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“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

**BOTUCATU – SP
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“Júlio de Mesquita Filho”

INSTITUTO DE BIOCIÊNCIAS DE BOTUCATU

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MAIRA DA SILVA RODRIGUES

DR. RAFAEL HENRIQUE NÓBREGA

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 μ g), 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 μ g 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*, *thrβ*, *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 somatotrophic 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



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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).

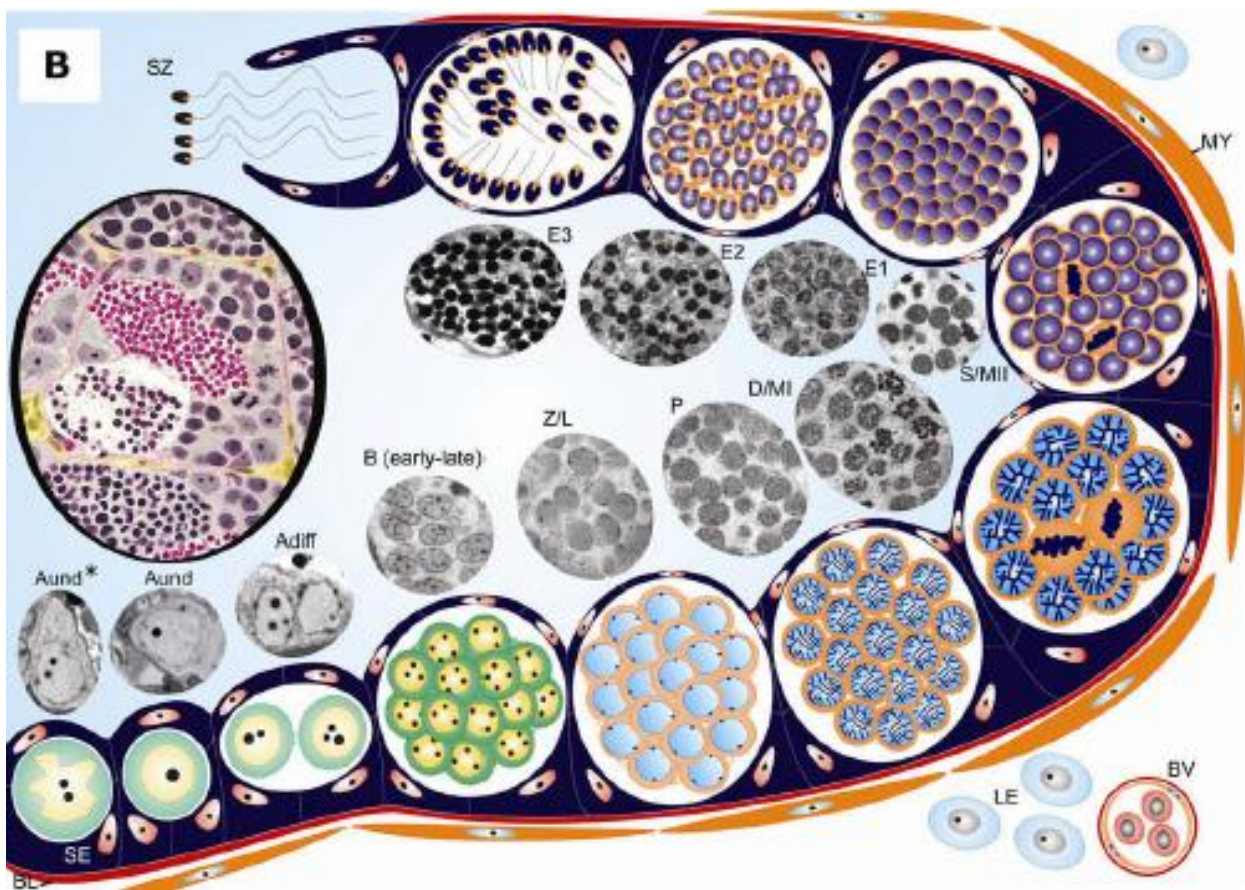
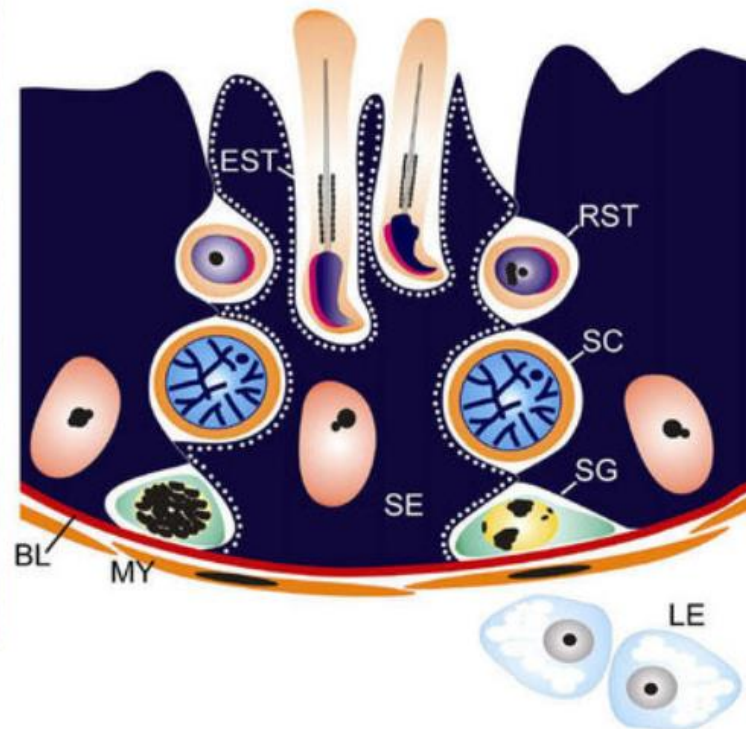
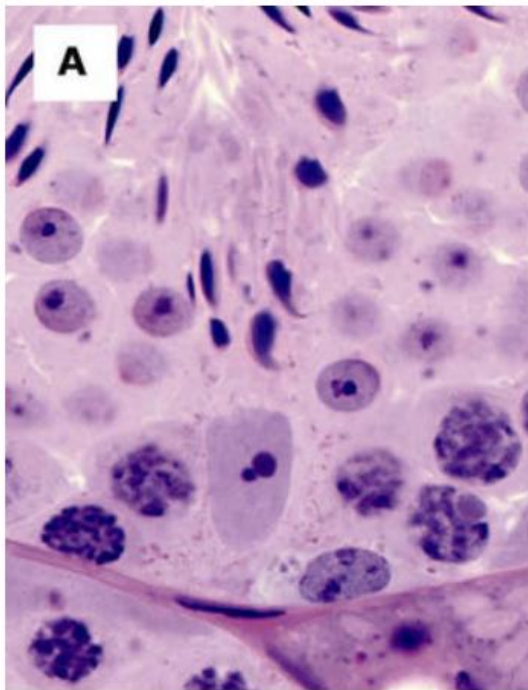


