The neuroendocrine control of feeding and reproduction in zebrafish (*Danio rerio*) and glass catfish (*Kryptopterus vitreolus*)

by:

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Abstract

Reproduction and feeding are two critical life processes in all vertebrates, the regulations of which involve a complex network of interactions. It has been suggested that appetite regulators, such as orexin, neuropeptide Y (NPY), and cocaine and amphetamine regulated transcript (CART), and reproductive hormones, like gonadotropin-releasing hormone (GnRH), gonadotropin-inhibitory hormone (GnIH), kisspeptin, and neurokinin B (NKB), interact to regulate both food intake and reproduction. However, this relationship is not well characterized in all vertebrate species, especially fish. The purpose of this study was to examine this relationship and uncover some of the regulating mechanisms underlying these two processes. Specifically, I investigated two species of freshwater fish; glass catfish (*Kryptopterus vitreolus*) and zebrafish (*Danio rerio*). In glass catfish, I was able to successfully isolate two reproductive hormones (GnRH1 and GnRH2) and three appetite regulators (orexin, NPY, and CART). I found that fasting affected the relative brain expression levels of all of these peptides, except for GnRH2, suggesting a link between nutritional status and endocrine regulation. In zebrafish, I examined how nutritional status, reproductive stage, gender, and strain affected the brain mRNA expression of certain appetite (orexin and NPY) and reproductive (GnRH, kisspeptin, GnIH, and NKB) hormones. To compare strains, I used both wild-type and transparent *Casper* zebrafish. My results suggest gender- and reproductive stage-specific, as well as strain-specific variations in the mechanisms that regulate feeding and reproduction in zebrafish. To further investigate these differences, I compared the brain mRNA expression of genes involved in the melanocortin system and melanin pathway between wild-type and *Casper* zebrafish. I found that the *Casper* zebrafish had lower levels of several of the genes examined, suggesting that

these strain-specific differences may be mediated by the melanocortin system. Overall, I was able to show a clear relationship between appetite and reproduction in both zebrafish and glass catfish, providing new insights into the endocrine mechanisms that regulate these two processes in fish.

Acknowledgements

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List of Abbreviations

ACTH	Adrenocorticotropic hormone
CART	Cocaine and amphetamine regulated transcript
Dyn	Dynorphin A
EF	Elongation factor
FSH	Follicle stimulating hormone
GnIH	Gonadotropin-inhibitory hormone
GnRH	Gonadotropin-releasing hormone
GSI	Gonadosomatic index
HPG	Hypothalamus-pituitary-gonadal
ICV	Intracerebroventricular
Kiss	Kisspeptin
LH	Luteinizing hormone
MITF	Microphthalmia-associated transcription factor
MSH	Melanocyte stimulating hormone
NKB	Neurokinin B
NPY	Neuropeptide Y
OX	Orexin
pomc	Proopiomelanocortin
qPCR	Quantitative polymerase chain reaction
Tac3	Tachykinin 3
TH	Tyrosine hydroxylase
Tyr	Tyrosinase

Chapter 1: General introduction

The relationship between food intake and reproduction has been clearly identified in some mammals. Generally speaking, abundance of food stores promotes reproduction, while low food availability inhibit reproduction (Schneider, 2004). When an animal is energetically stressed, it will focus its limited energy stores towards processes that are necessary for survival, and suspend those that are nonessential, such as reproduction, until adequate energy is available. Females are particularly susceptible to nutritional infertility, as they require large amounts of energy for egg development, conception, fetal maintenance and development, and in some cases, lactation (Wade et al., 1996; Hill et al., 2008; Allen & Ullrey, 2004). This has been well documented in many mammals, including humans. For example, menstrual irregularities and amenorrhea (complete loss of menstruation) are common in female athletes and those diagnosed with eating disorders, such as anorexia (Klentrou & Plyley, 2003). On the other hand, excessive energy stores can diminish reproductive ability, as illustrated in animals with obesity and certain metabolic diseases, such as diabetes (Hill et al., 2008). Thus, adequate food and energy supply is vital for initiation of puberty, sexual maturation, and reproductive behaviour in mammals. However, very little research has been done to investigate this relationship in fish.

Reproduction and feeding are two critical life processes in all vertebrates. Though a relationship between the two has been confirmed in mammals, it remains poorly understood in other vertebrates, including fish (Shahjahan et al., 2014). To date, only a relatively small number of fish species have been investigated with regards to endocrine regulation (Volkoff, 2016; Qi et al., 2013). Fish are the largest and most diverse vertebrate group, with teleost fish representing almost half of the vertebrate species in existence today (Volff, 2005). They display

broad variations in habitat, behaviour, and reproductive strategies. These wide variations in ecology and physiology can have substantial effects on the endocrine function of fish and the endocrine mechanisms regulating both feeding and reproductive behaviour appear to be species-specific, making it very difficult to generalize between fish species.

The purpose of the project was to examine the relationship between food intake and reproduction in freshwater fish models, specifically zebrafish (*Danio rerio*) and glass catfish (*Kryptopterus vitreolus*). I chose zebrafish as a model because they develop rapidly, spawn year-round, have a well-known genome, and various strains are commercially available, including the *Casper* mutant. The *Casper* zebrafish (*nacre*$^{w2/w2}$; *roy*$^{a9/a9}$) is a transgenic model that lacks both melanophores and iridophores, appearing completely transparent. This lack of pigmentation allows a clear view of the gonads, making identification of the gender and sexual stage of the fish much more straightforward. In addition, examining different strains of fish allowed us to assess possible strain-specific variations in the regulation of feeding and reproduction by comparing transgenic mutants with the wild-type fish. The glass catfish, another transparent species, was used as a model for the same reasons as the C*asper* fish, and because, to my knowledge, reproduction and appetite has never been investigated in this naturally transparent species. I focused on certain reproductive hormones, namely gonadotropin-releasing hormone (GnRH), kisspeptin, gonadotropin-inhibitory hormones (GnIH, and neurokinin B (NKB), as well as appetite regulating hormones, including orexin, cocaine- and amphetamine-regulated transcript (CART), and neuropeptide Y (NPY). and how factors like nutritional status, gender, and reproductive stage affect these hormones.

My objectives were to assess how feeding affects reproduction and how reproduction affects feeding, in order to uncover some of the underlying mechanisms that regulate these two processes. To assess how feeding affects reproduction, I manipulated feeding regime (e.g. fasting vs. satiation) and evaluated the relative mRNA expression levels of my target hormones. In zebrafish, I explored how sexual maturation affected the reproductive and feeding hormones in females, by comparing expression levels between reproductive stages. Due to the complexity of endocrine regulation, specifically in fish, the actions and interactions between these hormones thought to be involved in feeding and reproduction remain widely unknown. Through this research, I was able to better illustrate the regulating mechanisms of these two critical processes.

Chapter 2: The Regulation of Feeding in Glass Catfish (*Kryptopterus vitreolus*)

2.1 Abstract

Glass catfish (*Kryptopterus vitreolus*) are freshwater fish known for their natural transparency and for which the endocrine regulation of feeding has not yet examined. The regulation of feeding is a complex process that involves coordination between various signals, including a number of appetite-regulating hormones released by the brain. These hormones can be described as orexigenic (stimulate food intake, e.g. orexin and neuropeptide Y – NPY) or anorexigenic (inhibit food intake, e.g. cocaine and amphetamine regulated transcript – CART). Reproduction and energy homeostasis are closely linked, as factors that affect appetite have been shown to influence reproductive behaviours, likely by regulating reproductive hormones. Gonadotropin-releasing hormone (GnRH) is one of the most influential factors in reproduction. It stimulates gametogenesis and sexual behaviours through activation of the hypothalamus-pituitary-gonadal (HPG) axis. Most vertebrates have two forms of GnRH; GnRH1, which is found in the hypothalamus and is involved in the regulation of gonadotropin release, and GnRH2, which is generally present in the midbrain and is thought to have a neuromodulatory role. Although our understanding of the endocrine regulation of feeding and reproduction in fish has progressed in recent years, many gaps still remain, particularly with regards to catfish. I was able to successfully isolate cDNA encoding reproductive hormones (GnRH1, GnRH2) and appetite regulators (orexin, NPY, and CART) from glass catfish and examined their distribution in various tissues. I found wide distribution across various brain and peripheral tissues for all peptides, except CART, which was only present in whole brain. In order to uncover some of the mechanisms that regulate feeding in catfish, I assessed how nutritional status affected the

relative brain expression levels of these peptides. Fasting increased the expression the orexigenic hormones (i.e. orexin and NPY), which is expected as they both stimulate appetite. CART is an anorexigenic hormone, but was also increased during fasting. This was unexpected but may be due to an increase in foraging behaviours in starving fish, as CART has been shown to stimulate locomotion. Fasting decreased the expression levels of GnRH1, but did not affect GnRH2. The decrease in GnRH1 is consistent with the relationship seen in mammals and goldfish, where decreased food intake inhibits reproduction. The fact that GnRH2 was unaffected by fasting may indicate that it plays less of a role in reproduction and appetite than GnRH1. Overall, I have shown that nutritional status affects the expression of peptides involved in both feeding and reproduction. My study provides new insights on the endocrine mechanisms that regulate feeding and reproduction in catfish.

2.2 Introduction

Kryptopterus vitreolus are freshwater fish belonging to the Siluridae family in the order Siluriformes. They are commonly known as 'glass catfish' or 'ghost catfish' due to their lack of body pigmentation. Most silurids are found in Southeast Asia, with glass catfish being predominantly native to rivers in Thailand (Ng & Kottelat, 2013). The natural transparency of the glass catfish has not only led to its popularity in aquaria but also as an experimental subject, as they eliminate the need for genetic manipulation for producing transparent lines. They are often used as *in vivo* models for studies involving circulation (Rummer et al., 2014; Maruvada & Hynynen, 2004; Dahl Ejby Jensen et al., 2009), muscle function (Lieber et al., 1992; Yamada et al., 2009), pigment migration (Fujii et al., 1982; Hayashi & Fujii, 1994; Hayashi & Fujii, 2001), neural regeneration (Bever & Borgens, 1991), transplantation (Han et al., 2011), visual

processing (Wagner & Zeutzius, 1987), and DNA immunization (Dijkstra et al., 2001). The genus *Kryptopterus* contains several small catfish that exhibit similar morphologies; in fact, *K. vitreolus* is often misidentified as either *K. minor* or, more frequently, *K. bicirrhis*. In fact, all of the aforementioned studies describe the 'glass catfish' used as *Kryptopterus bicirrhis*. In 2013, this long-standing misidentification was resolved when Ng and Kottelat introduced the glass catfish as a distinct species, known as *Kryptopterus vitreolus*. Although similar, *K. bicirrhis* is larger and more opaque than *K. vitreolus*, which is small and completely transparent (Ng & Kottelat, 2013). This confusion warrants further studies in order to properly distinguish between species.

The regulation of feeding in fish is a complex process that involves specific coordination between various signals. The hypothalamus regulates homeostasis, including energy balance, by releasing a number of hormones that control appetite. These appetite regulators can be described as orexigenic (stimulate food intake, e.g. orexin, neuropeptide Y - NPY) or anorexigenic (inhibit food intake; e.g. cocaine and amphetamine regulated transcript - CART). Reproduction and feeding/energy homeostasis are closely linked, as factors that affect appetite, such as nutritional status, activity level, and stress have been shown to influence reproductive behaviours. Although our understanding of the endocrine regulation in fish has progressed in recent years, many gaps still remain, particularly with regards to Siluriformes.

Orexin is an orexigenic factor that consists of two forms, orexin A and orexin B (Sakurai, 2002). It was discovered in 1998 as a hypothalamic factor that stimulates feeding behaviour in mammals (Sakurai et al., 1998). The same action was later observed in fish, where orexin increased food intake in goldfish (*Carassius auratus*) (Nakamachi et al., 2006), zebrafish (*Danio rerio*) (Yokobori et al., 2011), and cavefish *Astyanax mexicanus* (Penney & Volkoff, 2014). Orexin

brain expression is increased during fasting in winter flounder (*Pleuronectes americanus*) (Buckley et al., 2010), red-bellied piranha (*Pygocentrus natereri*) (Volkoff, 2014), and dourado (*Salminus brasiliensis*) (Volkoff et al., 2016), suggesting a similar function. Yet, in some fish species, prolonged fasting decreases orexin levels, indicating species-specific variations (Babichuk & Volkoff, 2013). Orexin is known to interact with GnRH, a reproductive hormone. In mammals, orexin stimulates GnRH neurons to promote gonadotropin release (Sasson et al., 2006). In goldfish, however, orexin and GnRH negatively interact with each other, displaying the opposite relationship noted in mammals (Hoskins et al., 2008). To my knowledge, orexin has never been investigated in Siluriformes.

In addition to orexin, NPY and CART are two key players in the regulation of feeding. NPY is an orexigenic factor, promoting food intake in both mammals and fish, including channel catfish (*Ictalurus punctatus*) (Shahjahan et al., 2014; Silverstein & Plisetskaya, 2000). Orexin and NPY interact to control feeding behaviour in fish. Administration of orexin or NPY alone increases food intake in goldfish, while co-injection with NPY and orexin leads to an increase in food intake that is higher than NPY only. Blocking NPY or orexin receptors decreases both NPY- and orexin-induced feeding, and treatment with orexin leads to a greater expression of NPY mRNA (Volkoff & Peter, 2001). Unlike orexin, however, NPY seems stimulate GnRH and LH cells in catfish (Subhedar et al., 2005). It has been implicated in the production of sex steroids in the gonads of both male and female catfish (Priyadarshini & Lal, 2018; Singh & Lal, 2018). It remains unclear how NPY and orexin both stimulate appetite yet have opposite effects on the GnRH system in teleost fish.

CART was originally isolated in the rat brain (Douglass et al., 1995). It is known to suppress appetite in many vertebrate classes and has been described as the most potent anorexigenic factor discovered in fish to date (Kuhar et al., 2002; Volkoff et al., 2005). In goldfish, administration of CART decreases food consumption and increases locomotion, while fasting reduces CART expression. Its interactions with NPY and orexin have been well characterized, as CART inhibits both NPY- and orexin-induced feeding in goldfish (Volkoff & Peter, 2000). Fasting leads to a decrease in CART brain expression in African catfish (*Clarias gariepinus*) that is recovered upon refeeding (Subhedar et al., 2011; Kobayashi et al., 2008). Interestingly, CART is expressed in gonadotropin cells of the pituitary and in gonads of catfish (Kobayashi et al., 2008; Barsagade et al., 2010). There is much left to be discovered about CART, as the full scope of its functions, mechanisms, and interactions remains widely unknown, especially in fish species.

Nutritional status not only impacts appetite, but has been shown to cause varying effects on reproductive hormones. Gonadotropin-releasing hormone (GnRH) is arguably the most influential factor in reproduction. It is a critical component of the hypothalamus-pituitary-gonadal (HPG) axis responsible for controlling the reproductive cycle (Shahjahan et al., 2014). GnRH released from the hypothalamus stimulates the pituitary gland (specifically gonadotropic cells) to release gonadotropins, including follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (Hoskins et al., 2008). The gonadotropins then stimulate the gonads to develop and release sex steroids, which leads to gametogenesis and sexual behaviour. Most vertebrates have two forms of GnRH. The first is found in the hypothalamus and is involved in the regulation of gonadotropin release. This is a species-specific form that exhibits large variations in its structure, with over 20 homologues identified among vertebrate species to date (Millar

2005; Somoza et al., 2002). In catfish, this variant is known as GnRH1 or catfish GnRH (cfGnRH) and is distributed throughout the ventral forebrain (Zandbergen et al., 1995). The second form of GnRH is generally present in the midbrain and is highly conserved among vertebrate species. Although its exact function is unknown, it is thought to have a neuromodulatory role. In catfish, GnRH2 (also known as chicken GnRH-II or cGnRH-II) is found exclusively in the midbrain (Okubo & Nagahama, 2008; Zandbergen et al., 1995). Interestingly, the catfish GnRH receptor displays much higher affinity for GnRH2. However, this is compensated for by a 37-fold increase in the levels of GnRH1 compared to GnRH2 in the pituitary (Schulz et al., 1993). GnRH has been shown to be important not only in reproduction, but as a modulator of food intake through its interaction with orexin, an appetite stimulator. In goldfish, treatment with GnRH leads to an increase in spawning, and a decrease in both food intake and orexin brain mRNA levels. Injections of orexin, on the other hand, induce an inhibition of spawning, a decrease in brain GnRH expression, and an increase in food intake (Hoskins et al., 2008). This suggests that the inverse relationship between appetite and reproduction seen in some species is mediated by the actions and interactions of regulating hormones.

The objective of this study was to uncover some of the mechanisms underlying the regulation of feeding in catfish. I was able to successfully isolate cDNA encoding reproductive hormones (GnRH1, GnRH2) and "classic" appetite regulators (orexin, NPY, and CART) from glass catfish and examined their distributions in various tissues. I investigated the regulation of these peptides and their potential roles in appetite control by comparing relative expression levels between fed and fasted glass catfish.

2.3 Material and Methods

2.3.1 Experimental animals

Glass catfish (*Kryptopterus vitreolus*) (average length 4.64 $\pm$ 0.658 cm; average weight 0.408 $\pm$ 0.183 g) were obtained from Mirdo Importations Canada (Montreal, Quebec, Canada) and acclimated in 60 L tanks. Fish were maintained at a water temperature of 26^{o}C under a simulated photoperiod of 12H light:12H dark and fed once daily to satiety with tropical fish flakes (45% protein, 10% fat, 1.6% Omega-6 fatty acids, 1.6% Omega-3 fatty acids, 1.7% fibre, 7% moisture, 8.5% ash; Rolf C. Hagen Inc., Montreal, Quebec, Canada). Fish were acclimated under these conditions for one week prior to the start of any experimentation. All fish were anaesthetized in 0.05% tricaine methanesulfonate (MS222) and sacrificed by spinal section. Sex could not be determined, as I was unable to identify the gonads and no sexual dimorphism was observed. All experiments were carried out in accordance with the principles published in the Canadian Council on Animal Care's guide to the care and use of experimental animals.

2.3.2 RNA extraction and cDNA synthesis

Catfish were dissected and tissues were collected and preserved in RNA*later* (Qiagen, Mississauga, Ontario, Canada) at -20^{o}C. Total RNA was isolated using a *GeneJET*^{TM} RNA Purification Kit (Fermentas, Burlington, Ontario, Canada). Final RNA concentrations were quantified by optical density reading at 260 nm using a NanoDrop 2000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, Delaware, USA). All samples had absorbance ratios at 260 and 280 nm between 1.8 and 2.1. 200 ng of isolated RNA was then reverse transcribed

using a Verso cDNA Synthesis Kit (Thermo Fisher Scientific Lafayette, Colorado, USA) following the manufacturer's protocol. All cDNA was stored at -20°C until further use.

