The Role of Estrogens in Zebrafish Socio-Sexual Behaviors

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Abstract

Sex steroids are important hormones produced in the body to regulate an individual's physiology and behavior in preparation for reproduction. Aromatase (Cyp19a1) is the steroidogenic enzyme that converts aromatizable androgens into bioactive estrogens, and hence is in a pivotal position to mediate reproductive processes and sexual behavior. In mice, evidence from whole body aromatase knockout and estrogen receptor knockout lines has revealed the critical roles of estrogen signalling in ovarian development, gonadotropin signalling, ovulation, and sexual behavior. While the ovaries have high aromatase activity levels and are a major source of circulating estrogens in the female body, they can also be locally produced in tissues via tissue-specific aromatase expression. Of particular interest, the importance of brain-derived estrogens for reproductive processes and sexual behavior is still under study. Teleosts are an amenable model system for understanding the role of brain-derived estrogens in reproduction as they have two aromatase paralogs, *cyp19a1a* and *cyp19a1b*, that are highly expressed in the ovary and brain, respectively. In this thesis, I sought to identify the importance of brain-derived estrogens for sexual behaviors and reproductive health in zebrafish using *cyp19a1*$^{-/-}$ mutant lines and a transgenic *cyp19a1b*-GFP line. In dyadic sexual behavior assays, female *cyp19a1b*$^{-/-}$ mutants took 4.1 times longer to initiate spawning behavior with wildtype (WT) males compared to WT females. This suggested a potential impairment in mate identification and assessment in the female *cyp19a1b*$^{-/-}$ mutants. The importance of the *cyp19a1* paralogs for early larval development was revealed by 4 times higher progeny mortality from *cyp19a1b*$^{-/-}$ mutant female and *cyp19a1a*$^{-/-}$;*cyp19a1b*$^{-/-}$ mutant male pairings compared to WT pairings.

Gene expression analyses identified significantly lower levels of important neuroendocrine genes including the estrogen receptors and the nonapeptides, *arginine vasopressin* (*avp*) and *oxytocin*, in the telencephalon and hypothalamus of *cyp19a1b*$^{-/-}$ mutant female compared to WT female brains. I performed acute intraperitoneal injections of Avp and Oxytocin, as well as mixtures of their respective receptor antagonists, in *cyp19a1b*$^{-/-}$ mutant females to determine if the behavioral impairments could be rescued in adult fish. Arginine vasopressin was found to uniquely recover the delayed oviposition in female *cyp19a1b*$^{-/-}$ mutants. Immunohistochemistry experiments using the transgenic *cyp19a1b*-GFP zebrafish line with a zebrafish-validated Avp antibody revealed a close neuroanatomical proximity and contact points between radial glial cell Cyp19a1b-expressing fibres and Avp-immunopositive neurons in preoptic areas. These findings suggest that brain-derived estrogens, via Cyp19a1b activity, might affect female sexual behavior by diffusing to nearby neurons to regulate Avp signalling levels in preoptic areas. Together, these findings establish the importance of *cyp19a1b* for female zebrafish sexual behavior and identify a positive link between Cyp19a1b and Avp. Future study can help to characterize the estrogen-dependent pathways involved in regulating Avp signalling in the female brain and the extent of evolutionary conservation of this regulation pathway for female sexual behavior across vertebrates.

Résumé

Les stéroïdes sexuels sont des hormones importantes produites dans le corps pour réguler la physiologie et le comportement d'un individu en préparation à la reproduction. L'aromatase (Cyp19a1) est l'enzyme stéroïdogène qui convertit les androgènes aromatisables en œstrogènes bioactifs, et elle occupe ainsi une position cruciale dans la médiation des processus de reproduction et du comportement sexuel. Chez les souris, des preuves provenant de lignées de souris knockout de l'aromatase et des récepteurs d'œstrogènes ont révélé les rôles critiques de la signalisation des œstrogènes dans le développement ovarien, la signalisation des gonadotrophines, l'ovulation et le comportement sexuel. Bien que les ovaires présentent des niveaux d'activité aromatase élevés et constituent une source majeure d'œstrogènes circulants dans le corps féminin, ils peuvent également être produits localement dans les tissus via l'expression d'aromatase spécifique aux tissus. D'un intérêt particulier, l'importance des œstrogènes dérivés du cerveau dans les processus de reproduction et le comportement sexuel fait encore l'objet d'études. Les téléostéens constituent un système modèle adapté pour comprendre le rôle des œstrogènes dérivés du cerveau dans la reproduction, car ils possèdent deux paralogues d'aromatase, *cyp19a1a* et *cyp19a1b*, qui sont fortement exprimés dans l'ovaire et le cerveau, respectivement. Dans cette thèse, j'ai cherché à identifier l'importance des œstrogènes dérivés du cerveau pour les comportements sexuels et la santé reproductive chez le poisson zèbre en utilisant des lignées de mutants *cyp19a1*$^{-/-}$ et une lignée transgénique *cyp19a1b*-GFP. Dans les tests de comportement sexuel dyadique, les mutants femelles *cyp19a1b*$^{-/-}$ ont mis 4,1 fois plus de temps pour initier un comportement de ponte avec des mâles

de type sauvage (WT) par rapport aux femelles WT. Cela suggérait une altération potentielle de l'identification et de l'évaluation des partenaires chez les mutants femelles *cyp19a1b*$^{-/-}$. L'importance des paralogues de *cyp19a1* dans le développement larvaire précoce a été révélée par une mortalité des progénitures 4 fois plus élevée issue des accouplements de femelles mutantes *cyp19a1b*$^{-/-}$ et de mâles mutantes *cyp19a1a*$^{-/-}$;*cyp19a1b*$^{-/-}$ par rapport aux accouplements de type sauvage. Les analyses d'expression génique ont révélé des niveaux significativement plus bas de gènes neuroendocriniens importants, notamment des récepteurs d'œstrogènes et des nonapeptides, l'*arginine vasopressine* (*avp*) et l'*ocytocine*, dans le telencéphale et l'hypothalamus des mutants femelles *cyp19a1b*$^{-/-}$ par rapport aux cerveaux des femelles WT. J'ai réalisé des injections intrapéritonéales aiguës d'Avp et d'Ocytocine, ainsi que des mélanges de leurs antagonistes de récepteurs respectifs, chez les femelles mutantes *cyp19a1b*$^{-/-}$ pour déterminer si les altérations comportementales pouvaient être corrigées chez les poissons adultes. Il a été constaté que l'arginine vasopressine récupérait de manière unique la ponte retardée chez les femelles mutantes *cyp19a1b*$^{-/-}$. Des expériences d'immunohistochimie utilisant la lignée de poisson zèbre transgénique *cyp19a1b*-GFP avec un anticorps Avp validé chez le poisson zèbre ont révélé une proximité neuroanatomique étroite et des points de contact entre les fibres Cyp19a1b exprimant des cellules gliales radiales et les neurones immunopositifs à l'Avp dans les régions préoptiques. Ces résultats suggèrent que les œstrogènes dérivés du cerveau, via l'activité de Cyp19a1b, pourraient affecter le comportement sexuel des femelles en diffusant vers les neurones voisins pour réguler les niveaux de signalisation de l'Avp dans les régions préoptiques. Ensemble, ces résultats établissent l'importance de

cyp19a1b pour le comportement sexuel des femelles zèbres et identifient un lien positif entre Cyp19a1b et l'Avp. Des études futures peuvent aider à caractériser les voies dépendantes des œstrogènes impliquées dans la régulation de la signalisation de l'Avp dans le cerveau féminin et l'étendue de la conservation évolutive de cette voie de régulation du comportement sexuel féminin chez les vertébrés.

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Abbreviations Used

AC	adenylyl cyclase
AH	anterior hypothalamus
Ap1	activator protein 1
Aro KO	aromatase knockout
Avp	arginine vasopressin
bAro KO	brain aromatase knockout
BL	body length
bp	basepair
bw	bodyweight
cAMP	cyclic adenosine 3’,5’-monophosphate
cDNA	complementary DNA
CREB	cAMP response element binding protein
ddPCR	droplet digital PCR
dpf	days post-fertilization
EA	eye area
ELISA	enzyme-linked immunosorbent assay
EREs	estrogen response elements

Esr KO	estrogen receptor knockout
Esrs	estrogen receptors
mEsrs	membrane-bound estrogen receptors
EtOH	ethanol
E1	estrone
E2	estradiol
Fsh	follicle stimulating hormone
Gper	G protein-coupled estrogen receptor
Gnrh	gonadotropin-releasing hormone
hpf	hours post-fertilization
HPG	hypothalamic-pituitary-gonadal
Lh	luteinizing hormone
nERs	nuclear estrogen receptors
npba	neuropeptide B
Oxt	oxytocin
Oxtr	oxytocin receptor
PCR	polymerase chain reaction
PBS	phosphate-buffered saline

PKA	protein kinase A
POA	preoptic area
RGC	radial glial cell
RNA	ribonucleic acid
Scg2	secretogranin 2
SN	secretoneurin
Sp1	specificity protein 1
stAR	steroidogenic acute regulatory protein
T	testosterone
tAro KO	total aromatase knockout
TALEN	transcription activator-like effector nucleases
WT	wildtype
YSA	yolk sac area
11-KT	11-ketotestosterone

Chapter 1: General Introduction

Estrogenic Drive of Reproduction: The Interconnection of the Brain and the Gonads

This chapter is adapted for inclusion in:

Trudeau, V.L., Shaw, K., Spadacini, V., Hu, W. Reproductive neuroendocrinology in teleost fishes. Chapter 2. In *Hormones and Reproduction of Vertebrates*, *Vol 2*: *Fishes*. Second Edition. Eds. Norris, D.O. and Lopez, K.H.. Elsevier Publishing. Submitted August 2023.

1.1 Thesis Rationale

Estrogens have critical roles in preparing the female body for reproduction (Findlay et al., 2010). Aromatase (Cyp19a1) is the steroidogenic enzyme that functions in the final and rate limiting step for bioactive estrogen production in the steroidogenesis pathway (Gruber et al., 2002). While aromatase activity in the ovaries is a major tissue source of circulating (i.e., systemic) estrogens, they can also be locally produced in tissues via aromatase expression. There is growing recognition of the importance of tissue-specific aromatase expression for varying physiological and behavioral functions of estrogen signalling.

1.2 Thesis Hypotheses and Objectives

The goal of my thesis project is to establish the importance of brain aromatase (i.e., *cyp19a1b*) for sexual behavior in zebrafish (*Danio rerio*). I tested the hypothesis that mutation of *cyp19a1b* affects sexual behavior through reduced neuropeptide signalling levels in the brain. To address this hypothesis, I had the following objectives:

1) Assess changes in sexual behavior and reproductive health in *cyp19a1a*$^{-/-}$ and *cyp19a1b*$^{-/-}$ mutant zebrafish (Chapter 2).
2) Examine changes in the expression levels of *esr*s and neuropeptides known to be important for zebrafish sexual behavior in the brains of *cyp19a1b*$^{-/-}$ mutant zebrafish (Chapter 3).
3) Determine if the behavioral impairments can be rescued using acute intraperitoneal injections of hormones in adult fish (Chapter 3).
4) Identify the neuroanatomical distribution of Cyp19a1b relative to key regulators of teleost sexual behavior (Chapter 3).

1.3 Thesis Presentation

This doctoral thesis is composed of four chapters. An introductory chapter providing necessary background information for understanding the study topic (Chapter 1), two data chapters (Chapters 2-3), and a concluding chapter that features an overview of the thesis findings and discussion of future research opportunities to build on the findings (Chapter 4). Chapter 2 examines objective 1, and chapter 3 addresses objectives 2-4. Chapters 1-3 are presented as independent manuscripts. As such, there is some overlap in the content presented in each chapter, though I have attempted to keep this to a minimum, where possible.

The text follows the guidelines of the Zebrafish Information Network for nomenclature to designate teleost genes and their protein counterparts (https://zfin.atlassian.net/wiki/spaces/general/pages/1818394635/ZFIN+Zebrafish+Nomenclature+Conventions). Of note, the recently determined zebrafish nomenclature for *avp*/Avp and *oxt*/Oxt contrasts the historically used nomenclature of *avt*/Avt and *ist*/Ist. For mouse genes and their protein counterparts, the guidelines of the Mouse Genome Informatics Database were followed (https://www.informatics.jax.org/mgihome/nomen/gene.shtml#gnas) with slight modification to the gene symbols used for brain (*bAro* KO) and total aromatase knockout (*tAro* KO) lines to improve text clarity and reader comprehension.

1.4 Steroidogenic Pathway for Estrogen Production

Estrogens are 18-carbon steroids derived from cholesterol through a series of reactions. Firstly, cholesterol is taken into a steroidogenic cell through binding to lipoprotein receptors on the cell membrane and transported to the inner mitochondrial membrane via the cytoskeleton and intracellular carrier proteins such as the steroidogenic acute regulatory protein (stAR; Papadopoulos and Miller, 2012). Within the mitochondrial membrane, cholesterol is then converted into pregnenolone by the P450 side chain cleavage protein (Cyp11a1) and released into the mitochondrial matrix (Slominski et al., 2015). Pregnenolone is transported to the smooth endoplasmic reticulum where it can be converted into numerous steroid hormones through the activity of steroidogenic enzymes. The final rate-limiting step for bioactive estrogen production occurs in the smooth endoplasmic reticulum via the activity of the aromatase (Cyp19a1) enzyme that converts testosterone (T) and androstenedione into estradiol (E2) and estrone (E1), respectively (Gruber et al., 2002; Figure 1.1). Estrogens are metabolized through various pathways including hydroxylation, glucuronidation, sulfation, and O-methylation into hormonally inactive or less active water-soluble metabolites for elimination from the body (Zhu and Conney, 1998).

In birds and most mammals, there is only one gene encoding aromatase (*cyp19a1*). Differential tissue expression of aromatase, and consequently estrogen production, is established in the body via alternative splicing of the 5’ promoter region that constitutes the untranslated exon I of *cyp19a1* (Simpson et al., 1994). For example, ovarian aromatase expression is specified by the proximal promoter region II whilst brain expression is specified by the distal promoter region I.f (Simpson et al., 1997). In

contrast, as a consequence of the whole genome duplication event, most teleosts have two genes encoding aromatase: *cyp19a1a* and *cyp19a1b*, with distinct expression profiles predominately observed in the gonads and brain, respectively (Lin et al., 2020). Findings from teleost expression studies indicate that the paralogs represent a subfunctionalization of the ancestral *cyp19a1* gene (Zhang et al., 2014, but see Böhne et al., 2013 for novel functions observed in East African cichlids). For example, *cyp19a1a* is highly expressed in ovarian granulosa cells resulting in high estrogen production levels that are deposited into systemic circulation. Testes *cyp19a1a* expression is comparatively much lower in Leydig cells. Estrogen signalling is nonetheless believed to serve important functions in males through other local tissue-specific aromatase expression. In both sexes, *cyp19a1b* is highly expressed in the brain, and specifically in radial glial cells (RGCs). Teleost *cyp19a1b* RGC expression is due to the presence of a *cis*-acting regulatory glial x responsive element in the promoter region that recruits glial-specific transcription factors to upregulate *cyp19a1b* expression (Le Page et al., 2008). Aromatase expression has been identified in several other extra-gonadal tissues including the pituitary gland, fat, muscle, and bone, among others, providing a pathway for local estrogen production (Simpson et al., 2003).

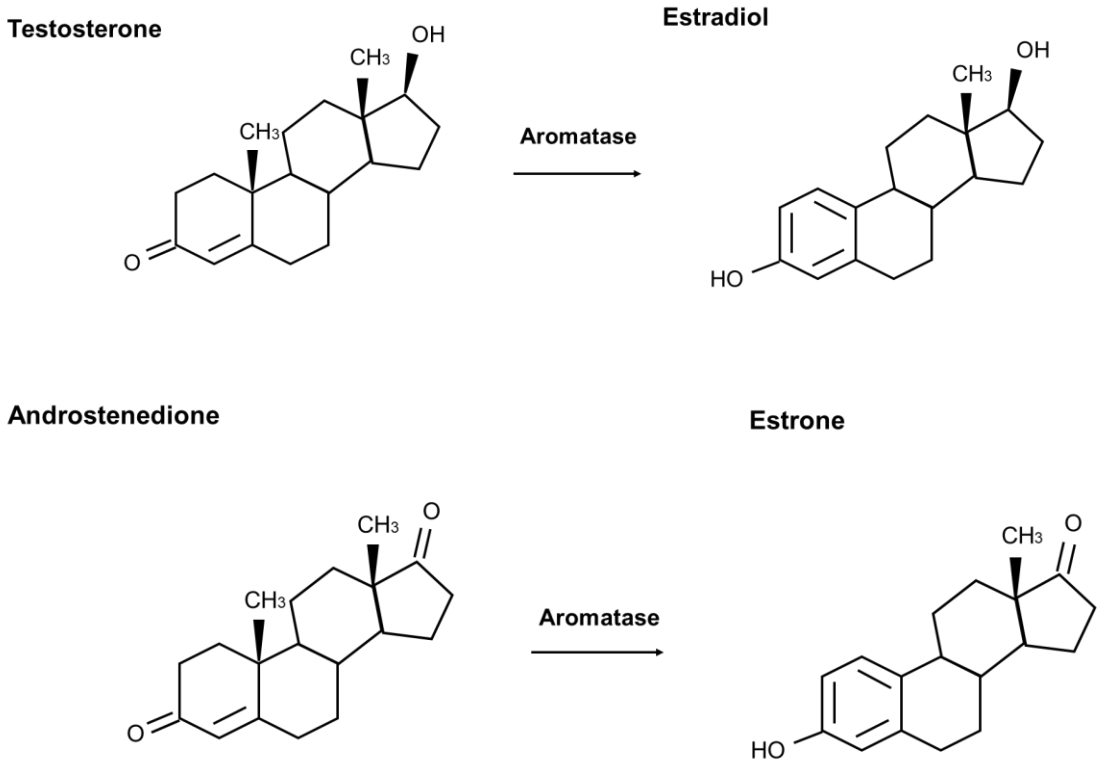


Figure 1.1- Aromatase pathway. The aromatase (Cyp19a1) enzyme functions to convert the aromatizable androgens, testosterone and androstenedione, into bioactive estrogens, estradiol and estrone, respectively.

1.5 Estrogen Signalling Pathways

Estrogen signalling is mediated by several pathways leading to tissue- and cell-specific effects. Intracellular nuclear estrogen receptors (nEsrs) activated by estrogen binding can dimerize to regulate gene transcription through binding to estrogen response elements (EREs) in gene promoter regions or through protein-protein interactions with transcription factors such as the activator protein 1 (Ap1) and specificity protein 1 (Sp1), when bound to their respective promoter elements (Figure 1.2; Klinge et al., 2001). These genomic signalling pathways can induce long term effects on diverse processes

including dendritic and synaptic structure, ion channel expression, neurotransmitter and neuropeptide signalling, among others (McEwen, 1991). While in birds and mammals there are two nEsrs named estrogen receptor 1 (Esr1) and estrogen receptor 2 (Esr2), most teleosts possess three nEsrs called Esr1*,* estrogen receptor 2a (Esr2a), and estrogen receptor 2b (Esr2b; Nelson and Habibi, 2013). Another pathway of estrogen signalling involves binding to extracellular membrane-bound estrogen receptors (mEsrs) that activate second messenger signalling pathways within the cell (Figure 1.2; Levin, 2009). These signalling pathways can lead to changes in protein activity, ionic currents and gene transcription, among others (Simoncini and Genazzani, 2003). In birds and mammals, several mEsrs have been identified including G protein-coupled estrogen receptor (Gper, formerly Gpr30) as well as recombinant mammalian isoforms, Esr-X and Gq-mEsr (Wojnarowski et al., 2022). In teleosts, a Gper has been identified, with more recent evidence revealing two paralogues, *gpera* and *gperb,* in some species (Pinto et al., 2018).

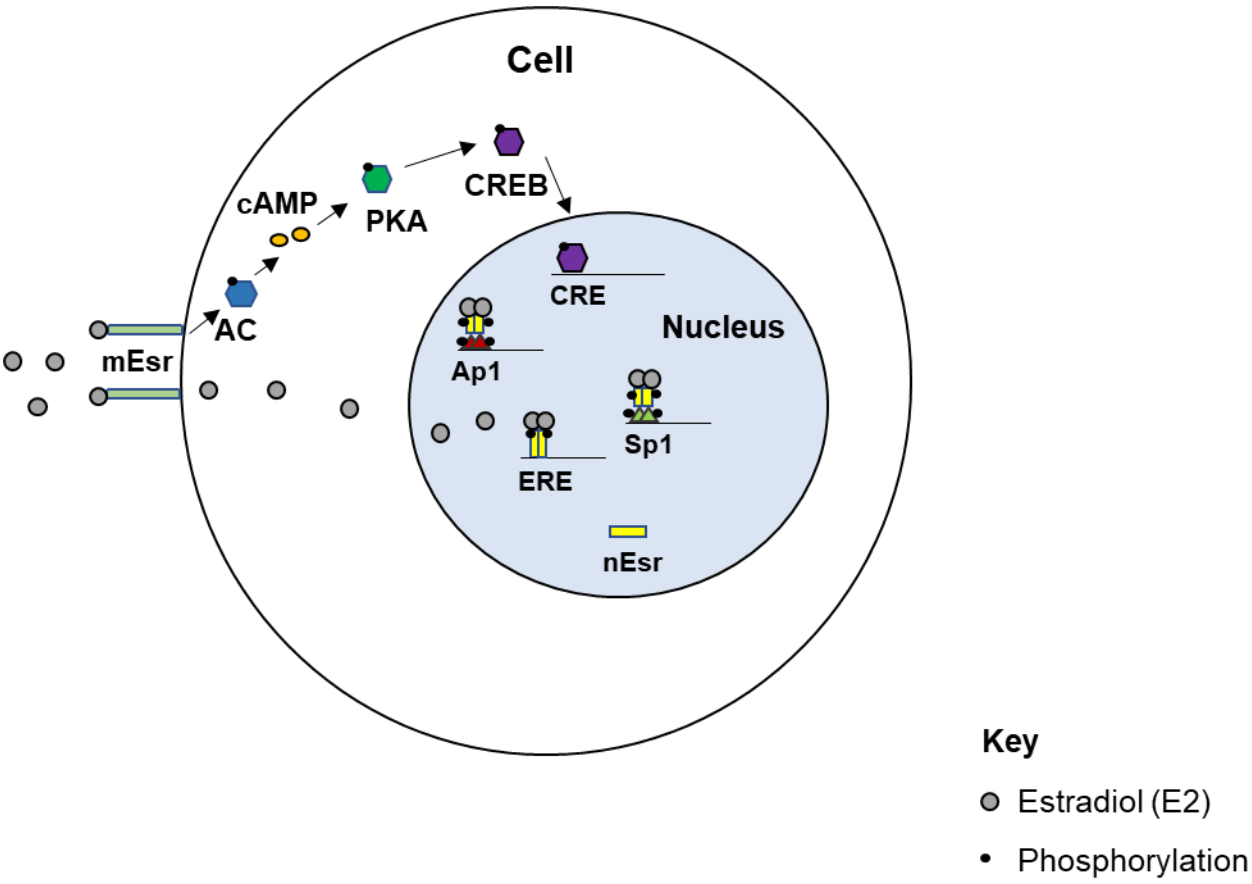


Figure 1.2- Simplified schematic of estrogen signalling pathways in the cell.

Estradiol (E2) can bind to activate extracellular membrane-bound estrogen receptors (mEsrs) on the cell surface. This leads to the activation of second messenger signalling pathways within the cell, such as the protein kinase A (PKA) signalling pathway involving adenylyl cyclase (AC), cyclic adenosine 3',5'-monophosphate (cAMP), PKA, and cAMP response element binding protein (CREB) that binds to cAMP response elements (CRE) in gene promoter regions to activate gene transcription. Another pathway of estrogen signalling involves E2 binding to activate nuclear estrogen receptors (nEsrs) in the cell nucleus, which can then dimerize to bind estrogen response elements (EREs) in gene promoter regions or interact with transcription

factors such as the activator protein 1 (Ap1) and specificity protein 1 (Sp1), when bound to their respective promoter elements.

1.6 The Hypothalamic-Pituitary-Gonadal Axis

The hypothalamic-pituitary-gonadal (HPG) axis represents a critical pathway for reproductive regulation in vertebrates. The hypothalamus, in the ventral forebrain, is composed of four regions named the preoptic, anterior, tuberal, and mammillary, that play important roles in regulating physiological homeostasis and behavior (Xie and Dorsky, 2017). The preoptic area (POA) and anterior hypothalamus (AH) are particularly important and well-studied brain regions in reproduction as they serve as major integration centers of sensory information from the peripheral nervous system with internal homeostatic cues to output signals that regulate neuronal activity in other brain regions (encephalotropic pathway) as well as the production and release of various endocrine hormones from the pituitary gland to target peripheral tissues (hypophysiotropic pathway; Alvarez-Bolado, 2019; Herget et al., 2014; Machluf et al., 2011). For example, the nonapeptides arginine vasopressin (Avp) and oxytocin (Oxt) produced in the POA and AH have been linked to numerous reproductive functions including neuromodulation of sensory processing, regulation of circadian rhythms, parturition/oviposition, lactation, and penile erection, among others (Banerjee et al., 2017). Another important signalling pathway in this region involves gonadotropin-releasing hormone (Gnrh) that is produced in the POA and AH and transported to the pituitary gland to stimulate luteinizing hormone (Lh) and follicle stimulating hormone (Fsh) release (Gore, 2002). The gonadotropins are transported to the gonads through

the systemic circulatory system where Lh functions to stimulate oocyte maturation and ovulation and Fsh induces gametogenesis and follicular growth (Zhang et al., 2015).

While mammals have a median eminence for transport of neuropeptides from the hypothalamus to the pituitary gland, this structure is absent in teleosts and instead neuropeptides are directly transported through neural fibres (Trudeau and Somoza, 2020). Another interesting difference in the HPG axis of mammals and teleosts is the necessary role of Gnrh for reproduction. In mammals, increased estrogen and progesterone levels produced by the ovaries during proestrus play a critical role in kisspeptin-mediated stimulation of Gnrh neurons in the POA to induce the Lh surge necessary for ovulation (Clarkson et al., 2008). Gnrh deficiency in female mice induces hypogonadotropic hypogonadism and infertility (Bliss et al., 2010; Mason et al., 1986). Interestingly, in some teleosts, Gnrh signalling is not required for reproduction. For example, there were no effects of *gnrh3* mutation, the main hypophysiotropic *gnrh*, on zebrafish reproduction or fertility (Feng et al., 2020; Marvel et al., 2018). The observed differences between mammals and teleosts may be a consequence of the direct pituitary innervation in teleosts that provides a pathway for independent stimulation of gonadotroph cells by numerous neuropeptides and neurotransmitters (Trudeau, 2018). Of particular interest, more recent findings in zebrafish mutant lines have demonstrated critical roles of the secretoneurins (SNs; the *secretogranin 2* (*scg2*)-derived peptides) and the nonapeptides for reproduction, which may represent additional regulatory pathways in teleosts. For example, in dyadic sexual behavior assays, female *scg2a*$^{-/-}$;*scg2b*$^{-/-}$ mutant zebrafish were found to have significant reproductive impairments characterized by reduced sexual behavior, ovulation, oviposition, and fertility (Mitchell et

al., 2020). It was also recently discovered that female zebrafish *avp*$^{-/-}$ mutants have lower sexual receptivity and fewer eggs spawned during sexual behavior assays (Ramachandran et al., 2023). Further description of the importance of the nonapeptides for teleost reproduction was covered more fully in a recent co-authored review paper (Appendix A; Mennigen et al., 2022).

1.7 The Role of Estrogens in Mouse Reproduction

Our early understanding of the importance of estrogens for reproduction was greatly advanced by the findings from mouse *Esr* knockout (*Esr* KO) and aromatase knockout (*Aro* KO) model systems. It is worthwhile to review the knowledge gained by studying these models to recognize the foundations of our understanding of estrogen signalling effects on reproduction and to help in recognizing the limitations for understanding brain-specific effects on sexual behavior compared to teleost model systems.

The developmental abnormalities and behavioral impairments observed in *Esr* KO and *Aro* KO mice identified the important estrogen-dependent physiological and behavioral changes necessary to prepare the body for reproduction. For example, *Esr1* KO females were infertile displaying smaller-sized uteri with impaired mitogenic activity, ovaries containing hemorrhagic and cystic follicles (i.e., impaired folliculogenesis), failed ovulation, and higher levels of *fsh* and *lh* receptors in the ovaries compared to wildtype (WT) females (Dupont et al., 2000; Emmen and Korach, 2003; Lubahn et al., 1993; Schomberg et al., 1999). Further study of the brain and pituitary gland in *Esr1* KO mice revealed that the ovarian abnormalities were, at least in part, due to disrupted negative feedback of Esr1 signalling in the hypothalamic-pituitary axis resulting in elevated serum Lh levels (Couse et al., 1999; Emmen and Korach, 2003; Scully et al., 1997). These

findings were supported by observations in a Lh transgenic mouse line that overexpressed the Lhb subunit. Mice with overexpression of Lhb displayed a similar phenotype of infertility with cyst formations observed in the ovary and disrupted ovulation revealing the important role of Esr1 signalling at the level of the hypothalamic-pituitary axis for ovarian function (Risma et al., 1995). In male *Esr1* KO mice, the reproductive tract developed normally at the prenatal stage; however, following puberty, males were infertile with significantly higher serum Lh levels and Leydig cell hyperplasia in the testis (Eddy et al., 1996). There were less dramatic effects of *Esr2* KO on mouse reproduction. Female *Esr2* KO mice were subfertile producing fewer pups and litters compared to WT females and possessed immature uteri and ovaries with fewer corpora lutea that indicated partial arrest of folliculogenesis and ovulatory dysfunction (Dupont et al., 2000; Emmen and Korach, 2003; Krege et al., 1998; Weihua et al., 2000). Male *Esr2* KO mice were fertile with no obvious morphological abnormalities (Emmen and Korach, 2003). In both sexes, *Esr1/2* KO mice were infertile displaying phenotypes largely reflective of the single *Esr* KO lines (Dupont et al., 2000; Emmen and Korach, 2003). The reproductive phenotypes of *Esr1/2* KO mice also resembled those of the total aromatase knockout (*tAro* KO) mice, which lack whole body aromatase expression (Britt et al., 2001; Fisher et al., 1998; Robertson et al., 2001). Together, these findings demonstrated that the loss of estrogen signalling dramatically impaired mouse reproduction through effects on reproductive tract development as well as through disruptions to the HPG axis.

Reproduction was also impaired in the *Esr* KO and *Aro* KO lines through effects on sexual behavior. Female *Esr1* KO and *tAro* KO mice produced fewer lordosis displays,

a measure of sexual receptivity, and increased rejection of male stud mice compared to WT females in sexual behavior assays (Bakker et al., 2002a, 2003; Ogawa et al., 1998). Male *Esr1* KO, *Esr1/2* KO, and *tAro* KO mice displayed impaired sexual behavior characterized by defects in mounting behavior and/or intromission and ejaculation when paired with WT females (Bakker et al., 2002b; Honda et al., 1998; Ogawa et al., 1997, 2000; Matsumoto et al., 2003; Wersinger et al., 1997). No effects of *Esr2* KO on female or male sexual behaviors were observed (Krege et al., 1998; Ogawa et al., 1999). While these impairments in sexual behavior could be the result of the identified disruptions in Lh signalling in the HPG axis, more recent evidence has indicated an impairment in social recognition abilities in the *Esr* KO and *Aro* KO mice that suggest defects in peripheral and central sensory processing pathways that are important for mate identification and assessment. For example, impairments in olfactory discrimination were identified in *Esr* KO and *Aro* KO mice that could underlie impaired mate recognition during sexual behavior since mice rely heavily on pheromonal cues for mating (Bakker et al., 2002a, 2002b; Choleris et al., 2003, 2006; Pierman et al., 2008). A summary figure of the effects of *Esr* KO and *Aro* KO on mouse reproductive physiology and sexual behavior is depicted in Figure 1.3.

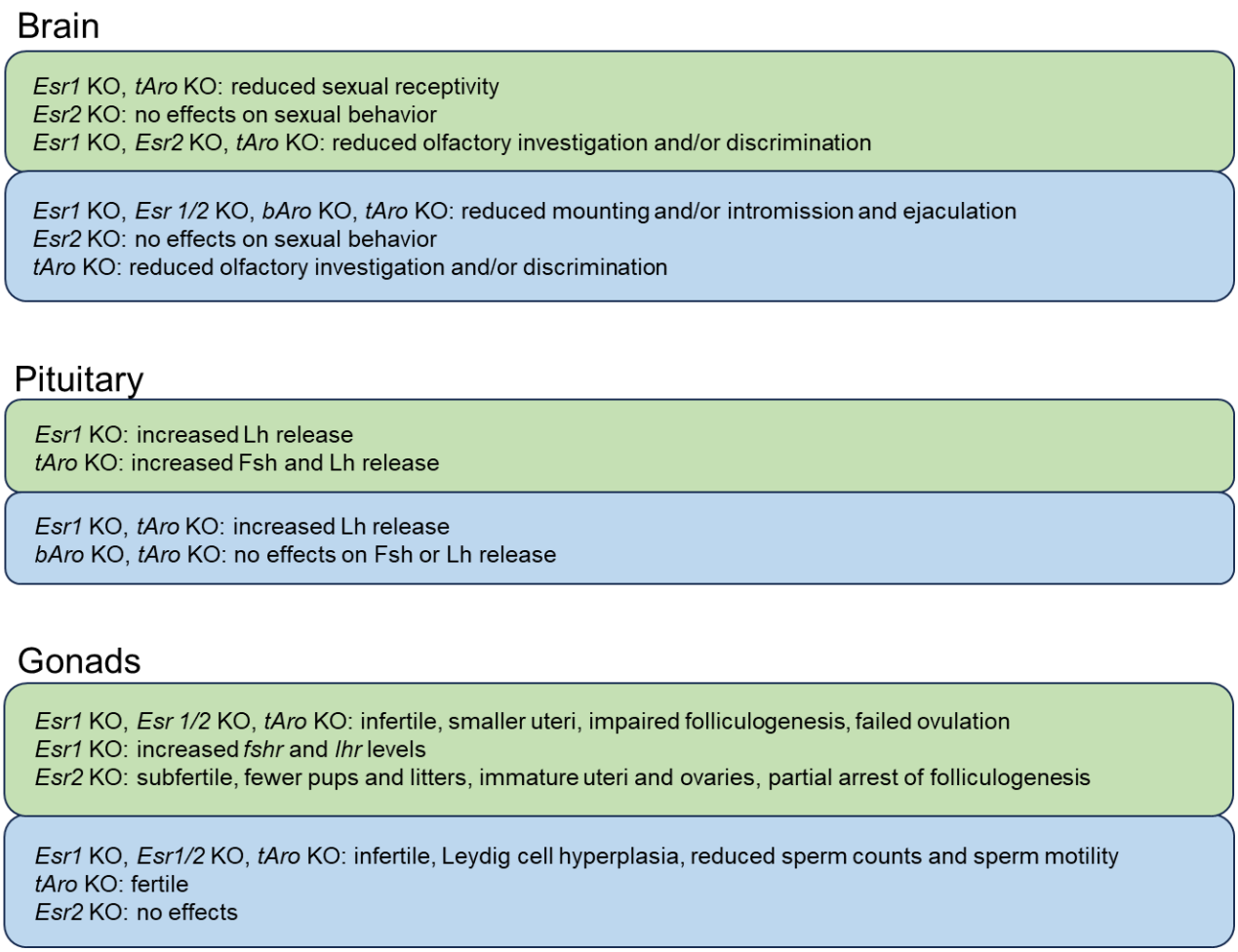


Figure 1.3- Summary of estrogen receptor knockout (*Esr* KO), total aromatase knockout (*tAro* KO), and brain aromatase knockout (*bAro* KO) effects on mouse reproductive physiology and sexual behavior post-puberty. Findings in female mice are boxed in green, while findings in males are boxed in blue. References used: Bakker et al., 2002a, 2002b, 2003; Britt et al., 2001; Brooks et al., 2020; Choleris et al., 2003, 2006; Couse et al., 1999; Dupont et al., 2000; Eddy et al., 1996; Emmen and Korach, 2003; Fisher et al., 1998; Honda et al., 1998; Krege et al., 1998; Lubahn et al., 1993; Matsumoto et al., 2003; Ogawa et al., 1997, 1998, 1999, 2000; Pierman et al., 2008; Robertson et al., 2001; Schomberg et al., 1999; Wersinger et al., 1997.

While there is strong evidence in mice identifying physiological and behavioral abnormalities following the loss of estrogen signalling pathways that significantly impair reproduction, it is difficult to disentangle the independent roles of gonadal- and brain-derived estrogens to the observed effects. Firstly, because effects on the HPG axis could be due solely to loss of estrogen signalling in one tissue, either the hypothalamus, pituitary, or gonads, but tissue-specific differences cannot be identified from whole-body KO lines. Secondly, because in females, large amounts of systemic estrogens supplied by the ovaries can cross the blood-brain barrier to affect estrogen signalling levels in the brain independent of local estrogen production (Diotel et al., 2018). The independent role of brain-derived estrogens in male sexual behavior was recently revealed through the creation of a brain aromatase knockout (*bAro* KO) model. Male *bAro* KO mice have significantly lower whole brain E2 levels compared to WT males and similar levels to *tAro* KO males, but do not display the significantly lower serum E2 levels observed in *tAro* KO males (Brooks et al., 2020). When tested in sexual behavior assays with a hormonally primed WT female, male *bAro* KO mice had a significantly longer latency to the first mount event compared to WT males. This finding confirmed an important role of brain-derived estrogens in male sexual behavior, whereby their absence significantly prolonged the initiation of mating behavior. The critical brain region of estrogen signalling underlying the behavioral impairments was not identified in the study.

While female *bAro* KO mice have not yet been assessed, growing evidence indicates that brain-derived estrogens likely serve an important role in coordinating female sexual behavior to ovarian readiness for reproduction. Neuroanatomical changes that serve critical roles in activating female sexual behavior have been identified in the mouse

brain and are linked to increased systemic estrogen levels during the estrous cycle (Gutierrez-Castellanos et al., 2022; Inoue et al., 2019, 2022; McHenry et al., 2017; Sica et al., 2009; Yin et al., 2022). Though changes in brain *cyp19a1* levels across the estrous cycle have not yet been studied, brain estrogen levels are likely a combined result of systemic estrogens that enter the brain through the blood-brain barrier as well as locally produced estrogens via upregulation of the positively estrogen-regulated mouse *cyp19a1* I.f promoter (Yilmaz et al., 2009). Indeed, brain regions with high aromatase activity levels have been documented to have higher E2 levels than those observed in the serum (i.e., systemic levels; Li and Gibbs, 2019). Brain aromatase activity alone was found to maintain transcription levels of estrogen-regulated genes in the female mouse brain following ovariectomy over short time periods (10 days); however, transcript levels of estrogen-regulated genes were significantly lower after long term (70-day) ovariectomy (Baumgartner et al., 2019). Together, these findings support the notion that ovarian estrogens could prime the brain for sexual behavior through effects on brain aromatase activity to coordinate the ovary and brain for reproduction. One of the important considerations when interpreting these findings, however, is that ovariectomy can affect numerous hormone signalling pathways that are independent of estrogens, since the ovaries produce many hormones that function in female reproduction, of particular note is progesterone. Therefore, the interpretation of support for this hypothesis is complicated by the confounding effects of ovariectomy on other hormone signalling pathways. To alleviate this concern requires the selective inhibition of aromatase expression independently in the ovary and brain to effectively

identify the independent contribution of ovarian- and brain-derived estrogens to female reproduction.

In this regard, teleost model systems offer significant advantages for assessing the independent importance of ovarian- and brain-derived estrogens for female sexual behavior. This is due to the presence of separate genes, *cyp19a1a* and *cyp19a1b*, that encode aromatase with distinct expression profiles, being highly expressed in the ovary and brain, respectively (Diotel et al., 2018). The large diversity of reproductive systems in teleosts also provide valuable opportunities for comparative analyses to identify evolutionary pathways of conserved and evolved differences in estrogen signalling effects on reproduction.

1.8 The Role of Estrogens in Teleost Reproduction

The recent creation of zebrafish *cyp19a1*$^{-/-}$ mutant lines identified the critical role of *cyp19a1a* in sexual differentiation of the ovaries, whilst *cyp19a1b* was not required for this process (Dranow et al., 2016; Lau et al., 2016; Yin et al., 2017). It was also observed that *cyp19a1a* was not required for sexual differentiation of the testes in male *cyp19a1a*$^{-/-}$ mutants and no developmental abnormalities were observed in male mutants. Rather, *cyp19a1a*$^{-/-}$ mutant testes were found to have higher spermatozoa counts and increased levels of spermatogenesis-related genes compared to WT males suggesting a potential reproductive benefit of *cyp19a1a* mutation in males (Tang et al., 2017). In addition to the importance of Cyp19a1a for ovarian development, ovarian-derived estrogens are also required for maintenance of the ovarian state in adulthood. If estrogen production is blocked via chemical inhibition of aromatase activity, female fertility is significantly impaired, and in some cases, the ovaries regress and

dedifferentiate to become testes signifying biological sex change (Rahaman et al., 2020; Takatsu et al., 2013).

Study of teleost *esr*$^{-/-}$ mutant lines has helped to identify the Esr signalling pathways underlying the observed effects on female fertility as well as identified compensatory *esr* expression in mutant lines during development and evolved differences in the importance of the *esr*s among species. In zebrafish, while there were no significant effects of single *esr1*, *esr2a* or *esr2b* mutation on female reproductive development or fertility at 120 days post-fertilization (dpf), male-biased sex ratios were observed in all mutant lines (Lu et al., 2017). Evidence of compensatory Esr signalling among the single mutant lines was suggested by the reproductive abnormalities observed in double mutant and triple mutant lines. For example, while double *esr1*$^{-/-}$;*esr2a*$^{-/-}$ mutant females displayed normal fertility, ovarian growth, and folliculogenesis, their ovaries contained multilayered follicle cells surrounding the oocyte at the previtellogenic stage compared to the single layered follicle observed in WT fish as well as hyperproliferation of stromal cells. The ovaries of double *esr2a*$^{-/-}$;*esr2b*$^{-/-}$ mutant and triple *esr1*$^{-/-}$;*esr2a*$^{-/-}$;*esr2b*$^{-/-}$ mutant lines displayed hyperproliferation of stromal cells and degenerating oocytes at 45 dpf. These mutant lines were also fully masculinized by 120 dpf demonstrating the importance of Esr2 signalling for ovarian differentiation. While study of *esr* compensation in the double mutant lines was not undertaken, female *esr2a*$^{-/-}$ ovaries were found to have increased *esr2b* levels suggesting compensation occurred during development to prevent the ovarian abnormalities observed in the double and triple mutants. No effects of single, double, or triple *esr* mutation on testicular development, spermatogenesis or male fertility were observed. Investigation of effects in the

hypothalamic-pituitary axis were not undertaken in the study. In another study, female *esr1*$^{-/-}$ mutant zebrafish were found to have enhanced fertility (i.e., higher egg production) at 90 dpf despite normal pituitary Lhb and Fshb levels but were infertile with significantly lower pituitary Lhb and Fshb levels and degenerated ovaries at 180 dpf (Chen et al., 2018). These findings indicated potential age-dependent effects of *esr1* mutation on female fertility. Male mutants were not assessed in the study. Together, these findings demonstrate a critical role of Esr2 signalling for ovarian development with evidence of *esr2* compensatory upregulation to prevent reproductive impairments in mutant lines, as well as Esr1 signalling effects on gonadotropin levels in the pituitary gland.

Species differences in Esr signalling importance for reproductive development is evidenced by differences in findings in female *esr*$^{-/-}$ mutant Japanese medaka (*Oryzias latipes*). Female and male medaka *esr1*$^{-/-}$ mutants did not display any significant impairments in gonadal development at 90 dpf and there was no evidence of compensatory upregulation identified in the mutant gonads (Tohyama et al., 2017). However, female *esr2a*$^{-/-}$ mutants aged 120-150 dpf displayed significantly higher pituitary *fshb* levels, unchanged pituitary *lhb* levels, and oviduct atresia resulting in failed oviposition and infertility (Kayo et al., 2019). No effects on ovarian or testicular development were observed in *esr2b*$^{-/-}$ mutant medaka; however, female mutants displayed increased rejection of WT males and did not spawn eggs during pairwise mating trials (Niishike et al., 2021). Together, these findings revealed important roles of Esr2a signalling for pituitary Fsh production and ovarian development, as well as Esr2b signalling effects on female sexual behavior in medaka. A summary figure of estrogen

receptor mutant (*esr*$^{-/-}$) and ovarian aromatase mutant (*cyp19a1a*$^{-/-}$) effects on zebrafish and medaka reproductive physiology and sexual behavior is depicted in Figure 1.4.

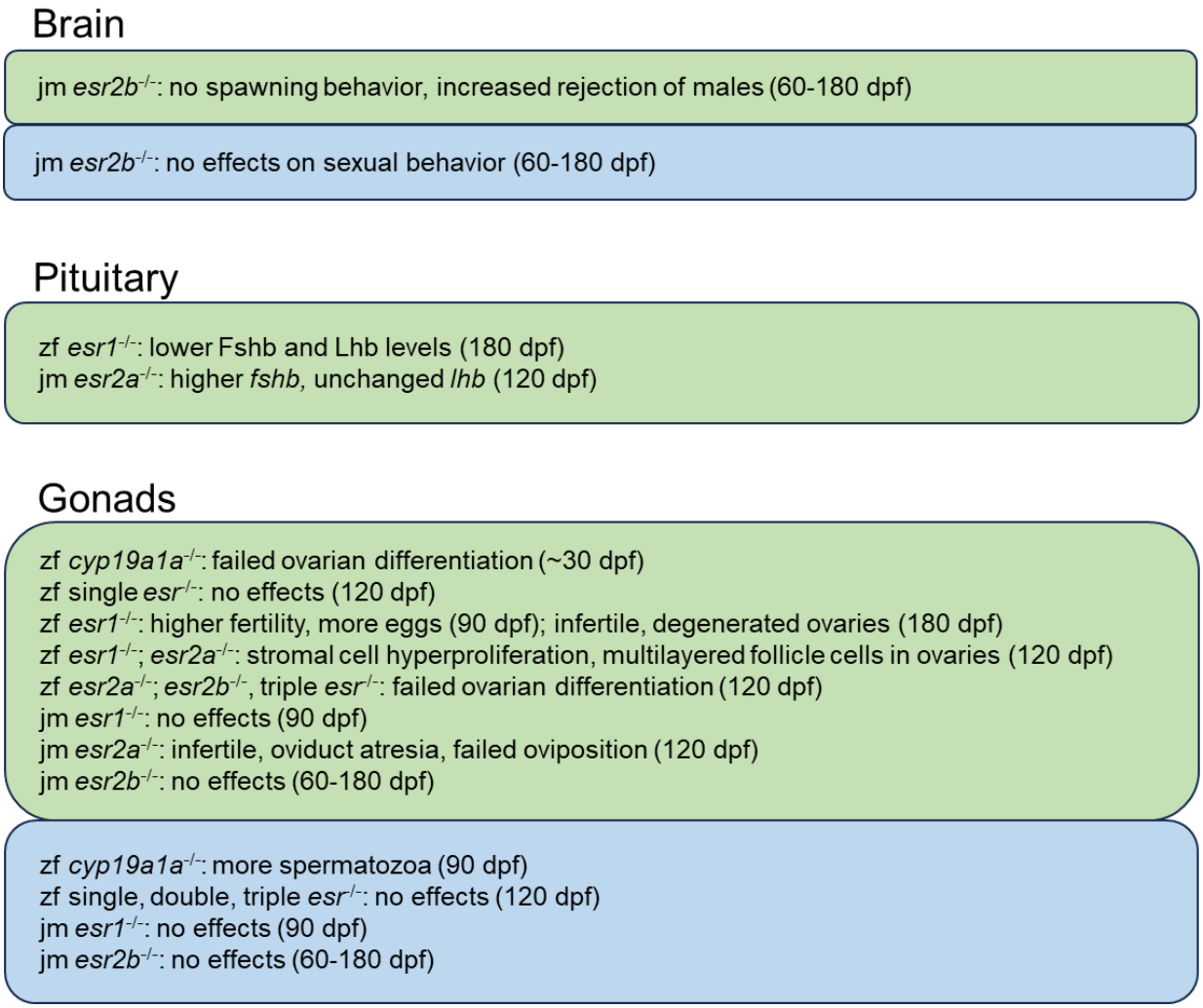


Figure 1.4- Summary of estrogen receptor mutant (*esr*$^{-/-}$) and ovarian aromatase mutant (*cyp19a1a*$^{-/-}$) effects on zebrafish (zf) and Japanese medaka (jm) reproductive physiology and sexual behavior. Findings in female fish are boxed in green, while findings in males are boxed in blue. Fish age at testing is indicated in days post-fertilization (dpf). References used: Chen et al., 2018; Dranow et al., 2016; Kayo et

al., 2019; Lau et al., 2016; Lu et al., 2017; Niishike et al., 2021; Tang et al., 2017; Tohyama et al., 2017; Yin et al., 2017.

Following sexual maturity, ovarian estrogens deposited into systemic circulation play important roles in preparing the body for reproduction. For example, systemic estrogens increase Esr1 signalling levels in the liver for vitellogenin production, which is an important yolk protein that nourishes the embryo during early development (Hara et al., 2016). Administration of chemical aromatase inhibitors significantly reduces systemic E2 and vitellogenin levels leading to decreased female fertility and fecundity (Ankley et al., 2002; Sun et al., 2007). Systemic estrogens also tune the peripheral nervous system for mate identification and assessment as evidenced in species that rely on auditory (Sisneros et al., 2004) and visual (Butler and Maruska, 2021; Friesen et al., 2017) communication signals.