Figure 1. Comparison between spermatogenesis in mouse **(A)** and zebrafish **(B)**. Sertoli cells (SE), basal lamina (BL), and peritubular myoid cells (MY); interstitial Leydig cells (LE) and blood vessels (BV) are shown. **(A)** - Spermatogonia (SG), spermatocyte (SC), round spermatid (EST), and elongated 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



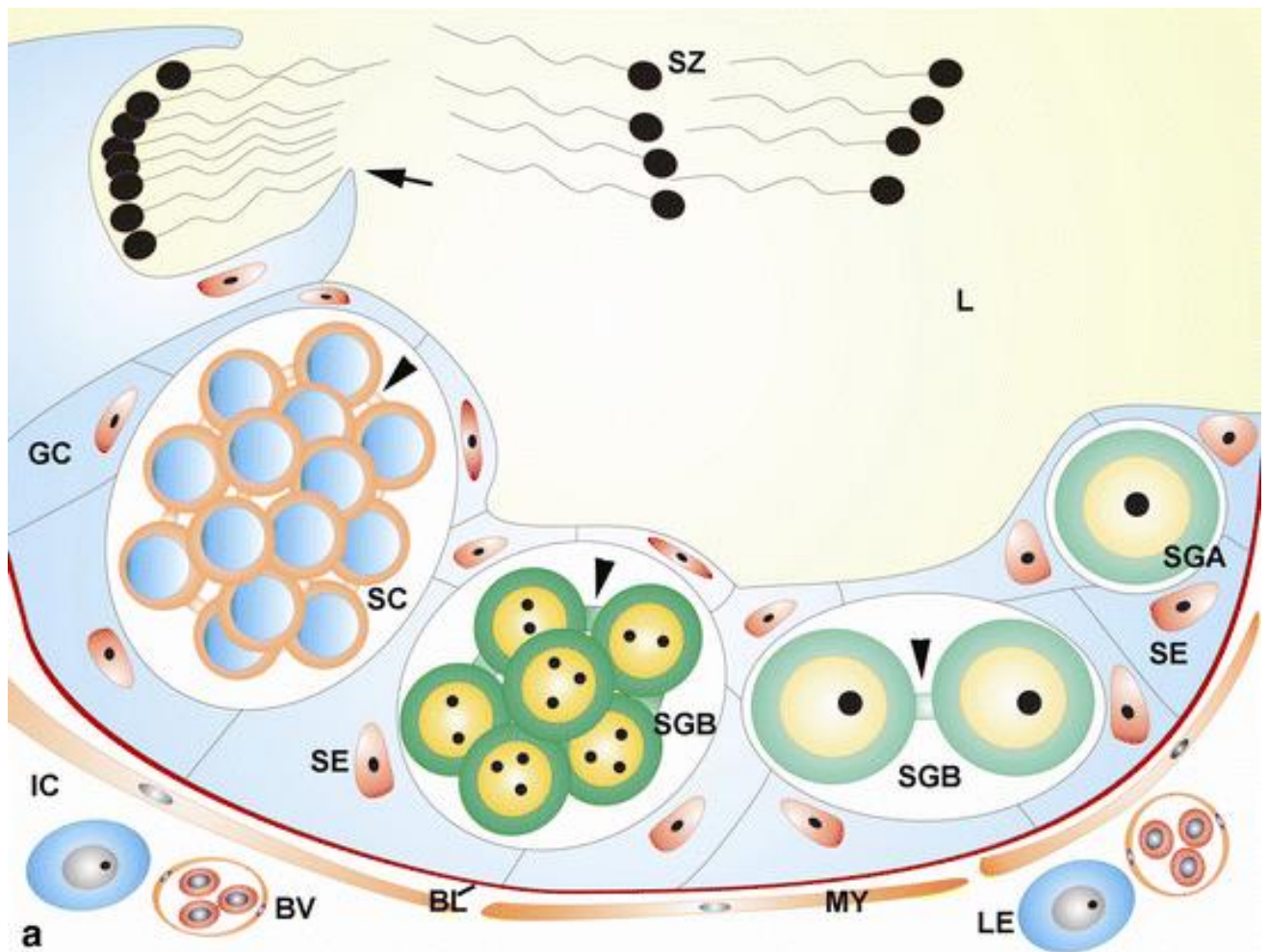
their nuclear envelope and greater nucleus-cytoplasm ratio. Additionally, by incorporation of BrdU (5'-bromo-2'-deoxyuridine), a synthetic nucleotide analogous to thymidine, during the synthesis phase (S phase), it is feasible to determine the presence of putative stem spermatogonia, known as "labeling-retaining 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