2.3.3 Cloning and sequence analysis

RNA from two fish was extracted and reverse transcribed as described above. DNA sequences from other *Siluriformes* species were used to design nucleotide primers for amplification (Table 2.1) for GnRH1, GnRH2, orexin, NPY, and CART. DNA was then subjected to PCR amplification using a Bio-Rad C1000 Touch Thermocycler (Bio-Rad, Mississauga, Ontario, Canada). The PCR reaction contained 1 μl of cDNA, 9.5 μl water, 12.5 μl 10x Go Taq Master Mix (Promega, Madison, Wisconsin, USA), and 1 μl of each primer (diluted to 10 μM). PCR conditions consisted of an initial denaturation step at 95°C for 3 minutes, followed by 30 cycles of: 1) Denaturation (95°C for 20 seconds), 2) Annealing (55°C for 30 seconds), 3) Elongation (72°C for 40 seconds); with a final elongation of 3 minutes at 72°C. Products were separated by electrophoresis in a 1.5% agarose gel stained with ethidium bromide and visualized using the Epichem Darkroom Bioimaging System (UVP, Upland, California, USA). Bands were excised and purified with the GeneJET™ Gel Extraction Kit (Fermentas), ligated in the pGEM-T easy vector (Promega) and sequenced by The Centre for Applied Genomics facility (TCAG; The Hospital for Sick Children, Toronto, Ontario, Canada)

DNA and deduced protein sequences were analyzed by the Basic Local Alignment Search Tool (BLAST; *https://blast.ncbi.nlm.nih.gov/Blast.cgi*). Multiple alignments of amino acid sequences were performed and phylogenetic trees were constructed with the maximum likelihood method and 1000 bootstraps using MEGA7 (*https://www.megasoftware.net/*).

2.3.4 Tissue distribution

RNA was extracted from brain, spleen, gill, barbel, intestine, stomach, liver, and muscle tissue of two catfish and reverse-transcribed as previously described. Gene-specific primers were used based on the sequences obtained from cloning (Table 2.1). A 25 μl PCR reaction was set up containing 12.5 μl 10x GoTaq Master Mix (Promega), 9.5 μl water, 1 μl of each primer (diluted to 10 μM), and 1 μl cDNA. PCR conditions included a 3-minute initial denaturation step at 95°C, followed by 30 cycles of 95°C for 20 seconds, 55°C for 30 seconds, and 72°C for 40 seconds, with a final extension of 3 minutes at 72°C. Products were separated by electrophoresis in a 1.5% agarose gel for 25 minutes at 125 Volts. A negative control containing water instead of cDNA was included to verify gene amplification and to test for contamination. Agarose gels were viewed in the Epichem Darkroom Bioimaging System (UVP). β-actin was used as a reference gene.

2.3.5 Fasting experiments

Twenty glass catfish were divided into two tanks (10 fish per tank) and acclimated for one week. Once acclimated, one tank was continually fed once daily to satiation, while the other tank was fasted. Fish were sampled after one week of experimental conditions. Brain tissues were collected and stored in RNA*later* at -20°C until RNA could be extracted. cDNA was synthesized as previously stated.

2.3.6 Quantitative PCR (qPCR)

In order to quantify gene expression, specific primer pairs (Table 2.1) were optimized for qPCR using a serial dilution of cDNA. Only primers with sufficiently high efficiency and linearity

of expression level were used in experimentation. Duplicate reactions were prepared using a mix containing 5 μl Sso Advanced™ Universal SYBR® Green Supermix (BioRad, Hercules, California, USA), 0.5 μl water, 0.25 μl 10 μM forward primer, 0.25 μl 10 μM reverse primer, and 4 μl cDNA (diluted 1:3 with water). The total reaction volume (10 μl) was then loaded into a 96-well plate. The following cycling conditions were performed using the CFX96 Touch™ Real-Time PCR Detection System (BioRad): 40 cycles; 1) Denaturation (94°C for 30 seconds), 2) Annealing (57°C for 45 seconds), and 3) Elongation (72°C for 60 seconds). Efficiencies, R2 values, and optimal annealing temperatures were determined for all primer pairs. A melting curve analysis was performed at the end of each qPCR to verify primer specificity. Several candidate genes were tested for use as reference genes including β-actin, 18S, and elongation factor 1-α (EF). Using NormFinder Software, it was determined that β-actin was the most stable reference gene across all groups.

2.3.7 qPCR analysis

Gene expression levels were measured and quantified using the CFX Maestro software (BioRad). The software compared expression levels using the ΔΔCt method. Gene expression levels were normalized to β-actin. Statistical analysis was then performed using Prism7 GraphPad Instat program (GraphPad Software Inc., San Diego, California, USA). Data was tested for normality using a Shapiro-Wilk normality test. An unpaired t-test was used to compare qPCR results for normal data, while non-normal data was analyzed using a non-parametric Mann-Whitney t-test. Results were compared between fed and fasted treatment groups. Significance was set at $p < 0.05$.

Table 2.1: Catfish primers used for cloning and qPCR analysis in fasting experiments with corresponding GenBank Accession ID (OX: orexin; NPY: neuropeptide Y; CART: cocaine and amphetamine regulated transcript; GnRH1: gonadotropin releasing hormone 1; GnRH2: gonadotropin releasing hormone 2).

Target Gene	Primer	Sequence (5'→3')	GenBank ID
OX	OX-F	5' GACCTGGATGGGAAACTCG 3'	KY770950
	OX-R	5' CTCTTGCCCATAGTAAGGATCC 3'	
NPY	NPY-F	5' GCGGAAGGATATCCAACAAA 3'	MH049870
	NPY-R	5' CACCACATCAAAGGGTCATC 3'	
CART	CART-F	5' CTATGTGCGACGTTGGAGAG 3'	KY770951
	CART-R	5' TGCAGTGCATGAACAGATGA 3'	
GnRH1	GnRH1-F	5' GTGAAGCGAGCACTCTGGTG 3'	MH049872
	GnRH1-R	5' TCAGCAGATCTTTCAGCCTGT 3'	
GnRH2	GnRH2-F	5' CTGTTGCTGGTTGCTGCCTT 3'	MH049871
	GnRH2-R	5' AGGATGCTCTTCAGGACGTTG 3'	
β-actin	Actin-F	5' CCCATCTATGAGGGTTATGCTCTG 3'	XM_017457500.1
	Actin-R	5' GCTCGGTCAGGATCTTCATCAG 3'	

2.4 Results

2.4.1 Cloning

Partial coding sequences for GnRH1, GnRH2, orexin, NPY, and CART (Fig. 2.1), as well as a partial sequence for the reference gene β-actin were successfully isolated from the brain tissues of glass catfish. When compared to other fish species, the sequences obtained for the catfish peptides were most similar to other Siluriformes (75-100%) as well as Perciformes (60-90%) and Cypriniformes (52-97%).

The glass catfish GnRH1 sequence identified showed 80-94% similarity with other Siluriformes (i.e. yellow catfish [84%], channel catfish [94%], and walking catfish [80%]), 63% with Perciformes (Nile tilapia), and 52-58% with Cypriniformes (i.e. common carp [57%], goldfish [58%], and zebrafish [52%]) (Fig. 2.1a; Fig. 2.2a). The isolated GnRH2 showed 98-100% similarity with other Siluriformes (i.e. yellow catfish [98%] and channel catfish [100%]), 88% similarity with Perciformes (Nile tilapia), 85% with Gasterosteiformes (three-spined stickleback), 73-88% with Cypriniformes (i.e. common carp [88%], naked carp [80%], zebrafish [83%], and goldfish 73%]), and 63% with Salmoniformes (Atlantic salmon) (Fig. 2.1b; Fig. 2.2b).

Glass catfish orexin showed 77-85% similarity to other Siluriformes (i.e. channel catfish [77%] and southern catfish [85%]), 78% with Perciformes (Nile tilapia), 78% with Beloniformes (Japanese rice fish), 70-79% with Cypriniformes (i.e. sheepshead minnow [70%], goldfish [75%], and zebrafish [79%]), and 68% Gadiformes (Atlantic cod) (Fig. 2.1c; Fig. 2.2c)

The isolated NPY fragment showed 90-95% similarity with other Siluriformes (i.e. yellow catfish [92%], channel catfish [95%], walking catfish [90%], and southern catfish [95%]), 81-82%

with Cypriniformes (i.e. goldfish [82%] and zebrafish [81%]), and 70% with Perciformes (Nile tilapia) (Fig. 2.1d; Fig. 2.2d).

The isolated CART sequence showed 99-100% similarity to other Siluriformes (i.e. southern catfish [100%] and channel catfish [99%]), 91% similarity with Perciformes (Nile tilapia), and 66-97% similarity with Cypriniformes (i.e. common carp [95%], goldfish CART1 [91%], goldfish CART2 [97%], zebrafish CART2 [95%] and zebrafish CART4 [66%]) (Fig. 2.1e; Fig. 2.2e).

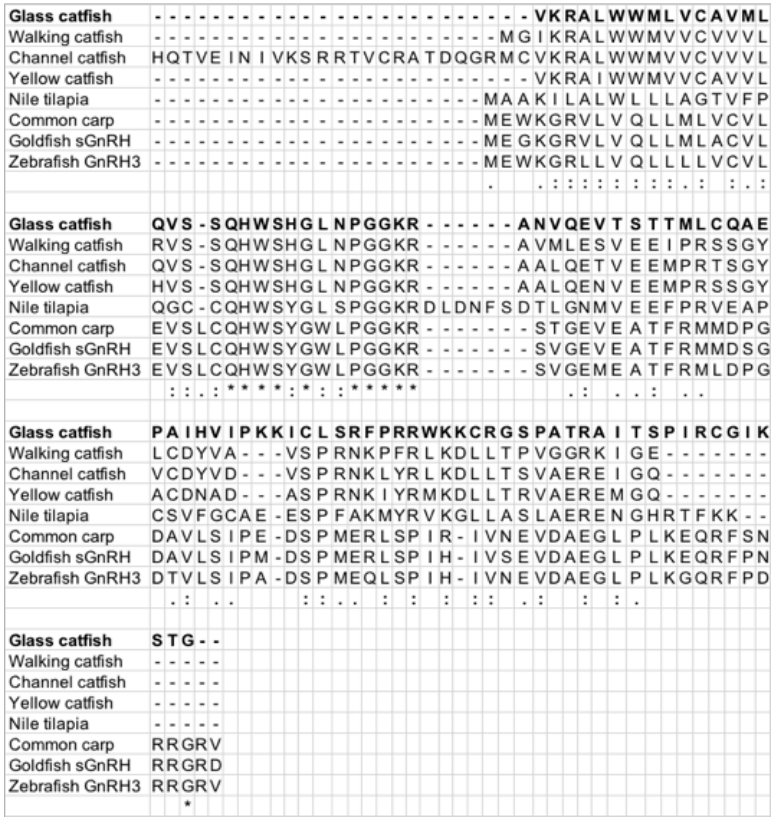
2.4.2 Tissue distribution

GnRH1 was detected in all tissue samples examined with similar apparent expression levels. GnRH2 was expressed in the brain, spleen, gill, and liver with the highest apparent expression being noted in the brain and liver. Orexin was detected in the brain, spleen, gill, intestine, and stomach, though all apparent expression levels were relatively low. NPY was expressed in the brain, spleen, gill, stomach, liver, and muscle tissue. The highest expression levels of NPY appeared to be in the stomach and in the brain. CART was only expressed in the brain, with no expression detected in any peripheral tissues. No mRNA expression was detected in the negative control samples for any of the genes investigated (Fig. 2.3).

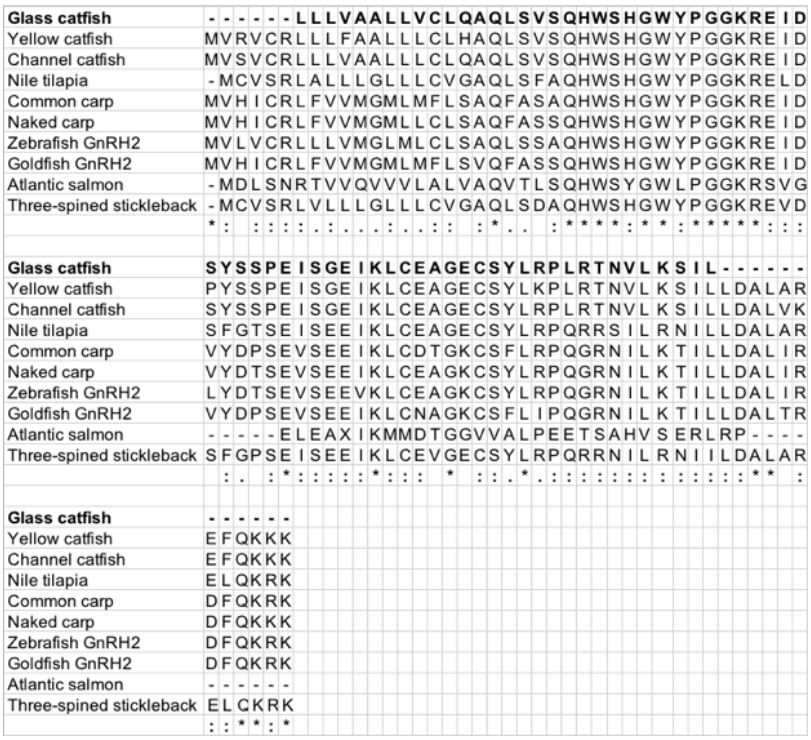
2.4.3 Fasting experiments

Fasting induced a decrease in the brain mRNA expression of GnRH1, but no change in GnRH2 expression. The expression levels of all three appetite regulators (orexin, NPY, and CART) were significantly increased during fasting (Fig. 2.4)

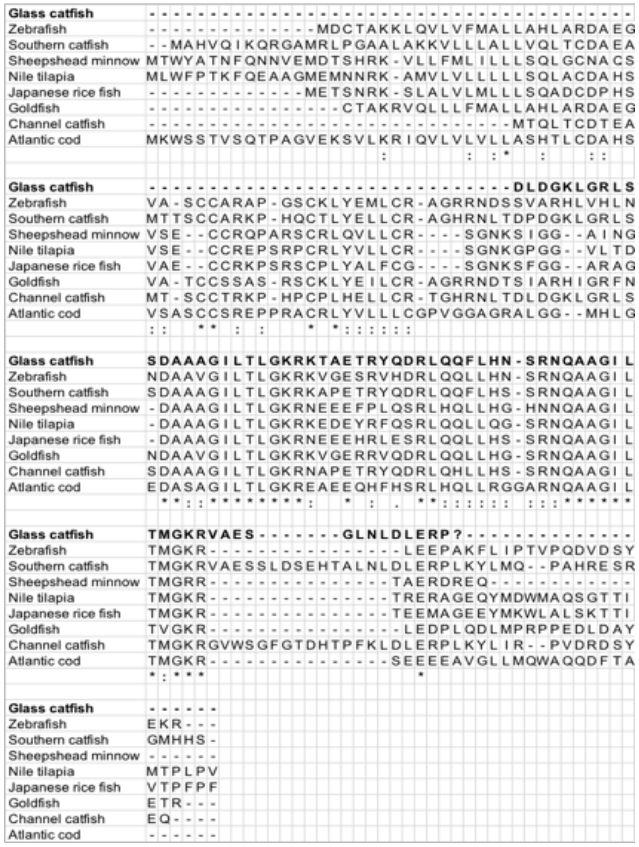
a) **GnRH1**



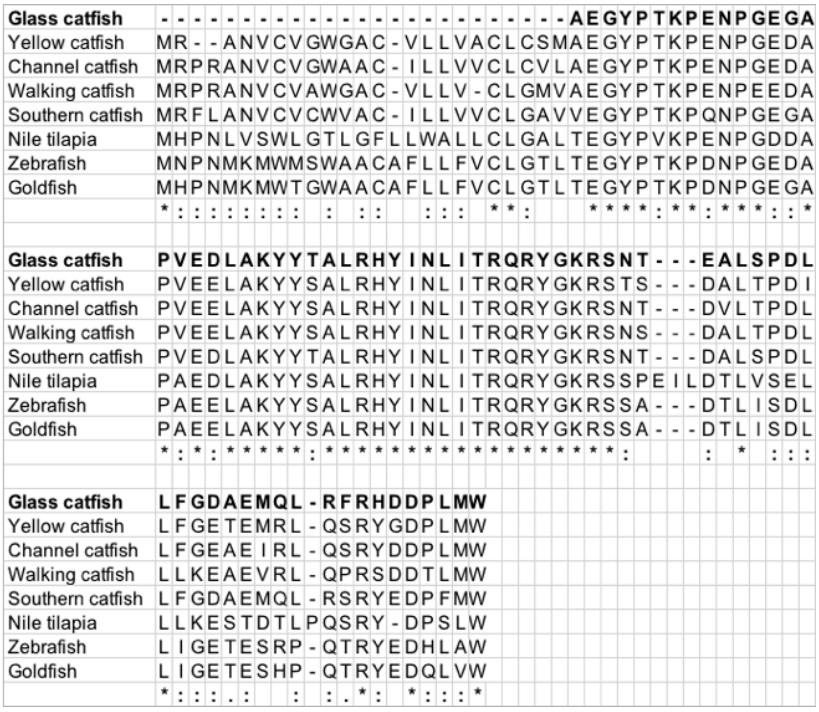
b) **GnRH2**



c) Orexin



d) NPY



e) **CART**

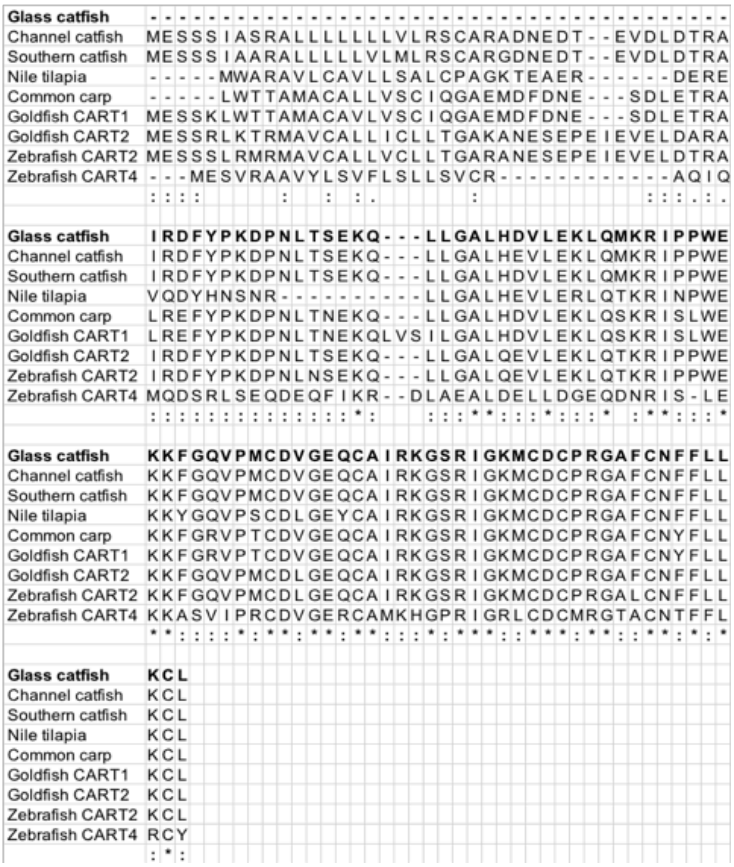
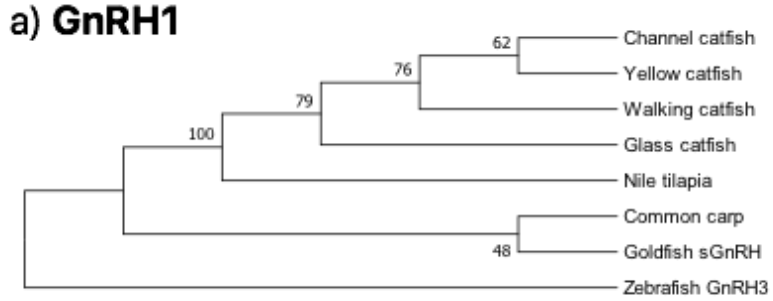