There is accumulating evidence identifying changes in the central nervous system that indicate a coordination of ovarian- and brain-derived estrogen production for reproduction. For example, levels of *cyp19a1b* and Cyp19a1b activity in the brain vary across the annual reproductive cycle with highest levels observed during the reproductive period corresponding to the timing, or shortly after the timing, of high systemic estrogens (i.e., high Cyp19a1a activity) in goldfish (*Carassius auratus*; Gelinas et al., 1998), the European sea bass (*Dicentrachus labrax*; González and Piferrer, 2003), the Asian Stinging catfish (*Heteropneustes fossilis*; Chaube et al., 2015), the channel catfish (*Ictalurus punctatus*; Kazeto et al., 2003), and the Indian climbing perch (*Anabas testudineus*; Pal et al., 2018). Support for an ovary-brain coordination of *cyp19a1a* and *cyp19a1b* levels also exists in species with shorter reproductive cycles

like the African cichlid (*Astatotilapia burtoni*; Maruska et al., 2020), and diurnal breeders with a 24-hour reproductive cycle such as zebrafish (Di Rosa et al., 2016), the Threespot Wrasse (*Halichoeres trimaculatus*; Oh et al., 2018), and medaka (Okubo et al., 2011). The *cyp19a1b* promoter contains an ERE providing a direct pathway for estrogenic regulation of *cyp19a1b* (Menuet et al., 2005) following entry of systemic estrogens into the brain that can bind and activate Esrs. Changes in brain estrogen signalling levels could represent important changes in the central nervous system to augment peripheral changes important for reproduction, and therefore, function to coordinate sexual behavior to the timing of ovarian preparation. Regions of conserved RGC *cyp19a1b* expression in the brain have been identified across teleosts suggesting potential conserved evolutionary pathways for estrogenic regulation of reproduction in the brain (Shaw, 2018). Of important note, *cyp19a1b* is exclusively expressed in RGCs in the teleost brain (Tong et al., 2009), which contrasts the constitutive neuronal expression in mice, under normal conditions (Beyer et al., 1994). Nonetheless, teleost RGCs are steroid synthetic cells that possess the full complement of steroidogenic enzymes for *de novo* steroidogenesis (Diotel et al., 2018). Therefore, if locally produced brain estrogens induce effects on sexual behavior, these changes are most likely the result of RGC-derived estrogens diffusing to bind Esrs in nearby neurons to modify levels of estrogen-regulated genes important for sexual behavior.

Despite strong indications of a likely role of *cyp19a1b* in teleost reproduction, there has been limited study of *cyp19a1*$^{-/-}$ and *esr*$^{-/-}$ mutant effects on teleost sexual behavior, other than the previously mentioned fertility assessments. To the best of my knowledge, only *esr2*$^{-/-}$ mutant medaka have been studied in sexual behavior assessments to date.

Female medaka *esr2a*$^{-/-}$ mutants displayed ovarian abnormalities that caused infertility despite displaying normal sexual behavior in pairwise mating trials (Kayo et al., 2019). Interestingly, female *esr2b*$^{-/-}$ mutant medaka displayed a different phenotype characterized by normal ovarian development but reduced sexual receptivity and therefore did not spawn eggs with WT males (Niishike et al., 2021). The effects of *esr2b* mutation were female-specific as male *esr2b*$^{-/-}$ mutant medaka displayed normal courtship behavior and achieved normal levels of spawning success with WT females. Further study determined that the female mutant behavioral phenotype was a consequence of reduced Esr2b signalling in *neuropeptide B* (*npba*)-expressing neurons in the ventral telencephalon and POA. These findings were supported by the previously described higher *npba* levels in these regions in female compared to male brains and reduced female sexual behavior in *npba*$^{-/-}$ mutant medaka (Hiraki-Kajiyama et al., 2019). Together, these findings demonstrated a sex-specific role of Esr2b signalling in *npba*-expressing neurons for the display of female medaka sexual behavior. Female medaka sexual behavior has also been linked to Oxt signalling. Female *oxt*$^{-/-}$ and *oxytocin receptor 1*$^{-/-}$ *(oxtr1*$^{-/-}$) mutants displayed reduced mate preference for familiar males compared to their WT counterparts (Yokoi et al., 2020).

Other important neuropeptides for female teleost sexual behavior that may be linked to brain estrogen signalling include Avp and the SNs. For example, female *avp*$^{-/-}$ mutant zebrafish displayed reproductive impairments characterized by lower female sexual receptivity and fewer eggs spawned during dyadic sexual behavior assays (Ramachandran et al., 2023). A functional link between brain estrogen signalling and Avp production was demonstrated in spawning capable female round gobies

(*Neogobius melanostomus*), whereby E2 administration in brain explants increased Avp release into the surrounding medium (Kalamarz-Kubiak et al., 2017). Female *scg2a*$^{-/-}$;*scg2b*$^{-/-}$ mutant zebrafish were also found to have reduced sexual behavior, ovulation, oviposition, and fertility (Mitchell et al., 2020). While intracerebroventricular injections of secretoneurin A (SNa; the Scg2a-derived peptide) downregulated *cyp19a1b* levels in goldfish telencephalon and hypothalamus (Da Fonte et al., 2018), the effects of brain estrogens on SN signalling are unknown.

In sum, teleosts are an ideal model system for studying brain-derived estrogen effects on reproduction due to the presence of an independent gene for local brain estrogen production (i.e., *cyp19a1b*). There is growing evidence that suggests an important role of *cyp19a1b* in female teleost sexual behavior that could represent a coordination of brain- and ovarian-derived estrogens for reproduction. Since *cyp19a1b* is exclusively expressed in RGCs in the teleost brain, this pathway would necessitate changes to important neuronal mediators of female sexual behavior. Together, these observations formed the foundation of this thesis.

1.9 Characterization of Zebrafish *cyp19a1*$^{-/-}$ Mutant Lines

The zebrafish *cyp19a1*$^{-/-}$ mutant lines used in this study were previously published in Yin et al. (2017). In brief, the mutant lines were created using a parental zebrafish AB strain and transcription activator-like effector nucleases (TALEN)-mediated technology to create indel mutations in each of the *cyp19a1* paralogs producing frameshift mutations in the corresponding proteins. A 31-basepair (bp) deletion was generated in exon 3 of *cyp19a1a* (Figure 1.5A) and a 13-bp deletion was created in exon 4 of *cyp19a1b* (Figure 1.5B). The target genomic regions in the *cyp19a1*$^{-/-}$ mutant lines were amplified

by polymerase chain reaction (PCR) and sequenced to verify the indel mutations at target sites. I further validated the *cyp19a1b*$^{-/-}$ mutant line using E2 enzyme-linked immunoassay (ELISA) tests to identify significantly lower brain E2 content in *cyp19a1b*$^{-/-}$ mutants compared to WT fish (see Chapter 2).

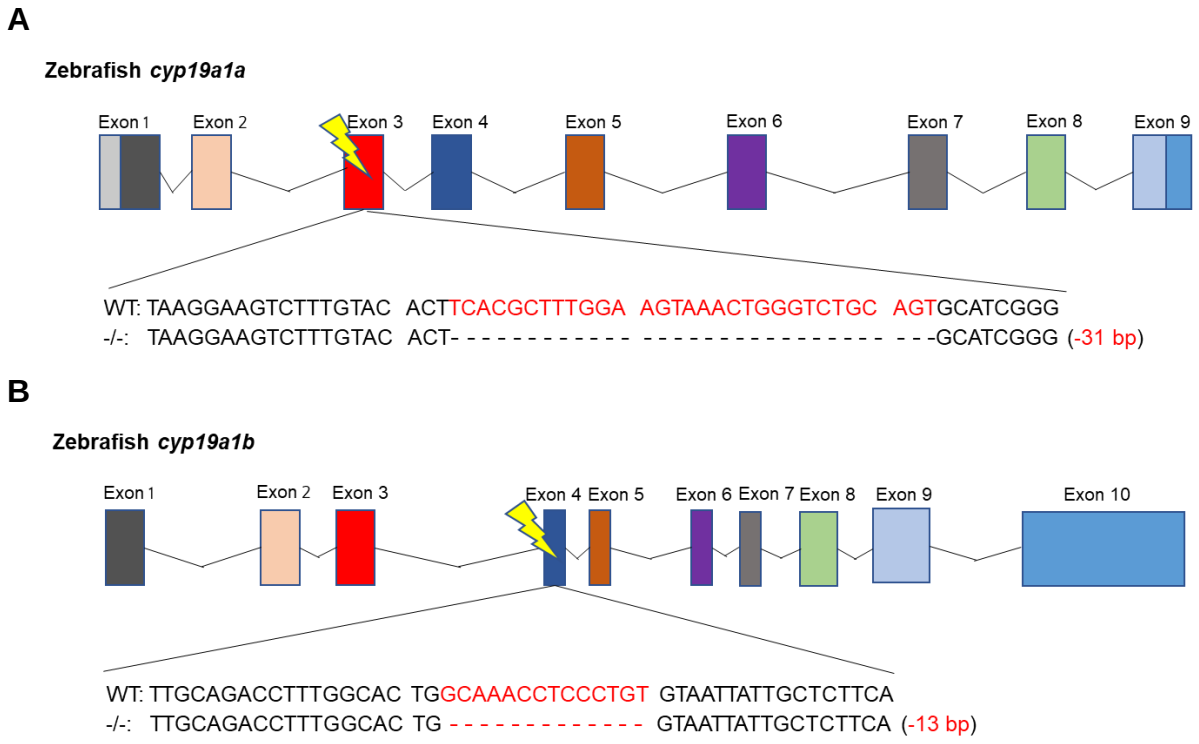


Figure 1.5- Zebrafish *cyp19a1*$^{-/-}$ mutant lines created by transcription activator-like effector nucleases (TALEN)-mediated gene deletions. The *cyp19a1a*$^{-/-}$ mutant line contains a 31-basepair (bp) deletion in exon 3 of *cyp19a1a* (**A**). The *cyp19a1b*$^{-/-}$ mutant line contains a 13-bp deletion in exon 4 of *cyp19a1b* (**B**). Locations of the gene deletions are indicated by red dashed lines. Figure adapted from Yin et al., 2017.

1.10 Ethics Statement

All experiments were approved by the University of Ottawa Protocol Review Committee and adhered to the guidelines of the Canadian Council on Animal Care for the use of animals in research and teaching.

Chapter 2: Mutation of Brain Aromatase Disrupts Spawning Behavior and Reproductive Health in Female Zebrafish

This chapter was adapted from:

Shaw, K., Therrien, M., Lu, C., Liu, X., Trudeau, V.L., 2023. Mutation of brain aromatase disrupts spawning behavior and reproductive health in female zebrafish. *Frontiers in Endocrinology*, *14*, p.1225199.

Study contributions: KS conceived of and conducted the experiments and wrote the manuscript, MT helped design and conducted experiments, CL provided methods and reagents, XL provided the *cyp19a1*$^{-/-}$ mutant lines, VLT helped design experiments, revised the manuscript, and provided funding for the study.

Abstract

Aromatase (Cyp19a1) is the steroidogenic enzyme that converts androgens into bioactive estrogens, and hence is in a pivotal position to mediate reproduction and sexual behavior. In teleosts, there are two aromatase paralogs: *cyp19a1a* that is highly expressed in granulosa and Leydig cells in the gonads with critical function in sexual differentiation of the ovary, and *cyp19a1b* that is highly expressed in radial glial cells in the brain with unknown roles in reproduction. *Cyp19a1*$^{-/-}$ mutant zebrafish lines were used to investigate the importance of the *cyp19a1* paralogs for sexual behavior and offspring survival and early development. Mutation of *cyp19a1b* was found to increase the latency to the first oviposition in females. Mutation of *cyp19a1b* in females also increased the number of eggs spawned; however, significantly more progeny died during early development resulting in no net increase in female fecundity. This finding suggests a higher metabolic cost of reproduction in *cyp19a1b*$^{-/-}$ mutant females. In males, the combined mutation of both *cyp19a1* paralogs resulted in significantly lower progeny survival rates, indicating a critical function of *cyp19a1* during early larval development. These data establish the specific importance of *cyp19a1b* for female sexual behavior and the importance of the *cyp19a1* paralogs for early larval survival.

2.1 Introduction

Androgens and estrogens are two major groups of sex steroids that play critical roles in vertebrates to coordinate the physiology and behavior of an individual with its environment. Aromatase (Cyp19a1) is the terminal steroidogenic enzyme that converts the aromatizable androgens, T and androstenedione, into E2 and E1, respectively. In birds and most mammals, there is only one aromatase gene, *cyp19a1*, whose

differential tissue distribution is attributed to differences in splicing of 5' untranslated promoter regions across tissues (Simpson et al., 1994). In contrast, teleosts possess two distinct *cyp19a1* genes, *cyp19a1a* and *cyp19a1b,* with differential tissue distribution. This is due to distinct regulatory elements in their promoter regions and differences in the presence of required transcription factors in specific cell types (Le Page et al., 2008). In many teleosts, *cyp19a1a* is the most highly expressed aromatase in granulosa and Leydig cells in the gonads; while *cyp19a1b* is expressed at much higher levels in the brain, and specifically in RGCs (Diotel et al., 2018).

The ovaries are a major source of estrogens that are released into systemic circulation to prepare the body for reproduction (Findlay et al., 2010). Loss of aromatase expression significantly impairs female fertility and fecundity, at least in part, through loss of ovarian estrogen production. For example, *tAro* KO female mice, which lack whole body aromatase expression, are infertile due to disrupted folliculogenesis and ovulation failure (Britt et al., 2001). In teleosts, short term chemical inhibition of aromatase reduces female fertility and fecundity through impaired oocyte development and reduced plasma vitellogenin levels (Ankley et al., 2002; Ayobahan et al., 2019; Sun et al., 2007). Long term aromatase inhibitor treatment induces more dramatic effects such as ovarian retraction followed by testes formation resulting in female-to-male sex change (Rahaman et al., 2020; Takatsu et al., 2013). Since chemical inhibitors are non-selective for the two Cyp19a1 isoforms, it only recently became possible to begin identifying their independent contributions to reproduction via the creation of zebrafish *cyp19a1*$^{-/-}$ mutant lines. It was discovered that *cyp19a1a* expression is critical for sexual differentiation of the ovaries while *cyp19a1b* expression is not required for this process

(Dranow et al., 2016; Lau et al., 2016; Yin et al., 2017). Though *cyp19a1a* is not required for testes differentiation, it is expressed at low levels and differences have been observed in the importance of its expression for male fertility in mice and teleosts. For example, the testes of male *tAro* KO mice were found to have arrested germ cell development at the spermatid stage as well as impaired sperm motility (Fisher et al., 1998; O'Donnell et al., 2001; Robertson et al., 1999, 2001). In contrast, there were no developmental abnormalities observed in the testes of zebrafish *cyp19a1a*$^{-/-}$ mutants compared to WT males (Tang et al., 2017). Rather, the testes of *cyp19a1a*$^{-/-}$ mutants had more spermatozoa and higher levels of spermatogenesis-related genes, and these mutant males displayed normal fertility levels. These findings reveal critical roles of ovarian aromatase expression for female fertility and fecundity in mice and teleosts, whilst differences have been observed for testicular aromatase importance in male fertility, with *cyp19a1a* being dispensable for male teleost fertility.

A second major source of estrogens is the brain. In birds and mammals, the *cyp19a1* gene contains a brain-specific promoter region that specifies constitutive neuronal expression (Honda et al., 1994, 1999; Ramachandran et al., 1999; Yilmaz et al., 2009). In contrast, teleosts have a second paralog, *cyp19a1b*, that is expressed exclusively in RGCs due to the presence of G x RE, the glial x responsive element, in the promoter region (Le Page et al., 2008). Increasing evidence has identified important roles for brain-derived estrogens in reproduction. For example, both female and male *tAro* KO mice display reduced sexual behavior (Bakker et al., 2002a, 2002b; Honda et al., 1998; Matsumoto et al., 2003) and impaired olfactory discrimination (Bakker et al., 2002b; Pierman et al., 2008) that together suggests an involvement of brain estrogens in social

recognition. The recent creation of a male *bAro* KO mouse line identified the important role of brain-derived estrogens in male sexual behavior. Male *bAro* KO mice were found to have a significantly longer latency to the first mount event and a trend, though not significant, towards a greater latency to the first intromission event when paired with a hormonally primed female compared to WT males in sexual behavior assays (Brooks et al., 2020). Female *bAro* KO mice have not yet been studied. In teleosts, studies have identified impairments in social recognition following chemical aromatase inhibition, such as reduced dominant male aggression in social behavior assays (Huffman et al., 2013; Jalabert et al., 2015). There has been comparatively less study, however, of effects on sexual behavior. This is likely due, at least in part, to the inability to identify specific Cyp19a1b- versus Cyp19a1a-induced effects on reproduction with the use of chemical aromatase inhibitors. There is strong evidence, however, to suggest that brain-derived estrogens likely play an important role in teleost sexual behaviors. Firstly, *cyp19a1b* is expressed in numerous brain regions important for sexual behavior (Shaw, 2018). Secondly, *cyp19a1b* is a known estrogen-regulated gene due to the presence of an ERE in its promoter region (Menuet et al., 2005). This observation suggests that increased systemic estrogen levels, via high ovarian Cyp19a1a activity, likely drive increased *cyp19a1b* expression to prepare the brain for sexual behavior at the time in which the ovaries are prepared for reproduction.

In this study, mutant *cyp19a1b*$^{-/-}$ females were found to have a longer latency to oviposition and released a significantly higher number of eggs during spawning compared to WT females. However, there was higher larval mortality in progeny from female *cyp19a1b*$^{-/-}$ mutant pairings, resulting in no net fecundity differences between

female *cyp19a1b*$^{-/-}$ mutant and WT pairings. A significantly higher larval mortality rate was found in progeny from *cyp19a1a*$^{-/-}$;*cyp19a1b*$^{-/-}$ mutant male pairings. These data reveal the importance of *cyp19a1b* for zebrafish reproduction.

2.2 Materials and Methods

2.2.1 Experimental animals

Procedures used in this study were approved by the University of Ottawa Animal Care Committee and followed the guidelines of the Canadian Council on Animal Care for the use of animals in research. All fish were reared at the University of Ottawa Aquatics Facility according to standard housing procedures. The *cyp19a1*$^{-/-}$ mutant lines and WT zebrafish used in the experiments were all derived from a parental zebrafish AB strain to ensure identical genetic backgrounds amongst the groups for assessing the effects of *cyp19a1* mutation on sexual behavior. The *cyp19a1*$^{-/-}$ mutant lines were generated using the TALEN genome editing system to create indel mutations at target sites in each of the *cyp19a1* paralogs producing frame-shift mutations (Yin et al., 2017). Mutation of *cyp19a1a* impairs sexual differentiation of the ovary resulting in all male populations of *cyp19a1a*$^{-/-}$ and *cyp19a1a*$^{-/-}$;*cyp19a1b*$^{-/-}$ mutant lines, thus only male mutants can be tested in these lines. These males have significantly lower serum E2 levels compared to WT males due to the contributions of gonadal aromatase expression to circulating estrogen levels. Mutation of *cyp19a1b* does not affect the sex ratio of the mutant line allowing testing of *cyp19a1b* mutation effects on both males and females, and serum E2 levels in these fish are not significantly different from their WT counterparts. Fish were housed in 10-L tanks with dechloraminated water at 28°C and maintained on a 14:10 light-dark cycle and fed twice daily. All fish tested in this

experiment were between the ages of 5-11 months post-fertilization and had no previous experience in the sexual behavior assay; with males and females separated into same-sex tanks at sexual maturation to prevent sexual interactions.

2.2.2 Behavioral tests

For all trials, mutant fish were size matched within 2 mm body length (BL; < 10% BL difference) to an opposite sex WT fish. The evening before each experiment, paired fish were transferred to a 1-L testing tank containing an insert at the bottom for egg collection and a divider in the middle of the tank to keep the male and female separate before testing. The testing pair was allowed to acclimate overnight in a ZebraCube (Viewpoint Behavior Technology, Inc., Lyon, France) with the camera (either a Panasonic 16GB HC-V700M Full HD camcorder, Osaka, Japan or a Canon VIXIA HF R800 camcorder, Tokyo, Japan) present. The next morning, the pair was transferred to a new 1-L testing tank containing clean system water and the divider was removed and video recording started at 0900h (lights on). The fish pair was allowed to interact for 150 mins, which was the most appropriate time determined in preliminary trials to capture the full timing of spawning behavior, particularly in the *cyp19a1b*$^{-/-}$ mutant lines. Videos were coded by date to ensure that the observer was blind to the treatment groups during viewing and video analysis using VLC media player (https://www.videolan.org/). Oviposition events were selected as the most appropriate measure to identify changes in sexual behavior in this study. This decision was made based on the similar descriptive measures for the ethograms of sexual and aggressive behaviors in zebrafish (Darrow and Harris, 2004; Oliveira et al., 2011) that prevented unambiguous identification of motivational state during initial interactions in the video recordings.

Since there is strong evidence identifying roles of brain derived E2, and therefore brain aromatase (i.e., Cyp19a1b), in both sexual and aggressive behaviors in vertebrates (Huffman et al., 2013; Jalabert et al., 2015; Shaw, 2018; Trainor et al., 2006), it was important to select a definitive measure of sexual behavior for analysis in this study. Oviposition events were characterized and were identified in the video recordings as the timing of gamete release during physical interaction. The oviposition times were manually recorded in an Excel spreadsheet for the later determination of the time to first and last spawning events, as well as the total number of spawning events in a trial.

2.2.3 Egg collection and eleutheroembryo rearing

Eggs were obtained from natural mating of a *cyp19a1*$^{-/-}$ mutant or WT fish paired with an opposite sex WT fish using the methods described above. Following the careful removal of the eggs from the bottom of the tank, they were then rinsed with clean system water and transferred to a Petri dish containing E3 medium at a density of 3 embryos/mL (Martinez et al., 2019). At 4 hours post-fertilization (hpf), unfertilized eggs were counted and removed from the Petri dish for the assessment of fertilization rates. All embryos were raised in an incubator at 28°C for the duration of the experiment. The following day (1 dpf), any dead embryos were counted and recorded, then removed, following which E3 medium was replaced, and dishes were returned to the incubator. On 2, 3, and 4 dpf, any dead eggs were again counted and removed. Eleutheroembryos (i.e., hatched embryos) were counted and placed into separate Petri dishes containing E3 medium for measurements, and the medium was then replaced for the incubation of the remaining live eggs.

2.2.4 Zebrafish eleutheroembryo morphometrics

On day 2 post-fertilization, five eleutheroembryos were randomly selected from each clutch for measurement. The five measurements were later averaged to obtain a single value to represent the clutch at the given time. The eleutheroembryos were anesthetized with Tricaine (100 mg/L; Syndel Canada, Nanaimo, BC, Canada, cat# 02168510) and positioned under a dissecting microscope (Leica Biosystems, Wetzlar, Germany, model# Wild M10) fitted with a phone mount and iPhone XS. Eleutheroembryos were gently positioned on their side to produce a lateral view for measurement of BL, eye area (EA), and yolk sac area (YSA) in photographs. A ruler was positioned under the microscope and photographed at the same magnification as used for Petri dishes to serve as a scale bar for image analysis. Following completion of the measurements, the eleutheroembryos were euthanized by immersion in an ice water bath.

2.2.5 Enzyme-linked immunosorbent assay

The brain and ovary steroid extraction protocol has been described previously (Lu et al., 2023). In brief, female *cyp19a1b*$^{-/-}$ mutant and WT fish were euthanized between 9h00 – 10h00 in an ice water bath, and brains and ovaries were immediately dissected, placed into individual labelled tubes, weighed, and tubes were then placed on ice. Homogenization buffer (90% methanol) was added to each tube, 150 µL per brain and 1000 µL per ovary sample, and samples were then sonicated for tissue dispersion and steroid release into solution. Samples were centrifuged (4°C, 13,200 rpm) for 10 mins, and the supernatant was carefully removed and transferred to a new labelled tube. The samples were then evaporated to dryness at 45°C (Labconco Centrivap Centrifugal

Vacuum Concentrator, Kansas City, USA, model# 7810014, 45°C, 1 hr), and stored at 4°C overnight. The following day, 100 µL resuspension buffer (0.2% formic acid, 5% acetonitrile in water) was added to each sample tube, vortexed, and the tubes were then placed in a sonic water bath for 15 mins to resuspend the samples. Following resuspension, the samples were run in C-18 solid-phase extraction columns (Dr. Maisch HPLC GmbH, Ammerbuch-Entringen, Germany, cat# r10.aq,; > 85% recovery rate in liquid chromatography tandem mass spectrometry) and the eluted samples were then evaporated to dryness (45°C, 3 hrs). The evaporated steroid residue was then resuspended in buffer, 200 µL for brain samples and 1000 µL for ovary samples, for 24 hrs at 4°C with intermittent vortexing prior to testing. Levels of E2 were measured using ELISA test kits (Cayman Chemical, Ann Arbor, MI, USA, cat# 501890,) according to the manufacturer's instructions. This assay has been extensively tested in teleost species (Chen et al., 2018; Converse and Thomas, 2019; Giroux et al., 2019; Kayo et al., 2020; Nishiike et al., 2021; Saravanan et al., 2019) with an assay range of 0.61 – 10,000 pg/mL and sensitivity limit of 20 pg/mL. This E2 assay has very low levels of cross reactivity to other steroids such as cortisol, progesterone and T (< 0.01%). All samples were run in duplicate on a single assay plate and only samples with intra-assay coefficient of variations < 10% were used in analyses.

2.2.6 Image analysis

Photos were analyzed using ImageJ (v1.53) software. All images were analyzed by the same observer to ensure consistent measurements (i.e., to prevent inter-observer variability), and each measurement was repeated three times, and the average value was calculated. Each image was measured for BL, EA, and YSA. Body length consisted

of a straight-line measurement from the posterior tip of the notochord to the most anterior tip of the head passing through the eye (Figure 2.1). Measurements of EA and YSA were conducted according to Martínez et al. (2019) and traced with the freehand tracing tool in ImageJ (Figure 2.1).

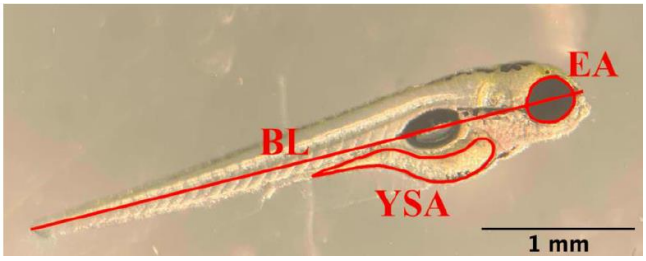


Figure 2.1- Depiction of morphometric analyses. The measurement of body length (BL), eye area (EA), and yolk sac area (YSA) in an eleutheroembryo on day 2 post-fertilization. Scale bar= 1 mm.

2.2.7 Statistical analyses

Statistical analyses were conducted using GraphPad Prism v9 (GraphPad Software, Inc., La Jolla, CA, USA) with normality and homoscedasticity assessed using Shapiro-Wilk and Levene's tests, respectively. For the behavior and eleutheroembryo analyses, normally distributed data were analyzed by either Student's T test or One-Way ANOVA followed by Dunnett's multiple comparisons tests for pairwise comparisons. Data that were not normally distributed were analyzed using either Mann-Whitney U or Kruskal-Wallis tests followed by Dunn's multiple comparisons tests for pairwise comparisons. Data are presented as boxplots with the horizontal lines representing mean or median values, boxes representing interquartile ranges, and whiskers representing min-max values. For the E2 measurements, data were log-transformed and analyzed using a

Two-Way ANOVA followed by Tukey's multiple comparisons tests. For all data, significance is defined at $p < 0.05$ and all tests were assessed as two-tailed.

2.3 Results

2.3.1 Time to the first oviposition event

Female *cyp19a1b*$^{-/-}$ mutant fish took significantly more time to the first oviposition event compared to WT females ($U(17,17)=70.50$, $p=0.0096$; Figure 2.2A). On average, *cyp19a1b*$^{-/-}$ mutant females took 4.1 times longer to the first oviposition event compared to WT females. There were no significant differences in the time to the first oviposition event among any of the male genotypes ($H(3)=7.005$, $p=0.0717$; Figure 2.2B).

2.3.2 Time to the last oviposition event

Female *cyp19a1b*$^{-/-}$ mutant fish took significantly more time to the last oviposition event compared to WT females ($U(17,17)=81.50$, $p=0.0293$; Figure 2.2C). On average, the last oviposition event in *cyp19a1b*$^{-/-}$ mutant female pairings occurred 2 times later than in WT pairings. There were no significant differences in the time to the last oviposition event among any of the male genotypes ($F(3)=1.657$, $p=0.1850$; Figure 2.2D).

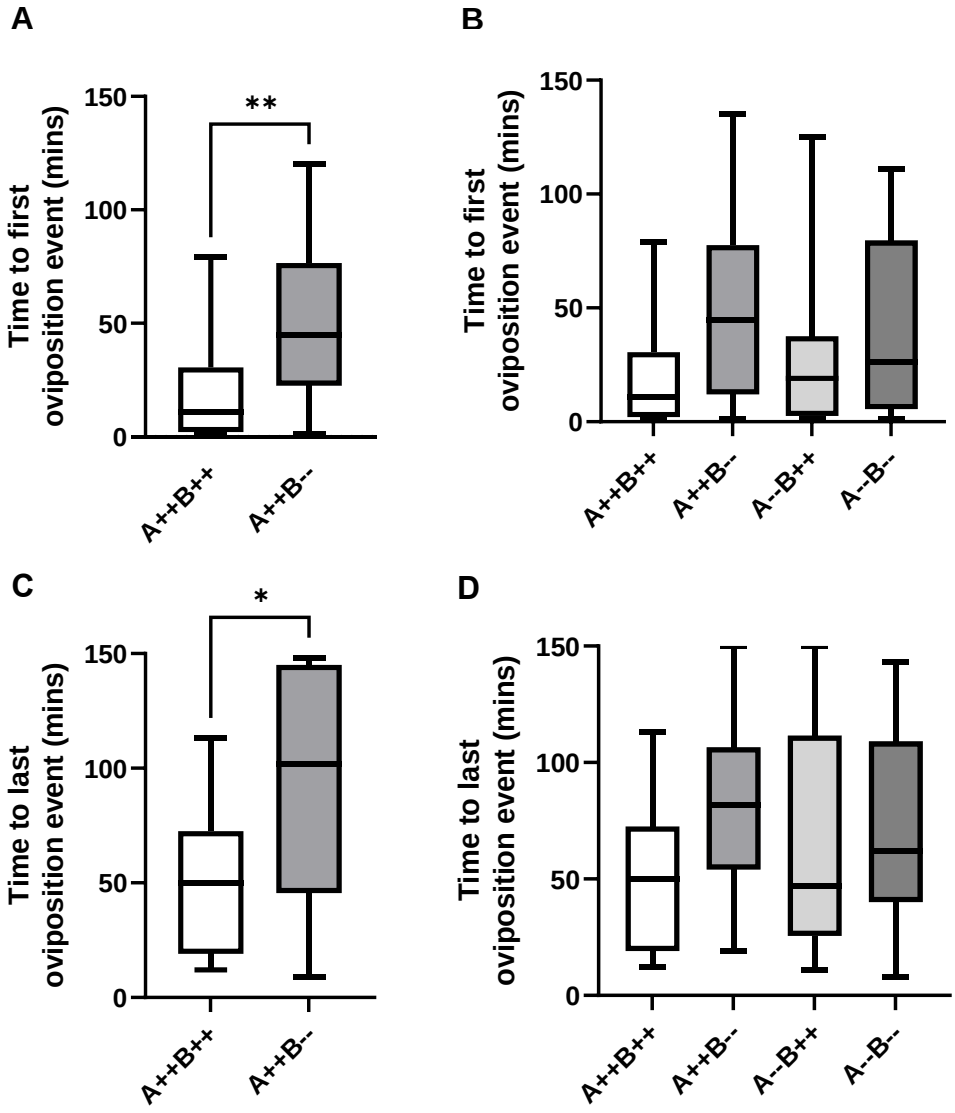
2.3.3 Spawning duration

There were no significant differences in the spawning duration between the *cyp19a1b*$^{-/-}$ mutant and WT female groups ($T(32)=1.130$, $p=0.2668$; Figure 2.2E) or between any of the mutant and WT male groups ($F(3)=0.3184$, $p=0.8120$; Figure 2.2F).

2.3.4 Number of oviposition events

Female *cyp19a1b*$^{-/-}$ mutant fish had significantly more oviposition events compared to WT females ($U(17,17)=68$, $p=0.0074$; Figure 2.2G). On average, *cyp19a1b*$^{-/-}$ mutant

females exhibited 2.7 times more oviposition events than WT females. There were no significant differences in the number of oviposition events among any of the male genotypes ($F(3)=0.7744$, $p=0.5125$; Figure 2.2H).



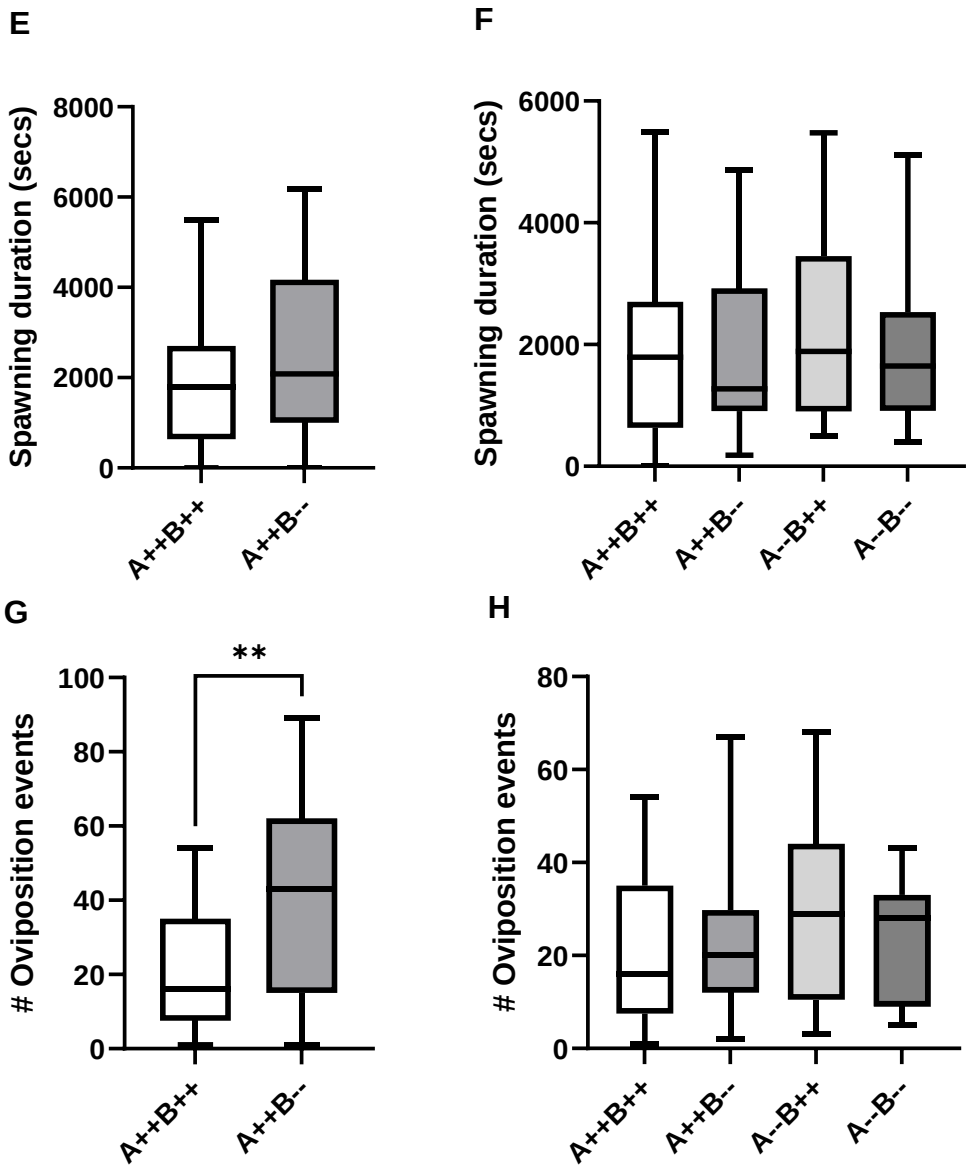


Figure 2.2- Timing and number of oviposition events during zebrafish pairwise mating trials. Test females (n=17 pairs) are displayed on the left **(A, C, E, G)** and test males (n=17-18 pairs) are displayed on the right **(B, D, F, H)**. Mann-Whitney U **(A, C, G)**, Student's T **(E)**, Kruskal-Wallis **(B)** and One-Way ANOVA **(D, F, H)** tests were performed. Horizontal lines represent mean or median values, boxes represent

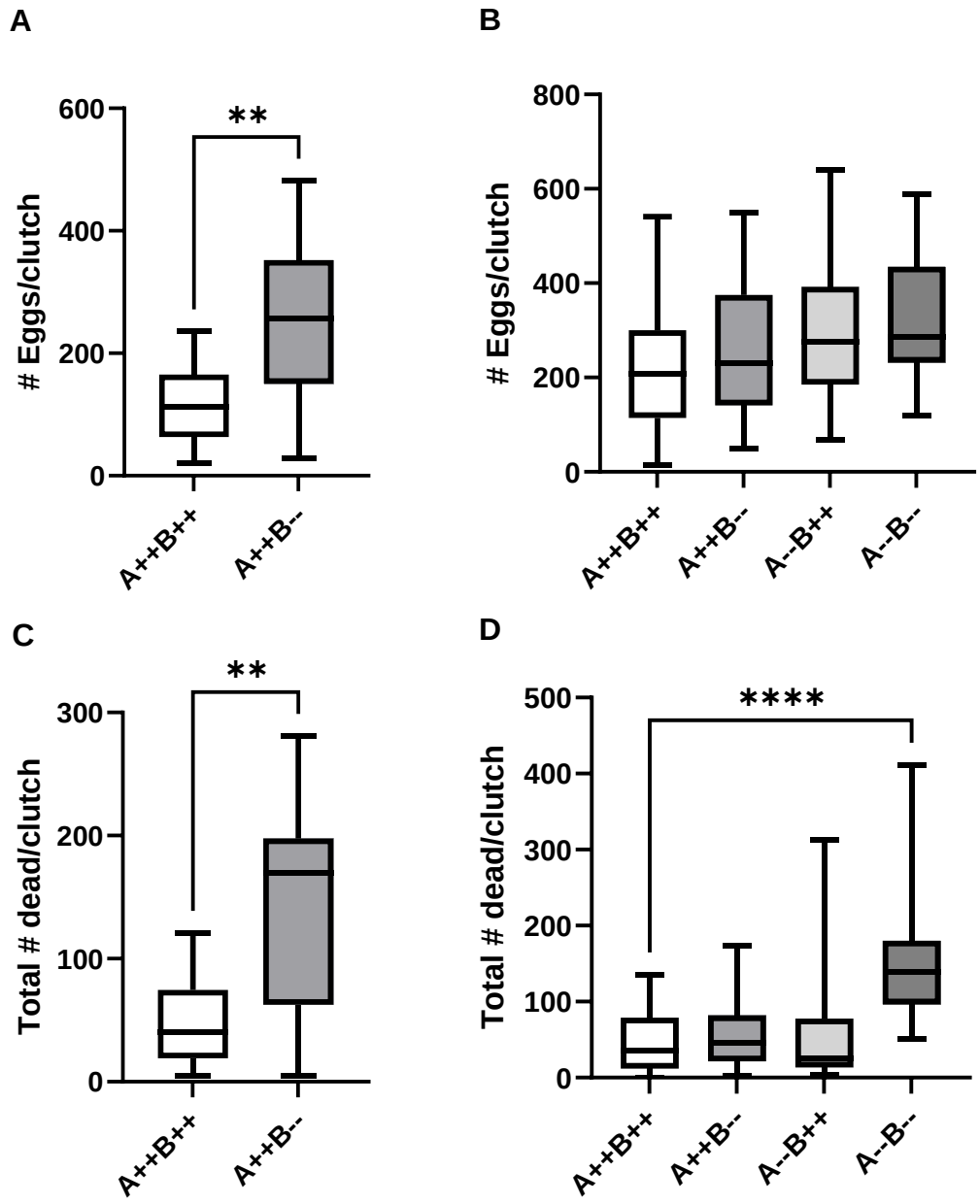
interquartile ranges, and whiskers represent min-max values. Key to genotypes: A=*cyp19a1a*, B=*cyp19a1b*. Asterisks denote $p<0.05$ (*) and $p<0.01$ (**).

2.3.5 Fecundity

There were no significant differences in fertilization rates between female *cyp19a1b*$^{-/-}$ mutant and WT female pairings ($U(14,16)=90$, $p=0.3557$, data not shown), with average fertilization rates of 99% and 93%, respectively. Female *cyp19a1b*$^{-/-}$ mutant fish spawned significantly more eggs per clutch compared to WT females ($U(14,17)=44$, $p=0.0022$, Figure 2.3A); however, significantly more eleutheroembryos died by Day 4 from the female *cyp19a1b*$^{-/-}$ mutant clutches compared to the WT clutches ($U(14,16)=45$, $p=0.0043$, Figure 2.3C), resulting in no net difference in fecundity between the female groups ($T(28)=0.1303$, $p=0.8973$, Figure 2.3E). On average, there were 2.3 times more eggs spawned by *cyp19a1b*$^{-/-}$ mutant females and 4.2 times more progeny died by Day 4 from these mutant females compared to WT females.

Fertilization rates for WT, *cyp19a1b*$^{-/-}$, *cyp19a1a*$^{-/-}$, and *cyp19a1a*$^{-/-}$;*cyp19a1b*$^{-/-}$ mutant group pairings were 99%, 98%, 95%, and 99%, respectively, with no significant differences observed among the male groups ($H(3)=3.039$, $p=0.3856$, data not shown). There were no significant differences in the number of eggs per clutch between any of the male group pairings ($F(3)=1.649$, $p=0.1857$, Figure 2.3B); however, there was a significant difference in the number of dead eleutheroembryos per clutch amongst the male genotypes ($H(3)= 23.91$, $p<0.0001$). Pairwise comparisons revealed that significantly more progeny died by Day 4 from the male *cyp19a1a*$^{-/-}$;*cyp19a1b*$^{-/-}$ mutant group pairings compared to the WT male pairings ($p<0.0001$, Figure 2.3D). There was also a significant difference in the survival rate of eleutheroembryos from the male

group pairings (H(3)=14.13, p=0.0027). Pairwise comparisons revealed that there was a significantly lower survival rate in the double mutant male offspring compared to the WT male offspring (p=0.0203, Figure 2.3F). The survival rate of larvae from male *cyp19a1a*$^{-/-}$;*cyp19a1b*$^{-/-}$ mutant pairings was 24% lower than WT pairings.



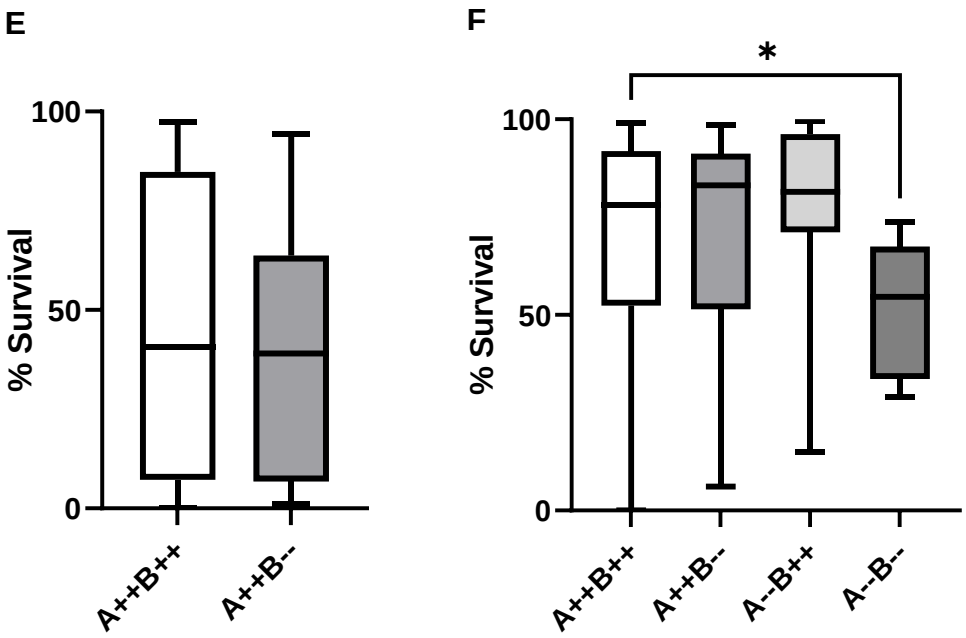
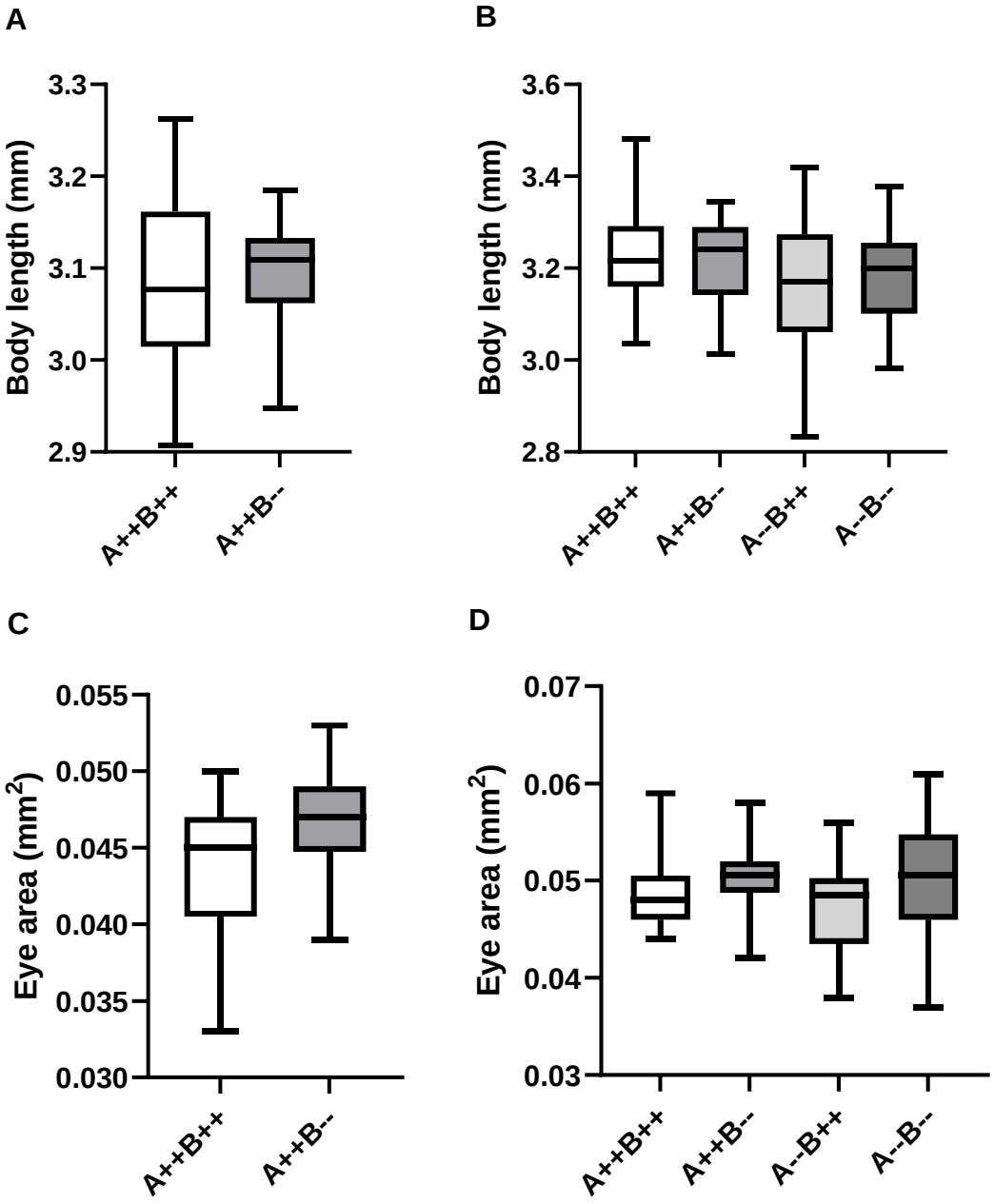


Figure 2.3- Number of eggs spawned per clutch and egg survival rate during zebrafish pairwise mating trials. Test females (n=14-16 pairs) are displayed on the left **(A, C, E)** and test males (n=16-23 pairs) are displayed on the right **(B, D, F)**. Mann-Whitney U **(A, C)**, Student's T **(E)**, One-Way ANOVA **(B)** and Kruskal Wallis **(D, F)** tests were performed. Horizontal lines represent mean or median values, boxes represent interquartile ranges, and whiskers represent min-max values. Key to genotypes: A=*cyp19a1a*, B=*cyp19a1b*. Asterisks denote p<0.05 (*), p<0.01 (**), p<0.0001 (****).

2.3.6 Eleutheroembryo morphometrics

There were no significant differences on day 2 between the BL ($T(21)=0.1487$, $p=0.8832$; Figure 2.4A), EA ($T(21)=1.647$, $p=0.1144$; Figure 2.4C), or YSA ($T(21)= 0.09161$, $p=0.9279$, Figure 2.4E) of offspring from female *cyp19a1b*$^{-/-}$ mutant and WT females. There were also no significant differences on day 2 between the BL

(F(3)=1.657, p=0.1844; Figure 2.4B), EA (H(3)=5.172, p=0.1596, Figure 2.4D), or YSA (H(3)=2.561, p=0.4644, Figure 2.4F) of offspring from any of the male genotypes.