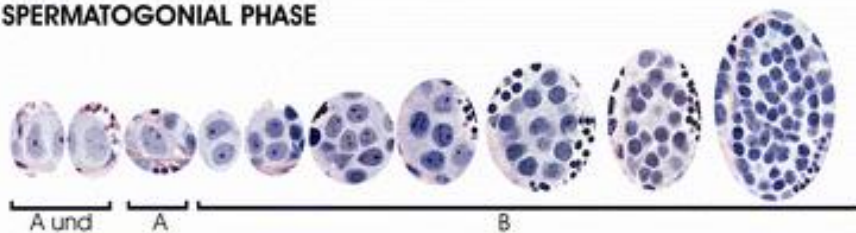
Spermatocytes have an average nuclear diameter of 5 μm and are at various stages of meiosis. They may be identified and distinguished by nuclear size, chromatin condensation, and meiotic figures. Leptotene/zygotene cells are more spherical than type B spermatogonia, and chromatin contains tiny spots 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

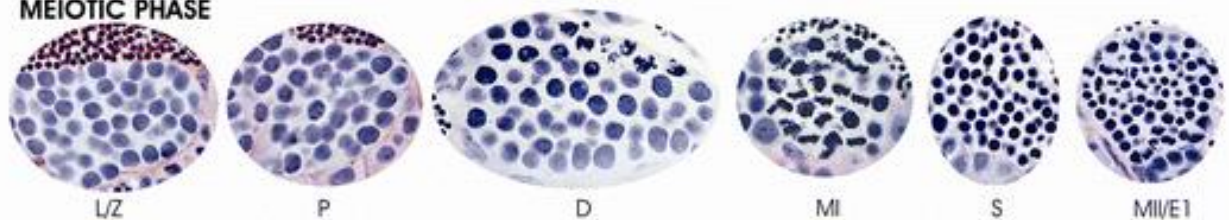
During spermiogenesis, the nuclear volume of the cells is reduced, and the classification of three types of spermatids: initial (E1), intermediate (E2), and final (E3) is based on the increase in nuclear compaction, decrease in cell volume, and spacing between cells due to the formation of flagella (Leal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



SPERMATOGONIAL PHASE



MEIOTIC PHASE



SPERMIOGENIC PHASE

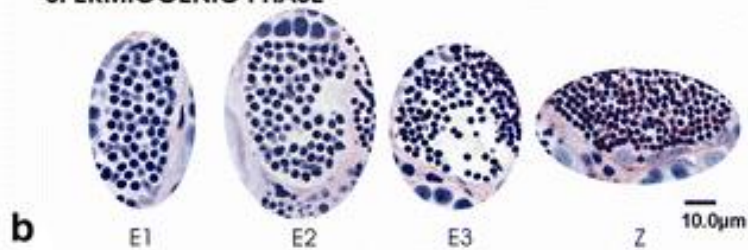


Figure 2. The basic organization of the fish testis **(a)** here was also observed in Figure 1B, and zebrafish testis showed the three phases of spermatogenesis in fish **(b)**. **(a)** – Tubular or germinal compartment (GC) and the intertubular or interstitial compartment (IC). Leydig cells (LE), blood 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).