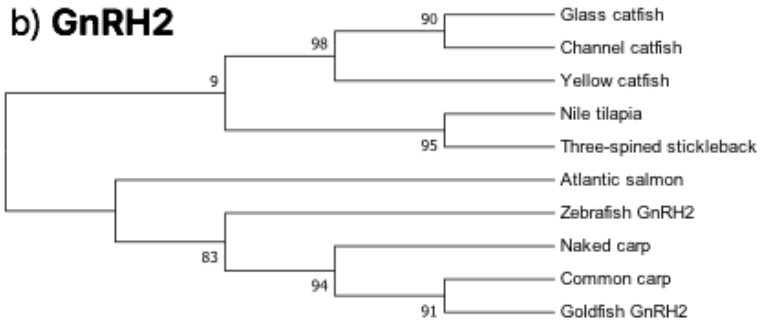
Fig. 2.1: Amino acid sequence alignment of: **a) GnRH1** [Glass catfish; *Kryptopterus vitreolus* (MH049872), Walking catfish; *Claria batrachus* (KM258877.1), Channel catfish; *Ictalurus punctatus* (XM_017468372.1), Yellow catfish; *Tachysurus fulvidraco* (KJ396288.1), Nile tilapia; *Oreochromis niloticus* (AF467291.1), Common carp; *Cyprinus carpio* (AY189960.1), Goldfish; *Carassius auratus* (U30301.1), Zebrafish GnRH3; *Danio rerio* (NM_182887.2)], **b) GnRH2** [Glass catfish; *Kryptopterus vitreolus* (MH049871), Yellow catfish; *Tachysurus fulvidraco* (KJ396290.1), Channel catfish; *Ictalurus punctatus* (XM_017493147.1), Nile tilapia; *Oreochromis niloticus* (XM_003442697.4), Common carp; *Cyprinus carpio* (AY148223.1), Naked carp; *Gymnocypris prezewalskii* (MF882998.1), Zebrafish GnRH2; *Danio rerio* (NM_181439.4), Goldfish; *Carassius auratus* (U40567.1), Atlantic salmon; *Salmo salar* (AY374335.1), Three-spined stickleback;

Gasterosteus aculeatus (JQ673490.1)], **c) Orexin** [Glass catfish; *Kryptopterus vitreolus* (KY770950), Zebrafish; *Danio rerio* (NM_001077392.2), Southern catfish; *Silurus meridionalis* (JQ277478.1), Sheepshead minnow; *Cyprinodon variegatus* (XM_015385951.1), Nile tilapia; *Oreochromis niloticus* (NM_001279703.1), Japanese rice fish; *Oryzias latipes* (XM_011477947.3), Goldfish; *Carassius auratus* (DQ923590.1), Channel catfish; *Ictalurus punctatus* (XM_017452947.1), Atlantic cod; *Gadus morhua* (DQ486137.1)], **d) NPY** [Glass catfish; *Kryptopterus vitreolus* (MH049870), Yellow catfish; *Tachysurus fulvidraco* (JX441993.1), Channel catfish; *Ictalurus punctatus* (NM_001200087.1), Walking catfish; *Clarias batrachus* (KX219789.1), Southern catfish; *Silurus meridionalis* (JX307653.1), Nile tilapia; *Oreochromis niloticus* (KJ778894.1), Goldfish; *Carassius auratus* (M87297.1), Zebrafish; *Danio rerio* (NM_131074.2)], **e) CART** [Glass catfish; *Kryptopterus vitreolus* (KY770951), Channel catfish; *Ictalurus punctatus* (XM_017473585.1), Southern catfish; *Silurus meridionalis* (JX478225.1), Nile tilapia; *Oreochromis niloticus* (XM_003449187.4), Common carp; *Cyprinus carpio* (AM498379.1), Goldfish; *Carassius auratus* CART1 (AY033816.1), Goldfish; CART2 (AY033817.1), Zebrafish; *Danio rerio* CART2 (NM_001017570.1), Zebrafish; CART4 (NM_001082932.1)]. An asterisk (*****) indicates a fully conserved amino acid residue, a colon (**:**) indicates a residue that is highly conserved between similar species, and a period (**.**) indicates partial conservation or conservation between weakly related species.

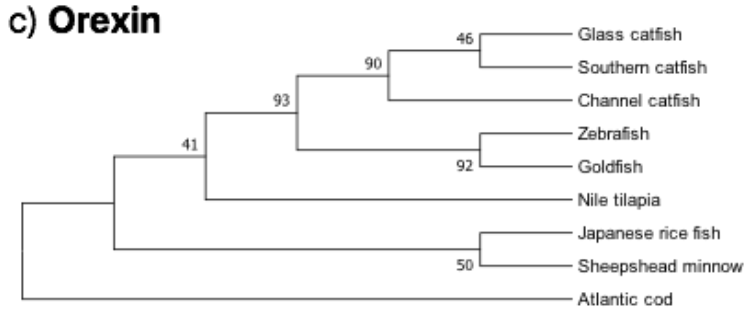
a) **GnRH1**



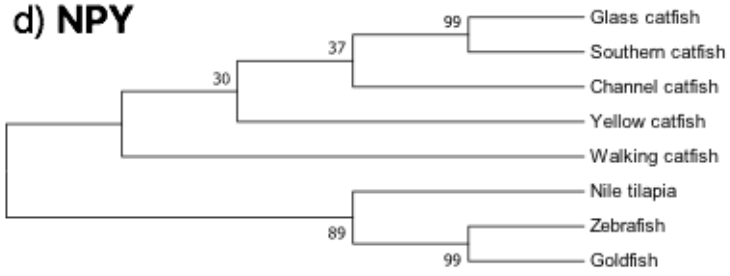
b) **GnRH2**



c) **Orexin**



d) **NPY**



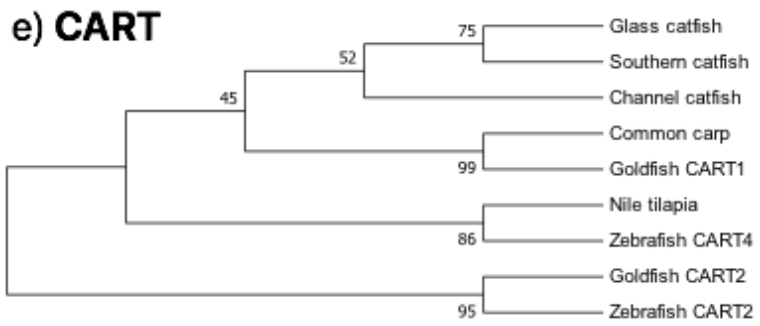


Fig. 2.2: Phylogenetic trees of GnRH1 (a), GnRH2 (b), Orexin (c), NPY (d), and CART (e) among sequences of various fish species (described in Fig. 2.1) using MEGA7 software (*https://www.megasoftware.net/*).

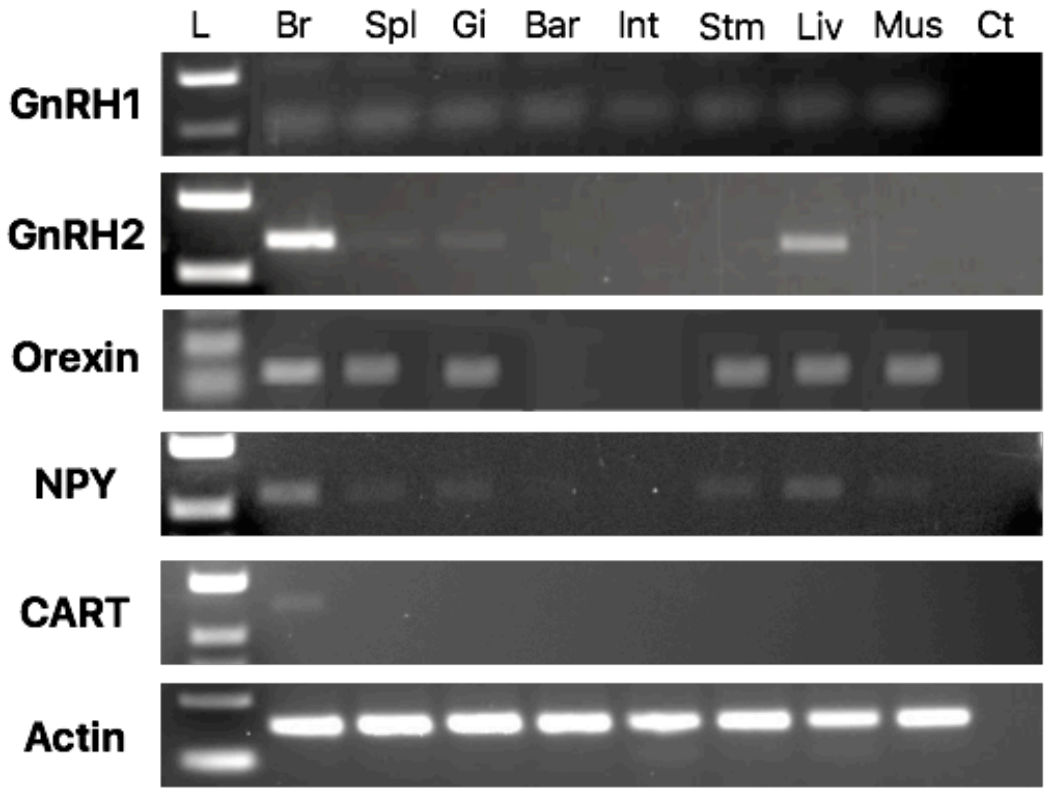


Fig. 2.3: Tissue distribution of GnRH1, GnRH2, Orexin, NPY, and CART in glass catfish (*Kryptopterus vitreolus*). cDNA fragments were amplified using PCR and visualized on a 1.5% agarose gel with ethidium bromide. Actin was used as a control gene. Lanes are labelled as follows (left to right): L (ladder), Br (brain), Spl (spleen), Gi (gill), Bar (barbel), Int (intestine), Stm (stomach), Liv (liver), Mus (muscle), and Ct (water control).

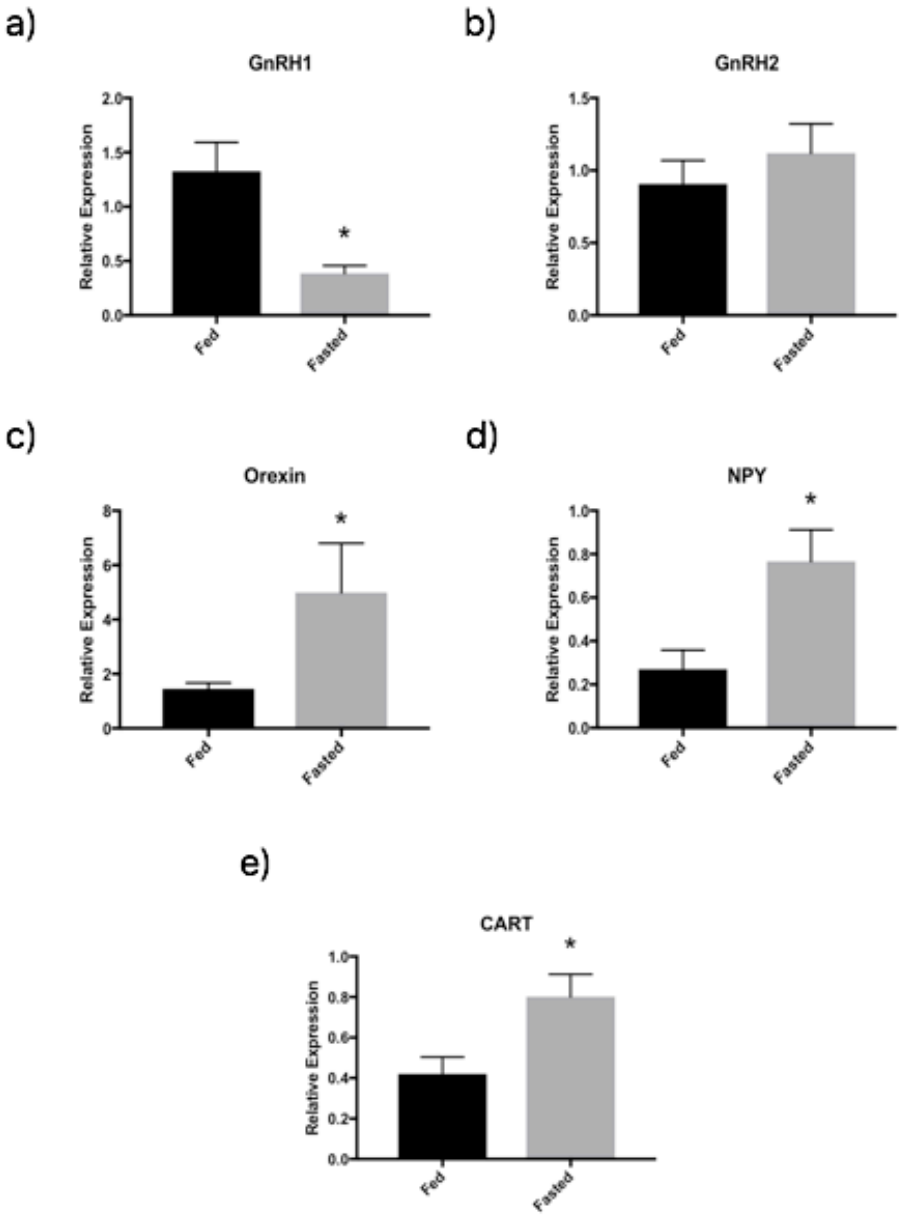


Fig. 2.4: Relative brain mRNA expression of GnRH1 (a), GnRH1 (b), Orexin (c), NPY (d), and CART (e) in glass catfish. Stars (*) indicate significant differences ($p < 0.05$) between fed and fasted conditions. Data are represented as mean $\pm$ SEM.

2.5 Discussion

The goals of this study were to uncover some of the mechanisms that regulate feeding in the glass catfish. I was able to successfully isolate partial mRNA sequences for GnRH1, GnRH2, orexin, NPY, and CART from the brain tissues of glass catfish. All of the isolated peptides were widely distributed among the tissues examined (i.e. brain, spleen, gill, barbel, intestine, stomach, liver and muscle), except for CART, which was only present in the brain. I also investigated the potential roles of these hormones in appetite control by comparing the brain mRNA expression levels between fed and fasted glass catfish. I found that both orexigenic (i.e. NPY and orexin) and anorexigenic (i.e. CART) hormones were up-regulated during fasting. GnRH1 was decreased during fasting, but GnRH2 was unaffected.

2.5.1 Cloning

When compared to sequences from other fish species, those obtained from glass catfish showed high similarity to sequences within the same order (Siluriformes, i.e. catfish), especially in the biologically active portion of the peptide. They were found to be quite similar to close phylogenetically related orders, including Cypriniformes (goldfish, zebrafish) and Perciformes (Nile tilapia).

GnRH1 controls gonadotropin release and often displays species-specific variations in its structure (Millar, 2005; Somoza et al., 2002). The structure of GnRH2, on the other hand, is well conserved among all vertebrate species (Okubo & Nagahama, 2008). This concept seems to be supported by the results of this cloning experiment, where GnRH2 sequence alignments showed a much higher similarity between unrelated species than GnRH1 sequences. The CART

sequence isolated from glass catfish was more similar to CART2 from both goldfish and zebrafish, compared to CART1 in goldfish and CART4 in zebrafish, which is consistent with CART sequences isolated from other catfish species (Kobayashi et al., 2008). Although multiple forms of CART are present in other fish species, it remains unclear if this is the case in catfish.

2.5.2 Tissue distribution

GnRH1 was expressed in all of the tissue types I investigated. The widespread distribution of GnRH1 is consistent with studies in other species of fish including cod (*Gadus morhua*) (Tuziak & Volkoff, 2013a), winter flounder (Tuziak & Volkoff, 2013b), zebrafish (Kuo et al., 2005), and killifish (*Fundulus heteroclitus)* (Ohkubo et al., 2010). The expression of GnRH1 in the brain is consistent with its role of coordinating the release of gonadotropins from the pituitary. The presence of GnRH1 among various peripheral tissues suggests that it may play a role in regulating processes outside of reproduction. For example, presence in the stomach and intestine may indicate involvement in feeding and absorption of nutrients.

In contrast to GnRH1, GnRH2 was expressed mostly in the brain with little to no expression in peripheral tissues. Similar results were found with GnRH3 in Atlantic cod and half-smooth tongue sole (*Cynoglossus semilaevis*). GnRH3 is expressed in the brain and heart but no other peripheral tissues in cod, and only in the brain and gonads in half-smooth tongue sole (Tuziak & Volkoff, 2013a; Zhou et al., 2012). The complete set of functions for GnRH2 is not known, but it is thought to be involved in neuromodulation and is found highly concentrated the midbrain in African catfish (Zandbergen et al., 1995). My results agree with this hypothesized role, as GnRH2 was highly expressed in the brain but not in other peripheral

tissues. This expression pattern, as opposed to GnRH1, may indicate less of a role in reproduction and feeding, and more of an involvement in regulating signal transduction within the brain.

Orexin was expressed in the brain, spleen, gill, intestine, and stomach. This distribution of orexin throughout the brain and peripheral tissues is consistent with that found in other species, including Nile tilapia (Chen et al., 2011), piranha (Volkoff, 2014), cunner (*Tautogolabrus adspersus*) (Babichuk & Volkoff, 2013), pirapitinga (Volkoff, 2015), winter flounder (Buckley et al., 2010), and Atlantic cod (Xu & Volkoff, 2007). Orexin was detected in several peripheral tissues in glass catfish, however expression levels were fairly low. Orexin is a hypothalamic peptide that stimulates feeding, therefore expression in the brain and digestive organs (i.e. intestine and stomach) would be expected and is consistent with the known action of orexin in vertebrates.

