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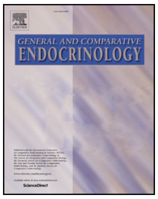

Knockout of Gnrh2 in zebrafish (*Danio rerio*) reveals its roles in regulating feeding behavior and oocyte quality

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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, has long been a mystery, despite its ubiquitous conservation in all jawed vertebrates except rodents. Previous behavioral studies in sparrows, musk shrews, mice, zebrafish, and goldfish show that Gnrh2 administrations both increase spawning behaviors and decrease feeding behaviors, suggesting a role of this peptide in metabolism regulation along with the canonical role in regulating reproduction. In order to more deeply explore the roles of Gnrh2, we used a cyprinid teleost, zebrafish, which has 2 forms of Gnrh, Gnrh2 and Gnrh3, to generate a knockout zebrafish line which contains a frameshift mutation and subsequent disruption of the coding for the functional Gnrh2 peptide. We examined differences in reproduction, feeding, growth, and mobility in this line, and discovered major differences in feeding and growth parameters, suggesting that Gnrh2 is a potent anorexigen in zebrafish. Additionally, there were no differences in mobility except for increased distances swam during feeding periods. There were no major differences in reproductive success, however, female *gnrh2*$^{-/-}$ zebrafish exhibited smaller oocytes and increased embryo mortality, indicating slightly decreased oocyte quality. Additionally, there were changes in the expression levels of many feeding, growth, and reproductive neuropeptides in *gnrh2*$^{-/-}$ zebrafish. Taken together, these findings suggest a role for Gnrh2 in controlling satiation in zebrafish along with a minor role in maintaining optimal oocyte quality in females.

1. 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). Up to three forms of GNRH exist in vertebrate brains, based on their location, named GNRH1, GNRH2, and GNRH3 (Kah et al., 2007). GNRH1 neurons are located in the preoptic area of the hypothalamus, GNRH2 neurons in the midbrain tegmentum, and the teleost-specific Gnrh3 neurons found along the olfactory bulb terminal nerve. In some species, such as cyprinids (Steven et al., 2003), salmonids (Okuzawa et al., 1990), and bony-tongue fishes (O'Neill et al., 1998), Gnrh1 has been lost, and Gnrh3, which is found in the same location, is believed to function as Gnrh1, regulating gonadotropin release from the pituitary (Abraham et al., 2008, Zohar et al., 2010).

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). The few studies that 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 in sparrows, goldfish, zebrafish, musk shrews, and mice showed that Gnrh2 both decreases feeding behavior and increases spawning behavior (Volkoff and Peter, 1999, Millar, 2003, Temple et al., 2003, Kauffman et al., 2005). 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.

In our study, we 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 feeding, growth, and reproductive systems. By generating a transgenic tg (Gnrh2:eGFP) zebrafish line, we have previously described a robust network of innervations throughout the brain, suggesting the involvement of this peptide in a multitude of systems (Xia et al., 2014). A particularly strong Gnrh2 presence was found along the spinal cord,

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suggesting a possible role in regulating mobility (Okuzawa, 2002). As alluded to earlier, studies on the administration of Gnrh2 have suggested that Gnrh2 may act as a satiety factor in zebrafish and goldfish (Hoskins et al., 2008, Matsuda et al., 2008, Nishiguchi et al., 2012). However, the extent and importance of these roles was not clear, particularly in light of the fact that rodent do not possess GnRH2 (Stewart et al., 2009). In order to study the loss-of-function of Gnrh2, we generated a knockout line where we created a targeted mutation in the *gnrh2* gene of zebrafish, 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. In the current study, we describe the effects of the loss of Gnrh2 on feeding, growth, and reproductive phenotypes and suggest that Gnrh2 is involved, to different degrees, in all three processes. Our findings corroborate the suggested role of Gnrh2 as a potent anorexigenic hormone in zebrafish, and demonstrate, for the first time, a minor role of Gnrh2 in maintaining oocyte quality. Overall, our results support the notion that Gnrh2 has roles in controlling both feeding and reproductive signals in the brain of zebrafish.

2. Material and methods

2.1. 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 Paramecium 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 µm 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 (IACUC) at the University of Maryland School of Medicine.

2.2. Reproductive parameter assessments

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 h 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 h 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.

2.3. Gonad histology

Ovaries and testes were dissected from zebrafish and fixed in 4% PFA in PBS overnight 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 from each sample to determine gametogenesis in young fish (Patiño and Sullivan, 2002).

2.4. Gene expression of adults

Differences in the gene expression levels of the following various feeding and growth peptides: *agouti-related peptide* (*agrp*), *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*), as well as reproductive peptides: *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)*, and *oxytocin* (*oxt*) 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 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 min 95 °C activation, 5 s 95 °C denaturation, and 30 s 60 °C annealing, with the last two steps repeating 40 times.

2.5. 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 and 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 s for a total of 7.5 h. After one hour, lights were turned off for 10 min to elicit a quick startle response, and analysis was then continued for another 1.5 h to allow the fish to reach basal swimming behavior. *Paramecium* or *Artemia* were added 2.5 h after the trial was initiated, and analysis continued for another 5 h to quantify mobility during feeding.

