APPROVAL SHEET

Title of Dissertation: The Roles of Gonadotropin-releasing Hormone 2 (Gnrh2) in Feeding and Reproduction in Zebrafish: A Potential Mediator of These Interlinked Processes

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ABSTRACT

Title of Document: THE ROLES OF GONADOTROPIN-RELEASING HORMONE 2 (GNRH2) IN FEEDING AND REPRODUCTION IN ZEBRAFISH: A POTENTIAL MEDIATOR OF THESE INTERLINKED PROCESSES

Miranda M. Marvel, Doctor of Philosophy, 2019

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Gonadotropin-releasing hormone (GNRH) is the neuropeptide in vertebrates most well-known for controlling reproduction and stimulating the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), from the pituitary. The gonadotropins circulate to the gonads and stimulate steroidogenesis and gametogenesis. There are three forms of GNRH, named for their location and function in the brain. GNRH1 is known to be the main hypophysiotropic isoform regulating gonadotropin release. GNRH2 is found in the midbrain and, despite being the most evolutionarily conserved and ubiquitous, is the least studied form among the three, most likely due to the absence of GNRH2 protein in common murine research models. Zebrafish, another common vertebrate model for genetic manipulation and biological studies, possesses Gnrh2 and is therefore an ideal organism to study this neuropeptide. Through a combination of gene knockout/knockdown, neuroanatomical imaging, and functional assays, the roles of Gnrh2 in zebrafish were comprehensively explored. Gnrh2 knockout (*gnrh2*$^{-/-}$) zebrafish demonstrated decreased *lhb* expression, compromised oocyte quality, increased feeding

and growth, and, most strikingly, major inhibition of spawning and oocyte maturation after long-term fasting. Neuroanatomical assays confirmed that Gnrh2 projected more neurons to gonadotropes under fasting conditions, whereas Gnrh3 was inhibited. Interestingly, Gnrh2 was unable to compensate for Gnrh3 loss, as double knockout (*gnrh2*$^{-/-}$*;gnrh3*$^{-/-}$) zebrafish displayed normal reproduction. Through transcriptomics and qPCR, novel differentially expressed reproductive factors were discovered in knockout zebrafish, with potential reproductive regulatory roles. Additionally, examination of Gnrh2 neuronal projections identified fibers innervating melatonin cells, neurons of the feeding factor, Agouti-related peptide 1 (Agrp1), and neurons of the reproduction regulator, Gonadotropin-inhibitory hormone (Gnih). Gnrh2 was also able to modulate *gnih* and *agrp1* expression. Additionally, a previously unstudied population of Gnrh2 was identified in the olfactory region and multiple experiments implicated these neurons in transducing pheromonal cues, and thus affecting reproductive behavior. Overall, Gnrh2 most likely has roles in stimulating *lhb* expression, maintaining oocyte quality, transducing pheromonal cues, and reducing feeding behavior through the modulation of *agrp1* and potentially melatonin. Under fasting conditions, Gnrh2 exhibits plasticity to become the main hypophysiotropic stimulator of gonadotropin secretion. Gnrh2 therefore appears to be an important upstream factor mediating feeding and reproductive processes in vertebrates.

# THE ROLES OF GONADOTROPIN-RELEASING HORMONE 2 (GNRH2) IN FEEDING AND REPRODUCTION IN ZEBRAFISH: A POTENTIAL MEDIATOR OF THESE INTERLINKED PROCESSES

By

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# Dedication

This dissertation is dedicated to my grandfather, Robert L. Marvel, who instilled in me a love for the environment and critters of the Chesapeake Bay, which motivated me to pursue a career in environmental sciences, and whom unfortunately passed away before his time in the midst of my graduate studies. I also dedicate this work to my biology teachers and professors, whose passion for science motivated me to continue a path in this field

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# CHAPTER 1: Introduction

## Neuroendocrine Control of Reproduction

### *Overview*

In vertebrates, the control of reproduction begins at the level of the brain, with major reproductive regulators usually located in the hypothalamic region. Hypothalamic neurons send neuropeptides to the pituitary, which then inhibit or stimulate release of the gonadotropins, Luteinizing hormone (LH) and Follicle-stimulating hormone (FSH) from gonadotropes. LH and FSH are then released into the bloodstream, where they circulate to the gonads and stimulate gametogenesis and steroidogenesis which controls gonadal growth, reproductive behaviors, and more. Since the control of reproduction involves many factors acting at the hypothalamus, pituitary, and gonadal level, it is named the hypothalamic-pituitary-gonad (HPG) axis. The main factor at the hypothalamus level regulating gonadotropin release in vertebrates is considered to be the neuropeptide, Gonadotropin-releasing hormone (GNRH), which stimulates gonadotropin release via being secreted into the median eminence, in mammals (Ojeda et al., 2008), or through direct neuronal innervation of gonadotropin cells in teleosts (Zohar et al., 2010). There are currently three main isoforms of GNRH in vertebrates, based on their location and function in the brain, named GNRH1, GNRH2, or Gnrh3. Mammals either only have GNRH1, located in the olfactory bulb and hypothalamus, considered to be the main gonadotropin regulator as it is hypophysiotropic, or GNRH1 and GNRH2 (Roch et al., 2011). GNRH2 has a conserved amino-acid sequence and is located in the midbrain tegmentum of all vertebrates that possess it. GNRH2 is not thought to be a major gonadotropin regulator, since its neurons do not reach the median eminence or pituitary in many vertebrates (Kah et al., 2007), although some species do contain

hypophysiotropic GNRH2 (Guzmán et al., 2009; Johnson et al., 1999). Gnrh3 is a teleost-specific form that is found in neurons of the olfactory bulb terminal nerve (OB-TN), and thought to have neuromodulatory functions (Okuzawa et al., 1990). In mammals, once GNRH neurons are activated during puberty, they exhibit a pulsatile release, in which the frequency of the pulses differentially stimulates LH and FSH release from the pituitary (Kaiser et al., 1997). Gonadal steroids which are stimulated by LH and FSH release circulate back to the brain through the bloodstream and can be stimulatory or inhibitory to GNRH depending on the reproductive state of the animal, in what is described as steroidal feedback (Moenter et al., 2009).

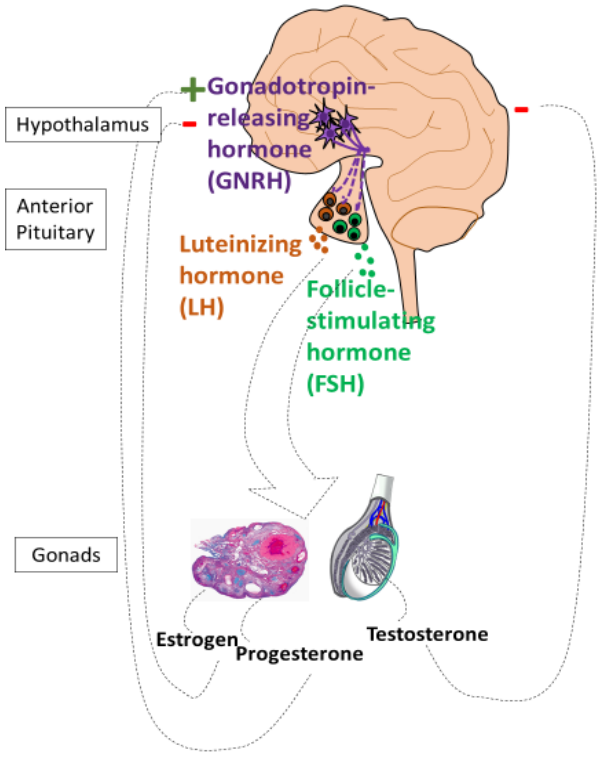


**Figure 1.1.** Graphical depiction of a typical hypothalamic-pituitary-gonad (HPG) axis in mammals. Gonadotropin-releasing hormone (GNRH) neurons in the hypothalamus reach the median eminence and secrete GNRH to the hypophyseal portal system, which reaches gonadotropes in the anterior pituitary and stimulates the production of Luteinizing hormone (LH) and follicle-stimulating hormone (FSH), which circulate to the gonads and

stimulate gametogenesis and steroidogenesis. The steroids then circulate back to the brain and may exhibit stimulatory or inhibitory effects on GNRH production

Since reproduction is a complex process, which is turned on only during sexual maturation (or puberty) and needs to be finely tuned with the time of day or appropriate environmental and social conditions, the hypothalamic GNRH neurons receives internal and external afferent signals. Alternatively, non-hypothalamic GNRH neurons may also be directly affected by photoperiod or environmental cues, although this is less studied and usually not thought to be the case. Some neuroendocrine factors which are known to be able to regulate GNRH activity include KNDy neurons, which contain Kisspeptin, Neurokinin B, and Dynorphin A (Wakabayashi et al., 2012), Gonadotropin-inhibitory hormone (GNIH) (Bentley et al., 2006), and melatonin (Roy and Belsham, 2002). Kisspeptin, NKB, and Dynorphin A are found in the arcuate nucleus and work together as pacemakers of GNRH neuronal pulses. Kisspeptin neurons in the preoptic area (POA) help to control the feedback of steroids (Maeda et al., 2007), puberty (Navarro et al., 2007) and seasonal patterns (Clarke et al., 2009) of GNRH activity. GNIH, also known as RFRP3 in mammals, is a hypothalamic RFamide peptide which has been recently found to have inhibitory effects on gonadotropin secretion in many animals and may control the release of LH and FSH through direct inhibitory actions at the pituitary level, or through inhibiting GNRH neurons in the hypothalamus (Tsutsui et al., 2010). Melatonin is the main hormone produced by the pineal gland, strongly peaking during nighttime hours to control circadian and seasonal processes and has been shown to directly inhibit GNRH expression and release, which may coordinate reproduction to the appropriate photoperiod cycle (Roy and Belsham, 2002). The neuroendocrine control of reproduction is complex and involves many players, but the importance of GNRH as a gonadotropin

regulator is assumed to be conserved amongst all vertebrate groups. However, one of the missing links in reproductive neuroendocrine research is knowledge on the roles of the midbrain GNRH2, which is the most conserved and widespread isoform, suggesting it has important biological roles (Kah et al., 2007).

## Neuroendocrine Control of Feeding

### *Overview*

In fish, as well as mammals, the hypothalamus of the brain is the central hub to coordinate and control feeding behaviors (Volkoff et al. 2004). A number of hormones which stimulate feeding behavior (orexigenic) or inhibit feeding behavior (anorexigenic) are expressed in the hypothalamus, as well as many receptors that integrate feeding signals from the periphery to coordinate feeding with input about body weight, food intake, and photoperiod (Volkoff et al. 2004). Many of the neuropeptides controlling feeding are conserved from mammals to fish. Two potent orexigenic hormones include Neuropeptide Y (NPY) and Agouti-related peptide (AGRP), which are co-expressed in some neurons in the hypothalamus, with other populations expressing solely NPY (Hahn et al., 1998, Kalra et al., 1999). NPY and AGRP are upregulated after fasting, and work differentially to regulate the anorexigenic proopiomelanocortin (POMC) system (Hahn et al., 1998). POMC is a precursor to the Alpha-melanocyte stimulating hormone (α-MSH), an anorexigenic hormone which acts through the MC3R and MC4R receptors (Biebermann et al., 2012). AGRP is an antagonist of α-MSH through binding to and inhibiting MC3R and MC4R (Bagnol et al., 1999) to exert long-lasting effects on food intake (Hagan et al., 2001), and is very sensitive to changes in feeding conditions in both mammals and fish (Song et al. 2003). A receptor for NPY, Y1R, is expressed on POMC neurons and can be inhibited via the binding of NPY (Broberger et al. 1997). Other

neuropeptides which can stimulate food intake in the hypothalamus include Orexin/Hypocretin (Magni et al., 2009, Volkoff 2016), Galanin (Magni et al., 2009, Volkoff 2016), and Melanin-concentrating hormone (MCH) in fish (in mammals it is thought to be an anorexigen (Presse et al., 1996)). Anorexigenic hormones, other than POMC/α-MSH, include Cocaine- and amphetamine-regulated transcript (CART), Corticotropin-releasing hormone (CRH), and Cholecystokinin (CCK) (Valassi et al. 2008, Magni et al., 2009). Many of these hormones are regulated via circulating peripheral signals of food intake, such as leptin, an adipocyte-produced hormone which can modulate POMC and NPY/AGRP expression (Bouret et al., 2004), insulin (Oomura and Kita, 1981), and ghrelin, a hormone produced both in the stomach and hypothalamus (Horvath et al., 2001).

***Coordination between systems***

The control of reproduction in all organisms needs to synchronized with environmental conditions that promote maximum survival of the progeny. In animals in which nutritionally rich environments are necessary to promote energy-consuming gonadal development, maintenance, and parental care, reproductive parameters are usually inhibited severely by short-term starvation and factors indicating a nutritionally poor state, such as in murine models (McClure, 1959, Bronson and Marstellar, 1985). In other animals, such as salmonids and mollusks, eggs are fertilized and laid after long periods of fasting, requiring an active HPG axis even in the absence of food (Miller et al., 2009). The crosstalk between feeding and reproductive systems will most likely vary widely between species based on the varying foraging, reproductive, and parental care behaviors they exhibit and environment they live in.

The mediators that allow coordination between feeding systems and reproduction are still widely unknown. Nevertheless, many feeding factors in the brain have been implicated in influencing certain levels of the reproductive HPG axis, and a few reproductive factors can also mediate feeding behaviors. For instance, Leptin, an adipocyte hormone and indicator of energy stores (Maffei et al., 1995; Swoap, 2008), can regulate gonadotropin secretion through both indirectly modulating GNRH neuronal secretion (Gamba and Pralong, 2006), and enhancing sensitivity of the gonadotrope cells to GNRH activation (Avelino-Cruz et al. 2009). There seems to be a similar role for Leptin in teleosts, with Leptin administration upregulating Gnrh in pike perch (*Sander lucioperca*) and zebrafish (*Danio rerio*) (Schaefer and Wuertz, 2016; Tuttle, 2017), suggesting a potential conserved mechanism for the stimulation of Gnrh and the HPG axis only after the appropriate growth and adipocyte levels seen in maturing animals. Another example of a nutritional signal interacting with the HPG axis is glucose, which normally increases shortly after food intake. In the teleost medaka (*Oryzias latipes*), low glucose levels have been shown to inhibit the neuronal activity of Gnrh1 in females; thus ensuring high reproductive activity only when nutrition and glucose levels are high.

On the other hand, some orexigenic factors, which stimulate feeding and are upregulated in fasted states, can inhibit the reproductive axis at the brain and pituitary level. Neuropeptide Y is a conserved orexigen found in mammals and teleosts, which is upregulated during fasting and can inhibit Gnrh neuronal activity. In rats, one of the receptors for NPY, Y5R, is expressed in GNRH neurons and the binding of NPY to Y5R suppresses GNRH neuronal activity (Xu et al. 2009). Withholding food led to suppression of LH levels in wild-type mice but not NPY knockout mice, determining that

NPY is a key feeding signal which inhibits LH under fasting conditions (Hill and Levine, 2003). Additionally, Kisspeptins may be a mediating factor that respond to feeding signals and convey these signals to GNRH neurons. KISS neurons in rats were shown to decrease in response to fasting, and Leptin receptors have also been found on KISS neurons, suggesting it may also respond to high nutritional states as well (Hill et al., 2008). Although the effects of circulating and central feeding signals on reproductive neuronal activity are relatively well known, the effect of reproductive factors on feeding neuropeptides in the brain have rarely been studied.

## Gonadotropin-releasing hormone family

### *Discovery*

The gonadotropin-releasing hormones (GNRHs) are a family of tropic neuropeptides found in the brains of all vertebrates, and some invertebrates, and have long been known to be an upstream regulator of reproduction, through stimulating the synthesis and secretion of the main reproductive circulatory hormones, the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH). GNRH was first discovered in mammals (Matsuo et al. 1971) and initially called LH-RH (luteinizing hormone releasing hormone) when it was determined to be able to induce the release of LH. It was later determined that it is able to induce the release of FSH as well, and later renamed gonadotropin-releasing hormone (GNRH).

### *Distribution and variance amongst species*

GNRH is conserved in all jawed vertebrates, along with also being present in the primitive jawless fish, the sea lamprey, and the invertebrate tunicates. The bioactive

peptide in the GNRH is a neurodecapeptide (consisting of 10 amino acids), with the conservation of the first four amino acids (pyro-glutamate at the N-terminus, histidine, tryptophan, and serine) and the last two amino acids (proline and amidated glycine at the C-terminus) being almost universal, with amino acids five through eight varying amongst species (King and Millar 1992, Sower et al. 1993, Powell et al. 1994, Montero and Dufour 1996). GNRH-like peptides also exist in other invertebrate groups, such as mollusks and annelids, with the conservation of the pyro-glutamate and some varying similarities within the other amino acids, suggesting an ancient evolutionary origin of GNRH. GNRH exists as a prohormone before cleavage. Upstream of the decapeptide GNRH gene is a signal peptide sequence, and downstream is the gene sequence for the GNRH-associated peptide (GAP). The signal peptide and GAP sequence varies significantly between the species, and the functions of the GAP are still largely unknown and understudied. However, the presence and conservation of GNRH in all vertebrates, and some invertebrates, suggests an important evolutionary and biological role.

***Multiplicity of Isoforms***

There are three main isoforms of GNRH found in vertebrates, with the number of isoforms varying between one (rodents) to three (in many teleosts). The nomenclature of the GNRH isoforms depends on the amino acid sequence and location of GNRH in the brain, with the hypothalamic preoptic GNRH deemed GNRH1, the midbrain form of GNRH deemed GNRH2, and the teleost-specific olfactory bulb/terminal nerve GNRH deemed Gnrh3. The amino-acid sequence of GNRH2 and Gnrh3 is invariant; however, the amino acid sequence of GNRH1 is species-specific, with varying residues at positions five through eight of the decapeptide. GNRH1 is the form of GNRH which reaches the

pituitary and is the main stimulator for the synthesis/secretion of the gonadotropin hormones. Most mammals possess a GNRH1 and GNRH2 isoform; however, some mammals have protein-disrupting amino-acid replacements or partial gene deletions which have inactivated this hormone, such as is the case in rats, mice, pika, rabbits, orangutans, lemurs, sheep, and cows, which only possess the GNRH1 isoform. The existence of three distinct GNRH isoforms in an organism was first discovered in sea bream (*Sparus aurata*) by Powell et al. in 1994 (Powell et al. 1994), and later found in other teleost species (mostly perciforms), such as the Pacific herring (*Clupea pallasii*) (Carolsfeld et al., 2000), European sea bass (*Dicentrarchus labrax*) (Rodríguez et al., 2000), and medaka (Oka, 2009). The additional GNRH isoform is most likely due to a teleost-specific unique gene duplication event, after the divergence of teleosts from tetrapods, which may have led to two additional GNRH-coding genes, followed by a loss of one of these genes over time (Okubo and Nagahama, 2008). In other fish species, as is the case in cyprinids (Steven et al., 2003) and most salmonids (Adams et al., 2002), Gnrh1 has been lost and Gnrh3 is found in similar locations as Gnrh1 and is thought to function as the major hypophysiotropic isoform. In other fishes, such as silurids (Schultz et al., 1993) and eels (Okubo and Nagahama, 2008), Gnrh3 has been lost and only the species-specific Gnrh1 isoform exists alongside the conserved Gnrh2 isoform. The importance of multiple isoforms in vertebrate brains is still unclear. The distinct locations of the different isoforms in vertebrate brains suggest they may also have distinct and separate roles.

### *Roles of Gnrh1 in mammals*

GNRH1 in mammals is well-classified as a major upstream stimulator of reproduction, through binding to Gnrh receptors on gonadotropin cells and stimulating the synthesis and secretion of luteinizing hormone, and follicle-stimulating hormone (Fink, 1988). These circulatory gonadotropins then act on a number of different tissues, but mainly target the gonads to induce gametogenesis, the development and maturation of gamete cells, and steroidogenesis from follicle cells. In most species, GNRH1 will increase in conjunction with sexual maturity, and trigger the development of the gonads through the additional stimulation and release of the gonadotropins. In mammals, GNRH is released in a pulsatile manner in the median eminence of the hypothalamus (Marshall and Kelch, 1986). From there, it travels through the hypophyseal portal system where it then reaches the anterior pituitary where the gonadotropes reside (Fink, 2012). The rate of GNRH pulsatility differentially regulates gonadotropin release, where slow GNRH pulses induces FSH release and folliculogenesis during the mammalian menstrual cycle, and fast rates of GNRH pulses induce the sudden spike in LH release (termed the LH surge) which is essential for ovulation (Ferris and Shupnik, 2006). A number of upstream factors are thought to transduce environmental cues in order to control the expression of GNRH, and activate the upregulation only at appropriate times during the age, or reproductive season, of the animal. Some of these factors include Kisspeptins (KISS) (Smith et al., 2011), Neurokinin B (NKB) (Ferris and Shupnik, 2006), Gonadotropin-inhibitory hormone (GNIH) (Tsutsui et al., 2010), and melatonin (Roy and Belsham, 2002). For instance, KISS neuronal innervation around Gnrh neuronal soma increases just prior to puberty, and its signaling is necessary to stimulate GNRH

activation during this period (Clarkson et al., 2010). The importance of GNRH in mammalian reproduction is exemplified by the fact that mutations in the GNRH1 (Bouligand et al., 2009; Mason et al. 1986), GNRH receptors (Roux et al. 1997), or upstream factors such as KISS (D'Anglemont de Tassigny et al., 2007) and TAC3 (Topaloglu et al., 2009) genes result in hypogonadotropic hypogonadism, a syndrome which is characterized by substantially reduced gonadotropin secretion, inhibited pubertal maturation, and infertility (Fraiella et al., 2013). The detrimental reproductive abnormalities, can be ameliorated through the pulsatile administration of GNRH, or gene therapy introducing a functional GNRH1 gene (Skarin et al. 1982, Mason et al. 1986, Fraiella et al. 2013).

***Roles of Gnrh1 in teleosts***

The roles of Gnrh1 in teleost seem to be homologous to those in mammals, as it is the main hypophysiotropic isoform and a potent stimulator of gonadotropins from the pituitary. Although in some species it is expressed in close proximity to the third Gnrh isoform, sGnrh or Gnrh3, the neuronal soma exist in the preoptic hypothalamus of teleost and normally exhibit the most abundant presence in the pituitary. In four different fish species which express three Gnrh isoforms (red seabream, *Pagrus major*; black seabream, *Acanthopagrus schlegeli*; striped knifejaw, *Oplegnathus fasciatus*; and Nile tilapia, *Oreochromis niloticus*), the Gnrh1 isoform was found in the pituitary at a 500 to 2,400-fold higher concentration than the other two Gnrh isoforms (Holland et al., 1998; Senthilkumaran et al., 1999). In dwarf gourami (*Trichogaster lalius*) and Nile tilapia, retrograde labeling of neurons from the pituitary show that only the Gnrh1 preoptic neurons project to the pituitary, unlike the midbrain and olfactory Gnrh populations

(Yamamoto et al., 1998). The expression of Gnrh1 also changes correspondingly with the spawning season. In male European sea bass (*Dicentrarchus labrax*), the protein content of Gnrh1, but not Gnrh2 or Gnrh3, in the pituitary increased during the spawning period (Rodríguez et al., 2000). Additionally, in red seabream (*Pagrus major*), Gnrh1 expression peaks just prior to the spawning period and is the only Gnrh isoform which increases its protein secretion to the pituitary (Okuzawa et al., 2003). In daily spawners, such as medaka, *lhb* expression increases subsequent to increases in *gnrh1*, and Gnrh1 has been shown to directly affect *lhb* expression from pituitary cultures (Karigo et al. 2012). The location of Gnrh1 neurons in the POA and pituitary, two areas associated with reproduction, suggest the neurons may regulate reproduction. Additionally, the Gnrh1 expression changes during the spawning period which correlate with important reproductive events, and the ability of Gnrh1 to stimulate gonadotropin expression and secretion, demonstrate that it is an important hypophysiotropic regulator of reproduction in teleosts.

***Roles of Gnrh3 in teleosts***

The role of Gnrh3, previously termed sGnrh for salmon Gnrh, as it was first discovered in salmon brain extracts (Sherwood et al. 1983), seem to vary depending on whether it is expressed in fish with two or three Gnrh isoforms. In fish with two Gnrh isoforms, Gnrh3 is expressed both in the olfactory region, as well as in the preoptic hypothalamus, with projections abundantly reaching the anterior pituitary. In these fish, it is considered a major hypophysiotropic hormone stimulating gonadotropin release, similar to the roles of Gnrh1 in mammals and other teleosts (Amano et al., 1995; Kobayashi et al., 1997; Peter and Yu, 1997, Somoza et al. 2002). However, the recent

surprising results that a Gnrh3 knockout zebrafish line (*gnrh3*$^{-/-}$) resulted in no compromised fertility, suggests that either Gnrh3 is not essential for reproduction and gonadotropin release in this species, or that there are redundant reproductive regulators which are upregulated after the genetic loss to compensate for its absence (Spicer et al. 2016, Liu et al. 2017, Marvel et al. 2018). Previous studies show that Gnrh3 neuronal ablation results in impeded ovarian development and infertility, suggesting that it is still a reproductively important peptide (Abraham et al. 2010). Studies in other teleost species which contain only two Gnrh isoforms need to be conducted to solidify the role of Gnrh3 in teleosts. Gnrh3 may also play a role in the transduction of pheromonal olfactory cues in fish with two Gnrh isoforms, such as zebrafish, as well, since the addition of olfactory cues stimulates the activation of these neurons, and subsequent increases in male reproductive behaviors was recorded in this species (Li et al. 2017). A recent study showed the involvement of Gnrh3 in sensing $CO_2$ signals and eliciting slow avoidance response to these molecules (Koide et al. 2018). In fish which express three Gnrh isoforms, Gnrh3 is localized only in the terminal nerve regions of the olfactory bulb and expressed throughout the telencephalon, but is not thought to have a major hypophysiotropic role, due to the low presence of Gnrh3 in the pituitary of these fish. Instead, Gnrh3 may have more of a neuromodulatory role in transducing olfactory and visual cues to modulate reproductive behaviors (Soga et al. 2006). In tilapia (*Oreochromis niloticus*), Gnrh3 only extends throughout the brain, and does not reach the pituitary (Soga et al. 2006), but neutralization of the protein through administration of Gnrh3 antisera resulted in inhibited reproductive behaviors in males, such as nest-building and aggression, pointing to an important role in modulating reproductive

behavior (Ogawa et al. 2006). In medaka, Gnrh3 neurons in the terminal nerve help to facilitate visual mate familiarization, which is necessary for female receptivity to male mating behaviors (Okuyama et al. 2014). The roles of Gnrh3 in fish appear to be important for maintaining normal reproductive behaviors and development, however, exhibit large species-specific differences.

## Current knowledge about the roles of Gnrh2

### *Discovery and evolution*

GNRH2 was first discovered in the midbrain tegmentum of chickens (Miyamoto, 1982), and was thus first called chicken Gnrh-II (cGnrh-II). With the discoveries of the presence of GNRH2 in mammals, other birds, and teleosts, chicken Gnrh-II was renamed GNRH2, and the other isoforms named after their location in the brain (Miyamoto et al., 2007). Unlike GNRH1, GNRH2 has a completely conserved amino-acid sequence, suggesting an important evolutionary and biological role. However, due to partial gene deletions, premature stop codon insertions, or amino acid alterations, GNRH2 has been lost in some mammalian species such as rats, mice, pika, rabbits, orangutans, lemurs, sheep, and cows, as stated above (Desaulniers et al., 2017). In all teleost species studied so far, GNRH2 is present and functional (Long, 2009; Amano et al., 1991; Lin and Peter, 1997; Steven et al., 2003), suggesting it may play a more important role in fish.

GNRH2 is thought to have evolved over 500 million years ago and may be the most ancestral form compared to the other isoforms, as GNRH1 is thought to have evolved only 350 million years ago. In support of this theory, GNRH2 has the highest homology with the ancient hormones in insects which are thought to have once formed a

superfamily with GNRH and include a common ancestor, including the corazonins (Crz) in insects and crustaceans and adipokinetic hormones (Akh) in insects, crustaceans, nematodes, and molluscs (Roch et al., 2011; Zandawala et al., 2018).

***Roles in reproduction***

GNRH2 has an abundant distribution of axonal fibers throughout the brain, suggesting a multitude of roles in different processes (Xia et al., 2014). Despite the fact that in many animals, GNRH2 fibers are not seen in the pituitary, GNRH2 does seem to have reproductive roles, including the control of female reproductive behaviors in birds, mammals, and fish. In female sparrows, administrations of GNRH2, but not GNRH1, increased female courtship solicitations (Maney et al., 2007). In female goldfish, injections of Gnrh2 and Gnrh3 stimulated increased female spawning acts (Volkoff and Peter, 1999). In mammals, both female musk shrews (Temple et al. 2003) and marmoset monkeys (Barnett et al. 2006) exhibited increased reproductive behaviors and receptivity after administrations of GNRH2. The presence of GNRH2 in the pituitary is extremely variable among vertebrates but seems to be more prevalent among teleosts. In some fish groups which contain two Gnrh isoforms, such as silurids (Schultz et al. 1993) and cyprinids (Lu et al. 1988, Steven et al. 2003), Gnrh2 is seen in the pituitary, whereas in others, such as salmonids, Gnrh2 is not found (Amano et al. 1991). The abundance of Gnrh2 in the pituitary compared to the other isoforms is also very variable depending on the species. In Senegalese sole (*Solea senegalensis*), a teleost with three Gnrh isoforms, Gnrh2 is found at the highest abundance in the pituitary (Guzmán et al. 2009); however, in other teleost species, such as chub mackerels (*Scomber japonicus*) (Selvaraj et al. 2009) and cichlids such as *Astatotilapia burtoni* (Powell et al. 1995), pituitary Gnrh2 is

not present. Gnrh2 can sometimes elicit gonadotropin release through activating the receptor at a much higher potency than the other isoforms, as in the cases of striped bass (*Morone saxatilis*) (Deoraj, 2000), gilthead sea bream (*Sparus aurata*) (Zohar et al. 1995), goldfish (*Carassius auratus*) (Johnson et al., 1999), and African catfish (*Clarias gariepinus*) (Schulz et al., 1993). In other mammals, such as monkeys (Okada et al. 2003), GNRH2 can bind to gonadotropin GNRH receptors, but activates them with less potency than GNRH1. This suggests that GNRH2 may have a hypophysiotropic role in some animals and has the ability to stimulate gonadotropin release. The extent and endogenous nature of gonadotropin stimulation by GNRH2 is not well characterized or documented, however, and the complete scope of the reproductive roles of GNRH2 still need to be studied.

***Roles in feeding regulation***

Out of the three vertebrate GNRH isoforms, GNRH2 appears to be the only isoform implicated in feeding behavior regulation. As GNRH2 administrations to most animals appears to increase female spawning behaviors, it also seems to have an almost universal role in decreasing feeding behaviors. Intracerebroventricular (ICV) injections to both zebrafish and goldfish significantly decreased food intake (Nishiguchi et al 2012, Matsuda et al 2008, Kang et al 2011). The same effect can be seen in some mammalian species as well, as central administrations of GNRH2, but not GNRH1, restricted food intake in musk shrews (*Suncus murinus*) (Kauffman and Rissman, 2003). Surprisingly, the same administrations of GNRH2 to mice, which do not endogenously produce a functional GNRH2 protein, had the same effect of decreasing food intake, suggesting a functional receptor for GNRH2 still exists in mice, with the same biological effects

(Kauffman and Rissman, 2004b). So far, almost all of the studies on the functions of Gnrh2 in feeding regulation examined the effects of additional GNRH2 administration, which may be higher than endogenous levels, and subsequent behavioral studies. Studies on the molecular mechanisms of the role of GNRH2 in controlling feeding behavior are scarce. Recent studies have shown preliminary evidence that Gnrh2 and the type 1 Gnrh receptor are downstream mediators of the anorexigenic hormones α-Msh and Crh (Kang et al. 2011). There also appears to be a mutually antagonistic relationship between Gnrh2 and Hypocretin in goldfish, where Gnrh2 injections can increase spawning behaviors and decrease *hypocretin* mRNA, whereas Hypocretin injections inhibit spawning and decrease *gnrh2* mRNA (Hoskins et al., 2008). Additionally, in pike perch (*Sander lucioperca*) and zebrafish, exogenous administrations of leptin can upregulate *gnrh2* expression, but not *gnrh1*, specifically linking this peptide to nutritional signals (Schaefer and Wuertz, 2016; Tuttle, 2017). Interestingly, the insect adipokinetic hormones, which are thought to have evolved from a common ancestor with GNRH2, and have a 4-5 amino acid sequence similarity, are responsible for lipid and carbohydrate mobilization during high-energy activities, and therefore have major roles in regulating energy homeostasis (Roch et al., 2011). As Gnrh2 is thought to be the most ancestral Gnrh, and has an ubiquitously conserved amino acid sequence, it may have preserved its ancient role in energy homeostasis and metabolism but more recently diverged to include further roles in neuromodulation and reproductive regulation.

### *Non-canonical roles of GNRH2*

As the axonal fibers of GNRH2 reach many different regions of the brain, it is possible that GNRH2 is pleiotropic, regulating multiple processes (Xia et al., 2014). In

addition to the roles in reproduction and feeding regulation, GNRH2 may have additional roles in other systems, a few of which are just now coming to light. For instance, in the European sea bass, *Dicentrarchus labrax*, afferent and efferent Gnrh2 neuronal fibers are seen penetrating the pineal gland, and Gnrh2 peptide can stimulate melatonin secretions from cultured pineal glands (Servili et al., 2010). This suggests a role for Gnrh2 in regulating the circadian cycle in fish and may also be able to respond to melatonin signals. However, melatonin treatments changed the expression of *gnrh1* and *gnrh3*, but not *gnrh2*, suggesting Gnrh2 may only be an upstream modulator of melatonin (Alvarado et al. 2015). Additionally, both Gnrh isoforms have been shown to be able to induce growth hormone secretion from pituitary somatotopes (Chang et al. 1990, Canosa et al, 2007). In addition to eliciting growth hormone secretion, evidence has been shown that in at least one fish species, the Mozambique tilapia (*Oreochromis mossambicus*), exogenous Gnrh2 was the most potent stimulator of pituitary prolactin secretion out of the Gnrh isoforms (Weber et al. 1997). The roles of prolactin are not as well characterized in fish as they are in mammals, but research shows it most likely has an abundance of roles ranging from osmolality regulation to reproductive development and brood care (Whittington and Wilson, 2013). The biological roles of Gnrh2 are most likely extensive and species-specific, but there appears to be common trends in upregulating reproductive and spawning behaviors and downregulating feeding amongst different vertebrate groups. A comprehensive and mechanistic study of the biological roles of Gnrh2 is imperative to advance our understanding of this hormone.

## Zebrafish as a model organism for studying Gnrh2

### *Advantages of using zebrafish in research*

The teleost model organism, zebrafish (*Danio rerio*) has recently emerged as an excellent model for studying biological systems, especially when using molecular and genetic approaches (Varshney and Burgess, 2013; Wyatt et al., 2015). Some of its advantage include a transparent epidermis in larval stages, which allows easy visualization of organs and tissues throughout early development for fast phenotyping, fast maturation and high progeny numbers, which allows for a statistically high number of experimental subjects (Dahm and Geisler, 2006), ease of genetic manipulation due to their sequenced and highly annotated genome available for reference (Varshney and Burgess, 2013; Howe et al., 2013), and externally fertilized eggs in which external small molecules or compounds can be transferred via microinjections or absorption. Zebrafish have been increasing in popularity as an ideal model for both forward and reverse genetics. Within the highly annotated genomic sequence, more than 26,000 genes have been identified (Howe et al., 2013). Gene-editing systems, knockdowns, and mRNA overexpression can be easily transferred to zebrafish eggs, and the fast development of the organism allows for the immediate identification of developmental phenotypes. The sequenced genome of the zebrafish means that any gene can be targeted for reverse genetic analysis, and forward or reverse genetic approaches can be applied to study the function of novel or unknown genes (Varshney and Burgess, 2013). The Zebrafish Information Network (ZFIN) database has been set up as a central hub of organization to keep track of all known mutant and transgenic zebrafish generated thus far (Spraque et al., 2006). Zebrafish have been used as great model organisms for studying human health

and diseases, considering the conserved biological systems in which 70% or more of human genes have a related zebrafish ortholog (Howe et al., 2013) and for aquaculture fish research, considering the highly conserved genes between zebrafish and other economically important aquaculture fish species (Dahm and Geisler, 2006).

***Previous studies analyzing Gnrh2 in zebrafish***

There have only been a few studies looking specifically at the role of Gnrh2 in zebrafish. The *gnrh2* gene was first cloned and localized in zebrafish in several labs, showing the location of Gnrh2 in the midbrain and surrounding the seminiferous tubules of the testes (Kuo et al., 2005). One of the first studies focusing on studying the roles of Gnrh2, used Gnrh2 ICV injections and subsequent behavioral and qPCR analyses to show that exogenous Gnrh2 injections decreased feeding behavior and downregulated *orexin* (also known as *hypocretin*) (Nishiguchi et al., 2012). Additionally, overfeeding resulted in upregulation of *gnrh2* and administration of the orexigenic hormone, Hypocretin, resulted in downregulated *gnrh2*, showing an overall anorexigenic role of Gnrh2 in zebrafish (Nishiguchi et al., 2012).

The zebrafish model was used in the Zohar lab to create a transgenic fish line which express green fluorescent protein specifically in Gnrh2 neurons (tg(Gnrh2:eGFP)), and was subsequently imaged using a confocal microscope to map out the neuronal projections throughout the brain and body, and begin examining its interactions with the Gnrh3 neuronal systems (Xia et al., 2014). Examining the brain centers in which Gnrh2 is located in and projecting to, allowed the beginning of hypotheses on which roles it may have. For instance, the finding that neuronal projections are seen through the spinal cord suggests Gnrh2 may be coordinating mobility, and the finding that Gnrh2 is also seen in

the pituitary suggests that it may coordinate reproductive gonadotropin control (Xia et al., 2014). The absence of a comprehensive analysis of Gnrh2 functions in zebrafish motivated me to further analyze its biological roles through genetic knockout and subsequent loss-of-function analyses, and further studies on the neuroanatomical interactions of Gnrh2 with other brain factors.

## Hypotheses and Objectives

Hypothesis 1: Gnrh2 may have a role in zebrafish reproduction, including gamete maintenance and development in both sexes.

Experimental Approach:

a. Studying the Gnrh2 loss-of-function effects on reproduction by generating a *gnrh2*$^{-/-}$ zebrafish line and examining any differences in reproductive parameters
b. Studying the neuroanatomical interactions of Gnrh2 neurons with other reproductive factors, such as Gnrh3 and Gnih, and examining its presence in reproductive centers of the brain and pituitary
c. Studying the functional effects of Gnrh2 treatments on expression of other reproductive factors, such as *gnrh3*, *gnih*, *lhb*, and *fshb*

Hypothesis 2: Gnrh2 has a role in zebrafish feeding regulation through downregulating feeding behaviors.

Experimental Approach:

a. Studying the Gnrh2 loss-of-function effects on feeding and growth by generating a *gnrh2*$^{-/-}$ zebrafish line and examining any differences in feeding and growth parameters

b. Studying the neuroanatomical interactions of Gnrh2 neurons with other feeding factors, such as Agrp1

c. Studying the functional effects of Gnrh2 treatments on expression of other feeding factors, such as *agrp1*, *pomca,* and *hypocretin*

Hypothesis 3: Gnrh2 has a role in mediating feeding and reproduction.

Experimental Approach:

a. Studying the Gnrh2 loss-of-function effects on reproduction in fish subjected to different feeding states
b. Studying the Gnrh2 neuronal projections to the pituitary and gonadotropin expression/secretion in wild-type and *gnrh2*$^{-/-}$ fish subjected to short-term and long-term fasting conditions
c. Studying the interactions of Gnrh2 with other factors that mediate both feeding and reproductive parameters, such as melatonin

Hypothesis 4: Gnrh2 compensates for Gnrh3 loss, such as in *gnrh3*$^{-/-}$ fish.

Experimental Approach:

a. Examining the differential expression of *gnrh2* in *gnrh3*$^{-/-}$ fish
b. Helping to generate a double knockout, *gnrh2*$^{-/-}$*;gnrh3*$^{-/-}$ zebrafish line and examining loss-of-function phenotypes of reproductive parameters

c. Examining the differential expression of other reproductive factors using transcriptomics approaches with *gnrh3*$^{-/-}$ fish, and targeted qPCR analyses with *gnrh2*$^{-/-}$;*gnrh3*$^{-/-}$ zebrafish

# CHAPTER 2: Studying the Roles of Gnrh2 in Reproduction


## Abstract

Many studies on Gnrh1, and the teleost Gnrh3, have elucidated the roles of these peptides in reproductive regulation. However, the role of the midbrain population of Gnrh, Gnrh2, in reproduction has long been ignored and overlooked, despite its ubiquitous conservation in all jawed vertebrates except rodents. Previous behavioral studies in sparrows, musk shrews, mice, zebrafish, and goldfish show that Gnrh2 exogenous administrations increases spawning behaviors, suggesting a role of this peptide in regulating female reproductive behaviors. Additionally, many *in vitro* studies show that Gnrh2 can bind to and activate Gnrh receptors in some fish even more potently than the other isoforms. However, the roles of endogenous Gnrh2 in reproduction still have not been comprehensively examined. In order to explore the roles of Gnrh2 in all aspects of reproduction, a knockout Gnrh2 zebrafish line (*gnrh2*$^{-/-}$) was generated, which contains a 21 base-pair (bp) deletion, two bp insertion (19 bp total deletion), resulting in a frameshift mutation and subsequent disruption of the coding for the peptide. Analysis of many reproductive parameters, including gonadal development, fecundity, fertility, gonadotropin levels, yielded no major impact of the loss of Gnrh2 on reproductive success. However, female *gnrh2*$^{-/-}$ zebrafish had slightly decreased oocyte quality, evident by smaller oocytes and increased embryo mortality. To begin understanding Gnrh2 mode of action in the regulation of reproduction, a comprehensive real-time PCR analysis was conducted, in which differentially expressed genes in the *gnrh2*$^{-/-}$ and wild type lines were identified. During development, zebrafish larvae exhibited decreased expression levels of *lhb*. At adulthood, there were changes in the expression levels of many reproductive neuropeptides in *gnrh2*$^{-/-}$ zebrafish, including decreased *lhb* mRNA in

adult males and increased transcript levels of Gonadotropin-inhibitory hormone (*gnih*) in adult males and females. *In vivo* and *in vitro* functional assays show that Gnrh2 can modulate both *lhb* and *gnih* expression. Furthermore, neuroanatomical studies have shown that Gnrh2 neurons and Gnih neurons are in close proximity to each other in the brain. Taken together, these findings implicate roles for Gnrh2 in maintaining optimal oocyte quality in females. These roles are probably relayed through a direct induction of Lh, as well as through indirect modulation via the interactions with several reproductive factors (Gnih, Nkb, and Scg2a), as well as with neuropeptides with additional metabolic roles, such as Spexin.

## Introduction

The neuropeptide Gonadotropin-releasing hormone (GNRH) is canonically known to be a major regulator of reproduction in vertebrates, inducing the expression and secretion of the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), from the pituitary (Amoss et al., 1971, Matsuo et al., 1971, Zohar et al., 2010). Of the three GNRH isoforms, the ones located in the preoptic area of the hypothalamus, either GNRH1 neurons or the teleost-specific Gnrh3 neurons, are known to be the population that is mainly responsible for regulating gonadotropin release from the pituitary (Abraham et al., 2008, Zohar et al., 2010). The fact that in most vertebrate species, GNRH2 is thought to be absent in the pituitary and non-hypophysiotropic, has led to most studies ignoring the role of Gnrh2 in reproduction (Selvaraj et al. 2009, Powell et al. 1995). However, the midbrain Gnrh2 does innervate the pituitary in some fish species, and occasionally, is even more abundant than the other isoforms (Guzmán et

al. 2009), suggesting the role and importance of Gnrh2 in reproduction is most likely variable and species-specific.

The exact conservation of the amino-acid sequence of GNHR2, suggests that it has a very important biological role. However, despite its conservancy and ubiquity in most vertebrates, except rodents, the exact roles of GNRH2 in reproduction are not fully known (Miyamoto et al., 1984, Kah et al., 1993, Zohar et al., 2010). The few studies that looked into the roles of GNRH2 in reproduction have postulated that it has a neuromodulatory role in stimulating reproductive behavior (Volkoff and Peter, 1999, Temple et al., 2003, Kauffman and Rissman, 2004, Hoskins et al., 2008). Studies in sparrows (*Passer domesticus*), goldfish (*Carassius auratus*), zebrafish, musk shrews (*Suncus murinus*), and mice (*Mus musculus*) showed that administration of GNRH2 peptide both decreases feeding and increases spawning behaviors (Volkoff and Peter, 1999, Millar, 2003, Temple et al., 2003, Kauffman et al., 2005), suggesting that this reproductive role may be conserved amongst all vertebrates. Additionally, these findings suggest a dual role in regulating, or alternatively, a mediating role of these important processes. However, the mechanism by which GNRH2 controls reproductive behavior and gonadotropin synthesis has not been well-studied.

Along with the hypophysiotropic GNRH, GNRH2 levels been shown to increase in conjunction with the spawning season, with increased protein content during gonadal recrudescence and just prior to spawning, for instance, in striped bass (*Morone saxatilis*) pituitaries (Holland et al., 2001), and expression increases just prior to the spawning period in red sea bream (*Pagrus major*) brains (Okuzawa et al., 2003). Surprisingly, as stated earlier in Chapter 1, in most fish species, Gnrh receptors on gonadotrope cells are

actually most sensitive to Gnrh2 (Alok et al., 2000; Zohar et al., 2010) and in some fish, such as goldfish, Gnrh2 is the most potent in eliciting release of gonadotropins compared to the other Gnrh isoforms (Khakoo et al., 1994), further pointing to an important role of Gnrh2 in reproductive processes in fish.

In the current study, I used the model organism zebrafish, a cyprinid that possesses two Gnrh isoforms, Gnrh2 and the hypophysiotropic Gnrh3 (Steven et al., 2003), to begin revealing the roles of GNRH2 in reproductive systems. Using a transgenic tg(Gnrh2:eGFP) zebrafish line previously generated in the lab, a robust network of innervations throughout the brain has been described, suggesting the involvement of this peptide in a multitude of systems (Xia et al., 2014). Surprisingly, Gnrh2 axon terminals were found also in the pituitary. This finding prompted the study into the potential roles of Gnrh2 in gonadotropin regulation and secretion and the generation of a Gnrh2 knockout line to study the loss of function effects of this peptide on reproductive parameters. This chapter describes the results of a holistic approach that includes loss-of-function studies, functional assays, and neuroanatomical interactions to comprehensively explore the roles of Gnrh2 in reproductive regulation and begin to elucidate the mechanism of action of Gnrh2. In this frame, both direct and indirect interactions (with other reproductive factors) were investigated in order to start understanding the molecular network involved in reproductive control.

The results of this study demonstrate, for the first time, a minor role of Gnrh2 in maintaining oocyte quality, probably due to the regulation of *lhb* expression, and interactions with another reproductive neuropeptide, Gnih. The loss of Gnrh2 resulted in

the upregulation, and downregulation, of a few reproductive genes, pointing to potential factors which may take part in the reproduction regulatory network of Gnrh2.

## Methods

### Zebrafish maintenance and husbandry

All zebrafish were kept in the in-house facility at the Institute of Marine and Environmental Technology and maintained in a 28° C recirculating system with a 14L:10D light cycle. Zebrafish larvae from 4 dpf to 30 dpf were kept on a nursery shelf in 300 mL tanks and fed *Paramecia ad libitum* twice daily until 15 dpf. From 15 to 30 dpf, zebrafish were fed *Artemia ad libitum* twice daily, and once large enough, fed 300 um Gemma diet pellets (Skretting) twice daily. After 30 dpf, zebrafish were moved into the recirculating water aquaria. Prior to tissue collections, adult zebrafish were euthanized in a cold ice-water bath and then promptly decapitated. Larval zebrafish were killed in tricaine (MS-222, Sigma-Aldrich). All experimental protocols were approved by the Institutional Animal Care and Use Committee at the University of Maryland School of Medicine.

### Generation of the *gnrh2*$^{-/-}$ zebrafish line

The first step in analyzing the roles of Gnrh2 was to generate a *gnrh2*$^{-/-}$ line using TALEN-mediated technology, and subsequently analyze the loss-of-function effects. The TALENs used to generate a *gnrh2* mutation were designed by Wei Xia to induce a double-stranded break at base pair 15,729,885 in the coding region of *gnrh2* gDNA on chromosome 21 (Genbank accession #: NC_007132.6) and generated through an NIH-sponsored initiative (NIH R01 GM088040). This base pair is located on the second exon

of the *gnrh2* gene on the first of ten amino acids in the signaling peptide coding region. The TALEN sequence was cloned into the JDS71 vector (Addgene) downstream of a T7 promoter site. One µg of linearized plasmid (using PmeI restriction enzyme) was transcribed into mRNA using the T7 RNA polymerase and mMessage mMachine Transcription Kit, treated with DNaseI, and then a polyA tail was added on using the PolyA Tailing Kit (all from Thermo Fisher Scientific). TALEN mRNAs (50-75 ng/µL) were micro-injected into wild-type (WT) zebrafish embryos at the 1-2 cell stage. Adult founder fish (F0 generation) were then crossed with wild-type fish to produce a heterozygous F1 generation. Screening for the presence of mutations was performed by PCR on gDNA extracted from clipped fin samples used as template and GoTaq DNA Polymerase (Promega). DNA extracts were cloned in pGEM-T vector (Promega) and sequenced, and the nucleotide sequences were analyzed and compared against wild-type to select those that result in the loss of a viable translated peptide (Fig. 2.1B). Fish positive for a 19 base-pair deletion within the Gnrh2 coding region (Fig. 2.1B) were selected to create homozygous lines of *gnrh2*$^{-/-}$ by crossing with wild-type fish from the same line as founder fish to get a 50% heterozygous F2 generation. Primers designed to specifically amplify either wild-type or mutant *gnrh2* sequences were used to screen fish with the 19 base-pair *gnrh2* mutation (G2WTFor, G2MutFor, and G2Rev, Table 2.1). Heterozygous fish were in-crossed to produce 25% homozygous *gnrh2*$^{-/-}$ offspring. The loss of Gnrh2 peptide in the *gnrh2*$^{-/-}$ brain was validated in the homozygous fish using immunohistochemistry and specific antibodies as described below.

**Validation of the *gnrh2*$^{-/-}$ zebrafish line through immunohistochemistry (IHC) analyses**

The loss of the Gnrh2 peptide in *gnrh2*$^{-/-}$ fish was verified using immunohistochemistry (IHC) with antibodies against the specific recombinant GAP region of Gnrh2 (GAP2) raised in rabbits, or the anti-decapeptide region (kindly received as a gift from the late Judy King). Brains from sexually mature *gnrh2*$^{-/-}$ and wild-type fish of the same age were dissected and fixed in 4% PFA overnight and immersed in 30% sucrose in PBS for four hours, or until brains sunk to the bottom of the vial. Brain samples were then frozen in OCT and stored at -80° C. Cryo-sectioning was conducted at -20° C using a Tissue-Tek Cryo3 cryostat. Brains were sectioned to 10 µM thickness, placed on Plus coated slides, and stored at -80°C until IHC was performed. Slides were briefly fixed in acetone and then washed in PBS. Slides were blocked for one hour in 5% normal goat serum, and incubated with a 1:1,000 dilution of Anti-GAP2 or Anti-Gnrh2 in 1% BSA and 0.3% Triton X-100 overnight at 4°C. Slides were then washed in PBS-Tw (0.05% Tween-20 in PBS) and incubated in fluorescein or Cy3-conjugated Goat anti-Rabbit antibody (Genscript) at a 1:1,000 dilution in 1% BSA and 0.3% Triton for one hour. Then, slides were washed in PBS-Tw 3X for 10 minutes each, and then mounted in 50% glycerol plus 10 µg/ml Hoescht 33342 (Sigma). Slides were viewed on a Zeiss Axioplan2 microscope and screened for the presence of Gnrh2 soma and fibers.

**Reproductive parameter assessments**

After the homozygous *gnrh2*$^{-/-}$ was generated and validated, any differences in reproduction associated with the loss of Gnrh2 were examined. In order to determine any differences in reproductive characteristics, gonadosomatic index (GSI), fecundity,

percentage of fertility, embryo survival rate, and oocyte diameters from wild-type and *gnrh2*$^{-/-}$ individuals were assessed. Six pairs of each genotype in the following combinations: wild-type male X wild-type female, wild-type male X *gnrh2*$^{-/-}$ female, *gnrh2*$^{-/-}$ male X wild-type female, and *gnrh2*$^{-/-}$ male X *gnrh2*$^{-/-}$ female, were set to spawn in separate containers. The number of eggs laid (collected 1 hr after spawning) by each female per spawn was counted and embryo survival rate was determined the next day. To evaluate fertility, six males of each genotype (wild-type and *gnrh2*$^{-/-}$) were crossed with wild-type female siblings. Spawned eggs in the 16-64-cell stage were counted to determine the percentage of fertilized eggs (eggs actively dividing). Oocyte size was determined by pairing nine *gnrh2*$^{-/-}$ and wild-type females with wild-type males and collecting the eggs after 1 hour of spawning. At least 20 eggs per female were imaged on a slide containing a scale bar using a CCD Olympus DP70 camera and Zeiss Axioplan2 microscope. Egg diameters were measured using ImageJ software. To determine GSI, six age and size-matched females and males from each genotype were weighed, and subsequently euthanized. The gonads were then dissected and weighed and quantified as a percentage of total body weight.

**Sperm motility and density analyses**

As Gnrh2 has been associated with testes development and sperm quality in some mammalian species (White et al., 2017), I wanted to determine whether Gnrh2 has a major role in maintaining sperm motility, quality, and density in zebrafish by comparing these factors in *gnrh2*$^{-/-}$ males and related wild-type male counterparts. In order to do so, sperm was extracted and densities were determined with a hemacytometer, and sperm motility/quality was analyzed with a computer-assisted sperm analysis (CASA) machine

in Dr. Curry Woods' laboratory at University of Maryland, with the kind assistance and guidance from Dr. L. Curry Woods and Dr. Tyler Frankel. To extract and analyze sperm from zebrafish, one µL of sperm was extracted from 9 mature males, of the same age, of wild-type and *gnrh2*$^{-/-}$ genotypes and added to 9 µL of sperm extender solution (consisting of 130 mM KCL, 50 mM NaCl, 2 mM CaCl2, 1 mM MgSO4, 10 mM D-(+)-Glucose, and 30 mM HEPES-KOH) on ice. Ten µL of sperm was then diluted in 90 µL of additional extender solution, and 10 µL added to a hemocytometer where the number of sperm cells was then counted on six different small grids (measuring 0.2 mm x 0.2 mm) per sample. The density numbers were then extrapolated to obtain average amounts of sperm per 100 ul for each sample. For sperm motility analysis, tubes of sperm in sperm extender solution, on ice, were immediately transported to the University of Maryland College Park and analyzed using CASA on a Makler counting chamber (Sefi Medical Instruments, Haifa, Israel), which was visualized by a negative phase contrast Olympus CX41 microscope and analyzed real-time using a Hamilton-Thorne CEROS™ (version 12, Hamilton-Thorne, Beverly, Maine) Computer-assisted sperm analysis (CASA) (Frankel, 2013). Zebrafish sperm was added to the Makler chamber and diluted and mixed in 1:10 in activating water to immediately begin activation. Immediate visualization and sperm motility analysis was conducted with the Hamilton-Thorne CEROS™, which calculated the sperm motility parameters of the in-frame sperm, including motility (percentage of sperm cells moving in-frame), progressive motility (percentage of sperm cells with a path velocity greater than 100 µm/s and straightness greater than 80%), path velocity (measured in µm/s), and percent linearity (percentage of sperm exhibiting a straight trajectory) were calculated for all sperm (Frankel, 2013).

Triplicate analyses of each individual sperm sample were conducted to get an average of sperm quality parameters. Average sperm quality numbers were then totaled for each genotype and compared via statistical analysis.

**Gonad histology**

To determine differences in gametogenesis and gonadal development in males and females, *gnrh2*$^{-/-}$ and wild-type females and males at different stages during development were collected and gonad morphology analyzed via hematoxylin and eosin (H&E) staining (Sabaliauskas et al., 2006). Ovaries and testes were dissected from zebrafish and fixed in 4% PFA in PBS overnight at 4°C and embedded in paraffin (Fischer et al., 2008). Sections, 5 µm thick, were mounted on Plus-coated slides and dried overnight at 50°C. The sections were then rehydrated and stained with hematoxylin and eosin according to the manufacturer's protocol (Sigma-Aldrich, St. Louis, MO, USA). Ovarian and testicular sections were viewed and analyzed using the bright field setting of a Zeiss Axioplan2 microscope and CCD Olympus DP70 camera and the most advanced stage of oogenesis and spermatogenesis were assessed, by identifying the presence of mature oocytes (greater than 500 µm and undergoing germinal vesicle migration or breakdown) or mature spermatozoa, from each sample to determine gametogenesis in young fish (Patiño et al., 2002) and gonadal developmental stages.

**Reproductive gene expression comparisons during development**

To determine the effect of Gnrh2 loss-of-function on reproduction, the gene expression of several reproductive neuropeptides, including *gnrh2*, *gnrh3*, and *gnih*, and the gonadotropin genes, *cga*, *lhb*, and *fshb* were compared between wild-type and Gnrh2

knockout (*gnrh2*$^{-/-}$) larvae throughout the first 30 days of zebrafish development. Samples collected to compare the developmental profile of reproductive genes included whole zebrafish larvae and juveniles at 1, 2, 3, 8, 12, 18, 24, and 30 dpf, which were quickly killed in tricaine, washed in distilled water, and then flash frozen in dry ice. Samples, in triplicate, for each genotype (wild-type and *gnrh2*$^{-/-}$) were collected and pooled (as follows: n=20 for 1, 2, and 3 dpf; n=15 for 8 and 12 dpf; n=10 for 18 and 24 dpf; and n=6 for 30 dpf). Total RNA from samples was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The Quantitect Reverse Transcription Kit (Qiagen, Valencia, CA, USA), that includes gDNA Wipeout to eliminate gDNA contamination, was used to reverse transcribe 1 µg of RNA from each sample. In each round, a non-RT control and no template control were added to determine gDNA and template contaminations. QPCR was conducted using 20 ng of cDNA for each sample in duplicate with SYBR Green qPCR mix and gene-specific primers for each gene measured (*gnrh2*, *gnrh3*, *gnih*, and the gonadotropin genes, *cga*, *lhb*, and *fshb)*, with CT values for each sample normalized against an internal *eef1α1* control. Amplification of cDNA was performed on a 7500 Fast Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA) and conditions included a 2 minute 95°C activation, 5 second 95°C denaturation, and 30 second 60°C annealing, with the last two steps repeating 40 times. For each gene, expression values were additionally normalized to the 1 dpf wild-type expression value for relative comparisons between *gnrh2*$^{-/-}$ and wild-type larvae.

**Reproductive gene expression comparisons in adults**

There are several reproductive neuropeptides that are involved in influencing reproduction in fish and may have relationships with the Gnrh system. To determine potential factors that may have relationships or interactions with Gnrh2, differences in the gene expression levels of the following various reproductive peptides in the brain: *gonadotropin-inhibitory hormone* (*gnih), secretogranin 2a* (*scg2a), tachykinin 3a* (*tac3a), arginine vasopressin* (*avp), gonadotropin-releasing hormone 3* (*gnrh3), kisspeptin 1* and *2* (*kiss1* and *kiss2), oxytocin* (*oxt*), and *vasoactive intestinal peptide* (*vip*), and the following gonadotropin subunits and receptor genes in the pituitary: *common glycoprotein subunit alpha* (*cga*), *follicle-stimulating hormone subunit beta* (*fshb*), *luteinizing hormone subunit beta* (*lhb*), *gonadotropin-releasing hormone receptor 1* (*gnrhr1*), *gonadotropin-releasing hormone receptor 3* (*gnrhr3*), and *gonadotropin-releasing hormone receptor 4* (*gnrhr4*) in adulthood were determined using qPCR and expression values compared between *gnrh2*$^{-/-}$ and wild-type females and males. Brains and pituitaries from six to eight *gnrh2*$^{-/-}$ and wild-type adults of the same age and sex were dissected, flash frozen in dry ice, and stored at -80° C until RNA extraction. RNA extraction and qPCR analysis of the reproductive genes were conducted using the same protocol as the previous section.

**Gnrh2 *in vivo* treatment studies**

The effect of Gnrh2 on *lhb*, *fshb*, *gnrh3*, and *gnih* transcription in *gnrh2*$^{-/-}$ males was analyzed *in vivo* with intracerebroventricular (ICV) injections of Gnrh2 peptide to the third ventricle of the brain of male *gnrh2*$^{-/-}$ and wild-type fish. ICV injections were conducted using a 50 µL Hamilton syringe equipped with a 30 gauge needle and a 2 mm

penetration stopper. Male fish (weighing 200 mg) were deeply anesthetized in Tricaine (MS-222), and then injected with Gnrh2 peptide at concentrations of 0 (control), 0.1, or 1 picomole (pmol)/µL in saline containing 0.05% Evans Blue dye. Fish were monitored for normal behavior, and after 6 hours, fish were euthanized, and brains and pituitaries dissected. Successful injections were verified by the presence of Evans Blue Dye in the third ventricle during dissections. Brains and pituitaries were separated and flash frozen in dry ice and stored at -80°C until RNA extraction was conducted, as discussed in previous sections (with the addition of 0.5 µL of glycol blue added during the isopropanol step for pituitary samples to improve detection and aggregation of pituitary RNA which is in lower quantities compared to brains), followed by qPCR analysis to compare *lhb*, *fshb*, *gnrh3*, and *gnih* mRNA levels.

