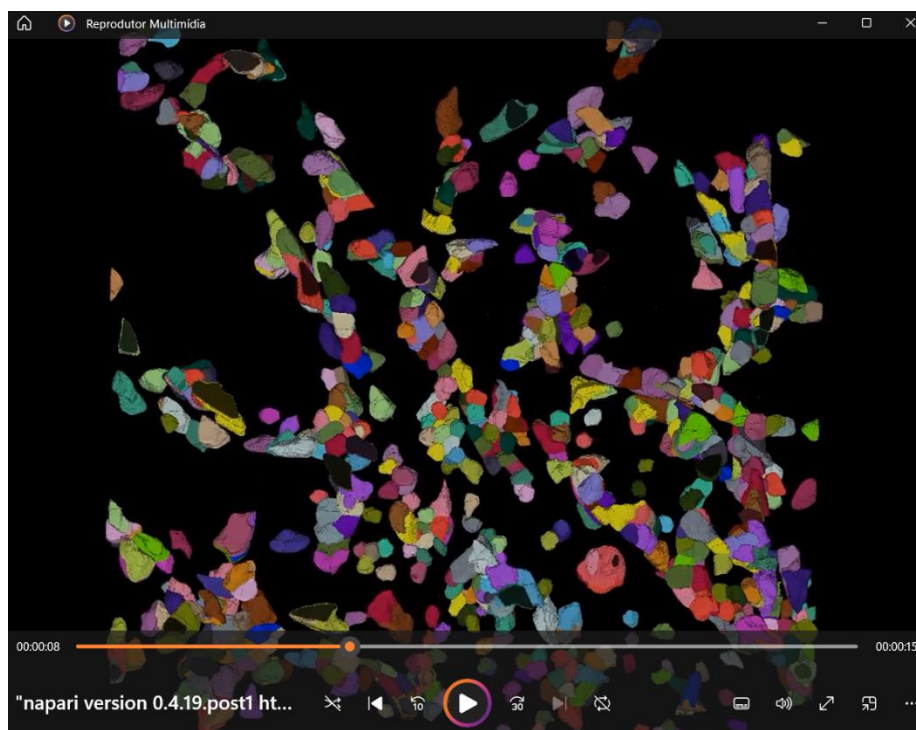


Conclusion

Our previous data demonstrated that 3D imaging techniques developed in the testis to better understand the spermatogenesis dynamics in fish represent a significant advancement over previous studies. These improvements are expected to greatly enhance the accuracy and efficiency of estimating different spermatogonial subpopulations, including the identification of single spermatogonial stem cells. Furthermore, the advances in artificial intelligence (AI) can be useful for optimizing image analysis in the reproduction field.

Supplementary Materials

Supplementary Video V1: Animation of a 3D zebrafish testis reconstruction. The animation has been reconstructed from light sheet microscopy images and analyzed by artificial intelligence (IA).



Author Contributions

Conception and design of experiments, J-J.L., M.S.R., N.C.; performance of experiments, M.S.R., N.C.; data analysis, M.S.R., N.C., R.H.N., J-J.L.; contribution of reagents/materials/analysis tools, J-J.L.; writing of the manuscript, M.S.R., N.C., R.H.N., J-J.L. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement

The French European Union regulation concerning the protection of experimental animals approved the animal study protocol. All the experimental procedures were conducted in



agreement with the guidelines for the care and use of animals at the National Veterinary School of Nantes, France.

Informed Consent Statement

Not applicable.

Data Availability Statement

Data is contained within the article or supplementary material. The data presented in this study are available in this manuscript and supplemental material.

Conflicts of Interest

The authors declare no conflict of interest.

5. References

BRAAT A, ZANDBERGEN T, VAN DE WATER S, GOOS H, ZIVKOVIC D: Characterization of zebrafish primordial germ cells: morphology and early distribution of vasa RNA. **Dev Dyn.** 216: 153-167. 1999.

BRAAT AK, VAN DE WATER S, GOOS H, BOGERD J, ZIVKOVIC D. Vasa protein expression and localization in the zebrafish. **Mech Dev.** 95(1-2):271-4. 2000.

BISWAS, L.; CHEN, J.; DE ANGELIS, J.; CHATZIS, A.; NANCHAHAL, J.; DUSTIN, M.L.; RAMASAMY, S.K.; KUSUMBE, A.P. SUMIC: A simple ultrafast multicolor immunolabelling and clearing approach for whole-organ and large tissue 3D imaging. **bioRxiv.** 2021.

CHUNG K, WALLACE J, KIM S-Y, KALYANASUNDARAM S, ANDALMAN AS, DAVIDSON TJ, MIRZABEKOV JJ, ZALOCUSKY KA, MATTIS J, DENISIN AK, PAK S, BERNSTEIN H. Structural and molecular interrogation of intact biological systems. **Nature.** 497:332–337. 2013.

COLLERY, R.F.; LINK, B.A. Dynamic smad-mediated BMP signaling revealed through transgenic zebrafish. **Dev. Dyn.** 240:712–22. 2011.

de MEDEIROS, G., ORTIZ, R., STRNAD, P., BONI, A., MOOS F., REPINA, N., MEYLAN, C.M., MAURER, F., LIBERALI, P. Multiscale light-sheet organoid imaging framework. **Nature Communications.** 2022.

de ROOIJ, D.G. Proliferation and differentiation of spermatogonial stem cells. **Reproduction-Cambridge,** 121, 347–354. 2001.



de ROOIJ, D.G. Organization of the seminiferous epithelium and the cycle, and morphometric description of spermatogonial subtypes (rodents and primates) **J.M. Oatley, M.D. Griswold (Eds.). The Biology of Mammalian Spermatogonia.** 2017.

EHMCKE, J.; WISTUBA, J.; SCHLATT, S. Spermatogonial stem cells: questions, models, and perspectives. *Human Reproduction Update*. 12: 275-282. 2006.

FAN L, MOON J, WONG TT, CRODIAN J, COLLODI P. Zebrafish primordial germ cell cultures derived from vasa::RFP transgenic embryos. **Stem Cells Dev**. PMC2741329. 2008.

FANG, C., YU, T., CHU, T., FENG, W., ZHAO, F., WANG, X., HUANG, Y., LI, Y., WAN, P., MEI, W., ZHU, D., FEI, P. Minutes-timescale 3D isotropic imaging of entire organs at subcellular resolution by content-aware compressed-sensing light-sheet microscopy. **Nat Commun** 12, 107. 2021.

FOLTS, L., MARTINEZ, A.S., McKEY, J. Tissue clearing imaging approaches for in toto analysis of the reproductive system. **Biology of Reproduction**. 110: 1041-1054. 2014.

FRANÇA, L.R., NÓBREGA, R.H., MORAIS, R.D.V.S., ASSIS, L.H.D.C., SCHULZ, R.W. Sertoli cell structure and function in anamniote vertebrates. **In Sertoli Cell Biology, Academic Press:** Oxford, UK, pp. 385–407. 2015.

FRETAUD, M., RIVIERE, L., JOB, E., GAY, S., LAREYRE, J-J., JOLY, J-S., AFFATICATI, P., THERMES, V. High-resolution 3D imaging of whole organ after clearing: taking a new look at the zebrafish testis. **Scientific Reports**. 7: 43012. 2017.

FUJIWARA Y, KOMIYA T, KAWABATA H, SATO M, FUJIMOTO H, FURUSAWA M, NOCE T: Isolation of a DEAD-family protein gene that encodes a murine homolog of *Drosophila* vasa and its specific expression in germ cell lineage. **Proc Natl Acad Sci USA**. 91: 12258-12262. 1994.

GAUTIER, A., SOHM, F., JOLY, J-S., LE GAC, F., LAREYRE, J-J. The proximal Promoter Region of the Zebrafish *gsdf* Gene Is Sufficient to Mimic the Spatio-Temporal Expression Pattern of the Endogenous Gene in Sertoli and Granulosa Cells. **Biology of Reproduction**. 2011.

GÓMEZ-GAVIRO, M.V.; SANDERSON, D.; RIPOLL, J.; DESCO, M. Biomedical applications of tissue clearing and three-dimensional imaging in health and disease. **iScience**. 23(8):101432. 2020.

GUSTAFSON, E.A.; WESSEL, G.M. Vasa genes: emerging roles in the germ line and in multipotent cells. **Bioessays**. 32(7):626-37. PMC3090673. 2010.

HAY B, JAN L, JAN Y: A protein component of *Drosophila* polar granules is encoded by vasa and has extensive sequence similarity to ATP-dependent helicases. **Cell**. 55: 577-587. 1988.

HAMA H, KUROKAWA H, KAWANO H, ANDO R, SHIMOGORI T, NODA H, FUKAMI K, SAKAUE-SAWANO A, MIYAWAKI A. Scale: a chemical approach for



fluorescence imaging and reconstruction of transparent mouse brain. **Nat Neurosci.** 14:1481–1488. 2011.

HAMA, H. et al. ScaleS: an optical clearing palette for biological imaging. **Nat. Neurosci.** 18, 1518–1529. 2015.

HUA-MING, X., YI-JIE., R., FA, R., YU, L., TIAN-YU, F., ZHI, W., YE-QING, Y., LI-KUN, Z., JIAN-HONG, H. Recent advances in isolation, identification, and culture of mammalian spermatogonial stem cells. **Asian Journal of Andrology.** 2022.

IBTISHAM, F., HONARAMOOZ, A. Spermatogonial Stem Cells for In Vitro Spermatogenesis and In Vivo Restoration of Fertility. **Cells.** 2020.

LEAL, M.C., CARDOSO, E.R., NÓBREGA, R.H., BATLOUN, I. S.R., BOGERD, J. et al. Histological and stereological evaluation of zebrafish (*Danio rerio*) spermatogenesis with an emphasis on spermatogonial generations. **Biol Reprod** 81(1). 2009.

LESAGE, M., THOMAS, M., BUGEON, J., BRANTHONNE, A., GAY, S., CARDONA, E., HAGHEBAERT, M., MAHÉ, F., BOBE, J., THERMES, V. C-ECi: a CUBIC-Eci combined clearing method for three-dimensional follicular content analysis in the fish ovary. *Biology of Reproduction.* 103: 1099-1109. 2020.

MAOUCHE, A., CURRAN, E., GOUPIL, A.S., SAMBRONI, E., BELLAICHE, J., LE GAC, F., LAREYRE, J.J. New insights into the evolution, hormonal regulation, and spatiotemporal expression profiles of genes involved in the *Gfra1/Gdnf* and *Kit/Kitlg* regulatory pathways in rainbow trout testis. **Fish Physiol Biochem** 44, 1599–1616. 2018.

MCKEY J, CAMERON LA, LEWIS D, BATCHVAROV IS, CAPEL B. Combined iDISCO and CUBIC tissue clearing and light-sheet microscopy for in toto analysis of the adult mouse ovary. **Biol Reprod.** 2020.

MORO, E.; OZHAN-KIZIL, G.; MONGERA, A.; BEIS, D.; WIERZBICKI, C. In vivo Wnt signaling tracing through a transgenic biosensor fish reveals novel activity domains. **Dev. Biol.** 366:327–40. 2012.

NAKATA H, ISEKI S, MIZOKAMI A. Three-dimensional reconstruction of testis cords/seminiferous tubules. **Reprod Med Biol.** 14;20(4):402-409. 2021.

NÓBREGA, R.H., BATLOUNI, S.R. & FRANÇA, L.R. An overview of functional and stereological evaluation of spermatogenesis and germ cell transplantation in fish. **Fish Physiol Biochem** 35, 197–206. 2009.

O'CONNELL, M.R.; OAKES, B.L.; STERNBERG, S.H.; EAST-SELETSKY, A.; KAPLAN, M.; DOUDNA, J.A. Programmable RNA recognition and cleavage by CRISPR/Cas9. **Nature.** 516:263–66. 2014.

PARSONS, M.J.; PISHARATH, H.; YUSUFF, S.; MOORE, J.C.; SIEKMANN, A.F. Notch-responsive cells initiate the secondary transition in larval zebrafish pancreas. **Mech. Dev.** 126:898–912. 2009.



QIN, P.; PARLAK, M.; KUSCU, C.; BANDARIA, J.; MIR, M. Live cell imaging of low- and non-repetitive chromosome loci using CRISPR-Cas9. **Nat. Commun.** 8:14725. 2017.