2.6. Growth measurements

Body lengths and dry weights of wild-type and *gnrh2*$^{-/-}$ zebrafish

Table 1
Forward and reverse QPCR primer sequences for the housekeeping gene: *eef1a*, reproductive neuropeptide genes: *avp*, *cga*, *fshb*, *gnih*, *gnrh2*, *gnrh3*, *kiss1*, *kiss2*, *lhb*, *oxt*, *scg2a*, *spx*, *tac3a*, and feeding and growth genes: *agrp1*, *igf1a*, *nesfatin1*, *npy*, *hypocretin*, *pmch2*, *pomca*, *pacap1*, and *pacap2*.

Primers	Sequences (5′ to 3′)
Reproductive Primers	
Avp	Fw: AGAGAGCTGCGCTGTAGACC
	Rv: CGGGTTGCCAGATTGAGCAG
Cga	Fw: TCCGGTCTATCAGTGCGT
	Rv: GGATATTCGTGGCAACCATTT
Eef1a	Fw: AAGACAACCCCAAGGCTCTCA
	Rv: CCTTTGGAACGGTGTGATTGA
Fshb	Fw: GCTGGACAATGGATCGAGTTTA
	Rv: CTCGTAGCTCTTGTACATCAAGTT
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
Lhb	Fw: GGCTGGAAATGGTGTCTTCT
	Rv: CCACCGATACCGTCTCATTTAC
Oxt	Fw: AAAGGCCTGCGGTTATGAGG
	Rv: TTGGCCGGTTGATTGACAGC
Scg2a	Fw: GCGTCCGTTCAGTGCTTAAA
	Rv: GGTCTTGCGAACTGGTGGTA
Spx	Fw: TGGAGCGCACCCAAGGGCA
	Rv: TGACTGCGGGATTCTAAGCGTATTG
Tac3a	Fw: TGGTTTTGGTGCTGGAAACC
	Rv: TCTGTTTCGGCGTTTCTGC
Feeding and Growth Primers	
Agrp1	Fw: CACAGACTCACTGCCTGAAA
	Rv: CTTTGCCCAGATCCTCATCATA
Igf1a	Fw: AGCGGTCATTTCTTCCAG
	Rv: CCTTACAGGAAGAGTGGCTAT
Nesfatin1	Fw: CAGACTCATCAAATCGGCTACA
	Rv: CCTTCTCTCGTGCTCTTTCATC
Npy	Fw: TCAAGCGCTGACACCTTAAT
	Rv: GATGAGATCACCATGCCAAATG
Hypocretin	Fw: CAACAACTGCTGCACAACTC
	Rv: GTACCGTAGGGATGAGGAATTTAG
Pmch2	Fw: ACGAAGAACATACGGACCAA
	Rv: TCCACCAATCTACCCTCTATGA
Pomca	Fw: GCCTTTAAACCCACTTGGAAAG
	Rv: GAGTGTGAGCAGTGGTTTCT
Pacap1	Fw: ATCCGTCCGCTGCCGCAGAATG
	Rv: GAGTGTGAGCAGTGGTTTCT
Pacap2	Fw: CCTACTGCACGCCTATTGG
	Rv: CGATTCGTCCTCCTCGC

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 starved for one day, then collected, blotted dry with a paper towel, weighed, and standard body lengths (nose to caudal peduncle) measured.

2.7. Larvae feeding assays

In order to quantify the amount of food ingested by zebrafish larvae, a fluorescent paramecium 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 (0.06 g sea salt in RO water), centrifuged, and resuspended to obtain varying concentrations in fish water. Fluorescently-dyed *Paramecium* were then fed to groups of *gnrh2*$^{-/-}$ and wild-type larvae aged 7–10 dpf. *Paramecium* densities were quantified by counting *Paramecium* cells in 8 different 10 μL droplets under a dissecting scope. The optimal feeding concentration of *Paramecium* was determined by assessing the highest fluorescent readings of individual fish fed during a one-hour period (Fig. S1B). Fish between 7 and 10 dpf, who were seen to be actively feeding, were selected for the experiment, starved for 24 h, placed in 5 mL wells (6 individuals per well in a 24 well plate), then fed the same concentration of fluorescently-stained *Paramecium*. Time course feeding trials were conducted for 15, 30, 60, and 120 min 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 extract the stomach contents (6 pulses of 0.6 s at 60% max output). Fluorescence in lysates was measured at 485 nm excitation and 535 nm emission, with larvae fed unstained Paramecium as the blank control in a 96 well plate reader (Spectramax Gemini XS, Molecular Devices, Sunnyvale, CA, USA).

2.8. 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 min during a one-hour period of time.

2.9. Gnrh2 treatment studies

The effect of Gnrh2 on *lhb* transcription in the *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 and *in vitro* by incubating whole pituitaries with Gnrh2 peptide. 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) or 1 picomole (pmol)/μL in saline containing 0.05% Evans Blue dye. Fish were monitored for normal behavior, and after 2 or 6 h, 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. Pituitaries were flash frozen in dry ice and stored at −80 °C until RNA extraction was conducted, as discussed in the "Gene expression profiles" section (with the addition of 0.5 μL of glycol blue added during the isopropanol step), followed by qPCR analysis to compare *lhb* mRNA levels.