NPY was detected in brain and peripheral tissues, including stomach. NPY is detected in peripheral tissues in winter skate (*Raja ocellate*) (MacDonald & Volkoff, 2009), Chinese perch (Liang et al., 2007), Atlantic salmon (Murashita et al., 2009), blunt snout bream (Ji et al., 2015), and northern snakehead (Yang et al., 2018), though in all species expression is highest in the brain. My results are consistent with the main function of NPY in appetite regulation, but its distribution among various tissues may explain the many other proposed actions of NPY including involvement in reproduction (Subhedar et al., 2005), learning and memory (Gøtzsche & Woldbye, 2016), neurodegenerative diseases (Duarte-Neves et al., 2016), circadian rhythms, pain, cardiovascular and blood pressure regulation, and catecholamine release (Silva et al., 2002).

CART was only detected in the brain, with no expression seen in any peripheral tissue. This restricted distribution is consistent with that obtained in pirapitinga (Volkoff, 2015) and channel catfish, in which CART is expressed in brain and testis only (Kobayashi et al., 2008). In other species, such as Atlantic salmon (Murashita et al., 2009), carp (Wan et al., 2012), cod (Kehoe & Volkoff, 2007), and cunner (Babichuk & Volkoff, 2013), although CART is present in peripheral tissues, its expression is highest within the brain. This discrepancy could be due to the presence of multiple forms of CART in some fish species. For example, goldfish have two CART peptides, medaka has at least six distinct CART genes, all displaying slightly different distribution patterns (Volkoff & Peter, 2000; Murashita & Kurokawa, 2011). It remains unclear if there are multiple forms of CART present in catfish.

One limitation in this study was exclusion of the gonads in the tissue distribution. Unfortunately, I was unable to identify or collect gonads during sampling.

2.5.3 Fasting experiments

During this experiment, glass catfish were either fed daily to satiation or subject to food deprivation for 7 days. Brain mRNA expression levels of GnRH1, GnRH2, orexin, NPY and CART were assessed and compared between groups in order to investigate the effects of fasting on the regulation of these neuropeptides.

Fasting significantly decreased brain expression levels of GnRH1 in glass catfish, while GnRH2 expression was unaffected by nutritional status. Many teleost species possess three forms of GnRH; GnRH1, GnRH2, and GnRH3. GnRH3 is an isoform unique to fish and is thought to be involved in other reproductive functions besides the well-established regulation of the

pituitary (Zohar et al., 2010). Catfish, however, do not have this third isoform, possessing only GnRH1 and GnRH2. Instead, it has been shown that they display two distinct populations of GnRH1 within the brain, in addition to GnRH2 in the midbrain (Dubois et al., 2001). Zebrafish and goldfish, on the other hand, have only GnRH2 and GnRH3 but not GnRH1. ICV injections of GnRH2 in both these species decreases food intake, while injection of GnRH3 in goldfish does not affect feeding (Matsuda et al., 2008; Nishiguchi et al., 2012). In winter flounder, fasting decreases expression of both GnRH2 and GnRH3 in certain brain areas but does not affect GnRH1 expression (Tuziak & Volkoff, 2013b). Similar to zebrafish and goldfish, Atlantic cod have two functional GnRHs (GnRH2 and GnRH3), and food deprivation in these fish does not affect expression of either GnRH isoform (Tuziak & Volkoff, 2013a).

My results are consistent with those found in *Astatotilapia burtoni*, a mouthbrooding African cichlid fish, for which fasting induced a decrease in GnRH1 expression but no change in GnRH2 levels (Grone et al., 2012). These results suggest that different forms of GnRH are affected by fasting differently in a species-specific manner. It has been suggested that fasting-induced changes may be region specific. In this study, I analyzed whole brain samples and could thus not assess the effects of fasting on particular brain regions. Therefore, it is possible that fasting affects GnRH2 levels in specific areas but this effect is too small to be detected when whole brains are examined. Another possibility is that GnRH1 is more directly involved in the coordination of reproduction and appetite in the catfish brain, while GnRH2 plays less of a role in nutrition and has more of a neuromodulatory or strictly reproductive function (Okubo & Nagahama, 2008; Tuziak & Volkoff, 2013b).

Orexin is a powerful appetite stimulator in both mammals and fish (Sakurai, 2002). In my study, fasting increased mRNA expression of orexin in glass catfish, which is consistent with results in other fish species including winter flounder (Buckley et al., 2010), goldfish (Nakamachi et al., 2006), red-bellied piranha (Volkoff, 2014) and zebrafish (Novak et al., 2005), as well as in mammals (Korczynski et al., 2006). These results are consistent with studies showing that administration of orexin increases food intake in goldfish (Volkoff et al., 1999), cavefish (Penney & Volkoff, 2014) and zebrafish (Yokobori et al., 2011).

NPY is an orexigenic factor in both fish (e.g. Volkoff & Peter, 2001). and mammals (e.g. Shahjahan et al., 2014) and its expression would be expected to increase during fasting. This was observed in glass catfish, with fasted fish expressing higher levels of NPY than fed fish. Comparable results have been shown in channel catfish (Peterson et al., 2012), blunt snout bream (Ji et al., 2015), and northern snakehead (Yang et al., 2018).

Unlike NPY and orexin, CART is an anorexigenic factor and is known to inhibit food intake in vertebrates (Kuhar et al., 2002). Based on this, expression of CART would be expected to decrease during fasting. This has been shown in channel catfish, where expression of CART was higher in fed compared to fasted fish (Peterson et al., 2012; Kobayashi et al., 2008). In my study, however, a significant increase in CART mRNA expression was observed in fasted glass catfish, suggesting that CART might not act as an anorexigenic factor in this species. In addition to appetite regulation, CART has been shown to affect locomotion. Treatment with CART not only decreases food intake, but also increases locomotor activity and enhances anxiety and fear responses in rats (Kimmel et al., 2000; Kask et al., 2000). In goldfish, administration of CART peptides increases swimming, searching behaviours, and aggression (Volkoff & Peter, 2000). An

increase in CART, therefore, could be indicative of increased foraging behaviours in fasted fish. Another possibility is the presence of other forms of CART that may be more sensitive to fasting. In this experiment, I only assessed the response of one CART to food restriction, however, there is likely multiple forms present and it could be that these other forms may be more involved in energy homeostasis.

2.6 Conclusion

Overall, I was able to successfully isolate two reproductive peptides (GnRH1 and GnRH2) and three appetite-regulating hormones (NPY, orexin, and CART) from the glass catfish. Their widespread distributions in catfish brain and peripheral tissues suggest involvement in several physiological processes, including the regulation of appetite and reproduction. I have shown that nutritional status affects the expression of these peptides, with the exception of GnRH2. The regulation of appetite and reproduction is very complex and involves the interactions of a large number of peptides and hormones. My study provides new insights on the endocrine mechanisms of these two important processes in fish, by showing that food restriction affects the regulation not only of supposed appetite-regulators, but also of reproductive hormones in the glass catfish.

Chapter 3: The Regulation of Feeding and Reproduction in Two Strains of Zebrafish (*Danio rerio*)

3.1 Abstract

Appetite and reproduction are closely related functions that are both regulated by brain hormones. Appetite stimulators include orexin and neuropeptide Y (NPY), and reproductive hormones include gonadotropin-releasing hormone (GnRH), gonadotropin-inhibitory hormone (GnIH), kisspeptin, and neurokinin B (NKB). GnRH stimulates the secretion of pituitary gonadotropes, and its release is modulated by kisspeptin and GnIH. Kisspeptin is further controlled by two co-localized peptides; NKB and dynorphin A (Dyn). To better understand the mechanisms regulating appetite and reproduction in fish, I examined the effects of nutritional status, reproductive stage, gender, and strain on the brain mRNA expression of appetite (orexin and NPY) and reproductive (GnRH, kisspeptin, GnIH, and NKB) hormones in zebrafish. In order to compare strains, I used both wild-type and transparent *Casper* zebrafish. In wild-type zebrafish, fasting increased the expression of all hormones investigated, with wild-type females generally being more affected than wild-type males. In *Casper* zebrafish, only GnIH and NKB in males were affected by fasting, suggesting that *Casper* fish may be more resistant to fasting. Females with more eggs had higher levels of GnRH and kisspeptin in wild-type zebrafish, and higher levels of NKB in *Casper* zebrafish. To investigate whether differences between *Casper* and wild-type fish were due to genes involved in pigmentation, I compared the brain mRNA expression of enzymes involved in melanin synthesis (tyrosinase and tyrosine hydroxylase – TH), melanocortin receptors (Mc3r and Mc4r), and the melanocortin precursor (proopiomelanocortin – pomc) between the two strains. *Casper* zebrafish had lower levels of

Mc3r, tyrosinase, TH1, TH2, and pomc than wild-type fish. Overall, my results suggest the existence of gender- and reproductive stage-specific, as well as strain-specific variations in the mechanisms regulating feeding and reproduction in zebrafish, and that the melanocortin system and melanin pathways may be underlying these differences between strains.

3.2 Introduction

The coordination between appetite and reproduction is controlled mainly by hormones, many of which are produced in the brain. Important reproductive hormones include gonadotropin-releasing hormone (GnRH), gonadotropin-inhibitory hormone (GnIH), kisspeptin, and neurokinin B (NKB). Appetite regulators are categorized as orexigenic (stimulate food intake, such as orexin and neuropeptide Y -NPY) or anorexigenic (inhibit food intake). Although the majority of these regulating hormones were first isolated in mammals, a number of them have been identified in fish.

Gonadotropin-releasing hormone (GnRH) is a critical component of the hypothalamus-pituitary-gonadal (HPG) axis responsible for controlling the reproductive cycle (Shahjahan et al., 2014). In fish, as in most other vertebrates, GnRH released from the hypothalamus stimulates the pituitary gland (specifically gonadotrope cells) to release gonadotropins, including follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (Shahjahan et al., 2014). The gonadotropins then stimulate the gonads to develop and release sex steroids, which leads to gametogenesis and sexual behaviour. Fish can have up to three forms of GnRH along with two receptor types (Roch et al., 2014; Carolsfeld et al., 2000). GnRH1 is found mainly in the brain, in particular the hypothalamus, and is involved in the regulation of gonadotropin release (Zohar et

al., 2010). GnRH2 is present in the midbrain, and is thought to have a neuromodulatory function, though its exact role is still unknown. In teleosts that possess three forms, GnRH3 is located around the olfactory bulb and is thought to be associated with reproductive behaviour (Okubo & Nagahama, 2008). Zebrafish (*Danio rerio*) have two forms of GnRH; GnRH2 and GnRH3. In this case, GnRH3 is found around the olfactory bulb and in the hypothalamus, and regulates pituitary function, suggesting that GnRH3 assumes the role of GnRH1 in zebrafish (Liu et al., 2017; Zohar et al., 2010).

The regulation of GnRH involves two important RFamide peptides (peptides containing a C-terminal Arg-Phe-NH_2 motif): kisspeptin and gonadotropin-inhibitory hormone (GnIH) (Fig. 3.1). In mammals, kisspeptin is a potent GnRH secretagogue, acting directly on GnRH neurons (Ohtaki et al., 2001; Roa et al., 2008). In teleost fish, such as zebrafish, there are two kisspeptin genes (*kiss1* and *kiss2*) and two receptors (Kiss-R1 and Kiss-R2), which regulate the HPG axis (Parhar et al., 2016; Ogawa et al., 2012; Kitahashi et al., 2009) and are involved in the initiation of sexual maturation (Kitahashi, 2009). A separate study, however, reports that zebrafish lacking *kiss1* and *kiss2* genes display no deficits in reproduction, suggesting that kisspeptins are not always required for reproduction in this species, as was previously thought (Liu et al, 2017).

GnIH was originally discovered in 2000 in Japanese quail (*Coturnix japonica*) as an LH inhibitor (Tsutsiu et al., 2000) and was later identified in almost all vertebrate classes (Tsutsiu et al., 2018). In mammals and birds, GnIH prevents gonadotropin release by inhibiting GnRH neurons, kisspeptin neurons, and pituitary gonadotropes directly (Parhar et al., 2016). The physiological function of GnIH in fish and its role in reproduction, is still widely unknown. Injections of GnIH in immature female goldfish (*Carassius auratus*) decrease GnRH, FSH, and LH

mRNA levels (Qi et al., 2013). However, administration of GnIH to pituitary cell cultures in mature female Nile tilapia (*Oreochromis niloticus*) increases LH and FSH release (Biran et al., 2014). These results suggest that the role of GnIH in fish may depend on sexual maturity.

GnRH is released in a pulsatile manner. In mammals, although the mechanism of this episodic secretion is not fully known, two neuropeptides have been implicated in its control; neurokinin B (NKB) and dynorphin A (Dyn) (Fig. 3.1) (Grachev et al., 2014). NKB and Dyn are co-localized with kisspeptin in neuronal populations referred to as KNDy neurons and they stimulate and inhibit kisspeptin release, respectively, creating an "autosynaptic feedback" within these KNDy neurons (Tng, 2015). This oscillation between stimulation and inhibition acts as a fine tuning of kisspeptin release and has been proposed as the underlying regulation of the GnRH pulses (Grachev et al., 2014). NKB is one of the peptides encoded by a tachykinin gene known as Tac3. Zebrafish express two *tac3* genes; *tac3a* and *tac3b* (both expressed in the brain and ovary) (Biran et al., 2012). However, it has been suggested that these "KNDy neurons" do not exist in fish, as there is no evidence of NKB and kisspeptin co-expression in zebrafish (Hu et al., 2014a; Ogawa et al., 2012). Injections of either NKBa and NKBb stimulate LH secretion in zebrafish, with NKBa being more potent than NKBb (Biran et al., 2012) and an increase in whole brain *tac3a* mRNA expression is seen during maturation along with an increase in kisspepting, suggesting that, as in mammals, kisspeptin and NKB play a role in the control of reproduction.

Feeding and reproduction are closely linked processes, as successful reproduction requires adequate resources in order to sustain the high-energy demands for the production of gametes and sexual behaviours (Volkoff and London, 2018). In fish, restricted feeding or fasting has been shown to affect the reproductive performance (Izquierdo et al., 2001) and, very often,

the expression of reproductive hormones. Fasting decreases GnRH2 mRNA expression in Ya fish (*Schizothorax prenanti*) (Wang et al., 2014), and winter flounder (*Pseudopleuronectes americanus*) (Tuziak and Volkoff, 2013a), but does not affect GnRH2 expression in the cichlid fish, *Astatotilapia burtoni* (Grone et al., 2012) or Atlantic cod (*Gadus morhua*) (Tuziak and Volkoff, 2013b), and in zebrafish, GnRH2 mRNA levels are higher in fish fed excess food than those in fish fed a normal ration (Nishiguchi et al., 2012). Fasting decreases *kiss1* expression in both rats (Castellano et al., 2005) and monkeys (Wahab et al., 2011), however in sea bass (*Dicentrarchus labrax*), food restriction induces an increase in kisspeptin expression in the male brain (Escobar et al., 2016).

Currently, there are very little published data on the involvement of GnIH in the regulation of feeding or how it might be affected by nutritional status in fish, however it has been shown to increase food intake in sheep and rats (Clarke et al., 2012). Fasting activates GnIH neurons and decreases sexual motivation in hamsters, though it is not known whether GnIH is causing this shift in behaviour or if the decrease in GnIH is an incidental response to some other change (Klingerman et al., 2011). In chicks, intracerebroventricular (ICV) injections of GnIH not only increase food intake, but increase NPY mRNA and decrease proopiomelanocortin (pomc) mRNA. NPY is a strong appetite stimulator, while pomc is a precursor for peptides that decrease appetite (McConn et al., 2014). These results indicate that GnIH may be involved in the control of feeding through its interaction with other neuropeptides.

In fish, orexin and NPY are two major regulators of feeding. Orexin is an orexigenic factor, that consists of two forms; orexin A and orexin B (Volkoff et al., 2005). It was discovered

in 1998 as a hypothalamic factor that stimulates feeding behaviour in mammals (Sakurai et al., 1998). The same action was later observed in fish, where orexin injections increased food intake in goldfish (Nakamachi et al., 2006), zebrafish (Yokobori et al., 2011), and cavefish (*Astyanax mexicanus*) (Penney & Volkoff, 2014). Orexin mRNA expression is increased during fasting in winter flounder (*Pseudopleuronectes americanus*) (Buckley et al., 2010), red-bellied piranha (*Pygocentrus nattereri*) (Volkoff, 2014), and freshwater dourado (*Salminus brasiliensis*) (Volkoff et al., 2016), suggesting a similar function. Yet, in some fish species, prolonged fasting decreases orexin levels, again indicating species-specific variations (Babichuk & Volkoff, 2013). In mammals, orexin stimulates GnRH neurons to promote gonadotropin release (Sasson et al., 2006). In goldfish, however, orexin negatively interacts with GnRH to inhibit reproduction (Hoskins et al., 2008).

NPY is an orexigenic factor, promoting food intake in both mammals and fish (Shahjahan et al., 2014). Orexin and NPY interact to control feeding behaviour in fish: administration of orexin or NPY alone increases food intake in goldfish, while co-injection with NPY and orexin leads to an increase in food intake that is higher than NPY only. Blocking NPY or orexin receptors decreases both NPY- and orexin-induced feeding, and treatment with orexin leads to a greater expression of NPY mRNA (Volkoff & Peter, 2001).

The *Casper* zebrafish (*nacre*$^{w2/w2}$; *roy*$^{a9/a9}$) is a transgenic model that lacks both melanophores and iridophores, due to mutations in the *mitfa* gene and *mpv17* (a gene encoding a mitochondrial protein), respectively (White et al., 2008). I chose to include these transparent fish to facilitate the identification of gender and sexual maturity. In zebrafish, skin melanophores produce the pigment melanin by conversion of tyrosine into L-DOPA and then

melanin (catalyzed by tyrosinase) (Braash et al., 2007). In catecholaminergic cells of the nervous system, L-DOPA is converted into dopamine [by hydroxylase (TH)], and to catecholamines (dopamine and epinephrine) (Nagatsu, T., 2006). Though very little is known about the physiological functions of melanin and its regulation in fish, several genes have been reported to be involved in the determination of skin color. The melanocortin system includes peptides that are derived from the melanocortin precursor, POMC. These melanocortin peptides act by binding melanocortin receptors, of which most fish have at least five (MC1R to MC5R) (Ronnestad et al., 2017). The melanocortin alpha-melanocyte stimulating hormone (α -MSH) acts on teleost fish skin mainly through melanocortin 1 receptors (MCR1) and stimulates the production (melanogenesis), release and dispersion of melanin within melanophores, causing the skin to darken (Metz et al., 2006; Cerdá-Reverter et al., 2011; Cal et al., 2017). MSH can also bind any of the other four receptors and has been implicated in the control of food intake through interaction with MC3R and MC4R (Ronnestad et al., 2017). POMC is also involved in appetite regulation as an anorexigenic factor in both mammals (Millington, 2007) and zebrafish (Cortés et al., 2018).