RAZ, E. The function and regulation of vasa-like genes in germ-cell development. **Genome Biol** 1, reviews. 1017.1. 2000.

SANTI, P.A. Light sheet fluorescence microscopy. **J Histochem Cytochem.** 59:129–138. 2011.

SCHINDELIN, J. et al. Fiji: an open-source platform for biological-image analysis. **Nat. Methods** 9, 676–682. 2012.

SCHULZ, R.W., FRANÇA, L.R., LAREYRE, J.J., LEGAC, F., CHIARINI-GARCIA, H., et al. Spermatogenesis in fish. **Gen Comp Endocrinol** 165(3): 390–411. 2010.

SPANGERBERG, P., HAGEMANN, N., SQUIRE, A., FÖRSTER, N., KRAUß, S.D., QI, Y., YUSUF, A.M., WANG, J., GRÜNEBOOM, A., KOWITZ, L., KORSTE, S., TOTZECK, M., CIBIR, Z., TUZ, A.A., SINGH, V., SIEMES, D., STRUENSEE, L., ENGEL, D.R., LUDEWIG, P., MELO, L.M.N., HELFRICH, I., CHEN, J., GUNZER, M., HERMANN, D.M., MOSIG, A. Rapid and fully automated blood vasculature analysis in 3D light-sheet image volumes of different organs. **Cell Reports Methods.** 3: 100436. 2023.

SUSAKI, E. A. ; TAINAKA, K. ; PERRIN, D. ; KISHINO, F. ; TAWARA, T. ; WATANABE, T.M.; YOKOYAMA, C.; ONOE, H.; EGUCHI, M.; YAMAGUCHI, S.; ABE, T.; KIYONARI, H.; SHIMIZU, Y.; MIYAWAKI, A.; YOKOTA, H.; UEDA, H.R. Whole-Brain Imaging with Single-Cell Resolution Using Chemical Cocktails and Computational Analysis. **Cell.** 157:726–739. 2014.

SUSAKI, E. ; TAINAKA, K. ; PERRIN, D. ; YUKINAGA, H. ; KUNO, A. ; UEDA, H.R. Advanced CUBIC protocols for whole-brain and whole-body clearing and imaging. **Nat. Protoc.** 10: 1709–1727. 2015.

TAINAKA, K.; KUBOTA, S.I.; SUYAMA, T.Q. ; SUSAKI, E.A. ; PERRIN, D. ; UKAI-TADENUMA, M. ; UKAI, H. ; UEDA, H.R. Whole-Body Imaging with Single-Cell Resolution by Tissue Decolorization. **Cell.** 159: 911–924. 2014.

TAINAKA, K., KUNO, A., KUBOTA, S. I., MURAKAMI, T.; UEDA, H. R. Chemical Principles in Tissue Clearing and Staining Protocols for Whole-Body Cell Profiling. **Annu. Rev. Cell Dev. Biol.** 32: 713–741. 2016.

XIE, X., NÓBREGA, R.H., PŠENIČKA, M. Spermatogonial Stem Cells in Fish: Characterization, isolation, enrichment, and recent advances of in vitro culture systems. **Biomolecules.** 10(4): 644. 2020.

WAN, Y.; McDOLLE, K.; KELLER, P.J. Light-sheet microscopy and its potential for understanding developmental process. **Annual Review of Cell and Developmental Biology.** 2019.



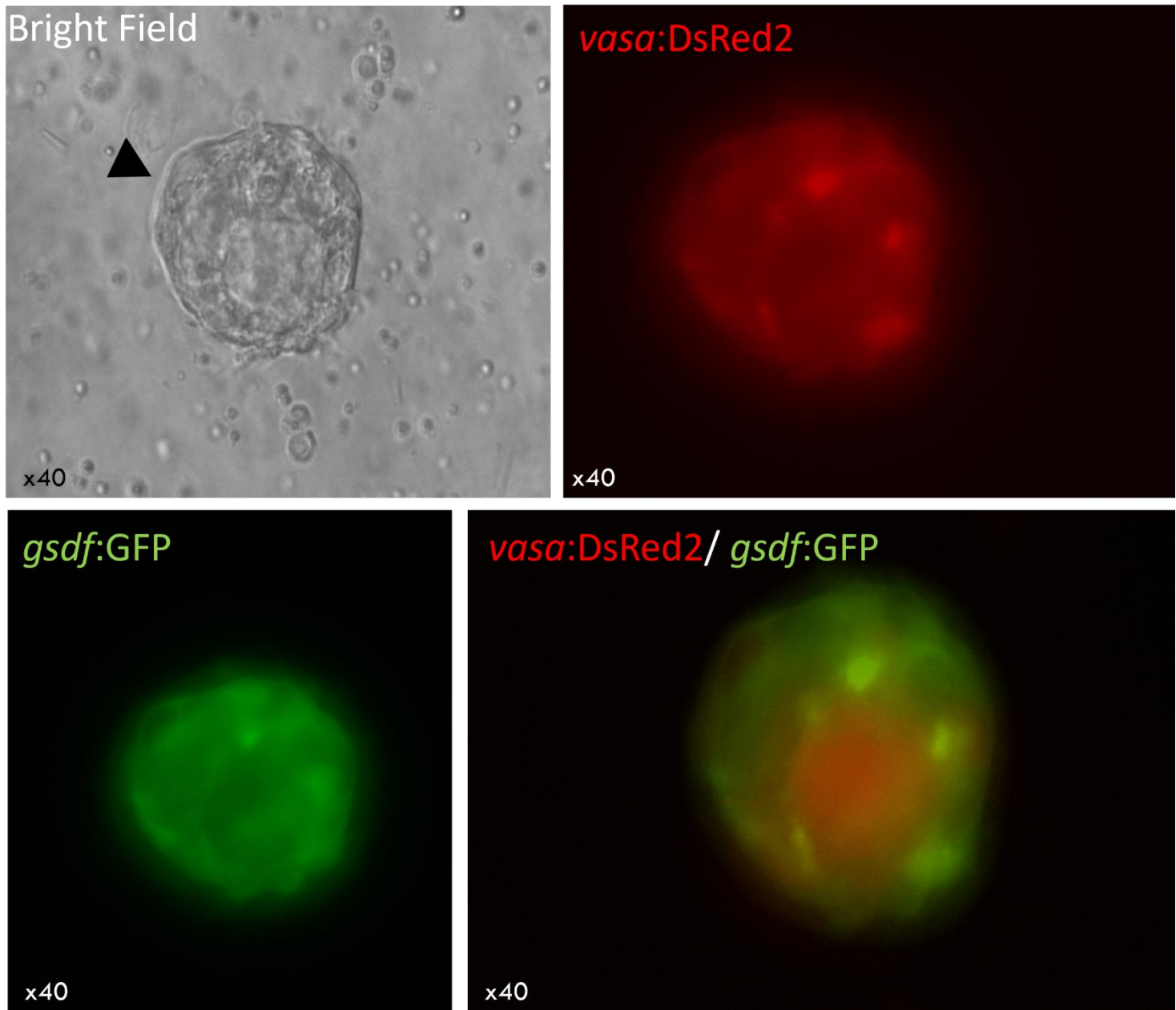
WEIDINGER G, WOLKE U, KOPRUNNER M, KLINGER M, RAZ E: Identification of tissues and patterning events required for distinct steps in early migration of zebrafish primordial germ cells. **Development**. 126: 5295-5307.1999.

ZHANG, D.; CLEVELAND, A.H.; KRIMITZA, E.; HAN, K.; YI, C.; STOUT, A.L.; ZOU, W.; DORSEY, J.F.; GONG, Y.; FAN, Y. Spatial analysis of tissue immunity and vascularity by light sheet fluorescence microscopy. **Nature protocols**. 19: 1053-1084. 2024.

ZHAO X, WAN W, ZHANG X, WU Z, YANG H. Spermatogonial Stem Cell Transplantation in Large Animals. **Animals**. 11:918. 2021.

Attachment 2: Development of testicular organoids as a model for studying fish spermatogenesis and developing innovative aquaculture applications

Manuscript in preparation



The cells from zebrafish testis (Dr_*gsdf*:eGFP/*vasa*: DsRed) were encapsulated in Matrigel and monitored for five days. Here we observed the aggregate development across day 1. Green fluorescence generated by the GFP was detected in Sertoli and red fluorescence was generated by DsRed2 in germ cells (Figure by the author).



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Development of testicular organoids as a model for studying fish spermatogenesis and developing innovative aquaculture applications

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Abstract

Organoids are three-dimensional (3D) cell agglomerates that recapitulate *de novo* tissue morphogenesis, and consequently tissue cytoarchitecture and biological function of a given organ. Therefore, a 3D culture system can open opportunities to better understand spermatogenesis, including spermatogonial differentiation, interactions between spermatogonial stem cells (SSCs) and somatic cells, as well as the mechanisms involved in male infertility and early puberty. Attempts to create organoids originating in several tissues have now been reported in mammals, including testis. However, there are no testicular organoids developed for fish, especially for valuable fish species. In this context, this current study aimed to develop 3D cultures allowing the renewal and/or differentiation of the germ stem cells. Given this context, the experimental design encompassed the following topics: 1) Improving a 3D organotypic culture system to optimize a culture medium to maintain the renewal of the germ stem cell, and 2) a 3D culture system to develop testicular organoids. These interactive topics give insight into the underlying SSCs renewal, and factors involved in the dynamic spermatogenesis in fish, and provide clues for further in-depth research on designing the cellular interaction in the niche using an improvement of models that better mimic the *in vivo* interactions.

Keywords: Teleost fish, testicular organoids, spermatogenesis, biotechnology, organotypic testis



1. Introduction

Organoids are three-dimensional (3D) structures that are comprised of multiple cell types, capable of self-organization to recapitulate *de novo* tissue morphogenesis (Clevers, 2016; Fatehullah et al., 2016; Ho et al., 2018; Sakib et al., 2019). A large number of organoids have been successfully obtained from different organs, including the kidney, brain, gut, thyroid gland, and testes (Clevers, 2016; Sakib et al., 2019; Romero Guevara et al., 2020; Chiaradia & Lancaster, 2020; Gomes & Boudreau, 2021; Van der Vaart et al., 2021).

Testicular organoids have been developed for human and animal models to study spermatogenesis (Pandergraft et al., 2017; Sakib et al., 2019, 2022). Two-dimensional (2D) culture systems are not optimal for studying the complex cell-cell interaction as observed *in vivo* (Kanatsu-Shinohara et al., 2003). Therefore, 3D testicular organoids can open new opportunities to understand testicular physiology, including the process involving spermatogonial differentiation, interactions between spermatogonial stem cells (SSCs) and somatic cells in the germinal niche, as well as the mechanisms involved in male infertility and early puberty (Strange et al., 2018; Sakib et al., 2022).

In fish, spermatogenesis is a complex process that is induced by autocrine/paracrine hormones, growth factors, and cell interactions (Schulz et al., 2010), which occurs in cysts that are formed when a single spermatogonia (primary or type A spermatogonia) is surrounded by the cytoplasmic projections of Sertoli cells (Nóbrega et al., 2009; Leal et al., 2009; Schulz et al., 2010). In fish, Sertoli cells can continuously proliferate even after the onset of puberty (França et al., 2015). SSCs support spermatogenesis throughout adult life by balancing between self-renewal and differentiation to sustain continuously the production of progenitor spermatogonia that differentiate into spermatozoa (de Rooij, 2017; Maouche et al., 2018; Ibtisham & Honaramooz, 2020; Zhao et al., 2021; Hua-Ming et al., 2022). This balance is crucial to avoid stem cell tumors or spermatogenesis depletion (de Rooij, 2001). In addition, this regulation is important for puberty and adult gonadal maturation in seasonal breeders. SSCs differentiation does not occur in juveniles, however, is activated during puberty or at the beginning of the reproductive season. This change in stem cell activity is relevant for aquaculture species (Ji et al., 2011). For example, in rainbow trout (*Oncorhynchus mykiss*) a member of the Salmonidae family, and an important economic species (Hardy, 2002), early puberty can lead to stunting of growth and decreased flesh quality, affecting the economic sector (Okuzawa, 2002). Testicular organoids could be useful tools to understand



spermatogenesis, and regulation of the spermatogonial stem cells in their niche, especially in aquaculture species, such as immature trout.