The effect of Gnrh2 on the expression of *lhb* in *gnrh2*$^{-/-}$ and wild-type fish was also tested by incubating pituitaries of wild-type and *gnrh2*$^{-/-}$ male fish with varying concentrations of the decapeptide. The protocol follows the methods outlined in Spicer et al. (2017). Briefly, pituitaries 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) (n = 6/treatment). Pituitaries were washed in the media for 2 h at 28 °C and 30 RPM and then incubated with media including 0, 10, or 100 nM of Gnrh2 peptide for 18 h 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) with the addition of 0.5 µL of GlycolBlue. Synthesis of cDNA and subsequent qPCR to measure *lhb* mRNA levels were conducted as described above (Section 2.4).

2.10. 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, and oocyte diameters and one-way ANOVA for *in vitro* and *in vivo* Gnrh2 treatments. Statistical analysis was performed using repeated measures ANOVA for feeding, mobility, and growth assessments. Statistical analysis for qPCR data was determined using MANOVA.

3. Results

3.1. Reproductive assessments and gonad histology

No significant differences were detected in the fecundity or fertility between *gnrh2*$^{-/-}$ and wild-type pairs (Fig. 1A & B). An average of 200 eggs were produced by all spawning pairs (Fig. 1A) with an average of 90% fertilized eggs for both wild-type and *gnrh2*$^{-/-}$ pairs (Fig. 1B). The GSI of *gnrh2*$^{-/-}$ females was decreased by 3% compared to WT females, but there were no differences between males (Fig. 1C). The average egg survival of *gnrh2*$^{-/-}$ females, spawned with wild-type males, was around 68%, slightly lower than that of wild-type pairings, which was around 84% (Fig. 1D). A 10% decrease in the egg diameter (fertilized and unfertilized) of *gnrh2*$^{-/-}$ females compared with wild-type eggs was observed (Fig. 1E). Gonad sections of *gnrh2*$^{-/-}$ and wild-type individuals from 95 to 240 dpf looked morphologically similar at all of the different life stages (Fig. 1F). Fully vitellogenic oocytes were present in both female *gnrh2*$^{-/-}$ and WT zebrafish (Fig. 1F, black stars) and spermatozoa in both *gnrh2*$^{-/-}$ and wild-type males (Fig. 1F, green stars).

3.2. Feeding and mobility comparison

Overall, *gnrh2*$^{-/-}$ zebrafish larvae ate significantly more Paramecium than wild-type (Fig. 2A). At individual time-points, a higher consumption of Paramecium was observed at 1 h of feeding in the *gnrh2*$^{-/-}$ larvae compared to wild-type larvae, which was doubled and significantly higher by 120 min (Fig. 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 min (Fig. 2B).

When movement was compared between wild type and *gnrh2*$^{-/-}$ larvae (between 7 and 18 dpf), there was no difference in the distances swam before food was introduced, but significantly higher mobility after feeding (Fig. 2C). *Gnrh2*$^{-/-}$ individuals swam ∼20% more distance than wild-type between 1 h and 3.5 h after food introduction before leveling out to the same swimming distances as wild-type (Fig. 2C).

3.3. Growth and body weight comparison

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. 3A). Pooled *gnrh2*$^{-/-}$ zebrafish larvae weighed significantly more than wild-type larvae at 12 dpf (Fig. 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. 3C), and weighed 28% more than wild-type females (Fig. 3D). *Gnrh2*$^{-/-}$ males showed no differences in length (Fig. 3C) or weight (Fig. 3D).

3.4. Temporal expression of the gonadotropins during development and in adulthood

Gnrh2$^{-/-}$ embryos had increased levels of *fshb* at 3, 8, and 18 dpf (Fig. 4B) and decreased levels of *lhb* at 4, 12, and 30 dpf (Fig. 4C) which continued into adulthood for males (Fig. 4F). There were no significant differences in *cga* expression levels throughout zebrafish development (Fig. 4A). In adults, there were no differences in the mRNA levels of *cga* or *fshb* in the pituitary (Fig. 4D and E).

3.5. The effect of Gnrh2 on lhb expression in vivo and in vitro

Intracerebroventricular (ICV) injections of Gnrh2 peptide (1 pmol/µL) to wild-type fish did not show any significant effect after 2 h, but increased the expression of *lhb* in the pituitary 3-fold compared to saline injected WT after 6 h (Fig. 5A). 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 h, and 7-fold at 6 h post injection (Fig. 5A). 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. 5B) 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. 5B).

3.6. Gene expression profiles of growth, feeding and reproductive related factors during adulthood

In adults, *gnrh2*$^{-/-}$ males exhibited significantly decreased expression levels of *agrp1*, and both males and females exhibited a slight reduction in *nesfatin1* (Fig. 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. 6A and B). *Gnrh2*$^{-/-}$ adult females had significantly higher levels of *hyporcretin*, *igf1a,* and *pacap1* compared to wild-type females (Fig. 6B), although there were no differences in the males (Fig. 6A). There was no difference in *npy*, *pmch2*, or *pacap2* mRNA levels between *gnrh2*$^{-/-}$ and wild type zebrafish brains (Fig. 6A and B).

