Alternate signalling pathways in the endocrine control of reproduction in zebrafish (*Danio rerio*).

Tesis entregada a la

UNIVERSIDAD DE VALPARAISO

En Cumplimento de los requisitos para optar al grado de

Doctor en Ciencias con Mención en Neurociencia

Facultad de Ciencias

Por

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Diciembre, 2024

Tesis dirigida por

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FACULTAD DE CIENCIAS

UNIVERSIDAD DE VALPARAÍSO

INFORME DE APROBACION DE TESIS DE DOCTORADO

Se informa a la Facultad de Ciencias que la Tesis de Doctorado presentada por:

Eugene Mbar TINE

Ha sido aprobada por la comisión de Evaluación de la tesis como requisito para optar al grado de Doctor en Ciencias con mención en Neurociencia, en el examen de Defensa de Tesis rendido el 25 de noviembre 2024.

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FUNDING

Fondo Nacional de Desarrollo Científico y Tecnológico (FONDECYT) 1160076 (**KEW**), 1221270 (**JE**), 11201118 **(RF**)

Centro Interdisciplinario de Neurociencia de Valparaíso (CINV) ICM ANID ICN09-022, CINV (**KEW**, **JE**, **EMT**)

Fondo de Fomento al Desarrollo Científico y Tecnológico (FONDEF) ID23ID10264 (**RF**)

Doctoral Fellowships FIB-UV Universidad de Valparaíso (**EMT**), ANID 21221268 (**IP-B**)

A mes mères Joséphine Ndew Diop et Brigitte Tine, à toute la Famille de Sang Olou et

à mon homonyme Pierre Eugène Faye

ACKNOWLEDGMENTS

First, I'd like to thank my supervisor Dr. Kathleen Whitlock for first welcoming me into your laboratory and making me feel at home. Thank you for your support, which has been fundamental, and the confidence you have shown in me throughout the whole thesis process, from brain-storming the thesis project during the pandemic to writing what I can call a work of art. And thank you for opening the doors of your house to me, with the barbecues, the celebrations, and for making the laboratory a convivial place to work.

I can't fail to thank Dr. John Ewer, who not only chaired my thesis committee, but also provided invaluable help and support throughout the process, especially in the genomics section.

Thanks to Dr. Luis Ricardo Fuentes for welcoming me in his laboratory for 6 weeks and teaching me about the many facets of fish gonadal development, and for your constructive criticism. To the lab members for welcoming me especially Ruth, Constanza and Indrig, but also Dr. Lorena Armijo.

To Dr. Nicola Illing for your support and constructive criticism, and for always bringing out the best in me.

Also, thanks to Dr. Ana Maria Cardenas, director of the Neuroscience PhD program, for her support.

To Trinidad Ordenes and Stephanie Alanis for taking care of the fish and keeping them happy, for their support and friendship.

Thanks to my former and current lab-mates, Maria Fernanda, Ricardo, Cristian, Valeria, Liliana, Angelina, Justin, Catalina, Javier, Maia, Constanza, Ana-Luiza, Alvaro and Paulina for your friendship, and support, and the good times spent together. Not forgetting my friends Benjamin Thiao, Victor Simon, Jorge Torres, Celine Lavergne Joaquin Araya, DNUV-Class, 2019, and Jorge Munoz for their support during this process.

Finally, I'd like to thank all my Family, who have been and always be my rock and carry me in their hearts “*Thiorkat*”. Legacy. Marc 12:30-31

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ABBREVIATIONS

Cck: cholecystokinin

CG: Cortical granules

CNS: Central Nervous System

CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats

Div: Diencephalic Ventricle

DNA: Deoxyribonucleic acid

Dpf: Day post fertilization

Fsh: Follicle stimulating hormone (protein)

Fshb: Follicle stimulating hormone beta (gene and messenger RNA)

GFP: Green Fluorescent Protein

Gnrh: Gonadotropin Releasing Hormone

Gnrh1: Gonadotropin Releasing Hormone 1

Gnrh2: Gonadotropin Releasing Hormone 2

Gnrh3: Gonadotropin Releasing Hormone 3

Gnrh-Rs: Gonadotropin Releasing Hormone Receptors

Gnrh-R1: Gonadotropin Releasing Hormone Receptor 1

Gnrh-R2: Gonadotropin Releasing Hormone Receptor 2

Gnrh-R3: Gonadotropin Releasing Hormone Receptor 3

Gnrh-R4: Gonadotropin Releasing Hormone Receptor 4

GPCR: G-Protein Coupled-Receptor

Gpr173: G-Protein Coupled-Receptor 173

Hpf: Hour post fertilization

HPG: Hypothalamic-Pituitary- Gonadal axis

Kiss: Kisspeptin

Lh: Luteinising hormone (protein)

Lhb: Luteinising hormone beta (gene and messenger RNA)

MITRAC: Mitochondrial translation regulation assembly intermediate of cytochrome c oxidase complex

mRNA: messenger Ribonucleic acid

OB: Olfactory bulb

OE: Olfactory Epithelia o Olfactory Epithelium

OO: Olfactory organ

PCR: Polymerase Chain Reaction

Pit: Pituitary

PM: Magnocellular pre-optic area

Pnx: Phoenixin

Pnx-20: Phoenixin 20

POA: Pre-optic area

Pomc: Pro-opiomelanocortin

Ppp: Parvocellular pre-optic area

qPCR: Quantitative Polymerase Chain Reaction

RF: Reticular formation or Raphe

RNA: Ribonucleic acid

RTqPCR: Reverse Transcription Quantitative Polymerase Chain Reaction

Smim20: Small Integral Membrane Protein 20

Sreb3: Super-conserved Receptor Expressed in the Brain 3

St1: Oogonia

St2: Early follicles stages

St3: Cortical alveolus stages

St4: Early vitellogenic stages

St5: Late vitellogenic stages

StA: Spermatogonia

StB: Leptotene stage

StC: Primary spermatocytes

StD: Secondary spermatocytes

StE: Spermatozoa

TALEN: Transcription activator-like effector nucleases

Tub-ac: Tubulin acetylated

WT: Wild-type

YV: Yolk vesicle

zPnx-20: zebrafish Phoenixin 20

SUMMARY

Gonadotropin-releasing hormone (Gnrh) is a neuroendocrine decapeptide highly conserved across vertebrate species that is essential not only for the onset of puberty but also for the maintenance of reproductive state. Results from recent studies indicate that endogenous Gnrh does not control reproduction in zebrafish and potentially other fishes. Because fishes use waterborne hormones to control reproduction, we tested whether exogenous Gnrh affects the hypothalamic- pituitary axis. Gnrh introduced into water housing individual fish isolated from their conspecifics induced sex-specific pituitary responses on gonadotropins expression. Furthermore, blocking the noses eliminated these responses. To determine whether modulation of the exogenous Gnrh signalling pathway is controlled at the receptor level, a CRISPR/Cas9 loss of function mutant in the *gonadotropin-releasing hormone receptor 3* (*gnrh-receptor3)* gene was generated. The *gnrh-r3*$^{-/-}$ mutants are infertile where males do not produce sperm, and females produce few mature oocytes. The data presented here suggest that zebrafish have a potential olfactory–portal system that underlies neuroendocrine communication controlling reproduction.

CHAPTER I: INTRODUCTION

Gonadotropin Releasing Hormones: The master control peptide

Reproduction is essential for the survival of organisms, where animals use different strategies to optimize and secure the success of their reproductive behavior and physiology to perpetuate their species. In vertebrates, the endocrine regulation of reproduction, that coordinates the physiological response in different organs, is through the release of gonadotropin-releasing hormone (Gnrh) from the central nervous system (CNS) (Campbell & Jialal 2019). In vertebrates, Gnrh is the principal neuroendocrine peptide controlling the release of gonadotropes from the pituitary. In mammals, this decapeptide hormone is secreted by neurons of the pre-optic area into the vasculature of the hypothalamic-hypophyseal portal system (Wray & Hoffman, 1986), where it stimulates the synthesis and release of gonadotropes luteinizing hormone (Lh) and follicle-stimulating hormone (Fsh) (Thompson et al., 2016; Stamatiades & Kaiser, 2018) from the anterior pituitary. In females, the release of Lh and Fsh plays a crucial role in regulating the ovarian cycle. Specifically, it stimulates the growth and development of ovarian follicles, ultimately leading to ovulation (Raju et al., 2013). In males, the actions of Lh and Fsh are vital for the process of spermatogenesis. Lh acts on the Leydig cells within the testes, stimulating the production of testosterone, which is essential for the development and maturation of sperm cells (Sansone et al., 2019). Thus, the pulsatile secretion of Gnrh and its subsequent effects on the release of Lh and Fsh from the pituitary gland play a central role in regulating reproductive processes in both females and males, ensuring the proper functioning of the reproductive system.

Like mammals, the release of Lh and Fsh from the pituitary gland in response to Gnrh secretion plays a crucial role in regulating various aspects of reproduction in teleost fishes, including gonadal development, gametogenesis and spawning (Hellqvist

et al., 2006; Zhang et al., 2015). In contrast to mammals, in fishes Gnrh is released through direct synaptic connections (Peter 1982; Zohar et al., 2010) where it activates the release of Lh and Fsh from different cells types within the pituitary (Parhar et al., 2003; Golan et al., 2016; Hollander-Cohen et al., 2021). Unlike mammals, who in general have to isoforms of Gnrh peptide (Gnrh1 and Gnrh2), teleosts have three isoforms of Gnrh coded for by distinct genes *gnrh1*, *gnrh2* and *gnrh3* where the Gnrh3 peptide is a fish specific hormone (Sherwood et al., 1983; Powell et al., 1994; Fernald & White, 1999; Okubo & Nagahama 2008; Tostivint 2011; Reyes-Tomassini 2013; Sukhan et al., 2013). The neurons expressing the Gnrh peptides are found in different locations in the brain and have varying functions (White and Fernald 1998; Gomes et al., 2013). In fishes neurons in the preoptic area that regulate gonadal maturation through stimulation of gonadotropin cells in the pituitary contain either Gnrh1 or Gnrh3 (Kim et al., 1995; Amano et al., 1998). In contrast, Gnrh2 the most highly conserved of the Gnrh peptides (Millar R.P. 2003; Temple et al., 2003), is found in neurons located in the midbrain and modulate sexual and feeding behavior (Kauffman & Rissman 2004). The Gnrh3 peptide, is found in neurons of the terminal nerve, and ventral forebrain, and has neuromodulatory functions (Amano et al., 1997).

Gonadotropin Releasing Hormones and zebrafish reproduction

In zebrafish (*Danio rerio*) it has been shown that the gene encoding Gnrh1 is not present in the genome (Whitlock et al., 2019) and Gnrh3 was thought to control reproduction (Karigo & Oka, 2013). Other fishes such as Goldfish (*Carassius auratus*) lack *gnrh1* but use Gnrh2 or Gnrh3 for reproduction (Marchant et al, 1989), and in salmonids, lack of *gnrh1* is compensated for duplications of *grnh3* (*gnrh3A* and *gnrh3B*) (Leder et al., 2004) where Gnrh3A is used to control reproduction (Ando & Urano 2005).

In zebrafish, *gnrh3*, is expressed primarily in cell bodies of the terminal nerve with limited expression in the ventral telencephalon (Gopinath et al., 2004; Whitlock K.E. 2005) but is not present in the hypothalamus (Whitlock et al., 2003; Whitlock et al., 2005). Most recently, the loss of function for *gnrh2* and *gnrh3* by TALEN-based knock-out techniques (Spicer et al., 2016; Marvel et al., 2018) as well as *kisspeptin 1* and *kisspeptin 2*, two upstream regulators of Gnrh neurons activation and Gnrh secretion (Liu et al., 2017), does not affect reproductive capacities of the mutant animals. Additionally, an analysis by Mass spectrometry of adult zebrafish hypothalamus done by our laboratory suggests that Gnrh2 and Gnrh3 peptides are not present in the zebrafish hypothalamus (Whitlock et al., 2019). These results taken together suggest the zebrafish do not use endogenous Gnrh to control reproduction.

Because the Gnrh peptides are part of an extremely conserved signalling pathway controlling reproduction, it is very unusual that zebrafish lack one form (*gnrh1*) and knock-out of the other two forms (*gnrh2* and *gnrh3*) does not result in defects in reproduction. Different possibilities can explain the finding that Gnrh peptides do not control reproduction in zebrafish: One possibility is that a) Zebrafish have co-opted a different peptide into the reproductive pathway; b) Reproduction in zebrafish is controlled by exogenous Gnrh released into the water by conspecific permitting the maintenance of reproductive capacity.

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CHAPTER II: THE PREPROHORMONE PHOENIXIN (PNX-20) CONTROLS REPRODUCTION IN ZEBRAFISH

I. INTRODUCTION

Our lab has proposed that the preprohormone Phoenixin (Pnx) is a potential candidate replacing the function of Gnrh1 in the brain-pituitary-gonad axis of zebrafish (Whitlock et al., 2019). Phoenixin was discovered by bioinformatics algorithm that used information from the Human Genome Project to predict unidentified, secreted and highly conserved peptide hormones. The algorithm eliminated proteins that contain transmembrane domain but included potential peptides that have signal peptide and potential peptides sequences that contain dibasic cleavage sites flanking a mature region (Yosten et al., 2013). Pnx is derived from a posttranslational proteolytic cleavage of the precursor Small Integral Membrane Protein 20 (Smim20) prohormone (Yosten et al., 2013; Palasz et al., 2018; Suszka-Switek et al., 2019). The Smim20 protein was also described as a part of the MITRAC (Mitochondrial translation regulation assembly intermediate of cytochrome c oxidase complex), which regulates cytochrome C oxidase assembly (Dennerlein et al., 2015). The Smim20/Pnx preprohormone has five predicted cleavage products where the amidated 14 amino-acid (Pnx-14) and 20 amino-acid (Pnx-20) products have been confirmed to be biological active (Yosten et al., 2013). In mammals, Pnx-14 and Pnx-20 are present in different organs of the reproductive axis as well as in a limited neural population in the rat hypothalamus (Yosten et al., 2013), and ovary (Kalamon et al., 2020). The full-length peptide is highly conserved across vertebrates including the zebrafish (Yosten et al., 2013) and was believed to be the ligand of the orphan metabotropic receptor Gpr173 or Super-conserved Receptor Expressed in the Brain 3 (Sreb3) (Treen et al., 2016), with an important role in the brain-pituitary-gonadal axis (Matsumoto et al., 2005). More recently studies have questioned whether Gpr173 is the recepor for Pnx based on phyletic patterns (Yanez-

Guerra et al., 2022) and negative pharmacological assays (He et al., 2023). Thus, the initial studies on Pnx signalling (Treen et al., 2016; Stein et al., 2016; Nguyen et al., 2019) showing a diminished effect of Pnx after a knockdown of *gpr173* mRNA may be an indirect signalling relation rather than a direct Gpr173/Pnx coupling.

In vitro experiments showed that Pnx-20 has an important role in the regulation of the reproduction by acting on the hypothalamic-pituitary axis to potentiate the expression of pituitary gonadotropes by increasing the expression of *gnrh-receptor* in rat (Yosten et al., 2013). Pnx-20 also stimulates follicular cell development by accelerating the proliferation of human granulosa cells and inducing Estradiol secretion (Nguyen et al., 2019). In teleost, in vitro and in vivo experiments showed that treatment with the human Pnx-20 significantly increased the expression of *gnrh-receptor*, *lhb* and *fshb* mRNA(s) in the pituitary of the spotted scat fish (Wang et al., 2019). Previous studies have shown Pnx-like expression in the gonads of zebrafish (Rajeswari & Unniappan 2020), with intraperitoneal injection of zebrafish Pnx-20 (zPnx-20) increasing *kiss* and *kiss*-receptor in the hypothalamus, *lhb*-receptor in the testis and *fshb*-receptor in the ovary of zebrafish (Rajeswari & Unniappan 2020). *In vitro* treatment of zPnx-20 in zebrafish liver cell line, increased the expression of *vitellogenin* and *estrogen-receptor* (Rajeswari & Unniappan 2020). Our laboratory showed that pnx is expressed in the hypothalamus of the adult zebrafish by *in situ* hybridization and confirmed low level of expression in larvae by RT-PCR (Ceriani et al., 2021). Pnx-like labeling of the hypothalamus was also confirmed using the available Pnx antibody directed against the human Pnx-14 and Pnx-20 peptides (Ceriani et al., 2021).

Because of the potential role of Pnx in the Gnrh signalling pathway, we proposed that zebrafish may have co-opted Pnx to replace Gnrh function in reproduction

(Whitlock et al., 2019). To confirm Pnx labeling in zebrafish, we characterized the expression of Pnx during development using an antibody directed against the zebrafish Pnx-20 peptide. While no labeling was detected in the larval stages, anti-zPnx-20 labeling was detected starting at the onset of puberty in the preoptic area of the hypothalamus and pituitary where it was maintained throughout adulthood. To test whether zPnx-20 could initiate a hypothalamic response, adult animals were injected intraperitoneally with zPnx-20. These animals showed significant increases in *lhb* mRNA in both females and males, and *fshb* mRNA only in females as judged by RTqPCR. Furthermore, using CRISPR/Cas9 we generated mutations in *phoenixin* to determine whether the loss of function of *phoenixin/smim20* affected reproduction and fertility. One mutation, the CRISPR/Cas9 mutant $pnx^{uv\Delta 10/uv\Delta 10}$, appeared to significantly reduce the ability of $pnx^{uv\Delta 10/uv\Delta 10}$ males to stimulate egg spawning in comparison to wild-type or heterozygote siblings. Strikingly, we did not isolate $pnx^{uv\Delta 10/uv\Delta 10}$ adult females suggesting that zPnx-20 is necessary for female fate in zebrafish

II. **MATERIALS AND METHODS**

Animals

Zebrafish were maintained in a recirculating system (Aquatic Habitats Inc., Apopka, FL) at 28°C on a light-dark cycle of 14 and 10 hours respectively. All fish were maintained in the Whitlock Fish Facility at the Universidad de Valparaiso. Wild-type fish of Cornell strain (derived from Oregon AB) and Valparaiso strain Wild-type from Whitlock laboratory were used to create all the mutant lines. All protocols and procedures employed were reviewed by the Institutional Committee of Bioethics for Research with Experimental Animals, University of Valparaiso (#BA084-2016). Bioethical Approval EMT (#CBC492022; University of Valparaiso). Embryos were obtained from natural spawning in laboratory conditions and raised at 28.5°C in Embryo medium as described (Westerfield, 2007). Staging of pubertal fish was done according to Singleman & Holtzman, 2014. The reporter line *Tg:(pomc:gfp*) (Liu et al, 2003) was used to visualize specific pituitary cell types. All animals are feed twice a day with artemia and dry food.

Zebrafish Pnx-20 antibody production

A polyclonal zebrafish Pnx-20 antibody was produced for the Whitlock Laboratory by Pacific Immunology (USA) https://www.pacificimmunology.com/, using the specific amino-acid sequence of zebrafish Pnx-20 peptide (AGVNQADIQPVGVKVWSDPYKPKS) as an immunogen and hosted in Rabbit.

Immunohistochemistry

Cryo-sectioning

Fish were selected from development stages were sacrificed and the heads or gonads collected and fixed in 4**%** paraformaldehyde for 24 hours at 4°C. The heads were then decalcified in 0.2 Molar ethylene-diamine-tetra-acetic acid (EDTA) solution pH 7.3 for 2 days at for 4°C. Tissues were then embedded in 5% sucrose/1.5% agarose in MilliQ H20. Blocks were submerged in 30% sucrose overnight, covered by O.C.T Compound (Tissue Tek®) and frozen in cryomolds at -20°C for at least 2 days prior the cryo-sectioning. Thirty micro-meter cryo-sections were processed for immunohistochemistry.

Antibodies

Cryosections were incubated with rabbit anti-zPnx-20 specific for zPnx-20 peptide (1:500, generated by Pacific Immunology, USA) or rabbit anti-Sreb3-N-terminal (Gpr173) (1:250, Abcam ab188765) and mouse anti-GFP (1:500, Invitrogen A-11120) or mouse anti-acetylated tubulin (1:500, Sigma T6793) primary antibodies overnight at 4°C and processed using VECTASTAIN elite ABC kit (Rabbit IgG, VECTOR; PK-6101) and DAB Peroxidase (HRP) Substrate (VECTOR; SK-4105), following manufacturer instructions, or Alexa Fluor 568 conjugated anti–rabbit antibody (goat 1:500, Molecular Probes) and Dylight 488–conjugated anti–mouse antibody (goat 1:500, Jackson Immuno Research) (Supplemental Table 2).

Microscopy and image processing

Bright field images were obtained using Leica DMR microscope (Leica Microsystems CMS GmbH, Wentzler, Germany) and a Leica DFC 480 camera (Leica Microsystems Ltd, Heerbrugg, Switzerland) and processed using the Leica Application Suite 2.3.3 software (Leica Microscope Ltd) Fluorescent images were acquired using a Spinning Disc microscope Olympus BX-DSU (Olympus Corporation, Shinjuku-ku, Tokyo, Japan) with ORCA IR2 Hamamatsu camera (Hamamatsu Photonics, Higashi-ku, Hamamatsu City, Japan) and Olympus CellR software (Olympus Soft Imaging Solutions, Munich, Germany). Images were deconvoluted in AutoQuantX 2.2.2 (Media Cybernetics, Bethesda, MD, USA) and processed using FIJI (National Institutes of Health, Bethesda, Maryland, USA; (Schindelin et al., 2012).