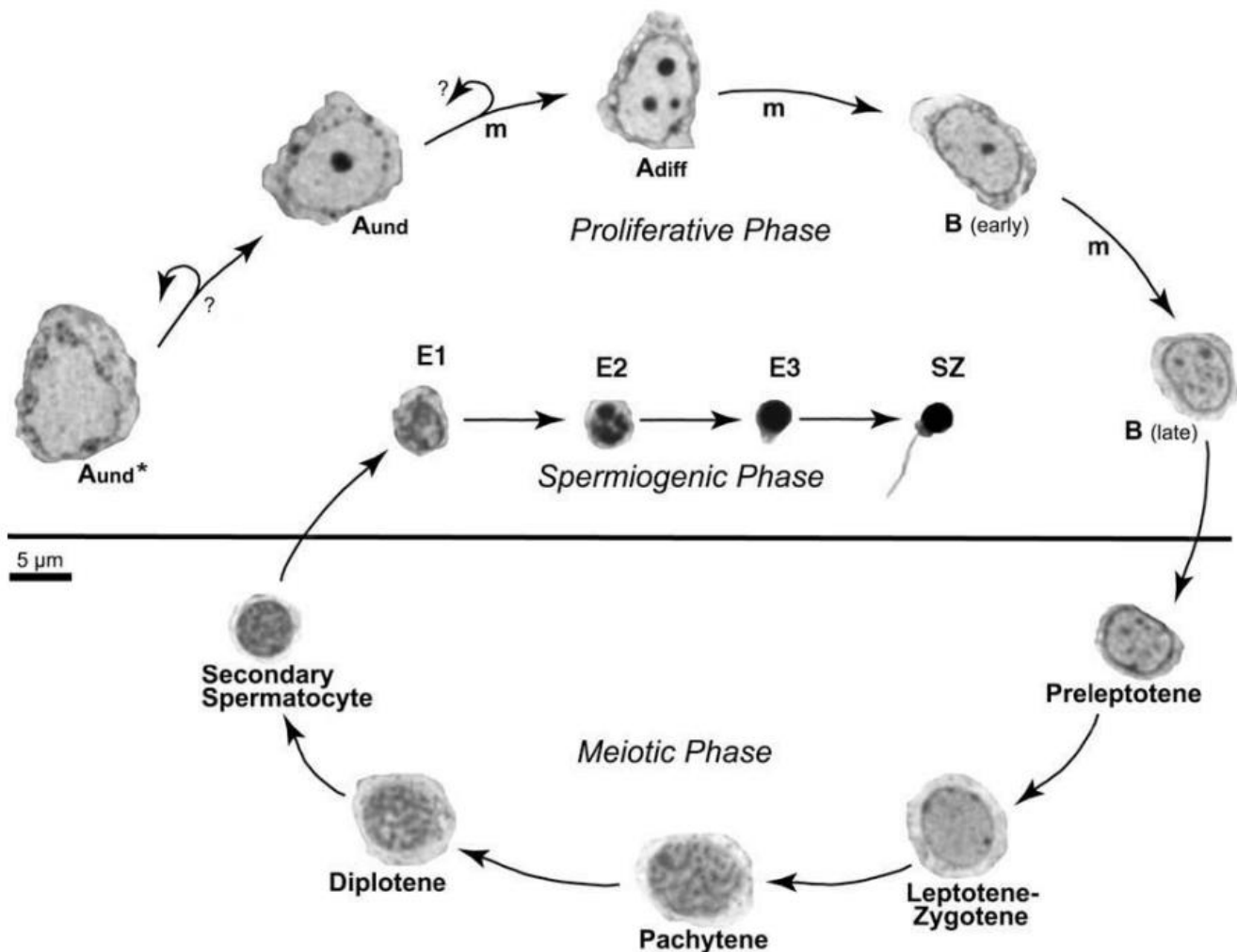


Figure 3. Detailed zebrafish testis showing the three phases of spermatogenesis in fish. Type A undifferentiated spermatogonia (Aund* and Aund), type A differentiated spermatogonia (Adiff), type B spermatogonia (B), self-renew (curved arrows), mitosis (m), early spermatids (E1), intermediate spermatids (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.

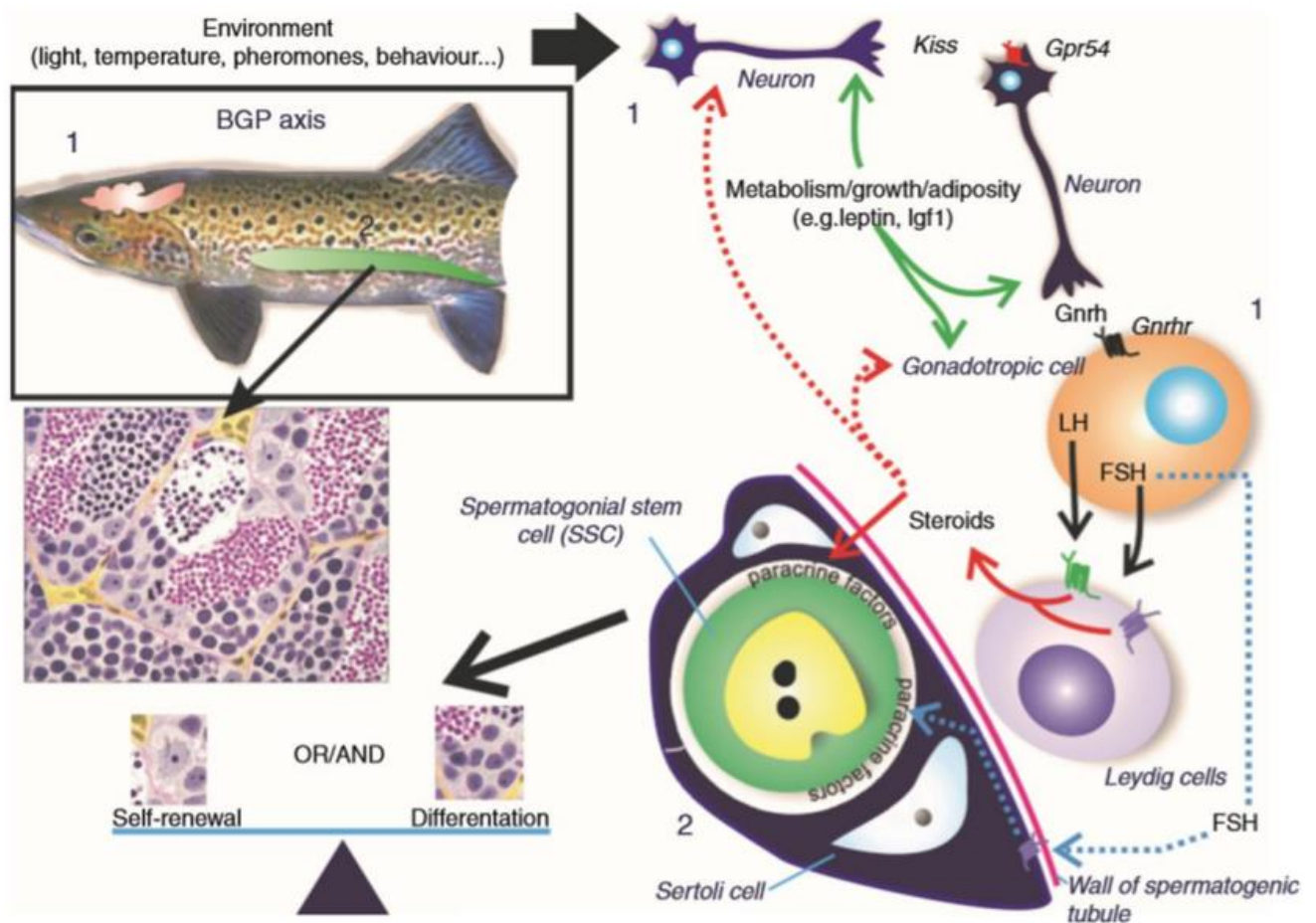


Figure 4. Illustration of the reproductive axis in fish. The initiation of the process is determined by environmental stimuli, such as water temperature and photoperiod, followed by a cascade of hormones along the hypothalamic-pituitary-gonadal (HPG) axis. This cascade begins with Kiss neurons in the 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).