In terms of reproductive related gene expression, in both *gnrh2*$^{-/-}$ males and females, mRNA levels of *gnih* and *spexin* were higher (Fig. 6C and D). Males had significantly higher expression levels of *tac3a* and *scg2a*, while females had significantly lower levels of *scg2a* (Fig. 6D). There were no significant differences in *avp*, *gnrh3*, *kiss1*, *kiss2*, or *oxt* (Fig. 6C and D).

4. Discussion

The exact functions and roles of the evolutionarily conserved midbrain GNRH2 has long been a mystery, with most studies focusing on studying the roles of the hypophysiotropic GNRH1 or teleost Gnrh3 form. In order to start understanding the roles of the Gnrh2 isoform in vertebrates, we used zebrafish that possess both Gnrh2 and the hypophysiotropic Gnrh3 (Steven et al., 2003), to generate the first and only organism with a targeted Gnrh2 genetic knockout. The absence of Gnrh2 peptide was previously verified in this line (Marvel et al., 2018). In the current study, we examined physiological, behavioral, and gene expression differences in reproductive, growth, and feeding systems between *gnrh2*$^{-/-}$ and WT fish. *Gnrh2*$^{-/-}$ fish exhibited major differences in feeding and growth, continuing from development into

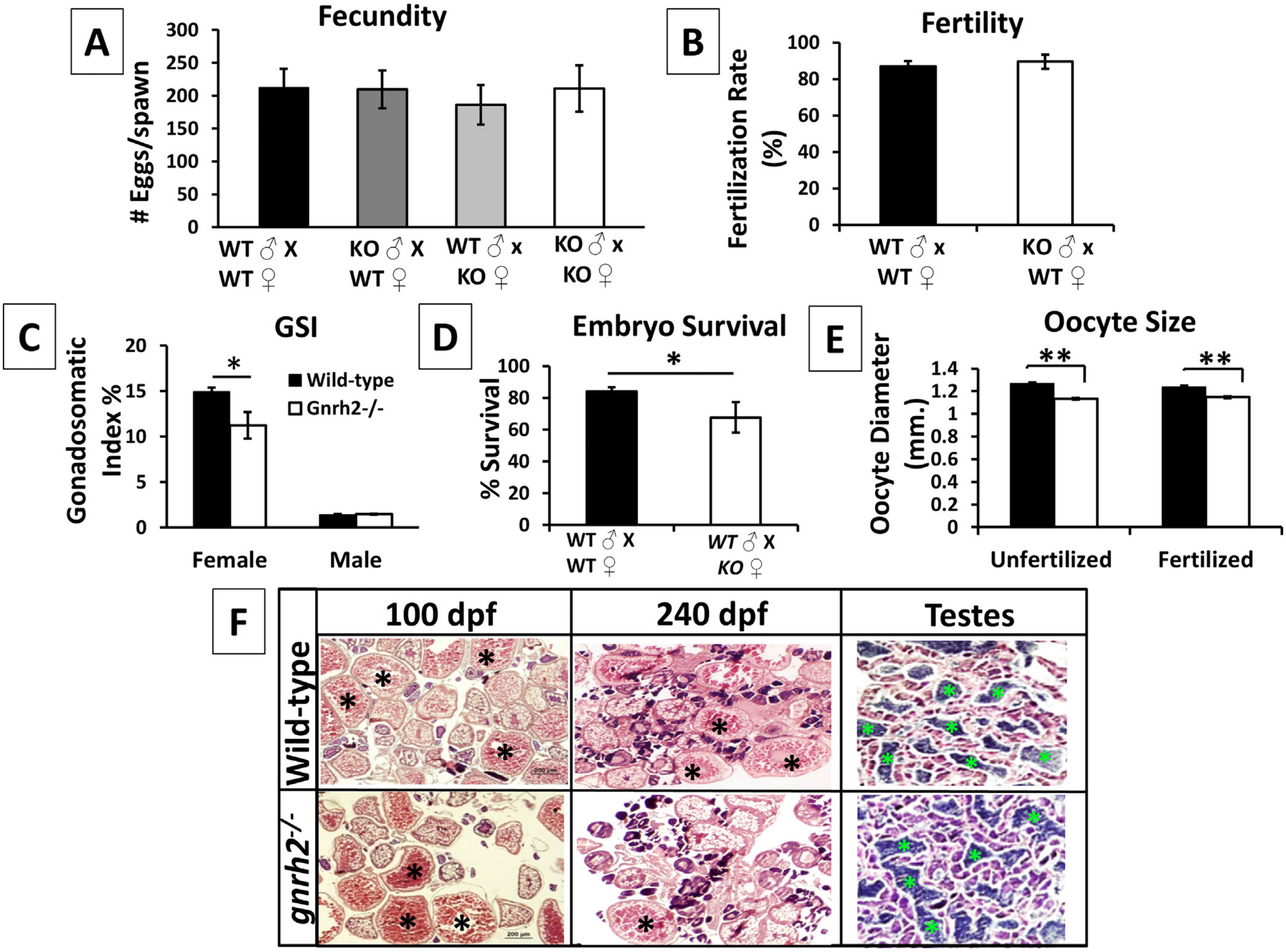

Fig. 1. The effect of the loss of Gnrh2 on reproductive characteristics: Comparison of the number of eggs per spawn (fecundity) of wild-type male and female spawning pairs, gnrh2$^{-/-}$ male and wild-type female spawning pairs, wild-type male and gnrh2$^{-/-}$ female spawning pairs, and gnrh2$^{-/-}$ 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 ($^{*}P < 0.05$, $^{**}P < 0.01$).