However, the development of testis organoids in fish research presents several limitations, including challenges in establishing the extracellular matrix, optimizing the cell culture medium, and identifying the most suitable developmental fish stage for obtaining stem cells to produce testis organoids. To address these challenges and devise an efficient protocol for the development of testicular organoids in fish, our approach involved the following steps: initially, we directed our efforts towards improving the culture medium composition for supporting the SSCs survival, self-renewal, proliferation, and differentiation using 3D culture system based on culturing whole zebrafish testis. This involved the tests of different commercial culture media and pH buffering systems. The insights gained from studying 3D organotypic testes were subsequently applied to the development of 3D testicular organoids in mature zebrafish and immature trout testis. By following this iterative approach, testis organoids in fish still hold promise as valuable tools for studying different aspects of reproductive biology, toxicology, and aquaculture. Despite these limitations the refining organoid culture protocols and enhancing tissue engineering techniques, we can understand the potential of testis organoid models in fish studies. With continued innovation and collaboration, these organoid systems will undoubtedly become indispensable tools for advancing research in reproductive biology, ultimately benefiting scientific knowledge, conservation, and aquaculture applications.

2. Methods

2.1 Animal husbandry

In the experiments, we utilized adult male zebrafish from the double transgenic line *Dr_gsdf:eGFP/vasa: DsRed* (Gautier et al., 2011; Lareyre et al, not published), aged between 5 to 12 months. The animals were bred and raised in our facilities (INRA LPGP, Rennes) and maintained under oxygen saturation in a recirculating water system at 28 °C ±, pH 7.5. Zebrafish were exposed to a photoperiod of 16 hours light/ 8 hours dark. For the dissection procedure, the zebrafish were euthanized by immersion in a lethal dose of Tricaine (MS-222) 300 mg/L. Male rainbow trout (*Oncorhynchus mykiss*) (12 months) used in this study were sourced from the INRA experimental fish farm situated in Drennec, France. This facility likely specializes in breeding and maintaining fish populations for research purposes. They were maintained under natural light conditions and a consistent photoperiod at a temperature of 10



°C, which reflects the typical environmental conditions for trout in their native habitats. For the dissection procedure, the animals were euthanized by immersion in a lethal dose of Tricaine (MS-222) 200 mg/L. Subsequently, the testes from transgenic zebrafish and trout were carefully excised from the body cavity and promptly used in the experiments below.

2.2 Testis tissue culture system

Using a previously established *ex vivo* culture system (Leal et al., 2009), adult zebrafish testis from the double transgenic line *Dr_gsd4:eGFP/vasa: DsRed* was used in this approach. Zebrafish ($n = 24$) testis were dissected out, and long-term incubations were performed in three conditions: with Leibovitz-15 (Invitrogen, Carlsbad, CA, USA), according to Leal and colleagues (2009). The culture medium was supplemented with 10 mM HEPES (Merck, Darmstadt, Germany), and the pH was adjusted to 7.4. Incubations were performed in a humidified air atmosphere at 28 °C without CO₂. In the other condition, testes were incubated in Advanced DMEM/F-12 medium (Gibco™) supplemented with 2mM (1x) GlutaMAX, 100x (ASG) antimycotic solution, 0.25 ug/mL streptomycin, 2 ug/mL amphotericin B, 50 mg/mL ampicillin, 2% fetal bovine serum (FBS), 1% rainbow trout plasma, and 40 ng/mL human bFGF (basal culture medium condition), while its contralateral one was incubated in Advanced DMEM/F-12 medium with 10 mM HEPES. In this condition, the organotypic culture was incubated in 5% CO₂, in a humidified air at 28 °C. In the last condition, the testis was incubated in Advanced DMEM/F-12 medium without HEPES, while its contralateral one was incubated in Advanced DMEM/F-12 medium with supplements containing different small molecules: A83-01 (1 μM), PD0325901 (1 μM), and CHIR99021 (3 μM). The main question was whether a cocktail of these small molecules could improve the self-renewal and proliferation of stem cell spermatogonia in the *ex vivo* system. Testes were placed on a nitrocellulose membrane measuring 0.25 cm² (25 μm of thickness and 0.22 μm of porosity) on top of a cylinder of agarose (1.5% w/v, Ringer's solution – pH 7.4) with 1 mL of culture medium into a 24-well plate, where the medium was changed on day 3 of culture. Following the period of incubation (7 days), testes were individually collected for histology analysis, and for future light-sheet (Figure 1).

2.3 Molecules used in the testis tissue culture system



In the current study, our hypothesis centers around achieving the most effective spermatogonia enrichment. We propose that optimal expansion occurs when we induce pathways related to cell proliferation, survival, and self-renewal while simultaneously inhibiting pathways associated with apoptosis and cellular differentiation. By promoting cell proliferation, we expect to stimulate the growth and multiplication of cells within the tissue. Enhanced survival pathways should prevent cell death, leading to a higher overall cell population. Moreover, by encouraging self-renewal pathways, we anticipate that the tissue will maintain its regenerative capacity and continue to replenish its cell pool. On the other hand, we aim to suppress apoptosis, the process of programmed cell death, to reduce cell loss and maintain tissue integrity. By inhibiting differentiation pathways, we seek to prevent cells from maturing into specialized types and prevent their multipotency and versatility. By simultaneously balancing these pathways, we believe we can achieve the most favorable conditions for spermatogonia expansion, leading to significant advancements in our understanding of testis biology. Therefore, through data mining, we have identified a set of small molecules that align with our hypothesis. These molecules can potentially induce the desired pathways for cell proliferation while inhibiting unwanted processes. The selected small molecules are as follows:

1. A 83-01 (70022, stemcell technologies) is a selective inhibitor of the transforming growth factor-beta (TGF- β) type I receptor (ALK5) kinase activity. It acts by blocking the downstream signaling of TGF- β , a cytokine that plays a crucial role in various cellular processes, including cell proliferation, differentiation, migration, and apoptosis. By inhibiting ALK5, A 83-01 effectively prevents the activation of the TGF- β signaling pathway, leading to the suppression of TGF- β -mediated cellular responses (Tojo et al., 2005; Wu et al., 2017);
2. PD0325901 (PD) (72182, stemcell technologies) is a small molecule inhibitor that specifically targets MEK (Mitogen-Activated Protein Kinase Kinase), an enzyme involved in the MAPK (Mitogen-Activated Protein Kinase) signaling pathway. By inhibiting MEK, this molecule effectively blocks the downstream activation of ERK (Extracellular Signal-Regulated Kinase), a key player in cell proliferation and survival (Sebolt-Leopold & Herrera, 2004; Zarrabi et al., 2020);
3. Chir9901 (Chir) (72052, stemcell technologies) is a small molecule that targets Glycogen Synthase Kinase 3 (GSK-3), an enzyme involved in the Wnt signaling pathway. By inhibiting GSK-3, Chir can activate the Wnt signaling cascade, leading to the stabilization and accumulation of β -catenin, a critical transcriptional coactivator. This activation promotes the



self-renewal and proliferation of stem cells and plays an essential role in embryonic development and tissue regeneration (Zarrabi et al., 2020);

2.4 Histological analysis

For histological analysis, testis after tissue organotypic culture were fixed in Bouin-Holland fluid for 3 hours at room temperature. Samples were subsequently dehydrated in aqueous ethanol 70 % and 95 %, then in ethanol/butanol (5:95), and finally butanol. Tissues were embedded in paraffin and sectioned at 6 μm thickness. These sections were stained with Hematoxylin & Eosin. Also, in some cultures, the testis was fixed in 2 % glutaraldehyde and 4 % paraformaldehyde in Sorensen buffer (0.1 M, pH 7.2) at 4 °C overnight. Subsequently, testes were dehydrated, embedded in GMA resin (Technovit 7100 – Heraeus Kulzer, Wehrheim, Germany), sectioned at 3 μm thickness, and stained with Hematoxylin & Eosin, and examined and documented using a Leica microscope (Leica, Heidelberg, Germany).

2.5 Generation of 3D testicular organoids in fish

Testes from sexually immature juvenile trout or sexually mature zebrafish were digested with a trypsin solution containing trypsin 0.15 % or 0,0375 % final concentration, respectively, and 40 mg/mL collagenase H, 200 nM L-Glutamine, 5 % fetal bovine serum (FBS), 50 mg/mL DNase I, and antibiotics (50 $\mu\text{g/mL}$ ampicillin, 50 U/mL penicillin, and 50 $\mu\text{g/mL}$ streptomycin) in L-15 (pH 7.4), at 20 °C for 4 h for trout testis or 28 °C for 1.15 h for zebrafish testis (Figures 2 and 3). During digestion, gentle pipetting was applied every 30 min to physically disperse any remaining intact portions of the testis. Dissociated testicular cells were washed three times by centrifugation with Leibovitz L-15 medium supplemented with 10 % FBS, 25 mM HEPES, and antibiotics to eliminate enzyme activity. The pellets were resuspended in 2 mL of the L-15 medium and were filtered through a nylon screen (42- μm -pore size) to eliminate large clumps of undissociated cells (Shikina & Yoshizaki, 2010; Iwasaki-Takahashi et al., 2020). Different commercially available hydrogels (Matrigel or VitroGel) were tested for establishing fish testicular organoids. Corning Matrigel Matrix for organoid culture is a solubilized basement membrane that supports organoid growth and differentiation of tissue, which has been tested for the ability to form and maintain stable 3D domes/droplets on multiwell plates. Matrigel is extracted from the Engelbreth-Holm-Swarm (EHS) mouse



sarcoma, a tumor rich in extracellular matrix (ECM) proteins, including Laminin (a major component), Collagen IV, heparan sulfate proteoglycans, entactin/nidogen, and several growth factors (Life Sciences). On the other hand, VitroGel is a tunable, xeno-free (animal origin-free) hydrogel system that allows maximum flexibility to manipulate the 3D cell culture environment for cell growth. The VitroGel 3D matrix structure is good for cell spheroid formation, suspension cells, or cells requiring low cell-matrix interactions (The Well Bioscience). Then, testicular cells were encapsulated and cultured for a long time. The forming organoids were maintained using advanced DMEM/F12 (Sigma-Aldrich) (pH 7.8) containing glutamax (200 nM), antibiotics, 10 % FBS, 100 μ l rainbow trout plasma, and 50 ng/mL human bFGF in domes at 18 °C (trout) or higher temperature (zebrafish), in air with medium change every two days (Figures 2 and 3).

3. Results

3.1 *ex vivo* analysis of cultured testicular explants in zebrafish

Previous studies described the utilization of adult zebrafish testicular explants cultured in L-15 supplemented with 10 mM HEPES under an air atmosphere (Leal et al., 2009). Unfortunately, the number of single germ stem cells and spermatogonial doublets remains unstimulated (Figure 4A), although meiotic and post-meiotic cells could be observed after 11-KT supplementation (Figure 4E). We have recently enhanced the culture conditions of testicular explants collected from adult zebrafish testes using an advanced DMEM medium under 5 % CO₂ atmospheric conditions. After 7 days of culture, we observed numerous large single germ stem cells and spermatogonial doublets, while meiotic and post-meiotic cells were dramatically reduced (Figure 4C). We used similar conditions but supplemented advanced DMEM with 10 nM HEPES under 5 % CO₂. We observed numerous large germ stem cells (Aund) (Figure 4D and 5A-F), asymmetrical (Figure 4D, 5A, indicated by black asterisk), and symmetrical (Figure 4D, 5A, indicated by black arrowhead) spermatogonial doublets. Asymmetrical doublets are characterized by two interconnected large cells, with one cell exhibiting a clear nucleus (low chromatin condensation) with a large nucleolus at the periphery of the nucleus, while the second cell displays a darker nucleus (higher chromatin condensation) with multiple lobes of the nucleolus, suggesting a differentiation process. The symmetrical spermatogonial doublets consist of two identical smaller cells with condensed chromatin and a fragmented nucleolus. No meiotic cells are observed in the cultured testis in the absence of 11-ketosterone (11-KT).



The analysis of zebrafish testis cultured with small molecules was inconclusive. Further data is needed to analyze the true effects of these different compounds in zebrafish testis (Figure 4F).