The objectives of this study were to uncover some of the mechanism that regulate appetite and reproduction in zebrafish. In order to compare between strains, I used both wild-type zebrafish and *Casper* zebrafish. I included wild-type zebrafish as a control to compare the two strains. First, I assessed how nutritional status affected the expression of certain reproductive hormones (GnRH, kisspeptin, GnIH, and NKB) and appetite regulators (orexin and NPY), by comparing fed and fasted groups. I compared males and females of each group, in order to examine gender-specific differences. I then investigated the effects of reproductive

stage on the brain expression levels of reproductive and appetite regulating hormones in females. During the course of my study, I noted significant differences in the *Casper* zebrafish compared to the wild-type. In order to understand the mechanisms responsible for these discrepancies between these two strains, and if changes in pigmentation were a possible cause, I compared the brain mRNA levels of enzymes involved in melanin synthesis (i.e. tyrosinase and TH), melanocortin receptors (Mc3r and Mc4r), and melanocortin precursor (pomc) between the two strains.

3.3 Materials and methods

3.3.1 Experimental animals

Wild-type and *Casper* zebrafish (*Danio rerio*) were obtained from the Zebrafish Core Facility at Dalhousie University (Halifax, Nova Scotia, Canada). Both strains had similar sizes (average total length of 3.068 ± 0.229 cm and average weight of 0.3416 ± 0.103 g for wild-type zebrafish; average of 3.268 ± 0.232 cm in length and 0.3297 ± 0.0686 g in weight for *Casper* zebrafish). A mixture of females and males from each strain were divided and acclimated into 4 separate 60 L tanks. Fish were maintained at a water temperature of 28^{o}C under a simulated photoperiod of 12H light:12H dark and fed to satiety once daily with tropical fish flakes (45% protein, 10% fat, 1.6% Omega-6 fatty acids, 1.6% Omega-3 fatty acids, 1.7% fibre, 7% moisture, 8.5% ash; Rolf C. Hagen Inc., Montreal, Quebec, Canada). Fish were acclimated under these conditions for one week prior to the start of any experimentation. All experiments were carried out in accordance with the principles published in the Canadian Council on Animal Care's guide to the care and use of experimental animals.

3.3.2 Fasting experiments

Fifty zebrafish of both strains (twenty-five females and twenty-five males) were randomly separated into 4 tanks (Table 3.1) and acclimated to these conditions for one week. After the acclimation period, two tanks continued to be fed once daily to satiation and two tanks were fasted for one week. Fish were sacrificed after 7 days by immersion in 0.05% tricaine methanesulfonate (MS 222) (Syndel Laboratories, Vancouver, British Columbia, Canada) followed by a spinal section. Gender was determined based on abdomen shape, colouring, and the presence or absence of ova. The length, width, weight and sex of each fish were recorded. For mature female fish with ova, gonadal mass was recorded. Whole brains were collected and preserved in RNA*later* (Qiagen, Mississauga, Ontario, Canada) at -20^{o}C. RNA extraction and cDNA synthesis were performed, and gene expression levels were assessed using quantitative PCR (qPCR), as described below. Brain mRNA expression was compared between fed and fasted groups, as well as between genders within each group.

3.3.3 Reproductive stage experiments

Female zebrafish from the above fasting experiments were separated into two groups based on their gonadosomatic index (GSI). GSI was calculated as the proportion of gonadal mass relative to the total body mass (i.e. $\frac{weight\ of\ eggs\ (g)}{total\ body\ weight\ (g)}$ x 100%) and was used as an indicator of reproductive stage. Male gonads (i.e. sperm) was not able to be collected during dissection, therefore only female fish were included in this experiment. Table 3.2 shows how *Casper* and wild-type females were subdivided based on GSI. Brain mRNA expression levels

were compared between reproductive stages among fed females, as well as fasted females, using qPCR (see below sections for full procedure).

3.3.4 Melanocortin expression

The expression levels of genes involved in the melanocortin system were compared between wild-type and *Casper* zebrafish, using fed and fasted fish of both sexes from the above fasting experiments. Brain mRNA expression was assessed and compared using qPCR (see below sections for full procedure).

3.3.5 RNA extraction and cDNA synthesis

RNA extractions were performed using a FastRNA Green Kit (MP Bio, Solon, Ohio, USA), following the manufacturer's protocol. Final RNA concentrations were quantified by optical density reading at 260 nm using a NanoDrop 2000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, Delaware, USA). All samples had absorbance ratios at 260 and 280 nm between 1.6 and 2.0.

200 ng of RNA from each brain tissue was reverse transcribed using a Verso cDNA Synthesis Kit (Thermo Fisher Scientific, Lafayette, Colorado, USA) following the manufacturer's protocol. All cDNAs were stored at -20^{o}C until further use.

3.3.6 Quantitative PCR (qPCR)

In order to quantify gene expression, specific qPCR primers were designed for GnRH2, GnRH3, kiss1, kiss2, GnIH, NKB, NPY, and orexin using Primer3 software (*http://bioinfo.ut.ee/primer3-0.4.0/*) based on available zebrafish sequences from the National

Center for Biotechnology Information (NCBI; *https://www.ncbi.nlm.nih.gov/*). Multiple primer sets were designed for each gene and optimized for qPCR using a serial dilution of cDNA. Primers were designed to anneal at approximately the same temperature (57-58^{o}C). Only primers with the highest efficiency and linearity were used in experimentation (see Table 3.3 for primers used in fasting and reproductive stage experiments and Table 3.4 for primers used for melanocortin expression). For fasting and reproductive stage experiments, duplicate reactions were prepared using a mix containing 0.2 μl 10 μl forward primer, 0.2 μl 10 μl reverse primer, 2.6 μl water, 5 μl SYBR FAST qPCR Master Mix (Kapa Biosystems, Boston, Massachusetts, USA), and 2 μl cDNA (diluted 1:3 with water) for a total volume of 10 μl. 96-well plates were loaded with an epMotion® 5070 automated pipetting system (Eppendorf, Mississauga, Ontario, Canada). The following cycling conditions were performed using a MasterCycler® Realplex 2S thermocycler (Eppendorf): 40 cycles; 1) Denaturation (94^{o}C for 30 seconds), 2) Annealing (57^{o}C for 45 seconds), and 3) Elongation (72^{o}C for 60 seconds). For the melanocortin expression, duplicate reactions were prepared containing 5 μl Sso Advanced™ Universal SYBR® Green Supermix (BioRad, Hercules, California, USA), 0.5 μl water, 0.25 μl 10 μM forward primer, 0.25 μl 10 μM reverse primer, and 4 μl cDNA (diluted 1:3 with water). The total reaction volume (10 μl) was then loaded into a 96-well plate. The following cycling conditions were performed using the CFX96 Touch™ Real-Time PCR Detection System (BioRad): 40 cycles of; 1) Denaturation (95^{o}C for 30 seconds), 2) Annealing (57^{o}C for 45 seconds0, and 3) Elongation (72^{o}C for 60 seconds). Efficiencies, R^2 values, and optimal annealing temperatures were determined for all primer pairs. A melting curve analysis was performed at the end of each qPCR to verify primer specificity. Several candidate genes were tested for use as reference

genes including 18S, actin, elongation factor α-1 (EF), and ribosomal protein L (RPL). Using NormFinder Software (*https://moma.dk/normfinder-software*), it was determined the EF was the most stable reference gene across all groups.

3.3.7 qPCR analysis

For the fasting and reproductive stage experiments, gene expression levels were measured and quantified using Realplex 1.5 software (Eppendorf). For the melanocortin expression experiment, gene expression levels were measured and quantified using the CFX Maestro software (BioRad). Both types of software compared expression levels using the $\Delta\Delta$Ct method. Gene expression levels were normalized to EF. Statistical analysis was then performed using Prism7 GraphPad Instat program (GraphPad Software Inc., San Diego, California, USA). Data was tested for normality using a Shapiro-Wilk normality test. An unpaired t-test was used to compare qPCR results for normal data, while non-normal data was analyzed using a non-parametric Mann-Whitney t-test. Results were compared between groups, with significance set at $p < 0.05$.

Table 3.1: Experimental setup for zebrafish fasting experiment showing numbers and gender of fish in each tank

Tank	Treatment	# of Females		# of Males	
		***Casper* Zebrafish**	**Wild-type Zebrafish**	***Casper* Zebrafish**	**Wild-type Zebrafish**
1	Fed	4	6	5	7
2	Fasted	6	7	4	6
3	Fed	4	6	6	6
4	Fasted	1	6	7	6

Table 3.2: Experimental setup of reproductive stage experiment, including treatment, GSI, and classification of female zebrafish used. Fed fish were classified based on a GSI higher or lower than 3.2% (i.e. "Small GSI" = GSI < 3.2%, "Large GSI" = GSI > 3.2%). Fasted fish were classified by GSI greater or less than 5% in wild-type fish and 7.5% in *Casper* zebrafish (i.e. For fasted wild-type fish: "Small GSI" = GSI < 5%, "Large GSI" = GSI > 5%; For fasted *Casper* zebrafish: "Small GSI" = GSI < 7.5%, "Large GSI" = GSI > 7.5%).

Wild-type Zebrafish				***Casper* Zebrafish**			
Treatment	**Grouping**	**Mean GSI**	**Number of Females**	**Treatment**	**Grouping**	**Mean GSI**	**Number of Females**
Fed	Small GSI	2.2%	5	Fed	Small GSI	2.78%	3
Fed	Large GSI	4.5%	3	Fed	Large GSI	5.4%	3
Fasted	Small GSI	3.02%	6	Fasted	Small GSI	5.62%	3
Fasted	Large GSI	7.95%	6	Fasted	Large GSI	7.99%	3

Table 3.3: Zebrafish primers used for qPCR analysis in fasting and reproductive stage experiments with corresponding GenBank Accession ID (GnRH2: gonadotropin releasing hormone 2; GnRH3: gonadotropin releasing hormone 3; Kiss1: kisspeptin 1; Kiss2: kisspeptin 2; GnIH: gonadotropin inhibitory hormone; NKB: neurokinin B; NPY: neuropeptide Y; OX: orexin; EF: elongation factor)

Target Gene	Primer	Sequence (5'→3')	GenBank ID
GnRH2	GnRH2-F	5' ACATCCTCAAGACAATACTGCTGGA 3'	NM_181439.4
	GnRH2-R	5' GAAAAGGCAGGCCAAATGTG 3'	
GnRH3	GnRH3-F	5' TGGTCCAGTTGTTGCTGTTAGTT 3'	NM_182887.2
	GnRH3-R	5' CCTGAATGTTGCCTCCATTTC 3'	
Kiss1	Kiss1-F	5' CCCTCTGGGCATTTTCAGTA 3'	NM_001113489.1
	Kiss1-R	5' ATGGAGAAGAGCGCTGAGAG 3'	
Kiss2	Kiss2-F	5' GCCTATGCCAGACCCCAAA 3'	NM_001142585
	Kiss2-R	5' TTTACTGCGTGCTAGTCGATGTTT 3'	
GnIH	GnIH-F	5' TCCTGAGCAGCTTCATGCTA 3'	NM_001082949.1
	GnIH-R	5' GGGGCCACATTAAGAGTGAA 3'	
NKB	NKB-F	5' GGAGCGCTACGACAAACGAT 3'	NM_001256390.1
	NKB-R	5' CACCACAGCAAAACCTCAGTC 3'	
NPY	NPY-F	5' CCAAACATGAAGATGTGGATGAG 3'	NM_131074.2
	NPY-R	5' CCAAGCAGACGAACAAGAGAAA 3'	
Orexin	OX-F	5' CTACGAGATGCTGTGCCGAG 3'	NM_001077392
	OX-R	5' CCAAGAGTGAGAATCCCGAC 3'	
EF	EF-F	5' ACCCTCCTCTTGGTCGCTTT 3'	NM_131263.1
	EF-R	5' CCGATTTTCTTCTCAACGCTCTT 3'	

Table 3.4: Zebrafish primers used for qPCR analysis in melanocortin expression experiment with corresponding GenBank Accession ID (Mc3r: melanocortin 3 receptor; Mc4r: melanocortin 4 receptor; Tyr: tyrosinase; pomc: proopiomelanocortin a; TH1: tyrosine hydroxylase 1; TH2: tyrosine hydroxylase 2).

Target Gene	Primer	Sequence (5'→3')	GenBank ID
Mc3r	Mc3r-F	5' GAGAATTGCAGCATTGCCCC 3'	NM_180972.2
	Mc3r-R	5' GAGCGGGTCAATCACAGAGT 3'	
Mc4r	Mc4r-F	5' ATTCATTTCGGAACCACAGC 3'	NM_173278.1
	Mc4r-R	5' CGAAGCATTGGAGACACTCA 3'	
Tyr	Tyr-F	5' CATCCTGGTGTCCGACCTTC 3'	NM_131013.3
	Tyr-R	5' TGAACCTCTGCCTCTCGGTA 3'	
Pomc	pomc-F	5' AGGGGAGTGAGGATGTTGTG 3'	NM_181438.3
	pomc-R	5' TCCGGCTCTATCTGTTCAGG 3'	
TH1	TH1-F	5' GAACATGGCGGGAGGTCTAC 3'	NM_131149.1
	TH1-R	5' GAGGAAGCGTGCCGTATGTA 3'	
TH2	TH2-F	5' AAAGGCTTATGGGGCTGGAC 3'	NM_001001829.1
	TH2-R	5' GCTGCAAGTGTAGGGGTCAT 3'	

3.4 Results

3.4.1 Effects of fasting on gene expression

3.4.1.1 Appetite-regulating hormones

In wild-type zebrafish, fasting significantly increased levels of NPY and orexin, with gender-specific differences: NPY expression was increased in males, while orexin was up-regulated in females only (Fig. 3.2a). Fasting did not affect either appetite-regulating hormone (i.e. NPY or orexin) in male or female *Casper* zebrafish (Fig. 3.2b).

3.4.1.2 Reproductive hormones

In wild-type fish, fasting resulted in a significant increase in all reproductive hormones in pooled males and females (Fig. 3.3a). When separated by gender, increases in GnRH2, GnRH3, Kiss1, and GnIH were significant only in females, while Kiss2 was significantly increased in male fish only. No gender differences were noted in NKB, as expression was significantly increased in both males and females. In *Casper* zebrafish, fasting increased GnIH expression when males and females were pooled, but no significant difference was seen in either gender individually (Fig. 3.3b). Fasting increased NKB in males but did not significantly alter the expression of any other reproductive hormone (GnRH2, GnRH3, Kiss1, Kiss2).

3.4.2 Effects of reproductive stage on response to fasting in females

3.4.2.1 Wild-type zebrafish

In fed wild-type females, GnRH2, GnRH3 and NPY mRNA expressions were higher in females with more eggs (Fig. 3.4). No significant differences were seen in the expression of

Kiss1, Kiss2, GnIH, NKB, or orexin. In fasted wild-type fish, females with more eggs had higher mRNA expression levels of GnRH2, Kiss1, GnIH, and orexin (Fig. 3.5). No significant differences were seen in the expression of GnRH3, Kiss2, NKB, or NPY between reproductive stages in females.

3.4.2.2 Casper zebrafish

In *Casper* females, fish with larger GSIs had higher expression of NKB (Fig. 3.6). No other significant differences were noted between reproductive stages. In female fasted *Casper* fish, none of the reproductive or appetite regulating peptides showed significant differences in expression between reproductive stages (Fig. 3.7).

3.4.3 Strain-specific differences in the melanocortin system

The brain mRNA expression of Mc3r, tyrosinase, pomc, TH1, and TH2 were significantly lower in *Casper* zebrafish compared to wild-type zebrafish. No significant difference was noted in the expression of Mc4r (Fig. 3.8). In fasted fish, the brain expression of Mc4r was significantly higher in *Casper* zebrafish compared to wild-type fish. No significant differences were noted in the expression of Mc3r and pomc (Fig. 3.9).

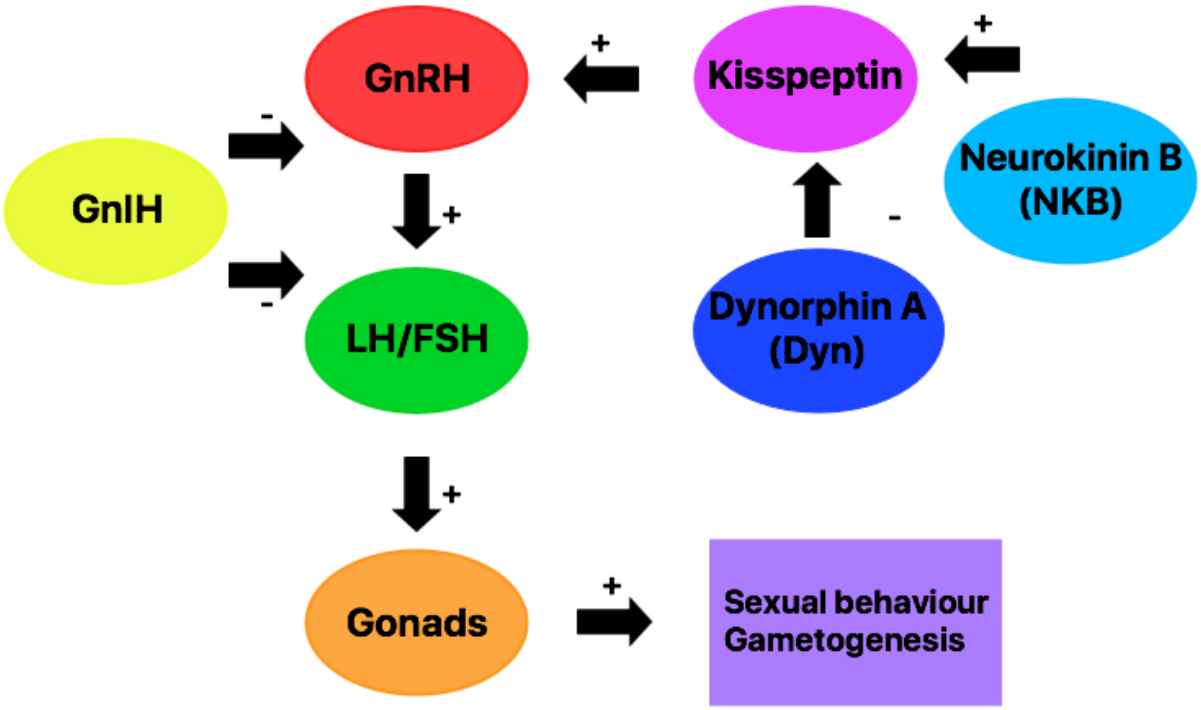


Figure 3.1: Summary of actions and interactions between major reproductive hormones, including GnRH, GnIH, kisspeptin, NKB, and Dyn.