adulthood, suggesting Gnrh2 is a major anorexigenic peptide and involved in controlling the satiation of zebrafish.

Gnrh2$^{-/-}$ fish exhibited overall increased long-term feeding compared to WT, 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 (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 to prevent overfeeding. 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, 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). Additionally, Gnrh2 has been shown to downregulate the orexigenic neuropeptide Hypocretin (Hoskins et al., 2008). as well as mediate 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 order to mediate 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 in order to limit the excessive food intake. 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 development as well (See Fig. 1, Marvel et al., 2019)

A more complex picture was obtained when reproductive characteristics were examined. Overall, *gnrh2*$^{-/-}$ adults were reproductively fertile, displaying no major differences in fecundity and fertility

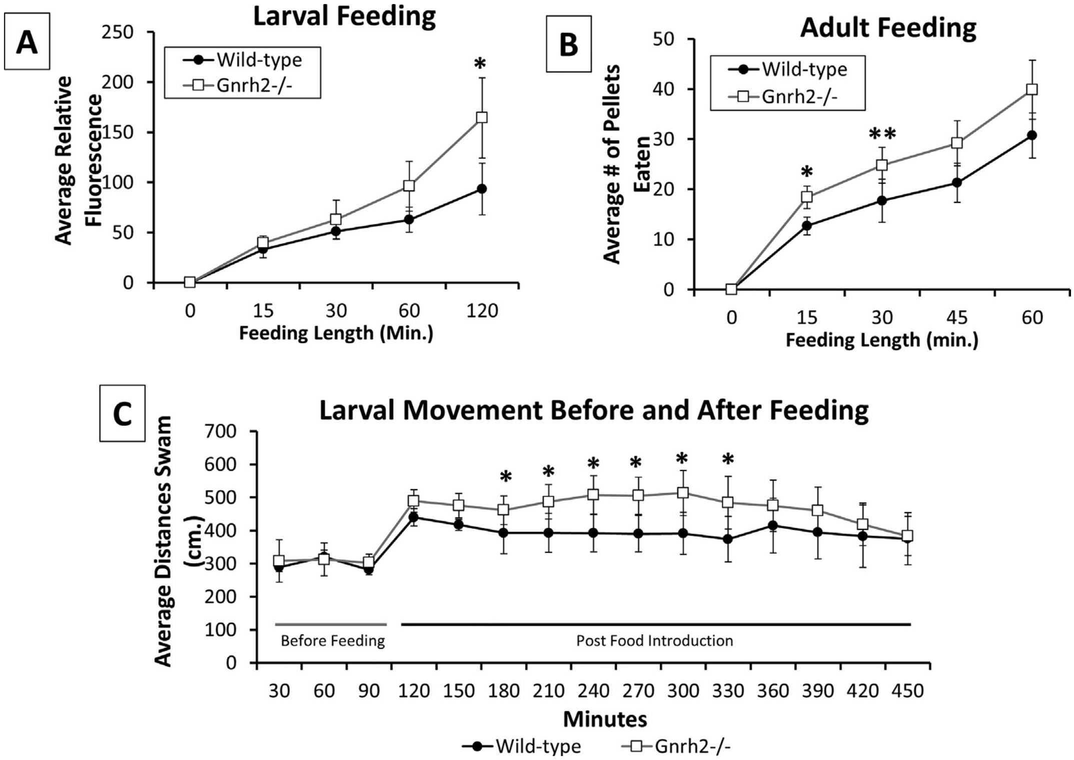

Fig. 2. Comparison of feeding between wild type and gnrh2$^{-/-}$ lines: Fluorescence readings from wild-type and gnrh2$^{-/-}$ embryos (7–10 dpf) fed fluorescently stained Paramecium (1000/5 mL) for 15, 30, 60, or 120 min (A). Comparison of the average number of pellets eaten by gnrh2$^{-/-}$ and wild-type adults (6–9 months old) fed Gemma Diet pellets (300 µm) for 15, 30, 45, or 60 min (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).

