Neuroendocrine Regulation of Reproduction in Fish

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By

Jithine Jayakumar Rajeswari

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OR

Dean
College of Graduate and Postdoctoral Studies
University of Saskatchewan
116 Thorvaldson Building, 110 Science Place
Saskatoon, Saskatchewan S7N 5C9 Canada

Abstract

Fish is an essential source of food, and it is important to improve fish yield in aquaculture to enhance food production and sustainability. Several lines of research are in progress to increase reproductive capacity in cultured fishes. Hormones play a critical role in fish reproductive success and endocrine approaches are being successfully tested in stimulating fish reproduction. In this context, my thesis research aimed to further characterize three relatively new orphan ligands and their role in the regulation of reproductive hormones and oocyte maturation in fish. Phoenixin-20 (PNX-20) is a newly identified peptide with endocrine-like functions in mammals. Nesfatin-1 is an 82 amino acid multifunctional peptide with hormone-like actions in vertebrates. Nesfatin-1-like peptide (NLP) is 77 amino acids long, resembles nesfatin-1, and share similar biological actions. Based on the available information from mammals and fish, I hypothesized that PNX is stimulatory, while both nesfatin-1 and NLP are inhibitory to reproductive hormones and oocyte maturation in teleosts. I tested this hypothesis using goldfish and zebrafish, two well-characterized models in neuroendocrinology. qPCR-based gene expression results indicated a widespread distribution of PNX in central and peripheral tissues of zebrafish. Immunohistochemical localization of PNX and its putative receptor (SREB3) in the gonads suggested a role of PNX in zebrafish reproduction. *In vivo* administration of PNX-20 upregulated reproductive regulatory hormonal transcripts in the hypothalamus and gonads of zebrafish. *In vitro* incubation of PNX-20 upregulated all vitellogenin transcripts in zebrafish liver cells. In addition, incubation of ovarian follicles with PNX-20 promoted oocyte maturation in zebrafish. Together, my research outcomes supported my hypothesis that PNX-20 promotes reproductive functions in zebrafish, by positively influencing hormones, vitellogenesis and oocytes. For both nesfatin-1 and NLP studies, I used male and female goldfish as a research model. Single intraperitoneal (IP) injection of synthetic gfnesfatin-1 and gfNLP (50 ng/g body weight) suppressed several reproductive regulatory hormonal mRNAs in both sexes. These include the gonadotropin-releasing hormones (hypothalamus), kisspeptin system (multiple tissues), gonadotropin β subunits (pituitary), gonadotropin receptors and genes involved in sex steroidogenic synthetic pathway (gonads). In addition, both peptides upregulated the expression of several reproductive suppressors in goldfish, including the gonadotropin inhibitory hormone and anti-Müllerian hormone. Both nesfatin-1 and NLP decreased serum testosterone and

estradiol levels in male and female goldfish at 60 minutes post-injection. Incubation of ovarian follicles with NLP suppressed oocyte maturation in zebrafish. Overall, my results suggested that both nesfatin-1 and NLP negatively influence aspects of reproductive biology in fish by suppressing the expression of critical hormonal mRNAs, gonadal steroids, and oocyte maturation. The results supported my hypotheses about nesfatin-1 and NLP. While limitations exist, my research identified PNX-20 as a positive regulator, and both nesfatin-1 and NLP as negative regulators of reproductive biology of fish. This is the major contribution of my thesis research. Future research to extend these findings to male fish and commercially cultured fish, and to understand the mechanism of action of these hormones is essential.

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A substantial part (data chapters) of this thesis has been published or under the submission process to consider for publication (introduction chapter) in peer-reviewed journals.

Dedication

I dedicate my Ph.D. thesis to everyone who has helped, guided and assisted me throughout my graduate studies. First, I would like to dedicate this thesis to my family members, who have always encouraged me to pursue my ambitions to date. This thesis is dedicated to my father, mother, and brothers who are eagerly waiting for my return home for the past three and a half years. Next, I would like to dedicate this thesis to my supervisor Dr. Suraj Unniappan for his continued guidance, encouragement and support throughout my graduate studies. You always serve as an inspiration to me and corrected me for the endless mistakes that I made with my research and writing. Your trust in me during the research program helped me in the completion of my program of study. I am deeply appreciative and thankful for your advice as a mentor, friend, science family member and a well-wisher over the past years at the University of Saskatchewan. You will always be a role model in my life, you and your family will be remembered in my whole life. I am privileged to complete my Ph.D. degree from your lab and also grateful to have worked under various operating grants that you have achieved over the years, which has helped me accomplish prompt completion of my thesis projects. I want to thank members of the Unniappan lab for their unconditional help and support. Final thanks to my friends in India and Canada who have always been part of my life journey.

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List of Abbreviations

AMH- Anti-Müllerian hormone

AR- Androgen receptor

Arc- Arcuate nucleus

AVPV- Arcuate and anteroventral periventricular nucleus

BSA- Bovine serum albumin

C4orf52- Chromosome 4 open reading frame 52 (also referred to as SMIM20, phoenixin precursor protein)

cGnRH-II- Chicken gonadotropin-releasing hormone-II (GnRH2)

CRISPR- Clustered regularly interspaced short palindromic repeats

CYP11a1- Cytochrome P450 family 11 subfamily A member 1 (Cholesterol side-chain cleavage enzyme, P450scc)

CYP11b1- Cytochrome P450 Family 11 Subfamily B Member 1 (also known as steroid 11β-hydroxylase, P450 11B1)

CYP17a- Cytochrome P450 Family 17 Subfamily A Member 1 (also known as steroid 17α-monooxygenase or 17α-hydroxylase/lyase)

CYP19a1a- Cytochrome P450, family 19, subfamily A, polypeptide 1a (gonadal aromatase)

CYP19a1b- Cytochrome P450, family 19, subfamily A, polypeptide 1b (brain aromatase)

DA- Dorsal hypothalamic area

DHEA- Dehydroepiandrosterone

DHP- Dihydroprogesterone (17α,20β-dihydroxy-4-pregnen-3-one)

DMRT1- Doublesex- and mab-3-related transcription factor 1

E1- Estrone

E2- Estradiol-17β

ELISA- Enzyme-linked immunosorbent assay

ER- Estrogen receptor

esr1- Estrogen receptor 1

esr2a- Estrogen receptor 2a

esr2b- Estrogen receptor 2b

FSH- Follicle-stimulating hormone

FSHβ- Follicle-stimulating hormone subunit beta

FSHr- Follicle-stimulating hormone receptor

GH-IGF-1- Growth hormone-insulin-like growth factor-1 system

GnIH- Gonadotropin Inhibitory Hormone

GnIHr- Gonadotropin Inhibitory Hormone receptor (GPR147)

GnRH- Gonadotropin-releasing hormone

GnRHr- GnRH receptor

GPα- Glycoprotein hormone α subunit

GPR54a- G-coupled protein receptor 54a (KISS1 receptor a)

GPR54b- G-coupled protein receptor 54b (KISS1 receptor b)

GPR173- G-coupled protein receptor 173 (phoenixin receptor, also known as SREB3)

GSDF- Gonadal soma-derived growth factor

GTHs- Gonadotropins

GVBD- Germinal vesicle breakdown

HPG axis- Hypothalamo-pituitary-gonadal axis

HPI axis- Hypothalamo–pituitary–interrenal axis

3β-HSD- 3β-Hydroxysteroid dehydrogenase

20β-HSD- 20β-hydroxysteroid dehydrogenase

HSD11b- 11β-Hydroxysteroid dehydrogenase

HSD17b/ 17-βHSD- 17β-Hydroxysteroid dehydrogenases

ICC- Immunocytochemistry

ICV- Intracerebroventricular injection

IHC- Immunohistochemistry

IP- Intraperitoneal injection

kiss-1a- Kisspeptin 1a (kiss1, in zebrafish)

kiss-1b- Kisspeptin 1b

kiss2- Kisspeptin 2

11-KT- 11-Ketotestosterone

LH- Luteinizing hormone

LHβ- Luteinizing hormone subunit beta

LHr- Luteinizing hormone receptor

LVI- Lipovitellin I

LVII- Lipovitellin II

mGnRHa- Mammalian GnRH analogue

MIH- Maturation inducing hormone

MIS- Maturation inducing steroids

MPF- Maturation-promoting factor

NEFA- Nucleic acid/DNA binding/EF-hand/Acidic amino acid rich region

Nesfatin-1- NEFA/nucleobindin-2-encoded satiety and fat-influencing protein-1

NLP- Nesfatin-1-like peptide

NUCB1- Nucleobindin 1(calnuc)

NUCB2- Nucleobindin 2

PCOS- Polycystic ovary syndrome

PD-ECGF- Platelet-derived endothelial cell growth factor

PeF- Perifornical area

PGC1α- Peroxisome proliferator-activated receptor gamma coactivator 1-alpha

PNX- Phoenixin

PV- Phosvitin

RIA- Radioimmunoassay

RT-qPCR- Real-time -quantitative polymerase chain reaction

20β-S- 17α,20β,21-trihydroxy-4-pregnen-3-one

sGnRH- Salmon gonadotropin-releasing hormone (GnRH3)

sGnRHa- Salmon gonadotropin-releasing hormone analogue

shbg- Sex hormone-binding globulin

siRNA- Small interfering RNA

SMIM20- Small Integral Membrane Protein 20

SOR- Supraoptic retrochasmatic nucleus

SREB3- Super conserved receptor expressed in brain 3 (Phoenixin receptor, also known as GPR173)

StAR- Steroidogenic acute regulatory protein

T- Testosterone

TALEN- Transcription activator-like effector nucleases

TGFβ- Transforming growth factor beta

VMH- Ventromedial hypothalamus

vtg- Vitellogenin

ZI- Zona incerta

ZFL cells- Zebrafish liver cells

Transition

The following chapter is the introduction to my thesis, which aims to place my research in the bigger scheme of fish endocrine physiology, reproduction and aquaculture. It highlights the importance of hormone-based reproductive strategies to increase aquaculture yield. The introduction will provide state-of-the-art knowledge on the neuroendocrine regulation of fish reproduction, major hormones, and their involvement in fish reproduction. It will specifically focus on the peptides studied under this thesis research. Towards the end of this chapter, I will provide the rationale for my research, the hypotheses and specific objectives.

Manuscript Details:

Some parts of the introduction chapter form selected sections in two review manuscripts under preparation for peer-review and publication.

1) Jithine Jayakumar Rajeswari and Suraj Unniappan. Neuroendocrine regulators of fish reproduction - a 2020 update.

2) Jithine Jayakumar Rajeswari, Kundanika Mukherjee and Suraj Unniappan. Phoenixin as a Metabolic and Reproductive Hormone: An Emerging Comparative Perspective.

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Review manuscript no.1 (final stage of preparation) - JJR prepared the outline, literature review and manuscript draft and revised it for submission. SU provided the original idea and helped in planning the outline, manuscript preparation and revisions.

Review manuscript no. 2 (in preparation) - Both JJR and KM equally contributed for the preparation of the original draft of the manuscript, revision, and submission. SU provided the original idea and helped in the organization of the contents, manuscript preparation and revisions.

Chapter 1
General Introduction

1.1. Introduction

Food security can be defined as secure access to enough food at all times for everyone (Food and Agriculture Organization, 2000). It was estimated that the world population in 2050 estimated to be between 9-11 billion (Röös et al., 2017; UN, 2013). According to the United Nations (UN), it will be a mammoth challenge to maintain food supply to meet the demand of the growing population and lowering the impact of it on the environment (United Nations, 2018). This will be an uphill task, especially for countries with a large population and are already suffering from environmental issues (FAO, 2017a; Suweis et al., 2015). Global agriculture is currently utilizing half of the available land area and 70% of freshwater available for human use (FAO, 2017b). The adverse environmental effects of current food production are very high. For instance, agriculture contributes one-third of the total greenhouse gas emission by any human activity (nearly 50% of which is contributed by livestock alone) (Schubel and Thompson, 2019). Surface runoff, impact and pollution by pesticides and fertilizer use, deforestation, loss of biodiversity and mass extinctions of species, are some of the other examples of impacts of agriculture on the environment. The UN predicts the food production should increase by 50% to meet the demands of the population by 2050 (FAO, 2017a). Studies suggest that it is highly unlikely to achieve this goal with existing practices and agriculture production rate (Schubel and Thompson, 2019). An effective solution to address this deficit is aquaculture, which should enable an increase in fish production for human consumption.

1.1.1. Aquaculture and food security

Fish and its availability are critical contributors to food security, which provide a relatively low-cost, reliable protein source for millions of people around the globe. Fish also provides additional nutrients, including omega-3 fatty acids, essential vitamins, and minerals (FAO, 2012; HELP, 2014). In terms of human health, fish is considered one of the healthiest food because of the low carbohydrate, saturated fat and cholesterol levels. According to the World Fish Center (WFC), in the last 50 years, the per capita fish consumption doubled (World Fish Center, 2002), and it is about 16.6% of the total animal proteins used for human consumption (FAO, 2012). Several studies suggest the potential of aquaculture and fisheries to

meet human needs with minimal impact on the environment (Froehlich et al., 2018; Springmann et al., 2016; Thilsted et al., 2016). In addition, aquaculture serves as a source of income and livelihood for about one billion people around the globe (FAO, 2018). Although wild and captured fish contribute to the major share of total fish production, to meet the demand of a growing world population, alternate strategies should be implemented. This could be achieved by improving aquaculture practices. It was projected that by 2030, about 60% of fish for direct human consumption would be contributed by aquaculture. To reach this goal, there is a need to introduce new strategies to improve aquaculture that eventually leads to more fish production. Application of biotechnology and transgenics (e.g. growth hormone transgenic fish, inter-specific hybrid fish with enhanced growth), enhancement of nutritional quality of fish food, improvement of breeding programs and hormonal treatments (reproductive strategy), and improved disease management are some of the potential approaches to enhance aquaculture yield.

1.1.2. Importance of reproductive strategies to improve aquaculture

The main goal of introducing reproductive manipulation in aquaculture is to successfully breed fish in captivity and thereby increase the availability of seed stock for largescale aquaculture. Although improvements in reproductive strategies could assist in aquaculture productivity, it remains one of the toughest areas to make progress in and is challenging particularly among captive populations (Zohar and Mylonas, 2001). This is because of the complex and species-specific reproductive features exhibited by different fish. An in-depth understanding of the reproductive strategy of fish (in some cases, specific-species of interest) is critical for the application of potential improvement measures and manipulations. The two most common problems associated with breeding fish in captivity include the following: female fails to undergo the final maturation of oocyte (Peter et al., 1993; Zohar, 1988, 1989a, 1989b), and the quality and quantity of milt is compromised in males (Billard, 1986, 1989). The current approaches to improve the reproductive potential of fish focus on environmental parameters (Munro, 1990; Yaron, 1995; Zohar, 1989b) and hormonal treatments, of which the latter is reliable and successful in many species (Donaldson and Hunter, 1983; Peter et al., 1988, 1993; Tucker, 1994). Successful use of hormonal therapies dates back to the 1930s, where the injection of crude pituitary extracts (from mature fish) to broodstock was used to induce spawning

(Houssay, 1930). Since then, both natural and synthetic hormones are being employed in fish farming to enhance reproductive strategies that promote aquaculture yield (Hoga et al., 2018).

Hormonal manipulations could be used to achieve synchronized breeding of fish in a broodstock, or for controlling the timing of spawning (accelerate or delay). This will enable us to control the availability of fish (greater flexibility in marketing), allows maximization of resource utilization, and ensures high turnover (Hoga et al., 2018). However, the biggest application of hormonal manipulation of aquaculture is effective breeding and mass production of seed stocks from a captive population (species with reproductive dysfunctions) that cannot be bred by other methods. The generally used mode of hormone administration is either intramuscular or IP injections (Hoga et al., 2018). Hoga and colleagues summarized the most commonly used hormones and their combination in artificial breeding to induce or suppress maturation and spawning in fish (Hoga et al., 2018). Injection of crude preparations of pituitary extracts (mature fish, mainly from carp and salmon) is one of the oldest and still prevailing method in this category. Another commonly used endocrine agent is the GnRH of both mammalian (mGnRH) and fish (sGnRH) origins. In addition, synthetic analogues of GnRH (GnRHa), dopamine antagonists (pimozide, domperidone, metoclopramide and reserpine) and synthetic gonadotropins are also commonly used to induce, suppress or delay breeding fish in captivity (Almeida, 2013; Araújo et al., 2014; Hoga et al., 2018; Zohar and Mylonas, 2001). Hormonal combinations, for example, a mixture of dopamine antagonists and GnRH are the most widely used, particularly in species in which GnRH alone cannot induce maturation/spawning (Almeida, 2013; Venturieri and Bernardino, 1999). The dopamine antagonist inhibits dopamine action by inhibiting the binding of dopamine to its receptor, thereby ensuring endogenous GnRH release (dopamine is a potent GnRH suppressor). The additional dose of GnRH in the combination will generate a surge in gonadotropin release and thus promote the maturation of gametes. Examples of commercially available combinations are Ovaprim™ and Ovopel™. Ovaprim™ is a hormonal combination of a fish GnRH analogue (inducer) sGnRHa and dopamine antagonist (inhibitor), domperidone. Ovopel™, on the other hand, is a combination of mammalian GnRH analogue (mGnRHa) and a dopamine antagonist (metoclopramide). Both of them are very successfully used in the captive (artificial) breeding of many fish species (Hoga et al., 2018). Other examples of synthetic hormonal agents to induce spawning are Dagin™ ([D-Arg6, Pro9-NEt]-sGnRH),

Aquaspawn™ (synthetic GnRH) and Ovatide™ (GnRHa + dopamine inhibitor, pimozide). Synthetic hormonal combinations are preferred over natural hormones due to their easy use with superlative results but, variability in their efficiency, concerns about environmental residue discharges and accumulation are limitations. Lack of basic knowledge of the influence of other major players in fish reproduction is a major reason for variability in action/ results. In addition, even though many of these hormones are 100% efficient in inducing spawning, fecundity and fertilization rates are very different (Ali et al., 2015). The half-life of many of these hormonal combinations is short, and their residues (metabolites) are detected in several occasions in many fish studies (Hoga et al., 2018). For instance, independent studies conducted in China, Spain, and Taiwan reported the presence of hormone residues in common river species, including minnows, carp, bogue and tilapia, which are exposed to aquaculture effluents (Chen et al., 2012; Guedes-Alonso et al., 2017; Liu et al., 2011b). The presence of hormonal residues with xenobiotic properties in food or environment is a potentially harmful to terrestrial as well as aquatic animals. This raises questions about safety standards and reflects on the need for in-depth research in reproductive physiology and neuroendocrine action of hormonal agents. Further research is needed to look for hormonal combinations that are of natural origin, which could be more efficient and eco-friendly and safe to use in fish breeding. In my research, the focus will be to enhance our understanding of additional hormonal/peptide regulators of reproduction in fish. Such hormones and/or peptides could be eventually used for the improvement of fish reproduction and aquaculture yield.

1.2. Introduction to fish reproduction

Among vertebrates, fishes form the largest group of organisms in terms of their number by means of population and species diversity. Among fishes, teleosts, or bony fishes are the most advanced with approximately 30,000 species that covers 96 percent of the entire fish class (Helfman et al., 2009). They are ubiquitous in the distribution in all aquatic environments. To be able to thrive in a wide variety of habitats, fishes have developed several adaptations, including those to the reproductive system/processes. One of the unique aspects of teleost reproduction is that they display almost all types of reproduction found in vertebrates (Desjardins and Fernald, 2009). Among fishes, 88% are gonochorists in which sexes are separate, with testis (sperm) in males and ovary (eggs) in females (Patzner, 2008). Some gonochoristic species in their earlier stages of growth and development are hermaphrodites and can develop into either male or female. In other words, many fishes have the bipotential ability to develop either testis or ovary, even after later developmental stages (Adkins-Regan, 2005). Although it is rare, parthenogenesis is also reported among fishes (Dudgeon et al., 2017; Holtcamp, 2009; Spurway, 1953). The vast majority of fishes follow external fertilization by releasing egg and sperm into water, and fertilization takes place externally in the water (Patzner, 2008). Several external and internal factors contribute to the reproductive success of teleosts, which makes the reproductive process complex and unique. External factors, including temperature, photoperiod, and nutrient availability are very important for their reproductive success, especially for those who shows seasonal cyclicity in reproduction. Internal factors include hormones, which play key roles in fish reproduction (Abraham et al., 2009; Nelson and Sheridan, 2006). The internal factors are fine-tuned with the external factors to ensure the ideal conditions for reproduction.

The complex environments where they dwell demands more complex regulatory and functional organization in fishes. One of the classic examples for such a complex interconnected system is the neuroendocrine axis, which is very ordered, hierarchical, and compartmentalized. In the neuroendocrine axis, the brain acts as the central regulator and endocrine organs or a group of cells or tissues that secrete hormones, which receive inputs from both brain and peripheral organs and secretes hormones which act on target organs to elicit specific responses and maintains homeostasis. Geoffery Harris (Harris, 1948) is the one who first proposed and identified the neuroendocrine axis through his studies on rats. He found that the hypophyseal

portal system and secretions from the hypothalamus influence the pituitary to release hormones that regulate the functions of other endocrine glands. Later similar neuroendocrine axis was reported in fish and other lower vertebrates as well, involved in the regulation of key functions. In vertebrates, the neuroendocrine axis regulates the key physiological functions such as growth, metabolism, immune function, and reproduction to maintain homeostasis.

1.3. Neuroendocrine regulation of reproduction in fish

1.3.1. The hypothalamo-pituitary-gonadal (HPG) axis in reproduction:

Vertebrates reproduce sexually (males and females) and employ different reproductive strategies. For instance, fishes and amphibians reproduce externally by releasing the gametes into the water followed by fertilization and development (except shark and ray species, which are ovoviviparous). In case of reptiles and birds, the fertilization is internal; but the development is external (inside a calcareous shell but outside the body of the female parent). In mammals, fertilization and development are internal and they give birth to young ones (except prototherians, in which development is external). Even though vertebrates are known for their remarkably diverse reproductive strategies, the central regulatory network is highly conserved across the subphylum. Neuroendocrine regulation of reproduction in vertebrates is under the control of the hypothalamo-pituitary-gonadal (HPG) axis (**Figure 1.1**), a classic example of the neuroendocrine axis. The HPG axis is comprised of the hypothalamus, pituitary and gonads (ovary and testis) which works as a single unit to coordinate the actions of endocrine, autocrine, paracrine, and intracrine factors in regulating reproduction (Unniappan, 2010). Hypothalamus releases the neurohormone, GnRH that controls the production and release of FSH and LH or the gonadotropins (GTHs) from the anterior pituitary (Ankley and Johnson, 2004; Weltzien et al., 2004; Yaron et al., 2003). Gonadotropins reach gonads through blood and regulate gametogenesis, sex steroid hormone production and the development of secondary sex characteristics. The mode of operation of HPG axis is identical among vertebrates and proper functioning of HPG axis is critical for reproductive success of vertebrates. The HPG axis helps to achieve this by coordinating both internal (central and peripheral inputs) and external (photoperiod, temperature, food, stress) cues.

In general, three means of hypothalamic (anatomic) control of pituitary functions are considered in vertebrates. First one is the diffusion of neurohormones to the pituitary from the

hypothalamus (agnathans). The second one is by the direct innervation of nerve endings to the pituitary from the hypothalamus without the involvement of the portal system, which operates in teleosts. And in the third mode through the portal system via the median eminence as found in tetrapods (Sower, 2015). Golan and colleagues reported that in addition to direct innervation, neurovascular control of gonadotropins is present in zebrafish providing additional information about the hypothalamo- hypophyseal system in teleost (Golan et al., 2015). It was believed that the hypothalamic regulation of reproduction via pituitary is absent in agnathans due to the lack of physiological connection between the hypothalamus and pituitary. However, this concept was refuted later (Nozaki, 2013). Based on the immunohistochemical studies, Nozaki proposed the presence of diffused as well as "pre-median eminence" control of pituitary gonadotropin secretion in the hagfish (Nozaki, 2013). All these pieces of evidence support the evolution of more complex regulatory mechanisms in tetrapods (portal system) compared to teleosts (direct innervation) and agnathans (diffusion), and the existence of evolutionary refinement of neuroendocrine regulation of reproduction.

1.3.1a. The GnRH system in fish

The GnRH is a decapeptide, which is considered as the master regulator of reproduction in vertebrates. It was first reported as an LH releasing factor from the hypothalamus of pigs and sheep by two independent research groups (Amoss et al., 1971; Matsuo et al., 1971). At first, GnRH was named as LH-releasing hormone (LHRH) and later found to stimulate FSH release as well. Hence, this hormone was renamed as GnRH. Two modes of GnRH secretion were reported in vertebrates, particularly in mammals: surge mode and pulsatile mode. The pulse mode is extensively studied in mammals and it is critical for the secretion of LH and FSH from the anterior pituitary (Okamura et al., 2013), which drives folliculogenesis, spermatogenesis and steroidogenesis. The surge mode controls preovulatory LH surge, which triggers ovulation (Maeda et al., 2010). The pulsatile and rhythmic release of GnRH in mammals, including humans and rodents is under the control of two neuropeptides, neurokinin B or NKB (product of tachykinin gene or Tac3) and dynorphin A or Dyn (product of prodynorphin gene or pdyn) (Grachev et al., 2014; Knobil, 1990). Even though the role of NKB and Dyn in GnRH release is lacking, it was reported that both these neuropeptides have roles in directly inducing

gonadotropin release and regulate reproduction in fish (Biran et al., 2012; Liu et al., 2018; Vijayalaxmi and Ganesh, 2020).

Multiple forms of GnRH are expressed in vertebrates. Teleosts for instance, exhibit a greater number and diversity of GnRH peptides and GnRH receptor (GnRHr) subtypes (Gothilf et al., 1995; Lethimonier et al., 2004; Okubo and Nagahama, 2008; Tostivint, 2011). Three forms of GnRH are found in fishes such as GnRH1, GnRH2 (GnRH-II) and GnRH3. GnRH1 is found in almost all vertebrates except some teleosts, including cyprinidae and salmonidae. GnRH2 is present in all vertebrates except rodents. GnRH3 is exclusively found in fishes and some agnathans.

Neurons that produce GnRH1 are found in the preoptic area of hypothalamus (POA) and project predominantly into the pituitary, where they regulate reproduction via gonadotropin release in many fish species including cichlids and flatfish (Amano et al., 2004; White et al., 2002). GnRH1 is very critical for the reproductive success of many species. In humans and mouse, mutations in GnRH1 leads to infertility (Bouligand et al., 2009; Mason et al., 1986). In addition to the reproductive role, GnRH1 also regulates the release of growth hormone (GH) (Marchant et al., 1989), prolactin (Weber et al., 1997) and somatolactin (Kakizawa et al., 1997) from the pituitary of teleost. In musk shrew, GnRH1 is shown to influence reproductive behavior (Schiml and Rissman, 2000). There are species-specific forms of GnRH1, including mammalian GnRH (mGnRH), sea bream GnRH (sbGnRH), pejerrey GnRH (pjGnRH), catfish GnRH (catGnRH), herring GnRH (hGnRH) and whitefish GnRH (wfGnRH). It is widely accepted that GnRH1 is critical for the release of gonadotropins from the pituitary and gonadal development (Kah et al., 2007; Nocillado et al., 2007).

GnRH2 or GnRH-II was first identified in the year 1984 from chicken brain (Miyamoto et al., 1984). Chicken GnRH-II (cGnRH-II) is the single representative present in the GnRH-II subtype and is expressed in a large number of fish species (Oka, 2010; Sower et al., 2009). cGnRH-II producing neurons are mainly concentrated on the midbrain tegmentum near the third ventricle and cGnRH-II neurons project throughout the brain, especially to midbrain, hindbrain and the third ventricle directly (González-Martínez et al., 2002; Steven et al., 2003). cGnRH-II has a role in reproductive behavior (Volkoff and Peter, 1999), feeding and metabolism (Kauffman and Rissman, 2004; Temple et al., 2003). Many authors reported that cGnRH-II

regulates feeding and sexual behavior in teleosts (Volkoff and Peter, 1999), birds (Maney et al., 1997) and mammals (Barnett et al., 2006; Kauffman and Rissman, 2004). Chang and colleagues reported that cGnRH-II could induce LH release from the pituitary of goldfish (Chang et al., 2009). The complex networking, expression pattern and the findings reported above indicate the possible role of cGnRH-II in balancing energy homeostasis, survival and reproductive success in vertebrates.

GnRH3 was first identified from the lamprey brain (Sower et al., 1993). GnRH3 neurons are located in the terminal nerve ganglion near the olfactory bulb and project primarily to the telencephalon but also widely into the whole brain, including the retina and olfactory epithelium (Grens et al., 2005; Wirsig-Wiechmann and Oka, 2002). In general, GnRH3 plays a role in sensory signal processing (Behrens et al., 1993; Kinoshita et al., 2007; Maruska and Tricas, 2007) and reproductive behavior (Karigo and Oka, 2013). In teleosts, GnRH3 controls nesting (Ogawa et al., 2006; Yamamoto et al., 1997), aggression (Ogawa et al., 2006), and spawning behaviors (Volkoff and Peter, 1999). In cyprinidae and salmonidae, GnRH3 is the main reproductive hormone (Muñoz-Cueto et al., 2020; Umatani and Oka, 2019). All these reports point towards the role of GnRH3 as a collective modulator of sensory processing, reproductive behavior, and a major reproductive hormone in fish without GnRH1. Gene knockout studies in zebrafish indicates that loss of GnRH3 and kisspeptin (kiss1, kiss2) (triple knockout, TKO) did not influence the normal reproductive potential (Liu et al., 2017). However, in medaka, GnRH1 knockout (KO) leads to infertility in female fish, but male KO develops normally and are fertile (Takahashi et al., 2016). This suggests that species-specific differences exist in the critical nature of the GnRH system for reproductive success, and mechanisms to compensate for GnRH absence might be in operation in some species.

Vertebrate GnRHrs are members of the G-protein coupled receptor (GPCR) family. They show overall structural similarity with an N-terminal extracellular region and seven hydrophobic transmembrane domains connected by alternating hydrophobic intercellular loops and terminated with a cytoplasmic C-terminal tail (which is missing in mammals) (Millar et al., 2004). Based on their nucleotide sequences, the GnRHr family is broadly divided into 3 groups: type 1, 2, 3 (Millar et al., 2004; Tello et al., 2008). Type 1 GnRHr is found in all vertebrates, type two is predominantly found in amphibians and mammals including humans, and type three is mainly

found in fish species particularly in Perciform species (Flanagan et al., 2007; Levavi-Sivan and Avitan, 2005; Tello et al., 2008). The number and distribution of GnRHrs are also varied among species. For instance, in zebrafish, there are four functional GnRHr (under type 1 and 3 groups) were reported and expressed in pituitary, eye and gonads (Tello et al., 2008). In mammals, two types of GnRHrs were reported, but the GnRHr1 is known to have biological activity and the GnRHr2 is silenced (Millar, 2005). The number of GnRHr isoforms is high among teleosts and is because of gene duplication among teleosts. Five GnRHrs were reported in pufferfish, cherry salmon and European sea bass (Kah et al., 2007; Tello et al., 2008). Kim and colleagues have subdivided the 3 types of GnRHrs further into five classes: non-mammalian type I (GnRHrn1 and GnRHrn1b), non-mammalian type II (GnRHrn2), and non-mammalian type III (GnRHrn3 and GnRHrn3b) (Kim et al., 2011). The diversity of GnRHrs uplifts the complexity and uncertainty to determine which receptor type is the most potent to evoke the gonadotropin secretion from the pituitary among fish. In fish, the presence of all GnRHr types (GnRHRn1, GnRHRn1b, GnRHRn2, GnRHRn3, and GnRHRn3b) has been documented in all compartments of the HPG-axis (Gopurappilly et al., 2013; Lethimonier et al., 2004) and studies suggest they are mostly dedicated to reproductive functions (Volkoff and Peter, 1999). These findings support the multispectral role of GnRH system in integrating physiological homeostasis to enable reproductive success of vertebrates.

1.3.1b. Pituitary gland and gonadotropins in fish

The pituitary in teleosts is divided into two compartments, the adenohypophysis, representing the non-neural part, and the neurohypophysis, which is of neural origin, derived from the ventral diencephalon. The adenohypophysis is divided into rostral pars distalis, proximal pars distalis, and pars intermedia. There is a lack of cleft separating the pars distalis and pars intermedia in teleosts. The unique feature of the teleost HPG axis is that they lack a hypothalamo-hypophyseal portal system, and the neurosecretory neurons in the hypothalamus directly innervate the pars distalis (PD), and the pars intermedia (PI) of the pituitary adenohypophysis (Zohar et al., 2010). GnRH from the hypothalamus binds to its receptors in the pituitary, and release gonadotropins. The gonadotropins are the follicle-stimulating hormone (FSH) and Luteinizing hormone (LH) from the pituitary, which are directly released into the blood and reach their target organs, the gonads, to regulate its function. The gonadotropins are

heterodimeric glycoproteins which have a common subunit termed the glycoprotein hormone α subunit (GPα) and specific subunit called FSHβ and LHβ (Pierce and Parsons, 1981). Until late 1980s, it was believed that there is only one type of gonadotropin (GtH) produced from the pituitary of fish dedicated to performing all reproductive functions. However, this concept was revised by the discovery of two gonadotropin types (GtHI and GtHII) from the pituitary of salmon (Kawauchi et al., 1989). The GtHII was considered as the common GtH previously (Kawauchi et al., 1989; Suzuki et al., 1988; Swanson, 1991). Later due to sequence identity, GtHII was renamed as FSHβ and GtHI as LHβ (Quérat et al., 2001). The FSHβ and LHβ are mainly transcribed in the proximal pars distalis and the periphery of pars intermedia from two unlinked genes. Even though the LHβ and FSHβ subunits markedly differ in their structure, the evolutionary analysis suggests they originate from a single ancestor gene via duplication (Li and Ford, 1998). In a primitive fish, lamprey, only one gonadotropin β subunit is cloned, which suggests and supports the existence of a single ancestor for gonadotropin β subunits in teleosts (Sower et al., 2009). The GPα is also transcribed in the same region but abundantly synthesized from the proximal pars distalis (Cerdà et al., 2008). In comparison to teleost FSHβ, LHβ exhibits high structural conservation (Levavi-Sivan et al., 2010). Recent knockout studies suggest that mutant zebrafish lacking LHβ or FSHβ develop normal testis and ovary, but fail to spawn and become infertile (Chu et al., 2014, 2015; Zhang et al., 2015). This indicates the importance of both gonadotropins in the final maturation of gametes in fish.

1.3.1c. The gonadal hormones and reproduction in fish

In response to the gonadotropins, the gonads produce steroid hormones, which facilitate reproductive success and have a role in the feedback control of pituitary gonadotropins. Fish FSH stimulates follicular growth in the ovary and spermatogenesis in the testis, whereas LH is involved in the control of the final steps leading to ovulation and spermiation in teleosts (Levavi-Sivan et al., 2010). In the ovary, FSH binds to the FSH receptor (FSHr) in the granulosa cells and promotes follicular maturation and aromatization of androgens to estrogen. In the testis, FSH binds to FSHr, which is concentrated in the Sertoli cells and stimulates the proliferation of Sertoli cells and spermatogenesis. The prime target site for LH in males is the Leydig cells of the testis, where the LHr is located and their main function is to control androgen synthesis (Hachfi et al., 2012). The main steroid hormones produced from the gonads in response to the

gonadotropins are 17β estradiol (E_2 or E) in females, and testosterone (T) mainly 11-ketotestosterone (11 KT or T, which is the main T in fish) in males. In both sexes, gonadotropins stimulate the synthesis of maturation inducing hormones/steroids (MIHs or MIS), which include 17α, 20β-dihydroxyprogesterone (17α, 20β-DHP) and 17α, 20β, 21-trihydroxy-4-pregnen-3-one (20β-S) (Kime, 1993). Gene knockout studies indicate that mutants lacking one or both gonadotropin receptors fail to reproduce, or show a reduction in gamete production in LHr mutants (Li and Cheng, 2018).

Compared to mammalian FSH and LHr, the teleost gonadotropin receptors show some marked differences in its expression. In mammals, LHr is limited to theca and granulosa cells of the ovary and in the Leydig cells of the testis. Meanwhile, FSHr is expressed in the testicular Sertoli cells and ovarian granulosa cells (Themmen and Huhtaniemi, 2000). However, FSHr and LHr are expressed in both Sertoli and Leydig cells of the testis, theca and granulosa cells of the ovary of fish (Andersson et al., 2009; García-López et al., 2009, 2010; Ogiwara et al., 2013; Ohta et al., 2007). The ligand-binding specificity of mammalian gonadotropins are very high (Moyle et al., 1994). For example, amago salmon and rainbow trout gonadotrophin receptors exhibit ligand specificity similar to mammals (Oba et al., 1999a, 1999b; Sambroni et al., 2007). Exceptions to this include African catfish and coho salmon, where the FSHr fail to distinguish both gonadotropins, but the LHr specifically binds to LH in both species (Vischer et al., 2003; Yan et al., 1992).

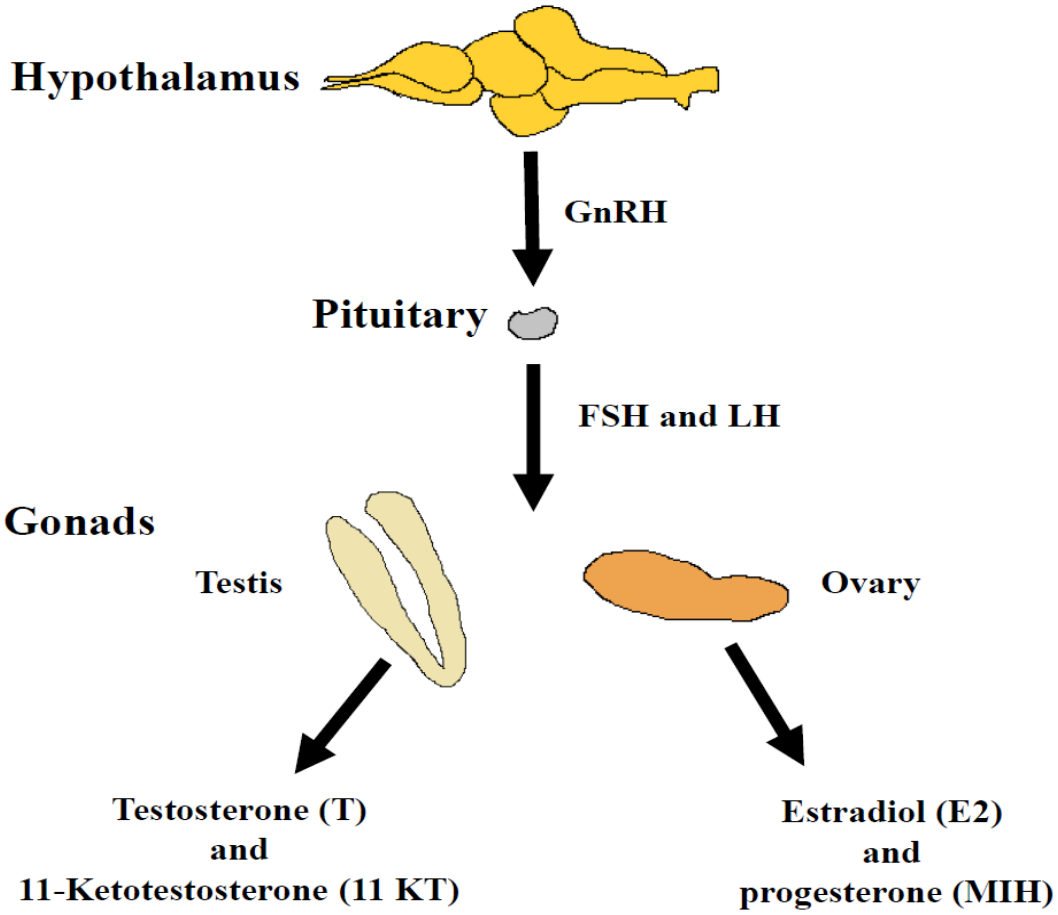

Figure 1.1: The hypothalamo-pituitary-gonadal (HPG) axis

1.3.2. Non-HPG axis hormones/peptides with reproductive functions in fish

Multiple hormones and regulatory factors that are primarily produced outside of the HPG axis (both central and peripheral origin) influence the function of the HPG axis. The list of hormones and peptides that have been reported to play a role in vertebrate reproduction includes kisspeptin, gonadotropin inhibitory hormone (GnIH), orexin, neuropeptide-Y (NPY), 26RFamide (QRFP), galanin (GAL), pituitary adenylate cyclase-activating polypeptide (PACAP), melanin-concentrating hormone (MCH), cocaine-and amphetamine-regulated transcript (CART), nesfatin-1, phoenixin, ghrelin, leptin, prolactin, cholecystokinin (CCK), thyroid hormone, and anti-Mullerian hormone (AMH). In the following sections, I will solely focus on the regulatory factors of central and peripheral origin that were pursued in my thesis research.

1.3.2a. Kisspeptin

Kisspeptin is a neuropeptide encoded by the KISS1/kiss1 gene, originally identified as a metastasis suppressor gene (Lee et al., 1996). In 2003, the receptor for kisspeptin, GPR54a or kiss1r (kiss1ra) was identified, and the role of kisspeptin on reproduction was reported (Seminara et al., 2003). Mutation or inactivation of GPR54 resulted in idiopathic hypogonadotropic hypogonadism in humans (de Roux et al., 2003; Seminara et al., 2003). GPR54 null mice failed to undergo puberty with reduced gonadal size, low levels of gonadotropins and steroid hormone production (Funes et al., 2003; Messager et al., 2005; Seminara et al., 2003). In mammals, kiss1ra and GPR54a mRNA are expressed in the hypothalamic arcuate nucleus, with key positive and negative feedback control of gonadotropin secretion by sex steroids and metabolic hormones. Many authors reported the critical role of the kisspeptin system in the control of pulsatile release of GnRH and gonadotropin secretion in mammals (Keen et al., 2008; Ohkura et al., 2009; Wakabayashi et al., 2010).

Two kisspeptin genes (kiss1 and kiss2) and 2 kisspeptin receptors (kiss1ra and kiss1rb [GPR54 a and b]) were reported in many teleosts including medaka, zebrafish and sea bass (Felip et al., 2009; Kitahashi et al., 2009). However, in some species including Senegalese sole (Mechaly et al., 2009), orange-spotted grouper (Shi et al., 2010), Atlantic halibut (Mechaly et al., 2010) and grass puffer (Shahjahan et al., 2010) only kiss2 and kiss1rb (previously known as kiss2r) are present. The expression of kisspeptin and its receptors in the HPG axis in fish indicates their importance in fish reproduction. For example, in medaka, kiss1 neurons are found

in the nucleus ventral tuberis (nVT) and the nucleus posterioris periventricularis (NPPv) (Kanda et al., 2008; Kitahashi et al., 2009), while kiss2 neurons are located in the dorsal zone of periventricular hypothalamus. In zebrafish, kiss2 mRNA is expressed in all hypothalamic populations and extensively in the subpallium, POA and ventral and caudal hypothalamus, and the mesencephalon (Servili et al., 2011). In Nile tilapia, all three GnRH neuronal populations express kiss1rb (Parhar et al., 2004), suggesting the possible role of kiss2 in regulating the HPG axis in Nile tilapia. However, in zebrafish, kiss1 mRNA is localized in the habenula and is connected to the ventral interpeduncular nucleus, which suggests a role for kiss1 in the serotonergic system instead of HPG axis in zebrafish (Kitahashi et al., 2009; Ogawa et al., 2012). Similar to mammals, there is a significant elevation in the kisspeptin system has been observed during the onset of puberty in many teleosts. For instance, in zebrafish, along with GnRH2 and GnRH3, both kiss1 and kiss2 mRNA levels were elevated during the onset of pubertal phase (Kitahashi et al., 2009). All these reports point towards the possible role of the kisspeptin system in the control of reproduction via HPG axis in fish.

1.3.2b. Gonadotropin inhibitory hormone (GnIH)

GnIH is a hypothalamic dodecapeptide (Ser-Ile-Lys-Pro-Ser-Ala-Tyr-Leu-Pro-Leu-Arg-Phe-NH2 (SIKPSAYLPLRF-amide) which is first isolated from the Japanese quail, *Coturnix japonica* (Tsutsui et al., 2000). Before its discovery, it was believed that there is no natural suppressor of GnRH, the master player of vertebrate reproduction. Expression of GnIH is reported in the brain, eye, testis and ovary of mammals and birds. It was reported that GnIH acts on the GnRH neurons and pituitary gonadotrophs to downregulate GnRH and gonadotropins, thereby negatively affecting reproduction in mammals and birds (Parhar et al., 2012; Tsutsui et al., 2012). In all avian species studied, GnIH downregulation of gonadotropin secretion is mediated via GPR147 receptor (GnIHr) (Ciccone et al., 2004; Osugi et al., 2004; Ubuka et al., 2006). Both avian and mammalian GnIH peptides (via central and peripheral administration) suppress gonadotropin release in rats (Johnson et al., 2007; Kriegsfeld et al., 2006; Rizwan et al., 2009). GnIH and its receptor are also expressed in the gonads of house sparrow, specifically in the cell types involved in the steroidogenic pathway. This indicates an additional role for GnIH in the gonads, as a local modulator of reproductive processes (McGuire and Bentley, 2010).