Intraperitoneal injection of zebrafish Pnx-20 peptide

Two weeks prior to the experiment, adult male and adult female zebrafish were separated and grouped (n=3/group; age and weight-matched). On the day of the experiment, fish were anesthetized with tricaine (400mg/100ml) (A5040 Sigma-Aldrich), immobilized with a sponge, and injected intraperitoneally using a #70 Hamilton syringe. 10 µL per gram of fish were injected intraperitoneally (Kinkel et al., 2010) with the peptide or Saline according to the assigned group: 100 ng/g body weight of custom synthesized zebrafish Phoenixin (zPnx-20), Pacific Immunology, Cat# 1711-PAC 21; ≥95% pure) diluted in saline. Controls were injected with saline (0.9% sodium chloride). Two 2 hours after the injections, the fish were sacrificed and tissues (pituitaries) were collected and stored at -80°C in TriZol Reagent (Ambion, Life Technologies) until further analysis.

Total RNA isolation and reverse transcription

The pituitaries and hypothalamus of each group were combined, and total RNA was extracted using TriZol Reagent (Ambion, Life Technologies) according to previous published protocol (Peterson & Freeman, 2009; Calfún et al., 2016). Briefly, the tissues were isolated from the adult male or female zebrafish under the dissecting microscope and homogenized in 1mL TriZol-Reagent. 200µL of Chloroform (Sigma-Aldrich) was added in each tube to a total volume of 1200µL per tube and the mixture was centrifuged at 4°C for 15 minutes at 12000 ×g in a centrifuge (Servall legend RT). After centrifugation, the aqueous layer (± 500µL) was transferred to a new tube and mixed with 500µL of 2-propanol (Sigma, Life Technologies) and centrifuged to precipitate the RNA. RNA was then washed with 75% ethanol 25% DEPC treated water. Total RNA was re-suspended with DEPC treated water and treated with DNAse I Amplification grade (Invitrogen), then quantified by the Qubit HS RNA Assay Kit (Invitrogen, ThermoFisher Scientific) using a Qubit 3.0 Fluorometer (Invitrogen, Life Technologies).

To synthesize cDNA from total RNA, reverse transcription was performed at 42°C for 1 hour in a reaction of 20µL containing x µL of total RNA at the initial concentration, 1µL of 10mM dNTP mix, 1µL of 0.5 µg of Oligo(dT) (Invitrogen), 50 U of SuperScript II reverse transcriptase (Invitrogen) and RNAse Out (Invitrogen). The cDNA was then used for qPCR.

Quantitative PCR of *lhb* and *fshb* mRNAs expression

To examine the mRNA expression level in the pituitaries genes *lhb* and *fshb*, a qPCR was performed using M×3000P thermocycler (Stratagene). A reaction mixture of 2µL of (cDNA), 0.75µL of each primer, and 12.5 µL of SYBR Green/ROX qPCR Master

Mix (Molecular Probes) in a final volume of 25µL. Relative quantification was done using the Ct-values and *beta actin-1* as housekeeping gene (**Supplemnetal Table 1**), and no RT or no template controls for each gene. The reaction profile consisted one cycle of 95°C for 10 minutes, then 40 cycles of 95°C for 20 sec, and 59°C for 20 sec, and 72°C for 20sec for signal detection. The data were analysed using the thermocycler software (MxPro-Mx3000P v4.10) and Prism8.

All gene expression values were normalized to housekeeping gene, *beta-actin* (Calfún et al., 2016). Relative expressions were calculated using the $2^{-\Delta Ct}$ method relative to wild-type levels. For statistical analysis, Student *t*-test comparing the average expression of each gene between experimental and wild-type groups was conducted and statistically significantly differentially expressed genes were identified as those with a *P*-value ≤ 0.05.

Generation of *pnx* mutant fish

CRISPR/Cas9 design

All gRNAs were designed were designed using CHOPCHOP (https://chopchop.cbu.uib.no/) and Integrated DNA Technologies (https://www.idtdna.com/site/order/designtool/index/CRISPR_CUSTOM). Specific sgRNAs and Constant oligonucleotide were purchased from Macrogen Inc (South Korea). Cas9 (TrueCut Cas9 Protein v2 5µg/µL #A36499) was purchased from ThermoFisher. To generate the sgRNAs we used approach from Gagnon et al., 2014. For each sgRNA, 60 base pair oligonucleotides (gene-specific oligo) were ordered containing a promoter for in vitro transcription (T7 promoter), the 20-base spacer region specific to the target gene and an overlap region that anneals to the constant

oligonucleotide. If the sgRNA did not start with 2 GG, theses bases are added to enhance the transcription reaction of the T7 polymerase. The gene-specific oligo using T7 has the architecture TAATACGACTCACTATA-N20-GTTTTAGAGCTAGAAATAGCAAG, with Ns replaced by the 20-base specific to the target and the constant-oligo: AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTA ACTTGCTATTTCTAGCTCTAAAAC. gRNAs and constant-oligos were suspended in 100µM stock solution. To anneal the sgRNA and the Constant-oligo, 1µL of each stock solution in a total volume of 10µL was used and incubated under these conditions 5 minutes at 95°C, 95°C ➔85°C for -2°C/second, then 85°C ➔ 25°C for -0.1°C/second and hold at 4°C. Finally, 10µL of solution containing 0,5µL of T4 NEB DNA polymerase, 2,5µL of dNTPs (10mM), 2µL of 10X NEB Buffer2, 0,2µL of 100X NEB BSA and 4.8µL of water was added and incubated at 12°C for 20 minutes to fill-in. The template was purified using PCR clean-up column (Nalgen) and verified by electrophoresis. *In vitro* transcription of the dsDNA was done overnight using 3µL of the purified template with T7 transcription Kit (New England Biolabs E2040S), the gRNAs were then purified using RNAeasy mini Kit (Qiagen 74104).

CRISPR/Cas9 injection and generation of mutants

To generate mutants, a 2nL mixture composed of 50ng/µL of each sgRNAs for *pnx* and 1µL of Cas9 protein (5µg/µL) and 1µL of phenol-red was injected into the yolk of 1 or 2 cells stage embryos. The mixture was heated at 37°C for 5 minutes prior to the injection to allow the formation of the sgRNA-Cas9 complex. The sgRNAs target sites for *pnx* (ensemble gene ID: ENSDARG00000112670) in exon 1 were 5'-GCTCACTCCACTTTGACAGT-3', 5'-AAGAGGATAACGCTCATATT-3', 5'-

ATATTTTTTCATCCTCTCAC-3' and exon 2 was 5'-GCAAGTGCAGAAGGTGAACC-3' (**Supplemental Table 3**). Founders were identified by PCR and out crossed to wild-type (F1), then in-crossed to generate heterozygote and homozygote F2. For each target site, specific primers were designed to genotype the mutations. *pnx* mutant lines were genotyped with primers Forward 5'-ACCCCGGAACAACTTGAGTG-3', reverse 5'-AAGTTTAGGCTGTGTCACGGT-3' for exon 1 and Forward 5'-CAACTGGGCCATTAGAAATCA-3', Reverse 5'-TACCAACAGGCTGTATGTCTGC-3' for exon 2 (**Supplemental Table 4**).

Characterization of reproductive capacities

Characterization of the reproductive capacities was carried-out by crossing fish. Briefly, sexually mature wild-type and mutant fish of the same age were selected for pair wise crossing. Pairs of wild-type males and females, mutant males and females, and combinations of one wild-type male paired with one mutant female, and one wild-type female paired with one mutant male were analyzed. In the morning, immediately after lights on, dividers were removed, and fish allowed to spawn for 1.50 h before eggs were collected from each container. The total number of eggs was counted for each pair to obtain fecundity numbers, and the percentage of eggs fertilized was quantified via embryonic development at 6 hours post fertilization (hpf). The experiment was repeated four times.

Gonadal morphology

Adult zebrafish (10 to 12 months), were sacrificed using a high dose of Tricaine (400mg/100ml) (#A5040 Sigma-Aldrich) (Wilson et al., 2009) and gonads were collected in Ringer (zebrafish book, Westerfield, 2007) for photography using Moticam

4000 True 4K Imaging (Motic) connected to a Leica MZ12.5 dissecting microscope (Leica Microsystems CMS GmbH, Wentzler, Germany).

III. RESULTS

zPnx-20 protein is expressed in zebrafish hypothalamus starting at the onset of puberty

Previously our laboratory has shown that Pnx-20 is expressed in the preoptic area of the adult zebrafish using the commercially available Pnx antibody (Ceriani et al., 2021). Using zebrafish specific antibody zPnx-20 (anti-Pnx20-zebrafish PAC: 15947/15948 Pacific Immunology USA, from Whitlock Laboratory), the expression pattern of zPnx-20 was examined previous to, during and after puberty, using the description of transitional phenotypes relative to zebrafish maturation (Singleman and Holtzman 2014). Antibody labeling using anti-zPnx-20 was analyzed in *Tg(pomc:gfp)* fish as a marker for corticotrophs cells (including Pomc cells and Acth cells) in the pituitary in zebrafish (Liu et al., 2003). The *Tg(pomc:gfp)* reporter was used to confirm pituitary structures in the adult sectioned tissues. Using the anti-zPnx-20 antibody no labeling was detected in the pituitary of the zebrafish at 15 dpf, 21dpf and 35dpf (**Supplemental Figure 1**). Labeling was first detected in the hypothalamic pre-optic area (POA) and the pituitary of the zebrafish at 47dpf in animals that have body size 14 to 17mm and color pattern corresponding to the onset of puberty in zebrafish (Chen and Ge 2013, Singleman and Holtzman 2014). zPnx-20 20 positive cells are present in the magnocellular preoptic nucleus (PM) and the parvocellular preoptic nucleus (Ppp) adjacent to the diencephalic ventricle (DiV) (**Figure 1 A, A', D and D'**) in agreement with previous work from our lab (Ceriani et al., 2021); additionally, expression was observed in the reticular formation **Figure 1 E"**) as shown previously in rat (Palasz et al., 2015; Prinz et al., 2017). Interestingly, the immuno-labelling in the pituitary is

consistent with what has been previously described and could be axon terminal labeling (Yosten et al., 2013; **Figure 1 B, B', E and E'**).

Using the commercially available mammalian SREB3 antibody we examined expression of Gpr173, the proposed orphan receptor of Pnx-20 (Stein et al., 2016; Palasz et al., 2017; Rajeswari and Unniappan 2020). We observed Sreb3-like labelling in cells in the pituitary of the adult zebrafish (**Supplemental Figure 2**) and the Sreb3-like positive cells do not localize with the GFP positive cells. Because subsequently it was reported that Gpr173/Sreb3 was unlikely be Pnx receptor (Yanez-Guerra et al., 2022) we did not continue with this characterization.

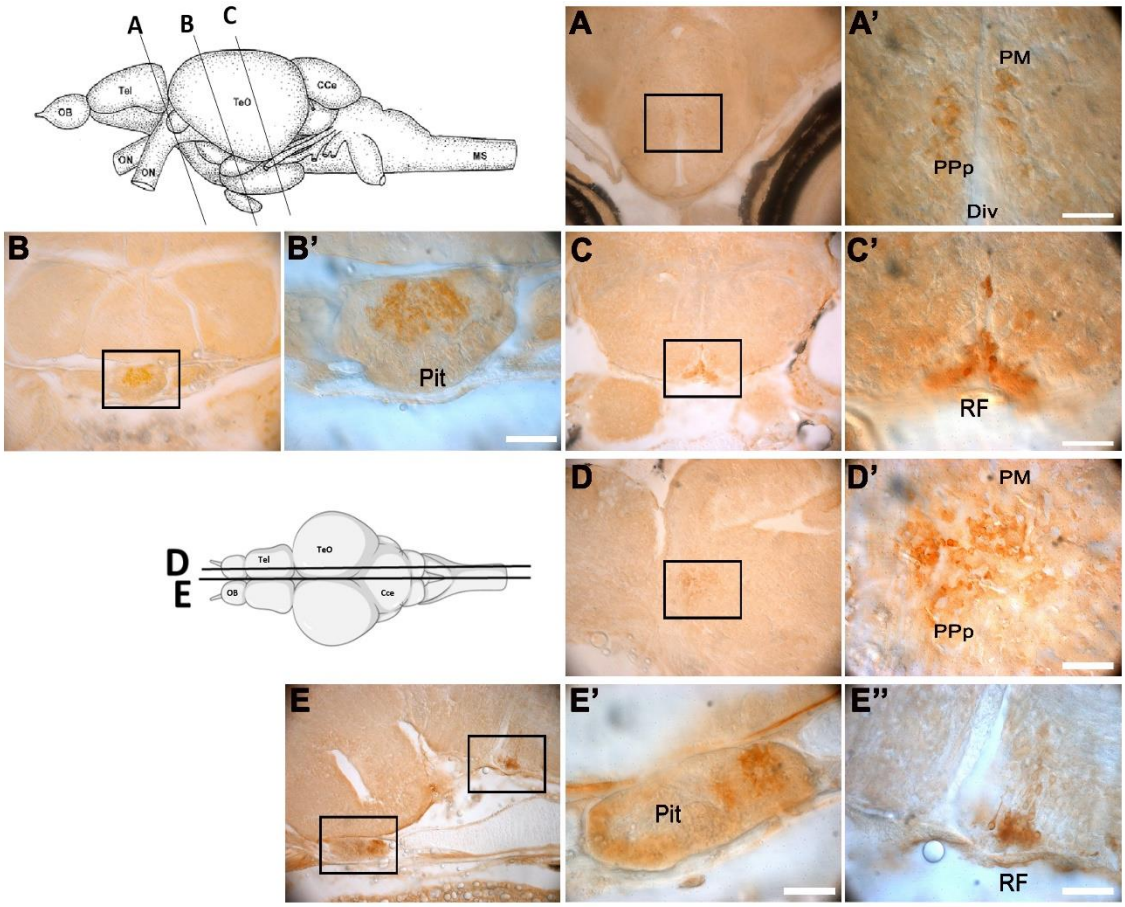

Figure 1: **Expression of zPnx-20 in zebrafish hypothalamus at the onset of puberty** (A–A'): Coronal cryostat section of 47dpf zebrafish (30uM, n=8 fish) with zPnx-20 immunoreactivity in the magnocellular pre-optic nucleus (PM) and the parvocellular pre-optic nucleus (PPp) adjacent to the diencephalic ventricle (DiV). (B–B') in the pituitary (Pit) (C-C') in the Raphe. A-C' are frontal sections. (D-D'): Sagittal cryostat section of 47dpf zebrafish with zPnx-20 immunoreactivity in the magnocellular pre-optic nucleus (PM) and the parvocellular pre-optic nucleus (PPp) adjacent to the diencephalic ventricle (DiV). (E-E'') in the pituitary and the Raphe (RF). (E') higher magnification of boxed in E (pit) and (E'') higher magnification of boxed in E (RF). D-E'' are sagittal sections. Scale-bar 50µM (A', B', C', D', E' and E'')

zPnx-20 peptide affects mRNA expression of pituitary hormones *lhb* and *fshb*

To determine the physiological role of zPnx-20 in the expression of pituitary gonadotropes mRNA (*lhb* and *fshb*), adult females and males were challenged by intraperitoneal injection of zPnx-20. In females, exposure to zPnx-20 resulted in the up-regulation of the expression of gonadotropes *lhb* (**Figure 2 A**) and *fshb* **(Figure 2 C**). The increase was more significant for *lhb* mRNA (P-values *<0.0002)* than for *fshb* mRNA (P-values *<0.05).* In males, exposure to zPnx-20 resulted in significant increases in the expression of *lhb* mRNA (**Figure 2 B**)*;* however, the expression of *fshb* mRNA was significantly decreased (**Figure 2 D**). Our results showing increased *lhb* mRNA and decreased *fshb* mRNA in the pituitary of males are consistent with previously published data showing increased *lh-receptor* and decreased *fshb-receptor* in the testis of zebrafish. In contrast, our results showing increased *lhb* mRNA in the pituitary of females are not consistent with decreased expression of *lhb*-receptor mRNA in ovaries (Rajeswari and Unniappan 2020). This may be because in response to Lh surge, the *lh-receptor* expression in the ovaries is down-regulated and undergoes accelerated degradation (Menon et al., 2007; Menon and Menon 2014).

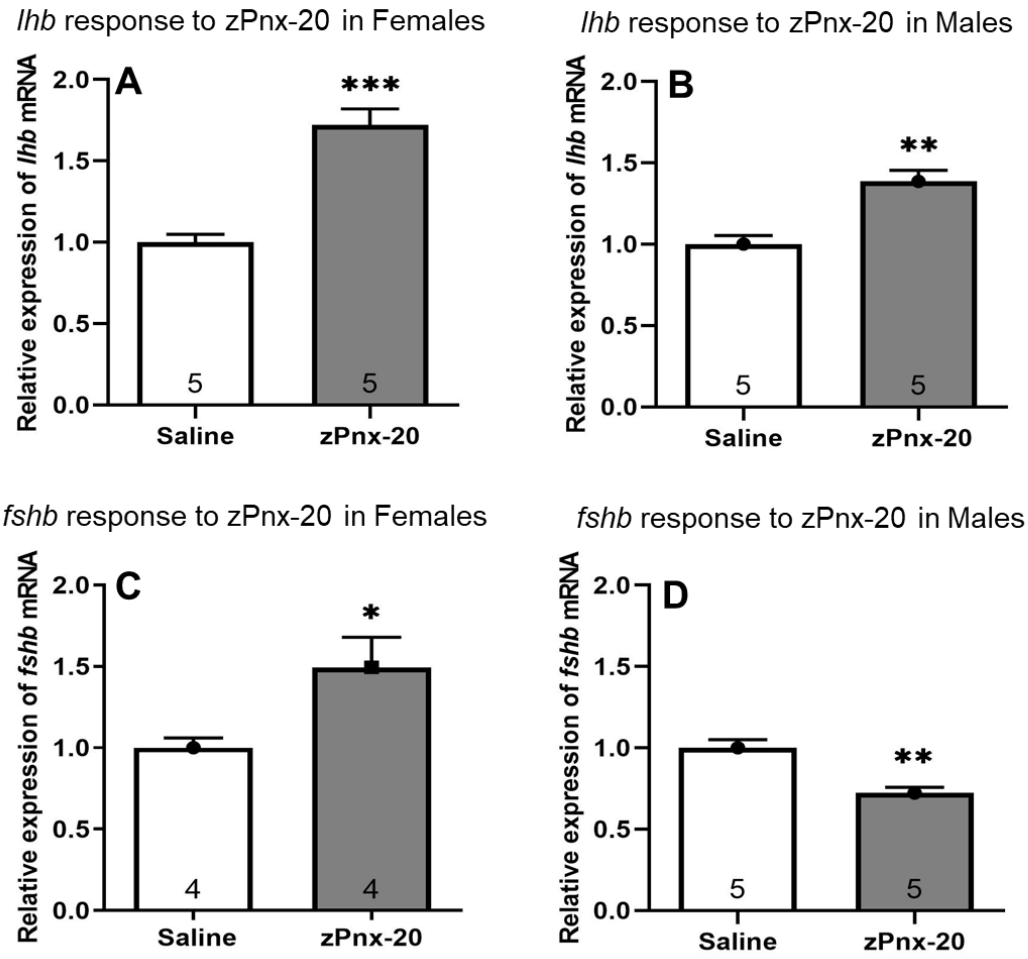

Figure 2: **Pituitary response to zPnx-20.** Relative expression of gonadotropin mRNAs in response to zPnx-20 peptide intraperitoneal injections. In adult females *lhb* mRNA (A) and *fshb* mRNA (B) are significantly up-regulated in response to the zPnx-20 peptide injection. In males only *lhb* mRNA(C) showed a significant increase of expression and *fshb* mRNA (D) is down-regulated in response to the zPnx-20 peptide injection. Housekeeping gene beta-actin. Data*:* expressed in Mean ±SEM with comparison of the control group. Statistics *One-sample t-test* (Prism8). **P< 0.05; **P<0.002; ***. P<0.0002*. N=5. For female *fshb* N=4. (n=1 represent grouped hypothalamus and pituitaries of 3 different fish).

Knockout of *phoenixin* using CRISPR/Cas9

To address the hypothesis that in zebrafish zPnx-20 replaces Gnrh into the control of reproduction, we generated CRISPR/Cas9 mutations in the *pnx* gene by injecting a total of 4 guide RNAs. All single guide RNAs (sgRNAs) were designed using CHOPCHOP (https://chopchop.cbu.uib.no/) and Integrated DNA Technologies (https://www.idtdna.com/site/order/designtool/index/CRISPR_CUSTOM).

Knockout of *phoenixin* in exon 1

The gRNAs target sites for *pnx* (ensemble gene ID: ENSDARG00000112670) in the exon 1 were 5'-GCTCACTCCACTTTGACAGT-3', 5'-AAGAGGATAACGCTCATATT-3', 5'-ATATTTTTTCATCCTCTCAC-3' located into the exon 1 (**Figure 3A, Supplemental Table 3**) were injected all together at one to two cells stages. Founders were identified by PCR and out crossed to wild-type. The F1 were then in-crossed to generate heterozygote and homozygote F2. *Pnx* mutant lines were genotyped using primers forward 5'-ACCCCGGAACAACTTGAGTG-3', reverse 5'-AAGTTTAGGCTGTGTCACGGT-3' for targets located into the exon 1 (**Figure 3A, red arrow, Supplemental Table 4**). The CRISPR/Cas9 based mutagenesis on *pnx* resulted on a 96bp ($pnx^{uv\Delta 96/uv\Delta 96}$) deletion in the exon 1(**Figure 3B**).

