Reproductive Consequences of CRISPR/Cas9-Based *avp* Knock-Out in Zebrafish (*Danio rerio)*

DIVYA RAMACHANDRAN

Thesis submitted to the University of Ottawa
in partial Fulfillment of the requirements for the
the Master of Science in Biology
with a specialization in Science, Society and Policy

Department of Science
Biology
University of Ottawa

Abstract

The nonapeptide family of hormones is deeply conserved in evolution. In teleost fishes, as in all vertebrates, two nonapeptide families exist. These are vasotocin (*avp*) and oxytocin (*oxt*). While vasotocin has been shown to regulate individual aspects of reproductive physiology in several teleost species, an integrative assessment of its role on male and female reproduction is currently lacking even in widely used fish models, such as the zebrafish (*Danio rerio*). Taking advantage of the genetic tractability of the zebrafish, and its emerging status as model to study reproductive physiology, I generated *avp* $^{-/-}$ mutants using a CRISPR/Cas9 based approach to determine reproductive consequences in female and male zebrafish. Following the identification of a female-specific reproductive phenotype which manifests as a reduction in oocyte release and decreased quivering behaviour, I investigated the potential mechanistic basis at the level of the gonad. In *avp* $^{-/-}$ ovaries, significantly fewer eggs were present compared to WT fishes. When comparing the distribution of oocyte maturation stages, a significantly lower percentage of stage I and higher percentage of stage V oocytes was present in *avp*$^{-/-}$ ovaries. The altered distribution in oocyte maturation stages coincided with significant decreases in ovarian transcript abundance of *nanos2*, a germ-cell specific marker suggesting a possible role for Avp in germ-cell maintenance. Additionally, I observed a decrease in the ovarian concentration of the prostaglandin $PGF_{2\alpha,}$ which coincided with a reduction in ovarian transcript abundance of *pla2g4ab*, a paralogue of the phospholipase A2 involved in mobilizing arachidonic acid, a precursor of $PGF_{2\alpha,}$. Together, these finding suggests a role for Avp in $PGF_{2\alpha}$ -mediated ovulation. Because Avp has pleiotropic effects and may thus affect female reproductive physiology indirectly, we assessed somatic growth, a key regulator of sexual maturation in zebrafish, as well as aspects of the endocrine stress axis known to affect oocyte growth in *avp* $^{-/-}$ mutants. While *avp* $^{-/-}$ mutants did not exhibit differences in somatic growth up to sexual maturation or GSI, mutants exhibited hypercortisolism. While other zebrafish knock-out mutants exhibiting persistent hypercortisolism do not share the observed reproductive phenotype, future studies investigating potential contributions of pleiotropic Avp effects are nevertheless warranted. Overall, I demonstrate that *avp*, while not essential, affects female reproductive success, at least

in part by regulating oocyte maturation. This finding is in line with the recent findings from other vertebrate and invertebrate species, suggesting an evolutionarily ancient role in these processes. It is anticipated that such novel insights into the regulation of female oocyte maturation have in addition to increasing our understanding of female reproduction, translational potential for captive breeding (aquaculture, species conservation) and ecotoxicology (insight into mode of action of specific EDCs).

Résumé

La famille des hormones nonapeptidiques est profondément conservée dans evolution. Chez les poissons téléostéens, comme chez tous les vertébrés, deux familles nonapeptidiques existent. Ce sont la vasotocine (*avp*) et l'oxytocine (*oxt*). Bien qu'il ait été démontré que la vasotocine régule des aspects individuels de la physiologie de la reproduction chez plusieurs espèces de téléostéens, une évaluation intégrée de son rôle sur la reproduction mâle et femelle fait actuellement défaut, même chez des modèles de poissons largement utilisés, tels que le poisson zèbre (*Danio rerio*). Profitant de la tractabilité génétique du poisson zèbre, et de son statut émergent de modèle pour étudier la physiologie de la reproduction, j'ai généré des mutants *avp* $^{-/-}$ en utilisant une approche basée sur CRISPR/Cas9 pour déterminer les conséquences sur la reproduction chez les femelles et les mâles du poisson zèbre. Suite à l'identification d'un phénotype reproducteur spécifique à la femelle qui se manifeste par une réduction de la libération des ovocytes et une diminution du comportement de frémissement, j'ai étudié la base mécanistique potentielle au niveau de la gonade. Dans les ovaires *avp* $^{-/-}$, il y avait significativement moins d'ovules que chez les poissons WT. En comparant la distribution des stades de maturation des ovocytes, un pourcentage significativement plus faible d'ovocytes de stade I et un pourcentage plus élevé d'ovocytes de stade V étaient présents dans les ovaires *avp* $^{-/-}$. La distribution altérée des stades de maturation des ovocytes coïncide avec des diminutions significatives de l'abondance des transcriptions ovariennes de *nanos2*, un marqueur spécifique des cellules germinales, suggérant un rôle possible de l'Avp dans la maintenance des cellules germinales. En outre, j'ai observé des diminutions des concentrations ovariennes de $PGF_{2\alpha}$ qui ont coïncidé avec une réduction de l'abondance des transcriptions ovariennes de *pla2g4ab*, un paralogue de la phospholipase A2 impliquée dans la mobilisation de l'acide arachidonique, un précurseur de $PGF_{2\alpha}$, à partir des phospholipides membranaires. L'ensemble de ces résultats suggère un rôle de l'Avp dans l'ovulation médiée par le $PGF_{2\alpha}$. Comme l'Avp a des effets pléiotropiques et peut donc affecter indirectement la physiologie de la reproduction chez la femelle, nous avons évalué la croissance somatique, un régulateur clé de la maturation

sexuelle chez le poisson zèbre, ainsi que les aspects de l'axe du stress endocrinien connus pour affecter la croissance des ovocytes chez les mutants *avp* $^{-/-}$. Alors que les mutants *avp* $^{-/-}$ ne présentaient pas de différences dans la croissance somatique jusqu'à la maturation sexuelle ou le GSI, les mutants présentaient un hypercortisolisme. Bien que d'autres mutants knock-out de poisson zèbre présentant un hypercortisolisme persistant ne partagent pas le phénotype reproducteur observé, des études futures examinant les contributions potentielles des effets pléiotropiques de l'Avp sont néanmoins justifiées. Dans l'ensemble, je démontre que l'Avp, bien que non essentielle, affecte le succès reproducteur des femelles, au moins en partie en régulant la maturation des ovocytes. Cette découverte est en accord avec les résultats récents d'autres espèces vertébrées et invertébrées, suggérant un rôle ancien dans ces processus. On s'attend à ce que ces nouvelles connaissances sur la régulation de la maturation des ovocytes femelles aient, en plus d'améliorer notre compréhension de la reproduction féminine, un potentiel de traduction pour la reproduction en captivité (aquaculture, conservation des espèces) et l'écotoxicologie (un aperçu mécaniste de perturbateurs endocriniens).

Acknowledgements

First and foremost, I would like to thank my supervisor Dr. Jan Mennigen who supported me wholeheartedly and always showed his support, dedication, and confidence in me during these last two years. I am extremely lucky to have a supervisor who helped me through both lab-related and personal issues for which I am extremely appreciative. I am extremely grateful for all of your kindness and support throughout my degree. I would also like to extend my thanks to my thesis advisory committee members Dr. Vance Trudeau and Dr. John Lewis who showed keen interest in my project and provided me with very helpful feedback and suggestions, as well as Dr. Kathleen Gilmour for joining my defence committee.

To my lab mates, both past and present, thank you for your endless help from experimental issues to little quick questions when I feel the need to double check if what I'm doing is correct. It has been a pleasure to get to know each member of the lab and I'll cherish the conversations and all the support throughout the past two years. I would also like to thank specifically, Kusum Sharma, Rita Wehbe, and Brooke Kattenbusch for all your time and energy helping me run experiments. I would also like to extend my sincerest thanks to Udeesha Erandani and Katherine Shaw for showing me the ropes when I first came to uOttawa and helping me to understand the complexities when genotyping fish as well as answering all my questions with experimental designs. I would also like to thank Vishal Saxena who helped design and microinject zebrafish without which the creation of the mutant lines would not have been possible. As well, thank you to the Gilmour lab for help and advice on zebrafish cortisol assay. I'd like to thank Ethan Ramsay and the entire ACVS staff for the excellent care and maintenance of my precious fish.

Finally, I would like to thank my family for all their support, their love and for being always enthusiastic about my project and throughout my academic journey and my friends, old and new, for making moving to a new city and school during the pandemic not as intimidating. I will cherish all of the food Fridays, F1 Sundays, and all the good times we have shared while living in Ottawa, it has truly been so much fun!

Table of Contents

Chapter 3: Assessment of pleiotropic roles of Avp with potential implications for the reproductive phenotype observed in female *avp* $^{-/-}$ mutants

List of Figures

Chapter 1:

Chapter 2:

Appendix

List of Tables

Chapter 1:

Chapter 2:

List of commonly used abbreviations

11-KT	11-Ketotestorone
11β-hsd2	11β-hydroxysteroid dehydrogenase type 2
17, 20β-DP	17α,20β-dihydroxy-4-pregnen-3,-dione
17-OHP4	17-hydroxyprogesterone
17α, 20-P	17α,20β-dihydroxy-4-pregnen-3-one
4R WGD	Tetraploidization
AA	Amino acid
ACTH	Adrenocorticotrophic hormone
aqp1b	Ovary-specific aquaporin 1ab
ar	Androgen receptor
AVP	Arginine vasopressin
AVPR	Arginine vasopressin receptor
AVPR1 antagonist	deamino-Pen^{1}, O-Me-Try^{2}, Arg^{8} vasopressin
AVPR2 antagonist	1-adamantane acetyl O-Et-D-Try^{2}Val^{4}, Abu^{6}, Arg^{8,9} vasopressin
BMI	Body-mass index
BPA	Bisphenol A
BPS	Bisphenol S
ciVP	[Ile^{4}]-VP
CRH	Corticotropin-releasing hormone
Cytb5	Cytochrom b5
ddx4	DEAD (Asp-Glu-Ala-Asp) box polypeptide 4
dpf	Days post fertilization
E2	Estradiol
E2	17-β-estradiol
EGFR	Epidermal growth factor receptor
ELISA assay	Enzyme-linked immunosorbent assay
EOD	Electric organ discharge
ER	Estrogen receptor
EtOH	Ethanol
FLX	Fluoxetine
FOM	Follicular and oocyte maturation
FSH	Follicle stimulating hormone
GABA	Gamma-aminobutyric acid
GnrH	Gonadotropin-releasing hormone
GSC	Germinal stem cells
GSI	Gonadal-stomatic index
GVBD	Germinal vesicle breakdown
hCG	Human Chorionic gonadotropin
HPG	Hypothalamic pituitary gonadal axis
HPI	Hypothalamic pituitary interrenal axis

HPLC	High-performance liquid chromatography
i.c.v.	Intracerebroventricular
i.p.	Intraperitoneal
LDT	Light and dark test
LH	Luteinizing hormone
lhcgr	Luteinizing hormone / choriogonadotropin receptor
MIS	Maturation-inducing steroid
mpf	Months post fertilization
mPR	Membrane progestin receptor
nanos2	Nanos homolog 2
NPO	Nucleus preopticus
NTT	Novel tank test
OXT	Oxytocin
OXTR	Oxytocin receptor
P4	Progesterone
PCB-77	Polychlorinated biphenyl congener 3,3′,4,4′-tetrachlorobiphenyl
PGE2	Prostaglandin E2
PGF2α	Prostaglandin F2α
pgr	Progesterone receptor
pgrmc1	Progesterone receptor membrane component 1
pgrmc2	Progesterone receptor membrane component 2
PGs	Prostaglandins
pla2g4ab	Phospholipase A2, group IV Ab (cytosolic, calcium-dependent)
POA	Preoptic Area
ptgs2α	Prostaglandin-endoperoxide synthase 2a
star	Steroidogenic acute regulatory protein
T	Testosterone
TALEN	Transcription activator-like effector nucleases
vasa	refer to ddx4
WT	Wildtype
ZGN	Zinc finger nucleases

Chapter I
Introduction

Partially based on:

Mennigen JA, **Ramachandran D**, Shaw K, Chaube R, Joy K, Trudeau VL. Reproductive roles of the vasopressin/oxytocin neuropeptide families in teleost fishes. *Frontiers in Endocrinology*, accepted.

Statement of contribution:

I conducted a detailed literature review to critically assess the current state of knowledge regarding the role of nonapeptides in teleost reproduction including courtship behaviours. All sections that are included in this chapter were initially drafted by me with the exception of Section 2.3:Endocrine and paracrine roles of nonapeptides in gonads, which were initially drafted by Dr. Chaube and Dr. Joy. I furthermore contributed to the retrieval of teleost nonapeptide and nonapeptide receptor genome locus information, mRNA coding sequences and derived AA sequences and their *in silico* analyses to provide a detailed analysis of the diversity and evolution of the teleost nonapeptide and nonapeptide receptor repertoire. While the latter results are not included in this chapter, the **Supplemental Figures 1.1 and 1.2** provide an overview of the most significant findings. In this chapter and all other chapters, the nomenclature used to designate teleost genes and their protein products was based on the recent Zebrafish Information Network (ZFIN, www.zfin.org) nomenclature, which uses a*vp*/Avp and *oxt*/Oxt.

1. Teleost fishes and their reproductive biology

1.1. What are teleosts?

Teleosts (Greek 'teleios' = complete and 'osteon' = bone) constitute a large and highly diverse infraclass of ray-finned fishes or Actinopterygii. Including more than 26.000 different species, teleosts make up ~96% of all extant fish species and half of all extant vertebrates in the world (Helfman, G.S., Collette, B.B. and Facey, 2009). Having rayed fins, a swim bladder and jaws, this infraclass is characterized by movable premaxilla and corresponding jaw modifications, which differentiate them from other (basal) bony fishes. The habitat ranges of teleosts are diverse and species in this infraclass are found throughout all aquatic environments (Wootton, 1998; Helfman, G.S., Collette, B.B. and Facey, 2009). A key event in the evolution of the teleost fishes was a whole genome duplication event (teleost-specific genome duplication) followed, in some instances, by subsequent lineage-specific genome duplication events, such as, for example, in salmonids (Amores et al., 1998; Finn and Kristoffersen, 2007; Santini et al., 2009). It is believed that this genomic complexity has provided the molecular substrate for the high diversity in teleosts. Perhaps unsurprisingly given their species richness and wide distribution in diverse habitats, teleost fishes are diverse in with regard to their reproductive biology, providing excellent (comparative) models to study reproductive physiology and behaviour (Smith and Wootton, 2016). This diversity is briefly reviewed here.

1.2. Teleost reproduction

Teleost fishes exhibit significant plasticity in terms of their reproductive biology and life history traits. Most teleost (about 88%) are gonochorists, meaning sex is determined by genetic sex determination or by environmental conditions and individuals are categorized as either male or female (Patzner, 2008; Shaw et al., 2016). 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 (Bass and Groberb, 2001; Shaw et al., 2016; Trudeau, 2021). Examples of protandrous, protogynous, and bidirectional sex changing teleosts are the clownfish, *Amphiprion spp.*, the bluehead wrasse, *Thalassoma bifasciatium*, and the monogamous coral-dwelling

gobies, *Gobiodon histrio*, respectively. Teleost fishes are mostly oviparous but can be viviparous, in which case eggs are fertilized and allowed to develop in the ovaries of the females, thus providing protection of the developing eggs (Shaw et al., 2016). This is exemplified by the guppy, *Poecilia reticulata*. Though rare, in one species, the mangrove killifish, *Kryptolebias marmoratus*, alternative forms to sexual reproduction exist in that under specific conditions, only the female genome is transmitted between generations of females, a process termed parthenogenesis (Tatarenkov et al., 2009; Shaw et al., 2016). In teleosts, there are usually two types of spawning dynamics that take place: semelparity and iteroparity (Roff, 1992; Stearns, 1992; Wootton, 1998; Shaw et al., 2016). Semelparity refers to the condition that strong physiological changes associated with reproduction will result in mortality following breeding, for example in migrating salmonids. In contrast, iteroparity refers to a reproductive process in which individuals generally survive post-reproduction. During the breeding season, female teleosts may either release all their eggs during a short period of time, after which no more eggs are released during that breeding season. Conversely, some female teleosts can release eggs in intervals throughout the breeding season. The latter can be further distinguished into determinate breeders, in which a set number of eggs are present in the ovaries for that breeding season, and indeterminate breeders, in which the number of eggs present in the ovary can be replenished during the breeding season (Shaw et al., 2016). Teleost show great diversity in social mating behaviour ranging in from more promiscuous behaviour to monogamy (Shaw et al., 2016). Once sexually mature, males and females may differ in appearance, either permanently or only in the breeding season (Helfman, Collette, and Facey, 2009; Wootton and Smith, 2015; Shaw et al., 2016). The majority of teleost show no post-fertilization care; however if present, the most common form is that of paternal care (Shaw et al., 2016), as described for the monogamous convict cichlid, *Amatitlania nigrofasciata* . Maternal care, has, however has also been reported in cichlids, for example in the form of egg incubation in the buccal cavity in some African cichlid species including the Tanganyikan cichlid, *Neolamprologus furcifer* (Kolm N., 2009; Butler et al., 2020).

1.3. The hypothalamic-pituitary-gonadal (HPG) axis

As in all vertebrates, the role of the (neuro)endocrine reproductive axis is fundamental in the regulation of reproduction in teleosts. The endocrine axis controlling reproduction comprises of three main tissues: the hypothalamus, pituitary, and the gonads. It is thus termed the hypothalamic-pituitary-gonadal (HPG) axis. From the hypothalamus, the release of gonadotropin-releasing hormone (GnrH) to the pituitary stimulates the release of gonadotropins such as luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Unlike in other vertebrates which possess a median eminence, hypophysiotropic neurons, such as gonadotropin releasing hormone (GnrH) neurons, directly innervate highly regionalized gonadotropic cells containing either LH or FSH in the pituitary *pars proximalis distalis* (PPD) in teleosts. This is thought to represent an early vertebrate evolutionary state that allows for a faster and more precise control in the pathway (Sherwood and Adams, 2005; Levavi-Sivan et al., 2010; Zohar et al., 2010; Kah and Dufour, 2011). Stimulation of gonadotropes by GnRH results in the secretion of LH and the FSH into the blood stream (Sherwood and Adams, 2005; Chauvigné et al., 2010), from where these peptide hormones act on gonadal receptors located in the ovaries (the thecal and granulosa cells of the ovarian follicle complex) and the testes (the Leydig and Sertoli cells) depending on the density of the receptors in each target cell (Wootton and Smith, 2015). Through their receptors, LH and FSH regulate gonadal growth and maturation, gametogenesis and steroidogenesis. The gonadal steroids, as well as nonsteroidal factors, such as activin, exert complex feedback effects at multiple levels of the HPG axis, fine-tuning its activity (Trudeau, 2021). The gonadal steroids also regulate reproductive behaviour in both female and teleost fishes (Munakata and Kobayashi, 2010; Zohar et al., 2010; Wootton and Smith, 2015). Interestingly, and in contrast to other vertebrate classes, recent knock-out studies in genetically tractable teleost model organisms, such as the zebrafish, *Danio rerio*, and the Japanese medaka, *Oryzias latipes* suggest that HPG axis components historically considered necessary for vertebrate reproduction most notably GnrH, are not essential for teleost reproduction (Trudeau, 2018, 2021; Zohar et al., 2022). It has been hypothesized that this is linked to a multimodal and thus possible redundant network of (stimulatory) neuroendocrine factors controlling pituitary hormone release. Given these recent paradigms shift in the field of endocrine control of

reproduction in teleost fishes, it is thus important to comparatively investigate the contribution of (phylogenetically ancient) neuroendocrine systems in (and given their diversity, among) teleost fishes.

1.4. Reproductive behaviour

Reproductive behaviour or 'courtship behaviour' is challenging to define in teleosts due to the diversity of behaviour patterns that exist in this infraclass. Due to this fact, researchers have broadly defined this term as behaviours performed by sexually mature males and females in order to synchronize gamete release and maximize the production of offspring (Munakata and Kobayashi, 2010). Control of reproductive behaviour is exerted centrally by the neuronal circuitry in the telencephalon, preoptic area (POA) and the hypothalamus, which are, in order to synchronize with the physiological reproductive state (e.g. gamete maturation and release) responsive to HPG axis cues in form of reproductive hormones (Wootton and Smith, 2015). A well-studied example illustrating the regulation of male and female courtship behaviour through the HPG axis is the goldfish, *Carassius auratus*, where 11 keto-testosterone (11-KT), the major androgen produced in the testes elicits male behaviour, while Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) released during ovulation, elicits female spawning behaviour (Kobayashi and Nakanishi, 1999; Kobayashi et al., 2002). Finally, for courtship behaviour to occur, males and female reproductive physiology needs to be synchronized to maximize reproductive success through timed release of milt and oocytes. As such, and as demonstrated in goldfish, female reproductive behaviour is dependent on oocytes to be fully mature and ovulated as a result of an LH surge, whereas male spermatogenesis is completed resulting in a sufficient amount of milt (Kobayashi et al., 2002). Finally, as elegantly demonstrated in goldfish which often mate in murky waters and are thus dependent on olfactory cues, final coordination between male and female reproductive behaviour can be dependent on pheromones (Munakata and Kobayashi, 2010). Indeed, key molecules involved in oocyte maturation and ovulation, such as 17α,20β-dihydroxy-4-pregnen-3-one (17,20P) and $PGF_{2\alpha}$ not only act to synchronize female behaviour with HPG axis activity, but are, through their secretion via urine, released into the water where they can be detected by males via the olfactory

epithelium in pM concentration ranges to stimulate male courtship behaviour and milt release (Kobayashi and Nakanishi, 1999; Kobayashi et al., 2002; Yabuki et al., 2016). The neuroendocrine pathways thus play a role in the synchrony of reproductive behaviour in teleost fishes, however the involvement of specific chemical mediators may differ between species (Evans, 1998; Munakata and Kobayashi, 2010). While some endogenous mediators, such as neurosteroids, may be produced locally in specific brain regions, other mediators, such as gonadal steroids, are produced in the gonads and transported across the blood brain barrier to regulate specific behavioural neurocircuits in the brain (Evans, 1998; Munakata and Kobayashi, 2010). Finally, both HPG axis as well as behaviours are modulated by exogenous cues such as, for example, photoperiod and temperature (Munakata and Kobayashi, 2010; Juntti and Fernald, 2016).

2. The teleost nonapeptide system

Among the candidate neuroendocrine systems whose role in teleost reproduction warrants renewed and detailed investigation given the recent findings revealing the dispensability of the GnrH system, the phylogenetically ancient nonapeptide systems merit detailed investigation. The vertebrate nonapeptide family consists of arginine vasopressin (AVP) and oxytocin (OXT), is phylogenetically ancient and regulates reproduction and sociosexual behavior. The peptides have additional pleiotropic effects on energy balance, the stress response, osmoregulation, the cardiovascular system, and circadian rhythms.

2.1. Nonapeptide structure and evolution

Nonapeptide-like peptides are present in invertebrates (Odekunle and Elphick, 2020), and studies in basal cephalochordates, such as the Florida lancelet, *Branchiostoma floridae*, and urochordates, such as the sea vase, *Ciona intestinalis*, have reported the presence of single nonapeptides, [Ile^4]-VP and Ciona (ciVP) (Gwee et al, 2009; Kawada et al, 2008; Matsubara et al, 2019). 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* (Banerjee et al., 2015, 2018). It is believed

that two rounds of whole genome duplication (2R WGD), one before and one after the separation of agnathans and gnathostomes (Panopoulou and Poustka, 2005), 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 (Acher, 1996). 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 (Acher and Chauvet, 1988). 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 (Acher, 1988). In teleost fishes, vasotocin (Avp) was originally isolated and characterized in pout, *Gadus luscus* (Chauvet J, . Chauvet-Lenci MT, 1961), pollock, *Polacchius virens* (Heller and Pickering, 1961), and European hake, *Mercluccius mercluccius* (Chauvet et al., 1961), and has since been identified in genomes of all teleost fishes studied to date (Gwee et al., 2009; Banerjee et al., 2015). Avp occurs in all vertebrates except for mammals, where it appears to have mutated to give rise to vasopressin (AVP) in which ^{3}Ile was substituted for ^{3}Phe (Gwee et al., 2009; Banerjee et al., 2015, 2017). 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 (Gwee et al., 2009; Banerjee et al., 2015, 2017). The OXT nonapeptides are characterized by a neutral amino acid (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 2, 3 and 4 in the ring structure and position 8 in the tail structure (**Table 1**).

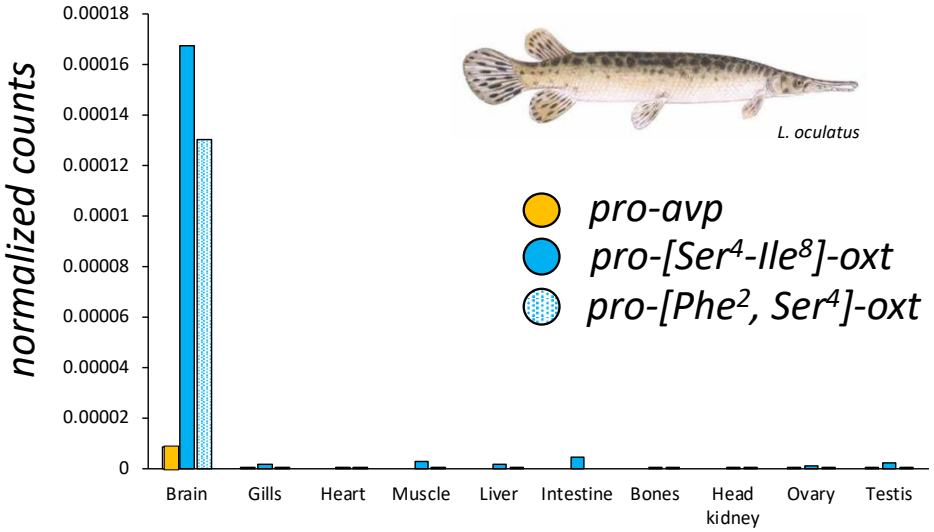
Table 1. *Oxt nonapeptide family orthologues in different fish groups. The AA sequences of the mature hormones are given. Coloured and underlined AA are modifications from the mammalian OXT AA sequence.*

Species/Groups	Historical name	AA sequence	Reference
Holocephali: *Callorhinchus milli* *Hydrolagus colliei*	Oxytocin	CYISNCPQG	(Gwee et al., 2009)
Skates: *Raja miraletus*	Glumitocin	CYISNCPQG	(Acher, 1996)
Sharks:			
Squalus acanthias	Aspargtocin Valitocin	CYINNCPLG CYIQNCPVG	(Acher, 1972)
Scyliorhinus caniculus	Asavatocin Phasavatocin	CYINNCPVG CYFNNCPVG	(Chauvet et al., 1994)
Triakis scyllium	Asavatocin Phasitocin	CYINNCPVG CYFNNCPIG	(Hyodo et al., 2004)
Rays: *Torpedo marmorata*	Isotocin b	CYISNCPIG	(Buchholz et al., 1995)
Spotted gar: *Lepisosteus oculatus*	[Phe^2, Ser^4]-Oxytocin (a variant of isotocin b) Isotocin b	CFISNCPIG CYISNCPIG	Genomic sources (Banerjee et al., 2018)
Most teleost fishes:	Isotocin b	CYISNCPIG	(Acher, 1996)
Catfishes: *Heteropneustes fossilis* + *Clarias batrachus*	Isotocin a (Sevatocin) Isotocin b	CYISNCPVG CYISNCPIG	(Banerjee et al., 2018)
Some lungfish, Coelacanth *Latimeria chalumnae*	Mesotocin	CYIQNCPIG	(Gwee et al., 2008)
Australian lungfish, *Neoceratodus forsteri*	[Phe^2]-Mesotocin	CFIQNCPG	(Acher, 1996)
Human: *Homo sapiens*	Oxytocin	CYIQNCPLG	

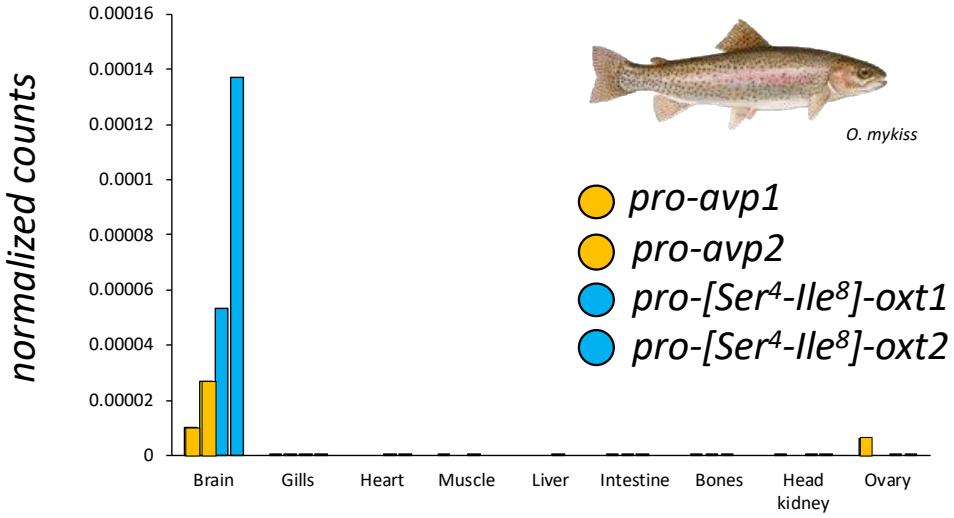
With reference to mammalian OXT, the substitutions are Tyr^2 by Phe^2, Ile^3 by Phe^3, Gln^4 by Ser^4 or Asp^4, and Leu^8 by Ile^8 or Val^8. In teleost fishes, a single OXT family peptide (Oxt; [Ser^4-Ile^8]-OXT) was first isolated in pout, pollock, and the European hake and originally termed isotocin due to the presence of Ile in position 8 (Acher, et al., 1962). While position 8 mutations are very common in cartilaginous fishes (Acher R, et al., 1999) specific Oxt family peptides have also been reported in Sarcopterygii, such as the Australian lungfish, *Neoceratodus forsteri*, which expresses [Phe^2-Ile^3] OXT (Hyodo et al., 1997). Based on more recent genomic data, it has become evident that non-teleost Actinopterygians also express specific Oxt peptides (Banerjee et al., 2018). 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 (Pasquier et al., 2016) does not reveal clear-cut differential expression of both *oxt* paralogues in spotted gar (**Fig. 1A**).

In teleost, 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 (Heierhorst et al., 1989; Suzuki and Hyodo, 1992; Hiraoka et al., 1993; Banerjee et al., 2018). 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 (Banerjee et al., 2018). In salmonids and catostomids, the multiple *oxt* copies may be due to tetraploidization (4R WGD) and/or gene conversion (Acharjee et al, 2018; Heierhorst et al., 1989; Suzuki et al, 1992). Regarding tissue expression profiles of paralogous *oxt* genes in salmonids, analysis of RNA-seq data (Pasquier et al., 2016) suggests similar expression profiles, at least in Atlantic salmon, *Salmo salar* (**Fig. 1B**), and brook trout, *Salvelinus fontinalis* (**Fig. 1C**).

A


normalized counts
0.00018
0.00016
0.00014
0.00012
0.0001
0.00008
0.00006
0.00004
0.00002
0
Brain
Gills
Heart
Muscle
Liver
Intestine
Bones
Head kidney
Ovary
Testis
L. oculatus
pro-avp
pro-[Ser4-Ile8]-oxt
pro-[Phe2, Ser4]-oxt

B


normalized counts
0.00016
0.00014
0.00012
0.0001
0.00008
0.00006
0.00004
0.00002
0
Brain
Gills
Heart
Muscle
Liver
Intestine
Bones
Head kidney
Ovary
O. mykiss
pro-avp1
pro-avp2
pro-[Ser4-Ile8]-oxt1
pro-[Ser4-Ile8]-oxt2

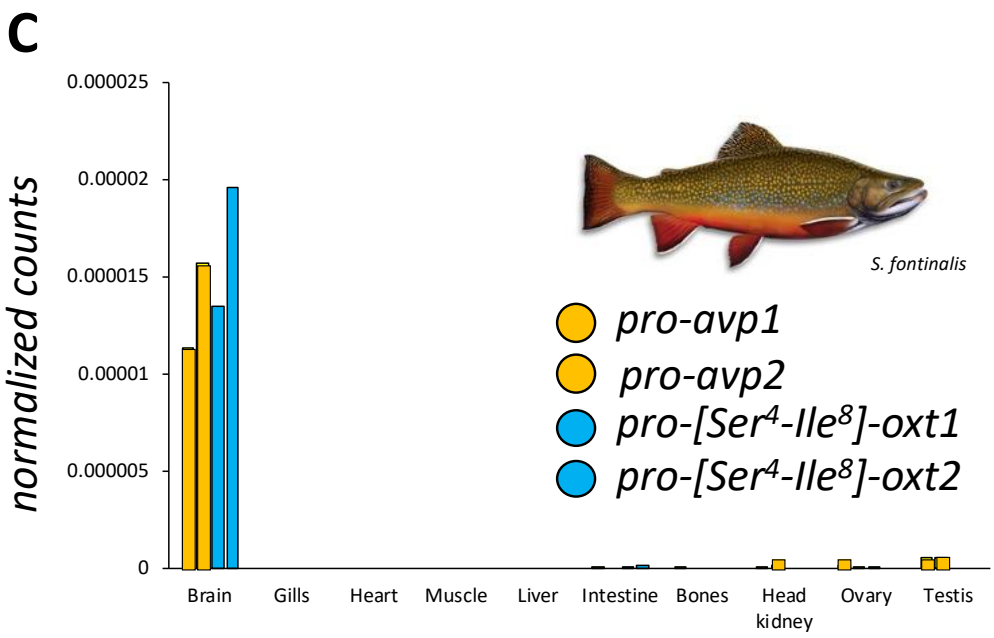


Figure 1. *Tissue-specific expression profiles of nonapeptide precursor coding genes in spotted gar (A), rainbow trout (B), and brook trout (C) derived from the Phylofish RNA-seq database (www.phylofish.signae.org) queried on June 1st, 2022.*

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* (Banerjee et al., 2018). 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 (Banerjee et al., 2018). 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 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 (Acharjee

et al., 2018; Banerjee et al., 2018). It is possible that Oxt peptides bind to the same receptors, but receptor characterization has not yet been undertaken.

Table 2. *General features of the cDNAs of an encoded precursor proteins of Oxta, Oxtb and Avp in the catfish Heteropneustes fossilis. The mature nonapeptide hormone moiety (9 AA), cleavage site (GKR), and the domain between signal peptide and neurophysin are not indicated.*

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

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.

2.2. 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 (Rodriguez-Santiago et al., 2017;

Loveland and Hu, 2018). 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 (Gutnick et al., 2011; Herget and Ryu, 2015; Kagawa et al., 2016; Herget et al., 2017). In teleost, 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 (Heierhorst et al, 1989; Hyodo et al, 1997; Loveland et al., 2018; Rodriguez- Santiago et al.,2017; Sukuzi et al, 1992, 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 (Macey et al., 1974; Demski and Sloan, 1985; Walton et al., 2010).

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, known to respond to female sex pheromones(Yabuki et al., 2016), fibers positive for Oxt have been identified in the olfactory bulb (Altmieme et al., 2019). In the plainfin midshipman, *Porichtys notatus*, which relies on vocalization as part of their courtship behaviour (Forlano and Bass, 2011), 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 (Goodson and Bass, 2000; Goodson et al., 2003). In the weakly electric gymnotiform bluntnose knifefish, *Brachyhypopomus gauderio*, which uses electric organ discharge (EOD) signals for mate selection (Silva et al., 2007), Avp innervation in the medulla was found to be in proximity of the pacemaker nucleus that controls EOD (Perrone et al., 2014). 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 (Batten,

1986; Groves and Batten, 1986). In some species, such as the dwarf gourami, *Colisa lalia*, a colocalization of parvocellular Oxt with gonadotropin releasing hormone (Gnrh) has been reported (Maejima et al., 1994). In the goldfish, Oxt colocalizes with secretoneurin a (Canosa et al., 2011), an important hypophysiotropic stimulator of the hypothalamo-pituitary-gonadal (HPG) axis (Zhao et al., 2009; Mitchell et al., 2020). 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 nonpeptidergic innervation principally forms contact with pituitary vasculature in the form of terminal release buttons which highlights an endocrine role of the nonapeptides (Batten, 1986; Groves and Batten, 1986; Batten et al., 1990, 1999). 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* (Sokołowska et al., 2020), and the air-breathing catfish, *Heteropneustes fossilis* (Singh and Joy, 2008). In addition to pituitary release, nonapeptide fibers innervating gonadotrophs in the proximal pars distalis through gaps in the basement lamina have been demonstrated in the sailfin molly (Batten, 1986; Batten et al., 1999). 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 (Saito et al., 2004), 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, (Herget and Ryu, 2015; Herget et al., 2017).

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 (Bobe et al., 2006). High *oxt* expression is also noted for whole zebrafish ovaries (Chou et al., 2011). 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 (Singh and Joy, 2008). 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 (Singh and Joy, 2008). Conversely, Avp has been located to interstitial cells in the testes of the chanchita, *Cichlasoma dimerus* (Ramallo et al., 2012). The expression of gonadal nonapeptide systems, and especially *avp*, appears to be a more widespread feature in teleost, as suggested by RNA-seq data mined from the Phylofish database (Pasquier et al., 2016) and presented here (**Figure 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.

2.3. Nonapeptide-dependent regulation of the HPG Axis

2.3.1. 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 (Trudeau, 1997). 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, *CIV*, 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* (Maruska et al., 2007; Kline et al., 2016; Rahman et al., 2020) must be investigated. Studies in female goldfish show that serotonin neurons, known to stimulate pituitary Lh release in this species (Somoza et al., 1988), 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 (Mennigen et al., 2017). 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 (Trudeau, 1997). This is supported by work in the walking catfish, which suggests that Oxt may stimulate Lh release via the inhibition of dopaminergic blockage (Singh et al., 2016). 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 (Singh et al., 2016).

In addition to hypothalamic interaction between nonapeptide systems and the neuronal circuitry involved in HPG axis regulation in several teleost models (Mennigen et al., 2017; Popesku et al., 2011; Semsar et al, 2001), 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 (Canosa et al., 2011), while colocalization of Oxt and GnRH was reported in the dwarf gourami (Maejima et al., 1994). 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 (Saito et al., 2003), and kisspeptin, which contact Oxt and Avp neurons in Japanese medaka (Kanda et al., 2013) and striped bass, *Morone saxatillis* (Zmora et al., 2015). 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 (Trudeau and Somoza, 2020). Given the reported roles of nonapeptides in teleost sociosexual and courtship behaviour, neuroanatomical evidence may also reflect synchronization of 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 (Chaube et al., 2012). 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 (Chaube et al., 2012). A male-specific stimulatory effect of androgens on parvocellular Oxt neurons in the medaka POA has also been reported (Yamashita et al., 2017). 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 (Altmieme et al., 2019; Demski et al., 1995). 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 (Kalamarz-Kubiak et al., 2017). 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 (Kalamarz-Kubiak et al., 2017). 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 (Kalamarz-Kubiak et al., 2017). 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 are at least based on immunohistochemical evidence, direct targets for estrogen actions, as they express the membrane estrogen receptor Gper1 (Mangiamele et al., 2013) and are surrounded by radial glia, the only cells in the teleost brain expressing *cyp19a1b* and capable of producing neuroestrogens (Da Fonte et al., 2018). 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 teleost. 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.