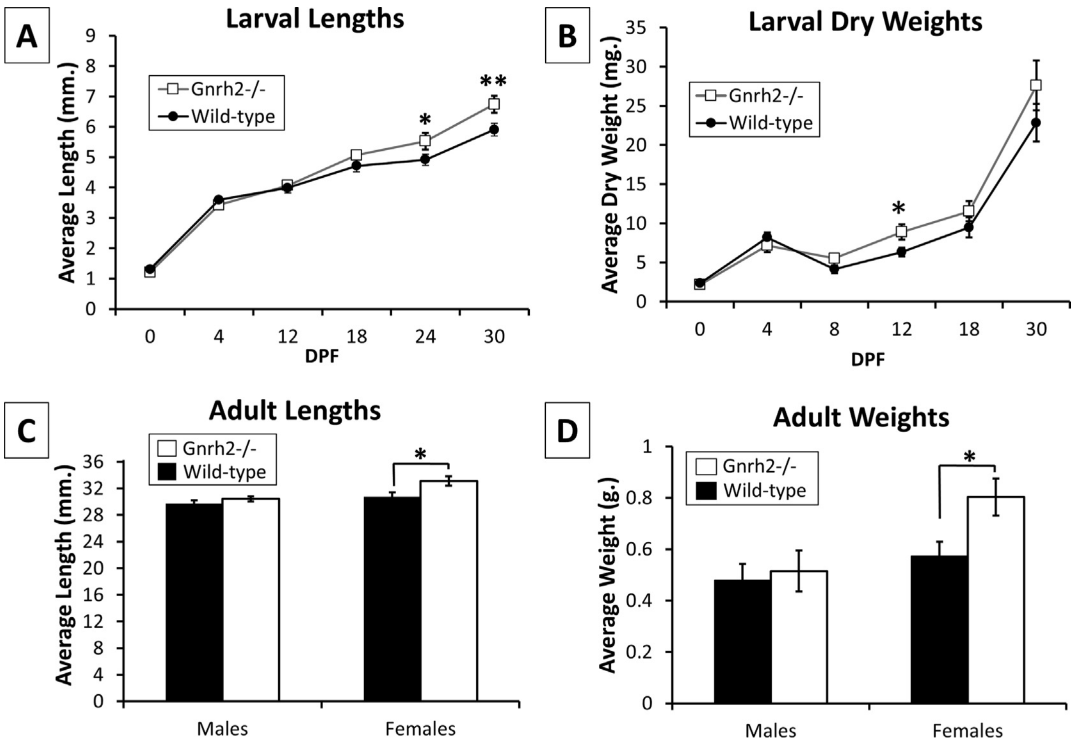

Fig. 3. Growth rate comparisons: Average body lengths (A) and dry weight (B) of wild-type and gnrh2$^{-/-}$ larvae between 0 and 30 dpf. Average standard body lengths (C) and total wet weights (D) of adult male (n = 16) and female (n = 10) wild-type and gnrh2$^{-/-}$ individuals (6 months old). All data 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)).