One of the interesting findings is that all putative mammalian, reptilian, amphibian and fish GnIH peptides have a common C- terminal LPXRF-amide (X=L or Q) motif similar to the avian GnIH or GnIH related peptides (Tsutsui, 2009, 2016). In fish, the presence of GnIH in the reproductive centers of the brain, and in the gonads was reported (Amano et al., 2006; Sawada et al., 2002). In goldfish, three GnIH peptides are reported, gfLPXRFa-1, 2, and 3 (Sawada et al., 2002). Unlike mammals and birds, the role of GnIH on fish reproduction is complicated. For instance, in goldfish GnIH peptides have both inhibitory and stimulatory effects on gonadotropin synthesis and release, which is dependent on the reproductive state of the fish (Amano et al., 2006; Moussavi et al., 2012, 2013; Qi et al., 2013a). In zebrafish, zfLPXRF-3 has an inhibitory effect on gonadotropin release (Zhang et al., 2010). IP injection of tilapia GnIH2 (tiGnIH) induced the secretion of gonadotropins in female tilapia (Biran et al., 2014). However, in male European sea bass, intracerebroventricular (ICV) injection of sea bass GnIH2 (sbGnIH) inhibited FSHβ and LHβ expression and decreased plasma LH levels (Paullada-Salmerón et al., 2016a). In conclusion, the action of GnIH in fish depends on the species, sex, physiological and reproductive status, dose and the route of administration.

13.2c. Nesfatin-1

Nesfatin-1 (Nucleobindin-2 Encoded Satiety- and FAT-Influencing Protein) was originally identified in 2006, as a novel hypothalamic neuropeptide with anorexigenic action, which is highly conserved among vertebrates (Oh-I et al., 2006). The precursor of nesfatin-1 is nucleobindin-2 (NUCB2) or (the Nucleic acid/DNA binding/EF-hand/Acidic amino acid rich region, NEFA), which is a calcium and DNA binding protein (Gonzalez et al., 2012a; Oh-I et al., 2006; Tulke et al., 2016). Post-translational proteolytic processing of NUCB2 (396 amino acid [aa]) by prohormone convertase (PC) 1/3 and PC2, produce three peptides: N-terminal nesfatin-1 (82 aa), and two C-terminal peptides namely, nesfatin-2 (79 aa), and nesfatin-3 (231 aa) (Oh-I et al., 2006). Among these three peptides, nesfatin-1 is the only one known to be biologically active, and it exhibits anorexigenic activity (Gonzalez et al., 2010; Oh-I et al., 2006). The highly conserved M30 or mid-segment (aa 30-59) is critical for the biological activity of nesfatin-1 (Shimizu et al., 2009). Cellular and molecular localization studies indicate nesfatin-1 has a wide spectrum of expression patterns in both central and peripheral tissues in both mammals and fish (Yuan et al., 2020). The receptor for nesfatin-1 is yet to be identified, and the peptide remains an

orphan ligand. However, it was widely accepted that nesfatin-1 act via a G-protein coupled receptor (GPCR) (Brailoiu et al., 2007). The potential candidate receptors for nesfatin-1 are GPCR3, 6 and 12, which need confirmation (Ishida et al., 2012; Osei-Hyiaman, et al., 2011).

As mentioned earlier, NUCB2 can bind to Ca^{2+}, which helps NUCB2 to interact with multiple biochemical pathways and cellular functions and indicates the potential of this peptide to regulate multiple biological functions. NUCB2 received its name due to the high sequence similarity to NUCB1, another member of the nucleobindin family. Even though NUCB2 was identified much earlier (Miura et al., 1992a), the peptide garnered more attention after the discovery of the satiety property of the N-terminal fragment of NUCB2 (nesfatin-1) (Oh-I et al., 2006). Followed by this, several studies in mammals and fish documented the anorexigenic property of nesfatin-1 (Gonzalez et al., 2010; Shimizu et al., 2009; Stengel et al., 2009a; Su et al., 2010). One of the structural features of NUCB2 is the presence of basic amino acid pairs (Lys-Arg or Arg-Arg) in their cleavage sites, which are conserved across species (Oh-I et al., 2006). Even though the antibodies used to detect the presence of NUCB2 cannot distinguish NUCB2 from nesfatin-1, the presence of these peptides was detected in both central and peripheral organs (Angelone et al., 2020; Schalla et al., 2020). In addition to feed intake regulation, NUCB2/nesfatin-1 is shown to influence adipocyte development (Ramanjaneya et al., 2010, 2015), gastrointestinal functions (Atsuchi et al., 2010; Schalla and Stengel, 2018; Stengel et al., 2009a), blood glucose homeostasis (Bonnet et al., 2013; Dong et al., 2013; Gonzalez et al., 2009, 2011), fat metabolism (Dong et al., 2013; Verdeguer et al., 2015; Yin et al., 2015), cardiovascular function (Osaki and Shimizu, 2014; Yosten and Samson, 2009), and reproduction (García-Galiano et al., 2012; Gonzalez et al., 2012b; Seon et al., 2017).

The understanding of the reproductive regulatory role of nesfatin-1 in mammals is in detail compared to fish. The expression of NUCB2 mRNA is detected in both testis and ovary of different mammals, including humans (García-Galiano et al., 2012; Kim et al., 2014; Stengel et al., 2009b). Nesfatin-1 expression was detected in the gonads of Japanese quail (*Coturnix japonica*) (Banerjee and Chaturvedi, 2015). It was reported that the levels of NUCB2/nesfatin-1 increased in rats and humans during pubertal transition (Çatlı et al., 2015; García-Galiano et al., 2010), which suggest a role for nesfstin-1 in mammalian reproduction. ICV administration of nesfatin-1 induces LH release in rats (García-Galiano et al., 2010). In addition, they also reported

that antisense oligonucleotide of NUCB2 resulted in or decrease in ovarian mass and delayed vaginal opening in rats. In male rats, nesfatin-1 suppresses the expression of GnRH and kisspeptin (hypothalamus), gonadotropin β subunits (pituitary) and steroidogenic acute regulatory protein (StAR, in the testis) post-ICV administration (Gao et al., 2016). This indicates sex-specific reproductive regulatory role of nesfatin-1 in rats. *In vitro* treatment of T increased the expression of NUCB2/nesfatin-1 in the hypothalamic and pituitary cells (Hatef and Unniappan, 2017). In addition, in castrated mice, a decrease in NUCB2/nesfatin-1 expression was observed, which is reversed by T treatment (Seon et al., 2017). Recently it was reported that nesfatin-1 induces the development and maturation of testis and facilitates testicular functions in pre-pubertal mice (Ranjan et al., 2019a). In male rats, ICV administration of nesfatin-1 alone or in combination with PNX-20 induces plasma LH, FSH and T levels (Guvenc et al., 2019). These results implicate NUCB2/nesfatin-1 in the regulation of reproductive function in mammals, in a sex and species-specific manner.

NUCB2 expression has been identified in different fish species including goldfish (Gonzalez et al., 2012b), zebrafish (Hatef et al., 2015a), Ya fish (Lin et al., 2014), Siberian sturgeon (Zhang et al., 2018a), *Schizothorax davidi* (Yuan et al., 2020). Two isoforms of NUCB2, NUCB2A and NUCB2B were reported in zebrafish (Hatef et al., 2015a) and *S. davidi* (Yuan et al., 2020). Similar to mammals, nesfatin-1 is reported as an anorexigenic peptide in fish (Blanco et al., 2018a; Gonzalez et al., 2010; Kerbel and Unniappan, 2012; Yuan et al., 2020; Zhang et al., 2018b, 2018a). Both nutrient status and energy state can alter the expression of nesfatin-1 in fish (Blanco et al., 2016; Hatef et al., 2015a; Montesano et al., 2019; Yuan et al., 2020), which indicates a potential metabolic role for nesfatin-1 in fish. The role of NUCB2/nesfatin-1 in fish reproduction is limited. Nesfatin-1 expression in the HPG axis also indicates its importance in fish reproduction (Gonzalez et al., 2012b). IP injection of nesfatin-1 suppresses GnRH and gonadotropin mRNAs and gonadotropin release (LH) in goldfish (Gonzalez et al., 2012b). This report indicates that unlike in mammals, nesfatin-1 suppresses reproductive hormones in fish. However, it has to be noted that this was not a sex-specific study, and they measured the reproductive hormones in a mixed-sex population. In mammals, it was generally observed that in females, nesfatin-1 induce reproductive hormone release (García-Galiano et al., 2010) and in males, it suppresses reproductive hormones (Gao et al., 2016).

Gonzalez and colleagues also found that nesfatin-1 suppresses oocyte maturation in zebrafish (Gonzalez et al., 2012b). Studies in goldfish indicate that similar to mammals, expression of NUCB2/nesfatin-1 can be modulated by sex steroids. IP implanted T increased NUCB2 mRNA in the forebrain of goldfish. However, both T and E2 decreases the gut and pituitary NUCB2 mRNA expression (Bertucci et al., 2016). The circulating levels of nesfatin-1 were increased by estradiol and were decreased by T in goldfish. This indicates that similar to mammals, sex-specific modulation of NUCB2/nesfatin-1 exists in fish as well. Studies in female rainbow trout observed elevated nesfatin-1 levels post-spawning (Caldwell et al., 2014). Future research on several species with specific attention on sex, age and reproductive stages are needed to understand the involvement of nesfatin-1 in fish reproductive regulation.

1.3.2d. Nesfatin-1-like peptide

Nucleobindin-1 or NUCB1 is another member of the nucleobindin family which was first isolated from B lymphocyte cell cultures from lupus-prone MRL/l mice (Miura et al., 1992a). Because of its Ca^{2+} binding ability, it was also called CALNUC. Miura and colleagues determined that NUCB1 has a role in apoptosis and autoimmunity (Miura et al., 1992a). Phylogenetic analysis suggests that NUCB1 is more ancestral in origin and more similar to invertebrate nucleobindin compared to NUCB2 (Leung et al., 2019). The NUCB1 sequence is very similar to the NUCB2, and it is highly conserved (62%) in humans, especially within the bioactive core region of nesfatin-1 (24-53 aa) (Miura et al., 1994; Mohan and Unniappan, 2013; Moncrief et al., 1990). It was suggested that similar to NUCB2/nesfatin-1, it is possible that PCs process NUCB1 to produce a bioactive peptide similar to nesfatin-1 (Gonzalez et al., 2012a). Ramesh and colleagues proposed that similar to NUCB2, NUCB1 also have prohormone convertase cleavage sites (Ramesh et al., 2015). They also reported that post-translational modification of NUCB1 by prohormone convertases (PC) 1/3 and PC2 to produce a bioactive peptide and named it as NLP. Like NUCB2/nesfatin-1, NUCB1/NLP also exhibits a wide range of expression patterns in mammals (Tsukumo et al., 2007; Tulke et al., 2016; Williams et al., 2014). This indicates a possible biological role for NLP, resembling that of nesfatin-1.

NLP induces insulin secretion from mouse insulinoma (MIN6) cells. This was the first documented functional characterization of NLP in any vertebrates (Ramesh et al., 2015). They also found that 77 aa long amino acid sequence integrity (including a potential M30 region) is

critical for its biological activity. Continuous infusion of NLP causes a decrease in dark phase food intake in rats (Gawli et al., 2017). An increase in respiratory exchange ratio and energy expenditure and a decrease in fat oxidation were also observed in NLP-infused rats. This report indicates a potential role of NLP in feeding and metabolism in mammals. NUCB1/NLP was also characterized in some fishes (Sundarrajan et al., 2016; Yuan et al., 2020). NUCB1/NLP is widely expressed in both central and peripheral tissues in fish (Sundarrajan et al., 2016; Yuan et al., 2020). Unlike many other peptides, the anorexigenic property of NUCB1/NLP was first reported from a non-mammalian model. Studies in goldfish found the anorexigenic property of NLP (Sundarrajan et al., 2016). They also reported that NLP expression could be modulated by circadian pattern and the macronutrient composition of diet in goldfish. Recently NLP was characterized in another fish model, *S. davidi* and they also reported an anorexigenic property for NLP (Yuan et al., 2020).

The role of NLP on reproduction in any vertebrate model is yet to be determined. However, tissue distribution and cellular localization results from mammals (mice and rats) and fish (goldfish and *S. davidi*) noted the expression of NLP in the HPG axis tissues (Sundarrajan et al., 2016; Tulke et al., 2016; Williams et al., 2014; Yuan et al., 2020). In mice, NUCB1 mRNA and protein expression were detected in the testicular seminiferous tubules and in the follicular cells of the ovary (Williams et al., 2014). NUCB1/NLP expression in both mRNA and protein levels were observed in the testis and ovary of goldfish (Sundarrajan et al., 2016). Tissue distribution results suggest moderate expression of NUCB1 in the gonads (sex unspecified, possibly mixed-sex group) of *S. davidi* (Yuan et al., 2020). Sundararajan and colleagues found that sex steroids could modulate the NUCB1 mRNA in goldfish. Treatment with estradiol suppresses the expression of NUCB1 mRNA in goldfish pituitary. Meanwhile, T upregulates NUCB1/NLP mRNA expression in the hypothalamus, gut and hindbrain of goldfish (Sundarrajan et al., 2016). All these reports support the hypothesis that NLP has a possible role in the regulation of reproduction in vertebrates, which is yet to be identified.

1.3.2e. Phoenixin

Phoenixin (PNX) is a more recently identified peptide, which was first isolated from the rat hypothalamus and bovine heart (Yosten et al., 2013). Yosten and colleagues utilized a bioinformatics algorithm previously used to discover neuronostatin (Samson et al., 2008) to

identify PNX (Yosten et al., 2013). The mature PNX peptide is produced from an uncharacterized protein called small integral membrane protein 20 (SMIM20) or Chromosome 4 open reading frame 52 (C4orf52). SMIM20 is an understudied peptide and the available literature indicates it is highly conserved across species and acts as a chaperone-like protein in the mitochondria (Dennerlein et al., 2015). It also plays an important role in cytochrome c oxidase assembly in the electron transport chain. Some of the notable characteristics of the SMIM20 protein are that it contains glycine residues which undergo C-terminal amidation (critical for the bioactivity of the peptide) and also a carboxypeptidase cleavage sites (Fricker, 2012). In the original discovery paper, the authors also identified and described information about the C-terminal glycine residue (which is the potential amidation site) as well as presence number of conserved dibasic residues (potential cleavage sites), suggest the possibility of production of peptides with different amino acid length from the precursor (Yosten et al., 2013).

Two predominant variants of PNX reported so far are a 20-amino acid residue named phoenixin-20 amide (AGIVQEDVQPPGLKVWSDPF-amide) or PNX-20 amide, and a 14 amino acid residue named phoenixin-14 amide (DVQPPGLKVWSDPF-amide) or PNX-14 amide in mammals. In fact, PNX-14 is a degraded product of PNX-20, which lacks the N-terminal six amino acids. Another degraded product of PNX-20, named PNX-17 amide (VQEDVQPPGLKVWSDPF-amide) was also reported (Cowan et al., 2015). PNX-14 is identical in humans, rats, mice, pigs and dogs. PNX-20 differs in one amino acid between the coding region of human, canine and porcine sequences. Phoenixin mRNA and protein are expressed mainly in tissues including the hypothalamus, brain stem, pituitary, spinal cord, thalamus, heart, thymus, stomach, spleen and gonads of rats with highest central expression in the hypothalamus and peripherally in the heart (Yosten et al., 2013). In the hypothalamus, PNX-immunoreactivity was identified in the dorsal hypothalamic area (DA), arcuate nucleus (Arc), zona incerta (ZI), supraoptic retrochasmatic nucleus (SOR) ventromedial hypothalamus (VMH), lateral hypothalamus, and perifornical area (PeF) (Yosten et al., 2013). The presence of PNX in the median eminence suggest its regulatory role in tropic hormone secretion from the pituitary. Further studies identified the role of PNX in GnRH mediated LH release from rat primary cell cultures (Yosten et al., 2013). The first information about the candidate receptor for PNX-20 is reported by Treen and colleagues (Treen et al., 2016). They used mHypoA-GnRH/GFP and

mHypoA-Kiss/GFP-3 hypothalamic cell lines to study the role of PNX-20 in GnRH and kisspeptin system. They observed a remarkably high expression of GPR173, a GPCR in both cell lines. In addition, the siRNA mediated gene knockdown of GPR173 halts the PNX-20 mediated upregulation of GnRH, GnRHr and kisspeptin in both cell lines (Treen et al., 2016). They also found that PNX-20 treatment potentiates GPR173 expression in the mHypoA-GnRH/GFP cell line. This suggests that GPR173 is a possible receptor for PNX-20. The GPR173 also called super conserved receptor expressed in the brain 3 (SREB3), which is a type of SREB abundantly expressed predominantly in the brain and gonads (Matsumoto et al., 2000). The involvement of GPR173 in PNX-20 action was also studied by Stein and colleagues in female rats and with ligand binding assay (Stein et al., 2016). They used deductive ligand-receptor matching strategy to identify GPR173 as the top candidate receptor for PNX-20. It is interesting to note that GPR173 is expressed in all compartments of the HPG axis (Fagerberg et al., 2014), suggesting a possible role for PNX-20 and GPR173 in the regulation of reproduction.

PNX (both PNX-20 and PNX-14) is now considered as a peptide with hormone-like actions and it is known to be involved in multiple regulatory functions in vertebrates. ICV injection of PNX-20 increased plasma LH level in a dose-related manner in female rats, while knockdown of GPR173 halted this and delayed the estrous cycle (Stein et al., 2016). Similar results were reported earlier in PNX knockdown rats, where the estrous cycle was found delayed (Yosten et al., 2013). This suggests PNX is a positive regulator of reproduction in rodents. In addition, GnRH antagonist (cetrorelix) and agonist (buserelin) treatments increased SMIM20 mRNAs in all compartments of the HPG axis in female rats (Suszka-Świtek et al., 2019). They also found a decrease in the expression of GPR173 mRNA in the hypothalamus and pituitary of female rats in both treatment groups. In humans with polycystic ovary syndrome (PCOS), high circulating levels of PNX-14 was reported (Ullah et al., 2017). They also reported that PNX levels are positively correlated to LH, FSH, T, progesterone and nesfatin-1 in circulation. Treatment with PNX-20 promotes follicular growth via GPR173 in human ovarian follicles (Nguyen et al., 2019). Comparing the results from diestrus female rats and human patients with PCOS suggests PNX-20 have an important role in the regulation of reproductive hormones and normal gonadal cycle in mammals. Recently it was reported that ICV administration of PNX

alone or in combination with nesfatin-1 induces plasma LH, FSH and T levels in male rats without any change in plasma GnRH levels (Guvenc et al., 2019).

In fish, reports elucidating the role of PNX are lacking. So far there are only a few reports available which studied the role of PNX-20 in a non-mammalian vertebrate. Wang and colleagues characterized PNX in spotted scat (*Scatophagus argus*) and reported that PNX is expressed in both central and peripheral tissues with the highest levels in the hypothalamus (Wang et al., 2018). They also reported that short-term fasting elevates the SMIM20 mRNA expression in the hypothalamus and indicate a possible role for PNX in feeding and metabolism in spotted scat. In another study, they characterized the mRNA expression at different ovarian/ follicular stages in spotted scat and found that the highest expression of SMIM20 observed in the hypothalamus and pituitary at stage IV oocyte stage (Wang et al., 2019). They reported that *in vitro* incubation of pituitary fragments with PNX-20 or PNX-14 significantly increased the expression of GnRHr, LH and FSH mRNAs and observed a similar effect in their *in vivo* injection study as well. In my study, it was observed that PNX-20 suppressed feeding, and modulated feeding and glucoregulatory genes in zebrafish (Rajeswari et al., 2020). This suggests that similar to mammals, PNX might have a possible regulatory role in feeding, metabolism and reproduction in fish.

1.4. Hormonal control of gametogenesis in fish

In fish, gametogenesis is under the control of the HPG axis. The gonadotropins from the pituitary and the sex steroids from the gonads mediate gametogenesis. In male and female fish, there are some marked differences in gametogenesis and the main hormonal factors controlling it.

1.4.1. Hormonal control of spermatogenesis

The testis of teleosts is composed of interstitial and lobular (tubular) compartments. The interstitial compartment separates individual lobules contain the interstitial cells, also called Leydig cells, blood cells and fibroblast connective tissue. The Leydig cells produce sex steroids (androgens) in response to the stimulation from gonadotropins (Nagahama, 1983). The lobular compartment is composed of germ cells that produce the male gametes and the somatic cells, known as Sertoli cells. The Sertoli cells have an intimate connection with, and ensheaths the spermatogonium, which produces spermatocytes around the developing gametes.

Spermatogenesis consists of a series of stages that resulted in sperm production. The main successive stages in spermatogenesis are differentiated spermatogonia, primary spermatocytes, secondary spermatocytes, spermatids and spermatozoa (Schulz et al., 2010).

In general, FSH controls the first part of spermatogenesis, and LH regulates the following stages. In vertebrates, FSH predominantly controls the Sertoli cell functions and LH controls the Leydig cell functions via their respective receptors. However, in fish as mentioned earlier receptor-ligand non-specificity is observed in many fish species (Schulz et al., 2010), so there are possible exceptions for the above statement in fish. In fish, both FSH and LH are considered as potent sex steroidogenic hormones (Planas et al., 1993; Schulz et al., 2010). In addition to FSH and LH, Sertoli cell and local gonadal-cell (Leydig cells for instance) derived factors also have a role in fish spermatogenesis. They are activin, inhibin (Miura and Miura, 2001), gonadal soma-derived growth factor (GSDF) (Sawatari et al., 2007); both activin and GSDF are members of the transforming growth factor (TGF)-β superfamily, AMH (Miura et al., 2002) and its receptor (Morinaga et al., 2007), the platelet-derived endothelial cell growth factor (PD-ECGF) (Miura et al., 2003) and Gdf9 (Chen et al., 2017). The expression of some of these factors are under the control of the androgens (activin, AMH) and estrogens (PD-ECDF) (Schulz et al., 2010). In addition to the gonadotropins, another pituitary derived hormone, the growth hormone (GH) also plays an important role in spermatogenesis in fish through the growth hormone-insulin-like growth factor-1 (GH-IGF-1 system) (Schulz et al., 2010; Singh et al., 1988). The role of GH-IGF-1 in promoting spermatogenesis and sperm production are documented in several fish species including Chinook salmon (Campbell et al., 2003), trout (Gac et al., 1996; Gomez et al., 1998; Le Gac et al., 1993; Loir and Gac, 1994), tilapia (Berishvili et al., 2006) and Japanese eel (Miura and Miura, 2001).

The role of sex steroids in teleost spermatogenesis is also documented in many fish species. Similar to other vertebrates, androgens (11-KT and T) play a critical role in spermatogenesis in fish as well. It was observed that there is a progressive increase in their level in the blood as the spermatogenesis continues, which decreased during spermiation (Schulz et al., 2010). Androgens promote the expression of genes that are known to regulate spermatogenesis in fish (Le Gac et al., 2008; Schulz et al., 2010). Some of these genes which are upregulated are the double-sex- and mab-3-related transcription factor 1 (DMRT1), transcription factor AP-1,

transcription factor E2F4 (E2F-4), peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α), and transcription factor SOX-8 (Schulz et al., 2010). One of the genes which is suppressed by both 11KT and T in the testis is the AMH, which is a potent suppressor of spermatogenesis and sex steroidogenesis in many teleost species (Halm et al., 2007; Pala et al., 2008; Rodríguez-Marí et al., 2005). All this information suggests the involvement of androgens in spermatogenesis. However, the role of estrogens in spermatogenesis is not clear in fish. Even though a lower dose of estrogens show some stimulatory effects, higher doses show inhibition of spermatogenesis in many fish species (Chaves-Pozo et al., 2007; Lahnsteiner et al., 2006; Song and Gutzeit, 2003). Another important steroid hormone involved in spermatogenesis is the 17α,20β-dihydroxy-4-pregnen-3-one (DHP), which is a progestin critical for the final maturation of spermatogenesis in many vertebrates including fish. High progestin titer is observed in the spermiation stage of many teleosts (Schulz et al., 2010). For instance, in salmonidae and cyprinidae, both DHP and 17α,20β,21-trihydroxy-4-pregnen-3-one (20β-S) induce spermiation (Ueda et al., 1985). In addition, they also have a role in milt production (Baynes and Scott, 1985; Yueh and Chang, 1997), sperm motility (Miura et al., 1992b; Tubbs and Thomas, 2008), and the initiation of spermatogenetic cell meiosis (Miura et al., 2006) in teleosts.

1.4.2. Hormonal control of oogenesis

The ovary of teleost may form in pairs, as in zebrafish, or single (paired ovary fused to form this) as in the case of medaka (Grier et al., 2009). Even though there are marked differences in their structural organization and pattern of gamete development, the ovary of teleost contains the following tissues and cell types. The main cell type of the ovary is the oogonia which give rise to oocytes that undergo different developing stages and follicle cells immediately surrounding the developing oocytes, supporting tissue also called stromal tissue and vascular well as nervous tissue (Nagahama, 1983). Oocytes in the early stage of development are surrounded by follicle cells, the granulosa cells in the inside, and thecal cells on the outside. Both of them are separated from each other by a basement membrane (Nagahama, 1983). In zebrafish, oogenesis or follicular development consists of five distinct stages (categorized based on the size of the follicle, content and stage of development) (**Figure 1.2**). They are stage I and stage II, called previtellogenic follicles (size less than 0.34 mm), stage III, the growing follicle (vitellogenin follicle, size range 0.35-0.69 mm), stage IV, called the maturation stage follicle

(greater than 0.69 mm), stage V, follicle which is the fully ovulated egg (Clelland and Peng, 2009; Clelland et al., 2007; Peng et al., 2009). Similar to spermatogenesis, oogenesis or formation of ova or egg also occurs through successive stages. They are follows: the formation of the oogonia from the primordial germ cells (PGCs), the transformation of oogonia to the primary oocytes by meiotic division, followed by an increase in the size of the primary oocyte (also called the growth phase) by the accumulation of reserve food (vitellogenesis, described separately) inside the oocyte by endocytosis. The growth phase is divided into primary (accumulation of maternal RNA and cortical alveoli formation happens and secondary (vitellogenin, vtg uptake) growth phases. Once the oocyte complete growth, the next phase in oocyte development called the maturation of oocyte begins. This was characterized by a stoppage of growth (reduction in the endocytosis of vtg) and the reinitiation of meiosis (oocytes until this stage are at meiotic arrest, at the diplotene stage of prophase-I). In the maturation phase, the developing oocyte completes the meiosis-1 division by the germinal vesicle breakdown (GVBD) and the formation of haploid secondary oocytes and a polar body (Lubzens et al., 2010; Nagahama, 1983). This was followed by the release of the egg to outside by the rupturing of the follicle in the oviduct, thus completing oogenesis. These are the main events during oogenesis in fish.

Similar to spermatogenesis, oogenesis is also under the control of the pituitary gonadotropins. In addition, several hormones and growth factors are known to be involved in this process. The role of endocrine and paracrine factors involved in the ovarian physiology in fish was documented by several authors (Chen et al., 2017; Clelland and Peng, 2009; Clelland et al., 2007; Kohli et al., 2003, 2005; Wu et al., 2000; Zhou et al., 2016). In general, the first phase of oogenesis is under the control of FSH which includes the growth and development of follicle and vitellogenesis, and the second phase is under the control of LH (oocyte maturation and ovulation) (Levavi-Sivan et al., 2010; Yaron et al., 2003). However, studies indicate that in many fish species both LH and FSH can potentiate early oogenesis including vitellogenesis (Aizen et al., 2012), the final maturation of oocytes included by the MIH which can only be achieved by the LH not FSH (Planas et al., 2000; Suzuki et al., 1988). The FSH dominates the early phase of oogenesis (even though this process can happen without gonadotropin regulation) and vitellogenesis partially via stimulating the estradiol biosynthesis from the ovarian follicles

(granulosa and thecal cells, please refer sex steroidogenesis section) which in turn regulate the production of vtg from the liver (Nagahama, 1983, 1994; Nagahama and Yamashita, 2008; Yaron and Levavi-Sivan, 2011). During the growth of oocytes, their nuclear division is continued to be arrested at prophase-1 of first meiotic division (Yaron and Levavi-Sivan, 2011). Once the growth completes, called post-vitellogenic oocyte, they may continue to stay like that for months in several fish species (Yaron and Levavi-Sivan, 2011). However, the oocyte will enter into the next phase of development called the maturation, which was triggered by an LH surge. LH binds to the LH receptors in the granulosa cells of the ovarian follicle and induces the production of maturation-inducing steroids (MIS) (Nagahama and Yamashita, 2008; Yaron and Levavi-Sivan, 2011). It was found that LH-induced oocyte maturation is achievable only in intact follicles, not in defolliculated oocytes, which indicates LH mediates oocyte maturation indirectly and led to the discovery of MIH (Goetz, 1983; Nagahama, 1987). There are two MIS identified in fish, 17α,20β-DP (17a,20B-dihydroxy-4-pregnen-3-one or 17βS, MIH), 17a,20B,21 -trihydroxy-4-pregnen-3-one, 20βS). The 17βS is more commonly known as MIH because of its high potency to induce oocyte maturation in many teleosts tested (Nagahama, 1983; Nagahama and Yamashita, 2008). The 20βS was first identified from Atlantic croaker (Nagahama and Adachi, 1985) and later in spotted sea trout, which is a potent inducer of oocyte maturation in this species (Thomas and Trant, 1989; Trant and Thomas, 1989; Trant et al., 1986). MIH production is similar to what was observed in case of estradiol, which supports the two-cell theory. In the oocyte growth phase, the follicular cells (theca and granulosa cells) involved in the biosynthesis of estradiol (theca produce T, which is converted to E2 by the granulosa cells), which control oocyte growth and vitellogenesis. The shift happens during the maturation stage in which instead of estradiol, the follicular cells produce more MIH (theca produce 17a hydroxyprogesterone, which is the precursor of MIH) for the final maturation of the oocyte.

During the maturation, the oocyte resumes and completes the first meiotic division, which is controlled by both LH and MIH. The MIH binds to its receptors on the oocyte surface and a cascade of G-protein coupled signal transduction is initiated (Oba et al., 1997; Pace and Thomas, 2005; Yoshikuni and Nagahama, 1994). It was reported that MIH is only able to induce oocyte maturation by acting on the cell surface, and injection of MIH into oocytes fails to induce oocyte maturation (Yamashita et al., 1992). MIH induces the production of a factor within the

cell, which mediates the final maturation events in the oocytes and it was named as maturation-promoting factor (MPF) (Masui, 1996; Masui and Markert, 1971). Earlier characterization studies in goldfish indicate that the active MPF consists of a complex of 34-kDa cdc2 kinases and cyclin B (Hirai et al., 1992; Katsu et al., 1993). It was also reported that MIH induces the production of cyclin B, and the activation of 35-kDa cdc2 (inactive) to 34-kDa cdc2 (active form). The active cdc2 binds to cyclin B to form the MPF (Nagahama, 1994). In conclusion, the MPF induces the final maturation of oocyte by GVBD followed by ovulation, which is also regulated by the MPF along with LH and MIH in fish.

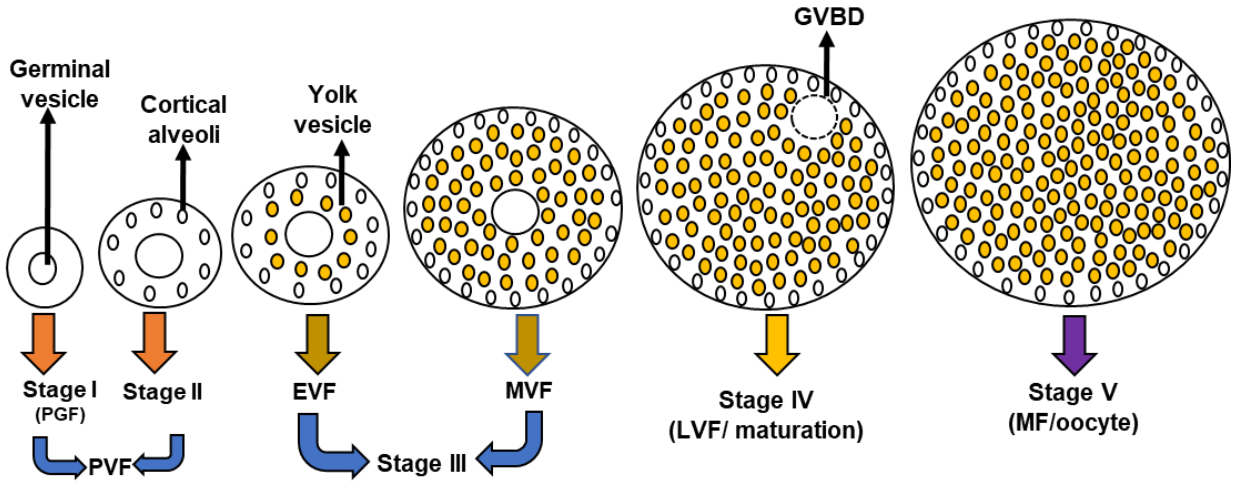

Figure 1.2: Schematic representation of development stages of zebrafish ovarian follicles, stage I to stage V: PGF: Primary-growth follicles; PVF: Previtellogenic follicle; EV: Early vitellogenic follicle; MVF; Mid vitellogenic follicle; LVF: Late vitellogenic follicle/ maturation follicle; MF: mature follicle/ oocyte; GVBD: Germinal vesicle breakdown.

1.4.3. Vitellogenesis in fish

Vitellogenesis involves the synthesis, transport, absorption and processing of vtg by the oocytes (Wootton and Smith, 2015), and it is a critical process in oogenesis. Vtg is the principal component of the egg yolk protein. It is predominantly synthesized by the liver in vertebrates, which is under the control of estrogen (estradiol-17β) (Polzonetti-Magni et al., 2004). Vtg acts as the critical nutrient reserve for the developing embryo. It is processed by the developing oocyte

as there components, namely lipovitellin I (LVI), phosvitin (PV), and lipovitellin II (LVII) (Wang et al., 2005). Estrogen acts by binding to the estrogen receptor (ER), which is a nuclear receptor which in turn binds to the estrogen response element (ERE) sequences in the nucleus and activates gene expression and protein synthesis (Gruber et al., 2004; Klinge et al., 2004). In vertebrates (mammals, birds and fish), there are at least two ERs, ERa and ERb. In zebrafish, ER subtypes are estrogen receptor 1 (ESR1), estrogen receptor 2a (ESR2a) and estrogen receptor 2b (ESR2b) (Hawkins et al., 2000; Menuet et al., 2002). Vtg is a large protein (phospholipoglycoprotein) of molecular weight ranging from 250-600 kDa with calcium-binding property (Levi et al., 2009). Previous research documented seven vtg transcripts expressed in the liver (Wang et al., 2005), but more recent proteome profiling revealed the presence of an additional (8^{th}) vtg transcript in zebrafish (Ziv et al., 2008) (**Figure 1.3**). The vtg gene transcripts are further divided into three families: vitellogenin (VTG) 1 or VtgAo1 (Vtg1, 4, 5, 6 and 7), VTG2 or VtgAo2 (two Vtg2 genes), and VTG3 or VTG C (only one member, Vtg3) (Levi et al., 2009). All genes except Vtg3 (chromosome no 11) were located in chromosome 22 (Finn and Kristoffersen, 2007). All vtg genes are expressed in the female liver, however, the expression of these genes in male and juvenile fish can be triggered by estradiol treatment in zebrafish (Wang et al., 2005). The expression abundance (mRNA) of vtg1 is very high compared to vtg2 (100 times) and vtg3 in zebrafish (1000 times) (Wang et al., 2005). In addition to its role as the main source of vtg, the liver is also responsible for the synthesis of critical ovarian molecules, including the zona pellucida proteins and the steroid hormone-binding globulin (SHBG), and have a role in zebrafish reproduction (Arukwe and Goksøyr, 2003; Levi et al., 2009).

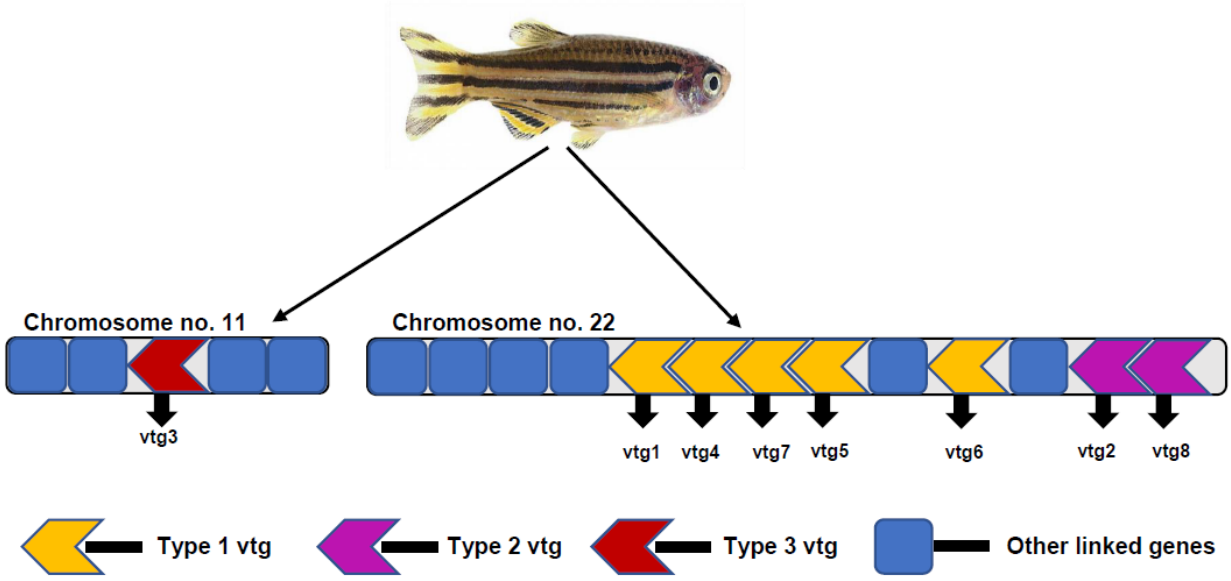

Figure 1.3: Syntenic organization of multiple vtg genes in the zebrafish genome: Pictorial representation of vtg genes in zebrafish genome in chromosome no 11 and 22. Three types of zebrafish vtg genes are represented in chevron (arrows) with different colors (orange, magenta and red). Other linked genes are represented in rectangles (blue).

1.4.4. Steroidogenesis in fish

Steroid hormones are important modulators of different functions, including development, stress response, reproduction, neuroprotection, and energy and mineral homeostasis in vertebrates (Rajakumar and Senthilkumaran, 2020; Tokarz et al., 2015). In teleosts, sex steroids and glucocorticoids play an important role in sex determination as well (Devlin and Nagahama, 2002; Guiguen et al., 2010; Hattori et al., 2009; Rajakumar and Senthilkumaran, 2020; Yamaguchi et al., 2010). Precursor for all steroid hormones is cholesterol, which is converted to pregnenolone, which is then subsequently converted into different steroid hormones (Miller, 2013). The main organs of steroid hormone synthesis are the gonads, the interrenal glands and the brain in teleosts (Tokarz et al., 2015). Steroidogenesis is under the control of two neuroendocrine axis, the hypothalamo–pituitary–gonadal (HPG) axis and the hypothalamo–pituitary–interrenal (HPI) axis in fish (Liley and Stacey, 1983; Liu et al., 2011a; Tokarz et al., 2015). The organs dedicated to steroidogenesis express a set of genes that encode critical processing enzymes (predominantly inside the mitochondria of a cell) that regulate steroid hormone biosynthetic pathway (Miller, 2013) (**Figure 1.4**).

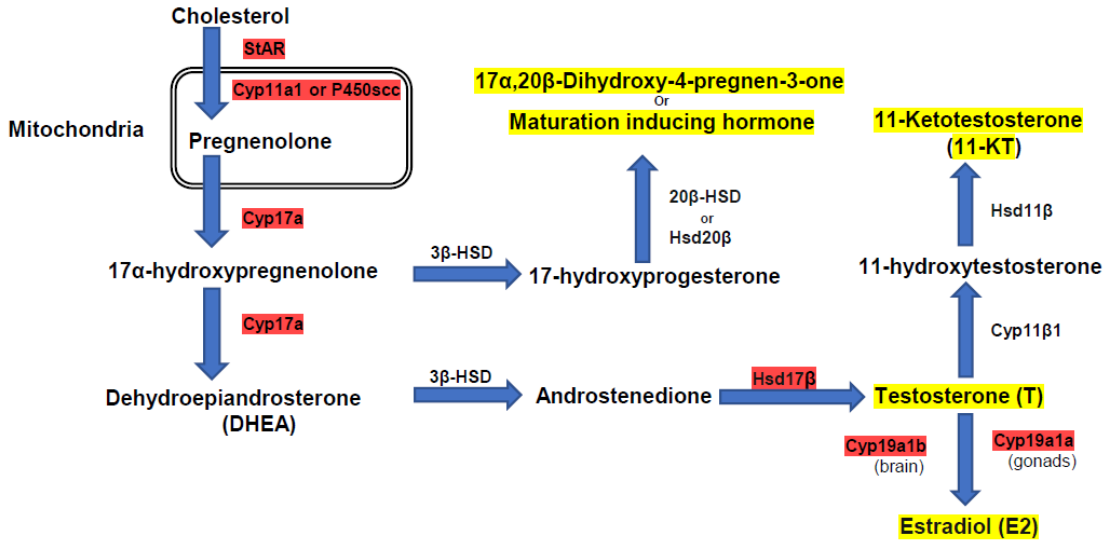

Figure 1.4: Sex steroid hormone biosynthetic pathway in fish: Representative flow diagram showing major enzymes, and the intermediate and final products of sex steroid biosynthetic pathway in fish. Genes studied in the thesis are highlighted in red and final products are highlighted in yellow.

In the steroidogenic pathway, there are some critical enzymes and protein transporters that play an important role. The transport of cholesterol from outside (cytoplasm) to the inner mitochondrial membrane is achieved predominantly by a non-vesicular cholesterol transport mechanism mediated by the StAR that has high affinity towards cholesterol (Miller and Bose, 2011). The second important regulator is CYP11a1 or P450scc, which is located inside the inner mitochondrial membrane. CYP11a1 convert the insoluble cholesterol (by cleaving the 20, 22 side chains of cholesterol) to soluble pregnenolone. This is a limiting step (Nematollahi et al., 2012) and is a slow process in the steroid hormone biosynthetic pathway (Mast et al., 2011). The expression of CYP11a1 is controlled by hormones (tropic and metabolic), and its expression pattern is varied during different physiological conditions in teleosts (Rajakumar and Senthilkumaran, 2014a). Subsequently, pregnenolone is converted into corticosteroids, testosterone (T), 11-ketotestosterone (11-KT), estradiol-17β (E2) and 17α, 20β-dihydroxy-4-pregnen-3-one (17α,20β-DP) by different enzymatic pathways (Rajakumar and Senthilkumaran, 2020). Some of the major enzymes involved in the sex steroidogenic pathway are explained in the next section which includes CYP17a, 17β hydroxysteroid dehydrogenase 1 (HSD17b), 3β-HSD, CYP19a1a (aromatase), CYP19a1b, CYP11b1, HSD11b, and 20β-HSD.

CYP17a is also called 17α-hydroxylase/lyase, which is one of the most studied enzymes because it catalyzes a critical step in the steroidogenic pathway. This is the only enzyme that can convert C21 steroids to C19 steroids and the major products of this process are 17α-hydroxyprogesterone and androstenedione, which cannot be produced otherwise (Tokarz et al., 2015). In fish, a gonad-specific form, CYP17a1, and a gonadal and extragonadal variant CYP17a2 was identified in several species (Rajakumar and Senthilkumaran, 2020). It was postulated that CYP17a1 and CYP17a2 are possibly involved in the steroidogenic shift during the development and maturation of gametes in teleosts (Rajakumar and Senthilkumaran, 2020). 3β-HSD is involved in the conversion of three steroid hormone intermediates: 1. pregnenolone, 17-hydroxypregnenolone, 2. dehydroepiandrosterone (DHEA) to progesterone, and 3. 17-hydroxyprogesterone and androstenedione (Diotel et al., 2011). HSD17b is involved in the regulation of specific substrate availability, catalyzing the conversion rate of 17β-hydroxy-(active) and 17-keto-(inactive) steroids and regulates E and T biosynthesis (Rajakumar and Senthilkumaran, 2014b, 2020). In teleosts, along with CYP19a1a, HSD17b1 is involved in the

conversion of estrone (E1) to estradiol (E2) (Zhou et al., 2005). HSD17b2 have a role in the inactivation of both estrogens and androgens and HSD17b3 is involved in androgen production in the testis (Adamski and Jakob, 2001; Moeller and Adamski, 2009). CYP19a1a (popularly known as the aromatase) is another critical enzyme involved in the sex steroidogenic pathway responsible for C18 steroid biosynthesis (predominantly estrogens). Gonadal steroids have important roles in sex determination and sex reversal in teleosts and this enzyme in particular play an inevitable role in it (Böhne et al., 2013; Mills et al., 2014; Rashid et al., 2007). For instance, CYP19a1a knockout in zebrafish (TALEN or CRISPR) resulted in all male offspring (Lau et al., 2016). In the ovarian follicles of fish, the theca cells produce T in response to gonadotropins , which is then transported to the granulosa cells where CYP19a1a convert T to E (Nagahama et al., 1995; Senthilkumaran et al., 2004). However, during the maturation phase, CYP19a1a was inhibited by the LH (promote 20β-hydroxysteroid dehydrogenase, 20β-HSD or HSD20 β expression instead) for a steroidogenic shift (E to DHP) (Nakamura et al., 2016; Senthilkumaran et al., 2004). The production of progestins (DHP) and estrogens (E1 and E2) in the ovarian follicle is an example for the two cell theory (Ryan et al., 1968), which explains the involvement of two cell compartments with enzyme machinery for the production of these sex steroids. CYP19a1b is involved in the conversion of T to E, which is abundantly expressed in the brain. CYP11b1 is involved in the production of 11-hydroxyandrostenedione (11-OHA) or 11-hydroxytestosterone in the testis of many teleosts, which is the precursor of 11-KT (Lokman et al., 2002; Rajakumar and Senthilkumaran, 2015). HSD11b is involved in the biosynthesis of androgens and glucocorticoids in fish (Moeller and Adamski, 2009; Rajakumar and Senthilkumaran, 2016). 20β-HSD, as mentioned earlier, is involved in the production of MIH or DHP in some fish species (Senthilkumaran et al., 2004; Sreenivasulu et al., 2012).