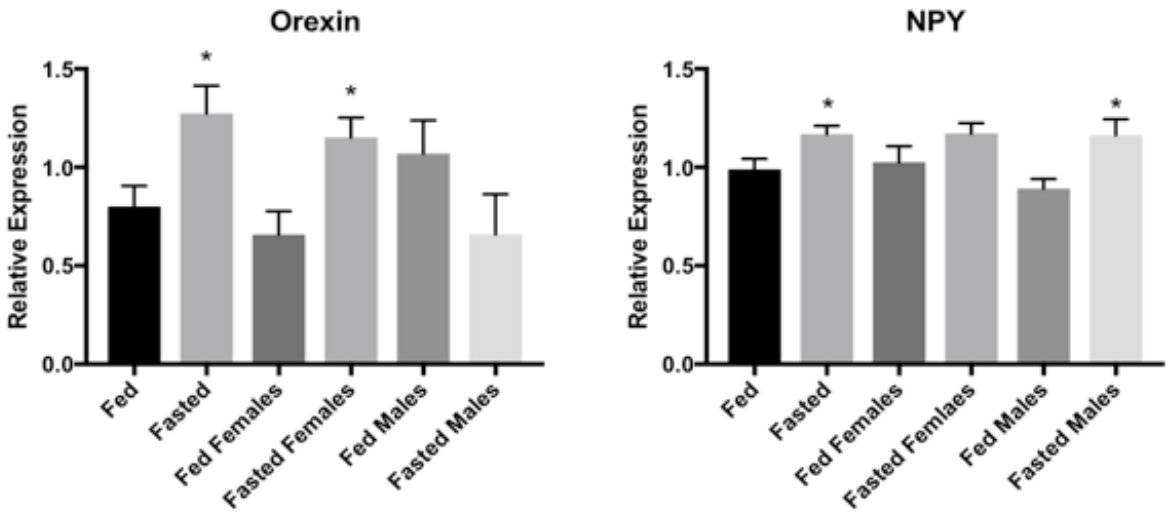


Figure 3.2a: Relative brain mRNA expression of orexin and NPY in wild-type zebrafish. Stars (*) indicate significant differences ($p < 0.05$) between fed and fasted conditions. Data are represented as mean $\pm$ SEM.

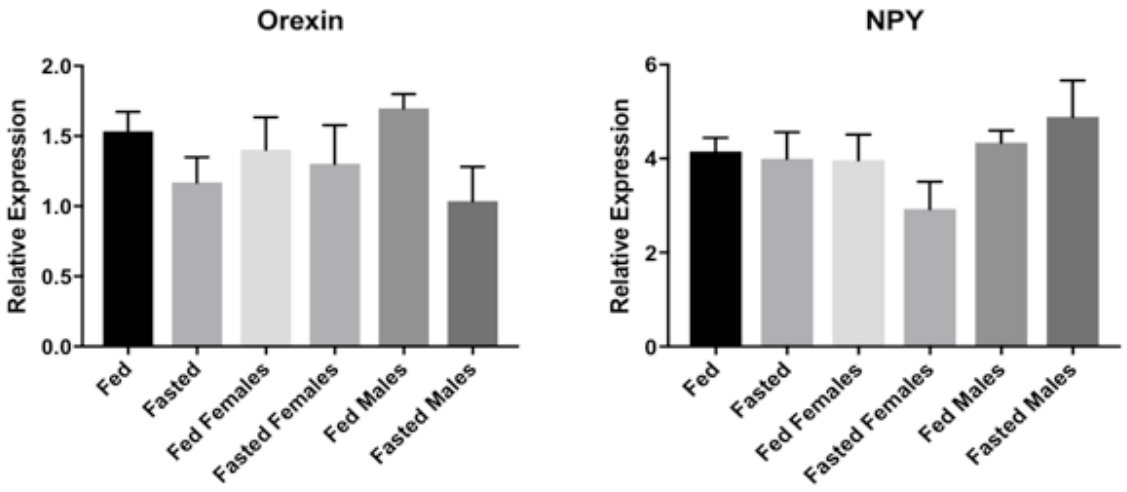


Figure 3.2b: Relative brain mRNA expression of orexin and NPY in *Casper* zebrafish. Data are represented as mean ± SEM.

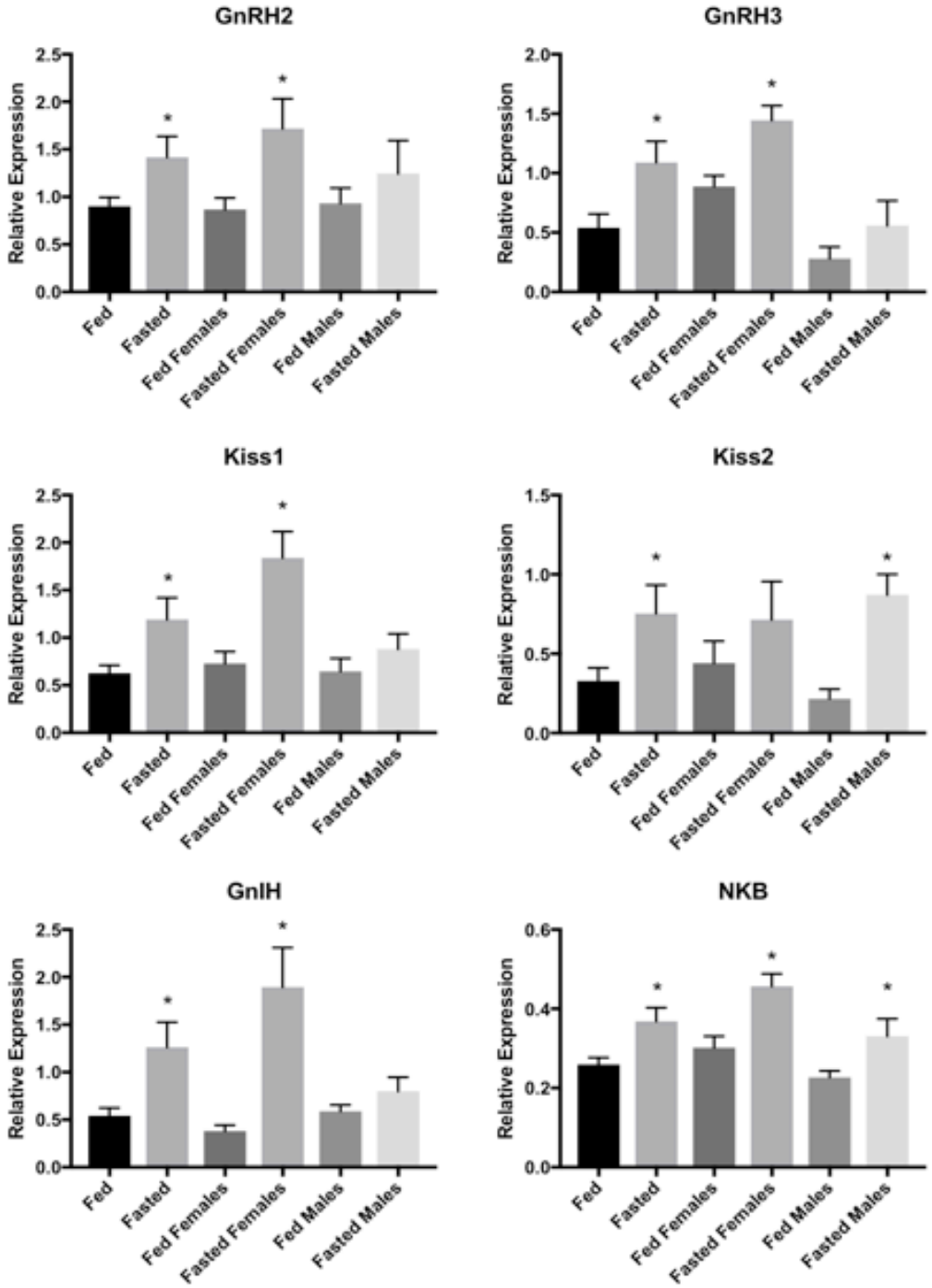


Figure 3.3a: Relative brain mRNA expression of GnRH2, GnRH3, Kiss1, Kiss2, GnIH and NKB in wild-type zebrafish. Stars (*) indicate significant differences ($p < 0.05$) between fed and fasted conditions. Data are represented as mean $\pm$ SEM.

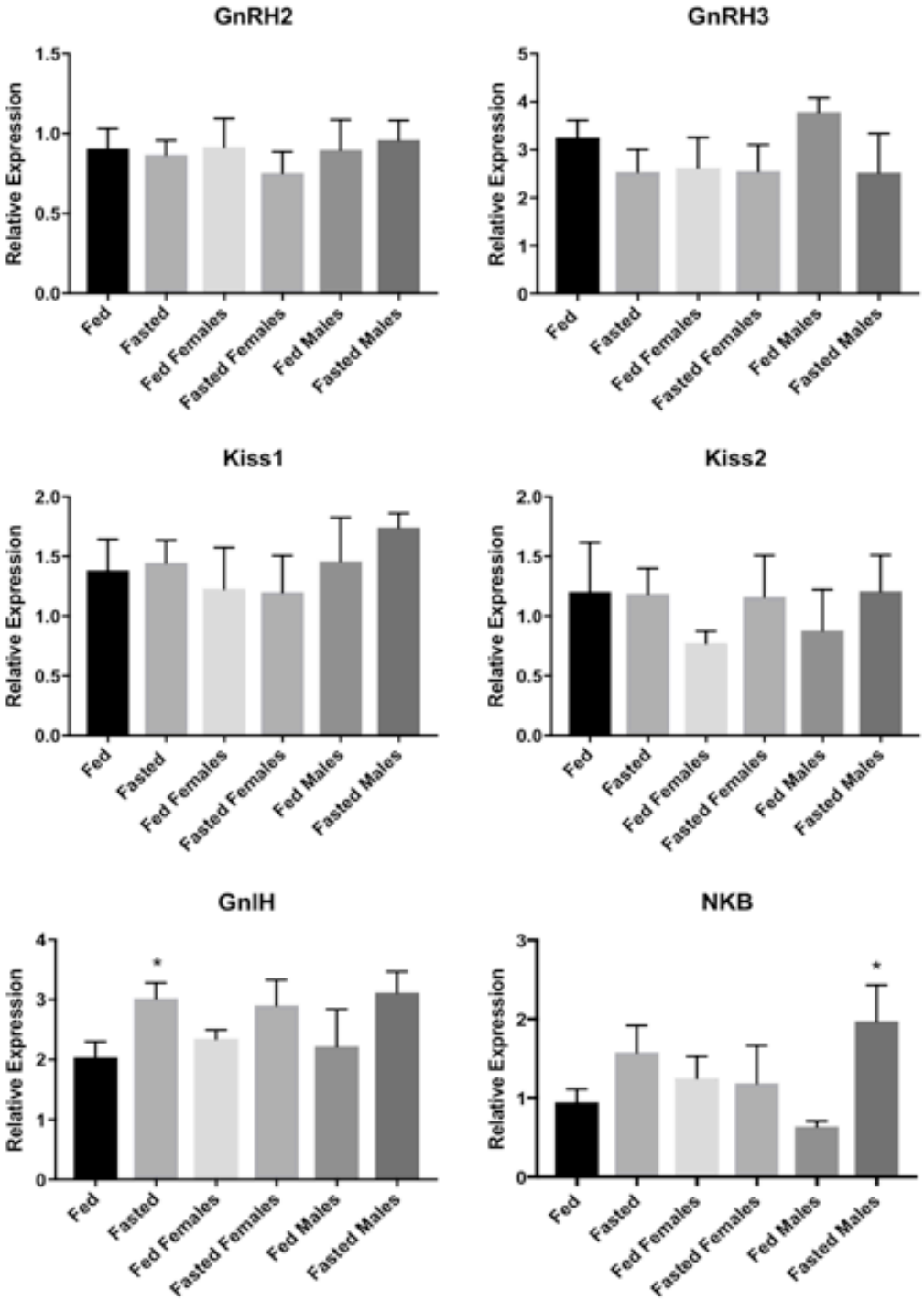


Figure 3.3b: Relative brain mRNA expression of GnRH2, GnRH3, Kiss1, Kiss2, GnIH and NKB in *Casper* zebrafish. Stars (*) indicate significant differences ($p < 0.05$) between fed and fasted conditions. Data are represented as mean $\pm$ SEM.

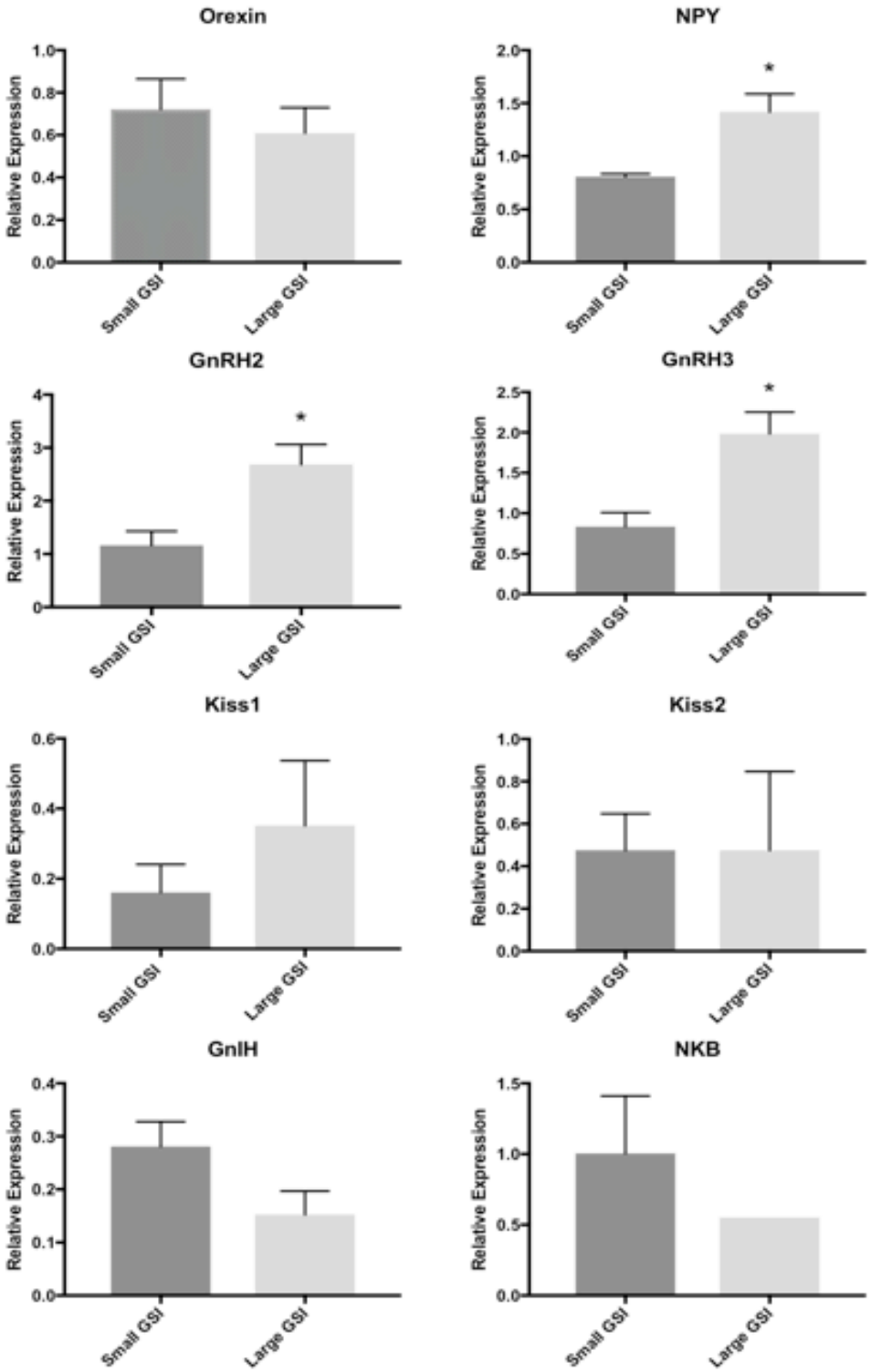


Figure 3.4: Relative brain mRNA expression of orexin, NPY, GnRH2, GnRH3, Kiss1, Kiss2, GnIH, and NKB in fed female wild-type zebrafish. Significant differences ($p < 0.05$) between females

with a small GSI (< 3.2%) and females with a large GSI (> 3.2%) are indicated by stars (*). Data are represented as mean ± SEM.

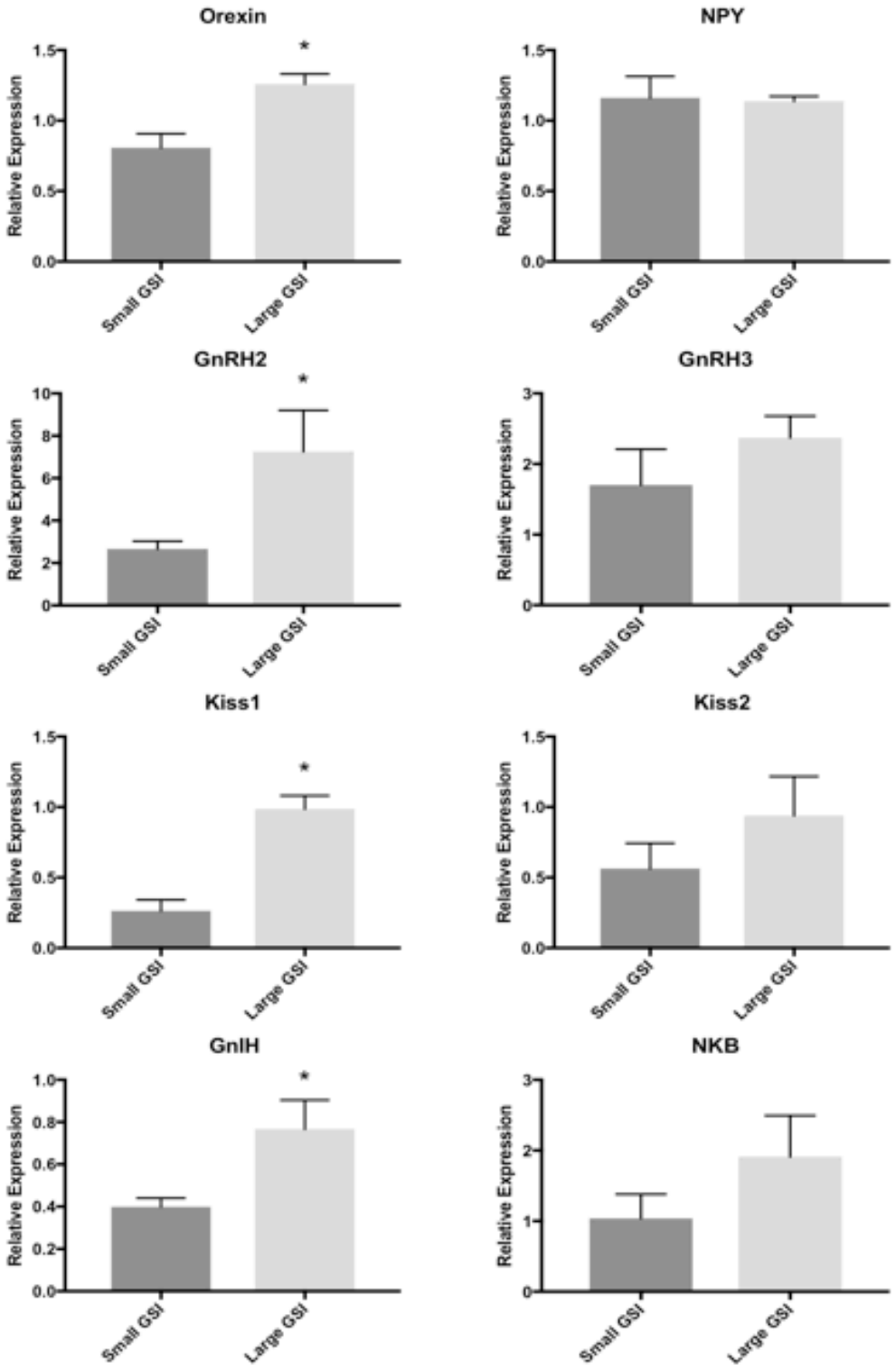


Figure 3.5: Relative brain mRNA expression of orexin, NPY, GnRH2, GnRH3, Kiss1, Kiss2, GnIH, and NKB in fasted female wild-type zebrafish. Significant differences ($p < 0.05$) between females

with a small GSI (< 5%) and females with a large GSI (> 5%) are indicated by stars (*). Data are represented as mean ± SEM.