**Gnrh2 *in vitro* treatment studies**

The effect of Gnrh2 on the expression of *lhb*, *fshb*, *gnrh3*, and *gnih* in *gnrh2*$^{-/-}$ and wild-type fish was also tested by incubating dissected, cultured brains or pituitaries of wild-type and *gnrh2*$^{-/-}$ male fish *in vitro* with varying concentrations of the decapeptide. Pituitaries or brains (n=6-8/treatment) were dissected and placed in 24-well cell culture plates with sterile cell culture inserts (Millipore, 70 µm pore size) containing 1 mL L-15 media (supplemented with 0.1% BSA, 20 mM sodium bicarbonate and antibiotic mixture). Tissues were washed in the media for 2 hr at 28°C and 30 RPM and then incubated with media including 0, 10, or 100 nM of Gnrh2 peptide for 18 hr before being collected and placed individually in 1.5 mL tubes and flash frozen at -80°C before RNA extraction was conducted (as described previously in section 2.4). Synthesis of cDNA and subsequent qPCR to measure *gnrh3, gnih, lhb,* and *fshb* mRNA levels were

conducted as described above. For each genotype, all values were additionally normalized to the lowest control concentration expression value to determine the relative value fold-change difference with increasing Gnrh2 concentrations.

**Gnrh2 and Gnih neuroanatomical interaction analyses**

Interactions between Gnrh2 and Gnih (Lpxrfa) in the brain were examined using double- label immunohistochemistry with antibodies against the zebrafish specific recombinant GAP region of Gnrh2 (GAP2) (Xia et al., 2014) or Gnih (Spicer et al., 2017) raised in rabbits. Brains from sexually mature *gnrh2*$^{-/-}$ and wild-type fish of the same age were dissected and fixed in 4% PFA overnight and immersed in 30% sucrose in PBS for four hours at 4°C, or until brains sunk to the bottom of the vial. Brain samples were then frozen in OCT and stored at -80° C. Cryo-sectioning was conducted at -20° C using a Tissue-Tek Cryo3 cryostat. Brains were sectioned to 10 µM thickness, placed on Plus coated slides, and stored at -80°C until immunohistochemistry (IHC) was performed. To perform double-staining IHC, slides were briefly fixed in acetone, and endogenous HRP was quenched in 0.3% $H_2O_2$ in PBS for 30 minutes. Slides were then washed in PBS, blocked for one hour in 5% normal goat serum, and incubated with a 1:1,000 dilution of Anti-GAP2 in 1% BSA and 0.3% Triton X-100 overnight at 4°C. Slides were then washed in TNT (100 mM Tris Ph 7.5, 150 mM NaCl, 0.5% Tween-20**)** and incubated in an HRP-conjugated Goat anti-Rabbit antibody (Genscript) at a 1:1,000 dilution in 1% BSA and 0.3% Triton for one hour. Then, slides were washed in TNT and incubated in a fluorescein dye from the Tyramide Signal Amplification Plus kit (TSA Plus kit, Perkin Elmer) at a 1:50 dilution for 5 minutes, washed in TNT, and HRP signal quenched with 0.02 N HCl for 10 minutes. After washing, the procedure for IHC delineated above was

repeated on the same slides, but with an Anti-Gnih primary antibody (1:750 dilution) and Cy3 dye from the TSA kit to label Gnih protein. Slides were mounted in 50% glycerol plus 10 µg/ml Hoescht 33342 (Sigma) and viewed on a Leica Sp6 confocal microscope and screened for the presence of Gnrh2 and Gnih soma and fibers.

**Statistical analysis**

Statistically significant differences between wild-type and *gnrh2*$^{-/-}$ groups were determined using one-way ANOVA followed by Student t-tests for fertility, fecundity, embryo survival, oocyte diameters, and sperm density and quality, and one-way ANOVA for *in vitro* and *in vivo* Gnrh2 treatments. Statistical analysis for qPCR data was determined using MANOVA.

# Results

## *qPCR and Screening Primer Sequences*

| Primers | Sequences (5' to 3') |
|---|---|
| ***QPCR Primers*** | |
| *Cga* | Fw: TCCGGTCTATCAGTGCGT |
| | Rv: GGATATTCGTGGCAACCATTT |
| *Fshb* | Fw: GCTGGACAATGGATCGAGTTTA |
| | Rv: CTCGTAGCTCTTGTACATCAAGTT |
| *Lhb* | Fw: GGCTGGAAATGGTGTCTTCT |
| | Rv: CCACCGATACCGTCTCATTTAC |
| *Gnih* | Fw: GGGGACAGTTTTCAGAGAATGCTCAAG |
| | Rv: CGTCCGAAGCGAAGAGGGAG |
| *Gnrh2* | Fw: CAGAGGTTTCAGAGGAAGTGAAGC |
| | Rv: TGAGGGCATCCAGCAGTATTG |
| *Gnrh3* | Fw: TGGAGGCAACATTCAGGATGT |
| | Rv: CCACCTCATTCACTATGTGTATTGG |
| *Kiss1* | Fw: CTTAGAAGATGAAACTCCAGAGGAA |
| | Rv: GAGAAGAGCGCTGAGAGTTTAG |
| *Kiss2* | Fw: CAGAGCCTATGCCAGACC |
| | Rv: CTAGTCGATGTTTGCAGGATATTT |
| *Mao* | Fw: GAGGAGGAAGATGCACCTATTG |
| | Rv: TGCCAGCTTCCGTGATTT |
| *Oxt* | Fw: AAAGGCCTGCGGTTATGAGG |
| | Rv: TTGGCCGGTTGATTGACAGC |
| *Scg2a* | Fw: GCGTCCGTTCAGTGCTTAAA |
| | Rv: GGTCTTGCGAACTGGTGGTA |
| *Tac3a* | Fw: TGGTTTTGGTGCTGGAAACC |
| | Rv: TCTGTTTCGGCGTTTCTGC |
| *Th* | Fw: GACGGAAGATGATCGGAGACA |
| | Rv: CCGCCATGTTCCGATTTCT |
| *Vip* | Fw: GCAGATGGCGGTGAAGAAATA |
| | Rv: TCATCGTAGCTCTCCCGATATG |
| *Aanat2* | Fw: GTGTCTGGTGAATGTCCTCTAAC |
| | Rv: CCAGCCAGATCCAATGATGAA |
| *Agrp1* | Fw: CACAGACTCACTGCCTGAAA |
| | Rv: CTTTGCCCAGATCCTCATCATA |
| *Igf1a* | Fw: AGCGGTCATTTCTTCCAG |
| | Rv: CCTTACAGGAAGAGTGGCTAT |
| *Npy* | Fw: TCAAGCGCTGACACCTTAAT |
| | Rv: GATGAGATCACCATGCCAAATG |
| *Orexin* | Fw: CAACAACTGCTGCACAACTC |

| | |
|---|---|
| | Rv: GTACCGTAGGGATGAGGAATTTAG |
| *Pmch2* | Fw: ACGAAGAACATACGGACCAA |
| | Rv: TCCACCAATCTACCCTCTATGA |
| *Pomca* | Fw: GCCTTTAAACCCACTTGGAAAG |
| | Rv: GAGTGTGAGCAGTGGTTTCT |
| *Pacap1* | Fw: ATCCGTCCGCTGCCGCAGAATG |
| | Rv: GAGTGTGAGCAGTGGTTTCT |
| *Pacap2* | Fw: CCTACTGCACGCCTATTGG |
| | Rv: CGATTCGTCCTCCTCGC |
| *Spexin* | Fw: TGGAGCGCACCCAAGGGCA |
| | Rv: TGACTGCGGGATTCTAAGCGTATTG |
| *Eef1a* | Fw: AAGACAACCCCAAGGCTCTCA |
| | Rv: CCTTTGGAACGGTGTGATTGA |
| *Sep15* | Fw: TATTGTTGATTGTTGCTGAGGG |
| | Rv: ACGCTGAGAGATGTACACAGGA |
| ***Screening Primers*** | |
| Gnrh3WT | Fw: GAAGGTTGCTGGTCCAGTTGTTGTTGCTG |
| | Rv: TGCACATGTACTTGCTGAATTA |
| Gnrh3Mut | Fw: TTAGTTTSGSTTTCAGCAGTTTTAGCATC |
| | Rv: TGCACATGTACTTGCTGAATTA |
| Gnrh2WT | Fw: GCGCTCAGCACTGGTCTCA |
| | Rv: CTCTTTAAGGGAGAGAAGAATATTTTCAA |
| Gnrh2Mut | Fw: GAGTGCTCAGTTGAGTACGGC |
| | Rv: CTCTTTAAGGGAGAGAAGAATATTTTCAA |

**Table 2.1.** List of primers used in qPCR analysis and primers used to screen mutant and wild-type genotypes

**Generation of the 19 bp deletion *gnrh2*$^{-/-}$ line**

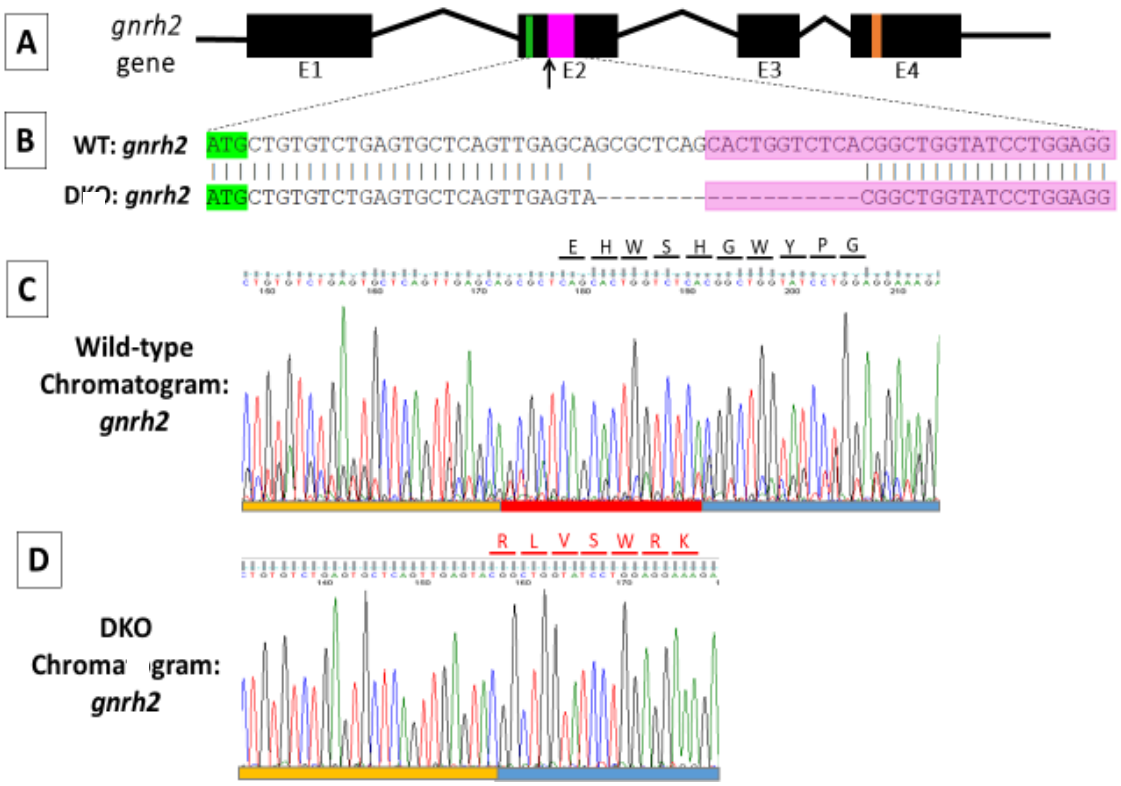


**Figure 2.1**. *Generation of the gnrh2*$^{-/-}$ *mutant line*. (A) A schematic illustration of the genetic structure of Gnrh2 and targeting site of the TALEN (black arrow) at the start of the decapeptide coding region (pink) in exon 2, with the start codon (green) and stop codon (orange) also depicted. Introns are depicted as lines, and exons as boxes. (B) The *gnrh2* nucleotide sequence of wild-type fish from the start codon (green) to the end of the decapeptide coding sequence (pink) compared to the KO nucleotide sequence, which contains a 19 base-pair deletion 8 base-pairs upstream of the decapeptide coding sequence (dashes). (C) Wild-type chromatogram showing the normal nucleotide sequence obtained after sequencing upstream (yellow) and downstream (blue) of the 19 base-pair deletion region (red), along with the encoded amino acid sequence (black letters). (D) Homozygous KO chromatogram shows the missing 19 base-pair region (red) seen in thewild-typechromatogram, and predicted encoded amino acid sequence (red letters).

The TALENs used to generate the *gnrh2*$^{-/-}$ knockout line targeted a specific site at exon 2 of the gnrh2 gDNA sequence at the beginning of the coding region of the decapeptide (pink, Fig. 2.1A). An F1 heterozygous fish, harboring a 19 base-pair deletion

(red, Fig. 2.1B) was selected to propagate the line. The deletion encompasses 8 base-pairs upstream of the peptide coding region (pink) and 11 base-pairs encoding the first four amino acids of the Gnrh2 peptide (Fig. 2.1B & C). The mutation also causes a frameshift and subsequent loss of the peptide and the downstream GAP (Fig. 2.1D). Fish were bred with wild-type and in-crossed to create a homozygous mutant and wild-type line, and only fish that displayed homozygosity for wild-type or the mutation were sequenced. The chromatograms from the homozygous knockout fish (Fig. 2.1D) showed single peaks and the loss of the 19 nucleotides (red region) found in wild-type fish chromatograms (Fig. 2.1C).

**Immunohistochemistry shows no Gnrh2 presence in *gnrh2*$^{-/-}$ fish**

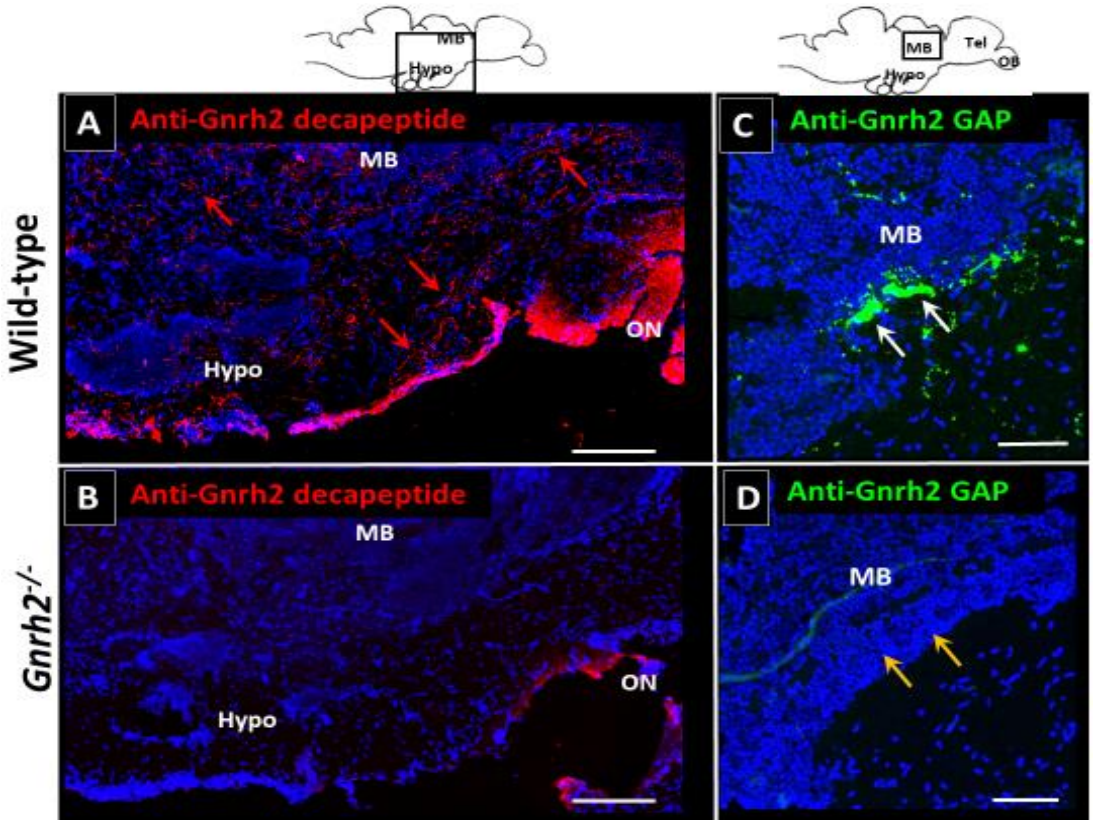


**Figure 2.2.** *Validation of the absence of Gnrh peptides in gnrh2*$^{-/-}$ *adult brains*. Immunostaining of Gnrh2 anti-decapeptide (red) and GAP2 (green) in sagittal brain

sections of adult zebrafish. Abundant neuronal fibers of Gnrh2 are seen throughout wild-type brain sections (red and white arrows) (A, C), but none are visible in *gnrh2*$^{-/-}$ brains (B, D). Gnrh2 soma is seen in the midbrain tegmentum region of wild-type brains (C, white arrows) but not in *gnrh2*$^{-/-}$ brains (D, yellow arrows). Hypo, hypothalamus; MB, midbrain tegmentum; POA, preoptic area; Tel, telencephalon; OB, olfactory bulb. Scale bars = 100 µm.

IHC was conducted to verify that the genetic mutation in Gnrh2 resulted in the loss of the peptide in *gnrh2*$^{-/-}$ brains. IHC staining of wild-type brains with Gnrh2 decapeptide antibodies resulted in the abundant appearance of Gnrh2 fibers throughout the entirety of the brain, most notably in the olfactory bulb, midbrain, hypothalamus, and hindbrain regions (Fig. 2.2A), however in *gnrh2*$^{-/-}$ fish, no Gnrh2 signal is seen using the same antibodies (Fig. 2.2B). Congruent with previous reports, Gnrh2 soma are found in the mid-brain tegmentum region of the brain (Fig. 2.2C). Staining of *gnrh2*$^{-/-}$ brains with GAP2 antibodies, however, did not result in the appearance of any Gnrh2 fibers or soma (Fig. 2.2D), confirming the gene knockout resulted in a loss of the Gnrh peptides.

**Reproductive assessments and gonad histology of *gnrh2*$^{-/-}$ and wild-type counterparts**

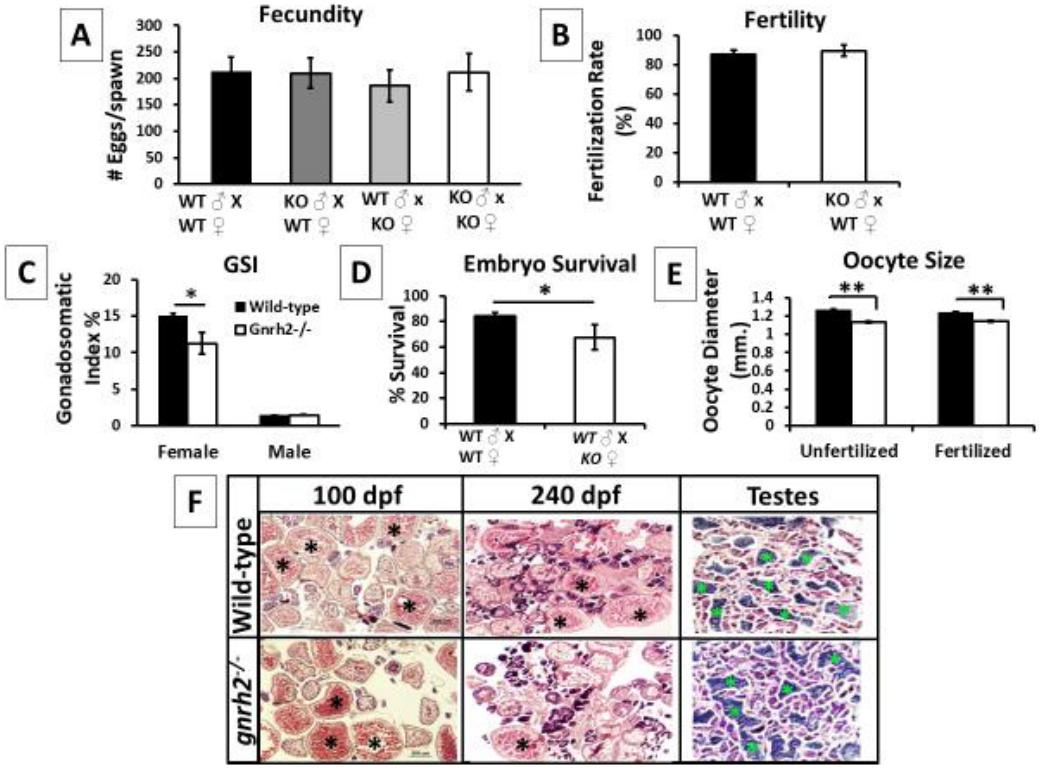


**Figure. 2.3.** *The effect of the loss of Gnrh2 on reproductive characteristics:* Comparison of the number of eggs per spawn (fecundity) of wild-type male and female spawning pairs, *gnrh2*$^{-/-}$ male and wild-type female spawning pairs, wild-type male and *gnrh2*$^{-/-}$ female spawning pairs, and *gnrh2*$^{-/-}$ male and female spawning pairs (A). Percentage of eggs fertilized from wild-type and *gnrh2*$^{-/-}$ males paired with wild-type females (B). Gonadosomatic index (percent of gonad weight to total body weight) between 6-month *gnrh2*$^{-/-}$ and wild-type females and males (C). Survival rate of 1 dpf embryos from wild-type and *gnrh2*$^{-/-}$ females spawned with wild-type males (D). Comparison of the oocyte diameter of wild-type and *gnrh2*$^{-/-}$ females 6-9 months old (n=10) before and after fertilization (E). Ovarian and testicular sections sampled at 100 and 240 dpf and stained with hematoxylin and eosin (H&E) (F). Black stars depict fully vitellogenic and mature oocytes and green stars indicate a cluster of mature spermatozoa. All data were expressed as means ± S.E.M. Stars indicate significant difference between wild-type and *gnrh2*$^{-/-}$ genotypes (* = $P < 0.05$, ** = $P < 0.01$).

No significant differences were detected in the fecundity or fertility rates between *gnrh2*$^{-/-}$ and wild-type pairs (Fig. 2.3A & B). An average of 200 eggs were produced by all spawning pairs (Fig. 2.3A) with an average of 90% fertilized eggs for both wild-type

and *gnrh2*$^{-/-}$ pairs (Fig 2.3B). The GSI of *gnrh2*$^{-/-}$ females was decreased by 3% compared to wild-type females, but there were no differences between males (Fig. 2.3C). The average egg survival of *gnrh2*$^{-/-}$ females, spawned with wild-type males, was around 68%, significantly lower than that of wild-type pairings, which was around 84% (Fig. 2.3D). A 10% decrease in the egg diameter (fertilized and unfertilized) of *gnrh2*$^{-/-}$ females compared with wild-type eggs was observed (Fig. 2.3E). Gonad sections of *gnrh2*$^{-/-}$ and wild-type individuals from 100 to 240 dpf looked morphologically similar at all of the different life stages, displaying the same developmental stages of oocyte and sperm (Fig. 2.3F). Fully vitellogenic oocytes were present in both female *gnrh2*$^{-/-}$ and wild-type zebrafish (Fig. 2.3F, black stars) and spermatazoa in both *gnrh2*$^{-/-}$ and wild-type males and in the same proportion (Fig. 2.3F, green stars).

**Sperm motility and density of *gnrh2*$^{-/-}$ compared to wild-type counterparts**

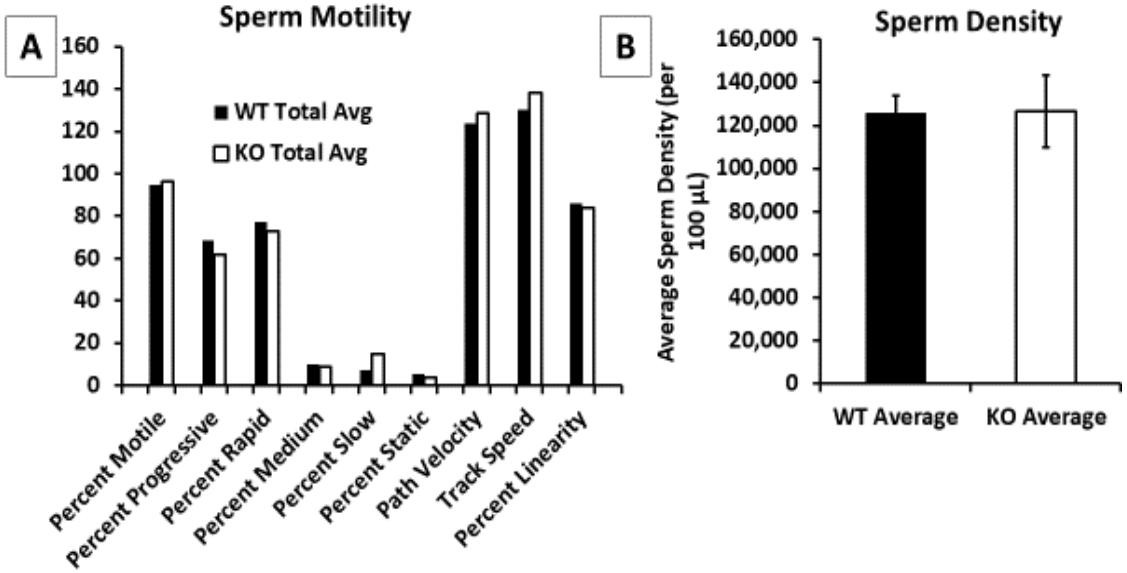


**Figure. 2.4.** *Effect of the loss of Gnrh2 on sperm motility and density.* The average percentages of sperm cells which exhibit motility, linearity, progressive motion, and speeds at a rapid, medium, slow, and static rate in wild-type and *gnrh2*$^{-/-}$ (KO) males

(n=9/genotype) (A). The average sperm density from 1 µL of sperm collected from wild-type and *gnrh2*$^{-/-}$ (KO) males (n=6/genotype) (B).

There were no significant differences in sperm motility characteristics (Fig. 2.4A) and sperm densities (Fig. 2.4B) between wild-type and *gnrh2*$^{-/-}$ males. There was an average of around 125,000 and 126,000 sperm cells/100 uL in wild-type and *gnrh2*$^{-/-}$ males, respectively. Additionally, the percentage of motile sperm cells was approximately 95% and 96%, progressively motile sperm cells 69% and 62%, and linear cells 86% and 84% for wild-type and *gnrh2*$^{-/-}$ males, respectively. The track speed and path velocities were also similar (an average of 125 and 135 µm/s respectively) and showed no significant differences between wild-type and *gnrh2*$^{-/-}$ males.

**Reproductive gene expression of *gnrh2*$^{-/-}$ and wild-type fish throughout development**

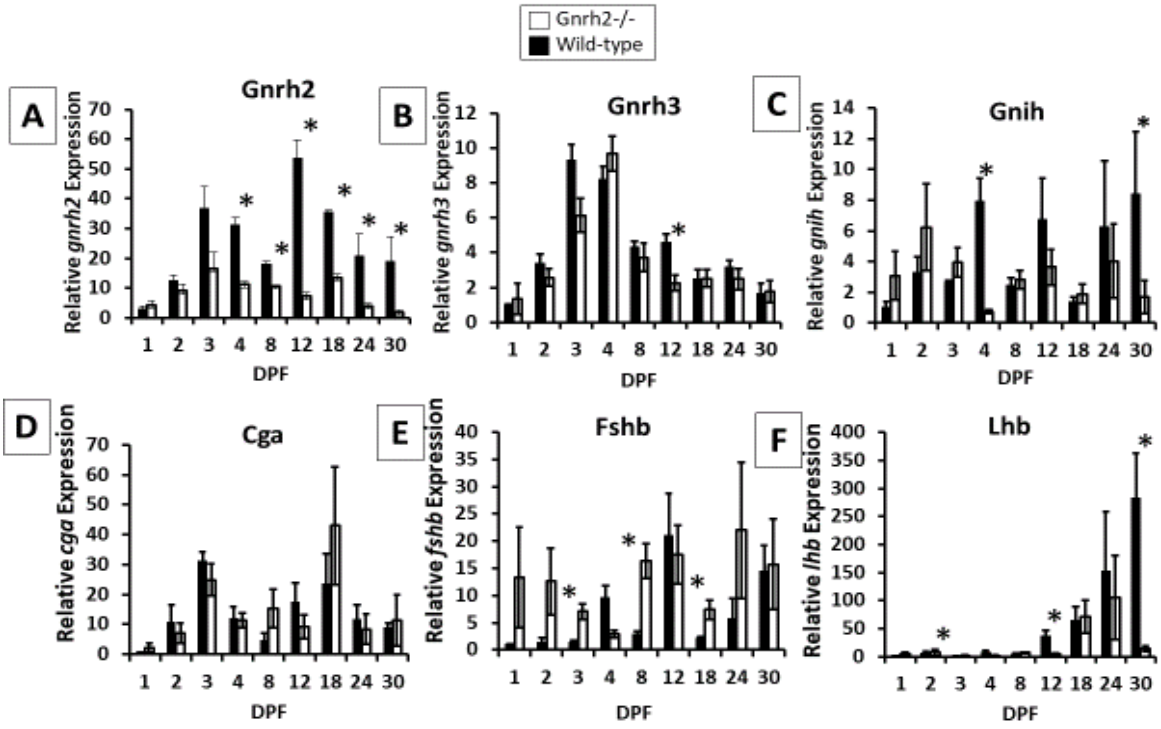


**Figure. 2.5.** *Gene expression of reproductive and gonadotropin genes during development in wild type and gnrh2*$^{-/-}$ *lines*: mRNA levels of *gnrh2* (A), *gnrh3* (B), *gnih* (C), *cga* (D), *fshb* (E) and *lhb* (F), of wild-type and *gnrh2*$^{-/-}$ larvae from 1 to 30 dpf,

relative to wild-type levels at 1 dpf (A). All data were expressed as means ± S.E.M. Stars Indicates significantly different mRNA levels between the genotypes (* = $P < 0.05$; ANOVA).

Throughout the first 30 days of development, there were no differences in the expression of *gnrh3* between *gnrh2*$^{-/-}$ and wild-type fish except for a reduction at 12 dpf (Fig. 2.5B), however, there was significantly reduced mRNA levels of *gnrh2* in *gnrh2*$^{-/-}$ larvae between 4 and 30 dpf, which progressively decreases over time (Fig. 2.5A). The only differences in *gnih* expression between *gnrh2*$^{-/-}$ and wild-type larvae is a decreased expression in *gnrh2*$^{-/-}$ fish at 4 and 30 dpf. *Gnrh2*$^{-/-}$ embryos had increased mRNA levels of *fshb* at 3, 8, and 18 dpf (Fig. 2.5E) and decreased levels of *lhb* at 4, 12, and 30 dpf (Fig. 2.5F). There were no significant differences in *cga* expression levels throughout zebrafish development (Fig. 2.5D).

**Reproductive gene expression of *gnrh2*$^{-/-}$ and wild-type fish in adult brains**

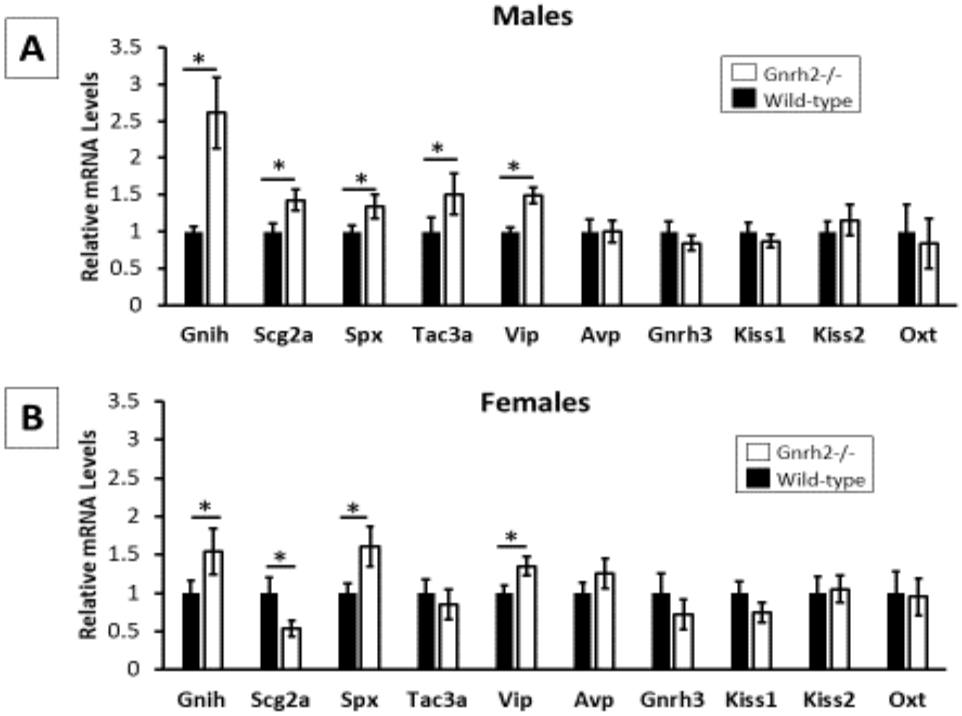

**Figure. 2.6.** *Effect of the loss of Gnrh2 reproductive-related gene expression:* mRNA levels of *gnih*, *scg2a*, *spx*, *tac3a*, *vip*, *avp*, *gnrh3*, *kiss1*, *kiss2*, and *oxt* male (A) and female (B) wild-type and *gnrh2*$^{-/-}$ brains at six months of age. All data were expressed as means ± S.E.M. Stars indicate significantly different mRNA levels between the genotypes (* = P < 0.05; MANOVA).

In terms of reproductive related gene expression, in both *gnrh2*$^{-/-}$ males and females, mRNA levels of *gnih*, *spexin*, and *vip* were higher (Fig. 2.6A & B). Males had significantly higher expression levels of *tac3a* and *scg2a* (Fig. 2.6A), while females had significantly lower levels of *scg2a* (Fig. 2.6B). There were no significant differences in *avp*, *gnrh3*, *kiss1*, *kiss2*, or *oxt* between *gnrh2*$^{-/-}$ and wild-type fish (Fig. 2.6A & B).

**Reproductive gene expression of the gonadotropin genes and Gnrh receptors in *gnrh2*$^{-/-}$ and wild-type adult pituitaries**

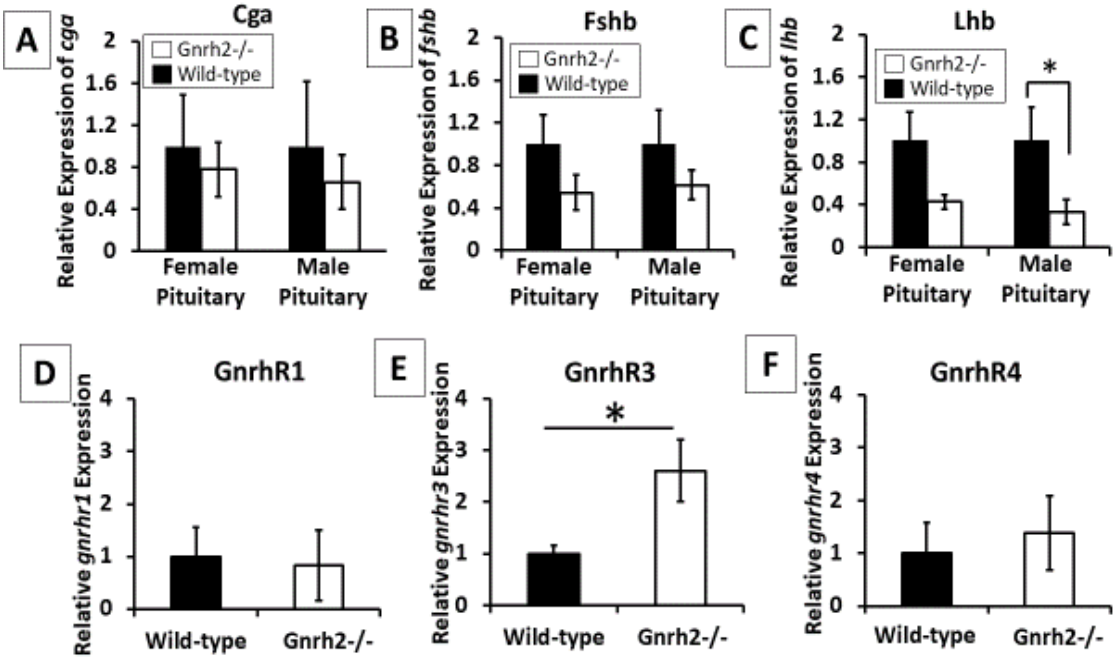


**Figure. 2.7.** *Gene expression of the gonadotropin genes and Gnrh receptors in adult wild type and gnrh2*$^{-/-}$ *pituitaries*: mRNA levels of the gonadotropin genes, *cga* (A), *fshb* (B) and *lhb* (C), and the Gnrh receptor genes, *gnrhr1* (D), *gnrhr3* (E), and *gnrhr4* (F) from

wild-type and *gnrh2*$^{-/-}$ pituitaries of 6-month old adults. All data were expressed as means ± S.E.M. Stars Indicates significantly different mRNA levels between the genotypes (* = P < 0.05; ANOVA).

In zebrafish pituitaries, there were no differences in the mRNA levels of *cga* or *fshb* in the pituitary (Fig. 2.7 A & B), however, there was a decrease in *lhb* expression, which was significant in males (Fig. 2.7C). Additionally, there were no differences in *gnrhr1* (Fig. 2.7A) and *gnrhr4* (Fig. 2.7F) expression between *gnrh2*$^{-/-}$ and wild-type individuals, but there was a significant 3-fold increase in *gnrhr3* expression in *gnrh2*$^{-/-}$ fish compared to wild-type (Fig. 2.7E).

**Neuroanatomical interactions of Gnrh2 and Gnih**

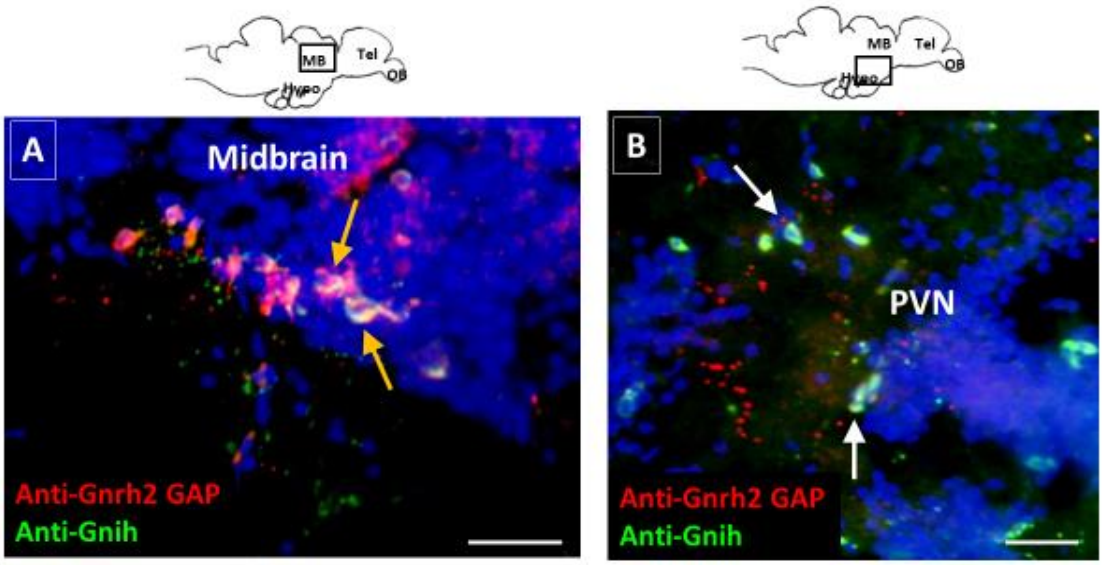


**Figure 2.8..** *Double-label immunohistochemistry of Gnrh2 and Gnih in adult wild-type brains*. Immunostaining of Gnrh2 GAP (red) and Gnih (green) in sagittal brain sections of adult zebrafish. In the midbrain tegmentum, abundant Gnih fibers can be seen in proximity to and innervating Gnrh2 soma (yellow arrows). In the paraventricular nucleus region of the hypothalamus, red Gnrh2 neuronal fibers can be seen in proximity to Gnih soma (white arrows). Hypo, hypothalamus; MB, midbrain tegmentum; POA, preoptic area; PVN, paraventricular nucleus; Tel, telencephalon; OB, olfactory bulb. Scale bars = 25 µm.

In the midbrain tegmentum, abundant Gnih fibers (as labelled by green fluorescence) can be seen reaching Gnrh2 neuronal soma (as labelled by red fluorescence), with the overlapping of the two colors seen in yellow, indicating innervation of Gnrh2 soma by Gnih fibers (Fig. 2.8A). In the periventricular nucleus, Gnih neuronal soma are surrounded by Gnrh2 fibers which come in close proximity to Gnih cells, but no direct contact is seen (Fig. 2.8B).

**Effect of *in vivo* and *in vitro* administration of Gnrh2 on *gnrh3* and *gnih* expression**

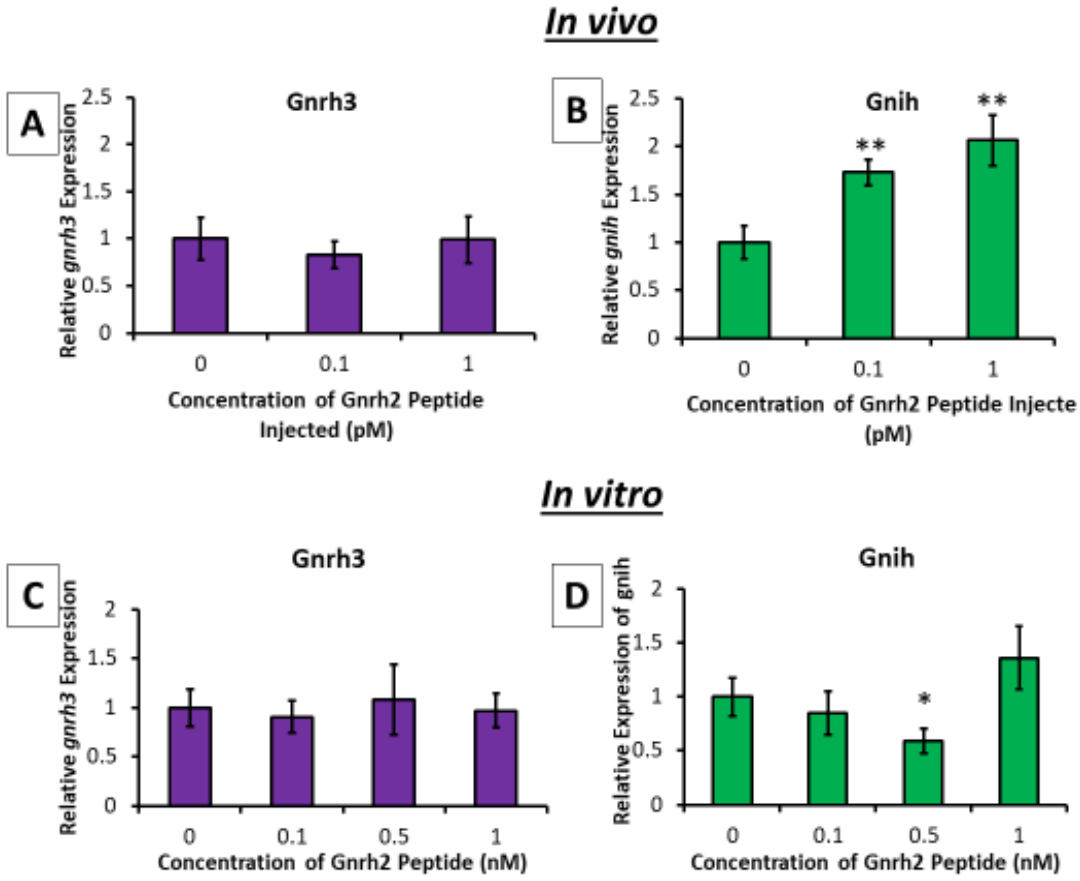


**Figure. 2.9** *Effect of in vivo and in vitro administration of Gnrh2 on gnrh3 and gnih expression* mRNA levels of *gnrh3* (A) and *gnih* (B) in wild-type and *gnrh2*$^{-/-}$ male brains after intracerebroventricular injections of Gnrh2 peptide (0, 0.1, or 1 pM). Expression levels of *gnrh3* and *gnih* after i*n vitro* incubations of wild-type and *gnrh2*$^{-/-}$ male brains with 0, 0.1, 0.5, or 1 nM concentrations of Gnrh2 peptide (B). All data were expressed as

means ± S.E.M. Stars indicate significantly different expression levels (** = P<0.01, * = P < 0.05, ANOVA).

Intracerebroventricular injections of Gnrh2 did not elicit any differences in *gnrh3* expression (Fig. 2.9A), however, Gnrh2 injections at concentrations of 0.1 pM induced a significant 1.7-fold increase in *gnih* expression, and injections at 1 pM induced a 2-fold increase (Fig. 2.9B). Incubations of dissected brains with Gnrh2 treatments at concentrations between 0.1 and 1 nM did not change the expression of *gnrh3* (Fig. 2.9C). Gnrh2 *in vitro* treatments of 0.1 and 1 nM concentrations did not affect the expression of *gnih*, but treatments of 0.5 nM downregulated *gnih* expression (Fig. 2.9D).

**Effect of *in vivo* and *in vitro* administration of Gnrh2 on gonadotropin expression**

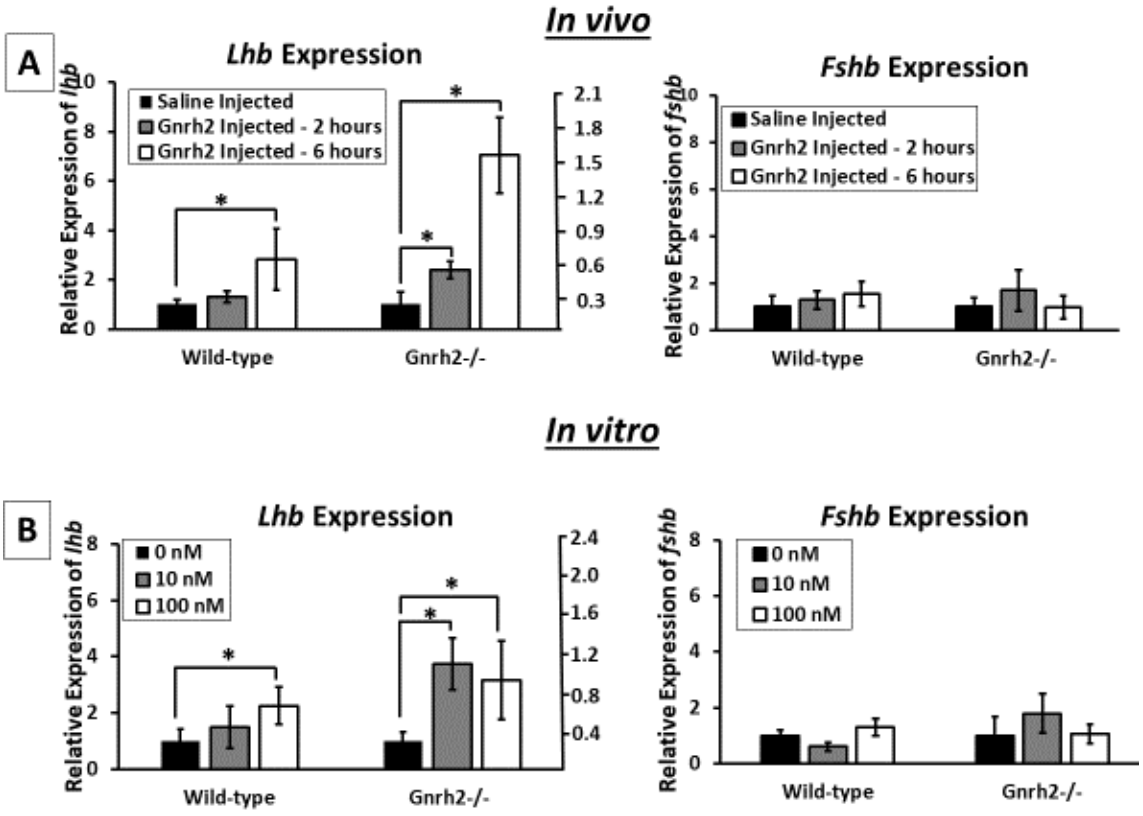


**Figure. 2.10.** *The effect of Gnrh2 on lhb and fshb expression in vivo and in vitro*: Expression levels of *lhb* and *fshb* in wild-type and *gnrh2*$^{-/-}$ male pituitaries after intracerebroventricular injections of Gnrh2 peptide (1 pmol/µL) (A). Expression levels of

*lhb* and *fshb* after i*n vitro* incubations of wild-type and *gnrh2*$^{-/-}$ male pituitaries with 0, 10, or 100 nM concentrations of Gnrh2 peptide (B). All data were expressed as means ± S.E.M. Stars indicate significantly different expression levels (* = P < 0.05, ANOVA).

Intracerebroventricular (ICV) injections of Gnrh2 peptide (1 pmol/µL) to wild-type fish did not show any significant effect after 2 hours, but increased the expression of *lhb* in the pituitary 3-fold compared to saline injected wild-type after 6 hours (Fig. 2.10A). ICV injections of Gnrh2 peptide (1 pmol/µL) to *gnrh2*$^{-/-}$ fish also increased the expression of *lhb* in these zebrafish. Gnrh2 injections elicited an even more pronounced increase of *lhb* expression in *gnrh2*$^{-/-}$ fish compared to wild-type fish, significantly increasing *lhb* expression 2-fold compared to saline injected *gnrh2*$^{-/-}$ as early as 2 hours, and 7-fold at 6 hours post injection (Fig. 2.10A). Congruently, *in vitro* incubations of wild-type pituitaries with Gnrh2 peptide resulted in increased expression levels of *lhb* only at the highest concentration (100 nM, Fig. 2.10B) while in *gnrh2*$^{-/-}$ fish, a 3-fold increase of *lhb* expression was obtained as low as 10 nM and also at 100 nM concentrations (Fig. 2.10B). Contrastingly, *in vitro* incubations of Gnrh2 of pituitaries from wild-type and *gnrh2*$^{-/-}$ males did not induce any differences in *fshb* expression (Fig. 2.10B).

## Discussion

This chapter describes the results of a comprehensive examination into the roles of Gnrh2 in reproduction and point to some potential roles of this peptide in reproductive maintenance, most likely via the regulation of gonadotropin expression in the pituitary and interactions with other brain reproductive factors. The exact functions and roles of the evolutionarily conserved midbrain GNRH2 in reproduction has long been a mystery,

with most studies focusing on studying the reproductive roles of the hypophysiotropic GNRH1 or teleost Gnrh3 form. The zebrafish model, which possess both Gnrh2 and the hypophysiotropic Gnrh3 (Steven et al., 2003), is a perfect organism to study and shed light on the reproductive roles of Gnrh2 in vertebrates. Through genetically editing zebrafish, the first and only organism with a targeted Gnrh2 genetic knockout was generated. To generate this knockout line, a targeted mutation in the *gnrh2* gene of zebrafish was induced using TALEN-mediated technology (Marvel et al., 2018). The mutation caused a frameshift and subsequent disruption of the coding of the Gnrh2 protein. Our knockout zebrafish line is the only model species that harbors such a targeted mutation and the first to demonstrate the consequences of Gnrh2 elimination.

The *gnrh2*$^{-/-}$ line contains a 19 base-pair deletion, which results in a frameshift mutation that also disrupts the coding of the protein. The amino-acid sequence encoded by the mutant gene bears no resemblance to the bioactive form of the Gnrh2 decapeptide. Immunostaining revealed no presence of the Gnrh2 protein in *gnrh2*$^{-/-}$ brains when using either the anti-decapeptide or anti-GAP antibodies, verifying that the *gnrh2* mutation resulted in a complete loss of Gnrh2. The presence of the mutated *gnrh2* cDNA in the *gnrh2*$^{-/-}$ line was verified by sequencing and found to contain no alternative splicing or additional endogenous mutations. Additionally, the *gnrh2*$^{-/-}$ fish are characterized by significantly lower *gnrh2* mRNA compared to wild-type, starting from 3 dpf, suggesting that the mutation causes for instability and possibly degradation of the mutated mRNA as well as preventing the decapeptide formation. Degradation or decay of mRNA is frequently observed after mutations in which a premature stop codon either prevents poly-adenylation or disrupts the translation process (Maquat, 2004). Another possibility

is that mRNA degradation can be induced after targeted-cleavage or nicking of the DNA gene-of-interest (Zebec et al., 2014).

Once the homozygous *gnrh2*$^{-/-}$ line was established, physiological and gene expression differences were determined in reproductive systems between *gnrh2*$^{-/-}$ and wild-type fish. A number of different physiological and histological reproductive parameters were compared between mature *gnrh2*$^{-/-}$ and wild-type fish. Overall, *gnrh2*$^{-/-}$ adults were reproductively fertile, displaying no major differences in fecundity and fertility compared to wild-type adults. Additionally, the gonadal morphology was examined at different stages with no major differences in ovarian and testicular development or gametogenesis. This suggests that Gnrh2 does not have a major role in gonadal development and gametogenesis in zebrafish. However, some minor differences in *gnrh2*$^{-/-}$ females were observed, in which they exhibited decreased GSI, smaller oocytes, and compromised embryo survival, indicating an overall lower oocyte quality. Additionally, even though the GSI of *gnrh2*$^{-/-}$ females was reduced, the body weights and lengths of *gnrh2*$^{-/-}$ females were, on average, larger than wild-type females (see Chapter 3), indicating that the GSI differences may be due to increased somatic weight which did not correspond with increased gamete size in the *gnrh2*$^{-/-}$ females. The decreased egg quality after the loss of Gnrh2 may be due to the increased feeding and somatic growth of the females (see Chapter 3), or the result of the slightly decreased *lhb* expression. The results suggest that Gnrh2 is involved, to some extent, in maintaining optimal oocyte quality in females.

I also examined the role of Gnrh2 in maintaining sperm density and quality due to its roles in spermatogenesis in mammals. Previous studies in boar (*Sus scrufa*) show that

the receptor specific for Gnrh2 in mammals, GnrhR2, is present on Leydig cells of the testes, and that GnrhR2 knockdown resulted in inhibitions of testes growth, sperm quality, and male steroidogenesis, suggesting that Gnrh2 has a role in maintaining testosterone production and spermatogenesis in some species (White et al., 2017). To test whether Gnrh2 has similar roles in zebrafish, I analyzed sperm quality and density in *gnrh2*$^{-/-}$ males compared to wild-type, but the loss of Gnrh2 did not result in any differences in sperm motility or density, clearly demonstrating that Gnrh2 is not a major regulator of spermatogenesis in zebrafish. The fact that the loss of Gnrh2 only results in only minor differences in reproductive characteristics, is most likely due to the abundant presence of Gnrh3 in the pituitary. The continued presence of Gnrh3 is probably also the reason for the observed normal testicular development and spermatogenesis. The results do suggest that Gnrh2 promotes oocyte growth, but is also not crucial for oocyte maturation and ovulation under normal feeding regimes.

Expression levels of a number of different reproductive factors at the brain and pituitary level, throughout development and adulthood, were explored in order to reveal the Gnrh2 reproduction regulatory network. In the first 30 days of development, the loss of Gnrh2 was associated with no differences in Gnrh3, suggesting that during early ontogenesis, Gnrh3 may not be compensating for Gnrh2 loss, and that Gnrh2 does not have a role in maintaining *gnrh3* expression. The loss of Gnrh2 was associated with decreased expression of *gnih* at 4 dpf and 30 dpf, however, the transcript levels of *gnih* were actually significantly higher in adult *gnrh2*$^{-/-}$ males and females, suggesting a possible differential regulatory role during the two life stages. Many factors have different roles depending on the developmental stage, and Gnrh2 and Gnrh3 may have a

completely different action than their function in reproduction in adults. For example, during early larval life stages of zebrafish, Gnrh2 and Gnrh3 may have roles in eye stalk formation and brain organization, considering that Gnrh2 and Gnrh3 knockdown resulted in the differential expression of many genes involved in these processes, as well as brain and eye morphology (Wu et al., 2006). It is possible that Gnrh2 also has differential roles throughout larval development, including regulating other gene transcript levels, such as *gnih*, which results in its abnormal expression after the loss of Gnrh2. GNIH, or LPXRFA, is an RFamide protein (Tsutsui et al., 2000) which exhibits a normally inhibitory role on GNRH and gonadotropin production in vertebrates (Tsutsui et al., 2009), although the role of Gnih in teleosts has been found to be either stimulatory or inhibitory to gonadotropin synthesis (Moussavi et al., 2014, Ogawa et al., 2016, Spicer at al., 2017). The roles of Gnih in reproduction and interactions with Gnrh3 in zebrafish has been closely examined previously by a previous graduate student in our lab (Spicer et al., 2017). She showed that Gnih and Gnrh3 interact neuroanatomically, and that Gnih is able to downregulate *cga* and *lhb* expression in the pituitary, and *gnrh3* expression in the brain (Spicer et al., 2017). During development, it is possible that *gnrh2* may be involved in *gnih* regulation, or the loss of Gnrh2 has indirect effects resulting in decreased *gnih* expression throughout this period.

The loss of Gnrh2 was also associated with lower levels of *lhb* throughout development, but higher levels of *fshb*. In adulthood, the expression of *lhb* continued to be lower in *gnrh2*$^{-/-}$ adults, and significantly so in males, whereas *fshb* and *cga* expression was similar between the two genotypes. This suggests that Gnrh2 may have more prominent roles in Lh regulation, and thus, late stage oocyte maturation and ovulation.

This is a similar phenomenon that has been studied in other species, where Gnrh regulated *lhb* but not *fshb* expression and protein secretion. In European sea bass (*Dicentrarchus labrax*), Gnrh treatments increased the expression of *lhb* and *cga*, as well as the plasma level of the Lh hormone, but had no effect on *fshb* expression or protein secretion (Mateos et al., 2002). Mammals also exhibit differential regulation of the gonadotropin transcripts, as the effect of Gnrh on gonadotropin secretion is dependent on the rate of pulsatile release of Gnrh, with fast Gnrh pulses upregulating *lhb* and *cga* transcript levels, and slow pulses upregulating *fshb* transcript levels (Haisenleder et al., 1991). As for zebrafish, they exhibit asynchronous ovaries, where oocytes at all stages of maturation are present, and random early stage oocytes are constantly being recruited into late stage mature oocytes. As Fsh is more involved in the early stages of follicle maturation (Zhang et al., 2015), and Lh is involved in final stages of maturation and ovulation (Chu et al., 2014), it is possible that Fsh is able to regulate itself or has multiple regulators, whereas the regulation of Lh is more specific in order to coordinate final maturation and ovulation only at specific times when it is beneficial for spawning and egg-laying. It is also possible that Gnrh2 selectively regulates *lhb* expression, whereas Gnrh3 regulates *lhb* and *fshb*, but this would need to be studied further to determine if it is the case, along with Gnrh receptor localization on gonadotrope cells.

In adulthood, Gnrh2 knockout resulted in differential expression of several genes. There was upregulated expression of *gnih* and *spx* in both sexes and *scg2a* and *tac3a* in males only. As all of these neuropeptides have been shown to be hypophysiotropic, with the abilities to modulate gonadotropin expression or release (Zhao et al., 2006, Spicer et al., 2017), it is possible that this upregulation is a compensatory mechanism to continue

regulating gonadotropin levels and reproduction in the absence of Gnrh2. The sex differences in *scg2a* expression may account for the sex-differences in the gamete quality of *gnrh2*$^{-/-}$ fish, as male gametes showed no differences in quality or density, whereas female oocytes appeared to have slightly diminished quality, and *scg2a* has previously been found to be hypophysiotropic and able to stimulate gonadotropin release in goldfish and mice pituitary cells (Zhao et al., 2006, 2011). The functional relationships and interactions of Gnrh2 with other reproduction neuropeptides will need to be examined further in the future. Interestingly, whereas the single *gnrh2* KO line displayed some differences in reproductive phenotypes, zebrafish lines harboring a null mutation in the *gnrh3* gene (both single and double Gnrh KO) displayed no differences in reproductive parameters (Spicer et al. 2016, Liu et al. 2017, Marvel et al. 2018), potentially due to the differences in the differential expression patterns in these lines. It is possible that the loss of the hypophysiotropic Gnrh3 activates a reproductive compensation that stimulates the reproductive HPG axis and, in turn, maintains normal gonadal development and reproduction. In support of the compensation theory, upregulation of other reproductive neuropeptides, including *avp*, *tac3a*, and *scg2a*, occurs in Gnrh3 KO lines, but does not occur with just the loss of Gnrh2 (Liu et al., 2017, Marvel et al., 2018). This difference may point to the relative higher importance of Gnrh3 in reproduction compared to Gnrh2, and suggests that Gnrh2 loss results in minor differences that do not require compensation, for the fish to be able to reproduce and development normally.