2.3.2. Nonapeptides directly regulate gonadotrophs in several species

In the goldfish, hypothalamic expression of *oxt* was found to peak seasonally in reproductively mature females (Zhang et al., 2009). Pharmacological investigations demonstrate that hypothalamic induction of goldfish *oxt* is dependent on GABAergic and dopaminergic signaling (Popesku et al., 2008), in line with an integration of this nonapeptide system with seasonally-regulated HPG neurocircuitry in this species (Trudeau, 1997). Intraperitoneal injection of 1 μg/g body weight Oxt in sexually recrudescent female goldfish significantly increased circulating Lh by 167% 5h post-injection (Popesku et at., 2011) with subsequent increases in circulating E_2 12h post injection (Popesku et al., 2008). 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 (Mennigen et al., 2017). In these preparations, Oxt significantly stimulated Lh release without affecting *lhb* or *fshb* subunit mRNAs, suggesting direct, transcription-independent stimulation of Lh release (Mennigen et al., 2017). These findings are in line with reported neuroanatomical evidence in other teleosts, such as the sailfin molly, the European bass, and the African sharptooth catfish, in which nonapeptidergic innervation of gonadotrophs has been reported (Batten, 1986; Batten et al., 1999). Similar to Oxt, a stimulatory effect of Avp on Lh release has been demonstrated in at least two teleost: in

the sailfin molly 18h pituitary incubation with Avp stimulated Lh synthesis and release, with lower dose-responsiveness and more consistent effects in male compared to female pituitaries (Batten, 1986). In the Asian stinging catfish, Avp, and to a much lesser extent Oxt and serotonin, stimulated *gpa, fshb* and *lhb* in pituitary cultures in a sex- and reproductive stage-dependent manner (Acharjee et al., 2018). 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 (Singh et al., 2012). In the ricefield eel, *Monopterus albus*, Oxt-stimulated Lh release from dispersed pituitaries via an Oxtra-activated IP_3/Ca^{2+} pathway (Yang et al., 2021). 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 (Ramallo et al., 2012) 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 (Evans et al., 1992; Robinson et al., 1992).

2.3.3. 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.

2.3.3.1. Steroidogenesis

Effects of nonapeptides on male steroidogenesis have been reported in testicular cultures of the rainbow trout (Rodríguez and Specker, 1991). 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 testosterone (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 (Ramallo et al., 2012). 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.

Table 1.3. 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	(Rawat et al., 2015; 2019)
	avpr1aa *avpr1ab* *avpr2aa* *avpr2ab* *oxtrb*	Pupfish, *Cyprinodon nevadensis amargosae*	*avpr1aa*, *avpr1ab*, *avpr2ab* and *oxtr* mRNA is expressed in telencehalon, hypothalamus and hindbrain	(Lema et al., 2015)
	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	(Iwasaki et al., 2013)
	avpr1ab	Atlantic Croaker	mRNA and protein localized to hypothalamic GnRH neurons	(Rahman et al., 2020)
	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	(Kline et al., 2011; 2016)
Pituitary	*avpr1aa*; *oxtra*	*Astatotilapia burtoni*	mRNA and protein expressed in telencephalon and hypothalamus	(Huffman et al., 2012)
	avpr1aa; *avpr1ab*; *avpr2ab*	Air-breathing catfish *Heteropneustes fossilis*	mRNA expressed in male and female rostral pars distalis and pars nervosa	(Rawat et al., 2015; 2019)
	oxtra	Rice-field eel	mRNA located to Lh but not Fsh cells	(Yang et al., 2021)

		Monopterus albus		
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	(Rawat et al., 2015; 2019)
	oxtra	Guppy	Ovaries, expressed in follicular layer	(Lyu et al., 2021)
	avpr1aa; *avpr1ab*	Bluehead wrasse	Ovaries, Testes	(Lema et al., 2012)

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* (Mennigen et al., 2017), it was not investigated whether these effects are linked to prior increases in Lh (Popesku et at., 2011) 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 (Singh and Joy, 2009b). 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 post-vitellogenic ovaries (Singh and Joy, 2009b). 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 post-vitellogenic ovary (Singh and Joy, 2009b). The inhibitory effect of Avp on the E_2 synthesis in the post-vitellogenic 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 (Mishra and Joy, 2006). The MIS reinitiates oocyte meiosis up to the second metaphase (Nagahama, 1994). Concurrent with the modulation of E_2 synthesis, Avp and, to a lesser extent, Oxt also stimulated progesterone (P_4) production (Singh and Joy, 2009b). 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 (Singh and Joy, 2009b). In late post-vitellogenic ovaries, concentrations as low as 1 nM Avp induced a 60% increase over baseline concentrations (Singh and Joy, 2009b). 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 8h and 16h incubation (Singh and Joy, 2009b). Avp stimulated the production of 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 (Singh and Joy, 2009b). The combination of Avp and hCG elicited a cumulative effect on the 17,20β-DP level

especially after 16h of incubation in the spawning phase (Singh and Joy, 2009b). 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 (Singh and Joy, 2011) 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* (Sirotkin et al., 1990).

2.3.3.2. 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 (Singh and Joy, 2011). In this experiment, post-vitellogenic 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^{2}Val^{4}, Abu^{6}, Arg^{8,9} vasopressin), or both. GVBD, ovulation and 17,20β-DP concentration was 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 (Lister and Van Der Kraak, 2008). The cyclooxygenase inhibitor indomethacin can block MIS-induced final oocyte maturation and ovulation in yellow perch, *Perca flavescens,* and Atlantic croaker, indicating dependence on PGs (Bradley and Goetz, 1994; Patiño et al., 2003). The functional relationship between Avp and PGs was investigated in air-breathing catfish (Joy and Singh, 2013): 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

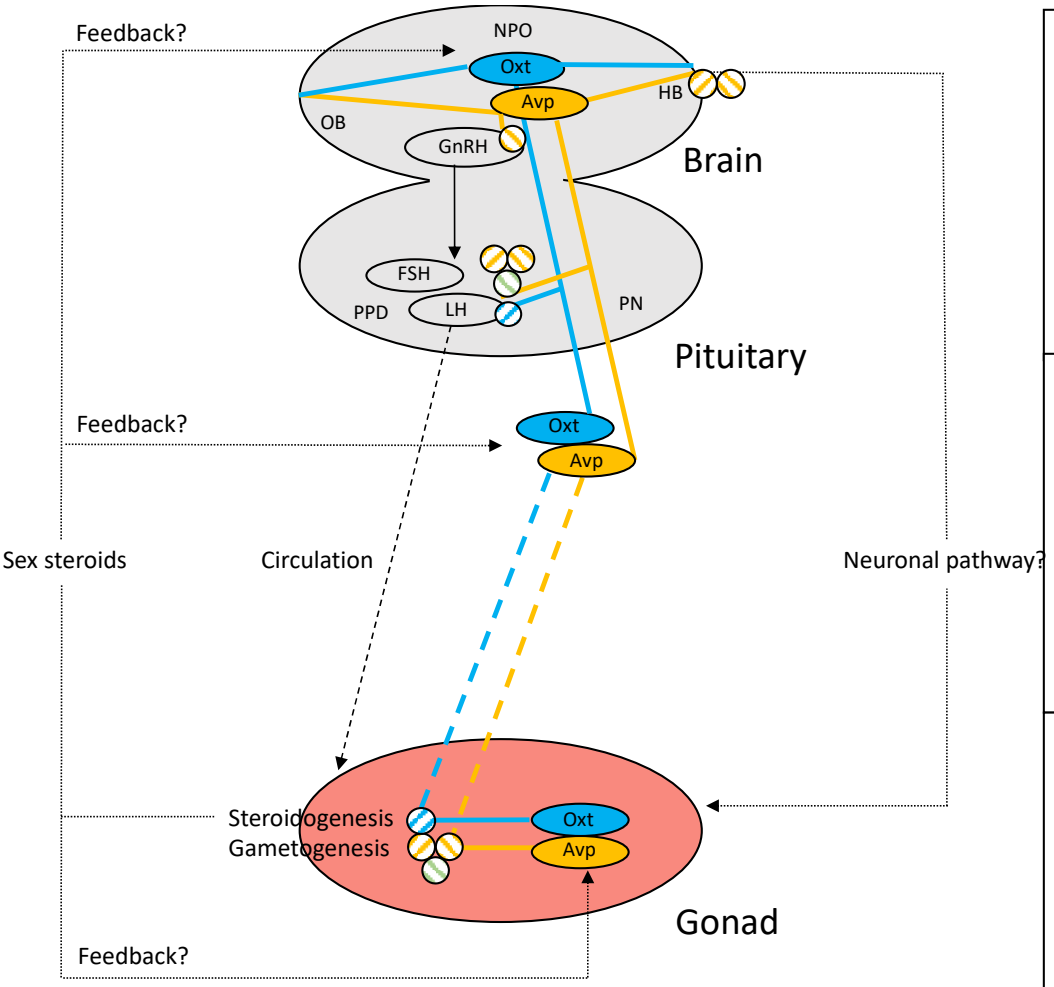
antagonist but not by the AVPR2 receptor antagonist. Indomethacin inhibited the Avp and hCG-induced GVBD and ovulation. Both $PGF_{2\alpha}$ and PGE_2 stimulated GVBD and ovulation in a dose- and time-dependent manner and $PGF_{2\alpha}$ was more effective than PGE_2. Taken together, these observations 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 (Cerdà, 2009), and this process is retained to some extent in freshwater and anadromous fishes. Singh and Joy (Singh and Joy, 2010) reported a 23% rise in oocyte water content during the FOM and ovulation in Asian stinging catfish 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 and colleagues reported that Avp regulates *aqp1ab,* (ovary-specific aquaporin 1ab) expression through an Avpr2 receptor (Acharjee et al., 2011; Chaube et al., 2011), 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, in which fish and mammalian neurohypophysial preparations as well as synthetic OXT initiated a spawning reflex response (Wilhelmi et al., 1955). 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 (Wilhelmi et al., 1955). 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 (Viveiros et al., 2003). 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 (Wisdom et al., 2022). 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* (Wisdom et al., 2022).

Regarding parturition in teleost, nonapeptides have been reported to stimulate premature parturition in the guppy, an ovoviviparous teleost (Venkatesh et al., 1992; Lyu et al., 2021). The injection of Avp, Oxt and PGs to guppy, a live-bearing teleost, induced premature parturition (Wisdom et al., 2022). 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 (Lyu et al., 2021). 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 (Lyu et al., 2021). 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 (Xiong et al., 2020). 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 (Peter, 1977).

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 2**. It is anticipated, however, that additional detailed comparative studies will uncover diversity of nonapeptide-dependent regulation of teleost reproductive physiology.



Brain / Neuromodulation

-Reproductive behaviour control via regulation of motor neurons (hindbrain - HB)

-Sensory modulation of reproductive cues via afferent modulation exogenous cues (e.g. olfactory bulb - OB)

Endocrine system

-Oxt and Avp release into circulation in pars nervosa of the pituitary (PN)

-Modulation of gonadotropin production and/or release via G-coupled receptors in pars proximalis distalis of the pituitary (PPD)

Gonadal system

-Gonadal production of nonapeptides with paracrine roles

-G-coupled protein respond to circulating and/or locally produced nonapeptides to regulate steroidogenesis and gametogenesis

Figure 1.2. 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 the **Supplemental Figures 1.1 and 1.2**. of this chapter.

2.4. Nonapeptide-dependent regulation of reproductive behaviour

The plainfin midshipman fish, displays sex- and morph-specific vocalization during mating. Oxt and Avp regulate these sex- and morph-specific effects on the vocal circuitry (Goodson and Bass, 2000). 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, knockout mutants, an essential role for Avp in male mate-guarding behaviours in this non-monogamous species has been demonstrated (Yokoi et al., 2015). 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 (Yokoi et al., 2015). 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 (Yokoi et al., 2020). 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, 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 (Semsar et al., 2001). 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 (Semsar et al., 2001). In male white perch, *Morone americana*, intracerebroventricular but not intraperitoneal administration of Avp significantly stimulated an important behavior termed 'attending' without affecting whole body or circulating androgens (Salek et al., 2002). 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 (Santangelo, 2015). Similarly, administration of Manning's compound significantly reduced male reproductive courtship behaviour and reproductive success in mating assays with female zebrafish, without affecting whole body androgen (T and 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 (Altmieme et al., 2019). In the weakly electric fish, 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 (Pouso et al., 2019). In the same species, Avp increases dominance in part via direct modulation of the EOD rate (Perrone and Silva, 2016). 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* (Carneiro et al., 2003). There is a need for detailed comparative studies of the roles of nonapeptides in teleost which exhibit the most diverse reproductive strategies and behaviours amongst the vertebrates (Godwin and Thompson, 2012).

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 ocellari*s 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 (DeAngelis et al., 2017). This suggests a specific action of Oxt in controlling male common clownfish parental care behaviours (DeAngelis et al., 2017); 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 (DeAngelis et al., 2020). Conversely, administration of an AVTR1 receptor antagonist increased male parental behaviours while reducing aggression towards intruders (DeAngelis et al., 2020), thus demonstrating antagonistic roles of the nonapeptide systems in common clownfish (DeAngelis et al., 2017, 2020). These data suggest a role for nonapeptides in paternal care. In the monogamous convict cichlid, 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 (O'Connell et al., 2012). Administration of a mammalian OXTR antagonist in biparental males inhibited paternal care behaviour, indicating a functional role for Oxt neurons (O'Connell et al., 2012).

3. Conclusion

The Avp nonapeptide reviewed above provides a good candidate to explore its necessity and function in fish reproduction. It is a phenotypically ancient neuromodulator/hormone system organized in a way to coordinate HPG axis function and reproductive behaviour. Previous literature indicates that this system has been shown to affect individual aspects of reproduction in diverse teleost models. However, what is currently lacking are studies in a single teleost fish model which interactively assesses the reproductive role of this nonapeptide *in vivo*.

3.1. Gaps in Knowledge

Firstly, there is a challenged paradigm in that GnrH is not necessary for teleost reproduction, thus begs the question of the master regulator system or at least the question of which ones are important. Secondly, due to the substantial amount evidence and the conserved, phylogenetically old status of the *avp* system, exploring this pathway using a knockout approach addresses a gap as there is limited evidence addressing integrability between behaviour and the HPG axis activity. Behavioural and endocrine roles of nonpeptides in teleost reproduction have largely been studied in isolation and in different fish species. This is in spite of the postulated need to consider courtship behaviour in the context of HPG axis function in teleost fishes

(Parolini et al., 2019). Thirdly, with the teleost family being so diverse there is a need in an increased need to comparatively study the plasticity of these nonapeptides and their role in reproduction (Godwin and Thompson, 2012; Joy and Chaube, 2015) however, it will be equally important to comprehensively study the reproductive roles of nonapeptides within a single species. The current knowledge investigating the role of nonapeptides across the different levels of the HPG axis with in a single teleost species has been limited to the Asian stinging catfish (Singh and Joy, 2008; Joy and Chaube, 2015) and, to a lesser degree, the canchita cichlid (Ramallo et al., 2012). In addition, the recent generation of nonapeptide and/or nonapeptide receptor knock-out models in genetically tractable model systems such as zebrafish (Wee et al., 2022) and medaka (Yokoi et al., 2015, 2020) may hold particular promise. Furthermore, they provide a great complementary model to study gene function in models outside of rodents where homozygous lethality occurs ex. Brca2 mice model (Evers and Jonkers, 2006) or not viable like with the Brattleboro rat model (Zelena et al., 2026). However, neither of these models have, to-date, been used to explore nonapeptide effects on the HPG axis. 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 (Altmieme et al., 2019), few studies have been conducted in this area. As evolutionarily conserved systems regulating reproduction, 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. The 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 (Zohar, 2021). Emerging evidence in ecotoxicology suggests that neuroendocrine disruption of teleost nonapeptides may be linked to decreased reproductive success in teleost fishes, an ecologically meaningful endpoint (Arcand-Hoy and Benson, 1998).

3.2. Zebrafish

Zebrafish belong to the *Cyprnidae*, which is the largest family within the infraclass of teleost fishes. Within the *Cyprinidae* family, zebrafish diverged approximatively 60 million years ago (Ota and Abe, 2016). Native to the Himalayan region, zebrafish were first described at the end of the 19th century (Spence et al., 2006) and have been used as a laboratory model since the 1930s (Creaser, 1934). Zebrafish grew in popularity as a research model following studies pioneered by George Streisinger at the University of Oregon in the 1970s and 1980s (Streisinger et al., 1981). The growing success of zebrafish as important fundamental and translational research model (Best et al., 2008; Li and Ge, 2020) is due to many of its well-described advantages at every stage of life. Briefly, these include rapid and easily observable development, small size, low cost, easy maintenance, large clutch size, a sequenced genome, and the associated feasibility of genome-editing techniques (Ye and Chen, 2020; Trompouki et al., 2018; Saleem and Kannan, 2018). Furthermore, the high conservation between the zebrafish and mammalian models such as rodent models and humans, allows researchers to use this fish model as a tool for screening in pharmacological studies or as a complementary model to study gene functions when there is homozygous lethality in rodent models (Ye and Chen, 2020; Saleem and Kannan, 2018). Furthermore, with a better understanding of the similarities and differences between various model species allows for deeper understanding of the evolution and diversity of physiological systems (Ye and Chen, 2020; Trompouki et al., 2018). In addition, as a representative of the largest family (*Cyprinidae*) within the largest infraclass of vertebrates studies in fish species like zebrafish will be of substantial value in developing improved methodology for captive breeding, of use in aquaculture and conservation biology applications (Sun and Zhu, 2019). Finally, zebrafish are emerging models in aquatic ecotoxicology, allowing to study responses to endocrine disrupting chemicals (Arcand-Hoy and Benson, 1998).

3.2.1. Zebrafish reproduction

Zebrafish in the laboratory setting are indeterminate breeders, and sexually mature fish (~ 3 months, 3mpf) are able to spawn approximately every 1-2 days, with ovulation occurring overnight and spawning occurring within the first 10 minutes after the onset of light (Spence et al., 2006; Juntti and Fernald, 2016). In the wild, both seasonal and extra-seasonal spawning have been described in zebrafish. However understanding of the reproductive biology in the wild remains limited, as it has been reported rather than seasonal cues or biological age, food availability and somatic growth are important determinants of reproductive success (Spence et al., 2006). During a single spawning event, male and female zebrafish follow a series of stepwise courtship behaviour that include chasing, nudging, and encircling for males and quivering in female zebrafish that ultimately releases 5-20 eggs periodically (Darrow and Harris, 2004). This event is coupled with male milt release to maximize external fertilization (Spence et al., 2006). Clutch sizes of single pairings can be variable, ranging from 50-300 plus eggs per clutch. Male and female reproductive behaviour are dependent on each other to synchronize individual reproductive status to coordinate courtship behaviours and maximize reproductive success.

3.2.2. Targeted Gene Knockout Methods in zebrafish

There are four main methods of genome editing technologies that can be used to preformed in zebrafish; morpholinos, ZGN, TALEN and CRISPR/Cas9 (Sakai et al., 2018; Zhu and Ge, 2018). Morpholinos create a knock-down effect when injected and used to analyze gene function in early development. They are low in costs however, there is a possibility of off target effects that can occur that limit the conclusions that can be made in these studies (Sakai et al., 2018; Zhu and Ge, 2018). With this introduction of targeted nuclease technologies (ZGN, TALEN), allowed for the study of knock-out effects of gene functions that offered increased flexibility and lower costs (Sakai et al., 2018; Zhu and Ge, 2018). These methods were soon replaced by CRISPR/Cas9 which offers superior flexibility and efficiency over earlier gene-editing methods (Hwang et al., 2013). This method works by having a small guide RNA guide the nuclease Cas9 towards the target DNA site. Once at the target site, the nuclease cleaves the site and allows for insertion-

deletion mutations with in the genome. Using these methods allows for the comprehensive study of a single gene. The zebrafish model is an ideal model to study the reproductive roles of the Avp system, since many successful gene knockout models have been used to link reproductive phenotypes to gene function in this model system using genome editing and the CRISPR/Cas9 system (Li and Ge, 2020). Secondly, there is substantial literature focused on reproductive behaviour and physiology in zebrafish, as well as the nonapeptide system (Yabuki et al., 2016; Li and Ge, 2020; Mitchell et al., 2020; Zohar, 2021). Lastly there has been some preliminary studies exploring the reproductive function of nonapeptides specifically using a zebrafish model through pharmacological studies (Altmieme et al., 2019).

4. Hypothesis and Objectives

As reviewed in detail (**Chapter 1**), the study of both behavioural and endocrine roles of Avp in teleost reproduction has largely been conducted in isolation. Nevertheless, evidence points to a stimulatory role on reproductive function both at the level of the HPG axis, as well as on (male) courtship behaviour. Using zebrafish, a genetically tractable model system, I generated *avp*$^{-/-}$ knockout models with the aim of testing the necessity or importance of the Avp system in a single teleost species *in vivo*. In this thesis, I specifically tested the hypothesis that *avp* (sex-dependently) contributes to reproductive success in zebrafish by regulating HPG axis function and/or reproductive behaviour. Given that the Avp nonapeptide system has the potential to affect reproductive physiology and success via central neuromodulation of courtship behaviour, and peripherally via endocrine mechanisms, I comprehensively investigated the importance of a functional Avp system on both endpoints in this model (**Chapter 2).** I specifically predicted that if the *avp* gene plays a necessary or important role in zebrafish reproduction, then I will observe a complete inhibition or decrease in reproductive success in *avp* $^{-/-}$ mating pairs compared to WT controls. Furthermore, if the role of the *avt* gene in contributing to reproductive success is sex-specific, I predicted that backcrosses of mutant of the sex in which Avp exerts important reproductive functions with WT of the opposite sex will recapitulate the reproductive deficit observed in *avp* $^{-/-}$ mating pairs. Conversely, I predicted that if the reproductive phenotype is mediated by a single sex in mutants, that the opposite backcross with wildtypes

would not be different from WT pairs. To determine a potential mechanistic basis of observable diminished reproductive success *avp* $^{-/-}$ mutants, I quantified reproductive behavior and the quantity of fertilized eggs. Because I found that the reduction in reproductive success was dependent on female mutants, I conducted subsequent mechanistic studies focused on ovaries (female gonads) using histological, metabolite and gene expression approaches to assess oocyte maturation and explore a potential mechanistic basis for alterations in courtship behaviour.

Because the possibility for pleiotropic effects of Avp in fishes exists (Balment RJ et al., 2006), I prioritized the study of potential roles of Avp in the regulation of two physiological systems, the endocrine stress axis on the one hand, and energy balance / somatic growth on the other (**Chapter 3**). While additional pleiotropic roles of Avp may contribute to reproductive consequences observed in *avp* $^{-/-}$ mutants, the study of aspects of these physiological processes were prioritized as comparatively more detailed evidence for their modulation by Avp exists in teleost fishes, and because their role in modulating aspects of reproductive physiology has been described in teleosts. Based on pharmacological evidence from acute studies conducted in adult teleost fishes, I hypothesized that *avp* $^{-/-}$ mutants would exhibit increased somatic growth up to sexual maturity based on a lack of central inhibition on feed-intake and decreased locomotory energy expenditure. Regarding the endocrine stress axis activity and 'anxiety-like' behaviours, I hypothesized that *avp* $^{-/-}$ mutants would exhibit hypocortisolism and a reduction in anxiety-like behaviours. I discuss my findings that despite a decrease in larval locomotion, *avp* $^{-/-}$ do not exhibit overall differences in somatic growth or gonadosomatic index (GSI), and that *avp* $^{-/-}$ exhibit hypercortisolism in the context of known literature to assess the possible contribution of these phenotypes to the described female reproductive phenotype.

Finally, I provide a general discussion of my findings in **Chapter 4**. In line with my requirements for a specialization in 'Science, Society and Policy', I place particular emphasis on the relevance and translational potential of the *avp* $^{-/-}$ zebrafish model, and zebrafish models in areas of societal interest. These are, firstly, the study of female reproduction in a biomedical context, secondly, the importance of studying teleost reproduction in the context of assisted breeding programs with relevance for aquaculture and species

conservation efforts, and thirdly, the application of novel insight into hormonal regulation of fish reproduction in an ecotoxicological context, with particular focus on endocrine-disrupting chemicals (EDCs).

Chapter II

Knock-out of arginine vasopressin reduces reproductive success in female zebrafish, Danio rerio

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

Ramachandran D, Sharma K, Saxena V, Mennigen JA. Knock-out of arginine vasopressin reduces reproductive success in female zebrafish, *Danio rerio*.

Statement of contribution:

With help from Vishal Saxena from the Aquatics Facility, I generated *avp* $^{-/-}$ knock-out zebrafish and established a stable homozygous mutant line using a CRISPR/Cas9 based approach. I co-developed and conducted all experimental work described in this chapter and coordinated and supervised Honour's student Kusum Sharma and volunteer Brooke Kattenbusch's contributions to this project, specifically their experimental contributions in genotyping, breeding assays, histological analyses, and blind analysis of courtship behaviours.

Knock-out of arginine vasopressin reduces reproductive success in female zebrafish, *Danio rerio*.

Divya Ramachandran[1], Kusum Sharma[1], Vishal Saxena[1], Jan A. Mennigen[1,2]

[1] Department of Biology, University of Ottawa, ON, Canada

[2] Corresponding author e-mail address: jan.mennigen@uottawa.ca

Key words: Nonapeptides, courtship behaviour, ovary, CRISPR/Cas9

1. Abstract

The nonapeptide vasopressin (Avp) is an evolutionarily conserved nonapeptide which acts as neuromodulator and endocrine/paracrine signaling molecule. Circumstantial and mechanistic evidence from pharmacological manipulations of the Avp system in several teleost fishes suggest sex- and species-specific reproductive roles. While effects on reproductive physiology have been documented to involve both courtship behaviours or effects on the hypothalamic-pituitary-gonadal (HPG) axes, comprehensive studies investigating physiological and behavioural reproductive consequences of genetic ablation of Avp in genetically tractable fish model, such as the zebrafish are currently lacking. Here, we report the generation of a CRISPR/Cas9-based *avp* $^{-/-}$ zebrafish mutant, which we used to investigate reproductive roles of Avp. Breeding pairs of *avp* $^{-/-}$ fish produce significantly fewer fertilized eggs per clutch compared to wildtypes (WT), an effect coincident with reduced female quivering courtship behaviour. Crossbreeding experiments showed the reproductive phenotype to be female-dependent, as *avp* $^{-/-}$ males reproduce normally when paired with female WT fish. Sectioning of female gonads revealed a reduction in overall oocytes, as well as fewer early-stage I oocytes but more stage V oocytes in the ovarian oocyte pool in *avp* $^{-/-}$ fish. Ovarian gene expression analysis revealed significant decreases in the germ cell marker *nanos2,* suggesting a potential role for Avp in germ-cell maintenance. Ovaries of Avp mutants also exhibited a significant reduction in concentrations of the prostaglandin, $PGF_{2\alpha}$, a known regulator of ovulation and female courtship behaviour in some female teleosts. This reduction coincided with significantly decreased transcript abundance of *pla2g4ab,* a phospholipase involved in mobilization of arachidonic acid, a precursor of $PGF_{2\alpha}$. Together, these findings provide further support for the emerging roles of Avp in female (teleost) reproduction and open translational research avenues in the domains of captive breeding in the context of aquaculture and species conservation. The findings are also anticipated to contribute to an improved understanding of possible modes of action of endocrine disrupting chemicals in the field of ecotoxicology.

2. Introduction

The nonapeptide arginine vasotocin (AVT) is highly conserved in vertebrate evolution and is found in all non-mammalian vertebrates (Gwee et al., 2008; Banerjee et al., 2017). In its mature form, AVT forms a circular nonapeptide with characteristic basic amino acid in position 8 (Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Arg-Gly-NH_2) which differentiates it from related oxytocin-lineage peptides considered to have evolved from ancestral vasotocin gene at the base of vertebrate evolution (Gwee et al., 2008; Banerjee et al., 2017). The vasotocin (*avp*) gene in teleost fish as in other non-mammalian vertebrate species and the homologous mammalian arginine vasopressin (AVP) gene encodes a precursor protein whose cleavage generated the mature nonapeptide, a carrier protein termed neurophysin 2, as well as a peptide fragment termed co-peptin (Gwee et al., 2008; Banerjee et al., 2017). In zebrafish and other teleosts studied to date, vasotocin is produced in giganto-, magno-, and parvocellular neurons of the preoptic area (POA), from where it innervates diverse hypothalamic regions and extrahypothalamic brain regions (Batten et al., 1990; Saito et al., 2004; Thompson and Walton, 2009; Herget and Ryu, 2015; Kagawa et al., 2016; Pouso et al., 2017). Innervation of extrahypothalamic regions includes sensory regions (Goodson et al., 2003; Perrone et al., 2014) and hindbrain motorneurons (Thompson and Walton, 2009; Iwasaki et al., 2013). These regions are directly relevant to the perception of environmental stimuli relevant to reproduction on the one hand and the control of courtship behaviour on the other. From the preoptic area (POA), vasotocinergic innervation also robustly extends to the pituitary gland, especially in the *pars nervosa*, where the nonapeptide is released into circulation via terminal buttons (Batten and Batten, 1986; Groves and Batten, 1986). Albeit to a lesser extent, vasotocinergic innervation has also been described to contact gonadotrophs at least in some teleost species (Batten and Batten, 1986; Batten et al., 1999). In the few teleost fishes investigated, peripheral vasotocin expression has been reported in the testes (Ramallo et al., 2012) and the ovary (Singh and Joy, 2008; Banerjee et al., 2018). Altogether, the (neuro)anatomy of the Avp system suggests possible roles in regulating behavioural and endocrine components of reproduction in teleost fishes by acting as central neuromodulator and/or endocrine or paracrine factor along the hypothalamus-pituitary-gonadal (HPG) axis.

Indeed, circumstantial as well as functional evidence from pharmacological, and more recently genetic ablation studies in different teleost fishes support this notion. For example, reproductive stage dependent *avt* expression and/or translated Avp protein in whole brain, circulation and the ovary have been shown reported in several fish species, including the round goby, *Neogobius melanostomus* (Sokołowska et al., 2015; Kalamarz-Kubiak et al., 2017), the three-spined stickleback, *Gasterosteus aculeatus* (Kleszczyńska et al., 2012; Kulczykowska and Kleszczyńska, 2014), and the Asian stinging catfish, *Heteropneustes fossilis* (Singh and Joy, 2008). Functionally, pharmacological studies (Semsar et al., 2001; Salek et al., 2002; Santangelo and Bass, 2010; Pouso et al., 2019) have implicated Avp in stimulating male courtship behaviour in several teleost fish species, including the zebrafish, *Danio rerio* (Altmieme et al., 2019). Genetic ablation of *avt* in male Japanese medaka, *Oryzias latipes* resulted in decreased sexual motivation and disrupted mate-guarding behaviour (Yokoi et al., 2015). However, evidence from investigations of Avp effects of courtship behaviour in the peacock blenny, *Salaria pavo* demonstrate effects in females and sneaker males, but not dominant males (Carneiro et al., 2003), underlining the importance of considering possible species effects of Avp between teleost fishes. Regarding the HPG axis, studies in teleost species such as the sailfin molly *Poecilia latipinna* (Groves and Batten, 1986), and the Asian stinging catfish (Acharjee et al., 2018), and the cichlid *Cichlasoma dimerus* (Ramallo et al., 2012), unequivocally point to hypo-physiotropic roles of AVP in the stimulation of LH and FSH subunit expression and/or LH release. Finally, endocrine and/or paracrine roles for Avp have been reported to regulate gametogenesis and steroidogenesis and gamete release in males and females. Regarding effects of Avp on male gonads, testes incubated with Avp *in vitro* stimulated testosterone (T) release in both rainbow trout, *Onchorhynchus mykiss* (Rodríguez and Specker, 1991) and the cichlid (Ramallo et al., 2012). Administration of Avp in the catfish, *Clarias magur*, increased spermatozoa concentration in strippable milt (Wisdom et al., 2022). Insight on the role of Avp on female gonads stem largely from a suite of studies conducted in the Asian stinging catfish (Singh and Joy, 2008, 2009a, 2009b, 2011; Acharjee et al., 2011; Joy and Singh, 2013). In this species, Avp has been shown to promote estradiol (E_2) production in previtellogenic follicles, and promotes final oocyte maturation via steroidogenic shift and characterized by

an inhibition in E_2 but induction of maturation-inducing steroid and progesterone (P_4) in post-vitellogenic follicles to promote germinal vesicle breakdown (Joy and Chaube, 2015). Finally, Avp has also been shown to contribute to oocyte hydration via aquaporin 1ab (*aqp1ab*) transcript abundance (Joy and Chaube, 2015).

While these studies reveal that Avp generally plays important reproductive roles in teleost fish, their involvement in zebrafish, a genetically and increasingly physiologically tractable model species to investigate reproductive function (Li and Ge, 2020) are only beginning to be understood (Altmieme et al., 2019). Importantly, in the reproductively diverse infraclass of teleost fishes, it has been postulated that sociosexual nonapeptide function is likely species-specific, creating a clear need for comparative studies (Godwin and Thompson, 2012; Joy and Chaube, 2015). Finally, few, if any, previous studies, including recent genetic ablation studies in Japanese medaka (Yokoi et al., 2015), have probed nonapeptide function in teleost reproduction comprehensively *in vivo* by investigating both behavioural and HPG axis consequences in a single species. This is however important, as a clear link between gamete production capacity and reproductive behaviour has been demonstrated in medaka knock-out fish (Padilla et al., 2021) and functional organismal consequences of Avp-dependent regulation of gametogenesis and steroidogenesis in tissues such as in the ovary (Joy and Chaube, 2015), remain to be evaluated *in vivo*.

To address these gaps, we here created a zebrafish *avp* $^{-/-}$ knock-out model to test the hypothesis that Avp contributes to reproductive success in zebrafish in either or both sexes through regulation of courtship behaviour and/or the HPG axis. Following the identification of a female-specific impairment of reproductive function, we subsequently assessed the potential mechanistic basis of this phenotype by assessing ovarian function using histological, biochemical, and molecular experimental approaches.

3. Material and Methods

3.1. Experimental animals and husbandry

All experiments were carried out in accordance with animal care guidelines provided by the Canadian Council on Animal Care and with prior approval from the University of Ottawa Animal Care Committee

(Protocol BL-3561). Wild-type (WT) zebrafish were sourced from the in-house stock at the University of Ottawa and maintained in 12 L tanks at a density of 3 fish/1 L n a recirculating system (Techniplast, Montréal, QC, Canada) supplied with salt-dosed (Instant Ocean, St. Blacksburg, VA, USA) RO water (hereafter "system water") maintained at a pH of 7.3, a conductivity of 400 μS, and a temperature of 28°C under a 14:10-h light–dark cycle. Fish were fed once or twice daily with GEMMA zebrafish diets (Skretting, Vancouver, BC, Canada) according to (eleuthero) embryonic and larval life stage. Adult fish were fed a mixed diet (Adult Zebrafish diet, Zeigler Bros Inc, Gardners, PA, USA; Larval AP-100, Zeigler Bros Inc, Gardners, PA, USA; Golden Pearls, Artemia International, Fairview, TX, USA) twice daily. Embryos (first for the development of the knockout line and later for maintenance of WT and *avp* $^{-/-}$ line and to supply animals for experiments) were attained by pairing individual male and female fish (4-8 months post fertilization; mpf) previously separated by visual inspection of pectoral fin breeding tubercles, body shape and coloring (Yossa et al., 2013; McMillan et al., 2015), and maintained in single sex groups. Individual males and females were then moved in a 1 L static breeding tanks with a perforated base insert and separated with a removable divider overnight. The following morning at the beginning of the light cycle, the barrier was removed, fish allowed to interact, and egg collected after spawning.