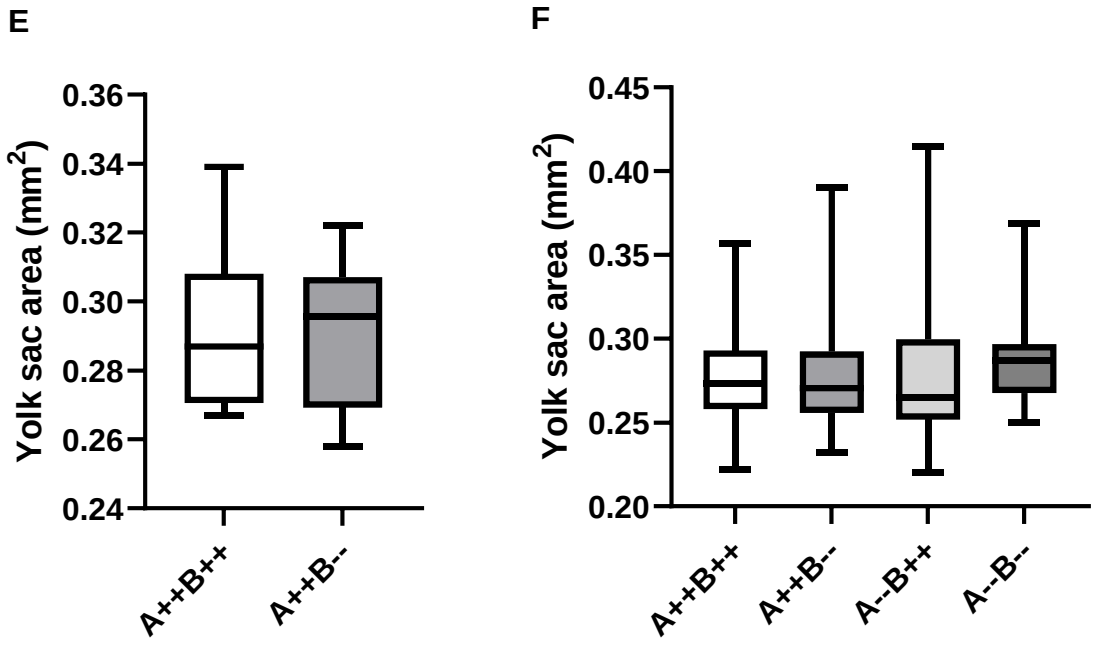


Figure 2.4- Body length, eye area, and yolk sac area measurements of eleutheroembryos from zebrafish pairwise mating trials on day 2 post-fertilization. Eleutheroembryos from test females (n=9-14) are displayed on the left **(A, C, E)** and from test males (n=16-21 pairs) are displayed on the right **(B, D, F)**. Student's T **(A, C, E)**, One-Way ANOVA **(B)** and Kruskal-Wallis **(D, F)** tests were performed. Horizontal lines represent mean or median values, boxes represent interquartile ranges, and whiskers represent min-max values. No statistical differences between genotypes were evident. Key to genotypes: A=*cyp19a1a*, B=*cyp19a1b*.

2.3.7 Estradiol content in female brain and ovary

There was a significant main effect of tissue type on E2 levels ($F(1,21)=11.40$, $p=0.0029$). Ovarian tissue had 1.6 times higher E2 levels compared to brain. There was no significant main effect of genotype on E2 levels ($F(1,21)=3.295$, $p=0.0838$). There was a significant tissue type X genotype interaction ($F(1,21)=15.36$, $p=0.0008$). The

brains of *cyp19a1b*$^{-/-}$ mutant females had 2.3 times lower E2 levels compared to the brains of WT females (p=0.0066; Figure 2.5). The brains of *cyp19a1b*$^{-/-}$ mutant females also had 3 and 2.2 times lower E2 levels compared to their ovaries (p=0.0002) and to the ovaries of WT females (p=0.0080), respectively (Figure 2.5).

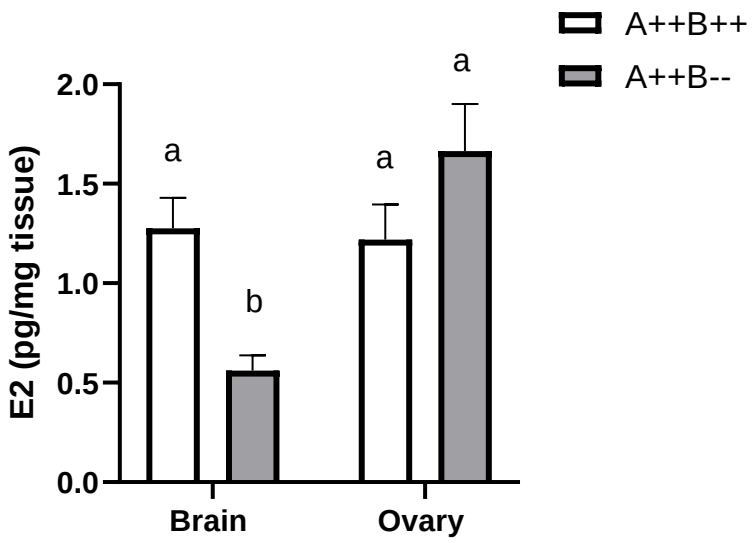


Figure 2.5- Estradiol (E2) levels in the brains and ovaries of adult WT (A++B++) and *cyp19a1b*$^{-/-}$ mutant (A++B--) female zebrafish (n=5-8 per group). Data were log-transformed and analyzed using a Two-Way ANOVA followed by Tukey's multiple comparisons tests. Data are plotted as means + standard error of the mean. Means with different letters a-b represent statistically significant differences ($p<0.05$). For clarity, the significant main effect of tissue type is not displayed. Key to genotypes: A=*cyp19a1a*, B=*cyp19a1b*.

2.4 Discussion

This study is the first assessment of the independent contribution of *cyp19a1b* and by implication, brain estrogen production, to female sexual behavior in a teleost species. It was found that *cyp19a1b*$^{-/-}$ mutant female zebrafish exhibited an increased latency to

initiate spawning and released higher numbers of eggs compared to WT females. These findings represent an important difference from the observed impairment of female fertility in chemical inhibitor studies in which both aromatase isoforms are inhibited in the body. For example, total aromatase inhibition reduced female fathead minnow (*Pimephales promelas*; Ankley et al., 2002), zebrafish (Ayobahan et al., 2019), and medaka (Sun et al., 2007) fertility and fecundity. These differences are likely due to the contribution of circulating estrogens produced by the ovaries via Cyp19a1a that function to maintain the integrity of the ovarian state and the reproductive capacity (i.e., fertility) of female zebrafish (Rahaman et al., 2020). The observed effects on female sexual behavior in the current study are linked to altered local brain estrogen production via Cyp19a1b, because brain E2 levels are significantly lower in *cyp19a1b*$^{-/-}$ mutant females compared to WT females, whilst ovarian E2 levels are similar between the groups. The time to the last spawning event, but not the spawning duration, was also longer in the *cyp19a1b*$^{-/-}$ mutant females. This suggests that the mutation of *cyp19a1b* affects the perception of reproductive cues important for timely mate identification and assessment, since zebrafish rely heavily on visual and pheromonal cues for reproductive behavior (Li et al., 2018). We note that *cyp19a1b* and *esr*s (*esr1*, *esr2a*, *esr2b*) are highly expressed at multiple levels of these sensory pathways including from the peripheral level of sensory nerve fibres to the levels of primary targets and sensory integration centres in the brain (Shaw, 2018). Future study of *cyp19a1b*$^{-/-}$ mutant females will need to account for these potential multiple levels of sensory impairments as well as to identify downstream neuronal mediators of RGC-derived estrogens.

Our results resemble those observed in the *tAro* KO female mice that display reduced sexual behavior when paired with WT males (Bakker et al., 2002b), likely due to impaired brain estrogen signalling (Cornil, 2018). There has been no study to date of specific *bAro* KO effects on female sexual behavior in mammalian models. However, male *bAro* KO male mice have impaired social recognition and a significantly longer latency to initiate mounting behavior with hormonally primed female mice compared to WT males (Brooks et al., 2020). Our data for male zebrafish contrast those in mice as we observed no significant differences in spawning success between male *cyp19a1b*$^{-/-}$ mutants and WT fish. Rather, increasing evidence indicates that brain androgen signalling is critical for male teleost sexual behavior. It was recently discovered that *cyp17a1*$^{-/-}$ mutant male zebrafish display reduced mating behaviors with WT females compared to WT males, which is likely a result of lower brain levels of T and 11-ketotestosterone (11-KT; Shu et al., 2020). The reduced contact time of *cyp17a1*$^{-/-}$ male mutants with WT females could be rescued following 11-KT administration, indicating that non-aromatizable androgens regulate male sexual behavior (Shu et al., 2020). Evidence from androgen receptor gene editing studies also demonstrate the important role for androgen signalling in male teleost sexual behavior (Alward et al., 2020; Yong et al., 2017).

An important observation in the current study is that female *cyp19a1b*$^{-/-}$ mutants paired with WT males spawned significantly more eggs compared to WT females; however, there was a concomitant decrease in larval survival so that the total number of viable larvae produced was similar in female mutants and WT fish. One possible but unlikely explanation for the observed higher larval mortality is reduced circulating E2 and

vitellogenin egg deposition in female *cyp19a1b*$^{-/-}$ mutants. Vitellogenin is an estrogen-regulated hepatic protein that nourishes the embryo during early development and is critical for embryo survival (Hara et al., 2016). Vitellogenin levels correlate with serum E2 and are significantly lower in females following administration of chemical aromatase inhibitors (Ankley et al., 2002; Ayobahan et al., 2019; Sun et al., 2007). The higher larval mortality in the current study is not due to changes in ovary-derived E2 in *cyp19a1b*$^{-/-}$ mutant females since the *cyp19a1b*$^{-/-}$ mutant and WT females have similar ovarian tissue (this study) and circulating E2 (Yin et al., 2017) levels. Moreover, there were no significant differences in yolk sac volume or BL at 2 dpf in progeny from *cyp19a1b*$^{-/-}$ mutant female and WT pairings. These two morphometrics are positively correlated in embryos and directly linked to maternal vitellogenin levels that are driven by systemic estrogens which bind to activate hepatic Esrs (Bugel et al., 2011; Jardine and Litvak, 2003).

The *cyp19a1b*$^{-/-}$ mutant females released more than twice the number of eggs compared to WT females. This suggests that the mutation of Cyp19a1b may increase the energetic cost of reproduction due to the substantial metabolic investment in large quantities of larvae that do not survive to adulthood. It is well known that spawning is one of the most metabolically demanding activities for a fish (McBride et al., 2015). For example, the rate of gamete biomass production is roughly proportional to whole-organism metabolic rate, with female fishes allocating approximately half of their energy reserves towards reproductive function (Hayward and Gilloly, 2011). It will be important for future studies to determine the contribution of Cyp19a1b to metabolic rate and long-term fitness costs associated with higher reproductive investment by mutant females.

Higher mortality rates in larvae from *cyp19a1a*$^{-/-}$;*cyp19a1b*$^{-/-}$ mutant pairings compared to WT pairings among the male genotypes also revealed a role for aromatase in males. While larval survival was similar between WT, *cyp19a1a*$^{-/-}$ and *cyp19a1b*$^{-/-}$ mutant males, survival of offspring from the double mutant males was 24% lower than WT offspring. This finding suggests that total aromatase activity of the father contributes to offspring survival. This idea is supported by previous studies reporting increased mortality in larval zebrafish during acute chemical aromatase inhibition (Alharthy et al., 2017; Allgood et al., 2013; Bernardo and Connaughton, 2022; Martínez et al., 2019; Santos et al., 2014). Moreover, both *cyp19a1* transcripts are expressed during the first 48 hpf in zebrafish embryos, which suggests a role in early development and survival (Sawyer et al., 2006). While overall survival rate was lower in larvae from *cyp19a1a*$^{-/-}$;*cyp19a1b*$^{-/-}$ mutant males, the size of the remaining survivors appeared relatively normal. At 2 dpf, BL, EA, and YSA were similar across all genotypes. These observations are similar to those of Gould et al. (2019), reporting no effect of aromatase inhibition on larval zebrafish development. However, our study contrasts those demonstrating chemical inhibition of both aromatases affect one or multiple of these morphometrics (Alharthy et al., 2017; Bernardo and Connaughton, 2022; Dang et al., 2018; Hamad et al., 2007; Martínez et al., 2019). It is possible that significant differences in these morphometrics might emerge during later development after 2 dpf.

2.5 Conclusions

We have demonstrated a role of brain aromatase in female sexual behavior. Female zebrafish carrying a frameshift mutation in *cyp19a1b* had a longer latency to initiate spawning behavior and had higher numbers of eggs spawned compared to WT

females. The importance of *cyp19a1b* for embryo survival was demonstrated by the increased mortality of progeny from female *cyp19a1b*$^{-/-}$ mutants and *cyp19a1a*$^{-/-}$;*cyp19a1b*$^{-/-}$ mutants compared to WT pairings. Further study will be needed to determine the downstream neuronal pathways through which brain estrogens produced in RGCs lead to the observed changes in female spawning behavior. It will also be important to determine the causes of increased mortality in eleutheroembryos from *cyp19a1b*$^{-/-}$ mutant females and *cyp19a1a*$^{-/-}$;*cyp19a1b*$^{-/-}$ mutant males.

Chapter 3: Arginine Vasopressin Injection Rescues Delayed Oviposition in *cyp19a1b*$^{-/-}$ Mutant Female Zebrafish

Formatted as a manuscript in preparation for submission to Frontiers in Endocrinology:

Shaw, K., Lu, C., Liu, X., Trudeau, V.L. Arginine vasopressin injection rescues delayed oviposition in *cyp19a1b*$^{-/-}$ mutant female zebrafish.

Study contributions: KS conceived of and conducted the experiments and wrote the manuscript, CL provided methods and reagents, XL provided the *cyp19a1*$^{-/-}$ mutant lines, VLT helped design experiments, revised the manuscript, and provided funding for the study.

Abstract

In zebrafish, estrogens produced in the ovaries via Cyp19a1a activity are required for both sexual differentiation of the ovary during early development as well as maintenance of the ovarian state during adulthood. The importance of Cyp19a1b that is highly expressed in the brain for female reproduction is still under study. We previously reported that female *cyp19a1b*$^{-/-}$ mutant zebrafish have significantly lower brain estradiol levels and impaired spawning behavior characterized by an increased latency to oviposition during dyadic sexual behavior encounters. In the current study, we provide evidence that the delayed oviposition in female *cyp19a1b*$^{-/-}$ mutants is linked to impaired arginine vasopressin (Avp) signalling. Droplet digital PCR experiments revealed that levels of the estrogen receptors, *avp*, and *oxytocin* (*oxt*) are lower in the hypothalamus of mutant females compared to wildtype fish. We then used acute intraperitoneal injections of Avp and Oxt, along with mixtures of their respective receptor antagonists, to determine that Avp can uniquely rescue the delayed oviposition in female *cyp19a1b*$^{-/-}$ mutants. Using immunohistochemistry, we demonstrated that Cyp19a1b-expressing radial glial cell (RGC) fibres surround and are in contact with Avp-immunopositive neurons in the preoptic areas of the brain. This could provide the neuroanatomical proximity for RGC-derived estrogens to diffuse and bind in Avp-immunopositive neurons to affect expression levels. Together these findings identify a positive link between Cyp19a1b and Avp for female zebrafish sexual behavior. They also suggest that the female *cyp19a1b*$^{-/-}$ mutant behavioral phenotype is likely a consequence of impaired processing of social cues important for mate identification and assessment.

3.1 Introduction

Estrogens are critical sex steroids for female reproduction (Findlay et al., 2010). The final and rate limiting step for bioactive estrogen production involves the aromatase (Cyp19a1) enzyme that converts the aromatizable androgens, T and androstenedione, into the bioactive estrogens, E2 and E1, respectively (Simpson et al., 1994). Teleosts have two paralogs encoding aromatase, *cyp19a1a* and *cyp19a1b*, that are highly expressed in the ovaries and brain, respectively, due to distinct regulatory elements in their promoter regions (Diotel et al. 2010; Le Page et al. 2008). Estrogens can exert diverse effects in the body by binding to different Esrs that initiate various signalling pathways with tissue- and cell-specific effects (Heldring et al., 2007). Gene expression can be regulated directly by nEsrs binding to EREs in gene promoter regions or through indirect pathways involving protein-protein interactions with transcription factors such as the Ap1 and the Sp1, when bound to their respective promoter elements (Klinge et al., 2001). Though comparatively less studied, mEsrs have also been shown to affect the expression levels of estrogen-regulated genes through second messenger signalling pathways (Maggiolini and Picard, 2010). Teleosts have three nEsrs named Esr1, Esr2a, and Esr2b (Nelson and Habibi, 2013), and the mEsr, Gper (Thomas, 2012).

A recent report indicated that *cyp19a1b*$^{-/-}$ mutant female zebrafish that have significantly lower brain E2 levels compared to WT females have altered female sexual behavior (Chapter 2; Shaw et al., 2023). Mutant *cyp19a1b*$^{-/-}$ females had delayed spawning behavior with WT males in sexual behavior assays compared to WT females. This delayed initiation of sexual behavior is reminiscent of the phenotype observed in *Aro* KO mice that have reduced sexual behavior (Bakker et al., 2002a, 2002b, 2003; Honda et

al., 1998; Matsumoto et al., 2003). The behavioral impairments in *Aro* KO mice are hypothesized to be linked to disrupted olfactory discrimination that reduces social recognition through effects on nEsrs and the nonapeptides, Oxt and Avp (Choleris et al., 2003, 2006, Pierman et al., 2008). The similar behavioral impairments between the *Aro* KO mice and *cyp19a1b*$^{-/-}$ mutant zebrafish suggest potential similarities in the disruption of the signalling pathways underlying the behavioral phenotypes despite differences in brain aromatase cellular localization. In mice, under normal conditions, aromatase is constitutively expressed in neurons (Honda et al., 1999). However, in teleosts, due to the presence of a cis-acting regulatory glial x responsive element in the *cyp19a1b* promoter region, aromatase is exclusively expressed in RGCs (Le Page et al., 2008). The previous finding that *cyp19a1b* mutation impairs female sexual behavior suggests that RGC-derived estrogens are likely diffusing to bind Esrs in nearby neurons to affect signalling pathways involving estrogen-regulated genes important for female sexual behavior. There are several candidate genes known to affect zebrafish sexual behavior that could be linked to the female *cyp19a1b*$^{-/-}$ mutant behavioral phenotype.

An involvement of the nonapeptides in female teleost sexual behavior is strongly suggested by the findings of changes in brain nonapeptide levels across female reproductive states (Butler et al., 2021; Gozdowska et al., 2006; Kulczykowska and Kleszczyńska, 2014; Maruska et al., 2007; Ohya and Hayashi, 2006; Singh and Joy, 2008; Sokolowska et al., 2015; Zhang et al., 2009) and observations that exogenous E2 administration upregulates brain nonapeptide levels (Chaube et al., 2012; Kalamarz-Kubiak et al., 2017; Nagarajan et al., 2015; Singh and Joy, 2009). The nonapeptides exert effects on reproduction through two main signalling pathways in the brain. Firstly,

through a hypophysiotropic pathway involving magnocellular neurons in the POA that project towards the pituitary gland for nonapeptide release into the circulatory system to regulate processes including osmoregulation, gonadal steroidogenesis, gametogenesis and parturition, amongst others (Herget et al. 2014, 2017; Mennigen et al., 2022). Secondly, through an encephalotropic pathway involving parvocellular and gigantocellular neurons that project to other brain regions including the telencephalon, prethalamus, hypothalamus, optic tectum, and the hindbrain (Herget et al. 2014, 2017). The encephalotropic pathway is hypothesized to underly the neuromodulatory roles of the nonapeptides in affecting the salience of social information processing in the brain that can affect social recognition and behaviors (Wacker and Ludwig, 2012). Indeed, recent findings from teleost studies have identified impairments in social recognition and behavior in *avp*$^{-/-}$ and *oxt*$^{-/-}$ mutant females that indicate important roles of nonapeptide signalling in female sexual behavior. For example, female *avp*$^{-/-}$ mutant zebrafish displayed reduced sexual behavior characterized by fewer quiver events produced, a measure of female sexual receptivity, when paired with WT males in sexual interactions (Ramachandran et al., 2023). In another study, the mate preference of female medaka for familiar males was absent in *oxt*$^{-/-}$ and *oxtr1*$^{-/-}$ mutants suggesting reduced social recognition of mates compared to WT females during pairwise mating trials (Yokoi et al., 2020). Given the well conserved expression pattern of *cyp19a1b* in RGCs lining the teleost diencephalic ventricle (Shaw, 2018), which is in close neuroanatomical proximity to the POA where high nonapeptide expression is observed (Banerjee et al., 2017), further study to characterize the interactions of *cyp19a1b* and nonapeptides in the female teleost brain in association with sexual behavior are well warranted.

Other important neuropeptides for female zebrafish sexual behavior are SNa and Secretoneurin B (SNb) that are derived from proteolytic processing of the precursor Scg2a and Secretogranin 2b (Scg2b) proteins, respectively. Analysis of *scg2a*$^{-/-}$ and *scg2b*$^{-/-}$ frameshift mutant zebrafish lines demonstrated the critical importance of SNa and SNb for female sexual behavior, ovulation, oviposition, and fertility (Mitchell et al., 2020). Interactions between the secretogranergic system and Cyp19a1b have been previously identified in the teleost brain in regions important for sexual behavior. For example, *scg2a* and *scg2b* transcripts are expressed in numerous brain regions important for reproduction that lie close to the diencephalic ventricle (Mitchell, 2018) where *cyp19a1b* is highly expressed (Shaw, 2018). A close neuroanatomical proximity between the soma of Scg2a-immunoreactive neurons and RGC fibres where Cyp19a1b is expressed was also identified in the POA of female goldfish (DaFonte et al., 2018). Intracerebroventricular injection of SNa downregulated *cyp19a1b* levels in the female goldfish telencephalon and hypothalamus, suggesting a regulatory pathway between SNa and neuroestrogen production (Da Fonte et al., 2018). Together, these findings suggest potential interactions between the SNs and *cyp19a1b* that might be important for the regulation of female sexual behavior.

The purpose of this study was to investigate the mechanistic pathways through which *cyp19a1b* mutation in female zebrafish disrupts sexual behavior. Based on the findings from the various studies of *Aro* KO mice as well as the recent discoveries of the importance of the nonapeptides and SNs for female zebrafish sexual behavior, we focused the study on identifying the potential interactions between *cyp19a1b*, the nEsrs and these neuropeptides. We first used ddPCR experiments to identify significantly

lower levels of *avp* and *oxt* in the hypothalamus of female *cyp19a1b*$^{-/-}$ mutants. We then conducted acute injections of the nonapeptides and observed that Avp uniquely rescued the delayed oviposition in female *cyp19a1b*$^{-/-}$ mutants and this effect was blocked with administration of the Avpr1a receptor antagonist. Using immunohistochemistry, the preoptic areas were identified as regions with close neuroanatomical proximity between Cyp19a1b-expressing RGCs and Avp-immunopositive neurons in the female zebrafish brain. Together, our findings demonstrate that the impaired sexual behavior in female *cyp19a1b*$^{-/-}$ mutant zebrafish is likely a consequence of disrupted estrogenic regulation of *avp* in preoptic areas and that these behavioral effects can be rescued by acute Avp administration in adult fish.

3.2 Materials and Methods

3.2.1 Experimental animals

Wildtype, *cyp19a1b*$^{-/-}$ mutant female (Yin et al., 2017), and *cyp19a1b*-GFP transgenic (Tg(*cyp19a1b*-GFP); Tong et al., 2009) zebrafish were raised in the University of Ottawa Aquatics Facility. Fish were housed in 10-L tanks of dechloraminated recirculating water maintained at 28°C on a 14:10 light:dark cycle, and fed twice daily. All fish tested in this experiment were between the ages of 5-11 months post-fertilization and had no previous experience in the sexual behavior assay; with males and females separated for at least two weeks prior to testing.

3.2.2 Total RNA extraction and cDNA synthesis

For gene expression measurements, the telencephalon and hypothalamus were selected for study because they contain many key brain areas for regulating sexual behavior and reproduction (Gonçalves and Oliveira, 2011). Female WT and *cyp19a1b*$^{-/-}$

mutant fish (n=6-9 per group) were euthanized in an ice water bath at two time points corresponding to during (10h00) and outside (14h00) the timing of zebrafish reproductive behavior. The brains were immediately removed, and the telencephalon was dissected at its connections to the olfactory bulb and hypothalamus and placed into a 1.5 mL RNase-free Eppendorf tube on dry ice. The brains were then inverted to dissect the hypothalamus region including the ventrolateral extending hypothalamic lobes and diencephalon tissue ventral to the optic tectum and placed into a separate 1.5 mL RNase-free Eppendorf tube on dry ice. This dissection method produces telencephalon samples containing the dorsal and ventral telencephalon regions as well as the anterior preoptic area encompassing the parvocellular preoptic neurons, whilst the hypothalamus samples contain the posterior preoptic area including the magnocellular and gigantocellular neurons and surrounding hypothalamic regions. Total ribonucleic acid (RNA) was isolated from samples using Trizol (Thermo Fisher Scientific, Waltham, MA, USA, cat# 15596018) according to the manufacturer's instructions with a few modifications. Briefly, 50 µL Trizol was added to each tube and tissues were homogenized using a pestle with intermittent vortexing. Samples were incubated for 5 mins at room temperature and then 10 µL chloroform was added to each tube, vortexed, and allowed to incubate for 3 mins. Following incubation, samples were centrifuged for 5 mins at 12,600 rpm at 4°C, the supernatant was carefully transferred to a new labelled RNase-free 1.5 mL tube and 25 µL isopropanol was added to each tube, and samples were vortexed. The tubes were incubated for 10 mins at room temperature and then centrifuged for 30 mins at 12,600 rpm at 4°C. The isopropanol was removed, and pellets were rinsed with 50 µL of 75% ethanol (EtOH) followed by centrifugation for

5 mins at 12,600 rpm at 4°C. The EtOH was then removed from tubes and the rinse step was repeated. The tubes were then left open for 2 mins in a fumehood to dry the samples. Samples were resuspended in 20 µL RNase-free ddH_2O and incubated for 10 mins at 60°C on a heat block. The samples were then vortexed and placed on ice before quality assessment. Total RNA concentration as well as 260/280 and 260/230 absorbance measurements were assessed using a spectrophotometer (Thermo Fisher Scientific, NanoDrop 2000). RNA integrity was verified using gel electrophoresis (1% weight/volume agarose). RNA was stored at -80°C until further usage. Total complementary DNA (cDNA) was synthesized using the iScript Reverse Transcription Supermix (Bio-Rad, Hercules, CA, USA, cat# 1708841), according to the manufacturer's instructions. Synthesis of cDNA used 600 ng of total RNA from telencephalon and hypothalamus samples and cDNA was stored at -20°C.

3.2.3 Primer design

Primers for gene expression analysis were designed using either NCBI Primer Blast Software (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) or obtained from previous gene expression analysis studies (Griffin et al., 2013; Mitchell, 2018; Mitchell et al., 2020). All primers were supplied by Integrated DNA Technologies (Coralville, IA, USA) and the specificity of primers for the genes of interest were confirmed by sanger sequencing of PCR products (Génome Québec, Montréal, QC, Canada) and sequences were matched to the zebrafish genome using the NIH Basic Local Alignment Search Tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Appropriate annealing temperatures and cDNA dilutions for ddPCR analysis were identified by performing thermal gradients (51-63°C) with cDNA collected from telencephalon and hypothalamus (1:2 – 1:320 dilution)

samples. Primer sequences, annealing temperatures, and amplicon sizes are presented in Table 3.1.

Table 3.1- Primer pairs and experimental conditions for gene expression analyses of telencephalon and hypothalamus samples.

Gene Name	Gene Abbreviation	Primer	Annealing Temperature (°C)	Amplicon Size (bp)
estrogen receptor 1	*esr1*	F:CACATCAGACACATGAGCAACAA R:CTGAAGACTGGAACCGCTGA	55.7	121
estrogen receptor 2a	*esr2a*	F:GCCTGCCGACTCCGAAA R:TTGTTGGTAGCTGCTACGATCTCT	55.7	77
estrogen receptor 2b	*esr2b*	F: CGACTCCGCAAGTGCTATGAA R: ACGATGTCGAGCACCTCGAT	55.7	83
arginine vasopressin	*avp*	F:AGGTCTGCATGGAAGAGGAG R:CTGCCTTCAGGACAGTCTGG	60.7	146
oxytocin	*oxt*	F:GATCTGCTGCTGAAGCTCCT R:TACAAAAGTGGGTGGCGAGT	60.7	134
secretogranin 2a	*scg2a*	F:CAGGACGTACGGGTTATGCT R:GCGTTGGTCTTTGGTTTTGT	60.7	138
secretrogranin 2b	*scg2b*	F:AAACAAAGCTCCGAGCAAAA R:AACTGGTGTCGGGATACTCG	55.7	116
tata-binding protein	*tbp*	F:TACCCACCAGCAGTTTAGCA R:TCTAACCTTGGCACCTGTGA	60.7	130

3.2.4 Droplet digital PCR protocol

The ddPCR procedures including experiment set-up, droplet generation, and transfer of emulsified samples to PCR plates, were performed according to the manufacturer's protocols (Bio-Rad, QX200 Droplet Digital PCR system). In brief, at the start of the experiment, telencephalon or hypothalamus cDNA samples were diluted to the appropriate concentration determined for each primer pair. The reaction mixtures were prepared, each containing 11.5 µL EvaGreen ddPCR Supermix (Bio-Rad, cat# 1864034), 0.23 µL of 10 µM gene-specific forward and revere primers, 6.04 µL nuclease-free water, and 5 µL of template cDNA at primer-specific dilution. 20 µL of the sample reactions were then carefully transferred into the wells of a droplet generation

cartridge (Bio-Rad, cat# 1864008) along with droplet generation oil (Bio-Rad, cat# 1864006) to the corresponding oil wells. The cartridge was sealed with a gasket (Bio-Rad, cat# 1863009) and then inserted into a droplet generator (Bio-Rad, model# QX200 Droplet Generator) for droplet generation. The emulsified samples were then carefully transferred to the wells of a 96-well ddPCR plate (Bio-Rad, cat# 12001925) and sealed with a heat foil (Bio-Rad, cat# 1814040). The PCR reactions were performed using a thermal cycler (Bio-Rad, model# C1000 Touch Thermal Cycler) with the cycle conditions: (95°C for 5 mins), 49 cycles of ((95°C for 30 secs), (annealing temperature for 1 min), (72°C for 30 secs), with a ramp rate of -2°C/sec), followed by (4°C for 5 mins), (90°C for 5 mins), and finally (12°C for 5 mins). The fluorescence of droplets was measured using a droplet reader (Bio-Rad, model# QX200 Droplet Reader) and data were analyzed using QuantaSoft Analysis Pro software (Bio-Rad, v1.0.596).

3.2.5 Brain estradiol enzyme-linked immunosorbent assays

For the E2 assays, methods used were similar to those previously described in Chapter 2, with the exception that brains were collected at two time points corresponding to 10h00 and 14h00 (n=4-5 per group). At each time point, both WT and *cyp19a1b*$^{-/-}$ mutant female fish were euthanized in an ice water bath, and brains were immediately dissected and placed into individual labelled 1.5 mL Eppendorf tubes. Homogenization buffer (150 µL per brain, 90% methanol) were added to each tube and tissues were then individually sonicated for steroid release into solution. Tubes were centrifuged (4°C, 13,200 rpm, 10 mins) and the supernatant was then carefully removed and transferred to a new labelled tube and evaporated to dryness (Labconco Centrivap Centrifugal Vacuum Concentrator, model# 7810014, 45°C, 1 hr), then stored at 4°C

overnight. The next day, samples were resuspended with 100 µL resuspension buffer (0.2% formic acid, 5% acetonitrile in water) added to each tube, vortexed, and tubes were sonicated in a water bath for 15 mins. Following resuspension, samples were run on C-18 solid-phase extraction columns (Dr. Maisch HPLC GmbH, cat# r10.aq; > 85% recovery rate in liquid chromatography tandem mass spectrometry) with a final elution volume of 120 µL. The eluted samples were then evaporated to dryness (45°C, 3 hrs). The evaporated steroid residue was then resuspended in 200 µL ELISA buffer for 24 hrs at 4°C with intermittent vortexing prior to testing. Levels of E2 were measured using ELISA test kits (Cayman Chemical, cat# 501890) according to the manufacturer's instructions. Samples were run in duplicate and only samples with intra-assay coefficient of variations < 10% were used in analyses.

3.2.6 Characterizing female *cyp19a1b*$^{-/-}$ mutant sexual behavior following peptide injection

The methods used in this experiment are similar to those describe in Chapter 2. In brief, for all trials, a *cyp19a1b*$^{-/-}$ mutant female was size-matched with a male WT fish (within 2 mm BL, < 10% BL difference). The evening before each experiment, paired fish were transferred to a 1-L testing tank containing an insert at the bottom for egg collection and a divider in the middle of the tank to keep the male and female separate before testing. The testing pair was allowed to acclimate overnight in the Zebracube (Viewpoint Behavior Technology, Inc.) with the camera present (either a Panasonic 16GB HC-V700M Full HD camcorder or a Canon VIXIA HF R800 camcorder). The following morning at 9h00, the pair was transferred to a new 1-L testing tank containing clean system water. The female was immediately removed and anesthetized with Tricaine

(Syndel, cat# 02168510) according to University of Ottawa zebrafish husbandry procedures, then weighed on an analytical balance, and transferred to a moist sponge with an insert cut for positioning the female for injection. Females (n=15 per group) were intraperitoneally injected with dosages based on bodyweight (bw) using a 32-gauge syringe (Hamilton Company, Reno, NV, USA, cat# CAL80308) with either 1) Ringer's solution (pH 7), 2) Oxt (0.5 µg/g bw), 3) Avp (0.5 µg/g bw), 4) mix of 0.5 µg/g bw Avp + 5 µg/g bw Manning Compound (an Avpr1a antagonist), or 5) mix of 0.5 µg/g bw Avp + 5 µg/g bw L-368,899 (an Oxtr antagonist). The zebrafish Oxt and Avp peptides were synthesized *in house* and sequences were confirmed by mass spectrometry. The peptide receptor antagonists, Manning Compound (Bio-Techne Canada, Toronto, ON, Canada, cat# 3377) and L-368,899 (Bio-Techne Canada, cat# 2641) were dissolved in water according to manufacturer's instructions. Following injection, the female was immediately transferred to a new housing tank containing clean system water to recover for 5 mins before being transferred back to the testing tank. The divider was then removed, and video recording started. All filming began within 15 mins of lights on (9h00). The fish pair was allowed to interact for 60 mins to determine whether the peptide injections could rescue the delayed time to the first oviposition event observed in the female *cyp19a1b*$^{-/-}$ mutants (see Chapter 2). Videos were coded by date and viewed by an observer blind to the treatment groups using VLC media player (https://www.videolan.org/) to record the time to the first oviposition event.

3.2.7 Immunohistochemistry

Fish (n= 20) from the Tg(*cyp19a1b*-GFP) line were euthanized using an ice water bath and carefully positioned under a dissection microscope for the careful removal of scales

on the skull cap using dissecting tweezers to expose the brain. Fish heads were then dissected away from the body and placed immediately in a falcon tube containing 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.4) for 24 hrs fixation on an orbital shaker at room temperature. Fixed samples were then washed with PBS (3 x 20 mins), following which the heads were placed in 0.5 M ethylenediaminetetraacetic acid for 7 days for decalcification. Following decalcification, the samples were then successively dehydrated for 30 mins each in 30%, 50%, 70%, 80% and finally 99% EtOH. Samples were cleared by 2 x 1 hr incubations in 100% xylene, incubated for 1 hr in melted paraffin at 60°C, and then transferred to new melted paraffin and left overnight at 60°C. The next day, the samples were embedded in a cassette containing newly melted paraffin with heads oriented for transverse sectioning and allowed to cool. The embedded heads were sectioned at 15 µm thickness using a motorized microtome (Leica Biosystems, model# RM2255). Sample sections were spread on a 40°C water bath to remove wrinkles and then mounted on Superfrost Plus slides (Thermo Fisher Scientific, cat# 22-037-246). Slides were allowed to air dry and then stored in a slide box at 4°C prior to use.

All wash steps were conducted using glass Coplin jars (Ted Pella, Inc., Redding, CA, USA, cat# 432-1). Slides were first heated for 1 hr at 60°C on a slide warmer to improve tissue adherence to the slides during wash steps. The slides were then deparaffinized by xylene washes (2 x 20 mins) and rehydrated using a series of washes including 2 x 100% EtOH, 2 x 95% EtOH, 1 x 70% EtOH, 1 x PBS, each for 10 mins. The slides were then transferred to Coplin jars containing 0.01 M sodium citrate (pH 6.0) and placed in a 90°C water bath for 30 mins for antigen retrieval. Following this, slides were allowed to

cool and washed in PBS (2 x 5 mins), then incubated in blocking buffer (1% bovine serum albumin in 0.3% Triton PBS) for 30 mins. Slides were then placed in a humid chamber at 4°C to incubate overnight with the primary antibodies: anti-Avp (rabbit, Immunostar, Hudson, WI, USA, cat# 20069) and anti-GFP (chicken, Abcam, Cambridge, UK, cat# ab13970), diluted 1:500 in blocking buffer. The anti-Avp antibody was validated and found to be specific for teleost Avp previously (Pouso et al., 2019) as well as in this study (see below). The following day, the slides were washed in PBS (2 x 10 mins), and subsequently incubated for 2 hrs in a dark humid chamber at room temperature with the secondary antibodies: donkey anti-rabbit IgG (H+L) Alexa Fluor 680 (Invitrogen, Waltham, MA, USA, cat# A10043) and goat anti-chicken IgY (H+L) Alexa Fluor 594 (Invitrogen, cat# A11042), diluted 1:500 in PBS. The next day, slides were washed in the dark in PBS (2 x 10 mins), then incubated in 200 µL Hoechst solution (1:10,000, Invitrogen, cat# H3570) in a dark chamber for 8 mins, followed by PBS washes (3 x 5 mins) in the dark, and mounted with SlowFade Diamond Antifade Mountant (Invitrogen, cat# S36963) and a cover slip (VWR, Radnor, PA, USA, cat# 48393-221). Slides were stored in the dark until visualization. Slides were imaged using an Olympus confocal microscope (Olympus, Richmond Hill, ON, Canada, model #FV1000) and FV10-ASW-4.2 Viewer software.

3.2.8 Antibody characterization

The Avp antibody has been previously validated for use in the teleost *Brachyhypopomus gauderio* (Pouso et al., 2019). To validate the antibody for use in zebrafish, sections were incubated with anti-Avp (1:500) pre-adsorbed for 24 hrs with an excess of zebrafish Avp (10 µM). No Avp labelling was observed in the sections

demonstrating primary antiserum specificity. Separate sections were incubated with anti-Avp (1:500) pre-adsorbed for 24 hrs with an excess of zebrafish Oxt (10 µM). Avp labelling was unaffected by pre-adsorption with Oxt, demonstrating antibody specificity for the Avp antigen. Control sections omitting the primary and secondary antibodies did not display any labelling.

3.2.9 Statistical analyses

Statistical analyses were conducted using Graphpad Prism v9 (GraphPad Software, Inc.). For both gene expression data and E2 measurements, normality of residuals and homoscedasticity were assessed using Shapiro-Wilk tests and Spearman's tests for heteroscedasticity, respectively. Data were then analyzed in Two-Way ANOVA tests followed by Tukey's multiple comparisons tests with the Holm-Šídák method used to correct for multiple comparisons. Gene expression data that were non-normally distributed were either log- or square root-transformed, and data that could not be normalized through transformation were grouped by genotype and assessed using Mann Whitney tests with the Holm-Šídák method to correct for multiple comparisons. The absolute transcript levels of target genes in the telencephalon and hypothalamus were normalized to *tata-binding protein* (*tbp*), an internal housekeeping gene that is not estrogen-regulated (Filby and Tyler, 2007). There were no significant differences observed in *tbp* levels across genotype groups or time points in the study confirming the appropriate usage of *tbp* as a housekeeping gene. Gene expression levels are presented as fold change relative to WT female levels at 10h00 with individual points and means or median values displayed. For the behavioral rescue experiments, normality and homoscedasticity were assessed using Shapiro-Wilk and Levene's tests,

respectively. If a pair did not spawn eggs in the 60-min test period, a maximum latency of 60 mins was assigned. Data were analyzed using a Kruskal-Wallis test followed by Dunn's multiple comparisons tests for pairwise comparisons. Data are presented as medians, with values for individual pairs displayed. For all data, significance was defined at $p < 0.05$ and all significance tests were assessed as two-tailed.

3.3 Results

3.3.1 Decreased expression levels of reproductive neuroendocrine genes in the telencephalon and hypothalamus of *cyp19a1b*$^{-/-}$ mutant females

Telencephalon

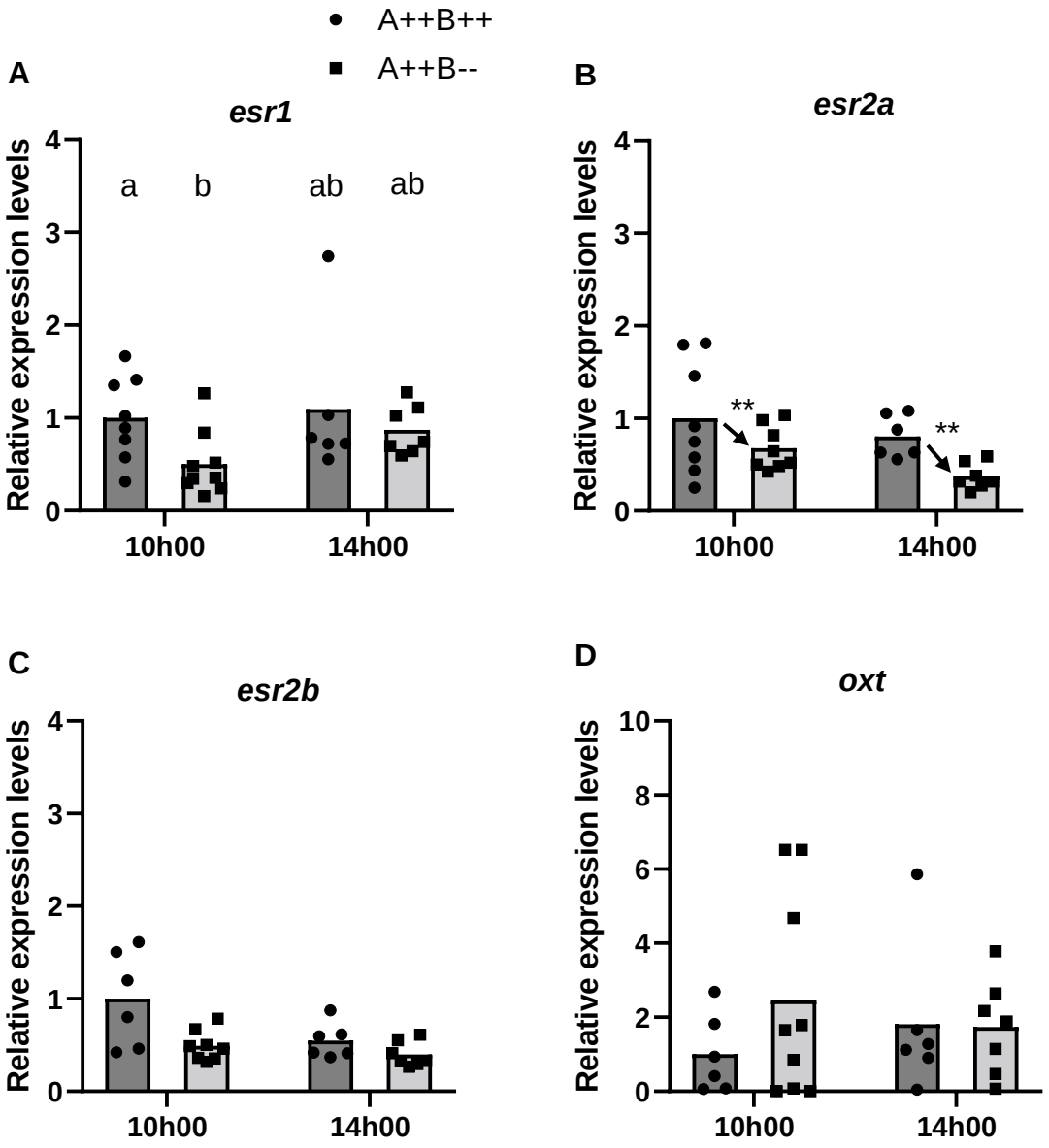
Two-Way ANOVA was used to assess the effects of time (10h00 and 14h00) and genotype (*cyp19a1b*$^{-/-}$ mutant and WT) on gene expression levels in the telencephalon.

There were small differences in the expression levels of the *esr*s between the telencephalon of female WT and *cyp19a1b*$^{-/-}$ mutants. While there were no significant main effects of time ($F(1,25)=0.9293$, $p=0.3443$) or genotype ($F(1,25)=2.668$, $p=0.1149$) on *esr1* levels, there was a statistically significant time X genotype interaction ($F(1,25)=5.554$, $p=0.0266$). Post-hoc tests revealed that the telencephalon of WT females had 1.9 times higher *esr1* levels compared to *cyp19a1b*$^{-/-}$ mutant females at 10h00 ($p=0.0217$; Figure 3.1A). There were no other significant interactions. A significant main effect of genotype ($F(1,25)=8.662$, $p=0.0069$), but not of time ($F(1,25)=3.400$, $p=0.0771$) or time X genotype interaction ($F(1,25)=2.375$, $p=0.1358$), on *esr2a* levels was observed. The telencephalon of WT females had 1.7 times higher *esr2a* levels compared to *cyp19a1b*$^{-/-}$ mutant females (Figure 3.1B). For *esr2b* levels,

there were no significant differences in *esr2b* levels at either 10h00 ($U(6,8)=21.00$, $p=0.2991$) or 14h00 ($U(5,6)=9.00$, $p=0.1925$; Figure 3.1C).

No significant main effects were observed of time ($F(1,24)=0.2294$, $p=0.6363$), genotype ($F(1,24)=0.5074$, $p=0.4831$), or time X genotype interaction ($F(1,24)=0.3781$, $p=0.5444$) on *oxt* levels (Figure 3.1D). There were no significant differences in *avp* levels at either 10h00 ($U(7,8)=34.00$, $p=0.8884$) or 14h00 ($U(5,6)=17.00$, $p=0.8618$; Figure 3.1E).

There were statistically significant main effects of time for both *scg2a* ($F(1,26)=7.717$, $p=0.0100$) and *scg2b* ($F(1,25)=14.21$, $p=0.0009$) levels in the telencephalon. Levels of *scg2a* and *scg2b* were 1.4 and 1.3 times higher, respectively, at 10h00 compared to 14h00 (Figure 3.1F, 3.1G). There was also a significant main effect of genotype ($F(1,25)=8.833$, $p=0.0065$) on *scg2b* levels. The telencephalon of WT females had 1.2 times higher *scg2b* levels compared to *cyp19a1b*$^{-/-}$ mutant females (Figure 3.1G). There was no significant main effect of genotype on *scg2a* levels ($F(1,26)=2.501$, $p=0.1258$) or time X genotype interaction on *scg2a* levels ($F(1,26)=0.4523$, $p=0.5072$) or *scg2b* levels ($F(1,25)=2.835$, $p=0.1047$).