The differential expression of *gnih* in *gnrh2*$^{-/-}$ fish, prompted a closer look at the interactions between Gnrh2 and Gnih neurons. In terms of relationships between Gnih and Gnrh2 in fish, interactions have been shown in the fish species, *Astyanax*

*altiparanae*, where zebrafish zGnih-3 peptide was able to increase *gnrh2* mRNA levels, but downregulated the ability of Gnrh2 to stimulate gonadotropin expression, showing a dual mode of regulation (Branco et al., 2019). However, the relationships of Gnrh2 and Gnih have not yet been studied in zebrafish, therefore IHC was conducted to double-label Gnrh2 and Gnih in the brain. Immunostaining revealed that Gnrh2 is abundantly found near Gnih soma in the periventricular nucleus of the hypothalamus and the peptide may be able to reach Gnih soma. Concurrently, Gnih neuronal fibers are also seen surrounding and interposed on (potentially contacting) Gnrh2 soma in the midbrain, suggesting a cross-talk between the two systems. It is also possible that Gnih is an upstream regulator of Gnrh2, due to its abundant innervations of Gnrh2 cells however, this possibility needs to be studied more in the future. A similar neuroanatomical relationship of Gnih and Gnrh2 exists in other models, as it has been shown that in mammalian models, GNIH is innervating GNRH2 cells (Tsutsui, 2009), and GNIH receptors are found in GNRH2 cells of European starlings (*Sturnus vulgaris*) (Ubuka et al., 2008). Data suggesting that this is also the case in zebrafish was obtained by Gnrh2 treatments *in vivo* and *in vitro*, that resulted in modulation of *gnih* transcript. This effect was dependent on the concentration of Gnrh2 and the mode of administration: with *in vitro* Gnrh2 administrations, moderate concentrations (0.5 nM) resulting in *gnih* downregulation, but higher concentrations of 1 nM resulted in no differences. When Gnrh2 was injected *in vivo* to live fish, both concentrations induced upregulation of *gnih*. We wanted to also examine the effect of Gnrh2 on gene expression levels *in vitro*, since *in vivo* approaches can allow many different external factors, including the potential for other brain factors outside of the Gnrh system, to influence expression values system-wide. *In vitro* experiments allowed

for a more direct approach to introduce Gnrh2 to only brains or pituitaries without additional factors being present to influence the outcome. There seems to be a complicated relationship between Lpxrfa and Gnrh systems, which needs to be further investigated, but may be part of a feedback system, where the two peptides, dependent on their concentrations, can mutually either down or upregulate the other in order to modulate reproductive outputs. Based on the functional and neuroanatomical results, Gnrh2 most likely has an indirect effect on Gnih. Gnrh2 may directly influence other factors or behaviors, which in turn, affect Gnih levels. It is also possible that Gnrh2 and Gnih are part of a multi-factor network of communication that mediates reproduction. In other animals, GNIH has been shown to inhibit GNRH2, such as in songbirds (Bentley et al., 2006), and this upstream control of Gnrh2 would need to be further investigated in zebrafish as well.

An interesting peptide, which has just begun to be studied in fish, Spexin, has been shown to have roles in both reproduction and feeding and in our results, showed differential expression with the loss of Gnrh2. Spexin has been shown to have inhibitory effects on reproduction in goldfish, as it can inhibit Lh secretion via *in vivo* and *in vitro* treatments, and *spx* mRNA levels decreases during the sexual maturation of goldfish (Liu et al., 2013). The gene for Spexin is located close to the *kiss2* loci and is thought to have coevolved with Kisspeptin and Galanin peptides (Kim et al., 2014). In zebrafish, Spexin is localized in both brain and ovary tissues, suggesting it has reproductive roles in this species as well (Liu et al., 2013). The upregulation of Spexin in *gnrh2*$^{-/-}$ fish suggests that Gnrh2 may play a role in regulating Spexin, and since Gnrh2 normally increases spawning behaviors (Volkoff and Peter, 1999) and *lhb* expression, may help to decrease

Spexin levels in order to stimulate reproduction. Spexin is additionally thought to also have major roles in feeding regulation in both mammals and fish (Ma et al., 2018; Wong et al., 2013). In zebrafish, Spexin knockout (*spx1*$^{-/-}$) resulted in increased food intake and dysregulated *agrp1* expression, suggesting that Spexin may function to decrease feeding in zebrafish through the downregulation of *agrp1* (Zheng et al., 2017). Since Gnrh2 has also been shown to be an anorexigenic hormone, able to downregulate feeding behaviors in zebrafish (Nishiguchi et al., 2012), it is possible that it works in conjunction with Spexin to enable these roles. A closer examination of the interactions between Gnrh2 and Spexin should be conducted in the future to determine these relationships, and may help elucidate knowledge on the mediation between reproduction and feeding.

The next step was to conclusively explore the ability of Gnrh2 to regulate gonadotropin expression in zebrafish. Using the tg(Gnrh2:eGFP) line generated in the Zohar lab, it was previously shown that some Gnrh2 neuronal projections innervate the pituitary of zebrafish (Xia et al., 2014). Gnrh2 has been shown to be hypophysiotropic in goldfish and able to elicit Lh release, and in some cases, is even more potent than Gnrh3 at upregulating *lhb* mRNA levels (Khakoo et al., 1994). In many teleosts, such as goldfish, gilthead seabream (*Sparus aurata*), and African catfish (*Clarias gariepinus*), Gnrh2 can bind to and activate Gnrh receptors on gonadotrope cells with greater potency than Gnrh3 or Gnrh1 (Zohar et al., 1995, Illing et al., 1999, Bosma et al., 2000). These observations, together with the finding that *gnrh2*$^{-/-}$ larvae and males exhibited significantly lower *lhb* expression levels prompted us to look into the ability of Gnrh2 to modulate gonadotropins also in zebrafish. Several different *in vivo* and *in vitro* approaches of administering exogenous Gnrh2 to zebrafish demonstrated that Gnrh2 can

elicit increased *lhb* expression in zebrafish pituitaries, however, the same treatment did not result in any changes in *fshb* expression. Together, with the finding of the overall lower levels of lhb in *gnrh2*$^{-/-}$, it seems that Gnrh2 specifically regulates *lhb*, but not *fshb*. In zebrafish, Lh and Fsh cells are discrete and separate cells (Golan et al., 2014), unlike in mammals in which certain cells express both gonadotropins (Herbert, 1976). Gnrh2 treatment elicited upregulation of *lhb* at a higher magnitude in *gnrh2*$^{-/-}$ fish compared to wild-type, potentially due to the fact that *gnrhr3* is also upregulated 3-fold in *gnrh2*$^{-/-}$ fish (Fig. 2.7), and this receptor has been proposed to be most specific to Gnrh2 over the other isoforms (Tello et al., 2008, Nishiguchi et al., 2012). Definitive proof for the specificity of Gnrh2 to Lh, but not Fsh, would be to determine whether colocalization of this receptor occurs in Lh but not Fsh gonadotropes. Taken together, the loss of Gnrh2 that was associated with significantly decreased levels of *lhb* in males, and the rescue of these low levels in *gnrh2*$^{-/-}$ fish by Gnrh2 peptide, infer that Gnrh2 most likely has a hypophysiotropic role in stimulating *lhb* expression.

In summary, knocking out *gnrh2* in zebrafish enabled a comprehensive study of the roles of Gnrh2 in reproduction. The functional assays demonstrated that Gnrh2 most likely has a role in maintaining egg quality and *lhb* expression. Furthermore, the loss of Gnrh2 was associated with differences in several different reproductive peptide expression levels, suggesting that Gnrh2 interacts with these differentially expressed factors, including Gnih and Scg2a, and is probably part of a broader neuropeptide network controlling reproduction. The fact that Spexin expression is also affected infers that Gnrh2 may be involved in additional networks controlling other physiological processes such as metabolism (Zheng et al., 2017).

# CHAPTER 3: Investigating the Roles of Gnrh2 in Feeding and Growth

## Abstract

The Gonadotropin-releasing hormone (GNRH) family has long been known to be major regulators of reproduction in vertebrates. Recently, studies in a few vertebrate species have shown that the midbrain isoform of GNRH, GNRH2, abundantly innervates areas of the brain involved in regulating feeding, including the melanocortin system, and may be a regulator of feeding behaviors and metabolism in addition to regulating reproduction. Previous behavioral studies in sparrows, musk shrews, mice, zebrafish, and goldfish show that exogenous GNRH2 both increases spawning behaviors and decreases feeding behaviors, suggesting a role of this peptide in metabolism regulation that may be conserved across many different vertebrate groups. The knockout Gnrh2 (*gnrh2*$^{-/-}$) zebrafish line was used to analyze the effect of its loss-of-function on feeding, growth, and mobility. The results revealed major differences in feeding and growth parameters, clearly demonstrating that Gnrh2 is a potent anorexigen in zebrafish. Mobility studies of the *gnrh2*$^{-/-}$ line support the anorexigenic effect of Gnrh2 as there were no differences in mobility except for increased distances swam during feeding periods. Further support for the anorexigenic role of Gnrh2 was evident from the array of differentially expressed feeding/metabolism genes in *gnrh2*$^{-/-}$ juveniles and adults, particularly *nesfatin1*, *proopiomelanocortin a*, *hypocretin*, and *agouti-related peptide 1* (*agrp1*). Focusing on Agrp1, the most important orexigenic peptide in zebrafish, further studies revealed robust neuroanatomical and functional interactions between Gnrh2 and Agrp1 neurons. Functional studies revealed that Gnrh2 brain treatments *in vivo* or *in vitro* downregulated *agrp1* gene expression. Knockdown studies revealed that treatment of embryos with *agrp1* or *gnrh2* morpholino antisense oligomer modulated each other's expression.

Neuroanatomical studies revealed mutual neuronal interactions and reciprocal receptor expression of Gnrh2 and Agrp1. Taken together, these findings suggest a role for Gnrh2 in controlling satiation in zebrafish, which may be enacted through its ability to directly act on Agrp1 neurons to downregulate *agrp1* expression and in turn to curb appetite. The study demonstrates dynamic relationships between Gnrh2 and Agrp1 under different feeding regimes overall suggesting that Gnrh2 may act together with Agrp1 to prevent overfeeding.

## Introduction

Despite its conservancy and ubiquity in most vertebrates, except rodents, the exact roles of GNRH2 are not fully known (Miyamoto et al., 1984, Kah et al., 1993, Zohar et al., 2010). A few studies that have looked into the roles of GNRH2 have suggested that GNRH2 acts as an anorexigenic factor (Hoskins et al., 2008, Nishiguchi et al., 2012), along with a role in stimulating reproductive behavior (Volkoff and Peter, 1999, Temple et al., 2003, Kauffman and Rissman, 2004, Hoskins et al., 2008). Studies conducted across many vertebrate groups, including birds, mammals, and fish, show that Gnrh2 administrations can ubiquitously attenuate feeding behavior (Volkoff and Peter, 1999, Millar, 2003, Temple et al., 2003, Kauffman et al., 2005). Even mice (*Mus musculus*), which do not possess Gnrh2 due to a partial gene deletion, exhibit decreased feeding after GNRH2 injections (Kauffman, 2004). The findings that GNRH2 is involved in the regulation of both feeding and reproduction suggests that it serves a role in both of these important processes. Therefore, after first establishing the reproductive roles of Gnrh2, the focus of this chapter was on investigating whether this neuropeptide is also involved in the regulation of feeding, and in turn, growth, in zebrafish.

Previous studies using the transgenic tg(Gnrh2:eGFP) zebrafish line, describe a robust network of innervations throughout the brain, suggesting a neuromodulatory role. Remarkably, Gnrh2 neuronal projections into the pituitary are extended when the fish are subjected to long fasting conditions (Xia et al., 2014). Additionally, the particularly strong Gnrh2 presence found along the spinal cord suggests a possible role for Gnrh2 in regulating mobility, which could be a way to manipulate behavioral aspects of feeding (Okuzawa et al., 2003). However, the extent and importance of these roles was not clear, mainly due to the fact that rodent do not possess GNRH2 (Stewart et al., 2009). Nevertheless, a few studies on the administration of Gnrh2 have suggested that Gnrh2 may act as a satiety factor in zebrafish and goldfish (*Carassius auratus*) (Hoskins et al., 2008, Matsuda et al., 2008, Nishiguchi et al., 2012). Additionally, interactions of Gnrh2 with other feeding neuropeptides in fish have been recently brought to light. Gnrh2 has been shown to have a mutual inhibitory relationship with the orexigenic neuropeptide, Hypocretin, in goldfish (Hoskins et al., 2008). Also, Gnrh2 has been shown to potentially mediate the actions of two anorexigenic neuropeptides, alpha Melanocyte-stimulating hormone (a-Msh) and Corticotropin-releasing hormone (Crh) in goldfish (Kang et al., 2011).

However, a detailed look at the interactions between Gnrh2 and one of the most potent orexigens in zebrafish, Agouti-related peptide 1 (Agrp1), has not yet been conducted in any organism. Previous studies show that Agrp1 may be of high importance in controlling feeding in zebrafish. Agrp1 has been shown to be the most highly upregulated factor after fasting in zebrafish, as *agrp1* transcripts were the only gene found to be upregulated after a fasting regime, and Agrp1-expressing cells were shown to

be more numerous in number in the lateral hypothalamus of fasted fish compared to fed fish (Jeong et al., 2018, Drew et al., 2008). Endogenous internal factors which can regulate Agrp1 have not yet been examined, and therefore this system was chosen to determine if Gnrh2 has any upstream control of Agrp1 to get a clearer picture of feeding regulatory networks in vertebrates.

This chapter describes the effects of the loss of Gnrh2 on feeding and growth phenotypes and suggests that Gnrh2 is able to influence these two systems. Additionally, possible mechanisms of action of Gnrh2 were examined by studying its roles in regulating and interacting with another potent orexigenic peptide, Agrp1. Mutual innervations of Gnrh2 and Agrp1 neuronal populations were seen and functional assays determined that Gnrh2 can modulate *agrp1* expression. Our findings corroborate the suggested role of Gnrh2 as a potent anorexigenic hormone in zebrafish. Overall, our results support the notion that Gnrh2 has roles in controlling both feeding and reproductive signals in the brain of zebrafish and may function to curb appetite and inhibit the appeal of food through downregulating *agrp1*.

## Methods

### Larval feeding assays

First, Gnrh2 loss-of-function effects on larval feeding were examined to determine whether Gnrh2 has roles in early-stage feeding regulation. In order to quantify the amount of food ingested by zebrafish larvae, a fluorescent paramecia feeding assay was developed based on the protocol by Shimada et al. (2012), with some minor modifications. *Paramecium* were stained with the fluorescent dye, 4-(4-

(didecylamino)styryl)-*N*-methylpyridinium iodide (4-di-10 ASP) (Molecular Probes, Eugene, OR, USA), for one hour, washed in fish water (0.6 g sea salt in 1 L RO water), centrifuged, and resuspended to obtain varying concentrations in fish water. Fluorescently-dyed paramecia were then fed to groups of *gnrh2*$^{-/-}$ and wild-type larvae aged 7-10 dpf. Paramecia densities were quantified by counting *Paramecium* cells in 8 different 10 µL droplets under a dissecting scope. The optimal feeding concentration of paramecia was determined by assessing the highest fluorescent readings of individual fish fed during a one-hour period. Fish between 7-10 dpf, who were seen to be actively feeding, were selected for the experiment, starved for 24 hours, placed in 5 mL wells (6 individuals per well in a 24 well plate), then fed the same concentration of fluorescently-stained paramecia. Time course feeding trials were conducted for 15, 30, 60, and 120 minutes with triplicate groups of six *gnrh2*$^{-/-}$ and wild-type larvae for each time point collected, washed in fish water, and quickly killed with tricaine. The larvae were then sonicated in 300 µL RO water using a Branson Sonifier 250 sonicator (Cole Parmer, Vernon Hills, IL, USA) to homogenize the samples (6 pulses of 0.6 seconds at 60% max output). Fluorescence in lysates was measured at 485 nm excitation and 535 nm emission, with larvae fed unstained paramecia as the blank control in a 96 well plate reader (Spectramax Gemini XS, Molecular Devices, Sunnyvale, CA, USA). The ability of the spectrophotometer to detect differences in paramecia densities was initially tested and verified by sonicating paramecia solutions at densities between 10 and 2,000 cells/mL.

**Adult feeding assays**

Adult food intake was also quantified in 18-hour fasted adults by placing six *gnrh2*$^{-/-}$ females and males in individual tanks alongside the same number of wild-type counterparts of the same size and age. A total of 60 Pellets (300 µm in size) were fed to each tank and the number of pellets eaten was counted every 15 minutes during a one-hour period of time.

**Mobility comparison**

To assess mobility of *gnrh2*$^{-/-}$ and wild-type larvae, 24 well plates containing zebrafish larvae were placed in a DanioVision observation chamber and EthoVision XT software (Version 8.0, Noldus Information Technology, Wageningen, The Netherlands) was used to track individual fish movements and quantify distances swam. Seven trials were conducted using fish between 7-10 dpf who were actively eating *Paramecium*, and three trials were conducted using fish at 18 dpf who were actively eating *Artemia.*

For each trial, 12 *gnrh2*$^{-/-}$ and 12 wild-type larvae were placed individually in wells containing 1.5 mL of fish water and allowed to swim freely. Distances and velocities of fish were quantified every 10 seconds for a total of 7.5 hours. After one hour, lights were turned off for 10 minutes to elicit a quick startle response, and analysis was then continued for another 1.5 hours to allow the fish to reach basal swimming behavior. *Paramecium* or *Artemia* nauplii were added 2.5 hours after the trial was initiated, and analysis continued for another 5 hours to quantify mobility during feeding.

**Growth measurements**

Body lengths and dry weights of wild-type and *gnrh2*$^{-/-}$ zebrafish larvae were determined throughout development in fish at 4, 8, 12, 16, 24, 30 dpf. Prior to collection, larvae were placed in tanks of identical densities and fed the same amount of *Paramecium* or *Artemia*, twice daily. To obtain body length measurements, an image of a slide containing a scale bar was captured using a CCD Olympus DP70 camera and Zeiss Axioplan2 microscope followed by imaging each wild-type and *gnrh2*$^{-/-}$ larvae on the same slide. Body lengths were then quantified using the ImageJ software (U.S. National Institutes of Health, Bethesda, MD, USA). A minimum of 10 larvae from three different spawning pairings were measured at each time point (for a total of 30 embryos/genotype/time point). To obtain dry body weight measurements, 15 larvae (at 4 and 8 dpf), 10 larvae (at 12, 16, and 24 dpf), or 6 juveniles (at 30 dpf) of each genotype were quickly killed in tricaine, washed in distilled water, and placed in pre-weighed tubes, in triplicate. The fish were then lyophilized overnight and weighed. To determine any differences in adult weights, ten adult females and sixteen males of each genotype at six months of age were fasted for one day, then collected, blotted dry with a paper towel, weighed, and standard body lengths (nose to caudal peduncle) measured.

**Feeding and growth gene expression comparisons during development**

To determine if Gnrh2 has a role in regulating expression of feeding factors during development, the gene expression of several feeding and growth neuropeptides, including *growth hormone* (*gh*), *insulin-growth factor 1a* (*igf1a*), *nesfatin1*, *agouti-related peptide 1* (*agrp1*), *corticotropin-releasing hormone* (*crh*), *neuropeptide y* (*npy*), *pro-melanin concentrating hormone 2* (*pmch2*), *hypocretin* (*hcrt*), and *proopiomelanocortin a*

(*pomca*) were compared between wild-type and Gnrh2 knockout (*gnrh2*$^{-/-}$) larvae throughout the first 30 days of zebrafish development. Samples collected to compare the developmental profile of feeding/growth genes included whole zebrafish larvae and juveniles at 1, 2, 3, 8, 12, 18, 24, and 30 dpf, which were quickly killed in tricaine, washed in distilled water, and then flash frozen in dry ice. Samples, in triplicate, for each genotype (wild-type and *gnrh2*$^{-/-}$) were collected and pooled (as follows: n=20 for 1, 2, and 3 dpf; n=15 for 8 and 12 dpf; n=10 for 18 and 24 dpf; and n=6 for 30 dpf). Total RNA from samples was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The Quantitect Reverse Transcription Kit (Qiagen, Valencia, CA, USA), that includes gDNA Wipeout to eliminate gDNA contamination, was used to reverse transcribe 1 µg of RNA from each sample. In each round, a non-RT control and no template control were added to determine gDNA and template contaminations. QPCR was conducted using 20 ng of cDNA for each sample in duplicate with SYBR Green qPCR mix and gene-specific primers, with CT values for each sample normalized against an internal eef1α1 control. Amplification of cDNA was performed on a 7500 Fast Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA) and conditions included a 2 minute 95°C activation, 5 second 95°C denaturation, and 30 second 60°C annealing, with the last two steps repeating 40 times. For each gene, expression values were additionally normalized to the 1 DPF wild-type expression value for relative comparisons between *gnrh2*$^{-/-}$ and wild-type larvae.

**Feeding and growth gene expression comparisons in adults**

Differences in the gene expression levels of the following various feeding and growth peptides: *agouti-related peptide 1* (*agrp1*), *neuropeptide y* (*npy), hypocretin* (*hcrt),*

*proopiomelanocortin a* (*pomca*), *pro-melanin concentrating hormone 2* (*pmch2*), *insulin growth factor 1a* (*igf1a), pituitary adenylate cyclase activating peptide 1* and *2* (*pacap1* and *pacap2*) in adulthood were determined using qPCR. Brains and pituitaries from six to eight *gnrh2*$^{-/-}$ and wild-type adults of the same age and sex were dissected, flash frozen in dry ice, and stored at -80° C until RNA extraction. Total RNA from samples was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The Quantitect Reverse Transcription Kit (Qiagen, Valencia, CA, USA) that includes gDNA Wipeout to eliminate gDNA contamination was used to reverse transcribe 1 µg of RNA from each sample. In each round, a non-RT control and no template control were added to determine gDNA and template contaminations. QPCR was conducted using 20 ng of cDNA for each sample in duplicate with SYBR Green qPCR mix and gene-specific primers (Table 2.1), with $C_T$ values for each sample normalized against an internal *eef1α1* control. Amplification of cDNA was performed on a 7500 Fast Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA) and conditions included a 2 minute 95°C activation, 5 second 95°C denaturation, and 30 second 60°C annealing, with the last two steps repeating 40 times.

**Morpholino injections and subsequent gene expression analysis of *gnrh2* and *agrp1* and growth assessments**

In order to determine if Gnrh2 and Agrp1 influence the expression of each other, injections of antisense morpholino oligomers (MO), to induce *gnrh2* or *agrp1* knockdown through blocking pre-mRNA splicing or translation, in zebrafish embryos was conducted. For *agrp1* knockdowns, morpholino oligos targeting and blocking Agrp1 translation were used, and for *gnrh2* knockdown, morpholino oligos targeting and

blocking *gnrh2* pre-mRNA splicing were used. Zebrafish eggs at the 1-2 cell stage were collected after spawning, and MOs diluted to a concentration of 0.5 mM, mixed with 0.1% phenol red (for visualization during injections), were microinjected at 1-2 nL/embryo directly to the cell of each embryo. Several hundred embryos were injected with either Agrp or Gnrh2-targetting MOs along with several hundred embryos (from the same spawning cohort) injected with control morpholinos of the same concentration. At 24, 48, and 96 hours post-injection, zebrafish embryos were dechorionated (if unhatched) and 20 Agrp or Gnrh2 MO-injected embryos collected per tube alongside 20 control MO-injected embryos. Three tubes of 20 embryos were collected at each time point, for each experimental treatment, and each experiment repeated at least once. Zebrafish embryo samples were immediately frozen on dry ice and stored at -80° C until RNA extraction was conducted. For RNA extraction, 100 uL of Trizol was added to each tube and RNA extraction conducted according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). The Quantitect Reverse Transcription Kit (Qiagen, Valencia, CA, USA) that includes gDNA Wipeout to eliminate gDNA contamination was used to reverse transcribe 500 ng of RNA from each sample. In each round, a non-RT control and no template control were added to determine gDNA and template contaminations. QPCR was conducted using 20 ng of cDNA for each sample in duplicate with SYBR Green qPCR mix and gene-specific primers (Table 2.1), with $C_T$ values for each sample normalized against an internal *eef1α1* control. Amplification of cDNA was performed on a 7500 Fast Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA) and conditions included a 2 minute 95°C activation, 5 second 95°C denaturation, and 30 second 60°C annealing, with the last two steps repeating 40 times.

For growth measurements, 10 Gnrh2 MO or control MO embryos at 2, 4, and 30 dpf were anesthetized in tricaine (MS-222) and imaged alongside a scale bar image for reference. Measurements for each embryo were quantified using ImageJ and averaged for each time point/genotype.

### *Gnrh2* and *agrp1* knockdown verification

In order to assess whether morpholino injections targeting Agrp1 translation were efficient at knocking down its intended target, whole-mount immunohistochemistry using antibodies against Agrp were used on Agrp and control MO-injected embryos at 3 dpf. Embryos were fixed in 4% PFA (paraformaldehyde) overnight at 4°C, washed twice in PBS, and then transferred to 25%, 50%, and then 100% methanol (MeOH) solutions and stored at -20°C until IHC conducted. For IHC, fixed embryos were washed in cold acetone for 8 min in -20°C. then washed in 100% MeOH for 5 min at room temp. Samples were washed in 50% MeOH/50% PBS-Tr (PBS + 0.5% Tritonx100) for 5 min, and then washed in PBS-Tr 3X for 5 min. In order to increase antibody permeation, 3 dpf embryos were treated with proteinase K (10 µg/mL) for 30 minutes. Samples were washed twice in PBS-Tr and fixed in 4% PFA for 20 min. Samples were washed 3 X in PBS-Tr for min, and then blocked in blocking solution (10% Goat Serum and 1% DMSO in PBS-Tr) for 1 hour on a rotating shaker at 60 RPM. Samples were then incubated with a commercially available primary antibody against Agrp1 (Phoenix Pharmaceuticals, Inc, Burlingame, CA, USA), previously used to immunostain Agrp1 in zebrafish brains (Forlano and Cone, 2007) diluted 1:400 in blocking solution for 1-2 hours at RT on a rotating shaker, and then overnight at 4°C. The next day, samples are washed 4-6 X in PBS-Tr for 30 min and then incubated in secondary antibody (Goat Anti-Rabbit IgG

H&L, Alexa Fluor 555) at 1:100 in blocking solution for 2 hours on a rotating shaker. Samples were then washed 4X in PBS-Tr for 20 min, then mounted in 1.5% low-melting agarose for imaging.

In order to verify the blocked splicing and translation of Gnrh2 after Gnrh2 MO injections, Gnrh2-targeting morpholinos and control morpholinos were injected to transgenic tg(Gnrh2:eGFP) eggs at the 1-2 cell stage (see previous section for morpholino injection protocol). At 6 dpf, when transgenic fluorescence signal is bright and clear in both the midbrain and olfactory epithelium, ten control MO-injected embryos, and ten Gnrh2-MO injected embryos were collected and anesthetized in MS-222. Embryos were mounted in 1.5% low-melting point agarose and imaged on a Zeiss Axioplan2 microscope and CCD Olympus DP70 camera and screened for the presence of Gnrh2-specific fluorescence in the brain.

**Gnrh2 *in vivo* treatment studies**

We wanted to examine if Gnrh2 treatments could directly affect the levels of *agrp1* transcript. The effect of Gnrh2 on *agrp1* in wild-type males was analyzed *in vivo* with intracerebroventricular (ICV) injections of Gnrh2 peptide to the third ventricle of the brain of male zebrafish. ICV injections were conducted using a 50 µL Hamilton syringe equipped with a 30-gauge needle and a 2 mm penetration stopper. Male fish (weighing 200 mg) were deeply anesthetized in Tricaine, and then injected with Gnrh2 peptide at concentrations of 0 (control), 0.1 pM, 1 pM, 0.1 nM, and 1 nM in saline containing 0.05% Evans Blue dye (six fish per treatment concentration). Fish were monitored for normal behavior, and after 6 hours, fish were euthanized, and brains dissected. Successful injections were verified by the presence of Evans Blue Dye in the third ventricle during

dissections. Brains were flash frozen in dry ice and stored at -80°C until RNA extraction was conducted, as discussed in previous sections), followed by qPCR analysis to compare *agrp1* mRNA levels.

**Gnrh2 *in vitro* treatment studies**

In order to see if Gnrh2 treatments *in vitro* can affect *agrp1* expression, brains of size and age-matched wild-type adult male zebrafish were dissected and placed in sterile cell-culture 12-well plates containing 2 mL of cold DMEM media (supplemented with 25 mM D-gluocose, HEPES, 50 µM bacitracin, 50 µM ascorbic acid, and 0.1% BSA, pH adjusted to 7.4) and filter sterilized. Brains were chopped into 200 uM thick sections with a mechanical tissue chopper (McIlwain, the Vibratome Company) and placed back into wells. Plates were rotated at 45 RPM at 28° C, and media changed out every hour for 3 hours. Subsequently, media with varying concentrations of Gnrh2 peptide (0, 0.1, 0.5, and 1 nM) replaced the washing media (n=6 wells/concentration) and brains incubated in the varying concentrations of Gnrh2 for 6 hours. After 6 hours, brain tissues were collected in 1.5 mL tubes and flash frozen at -80C until RNA extraction. RNA extraction was conducted with Trizol reagent, as described above, and qPCR analysis of *agrp1* expression conducted. All values were normalized to a housekeeping gene, *eef1a*, expression values and further normalized relative to the control (0 nM) expression values for fold-change analysis.

**Gnrh2 and Agrp1 neuroanatomical interaction analyses**

Interactions between Gnrh2 and Agrp1 in the brain were examined using double-label immunohistochemistry with antibodies against the specific recombinant GAP

region of Gnrh2 (GAP2) raised in rabbits or using a commercially available Agrp antibody (Phoenix Pharmaceuticals, Inc, Burlingame, CA, USA), previously used to localize Agrp1 in zebrafish brains (Forlano and Cone, 2007). Brains from 6-month old wild-type fish were dissected and fixed in 4% PFA overnight and immersed in 30% sucrose in PBS for four hours, or until brains sunk to the bottom of the vial. Brain samples were then frozen in OCT and stored at -80° C. Cryo-sectioning was conducted at -20° C using a Tissue-Tek Cryo3 cryostat. Brains were sectioned to 10 µM thickness, placed on Plus coated slides, and stored at -80°C until immunohistochemistry (IHC) was performed. To perform double-staining IHC, slides were briefly fixed in acetone, and quenched in 0.3% $H_2O_2$ in PBS for 30 minutes. Slides were then washed in PBS, blocked for one hour in 5% normal goat serum, and incubated with a 1:1,000 dilution of Anti-GAP2 in 1% BSA and 0.3% Triton X-100 overnight at 4°C. Slides were then washed in TNT (100 mM Tris Ph 7.5, 150 mM NaCl, 0.5% Tween-20) and incubated in an HRP-conjugated Goat anti-Rabbit antibody (Genscript) at a 1:1,000 dilution in 1% BSA and 0.3% Triton for one hour. Then, slides were washed in TNT X 3 and incubated in a fluorescein dye from the Tyramide Signal Amplification Plus kit (TSA Plus kit, Perkin Elmer) at a 1:50 dilution for 5 minutes, washed in TNT, and HRP signal quenched with 0.02 N HCl for 10 minutes. After washing, the procedure for IHC delineated above was repeated on the same slides, but with an Anti-Agrp1 primary antibody at a 1:400 dilution and Cy3 dye from the TSA kit to label Agrp1 protein. Slides were mounted in 50% glycerol plus 10 µg/ml Hoescht 33342 (Sigma) and viewed on a Leica Sp6 confocal microscope and screened for the presence of Gnrh2 and Agrp1 soma and fibers.

**In situ hybridization (ISH) of *mc4r* and *gnrhr1***

In situ hybridization to detect *mc4r* expression in Gnrh cells or *gnrhr1* expression in Agrp1 cells was conducted on wild-type adult brain sections at 10 uM thickness, fixed and sectioned as depicted in previous immunohistochemistry Methods sections above. Slides with brain sections were briefly dried at 55°C for 5 minutes, and then washed twice in DEPC-treated PBS. Slides were fixed in 4% PFA for 15 minutes, and then washed 3X in PBS. To eliminate endogenous peroxidases, brain sections were quenched with 0.3% H2O2 in methanol for 30 minutes, and then washed in PBS. Slides were incubated in 0.1 M TAE (with 0.25% acetic anhydrate and 0.05N NaOH) for 10 minutes, and then washed with 2X SSC (saline sodium citrate) for 5 min. Slides were then incubated in pre-hybridization buffer (50% Formamide, 3X SSC, 0.5% calf thymus DNA (CTD)) for 2 hours at 62°C. Slides were then incubated in hybridization buffer containing 300 ng/mL of DIG-labelled riboprobe (targeting Mc4r or Gnrhr1) overnight at 62°C. The next day, slides were washed with 2X SSC at RT for 30 min, and then washed with 2X SSC, 0.4X SSC, and 0.1X SSC each for 30 minutes at 60°C. Slides were then incubated in 1X SSC for 10 min and then buffer 1 (150 mM NaCl, 10 mM Tris, pH 7.5) for 10 min. Slides were blocked in TNB blocking buffer (0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, 0.5% (w/v) Blocking reagent) for 30 minutes, and then incubated in the secondary antibody, Anti-Digoxigen-POD, at 1:200 in TNB for 1 hour. Slides were washed in TNT (0.1 M Tris-Cl, 150 mM NaCl, 0.1% (v/v) Tween-20) 3X for 20 minutes and then incubated in a fluorescein dye from the Tyramide Signal Amplification Plus kit (TSA Plus kit, Perkin Elmer) at a 1:50 dilution for 5 minutes, before being washed in TNT 3X for 20 min once again, and HRP signal quenched with 0.02 N HCl for 10 minutes . After

ISH was conducted, the same slidered underwent immunohistochemistry analyses using Anti-GAP2, Anti-GAP3, or Anti-Agrp antibodies using the methods described above. After IHC, slides were mounted in 50% glycerol plus 10 µg/ml Hoescht 33342 (Sigma) and imaged on a Leica Sp6 confocal microscope.

## Results

**Fluorescent paramecia feeding assay optimization**.

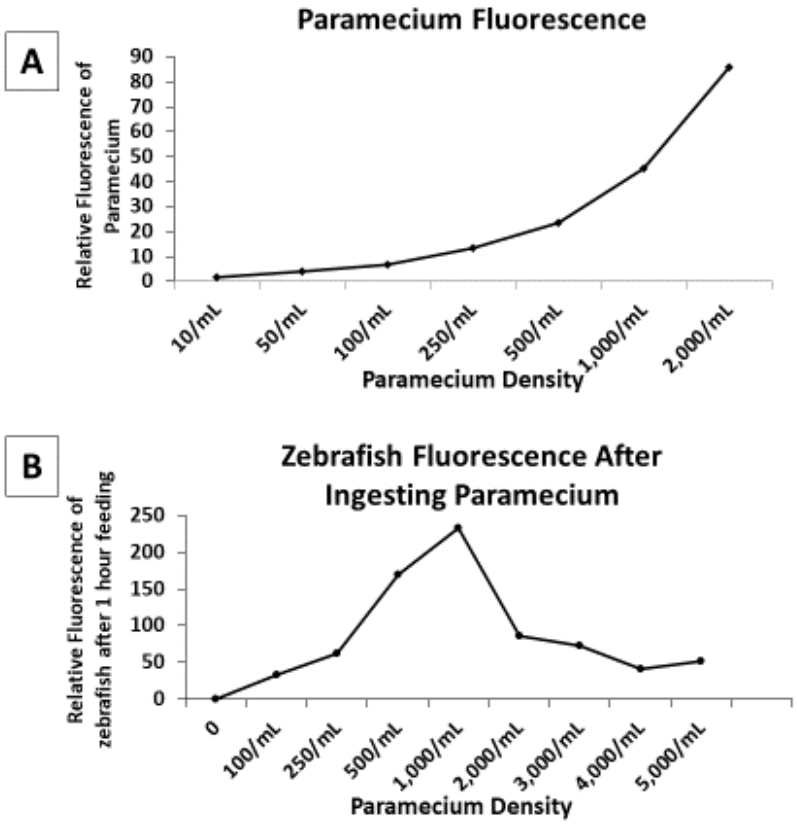


**Figure. 3.1.** *Fluorescent paramecia feeding assay optimization*. The ability to detect increases in paramecia densities via fluorescence readings was tested and quantified in densities between 10 cells/mL to 2,000/mL with a Spectramax Gemini XS spectrofluorometer (A). Fluorescence readings were assessed with sonicated zebrafish fed paramecia densities ranging from 100 – 5,000/mL to determine the optimal paramecia density for subsequent feeding trials (B).

The feeding trial experiments were optimized and tested through first assessing the ability of the spectrofluorometer to detect differences in fluorescently-stained

paramecia densities. The quantification protocol was verified as there was a linear increase in fluorescence readings associated with a linear increase in paramecia densities (Fig. 3.1A). Subsequently, the optimal concentration of paramecia to feed zebrafish for a one-hour feeding trial was assessed through determining the highest fluorescent reading (associated with the highest food intake) of varying paramecia densities fed to zebrafish. The highest reading was associated with 1,000 Paramecium/mL, and there was a continual decline at higher concentrations, verifying that the highest concentration fed could not exceed this value (Fig. 3.1B).

**Feeding and mobility comparisons**

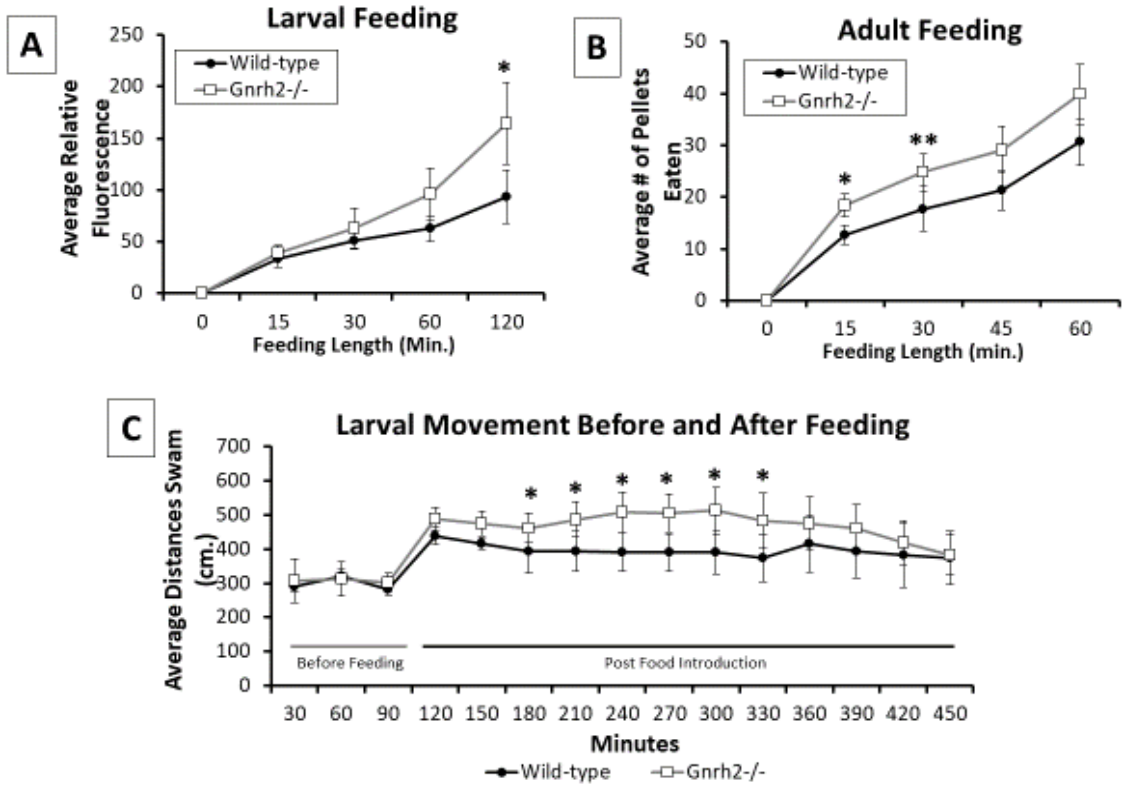


**Figure. 3.2.** *Comparison of feeding between wild type and gnrh2$^{-/-}$ lines:* Fluorescence readings from wild-type and *gnrh2*$^{-/-}$ embryos (7-10 dpf) fed fluorescently stained *Paramecium* (1000/5 ml) for 15, 30, 60, or 120 minutes (A). Comparison of the average number of pellets eaten by *gnrh2*$^{-/-}$ and wild-type adults (6-9 months old) fed Gemma Diet pellets (300 µm) for 15, 30, 45, or 60 minutes (B). Average total distances swam in

centimeters (cm.) from five trials of 12 each wild-type and *gnrh2*$^{-/-}$ larvae, before and after food introduction (C). All data were expressed as means ± S.E.M. Stars indicate significant difference between *gnrh2*$^{-/-}$ and wild-type zebrafish (* = $P < 0.05$, ** = $P < 0.01$; Repeated measures ANOVA with post-hoc).

The role of Gnrh2 in feeding was analyzed through comparing food intake of *gnrh2*$^{-/-}$ and wild-type larvae and adults. Overall, *gnrh2*$^{-/-}$ zebrafish larvae ate significantly more paramecia than wild-type (Fig. 3.2A). At individual time-points, a higher consumption of paramecia was observed at 1 hour of feeding in the *gnrh2*$^{-/-}$ larvae compared to wild-type larvae, which was doubled and significantly higher by 120 min (Fig. 3.2A). Similarly, adult *gnrh2*$^{-/-}$ fish ingested significantly more pellets compared to wild-type fish, with both males and females eating significantly more at the time-points of 15 and 30 minutes (Fig. 3.2B).

When movement was compared between wild type and *gnrh2*$^{-/-}$ larvae (between 7-18 dpf), there was no difference in the distances swam before food was introduced, but significantly higher mobility after feeding (Fig. 3.2C). *Gnrh2*$^{-/-}$ individuals swam ~ 20% more distance than wild-type between 1 hour and 3.5 hours after food introduction before leveling out to the same swimming distances as wild-type (Fig. 3.2C).

**Growth and body weight comparison**

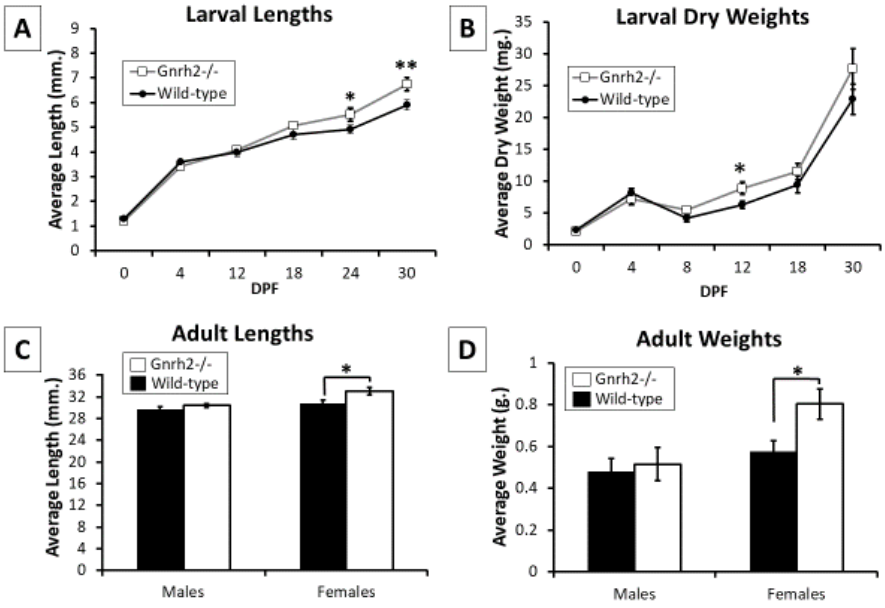


**Figure. 3.3.** *Growth rate comparisons:* Average body lengths (A) and dry weight (B) of wild-type and *gnrh2*$^{-/-}$ larvae between 0 and 30 dpf. Average standard body lengths (C) and total wet weights (D) of adult male (n=16) and female (n=10) wild-type and *gnrh2*$^{-/-}$ individuals (6 months old). All data were expressed as means ± S.E.M. Stars indicate significantly increased lengths or weights of *gnrh2*$^{-/-}$ zebrafish (* = $P < 0.05$, ** = $P<0.01$, Repeated measures ANOVA (A,B), Student's t-test (C,D)).

In general, *gnrh2*$^{-/-}$ zebrafish larvae were significantly larger than wild-type larvae in terms of dry weight and length. Larvae between 18 and 30 dpf, showed a 5% increase in body length at 18 dpf and 27% increase at 30 dpf (Fig. 3.3A). Pooled *gnrh2*$^{-/-}$ zebrafish larvae weighed significantly more than wild-type larvae at 12 dpf (Fig. 3.3B). This increase in growth continued into adulthood with females, as *gnrh2*$^{-/-}$ females measured an average of 3 mm. more, a 9% increase compared to wild-type.(Fig. 3.3C), and weighed 28% more than wild-type females (Fig. 3.3D).*Gnrh2*$^{-/-}$ males showed no differences in length (Fig. 3.3C) or weight (Fig. 3.3D).

**Expression of feeding/growth neuropeptides throughout development**

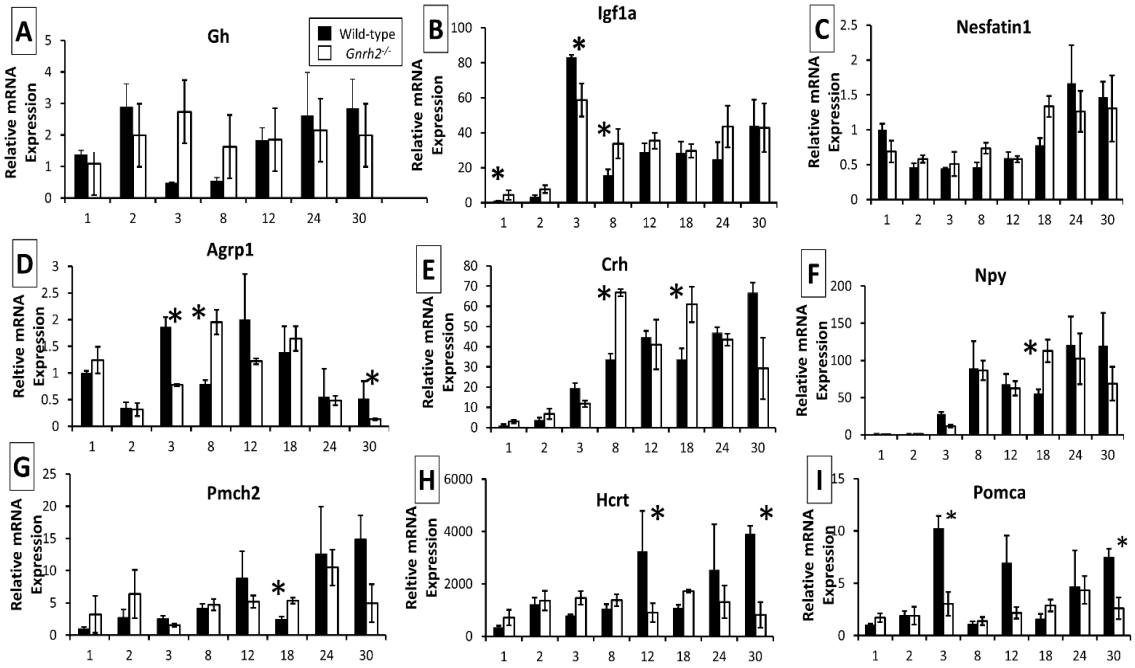


**Figure 3.4.** *Gene expression of feeding and growth genes during development in wild type and gnrh2$^{-/-}$ lines*: mRNA levels of *gh* (A), *igf1a* (B), *nesfatin1* (C), *agrp1* (D), *crh* (E) *npy* (F), *pmch2* (G), *hcrt* (H), and *pomca* (I) of wild-type and *gnrh2*$^{-/-}$ larvae from 1 to 30 dpf, relative to wild-type levels at 1 dpf. All data were expressed as means ± S.E.M. Stars Indicates significantly different mRNA levels between the genotypes (* = $P < 0.05$; ANOVA).

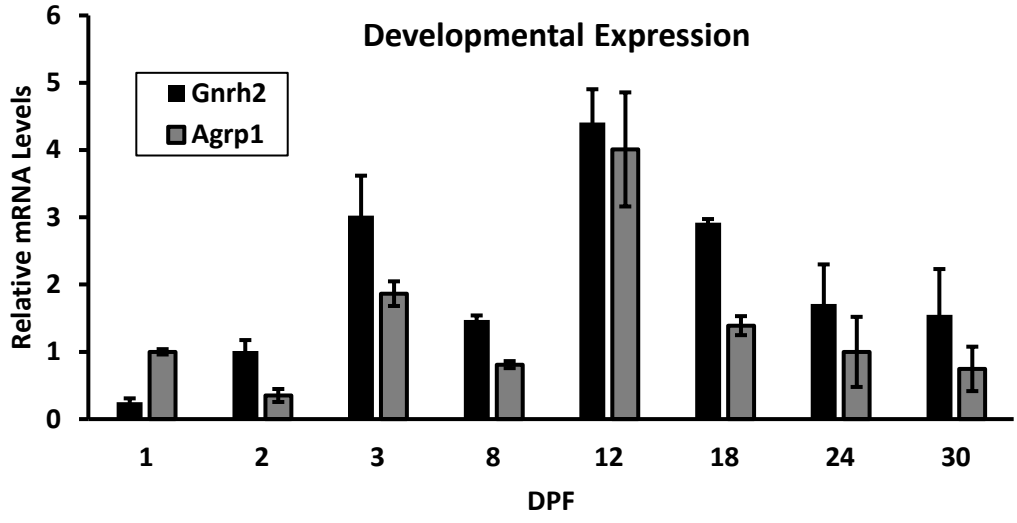


**Figure 3.5.** Relative expression of gnrh2 and agrp1 throughout the first 30 days of development. Black bars = relative gnrh2 expression, grey bars = relative agrp1 expression.

*Gnrh2*$^{-/-}$ embryos had a 1.5-fold higher expression level of growth hormone (*gh*) at 8 dpf, and 12 to 30 dpf, although not significant (Fig. 3.4A). *Gnrh2*$^{-/-}$ larvae had significantly higher mRNA levels of *igf1a* at 2 and 8 dpf, although it was significantly lower at 3 dpf (Fig. 3.4B). The transcript levels of feeding peptide genes in *gnrh2*$^{-/-}$ and wild-type embryos (0 to 30 dpf) varied widely. *Gnrh2*$^{-/-}$ fish have significantly lower levels of *agrp1* mRNA at 3 and 30 dpf and significantly higher levels at 8 dpf (Fig. 3.4D). *Gnrh2*$^{-/-}$ larvae had significantly higher levels of *crh* at 8 and 18 dpf (Fig. 3.4E). *Gnrh2*$^{-/-}$ fish had significantly higher levels of *npy* at 18 dpf (Fig. 3.4F). *Gnrh2*$^{-/-}$ had significantly lower levels of pmch2 at 18 dpf (Fig. 3.4G). *Gnrh2*$^{-/-}$ had significantly lower levels of orexin (*hcrt*) at 12 dpf and 30 dpf (Fig. 3.4H). *Gnrh2*$^{-/-}$ had significantly lower levels of *pomca* at 3 and 30 dpf (Fig. 3.4I). There were no differences in *nesfatin1* (Fig. 3.4C) or *gh* (Fig. 3.4A).

An interesting pattern emerges when looking at *gnrh2* and *agrp1* developmental expression, where the two gene expression levels followed the exact same trends throughout the first 30 days of zebrafish development, with increased expression at 3 dpf, decrease at 8 dpf, peak at 12 dpf, and gradual decrease from 12 dpf to 30 dpf occurs (Fig. 3.5)

**Gene expression profiles of feeding and growth-related factors during adulthood**

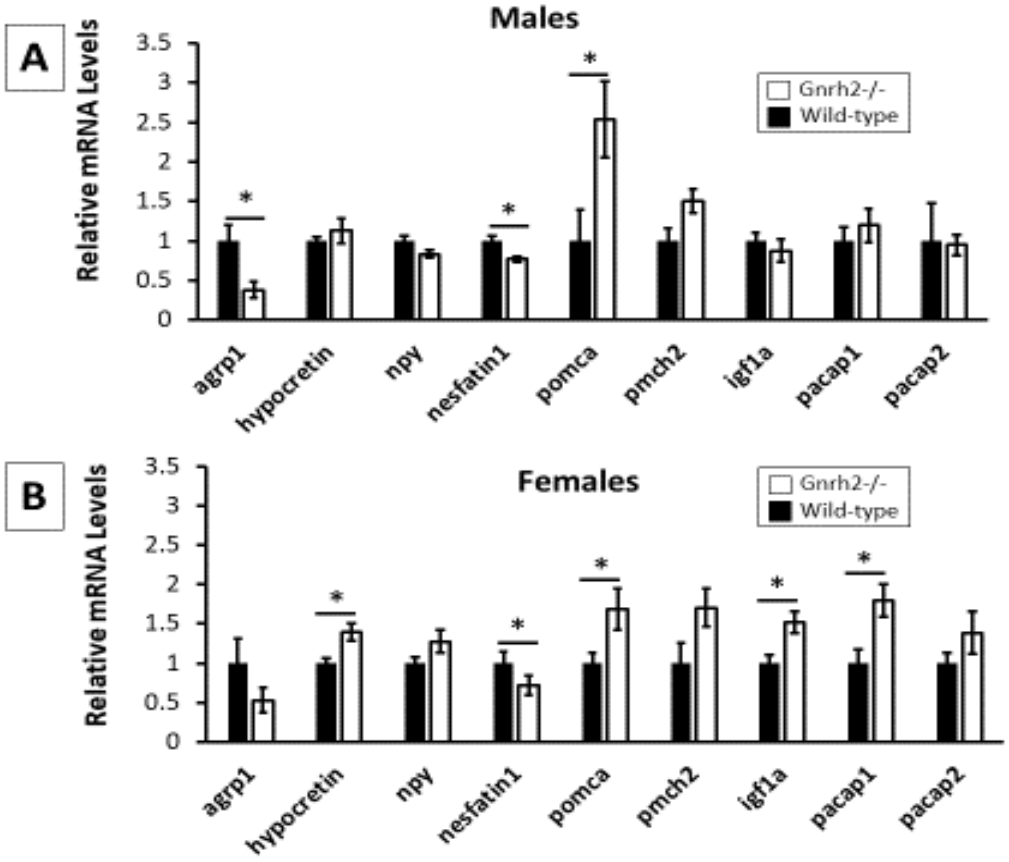


**Figure 3.6.** *Effect of the loss of Gnrh2 on feeding, growth, and reproductive-related gene expression:* mRNA levels of feeding and growth-related neuropeptides, *agrp1, hypocretin, npy, nesfatin1, pomca, pmch2, igf1a, pacap1, and pacap2* of male (A) and female (B) wild-type and *gnrh2*$^{-/-}$ brains at six months of age (n=8/sex/genotype). All data were expressed as means ± S.E.M. Stars indicate significantly different mRNA levels between the genotypes (* = $P < 0.05$; MANOVA).

In adults, *gnrh2*$^{-/-}$ males exhibited significantly decreased expression levels of *agrp1*, and both males and females exhibited a 20% reduction in *nesfatin1* (Fig. 3.6A and B). *Gnrh2*$^{-/-}$ fish exhibited a 2.5-fold and 1.7-fold increase of *pomca* mRNA levels for males and females, respectively (Fig. 3.6A and B). *Gnrh2*$^{-/-}$ adult females had significantly higher levels of *hypocretin, igf1a,* and *pacap1* compared to wild-type females (Fig. 3.6B), although there were no differences in the males (Fig. 3.6A). There

was no difference in *npy*, *pmch2*, or *pacap2* mRNA levels between *gnrh2*$^{-/-}$ and wild type zebrafish brains (Fig. 3.6A and B).

**Verification of the *agrp* and *gnrh2* knockdown**

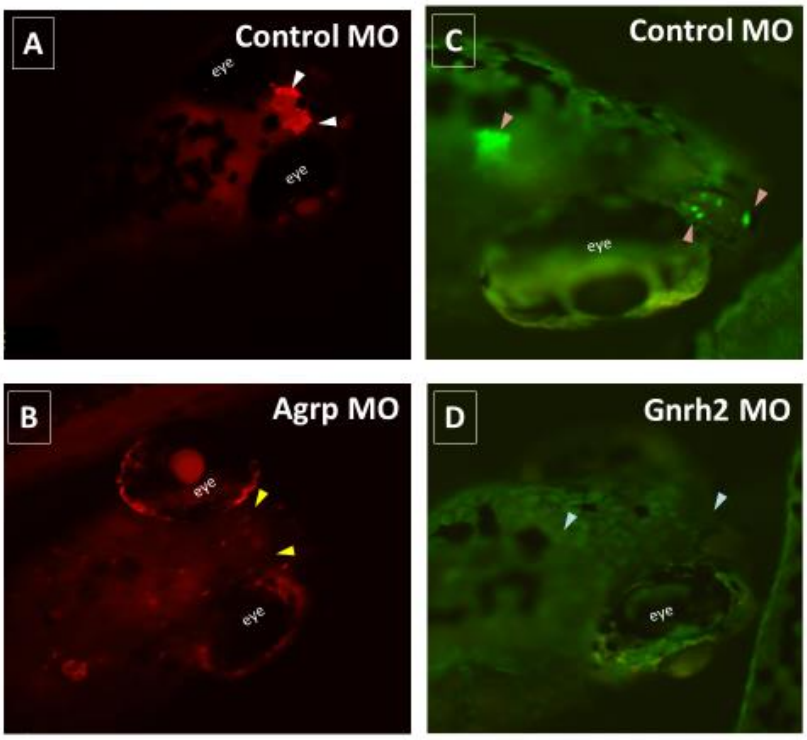


**Figure 3.7.** Whole-mount immunohistochemistry of Agrp1 in 3 dpf embryos injected with control morpholino (A) or Agrp morpholino (B). Transgenic *gnrh2* signal in 3 dpf tg(Gnrh2:eGFP) embryos injected with control morpholino (C) or Gnrh2 morpholino (D).

Whole-mount immunohistochemistry, using antibodies specific to Agrp, on control morpholino injected embryos resulted in immunolabeled Agrp signal in the forebrain and preoptic region (Fig. 3.7A, white arrow). Immunostaining of Agrp in Agrp1 morpholino-injected embryos resulted in no fluorescent signal in the brain (Fig. 3.7B, yellow arrow). Transgenic tg(Gnrh2:eGFP) fish injected with control morpholinos showed normal expression of Gnrh2 in the midbrain and olfactory regions of the brain

(Fig. 3.7C, light pink arrows). Transgenic tg(Gnrh2:eGFP) fish injected with Gnrh2 morpholinos showed no Gnrh2 signal in the brain (Fig. 3.7D, light blue arrows).

**Effect of *agrp* knockdown on *gnrh2* expression and *gnrh2* knockdown on *agrp1* expression and larval growth**

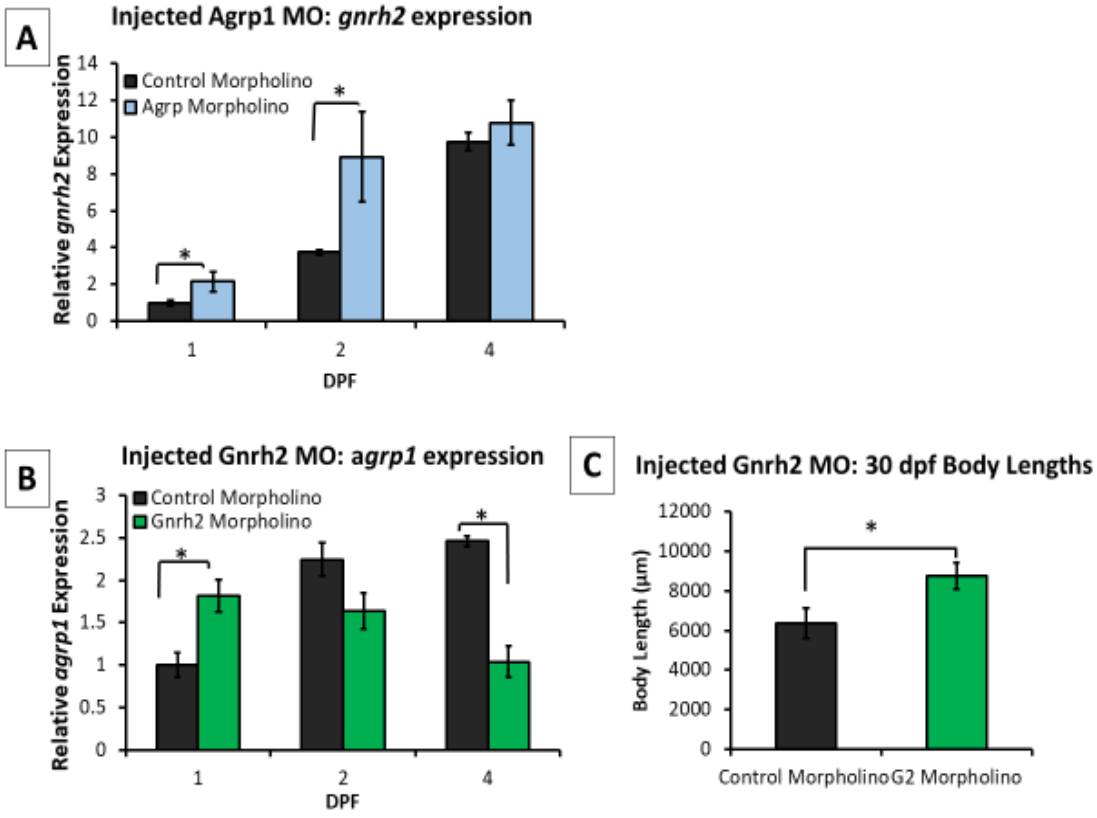


**Figure 3.8.** Expression of *gnrh2* at 1, 2, and 4 days after control morpholino or Agrp morpholino (knockdown) injections (A). Expression of *agrp1* for 1, 2, and 4 days after Gnrh2 morpholino injections (B). Body lengths of 30 dpf juveniles which were previously injected with control morpholino or Gnrh2 morpholino (C). All data were expressed as means ± S.E.M. Stars indicate significantly different mRNA levels or body lengths between the genotypes (* = $P < 0.05$; ANOVA).

Agrp1 morpholino injected embryos exhibited upregulation of *gnrh2* at 1 and 2 dpf compared to control morpholino injected siblings (Fig. 3.8A). Embryos injected with Gnrh2 morpholino exhibited upregulation of *agrp1* expression at 1 dpf, but downregulation of *agrp1* at 4 dpf compared to control morpholino injected siblings (Fig.

3.8B). Additionally, fish injected with Gnrh2 morpholinos had a longer body length of 2 mm, on average, than control morpholino injected fish (Fig. 3.8C).