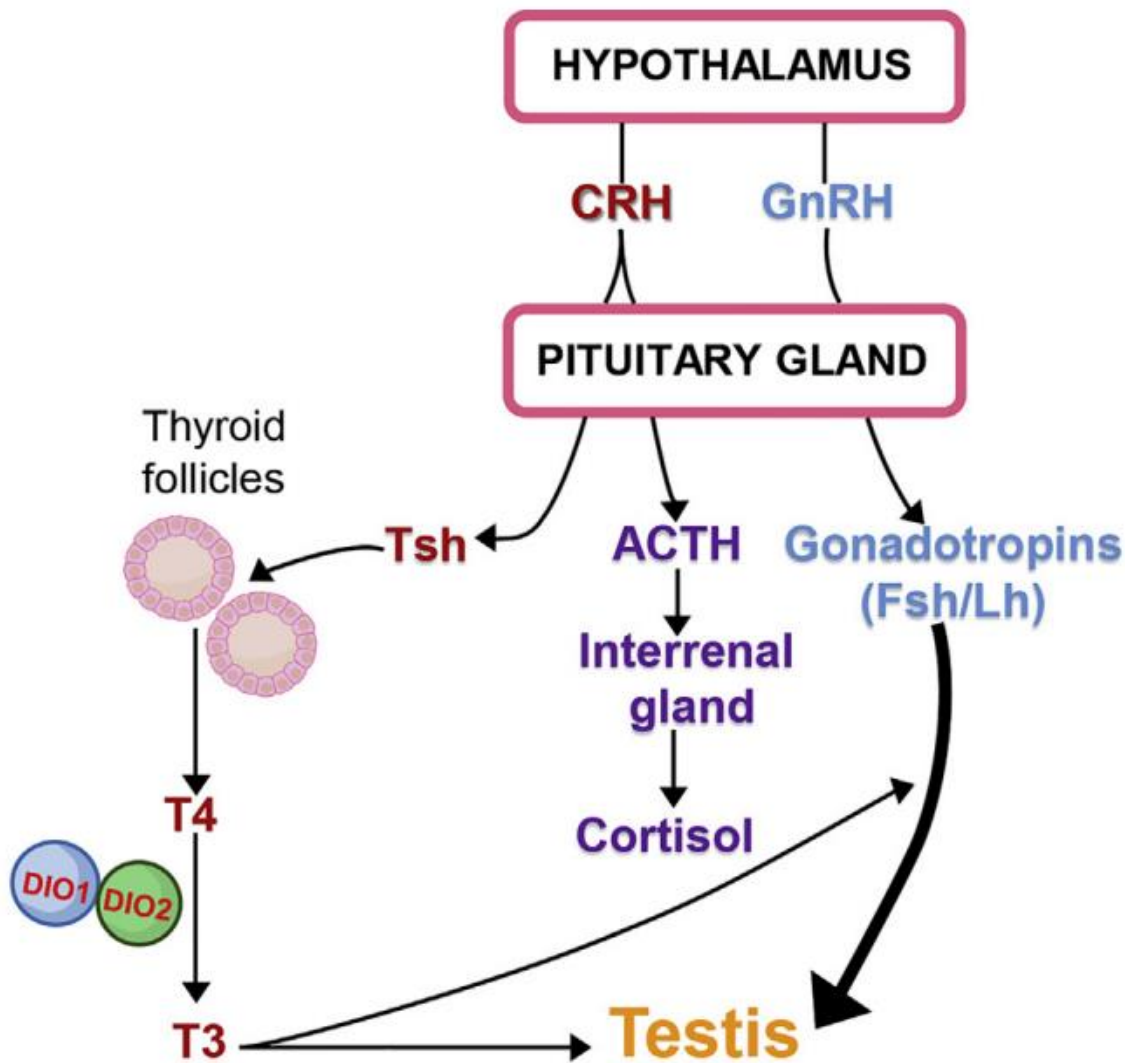


Figure 5. hypothalamic-pituitary-thyroid (HPT) axis in fish. The pituitary gland releases the thyroid-stimulating hormone (Tsh), which is crucial in regulating the thyroid gland. Additionally, the corticotropin-releasing hormone (Crh) stimulates adrenocorticotrophic hormone (Acth) production. Acth, in 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).