A++B++
A++B--
A
esr1
Relative expression levels
a
b
ab
ab
10h00
14h00
B
esr2a
**
**
C
esr2b
D
oxt

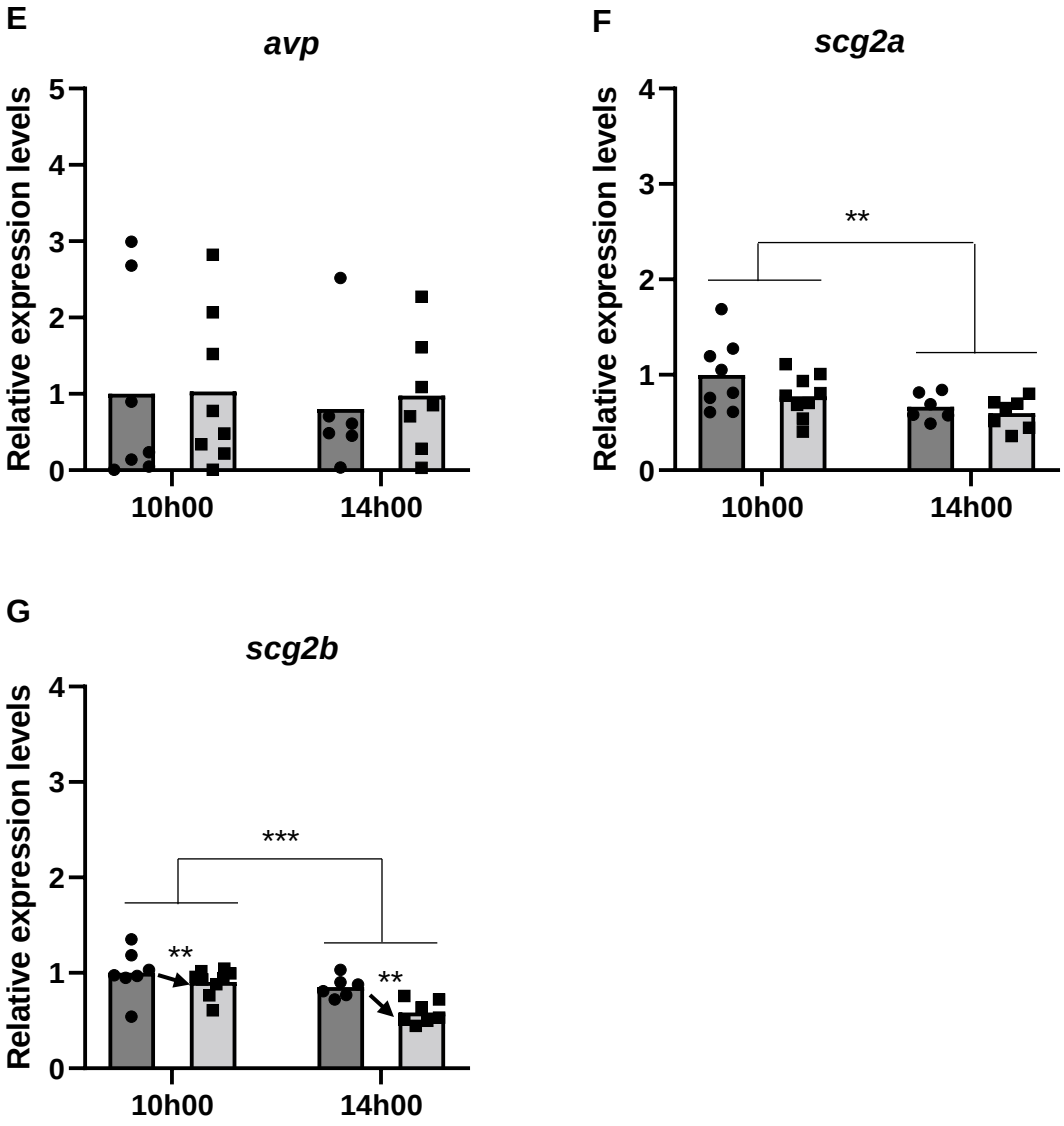


Figure 3.1- Gene expression in the telencephalon of adult wildtype (A++B++) and *cyp19a1b*$^{-/-}$ mutant (A++B--) female zebrafish in the morning (10h00) and afternoon (14h00). The abundance of mRNAs (copies/µL) for all genes measured was normalized by tata-binding protein levels within the sample and data are plotted as fold change relative to the wildtype female levels at 10h00. Parametric data were analyzed by Two-Way ANOVA tests followed by Tukey's multiple comparisons tests and individual values are plotted with bars representing mean values (**A**,**B**,**D**,**F**,**G**), whilst

non-parametric data were analyzed using multiple Mann-Whitney U tests with a Holm-Šídák correction for multiple comparisons and plotted as individual points with bars representing median values (**C**,**E**). Error bars are not displayed for clarity. Significant main effects of time (horizontal bars) and genotype (arrows) are indicated by asterisks (** p<0.01; *** p<0.001). When there is a statistically significant interaction effect, means with different letters a-b represent statistically significant differences (p<0.05).

Hypothalamus

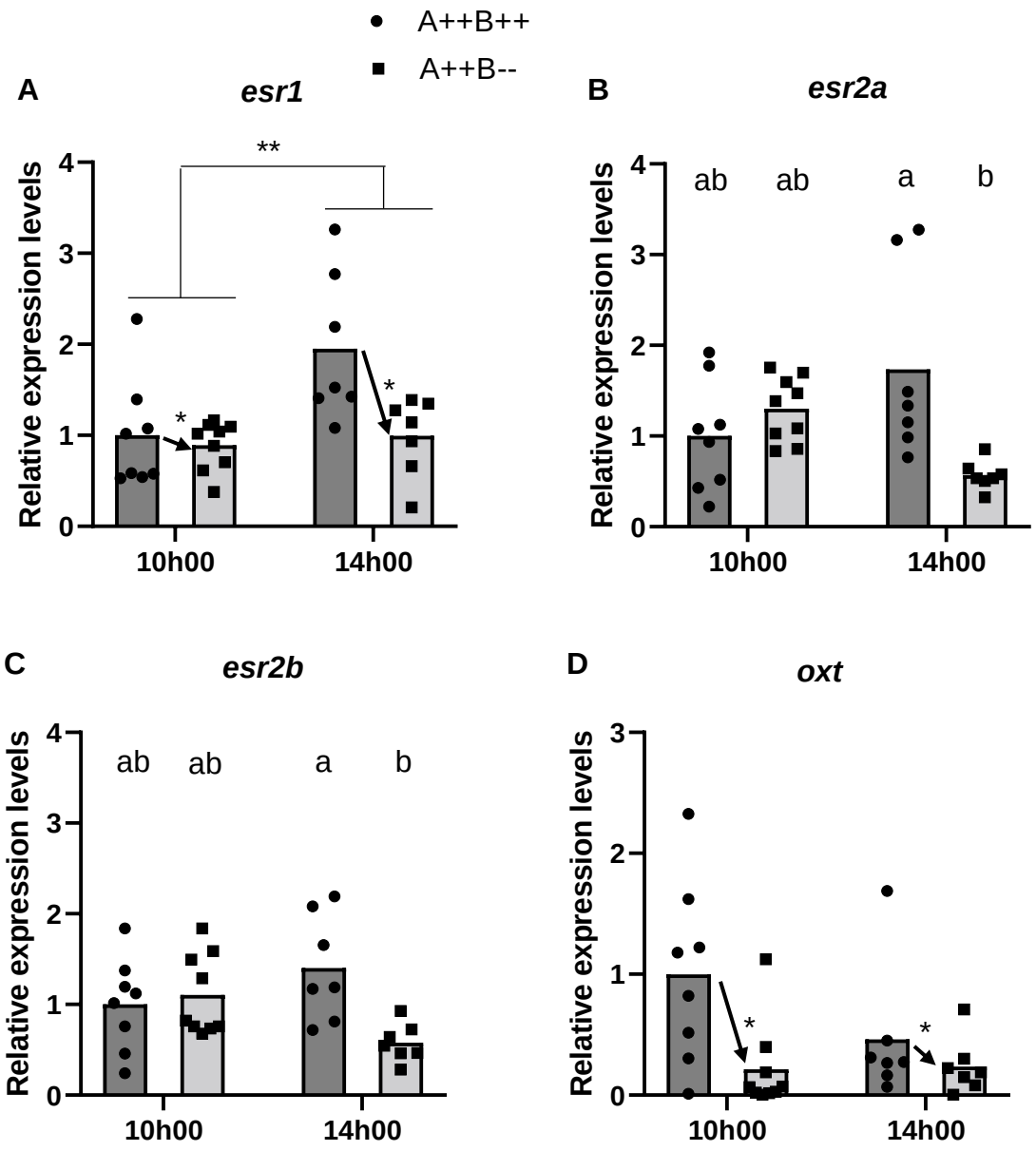
Two-way ANOVA was performed to assess the effects of time (10h00 and 14h00) and genotype (*cyp19a1b*$^{-/-}$ mutant and WT) on gene expression levels in the hypothalamus.

There were significant differences in *esr* levels between the hypothalamus of WT and *cyp19a1b*$^{-/-}$ mutants. There were significant main effects of time ($F(1,26)=8.967$, $p=0.0060$) and genotype ($F(1,26)=5.475$, $p=0.0272$) on *esr1* levels. Levels of *esr1* were 1.6 times higher at 14h00 compared to 10h00 (Figure 3.2A), and WT females had 1.5 times higher *esr1* levels compared to *cyp19a1b*$^{-/-}$ mutant females (Figure 3.2A). There was no significant time X genotype interaction ($F(1,26)=3.758$, $p=0.0635$). No significant main effects of time on *esr2a* ($F(1,25)=2.139$, $p=0.1560$) or *esr2b* ($F(1,27)=0.4068$, $p=0.5290$) levels, or main effects of genotype on *esr2a* ($F(1,25)=0.6108$, $p=0.4418$) or *esr2b* ($F(1,27)=3.782$, $p=0.0623$) levels were observed. However, there were significant time X genotype interactions for *esr2a* ($F(1,25)=10.65$, $p=0.0032$) and *esr2b* ($F(1,27)=9.103$, $p=0.0055$) levels. Post-hoc tests revealed that the hypothalamus of WT females had 3.1 times higher *esr2a* levels compared to *cyp19a1b*$^{-/-}$ mutant females at 14h00 ($p=0.0076$; Figure 3.2B). Levels of *esr2b* were 2.4 times higher in the

hypothalamus of WT females compared *cyp19a1b*$^{-/-}$ mutant females at 14h00 (p=0.0120; Figure 3.2C). No other significant interactions were evident.

There were large differences in *oxt* and *avp* levels in the hypothalamus of WT and *cyp19a1b*$^{-/-}$ mutant females. Significant main effects of genotype on both *oxt* ($F(1,27)=7.300$, $p=0.0118$) and *avp* ($F(1,27)=6.848$, $p=0.0144$) levels were observed. The hypothalamus of WT females had 3.2 and 1.8 times higher *oxt* and *avp* levels, respectively, compared to *cyp19a1b*$^{-/-}$ mutant females (Figure 3.2D, 3.2E). There were no significant main effects of time on *oxt* ($F(1,27)=0.0019$, $p=0.9656$) or *avp* ($F(1,27)=0.0848$, $p=0.7731$) levels, or time X genotype interactions on *oxt* ($F(1,27)=1.373$, $p=0.2515$) or *avp* ($F(1,27)=0.0083$, $p=0.9281$) levels.

No significant differences in *scg2a* levels at either 10h00 ($U(6,7)=22.00$, $p=0.3282$) or 14h00 ($U(6,7)=11.00$, $p=0.1852$; Figure 3.2F) were observed. There was a significant time X genotype interaction on *scg2b* levels ($F(1,26)=6.704$, $p=0.0156$), with post-hoc tests revealing that *cyp19a1b*$^{-/-}$ mutant females had 1.7 times higher *scg2b* levels at 10h00 compared to 14h00 ($p=0.0153$; Figure 3.2G). There were no other significant interactions and no main effects of time ($F(1,26)=4.097$, $p=0.0537$) or genotype ($F(1,26)=0.8427$, $p=0.3671$) on hypothalamic *scg2b* levels.


A++B++
A++B--
A
esr1
B
esr2a
C
esr2b
D
oxt
Relative expression levels
10h00
14h00
ab
ab
a
b
**
*

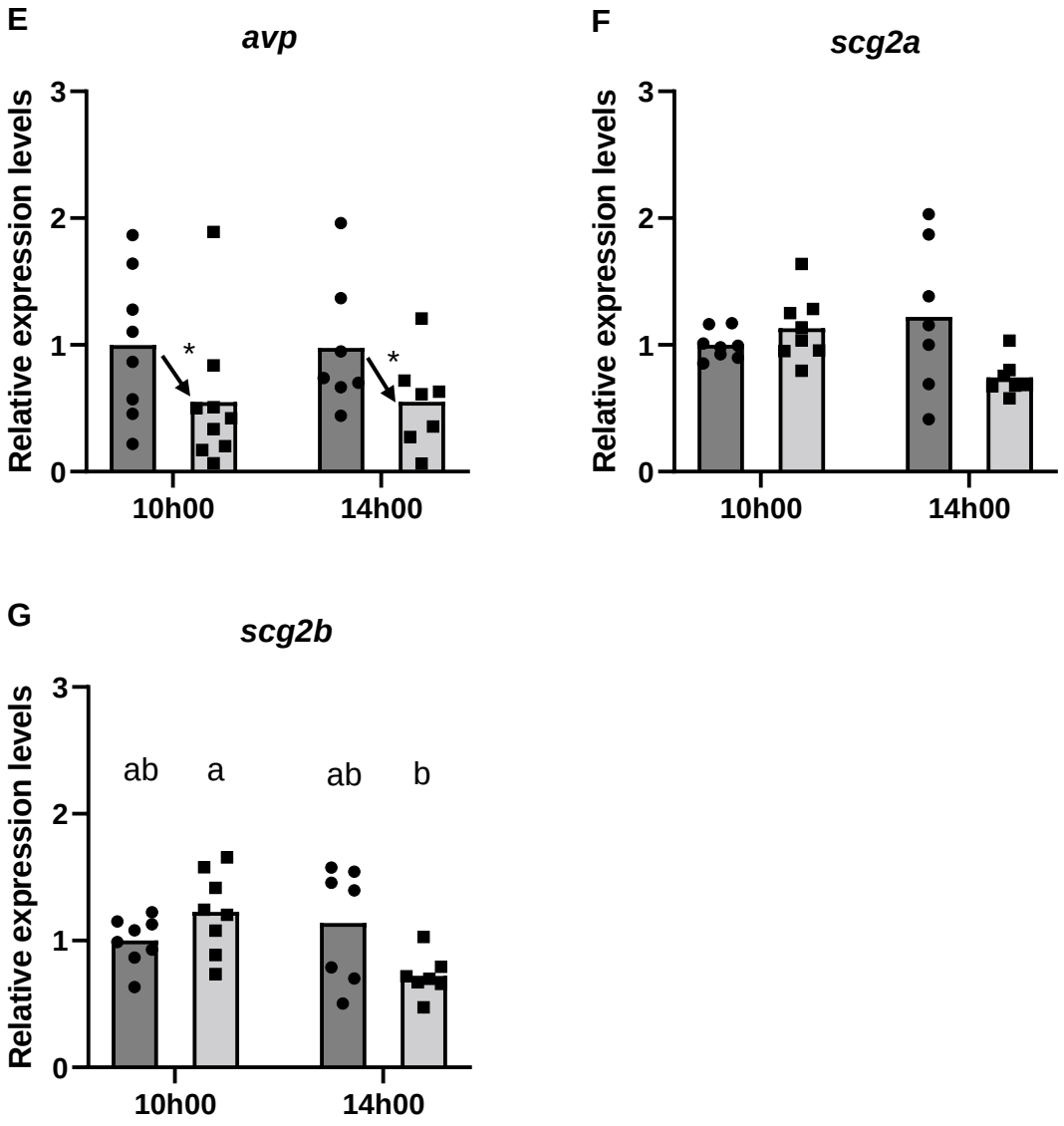


Figure 3.2- Gene expression in the hypothalamus of adult wildtype (A++B++) and *cyp19a1b*$^{-/-}$ mutant (A++B--) female zebrafish in the morning (10h00) and afternoon (14h00). The abundance of mRNAs (copies/µL) for all genes measured were normalized by tata-binding protein levels within the sample and data are plotted as fold change relative to the wildtype female levels at 10h00. Parametric data were analyzed by Two-Way ANOVA tests followed by Tukey's multiple comparisons tests and individual values are plotted with bars representing mean values (**A**,**B**,**C**,**D**,**E**,**G**), whilst non-parametric data were analyzed using multiple Mann-Whitney U tests with a Holm-

Šídák correction for multiple comparisons and plotted as individual points with bars representing median values (**F**). Error bars are not displayed for clarity. Significant main effects of time (horizontal bars) and genotype (arrows) are indicated by asterisks (* p<0.05; ** p<0.01). When there is a statistically significant interaction effect, means with different letters a-b represent statistically significant differences (p<0.05).

3.3.2 Brain estradiol content fluctuates between the morning and afternoon

There was a significant main effect of time on brain E2 levels (F(1,13)=10.14, p=0.0072). Brain E2 levels were 1.4 times higher at 14h00 compared to 10h00 (Figure 3.3). There was also a significant main effect of genotype (F(1,13)=23.04, p=0.0003). WT brains had 1.7 times higher E2 levels compared to *cyp19a1b*$^{-/-}$ mutant brains (Figure 3.3). There was no significant time X genotype interaction (F(1,13)=2.291, p=0.1541).

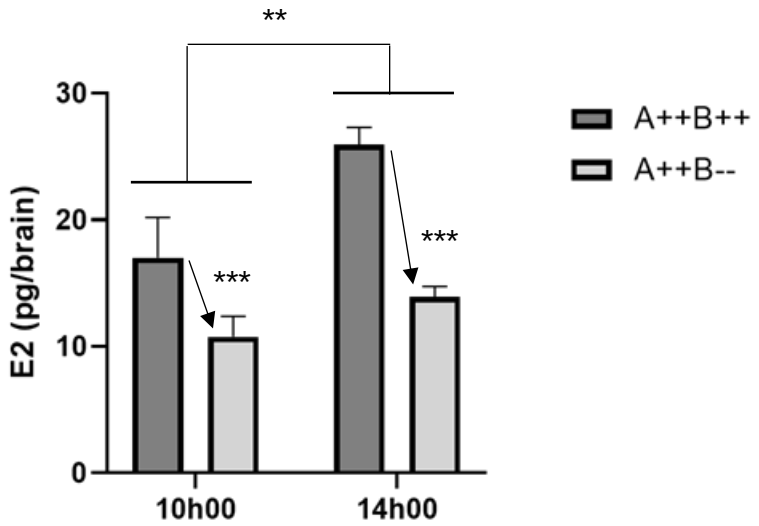


Figure 3.3- Estradiol (E2) levels in the brains of adult wildtype (A++B++) and *cyp19a1b*$^{-/-}$ mutant (A++B--) female zebrafish in the morning (10h00) and afternoon (14h00). Data were analyzed using a Two-Way ANOVA followed by Tukey's multiple comparisons tests. Significant main effects of time (**, p=0.0072) and genotype

(*** with arrow; p=0.0003) are indicated. There was no significant interaction effect. Data are plotted as means + standard error of the mean (n=4-5 per group).

3.3.3 Arginine vasopressin injection rescues the behavioral phenotype in *cyp19a1b*$^{-/-}$ mutant females

There was a significant difference in the time to the first oviposition event amongst the *cyp19a1b*$^{-/-}$ mutant female groups (H(4)=14.87, p=0.005; Figure 3.4), confirming the previous study findings (see Chapter 2; Shaw et al., 2023). Pairwise comparisons revealed that both Avp (p=0.0221) and Avp + L-368,899 (p=0.0155) groups took significantly less time to the first oviposition event compared to the saline-injected group. Saline-injected *cyp19a1b*$^{-/-}$ mutant females took 5.5 and 4.3 times longer to the first oviposition event compared to Avp- and Avp + L-368,899-injected *cyp19a1b*$^{-/-}$ mutant females, respectively. There were no significant differences in the time to the first oviposition event in the Avp + Manning Compound (p=0.7986) or Oxt (p>0.9999) groups compared to saline-injected fish.

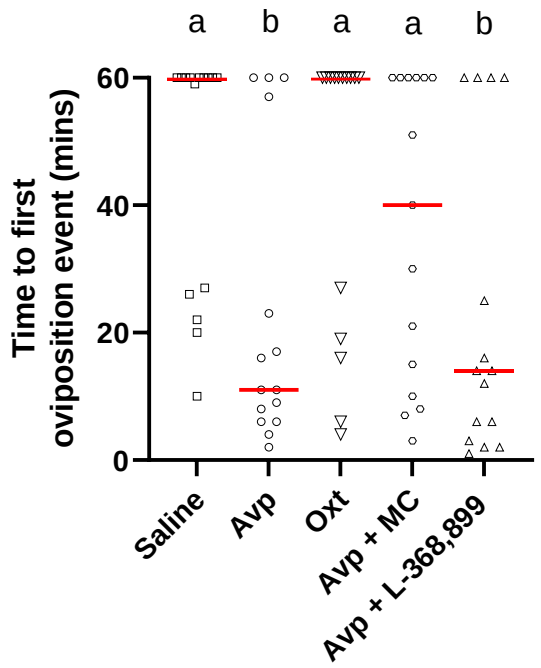


Figure 3.4- Time to first oviposition event during zebrafish pairwise mating trials with female *cyp19a1b*$^{-/-}$ mutant fish intraperitoneally injected with nonapeptides and mixed nonapeptides with receptor antagonists (n=15 per group). Significant differences were assessed using a Kruskal Wallis test followed by a Dunn's multiple comparisons test. Individual data points are displayed with red bars representing median values. Different letters a-b represent statistically significant differences. Statistical significance is defined at $p<0.05$.

3.3.4 Cyp19a1b-immunopositive radial glial cell fibres contact arginine vasopressin cells in preoptic areas

Avp immunolabelling in POA neurons (Figure 3.5A) was absent in sections pre-adsorbed with Avp (Figure 3.5B), but not when pre-adsorbed with Oxt (Figure 3.5C). Subpopulations of POA neurons were identified based on their neuroanatomical positions in the rostral-caudal and ventral-dorsal axes as previously described (Pouso et al., 2017). Avp-immunopositive neurons were found exclusively in the POA in

periventricular locations. In the anterior POA, Cyp19a1b-expressing RGCs in the peripheral layer of the ventral telencephalon were observed in contact with the soma of parvocellular Avp-immunopositive cell bodies (Figure 3.6). In the posterior POA, probable contact points between Cyp19a1b-expressing RGC fibres lining the diencephalic ventricle and cell bodies of magnocellular and gigantocellular Avp-immunopositive neurons were observable indicating potential functional relationships (Figure 3.7).

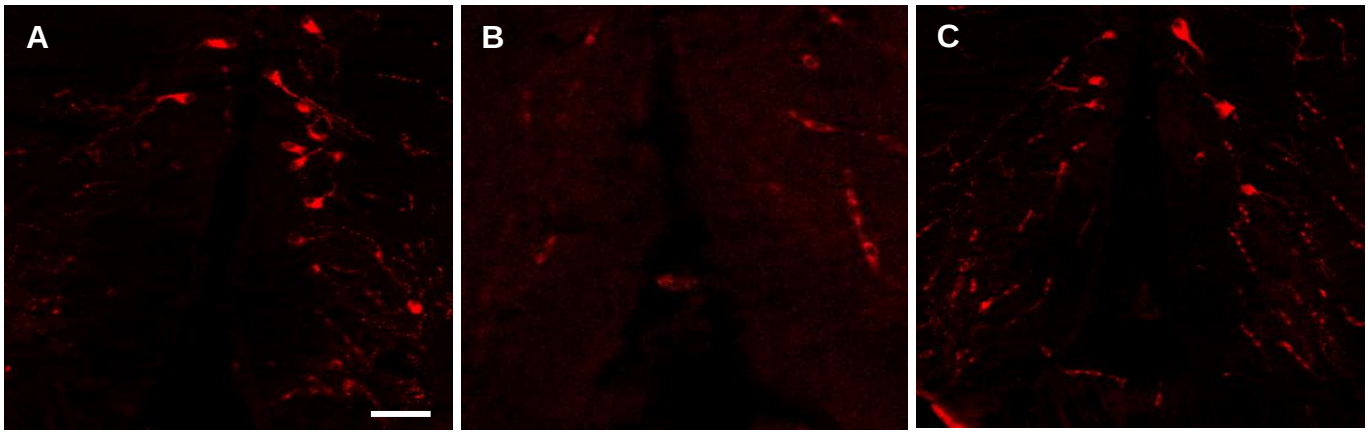


Figure 3.5- Arginine vasopressin (Avp) immunolabelling in pre-adsorption tests with Avp and Oxytocin (Oxt) in a transverse section of a female wildtype zebrafish preoptic area. The confocal images show Avp-immunopositive cell bodies surrounding the diencephalic ventricle (**A**). No Avp-immunopositive neurons were present in sections pre-adsorbed with Avp (**B**). Avp immunolabelling was unaffected in sections pre-adsorbed with Oxt (**C**). Scale bar=20 µm.

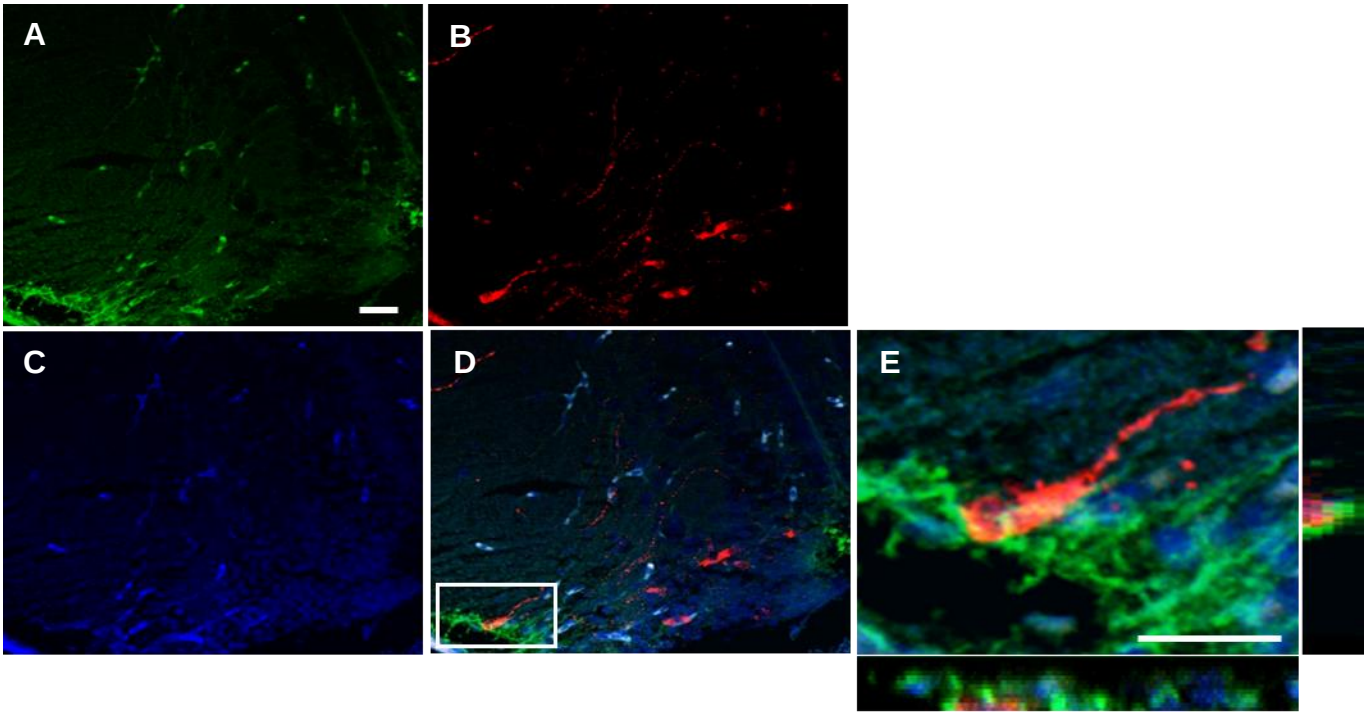


Figure 3.6- Double immunofluorescence against Cyp19a1b (green) and Arginine vasopressin (Avp; red) in a female Tg(*cyp19a1b*-GFP) zebrafish anterior preoptic area. In this transverse brain section, Cyp19a1b-positive radial glial cells fibres lining the peripheral layer of the ventral telencephalon (**A**) surround an Avp-immunopositive parvocellular cell body (**B**). The nuclear stain Hoescht (blue) is also shown (**C**). Single slice scanning view of the boxed area in panel **D** shows Cyp19a1b-positive fibres in contact with the Avp-immunopositive cell body (**E**). Scale bar=20 µm.

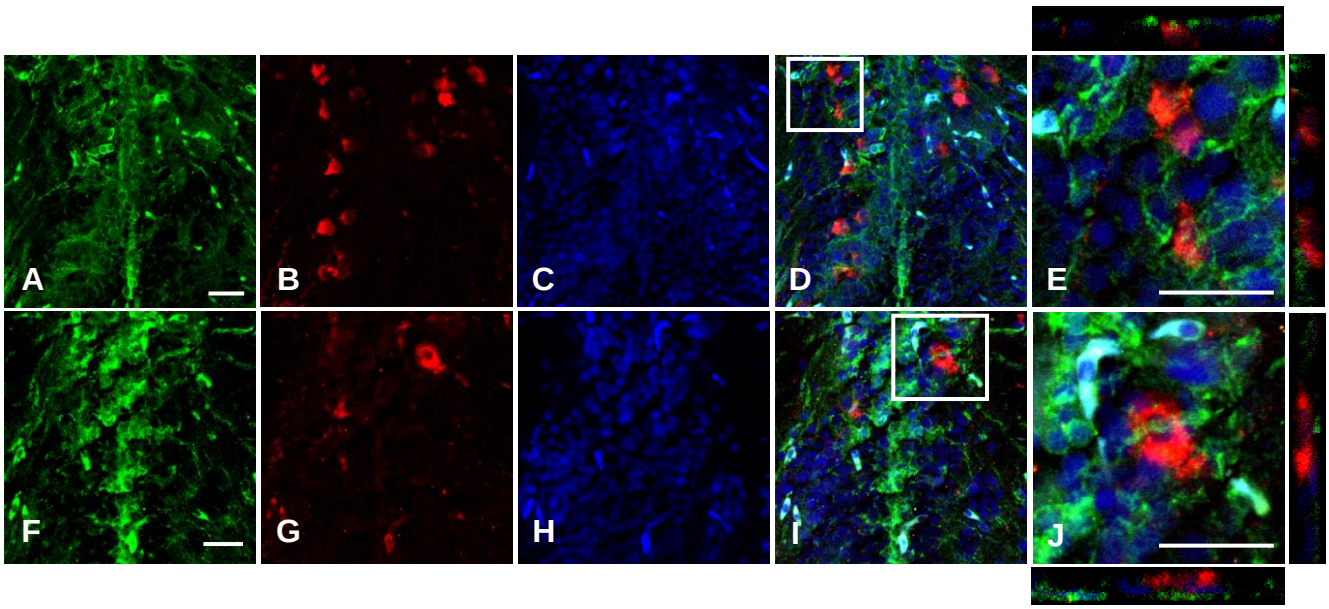


Figure 3.7- Double immunofluorescence against Cyp19a1b (green) and Arginine vasopressin (Avp; red) in a female Tg(*cyp19a1b*-GFP) zebrafish posterior preoptic area. In this transverse brain section, Cyp19a1b-positive radial glial cell fibres (**A**,**F**) surround Avp-immunoreactive magnocellular (**B**) and gigantocellular (**G**) cell bodies. The nuclear stain Hoescht (blue) is also shown (**C**,**H**). Single slice scanning view of the boxed areas in panels **D** and **I** show Cyp19a1b-positive fibres in contact with the Avp-immunopositive cell bodies (**E**,**J**). Scale bar=20 µm.

3.4 Discussion

It was previously reported that female *cyp19a1b*$^{-/-}$ mutant zebrafish paired with WT males have a significantly longer latency to initiate spawning compared to WT females during dyadic sexual behavior assays (see Chapter 2; Shaw et al., 2023). This is suggestive of a potential impairment in the ability of a female mutant to identify and/or assess a potential mate. Since *cyp19a1b* is exclusively expressed in RGCs in the teleost brain (Shaw, 2018), these findings indicate that locally produced E2 in RGCs is

likely diffusing to activate Esrs in nearby neurons to affect the expression of behaviorally relevant estrogen-regulated genes. Indeed, teleost RGCs have been previously shown to have the full complement of steroidogenic enzymes necessary for *de novo* steroidogenesis and the importance of RGC-derived steroids in the teleost brain for diverse functions from regulating neurogenesis to various aspects of social behavior has been previously proposed (Diotel et al., 2018). To identify the potential neuroendocrine mechanism underlying the behavioral impairment observed in the *cyp19a1b*$^{-/-}$ mutant females, we used ddPCR experiments to first quantify changes in the *esr*s (*esr1*, *esr2a*, *esr2b*), the nonapeptides (*avp*, *oxt*), and the *scg*s (s*cg2a*, *scg2b*). Telencephalon and hypothalamus samples were analyzed separately at two time points corresponding to during (10h00) and outside (14h00) the time of zebrafish reproductive activity. The findings from the ddPCR experiments revealed significant effects of *cyp19a1b* mutation on the expression levels of *esr*s, *avp*, and *oxt* in the hypothalamus.

The telencephalon and hypothalamus of *cyp19a1b*$^{-/-}$ mutant females had significantly lower *esr* levels compared to WT females. Levels of *esr1* were 1.5 times higher in the hypothalamus of WT fish compared to *cyp19a1b*$^{-/-}$ mutants. This finding is supported by the previously observed lower brain E2 levels in mutant females (see Chapter 2; Shaw et al., 2023) and the known positive estrogenic regulation of *esr1* due to the presence of an ERE in its promoter region. Time-dependent expression profiles of *esr1* were also observed. Levels were 1.6 times higher in the hypothalamus at 14h00 compared to 10h00, and 1.9 times higher levels specifically at 10h00 in the telencephalon of WT females compared to *cyp19a1b*$^{-/-}$ mutants. Lower levels of *esr2a* and *esr2b* were also identified in female *cyp19a1b*$^{-/-}$ mutants compared to WT females with region- and time-

specific effects. Levels of *esr2a* were 1.7 times higher in the telencephalon of WT females compared to *cyp19a1b*$^{-/-}$ mutant females, whilst in the hypothalamus both *esr2a* and *esr2b* levels were 3.1 and 2.4 times higher, respectively, in WT females compared to mutants specifically at 14h00. Together, these findings identify lower levels of *esr1* in the mutants during the morning when fish are sexually active, whilst *esr2a* and *esr2b* levels are lower in the afternoon, which may represent impaired establishment of estrogen-regulated gene expression profiles to subsequently prepare for sexual activity the following morning.

There has been surprisingly little study of the importance of brain Esr signalling for female teleost sexual behavior, outside of fertility assessments. Of the few studies conducted, no effects of *esr2a* mutation were observed on female medaka sexual behavior (Kayo et al., 2019); however, *esr2b* mutation affected female sexual receptivity. In pairwise mating trials of medaka, female *esr2b*$^{-/-}$ mutants were unreceptive to males and therefore did not spawn eggs despite normal ovarian function (Nishiike et al., 2021). The study revealed that the female-biased sexual dimorphism in *esr2b*-expressing neurons in the ventral telencephalic area and the magnocellular/gigantocellular portion of the posterior POA decreased following water-borne aromatase inhibitor treatment indicating that aromatase activity positively regulates brain *esr2b* levels. Medaka *esr2b* was shown to be positively estrogen-regulated using a luciferase reporter cell line in combination with E2 treatment. Together, these findings demonstrate that aromatase activity positively regulates Esr2b signalling levels in female medaka in brain regions important for sexual behavior. Our study findings suggest that brain aromatase activity likely regulates *esr2b* levels in the

female zebrafish brain and that these effects may be linked to female sexual behavior. While in-depth study of *esr1*$^{-/-}$ mutant effects on female teleost sexual behavior have not been investigated, we observed that *esr1* levels were lower in the hypothalamus of *cyp19a1b*$^{-/-}$ mutants compared to WT females at the time of reproductive activity. Further study of Esr1 signalling effects on female sexual behavior seem particularly well warranted given the similar behavioral phenotypes of female *Aro* KO and *Esr1* KO mice that display increased rejection of male stud mice during sexual behavior assays (Bakker et al., 2002a, 2003; Ogawa et al., 1998). In zebrafish, all three nEsrs are expressed in important neuroendocrine regions for female sexual behavior and their expression profiles parallel regions of *cyp19a1b* expression (Menuet et al., 2002; Pellegrini et al., 2005). Our current study findings of lower *esr* levels in the telencephalon and hypothalamus of female *cyp19a1b*$^{-/-}$ mutants that have abnormal spawning behavior draws attention to the need for further study of *esr*$^{-/-}$ mutant effects on female sexual behavior.

Both *avp* and *oxt* were expressed at significantly lower levels in the hypothalamus of *cyp19a1b*$^{-/-}$ mutant females compared to WT fish. The hypothalamus of WT females had 1.8 times and 3.2 times higher levels of *avp* and *oxt*, respectively, compared to *cyp19a1b*$^{-/-}$ mutants. These findings are supported by other teleost studies demonstrating that E2 administration increases brain *avp* and/or *oxt* levels (Ayoub, 1998; Chaube et al., 2012; Kalamarz-Kubiak et al., 2017; Nagarjayan et al., 2015; Singh et al., 2009; Wang et al., 1995). Our study finding of lower nonapeptides levels in the hypothalamus of female *cyp19a1b*$^{-/-}$ mutants is particularly interesting considering the important role of Avp and Oxt signalling in modifying the salience of social information

processing in the brain to affect behavior (Wacker and Ludwig, 2012). Recent teleost studies using nonapeptide mutant lines have identified important roles of Avp and Oxt signalling for mate assessment. Female *avp*$^{-/-}$ mutant zebrafish produced significantly fewer quiver events with WT males compared to WT females during sexual interactions (Ramachandran et al., 2023). In another study, *oxt*$^{-/-}$ and *oxtr1*$^{-/-}$ mutant Japanese medaka showed reduced social recognition of familiar males compared to WT females during sexual behavior assays (Yokoi et al., 2020). An impairment of sensory information processing that functions in mate identification and assessment, such as might occur by reduced *avp* and/or *oxt* levels, could represent a link to the behavioral phenotype observed in the *cyp19a1b*$^{-/-}$ mutant females during sexual behavior assays (see Chapter 2). Together, these findings suggest that the longer latency to initiate spawning behavior in the *cyp19a1b*$^{-/-}$ mutant females could be a consequence of impaired processing of sensory information due to reduced positive estrogenic regulation of Avp and/or Oxt signalling in the brain.

There were significant main effects of time on *scg2a* and *scg2b* levels in the telencephalon that confirm their high expression levels in the morning during the time at which zebrafish are sexually active. However, there were minor differences in expression levels between the brains of *cyp19a1b*$^{-/-}$ mutant and WT females. For example, *scg2b* levels in the telencephalon of WT females were 1.2 times higher than those observed in *cyp19a1b*$^{-/-}$ mutants. Though these data indicate minor changes in *scg2b* levels between *cyp19a1b*$^{-/-}$ mutant and WT females, they are unlikely to explain the significant behavioral phenotype of the *cyp19a1b*$^{-/-}$ mutant females. Of note, *scg2a*$^{-/-}$ and *scg2b*$^{-/-}$ mutant female zebrafish are characterized by an ovulatory rather than

behavioral dysfunction that underlies their impaired reproductive capacity (Mitchell et al., 2020). Therefore, the behavioral dysfunction in the *cyp19a1b*$^{-/-}$ mutant females seems unlikely to be directly associated with impaired brain SN signalling.

Based on the differences observed in the expression levels of genes between the morning and afternoon sampling times, we then assessed E2 brain content in *cyp19a1b*$^{-/-}$ mutants and WT females to determine if there were significant differences at the sampling time points. Female WT brains had 1.7 times higher E2 content compared to *cyp19a1b*$^{-/-}$ mutant brains confirming our previous study finding (see Chapter 2; Shaw et al., 2023). The afternoon sampling time had 1.4 times higher brain E2 content compared to the morning. This time difference in brain E2 content most likely represents E2 that crosses the blood-brain barrier from systemic circulation due to increased Cyp19a1a activity in the ovary at this time. Since no significant differences in ovarian E2 content (see Chapter 2) or serum E2 levels (Yin et al., 2017) were previously observed between *cyp19a1b*$^{-/-}$ mutants and WT females, the larger increase in brain E2 content from 10h00 to 14h00 in WT females compared to *cyp19a1b*$^{-/-}$ mutant females could represent the onset of brain *cyp19a1b* upregulation in WT females that does not occur in the *cyp19a1b*$^{-/-}$ mutants. Support for this hypothesis is provided by a previous gene expression study that identified a peak in ovarian *cyp19a1a* levels at the mid-point of the light phase that was followed by later increased brain *cyp19a1b* levels (Di Rosa et al., 2016). This daily rhythm of gene expression is hypothesized to serve important functions in regulating reproductive activity in diurnal spawners similar to that observed in seasonally breeding species. In seasonally breeding teleosts, increased circulating E2 levels in females, driven by ovarian recrudescence and increased Cyp19a1a activity

in the ovary, peak prior to the breeding season. This increases brain *cyp19a1b* levels and Cyp19a1b activity in the breeding months, due to the positive estrogenic regulation of *cyp19a1b* (Chaube et al., 2015; Gelinas et al., 1998; Pal et al., 2018; Pasmanik and Callard, 1988). The expected increase in brain *esr1* levels and E2 content in the afternoon was absent in female *cyp19a1b*$^{-/-}$ mutants. While speculative, it is possible that this altered expression profile is part of the mechanism underlying the female *cyp19a1b*$^{-/-}$ mutant behavioral impairment.

We conducted acute intraperitoneal injections of teleost Avp and Oxt to see if either nonapeptide could rescue the behavioral phenotype in the *cyp19a1b*$^{-/-}$ mutant females. The nonapeptides were selected for study in the rescue experiment based on their known effects on social recognition and the findings that *avp* and *oxt* levels were ~45% and ~68% lower, respectively, in the hypothalamus of *cyp19a1b*$^{-/-}$ mutants compared to WT fish. Acute intraperitoneal Avp injections rescued the time to the first oviposition event in female *cyp19a1b*$^{-/-}$ mutants. This behavioral rescue was receptor-dependent because antagonism of Avpr1a receptor signalling by coadministration of Manning Compound blocked the effect of Avp to advance oviposition in female mutants. On the other hand, coadministration of the Oxtr antagonist L-368,899 had no effect on Avp action. This demonstrates that the rescue effects observed are specific to Avp signalling pathways.

These findings are supported by a previous study in female Asian stinging catfish, whereby low dosage 0.1 µg/g bw E2 intraperitoneal injections given daily for three days in ovariectomized females increased brain Avp levels (Chaube et al., 2012). A functional

relationship between E2 and Avp has also been previously observed in female round gobies during the spawning-capable phase whereby brain explants perfused *in vitro* with E2 released higher concentrations of Avp into the surrounding medium (Kalamarz-Kubiak et al., 2017). Brain explants perfused with E2 also increased Oxt release. Nonapeptide release induced by E2 could be blocked by administration of actinomycin D, demonstrating the dependence of E2 action on *de novo* RNA synthesis. It is interesting to note that Avp release also occurred rapidly, within 20 mins of E2 administration, suggesting an additional non-genomic pathway of estrogenic regulation. It would be interesting in a future study to measure the levels of bioactive nonapeptides in the brains of *cyp19a1b*$^{-/-}$ mutants to complement the findings in the current study. Indeed, previous studies in other teleosts have demonstrated links between oviposition and bioactive brain nonapeptide levels in the females (Kulczykowska and Kleszczyńska, 2014; Kulczykowska et al., 2014). It will also be important in future study to identify the potential additional contributions of Gper (the teleost mEsr) signalling to the observed behavioral phenotype in the *cyp19a1b*$^{-/-}$ mutants. Since significantly lower levels of nuclear *esr*s were identified in addition to *avp* in the hypothalamus of *cyp19a1b*$^{-/-}$ mutants compared to WT females, the current findings suggest at least a genomic regulatory mechanism underlying the behavioral phenotype observed in the mutant fish. The contributions of non-genomic estrogenic regulation of female zebrafish sexual behavior should be further explored.

Since the rescue experiment demonstrated that acute Avp injection uniquely rescued the delayed oviposition in *cyp19a1b*$^{-/-}$ mutants, we wanted to establish the neuroanatomical basis for Cyp19a1b-derived estrogenic communication with Avp

neurons. To investigate the neuroanatomical proximity for brain-derived E2 induction of *avp* levels, we used immunohistochemistry methods with a teleost-validated Avp antibody and the Tg(*cyp19a1b*-GFP) zebrafish line (Tong et al., 2009). Close neuroanatomical proximities between Cyp19a1b-positive RGC fibres and Avp-immunopositive cell bodies were identified in the anterior and posterior parts of the POA. This could allow RGC-derived E2 to diffuse into nearby Avp-immunopositive neurons to regulate *avp* levels in the female zebrafish brain. A close association between Cyp19a1b-immunopositive RGC fibres and *avp*/Avp-expressing neurons in the POA has also been previously demonstrated in the orange-spotted grouper (*Epinephelus coioides*; Nagarjayan et al., 2015) and the bluehead wrasse (*Thalassoma bifasciatum*; Marsh et al., 2006). In the orange-spotted grouper, *avp* colocalized with *esr1*, *esr2b* and *gper* in all three preoptic neuron populations- the parvocellular, magnocellular, and gigantocellular neurons, indicating the potential for direct estrogenic regulation of *avp* levels (Nagarjayan et al., 2015). In the bluehead wrasse, Cyp19a1b-positive RGC fibres were observed in close association to Avp-immunopositive cell bodies in the POA indicating a potential functional relationship (Marsh et al., 2006). Though the functional importance of the individual Avp subpopulations in teleost social behavior is currently unknown, evidence has suggested a potential role of gigantocellular neurons in female reproduction. A previous study in the half-spotted goby (*Asterropteryx semipunctata*) identified significantly high numbers of gigantocellular neurons in the peak-spawning period compared to the pre- and post-spawning periods, which was not seen similarly in the parvocellular or magnocellular neurons (Maruska et al., 2007). These differences were also observed, however, in the

non-spawning season and thus it is unclear whether the gigantocellular Avp-immunopositive neurons contribute to female reproduction. It will be important in future to identify if nEsrs colocalize with Avp in the female zebrafish brain to determine if there is a direct pathway for estrogenic regulation of *avp*. Future study should also identify which Avp-immunopositive subpopulations in the POA are important for the observed effects on female sexual behavior.

3.5. Conclusions

We have demonstrated that Avp rescues the behavioral phenotype of female *cyp19a1b*$^{-/-}$ mutant zebrafish. Our data indicate that lower brain E2 levels in the *cyp19a1b*$^{-/-}$ mutant females reduces Avp signalling in the hypothalamus leading to an increased latency to the first oviposition event during pairwise mating trials. Contact points between Cyp19a1b-expressing RGCs and Avp-immunopositive cell bodies in the POA identify the neuroanatomical proximity and region of interest for locally produced E2 to regulate *avp* levels. Rescue of delayed oviposition with a single injection of Avp indicates the importance of the RGC-Avp network in the regulation of female behavior. Further study will be needed to identify nEsr and Avp colocalization and the neuronal subpopulations in the POA responsible for the observed effects on female spawning behavior.

Chapter 4: General Discussion and Conclusions

4.1 Thesis Overview

The goal of my thesis research was to investigate the importance of brain aromatase (i.e., *cyp19a1b*) for zebrafish sexual behaviors. The findings contribute to our understanding of the importance of brain-derived estrogens to teleost reproduction. This builds on the well characterized importance of ovarian-derived estrogens for ovarian development and functioning as well as in preparing the female body for reproductive activity. Numerous observations from previous teleost studies suggest a coordination of ovarian- and brain-derived estrogens to stimulate sexual behavior at the time of ovarian readiness. Firstly, *cyp19a1b* is expressed in RGCs surrounding brain regions important for reproduction (Shaw, 2018). Secondly, *cyp19a1b* is positively estrogen-regulated via the presence of an ERE in its promoter region (Menuet et al., 2005). Finally, *cyp19a1b* levels are upregulated during the reproductive period following the timing of ovarian estrogen production in many species (Chaube et al., 2015; Di Rosa et al., 2016; Gelinas et al., 1998; González and Piferrer, 2003; Kazeto et al., 2003; Maruska et al., 2020; Oh et al., 2018; Okubo et al., 2011; Pal et al., 2018). Together, these findings suggest an important role of brain-derived estrogens in female reproduction. My findings demonstrate that brain-derived estrogens have a critical role in spawning behavior in female zebrafish through positive regulation of Avp signalling in the POA.

4.2 Thesis Summary

My thesis project is composed of four objectives that together provide evidence to demonstrate an important role of brain-derived estrogens for female zebrafish sexual behavior.

The first objective of my research was to identify changes in sexual behavior and reproductive health in *cyp19a1a*$^{-/-}$ and *cyp19a1b*$^{-/-}$ mutant zebrafish (Chapter 2). For this study, sexual behavior assays were conducted involving a mutant fish paired with an opposite-sex WT fish and spawning behavior was analyzed using video recordings. The eggs spawned during trials were collected and raised for 4 dpf to identify the effects of parental *cyp19a1* mutation on early embryo development and survival. The study findings revealed that female *cyp19a1b*$^{-/-}$ mutants displayed 4.1 times longer latency to initiate spawning compared to WT females. This behavioral impairment is reminiscent of the reduced sexual behavior observed in *Aro* KO mice (Bakker et al., 2002a, 2002b; Brooks et al., 2020; Honda et al., 1998; Matsumoto et al., 2003). Female *cyp19a1b*$^{-/-}$ mutants also spawned 2.3 times more eggs than WT females; however, despite normal fertilization rates, 4.2 times more progeny died during early development. There were no obvious morphological abnormalities in the embryos or differences in morphometric measurements of BL, EA, or YSA that might suggest a cause of the increased mortality. Rather, these findings in combination with the previous identification of high *cyp19a1b* levels in unfertilized eggs (Sawyer et al., 2006) indicates potentially important roles of maternal *cyp19a1b* deposition in eggs for early embryo survival. Embryo survival rates were 24% lower in progeny from male *cyp19a1a*$^{-/-}$;*cyp19a1b*$^{-/-}$ mutant pairings

compared to WT pairings, which further suggests a critical role of the *cyp19a1* paralogs for early embryo survival.

The second thesis objective was to assess changes in the levels of *esr*s, *avp*, *oxt*, *scg2a* and *scg2b* in the telencephalon and hypothalamus of *cyp19a1b*$^{-/-}$ mutant females that might be linked to the observed impairments in sexual behavior (Chapter 3). The telencephalon of female *cyp19a1b*$^{-/-}$ mutants had 47% lower *esr1* levels compared to WT females in the morning when fish are reproductively active. In the hypothalamus, *esr1* levels were 33% lower in *cyp19a1b*$^{-/-}$ mutants compared to WT fish, and 37% lower in the morning compared to the afternoon. These findings are supported by the known positive estrogenic regulation of *esr1* due to an ERE in its promoter (Menuet et al., 2004), and data indicating 44% lower E2 levels in *cyp19a1b*$^{-/-}$ mutant brains compared to WT brains (Chapter 3) and 33% lower brain E2 levels in the morning compared to the afternoon (Chapter 3). The telencephalon of female mutants had 41% lower *esr2a* levels compared to WT fish. The female mutant hypothalamus had 68% and 58% lower *esr2a* and *esr2b* levels, respectively, compared to WT fish in the afternoon. This could indicate that lower Esr2 signalling in the *cyp19a1b*$^{-/-}$ mutant hypothalamus impairs establishment of estrogen-regulated gene expression profiles in the afternoon for reproductive activity the following morning. There were 45% and 68% lower levels of *avp* and *oxt*, respectively, in the hypothalamus of mutant females compared to WT fish. Together, these observations suggest disruptions to neuroendocrine signalling pathways in the brains of *cyp19a1b*$^{-/-}$ mutant females that might underlie the behavioral impairments.