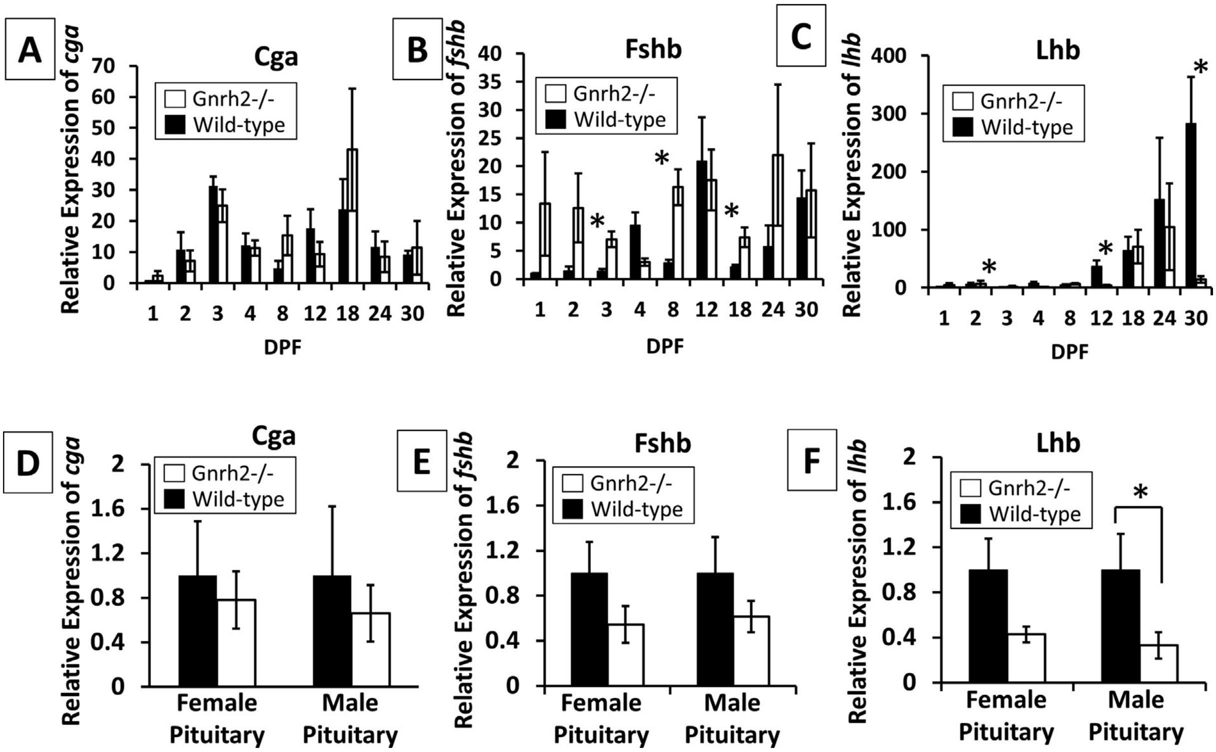

Fig. 4. Gene expression of the gonadotropin genes during development and adulthood in wild type and gnrh2$^{-/-}$ lines: mRNA levels of cga (A), lhb (B) and fshb (C), of wild-type and gnrh2$^{-/-}$ larvae from 1 to 30 dpf, relative to wild-type levels at 1 dpf (A). mRNA levels of cga (D), lhb (E), and fshb (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).

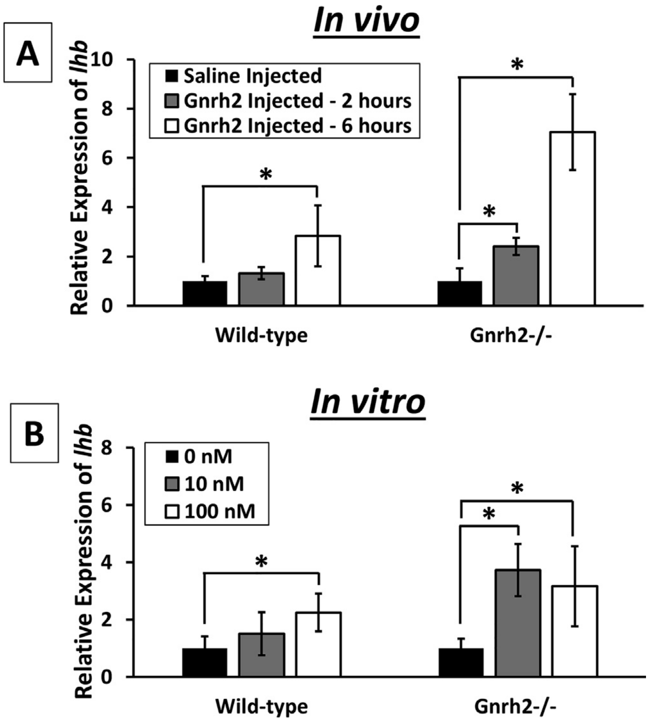

Fig. 5. The effect of Gnrh2 on lhb expression *in vivo* and *in vitro*: Expression levels of lhb in wild-type and gnrh2$^{-/-}$ male pituitaries after intracerebroventricular injections of Gnrh2 peptide (1 pmol/μL) (A) and *in vitro* incubations of wild-type and gnrh2$^{-/-}$ male pituitaries with 0, 10, or 100 nM concentrations of Gnrh2 peptide. All data were expressed as means ± S.E.M. Stars indicate significant differences in expression ($^{*}P < 0.05$, ANOVA).

compared to WT. However, *gnrh2*$^{-/-}$ females 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 WT females, 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. Taken together, our results suggest that Gnrh2 is involved, to some extent, in maintaining optimal oocyte quality in females. 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. 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, 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, 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. Further studies should be conducted in order to determine whether Gnrh2 is mediating and integrating feeding and reproductive systems in order to maintain optimal gonadal growth and reproductive performance.

We have previously shown that some Gnrh2 neuronal projections are found in the pituitary of the tg(Gnrh2:eGFP) line (Xia et al., 2014). Gnrh2 has been shown to be hypophysiotropic in goldfish and able to