3.2 Trout testicular cells encapsulated in commercial matrix VitroGel

In our preliminary experiments involving the dissociation of immature trout testis using the protocol described above, we observed that approximately 97 % (gate 1, is IP negative) of the cells remained viable by CMF (Figure 6A). This high level of cell viability was confirmed through the use of calcein fluorescent dye (Figure 6B). We observed that alive cells incorporated calcein, a fluorescent dye, indicating cell viability (Figure 7B-C). 3D testicular cell cultures have been successful in mammals because they preserve the stem cell niche and offer the ability to access and manipulate single cells (Pandergraft et al., 2017; Sakib et al., 2019, 2022). However, this system remains poorly studied in fish. To our knowledge, this is one of the few studies within the France-Brazil research group that demonstrated efforts to manipulate fish testis to develop organoids. Despite our efforts to develop fish testicular organoids, we observed that the aggregates from trout testis cells encapsulated into different VitroGel and cultured in DMEM displayed challenges in their growth. We managed to maintain the aggregates for three days. After this time, they do not present growth/development, leading us to hate the cultures. The cells encapsulated in VitroGel versions 1 and 2 displayed different shapes, with structures that did not grow in size or increase in cell density during the observation period. The aggregates stayed small, not exceeding 50 μm . Additionally, we did not observe complex structures or bud formation (Figure 7A-B). In contrast, cells encapsulated in VitroGel versions 3 and 4 formed a thin line/envelope around the small aggregates, but these structures remained disorganized (Figure 7C-D).

3.3 Zebrafish testicular cells encapsulated in commercial matrix Matrigel

After cell dissociation from zebrafish testis, we observed that approximately 87 % (gate 1, IP negative) of the cells remained viable by CMF (Figure 8). We observed different round-shaped structures forming. An epithelial envelope was apparent at the periphery of these structures. The fluorescence intensity was still present on day three, but it decreased by day five. We decided to stop the culture at that point as the aggregates were losing their shape (disaggregating), and the fluorescence signal had weakened (Figure 9A-C). In general, we noted



limited growth of the testicular cell aggregates regardless of the species, including zebrafish and trout, and the extracellular matrix used. We hypothesized that the culture medium or physical conditions were not conducive to germ cell survival and proliferation.

4. Discussion

When designing a 3D culture system, it is crucial to ensure that it can replicate the diverse *in vivo* conditions to accurately mimic physiology, including spermatogenesis in vertebrates, such as fish. Such a system would provide an authentic platform that replicates the dynamic physiological conditions necessary for the homeostatic maintenance of both cells and organs while preserving their functionality. For that reason, testicular organoid system development in fish could be useful tools to understand spermatogenesis, and regulation of the spermatogonial stem cells in their niche, especially in aquaculture species, such as immature trout. However, the development of testis organoids in fish research presents several challenges, such as establishing the extracellular matrix, optimizing the cell culture medium, and identifying the most suitable developmental fish stage to obtain stem cells to produce testis organoids. In our current study, we first evaluated the improvement of the culture medium composition for supporting the SSCs' survival, self-renewal, proliferation, and differentiation using a 3D culture system based on culturing whole zebrafish testis. This involved the tests of different commercial culture media and pH buffering systems.

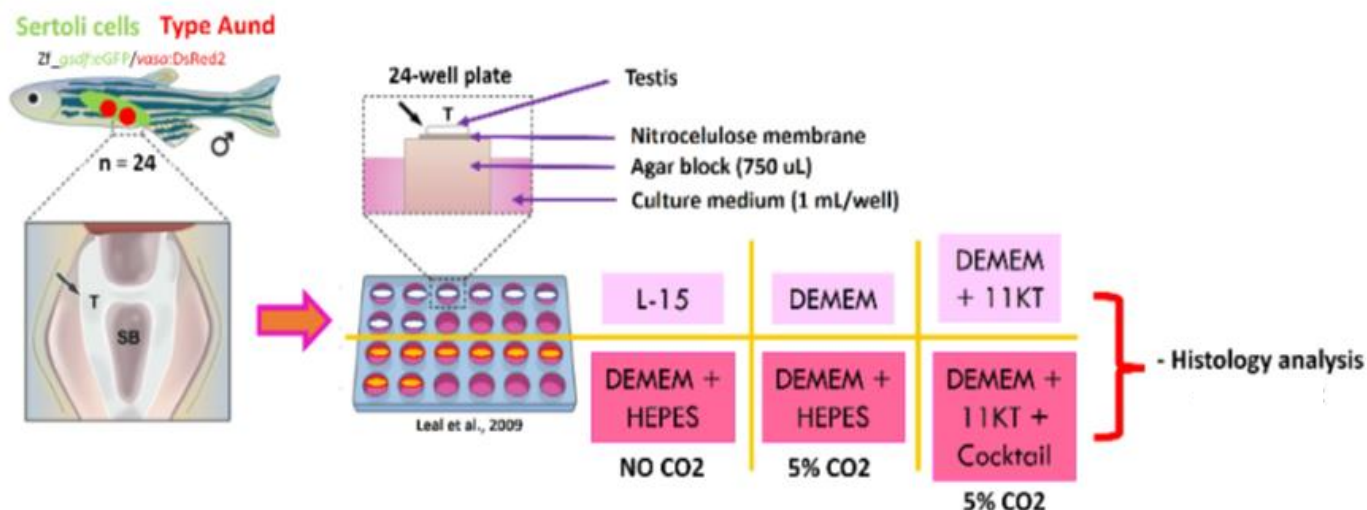
Organ cultures of testicular tissue fragments have been successfully used for zebrafish testis to study spermatogenesis in different contexts (Leal et al., 2009; Tovo-Neto et al., 2020; Doretto et al., 2020; Rodrigues et al., 2022). Therefore, we decide to optimize the culture medium with supplements using an organotypic culture system for zebrafish testis (Leal et al., 2009). Here, we tested advanced DMEM in our organotypic culture because this medium is the most commonly used in 3D culture. We observed an increase in the number of single germ stem cells and spermatogonial doublets compared to the testis cultured in L-15. We hypothesize that DMEM is a superior medium, providing a more stable and balanced environment for cell growth and proliferation than L-15, due to its composition of essential nutrients, vitamins, and minerals.

DMEM can be customized by adjusting factors such as pH. We used DMEM supplemented with 10 nM HEPES under 5 % CO₂. After 7 days of culture, we observed numerous large germ stem cells throughout all the areas of the evaluated testes, as well as



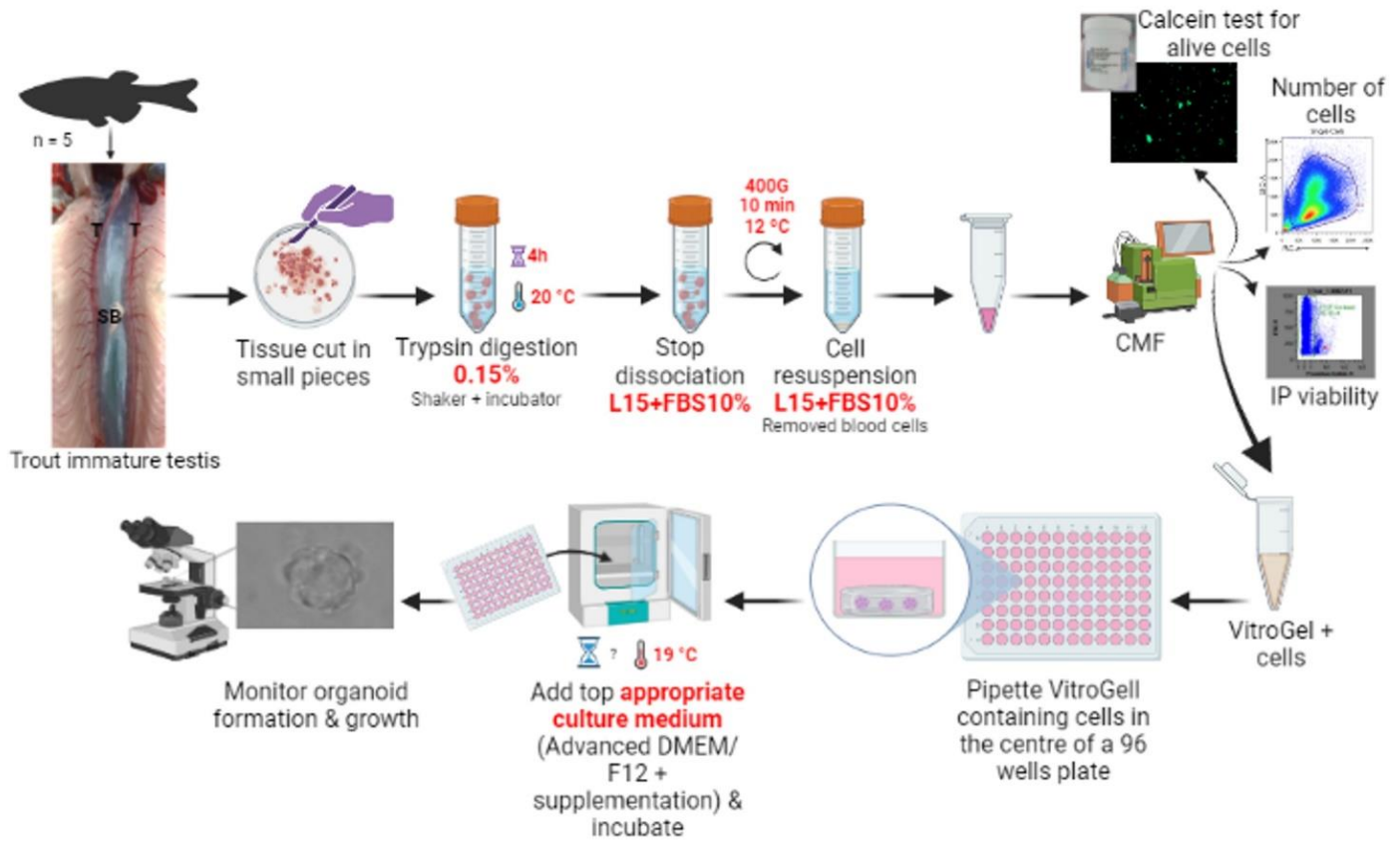
asymmetrical and symmetrical spermatogonial doublets, suggesting a differentiation process. HEPES (acid N-2-hydroxyéthylpipérazine-N-2-éthanesulfonique), is a zwitterionic organic chemical buffer commonly used in cell culture medium. The addition of 10-25 nM HEPES provides extra buffering capacity in the culture outside of a CO₂ incubator, has a limited effect on biochemical reactions, and is chemically and enzymatically stable (Sigma-Aldrich). Despite the positive stimulatory effects on the proliferation of isolated spermatogonial doublets, the use of DMEM medium culture combined with HEPES under 5 % CO₂ in organotypic cultures of zebrafish testis remains uncertain. Further tests should be investigated to understand its role in germ stem cell populations.

Figures



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Figure 1: Here is the schematic diagram of the *ex vivo* zebrafish testicular culture model incubated for 7 days (long-term exposition) under different medium culture conditions, in the presence or absence of 5 % CO₂: basal medium with L-15 with the contralateral testis incubated with DEMEM plus HEPES; DEMEM culture medium compared with DEMEM with HEPES, and DEMEM plus 11- Ketosterone (11-KT) (100 nM) compared with DEMEM plus 11-KT (100 nM) supplemented with cocktails of small molecules. This setup allows for the evaluation of the effects of different culture mediums, pH buffering systems, and additives on zebrafish testis culture over an extended period.



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Figure 2: A diagram illustrating the protocol of testis dissociation for testicular organoid 3D models using xeno-free VitroGel. Immature trout testes were dissected into small pieces and subjected to digestion with 0.15 % trypsin at 20 °C for 4 h. The digestion process was halted by the addition of 10 % FBS in the L-15 medium. Following this, blood cells were removed, and the remaining cells were resuspended. The resuspended solution was then subjected to cell counting using flow cytometry (CMF). The viability of cells was assessed using calcein fluorescent dye, and Iodide Propidium (IP). Subsequently, 1.5×10^6 cells were encapsulated in four different VitroGel formulations (V-ORG3 > V-ORG4 > V-ORG2 > V-ORG1) based on mechanical properties and degradability. The elastic modulation (G') ranged from 50 to 300 Pa, similar to that of a native matrix and Matrigel with some differences in the formulations. Finally, the encapsulated cells were incubated in advanced DMEM/F12 supplemented as described above. Aggregate formation and growth were monitored by optical microscopy over three days.