3.2. Generation of avp $^{-/-}$ knock-out zebrafish

A current zebrafish genome annotation (GRCZ11) in Ensembl was used for zebrafish gene coding and transcript information. Sequences were retrieved and gene structures mapped using Snapgene (Dotmatics, Boston, MA, USA). To identify CRISPR target sites, 5 kb sequence flanking translation start (5') and stop site (3') were used in CHOPCHOP software. A protocol published by Gagnon et al. (2014) was followed to generate templates for sgRNA transcription by annealing gene specific oligonucleotides containing the T7 promoter sequence, the 20 base pairs (bp) target site (**Table 1**) followed by the PAM sequence, and a complementary region of 80 bp constant oligonucleotide. All sgRNAs were transcribed using the HighScribe™ T7 Quick High Yield RNA synthesis kit (New England BioLabs, Ipswich, MA, USA), and the resulting RNA was purified using 5M ammonium acetate and ethanol precipitation. RNA

bands were detected by electrophoresis on a 1% agarose gel and RNA purity and concentration were measured using NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The CRISPR/Cas9 based approach (**Fig. 1A**) was designed to completely delete the *avp* $^{-/-}$ gene locus (NCBI Gene ID: 352922, genomic region NC_007119.7, Chr. 8), excising a region of 18832 nt including all three *avp* exons (**Fig. 1B**). Briefly, microinjections were performed in 1-cell stage embryos. The injection solution comprised of the following: 40 ng/µl for each sgRNA, 20 ng/µl Cas9 protein (New England BioLabs, Ipswich, MA, USA), and 0.1% Phenol Red (Sigma, Burlington, MA, USA) suspended Danieau buffer (in mmol/L: 58 NaCl, 0.7 KCl, 0.4 $MgSO_4$, 0.6 $Ca(NO_3)_2$, and 5.0 Hepes; pH 7.6). At 1 day post fertilization (dpf), 20 embryos (5 embryos/tube, 4 tubes) were genotyped. Following confirmation of whole gene deletion 1 dpf embryos, the rest of the embryos were reared to sexual maturity (60–90 dpf). For germ-line mutant screening, adults reared form injected embryos were mated with WT, and 40 offspring embryos (5 embryos/tube, 8 tubes) collected for genotyping of potential founders (F0). Following detection of whole gene deletion, mutant offspring (F1) were raised to 1 mpf, at which stage they were genotyped via DNA extracted from fin-clipping (collected following brief anesthesia in buffered tricaine mesylate (MS-222; Syndel Laboratories, Nanaimo, BC, Canada; 100 mg/l in 20 µl of 50 mmol/l NaOH at 95°C for 10 minutes (min) followed by neutralization with 2 µl of 1 mol/l Tris–HCl (pH 8). Heterozygous F1 were then crossed to generate homozygous F2 mutants, which were again genotyped as described above. For each genotyping, the PCR amplification reaction mix consisted of 1 µl of fin clip DNA digest template in 24µl PCR reaction master mix, which contained 12.5 µl 2X GoTaq Green Master Mix (Promega, Madison, WI, USA), 1 µl forward primer 1, 1 µl forward primer 2, 1 µl reverse primer (all IDT, Coralville, IA, USA, **Table 1**) and 8.5 µl ddH_2O. The PCR reactions were run with a temperature cycle of 95°C for 3 min followed by 35 cycles of 95°C for 30 s (denaturation), 60°C for 30 s (annealing), 72°C (extension) for 1 min. Following the last cycle, a final 72°C step for 10 min and samples maintained at 4°C until analysis. Gel electrophoresis using a 1% Agarose gel containing Red Safe dye (Froggabio, North York, ON, Canada) run for 30 min at 100 V was used to visualize amplicons. Using three primers listed in **Table 1,** this reaction produced a single 376

bp band for WT, two bands (376 bp and 138 bp) for heterozygous *avp* $^{+/-}$ mutants, or a single band (138 bp) for homozygous *avp* $^{-/-}$ mutants. Following identification of heterozygous mutants in somatic tissue of the founder generation, presence of the mutation in germline was confirmed by consistent appearance of *avp* $^{+/-}$ genotypes in fin clips of a fraction in F1 offspring following crosses of identified F0 *avp* $^{+/-}$ and WT. A mutant line was then established by incrossing heterozygous F1 *avt* $^{+/-}$ carrying the same deletion to generate homozygous F_2 *avp* $^{-/-}$ (**Fig. 1A**). In F2 *avp* $^{-/-}$ 376 bp fragments amplified from fin clips of fish carrying the homozygous deletion of all three *avp* exon by PCR described above was sent for Sanger sequencing (Genome Quebec, McGill University, Montreal, Canada) of the PCR product and alignment with the wild-type genomic sequence confirmed an 18832 bp deletion encompassing all three *avp* exons (**Fig. 1C).** Finally, absence of the *avp* mRNA transcript was confirmed in adult F2 *avp* $^{-/-}$ using total RNA extraction from brain and ovary using the Trizol Reagent (Invitrogen, Burlington, ON, Canada), followed by cDNA synthesis using the Quantitect reverse transcriptase kit (Qiagen, Hilden, Germany) according to the manufacturers' instructions. cDNA synthesized from total RNA extracted from brain and ovaries of WT and *avp* $^{-/-}$ was then used as template in PCR reactions prepared and run as described for fin clip DNA analysis with the exception that 11 µl cDNA template from brain or ovary, 1 µl *avp* FW and 1 µl *avp* RV primer (IDT, **Table 1**) and 8.5µl ddH2) were used. Gel electrophoresis using a 1% Agarose gel containing Red Safe dye (Froggabio, North York, ON, Canada) run for 30 min at 100 V was used to visualize *avp* amplicons (121 bp) (**Fig. 1D**).

Table 1. CRISPR/Cas9 constructs, genome target sequences, and *avp* $^{-/-}$ genotyping primer sequences.

Sequence ID	**Target and location according to NCBI zebrafish genome GRCz11 Primary Assembly**	**Sequence**
T1/PAM	Genomic downstream of *avt* locus NC_007119.7:1144047	GACATGTAGACGGACGC*CAG/GGG
PAM/T2	Genomic upstream of *avt* locus NC_007119.7:116359	CCA/GGA*CAGATCTCTAATGGACC
AVTF1	Genomic upstream of *avt* locus NC_007119.7:1143996	AGGATCTGTGACTACGCAATC
AVTF2	Genomic upstream of *avt* locus NC_007119.7:116250	AGTGACGTGTCAACACTAGT
AVTR1	Genomic upstream of *avt* locus NC_007119.7:116405	GTAATGCGGATAACTCTCAGG
AVTFW	*avp* mRNA NM_178293.2 nucleotides 108-128	CCCAGCCGGAGCCCATCAGA
AVTRV	*avt* mRNA NM_178293.2 nucleotides 219-239	CCATGCAGACCTGCGCCTCC

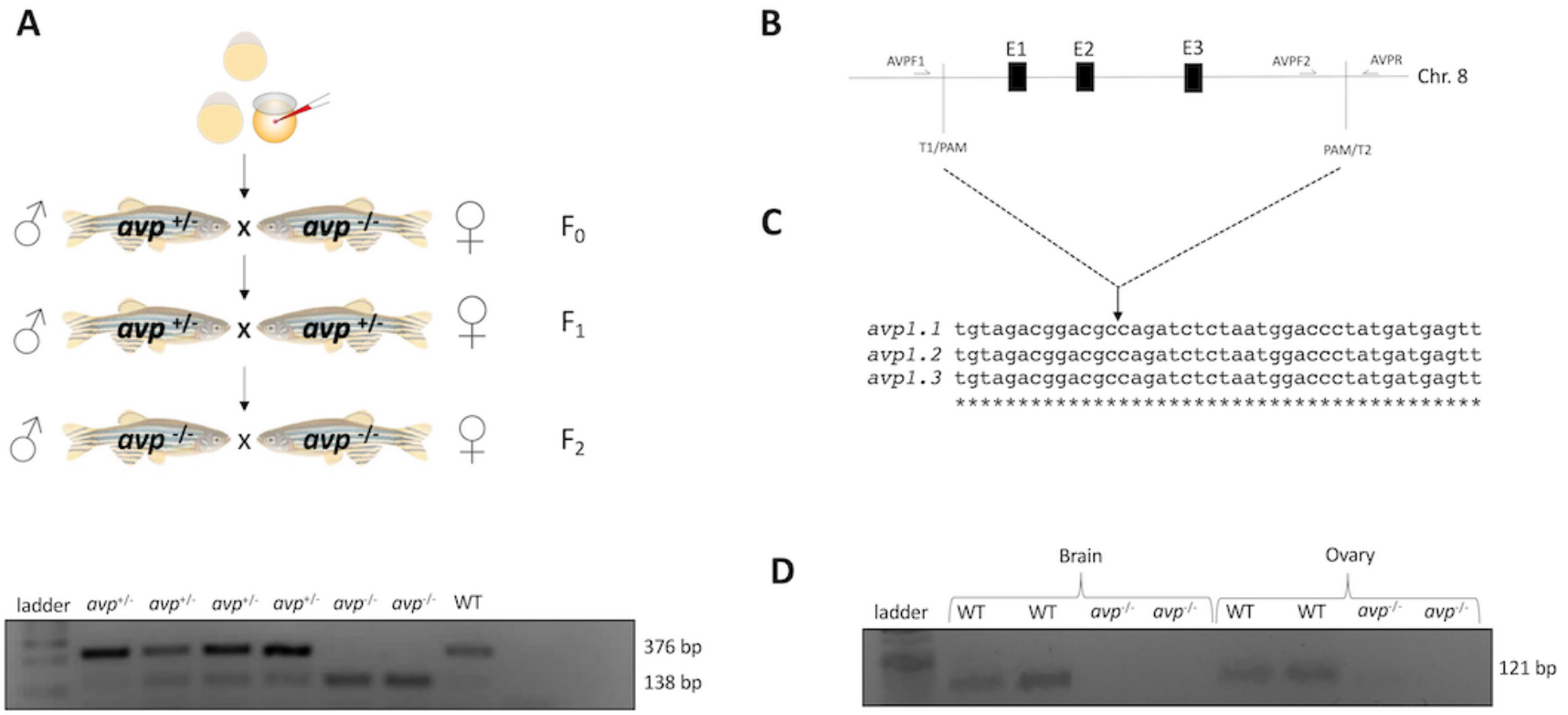


Figure 1. Validation of *avp* $^{-/-}$ zebrafish. CRISPR/Cas9 construct, and target sequence used for zebrafish embryo microinjections (**A**). DNA sequencing of *avp* locus in *avp* $^{-/-}$ fish (**B**), cDNA Agarose gel electrophoresis analyzing *avp* $^{-/-}$ mRNA specific amplicons in brain and ovaries obtained by PCR (**C**), and real-time RT-PCR quantification of *avp* transcript in WT and *avp* $^{-/-}$ fish.

3.3. Experimental design

The reproductive phenotype and potential underlying mechanisms were assessed using a three-tiered approach (**Fig. 2A-C)**. First, reproductive success was assessed in breeding assays using all four genotype combinations of male and female WT and *avp* $^{-/-}$ zebrafish (**Fig. 2A**). Following the identification of a female-specific phenotype, underlying mechanisms were assessed at the level of the ovary using histology, gene expression and hormone quantification assays (**Fig. 2B**). Finally, rescue experiments were conducted to assess whether activational or organizational effects of Avp are responsible for the observed phenotype (**Fig. 2C**).

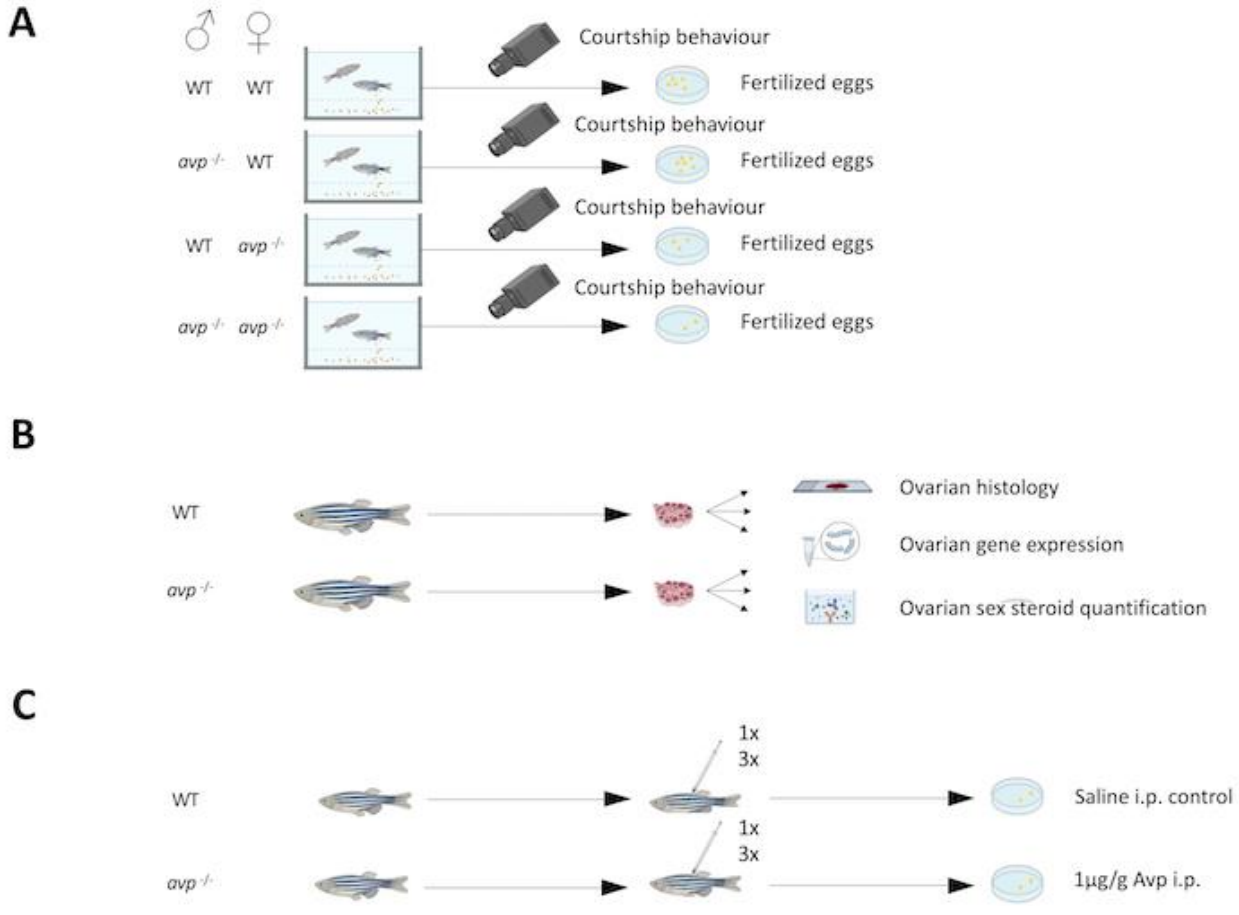


Figure 2. Schematic representation of experimental designs.

3.3.1. Zebrafish breeding assays, quantification of reproductive success, and courtship behaviour

Single male and female WT and male and female *avp* $^{-/-}$ zebrafish were combined as breeding pairs in a new 2 L breeding tanks to assess reproductive success. Following the observation of decreased reproductive success in *avp* $^{-/-}$ pairs compared to wildtype, additional breeding pairs in which WT of one sex was paired with the *avp* $^{-/-}$ fish of the other sex were investigated to assess sex-specific contributions of Avp to reproductive success in zebrafish. Thus, four groups were investigated (**Fig. 2A**): male WT x female WT (n=35), male *avt* $^{-/-}$ x female WT (n=12), male WT x female *avt* $^{-/-}$ (n=16) and male *avt* $^{-/-}$ x female *avt* $^{-/-}$ (n=35). Pairs received additional feeding in the afternoon the day before the mating assay and separated by divider overnight. The following day, before the onset of first light in the facility at 8:00h, fish were transferred to a new 2 L breeding tank with fresh system water, and the divider was removed at the onset of the light cycle. Breeding pairs were allowed to interact for 2h in the Zebracube and Zebrafish Behaviour platform (Viewpoint, Montréal, QC, Canada), allowing for a controlled environment and video recording of courtship behaviours using a Dragonfly2 DR2-HIBW camera with 30 frames/s (Point Grey Research, Richmond, BC, Canada). Following the 2h breeding period, eggs, if present, were collected using a strainer and placed in a petri dish filled with methylene blue system water. The total number of fertilized eggs (translucent, symmetrical with increased perivitelline space) were then quantified as measure of reproductive success. If present, unfertilized eggs (slightly yellow in colour and with granular appearance) and/or dead eggs (white in colour and often broken down) were counted separately and removed from the petri dish. Following consistent demonstration that irrespective of genotype, all analyzed courtship behaviours declined after initial interaction, the first 10 min of interaction were analyzed for each video in a subset of animals for which the interaction was recorded. To minimize confounding variables when courtship behaviours was analyzed, all videos were randomized and named using a code and a single observer was trained using a different set of videos to have consistency. Specifically, and as previously described (Yabuki et al., 2016; Altmieme et al., 2019; Mitchell et al., 2020), the number of individual male-initiated chasing events, the total duration of these chasing events, the number of nudging (targeted physical contacts of the male's head with the female flank), encircling (male swimming a full close circle around

the female), and quivering events (close flank by flank contact and parallel swimming of male and female in a jagged line, followed by female flexing away from the male) were analyzed by an experimenter blind to the crossed genotypes.

3.3.2. Investigation of the mechanistic basis of the reproductive phenotype

3.3.2.1. Ovarian Histology

The gonadosomatic index (GSI) was assessed from total WT and *avp*$^{-/-}$ fish sampled across experiments. Abdominal sections containing the ovary were collected from WT (n = 4) and *avp* $^{-/-}$ (n = 6) by terminally anesthetizing each fish, weighing the fish, and then collecting the abdominal section. Each section was placed in a 1.5 ml Eppendorf tube. The samples were then placed in 4% PFA PBS solution and incubated at 4°C overnight, the samples were washed for 3 x 15 min in PBS the following day. The samples were then transferred to a 0.5M EDTA (pH=8.0) solution to decalcify tissue for one week at 4°C. The following week, the samples were washed for 3 x 15 min in PBS, before successive dehydration steps in 10%, 20% and 30% sucrose in PBS solutions. Samples were placed in each sucrose solution until samples sunk. Finally, samples were embedded in molds with OCT (Thermo Fisher Scientific) and stored at -80°C until sectioning. Each sample was sectioned across the sagittal plane using a Cryostat (LEICA CM 3050 S, Leica, Concord, ON, Canada) and serially cut into thirty 12 μm sections (OT = -20°C, CT = -26 °C) collected on Fisherbrand Superfrost Plus microscope slides (Thermo Fisher Scientific). All slides were then rinsed in 1 x PBS for 3 min, then placed under a gentle stream of tap water for 3 min to wash off the OCT. For H&E staining, the slides were incubated for 30 seconds (s) in Mayer's Hematoxylin, then placed in 1 x PBS for 20 seconds and washed under a gentle stream of tap water for 1 min. The slides were then dipped in 70% and 95% EtOH for 30 s consecutively and placed in the counterstain Alcoholic Eosin for 30 s. Once removed, the slides were dehydrated through 2 x 15 seconds in 95% EtOH and 3 x 15 seconds in 99% EtOH. To ensure the tissue is cleared of any other residue, slides were immersed in xylene. 3 x for 1 min. Ovary sections were imaged using a dissection microscope (Olympus SZ2-ILST, Richmond Hill, ON, Canada) using a 6.7x magnification to determine the number of eggs and largest diameter of each oocyte.

The total number of oocytes per section and oocyte stages (I-V) were determined for each of the eggs measured according to Li and Ge (2020). Oocyte stages were expressed as percentage of total oocytes within WT or *avp* $^{-/-}$ ovaries respectively.

3.3.2.2. Quantification of ovarian reproductive hormone concentrations

Whole ovaries from WT (n=18) and *avp* $^{-/-}$ (n=18) female zebrafish were carefully extracted following terminal anesthesia. Both whole fish weight before the dissection, as well as ovary mass following the dissection were measured. In addition, 1.5 ml Eppendorf tube were weighed before and after the ovarian tissues were placed in side them to ensure consistency of weights for GSI calculations. Tissues were then stored at -80°C until processing. To extract ovarian hormones, ovarian tissue samples were thawed on ice and 200 µl ELISA buffer (Cayman Chemicals, Ann Arbor, MI, USA) was added to each tube. The samples were then sonicated, ensuring the probe was cleaned with 75% EtOH and RNAse free water in between each sample. Following homogenization, 1 ml of diethyl ether was placed into each sample, vortexed and allowed to sit for 30 min. The samples were then centrifuged at 3000 g for 5 min and flash frozen at -80°C for 30 min. The liquid phase was removed and placed into new microcentrifuge tubes. The ether was evaporated under a gentle stream of nitrogen under the fume hood, and the extraction steps were then repeated two more times. After the final extraction step, 250 µl extraction buffer was added to the tubes, vortexed and placed in a heat block for 5 min at 65°C. The samples were vortexed, placed on the heat block for additional 5 min and vortexed for a final time. The extracts were then placed at -80 °C until use. Extracts were used for the quantification of 17-β estradiol (E_2), Progesterone (P_4) and Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) using ELISA kits #501890, #582601 and #516011 (all Cayman Chemicals) according to manufacturer's instructions. Assay sensitivities (defined as 80% B/B_0) were 9.43 pg/ml for E_2, pg/ml 7.65 pg/ml for P_4 and 19.20 pg/ml $PGF_{2\alpha}$, respectively. All samples were run in duplicate on a single plate and a cut-off of <20% was applied for replicates. All analyte concentrations were corrected for sample dilution factors and normalized by ovarian weight prior to analysis.

3.3.2.3. Ovarian gene expression assays

Ovaries from female WT (n=7) and *avp* $^{-/-}$ mutants (n=7) were collected and immediately stored at -80 °C until processing as previously described. To extract total ovarian RNA, frozen tissues were homogenized in 250 µl of TRIzol reagent (Invitrogen) using a sonicator. The probe was cleaned with 75% EtOH and RNAse-free water in between each sample to avoid cross-contamination. Total RNA was extracted according to the manufacturer's instruction and the total RNA pellet resuspended in 30 µl of DEPC water. The purity and concentration of total RNA was then assessed using a NanoDrop and was used to generate cDNA. cDNA was synthesized using the QuantiTech Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions, using 1 µg of total RNA per sample. Controls devoid of reverse transcriptase enzyme were used to monitor potential contamination during subsequent gene expression assays. All samples were stored at -20°C until used as templates in gene expression assays.

Two-step SYBR green-based, semi-quantitative real-time RT polymerase chain reaction (RT-qPCR) was used to quantify relative fold-changes of 11 ovarian gene transcripts with known function in different stages of zebrafish oocyte maturation, as reviewed by Li and Ge (2020). Briefly, the assay profiled the expression of two genes enriched in germ cells (*vasa*, *nanos2*), the gonadotropin receptors (*lhcgr*, *fshr)*, the lipoprotein *vtg1,* nuclear and membrane receptors for steroids (*ar*, *pgr*, *pgrmc1*, *pgrmc2*) and genes involved in $PGF_{2\alpha}$ synthesis pathway (*pla2g4ab*, *ptgs2*). A complete list of gene IDs, full gene names, primer sequences used in gene expression assays and annealing temperatures can be found in **Table 2**. All assays were run on a BioRad CFX96 machine (Bio-Rad, Mississauga, ON, Canada) and consisted of a serially diluted standard curve consisting of pooled cDNA, a negative no-RT control, and individual samples run in duplicates. For each sample, the total reaction volume was 20 µl, which consisted of 1 µl of diluted cDNA template, 1 µl of 10 nM specific forward and 1 µl of 10 nM specific reverse primer (**Table 2**), 10 µl of SsoAdvanced Universal Inhibitor-Tolerant SYBR Green Supermix (Bio-Rad) and 7 µl of DEPC-water. For each assay, parameters were an initial two min activation step at 95°C, followed by 40 cycles consisting of a 20 s denaturation step at 95°C combined with a 30s annealing and extension step at

a primer specific temperature between 58-63°C. After each run, melting curves were produced by gradually increasing the temperature and final curves were assessed for single peaks to confirm specificity of the reactions. The acceptable range for amplification efficiency calculated from serially diluted curved was 90-110%, with an $R^2 > 0.95$. Relative gene expression was subsequently normalized using the NORMA-gene method (Heckmann et al., 2011) and relative transcript changes normalized to WT control zebrafish to determine relative fold-change for a given transcript in the *avp*$^{-/-}$ compared to WT.

Table 2. Profiled transcripts, primer sequences, and real-time RT-PCR reaction conditions used to quantify gene expression in WT and *avp* $^{-/-}$ ovarian tissue.

Gene symbol	NCBI Gene ID	Gene name	Forward Primer (5'3')	Reverse Primer (3'5')	Annealing temperature (° C)	Efficiency (%)	R^2
ddx4	30263	DEAD (Asp-Glu-Ala-Asp) box polypeptide 4 / Vasa	GCTGCAATGTTCTGTGTGCT	CATGAGGGTTTGTCGTTCCT	60	111.4	0.98
nanos2	101885179	nanos homolog 2	ACGGCTGTTTCCTGATGTG	CCCTTGCCTTTAGTCTGTGG	58	104.9	0.96
lhcgr	402920	luteinizing hormone / choriogonadotropin receptor	AAAAGGACGAGTCGCTGAAA	AAAACAAGAAGCAGGGCAGA	60	92.7	0.96
fshr	195820	follicle stimulating hormone receptor	TACCCCATCAATTCCTGCTC	CATCCAGATTCCACGCTTTT	60	99.3	0.92
vtg1	559475	vitellogenin 1	TTCAGACCCCCATTCAACTC	TTTCTCCAAGGAGGCAACAC	60	107.4	0.99
ar	100005148	androgen receptor	AGGGAGTTTTCCGACGAGTT	TTGGCAGGGTAAAAGTGAGG	60	111.08	0.97
pgr	569575	progesterone receptor	GAGTCCTTCGCTGATGTTCC	CTCTGGCTGTGTGTTGTCGT	58	95.2	0.99
pgrmc1	10857	progesterone receptor membrane component 1	CCTGGCTACGTTCTGTTTGG	GGGTCCGCTCTAATCCTTCT	58	112.7	0.99
pgrmc2	406378	progesterone receptor membrane component 2	TTCACGTCTGTGAGCGAAAC	AGAGGGAAACGGATGGAAAC	58	108.2	0.99
pla2g4ab	559087	phospholipase A2, group IV Ab (cytosolic, calcium-dependent)	ACAGGTGAACAAGGGCAGAG	ACAGGTGAACAAGGGCAGAG	60	96.5	0.98
ptgs2a	246227	prostaglandin-endoperoxide synthase 2a	GAGCTTCTCACACGCATCAA	ATGGGACCTTGACAACAGGA	63	86.4	0.98

3.3.3. Preliminary Avp rescue experiments

Acute single intraperitoneal (i.p.) Avp injection and repeated i.p. Avp injection experiments were conducted to assess whether the observed female reproductive phenotype in *avp*$^{-/-}$ zebrafish could be rescued through activational effects of Avp (**Fig. 2).** For the acute injection experiment, female WT and *avp* zebrafish were separated and held in single-sex tanks for two weeks prior to the start of the experiment. At the onset of the light the next morning, female WT fish (n=7), female *avp*$^{-/-}$ fish (n=7) and female *avp*$^{-/-}$ fish (n=7) were anesthetized using 0.24 mg/ml of tricaine (Syndel Laboratories) and once confirmation of deep anesthesia was confirmed, i.p. injected with either physiological saline (WT) for both WT and *avp*$^{-/-}$ female fish and or 1 μg/g body weight AVP in physiological saline (*avp* $^{-/-}$) for the remaining *avp*$^{-/-}$ female fish group. Following recovery from anesthesia (~10 min) female fish were set up for breeding with corresponding male zebrafish and mating success quantified as previously described. Using the same experimental parameters, a second repeated injection rescue experiment was conducted using WT (n=9) with saline, avp $^{-/-}$ (n=9) with saline and avp $^{-/-}$ (n=9) with 1 μg/g body weight AVP in physiological saline. Zebrafish spawned at the beginning of the experiment to allow liberation of eggs and subsequently injected on day 3, 6 and 9 after before quantification of reproductive success on the morning of day 10.

3.4. Statistical analysis

For each experiment, normal distribution of data was assessed using the Shapiro-Wilk test and homoscedasticity and Levene's test, respectively. In cases where the raw data were not parametric, standard transformations (log, sqrt, inversion, arcsin) were used to improve normality and/or homoscedasticity. Single outliers were identified in normally distributed data using Grubb's test. For parametric and homoscedastic data, a t-test was used in case of two comparison groups and univariate ANOVAs (one-way or two-way, as appropriate) followed by Tukey's post-hoc tests in cases of multiple comparison groups. In cases where data not normally distributed, Mann-Whitney U tests were used for comparisons of two groups, and Kruskal-Wallis tests for multiple groups followed by Dunn's post-hoc test. In cases were the

investigation of interaction of independent variables was warranted for non-parametric datasets, an ANOVA based on ranks followed by the Scheirer-Ray-Hare extension (Scheirer et al., 1976) was used. In all cases, a *P*-value <0.05 was considered as cut-off for significance. All analyses were conducted using SPSS Version 28 and graphs plotted using Graphpad Prism Version 9 (Graphpad Software, LaJolla, CA, USA).

4. Results

4.1. The reduced reproductive success in avp $^{-/-}$ fish is dependent on the female genotype

When comparing WT and *avp* $^{-/-}$ breeding pairs, the number of successful breeding was reduced from 53% in WT to 35.4% in *avp* $^{-/-}$ (**Fig. 3A)**. However, this reduction was not significantly different when assessed by Fisher's exact test (*P*=0.097). Conversely, the median number of viable eggs produced was significantly reduced (Mann-Whitney U= 741.5, *P*=0.004), reaching a median of 40 in WT and 0 in *avp* $^{-/-}$ pairs (**Fig. 3B**). When comparing average clutch size of successful breeding pairs, a significant (df=39, t=3.559, *P*=0.001), a more than 3-fold reduction was found in *avp* $^{-/-}$ breeding pairs compared to WT breeding pairs (**Fig. 3C**). Mean clutch sizes were 190 eggs in WT and 56 in *avp* $^{-/-}$ mutants. To delineate potential sex-specific genotype contributions, additional backcrosses between male WT and female *avp* $^{-/-}$ as well as female WT and male *avp* $^{-/-}$ were generated and the percentage of successful breeding events (**Fig. 3D**), the number of viable eggs produced (**Fig. 3E**) and clutch sizes (**Fig. 3F**) analyzed in conjunction with data obtained from homozygous breeding pairs presented above. The median number of viable eggs produced (**Fig. 3E)** depended significantly on the female (df=1 H=16.5551 *P*<0.001), but not male (df=1 H=0.320 *P*=0.572), genotype and was not dependent on the interaction of both genotypes (df=1 H=0.364 P=0.55)**.** Regarding clutch size, a significant effect of female genotype (df=1 F=17.062; *P*<0.01), but not male genotype (df=1 F=1.550; *P*=0.218) or their interaction (df=1; F=0.99; *P*=0.754) was observed (**Fig. 3F**). For the female genotypes, median number of eggs produced, and mean clutch size was significantly lower for *avp* $^{-/-}$ compared to WT (*P*<0.01).

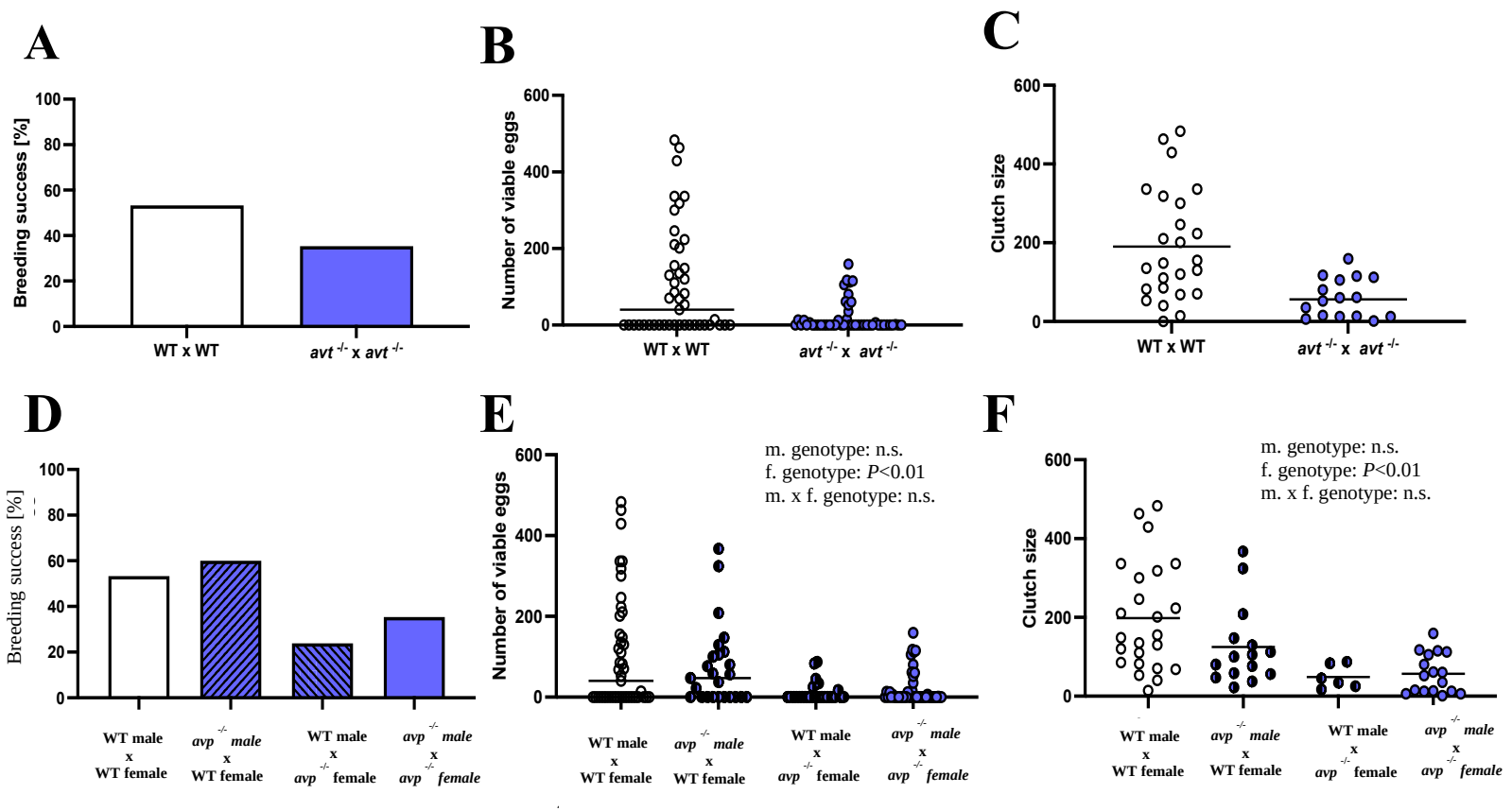


Figure 3. Percent successful breeding trials in WT (n=35) and *avp*$^{-/-}$ breeding pairs (n=35) analyzed by Fisher's exact test (**A**). Number of fertilized eggs in WT and *avp* $^{-/-}$ in all breeding trials analyzed by Mann-Whitney U test (**B**). Clutch size expressed as the number of fertilized eggs in successful breeding trials analyzed by (**C**). Full-factorial crossbreeding experiments to delineate sex-specific contributions of *avp* to zebrafish reproductive success. Four crosses were bred: WT females x WT males; WT females x *avp* $^{-/-}$ males (n=12) ; WT males x *avp* $^{-/-}$ females (n=16); *avp* $^{-/-}$ males x *avp* $^{-/-}$ females (**D**). Number of fertilized eggs of each breeding trials across breeding groups (**E**). Clutch size expressed as the number of fertilized eggs in successful breeding trials (**F**).

4.2. Quivering, a courtship behaviour linked to egg release is reduced in avp $^{-/-}$ females

Analysis of several aspects of courtship behaviours in breeding pairs revealed no or only marginally significant effects of male or female genotypes or their interaction. Specifically, within the first ten minutes of interaction of males and females during breeding assays, the number of male chasing events (**Fig. 4A**) did not significantly depend on male genotype (df=1 F=2.571; *P*=0.118), female genotype (df=1 F=1.017 *P*=0.320), or their interaction (df=1 F=0.180 *P*=0.647). Similarly, the duration of male chasing events (**Fig. 4B**) was not significantly affected by male genotype (df=1 F=4.096 *P*=0.051), female genotypes (df=1 F=1.032 *P*=0.317), or their interaction (df=1 F=0.438 *P*=0.513). The number of times males circled the females in breeding pairs (**Fig. 4C**) was neither dependent on male (df=1 H=0.676 P=0.411) or female (df=1 H=0.689 *P*=0.407) genotype, nor their interaction (df=1 H=0.012 *P*=0.914). Similarly, the number of times the male nudged the female's flank (**Fig. 4D**) was neither affected by male (df=1 H=1.915 *P*=0.166) nor female genotype (df=1 H=1.788 *P*=0.118) or their interaction (df=1 H=0.016 *P*=0.898). Conversely, quivering behaviour was significantly affected by female (df=1 H=8.042 P<0.01), but not male (df=1 H=1.35 *P*=0.244) genotype and was significantly reduced in mating pairs with *avp* $^{-/-}$ females compared to WT females (**Fig. 4E**). Quivering was not dependent on interaction of male and female genotypes (df=1 H=0.413 P=0.520).

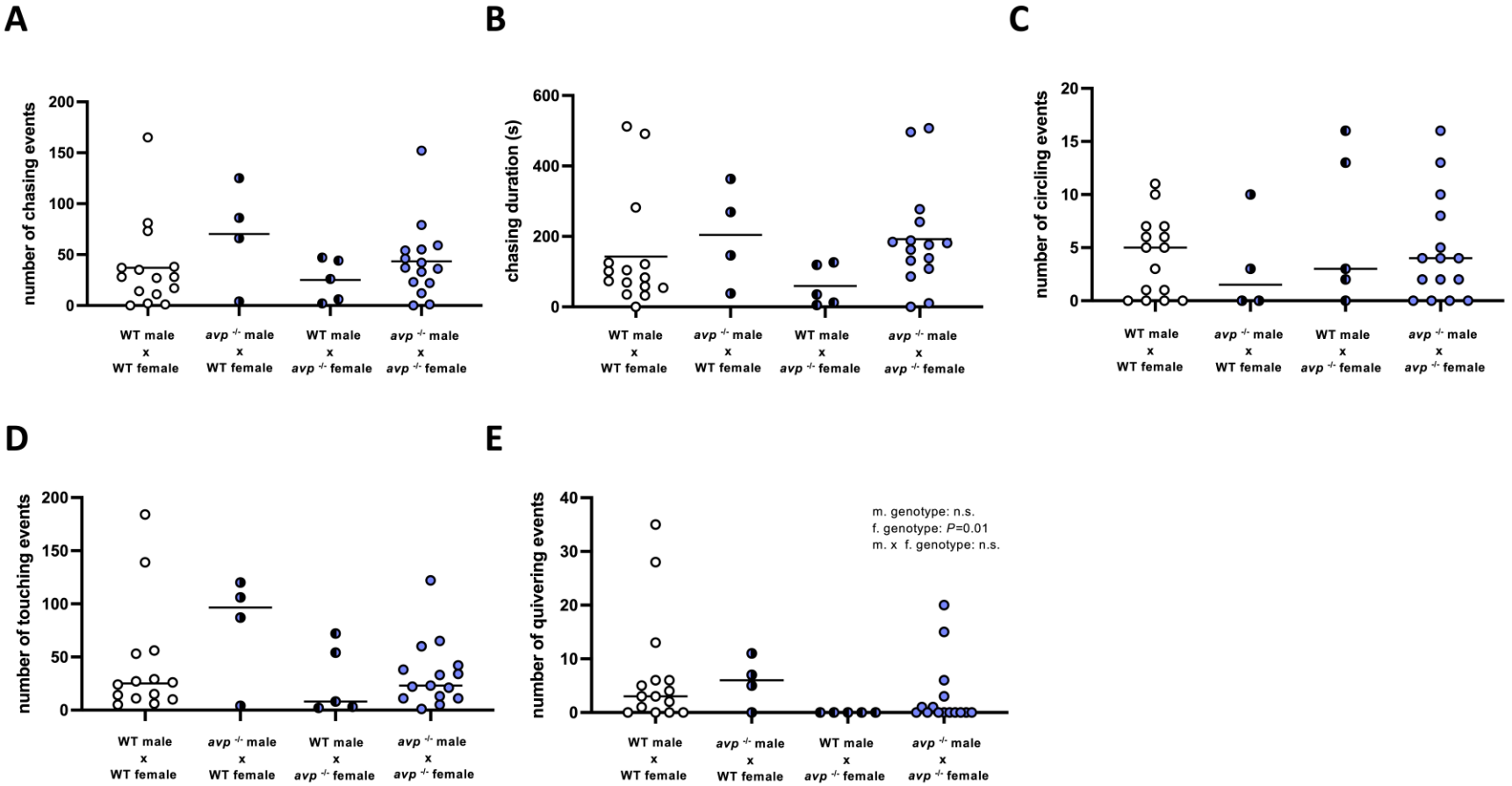


Figure 4. Quantification of courtship behaviours in WT and *avp* $^{-/-}$ fish breeding pairs. Full-factorial crossbreeding experiments to delineate sex-specific contributions of *avp* to zebrafish reproductive success. Four crosses were bred: WT females x WT males (n=14); WT females x *avp* $^{-/-}$ males (n=4) ; WT males x *avp* $^{-/-}$ females (n=5); *avp* $^{-/-}$ males x *avp* $^{-/-}$ females (n=15). A total of 2h of interaction after barrier removal were videotaped, and 1-10 min, 60-70 min, and 110-120 min were analyzed by a researcher blind to genotype of breeding pairs with only the data for the first 10 minutes displayed here. Results for number of nudges (**A**), number of chasing events (**B**), duration of chasing events (**C**), number of circling events (**D**), number of quivering events (**E).**

4.3. Reduction in female reproductive success is linked to retention of mature oocytes and decreased recruitment of stage I oocytes

The GSI was not significantly different between WT and *avp* $^{-/-}$ females (df=49 t=1.650 *P*=0.105; **Fig. 5A**). The median number of oocytes in ovarian sections were reduced ~2-fold in *avp* $^{-/-}$ compared to WT sections (Mann-Whitney U= 4959 *P*<0.001: **Fig. 5B**). This reduction is also significant when averaging all section counts for each animal to compare WT and avp$^{-/-}$ individual animal ovaries as biological replicates (Mann-Whitney U=1 *P*=0.0317; **Supplemental Figure 2.1**). When comparing the distribution of oocyte stages as percentage of total oocytes within each stage (**Fig. 5C**), comparisons revealed a significantly reduced percentage of stage I oocytes in *avp* $^{-/-}$ ovarian sections compared to WT ovarian sections (df=7 t=2.675 *P*=0.0368) and a significant increase in stage V oocytes (df=7 t=2.374 *P*=0.0493). No significant differences in the percentage of stage II (Mann Whitney U=4, *P*=0.343), III (df=3 t=1.008 *P*= 0.383) and IV oocytes (df=5.345 t=1.299 *P*=0.964) were found. Examples of stained ovary sections used for quantification of oocytes in WT and *avp* $^{-/-}$ ovaries are shown in **Fig. 5D**.