**Effect of Gnrh2 *in vivo* and *in vitro* treatments on *agrp1* expression**

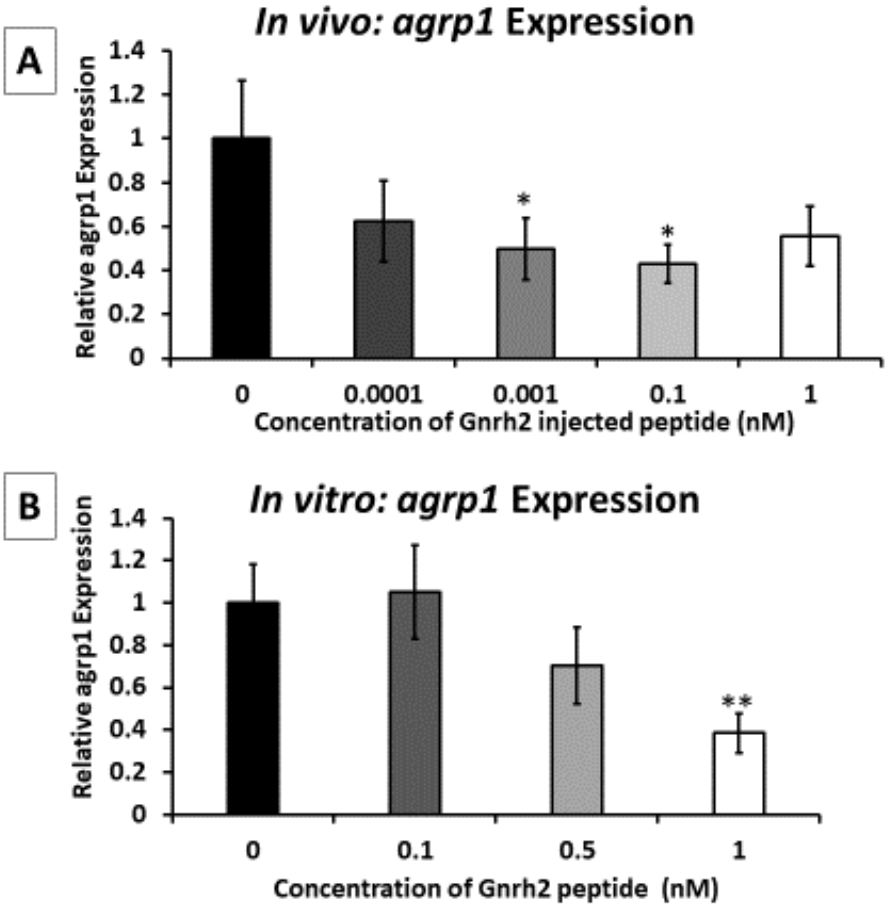


**Figure 3.9.** The effect of varying concentrations of Gnrh2 peptide injected to the third ventricle of male brains on *agrp1* expression levels (A). The effect of varying concentrations of Gnrh2 peptide incubated with dissected cultures of zebrafish brains on *agrp1* expression levels (B). All data were expressed as means ± S.E.M. Stars indicate significantly different mRNA levels (* = $P < 0.05$; ANOVA).

To determine whether Gnrh2 can modulate the expression of *agrp1*, Gnrh2 was administered *in vivo* to live male zebrafish, or *in vitro* to dissected zebrafish brain cultures. Concentrations of Gnrh2 ranging from 1 pM to 0.1 nM resulted in significant

downregulation of *agrp1* expression in brains dissected 6 hours after injections (Fig. 3.9A). *In vitro* incubations of Gnrh2 had similar effects on *agrp1* expression. Lower concentrations of Gnrh2 treatments resulted in no differences in *agrp1* expression, but the higher concentration of 1 nM resulted in significant decreased in *agrp1* expression (Fig. 3.9B).

**Neuroanatomical interactions of Gnrh2 and Agrp1 in zebrafish brains**

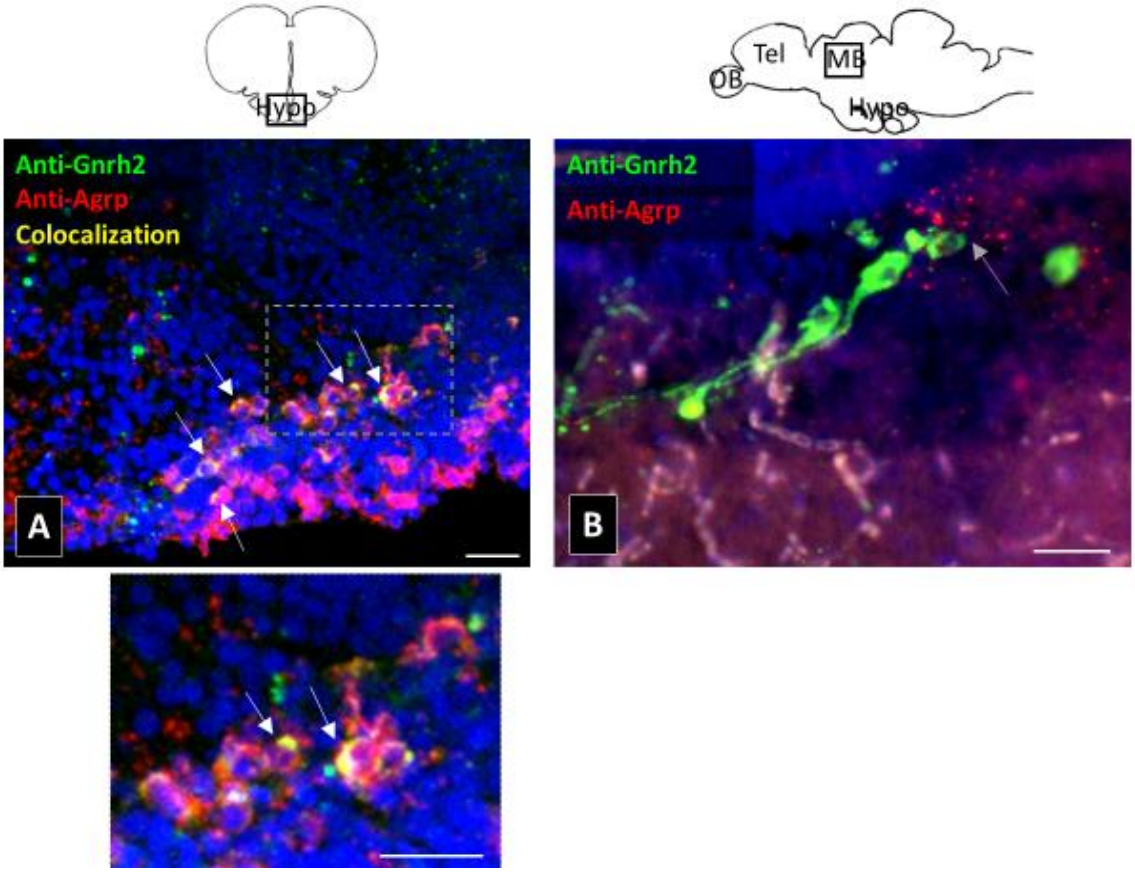


**Figure 3.10.** *Double-label immunohistochemistry of Gnrh2 and Agrp1.* Immunolabelling of Gnrh2 (green) and Agrp1 (red) in the ventral periventricular hypothalamic region, focusing on agrp1-expressing cells innervated by Gnrh2 fibers (A). Immunolabelling of Gnrh2 soma (green) and Agrp1 neuronal fibers (red) in the midbrain tegmentum region (B). Hypo, hypothalamus; MB, midbrain; Tel, telencephalon, OB, olfactory bulbs. Scale bars = 25 µm.

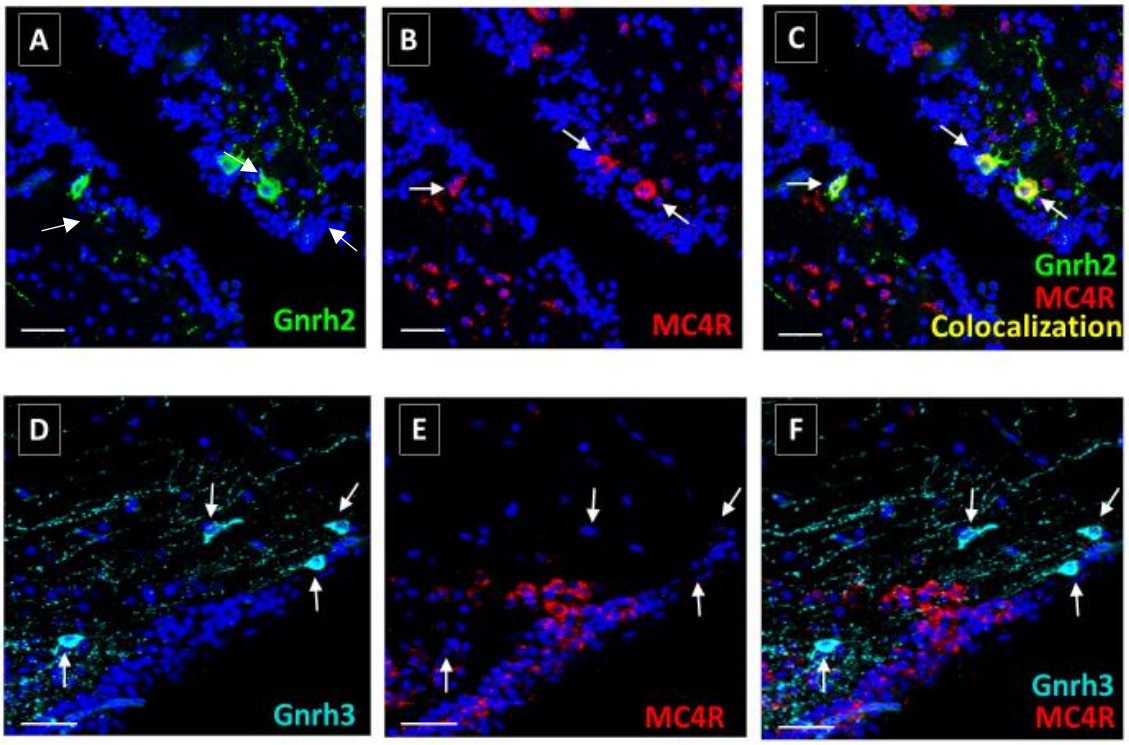


**Figure 3.11.** *In situ hybridization of MC4R and IHC of Gnrh2 or Gnrh3.* Immunolabelling of Gnrh2 (green) and Gnrh3 (cyan) on brain sections subsequent to MC4R (red) *in situ* hybridization. Single Gnrh2 signal (A), MC4R signal (B), and the overlay of Gnrh2 and MC4R (C). Single-label Gnrh3 (D), Mc4R (E), and the overlay of Gnrh3 and MC4R (F). Scale bars = 25 µm.

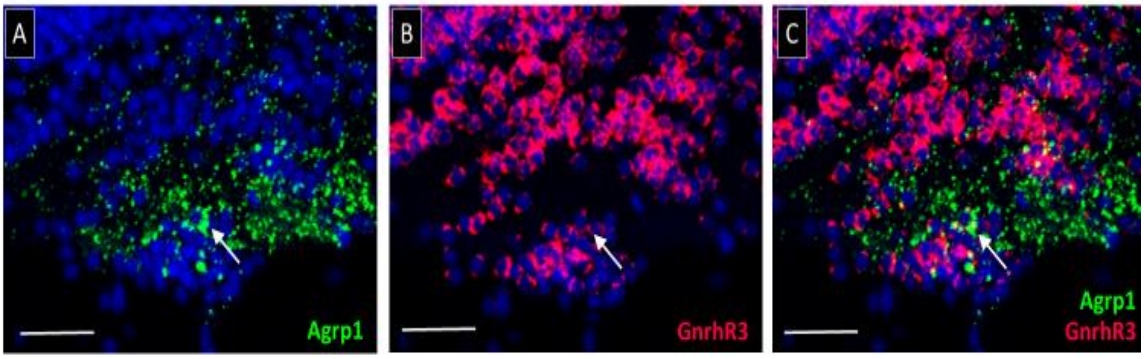


**Figure 3.12.** *In situ hybridization of GnrhR3 and IHC of Agrp1.* Immunolabelling of Agrp1 (green) on brain sections subsequent to GnrhR2 (red) *in situ* hybridization. Single Agrp1 signal (A), GnrhR3 signal (B), and the overlay of Agrp1 and GnrhR3 (C). Scale bars = 25 µm.

Brain sections of adult zebrafish were stained with antibodies against Gnrh2 and Agrp1 in order to analyze the interactions between the two neuronal systems. In the ventral region of the periventricular hypothalamus, dense clusters of agrp1-expressing cells are seen, with green Gnrh2 fibers in close proximity to, and innervating the cells, with the colocalization being represented with a yellow color where the green and red intersect with some of the more dorsal Agrp1 soma (Fig. 3.10A, white arrows). In the midbrain tegmentum, Agrp1 neuronal fibers are seen near and around Gnrh2 soma, although no direct contact is seen (Fig. 3.10B, light grey arrow).

GnrhR3 (Gnrh2 receptor) and MC4R (Agrp1 receptors) were also analyzed to see if they were expressed in Agrp1 or Gnrh2/Gnrh3 cells, respectively, via specific riboprobes with *in situ* hybridization. MC4R signal is seen specifically in several Gnrh2 cells in the midbrain (Fig. 3.11A, B, & C), but not in Gnrh3 cells in the preoptic area (Fig. 3.11D, E, & F). Signal of GnrhR3 mRNA is also shown in the neuronal soma of Agrp1 (Fig. 3.12A, B, & C).

**Effects of the loss of Gnrh2 on *agrp1* expression in normally fed and overfed fish**

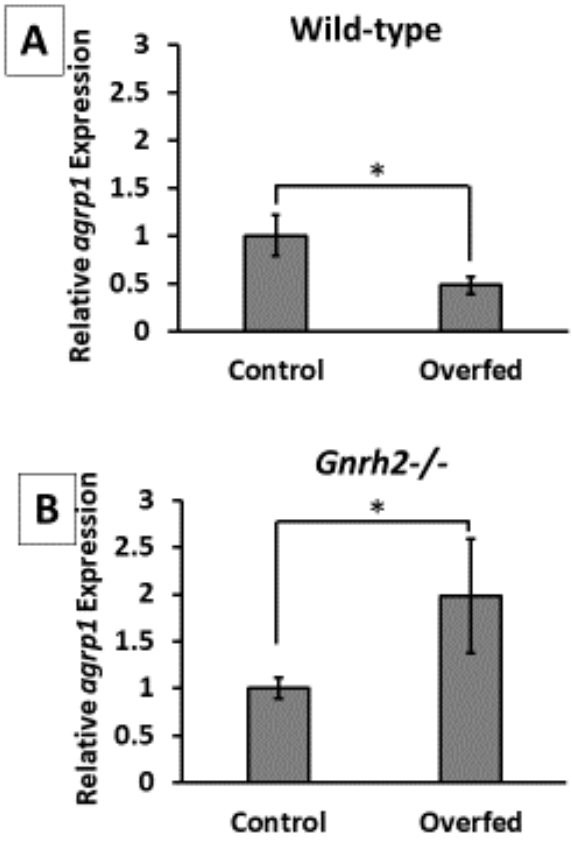


**Figure 3.13.** *Effects of overfeeding on agrp1 expression in gnrh2$^{-/-}$ and wild-type fish.* Relative expression levels of *agrp1* in normally-fed fish (3% body weight/feeding) or overfed fish (10% body weight/day) after 14 days in wild-type fish brains (A) and *gnrh2*$^{-/-}$ brains (B).

In wild-type fish, fish fed an overabundance of food (weighing 10% body weight/feeding) had a significant 2-fold decrease in *agrp1* expression levels, compared to fish fed a normal diet (3% body weight/ feeding) (Fig. 3.13A). In *gnrh2*$^{-/-}$ fish, an opposite effect occurred where overfed fish exhibited a 2-fold increase in the expression of *agrp1* compared to normally fed fish (Fig. 3.13B).

## Discussion

In this chapter, we comprehensively examined the roles that Gnrh2 plays in regulating feeding and growth. We employed loss-of-function experiments to determine differences in feeding and growth associated with the loss of Gnrh2, and functional and neuroanatomical experiments to examine whether Gnrh2 interacts with Agrp1 to enact its feeding regulation.

Using our knockout line, we show that *gnrh2*$^{-/-}$ fish exhibited major differences in feeding and growth, continuing from development into adulthood, granting Gnrh2 a major anorexigenic role in controlling the satiation of zebrafish. *Gnrh2*$^{-/-}$ fish exhibited overall increased long-term feeding compared to wild-type, and this was associated with increased body weight and length during development and at adulthood in females. These results further support the role of Gnrh2 as a potent anorexigen in zebrafish, which was previously suggested when Gnrh2 ICV injections induced reduced food intake in zebrafish and goldfish (*Carassius auratus*) (Matsuda et al., 2008, Nishiguchi et al., 2012). Although there were no differences in short-term food intake between *gnrh2*$^{-/-}$ and wild-type larvae, long-term feeding was significantly higher in fish lacking Gnrh2, suggesting that Gnrh2 acts as a satiation factor. The role of Gnrh2 as an anorexigen is supported by the fact that excessive feeding of adult zebrafish causes an upregulation of *gnrh2* (Nishiguchi et al., 2012). In pike perch (*Sander lucioperca*), administrations of Leptin leads to significant upregulation of *gnrh2*, but not *gnrh1*, in the hypothalamus, suggesting that leptin may be the signal Gnrh2 is responding to in order to enact its anorexigenic functions (Schaefer and Wuertz, 2016). Leptin is an anorexigenic hormone in which the plasma concentration usually parallels the total adipose mass (Klein et al., 1996), and in

mammals, increases after food intake (Barrachina et al., 1997). Since this study focused on the Gnrh2 loss-of-function effects on other neuropeptides in the brain, and Leptin is a hormone primarily synthesized in the liver of zebrafish (Denver et al., 2011), Leptin expression differences were not studied in this chapter, however, the effect of Gnrh2 loss on Leptin expression would be an interesting future experiment to conduct. In goldfish, Gnrh2 has been shown to downregulate the orexigenic neuropeptide Hypocretin (Hoskins et al., 2008), as well as mediate the feeding actions of α-Msh and Crh, which function as anorexigens in goldfish (Kang et al., 2011), proposing a complex interaction of Gnrh2 with other feeding peptides in mediating feeding behavior. The fact that Hypocretin expression was slightly increased in *gnrh2*$^{-/-}$ females further supports the role of Gnrh2 in down-regulating *hypocretin* levels in fish to modulate feeding behaviors. Further studies on the interactions of Gnrh2 with other feeding peptides will need to be conducted to understand how the entire network is regulating feeding behavior. In this study, the loss of Gnrh2 and increase in feeding was associated with differences in the expression levels of feeding neuropeptides, including a downregulation of *agrp1*, an orexigen, in males, and, on the other hand, upregulation of anorexigens such as *pomca*. The expression levels of these feeding factors in *gnrh2*$^{-/-}$ fish may be changing in response to the increased feeding to limit the excessive food intake and suggests that Gnrh2 acts to prevent overfeeding. Throughout the first 30 days of development, the loss of Gnrh2 also leads to a variable downregulation and upregulation of some feeding peptides (*agrp1*, *crh*, *npy*, *hcrt*, *pmch2*, and *pomca*) and growth-related hormones (*igf1a*), pointing to potential relationships of Gnrh2 with these peptides during ontogeny, which may be associated with different events occurring during this intense development period.

It is possible that the loss of Gnrh2 and subsequent increase in body growth rates of *gnrh2*$^{-/-}$ females is a result of more energy being shuttled toward somatic growth at the expense of gonadal growth, as exhibited by the decreased GSI shown in Chapter 2. The period coinciding with gonadal development in fish is normally characterized by decreased growth rates, and these two processes are known to be mutually antagonistic (Taranger et al., 2010). For instance, in the Atlantic cod (*Gadus morhua*), the onset of gonadal development results in reduced whole body dry matter (Hemre et al., 2002), and on the other hand, photoperiod manipulations in Nile tilapia (*Oreochromis niloticus*), which delayed gonadal growth, led to increased somatic growth (Rad et al., 2006), demonstrating a trade-off between somatic and gonadal growth and maturation. A similar phenomenon appears to be occurring in the *gnrh2*$^{-/-}$ females, where decreased gonadal growth is coinciding with increased somatic growth, resulting in heavier females with a lower GSI. This finding suggests that Gnrh2 is involved in the energy channeling between gonadal development and somatic growth. Gnrh2 is mediating and integrating feeding and reproductive systems in order to maintain optimal gonadal growth and reproductive performance.

We also explored the interactions of Gnrh2 with the potent orexigenic neuropeptide, Agrp1. Agrp1 in zebrafish is located in the ventral region of the periventricular hypothalamus (Shainer et al., 2017). In mammals, AGRP has been shown to be an orexigenic hormone and AGRP neurons are critically important in stimulating feeding behaviors (Gropp et al., 2005). When examining transcriptomes of fish undergoing fasting, Agrp1 is the only factor showing differential expression with a potent upregulation (Song et al., 2003) and an increased number of cells expressing *agrp1* can

be seen in brains of fasted zebrafish (Jeong et al., 2018). Due to the suggested importance of Agrp1 in zebrafish feeding, we wanted to see whether Gnrh2 is an upstream or downstream factor of Agrp1 to enact its feeding regulation. Interestingly, the developmental expression pattern of both *gnrh2* and *agrp1* was correlated in the first 30 days of zebrafish development, suggesting that they are either regulated by the same upstream factor, or mutually influencing the expression of each other. Using antisense morpholino in embryos, *gnrh2* or *agrp1* knockdown induced short-term upregulation of the other gene. Interestingly, after 4 dpf, agrp1 expression was decreased compared to control morpholinos. Due to the fact that we see increased feeding associated with Gnrh2 loss, it is possible that the *gnrh2* knockdown embryos are exhibiting higher metabolic activity, during which *agrp1* expression is decreased in order to regulate food consumption.

The ability of Gnrh2 to directly affect *agrp1* expression was also tested in adults with *in vivo* and *in vitro* exogenous Gnrh2 treatments. Both types of administrations induced downregulation of *agrp1* at most of the concentrations tested, confirming the findings from the morpholino treatments that Gnrh2 presence can influence *agrp1* expression values. The fact that *in vitro* analyses also resulted in *agrp1* downregulation, in addition to the *in vivo* experiments, suggests that Gnrh2 has a direct effect on controlling *agrp1* expression. However, in order to determine if Gnrh2 is able to directly contact and influence *agrp1*-expressing cells, it needed to be determined whether the endogenous neuronal cells of Gnrh2 and Agrp1 come in contact with each other and in order to do so, double-label immunohistochemistry was conducted to visually stain these neurons. Through antibody staining, it was observed that there are abundant Gnrh2

neuronal fibers innervating Agrp1 soma in the hypothalamus. The innervation of the soma suggest that Gnrh2 may be able to bind to Agrp1 cells. In support of this, Gnrh receptor 1, one of the four GnrhRs with high affinity to Gnrh2 in zebrafish (Tello et al., 2008), is expressed in Agrp1 cells in our studies, signifying that Gnrh2 may enact its feeding modulatory roles through binding to the Gnrh receptors on these cells to inhibit Agrp1. Concurrently, the receptors to which Agrp1 bind, Mc4r, are also expressed in Gnrh2 cells, but not Gnrh3 in our studies on zebrafish, suggesting a feedback system that is selective with Gnrh2. This finding also suggests that Gnrh2 is able to respond to feeding signals, such as those from the melanocortin system, as both Agrp1 and Pomca are able to bind to and inhibit, or activate, Mc4r (respectively).

The importance of Gnrh2 in regulating *agrp1* expression was also studied through subjecting *gnrh2*$^{-/-}$ and wild-type fish to different feeding regimes, including normally fed and overfed conditions, and determining subsequent *agrp1* expression levels. Previously, studies showed that *agrp1* levels only increase in fasted states (Hahn et al., 1998), and we show that overfed states induce a decrease in *agrp1* mRNA levels. In wild-type zebrafish, *agrp1* expression was decreased compared to normally fed siblings, as to be expected. In *gnrh2*$^{-/-}$ fish, however, the response of *agrp1* expression to overfeeding was opposite than expected. After overfeeding for 14 days, *gnrh2*$^{-/-}$ fish surprisingly had increased levels of *agrp1* as opposed to the expected decrease, suggesting there is a dysregulation of *agrp1* expression during this state. Interestingly, we also see that *gnrh2*$^{-/-}$ fish also exhibit much longer periods of feeding than wild-type, suggesting that the dysregulation of agrp1 may be one of the reasons for this overfeeding phenotype. It is possible that, since *gnrh2* expression increases by a 4.5-fold change in excessively fed zebrafish (Nishiguchi et al.,

2012), this *gnrh2* upregulation is directly causing the downregulation of *agrp1* to prevent continued overfeeding.

Our studies corroborate previous research which suggest that Gnrh2 has a definitive role in regulating feeding behavior. Through analyzing the interactions of Gnrh2 with Agrp1, we propose that this regulatory role may be enacted in part through its modulation of and downregulation of Agrp1 in high nutritional or "overfed" states. We do show that Gnrh2 can downregulate Agrp1, which express Gnrh receptors. The complete network of signals involved in controlling feeding behavior, and communicating with reproductive signals, still need to be studied further to get a conclusive picture of the neuroendocrine control of feeding and reproduction.

# CHAPTER 4: Examining the Mediation of Feeding and Reproduction by Gnrh2

## Abstract

In this chapter, the roles of Gnrh2 in mediating reproduction dependent on the nutritional status of zebrafish are analyzed and potential pathways that Gnrh2 takes in order to enact these roles are explored. Research on the hypophysiotropic Gnrh in teleosts, either Gnrh1 or Gnrh3, show that this neuropeptide is normally inhibited by fasting states. However, many teleosts are able to continue spawning successfully during fasting periods, suggesting another reproductive factor may be involved in regulating reproduction during this period. Additionally, previous studies in musk shrews and mice show that Gnrh2, but not Gnrh1, reinstates reproduction in underfed mammals, suggesting a specific and nutrition-dependent role of Gnrh2 in reproduction regulation. In previous studies and the previous chapters, it was established and determined that Gnrh2 regulates these two processes and, more importantly, is modulated by feeding/metabolism signals such as Agrp1 and Hypocretin. In order to test if and how Gnrh2 regulates reproduction in zebrafish, spawning, male courting behaviors, gametogenesis, gonadotropin expression and synthesis differences in *gnrh2*$^{-/-}$ and wild-type zebrafish undergoing different feeding conditions were examined. Additionally, Gnrh2 and Gnrh3 projections to the pituitary and gonadotropes in varying feeding states were analyzed. Potential pathways and indirect effects of Gnrh2 on mediating reproduction and feeding through the melatonin pathway by analyzing the innervation of the pineal gland and control of melatonin by Gnrh2 were also explored. It is shown that Gnrh2 is important in maintaining spawning, gonadotropin synthesis, and oocyte maturation and ovulation in long-term fasted zebrafish, associated with increased neuronal projections to the pituitary

and both Lh and Fsh gonadotropes during these fasted states. As a potential route of regulation, it is shown that Gnrh2 directly innervates pineal gland melatonin cells and is able to stimulate melatonin release, potentially to induce gametogenesis and feeding changes in turn. These findings suggest that in zebrafish, Gnrh2 assumes the role of the inhibited Gnrh3 to maintain reproduction even in nutritionally deprived conditions and may coordinate reproduction and feeding behaviors through the control of melatonin.

## Introduction

Reproduction and feeding are two processes which have been known to be intricately linked. With reproductive activities and gamete maintenance being energetically costly in some species, appropriate energy intake is essential to maintain optimal reproductive health in many animals. It has been shown that one of the major upstream regulators of reproduction in the vertebrate brain, the hypophysiotropic gonadotropin releasing hormone (Gnrh1 or Gnrh3 in some teleosts), can be inhibited by fasting, and may be inhibited by metabolic factors such as Neuropeptide Y (Npy, an orexigenic neurohormone) during malnourished states (Kalra and Kalra, 1996), Adiponectin (Wen et al. 2008), and reduced Leptin (a circulating hormone and signal of nutritional state produced from adipocytes) levels (Nagatani et al. 1998). However, these pathways are complicated and still not well understood. Some species, such as mice (*Mus musculus*) and rats (*Rattus*), which only have one form of Gnrh, GNRH1, experience severely disrupted reproduction after fasting (McClure, 1959, Bronson and Marstellar, 1985). On the other hand, many fish species, which possess multiple Gnrh isoforms, still reproduce after long-term starvation, despite the inhibition of Gnrh1 or Gnrh3 by fasting, with many purposely undergoing a fasting period before reproductive activities occur.

For instance, adult salmon cease feeding on their migratory spawning run, where they return to their natal location of birth to spawn (Miller et al., 2009), and Atlantic cod (*Gadus morhua*) and winter flounder (*Pseudopleuronectes americanus*) both decrease their food intake in coordination with the spawning period, and only reestablish feeding once the spawning season is finished (Fordham and Trippel, 1999; Volkoff et al., 2009; Scott and Scott, 1988). This suggests that the possession of multiple Gnrh isoforms may allow for the continuation of reproductive processes in the face of nutritional deprivation. However, the specific Gnrh isoforms involved in this process, the mechanisms of actions, and the factors linking metabolism and reproduction are still mostly unknown. With reproductive and foraging activities differing widely between species, the variation and similarities between vertebrate groups need to be further explored.

As the Gnrh neuropeptides directly stimulate gonadotropins and are located in areas of the brain alongside many different feeding neuropeptides, they are candidates for integrating and mediating both feeding and reproductive processes. The second isoform of Gnrh, Gnrh2, has been shown with several behavioral studies to be able to modulate feeding behaviors as well as reproductive activities (Kauffman and Rissman, 2004a; Kauffman and Rissman, 2004b; Matsuda et al., 2008; Nishiguchi et al., 2012). Gnrh2 injections to goldfish (*Carassius auratus*) and zebrafish both decreases feeding behaviors and increases spawning behaviors (Volkoff and Peter, 1999; Nishiguchi et al., 2012). Additionally, Gnrh2 has been shown to respond to Hypocretin and Agrp1 peptides, but other factors, such as Leptin, which inhibit Gnrh3, do not affect Gnrh2 (Chapter 3). This suggests that Gnrh2 is involved in mediating both reproduction and feeding. In support, it has been shown that Gnrh2 is involved in the control of female spawning behaviors in

mammals specifically in fasted states, but not in well-fed conditions. For instance, in musk shrews (*Suncus murinus*) that were food deprived, only the Gnrh2 isoform, but not Gnrh1, was able to stimulate spawning activities (Kauffman and Rissman, 2004a). Interestingly, food-restricted musk shrew females actually show an increased number of Gnrh2 cell bodies in the anterior midbrain and a higher density of Gnrh2 fibers in the median eminence, the area of the brain right above the pituitary, in food-restricted conditions compared to fed conditions (Temple et al., 2003). Similarly, for the first time, starved zebrafish were shown to exhibit a higher abundance of extended Gnrh2 neuronal projections in the pituitary compared to fed fish, showing the differential proliferation of Gnrh2 depending on the energy level of the fish (Xia et al. 2014). The increased involvement of Gnrh2 in reproductive areas of the brains after food deprivation appears to be a similar occurrence across vertebrate groups. This implies that although the Gnrh isoform in the preoptic area has mainly reproductive roles, the midbrain Gnrh2 may be able to mediate both feeding and reproductive processes and has a differential role in reproduction that depends on nutritional status.

The mechanisms in which Gnrh2 enacts its roles in feeding and reproduction are still unknown. It is possible that Gnrh2 has upstream control of factors involved in both feeding and reproduction. We showed in earlier chapters that the genetic loss of Gnrh2 induces a large upregulation of Spexin, in both males and females. Spexin has roles in both the negative regulation of feeding and gonadotropin secretion, suggesting it may help to mediate the actions of Gnrh2 in these systems. Additionally, the loss of Gnrh2 resulted in the differential expression of many feeding factors, including Hypocretin, Agrp1, and Pomca. The mutual negative relationship of Gnrh2 with Hypocretin has been

shown in goldfish, suggesting a similar relationship may also be occurring in zebrafish (Hoskins et al., 2008). We also showed that Gnrh2 may enact its roles in feeding via the regulation of Agrp1, since Gnrh2 treatments *in vivo* and *in vitro*, caused downregulation of *agrp1* transcripts. Studies on the relationships of Gnrh2 with other feeding factors have given us a clearer picture on how it enacts it roles in feeding, however, the mechanisms of action of Gnrh2 in regulating reproduction and spawning behaviors is still unexplored. Gnrh2 most likely has a direct role in regulating *lhb* secretion (as seen in Chapter 2) through contacting gonadotrope cells, however, under normally fed conditions, the neuronal projections to the pituitary are sparse (Xia et al., 2014). The extensive projections of Gnrh2 throughout other parts of the brain, and its roles in stimulating spawning behaviors (Volkoff and Peter, 1999), are most likely enacted in other ways and with other reproductive factors. Melatonin is a hormone produced in the retina and pineal gland of fish during the night, and has been shown to have feeding, growth, and reproductive functions. In zebrafish, melatonin promotes follicle maturation, with melatonin administrations causing increased GSI, vitellogenin production, increased *lhb* expression, and an increased proportion of oocytes undergoing germinal vesicle breakdown (GVBD), a process indicating the late stages of oocyte maturation (Carnevali et al., 2011). As zebrafish are daily spawners, and normally spawn in the early morning, melatonin promotion of follicle maturation may be one pathway to ensure overnight gametogenesis and steroidogenesis to prepare the gonads for spawning. Additionally, melatonin administrations to zebrafish significantly decrease food intake, as well as modulate a number of different feeding hormone transcripts, very similar to the effect of Gnrh2 treatments to zebrafish (Piccinetti et al., 2010). A previous study in European sea

bass (*Dicentrarchus labrax*) shows that Gnrh2 penetrates the pineal gland, innervates melatonin cells, and is able to elicit melatonin release (Servili et al., 2010). It is possible that Gnrh2 is also enacting its roles in mediating reproduction and feeding in zebrafish partly though the melatonin system. In this study, we were able to examine if this is the case in zebrafish, and analyze if Gnrh2 has downstream or upstream relationships with melatonin in order to enable its differential role in reproduction and feeding.

In this chapter, the role of Gnrh2 in mediating reproduction dependent on nutritional status is analyzed and whether Gnrh2 is the missing link between feeding and reproductive processes is examined. The Gnrh2 loss-of-function effects on reproduction of fed and fasted fish are examined and it is shown that the loss of Gnrh2 results in major inhibitions of reproduction at the pituitary and behavioral level in fasted fish. This suggests that, similar to musk shrews, Gnrh2 is a major reproductive regulator in fish undergoing fasted conditions and may be a backup mechanism to compensate for the inhibited Gnrh3. Additionally, one pathway in which Gnrh2 may take to enact its dual regulation of feeding and reproduction is explored, through examining the regulation of melatonin by Gnrh2.

## Methods

### *Gnrh2*$^{-/-}$, *gnrh3*$^{-/-}$, and wild-type spawnings in fed and fasted states

Groups of zebrafish underwent three different feeding regimes (0, 7, and 14 day fasting) to determine if starvation conditions differentially affect the spawning of *gnrh2*$^{-/-}$, *gnrh3*$^{-/-}$, and wild-type individuals. Knockout and wild-type zebrafish groups were age-matched (between 7 and 8-months old) and size-matched and for each trial, 6 to 8

females from each genotype, and 8 to 10 males from each genotype were selected. All individuals studied (fed and starved *gnrh2*$^{-/-}$, fed and starved *gnrh3*$^{-/-}$, fed and starved wild-type) were paired with a fed wild-type partner from the same cohort to reduce variations in partner spawning variation. *Gnrh2*$^{-/-}$ and *gnrh3*$^{-/-}$ fish were compared with their cousin wild-type fish, with which they shared grandparents, to reduce strain variation. All fish were paired together with a fed wild-type partner the night before experimentation, separated by a divider. After the lights were turned on the next morning, spawning dividers were lifted, and fish allowed to spawn for one hour. A successful spawn was counted as one where females released eggs. After the initial spawning period, fish which successfully spawned were then placed in tanks without access to food, and the experiment repeated after 7 and 14 days. The experiment was repeated three times with different *gnrh2*$^{-/-}$ and wild-type fish, and twice with *gnrh3*$^{-/-}$ and wild-type.

**Male reproductive behavior**

To determine if changing feeding regimes affects zebrafish reproductive behaviors in male *gnrh2*$^{-/-}$ and wild-type individuals, reproductive behaviors were recorded and analyzed before (normally fed, 3% b/w) and after a 14-day starvation regime for 10 minute periods of time. Eight male fish, between 7 and 8 months of age and size-matched to reduce variation, were used for each genotype group for the experiment. All male fish (*gnrh2*$^{-/-}$and wild-type) were paired with a fed wild-type female partner from the same cohort to reduce variations in partner spawning variation. Tanks were filmed with a GoPro Hero 3 camera for the 10 minute period of time directly after spawning dividers were removed, and videos later analyzed. As males usually initiate the

courtship during spawning, the number of male encounters with females was counted for each spawning tank.

**Gonad histology**

Gonad morphology was examined and compared between normally fed and 14-day starved wild-type and *gnrh2*$^{-/-}$ males and females with gonad histology via hematoxylin and eosin (H&E) staining. Ten ovaries and testes were dissected from zebrafish of each genotype (*gnrh2*$^{-/-}$ or wild-type) at each feeding state (fed or 14-day fasted) and fixed in 4% PFA in PBS overnight and then embedded in paraffin (Fischer et al., 2008). Sections, 5 µm thick, were mounted on Plus-coated slides and dried overnight at 50°C. The sections were then rehydrated and stained with hematoxylin and eosin according to the manufacturer's protocol (Sigma-Aldrich, St. Louis, MO, USA). Ovarian and testicular sections were viewed and imaged using the bright field setting of a Zeiss Axioplan2 microscope and CCD Olympus DP70 camera with a scale bar. Oocytes were classified into three different maturational stages, related to the five stages of maturation classified by Selman previously (Selman et al., 1993; Clelland and Peng, 2009). Oocytes at stages 1 and 2 (primary growth and cortical alveolus stages) were classified as "previtellogenic" by their small size, under 250 mm, and the absence of vitellogenin uptake. Oocytes at stages 3 (vitellogenin uptake stage) are classified as "vitellogenic" by their size, between 250 and 520 mm, presence of vitellogenin, and nuclear membrane. Oocytes at stages 4 and 5 (oocyte maturation and ovulated stage) are classified as "fully mature" by the presence of germinal vesicle breakdown (GVBD) and sizes greater than 520 mm. The number of oocytes at each stage was counted for each section, and then

summed, averaged, and quantified as a proportion of the total oocyte population for each ovary.

**Gene expression of *lhb* and *fshb* in fed and fasted states**

Differences in the gene expression levels of *lhb* and *fshb* in fed and 14-day fasted adult *gnrh2*$^{-/-}$ and wild-type male pituitaries were determined using qPCR. Pituitaries from eight *gnrh2*$^{-/-}$ and wild-type adults of the same age and sex were dissected, flash frozen in dry ice, and stored at -80° C until RNA extraction. Pituitaries were homogenized by adding 150 µL DEPC-treated sterile milliQ water to each frozen pituitary and sonicating each sample for 20 seconds on ice (Sonifier 450, Branson Inc., Danbury, CT) (duty cycle 100%, output control at 3). To 50 µL of each homogenized sample, 100 µL of Trizol reagent was added and total RNA from samples was extracted according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). The Quantitect Reverse Transcription Kit (Qiagen, Valencia, CA, USA) that includes gDNA Wipeout to eliminate gDNA contamination was used to reverse transcribe 1 µg of RNA from each sample. In each round, a non-RT control and no template control were added to determine gDNA and template contaminations. QPCR was conducted using 20 ng of cDNA for each sample in duplicate with SYBR Green qPCR mix and gene-specific primers (Table 2.1), with $C_T$ values for each sample normalized against an internal *eef1α1* control. Amplification of cDNA was performed on a 7500 Fast Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA) and conditions included a 2 minute 95°C activation, 5 second 95°C denaturation, and 30 second 60°C annealing, with the last two steps repeating 40 times.

**Lh and Fsh ELISA**

For gonadotropin protein extraction, pituitary tissues were dissected from adult zebrafish, placed in a 1.5 mL tube, and quickly frozen on dry ice. Tubes were then placed on ice, 200 µL of PBST (0.05% Tween 20 in PBS) solution was added, and pituitaries were homogenized via sonication for 20 seconds each (Sonifier 450, duty cycle 100%, and output cycle at 3). 140 µL of the homogenized pituitary solution was added to another 1.5 mL tube containing 140 µL of 4N acetic acid, for a final concentration of 2N acetic acid. Tubes were immediately vortexed and placed on ice for 15 minutes. Samples were centrifuged for 30 minutes at 14,000 RPM, and the supernatants collected and frozen at – 80° C until the ELISA was conducted. The Lh and Fsh ELISA protocol was developed using recombinant hormones and optimized in Dr. Berta-Levavi-Sivan's lab, from the Hebrew University of Jerusalem, with minor modifications for our use (Hollander-Cohen et al., 2018). Just prior to the ELISA assay, samples were lyophilized overnight, reconstituted in 250 µL of sterile milliQ water, briefly centrifuged, and 125 µL supernatant transferred to 2 new tubes (one for Fsh ELISA, one for Lh ELISA). For the ELISA, 96-well ELISA plates were coated with either 100 µL of beta-FSH in 50 mM $NaHCO_3$ (pH 9.6) (at a final concentration of 2.5 ng/mL of beta-FSH) or 100 µL of beta-LH in 50 mM $NaHCO_3$ (pH 9.6) (at a final concentration of 20 ng/mL of beta-Lh). Standards were set up by diluting beta-alpha FSH to 20ng/ml in 250 uL assay buffer (PBS, with 0.05% Tween 20 and 0.01% BSA) with 10 subsequent two-fold serial dilutions (11 tubes total, ranging from 20 ng/mL to 9.7 pg/mL) or beta-alpha LH to 210 ng/ml in 250 uL assay buffer with 10 subsequent three-fold serial dilutions (11 tubes total, ranging from 210 ng/mL to 1.2 pg/mL). After standards and samples were set up,

50 uL of Fsh-antibody or Lh antibody (each diluted 1:1,000 in assay buffer) were added to each standard or sample tube. All tubes and coated plates were covered and incubated overnight at RT. The next day, coated plates were washed 3X in PBS-T (0.05% Tween 20 in PBS) at 200 µL/well. 200 µL of blocking buffer (PBS-T plus 1% BSA) was added to each well, and plates then covered and incubated for 30 minutes at 37° C. Plates were then washed 3X and then 100 µL of standard or sample added to each well of the plates. Plates were incubated at 37° C for 1.5 hours and then washed 3X. Secondary antibody consisting of 100 uL of GAR-HRP (at a 1:10,000 dilution or 1 uL in 10 mL PBS-T 1% BSA) was added to each well and plates incubated at 37° C for 1 hour. Plates were washed 3X and then 100 uL of 1:2 diluted 3,3',5,5'-Tetramethylbenzidine (TMB ELISA Substrate, Abcam) added to each well. Plates were then covered and placed in a dark location for approximately 1 hour (for Lh ELISA) or 20 minutes (For Fsh ELISA), and once the color developed to the appropriate opacity, the reaction was stopped with 100 µL of 1N phosphoric acid. OD readings were subsequently conducted using a spectrophotometer at 450 nm.

**Gnrh2 ELISA**

Pituitary tissues were homogenized and extracted according to the same procedures above (Lh and Fsh ELISA protocol) and stored at -80° C until ready for the ELISA. Just prior to the ELISA assay, samples were lyophilized overnight, reconstituted in 250 µL of sterile milliQ water, briefly centrifuged, and supernatant transferred to a new tube. For the ELISA, 96-well plates were coated with goat anti-rabbit IgG (200 µL/well) overnight at a concentration of 10 µg/mL in potassium phosphate buffer. Blocking buffer (0.3% BSA, 0.9% NaCl, 1.8% $K_2HPO_4$, `,3% $KH_2PO_4$, 1mM EDTA, 5

mM sodium azide) was then added at a volume of 200 µL/well, and plates stored for a minimum of 4 hours at room temperature and up to 5 weeks at 4° C. Plates were washed 3X in PBST, and 50 µL of samples or standards added to each well. Standards consisted of Gnrh2 peptide diluted to known concentrations of 10 ng/mL to 20 pg/mL. Lyophilized cGnrh2-aCHE (AchE- Acetylcholine Estherase) tracer (produced by Cayman Chemicals Inc.) was reconstituted to 8 ng/mL in 5 mL of assay buffer (0.9% NaCl, 1.8% $K_2HPO_4$, 3% $KH_2PO_4$, 0.1% BSA, 0.15 mM sodium azide) and 50 µL added to all wells but one (NSB- non-specific binding) control well. Gnrh2 anti-decapeptide antibody, (kindly provided from the late Dr. Judy King), was diluted to a final concentration of 1:15,000 in assay buffer and 50 uL added to each well but one control well. Plates were covered and incubated for 3 days at 4°C. ELISA plates were then washed 3X with 200 µL/well of PBST, and then 200 µL of Ellmann's reagent (0.9% NaCl, 1.8% $K_2HPO_4$, 3% $KH_2PO_4$, 215 mg DNTB (5,5'-Dithiobis(2-nitro-benzoic acid)) (sigma-D8120), 200 mg acetyl thiocholine) was added to each well. Plates were covered and placed in a dark location for approximately 3 hours, or until the color developed to 1.5-2.0 OD. OD readings were subsequently quantified using a spectrophotometer at 405 nm.

**Confocal microscopy and characterization of transgenic zebrafish pituitaries**

In order to characterize Gnrh2 and Gnrh3 neuronal projections, and examine Gnrh2 neuronal innervations with Lhb cells, we dissected and imaged whole transgenic zebrafish pituitaries, which specifically express fluorescent proteins in Gnrh2 (tg(Gnrh2:eGFP)), Gnrh3 (tg(Gnrh3:tdtomato)), or Lh cells (tg(Gnrh2:eGFP;Lh:mCherry)), with a confocal microscope. The tg(Lh:mCherry) zebrafish line was generated and kindly provided to us by Dr. Berta Levavi-Sivan's lab,

from Hebrew University of Jerusalem (Golan et al., 2014). A minimum of six pituitaries from each transgenic line at each feeding condition (fed or 14-day fasted) were dissected. Zebrafish were euthanized in an ice water bath and spinal cord dislocated, and then transferred to a solution which mimics *in vivo* conditions in the brain, artificial cerebrospinal fluid (ACSF: 119 mM NaCl, 26.2 mM $NaHCO_3$, 2.5 mM KCl, 1 mM $NaH_2PO_4$, 1.3 mM $MgCl_2$, 10 mM glucose, 2.5 mM CaCl2, plus 95% $O_2$/5% $CO_2$) bath for pituitary dissection. Whole pituitaries were dissected and quickly transferred to a glass bottomed culture dish and covered with fresh 1X ACSF. A small piece of coverslip was gently placed on top of pituitaries to flatten them for subsequent imaging on a Leica SP6 confocal microscope. Entire pituitaries were then imaged at 5X and 20X magnification. Z-stack projections of pituitary images were conducted using ImageJ. In ImageJ, a scale bar was used to set measurement parameters, and Gnrh2 and Gnrh3 neuronal fiber lengths were measured for each pituitary. Additionally, for double transgenic tg(Gnrh2:eGFP; Lh:mCherry) pituitaries, the number of Lh cells next to Gnrh2 fibers was counted for each pituitary and then averaged.

**Double-label immunohistochemistry of Fsh and Gnrh2 in zebrafish pituitaries**

Neuroanatomical interactions of Gnrh2 with Fsh cells in fed and fasted pituitaries were analyzed using immunohistochemistry with antibodies against the specific recombinant GAP region of Gnrh2 (GAP2) raised in rabbits, and with antibodies generated against carp-Fsh, which shares a large amount of homology with zebrafish Fsh, kindly provided to us by Dr. Berta Levavi-Sivan (Xia et al., 2014; Hollander-Cohen et al., 2015). Pituitaries from six sexually mature *gnrh2*$^{-/-}$ and wild-type fish of the same age after normally fed or 14-day fasted conditions, were dissected and fixed in 4% PFA

overnight and immersed in 30% sucrose in PBS for four hours, or until brains sunk to the bottom of the vial. Brain samples were then frozen in OCT and stored at -80° C until sectioning. Cryo-sectioning was conducted at -20° C using a Tissue-Tek Cryo3 cryostat. Brains were sectioned to 10 µM thickness, placed on Plus coated slides, and stored at -80°C until immunohistochemistry (IHC) was performed. To perform double-staining IHC, slides were briefly fixed in acetone, and quenched in 0.3% $H_2O_2$ in PBS for 30 minutes. Slides were then washed in PBS, blocked for one hour in 5% normal goat serum, and incubated with a 1:1,000 dilution of Anti-GAP2 in 1% BSA and 0.3% Triton X-100 overnight at 4°C. Slides were then washed in TNT (100 mM Tris Ph 7.5, 150 mM NaCl, 0.5% Tween-20) and incubated in an HRP-conjugated Goat anti-Rabbit (GAR-HRP) antibody (Genscript) at a 1:1,000 dilution in 1% BSA and 0.3% Triton for one hour. Then, slides were washed in TNT and incubated in a fluorescein dye from the Tyramide Signal Amplification Plus kit (TSA Plus kit, Perkin Elmer) at a 1:50 dilution for 5 minutes, washed in TNT, and HRP signal quenched with 0.02 N HCl for 10 minutes. After washing, the procedure for IHC delineated above was repeated on the same slides, but with a carp-Fsh primary antibody and Cy3 dye from the TSA kit to label Fsh protein at a dilution of 1:200. Slides were mounted in 50% glycerol plus 10 µg/ml Hoescht 33342 (Sigma) and viewed on a Leica SP6 confocal microscope and screened for the presence of Gnrh2 fibers and Fsh cells. Images were then taken at 5X and 20X magnification and images compiled and colors modified to green for Gnrh2, and magenta for Fsh using ImageJ. For each pituitary, number of Fsh cells with Gnrh2 fiber contacts were quantified for each section and summed overall.

**Confocal imaging of Gnrh2 and melatonin cells in larvae exposed to different photoperiods**

In order to characterize if photoperiod differences resulted in differences in Gnrh2 neuronal projections to melatonin cells, eggs from the same spawning pair of transgenic tg(Aanat2:mCherry;Gnrh2:eGFP) parents were collected and divided into three groups of equal densities 1 hour after spawning. One group (LL) was exposed to an all-light photoperiod, where embryos were constantly placed under a lamp mimicking natural sunlight. One group (LD) was exposed to a natural photoperiod, where the embryos were exposed to a lamp mimicking natural sunlight that was on a 12 hour light; 12 hour dark timer to establish a natural photoperiod. One group (DD) was exposed to an all dark photoperiod and placed in a light-blocking dark container which prevented any light-source from reaching the embryos. At 3 or 6 dpf, embryos were collected and euthanized in an ice-bath also containing saturated tricaine (MS-222). Zebrafish larvae were then mounted in a sagittal orientation in 1.5% low-melting point agarose, and then covered in freshly oxygenated 1X ACSF solution to keep the tissues alive and fluorescent signal strong. Pineal gland regions were located and images were taken on a Leica SP6 confocal microscope at 5X and 20X magnification.

**Gnrh2 *in vitro* treatments to pineal glands**

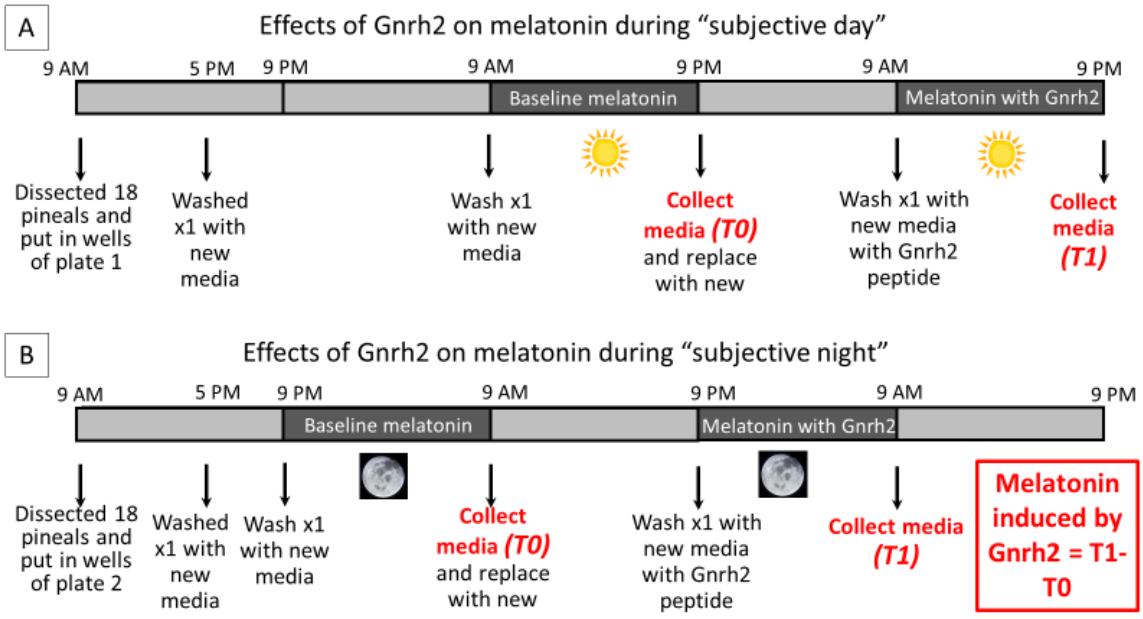


**Figure 4.1.** Timeline of pineal gland culture media collections to determine the effect of Gnrh2 on melatonin secretion during daytime (A) and nighttime (B) hours

In order to determine if Gnrh2 could induce melatonin changes, Gnrh2 peptide treatments of pineal gland cultures was conducted in Dr. Yoav Gothilf's laboratory at Tel Aviv University, with the help of Dr. Laura Gabriela. First, pineal glands were dissected in the morning from 36 transgenic zebrafish, (tg(Aanat2:RFP)), using a fluorescent dissecting microscope to ensure the transgenic pineal gland signal was intact and whole for each sample. Pineal glands were placed in two 18-well plates (one plate for daytime melatonin analysis, and one for nighttime melatonin analysis), with 1.5 mL of culture media per well (Eagle's Minimal Media, containing 1% fungicide, 1% antibiotic treatment, 2.2% $NaHCO_3$, and 0.01% L-tryptophan), and kept in the dark for the duration of the experiments. Samples were washed in media once, and then media replaced in the evening before incubating overnight for 12 hours. For daytime samples, samples were washed again with new media in the morning, incubated for 12 hours, and then media

collected to determine baseline T0 (daytime) melatonin levels. Media samples were collected in 1.5 mL tubes and flash frozen in liquid nitrogen, before being stored at -80°C until High Performance Liquid Chromatography (HPLC) was conducted. The next morning, samples were replaced with media containing either 0, 1 µM, or 10 µM of Gnrh2 peptide (6 samples per treatment) and let incubate for 12 hours, before media was again collected and frozen to determine T1 (daytime) melatonin concentrations after Gnrh2 treatments (Fig. 4.1A). For nighttime samples, media was replaced in the evening, and then pineal glands incubated for 12 hours before media was collected for baseline T0 (nighttime) melatonin analysis. The next evening, nighttime samples were replaced with media containing either 0, 1 µM, or 10 µM (6 samples per treatment) and let incubate for 12 hours. After the incubation, media was collected to determine T1 (nighttime) melatonin analysis of varying Gnrh2 treatments (Fig. 4.1B). To determine melatonin concentrations, High Performance Liquid Chromatography (HPLC) was conducted by Dr. Laura Gabriela Nisembaum at the Observatoire océanologique de Banyuls-sur-Mer using a reverse-phased analytic column, as outlined previously (Nisembaum et al., 2015).

**Nighttime *arylalkylamine-N-acetyltransferase 2* (*aanat2*) expression of *gnrh2*$^{-/-}$ and wild-type fish**

To obtain tg(Aanat2:mCherry) fish with and without endogenous Gnrh2, we first crossed our Gnrh2 knockout zebrafish line (*gnrh2*$^{-/-}$) with the transgenic tg(Aanat2:mCherry) zebrafish (Aanat2 is the major penultimate enzyme in the biosynthesis of melatonin and a good marker for melatonin concentrations, the line was generated and provided by Dr. Yoav Gothilf (Gothilf et al., 2002) to obtain heterozygous *gnrh2*$^{+/-}$; Aanat2:mCherry$^{+/-}$ lines). These fish were then in-crossed to generate 25%

*gnrh2*$^{-/-}$, 25% *gnrh2*$^{+/+}$, and 50% *gnrh2*$^{+/-}$ fish. All fish were screened as embryos (1-4 dpf) for the presence of positive Aanat2:mCherry signal. All positive tg(Aanat2:mCherry) zebrafish were then grown to adulthood, and fins clipped to obtain DNA extracts to screen for the Gnrh2 genotype (as described in the Chapter 2 methods section). *Gnrh2*$^{-/-}$, *gnrh2*$^{+/+}$, and *gnrh2*$^{+/-}$ fish were then separated and, the next night, at least 6 fish per genotype were collected 5 hours after the light was turned off, at 3 AM, during the highest peak of melatonin production (Zhdanova et al., 2008). Zebrafish were euthanized in an ice water bath with subsequent spinal cord dislocation. The pineal glands were dissected and placed in a 1.5 mL tube and quickly flash frozen in dry ice and stored at -80°C until RNA extraction. RNA was extracted, and qPCR conducted according to the methods described above, and using Aanat2 primers to determine *aanat2* expression levels.

**Sleep/wake (photoperiod dependent) mobility comparisons**

To determine if the loss of Gnrh2 resulted in any differences in photoperiodic mobility, *gnrh2*$^{-/-}$ and wild-type larvae between the ages of 12-18 dpf, were analyzed to determine daytime and nighttime mobility. For each trial, 12 *gnrh2*$^{-/-}$ larvae and 12 wild-type counterparts of the same age were placed individually in wells of a 24-well plate containing 1.5 mL fish water (0.06 g salinity) in the morning. Plates were placed in the DanioVision Observation Chamber, with 75% light output and after one-hour, movement parameters such as distances swam and velocity were tracked using the EthoVision XT version 11 software. Mobility was calculated every 30 seconds over a 24 hour period of time, with a light source turned on for eight hours (to emulate daytime), and then the light was turned off for 11 hours (to emulate nighttime), before the light was turned on again

to analyze daytime mobility for the second time. Six trials were conducted, comparing different *gnrh2*$^{-/-}$ and wild-type groups, and total averages of distanced swam were calculated for every 30 minutes. Statistical analysis was conducted using a repeated-measures ANOVA to determine differences between *gnrh2*$^{-/-}$ and wild-type mobility.

## Results

### Comparing reproduction in *gnrh2*$^{-/-}$, *gnrh3*$^{-/-}$, and wild-type zebrafish exposed to varying fasting periods

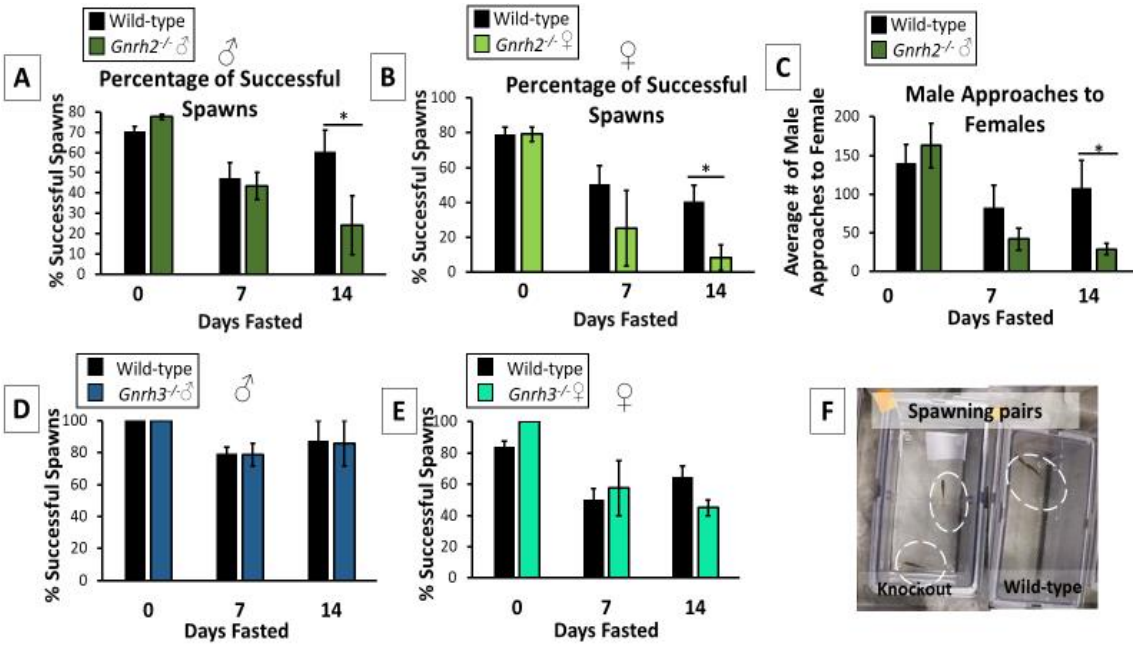


**Figure 4.2.** Percentage of successful spawns of *gnrh2*$^{-/-}$ (green bars) males compared to wild-type (black bars) males after 0, 7, and 14 days fasting (A), and *gnrh2*$^{-/-}$ females compared to wild-type females after 0, 7, and 14 days fasting (B). Percentage of successful spawns of *gnrh3*$^{-/-}$ (teal bars) males compared to wild-type (black bars) males after 0, 7, and 14 days fasting (C), and *gnrh2*$^{-/-}$ females compared to wild-type females after 0, 7, and 14 days fasting (D). Average number of times *gnrh2*$^{-/-}$ and wild-type males initiated courtship approaches to females for ten minutes after spawning dividers were removed, 0, 7 and 14 days after fasting (E). All data were expressed as means ± S.E.M. Stars indicate significantly different spawning percentages or courtship approaches between the genotypes (* = $P < 0.05$; repeated measures ANOVA).