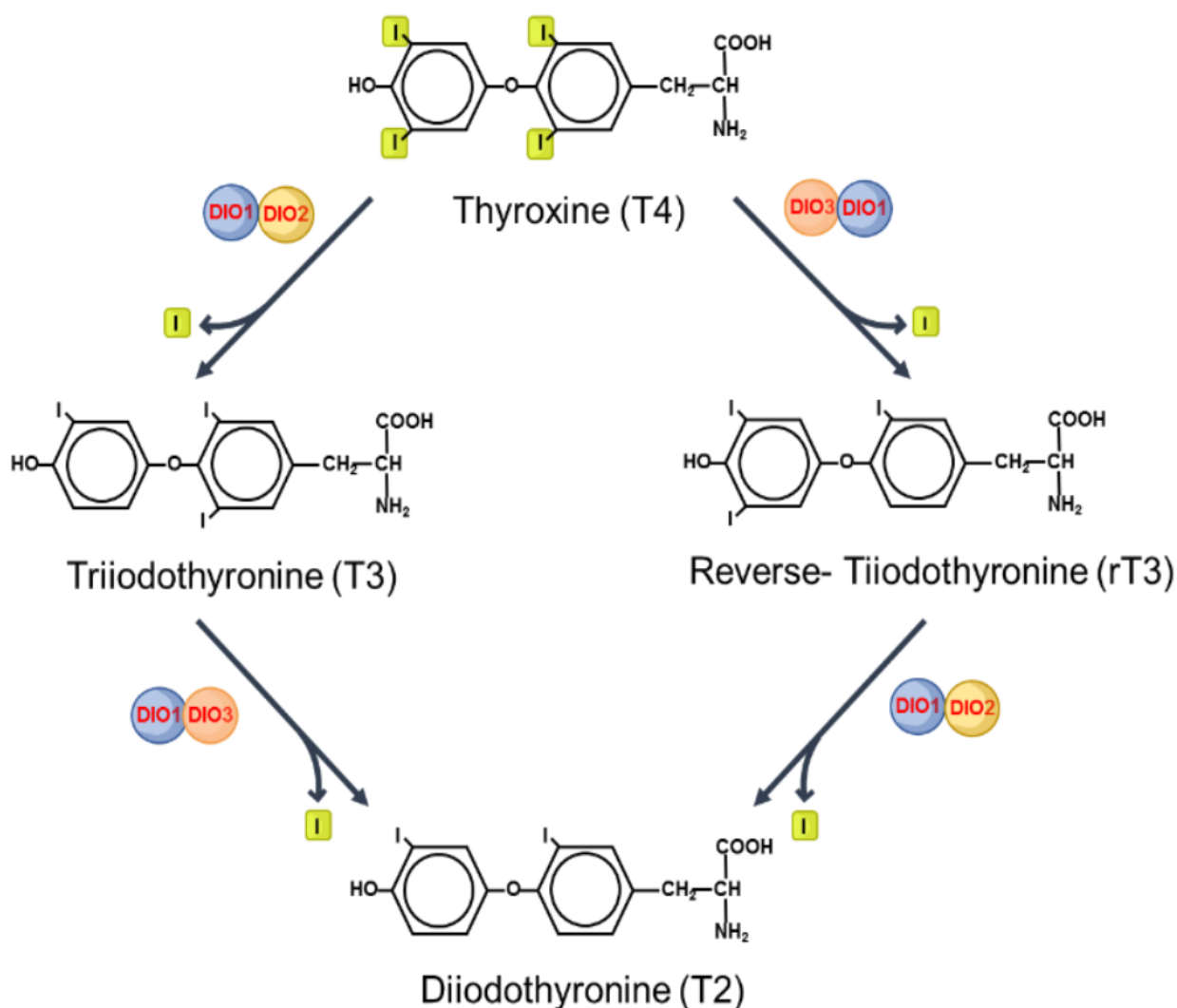


Figure 6. Deiodinases play an important role in the metabolism of the thyroid hormone T3. Type I and type II iodothyronine deiodinases (DIO1 and DIO2) convert thyroxine (T4), the main product released by thyroid follicles, into the bioactive T3. Furthermore, DIO1 also plays a crucial role in the production 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.