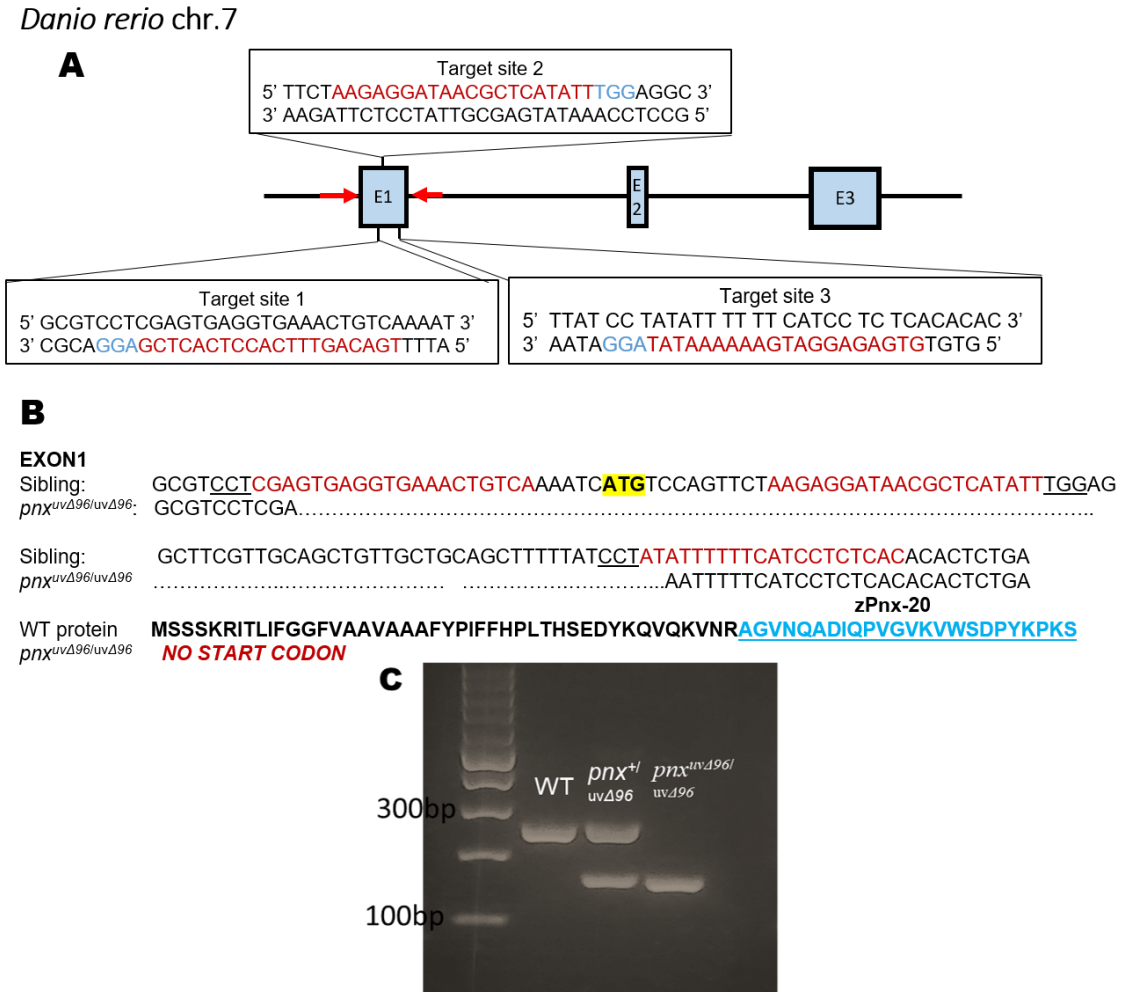

Figure 3: Generation of *pnx*$^{uv\Delta96/\ uv\Delta96}$ mutation in exon 1. (A): Localization of the guides RNAs used to induce mutations in *pnx* by CRISPR and the primers used to screen the injected fish by PCR (red arrows). (B): Comparison of the sequences between a wild-type fish and a *pnx*$^{uv\Delta96/uv\Delta96.}$ (C): Gel electrophoresis of a wild-type fish and a *pnx*$^{+/uv\Delta96}$ showing the difference between the wild-type band and the mutant band.

***pnx*$^{uvΔ96/uvΔ96}$ mutants have an alternative start-codon**

The CRISPR/Cas9 based mutant *pnx*$^{uvΔ96/uvΔ96}$ resulted in a deletion in exon 1 (**Figure 3B**). Analysis of the sequences of the *pnx*$^{uvΔ96/uvΔ96}$ mutants showed that despite the loss of the canonical start-codon ATG, the *pnx*$^{uvΔ96/uvΔ96}$ mutation has an alternate start-codon ATT allowing for the translation of a truncated protein that still expresses the twenty amino-acid at the carboxyl terminal of the protein encoding the Pnx-20 peptide (**Figure 4**)

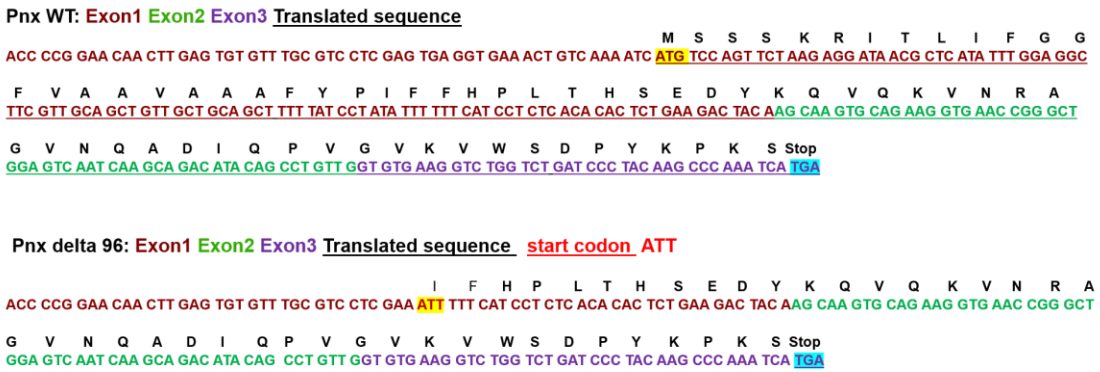

Figure 4: Analysis and comparison of the sequences of a wild-type and the *pnx*$^{uvΔ96/uvΔ96}$. *pnx*$^{uvΔ96/uvΔ96}$ mutants have an alternate start-codon ATT allowing the production of the twenty amino-acid carboxyl terminal of the zPnx-20 peptide.

pnx$^{uvΔ96/uvΔ96}$ **mutants express zPnx-20 protein**

To confirm whether the *pnx$^{uvΔ96/uvΔ96}$* mutants still expressed Pnx-20 protein, we used the anti-zPnx-20 antibody to determine whether a signal was detected in the hypothalamus and the pituitary. Consistent with the second site start-codon initiation of a targeted protein still containing the Pnx-20 protein, positive labeling of zPnx-20 antibody was observed in the magnocellular pre-optic nucleus (PM) and the parvocellular pre-optic nucleus (PPp) adjacent to the diencephalic ventricle (DiV) (**Figure C and C'**) as compared to the wild-type fish (**Figure 5 A, A'**). The *pnx$^{uvΔ96/uvΔ96}$* were positives for zPnx-20 immunolabelling in the pituitary (**Figure 4 D, D'**), as showed in the wild-type (**Figure 5 B, B')**. After pre-absorption of the antibody by the zPnx-20 peptide, we did not see any positive cells labeled by the zPnx-20 antibody in the preoptic area (**Figure 5 E, E'**) and in the pituitary (**Figure 5 F, F'**). Thus confirming the specificity of our zPnx-20 antibody made specifically for the zebrafish Pnx-20 peptide. The presence of immunoreactivity in our *pnx$^{uvΔ96/uvΔ96}$* confirms the genetic evidence that the deletion leaves the Pnx-20 coding sequence and protein intact.

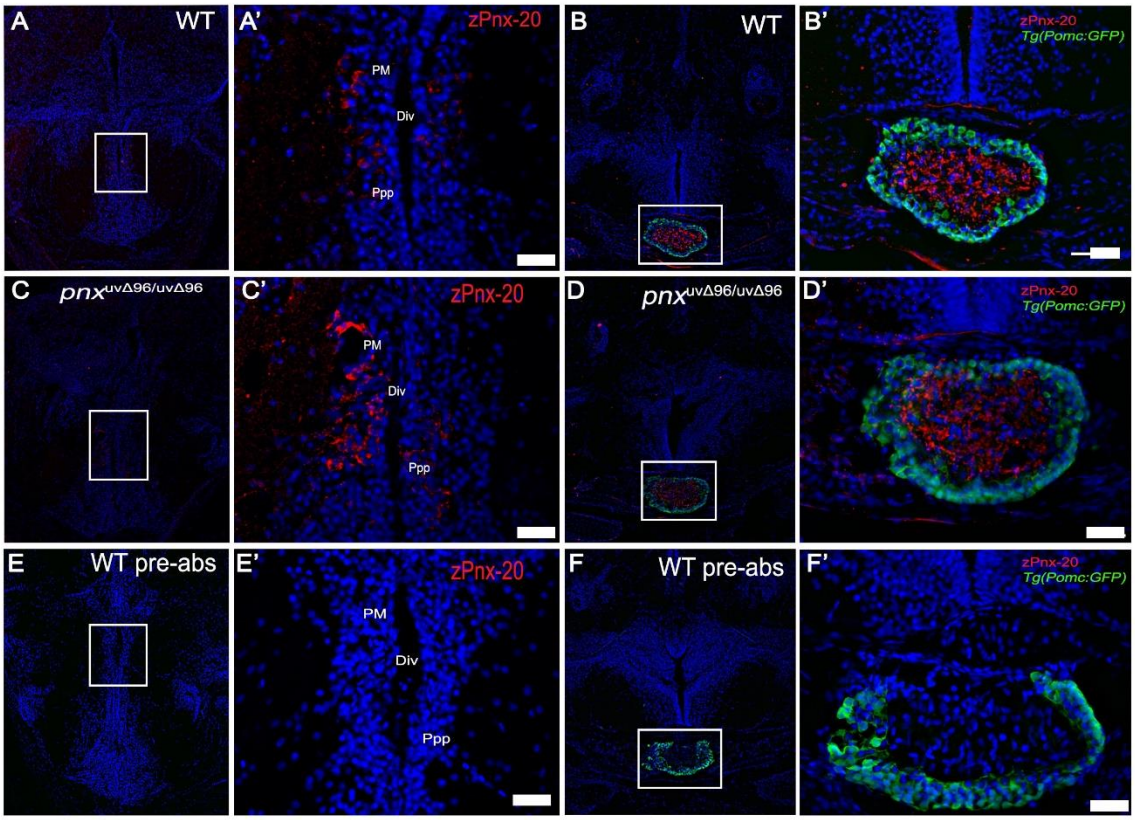

Figure 5: **Labeling of zPnx-20 in zebrafish hypothalamus and pituitary of the *pnx*$^{uv\Delta 96/uv\Delta 96}$ adults line**. Cryostat section of wild-type zebrafish (n=4 fish) where zPnx-20 immunoreactive cells are seen in the magnocellular pre-optic nucleus (PM) and the parvocellular pre-optic nucleus (PPp) adjacent to the diencephalic ventricle (DiV) (A–A') and in the pituitary (Pit) (B-B'). A' and B' are higher magnification of boxed in A and B respectively. (C-D) shows labeling of zPnx-20 in the preoptic area (C and C') and the pituitary (D and D') of the *pnx*$^{uv\Delta 96/uv\Delta 96}$ mutant line (n=4)**.** (E-F) negative control after pre-absorption of zPnx-20 antibody with the peptide. All images are from cryostat cross-section with z-stack of 9µm. Scale-bar 25µM (A' B' C' D', E', F').

Knockout of *phoenixin* using CRISPR/Cas9 in exon 2

The second CRISPR/Cas9 based mutant was a 10 bp deletion in exon 2 (**Figure 6B**) and according to zebrafish nomenclature guidelines named, *pnx*$^{uv\Delta 10/\ uv\Delta 10}$. The gRNAs target sites for *pnx* (ensemble gene ID: ENSDARG00000112670) in exon 2 was 5'-GCAAGTGCAGAAGGTGAACC-3' (**Figure 6A, Supplemental Table 3**). *Pnx* mutant lines were genotyped using primers forward 5'-CAACTGGGCCATTAGAAATCA-3', reverse 5'-TACCAACAGGCTGTATGTCTGC-3' for the exon 2 (**Figure 6A red arrow, Supplemental Table 4**). The gRNA targeting exon 2 resulted in a 10bp deletion (*pnx*$^{uv\Delta 10/uv\Delta 10}$), inducing a frameshift in the open reading frame of the protein with a predicted loss of the zPnx-20 function. In this mutant background adult *pnx*$^{uv\Delta 10/uv\Delta 10}$ males were generated but no *pnx*$^{uv\Delta 10/uv\Delta 10}$ female were ever produced.

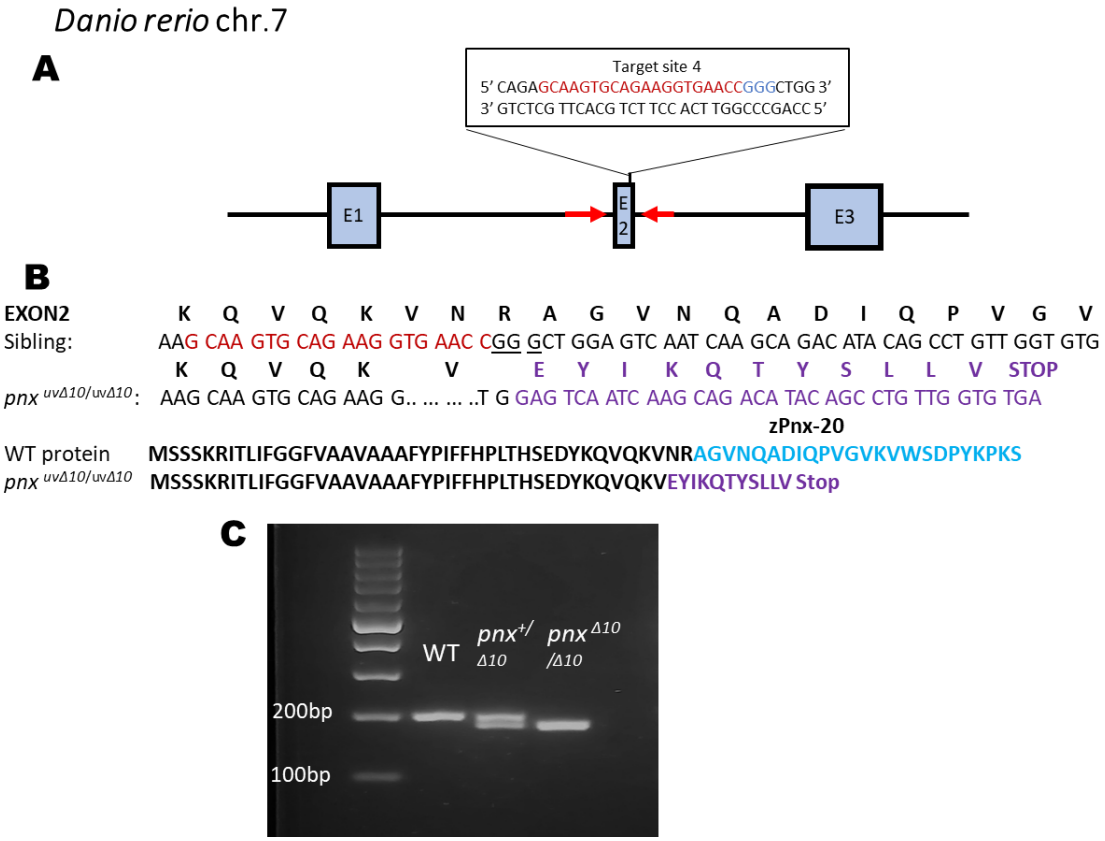

Figure 6: Generation of *pnx*$^{uvΔ10/uvΔ10}$ mutation in exon 2. (A): Localization of the guides RNAs used to knock-out the *pnx* gene by CRISPR and the primers used to screen the injected fish by PCR (red arrows). (B): Comparison of the sequences between a wild-type fish and a *pnx*$^{uvΔ10/uvΔ10.}$ (C): Gel electrophoresis of a wild-type fish a *pnx*$^{+/uvΔ10}$ and a *pnx*$^{uvΔ10/uvΔ10}$ fish showing the difference between the wild-type and mutant product.

pnx$^{uv\Delta 10/uv\Delta 10}$ **do not express zPnx-20 and do not produce female homozygote**

To characterize the phenotype of the pnxuv10$^{/uv\Delta 10}$ mutants immunofluorescence was performed using the anti- zPnx-20 antibody in sectioned adult tissues. The *pnx$^{uv10/uv\Delta 10}$* mutants did not show any labeling of zPnx-20 antibody in the magnocellular pre-optic nucleus (PM) and the parvocellular pre-optic nucleus (PPp) adjacent to the diencephalic ventricle (DiV) (**Figure 7 B, B'**) as seen in the PM and the PPp in the preoptic area of wild-type fish (**Figure 7A, A' red**). Unlike wild-type fish that showed zPnx-20 immuno reactivity in the pituitary gland (**Figure 7 C, C' red**), the *pnx$^{uv\Delta 10/uv\Delta 10}$* mutants lacked of immuno-labeling in the pituitary gland (**Figure 7 D, D'**), where only anti-GFP labeling of the (*Tg:pomc:gfp*) line is observed (**Figure 7 D, D' green**). The absence of zPnx-20 immunoreactivity in *pnx$^{uv10/uv\Delta 10}$* mutant is in agreement with the elimination of the zPnx-20 coding sequence generated by the frameshift mutation.

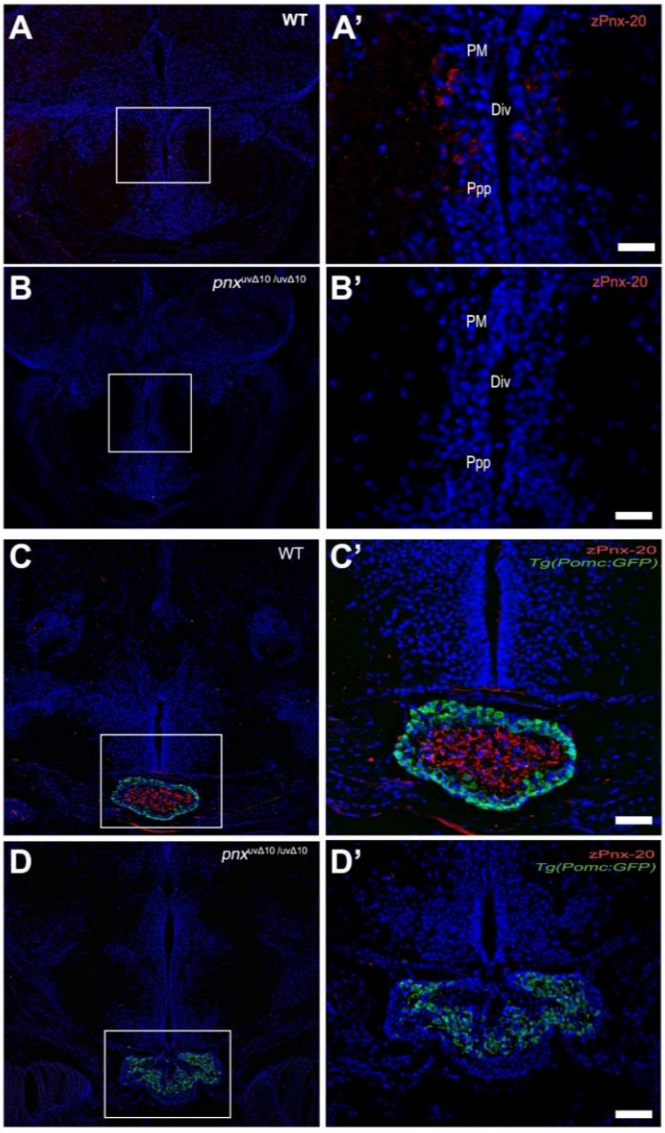

Figure 7: **Characterization of zPnx-20 antibody labeling in zebrafish *pnx$^{uv10/uvΔ10}$* mutant**. (A–A'): Cryostat section of a wild-type zebrafish (n = 4) with zPnx-20 immunoreactivity in the magnocellular pre-optic nucleus (PM) and the parvocellular pre-optic nucleus (PPp) adjacent to the diencephalic ventricle (DiV), and in the pituitary (Pit) (C-C'). (B-B') Cryostat section of a *pnx$^{uv10/uvΔ10}$* mutant zebrafish (n = 3) with no zPnx-20 immunoreactivity in pre-optic area or in the pituitary (D-D'). All images are form cryostat cross-section with z-stack of 9µm.Scale-bar 25µM (A', B', C' and D').

Analysis of reproductive capacities of the *pnx*$^{uv\Delta10/uv\Delta10}$ males through quantification of egg production and fertilization

To characterize the reproductive capacities of *pnx*$^{uv10/uv\Delta10}$ mutants, pairwise crosses were made, and the number and fertility of the eggs scored. The analysis of the reproductive capabilities showed no significant differences between heterozygotes and their wild-type siblings. However, for *pnx*$^{uv\Delta10/uv\Delta10}$ homozygous males, the female (*pnx*$^{+/uv\Delta10}$ or wild-type) crossed with these males produce significantly fewer eggs than the crosses between male *pnx*$^{+/uv\Delta10}$ and female *pnx*$^{+/uv\Delta10}$ or wild-type or control wild-type (**Figure 8**) suggesting that *pnx*$^{uv\Delta10/uv\Delta10}$ males did not stimulate reproductive behaviors. However, while fewer eggs were produced, there were not significant differences in the percentage of fertilized eggs per spawn (**Figure 9**).