When examining reproduction parameters at 0, 7, and 14 days of fasting, there were no significant differences in female or male spawning percentages at 0 and 7 days fasting (Fig. 4.2A & B). However, after 14 days of fasting, fed wild-type females paired with starved *gnrh2*$^{-/-}$ males exhibited decreased spawning compared to those paired with starved wild-type males (Fig. 4.2A). Although both genotypes showed decreased spawning success at 7 days fasting, wild-type males, but not *gnrh2*$^{-/-}$ males, recovered spawning after 14 days starvation (Fig. 4.2A). Additionally, *gnrh2*$^{-/-}$ females had significantly decreased rates of spawning compared to wild-type females after 14 days of starvation, with only one out of the three experiments exhibiting any spawns from *gnrh2*$^{-/-}$ females (Fig. 4.2B). When comparing *gnrh3*$^{-/-}$ male spawns with wild-type counterparts, there was no significant difference in the percentage of spawnings (Fig. 4.2D). Similarly, there were no significant differences between *gnrh3*$^{-/-}$ and wild-type female spawning percentages, although there was a small decrease in *gnrh3*$^{-/-}$ female spawns compared to wild-type females at 14 days fasting, it was not significant. Unlike the wild-type males, wild-type, *gnrh2*$^{-/-}$, and *gnrh3*$^{-/-}$ females both showed a continuous decrease in spawning success correlated with longer periods of starvation (Fig. 4.2B & E).

**Male reproductive behaviors**

When examining reproductive behavior, normally fed *gnrh2*$^{-/-}$ and wild-type males displayed courtship meetings with females at the same rate and frequency. However, after 14 days of fasting, *gnrh2*$^{-/-}$ males initiated courtship behavior significantly less than wild-type males (Fig. 4.2C)**.**

**Gonad morphology and oocyte maturation of *gnrh2*$^{-/-}$ and wild-type fish in fed and fasted conditions**

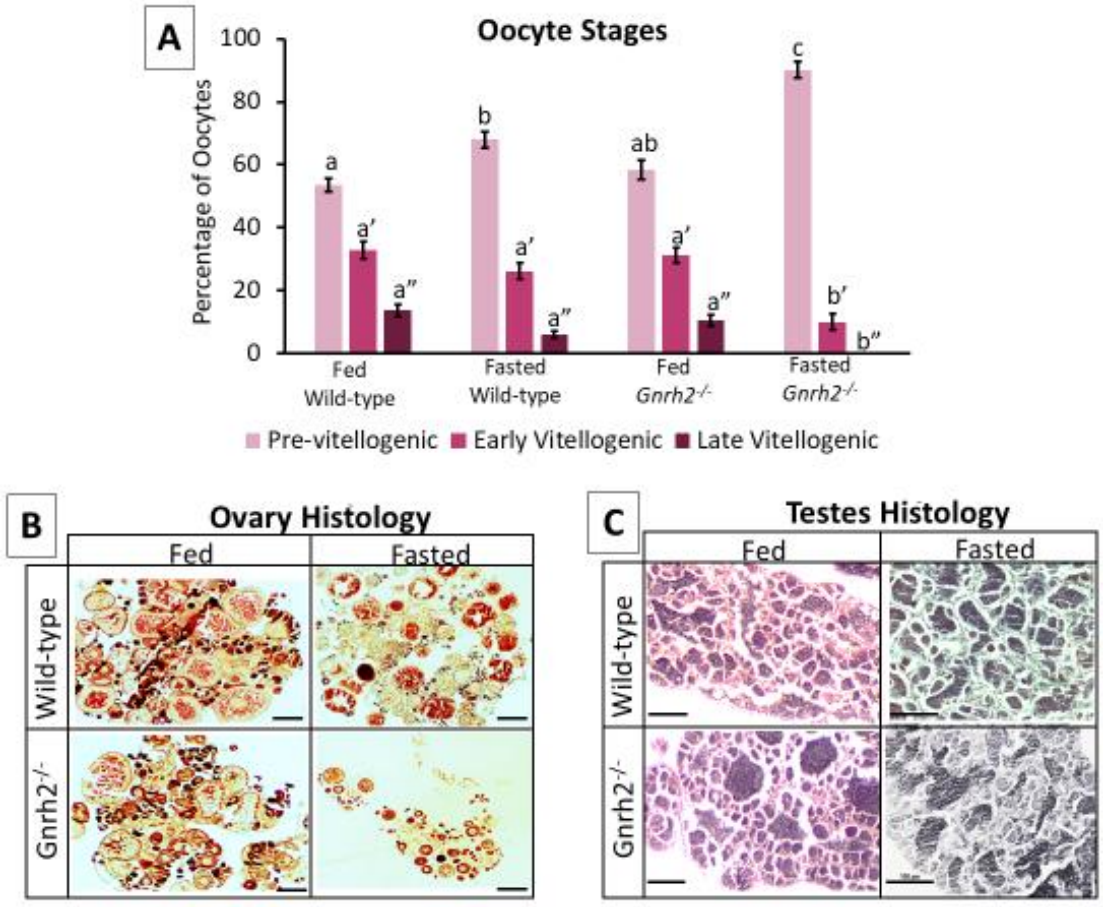


**Fig. 4.3.** *Gonad histology of gnrh2*$^{-/-}$ *and wild-type ovaries and testes in fed and fasted conditions* Percentage of oocytes at the pre-vitellogenic, early vitellogenic, or late vitellogenic (fully mature) stage in fed and fasted wild-type and *gnrh2*$^{-/-}$ females (A) (n=10/genotype). Representative images of the hematoxylin & eosin stained ovary section from normally fed or 14-day fasted wild-type and *gnrh2*$^{-/-}$ female (B) and representative images of the hematoxylin & eosin stained testes section from normally fed or 14-day fasted wild-type and *gnrh2*$^{-/-}$ males (C). Scale bars = 500 µm (ovary histology, B) and 100 µm (testes histology, C).

When examining testes morphology between wild-type and *gnrh2*$^{-/-}$ individuals, both wild-type and *gnrh2*$^{-/-}$ males exhibited mature spermatozoa, showing no differences in the overall gonadal morphology (Fig. 4.3C). A different picture was obtained when looking at starved females, where after 14 days of fasting, *gnrh2*$^{-/-}$ females exhibited significantly decreased percentages of fully mature oocytes and increased percentages of

pre-vitellogenic and early vitellogenic oocytes compared to wild-type and fed *gnrh2*$^{-/-}$ females (Fig. 4.3A). Additionally, starvation also decreased the overall size of the oocytes of wild-type and *gnrh2*$^{-/-}$ females, with *gnrh2*$^{-/-}$ females showing a larger reduction in ovary size after food restriction (Fig. 4.3B).

**Expression and protein content of Lh and Fsh in 0, 7, and 14 day fasted *gnrh2*$^{-/-}$ and wild-type pituitaries**

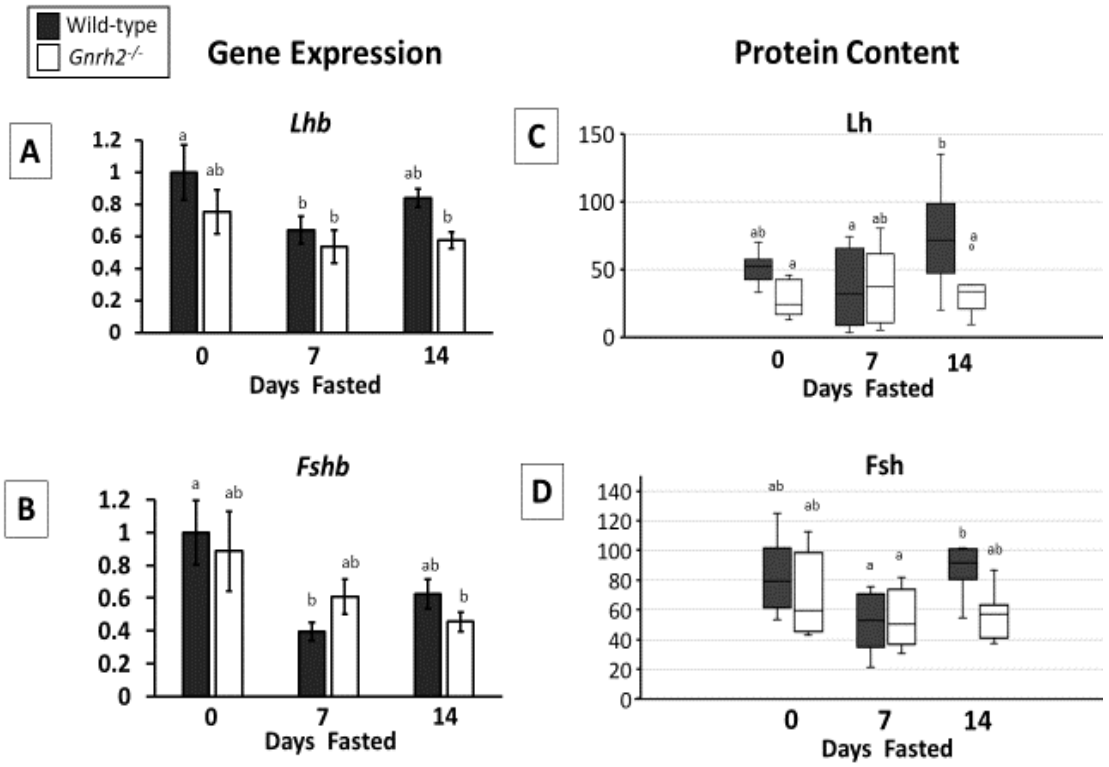


**Figure 4.4.** Relative mRNA levels of *lhb* (A) and *fshb* (B) from pituitaries of 0, 7, and 14 day fasted *gnrh2*$^{-/-}$ and wild-type zebrafish (n=8/genotype/time point). Lh (C) and Fsh (D) protein content from pituitaries of *gnrh2*$^{-/-}$ and wild-type males after 0, 7, and 14 days fasting. Black bars = wild-type, open bars = *gnrh2*$^{-/-}$. All data were expressed as means ± S.E.M. Different letters indicate significantly different expression levels or protein contents, i.e. a is significantly different from b ($P < 0.05$; ANOVA).

After 7 days of fasting, both *gnrh2*$^{-/-}$ and wild-type males exhibited significantly decreased levels of *lhb* compared to wild-type basal levels (Fig. 4.4A). After 14 days of fasting, *gnrh2*$^{-/-}$ *lhb* expression was still significantly decreased, whereas wild-type *lhb*

expression increased to wild-type basal levels (Fig. 4.4A). After 7 days of fasting, wild-type, but not *gnrh2*$^{-/-}$, pituitaries expressed significantly decreased *fshb* mRNA levels (Fig. 4.4A). After 14 days of fasting, *gnrh2*$^{-/-}$ pituitaries expressed significantly decreased *fshb* levels, but wild-type *fshb* expression was no different from normally fed *fshb* levels (Fig. 4.4A). As for Lh protein content, 7 days of fasting did not affect the levels in wild-type or *gnrh2*$^{-/-}$ pituitaries, however at 14 days of fasting, wild-type pituitaries contained significantly more Lhb protein than *gnrh2*$^{-/-}$ pituitaries (Fig. 4.4C). Although Fsh protein content was lower in both wild-type and *gnrh2*$^{-/-}$ pituitaries, it was not a significant decrease. However, at 14 days fasting, wild-type pituitaries contained significantly more Fsh protein than 7-day fasted pituitaries, whereas *gnrh2*$^{-/-}$ pituitaries remained low (Fig. 4.4D).

**Effect of fasting on Gnrh2 and Gnrh3 neuronal projections to pituitaries**

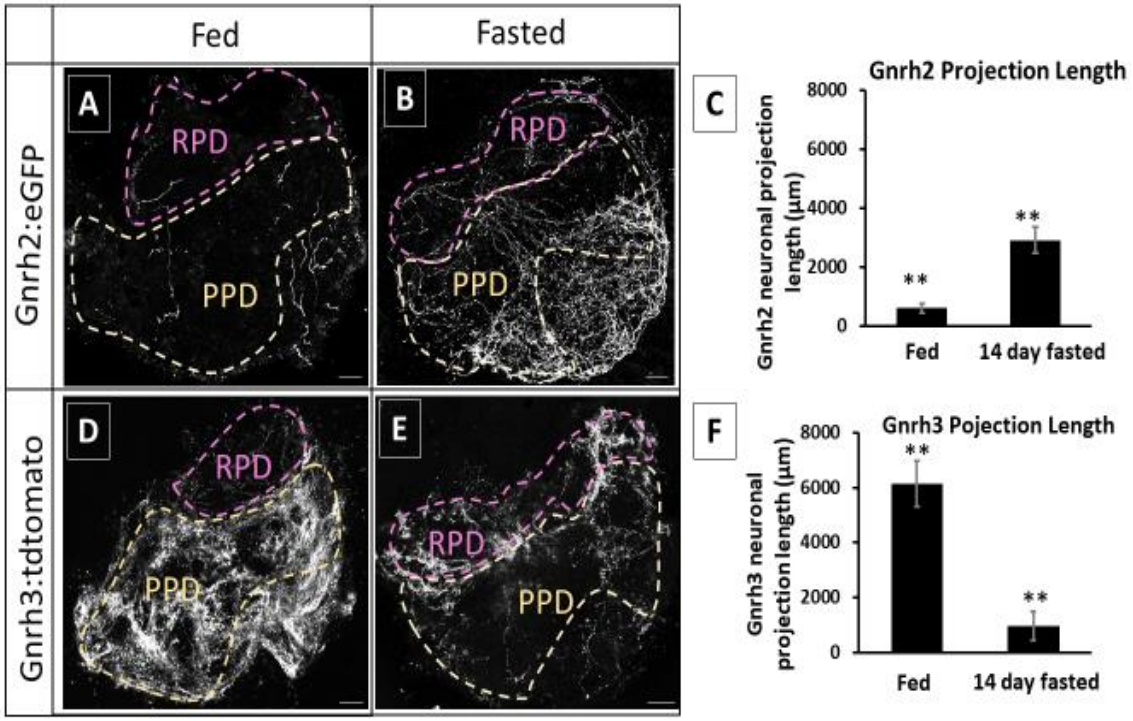


**Figure 4.5.** Representative images of whole pituitaries from tg(Gnrh2:eGFP) or tg(gnrh3:tdTomato) in fed (A) or 14-day fasted (B) conditions, and Gnrh3 in fed (D) or

14-day fasted (E) conditions. Neuronal projection lengths of Gnrh2 in fed and 14-day fasted pituitaries (C) and of Gnrh3 in fed and 14 day fasted pituitaries (F, n=8/group). Bar graphs are expressed as means ± S.E.M. Stars indicate significantly different projection lengths between fed and fasted conditions (** = P < 0.01; ANOVA) RPD = rostral pars distalis, PPD = proximal pars distalis. Scale bars = 50 µm.

Transgenic Gnrh2:eGFP pituitary images show that few, but not many, Gnrh2 neurons are present in fed pituitaries (Fig. 4.5A), whereas 14-day fasted pituitaries contain many Gnrh2 neuronal projections, also extending to the PPD where gonadotropes are located, (Fig. 4.5B), of which the measured lengths and abundance of projections are significantly higher than in fed pituitaries (Fig. 4.5C). In transgenic tg(Gnrh3:tdtomato) pituitaries, there is an abundant presence of Gnrh3 neurons in fed conditions (Fig. 4.5D). In fasted tg(Gnrh3:tdtomato) pituitaries, Gnrh3 neurons are still present (Fig. 4.5E), but in a decreased abundance compared to fed pituitaries, barely reaching the PPD (Fig. 4.5F).

**Effect of starvation on Gnrh2 protein secretion to pituitaries of males and females**

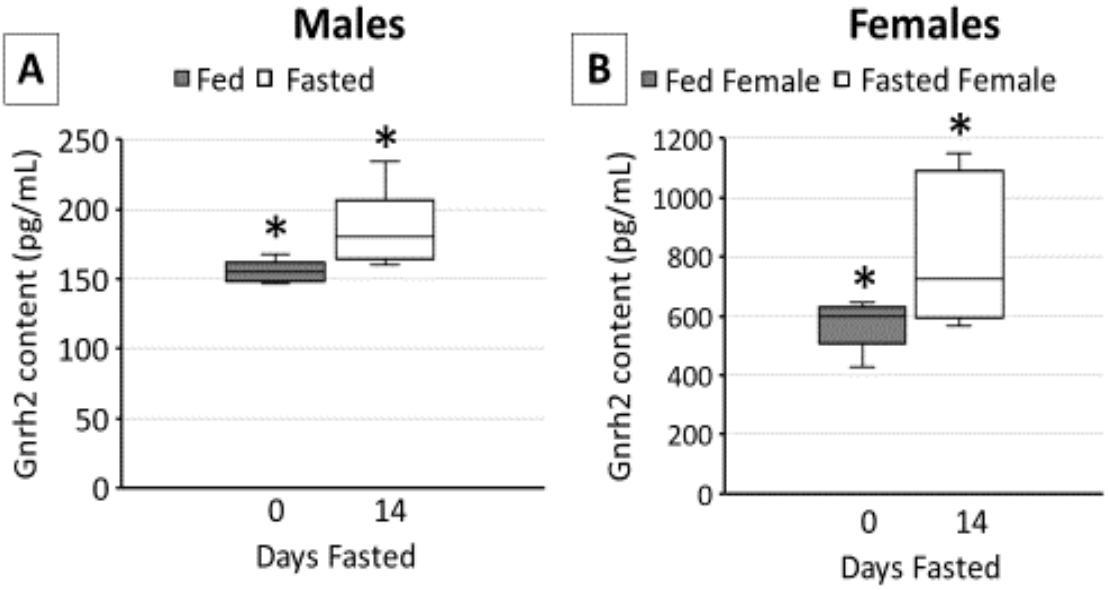


**Figure 4.6.** Average Gnrh2 protein content in pituitaries from fed and 14-day fasted pituitaries of males (A) and females (B) (n=8/condition). Stars indicate significantly

different Gnrh2 protein content amounts between fed and fasted conditions (* = $P < 0.05$; ANOVA).

The average Gnrh2 protein content in pituitaries of males which were fasted for 14 days was significantly higher than in male pituitaries which were normally fed (Fig. 4.6A). The protein content increased from an average of 150 pg/mL to 190 pg/mL, a 17% increase (Fig. 4.6A). The average Gnrh2 protein content of females also increased in pituitaries which had undergone 14 days of fasting compared to fed pituitaries, with fed pituitaries containing, on average, 600 pg/mL of Gnrh2 protein and fasted pituitaries 860 pg/mL, a 30% increase (Fig. 4.6B). Gnrh2 content in females was about 4-fold higher on average than male Gnrh2 content.

**Effects of fasting on the number of Gnrh2 neurons contacting Lh cells in the zebrafish pituitary**

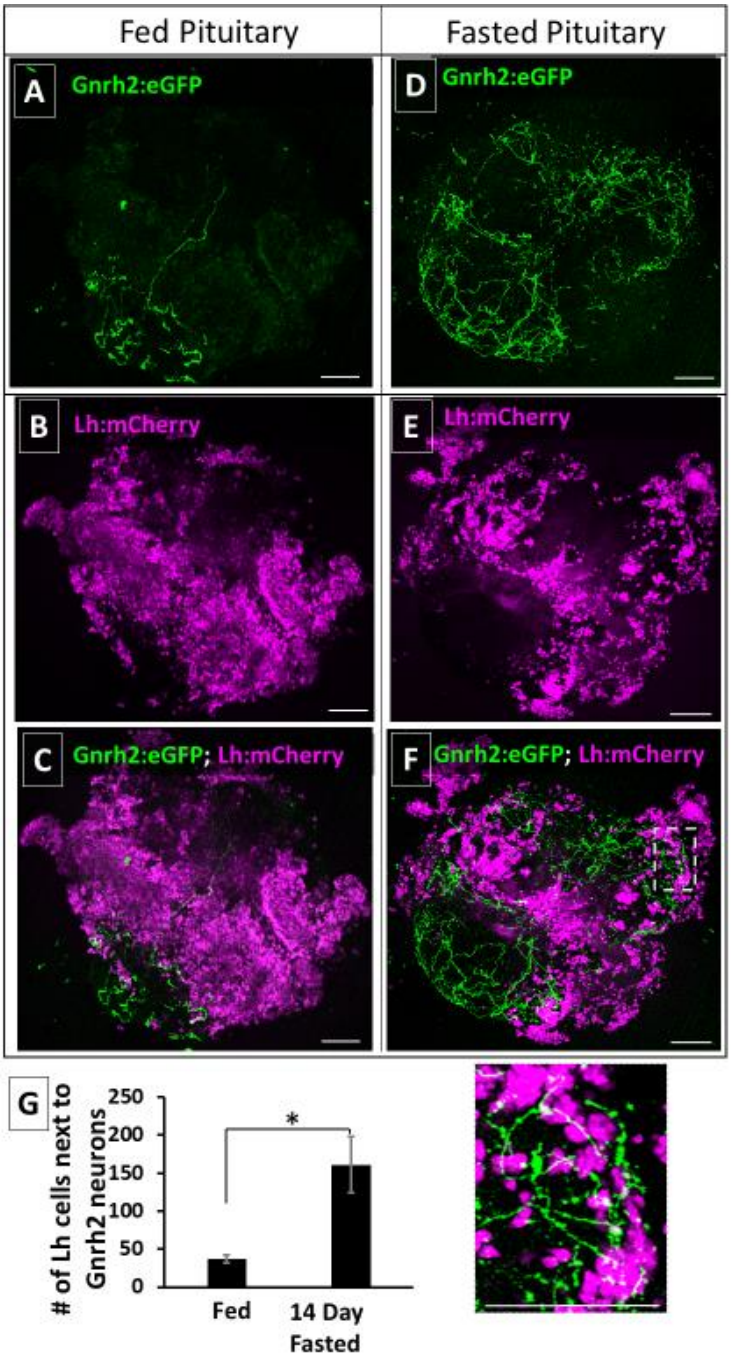


**Figure 4.7.** Representative images of whole double transgenic tg(Gnrh2:eGFP; Lh:mcherry) pituitaries from fed (A, B, C) and 14-day fasted zebrafish (D, E, F) with a subset of a fasted pituitary image shown at 20X magnification. The average number of Lh cells contacted by Gnrh2 neurons from fed and 14-day fasted pituitaries (G, n=6/group). Bar graphs are represented as mean ± SEM. Stars indicate significantly

different number of Lh-Gnrh2 contacts between fed and fasted conditions ( * = P < 0.05; ANOVA). Scale bars = 100 µm.

In fed pituitaries, Gnrh2 neurons are most present in the neurohypophysis, with very few penetrating into the adenohypophysis, where the Lh cells are present (Fig. 4.7A, B, and C). In fasted pituitaries, Gnrh2 neurons are abundantly innervating the neurohypophysis and extend into the adenohypophysis, in close proximity to many Lh cells (Fig. 4.7D, E, and F). When quantifying the number of Lh cells contacted by Gnrh2 neurons, there are significantly more innervations of Lh by Gnrh2 in fasted pituitaries compared to fed, with an almost 4-fold increase (Fig. 4.7G).

**Effects of fasting on the number of Fsh cells in proximity to Gnrh2 neurons**

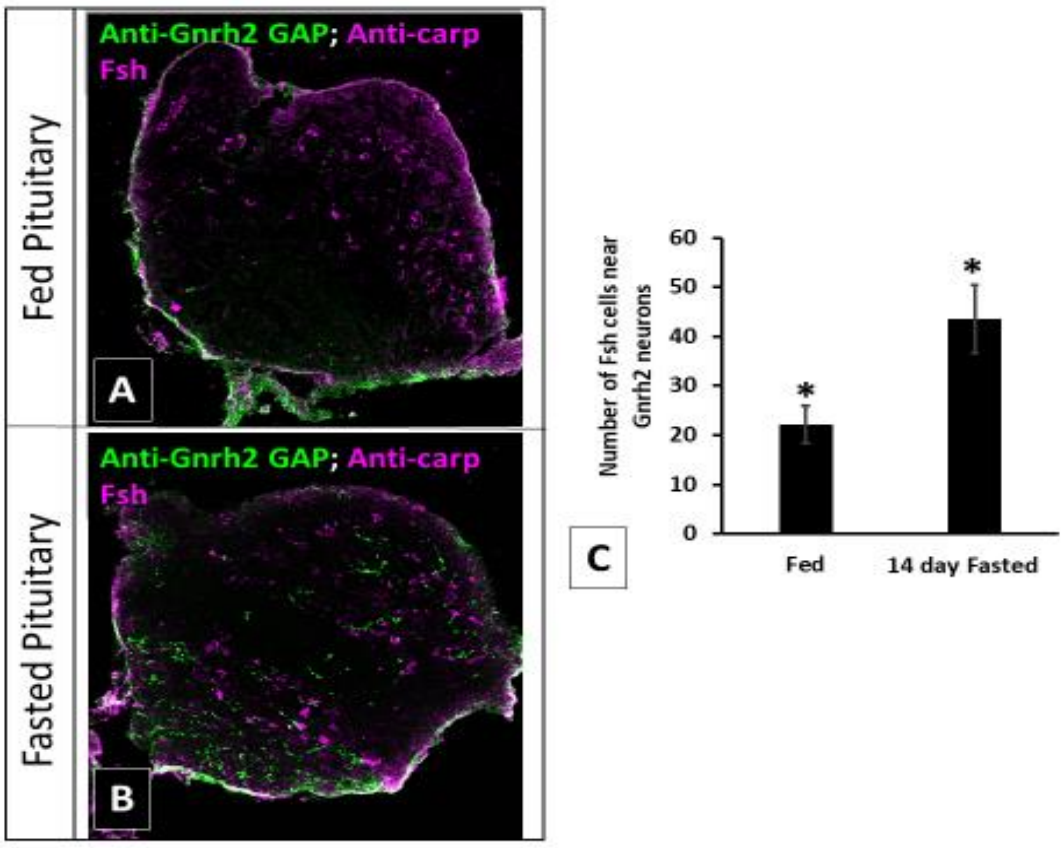


**Figure 4.8**. Double immunostaining of Gnrh2 (green) using antibodies against GAP2, and Fsh (magenta) using antibodies against carp-Fsh in fed (A) and 14-day fasted (B)

pituitary sections (10 µm thick). Average number of Fsh cells contacted by Gnrh2 neurons in fed and 14-day fasted pituitaries (C, n=6/group). Bar graphs are represented as mean ± SEM. Stars indicate significantly different number of Fsh-Gnrh2 contacts between fed and fasted conditions ( * = P < 0.05; ANOVA).

Pituitaries from normally fed zebrafish with immunolabelled Gnrh2 and Fsh, show few Gnrh2 fibers with Gnrh2 protein and, on average, exhibit around 20 Fsh cells in close proximity to Gnrh2 neurons (Fig. 4.8A & C). Pituitaries from 14-day fasted zebrafish which were immunolabelled with Gnrh2 and Fsh, show a higher abundance of Gnrh2 protein in and around the adenohypophysis region, and next to Fsh cells (Fig. 4.8B). There was, on average, about 45 Fsh cells innervated by Gnrh2 in fasted pituitaries, a 2-fold increase from fed pituitaries (Fig. 4.8C).

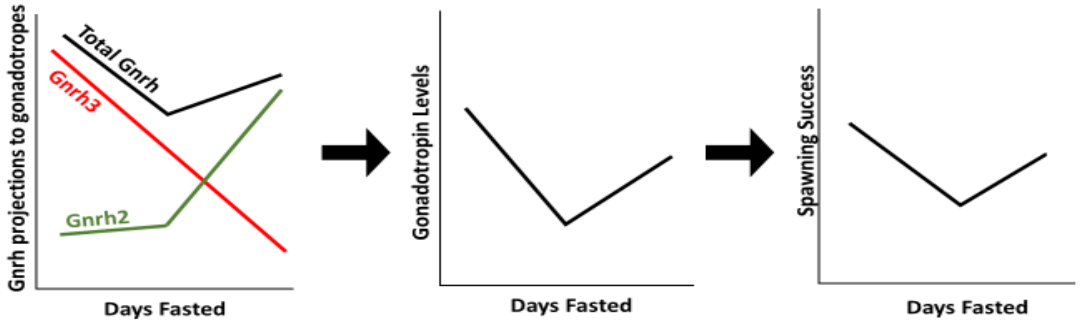


**Figure 4.9.** Summarized pattern of Gnrh levels in the pituitary, gonadotropin levels, and reproductive outputs throughout a fasting period in male zebrafish

**Confocal imaging of Gnrh2 and Gnrh3 neuronal innervations with melatonin cells**

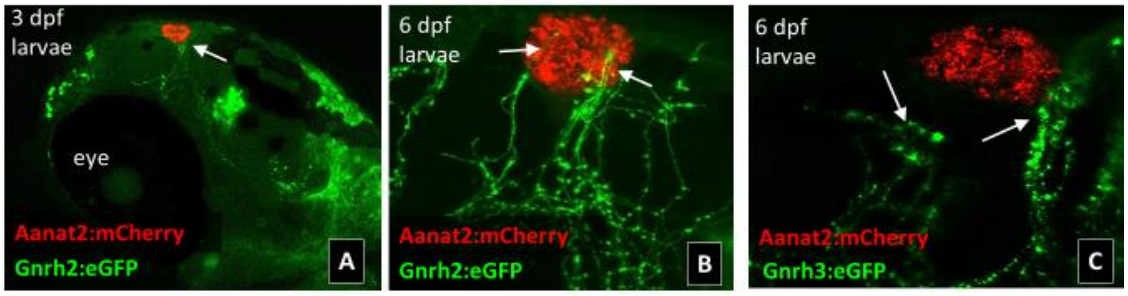


**Figure 4.10.** Sagittal image of a double transgenic tg(Gnrh2:eGFP;Aanat2:mCherry) zebrafish larvae at 3 dpf (A) and 6 dpf at 20X magnification (B) showing Gnrh2

innervations of Aanat2 cells (white arrows). Sagittal image of a double transgenic tg(Gnrh3:eGFP; Aanat2:mCherry) zebrafish at 6 dpf (C) showing the closest projections to Aanat2 cells (white arrows).

As early as 3 dpf, Gnrh2 neurons (green) are seen in very close proximity to Aanat2 or melatonin cells (red) in the pineal gland (Fig. 4.10A). By 6 dpf, Gnrh2 neurons are seen innervating melatonin cells in the anterior and posterior pineal gland (Fig. 4.10B). Gnrh3 neurons in larvae at 6 dpf are seen coming close to the pineal gland, but do not contact melatonin cells (Fig. 4.10C).

**Confocal imaging of Gnrh2 and melatonin cells in larvae exposed to different photoperiods**

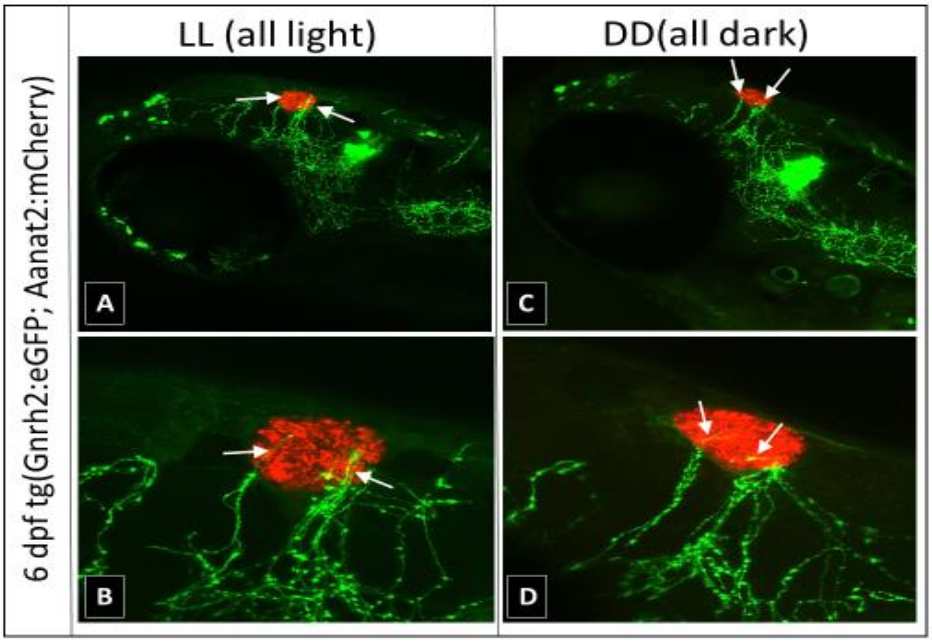


**Figure 4.11.** Sagittal images of whole transgenic zebrafish larvae (tg(Gnrh2:eGFP;Aanat2:mCherry)) at 6 dpf exposed to all light photoperiods at 5X (A) and 20X (B) magnifications, and larvae exposed to all dark photoperiods at 5X (C) and 20X (D) magnifications, depicting Gnrh2 neurons innervating Aanat2 cells (white arrows).

Zebrafish larvae at 6 dpf exposed to all light photoperiods show projections to the pineal gland, with innervations of melatonin-producing cells in the anterior and posterior region of the gland at 5X (Fig. 4.11A) and 20X magnifications (Fig. 4.11B). Zebrafish

larvae at 6 dpf exposed to all dark photoperiods do not show any different patterns of Gnrh2 neuronal projections, with similar innervations of melatonin-producing cells in the anterior and posterior region portion of the pineal gland at 5X (Fig. 4.11C) and 20X (Fig. 4.11D) magnifications.

**Immunohistochemistry of Gnrh2 in adult Aanat2:mCherry pineal glands**

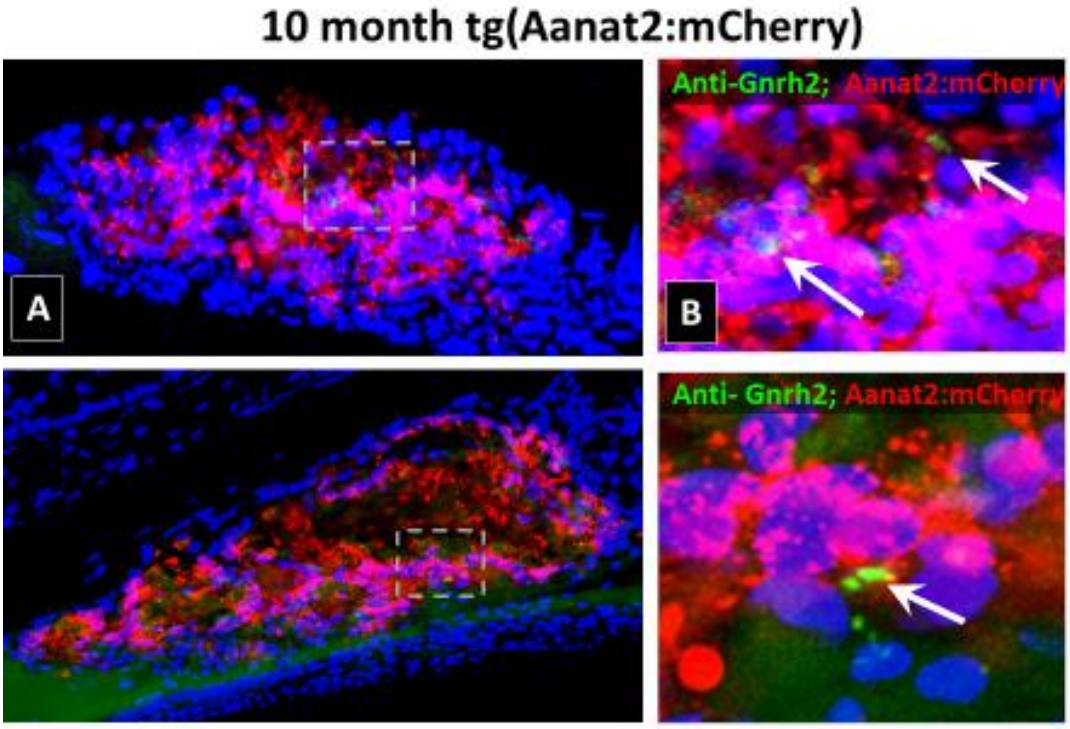


**Figure 4.12.** Pineal gland sections of adult zebrafish, depicting transgenic signal of Aanat2 (Aanat2:mCherry) with immunolabelled Gnrh2 neurons (white neurons) and DAPI-stained nuclei at 5X (A) and 20X (B) magnifications.

Cryosections of adult zebrafish pineal glands show the presence of immunolabelled Gnrh2 neurons. Although they are few in number, some of the innervations are juxtaposed to melatonin-producing (Aanat2:mCherry) cells, visualized at 5X (Fig. 4.12A) and 20X (Fig. 4.12B) magnifications.

**Effects of *in vitro* treatments of Gnrh2 on melatonin production of pineal gland cultures**

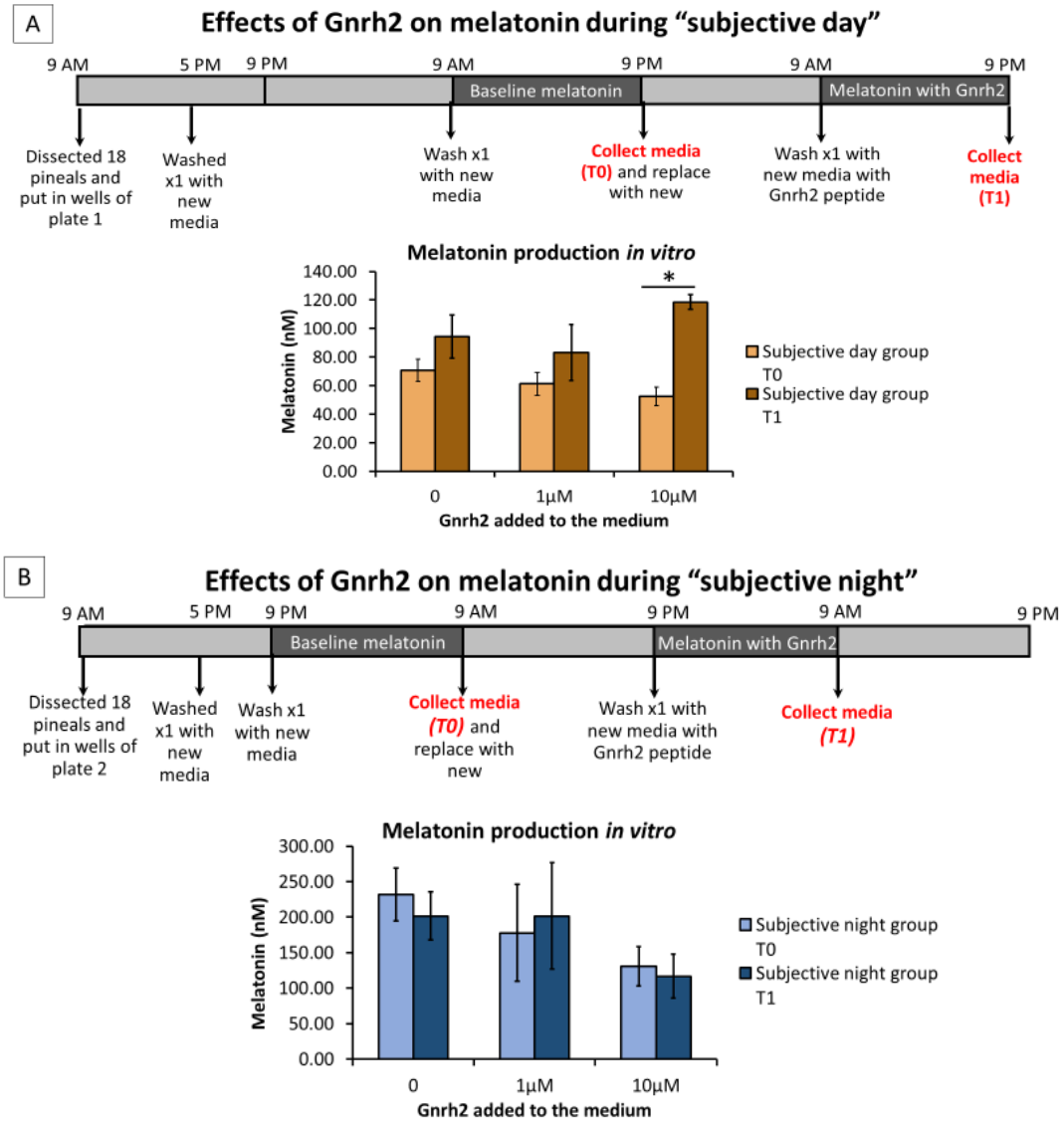


**Figure 4.13.** Concentrations of melatonin secreted from pineal gland cultures during the daytime, before and after different concentrations of 12-hour Gnrh2 peptide treatments (A), and concentrations of melatonin secreted from pineal gland cultures during the nighttime, before and after different concentrations of 12-hour Gnrh2 peptide treatments (B). All data are represented as mean ± SEM . Stars (*) depict significantly different melatonin concentrations after Gnrh2 treatment. (* = $P < 0.05$; ANOVA).

Over a 12-hour period of pineal gland culture, between 50 and 70 nM of melatonin is secreted to the media during the subjective daytime period (T0, Fig. 4.13A).

After Gnrh2 treatments of 10 µM concentrations, melatonin production increased 2-fold to an average concentration of 120 nM (Fig. 4.13A). Lower concentrations of 0 and 1 µM did not induce an increase in daytime melatonin secretion from pineal glands (Fig. 4.13A). Over a 12-hour period of subjective nighttime, pineal gland cultures secreted and average of 130 to 230 nM of melatonin (Fig. 4.13B). Treatments of Gnrh2 peptide did not induce any changes in melatonin secretion from pineal glands during the subjective nighttime (Fig. 4.13B).

**Photoperiodic mobility differences of *gnrh2*$^{-/-}$ and wild-type larvae**

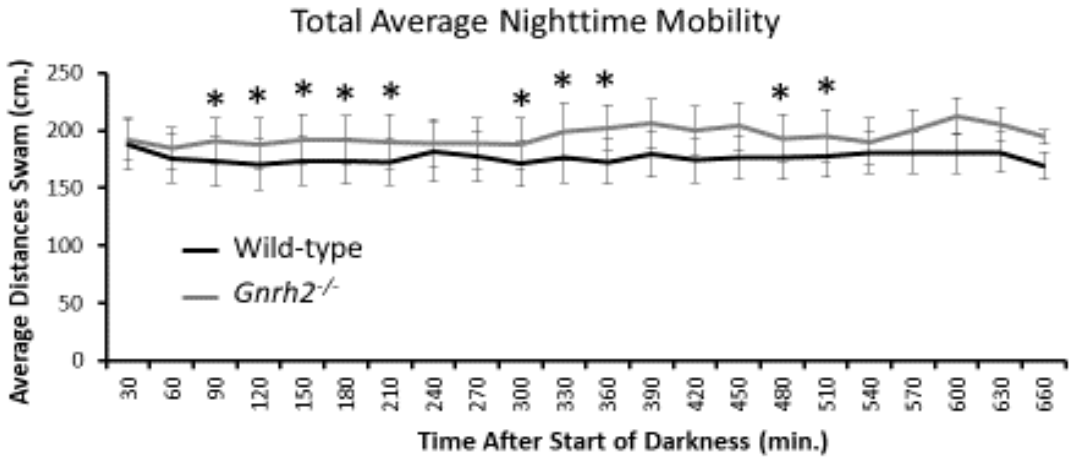


**Figure 4.14.** Average total distances swam in centimeters (cm.) from six trials of 12 each wild-type and *gnrh2*$^{-/-}$ larvae during the 11-hour nighttime photoperiod. All data were expressed as means ± S.E.M. Stars indicate significant difference between *gnrh2*$^{-/-}$ and wild-type zebrafish (* = $P < 0.05$; Repeated measures ANOVA with post-hoc).

During the nighttime hours, *gnrh2*$^{-/-}$ larvae averaged around 190 cm. distances swam over a 30 minute period of time, whereas wild-type larvae averaged around 20 cm. less per each 30 minute period, a significant decrease (Fig. 4.14). Over certain 30-minute stretches, *gnrh2*$^{-/-}$ larvae swam significantly longer distances than wild-type, however, on

average, wild-type larvae did not ever swim more distances than *gnrh2*$^{-/-}$ larvae (Fig. 4.14).

**Relative expression of *aanat2* in sibling wild-type, heterozygous, and Gnrh2 knockout counterparts**

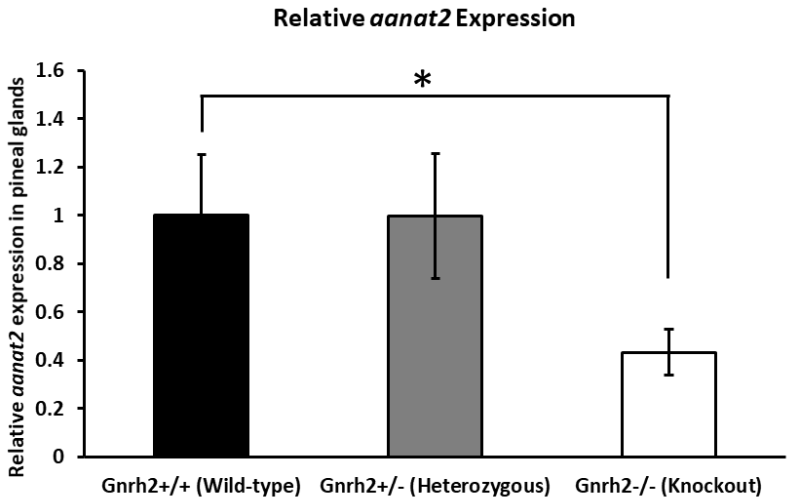


**Fig. 4.15.** Relative mRNA levels of *aanat2* in wild-type, heterozygous, and Gnrh2 knockout siblings from the same parents. All data were expressed as means ± S.E.M. Different letters indicate significantly different expression levels or protein contents, i.e. a is significantly different from b ($P < 0.05$; ANOVA).

Expression of *aanat2* in Gnrh2 knockout fish is at a 2-fold lower level compared to their heterozygous, and wild-type siblings. Heterozygous fish and wild-type fish on average contain the same mRNA levels of *aanat2*.

## Discussion

In this chapter, the roles of Gnrh2 in mediating reproduction and feeding are explored, by first focusing on the role of Gnrh2 in reproduction dependent on the nutritional status of zebrafish, and then exploring potential pathways Gnrh2 takes to enact its dual mode of regulation on feeding and reproduction. Surprisingly, a very strong inhibition of reproduction is seen in fasted *gnrh2*$^{-/-}$ fish, compared to wild-type fish,

giving us clues for the first time that Gnrh2 may be involved in maintaining reproduction in nutritionally deprived teleosts. In previous chapters, we show that Gnrh2 has minor roles in reproduction (such as a possible role in regulating oocyte quality and *lhb* expression in Chapter 2) and a potentially major role in regulating feeding behavior (Chapter 3). In this chapter, we show that the minor role of Gnrh2 in regulating reproduction in fed states becomes more prominent in fasted states, where Gnrh3 is inhibited. I also explored an additionally pathway in which Gnrh2 can enact its dual role in downregulating feeding and increasing spawning and reproductive parameters by showing the Gnrh2 innervation of cells producing melatonin, a hormone which is involved in downregulating feeding and promoting gametogenesis in zebrafish, and potential ability to induce melatonin release. To date, Gnrh2 innervation of the pineal gland and modulation of melatonin was shown in only the European sea bass, a perciform belonging to the Moronidae family (Servili et al., 2010). The finding of a similar phenomenon in a cyprinid suggests this pathway may be conserved in other teleosts as well.

Previous studies in musk shrews and mice, show that specifically treatments of Gnrh2, but not Gnrh1, can induce reproduction of these animals in starved/fasted states (Kauffman et al., 2005), suggesting an isoform-specific role of Gnrh2 in promoting reproduction in fasted states. In order to determine if Gnrh2 has a similar role in zebrafish, fish with and without endogenous production of Gnrh2 and Gnrh3 were subjected to short and long-term starvation to determine if this is a universal role for the neuropeptide. A very striking difference was seen in reproductive outputs in *gnrh2*$^{-/-}$ compared to wild-type counterparts in fasted states, where *gnrh2*$^{-/-}$ females and males

exhibited significantly less successful spawns after 14 days of fasting compared to wild-type. However, the same case was not seen in *gnrh3*$^{-/-}$ fish, where their reproductive outputs were the same as wild-type counterparts in both fed and fasted states. This shows that the loss of Gnrh2 but not Gnrh3 results in inhibited reproduction after long-term fasting, suggesting that Gnrh2 is an important reproductive regulator during this state. There were sex-specific differences in the reproductive response to fasting. Male wild-type and *gnrh3*$^{-/-}$ zebrafish showed slightly decreased spawning rates at 7-days fasting, but 14-days fasting the reproduction rates were similar to normally fed states again, implying a recovery occurs. Female zebrafish of all genotypes show continuously decreased reproductive rates associated with longer periods of fasting. This phenomenon is similar to that shown in medaka (*Oryzias latipes*), where female reproduction was more negatively impacted by food depletion than males, and the Gnrh1 neuronal firing was inhibited by food-depleted, low-glucose conditions in females more than males (Hasebe et al. 2016).

The fact that *gnrh3*$^{-/-}$ fish were able to reproduce after long-time fasting implies that the presence of just Gnrh2 is enough to stimulate reproduction and gametogenesis for successful spawns. The presence of only Gnrh3 in fasted states was not enough to maintain reproduction, suggesting that this peptide is specifically inhibited by fasting in zebrafish. The specific inhibition of Gnrh3 or the main hypophysiotropic Gnrh, Gnrh1, by fasting has been shown in other species. In glass catfish (*Kryptopterus vitreolus*), fasting decreased the expression levels of *gnrh1*, the main hypophysiotropic Gnrh in this species, but did not affect the expression of *gnrh2* (London and Volkoff, 2019). Additionally, Gnrh3 soma in the hypothalamus region of adult zebrafish express the

receptor for orexigen/hypocretin (Hcrtr), a feeding hormone which is elevated after fasting, and the neuronal firing rate of Gnrh3 is inhibited after treatment with Hcrt (Zhao et al. 2016). The presence of Gnrh2, but not Gnrh3, being able to maintain reproduction in zebrafish undergoing fasting, is a similar phenomenon as seen in two different mammalian species, mice and musk shrews. As stated earlier, starved mice and musk shrews were able to be stimulated to reproduce after administrations of Gnrh2, but not Gnrh1, showing that the organism is more responsive to Gnrh2 during this fasted state (Kauffman and Rissman, 2004a; Kauffman and Rissman, 2004b). In animals in which reproduction is still important even under food depleted conditions, such as teleosts, Gnrh2 may be an important backup system to continue maintaining reproduction after the inhibition of Gnrh1 or Gnrh3. In support of this theory, rodents which have lost Gnrh2 have severe reproductive defects after short-term starvation, such as in female mice which exhibit infertility after just 48 hours of starvation (McClure, 1959, Bronson and Marstellar, 1985). It is possible that the evolutionary loss of Gnrh2 in these animals also resulted in the loss of the ability to maintain gonadal maturation and reproductive performance after longer periods of starvation. This may be an evolutionarily beneficial trait to ensure that reproduction only occurs in nutritionally abundant states, since parental care requires a lot of energy, as opposed to teleosts in which many species do not exhibit energetically costly care for their young (Balshine et al., 2002).

For the first time, the failure of reproduction in zebrafish after the loss of Gnrh2 in nutritionally deprived states was characterized at the gonadal level, by examining gonad morphology and gametogenesis of *gnrh2*$^{-/-}$ and wild-type males and females. In male zebrafish, the testes exhibited slight decreases in size, but the presence of mature

spermatozoa was seen in both wild-type and *gnrh2*$^{-/-}$ males, with no differences seen in gametogenesis, suggesting that Gnrh2 is not a main regulator of spermatogenesis in fasted states. This is similar to the case in fed zebrafish, where the loss of Gnrh2 was not associated with any differences in testes morphology or spermatogenesis, but slight changes in oocyte quality were seen (discussed in detail in Chapter 2). However, in fasted states, a much more dramatic inhibition of oocyte maturation is seen in fasted *gnrh2*$^{-/-}$ females compared to fasted wild-type females. Whereas only slight differences are seen in fed states, after fasting, *gnrh2*$^{-/-}$ females fail to produce any mature oocytes, whereas wild-type females still exhibit some mature oocyte formation. Additionally, the GSI of fasted *gnrh2*$^{-/-}$ females is significantly decreased compared to fasted wild-type females. These results suggest that, where the role of Gnrh2 in maintaining oocyte maturation in fed states is minimal, it becomes a crucial regulator of final oocyte maturation in fasted conditions. It is possible the reduced ovulation and oocyte maturation in *gnrh2*$^{-/-}$ females are due to decreased levels of Lh secretion after fasting, since Gnrh2 may be an important stimulator of Lh synthesis and secretion (as discussed in Chapter 2). *Lhb* knockout studies in zebrafish demonstrate the importance of Lh in the final maturation of oocytes and ovulation in females (Chu et al., 2014). In medaka, Gnrh2 has been shown to potently activate the Lh gonadotropes through extracellular and intracellular calcium uptake (Strandabø et al., 2013), and in zebrafish, Gnrh2 can activate all four Gnrh receptors (Tello et al., 2008), suggesting that Gnrh2 may have critical roles in promoting Lh secretion. Additionally, in previous chapters, it was shown that the loss of Gnrh2 was associated with decreased Lhb levels throughout development and in adult males, suggesting that Gnrh2 promotes *lhb* expression. Additionally, we showed in previous

chapters that administration of Gnrh2 *in vivo* and *in vitro* upregulate *lhb* expression, further supporting the role of Gnrh2 as an administrator of Lh production. Fasting conditions in the *gnrh2*$^{-/-}$ fish results in a brain depleted of both Gnrh isoforms, and in turn, a reduction in Lh output, and consequently, failed final oocyte maturation and ovulation of which Lh is responsible (Chu et al., 2014).

It was previously shown that Gnrh2 may have minor roles in Lh regulation (Chapter 2), but with the suggested change to a more prominent role in reproduction regulation after fasting, I wanted to examine if Gnrh2 also had a more major role in gonadotropin expression and synthesis regulation, and examine the neuroanatomical innervations of the Gnrh neurons to gonadotrope cells in fed and fasted states. Since males exhibit a decrease, then recovery of reproduction after long-term fasting, the pituitaries of males were used to examine if the gonadotropin levels exhibited similar or different patterns after fasting. Similar to the spawning rate patterns, wild-type males did indeed exhibit slight decreases in *lhb* and *fshb* expression, and protein synthesis, and then a recovery to basal levels, suggesting the changes in gonadotrope levels were the cause for the reduced reproduction rates after 14-day fasting. Conversely, the lack of Gnrh2 in the *gnrh2*$^{-/-}$ line, eliminated this recovery, with *lhb* and *fshb* expression and protein content staying at decreased levels after 14-day fasting. Hence, the presence of Gnrh2 is necessary to stimulate Lh and Fsh expression and synthesis in these fasted states. In order to study how the Gnrh neuronal projections are neuroanatomically interacting with gonadotrope cells during the fasting period, immunohistochemistry and transgenic fish were used to examine the Gnrh neurons and their proximity to gonadotropes. An interesting plasticity was seen where Gnrh3 neuronal projections dramatically decrease

their presence in the pituitary over the fasting period, with fewer nerve terminals seen in the proximal pars distalis (PPD) and next to gonadotropes, whereas Gnrh2 neurons increase their presence in the PPD and their innervations to gonadotrope cells. The increased presence of Gnrh2 to the pituitary after fasting has been seen before in the Zohar lab (Xia et al., 2014), and intriguingly, is also a similar pattern seen in the brain of the mammalian musk shrew (Temple et al., 2003). As stated earlier, after fasting, Gnrh2 immunoreactive fibers increased in abundance in the median eminence, the area of the mammalian brain with vascular connections to the pituitary (Temple et al., 2003). In mammals, the hypophysiotropic Gnrh neurons terminate in the median eminence and secrete the Gnrh protein to the hypophyseal portal system, which then travels to the pituitary (Clarke et al., 1987), a different phenomenon from teleosts, where Gnrh neurons project into the pituitary and directly terminate on gonadotropin cells, or around vasculature near the gonadotropes (Golan et al., 2014). The increase of Gnrh2 neurons in the median eminence of musk shrews after fasting is thus similar to the increase of Gnrh2 neurons near the pituitary gonadotropes in zebrafish. Since the same pattern is seen in a teleost and mammalian model, the plasticity of Gnrh2 in nutritionally deprived states may be conserved amongst many vertebrate groups. The ability of Gnrh2 to exhibit plasticity in nutritionally deprived states suggests that it may be responding to nutritional signals, which are upregulated during this period. We previously showed that *agrp1* knockdown resulted in differential expression of *gnrh2* (Fig.3.8, Chapter 3), and that Mc4r receptors are expressed in Gnrh2 neurons, but not Gnrh3 (Fig. 3.11, Chapter 3), suggesting that the melanocortin peptides Agrp and Pomca may be the factors that Gnrh2 is responding to. Another possible feeding signal that Gnrh2 may respond to is Leptin, a hormone which is

thought to be a major peripheral signal of lipid levels and involved in food and reproduction regulation (Copeland et al., 2011). In mammals, Leptin is a potent peripheral feeding coordinating hormone, produced in adipose cells and circulating through the body via the bloodstream, binding to many different feeding-related neurons in the hypothalamus and modulating appetite and fat metabolism (Maffei et al., 1995; Swoap, 2008). In teleosts, many of which possess two genes for Leptin, the roles of this hormone in appetite-regulation is not as well known, and appear to be species-specific, but is still thought to have conserved roles involved in reproductive regulation and fat metabolism (Copeland et al., 2011). In pike perch, administrations of Leptin, resulted in differential expression of *gnrh2*, but not *gnrh1* (Schaefer and Wuertz, 2016), providing another example of Gnrh2 specifically responding to nutritional signals in fish. In zebrafish, Leptin receptors are seen in the hypothalamic region, but the relationships of this hormone with the Gnrh isoforms still need to be studied (Liu et al., 2010). Upstream feeding factors which can modulate Gnrh2 in zebrafish will need to be further studied in order to determine the entire mediating network between feeding and reproduction.

Overall, a similar pattern can be seen when taking into account the amount of total Gnrh projections to the pituitary, gonadotropin expression/protein amount, and reproductive outputs over the 14-day fasting period (Fig. 4.9). During short-term starvation, Gnrh projections to the pituitary are low, corresponding similarly with low gonadotropin expression, gonadotropin protein content, and reproductive outputs After long-term, 14-day starvation, Gnrh2 projections have increased to the pituitary, corresponding similarly with a recovery of increased gonadotropin expression, gonadotropin protein content, and spawning success (Fig. 4.9). These results strongly

suggest that the presence of Gnrh2 is necessary to stimulate gonadotropin expression/secretion, oocyte maturation, and spawning in fasted states. In normally fed states, we previously showed that Gnrh2 has roles in *lhb* but not *fshb* expression, but in fasted states, it seems that Gnrh2 also is important for *fshb* expression and synthesis as well. After fasting, Gnrh2 appears to be one of the main factors regulating the brain-pituitary-gonad (BPG) axis, in which the loss of Gnrh2 in the knockout *gnrh2*$^{-/-}$ line resulted in a disruption of this axis at both the pituitary and gonad level (in females). The presence of normal spermatogenesis in fasted male *gnrh2*$^{-/-}$ fish, suggests that the decreased levels of Gnrh3 in the pituitary and lower levels of *fshb* and *lhb* were enough to still maintain spermatogenesis, but not enough for the more energetically costly oogenesis. Female reproduction is usually much more inhibited by fasting states than males in other animals (Hasebe et al., 2011). Even so, spawning is still seen in 14-day fasted wild-type females with Gnrh2, suggesting that Gnrh2 has a major reproductive role depending on the nutritional condition.

Our results show that Gnrh2 has a dual role in mediating feeding and reproduction. To analyze the mechanisms in which Gnrh2 takes to enact this role, the ability of Gnrh2 to modulate melatonin in zebrafish was examined. Melatonin-producing cells are found in the pineal gland and exhibit nighttime peaks (Zhdanova et al., 2008), with the ability to affect both feeding behaviors and reproductive behaviors in many vertebrates (Lima-Cabello et al., 2014). In zebrafish, melatonin can decrease feeding behaviors via the modulation of orexigenic and anorexigenic gene expression in the brain (Piccinetti et al., 2010), and also induces oocyte maturation and increased fecundity (Carnevali et al., 2011) suggesting that the roles of melatonin parallel the roles of Gnrh2.

In European sea bass, Gnrh2 neuronal projection were seen to contact and innervate the pineal gland, with both afferent and efferent neurons determined through immunohistochemistry and retrograde tracing, and Gnrh2 treatments elicited melatonin secretion from pineal gland cultures, suggesting that Gnrh2 regulates melatonin in this species (Servili et al., 2010). In the double transgenic tg(Aanat2:mCherry;Gnrh2:eGFP) larvae, we also saw the presence of numerous Gnrh2 neurons in the pineal region, and saw Gnrh2 neurons at 3 dpf coming in very close contact with melatonin-producing cells. By 6 dpf, a few Gnrh2 fibers were seen to contact melatonin cells in the anterior and posterior pineal gland. Conversely, the double transgenic tg(Aanat2:mCherry; Gnrh3:eGFP) larvae show that Gnrh3 neurons come in close proximity to, but do not innervate melatonin cells, showing that this is a specific interaction of Gnrh2 with melatonin cells. Gnrh2 immunoreactive neurons were also seen in transgenic adult pineal glands, suggesting that Gnrh2 is innervating melatonin cells in both larval and adult stages. Gnrh2 patterns to the pineal gland did not change when larval zebrafish were subjected to all light (LL, melatonin-inhibiting), normal photoperiod (LD), or all dark (DD, melatonin-promoting) photoperiods, suggesting that different photoperiods, corresponding with different melatonin levels, do not affect Gnrh2, which may mean that Gnrh2 is upstream to the control of melatonin. The ability of Grnh2 to directly regulate melatonin from pineal glands was tested and shown that Gnrh2, at higher concentrations, can induce increases in melatonin during the daytime, but not nighttime hours. This may suggest that Gnrh2 has a photoperiod-specific role of eliciting melatonin increases at certain times of the day. Since melatonin production induces increased GSI, follicle growth, and maturation, most likely to prepare the ovaries for a morning spawn

(Carnevali et al., 2011), Gnrh2 may help to begin this process at the evening hours, by stimulating melatonin increases. Gnrh2 expression increases during the evening, peaking around the time of lights turning off, supporting the theory that it may begin to stimulate melatonin during this period (Paredes et al., 2019). To concretely determine whether Gnrh2 has roles in melatonin production and photoperiodic behaviors, both *aanat2* expression and circadian mobility was compared between *gnrh2*$^{-/-}$ and wild-type larvae, and *gnrh2*$^{-/-}$ larvae were shown to have significantly increased levels of mobility during the nighttime hours compared to wild-type. Since melatonin normally induces a sleeping pattern and reduced mobility in fish (Zhdanova et al, 2001), it is most likely the loss of Gnrh2 leading to decreased melatonin which results in the higher nighttime activity in these animals. These results suggest that Gnrh2 does have a role in stimulating melatonin through innervating melatonin cells and possible stimulating differential regulation of *aanat2*. The regulation of melatonin may be one indirect method Gnrh2 enacts in order to regulate feeding and reproduction in a photoperiodic-specific manner. Under normally fed conditions, the loss of Gnrh2 resulted in the loss of oocyte quality, despite only sparse neuronal projections to the gonadotropin cells in the pituitary. Gnrh2 may also control oocyte maturation overnight through beginning to stimulate melatonin in the evening hours, which directly circulates to the brain, pituitary, and gonad-level to induce oocyte maturation (Carnevali et al., 2010; Zohar et al., 2010; Lima-Cabello et al., 2014). This pathway will need to be further examined to see if this is the case. The presence of Gnrh2 innervations in both European sea bass and zebrafish, suggest that the ability of Gnrh2 in regulating melatonin may occur in other teleost species as well.