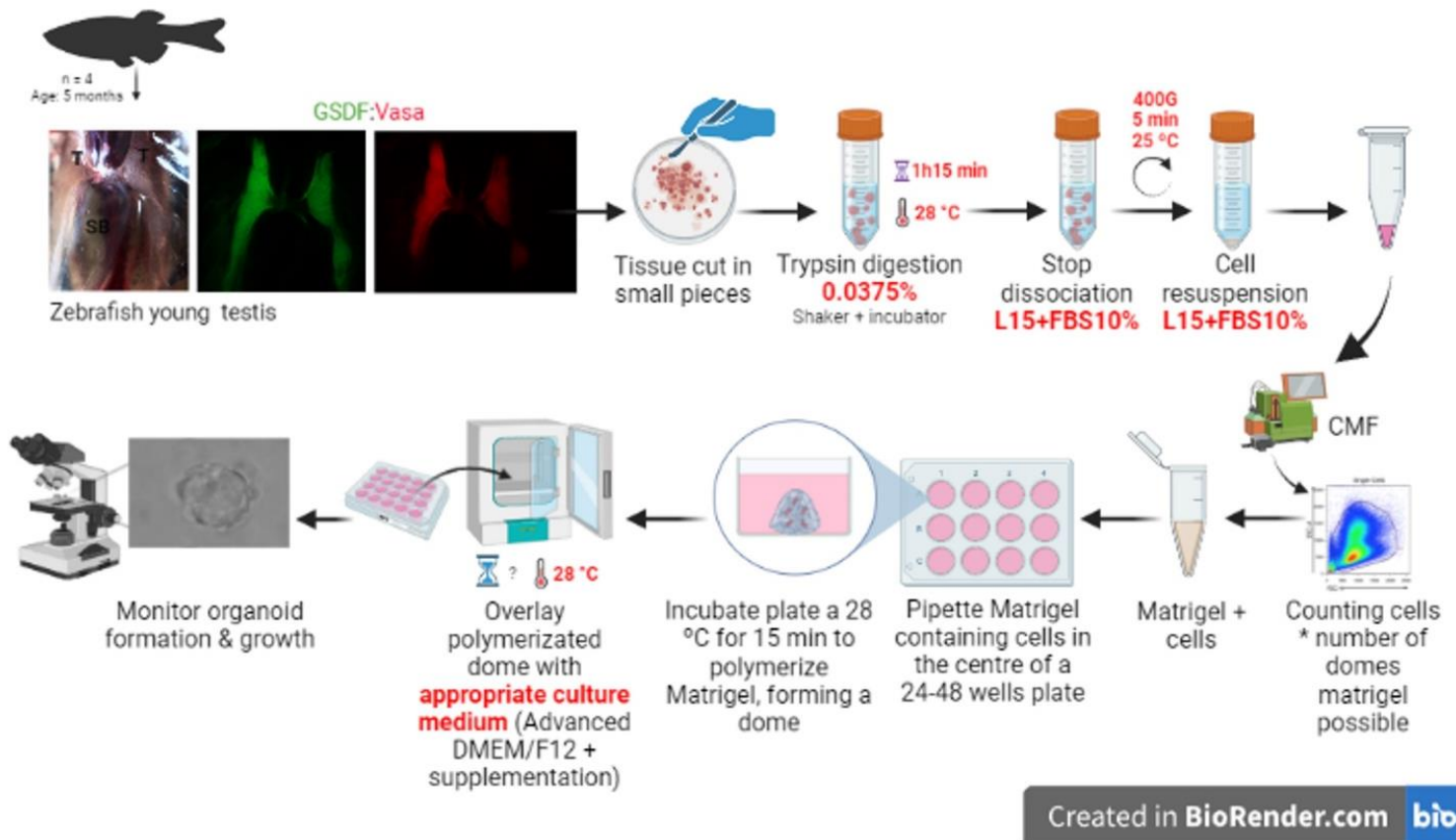


Figure 3: A diagram illustrating the protocol of testis dissociation for testicular organoid 3D models using commercial ECM hydrogel Matrigel. Zebrafish testes were dissected into small pieces and subjected to digestion with 0.0375 % trypsin at 28 °C for 1.15 h. The digestion process was halted by the addition of 10 % FBS in the L-15 medium. Following this, blood cells were removed, and the remaining cells were resuspended. The resuspended solution was then subjected to cell counting using flow cytometry (CMF) to determine the appropriate number of domes was feasible. Subsequently, 0.5×10^6 cells were encapsulated in Matrigel domes. Finally, the encapsulated cells were incubated in advanced DMEM/F12 supplemented as described above. Aggregate formation and growth were monitored by fluorescent microscopy over three days.

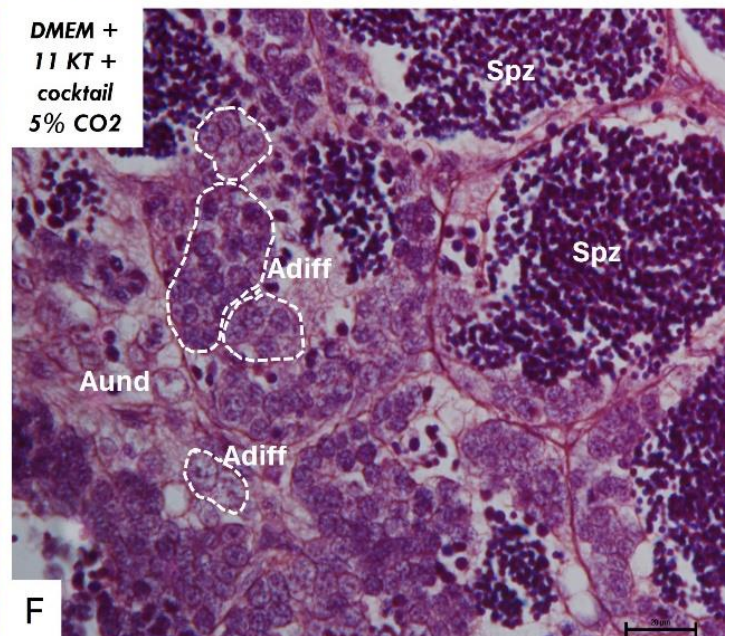
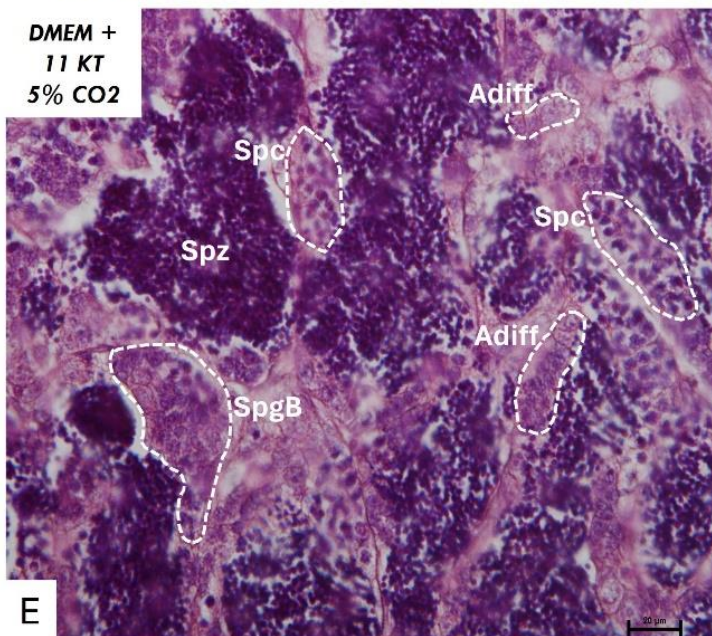
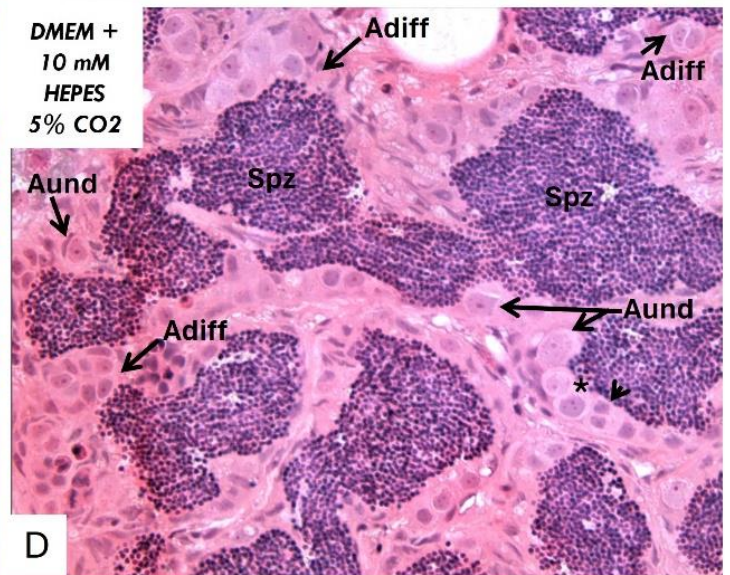
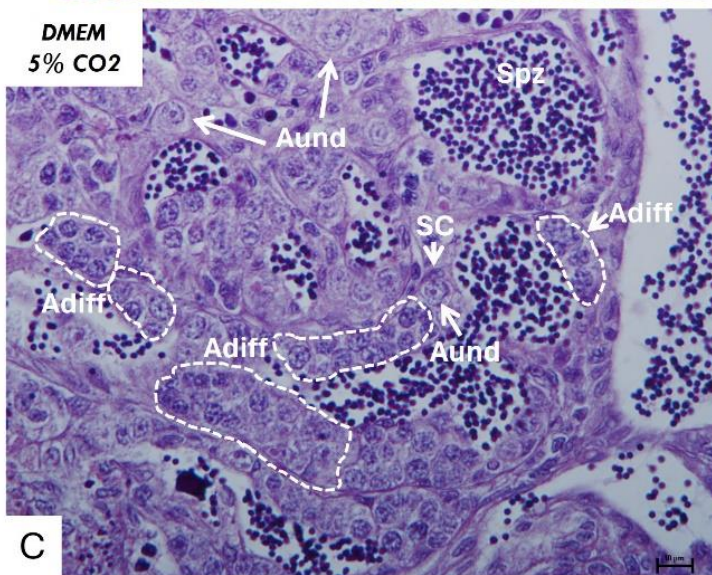
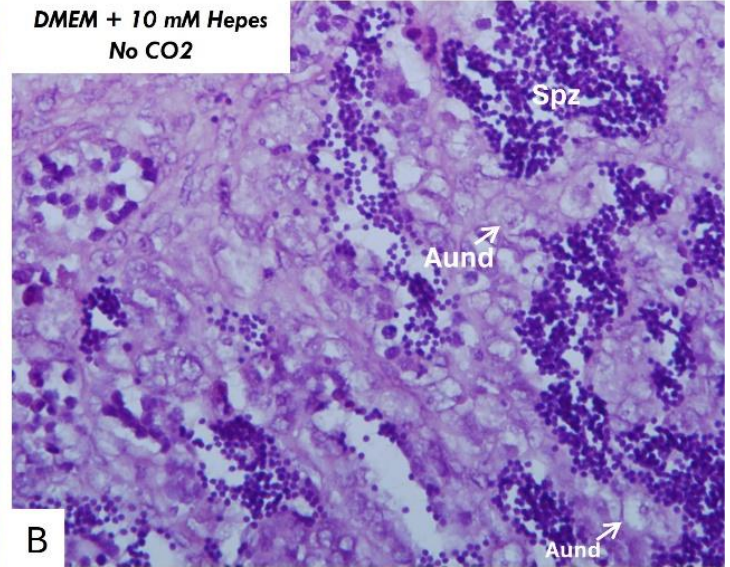
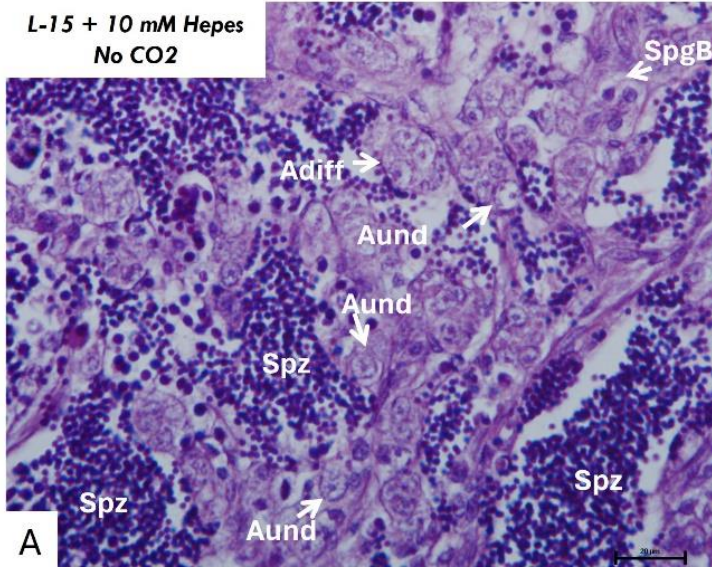




Figure 4: The adult zebrafish testis was cultured for 7 days using different culture mediums, with or without 10 mM HEPES, and with or without 5 % CO₂: one testis was incubated in L-15 **(A)** and the contralateral was cultured in advanced DMEM supplemented with HEPES **(B)** without CO₂. In another group, the testes were incubated in DMEM **(C)** or DMEM supplemented with HEPES **(D)**, in the presence of CO₂. In the last test, we incubated the testes in DMEM supplemented with 11-KT **(E)**, and the contralateral in DMEM with 11-KT supplemented with small molecules **(F)** described in the materials and methods, in the presence of CO₂. Scale bar: 20 µm. Staining: Hematoxylin & Eosin.