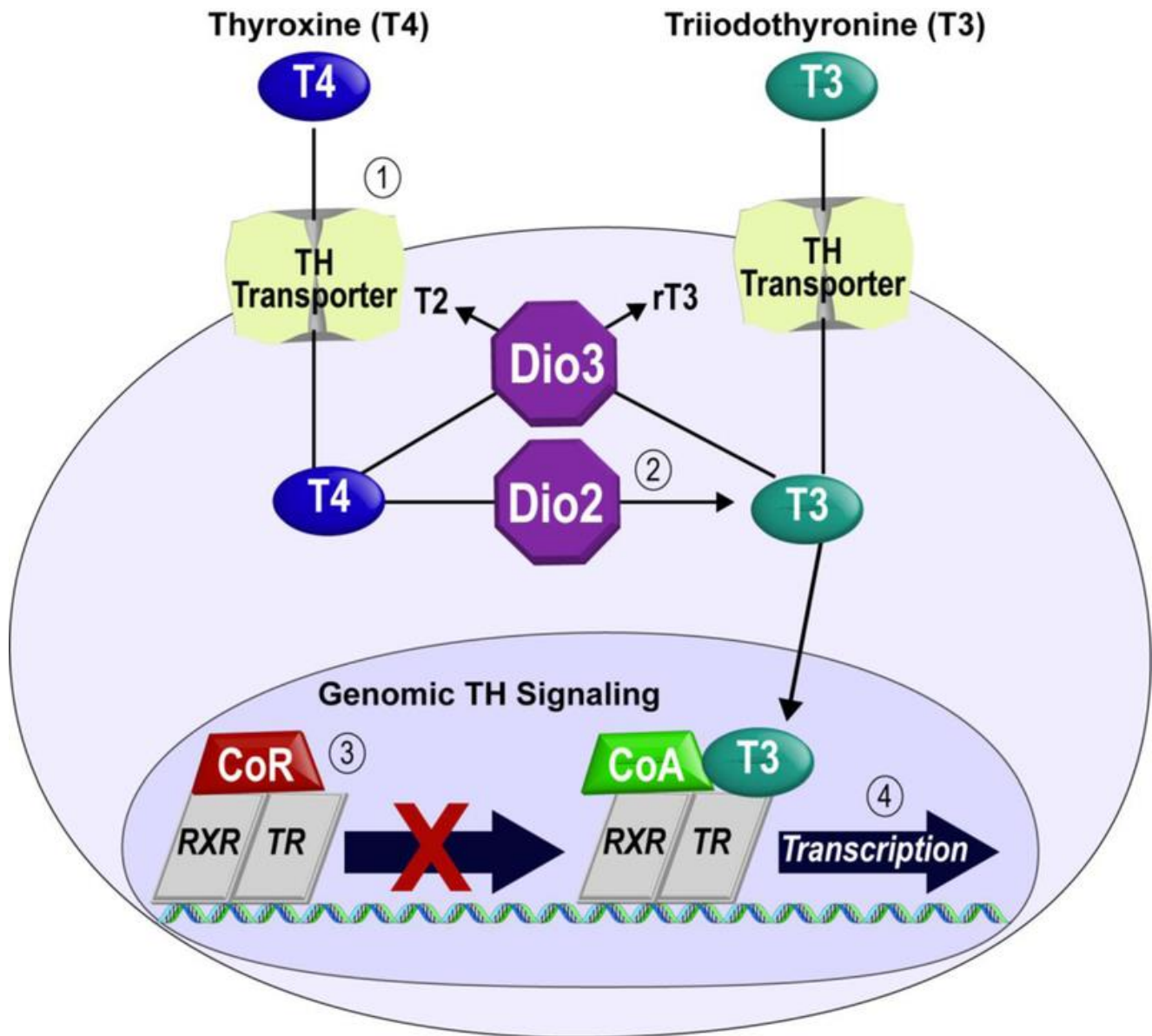


Figure 7. TH signaling. (1) Circulating levels of T4 and T3 are transported to target cells by specific transporters. (2) T4 is converted to the active T3 by deiodinase type II (DIO2). T4 and T3 can also be converted to rT3 and T2, respectively, by DIO3. (3) In the nucleus, the thyroid hormone receptor (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 *lhβ* and *fshβ* 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 somatotrophic 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 somatotrophic 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).

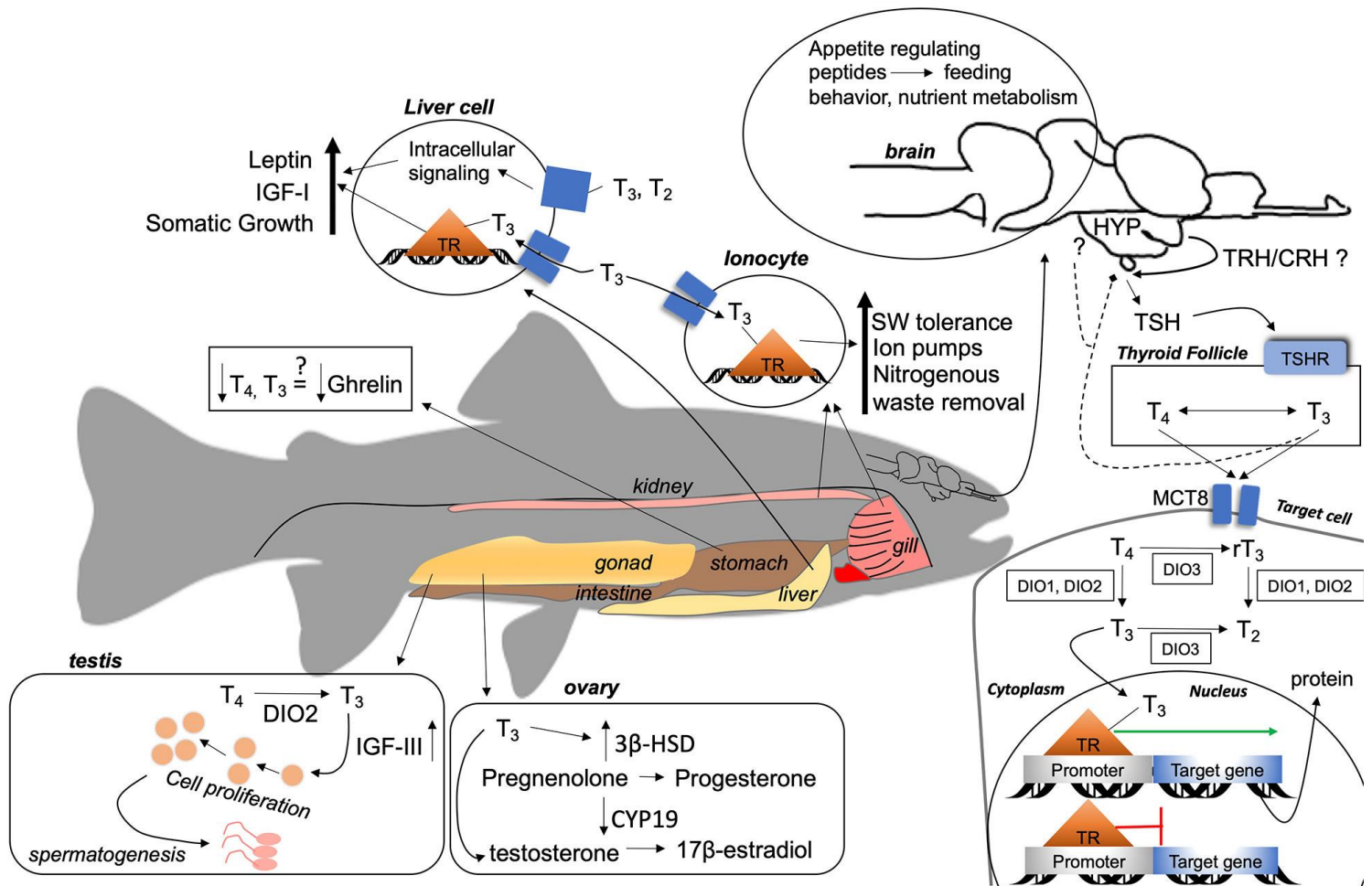


Figure 8. An overview of the general actions of THs in fish. Corticotropin-releasing hormone (CRH) stimulates the pituitary to release TSH, which binds to the TSH receptor (TSHR) on the thyroid follicles. Intracellular processes produce T4 and T3 that enter the circulation to target cells (solid line) 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.