In summary, in this chapter we show for the first time that Gnrh2 has major roles in mediating reproduction and feeding and is crucial to maintain reproduction in fasted zebrafish. The plasticity of Gnrh3 and Gnrh2 neuronal projections in the pituitary, based on the nutritional status of zebrafish, is shown. We show that the gonadotropin expression patterns and protein content is consistent with this pattern of Gnrh3 loss and Gnrh2 increase, where the increase of Gnrh2 at two weeks of fasting is enough to recover decreased gonadotropin levels which occur during short-term fasting, suggesting that Gnrh2 is a backup system to continue reproduction even under food-depleted states. To support this theory, we see that fish which do not contain Gnrh2 (*gnrh2*$^{-/-}$) exhibit severely reduced spawning rates after long-term starvation, unlike wild-type and *gnrh3*$^{-/-}$ fish. It is possible that Gnrh2 is the key to continuing reproductive success during a fasted state in many vertebrates, however, other species will need to be studied to see if this is the case. Additionally, we suggest an additional pathway in which Gnrh2 is enacting its dual role in controlling feeding and reproductive behaviors, through the potential modulation of melatonin from the pineal gland in zebrafish, which may help to maintain oocyte maturation and decrease feeding behaviors overnight.

# CHAPTER 5: Studying the Roles of Gnrh2 in Olfactory Transduction


## Abstract

The gonadotropin releasing hormone (GNRH) family is the main upstream neuropeptide coordinating reproduction and thus, integrates many different signals in order to synchronize gonadotropin secretion and reproduction based on optimal environmental and internal conditions. The teleost-specific Gnrh3 present in the olfactory bulb is known to be able to transduce pheromonal signals. A thorough re-examination of the Gnrh2 transgenic line (tg(Gnrh2:eGFP)) in zebrafish identified a previously uncharacterized population of Gnrh2 neurons in this region, in close proximity to and colocalized in some cases with Gnrh3 neurons. The colocalization occurs in only a few neurons in larval stages, but in adulthood, numerous cells appear to coexpress both isoforms of Gnrh. Although previous studies in mammalian cells and sea lamprey demonstrate coexpression of multiple isoforms in GNRH cells, the presence and function of Gnrh2 in the olfactory bulb of teleosts have never before been studied. The localization of the Gnrh2 cells potentially exhibit a sex-specific difference, with females and males expressing colocalized cells in slightly different regions of the olfactory bulb. The involvement of olfactory bulb Gnrh2 in transducing chemosensory signals in adult zebrafish is demonstrated through expression changes between monosex and mixed sex tanks. Functional assays and qPCR have determined that these social cues, but not feeding differences, induced similar changes in *gnrh2* and *gnrh3* expression in the olfactory region, suggesting that Gnrh2 is transducing similar pheromonal cues as Gnrh3 and may help to coordinate chemosensory cues with reproductive behaviors.

## Introduction

The gonadotropin-releasing hormone (GNRH) peptide family is one of the main regulators of gonadotropin release from the pituitary, resulting in gametogenesis and steroidogenesis (Zohar et al., 2010). GNRH neuronal activity must be highly coordinated with environmental, external signals, along with internal signals in order to coordinate reproduction and steroid production at the optimal time. Some upstream neuropeptides, such as Kisspeptins, are known to help integrate photoperiod and seasonal cues to upregulate GNRH at the appropriate time of the day or year (Revel et al., 2006). GNRH may also be able to directly integrate external signals, such as pheromonal cues and photoperiod signals, to coordinate reproductive activities (Servili et al., 2010; Espigares et al., 2017; Li et al., 2017).

The preoptic area (POA) hypophysioptropic GNRH in mammalian vertebrates has a well characterized presence in the olfactory region of the brain, with GNRH disrupting mutations causing an anosmic syndrome, called Kallman's syndrome, which results in both reproductive and olfactory defects (Forni and Wray, 2015). In zebrafish, the olfactory Gnrh, called Gnrh3, is thought to be involved in regulating the Gnrh3 migration patterns from the nasal placode to the preoptic area, with many neurons staying in the olfactory bulb (Palevitch et al., 2009). For many years, the teleost Gnrh3 neurons in the olfactory bulb/terminal nerve (OB/TN) of adults were believed to carry unspecified neuromodulatory functions (Zohar et al., 2010). The sensory cues, whether internal or external, in which the Gnrh3 neurons are transducing have yet to be fully characterized, with some controversial and potentially species-specific results. One study in goldfish (*Carassius auratus*) suggests that sex pheromones are signaling through the medial

olfactory system, and only tactile stimulations inhibit the terminal nerve, suggesting a role of Gnrh3 terminal nerves in transducing physical signals (Fujita et al., 1991). However, another study in goldfish show that males exposed to females exhibiting spawning behaviors increases Gnrh expression levels in the olfactory bulb for several hours, suggesting the female pheromones are inducing this increase (Yu and Peter, 1990). In axolotls (*Ambystoma mexicanum*), terminal nerve Gnrh is thought to inhibit the olfactory response to food cues, potentially to decrease the desirability of food during courtship and mating sessions (Park and Eisthen, 2003). In medaka (*Oryzias latipes*), a fish which contain three different isoforms of Gnrh, the Gnrh3 isoform which is only seen in the olfactory/terminal nerve, but not in the hypothalamic region, was found to have regular pacemaker activity and thought to be more involved in neuromodulation than chemical pheromone transduction (Karigo and Oka, 2013). However, receptors of Gnrh have been shown to increase during the reproductive breeding season, and ablation of the terminal nerve abolishes male dwarf gourami (*Trichogaster lalius*) nest-building behaviors, giving further clues that these neurons are involved in reproductive functions in many fish (Wirsig-Wiechmann, 2001). Additionally, a recent study in zebrafish has shown that the Gnrh3 olfactory bulb neurons are important for integrating female pheromone signals and activating male reproductive behaviors (Li et al., 2017), suggesting a role in pheromonal detection for olfactory bulb Gnrh3 as well.

Interestingly, a previous study in zebrafish Gnrh localization showed abundant Gnrh2 neuronal innervation in the olfactory regions (Xia et al., 2014). Relying on common consensus that Gnrh2 soma are present only in the midbrain tegmentum, the study concluded that Gnrh2 axons, originating in the midbrain, are forming bundles of

axon terminals in the olfactory bulbs next to Gnrh3 soma. However, using improved imaging techniques to document the Gnrh2 neurons in the tg(Gnrh2:eGFP) and the double transgenic tg(Gnrh2:eGFP;Gnrh3:tdTomato) lines, it was suspected that Gnrh2 soma are also present in the forebrain. This has raised the possibility that these Gnrh2 neurons, if indeed are genuine forebrain populations, may also be involved in integrating olfaction cues. Concrete proof of Gnrh2 soma in olfactory regions has not been shown before, however, previous studies give clues that Gnrh2-producing cells are present in this location. Several studies in goldfish propose the localization of Gnrh2 in the olfactory bulb, with one group suggesting Gnrh2 soma are present in the olfactory bulb, but in fewer number, than Gnrh3, along with immunoreactive fibers (Kim et al., 1995), and other groups showing the presence of *gnrh2* mRNA in olfactory bulb and olfactory tracts (Lin and Peter, 1997; Yu et al., 1998). Contrastingly, one group refutes their claims by showing that specific mRNA probes only detect *gnrh3* in the olfactory bulb, but not *gnrh2* (Kawai et al., 2010). It is possible that the Gnrh2 neurons are highly plastic and their presence in goldfish is dependent on the age, sex, or environmental conditions of the individual, providing a potential explanation for why some groups find Gnrh2 in the olfactory terminal nerve and others do not. It was shown in zebrafish, by using qPCR, that Gnrh2, Gnrh3, and Gnrh receptors mRNA are found in the olfactory epithelium, including the receptor specifically activated by Gnrh2, GnrhR3 (Tello et al., 2008; Corchuelo et al., 2017). Moreover, olfactory *gnrhr3* expression peaked and *gnrh2* expression had a 10-fold increase in mid-vitellogenic stages of ovarian maturation, suggesting that Gnrh2 may have a role alongside Gnrh3 in coordinating olfaction and reproductive development (Corchuelo et al., 2017). In tilapia (*Oreochromis niloticus*),

both subtypes of Gnrh receptors, GnrhR1 and GnrhR2, are expressed in the olfactory bulbs, indicating that all Gnrh isoforms are capable of binding to and modulating olfactory cells (Maruska and Fernald, 2010).

The concrete roles that the Gnrh isoforms play in signal transduction in the olfactory region have yet to be fully characterized. However, a role in pheromone transduction seems to be likely, as external pheromonal signals have previously been shown to affect Gnrh in vertebrates. In female goats (*Capra aegagrus hircus*), just a few minutes exposure to male pheromones is enough to stimulate increases in the frequency of POA GNRH pulses in the female (Ichimaru et al., 1999). A feedback loop has been suggested between GNRH and pheromonal cues, where pheromonal signals can modulate GNRH activity, but GNRH can also modulate the sensitivity of the brain to pheromonal cues (Boehm et al., 2005). The organization of the fish olfactory bulb is thought to consist of three different cell populations, based on their cell type and location, which transduce three different signals: sex pheromones, food odors, and social cues/migratory signals (Hamdani and Døving. 2007). The sexual pheromone transduction in goldfish is thought to occur in the medial olfactory nerves (Hasegawa et al., 1994), and as Gnrh nerves are found in the medial olfactory tract of the olfactory bulb, along with the terminal nerves, they may help transduce these signals as well (Kim et al., 1995).

In this study, the roles of Gnrh2 in transducing olfactory signals were explored through localization of Gnrh2 in olfactory regions of the brain, including examining its presence and interactions in the olfactory epithelium and olfactory bulbs, identifying sex-specific coexpression patterns with Gnrh3 soma, and beginning to identify which odorant cues can modulate Gnrh2 expression. Unexpectedly, examining closely Gnrh soma in the

olfactory bulbs and olfactory epithelium of double transgenic zebrafish tg(Gnrh2:eGFP; Gnrh3:tdtomato) in *in vivo* conditions, evidence of both Gnrh2 and Gnrh3-expressing cells are seen. Both immunohistochemistry and *in situ* hybridization were used to verify the presence of Gnrh2 in the olfactory bulbs and its interactions with Gnrh3 soma. As a result of this study, we propose that Gnrh2 may be involved in coordinating pheromonal signals in the olfactory.

## Methods

### Zebrafish maintenance and husbandry

All zebrafish were kept in the in-house facility at the Institute of Marine and Environmental Technology and maintained at a 14L:10D cycle in a 28°C recirculating water system. Zebrafish larvae were fed live *Paramecium* from 5 days-post-fertilization (dpf) to 15 dpf and then fed live *Artemia nauplii* until they were large enough to feed on 300 µm Gemma diet pellets (Skretting), which they were fed *ad libitum* twice daily. Zebrafish larvae were kept in 300 mL tanks on a nursery shelf until 30 dpf, and then moved to aquaria in the recirculating water system. Prior to tissue collections, adult zebrafish were euthanized in a cold ice-water bath and then promptly decapitated. Larval zebrafish were killed in tricaine (MS-222, Sigma-Aldrich). All experimental protocols were approved by the Institutional Animal Care and Use Committee at the University of Maryland School of Medicine.

### Confocal imaging of Gnrh2 and Gnrh3 in olfactory bulbs of larvae and adults

In order to characterize Gnrh2 and Gnrh3 neuronal soma and projections in olfactory bulbs and epitheliums, whole zebrafish larvae or adult zebrafish brains of

double transgenic zebrafish (tg(Gnrh2:eGFP;Gnrh3:tdtomato)), were imaged in 1X artificial cerebrospinal fluid (ACSF: 119 mM NaCl, 26.2 mM $NaHCO_3$, 2.5 mM KCl, 1 mM $NaH_2PO_4$, 1.3 mM $MgCl_2$, 10 mM glucose, 2.5 mM CaCl2) solution with a confocal microscope. For developmental analysis of Gnrh2 and Gnrh3 in young zebrafish larval olfactory bulbs, zebrafish eggs at 1 DPF were raised in water containing Phenylthiourea (PTU) to prevent pigmentation, and at 3 and 5 DPF, larvae were euthanized in an ice-bath also containing saturated tricaine (MS-222). Zebrafish larvae were then mounted in 1.5% low-melting point agarose, and then covered in freshly oxygenated 1X ACSF solution to keep the tissues alive. Olfactory brain regions were located and images were taken on a Leica SP8 confocal microscrope at 5X and 20X magnification.

For adult olfactory bulb Gnrh2 and Gnrh3 imaging, male and female adult zebrafish, between 6-8 months old, were selected and euthanized in an ice water bath and subsequent spinal cord dislocation. Brains were dissected in an ACSF bath to keep the tissues alive, and then transferred to a circular slide with a glass bottom cover, and immersed in freshly oxygenated 1X ACSF, with the ventral side of the olfactory bulbs touching the bottom where the imaging plane would be captured. A minimum of five female and five male brains were dissected. Samples were then imaged using a Leica SP6 confocal microscope at 5X, 20X, and occasionally 40X magnification, with Gnrh2 captured using selective wavelengths for eGFP emission and excitation and Gnrh3 images captured with selective wavelengths for mCherry emission and excitation. Z-stack projections of olfactory bulb images were conducted using ImageJ.

**Double label immunohistochemistry of Gnrh2 and Gnrh3 in olfactory bulbs**

In order to determine if Gnrh2 is present in olfactory bulbs of zebrafish and colocalized with Gnrh3, immunohistochemistry (IHC) with antibodies against the specific recombinant GAP region of Gnrh2 (GAP2) raised in rabbits was used with wild-type or transgenic tg(Gnrh3:tdtomato) brains. Double immunohistochemistry was conducted with wild-type brains and with antibodies generated against the specific GAP2 or recombinant GAP region of Gnrh3 (GAP3) raised in rabbits. Brains from sexually mature wild-type or tg(Gnrh3:tdtomato) fish were dissected and fixed in 4% PFA overnight and immersed in 30% sucrose in PBS for four hours, or until brains sunk to the bottom of the vial. Brain samples were then frozen in OCT and stored at -80° C until sectioning. Cryo-sectioning was conducted at -20° C using a Tissue-Tek Cryo3 cryostat. Brains were sectioned to 10 µM thickness, placed on Plus coated slides, and stored at -80°C until immunohistochemistry was performed. To perform single IHC on tg(Gnrh3:tdtomato brains), slides were briefly fixed in acetone and then washed in PBS 3X. Slides were then blocked for one hour in 5% normal goat serum, and incubated with a 1:750 dilution of Anti-GAP2 in 1% BSA and 0.3% Triton X-100 overnight at 4°C. Slides were then washed in PBS-Tw (PBS + 0.05% Tween-20) and incubated in a fluorescent Alexa Fluor® 488-conjugated Goat anti-Rabbit IgG secondary antibody (Abcam) at a 1:400 dilution in 1% BSA and 0.3% Triton for one hour. Then, slides were washed in PBS-Tw and mounted in 50% glycerol plus 10 µg/ml Hoescht 33342 (Sigma) to stain nuclei.

To perform double IHC for Gnrh2 and Gnrh3 in wild-type brains, slides were briefly fixed in acetone, and quenched in 0.3% $H_2O_2$ in PBS for 30 minutes. Slides were

then washed in PBS, blocked for one hour in 5% normal goat serum, and incubated with a 1:1,000 dilution of Anti-GAP2 in 1% BSA and 0.3% Triton X-100 overnight at 4˚C. Slides were then washed in TNT (100 mM Tris Ph 7.5, 150 mM NaCl, 0.5% Tween-20) and incubated in an HRP-conjugated Goat anti-Rabbit (GAR-HRP) antibody (Genscript) at a 1:1,000 dilution in 1% BSA and 0.3% Triton for one hour. Then, slides were washed in TNT and incubated in a fluorescein dye from the Tyramide Signal Amplification Plus kit (TSA Plus kit, Perkin Elmer) at a 1:50 dilution for 5 minutes, washed in TNT, and HRP signal quenched with 0.02 N HCl for 10 minutes. After washing, the procedure for IHC delineated above was repeated on the same slides, but with a GAP3 primary antibody and Cy3 dye from the TSA kit to label Gnrh3 neurons. Slides were mounted in 50% glycerol plus 10 µg/ml Hoescht 33342 (Sigma) and viewed on a Leica SP6 confocal microscope and screened for the presence of Gnrh2 and Gnrh3 neurons in the olfactory bulb. Images were then taken at 5X and 20X magnification and image files were compiled using ImageJ (NIH, Bethesda, MD, USA).

### *In situ* hybridization of Gnrh2 and Gnrh3 in olfactory bulbs

Brains of adult wild-type females and males were dissected, fixed, cryoprotected, frozen in OCT at -80˚C, and sectioned at 10 µM thickness in the same manner as described above for IHC. Digoxigenin or fluorescein labeled riboprobes were prepared from the full length cDNAs of the corresponding gene (*gnrh2* or *gnrh3*) cloned in pGEMT vector, using T7 or SP6 RNA polymerases (New England Biolabs) to generate the anti-sense and sense riboprobes. For ISH, slides were briefly dried at 55˚C for 8 minutes and post-fixed with 4% PFA for 15 minutes. Slides were washed with PBS and incubated with 0.3% $H_2O_2$ in methanol for 30 minutes to quench endogenous

peroxidases. Slides were washed in PBS, incubated in 0.1M TEA buffer pH 8 (Triethanolamine), and washed in 2X sodium-saline citrate buffer (SSC). Pre-hybridization occurred at 62°C in a solution containing 50% Formamide, 3X SSC, and 10 mg/ml denatured calf thymus DNA. After 2 hours, slides were incubated at 62°C overnight in a buffer containing calf thymus DNA, and 300 ng/mL of *gnrh2* anti-sense RNA probe (DIG-labeled). Consecutive slides of brains were incubated with sense RNA probe of each gene as a negative control. Slides were then washed in 2X SSC at room temperature for 30 minutes, and 2X SSC, 0.4X SSC, and 0.1X SSC at 65°C for 30 minutes each, and 1x SSC for 10 minutes at room temperature. Slides were blocked with TNB (100 mM Tris pH 7.5, 150 mM NaCl, 0.5% Blocking reagent, from the TSA kit) and followed the same procedures delineated above for the IHC, except with the secondary antibody, Anti-DIG HRP-conjugated (Roche) diluted 1:200, and with slides labelled with Fluorescein from the TSA kit at 1:50 dilution. Slides were then washed 4X for 20 minutes each, and the HRP signal quenched with 0.02 N HCl for 15 minutes. After washing, the procedure for ISH was repeated, except using 300 ng/mL of *gnrh3* anti-sense RNA probe (DIG-labeled), and the Cy3 dye from the TSA kit to localize *gnrh3* expression. After mounting slides with 50% glycerol plus Hoechst 33342, they were viewed and analyzed for Cy3 and/or fluorescein signal using a Leica SP6 confocal microscope and pictures taken at 5X and 20X magnification. Images were then compiled using ImageJ (NIH).

**QPCR of *gnrh2* and *gnrh3* expression in olfactory bulbs exposed to different feeding and social conditions**

In order to determine if feeding or pheromonal differences influence *gnrh2* and *gnrh3* expression in olfactory bulbs, groups of fish from the same cohort were separated into 4 different groups. To determine if the presence or absence of pheromones affected *gnrh2*/*gnrh3* expression, one group contained 8 males and 8 females together in the same tank, and another group contained 8 males and 8 females separated into individual containers, where they could only see, but were not exposed to zebrafish of the opposite sex, for one week. To determine whether feeding differences affected gnrh2/gnrh3 expression, one group of 8 males and 8 females was normally fed for one week, whereas another group, also containing 8 males and 8 females was fasted for one week. After the one week period of time, all fish were quickly euthanized via an ice water bath and spinal cord dislocated, and then olfactory bulbs and tracts dissected and separated from the rest of the brain, and quickly flash frozen in liquid nitrogen and stored at -80°C until RNA extraction and qPCR was conducted.

To each sample, 150 µL of Trizol reagent was added and total RNA from samples was extracted according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). The Quantitect Reverse Transcription Kit (Qiagen, Valencia, CA, USA) that includes gDNA Wipeout to eliminate gDNA contamination was used to reverse transcribe 1 µg of RNA from each sample. In each round, a non-RT control and no template control were added to determine gDNA and template contaminations. QPCR was conducted using 20 ng of cDNA for each sample in duplicate with SYBR Green qPCR mix and gene-specific primers (Table 2.1), with $C_T$ values for each sample normalized against an internal

*eef1α1* control. Amplification of cDNA was performed on a 7500 Fast Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA) and conditions included a 2 minute 95°C activation, 5 second 95°C denaturation, and 30 second 60°C annealing, with the last two steps repeating 40 times.

## Results

### Localization of Gnrh2 and Gnrh3 in olfactory regions of zebrafish larvae

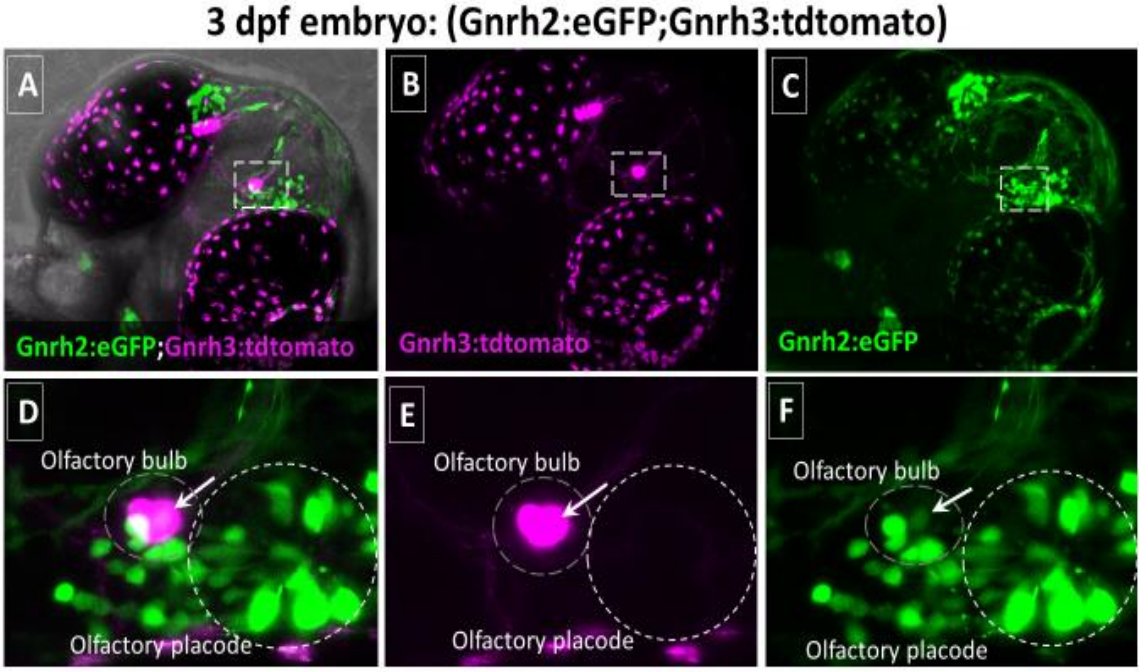


**Figure 5.1.** Confocal images of Gnrh2 (green) and Gnrh3 (magenta) in the anterior olfactory region of 3 dpf zebrafish larvae. Merged Gnrh2 and Gnrh3 images at 5X magnification (A), with the focus on the olfactory bulb (dashed square). The 20X magnification image is shown with Gnrh3 expressed in the olfactory bulb (grey circle) and Gnrh2 soma abundantly expressed in the olfactory epithelum and near the olfactory bulb (white circle), with one cell showing colocalization (D, white arrow). Single Gnrh3 signal in the olfactory region is shown at 5X (B) and 20X (E) magnification, and single Gnrh2 signal is shown in the olfactory region at 5X (C) and 20X (F) magnification.

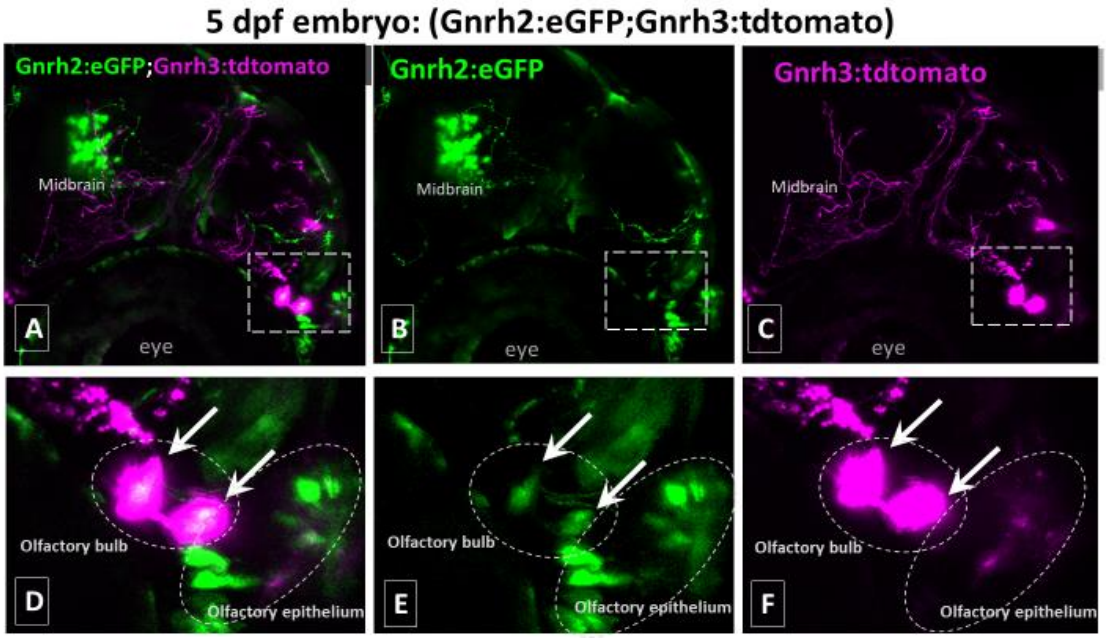


**Figure 5.2.** Confocal images of Gnrh2 (green) and Gnrh3 (magenta) in the anterior olfactory region of 5 dpf zebrafish larvae. Merged Gnrh2 and Gnrh3 images at 5X magnification (A) and 20X magnification image is shown with two cells showing colocalization (D, white arrow). Single Gnrh2 signal in the olfactory region is shown at 5X (B) and 20X (E) magnification, and single Gnrh3 signal is shown in the olfactory region at 5X (C) and 20X (F) magnification.

As early as 3 dpf, zebrafish larvae express a few Gnrh3 soma in the olfactory bulbs (Fig. 5.1A & D), and Gnrh2 soma in the olfactory epithelium (Fig. 5.1C & F). One neuronal soma can be seen in 3 dpf larvae co-expressing both Gnrh2 and Gnrh3 (Fig. 5.1D, E, & F). At 5 dpf, two neuronal soma can be seen strongly expressing Gnrh3 in the olfactory bulb (Fig. 5.2C & F). Gnrh2 soma are present in the midbrain and olfactory epithelium (Fig. 5.2B & E), with two neuronal soma in the olfactory bulb also expressing Gnrh2 along with Gnrh3 (Fig. 5.2A & D).

**Localization of Gnrh2 and Gnrh3 in adult olfactory bulbs**

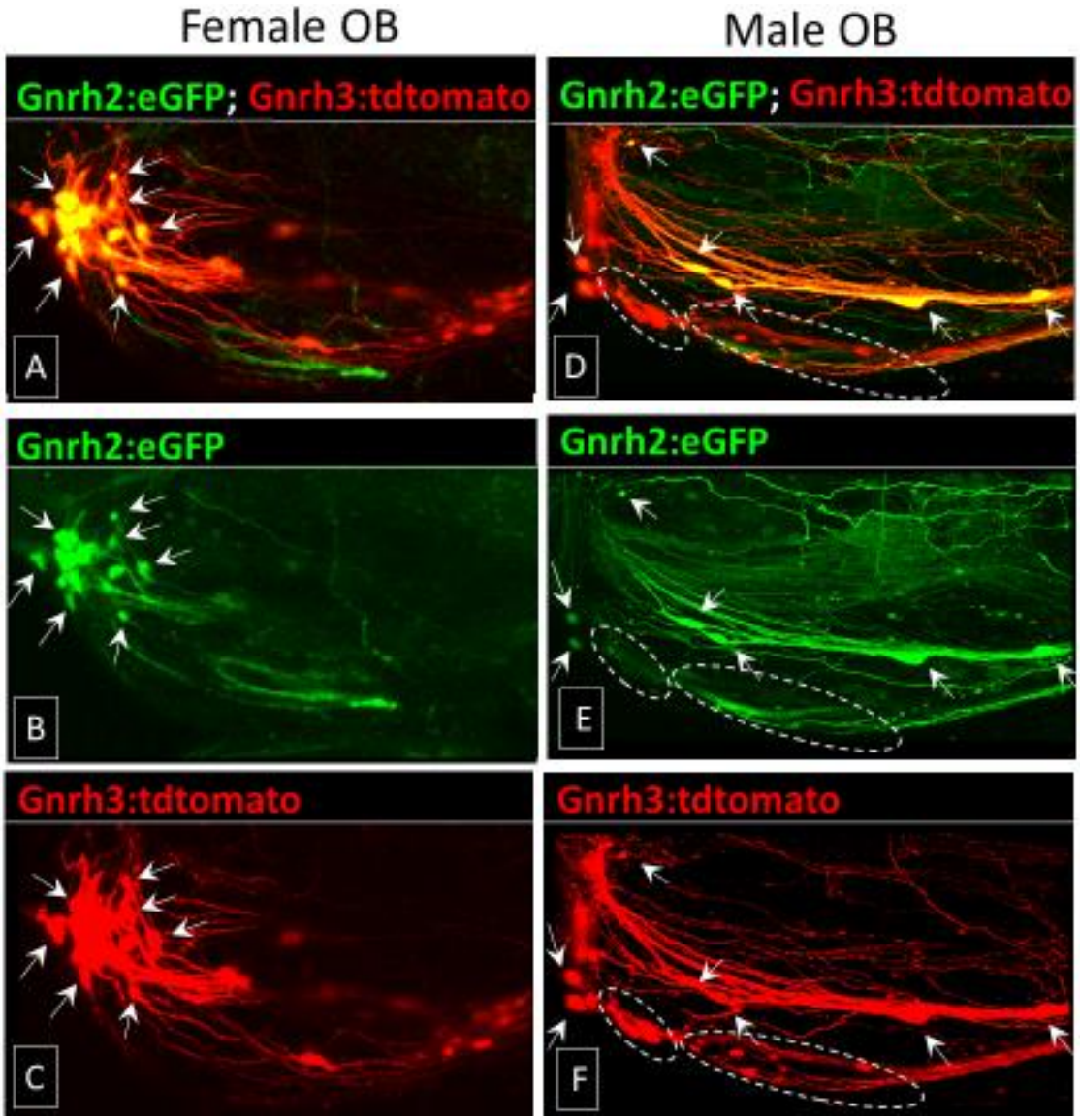


**Figure 5.3.** Confocal images of Gnrh2 (green) and Gnrh3 (red) in the olfactory bulbs. Merged Gnrh2 and Gnrh3 images at 10X magnification of two olfactory bulbs from female zebrafish, showing the ventral side (A), with single Gnrh2 signal (B), and single Gnrh3 signal (C), showing colocalization in numerous cells (white arrows). Merged Gnrh2 and Gnrh3 images at 10X magnification of one olfactory bulb from male zebrafish, showing the ventral side (D), with single Gnrh2 signal (E), and single Gnrh3 signal (F), showing colocalization in several cells (white arrows), and cells expressing only Gnrh3, but not Gnrh2 (dashed circles).

In the ventral anterior region of female olfactory bulbs, multiple Gnrh2 soma are seen (Fig. 5.3B), along with dense bundles of neuronal fibers. Very strongly expressed

and abundant Gnrh3 soma are seen in the same region of female olfactory bulbs (Fig. 5.3C). Many neuronal soma express both Gnrh2 and Gnrh3, with the overlay of the two colors being expressed as yellow (Fig. 5.3A). In male olfactory bulbs, a few Gnrh2 soma are seen in the most anterior region of the ventral olfactory bulb (Fig. 5.3E), with several cells also present in the more posterior ventral region. Abundant Gnrh3 soma are seen in the anterior, mid, and posterior region of the ventral olfactory bulb, with many fibers also projecting throughout (Fig. 5.3F). Some of the neuronal cells co-express Gnrh2 and Gnrh3, and depicted as a yellow color due to the overlay of the two signals (Fig. 5.3D).

**Localization of Gnrh2 and Gnrh3 in non-olfactory regions of the brain**

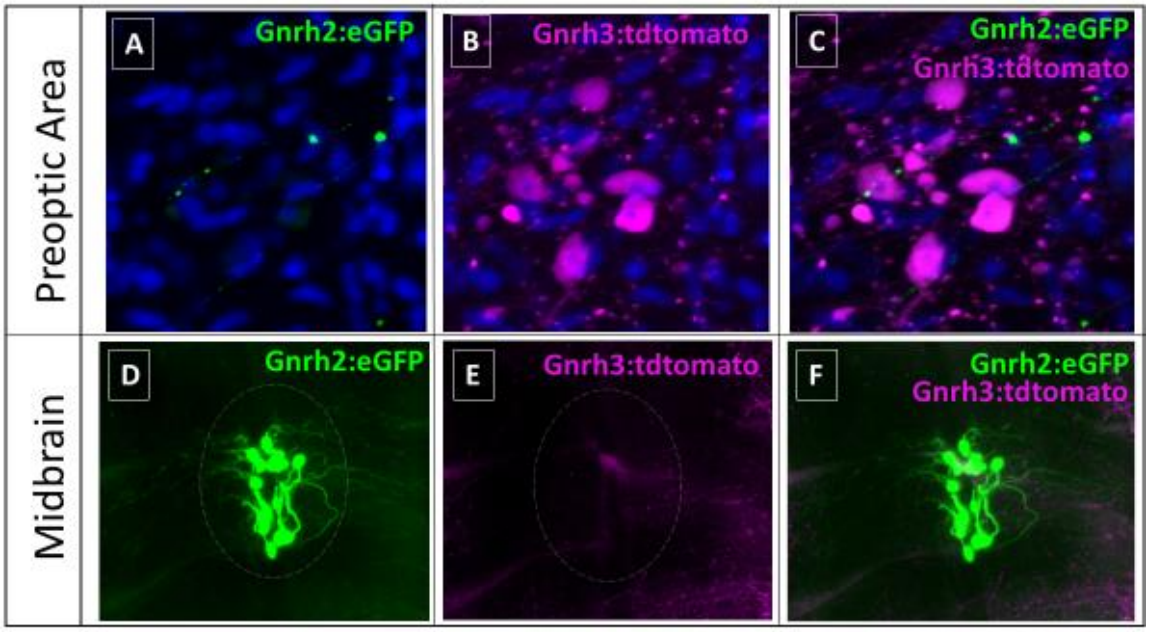


**Figure 5.4.** Confocal images of Gnrh2 (green) and Gnrh3 (magenta) in the preoptic area of the hypothalamus and midbrain from a transverse oriented brain. Merged Gnrh2 and Gnrh3 images at 20X magnification in the preoptic area of the hypothalamus (C), with single Gnrh2 signal (A), and single Gnrh3 signal (B). Merged Gnrh2 and Gnrh3 images at 10X magnification of the midbrain tegmentum (F), with single Gnrh2 signal (D), and single Gnrh3 signal (E).

In the preoptic hypothalamus region of the brain, many Gnrh3 cells are seen (Fig. 5.4B), and a few, but not many, Gnrh2 fibers can be seen around the same region (Fi.g 5.4A), however, no Gnrh2 neuronal soma or colocalization is seen in this region of the brain (Fig. 5.4C). In the midbrain tegmentum, a dense bundle of Gnrh2 neuronal cells is seen (Fig. 5.4D), and Gnrh3 neuronal projections are also seen around and close to Gnrh2 cells (Fig. 5.4E), however, no Gnrh3 neuronal soma or colocalization is seen in the midbrain (Fig. 5.4F).

**Immunohistochemistry of Gnrh2 in adult olfactory epithelium**

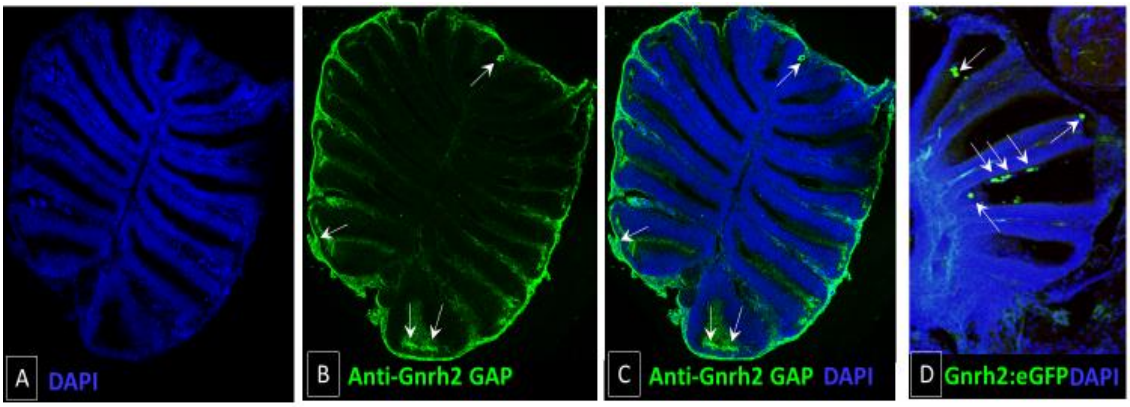


**Figure 5.5.** Sections of an adult olfactory epithelium, stained with DAPI (A) and immunoreactive Gnrh2 (B), and the merged image showing both DAPI and immunostained Gnrh2 (C), exhibit signs of several Gnrh2 soma. Sections of the olfactory epithelium from transgenic tg(Gnrh2:eGFP) zebrafish also exhibit signs of Gnrh2 soma (D).

Immunostaining of Gnrh2 in sections of olfactory epitheliums, reveal the presence of numerous Gnrh2 soma around the edges of the olfactory region (Fig. 5.5A, B, & C). Sections of transgenic tg(Gnrh2:eGFP) also show signal of multiple Gnrh2 soma in the olfactory epithelium (Fig. 5.5D).

**Immunohistochemistry of Gnrh2 in Gnrh3:tdtomato olfactory bulbs**

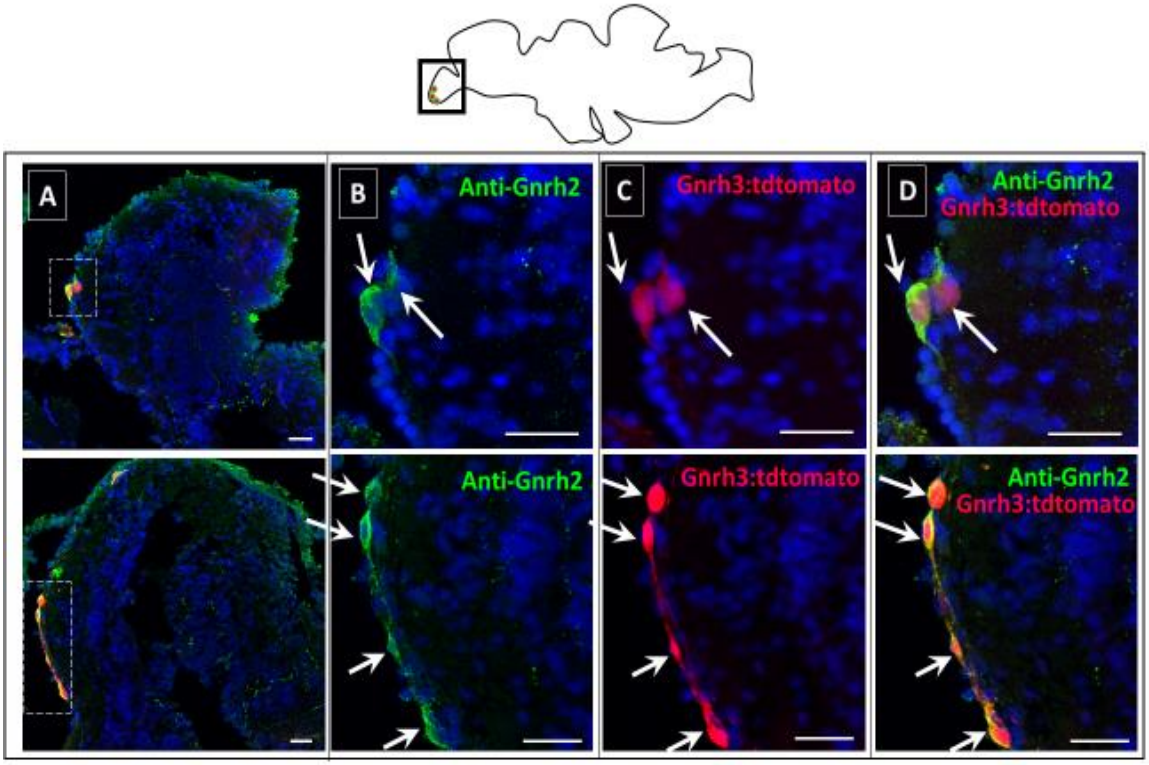


**Figure 5.6.** Female olfactory bulb sections at 10X magnification showing transgenic Gnrh3:tdtomato signal (red) and immunolabelled Gnrh2 (green) (A). Immunolabelled Gnrh2 in the ventral region of the olfactory bulbs at 20X magnification (B), transgenic signal of Gnrh3:tdtomato in the same regions (C), and a merged image of Gnrh2 and Gnrh3 signal (D), with colocalized soma apparent (white arrows).

Cryosections of olfactory bulbs show evidence of Gnrh cells in the ventral region (Fig. 5.6A). Immunolabelled Gnrh2 protein is seen in multiple cells along the ventral side of olfactory bulbs (Fig. 5.6B). Cells expressing Gnrh3:tdtomato are found along the ventral olfactory bulb as well (Fig. 5.6C) and many of the same cells expressing Gnrh3 are also immunolabelled with Gnrh2 after immunohistochemistry (Fig. 5.6D).

***In situ* hybridization of *gnrh2* and *gnrh3* in olfactory bulbs**

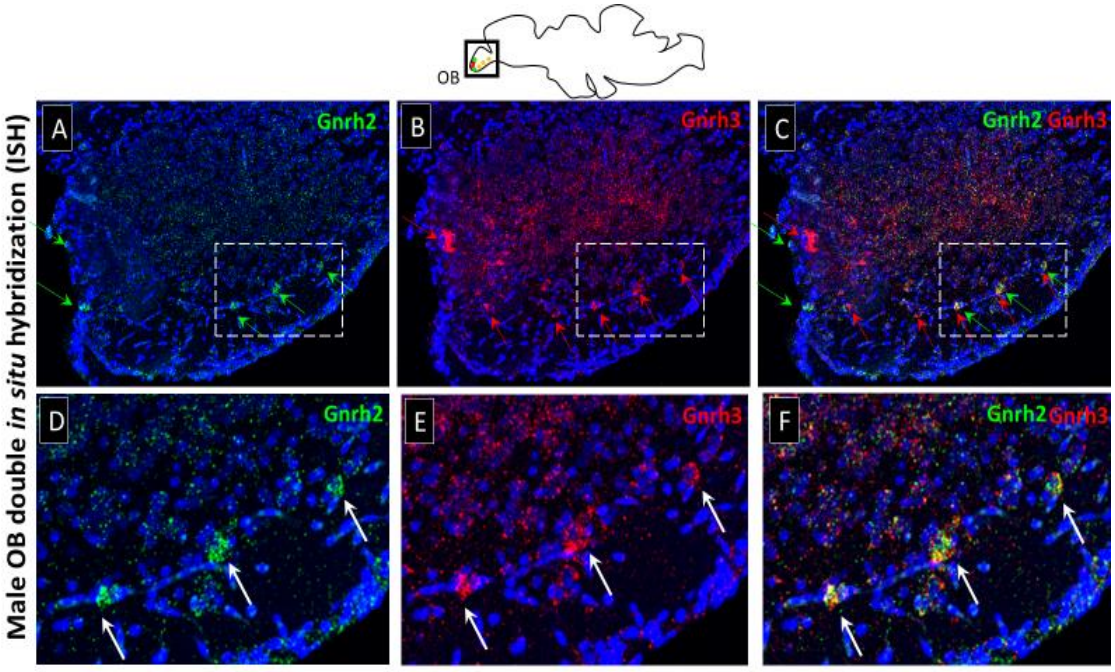


**Figure 5.7.** Male olfactory bulbs with gnrh2-expressing cells (green arrows) at 5X (A) and 10X (white arrows) (D) magnification after ISH. Gnrh3-expressing cells (red arrows) at 5X (B) and 10X (white arrows) (E) after ISH. Overlay of Gnrh2 (green arrows) and Gnrh3 signal (red arrows) (C) with several cells expressing both Gnrh2 and Gnrh3 (F, white arrows).

Similar to patterns seen in transgenic male olfactory bulbs (Fig 5.3), two cells are shown expressing Gnrh2 in the anterior part of olfactory bulb sections, along with cells along the ventral side of the bulb (Fig. 5.7A & D). Many cells express Gnrh3, also in the anterior portion and along the ventral side of olfactory bulb sections, (Fig. 5.7B&E). When looking at the overlay of *in situ* hybridization stained Gnrh2 and Gnrh3, separate cells appear to be expressing Gnrh2 and Gnrh3 in the anterior region, but the ventral side shows around 3 cells expressing both Gnrh2 and Gnrh3 in the same location (Fig. 5.7C & F).

**Double immunohistochemistry and *in situ* hybridization of Gnrh2 and Gnrh3 in female olfactory bulbs**

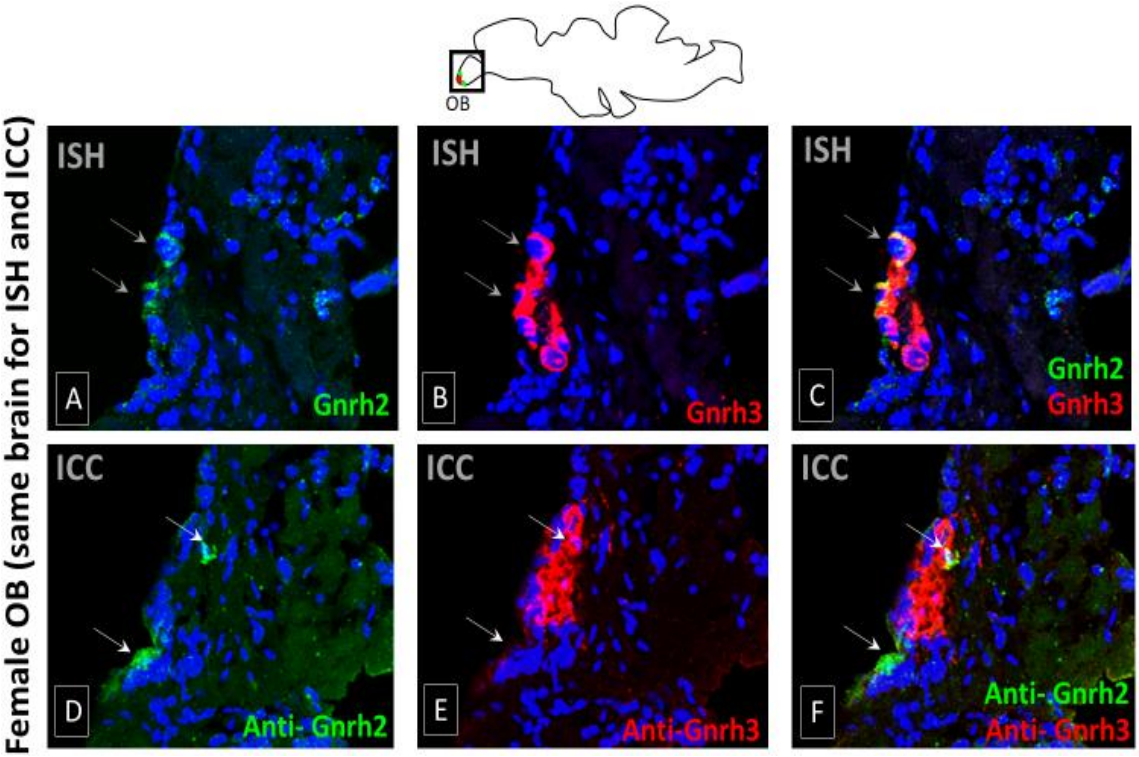


**Figure 5.8.** Anterior, ventral section of a female olfactory bulb with Gnrh2 labelled with riboprobes via ISH (A, grey arrows) and immunolabelled Gnrh2 via ICC (D, white arrows) in the same region. Riboprobe-labelled Gnrh3 (B) and immunolabelled Gnrh3 (E) in the same region of the olfactory bulb. Overlay of gnrh2 and gnrh3-expressing cells (C) and double immunolabelled Gnrh2 and Gnrh3 (F) show signal in the same region.

To determine if there were sex-specific and expression and translation differences in Gnrh2 and Gnrh3 expression and colocalization in female olfactory bulbs, consecutive sections of olfactory bulbs from the same female brain were stained using in situ hybridization or immunohistochemistry. Both *in situ* hybridization (ISH) (Fig. 5.8A) and immunohistochemistry (IHC) (Fig. 5.8D) labelled Gnrh2, show Gnrh2 presence in the anterior portion of the olfactory bulb. Similarly, Gnrh3 is also seen after both ISH (Fig. 5.7B) and IHC (Fig. 5.8E) but with many more cells expressing and translating Gnrh3 in these regions. When looking at Gnrh2 and Gnrh3 together, two *gnrh2*-expressing cells are shown in the same cell as *gnrh3*-expressing cells (Fig. 5.8C). Gnrh2 protein is stained

in cells next to, but not colocalized with Gnrh3, along with one cell immunolabelled for both Gnrh2 and Gnrh3 (Fig. 5.8F).

**Effects of feeding and social differences on *gnrh2* and *gnrh3* expression in olfactory bulbs**

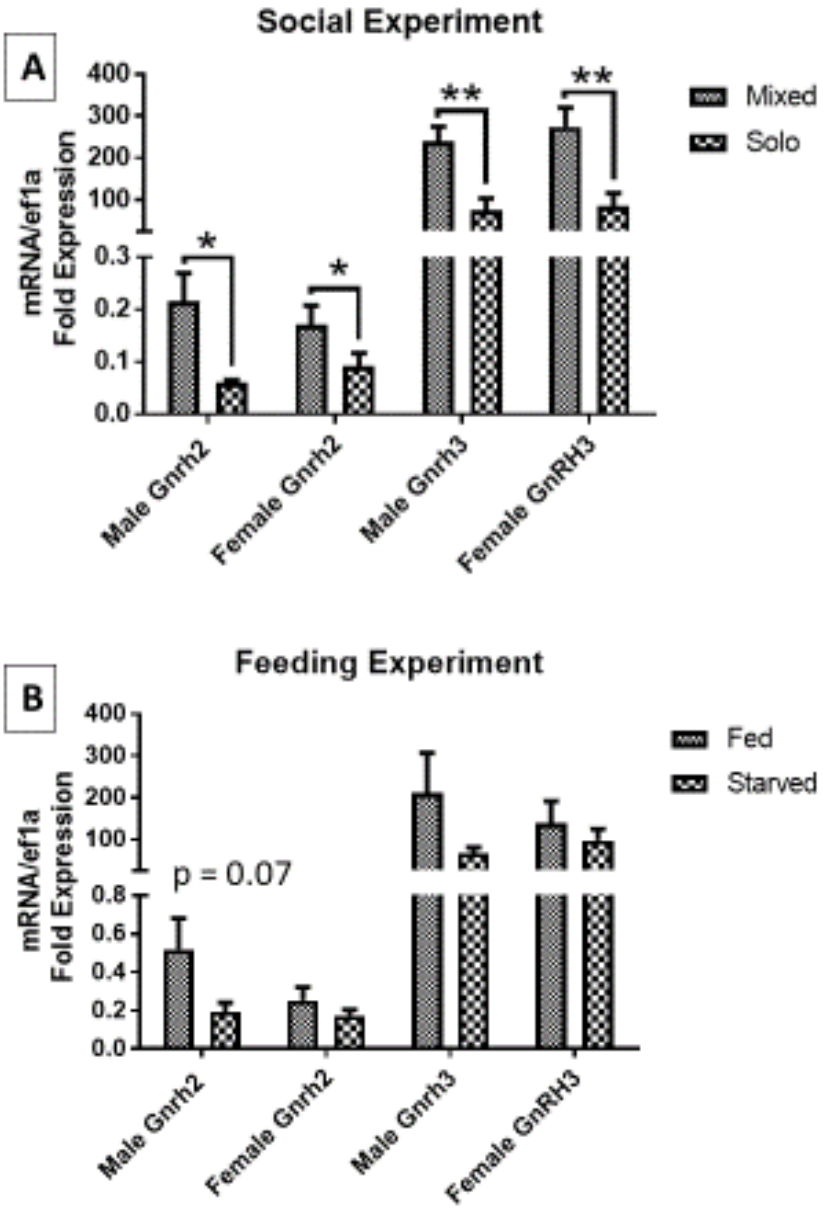


**Figure 5.9.** Relative expression of *gnrh2* and *gnrh3* in male and female zebrafish exposed to differing social conditions for one week (A) and relative expression of *gnrh2* and *gnrh3* in male and female zebrafish exposed to different feeding conditions for one

week (B). All data are expressed as means ± SEM. Stars (**) indicate statistically different expression levels between treatments. (** = P < 0.01, ANOVA).

Both *gnrh2* and *gnrh3* expression was present in olfactory bulbs, measured via qPCR, but expression of *gnrh3* was around 1,000-fold higher than *gnrh2*. Males and females showed similar expression values of *gnrh2* and *gnrh3*. Both sexes and Gnrh isoforms showed similar patterns of significantly decreased expression values in solo conditions (able to see but not smell conspecifics), compared to mixed conditions (with the presence of conspecific pheromones) (Fig. 5.9A). In olfactory bulbs, fasting conditions did not significantly change the expression of *gnrh2* or *gnrh3* in males or females (Fig. 5.9B).

## Discussion

In this study, it is shown for the very first time that Gnrh2 soma may be present in the olfactory bulbs and olfactory epithelium of zebrafish. The localization of Gnrh2 in olfactory-sensing regions of the brain was characterized and validated through the use of the transgenic zebrafish line (tg(Gnrh2:eGFP)), immunohistochemistry, and *in situ* hybridization. Functional assays were also conducted to narrow down the specific olfactory cues in which Gnrh2 may be transducing in these regions. In terms of olfactory transduction, it was determined that Gnrh2 is located in the olfactory epithelium and olfactory bulbs, and potentially able to transduce pheromonal cues. Because Gnrh2 axons extend from the olfactory bulbs along the telencephalon tract, it is possible that Gnrh2 also transduces olfactory cues in order to coordinate reproductive and feeding regulation during the appropriate presence of social and breeding conspecifics.

I first investigated the neuroanatomical presence of Gnrh2 in the olfactory regions of the brain and its interactions with Gnrh3 neuronal soma in the ventral olfactory bulb.

Visualizing transgenic zebrafish, the presence of Gnrh2 soma in the olfactory epithelium, and one or two soma in the olfactory bulb area, is very clear. This is a surprising result, considering the fact that Gnrh2 has not often been characterized or seen in the olfactory bulb in previous studies of zebrafish. However, *gnrh2* expression in zebrafish olfactory bulbs has been shown by PCR in other labs, along with the receptor most specific for Gnrh2, *gnrhr3* (Corchuelo et al., 2017). Additionally, *gnrh2* expression was shown to change in female zebrafish based on their maturational stage, indicating that it may have a reproductive coordinating role in this location (Corchuelo et al., 2017). Moreover, multiple groups have shown the presence of *gnrh2* mRNA in goldfish olfactory bulbs and tracts, further determining that Gnrh2-expressing cells are present in this region (Lin and Peter, 1997; Yu et al., 1998). One group even showed that Gnrh2 immunoreactive soma and fibers are seen in the same locations as Gnrh3 in the terminal nerve and medial olfactory tract, suggesting that Gnrh2 and Gnrh3 co-expression also occurs in goldfish (Kim et al., 1995). However, this phenomenon is controversial as a more recent study failed to detect any *gnrh2* mRNA in the goldfish olfactory bulb, although they propose that the expression of Gnrh2 may be present, but too low to be detected (Kawai et al., 2010). The fact that Gnrh2 is expressed at a much lower level, and in fewer soma, than Gnrh3, may be another reason that studies have focused on the roles of Gnrh3, or other hypophysiotropic Gnrh isoforms, in olfactory transduction. In order to determine the developmental presence of Gnrh2 in olfactory bulbs, we imaged zebrafish at different developmental stages, and showed that as early as 3 dpf, Gnrh2 is present in the olfactory bulb and colocalized with one Gnrh3 neuronal soma, and at 5 dpf, colocalized with two Gnrh3 soma. The roles of Gnrh in the olfactory region in developing zebrafish have not

been well studied, but one study did show that Gnrh3 most likely has roles in $CO_2$-avoidance behavioral responses in the olfactory bulb region in larval zebrafish, suggesting that Gnrh2 may also have these similar roles during developmental stages (Koide et al., 2018). Additionally, Gnrh3 neurons express a factor which seems to help control the migration of some Gnrh3 neurons from the olfactory region to the hypothalamus during very early stages of development, although whether these neurons are the same ones co-expressed with Gnrh2 are yet to be determined (Abraham et al., 2008; Palevitch et al., 2010). In adult zebrafish, the number of neurons co-expressing both Gnrh2 and Gnrh3 are more numerous in number. In other regions of the brain, including the preoptic area, hypothalamus, and midbrain tegmentum, we do not see any signs of colocalization of Gnrh2 and Gnrh3, indicating that this is probably a specific occurrence in the olfactory region. This is one of the first studies suggesting a colocalization of Gnrh2 with other Gnrh isoforms in specific regions of the brain. Nevertheless, in sea lamprey (*Petromyzon marinus*), multiple Gnrh isoforms are seen to be colocalized throughout the brain, with lamprey Gnrh1 and 2 (lGnrh1 and lGnrh2) colocalized in larval olfactory bulbs, preoptic area, hypothalamus, and rhombencephalon, and lGnrh1, lGnrh2, and lGnrh3 triple colocalized in the adult preoptic area (Van Gulick et al., 2018). Colocalization of multiple Gnrh isoforms may occur in other vertebrate groups as well, since both human and mouse neuronal cell lines were shown to co-express both GNRH1 and GNRH2 (Chen et al., 2001).

To concretely determine whether Gnrh2 is truly expressed and translated in olfactory bulb cells, and to determine whether it is expressed in the same cells as Gnrh3, immunohistochemistry was conducted to stain Gnrh2 and Gnrh3 protein in brain sections,

and *in situ* hybridization was used to label *gnrh2* and *gnrh3* mRNA. Thin brain sections of 10 µm were used to identify if the Gnrh isoforms were adjacent or in the same cells in the olfactory bulb. Immunohistochemistry clearly showed Gnrh2 protein labelled in the same cells expressing Gnrh3, from transgenic tg(Gnrh3:tdtomato) zebrafish. The antibody used in this analysis is specific for the GAP region of Gnrh2, which does not share homology with any portions of Gnrh3 (Xia et al., 2014). Additionally, double-labelled immunohistochemistry and *in situ* hybridization show that some cells produce both Gnrh2 and Gnrh3 protein in the ventral olfactory bulb, whereas some cells produce only Gnrh2 or Gnrh3. These neuroanatomical patterns are similar to the signals seen in transgenic zebrafish olfactory bulbs, suggesting that the colocalization is truly happening and not just an artifact of color bleedover from the microscopy imaging. Comparing *in situ* hybridization, which represents *gnrh2* and *gnrh3* mRNA localization, with immunohistochemistry analyses, which represents Gnrh2 and Gnrh3 protein localization, from brain sections of the same individual, it appears that Gnrh2 and Gnrh3 protein may be secreted outside of the cells, since more cells appear to show the immunoreactive Gnrh protein compared to the mRNA expression, with the Gnrh2 appearing in cells just outside of the Gnrh3 cells. This is consistent with previous studies which show that terminal nerve Gnrh3 appear to be exocytosed from the neuronal cell body, and exhibit autocrine functions by communicating via gap junctions to coordinate firing activity with each other, therefore playing roles in both paracrine and autocrine regulation (Oka and Ichikawa, 1991; Karigo and Oka, 2013). Our results suggest that Gnrh3 also exists in tight bundles and may exhibit the same paracrine/autocrine function as well, although this would need to be studied further to concretely determine whether this is the case.