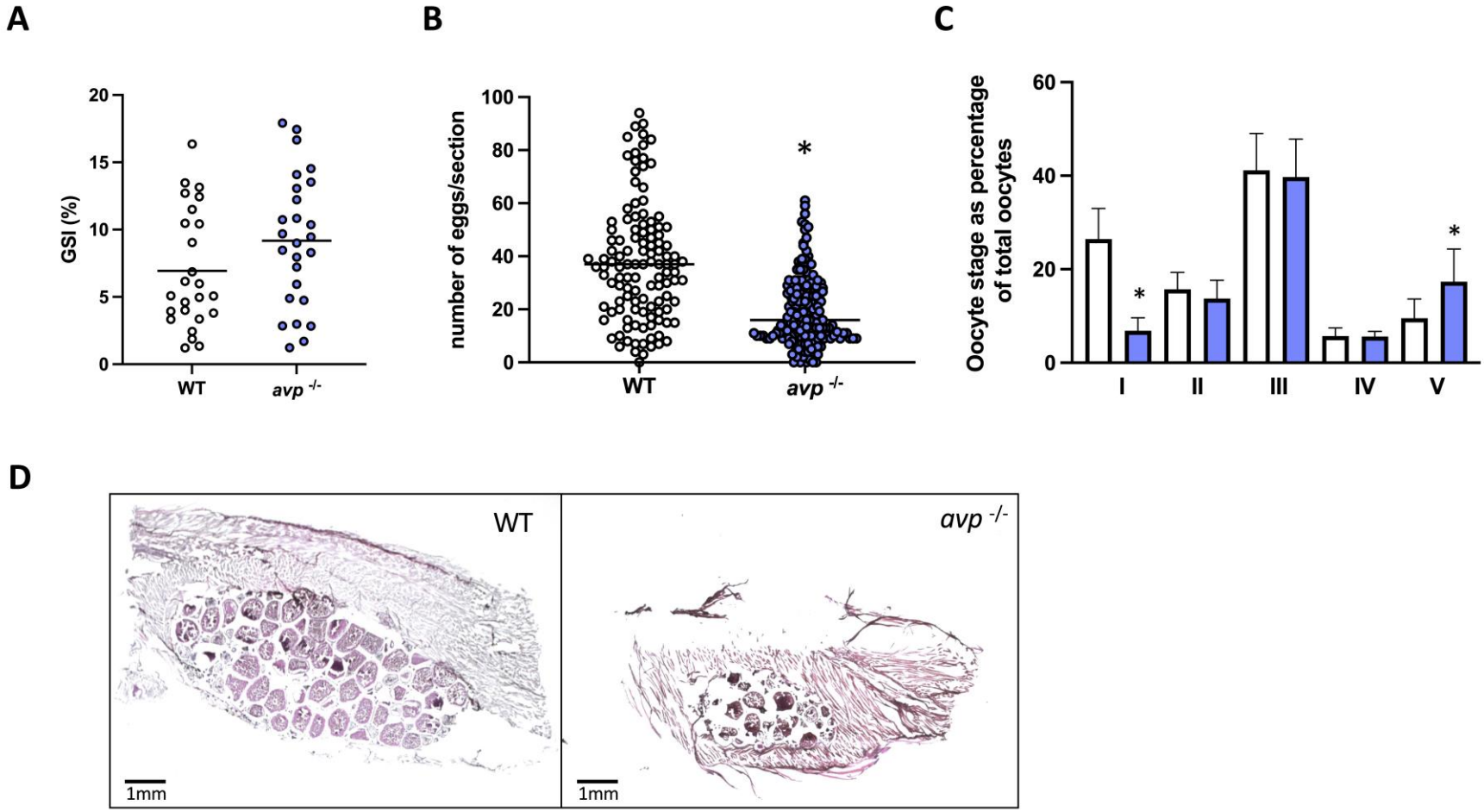


Figure 5. Ovarian histology and distribution analysis. Representative images of paraffin sections of WT (n=118) and *avp* $^{-/-}$ (n=172) ovaries (**A**). Using length measurements as specified by Li and Ge (2020) oocytes were staged, and distributions determined (**B**).

4.4. Ovarian $PGF_{2\alpha}$ is significantly reduced in avp $^{-/-}$ mutants

Ovarian concentrations of 17-β estradiol (**Fig. 6A)** were not significantly different between WT and *avp*$^{-/-}$ mutants (df=29 t=0.874 *P*=0.389) with an intra-assay coefficient of variability (CV) of 13.96% and a sensitivity of 20pg/ml. Similarly, ovarian tissue concentrations of progesterone (**Fig. 6B**) did not differ significantly between WT and *avp* $^{-/-}$ (df=32 t=0.6461 *P*=0.523), intra-assay CV of 15.75% and a sensitivity of 10pg/ml. Conversely, $PGF_{2\alpha}$ (**Fig. 6C**) was significantly reduced in *avp* $^{-/-}$ ovaries compared to WT ovaries (df=26 t=2.518 *P*=0.018), intra-assay CV of 13.4% and a sensitivity of 10pg/ml..

4.5. Ovarian transcripts coding for proteins involved in $PGF_{2\alpha}$ synthesis is reduced in avp $^{-/-}$ mutants

The ovarian transcript abundances of *nanos2* (df=11 t=2.598 *P*=0.025), and *pla2g4ab* (df=12 t=3.087 *P*=0.009) were significantly reduced, while the ovarian transcript abundances of *pgrmc1* (df=7.511 t=2.809 P=0.0244) and *pgrmc2* (df=12 t=2.276 P=0.042) were significantly increased in *avp* $^{-/-}$ mutants compared to WT **(Fig. 7).** The ovarian transcript abundance of *ddx4* (df=7.403 t=0.8331 P=0.43) *lhcgr* (df=12 t=1.770 P=0.102), *fshr* (df=12 t=0.9722 *P*=0.350) *vtg1* (df=12 t=1.189 *P*=0.257), *ar* (df=12 t=1.306 *P*=0.216), *pgr* (df12 t=1.452 *P*=0.172) and *ptgs2* (df=12 t=2.027 *P*=0.066) was not significantly different between *avp* $^{-/-}$ mutants and WT.

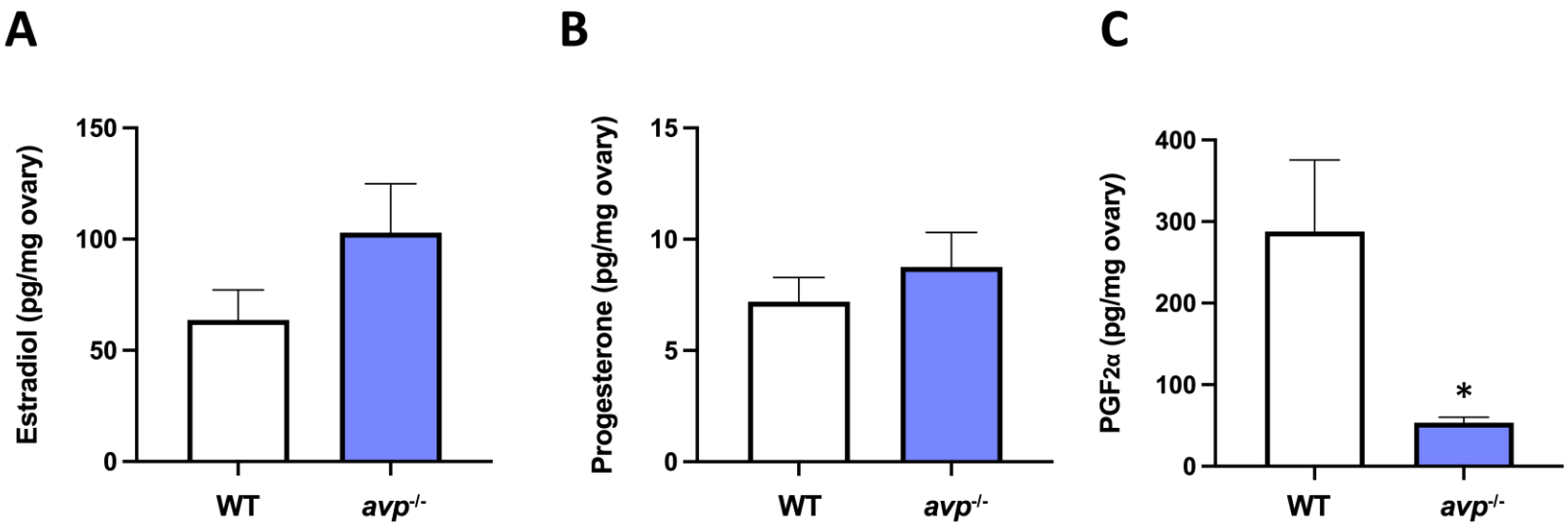


Figure 6. Ovarian sex steroid concentrations of E_2 (**A**), T (**B**), and $PGF_{2\alpha}$ (**C**) in WT (n=18) and avp $^{-/-}$ fish (n=18).

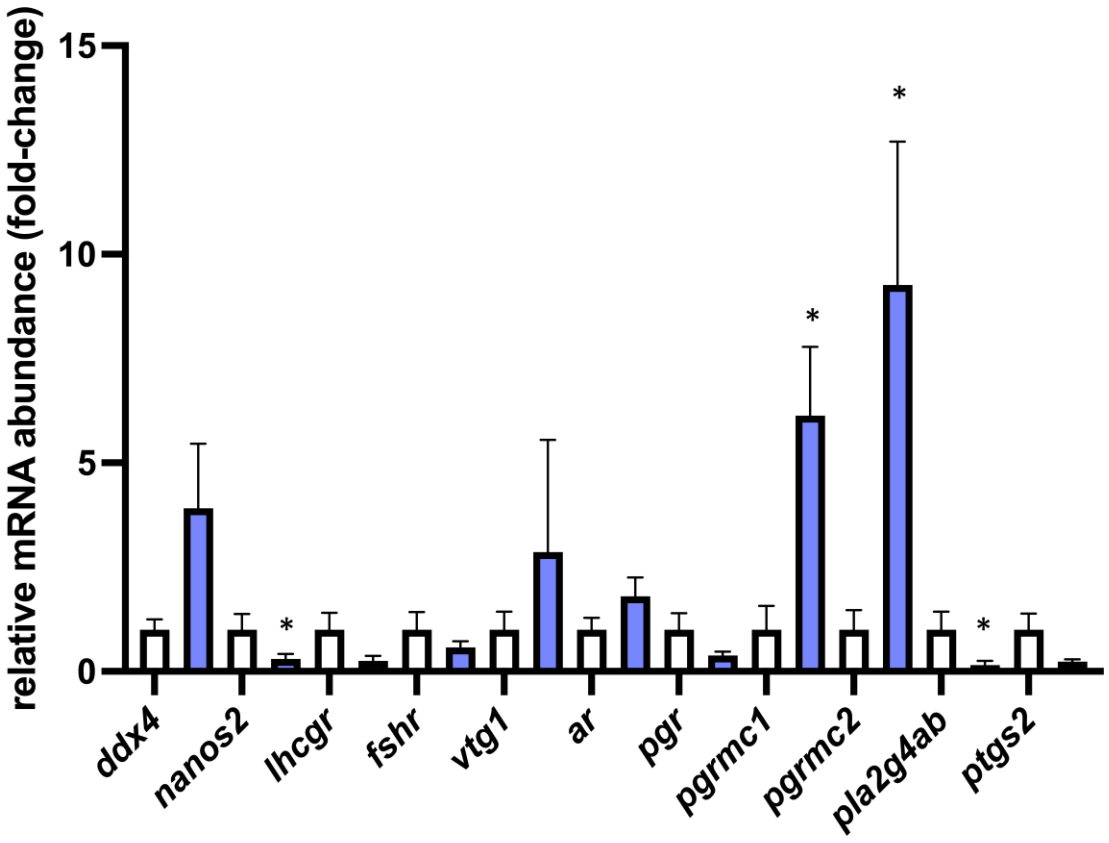


Figure 7. Targeted gene expression of selected genes with characterized roles in different stages of oocyte development in WT (n=7) and *avp* $^{-/-}$ (n=7) ovaries

4.6. Acute or repeated Avp injection does not attenuate the reduced female reproductive success in avp $^{-/-}$ *mutants*

After acute Avp injection, no significant differences of total spawned egg number (df=2, H=1.943, P=0.39; **Fig. 8B**) or clutch size (df=2, H=1.61, P=0.50; **Fig. 8C**) were observed. However, pairings that included saline-injected WT females were significantly different suggesting no rescue (Post-Hoc, P<0.05). Repeated Avp injection in *avp* $^{-/-}$ females revealed no significant differences in total spawned eggs (df=2, H=4.542, P=0.103; **Fig. 8E**) or clutch size (df=2, H=2.961, P=0.0.226; **Fig. 8F**) in homozygous breeding pairs. In both experiments, more assays are needed to determine if the phenotype has been rescued or if the lack of significant differences is only due to low sample size and variability.

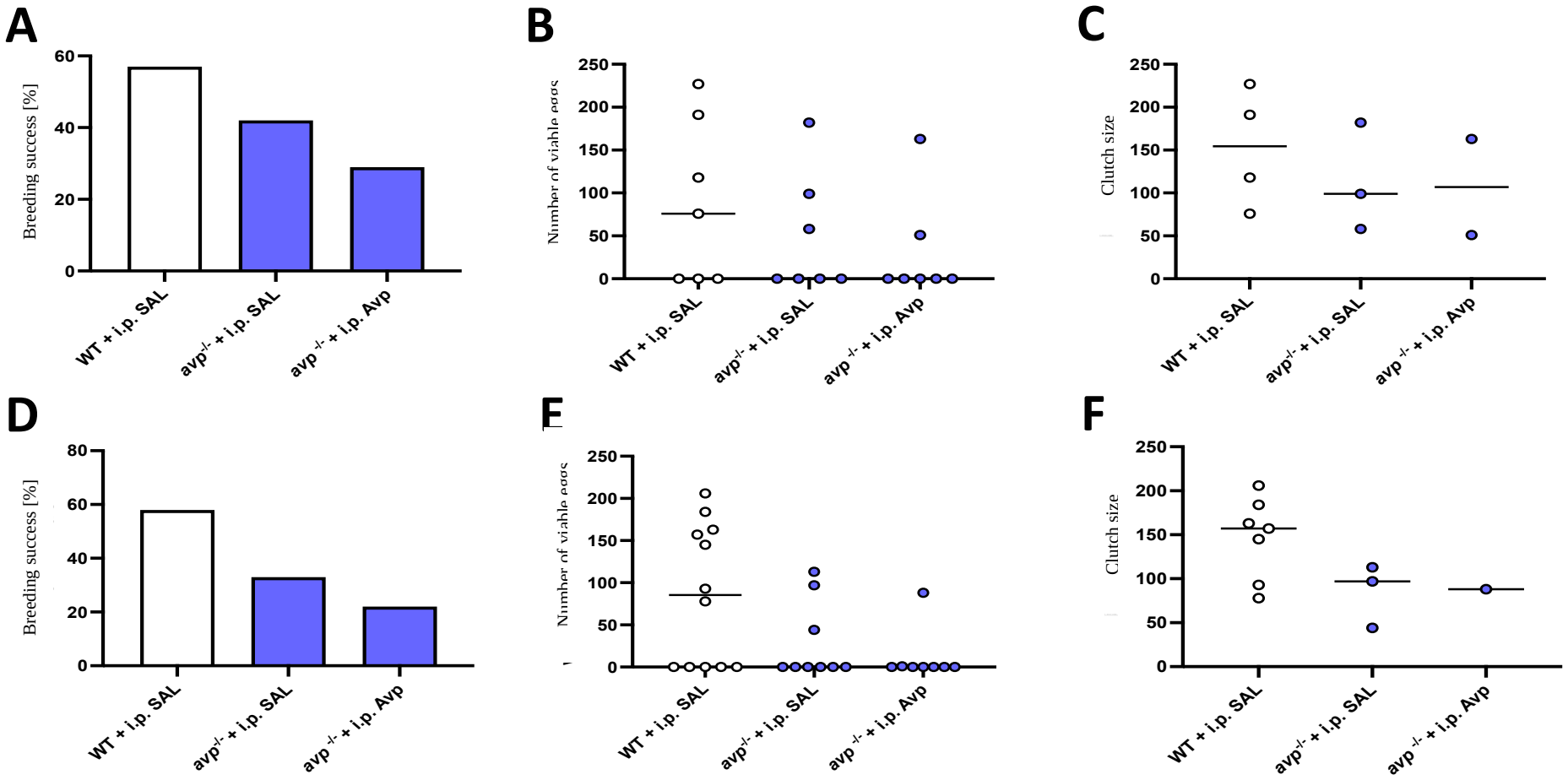


Figure 8. Rescue experiments assessing spawning success in i.p. saline-injected WT, saline-injected *avp* $^{-/-}$ fish, and 1 µg/g Avp injected *avp* $^{-/-}$ fish injected acutely on the morning of breeding assays (n=7 for each group, **A**) and repeatedly on every 3rd day for a period of 10 d with the final injection the day before the breeding assay (n=9 for each group, **D**). Number of fertilized eggs of each breeding trials across breeding groups that were acutely injected on the morning of breeding assays (**B**) and trice over a 10 d period (**E**). Clutch size expressed as the number of fertilized eggs in successful breeding trials for the acutely injected experiment (**C**) and repeated experiment (**F**).

5. Discussion

5.1. avp $^{-/-}$ mutants exhibit a female-specific reduction in reproductive success

We identified a significant reduction in reproductive success in *avp* $^{-/-}$ breeding pairs which manifested itself by reduced egg numbers and clutch sizes, but not a significant reduction in spawning success. Backcrosses of *avp* $^{-/-}$ males and females with WT females and males, respectively revealed that the observed reduction in these parameters was dependent on female, but not male *avp* $^{-/-}$ genotypes, as the latter reproduced similarly to WT pairs when crossed with WT females. The identification of reduced reproductive success in *avp* $^{-/-}$ breeding pairs compared to WT breeding pairs is in line with circumstantial evidence and mechanistic studies conducted in fish reviewed in the introduction of this study, although these studies report effects on both female and male reproductive function, especially regarding courtship behaviour, in several teleost species (Yokoi et al., 2015; Altmieme et al., 2019). Indeed, a recent study in male zebrafish revealed that acute pharmacological inhibition of Avp signaling via through the i.p. injection Manning's compound significantly reduced reproductive success compared to saline injected males when crossed with untreated females, an effect linked to inhibition of courtship behaviour (Altmieme et al., 2019). Similarly, the recently generated and only teleost *avp* $^{-/-}$ knock-out model to date, *avp* $^{-/-}$ Japanese medaka (Yokoi et al., 2015) exhibited a reduction in male dominance associated with mate-guarding, however, female reproductive function was not addressed in this study. In another, albeit seasonally spawning cyprinid, the Asian stinging catfish, a suite of in vitro studies revealed a role for Avp in oocyte maturation by promoting germinal vesicle breakdown (GVBD), modulating steroidogenesis, and promoting ovulation (Joy and Chaube, 2015). Early studies in natural Avp mutants in mammals (Altmieme et al., 2019), and recent studies of Ciona vasopressin (CiVP) in the vase tunicate, *Ciona intestinalis*, the closest existent vertebrate sister group (Matsubara et al., 2019; Kawada et al., 2021) suggest that a role for vasopressin on female reproductive physiology, and ovarian function in particular, may be evolutionarily deeply conserved in chordates. In the Brattleboro Long-Evans rats, an early natural mutant model characterized by the inability to produce central, but not peripheral, Avp aberrant estrous cycle length and smaller litter size have been reported (Boer et al., 1981). In the vase tunicate, Avp stimulated oocyte maturation via promotion

of germinal vesicle breakdown and ovulation via induction of metalloproteinases (Matsubara et al., 2019; Kawada et al., 2021).

5.2. Reduced female reproductive success in avp $^{-/-}$ mutants is linked to retention of mature eggs

To address the mechanistic basis of decreased egg release and clutch size in female *avp* $^{-/-}$ mutants, we investigated courtship behaviour and the HPG axis, specifically at the ovary level. In contrast to previous studies demonstrating an acute role for Avp in the modulation of male courtship behaviours following pharmacological inhibition of Avp signaling via Manning's compound, a V1aR antagonist (Altmieme et al., 2019) male courtship behaviours such as chasing, circling and nudging remained unaffected in any breeding combination of male or female *avp* $^{-/-}$ mutants and male or female WT. Such differences may be linked to the different temporal timescales in genetic and pharmacological disruption of Avp signaling, as *avp* $^{-/-}$ mutants may experience an alteration in organisational effects of Avp and/or compensatory effects, while acute pharmacological manipulation of receptor specific Avp signaling targets activational effects of Avp on a naturally matured Avp system. Potential organizational effects of Avp systems are only beginning to be investigated in different vertebrate species including birds (Baran, 2017) and mammals (Patisaul, 2017), but remain completely uncharacterized in fish. Thus, future studies are warranted to discern potential organisational an activational roles of Avp in fishes. Interestingly, quivering, a zebrafish courtship behaviour linked to acute egg release and simultaneous male milt release to maximize fertilization (Zempo et al., 2021), was significantly reduced based on female, but not male *avp* $^{-/-}$ genotype.

In line with this behavioural phenotype, histological analysis of *avp* $^{-/-}$ ovaries revealed a significantly higher percentage of stage V oocytes when quantifying oocyte stage distribution (stages I-V) as percentage of total oocytes. This finding suggests that retention of mature oocytes contributed, at least in part, to the female reproductive phenotype in *avp* $^{-/-}$ mutants. Because the endocrine control of the ovulation process is well known to include $PGF_{2\alpha}$ in zebrafish (Lister and Van Der Kraak, 2008, 2009), other cyprinids, such as goldfish, *Carassius auratus* (Sorensen et al., 2018), and several other teleost fishes (Takahashi et al., 2018), we quantified ovarian $PGF_{2\alpha}$ concentrations and ovarian gene targets coding for

enzymes involved in its synthesis. Indeed, ovarian concentrations were $PGF_{2\alpha}$ were significantly reduced by ~80% in *avp* $^{-/-}$ compared to WT ovaries, suggesting that Avp is a physiological regulator of mature egg release through the stimulation of $PGF_{2\alpha}$ in zebrafish. This is in line with detailed *in vitro* studies in another, albeit seasonally breeding cyprinid, the Asian stinging catfish, where a suite of studies demonstrated that Avp acutely and potently stimulates $PGF_{2\alpha}$ synthesis in post-vitellogenic follicles (Joy and Singh, 2013; Joy and Chaube, 2015). This effect was biphasic eliciting a maximal stimulation at a concentration of 100 nM Avp which is stronger than the increase in $PGF_{2\alpha}$ synthesis elicited by incubation with 20 IU/ml hCG (Joy and Singh, 2013; Joy and Chaube, 2015). Concurrent incubation with Avp and hCG at aforementioned concentrations further increased ovarian $PGF_{2\alpha}$ synthesis, suggesting additive effects (Joy and Singh, 2013; Joy and Chaube, 2015). Finally, Avp stimulation of $PGF_{2\alpha}$ induced final oocyte maturation and ovulation *in vitro*, an effect that was reduced by administration of a V1aR antagonist and indomethacin, a non-selective cyclooxygenase inhibitor. In light of these studies, our findings suggest that the observed reproductive phenotype in female *avp* $^{-/-}$ mutants are, at least in part, mediated at the level of the ovary and that the observed roles of Avp in oocyte maturation and ovulation *in vitro* in a seasonal spawning fish extend to asynchronous zebrafish *in vitro*. It remains, however unclear to which extent central and/or peripheral ovarian Avp systems are involved in this system and future studies using *in vitro* incubations of *avp* $^{-/-}$ ovarian tissue are warranted to dissect the role of the peripheral ovarian Avp system on $PGF_{2\alpha}$ oocyte maturation and ovulation in detail.

At the molecular level, the reduced ovarian concentration of $PGF_{2\alpha}$ is linked to a significant or marginally significant reduction in two key transcripts coding for enzymatic components of the $PGF_{2\alpha}$ biosynthesis pathway. Specifically, the transcript abundance of *pla2g4ab*, a paralogue of the phospholipase A_2, which cleaves the prostaglandin precursor arachidonic acid (AA) from membrane phospholipids (Takahashi et al., 2018) is significantly reduced by ~80% in *avp* $^{-/-}$ ovaries compared to WT ovaries. A similarly strong, albeit marginally significant, ~80% reduction in the transcript abundance of *ptgs2*, a paralogue coding for prostaglandin-endoperoxide synthase also known as cyclooxygenase (Takahashi et

al., 2018). These findings suggest that ovarian Avp signaling acts to stimulate $PGF_{2\alpha}$ biosynthesis by inducing transcripts involved in the two regulatory steps of the $PGF_{2\alpha}$ biosynthesis, AA precursor mobilization and its oxidation to prostaglandin H_2 (PGH_2), which is further converted to mature prostaglandins including $PGF_{2\alpha}$.. The physiological importance of both steps in ovarian zebrafish $PGF_{2\alpha}$ synthesis has been demonstrated in detail (Lister and Van Der Kraak, 2008). Firstly, the importance of AA mobilization in ovarian $PGF_{2\alpha}$ synthesis was demonstrated by the finding that increasing concentrations of AA dose-dependently stimulate $PGF_{2\alpha}$ irrespective of ovarian follicle stage (Lister and Van Der Kraak, 2008). Secondly, the importance of ovarian cyclooxygenase in ovarian $PGF_{2\alpha}$ in zebrafish ovarian follicles in vitro was demonstrated by the fact that indomethacin, a non-selective cyclooxygenase 2 inhibitor significantly reduced AA stimulated $PGF_{2\alpha}$ synthesis in vitellogenic follicles *in vitro*. Conversely, no changes were observed in ovarian E_2 and P_4, which is in contrast of reported regulation of both steroids by Avp in oocytes of a seasonal breeder the Asian stinging catfish (Joy and Chaube, 2015). However, because season-dependent stimulatory and inhibitory effects of Avp on these sex steroids were observed in catfish oocytes, it is possible that ovarian cell specific effects were masked in ovaries of asynchronous zebrafish, which contain multiple oocyte maturation stages at a given point in time. Because ovarian $PGF_{2\alpha}$ released during ovulation into female circulation is a potent regulator of female reproductive behaviour in several (cyprinid) fishes (Stacey and Peter, 1979; Juntti et al., 2016; Sorensen et al., 2018) which through secretion via urine and action as a pheromone furthermore elicits male courtship to synchronize mature gamete release (Sorensen et al., 1988; Kobayashi et al., 2002; Yabuki et al., 2016), it is likely that the described reduction in quivering behaviour in female *avp* $^{-/-}$ mutants is a consequence of reduced Avp-dependent $PGF_{2\alpha}$ release. Future i.p. $PGF_{2\alpha}$ rescue studies in *avp* $^{-/-}$ mutant females are clearly warranted to discern which aspects of the reproductive phenotype female *avp* $^{-/-}$ are dependent on disrupted ovarian $PGF_{2\alpha}$ synthesis.

5.3. Additional aspects of the female reproductive phenotype in avp $^{-/-}$ mutants

In addition to the described integrative evidence suggesting that female reproductive consequences of *avp* $^{-/-}$ knockouts are, at least in part, dependent on Avp-dependent stimulation of ovarian $PGF_{2\alpha}$, our mechanistic studies point to additional, previously unidentified mechanisms. When considering the distribution of oocyte stages as percentage of total oocytes in *avt* $^{-/-}$ compared to WT, a significantly lower percentage of stage I oocytes are observed in *avp* $^{-/-}$ compared to WT ovarian sections. This suggests that Avp may, in addition to previously discussed roles in oocyte maturation and ovulation, play a role in germline stem cell maintenance, proliferation and/or differentiation into stage I follicles. In zebrafish, and in contrast to mammals which possess a finite number of oocytes following embryogenesis, zebrafish can produce new oocytes throughout their lifetime due to the presence of self-renewing germline stem cells (Liu et al., 2022). Indeed, this possibility is supported by the finding that transcripts of *nanos2* are significantly reduced in *avp* $^{-/-}$ ovaries compared to WT ovaries. The *nanos2* transcript encodes an RNA-binding protein that is specifically expressed in zebrafish GSCs (Beer and Draper, 2013). In a recent detailed single-cell RNA-seq analysis of ovarian cells in zebrafish, it has specifically been shown to be present in a single, temporally early subcluster of GSC, which is in contrast to another germinal stem cells (GSC) marker, *ddx4* (formerly *vasa*), which is present in all nine identified subclusters of GSC as well as stage 1A oocytes (Liu et al., 2022). Such detailed single cell expression analyses of ovarian cell populations in zebrafish provide an explanation for the observed discrepancy in GSC marker expression observed in *avp* $^{-/-}$ compared to WT ovaries, as expression of *ddx4*, an ATP-dependent RNA helicase identified in zebrafish GSCs across development (Hartung et al., 2014) did not change significantly between genotypes and revealed a tendency for an increase in transcript abundance in *avp* $^{-/-}$ compared to WT. Together, these findings may suggest a specific reduction of early pool GSC in the absence of Avp in the zebrafish ovary. While functionally *nanos2* has been shown to maintain ovarian GSCs and to drive zebrafish ovarian regeneration following ablation oocytes (Liu et al., 2022), it is currently unknown whether nanos2 positive GSC are directly responsive to Avp via expression of receptors or whether indirect effects manifested in the *avp* $^{-/-}$ ovaries, such as the retention of final stage oocytes and/or a reduction in ovulation are involved

in this phenotype. Interestingly, using the searchable single cell RNA-seq database published in (Liu et al., 2022), *avp*, *avpr1ab* and to. Lesser extent *avpr2ab* are while not highly abundant, mostly expressed in GSCs, and specifically, early GSCs to which *nanos2* expression is restricted (**Supplemental Figure 2.2**). Thus, it is feasible that Avp directly regulates a specific pool of undifferentiated GSCs in zebrafish. This possibility clearly warrants further investigation, as regulation of (ovarian) GSCs by Avp have not been described in any species.

Finally, ovarian gene expression analysis revealed a significant increase in progestin receptor membrane components 1 and 2 transcripts (*pgrmc1*, *pgrmc2*), which code for single transmembrane heme-binding protein containing a cytochrome b5 (Cytb5) motif and have which has been proposed as a progesterone binding protein in vertebrates, but also can also interact with various molecules such as heme, fatty acid 2-hydroxylase, cytochrome P-450 enzymes, insulin receptor, epidermal growth factor receptor (EGFR), Erbb2, and membrane progestin receptor α (mPRα), among others, rendering its precise physiological roles difficult to dissect (Wu et al., 2018). In female zebrafish, *pgrmc1 and pgrmc2* are most abundantly expressed in ovarian tissue, with a reported expression peak during oogenesis in stage I follicles (Wu et al., 2018, 2019). Recent single cell sequencing of ovarian cells in mature females provided a higher resolution of cell-type sand stage specific expression profiles, revealing a more widespread expression in ovarian cells with the highest expression found located in stromal and thecal cells for *pgrmc1* and differentiating GSCs and follicles for *pgrmc2* (Liu et al., 2022). Irrespective of distribution, global functional roles in ovaries have been assessed for both *pgrmc1* and *pgrmc2* using knock-out approaches in zebrafish in vivo as well as pharmacological approaches in oocytes *in vitro* (Wu et al., 2018, 2019; Wu and Zhu, 2020). These studies revealed, among other effects, a role for Pgrmc in the promotion of meiotic maturation in oocytes via stabilization of mPr and thus progestin sensitivity *in vivo* and *in vitro* (Wu et al., 2018). Thus, while the precise role of Pgrmc1 and Pgrmc2 is difficult to disentangle due to the widespread expression in ovarian cell types (Liu et al., 2022), it is possible that the observed increase in matured

oocytes observed in *avp* $^{-/-}$ ovaries compared to WT is partially dependent on the promotion of meiotic oocyte maturation in zebrafish.

5.4. Potential role of pleiotropic effects of Avp on the female reproductive phenotype in avp $^{-/-}$ *mutants*

Because our study investigated the reproductive consequences of Avp using our newly created *avp* $^{-/-}$ mutant *in vivo*, it is important to at least consider the possibility of indirect pleiotropic effects of Avp. In teleost fishes, several physiological roles for Avp have been described, including, but not limited to energy balance, regulation of the endocrine stress axis, osmoregulation and cardiovascular effects, as well as circadian regulation (Balment et al., 2006). While it is impossible to assess all phenotypic characteristics in the *avp* $^{-/-}$ knock out model in the context of this study, we elucidated potential consequences on somatic growth, a more important determinant of reproductive maturity in developing female zebrafish compared to age (Chen and Ge, 2013). While potent effects of Avp on the regulation of feed-intake have been described in adult teleost fishes (Gesto et al., 2014), no difference in body mass, body length or body mass index (BMI) was found between *avp* $^{-/-}$ and WT fishes up to sexual maturity 3 mpf (**Chapter 3**), suggesting that the observed reproductive effects are not secondary effects linked to somatic growth and different timelines to achieve sexual maturity. Secondly, because Avp has been characterized to be involved in the regulation of the endocrine stress axis in teleost fishes (Balment RJ et al., 2006; Gesto et al., 2014), we assessed larval and adult cortisol concentrations in *avp* $^{-/-}$ mutants and WT. Both larvae and adult zebrafish exhibited an elevated concentration of cortisol (**Chapter 3**), raising the possibility that hypercortisolism may contribute the described female reproductive phenotype in *avp* $^{-/-}$ mutants. Increased cortisol has been shown to affect zebrafish ovarian function and oogenesis *in vitro* and *in vivo* (Sousa et al., 2015; Faught et al., 2020; Maradonna et al., 2020). Briefly, direct incubation of stage I and especially stage II follicles with 1 μM cortisol induced increased DNA damage as assessed by comet assay (Sousa et al., 2015). In female *gr* $^{-/-}$ zebrafish characterized by hypercortisolism, an increase in oocyte cortisol and a reduction in ovulated eggs has been identified (Maradonna et al., 2020). Unfortunately, no oocyte stage distribution has been reported in this study (Maradonna et al., 2020). In females of another *gr* $^{-/-}$ knock-out model, increased

fecundity was observed in early adult females, while decreased fecundity was observed in adult females, indicative of early ovarian senescence (Faught et al., 2020). In both age-groups, increased follicular atresia was observed. However, especially because a buffering capacity with regard to cortisol load that is mediated via induction of 11β-hydroxysteroid dehydrogenase type 2 (*11β-hsd2)*, a cortisol inactivating enzyme, has been described in zebrafish oocytes (Faught et al., 2016), additional studies are necessary to specifically address potential contributions of cortisol to the observed reproductive phenotype in female *avp* $^{-/-}$ fish.

6. Conclusion

In sum, our study using newly created *avp* $^{-/-}$ knock-out zebrafish shows that reproductive function, as quantified egg release and clutch size, is significantly reduced and that this reduction is dependent on female mutants. Integrative mechanistic investigation of the underlying mechanisms of this phenotype strongly suggest that this phenotype is at least in part dependent on reduced ovulation mediated by a disrupted $PGF_{2\alpha}$ synthesis, which may also underlie the reduction in quivering behaviour associated with ovulation. These findings in asynchronously spawning catfish are in line with previous findings from in vitro studies investigating the role of Avp on oocytes maturation and function *in vitro* (Joy and Chaube, 2015), and furthermore contribute to the emerging picture of an evolutionary conserved role for Avp peptides in oocyte maturation and release in chordates (Matsubara et al., 2019). Mechanistically, our study additionally uncovers a potentially new mechanism for Avp on oocyte maturation, revealing a potentially direct regulation of undifferentiated *nanos2* pools of GSCs by Avp which merit further investigation. While no differences in overt *avp* $^{-/-}$ female reproductive phenotypes following acute but not repeated Avp administration may suggest that the phenotype can be rescued, a larger number of individuals should be injected acutely to assess whether the female reproductive phenotype is dependent on organizational or activational effects of Avp. Finally, our study suggests that *avp* $^{-/-}$ zebrafish may be a promising *in vivo* model to dissect pleiotropic Avp effects on additional physiological systems.

7. Acknowledgements

The authors gratefully acknowledge funding support in form of a NSERC-DG (#147476) and CFI-JELF (#148035) awarded to JAM. The authors are grateful for excellent animal care by ACVS at the University of Ottawa and would like to acknowledge the help of Brooke Kattenbusch in the analysis of courtship behaviours.