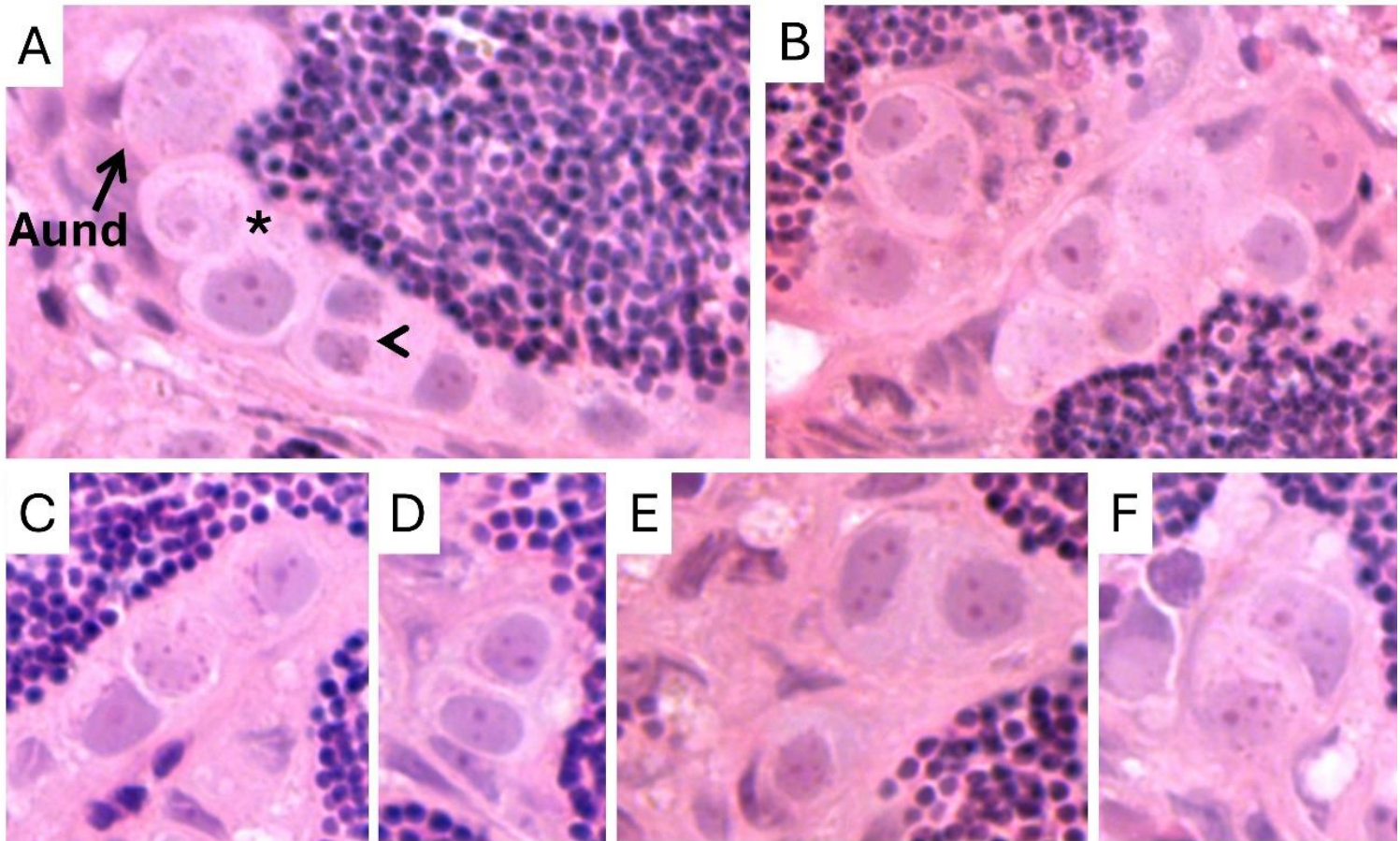


Figure 5: Inset of figure 4D (testes incubated with advanced DMEM supplemented with 10 nM HEPES under 5 % CO₂ atmospheric conditions) showing numerous large single germ stem cells and spermatogonial doublets (A-F). Note the presence of a large single germ stem cell (Aund), asymmetrical (black asterisk), and symmetrical (black arrowhead) (A). Staining: Hematoxylin & Eosin.

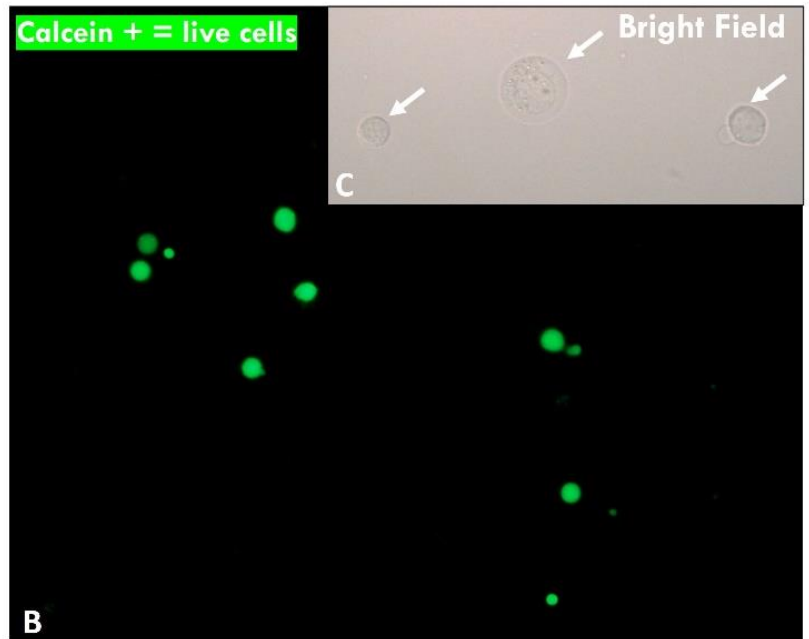
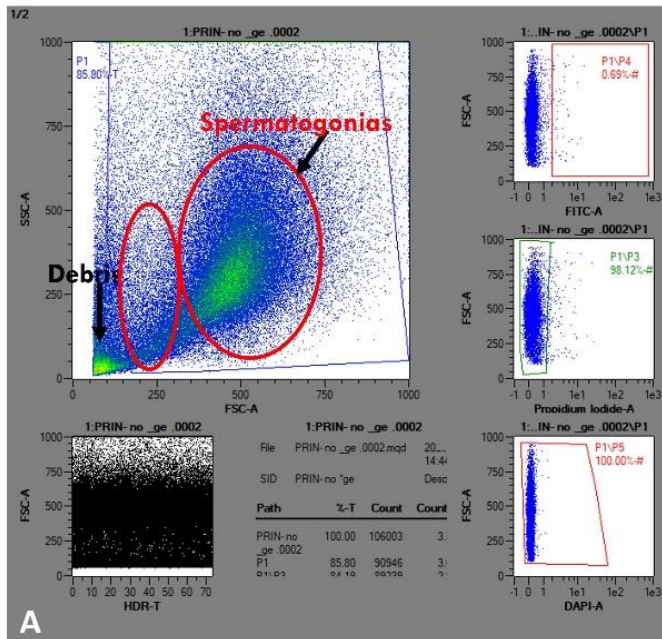


Figure 6: Flow cytometry results indicated that cells from immature testes within Gate 1 were Iodide Propidium (IP) negative, suggesting that the cells were alive (A). We confirm cell viability using calcein fluorescent dye, which was incorporated by live cells, resulting in the expression of green fluorescence (B). The inset of Figure B shows the live cells in a bright field (C).

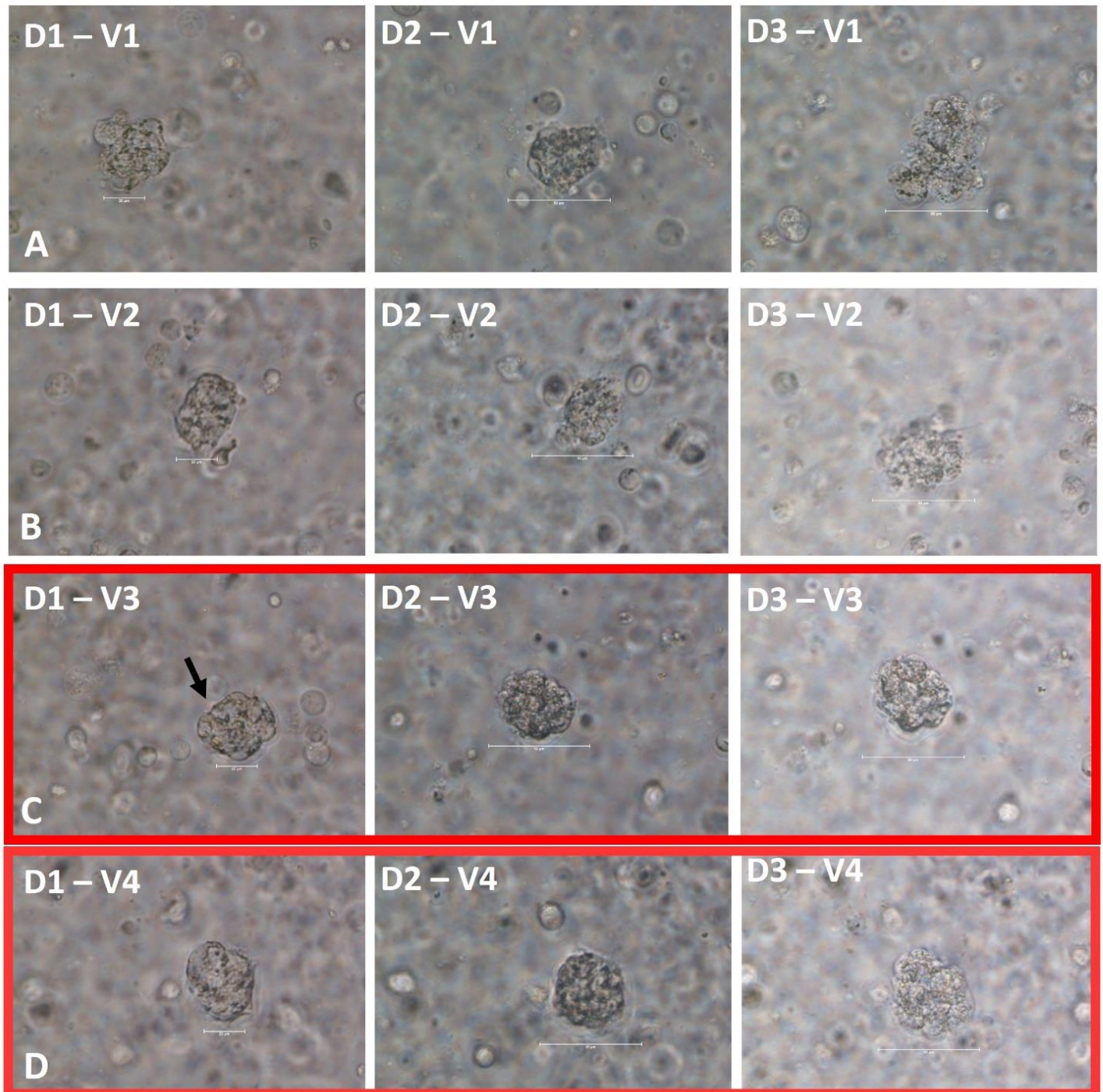


Figure 7: The cells from immature trout testis were encapsulated in different Vitrogel versions (V1, V2, V3, and V4) (A-D), and monitored for three days (D1, D2, and D3). Although the cells encapsulated in Vitrogel versions 3 and 4 (red squares) formed a thin line/envelope around the small aggregates, these structures remained small and disorganized.