There does seem to be some sex-specific differences in the localization and co-expression of Gnrh2 in male and female olfactory bulbs. Although both sexes do show the presence of both solo Gnrh3-expressing cells and colocalized Gnrh2 and Gnrh3 expressing cells, females appear to have more colocalized cells in the most rostral anterior part of the olfactory bulb, near the edges of the tissue, whereas males appear to have more *gnrh2* and *gnrh3* expressing cells in the medial olfactory tract, in more ventral and posterior regions. The location of Gnrh3-expressing cells does not appear to be too different between males and females, showing that it may only be the expression of Gnrh2 in olfactory bulbs which show minor sex-specific differences. Since pheromonal release and reproductive behaviors exhibit strong sex-specific differences in fish, the pheromonal transduction differences may be the cause for the different localization of the colocalized cells in the olfactory bulbs. The teleost pheromonal control of reproduction is best characterized in goldfish. It has been shown that males are very sensitive to both male and female pheromones, with mature male pheromones, such as androstenedione, increasing aggressive behaviors in males, and female-specific pheromones, such as 17,20beta-dihydroxy-4-pregnen-3-one-20-sulfate which is released by preovulatory goldfish, inducing courtship behaviors in males (Poling et al., 2001). Some hormones, such as prostaglandin F2α ($PGF_{2\alpha}$), which is synthesized in the oviduct and released in ovulating females, stimulate spawning behaviors in both male and female goldfish (Kobayashi et al., 2002). These responses indicate sex-specific differences and similarities in pheromonal detection (Kobayashi et al., 2002), signifying that the organization and cell-types of pheromone transduction in the olfactory region will probably also contain both similarities and differences. The differences in pheromonal

detection may be the reason for the slight differences in Gnrh2 and Gnrh3 cell organization in the olfactory bulbs of males and females.

Previous studies showed that Gnrh3 in the olfactory bulb have multiple and species-specific roles, including integrating carbon dioxide ($CO_2$) signals into avoidance responses (Koide et al., 2018), modulating olfactory bulb sensitivity to food odors (Park and Eisthen, 2003), and initiating male reproductive behaviors in the presence of pheromonal odors from females (Li et al., 2017). Since Gnrh2 has been shown to have roles in mediating both reproductive and feeding behaviors (Kauffman and Rissman, 2004a, Kauffman and Rissman, 2004b, Matsuda et al., 2008, Nishiguchi et al., 2012), a functional assay was initiated to determine if Gnrh2 and Gnrh3 specifically in the olfactory bulbs responded more to social or feeding differences. There was only differential expression of *gnrh2* and *gnrh3* in olfactory bulbs when fish were exposed to different social conditions, which may correspond to the presence or absence of pheromones. In our experiment, fish were able to visualize conspecifics, but not exposed to any olfactory signals, such as pheromones, from conspecifics, suggesting that it was the pheromonal differences which induced the change of *gnrh2* and *gnrh3*. The ability of pheromones to modulate *gnrh* expression has been shown in numerous studies. For instance, the presence of male pheromones induces an instant change in Gnrh pulse frequencies in female ruminants (Murata et al., 2009). Exposing female prairie voles (*Microtus ochrogaster*) to male urine increases their olfactory Gnrh levels (Dluzen et al., 1981), and the presence of a female exhibiting spawning behaviors increases Gnrh olfactory levels in male goldfish , a phenomenon which is abolished when the nerve tracts that transduce female sex pheromones are cut (Yu and Peter, 1990; Kawai et al.,

2009). The response of both Gnrh2 and Gnrh3 to feeding and social condition differences were the same (in both genders), strongly indicating that Gnrh2 may have similar roles to Gnrh3 in transducing chemical cues to coordinate spawning behaviors. Additionally, the presence of *gnrh2* mRNA, verified by qPCR, is another validation that Gnrh2-expressing cells exist in the olfactory bulbs. The fact that *gnrh2* transcript levels also exhibit the same response as *gnrh3* transcripts in these tissues strongly suggests that this co-localization is occurring, and the cells they are expressed in are responding to the same signals. In terms of pheromones specifically studied in zebrafish species, $PGF_{2\alpha}$ has been shown to strongly attract male zebrafish and initiate courtship behavior (Yabuki et al., 2016). Recently, the olfactory receptor OR114-1 has been determined to be a strong candidate as a receptor of $PGF_{2\alpha}$ (Yabuki et al., 2016). Future studies determining if this receptor is present on Gnrh2 cells in the olfactory epithelium, or Gnrh2 and Gnrh3 cells in the olfactory bulb, would solidify whether these cells are transducing $PGF_{2\alpha}$ signals.

In summary, it is shown that Gnrh2 is present and expressed in olfactory regions of zebrafish brains, suggesting that this neuropeptide is involved in transducing external olfactory signals. Since previous studies have shown that Gnrh2 can modulate feeding behaviors and reproductive parameters, it is possible that this neuropeptide is directly integrating external olfactory cues to coordinate its activation of these roles. For the first time, it is shown that Gnrh2 may be colocalized with some Gnrh3 soma in the olfactory bulb and may help to transduce pheromonal cues in these regions. The response of Gnrh2 and Gnrh3 to different feeding and social conditions were also the same, suggesting that Gnrh2 is transducing the same signals of Gnrh3 and may help to coordinate reproductive behaviors.

# CHAPTER 6: Determining Whether Gnrh2 or Other Reproductive Factors Compensate for Gnrh3 Loss


## Abstract

Although gonadotropin-releasing hormone (GNRH) is known as a pivotal upstream regulator of reproduction in vertebrates, reproduction is not compromised in the hypophysiotropic Gnrh3 knockout line in zebrafish (*gnrh3*$^{-/-}$). In order to determine if Gnrh2, the only other Gnrh isoform in zebrafish brains, is compensating for the loss of Gnrh3, a double Gnrh knockout zebrafish line (*gnrh2*$^{-/-}$*;gnrh3*$^{-/-}$) was generated. Surprisingly, the loss of both Gnrh isoforms resulted in no major impact on reproduction, indicating that a compensatory response, outside of the Gnrh system, was evoked. A plethora of factors acting along the reproductive hypothalamus-pituitary axis were evaluated as possible compensators for Gnrh3, based on neuroanatomical assays, and RNA-Seq differential gene expression studies in *gnrh3*$^{-/-}$ fish, and targeted differential gene expression studies in double knockout (*gnrh2*$^{-/-}$*;gnrh3*$^{-/-}$) fish. In addition, we also examined the involvement of feeding factors in the brain as potential compensators for Gnrh2, which has known anorexigenic effects. When looking at whole-transcriptome changes in single Gnrh3 knockout zebrafish, we determined upregulation of several neuropeptide, neuronal plasticity, and neuronal activity genes, and a general downregulation of the dopamine system. In double knockout fish, we found that loss of both Gnrh isoforms resulted in upregulation of several neuropeptide genes in the brain, specifically *gonadotropin-inhibitory hormone* (*gnih*), *secretogranin 2* (*scg2*), *tachykinin 3a* (*tac3a*), *vasoactive intestinal peptide* (*vip*), and *pituitary adenylate cyclase-activating peptide 1* (*pacap1*), and downregulation of *agouti-related peptide 1* (*agrp1*), indicating the compensation occurs outside of Gnrh cells and therefore is a non-cell autonomous

response to the loss of Gnrh. While the differential expression of *gnih* and *agrp1* in the double knockout line was confined to the periventricular nucleus and hypothalamus, respectively, the upregulation of *scg2a* corresponded with a broader neuronal redistribution in the lateral hypothalamus and hindbrain and upregulation of *vip* corresponded with an extended neuronal distribution to more anterior regions of the preoptic area, similar to where Gnrh3 neurons are normally found. In conclusion, our results demonstrate the existence of a redundant reproductive regulatory system that comes into play when Gnrh2 and Gnrh3 are lost.

## Introduction

It is well known that the main upstream factor in the hypothalamus controlling the reproductive brain-pituitary-gonad (BPG) axis in vertebrates is the gonadotropin-releasing hormone (GNRH). It has been previously well established that this neuropeptide is essential for the synthesis and secretion of the pituitary gonadotropins, luteinizing hormone (Lh) and follicle-stimulating hormone (Fsh) (Schally et al., 1971; Zohar et al., 2010). Although vertebrates possess multiple isoforms of Gnrh in the brain, the isoform found in the preoptic region of the hypothalamus is the hypophysiotropic one, responsible for releasing the gonadotropins, and often referred to as GNRH1 (Fernald and White, 1999). However, in many teleosts, such as in some cyprinids, salmonids, and bony-tongue fishes (Okuzawa et al., 1990; Steven et al., 2003; O'Neill et al., 1998) they also have a third isoform, Gnrh3, which is additionally found in the olfactory bulb/terminal nerve area along with the preoptic hypothalamus and has taken over the hypophysiotropic roles of the evolutionarily lost Gnrh1 form. A genetic defect in GNRH1, or a disruption of its neuronal migration during ontogeny, has been well-studied

in mammals and is known to lead to hypogonadotropic hypogonadism and infertility in humans and rodents (Bouligand et al., 2009; Schwanzel-Fukuda et al., 1989; Mason et al., 1986). However, the effects of a mutation or loss of the hypophysiotropic Gnrh in teleosts has only just begun to be studied, with interesting and surprising results.

To better characterize the roles of the hypophysiotropic Gnrh in teleosts, we previously generated a knockout model in zebrafish, which have a loss of the hypophysiotropic Gnrh3 (*gnrh3*$^{-/-}$) due to a targeted mutation in the *gnrh3* gene (Spicer et al., 2016). The loss of Gnrh3 resulted in no changes in reproductive success, an unexpected finding because genetic mutations of *Gnrh1* results in disrupted puberty and infertility in mice and humans (Bouligand et al., 2009; Au et al., 2011), and Gnrh3 neuronal ablation results in infertile female zebrafish (Abraham et al., 2010). This suggested that a compensatory mechanism is activated to maintain normal execution of reproduction in zebrafish in response to the genetic mutation of Gnrh3. This finding was reinforced by the discoveries of other groups, where the knockout of Gnrh3 and other upstream reproductive neuropeptides, Kisspeptins 1&2 (Tang et al., 2015, Liu et al., 2017), in zebrafish resulted in no phenotypic differences in reproduction despite their crucial role in more evolved vertebrates (Mason et al., 1986; Clarkson et al., 2010). Gnrh2 was the predicted candidate to compensate for the loss of Gnrh3 because its neurons innervate the pituitary in zebrafish (Xia et al., 2014), it has the ability to stimulate gonadotropin release in vivo, and can activate all four Gnrh receptors in zebrafish with equal or greater potency than Gnrh3 (Tello et al., 2008). Thus, we first used the single *gnrh3*$^{-/-}$ knockout fish to obtain whole transcriptome data of brains and pituitaries, comparing knockout and wild-type counterparts, to begin examining

differentially expressed genes. Then, we generated a double knockout (DKO) zebrafish line (*gnrh2*$^{-/-}$*;gnrh3*$^{-/-}$) which contains a null mutation in both the *gnrh2* and *gnrh3* genes, resulting in a complete loss of all Gnrh peptides in the zebrafish brain, (and is the first vertebrate species studied where two Gnrh isoforms are knocked out) to determine if Gnrh2 is compensating for the loss of Gnrh3, and if not, determining what other factors may be compensating for Gnrh loss, illuminating potentially previously unknown reproductive regulators.

We comprehensively characterized the reproductive functions in this line, however, as will be entailed below, this line exhibits normal reproduction and fertility. Therefore, in order to understand the underlying reason for the lack of the expected reproductive phenotype, we began to look for alternative compensating pathways, based on the differential expression of neuropeptide-coding genes. Additionally, because Gnrh2 is implicated in regulating feeding behaviors in mammals, birds, and fish, and can modulate certain feeding peptides in goldfish (*Carassius auratus*) (Hoskins et al., 2008; Kang et al., 2011), we examined representative feeding-related genes as potential compensators for the loss of Gnrh2. The findings from this study point to several potential neuropeptides that change their expression profile in the Gnrh3 KO and DKO line, and together may participate in what seems to be a multi-factorial type of compensation for the loss of the Gnrh ligands.

## Methods

### Zebrafish Maintenance and Husbandry

All zebrafish were kept in the in-house facility at the Institute of Marine and Environmental Technology and maintained at a 14L:10D cycle in a 28°C recirculating

water system. Zebrafish larvae were fed live *Paramecium* from 5 days-post-fertilization (dpf) to 15 dpf and then fed live *Artemia nauplii* until they were large enough to feed on 300 µm Gemma diet pellets (Skretting), which they were fed *ad libitum* twice daily. Zebrafish larvae were kept in 300 mL tanks on a nursery shelf until 30 dpf, and then moved to aquaria in the recirculating water system. Prior to tissue collections, adult zebrafish were euthanized in a cold ice-water bath and then promptly decapitated. Larval zebrafish were killed in tricaine (MS-222, Sigma-Aldrich). All experimental protocols were approved by the Institutional Animal Care and Use Committee at the University of Maryland School of Medicine.

**RNA-Seq analysis to determine differentially expressed genes in *gnrh3*$^{-/-}$ and wild-type brains and pituitaries**

In order to obtain RNA-Seq transcriptome reads of brains and pituitaries from *gnrh3*$^{-/-}$ and wild-type zebrafish, ten brains from *gnrh3*$^{-/-}$ males and ten brains from wild-type males at 8 months of age were dissected and 5 brains were put in each tube containing RNA*later* stabilization solution (Thermofisher Scientific) and flash frozen at -80°C. Pituitaries were dissected from 20 *gnrh3*$^{-/-}$ males and 20 wild-type males at 8 months of age and 10 pituitaries were put in each tube containing RNA*later* stabilization solution (Thermofisher Scientific) and then flash frozen at -80°C. Samples were then sent to Omega Bioservices, who extracted the RNA and used poly-T primers for exome sequencing, and generated around 10 million transcript reads per sample, containing 150 bp paired-end reads. The reads were saved and sent to our lab in fastq form for subsequent analysis.

In order to conduct RNA-Seq analysis, all programs and transcriptome data were transferred to a remote server. Quality control was first conducted on the fastq transcriptome reads. FastQC software (Babraham Bioinformatics) was used to determine adapter sequences and base-pair quality analysis. The first 5 base-pairs of all transcript reads were determined to be of low quality and trimmed from each read using the Trimmomatic program, from Usadel Lab (Bolger et al., 2014). Additionally, the Trimmomatic program was used to dispose of low-quality reads, in which a sliding window analysis disposed of low-quality base-pairs in all reads. The next step was to map the fastq reads to a reference genome. The GRCz10 zebrafish genome (as .fasta files) and annotation file (in .gtf form) were downloaded from the NCBI website (sequenced as part of Zebrafish Genome Project at the Wellcome Trust Sanger Institute). To construct a reference genome, the Bowtie2 program was used to compile and build the GRCz10 zebrafish reference genome from the downloaded fasta files (Langmead and Salzberg, 2012). The Tophat2 program, from Johns Hopkins University (Kim et al., 2013), was then used to map all fastq transcriptome reads to the reference genome (one sample was mapped at a time), and all properly mapped reads were merged into an "accepted_hits.bam" file for each sample. The Cufflinks program (Trapnell et al., 2012) was then used to assemble the mapped reads into transcript files and estimate the abundances for each transcript. The Cuffmerge program was used to assemble all of the sample transcript files together, and the Cuffdiff program conducted the differential expression analysis using all of the assembled files. Files from the Tophat mapping and assembly were also used in DeSeq2 software analysis in R for further differential gene expression analysis and visualization of the DEG output quality data for the analyzed

samples was also conducted in SeqMonk (Babraham Bioinformatics). Significant differentially expressed genes were compared between Cuffdiff and DeSeq2 output, and genes which were determined to be differentially expressed in both software analyses were kept and determined as significant.

For gene ontology analysis, to determine biological processes which were significantly differentially expressed between the genotypes, the Goseq program (version 3.6, Bioconductor) was used with inputs from the differential gene expression analysis in DeSeq2, and gene annotation files from the NCBI Danio rerio Grcz10 assembly (Young et al., 2010).

**Generating the *gnrh2*$^{-/-}$;*gnrh3*$^{-/-}$ zebrafish lines**

The double knockout line was generated by crossing the homozygous *gnrh3*$^{-/-}$ line with the homozygous *gnrh2*$^{-/-}$ line (Spicer et al., 2016; Marvel et al., 2018). Double heterozygous (*gnrh2*$^{+/-}$;*gnrh3*$^{+/-}$) offspring from the homozygous mutant crosses were then in crossed to produce the homozygous *gnrh2*$^{-/-}$;*gnrh3*$^{-/-}$ progeny. Fish were screened with amplicons of wild-type and *gnrh3*$^{-/-}$ DNA selectively amplified with specific corresponding primers (Table 2.1). Screening of the *gnrh2*$^{-/-}$ mutation was conducted similarly using specific wild-type and mutant primers (Table 2.1). Verification of the loss of Gnrh2 and Gnrh3 protein was conducted using immunohistochemistry with antibodies against the specific recombinant zebrafish (ZF) Gnrh2-associated peptide (GAP2) (Xia et al., 2014) or ZF Gnrh3-associated peptide region (GAP3) (Spicer et al., 2016) downstream of the decapeptide coding region, as well as with antibodies against the Gnrh2 and Gnrh3 decapeptide coding regions (Spicer et al., 2016, (kindly provided by the late Dr. Judy King), to verify that both the decapeptide and GAP regions were absent.

Homozygous double knockout (DKO) *gnrh2*$^{-/-}$;*gnrh3*$^{-/-}$ and wild-type (*gnrh2*$^{+/+}$;*gnrh3*$^{+/+}$) offspring from these crosses were kept to propagate the lines and compare reproductive characteristics and expression levels of reproductive and feeding-related genes.

**Validating the *gnrh2*$^{-/-}$;*gnrh3*$^{-/-}$ zebrafish lines using immunohistochemistry**

The loss of the Gnrh2 and Gnrh3 peptide in DKO fish was verified using immunohistochemistry with antibodies against the specific recombinant GAP (GAP2 and GAP3) and decapeptide regions of Gnrh2 or Gnrh3 raised in rabbits. Brains from sexually mature DKO and wild-type fish of the same age were dissected and fixed in 4% PFA overnight and immersed in 30% sucrose in PBS for four hours, or until brains sunk to the bottom of the vial. Brain samples were then frozen in OCT and stored at -80°C. Cryo-sectioning was conducted at -20°C using a Tissue-Tek Cryo3 cryostat. Brains were sectioned to 10 µM thickness, placed on Plus coated slides, and stored at -80°C until immunohistochemistry (IHC) was performed. To perform double-staining IHC, slides were briefly fixed in acetone, and quenched in 0.3% $H_2O_2$ in PBS for 30 minutes. Slides were then washed in PBS, blocked for one hour in 5% normal goat serum, and incubated with a 1:1,000 dilution of Anti-GAP2 in 1% BSA and 0.3% Triton X-100 overnight at 4°C. As a negative control, consecutive slides were incubated with pre-immune serum, instead of a primary antibody, from the same animal the antibodies originated from. Slides were then washed in 100 mM Tris Ph 7.5, 150 mM NaCl, and 0.5% Tween-20 (TNT) and incubated in an HRP-conjugated Goat anti-Rabbit antibody (Genscript) at a 1:1,000 dilution in 1% BSA and 0.3% Triton for one hour. Then, slides were washed in TNT and incubated in a fluorescein dye from the Tyramide Signal Amplification Plus kit (TSA Plus kit, Perkin Elmer) at a 1:50 dilution for 5 minutes, washed in TNT, and HRP

signal quenched with 0.02 N HCl for 10 minutes. After washing, the procedure for IHC delineated above was repeated on the same slides, but with an Anti-GAP3 primary antibody and Cy3 dye from the TSA kit to label Gnrh3 protein. Slides were mounted in 50% glycerol plus 10 µg/ml Hoescht 33342 (Sigma) and viewed on a Zeiss Axioplan2 microscope and screened for the presence of Gnrh2 and Gnrh3 soma and fibers.

**Reproductive parameter assessments**

Sexually mature wild-type and DKO fish of the same age were selected for spawning, and fecundity, fertility, embryo survival, egg diameter, and gonad morphology characteristics were measured and compared between the groups. The spawning procedure follows a routine protocol as described by Westerfield, 2007 (Westerfield, 2007). Six pairs of each genotype combination were placed in spawning containers and separated by a divider overnight. Pairs of wild-type males and females, DKO males and females, and combinations of one wild-type male paired with one DKO female, and one wild-type female paired with one DKO male were analyzed. In the morning, immediately after the light was turned on, dividers were removed, and fish allowed to spawn for one hour before eggs were collected from each container. The total number of eggs was counted for each pair to obtain fecundity numbers, and the percentage of eggs fertilized was quantified via embryonic development at 6 hours post fertilization (hpf). The next day, the number of surviving and dead eggs were counted to obtain survival percentages. Gonadosomatic index (GSI) was determined as percentage of gonadal weight/total body weight in six age-matched DKO and wild-type males and females at 5 months of age.

**Gonad histology**

To examine gonadal morphology, DKO and wild-type females and males of 4 months of age were sacrificed and gonads dissected and fixed in 4% PFA overnight at 4˚C. The gonads were then dehydrated through a series of increasing ethanol concentrations, from 50% to 100%, and xylene washes at room temperature (RT), and infused in paraffin at 60˚C overnight. Samples were embedded in paraffin blocks, sectioned to 5 µm thickness using a microtome, and dried overnight at 38˚C. Sections were stained with hematoxylin and eosin and mounted with Permount according to the manufacturer's protocol (Sigma). Sections were examined using a Zeiss Axioplane2 microscope, and pictures taken at 5X and 20X magnification with a CCD Olympus DP70 camera. The most advanced stage of gametogenesis was noted for each individual fish based on the oocyte size and yolk morphology (Selman et al., 1993), and sperm size, opacity, and distribution (Leal et al., 2009).

**Reproductive gene expression differences**

The expression levels of multiple reproductive (*avp, cga, fshb, gnih, kiss1, kiss2, lhb, pacap1, scg2, spx, tac3a*) and metabolic related genes (*agrp1, npy, orexin, pmch2, pomca*) were measured in whole DKO and WT fish throughout the first 30 days of development and in adult brains using QPCR (Table 2.1). We focused on examining the gene expression of peptides that have previously been shown to have potential reproductive regulatory roles or are hypophysiotropic. For the developmental assay, pools of embryos and larvae were sampled in triplicate at 1,2, and 3 dpf (n=20/sample), 8, 12 dpf, and 18 dpf (n=10/sample), 24 dpf (n=8/sample), and 30 dpf (n=6/sample), euthanized in MS-222, flash frozen on dry ice, and stored at -80°C until RNA extraction.

For adults, individual brains and pituitaries of 8 sexually mature and age-matched male DKO and WT fish and 6 female DKO and WT fish were dissected, flash frozen on dry ice, and stored at -80°C until RNA extraction was conducted. RNA was extracted using Trizol reagent according to the manufacturer's protocol (Promega). The RNA quantity of each sample was measured using Nanodrop, and only samples with 280/260 ≥1.8 were used for cDNA synthesis. 1 microgram of RNA was reverse transcribed using the Quantitect Reverse Transcription kit, including a gDNA elimination step (Qiagen). All gene expression values were normalized to two housekeeping genes, *eef1a* and *sep15* (Xu et al., 2016). Expression values and relative expressions were calculated using the $2^{-\Delta Ct}$ method relative to WT levels. For statistical analysis, a one-way ANOVA followed by student's t-test comparing the average expression of each gene between DKO and wild-type groups was conducted and statistically significantly differentially expressed genes were identified as those with a P-value ≤ 0.05.

### *In situ* hybridization of *gnih agrp1* and *scg2a*

Genes that showed significantly different expression levels via QPCR between DKO and WT fish were selected to examine changes in distribution and abundance using in situ hybridization. Brains of sexually mature, age-matched, DKO and wild-type females and males were dissected, fixed, cryoprotected, frozen in OCT at -80°C, and sectioned at 10 µM thickness in the same manner as described above for IHC. Male brains were used to analyze the differential expression of secretogranin 2a (*scg2a*, gene accession # NM_001077748), vasoactive intestinal peptide (*vip*, gene accession # NM_001114553.3), and gonadotropin-inhibiting hormone (*gnih*, gene accession # GU290218) and female brains were used to analyze the differential expression of agouti-

related peptide 1 (*agrp1*, accession #NM_001328012) as these genes showed sex-specific differences. Digoxigenin or fluorescein labeled riboprobes were prepared from the full length cDNAs of the corresponding gene cloned in pGEMT vector, using T7 or SP6 RNA polymerases (New England Biolabs) to generate the anti-sense and sense riboprobes. For ISH, slides were briefly dried at 55˚C for 8 minutes and post-fixed with 4% PFA for 15 minutes. Slides were washed with PBS and incubated with 0.3% $H_2O_2$ in methanol for 30 minutes to quench endogenous peroxidases. Slides were washed in PBS, incubated in 0.1M TEA buffer pH 8 (Triethanolamine), and washed in 2X sodium-saline citrate buffer (SSC). Pre-hybridization occurred at 65˚C in a solution containing 50% Formamide, 3X SSC, and 10 mg/ml denatured calf thymus DNA. After 2 hours, slides were incubated at 65˚C overnight in a buffer containing calf thymus DNA, and 250 ng/ml of *scg2* anti-sense RNA probe (DIG-labeled), 500 ng/ml of *gnih* anti-sense RNA probe, or 500 ng/mL *agrp1* anti-sense RNA probe (Fluorescein-labeled). Consecutive slides of WT and DKO brains were incubated with sense RNA probe of each gene as a negative control. Slides were then washed in 2X SSC at room temperature for 30 minutes, and 2X SSC, 0.4X SSC, and 0.1X SSC at 65˚C for 30 minutes each, and 1x SSC for 10 minutes at room temperature. Slides were blocked with TNB (100 mM Tris pH 7.5, 150 mM NaCl, 0.5% Blocking reagent, from the TSA kit) and followed the same procedures delineated above for the IHC, except with the secondary antibody being either Anti-DIG HRP-conjugated (Roche) or Anti-Fluorescein HRP-conjugated diluted 1:200, and with slides labelled with Cy3 from the TSA kit. After mounting slides with 50% glycerol plus Hoechst 33342, they were viewed and analyzed for Cy3 and/or fluorescein signal using a Zeiss Axioplan2 fluorescent microscope and pictures taken at 5x and 20x magnification.

The distribution of all genes was analyzed using the Zebrafish brain atlas (Wullimann et al., 1996) and compared between genotype groups, and the total number of soma counted for each brain (n=6 brains for each gene and genotype).

## Results

### Overview of differential gene expression analysis

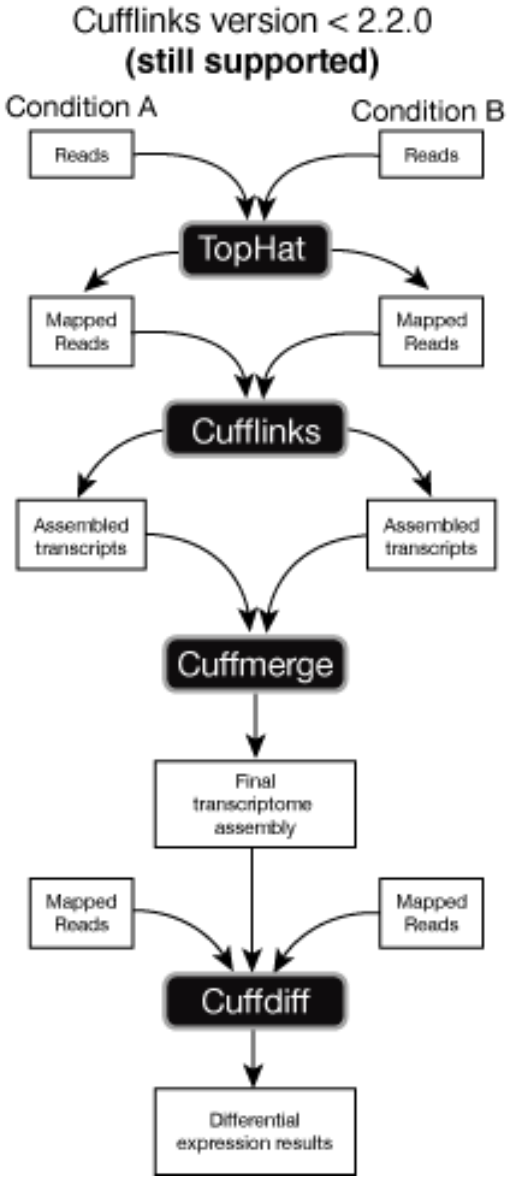


**Figure 6.1.** Pipeline used for initial differential expression analysis of *gnrh3*$^{-/-}$ and wild-type RNA-Seq transcriptome data (from Trapnell et al., 2012).

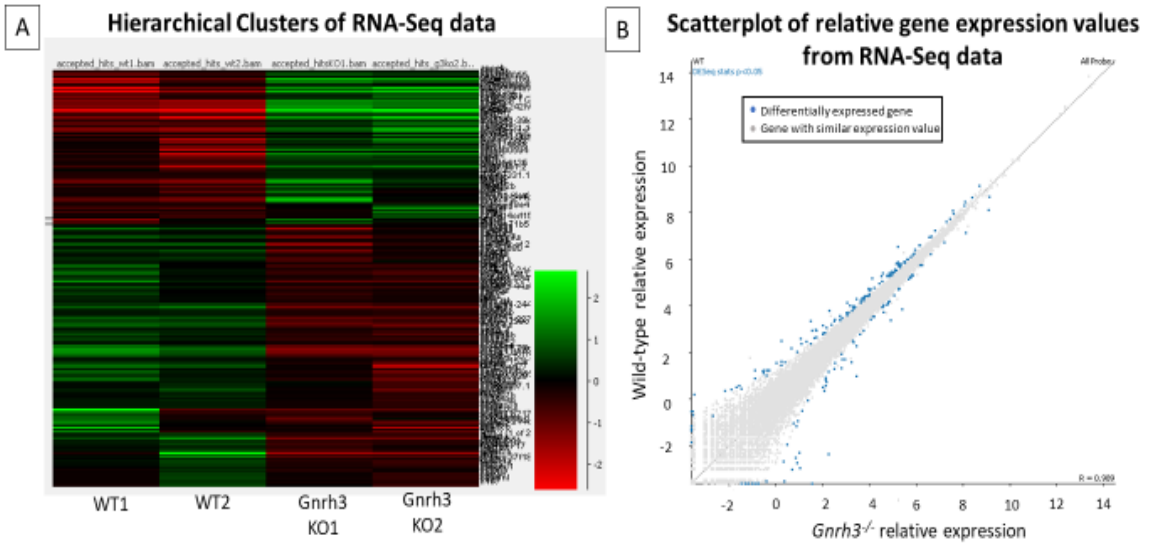


**Figure 6.2.** Hierarchical cluster analysis values for the differentially expressed genes of wild-type technical replicates and *gnrh3*$^{-/-}$ technical replicates (A), and a scatterplot of relative expression values for wild-type and *gnrh3*$^{-/-}$ genes, with differentially expressed genes (blue) and similarly expressed genes (grey) both represented.

Hierarchical clusters of statistically differentially expressed gene values show that the two wild-type technical samples exhibit similar expression patterns (green or red) to each other, but opposite from the other genotype, whereas the two *gnrh3*$^{-/-}$ technical replicates exhibit similar expression patterns to each other (green or red), suggesting decent quality data and verifying statistical differential expression (Fig. 6.2A). From the DeSeq2 analysis, there were 219 significantly differentially expressed genes of *gnrh3*$^{-/-}$ brains compared to wild-type, with 88 genes upregulated in *gnrh3*$^{-/-}$ brains compared to wild-type, and 131 genes significantly downregulated in *gnrh3*$^{-/-}$ brains compared to wild-type (Fig. 6.2B).

**Differentially expressed neuropeptides and dopamine genes in *gnrh3*$^{-/-}$ brains compared to wild-type**

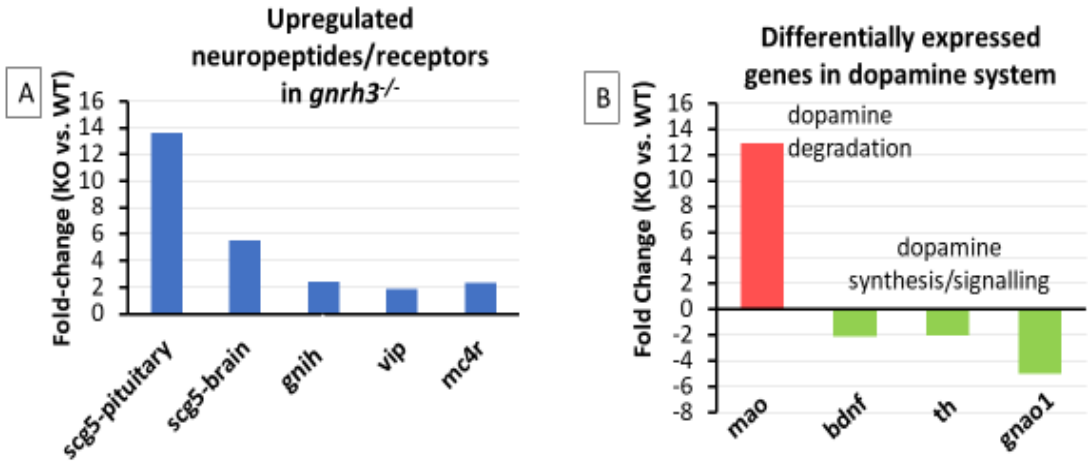


**Figure 6.3.** Upregulated neuropeptide and neuropeptide receptor genes in *gnrh3*$^{-/-}$ brain and pituitary tissues compared to wild-type (represented as fold-change of *gnrh3*$^{-/-}$ samples compared to wild-type) (A). Upregulated and downregulated dopamine processing and signaling genes in *gnrh3*$^{-/-}$ brains compared to wild-type (B).

When focusing on differentially expressed genes encoding for potentially reproductive neuropeptides in the brain, there are several which are upregulated in *gnrh3*$^{-/-}$ brains compared to wild-type, with the gene encoding the chaperone Secretogranin 5 (*scg5*) expressing a 5-fold increase in *gnrh3*$^{-/-}$ brains, and 13-fold increase in *gnrh3*$^{-/-}$ pituitaries compared to wild-type (Fig. 6.3A). Other upregulated genes include *gonadotropin-inhibitory hormone* (*gnih*), *vasoactive intestinal peptide* (*vip*), and *melanocortin 4 receptor* (*mc4r*), which all exhibit a 2-fold expression increase in *gnrh3*$^{-/-}$ brains compared to wild-type (Fig. 6.3A). The *monamine oxidase* gene (*mao*), which expresses an enzyme involved in dopamine degradation, exhibits a 13-fold increase in *gnrh3*$^{-/-}$ brains compared to wild-type, whereas the dopamine synthesizing enzyme, *tyrosine hydroxylase* gene (*th*), along with dopaminergic signaling factors, *bdnf* and *gnao1*, are expressed at a 2-fold to 5-fold decreased amount in *gnrh3*$^{-/-}$ brains compared to wild-type brains (Fig. 6.3B).

**Gene ontology analysis**

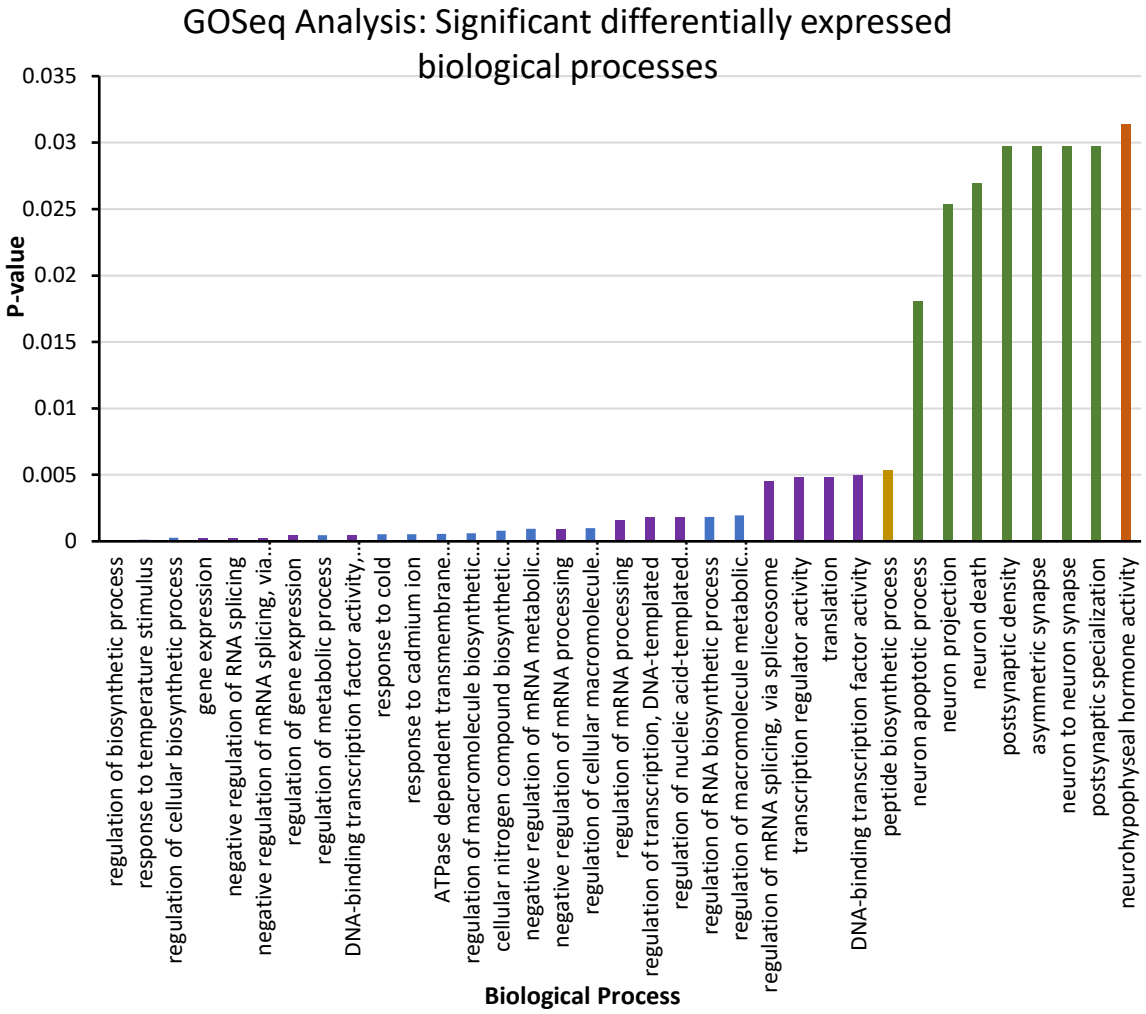


**Figure 6.4.** Results from gene ontology analysis, displaying significantly overrepresented biological processes from differential gene expression analysis of *gnrh3*$^{-/-}$ and wild-type brain samples. Purple = transcription or translation regulatory processes, yellow = peptide biosynthetic processes, green = neuronal regulatory processes, orange = neurohypophyseal hormone activity processes, blue = other biological process.

Gene ontology analysis using GOseq determined 196 significantly overrepresented biological processes of the differentially expressed genes between *gnrh3*$^{-/-}$ and wild-type brains from DeSeq2. A representation of the most statistically significant processes demonstrates an abundance of metabolic, transcription, and translation regulation differences (blue and purple bars, Fig. 6.4). Additionally, there are many

differences in neuronal and synaptic regulation processes between genotypes (green bars, Fig. 6.4). There is also an overrepresentation of peptide biosynthetic processes (yellow) and processes involved in neurohypophyseal hormone activity (orange) (Fig. 6.4).

**Validating the absence of Gnrh2 and Gnrh3 peptides in *gnrh2*$^{-/-}$;*gnrh3*$^{-/-}$ brains**

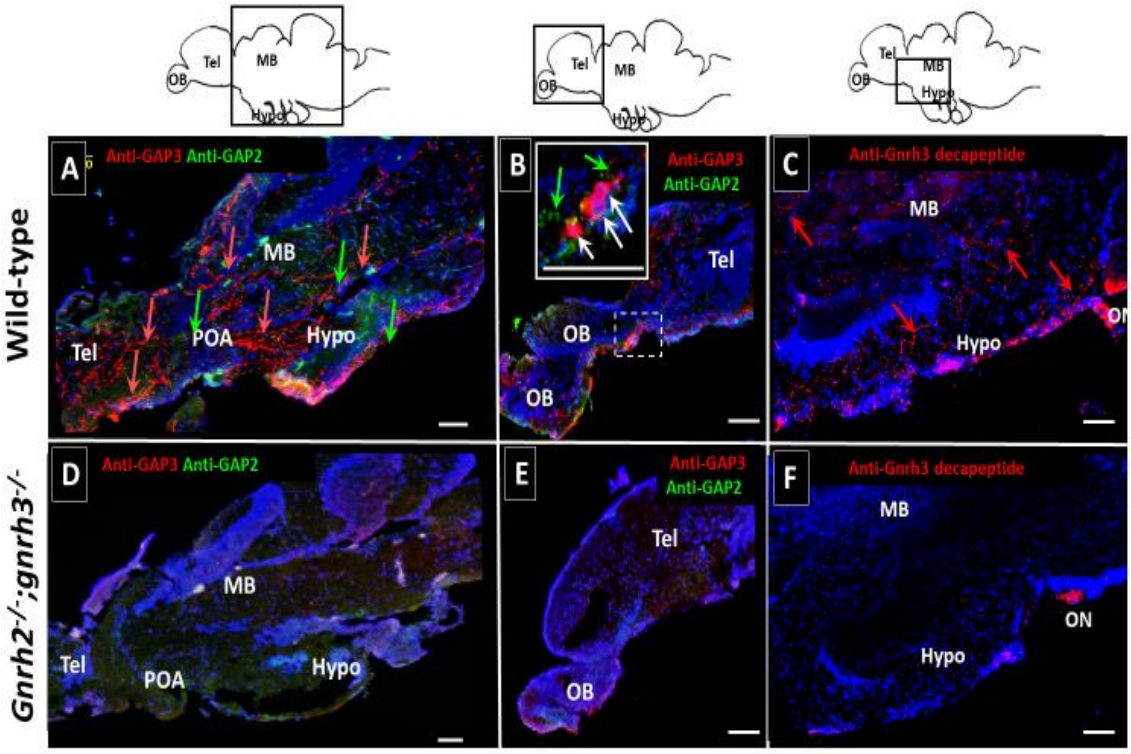


**Figure 6.5.** Validation of the absence of the Gnrh peptides in DKO adult brains using antibodies against the Gnrh2-associated peptide, Gnrh3-associated peptide, and Gnrh3 decapeptide. Immunostaining of Gnrh2 (green) and Gnrh3 (red) in sagittal brain sections of adult male fish. Abundant neuronal fibers of Gnrh3 and Gnrh2 are seen throughout wild-type brain sections (red and green arrows) (A), but none are visible in DKO brains (D). The olfactory bulb terminal nerve in wild-type brains contains red Gnrh3 soma (white arrows) and green Gnrh2 fibers (neon green arrows) (B), whereas DKO olfactory bulbs do not show any signals (E). Gnrh3 fibers are seen abundantly throughout the wild-type hypothalamus (C, red arrows) using antibodies targeting the Gnrh3 decapeptide region, but no signal is apparent in DKO (F). Hypo, hypothalamus; MB, midbrain tegmentum; POA, preoptic area; Tel, telencephalon; OB, olfactory bulb. Scale bars = 100 μm.

IHC was conducted to verify that the genetic mutations in Gnrh2 and Gnrh3 resulted in the loss of the peptide in DKO brains. IHC staining of wild-type brains with

GAP2 and GAP3 antibodies resulted in the abundant appearance of Gnrh2 and Gnrh3 fibers throughout the entirety of the brain, most notably in the olfactory bulb, telencephalon, midbrain, and hypothalamus (Fig. 6.5A). Congruent with previous reports (Spicer et al., 2016, Xia et al., 2014), Gnrh3 soma are found in the preoptic and terminal nerve areas (Fig. 6.5B). Staining of DKO brains with GAP2 and GAP3 antibodies, however, did not result in the appearance of any Gnrh2 or Gnrh3 fibers (Fig. 6.5D). The terminal nerve region of DKO brains (Fig. 6.5E) also did not contain any signal of Gnrh3 or Gnrh2 soma, confirming the gene knockout resulted in a loss of the Gnrh peptides. IHC staining of WT brains with the Gnrh3 anti-decapeptide antibodies showed abundant signal throughout the brain, especially in the preoptic area, midbrain, and hypothalamic regions (Fig. 6.5C). IHC staining of DKO brains with the same antibodies did not result in any signal, further verifying that the Gnrh2 and Gnrh3 decapeptides were not being produced in this line (Fig. 6.5F).

**Comparing reproductive outputs of *gnrh2*$^{-/-}$;*gnrh3*$^{-/-}$ and wild-type adults**

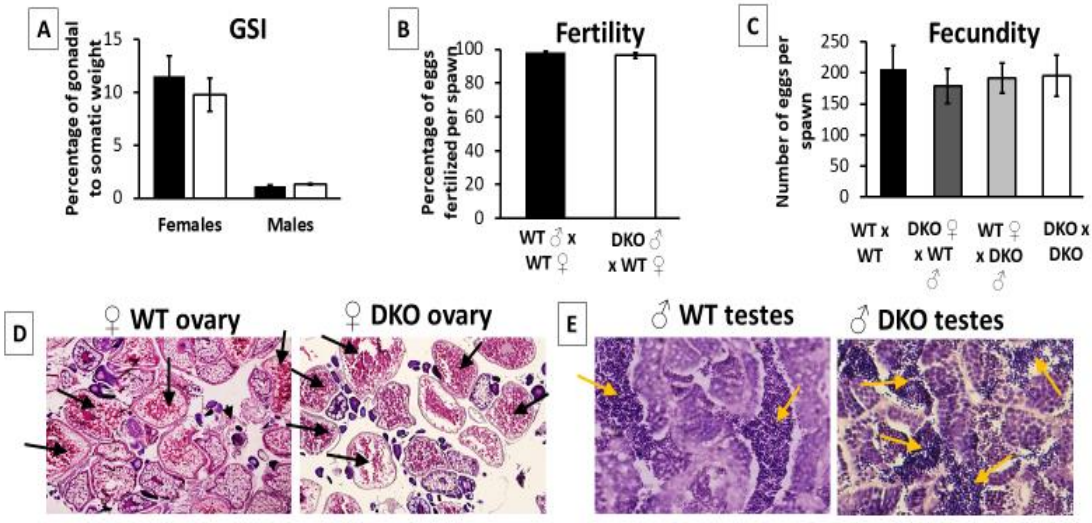


**Figure 6.6.** Comparison of reproductive outputs between wild type and DKO fish. (A) GSI comparison of WT and DKO females and males (n=6 of each). Number of eggs fertilized per spawn, fertility (B), and number of eggs produced per spawn of one male

paired with one female, fecundity (C) (n=6). Gonad morphology comparison of wild-type and DKO ovaries at 4 months of age, displaying fully mature oocytes (black arrows) (G), and wild-type and DKO testes at 4 months of age, displaying fully mature spermatozoa (yellow arrows) (H). Closed bars = wild-type, open bars= *gnrh2*$^{-/-}$*;gnrh3*$^{-/-}$ (DKO). All data are presented as mean ±S.E.M. Statistical significance (*) is evaluated as $P \leq 0.05$.

To determine reproductive characteristics of fish after the loss of Gnrh2 and Gnrh3, measurements of GSI, fertility, and fecundity were compared between the wild-type and the DKO in different crossing combinations. There were no differences in the GSI of wild-type and DKO females or males (Fig. 6.6A). Male fertility was not significantly different between wild-type and DKO males (Fig. 6.6B), and the number of eggs produced per spawn (fecundity) was also not significantly different between genotype pairings (Fig. 6.6C). There were no differences in the morphology of the gonads between DKO and wild-type individuals. At 4 months of age, both DKO and wild-type ovaries contained fully mature oocytes (Fig. 6.6D, black arrows) and DKO and wild-type testes displayed fully mature spermatozoa (Fig. 6.6E, yellow arrows).

**Gonadotropin gene expression comparison throughout development and adulthood of DKO and wild-type zebrafish**

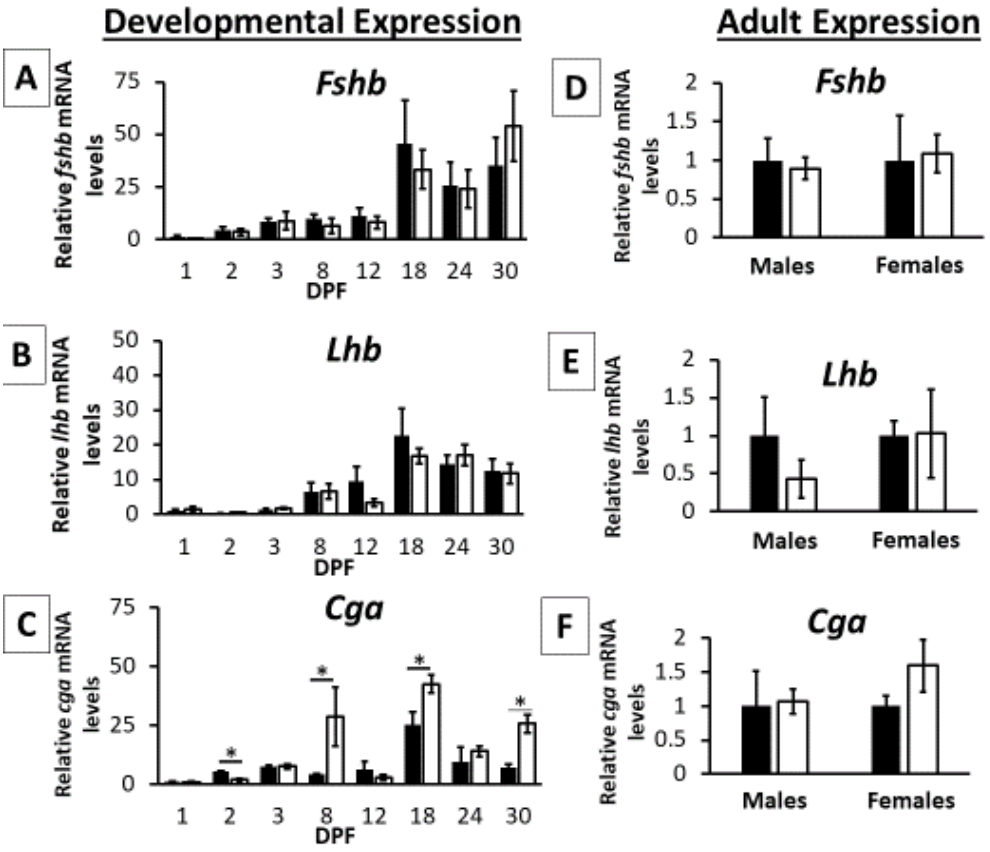


**Figure 6.7.** The effect of the loss of Gnrh2 and Gnrh3 on the expression of reproductive pituitary genes throughout development and in adulthood. Comparisons of the expression levels of gonadotropin genes in whole larvae between 1 and 30 dpf and adult pituitaries were measured via qPCR. Relative expression levels of the gonadotropin genes, *fshb* (A), *lhb* (B), and *cga* (C) in fish throughout the first 30 days of development and relative expression levels of the gonadotropin genes, *fshb* (D), *lhb* (E), and *cga* (F) in adult pituitary samples. Closed bars = wild-type, open bars= *gnrh2*$^{-/-}$*;gnrh3*$^{-/-}$(DKO). All data are presented as mean ± S.E.M. Statistical significance (*) was evaluated as $P \leq 0.05$.

Expression levels of *fshb* and *lhb* throughout the first 30 days of development were not significantly different between wild-type and DKO embryos and larvae (Fig. 6.7A&B). Similar to what was reported for the *gnrh3*$^{-/-}$ line [16], mRNA levels of the common gonadotropin-alpha subunit (*cga*) was significantly lower in DKO embryos at 2 dpf, but significantly higher at several points later in development, including 8 dpf, 18 dpf, and 30 dpf (Fig. 6.7C). In adults, the expression levels of *fshb* (Fig. 6.7D), *lhb* (Fig.

6.7E), and *cga* (Fig. 6.7F) in the pituitaries did not show any significant differences between DKO and WT, despite the upregulation of *cga* in DKO fish during development.

**Reproductive gene expression analysis of DKO and wild-type adult zebrafish**

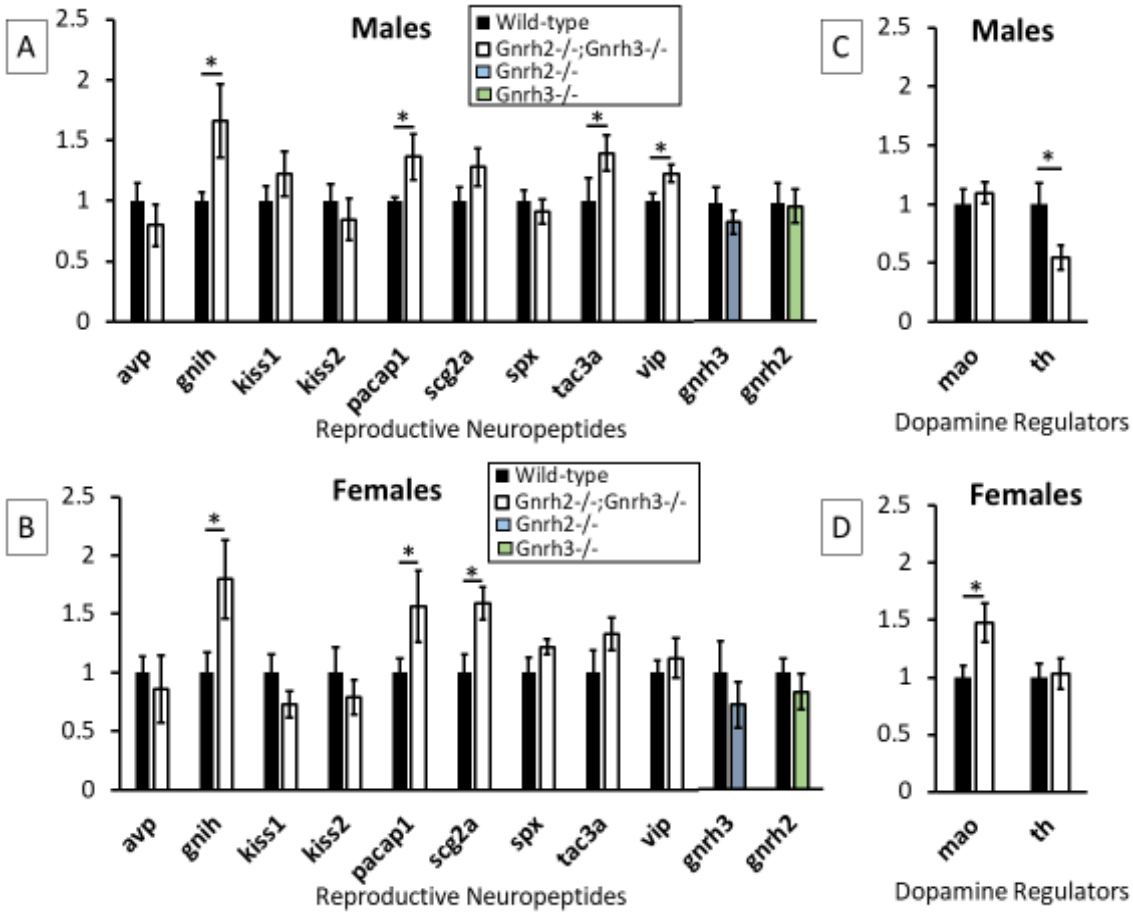


**Figure 6.8** The effect of the loss of Gnrh2 and Gnrh3 on reproductive gene expression. The relative expression levels of reproductive genes were measured and compared between DKO and WT fish brains via qPCR. Relative expression levels of the neuropeptides, *arginine vasopressin* (*avp*), *gonadotropin-inhibiting hormone* (*gnih*), *kisspeptin 1* (*kiss1*), *kisspeptin 2* (*kiss2*), *pituitary adenylate cyclase-activating peptide 1* (*pacap1*), *secretogranin 2a* (*scg2a*), *spexin* (*spx*), *tachykinin 3a* (*tac3a*) and *vasoactive intestinal peptide* (*vip*) of brains from DKO and WT males (A) and females (B) and relative expression levels of *gnrh3* in wild-type male and female brains compared to the single KO line, *gnrh2*$^{-/-}$, and relative expression levels of *gnrh2* in wild-type male and female brains compared to the single KO line, *gnrh3*$^{-/-}$. Relative expression levels of the dopamine regulatory genes, *monamine oxidase* (*mao*), and *tyrosine hydroxylase* (*th*), of brains from DKO and WT males (C) and females (D). Closed bars= wild-type, open bars= *gnrh2*$^{-/-}$*;gnrh3*$^{-/-}$(DKO), blue bars= *gnrh2*$^{-/-}$, or green bars= *gnrh3*$^{-/-}$. All data are presented as mean ± S.E.M. Statistical significance (*) was evaluated as $P \leq 0.05$.

*Gnih* expression levels displayed a 1.9 and 1.6-fold increase in male and female DKO brains, respectively, compared to WT (Fig. 6.8A and B). The expression levels of *pituitary adenylate cyclase-activating peptide 1 (pacap1)* were significantly higher in DKO male and female brains, with a 1.5 and 1.4-fold increase, respectively (Fig. 6.8A and B). There were sex specific differences in *scg2a*, which showed a 1.6-fold increase in female DKO brains compared to WT (Fig. 6.8B), and *tac3a*, the gene encoding for Neurokinin B, which showed a 1.4-fold increase in DKO male brains compared to WT, and *vip*, which showed a 1.25-fold increase in DKO male brains compared to WT (Fig. 6.8A). There were no significant differences in the expression levels of *kiss1*, *kiss2*, *spexin*, or *avp* (Fig. 6.8A and B), nor in *gnrh3* (Fig. 6.8A and B) or *gnrh2* (Fig. 6.8A and B) in the single KO lines compared to wild-type. DKO male brains show a 2-fold decrease in *th*, the gene for the dopamine synthesizing enzyme (Fig. 6.8C), whereas DKO female brains show a 1.5-fold increase in *mao*, the gene for the enzyme degrading dopamine (Fig. 6.8D).

***In situ* hybridization analysis of *scg2a* in DKO and WT brains**

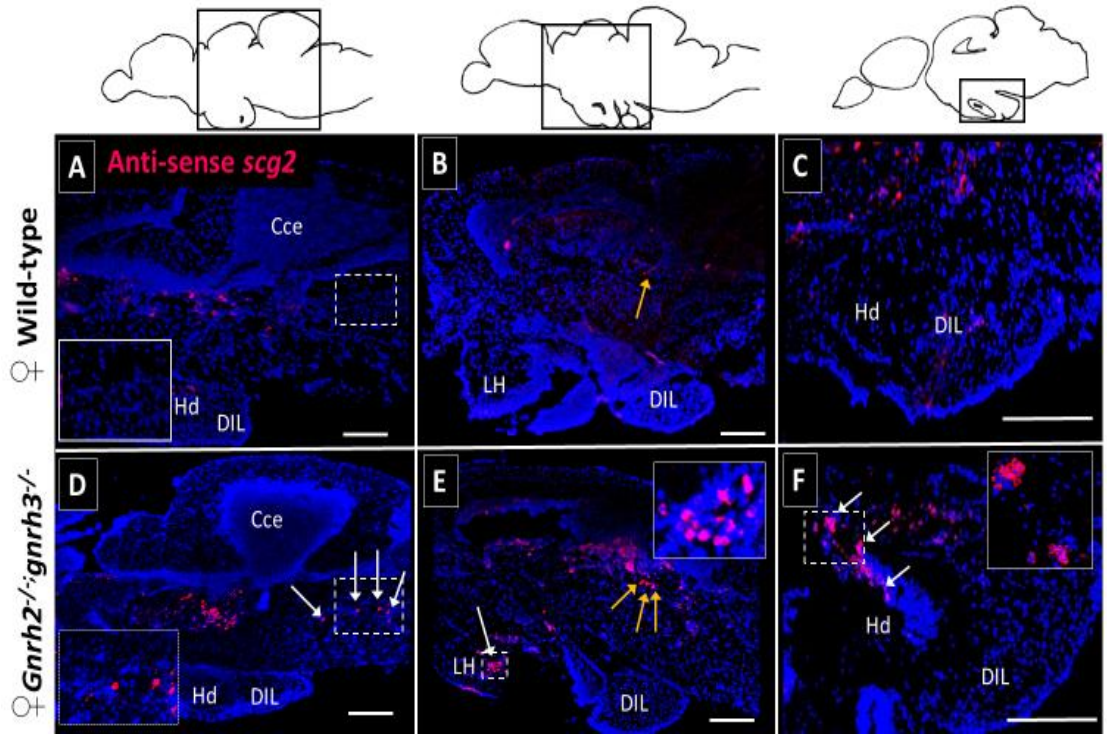


**Figure 6.9.** Comparison of *secretogranin 2a* neuronal distribution. *In situ* hybridization of *scg2a* was conducted on sagittal sections of female wild-type and DKO brains. An abundant number of cells expressing *scg2a* are found just ventral of the posterior Cce in DKO brains (D), whereas a few cells express *scg2a* in this region of the WT brain (A). DKO brains show expression of *scg2a* in the hindbrain, close to the commisura ventralis rhombencephali, region (D, dotted box), whereas no hindbrain expression of *scg2a* is seen in WT brains (A, dotted box). Additionally, *scg2a* is expressed in the lateral hypothalamus region of DKO brains (E) but not WT brains (B), and displays abundant expression in the nucleus lateralis valvulae (yellow arrows). In more lateral sagittal sections, *scg2a* expression is seen in the dorsal hypothalamus region of DKO brains (F) but not WT (C). All dotted boxes are presented at a higher magnification in the white boxed insets present in each figure. Cce= corpus cerebellum, DIL= diffuse inferior lobe, Hd= dorsal hypothalamus, LH = lateral hypothalamus. Scale bars = 100 µm

Since *scg2a* expression levels increased in DKO female brains, *in situ* hybridization was conducted to determine how this upregulation is manifested neuroanatomically in the brain. Wild-type and DKO brains displayed a similar abundant distribution of *scg2a* neurons in the medial sagittal regions, including in the ventral

telencephalon, preoptic area, midbrain, and dorsal hypothalamus (Fig. 3A and D). However, the DKO brains also displayed an additional specific neuronal subpopulation expressing *scg2* ventral to the corpus cerebellum (CCE), close to the reticular formation and commisura ventralis rhombencephali (Fig. 6.9D). Lateral regions of the brain had a higher abundance of *scg2a* neuronal soma in DKO compared to wild-type (yellow arrows), with specific *scg2a* soma in the lateral hypothalamus (LH) (Fig. 6.9E) and lateral dorsal hypothalamus (Hd) (Fig. 6.9F) (white arrows) that are missing in wild-type brains (Fig. 6.9B and C).