Because of the reduction in the number of eggs produced, the morphology of the gonads in the mutants was analyzed (**Figure 10**). No differences in morphology between the wild-type, heterozygotes and homozygotes mutant for *pnx*$^{uv\Delta10/uv\Delta10}$ (**Figure 10**) were observed. We then examined the gonads at the microscopic level (**Figure 11**) and no differences were observed in the area occupied by the cyst within the testis (**Figure 11A, B, C**), but there is a difference in the organization of the spermatozoids where the wild-type (**Figure 11D**) have a regular compaction of the spermatozoids. However, the homozygotes mutant males have hole-like spaces within space occupied by the spermatozoids (**Figure 11E and E', white arrows**).

We then analyzed zPnx-20 labeling in the testis of a wild-type zebrafish and showed that, zPnx-20 is highly expressed in the spermatogonia (**Figure 12, stA**), and the first steps of spermatogenesis such as leptotene cells (**Figure 12, stB**), primary (**Figure 13, stC**) and secondary spermatocytes (**Figure 12, stD**), but not in the

spermatozoa (**Figure 13, stE**). In females, the expression of zPnx-20 was restricted in the granulosa cells surrounding the oocytes (**Figure 13, white arrows**). These results indicate that zPnx-20 may play an important role in spermatogenesis and may be necessary for oocyte maturation.

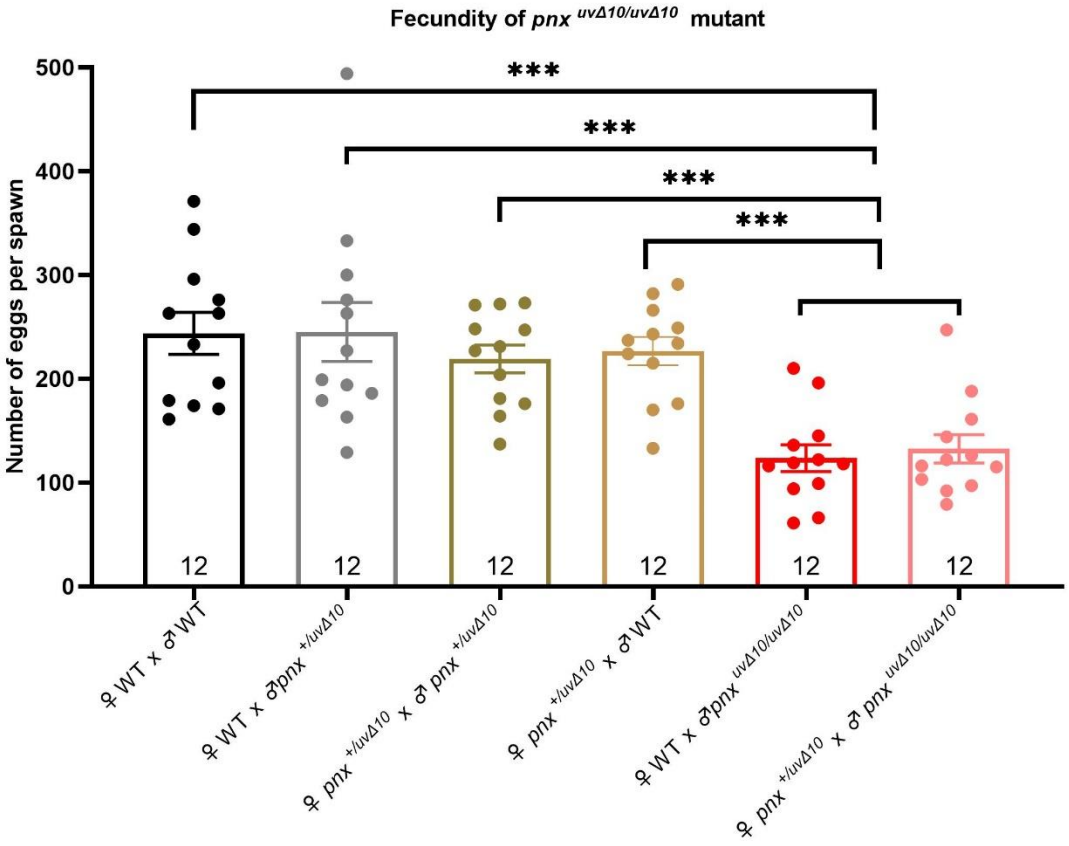

Figure 8: Comparison of egg production in wild-type and *pnx*$^{uvΔ10/uvΔ10}$ fish. (A) Number of eggs produced per spawn of one female paired with one male (n = 12). Mating was between *pnx*$^{+/uvΔ10}$ or wild-type females with male *pnx*$^{uvΔ10/uvΔ10}$ mutant or *pnx*$^{+/uvΔ10}$ or wild-type siblings. Data (n=12 crosses) are presented as Mean ±SEM. Statistical significance P-values are calculated with *One-sample t-test* and Ordinary one-way ANOVA (Prism8). **P< 0.05; **P<0.002 ***; P<0.001.*

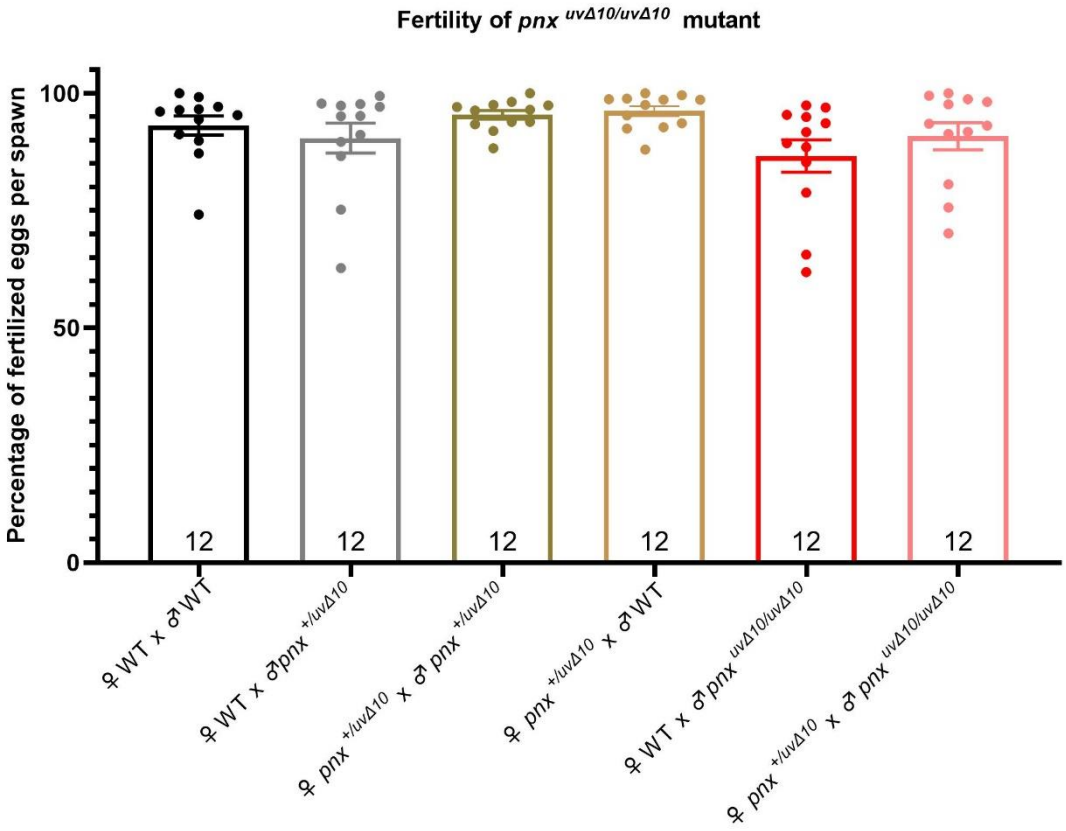

Figure 9: Comparison of fertilization rate in wild-type and *pnx*$^{uv\Delta10/uv\Delta10}$ fish. (A) Percentage of fertilized eggs per spawn of one male paired with one female (n = 12). Mating was between *pnx*$^{+/uv\Delta10}$ or wild-type females with male *pnx*$^{uv\Delta10/uv\Delta10}$ mutant or *pnx*$^{+/uv\Delta10}$ or wild-type siblings. Data (n=12 crosses) are presented as mean ±SEM. Statistical significance P-values are calculated with *One-sample t-test* and Ordinary one-way ANOVA (Prism8). **P< 0.05; **P<0.002 ***; P<0.001.*

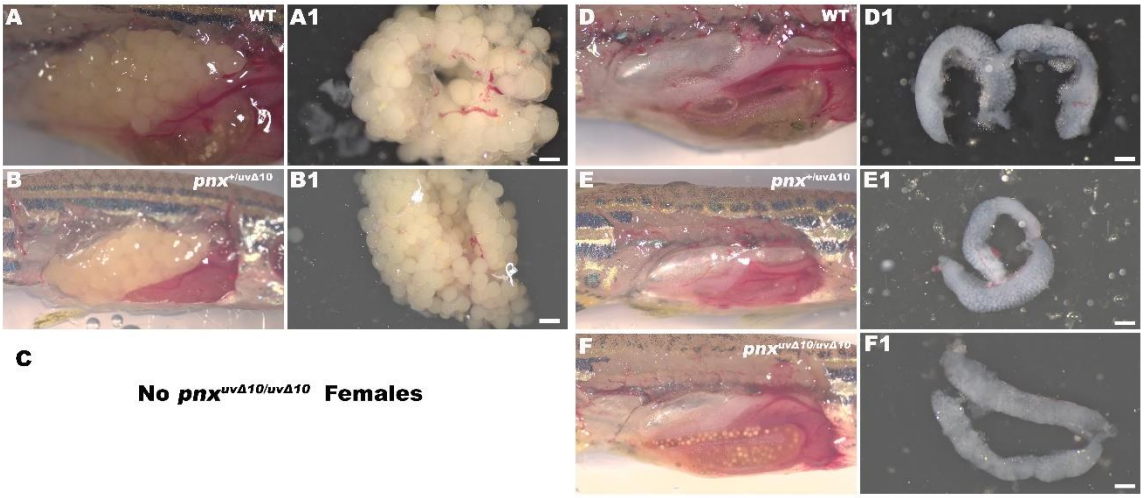

Figure 10: Morphology of gonads from wild-type and *pnx* mutant fish. (**A, A1**): ovaries from wildtype females and from $pnx^{+/uv\Delta 10}$ heterozygous mutant females (**B, B1**) showing normal ovary and oocyte structure. **C**) Homozygous $pnx^{uv\Delta 1/uv\Delta 10}$ mutant females were never observed. (**D, D1**) Wildtype male testis showing normal structure and extension. (**E, E1**) testis of heterozygous $pnx^{+/uv\Delta 10}$ and (**F, F1**) homozygous $pnx^{uv\Delta 10/uv\Delta 10}$ mutant males showing normal structure. Scale bars 500uM.

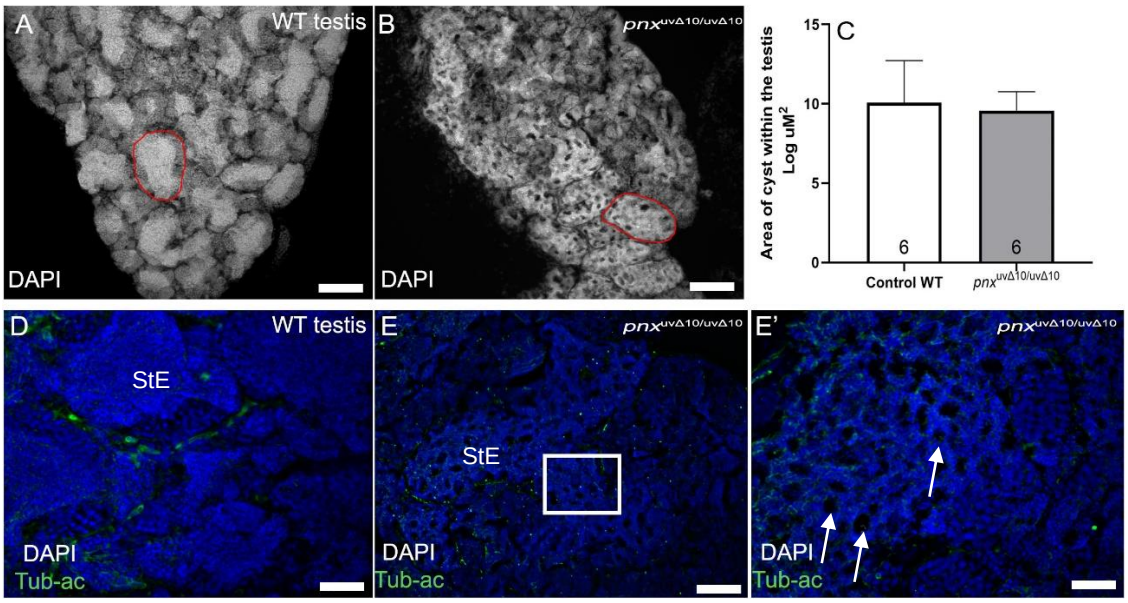

Figure 11: **Comparison of structure of *pnx*$^{uvΔ1/uvΔ10}$ and wild-type testis** Cryostat section of zebrafish wild-type (A) and *pnx*$^{uvΔ1/uvΔ10}$ (B) testis (n=3 animals). (C) Comparison of area of cyst within the testis as shown in red circles in (A and B) no significance between the wild-types and mutants (*P-values* = 0.7269). (D-E') Testis of wild-type (D) where the spermatozoids (StE) are compacted and *pnx*$^{uvΔ1/uvΔ10}$ (E, E') where there are holes within the space occupied by the spermatozoids (StE) as shown by the whites arrows. All images are from cryostat cross-section of 10µm. Z-stack: 6µm Scale-bar 25µM (E'), 50µM (D, E). 100µM (A, B). C: Data (n=6 slides) are presented as mean ±SEM. Statistical significance P-values are calculated with *One-sample t-test* (Prism8). **P< 0.05.*

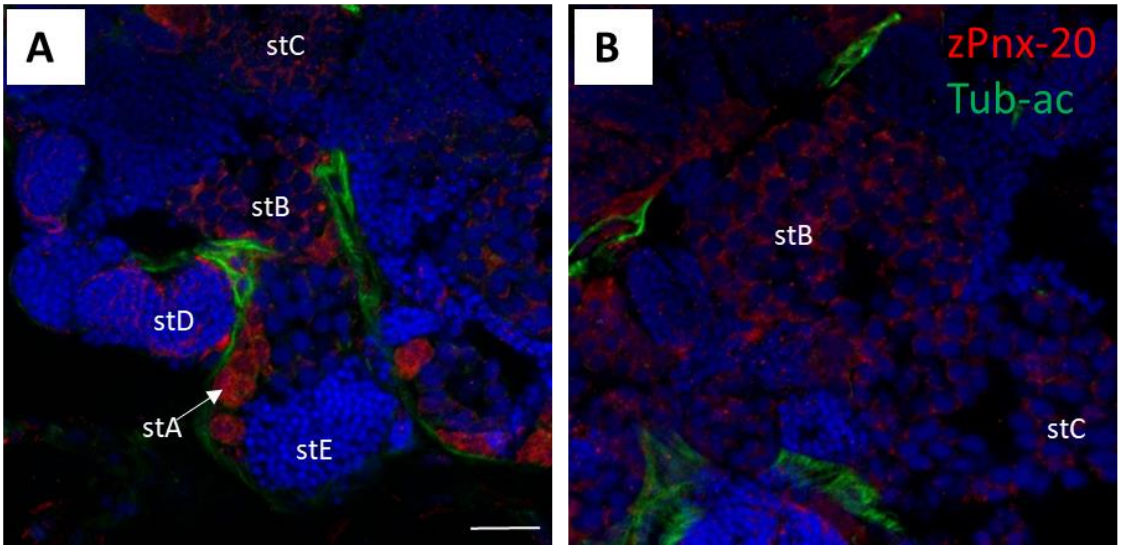

Figure 12: **Expression of zPnx-20 in adult zebrafish testis** (A and B): Cryostat section of zebrafish testis (n=3 animals) with zPnx-20 immunoreactivity in the spermatogonia (stA), leptotene cells (stB), primary (stC) and secondary spermatocytes (stD) but not in the spermatozoa (stE). All images are from cryostat cross-section of 10µm. Z-stack: 3µm Scale-bar 50µM.

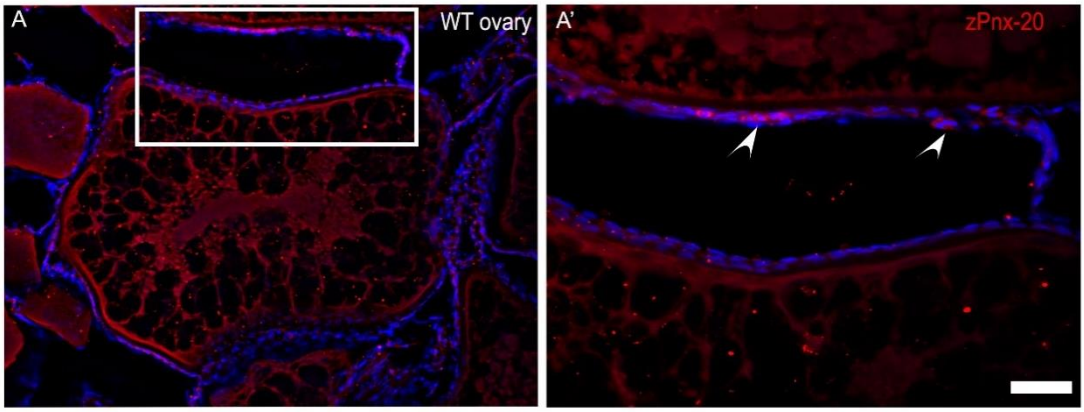

Figure 13: **Expression of zPnx-20 in adult zebrafish ovary** (A-A'): Cryostat section of zebrafish ovary (n=3 animals) with zPnx-20 immunoreactivity in the granulosa cells surrounding the oocytes. (A') hyper magnification of boxed in A. All images are from cryostat cross-section of 10µm. Z-stack: 3µm Scale-bar 25µM.

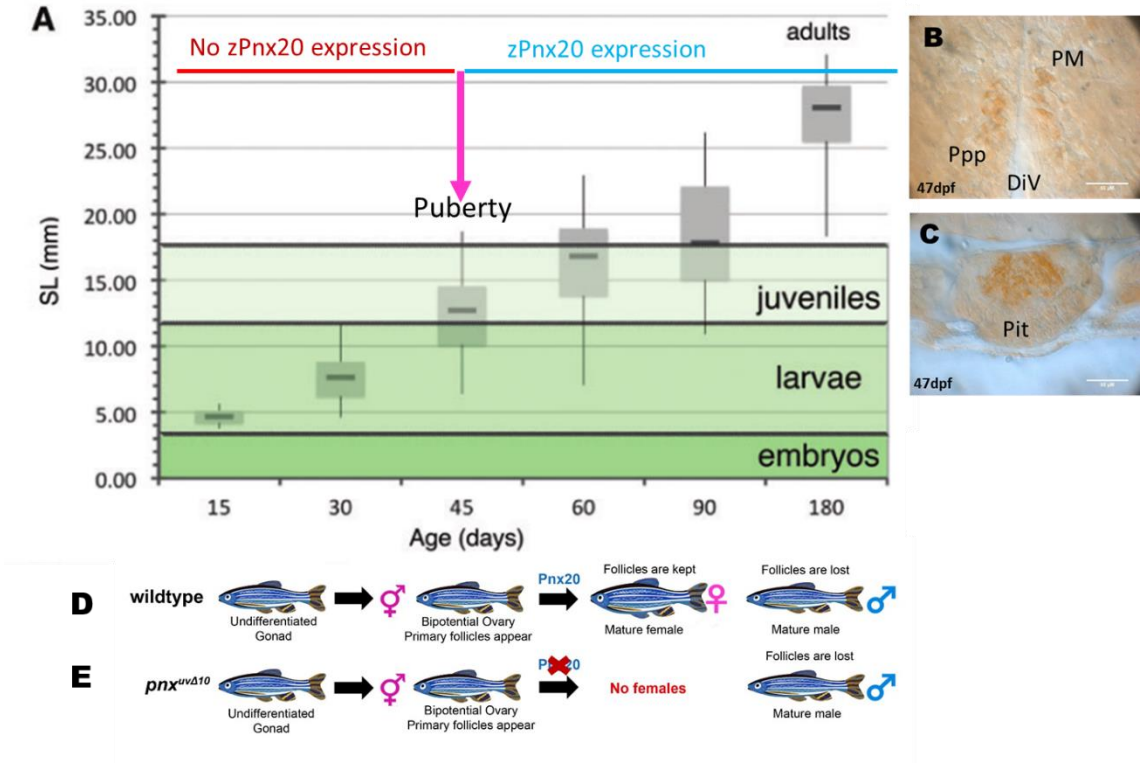

Figure 14: Pnx-20 may play a role in female fate decision. (A) Developmental staging of zebrafish *Singleman and Holtzman 2014*. (**B, C**) zPnx-20 labeling is detected in the hypothalamus at the onset of puberty ~47 dpf: (B). magnocellular preoptic nucleus (PM) and the parvocellular preoptic nucleus (Ppp) adjacent to the diencephalic ventricle (DiV) and (C) pituitary at the onset of puberty. (**D**) Zebrafish bipotential ovary can switch to produce males or stays female at puberty. **E**). No adult *pnx*$^{uvΔ1/uvΔ10}$ females were isolated. (*Adapted from Singleman and Holtzman 2014*)

IV. DISCUSSION

zPnx-20 peptide is present at the onset of in zebrafish

Previous research from our laboratory demonstrated that zebrafish Phoenixin 20 (zPnx-20) peptide is expressed in the preoptic area and the pituitary of adult zebrafish (Ceriani et al., 2021). This current study extends those findings by examining zPnx-20 immunolabeling during different developmental stages. We observed that zPnx-20 is absent from the pituitary at earlier developmental stages (15 dpf, 21 dpf, and 35 dpf; **Supplemental Figure 1**). However, zPnx-20 immunolabelling was detected in the hypothalamus and pituitary by 47 dpf, which coincides with physical markers of puberty, such as increased body size and colour pattern changes (Chen & Ge, 2013; Singleman & Holtzman, 2014). This timing aligns with prior studies indicating that puberty in zebrafish is associated with specific gene expression changes, including *kisspeptin* (Kitahashi et al, 2009) and *lhb* (Chen & Ge 2012), as well as increased hormonal activity (estradiol; Tang et al, 2017), particularly within the hypothalamic-pituitary axis.