To study the pathway underlying the observed behavioral impairments in mutant females, the third objective of the project was to determine if the abnormal spawning behavior in *cyp19a1b*$^{-/-}$ mutant females could be rescued by hormone treatment in adult fish (Chapter 3). The nonapeptide pathways were of particular interest in the female mutants because Avp and Oxt signalling are involved in modifying the salience of sensory information processing in the brain for social recognition and behavior (Wacker and Ludwig, 2012) and because the female mutants had delayed spawning that might reflect impaired mate identification. Moreover, accumulating evidence has demonstrated positive estrogenic regulation of brain Avp and Oxt signalling pathways in female teleosts (Chaube et al., 2012; Kalamarz-Kubiak et al., 2017; Nagarajan et al, 2015; Singh and Joy, 2009). In the current study, acute intraperitoneal injections of Avp and Oxt, along with coadministration of their respective receptor antagonists, were conducted in female mutants to determine if the observed impairments in spawning behavior could be rescued. Administration of Avp was found to uniquely rescue the time to the first oviposition event in female *cyp19a1b*$^{-/-}$ mutants and these effects were blocked with coadministration of an Avpr1a antagonist. Together these findings indicate an important association between brain-derived estrogens and Avp signalling for female sexual behavior.

The final objective in the thesis was to identify the neuroanatomical relationship between Cyp19a1b-expressing RGCs and Avp-immunopositive neurons in the female zebrafish brain (Chapter 3). This study was a necessary step to determine if brain-derived estrogens, via Cyp19a1b activity, could potentially diffuse to and affect nearby Avp-immunopositive neurons to regulate *avp* levels as suggested by the gene

expression data. Using immunohistochemistry methods, Cyp19a1b-positive RGC fibres were found in close neuroanatomical proximity and in contact with Avp-immunopositive neurons in both the anterior and posterior POA. These findings indicate that brain-derived estrogens from RGCs lining the diencephalic ventricle and in the POA could regulate Avp signalling levels by binding in nearby Avp-immunopositive neurons involved in the neural circuit for female sexual behavior.

While my findings are novel and have shown the importance of Cyp19a1b for female sexual behavior, many questions remain, some of which are addressed in the following section.

4.3 Future Directions

4.3.1 Determine the effects of *cyp19a1b* mutation on gonadotropin levels

The study findings in Chapter 2 identified an increased number of eggs spawned by *cyp19a1b*$^{-/-}$ mutant females in sexual behavior assays. This suggests that *cyp19a1b* mutation may affect the hypophysiotropic pathway that regulates gamete production and release. These processes are controlled by the gonadotropins that are produced in the pituitary gland and released into systemic circulation to bind receptors in the ovary where Lh stimulates oogenesis and ovulation and Fsh promotes gametogenesis and follicular growth (Zhang et al., 2015).

Future study should assess changes in *lhb* and *fshb* levels in the pituitary gland of female *cyp19a1b*$^{-/-}$ mutants to determine if there are higher gonadotropin production levels in the mutants that may be linked to the increased number of eggs spawned. There are no specific and validated immunoassays for zebrafish Lh and Fsh. Thus, measuring hormone levels in the pituitary gland and in blood samples would first require

development and validation of these zebrafish immunoassays. This method would help to determine if there are effects on the levels of gonadotropin release from the pituitary gland which would be a stronger indication of the hormone levels that bind receptors in the ovaries. Adopting an assay approach similar to that of another small teleost model, the medaka, seems most appropriate (Burow et al., 2019). Interestingly, Cyp19a1b was shown to colocalize with Lhb, but not Fshb, in pituitary cells of the ricefield eel (*Monopterus albus*) and the Japanese puffer (*Takifugu rubripes*), which suggests a potential involvement of Cyp19a1b-derived estrogens in Lh regulation (Yamaguchi et al., 2022; Zhang et al., 2014). High levels of *cyp19a1b* in the pituitary gland have been identified across numerous teleosts (Shaw, 2018), suggesting an important role of Cyp19a1b-derived estrogens in the pituitary that warrants further study. Study of *in vitro* pituitary gland preparations and targeted pituitary cell-specific *cyp19a1b*$^{-/-}$ mutant lines could help in determining the independent actions of Cyp19a1b-derived estrogens in the pituitary gland from those in the hypothalamus for reproduction.

4.3.2 Determine the metabolic and fitness consequences of *cyp19a1b* mutation

While female *cyp19a1b*$^{-/-}$ mutants spawned significantly more eggs per clutch compared to WT females, there were significantly higher progeny mortality from female *cyp19a1b*$^{-/-}$ mutant pairings (Chapter 2). This suggests a potentially higher energetic investment in reproduction by mutant females because gamete biomass production requires substantial energetic reserves (Hayward and Gilloly, 2011). It is interesting to note that in female mice, estrogen signalling in the hypothalamus is involved in maintaining energy homeostasis by suppressing food intake and increasing energy expenditure (Saito et al., 2015; Xu and López, 2018). This occurs through Esr1-mediated stimulation

of pro-opiomelanocortin neurons that inhibit food intake and the inhibition of neuropeptide Y and agouti-related peptide neurons that stimulate food intake in the acuate nucleus (Olofsson et al., 2009; Xu et al., 2011). In the ventromedial hypothalamus, Esr1 signalling stimulates physical activity and thermogenesis resulting in increased energy expenditure (Musatov et al., 2007). The hypothalamus serves as an important region where the metabolic system can modify neuroendocrine signalling pathways involved in reproduction to ensure that it occurs when there are sufficient energetic reserves in the body (Hill et al., 2008; Navarro and Kaiser, 2013; Shahjahan et al., 2014).

Considering these observations, it would be interesting in future studies to determine the metabolic rate in *cyp19a1b*$^{-/-}$ mutant females and the long-term fitness consequences associated with higher reproductive investment. To address these questions, one could determine energetic stores and metabolic rates under basal conditions and during reproduction in mutant females to see if their metabolic rates are higher during reproduction favouring increased gamete production levels. Future studies could also compare the lifespan of *cyp19a1b*$^{-/-}$ mutant and WT fish on a food-restricted diet to determine if the higher reproductive investment is reduced or if a mutant's lifespan is shorter when food is limited.

4.3.3 Assess the mechanism of *cyp19a1* mutation effects on increased progeny mortality

Important roles of the *cyp19a1* paralogs for early embryo development and survival were evidenced by the higher progeny mortality from female *cyp19a1b*$^{-/-}$ mutant and male *cyp19a1a*$^{-/-}$*;cyp19a1b*$^{-/-}$ mutant pairings (Chapter 2). This finding is supported by

previously identified time-dependent changes in the expression profiles of *cyp19a1a* and *cyp19a1b* during the first 72 hpf in zebrafish embryos indicating critical roles in early development (Sawyer et al., 2006). Despite recognition of the importance of estrogen signalling in vertebrate development and survival (Bondesson et al., 2015), there has been little follow up to identify the key roles of the *cyp19a1* paralogs in zebrafish embryos. While the findings in the current study demonstrate critical roles of the *cyp19a1* paralogs for zebrafish embryo survival, there were no observable differences in BL, EA, or YSA between progeny groups that would suggest a link to the increased mortality. Thus, further study is warranted to determine the mechanistic pathways underlying the increased mortality in *cyp19a1*$^{-/-}$ mutant progeny.

Two areas for future study in the *cyp19a1*$^{-/-}$ mutant progeny are to assess the potential effects of *cyp19a1* mutation on embryo brain and heart development. These study questions are of interest based on previous findings in larval zebrafish. Firstly, through study of a transgenic ERE-GFP zebrafish line, the brain and heart were identified as estrogen-responsive tissues in 3-5 dpf larval zebrafish (Gorelick and Halpern, 2011). Secondly, estrogen-regulated genes involved in biological processes including apoptosis, cell proliferation, multicellular organismal development, metabolism, and immune response, among others, were identified using a microarray analysis of zebrafish embryos treated with E2 from 3 hpf to 4 dpf (Hao et al., 2013). Finally, study of morpholino-mediated knockdown of *cyp19a1b* identified lower heart rates in larval zebrafish that were correlated with reduced serotonergic neuron immunofluorescence (Ulhaq and Kishida, 2018). This suggests important functions of Cyp19a1b in brain development of the serotonergic system and heart functioning in larval zebrafish. There

is also strong evidence in adult zebrafish identifying an important role of Cyp19a1b in regulating neurogenesis (Pellegrini et al., 2016), though effects in larvae are currently unknown. Together, these findings suggest potential roles of the *cyp19a1* paralogs in brain and heart development in zebrafish embryos. Future study could assess differences in neurogenesis rates using the exogenous cell tracer 5'-bromo-2'-deoxyuridine (BrdU) and heart rates in progeny of *cyp19a1*$^{-/-}$ mutants and WT fish to determine if there are differences that might underlie the increased mutant progeny mortality.

4.3.4 Identify the estrogen receptors involved in the regulation of *arginine vasopressin* levels in the hypothalamus

Gene expression analyses in the hypothalamus of female *cyp19a1b*$^{-/-}$ mutants revealed significantly lower levels of *esr*s and *avp* compared to WT fish (Chapter 3). Immunohistochemistry revealed a close neuroanatomical proximity and contact points between Cyp19a1b-expressing RGC fibres and Avp-immunopositive cell bodies in the POA (Chapter 3). Together, these findings suggest positive estrogenic regulation of *avp* levels in the female brain.

Future study should determine if *esr*s colocalize with *avp* in preoptic areas to provide a direct pathway for estrogenic regulation of *avp*. Since zebrafish *avp* does not contain an ERE, activated Esrs would be predicted to positively regulate the *avp* promoter through protein-protein interactions with Ap1 when bound to its response element (Figure 4.1) as occurs in the estrogenic regulation of rat *avp* (Shapiro et al., 2000). Future study is also needed to determine the contribution of reduced Gper signalling to the reproductive impairments identified in the female *cyp19a1b*$^{-/-}$ mutants.

4.3.5 Identify the estrogen-regulated Avp neurons involved in female sexual behavior

The rescue experiment determined that acute Avp administration can rescue the delayed oviposition in female *cyp19a1b*$^{-/-}$ mutants (Chapter 3). While the distribution of Avp-expressing cells is well conserved in the POA across teleost species (Banerjee et al., 2017), the function of the individual parvo-, magno-, and gigantocellular populations in reproduction is not well characterized. It is therefore difficult to comment on the POA subpopulation of Cyp19a1b-positive RGCs and Avp-immunopositive neurons involved in the regulation of female sexual behavior. Future study could use immediate-early gene expression to identify neurons activated during sexual behavior (Guzowski et al., 2005) and single-cell ablation to selectively inhibit neuron functioning (Liu et al., 2019) within POA subpopulations to help identify the important regions of estrogen-regulated Avp neurons involved in female sexual behavior.

The finding that Manning Compound coadministration blocked the Avp-induced rescue of the delayed oviposition in female mutants reveals the critical role of Avpr1a signalling (Chapter 3). The neuroanatomical distribution of *avpr1a* in adult zebrafish is currently uncharacterized. In larval zebrafish, *avpr1a* is expressed in forebrain neurons with axons projecting into the postoptic commissure and in hindbrain neurons that send axons into the medial longitudinal fasciculus (Iwasaki et al., 2013). The hindbrain *avpr1a*-expressing neurons are hypothesized to function in sensorimotor processing to enhance sensitization of motor responses to sensory stimulation. This is based on the observations that many neurons projecting axons into the medial longitudinal fasciculus are involved in motor control and that the *avpr1a*-expressing hindbrain neurons receive axonal contacts from *avp*-expressing neurons in the POA as well as axons from sensory

neurons in the lateral longitudinal fasciculus (Iwasaki et al., 2013). The *avpr1a*-expressing hindbrain neurons are therefore well situated to integrate sensory information from the forebrain with hindbrain motor responses to control behavior. It would be interesting in future study to determine if the delayed oviposition in female mutants is linked to disruptions in this sensorimotor processing pathway. This could be assessed through study of zebrafish with targeted hindbrain *avpr1a* mutation in sexual behavior assays to determine if the mutants display a similar delayed oviposition phenotype as observed in the *cyp19a1b*$^{-/-}$ mutant females.

4.4 Concluding Remarks

The research presented in this thesis demonstrates the important role of brain-derived estrogens for female zebrafish reproduction. Brain-derived estrogens, via Cyp19a1b activity, play a critical role in female spawning behavior through estrogenic regulation of neuroendocrine signalling pathways. Mutation of *cyp19a1b* in females reduces Avp signalling levels in the brain leading to impairments in female sexual behavior. A proposed model of Cyp19a1b-derived estrogenic regulation of *avp* levels in the female zebrafish brain is depicted in Figure 4.1. The knowledge gained from these thesis findings contributes to our understanding of the role of Cyp19a1b in the control of teleost sexual behavior (Figure 4.2).

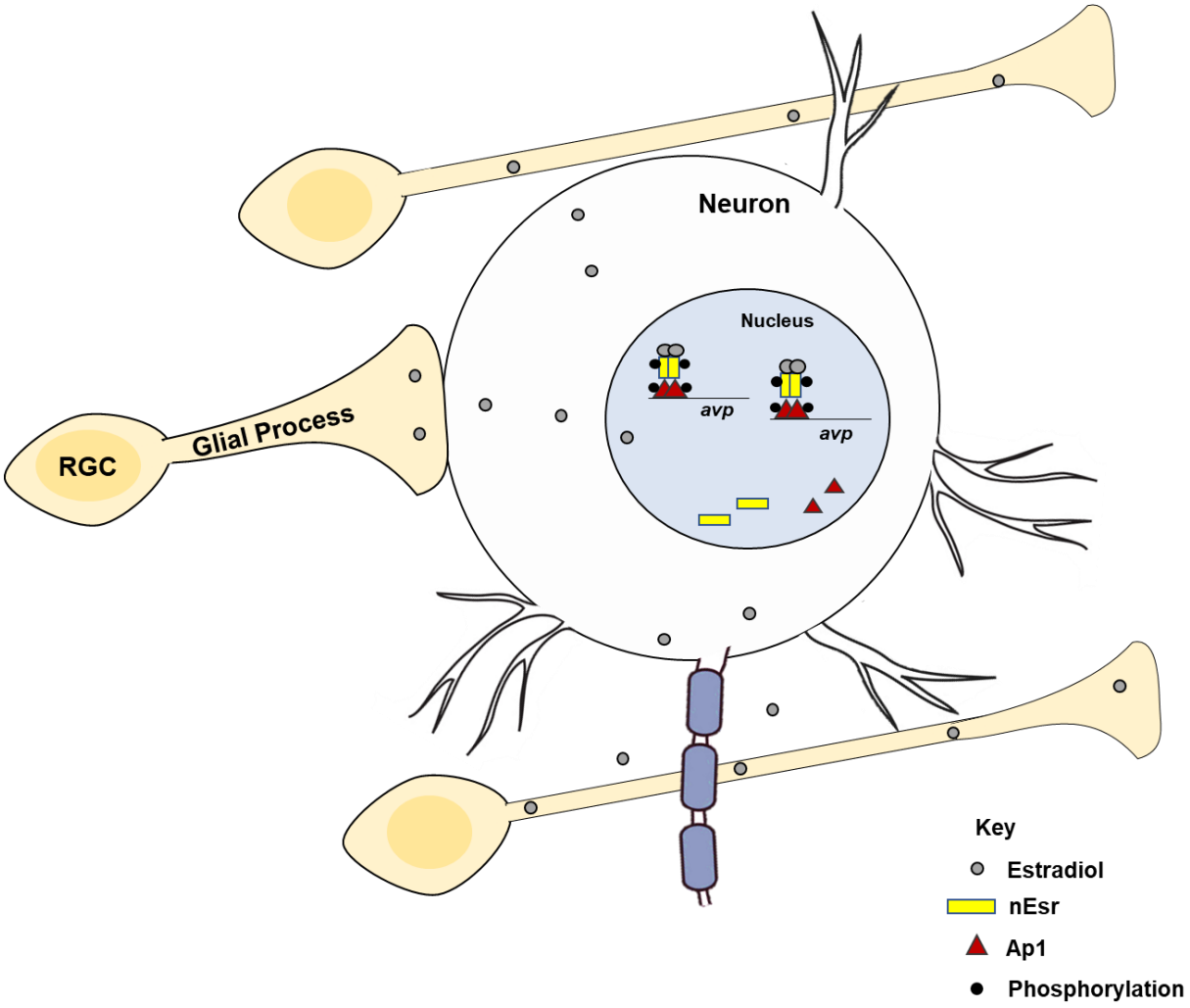


Figure 4.1- Proposed model of Cyp19a1b-derived estrogenic regulation of *avp* levels in the female zebrafish brain. Radial glial cells (RGCs) expressing Cyp19a1b line the diencephalic ventricle and are present in preoptic areas with processes extending laterally that surround and contact nearby neurons. Estradiol produced via Cyp19a1b activity could diffuse into neurons to bind and activate nuclear estrogen receptors (nEsrs) in the cell nucleus. Activated nEsrs could then dimerize and bind activator protein 1 (Ap1) bound to its response element in the *arginine vasopressin* (*avp*) promoter to upregulate *avp* levels. This pathway is hypothesized to play an important role in female zebrafish spawning behavior.

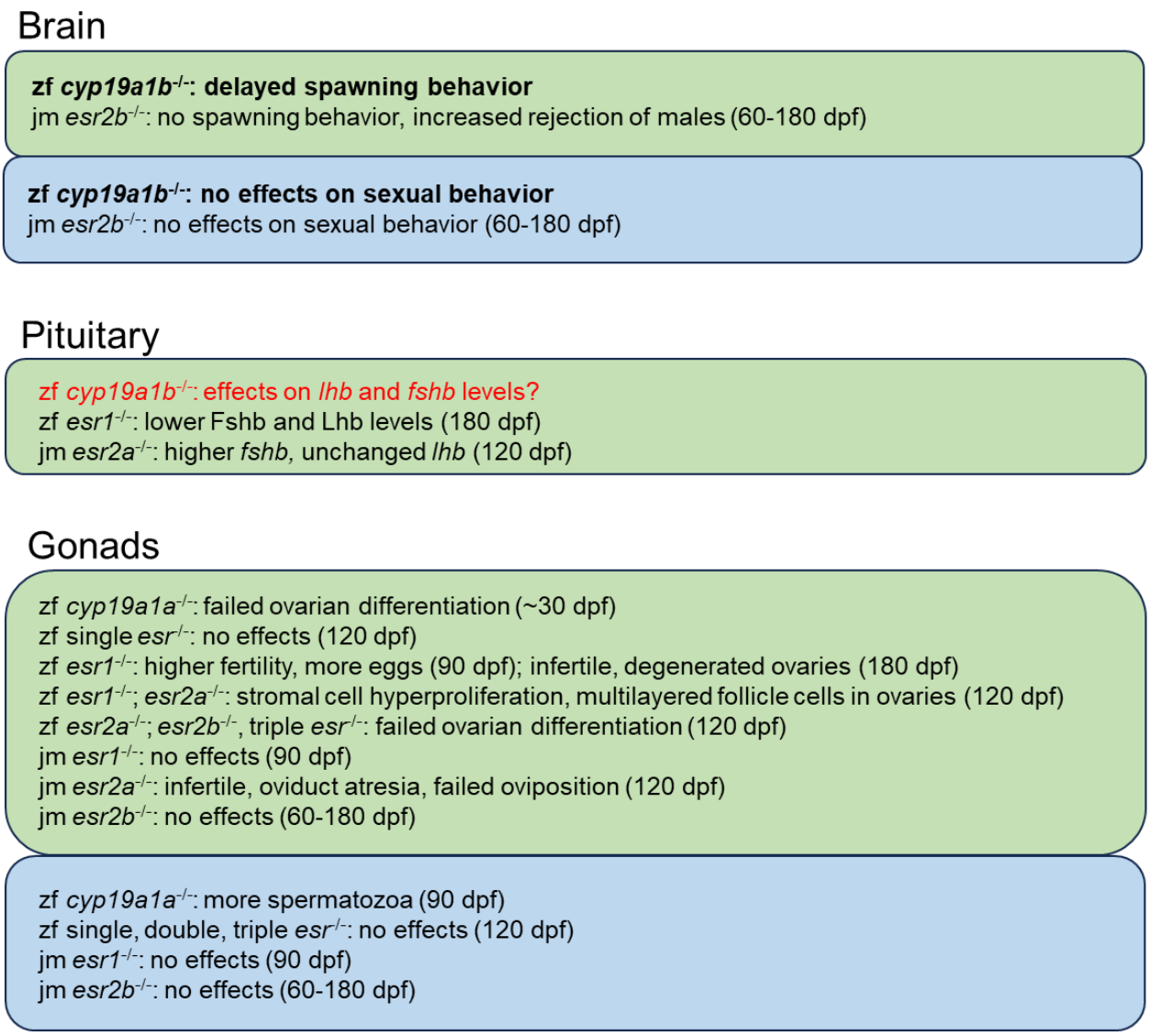


Figure 4.2- Summary of estrogen receptor mutant (*esr*$^{-/-}$), ovarian aromatase mutant (*cyp19a1a*$^{-/-}$), and brain aromatase mutant (*cyp19a1b*$^{-/-}$) effects on zebrafish (zf) and Japanese medaka (jm) reproductive physiology and sexual behavior. Findings in female fish are boxed in green, while findings in males are boxed in blue. Fish age at testing is indicated in days post-fertilization (dpf). The contributions of the thesis findings are indicated in bold. Future study to build on the current thesis findings is indicated in red. References used: Chen et al., 2018; Dranow et al., 2016;

Kayo et al., 2019; Lau et al., 2016; Lu et al., 2017; Niishike et al., 2021; Tang et al., 2017; Tohyama et al., 2017; Yin et al., 2017.

References

Alharthy, K.M., Albaqami, F.F., Thornton, C., Corrales, J. and Willett, K.L., 2017. Mechanistic evaluation of benzo [a] pyrene's developmental toxicities mediated by reduced Cyp19a1b activity. *Toxicological Sciences*, *155*(1), pp.135-147.

Allgood Jr, O.E., Hamad, A., Fox, J., DeFrank, A., Gilley, R., Dawson, F., Sykes, B., Underwood, T.J., Naylor, R.C., Briggs, A.A. and Lassiter, C.S., 2013. Estrogen prevents cardiac and vascular failure in the 'listless' zebrafish (*Danio rerio*) developmental model. *General and Comparative Endocrinology*, *189*, pp.33-42.

Alvarez-Bolado, G., 2019. Development of neuroendocrine neurons in the mammalian hypothalamus. *Cell and Tissue Research*, *375*(1), pp.23-39.

Alward, B.A., Laud, V.A., Skalnik, C.J., York, R.A., Juntti, S.A. and Fernald, R.D., 2020. Modular genetic control of social status in a cichlid fish. *Proceedings of the National Academy of Sciences*, *117*(45), pp.28167-28174.

Ankley, G.T., Kahl, M.D., Jensen, K.M., Hornung, M.W., Korte, J.J., Makynen, E.A. and Leino, R.L., 2002. Evaluation of the aromatase inhibitor fadrozole in a short-term reproduction assay with the fathead minnow (*Pimephales promelas*). *Toxicological Sciences*, *67*(1), pp.121-130.

Ayobahan, S.U., Eilebrecht, E., Kotthoff, M., Baumann, L., Eilebrecht, S., Teigeler, M., Hollert, H., Kalkhof, S. and Schäfers, C., 2019. A combined FSTRA-shotgun proteomics approach to identify molecular changes in zebrafish upon chemical exposure. *Scientific Reports*, *9*(1), p.6599.

Ayoub, F., 1998. Estradiol regulation of the neurohypophysial hormones in the goldfish, *Carassius auratus*. University of Ottawa (Canada), pp.1-96.

Bakker, J., Honda, S.I., Harada, N. and Balthazart, J., 2002a. The aromatase knock-out mouse provides new evidence that estradiol is required during development in the female for the expression of sociosexual behaviors in adulthood. *Journal of Neuroscience*, *22*(20), pp.9104-9112.

Bakker, J., Honda, S., Harada, N. and Balthazart, J., 2002b. Sexual partner preference requires a functional aromatase (*cyp19*) gene in male mice. *Hormones and Behavior*, *42*(2), pp.158-171.

Bakker, J., Honda, S., Harada, N. and Balthazart, J., 2003. The aromatase knockout (ArKO) mouse provides new evidence that estrogens are required for the development of the female brain. *Annals of the New York Academy of Sciences*, *1007*(1), pp.251-262.

Banerjee, P., Joy, K.P. and Chaube, R., 2017. Structural and functional diversity of nonapeptide hormones from an evolutionary perspective: a review. *General and Comparative Endocrinology*, *241*, pp.4-23.

Baumgartner, N.E., Grissom, E.M., Pollard, K.J., McQuillen, S.M. and Daniel, J.M., 2019. Neuroestrogen-dependent transcriptional activity in the brains of ERE-luciferase reporter mice following short-and long-term ovariectomy. *Eneuro*, *6*(5), pp.1-11.

Bernardo, R.C. and Connaughton, V.P., 2022. Transient developmental exposure to tributyltin reduces optomotor responses in larval zebrafish (*Danio rerio*). *Neurotoxicology and Teratology*, *89*, p.107055.

Beyer, C., Green, S.J., Barker, P.J., Huskisson, N.S. and Hutchinson, J.B., 1994. Aromatase-immunoreactivity is localised specifically in neurones in the developing mouse hypothalamus and cortex. *Brain Research*, *638*(1-2), pp.203-210.

Bliss, S.P., Navratil, A.M., Xie, J. and Roberson, M.S., 2010. GnRH signaling, the gonadotrope and endocrine control of fertility. *Frontiers in Neuroendocrinology*, *31*(3), pp.322-340.

Böhne, A., Heule, C., Boileau, N. and Salzburger, W., 2013. Expression and sequence evolution of aromatase cyp19a1 and other sexual development genes in East African cichlid fishes. *Molecular Biology and Evolution*, *30*(10), pp.2268-2285.

Bondesson, M., Hao, R., Lin, C.Y., Williams, C. and Gustafsson, J.Å., 2015. Estrogen receptor signaling during vertebrate development. *Biochimica et Biophysica Acta (BBA)-Gene Regulatory Mechanisms*, *1849*(2), pp.142-151.

Britt, K.L., Drummond, A.E., Dyson, M., Wreford, N.G., Jones, M.E., Simpson, E.R. and Findlay, J.K., 2001. The ovarian phenotype of the aromatase knockout (ArKO) mouse. *The Journal of Steroid Biochemistry and Molecular Biology*, *79*(1-5), pp.181-185.

Brooks, D.C., Coon V, J.S., Ercan, C.M., Xu, X., Dong, H., Levine, J.E., Bulun, S.E. and Zhao, H., 2020. Brain aromatase and the regulation of sexual activity in male mice. *Endocrinology*, *161*(10), p.bqaa137.

Bugel, S.M., White, L.A. and Cooper, K.R., 2011. Decreased vitellogenin inducibility and 17β-estradiol levels correlated with reduced egg production in killifish (*Fundulus heteroclitus*) from Newark Bay, NJ. *Aquatic Toxicology*, *105*(1-2), pp.1-12.

Burow, S., Fontaine, R., von Krogh, K., Mayer, I., Nourizadeh-Lillabadi, R., Hollander-Cohen, L., Cohen, Y., Shpilman, M., Levavi-Sivan, B. and Weltzien, F.A., 2019. Medaka follicle-stimulating hormone (Fsh) and luteinizing hormone (Lh): Developmental profiles of pituitary protein and gene expression levels. *General and Comparative Endocrinology*, *272*, pp.93-108.

Butler, J.M., Anselmo, C.M. and Maruska, K.P., 2021. Female reproductive state is associated with changes in distinct arginine vasotocin cell types in the preoptic area of *Astatotilapia burtoni*. *Journal of Comparative Neurology*, *529*(5), pp.987-1003.

Butler, J.M. and Maruska, K.P., 2021. Opsin expression varies with Reproductive state in the cichlid fish *Astatotilapia burtoni*. *Integrative and Comparative Biology*, *61*(1), pp.240-248.

Chaube, R., Rawat, A. and Joy, K.P., 2015. Molecular cloning and characterization of brain and ovarian cytochrome P450 aromatase genes in the catfish *Heteropneustes fossilis*: Sex, tissue and seasonal variation in, and effects of gonadotropin on gene expression. *General and Comparative Endocrinology*, *221*, pp.120-133.

Chaube, R., Singh, R.K. and Joy, K.P., 2012. Estrogen regulation of brain vasotocin secretion in the catfish *Heteropneustes fossilis*: an interaction with catecholaminergic system. *General and Comparative Endocrinology*, *175*(1), pp.206-213.

Chen, Y., Tang, H., Wang, L., He, J., Guo, Y., Liu, Y., Liu, X. and Lin, H., 2018. Fertility enhancement but premature ovarian failure in esr1-deficient female zebrafish. *Frontiers in Endocrinology*, *9*, p.567.

Choleris, E., Gustafsson, J.Å., Korach, K.S., Muglia, L.J., Pfaff, D.W. and Ogawa, S., 2003. An estrogen-dependent four-gene micronet regulating social recognition: a study with oxytocin and estrogen receptor-α and-β knockout mice. *Proceedings of the National Academy of Sciences*, *100*(10), pp.6192-6197.

Choleris, E., Ogawa, S., Kavaliers, M., Gustafsson, J.Å., Korach, K.S., Muglia, L.J. and Pfaff, D.W., 2006. Involvement of estrogen receptor α, β and oxytocin in social discrimination: a detailed behavioral analysis with knockout female mice. *Genes, Brain and Behavior*, *5*(7), pp.528-539.

Clarkson, J., de Tassigny, X.D.A., Moreno, A.S., Colledge, W.H. and Herbison, A.E., 2008. Kisspeptin–GPR54 signaling is essential for preovulatory gonadotropin-releasing hormone neuron activation and the luteinizing hormone surge. *Journal of Neuroscience*, *28*(35), pp.8691-8697.

Converse, A. and Thomas, P., 2019. Androgens regulate follicle stage-dependent pro- and anti-apoptosis in teleost ovaries through ZIP9 activation of different G proteins. *Biology of Reproduction*, *101*(2), pp.377-391.

Cornil, C.A., 2018. On the role of brain aromatase in females: why are estrogens produced locally when they are available systemically?. *Journal of Comparative Physiology A*, *204*(1), pp.31-49.

Couse, J.F. and Korach, K.S., 1999. Estrogen receptor null mice: what have we learned and where will they lead us?. *Endocrine Reviews*, *20*(3), pp.358-417.

Da Fonte, D.F., Xing, L., Mikwar, M. and Trudeau, V.L., 2018. Secretoneurin-A inhibits aromatase B (cyp19a1b) expression in female goldfish (*Carassius auratus*) radial glial cells. *General and Comparative Endocrinology*, *257*, pp.106-112.

Dang, Y., Sun, Q., Letcher, R.J. and Liu, C., 2018. Unexpected observations: Exposure to aromatase inhibitor prochloraz did not alter the vitellogenin content of zebrafish ova but did inhibit the growth of larval offspring. *Environmental Science & Technology Letters*, *5*(11), pp.629-634.

Darrow, K.O. and Harris, W.A., 2004. Characterization and development of courtship in zebrafish, *Danio rerio*. *Zebrafish*, *1*(1), pp.40-45.

Di Rosa, V., López-Olmeda, J.F., Burguillo, A., Frigato, E., Bertolucci, C., Piferrer, F. and Sánchez-Vázquez, F.J., 2016. Daily rhythms of the expression of key genes involved in steroidogenesis and gonadal function in zebrafish. *PloS One*, *11*(6), p.e0157716.

Diotel, N., Charlier, T.D., Lefebvre d'Hellencourt, C., Couret, D., Trudeau, V.L., Nicolau, J.C., Meilhac, O., Kah, O. and Pellegrini, E., 2018. Steroid transport, local synthesis, and signaling within the brain: roles in neurogenesis, neuroprotection, and sexual behaviors. *Frontiers in Neuroscience*, *12*, p.84.

Diotel, N., Le Page, Y., Mouriec, K., Tong, S.K., Pellegrini, E., Vaillant, C., Anglade, I., Brion, F., Pakdel, F., Chung, B.C. and Kah, O., 2010. Aromatase in the brain of teleost fish: expression, regulation and putative functions. *Frontiers in neuroendocrinology*, *31*(2), pp.172-192.

Dranow, D.B., Hu, K., Bird, A.M., Lawry, S.T., Adams, M.T., Sanchez, A., Amatruda, J.F. and Draper, B.W., 2016. Bmp15 is an oocyte-produced signal required for maintenance of the adult female sexual phenotype in zebrafish. *PLoS Genetics*, *12*(9), p.e1006323.

Dupont, S., Krust, A., Gansmuller, A., Dierich, A., Chambon, P. and Mark, M., 2000. Effect of single and compound knockouts of estrogen receptors alpha (ERalpha) and beta (ERbeta) on mouse reproductive phenotypes. *Development*, *127*(19), pp.4277-4291.

Eddy, E.M., Washburn, T.F., Bunch, D.O., Goulding, E.H., Gladen, B.C., Lubahn, D.B. and Korach, K.S., 1996. Targeted disruption of the estrogen receptor gene in male mice causes alteration of spermatogenesis and infertility. *Endocrinology*, *137*(11), pp.4796-4805.

Emmen, J.M.A. and Korach, K.S., 2003. Estrogen receptor knockout mice: phenotypes in the female reproductive tract. *Gynecological Endocrinology*, *17*(2), pp.169-176.

Feng, K., Cui, X., Song, Y., Tao, B., Chen, J., Wang, J., Liu, S., Sun, Y., Zhu, Z., Trudeau, V.L. and Hu, W., 2020. Gnrh3 regulates PGC proliferation and sex differentiation in developing zebrafish. *Endocrinology*, *161*(1), p.bqz024.

Filby, A.L. and Tyler, C.R., 2007. Appropriate 'housekeeping' genes for use in expression profiling the effects of environmental estrogens in fish. *BMC Molecular Biology*, *8*(1), pp.1-13.

Findlay, J.K., Liew, S.H., Simpson, E.R. and Korach, K.S., 2010. Estrogen signaling in the regulation of female reproductive functions. *Fertility Control*, pp.29-35.

Fisher, C.R., Graves, K.H., Parlow, A.F. and Simpson, E.R., 1998. Characterization of mice deficient in aromatase (ArKO) because of targeted disruption of the cyp19 gene. *Proceedings of the National Academy of Sciences*, *95*(12), pp.6965-6970.

Friesen, C.N., Ramsey, M.E. and Cummings, M.E., 2017. Differential sensitivity to estrogen-induced opsin expression in two poeciliid freshwater fish species. *General and Comparative Endocrinology*, *246*, pp.200-210.

Gelinas, D., Pitoc, G.A. and Callard, G.V., 1998. Isolation of a goldfish brain cytochrome P450 aromatase cDNA:: mRNA expression during the seasonal cycle and after steroid treatment. *Molecular and Cellular Endocrinology*, *138*(1-2), pp.81-93.

Giroux, M., Vliet, S.M., Volz, D.C., Gan, J. and Schlenk, D., 2019. Mechanisms behind interactive effects of temperature and bifenthrin on the predator avoidance behaviors in parr of Chinook salmon (*Oncorhynchus tshawytscha*). *Aquatic Toxicology*, *216*, p.105312.

Gonçalves, D.M. and Oliveira, R.F., 2011. Hormones and sexual behavior of teleost fishes. In *Hormones and reproduction of vertebrates* (pp. 119-147). Academic Press.

González, A. and Piferrer, F., 2003. Aromatase activity in the European sea bass (*Dicentrarchus labrax L.*) brain. Distribution and changes in relation to age, sex, and the annual reproductive cycle. *General and Comparative Endocrinology*, *132*(2), pp.223-230.

Gore, A.C., 2002. Neuroanatomy of the GnRH-1 System. In *GnRH: the master molecule of reproduction* (pp.9-26). Springer Science & Business Media.

Gorelick, D.A. and Halpern, M.E., 2011. Visualization of estrogen receptor transcriptional activation in zebrafish. *Endocrinology*, *152*(7), pp.2690-2703.

Gould, C.J., Saldanha, C.J. and Connaughton, V.P., 2019. Acute exposure to 4-OH-A, not PCB1254, alters brain aromatase activity but does not adversely affect growth in zebrafish. *Environmental Toxicology and Pharmacology*, *68*, pp.133-140.

Gozdowska, M., Kleszczyńska, A., Sokołowska, E. and Kulczykowska, E., 2006. Arginine vasotocin (Avt) and isotocin (It) in fish brain: diurnal and seasonal variations. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, *143*(3), pp.330-334.

Griffin, L. B., January, K. E., Ho, K. W., Cotter, K. A., and Callard, G. V., 2013. Morpholino-mediated knockdown of ERα, ERβa, and ERβb mRNAs in zebrafish (*Danio rerio*) embryos reveals differential regulation of estrogen-inducible genes. *Endocrinology*, *154*(11), pp.4158-4169.

Gruber, C.J., Tschugguel, W., Schneeberger, C. and Huber, J.C., 2002. Production and actions of estrogens. *New England Journal of Medicine*, *346*(5), pp.340-352.

Gutierrez-Castellanos, N., Husain, B.F., Dias, I.C. and Lima, S.Q., 2022. Neural and behavioral plasticity across the female reproductive cycle. *Trends in Endocrinology & Metabolism*, *33*(11), pp.769-785.

Guzowski, J.F., Timlin, J.A., Roysam, B., McNaughton, B.L., Worley, P.F. and Barnes, C.A., 2005. Mapping behaviorally relevant neural circuits with immediate-early gene expression. *Current Opinion in Neurobiology*, *15*(5), pp.599-606.

Hamad, A., Kluk, M., Fox, J., Park, M. and Turner, J.E., 2007. The effects of aromatase inhibitors and selective estrogen receptor modulators on eye development in the zebrafish (*Danio rerio*). *Current Eye Research*, *32*(10), pp.819-827.

Hao, R., Bondesson, M., Singh, A.V., Riu, A., McCollum, C.W., Knudsen, T.B., Gorelick, D.A. and Gustafsson, J.Å., 2013. Identification of estrogen target genes during zebrafish embryonic development through transcriptomic analysis. *PloS One*, *8*(11), p.e79020.

Hara, A., Hiramatsu, N. and Fujita, T., 2016. Vitellogenesis and choriogenesis in fishes. *Fisheries Science*, *82*, pp.187-202.

Hayward, A. and Gillooly, J.F., 2011. The cost of sex: quantifying energetic investment in gamete production by males and females. *PLoS One*, *6*(1), p.e16557.

Heldring, N., Pike, A., Andersson, S., Matthews, J., Cheng, G., Hartman, J., Tujague, M., Strom, A., Treuter, E., Warner, M. and Gustafsson, J.A., 2007. Estrogen receptors: how do they signal and what are their targets. *Physiological Reviews*, *87*(3), pp.905-931.

Herget, U., Gutierrez-Triana, J.A., Thula, O.S., Knerr, B. and Ryu, S., 2017. Single-cell reconstruction of oxytocinergic neurons reveals separate hypophysiotropic and encephalotropic subtypes in larval zebrafish. *ENeuro*, *4*(1), pp.1-16.

Herget, U., Wolf, A., Wullimann, M.F. and Ryu, S., 2014. Molecular neuroanatomy and chemoarchitecture of the neurosecretory preoptic-hypothalamic area in zebrafish larvae. *Journal of Comparative Neurology*, *522*(7), pp.1542-1564.

Hill, J.W., Elmquist, J.K. and Elias, C.F., 2008. Hypothalamic pathways linking energy balance and reproduction. *American Journal of Physiology-Endocrinology and Metabolism*, *294*(5), pp.E827-E832.

Hiraki-Kajiyama, T., Yamashita, J., Yokoyama, K., Kikuchi, Y., Nakajo, M., Miyazoe, D., Nishiike, Y., Ishikawa, K., Hosono, K., Kawabata-Sakata, Y. and Ansai, S., 2019. Neuropeptide B mediates female sexual receptivity in medaka fish, acting in a female-specific but reversible manner. *Elife*, *8*, p.e39495.

Honda, S.I., Harada, N., Abe-Dohmae, S. and Takagi, Y., 1999. Identification of cis-acting elements in the proximal promoter region for brain-specific exon 1 of the mouse aromatase gene. *Molecular Brain Research*, *66*(1-2), pp.122-132.

Honda, S.I., Harada, N., Ito, S., Takagi, Y. and Maeda, S., 1998. Disruption of sexual behavior in male aromatase-deficient mice lacking exons 1 and 2 of the cyp19 gene. *Biochemical and Biophysical Research Communications*, *252*(2), pp.445-449.

Honda, S.I., Harada, N. and Takagi, Y., 1994. Novel exon 1 of the aromatase gene specific for aromatase transcripts in human brain. *Biochemical and Biophysical Research Communications*, *198*(3), pp.1153-1160.

Huffman, L.S., O'Connell, L.A. and Hofmann, H.A., 2013. Aromatase regulates aggression in the African cichlid fish *Astatotilapia burtoni*. *Physiology & Behavior*, *112*, pp.77-83.

Inoue, S., 2022. Neural basis for estrous cycle-dependent control of female behaviors. *Neuroscience Research*, *176*, pp.1-8.

Inoue, S., Yang, R., Tantry, A., Davis, C.H., Yang, T., Knoedler, J.R., Wei, Y., Adams, E.L., Thombare, S., Golf, S.R. and Neve, R.L., 2019. Periodic remodeling in a neural circuit governs timing of female sexual behavior. *Cell*, *179*(6), pp.1393-1408.

Iwasaki, K., Taguchi, M., Bonkowsky, J.L. and Kuwada, J.Y., 2013. Expression of arginine vasotocin receptors in the developing zebrafish CNS. *Gene Expression Patterns*, *13*(8), pp.335-342.

Jalabert, C., Quintana, L., Pessina, P. and Silva, A., 2015. Extra-gonadal steroids modulate non-breeding territorial aggression in weakly electric fish. *Hormones and Behavior*, *72*, pp.60-67.

Jardine, D. and Litvak, M.K., 2003. Direct yolk sac volume manipulation of zebrafish embryos and the relationship between offspring size and yolk sac volume. *Journal of Fish Biology*, *63*(2), pp.388-397.

Kalamarz-Kubiak, H., Gozdowska, M., Guellard, T. and Kulczykowska, E., 2017. How does oestradiol influence the AVT/IT system in female round gobies during different reproductive phases?. *Biology Open*, *6*(10), pp.1493-1501.

Kayo, D., Oka, Y. and Kanda, S., 2020. Examination of methods for manipulating serum 17β-Estradiol (E2) levels by analysis of blood E2 concentration in medaka (*Oryzias latipes*). *General and Comparative Endocrinology*, *285*, p.113272.

Kayo, D., Zempo, B., Tomihara, S., Oka, Y. and Kanda, S., 2019. Gene knockout analysis reveals essentiality of estrogen receptor β1 (Esr2a) for female reproduction in medaka. *Scientific Reports*, *9*(1), p.8868.

Kazeto, Y., Goto-Kazeto, R., Place, A.R. and Trant, J.M., 2003. Aromatase expression in zebrafish and channel catfish brains: changes in transcript abundance associated with the reproductive cycle and exposure to endocrine disrupting chemicals. *Fish Physiology and Biochemistry*, *28*, pp.29-32.

Klinge, C.M., 2001. Estrogen receptor interaction with estrogen response elements. *Nucleic Acids Research*, *29*(14), pp.2905-2919.

Krege, J.H., Hodgin, J.B., Couse, J.F., Enmark, E., Warner, M., Mahler, J.F., Sar, M., Korach, K.S., Gustafsson, J.Å. and Smithies, O., 1998. Generation and reproductive phenotypes of mice lacking estrogen receptor β. *Proceedings of the National Academy of Sciences*, *95*(26), pp.15677-15682.

Kulczykowska, E., Kalamarz-Kubiak, H., Nietrzeba, M. and Gozdowska, M., 2014. Brain nonapeptide and gonadal steroid responses to deprivation of heterosexual contact in the black molly. *Biology Open*, *4*(1), pp.69-78.

Kulczykowska, E. and Kleszczyńska, A., 2014. Brain arginine vasotocin and isotocin in breeding female three-spined sticklebacks (*Gasterosteus aculeatus*): the presence of male and egg deposition. *General and Comparative Endocrinology*, *204*, pp.8-12.

Lau, E.S.W., Zhang, Z., Qin, M. and Ge, W., 2016. Knockout of zebrafish ovarian aromatase gene (*cyp19a1a*) by TALEN and CRISPR/Cas9 leads to all-male offspring due to failed ovarian differentiation. *Scientific Reports*, *6*(1), pp.1-14.

Le Page, Y., Menuet, A., Kah, O. and Pakdel, F., 2008. Characterization of a cis-acting element involved in cell-specific expression of the zebrafish brain aromatase

gene. *Molecular Reproduction and Development: Incorporating Gamete Research*, *75*(10), pp.1549-1557.

Levin, E.R., 2009. Plasma membrane estrogen receptors. *Trends in Endocrinology & Metabolism*, *20*(10), pp.477-482.

Li, J. and Gibbs, R.B., 2019. Detection of estradiol in rat brain tissues: Contribution of local versus systemic production. *Psychoneuroendocrinology*, *102*, pp.84-94.

Li, J., Hubbard, P.C. and Canário, A.V., 2018. Male zebrafish (*Danio rerio*) odorants attract females and induce spawning. *Aquaculture and Fisheries*, *3*(4), pp.139-144.

Lin, C.J., Maugars, G., Lafont, A.G., Jeng, S.R., Wu, G.C., Dufour, S. and Chang, C.F., 2020. Basal teleosts provide new insights into the evolutionary history of teleost-duplicated aromatase. *General and Comparative Endocrinology*, *291*, p.113395.

Liu, F., Dai, S., Feng, D., Peng, X., Qin, Z., Kearns, A.C., Huang, W., Chen, Y., Ergün, S., Wang, H. and Rappaport, J., 2019. Versatile cell ablation tools and their applications to study loss of cell functions. *Cellular and Molecular Life Sciences*, *76*, pp.4725-4743.

Lu, H., Cui, Y., Jiang, L. and Ge, W., 2017. Functional analysis of nuclear estrogen receptors in zebrafish reproduction by genome editing approach. *Endocrinology*, *158*(7), pp.2292-2308.

Lu, C., Peng, D., Erandani, U., Mitchell, K., Martyniuk, C.J. and Trudeau, V.L., 2023. Simultaneous extraction and detection of peptides, steroids, and proteins in small tissue samples. *Frontiers in Endocrinology*, *14*, p.1266985.

Lubahn, D.B., Moyer, J.S., Golding, T.S., Couse, J.F., Korach, K.S. and Smithies, O., 1993. Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene. *Proceedings of the National Academy of Sciences*, *90*(23), pp.11162-11166.

Machluf, Y., Gutnick, A. and Levkowitz, G., 2011. Development of the zebrafish hypothalamus. *Annals of the New York Academy of Sciences*, *1220*(1), pp.93-105.

Maggiolini, M. and Picard, D., 2010. The unfolding stories of GPR30, a new membrane-bound estrogen receptor. *Journal of Endocrinology*, *204*(2), pp.105-114.

Marsh, K.E., Creutz, L.M., Hawkins, M.B. and Godwin, J., 2006. Aromatase immunoreactivity in the bluehead wrasse brain, *Thalassoma bifasciatum*: immunolocalization and co-regionalization with arginine vasotocin and tyrosine hydroxylase. *Brain research*, *1126*(1), pp.91-101.

Martínez, R., Navarro-Martín, L., Luccarelli, C., Codina, A.E., Raldúa, D., Barata, C., Tauler, R. and Piña, B., 2019. Unravelling the mechanisms of PFOS toxicity by combining morphological and transcriptomic analyses in zebrafish embryos. *Science of the Total Environment*, *674*, pp.462-471.

Maruska, K.P., Butler, J.M., Anselmo, C. and Tandukar, G., 2020. Distribution of aromatase in the brain of the African cichlid fish *Astatotilapia burtoni*: Aromatase expression, but not estrogen receptors, varies with female reproductive state. *Journal of Comparative Neurology*, *528*(15), pp.2499-2522.

Maruska, K.P., Mizobe, M.H. and Tricas, T.C., 2007. Sex and seasonal co-variation of arginine vasotocin (AVT) and gonadotropin-releasing hormone (GnRH) neurons in the

brain of the halfspotted goby. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, *147*(1), pp.129-144.

Marvel, M., Spicer, O.S., Wong, T.T., Zmora, N. and Zohar, Y., 2018. Knockout of the Gnrh genes in zebrafish: effects on reproduction and potential compensation by reproductive and feeding-related neuropeptides. *Biology of Reproduction*, *99*(3), pp.565-577.

Mason, A.J., Hayflick, J.S., Zoeller, R.T., Young III, W.S., Phillips, H.S., Nikolics, K. and Seeburg, P.H., 1986. A deletion truncating the gonadotropin-releasing hormone gene is responsible for hypogonadism in the hpg mouse. *Science*, *234*(4782), pp.1366-1371.

Matsumoto, T., Honda, S.I. and Harada, N., 2003. Alteration in sex-specific behaviors in male mice lacking the aromatase gene. *Neuroendocrinology*, *77*(6), pp.416-424.