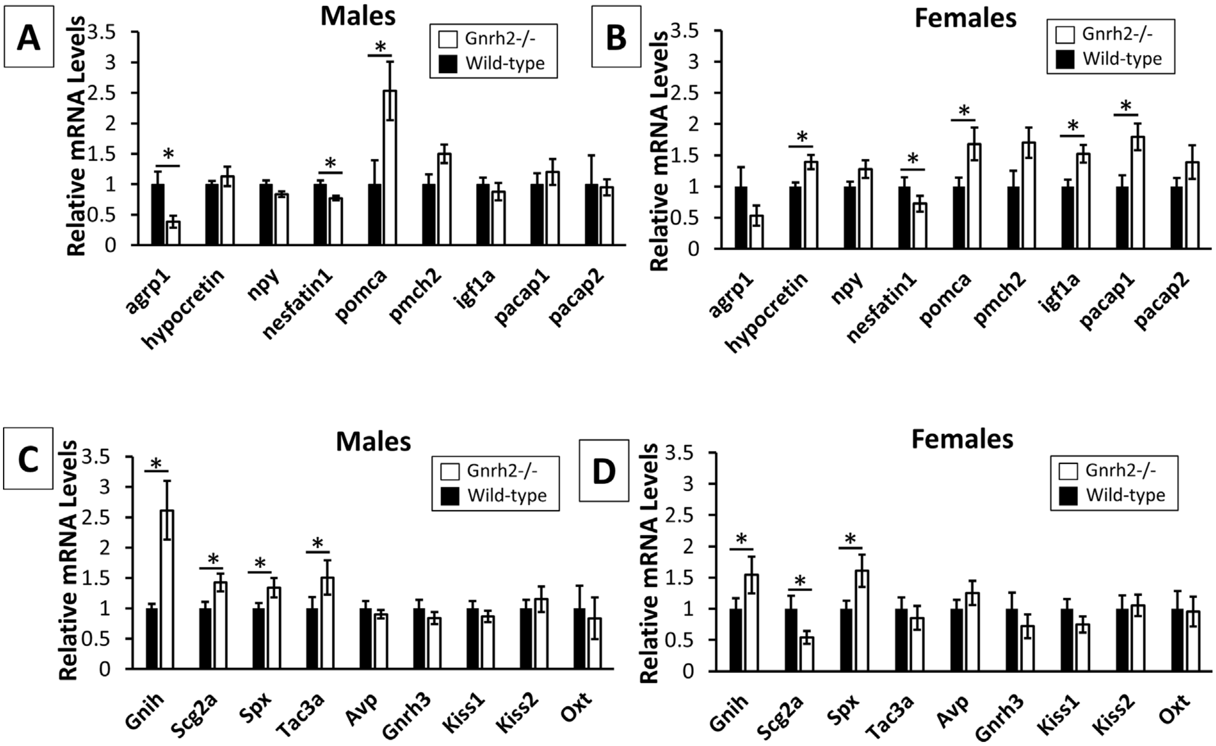

Fig. 6. Effect of the loss of Gnrh2 on feeding, growth, and reproductive-related gene expression: mRNA levels of feeding and growth-related neuropeptides, agrp1, hypocretin, npy, nesfatin1, pomca, pmch2, igf1a, pacap1, and pacap2 of male (A) and female (B) wild-type and gnrh2$^{-/-}$ brains at six months of age (n = 8/sex/genotype). mRNA level of gnih, scg2a, spx, tac3a, avp, gnrh3, kiss1, kiss2, and oxt of male (C) and female (D) 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).

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, and African catfish, Gnrh2 can bind to and activate Gnrh receptors with greater potency than Gnrh3 or Gnrh1 (Zohar et al., 1995, Illing et al., 1999, Bosma et al., 2000). We showed that Gnrh2 treatments, both *in vivo* and *in vitro*, can elicit increased *lhb* expression in zebrafish pituitaries, suggesting a role of Gnrh2 in stimulating the expression of this gonadotrope in zebrafish. Gnrh2 treatment elicited upregulation of *lhb* at a higher magnitude in *gnrh2*$^{-/-}$ fish compared to WT, potentially due to the fact that *gnrhr3* is also upregulated 3-fold in *gnrh2*$^{-/-}$ fish (see Fig. 2, Marvel et al., 2019), and this receptor has been proposed to be most specific to Gnrh2 over the other isoform (Tello et al., 2008, Nishiguchi et al., 2012). The loss of Gnrh2 was associated with significantly decreased levels of *lhb* in males, further signifying that Gnrh2 has a hypophysiotropic role in stimulating *lhb* expression. The loss of Gnrh2 did not result in any differences in sperm motility or density, however, suggesting that Gnrh2 is not a major regulator of spermatogenesis in zebrafish (see Fig. 3, Marvel et al., 2019). The fact that the loss of Gnrh2 only results in minor differences in reproductive characteristics is most likely due to the abundant presence of Gnrh3 in the pituitary continuing to stimulate gonadotropin release. In summary, our results suggest that Gnrh2 promotes oocyte growth and may have a role in regulating Lh gonadotrophs, but is not crucial for oocyte maturation and ovulation under normal feeding regimes.

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). 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, we, and other labs, have detected upregulation of other reproductive neuropeptides in the Gnrh3 KO lines, including *avp*, *tac3a*, and *scg2a*, which doesn't 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. The loss of Gnrh2, instead, was associated with changes in other reproductive neuropeptides, such as an upregulation of *spx* and *gnih* in both sexes, an upregulation of *scg2a* and *tac3a* in males, and a downregulation of *scg2a* in females. 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.

5. Conclusion

By knocking out *gnrh2* in zebrafish, we were able to comprehensively study the potential roles of Gnrh2 in reproduction, feeding, and growth. We showed that Gnrh2 most likely has a role in maintaining egg quality and *lhb* expression. Additionally, we determined that Gnrh2 has roles in controlling feeding behavior as an anorexigenic hormone, with substantial differences in food intake and growth being seen in both larvae and adults that have lost Gnrh2. Furthermore, the loss of Gnrh2 was associated with differences in several different feeding, growth, and reproductive peptide expression levels. Gnrh2 appears to have a multiplicity of roles in zebrafish and may act as a mediator of feeding and reproductive behaviors and characteristics in order to maintain optimal gonadal growth and reproduction.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2019.04.002.

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