1.5. Animal models used in my research

In biomedical research, different animal models are employed to answer research questions. For my thesis research, goldfish *(Carassius auratus)* and zebrafish *(Danio rerio)* were used. Although mouse, rat and primates are important research models, the percentage usage of fish as a research model has been gradually increasing since 1960s (Bolis et al., 2001). Some of the notable qualities of fish that makes it a desirable model include its relatively small size, short generation time, low maintenance cost, amenability to transgenesis and mutagenesis, the capability to produce a large number of eggs/offspring at a time, transparent embryos for developmental studies, and conserved structural and functional organ systems. In addition to its use in biomedical and fundamental biological studies, several fish species are used to answer aquaculture related questions. Both animal models I have used here are not commercially cultured for human consumption. I use these two models to gather information about fundamental research questions that can benefit the aquaculture improvement. One of the best examples for the successful application of small fish models for the improvement of aquaculture production is the formulation of Ovaprim™, a commercially used spawning inducer. It was formulated based on research conducted in goldfish (Omeljaniuk et al., 1987; Peter et al., 1987, 1988). As the neuroendocrine regulation of fish reproduction is relatively well-characterized, and the basic knowledge garnered using such models are possibly translatable to other species, I am using goldfish and zebrafish, members of the cyprinidae family.

1.5.1. Goldfish

Goldfish (*C. auratus*) is a freshwater fish that belongs to the cyprinidae (carp) family. It is a world famous ornamental fish, domesticated about 1000 years ago in South China (Omori and Kon, 2019). About 180 variants in 70 genetic strains are currently bred across the globe. In addition to its ornamental value, it is widely used as a research model because of its unique advantages over other fishes. The FAO statistics indicates that among all cultured fish species in aquaculture, members from cyprinidae are the most widely cultured in terms of the number of countries employing them and the production numbers (FAO, 2017c). The most widely used cyprinid in research is zebrafish, which is a very close relative of goldfish. Goldfish have some advantages over zebrafish in studying many research questions. Goldfish is a suitable model organism to study research related to pigmentation and body coloration in vertebrates, to study

the diversity of molecular basis of organ morphology and skeletal system, and artificial selection (Omori and Kon, 2019). The size of goldfish is another advantage compared to zebrafish. This allows more access to some tissue samples (for instance, pituitary tissue samples in my research) and serum collection (multiple blood collection). Goldfish is suitable for developmental studies due to the bigger larval size compared to zebrafish larvae (Tsai et al., 2013). In addition, the whole genome sequencing of goldfish was completed (Chen et al., 2019). The common and comet varieties of goldfish are widely used in research. Goldfish can be maintained relatively easily when compared to many other cultured fish models, especially trout and salmon. They can survive in a wide range of temperatures (20-41 ^{o}C), turbidity, pH (5.5-7.0) and salinity (up to 6 ppm) conditions (Blanco et al., 2018b). Goldfish is also widely used in neurology and neuroendocrinology due to the availability of the stereotaxic atlas (Peter and Gill, 1975). It was found that goldfish is also a suitable model for both *in vivo* and *in vitro* studies including growth, behavior, comparative physiology, hormonal actions, cell and organ culture studies (Bertucci et al., 2017; Grey and Chang, 2011; Maximino et al., 2015; Nakamachi et al., 2014; Tinoco et al., 2015).

Goldfish possess all major internal organs found in vertebrates, which includes the brain, pituitary, heart, gut, liver (hepatopancreas), adipose tissue, kidney (head and caudal compartments), gonads and also gill (as compared to mammalian lungs). As my research is focused on the neuroendocrine regulation of reproduction, the main organs of interest will be the brain (especially, the hypothalamus), pituitary, and the gonads. Goldfish is a very widely used model in neurobiology because of its large sized brain. This feature makes it easy for the collection, processing and study of tissues/organs, and the measurement of physiological and biochemical parameters in individual lobes or portions of the brain. The aim of my thesis research is to study hormonal mediators of reproduction, which are expected abundantly in the goldfish brain. Therefore, the bigger size makes it easy to confidently separate and study the brain and the hypothalamus separately in case of goldfish. For instance, there are two GnRHs expressed in cyprinidae, salmon and chicken GnRH, of which one is predominantly expressed in the hypothalamus (sGnRH) and the other one (cGnRH2) is in the midbrain (more details are in the HPG axis section). This makes it is easy to localize and quantify the expression of both GnRH types in goldfish separately. Another advantage of the use of goldfish in my study is the

availability of a larger pituitary. Gonadotropins are inevitable for reproduction in vertebrates and quantifying their expression is important for the full understanding of neuroendocrine regulation of reproduction. The third most important organ of my research interest is the gonads (the testis and ovary). Goldfish is a seasonal breeder (reproductively active during spring-summer). Because of this reason, goldfish is widely used to study the seasonal changes in the hormonal milieu and gonadal morphology related to reproduction in fish (Ge et al., 1993; Habibi et al., 1989; Peter and Crim, 1978; Trudeau, 1997). They are also used in studies related to the influence of the environmental factors on gonadal growth and reproduction in fish (Delahunty and Vlaming, 1980; Spieler et al., 1977). Previous research from our lab characterized the mRNA and protein expression of several peptides with hormone-like actions in the HPG axis of goldfish (Gonzalez et al., 2012b; Sundarrajan et al., 2016).

Although there are several advantages of using goldfish in research related to neuroendocrinology, there are some drawbacks and challenges as well. One of them is the presence of multiple variants of the same gene due to genome duplication and tetraploidy of the goldfish genome (Blanco et al., 2018b; Larhammar and Risinger, 1994). A second challenge is the seasonal reproductive nature of goldfish, which limits certain studies to a limited period during the year. Some of the hormones involved in reproduction act in a reproductive-stage specific manner and this makes it hard to make conclusions in goldfish (Moussavi et al., 2009, 2012, 2013). For reproducible and reliable results, consistency in seasonal experimentation is very important when goldfish is used for reproduction research.

1.5.2. Zebrafish

Zebrafish, a tropical cyprinid is one of the most widely used vertebrate model used in biomedical research. Zebrafish has become a very popular animal model during the last 30 years. The founding father of zebrafish model-based research is George Stresinger, who was interested in this because of its small size (up to 5 cm), transparent embryos and larvae, and an impressive fecundity rate (Grunwald and Streisinger, 1992). Zebrafish is a very popular animal model for genetic and developmental research because of its small size, need for less space to house, ease in mutation studies in vertebrate development (due to its transparent embryo), and the large size of egg mass (approximately 200 eggs per week per female fish). This is an additional benefit for large-scale screening and statistical analysis compared to mammalian models these features

made zebrafish a favorite model for biomedical research. Forward genetics and mutation studies in zebrafish led to understand the complex orchestration of vertebrate early developmental events (Poon and Brand, 2013).

Fast development is another important feature of zebrafish. The fertilized egg initiates developmental signs and starts the formation of the primordial organs at about 24 hr post-fertilization (hpf) (Poon and Brand, 2013). The embryo will hatch at about 48 hpf and become a larva 72 hpf. Metamorphosis of zebrafish happens at about 30 days post fertilization (dpf) and become adults capable of reproduction on 90 dpf. The complete genome sequence was made available to the scientific world (Howe et al., 2013). This development further helped the usage of zebrafish as a human disease model. It was found that 70% of all human genes have at least one orthologue representative in the zebrafish genome (Howe et al., 2013).

In addition to human disease and biomedical research, many studies and reviews suggest that zebrafish is a suitable model for aquaculture and fish husbandry as well (Aleström and Winther-Larsen, 2016; Ribas and Piferrer, 2014; Ulloa et al., 2014). One of the main areas where zebrafish is used as a model system for aquaculture research is in feed formulation (Hedrera et al., 2013; Ulloa et al., 2011, 2013, 2014). Zebrafish also used as a model organism to study the growth performance, reproduction, disease resistance, environmental toxicology and stress response (Aleström and Winther-Larsen, 2016; Gerhart and Janz, 2019; Pettem et al., 2017). Another important area were zebrafish is widely used is gene manipulation studies. They are very suitable for both gene knockdown (siRNA or ShRNA) or gene knockout (TALEN and CRISPR) platforms which was established for several human and biomedical research programs and evolutionary and comparative endocrinology studies (Irion et al., 2014; Seth et al., 2013). It was reported that the efficiency of CRISPR/Cas system is 75-99% in zebrafish models (Cade et al., 2012; Hwang et al., 2013). As the development is external, non-invasive collection and manipulation of embryonic gene manipulations are possible in zebrafish compared to mammalian models.

Zebrafish attain maturity approximately 90 days post fertilization and are capable of reproduction after this. However, unlike goldfish, zebrafish reproduction is continuous, and they breed throughout the year (irrespective of the season), particularly in laboratory conditions. In zebrafish, the sexes are not distinguishable until 3 months of age and the gonads are primarily

developed with ovarian tissue. However, when they reach 11 weeks old, a transition from female to male gonadal tissue becomes more prominent, and ovarian tissue in male fish disintegrates by apoptosis (Maack and Segner, 2003; Nasiadka and Clark, 2012). Research suggests the best age range of zebrafish for reproductive related studies and breeding programs is between 6 months to 2 years (Westerfield, 2000). They are suitable for both *in vitro* (e.g. oocyte maturation and gonadal organ culture) and *in vivo* (e.g. IP injection) studies to study reproduction.

One of the notable disadvantages of zebrafish is the small pituitary, which is extremely difficult to collect and use for gene expression and morphological studies. Another important limitation is the absence of large blood volume for serum or plasma samples for multiple hormone analysis. While such limitations exist, zebrafish is still considered and widely used as an important research model in both human and aquatic research, including reproductive biology and neuroendocrinology.

1.6. Rationale:

PNX is emerging as a potent regulator of reproduction in mammals (Stein et al., 2016; Yosten et al., 2013). Recent studies suggest possible roles for PNX in fish reproduction (Wang et al., 2019). However, it is unclear whether PNX-20 is indeed a reproductive regulator in fish. The role of nesfatin-1 in the regulation of fish reproductive hormones from the hypothalamus and pituitary has been identified (Gonzalez et al., 2012b). Whether nesfatin-1 influences gonadal biology as well, and that again, in a sex-specific manner is yet to be determined. Characterization studies conducted in goldfish identified NLP and localized NUCB1/NLP-like immunoreactivity in the supporting cell layers of goldfish gonads. It was also found that sex steroids modulate NUCB1 mRNA expression in goldfish (Sundarrajan et al., 2016). These results suggest that NLP has a regulatory role in goldfish reproduction but require additional research to identify potential roles of NLP on reproduction. I used goldfish and zebrafish to conduct research that aims to gain a deeper understanding on the biology of PNX, nesfatin-1 and NLP in fish reproductive biology. To achieve this, my research focused primarily on measuring reproductive hormones or mRNAs encoding these hormones, their receptors and/or processing enzymes. In some studies, mRNAs encoding vtg were measured, and oocyte maturation was studied *in vitro*.

1.7. Hypotheses:

1. PNX-20 promotes, while nesfatin-1 and NLP suppress reproductive hormones primarily derived from the HPG axis of fish in a sex-specific manner.
2. PNX-20 acts directly on the liver and stimulate liver vitellogenic processes.
3. PNX-20 increases, but NLP suppresses oocyte maturation in zebrafish.

1.8. Specific objectives of my thesis research:

1. Elucidate the reproductive endocrine and ovarian roles of PNX-20 in zebrafish.
2. Determine the role of NUCB2/nesfatin-1 in the control of HPG axis, especially gonadal steroid hormones in goldfish.
3. To study whether NLP has any role in the regulation of reproduction through its actions on HPG hormones and ovarian functions in fish.

Transition

The following chapter will focus on the first objective of my thesis research. The reproductive regulatory role of novel peptide phoenixin is established in mammals (Stein et al., 2016; Treen et al., 2016; Yosten et al., 2013). PNX-20 promotes reproduction in mammals by positively influencing the HPG axis (Stein et al., 2016; Yosten et al., 2013). However, whether PNX is a reproductive hormone in non-mammals, especially fish, is unknown.

Hypothesis: I hypothesized that PNX-20 stimulates reproductive hormones of the HPG axis and promotes oocyte maturation in zebrafish follicles.

Specific objectives:

1. Characterize PNX-20 and determine whether it is involved in the regulation of reproduction in zebrafish via influencing the HPG axis.
2. Determine whether PNX-20 has any role in zebrafish vitellogenesis.
3. Study the role of PNX-20 on oocyte maturation in zebrafish.

Result highlights: My *in vivo* injection studies show an upregulation of both GnRH isoforms in the hypothalamus of male and female zebrafish. In the gonads, the expression of mRNAs encoding the gonadotropin receptor and sex steroidogenic proteins was upregulated. *In vitro* experiments using ZFL cells suggest that PNX-20 promotes vitellogenesis and that endogenous PNX-20 is involved in vitellogenesis in zebrafish. In addition, PNX-20 has acts directly on follicles to promote oocyte maturation in zebrafish. My results suggest a positive reproductive regulatory role for PNX in zebrafish, which appears to be conserved across species in which PNX-20 has been studied to date.

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Chapter 2

Phoenixin-20 Stimulates mRNAs Encoding Hypothalamo-Pituitary-Gonadal Hormones, is Pro-Vitellogenic, and Promotes Oocyte Maturation in Zebrafish

2.1. Abstract

Phoenixin-20 (PNX-20) is a bioactive peptide with hormone-like actions in vertebrates. In mammals, PNX stimulates hypothalamo-pituitary-gonadal hormones and regulates reproductive processes. My immunohisto/cytochemical studies show PNX-like and the putative PNX receptor, SREB3-like immunoreactivity in the gonads of zebrafish, and ZFL cell line. IP injection of zebrafish PNX-20 upregulates mRNAs encoding sGnRH and cGnRH-II, and kisspeptin and its receptor in the zebrafish hypothalamus. Similarly, LHr mRNA expression in the testis, FSHr in the ovary, and the kisspeptin system were upregulated in the gonads of PNX-20 injected fish. I also observed the upregulation of genes involved in the sex steroidogenic pathway (CYP11a1, CYP17a1, , CYP19a1a) in the gonads of PNX-20 administered fish. PNX-20 upregulates the expression of vtg isoforms and esr2a and 2b mRNAs in ZFL cells *in vitro*. Meanwhile, siRNA-mediated knockdown of PNX-20 resulted in the downregulation of all vtg transcripts, further suggesting its possible role in vitellogenesis. PNX-20 treatment resulted in a significant increase in GVBD in zebrafish follicles *in vitro*. Collectively, these results provide strong evidence for PNX-20 effects on the HPG axis and liver to promote reproduction in zebrafish.

2.2. Introduction

Advances in computational biology, especially in the use of bioinformatics tools, helped the discovery of many regulatory molecules with hormone-like actions. A recent example is phoenixin-20 (PNX-20), which was originally identified as a reproductive regulatory peptide (Yosten et al., 2013). Phoenixin was first isolated from the rat hypothalamus and bovine heart by two independent research groups (Lyu et al., 2013; Yosten et al., 2013). PNX stimulates reproductive functions via acting on the HPG axis (Yosten et al., 2013). Later it was reported that PNX mediates its reproductive regulatory effects through GPR173, and cAMP-PKA dependent pathway, acting on both GnRH and kiss1 expressing neurons (Treen et al., 2016). In mice, PNX positively influences Kiss1 transcription and GnRH mediated gonadotropin release (Pałasz et al., 2015; Yosten et al., 2013). ICV injection of PNX-20 significantly increases plasma LH levels in a dose-related manner, and potentiate GnRH-induced LH secretion from cultured anterior pituitary cells *in vitro* (Stein et al., 2016).

PNX in non-mammals is poorly understood. In fish (spotted scat; *S. argus*), short term fasting increases the expression of PNX-20 in the hypothalamus and refeeding attenuates it (Wang et al., 2018). It was also reported that both *in vitro* and *in vivo* administration of PNX upregulates the expression of GnRHr, LH and FSH in the pituitary of spotted scat (Wang et al., 2019). These results suggest PNX appears to have a regulatory role in reproduction in fish. My research aimed to determine whether PNX-20 has a reproductive regulatory role in zebrafish, a well-characterized model system in comparative endocrinology. I tested for the possible effects of PNX-20 on tissue-specific expression of mRNAs encoding reproductive regulatory peptides, and vtg genes in the liver and oocyte maturation in zebrafish. Results from my studies indicate that PNX-20 is a positive modulator of the HPG axis and vitellogenesis and promotes oocyte maturation in zebrafish.

2.3. Materials and methods

2.3.1. Animals

Adult male and female zebrafish (*D. rerio*; 12 months old; body weight: ~1 g) were obtained from the Aquatic Toxicology Research Facility at the University of Saskatchewan. Fish were maintained in constantly aerated freshwater aquaria (10 L capacity), under a simulated 12 h light:12 h dark photoperiod (lights on at 07:00 AM). Fish fed once daily (4% body weight ration) with slow sinking pellets (Aqueon, Franklin, WI, United States) at a scheduled feeding time. Tricaine methanesulfonate-222 (TMS-222, Syndel Laboratories, BC, Canada, 0.5%) was used for anesthetizing the fish (for IP injection) and euthanasia by spinal transection, followed by tissue collection. All animal studies were carried out according to the policies of the Canadian Council for Animal Care and were approved by the University of Saskatchewan Animal Research Ethics Board (Animal Use Protocol # 2012-0082).

2.3.2. *In silico* analysis

SMIM20 amino acid sequences of various vertebrates were obtained from GenBank (https://www.ncbi.nlm.nih.gov/protein/). Clustal Omega multiple sequence analysis tool (https://www.ebi.ac.uk/Tools/msa/clustalo/) was used to align the sequences. I used ProtParam (https://web.expasy.org/protpara) bioinformatics data analysis tool for the computation of various physical and chemical parameters of zebrafish SMIM20.

2.3.3. Tissue distribution of SMIM20 and SREB3

To study the tissue distribution of SMIM20 and SREB3 mRNAs, the following tissues were collected from six zebrafish: brain (without the hypothalamus), hypothalamus, eye, skin, gills, heart, foregut (intestinal bulb and the anterior-most portion of the intestine, equivalent of the J loop region in stomach less fish), hindgut (the posterior-most portion of the intestine), liver, spleen, muscle, testis and ovary. Tissues were immediately frozen in liquid nitrogen and stored at -80 ºC until quantification of gene expression (described in detail in the qPCR section).

2.3.4. Immunohistochemical and immunocytochemical localization of PNX and SREB3 in zebrafish gonads and ZFL cell line

Identifying and localizing PNX and SREB3 immunoreactive cells in the gonads will assist us in predicting possible reproductive regulatory roles of PNX in zebrafish. Immunohistochemical studies (IHC) were conducted using methods described earlier (Hatef et

al., 2015b). The details of antibodies used in my research are rabbit anti-PNX-14 primary antibody (Phoenix Pharmaceuticals, Burlingame, CA, United States, 1:1000 dilution) and rabbit anti-SREB3 antibody - N-terminal (Abcam, Cambridge, UK, 1:500 dilution). The secondary antibody used was Texas Red Goat anti-rabbit antibody (Vector Laboratories, Burlingame, CA, United States, 1:3000 dilution). All antibodies diluted using antibody diluent (Abcam, UK). Antigen retrieval step was included for slides stained with SREB3 (1 mM EDTA, pH 8.0, and heat treatment, antigen retrieval protocol, Abcam). Preabsorption and secondary antibody alone controls were conducted as previously described (Sundarrajan et al., 2017). Briefly, for the preabsorption control, zebrafish PNX-20 (custom synthesized, 10 μg) was preabsorbed with PNX-14 primary antibody overnight and this cocktail was used instead of the primary antibody for immunostaining. The negative controls were run only with the secondary antibody (no primary antibody incubation, used antibody diluent). Only the latter was used as a control for the SREB3 immunoreactivity. Vectashield medium containing DAPI dye (Vector Laboratories, Burlingame, CA, United States), which stain the nucleus of the cells blue, was used to mount the slides. The immunocytochemical (ICC) studies were conducted to localize PNX and SREB3 immunoreactivity in the zebrafish liver cell line (ZFL, Cat# ATCC® CRL-2643™, ATCC, Manassas, VA, United States). The ICC was conducted as previously described (Hatef and Unniappan, 2017) with slight modifications. Briefly, upon 60-70% confluency, cells grown in chamber slides (Corning, VWR, Canada) were fixed using ice-cold methanol (kept overnight at -20) for 2-5 minutes. The cells were then incubated with PBS-PF, followed by cell permeabilization using PBS-Triton X-100 (0.2%) for 10 minutes. The slides were then incubated with blocking solution containing 5% protein block (Abcam) and BSA (5%) in PBS to prevent any non-specific antibody binding. The cells were then incubated with the primary antibody in antibody diluent solution (Abcam, UK) at 4 ^{0}C overnight. I used the same antibodies and dilutions as described above in the IHC section. No antigen retrieval steps were used for SREB3 stained slides (paraformaldehyde fixative is not used). Slides were mounted using Vectashield medium containing DAPI (Vector Laboratories). Both IHC and ICC slides were imaged using Olympus BX 51 microscope (Olympus Corporation, Tokyo, Japan) and analyzed using the DP controller software (Olympus Corporation, Japan). Since heterologous antibodies are used here,

the immunostaining obtained is referred to as phoenixin-like and SREB-like, to consider for the possible non-specific binding of antibodies.

2.3.5. Effect of exogenously administered PNX-20 on reproductive regulatory genes in the hypothalamus and gonads of zebrafish (*In vivo* injection studies)

Male and female zebrafish (age and weight-matched) were grouped (n=6/group) in fish aquaria and were acclimated for 2 weeks. On the experiment day, fish were anesthetized (TMS-222, 0.5%) and IP injected with 0.9% sterile saline (control) or 100 or 1000 ng/g body weight (BW) custom synthesized zebrafish PNX-20 (AGVNQADIQPVGVKVWSDPY, Pacific Immunology, Ramona, CA, United States, Cat# 1711-PAC-21; ≥95% pure). The total injection volume used was 4 microliters (µl)/fish. This dose was decided based on my unpublished results from preliminary studies. Tissues (including the hypothalamus, and gonads) were collected 1 hour post-injection and stored at -80 ^{o}C until further analysis.

2.3.6. ZFL cell line maintenance and PNX-20 treatment (*In vitro* studies)

The liver is the primary site of synthesis of vtg, the principal component of yolk protein critical for the development and maturation of egg or ova. To study whether PNX-20 has any role in vtg system in zebrafish, I incubated liver cells with zebrafish PNX-20. ZFL cell line was purchased from ATCC (Manassas, VA, United States) and was cultured in a complete growth media [50 % L-15 (ATCC, VA, United States), 35 % DMEM HG (Gibco, Thermo Fisher Scientific, Sunnyvale, CA, United States), 15 % Ham's F12 (Gibco, Thermo Fisher Scientific, CA, United States), 0.15 g/L sodium bicarbonate (BioShop, Mainway, Burlington, ON, Canada), 15 mM HEPES (Millipore Sigma, Burlington, Massachusetts, United States), 0.01mg/ml bovine insulin (Sigma-Aldrich, St. Louis, Missouri, United States), 50 ng/ml mouse EGF (Sigma-Aldrich, St. Louis, Missouri, United States), 5% heat-inactivated fetal bovine serum (Gibco, Thermo Fisher Scientific, CA, United States), 0.5% trout serum (Caisson Laboratories, Smithfield, Utah, United States)] as suggested by ATCC. The cells were cultured in T25 ml flasks (Greiner CellSTAR, VWR, PA, United States) and maintained at 28 degrees in an incubator. Cells were sub-cultured every 3 days, or when they reach 90% confluency. Passages 4-6 were used for all my experiments. For the *in vitro* peptide exposure studies, ZFL cells were cultured in 24 well plates (Greiner CellSTAR, VWR, PA, United States) for all experiments.

Briefly, approximately $1X10^6$ cells/well were allowed to grow in a 24 well plate for 2 days or until they reach 60-70% confluency. This was followed by replacement of the complete growth media with media containing different doses of PNX-20 (0, 1, 10, 100 nM zebrafish PNX-20). All my experiments were run with n=6 wells/treatment, and all experiments were repeated twice (total three times). Control wells received complete growth media without any peptides (received an equal amount of sterile water instead) in it. The time points considered are 1, 2, 6, and 24 hr. At each time point, media from cells were removed and 500 µl of RiboZol RNA isolation reagent (aMReSCO, VWR, PA, United States) was added and incubated at room temperature for 5 minutes. This was followed by collecting the samples and stored them at -80 ^oC for total RNA extraction.

2.3.7. Total RNA extraction, cDNA synthesis, RT-PCR, and real-time quantitative PCR

Total RNA extraction from samples (tissues and cells), cDNA synthesis and RT-qPCR were conducted as described earlier (Rajeswari et al., 2019). RiboZol RNA isolation reagent (aMReSCO, VWR, Radnor, PA, United States) was used for total RNA was extraction, followed by DNase I (Thermo Fisher Scientific, CA, United States) treatment following the manufactures instructions. The purity and quantity of the total RNA extracted were determined by optical density (OD) absorption ratio (OD 260 nm/OD 280 nm) using a Nanodrop 2000 (Thermo Fisher Scientific, CA, United States). iScript cDNA synthesis kit (BioRad Laboratories, Hercules, CA, United States) was used for cDNA synthesis, following the manufacturer's instructions. The CFX Connect Real-Time-quantitative PCR Detection System (Bio-Rad, CA, United States) and SensiFAST™ SYBR® No-ROX Kit (Biolne, Froggabio, Toronto, ON, Canada,) were used for gene expression analysis. Please refer **Table 2.1** for qPCR reaction mix details. Livak method (Livak and Schmittgen, 2001) was used for the relative data analysis ($2^{-\Delta\Delta CT}$ method). The quantitative gene expression of different genes and are normalized to the expression of the housekeeping genes, β-actin and 18S rRNA. Validation and optimization of the primers were carried out prior to qPCR to find the highest efficiency annealing temperatures. The qPCR conditions used were as follows: initial denaturation 95 °C (2 min), 35 cycles of denaturation: 95 °C (5 s), annealing: specific to each gene (25 s), elongation: 72 °C (20 s, when annealing temperature was less than 55 °C). A melting curve analysis was performed at 65 °C to 95 °C (5 s) was run to confirm the absence of any dimer formation or artifacts for each primer set. All

samples were run in duplicates. Negative controls with no template DNA (used nuclease-free water instead) in the PCR mix were also run for each gene. The primer sequences and annealing temperatures used for each primer set for quantifying mRNA expression are listed in **Table 2.2**. Other than primers used for vtg genes (except vtg1), all primers are custom designed. Primer sequences for vtg genes (vtg2, 3, 4, 5, & 7) were resynthesized as described earlier (Eide et al., 2014).

Table 2.1: Details of RT-qPCR reaction mix

Component	Volume
SensiFAST™ SYBR® No-ROX Kit	5 µl
Forward Primer	0.5µl
Reverse Primer	0.5 µl
cDNA	2 µl
Millipore Water	2 µl
Total Volume of the Sample	10 µl

Table 2.2: Primer sequence details and annealing temperature used in the RT-qPCR analyses of the expression of mRNAs

Gene	Accession no.	Primer sequence (5'-3')		Annealing temperature (°C)
		Forward	Reverse	
18S rRNA	NM_173234.1	GGCGAGGGTTCTGCATAATA	CATCCTTCGTGTCCTCAACA	60
β-actin	NM_131031.2	TTCAAACGAACGACCAACCT	TTCCGCATCCTGAGTCAATG	60
sGnRH	NM_182887.2	CCCGGTGGAAAAAGAAGCGT	CCCCGTCTGTCTGGAAATCTT	60
cGnRH-II	NM_181439.4	AATGCAGTTACCTGAGACCG	AATCACGAATGAGGGCATCC	60
kiss1	NM_001113489.1	CTTCTCCATGGGTGCAGGTC	AATCGTGTGAGCATGTCCTGT	60
GPR54a	NM_001110531.1	CCTTCTGTGCTGAAGACGTG	CTCGGTGCTCCTCCTTTTGA	60
kiss2	NM_001142585.1	ATGGAGCGAAGGCAGTTTGA	TGTCAGAGTCGCTGGTTGTC	60
FSHr	NM_001001812.1	TTCCTGCTCAAACCCATTCC	GCATCCAAATCGGCTAGTCA	60
LHr	AY714133.1	TGAAAGAGCAGCCAGGTACT	TGCTAAATTTCCTTTCGCCG	60
CYP11a1	NM_152953.2	ACCCTGCATAAATGAGCGTC	GACAGTGGAGTTTTGCGGTG	60
CYP17a1	NM_212806.3	AGACCCACCACAGACCTTTA	GCACAATCGGCCACTTAAAC	60
CYP19a1a	NM_131154.3	ACCTCCACAAACTCTCACCT	TTGAGCGGGACTCCTAGAAA	60
CYP19a1b	NM_131642.2	TTGGACGCATGCATAAGACA	ACCGAATGGCTGGAAGTAAC	60
StAR	NM_131663.1	CTGTTTTCTGGCTGGGATGT	TCGCATGACAATACAGGTGG	60
17β-HSD	NM_205584.2	TGTATCTGGAGGCGATGGAA	TGGAGGTGAGTTTCAGGCTA	60
DMRT	NM_205628.2	CAGTCGCTCCATGTTGTCTT	AGTGGGCTGGTAAAGGTTGT	60
AMH	NM_001007779.1	TTAAGGGACATCCTGCCTCA	AACACAAATACAGTCGGCGT	60
AR	NM_001083123.1	CTCCAAAGCAAAGGACACCT	TTCGCCCATCTCGTTTTTCA	60
ER	NM_152959.1	GATGTCCCTGCTCACCAACA	CAACACTTCCAGCCAAGAGC	60
SMIM20	NM_001302624.1	TTTGGAGGCTTCGTTGCAG	GGCTTGTAGGGATCAGACCA	57
SREB3	NM_131498.1	CCATTGCCCACCATCGTTTC	ATGAAGCCTAGCGTGTCGTT	60
vtg1	NM_001044897.3	GAAGACTTTCTCGCCTGGAT	GCAGTACAGCAGTGGTCTAA	60
vtg2	NM_001044913.1	GGTGACTGGAAGATCCAAG	TCATGCGGCATTGGCTGG	57
vtg3	NM_131265.1	TTAGAACCAGCAAAGGATGC	CATCTCTTTTCTCCTTAAATAC	57
vtg4	NM_001045294.2	GAGAGACTGCCAAAAATTGCCT	GGAGAGAAAATCCTTATCAATGGTG	57
vtg5	NM_001025189.2	CCAAAAATTGTCACCACTTATGCT	CTTCATTCCTCCATGATATGCTTA	60
vtg7	NM_001102671.1	CCATCTCAGAAGTCCTTTG	CAAACATTCTTGTGAAAATGAAGATA	60
esr1	NM_152959.1	GACAGAAACCGTCGAAAGAG	CCGCGATCTTTACGAATACC	58
esr2a	NM_180966.2	GTCCGAGGTCTCAAGAGATA	AGGGCTTCTTCATGTCTTTC	57
esr2b	NM_174862.3	TCTCAGCACCTCTTTCCT	CATGCTAGCCTCAGTGTATG	60
shbg	NM_001007151.3	GAGCAGCAGGTGATCAAATA	CAAGGGTTAGGAACTCAA	57

2.3.8. siRNA mediated knockdown of PNX-20 (*In vitro* studies)

To study whether endogenous PNX-20 has any effect on the vtg system in zebrafish liver, I employed siRNA mediated gene knockdown approach using the ZFL cell line. Zebrafish PNX-20 siRNA and scrambled siRNA are custom synthesized (Dharmacon, Horizon Discovery,

Waterbeach, United Kingdom). The scrambled siRNA represents the same composition of nucleotides; however, the arrangement of nucleotides in the sequence is highly dissimilar. Scrambled siRNA is to check or confirm whether the order of nucleotide or nucleotide integrity is critical for siRNA action. Both siRNAs and its scrambled controls were designed using online design tools (Genscript sequence scramble tool). Details of siRNAs are provided in **Table 2.3**.

The ZFL cells grown in 24 well plates (as described above) were treated with either different doses of siRNA (0, 1, 10, 100 nM) or scrambled siRNA. I used Lipofectamine RNAiMAX (Invitrogen, Thermo Fisher scientific CA, United States) mediated siRNA transfection method in my experiments. The Lipofectamine-siRNA mix was prepared in accordance with the manufacturer's instructions. Briefly, Lipofectamine was prepared using Opti-MEM serum-free media (Gibco, Thermo Fisher Scientific, CA, United States) and was mixed and incubated with siRNA, which devoid of any serum. The final concentrations for the treatments were prepared by adding the appropriate volume of the Lipofectamine-siRNA mix to complete growth media containing serum. This mixture was added to cells, which is about 50-60% confluent. The time points I used to check the level of knockdown were 24, 48, 72 and 96 hr post-siRNA treatment. The total RNA extraction was carried out as described below. Cell viability and growth rate were closely monitored during the entire treatment period.

Table 2.3: Details of siRNA and scrambled siRNA sequences used for the gene knockdown experiment to study the role of PNX in vitellogenesis in zebrafish

Sense sequence	**Antisense sequence**
UACCUACUUUUCCUGCUUACGCUU	GCGUAAGCAGGAAAAGUAGGUAUU
Scrambled Sense sequence	**Scrambled Antisense sequence**
AGUACUUCGCUUCCUUCCUCUUU	AGAGGAAGGAAGCGAAGUACUUU

2.3.9. Oocyte maturation assay

Oocyte maturation assay was conducted as described earlier (Nair et al., 2013; Welch et al., 2017). Briefly, 3-4 female zebrafish were euthanized as described above and ovaries were collected in sorting media containing 90% Leibovitz's L-15 medium (Sigma, Cat# L1518) at pH 9.0 containing 10% bovine serum albumin (BSA). Individual oocytes or follicles in stage III were carefully separated from others under a dissection microscope. Follicle with size ranges from 0.55–0.62 mm in diameter were chosen for the study (oocytes at this stage respond to the MIH) as previously described (Clelland and Peng, 2009; Gonzalez et al., 2012b; Shepperd et al., 2012). Collected stage III follicles were either incubated in a 24 well plate with media (90% L-15 media p^H 9+10% BSA) containing either 100 ng/mL maturation-inducing hormone (MIH; 4-Pregnen-17α,20β-diol-3-one) or 10 or 100 ng/mL ZF PNX-20. The control group was run without any hormones. To each well, 5-7 oocytes were added (n=6 wells per treatment) and the experiment was repeated 4 times. The maturation rate was scored under a dissection microscope 24 hr post-incubation and the GVBD, followed by changes in the appearance of the follicle from opaque to translucent is used to confirm maturation (fifth stage).

2.3.10. Statistical analysis

Quantitative qPCR gene expression data were analyzed using one-way ANOVA followed by Tukey's multiple comparison test or Student–Newman–Keuls (SNK) test. $P < 0.05$ was considered statistical significance. PRISM version 5 (GraphPad Inc., United States) and IBM SPSS™ version 21 (IBM, United States) software was used for statistical analysis. PRISM version 5 (GraphPad Inc., United States) was used for generating graphs. Data are represented as mean ± SEM.

2.4. Results

2.4.1. SMIM20/PNX-20 amino acid sequence is conserved among vertebrates.

I found high conservation of amino acids in the PNX-20 region of SMIM20 in vertebrates (**Figure 2.4.1 A**). Zebrafish mature PNX-20 exhibits the highest sequence identity with alligator (70%), followed by mouse, chicken, human (65%) and frog (50%). The zebrafish precursor (SMIM20) amino acid sequence exhibits 65.67% sequence identity with frog, followed by alligator (58.21%), chicken, human (56.72%) and mouse (55.22%).

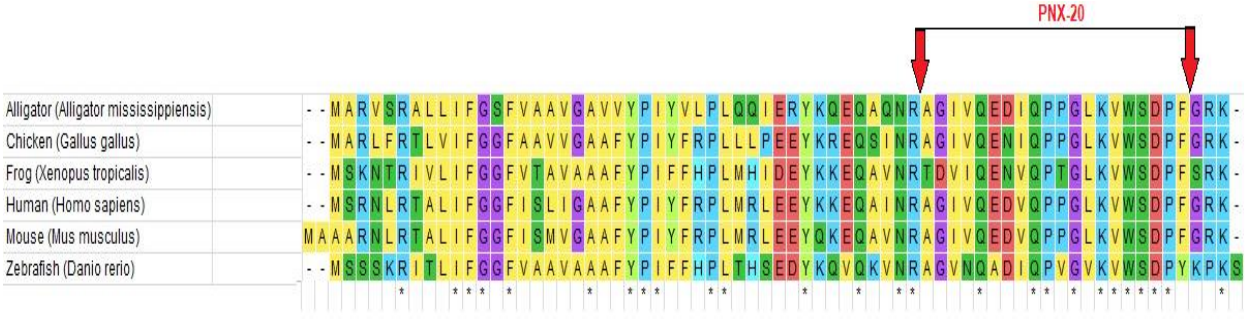

Figure 2.4.1: Multiple sequence alignment of vertebrate preprophoenixin (SMIM20) amino acid sequences. Alignment of zebrafish SMIM20 amino acid sequence with sequences from other vertebrates. Active peptide regions are within the arrows. Shared amino acids among sequences are denoted by an asterisk. GenBank ID of sequences used for the alignment are as follow: Alligator (XP_006260495.1), Chicken (NP_001138902.1), Frog (XP_002939144.1), Human (NP_001138904.1), Mouse (NP_001138905.1), and Zebrafish (NP_001289553.1).

2.4.2. SMIM20 and SREB3 mRNAs are widespread within zebrafish tissues.

Expression of SMIM20 mRNAs was found to be more prominent in the ovary, but was detected in several tissues of zebrafish, including the skin, eye, gill, foregut and liver. The lowest SMIM20 mRNA expression was observed in the muscle tissue (**Figure 2.4.2 A**). A widespread distribution was also found for mRNAs encoding SREB3. Tissues showing a prominent expression include the whole brain, hypothalamus and spleen (**Figure 2.4.2 B**).

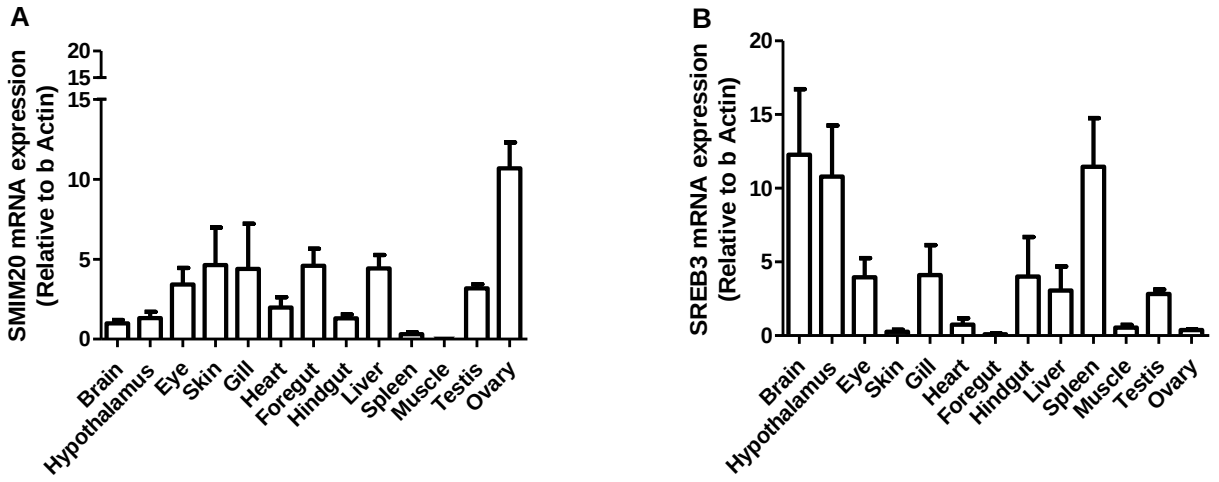

Figure 2.4.2: Tissue distribution of SMIM20 (A) and SREB3 (B) mRNAs in zebrafish. Quantitative analysis of mRNAs encoding SMIM20 (**A**) and SREB3 (**B**) in zebrafish tissues. Data obtained by RT-qPCR were normalized to β-actin and are expressed as mean + SEM (n=6), relative to the tissue with the lowest mRNA expression.

2.4.3. PNX-like and SREB3-like immunoreactivity (ir) was detected in the gonads of zebrafish

In the gonads of both male (**Figure 2.4.3 A**) and female (**Figure 2.4.3 B**) zebrafish, I found PNX-like and SREB3-like immunoreactivity in the supporting cell layers. No immunoreactive signals were found in the preabsorption or secondary antibody alone negative controls in both testis and ovary.

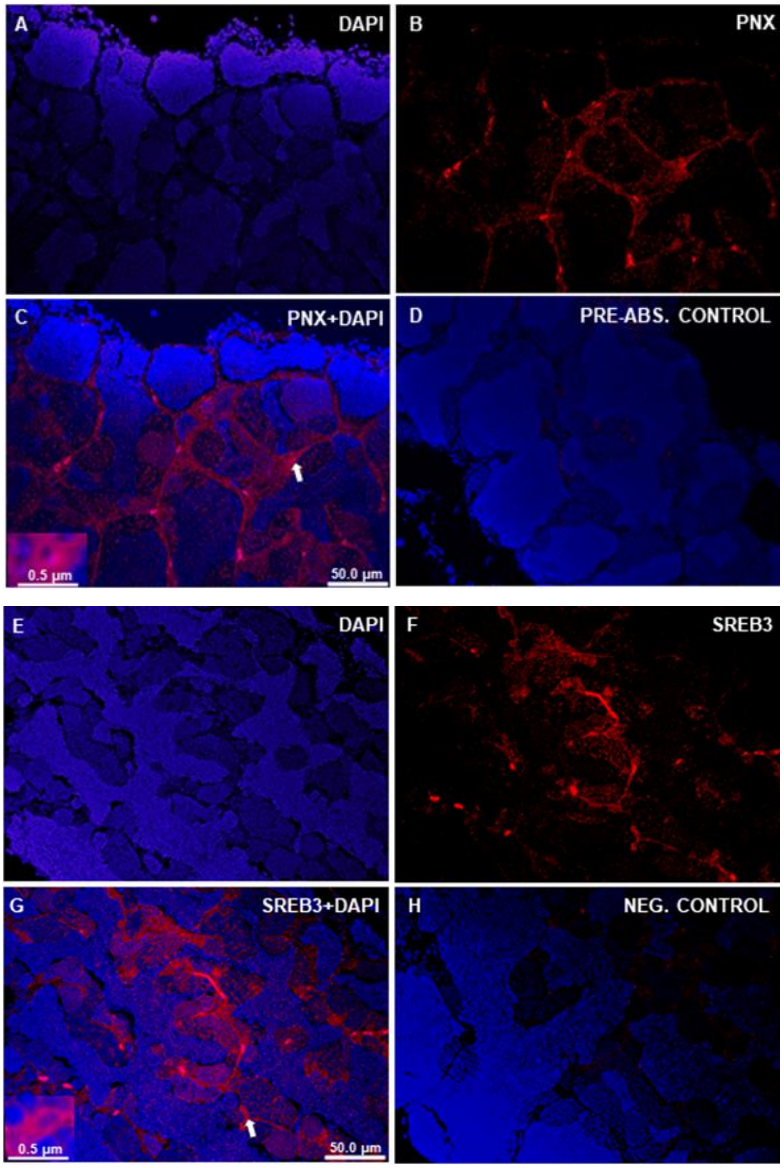

Figure 2.4.3 A: Immunohistochemical localization of PNX-like and SREB3-like- ir in the testis of zebrafish. Figure shows representative sections of zebrafish testis (**A-H**) showing PNX-like (red; **B-C**) and SREB3-like (red; **F-G**) immunoreactivity. No PNX/SREB-3-like-ir was observed in both pre-absorption (**D**) and secondary antibody alone negative control (**H**). Nuclei shown in blue are stained with DAPI. Scale bars are indicated in each image.