McBride, R.S., Somarakis, S., Fitzhugh, G.R., Albert, A., Yaragina, N.A., Wuenschel, M.J., Alonso-Fernández, A. and Basilone, G., 2015. Energy acquisition and allocation to egg production in relation to fish reproductive strategies. *Fish and Fisheries*, *16*(1), pp.23-57.

McEwen, B.S., 1991. Non-genomic and genomic effects of steroids on neural activity. *Trends in Pharmacological Sciences*, *12*, pp.141-147.

McHenry, J.A., Otis, J.M., Rossi, M.A., Robinson, J.E., Kosyk, O., Miller, N.W., McElligott, Z.A., Budygin, E.A., Rubinow, D.R. and Stuber, G.D., 2017. Hormonal gain control of a medial preoptic area social reward circuit. *Nature Neuroscience*, *20*(3), pp.449-458.

Mennigen, J.A., Ramachandran, D., Shaw, K., Chaube, R., Joy, K.P. and Trudeau, V.L., 2022. Reproductive roles of the vasopressin/oxytocin neuropeptide family in teleost fishes. *Frontiers in Endocrinology*, *13*, p.1005863.

Menuet, A., Le Page, Y., Torres, O., Kern, L., Kah, O. and Pakdel, F., 2004. Analysis of the estrogen regulation of the zebrafish estrogen receptor (ER) reveals distinct effects of ERalpha, ERbeta1 and ERbeta2. *Journal of Molecular Endocrinology*, *32*(3), pp.975-986.

Menuet, A., Pellegrini, E., Anglade, I., Blaise, O., Laudet, V., Kah, O. and Pakdel, F., 2002. Molecular characterization of three estrogen receptor forms in zebrafish: binding characteristics, transactivation properties, and tissue distributions. *Biology of Reproduction*, *66*(6), pp.1881-1892.

Menuet, A., Pellegrini, E., Brion, F., Gueguen, M.M., Anglade, I., Pakdel, F. and Kah, O., 2005. Expression and estrogen-dependent regulation of the zebrafish brain aromatase gene. *Journal of Comparative Neurology*, *485*(4), pp.304-320.

Mitchell, K., 2018. *Characterization and Role of Secretogranin-II/Secretoneurin in Zebrafish Reproduction* (Doctoral dissertation, Université d'Ottawa/University of Ottawa), pp.1-165.

Mitchell, K., Zhang, W.S., Lu, C., Tao, B., Chen, L., Hu, W. and Trudeau, V.L., 2020. Targeted mutation of secretogranin-2 disrupts sexual behavior and reproduction in zebrafish. *Proceedings of the National Academy of Sciences*, *117*(23), pp.12772-12783.

Musatov, S., Chen, W., Pfaff, D.W., Mobbs, C.V., Yang, X.J., Clegg, D.J., Kaplitt, M.G. and Ogawa, S., 2007. Silencing of estrogen receptor α in the ventromedial nucleus of

hypothalamus leads to metabolic syndrome. *Proceedings of the National Academy of Sciences*, *104*(7), pp.2501-2506.

Nagarajan, G., Aruna, A. and Chang, C.F., 2015. Neuropeptide arginine vasotocin positively affects neurosteroidogenesis in the early brain of grouper, *Epinephelus coioides*. *Journal of Neuroendocrinology*, *27*(9), pp.718-736.

Navarro, V.M. and Kaiser, U.B., 2013. Metabolic influences on neuroendocrine regulation of reproduction. *Current Opinion in Endocrinology, Diabetes, and Obesity*, *20*(4), pp.335-341.

Nelson, E.R. and Habibi, H.R., 2013. Estrogen receptor function and regulation in fish and other vertebrates. *General and Comparative Endocrinology*, *192*, pp.15-24.

Nishiike, Y., Miyazoe, D., Togawa, R., Yokoyama, K., Nakasone, K., Miyata, M., Kikuchi, Y., Kamei, Y., Todo, T., Ishikawa-Fujiwara, T. and Ohno, K., 2021. Estrogen receptor 2b is the major determinant of sex-typical mating behavior and sexual preference in medaka. *Current Biology*, *31*(8), pp.1699-1710.

O'Donnell, L., Robertson, K.M., Jones, M.E. and Simpson, E.R., 2001. Estrogen and spermatogenesis. *Endocrine Reviews*, *22*(3), pp.289-318.

Ogawa, S., Chan, J., Chester, A.E., Gustafsson, J.Å., Korach, K.S. and Pfaff, D.W., 1999. Survival of reproductive behaviors in estrogen receptor β gene-deficient (βERKO) male and female mice. *Proceedings of the National Academy of Sciences*, *96*(22), pp.12887-12892.

Ogawa, S., Chester, A.E., Hewitt, S.C., Walker, V.R., Gustafsson, J.Å., Smithies, O., Korach, K.S. and Pfaff, D.W., 2000. Abolition of male sexual behaviors in mice lacking estrogen receptors α and β (αβERKO). *Proceedings of the National Academy of Sciences*, *97*(26), pp.14737-14741.

Ogawa, S., Eng, V., Taylor, J., Lubahn, D.B., Korach, K.S. and Pfaff, D.W., 1998. Roles of estrogen receptor-α gene expression in reproduction-related behaviors in female mice. *Endocrinology*, *139*(12), pp.5070-5081.

Ogawa, S., Lubahn, D.B., Korach, K.S. and Pfaff, D.W., 1997. Behavioral effects of estrogen receptor gene disruption in male mice. *Proceedings of the National Academy of Sciences*, *94*(4), pp.1476-1481.

Oh, D.J., Hur, S.P., Bouchekioua, S., Takeuchi, Y., Udagawa, S., Aluru, N., Park, Y.J., Park, J.G., Kim, S.J., Moon, T.W. and Vijayan, M.M., 2018. Tide-related changes in mRNA abundance of aromatases and estrogen receptors in the ovary and brain of the threespot wrasse *Halichoeres trimaculatus*. *Ocean Science Journal*, *53*, pp.239-249.

Ohya, T. and Hayashi, S., 2006. Vasotocin/isotocin-immunoreactive neurons in the medaka fish brain are sexually dimorphic and their numbers decrease after spawning in the female. *Zoological Science*, *23*(1), pp.23-29.

Okubo, K., Takeuchi, A., Chaube, R., Paul-Prasanth, B., Kanda, S., Oka, Y. and Nagahama, Y., 2011. Sex differences in aromatase gene expression in the medaka brain. *Journal of Neuroendocrinology*, *23*(5), pp.412-423.

Oliveira, R.F., Silva, J.F. and Simoes, J.M., 2011. Fighting zebrafish: characterization of aggressive behavior and winner–loser effects. *Zebrafish*, *8*(2), pp.73-81.

Olofsson, L.E., Pierce, A.A. and Xu, A.W., 2009. Functional requirement of AgRP and NPY neurons in ovarian cycle-dependent regulation of food intake. *Proceedings of the National Academy of Sciences*, *106*(37), pp.15932-15937.

Pal, P., Moulik, S.R., Gupta, S., Guha, P., Majumder, S., Kundu, S., Mallick, B., Pramanick, K. and Mukherjee, D., 2018. Estrogen-regulated expression of P450arom genes in the brain and ovaries of adult female Indian climbing perch, *Anabas testudineus*. *Journal of Experimental Zoology Part A: Ecological and Integrative Physiology*, *329*(1), pp.29-42.

Papadopoulos, V. and Miller, W.L., 2012. Role of mitochondria in steroidogenesis. *Best practice & research Clinical Endocrinology & Metabolism*, *26*(6), pp.771-790.

Pasmanik, M. and Callard, G.V., 1988. Changes in brain aromatase and 5α-reductase activities correlate significantly with seasonal reproductive cycles in goldfish (*Carassius auratus*). *Endocrinology*, *122*(4), pp.1349-1356.

Pellegrini, E., Diotel, N., Vaillant-Capitaine, C., Maria, R.P., Gueguen, M.M., Nasri, A., Nicolau, J.C. and Kah, O., 2016. Steroid modulation of neurogenesis: Focus on radial glial cells in zebrafish. *The Journal of Steroid Biochemistry and Molecular Biology*, *160*, pp.27-36.

Pellegrini, E., Menuet, A., Lethimonier, C., Adrio, F., Gueguen, M.M., Tascon, C., Anglade, I., Pakdel, F. and Kah, O., 2005. Relationships between aromatase and estrogen receptors in the brain of teleost fish. *General and Comparative Endocrinology*, *142*(1-2), pp.60-66.

Pierman, S., Sica, M., Allieri, F., Viglietti-Panzica, C., Panzica, G.C. and Bakker, J., 2008. Activational effects of estradiol and dihydrotestosterone on social recognition and the arginine-vasopressin immunoreactive system in male mice lacking a functional aromatase gene. *Hormones and Behavior*, *54*(1), pp.98-106.

Pinto, P.I., Andrade, A.R., Estêvão, M.D., Alvarado, M.V., Felip, A. and Power, D.M., 2018. Duplicated membrane estrogen receptors in the European sea bass (*Dicentrarchus labrax*): Phylogeny, expression and regulation throughout the reproductive cycle. *The Journal of Steroid Biochemistry and Molecular Biology*, *178*, pp.234-242.

Pouso, P., Cabana, Á., Goodson, J.L. and Silva, A., 2019. Preoptic area activation and vasotocin involvement in the reproductive behavior of a weakly pulse-type electric fish, *Brachyhypopomus gauderio*. *Frontiers in Integrative Neuroscience*, *13*, p.37.

Rahaman, M.M., Kumagai, R.I. and Tokumoto, T., 2020. Rapid induction of female-to-male sex change in adult zebrafish by injection of an aromatase inhibitor. *Zebrafish*, *17*(4), pp.261-267.

Ramachandran, B., Schlinger, B.A., Arnold, A.P. and Campagnoni, A.T., 1999. Zebra finch aromatase gene expression is regulated in the brain through an alternate promoter. *Gene*, *240*(1), pp.209-216.

Ramachandran, D., Sharma, K., Saxena, V., Nipu, N., Rajapaksha, D.C. and Mennigen, J.A., 2023. Knock-out of vasotocin reduces reproductive success in female zebrafish, Danio rerio. *Frontiers in Endocrinology*, *14*.

Risma, K.A., Clay, C.M., Nett, T.M., Wagner, T., Yun, J. and Nilson, J.H., 1995. Targeted overexpression of luteinizing hormone in transgenic mice leads to infertility, polycystic ovaries, and ovarian tumors. *Proceedings of the National Academy of Sciences*, *92*(5), pp.1322-1326.

Robertson, K.M., O'Donnell, L., Jones, M.E., Meachem, S.J., Boon, W.C., Fisher, C.R., Graves, K.H., McLachlan, R.I. and Simpson, E.R., 1999. Impairment of spermatogenesis in mice lacking a functional aromatase (*cyp 19*) gene. *Proceedings of the National Academy of Sciences*, *96*(14), pp.7986-7991.

Robertson, K.M., Simpson, E.R., Lacham-Kaplan, O.R.L.Y. and Jones, M.E., 2001. Characterization of the fertility of male aromatase knockout mice. *Journal of Andrology*, *22*(5), pp.825-830.

Saito, K., Cao, X., He, Y. and Xu, Y., 2015. Progress in the molecular understanding of central regulation of body weight by estrogens. *Obesity*, *23*(5), pp.919-926.

Santos, D., Matos, M. and Coimbra, A.M., 2014. Developmental toxicity of endocrine disruptors in early life stages of zebrafish, a genetic and embryogenesis study. *Neurotoxicology and Teratology*, *46*, pp.18-25.

Saravanan, M., Nam, S.E., Eom, H.J., Lee, D.H. and Rhee, J.S., 2019. Long-term exposure to waterborne nonylphenol alters reproductive physiological parameters in economically important marine fish. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, *216*, pp.10-18.

Sawyer, S.J., Gerstner, K.A. and Callard, G.V., 2006. Real-time PCR analysis of cytochrome P450 aromatase expression in zebrafish: gene specific tissue distribution,

sex differences, developmental programming, and estrogen regulation. *General and Comparative Endocrinology*, *147*(2), pp.108-117.

Schomberg, D.W., Couse, J.F., Mukherjee, A., Lubahn, D.B., Sar, M., Mayo, K.E. and Korach, K.S., 1999. Targeted disruption of the estrogen receptor-α gene in female mice: characterization of ovarian responses and phenotype in the adult. *Endocrinology*, *140*(6), pp.2733-2744.

Scully, K.M., Gleiberman, A.S., Lindzey, J., Lubahn, D.B., Korach, K.S. and Rosenfeld, M.G., 1997. Role of estrogen receptor-α in the anterior pituitary gland. *Molecular Endocrinology*, *11*(6), pp.674-681.

Shahjahan, M., Kitahashi, T. and Parhar, I.S., 2014. Central pathways integrating metabolism and reproduction in teleosts. *Frontiers in Endocrinology*, *5*, pp.36.

Shapiro, R.A., Xu, C. and Dorsa, D.M., 2000. Differential transcriptional regulation of rat vasopressin gene expression by estrogen receptor α and β. *Endocrinology*, *141*(11), pp.4056-4064.

Shaw, K., 2018. Aromatase expression and function in the brain and behavior: A comparison across communication systems in teleosts. *Journal of Chemical Neuroanatomy*, *94*, pp.139-153.

Shaw, K., Therrien, M., Lu, C., Liu, X. and Trudeau, V.L., 2023. Mutation of brain aromatase disrupts spawning behavior and reproductive health in female zebrafish. *Frontiers in Endocrinology*, *14*, p.1225199.

Shu, T., Zhai, G., Pradhan, A., Olsson, P.E. and Yin, Z., 2020. Zebrafish cyp17a1 knockout reveals that androgen-mediated signaling is important for male brain sex differentiation. *General and Comparative Endocrinology*, *295*, p.113490.

Sica, M., Martini, M., Viglietti-Panzica, C. and Panzica, G., 2009. Estrous cycle influences the expression of neuronal nitric oxide synthase in the hypothalamus and limbic system of female mice. *BMC Neuroscience*, *10*, pp.1-14.

Simoncini, T. and Genazzani, A.R., 2003. Non-genomic actions of sex steroid hormones. *European Journal of Endocrinology*, *148*(3), pp.281-292.

Simpson, E.R., 2003. Sources of estrogen and their importance. *The Journal of Steroid Biochemistry and Molecular Biology*, *86*(3-5), pp.225-230.

Simpson, E.R., Mahendroo, M.S., Means, G.D., Kilgore, M.W., Hinshelwood, M.M., Graham-Lorence, S., Amarneh, B., Ito, Y., Fisher, C.R., Michael, M.D. and Mendelson, C.R., 1994. Aromatase cytochrome P450, the enzyme responsible for estrogen biosynthesis. *Endocrine Reviews*, *15*(3), pp.342-355.

Simpson, E.R., Michael, M.D., Agarwal, V.R., Hinshelwood, M.M., Bulun, S.E. and Zhao, Y., 1997. Expression of the CYP19 (aromatase) gene: an unusual case of alternative promoter usage. *The FASEB journal*, *11*(1), pp.29-36.

Singh, V. and Joy, K.P., 2008. Immunocytochemical localization, HPLC characterization, and seasonal dynamics of vasotocin in the brain, blood plasma and gonads of the catfish *Heteropneustes fossilis*. *General and Comparative Endocrinology*, *159*(2-3), pp.214-225.

Singh, V. and Joy, K.P., 2009. Effects of hCG and ovarian steroid hormones on vasotocin levels in the female catfish *Heteropneustes fossilis*. *General and Comparative Endocrinology*, *162*(2), pp.172-178.

Sisneros, J.A., Forlano, P.M., Deitcher, D.L. and Bass, A.H., 2004. Steroid-dependent auditory plasticity leads to adaptive coupling of sender and receiver. *Science*, *305*(5682), pp.404-407.

Slominski, A.T., Li, W., Kim, T.K., Semak, I., Wang, J., Zjawiony, J.K. and Tuckey, R.C., 2015. Novel activities of CYP11A1 and their potential physiological significance. *The Journal of Steroid Biochemistry and Molecular Biology*, *151*, pp.25-37.

Sokołowska, E., Kleszczyńska, A., Nietrzeba, M. and Kulczykowska, E., 2015. Annual changes in brain concentration of arginine vasotocin and isotocin correspond with phases of reproductive cycle in round goby, *Neogobius melanostomus*. *Chronobiology International*, *32*(7), pp.917-924.

Sun, L., Zha, J., Spear, P.A. and Wang, Z., 2007. Toxicity of the aromatase inhibitor letrozole to Japanese medaka (*Oryzias latipes*) eggs, larvae and breeding adults. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, *145*(4), pp.533-541.

Takatsu, K., Miyaoku, K., Roy, S.R., Murono, Y., Sago, T., Itagaki, H., Nakamura, M. and Tokumoto, T., 2013. Induction of female-to-male sex change in adult zebrafish by aromatase inhibitor treatment. *Scientific Reports*, *3*(1), pp.1-7.

Tang, H., Chen, Y., Liu, Y., Yin, Y., Li, G., Guo, Y., Liu, X. and Lin, H., 2017. New insights into the role of estrogens in male fertility based on findings in aromatase-deficient zebrafish. *Endocrinology*, *158*(9), pp.3042-3054.

Thomas, P., 2012. Rapid steroid hormone actions initiated at the cell surface and the receptors that mediate them with an emphasis on recent progress in fish models. *General and Comparative Endocrinology*, *175*(3), pp.367-383.

Tohyama, S., Ogino, Y., Lange, A., Myosho, T., Kobayashi, T., Hirano, Y., Yamada, G., Sato, T., Tatarazako, N., Tyler, C.R. and Iguchi, T., 2017. Establishment of estrogen receptor 1 (ESR1)-knockout medaka: ESR 1 is dispensable for sexual development and reproduction in medaka, *Oryzias latipes*. *Development, Growth & Differentiation*, *59*(6), pp.552-561.

Tong, S.K., Mouriec, K., Kuo, M.W., Pellegrini, E., Gueguen, M.M., Brion, F., Kah, O. and Chung, B.C., 2009. A cyp19a1b-gfp (aromatase B) transgenic zebrafish line that expresses GFP in radial glial cells. *Genesis*, *47*(2), pp.67-73.

Trainor, B.C., Kyomen, H.H. and Marler, C.A., 2006. Estrogenic encounters: how interactions between aromatase and the environment modulate aggression. *Frontiers in Neuroendocrinology*, *27*(2), pp.170-179.

Trudeau, V.L., 2018. Facing the challenges of neuropeptide gene knockouts: why do they not inhibit reproduction in adult teleost fish?. *Frontiers in Neuroscience*, *12*, p.302.

Trudeau, V.L. and Somoza, G.M., 2020. Multimodal hypothalamo-hypophysial communication in the vertebrates. *General and Comparative Endocrinology*, *293*, p.113475.

Ulhaq, Z.S. and Kishida, M., 2018. Brain aromatase modulates serotonergic neuron by regulating serotonin levels in zebrafish embryos and larvae. *Frontiers in Endocrinology*, *9*, p.230.

Wacker, D.W. and Ludwig, M., 2012. Vasopressin, oxytocin, and social odor recognition. *Hormones and Behavior*, *61*(3), pp.259-265.

Wang, H., Ward, A.R. and Morris, J.F., 1995. Oestradiol acutely stimulates exocytosis of oxytocin and vasopressin from dendrites and somata of hypothalamic magnocellular neurons. *Neuroscience*, *68*(4), pp.1179-1188.

Weihua, Z., Saji, S., Mäkinen, S., Cheng, G., Jensen, E. V., Warner, M., and Gustafsson, J. A. (2000). Estrogen receptor (ER) beta, a modulator of ERalpha in the uterus. *Proceedings of the National Academy of Sciences of the United States of America*, *97*(11), pp.5936–5941.

Wersinger, S.R., Sannen, K., Villalba, C., Lubahn, D.B., Rissman, E.F. and De Vries, G.J., 1997. Masculine sexual behavior is disrupted in male and female mice lacking a functional estrogen receptor α gene. *Hormones and Behavior*, *32*(3), pp.176-183.

Wojnarowski, K., Cholewińska, P., Palić, D., Bednarska, M., Jarosz, M. and Wiśniewska, I., 2022. Estrogen Receptors Mediated Negative Effects of Estrogens and Xenoestrogens in Teleost Fishes. *International Journal of Molecular Sciences*, *23*(5), p.2605.

Xie, Y. and Dorsky, R.I., 2017. Development of the hypothalamus: conservation, modification and innovation. *Development*, *144*(9), pp.1588-1599.

Xu, Y. and López, M., 2018. Central regulation of energy metabolism by estrogens. *Molecular Metabolism*, *15*, pp.104-115.

Xu, Y., Nedungadi, T.P., Zhu, L., Sobhani, N., Irani, B.G., Davis, K.E., Zhang, X., Zou, F., Gent, L.M., Hahner, L.D. and Khan, S.A., 2011. Distinct hypothalamic neurons mediate estrogenic effects on energy homeostasis and reproduction. *Cell Metabolism*, *14*(4), pp.453-465.

Yamaguchi, A., Tsunematsu, T., Motojima, Y., Toriyama, K., Horinouchi, A., Ishii, Y., Murata, H., Yoshikawa, S., Nyuji, M. and Shimizu, A., 2022. Pituitary luteinizing hormone synthesis starts in aromatase (cyp19a1b)-positive cells expressing esr1 and esr2b at the onset of puberty in *Takifugu rubripes* (fugu). *Cell and Tissue Research*, *389*(2), pp.259-287.

Yilmaz, M.B., Wolfe, A., Cheng, Y.H., Glidewell-Kenney, C., Jameson, J.L. and Bulun, S.E., 2009. Aromatase promoter If is regulated by estrogen receptor alpha (ESR1) in mouse hypothalamic neuronal cell lines. *Biology of Reproduction*, *81*(5), pp.956-965.

Yin, L., Hashikawa, K., Hashikawa, Y., Osakada, T., Lischinsky, J.E., Diaz, V. and Lin, D., 2022. VMHvllCckar cells dynamically control female sexual behaviors over the reproductive cycle. *Neuron*, *110*(18), pp.3000-3017.

Yin, Y., Tang, H., Liu, Y., Chen, Y., Li, G., Liu, X. and Lin, H., 2017. Targeted disruption of aromatase reveals dual functions of cyp19a1a during sex differentiation in zebrafish. *Endocrinology*, *158*(9), pp.3030-3041.

Yokoi, S., Naruse, K., Kamei, Y., Ansai, S., Kinoshita, M., Mito, M., Iwasaki, S., Inoue, S., Okuyama, T., Nakagawa, S. and Young, L.J., 2020. Sexually dimorphic role of

oxytocin in medaka mate choice. *Proceedings of the National Academy of Sciences*, *117*(9), pp.4802-4808.

Yong, L., Thet, Z. and Zhu, Y., 2017. Genetic editing of the androgen receptor contributes to impaired male courtship behavior in zebrafish. *Journal of Experimental Biology*, *220*(17), pp.3017-3021.

Zhang, D., Xiong, H., Mennigen, J.A., Popesku, J.T., Marlatt, V.L., Martyniuk, C.J., Crump, K., Cossins, A.R., Xia, X. and Trudeau, V.L., 2009. Defining global neuroendocrine gene expression patterns associated with reproductive seasonality in fish. *PloS One*, *4*(6), p.e5816.

Zhang, S., Zhang, Y., Chen, W., Wu, Y., Ge, W., Zhang, L. and Zhang, W., 2014. Aromatase (Cyp19a1b) in the pituitary is dynamically involved in the upregulation of lhb but not fshb in the vitellogenic female ricefield eel *Monopterus albus*. *Endocrinology*, *155*(11), pp.4531-4541.

Zhang, Y., Zhang, S., Lu, H., Zhang, L. and Zhang, W., 2014. Genes encoding aromatases in teleosts: evolution and expression regulation. *General and Comparative Endocrinology*, *205*, pp.151-158.

Zhang, Z., Zhu, B. and Ge, W., 2015. Genetic analysis of zebrafish gonadotropin (FSH and LH) functions by TALEN-mediated gene disruption. *Molecular Endocrinology*, *29*(1), pp.76-98.

Zhu, B.T. and Conney, A.H., 1998. Functional role of estrogen metabolism in target cells: review and perspectives. *Carcinogenesis*, *19*(1), pp.1-27.

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Reproductive roles of the vasopressin/oxytocin neuropeptide family in teleost fishes

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The vertebrate nonapeptide families arginine vasopressin (AVP) and oxytocin (OXT) are considered to have evolved from a single vasopressin-like peptide present in invertebrates and termed arginine vasotocin in early vertebrate evolution. Unprecedented genome sequence availability has more recently allowed new insight into the evolution of nonapeptides and especially their receptor families in the context of whole genome duplications. In bony fish, nonapeptide homologues of AVP termed arginine vasotocin (Avp) and an OXT family peptide (Oxt) originally termed isotocin have been characterized. While reproductive roles of both nonapeptide families have historically been studied in several vertebrates, their roles in teleost reproduction remain much less understood. Taking advantage of novel genome resources and associated technological advances such as genetic modifications in fish models, we here critically review the current state of knowledge regarding the roles of nonapeptide systems in teleost reproduction. We further discuss sources of plasticity of the conserved nonapeptide systems in the context of diverse reproductive phenotypes observed in teleost fishes. Given the dual roles of preoptic area (POA) synthesized Avp and Oxt as neuromodulators and endocrine/paracrine factors, we focus on known roles of both peptides on reproductive behaviour and the regulation of the hypothalamic-pituitary-gonadal axis. Emphasis is placed on the identification of a gonadal nonapeptide system that plays critical roles in both steroidogenesis and gamete maturation. We conclude by highlighting key research gaps including a call for translational studies linking new mechanistic understanding of nonapeptide regulated physiology in the context of aquaculture, conservation biology and ecotoxicology.

KEYWORDS

endocrine system, hormone, hypothalamic–pituitary–gonadal axis, isotocin, neuromodulator, courtship behaviour, paracrine, vasotocin

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1 Introduction

Members of the arginine vasopressin (AVP)/oxytocin (OXT) molecular family and their receptors are phylogenetically ancient, originating in a common ancestor evolved from a common ancestor of the Bilateria (1). As conserved cyclic nonapeptides, they exert pleiotropic functions in reproduction, sociosexual behavior, energy balance, osmoregulation, and the cardiovascular system, among others. In this review we take a focussed approach and examine their importance in the reproductive physiology of teleost fishes.

1.1 The teleost nonapeptide system

1.1.1 Nonapeptide structure and evolution

In basal cephalochordates, such as the Florida lancelet, *Branchiostoma floridae*, and urochordates such as the sea vase, *Ciona intestinalis*, single nonapeptides, [Ile^4]-VP and Ciona (ciVP) have been reported (2–4). The presence of a single AVP family peptide extends to basal vertebrate agnathans, including lampreys such as the Japanese lamprey, *Lethenteron japonicum*, and hagfishes such as *Eptatretus burgeri* (5, 6). It is believed that two rounds of whole genome duplication (2R WGD), one before and one after the separation of agnathans and gnathostomes (7), led to a duplication of the ancestral arginine vasotocin (*avp)* gene. This duplication gave rise to two nonapeptide families conserved in vertebrates; the AVP and OXT family peptides (8). In vertebrates, both genes code for precursor proteins which include a 5' signal sequence, a highly conserved nonapeptide and neurophysin, and, in the case of the AVP family, a C-terminal glycoprotein termed copeptin without known biological function (9). Processing into the mature nonapeptides occurs *via* prohormone convertases, and the acidic neurophysins I (OXT family) and neurophysin II (AVP family) associate with mature nonapeptides acting as carrier molecules within the neurosecretory system (9).

In teleost fishes, vasotocin (Avp) was originally isolated and characterized in pout, *Gadus luscus* (10), pollock, *Polacchius virens* (11), and European hake, *Mercluccius mercluccius* (12), and has since been identified in genomes of all teleost fishes studied to date (3, 6). Avp occurs in all verebrates except for mammals, where it appears to have mutated to give rise to vasopressin (AVP) in which ^{3}Ile was substituted for ^{3}Phe (3, 5, 6). All AVP family members are basic nonapeptides due to a basic amino acid (AA) at position 8.

In contrast to the AVP family peptides, the duplicated nonapeptide gene appears to have accumulated more mutations giving rise to the OXT family (3, 5, 6). The OXT nonapeptides are characterized by a neutral AA at position 8 (Leu, Ile, Gln or Val). In fishes alone, as many as 12 Oxt family peptides are known and likely arose due to lineage-specific duplications followed by substitutions in AA positions 3, 4 and 5 in the ring structure and position 8 in the tail structure (Table 1). With reference to mammalian OXT, the substitutions are Tyr^2 by Phe^2, Ile^3 by Phe^3, Gln^4 by Ser^4 or Asp^4, and Leu^8 by Ile^8 or Val^8. In teleost fishes, a single OXT family peptide (Oxt; [Ser^4-Ile^8]-OXT) was first isolated in pout, pollock, and the European hake and originally termed isotocin due to the presence of Ile in position 8 (18).

While position 8 mutations are very common in cartilaginous fishes (19) specific Oxt family peptides have also been reported in Sarcopterygii, such as the Australian lungfish, *Neoceratodus forsteri*, which expresses [Phe^2-Ile^3] OXT (20). Based on more recent genomic data, it has become evident that non-teleost Actinopterygians also express specific Oxt peptides (6). Indeed, in the spotted gar, *Lepisosteus oculatus*, which possess a pre-3R genome, two gene paralogues coding for Oxt were identified - one coding for a pro-[Ser^4-Ile^8]-OXT with a long C-terminal (NCBI Accession No. XM_006626499.1) and the other coding for a novel pro-[Phe^2, Ser^4]-OXT, which has a short C-terminal like other vertebrate neutral OXT family peptide precursors (NCBI Accession no. XM_006626523.1). An analysis of tissue expression profiles based on a fish RNA-seq expression database (21) does not reveal clear-cut differential expression of both *oxt* paralogues in spotted gar (Figure 1A).

In teleosts, characterized by a 3R genome condition, occurrence of two copies each of pro-*avp* (pro-a*vp*1 and pro-*avp2*) and pro-*oxt* (pro-*oxt*1 and pro-*oxt*2) have been reported in the blind cave fish, *Astyanax mexicanus*, based on genomic information and in salmonids and catastomids based on cloning studies (6, 22–24). In the blind cave fish, a diploid fish, synteny analysis suggests that the gene duplications may be due to 3R without subsequent gene loci losses (6). In salmonids and catostomids, the multiple *oxt* copies may be due to tetraploidization (4R WGD) and/or gene conversion (22–24). Regarding tissue expression profiles of paralogous *oxt* genes in salmonids, analysis of RNA-seq data (21) suggests similar expression profiles, at least in, *rainbow trout, Oncorhynchus mykiss* (Figure 1B), and brook trout, *Salvelinus fontinalis* (Figure 1C).

More recently however, the paradigm that teleost fishes, while encoding multiple gene loci for neutral nonapeptides in some cases, nevertheless exclusively express Oxt ([Ser^4-Ile^8]-OXT) was challenged by the cloning and discovery of two different neutral Oxt family peptides in the Asian stinging catfish, *Heteropneustes fossilis*, and the walking catfish, *Clarias batrachus* (6). In both species, a conventional *oxt* gene coding for a [Ser^4-Ile^8]-OXT and a second *oxt* gene coding for the novel [Ser^4-Val^8]-OXT, coined sevatocin, are expressed in addition to a single *avt* gene (6). Like pro-[Ser^4-Ile^8]-OXT, the peptide precursor encoding for [Ser^4-Val^8]-OXT has a similar organization (Table 2), with an extended C-terminal and a Leu-rich region. The functional implications of the *oxt* genes in these catfish species deserve special mention. The substitutions in the hormone moiety (Ile^8/Val^8) may lead to

TABLE 1 Oxt nonapeptide family orthologues in different fish groups.

Species/Groups	Historical name	AA sequence	Reference
Holocephali: *Callorhinchus milli* *Hydrolagus colliei*	Oxytocin	CYISNCPQG	(3)
Skates: *Raja miraletus*	Glumitocin	CYISNCPQG	(8)
Sharks: *Squalus acanthias*	Aspargtocin Valitocin	CYINNCPLG CYIQNCPVG	(13)
Scyliorhinus caniculus	Asavatocin Phasavatocin	CYINNCPVG CYFNNCPVG	(14)
Triakis scyllium	Asavatocin Phasitocin	CYINNCPVG CYFNNCPIG	(15)
Rays: *Torpedo marmorata*	Isotocin b	CYISNCPIG	(16)
Spotted gar: *Lepisosteus oculatus*	[Phe^2, Ser^4]-Oxytocin (a variant of ITb) Isotocin b	CFISNCPIG CYISNCPIG	Genomic sources (6)
Most teleost fishes	Isotocin b	CYISNCPIG	(8)
Catfishes: *Heteropneustes fossilis, Clarias batrachus*	Isotocin a (Sevatocin) Isotocin b	CYISNCPVG CYISNCPIG	(6)
Some lungfish, Coelacanth, *Latimeria chalumnae*	Mesotocin	CYIQNCPIG	(17)
Australian lungfish, *Neoceratodus forsteri*	[Phe^2]-Mesotocin	CFIQNCPG	(8)
Human *Homo sapiens*	Oxytocin	CYIQNCPLG	

The AA sequences of the mature hormones are given. Coloured and underlined AA are modifications from the mammalian OXT AA sequence.

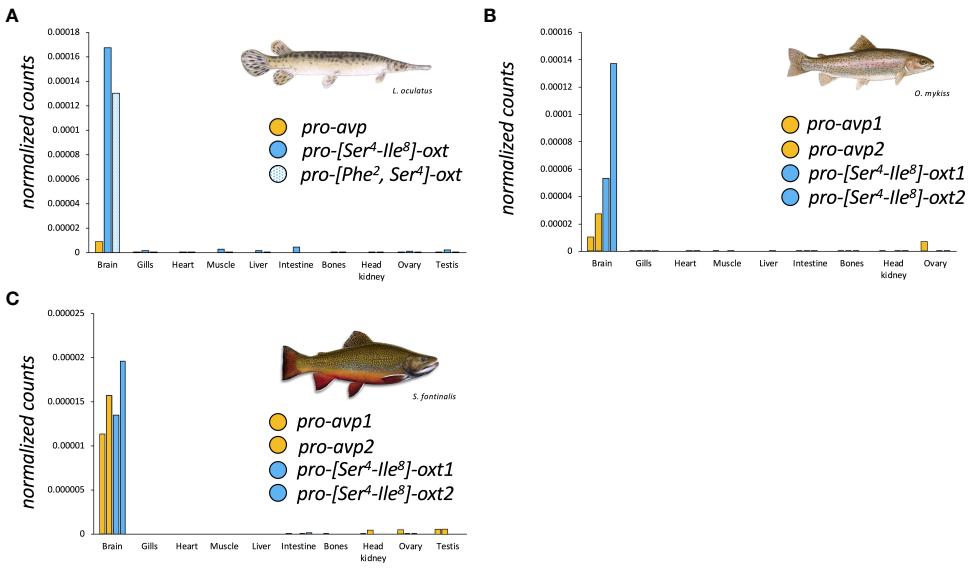

FIGURE 1
Tissue-specific expression profiles of nonapeptide precursor coding genes in spotted gar **(A)**, rainbow trout, *Oncorhynchus mykiss* **(B)**, and brook trout, *Salvelinus fontinalis* **(C)** derived from the Phylofish RNA-seq database (www.phylofish.signae.org) queried on June 1st, 2022 (20).

TABLE 2 General features of the cDNAs of an encoded precursor proteins of Oxta, Oxtb and Avp in the catfish Heteropneustes fossilis.

Nonapeptide	cDNA (bp)	Coding sequence (bp)	Precursor protein (AAs)	Signal peptide (AAs)	Neurophysin peptide (NP)(AAs)	Cys in NP (AA)	Leucine-rich core
Oxta	619	1-462	153	19	122	14	LLRKLLHL
Oxtb	708	56-508	151	29	118	14	LLKLLHL
Avp	618	60-524	155	20	122	14	LLLRILH

The mature nonapeptide hormone moiety (9 AA), cleavage site (GKR), and the domain between signal peptide and neurophysin are not indicated.

altered receptor-ligand interaction with possible sub- or neo-functionalization. Both *oxt*-like genes are similarly expressed in the preoptic area (POA), and functional studies show that both synthetic [Ser^4-Ile^8]-OXT and [Ser^4-Val^8]-OXT similarly regulate *fshb, lhb* and *gpa* expression in the catfish pituitary (6, 24). It is possible that both Oxt peptides bind to the same receptors, but receptor characterization has not yet been undertaken.

For the purpose of this review, we follow the recent Zebrafish Information Network (ZFIN, www.zfin.org) nomenclature, which uses a*vp*/Avp and *oxt*/Oxt to designate teleost genes and their protein products. The reasoning for this recent change in the literature is to highlight the homologous nature of *avp* and *oxt* family genes and their products in vertebrates. Consequently, this nomenclature no longer uses the historical distinction between teleost and mammalian nonapeptides (arginine vasotocin/arginine vasopressin and isotocin/oxytocin) which is reflective of their AA composition. Thus, while the historically widely used nomenclature is not used in this review, it is implicitly understood that the teleost *avp*/Avp and *oxt*/Oxt differ from mammalian and other vertebrate nonapeptide homologues in their AA residues as described.

1.1.2 Anatomy of the nonapeptide system in the context of reproduction

The distribution of Avp and Oxt has been examined in many teleosts and will not be covered in detail here. Controversies arise largely because of differing sensitivities of the neuroanatomical methods used, variable control experiments and other technical challenges (25, 26). Nevertheless, the use of transgenic approaches is helping to firmly establish the key locations of neuronal soma, and a new appreciation for their wide projection fields (27–30). In teleosts, Avp and Oxt neurons are intermingled and have been classified into three populations in the POA based on soma size. These are the giganto-, magno- and parvocellular neurons. Preoptic Avp and Oxt neurons send their fibers into diverse regions of the brain such as the hypothalamus, ventral telencephalon, mesencephalon and diencephalon, as well as the hindbrain and the spinal cord (20–30, amongst many others). Through the latter two systems, a role for POA-derived nonapeptides in modulating motor output related to reproductive behaviour and gamete release has been postulated in some, but not all teleost species (31–33).

In fish species in which specific sensory modalities have been shown to play a key role in reproductive behaviours, nonapeptide innervation has been described for distinct brain regions involved in sending and receiving sensory information. For example, in male zebrafish, *Danio rerio*, known to respond to female sex pheromones (34), fibers positive for Oxt have been identified in the olfactory bulb (35). In the plainfin midshipman, *Porichtys notatus*, which relies on vocalization as part of their courtship behaviour (36), especially Avp but also Oxt innervation was found in fore- and mid-brain regions involved in vocalization, and diencephalic regions of the ascending auditory pathway (37, 38). In the weakly electric gymnotiform bluntnose knifefish, *Brachyhypopomus gauderio*, which uses electric organ discharge (EOD) signals for mate selection (39), Avp innervation in the medulla was found to be in proximity of the pacemaker nucleus that controls EOD (40). Together, neuroanatomical evidence thus suggests a role for nonapeptides in modulating both emitting and receiving pathways of diverse sensory signals linked to reproductive behaviours in various teleost fishes.

In all teleost species studied to date, prominent preoptic and ventral hypothalamic projections terminating in the posterior pituitary for release to the pituitary vasculature have been reported (41, 42). In some species, such as the dwarf gourami, *Colisa lalia*, a colocalization of parvocellular Oxt with gonadotropin releasing hormone (Gnrh) has been reported (43). In the goldfish, *Carassius auratus*, Oxt colocalizes with secretoneurin a (44), an important hypophysiotropic stimulator of the hypothalamo-pituitary-gonadal (HPG) axis (45, 46). Such neuroanatomical data suggest that nonapeptides may regulate the HPG axis by co-release with other neuropeptides.

Detailed neuroanatomical studies of the pituitary in the sailfin molly, *Poecilia lattipinna*, the European bass, *Dicentrarchus labrax*, and the African sharptooth catfish, *Clarias gariepinus*, revealed that nonapeptidergic innervation principally forms contact with pituitary vasculature in the form of terminal release buttons which highlights an endocrine role of the nonapeptides (41, 42, 47, 48). Central and circulating Avp and Oxt concentrations in the nM range have been reported to be sex-specific, correlated, and reproductive stage-dependent in at least some species, such as the round goby, *Neogobius melanostomus* (49), and the air-breathing catfish, *Heteropneustes fossilis* (50). In addition to pituitary release,

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nonapeptide fibers innervating gonadotrophs in the proximal pars distalis through gaps in the basement lamina have been demonstrated in the sailfin molly, *Poecilia latipinna* (41, 48). While there are far fewer contact sites of nonapeptide innervation of gonadotrophs compared to pituitary blood vessels, this evidence does nevertheless suggest that the neuronal organization of nonapeptide fibers in the pituitary also provides a basis for paracrine effects on gonadotrophs. Whether a single nonapeptide neuron originating from the teleost POA can be both encephalotropic and hypophysiotropic, and thus simultaneously regulate brain function as a neuromodulator and peripheral function as a paracrine factor or hormone, remains an open question. Evidence to-date suggests that this may be species-dependent. While single nonapeptide neurons originating in the POA have been shown to extend to both extrahypothalamic brain regions and the pituitary in Atlantic salmon, *Salmo salar* (51), clearly distinct Avp and Oxt neurons originating in the POA have been shown to innervate either extrahypothalamic brain regions or the pituitary, but not both, in zebrafish, *Danio rerio* (28, 29).

Transcript and/or protein abundance of nonapeptides in female and, to a lesser extent, male gonads has also been reported in several species. In rainbow trout, *Onchorhynchus mykiss*, ovarian expression of both *avp* and *oxt* has been reported (52). High *oxt* expression is also noted for whole zebrafish ovaries (53). In the air-breathing catfish, high-performance liquid chromatography (HPLC) analysis indicated the presence of Avp in ovaries and, albeit to a much lower extent, in testes (50). Immunohistochemistry approaches localized Avp to the ovarian follicular cell layer, with positive staining in both theca and granulosa cells, but failed to locate Avp in testes (50). Conversely, Avp has been located to interstitial cells in the testes of the chanchita, *Cichlasoma dimerus* (54). The expression of gonadal nonapeptide systems, and especially *avp*, appears to be a more widespread feature in teleosts, as suggested by RNA-seq data mined from the Phylofish database (20) and presented here (Figures 1A-C). Together, evidence of expression of a gonadal nonapeptide system in female and male gonads provides the anatomical basis for an additional reproductive role of nonapeptides thorough paracrine modulation of processes such as gametogenesis, steroidogenesis, and gamete release.

1.1.3 The teleost nonapeptide receptor repertoire

Nonapeptide systems can regulate reproductive physiology *via* central neuromodulatory and/or peripheral endocrine and/or paracrine pathways *via* G-protein coupled membrane-bound AVP and OXT peptide family receptors (AVPRs and OXTRs). Since newly available genome sequences have recently allowed for a comprehensive description of the vertebrate nonapeptide receptor repertoires and their evolution resulting in proposed nomenclature changes (55–59), we here review the repertoire of teleost receptors considering these findings in detail. For the purpose of this review, we once again follow the ZFIN nomenclature, which is largely reflective of these changes. This focused review provides the basis to critically review relevant information regarding specific teleost Avpr and Oxtr function in mediating central and peripheral nonapeptide effects on teleost reproduction.

The teleost nonapeptide receptor repertoire consists of five distinct gene family members, although not all are present in every teleost species. These receptors include *avpr1a, avpr2, avprl* as well as *oxtr* and their paralogues. A fifth vertebrate nonapeptide receptor family member, *avpr1b* is present in basal ray-finned fishes as well as tetrapods but is not present in elasmobranchs and teleost fishes (55–59) (Figures 2A-C). With regard to intracellular signaling mechanisms, Avprs and Oxtrs use diacylglycerol (DAG), inositol triphosphate (IP_3) and calcium (Ca^{2+}) as second-messengers with the exception of Avpr2l, which uses cyclic adenosine monophosphate (cAMP) (60). Avpr2b signaling has not directly been assessed in teleost fishes but may be similar to Avpr2aa, based on intracellular domain AA sequence similarities (57). For *avpr1*, all teleost genomes analyzed to date possess two paralogues, termed *avpr1aa* and *avpr1ab* (Figure 2A). Additionally, all teleost fishes appear to possess *avpr2aa* and *avpr2ab* paralogues (Figure 2B). The presence and retention of these paralogues is in line with the teleost-specific genome duplication, as single *avpr1a* and *avpr2a* genes exist in early non-teleost fishes (Figures 2A, B). Conversely, the situation appears to be more complex with regard to *avpr2b* and *avpr2l* genes which are retained in different paralogue numbers and lost in some but not all teleost fishes in no obvious evolutionary pattern (Figures 2A, B). The *oxtra* and *oxtrb* paralogues are found in all teleosts assessed to date, again indicative of a retention of paralogues following genome duplication at the base of teleost evolution (Figure 2C). Teleost-specific changes in the nonapeptide receptor repertoires have been reported, especially regarding possible intracellular AA sequences involved in cell signalling. For example, the teleost Avpr2aa AA sequence harbours extensions of the intracellular loop 3 region, which include relatively well-conserved Tyr, Ser and Thr residues which can be phosphorylated by a variety of intracellular kinases to alter intracellular signaling cascades as well as G-protein coupled receptor attenuation/desensitization, endocytosis, and intracellular trafficking (59). Lineage-specific changes in the nonapeptide repertoires have also been reported even within the infraclass of teleost fishes. For example, otocephalans lack an *avpr2bb* paralogue found in early teleosts as well as euteleosts (59), as shown in Figure 2B. In our current microsynteny analysis, we found additional otocephalan-specific changes for the *oxtrb* nonapeptide receptor locus (Figure 3A). These microsynteny changes translate into C-terminal AA sequence changes in an intracellular domain which, based on annotation from functional mutation studies in the human OXTR (61), is

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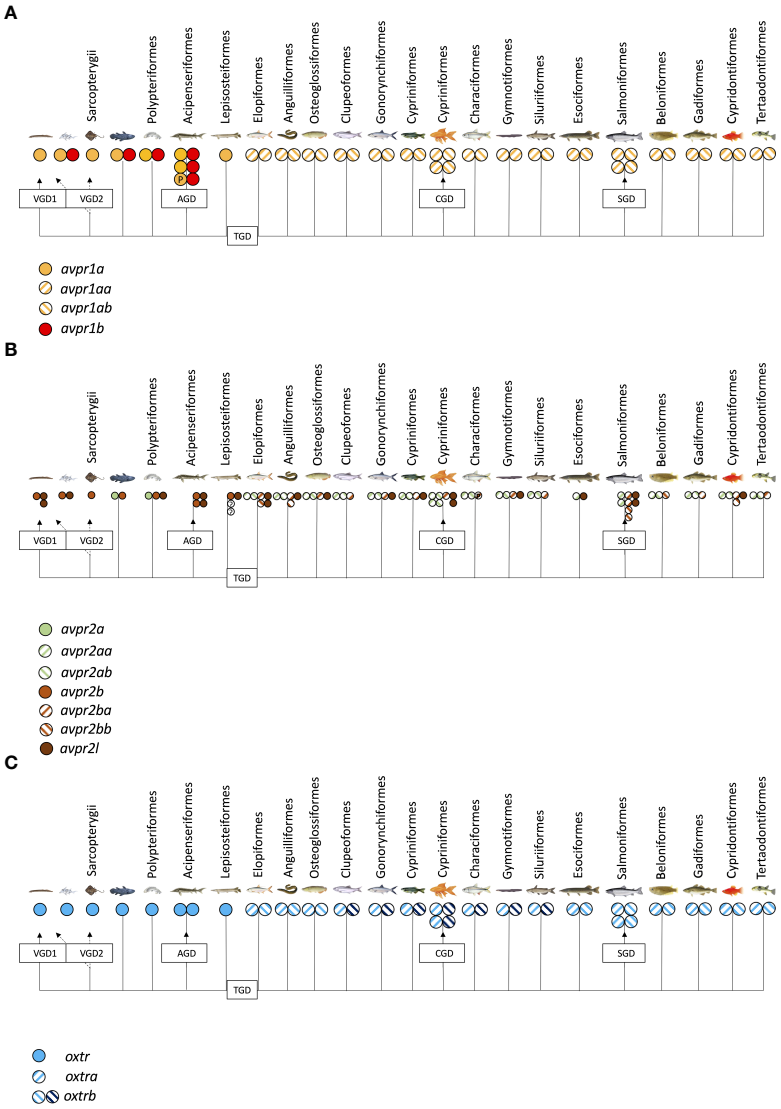

FIGURE 2
Presence of *avpr1* **(A)**, *avpr2/avpr2l* **(B)** and *oxtr* **(C)** genes in published fish genomes accessed on NCBI (www.pubmed.com) on June 1st, 2022.

linked to G_q-protein recruitment and cell signaling (Figure 3B). Otocephala, which diverged from Euteleostei during the Jurassic ~220 million years ago, are characterized by innovations in communication and sensing, notably through Schreckstoff, the alarm substance released through skin injury from conspecifics, and auditory capacity through a link between the swim bladder and inner ear (otophysic link) (62). It is thus tempting to speculate that such rearrangements, with possible functional implications, represent changes which facilitated sub- or neo-functionalization linked to increased social cues relevant to reproduction in otocephalans. In the case of otocephalan Oxtrb, a loss of highly conserved intracellular domain AA residues was observed (Figure 3B). These are specifically located in the C-terminal sequence and have been shown to be required for $G_{q/11}$ and subsequent IP_3, DAG and Ca^{2+} signaling (61). They also include consecutive Cys residues, which through palmitoylation, have been shown to anchor the C-terminal region to the membrane (61). Finally, it includes consecutive Ser residues believed to be involved in receptor retention for receptors following ligand-dependent internalization (61). Investigation of *oxtra* and *oxtrb* expression using an RNA-seq-based fish tissue database (21) reveal that in the otocephalans Allis shad, *Alosa alosa*, and Mexican cavefish, expression pattern for both *oxtra* and *oxtrb* receptor paralogues are similar in most tissues, apart from the brain where *oxtra* expression exceeded *oxtrb* expression (Figure 3C). In zebrafish ovary, *oxtrb* appears to be more strongly expressed compared to *oxtra* (53), suggesting possible differential roles in regulating ovarian function (53).