Chapter III

Assessment of pleiotropic roles of Avp with potential implications for the reproductive phenotype in *avp* $^{-/-}$ mutants

Statement of contribution:

I conducted a series of experiments focused on elucidating the role of the nonapeptide Avp in regulating growth, the endocrine stress axis, and specific 'anxiety-like' behaviours and locomotion at different stages of the zebrafish life cycle. Specifically, I quantified somatic growth by measuring body mass and length monthly up to sexual maturity (1-3 mpf). In larvae, I quantified basal whole body cortisol concentrations, whereas I quantified blood cortisol concentrations at baseline and following acute exposure to netting and air-exposure to assess endocrine stress axis activity in *avp* $^{-/-}$ mutants and wild-type fish. In larvae, I further quantified locomotion, an energy expending process, and assessed 'anxiety-like' behaviours in zebrafish using the novel-tank test paradigm in adult zebrafish. Two Honour's students in the lab, Rita Wehbe and Kusum Sharma were involved in stress axis experimental protocols, steroid extractions, and anxiety-like behaviour phenotyping. I would like to thank Dr. Emily Standen and her lab for the use of the light microscope and Dr. Katie Gilmour and her lab for the training and generous donation of the ELISA cortisol assays.

1. Evolutionary conservation of the Avp system and pleiotropic roles in animal physiology

In all non-mammalian vertebrates, the nonapeptide arginine vasotocin (*avp*) has been found due it being highly conserved in vertebrate evolution (Gwee et al., 2008; Banerjee et al., 2017). The *avp* gene in teleost fishes as in other non-mammalian vertebrate species and the homologous mammalian arginine vasopressin (AVP) gene encodes a precursor protein whose cleavage generates the mature nonapeptide, a carrier protein termed neurophysin 2, as well as a peptide fragment termed co-peptin (Gwee et al., 2008; Banerjee et al., 2017). In zebrafish and other teleost studied to date, vasotocin is produced in giganto-, magno-, and parvocellular neurons of the preoptic area (POA), from where it innervates diverse hypothalamic regions and extrahypothalamic brain regions (Batten et al., 1990; Saito et al., 2004; Thompson and Walton, 2009; Herget and Ryu, 2015; Kagawa et al., 2016; Pouso et al., 2017). Innervation of extrahypothalamic regions includes sensory regions (Goodson et al., 2003; Perrone et al., 2014) and hindbrain motoneurons (Batten, 1986; Iwasaki et al., 2013). These regions are directly relevant to the perception of environmental stimuli relevant and the regulation of appropriate behaviour. Due to this nature, *avp* may act centrally within the central nervous system as a neuromodulator or peripherally in various targeted organs as this nonapeptide is involved in the regulation of numerous aspects of teleost physiology, including growth and metabolism, stress axis regulation, cardiovascular function, osmoregulation and circadian and seasonal rhythms (Balment RJ et al., 2006; Gesto et al., 2014). The pleiotropic roles of Avp are not exclusive to teleost fishes. Studies in an invertebrate models, for example, have shown an evolutionary conserved and ancient role for Avp in the regulation of feeding and reproduction (Odekunle and Elphick, 2020). Similarly, pleiotropic roles of Avp extend to other vertebrate species, and roles for Avp in the regulation of growth and energy metabolism, the endocrine stress axis, and diverse behaviours have been described in most vertebrate classes (Goodson and Bass, 2001; Albers, 2012; Hasunuma et al., 2013; Rotondo et al., 2016; Yoshimura et al., 2021).

2. Pleiotropic roles of Avp in teleost physiology

Here, I briefly review the role of Avp in teleost physiology outside of reproduction. Following a brief discussion of the receptor distribution to describe the ability of different tissues to respond to circulating and/or locally produced Avp, I will discuss the current state of knowledge in Avp-dependent regulation of physiological processes in teleost fishes. Emphasis is placed on energy metabolism / growth and the endocrine stress axis, as both processes are known to affect reproductive physiology in teleost fishes (Chen and Ge, 2013; Sousa et al., 2015; Faught et al., 2020) and may thus contribute to the observed reproductive phenotype described in detail in **Chapter 2**.

2.1. Different Avp receptors are widely expressed in brain regions and peripheral tissues relevant to the regulation of energy balance and metabolism, the endocrine stress axis activity and behaviour

The investigation of tissue-specific expression of avp receptors (*avpr*) provide additional support for the pleotropic roles of this nonapeptide. In general, the teleost *avp* receptor repertoire consists of three receptor families: *avpr1a, avpr2, avprl* and their paralogues, although not all are present in every teleost species. In addition, another member of the *avp* receptor family member, *avpr1b* is present in basal ray-finned fishes as well as tetrapod, but is not present in elasmobranchs and teleost fishes (Ocampo Daza et al., 2012; Banerjee et al., 2017; Mayasich and Clarke, 2020; Theofanopoulou, 2021; Theofanopoulou et al., 2021). Through genomic analysis conducted (**Chapter 1**, **Supplemental Figure 1.1**) all teleost genomes analyzed at the time of the review possess two paralogues, termed *avpr1aa* and *avpr1ab* from the *avpr1a* receptor family as well as *avpr2aa* and *avpr2ab* paralogues. While variation between teleost species exists regarding receptor subtype-specific expression, several studies in teleost species reveal expression in brain regions involved in the central regulation of energy balance via regulation of feed-intake. For example, expression of different subtypes of *avpr* in the hypothalamus and hindbrain, two tissues involved in the regulation of feeding behaviour and other behaviours such as locomotion, have been reported in the air-breathing catfish, *Heteropneustes fossilis* (Rawat et al., 2015, 2019), the Armargosa pupfish, *Cyprinodon nevadensis amargosae* (Lema et al., 2015), and zebrafish, *Danio rerio* (Iwasaki et al., 2013). Additionally,

avpr receptors have, at least in some teleost fish such as the Armargosa pupfish, been reported in peripheral tissues including tissues with key functions in the regulation of organismal energy metabolism, such as the liver (Sean C. Lema, 2010). Together, this distribution pattern demonstrates that Avp may exert effects on central and peripheral tissues to modulate different aspects of somatic growth and energy balance.

Regarding components of the stress axis in the hypothalamus, corticotropes of the pituitary gland and inter-renal tissue, several studies in fish have demonstrated the potential for Avp-dependent modulation in teleost fishes. Significant co-expression of Avp and corticotrophin-releasing Hormone (CRH) has been reported in the nucleus preopticus (NPO) in zebrafish (Herget and Ryu, 2015), suggesting potential co-regulation of downstream components of the stress axis. Similarly, colocalization of Avp and CRH has been reported in eel (Olivereau and Olivereau, 1990), where the extent of detected colocalization is furthermore responsive to modulation of cortisol concentrations. Evidence from the seabass, *Dicentrarchus labrax*, reveals that Avp binding sites exist in diverse hypothalamic nuclei, especially the NPO, but also the pars distalis of the pituitary, an area to which corticotropes are localized in teleost fish (Moons et al., 1989). Such evidence clearly suggests the potential for a pituitary level modulation of ACTH release. While detailed investigation regarding the expression of *avpr* in teleost inter-renal steroidogenic cells has to my knowledge not been reported in the literature, their reported expression in macro-dissected kidneys may suggest a role at the level of inter-renals. However, given the well-reported osmoregulatory properties of Avp at the level of the kidney, but also the gills (Lema et al., 2012, 2019), detailed studies would be needed to directly address this possibility in teleost fishes. Expression of *avprs* has recently also been reported in the head kidney of the seabream, *Sparus aurata* (Skrzynska et al., 2018). Overall, the Avp system distribution in several teleost fishes is in line with the reported pleiotropic effects, and specifically provides a basis for central and peripheral regulation of energy balance and metabolism, endocrine the stress axis, as well as (associated) behaviours.

2.2. Evidence for roles of Avp in the regulation of energy balance and somatic growth in teleost fishes

In teleost fishes, evidence from pharmacological studies in adults suggests a role for Avp in the (central) regulation of energy balance. Intra-cerebroventricular (i.c.v.) administration of 1 and 4 ng/g Avp in rainbow led to potent, anorexigenic effects lasting from 4 h-10 h (Gesto et al., 2014). Mechanistically, this decrease was linked to increased serotonergic activity in whole brain serotonin concentration and increased hypothalamic expression of cocaine and amphetamine regulated transcript (*cart*) as well as pro-opiomelanocortin (*pomc*), known anorexigenic factors in several teleost fishes (Mennigen et al., 2009; Volkoff, 2016). Similarly, a study in goldfish, *Carassius auratus*, testing intra-peritoneal (i.p.) and intra-cerebroventricular (i.c.v.) routes of administration found that only the latter significantly reduced feed-intake, suggesting that Avp exerts a central anorexigenic role. These findings are similar compared to reports of central anorexigenic effects in mammals (Yoshimura et al., 2021), where they appear to be mediated by vasopressin receptor 1 types. Thus, it is possible that central anorexigenic effects of Avp are conserved, at least in vertebrates (Odekunle and Elphick, 2020). In addition to these central effects, peripheral effects for Avp on liver energy metabolism have been reported, at least *in vitro* (Moon and Mommsen, 1990). These effects appeared to be highly species-specific: Avp stimulated gluconeogenesis and glycogenolysis in American eel, *Anguilla rostrata*, possibly through a Avpr2-type receptor. Conversely, no effects of Avp on these pathways were identified in hepatocytes from brown bullhead, *Ameiurus nebulosus*, or rainbow trout (Moon and Mommsen, 1990).

2.3. Evidence for a role of Avp in the regulation of the teleost endocrine stress axis

The endocrine stress axis in teleost fish is regulated at different tissue levels. At the level of the preoptic area, CRH is released to the distal part of the pituitary, form where ACTH is released into circulation. ACTH then stimulates cortisol synthesis (and subsequent passive release) at the level of steroidogenic internal cells. As is the case for many endocrine axes, cortisol exerts feedback at different

levels to regulate the hypothalamus-pituitary-interrenal (HPI) axis activity (Best and Gilmour, 2022). Several lines of evidence suggest a stimulatory role for Avp on HPI activity. Central (i.c.v.) administration of Avp induces significant increases in circulating cortisol and stimulates hyperglycemia (Gesto et al., 2014), providing direct evidence for a endocrine stress axis activation. Evidence from goldfish pituitary cell incubations *in vitro* suggests that this stimulation occurs, at least in part via stimulation of pituitary ACTH release (Fryer, 1989). To my knowledge, no direct studies of Avp effects on cortisol synthesis in interrenal cells have been conducted in teleost fish. Finally, the central Avp system as well as peripheral receptor expression have been demonstrated to be responsive to stress and/or cortisol manipulations (Gilchriest et al., 2001; Skrzynska et al., 2018), suggesting a role in mediating central feedback on the HPI axis. However, the interpretation of these findings is complex, due to experimental differences in stressors and duration of cortisol manipulations. Irrespective of the site of modulation, these teleost studies suggest a stimulatory action of Avp on the HPI axis, suggesting evolutionary conservation between vertebrates (Cádiz et al., 2014; Zelena et al., 2015).

2.4. Evidence for Avp-dependent regulation of teleost behaviour

Investigations of behavioural roles of Avp in teleost fishes have largely focused on social hierarchies and dominance status (Almeida and Oliveira, 2015; Lema et al., 2015). Conversely, while the (circadian) Avp-dependent regulation of energy-expending locomotor behaviour has been studied in mammals, comparative studies are lacking in teleost fishes, even though circadian expression of central *avp* has been reported in several species (Balment RJ et al., 2006; Altmieme et al., 2019). Similarly, while a role for central Avp signalling in anxiety behaviour has been reported particularly in rodent models (Bielsky et al., 2004), only few studies have explored the role of Avp on anxiety-like behaviours in teleost fishes, revealing complex, dose-dependent responses (Braida et al., 2012). Because energy balance, activation of the endocrine stress axis, as well as behavioural disruption are reported to affect reproductive success in teleost fishes in general (Schreck et al., 2001; Lester et al., 2004), and zebrafish in particular (Sousa et al.,

2015; Faught et al., 2020), we here assess apical endpoints (somatic growth, basal and stimulated cortisol concentration, exploration of a novel environment) to assess the possibility that the described female reproductive phenotype in (female) *avp* $^{-/-}$ fish may, at least in part, be linked to wider physiological roles of Avp. While such additional assessments represent an improvement over knock-out studies investigating widely expressed genes in the context of reproductive phenotypes, it is acknowledged that the selection of endpoints is, in the context of the timeframe for this thesis, limited.

2.5. Specific hypothesis

Zebrafish provide the advantage of rapid maturation of stress axis function (Alderman and Bernier, 2009), neurocircuitry controlling locomotory behaviour (Roussel et al., 2021), and rapid commencement of exogenous feeding and subsequent somatic growth to sexual maturity (Martínez et al., 2020). Thus, relevant endpoints described in detail can be assessed in *avp* $^{-/-}$ and WT mutants at larval and/or adult stages. Based on acute pharmacological studies in adult teleost fish species containing a normally developed Avp system reviewed above, I hypothesized that *avp* $^{-/-}$ mutants would exhibit increased somatic growth due to increased feed-intake and/or decreased locomotor activity, a reduction in the activation of the endocrine stress axis, and a reduction in anxiety-like behaviour.

3. Materials and methods

3.1. Experimental animals and husbandry

All experiments were carried out in accordance with animal care guidelines provided by the Canadian Council on Animal Care and with prior approval from the University of Ottawa Animal Care Committee (Protocol BL-3561). Wild-type (WT) zebrafish were sourced from the in-house stock at the University of Ottawa and maintained in 12 L tanks at a density of 3 fish/1L in a recirculating system

(Techniplast, Montréal, QC, Canada) supplied with salt-dosed (Instant Ocean, St. Blacksburg, VA, USA) RO water (hereafter "system water") maintained at a pH of 7.3, a conductivity of 400 μS, and a temperature of 28 °C under a 14:10h light–dark cycle. Fish were fed once or twice daily with GEMMA zebrafish diets (Skretting, Vancouver, BC, Canada) according to (eleuthero)embryonic and larval life stage. The *avp* $^{-/-}$ line generation has been described previously in Chapter 2, and was maintained at a density of 3 fish/1L. Adult fish were fed a mixed diet (Adult Zebrafish diet, Zeigler Bros Inc, Gardners, PA, USA; Larval AP-100, Zeigler Bros Inc, Gardners, PA, USA; Golden Pearls, Artemia International, Fairview, TX, USA) fed twice daily. Maintenance of WT and *avp* $^{-/-}$ lines and supply of animals for experiments were attained through the pairing of individual male and female fish as described previously in Chapter 2.

3.2. Experimental design

Three phenotypes that may contribute to the reproductive phenotype described in Chapter 2 were assessed in *avp*$^{-/-}$ mutants (**Fig. 1)**. Effects on somatic growth and energy balance were assessed by quantifying growth trajectories (body mass, length) monthly up to sexual maturity at 3 months post fertilization (3 mpf) (**Fig. 1A)**. At the larval stage, a locomotor assay was used to assess possible effects on locomotor-dependent energy expenditure(**Fig. 1C)**. Effects on the endocrine stress axis were assessed by quantifying basal cortisol concentrations in larvae and both basal and acutely stimulated cortisol concentrations in sexually mature fish (**Fig. 1B)**. Finally, anxiety-like behaviours were assessed the novel tank test assay (**Fig. 1C)**.

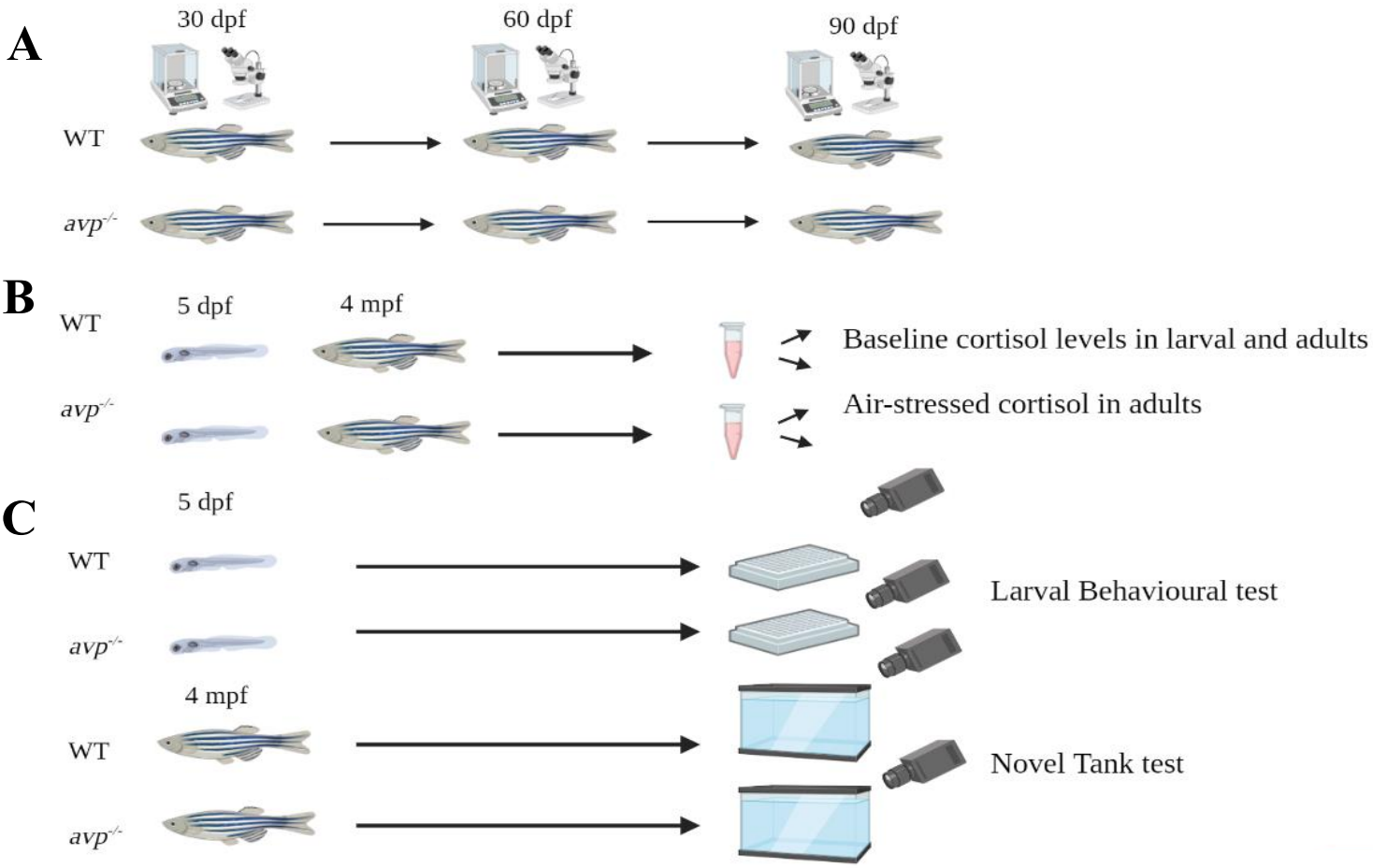


Figure 1: Schematic representation of experimental designs.

3.3. Assessment of somatic growth and larval locomotory energy expenditure

3.3.1. Growth trajectories, length and weight measurements

The body mass index (BMI) was assessed from WT (n=20) and *avp*$^{-/-}$ (n=20) at 30, 60 and 90 days post fertilization (dpf). Each fish was anesthetized using 0.24 mg/ml of tricaine (Syndel Laboratories) and once deep anesthesia was confirmed, each fish was lightly dried, weighed using an analytical scale to determine body mass, and imaged using a digital light microscope to determine body length (BL).

Following recovery from anesthesia (~10 min), each fish was placed back in the housing tank. Images were scaled and measured by following the notochord on laterally positioned individuals on acquired images.

3.3.2. Larval locomotion behaviour test

The locomotor behaviour of WT (n=164) and *avp*$^{-/-}$ (n=65) larvae of eleuthero-embryos (5 dpf) zebrafish were assessed in a 96-multiwell clear plate with 500 µl of system water using Zebrabox instrument (Viewpoint Life Science, Montreal, QC, Canada). Zebrafish were first acclimated for 30 min to reduce sampling stress and then placed in the Zebra box instrument. The larvae were then exposed to a series of instantaneous 100% light/dark conditions in the following pattern: 20 min light, 5 min dark, 5 min light, 5min dark, 5min light, 5 min dark, 5min light. The Viewpoint Zebrafish quantification software was used to track individual movements and preform an automated behavioural analysis. Movement time and distance of a given larvae was defined as three different movement ranges: inactivity (<3mm/s), small (3-6mm/s) and large (6mm/s) movements. Total movement was calculated by adding short and long movements together and velocity for small, large, and total movements was calculated by dividing number of movement events by time spent moving within a binned time period of 1 min.

3.4. Assessment of basal and stimulated endocrine stress axis in avp $^{-/-}$

3.4.1. Quantification of basal cortisol hormone levels in larvae

Larvae (n=25 per replicate) from WT (n=3 replicates) and *avp* $^{-/-}$ (n=3 replicates) were carefully collected and placed in 1.5 ml microcentrifuge tubes and stored at -80°C until processing. To extract cortisol, larval tissue samples were thawed on ice and 200 µl extraction buffer was added to each tube. The samples were then sonicated, ensuring the probe was cleaned with 75% EtOH and RNAse free water in between each sample. Following homogenization, 1ml of diethyl ether was placed into each sample,

vortexed and allowed to sit for 30 min. The samples were then centrifuged at 3000 g for five min and flash frozen at -80°C for 30 min. The liquid phase was removed and placed into new microcentrifuge tubes. The ether was evaporated under a gentle stream of air under the fume hood and the extraction steps were then repeated two more times. After the final extraction step, 250 µl extraction buffer was added to the tubes, vortexed and placed in a heat block for five minutes at 65°C. The samples were vortexed, placed on the heat block for additional 5 min and vortexed for a final time. The extracts were then placed at -80°C until use. Extracts were used for the quantification of cortisol using ELISA kit #402710 (Neogen Diagnostics, Edmonton, AB, Canada) according to the manufacturer's instructions. All samples were run in duplicates, on a single plate, and a cut-off of <20% was applied for replicates. All samples were normalized by number of larvae in each microcentrifuge tube prior to analysis.

3.4.2. Quantification of basal and acutely stimulated blood cortisol hormone levels in adults

Twenty fish from WT and *avp*$^{-/-}$ were used to assess basal and stimulated cortisol levels in zebrafish. The fish were separated into four different housing tanks: WT unstressed (n=10), WT stressed (n=10), *avp*$^{-/-}$ unstressed (n=10) and *avp*$^{-/-}$ stressed (n=10). Fish placed in the unstressed treatment groups were terminally anesthetized. Fish placed in the stressed treatment groups were placed in a 2L static breeding tank. For each treatment group, all fish were simultaneously netted, suspended in the air for 3 min, placed back into the tank for 3 min and then re-suspended in the air for an additional 3 min. The fish were then returned to the housing tank for a 10 min period prior to being terminally anesthetized. Whole blood from each treatment group was carefully extracted following terminal anesthesia and weighed, placed in 1.5ml microcentrifuge tubes, and stored at -80°C until processing. To extract cortisol, whole blood samples were thawed on ice and 200 µl extraction buffer was added to each tube. The samples were then sonicated, ensuring the probe was cleaned with 75% EtOH and RNAse-free water in between each sample. Following homogenization, 1ml of diethyl ether was placed into each sample, vortexed and allowed to sit for 30 minutes. The samples were then centrifuged at 3000 g for five minutes and flash frozen at -80 °C for 30

min. The liquid phase was removed and placed into new microcentrifuge tubes. The ether was evaporated under a gentle stream of air under the fume hood and the extraction steps were then repeated two more times. After the final extraction step, 250 µl extraction buffer was added to the tubes, vortexed and placed in a heat block for five minutes at 65 °C. The samples were vortexed, placed on the heat block for additional 5 min and vortexed for a final time. The extracts were then placed at -80°C until use. Extracts were used for the quantification of cortisol using ELISA kit #402710 (Neogen) according to manufacturer's instructions. All samples were run in duplicates and a cut-off of <20% was applied for replicates. All samples were corrected for sample dilution factors and rerun if necessary prior to analysis.

3.5 Novel tank test in adult zebrafish

Wildtype (n=30) and *avp*$^{-/-}$ (n=32) were netted from their housing tanks and transferred into temporary transport tanks, used to move fish into the behaviour room between the hours of 9:30h – 11:30h. Each individual fish was then released into an observational 3.5L (novel tanks) with a black background using the same net previously used. Behavioural movements of the fish were recorded using a Canon Vixia HF R400 camera positioned at ~1 m from the observation tank for a period of 10 min in a close room with minimal interaction with any outside stimulus. Each video was divided into 1 min intervals and analyzed determining the total time (s) spent in the bottom 1/3 of the tank, in the top 2/3, and the number of crossing upwards to the top 2/3 of the tank.

3.6 Statistical analyses

For each experiment, the raw data was tested for normality using the Shapiro-Wilk test and homoscedasticity using Levene's test, respectively. In cases where the raw data were non-parametric, standard transformations (log, sqrt, inversion) were used to improve normality and/or homoscedasticity. Single outliers were identified in normally distributed data using Grubb's test. For parametric and

homoscedastic data, a t-test was used in case of two comparison groups and univariate ANOVAs (one-way or two-way, as appropriate) followed by Tukey's post-hoc tests in cases of multiple comparison groups. In cases where data not normally distributed, Mann-Whitney U tests were used for comparisons of two groups, and Kruskal-Wallis tests for multiple groups followed by Dunn's post-hoc test. In all cases, a *P*-value <0.05 was considered as cut-off for significance. These analyses were conducted, and graphs plotted using Prism Version 9 (Graphpad Software, LaJolla, CA, USA).

4. Results

4.1. Somatic growth up to sexual maturity is not altered in avp $^{-/-}$ mutants

Neither body mass (df=1, F=0.27, *P*=0.607; **Fig. 2A**), body length (df=2, F=0.011, *P*=0.916; **Fig. 2B**) were significantly different based on genotype. Conversely and expectedly, body mass (df=1.704, F=432.164, *P*<0.01; **Fig. 2A**), body length (df=1.658 F=310.825 *P*<0.01; **Fig. 2B**) were dependent on time, with significant increases each month up to 3 mpf (*P*>0.05). No interaction effect between genotype and time was observed for body mass (df=1.549 F=0.014 *P*=0.968; **Fig. 2A**), body length (df=1.511 F= 0.631 *P*=0.508; **Fig. 2B**).

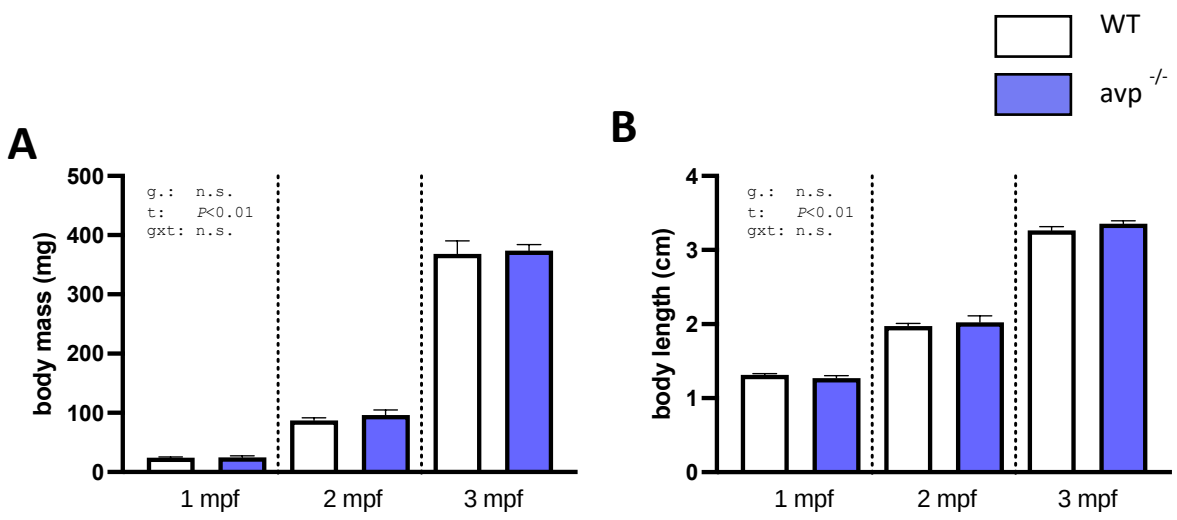


Figure 2: Developmental trajectories of body mass (A) and body length (B) in WT (n=20) and *avp* $^{-/-}$ (n=20) zebrafish. Developing zebrafish were quantified monthly until reaching sexual maturity at 3 mpf. All data are presented as mean ± S.E.M. and were analyzed using repeated measurement ANOVAs. Significant effects of genotype, time or their interaction were assessed at $P<0.05$ and are indicated within the graphs.

4.2. Avp $^{-/-}$ *mutant larvae exhibit hypercortisolism*

Cortisol levels in *avp* $^{-/-}$ larvae was significantly higher than that of WT of controls (df=4, t=4.051, p=0.0155, **Fig. 3A**) with an intra-assay coefficient of variability (CV) of 4.68% . In adults (**Fig. 3B**), cortisol concentrations were dependent on genotype (df=1 H=4.338 $P=0.028$) and on acute stress exposure (df=1 H=4.338 $P=0.037$), with significantly elevated blood cortisol concentrations following exposure to netting and air-exposure ($P<0.05$). However, there was no significant interaction between genotype and stress exposure (df=1 H=0.047 $P=0.829$). There was an intra-assay CV of 6.68% and an inter-assay CV of 7.66%.

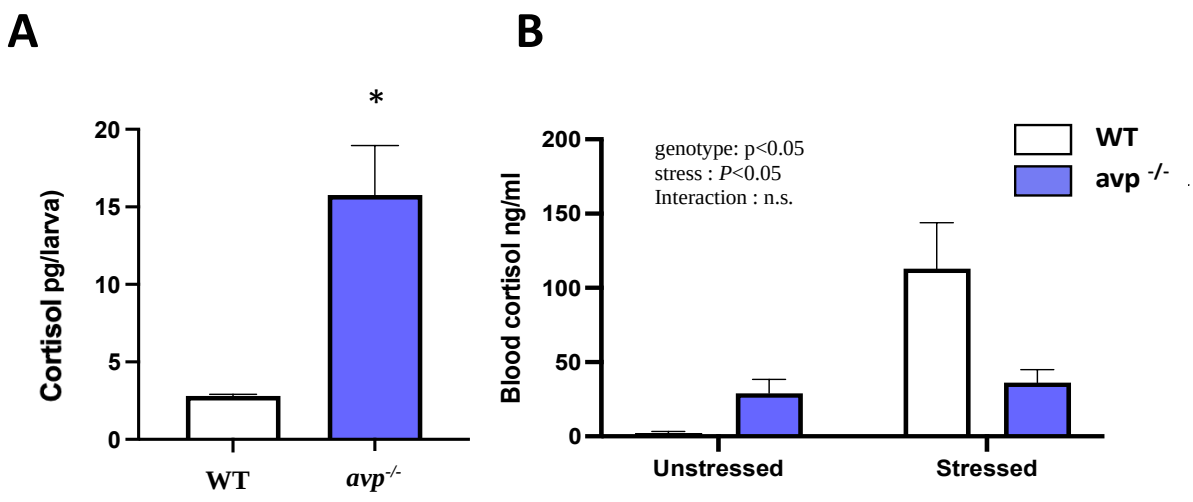


Figure 3. Larval cortisol concentration at 5 dpf (**A**) and adult blood cortisol concentration at baseline and following an acute air-stress exposure (B). Data are presented as means ± S.E.M. and were analyzed using a t-test (**A**), an ANOVA on ranks followed by Ray-Scheirer-Hare extension tests (**B**). A threshold of $P<0.05$ was used to discern significant differences, which are indicated by asterisk (**A**) or within the graph (**B**). Larval cortisol was determined in WT and *avp* $^{-/-}$ 5 dpf larvae pools (n=3 per genotype, 25 larvae per pool) according to published protocols (Hare et al., 2021). Adult whole blood cortisol was analyzed in adults of mixed sex and analyzed under baseline conditions (n=10 for both WT and *avp* $^{-/-}$ mutants), as well as following repeated air-exposure (n=10 for both WT and *avp* $^{-/-}$ mutants) according to published protocols (Ramsay et al., 2009) with the modification that cortisol was extracted from whole blood.

4.3. Avp $^{-/-}$ mutant larvae exhibit hypolocomotion and increased adult exploratory behaviour in the novel tank test paradigm

4.3.1. Locomotion of larval zebrafish is significantly decreased in avp$^{-/-}$ mutants

In 5 dpf zebrafish larvae, there is a significant effect of genotype (df= 1, H=77.26 P<0.01), lighting condition (df=2 H=44.58 *P*<0.01) and their interaction (df=2 H=8.41 *P*<0.01) when considering the average total distance travelled; **Fig. 4A+D**). These findings translate into significant reductions in total distance travelled in either lighting condition (**Fig. 4D**), as well as indicated by the significant interaction quantitatively different increases in total locomotion in response to darkness. Nevertheless, both WT and *avp* $^{-/-}$ fish significantly increase total distance travelled in darkness compared to light. Movement time dedicated to total distance covered (**Fig. 4B+E**) was significantly dependent on genotype (df=1 H=75.77 P<0.01), and lighting condition (df=1 H=27.93 *P*<0.01), but not their interaction (df=1 H=1.20 *P*=0.27). Post-hoc analyses revealed an overall reduction in time spent moving in *avp* $^{-/-}$ compared to WT fish (*P*<0.05) and an overall increase in time spent moving in dark compared to light conditions. Finally calculated velocity of total distance travelled over total time spent moving (**Fig. 4C+F**), was dependent on genotype (df=1 H=23 *P*<0.01), lighting condition df=1 H=33.27 P<0.01) and their interaction (df=1 H=18.87, *P*<0.01). Post-hoc analysis revealed that this interaction translated into a lack of increase in velocity in response to darkness in *avp* $^{-/-}$ larvae, which resulted in maintenance of baseline velocity observed in both WT and *avp* $^{-/-}$ mutants. The overall measurements of total distance travelled, time spent moving and calculated velocity were further analyzed independently for long (**Fig. 5**) and short (**Fig. 6**) distance movements. For long movement distance covered (**Fig. 5A+D**), significant effects of genotype (df=1 H=29.88 P<0.01), lighting (df=1 H=35.08 P<0.01) and their interaction (df=1 H=35.08 P<0.01) were found . For time spend covering long distances (**Fig. 5B+E**), significant effects of genotype (df=1 H=32.13 P<0.01), lighting (df=1 H=36.03 P<0.01) and their interaction (df=1 H=14.38 P<0.01) were found. For velocity during long distance movement (**Fig. 5C+F**), significant effects of genotype (df=1 H=11.68 P<0.01), lighting (df=1 H=21.98 P<0.01) and their interaction (df=1 H=24.05 P<0.01) were found. In all

instances, post-hoc analyses revealed significantly higher measures in WT under dark conditions compared to all other measures. For short distances covered (**Fig. 5A+D**), no significant effects of genotype (df=1 H=2.62 P=0.11), lighting (df=1 H=2.30 P=0.13) Their interaction was not significant (df=1 H=1.83 *P*=0.18). For time spend covering short distances (**Fig. 6B+E**), no significant effect of genotype (df=1 H=2.94 *P*<0.01), but of lighting (df=1 H=7.57 *P*<0.01) was found, with significantly decreased short distance movement time in the dark compared to light (*P*<0.05). For velocity during short distance movement (**Fig. 6C+F**), no significant effects of genotype (df=1 H=1.95 P=0.16), lighting (df=1 H=0.65 P=0.42) or their interaction (df=1 H=0.001 *P*=0.97) were found. Finally, inactivity time (**Fig. 7**), was significantly dependent on genotype (df=1 H=4.76 *P*=0.029), lighting condition (df=1 H=10.33 *P*=0.001) and their interaction (df=1 H=6.84 *P*=0.009). Post-hoc analysis revealed a significant reduction in inactivity time in WT under dark conditions compared to all other groups.

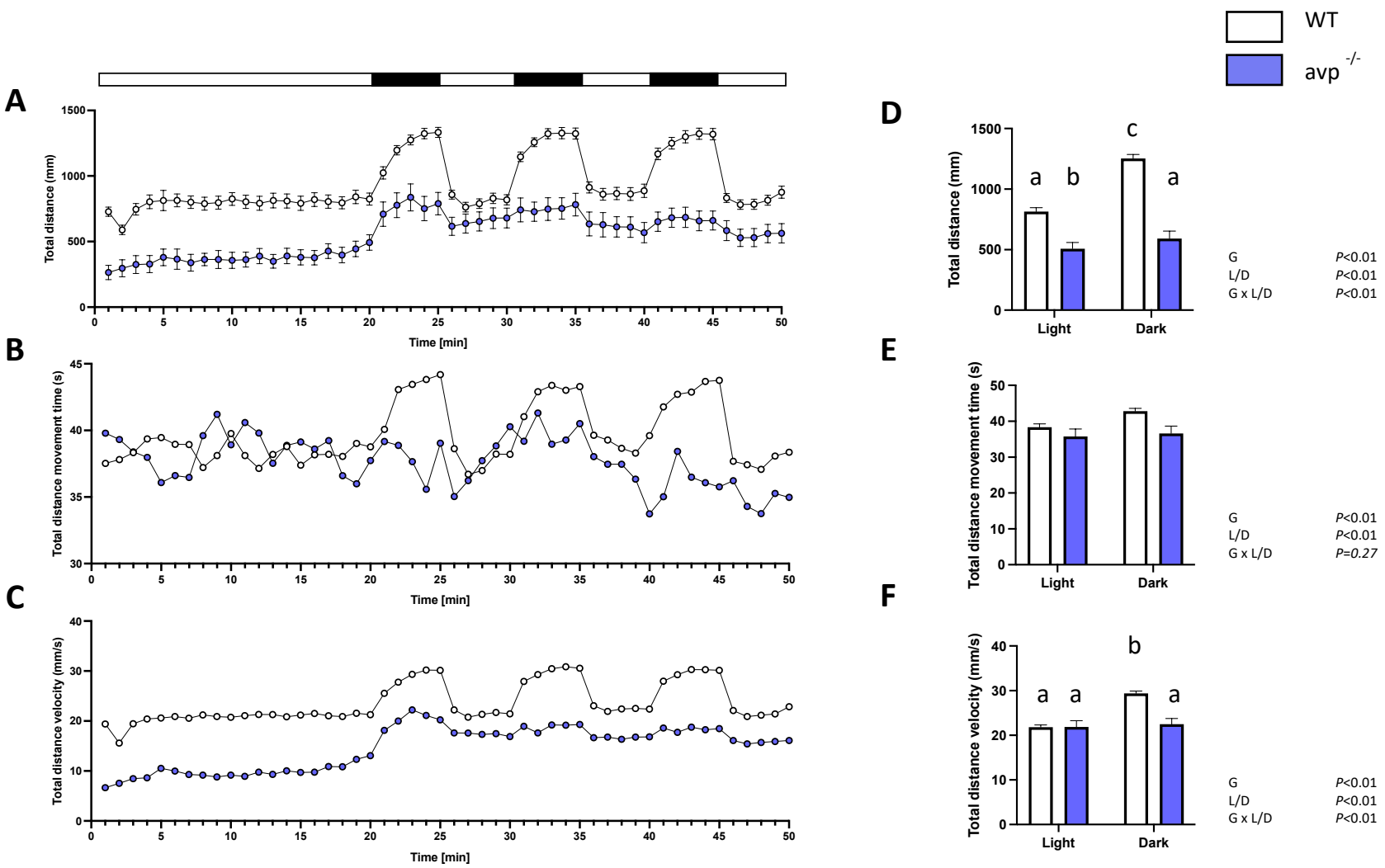

WT
avp -/-
A
Total distance (mm)
Time [min]
B
Total distance movement time (s)
Time [min]
C
Total distance velocity (mm/s)
Time [min]
D
Total distance (mm)
a b c a
Light
Dark
G P<0.01
L/D P<0.01
G x L/D P<0.01
E
Total distance movement time (s)
Light
Dark
G P<0.01
L/D P<0.01
G x L/D P=0.27
F
Total distance velocity (mm/s)
a a b a
Light
Dark
G P<0.01
L/D P<0.01
G x L/D P<0.01

Figure 4. Average total distance (mm) (A), total distance movement time(s) (B) and total distance velocity (mm/s)(C) shown throughout experimental quantification period at each time point (min) for 5dpf *avp* $^{-/-}$ (n= 65) and WT (n=164) larval zebrafish. Average total distance (D), total distance movement time (E) and total distance velocity for 5dpf *avp* $^{-/-}$ and WT larval zebrafish at light and dark conditions. Individual data for each group are shown and average values S.E.M. with different letters indicating significant difference between WT control and *avp* $^{-/-}$ groups in each treatment group, determined by two-way ANOVA.