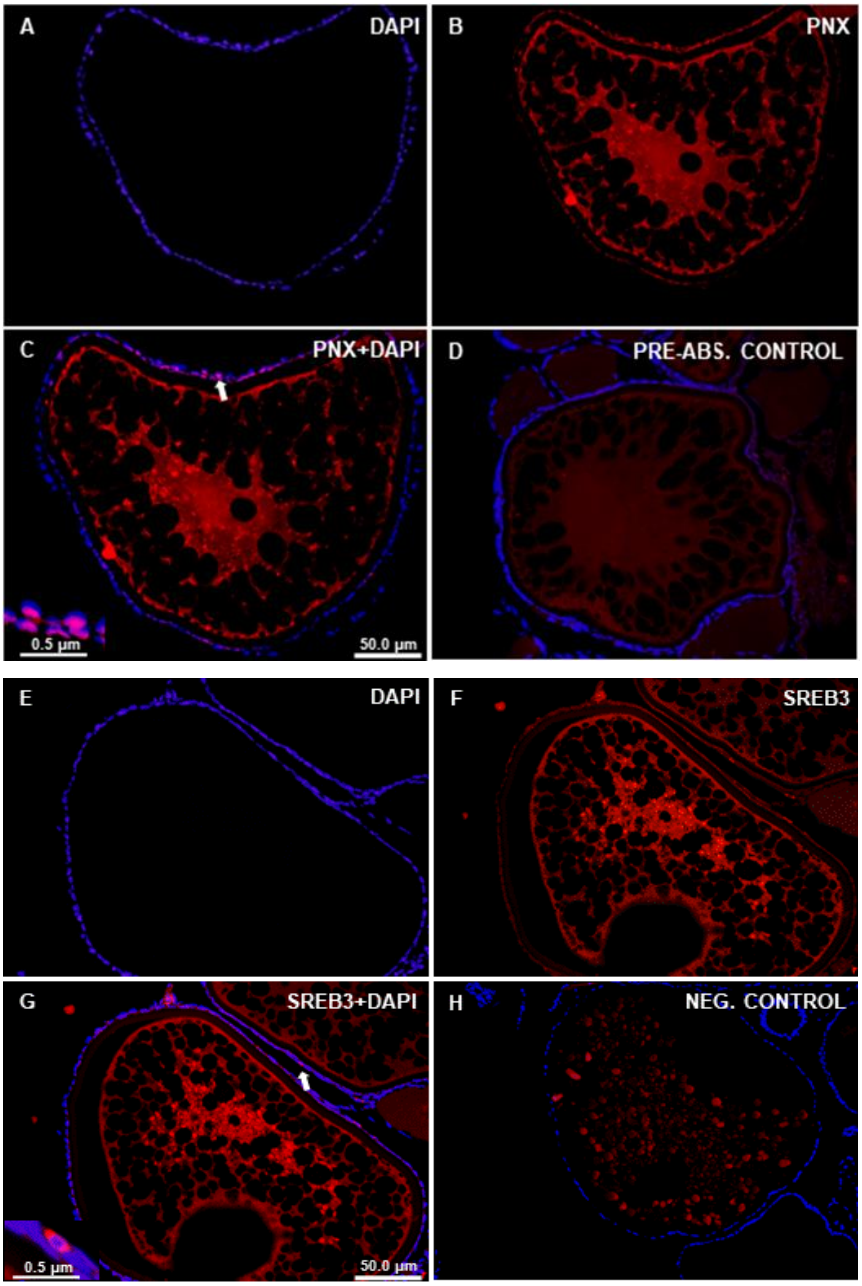

Figure 2.4.3 B: Immunohistochemical localization of PNX-like and SREB3-like- ir in the ovary of zebrafish. Figure shows representative sections of zebrafish ovary (**A-H**) showing PNX-like (red; **B-C**) and SREB3-like (red; **F-G**) immunoreactivity. No PNX/SREB-3-like-ir was observed in both pre-absorption (**D**) and secondary antibody alone-negative control (**H**). Nuclei shown in blue are stained with DAPI. Scale bars are indicated in each image.

2.4.4. PNX-like and SREB3-like immunoreactivity (ir) was detected in the ZFL cell line

Both PNX-like and SREB3-like immunoreactivity was detected in the cytoplasm of ZFL cells (**Figure 2.4.4**). No immunostaining was found in preabsorption and negative controls.

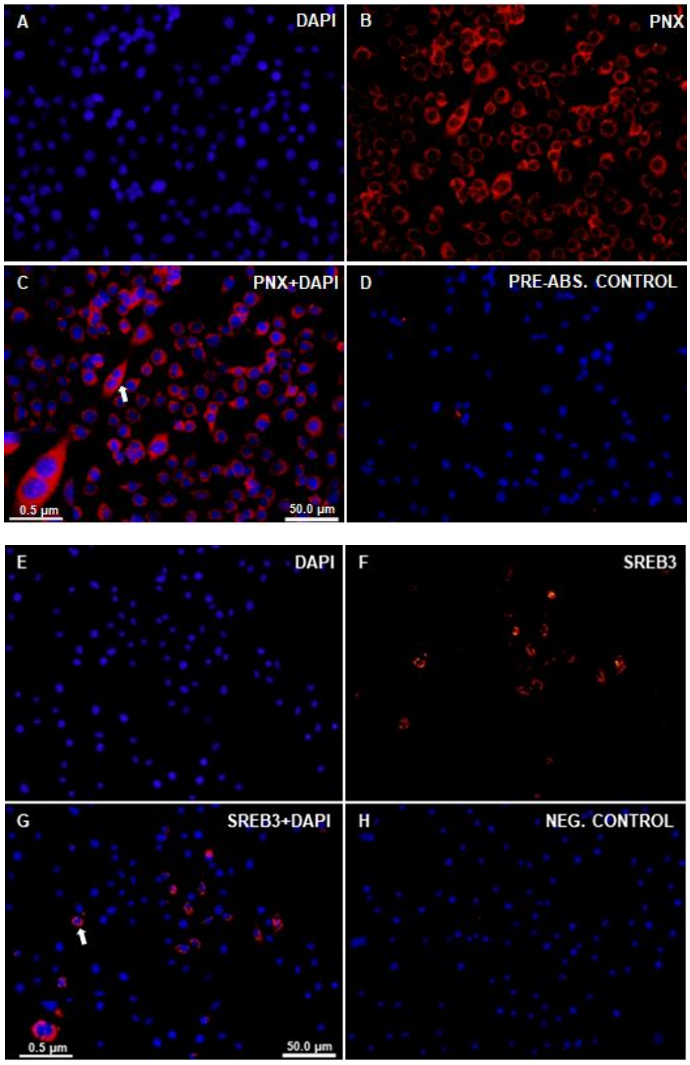

Figure 2.4.4: Immunohistochemical localization of PNX-like and SREB3-like- ir in the ZFL cell line. Figure shows representative sections of ZFL cell line (**A-H**) showing PNX-like (red; **B-C**) and SREB3-like (red; **F-G**) immunoreactivity. No PNX/SREB-3-like-ir was observed in both pre-absorption (**D**) and secondary antibody alone-negative control (**H**). Nuclei are shown in blue are stained with DAPI. Scale bars are indicated in each image.

2.4.5. Intraperitoneally administered PNX-20 upregulated GnRH, kisspeptin and CYP19a1b mRNA expression in the hypothalamus of zebrafish

A significant increase in sGnRH and cGnRH-II mRNA expression was noted in the hypothalamus of both male and female zebrafish post-PNX-20 administration (**Figure 2.4.5 A-D**). A significant increase in the expression of kiss1 in both male and female treatment groups (**Figure 2.4.5 E-F**) and GPR54a (only in the male fish) (**Figure 2.4.5 G**) mRNAs in zebrafish hypothalamus was observed. However, I did not observe any significant changes in the mRNA expression of GPR54a in the hypothalamus of female fish group (**Figure 2.4.5 H**). I also observed a significant increase in the expression of kiss2 mRNA in the hypothalamus of both male (1000 ng/g PNX-20) and female (both 100 and 1000 ng/g PNX-20) treatment groups (**Figure 2.4.5 I-J**). CYP19a1b mRNA expression was also significantly increased in the hypothalamus of female fish injected with 1000 ng/g PNX-20 (**Figure 2.4.5 L**), but no significant changes were found in the male fish group treated with PNX-20 (**Figure 2.4.5 K**).

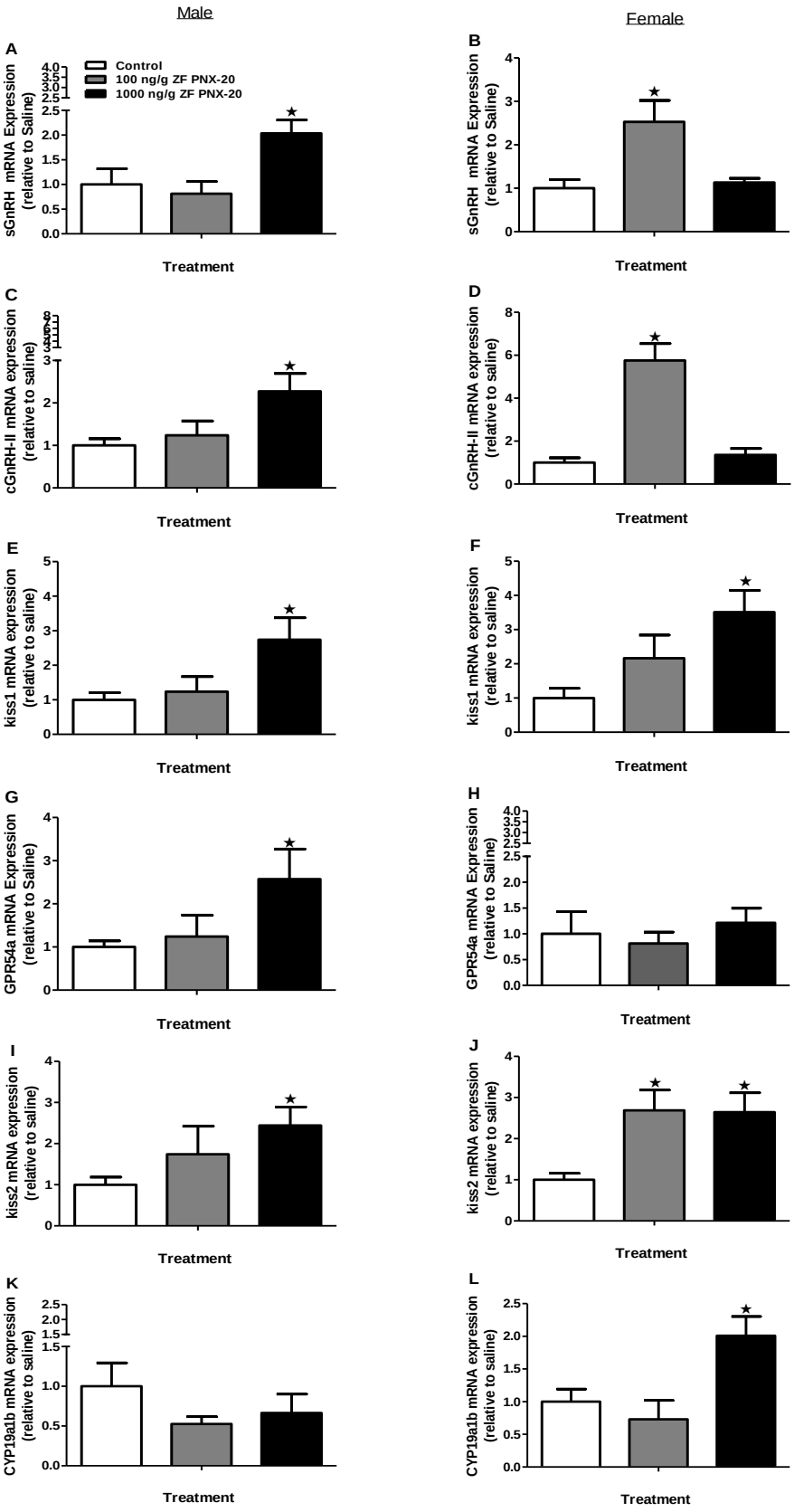
Male
Female
A
B
C
D
E
F
G
H
I
J
K
L
Control
100 ng/g ZF PNX-20
1000 ng/g ZF PNX-20
sGnRH mRNA Expression (relative to Saline)
cGnRH-II mRNA expression (relative to saline)
kiss1 mRNA expression (relative to saline)
GPR54a mRNA Expression (relative to Saline)
kiss2 mRNA expression (relative to saline)
CYP19a1b mRNA expression (relative to saline)
Treatment

Figure 2.4.5: Expression levels of mRNAs encoding reproductive regulatory genes in the hypothalamus (A-L) of male (left panel) and female (right panel) zebrafish 1 hr after IP administration of 100 and 1000 ng/g BW of PNX-20. PNX administration significantly upregulates the mRNA expression of sGnRH (**A-B**), cGnRH-II (**C-D**), kiss1 (**E-F**) and kiss2 (**I-J**) in both male and female fish group. The GPR54a (**G**) significantly upregulated in the male treatment group only. CYP19a1b mRNA expression significantly upregulated in the female treatment group (**L**). Data obtained by RT-qPCR are represented as mean + SEM (n=6 fish/group). One-way ANOVA followed by Tukey's multiple comparison test or Student–Newman–Keuls (SNK) test was used for statistical analysis. Asterisks denote significant differences between control and treated groups. $p < 0.05$ was considered statistical significance.

2.4.6. PNX-20 influences the expression of reproductive genes in the gonads of zebrafish

Sex-specific expression of LH receptor (LHr) and FSH receptor (FSHr) mRNAs was observed in the gonads of zebrafish post-PNX-20 administration. LHr mRNA expression significantly increased in the testis (100 ng/g PNX-20), and significantly decreased (1000 ng/g PNX-20) in the ovary of zebrafish (**Figure 2.4.6 A-B**). The FSHr mRNA expression was significantly downregulated in the testis and significantly upregulated in the ovary of zebrafish injected with 1000 ng/g PNX-20 (**Figure 2.4.6 C-D**). A significant increase kiss1 and GPR54a mRNA expression in the testis and ovary of zebrafish treatment groups (**Figure 2.4.6 E-H**) and kiss2 mRNA expression in the testis of zebrafish injected with 100 ng/g PNX-20 (**Figure 2.4.6 I**) was detected. However, I did not observe any significant changes in the expression of kiss2 mRNA expression in the ovary of PNX-20 treated fish (**Figure 2.4.6 J**).

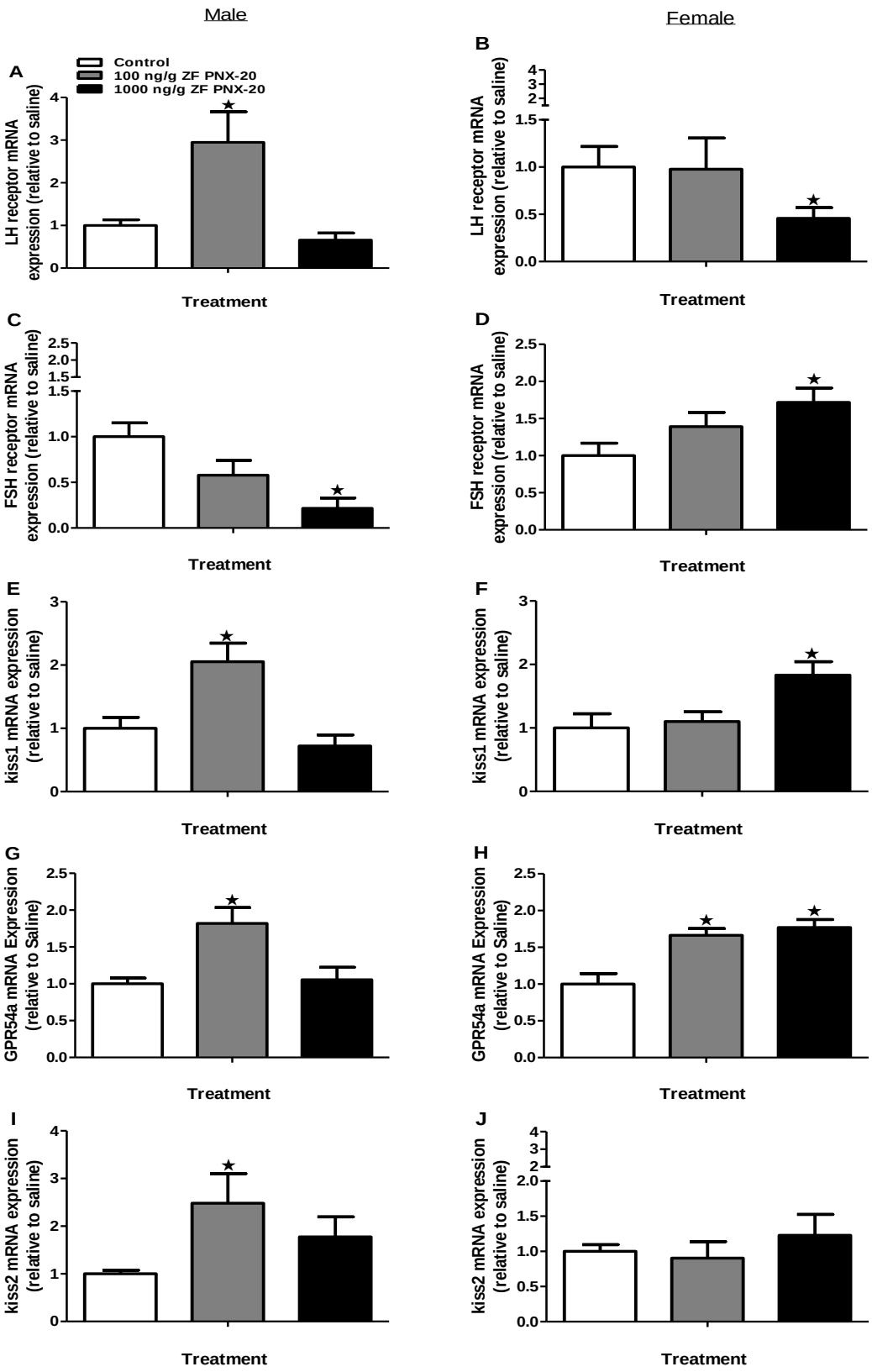
Male
Female
A
Control
100 ng/g ZF PNX-20
1000 ng/g ZF PNX-20
LH receptor mRNA expression (relative to saline)
B
C
FSH receptor mRNA expression (relative to saline)
D
E
kiss1 mRNA expression (relative to saline)
F
G
GPR54a mRNA Expression (relative to Saline)
H
I
kiss2 mRNA expression (relative to saline)
J
Treatment

Figure 2.4.6: Expression levels of mRNAs encoding gonadotropin receptors and kisspeptin system in the gonads (A-J) of male (left panel) and female (right panel) zebrafish post-PNX-20 administration. Upregulation of the mRNA expression of LHr in the testis (**A**) and downregulation in the ovary (**B**) of zebrafish was observed post-PNX-20 injection. However, FSHr mRNA expression downregulated in the testis (**C**) and upregulated in the ovary (**D**) post-PNX administration. The kiss1 (**E-F**) and GPR54a (**G-H**) mRNA expression significantly upregulated in both sex groups. A significant increase in the kiss2 mRNA expression observed only in the testis of the treatment group (**I**). Data obtained by RT-qPCR are represented as mean + SEM (n=6 fish/group). One-way ANOVA followed by Tukey's multiple comparison test or Student–Newman–Keuls (SNK) test was used for statistical analysis. Asterisks denote significant differences between control and treated groups. $p < 0.05$ was considered statistical significance.

2.4.7. PNX-20 influenced the genes involved in the gonadal sex steroidogenic pathway in zebrafish

A significant decrease in StAR mRNA expression in the testis of 1000 ng/g PNX-20 treated fish group (**Figure 2.4.7 A**) was found. However, no significant changes in StAR mRNA expression were observed in the ovary of PNX treated fish (**Figure 2.4.7 B**). The mRNA expression of CYP11a1, (**Figure 2.4.7 C-D**) and CYP17a1 (**Figure 2.4.7 E-F**) were significantly elevated in the gonads post-PNX-20 (1000 ng/g) administration. A significant increase in the expression of 17β-HSD was observed in the testis of zebrafish treated with 100 ng/g PNX-20 (**Figure 2.4.7 G**), but no significant changes were observed in the female fish group (**Figure 2.4.7 H**). Similarly, the expression of CYP19a1a mRNA in the gonads of both male and female zebrafish was upregulated post 100 ng/g PNX-20 administration (**Figure 2.4.7 I-J**).

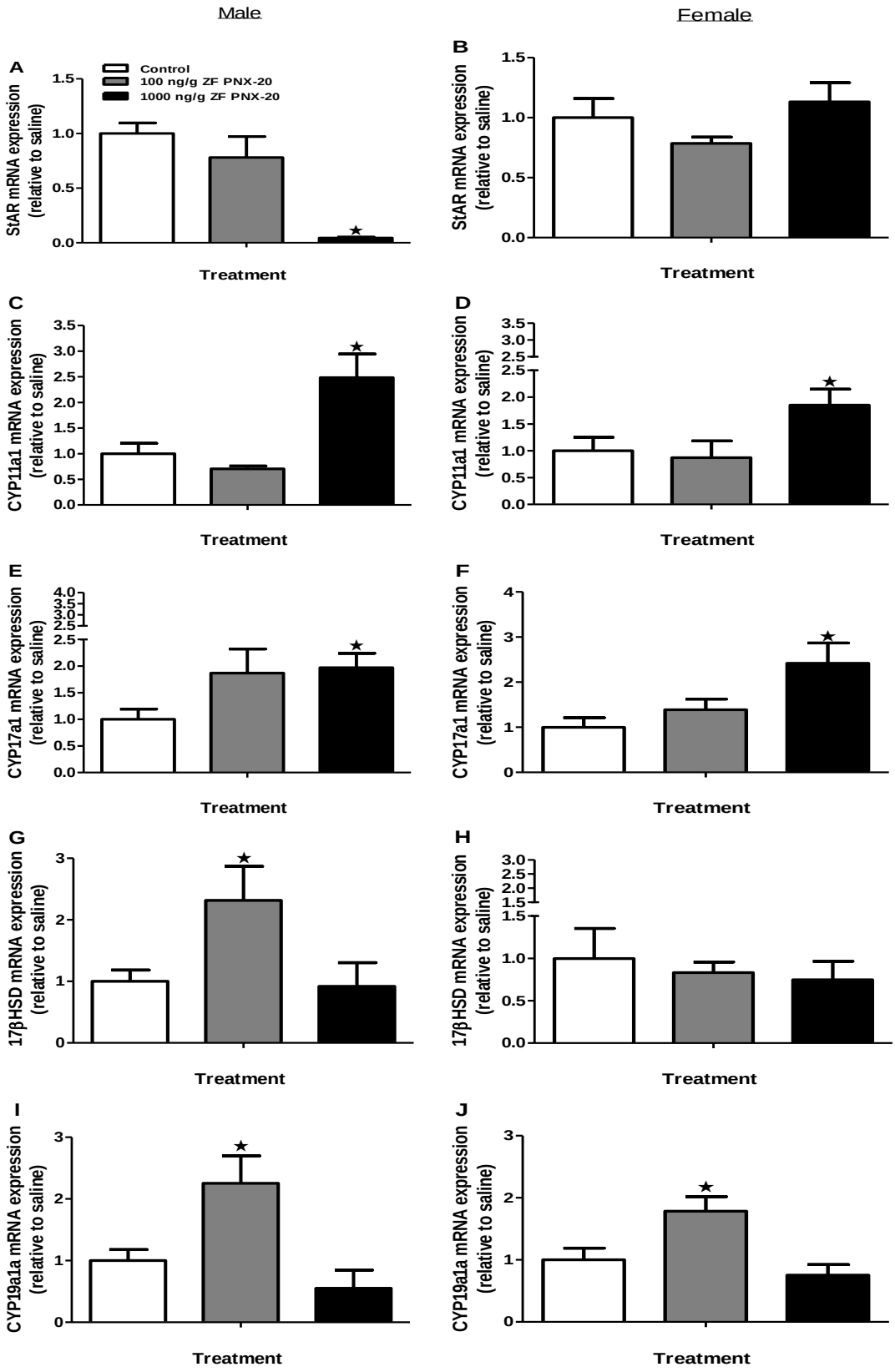
Male
Female
A
B
C
D
E
F
G
H
I
J
Control
100 ng/g ZF PNX-20
1000 ng/g ZF PNX-20
StAR mRNA expression (relative to saline)
CYP11a1 mRNA expression (relative to saline)
CYP17a1 mRNA expression (relative to saline)
17βHSD mRNA expression (relative to saline)
CYP19a1a mRNA expression (relative to saline)
Treatment

Figure 2.4.7: Expression pattern of genes involved in the sex steroidogenic pathway in the gonads (A-J) of male (left panel) and female (right panel) zebrafish post-PNX-20 administration. A significant decrease in StAR mRNA expression was observed in the testis post-PNX-20 injection (**A**). PNX-20 upregulates the mRNA expression of CYP11a1 (**C-D**), CYP17a1 (**E-F**), CYP19a1a (**I-J**) in the gonads of male and female zebrafish. The 17β-HSD upregulated only among the testis of treatment groups (**G**) and no changes were observed in ovary (**H**). Data obtained by RT-qPCR are represented as mean + SEM (n=6 fish/group). One-way ANOVA followed by Tukey's multiple comparison test or Student–Newman–Keuls (SNK) test was used for statistical analysis. Asterisks denote significant differences between control and treated groups. $p < 0.05$ was considered statistical significance.

2.4.8. PNX-20 modulated the mRNA expression of AMH, DMRT1, AR and ER in the gonads of zebrafish

Among gonadal genes that are important in zebrafish reproduction, AMH mRNA expression was significantly downregulated in the testis of zebrafish injected with 1000 ng/g PNX-20 (**Figure 2.4.8 A**). However, no significant changes in AMH mRNA expression were observed in female fish injected with PNX-20 (**Figure 2.4.8 B**). Meanwhile, DMRT1 mRNA expression in the testis (100ng/g) was increased but was decreased (100 & 1000ng/g) in the ovary (**Figure 2.4.8 C-D**). In the testis of 1000 ng/g PNX-20 treatment groups, both AR and ER mRNA expression was significantly downregulated (**Figure 2.4.8 E** & **G**). However, in the ovary of 1000 ng/g PNX-20 treated fish, both AR and ER mRNA expression was significantly increased (**Figure 2.4.8 F** & **H**).

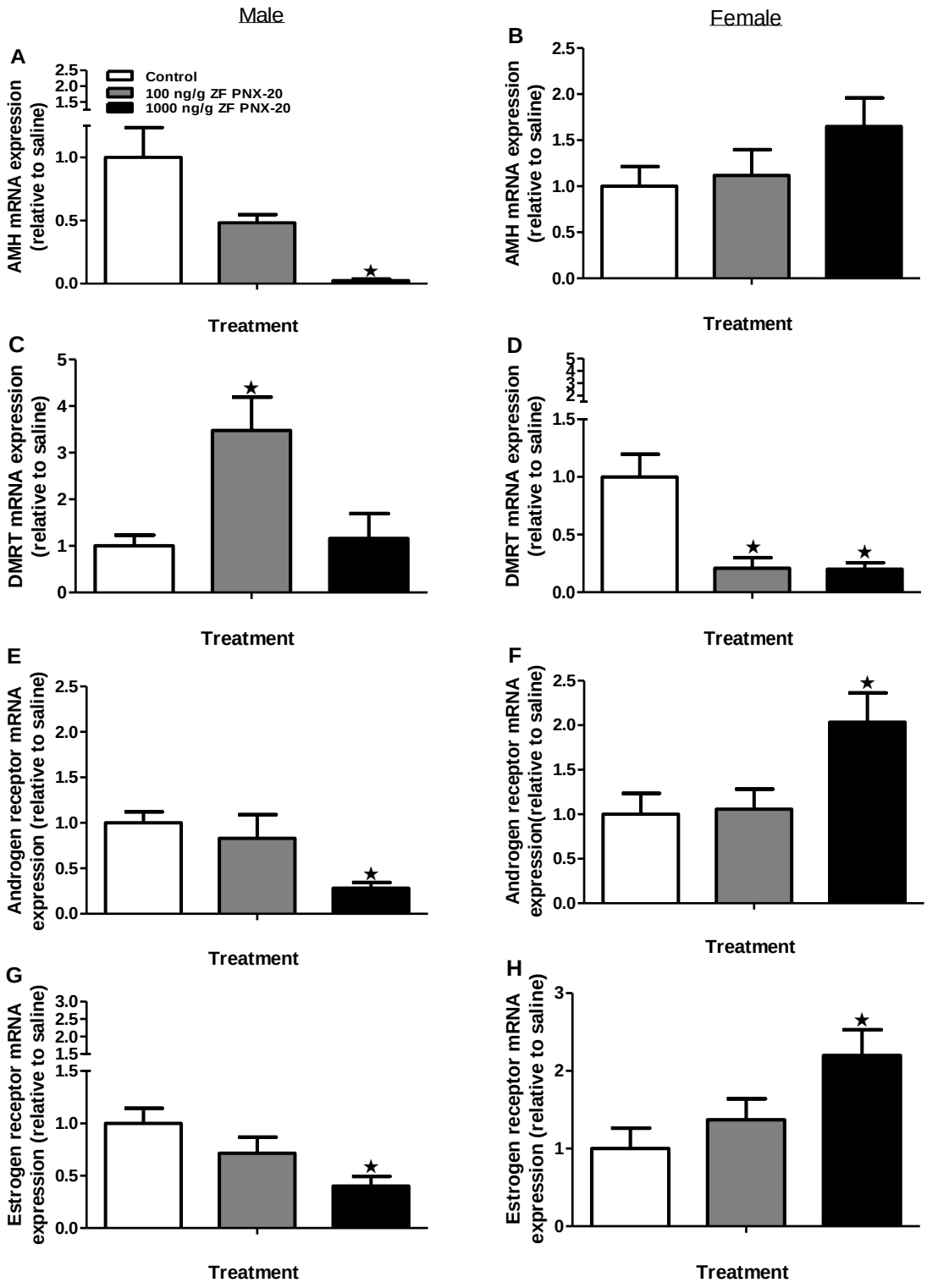
Male
Female
A
B
C
D
E
F
G
H
Control
100 ng/g ZF PNX-20
1000 ng/g ZF PNX-20
AMH mRNA expression (relative to saline)
DMRT mRNA expression (relative to saline)
Androgen receptor mRNA expression (relative to saline)
Androgen receptor mRNA expression(relative to saline)
Estrogen receptor mRNA expression (relative to saline)
Estrogen receptor mRNA expression (relative to saline)
Treatment

Figure 2.4.8: mRNA expression pattern of AMH, DMRT1 and sex-steroid hormone receptors in the gonads (A-H) of male (left panel) and female (right panel) zebrafish post-PNX-20 administration. The AMH mRNA expression significantly decreased in the testis (**A**) of PNX injected fish group. However, DMRT1 mRNA significantly increased in the testis (**C**) and significantly decreased in the ovary (**D**). Both AR and ER mRNAs were downregulated in the testis (**E** & **G**). In the ovary of PNX injected fish AR and ER mRNAs upregulated (**F** & **H**). Data obtained by RT-qPCR are represented as mean + SEM (n=6 fish/group). One-way ANOVA followed by Tukey's multiple comparison test or Student–Newman–Keuls (SNK) test was used for statistical analysis. Asterisks denote significant differences between control and treated groups. $p < 0.05$ was considered statistical significance.

2.4.9. *In vitro* treatment of PNX-20 upregulated the expression of vtg system mRNAs in ZFL cells

A significant increase in the vtg1 mRNA expression 2, 6 and 24 hr post-PNX-20 treatment (**Figure 2.4.9 A-B**). As for vtg2, I observed a significant increase in the expression at 6 and 24 hr post-PNX-20 treatment groups only (**Figure 2.4.9 D**), but not at 1 and 2 hr after PNX-20 treatment (**Figure 2.4.9 C**). However, similar to what I observed in case of vtg1, the mRNA expression of vtg3, 4, 5 and 7 were significantly upregulated at 2, 6 and 24 hr post-PNX-20 treatment (**Figure 2.4.9 E-L**).

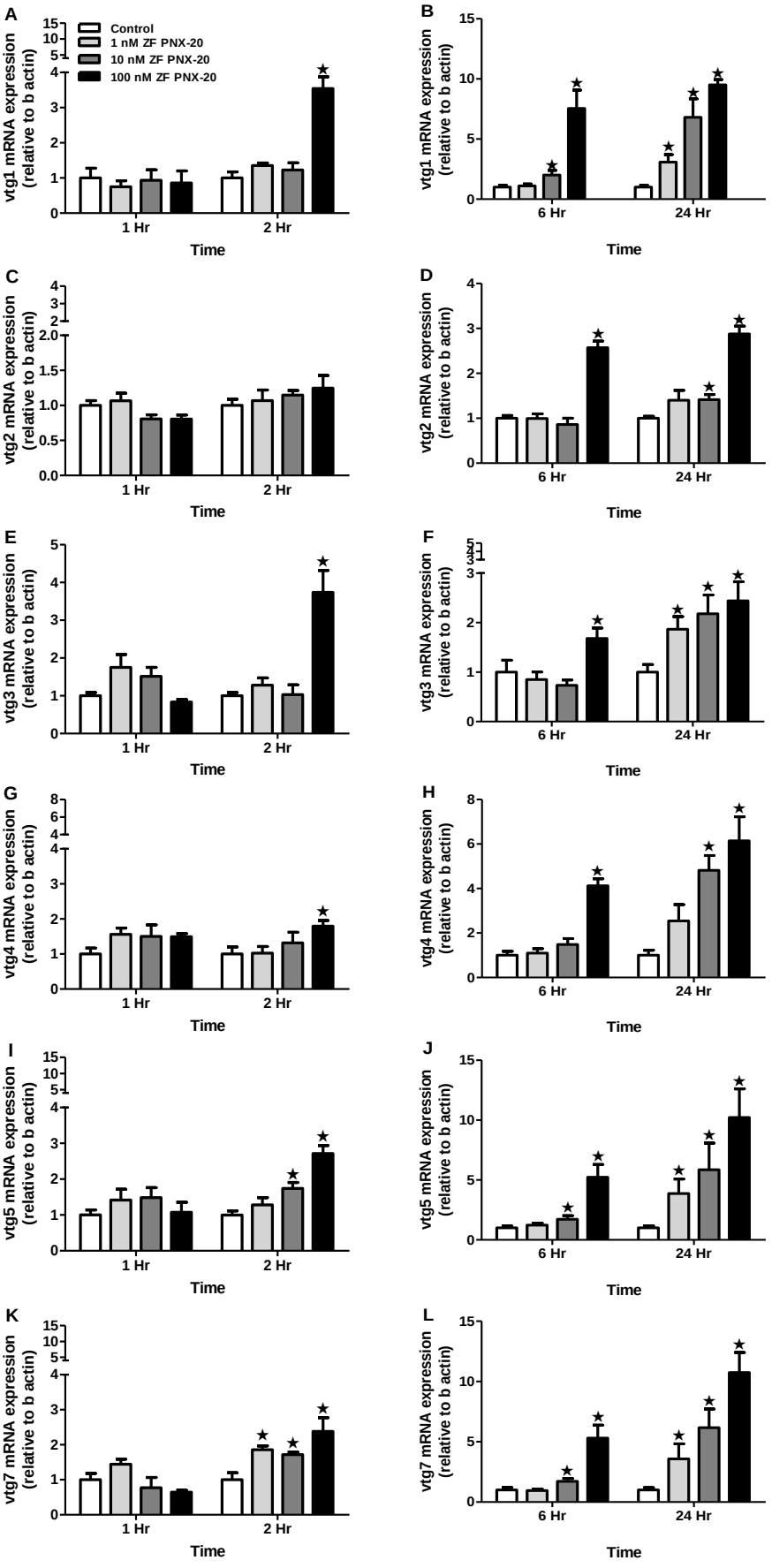
A
Control
1 nM ZF PNX-20
10 nM ZF PNX-20
100 nM ZF PNX-20
vtg1 mRNA expression (relative to b actin)
1 Hr
2 Hr
Time
B
vtg1 mRNA expression (relative to b actin)
6 Hr
24 Hr
Time
C
vtg2 mRNA expression (relative to b actin)
1 Hr
2 Hr
Time
D
vtg2 mRNA expression (relative to b actin)
6 Hr
24 Hr
Time
E
vtg3 mRNA expression (relative to b actin)
1 Hr
2 Hr
Time
F
vtg3 mRNA expression (relative to b actin)
6 Hr
24 Hr
Time
G
vtg4 mRNA expression (relative to b actin)
1 Hr
2 Hr
Time
H
vtg4 mRNA expression (relative to b actin)
6 Hr
24 Hr
Time
I
vtg5 mRNA expression (relative to b actin)
1 Hr
2 Hr
Time
J
vtg5 mRNA expression (relative to b actin)
6 Hr
24 Hr
Time
K
vtg7 mRNA expression (relative to b actin)
1 Hr
2 Hr
Time
L
vtg7 mRNA expression (relative to b actin)
6 Hr
24 Hr
Time

Figure 2.4.9: mRNA expression pattern of vtg in the ZFL cells post-PNX-20 treatment (A-L). The left panel represents 1 and 2 hr and the right panel represents 6 and 24 hr treatment groups. No significant changes in any of the vtg mRNA expression were observed in 1 hr treatment groups (1hr- A, C, E, G, I, K). Among 2 hr treatment groups, vtg1, vtg3, vtg4, vtg5 and vtg7 mRNA expression was upregulated (2 hr- A, E, G, I, K). However, significant upregulation of all vtg mRNAs was observed in the 6 and 24 hr treatment groups (B, D, F, H, J, L). Graphs represent pooled data from 2 independent studies (n=6 wells/treatment). One-way ANOVA followed by Tukey's multiple comparison test or Student–Newman–Keuls (SNK) test was used for statistical analysis. Asterisks denote significant differences between control and treated groups. $p < 0.05$ was considered statistical significance.

2.4.10. PNX-20 upregulated the mRNA expression of esr2 and shbg in ZFL cells

PNX-20 treated caused a significant increase in esr2a and esr2b mRNA expression (**Figure 2.4.10 C-F**). However, I did not observe any significant changes in the expression of esr1 mRNA expression in any treatment group (**Figure 2.4.10 A-B**). PNX-20 upregulated shbg mRNA expression at 2, 6 and 24 h (**Figure 2.4.10 G-H**).

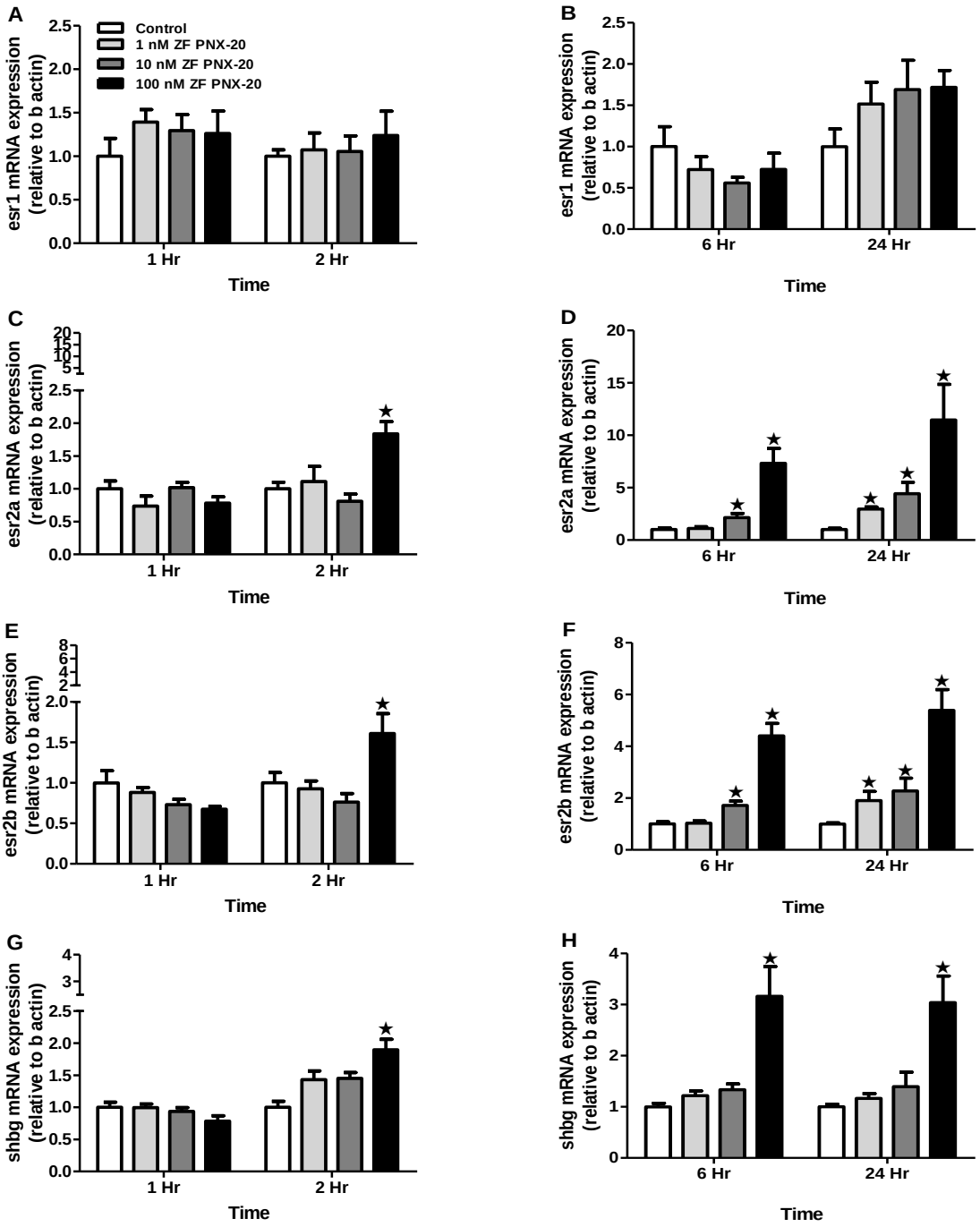

Figure 2.4.10: Expression pattern of esr and shbg mRNA post-PNX-20 incubation in ZFL cells. The left panel represents 1 and 2 hr and the right panel represents 6 and 24 hr treatment groups. The mRNA expression of esr1 was not changed post-PNX-20 treatment under any of the time points considered (**A-B**). The expression of esr2a, esr2b and shbg mRNAs was upregulated (**C-H**) in 2, 6 and 24 hr PNX-20 treatment groups. Graphs represent pooled data from 2 independent studies (n=6 wells/treatment). One-way ANOVA followed by Tukey's multiple comparison test or Student–Newman–Keuls (SNK) test was used for statistical analysis. Asterisks denote significant differences between control and treated groups. $p < 0.05$ was considered statistical significance.

2.4.11. Significant reduction in SMIM20 mRNA expression was observed in the ZFL cells post-siRNA treatments

The siRNA-mediated knockdown resulted in a significant reduction in SMIM20 mRNA in ZFL cell line 72 hr post-incubation (**Figure 2.4.11 B**). However, there were no significant changes in SMIM20 mRNA expression were observed among 24 (data not included), 48 and 96 hr scrambled siRNA treatment groups (**Figure 2.4.11 A** & **C**). In addition, I did not observe any significant changes in SMIM20 mRNA expression in the scrambled siRNA group at 72 hr time point (**Figure 2.4.11 D**).

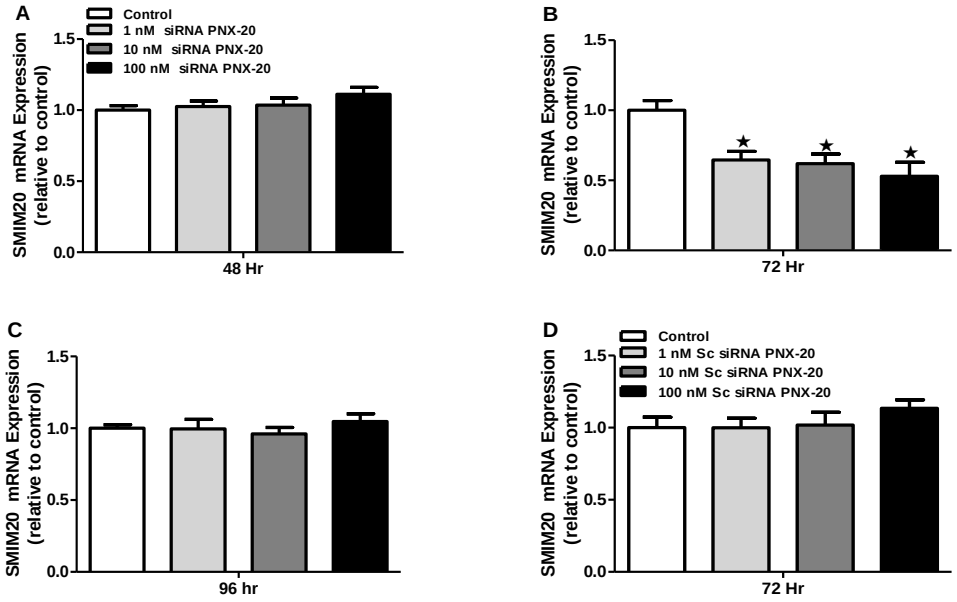

Figure 2.4.11: mRNA expression of SMIM20 in the ZFL cells post-siRNA and scrambled siRNA treatment. No significant changes in SMIM20 mRNA expression was observed in siRNA treated cells at 48 and 96 hr time points (**A** & **C**). A significant decrease in SMIM20 mRNA expression was observed at 72 hr post-siRNA treatment (**B**) in all concentrations I tested compared to control. No significant changes in the mRNA expression of SMIM20 were observed among the scrambled siRNA treated groups (**D**). Graphs represent pooled data from 2 independent studies (n=6 wells/treatment). One-way ANOVA followed by Tukey's multiple comparison test or Student–Newman–Keuls (SNK) test was used for statistical analysis. Asterisks denote significant differences between control and treated groups. $p < 0.05$ was considered statistical significance.

2.4.12. siRNA mediated PNX-20 knockdown downregulated all vtg mRNAs in ZFL cells

All vtg (vtg1, 2, 3, 4, 5 & 7) mRNAs were significantly downregulated in the siRNA treated group (**Figure 2.4.12 A-F**).