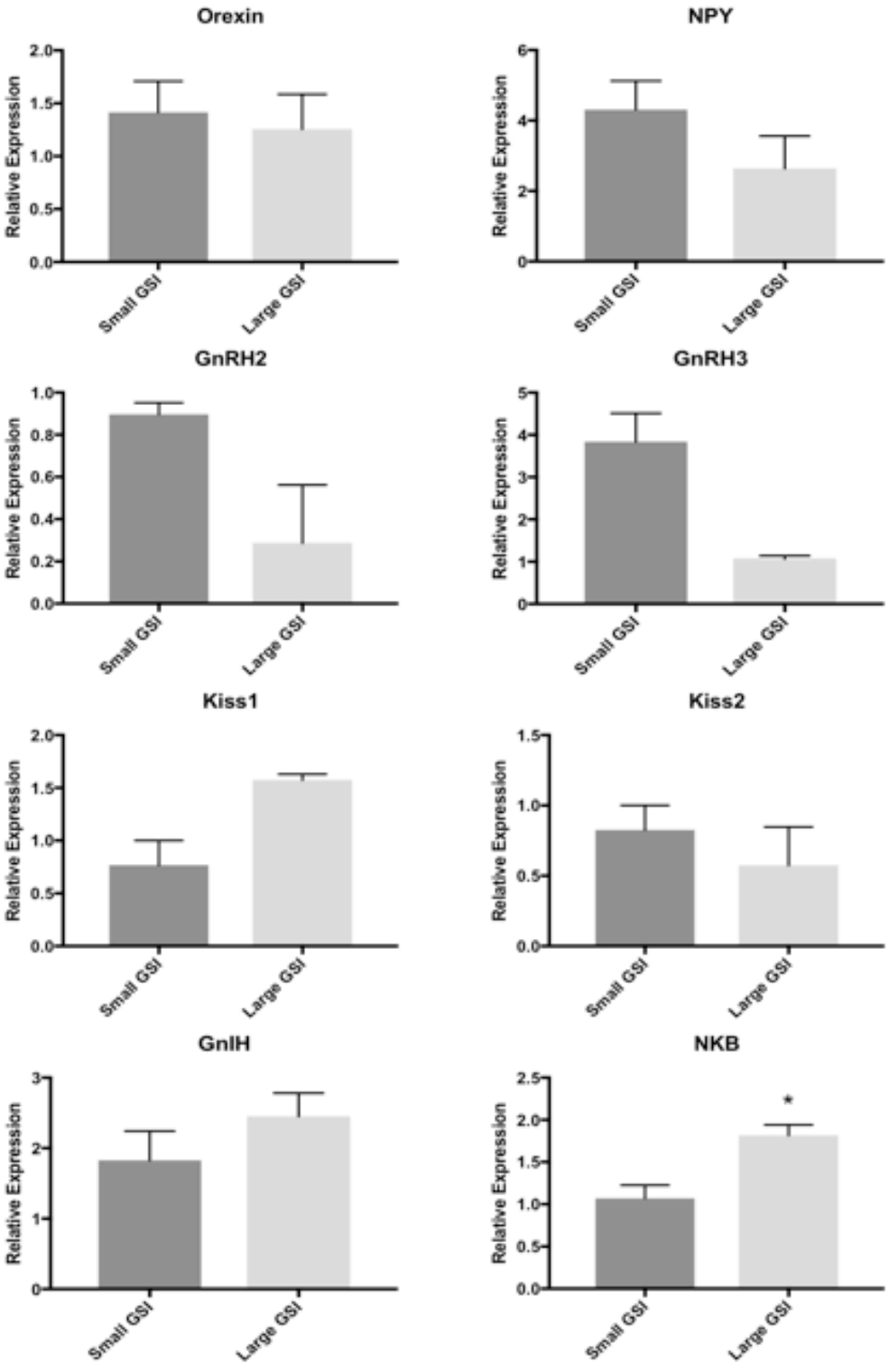


Figure 3.6: Relative brain mRNA expression of orexin, NPY, GnRH2, GnRH3, Kiss1, Kiss2, GnIH, and NKB in fed female *Casper* zebrafish. Significant differences ($p < 0.05$) between females with

a small GSI (< 3.2%) and females with a large GSI (> 3.2%) are indicated by stars (*). Data are represented as mean ± SEM.

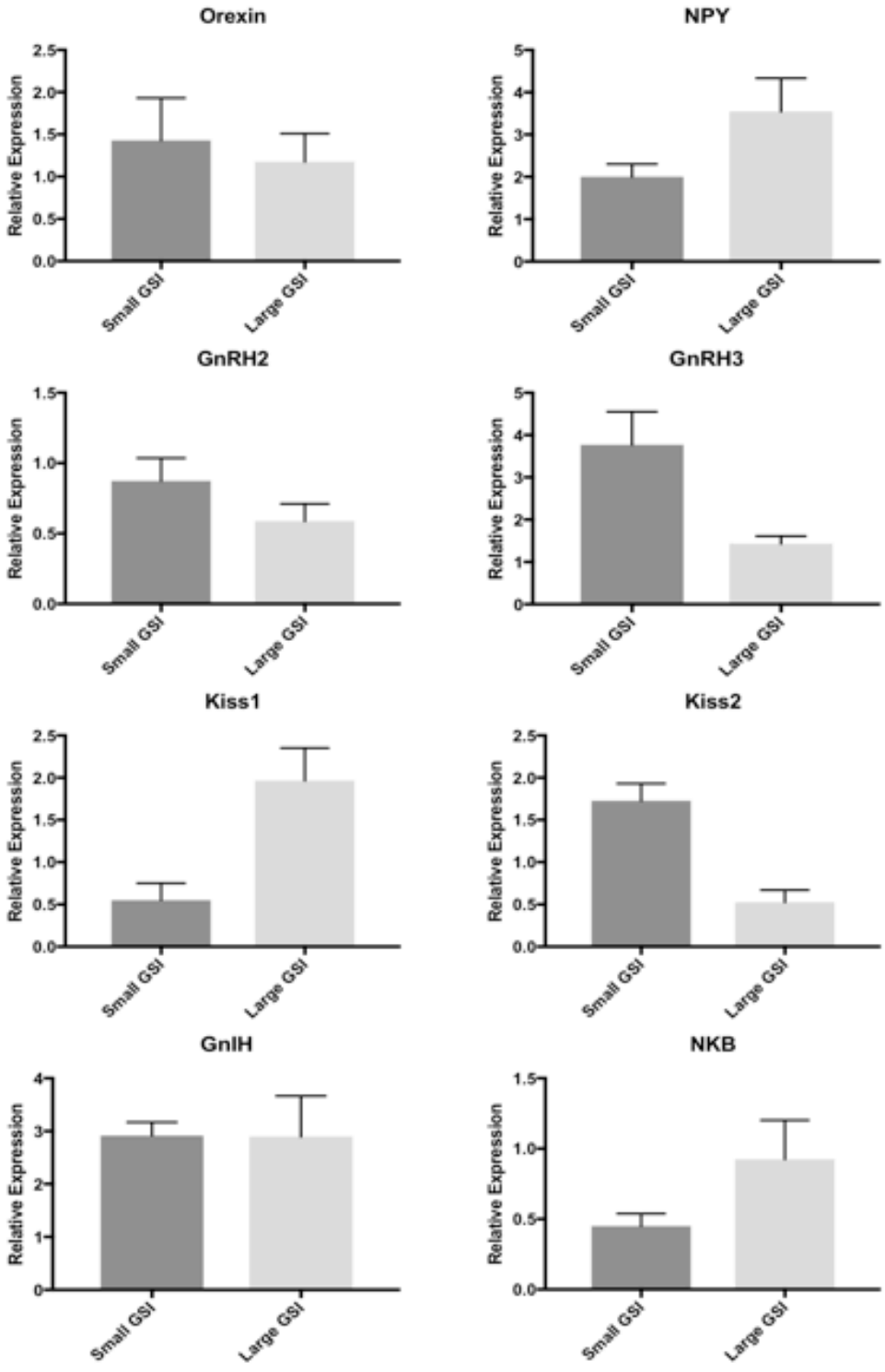


Figure 3.7: Relative brain mRNA expression of orexin, NPY, GnRH2, GnRH3, Kiss1, Kiss2, GnIH, and NKB in fasted female *Casper* zebrafish. Significant differences ($p < 0.05$) between females

with a small GSI (< 7.5%) and females with a large GSI (> 7.5%) are indicated by stars (*). Data are represented as mean ± SEM.

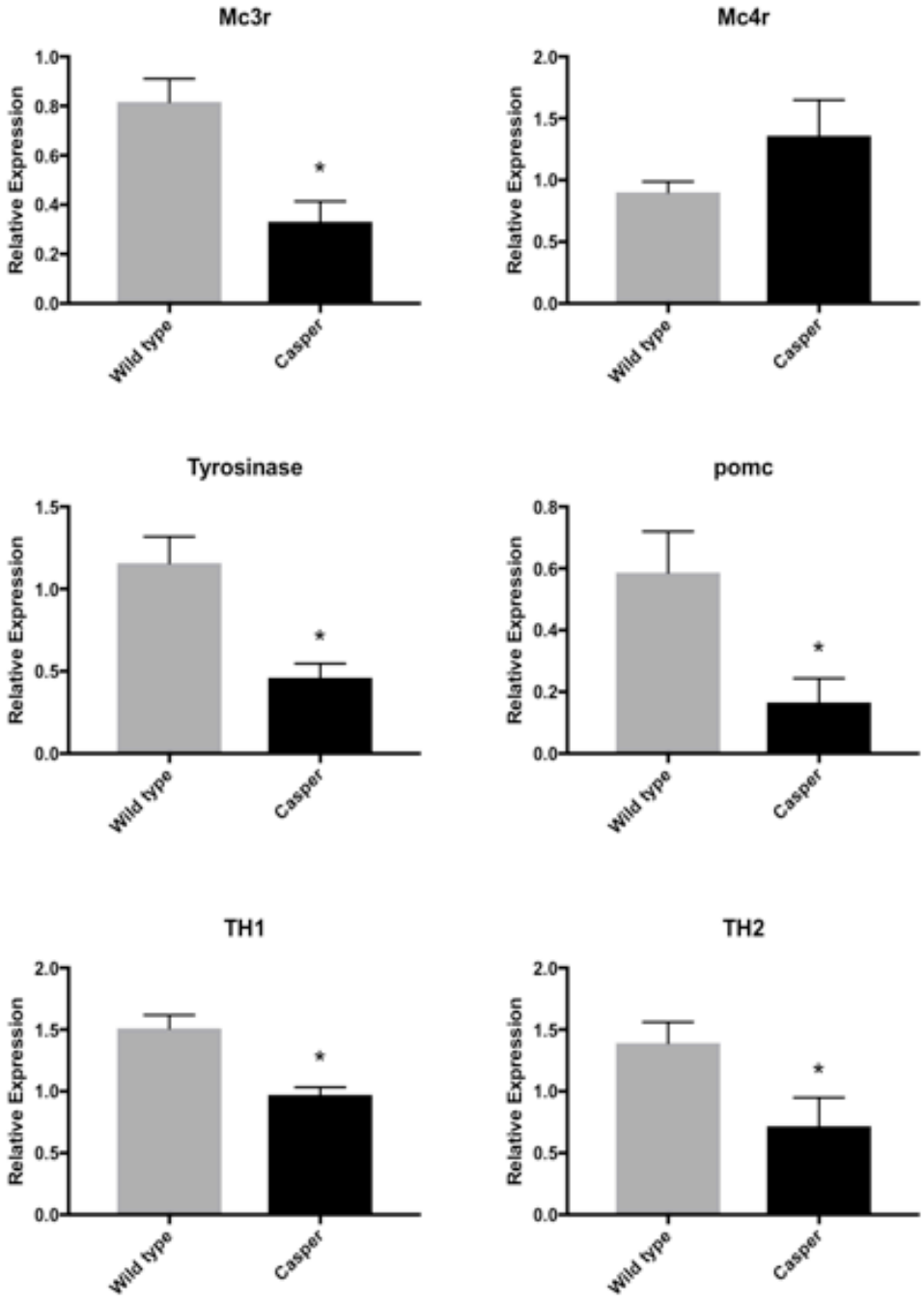


Figure 3.8: Relative brain mRNA expression of Mc3r, Mc4r, Tyrosinase, pomc, TH1, and TH2 in fed zebrafish. Significant differences ($p < 0.05$) between wild-type and *Casper* zebrafish indicated by stars (*). Data are represented as mean $\pm$ SEM.

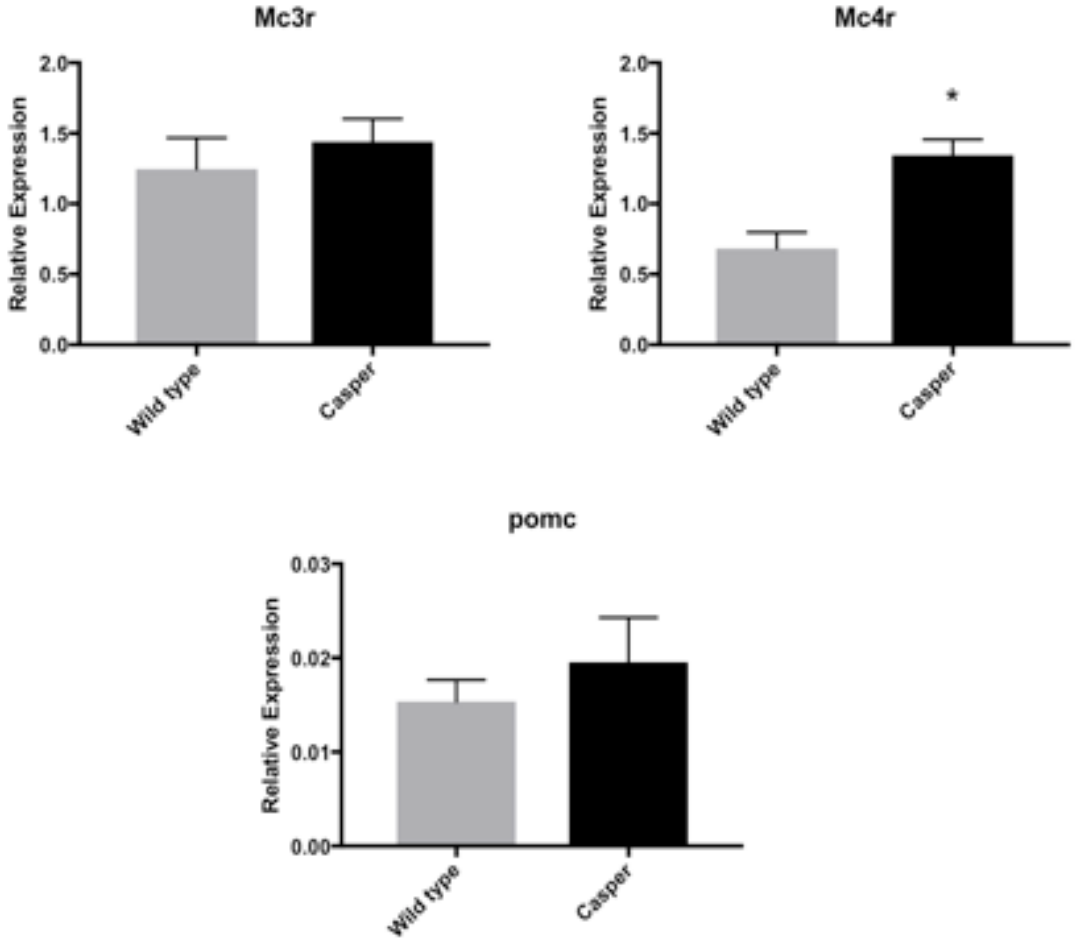


Figure 3.9: Relative brain mRNA expression of Mc3r, Mc4r, and pomc in fasted zebrafish. Significant differences ($p < 0.05$) between wild-type and *Casper* zebrafish indicated by stars (*). Data are represented as mean $\pm$ SEM.

3.5 Discussion

3.5.1 Wild-type zebrafish

3.5.1.1 Effects of fasting on hormone expression

3.5.1.1.1 Appetite-regulating hormones

The two appetite-regulating hormones investigated (NPY and orexin) were significantly increased during fasting. This is consistent with the known orexigenic function of these hormones, and with results from other studies. Food deprivation increases orexin expression in both mammals (e.g. Korczynski et al., 2006), and fish [e.g. winter flounder (Buckley et al., 2010), dourado (Volkoff et al., 2016), and goldfish (Nakamachi et al., 2006)]. Previous studies in zebrafish have found that orexin mRNA was higher in fasted fish compared to fed fish, that the number of orexin neurons increases during fasting and that administration of orexin stimulates food intake (Yokobori et al., 2011). Long-term food deprivation increases orexin expression in zebrafish, whereas short-term (i.e. 4 days) is not associated with changes in orexin mRNA or activity levels (Novak et al., 2005). Orexin has been implicated in sleep/wake cycles in both humans and zebrafish. Overexpression of orexin in zebrafish results in wakefulness and increased locomotion (Prober et al., 2006). This may indicate an involvement in the stress response of zebrafish, not only in terms of nutritional constraints, but perhaps other stressors as well.