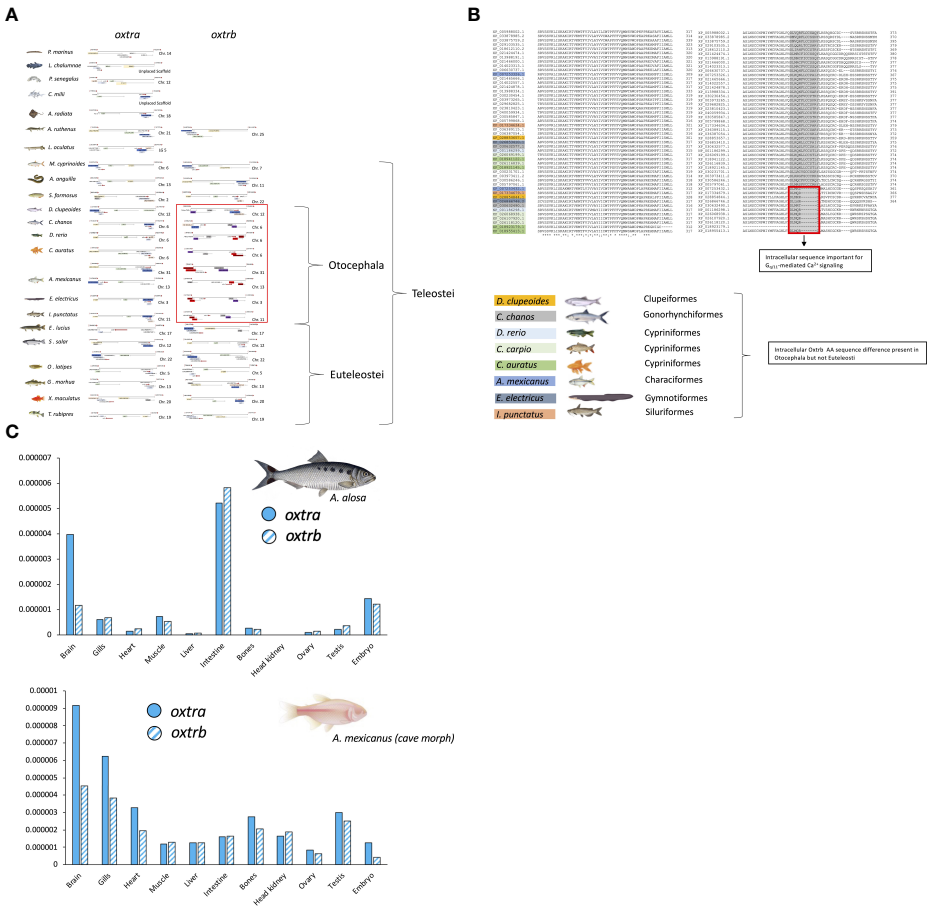

FIGURE 3
Microsynteny analysis **(A)**, predicted AA sequence alignment **(B)** and RNA-seq based tissue expression profiles **(C)** of *oxtra* and *oxtrb* paralogues in select teleost fishes. Micro-synteny analysis of *oxtr* paralogue gene loci was manually conducted on NCBI (www.pubmed.com) derived genome sequences retrieved on June 1st, 2022, while *oxtr* gene paralogue sequence-predicted AA sequences were aligned using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). RNA-seq based tissue expression profiles of *oxtr* paralogues was created derived from the Phylofish RNA-seq database queried on June 1st, 2022 (20).

In addition to *oxtr* paralogues, the expression of other teleost nonapeptide receptors and in some instances specific paralogues have been localized to tissues relevant to teleost reproduction (63–73) and are summarized in Table 3. Much of our current information on reproductive roles of nonapeptides has been derived from studies of otocephalan species including the air-breathing catfish (4, 5, 24, 84), goldfish (85–87), and zebrafish (35). Given that the nonapeptide repertoire is characterized by genomic rearrangements and potentially important AA sequence changes in this clade, caution is warranted when attempting to generalize functionality across all teleosts.

In contrast to the detailed evolutionary history, the functional characterization of nonapeptide receptors remains limited, and in some instances, such as for *avpr2l*, virtually unexplored in teleost fishes. Early studies in a cyprinid, the white sucker, *Catostomus commersonii*, investigated binding kinetics and specificity of both Avpr (88, 89) and Oxtr (90). Based on teleost genome sequences available today, these can retroactively be classified as Avpr1ab and Oxtra, respectively. These studies revealed that the Avpr1ab is highly selective for Avp (EC_{50} 13 nM ± 6 nM) over Oxt. Both nonapeptides bound to and activated cell signaling of a heterologously expressed Oxtra receptor with 3- to 4-fold higher affinity for Oxt (80 ± 30 nM) compared to Avp (300 ± 90 nM). More recent studies in zebrafish assessed specificity of nonapeptides and a mammalian OXT receptor antagonist (L-368,899) in heterologous expression assays (91). It was reported that Oxt exhibits similar affinities for both Oxtr parlogues with EC_{50} values of 2.99 ± 0.93 nM for Oxtra and 3.14 ± 1.10 nM for Oxtrb, respectively. In comparison, Avp exhibited slightly lower affinities to Oxtrs with EC_{50} values of 11.0 ± 3.0 nM for Oxtra and 27.0 ± 9.5 nM for Oxtrb, respectively. Both Avp and Oxt had low affinities for Avpr1aa (EC_{50} 727 ± 338 nM and 317 ± nM, respectively) and high affinities for Avpr1ab (2.79 ± 1.4 nM and

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TABLE 3 Nonapeptide receptor expression in central and peripheral teleost tissues relevant to reproduction.

Tissue	Receptor type	Species	Description	Reference
Brain	*avpr1aa avpr1ab avpr2ab*	Air-breathing catfish *Heteropneustes fossilis*	*avpr1aa* type receptor mRNA is expressed in major neuroendocrine hypothalamic and telencephalic nuclei including the POA and sensorimotor centres; *avpr2ab* type receptor mRNA is largely confined to subependymal telencephalon	(74, 75)
	avpr1aa avpr1ab avpr2aa avpr2ab oxtrb	Pupfish, *Cyprinodon nevadensis amargosae*	*avpr1aa*, *avpr1ab*, *avpr2ab* and *oxtr* mRNA is expressed in telencehalon, hypothalamus and hindbrain	(76)
	avpr1aa; avpr1ab	Zebrafish, *Danio rerio*	mRNAs are expressed in forebrain, midbrain, and hindbrain. *avpr1aa* positive hindbrain neurons are contacted by *avp* neurons originating from POA and lateral longitudinal fasciculus and extending to sensorimotor areas such as the medial longitudinal fasciculus	(77)
	avpr1ab	Atlantic Croaker	mRNA and protein localized to hypothalamic GnRH neurons	(78)
	avpr1ab	Rock hind, *Epinephelus adscensionis*	mRNA is widely distributed in brain areas linked to reproductive and sensorimotor control including hypothalamic GnRH neurons, POA and olfactory bulb	(63, 79)
	avpr1aa; oxtra	*Astatotilapia burtoni*	mRNA and protein expressed in telencephalon and hypothalamus	(80)
Pituitary	*avpr1aa; avpr1ab; avpr2ab*	Air-breathing catfish *Heteropneustes fossilis*	mRNA expressed in male and female rostral pars distalis and pars nervosa	(74, 75)
	oxtra	Rice-field eel *Monopterus albus*	mRNA located to Lh but not Fsh cells	(81)
Gonad	*avpr1aa; avpr1ab; avpr2ab*	Air-breathing catfish *Heteropneustes fossilis*	In testes, *avpr1ab* and *avpr2ab* receptor mRNA are localized to interstitial tissue seminiferous epithelium. In ovaries, *avpr1aa* and *avpr1ab* receptors are localized to the follicular layer and an *avpr2ab* receptor to the oocyte membrane	(74, 75)
	oxtra	Guppy	Ovaries, expressed in follicular layer	(82)
	avpr1aa; avpr1ab	Bluehead wrasse	Ovaries, Testes	(83)

3.52 ± 0.94 nM, respectively). The guppy Oxtra was shown to be activated by both Oxt and Avp (82). While Oxt induced a strong dose-response (10 nM-1 µM) induction of co-transfected luciferase CRE-element, Avp induced a weaker, yet significant response at all concentrations tested (82). Together these data show that at physiological concentrations, it is likely that some degree of cross activation between Avp and Oxt ligands and the nonapeptides receptors occurs. It is therefore important to consider that reproductive roles of Avp and Oxt may, at least in part, be dependent on receptor cross-activation.

2 External and internal reproductive cues regulate teleost central nonapeptide systems

In several seasonal teleost fishes, nonapeptide expression and/or protein abundance in the POA is positively correlated with mature reproductive status (49, 50), which in turn, is dependent on environmental cues such as photoperiod and temperature (92). In female goldfish, hypothalamic *oxt* mRNA abundance peaks in the seasonal breeding period when fish have maximal gonadosomatic index (GSI) and is likely photoregulated (93). In male goldfish, exposure to the female releaser pheromone $PGF_{2\alpha}$, an important olfactory reproductive cue for males, increases *oxt* and *avp* mRNA levels in the telencephalon while stimulating circulating testosterone (T) concentrations and strippable milt volumes (94). Together, such association of nonapeptide expression in the neuroendocrine brain and enhanced pituitary and gonadal activity during seasonal cycles and after pheromone exposure is supportive of a physiological role in reproduction. Exposure to androstenedione, another pheromone known to play an important role in male-male competition in a reproductive context in goldfish (95), increases parvocellular *avp* (96). Thus, it appears that reproductive pheromones represent important environmental cues to regulate male goldfish nonapeptide expression. In male and female round goby, *Neogobius melanostomus*, maximal seasonal brain Avp concentration was observed just before spawning in March-April, whereas that of Oxt peaked during spawning in May-June (97). The lowest brain Avp level was noted in the non-breeding season from November

to January, while the level of Oxt decreased immediately at the end of the spawning. The results show that high Avp levels correlate with pre-spawning period whereas the highest Oxt levels correspond to spawning. In female round gobies, these increases appear, at least in part, to be dependent on estrogens acting *via* genomic (nuclear receptors), and genomic and non-genomic mechanisms in the case of Avp and Oxt, respectively (98). This suggests that season-dependent gonadally-derived positive sex steroid feedback mechanisms may reinforce the seasonal activation of hypothalamic nonapeptide systems. In the half-spotted goby, *Asterropteryx semipunctata*, Avp and Gnrh protein abundances were positively correlated and exhibited significant peaks in their abundances in sexually mature females (99). In male sticklebacks, the highest brain concentrations of Avp were observed in the most aggressive males that cared for eggs and nuptial-colored subordinates that fought to change their social status. Oxt was significantly higher in brains of aggressive dominant males (100). In the stickleback, the highest Avp levels were found in brains of females that did not deposit eggs, regardless of whether they were kept with courting or non-courting males and whether they had a nest or not. The highest Oxt levels were observed in females that did not deposit eggs but were kept with a courting male. The presence of courting or non-courting males that somehow activate Oxt- or/and Avp-producing neurones may be decisive for both behaviour and/or final oocyte maturation or egg deposition, because brain levels of both nonapeptides decreased sharply after egg deposition (101).

In the anadromous chum salmon, *Oncorhynchus keta*, dynamic changes in nonapeptide transcript and protein abundance have been reported during the reproductive migration period (102, 103). Lower transcript but higher immunoreactivity of both Oxt and Avp in the POA of fish collected upstream in a freshwater system compared to those caught in the marine bay, which serves as an entry point of the reproductive migration (102, 103). Based on these results it is tempting to speculate that due to their well-described role in osmoregulation, nonapeptide changes may act to integrate relevant environmental signals, such as the change in salinity, to subsequently change aspects of reproductive physiology in these anadromous fish. It has, however, been shown that salinity changes are not consistently the principal factor mediating changes in the nonapeptide systems of migrating salmonids and temperature and endogenous sex steroid concentrations also act as important regulators of these systems (104). Nevertheless, these data demonstrate a reproductive phase-dependent regulation of the nonapeptide system in chum salmon. Within the context of salmonid reproduction and migration, it is important to note that alternative reproductive tactics exist among individuals. In addition to males migrating to the ocean to grow and mature, some precocious parr achieve sexual maturity quickly before migrating to the ocean and can thus fertilize female eggs as 'sneaker males' (105, 106). Two transcriptomic and targeted gene expression studies investigating differential gene expression in the whole brain of maturing males and precocious parr of Atlantic salmon identified nonapeptides as being differentially regulated (107). The functional relevance of the differential expression with regard to reproduction remains, however, unknown.

In some group-living cichlid species in which HPG axis activity and reproductive behaviour is linked to social dominance, *avp* and Avp abundance has been investigated (108). The results show differential effects on *avp* mRNA levels and Avp neuron size in different POA subpopulations, with higher levels in gigantocellular neurons and, conversely, lower levels in parvocellular neurons of dominant fish. While underlining the responsiveness of the nonapeptide system to social status, a determinant of reproductive status in at least some cichlids, these findings also highlight the importance of considering potentially differential effects on nonapeptide subpopulations in the POA. However, the finding that *avp* expression in the POA of dominant and reproductively active African cichlid fish, *Astatotilapia burtoni*, is significantly reduced compared to subordinates who are reproductively supressed (109), clearly suggests caution is warranted to avoid simplified and global paradigms regarding the role of nonapeptides in species in which social dominance is linked to increased reproductive capacity.

Teleost fish exhibit significant plasticity in terms of their reproductive biology and life history traits. Some species are sequential hermaphrodites, beginning life as one sex, and changing sometime later to the other. Such species are capable of protandrous (male-to-female), protogynous (female-to-male), or serial (bidirectional) sex change (110). Changes in nonapeptide systems have been observed in response to social context-induced sexual plasticity. For example, the bluehead wrasse, *Thalassoma bifasciatum*, exhibits specific increases in Avp immunoreactivity in the magnocellular POA when undergoing behavioural female-to-male sex change that occurs rapidly following the removal of a large terminal colour male and is independent of the gonads (111). The increase in magnocellular *avp* expression coincides with a rapid increase in dominant and male courting behaviour, suggesting a functional link (111, 112). Serial adult sex change in the marine goby, *Trimma okinawae*, is associated with significant and reversible changes in the size of Avp-producing forebrain cells, which are higher in males and coincide with increased male mating behaviour (113). In overcrowded single sex groups of female black mollies, *Poecilia sphenops*, which do not form social hierarchies, masculinization of reproductive behaviour occurs and is linked to a decrease of higher, female-typical Avp concentrations in this species to male typical concentrations (114). In a transcriptomic analysis of the bluehead wrasse forebrain, increased *oxt* transcript abundance was one of the few statistically significant changes detected across female-to-male sex change (115). The opposite trend was observed in the bluebanded goby, *Lythrypnus dalli*, where lower Oxt

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immunoreactivity was observed in the POA of males and late-stage female-to-male sex-changers compared to females (116).

Overall, these studies reveal context-dependent regulation of central nonapeptide systems across several species with diverse reproductive strategies. This strongly implicates nonapeptides in the regulation of teleost reproductive physiology. However, caution is clearly warranted to avoid oversimplified paradigms for specific roles of nonapeptides across all teleost species (117), as at least some species-specific roles are likely to have evolved among teleosts with such varied reproductive strategies.

3 Reproductive function of nonapeptides in teleost fishes

3.1 Nonapeptide-dependent regulation of reproductive behaviour

The plainfin midshipman fish, *Porychthys notatus*, displays sex- and morph-specific vocalization during mating. Oxt and Avp regulate these sex- and morph-specific effects on the vocal circuitry (37). Type I males mating call are stimulated by Avp, whereas female and type II males' grunting sounds are stimulated by Oxt. Using homozygous Japanese medaka, *Oryzias latipes*, knockout mutants, an essential role for Avp in male mate-guarding behaviours in this non-monogamous species has been demonstrated (118). For example, under natural conditions, two medaka males kept in triads with a female are in competition and the dominant medaka male that is 'guarding' the female exhibits increased reproductive success measured as increased paternity in offspring. Males harbouring mutations in a*vp* and *avpr1aa* exhibit significantly reduced male guarding behaviour indicating a key role for *avp* in dominant status-dependent reproductive success (118). Similarly, *oxt* and *oxtra*, exert sex-specific effects in Japanese medaka: mutant female fish exhibit a lack of mate preference for familiar males and mutant male fish have reduced courtships displays to unfamiliar females, but exhibit increased mate-guarding behaviour towards familiar females (64). Since the potential effects on the HPG axis were not quantified in these studies, it is not clear whether these effects are entirely mediated by the nonapeptides, or whether altered HPG axis regulation also contributes to the behavioural observations.

In male bluehead wrasse, *Thalassoma bifasciatum*, a species with alternate male reproductive tactics (territorial and non-territorial), Avp intraperitoneal injection increased courtship behaviour in the field irrespective of male reproductive tactic and promoted a territorial-like phenotype in non-territorial males (65). An opposite effect was observed following administration of Manning's compound, a mammalian AVPR1 receptor antagonist, suggesting that this effect is mediated *via* this nonapeptide receptor subtype (65). In male white perch, *Morone americana*, intracerebroventricular but not intraperitoneal administration of Avp significantly stimulated an important courtship behavior termed 'attending' without affecting whole body or circulating androgens (119). These data suggest that central rather than peripheral HPG axis actions are involved in mediating the effects of Avp on the male white perch courtship behaviour. In male beaugregory damselfish, *Stegastes leucostictus*, Manning's compound significantly lowered male courtship behaviour, while exogenous Avp administration did not affect male courtship behaviour (120). Similarly, administration of Manning's compound significantly reduced male reproductive courtship behaviour and reproductive success in mating assays with female zebrafish, *Danio rerio* without affecting whole body androgen (T and 11-keto-testosterone; 11-KT) levels, suggesting Avp acutely regulates male zebrafish courtship behaviour including chasing, nudging, and circling *via* central Avpr1a receptors and independently of HPG axis regulation (35). In the weakly electric fish, *Brachyhypopomus gauderio*, male courtship behaviour observed in male-female dyads resulted in a higher degree of Avp neuron activation in the nucleus preopticus ventricularis anterior compared to isolated males (121). In the same species, Avp increases dominance in part *via* direct modulation of the EOD rate (122). Together, these findings raise the possibility that male reproductive behaviour *via* electric signaling may be under Avp control in this species. Despite the reviewed evidence, a universal Avp-dependent stimulation of male reproductive behaviour in teleost fishes is unlikely, as the reproductive phenotype of females and sneaker males, but not dominant males, is sensitive to Avp in the peacock blenny, *Salaria pavo* (123). There is a need for detailed comparative studies of the roles of nonapeptides in teleosts which exhibit the most diverse reproductive strategies and behaviours amongst the vertebrates (117).

Several studies have provided evidence for regulatory roles of nonapeptides in teleost species with parental care. In the primarily paternal teleost the common clownfish, *Amphiprion ocellaris* for example, administration of an OXTR antagonist abolished paternal behaviours such as nips, fanning the eggs, and proportion of time in the nest, without affecting aggressive behaviours in paired non-reproductive fish (124). This suggests a specific action of Oxt in controlling male common clownfish parental care behaviours (124); however, whether the high selectivity of antagonist (desGly-NH2-d(CH2)5[D-Tyr2, Thr4]OVT) for mammalian OXTR also applies to teleosts has not been formally investigated. When introducing domino damselfish, *Dascyllus trimaculatus*, as non-conspecific intruders, administration of the OXTR antagonist reduced paternal care behaviour in clownfish, but increased aggression towards the non-conspecific intruder, demonstrating the importance of social context in behavioural responses (125). Conversely, administration of an AVTR1 receptor antagonist increased male parental behaviours while reducing aggression towards intruders (125), thus demonstrating antagonistic roles of the nonapeptide systems in common clownfish (124, 125). These data suggest a role for nonapeptides in paternal care. In

the monogamous convict cichlid, *Amatitlania nigrofasciata*, single fathers increase paternal care behaviours quickly after removal of the female partner, and this increase coincides with increased activation of parvocellular Oxt neurons in the POA (126). Administration of a mammalian OXTR antagonist in biparental males inhibited paternal care behaviour, indicating a functional role for Oxt neurons (126).

3.2 A role for central nonapeptides in regulating saliency to reproductive cues in teleost fishes

In addition to the direct modulation of teleost reproductive behaviours, central roles for nonapeptides may not be restricted to the role of transducing and integrating reproductive cues, but also to act as a filter and/or amplifier of exogenous or endogenous cues. This latter concept has recently gained more traction as the 'salience hypothesis', especially in higher vertebrate species, including humans. The salience hypothesis is based on the notion that an individual is being constantly inundated with sensory information in its environment and therefore needs to be able to filter the information to identify what is relevant and important (i.e., signal) from that which is unimportant (i.e., background noise). The nonapeptides OXT and AVP have been proposed to serve as two important neuromodulators in the central nervous system that can help to increase the salience of sensory information, such as conspecific olfactory cues, to induce effects on endocrine and behavioural responses (127). In fish, however, this concept remains comparatively poorly explored, especially in the context of the diverse reproductive strategies in different environmental conditions and cues. Olfactory stimuli in particular play important roles in at least some groups of teleost fish such as cyprinids (35, 94, 95). Future studies exploring sensitizing roles of nonapeptides to reproductive pheromonal and visual cue detection are thus clearly warranted. A recent study in zebrafish identified Oxt-like innervation in the male olfactory bulb (35), providing a neuroanatomical basis for functional studies. Similarly, the widely studied electrical and vocal communication systems in some teleost fishes (128) should provide excellent models to investigate possible modulatory roles of nonapeptides in the reception and integration rather than production of reproductive signals.

3.3 Nonapeptide-dependent regulation of the HPG axis

3.3.1 Nonapeptides are an integral part in the hypothalamic circuitry controlling the HPG axis

Both stimulatory and inhibitory factors regulating the teleost HPG axis have been well described (92). Nonapeptides may affect the HPG axis *via* modulation of stimulatory and/or inhibitory hypophysiotropic systems. To date, little evidence exists for potential roles of nonapeptides in directly affecting Gnrh in teleost fish. In rock hind, *Epinephelus adscensionis*, and in Atlantic Croaker, *Micropogonias undulatus*, Avpr1ab receptors are co-localized with Gnrh1 in preoptic anterior hypothalamic neurons. The functional relevance of this crosstalk other than reported concordant regulation of gene expression between *avpr1ab* and *gnrh1* (78, 79, 99) must be investigated. Studies in female goldfish show that serotonin neurons, known to stimulate pituitary Lh release in this species (129), are found in proximity to Oxt neurons in the POA and Oxt fibres in the pars nervosa of the pituitary gland, suggesting a possible interaction between them (87). However, the functional relevance of possible Oxt dependent modulation of serotonin-dependent gonadotropin release has not been investigated.

Nonapeptides may ostensibly also act to promote pituitary gonadotropin release *via* reduction of potent dopaminergic inputs on Lh release (92). This is supported by work in the walking catfish, *Clarias batrachus*, which suggests that Oxt may stimulate Lh release *via* the inhibition of dopaminergic blockage (66). In walking catfish Oxt immunoreactivity was greatly enhanced in the POA in female pre-spawning and spawning fish, and superfusion of brain slices with Oxt resulted in a ~50% reduction of tyrosine hydroxylase staining, suggesting rapid inhibitory effects on dopamine or other catecholaminergic neurons (66).

In addition to hypothalamic interaction between nonapeptide systems and the neuronal circuitry involved in HPG axis regulation in several teleost models (66, 86, 87), a few lines of evidence also demonstrated co-expression of nonapeptides with neuropeptides with known stimulatory function on gonadotropin release. For example, Oxt and secretoneurin were found to be colocalized in the POA and fibers innervating the pituitary in goldfish, *Carassius auratus* (44), while colocalization of Oxt and GnRH was reported in the dwarf gourami, *Colisa lalia* (43). Together, these studies suggest that potential co-release of nonapeptides with other neuropeptides known to stimulate gonadotropin release represent an understudied aspect of HPG axis regulation.

In addition to targeting neuronal circuitry involved in the regulation of the HPG axis, POA nonapeptide neurons in teleost fish themselves receive neuronal input from reproductive neuropeptides. These include Gnrh, which contact Oxt neurons in rainbow trout, *Oncorhynchus mykiss* (130), and kisspeptin, which contact Oxt and Avp neurons in Japanese medaka (131) and striped bass, *Morone saxatillis* (132). Together, this neuroanatomical evidence points to potential neuromodulatory roles for nonapeptides in HPG stimulation in the context of multimodal signaling systems regulating gonadotrophs in teleosts (133). Given the reported roles of nonapeptides in teleost sociosexual and courtship behaviour, neuroanatomical evidence may also reflect synchronization of

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HPG axis activation stimulation with nonapeptidergic behavioural pathways to maximize reproductive success.

Several studies have also demonstrated the sensitivity of both Oxt and Avp neuronal populations to sex steroids. Administration of both low (0.1 µg/g body weight) and high (0.5 µg/g body weight) estradiol (E_2) doses (0.5 µg/g body weight) normalizes ovariectomy-induced decreases in brain and plasma Avp concentrations in Asian stinging catfish (134). This effect appears to be, at least in part, indirectly mediated *via* the modulation of dopaminergic control, as treatment with α-methyl-para-tyrosine, a tyrosine hydroxylase inhibitor, partially abolished the restorative effect of the low E_2 dose on Avp abundance in the ovariectomized fish (134). A male-specific stimulatory effect of androgens on parvocellular Oxt neurons in the medaka POA has also been reported (135). This regulation also appears to be indirect, as androgen receptor expression was not found in Oxt neurons but on kisspeptin neurons known to stimulate Oxt neurons in this species (131, 135). In female round gobies, circulating E_2 levels are higher in the spawning phase compared to non-spawning phase and coincide with high circulating Avp and Oxt concentrations (98). Brain explant exposure to E_2 in spawning and non-spawning phases stimulated Avp and Oxt release in this species; however, pharmacological studies using the estrogen receptor (ER) antagonist fulvestrant and the transcription inhibitor actinomycin D showed that the effect of E_2 on Avp and Oxt release was mediated by different signaling pathways (98). E_2-dependent Avp release was mediated by ERs *via* both genomic and non-genomic pathways, while Oxt release was mediated through ERs *via* a genomic pathway only (98). Whether E_2 acted directly on nonapeptide neurons in female gobies was not resolved, as the study did not investigate whether nonapeptide neurons express ERs. Oxt neurons in the POA of goldfish, *Carassius auratus*, are, at least based on immunohistochemical evidence, direct targets for estrogen actions, as they express the membrane estrogen receptor Gper1 (96) and are surrounded by radial glia, the only cells in the teleost brain expressing *cyp19a1b* and capable of producing neuroestrogens (136). While these studies provide evidence for effects of steroids on nonapeptide systems, future studies are warranted to delineate direct and indirect mechanisms of action, and whether effects are mediated by gonadal steroids and feedback regulation and/or local neurosteroids.

In sum, POA nonapeptide systems have been shown to be integrated into neuronal circuits involved in the regulation of the HPG axis in several teleosts. Additionally, recent evidence suggests that nonapeptide neurons are also responsive to sex steroids, suggesting the potential for endocrine feedback and/or modulation *via* neurosteroids. Additional studies investigating nonapeptide crosstalk with other hypothalamic regulators of the HPG axis are clearly warranted, as are careful studies investigating the direct or indirect regulation of hypothalamic nonapeptide systems to sex steroids, the critical endogenous reproductive signals.

3.3.2 Nonapeptides exert direct hypophysiotropic effects on gonadotrophs in several species

In the goldfish, hypothalamic expression of *oxt* was found to peak seasonally in reproductively mature females (93). Pharmacological investigations demonstrate that hypothalamic induction of goldsfish *oxt* is dependent on GABAergic and dopaminergic signaling (137), in line with an integration of this nonapeptide system with seasonally-regulated HPG neurocircuitry in this species (92). Intraperitoneal injection of 1 µg/g body weight Oxt in sexually recrudescent female goldfish significantly increased circulating Lh by 167% 5h post-injection (86) with subsequent increases in circulating E_2 12h post injection (137). Unfortunately, Oxt-dependent stimulation of Lh release in sexually mature female goldfish was not investigated. Subsequent examination of potential direct effects of Oxt on gonadotrophs were investigated using primary goldfish dispersed pituitary cell cultures (87). In these preparations, Oxt significantly stimulated Lh release without affecting *lhb* or *fshb* subunit mRNAs, suggesting direct, transcription-independent stimulation of Lh release (87). These findings are in line with reported neuroanatomical evidence in other teleosts, such as the sailfin molly, *Poecilia latipinna*, the European bass, *Dicentrarchus labrax*, and the African sharptooth catfish, *Clarias gariepinus*, in which nonapeptidergic innervation of gonadotrophs has been reported (41, 48). Similar to Oxt, a stimulatory effect of Avp on Lh release has been demonstrated in at least two teleosts: in the sailfin molly 18 h pituitary incubation with Avp stimulated Lh synthesis and release, with lower dose- responsiveness and more consistent effects in male compared to female pituitaries (41). In the Asian stinging catfish, Avp, and to a much lesser extent Oxt and servatocin, stimulated *gpa*, *fshb* and *lhb* in pituitary cultures in a sex- and reproductive stage-dependent manner (24). The less potent effects of Oxt and servatocin where largely limited to *lhb* stimulation in pre-spawning females, with no effect on *fshb*. In female walking catfish, *Clarias batrachus*, pituitaries superfused with 20 nM Oxt for 1h displayed a significant decrease in Lhb staining reflective of increased Lh release was reported (138). In the ricefield eel, *Monopterus albus*, Oxt-stimulated Lh release from dispersed pituitaries *via* an Oxtra-activated IP_3/Ca^{2+} pathway (81). In males of the Chanchita, *Cichlasoma dimerus*, Avp-stimulated gonadotropin secretion in single pituitary culture, with biphasic stimulation of Lh release at the lowest (0.1 µM) and highest (10 µM) concentration of Avp tested (54) and a stimulation of Fsh release at the highest Avp concentration tested.

These data establish a stimulatory role of nonapeptides on gonadotrophs, with sex-, reproductive stage- and species-dependent differences in potency and gonadotropin specificity. While it is important to keep in mind that observations are limited to only a few teleost species, data to date suggest that hypophysiotropic nonapeptide systems stimulate Lh release in

teleost fishes similar to the situation reported in rodents and humans (139, 140).

3.3.3 Endocrine and paracrine roles of nonapeptides in gonads

Both nonapeptides and their receptors have also been identified in ovaries and testes, and roles for nonapeptides in the regulation key gonadal functions *via* endocrine and paracrine signaling have been reported.

3.3.3.1 Steroidogenesis

Effects of nonapeptides on male steroidogenesis have been reported in testicular cultures of the rainbow trout (141). Testosterone production stimulated by Avp and Oxt has been observed in immature but not mature testes *in vitro*. Exposure to Avp elicited a stronger maximal response in T production compared baseline production (6-fold) than Oxt (4-fold). The maximally active concentration of 100 nM Avp was furthermore found to augment dose-dependent Lh-stimulated T production, suggesting a synergistic role. In a similar study in chanchita, Avp-stimulated T production in testes incubated *in vitro* dose-dependently, reaching a significant, 2-fold increase at 50 nM (54). A limitation of these two studies is that the authors measured T, which is the prohormone for the more potent teleost sex steroids: 11-KT and E_2. Unfortunately, nonapeptide receptor antagonists were not used in these experiments to probe specific receptor involvement. Since recent gene expression and *in-situ* hybridization data suggests a role for *avpr1aa* and *avpr2aa* receptors (Table 3), additional studies with teleost-validated antagonists are warranted to probe the molecular mechanistic basis involved in Avp-induced T production in testes.

Several studies have investigated the role of nonapeptides on ovarian steroidogenesis. While a stimulatory role for Oxt on circulating E_2 has been reported in female goldfish *in vivo* (87), it was not investigated whether these effects are linked to prior increases in Lh (86) or mediated *via* direct action at the ovary. In contrast, a comprehensive study determining the role of Avp and Oxt on *in vitro* ovarian steroidogenesis at different seasonal reproductive developmental stages was conducted in the air-breathing catfish (142). Dose-dependent, biphasic stimulatory effects of Avp on E_2 production for pre-vitellogenic ovarian tissue were reported. In contrast, Avp produced a dose- and time-dependent inhibition of E_2 production in early postvitellogenic ovaries (142). In comparison, Oxt produced a low, yet significant, stimulation of E_2 production without any dose effect in the previtellogenic ovaries, and a dose- and time-dependent inhibition like Avp in the early postvitellogenic ovary (142). The inhibitory effect of Avp on the E_2 synthesis in the postvitellogenic ovary may be part of a trigger for the steroidogenic shift to decreased E_2 in favour of synthesis of the maturation-inducing steroid (MIS) 17, 20β-dihydroxy-4-pregnen-3, 20-dione (17, 20β-DP) in air-breathing catfish (143). The MIS reinitiates oocyte meiosis up to the second metaphase (144).

Concurrent with the modulation of E_2 synthesis, Avp and, to a lesser extent, Oxt also stimulated progesterone (P_4) production (142). The Avp-stimulated increase in P_4 was generally dose-dependent in pre- and early post-vitellogenic ovaries, reaching approximately a 30% increase in production compared to baseline at high doses (142). In late postvitellogenic ovaries, concentrations as low as 1 nM Avp induced a 60% increase over baseline concentrations (142). This effect was similar to that of hCG, and combined administration of 20 IU hCG and Oxt was found to be additive at least after 16 h incubation. Avp stimulated 17-hydroxyprogesterone (17-OHP_4) synthesis in the previtellogenic phase ovaries following both 8 h and 16 h incubation (142). Avp stimulated the production of 17,20β-Dihydroxy-4-pregnen-3-one (17,20β-DP), which acts as maturation induced steroid in this species, about 2-fold more in the spawning phase than pre-spawning phase, similar to hCG. The stimulatory effect of Oxt was several-fold lower compared to Avp and occurred at higher concentrations (142). The combination of Avp and hCG elicited a cumulative effect on the 17,20β-DP level especially after 16 h of incubation in the spawning phase (142). The authors concluded that Avp was more potent than Oxt to stimulate the progestin pathway, and that Avp paralleled the actions of hCG. The finding that hCG and steroid hormones (E_2, P_4 and 17, 20β-DP) stimulate ovarian Avp production suggests a positive feedback loop (145) underscoring the functional significance of Avp in follicular growth, maturation, and ovulation. The stimulatory effect of Avp on ovarian P_4 secretion is conserved as similar actions for AVP family peptides have been reported for chicken, mouse, and cow *in vitro* (146).

3.3.3.2 Gametogenesis, gamete release and parturition

In the air-breathing catfish ovaries, Avp induces germinal vesicle breakdown (GVBD) and ovulation in a dose- and time-dependent manner (145). In this experiment, postvitellogenic follicles were co-incubated with Avp and an AVPR1 antagonist (deamino-Pen^1, O-Me-Try^2, Arg^8 vasopressin), an AVPR2 antagonist (1-adamantane acetyl O-Et-D-Try^2Val^4, Abu^6, $Arg^{8,9}$ vasopressin), or both. GVBD, ovulation and 17,20β-DP concentration were inhibited or reduced by 92-94% after 24h co-incubation with both antagonists. The AVPR1 antagonist inhibited GVBD and ovulation by 82-83%, and the MIS concentration by 70%. The AVPR2 antagonist inhibited GVBD, ovulation and MIS concentration by 29%, 26% and 15%, respectively. The results show that the effects of Avp are mediated mainly by Avpr1 receptors with a minor role for Avpr2 receptors.

Prostaglandins (PGs) have a critical role in diverse aspects of reproduction in vertebrates (147). The cyclooxygenase inhibitor

indomethacin can block MIS-induced final oocycte maturation and ovulation in yellow perch, *Perca flavescens*, and Atlantic croaker, indicating dependence on PGs (148, 149) The functional relationship between Avp and PGs was investigated in air-breathing catfish (150): Avp stimulated $PGF_{2\alpha}$ and PGE_2 levels in a dose- and time-dependent manner *in vitro* and the effects were similar to that produced by hCG. Both Avp and hCG-induced stimulation of PG levels were inhibited by indomethacin, supporting involvement of cyclooxygenase. The Avp stimulation of PG levels was strongly inhibited by the AVPR1 receptor antagonist but not by the AVPR2 receptor antagonist. Indomethacin inhibited the Avp and hCG-induced GVBD and ovulation. Both $PGF_{2\alpha}$ and PGE_2 stimulated GVBD and ovulation in a dose- and time-dependent manner and $PGF_{2\alpha}$ was more effective than PGE_2. Taken together, these observations highlight a relationship between Avp and PGs, and their interaction in the control of oocyte maturation and ovulation.

Follicular or oocyte hydration is a phenomenon conspicuous and widespread in marine and catadromous fish eggs associated with follicular and oocyte maturation (FOM) and ovulation (151), and this process is retained to some extent in freshwater and anadromous fishes. Singh and Joy (152) reported a 23% rise in oocyte water content during the FOM and ovulation in *H. fossilis* with Avp eliciting a significant effect on oocyte water content, diameter, volume, osmolality, Na^+K^+ ATPase activity, Na^+, K+, Mg^{2+}, Ca^{2+} concentration, GVBD and ovulation, similar to hCG. The combination of Avp and hCG produced a higher effect. In a further study, Acharjee et al. reported that Avp regulates *aqp1ab*, (ovary-specific aquaporin 1ab) expression through an Avpr2 receptor (153, 154), which is linked to the cAMP-PKA pathway, similar to AVP in mammalian kidney tubules.

The involvement of nonapeptides in sperm release was first demonstrated in the killifish, *Fundulus heteroclitus*, in which fish and mammalian neurohypophyseal preparations as well as synthetic OXT initiated a spawning reflex response (155). The relative effectiveness of the nonapeptides to induce spawning reflex in the killifish was estimated to be the highest and equipotent for AVP and Avp, followed by Oxt and OXT (155). However, concentrations used in these original studies were high compared to physiological concentrations. In male African sharptooth catfish testes slices 30-min incubation with OXT (at 10 IU), but not Oxt, AVP, epinephrine, $PGF_{2\alpha}$, LH and pituitary extracts increased milt release (156). In male walking catfish, nonapeptides and their nanotube composites designed for slower release were tested for their efficacy to promote stripping of milt by abdominal massage (157). Both naked or nano-conjugated nonapeptides increased strippable milt concentrations without altering reproductive success of fertilized eggs and increased the expression of the steroidogenesis pathway enzymes *star*, *3bhsd*, *17bhsd*, *cyp17a1a*, ands *cyp11a1a* (157).

Regarding parturition in teleosts, nonapeptides have been reported to stimulate premature parturition in the guppy, *Poecilia reticulata*, an ovoviviparous teleost (82, 158). The injection of Avp, Oxt and PGs to guppy, *Poecilia reticulata*, a live-bearing teleost, induced premature parturition (157). Both Avp and Oxt stimulated *cox2* mRNA expression in guppy ovaries *in vitro*, which in the case of Avp, but not Oxt, translated into increased PG concentrations (82). Together this data suggests that the nonapeptide-dependent stimulation of premature parturition in guppies is mediated by PG. Interestingly, both Avp and Oxt exposure upregulated a guppy *oxtr* paralogue, suggesting interaction between ovarian nonapeptide systems in the guppy ovary (82). In zebrafish, a recent study investigating downstream effectors linked to a reduced ovulation phenotype observed in female chromosome 23 miR-200 cluster KO mutants showed that co-injection of hCG, Avp and Oxt, but not injection of synthetic human GnRH and LH analogues were able to partially rescue the phenotype (159). Together, this data suggests a Gnrh-independent role for Avp and Oxt in zebrafish ovulation. Nevertheless, early comparative evidence from teleost fishes has demonstrated that roles for nonapeptides on male and female spawning cannot be generalized in teleost fish and are possibly indirect following application of supraphysiological concentrations (160).

While we acknowledge a generally high degree of evolutionary conservation of nonapeptide genes and the gross (neuro)anatomical distribution of their expression, differences in reproductive function of nonapeptides between teleost species certainly exist. We have integrated the current state of knowledge of nonapeptides on central and HPG axis components of teleost reproduction in Figure 4. It is anticipated, however, that additional detailed comparative studies will uncover diversity of nonapeptide-dependent regulation of teleost reproductive physiology.

4 Translational aspects

As evolutionarily conserved systems regulating reproduction (3), research investigating roles of nonpeptides in teleost fish reproduction in detail have translational relevance in the areas of aquaculture and species conservation; both of which rely on methods informed by mechanistic understanding of reproductive physiology. As such, we anticipate that modulation of nonapeptide function in teleost species has significant potential to stimulate and possibly coordinate behavioural and endocrine processes necessary to promote reproduction for species in captivity (161). A third area of translational relevance is ecotoxicology. We review emerging evidence suggesting that neuroendocrine disruption of teleost nonapeptides may be linked to decreased

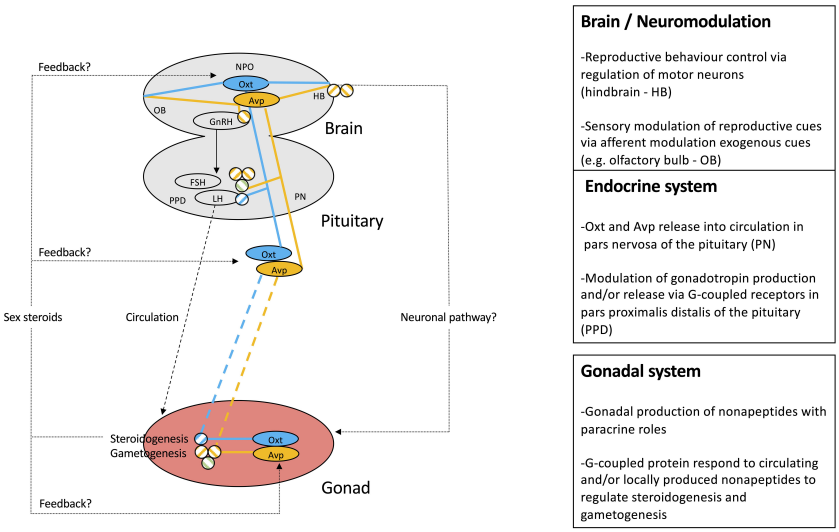

FIGURE 4
Schematic representation of mechanistic knowledge of nonapeptide roles in teleost reproduction. The Avp system is highlighted in yellow, while the Oxt system is represented in blue. Circled nonapeptides indicate sites of synthesis, while connecting lines represent neuronal, endocrine, and paracrine pathways. Specific receptor involvement in regulatory function of HPG axis components is symbolized as defined in Figure 2.

reproductive success in teleost fishes, an ecologically meaningful endpoint (162).

4.1 Neuroendocrine disruption

In teleost fish, relatively recent studies have investigated Avp and Oxt nonapeptide systems as targets of different groups of aquatic contaminants. Histological studies of POA magnocellular neurons revealed that a six-month exposure of channel catfish, *Channa punctatus*, to inorganic mercury at a concentration of 10 µg/L resulted in smaller and less active Avp-secreting neurons (163). Persistent organic pollutants have also been shown to affect nonapeptide systems in teleost fishes: in Atlantic Croaker, a four week daily dietary exposure (2 and 8 µg/g body weight) to the planar polychlorinated biphenyl congener 3,3′,4,4′-tetrachlorobiphenyl (PCB-77) significantly reduced hypothalamic expression of *avpr1a* mRNA and Avpr1a protein levels, as well as (co-localized) *gnrh1* mRNA levels in the brain (78).

Pharmaceuticals and plasticizers are other major environmental contaminants with reported effects on nonapeptide systems. Repeated intraperitoneal injections of pharmacological doses (5 µg/g) of the selective serotonin reuptake inhibitor and aquatic contaminant fluoxetine (FLX) significantly reduced *oxt* mRNA levels in female goldfish telencephalon and hypothalamus, an effect that was linked to reduced circulating E_2 concentrations (85). Subsequent waterborne FLX exposure studies in both female and male goldfish revealed that *oxt* transcript abundance in the same tissues was not affected by a two- week exposure to FLX at 540 ng/L and 54 µg/L concentrations, but that the same two-week exposure to waterborne fluoxetine significantly diminished releaser pheromone $PGF_{2\alpha}$-induced increases in *oxt* mRNA (94, 164). Similarly, targeted gene expression analysis of zebrafish larvae acutely exposed to 50 and 500 ng/L FLX for 96 h (165) as well as transcriptomic screens of whole brains collected from a wild zebrafish population exposed to 100 µg/L FLX at a concentration for two weeks (166) identified *oxt* transcripts as being differentially expressed in FLX-exposed fish compared to unexposed control fish. Together, these studies reveal that the Oxt system is responsive to FLX at both early developmental and adult life-stages, raising the possibility of mediating organisational as well as activational effects. Repeated injection of pharmacological concentrations of 6 µg/g body weight FLX over a period of two weeks significantly reduced *avt* transcript abundance in giganto-, magno- and parvocellular neurons of the POA in male bluehead wrasses, an effect that correlated with decreased territorial aggression (167). The responsiveness of teleost nonapeptide systems to FLX corresponds to several observations in mammalian models (168, 169) and suggests an evolutionarily conserved serotonin-dependent regulation of these systems (87, 170, 171).

In Japanese medaka chronically exposed to environmentally relevant and high concentrations of waterborne metamphetamine for a period of 90 days, a dose-dependent, significant increase in whole brain *oxtr* mRNA and Oxt peptide were observed (172). Because both FLX and metamphetamine affect neurotransmitter

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systems and neuroactive contaminants may exert reproductive effects, at least in part, *via* nonapeptidergic systems, these findings support the concept of neuroendocrine disruption (173). In line with this interpretation, a meta-analysis of transcriptomic screens of the goldfish hypothalamus identified *oxt* as the single transcript affected by drugs modulating serotonergic, dopaminergic, and GABAergic systems, all of which have established roles in goldfish reproduction (137). Concurrent with previously described responsiveness of teleost POA nonapeptide systems to sex steroids, zebrafish chronically exposed to 1,10 and 30 µg/L Bisphenol A (BPA), a weakly estrogenic compound, as well as 1 µg/L E_2, exhibited complex dose-dependent and sex-specific effects on whole brain nonapeptide and nonapeptide receptor gene expression levels, which corresponded with alterations in social but not overall locomotor behaviour (174, 175). Similarly, developmental (2-5dpf) exposure to BPA and its replacement compound Bisphenol S (BPS) in the low µM range revealed non-linear alteration of *oxt* and Oxt protein abundance in association with quantifiable behavioural disruptions at 21dpf (176, 177). There is a need to study the involvement of nonapeptide systems in mediating organizational and/or activational effects of endocrine disrupting chemicals. This is an area understudied in teleost fish (178) compared to rodent models (179, 180), and will inform the possible development of teleost nonapeptides as functional biomarkers relevant to reproductive function in teleost fishes.

5 The state of the art and current limitations

Having critically reviewed the current state of knowledge regarding reproductive roles of teleost nonapeptides, we conclude by briefly discussing key insights and current limitations in the field. We furthermore suggest conceptual and technical approaches in the hope of stimulating collaborative research in the field.

1. It is well-appreciated that teleosts are champions of reproductive plasticity and diversity. There is a high degree of evolutionary conservation of nonapeptides and their gross (neuro)anatomical distribution. However, novel resources such as genomic sequences and detailed comparative investigation increasingly reveal areas of plasticity of teleost nonapeptide systems. A point in case is the nonapeptide receptor inventory, which exhibits differences between teleost fishes and other vertebrates, but, importantly, also within teleost fish lineages such as the otocephala and euteleosti (59). It will thus be important to address functional differences in nonapeptide receptor paralogue expression, regulation, and function to assess their potential roles in mediating reproductive functions in a reproductively diverse fishes.
2. While increasing comparative studies to delineate plasticity of nonapeptides in regulating reproduction in the diverse group of teleost fishes will be important, it will be equally crucial to comprehensively study the reproductive roles of nonapeptides within single teleost fish species. In reviewing the current knowledge of the role of nonapeptides in teleost reproduction, it has become clear that, apart from detailed studies in the Asian stinging catfish (50, 84) and, to a lesser degree, the canchita cichlid (54), few if any studies have investigated the role of nonapeptides across the different levels of the HPG axis within a single teleost species. The need for detailed studies within single fish species becomes even more clear when considering that research investigating behavioural and endocrine roles of nonpeptides in teleost reproduction has largely co-existed in isolation focussing on separate fish species. However, there is a need to consider (nonapeptide-regulated) courtship behaviour in the context of HPG axis function (165). Investigation of the salience hypothesis and possible roles for nonapeptides in the integration of reproductive cues are also warranted.
3. The recent generation of nonapeptide and/or nonapeptide receptor knock-out models in genetically-tractable model systems such as zebrafish (67) and medaka (64, 118) may hold particular promise. However, neither of these models have, to-date, been used to explore nonapeptide effects on the HPG axis. Receptor-specific knock-outs may furthermore prove fruitful to probe the role of virtually uncharacterized nonapeptide receptors such as *avtr2l* and generally circumvent possible specificity issues linked to the use of mammalian nonapeptide agonists and antagonists. Furthermore, GFP-reporter lines have been described in zebrafish, at least for Oxt (68, 69). While such lines represent powerful tools to investigate regulation and activation of central and peripheral nonapeptide systems in response to environmental and endogenous cues relevant to reproduction (35), few studies have been conducted in this area. Transgenic reporter lines may also allow endocrine disrupting) chemical screening for effects on nonapeptide systems. Adult-specific transgenic ablation studies and optogenetic approaches may in the future permit specific investigation of organisational and activational reproductive effects of the nonapeptides.
4. Previous co-localization experiments (43, 44) and more recent single-cell techniques (28) have demonstrated co-localization of nonapeptides with other neuropeptide regulators of reproduction in neuroendocrine neurons. Thus, future functional studies should investigate co-release and interactions between nonapeptides and co-expressed reproductive regulators to quantify potential

combinatorial effects on courtship behaviour and HPG axis function.