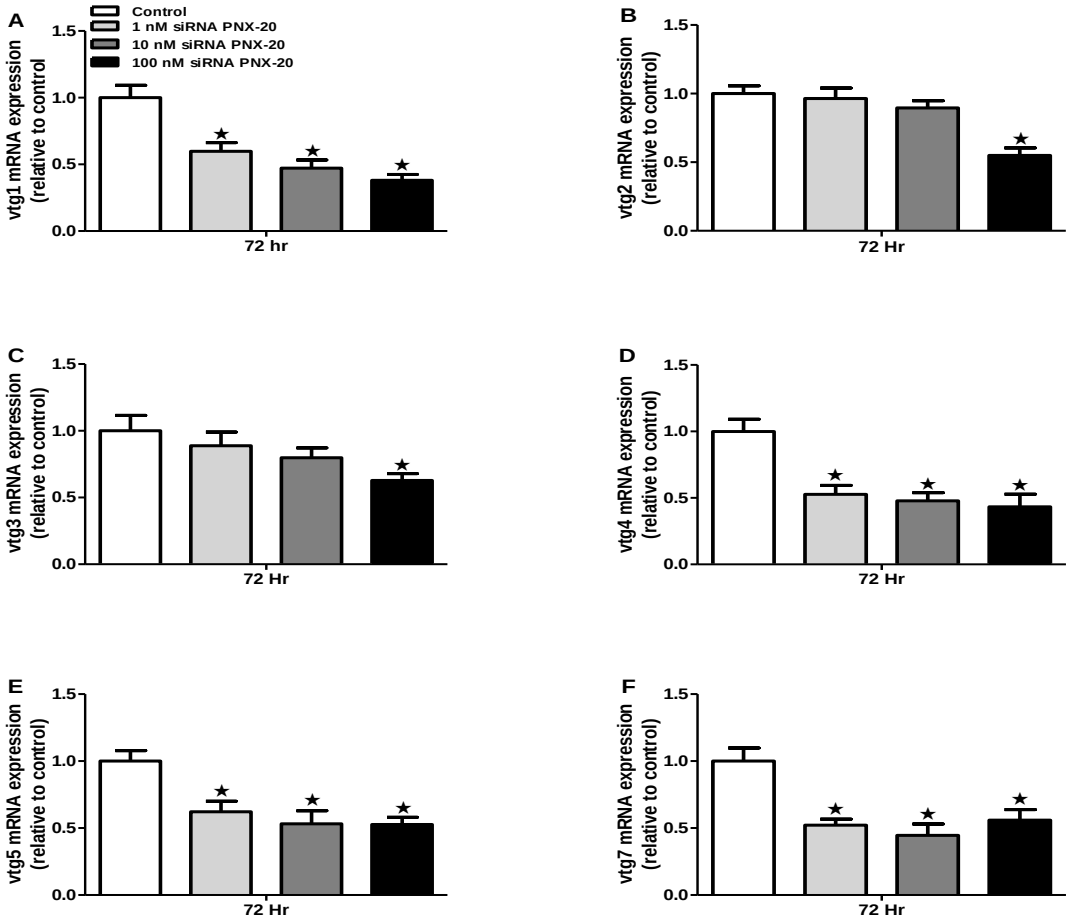

Figure 2.4.12: siRNA mediated gene knockdown of SMIM20 downregulates the vtg transcripts levels in ZFL cells. Reduction in the mRNA expression of SMIM20 downregulates the expression of all vtg mRNAs in the ZFL cells (**A-F**). Graphs represent pooled data from 2 independent studies (n=6 wells/treatment). One-way ANOVA followed by Tukey's multiple comparison test or Student–Newman–Keuls (SNK) test was used for statistical analysis. Asterisks denote significant differences between control and treated groups. $p < 0.05$ was considered statistical significance.

2.4.13. Scrambled SiRNA treatment did not affect the vtg mRNAs expression in ZFL cells

No significant change in the expression of any of the vtg mRNAs was observed in cells treated with the scrambled siRNA (**Figure 2.4.13 A-F**).

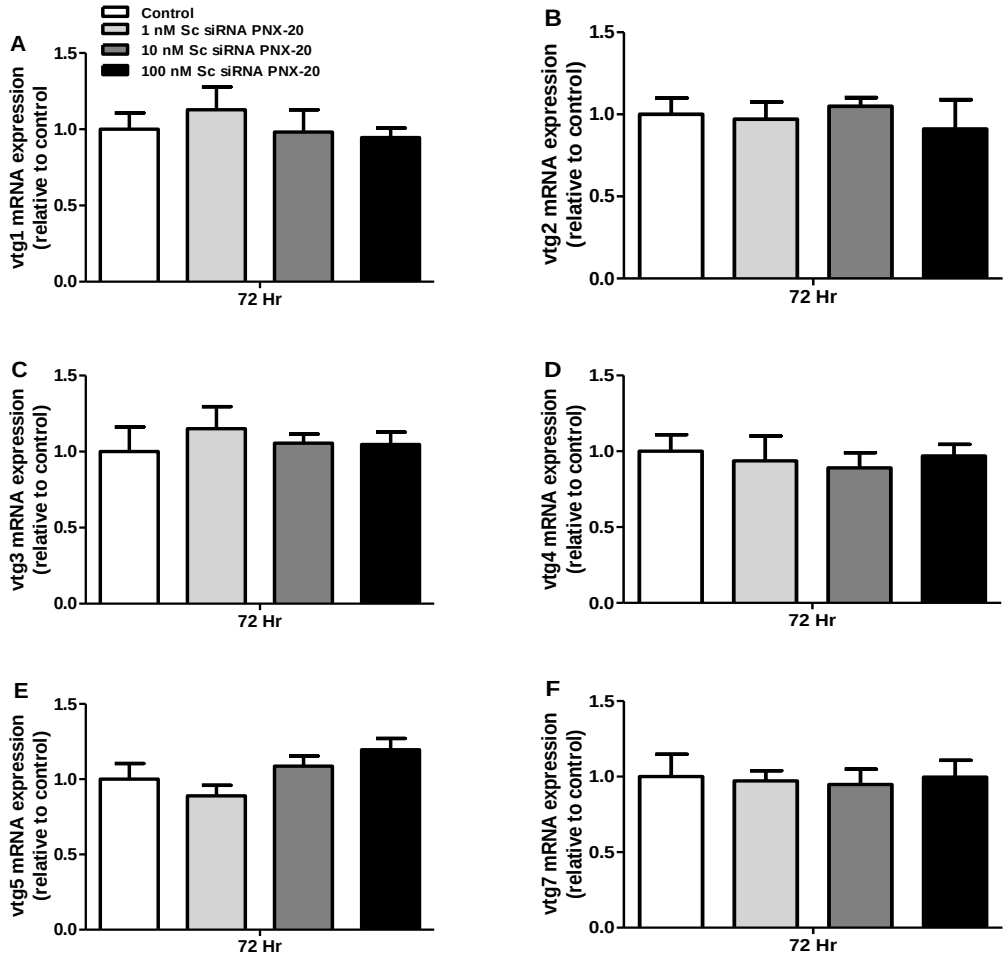

Figure 2.4.13: No significant changes in the vtg mRNA expression profiles were observed in scrambled siRNA treated (72 hr) ZFL cells. No significant changes in the mRNA expression profile of any of the vtg mRNAs were observed in ZFL cells incubated with scrambled siRNA at 72 hr (**A-F**). Graphs represent pooled data from 2 independent studies (n=6 wells/treatment). One-way ANOVA followed by Tukey's multiple comparison test or Student–Newman–Keuls (SNK) test was used for statistical analysis. Asterisks denote significant differences between control and treated groups. $p < 0.05$ was considered statistical significance.

2.4.14. PNX-20 increased GVBD and oocyte maturation in zebrafish

Incubation of zebrafish follicles with PNX-20 significantly increased GVBD compared to control (**Figure 2.4.14**). MIH (positive control) showed the highest response compared to both control and PNX-20 treated groups.

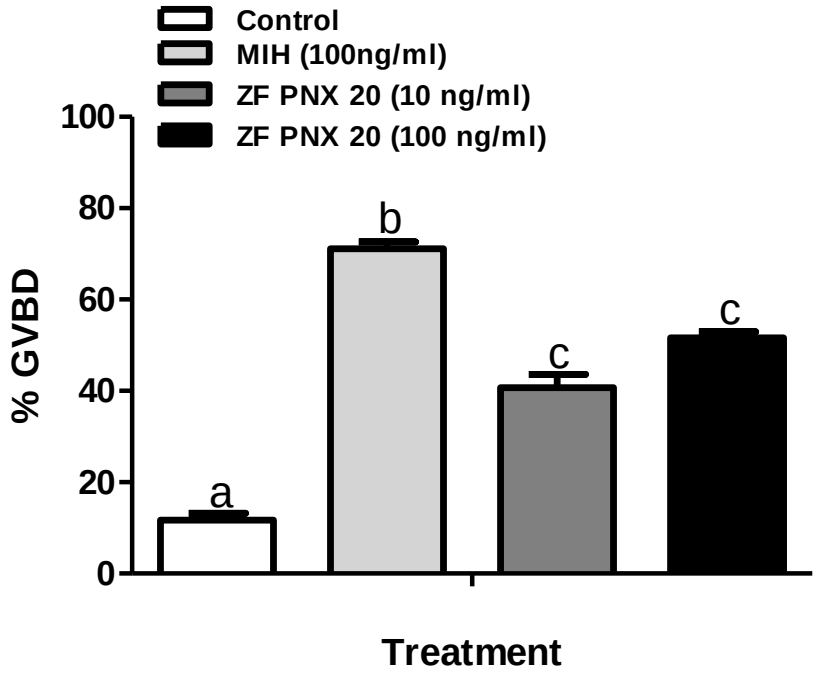

Figure 2.4.14: PNX-20 promotes GVBD and oocyte maturation in zebrafish. A significant increase in the maturation of oocytes were observed in ovarian follicles incubated with PNX-20 compared to controls. However, follicles treated with MIH showed the highest maturation rates compared to control and PNX treated group. Graphs represent pooled data from four independent studies (n=6 wells/treatment, 5-7 oocytes /well). One-way ANOVA followed by Tukey's multiple comparison test was used for statistical analysis. Different letters (a, b, c) denote statistical differences between groups. $p < 0.05$ was considered statistical significance.

2.5. Discussion

PNX is a novel peptide with hormone-like actions to regulate reproduction in mammals. The research outlined here addressed whether PNX-20 has any role in the regulation of reproduction in zebrafish. My research determined the effects of PNX-20 on the hypothalamus and gonads of the HPG axis, and liver, a major source of vtg that is critical for ovarian follicle biology. The *in silico* sequence analysis indicates conservation of amino acid sequences in the PNX-20 region of SMIM20 sequence suggests identical function across species. I found that the mRNA encoding PNX-20 are widely expressed within zebrafish tissues, and the highest levels of expression were detected in the ovary, which is in accordance with the well-known modulatory role of PNX on female reproduction (Stein et al., 2016; Treen et al., 2016; Wang et al., 2019). Expression of PNX-20 receptor mRNA, SREB3, was also observed in several zebrafish tissues, which were previously reported as sites of this receptor expression in mammals (Matsumoto et al., 2000; Stein et al., 2016; Suszka-Świtek et al., 2019). The immunohistochemical studies found PNX-like and SREB3-like immunoreactivity in the gonads, specifically in the supporting cell layers of developing ovarian follicles and spermatocytes. This provides new evidence for testis and ovary as a source of PNX-20 and sites of its action. The local expression and tissue distribution suggest a possible role of PNX-20 in zebrafish reproduction. I then used 100 and 1000 ng/g BW PNX-20 to study whether PNX-20 has any role in zebrafish reproduction. Single IP injection of PNX-20 significantly upregulated the expression of both sGnRH and cGnRH-II mRNAs in the hypothalamus of zebrafish. Both the GnRH isoforms are expressed in zebrafish with sGnRH has a pivotal role in the gonadotropin secretion from the pituitary (Steven et al., 2003). Results suggest that PNX-20 directly acts on the GnRH system and upregulates the expression of GnRH isoforms in both male and female zebrafish. This is consistent with results obtained from mammals (Pałasz et al., 2015; Stein et al., 2016; Treen et al., 2016; Yosten et al., 2013) and non-mammals (Wang et al., 2019). Based on this, it is likely that the stimulatory effect of PNX-20 on the GnRH system is conserved across species. Similarly, I found an upregulation of kiss1, kiss2 (both male and female fish) and GPR54a (only in the male fish group) mRNA expression in the hypothalamus of zebrafish. The kisspeptin system is primarily involved in the regulation of reproduction in vertebrates (Ogawa and Parhar, 2013; Tena-Sempere et al., 2012). Increased expression of kisspeptin system in PNX-20 treated fish suggests that in addition to the

GnRH, PNX-20 influences another potent regulator of reproduction in the hypothalamus of zebrafish. It was reported that in the rat hypothalamus, PNX stimulates kiss1 transcription in both arcuate and anteroventral periventricular (AVPV) nucleus (Treen et al., 2016). kiss1 is abundantly expressed in the teleost habenula, a region involved in serotonin mediated behavior responses (Ogawa and Parhar, 2018). Studies in mammals reported that in addition to its reproductive roles, PNX-20 is a potent regulator of behavior response as well (Cowan et al., 2015; Friedrich et al., 2019; Hofmann et al., 2017; Jiang et al., 2015). Upregulation of kiss1 post-PNX-20 administration suggests in addition to the reproductive regulatory role, PNX might be involved in the regulation of behavior response in teleost. These results present a generally stimulatory role for PNX-20 on the hypothalamic reproductive hormones. ICV injection of PNX-20 significantly increased the plasma LH levels in a dose-related manner to potentiate GnRH-induced LH secretion from cultured anterior pituitary cells of rat *in vitro* (Stein et al., 2016). Due to the extremely small size of zebrafish pituitary gland, I was unable to collect this tissue and determine PNX-20 on pituitary hormones.

The effects of PNX-20 on zebrafish gonads were also positive. LHr mRNA expression in the testis increased, while its expression in the ovary decreased in response to PNX-20. Meanwhile, FSHr expression was downregulated in the testis and upregulated in the ovary of the treatment group. This differential effect is likely due to the sex-specific variation in the effects of PNX-20. In agreement with the observations in the hypothalamus, an upregulation of kiss1, GPR54a and kiss2 (only in the male treatment group) mRNAs was identified in the gonads of zebrafish treated with PNX-20. Studies conducted in different fish species suggest that kisspeptin and gonadal development are positively correlated (Ohga et al., 2018). The upregulation of the kisspeptin system in the gonads of zebrafish is another possible mechanism by which PNX-20 influences zebrafish reproduction.

Gonadal sex steroids are critical endocrine regulators of reproduction in vertebrates, and are critical for the maturation of gametes and the development of secondary sexual characters (Yaron and Levavi-Sivan, 2011). The gene quantification results indicate that PNX-20 upregulates the expression of critical genes involved in gonadal sex steroidogenesis in both male and female zebrafish. This includes the CYP11a1 (P450ssc), CYP17a1 and CYP19a1a in which their expression was significantly increased in both male and female treatment groups. In

addition, 17β-HSD mRNA expression significantly increased in the testis of zebrafish injected with PNX-20. My results suggest that PNX-20 has a role in the sex steroidogenesis in zebrafish. The only exception was the expression of StAR, which was significantly low in the testis of the 1000 ng/g treatment group in my study. I also measured other reproduction-related genes. One among them is AMH, which has a role in sex determination, gonadal development and gametogenesis in fishes (Pfennig et al., 2015). Decreased expression of AMH was observed in the testis of zebrafish treated with PNX-20. AMH is a steroidogenic suppressor in the gonads (Pfennig et al., 2015). Decreased AMH mRNA expression is additional evidence for the positive role of PNX-20 on sex steroidogenesis in male zebrafish. Another key regulator involved in vertebrate sex determination and gonadal development and gametogenesis is DMRT1 (Matson and Zarkower, 2012). I observed a significant increase in the mRNA expression of DMRT1 in the testis, but it was decreased in the ovary of the PNX injected fish groups. This stands as another example for a sex-specific influence of PNX-20 on gonadal gene expression. It was reported that DMRT1 mutant zebrafish fail to develop into male fish or become sterile males, but ovary develops normally (Webster et al., 2017). In addition, loss of DMRT1 leads to conditions including gonadal dysgenesis, sex reversal syndromes, and reduced germ cells in the testis in vertebrates (Ottolenghi et al., 2000; Õunap et al., 2004; Raymond et al., 1999; Wu et al., 2012). Upregulation of potent male reproductive promoter DMRT1 in the testis is additional evidence for the positive influence of PNX-20 in zebrafish gamete biology. Both AR and ER mRNA expression were significantly higher in the ovary, but opposite effects were found in the case of testis. It was reported that in medaka, treatment with estradiol (E2) significantly increases the expression of both ER and AR, but treatment with 11-KT suppresses both AR and ER in the brain (Hiraki et al., 2012). It is possible that a similar kind of regulation happens in the gonads of the zebrafish, where the expression of steroidogenic genes and circulating steroid hormones were elevated, but the receptors (AR and ER) were decreased.

In addition to the HPG axis and related hormones, many other factors and biomolecules are important for reproductive success in zebrafish. One among them is vtg, the principal yolk protein, synthesized and secreted predominantly by the liver. Vitellogenin has a critical role in the development and maturation of egg or ova in vertebrates (Li and Zhang, 2017). I used ZFL cells to study the *in vitro* effects of PNX-20 on vtg mRNA expression. I observed that all vtg

mRNAs (vtg1, 2, 3, 4, 5 and 7) were significantly upregulated post-PNX-20 treatment in the ZFL cell line. My results suggest that the upregulation of the vtg mRNA expression may lead to elevated vtg secretion, which leads to adequate availability of circulating vtg, which then promotes the maturation of egg or developing ova. This is another possible mechanism by which PNX-20 influence (positively) zebrafish reproduction (in female fish). Liver vtg synthesis is under the control of estrogen, which acts via estrogen receptors (esr's). In my research, I observed that PNX-20 treatment leads to an increased expression of esr2a and esr2b, which is an additional possible mechanism by which PNX-20 influence vitellogenesis in zebrafish. I also found elevated expression of shbg a liver-derived binding protein involved in the regulation of circulating sex steroid levels in the ZFL cell line post-PNX-20 treatment. In an alternate approach, I used siRNA mediated knockdown to study whether endogenous PNX-20 has any role in vtg synthesis in the ZFL cell line. I found that the siRNA mediated gene knockdown of SMIM20 leads to decreased expression of all vtg gens in the ZFL cell line. This suggests endogenous PNX-20 is a regulator of vtg synthesis in zebrafish liver.

To study whether PNX-20 has any role in the maturation of gametes (ova), I used oocyte maturation assay, which is a well-established method to assess direct action of ligand and hormones on oogenesis/oocyte development (Clelland et al., 2007; Gonzalez et al., 2012b; Shepperd et al., 2012; Welch et al., 2017). Incubation with zebrafish PNX-20 (both 100 and 100 ng/mL PNX-20) upregulated GVBD and oocyte maturation in zebrafish follicles. This is in addition to the results I observed in both my *in vivo* and *in vitro* effect of PNX-20 on the overall increase in gene expression. This additional evidence for the direct action of PNX-20 on gamete maturation in zebrafish suggests additional ways in which PNX-20 plays a positive modulatory role in fish reproduction.

2.6. Conclusion

The outcomes of this research provide several lines of novel information on exogenous PNX-20 in regulating reproduction. These include the regulation of vtg, upregulation of steroidogenic mRNAs in the gonads, and stimulation of oocyte maturation in zebrafish. In addition, I discovered that endogenous PNX is also important for vtg mRNA expression. My results on the effects of PNX-20 on hypothalamic GnRH and kisspeptin mRNAs also concur with what has been reported in mammalian models, and at least in one fish species. These results help to conclude that the regulatory role (generally stimulatory) of PNX on reproduction is conserved across species. However, further studies are needed to confirm that the mRNA expression changes are also reflected at the protein level, both in tissues (enzymes) and in circulation (hormones). While such limitations exist, this research and its outcomes have provided substantial new information to implicate PNX-20 in fish reproduction. Future research on its role in other fish species, especially those cultured as a food source, is strongly expected to benefit aquaculture.

Transition

The following chapter will focus on the second objective of my thesis research, which aims to gain a deeper understanding on the role of nesfatin-1 in goldfish reproduction. Nesfatin-1 is a multifunctional peptide with hormone-like actions in vertebrates. Its role in energy homeostasis and reproduction are addressed in detail in many vertebrates, particularly in mammals. Previous work from our lab indicates that nesfatin-1 is anorexigenic and suppresses hypothalamic and pituitary reproductive hormones in goldfish (Gonzalez et al., 2012b). Mammalian studies suggest that nesfatin-1 possibly exerts its effects in a sex-specific manner (Gao et al., 2016; García-Galiano et al., 2010).

Hypothesis: Nesfatin-1 is a suppressor of HPG hormones, especially gonadal steroids, of both male and female goldfish.

Specific objectives:

1. Determine how exogenously administered nesfatin-1 influences critical genes encoding both HPG and non-HPG axis derived hormonal regulators of reproduction.
2. To study whether nesfatin-1 is involved in the regulation of gonadal steroidogenesis and its secretion in goldfish.

Result highlights: Nesfatin-1 suppresses expression of mRNAs encoding GnRH, gonadotropin subunits and kisspeptin system in the hypothalamus and pituitary, and both LH and FSH receptors in the gonads of both male and female goldfish. An overall suppression of sex steroidogenic genes was also observed, which resulted in corresponding changes in the circulating levels of testosterone (T) and estradiol (E) in male and female goldfish.

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Contributions: JJR planned and conducted the studies, analyzed and interpreted data, prepared the manuscript draft and revised it for submission. SU provided the original idea and funding, and helped in the planning, designing of experiments and assisted in tissue sampling, data analysis and interpretation, manuscript preparation and revisions.

Chapter 3
Nesfatin-1 Suppresses Fish Reproductive Axis and Gonadal Steroidogenesis

3.1. Abstract

Nesfatin-1 is a naturally occurring orphan ligand in fish and mammals. Research in our lab resulted in the identification of an inhibitory role for nesfatin-1 on pituitary hormones (goldfish) and oocyte maturation (zebrafish). The present study is an extension of these original findings and aimed to determine whether nesfatin-1 has any additional effects on HPG genes in male and female goldfish. I found that a single IP injection of synthetic nesfatin-1 (50 ng/g body weight) downregulated the expression of sGnRH, cGnRH-II, GPR54a and CYP19a1b mRNAs in the hypothalamus of both male and female goldfish at 15 minutes post-administration. In the pituitary of both males and females, nesfatin-1 reduced LHβ and FSHβ mRNA expression at 60 minutes, and GPR54a mRNA at 15 minutes. Similarly, the gonadotropin receptors LHr and FSHr were downregulated in the gonads. Meanwhile, GnIH, GnIHr, kiss-1a and GPR54a mRNA expression were in the gonads were increased post-nesfatin-1 treatment. Nesfatin-1 negatively influences the StAR, CYP11a, AMH and CYP19a1a mRNAs in the gonads of goldfish. In agreement with these results, nesfatin-1 reduced plasma estradiol and testosterone in female and male goldfish circulation at 60 minutes post-injection. The information generated through this research further solidified nesfatin-1 as an inhibitor of reproductive hormones in fish. Targeting nesfatin-1 and related peptides could yield beneficial effects in fish reproduction and aquaculture.

3.2. Introduction

The first evidence for the regulatory role of NUCB2/nesfatin-1 on reproduction in a non-mammalian vertebrate was reported in goldfish (Gonzalez et al., 2012b). IP injection of goldfish nesfatin-1 significantly reduced the expression of GnRH, LH and FSH, and serum LH in goldfish. In addition, incubation of ovarian follicles with nesfatin-1 significantly reduced GVBD and maturation of oocyte in zebrafish. Nesfatin-1 appears to be another linker of energy homeostasis and reproduction in fish. However, studies in female rainbow trout did not show any significant changes in circulating nesfatin-1 levels during long-term fasting, and no positive correlation between circulating nesfatin-1 levels and seasonal maturation was found (Caldwell et al., 2014). This suggests that nesfatin-1 possibly has species and sex-specific regulatory effects on reproduction and warrants further research on nesfatin-1 and reproduction.

While most of the above research in mammals (introduction section) and fish focused on females, Gao and colleagues reported a reduction in the expression of many mRNAs and proteins involved in the reproductive axis of adult male rats post-nesfatin-1 administration (Gao et al., 2016). This includes GnRH, kiss1, LHβ, FSHβ and StAR, 3β-HSD, 17β-HSD, and CYP11a1, mRNA transcripts and serum levels of LH, FSH and testosterone. This finding further suggests a sex-specific role for nesfatin-1 on reproduction in vertebrates. I hypothesized that nesfatin-1 suppresses the expression of HPG, and non-HPG genes involved in reproduction, and circulating levels of gonadal hormones in a sex-specific manner in fish. This research is a logical extension of our own studies that discovered a role for nesfatin-1 in fish reproduction (Gonzalez et al., 2012b). It determined how exogenously administered nesfatin-1 influences critical genes involved in goldfish reproduction (HPG and non-HPG axis regulators of reproduction). Further, I tested whether there are any effects for nesfatin-1 on gonadal steroids in goldfish circulation.

3.3. Materials and methods

3.3.1. Animals

Mature male and female goldfish *(C. auratus)* of the common variety, 20 ± 3 g, were obtained from a local vendor through the institutional animal order desk. Fish were housed in freshwater aquaria (60 L capacity) at 23± 2 °C, under a simulated 12 h light: 12 h dark photoperiod (lights on at 07:00 AM). Once daily, at a scheduled feeding time (11:00 h), fish were fed with commercial slow sinking pellets (Aqueon, Franklin, USA). All studies using fish complied with the Canadian Council for Animal Care regulations and were approved by the Animal Research Ethics Board (Animal Use Protocol # 2012-0082) of the University of Saskatchewan.

3.3.2. Experimental design and sampling

All experiments were completed between late spring and early summer (May-June) when goldfish were reproductively active. Male and female goldfish were randomly selected (weight matched) and were acclimated to 10 L experimental aquaria (n=2/ aquaria), maintained in a constantly aerated, continuous flow, temperature (23± 2 °C) controlled water current system. The objective of this research was to further determine the effects of nesfatin-1 on reproduction. Therefore, for the *in vivo* injection study, I used the same dose of nesfatin-1 and time points that were previously found effective in our own research (Gonzalez et al., 2012b). Briefly, male and female goldfish were anesthetized by using tricaine methanesulfonate-222 (TMS, 0.5%, Syndel Laboratories, BC, Canada) and injected IP with 100 µl of 0.9% sterile saline containing (50 ng/g body weight) goldfish synthetic nesfatin-1. The goldfish nesfatin-1 (1–82) (gfnesfatin-1; VPISIDKTKVKLPEETVKESPQNVDTGLHYDRYLREVIDFLEKDQHFREKLHNTDMEDI-KQGKLAKELDFVSHHVRTKLDEL) was custom synthesized (Genscript, Piscataway, NJ) and the purity (>95%) of the synthetic peptide was confirmed by mass spectrometry and MALDI-TOF (Gonzalez et al., 2010). Although the circulating levels of nesfatin-1 varies due to physiological conditions, the dose used here is likely supraphysiological based on the serum NUCB2/nesfatin-1 concentrations reported in goldfish (Gonzalez et al., 2012b). The control group was injected with 0.9% sterile saline (100 µl). Six male and 6 female fish from each of the groups (control and treatment) were anesthetized, euthanized at 15 minutes and 60 minutes post-nesfatin-1 administration. Tissues including hypothalamus, pituitary and gonads were collected

and flash- frozen immediately in liquid nitrogen and stored at -80 ^{0}C until further processing. Blood was collected from the caudal vein and serum was separated and stored at -20 ^{0}C for ELISA.

3.3.3. RT-qPCR based mRNA expression analysis

Total RNA extraction and cDNA synthesis Real-Time PCR (RT-qPCR) were conducted as described in chapter 2. Primer validation and efficiency of primers were confirmed for each primer set as described earlier (Gonzalez et al., 2012b). Genes quantified in this study were all previously documented to have a role in goldfish reproduction. Details of primers and primer-specific annealing temperatures used are provided in **Table 3.1**. qPCR cycle conditions were same as described in chapter 2, except for specific annealing temperatures and primer sequences, which is provided in **Table 3.1**. A melting curve analysis was performed as described earlier. Livak method (Livak and Schmittgen, 2001) was used for the qPCR data analysis, and the data were normalized to the housekeeping genes (18s rRNA, β-actin).

Table 3.1: Primer and annealing temperature details used for QPCR gene expression

Gene	Accession no.	Primer sequence (5'-3')		Annealing temperature (°C)
		Forward	Reverse	
18SrRNA	FJ710819.1	GGATGCCCTTAACTGGGTGT	CTAGCGGCGCAATACGAATG	60
β-actin	AF079831.1	CTACTGGTATTGTGATGGACT	TCCAGACAGAGTATTTGCGCT	60
sGnRH	AB020243.1	GGATGATGGATTCTGGTGATG	CTATGTGTATCGGTGAAAGC	59
cGnRH-II	U30386.1	TACGATTCCTCAGAGGTTTCAG	CATCCAGCAGTATTGTCTTCAG	59
kiss-1a	FJ465138.1	GACTCCAAGCAGCACTATC	CCCAAACGGGTTGTAGTT	61
kiss2	GQ141877.1	GACTCCAAGCAGCACTATC	CCCAAACGGGTTGTAGTT	60
GPR54a	FJ465139.1	CTGGTGCCCCTTTTCTTCTC	GGGTAGAGGGTAGCAGTGAA	61
GnIH	AB078976.1	CTCCCACCATCCTGCGACTT	CTTTGCGGTAGGGTGGCTGA	57
GnIHr	JN204438.1	CCAGCGTCAACCCTATTATC	AGCAGCCGTATTCAAAGG	59
LHβ	MH536845.1	GCTTGCCAGACTGTCCTC	GTCAGATGTGTCCATAGTGC	59
FSHβ	MH536843.1	GTTGTTATGGTGATTCTGTTG	ACGGTGATGGAGATATTGG	60
FSHr	HM347775.1	CGTCCACAATCCTACCTTCG	TGAGAAACGGTGATTAAGCGG	57
LHr	HM347776.1	CCTCTGCATCGGTGTGTATC	TAGACAGATGATTCGCCCGCC	60
CYP11a1	JQ340311.1	TACATTGATCCAGACGCCCA	TTAAAGATGCCGTCCCAAGC	57
CYP19a1a	JN620808.1	GTGCTCAAGACAATGTGTGC	AAATGAACCCTACCAACCCG	60
CYP19a1b	AB009335.1	AGGCGAGCGGGATGTAGAGT	CGTCCGATGTTCAGGATGAGG	59
StAR	AY877430.1	ATGGCTGGCAAACTGAGATCGAGA	TCCATGTTATCCACCAGCTCCTCA	58
AMH	KF640083.1	TCTCCGAAAGGAGAGGACGA	TTCATCCGCTCTGACTGCTC	57
AR	AY090897.1	TGGCTCGAACTTTTCCAAGG	TCTGTGGAAAACTGCGTCTG	58
ER	AY055725.1	ACAGGTCCAGTGTTGTGTGT	ATGATAGCCGGACGCATAGT	58

3.3.4. Serum testosterone and estradiol measurement using ELISA

Serum gonadal steroids were measured using testosterone (human) and estradiol (human) ELISA kits (Eagle Biosciences Inc., USA) previously validated for quantifying goldfish sex steroids by our lab (Bertucci et al., 2016). ELISAs were carried out according to the manufacturer's instructions.

3.3.5. Statistical analysis

Statistical analysis was performed using Student's *t*-test and the levels of significance were set at $p < 0.05$. PRISM version 5 (GraphPad Inc., USA) was used for generating graphs and statistical analysis. All data are presented as mean + SEM.

3.4. Results

3.4.1. Nesfatin-1 administration downregulated the expression of GnRH and kisspeptin system in the hypothalamus of goldfish

I observed a significant reduction in the expression of sGnRH and cGnRH-II mRNA expression in the hypothalamus of male and female goldfish 15 minutes post-nesfatin-1 administration (**Figure 3.4.1 A-D**). A significant reduction in GPR54a mRNA expression was found in male and female treatment groups 15 minutes post-nesfatin-1 administration (**Figure 3.4.1 I-J**). No significant changes in kiss-1a and kiss2 mRNA expression were observed in the hypothalamus of male and female treatment groups compared to control (**Figure 3.4.1 E-H**).

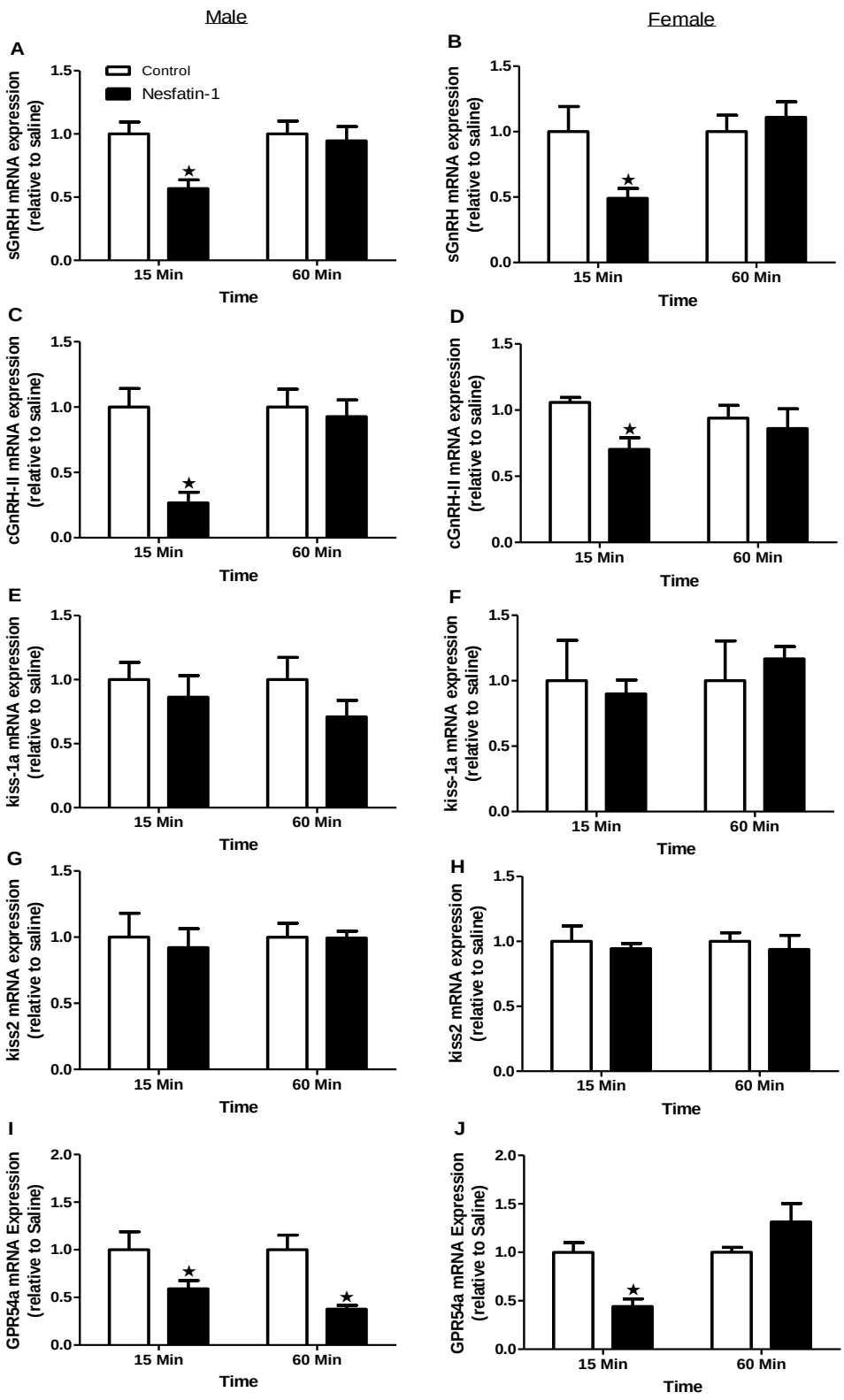
Male
Female
A
B
C
D
E
F
G
H
I
J
Control
Nesfatin-1
sGnRH mRNA expression (relative to saline)
cGnRH-II mRNA expression (relative to saline)
kiss-1a mRNA expression (relative to saline)
kiss2 mRNA expression (relative to saline)
GPR54a mRNA Expression (relative to Saline)
GPR54a mRNA expression (relative to saline)
15 Min
60 Min
Time
0.0
0.5
1.0
1.5
2.0

Figure 3.4.1: The mRNA expression profile of sGnRH, cGnRH-II, and kisspeptin system in the hypothalamus of male (left panel) and female (right panel) goldfish post-nesfatin-1 administration (**A-J**). Nesfatin-1 suppressed the mRNA expression of sGnRH (**A-B**), cGnRH-II (**C-D**), and GPR54a (**I-J**), in the hypothalamus of male and female goldfish. No significant changes in the mRNA expression of kiss-1a (**E-F**) and kiss2 (**G-H**) were observed in the hypothalamus of goldfish post-nesfatin-1 injection. Data obtained by RT-qPCR are represented as mean + SEM (n=6 fish/group). Student's *t*-test was used for statistical analysis, and the level of significance was set at $p < 0.05$. Asterisks denote significant differences between control and treated groups.

3.4.2. Nesfatin-1 suppressed the mRNA expression of CYP19a1b in the hypothalamus of goldfish

The mRNA expression of CYP19a1b were attenuated in both male and female treatment groups at 15 minutes post-injection (**Figure 3.4.2 E-F**). However, no significant changes in GnIH and GnIHr mRNA expression were detected in the hypothalamus of goldfish post-nesfatin-1 administration (**Figure 3.4.2 A-D**).

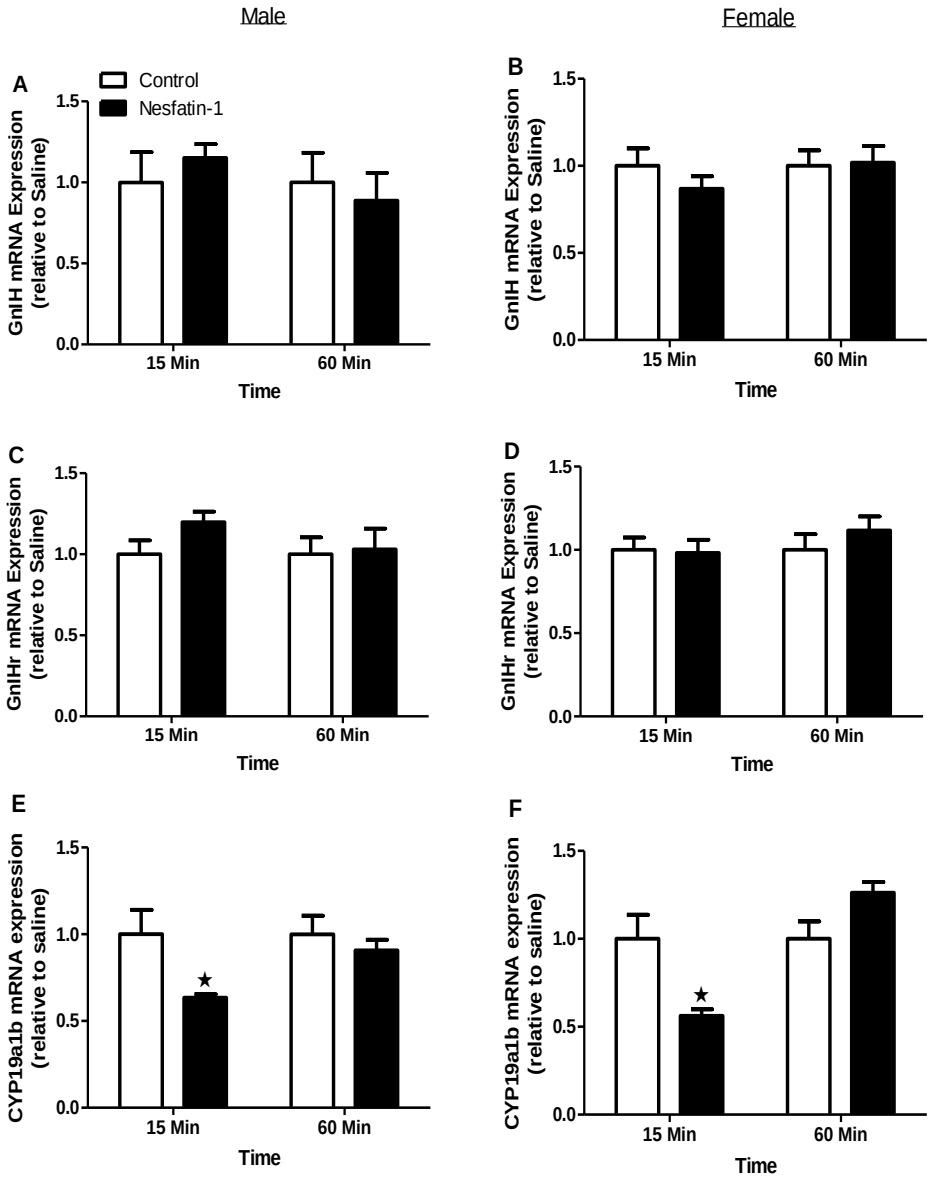

Figure 3.4.2: The mRNA expression profile of GnIH system and CYP19a1b post-nesfatin-1 administration in the hypothalamus of male (left panel) and female (right panel) goldfish (A-F). No significant changes in GnIH and GnIHr mRNA expression were observed in the hypothalamus of both male and female goldfish post-nesfatin-1 administration (**A-D**). However, the mRNA expression of CYP19a1b was significantly downregulated in male and female treatment groups at 15 minutes post-injection (**E-F**). Data obtained by RT-qPCR are represented as mean + SEM (n=6 fish/group). Student's *t*-test was used for statistical analysis, and the level of significance was set at $p < 0.05$. Asterisks denote significant differences between control and treated groups.

3.4.3. Nesfatin-1 suppressed the expression of the gonadotropin β subunits and kisspeptin system in the pituitary of goldfish

Nesfatin-1 downregulated the expression of LHβ, FSHβ mRNA expression in the pituitary of both male and female goldfish groups at 60 minutes post-injection (**Figure 3.4.3 A-D**). Nesfatin-1 caused a significant decrease in GPR54a mRNA expression in the pituitary of both treatment groups at 15 minutes post-injection (**Figure 3.4.3 I-J**). However, there were no significant changes in kiss-1a and kiss2 mRNA expression in male or female treatment groups post-nesfatin-1 administration (**Figure 3.4.3 E-H**).

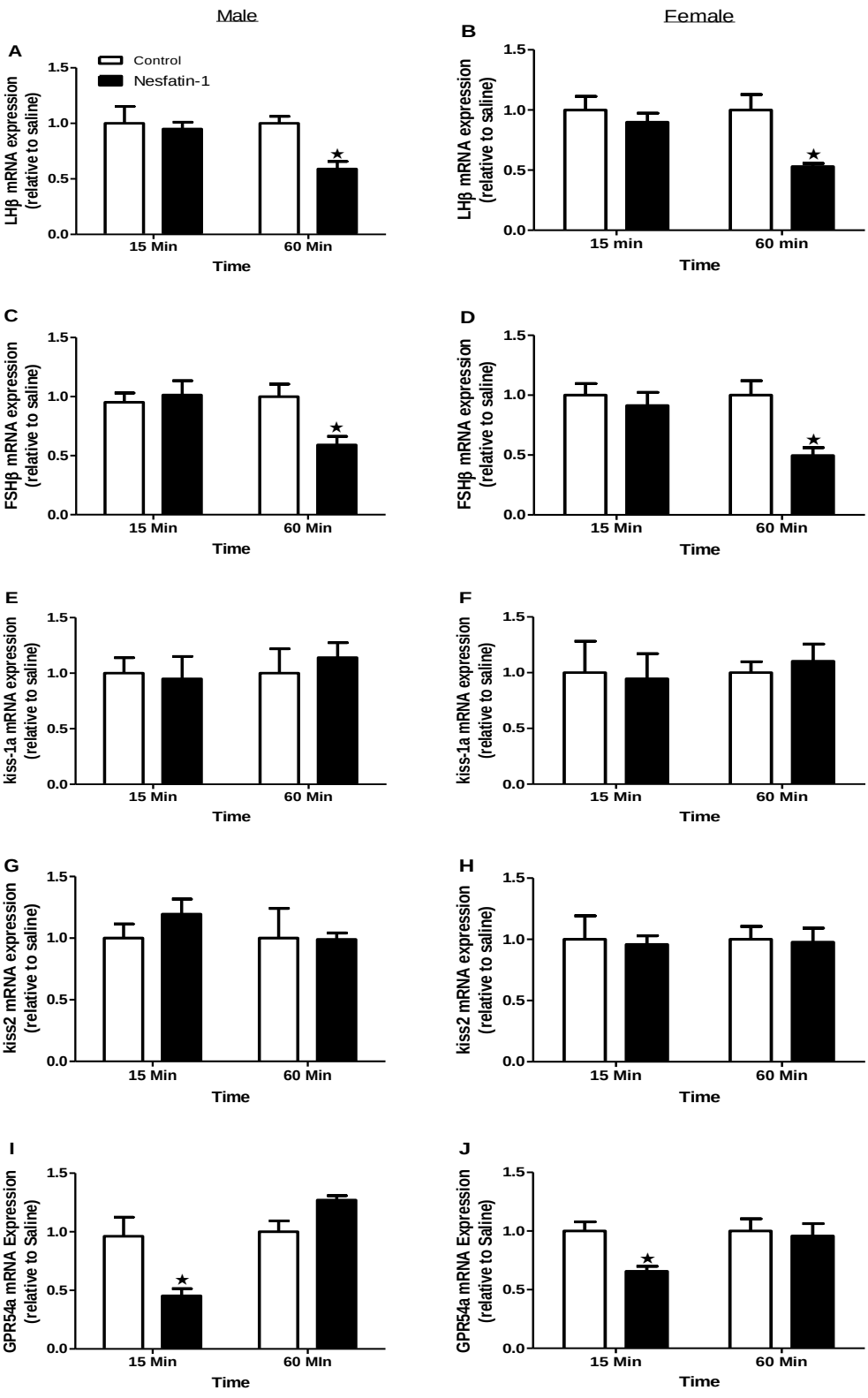
Male
Female
A
B
C
D
E
F
G
H
I
J
Control
Nesfatin-1
LHβ mRNA expression (relative to saline)
FSHβ mRNA expression (relative to saline)
kiss-1a mRNA expression (relative to saline)
kiss2 mRNA expression (relative to saline)
GPR54a mRNA Expression (relative to Saline)
GPR54a mRNA Expression (relative to Saline)
1.5
1.0
0.5
0.0
15 Min
60 Min
15 min
60 min
60 MIn
Time
★

Figure 3.4.3: Expression levels of mRNAs encoding gonadotropin β subunits and kisspeptin system in the pituitary of male (left panel) and female (right panel) goldfish post-nesfatin-1 administration (A-J). Nesfatin-1 suppressed the mRNA expression of LHβ (**A-B**) and FSHβ (**C-D**) in the pituitary of both male and female goldfish at 60 minutes post-injection. I observed a decrease in GPR54a (**I-J**) mRNA expression at 15 minutes post-nesfatin-1 administration in both sex groups. However, I did not observe any significant changes in the expression of kiss-1a (**E-F**) and kiss2 (**G-H**) in the pituitary of both sex groups. Data obtained by RT-qPCR are represented as mean + SEM (n=6 fish/group). Student's *t*-test was used for statistical analysis, and the level of significance was set at $p < 0.05$. Asterisks denote significant differences between control and treated groups.