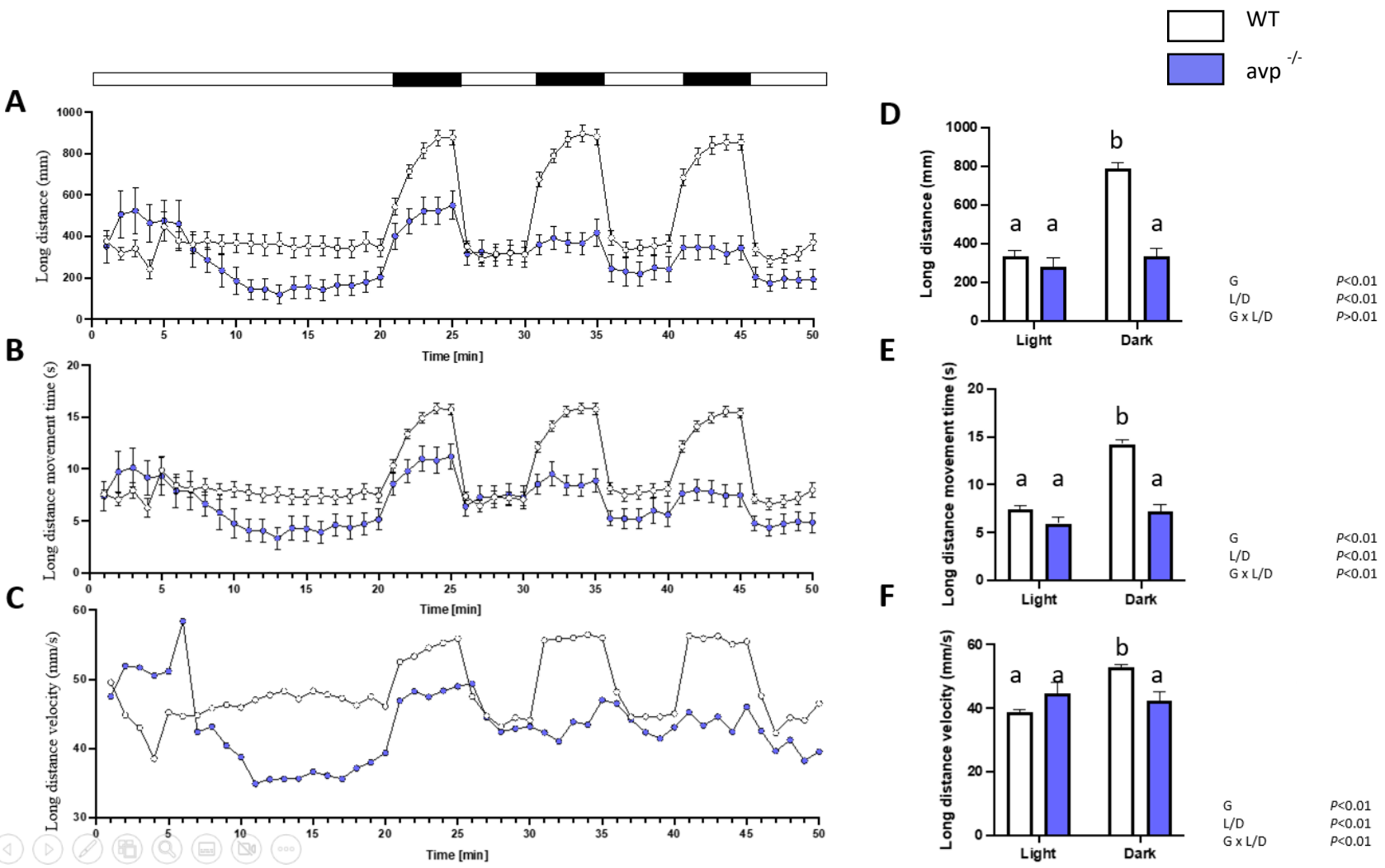

WT
avp -/-
A
Long distance (mm)
Time [min]
B
Long distance movement time (s)
Time [min]
C
Long distance velocity (mm/s)
Time [min]
D
Long distance (mm)
Light
Dark
G P<0.01
L/D P<0.01
G x L/D P>0.01
E
Long distance movement time (s)
Light
Dark
G P<0.01
L/D P<0.01
G x L/D P<0.01
F
Long distance velocity (mm/s)
Light
Dark
G P<0.01
L/D P<0.01
G x L/D P<0.01

Figure 5. Average long total distance (mm) (A), long distance movement time (s) (B) and long distance velocity (mm/s) (C) shown throughout experimental quantification period at each time point (min) for 5dpf *avp* $^{-/-}$ (n=65) and WT (n=164) larval zebrafish. Average long distance (D), long distance movement time (E) and long distance velocity (F) for 5dpf *avp* $^{-/-}$ and WT larval zebrafish at light and dark conditions. Individual data for each group are shown and average values S.E.M. with different letters indicating significant difference between WT control and *avp* $^{-/-}$ groups in each treatment group, determined by two-way ANOVA.

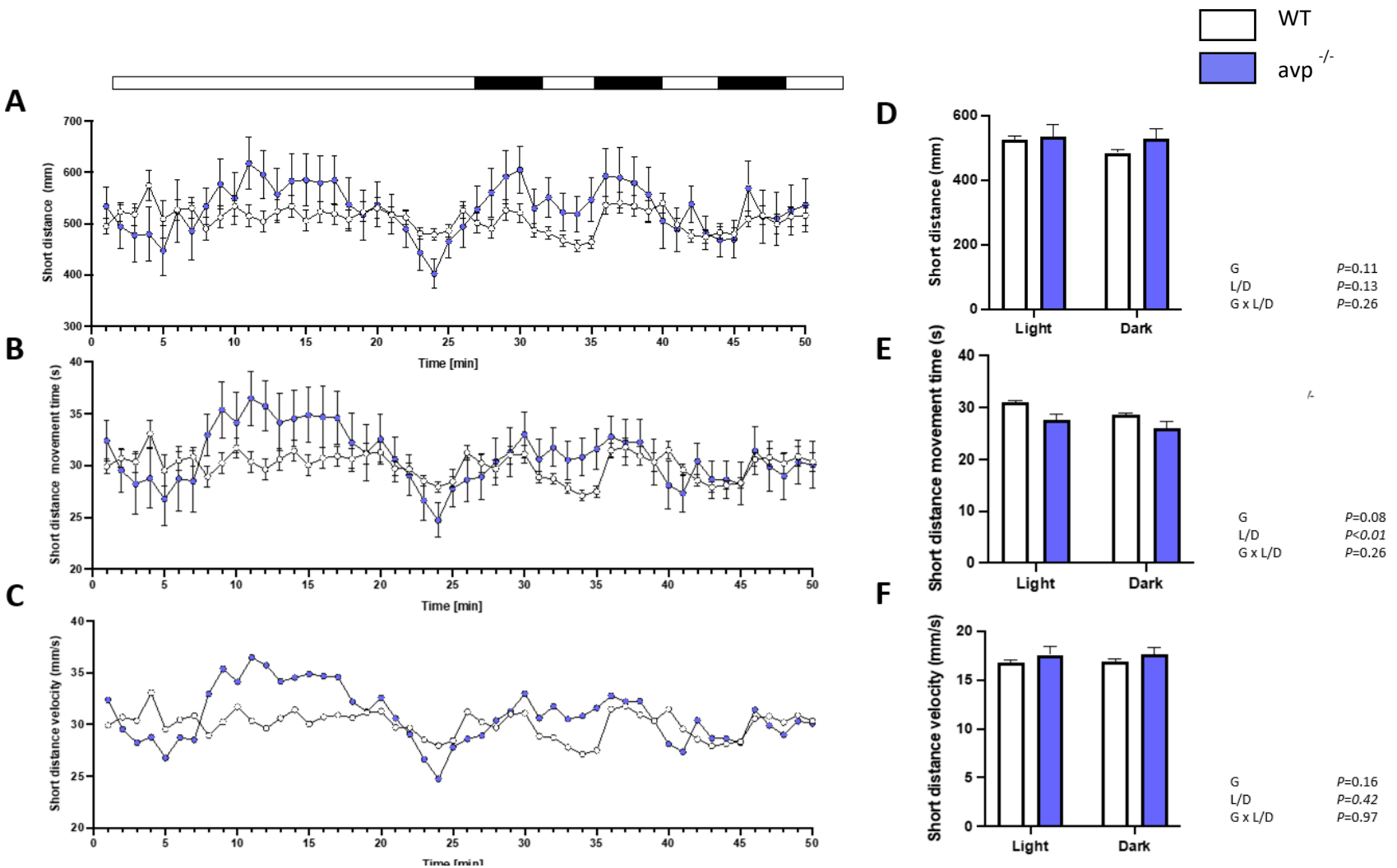

WT
avp -/-
A
Short distance (mm)
Time [min]
B
Short distance movement time (s)
Time [min]
C
Short distance velocity (mm/s)
Time [min]
D
Short distance (mm)
Light
Dark
G P=0.11
L/D P=0.13
G x L/D P=0.26
E
Short distance movement time (s)
Light
Dark
G P=0.08
L/D P<0.01
G x L/D P=0.26
F
Short distance velocity (mm/s)
Light
Dark
G P=0.16
L/D P=0.42
G x L/D P=0.97

Figure 6. Short total distance (mm) (A), short distance movement time(s) (B) and short distance velocity (mm/s) (C) shown throughout experimental quantification period at each time point (min) for 5dpf *avp* $^{-/-}$ (n=65) and WT (n=164) larval zebrafish. Average short distance (D), short distance movement time (E) and short distance velocity (F) for 5dpf *avp* $^{-/-}$ and WT larval zebrafish at light and dark conditions. Individual data for each group are shown and average values S.E.M. with different letters indicating significant difference between WT control and *avp* $^{-/-}$ groups in each treatment group, determined by two-way ANOVA.

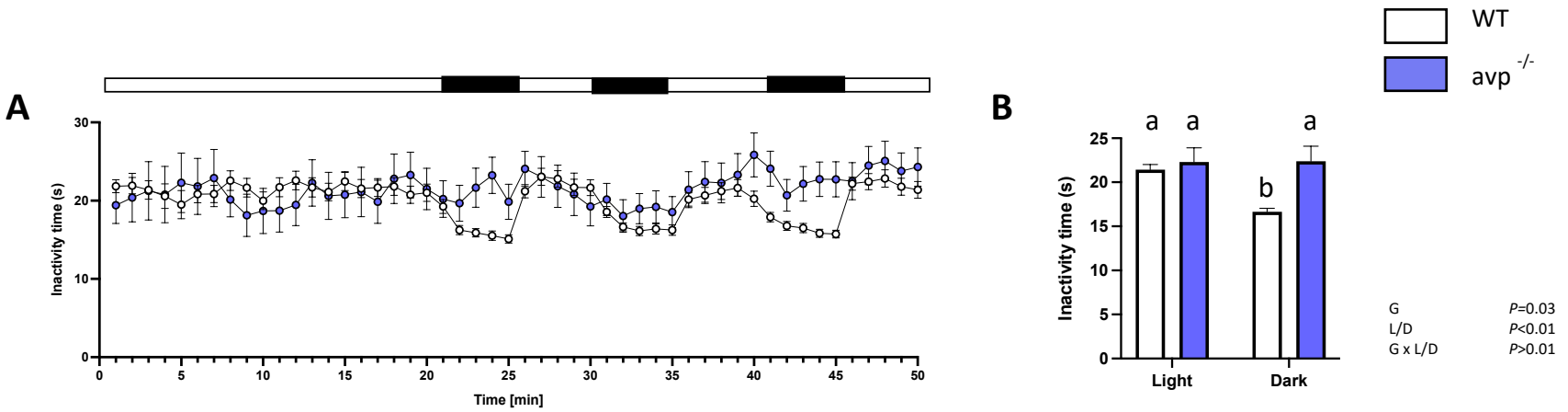


Figure 7. Average inactivity time (s) shown throughout experimental quantification period at each time point (min) for 5dpf *avp* $^{-/-}$ (n=65) and WT (n=164) larval zebrafish (A) and average inactivity time (s) for 5dpf *avp* $^{-/-}$ and WT larval zebrafish at light and dark conditions (B). Individual data for each group are shown and average values S.E.M. with different letters indicating significant difference between WT control and *avp* $^{-/-}$ groups in each treatment group, determined by two-way ANOVA.

4.3.2 Novel tank exploration is increased in avp $^{-/-}$ mutants

On average *avp* $^{-/-}$ zebrafish when exposed to a novel tank spent significantly more time in the upper two-thirds of the tanks compared to WT (df=59 t=2.564 *P*=0.0129; **Fig. 8A).** Conversely, the number of crossover events from the bottom one-third of the tank to the top two-thirds of the tank was not significantly different in the two groups (Mann-Whitney U=460.5, P=0.9628; **Fig. 8B**).

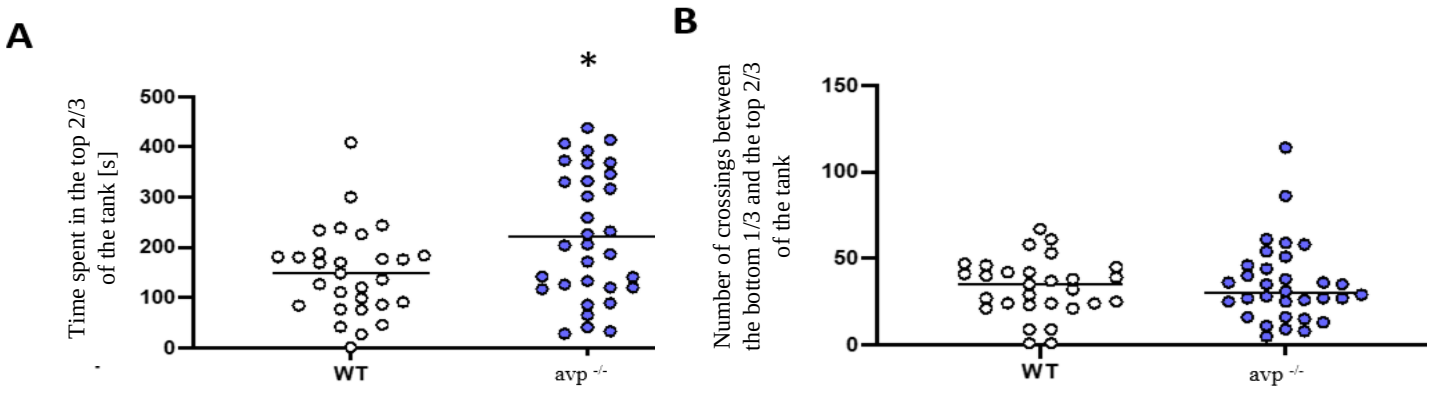


Figure 8. Novel tank test for WT (n=30) and *avp* $^{-/-}$ (n=32) adult zebrafish measuring time spent in the top two-thirds of the novel tank (A) and number of crossings between the bottom and top of the tank (B). Individual data for each group is shown with the average value, significant difference between the groups indicated, determined by using a two-way ANOVA (A) and Mann Whitney U (B).

5. Discussion

Due to the pleiotropic nature of Avp with possible effects on physiological systems that may contribute indirectly to reproductive effects, I assessed endpoints relevant to somatic growth and energy expenditure, the endocrine stress axis and anxiety-like /exploratory behaviour. The key results are discussed below.

5.1. Avp $^{-/-}$ mutants exhibit no change in overt somatic growth indices during sexual maturation

I identified no significant change in body weight or body length between the *avp* $^{-/-}$ mutants and control WT fish that were measured a three developmental time points until sexual maturity. The lack of change in these measurements, was in contrast to described central anorexigenic effects of Avp in teleost fishes (Gesto et al., 2014) and contrary to my prediction. While the differences to previous literature in teleost fishes might be linked to species-specificity of effects or different technical approaches (*avp* $^{-/-}$ knock outs may have organisational and/or compensatory effects as opposed to pharmacological studies in adults targeting a mature Avp system), these findings suggest that overt somatic growth effects did not affect the reproductive phenotype in *avp* $^{-/-}$ knock out fish. This is further underlined by the lack of difference in GSI in adult *avp* $^{-/-}$ females compared to WT, which suggest no difference in investment in gonadal tissue.

5.2. *Decreased locomotor behaviour in* avp $^{-/-}$ *larvae*

Several aspects of locomotor behaviour, especially large distance movements were reduced in *avp*$^{-/-}$ larvae compared to wild-type, sometimes in a lighting condition dependent manner. Conversely mutant larvae spent significantly more time being inactive. While these measurements are restricted to very early development and may thus change over the larger developmental timescales assessed, it is clear that these changes in energy expenditure do not affect overall somatic growth. Furthermore, since male specific reproductive behaviour was quantified and not found to differ compared to WT as described in **Chapter 2**, it is unlikely that overt effects on locomotor output contributed to alterations in reproductively important courtship behaviour via global reductions in movement.

5.3. Hypercortisolism in avp $^{-/-}$ larvae

To determine the role of Avp in zebrafish HPI axis function, I investigated baseline levels of cortisol in pooled whole larvae and blood samples of adult *avp* $^{-/-}$ mutants. I additionally tested changes in adult blood cortisol levels in response to an acute stressor. Contrary to my predictions, larval zebrafish exhibited significant hypercortisolism, while the stress dependent increase in adult zebrafish blood samples was also dependent on the genotype. It is currently unclear whether these findings reveal direct modulatory roles of Avp on the HPI axis in zebrafish, or whether other (developmental) effects of Avp, for example on osmoregulation, may contribute to the observed effects. Regardless of the nature of the hypercortisolism, it is thus possible that this phenotype indirectly contributes, at least in part, to reproductive effects described in **Chapter 2**. While in contrast to female *avp* $^{-/-}$ mutants, male mutants did not exhibit reproductive defects (**Chapter 2**), our adult sampling unfortunately did not distinguish between sexes. Such analyses are clearly warranted in the future, especially given the sexually dimorphic nature in whole body cortisol responses following exposure to an acute stressor (Vera-Chang et al., 2018).

The effects of cortisol on ovarian function and oogenesis has been studied directly on oocytes *in vitro* and through knock out models exhibiting hypercortisolism *in vivo* (Sousa et al., 2015; Faught et al., 2020; Maradonna et al., 2020). Direct incubation of stage I and especially stage II follicles with 1uM cortisol induced increases in DNA damage as assessed by comet assay (Sousa et al., 2015). In female *gr* $^{-/-}$ zebrafish characterized by hypercortisolism, an increase in oocyte cortisol concentration and a reduction in ovulated eggs have been identified however, no oocyte stage distribution has been reported in this study (Maradonna et al., 2020). The females of another *gr* $^{-/-}$ knock-out model, increased fecundity was observed in early adult females comparable in age to animals used in reproductive assays in **Chapter 2**, while decreased fecundity was observed older adult females, indicative of advancement of ovarian senescence (Faught et al., 2020). In both age-groups, increased follicular atresia was observed. However, especially because a buffering capacity with regard to cortisol load that is mediated via induction of 11β-hydroxysteroid dehydrogenase type 2 (*11β-hsd2)*, a cortisol inactivating enzyme, has been described in zebrafish oocytes (Faught et al.,

2016), additional studies are necessary to specifically address potential contributions of cortisol to the observed reproductive phenotype in female *avp* $^{-/-}$ fish.

5.4. avp $^{-/-}$ *mutants exhibit increased exploratory behaviour*

Adult mutant *avp* $^{-/-}$ fish increased exploration of the bottom tank area in the novel tank test paradigm, an assay described to measure 'anxiety-like' behaviour in zebrafish. While this finding is in line with reported anxiogenic roles of Avp in rodent models with a wider behavioural repertoire (Bielsky et al., 2004), is unclear whether this behaviour may be linked to hypercortisolism also found in these mutants. Indeed, the relationship between cortisol and novel tank test exploration has largely been studied in conjunction with fluoxetine, which – among other effects- lowers cortisol in zebrafish (Egan et al., 2009; Vera-Chang et al., 2018). However, different relationships between measured cortisol and behavioural phenotypes in the novel tank test have been reported: While acute adult exposure to fluoxetine reduces cortisol in wildtype zebrafish and elicits increased exploration of the upper tank area in a novel tank test paradigm (Egan et al., 2009), developmental fluoxetine exposure is linked to opposite (transgenerational) effects with increased bottom dwelling and decreased cortisol concentrations, an effect rescued by cortisol exposure (Vera-Chang et al., 2018). Interestingly, an effect of cortisol administration during early stress axis development in zebrafish around 3 dpf on early larval locomotion has also been reported (Best and Vijayan, 2018), with cortisol causing significant increases in overall locomotion. Future studies may thus be warranted to assess the contribution of hypercortisolism on zebrafish behaviours in *avp* $^{-/-}$ knock-out fish.

6.Conclusion

In sum, our study using newly created *avp* $^{-/-}$ knock-out zebrafish reveals non-reproductive effects, particularly with regard to behaviour and endocrine stress axis activation. The additional investigation of the phenotype suggests that this there is neither a significant difference in somatic growth during sexual maturation, a key indicator of reproductive ages (Frederickson et al., 2021), nor GSI in adult females, a key

indicator of gonadal investment. However, the observed increase in cortisol levels in mutant fish warrants additional study with regard to possible reproductive consequences, as *in vitro* and *in vivo* evidence raise the possibly of (complex) effects of cortisol on female gonadal function in zebrafish (Faught et al., 2020; Maradonna et al., 2020).

Chapter IV

General discussion

Partially based on:

Mennigen JA, **Ramachandran D**, Shaw K, Chaube R, Joy K, Trudeau VL. Reproductive roles of the vasopressin/oxytocin neuropeptide families in teleost fishes. *Frontiers in Endocrinology*, accepted.

1. Outline

Through the experiments that were discussed previously, I investigated the specific roles of the conserved arginine vasotocin system using a CRISPR/Cas9 knockout zebrafish model. While specific discussion of the experimental results obtained in from each experiment are contained within the relevant data chapters, this conclusion chapter serves to summarize the novel findings in the context of my overall hypothesis and in the context of the current state of knowledge in the field. I will then discuss possible limitations of the experimental approaches and identify specific research questions for future investigations of the role of nonapeptides in teleost fishes. Additionally, I will be discussing the importance of this research in a larger societal context by highlighting possible areas for application of my research findings.

2. Summary of novel findings

2.1 Avp $^{-/-}$ mutants reveals sex-specific roles for Avp on zebrafish reproduction

My experimental results clearly identify a role for Avp in female zebrafish reproductive physiology and success. With the creation of an *avp*$^{-/-}$ mutant line, I was able to observe a significant reduction in reproductive success, as determined by reduced egg release and clutch sizes. Through backcross experiments, I was able to link this outcome specifically to the female genotype, which was further substantiated by a female mutant dependent reduction in quivering courtship behaviour associated with egg release. With regard to the potential mechanistic basis of this phenotype, *avp* mutant female zebrafish had a significant decrease in the number of oocytes found in the ovaries, and an altered distribution of oocyte stages in the total oocyte pool. Specifically, a decreased amount of stage I oocytes and an increased in large stage V oocytes was observed. Ovarian gene expression and metabolite analyses further corroborated these findings and provided additional insight into the potential mechanistic basis. On the one hand a significant reduction in the germ-stem-cell specific marker *nanos2* suggests a role for Avp on germ cell maintenance and thus recruitment into stage I oocytes. This novel finding deserves further exploration, as novel zebrafish ovary single cell sequencing data reveals specific expression of *avpr* receptor subtypes in this group of ovarian cells. On the other hand, *avp* $^{-/-}$ ovaries exhibited a significant decrease in $PGF_{2\alpha}$, which coincided

with decreased transcript abundance in enzymes involved in its biosynthesis pathway. As $PGF_{2\alpha}$ is linked to ovulation and coincident activation of female courtship behaviour, this suggests that Avp deficiency may be involved in increased stage V oocyte retention and decreased female quivering behaviour. Interestingly, while this is the first finding in an asynchronously spawning fish species, pharmacological modulation of Avp in catfish ovaries in vitro has similarly shown a role in stimulating $PGF_{2\alpha}$ as well as effects on oocyte maturation and ovulation. Thus, while other aspects of the HPG axis remain to be assessed in female *avp* $^{-/-}$ zebrafish, it appears that Avp regulates female reproductive function at least in part, via stimulation of ovarian $PGF_{2\alpha}$. Nevertheless, Avp rescue experiments of different length did not rescue the observed phenotype. While a longer-term repeated administration of Avp suffered from the possible drawback that the final injection was placed 24h prior to mating assays and may thus have been degraded as its mammalian half-life is reported to be in the range of 30 min, the acute rescue experiment is inconclusive, as more mutants needed to be grown to reliably test a possible rescue using sufficient sample size and thus statistical power. Rescue experiments with $PGF_{2\alpha}$ and hCG are furthermore warranted in the future to assess whether the female *avp* $^{-/-}$ phenotype is linked to antinational, or – possibly- organisational effects of Avp. Rescue experiments that are stage-specific in addition to histological analysis of ovarian development across different times during sexual differentiation and maturation, may furthermore be useful to delineate these possibilities.

2.2 Effects of avp on other systems

The study using *avp* $^{-/-}$ knock-out zebrafish touches upon a few pleotropic endpoints and the relationship with the reproductive phenotype displaced by the *avp* $^{-/-}$ knock-out zebrafish. The investigation into the phenotype suggests that this there is no significance in growth rate which is a key indicator of reproductive maturation (Frederickson et al., 2021). However, there is an increase in baseline cortisol levels in these mutant fish that may play a role in the retention of oocytes and resulting in decreased quality of oocytes and embryo viability exemplified in other *gr* $^{-/-}$ knock-out models (Faught et al., 2020; Maradonna

et al., 2020). Though these endpoints touch upon only a few physiological systems related specifically to reproductive success, this study suggests that that *avp* $^{-/-}$ zebrafish may be a promising *in vivo* model to dissect pleiotropic *avp* effects on female reproductive function through additional studies in the future.

3. Relevance of the study of reproductive function using zebrafish knock-out models

Interest in using zebrafish as a model for both medical and environmental research is growing. While zebrafish are a good laboratory model due to it easy upkeep and low costs, another positive of this model is that the research can be translated to other models due to evolutionary conservation between systems. I will here discuss specific translational areas, ranging from the application of the *avp* $^{-/-}$ mutants and zebrafish knock-outs insight into female reproductive biology in a biomedical context to harnessing novel insight into regulators of teleost reproduction to facilitate breeding in captivity. The latter is, for example of relevance to aquaculture operations, as well as assisted breeding to rear species in captivity. Finally, I will discuss emerging evidence for the teleost Avp system in the context of endocrine disruption in the context of ecotoxicology, an area which will allow to translate novel mechanistic insight of regulation and function of the Avp system in the context of reproduction to develop novel monitoring approaches and better assess population level consequences of exposure to aquatic endocrine active substances.

3.1. Biomedical research

Zebrafish is growing as a model for medical research as there many shared systems between zebrafish and mammalian models (Ye and Chen, 2020). When studying gene function, zebrafish models in general provide a good complementary model when homozygous lethality occurs in mammalian models, examples include the Brac2 mouse mutants that were lethal or not viable (Ye and Chen, 2020), like the Barbaro mouse model. Additionally many behaviours are evolutionary conserved and are comparable to characteristics exhibited in humans and other mammals such as age-related changes across the cognitive and neurobiological spectrums (Adams and Kafaligonul, 2018). When researching neurodevelopmental disorders, zebrafish had become an emerging model as similarities between behaviors exhibited in zebrafish

to that of humans. Zebrafish also is highly suitable for pharmacological studies due to similar if not identical receptors and biochemical mechanisms being affected being comparable to those of mammals (Shams et al., 2018). In addition, the effects of drugs in the brain can be evaluated using larval zebrafish up to 8 dpf due to the incomplete nature of the blood-brain barrier until 8dpf (Meshalkina et al., 2018). While the brain region of zebrafish is less complex than mammalian models, fundamental mechanisms can be analysed (Shams et al., 2018). For example, in the *avp*-knockout model, specific behaviours such as locomotion, novel tank exploration and female courtship behaviour , were altered in these fishes. Because dysfunction of nonapeptide systems have been found to play a role in several neuro-behavioural disorders (Salahinejad et al., 2020) in mammalian rodent models and humans, zebrafish nonapeptide knock-out models can be assessed for relevant (social) behaviour alterations in future studies. Should a relevant behavioural phenotype be present, such models can successfully an in large scale be used to assess the efficacity of drug treatments (Shams et al., 2018).

Another key area, in which the zebrafish model holds promise is addressing of challenges to global female health. Female animals share biological characteristics that can place them at an increased risk for similar diseases and disorders (Natterson-Horowitz et al., 2022). With anthropogenic changes in the environments, there is an unprecedented significance in the health of female animals and that of female humans (Natterson-Horowitz et al., 2022). There is significant evidence that climate change and environmental degradation disproportionately impact women and girls, reinforces the importance and urgency of a female focus (Natterson-Horowitz et al., 2022). Additionally, examining the unique health vulnerabilities of female animals such as threats to female fertility also align with conservation efforts and the goal of mitigating high rate of species extinction and loss of global biodiversity (Comizzoli et al., 2010; Comizzoli and Holt, 2019). Indeed, several funding agencies in many countries, such as the National Institute of Heath in the US, or the National Science and Engineering Council in Canada, have now prioritized the study of female biology, a variable historically neglected in areas such as drug development due to variability in experimental outcomes linked to hormonal cycles.

Zebrafish is considered as a promising model for reproductive research due to its similarity to humans as there are similar neurons regulating the reproductive system, as well as similar reproductive hormones and responses (Hoo et al., 2016). Specifically, the *avp* knockout model addresses sex-specific roles of Avp in regard to reproduction. With limited research looking specifically at female sex-specific effects of this nonapeptide, this model provides novel understanding.

3.2. Teleost reproduction in captivity

Insight from basic research exploring the mechanistic basis of teleost reproduction has a long history of translational potential. For example, the initial study of the pituitary and gonadotropin-dependent regulation of fish reproduction pioneered by Dr. Zohar has led to cloning and purification of functional analogues effectively allowing to 'close the cycle' of aquaculture species previously difficult to breed in captivity (Zohar, 2021). This has notably had significant impact in advancing the aquaculture industry in. Similarly, the discovery of tonic dopamine dependent inhibition of the reproductive axis at the level of the pituitary of many, but not all, teleost species has led to the development of domperidone, a dopamine receptor 2 antagonist used in the aquaculture industry in conjunction with sGnrHa under the brand name Ovaprim ™ to induce spawning (Peter et al., 1988). Indeed, along the same lines, studies probing the application of nonapeptides, including vasotocin, in facilitating extraction of gametes in aquaculture species such as the walking catfish, *Clarias magur* has recently been reported (Wisdom et al., 2022). Given the descried roles of Avp in teleost courtship behaviour and HPG axis regulation, further investigation of Avp agonists represents a potentially promising area of investigation in this regard. Finally, while Avp specific roles may differ within species in the highly diverse infraclass of teleost fishes, comparative investigation of Avp-dependent regulation of teleost reproduction and pharmacological modulation of the Avp system in endangered species may represent a potentially promising avenue in captive breeding of species of concern.

3.3. Ecotoxicology

In teleost fish, relatively recent studies have investigated Avp (and Oxt) nonapeptide systems as targets of different groups of aquatic contaminants, rendering them a mechanistic target of endocrine disruption chemicals. 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 (Wong et al., 2013). 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 (Kline et al., 2016).

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 (Mennigen et al., 2008). 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 (Semsar et al., 2004; Mennigen et al., 2010). Similarly, targeted gene expression analysis of zebrafish larvae acutely exposed to 50 and 500 ng/L FLX for 96 h (Cantor et al., 1999) 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 (de Jong et al., 2007) 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 (S., 2007). The responsiveness of teleost nonapeptide systems to FLX corresponds to several observations in mammalian models (León-Olea et al., 2014; Wang et al., 2020) and suggests an evolutionarily conserved serotonin-dependent regulation of these systems (Mennigen et al., 2017; Salahinejad et al., 2020, 2022)

In Japanese medaka chronically exposed to environmentally relevant and high concentrations of waterborne methamphetamine for a period of 90 days, a dose-dependent, significant increase in whole brain *oxtr* mRNA and Oxt peptide were observed (Naderi et al., 2022). Because both FLX and methamphetamine affect neurotransmitter systems and neuroactive contaminants may exert reproductive effects, at least in part, via nonpeptidergic systems, these findings support the concept of neuroendocrine disruption (Naderi et al., 2019). 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 (Popesku et al., 2008). 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 (Patisaul, 2017; Kalamarz-Kubiak, 2022). 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 (Reilly et al., 2022; Wee et al., 2022). 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 (Blechman et al., 2011) compared to rodent models (Coffey et al., 2013; Hughes et al., 2018) and will inform the possible development of teleost nonapeptides as functional biomarkers relevant to reproductive function in teleost fishes. Given the

emerging evidence revealing the susceptibility of teleost Avp and related Oxt nonapeptide systems to endocrine disrupting chemicals, the development of transgenic reporter lines of Avp in genetically tractable fish models represents a feasible application to assess endocrine disruption of the Avp system in a fish species *in vivo*. Similar approaches have successfully been developed to assess estrogenic responses of contaminants in zebrafish (Takesono et al., 2022). Such in vivo systems have the advantage that compared to in vitro assays, organismal level responses in responses can be assessed in a fully functional organism thus accounting for uptake and depuration kinetics and internal tissue exposures and potentially differential responsiveness. As these assays can readily be conducted in *real-time* and in response to single aquatic contaminants, aquatic contaminant mixtures, or sediment exposures, they hold enormous potential to Avp or Oxt mediated endocrine disrupting effects across life stages. Finally, due to the capacity to develop high throughput approaches, such models are attractive in dose-response modelling and screening of a plethora of structurally related compounds with possible effects on the nonapeptide system.

References

Acharjee, A., Chaube, R., and Joy, K. (2011). Hormonal Regulation of Aquaporin-1ab in Heteropneustes fossilis Oocytes in vitro. 6–8.

Acharjee, A., Chaube, R., and Joy, K. P. (2018). 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.* 260, 80–89. doi: 10.1016/j.ygcen.2018.01.001.

Acher R, Chauvet J, Chauvet MT, C. D. (1962). Isolation of a new neurohypophysial hormone, isotocin, present in bony fish. *Biochim Biophys Acta* 58, 624–625.

Acher R, Chauvet J, Chauvet MT, R. Y. (1999). Unique evolution of neurohypophysial hormones in cartilaginous fishes: possible implications for urea-based osmoregulation. *J Exp Zool* 284, 475–484. doi: 10.1002/(sici)1097-010x.

Acher, R. (1996). Molecular evolution of fish neurohypophysial hormones: Neutral and selective evolutionary mechanisms. *Gen. Comp. Endocrinol.* 102, 157–172. doi: 10.1006/gcen.1996.0057.

Acher, R., and Chauvet, J. (1988). Structure, processing and evolution of the neurohypophysial hormone-neurophysin precursors. *Biochimie* 70, 1197–1207. doi: 10.1016/0300-9084(88)90185-X.

Adams, M. M., and Kafaligonul, H. (2018). Zebrafish-A model organism for studying the neurobiological mechanisms underlying cognitive brain aging and use of potential interventions. *Front. Cell Dev. Biol.* 6, 1–5. doi: 10.3389/fcell.2018.00135.

Albers, H. E. (2012). The regulation of social recognition, social communication and aggression: Vasopressin in the social behavior neural network. *Horm. Behav.* 61, 283–292. doi: 10.1016/j.yhbeh.2011.10.007.

Alderman, S. L., and Bernier, N. J. (2009). Ontogeny of the corticotropin-releasing factor system in zebrafish. *Gen. Comp. Endocrinol.* 164, 61–69. doi: 10.1016/J.YGCEN.2009.04.007.

Almeida, O., and Oliveira, R. F. (2015). Social Status and Arginine Vasotocin Neuronal Phenotypes in a Cichlid Fish. *Brain. Behav. Evol.* 85, 203–213. doi: 10.1159/000381251.

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

Amores, A., Force, A., Yan, Y.-L., Joly, L., Amemiya, C., Fritz, A., et al. (1998). Zebrafish hox Clusters and Vertebrate Genome Evolution. *Science (80-.).* 282, 1711–1714. doi: 10.1126/science.282.5394.1711.

Arcand-Hoy, L. D., and Benson, W. H. (1998). Fish reproduction: An ecologically relevant indicator of endocrine disruption. *Environ. Toxicol. Chem.* 17, 49–57. doi: 10.1897/1551-5028(1998)017<0049:FRAERI>2.3.CO;2.

Balment RJ, lu, W., Weybourne, E., and Warne, J. (2006). Arginine vasotocin a key hormone in fish physiology and behavior: a review with insights from mammalian models. *Gen. adn Comp. Endocrinol.*, 9–16. Available at: https://reader.elsevier.com/reader/sd/pii/S0016648006000165?token=11450DB678923DEE395219BB508FE79B1C3959B07EC3B75916F473806C817EB3E17C5B5571283A5654183B9B7702AD32 [Accessed September 17, 2020].

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

Banerjee, P., Chaube, R., and Joy, K. P. (2018). 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.* 30, e12647. doi: 10.1111/jne.12647.

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

Baran, N. M. (2017). Sensitive Periods, Vasotocin-Family Peptides, and the Evolution and Development of Social Behavior. *Front. Endocrinol. (Lausanne).* 8. doi: 10.3389/fendo.2017.00189.