Author contributions

JM, DR, KS,RC, KJ, and VLT contributed to conception and design of the review. DR conducted in silico analyses. JM wrote the first draft of the manuscript. DR, KS, RC, KJ and VLT wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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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.

Publisher's note

References

1. Odekunle EA, Elphick MR. Comparative and evolutionary physiology of Vasopressin/Oxytocin type neuropeptide signaling in invertebrates. *Front Endocrinol* (2020) 11:225. doi: 10.3389/fendo.2020.00225

2. Kawada T, Sekiguchi T, Itoh Y, Ogasawara M, Satake H. Characterization of a novel vasopressin/oxytocin superfamily peptide and its receptor from an ascidian, ciona intestinalis. *Peptides* (2008) 29:1672–8. doi: 10.1016/j.peptides.2008.05.030

3. Gwee P-C, Tay B-H, Brenner S, Venkatesh B. Characterization of the neurohypophysial hormone gene loci in elephant shark and the Japanese lamprey: origin of the vertebrate neurohypophysial hormone genes. *BMC Evol Biol* (2009) 9:47. doi: 10.1186/1471-2148-9-47

4. Matsubara S, Shiraishi A, Osugi T, Kawada T, Satake H. The regulation of oocyte maturation and ovulation in the closest sister group of vertebrates. *Elife* (2019) 8:e49062. doi: 10.7554/eLife.49062

5. Banerjee P, Chaube R, Joy KP. Molecular cloning, sequencing, and tissue expression of vasotocin and isotocin precursor genes from ostariophysian catfishes: phylogeny and evolutionary considerations in teleosts. *Front Neurosci* (2015) 9:166. doi: 10.3389/fnins.2015.00166

6. Banerjee P, Chaube R, Joy KP. Molecular cloning and characterisation of an isotocin paralogue ([V8] isotocin) in catfishes (superorder ostariophysi): Origin traced likely to the fish-specific whole genome duplication. *J Neuroendocrinol* (2018) 30:e12647. doi: 10.1111/jne.12647

7. Panopoulou G, Poustka AJ. Timing and mechanism of ancient vertebrate genome duplications – the adventure of a hypothesis. *Trends Genet* (2005) 21:559–67. doi: 10.1016/j.tig.2005.08.004

8. Acher R. Molecular evolution of fish neurohypophysial hormones: Neutral and selective evolutionary mechanisms. *Gen Comp Endocrinol* (1996) 102:157–72. doi: 10.1006/gcen.1996.0057

9. Acher R, Chauvet J. Structure, processing, and evolution of the neurohypophysial hormone-neurophysin precursors. *Biochimie* (1988) 70:1197–207. doi: 10.1016/0300-9084(88)90185

10. Acher R, Chauvet J, Chauvet MT, Crepy D. Neurohypophysial hormones from fish: isolation of a vasotocin from the whiting-pour (Gadus luscus l.). *Biochim Biophys Acta* (1961) 51:419–20. doi: 10.1016/0006-3002(61)90198-6

11. Heller H, Pickering BT. Neurohypophysial hormones of non-mammalian vertebrates. *J Physiol* (1961) 155:98–114. doi: 10.1113/jphysiol.1961.sp006616

12. Chauvet J, Chauvet-Lenci MT, Acher R. Some observations on the neurohypophysial hormones of a teleostfish, the hake (Merluccius merluccius l.). *C R Seances Soc Biol Fil* (1961) 252:2145–7.

13. Acher R, Chauvet J, Chauvet MT. Phylogeny of the neurohypophysial hormones. *Two New active pept isolated cartilaginous fish Squalus acanthias. Eur J Biochem* (1972) 29:12–9. doi: 10.1111/j.1432-1033.1972.tb01951.x

14. Chauvet J, Rouille Y, Chauveau C, Chauvet MT, Acher R. Special evolution of neurohypophysial hormones in cartilaginous fishes: asvatocin and phasvatocin, two oxytocin-like peptides isolated from the spotted dogfish (Scyliorhinus caniculus). *PNAS* (1994) 91:11266–70. doi: 10.1073/pnas.91.23.11266

15. Hyodo S, Tsukada T, Takei Y. Neurohypophysial hormones of dogfish, triakis scyllium: structures and salinity-dependent secretion. *Gen Comp Endocrinol* (2004) 138:97–104. doi: 10.1016/j.ygcen.2004.05.009

16. Buchholz H, Schönrock C, Fehr S, Richter D. Sequence analysis of a cDNA encoding an isotocin precursor and localization of the corresponding mRNA in the brain of the cartilaginous fish torpedo marmorata. *Mol Mar Biol Biotechnol* (1995) 4:179–84.

17. Gwee P-C, Amemiya CT, Brenner S, Venkatesh B. Sequence and organization of coelacanth neurohypophysial hormone genes: evolutionary history of the vertebrate neurohypophysial hormone gene locus. *BMC Evol Biol* (2008) 8:93. doi: 10.1186/1471-2148-8-93

18. Acher R, Chauvet J, Chauvet MT, Crepy D. Isolation of a new neurohypophysial hormone, isotocin, present in bony fish. *Biochim Biophys Acta* (1962) 58:624–5. doi: 10.1016/0006-3002(62)90085-9

19. Acher R, Chauvet J, Chauvet MT, Rouille Y. Unique evolution of neurohypophysial hormones in cartilaginous fishes: possible implications for urea-based osmoregulation. *J Exp Zool* (1999) 284:475–84. doi: 10.1002/(sici)1097-010x

20. Hyodo S, Ishii S, Joss JMP. Australian Lungfish neurohypophysial hormone genes encode vasotocin and [Phe^2] mesotocin precursors homologous to tetrapod-type precursors. *Proc Natl Acad Sci USA* (1997) 94:13339–44. doi: 10.1073/pnas.94.24.13339

21. Pasquier J, Cabau C, Nguyen T, Jouanno E, Severac D, Braasch I, et al. Gene evolution and gene expression after whole genome duplication in fish: the PhyloFish database. *BMC Genomics* (2016) 17:368. doi: 10.1186/s12864-016-2709-z

22. Suzuki M, Hyodo S, Urano A. Cloning and sequence analyses of vasotocin and isotocin precursor cDNAs in the masu salmon, oncorhynchus masou: evolution of neurohypophysial hormone precursors. *Zoolog Sci* (1992) 9:157–67. doi: 10.1006/gcen.1993.1165

23. Heierhorst J, Morley SD, Figueroa J, Krentler C, Lederis K, Richter D. Vasotocin and isotocin precursors from the white sucker, catostomus commersoni: cloning and sequence analysis of the cDNAs. *Proc Natl Acad Sci USA* (1989) 86:5242–6. doi: 10.1073/pnas.86.14.5242

24. Acharjee A, Chaube R, Joy KP. Reproductive stage- and sex-dependant effects of neurohypophyseal nonapeptides on gonadotropin subunit mRNA expression in the catfish heteropneustes fossilis: An *in vitro* study. *Gen Comp Endocrinol* (2018) 260:80–9. doi: 10.1016/j.ygcen.2018.01.001

25. Rodriguez-Santiago M, Nguyen J, Winton LS, Weitekamp CA, Hofmann HA. Arginine vasotocin preprohormone is expressed in surprising regions of the teleost forebrain. *Front Endocrinol (Lausanne)* (2017) 8:195. doi: 10.3389/fendo.2017.00195

26. Loveland JL, Hu CK. Commentary: Arginine vasotocin preprohormone is expressed in surprising regions of the teleost forebrain. *Front Endocrinol (Lausanne)* (2018) 9:63. doi: 10.3389/fendo.2018.00063

27. Gutnick A, Blechman J, Kaslin J, Herwig L, Belting H-G, Affolter M, et al. The hypothalamic neuropeptide oxytocin is required for formation of the neurovascular interface of the pituitary. *Dev Cell* (2011) 21:642–54. doi: 10.1016/j.devcel.2011.09.004

28. Herget U, Ryu S. Coexpression analysis of nine neuropeptides in the neurosecretory preoptic area of larval zebrafish. *Front Neuroanat* (2015) 9:2. doi: 10.3389/fnana.2015.00002

29. Herget U, Gutierrez-Triana JA, Salazar Thula O, Knerr B, Ryu S. Single-cell reconstruction of oxytocinergic neurons reveals separate hypophysiotropic and encephalotropic subtypes in larval zebrafish. *eNeuro* (2017) 4:1–16. doi: 10.1523/ENEURO.0278-16.2016

30. Kagawa N, Honda A, Zenno A, Omoto R, Imanaka S, Takehana Y, et al. Arginine vasotocin neuronal development and its projection in the adult brain of the medaka. *Neurosci Lett* (2016) 613:47–53. doi: 10.1016/j.neulet.2015.12.049

31. Demski LS, Sloan HE. A direct magnocellular-preopticospinal pathway in goldfish: Implications for control of sex behavior. *Neurosci Lett* (1985) 55:283–8. doi: 10.1016/0304-3940(85)90449-5

32. Walton JC, Waxman B, Hoffbuhr K, Kennedy M, Beth E, Scangos J, et al. Behavioral effects of hindbrain vasotocin in goldfish are seasonally variable but not sexually dimorphic. *Neuropharmacology* (2010) 58:126–34. doi: 10.1016/j.neuropharm.2009.07.018

33. Macey MJ, Pickford GE, Peter RE. Forebrain localization of the spawning reflex response to exogenous neurohypophysial hormones in the killifish. *Fundulus heteroclitus. J Exp Zool* (1974) 190:269–79. doi: 10.1002/jez.1401900303

34. Yabuki Y, Koide T, Miyasaka N, Wakisaka N, Masuda M, Ohkura M, et al. Olfactory receptor for prostaglandin F2α mediates male fish courtship behavior. *Nat Neurosci* (2016) 19:897–904. doi: 10.1038/nn.4314

35. Altmieme Z, Jubouri M, Touma K, Coté G, Fonseca M, Julian T, et al. A reproductive role for the nonapeptides vasotocin and isotocin in male zebrafish (Danio rerio). *Comp Biochem Physiol B Biochem Mol Biol* (2019) 238:110333. doi: 10.1016/j.cbpb.2019.110333

36. Forlano PM, Bass AH. Neural and hormonal mechanisms of reproductive-related arousal in fishes. *Horm Behav* (2011) 59:616–29. doi: 10.1016/j.yhbeh.2010.10.006

37. Goodson JL, Bass AH. Forebrain peptides modulate sexually polymorphic vocal circuitry. *Nature* (2000) 403:769–72. doi: 10.1038/35001581

38. Goodson JL, Evans AK, Bass AH. Putative isotocin distributions in sonic fish: Relation to vasotocin and vocal-acoustic circuitry. *J Comp Neurol* (2003) 462:1–14. doi: 10.1002/cne.10679

39. Silva A, Perrone R, Macadar O. Environmental, seasonal, and social modulations of basal activity in a weakly electric fish. *Physiol Behav* (2007) 90:525–36. doi: 10.1016/j.physbeh.2006.11.003

40. Perrone R, Migliaro A, Comas V, Quintana L, Borde M, Silva A. Local vasotocin modulation of the pacemaker nucleus resembles distinct electric behaviors in two species of weakly electric fish. *J Physiol Paris* (2014) 108:203–12. doi: 10.1016/j.jphysparis.2014.07.007

41. Batten Trevor FC. Ultrastructural characterization of neurosecretory fibres immunoreactive for vasotocin, isotocin, somatostatin, LHRH and CRF in the pituitary of a teleost fish, poecilia latipinna. *Cell Tissue Res* (1986) 244: 661–72. doi: 10.1007/BF00212547

42. Groves DJ, Batten TFC. Direct control of the gonadotroph in a teleost, poecilia latipinna. *Gen Comp Endocrinol* (1986) 62:315–26. doi: 10.1016/0016-6480(86)90122-X

43. Maejima K, Oka Y, Park MK, Kawashima S. Immunohistochemical double-labeling study of gonadotropin-releasing hormone (GnRH)-immunoreactive cells and oxytocin-immunoreactive cells in the preoptic area of the dwarf gourami, colisa lalia. *J Neurosci Res* (1994) 20:189–93. doi: 10.1016/0168-0102(94)90037-X

44. Canosa LF, Lopez GC, Scharrig E, Lesaux-Farmer K, Somoza GM, Kah O, et al. Forebrain mapping of secretoneurin-like immunoreactivity and its colocalization with isotocin in the preoptic nucleus and pituitary gland of goldfish. *J Comp Neurol* (2011) 519:3748–65. doi: 10.1002/cne.22688

45. Zhao E, Basak A, Wong AOL, Ko W, Chen A, López GC, et al. The secretogranin II-derived peptide secretoneurin stimulates luteinizing hormone secretion from gonadotrophs. *Endocrinology* (2009) 150:2273–82. doi: 10.1210/en.2008-1060

46. Mitchell K, Zhang WS, Lu C, Tao B, Chen L, Hu W, et al. Targeted mutation of secretogranin-2 disrupts sexual behavior and reproduction in zebrafish. *Proc Natl Acad Sci USA* (2020) 117:12772–83. doi: 10.1073/pnas.2002004117

47. Batten TFC, Cambre ML, Moons L, Vandesande F. Comparative distribution of neuropeptide-immunoreactive systems in the brain of the green molly, poecilia latipinna. *J Comp Neurol* (1990) 302:893–919. doi: 10.1002/cne.903020416

48. Batten TFC, Moons L, Vandesande F. Innervation and control of the adenohypophysis by hypothalamic peptidergic neurons in teleost fishes: EM immunohistochemical evidence. *Microsc Res Tech* (1999) 44:19–35. doi: 10.1002/(SICI)1097-0029(19990101)44:1<19::AID-JEMT4>3.0.CO;2-L

49. Sokołowska E, Gozdowska M, Kulczykowska E. Nonapeptides arginine vasotocin and isotocin in fishes: Advantage of bioactive molecules measurement. *Front Mar Sci* (2020) 7:610. doi: 10.3389/fmars.2020.00610

50. Singh V, Joy KP. Immunocytochemical localization, HPLC characterization, and seasonal dynamics of vasotocin in the brain, blood plasma and gonads of the catfish heteropneustes fossilis. *Gen Comp Endocrinol* (2008) 159:214–25. doi: 10.1016/j.ygcen.2008.09.003

51. Saito D, Komatsuda M, Urano A. Functional organization of preoptic vasotocin and isotocin neurons in the brain of rainbow trout: central and neurohypophysial projections of single neurons. *Neuroscience* (2004) 124:973–84. doi: 10.1016/j.neuroscience.2003.12.038

52. Bobe J, Montfort J, Nguyen T, Fostier A. Identification of new participants in the rainbow trout (Oncorhynchus mykiss) oocyte maturation and ovulation processes using cDNA microarrays. *Reprod Biol Endocrinol* (2006) 4:39. doi: 10.1186/1477-7827-4-39

53. Chou M-Y, Hung J-C, Wu L-C, Hwang S-PL, Hwang P-P. Isotocin controls ion regulation through regulating ionocyte progenitor differentiation and proliferation. *Cell Mol Life Sci* (2011) 68:2797–809. doi: 10.1007/s00018-010-0593-2

54. Ramallo MR, Grober M, Cánepa MM, Morandini L, Pandolfi M. Arginine-vasotocin expression and participation in reproduction and social behavior in males of the cichlid fish cichlasoma dimerus. *Gen Comp Endocrinol* (2012) 179:221–31. doi: 10.1016/j.ygcen.2012.08.015

55. Banerjee P, Joy KP, Chaube R. Structural and functional diversity of nonapeptide hormones from an evolutionary perspective: A review. *Gen Comp Endocrinol* (2017) 241:4–23. doi: 10.1016/j.ygcen.2016.04.025

56. Mayasich SA, Clarke BL. Vasotocin and the origins of the vasopressin/oxytocin receptor gene family. *Vitam Horm* (2020) 113:1–12. doi: 10.1016/bs.vh.2019.08.018

57. Theofanopoulou C. Reconstructing the evolutionary history of the oxytocin and vasotocin receptor gene family: Insights on whole genome duplication scenarios. *Dev Biol* (2021) 479:99–106. doi: 10.1016/j.ydbio.2021.07.012

58. Theofanopoulou C, Gedman G, Cahill JA, Boeckx C, Jarvis ED. Universal nomenclature for oxytocin–vasotocin ligand and receptor families. *Nature* (2021) 592:747–55. doi: 10.1038/s41586-020-03040-7

59. Ocampo Daza D, Bergqvist CA, Larhammar D. The evolution of oxytocin and vasotocin receptor genes in jawed vertebrates: A clear case for gene duplications through ancestral whole-genome duplications. *Front Endocrinol* (2022) 12:792644. doi: 10.3389/fendo.2021.792644

60. Yamaguchi Y, Kaiya H, Konno N, Iwata E, Miyazato M, Uchiyama M, et al. The fifth neurohypophysial hormone receptor is structurally related to the V2-type receptor but functionally similar to V1-type receptors. *Gen Comp Endocrinol* (2012) 178:519–28. doi: 10.1016/j.ygcen.2012.07.008

61. Gimpl G, Fahrenholz F. The oxytocin receptor system: Structure, function, and regulation. *Physiol Rev* (2001) 81:629–83. doi: 10.1152/physrev.2001.81.2.629

62. Helfman G, Collette B, Facey D, Bowen BW. *The diversity of fishes: biology, evolution, and ecology. 2nd ed.* Chichester: Blackwell (2009).

63. Kline RJ, O'Connell LA, Hofmann HA, Holt GJ, Khan IA. The distribution of an AVT V1a receptor in the brain of a sex changing fish, epinephelus adscensionis. *J Chem Neuroanat* (2011) 42:72–88. doi: 10.1016/j.jchemneu.2011.06.005

64. Yokoi S, Naruse K, Kamei Y, Ansai S, Kinoshita M, Mito M, et al. Sexually dimorphic role of oxytocin in medaka mate choice. *Proc Natl Acad Sci USA* (2020) 117:4802–8. doi: 10.1073/pnas.1921446117

65. Semsar K, Kandel FLM, Godwin J. Manipulations of the AVT system shift social status and related courtship and aggressive behavior in the bluehead wrasse. *Horm Behav* (2001) 40:21–31. doi: 10.1006/hbeh.2001.1663

66. Singh O, Kumar S, Singh U, Bhute Y, Singru PS. Role of isotocin in the regulation of the hypophysiotropic dopamine neurones in the preoptic area of the catfish, clarias batrachus. *J Neuroendocrinol* (2016) 28:1–11. doi: 10.1111/jne.12441

67. Wee CL, Song E, Nikitchenko M, Herrera KJ, Wong S, Engert F, et al. Social isolation modulates appetite and avoidance behavior *via* a common oxytocinergic circuit in larval zebrafish. *Nat Commun* (2022) 13:2573. doi: 10.1038/s41467-022-29765-9

68. Blechman J, Amir-Zilberstein L, Gutnick A, Ben-Dor S, Levkowitz G. The metabolic regulator PGC-1α directly controls the expression of the hypothalamic

neuropeptide oxytocin. *J Neurosci* (2011) 31:14835–40. doi: 10.1523/JNEUROSCI.1798-11.2011

69. Coffey CM, Solleveld PA, Fang J, Roberts AK, Hong S-K, Dawid IB, et al. Novel oxytocin gene expression in the hindbrain is induced by alcohol exposure: transgenic zebrafish enable visualization of sensitive neurons. *PloS One* (2013) 8: e53991. doi: 10.1371/journal.pone.0053991

70. Canosa LF, Chang JP, Peter RE. Neuroendocrine control of growth hormone in fish. *Gen Comp Endocrinol* (2007) 151:1–26. doi: 10.1016/j.ygcen.2006.12.010

71. Pouso P, Perrone R, Silva A. Immunohistochemical description of isotocin neurons and the anatomo-functional comparative analysis between isotocin and vasotocin systems in the weakly electric fish, gymnotus omaroum. *Gen Comp Endocrinol* (2021) 313:113886. doi: 10.1016/j.ygcen.2021.113886

72. Pouso P, Radmilovich M, Silva A. An immunohistochemical study on the distribution of vasotocin neurons in the brain of two weakly electric fish, gymnotus omarorum and brachyhypopomus gauderio. *Tissue Cell* (2017) 49:257–69. doi: 10.1016/j.tice.2017.02.003

73. Arias Padilla LF, Castañeda-Cortés DC, Rosa IF, Moreno Acosta OD, Hattori RS, Nóbrega RH, et al. Cystic proliferation of germline stem cells is necessary to reproductive success and normal mating behavior in medaka. *Elife* (2021) 10:e62757. doi: 10.7554/eLife.62757

74. Rawat A, Chaube R, Joy KP. *In situ* localization of vasotocin receptor gene transcripts in the brain-pituitary-gonadal axis of the catfish heteropneustes fossilis: a morpho-functional study. *Fish Physiol Biochem* (2019) 45:885–905. doi: 10.1007/s10695-018-0590-1

75. Rawat A, Chaube R, Joy KP. Molecular cloning, sequencing, and phylogeny of vasotocin receptor genes in the air-breathing catfish heteropneustes fossilis with sex dimorphic and seasonal variations in tissue expression. *Fish Physiol Biochem* (2015) 41:509–32. doi: 10.1007/s10695-015-0026-0

76. Lema SC, Sanders KE, Walti KA. Arginine vasotocin, isotocin and nonapeptide receptor gene expression link to social status and aggression in sex-dependent patterns. *J Neuroendocrinol* (2015) 27:142–57. doi: 10.1111/jne.12239

77. Iwasaki K, Taguchi M, Bonkowsky JL, Kuwada JY. Expression of arginine vasotocin receptors in the developing zebrafish CNS. *Gene Expr Patterns* (2013) 13:335–42. doi: 10.1016/j.gep.2013.06.005

78. Rahman MS, Kline RJ, Vázquez OA, Khan IA, Thomas P. Molecular characterization and expression of arginine vasotocin V1a2 receptor in Atlantic croaker brain: Potential mechanisms of its downregulation by PCB77. *J Biochem Mol Toxicol* (2020) 34: 1–12. doi: 10.1002/jbt.22500

79. Kline RJ, Holt GJ, Khan IA. Arginine vasotocin V1a2 receptor and GnRH-I co-localize in preoptic neurons of the sex changing grouper, epinephelus adscensionis. *Gen Comp Endocrinol* (2016) 225:33–44. doi: 10.1016/j.ygcen.2015.07.013

80. Huffman LS, O'Connell LA, Kenkel CD, Kline RJ, Khan IA, Hofmann HA. Distribution of nonapeptide systems in the forebrain of an African cichlid fish, astatotilapia burtoni. *J Chem Neuroanat* (2012) 44:86–97. doi: 10.1016/j.jchemneu.2012.05.002

81. Yang W, Zhang N, Wu Y, Zhang L, Zhang L, Zhang W. Oxytocin-like signal regulates lh cells directly but not fsh cells in the ricefield eel *Monopterus albus* †. *Biol Reprod* (2021) 104:399–409. doi: 10.1093/biolre/ioaa202

82. Lyu LK, Li JS, Wang XJ, Yao YJ, Li JF, Li Y, et al. Arg-vasotocin directly activates isotocin receptors and induces COX2 expression in ovoviviparous guppies. *Front Endocrinol* (2021) 12:617580. doi: 10.3389/fendo.2021.617580

83. Lema SC, Slane MA, Salvesen KE, Godwin J. Variation in gene transcript profiles of two V1a-type arginine vasotocin receptors among sexual phases of bluehead wrasse (Thalassoma bifasciatum). *Gen Comp Endocrinol* (2012) 179:451–64. doi: 10.1016/j.ygcen.2012.10.001

84. Joy KP, Chaube R. Vasotocin – a new player in the control of oocyte maturation and ovulation in fish. *Gen Comp Endocrinol* (2015) 221:54–63. doi: 10.1016/j.ygcen.2015.02.013

85. Mennigen JA, Martyniuk CJ, Crump K, Xiong H, Zhao E, Popesku J, et al. Effects of fluoxetine on the reproductive axis of female goldfish (*Carassius auratus*). *Physiol Genomics* (2008) 35:273–82. doi: 10.1152/physiolgenomics.90263.2008

86. Popesku JT, Mennigen JA, Chang JP, Trudeau VL. Dopamine d $_1$ receptor blockage potentiates AMPA-stimulated luteinising hormone release in the goldfish: D $_1$ r blockage potentiates AMPA-stimulated LH release. *J Neuroendocrinol* (2011) 23:302–9. doi: 10.1111/j.1365-2826.2011.02114.x

87. Mennigen JA, Volkoff H, Chang JP, Trudeau VL. The nonapeptide isotocin in goldfish: Evidence for serotonergic regulation and functional roles in the control of food intake and pituitary hormone release. *Gen Comp Endocrinol* (2017) 254:38–49. doi: 10.1016/j.ygcen.2017.09.008

88. Mahlmann S, Meyerhof W, Hausmann H, Heierhorst J, Schönrock C, Zwiers H, et al. Structure, function, and phylogeny of [Arg8] vasotocin receptors from teleost fish and toad. *PNAS* (1994) 91:1342–5. doi: 10.1073/pnas.91.4.1342

89. Hausmann H, Richters A, Kreienkamp HJ, Meyerhof W, Mattes H, Lederis K, et al. Mutational analysis and molecular modeling of the nonapeptide hormone binding domains of the [Arg8] vasotocin receptor. *Proc Natl Acad Sci USA* (1996) 93:6907–12. doi: 10.1073/pnas.93.14.6907

90. Hausmann H, Meyerhof W, Zwiers H, Lederis K, Richter D. Teleost isotocin receptor: structure, functional expression, mRNA distribution and phylogeny. *FEBS Lett* (1995) 370:227–30. doi: 10.1016/0014-5793(95)00832-T

91. Landin J, Hovey D, Xu B, Lagman D, Zettergren A, Larhammar D, et al. Oxytocin receptors regulate social preference in zebrafish. *Sci Rep* (2020) 10:5435. doi: 10.1038/s41598-020-61073-4

92. Trudeau V. Neuroendocrine regulation of gonadotrophin II release and gonadal growth in the goldfish, carassius auratus. *Rev Reprod* (1997) 2:55–68. doi: 10.1530/ror.0.0020055

93. Zhang D, Xiong H, Mennigen JA, Popesku JT, Marlatt VL, Martyniuk CJ, et al. Defining global neuroendocrine gene expression patterns associated with reproductive seasonality in fish. *PloS One* (2009) 4:e5816. doi: 10.1371/journal.pone.0005816

94. Mennigen JA, Lado WE, Zamora JM, Duarte-Guterman P, Langlois VS, Metcalfe CD, et al. Waterborne fluoxetine disrupts the reproductive axis in sexually mature male goldfish, carassius auratus. *Aquat Toxicol* (2010) 100:354–64. doi: 10.1016/j.aquatox.2010.08.016

95. Sorensen PW, Pinillos M, Scott AP. Sexually mature male goldfish release large quantities of androstenedione into the water where it functions as a pheromone. *Gen Comp Endocrinol* (2005) 140:164–75. doi: 10.1016/j.ygcen.2004.11.006

96. Mangiamele LA, Keeney ADT, D'Agostino EN, Thompson RR. Pheromone exposure influences preoptic arginine vasotocin gene expression and inhibits social approach behavior in response to rivals but not potential mates. *Brain Behav Evol* (2013) 81:194–202. doi: 10.1159/000350589

97. Sokołowska E, Kleszczyńska A, Nietrzeba M, Kulczykowska E. Annual changes in brain concentration of arginine vasotocin and isotocin correspond with phases of reproductive cycle in round goby, neogobius melanostomus. *Chronobiol Int* (2015) 32:917–24. doi: 10.3109/07420528.2015.1052142

98. Kalamarz-Kubiak H, Gozdowska M, Guellard T, Kulczykowska E. . doi: 10.1242/bio.024844

99. Maruska KP, Mizobe MH, Tricas TC. Sex and seasonal co-variation of arginine vasotocin (AVT) and gonadotropin-releasing hormone (GnRH) neurons in the brain of the halfspotted goby. *Comp Biochem Physiol Part A Mol Integr* (2007) 147:129–44. doi: 10.1016/j.cbpa.2006.12.019

100. Kleszczyńska A, Sokołowska E, Kulczykowska E. Variation in brain arginine vasotocin (AVT) and isotocin (IT) levels with reproductive stage and social status in males of three-spined stickleback (Gasterosteus aculeatus). *Gen Comp Endocrinol* (2012) 175:290–6. doi: 10.1016/j.ygcen.2011.11.022

101. Kulczykowska E, Kleszczyńska A. Brain arginine vasotocin and isotocin in breeding female three-spined sticklebacks (Gasterosteus aculeatus): The presence of male and egg deposition. *Gen Comp Endocrinol* (2014) 204:8–12. doi: 10.1016/j.ygcen.2014.04.039

102. Ota Y, Ando H, Ban M, Ueda H, Urano A. Sexually different expression of neurohypophysial hormone genes in the preoptic nucleus of pre-spawning chum salmon. *Zool Sci* (1996) 13:593–601. doi: 10.2108/zsj.13.593

103. Hiraoka S, Ando H, Ban M, Ueda H, Urano A. Changes in expression of neurohypophysial hormone genes during spawning migration in chum salmon, oncorhynchus keta. *J Mol Endocrinol* (1997) 18:49–55. doi: 10.1677/jme.0.0180049

104. Makino K, Onuma TA, Kitahashi T, Ando H, Ban M, Urano A. Expression of hormone genes and osmoregulation in homing chum salmon: A minireview. *Gen Comp Endocrinol* (2007) 152:304–9. doi: 10.1016/j.ygcen.2007.01.010

105. Aubin-Horth N, Letcher BH, Hofmann HA. Gene-expression signatures of Atlantic salmon's plastic life cycle. *Gen Comp Endocrinol* (2009) 163:278–84. doi: 10.1016/j.ygcen.2009.04.021

106. Guiry A, Flynn D, Hubert S, O'Keeffe AM, LeProvost O, White SL, et al. Testes and brain gene expression in precocious male and adult maturing Atlantic salmon (Salmo salar). *BMC Genomics* (2010) 11:211. doi: 10.1186/1471-2164-11-211

107. Ota Y, Ando H, Ueda H, Urano A. Differences in seasonal expression of neurohypophysial hormone genes in ordinary and precocious Male masu salmon. *Gen Comp Endocrinol* (1999) 116:40–8. doi: 10.1006/gcen.1999.7344

108. Maruska KP. Social regulation of reproduction in male cichlid fishes. *Gen Comp Endocrinol* (2014) 207:2–12. doi: 10.1016/j.ygcen.2014.04.038

109. Greenwood AK, Wark AR, Fernald RD, Hofmann HA. Expression of arginine vasotocin in distinct preoptic regions is associated with dominant and subordinate behaviour in an African cichlid fish. *Proc R Soc B* (2008) 275:2393–402. doi: 10.1098/rspb.2008.0622

110. Bass AH, Grober MS. Social and neural modulation of sexual plasticity in teleost fish. *Brain Behav Evol* (2001) 57:293–300. doi: 10.1159/000047247

111. Semsar K, Godwin J. Social influences on the arginine vasotocin system are independent of gonads in a sex-changing fish. *J Neurosci* (2003) 23:4386–93. doi: 10.1523/JNEUROSCI.23-10-04386.2003

112. Godwin J, Sawby R, Warner RR, Crews D, Grober MS. Hypothalamic arginine vasotocin mRNA abundance variation across sexes and with sex change in a coral reef fish. *Brain Behav Evol* (2000) 55:77–84. doi: 10.1159/000006643

113. Grober MS, Sunobe T. Serial adult sex change involves rapid and reversible changes in forebrain neurochemistry. *Neuroreport* (1996) 7:2945–9. doi: 10.1097/00001756-199611250-00029

114. Kulczykowska E, Kalamarz-Kubiak H, Nietrzeba M, Gozdowska M. Brain nonapeptide and gonadal steroid responses to deprivation of heterosexual contact in the black molly. *Biol Open* (2015) 4:69–78. doi: 10.1242/bio.20149597

115. Liu H, Lamm MS, Rutherford K, Black MA, Godwin JR, Gemmell NJ. Large-Scale transcriptome sequencing reveals novel expression patterns for key sex-related genes in a sex-changing fish. *Biol Sex Differ* (2015) 6:26. doi: 10.1186/s13293-015-0044-8

116. Black MP, Reavis RH, Grober MS. Socially induced sex change regulates forebrain isotocin in lythrypnus dalli. *NeuroReport* (2004) 15:185–9. doi: 10.1097/00001756-200401190-00036

117. Godwin J, Thompson R. Nonapeptides and social behavior in fishes. *Horm Behav* (2012) 61:230–8. doi: 10.1016/j.yhbeh.2011.12.016

118. Yokoi S, Okuyama T, Kamei Y, Naruse K, Taniguchi Y, Ansai S, et al. An essential role of the arginine vasotocin system in mate-guarding behaviors in triadic relationships of medaka fish (Oryzias latipes). *PloS Genet* (2015) 11:e1005009. doi: 10.1371/journal.pgen.1005009

119. Salek SJ, Sullivan CV, Godwin J. Arginine vasotocin effects on courtship behavior in male white perch (Morone americana). *Behav Brain Res* (2002) 133:177–83. doi: 10.1016/S0166-4328(02)00003-7

120. Santangelo N, Bass AH. Individual behavioral and neuronal phenotypes for arginine vasotocin mediated courtship and aggression in a territorial teleost. *Brain Behav Evol* (2010) 75:282–91. doi: 10.1159/000316867

121. Pouso P, Cabana Á, Goodson JL, Silva A. Preoptic area activation and vasotocin involvement in the reproductive behavior of a weakly pulse-type electric fish, brachyhypopomus gauderio. *Front Integr Neurosci* (2019) 13:37. doi: 10.3389/fnint.2019.00037

122. Perrone R, Silva A. Vasotocin increases dominance in the weakly electric fish brachyhypopomus gauderio. *J Physiol-Paris* (2016) 110:119–26. doi: 10.1016/j.jphysparis.2016.12.004

123. Carneiro LA, Oliveira RF, Canário AVM, Grober MS. The effect of arginine vasotocin on courtship behaviour in a blenniid fish with alternative reproductive tactics. *Fish Physiol Biochem* (2003) 28:241–3. doi: 10.1023/B:FISH.0000030542.31395.8a

124. DeAngelis R, Gogola J, Dodd L, Rhodes JS. Opposite effects of nonapeptide antagonists on paternal behavior in the teleost fish amphiprion ocellaris. *Horm Behav* (2017) 90:113–9. doi: 10.1016/j.yhbeh.2017.02.013

125. DeAngelis R, Dodd L, Rhodes J. Nonapeptides mediate trade-offs in parental care strategy. *Horm Behav* (2020) 121:104717. doi: 10.1016/j.yhbeh.2020.104717

126. O'Connell LA, Matthews BJ, Hofmann HA. Isotocin regulates paternal care in a monogamous cichlid fish. *Horm Behav* (2012) 61:725–33. doi: 10.1016/j.yhbeh.2012.03.009

127. Wacker DW, Ludwig M. Vasopressin, oxytocin, and social odor recognition. *Horm Behav* (2012) 61(3):259–65. doi: 10.1016/j.yhbeh.2011.08.014

128. Dunlap KD, Koukos HM, Chagnaud BP, Zakon HH, Bass AH. Vocal and electric fish: Revisiting a comparison of two teleost models in the neuroethology of social behavior. *Front Neural Circuits* (2021) 15:713105. doi: 10.3389/fncir.2021.713105

129. Somoza GM, Yu KL, Peter RE. Serotonin stimulates gonadotropin release in female and male goldfish, *Carassius auratus* l. *Gen Comp Endocrinol* (1988) 72:374–82. doi: 10.1016/0016-6480(88)90159-1

130. Saito D, Hasegawa Y, Urano A. Gonadotropin-releasing hormones modulate electrical activity of vasotocin and isotocin neurons in the brain of rainbow trout. *Neurosci Lett* (2003) 351:107–10. doi: 10.1016/j.neulet.2003.08.017

131. Kanda S, Akazome Y, Mitani Y, Okubo K, Oka Y. Neuroanatomical evidence that kisspeptin directly regulates isotocin and vasotocin neurons. *PloS One* (2013) 8:e62776. doi: 10.1371/journal.pone.0062776

132. Zmora N, Stubblefield JD, Wong T-T, Levavi-Sivan B, Millar RP, Zohar Y. Kisspeptin antagonists reveal kisspeptin 1 and kisspeptin 2 differential regulation of reproduction in the teleost, morone saxatilis. *Biol Reprod* (2015) 93:1–12. doi: 10.1095/biolreprod.115.131870

133. Trudeau VL, Somoza GM. Multimodal hypothalamo-hypophysial communication in the vertebrates. *Gen Comp Endocrinol* (2020) 293:113475. doi: 10.1016/j.ygcen.2020.113475

134. Chaube R, Singh RK, Joy KP. Estrogen regulation of brain vasotocin secretion in the catfish heteropneustes fossilis: An interaction with catecholaminergic system. *Gen Comp Endocrinol* (2012) 175:206–13. doi: 10.1016/j.ygcen.2011.11.012

135. Yamashita J, Kawabata Y, Okubo K. Expression of isotocin is male-specifically up-regulated by gonadal androgen in the medaka brain. *J Neuroendocrinol* (2017) 29:e12545. doi: 10.1111/jne.12545

136. Da Fonte DF, Xing L, Mikwar M, Trudeau VL. Secretoneurin-a inhibits aromatase b (cyp19a1b) expression in female goldfish (*Carassius auratus*) radial glial cells. *Gen Comp Endocrinol* (2018) 257:106–12. doi: 10.1016/j.ygcen.2017.04.014

137. Popesku JT, Martyniuk CJ, Mennigen J, Xiong H, Zhang D, Xia X, et al. The goldfish (Carassius auratus) as a model for neuroendocrine signaling. *Mol Cell Endocrinol* (2008) 293:43–56. doi: 10.1016/j.mce.2008.06.017

138. Singh U, Kumar S, Singru PS. Interaction between dopamine- and isotocin-containing neurones in the preoptic area of the catfish, clarias batrachus: role in the regulation of luteinising hormone cells. *J Neuroendocrinol* (2012) 24:1398–411. doi: 10.1111/j.1365-2826.2012.02350.x

139. Robinson G, Evans JJ, Catt KJ. Oxytocin stimulates LH production by the anterior pituitary gland of the rat. *J Endocrinol* (1992) 132:277–83. doi: 10.1677/joe.0.1320277

140. Evans JJ, Robinson G, Catt KJ. Luteinizing hormone response to oxytocin is steroid-dependent. *Neuroendocrinology* (1992) 55:538–43. doi: 10.1159/000126167

141. Rodríguez M, Specker JL. *In vitro* effects of arginine vasotocin on testosterone production by testes of rainbow trout (Oncorhynchus mykiss). *Gen Comp Endocrinol* (1991) 83:249–57. doi: 10.1016/0016-6480(91)90028-5

142. Singh V, Joy KP. Relative *in vitro* seasonal effects of vasotocin and isotocin on ovarian steroid hormone levels in the catfish heteropneustes fossilis. *Gen Comp Endocrinol* (2009) 162:257–64. doi: 10.1016/j.ygcen.2009.03.024

143. Mishra A, Joy KP. Effects of gonadotrophin *in vivo* and 2-hydroxyoestradiol-17β *in vitro* on follicular steroid hormone profile associated with oocyte maturation in the catfish heteropneustes fossilis. *J Endocrinol* (2006) 189:341–53. doi: 10.1677/joe.1.06686

144. Nagahama Y. Endocrine regulation of gametogenesis in fish. *Int J Dev Biol* (1994) 38:217–29.

145. Singh V, Joy KP. Vasotocin induces final oocyte maturation and ovulation through the production of a maturation-inducing steroid in the catfish heteropneustes fossilis. *Gen Comp Endocrinol* (2011) 174(1):15–21. doi: 10.1016/j.ygcen.2011.07.009

146. Sirotkin AV, Gerasimova GG, Golubev AK, Dmitriev VB. The effect of arginine-vasotocin on the production of steroid hormones by mouse, cow, and chicken ovarian tissues. *vitro. BioMed Sci* (1990) 1:308–10.

147. Lister AL, van der Kraak G. An investigation into the role of prostaglandins in zebrafish oocyte maturation and ovulation. *Gen Comp Endocrinol* (2008) 159:46–57. doi: 10.1016/j.ygcen.2008.07.017

148. Bradley JA, Goetz FW. The inhibitory effects of indomethacin, nordihydroguaiaretic acid, and pyrrolidinedithiocarbamate on ovulation and prostaglandin synthesis in yellow perch (Perca flavescens) follicle incubates. *Prostaglandins* (1994) 48:11–20. doi: 10.1016/0090-6980(94)90092-2

149. Patiño R, Yoshizaki G, Bolamba D, Thomas P. Role of arachidonic acid and protein kinase c during maturation-inducing hormone-dependent meiotic resumption and ovulation in ovarian follicles of Atlantic croaker. *Biol Reprod* (2003) 68:516–23. doi: 10.1095/biolreprod.102.009662

150. Joy KP, Singh V. Functional interactions between vasotocin and prostaglandins during final oocyte maturation and ovulation in the catfish heteropneustes fossilis. *Gen Comp Endocrinol* (2013) 186:126–35. doi: 10.1016/j.ygcen.2013.02.043

151. Cerdà J. Molecular pathways during marine fish egg hydration: the role of aquaporins. *J Fish Biol* (2009) 75:2175–96. doi: 10.1111/j.1095-8649.2009.02397.x

152. Singh V, Joy KP. An involvement of vasotocin in oocyte hydration in the catfish heteropneustes fossilis: A comparison with effects of isotocin and hCG. *Gen Comp Endocrinol* (2010) 166:504–12. doi: 10.1016/j.ygcen.2010.02.020

153. Chaube R, Chauvigné F, Tingaud-Sequeira A, Joy KP, Acharjee A, Singh V, et al. Molecular and functional characterization of catfish (Heteropneustes fossilis) aquaporin-1b: changes in expression during ovarian development and hormone-induced follicular maturation. *Gen Comp Endocrinol* (2011) 170:162–71. doi: 10.1016/j.ygcen.2010.10.002

154. Acharjee A, Chaube R, Joy KP, Cerda J. Hormonal regulation of aquaporin-1ab in heteropneustes fossilis oocytes *in vitro*. *Indian J Sci Technol* (2011) 4: 165–166.

155. Wilhelmi AE, GE P, Sawyer WH. Initiation of the spawning reflex response in fundulus by the administration of fish and mammalian neurohypophysial

preparations and synthetic oxytocin. *Endocrinology* (1955) 57:243–52. doi: 10.1210/endo-57-2-243

156. Viveiros ATM, Jatzkowski A, Komen J. Effects of oxytocin on semen release response in African catfish (Clarias gariepinus). *Theriogenology* (2003) 59:1905–17. doi: 10.1016/s0093-691x(02)01290-6

157. Wisdom KS, Bhat IA, Pathan MA, CT I, Kumar P, Babu PG, et al. Teleost nonapeptides, isotocin and vasotocin administration released the milt by abdominal massage in Male catfish, clarias magur. *Front Endocrinol* (2022) 13:899463. doi: 10.3389/fendo.2022.899463

158. Venkatesh B, Tan CH, Lam TJ. Prostaglandins and teleost neurohypophyseal hormones induce premature parturition in the guppy, poecilia reticulata. *Gen Comp Endocrinol* (1992) 87:28–32. doi: 10.1016/0016-6480(92)90146-b

159. Xiong S, Tian J, Ge S, Li Z, Long Z, Guo W, et al. The microRNA-200 cluster on chromosome 23 is required for oocyte maturation and ovulation in zebrafish. *Biol Reprod* (2020) 103:769–78. doi: 10.1093/biolre/ioaa125

160. Peter RE. The preoptic nucleus in fishes: A comparative discussion of function-activity relationships. *Amer Zool* (1977) . 17:775–85. doi: 10.1093/icb/17.4.775

161. Zohar Y. Fish reproductive biology - reflecting on five decades of fundamental and translational research. *Gen Comp Endocrinol* (2021) 300:113544. doi: 10.1016/j.ygcen.2020.113544

162. Arcand-Hoy LD, Benson WH. Fish reproduction an ecologically relevant indicator of fish endocrine disruption. *Env Toxicol Chem* (1998) 17:49–57. doi: 10.1002/etc.5620170108

163. Ram RN, Joy KP. Mercurial induced changes in the hypothalamo-neurohypophysical complex in relation to reproduction in the teleostean fish, channa punctatus (Bloch). *Bull Environ Contam Toxicol* (1988) 41:329–36. doi: 10.1007/BF01688875

164. Mennigen JA, Zamora JM, Chang JP, Trudeau VL. Endocrine disrupting effects of waterborne fluoxetine exposure on the reproductive axis of female goldfish, carassius auratus. *Comp Biochem Physiol Part-C: Toxicol Pharmacol* (2017) 202:70–8. doi: 10.1016/j.cbpc.2017.08.003

165. Parolini M, Ghilardi A, De Felice B, Del Giacco L. Environmental concentration of fluoxetine disturbs larvae behavior and increases the defense response at molecular level in zebrafish (Danio rerio). *Environ Sci pollut Res Int* (2019) 26:34943–52. doi: 10.1007/s11356-019-06619-4

166. Wong RY, Oxendine SE, Godwin J. Behavioral and neurogenomic transcriptome changes in wild-derived zebrafish with fluoxetine treatment. *BMC Genomics* (2013) 14:348. doi: 10.1186/1471-2164-14-348

167. Semsar K, Perreault HAN, Godwin J. Fluoxetine-treated male wrasses exhibit low AVT expression. *Brain Res* (2004) 1029:141–7. doi: 10.1016/j.brainres.2004.09.030

168. Cantor JM, Binik YM, Pfaus JG. Chronic fluoxetine inhibits sexual behavior in the male rat: reversal with oxytocin. *Psychopharmacol (Berl)* (1999) 144:355–62. doi: 10.1007/s002130051018

169. de Jong TR, Veening JG, Olivier B, Waldinger MD. Oxytocin involvement in SSRI-induced delayed ejaculation: a review of animal studies. *J Sex Med* (2007) 4:14–28. doi: 10.1111/j.1743-6109.2006.00394.x

170. Jørgensen HS. Studies on the neuroendocrine role of serotonin. *Dan Med Bull* (2007) 54:266–88.

171. Mennigen JA, Stroud P, Zamora JM, Moon TW, Trudeau VL. Pharmaceuticals as neuroendocrine disruptors: lessons learned from fish on Prozac. *J Toxicol Environ Health B Crit Rev* (2011) 14:387–412. doi: 10.1080/10937404.2011.578559

172. Wang Z, Mao K, Du W, Cai M, Zhang Z, Li X. Diluted concentrations of methamphetamine in surface water induce behavior disorder, transgenerational toxicity, and ecosystem-level consequences of fish. *Water Res* (2020) 184:116164. doi: 10.1016/j.watres.2020.116164

173. León-Olea M, Martyniuk CJ, Orlando EF, Ottinger MA, Rosenfeld C, Wolstenholme J, et al. Current concepts in neuroendocrine disruption. *Gen Comp Endocrinol* (2014) 203:158–73. doi: 10.1016/j.ygcen.2014.02.005

174. Salahinejad A, Naderi M, Attaran A, Meuthen D, Niyogi S, Chivers DP. Effects of chronic exposure to bisphenol-s on social behaviors in adult zebrafish: Disruption of the neuropeptide signaling pathways in the brain. *Environ pollut* (2020) 262:113992. doi: 10.1016/j.envpol.2020.113992

175. Salahinejad A, Attaran A, Meuthen D, Rachamalla M, Chivers DP, Niyogi S. Maternal exposure to bisphenol s induces neuropeptide signaling dysfunction and oxidative stress in the brain, and abnormal social behaviors in zebrafish (Danio rerio) offspring. *Sci Total Environ* (2022) 830:154794. doi: 10.1016/j.scitotenv.2022.154794

176. Naderi M, Puar P, JavadiEsfahani R, Kwong RWM. Early developmental exposure to bisphenol a and bisphenol s disrupts socio-cognitive function, isotocin equilibrium, and excitation-inhibition balance in developing zebrafish. *Neurotoxicology* (2022) 88:144–54. doi: 10.1016/j.neuro.2021.11.009

177. Naderi F, Miguez JM, Soengas JL, López-Patiño MA. SIRT1 mediates the effect of stress on hypothalamic clock genes and food intake regulators in rainbow trout, oncorhynchus mykiss. *Comp Biochem Physiol A Mol Integr Physiol* (2019) 235:102–11. doi: 10.1016/j.cbpa.2019.05.021

178. Kalamarz-Kubiak H. Endocrine-disrupting compounds in fish physiology, with emphasis on their effects on the arginine Vasotocin/Isotocin system. *Endocr Metab Immune Disord Drug Targets* (2021) 22(7):738–47. doi: 10.2174/1871530321666210202150947

179. Patisaul HB. Endocrine disruption of vasopressin systems and related behaviors. *Front Endocrinol (Lausanne)* (2017) 8:134. doi: 10.3389/fendo.2017.00134

180. Reilly MP, Kunkel MN, Thompson LM, Zentay A, Weeks CD, Crews D, et al. Effects of endocrine-disrupting chemicals on hypothalamic oxytocin and vasopressin systems. *J Exp Zool A Ecol Integr Physiol* (2022) 337:75–87. doi: 10.1002/jez.2475