3.4.4. Nesfatin-1 did not influence the pituitary GnIH and GnIHr transcripts in goldfish

No significant changes in the GnIH and GnIHr mRNAs were observed in the pituitary of goldfish post-nesfatin-1 injection (**Figure 3.4.4 A-D**).

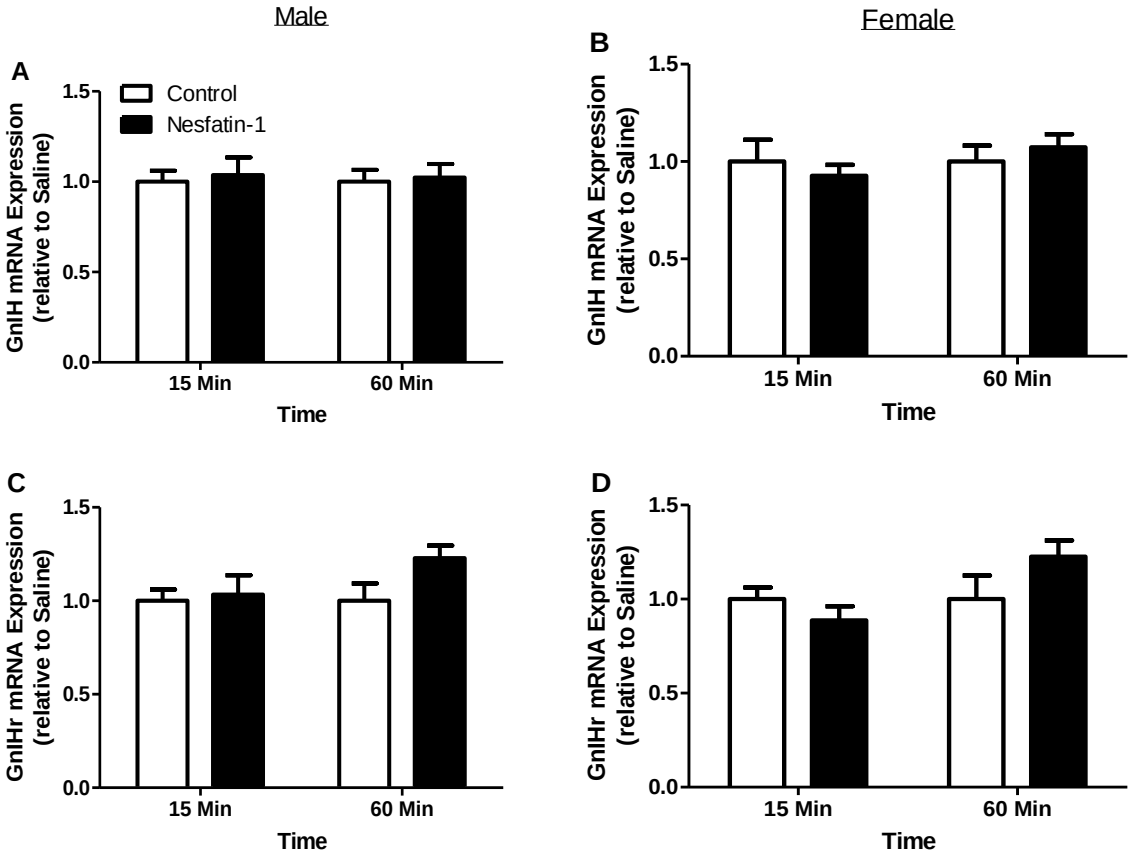

Figure 3.4.4: Expression profile of GnIH system post-nesfatin-1 administration in the pituitary of male (left panel) and female (right panel) goldfish (A-D). No significant changes in the mRNA expression of GnIH (**A-B**) and GnIHr (**C-D**) were observed in the pituitary of both male and female goldfish post-nesfatin-1 injection. Data obtained by RT-qPCR are represented as mean + SEM (n=6 fish/group). Student's *t*-test was used for statistical analysis, and the level of significance was set at $p < 0.05$.

3.4.5. Nesfatin-1 modulated the expression of gonadotropin receptor and sex steroid receptor mRNA expression in the gonads of goldfish

In the gonads of both male and female goldfish, I found a reduction in the expression of LHr mRNA expression at 15 minutes post-nesfatin-1 administration (**Figure 3.4.5 A-B**). However, the FSHr mRNA shows a differential expression pattern. In the testis of goldfish, a significant decrease in FSHr was observed at 15 minutes (**Figure 3.4.5 C**) post-injection. In the ovary of goldfish, I found a significant reduction in FSHr mRNA expression only at 60 minutes post-nesfatin-1 administration (**Figure 3.4.5 D**). AR mRNA expression was significantly upregulated in the testis (at 15 minutes) and downregulated in the ovary of goldfish (at 15 minutes) post-nesfatin-1 administration (**Figure 3.4.5 E-F**). However, I did not observe any significant changes in ER mRNA expression in the gonads of goldfish post-nesfatin-1 administration (**Figure 3.4.5 G-H**).

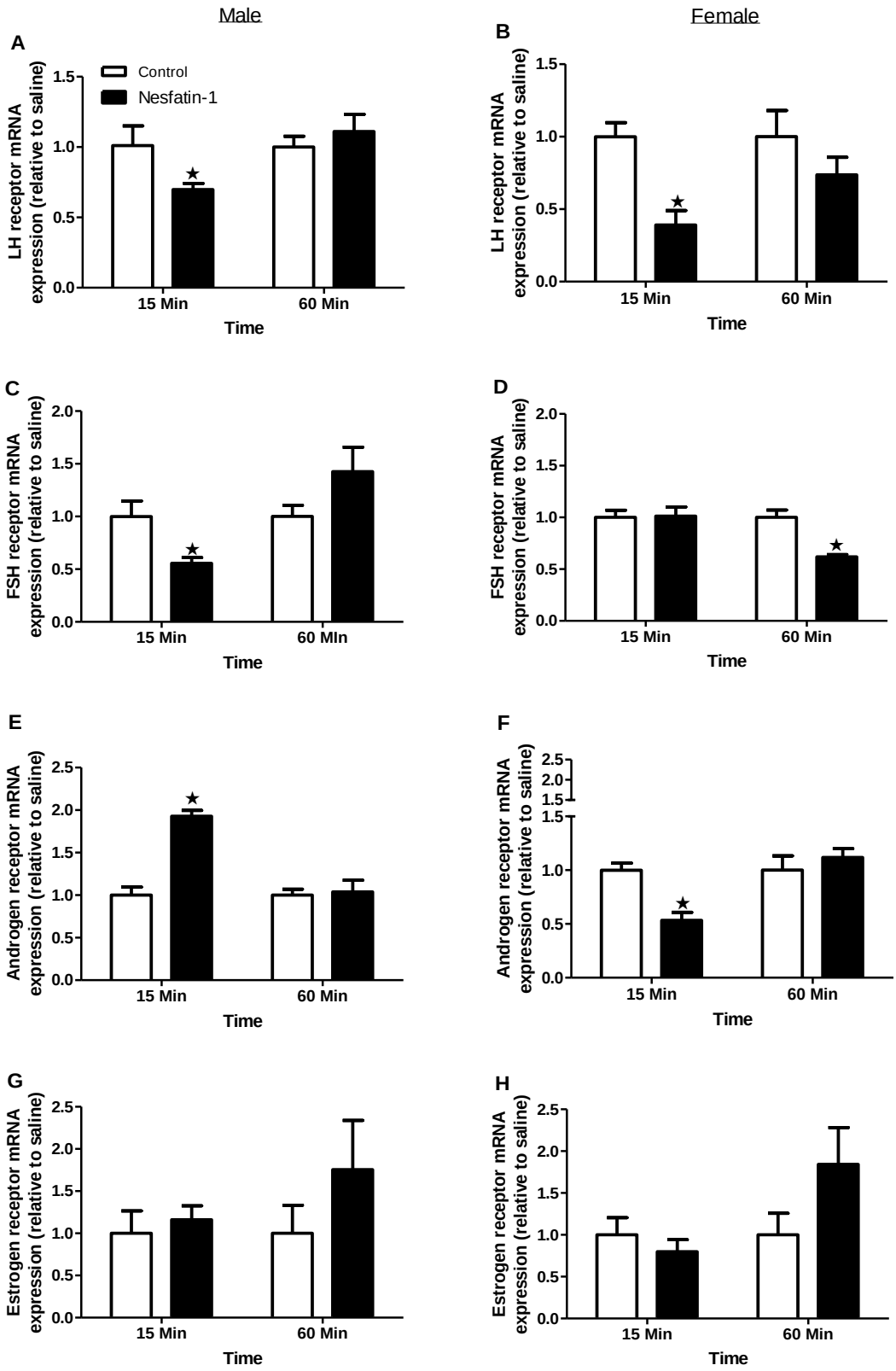
Male
Female
A
B
C
D
E
F
G
H
Control
Nesfatin-1
LH receptor mRNA expression (relative to saline)
FSH receptor mRNA expression (relative to saline)
Androgen receptor mRNA expression (relative to saline)
Estrogen receptor mRNA expression (relative to saline)
15 Min
60 Min
60 MIn
Time

Figure 3.4.5: The mRNA expression profile of gonadotropin and sex steroid receptor mRNAs in the testis (left panel), and ovary (right panel) of goldfish post-nesfatin-1 administration (A-H). In the gonads of goldfish, a significant decrease in LHr (**A-B**) and FSHr (**C-D**) mRNA expression was observed post-nesfatin-1 administration. In the testis and ovary of goldfish, a significant increase in AR mRNA expression was observed at 15 minutes post-nesfatin-1 injection (**E-F**). I did not observe any significant changes in the expression of ER (**G-H**) mRNA expression in the gonads of both sex groups post-nesfatin-1 injection. Data obtained by RT-qPCR are represented as mean + SEM (n=6 fish/group). Student's *t*-test was used for statistical analysis, and the level of significance was set at $p < 0.05$. Asterisks denote significant differences between control and treated groups.

3.4.6. Nesfatin-1 modulated the expression of GnIH, GnIHr, kiss-1a and GPR54a mRNA expression in the gonads of goldfish

A significant increase in GnIH mRNA expression was observed in the testis (only at 60 min) and ovary (both at 15 and 60 minutes) post-nesfatin-1 injection (**Figure 3.4.6 A-B**). In addition, GnIHr mRNA expression was also upregulated at both 15 and 60 minutes post-nesfatin-1 injection (**Figure 3.4.6 C-D**). I found a significant increase in kiss-1a (**Figure 3.4.6 E-F**) (at 15 minutes) and GPR54a (at 60 minutes in male and 15 minutes in female) (**Figure 3.4.6 I-J**) mRNA expression in the gonads of both treatment groups. However, I did not observe any significant changes in the kiss2 mRNA expression in both treatment groups post-nesfatin-1 injection (**Figure 3.4.6 G-H**).

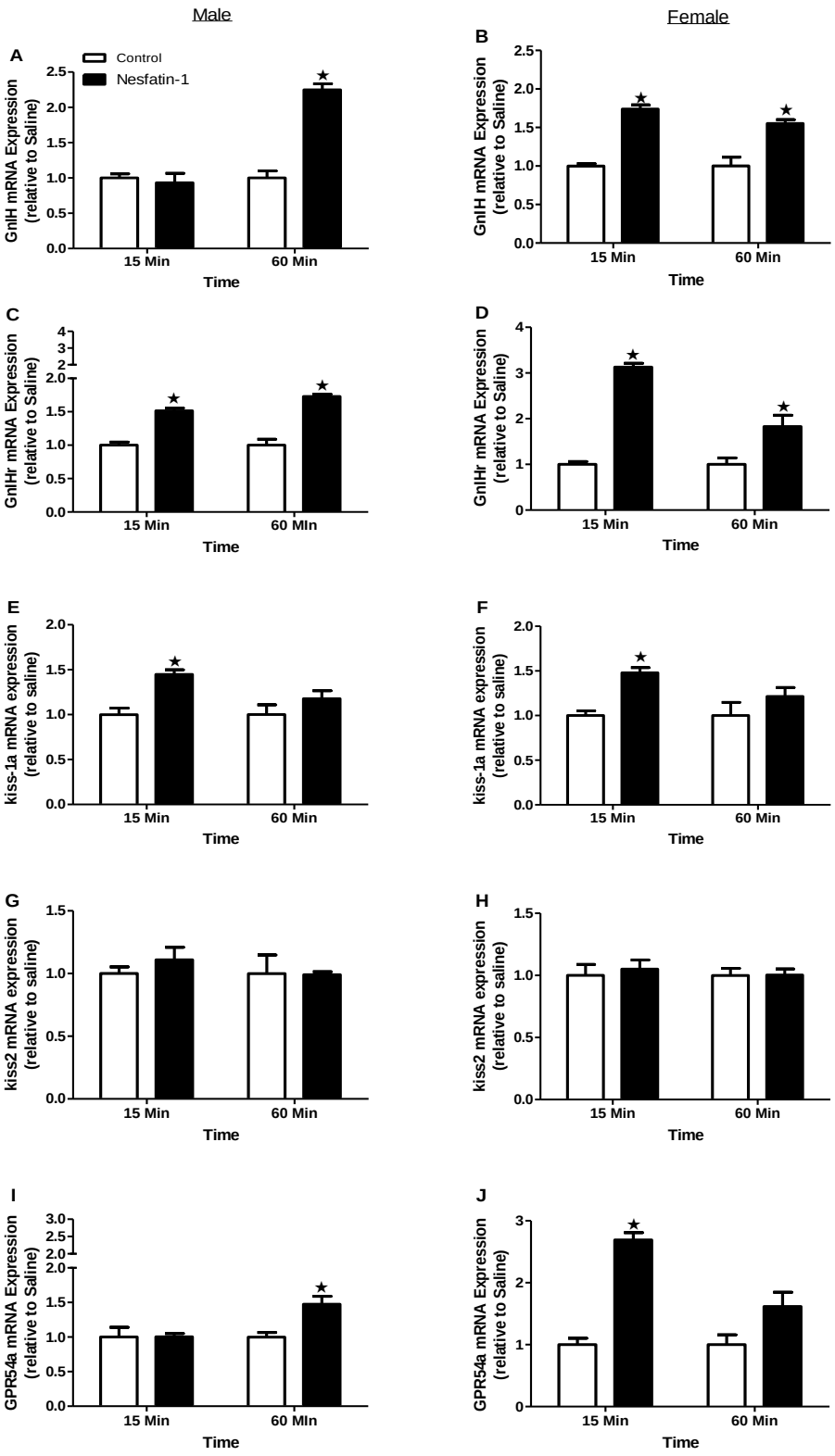
Male
Female
A
Control
Nesfatin-1
GnIH mRNA Expression (relative to Saline)
B
GnIH mRNA Expression (relative to Saline)
C
GnIHr mRNA Expression (relative to Saline)
D
GnIHr mRNA Expression (relative to Saline)
E
kiss-1a mRNA expression (relative to saline)
F
kiss-1a mRNA expression (relative to saline)
G
kiss2 mRNA expression (relative to saline)
H
kiss2 mRNA expression (relative to saline)
I
GPR54a mRNA Expression (relative to Saline)
J
GPR54a mRNA Expression (relative to Saline)
15 Min
60 Min
Time

Figure 3.4.6: Expression profile of GnIH and kisspeptin system mRNAs in the testis (left panel) and ovary (right panel) of goldfish post-nesfatin-1 administration (A-J). A significant increase in GnIH mRNA expression was observed in the testis (only at 15 minutes) (**A**) and ovary (at both 15 and 60 minutes) (**B**) of goldfish post-nesfatin-1 administration. Nesfatin-1 elevates GnIHr mRNA expression at both 15 and 60 minutes time points in the testis and ovary of goldfish (**C-D**). The mRNA expression of kiss-1a (**E-F**) and GPR54a (**I-J**) mRNAs were significantly increased in the gonads of both male and female goldfish. However, I did not observe any significant changes in the mRNA expression of kiss2 in the testis and ovary post-nesfatin-1 administration (**G-H**). Data obtained by RT-qPCR are represented as mean + SEM (n=6 fish/group). Student's *t*-test was used for statistical analysis, and the level of significance was set at $p < 0.05$. Asterisks denote significant differences between control and treated groups.

3.4.7. Nesfatin-1 influenced the expression of genes involved in sex steroidogenesis in the gonads of goldfish

In the testis, a significant reduction in StAR mRNA expression was observed (**Figure 3.4.7 A**) at 15 minutes post-nesfatin-1 injection. However, in the ovary a significant increase in StAR mRNA expression was observed at 15 minutes post-nesfatin-1 administration (**Figure 3.4.7 B**). CYP11a1 expression was significantly downregulated in both testis and ovary of goldfish at 15 minutes post-nesfatin-1 administration (**Figure 3.4.7 C-D**). CYP19a1a mRNA expression was significantly downregulated at 60 minutes post-nesfatin-1 administration in the gonads of goldfish (**Figure 3.4.7 E-F**). In the testis, I observed a significant increase in AMH mRNA expression at 60 minutes post-injection (**Figure 3.4.7 G**). However, in the ovary AMH mRNA expression was upregulated only during the 15 minutes post-nesfatin-1 injection (**Figure 3.4.7 H**) time point.

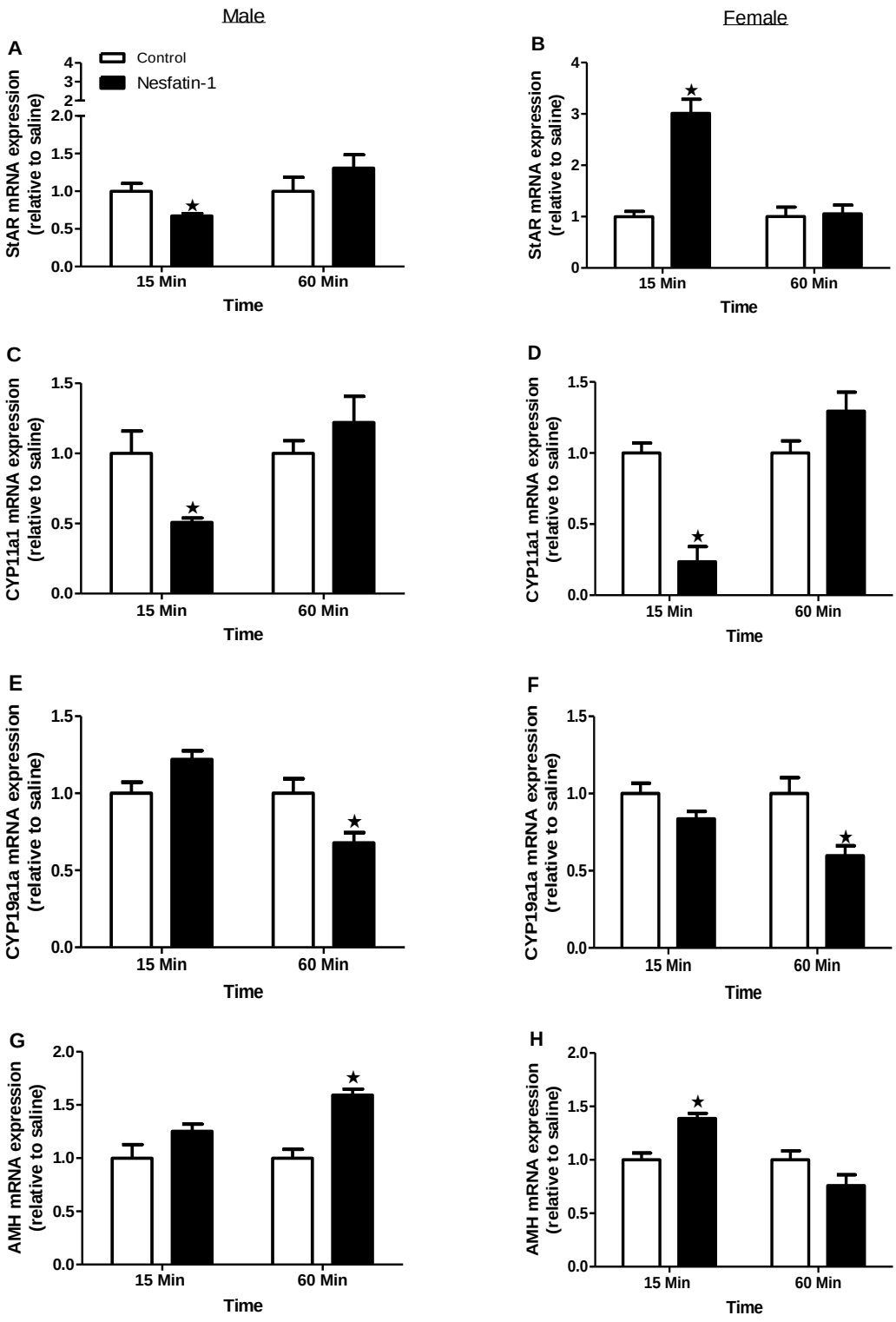
Male
Female
A
B
C
D
E
F
G
H
Control
Nesfatin-1
StAR mRNA expression (relative to saline)
CYP11a1 mRNA expression (relative to saline)
CYP19a1a mRNA expression (relative to saline)
AMH mRNA expression (relative to saline)
15 Min
60 Min
Time

Figure 3.4.7: Expression profile of genes involved in the sex steroidogenic pathway in the testis (left panel) and ovary (right panel) of goldfish post-nesfatin-1 administration (A-H). Nesfatin-1 suppressed the expression of StAR mRNA in the testis (**A**) and elevated it in the ovary (**B**) of goldfish at 15 minutes post-injection. A significant decrease in CYP11a1 (at 15 minutes) and CYP19a1a (at 60 minutes) was found in the testis (**C** & **E**) and ovary (**D** & **F**) of goldfish. A significant increase in the mRNA expression of AMH was observed in the testis (at 60 minutes) and ovary (at 15 minutes) of goldfish post-nesfatin-1 administration (**G-H**). Data obtained by RT-qPCR are represented as mean + SEM (n=6 fish/group). Student's *t*-test was used for statistical analysis, and the level of significance was set at $p < 0.05$. Asterisks denote significant differences between control and treated groups.

3.4.8. Nesfatin-1 downregulated the circulating levels of sex steroids in both male and female goldfish

I found a significant decrease in circulating testosterone (T) levels in the male treatment group at 60 minutes post-nesfatin-1 administration (**Figure 3.4.8 A**). Similarly, a significant decrease in plasma estradiol (E) levels in females was observed at 60 minutes post-nesfatin-1 administration (**Figure 3.4.8 B**).

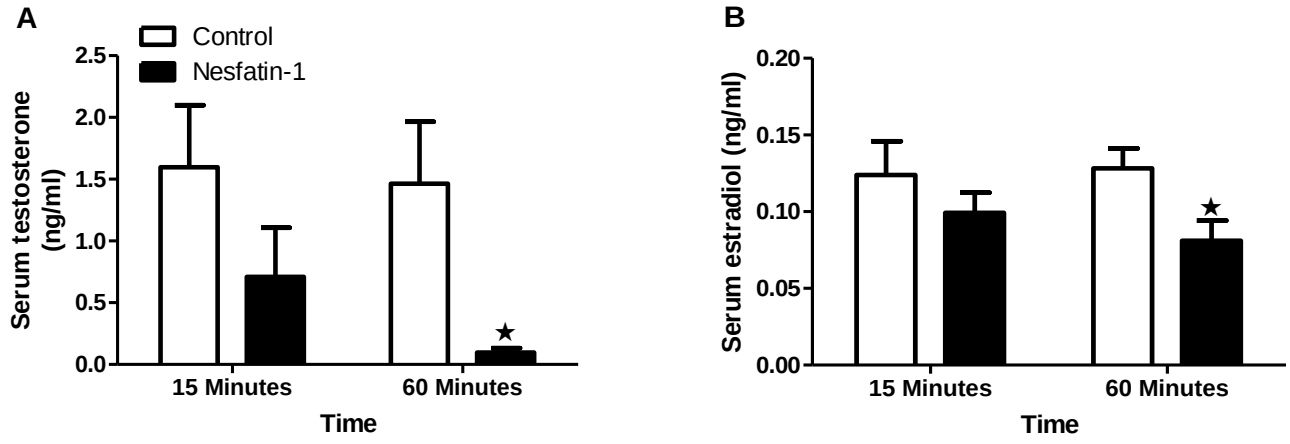

Figure 3.4.8: Serum testosterone and estradiol profiles in male and female goldfish post-nesfatin-1 administration. A significant decrease in serum levels of testosterone was observed in male fish group at 60 minutes post-nesfatin-1 administration (**A**). A significant decrease in serum estradiol was observed in female fish groups at 60 minutes post-nesfatin-1 administration (**B**). Data are represented as mean + SEM (n=6 fish/group). Student's *t*-test was used for statistical analysis, and the level of significance was set at $p < 0.05$. Asterisks denote significant differences between control and treated groups.

3.5. Discussion

Nesfatin-1 was originally identified as a metabolic regulator involved in feed intake regulation and energy homeostasis (Gonzalez et al., 2010; Oh-I et al., 2006). Recent evidences indicate that nesfatin-1 also has sex-specific regulatory roles in metabolism and reproduction (Bloem et al., 2012; Hofmann et al., 2015; Schalla and Stengel, 2018). As an extension of our own previous findings (Gonzalez et al., 2012b) on nesfatin-1 and reproduction, this research explored how nesfatin-1 affects goldfish HPG axis. In goldfish, two GnRH isoforms are expressed in the brain (including the hypothalamus), namely sGnRH and cGnRH-II, and both are shown to potentiate gonadotropin release in goldfish (Kim et al., 1995; Peter et al., 1985). I found that nesfatin-1 suppresses the expression of sGnRH and cGnRH-II mRNAs in the hypothalamus of both male and female goldfish. This is in agreement with the earlier report by (Gonzalez et al., 2012b), and adds on to the original findings to indicate that in both sexes, nesfatin-1 applies the same effect on GnRH. I observed a significant reduction in the expression of GnRH mRNAs within 15 minutes of nesfatin-1 administration, and this suggests that the action of nesfatin-1 in the brain after acute administration is very rapid. It was documented in mammalian studies that nesfatin-1 administered peripherally reach the brain roughly 10 minutes, can cross the blood-brain barrier in a non-saturable way (Pan et al., 2007; Price et al., 2007). My results suggest a similar kind of mechanism is in operation in fish as well. The doses and time-points chosen here were previously validated in goldfish and are known to affect feeding and reproductive hormones (Gonzalez et al., 2010, 2012b). The circulating levels of nesfatin-1 in goldfish is 2.52±0.26 ng/ml (Gonzalez et al., 2010). Based on this, the dose tested here appears to be supraphysiological or pharmacological. A significant reduction in the expression of GPR54a (kiss-1a receptor) mRNA expression in the hypothalamus of both male and female treatment groups at 15 minutes post-injection was observed. The role of nesfatin-1 in the expression of the kisspeptin system is not addressed in any teleosts so far. Incubation of murine hypothalamic (GT1-7) and pituitary (LβT2) cell lines with nesfatin-1 increased the expression of GPR54a (Hatef and Unniappan, 2017). This study points to the ability of nesfatin-1 to act directly on both hypothalamic neurons and gonadotrophs derived from mice. Direct action of nesfatin-1 on fish hypothalamic neurons and pituitary gonadotrophs is yet to be determined. In mammals, nesfatin-1 is generally considered as a positive regulator of reproduction (García-Galiano et al., 2010, 2012). However, ICV injection

of nesfatin-1 decreases the expression of GnRH and kisspeptin in male rats (Gao et al., 2016). This suggests that the sex of the animal and mode of administration might affect the action of nesfatin-1. I also found that nesfatin-1 suppresses the expression of CYP19a1b mRNA in the hypothalamus of male and female goldfish. Aromatase plays an important role in sexual behavior in both male and female goldfish (Lord et al., 2009). Reduction in CYP19a1b transcript suggests a possible role for nesfatin-1 in goldfish sexual behavior. Nesfatin-1 effects on feeding, anxiety and depression-like behaviors is documented in fish and mammals (Kühne et al., 2018; Lin et al., 2014). A role for nesfatin-1 in sexual behavior is not yet documented, but the results provided here raises a possible role for nesfatin-1 on sexual behavior. In addition, it was reported that nesfatin-1 influence the genes involved in sex steroidogenesis in males rats (Gao et al., 2016), which suggest regulatory mechanism of nesfatin-1 in vertebrates conserved across species. Overall, my data provides additional support to the notion that nesfatin-1 negatively influences hypothalamic hormones in fish.

In the pituitary, FSHβ, LHβ and GPR54a mRNA expression was decreased after nesfatin-1 administration. However, I did not observe any significant changes in kiss-1a mRNA expression, which is similar to what I found in the hypothalamus. It was reported earlier by immunohistochemical analysis that nesfatin-1 is abundantly expressed in the pituitary (Gonzalez et al., 2012b), suggesting its possible regulatory role in pituitary functions. The suppression of gonadotropin mRNA expression, the key regulators of vertebrate reproduction (Gharib et al., 1990; Zhang et al., 2015) is additional evidence for the negative effect of nesfatin-1 on goldfish reproduction. More evidence for the negative influence of nesfatin-1 was observed in the gonads of goldfish. In both sexes, LH and FSH receptor mRNA expressions were suppressed after nesfatin-1 administration. Contrary to what was observed in the hypothalamus and pituitary, I found an increase in both kiss-1a and GPR54a mRNA expression in both male and female gonads. My results from gonadal mRNA expression suggest that nesfatin-1 has tissue-specific effects on goldfish reproduction. Interestingly I did not observe any significant changes in kiss2 mRNA expression post-nesfatin-1 administration in all compartments of the HPG axis. Further studies are needed to confirm whether elevated kiss-1a and GPR54a is a feedback response or not. While most of the stimulatory hormones were downregulated, an opposite (upregulation) effect was found in the case of inhibitory hormones. A significant increase in both GnIH and GnIHr

mRNA expression, which is a potent inhibitor of reproduction in vertebrates (Tsutsui et al., 2010) was found in the gonads of goldfish. This possibly is an additional mechanism by which nesfatin-1 exerts its negative effects on goldfish reproduction. However, I did not observe any significant changes in GnIH and its receptor in both hypothalamus and pituitary of males and females. Together, these results suggest that nesfatin-1 influences all three components of the HPG axis to modulate reproductive hormones.

In addition to the overall negative effects of nesfatin-1 on the HPG axis genes that promote reproduction, I observed the influence of nesfatin-1 on genes involved in sex steroidogenesis. A reduction in StAR (in testis only), CYP11a1, and CYP19a1a mRNA expression was found after nesfatin-1 administration. This suggests that nesfatin-1 effects on sex steroidogenesis is limited not only to mammals as previously described (Gao et al., 2016), but a similar regulatory pattern is in operation in fish as well. I also found an increase in the expression of AMH, a potent inhibitor of sex steroidogenesis in vertebrates including fish (Halm et al., 2007; Lambeth et al., 2016; Pala et al., 2008; Pfennig et al., 2015; Rodríguez-Marí et al., 2005; Xu et al., 2019). This furnishes additional support for the suppressive effect of nesfatin-1 on goldfish reproduction. A sex-specific expression pattern of AR was found in nesfatin-1 treated fish. In the final study, I determined whether the changes in mRNA expression would eventually result in alterations in hormone levels. In fact, a significant reduction in serum T and E levels was found at 60 minutes after nesfatin-1 injection. These results, for the first time, show that gonadal sex steroids are targets of nesfatin-1. Nesfatin-1 influences male and female sex steroidogenic enzymes in a time-dependent manner. However, it is unclear whether nesfatin-1 affects gonadal steroid synthesis, secretion and/or clearance, or all of these processes that determine circulating levels. A significant decrease in StAR and CYP11a1 found in male goldfish possibly contributes to the reduction in circulating testosterone levels. It is possible that elevated AMH is another factor affecting circulating testosterone levels in male fish treated with nesfatin-1. In the ovary, a significant decrease found in both CYP11a1, and CYP19a1a likely mediates the negative effects of nesfatin-1 on circulating estradiol levels. The increase in StAR mRNA at 15 minutes post-injection in the ovary is possibly a feedback response to induce cholesterol uptake and thereby increase substrate availability. The mechanism of action of nesfatin-1 in causing these steroidogenic responses requires additional studies. For example, the significance of nesfatin-1

suppression of gonadal CYP19a1a and CYP19a1b expression in male fish and its implications in male reproductive physiology is lacking. Since the circulating levels of estradiol in male fish was not measured, I am unsure to what extend the changes in aromatase (CYP19a1a) gene expression will reflect in circulating estradiol levels. During development, majority of fish species exhibit gonadal expression of aromatase in males (reduced expression) and females (elevated expression) (Guiguen et al., 2010). Additional studies to understand the physiological relevance of such patterns of gonadal aromatase expression are essential. In addition, I did not quantify CYP17a1a expression in the gonads of goldfish due to the lack of sequence information (to design primers). Further studies are required to clearly understand the exact processes in steroid production and secretion that are influenced by nesfatin-1 in both male and female fish. Together, my results indicate nesfatin-1 influence all tissues of the HPG axis and influence many critical hormonal regulators of fish reproduction.

3.6. Conclusion

In conclusion, I reconfirmed some of the acute effects of nesfatin-1 on goldfish reproduction (Gonzalez et al., 2012b) in both males and females separately. In addition, I extended these results into additional hypothalamic and pituitary hormones including kisspeptin and GnIH, and gonadal steroids. Most of the *in vivo* effects of nesfatin-1 found in this study ceased within 60 minutes of administration. Additional research is necessary to determine the chronic effects of nesfatin-1 on fish reproductive hormones and reproduction. The novelty of this research lies in the findings of nesfatin-1 effects on kisspeptin, GnIH and steroidogenic enzymes, and hormone receptors, as well as the circulating sex steroids. My results suggest sex- and tissue-specific effects for nesfatin-1 in goldfish. Nesfatin-1 in goldfish is a suppressor of pro-reproductive hormones (GnRH, gonadotropins, kisspeptin), while it promotes inhibitors (GnIH, AMH) of reproduction. Additional studies are required to understand the actions and mechanism of actions of nesfatin-1 on steroidogenic cells. While I only studied mRNA expression in tissues, the measurement of sex steroids provided a meaningful endpoint reflective of reproductive outcomes in fish. The results provided here enabled us to reach an overall conclusion that by acting at all levels of the HPG axis, nesfatin-1 acts as a suppressor of reproduction in fish.

Transition

Nucleobindins (NUCBs) are a class of DNA and Ca^{2+} binding proteins. NUCB1 and NUCB2 produce NLP and nesfatin-1, respectively, in vertebrates. Nesfatin-1 is a metabolic peptide with reproductive roles in mammals and fish (Gao et al., 2016a; García-Galiano et al., 2010; Gonzalez et al., 2012b). Recent work from our lab suggests similar biological actions for NLP.

Hypothesis: I hypothesized that NLP suppresses HPG axis hormones and oocyte maturation in fish.

Specific objectives:

1. Study whether NLP has any role in the regulation of reproductive hormones in fish in a sex-specific manner.
2. Determine whether NLP influences gonadal steroidogenesis and circulating sex steroid hormones in goldfish.
3. Explore the potential roles of NLP in the maturation of oocytes in zebrafish.

Result highlights: Single IP injection of gfNLP suppressed the expression of both GnRH isoform mRNAs and kisspeptin system mRNAs in the hypothalamus of male and female goldfish. In addition, I observed a reduction in LH and FSHβ and kisspeptin system mRNAs in the pituitary of NLP-treated fish. In the gonads of NLP-treated goldfish, suppression of critical genes involved in sex steroidogenesis and reduction in the circulating levels of T and E were observed. NLP suppresses oocyte maturation in goldfish, which provides additional evidence for the suppressive effects of NLP in goldfish reproduction. Our results suggest that the feeding and reproductive effects of NLP and nesfatin-1 are conserved in goldfish.

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Contributions: JJR planned and conducted the studies, analyzed and interpreted data, prepared the manuscript draft and revised it for submission. AH helped in conducting the experiments and tissue sampling and revision of the manuscript. SU provided the original idea and funding, and helped in the planning, designing of experiments and assisted in tissue sampling, data analysis and interpretation, manuscript preparation and revisions.

Chapter 4
Nesfatin-1-Like Peptide Suppresses Hypothalamo-Pituitary-Gonadal mRNAs, Gonadal Steroidogenesis and Oocyte Maturation in Fish

4.1. Abstract

Nucleobindin (NUCB)-1 and NUCB2 are DNA and Ca^{2+} binding proteins with multiple functions in vertebrates. Prohormone convertase-mediated processing of NUCB2 results in the production of biologically active nesfatin-1. Nesfatin-1 is involved in the regulation of reproduction in many vertebrates, including fish. Our lab originally reported NLP encoded in NUCB1 that exhibits nesfatin-1-like metabolic effects. I hypothesized that NLP has a suppressive role in the reproductive physiology of fish. In this research, whether NLP regulates reproductive hormones and oocyte maturation in fish were determined. Single IP injection of goldfish NLP (50 ng/g body weight) suppressed sGnRH and cGnRH-II, GnIH, GnIHr, kisspeptin and CYP19a1b mRNA expression in the hypothalamus of both male and female goldfish. In the pituitary, NLP decreased mRNAs encoding LHβ, FSHβ and kisspeptin and its receptor, while a significant increase in GnIH and GnIHr was observed. In the gonads, LHr (only in male fish) and FSHr mRNAs were also significantly downregulated in NLP injected fish. Sex-specific modulation of GnIH, GnIHr and kisspeptin system in the gonads was also observed. NLP decreased sex steroidogenic enzyme encoding mRNAs and circulating levels of testosterone and estradiol. In addition, incubation of zebrafish ovarian follicles with NLP resulted in a reduction in oocyte maturation. These results provide evidence for a robust role for NLP in regulating reproductive hormones in goldfish, and oocyte maturation in zebrafish, and these effects resemble that of nesfatin-1.

4.2. Introduction

It was previously reported from our lab that NUCB1 encodes a peptide that could be processed by prohormone convertases, which is structurally similar to nesfatin-1 in mammals and fish, and named it NLP (Gonzalez et al., 2012a; Ramesh et al., 2015; Sundarrajan et al., 2016). The bioactive core of nesfatin-1 and NLP are highly conserved across species, and this suggests action through the same receptors and/or comparable mechanisms. It was reported that the amino acid sequence of both goldfish/zebrafish nesfatin-1 share up to 74% sequence identity with goldfish/zebrafish NLP (Sundarrajan et al., 2016). Similar to nesfatin-1, NLP is also anorexigenic in mammals and fish (Gawli et al., 2017; Sundarrajan et al., 2016). NUCB1 is also involved in autoimmunity (Kubota et al., 2001), calcium homeostasis (Lin et al., 2000), apoptosis (Valencia et al., 2008), insulin secretion (Ramesh et al., 2015), bone mineralization (Petersson et al., 2004), and amyloid fibril formation (Gupta et al., 2012; Lin et al., 2007). Research in our lab found NUCB1/NLP–like immunoreactive cells in the gonads of goldfish (Sundarrajan et al., 2016). This suggests gonadal production, and a possible regulatory role for NLP in goldfish reproduction. NUCB1 mRNA expression was upregulated by testosterone (in gut, hindbrain and hypothalamus) and downregulated by estradiol (pituitary) in female goldfish (Sundarrajan et al., 2016). These results suggest a possible role for NUCB1/NLP in the regulation of reproduction in fish. The close structural relationship of NLP with nesfatin-1 (especially the M30 region) further strengthens this proposed role. I hypothesized that NLP, like nesfatin-1, is a negative regulator of reproductive hormones from the HPG axis, and oocyte maturation in fish. Does acute administration of NLP influence the HPG axis and regulate oocyte maturation in fish? I addressed this question using goldfish and zebrafish (previously used to characterize nesfatin-1 effects on reproductive hormones and oocyte maturation, respectively), and both *in vitro* and *in vivo* approaches. Results from my studies indicate that NLP has a suppressive role in HPG hormone encoding mRNAs, steroidogenic enzyme mRNAs, gonadal steroids in circulation and oocyte maturation. These data support the notion that NLP is indeed a nesfatin-1-like peptide when it comes to its effects on reproductive hormones and oocyte maturation.

4.3. Materials and methods

4.3.1. Animal and ethics

I employed the same model organisms and conditions previously used to characterize the reproductive roles of nesfatin-1 in fish. I used goldfish for my *in vivo* IP injection study because of the availability of relatively larger sized HPG axis tissues (especially pituitary gland). Zebrafish (female, 12-month-old) was used for oocyte maturation assay due to its easy availability and continuous breeding nature throughout the year. Details of animal housing and physical parameters are same as described earlier in chapter 2 (for zebrafish) and chapter 3 (for goldfish).

4.3.2. Experimental design and sampling

All experiments were conducted as described earlier (*in vivo* [chapter 3] and *in vitro* [chapter 2]). Briefly, for the *in vivo* studies, weight-matched male and female goldfish were randomly selected and acclimated to 10L aquaria (n=2 fish /aquaria), under the conditions described earlier in chapter 3. The time points and the doses were selected based on preliminary studies, as well as based on the times and doses that were found to be effective for nesfatin-1 in regulating goldfish reproductive hormones and zebrafish oocyte maturation (Gonzalez et al., 2012b; Sundarrajan et al., 2016). Male and female goldfish were anesthetized (as described in chapter 3) and injected IP with 100 µl of 0.9% saline (sterile) containing (50 ng/g body weight) custom synthesized gf/zf NLP (VPIDRNPDPPQEEKAEENVDTGLYYDRYLREVIEVLETDP-HFREKLQTANTEDIKNGRLSKELDLVGHHVRTRLDEL) (Pacific Immunology, Ramona, CA, USA). The purity (>95%) of the synthetic peptide was confirmed by mass spectrometry and MALDI-TOF analysis. The control groups were IP injected with 100 µl sterile saline (0.9% NaCl). At 15 minutes and 60 minutes time points, 6 males and 6 females from both control and NLP injected groups were anesthetized (as described above), euthanized (spinal transection under deep anesthesia), and hypothalamus, pituitary and gonads were collected. Tissues were immediately flash-frozen in liquid nitrogen (LN_2) and stored at -80 ^{0}C until further processing. Blood was collected from the caudal vein, samples were allowed to clot on ice, serum separated by centrifugation at 7000 rpm for 9 min, and samples were stored at -20 ^{0}C for ELISA.

4.3.3. Total RNA extraction, cDNA synthesis, and real-time quantitative PCR

Total RNA extraction and cDNA synthesis and RT-qPCR gene quantification were carried out as previously described in chapter 2. Primer validation, efficiency and annealing temperature were checked as described earlier (Gonzalez et al., 2012b). Details of primers used, and specific annealing temperatures of each primer set are the same as described in **Table 3.1,** except 2 for primer sets. They are follows; kiss-1b (Accession no. FJ465138.1) F-GACTCCAAGCAGCACTATC, R-CCCAAACGGGTTGTAGTT and GPR54b (Accession no. FJ465140.1) F-TAGGCTTGGTGGGTAACT, R-GTGGCAGTGAAAGGAACA. The annealing temperature for both primer sets is 60 ^{0}C. The qPCR cycle conditions were the same as detailed in chapter 2. Livak method (Livak and Schmittgen, 2001) was used for the relative data analysis, data were normalized to the housekeeping genes (18s rRNA and β-actin).

4.3.4. Serum testosterone and estradiol measurement using ELISA

Serum testosterone and estradiol levels were measured using testosterone (human) and estradiol (human) ELISA kits (Eagle Biosciences Inc., USA) employed in chapter 3.