The localization of zPnx-20 positive cells in the magnocellular preoptic nucleus (PM) and the parvocellular preoptic nucleus (Ppp), as well as the reticular formation, is consistent with earlier studies in zebrafish (Ceriani et al., 2021) and other mammals (Palasz et al., 2015; Prinz et al., 2017). This suggests a conserved role for Pnx-20 in these brain regions across vertebrates. The presence of zPnx-20 in the reticular formation could indicate a broader neuromodulatory functions, potentially linked to behavioral changes. This idea is supported by reports of *pnx* mutant rats displaying significantly reduced locomotion in open field tests (McIlwraith et al., 2024).

Overall, these findings highlight zPnx-20 expression as a marker of puberty in zebrafish and suggests that zPnx-20 plays a critical role in the regulation of reproductive maturation in fishes.

zPnx-20 have a role in determination of sex at puberty

The role of Pnx-20 in regulating pituitary gonadotropins expression was revealed by Yosten et al., (2013) and subsequently intraperitoneal injection of zPnx-20 significantly increased *lhb* and *fshb* mRNA expression in female zebrafish. This up-regulation of gonadotropins indicates that zPnx-20 enhances follicle development and the number oocytes; processes driven by Fsh; but also stimulate the production of Lh in females, which is essential for ovulation and the control of the estrus cycle not only in mammals (Ngueyen et al, 2019, Stein et al, 2016, Billert et al., al 2020) but also in zebrafish.

Similarly, *lhb* mRNA expression increased significantly in male zebrafish, supporting the role of zPnx-20 in activating *lhb-receptor* (an upstream factor of Lh hormone) in zebrafish testis as shown by Rajeswari & Unniappan (2020). This activation of *Lhb* by zPnx-20 is linked to increased testosterone production and spermatogonia as seen in rat (Yilmaz et al., 2024). Interestingly, *fshb* mRNA was down-regulated in males, suggesting a negative regulatory effect of zPnx-20 in *fshb* expression. This result is consistent with the findings of Rajeswari & Unniappan (2020) in *fshb-receptor* mRNA expression in male zebrafish testes. While Lh is crucial for testosterone production, Fsh in may play a secondary role in male reproduction (Simoni et al., 2020). For instance, mice with Fsh deficiencies exhibit no reproduction deficits (Kumar et al., 1997, Dierich et al., 1998) and Fsh alone fail to induce spermatogenesis

in patient with secondary hypogonadism due either to idiopathic hypogonadotrophic hypogonadism (IHH) and Kallmann's syndrome or to hypo-pituitarism (Schison et al., 1993). Overall, these findings support the proposed modulatory role of Pnx-20 on gonadotropin expression in vertebrates (Yosten et al., 2013) particularly in teleosts (Wang et al., 2019) such as zebrafish (**Figure 2**), with distinct sex-specific effects on gonadotropins receptors (Rajeswari & Unniappan 2020).

zPnx-20 and female fate in zebrafish

Our observation that no female *pnx*$^{uv\Delta 10/uv\Delta 10}$ animals were produced is particularly striking and suggests that zPnx-20 is crucial for female sex differentiation. In zebrafish, during the early stages of development all animals develop an immature ovarian tissue and the ovarian development is the default pathway (Uchida et al., 2002; Von Hofsten and Olsson, 2005), then at puberty differentiation of mature ovaries or sex reversal into testes begin. The continuous development of the immature ovary to a mature ovary is based on an interplay of a variety of genetic factors. The most important factor is the inhibition of apoptosis to maintain oocyte survival and mature ovary development (Uchida et al., 2002; Luzio et al., 2016). This mechanism involves the inhibition of *tumor protein p53* (Rodriguez-Mari et al., 2010) and up-regulation of the genes such as gonadal aromatase *cyp19a1a,* (Yin et al., 2017) shown to be elevated in female zebrafish injected with Pnx-20 (Rajeswari and Unniappan 2020). Furthemore, *sox9b*, *foxl2a/foxl2b* are also important for female transition (Siegfried and Nüsslein-Volhard 2008; Carver and Zhu 2023). Most recently, cholecystokinin (Cck) and Cck-receptors have been shown to promote folliculogenesis (Uehara et al., 2024; Cohen et al., 2024). To date there is no evidence that Pnx has an anti-apoptotic role.

One possible mechanism where Pnx-20 might be involved is in blocking female development is through the granulosa cells. Granulosa cells are part of the ovary. They surround the primary follicle and play important role in oocyte growth, replication and acquisition of gonadotropic responsiveness, transferring essential molecules to the follicles (Klinger, & De Felici, 2002; Matsuda et al., 2012, Hatzirodos et al., 2014) and promoting maturation through Fsh (Rehnitz et al., 2017) and Fsh-receptor. Both are necessary for female fate in zebrafish (Xie et al., 2017). zPnx-20 is expressed in granulosa-cells in zebrafish (**Figure 13**; Rajeswari and Unniappan 2020) and mammals (Ngueyen et al., 2019; Kurowska et al., 2024), thus is likely to play a pivotal role in the function of granulosa cells. Pnx-20 is not only shown to promote proliferation of granulosa cells, but also increases estradiol production and potentiates the expression of genes related to follicle development in mammal granulosa cells (Ngueyen et al., 2019; Kurowska et al., 2024), indicating the potential role of Pnx-20 in promoting oocyte development (Billert et al, 2020). Thus Pnx-20 depletion may have altered granulosa cell function and therefore leading to inhibited female gonadal development during zebrafish puberty.

Male homozygote of *pnx*$^{uv\Delta 10/uv\ \Delta 10}$ are less prone to mating but still reproduce

Analysis of reproduction shows that females crossed with male zebrafish lacking zPnx-20 produce fewer eggs. Fertility in male depend mostly on intra-testicular testosterone level (Dohle et al., 2003) and testosterone level is positively associated with Pnx level in mammals (Cundubey, & Cam, 2023; Yilmaz et al., 2024). We have shown that zPnx-20 can activate the expression of *lhb*, which in male, is the hormone upstream of testosterone production (**Figure 6**), suggesting a role in the process of spermatogenesis. Males mutant for *pnx* seem to have less spermatozoids than wild-

type as they have hole-like spaces within their testis (**Figure 12**). This phenotype is consistent with data from studies in rats where chronic unpredictable stress causes very low level of Pnx and spaces between the seminiferous tubules and lesser spermatogenesis (Mohamed et al., 2024). Because Pnx increased the sperm production in rats (Yilmaz et al., 2024), its absence in male zebrafish may have led to reduced sperm production inducing a lower fecundity in comparison to the wild-type.

In conclusion, Pnx-20 has an important role in male zebrafish fecundity and is essential for female sex determination. Our *pnx*$^{uv\Delta 10/uv\ \Delta 10}$ mutant line exhibited no female homozygotes for the mutation indicating it's necessary in female fate. An inducible conditional knockout model of *pnx* could help to understand the important role of Pnx-20 in female development in zebrafish.

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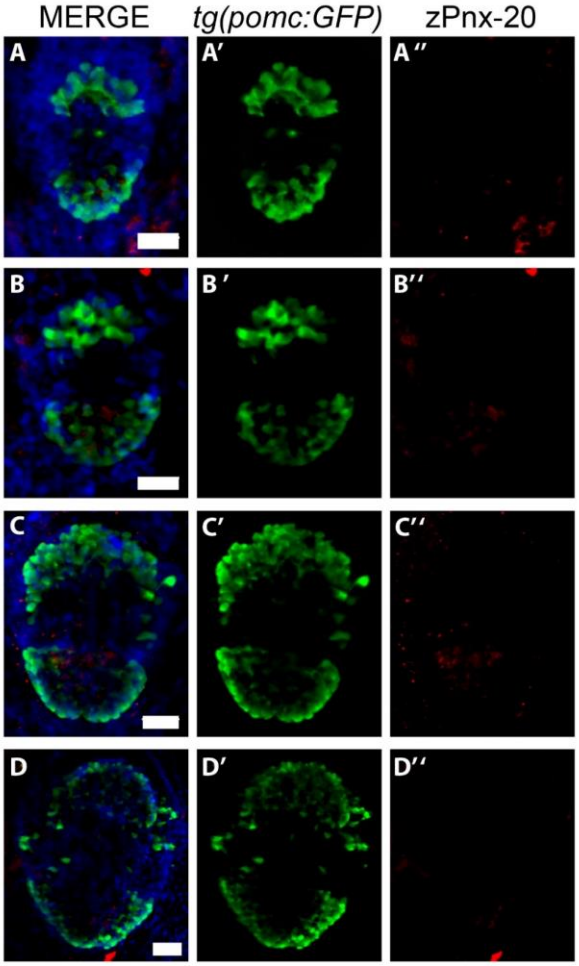
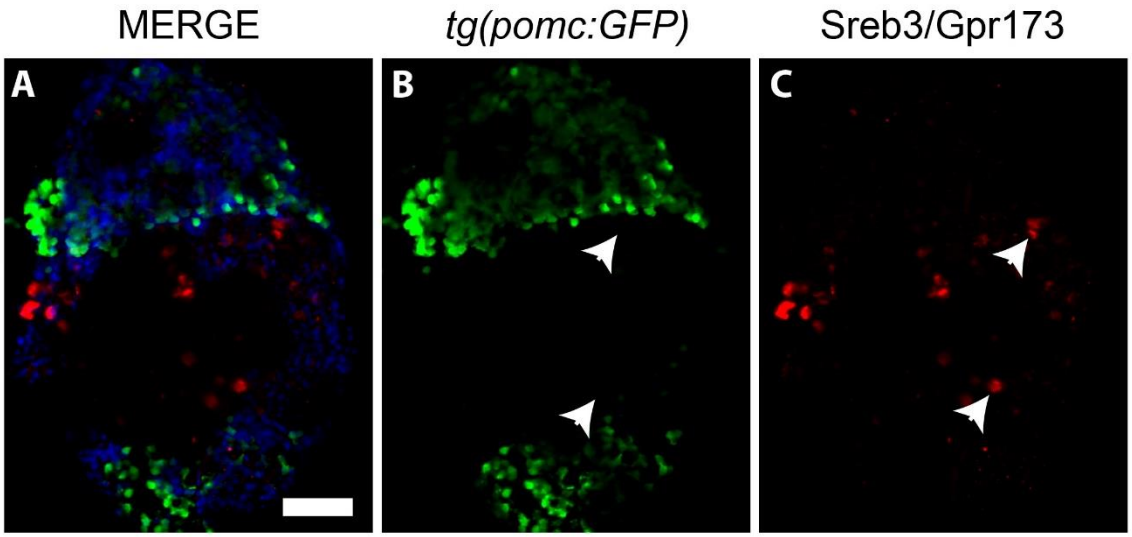
VI. ANNEX



Supplemental Figure 1: **Labeling of zPnx-20 in zebrafish pituitary during larval development.** At 15 dpf (A-A" n = 15) zPnx-20 labeling is not detected in the pituitary of the zebrafish as shown by the GFP expressing cells (A', B', C', D'). At 21dpf we did not detect Pnx labeling in the pituitary (B-B" n=15) and at 35 dpf (C-C" n=12). Consistent after a pre-absorption of the antibody with zPnx-20 peptide for 3 hours (D-D" n=3). All the images are ventral view, anterior in the top. Scale bars 25uM. Z-stack A-A": 15µm; B-D": 18µm.



Supplemental Figure 2: **Localization of Gpr173/Sreb3-like labeling in cells of the pituitary in the adult zebrafish**. The Sreb3-like labelled cells (A and C, n = 3) do not localize with the GFP expressing cells (A and B). Scale bars 50uM. All images are ventral view, anterior in the top. Z-stack: 30µm

Supplemental Table 1: List of primers of used of qPCR

Gene	Forward	Reverse	Length
lhb	GGCTGGAAATGGTGTCTTCT	CCACCGATACCGTCTCATTTAC	107 BP
fshb	GCTGGACAATGGATCGAGTTTA	CTCGTAGCTCTTGTACATCAAGTT	92 BP
beta-actin1	CGAGCAGGAGATGGGAACC	CAACGGAAACGATCATTGC	102 BP

Supplemental table 2: Primary antibodies

Antigen	Host	Dilution	Manufacturer	Cat. No	Immunogen organism	Used in zebrafish
Acetylated tubulin	Mouse	1:500	Sigma	T6793	Sea urchin	Subkhankulova et al., 2023
GFP	Mouse	1:500	Invitrogen	A-11120	*Aequorea victoria*	Ceriani et al., 2021
Sreb3	Rabbit	1:250	Abcam	Ab188765	Human	Rajeswari & Unniappan 2020
Zebrafish Phoenixin 20	Rabbit	1:500	Pacific Immunology	PAC 15947/15948	Zebrafish	no

Supplemental table 3: Guide RNAs for *pnx* CRISPR/Cas9 mutant generation

Gene	Id	Exon	Target sequence for CRISPR/Cas9
pnx	ENSDARG00000112670	Exon 1	5'-GCTCACTCCACTTTGACAGT-3'
pnx	ENSDARG00000112670	Exon 1	5'-AAGAGGATAACGCTCATATT-3'
pnx	ENSDARG00000112670	Exon 1	5'-ATATTTTTTCATCCTCTCAC-3'
pnx	ENSDARG00000112670	Exon 2	5'-GCAAGTGCAGAAGGTGAACC-3'

Supplemental table 4: List of primers for *pnx* CRISPR/Cas9 mutant genotyping

Gene	Id	Exon	Primers of CRISPR/Cas9 genotyping
pnx	ENSDARG00000112670	Exon 1	Forward 5'-ACCCCGGAACAACTTGAGTG-3'
pnx	ENSDARG00000112670	Exon 1	Reverse5'-AAGTTTAGGCTGTGTCACGGT-3'
pnx	ENSDARG00000112670	Exon 1	
pnx	ENSDARG00000112670	Exon 2	Forward 5'- CAACTGGGCCATTAGAAATCA-3'

			Reverse 5’-TACCAACAGGCTGTATGTCTGC-3’

CHAPTER III: REPRODUCTION IN ZEBRAFISH IS CONTROLLED AT THE LEVEL OF GNRH RECEPTORS

I. INTRODUCTION

The lack of *gnrh1* (Whitlock et al., 2019) and the loss of function of *gnrh2* and *gnrh3* (Spicer et al., 2016; Marvel et al., 2018) do not result in loss of reproductive capacity which is a surprising observation given that Gnrh peptides are part of an extremely conserved signalling pathway controlling reproduction in a wide variety of vertebrates. And while Pnx-20 plays an important role in zebrafish sexual differentiation, it is not the “master” peptide controlling reproduction. Thus we investigated other potential sources of hormonal cues specifically exogenous signals. In fish, the olfactory cues play an important role in reproduction (Kobayashi et al., 2002; Stacey N. 2003; Stacey and Sorensen 2011) including the use of hormones as pheromones. In goldfish hormones released into the water by ovulating females trigger milt production in males and the initiation of reproductive behaviors (Sorensen and Stacey 2004). Similarly, in zebrafish, it has been shown that hormones are also used as pheromones to initiate reproductive behaviors (Hurk and Lambert 1983).

Additionally, in zebrafish, receptors of sex hormones are expressed in the olfactory epithelia (OE) such as androgen receptors (Gorelick et al., 2008), Gnrh-R3 (Whitlock et al., 2006; Corchuelo et al., 2017, and prostaglandin receptors (Yabuki et al., 2016). The expression of these receptors suggests that exogenous Gnrh could act via the olfactory organ. To date there is no evidence that exogenous Gnrh acts on the olfactory sensory system. But it is known that the response to hormones used as pheromones in goldfish is mediated by the olfactory epithelia because cutting the olfactory nerve abolishes the response (Sorensen and Sato 2005). Furthermore, in zebrafish exposure to prostaglandin activates specific OSNs that send neural information via the olfactory bulb into the ventral telencephalon and the hypothalamic

preoptic area (Yabuki et al., 2016). Importantly the latter study uncovered a potential neural circuit connecting the olfactory response to a pheromone (prostaglandin) with centrally located endocrine control centre.

To determine whether Gnrh peptide can act as a pheromone triggering reproduction in zebrafish, we first isolated the animals to avoid any stimulation from other fish. Then we characterized the effect of exogenous Gnrh3 peptide on the mRNA expression of genes important in the control of reproduction (*lhb* and *fshb*) by RT-qPCR. Our results show that exogenous Gnrh exposure triggers a statistically significant increase in expression of pituitary gonadotropins *fshb* and *lhb* mRNA where females and males show different responses. When the openings of nose were blocked, the *fshb* and *lhb* response to exogenous Gnrh dropped drastically in both sexes. Thus, we proposed that Gnrh3 can act like a pheromone through the olfactory epithelia of zebrafish to influence and trigger gonadotropin hormone production in the pituitary

Reproduction in zebrafish is controlled at the level of the Gnrh-receptors

Gnrh-Rs are part of the family 1b (rhodopsin-like) of the seven-transmembrane-helix, guanine nucleotide-binding (G) protein coupled receptors (GPCRs), the largest family of signal-transducing molecules known (Arvanitakis et al., 1998). However, less is known about the classification of the Gnrh-R sub-family. Zebrafish have four Gnrh-Rs (Tello et al., 2008), with Gnrh-R1 and Gnrh-R3 belonging to the Gnrh-RIIb that is evolutionarily related to mammal Gnrh-R2, while Gnrh-R2 and Gnrh-R4 belong to the Gnrh-R IIa group that has no homologue in mammals (Roch et al., 2011). The four zebrafish Gnrh-rs 1, 2, 3 and 4 are expressed throughout the CNS including the hypothalamus (Tello et al., 2008).

In mammals, the pulsatile secretion of Gnrh results in the activation of Gnrh-Rs, and reproduction does not occur in the absence of Gnrh (Sherwood and Harvey. 1986; Anderson and Millar 2022; Prevot et al., 2023). This episodic release of Gnrh is essential for the maintenance of the Gnrh-R sensitivity (Schang et al., 2012; Constantin et al., 2021), because a sustained administration of Gnrh agonist (Gnrh-a) induced a down-regulation of the receptor (Nederpelt et al., 2016), resulting in decreased gonadotropin release (Okada et al., 1994; Huang et al., 2015).

In contrast to mammals, there is no evidence that pulsatile Gnrh release controls reproduction in teleosts (Fontaine et al., 2020). However, a daily circadian cycle of luteinizing hormone (Lh) secretion was found in female rainbow trout (Zohar et al., 1986) and goldfish (Hontela and Peter 1978). In fact, continuous administration of a synthetic Gnrh agonist (Gnrh-a), when applied via capsule under the skin triggers a permanent and high-level release of Lh (Mylonas and Zohar 2000). Because, unlike many fishes, zebrafish are continuous spawners (can spawn in a weekly basis) their physiology may be favored by Gnrh-Rs to control their reproductive capacities.

To determine whether exogenous Gnrh is acting through the Gnrh-Rs, we generated a loss of function mutant in Gnrh-R3 using CRISPR/Cas9. We focused on the non-mammalian Gnrh-Rs (Gnrh-R1 and Gnrh-R3) because they are more sensitive to the fish specific Gnrh3 (Tello et al., 2008), and apart of being expressed in most of the brain including the hypothalamus and the pituitary of teleost such as goldfish (Illing et al., 1999), zebrafish (Tello et al., 2008; Cortes-Campos et al, 2015; Marvel et al, 2021) and salmon (Ciani et al., 2020)*; gnrh-r3*/Gnrh-R3 gene and protein is also expressed in the OE of zebrafish (Whitlock et al., 2006; Corchuelo et al., 2017). We analyzed the amino-acid sequences of the zebrafish Gnrh-R1 and Gnrh-R3, comparing them with medaka and goldfish, two fish in which Gnrh controls reproduction, we

showed that zebrafish Gnrh-R1, in the contrast to Gnrh-R3, has a substitution of valine (V) in the place of the glutamic acid (E) at the end of the Extracellular Loup 3 (ECL3) (**Supplemental Figure 1**). The glutamic acid (E) in the ECL3 is important for the ligand binding in fish and studies have shown that zGnrh-R1 is less sensitive to physiological doses of Gnrh peptides in comparison to the other zGnrh-Rs (Tello et al, 2008). Thus because this substitution may impair Gnrh-R1 activation, the *gnrh-r3* gene was chosen to target with CRISPR/Cas9.