### *In situ* hybridization analysis of *vip* in DKO and WT brains

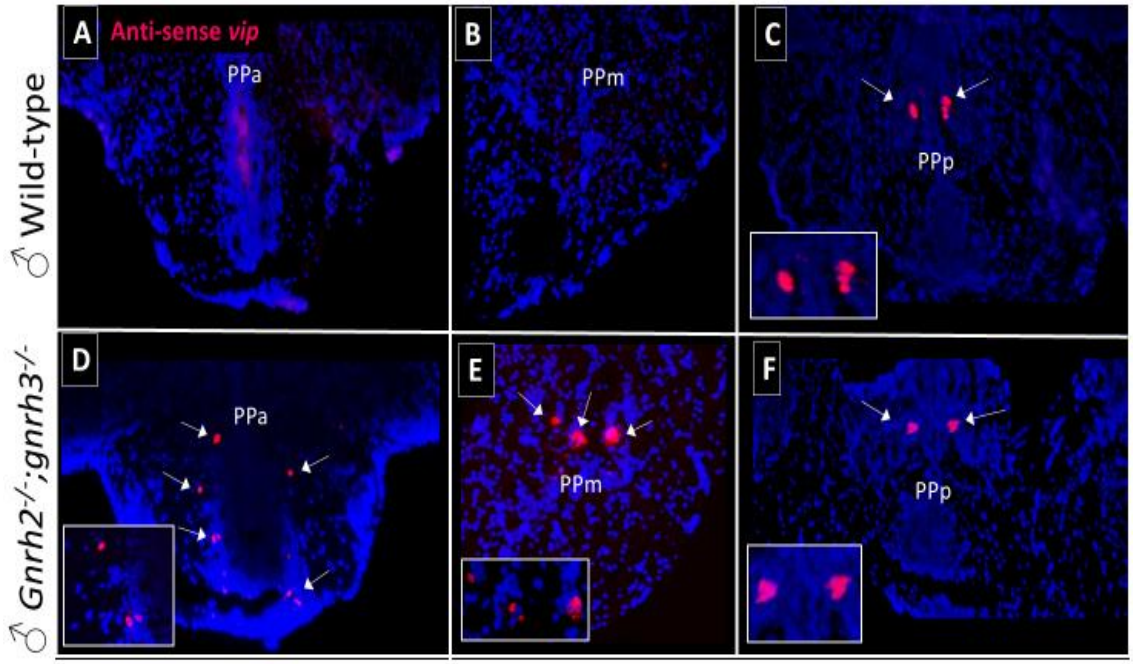


**Figure 6.10.** Comparison of *vasoactive intestinal peptide* neuronal distribution. *In situ* hybridization of *vip* was conducted on coronal sections of male wild-type and DKO brains. In the anterior preoptic area, *vip* is abundantly expressed in DKO brains (D, white arrows), but no signal shown in WT brains (A). In the medial preoptic area, WT brains again show no *vip* expressing cells (B), whereas DKO brains express several cells expressing *vip* (E, white arrows). In the posterior preoptic area, both wild-type (C) and DKO brains (F) show similar patterns of *vip*. PPa= anterior preoptic area, PPm= medial preoptic area, PPp= posterior preoptic area

Since *vip* expression levels significantly increased in DKO male brains, *in situ* hybridization was conducted to determine how this upregulation is manifested neuroanatomically in the brain. In male wild-type brains, signal of *vip* mRNA was present in the most posterior part of the preoptic area (PPp) (Fig. 6.10C), however, there was no signal in the anterior preoptic area (PPa) (Fig. 6.10A) or mid preoptic area (PPm) (Fig. 6.10B). In male DKO brains, there was abundant signal of *vip* mRNA in the PPa (Fig. 6.10D) and PPm (Fig. 6.10E) unlike in wild-type brains. The PPp signal of *vip* in DKO brains (Fig. 6.10F) was of similar distribution and abundance in WT brains (Fig. 6.10C). The hypothalamic signal of *vip* (not shown) was similar between wild-type and DKO brains, with expression in the dorsal hypothalamus.

***In situ* hybridization analysis of *gnih* in DKO and WT brains**

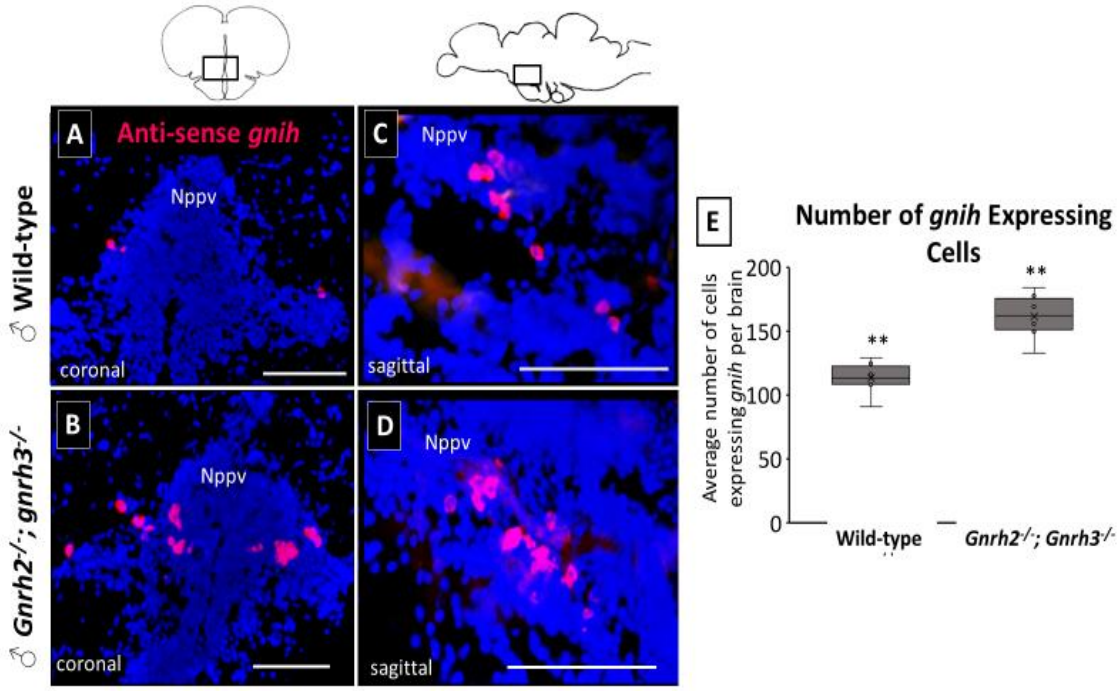


**Figure 6.11.** Comparison of *gnih* neuronal distribution and abundance. *In situ* hybridization of *gnih* in male zebrafish brain sections show neuronal soma expressing *gnih* in the Nppv in coronal sections of WT brains (A) and DKO brains (B). An abundance of neuronal soma expressing *gnih* is notable in DKO (D) compared to WT

sections (C) in the Nppv. The number of cells expressing *gnih* was counted for every section encompassing the entire Nppv, (n=6 brains per genotype), and compared between WT and DKO fish (E). Nppv = posterior periventricular nucleus, Scale bars = 100 µm. Statistical significance (**) was evaluated as $P \leq 0.01$.

Using *in situ* hybridization on male wild-type brain sections (Fig. 6.11A and C), Gnih neuronal soma were detected in the posterior periventricular nucleus (Nppv), dorsal to the ventral hypothalamic region. In male DKO brains, Gnih neuronal soma were found in the same region of the ventral hypothalamus (Fig. 6.11B and D), however with a 1.5-fold higher prevalence compared to WT (Fig. 6.11E).

**Feeding gene expression analysis of DKO and wild-type adult zebrafish**

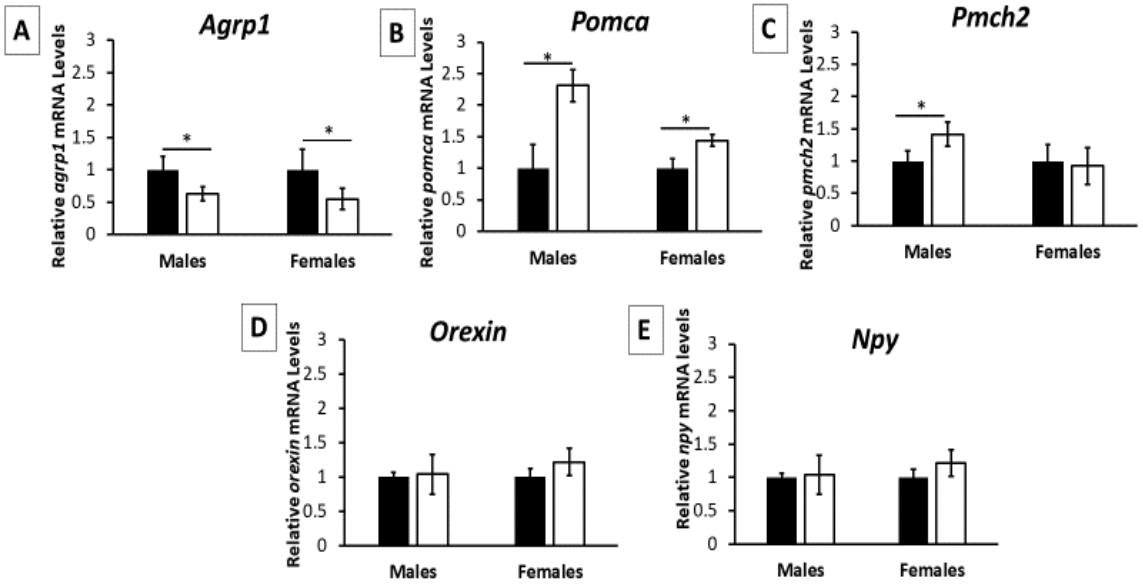


**Figure 6.12.** The effect of the loss of Gnrh2 and Gnrh3 on the expression of neuropeptide feeding genes. The relative expression levels of feeding genes were measured and compared between DKO and WT fish brains via QPCR. The relative mRNA levels of *agouti-related peptide 1* (*agrp1*) (A), *proopiomelanocortin a* (*pomca*) (B), *pro melanin concentrating hormone 2* (*pmch2*) (C), *orexin* (*orx*) (D), and *neuropeptide y* (*npy*) (E) of DKO and WT males and females. Closed bars= wild-type, open bars= *gnrh2*$^{-/-}$*;gnrh3*$^{-/-}$ (DKO). All data are presented as mean ± S.E.M. Statistical significance (*) was evaluated as $P \leq 0.05$.

As Gnrh2 was shown to function as an anorexigen (Nishiguchi et al., 2012; Hoskins et al., 2008; Kang et al., 2011), we also compared the expression levels of feeding genes between DKO and wild-type fish. The relative mRNA levels of *agrp1*, an orexigenic peptide, demonstrated a 2-fold decrease in DKO brains compared to WT (Fig. 6.12A), whereas mRNA levels of *proopiomelanocortin a* (*pomca*), the precursor of alpha-melanocyte stimulating hormone (α-Msh), an anorexigenic peptide, had a 2.3 and 1.45-fold increase in DKO male and female brains, respectively (Fig. 6.12B). The expression levels of *pro melanin concentrating hormone 2* (*pmch2*), an orexigenic peptide, also exhibited a 1.4-fold increase in DKO male brains, although there were no differences in females (Fig. 6.12C). Transcript levels of *orexin* (*orx*) and *neuropeptide y* (*npy*), both orexigenic factors, showed no significant differences between DKO and WT fish (Fig. 6.12D and E).

**Comparison of *agrp1*-expressing cells in DKO and WT brains**

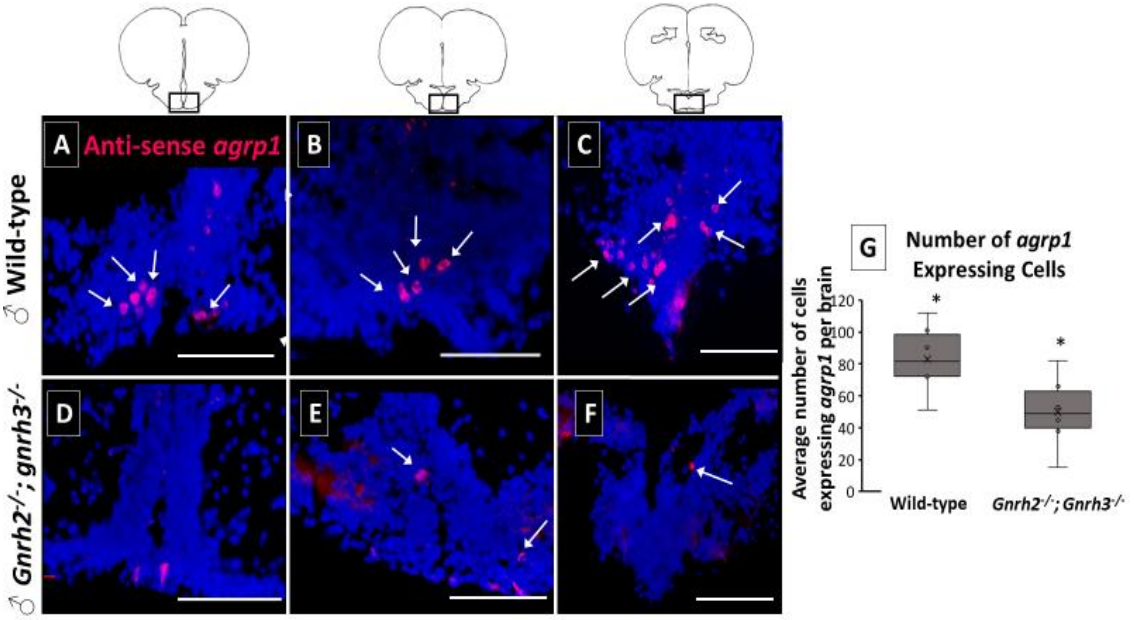


**Figure 6.13.** Comparison of *agrp1* neuronal distribution and abundance. *In situ* hybridization of *agrp1* in the anterior (A), mid (B), and posterior (C) regions of the ventral periventricular hypothalamus in wild-type male fish coronal brain sections show

abundant expression in numerous cells. There are fewer *agrp1* expressing neurons in the anterior (D), mid (E), and posterior (F) regions of the ventral periventricular hypothalamus of DKO male coronal brain sections compared to wild-type. Graphic presentation of the number of cells expressing *agrp1*, counted in slides representing the entirety of DKO (n=6) and WT (n=6) brains (G). Scale Bars= 100 µm.

To further validate the qPCR results and determine how the decreased expression of *agrp1* transpires in DKO brains, we conducted ISH of *agrp1* on DKO and WT male brains. Wild-type brains exhibit numerous soma expressing *agrp1* via ISH in the ventral lateral hypothalamus of coronal brain sections (Fig. 6.13A-C), with DKO brains displaying only a few *agrp1* neurons in the same area (Fig. 6.13D-F). The neuronal soma from whole sections containing Agrp1 neurons were counted in both DKO and wild type brains. DKO brains contained almost half the number of Agrp1 neuronal soma as seen in WT brains (Fig. 6.13G).

**Discussion**

In this study, transcriptomic differential expression analysis was first used to determine certain neuropeptide encoding genes and biological processes which were upregulated in Gnrh3 knockout brains and pituitaries and may be involved in compensating for the loss of Gnrh3. After this analysis, certain unexpected and novel factors were illuminated which may be more involved in reproduction than previously thought. After the analysis, the only other Gnrh isoform in zebrafish, Gnrh2, was knocked out to determine if it was compensating for Gnrh3 loss. This study is the first to genetically knockout all Gnrh genes in an organism and comprehensively analyze the subsequent loss-of-function effects. The initial postulation that Gnrh2 is compensating for the loss of the hypophysiotropic Gnrh3 was ruled out in light of the surprising findings that the DKO line reproduced normally. Therefore, potential alternative

compensators were searched for that display differential expression in the DKO line, focusing on certain genes which were found to be differentially expressed in the RNA-Seq analysis. The search was focused on major reproductive genes encoding neuropeptides in the brain and gonadotropins in the pituitary as potential compensators for Gnrh3 and on genes encoding feeding-related neuropeptides as potential compensators for Gnrh2.

Whole transcriptome RNA-Seq analysis is a powerful new tool to enable the mapping of the entire transcriptome and comprehensively determine expression changes due to environmental changes, experimental treatments, or, in our case, gene knockouts (Wang et al., 2009). Considering the fact that any mutation in the hypophysiotropic GNRH (Bouligand et al., 2009; Mason et al., 1986), GNRH receptor (Karges et al., 2003), or upstream GNRH activators, such as KISS (Kirilov et al., 2013), results in infertility and compromised reproduction in mammals, it was thought that the same would be true with teleosts. However, knocking out the hypophysiotropic Gnrh in zebrafish, Gnrh3, resulted in no differences in reproduction or gonadotropin levels in adulthood (Spicer et al., 2016). This led to the hypothesis that a compensatory mechanism is activated, in which other reproductive regulators may be upregulated after Gnrh3 mutation to take over its role in stimulating gonadotropin release. In order to begin investigating potential compensators for Gnrh3, differential gene expression analysis was conducted on *gnrh3*$^{-/-}$ and wild-type brain transcriptomes. Several neuropeptides and chaperone proteins were identified to be upregulated in *gnrh3*$^{-/-}$ brains, including Secretogranin 5 (Scg5) in the brain and pituitary, Gonadotropin-inhibitory hormone (Gnih), and Vasoactive intestinal peptide (Vip). The upregulation of these genes may be

part of an activated compensatory mechanism, after the genetic loss of Gnrh3, which acts together to take over the role of Gnrh3 in stimulating gonadotropin release and maintaining reproduction. The gene ontology analysis shows the upregulation of many processes involved in differential transcription and translation control, neuronal and synaptic organization, and biohormone processing, suggesting that there is an increased activity of neuronal reorganization and differential expression which may be compensating for Gnrh3 loss. In terms of the differentially expressed factors, Scg5 (also known as 7B2), which was highly upregulated, is part of the Chromogranin family, in which some members, such as the Secretoneurin protein derived from the Secretogranin 2a (Scg2a) precursor, have been shown to be able to stimulate Lh release from gonadotropes (Zhao et al., 2009; Trudeau et al., 2012). Unfortunately, not many studies have looked at the roles of Scg5, especially in teleosts. Two studies analyzing genes involved in puberty of bovine, discovered *scg5* as a gene which may be involved in puberty regulation and fertility (Deatley, 2012; Dias et al., 2017). Scg5 is also involved in the processing of proteins, as it is a potent inhibitor of Prohormone convertase 2 (PC2) (Apletalina et al., 2000). Interestingly, human 7B2 release in pituitary cells have been shown to be induced by GNRH (Leduc et al., 1987), with a potential colocalization in Lh and Fsh cells (Deng et al., 1986; Leduc et al., 1987), suggesting a reproductive role for this protein. Furthermore, the C-terminal region of the protein has been previously shown to be able to act as a neuropeptide (Senatorov et al., 1993). Our results suggest that Scg5 may be involved in compensating for Gnrh3 loss and could have potential roles in fish reproduction, potentially through controlling the processing of neurohormones or through the neuropeptide actions of the C-terminal peptide promoting reproduction, however,

further studies will need to identify if this is the case and what these roles are. The roles of the RFamide peptide, Gnih, in reproductive regulation have been studied in many species, showing that it can have inhibitory and stimulatory effects on gonadotropin release of fish (Moussavi et al., 2012, Spicer et al., 2017), and its upregulation in *gnrh3*$^{-/-}$ may suggest that it has differential roles in the gonadotropin regulation in zebrafish as well. Interestingly, the upregulation of the peptide, Vip in *gnrh3*$^{-/-}$ brains was unexpected, since its roles in fish reproduction have not well been studied. However, previous studies show that Vip is an important suprachiasmatic nucleus (SCN) neuropeptide in the brains of vertebrates, and may be involved in mammalian sexual potency (Gozes and Brenneman, 1989), prolactin release in hens (Mauro et al., 1989), Gnrh neuronal innervation in rats (Kriegsfeld et al., 2002), and is hypophysiotropic and known to modulate prolactin in the teleost, gilthead sea bream (*Sparus aurata*) (Brinca et al., 2003). The roles of Vip in regulating gonadotropin release in zebrafish will need to be further studied to determine if it can compensate for Gnrh3 loss, but Vip most likely has important roles in teleost reproduction which should be studied in the future. Additionally, according to the RNA-Seq analysis, the dopamine synthesizing enzyme, Tyrosine hydroxylase (Th), was downregulated in *gnrh3*$^{-/-}$ brains, whereas a dopamine degrading enzyme, Monamine oxidase (Mao), was upregulated, suggesting that the dopamine system was downregulated overall. Dopamine has been shown to potently inhibit reproduction in teleosts through direct synapses on gonadotropes and inhibiting gonadotropin release (Dufour et al., 2010; Fontaine et al., 2013). Additionally, in order to stimulate spawning in some fish species in aquaculture settings, a dopamine antagonist must be introduced in order to allow for reproduction (Zohar, 1989; Aizen et al., 2005).

Therefore, the downregulation of dopamine in *gnrh3*$^{-/-}$ brains may be a compensating mechanism to decrease reproductive inhibition in order to allow for normal reproduction to be maintained. In *gnrh3*$^{-/-}$ brains, the *gnrh2* gene was surprisingly not upregulated, despite being the most obvious potential compensator for Gnrh3 due to having a similar decapeptide sequence, and the ability to bind to Gnrh receptors at the same potency as Gnrh3 (Tello et al., 2008). The presence of Gnrh2 alone may be able to compensate for Gnrh3, so in order to determine if this is the case, the Gnrh2 knockout line, *gnrh2*$^{-/-}$, was crossed with the *gnrh3*$^{-/-}$ knockout line to determine if reproduction is compromised with the loss of all Gnrh isoforms.

The *gnrh3*$^{-/-}$ line used to generate the DKO line was described in Spicer et al. 2016., where IHC immunolabelling of Gnrh3 in *gnrh3*$^{-/-}$ brains showed no evidence of the presence of GAP3 as well as the decapeptide, validating the complete loss of Gnrh3 (Spicer et al., 2016). In the *gnrh2*$^{-/-}$ line that was generated, a 21 base-pair deletion, 2 base-pair insertion resulted in a frameshift mutation that also disrupted the coding of the protein (Marvel et al., 2018). Immunostaining revealed no presence of the Gnrh2 decapeptide or GAP in DKO brains, verifying that the *gnrh2* mutation resulted in a complete loss of Gnrh2. The presence of the mutated *gnrh2* cDNA in the *gnrh2*$^{-/-}$ line was verified by sequencing and found to contain no alternative splicing or additional endogenous mutations. Surprisingly, unlike in mammals where mutations in the hypophysiotropic *Gnrh1* gene results in infertility (Mason et al., 1986; Schwanzel-Fukuda et al., 1989; Bouligand et al., 2009), the complete loss of Gnrh2 and Gnrh3 resulted in no major differences in reproduction in zebrafish. The DKO fish were still able to successfully spawn, and all reproductive parameters, e.g. gametogenic

development, GSI, fertility and fecundity were unaffected in DKO fish and similar to those in wild-type fish. Additionally, when examining gametogenesis and gonadal morphology, the development and maturation of gametes was not impeded, and fully mature oocytes in DKO females and mature spermatozoa in DKO males was present at the same developmental stages as WT. These results demonstrate that the complete loss of both Gnrh ligands did not cause any effects on reproductive development and performance. The *gnrh3*$^{-/-}$ knockout line, generated by our lab, and the *gnrh3*$^{-/-}$*;kisspeptin1*$^{-/-}$*;kisspeptin2*$^{-/-}$ fish, described in Liu et al., 2017, also displayed similar phenotypes where the loss of Gnrh3, and the combined loss of Gnrh3 and the Kisspeptin ligands, resulted in no differences in fecundity and fertility (Spicer et al., 2016; Liu et al., 2017). Similar to the *gnrh3*$^{-/-}$ lines in zebrafish generated from different lab groups (Spicer et al., 2016, Liu et al., 2017), *gnrh1*$^{-/-}$ medaka (*Oryzias latipes*) also did not exhibit any differences in gametogenesis (Takahashi et al., 2016). However, unlike *gnrh3*$^{-/-}$ and *gnrh2*$^{-/-}$*; gnrh3*$^{-/-}$ zebrafish, *gnrh1*$^{-/-}$ medaka exhibited compromised ovulation. Disparate from zebrafish, medaka have three isoforms of Gnrh, with Gnrh1 expressed only in the hypothalamus, and Gnrh3 expressed only in the forebrain terminal nerve. In the medaka, knockout of *gnrh1* eliminates only the hypothalamic isoform, leaving in place the Gnrh3. Considering the common origination of Gnrh1 and Gnrh3 neurons from the olfactory placode, it may well be that the presence of Gnrh3 in the developing medaka prevents the activation of the compensation. Another possibility is that the compensatory mechanism to the loss of the Gnrh isoforms evolved with the evolution of the loss of the Gnrh1 isoform in these fish.

These results clearly demonstrate that neither Gnrh2 nor Kiss1 or Kiss2 compensate for the loss of Gnrh3 in zebrafish. Consequently, we set out to identify other factors along the HP axis that may fulfill this role based on differential gene expression and neuroanatomical changes in the brain during early development and adulthood. The expression patterns of the gonadotropin genes were analyzed and compared between DKO and WT whole fish between the ages of 1 and 30 dpf, as the gonadotropin gene expression levels increase during this period, coinciding with the beginning of sexual differentiation and early gonadal development (Clelland and Peng, 2009). The expression patterns of the specific gonadotropin beta subunits, *lhb* and *fshb*, did not differ between wild-type and DKO genotypes, whereas *cga* expression was upregulated in DKO fish at 8 dpf, 18 dpf, and 30 dpf. These expression patterns are very similar to what is reported in the single knockout, *gnrh3*$^{-/-}$ in the first 30 days of development, where *lhb* and *fshb* expression were unchanged, but *cga* was upregulated at 3, 8, 12, and 24 dpf (Spicer et al., 2016). This is different from the single knockout *gnrh2*$^{-/-}$ zebrafish line, which demonstrate no changes in *cga* but an upregulation of *fshb* and downregulation of *lhb*, suggesting the DKO expression patterns are more similar to *gnrh3*$^{-/-}$ than *gnrh2*$^{-/-}$ fish. Additionally, in both *gnrh3*$^{-/-}$ and *gnrh2*$^{-/-}$*;gnrh3*$^{-/-}$ lines, the expression levels of the gonadotropin genes in adult pituitaries were similar between wild-type and knockout lines. The fact that the additional loss of Gnrh2 did not affect the expression patterns of the gonadotropin genes in our DKO line further supports our conclusion that Gnrh2 is not the compensating factor for the loss of Gnrh3. Moreover, the similarity in the temporal expression of *lhb* and *fshb* at all developmental stages suggests that the compensation occurs upstream of the gonadotropins to maintain their normal levels. The higher levels

of *cga* mRNA during early development were unexpected because the Lh and Fsh are the rate-limiting subunits (Choi and Smitz, 2014). Nevertheless, it may be the result of an indirect effect induced by other compensatory events occurring during the establishment of the nascent reproductive system, granting *cga* a possible role in early developmental processes. Another possibility is the upregulation of thyroid stimulating hormone (Tsh) that shares the Cga subunit with Lh and Fsh and is reported to have a regulatory role in reproduction (Ikegami and Yoshimura, 2017). In support, some studies have reported the detection of the free alpha subunit in the plasma, which has several non-reproductive related functions (Moy et al., 1996; Blithe et al., 1991).

When comparing the expression pattern of additional reproductive neuropeptides between DKO and WT, we detected a number of genes that displayed upregulation in DKO zebrafish, albeit some of the genes exhibited a sex specific difference. The expression of *kiss1*, *kiss2*, *spexin*, and *avp* showed no differences in DKO brains, suggesting they do not participate in the compensation to the loss of Gnrh. The expression of *scg2a* and *mao* were upregulated in female DKO brain, whereas *tac3a* and *vip* were upregulated in male DKO brains, and the expression of *gnih* and *pacap1* was upregulated in both sexes. Currently, it is not clear why some genes display a sex specific response. A possible explanation is a sex specific regulation of these genes by Gnrh2 or Gnrh3 that drives the different reproductive cycles and strategies of the two sexes. Interestingly, the upregulation of *tac3a* and *scg2a* is also seen in the triple knockout (*gnrh3*$^{-/-}$*;kisspeptin1*$^{-/-}$*;kisspeptin2*$^{-/-}$) line from Liu et al., suggesting that the loss of Gnrh3 triggers the upregulation of these specific genes (Tang et al., 2015). Most of the upregulated neuropeptide genes in the DKO line have been previously shown to have

reproductive roles. *Tac3a* encodes for Neurokinin b (NKB) which induces the release of Lh in zebrafish and Fsh and Lh in tilapia (*Oreochromis niloticus*), and the receptors *tac3ra* and *tac3rb* are expressed in tilapia gonadotropin cells (Spicer et al., 2017; Zhou et al., 2012; Biran et al., 2012; Biran et al., 2014). Scg2a can stimulate the release of Lh in mice and goldfish, and therefore may be involved in inducing gonadotropin release in zebrafish as well (Zhao et al., 2006; Zhao et al., 2011). Vip has been shown to induce Lh release from cultured teleost pituitaries (Matsuda and Maruyama, 2007). Pacap1 has also been shown to be hypophysiotropic and may have gonadotropin-releasing functions in goldfish, and Gnih can stimulate gonadotropin synthesis in goldfish (Chang et al., 2001; Moussavi et al., 2013), implying that these neuropeptides may have hypophysiotropic roles in zebrafish as well. Altogether, the upregulation of these genes strongly suggests that the compensation involves multiple factors that collectively stimulate gonadotropin synthesis and release in response to the loss of both Gnrh isoforms. It is possible these compensating peptides are either activating their own receptors or Gnrh receptors on gonadotropes to stimulate gonadotropin release. However, there is no evidence in the literature of any peptides outside of the Gnrh family activating Gnrh receptors. The receptors for Nkb (Tac3ra and Tac3rb) and for Gnih (Lpxrf-r) are colocalized with tilapia gonadotropes (Biran et al., 2014), PACAP receptors are found on mice gonadotropes (Counis et al., 2007), and VIP receptors are found in avian pituitaries (Rozenboim et al., 1993), but VIP receptor localization on gonadotropes and knowledge on the Secretoneurin receptors are not well characterized. It is still unknown whether these peptides are able to activate any of the four zebrafish Gnrh receptors. The ability of these peptides to bind and activate these receptors will need to be studied in the future to

elucidate the mechanisms of this compensatory response. The localization of the four individual Gnrh receptors in the pituitary have not been characterized, but distribution expression studies show that all four Gnrh receptors are found in the brain and that both Gnrh isoforms activate the IP pathway, albeit with different potencies (Tello et al., 2008). The upregulation of these genes in the DKO line also suggests that they are attenuated by Gnrh3 and/or Gnrh2, however, it is not clear whether this attenuation is a direct or an indirect effect via other factors that directly respond to the loss of Gnrh3 or Gnrh2. Some of the intermediate factors may be involved in the inhibition of the reproductive axis, such as dopamine (Fountaine et al., 2013).

A compensatory response to a targeted gene knockout is not unique and has been reported in other systems as well. For instance, in the case of the genetic knockout of EGF Like Domain Multiple 7 (*egfl7*$^{-/-}$), a gene involved in vascular formation, no phenotypes were notable in the knockout line. Consequently, multiple extracellular matrix genes were identified as compensators as they displayed an upregulation in *egf7*$^{-/-}$ fish (Roussi et al., 2015). Interestingly, targeted knockouts of genes upstream of Gnrh3, such as Kisspeptins, resulted in no phenotypes in zebrafish (Tang et al., 2015). However, knockouts of genes acting downstream of Gnrh3, such as the gonadotropin genes (*lhb*$^{-/-}$ and *fshb*$^{-/-}$), resulted in moderate reproductive abnormalities (Zhang et al., 2015), and the gonadotropin receptor knockouts (*fshr*$^{-/-}$ and *lhr*$^{-/-}$;*fshr*$^{-/-}$) resulted in severe reproductive effects or infertility (Chu et al., 2015). This suggests that redundant regulatory pathways exist upstream in the reproductive HPG axis that can maintain reproduction when one or more of the genes are lost, whereas downstream factors are more specialized with less redundancies and are not replaceable by other factors.

Our neuroanatomical studies demonstrated that the difference in *scg2a* mRNA levels in female DKO fish is not simply a result of a change in magnitude but is also attributable to a more widespread expression and distribution of its neurons in areas of the brain that are not found in wild-type fish. *Scg2a* neuronal expression in DKO brains is more prevalent in the griseum centrale region, and *scg2a* expression is detected in the lateral hypothalamus and hindbrain regions only in DKO brains, but not wild-type brains. The distribution of immunoreactive neurons of the peptide derived from the Scg2a precursor protein, Secretoneurin (SN) (Canosa et al., 2011), displayed similar locations throughout the telencephalon and hypothalamus in goldfish, but not in the hindbrain as seen in the DKO fish brains. Scg2a, a member of the chromogranin family, was reported to have potential reproductive roles. Scg2a is located in reproductive areas of the brain and pituitary, and can stimulate gonadotropin release in cultured pituitary cells and in response to intraperitoneal injections in goldfish, and has an autocrine role in Lh secretion in mice gonadotrophs (Zhao et al., 2006; Zhao et al., 2011; Canosa et al., 2011; Trudeau et al., 2012; Zhao et al., 2010). Additionally, Scg2a expressing neurons directly innervate and regulate the brain aromatase gene (*cyp19a*) in radial glial cells (RGCs) in the preoptic region of the goldfish brain, in the vicinity of Gnrh3 soma (Da Fonte et al., 2018). RGCs are pluripotent neuronal progenitors and Scg2a induces their differentiation and in turn, neurogenesis and neurosteroidogenesis (Xing et al., 2014). Thus, the observation that Scg2a neurons appear in multiple brain regions suggests that reproduction-specific neurogenesis and neurosteroidogenesis may be activated as part of the overall response to the loss of Gnrh. Recently, EM66, another peptide derived from the Scg2a precursor, was shown to display similar roles to Gnrh2 as an anorexigenic

peptide. EM66 is abundant in the hypothalamus and acts to regulate feeding via Pomc and the Melanocortin-3 receptor, but not NPY (Trebak et al., 2017), suggesting the upregulation of *scg2a* in the DKO line may also be compensating for the loss of Gnrh2.

Similar to the neuronal redistributation of Scg2a neurons, Vip neurons are found in additional and novel areas of the DKO brain. We chose to focus on examining the neuronal distribution of Vip in DKO brains compared to WT, considering this peptide is upregulated in both *gnrh3*$^{-/-}$ brains, demonstrated via the RNA-Seq analysis, and in male DKO brains, demonstrated by targeted qPCR, suggesting it may be compensating for the loss of Gnrh3. Additionally, Vip has been previously shown to be able to induce Lh secretion in teleost pituitary tissues, *in vitro,* suggesting it may have reproductive roles (Matsuda and Maruyama, 2007). Unlike the case in Scg2a, the redistribution of Vip is found solely in the preoptic area, but other areas of the brain show similar distributions between DKO and WT fish. Vip is normally found in the posterior region of the preoptic area and the hypothalamus of wild-type zebrafish. However, in DKO brains, we see additional neurons expressed *vip* in the anterior (PPa) and medial region of the posterior brain (PPm), whereas the posterior preoptic area (PPp) signal is similar to WT brains. The preoptic area of the brain is an important location for reproductive regulation, as it is so close to the hypothalamus and pituitary and is the location where Gnrh3 neurons are normally located. Whether Vip is expanding to other regions of the preoptic area to take over the role of the lost Gnrh3, will need to be further explored, but its plasticity and location in this area, where Gnrh3 neurons are also found, is promising.

Unlike Scg2a and Vip neurons, Gnih soma distribution did not change between the wild-type and DKO brains and remained confined in the Nppv. However, in

agreement with the upregulation of *gnih* expression in DKO brains seen in our QPCR results, the number of *gnih* expressing soma increased 1.5-fold in DKO brains compared to wild-type. Gnih, also known as Lpxrfa, is an RFamide peptide, which inhibits gonadotropin release in mammals and birds, but can be either stimulatory or inhibitory to gonadotropin production in teleost species (Zhou et al., 2012; Biran et al., 2014; Paullada-Salmerón et al., 2016). Hence, this upregulation may be a part of the overall response to the lack of both Gnrh isoforms in order to regulate gonadotropin synthesis and release.

In light of the suggested anorexigenic role of Gnrh2 in regulating vertebrate feeding behavior, we also examined the expression of feeding related genes in the DKO line. In goldfish, Gnrh2 was shown to be a mediator of α-Msh and Corticotropin-releasing hormone (Crh) (Hoskins et al., 2008), and have a mutual inhibitory relationship with Orexin (Kang et al., 2011). In the DKO line, the downregulation of *agrp1*, an orexigenic factor, and upregulation of *pomca*, an anorexigenic peptide, demonstrate that the melanocortin system was affected, directly or indirectly, by the loss of the Gnrh peptides. Agrp1 neurons inhibit Pomc neurons and Agrp1 antagonizes the Melanocortin-receptor 4 (Mc4r) (Ollmann et al., 1997), hence the *pomca* upregulation may be a response to the downregulation and decreased inhibition by Agrp1, that together may take over the feeding-related roles of Gnrh2. The number of *agrp1* neurons in the hypothalamus of DKO was almost 2-fold lower compared to WT brains. In addition to its functions in feeding, AGRP1 has been reported to be a negative regulator of reproduction in mice through inhibiting KISS1 neurons and increasing infertility (Wu et al., 2012; Padilla et al., 2017). Therefore, the decreased *agrp1* mRNA levels in the DKO brain may

be a part of the combined compensatory response to the lack of both Gnrh3 and Gnrh2. Pmch2, another orexigenic peptide, was upregulated in DKO males, suggesting that the Gnrh system may have interactions with multiple feeding-related factors in the zebrafish brain. Interestingly, GNRH1 neurons were recently found to express MC4R in mice, underscoring the tight relationships between reproduction and feeding/energy balance (Israel et al., 2012).

Despite the reported upregulation of *npy* in the Gnrh3/Kisspeptin knockout line (Liu et al., 2017), our study did not detect any differences in *npy* expression in the DKO line. Npy is an orexigenic neuropeptide co-expressed with Agrp1 in hypothalamic neurons and implicated in regulating both reproduction and feeding pathways. NPY can interact directly with Y receptors on hypophysiotropic GNRH1 neurons to inhibit its activity in mammalian models (Xu et al., 2009; Klenke et al., 2010). The differences in the expression of *npy* in the Gnrh3/Kisspeptin knockout line may be attributable to the loss of the Kisspeptins rather than of Gnrh. This suggestion is supported by the fact that Kisspeptin has a direct inhibiting effect on *npy* expression (Fu and Van den Pol, 2010). The relationships between Gnrh2/Gnrh3 and feeding behavior/energy expenditure in the zebrafish warrants further investigation in order to better understand the link between feeding and reproduction.

Overall, our findings demonstrate that the genetic loss of both Gnrh isoforms does not compromise reproduction in zebrafish, unlike the situation in mammals. Therefore, it is possible that the inherited loss of the Gnrh isoforms in the DKO line activates a compensatory response unique to non-mammalian vertebrates. Our results clearly demonstrate that Gnrh2 is not compensating for the loss of Gnrh3 in the single KO line,

as the additional loss of Gnrh2 did not reveal any major changes in reproductive functions. The compensation, instead, appears to involve the upregulation of several different reproductive and feeding neuropeptides in the single KO and DKO line, along with the downregulation of certain feeding factors that may inhibit reproduction. These players act throughout development and adulthood to ensure normal reproduction in the absence of Gnrh2 and Gnrh3. This finding elucidates important features of compensatory mechanisms, as the loss of the two Gnrh isoforms appears to trigger a non-cell autonomous response, with changes to neuropeptides outside of the Gnrh cells. This suggests a cross-talk occurs between Gnrh and the compensatory peptides under normal circumstances. The loss of Gnrh2 and Gnrh3 also triggers plasticity of certain neuropeptide neurons that emerge in additional brain areas. Most of the upregulated reproductive peptides in this study have been shown to be hypophysiotropic in zebrafish or other animals. Whether the compensation involves one or more of these peptides to directly stimulate gonadotropin release is unknown and will be investigated further in the future. The DKO zebrafish line is a great system to discover circuits outside of the Gnrh system that are involved in reproduction which were previously unknown, and we have already begun to identify these factors in this study. This study likely revealed only part of the many changes comprising the compensatory response to the loss of Gnrh2 and Gnrh3 in zebrafish and may be only the tip of the iceberg. Further studies are underway to reveal the full extent of these changes and decipher the underlying mechanisms involved in maintaining reproduction in the absence of Gnrh2 and Gnrh3.

# CHAPTER 7: Conclusions and Future Directions

## Conclusions

The midbrain population of Gonadotropin-releasing hormone, GNRH2, has been greatly overlooked in favor of research focusing on the major hypophysiotropic isoforms in vertebrates, GNRH1, or the teleost-specific Gnrh3. One of the reasons for the lack of research on GNRH2 may be due to the partial gene deletion, and therefore absence, of GNRH2 in murine models, resulting in a loss of a great laboratory model organism for studying this peptide. The functional roles of GNRH2 are therefore still very much unknown, despite the conservation of GNRH2 decapeptide amino-acid sequence amongst all vertebrates that express it. Its conservation and relatively high abundance among species signifies it has very important conserved biological and evolutionary roles. The emergence of zebrafish as a useful and easy laboratory model for genetic and biological studies has provided a new model organism to study Gnrh2 and finally comprehensively elucidate the roles of this peptide and the molecular mechanisms involved in feeding and reproductive pathways involving Gnrh2. In my studies, I was able to use the zebrafish model to generate a Gnrh2 knockout zebrafish line (*gnrh2*$^{-/-}$) and double knockout zebrafish line (*gnrh2*$^{-/-}$*;gnrh3*$^{-/-}$) to study the loss-of-function phenotypes involved in feeding, growth, mobility, and reproduction to elucidate the functions of Gnrh2 in these processes. I was also able to use transgenic tg(Gnrh2:eGFP) zebrafish, along with immunohistochemistry and *in situ* hybridization techniques, to visualize the neuronal projections of Gnrh2, understand the plasticity of the system in different conditions, and begin identifying interactions of Gnrh2 with other feeding and reproductive neurons in the brain. Combining knockout and transgenic techniques with high resolution confocal microscopy imaging and functional assays, I was able to determine numerous new

findings on the roles of Gnrh2 in reproduction, feeding, and olfactory transduction, as well as identify novel players in the neurocircuits regulating reproduction after the loss of Gnrh2 and Gnrh3. These findings begin to scratch the surface at determining the functions and networks in play in which Gnrh2 is a part of, paving new potentials paths of research to study Gnrh2 in future studies.

My studies first delved into studying the role Gnrh2 plays in regulating reproduction and feeding through the use of the Gnrh2 knockout line. By determining phenotypic differences in reproductive parameters, the reproductive processes in which Gnrh2 may be involved under normal conditions were revealed. Overall, reproduction was not compromised in the *gnrh2*$^{-/-}$ fish, and the loss of Gnrh2 was not associated with any prominent differences in gametogenesis or spawning, suggesting that Gnrh2 is not a major regulator of reproduction in zebrafish. This is not surprising, considering the more abundant hypophysiotropic Gnrh, Gnrh3, is still present in this line, and most likely able to stimulate gonadotropin synthesis on its own. Surprisingly, recent studies in multiple labs showed that Gnrh3 knockout zebrafish (*gnrh3*$^{-/-}$), also do not exhibit any major differences in reproduction (Spicer et al., 2016; Liu et al., 2017), implying that Gnrh2 and Gnrh3 are not necessary for reproduction in zebrafish. This is an unexpected finding considering that targeted ablation of Gnrh3 neurons in zebrafish resulted in compromised oogenesis and infertile female zebrafish (Abraham et al., 2010). Neurons occasionally express multiple factors, neuropeptides, neurotransmitters and growth factors, some of which may reach the pituitary, or indirectly affect other neurons to regulate gonadotropin release. With the cutting-edge molecular tools available at our disposal, future studies will be able to isolate Gnrh3 neurons from the brain and determine, through

transcriptomics or other tools, all of the genes and peptides expressed in conjunction with Gnrh3. These results will determine, for the first time, factors which are co-expressed with Gnrh3, and potentially novel factors involved in reproduction. In my studies, I characterized the *gnrh2*$^{-/-}$ line; however, targeted conditional chemical ablation of Gnrh2 neurons has not been conducted, and would be an interesting experiment to conduct in the future to determine if, similar to Gnrh3, the genetic and neuronal loss results in different phenotypes. Nevertheless, the *gnrh2*$^{-/-}$ and *gnrh3*$^{-/-}$ lines were crossed together to determine if Gnrh2 is compensating for Gnrh3, and if different reproductive phenotypes occurred with the combined genetic loss of all Gnrh isoforms. Similar to the single knockout lines, the double knockout (DKO) *gnrh2*$^{-/-}$*;gnrh3*$^{-/-}$ lines exhibited no major reproductive defects, with similar gonadal morphology, gametogenesis, and spawning outputs as wild-type fish. This indicates that Gnrh2 is not compensating for Gnrh3, and that either the Gnrh system is not necessary for reproduction in zebrafish, or the genetic loss of the two Gnrh isoforms results in the activation of a compensatory backup mechanism to take over the roles of Gnrh in gonadotropin stimulation. The upregulation of compensatory genes has been seen in other knockout lines, such as in adipocyte fatty acid binding protein (AFABP) knockout mice, which exhibit upregulation of keratinocyte fatty acid binding protein (KFABP) (Shaughnessy et al., 2000), *egfl7*$^{-/-}$ zebrafish which exhibit upregulation of extracellular matrix genes and proteins, and *vegfaa*$^{-/-}$ zebrafish which exhibit upregulation of *vegfab* (Rossi et al., 2015). Similar to a triple Gnrh3/Kiss1/Kiss2 mutant (TKO), *gnrh2*$^{-/-}$*;gnrh3*$^{-/-}$ zebrafish exhibited upregulation of several genes, including *tac3a* and *scg2a*, but also upregulation of other genes not seen in the TKO, such as *gnih*, *pacap1*, *vip*, and *mao*. Genes encoding for Vasoactive intestinal

peptide (*vip*), Secretogranins (*scg2a*), and dopamine synthesizing Tyrosine hydroxylase (*th*) or dopamine degrading Monamine oxidase (*mao*) enzymes were selected based on transcriptomic comparisons where I saw upregulations of these genes in the single knockout *gnrh3*$^{-/-}$. Additionally, genes such as *scg2a* and *vip* showed a reorganization in additional parts of the brain, whereas genes such as *agrp1* and *gnih*, showed an increase in cell number expressing them associated with the loss of *gnrh2* and *gnrh3*, demonstrating the plasticity of the brain associated with changes in gene expression patterns and neuronal reorganization which occurs after genetic mutations. Altogether, these results suggest that redundant reproductive regulators exist and have the potential to compensate for Gnrh loss in zebrafish brains, by inducing upregulation of peptides such as Scg2a and Vip and downregulation of inhibitory peptides such as dopamine and Agrp1. This compensation makes it hard to determine if Gnrh2 and Gnrh3 are major regulators of reproduction under normal conditions in zebrafish, but previous studies in numerous fish species, including zebrafish, and other vertebrates, all indicate the functional importance of the Gnrh system in reproduction. It is shown in my studies that Gnrh2 is not a major regulator of reproduction, but the results of this study do point to potentially novel and exciting new factors which may have more reproductive relevance than was previously known and should be further studied in zebrafish.

Although there were no major reproductive inhibitions with the loss of Gnrh3, there were a few minor reproductive differences with the loss of Gnrh2, which gives some clues as to the roles Gnrh2 plays in reproductive regulation. The two most notable differences included decreased transcript levels of *lhb* in developing fish and adult males, along with decreased oocyte quality, indicated by decreased oocyte sizes, GSI, and

embryo survivability in females. The role of Gnrh2 in *lhb* regulation was directly tested through *in vivo* injections and *in vitro* incubations of the Gnrh2 peptide to zebrafish pituitaries, which resulted in increased *lhb* levels, but not *fshb* levels, in wild-type and *gnrh2*$^{-/-}$ fish, also showing that Gnrh2 rescue can salvage the low levels in knockout fish. The low *lhb* levels and decreased oocyte quality in *gnrh2*$^{-/-}$ fish, ability of Gnrh2 to increase *lhb* expression, and the presence of Gnrh2 neurons in the pituitary of zebrafish (Xia et al., 2014) all point to a role of Gnrh2 in regulating *lhb* expression, probably assisting to maintain optimal oocyte maturation in zebrafish. The loss of Gnrh2 also resulted in differential expression of several neuropeptide genes, including *gnih*, *tac3a*, and *scg2a*, which also show upregulation in the DKO line as well, suggesting that these neuropeptides may be compensating for the loss of Gnrh2 and participate in the network maintaining gonadotropin secretion along with Gnrh3, however are not capable to completely rescue the low levels of *lhb*. The relationship of Gnrh2 with Gnih was explored further, and Gnrh2 was shown to be able to indirectly modulate Gnih, as mutual innervations between Gnih and Gnrh2 neurons were detected. Additionally, Gnrh2 administrations elicited dose-dependent and administration-dependent differences in *gnih* gene expression. Additionally, relationships of Gnrh2 with melatonin were explored to determine another indirect pathway Gnrh2 may take to maintain oogenesis. Gnrh2 innervations clearly penetrated the pineal gland and innervate melatonin cells, strongly indicating it may have a role in melatonin regulation. As melatonin has been shown to potently induce follicle maturation, fecundity, and GSI in zebrafish (Carnevali et al., 2011), and in Gnrh2 knockout fish we see small differences in follicle maturation, the reason for the inhibited oocyte growth could be due to reduced melatonin secretion. In

order to determine if Gnrh2 could directly regulate melatonin synthesis, as is the case in European sea bass (Servili et al., 2010), the effect of Gnrh2 on melatonin secretion from pineal glands was determined. Gnrh2 was able to augment melatonin secretion during the daytime, when melatonin levels are low. Since Gnrh2 levels peak in zebrafish brains just prior to the time of night when melatonin peaks, it may be one of the factors triggering melatonin expression (Paredes et al., 2019). Additionally, nighttime mobility was higher in *gnrh2*$^{-/-}$ larvae, an activity usually resulting from lower melatonin levels, supporting the idea that the loss of Gnrh2 may result in lower melatonin production. Therefore, melatonin regulation may be one indirect pathway that Gnrh2 takes to enact its role in promoting oocyte growth and optimal quality.

In the framework of the current study, a previously unknown, novel population of Gnrh2 neurons was identified in the olfactory bulbs of both males and females, some of which are colocalized with Gnrh3 neurons. These neurons display a sexual dimorphism as in males, the colocalization with Gnrh3 primarily takes place in more of a medial and ventral olfactory tract, whereas in females, the most rostral portion of the olfactory bulb appeared to have more colocalized cells. As there have been very few studies suggesting a localization of Gnrh2 in the olfactory bulbs, the presence of Gnrh2 neurons in this region was verified using transgenic zebrafish imagery tools, immunohistochemistry, and *in situ* hybridization to determine the presence of both mRNA and Gnrh2 protein in olfactory regions, thus providing compelling data of the location of Gnrh2 in the olfactory region. Social studies have shown that changes in both *gnrh2* and *gnrh3* expression correspond with changes in social conditions, but not feeding differences, indicating that Gnrh2 in the olfactory region may transduce pheromone cues along with

Gnrh3. Consequently, an additional pheromone transducing role for Gnrh2 in reproduction is suggested for the olfactory bulb and olfactory epithelium population. Similarly, the medial olfactory tract has been shown in goldfish (*Carassius auratus*) to be involved in transducing pheromonal cues (Hasegawa et al., 1994; Kim et al., 1995), supporting that male Gnrh2/Gnrh3 colocalization in this location may be involved in pheromonal cues perception in this sex.

The roles of Gnrh2 in regulating feeding were also explored by examining Gnrh2 loss-of-function effects on food intake and growth. Significantly increased long-term food intake was seen in *gnrh2*$^{-/-}$ larvae, compared to wild-type, which continued and in turn corresponded with increases in somatic, but not gonadal, growth in adult females. These results strongly suggest that Gnrh2 is a potent anorexigenic hormone, supporting previous studies which show that Gnrh2 administrations lead to decreased feeding in zebrafish (Nishiguchi et al., 2012), goldfish (Hoskins et al., 2008; Matsuda et al., 2008), musk shrews (*Suncus murinus*) (Kauffman and Rissman, 2004a), and mice (Kauffman and Rissman, 2004b). The loss of Gnrh2 was associated with changes in several feeding neuropeptide expression levels, including *nesfatin1*, *hypocretin*, *pomca*, and *spexin* as well as several growth-related genes, such as *igf1a* and *pacap1* in females. The differential expression indicates these genes may be regulated by Gnrh2 or be changing indirectly as a result of the increased feeding behaviors. The fact that *gnrh2* expression in zebrafish peaks during the evening and overnight hours (Paredes et al., 2019), suggests that Gnrh2 may work to suppress feeding during the nighttime. Additionally, *gnrh2* expression increases in overfed conditions, suggesting it is a satiety signal in fish, and decreases feeding through the downregulation of *agrp1* (Nishiguchi et al., 2012). Agrp1

has been suggested to be the most potent orexigen, strongly increasing under any fasting condition, and decreasing in overfed conditions (Song et al., 2003; Jeong et al., 2018). This study demonstrates that Gnrh2 neurons innervate Agrp1 cells, and Gnrh2 administrations significantly downregulate *agrp1*, suggesting a pathway in which Gnrh2 takes to downregulate feeding behaviors. Another pathway may be through the regulation of melatonin production, since melatonin treatments have been shown to reduce food intake and significantly change the expression of many feeding genes in zebrafish (Piccinetti et al., 2010). The current study suggest that this pathway is also feasible as Gnrh2 neurons innervate pineal gland melatonin cells and can stimulate melatonin secretion, depending on the time of the day. Interestingly, the levels of Gnrh2 transcript display a circadian nighttime peak, further supporting the notion that Gnrh2 indeed acts through melatonin to curb feeding at night.

Furthermore, the role of Gnrh2 as a mediator between reproduction and feeding conditions was studied. By examining reproductive parameters in *gnrh2*$^{-/-}$ fish, it was determined that the most striking difference was exhibited by inhibited spawning and oocyte maturation under long-term fasting conditions. This led to a hypothesis that Gnrh2 may be involved in maintaining reproduction in long-term fasting conditions which normally inhibit Gnrh3. This hypothesis was supported by previous studies, where Gnrh2, but not Gnrh1, induced reproduction in fasted musk shrews and mice (Kauffman and Rissman 2004, Kauffman et al., 2005). In my studies, the role of Gnrh2 was studied at the pituitary and gonadal level, and the loss of Gnrh2 impaired male and female spawning, oocyte final maturation, male reproductive behaviors, and decreased gonadotropin expression and secretion after fasting. The importance of Gnrh2 in pituitary

gonadotropin secretion was examined through neuroanatomical studies of transgenic zebrafish pituitaries, which clearly showed decreased Gnrh3 and increased Gnrh2 projections to the pituitary and gonadotrope cells. This noticeable dynamic pattern in Gnrh projections strongly support the hypothesis that Gnrh2 is critical for gonadotropin regulation in fasted conditions. Interestingly, a similar case is seen in mammalian models, since musk shrews exhibited increased Gnrh2 soma abundance and fiber density in the median eminence, the equivalent of the increased Gnrh2 projections to the pituitary in teleosts (Temple et al., 2003). This suggests that the role of Gnrh2 in reproduction in fasting animals is conserved between different vertebrate groups.

Overall, combined Gnrh2 knockout, imagery techniques, and functional assays determined that Gnrh2 most likely has minor roles in *lhb* expression, oocyte quality regulation, and pheromone transduction under normal feeding conditions. These functions become more prominent under more drastic, long-term fasting conditions that inhibit Gnrh3 and simultaneously induce proliferation and extensions of Gnrh2 neurons to project more densely to gonadotropes. Additionally, it was determined that Gnrh2 is most likely a potent anorexigenic hormone, which may enact its roles overnight through both direct downregulation of Agrp1, and indirect stimulation of melatonin, which subsequently also downregulates feeding. Gnrh2 may be a mediator of feeding and reproduction, ensuring the correct balance of energy allocation towards gonadal growth and gametogenesis through downregulating feeding, under certain conditions, and promoting reproductive maintenance.

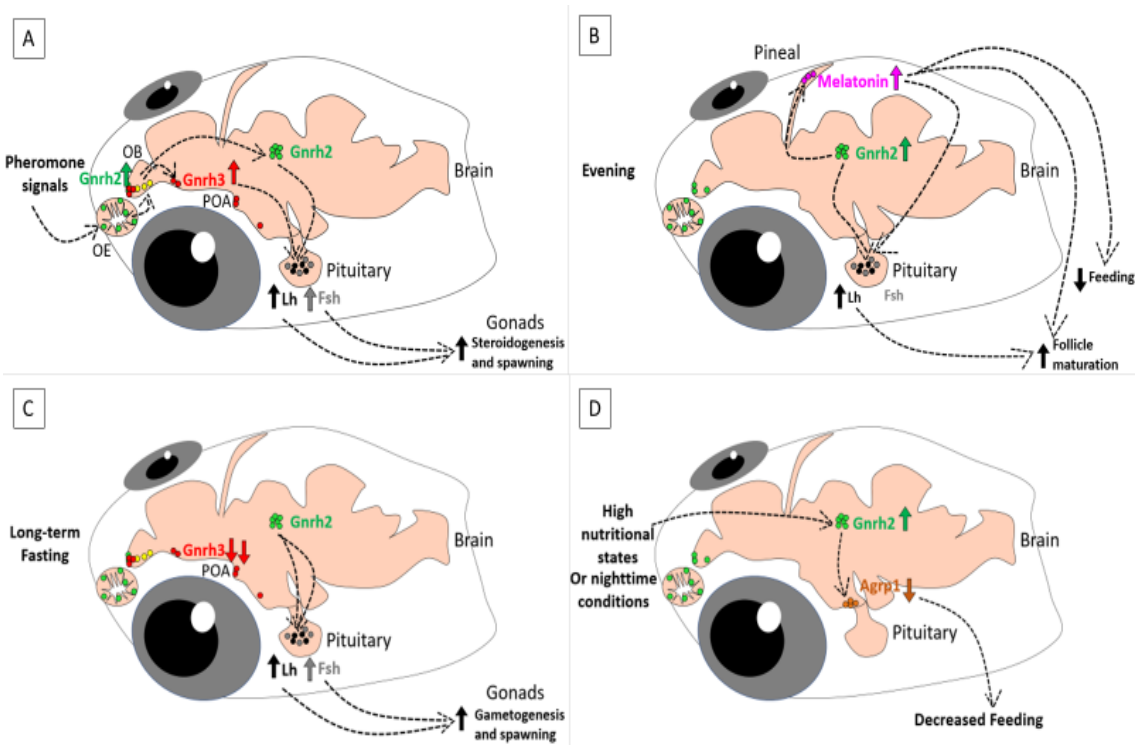


**Figure 7.1.** Summarized proposed roles of Gnrh2 in zebrafish reproduction and feeding, including transducing pheromonal cues from the olfactory bulb with Gnrh3 (A), inducing melatonin secretion to decrease feeding and stimulate follicle maturation overnight (B), increasing projections to the pituitary gonadotropes and stimulating gonadotropin release after long-term fasting (C), and downregulating *agrp1* to decrease feeding in high nutritional conditions or overnight (D). Fsh = follicle-stimulating hormone, Lh = luteinizing hormone, OB = olfactory bulbs, OE = olfactory epithelium.

To summarize the overall conclusions, Gnrh2 most likely has reproductive roles in:

<u>*Lhb* transcript regulation in normal feeding conditions via:</u>

- Direct innervations to Lh gonadotropes
- Stimulating melatonin production, which promotes *lhb* synthesis at the pituitary level
- Inhibitory or stimulatory regulation of *gnih* depending on the condition

Maintaining optimal oocyte quality in normal feeding conditions via:

- Stimulating *lhb* expression
- Stimulating melatonin which may directly stimulate gonadotropes in the pituitary and follicles at the gonad level

Gonadotropin regulation in fasting conditions via:

- Increased direct innervations of Lh and Fsh cells

Transducing pheromonal signals via:

- Pheromonal activation of Gnrh2/3 neurons in the olfactory region which in turn upregulates Gnrh2 and Gnrh3 neurons in the midbrain/hypothalamus, which increases Lh/Fsh synthesis and reproductive behaviors

Gnrh2 most likely has feeding roles in:

Downregulating feeding behaviors via:

- Downregulating *agrp1* in overfed conditions
- Indirect or direct regulation of Hypocretin, Nesfatin1, Spexin, and Pomca
- Stimulating melatonin production, which in turn downregulates feeding

## Future Directions

This research has led to several new findings on the roles of Gnrh2 and potential novel reproductive regulators, as well as supporting previous hypotheses on the roles of Gnrh2 in negative feeding regulation. Further research will need to be conducted to determine more clearly the roles that Gnrh2 plays in teleosts, as well as identifying upstream and downstream factors working with Gnrh2 in the reproductive and feeding networks in the brain. Based off the research conducted in my studies, there are multiple paths future research could take which would enable a deeper understanding of feeding and reproductive neuroendocrinology. Some proposed future research topics include:

1. Investigating relationships between Gnrh2 and Spexin, another neuropeptide which has roles in regulating *lhb* expression and feeding behaviors (both inhibitory) and showed to have differential expression with the loss of Gnrh2.
2. Investigating relationships of Gnrh2 with other feeding peptides (Hypocretin, Pomca, Nesfatin1, etc.) which showed differential expression with the loss of Gnrh2, to determine more mediating links between feeding and reproduction.
3. Investigating the role of Gnrh2 in reproduction in fasting conditions in other fish species and other vertebrate groups to determine the evolutionary conservation of this role.
4. Investigating the reproductive functions of novel neuropeptides or factors which were determined to be upregulated with the loss of Gnrh2 and Gnrh3 (i.e., such as Scg5 and Vip) through identifying their receptor localization in pituitary cells and ability to stimulate gonadotropes.

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