4.3.5. Oocyte maturation assay

I used zebrafish for the oocyte maturation assay and was conducted as previously described in chapter 2. The switch in model organism (from goldfish to zebrafish) for this study is due to several reasons. Goldfish is a seasonal breeder and the availability of oocytes is limited. Previous studies suggest that zebrafish is a better model for this assay compared to goldfish (Tokumoto et al., 2004). There is no marked difference between goldfish and zebrafish oocyte maturation. Finally, the original hypothesis of this research is based on previous studies from our lab, which explored the reproductive role of nesfatin-1 (Gonzalez et al., 2012b), in which zebrafish used for oocyte maturation assay. The collection of oocytes/ovarian follicles and the media composition for the analysis were explained in chapter 2 (methods, oocyte maturation assay). Briefly, Follicles at stage III were incubated in a 24 well plate either with maturation media (90% L-15 media pH 9+10% BSA+100 ng/mL MIH; 4-Pregnen-17α,20β-diol-3-one [Toronto Research Chemicals, Ontario, Canada]) or 10 or 50 ng/mL zebrafish/goldfish NLP. No peptides were added to control groups. Each well (n=6 wells per treatment) contained 5-7 oocytes (total number of oocytes used per treatment is 30-38). Each experiment was repeated 3 times, and thus, more than 90-114 oocytes were included in each treatment group. GVBD and the change in

the appearance of the follicle from opaque to translucent were used to confirm maturation (fifth stage) and to score the maturation rate for each treatment.

4.3.6. Statistical analysis

Both mRNA expression and ELISA data were analyzed using Student's *t*-test and the level of significance was set at $p < 0.05$. For the oocyte maturation assay, one-way ANOVA followed by Tukey's multiple comparison test were used. $P < 0.05$ was considered statistical significance (indicated by letters a, b, and c in graphs). PRISM version 5 (GraphPad Inc., USA) was used for generating graphs and statistical analyses. All data are presented as mean + SEM.

4.4. Results

4.4.1. NLP suppressed GnRH, GnIH, GnIH receptor and CYP19a1b in the hypothalamus of goldfish

A significant decrease in sGnRH mRNA expression was observed in the hypothalamus of male and female goldfish at 15 minutes post-NLP administration (**Figure 4.4.1 A-B**). Meanwhile, cGnRH-II mRNA expression was significantly downregulated at 15 and 60 minutes post-NLP injected male and female fish (**Figure 4.4.1 C-D**). The GnIH mRNA expression was reduced in the hypothalamus of male goldfish at 60 minutes (**Figure 4.4.1 E**). However, a significant reduction GnIH mRNA expression at 15 minutes and a significant increase at 60 minutes was observed in the hypothalamus of female goldfish injected with NLP (**Figure 4.4.1 F**). The GnIH receptor (GnIHr) mRNA was significantly downregulated in the hypothalamus of both male and female goldfish at 60 minutes (**Figure 4.4.1 G-H**). Similarly, CYP19a1b mRNA expression was significantly decreased in the hypothalamus of both male and female goldfish at 15 minutes, but not at 60 minutes (**Figure 4.4.1 I-J**).

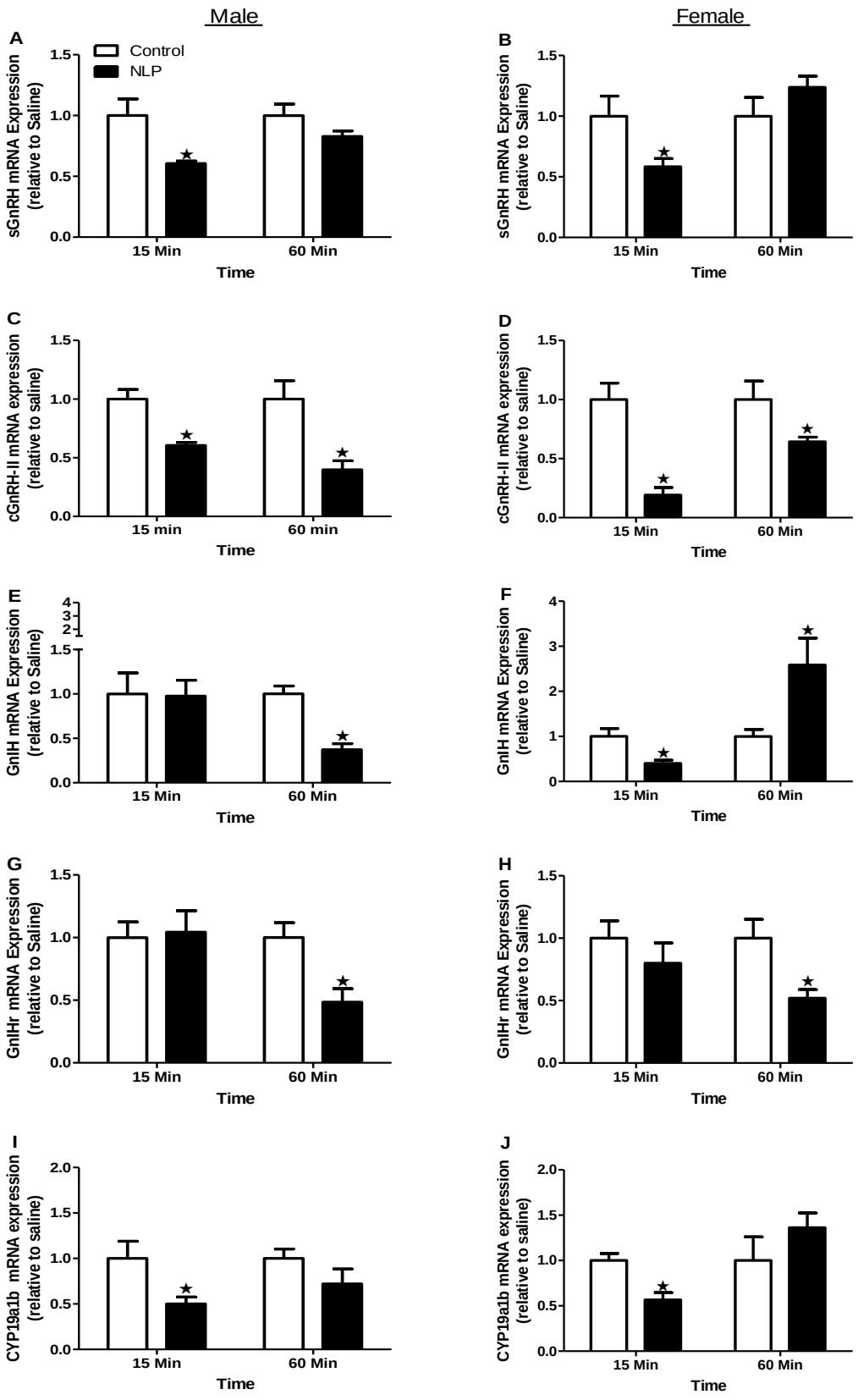

Male
Female
A
B
C
D
E
F
G
H
I
J
Control
NLP
sGnRH mRNA Expression (relative to Saline)
cGnRH-II mRNA expression (relative to saline)
GnIH mRNA Expression (relative to Saline)
GnIHr mRNA Expression (relative to Saline)
CYP19a1b mRNA expression (relative to saline)
15 Min
60 Min
15 min
60 min
Time

Figure 4.4.1: Expression profile of sGnRH, cGnRH-II, GnIH, GnIHr and CYP19a1b mRNAs in the hypothalamus of male (left panel) and female (right panel) goldfish post-NLP administration (**A**-**J**). Administration of NLP downregulated the expression of sGnRH (**A**-**B**), cGnRH-II (**C**-**D**), GnIH (**E**-**F**), GnIHr (**G**-**H**), and CYP19a1b (**I**-**J)** mRNAs. GnIH mRNA was upregulated in the female hypothalamus at 60 minutes (**F**). Data obtained by RT-qPCR are represented as mean + SEM (n=6 fish/group). Student's *t*-test was used for statistical analysis, and the level of significance was set at $p < 0.05$. Asterisks denote significant differences between control and treated groups.

4.4.2. NLP downregulated kisspeptin and kisspeptin receptor mRNAs in the hypothalamus of male and female goldfish

The mRNA expression of kiss-1b in the hypothalamus of female goldfish was reduced at 60 minutes post-NLP administration (**Figure 4.4.2 D**). However, no such changes in kiss-1a (in both sex groups) (**Figure 4.4.2 A-B**) and kiss-1b (in male fish) (**Figure 4.4.2 C**) mRNAs were found in the hypothalamus. NLP administration caused a significant decrease in kiss2 mRNA expression in the hypothalamus of both male (at 15 minutes) and female (at 60 minutes) fish groups (**Figure 4.4.2 E-F**). GPR54a expression was significantly decreased in the hypothalamus (at both 15 and 60 minutes) of both male and female goldfish (**Figure 4.4.2 G-H**). GPR54b mRNA expression was also significantly downregulated in male fish at both 15 and 60 minutes (**Figure 4.4.2 I**). However, there were no significant changes in GPR54b mRNA expression in the hypothalamus of female fish (**Figure 4.4.2 J**).

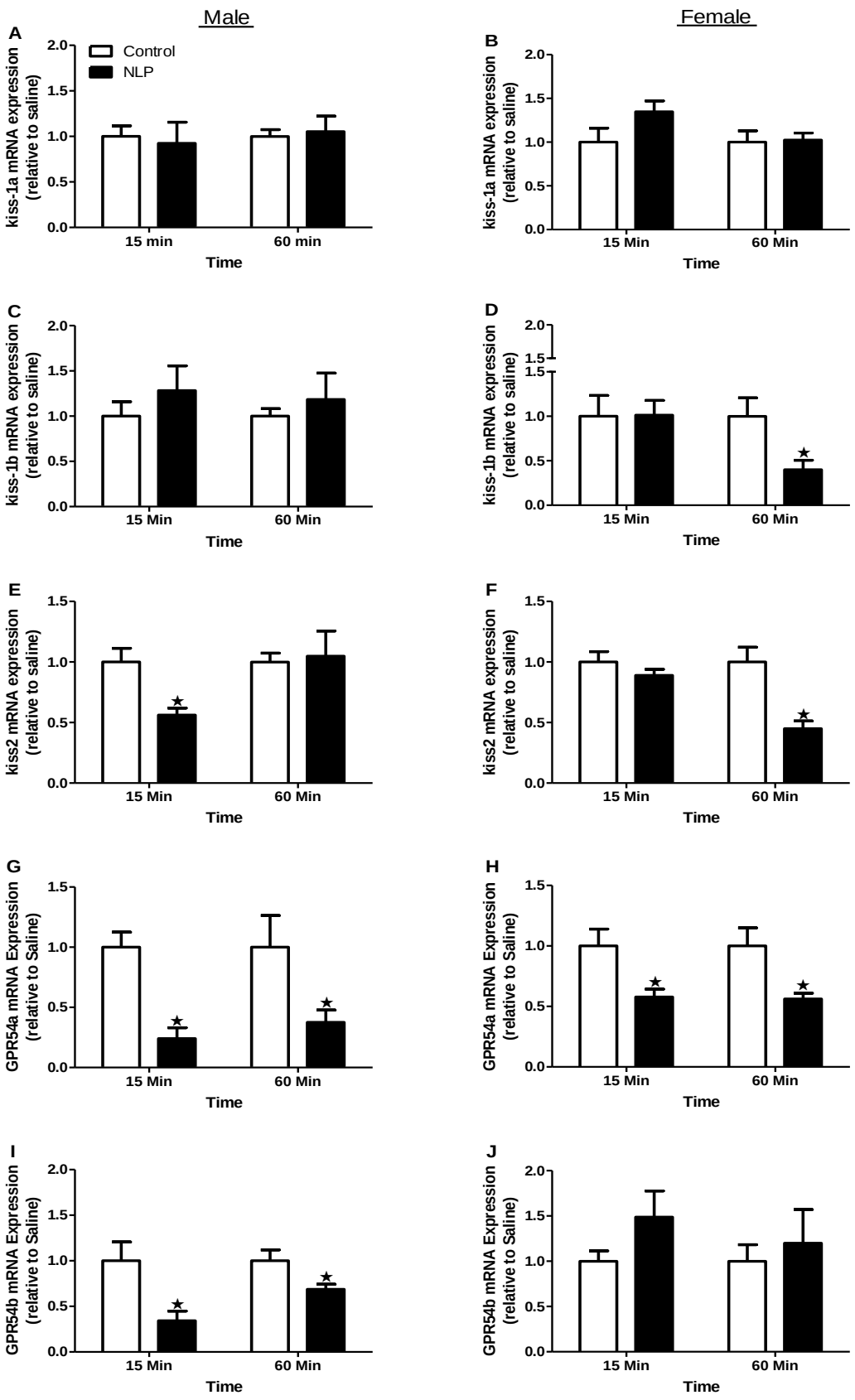

Male
Female
A
B
C
D
E
F
G
H
I
J
Control
NLP
kiss-1a mRNA expression (relative to saline)
kiss-1b mRNA expression (relative to saline)
kiss2 mRNA expression (relative to saline)
GPR54a mRNA Expression (relative to Saline)
GPR54b mRNA Expression (relative to Saline)
15 min
60 min
15 Min
60 Min
Time
0.0
0.5
1.0
1.5
2.0

Figure 4.4.2: Expression profile of the kisspeptin system mRNAs in the hypothalamus of male (left panel) and female (right panel) goldfish post-NLP administration (A-J). No significant changes in kiss-1a mRNA expression were observed in the hypothalamus of male and female fish groups post-NLP administration (**A**-**B**). The kiss-1b mRNA expression significantly downregulated in the female fish (**D**) and no significant changes were found in the male fish (**C**). The mRNA expression of kiss2 and GPR54a was downregulated in both sexes (**E**-**H**). The GPR54b mRNA expression downregulated only in the male fish group (**I**) with no significant changes in the female treatment groups (**J**). Data obtained by RT-qPCR are represented as mean + SEM (n=6 fish/group). Student's *t*-test was used for statistical analysis, and the level of significance was set at $p < 0.05$. Asterisks denote significant differences between control and treated groups.

4.4.3. NLP suppressed gonadotropin β subunits and increased GnIH and GnIHr mRNAs in the pituitary of goldfish

LHβ mRNA was significantly downregulated in the pituitary of male and female goldfish at 60 minutes post-NLP administration (**Figure 4.4.3 A-B**). The FSHβ mRNA expression was significantly downregulated at both 15 and 60 minutes in fish injected with NLP (**Figure 4.4.3 C-D**). In the pituitary of male and female fish, GnIH and GnIHr mRNAs were significantly upregulated at 15 minutes post-NLP administration (**Figure 4.4.3 E-H**).

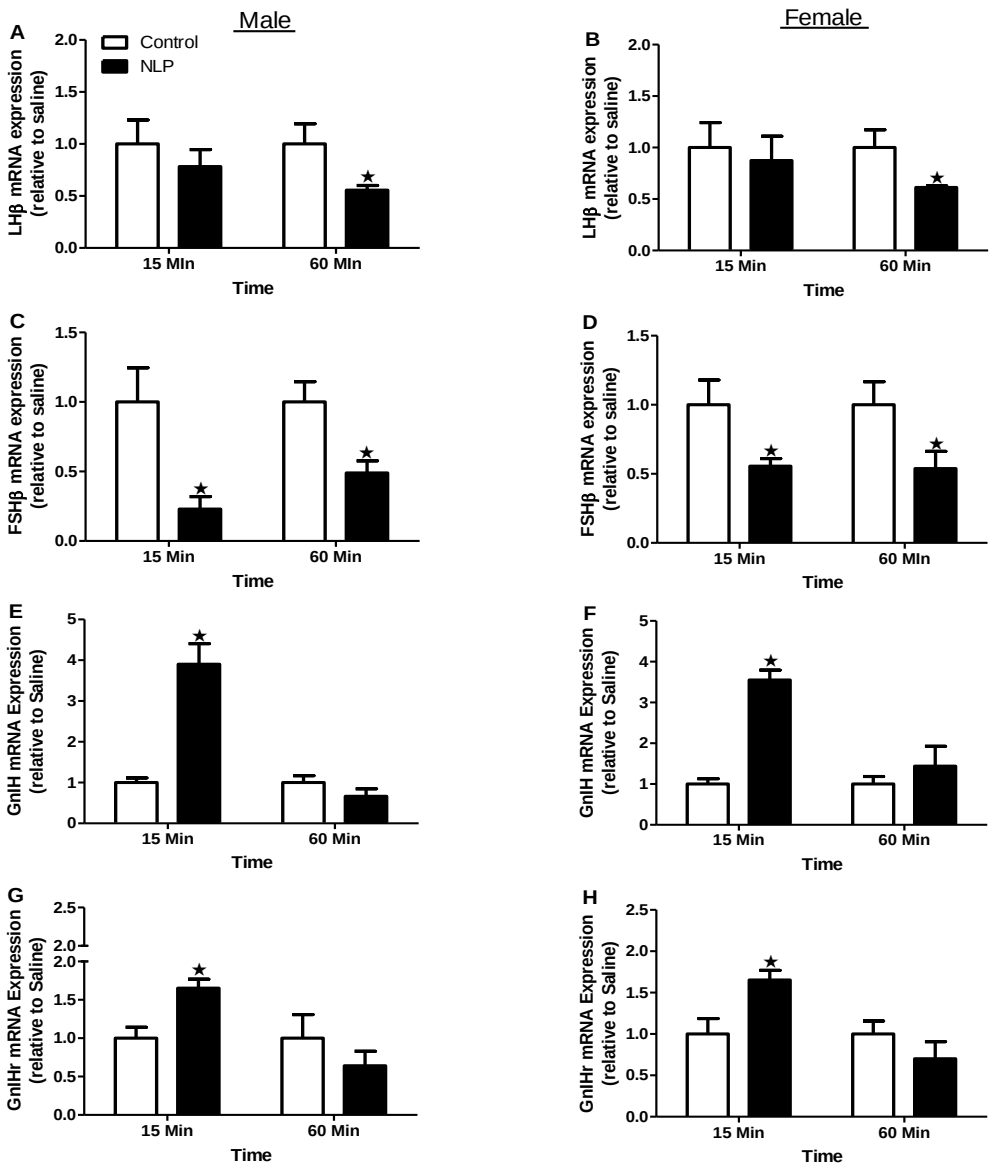


Figure 4.4.3: mRNA expression levels of gonadotropin β subunits and the GnIH system in the pituitary of male (left panel) and female (right panel) goldfish post-NLP administration (**A**-**H**). In the pituitary of both male and female fish groups, NLP suppresses both LHβ and FSHβ mRNA expression (**A-D**). The mRNA expression of both GnIH and GnIHr were upregulated post-NLP injection in the pituitary of both sex groups (**E-H**). Data obtained by RT-qPCR are represented as mean + SEM (n=6 fish/group). Student's *t*-test was used for statistical analysis, and the level of significance was set at $p < 0.05$. Asterisks denote significant differences between control and treated groups.

4.4.4. NLP downregulated kisspeptin system mRNAs in the pituitary of goldfish

A significant decrease in kiss-1a mRNA was observed in the pituitary of male and female goldfish 15 minutes post-NLP administration (**Figure 4.4.4 A-B**). Similar to this, kiss-1b mRNA expression was also significantly downregulated in the pituitary of male (both at 15 and 60 minutes time points) and female (only at 60 minutes time point) goldfish injected with NLP (**Figure 4.4.4 C-D**). NLP administration significantly downregulated kiss2 mRNA in the pituitary of male goldfish at 15 and 60 minutes after NLP injection (**Figure 4.4.4 E**). However, in the female goldfish pituitary, kiss2 mRNA expression was upregulated at 15 minutes followed by a significant decrease in expression was observed at the 60 minutes time point (**Figure 4.4.4 F**). The expression of GPR54a (at 15 minutes) and GPR54b (at 60 minutes) mRNAs was also downregulated in the pituitary of male and female goldfish (**Figure 4.4.4 G-J**).

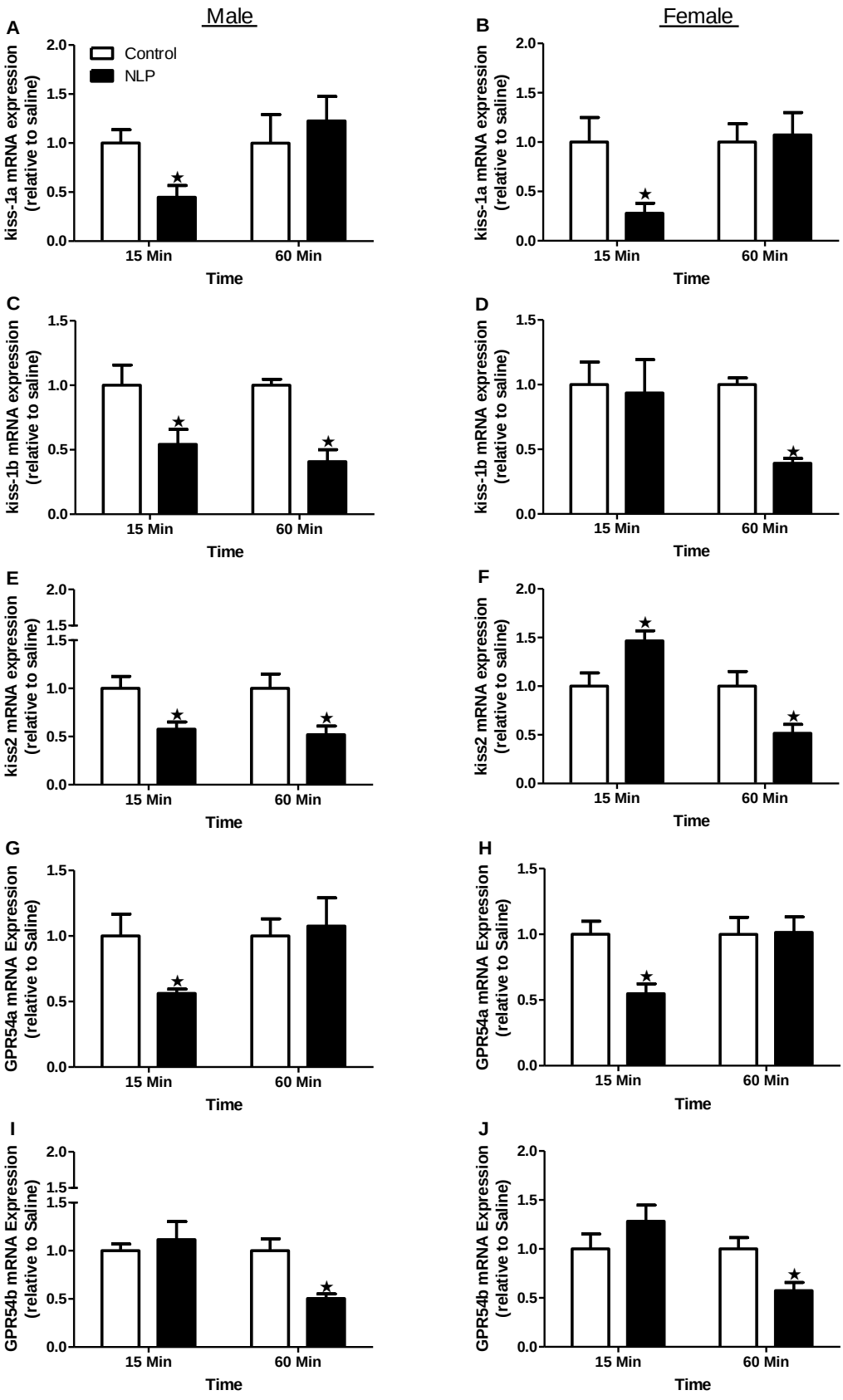

Male
Female
A
B
C
D
E
F
G
H
I
J
Control
NLP
kiss-1a mRNA expression (relative to saline)
kiss-1b mRNA expression (relative to saline)
kiss2 mRNA expression (relative to saline)
GPR54a mRNA Expression (relative to Saline)
GPR54b mRNA Expression (relative to Saline)
15 Min
60 Min
Time

Figure 4.4.4: The expression profile of the kisspeptin system mRNAs in the pituitary of male (left panel) and female (right panel) goldfish post-NLP administration (**A**-**J**). NLP administration significantly downregulated the mRNA expression of all kisspeptin genes in the pituitary of both male and female goldfish (**A**-**J**). Only at 15 minutes time point, kiss2 mRNA expression was significantly upregulated in the pituitary of the female fish (**F**). Data obtained by RT-qPCR are represented as mean + SEM (n=6 fish/group). Student's *t*-test was used for statistical analysis, and the level of significance was set at $p < 0.05$. Asterisks denote significant differences between control and treated groups.

4.4.5. NLP caused sex-specific changes in gonadal mRNAs

NLP administration significantly downregulated LHr mRNA expression in the testis of goldfish 60 minutes after NLP administration (**Figure 4.4.5 A**). But a significant increase in LHr was observed in the ovary of goldfish both at 15 and 60 minutes (**Figure 4.4.5 B**). However, FSHr mRNA expression was significantly downregulated in the testis (at 60 minutes) and ovary (at 15 minutes) of goldfish post-NLP administration (**Figure 4.4.5 C-D**). The GnIH mRNA expression was significantly downregulated in the testis of goldfish at 15 minutes (**Figure 4.4.5 E**). However, in the ovary of goldfish, a significant increase in GnIH mRNA expression at 15 minutes followed by a significant decrease at 60 minutes was observed (**Figure 4.4.5 F**). The GnIHr expression was significantly increased in the testis (at 15 minutes) and ovary (at 60 minutes) post-NLP injection (**Figure 4.4.5 G-H**). The expression of AMH mRNA was significantly upregulated in the testis of goldfish at 15 minutes (**Figure 4.4.5 I**). In the ovary, AMH mRNA expression was downregulated at 15 minutes and upregulated at 60 minutes in NLP injected fish (**Figure 4.4.5 J**).

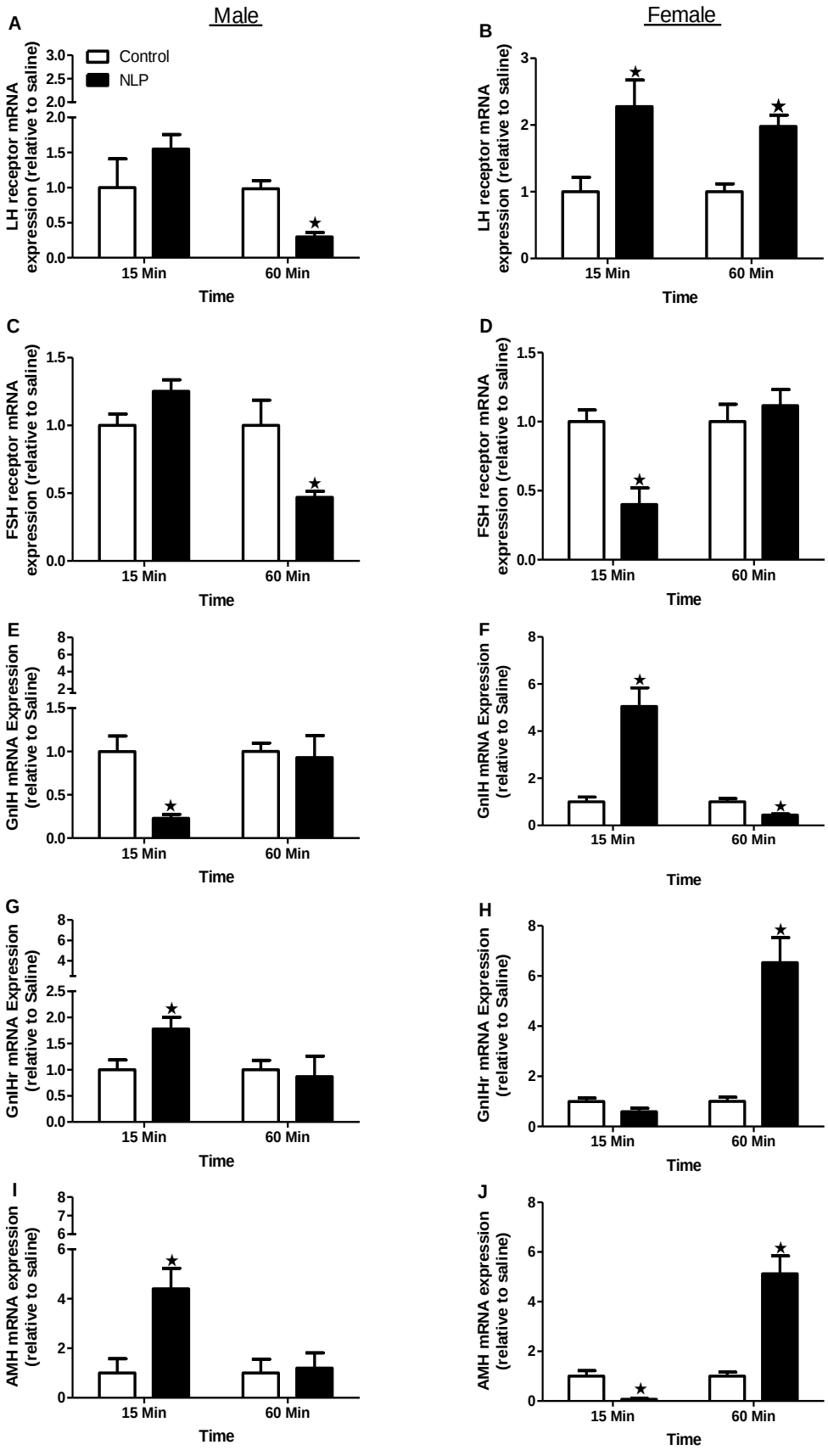

Male
Female
A
B
C
D
E
F
G
H
I
J
Control
NLP
LH receptor mRNA expression (relative to saline)
FSH receptor mRNA expression (relative to saline)
GnIH mRNA Expression (relative to Saline)
GnIHr mRNA Expression (relative to Saline)
AMH mRNA expression (relative to saline)
15 Min
60 Min
Time

Figure 4.4.5: LHr, FSHr, GnIH, GnIHr and AMH mRNA expression in the testis (left panel), and ovary (right panel) of goldfish post-NLP administration (**A**-**J**). Significant downregulation of mRNA transcript of LHr in the testis (**A**) and upregulation in the ovary (**B**) of goldfish was observed post-NLP injection. The FSHr mRNA expression was downregulated in both testis and ovary of goldfish (**C**-**D**). The GnIH mRNA was downregulated at 15 minutes post-NLP injection in the testis (**E**). In the ovary, GnIH mRNA upregulated at 15 minutes followed by a significant downregulation at 60 minutes in NLP injected fish (**F**). The NLP administration upregulates GnIHr mRNA expression in both sex groups (**G**-**H**). The mRNA expression of AMH was upregulated in the testis of goldfish (**I**). In the ovary, AMH mRNA downregulated at 15 minutes followed by a significant increase at 60 minutes (**J**). Data obtained by RT-qPCR are represented as mean + SEM (n=6 fish/group). Student's *t*-test was used for statistical analysis, and the level of significance was set at $p < 0.05$. Asterisks denote significant differences between control and treated groups.

4.4.6. NLP modulated the expression of kisspeptin system mRNAs in the gonads of goldfish

IP injection of NLP downregulated the expression of kiss-1a mRNA in the testis of goldfish at 15 and 60 minutes (**Figure 4.4.6 A**). But in the ovary, kiss-1a mRNA expression was upregulated at 15 minutes post-NLP administration (**Figure 4.4.6 B**). kiss-1b was downregulated in the testis of goldfish at 15 minutes after NLP injection (**Figure 4.4.6 C**). However, in the ovary a significant increase in kiss-1b mRNA expression at 15 minutes and a significant decrease at 60 minutes was observed (**Figure 4.4.6 D**). The kiss2 mRNA expression was downregulated at 15 and 60 minutes in the testis of goldfish (**Figure 4.4.6 E**). In the ovary, NLP injection significantly increased kiss2 mRNA expression at 15 and 60 minutes (**Figure 4.4.6 F**). The expression of GPR54a and GPR54b mRNAs was significantly decreased in the testis of goldfish at both 15 and 60 minutes (**Figure 4.4.6 G** & **I**). However, in the ovary of goldfish, a significant increase in both GPR54a and GPR54b was observed at 15 minutes (**Figure 4.4.6 H** & **J**). But GPR54b mRNA in the ovary was significantly downregulated at 60 minutes in the ovary (**Figure 4.4.6 J**).

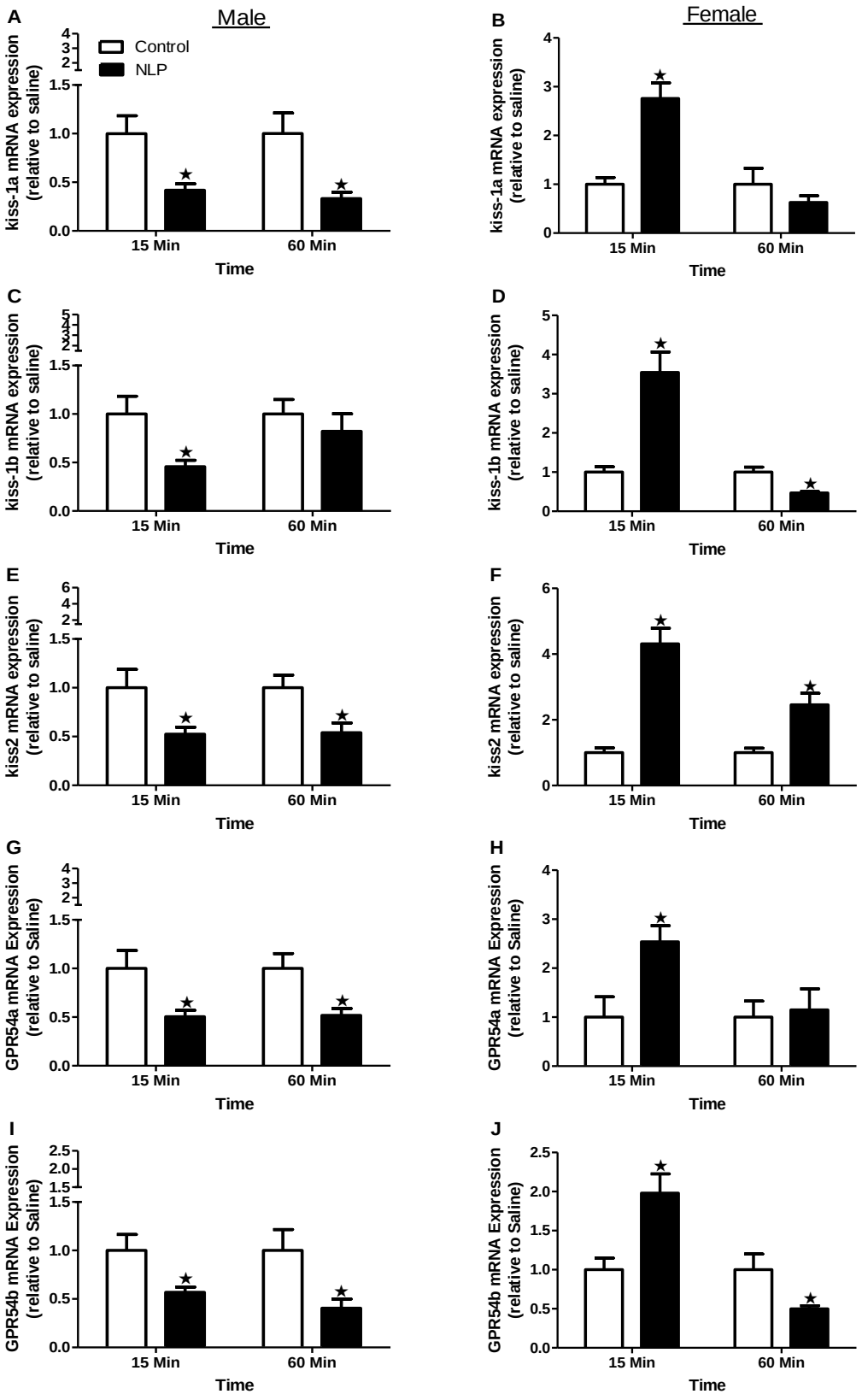

A
Male
Control
NLP
kiss-1a mRNA expression (relative to saline)
B
Female
kiss-1a mRNA expression (relative to saline)
C
kiss-1b mRNA expression (relative to saline)
D
kiss-1b mRNA expression (relative to saline)
E
kiss2 mRNA expression (relative to saline)
F
kiss2 mRNA expression (relative to saline)
G
GPR54a mRNA Expression (relative to Saline)
H
GPR54a mRNA Expression (relative to Saline)
I
GPR54b mRNA Expression (relative to Saline)
J
GPR54b mRNA Expression (relative to Saline)
15 Min
60 Min
Time

Figure 4.4.6: NLP effects on kisspeptin system in the testis (left panel), and ovary (right panel) of goldfish (**A-J**). The mRNA expression of kiss-1a was downregulated in the testis (**A**) and upregulated in the ovary (**B**) of NLP injected fish. In the testis of goldfish, the mRNA expression of kiss-1b (**C**), kiss2 (**E**), GPR54a (**G**) and GPR54b (**I**) were significantly reduced post-NLP administration. In the ovary, the mRNA expression of kiss-1b (**D**) and GPR54b (**J**) were upregulated at 15 minutes followed by downregulated at 60 minutes post-NLP injection. The mRNA expression of kiss2 (both at 15 and 60 min) and GPR54a (at 15 minutes) was upregulated in the ovary of goldfish post-NLP injection (**F** & **H**). Data obtained by RT-qPCR are represented as mean + SEM (n=6 fish/group). Student's *t*-test was used for statistical analysis, and the level of significance was set at $p < 0.05$. Asterisks denote significant differences between control and treated groups.

4.4.7. NLP influenced sex hormone receptors and genes involved in sex steroidogenesis in the gonads of goldfish

StAR mRNA expression was significantly downregulated in the testis (both 15 and 60 minutes) of goldfish post-NLP injection (**Figure 4.4.7 A**). In the ovary, StAR mRNA expression was downregulated at 15 minutes and upregulated at 60 minutes (**Figure 4.4.7 B**). The mRNA expression of CYP11a1 was significantly downregulated in both testis (both at 15 and 60 minutes) and ovary (only at 15 minutes) of goldfish (**Figure 4.4.7 C-D**). CYP19a1a mRNA expression was significantly decreased in the testis (both at 15 and 60 minutes) and ovary (only at 60 minutes) of goldfish injected with NLP (**Figure 4.4.7 E-F**). AR mRNA expression was downregulated in the testis and ovary of goldfish at both 15 and 60 minutes post-NLP administration (**Figure 4.4.7 G-H**). Similarly, ER mRNA was also downregulated in the testis (both 15 and 60 minutes) and ovary (only at 15 minutes) of goldfish injected with NLP (**Figure 4.4.7 I-J**).

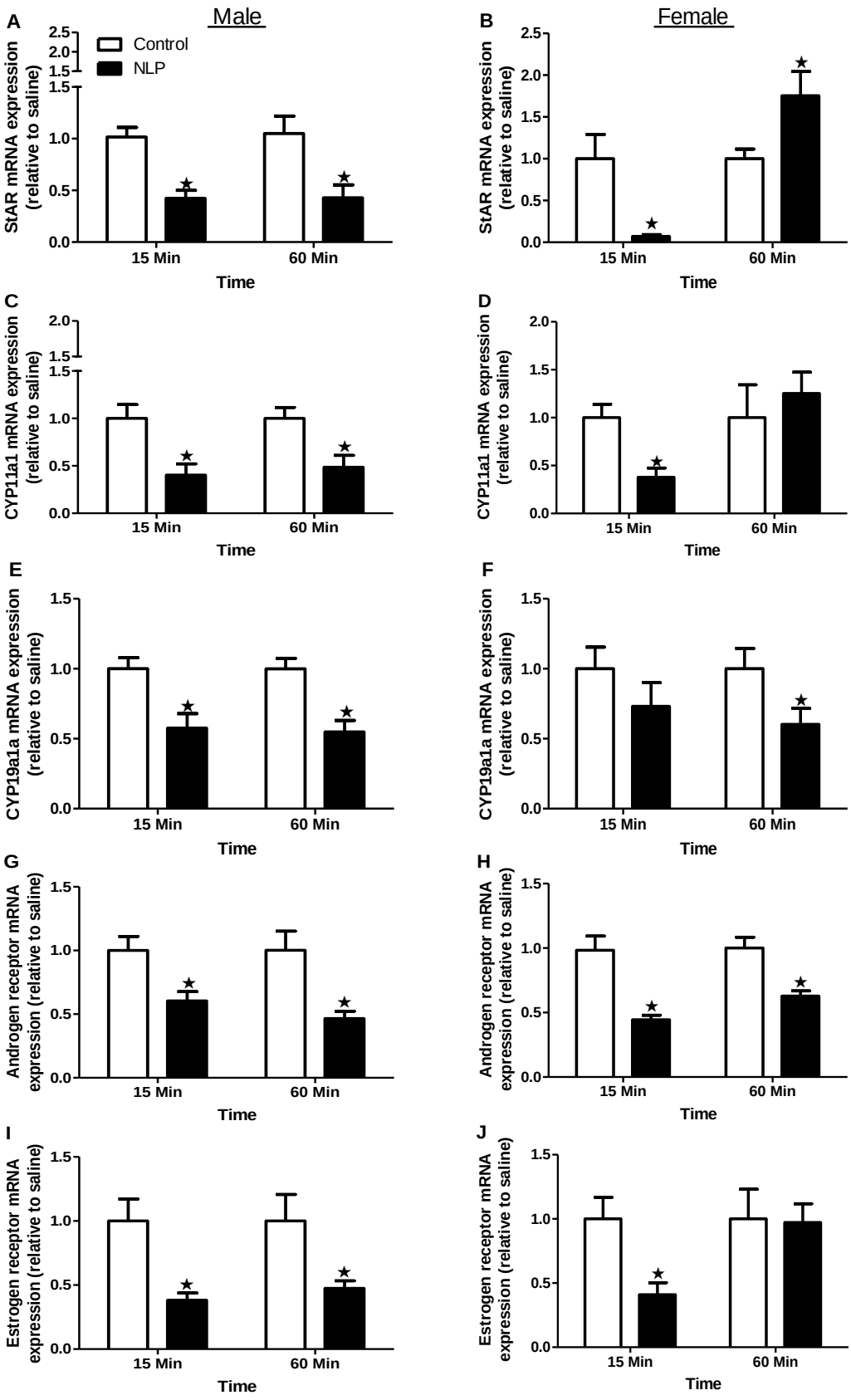

Male
Female
A
B
C
D
E
F
G
H
I
J
Control
NLP
StAR mRNA expression (relative to saline)
CYP11a1 mRNA expression (relative to saline)
CYP19a1a mRNA expression (relative to saline)
Androgen receptor mRNA expression (relative to saline)
Estrogen receptor mRNA expression (relative to saline)
15 Min
60 Min
Time

Figure 4.4.7: The mRNA expression profile of sex steroidogenic genes, AR and ER in the testis (left panel) and ovary (right panel) of goldfish post-NLP administration (**A-J**). Administration of NLP downregulates the expression of StAR mRNA in the testis of goldfish at 15 and 60 minutes time points (**A**). In the ovary, NLP injection downregulates StAR mRNA at 15 minutes followed by an increase at 60 minutes (**B**). The mRNA expression of CYP11a1 (**C-D**), CYP19a1a (**E-F**), AR (**G-H**) and ER (**I-J**) were significantly decreased post-NLP administration in the testis and ovary of goldfish. Data obtained by RT-qPCR are represented as mean + SEM (n=6 fish/group). Student's *t*-test was used for statistical analysis, and the level of significance was set at $p < 0.05$. Asterisks denote significant differences between control and treated groups.

4.4.8. NLP reduced circulating levels of sex steroids in goldfish

A significant decrease in circulating levels of testosterone (T) was observed in male goldfish at 60 minutes after NLP injection (**Figure 4.4.8 A**). A similar decrease in circulating levels of estradiol (E) was observed at 60 minutes of NLP treatment (**Figure 4.4.8 B**).

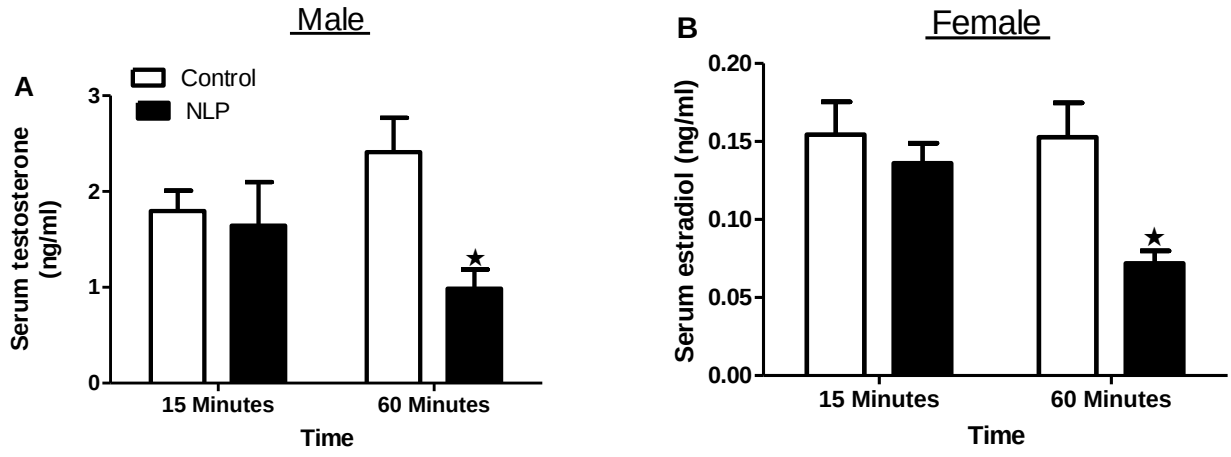


Figure 4.4.8: Serum testosterone and estradiol levels in male and female goldfish post-NLP administration. A significant decrease in serum levels of testosterone was observed in the male fish at 60 minutes post-NLP administration (**A**). In the female fish group, a significant decrease in serum estradiol was observed at 60 minutes post-NLP injection (**B**). Data represented as mean + SEM (n=6 fish/group). Student's *t*-test was used for statistical analysis, and the level of significance was set at $p < 0.05$. Asterisks denote significant differences between control and treated groups.