Bass, A. H., and Groberb, M. S. (2001). Social and Neural Modulation of Sexual Plasticity in Teleost Fish. *Brain. Behav. Evol.* 57, 293–300. doi: 10.1159/000047247.

Batten, T. C. (1986). 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.* 244. doi: 10.1007/BF00212547.

Batten, T. F. C., and Batten, T. F. C. (1986). 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 2443* 244, 661–672. doi: 10.1007/BF00212547.

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

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

Beer, R. L., and Draper, B. W. (2013). nanos3 maintains germline stem cells and expression of the conserved germline stem cell gene nanos2 in the zebrafish ovary. *Dev. Biol.* 374, 308–318. doi: 10.1016/j.ydbio.2012.12.003.

Best, J. D., Berghmans, S., Hunt, J. J. F. G., Clarke, S. C., Fleming. A., Goldsmith, P., et al. (2008). Non-associative learning un larval zebrafish. *Neuropsychopharmacology* 33, 1206-1215. doi:10.1038/sj.npp.1301489.

Best, C., and Gilmour, K. M. (2022). Regulation of cortisol production during chronic social stress in rainbow trout. *Gen. Comp. Endocrinol.* 325, 114056. doi: 10.1016/j.ygcen.2022.114056.

Best, C., and Vijayan, M. M. (2018). Cortisol elevation post-hatch affects behavioural performance in zebrafish larvae. *Gen. Comp. Endocrinol.* 257, 220–226. doi: 10.1016/j.ygcen.2017.07.009.

Bielsky, I. F., Hu, S.-B., Szegda, K. L., Westphal, H., and Young, L. J. (2004). Profound Impairment in Social Recognition and Reduction in Anxiety-Like Behavior in Vasopressin V1a Receptor

Knockout Mice. *Neuropsychopharmacology* 29, 483–493. doi: 10.1038/sj.npp.1300360.

Blechman, J., Amir-Zilberstein, L., Gutnick, A., Ben-Dor, S., and Levkowitz, G. (2011). The Metabolic Regulator PGC-1 Directly Controls the Expression of the Hypothalamic Neuropeptide Oxytocin. *J. Neurosci.* 31, 14835–14840. doi: 10.1523/JNEUROSCI.1798-11.2011.

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

Boer, K., Boer, G. J., and Swaab, D. F. (1981). Reproduction in Brattleboro rats with diabetes insipidus. *Reproduction* 61, 273–280. doi: 10.1530/jrf.0.0610273.

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

Braida, D., Donzelli, A., Martucci, R., Capurro, V., Busnelli, M., Chini, B., et al. (2012). Neurohypophyseal hormones manipulation modulate social and anxiety-related behavior in zebrafish. *Psychopharmacology (Berl).* 220, 319–330. doi: 10.1007/s00213-011-2482-2.

Butler, J. M., Herath, E. M., Rimal, A., Whitlow, S. M., and Maruska, K. P. (2020). Galanin neuron activation in feeding, parental care, and infanticide in a mouthbrooding African cichlid fish. *Horm. Behav.* 126. doi: 10.1016/J.YHBEH.2020.104870.

Cádiz, L., Román-Padilla, J., Gozdowska, M., Kulczykowska, E., Martínez-Rodríguez, G., Mancera, J. M., et al. (2014). Cortisol modulates vasotocinergic and isotocinergic pathways in the gilthead sea bream (Sparus aurata , Linnaeus 1758). *J. Exp. Biol.* doi: 10.1242/jeb.113944.

Canosa, L. F., Lopez, G. C., Scharrig, E., Lesaux-Farmer, K., Somoza, G. M., Kah, O., et al. (2011). Forebrain mapping of secretoneurin-like immunoreactivity and its colocalization with isotocin in the preoptic nucleus and pituitary gland of goldfish. *J. Comp. Neurol.* 519, 3748–3765. doi: 10.1002/cne.22688.

Cantor, J. M., Binik, Y. M., and Pfaus, J. G. (1999). Chronic fluoxetine inhibits sexual behavior in the male rat: reversal with oxytocin. *Psychopharmacology (Berl).* 144, 355–362. doi: 10.1007/s002130051018.

Carneiro, L. A., Oliveira, R. F., Canário, A. V. M., and Grober, M. S. (2003). The effect of arginine vasotocin on courtship behaviour in a blenniid fish with alternative reproductive tactics. *Fish Physiol. Biochem.* 28, 241–243. doi: 10.1023/B:FISH.0000030542.31395.8a.

Cerdà, J. (2009). Molecular pathways during marine fish egg hydration: the role of aquaporins. *J. Fish Biol.* 75, 2175–2196. doi: 10.1111/j.1095-8649.2009.02397.x.

Chaube, R., Chauvigné, F., Tingaud-Sequeira, A., Joy, K. P., Acharjee, A., Singh, V., et al. (2011). Molecular and functional characterization of catfish (Heteropneustes fossilis) aquaporin-1b: Changes in expression during ovarian development and hormone-induced follicular maturation. *Gen. Comp. Endocrinol.* 170, 162–171. doi: 10.1016/j.ygcen.2010.10.002.

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. *Gen. Comp. Endocrinol.* 175, 206–213. doi: 10.1016/j.ygcen.2011.11.012.

Chauvet J, . Chauvet-Lenci MT, A. R. (1961). Some observations on the neurohypophysial hormones of a teleostfish, the hake (Merluccius merluccius L.). *TC R Seances Soc Biol Fil* 252, 2145–2147. doi: 10.1113/jphysiol.1961.sp006616.

Chauvigné, F., Tingaud-Sequeira, A., Agulleiro, M. J., Calusinska, M., Gómez, A., Finn, R. N., et al. (2010). Functional and Evolutionary Analysis of Flatfish Gonadotropin Receptors Reveals Cladal- and Lineage-Level Divergence of the Teleost Glycoprotein Receptor Family1. *Biol. Reprod.* 82, 1088–1102. doi: 10.1095/biolreprod.109.082289.

Chen, W., and Ge, W. (2013). Gonad differentiation and puberty onset in the zebrafish: Evidence for the dependence of puberty onset on body growth but not age in females. *Mol. Reprod. Dev.* 80, 384–392. doi: 10.1002/mrd.22172.

Chou, M.-Y., Hung, J.-C., Wu, L.-C., Hwang, S.-P. L., and Hwang, P.-P. (2011). Isotocin controls ion

regulation through regulating ionocyte progenitor differentiation and proliferation. *Cell. Mol. Life Sci.* 68, 2797–2809. doi: 10.1007/s00018-010-0593-2.

Coffey, C. M., Solleveld, P. A., Fang, J., Roberts, A. K., Hong, S.-K., Dawid, I. B., et al. (2013). Novel Oxytocin Gene Expression in the Hindbrain Is Induced by Alcohol Exposure: Transgenic Zebrafish Enable Visualization of Sensitive Neurons. *PLoS One* 8, e53991. doi: 10.1371/journal.pone.0053991.

Comizzoli, P., and Holt, W. V. (2019). Breakthroughs and new horizons in reproductive biology of rare and endangered animal species. *Biol. Reprod.* 101, 514–525. doi: 10.1093/BIOLRE/IOZ031.

Comizzoli, P., Songsasen, N., and Wildt, D. E. (2010). Protecting and Extending Fertility for Females of Wild and Endangered Mammals. *Cancer Treat. Res.* 156, 87. doi: 10.1007/978-1-4419-6518-9_7.

Creaser, C. W. (1934). The technique of handling the zebrafish (Brachydanio rerio) for the production of eggs which are favourable for embryological research and are available at any specified time throughout the year. *Copeia*, 159–161.

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. *Gen. Comp. Endocrinol.* 257, 106–112. doi: 10.1016/j.ygcen.2017.04.014.

Darrow, K. O., and Harris, W. A. (2004). Characterization and Development of Courtship in Zebrafish, Danio rerio . *Zebrafish* 1, 40–45. doi: 10.1089/154585404774101662.

de Jong, T. R., Veening, J. G., Olivier, B., and Waldinger, M. D. (2007). Oxytocin Involvement in SSRI-Induced Delayed Ejaculation: A Review of Animal Studies. *J. Sex. Med.* 4, 14–28. doi: 10.1111/j.1743-6109.2006.00394.x.

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

DeAngelis, R., Gogola, J., Dodd, L., and Rhodes, J. S. (2017). Opposite effects of nonapeptide antagonists on paternal behavior in the teleost fish Amphiprion ocellaris. *Horm. Behav.* 90, 113–119. doi: 10.1016/j.yhbeh.2017.02.013.

Demski, L. S., and Sloan, H. E. (1985). A direct magnocellular-preopticospinal pathway in goldfish: Implications for control of sex behavior. *Neurosci. Lett.* 55, 283–288. doi: 10.1016/0304-3940(85)90449-5.

Egan, R. J., Bergner, C. L., Hart, P. C., Cachat, J. M., Canavello, P. R., Elegante, M. F., et al. (2009). Understanding behavioral and physiological phenotypes of stress and anxiety in zebrafish. *Behav. Brain Res.* 205, 38–44. doi: 10.1016/J.BBR.2009.06.022.

Evans, D. H. (1998). *The Fish Physiology, second ed*. 2nd Editio. Boca Raton, FL: CRC Press.

Evans, J. J., Robinson, G., and Catt, K. J. (1992). Luteinizing Hormone Response to Oxytocin Is Steroid-Dependent. *Neuroendocrinology* 55, 538–543. doi: 10.1159/000126167.

Evers, B., and Jonkers, J. (2006). Mouse models of BRCA1 and BRCA2 deficiency: past lessons, current understanding and future prospects. *Oncogene* 25, 5885–5897. doi: 10.1038/sj.onc.1209871.

Faught, E., Best, C., and Vijayan, M. M. (2016). Maternal stress-associated cortisol stimulation may protect embryos from cortisol excess in zebrafish. *R. Soc. Open Sci.* 3, 160032. doi: 10.1098/rsos.160032.

Faught, E., Santos, H. B., and Vijayan, M. M. (2020). Loss of the glucocorticoid receptor causes accelerated ovarian ageing in zebrafish. *Proc. R. Soc. B* 287, 20202190. doi: 10.1098/RSPB.2020.2190.

Finn, R. N., and Kristoffersen, B. A. (2007). Vertebrate Vitellogenin Gene Duplication in Relation to the "3R Hypothesis": Correlation to the Pelagic Egg and the Oceanic Radiation of Teleosts. *PLoS One* 2, e169. doi: 10.1371/journal.pone.0000169.

Forlano, P. M., and Bass, A. H. (2011). Neural and hormonal mechanisms of reproductive-related arousal in fishes. *Horm. Behav.* 59, 616–629. doi: 10.1016/j.yhbeh.2010.10.006.

Frederickson, S. C., Steinmiller, M. D., Blaylock, T. R., Wisnieski, M. E., Malley, J. D., Pandolfo, L. M., et al. (2021). Comparison of Juvenile Feed Protocols on Growth and Spawning in Zebrafish. *J. Am. Assoc. Lab. Anim. Sci.* 60, 298–305. doi: 10.30802/AALAS-JAALAS-20-000105.

Fryer, J. N. (1989). Neuropeptides regulating the activity of goldfish corticotropes and melanotropes. *Fish*

Physiol. Biochem. 7, 21–27. doi: 10.1007/BF00004686.

Gagnon, J. A., Valen, E., Thyme, S. B., Huang, P., Ahkmetova, L., Pauli, A., et al. (2014). Efficient Mutagenesis by Cas9 Protein-Mediated Oligonucleotide Insertion and Large-Scale Assessment of Single-Guide RNAs. *PLoS One* 9, e98186. doi: 10.1371/journal.pone.0098186.

Gesto, M., Soengas, J. L., Rodríguez-Illamola, A., and Míguez, J. M. (2014). Arginine Vasotocin Treatment Induces a Stress Response and Exerts a Potent Anorexigenic Effect in Rainbow Trout, Oncorhynchus mykiss. *J. Neuroendocrinol.* 26, 89–99. doi: 10.1111/JNE.12126.

Gilchriest, B. J., Tipping, D. R., Hake, L., Levy, A., and Baker, B. I. (2001). The Effects of Acute and Chronic Stresses on Vasotocin Gene Transcripts in the Brain of the Rainbow Trout (Oncorhynchus mykiss). *J. Neuroendocrinol.* 12, 795–801. doi: 10.1046/j.1365-2826.2000.00522.x.

Godwin, J., and Thompson, R. (2012). Nonapeptides and Social Behavior in Fishes. *Horm. Behav.* 61, 230–238. doi: 10.1016/J.YHBEH.2011.12.016.

Goodson, J. L., and Bass, A. H. (2000). Forebrain peptides modulate sexually polymorphic vocal circuitry. *Nature* 403, 769–772. doi: 10.1038/35001581.

Goodson, J. L., and Bass, A. H. (2001). Social behavior functions and related anatomical characteristics of vasotocin/vasopressin systems in vertebrates. *Brain Res. Rev.* 35, 246–265. doi: 10.1016/S0165-0173(01)00043-1.

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

Groves, D. J., and Batten, T. F. C. (1986). Direct control of the gonadotroph in a teleost, Poecilia latipinna. *Gen. Comp. Endocrinol.* 62, 315–326. doi: 10.1016/0016-6480(86)90122-X.

Gutnick, A., Blechman, J., Kaslin, J., Herwig, L., Belting, H.-G., Affolter, M., et al. (2011). The Hypothalamic Neuropeptide Oxytocin Is Required for Formation of the Neurovascular Interface of the Pituitary. *Dev. Cell* 21, 642–654. doi: 10.1016/j.devcel.2011.09.004.

Gwee, P.-C., Amemiya, C. T., Brenner, S., and Venkatesh, B. (2008). Sequence and organization of

coelacanth neurohypophysial hormone genes: Evolutionary history of the vertebrate neurohypophysial hormone gene locus. *BMC Evol. Biol.* 8, 93. doi: 10.1186/1471-2148-8-93.

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

Hare, A. J., Zimmer, A. M., LePabic, R., Morgan, A. L., and Gilmour, K. M. (2021). Early-life stress influences ion balance in developing zebrafish (Danio rerio). *J. Comp. Physiol. B* 191, 69–84. doi: 10.1007/s00360-020-01319-9.

Hartung, O., Forbes, M. M., and Marlow, F. L. (2014). Zebrafish vasa is required for germ-cell differentiation and maintenance. *Mol. Reprod. Dev.* 81, 946–961. doi: 10.1002/mrd.22414.

Hasunuma, I., Toyoda, F., Okada, R., Yamamoto, K., Kadono, Y., and Kikuyama, S. (2013). *Roles of arginine vasotocin receptors in the brain and pituitary of submammalian vertebrates*. 1st ed. Elsevier Inc. doi: 10.1016/B978-0-12-407696-9.00004-X.

Heckmann, L.-H., Sørensen, P. B., Krogh, P. H., and Sørensen, J. G. (2011). NORMA-Gene: A simple and robust method for qPCR normalization based on target gene data. *BMC Bioinformatics* 12, 250. doi: 10.1186/1471-2105-12-250.

Heierhorst, J., Morley, S. D., Figueroa, J., Krentler, C., Lederis, K., and Richter, D. (1989). Vasotocin and isotocin precursors from the white sucker, Catostomus commersoni: cloning and sequence analysis of the cDNAs. *Proc. Natl. Acad. Sci.* 86, 5242–5246. doi: 10.1073/pnas.86.14.5242.

Helfman, G.S., Collette, B.B. and Facey, D. E. (2009). *The Diversity of Fishes*. 2nd ed. Oxford: Wiley-Blackwell.

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

Herget, U., Gutierrez-Triana, J. A., Salazar Thula, O., Knerr, B., and Ryu, S. (2017). Single-Cell Reconstruction of Oxytocinergic Neurons Reveals Separate Hypophysiotropic and Encephalotropic Subtypes in Larval Zebrafish. *eneuro* 4, ENEURO.0278-16.2016. doi: 10.1523/ENEURO.0278-

16.2016.

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

Hiraoka, S., Suzuki, M., Yanagisawa, T., Iwata, M., and Urano, A. (1993). Divergence of Gene Expression in Neurohypophysial Hormone Precursors among Salmonids. *Gen. Comp. Endocrinol.* 92, 292–301. doi: 10.1006/gcen.1993.1165.

Hoo, J. Y., Kumari, Y., Shaikh, M. F., Hue, S. M., and Goh, B. H. (2016). Zebrafish: A Versatile Animal Model for Fertility Research. doi: 10.1155/2016/9732780.

Huffman, L. S., O'Connell, L. A., Kenkel, C. D., Kline, R. J., Khan, I. A., Hofmann, H. A. (2012). Distribution of nonapeptide systems in the forebrain of an African cichlid fish, *astatotilapia burtoni*. *J Chem Neuroanat.* 44, 86-97. doi: 10.1016/j.jchemneau.2012.05.002

Hughes, L. C., Ortí, G., Huang, Y., Sun, Y., Baldwin, C. C., Thompson, A. W., et al. (2018). Comprehensive phylogeny of ray-finned fishes (Actinopterygii) based on transcriptomic and genomic data. *Proc. Natl. Acad. Sci. U. S. A.* 115, 6249–6254. doi: 10.1073/pnas.1719358115.

Hwang, W. Y., Fu, Y., Reyon, D., Maeder, M. L., Tsai, S. Q., Sander, J. D., et al. (2013). Efficient genome editing in zebrafish using a CRISPR-Cas system. *Nat. Biotechnol.* 31, 227–229. doi: 10.1038/nbt.2501.

Hyodo, S., Ishii, S., and Joss, J. M. P. (1997). Australian lungfish neurohypophysial hormone genes encode vasotocin and [Phe 2]mesotocin precursors homologous to tetrapod-type precursors. *Proc. Natl. Acad. Sci.* 94, 13339–13344. doi: 10.1073/pnas.94.24.13339.

Iwasaki, K., Taguchi, M., Bonkowsky, J. L., and Kuwada, J. Y. (2013). Expression of arginine vasotocin receptors in the developing zebrafish CNS. *Gene Expr. Patterns* 13, 335–342. doi: 10.1016/j.gep.2013.06.005.

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

Joy, K. P., and Singh, V. (2013). Functional interactions between vasotocin and prostaglandins during

final oocyte maturation and ovulation in the catfish Heteropneustes fossilis. *Gen. Comp. Endocrinol.* 186, 126–135. doi: 10.1016/j.ygcen.2013.02.043.

Juntti, S. A., and Fernald, R. D. (2016). Timing reproduction in teleost fish: Cues and mechanisms. *Curr. Opin. Neurobiol.* 38, 57–62. doi: 10.1016/j.conb.2016.02.006.

Juntti, S. A., Hilliard, A. T., Kent, K. R., Kumar, A., Nguyen, A., Jimenez, M. A., et al. (2016). A Neural Basis for Control of Cichlid Female Reproductive Behavior by Prostaglandin F 2α. *Curr. Biol.* 26, 943–949. doi: 10.1016/j.cub.2016.01.067.

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

Kah, O., and Dufour, S. (2011). 'Conserved and Divergent Features of Reproductive Neuroendocrinology in Teleost Fishes', in *Hormones and Reproduction of Vertebrates* (Elsevier), 15–42. doi: 10.1016/B978-0-12-375009-9.10002-5.

Kalamarz-Kubiak, H. (2022). Endocrine-Disrupting Compounds in Fish Physiology, with Emphasis on their Effects on the Arginine Vasotocin/Isotocin System. *Endocrine, Metab. Immune Disord. - Drug Targets* 22, 738–747. doi: 10.2174/1871530321666210202150947.

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? doi: 10.1242/bio.024844.

Kanda, S., Akazome, Y., Mitani, Y., Okubo, K., and Oka, Y. (2013). Neuroanatomical Evidence That Kisspeptin Directly Regulates Isotocin and Vasotocin Neurons. *PLoS One* 8, 62776. doi: 10.1371/journal.pone.0062776.

Kawada, T., Shiraishi, A., Matsubara, S., Hozumi, A., Horie, T., Sasakura, Y., et al. (2021). Vasopressin Promoter Transgenic and Vasopressin Gene-Edited Ascidian, Ciona intestinalis Type A (Ciona robusta): Innervation, Gene Expression Profiles, and Phenotypes. *Front. Endocrinol. (Lausanne).* 12. doi: 10.3389/fendo.2021.668564.

Kleszczyńska, A., Sokołowska, E., and Kulczykowska, E. (2012). 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.* 175, 290–296. doi: 10.1016/j.ygcen.2011.11.022.

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

Kobayashi, M., and Nakanishi, T. (1999). 11-Ketotestosterone Induces Male-Type Sexual Behavior and Gonadotropin Secretion in Gynogenetic Crucian Carp, Carassius auratus langsdorfii. *Gen. Comp. Endocrinol.* 115, 178–187. doi: 10.1006/GCEN.1999.7314.

Kobayashi, M., Sorensen, P. W., and Stacey, N. E. (2002). Hormonal and pheromonal control of spawning behavior in the goldfish. *Fish Physiol. Biochem.* 26, 71–84. doi: 10.1023/A:1023375931734.

Kolm N. (2009). *Reproductive Biology and Phylogeny of Fishes (Agnathans and Bony Fishes)*. B.G.M. Jam. Enfield, NH: Science Publishers doi: 10.1201/9781482280609.

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. *Gen. Comp. Endocrinol.* 204, 8–12. doi: 10.1016/j.ygcen.2014.04.039.

Lema, S. C., Sanders, K. E., and Walti, K. A. (2015). Arginine Vasotocin, Isotocin and Nonapeptide Receptor Gene Expression Link to Social Status and Aggression in Sex-Dependent Patterns. *J. Neuroendocrinol.* 27, 142–157. doi: 10.1111/jne.12239.

Lema, S. C., Slane, M. A., Salvesen, K. E., and Godwin, J. (2012). Variation in gene transcript profiles of two V1a-type arginine vasotocin receptors among sexual phases of bluehead wrasse (Thalassoma bifasciatum). *Gen. Comp. Endocrinol.* 179, 451–464. doi: 10.1016/j.ygcen.2012.10.001.

Lema, S. C., Washburn, E. H., Crowley, M. E., Carvalho, P. G., Egelston, J. N., and McCormick, S. D. (2019). Evidence for a role of arginine vasotocin receptors in the gill during salinity acclimation by

a euryhaline teleost fish. *Am. J. Physiol. - Regul. Integr. Comp. Physiol.* 316, R735–R750. doi: 10.1152/ajpregu.00328.2018.

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

Lester, N. P., Shuter, B. J., and Abrams, P. A. (2004). Interpreting the von Bertalanffy model of somatic growth in fishes: the cost of reproduction. *Proc. R. Soc. London. Ser. B Biol. Sci.* 271, 1625–1631. doi: 10.1098/rspb.2004.2778.

Levavi-Sivan, B., Bogerd, J., Mañanós, E. L., Gómez, A., and Lareyre, J. J. (2010). Perspectives on fish gonadotropins and their receptors. *Gen. Comp. Endocrinol.* 165, 412–437. doi: 10.1016/j.ygcen.2009.07.019.

Li, J., and Ge, W. (2020). Zebrafish as a model for studying ovarian development: Recent advances from targeted gene knockout studies. *Mol. Cell. Endocrinol.* 507. doi: 10.1016/J.MCE.2020.110778.

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

Lister, A. L., and Van Der Kraak, G. J. (2009). Regulation of prostaglandin synthesis in ovaries of sexually-mature zebrafish (Danio rerio). *Mol. Reprod. Dev.* 76, 1064–1075. doi: 10.1002/mrd.21072.

Liu, Y., Kossack, M. E., McFaul, M. E., Christensen, L. N., Siebert, S., Wyatt, S. R., et al. (2022) Single-cell transcriptome reveals insights into the development and function of the zebrafish ovary. *eLife* 11, e76014. doi: 10.7554/eLife.76014.

Loveland, J. L., and Hu, C. K. (2018). Commentary: Arginine Vasotocin Preprohormone Is Expressed in Surprising Regions of the Teleost Forebrain. *Front. Endocrinol. (Lausanne).* 9. doi: 10.3389/fendo.2018.00063.

Lyu, L. K., Li, J. S., Wang, X. J., Yao, Y. J., Li, J. F., Li, Y., et al. (2021). Arg-Vasotocin Directly

Activates Isotocin Receptors and Induces COX2 Expression in Ovoviviparous Guppies. *Front. Endocrinol. (Lausanne).* 12. doi: 10.3389/fendo.2021.617580.

Macey, M. J., Pickford, G. E., and Peter, R. E. (1974). Forebrain localization of the spawning reflex response to exogenous neurohypophysial hormones in the killifish,Fundulus heteroclitus. *J. Exp. Zool.* 190, 269–279. doi: 10.1002/jez.1401900303.

Maejima, K., Oka, Y., Park, M. K., and Kawashima, S. (1994). 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. *Neurosci. Res.* 20, 189–193. doi: 10.1016/0168-0102(94)90037-X.

Mangiamele, L. A., Keeney, A. D. T., D'Agostino, E. N., and Thompson, R. R. (2013). Pheromone Exposure Influences Preoptic Arginine Vasotocin Gene Expression and Inhibits Social Approach Behavior in Response to Rivals but Not Potential Mates. *Brain. Behav. Evol.* 81, 194–202. doi: 10.1159/000350589.

Maradonna, F., Gioacchini, G., Notarstefano, V., Fontana, C. M., Citton, F., Dalla Valle, L., et al. (2020). Knockout of the Glucocorticoid Receptor Impairs Reproduction in Female Zebrafish. *Int. J. Mol. Sci.* 21, 9073. doi: 10.3390/ijms21239073.

Martínez, R., Tu, W., Eng, T., Allaire-Leung, M., Piña, B., Navarro-Martín, L., et al. (2020). Acute and long-term metabolic consequences of early developmental Bisphenol A exposure in zebrafish (Danio rerio). *Chemosphere* 256, 127080. doi: 10.1016/j.chemosphere.2020.127080.

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. *Comp. Biochem. Physiol. Part A Mol. Integr. Physiol.* 147, 129–144. doi: 10.1016/j.cbpa.2006.12.019.

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

Mayasich, S. A., and Clarke, B. L. (2020). *Vasotocin and the origins of the vasopressin/oxytocin receptor*

gene family. 1st ed. Elsevier Inc. doi: 10.1016/bs.vh.2019.08.018.

McMillan, S. C., Géraudie, J., and Akimenko, M.-A. (2015). Pectoral Fin Breeding Tubercle Clusters: A Method to Determine Zebrafish Sex. *Zebrafish* 12, 121–123. doi: 10.1089/zeb.2014.1060.

Mennigen, J. A., Harris, E. A., Chang, J. P., Moon, T. W., and Trudeau, V. L. (2009). Fluoxetine affects weight gain and expression of feeding peptides in the female goldfish brain. *Regul. Pept.* 155, 99–104. doi: 10.1016/j.regpep.2009.01.001.

Mennigen, J. A., Lado, W. E., Zamora, J. M., Duarte-Guterman, P., Langlois, V. S., Metcalfe, C. D., et al. (2010). Waterborne fluoxetine disrupts the reproductive axis in sexually mature male goldfish, Carassius auratus. *Aquat. Toxicol.* 100, 354–364. doi: 10.1016/j.aquatox.2010.08.016.

Mennigen, J. A., Martyniuk, C. J., Crump, K., Xiong, H., Zhao, E., Popesku, J., et al. (2008). Effects of fluoxetine on the reproductive axis of female goldfish (Carassius auratus). *Physiol. Genomics* 35, 273–282. doi: 10.1152/physiolgenomics.90263.2008.

Mennigen, J. A., Volkoff, H., Chang, J. P., and Trudeau, V. L. (2017). 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.* 254, 38–49. doi: 10.1016/j.ygcen.2017.09.008.

Meshalkina, D. A., N. Kizlyk, M., V. Kysil, E., Collier, A. D., Echevarria, D. J., Abreu, M. S., et al. (2018). Zebrafish models of autism spectrum disorder. *Exp. Neurol.* 299, 207–216. doi: 10.1016/j.expneurol.2017.02.004.

Mishra, A., and Joy, K. P. (2006). 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.* 189, 341–353. doi: 10.1677/joe.1.06686.

Mitchell, K., Su Zhang, W., Lu, C., Tao, B., Chen, L., Hu, W., et al. (2020). Targeted mutation of secretogranin-2 disrupts sexual behavior and reproduction in zebrafish. 117. doi: 10.1073/pnas.2002004117/-/DCSupplemental.

Moon, T. W., and Mommsen, T. P. (1990). Vasoactive peptides and phenylephrine actions in isolated teleost hepatocytes. *https://doi.org/10.1152/ajpendo.1990.259.5.E644* 259. doi:

10.1152/AJPENDO.1990.259.5.E644.

Moons, L., Cambré, M., Batten, T. F. C., and Vandesande, F. (1989). Autoradiographic localization of binding sites for vasotocin in the brain and pituitary of the sea bass (Dicentrarchus labrax). *Neurosci. Lett.* 100, 11–16. doi: 10.1016/0304-3940(89)90652-6.

Munakata, A., and Kobayashi, M. (2010). Endocrine control of sexual behavior in teleost fish. *Gen. Comp. Endocrinol.* 165, 456–468. doi: 10.1016/j.ygcen.2009.04.011.

Naderi, F., Míguez, J. M., Soengas, J. L., and López-Patiño, M. A. (2019). SIRT1 mediates the effect of stress on hypothalamic clock genes and food intake regulators in rainbow trout, Oncorhynchus mykiss. *Comp. Biochem. Physiol. Part A Mol. Integr. Physiol.* 235, 102–111. doi: 10.1016/j.cbpa.2019.05.021.

Naderi, M., Puar, P., JavadiEsfahani, R., and Kwong, R. W. M. (2022). Early developmental exposure to bisphenol A and bisphenol S disrupts socio-cognitive function, isotocin equilibrium, and excitation-inhibition balance in developing zebrafish. *Neurotoxicology* 88, 144–154. doi: 10.1016/j.neuro.2021.11.009.

Nagahama, Y. (1994). Endocrine regulation of gametogenesis in fish. *Int. J. Dev. Biol.* 38, 217–229.

Natterson-Horowitz, B., Boddy, A. M., and Zimmerman, D. (2022). Female Health Across the Tree of Life: Insights at the Intersection of Women's Health, One Health and Planetary Health. *PNAS Nexus* 1, 1–7. doi: 10.1093/pnasnexus/pgac044.

O'Connell, L. A., Matthews, B. J., and Hofmann, H. A. (2012). Isotocin regulates paternal care in a monogamous cichlid fish. *Horm. Behav.* 61, 725–733. doi: 10.1016/j.yhbeh.2012.03.009.

Ocampo Daza, D., Lewicka, M., and Larhammar, D. (2012). The oxytocin/vasopressin receptor family has at least five members in the gnathostome lineage, inclucing two distinct V2 subtypes. *Gen. Comp. Endocrinol.* 175, 135–143. doi: 10.1016/j.ygcen.2011.10.011.

Odekunle, E. A., and Elphick, M. R. (2020). Comparative and Evolutionary Physiology of Vasopressin/ Oxytocin-Type Neuropeptide Signaling in Invertebrates. *Front. Endocrinol. (Lausanne).* 11. doi: 10.3389/fendo.2020.00225.

Olivereau, M., and Olivereau, J. (1990). Effect of pharmacological adrenalectomy on corticotropin-releasing factor-like and arginine vasotocin immunoreactivities in the brain and pituitary of the eel: Immunocytochemical study. *Gen. Comp. Endocrinol.* 80, 199–215. doi: 10.1016/0016-6480(90)90165-I.

Ota, K. G., and Abe, G. (2016). Goldfish morphology as a model for evolutionary developmental biology. *WIREs Dev. Biol.* 5, 272–295. doi: 10.1002/wdev.224.

Padilla, L. F. A., Castañeda-Cortés, D. C., Rosa, I. F., Acosta, O. D. M., Hattori, R. S., Nóbrega, R. H., et al. (2021). Cystic proliferation of germline stem cells is necessary to reproductive success and normal mating behavior in medaka. *Elife* 10. doi: 10.7554/ELIFE.62757.

Panopoulou, G., and Poustka, A. (2005). Timing and mechanism of ancient vertebrate genome duplications – the adventure of a hypothesis. *Trends Genet.* 21, 559–567. doi: 10.1016/j.tig.2005.08.004.

Parolini, M., Ghilardi, A., De Felice, B., and Del Giacco, L. (2019). Environmental concentration of fluoxetine disturbs larvae behavior and increases the defense response at molecular level in zebrafish (Danio rerio). *Environ. Sci. Pollut. Res.* 26, 34943–34952. doi: 10.1007/s11356-019-06619-4.

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

Patiño, R., Yoshizaki, G., Bolamba, D., and Thomas, P. (2003). Role of Arachidonic Acid and Protein Kinase C During Maturation-Inducing Hormone-Dependent Meiotic Resumption and Ovulation in Ovarian Follicles of Atlantic Croaker1. *Biol. Reprod.* 68, 516–523. doi: 10.1095/biolreprod.102.009662.

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

Patzner, R. . (2008). *Reproductive strategies in fish. In: Fish Reproduction*. M.J. Roche. Enfield, NH:

Science Publishers.

Perrone, R., Migliaro, A., Comas, V., Quintana, L., Borde, M., and Silva, A. (2014). Local vasotocin modulation of the pacemaker nucleus resembles distinct electric behaviors in two species of weakly electric fish. *J. Physiol.* 108, 203–212. doi: 10.1016/j.jphysparis.2014.07.007.

Perrone, R., and Silva, A. (2016). Vasotocin increases dominance in the weakly electric fish Brachyhypopomus gauderio. *J. Physiol.* 110, 119–126. doi: 10.1016/j.jphysparis.2016.12.004.

Peter, R. E. (1977). The preoptic nucleus in fishes: A comparative discussion of function-activity relationships. *Integr. Comp. Biol.* 17, 775–785. doi: 10.1093/icb/17.4.775.

Peter, R. E., Lin, H.-R., and Van Der Kraak, G. (1988). Induced ovulation and spawning of cultured freshwater fish in China: Advances in application of GnRH analogues and dopamine antagonists. *Aquaculture* 74, 1–10. doi: 10.1016/0044-8486(88)90080-4.

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

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. *Front. Integr. Neurosci.* 13. doi: 10.3389/fnint.2019.00037.

Pouso, P., Perrone, R., and Silva, A. (2021). 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.* 313, 113886. doi: 10.1016/j.ygcen.2021.113886.

Pouso, P., Radmilovich, M., and Silva, A. (2017). An immunohistochemical study on the distribution of vasotocin neurons in the brain of two weakly electric fish, Gymnotus omarorum and Brachyhypopomus gauderio. *Tissue Cell* 49, 257–269. doi: 10.1016/j.tice.2017.02.003.

Rahman, M. S., Kline, R. J., Vázquez, O. A., Khan, I. A., and Thomas, P. (2020). Molecular characterization and expression of arginine vasotocin V1a2 receptor in Atlantic croaker brain:

Potential mechanisms of its downregulation by PCB77. *J. Biochem. Mol. Toxicol.* 34. doi: 10.1002/jbt.22500.

Ramallo, M. R., Grober, M., Cánepa, M. M., Morandini, L., and Pandolfi, M. (2012). Arginine-vasotocin expression and participation in reproduction and social behavior in males of the cichlid fish Cichlasoma dimerus. *Gen. Comp. Endocrinol.* 179, 221–231. doi: 10.1016/j.ygcen.2012.08.015.

Ramsay, J. M., Feist, G. W., Varga, Z. M., Westerfield, M., Kent, M. L., and Schreck, C. B. (2009). Whole-body cortisol response of zebrafish to acute net handling stress. *Aquaculture* 297, 157–162. doi: 10.1016/j.aquaculture.2009.08.035.

Rawat, A., Chaube, R., and Joy, K. P. (2015). 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.* 41, 509–532. doi: 10.1007/s10695-015-0026-0.

Rawat, A., Chaube, R., and Joy, K. P. (2019). 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.* 45, 885–905. doi: 10.1007/s10695-018-0590-1.

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

Robinson, G., Evans, J. J., and Catt, K. J. (1992). Oxytocin stimulates LH production by the anterior pituitary gland of the rat. *J. Endocrinol.* 132, 277–283. doi: 10.1677/joe.0.1320277.

Rodriguez-Santiago, M., Nguyen, J., Winton, L. S., Weitekamp, C. A., and Hofmann, H. A. (2017). Arginine Vasotocin Preprohormone Is Expressed in Surprising Regions of the Teleost Forebrain. *Front. Endocrinol. (Lausanne).* 8. doi: 10.3389/fendo.2017.00195.

Rodríguez, M., and Specker, J. L. (1991). In vitro effects of arginine vasotocin on testosterone production by testes of rainbow trout (Oncorhynchus mykiss). *Gen. Comp. Endocrinol.* 83, 249–257. doi: 10.1016/0016-6480(91)90028-5.

Roff, D. . (1992). *The Evolution of Life Histories*. London: Chapman and Hall.

Rotondo, F., Butz, H., Syro, L. V., Yousef, G. M., Di Ieva, A., Restrepo, L. M., et al. (2016). Arginine vasopressin (AVP): a review of its historical perspectives, current research and multifunctional role in the hypothalamo-hypophysial system. *Pituitary* 19, 345–355. doi: 10.1007/S11102-015-0703-0.

Roussel, Y., Gaudreau, S. F., Kacer, E. R., Sengupta, M., and Bui, T. V (2021). Modeling spinal locomotor circuits for movements in developing zebrafish. *Elife* 10. doi: 10.7554/eLife.67453.

S., J. H. (2007). Studies on the neuroendocrine role of serotonin. *Dan. Med. Bull.* 54, 266–288.

Saito, D., Hasegawa, Y., and Urano, A. (2003). Gonadotropin-releasing hormones modulate electrical activity of vasotocin and isotocin neurons in the brain of rainbow trout. *Neurosci. Lett.* 351, 107–110. doi: 10.1016/j.neulet.2003.08.017.

Saito, D., Komatsuda, M., and Urano, A. (2004). Functional organization of preoptic vasotocin and isotocin neurons in the brain of rainbow trout: central and neurohypophysial projections of single neurons. *Neuroscience* 124, 973–984. doi: 10.1016/j.neuroscience.2003.12.038.

Sakai, C., Ijaz, S., and Hoffman, E. J. (2018). Zebrafish Models of Neurodevelopmental Disorders: Past, Present, and Future. *Front. Mol. Neurosci.* 11. doi: 10.3389/fnmol.2018.00294.

Salahinejad, A., Attaran, A., Meuthen, D., Rachamalla, M., Chivers, D. P., and Niyogi, S. (2022). 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.* 830, 154794. doi: 10.1016/j.scitotenv.2022.154794.

Salahinejad, A., Naderi, M., Attaran, A., Meuthen, D., Niyogi, S., and Chivers, D. P. (2020). Effects of chronic exposure to bisphenol-S on social behaviors in adult zebrafish: Disruption of the neuropeptide signaling pathways in the brain. *Environ. Pollut.* 262, 113992. doi: 10.1016/J.ENVPOL.2020.113992.