Using CRISPR/Cas9 we generated a loss of function mutant in *gnrh-r3* that resulted in complete infertility in the males and almost complete infertility in females. The female can produce eggs but most of them are non-fertilizable and abnormal with defects in oocyte maturation. The males are totally infertile, do not initiate a mating response from the female, and analysis of their gonads showed a severe malformation of their testes. Thus, zebrafish clearly used Gnrh signalling to control reproduction and our results that it is exogenous Gnrh that activates the reproductive pathways via the olfactory organ.

II. MATERIALS AND METHODS

Gnrh3 exposure

Ten days before exposing the zebrafish to exogenous Gnr3, the animals (n=3 per group; age and weight-matched) were housed individually in 2L tanks outside the circulating water system of the fish facility to avoid stimulation of external hormones from other fish in the water system. The water was changed every day and the fish were fed twice a day with dry food. Prior the Gnrh3 exposure, the fish were acclimated for 1 hour in 450mL water in a new tank to minimize the background activation of the olfactory epithelium (Yabuki et al., 2016). After the acclimation, 10^{-9}M of synthetic Gnrh3 (Sigma-Aldrich #L4897) or dimethylsulfoxide (DMSO: control, Sigma-Aldrich) were added to the water and fish were exposed for 30 minutes. The fish were sacrificed, and the pituitaries were collected and stored at -80°C in TriZol Reagent (Ambion, Life Technologies) until further analysis. To ovoid interaction or overlapping between the endogenous peak of Gnrh, that occurs between 14:00 to 16:00, or the transcription of *lhb* and *fshb,* mRNAs between 16:00 to 18:00, and our experiments, the exposures were done in the morning between 09:00 to 11:00, time when there is no Gnrh surge that would trigger gonadotropes transcription (Karigo et al., 2012)

Temporary nares block

One day before the Gnrh3 exposure, adult fish were anesthetized in Tricaine (400mg/100ml) (A5040 Sigma-Aldrich) immobilized with a wet sponge in a Petri dish and put under the dissecting microscope. The nares were then dried with Kim-wipes. A small drop of tissue adhesive Leukosan® Adhesive (Chemence Mediccal Inc, USA) was applied to seal both anterior and the posterior nares of both noses (Mathuru et al.,

2012). Thirty seconds after the application of the adhesive, fish were allowed to recover in their individual tanks for overnight and were then exposed to Gnrh3 peptide as described above

Total RNA isolation and reverse transcription

The hypothalamus and pituitaries of each group (control group exposed with DMSO and experimental group exposed with Gnrh3) were combined, and total RNA was extracted using TriZol Reagent (Ambion, Life Technologies) according to previous published protocol (Calfún et al., 2016). Briefly, the tissues were isolated from the adult male or female zebrafish under the dissecting microscope and homogenized in 1mL TriZol-Reagent. 200µL of Chloroform (Sigma-Aldrich) was added in each tube to a total volume of 1200µL per tube and the mixture was centrifuged at 4°C for 15 minutes at 12000 ×g in a centrifuge (Servall legend RT). After centrifugation, the aqueous layer (± 500µL) was transferred to a new tube and mixed with 500µL of 2-propanol (Sigma, Life Technologies) and centrifuged to precipitate the RNA. RNA was then washed with 75% ethanol 25% DEPC water. Total RNA was resuspended with DEPC water and treated with DNAse I Amplification grade (Invitrogen), then quantified by the Qubit HS RNA Assay Kit (Invitrogen, ThermoFisher Scientific) using the Qubit 3.0 Fluorometer (Invitrogen, Life Technologies).

To synthesize cDNA from total RNA, reverse transcription was performed at 42°C for 1 hour in a reaction of 20µL containing x µL of total RNA at the initial concentration, 1µL of 10mM dNTP mix, 1µL of 0.5 µg of Oligo(dT) (Invitrogen) and 50 U of SuperScript II reverse transcriptase (Invitrogen) and RNAse Out (Invitrogen). The cDNA was then used for qPCR.

Quantitative PCR of *lhb* and *fshb* mRNAs expression

To examine the mRNA expression level of the pituitary genes *lhb* and *fshb*, qPCR was performed using M×3000P thermocycler (Stratagene). A reaction mixture of 2µL of (cDNA), 0.75µL of each primer, and 12.5 µL of SYBR Green/ROX qPCR Master Mix (Molecular Probes) in a final volume of 25µL. Relative quantification was done using the Ct-values and *beta actin-1* as housekeeping gene, and no RT or no template controls for each gene. The reaction profile consisted one cycle of 95°C for 10 minutes, then 40 cycles of 95°C for 15 sec, and 59°C for 15 sec, and 72°C for 15sec for signal detection. The data were analyzed using the thermocycler software (MxPro-Mx3000P v4.10) and Prism8.

Generation of *gnrh-r3* mutant fish

CRISPR/Cas9 design

All gRNAs were designed were designed using CHOPCHOP (https://chopchop.cbu.uib.no/) and Integrated DNA Technologies (https://www.idtdna.com/site/order/designtool/index/CRISPR_CUSTOM). Specific sgRNAs and Constant oligonucleotide were purchased from Macrogen Inc (South Korea). Cas9 (TrueCut Cas9 Protein v2 5µg/µL #A36499) were purchased from ThermoFisher. To generate the sgRNAs we used approach from Gagnon et al., 2014. For each sgRNA, we ordered 60 base pairs oligonucleotides (gene-specific oligo) containing a promoter for in vitro transcription (T7 promoter), the 20-base spacer region specific to the target gene and an overlap region that anneals to the constant oligonucleotide. If the sgRNA does not start with 2 GG, theses bases are added to enhance the transcription reaction of the T7 polymerase. The gene-specific oligo using

T7 have the architecture TAATACGACTCACTATA-N20-GTTTTAGAGCTAGAAATAGCAAG, with Ns replaced by the 20-base specific to the target and the constant-oligo: AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTA ACTTGCTATTTCTAGCTCTAAAAC. gRNAs and Constant-oligos were suspended in 100µM stock solution. To anneal the sgRNA and the Constant-oligo, 1µL of each stock solution in a total volume of 10µL was used and incubated under these conditions 5 minutes at 95°C, 95°C ➔85°C for -2°C/second, then 85°C ➔ 25°C for -0.1°C/second and hold at 4°C. Finally, 10µL of mixture containing 0,5µL of T4 NEB DNA polymerase, 2,5µL of dNTPs (10mM), 2µL of 10X NEB Buffer2, 0,2µL of 100X NEB BSA and 4.8µL of water was added into the mixture and incubate at 12°C for 20 minutes to fill-in. The template was purified using PCR clean-up column (Nalgen) and verified by electrophoresis. *In vitro* transcription of the dsDNA was done using 3µL of the purified tamplate with T7 transcription Kit (New England Biolabs E2040S) overnight, the gRNAs were then purified using RNAeasy mini Kit (Qiagen 74104).

CRISPR/Cas9 injection and generation of mutants

To generate mutations in *gnrh-r3*, 2nL of an injection mixture composed of 25ng/µL of each sgRNAs for *gnrh-r3* and 1µL of Cas9 protein (5µg/µL) and 1µL of phenol-red were injected into the yolk of 1 to 2 cells stage embryos. The mixture was heated at 37°C for 5 minutes prior to the injection to allow the formation of the sgRNA-Cas9 complex.

The sgRNAs target sites for *gnrh-r3* (GeneBank accession number: NC_007127) located in the exon 1 was 5'-TGCTGCTCAGGCCCGTGTGG-3', in the

exon 2 5'-TCACGTACAGCGTCACAAAG-3', and in the exon 3 was 5'-AGCAGGTGGTGGACGTACTC-3' (**Supplemental Table 2**) and were injected all together at one to two cells. Founders were identified by PCR and out crossed to wild-type animals (F1), then in-crossed to generate heterozygote and homozygote F2. For each target site specific primers were designed to genotype the mutations. *Gnrh-r3* mutant lines were genotyped with primers Forward 5'-GTGAAACTGGATCTCTCTGTCCTT-3' reverse 5'-GGTGTCTGTCCAGACTGATG -3' (**Figure 4, Supplemental Table 3**)

Characterization of reproductive capacities

Analysis of the reproductive capacities of the fish were carried-out where four pairs of sexually mature fish of the same age were placed in spawning containers and separated by a divider overnight. Pairs of wild-type males and females, mutant males and females, and combinations of one wild-type male paired with one mutant female, and one wild-type female paired with one mutant male were analyzed. In the morning, immediately after the lights on, dividers were removed, and fish allowed to spawn for 1.30 h before eggs were collected from each container. The total number of eggs was counted for each pair to obtain fecundity numbers, and the percentage of eggs fertilized was quantified via embryonic development at 6 hours post fertilization (hpf). The experiment was repeated four times.

Gonadal morphology with stain in paraffin sections

Adult zebrafish aged 10 to 12 months were sacrificed using a high dose of Tricaine (400mg/100ml) (#A5040 Sigma-Aldrich) (Wilson et al., 2009) and gonads were collected. The gonads were fixed in Bouin's solution for 48 h at 4°C. The gonads were

then rinsed in 70% ethanol and dehydrated in an increasing ethanol series until 100% ethanol, cleared in Histoclear (#H2779, Sigma-Aldrich) and embedded in Paraplast Plus (Sigma Chemical Co., St. Louis, MO, USA). Serial sections 5 µm thick of the gonads were obtained with Leica RM 2155 microtome, mounted on slides, de-paraffinized and rehydrated. Sections processed for histology were stained with a Hematotoxylin of Harris (Sigma Chemical Co, USA) and Chromotrope 2R) (Sigma Chemical Co, USA). The sections were then dehydrated and mounted with Entellan (107961- Merck Millipore).

III. RESULTS

IIIa. Exogenous Gnrh3 exposure affects gonadotropins expression in sex-specific manner

To characterize the effect of exogenous Gnrh3, on the hypothalamic reproductive response we isolated fish for 10 days in artificial water before introducing Gnrh3 (10^{-9}M) for 30 minutes directly in the water. The addition of Gnrh3 resulted in a significant increase in the expression of pituitary gonadotropins *fshb* (**Figure 1 A**) and *lhb* (**Figure 1 C**) mRNA in females in comparison to the control group (n=3). The increase was higher for *fshb* with a 6-fold increase when compared to the control, than for *lhb.* In contrast, for males exposure to exogenous Gnrh3 did not change the level of expression of the pituitary gonadotropins *fshb* (**Figure 1 B**) and *lhb* (**Figure 1 D**) in comparison to the control group.

To determine whether the response to exogenous Gnrh was mediated via the olfactory organ, we next blocked the nares of the fish with tissue glue, waited one day for recovery and then exposed the animals to exogenous Gnrh3. In both females (**Figure 2A**, f*shb*, **C**, *lhb*) and in males (**Figure 2 B**, f*shb*, **D**, *lhb*) there was a significant decrease in gonadotropin expression in nares blocked animals in comparison to their control. The results indicate that the olfactory organ play a significant role in the response to Gnrh3 and the resulting expression of pituitary gonadotropins and ultimately in reproduction.

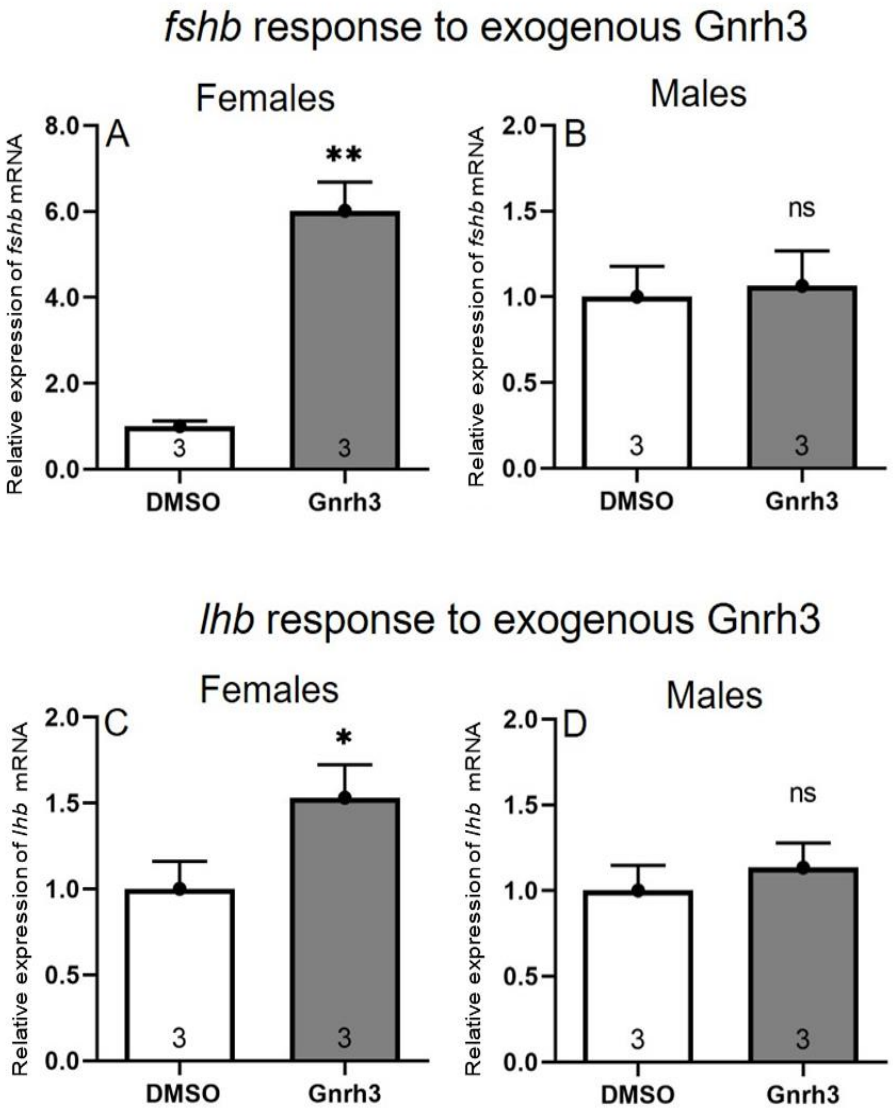


Figure 1: Exogenous application of Gnrh3 in water affects the expression of pituitary gonadotropes. Exogenous Gnrh3 significantly increases the expression of *fshb* (A) and *lhb* (C) mRNA in female zebrafish. In male zebrafish exogenous Gnrh3 did not affect mRNA expression of *fshb* (B) or *lhb* (D) in comparison the control group. **Data***:* expressed in Mean ±SEM with comparison of the control group. Housekeeping gene *beta-actin*. **Statistics** One-sample t-test (Prism8). *P< 0.05; **P<0.002; ***. P<0.0002. N=3 (N=1 represent grouped hypothalamus and pituitaries of 3 different fish).

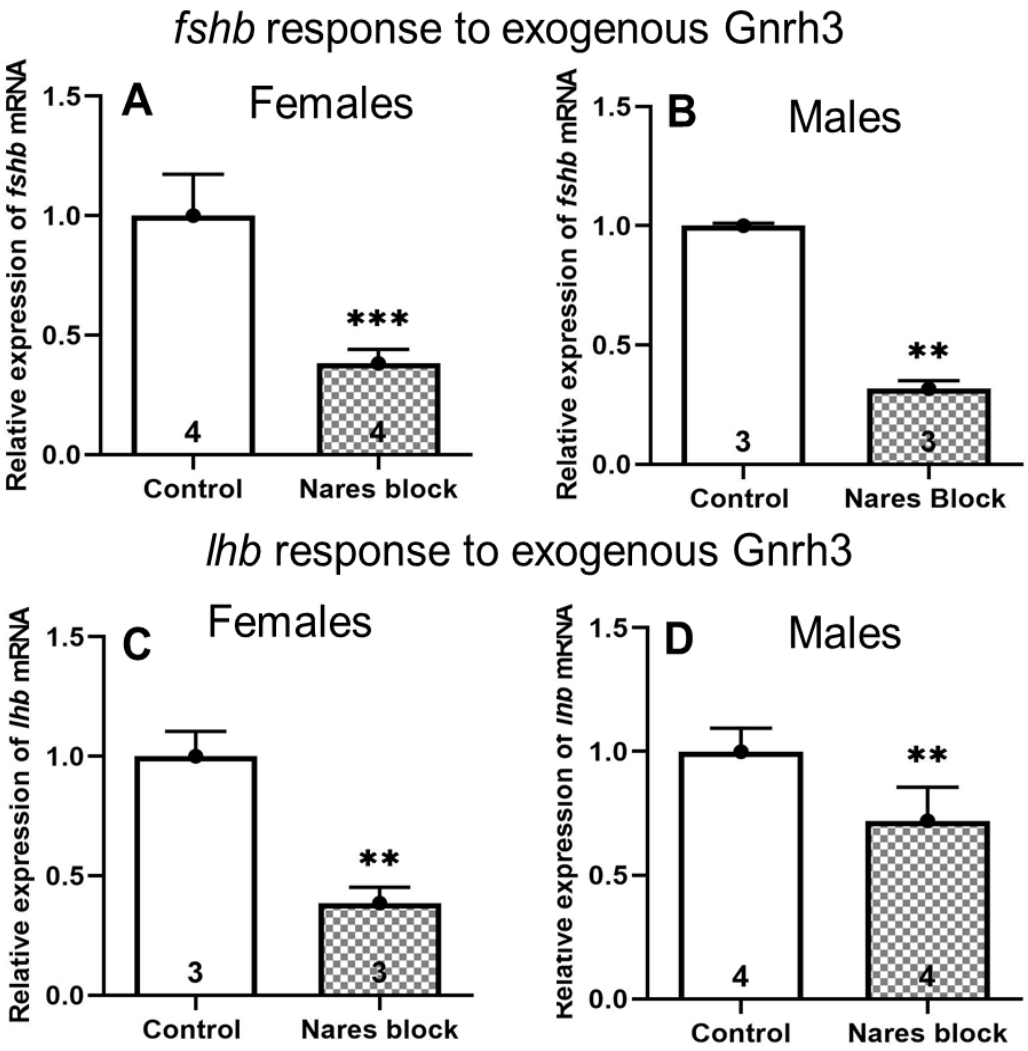


Figure 2: Nares block inhibit exogenous effect of Gnrh3 in pituitary gonadotropins expression. Nares block significantly decreased the expression of *fshb* in female (A) and male (B) but also *lhb* in female (C) and male (D). Data*:* expressed in Mean ±SEM with comparison of the control group. Housekeeping gene *beta-actin*. Statistics One-sample t-test (Prism8). *P< 0.05; **P<0.002; ***. P<0.0002. N=3 for males; N=4 for female (N=1 represent grouped hypothalamus and pituitaries of 3 different fish).

Knockout of *Gonadotropin Releasing Hormone Receptor-3* (*gnrh-r3*).

In order to generate mutations in *gnrh-r3* we designed sgRNAs targeting the following sites for *gnrh-r3* (GeneBank accession number: NC_007127) were 5'-TGCTGCTCAGGCCCGTGTGG-3' located into the exon1, 5'-TCACGTACAGCGTCACAAAG-3' located into the exon2 and 5'-AGCAGGTGGTGGACGTACTC-3' into the exon 3 (**Figure 3, Supplemental Table 2**) were injected all together at one or two cells. For each target site specific primers were designed to genotype the mutations. Putative *gnrh-r3* mutants was genotyped with primers forward: 5'- AGCTGTTCATCTTCCATACGGT-3', Reverse 5'-CCAGATGCTGTGTGTGTACCTT-3' for the exon 2 and Forward: 5'-GAAGATGACCCTCATCATTGTG-3' reverse: 5'-TCTCCAGTGTTCCTCCTTTGTT -3' for exon 3 (**Supplemental Table 3)**. Our sequencing of exon 2 and 3 did not show any CRISPR-based mutagenesis in *gnrh-r3*. (**Figure 3**) For exon 1 our primer mapping analysis show that there is a large deletion 750 base pair removing a large part of exon one and upstream region making it difficult to design. Ideal primer sequences for amplification as indicated in our primer mapping (**Figure 4, Table 1, and Supplemental Table 3**).