4.4.9. NLP suppresses oocyte maturation in zebrafish

NLP caused a significant decrease in GVBD in zebrafish follicles, compared to untreated controls (**Figure 4.4.9**). Follicles incubated with MIH (positive control) showed the highest maturation rate compared to both control and NLP treated groups.

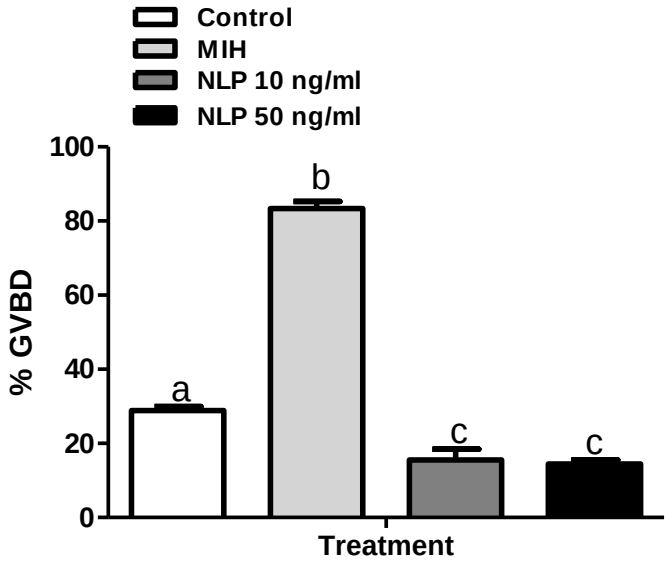


Figure 4.4.9: NLP suppressed GVBD and oocyte maturation in zebrafish. A significant decrease in the maturation of oocytes was observed in ovarian follicles incubated with NLP compared to controls. Follicles incubated with MIH showed the highest maturation rates compared to both control and NLP treated group. Graphs represent pooled data from three independent study (n=6 wells/treatment, 5-7 follicles/well). One-way ANOVA followed by Tukey's multiple comparison test was used for statistical analysis. Different letters (a, b, c) denote statistical differences between groups. $P < 0.05$ was considered statistical significance.

4.5. Discussion

The vertebrate neuroendocrine regulation of reproduction is primarily under the control of the HPG axis. Many non-HPG axis hormones also play important roles in the regulation of reproduction. Among them, metabolic hormones play a major role in vertebrate reproduction (Ahmed et al., 2012; Hatef and Unniappan, 2019; Seli et al., 2014; Shahjahan et al., 2014). Nesfatin-1 is a circulating orphan ligand with hormone-like actions in regulating metabolism and reproduction in mammals and fish (Gao et al., 2016; García-Galiano and Tena-Sempere, 2013; Gonzalez et al., 2012b; Ranjan et al., 2019b; Schalla and Stengel, 2018). Another member of the nucleobindin family is NUCB1, encodes NLP, which is reported to have similar structural and functional properties like nesfatin-1 (Gawli et al., 2017; Sundarrajan et al., 2016). This research addressed whether NLP has any or similar acute effects in the regulation of reproduction in fish. I used goldfish/zebrafish in my studies described here, the same model organisms that were used to characterize NLP in fish (Sundarrajan et al., 2016), and to study the role of nesfatin-1 in fish reproduction (Gonzalez et al., 2012b).

My results indicate that NLP affects all three tissues of the HPG axis. Single IP injection of synthetic NLP significantly reduced the expression of both GnRH types (sGnRH and cGnRH-II) in the hypothalamus of both sexes. This is in agreement with the results of Gonzalez and colleagues that nesfatin-1 suppresses both GnRH types in the hypothalamus of goldfish (Gonzalez et al., 2012b). In their study, single IP injection of nesfatin-1 suppressed both sGnRH and cGnRH-II (~75%) at 15 minutes post-injection. The time points, doses and injection volume were chosen based on my preliminary studies and the previous reports on nesfatin-1 research from our lab (Gonzalez et al., 2012b; Sundarrajan et al., 2016). Single IP injection of NLP suppresses food intake only at 60 minutes time point (not at 2 and 4 hr) in goldfish. It was reported that the half-life of nesfatin-1 is about 20 minutes, and it can cross the blood-brain barrier and reach the brain in less than 10 minutes (Pan et al., 2007; Price et al., 2007). It is possible that NLP exhibits a similar half-life and ability to cross the blood-brain barrier. The current research only addressed the acute effects of NLP, and further studies to determine chronic effects are warranted. Previous reports conducted on goldfish from our lab suggest the same possibility (Sundarrajan et al., 2016). In my study, I observed a similar effect in both sex groups (~50%), which is a comparable effect that suggests a similar influence for the NLP on the hypothalamus to regulate GnRH. While the

study on nesfatin-1 and GnRH did not consider sex, I found that this effect occurs in both males and females. Fish are shown to express multiple GnRH isoforms, and in goldfish, both sGnRH and cGnRH-II are shown to induce gonadotropin secretion from the pituitary (Kim et al., 1995; Peter et al., 1985). Downregulation of GnRH suggests a possible NLP suppression of reproduction in goldfish, mediated via GnRH. Another brain-derived hormone involved in the regulation of reproduction in vertebrates is GnIH, which suppresses the release of gonadotropin from the pituitary (Kriegsfeld et al., 2010; Moussavi et al., 2013; Pineda et al., 2010; Zhang et al., 2010). However, the contrary effects of GnIH was reported in many fish species (Amano et al., 2006; Moussavi et al., 2013). In my study, both GnIH and GnIHr mRNA expression was significantly decreased in both male and female treatment groups except for GnIH in the female fish group at 60 minutes time point. My results suggest that in addition to the GnRH system, NLP influences other major central regulators of reproduction, including kisspeptin and GnIH. I observed a decrease in GnRH and kisspeptin in the goldfish hypothalamus, and both of these promote gonadotropin release and reproduction in vertebrates. Meanwhile, GnIH, a central inhibitor was also found to decreased. This effect is likely due to a feedback effect to balance the negative effect of NLP. This appears to be the case as GnIH and its receptor were found elevated in the pituitary (see discussion below). I also observed a significant decrease in CYP19a1b in both sexes, which suggests additional evidence for the suppressive effects of NLP on goldfish reproduction. CYP19a1b is known to be involved in the regulation of reproduction and sexual behaviour (Amano et al., 2006; Gao et al., 2016; Pineda et al., 2010; Zhang et al., 2010). A reduction in its expression suggests a possible impairment in the reproductive physiology of goldfish. Another important gene system involved in the regulation of reproduction is the kisspeptin system (Gopurappilly et al., 2013; Lee et al., 2009; Rather et al., 2020). In general (with some exceptions), the expression of kiss-1a, kiss-1b, kiss2, and their receptors GPR54a and GPR54b was significantly downregulated in the hypothalamus of NLP injected male and female fish. My results support a negative regulatory role for NLP on the hypothalamic expression of two major endocrine factors (GnRH and kisspeptin system) that regulate reproduction in goldfish.

Next, I studied NLP actions on the pituitary. In the pituitary, two critical reproductive hormone components encoded, LHβ and FSHβ, also known as the gonadotropin β subunits (Pierce and Parsons, 1981). A significant reduction in the expression of both LHβ (~50%

reduction) and FSHβ (~80%) subunits was found in the pituitary of male and female goldfish injected with NLP. Similar to the NLP effects on the GnRH system, a reduction in gonadotropin β mRNA expression was elicited by NLP. This result is also on par with what was reported in the case of nesfatin-1 (Gonzalez et al., 2012b), where nesfatin-1 injection suppressed LHβ and FSHβ mRNA (~80% reduction) expression at 60 minutes post-injection. In contrast to what was found in the hypothalamus, NLP caused a significant increase in GnIH and its receptor mRNA expression in the pituitary of both male and female fish. Here, a suppressor was enhanced by NLP. This points towards the tissue-specific effects of NLP on reproductive hormone expression. Similar to what was found in the hypothalamus, I observed a significant reduction in the mRNA expression of the kisspeptin system (except for kiss2 at 15 minutes time point, in females) in the pituitary of both treatment groups. This is the first extensive study on the effects of nucleobindins /NLP on the gonadotropin, GnIH and kisspeptin system in a sex-specific manner in the pituitary of any species. These effects on the pituitary form another set of evidence on the negative influence of NLP on goldfish reproduction. A limitation is that I did not measure the serum levels of gonadotropins in this research, but hormones downstream (gonadal steroids) were quantified as discussed in the next paragraph.

The third tissue studied was the gonad. In line with my findings on NLP action in the hypothalamus and pituitary, I observed sex-specific regulatory (mainly inhibitory) effects of NLP on gonadal gene expression and reproductive function. Receptors for both LH and FSH were significantly downregulated in NLP treated fish. This is another example of sex- and tissue-specific regulation of gene expression by NLP. GnIH is known to regulate sex steroidogenesis in many vertebrates, including fish (Paullada-Salmerón et al., 2016b; Qi et al., 2013b) through its local expression and paracrine/autocrine action. I observed a significant reduction in the expression of GnIH in the testis of goldfish, but it was found increased in the ovary. This divergent effect in my study suggests that NLP could cause gonadal function by its sex-specific action on GnIH in goldfish. In both sexes, GnIHr expression was upregulated. Previous studies showed sex-specific expression and regulatory pattern of GnIH in the gonads of goldfish (Qi et al., 2013b). The NLP regulation of GnIH in gonads warrants further consideration. Sex-and tissue-specific regulatory effect of NLP on kisspeptin system was observed in the gonads of goldfish. NLP suppresses kisspeptin system in the testis, and this effect was time dependent.

Kisspeptin is a potent inducer of reproduction by stimulating the GnRH-LH/FSH system in vertebrates including fish (Ohga et al., 2017, 2018; Parhar et al., 2004; Tena-Sempere, 2010). Kisspeptin also regulates gonadal physiology directly by its local action in autocrine and paracrine manner. In many fish, it was reported that the local expression of both kisspeptin and its receptors in the gonads were elevated during gonadal maturation, spermiation and vitellogenesis (Felip et al., 2015; Nocillado et al., 2007; Ohga et al., 2018; Saha et al., 2016; Shahi et al., 2017; Tovar Bohórquez et al., 2017). My results point to a suppressive effect for NLP on gonadal development by restricting the local action of kisspeptin system, a potent stimulator of gonadal/gamete development. Future studies should focus on the gonadal regulation by NLP, its hormonal mediators and mechanism of action in fish.

Nesfatin-1 is involved in the regulation of sex steroidogenesis in rats (Gao et al., 2016). My studies revealed that the expression of AMH mRNA, which is a potent inhibitor of sex steroidogenesis in vertebrates (Lambeth et al., 2016; Pfennig et al., 2015; Xu et al., 2019), was upregulated in the gonads of NLP treated goldfish. This suggests a possible influence of NLP on goldfish sex steroidogenesis. To further understand the role of NLP on gonadal steroidogenesis, I looked at the expression pattern of critical genes (StAR, CYP11a1, CYP19a1a) involved in this process, and a significant reduction in its expression was observed, except for StAR at 60 minutes in the ovary. This suggests a possible suppressive effect for NLP on gonadal steroidogenesis. In support of this, I found a reduction in circulating levels of both T and E. This further confirms a more convincing outcome of NLP action on steroid hormone synthesis and secretion, confirming results from the gene expression studies. To study whether NLP has any direct role in gamete development in addition to its suppressive effects on gonadal hormones, I incubated zebrafish follicles with NLP. My results (reduction in GVBD) furnish additional line of evidence for the negative effects of NLP on fish reproduction. The oocyte maturation results (inhibition) are similar to what reported for nesfatin-1 (Gonzalez et al., 2012b).

4.6. Conclusion

Overall, my results indicate that NLP, similar to nesfatin-1, suppresses reproductive hormones in goldfish via acting on the HPG axis and the gonadal sex steroidogenic pathway and inhibits maturation of oocytes. The data support my hypothesis. Both goldfish NLP and nesfatin-1 show 74% amino acid sequence identity (Sundarrajan et al., 2016), and both exhibit an anorexigenic role in goldfish (Gonzalez et al., 2010; Sundarrajan et al., 2016). My results suggest that similar to feeding regulation, the reproductive regulatory roles of both NLP and nesfatin-1 are highly comparable. This suggests both NLP and nesfatin-1 not only share a common structural identity, their regulatory role in the control of metabolism and reproduction is also highly conserved. There is a strong association between reproductive success and energy balance. Both nesfatin-1 and NLP are primarily known as metabolic peptides. It is possible that the nuclobindins and encoded peptides integrate both metabolism and reproduction, which are tightly interlinked. I used fed goldfish or zebrafish for my studies, but it will be of interest to determine what roles these peptides play in energy-deficient states. A limitation of this research is that only mRNA expression was analyzed to check NLP effects on hormones and receptors. However, the measurement of steroid hormones and oocyte maturation provided endpoints/outcomes that are more convincing. Additional studies to obtain a deeper understanding on nucleobindins, nesfatin-1 and NLP on reproduction in male fish, as well as its species-, sex-, tissue-, cell- and ontogeny-specific effects are required. In addition, the identification of NLP receptors and signalling mechanisms that mediate its effects warrant consideration.

Chapter 5
General Discussion

The overarching goal of my thesis research was to gain better understanding on the reproductive and endocrine roles of three recently identified peptides, PNX-20, nesfatin-1 and NLP in teleost fish. Keeping that in mind, I pursued these peptidyl orphan ligands to study their role in the neuroendocrine regulation of fish reproduction. At the onset of my Ph.D. program, the role of PNX-20 and NLP on reproductive functions were not documented in fish. Some information was available on nesfatin-1 regulation of fish reproduction. This chapter will summarize and discuss the overall outcomes of my research and its contributions to the field of neuroendocrine regulation of reproduction in teleosts. This chapter will also discuss the major limitations of my research and will consider some future directions.

5.1. Phoenixin-20 – a novel neuroendocrine regulatory peptide in fish

One of the main objectives of chapter 2 research was to characterize PNX-20 in fish. PNX is a very recently identified peptide with multiple functions in mammals (Yuan et al., 2017). PNX was only characterized in one non-mammalian species, spotted Scat (*S. argus*) (Wang et al., 2018, 2019). In my *in silico* study, it was found that zebrafish PNX-20 and its precursor, SMIM20 exhibit high sequence similarity with other vertebrates. This suggests conservation in their function and the possibility of having similar regulatory responses across species. In addition, qPCR based gene expression analysis revealed a wide tissue distribution for both PNX-20 (SMIM20) and its putative receptor GPR173 (SREB3) in zebrafish central and peripheral tissues. My immunohisto/cytochemical studies identified and localized PNX-20 and SREB-3-like immunoreactivity (ir) in the testis, ovary and in ZFL cells. This further proposes possible multiple regulatory roles for PNX in zebrafish, especially in the regulation of reproduction.

In agreement with this, my *in vivo* IP injection study found that PNX-20 upregulates the expression of sGnRH and cGnRH-II mRNAs in the hypothalamus of male and female zebrafish. Similarly, PNX-20 stimulated the hypothalamic kisspeptin system (kiss1 and kiss2 mRNAs) and CYP19a1b (in female fish only) as well in zebrafish. In the gonads, PNX upregulated LHr (testis) and FSHr (ovary) and the kisspeptin system in zebrafish. Furthermore, PNX promoted the expression of many critical genes involved in sex steroidogenesis, which includes CYP11a1,

CYP17a1, 17β-HSD (only in the testis), CYP19a1a in both male and female zebrafish. I also observed a sex-specific regulation of many critical genes, which includes the AMH (reduced in the testis), DMRT (upregulated in the testis but downregulated in the ovary) and the sex steroid hormone receptors (AR and ER, downregulated in the testis and upregulated in the ovary) by PNX-20. The results provide new information supporting the hypothalamic and gonadal hormone regulation of PNX-20 in fish. These results, although not complete without circulating HPG hormone measurement, very strongly suggest a positive role for PNX-20 on reproduction by stimulating critical neuroendocrine modulators.

Having found some HPG axis effects for PNX-20, the next step of my research was to identify the possible role of PNX-20 on female gametes and processes facilitating oocyte development. *In vitro* studies using ZFL cell line were conducted to determine whether PNX has any role on vitellogenesis in zebrafish. My results suggest that exogenous PNX-20 directly acts on liver cells to upregulate all vtg transcripts (vtg1, 2, 3, 4, 5 and 7). PNX-20 also caused a significant increase in esr2a and 2b and shbg in ZFL cells. Estrogen is critical for liver vitellogenesis (Polzonetti-Magni et al., 2004; Subramoniam, 2017). siRNA mediated gene knockdown of PNX showed a reduction of all vtg transcripts in the ZFL cells further confirming the role of endogenous PNX on vitellogenesis in zebrafish. If these mRNA expression changes are any indication, PNX-20 possibly contributes to vtg availability and thereby induce oocyte production in the zebrafish ovary.

I observed that the incubation of zebrafish ovarian follicles (stage III) with PNX-20 promotes oocyte maturation in treated follicles. Oocyte maturation is a final phase in oocyte development and is under the control of hormones. My results suggest, in addition to the influence of PNX-20 on reproductive hormone and vtg transcripts, it directly modulates gametogenesis (final maturation of oocytes) in zebrafish. Collectively, my results suggest that PNX has an important role in multiple processes involved in zebrafish reproduction (Rajeswari and Unniappan, 2020). My results suggest that the positive reproductive functions of PNX are conserved in mammals and fish. Together, these results (summarized in **Table 5.1**) add PNX-20 as a new entrant into the growing number of neuroendocrine regulators of fish reproduction.

Summary table 5.1: PNX-20 and reproductive functions in fish

Gene	PNX-20	siRNA
Vtg1	↑	↓
Vtg2	↑	↓
Vtg3	↑	↓
Vtg4	↑	↓
Vtg5	↑	↓
Vtg7	↑	↓

Gene	Male	Female
sGnRH	↑	↑
cGnRH2	↑	↑
kiss1	↑	↑
kiss2	↑	↑
GPR54a	↑	↔
CYP19a1b	↔	↑

Liver

Brain

PNX-20

Ovary

Testis

Gene	PNX-20
LHr	↓
FSHr	↑
kiss1	↑
kiss2	↔
GPR54a	↑
STAR	↔
CYP11a1	↑
CYP17A1	↑
17βHSD	↔
CYP19a1a	↑
AHH	↔
DMRT1	↓
AR	↑
ER	↑
Oocyte maturation	↑

Gene	PNX-20
LHr	↑
FSHr	↓
kiss1	↑
kiss2	↑
GPR54a	↑
STAR	↓
CYP11a1	↑
CYP17A1	↑
17βHSD	↑
CYP19a1a	↑
AHH	↓
DMRT1	↑
AR	↓
ER	↓

↑ Upregulated; ↓ Downregulated; ↔ No Change

Limitations and future directions:

Gonadotropins are critical for reproductive success, and the loss of gonadotropins (via knockout) results in reproductive impairments and sterility in many species, including zebrafish (Chu et al., 2014, 2015; Zhang et al., 2015). I was unable to determine whether PNX-20 affects gonadotropin transcripts in zebrafish due to the very small size of the pituitary. Most of my results are at the mRNA level and lacks information on protein and circulating levels. The relatively small size of the animal and the restrictions in the obtainable amount of protein/serum made it difficult to conduct protein measurements. In future, pooling samples from more fish, and using larger fish or repeating these studies in goldfish, a larger cyprinid might help tissue collection for protein and serum hormone measurements. I had used a very high dose of PNX-20 (lower doses were non-effective) for my *in vivo* injection studies. The physiological levels of PNX-20 in zebrafish are currently unknown, and it is possible that the doses found effective here are supraphysiological. The mRNA expression results suggest a high abundance of PNX-20 receptors in the brain compared to other tissues. ICV administration of PNX-20 possibly will induce a stronger response due to this reason, and such approaches must be tested in future. My results did not provide any information regarding the regulatory role of PNX beyond 1-hour time point. Further studies are needed to confirm the long-term effects of PNX in fish reproduction. I used ZFL cells for my studies and it was reported that these cell lines are not the best for estrogen response and vitellogenesis related studies (Eide et al., 2014). However, as it is the sole ZFL cell line currently available, I selected the same for my studies. To further improve my findings, the role of PNX in vitellogenesis should be studied using primary cell lines or via an *in vivo* approach. I did not study PNX-20 and its role in male gametes. Lack of expertise and unavailability of sufficient number of sperm from zebrafish stopped my study/results at the preliminary stage. I could be overcome this by using other species (goldfish for instance) to study the role of PNX in male gamete function in fish. Another major limitation of this study is that it did not provide any information about the role of PNX (if any) in the *in vivo* gamete production and breeding performance of zebrafish. Although I provided evidences for the role of endogenous PNX in fish reproduction, that was limited to its role in vitellogenesis. Further studies are needed to confirm the role of endogenous PNX in fish reproduction and this could be achieved by studying the effects of genetic loss of PNX-20 on fish reproduction. PNX is a

known regulator of behavior in mammals (Haddock et al., 2020; Jiang et al., 2015; Lyu et al., 2018). Both GnRH (both sGnRH and cGnRH-II) and kisspeptin (kiss1) are known regulators of behavioral responses (feeding and mating behavior) in fish (Kitahashi et al., 2009; Ogawa and Parhar, 2018; Volkoff and Peter, 1999). PNX-20 and fish reproductive behavior is another topic of interest for future research. My research (not part of this thesis research) also found a role for PNX-20 in feeding and glucose metabolism (Rajeswari et al., 2020) in fish. It will be interesting to determine whether PNX-20 acts as a link between metabolism and reproduction in fish.

5.2. Nesfatin-1–additional evidence for a suppressive role on fish reproductive axis

A role for nesfatin-1 in reproduction, although limited, was reported in rodents and in fish (Dore et al., 2017). Chapter three of my thesis primarily addresses a paucity in information and provides further evidence for the role of nesfatin-1 in fish reproduction. It is a continuation and expansion of work published earlier from our lab (Gonzalez et al., 2012b). I found that a single IP injection of nesfatin-1 suppresses the hypothalamic GnRH transcripts (sGnRH and cGnRH-II) in male and female goldfish at the 15 minutes time point. In addition, the GPR54a and CYP19a1b transcripts were also downregulated in male and female fish. The gonadotropin beta (LHβ and FSHβ) mRNAs were downregulated at 60 minutes post-nesfatin-1 administration. The mRNA transcript results of the GnRH system in the hypothalamus, and the gonadotropin beta in the pituitary all agree with the previous report from our lab (Gonzalez et al., 2012b). GPR54a was suppressed by nesfatin-1 in the pituitary of both sex groups. Similarly, in the gonads, the gonadotropin receptor mRNAs (LHr and FSHr) were downregulated in both sex groups. Meanwhile, both GnIH and its receptor (GnIHr) were significantly upregulated in both sex groups. GnIH is a potent suppressor of reproductive function in many vertebrates, including teleosts (Osugi et al., 2014; Tsutsui et al., 2018). Unlike what I observed in the hypothalamus and pituitary, the mRNA expression levels of kisspeptin and GPR54a were upregulated in the testis and ovary of goldfish post-nesfatin-1 injection. The role of nesfatin-1 in the sex steroid hormone synthetic pathway is documented in mammals (Gao et al., 2016). I found that nesfatin-1 suppresses the mRNA expression of key enzymes involved in the sex steroidogenesis in fish. These include StAR (only in testis, upregulated in the ovary), CYP11a1, and CYP19a1a. I also found a significant increase in AMH mRNA, which is a potent suppressor of gametogenesis and sex steroidogenesis in many teleosts (Halm et al., 2007; Pala et al., 2008; Rodríguez-Marí et al., 2005). In agreement with this, I observed a significant decrease in the circulating levels of testosterone and estradiol levels in both male and female goldfish groups. This is the first time in any teleost, the influence of nesfatin-1 on the expression and secretion of sex steroid levels were studied. Overall, my results suggests (**Table 5.2**) nesfatin-1 negatively influences reproductive function by suppressing the expression of critical genes (HPG and non-HPG hormones) involved in goldfish reproduction, and sex steroid hormone secretion.

Summary table 5.2: Nesfatin-1 and reproductive functions in fish

Gene	Male	Female
LHβ	↓	↓
FSHβ	↓	↓
kiss-1a	↔	↔
kiss2	↔	↔
GPR54a	↓	↓
GnIH	↔	↔
GnIHr	↔	↔

Gene	Male	Female
sGnRH	↓	↓
cGnRH2	↓	↓
kiss-1a	↔	↔
kiss2	↔	↔
GPR54a	↓	↓
GnIH	↔	↔
GnIHr	↔	↔
CYP19a1b	↓	↓

Gene	Ovary
LHr	↓
FSHr	↓
kiss-1a	↑
kiss2	↔
GPR54a	↑
GnIH	↑
GnIHr	↑
STAR	↑
CYP11a1	↓
CYP19a1a	↓
AHH	↑
AR	↓
ER	↔
Serum estradiol (E)	↓

Gene	Testis
LHr	↓
FSHr	↓
kiss-1a	↑
kiss2	↔
GPR54a	↑
GnIH	↑
GnIHr	↑
STAR	↓
CYP11a1	↓
CYP19a1a	↓
AHH	↑
AR	↑
ER	↔
Serum Testosterone (T)	↓

↑ Up regulated; ↓ Down regulated; ↔ No Change

Limitations and future directions:

This study limited to just two-time points (15 and 60 minutes) and long-term effects of nesfatin-1 on goldfish reproduction need to be studied. I used a dose (50 ng/g BW), which was previously tested and found effective to induce anorexigenic properties, and reproductive hormone suppression in goldfish (Gonzalez et al., 2010, 2012b) via IP administration. However, it was found that the ICV administration of 5 ng/g BW nesfatin-1 is more effective (50% compared to 23% in IP) in suppressing food intake in goldfish (Gonzalez et al., 2010). Similar studies using the ICV route should be used to study the reproductive effects of nesfatin-1. I conducted my studies only during the reproductively active stage of goldfish. The role of nesfatin-1 on reproductive hormone regulation in different sexual stages (regressed, early recrudescence, late recrudescence) in goldfish needs to be studied. There are no studies on the role of nesfatin-1 in male gamete functions (e.g. sperm motility, survival) in fish. Further studies are needed to confirm the effects that I observed here are not transient but can induce long-term effects on fish reproduction. The role of endogenous nesfatin-1 in fish reproduction and the effects of genetic loss of nesfatin-1 in fish reproduction are important areas to be explored and addressed.

5.3. Nesfatin-1-like peptide (NLP) – a new orphan ligand with significant effects on the reproductive endocrine axis and ovarian follicular biology

Similar to nesfatin-1, NLP is identified as a functional peptide in vertebrates (Gawli et al., 2017; Ramesh et al., 2015; Sundarrajan et al., 2016). Research from our lab reported that like nesfatin-1, NLP is also anorexigenic in goldfish (Sundarrajan et al., 2016), which suggests conservation in their function. In chapter four, I explored the role of NLP on fish reproductive axis in sex-specific manner. I used the same dose and time points (50 ng/g BW; 15 and 60 minutes) employed earlier in the nesfatin-1 study (chapter 3) to directly compare the effects of nesfatin-1 and NLP. Single IP injection of gfNLP suppresses sGnRH and cGnRH-II mRNAs in the hypothalamus of goldfish. This was similar to what I observed in case of nesfatin-1 and indicates conservation in their action on metabolism and reproduction. The expression of GnIH and its receptor mRNAs was also suppressed in the hypothalamus of both sex groups (except for GnIH in female fish at 60 minutes, which was increased). The expression of CYP19a1b was downregulated in the hypothalamus of both sexes. Similarly, the expression of kisspeptin system (kiss-1a, kiss-1b, kiss2, GPR54a and GPR54b) was also downregulated (except kiss-1a) in the hypothalamus of goldfish post-NLP injection. In the pituitary, the LHβ (at 60 minutes) and FSHβ (both 15 and 60 minutes) mRNA levels were decreased in NLP-treated fish. This result is also similar to the results observed in case of nesfatin-1 (except for FSHβ suppression only at 60 minutes in case of nesfatin-1). Unlike what I observed in the hypothalamus, both GnIH and GnIHr mRNAs were upregulated in the pituitary of both male and female goldfish. All kisspeptin system genes were downregulated in the pituitary of both sexes post-NLP injection. Overall, NLP has nesfatin-1-like action on the pituitary and hypothalamus.

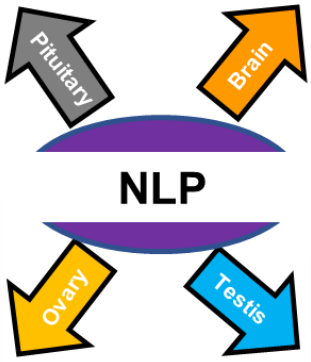
Except in the ovary (LHr upregulated), both LHr and FSHr mRNAs were downregulated in the gonads of NLP injected goldfish. GnIH mRNA was downregulated and GnIHr upregulated in the testis of goldfish. In the ovary, both GnIH (except at 60 minutes, downregulated) and GnIHr were upregulated. Similarly, AMH, a potent steroidogenic and gametogenic suppressor, was upregulated (except at 15 minutes time point in the ovary, where it was downregulated) in the gonads of NLP injected fish. The kisspeptin system shows a sex- and time point-dependent expression pattern in the gonads of goldfish. In the testis of goldfish, all kisspeptin system genes were downregulated. But in the ovary, all kisspeptin genes were upregulated at 15 minutes but

were downregulated (except kiss-1a and GPR54a, no change) at 60 minutes. This is an example of the sex-specific expression of NLP in the regulation of gene expression. Genes involved in the sex steroidogenic pathway (StAR, CYP11a1, CYP19a1a) and steroid hormone receptors (AR and ER) were downregulated (except StAR at 60 minutes, upregulated in the ovary) in the gonads of NLP injected fish. In addition, the serum levels of both testosterone and estradiol were downregulated in NLP injected male and female goldfish at 60 minutes time point. This is direct evidence for the role of NLP on reproductive hormone secretion. To confirm that the negative effects of NLP is not limited to reproductive hormone mRNA expression and secretion, I extended my studies to gamete maturation assay (here we used oocyte maturation assay). Incubation of ovarian follicles with NLP suppressed oocyte maturation in zebrafish. This result is also similar to what reported in the case of nesfatin-1 (Gonzalez et al., 2012b), and provides additional evidence for the conservation in the regulatory effects of both peptides. Collectively, my results (**Table 5.3**) furnish significant new evidences for NLP as a negative influencer of reproductive hormone expression (mRNA transcripts) and secretion (sex steroids), and gamete function (oocyte maturation) in fish.

Summary table 5.3: NLP and reproductive functions in fish

Gene	Male	Female
LHβ	↓	↓
FSHβ	↓	↓
kiss-1a	↓	↓
kiss-1b	↓	↓
kiss2	↓	↑/↓
GPR54a	↓	↓
GPR54b	↓	↓
GnIH	↑	↑
GnIHr	↑	↑

Gene	Male	Female
sGnRH	↓	↓
cGnRH2	↓	↓
kiss-1a	↔	↔
kiss-1b	↔	↓
kiss2	↓	↓
GPR54a	↓	↓
GPR54b	↓	↔
GnIH	↓	↓/↑
GnIHr	↓	↓
CYP19a1b	↓	↓



Gene	Ovary
LHr	↓
FSHr	↓
kiss-1a	↑
kiss-1b	↑/↓
kiss2	↑
GPR54a	↑
GPR54b	↑/↓
GnIH	↑/↓
GnIHr	↑
STAR	↓/↑
CYP11a1	↓
CYP19a1a	↓
AHH	↓/↑
AR	↓
ER	↓
Serum estradiol (E)	↓
Oocyte maturation	↓

Gene	Testis
LHr	↓
FSHr	↓
kiss-1a	↓
kiss-1b	↓
kiss2	↓
GPR54a	↓
GPR54b	↓
GnIH	↓
GnIHr	↑
STAR	↓
CYP11a1	↓
CYP19a1a	↓
AHH	↑
AR	↓
ER	↓
Serum Testosterone (T)	↓

↑ Upregulated; ↓ Downregulated; ↔ No Change; ↑/↓ Time depended increase and decrease

Limitations and future directions:

Similar to chapter 3, under this chapter, I studied the role of NLP in reproductive hormone expression, secretion and gamete function in fish. Even though NLP suppresses the circulating levels of sex steroids, which is an example of the physiological response, all other research were limited to the mRNA expression. No information is available to what extend the mRNA transcript effects observed here are reflected at the protein level. Unavailability of fish-specific antibodies for the Western blot study is a limitation for the lack of this particular set of data. In future, when fish-specific antibodies are available, this should be addressed. While this study is based on research conducted previously in our lab (Gonzalez et al., 2012b) that measured gonadotropin (LH) in fish after nesfatin-1 in injection, I did not measured serum LH in this study. Future research should consider measuring brain (hypothalamus) and pituitary reproductive hormones in response to NLP in fish. Similar to the nesfatin-1 study illustrated in chapter three, this work also lacks any information regarding the role of NLP on male gamete function. To understand the role of NLP in fish reproductive function on a long-term basis, longer duration (this study limited to 15 and 60 minutes time points) and multiple injections or infusion (using osmotic mini-pumps) should be employed in the future. As for the practical application approaches (improving the reproductive outcome), NLP injection and breeding performance studies should be carried out.

5.4. A comparison of reproductive regulatory effects of nesfatin-1 and NLP in fish

As previously detailed (chapter 1), both NLP and nesfatin-1 are very similar in their amino acid sequences, particularly in the bioactive core (Gawli et al., 2017; Gonzalez et al., 2012a; Ramesh et al., 2015; Sundarrajan et al., 2016), which reflects in their similar functional properties as well. For instance, both NLP and nesfatin-1 are anorexigenic in mammals and fish (Gawli et al., 2017; Gonzalez et al., 2010; Oh-I et al., 2006; Sundarrajan et al., 2016). Similarly, here I have observed that, similar to nesfatin-1, NLP also applies a suppressive role on reproduction in fish. Even though there are remarkable similarities in their actions, there are also several differences in their regulatory effects. In the hypothalamus and pituitary of male and female goldfish, both GnIH and GnIHr were unaffected by nesfatin-1, but their expression was downregulated in the hypothalamus, and upregulated in the pituitary of NLP injected fish. Similarly, the effects of nesfatin-1 are limited to kiss-1a and GPR54a in all compartments of the HPG axis. However, NLP downregulates (in general; in a sex and time-dependent manner) all kisspeptin system genes in all compartments of the HPG axis. This suggests that NLP likely has a wider reach than nesfatin-1, and it possibly reflects on the cell-specific expression of receptors that mediate their actions. NLP suppresses the mRNA expression of FSHβ at both 15 and 60 minutes time points compared to nesfatin-1 found to have the same effect only at 60 minutes time point. This is possibly a result of complex functional regulation of NLP in goldfish gonadotropin expression. Noticeably in the gonads of both male and female fish, NLP suppresses both sex steroid receptor (AR and ER) mRNA expression. However, nesfatin-1 shows a sex-specific regulation for AR (upregulated in the testis and suppressed in the ovary) with no changes in ER expression. This is also a notable difference in the regulatory effects of both NLP and nesfatin-1. But in general, both are suppressors of reproduction by influencing many critical reproductive hormone expression, secretion and gamete function in fish. In future, by the application of gene manipulation platforms (gene knockdown and gene knockout), the role of endogenous nesfatin-1 and NLP in fish reproduction should be studied. Fish are known to have compensatory regulatory mechanisms. Gene knockout studies will help to understand if there are any compensatory mechanisms in operation in case of the genetic loss of either of these peptides in fish reproduction. In conclusion, my research placed these two peptides into the growing list

of metabolic regulators of reproductive functions in fish. Both nesfatin-1 and NLP are orphan ligands, and the identification of their receptors and signaling mechanisms on HPG tissues are also important future directions. It is possible that both nesfatin-1 and NLP share same receptor mechanism for their action, which also needs to be studies. In addition, recent reports suggest a possible involvement of PNX and nesfatin-1 in the regulation of multiple functions including anxiety, reproduction, and feeding behavior in mammals (Friedrich et al., 2019, 2020; Pałasz et al., 2015, 2018; Ullah et al., 2017). It is expected to have similar functional relationship in fish as well. My results indicate that PNX-20 is stimulatory, while nesfatin-1 and NLP are suppressors of reproductive hormones and processes studied here **(Table 5.4)**. Both *in vivo* and *in vitro* hormonal combination (PNX-20 vs. nesfatin-1/NLP or both; PNX-20 plus other stimulators of reproductive hormones) studies should be conducted to establish interactions (if any) between these three peptides.

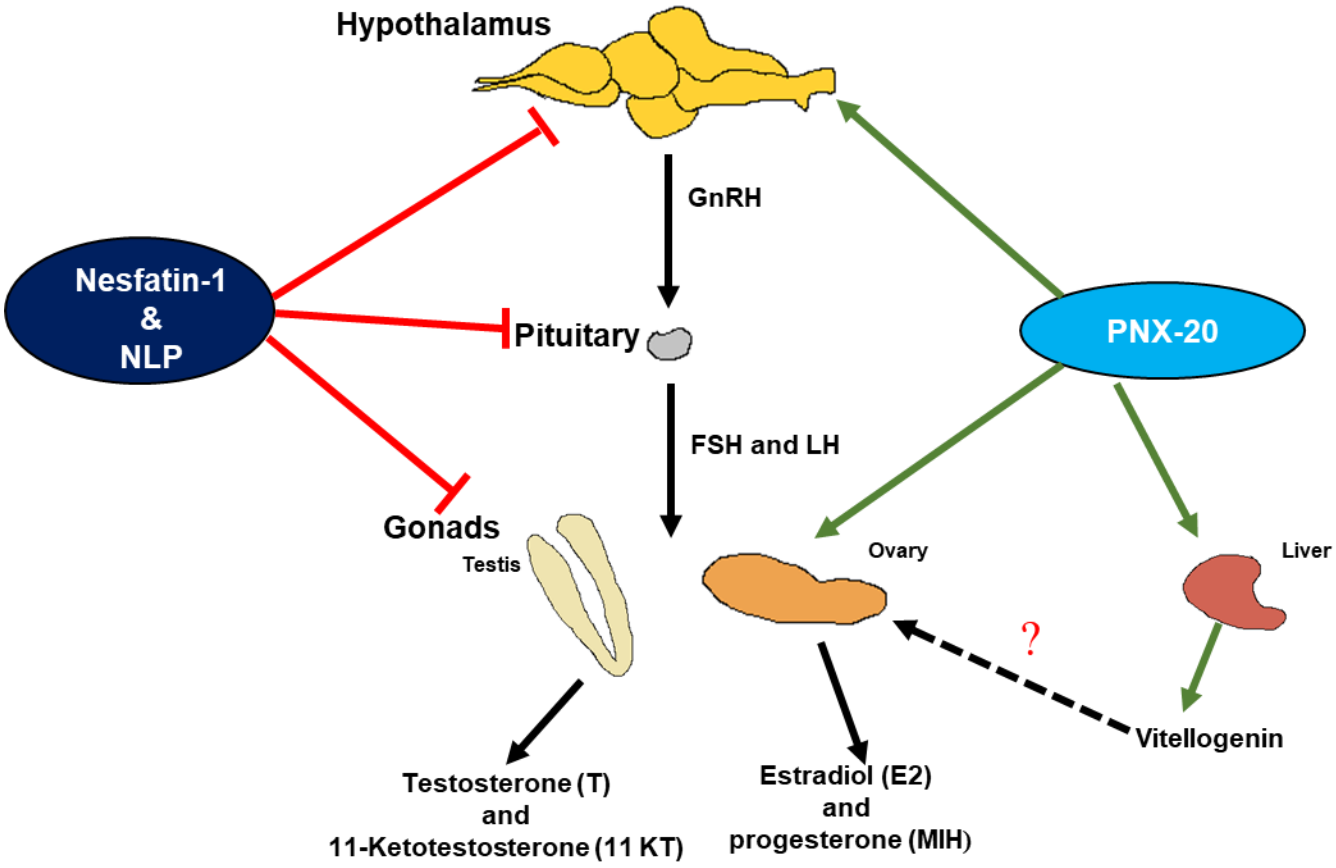
Summary table 5.4: PNX-20, nesfatin-1 and NLP and their role in fish reproduction

Tissues	PNX-20	Nesfatin-1	NLP
Hypothalamus	↑ GnRH, kisspeptin & CYP19a1b	↓ GnRH, kisspeptin & CYP19a1b	↓ GnRH, kisspeptin & CYP19a1b
Pituitary	↔	↓ LHβ, FSHβ & kisspeptin	↓ LHβ, FSHβ, & kisspeptin ↑ GnIH & GnIHr
Testis	↑ LHr, kisspeptin & sex steroidogenic genes	↓ LHr, FSHr, & sex steroidogenic genes ↑ GnIH, AMH & kisspeptin ↓ T levels (serum)	↓ LHr, FSHr, AR, ER & sex steroidogenic genes ↑ GnIHr & AMH ↓ T levels (serum)
Ovary	↑ FSHr, kisspeptin & sex steroidogenic genes ↑ Oocyte maturation	↓ LHr, FSHr, ER & sex steroidogenic genes ↓ E2 levels (serum)	↓ FSHr, AR, ER & sex steroidogenic genes ↑ LHr, kisspeptin, GnIH & AMH ↓ E2 levels (serum) ↓ Oocyte maturation
Liver	↑ Vitellogenin transcripts	↔	↔

↔ Not studied ↓ Downregulated ↑ Upregulated

5.5. Revisiting the Hypothalamo-Pituitary-Gonadal Axis: An update based on my thesis results

The outcomes of this thesis research support my hypotheses and enabled to redraw our knowledge on the HPG axis in fish by including three additional, hormone-like regulators: PNX-20, nesfatin-1, and NLP. Whether these peptides and their precursors are indeed reproductive regulators in other fishes remain unknown. Despite this, the major outcome of this research is that these three relatively new peptides are evolutionarily conserved both structurally and functionally in fish. PNX-20 has positive effects, both nesfatin-1 and NLP are negative regulators of reproductive hormone mRNAs and oocyte maturation. Gonadal steroids in circulation were suppressed by both nesfatin-1 and NLP. These are the only *in vivo* or whole animal hormonal outcome measurements I obtained in this research. Although the hypothalamic and pituitary hormones were not quantified, the changes found in gonadal sex steroids *in vivo* (the end result of hypothalamic and pituitary hormone action), provide very strong evidence for the role of these peptides on HPG axis function. In addition to its role in the HPG axis, phoenixin is possibly involved in vitellogenesis and NLP and PNX-20 influence oocyte maturation in zebrafish. **Figure 5.1** summarizes the revised HPG axis to include the three peptides characterized in my research.



Summary figure 5.1: Overall effects of PNX-20, nesfatin-1 and NLP in the HPG axis functions. Green arow indicates upregulation of reproductive functions and blunt-end red lines represent suppressive effects.

5.6. Significance and implications of my thesis research outcomes

As indicated in the Introduction chapter, there is a growing demand for fish as food. This demand can only be met with enhanced aquaculture practices that could lead to increased yield. Better fish farming approaches, including the improvement of fish reproductive potential, could help to accelerate fish yield. The use of endocrine factors (hormones, bioactive peptides) is an essential way of enhancing yield, especially those that regulate metabolism and reproduction. Only one of the peptides studied (PNX-20) was stimulatory in nature. This peptide could be elevated via long-term administration or genetic approaches (transgenic fish) to enhance reproductive capabilities. Meanwhile, nesfatin-1 and NLP are suppressors of reproductive hormones, and oocyte maturation. Therefore, approaches to suppress these hormones must be employed. These are theoretical possibilities, and the cost and ease of implementing approaches to target these peptides currently remain unknown. In addition, there appears to be species-specificity in the action of many hormones and bioactive peptides. None of the species studied in my research are commercially cultured in aquaculture for human consumption. Therefore, such efforts for future translation of my findings require additional knowledge on the peptides in aquaculture species (e.g. rainbow trout, salmon, sea bass, tilapia).

5.7. Conclusions

The main aim of this research was to understand more about the neuroendocrine regulators of fish reproduction. The results are generally supportive of my hypotheses. All my studies were focused on the neuroendocrine regulation of fish reproduction by covering the HPG axis hormones and receptor system, main non-HPG axis regulators of fish reproduction, sex steroidogenesis and gamete function. By carefully planning and utilizing the advantages of two different model organisms, my research added new knowledge into the neuroendocrine regulators of fish reproduction. This research indicates that PNX-20, nesfatin-1 and NLP are having an important role in the regulation of fish reproduction. My research placed PNX-20 as a positive regulator of fish reproduction, and which also indicates that the reproductive regulation of PNX-20 is conserved across species. Both *in vitro* and *in vivo* experiments indicate that PNX-20 promotes reproduction by influencing the HPG axis functions and other critical players in fish reproduction along with influencing other reproductive processes including gamete maturation, vitellogenesis, and sex steroidogenesis. These results indicate the reproductive effects of PNX-20, nesfatin-1 and NLP are generally the same in both sexes in goldfish, and both peptides elicit their effects in all tissues of the neuroendocrine axis and female gamete biology. As adequate energy reserve is critical to meet the high energy demand of reproductive functions, metabolic regulatory peptides work as molecular switches to maintain energy balance and body functions. I studied *ad libitum* fed fish in this research. What roles these peptides play when the fish are deprived of food or are in chronic starvation will be interesting. The addition of three novel molecules to the growing list of peptides with both reproductive and metabolic functions suggests the need for deeper understanding of genes with unknown functions. Fish lives in a complex environment, and a multiple, redundant milieu of endogenous endocrine factors assist them to survive adversities and maintain energy and reproductive homeostasis. Through my thesis, I include PNX-20, nesfatin-1 and NLP to that list of complex regulators in fish reproduction.

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