Saleem, S., and Kannan, R. R. (2018)., Zebrafish: an emerging real-time model system to study Alzheimer's disease and neurospcific drug discovery. *Cell Deaktg Discov.* 4, 45. doi: 10.1038/s41420-018-0109-7.

Salek, S. J., Sullivan, C. V, and Godwin, J. (2002). Arginine vasotocin effects on courtship behavior in male white perch (Morone americana). *Behav. Brain Res.* 133, 177–183. doi: 10.1016/S0166-4328(02)00003-7.

Santangelo, N. (2015). Female breeding experience affects parental care strategies of both parents in a monogamous cichlid fish. *Anim. Behav.* 104, 31–37. doi: 10.1016/j.anbehav.2015.03.004.

Santangelo, N., and Bass, A. H. (2010). Individual Behavioral and Neuronal Phenotypes for Arginine Vasotocin Mediated Courtship and Aggression in a Territorial Teleost. *Brain. Behav. Evol.* 75, 282–291. doi: 10.1159/000316867.

Santini, F., Harmon, L. J., Carnevale, G., and Alfaro, M. E. (2009). Did genome duplication drive the origin of teleosts? A comparative study of diversification in ray-finned fishes. *BMC Evol. Biol.* 9, 194. doi: 10.1186/1471-2148-9-194.

Scheirer, C. J., Ray, W. S., and Hare, N. (1976). The Analysis of Ranked Data Derived from Completely Randomized Factorial Designs. *Biometrics* 32, 429. doi: 10.2307/2529511.

Schreck, C. B., Contreras-Sanchez, W., and Fitzpatrick, M. S. (2001). Effects of stress on fish reproduction, gamete quality, and progeny. *Aquaculture* 197, 3–24. doi: 10.1016/S0044-8486(01)00580-4.

Sean C. Lema (2010). Identification of multiple vasotocin receptor cDNAs in teleost fish: Sequences, phylogenetic analysis, sites of expression, and regulation in the hypothalamus and gill in response to hyperosmotic challenge | Elsevier Enhanced Reader. *Mol. Cell. Ebdocrinology*, 215–230. Available at: https://reader.elsevier.com/reader/sd/pii/S0303720710000912?token=BC861231B6CC0B3AF32C2D3823BF7EA94781FE96A567B0054AE415556DDE1B42C9AF61DA3BBE78BEC86DD218EB569D2C [Accessed September 17, 2020].

Semsar, K., Kandel, F. L. M., and Godwin, J. (2001). Manipulations of the AVT System Shift Social Status and Related Courtship and Aggressive Behavior in the Bluehead Wrasse. *Horm. Behav.* 40, 21–31. doi: 10.1006/hbeh.2001.1663.

Semsar, K., Perreault, H. A. N., and Godwin, J. (2004). Fluoxetine-treated male wrasses exhibit low AVT expression. *Brain Res.* 1029, 141–147. doi: 10.1016/j.brainres.2004.09.030.

Shams, S., Rihel, J., Ortiz, J. G., and Gerlai, R. (2018). The zebrafish as a promising tool for modeling human brain disorders: A review based upon an IBNS Symposium. *Neurosci. Biobehav. Rev.* 85, 176–190. doi: 10.1016/j.neubiorev.2017.09.002.

Shaw, G. B., Smith, C., and Wootton, R. J. (2016). The remarkable reproductive diversity of teleost fishes. doi: 10.1111/faf.12116.

Sherwood, N. M., and Adams, B. A. (2005). 'Gonadotropin-Releasing Hormone in Fish: Evolution, Expression and Regulation of the GnRH Gene', in, 1–39. doi: 10.1142/9789812569189_0001.

Silva, A., Perrone, R., and Macadar, O. (2007). Environmental, seasonal, and social modulations of basal activity in a weakly electric fish. *Physiol. Behav.* 90, 525–536. doi: 10.1016/j.physbeh.2006.11.003.

Singh, O., Kumar, S., Singh, U., Bhute, Y., and Singru, P. S. (2016). Role of Isotocin in the Regulation of the Hypophysiotropic Dopamine Neurones in the Preoptic Area of the Catfish, Clarias batrachus. *J. Neuroendocrinol.* 28. doi: 10.1111/jne.12441.

Singh, U., Kumar, S., and Singru, P. S. (2012). 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.* 24, 1398–1411. doi: 10.1111/j.1365-2826.2012.02350.x.

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. *Gen. Comp. Endocrinol.* 159, 214–225. doi: 10.1016/j.ygcen.2008.09.003.

Singh, V., and Joy, K. P. (2009a). Effects of hCG and ovarian steroid hormones on vasotocin levels in the female catfish Heteropneustes fossilis. *Gen. Comp. Endocrinol.* 162, 172–178. doi: 10.1016/j.ygcen.2009.03.016.

Singh, V., and Joy, K. P. (2009b). Relative in vitro seasonal effects of vasotocin and isotocin on ovarian steroid hormone levels in the catfish Heteropneustes fossilis. *Gen. Comp. Endocrinol.* 162, 257–264.

doi: 10.1016/j.ygcen.2009.03.024.

Singh, V., and Joy, K. P. (2010). An involvement of vasotocin in oocyte hydration in the catfish Heteropneustes fossilis: A comparison with effects of isotocin and hCG. *Gen. Comp. Endocrinol.* 166, 504–512. doi: 10.1016/j.ygcen.2010.02.020.

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

Sirotkin, A. V, Gerasimova, G. G., Golubev, A. K., and Dmitriev, V. B. (1990). The effect of arginine-vasotocin on the production of steroid hormones by mouse, cow, and chicken ovarian tissues in vitro. *Biomed. Sci.* 1, 308–10. Available at: http://www.ncbi.nlm.nih.gov/pubmed/2103834.

Skrzynska, A. K., Maiorano, E., Bastaroli, M., Naderi, F., Míguez, J. M., Martínez-Rodríguez, G., et al. (2018). Impact of Air Exposure on Vasotocinergic and Isotocinergic Systems in Gilthead Sea Bream (Sparus aurata): New Insights on Fish Stress Response. *Front. Physiol.* 9. doi: 10.3389/fphys.2018.00096.

Smith, C., and Wootton, R. J. (2016). The remarkable reproductive diversity of teleost fishes. *Fish Fish.* 17, 1208–1215. doi: 10.1111/faf.12116.

Sokołowska, E., Gozdowska, M., and Kulczykowska, E. (2020). Nonapeptides Arginine Vasotocin and Isotocin in Fishes: Advantage of Bioactive Molecules Measurement. *Front. Mar. Sci.* 7, 1–8. doi: 10.3389/fmars.2020.00610.

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. *Chronobiol. Int.* 32, 917–924. doi: 10.3109/07420528.2015.1052142.

Somoza GM, Yu KL, P. R. (1988). Serotonin stimulates gonadotropin release in female and male goldfish, Carassius auratus L. *Gen Comp Endocrinol* 72, 374–382.

Sorensen, P. W., Appelt, C., Stacey, N. E., Goetz, F. W., and Brash, A. R. (2018). High levels of

circulating prostaglandin F2α associated with ovulation stimulate female sexual receptivity and spawning behavior in the goldfish (Carassius auratus). *Gen. Comp. Endocrinol.* 267, 128–136. doi: 10.1016/j.ygcen.2018.06.014.

Sorensen, P. W., Hara, T. J., Stacey, N. E., and Goetz, F. W. (1988). F Prostaglandins Function as Potent Olfactory Stimulants that Comprise the Postovulatory Female Sex Pheromone in Goldfish1. *Biol. Reprod.* 39, 1039–1050. doi: 10.1095/biolreprod39.5.1039.

Sousa, M. L., Figueiredo, F., Pinheiro, C., Silva, A., Malhão, F., Rocha, M. J., et al. (2015). Morphological and molecular effects of cortisol and ACTH on zebrafish stage I and II follicles. *Reproduction* 150, 429–436. doi: 10.1530/REP-15-0271.

Spence, R., Fatema, M. K., Reichard, M., Huq, K. A., Wahab, M. A., Ahmed, Z. F., et al. (2006). The distribution and habitat preferences of the zebrafish in Bangladesh. *J. Fish Biol.* 69, 1435–1448. doi: 10.1111/J.1095-8649.2006.01206.X.

Stacey, N. E., and Peter, R. E. (1979). Central action of prostaglandins in spawning behaviour of female goldfish. *Physiol. Behav.* 22, 1191–1196. doi: 10.1016/0031-9384(79)90275-0.

Stearns, S. C. (1992). *The Evolution of Life Histories*. Oxford: Oxford University Press.

Streisinger, G., Walker, C., Dower, N., Knauber, D., and Singer, F. (1981). Production of clones of homozygous diploid zebra fish (Brachydanio rerio) Production of homozygous diploids.

Suzuki M, Hyodo S, U. A. (1992). Cloning and sequence analyses of vasotocin and isotocin precursor cDNAs in the masu salmon, Oncorhynchus masou: evolution of neurohypophysial hormone precursors. *Zool. Sci* 9, 157–167.

Takahashi, T., Hagiwara, A., and Ogiwara, K. (2018). Prostaglandins in teleost ovulation: A review of the roles with a view to comparison with prostaglandins in mammalian ovulation. *Mol. Cell. Endocrinol.* 461, 236–247. doi: 10.1016/J.MCE.2017.09.019.

Takesono, A., Kudoh, T., and Tyler, C. R. (2022). Application of Transgenic Zebrafish Models for Studying the Effects of Estrogenic Endocrine Disrupting Chemicals on Embryonic Brain Development. *Front. Pharmacol.* 13. doi: 10.3389/fphar.2022.718072.

Tatarenkov, A., Lima, S. M. Q., Taylor, D. S., and Avise, J. C. (2009). Long-term retention of self-fertilization in a fish clade. *Proc. Natl. Acad. Sci. U. S. A.* 106, 14456–14459. doi: 10.1073/PNAS.0907852106/ASSET/6BE1F936-16E5-471D-B39B-B6B54E98EA1B/ASSETS/GRAPHIC/ZPQ9990993240002.JPEG.

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

Theofanopoulou, C., Gedman, G., Cahill, J. A., Boeckx, C., and Jarvis, E. D. (2021). Universal nomenclature for oxytocin–vasotocin ligand and receptor families. *Nature* 592, 747–755. doi: 10.1038/s41586-020-03040-7.

Thompson, R. R., and Walton, J. C. (2009). Vasotocin Immunoreactivity in Goldfish Brains: Characterizing Primitive Circuits Associated with Social Regulation. *Brain. Behav. Evol.* 73, 153–164. doi: 10.1159/000219485.

Trompouki, E., Viabba Maurwr-Morekku, C., Adama, M. M., Kafaligonul, H. (2018). Zebrafish- A model organism for studying the neurobiological mechanisms underlying cognitive brain aging and use of potential interventions. *Front. Cell Dev. Biol.* 1, 135. doi:10.3389/fcell.2018.00135.

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

Trudeau, V. L. (2018). Facing the challenges of neuropeptide gene knockouts: Why do they not inhibit reproduction in adult teleost fish? *Front. Neurosci.* 12, 302. doi: 10.3389/FNINS.2018.00302/BIBTEX.

Trudeau, V. L. (2021). Neuroendocrine Control of Reproduction in Teleost Fish: Concepts and Controversies. doi: 10.1146/annurev-animal-020420.

Trudeau, V. L., and Somoza, G. M. (2020). Multimodal hypothalamo-hypophysial communication in the vertebrates. *Gen. Comp. Endocrinol.* 293, 113475. doi: 10.1016/j.ygcen.2020.113475.

Venkatesh, B., Tan, C. H., and Lam, T. J. (1992). Prostaglandins and teleost neurohypophyseal hormones

induce premature parturition in the guppy, Poecilia reticulata. *Gen. Comp. Endocrinol.* 87, 28–32. doi: 10.1016/0016-6480(92)90146-B.

Vera-Chang, M. N., St-Jacques, A. D., Gagné, R., Martyniuk, C. J., Yauk, C. L., Moon, T. W., et al. (2018). Transgenerational hypocortisolism and behavioral disruption are induced by the antidepressant fluoxetine in male zebrafish Danio rerio. *Proc. Natl. Acad. Sci.* 115. doi: 10.1073/pnas.1811695115.

Viveiros, A. T. M., Jatzkowski, A., and Komen, J. (2003). Effects of oxytocin on semen release response in African catfish (Clarias gariepinus). *Theriogenology* 59, 1905–1917. doi: 10.1016/S0093-691X(02)01290-6.

Volkoff, H. (2016). The Neuroendocrine Regulation of Food Intake in Fish: A Review of Current Knowledge. *Front. Neurosci.* 10. doi: 10.3389/fnins.2016.00540.

Walton, J. C., Waxman, B., Hoffbuhr, K., Kennedy, M., Beth, E., Scangos, J., et al. (2010). Behavioral effects of hindbrain vasotocin in goldfish are seasonally variable but not sexually dimorphic. *Neuropharmacology* 58, 126–134. doi: 10.1016/j.neuropharm.2009.07.018.

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

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

Wilhelmi A. E., Pickford, G. E., and Sawyer, W.H. (1955). Initiation of the spawning reflex response in fundulus by the administration of fish and mammalian neurohypophysial preparations and synthetic oxytocin. *Endocrinology* 57, 243–252. doi: 10.1210/endo-57-2-243.

Wisdom, K. S., Bhat, I. A., Pathan, M. A., I., C. T., Kumar, P., Babu P., G., et al. (2022). Teleost Nonapeptides, Isotocin and Vasotocin Administration Released the Milt by Abdominal Massage in Male Catfish, Clarias magur. *Front. Endocrinol. (Lausanne).* 13. doi: 10.3389/fendo.2022.899463.

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

Wootton, R. J. (1998). *Ecology of Teleost Fishes*. 2nd ed. Dordrecht: Elsevier.

Wootton, R. J., and Smith, C. (2015). *Reproductive Biology of Teleost Fishes*. Available at: www.wiley.com/wiley-blackwell.

Wu, X. J., Thomas, P., and Zhu, Y. (2018). Pgrmc1 knockout impairs oocyte maturation in zebrafish. *Front. Endocrinol. (Lausanne).* 9, 560. doi: 10.3389/FENDO.2018.00560/XML/NLM.

Wu, X. J., Williams, M. J., Patel, P. R., Kew, K. A., and Zhu, Y. (2019). Subfertility and reduced progestin synthesis in Pgrmc2 knockout zebrafish. *Gen. Comp. Endocrinol.* 282, 113218. doi: 10.1016/J.YGCEN.2019.113218.

Wu, X. J., and Zhu, Y. (2020). Downregulation of nuclear progestin receptor (Pgr) and subfertility in double knockouts of progestin receptor membrane component 1 (pgrmc1) and pgrmc2 in zebrafish. *Gen. Comp. Endocrinol.* 285, 113275. doi: 10.1016/J.YGCEN.2019.113275.

Xiong, S., Tian, J., Ge, S., Li, Z., Long, Z., Guo, W., et al. (2020). The microRNA-200 cluster on chromosome 23 is required for oocyte maturation and ovulation in zebrafish†. *Biol. Reprod.* 103, 769–778. doi: 10.1093/biolre/ioaa125.

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

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

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

Ye, M., and Chen, Y. (2020). Zebrafish as an emerging model to study gonad development. *Comput. Struct. Biotechnol. J.* 18, 2373–2380. doi: 10.1016/j.csbj.2020.08.025.

Yokoi, S., Naruse, K., Kamei, Y., Ansai, S., Kinoshita, M., Mito, M., et al. (2020). Sexually dimorphic role of oxytocin in medaka mate choice. *Proc. Natl. Acad. Sci.* 117, 4802–4808. doi: 10.1073/pnas.1921446117.

Yokoi, S., Okuyama, T., Kamei, Y., Naruse, K., Taniguchi, Y., Ansai, S., et al. (2015). An Essential Role of the Arginine Vasotocin System in Mate-Guarding Behaviors in Triadic Relationships of Medaka Fish (Oryzias latipes). *PLOS Genet.* 11, e1005009. doi: 10.1371/journal.pgen.1005009.

Yoshimura, M., Conway-Campbell, B., and Ueta, Y. (2021). Arginine vasopressin: Direct and indirect action on metabolism. *Peptides* 142, 170555. doi: 10.1016/j.peptides.2021.170555.

Yossa, R., Sarker, P. K., Proulx, E., Saxena, V., Ekker, M., and Vandenberg, G. W. (2013). A Practical Approach for Sexing Zebrafish, Danio rerio. *J. Appl. Aquac.* 25, 148–153. doi: 10.1080/10454438.2013.792170.

Zelena, D., Pintér, O., Balázsfi, D. G., Langnaese, K., Richter, K., Landgraf, R., et al. (2015). Vasopressin signaling at brain level controls stress hormone release: the vasopressin-deficient Brattleboro rat as a model. *Amino Acids* 47, 2245–2253. doi: 10.1007/s00726-015-2026-x.

Zelena, D., Pintér, O., Gabriella Balázsfi, D., Langnaese, K., Richter, K., Landgraf, R., et al. (2015). Vasopressin signaling at brain level controls stress hormone release: the vasopressin-deficient Brattleboro rat as a model. doi: 10.1007/s00726-015-2026-x.

Zempo, B., Tanaka, N., Daikoku, E., and Ono, F. (2021). High-speed camera recordings uncover previously unidentified elements of zebrafish mating behaviors integral to successful fertilization. *Sci. Rep.* 11. doi: 10.1038/s41598-021-99638-6.

Zhang, D., Xiong, H., Mennigen, J. A., Popesku, J. T., Marlatt, V. L., Martyniuk, C. J., et al. (2009). Defining Global Neuroendocrine Gene Expression Patterns Associated with Reproductive Seasonality in Fish. *PLoS One* 4, e5816. doi: 10.1371/journal.pone.0005816.

Zhao, E., Basak, A., Wong, A. O. L., Ko, W., Chen, A., López, G. C., et al. (2009). The Secretogranin II-

Derived Peptide Secretoneurin Stimulates Luteinizing Hormone Secretion from Gonadotrophs. *Endocrinology* 150, 2273–2282. doi: 10.1210/en.2008-1060.

Zhu, B., and Ge, W. (2018). Genome editing in fishes and their applications. *Gen. Comp. Endocrinol.* 257, 3–12. doi: 10.1016/j.ygcen.2017.09.011.

Zmora, N., Stubblefield, J. D., Wong, T.-T., Levavi-Sivan, B., Millar, R. P., and Zohar, Y. (2015). Kisspeptin Antagonists Reveal Kisspeptin 1 and Kisspeptin 2 Differential Regulation of Reproduction in the Teleost, Morone saxatilis1. *Biol. Reprod.* 93. doi: 10.1095/biolreprod.115.131870.

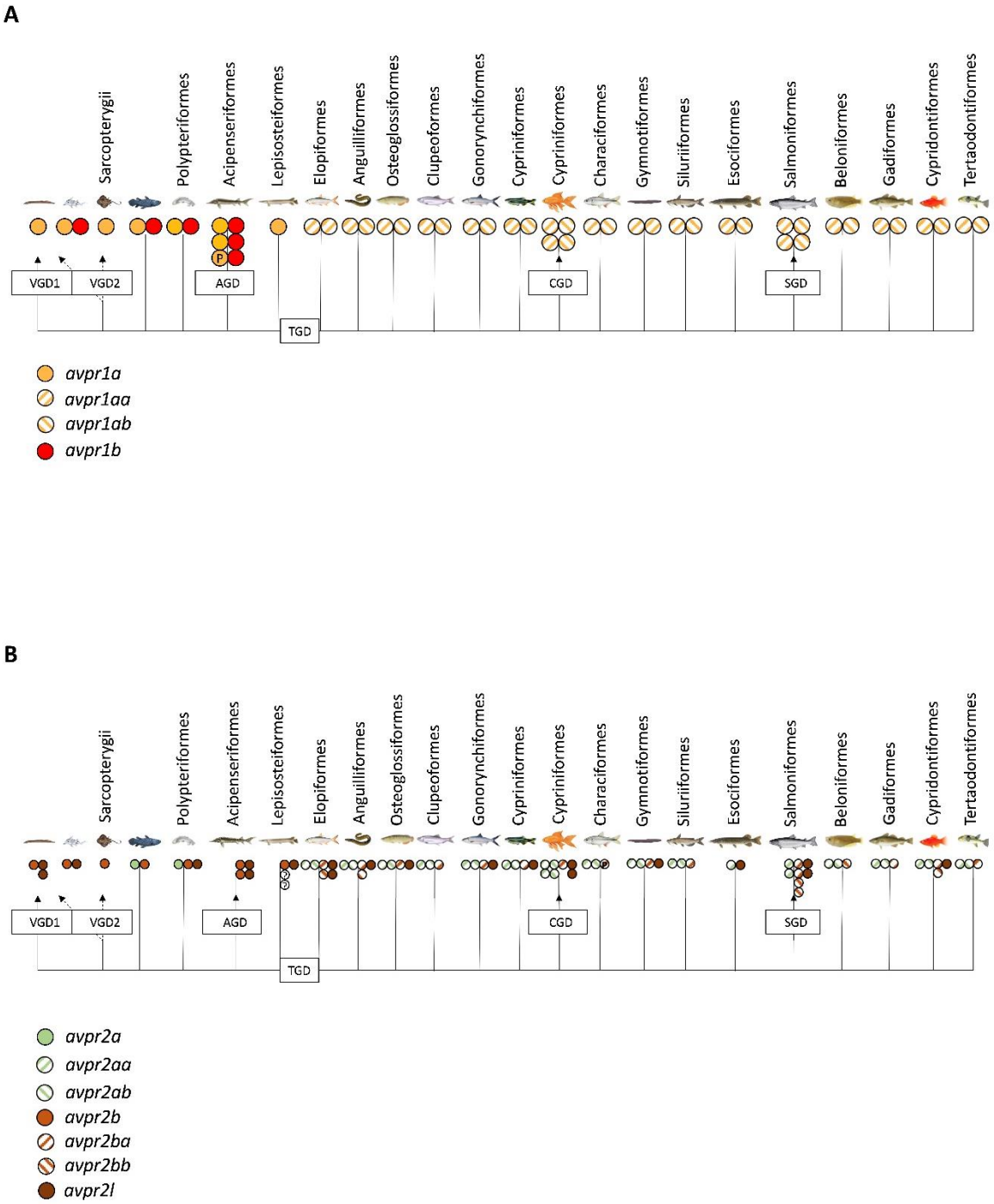
Zohar, Y. (2021). Fish reproductive biology – Reflecting on five decades of fundamental and translational research. *Gen. Comp. Endocrinol.* 300, 113544. doi: 10.1016/j.ygcen.2020.113544.

Zohar, Y., Muñoz-Cueto, J. A., Elizur, A., and Kah, O. (2010). Neuroendocrinology of reproduction in teleost fish. *Gen. Comp. Endocrinol.* 165, 438–455. doi: 10.1016/J.YGCEN.2009.04.017.

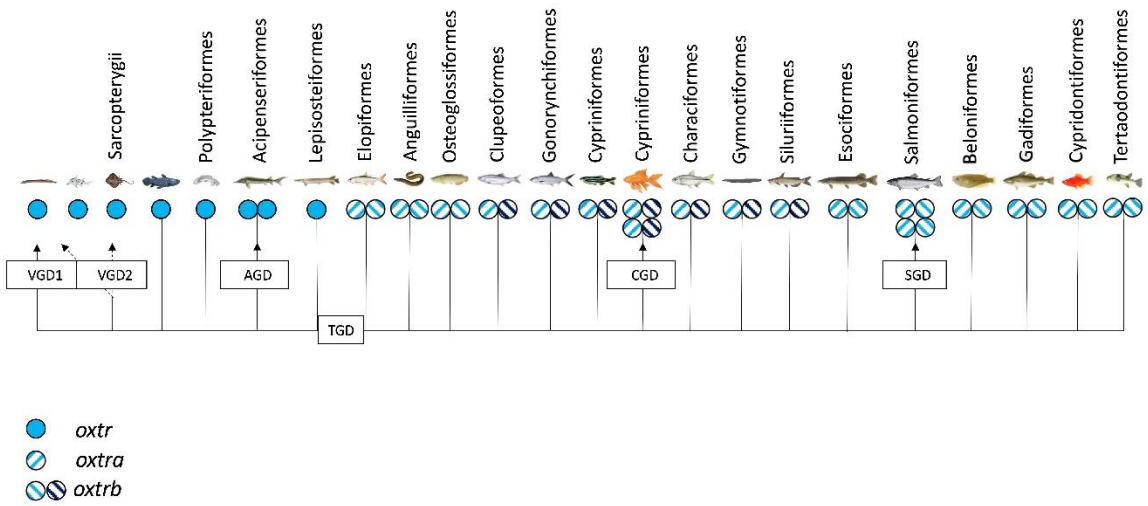
Zohar, Y., Zmora, N., Trudeau, V. L., Muñoz-Cueto, J. A., and Golan, M. (2022). A half century of fish gonadotropin-releasing hormones: Breaking paradigms. *J. Neuroendocrinol.* 34. doi: 10.1111/JNE.13069.

Appendix

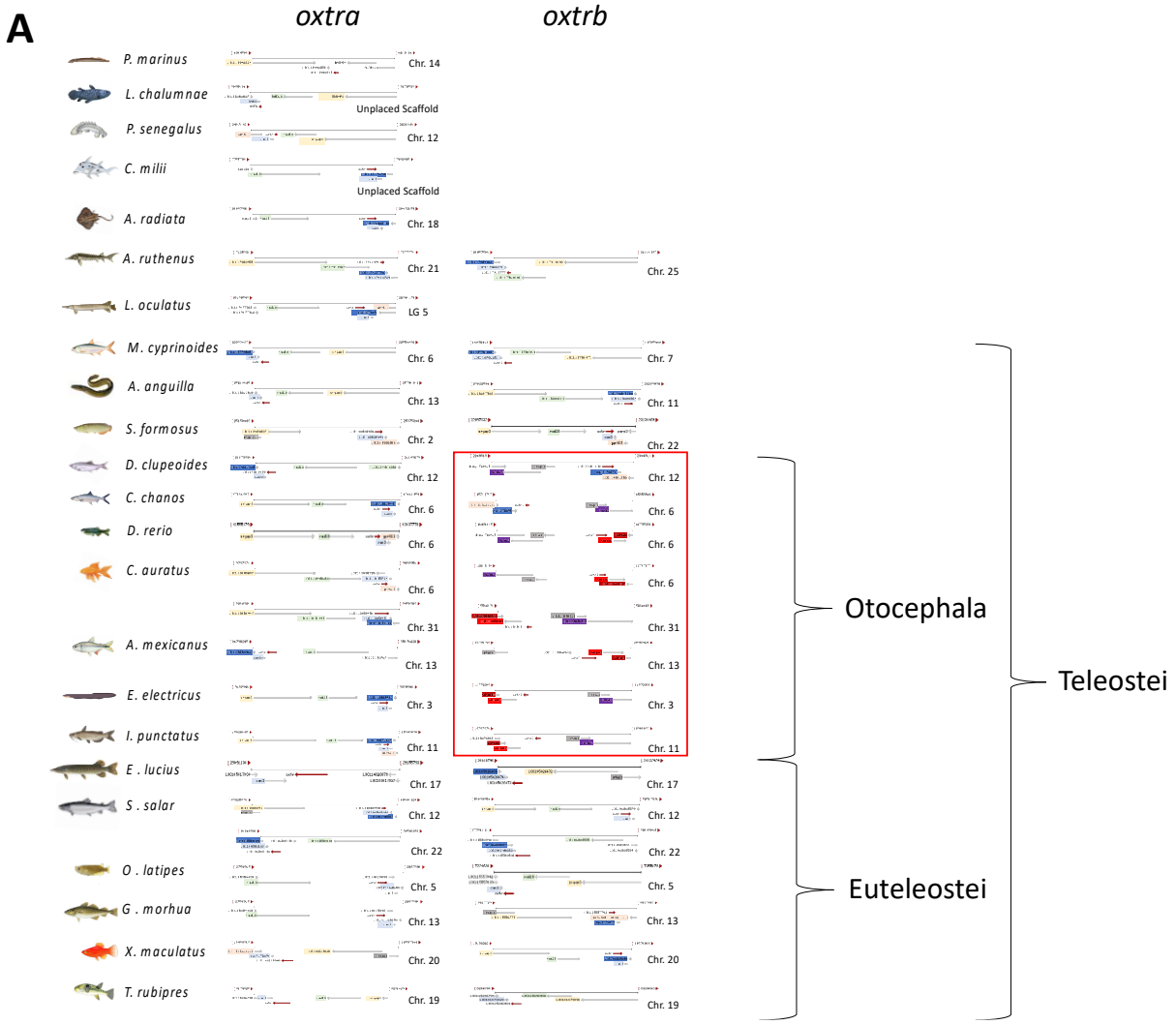
Supplemental Figure 1.1. *Presence of* avpr1 ***(A)***, avpr2/avpr2l ***(B)*** *and oxtr* ***(C)*** *genes in published fish genomes accessed on NCBI (www.pubmed.com) on June 1st, 2022.*



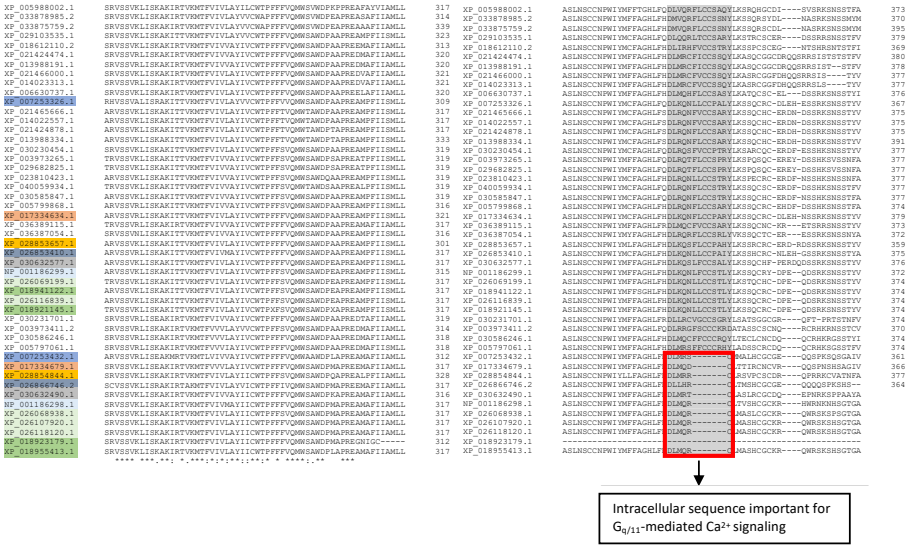
C


Sarcopterygii
Polypteriformes
Acipenseriformes
Lepisosteiformes
Elopiformes
Anguilliformes
Osteoglossiformes
Clupeoformes
Gonorynchiformes
Cypriniformes
Cypriniformes
Characiformes
Gymnotiformes
Siluriiformes
Esociformes
Salmoniformes
Beloniformes
Gadiformes
Cypridontiformes
Tertaodontiformes
VGD1
VGD2
AGD
TGD
CGD
SGD
oxtr
oxtra
oxtrb

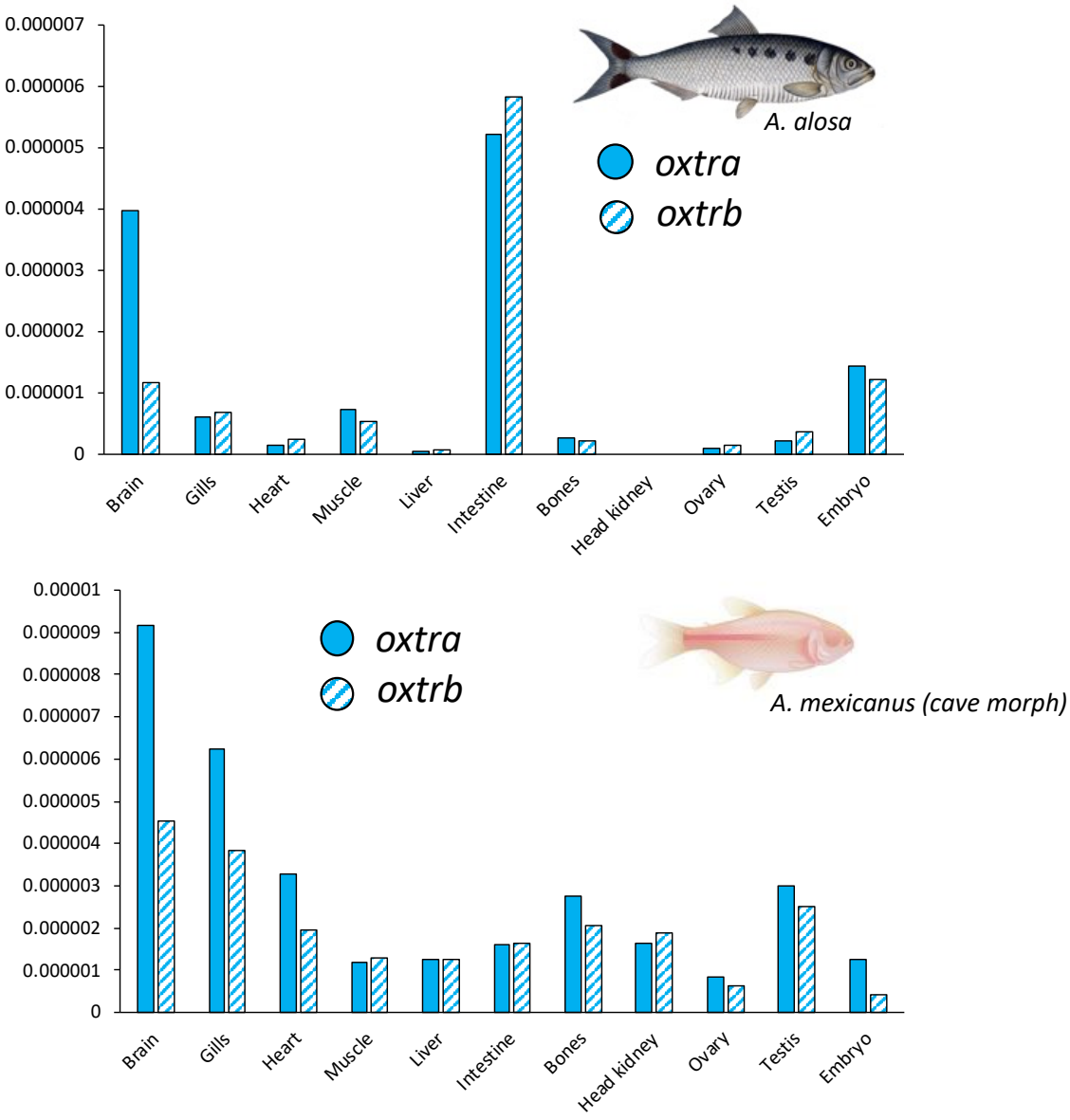
Supplemental Figure 1.2. *Microsynteny analysis (A), predicted AA sequence alignment (B) and RNA-seq based tissue expression profiles (C) of oxtra and oxtrb paralogues in select teleost fishes. Micro-synteny analysis of oxtr paralogue gene loci was manually conducted on NCBI (www.pubmed.com) derived 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 (www. http://phylofish.sigenae.org/index.html) queried on June 1st, 2022.*



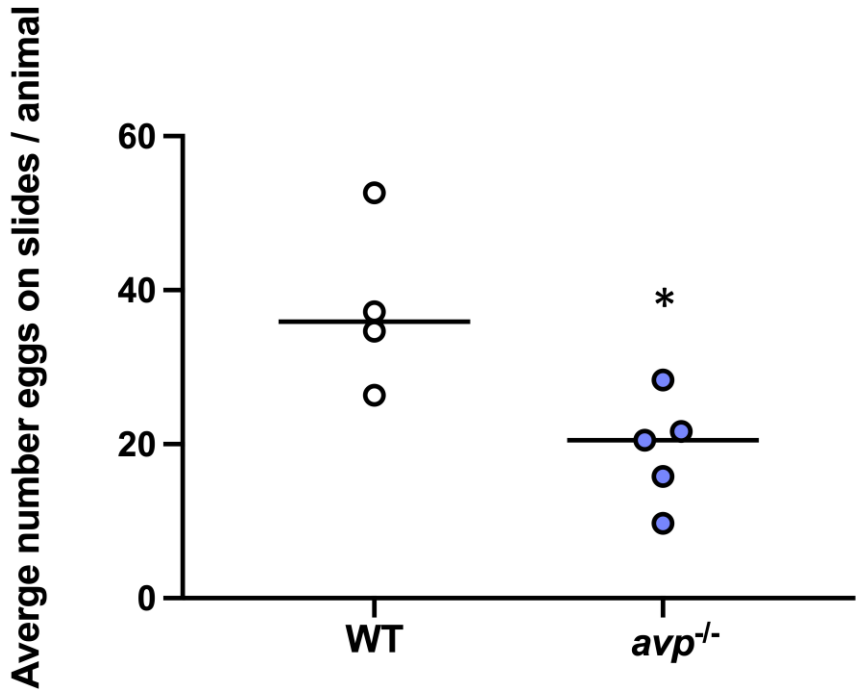
B


Intracellular sequence important for $G_{q/11}$-mediated Ca^{2+} signaling
D. clupeoides
C. chanos
D. rerio
C. carpio
C. auratus
A. mexicanus
E. electricus
I. punctatus
Clupeiformes
Gonorhynchiformes
Cypriniformes
Cypriniformes
Cypriniformes
Characiformes
Gymnotiformes
Siluriformes
Intracellular Otrb AA sequence difference present in Otocephala but not Euteleosti

C


0.000007
0.000006
0.000005
0.000004
0.000003
0.000002
0.000001
0
Brain
Gills
Heart
Muscle
Liver
Intestine
Bones
Head kidney
Ovary
Testis
Embryo
A. alosa
oxtra
oxtrb
0.00001
0.000009
0.000008
0.000007
0.000006
0.000005
0.000004
0.000003
0.000002
0.000001
0
Brain
Gills
Heart
Muscle
Liver
Intestine
Bones
Head kidney
Ovary
Testis
Embryo
oxtra
oxtrb
A. mexicanus (cave morph)

Supplemental Figure 2.1 Average number of ovarian oocytes irrespective of stage per WT and *avp* $^{-/-}$ fish (n=4 per group).



Supplemental Figure 2.2 *In silico* analysis of expression of *avp* and detected receptors (*avpr1ab*, *avpr2ab*) as well as *nanos2* in all ovarian cell types and differentiation stages (top row) and differentiating GSCs (bottom row) as described by single cell sequencing of 40 dpf female zebrafish ovaries (Liu et al., 2022). The database (https://singlecell.broadinstitute.org) was accessed on Sep 5th, 2022. The following manual annotations were added: GSC – germinal stem cells; EOO- early oogenesis; THE – thecal cells; FOL – follicles; STR -stroma cells; VAS -vasculature; IMM – immune cells; MEI- meiotic division stage