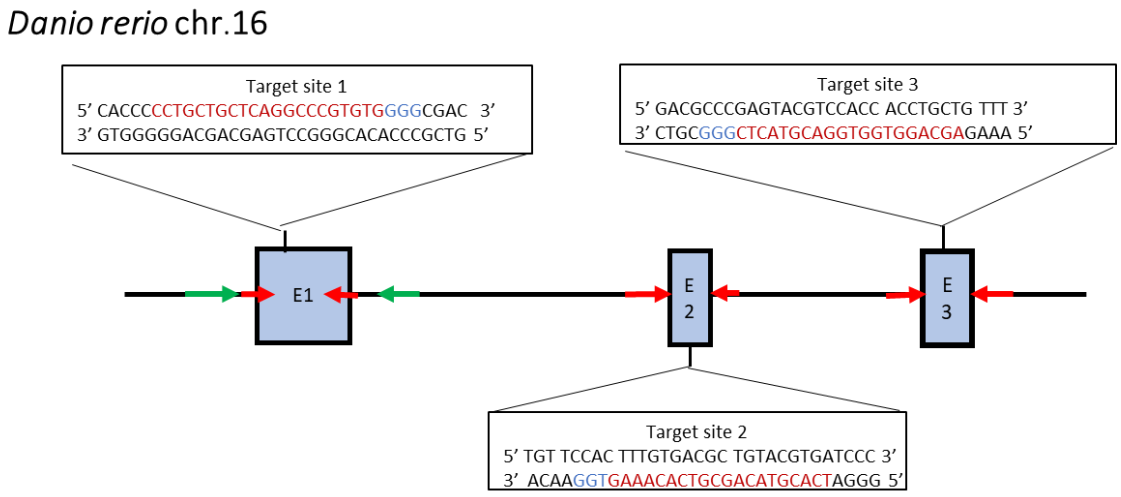
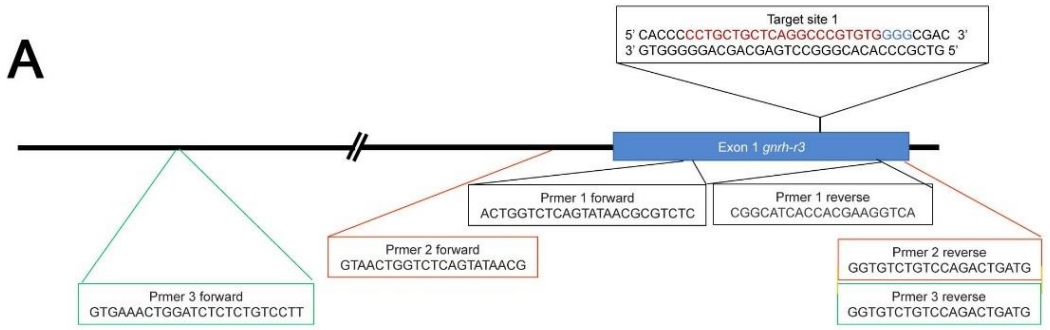


Figure 3: Localisation of the guides RNAs used (target sites) to induce mutations in *gnrh-r3* by CRISPR and the primers used to screen the injected fish by PCR (red arrows)



B

genotype	Primers 1	Primers 2	Primers 3
WT	Amplification 230 bp	Amplification 363 bp	Amplification 1379 bp
gnrh-r3$^{+/uv\Delta750}$	Amplification 230 bp	Amplification 363 bp	Amplification 600 bp and 1379 bp
gnrh-r3$^{uv\Delta750/uv\Delta750}$	No amplication	No amplification	Amplification 600 bp

Figure 4: Primer mapping of *gnrh-r3*$^{uv\Delta750/uv\Delta750}$ mutant. A) Location in wild-type genome (chromosome 16) of primer pairs used to map *gnrh-r3* deletion. **B**) Table showing amplicons for primer pair 1 (black), pair 2 (red) and pair 3 (green) in wildtype (WT), and mutant *gnrh-r3*$^{+/uv\Delta750}$ and *gnrh-r3*$^{uv\Delta750/uv\Delta750}$ animals.

Characterization of the CRISPR/Cas9-induced mutants by analysing their reproductive capacities through quantification of egg production and fertilization.

To characterize the reproductive capacities of the *gnrh-r3*$^{uv\Delta750/uv\Delta750}$ fish we performed pair-wise crosses of individuals fish and analyzed the resulting embryos for reproductive defects. Because animals heterozygous for the *gnrh-r3* mutation showed no reproductive defects, we focused on crosses using animals homozygous for the mutation. No difference in the number of eggs produced was observed between wild-type female or mutant female when crossed with wild-type males (**Figure 5**). However, there was a significant decrease in the fertilizable eggs produced by the *gnrh-r3*$^{uv\Delta750/uv\Delta750}$ females in comparison to the wild-type (**Figure 6**). The *gnrh-r3*$^{uv\Delta750/uv\Delta750}$ mutant females produce eggs (**Figure 5)** but most them are not mature and thus are not fertilizable (**Figure 6**), furthermore they cannot expand their chorions as evidenced by the size of the eggs (**Figure 7**).

When wild-type females were crossed with male *gnrh-r3*$^{uv\Delta750/uv\Delta750}$ mutants, no eggs were produced suggesting that mutant males do not initiate a mating response from the females (**Figure 5**), thus, making then behaviorally infertile.

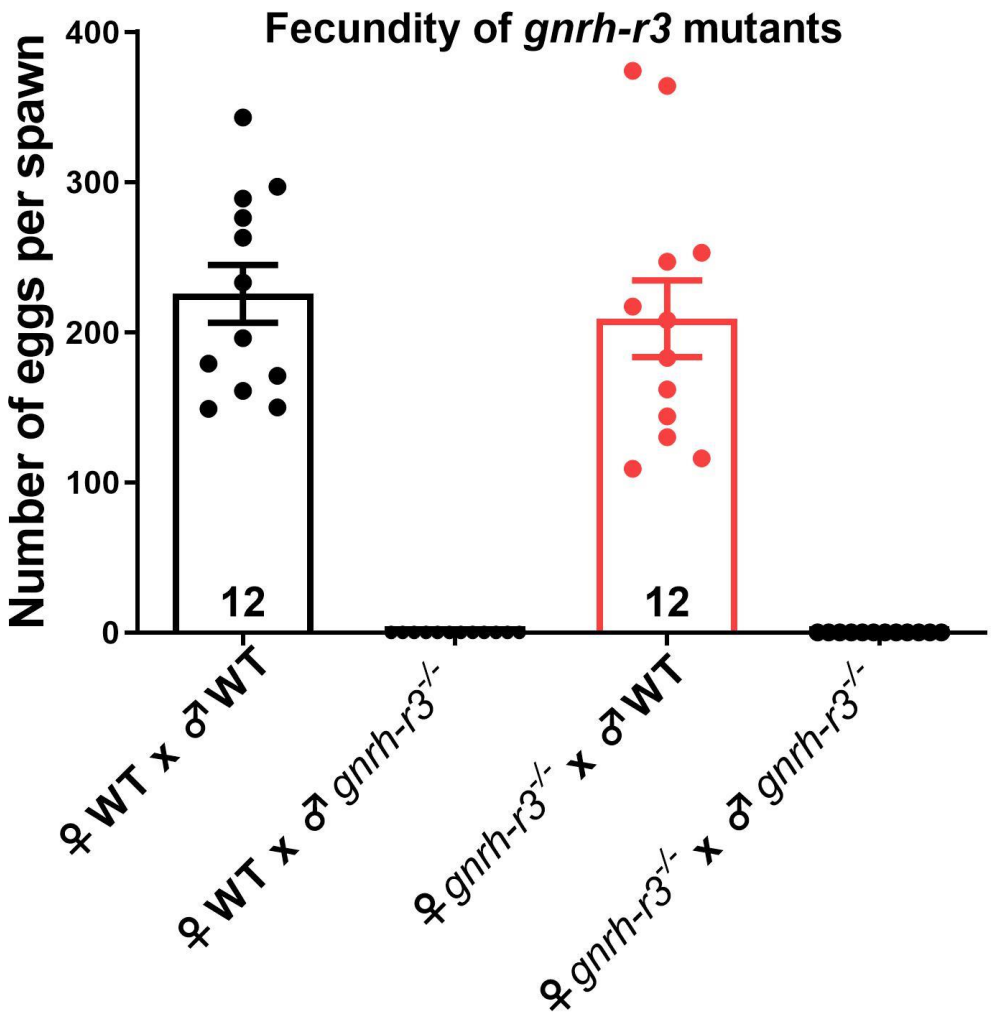


Figure 5: Number of eggs produced by individual crosses of wild-type and *gnrh-r3*$^{uv\Delta750/uv\Delta750}$ fish. (A) Number of eggs produced per spawn of one female paired with one male (n = 12). Mating was between *gnrh-r3*$^{uv\Delta750/uv\Delta750}$ or wild-type females with male *gnrh-r3*$^{uv\Delta750/uv\Delta750}$ or wild-type siblings. Data (n=12 crosses) are presented as mean ± SEM. Statistical significance P-values are calculated with *One-sample t-test* (Prism8). **P< 0.0332.*

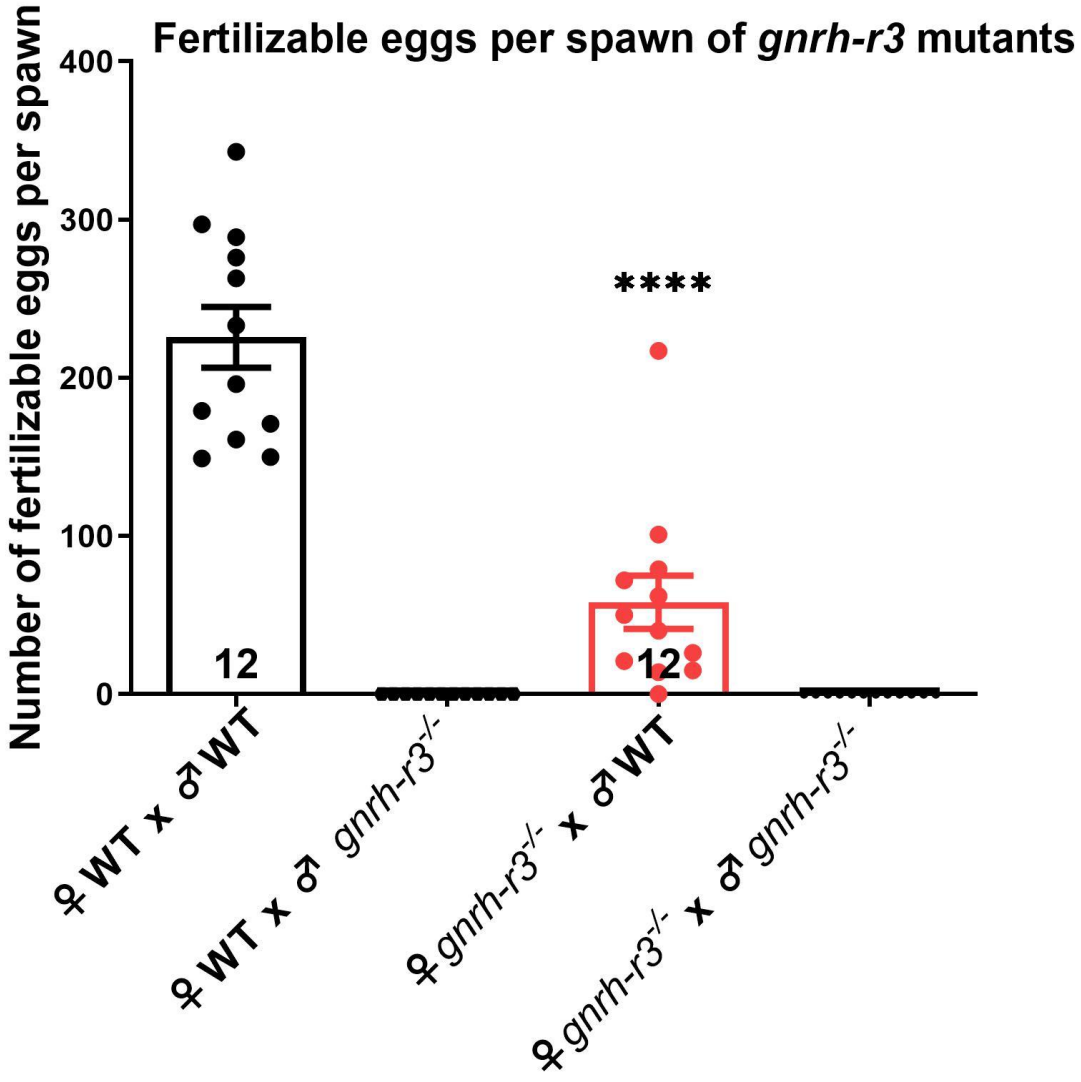


Figure 6: **Comparison of the ability to produce normal eggs between wild-type and *gnrh-r3*$^{uv\Delta750/uv\Delta750}$ females**. (A) Number of fertilizable eggs per spawn produced by wild-type (WT) females in comparison to the *gnrh-r3*$^{uv\Delta750/uv\Delta750}$ females. Mating was between *gnrh-r3*$^{uv\Delta750/uv\Delta750}$ or wild-type females with male *gnrh-r3*$^{uv\Delta750/uv\Delta750}$ or wild-type siblings. Data (n=12 crosses) are presented as mean ± SEM. Statistical significance P-values are calculated with *One-sample t-test* (Prism8). **P< 0.0332; **P<0.0021; ***. P<0.0002; ****P<0.0001*

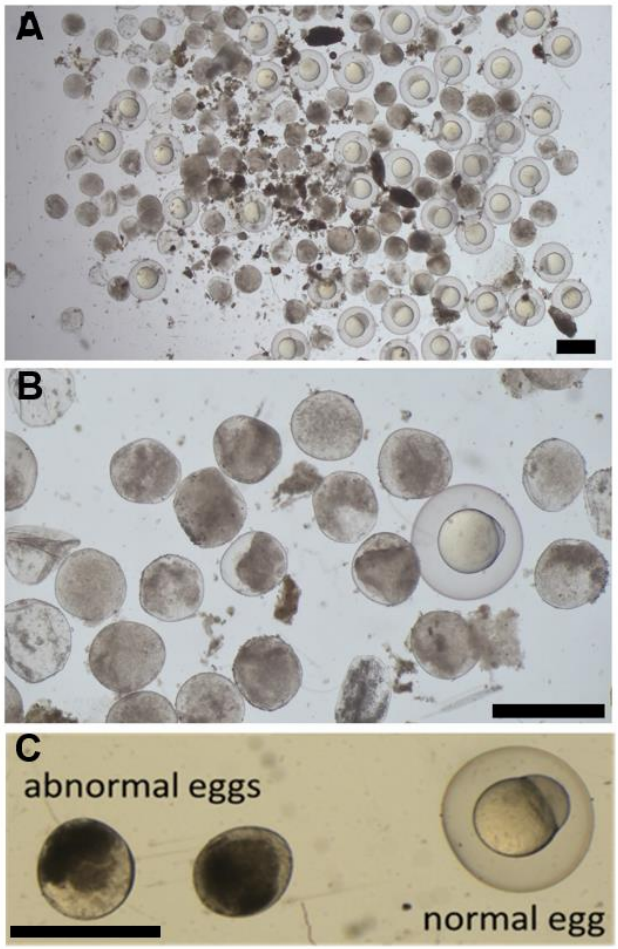


Figure 7: Sample of eggs produced by *gnrh-r3*$^{uv\Delta750/uv\Delta750}$ females where most of the eggs are abnormal. (A). Shows sample of eggs collected after a cross of *gnrh-r3*$^{uv\Delta750/uv\Delta750}$ female. (B, C) higher magnification where abnormal eggs can be seen without chorion extension in comparison to normal egg with chorion extension. Scale bar = 1000uM

IIId: Analysis of the morphology of the *gnrh-r3*$^{uv\Delta750/uv\Delta750}$ gonads

To better understand the drastic reproductive phenotypes, we next analyzed the morphology of the gonads of *gnrh-r3*$^{uv\Delta750/uv\Delta750}$ mutants. The dissection of ovaries showed that in comparison to the wild-type (**Figure 8 A, A1**) *gnrh-r3*$^{uv\Delta750/uv\Delta750}$ mutant ovaries have disintegrated later stages oocytes and are defective in their maturation (**Figure 8B, B1)**. In contrast, *gnrh-r3*$^{uv\Delta750/uv\Delta750}$ males that are totally infertile and do not reproduce showed that in comparison to the wild-type males (**Figure 8C, C1**), the *gnrh-r3*$^{uv\Delta750/uv\Delta750}$ mutants showed malformed testis that prevented connections with the cloaca of the animal (**Figure 8C, D, D1**).

To better understand these malformations, the morphology and phenotype of the different cell-type of the gonads were examined using haematoxylin-chromotrope 2R to label of the ovaries and testis. The analyses of the ovaries showed that the *gnrh-r3*$^{uv\Delta750/uv\Delta750}$ females have finer perivitellin membrane in the later stages of oocytes maturation (**Figure 9B),** in concordance with the observations shown in **Figure 8B1** of disintegrated oocytes, compared to the wild-type (**Figure 9A**). The *gnrh-r3*$^{uv\Delta750/uv\Delta750}$ males have mostly spermatogonia and do not show organized testis (**Figure 9 D**) in comparison the wild-type (**Figure 9C**). Thus, these results show shed light on the crucial role of *gnrh-r3* gene in the regulation of zebrafish reproduction. The knockout experiments provided valuable insights into how *gnrh-r3* influence gonadal development and maturation and reproduction in general in zebrafish.

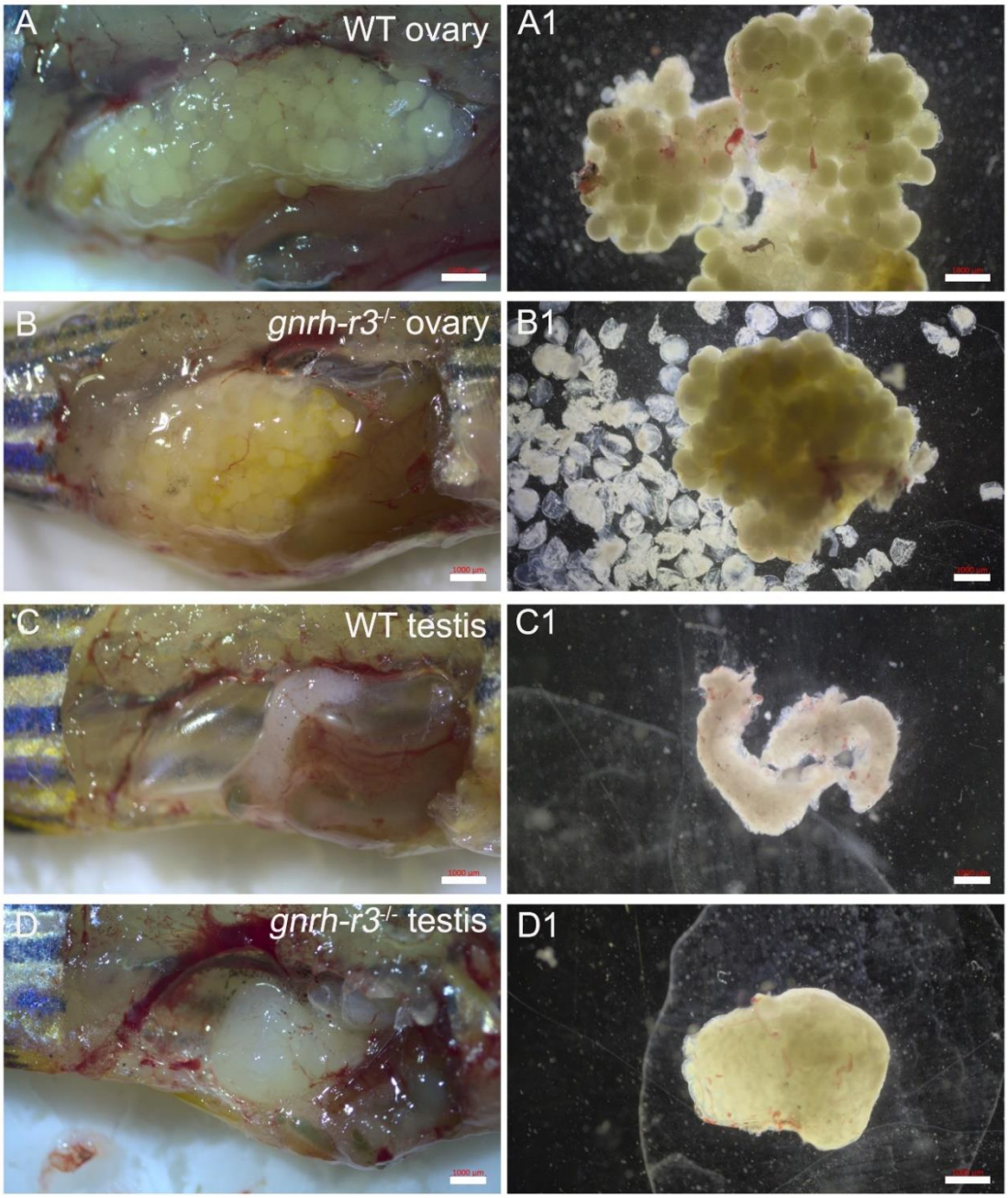


Figure 8: Phenotypes of wild-type and *gnrh-r3* mutant gonads (A, A1): wild-type ovaries and (B, B1) g*nrh-r3* mutant female ovaries showing disintegrated oocytes in the later stages with small ovary overall compared to the wild-type female. (C, C1): wild-type male testis showing the extension of the testis through the cloaca (red arrow) and (D, D1)**:** *gnrh-r3* mutant male testis showing a malformed testis without extension. Data: n=3 fish, scale bar 1000uM.