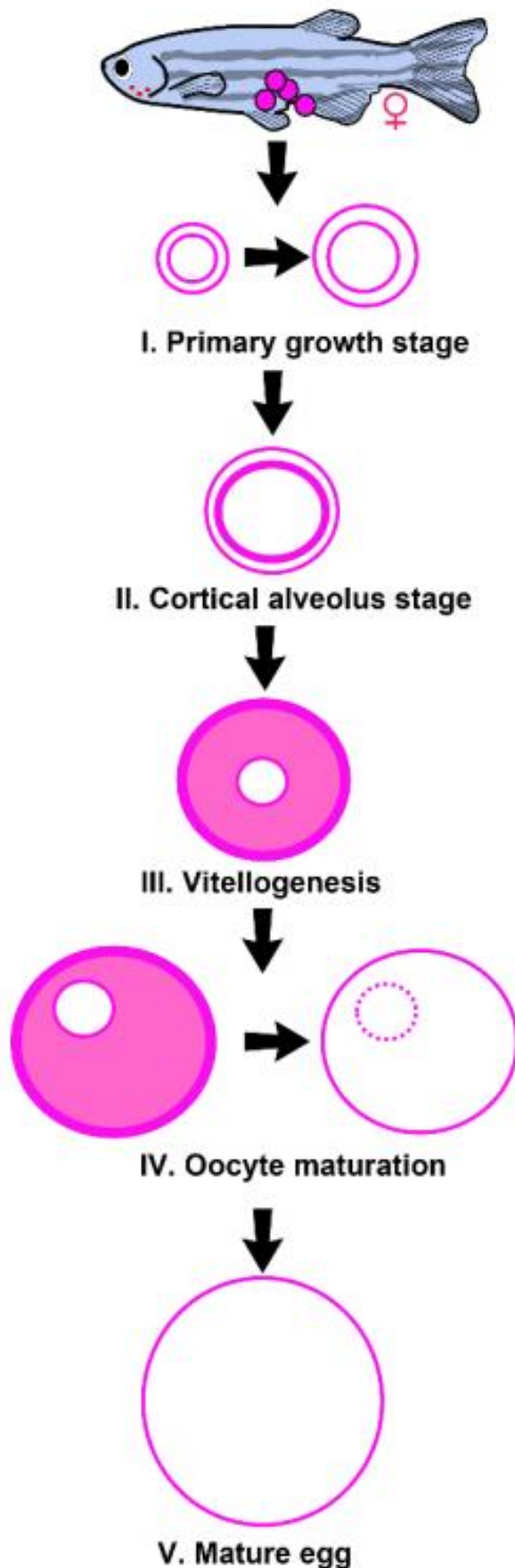


Figure 9. Schematic drawing of stages of follicle development in zebrafish. **Stage I/Primary growth stage:** the oocytes begin to enlarge, and follicles start to form. **Stage II/cortical alveolus stage:** cortical alveolus accumulates within the oocytes. **Stage III/Vitellogenesis:** follicles undergo 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).



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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

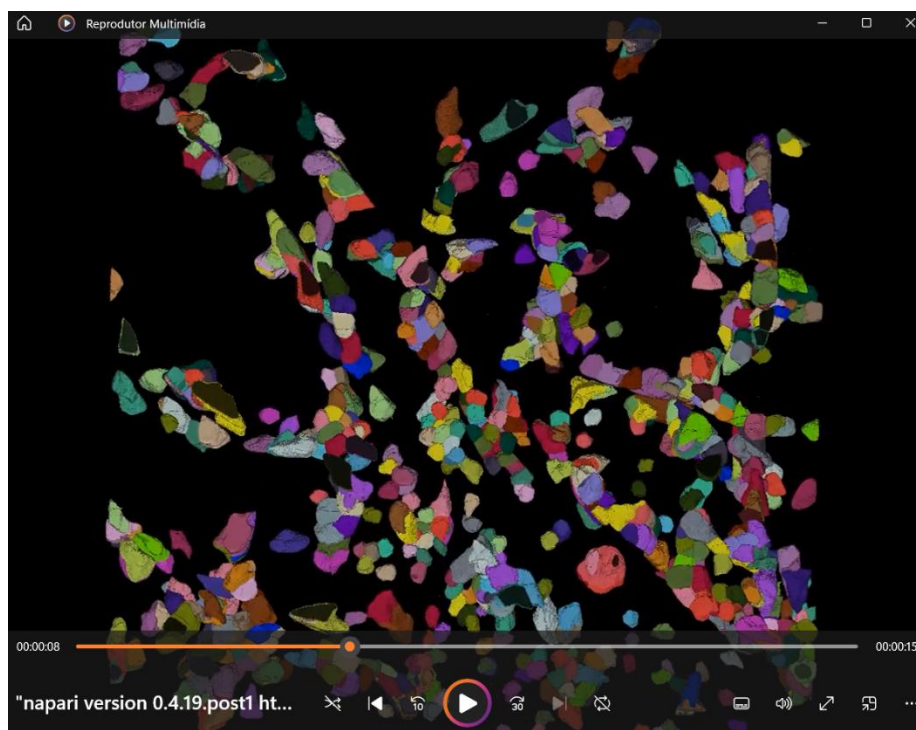
Manuscript in preparation

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

Manuscript in preparation

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.

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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.

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