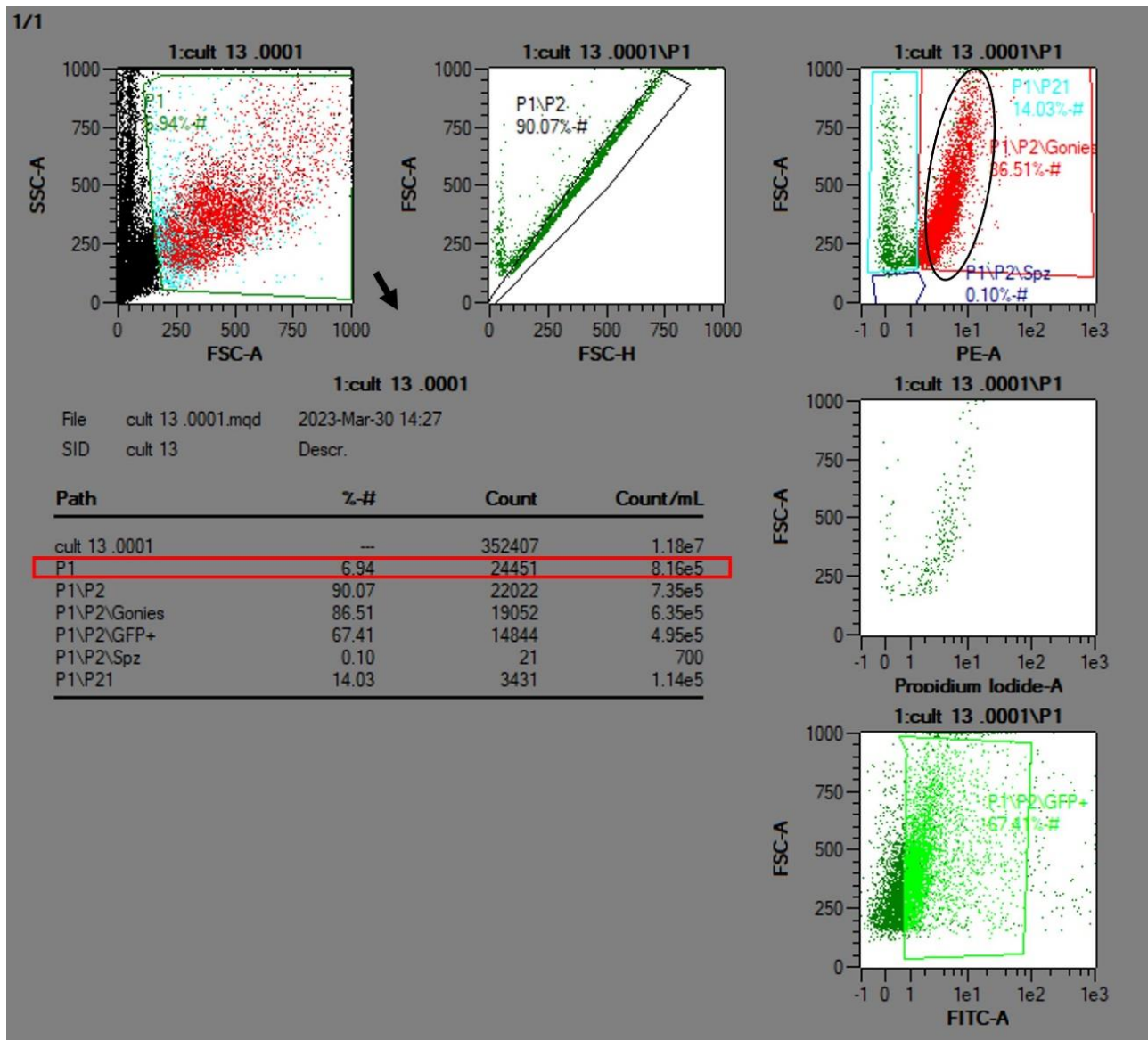


Figure 8: Flow cytometry results indicated that cells from zebrafish testis within Gate 1 were Iodide Propidium (IP) negative, suggesting that the cells were alive.

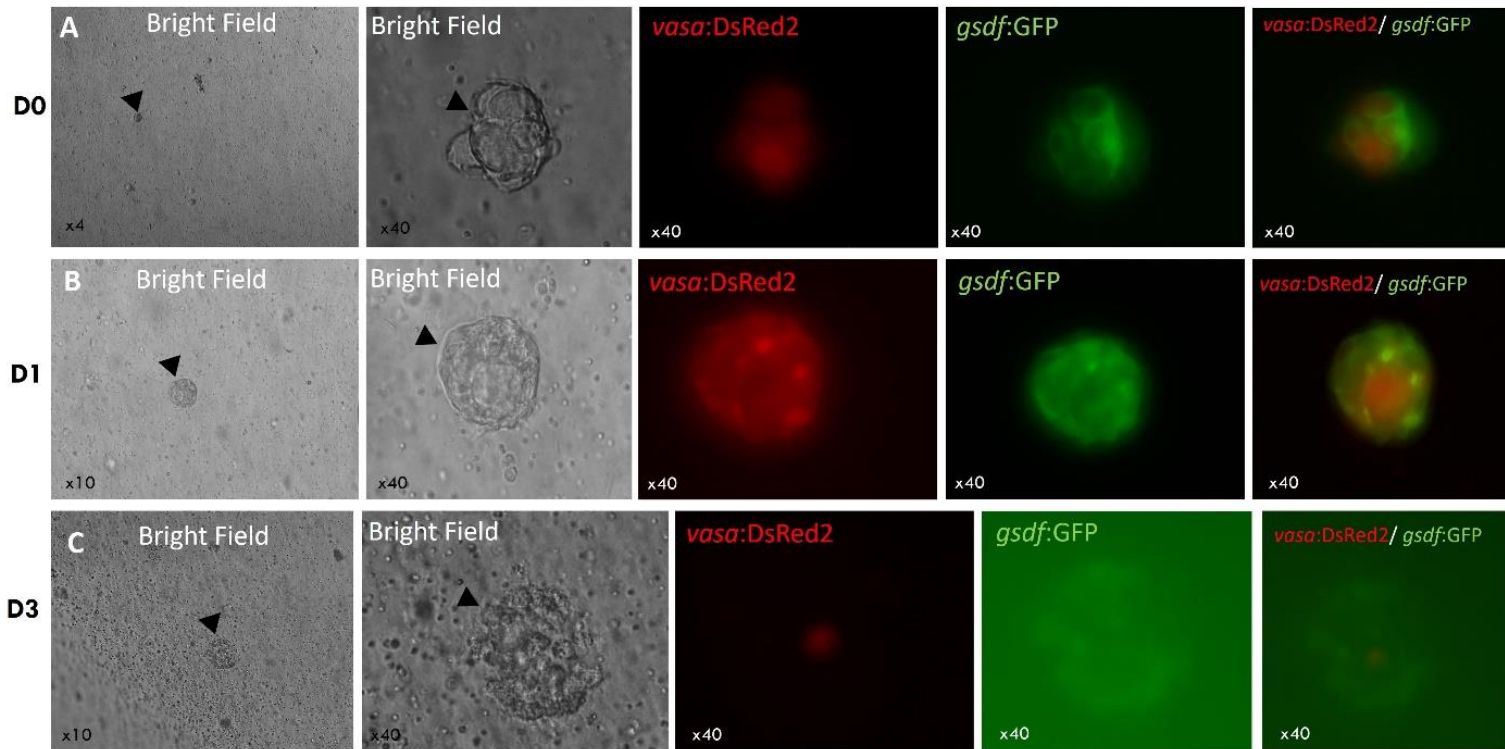


Figure 9: The cells from zebrafish testis were encapsulated in Matrigel and monitored for five days. Here, we observed the same aggregate development across day 0 (D0), day 1 (D1), and day 3 (D3) (A-C). Notably, both fluorescence signals (*Dr_gsdf:eGFP/vasa: DsRed*) were lost by D3 when the aggregates began to disaggregate.



Conclusion and perspective

In this study, we optimized culture conditions by using advanced DMEM/F12 culture medium, pH buffering systems, and additives, along with a 3D culture system designed for whole zebrafish testis. Furthermore, we tested different commercial matrices, such as VitroGel and Matrigel, to develop organoids from immature trout testis and mature zebrafish testis, respectively. We aimed to study the renewal and/or differentiation of germ stem cells. However, for this objective, more tests are needed to evaluate additives in the culture medium, including pH systems, select the optimal fish age and cell types for cultivation to form stable aggregates and eventually organoids, and determine the best matrix for this purpose.

Author Contributions

Conception and design of experiments, J-J.L., M.S.R., N.C.; performance of experiments, M.S.R., N.C.; data analysis, M.S.R., N.C., R.H.N., J-J.L.; contribution of reagents/materials/analysis tools, J-J.L.; writing of the manuscript, M.S.R., N.C., R.H.N., J-J.L. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement

The French European Union regulation concerning the protection of experimental animals approved the animal study protocol. All the experimental procedures were conducted in agreement with the guidelines for the care and use of animals at the National Veterinary School of Nantes, France.

Informed Consent Statement

Not applicable.

Data Availability Statement



Data is contained within the article or supplementary material. The data presented in this study are available in this manuscript and supplemental material.

Conflicts of Interest

The authors declare no conflict of interest.

5. References

- BRAAT A, ZANDBERGEN T, VAN DE WATER S, GOOS H, ZIVKOVIC D: Characterization of zebrafish primordial germ cells: morphology and early distribution of vasa RNA. **Dev Dyn.** 216: 153-167. 1999.
- BRAAT AK, VAN DE WATER S, GOOS H, BOGERD J, ZIVKOVIC D. Vasa protein expression and localization in the zebrafish. **Mech Dev.** Jul;95(1-2):271-4. 2000.
- CHIARADIA, I., LANCASTER, M.A. Brain organoids for the study of human neurobiology at the interface of *in vitro* and *in vivo*. **Nat Neurosci** 23, 1496–1508. 2020.
- CLEVERS, H. Modeling development and disease with organoids. **Cell** 165. 2016.
- de ROOIJ, D.G. Proliferation and differentiation of spermatogonial stem cells. **Reproduction-Cambridge**, 121, 347–354. 2001.
- de ROOIJ, D.G. Organization of the seminiferous epithelium and the cycle, and morphometric description of spermatogonial subtypes (rodents and primates) J.M. Oatley, M.D. Griswold (Eds.). **The Biology of Mammalian Spermatogonia**. 2017.
- FAN L, MOON J, WONG TT, CRODIAN J, COLLODI P. Zebrafish primordial germ cell cultures derived from vasa::RFP transgenic embryos. **Stem Cells Dev.** Jun;17(3):585-97. doi: 10.1089/scd.2007.0178. PMID: 18576915; PMCID: PMC2741329. 2008.
- FATEHULLAH, A., TAN, S.H., BARKER, N. Organoids as an *in vitro* model of human development and disease. **Nat Cell Biol.**18(3):246-254. 2016.
- FRANÇA, L.R., NÓBREGA, R.H., MORAIS, R.D.V.S., ASSIS, L.H.D.C., SCHULZ, R.W. Sertoli cell structure and function in anamniote vertebrates. **In Sertoli Cell Biology, Academic Press: Oxford, UK**, pp. 385–407. 2015.
- FRETAUD, M., RIVIERE, L., JOB, E., GAY, S., LAREYRE, J-J., JOLY, J-S., AFFATICATI, P., THERMES, V. High-resolution 3D imaging of whole organ after clearing taking a new look at the zebrafish testis. **Scientific Reports**, 7, 43012. 2017.
- FUJIWARA Y, KOMIYA T, KAWABATA H, SATO M, FUJIMOTO H, FURUSAWA M, NOCE T: Isolation of a DEAD-family protein gene that encodes a murine homolog of *Drosophila vasa* and its specific expression in germ cell lineage. **Proc Natl Acad Sci USA.** 91: 12258-12262. 1994.