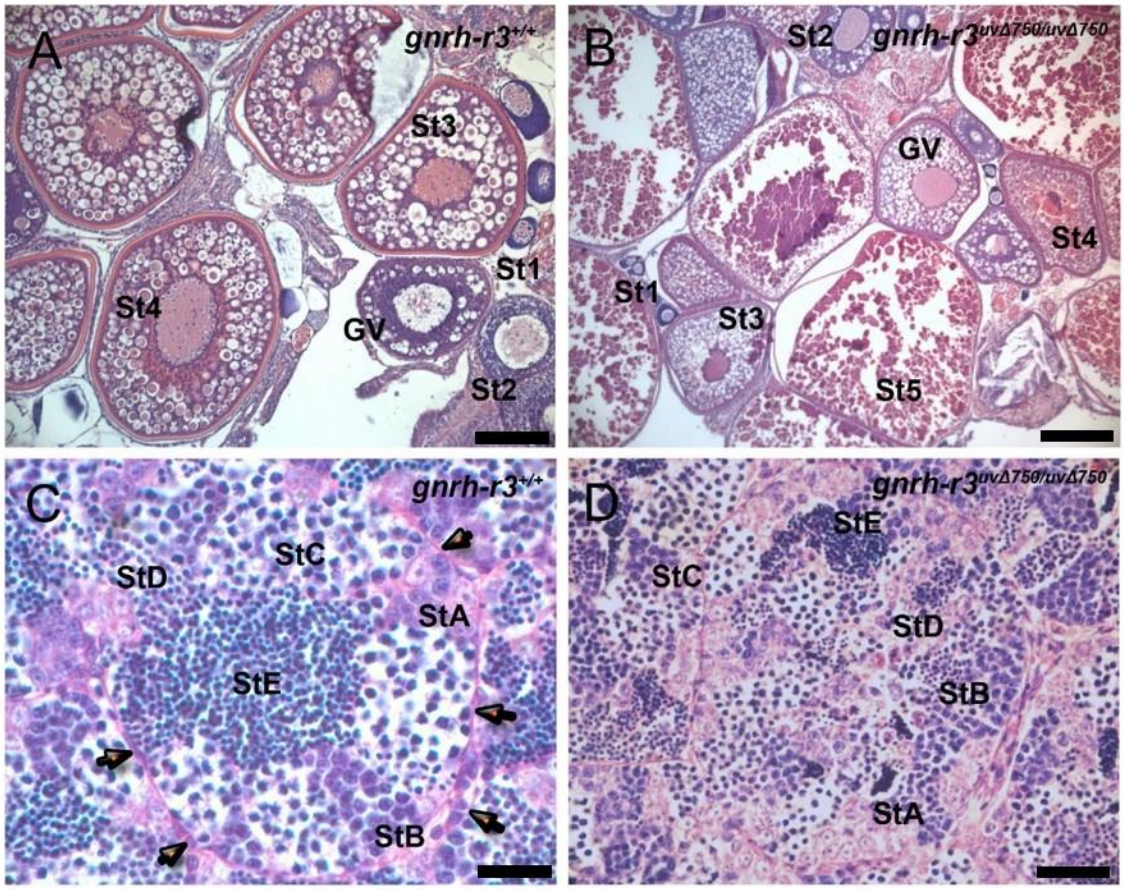


Figure 9: Morphology of gonads of *gnrh-r3*$^{uvΔ750/uvΔ750}$ mutants. **A**): Ovary of wild-type female (n=3 fish) showing all the stages of oocyte development from the early stages: oogonia (St1) and early follicles stages (St2), recognizable by visible germinal vesicle (GV), to the transitional stage or cortical alveolus stages (St3) with cortical granules (CG, red arrow) and the late stages or vitellogenic stages (St4) with the appearance of yolk vesicle (YV) surrounding the germinal vesicle. **B**): Ovary of *gnrh-r3*$^{uvΔ750/uvΔ750}$ female (n=3 fish), where later stages are affected: vitellogenic stages (St4) and late vitellogenic stages (St5) have finer and/or disintegrated membranes. **C**): Testes of wild-type males (n=3 fish) showing normal organization. Early stages of spermatogenesis: spermatogonia (StA), characterized by large nuclei, are organized in

two clusters located in the periphery. Near to the spermatogonia are the leptotene stage cells (StB), with small nuclei but which are larger those of the primary spermatocytes (StC). Later stages of spermatogenesis: the secondary spermatocytes (StD) and more visibly the spermatozoa (StE) are located in the middle of the circle. **D**): Testis of *gnrh-r3*$^{uv\Delta750/uv\Delta750}$ mutant male (n=3 fish) show abnormal organization of the cells at different stages of maturation (arrowheads), with visible earlier stages of spermatogenesis: spermatogonia (StA), the leptotene stage (StB) and the primary spermatocytes (StC). The spermatogonia occupy the majority of the circle and are located throughout the testes. StA: spermatogonia, StB: leptotene stage, StC: zygotene stage primary spermatocytes, StD: secondary spermatocytes, StE: spermatozoa. Data: n=3 fish, scale bar 30uM.

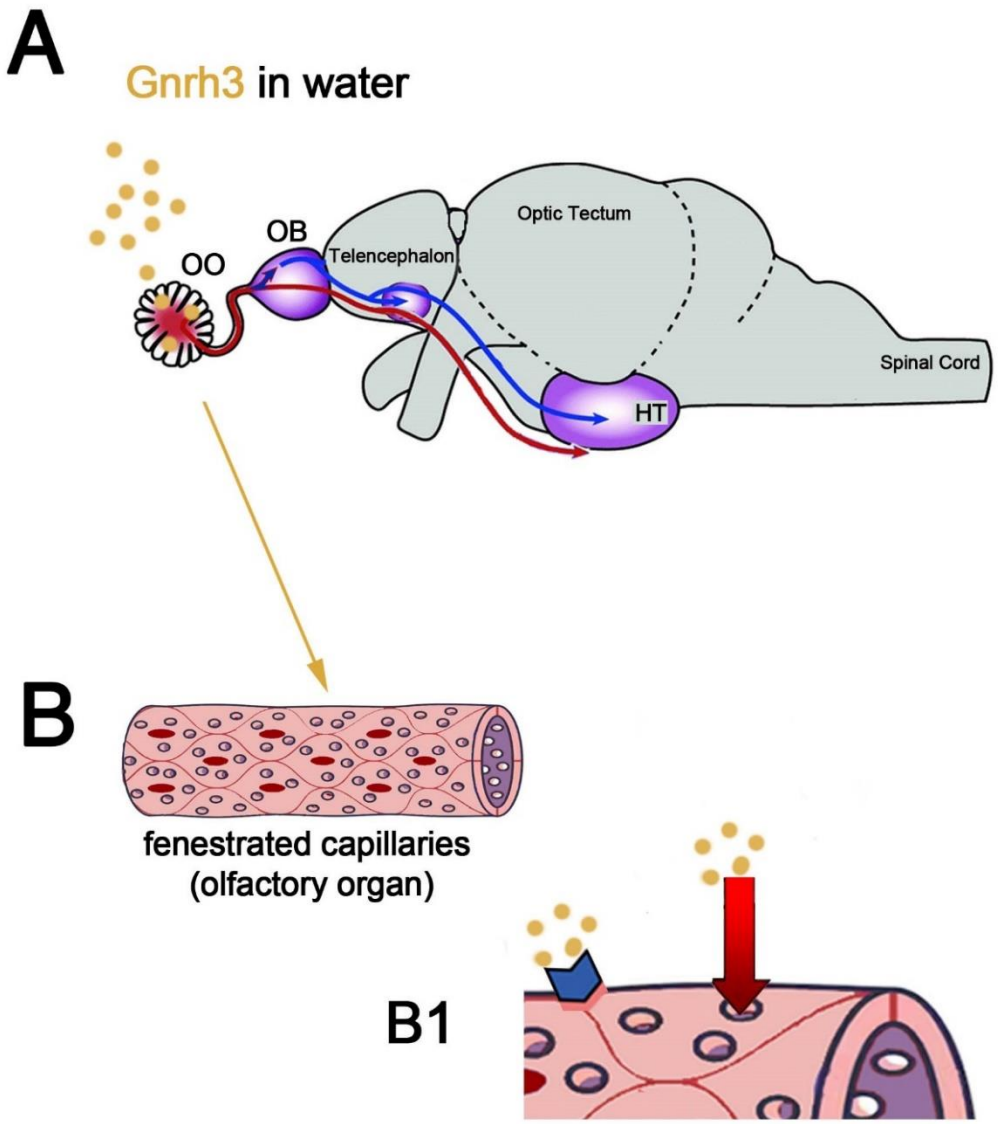


Figure 10. Model of Gnrh-R3 control of reproduction in zebrafish (A) Gnrh3 is released into the water from conspecifics and enters the olfactory organ. Gnrh3 interacts with the fenestrated blood vasculature of the olfactory organ (OO) via Gnrh-r3 (B1, blue) or passes through the blood vessels BV (B1, red arrow), and acts on the pituitary via blood vasculature (red tract). The potential for activation of neural pathways via the hypothalamus is not discounted (A, blue tract; see Yabuki et al., 2016 for example).

IV. DISCUSSION

Exogenous Gnrh3 exposure has sex-specific response in zebrafish

The exposure to exogenous Gnrh3 significantly upregulated both *fshb* and *lhb* mRNA expression in female zebrafish. The significant increase in *fshb* and *lhb* mRNA expression following the exogenous exposure indicates that Gnrh3 directly stimulates the pituitary to upregulate these gonadotropins via the olfactory organ. The 6-fold increase in *fshb* mRNA expression compared to the control group, indicate a potent stimulatory effect of Gnrh3 on this gonadotropin in particular. This differential sensitivity reflects the extensive vascularization of the zebrafish olfactory organ (Palominos and Whitlock, 2021; Palominos et al., 2022) but more importantly the vascularization pattern of the pituitary, where Fshb-expressing cells are more directly exposed to blood flow, enhancing their responsiveness to circulating factors (Golan et al., 2015). Additionally, the distinct roles of Fsh and Lh in ovarian follicle development and ovulation could account for the more significant *fshb* upregulation, as Fsh primarily governs folliculogenesis, and Gnrh plays an important role in the switching male to female in the gilthead seabream (Soverchia et al., 2007) while Lh initiates ovulation (Takahashi et al., 2019).

In contrast to females, exogenous Gnrh3 exposure did not alter *fshb* or *lhb* expression in male zebrafish. This sex-specific difference may be attributable to variations in the regulation of the hypothalamic pituitary gonadal (HPG) axis. Studies have shown that male and female fish exhibit sex-specific responses to pheromones where for example in zebrafish males but not females respond to exogenous prostaglandin (Yabuki et al., 2016). The absence of a significant response to

exogenous Gnrh3 in males could indicate that, under basal conditions, male gonadotropin expression may be regulated through alternate pathways or hormonal regulation. Yet the olfactory system remains integral to maintaining basal or regulated levels of these hormones by sensing the level of Gnrh3 and triggering a feedback loop that modulate pituitary gonadotropin release on the hypothalamic pituitary axis (Trudeau et al., 1991; Zhai et al., 2018) thus shutting down the release of gonadotropins until it is needed to produce steroids. The olfactory organ's ability to detect hormonal signals suggests a pathway by which external stimulation is transduced into neuroendocrine signals can modulate the hypothalamic pituitary axis (Bagade and Suresh 2022), influencing both physiological and behavioral responses for reproduction (Doving 2007; Abraham et al., 2010).

Blocking the nares of females eliminated the hypothalamic responses to exogenous Gnrh3 supporting the hypothesis that the olfactory organ mediates this sex-specific response. And, blocking the nares of males showed a significant decrease in the basal level of *lhb* and *fshb*, suggesting a modulation of the reproductive axis. These findings are consistent with previous research demonstrating that olfactory inputs, such as pheromones, modulate gonadotropin release and stimulate reproductive behaviors in fish (Stacey et al., 2003; Yabuki et al., 2016).

Our observations underscore the pivotal role of the olfactory system in detecting environmental and hormonal cues essential for regulating reproduction. The role of exogenous Gnrh3 may have in fact "rescued" the *gnrh2* and *gnrh3* loss of function mutants (Spicer et al., 2016; Marvel et al., 2018) because if the mutant animals were not isolated from their wildtype and heterozygous conspecifics, the females would clearly have been able to mount a gonadotropin response, and the males' response may in fact be secondary to female pheromone release as observed in goldfish.

Loss of Gnrh-R3 *gnrh-r3*$^{uv\Delta750}$ affects fecundity and fertility in zebrafish

Our targeting of Gnrh receptor function using CRISPR/Cas9 to produce a loss of function (LOF) mutant in *gnrh-r3* revealed the absolute necessity of Gnrh-R signaling for reproduction in zebrafish as the *gnrh-r3*$^{uv\Delta750}$ mutation affects both female and male fecundity and fertility. Gnrh and Gnrh-receptors are shown to be expressed in the fish gonads (Yu et al., 1998). In zebrafish it was shown that *gnrh3* and *gnrh-r3* transcripts are significantly more highly expressed in the gonads than *gnrh2* or the others *gnrh-rs* respectively (Fallah et al., 2020). The expression of *gnrh3* and *gnrh-r3* in the gonads likely have a direct role in regulating local hormone signaling that is essential for gametogenesis (Soverchia et al., 2007; Fallah et al., 2020). Because Gnrh-R3 by the action of Gnrh3 ligand is involved in promoting spermatogenesis (Fallah et al., 2020), its absence could explain the disorganized testis structure with high number of spermatogonia and the reduced number of spermatozoids seen in *gnrh-r3*$^{uv\Delta750/uv\Delta750}$ males.

In females, *gnrh-r3* expression in the ovaries may be necessary for proper oocyte maturation, because *gnrh-r3* is the first of the *gnrh-receptors* to be expressed in the ovary (Corchuelo et al., 2017). The observed defects in oocyte maturation in *gnrh-r3*$^{uv\Delta750/uv\Delta750}$ mutants indicate that and Gnrh-R3 signaling in the gonads contributes to the hormonal regulation necessary for full maturation of oocytes, possibly by influencing Fsh and Lh action in the ovaries, two hormones essential for oocyte maturation as mutation on *fshb* and *fshb-r* incudes lack of female in zebrafish (Xie et al., 2017).

To further explore the role of exogenous Gnrh signaling, we next targeted Gnrh receptor function. We used CRISPR/Cas9 to produce a 750 bp deletion in *gnrh-r3* gene that included 5' untranslated sequences as well as exon1. Mutation in *gnrh-r3* gene

affects both female and male fecundity and fertility in zebrafish. Gnrh and Gnrh-receptors are shown to be expressed in the fish gonads (Yu et al., 1998). In zebrafish its was shown that *gnrh3* and *gnrh-r3* transcripts was significantly more present in the gonads than *gnrh2* or the others *gnrh-rs* respectively (Fallah et al., 2020) Their expression in the gonads likely have a direct role in regulating local hormone signaling that is essential for gametogenesis (Soverchia et al., 2007; Fallah et al., 2020). Because Gnrh-R3 by the action of their ligand is involved in promoting spermatogenesis (Fallah et al., 2020). Its absence could explain the disorganized testis structure with high number of spermatogonia and the reduced number of spermatozoids seen in *gnrh-r3*$^{uv\Delta 750/uv\Delta 750}$ males.

In females, Gnrh-r3 expression in the ovaries may be necessary for proper oocyte maturation. Because *Gnrh-r3* is the first to be expressed in the ovary (Corchuelo et al., 2017). The observed defects in oocyte maturation in *gnrh-r3*$^{uv\Delta 750/uv\Delta 750}$ mutants female indicate that while Cck signaling in the brain are important for normal folliculogenesis Uehara et al., 2024; Cohen et al., 2024) in zebrafish, Gnrh3 and Gnrh-R3 signaling in the gonads contributes to the regulation of oocytes maturation (Fallah and Habibi 2020), possibly by influencing Fsh and Lh action in the ovaries, two hormones essential for oocyte maturation as mutation on *fshb* and *fshb-r* incudes lack of female in zebrafish (Xie et al., 2017).

Gnrh-R3 and the olfactory organ: Role in Reproductive Behavior

In many fish species, olfactory signals (pheromones) play a critical role in triggering reproductive behaviors, such as courtship, mate recognition, and synchronization of spawning (Hurk and Lambert 1983; Sorensen and Stacey 2004).

The expression of Gnrh-R3 in the olfactory organ suggests that this receptor play a crucial role that allows fish to detect and respond to exogenous reproductive pheromones such as Gnrh3.

Our results support the role of exogenous Gnrh3 in controlling reproduction via the olfactory epithelium (**Figure 10A, red**) acting through blood capillaries (**Figure 10B**) either via Gnrh-R3 (**Figure 10B1, blue**) or the fenestrations of the capillary system (**Figure 10B1, red arrow**). A characteristic of endocrine systems, such as the hypothalamic portal system, is the evolution of mechanisms that tightly regulate blood perfusion and oxygenation via fenestrated capillaries (Schaeffer et al., 2011). In mammals their presence has been reported in the olfactory organ (Grevers and Herrmann1987), and olfactory capillary systems have been shown to respond to odor stimuli (Chaigneau et al., 2003).

Adult zebrafish also have an extensive blood vasculature associated with the olfactory organ (Palominos et al, 2022), which is capable of taking up large molecules such as Gnrh peptides (Golan et al, 2015)**.** The highly vascularized olfactory organ permits nasally administrated molecules to enter the vasculature and rapidly reach the pituitary (Thorne et al. 2004) as evidenced by studies in, for example, frogs (Julien et al, 2019) and humans (Iwamoto et al, 2009), where nasal application of Gnrh analogues can trigger sperm production and increase serum gonadotropin. But also, Gnrh is shown in mammals to increase blood flow (Kanazawa et al, 2017) thus allowing the peptide to reach the pituitary gland more quickly. Thus, the blood vasculature associated with the olfactory organ could be the mechanism by which exogenous Gnrh acts on the pituitary, or in other words an “olfacto-portal system”. Receptors for Gnrh and Fsh are expressed within the vasculature of humans (Chegini et al., 1996, Stilley & Segaloff 2018) and Gnrh analogues used to treat sex steroid sensitive-malignancies are

associated with a high prevalence of cardiovascular mortality (Sturgeon et al., 2019). In zebrafish, Gnrh-R3 has been localized to the olfactory organ by antibody staining (Whitlock et al., 2006) and mRNA expression (Corchuelo et al., 2017), thus supporting a role for Gnrh signaling from the olfactory organs themselves.

In conclusion, our results have uncovered a new interaction between endogenous and exogenous Gnrh in fishes where the exogenous Gnrh, acting through an “olfacto-portal system”, can act to regulate reproduction. Future studies are needed to determine whether this situation is linked to the life history of fishes (schooling versus non-schooling) and whether there is also a neural component involved in the olfactory organ-mediated response.

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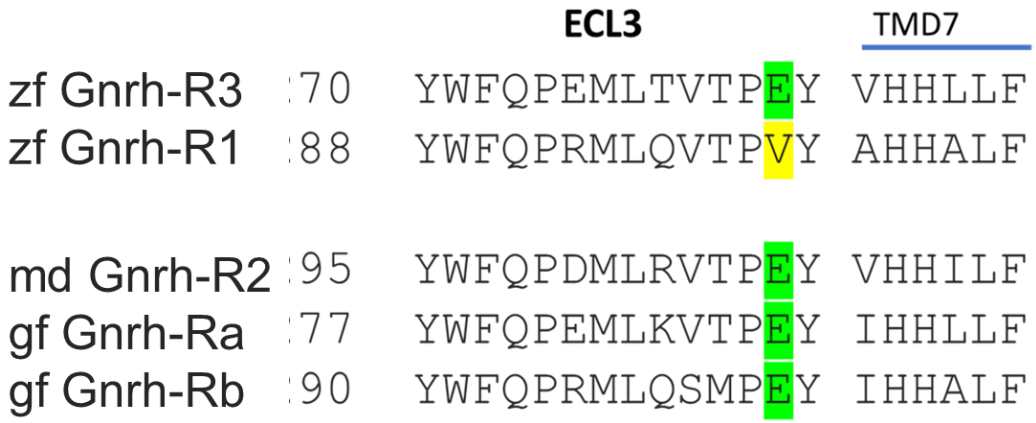
VI. ANNEX



Supplemental Figure 1: **Alignment of the Gnrh-Rs (non-mammalian type 1)** amino acid sequences highlighting the substitution of Glutamic acid (E) into Valine (V) by zfGnrh-r1 the extracellular loop 3 (ECL3). zf: zebrafish; md: medaka fish; gf: goldfish.

Supplemental Table 1: List of primers of used of qPCR

Gene	Forward	Reverse	Length
lhb	GGCTGGAAATGGTGTCTTCT	CCACCGATACCGTCTCATTTAC	107 BP
fshb	GCTGGACAATGGATCGAGTTTA	CTCGTAGCTCTTGTACATCAAGTT	92 BP
beta-actin1	CGAGCAGGAGATGGGAACC	CAACGGAAACGATCATTGC	102 BP

Supplemental table 2: List of guides RNAs for *gnrh-r3* CRISPR/Cas9 mutant generation

Gene	Id	Exon	Target sequence for CRISPR/Cas9
gnrh-r3	GeneBank NC_007127	Exon 1	5'-TGCTGCTCAGGCCCGTGTGG-3'
gnrh-r3	GeneBank NC_007127	Exon 2	5'-TCACGTACAGCGTCACAAAG-3'
gnrh-r3	GeneBank NC_007127	Exon 3	5'-AGCAGGTGGTGGACGTACTC-3'

Supplemental table 3: List of primers for *pnx* CRISPR/Cas9 mutant genotyping

Gene	Id	Exon	Target sequence for CRISPR/Cas9
gnrh-r3	GeneBank NC_007127	Exon 1	Primer 1 forward 5'-ACTGGTCTCAGTATAACGCGTCTC-3' Primer 1 reverse 5'-CGGCATCACCACGAAGGTCA-3' Primer 2 forward 5'-GTAACTGGTCTCAGTATAACG-3' Primer 2 reverse 5'-GGTGTCTGTCCAGACTGATG-3' Primer 3 forward 5'- GTGAAACTGGATCTCTCTGTCCTT-3' Primer 3 reverse 5'-GGTGTCTGTCCAGACTGATG -3'
gnrh-r3	GeneBank NC_007127	Exon 2	Forward 5'-AGCTGTTCATCTTCCATACGGT-3' Reverse 5'-CCAGATGCTGTGTGTGTACCTT-3'
gnrh-r3	GeneBank NC_007127	Exon 3	Forward 5'-GAAGATGACCCTCATCATTGTG-3' Reverse 5'-TCTCCAGTGTTCCTCCTTTGTT-3'