GAUTIER, A., SOHM, F., JOLY, J-S., LE GAC, F., LAREYRE, J-J. The proximal Promoter Region of the Zebrafish *gsdf* Gene Is Sufficient to Mimic the Spatio-Temporal Expression Pattern of the Endogenous Gene in Sertoli and Granulosa Cells. **Biology of Reproduction**. 2011.

GÓMEZ-BOUDREAU. Organoids and Their Use in Modeling Gut Epithelial Cell Lineage Differentiation and Barrier Properties During Intestinal Diseases. *Front. Cell Dev. Biol.*, 13 August. 2021.

GUSTAFSON EA, WESSEL GM. Vasa genes: emerging roles in the germ line and in multipotent cells. **Bioessays**. Jul;32(7):626-37. 2010.

HARDY, R.W., GATLIN III, D.M. Nutritional strategies to reduce nutrient losses in intensive aquaculture. J.R. Tomasso (Ed.), *Aquaculture and the Environment in the United States*, World **Aquaculture Society**, Baton Rouge, LA, 155-165. 2002.

HUA-MING, X., YI-JIE., R., FA, R., YU, L., TIAN-YU, F., ZHI, W., YE-QING, Y., LI-KUN, Z., JIAN-HONG, H. Recent advances in isolation, identification, and culture of mammalian spermatogonial stem cells. **Asian Journal of Andrology**. 2022.

HAMA, H. et al. ScaleS: an optical clearing palette for biological imaging. **Nat. Neurosci.** 18, 1518–1529. 2015.

HAY B, JAN L, JAN Y: A protein component of Drosophila polar granules is encoded by vasa and has extensive sequence similarity to ATP-dependent helicases. **Cell**. 55: 577-587. 1988.

HO, B.X., PEK, N.M.Q., SOH, B.S. Disease Modeling Using 3D Organoids Derived from Human Induced Pluripotent Stem Cells. **Int J Mol Sci**. 2018.

IBTISHAM, F., HONARAMOOZ, A. Spermatogonial Stem Cells for In Vitro Spermatogenesis and In Vivo Restoration of Fertility. **Cells**. 2020.

JI X., LIU H., CHEN S., JIANG Y. & TIAN Y. Growth differences and dimorphic expression of growth hormone (GH) in female and male *Cynoglossus semilaevis* after male sexual maturation. **Marine Genomics**, 4, 9–16. 2011.

KANATSU-SHINOHARA, M., OGONUKI, N., INOUE, K., MIKI, H., OGURA, A., TOYOKUNI, S., SHINOHARA, T. Long-term proliferation in culture and germline transmission of mouse male germline stem cells. **Biol Reprod** 69(2):612–616. 2003.

LEAL, M.C., CARDOSO, E.R., NÓBREGA, R.H., BATLOUN, I S.R., BOGERD, J. et al. Histological and stereological evaluation of zebrafish (*Danio rerio*) spermatogenesis with an emphasis on spermatogonial generations. **Biol Reprod** 81(1). 2009.

MAOUCHE, A., CURRAN, E., GOUPIL, AS., SAMBRONI, E., BELLAICHE, J., LE GAC, F., LAREYRE, J.J. New insights into the evolution, hormonal regulation, and spatiotemporal expression profiles of genes involved in the *Gfra1/Gdnf* and *Kit/Kitlg* regulatory pathways in rainbow trout testis. **Fish Physiol Biochem** 44, 1599–1616. 2018.



NAKATA H, ISEKI S, MIZOKAMI A. Three-dimensional reconstruction of testis cords/seminiferous tubules. **Reprod Med Biol**. 14;20(4):402-409. 2021.

NÓBREGA, R.H., BATLOUNI, S.R. & FRANÇA, L.R. An overview of functional and stereological evaluation of spermatogenesis and germ cell transplantation in fish. **Fish Physiol Biochem** 35, 197–206. 2009.

OKUZAWA, K. Puberty in teleost. **Fish Physiology and Biochemistry** 26, 31–41. 2002.

PENDERGRAFT, S.S., SADRI-ARDEKANI, H., ATALA, A., BISHOP, C.E. Three-dimensional testicular organoid: a novel tool for the study of human spermatogenesis and gonadotoxicity *in vitro*. **Biol Reprod**. 96(3):720-732. 2017.

RAZ, E. The function and regulation of vasa-like genes in germ-cell development. **Genome Biol** 1, reviews. 1017.1. 2000.

RODRIGUES, M.S., TOVO-NETO, A., ROSA, I.F., DORETTO, L. B., FALLAH, H.P., HABIBI, H.R., NÓBREGA, R.H. Thyroid Hormones Deficiency Impairs Male Germ Cell Development: A Cross Talk Between Hypothalamic-Pituitary-Thyroid, and—Gonadal Axes in Zebrafish. **Front. Cell Dev. Biol. Sec. Molecular and Cellular Pathology**. 2022.

ROMERO-GUEVARA, R., LOANNIDES, A., XINARIS, C. Kidney Organoids as Disease Models: Strengths, Weaknesses and Perspectives. **Frontiers**. 2020.

SAKIB, S., GOLDSMITH, T., VOIGT, A., & DOBRINSKI, I. Testicular organoids to study cell-cell interactions in the mammalian testis. **Andrology**. 2019.

SAKIB, S., LARA, N.L.M., HUYNH, B.C., DOBRINSKI, I. Organotypic Rat Testicular Organoids for the Study of Testicular Maturation and Toxicology. **Front. Endocrinol. Reproduction**. 2022.

SCHINDELIN, J. et al. Fiji: an open-source platform for biological-image analysis. **Nat. Methods** 9, 676–682. 2012.

SCHULZ, R.W., FRANÇA, L.R., LAREYRE, J.J., LEGAC, F., CHIARINI-GARCIA, H., et al. Spermatogenesis in fish. **Gen Comp Endocrinol** 165(3): 390–411. 2010.

SEBOLT-LEOPOLD, JS., HERRERA, R. Targeting the mitogen-activated protein kinase cascade to treat cancer **Nat Rev Cancer**, 4. pp. 937-947.2004.

STRANGE, D.P., ZARANDI, N.P., TRIVEDI, G., ATALA, A., BISHOP, C.E., SADRI-ARDEKANI, H. & VERMA, S. Human testicular organoid system as a novel tool to study Zika virus pathogenesis. **Emerging Microbes and Infections** 7 82. 2018.

SUSAKI, E. A. et al. Whole-Brain Imaging with Single-Cell Resolution Using Chemical Cocktails and Computational Analysis. **Cell** 157, 726–739. 2014.

SUSAKI, E. A. et al. Advanced CUBIC protocols for whole-brain and whole-body clearing and imaging. **Nat. Protoc**. 10, 1709–1727. 2015.



TAINAKA, K. et al. Whole-Body Imaging with Single-Cell Resolution by Tissue Decolorization. **Cell** 159, 911–924. 2014.

TAINAKA, K., KUNO, A., KUBOTA, S. I., MURAKAMI, T. & UEDA, H. R. Chemical Principles in Tissue Clearing and Staining Protocols for Whole-Body Cell Profiling. **Annu. Rev. Cell Dev. Biol.** 32, 713–741. 2016.

TOJO M, HAMASHIMA Y, HANYU A, KAJIMOTO T, SAITOH M, MIYAZONO K, NODE M, IMAMURA T. The ALK-5 inhibitor A-83-01 inhibits Smad signaling and epithelial-to-mesenchymal transition by transforming growth factor-beta. **Cancer Sci.** 96(11):791-800. 2005.

VAN DER VAART, J., BOSMANS, L., SIJBESMA, S.F., KNOOPS, K., VAN DE WETERING, W. J., OTTEN, H. G., BEGTHEL, H., RINKES, I. H. M. B., KORVING, J., LENTJES, E. G. W. M., LOPEZ-IGLESIAS, C., PETERS, P. J., VAN SANTEN, H.M., VRIENS, M.R., CLEVERS, H. Adult mouse and human organoids derived from thyroid follicular cells and modeling of Graves' hyperthyroidism. **PNAS.** 2021.

WEIDINGER G, WOLKE U, KOPRUNNER M, KLINGER M, RAZ E: Identification of tissues and patterning events required for distinct steps in early migration of zebrafish primordial germ cells. **Development.** 126: 5295-5307.1999.

WU Z.J., LI Y., WU Y.Z., WANG Y., NIAN W.Q., WANG L.L., LI L.C., LUO H.L., WANG D.L. Long non-coding RNA CCAT2 promotes the breast cancer growth and metastasis by regulating TGF- β signaling pathway. **Eur. Rev. Med. Pharmacol. Sci.** 21:706–714. 2017.

ZARRABI M, AFZAL E, ASGHARI MH, EBRAHIMI M. Combination of SB431542, Chir9901, and Bpv as a novel supplement in the culture of umbilical cord blood hematopoietic stem cells. **Stem Cell Res Ther.** 9;11(1):474. 2020.

ZHAO X, WAN W, ZHANG X, WU Z, YANG H. Spermatogonial Stem Cell Transplantation in Large Animals. **Animals.** 11:918. 2021.



Appendix

CERTIFICADO

Certificamos que a proposta intitulada "Hormônios tireoidianos e sua relação com o eixo reprodutivo-somatotrófico e seu possível papel no desenvolvimento embrionário em resposta ao estresse materno em zebrafish (*Danio rerio*)", protocolada sob o CEUA nº 8520250320 (ID 000055), sob a responsabilidade de **Prof. Dr. Rafael Henrique Nóbrega** e equipe; *Maira da Silva Rodrigues* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Universidade Estadual Paulista (IBB/UNESP) na reunião de 03/04/2020.

We certify that the proposal "Thyroid hormones and the relation to the reproductive-somatotropic axis and their possible role in embryonic development in response to maternal stress in zebrafish (*Danio rerio*)", utilizing 82 Fishes (males and females), protocol number CEUA 8520250320 (ID 000055), under the responsibility of **Prof. Dr. Rafael Henrique Nóbrega** and team; *Maira da Silva Rodrigues* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the São Paulo State University (IBB/UNESP) in the meeting of 04/03/2020.

Finalidade da Proposta: Pesquisa (Acadêmica)

Vigência da Proposta: de 04/2020 a 06/2023

Área: Ciências Biológicas

Origem: Animais provenientes de estabelecimentos comerciais

Espécie: Peixes

sexo: Machos e Fêmeas

idade: 4 a 12 meses

N: 82

Linhagem: *Danio rerio* (Zebrafish)/AB

Peso: 0200 a 0300 g

Local do experimento: Todos os experimentos serão conduzidos no Laboratório de Biologia Molecular e Reprodutiva, sob orientação do Prof. Dr. Rafael Henrique Nóbrega - Departamento de Biologia Estrutural e Funcional - Instituto de Biociências de Botucatu.

Botucatu, 03 de abril de 2020

Prof. Dr. Wellerson Rodrigo Scarano
Coordenador da Comissão de Ética no Uso de Animais
Universidade Estadual Paulista

Prof. Dr. Bruno César Schimming
Vice-Coordenador da Comissão de Ética no Uso de Animais
Universidade Estadual Paulista



**República Federativa do Brasil
Universidade de São Paulo
Instituto de Ciências Biomédicas**

A Diretora do Instituto de Ciências Biomédicas, nos termos do artigo 74, parágrafo único, inciso 5, alínea "b", do Estatuto da Universidade de São Paulo, certifica que

Maira da Silva Rodrigues



de nacionalidade brasileira,
portadora da cédula de identidade RG nº 489839903 SP,
nascida a 3 de maio de 1993 e natural do Estado de São Paulo,
concluiu o



**Curso de Extensão Universitária na modalidade de Difusão:
Princípios éticos e manejo de animais em pesquisa**

E, para que possa gozar de todos os direitos e prerrogativas legais, outorga-lhe o presente
Certificado.

São Paulo, 28 de junho de 2024

**Presidente da Comissão de Cultura e
Extensão Universitária**
Carla Roberta de Oliveira Carvalho

Diretora
Patrícia Gama

Código de controle: 285N-JTN4-WU5P-TTTL

Período do Curso:	11/04/2024 a 24/06/2024
Carga Horária:	40:00 horas
Frequência:	100.00%
Área de Conhecimento:	Manejo de Animais
Avaliação Final:	Aprovado

Recredenciamento junto ao MEC através da Portaria nº 503, de 19 de julho de 2022.