The increase in NPY during fasting is consistent with results in mammals (Marks et al., 1992) and fish models, including winter skate (*Raja ocellata*) (MacDonald & Volkoff, 2009a), channel catfish (*Ictalurus punctatus*) (Peterson et al., 2012), blunt snout bream (*Megalobrama*

amblycephala) (Ji et al., 2015), and winter flounder (MacDonald & Volkoff, 2009b). Injection of NPY in mice, goldfish, and zebrafish leads to an increase in feeding, and repeated injection leads to obesity in mice (Yokobori et al., 2012; Volkoff & Peter, 2001; Segal-Lieberman et al., 2003). NPY has been shown to increase growth hormone, gonadotropin, and GnRH release in goldfish (Peng et al., 1993). In contrast to orexin, this suggests an activation of reproduction in addition to its stimulatory role in appetite. This concept seems to agree with my results, as I not only found an increase in NPY during fasting, but also of GnRH, kisspeptin, and NKB.

3.5.1.1.2 Reproductive hormones

In wild-type zebrafish, all of the reproductive hormones investigated were significantly up-regulated during fasting. This was an unexpected result based on the inverse correlation between feeding and reproduction characterized in mammals and goldfish. However, fasting increased the expression of GnIH, which is seemingly consistent with relationship I expected. Several studies have identified an orexigenic action of GnIH in mammals and birds. Injections of GnIH increases food intake in rats (Johnson et al., 2007), sheep (Clarke et al., 2012), chickens (Tachibana et al., 2005), and ducks (Fraley et al., 2013). Fasting increases GnIH mRNA in chickens and increases the activity of GnIH neurons in ducks (McConn et al., 2016; Fraley et al., 2013). Although most of the current literature suggests that GnIH inhibits reproduction, there have been a few reports of stimulation of the HPG axis by GnIH. A study in mice found that only some GnRH neurons (about 53%) were responsive to GnIH, and of those responsive neurons, a small portion were stimulated by GnIH administration, though the majority were inhibited (Ducret et al., 2009). In hamsters, the activity of GnIH seems to depend on photoperiod, as it was discovered that “short-day” hamsters (8H light: 16H dark) expressed less GnIH mRNA than

"long-day" hamsters (16H light: 8H dark). More than that, ICV injection of GnIH decreased plasma LH in "long-day" hamsters, but increased plasma LH levels in "short-day" hamsters after 30 minutes (Ubuka et al., 2012). A similar situation has been shown in fish, where certain factors, such as sex, species, and maturation, seem to dictate whether GnIH displays stimulatory or inhibitory actions. For example, in goldfish GnIH was found to have varying effects on gonadotropin mRNA depending on gonadal stage and method of administration (i.e. *in vivo* vs. *in vitro*) (Moussavi et al., 2012). In sea bass (*Dicentrarchus labrax*), different GnIH peptides appear to have different functions; GnIH-1, but not GnIH-2, decreases FSH levels, while GnIH-2 increases the brain mRNA expression of GnRH2 and the kiss1 receptors (Paullada-Salmerón et al., 2016). Though the exact mechanisms of these contradictory roles are not well understood, several theories have been proposed (Ubuka & Parhar, 2018). The possibility that GnIH could be activating the HPG axis may help explain why the rest of the reproductive hormones that I investigated were all up-regulated, as they are all positive regulators of reproduction.

Kisspeptin and NKB both control reproduction through the modulation of GnRH release and were both increased during fasting in this experiment. In contrast to my results, fasting decreases kisspeptin expression in mammals, including rats (Kalamatianos et al., 2008; Ladyman & Woodside, 2014), lambs (Polkowska et al., 2015), and monkeys (Shamas et al., 2015; Wahab et al., 2011). Very few studies have examined non-mammalian kisspeptin with regards to appetite regulation and these suggest a very different situation in fish compared to mammals. In both male sea bass (Escobar et al., 2016) and Senegalese sole (*Solea senegalensis*) (Mechaly et al., 2011), food restriction increases hypothalamic kiss1 and kiss2 expressions. It is

interesting to note that Kiss1 receptor knockout mice eat less than wild-type mice, despite being obese, and display decreased locomotor activity and respiratory rates (Tolson et al., 2014). In addition to this, it has been found that patients with type-2 diabetes have increased production of kisspeptin in the liver and more circulating kisspeptins than healthy patients (Hussain et al., 2016). These two observations illustrate the need to further investigate the function of kisspeptin and how it interacts with other hormones to regulate both reproduction and appetite. Not only is the role of kisspeptin in appetite regulation variable, there has been recent evidence suggesting that its role in reproduction may not be as crucial in fish as was once assumed. It is well known that kisspeptin is required for sexual maturation in mammals, as disruption of the kisspeptin system can lead to a delay or complete lack of puberty (Terasawa et al., 2013). However, knockout zebrafish lacking kiss1, kiss2, both kiss1 and kiss2, or any combination of the kisspeptin receptors still develop normally and are fully fertile (Tang et al., 2015). This provides strong evidence that kisspeptin is not required for functional reproduction in some fish, as opposed to what was previously expected based on the mammalian system.

NKB is associated with reproductive success in mammals. Mutations in Tac3 or the Tac3 receptor in humans leads to hypogonadism and infertility (Young et al., 2010). There is currently not much known about the NKB system in fish, although two Tac3 genes have been cloned and characterized in zebrafish (Biran et al., 2012). In mammals, NKB is co-expressed on KNDy neurons with kisspeptin and is thought to modulate kisspeptin as an upstream regulator of GnRH release (Grachev et al., 2014). However, this does not appear to be the case in zebrafish as NKB and kisspeptin are expressed in separate neurons (Ogawa et al., 2012). Although the coordination between NKB and kisspeptin cannot be eliminated as a possible

regulator of GnRH release, it seems that these two peptides may act through independent pathways in zebrafish. The action of NKB may be different depending on which Tac3 gene it is encoded by. NKBa (encoded by Tac3a) has been implicated in sexual maturation and found to stimulate LH secretion in zebrafish, but not in grass carp (*Ctenopharyngodon idellus*) (Biran et al., 2012; Hu et al., 2014b). NKBb (encoded by Tac3b) was shown to increase LH secretion in zebrafish, though to a lesser extent, however its exact role is still unclear. In goldfish, Tac3a, but not Tac3b, is positively affected by ovariectomy and negatively affected by estradiol treatment (Qi et al., 2015). My current study, in which I investigated Tac3b, suggests that NKBb is involved in appetite regulation. There are very few studies involving NKB in fish and, to my knowledge, no published study exploring its role in nutrition. Although there is much left to be discovered, these results suggest that NKB is affected by nutritional status. This may support the stimulatory role of NKB in GnRH secretion, as the up-regulation of NKB corresponded to a similar up-regulation in both forms of GnRH, at least in females.

GnRH is a key component in the HPG axis and one of the major hormonal triggers for reproduction. Several studies involving different species, both mammalian and piscine, have examined the relationship between GnRH and nutritional status. Fasting decreases GnRH expression in rats and decreases expression of the GnRH receptor in rabbits (Gruenewald & Matsumoto, 1993; Parillo et al., 2014). Similar results are seen in fish species. In goldfish, injections of GnRH2 decrease food intake, while an increase in food intake associated with orexin treatment leads to decreased GnRH2 expression (Hoskins et al., 2008). In winter flounder, which have three isoforms of GnRH, fasting decreases GnRH2 and GnRH3, but not GnRH1 expression (Tuziak & Volkoff, 2013a). However, GnRH2 and GnRH3 expression levels are

unaffected by fasting in Atlantic cod (*Gadus morhua*) (Tuziak & Volkoff, 2013b). In zebrafish, GnRH2 suppresses food intake and although fasting was not investigated directly, overfed zebrafish expressed higher levels of GnRH2 (Nishiguchi et al., 2012). The results of this study do not agree with these previous findings, as I observed increases in both GnRH2 and GnRH3 expressions during fasting. GnRH has long been considered the ultimate driving force behind sexual maturation and reproduction in humans and mice, as GnRH mutations cause infertility in both (Chan et al., 2009; Cattanach et al., 1977). However, recent reports suggest that this is not necessarily true in zebrafish. GnRH3 knockout zebrafish express no deficits in development, reproduction, or fertility (Spicer et al., 2016). Moreover, double knockouts (lacking both GnRH2 and GnRH3) are normal (Marvel et al., 2018), suggesting that although some compensatory mechanism is present in these knockout fish, it is outside the GnRH system. Interestingly, double knockout zebrafish have upregulation of other reproductive hormones, including GnIH and Tac3a, as well as some feeding hormones (Marvel et al., 2018). Another factor to consider is the natural spawning cycle of zebrafish. Like many other tropical fish, zebrafish spawn year-round and are dependent on photoperiod; they naturally breed at first light. Since I chose to sample the fish first thing in the morning, it is possible that the high levels of reproductive hormones could be due to the natural reproductive cycle of the zebrafish. All of these findings point towards a highly interactive relationship between appetite and reproduction, though the mechanism of which appear to be species-specific.

3.5.1.1.3 Nutritional stress and reproduction

The effects of nutritional stress on reproduction are quite variable, especially in fish, and often depend on species, gender, reproductive stage, and the duration of deprivation. For

example, in yellowtail (*Seriola quinqueradiata*), the effects of fasting were different depending on the timing and maturity of the fish. In immature fish, long-term deprivation resulted in a delay in sexual maturation and reproduction corresponding with a decrease in gonadotropin synthesis. However, in more mature females, restricted feeding did not affect gonadotropin levels, even though oocyte development was impaired. The same group found that the impact of nutritional stress on reproduction depends on the duration and degree of deprivation (Higuchi et al., 2017; Higuchi et al., 2018). In general, nutritional stress experienced at an earlier life stage affects maturation and development, while nutritional stress later in life can alter spawning or time between spawning events (Schreck et al., 2001). Other factors that may affect how an organism responds to food deprivation, especially in fish, include reproductive strategy and spawning frequency. Energy allocation differs depending on how often a species will spawn (i.e. once vs. year-round) and how they reproduce (i.e. oviparity vs. viviparity, capital vs. income breeding, etc.). The type and severity of stress is important in predicting its impact on reproduction, as mild deprivation is often less detrimental than more severe stress (Contreras-Sánchez et al., 1998; Higuchi et al., 2018). This concept is further explained by the idea of "hormesis"; where low levels of stress, such as food restriction, can stimulate reproduction, whereas high levels, such as hypoxia, is inhibitory (Schreck, 2010). Although there is much left to be discovered about nutritional stress and reproduction and how they influence each other, the current perspective seems to propose that the relationship is not as straightforward as was once believed.

3.5.1.1.4 Gender differences

Several gender-specific differences were noted in my experiments. In general, I found that females were more affected by fasting than male fish. In almost all species, females invest more energy into elements of reproduction, including gamete production and parental care, than males (Hayward & Gillooly, 2011; Lodé, 2012; Penn & Smith, 2007). While males typically expend more energy on reproductive behaviours, such as courting, chasing, or fighting (Andersson, 1994). The effects of food deprivation, tends to differ between the sexes, with females being more sensitive than males, likely because they require more energy for reproduction (Øverli et al., 2006; Ray & Hansen, 2004; Perrigo & Bronson, 1985). In my study, the expression of orexin was affected by fasting in females, but not in males whereas the expression of NPY was affected in males but not in females. Other studies have noted gender-differences in the regulation of appetite. For example, in rats, fasting increases the number of orexin neurons and the expression of the orexin receptor in females but not in males (Funabashi et al., 2009; Isawa et a., 2015). In female African cichlids (*Astatotilapia burtoni*), which undergo a natural change between feeding and fasting states during their reproductive cycle, the size of NPY neurons was larger during the fasting state but there was no significant change in NPY expression (Porter et al., 2017). In a separate study involving both sexes of cichlid fish (*Cichlasoma dimerus*), injections of NPY increased growth hormone and gonadotropin levels, but in a sex-specific manner, with females generally requiring more NPY to elicit a response (Di Yorio et al., 2015). In terms of reproductive hormones, I found that GnRH2, GnRH3, and GnIH were only affected by fasting in females, but not males. Kisspeptin exhibited differential changes in each sex depending on the form, where kiss1 was increased in fasted

females only but kiss2 was increased in fasted males only. Kisspeptin exhibits gender-specific distributions within the brain of the Senegalese sole throughout the reproductive cycle, and a study in sea bass found that fasting increased levels of kiss2 more than kiss1 in males (Mechaly et al., 2012; Escobar et al., 2016). All of these studies, including the present one, support the idea that reproduction and appetite are regulated differently depending on gender, which agrees with the differences in morphology, physiology, and energetic requirements between males and females.

3.5.1.2 Influence of reproductive stage on the response to fasting in females

In fed female wild-type zebrafish, GnRH2, GnRH3, and NPY expression levels increased as GSI increased. GSI was used in this experiment as an indicator of reproductive stage, with the theory that as females mature, they produce more eggs and their GSI increases. As the primary function of GnRH is to stimulate the release of LH and FSH from the pituitary, which then causes gonadal development and production of gametes, it would be expected that females with a higher GSI (i.e. those producing more eggs) would express higher levels of GnRH. NPY has been shown to increase GnRH and gonadotropin levels in goldfish and cichlids (Peng et al., 1993; Di Yorio et al., 2015), suggesting that higher NPY expression in females producing more egg might be relate to its positive actions on GnRH.

In fasted wild-type zebrafish, GnRH, kisspeptin, GnIH, and orexin levels were significantly higher in females with higher GSIs. The increase in GnRH and kisspeptin likely correlates with the increase in gametogenesis. It is interesting to note that GnRH2, but not GnRH3, expression was affected by GSI in fasted fish. This may indicate that GnRH2 is more

involved in stimulating gametogenesis in female zebrafish than GnRH3. The increase in GnIH seen in mature fasted but not fed females potentially indicates an attempt to counteract the increase in GnRH that occurs during egg production. It is possible that, although these females are mature enough to be producing eggs, which is associated with higher GnRH levels, their food intake is restricted. Therefore, GnIH levels may be increased to compensate for the high GnRH levels in an effort to conserve energy during starvation. The higher fasting-induced increase in orexin expression in females with more eggs agrees with the role of orexin as an appetite stimulator. My results show that orexin levels increase as sexual maturation progresses. Females at later reproductive stages would require more energy to allocate towards gametogenesis and hormone production in order to sustain their reproductive capacity and be able to spawn. Food restriction would likely then be more detrimental in these females than in females at an earlier reproductive stage and would lead to a higher production of orexin in order to combat that energy deficit.

3.5.2 Casper zebrafish

In *Casper* zebrafish, most of the hormones examined were not affected by fasting or reproductive stage. Only GnIH and NKB levels were affected by fasting. The increases in NKB in male fish and GnIH overall are consistent with results of the wild-type zebrafish. Other than NKB, none of the reproductive or appetite-regulating hormone were affected by reproductive stage in female *Caspers*. This was highly unexpected since they are the same species and were subjected to the same experimentation as the wild-type fish. The mutation underlying the *Casper* phenotype is a knockout of two genes that results in the complete lack of melanophores

and iridophores. To my knowledge, neither one of these genes are directly involved in nutrition or reproduction.

3.5.2.1 Strain-specific differences in the melanocortin system

In order to assess if mutations of genes/cells involved in pigmentation might affect feeding and reproduction in *Casper* zebrafish, I decided to investigate some of the enzymes and peptides involved in the melanocortin system. My results show major differences between the wild-type and *Casper* zebrafish regarding melanocortin expression. Fed *Casper* fish displayed lower Mc3r, tyrosinase, TH1, TH2, and pomc expression levels than fed wild-type fish.

Tyrosinase and TH are both enzymes involved in the melanin synthesis pathway. The first, and rate-limiting, step in melanin synthesis is the conversion of tyrosine into L-DOPA, which is catalyzed by tyrosinase in melanosomes and TH in catecholaminergic cells (Morris et al., 2002). The fact that both of these enzymes are down-regulated in *Casper* zebrafish indicates a decrease in melanin synthesis compared to the wild-type. This is not surprising, as these transgenic fish are fully transparent and lack pigmentation. L-DOPA is subsequently oxidized to dopaquinone by tyrosinase in both cell types (i.e. melanosomes and catecholaminergic cells), which can then produce different forms of melanin. L-DOPA can alternatively be converted to dopamine by DOPA decarboxylase. Since, dopamine is an inhibitor of reproduction in many fish species, including zebrafish (Fontaine et al., 2013), I hypothesized that this could potentially explain the differences between the two strains, as a restriction of melanin synthesis might lead to an increase in dopamine synthesis. However, the decrease in both tyrosinase and TH expressions, goes against such a theory as lower expression of both of these enzymes would

result in lower levels of L-DOPA. I examined only mRNA expression of these enzymes, which does not necessarily predict post-transcriptional changes. Unfortunately, I was unable to compare dopamine levels between the two strains, therefore this theory cannot be fully excluded as a possibility.

Although zebrafish have six melanocortin receptors, I chose to include only Mc3r and Mc4r in my study, as those receptors have been shown to be involved in energy balance in mammals (Cone, 2006). I did note a significant difference in Mc3r, but not Mc4r, expression between *Casper* and wild-type zebrafish, suggesting that energy homeostasis may be regulated differently between these two strains. When fish were fasted, no difference in Mc3r expression was noted between strains. However, Mc4r was significantly increased in *Casper* fish compared to wild-type fish when both strains were fasted. Mc4r has been particularly implicated in appetite, where mice and humans with Mc4r mutations exhibit severe obesity and increased food intake, indicating an anorexigenic function (Huszar et al., 1997; Yeo et al., 1998). This relative increase in Mc4r between *Casper* and wild-type fish during fasting therefore seems counterintuitive but could be due to a decrease in Mc4r in the wild-type zebrafish during fasting but no change in the *Casper* Mc4r. This agrees with my results from the previous experiments and would similarly indicate that the *Casper* zebrafish are not responding to changes in nutritional status, associated with a decrease in Mc3r mRNA and a potentially defective Mc4r.

I found a decrease in pomc expression in *Casper* zebrafish compared to wild-type fish, which is a precursor for several peptides including MSH and ACTH. MSH is involved in melanin production, therefore a decrease in this peptide would be expected. However, if only melanin was being affected, pomc expression as a whole should not be significantly different than the

wild-type. In addition, pomc itself is involved in the regulation of feeding. In mice, activation of pomc neurons suppresses food intake and chicks injected with GnIH show an increase in feeding corresponding to higher NPY and lower pomc levels (Zhan et al., 2013; McConn et al., 2014). In this study, I found lower pomc levels in fed *Casper* zebrafish compared to wild-type, but no difference in pomc when both strains were fasted. This likely indicates that pomc expression is decreasing during fasting in the wild-type zebrafish, supposedly due to its anorexigenic action, but is remaining relatively stable in the *Casper* zebrafish independent of dietary condition. It suggests that the stress response in these fish may be impaired, as they express lower levels of pomc and do not respond to energetic or nutritional stress.

3.6 Conclusion

Overall, my results suggest a link between reproduction and nutritional status in zebrafish, though the relationship seems to differ from that of mammals in some aspects. I uncovered some gender- and reproductive stage-specific differences in the mechanisms regulating these two processes. Fasting caused several changes in the expression of both appetite and reproductive hormones in wild-type zebrafish, with females being more significantly affected than male fish. The mutant *Casper* zebrafish seemed resistant to the effects of fasting in all of the experiments conducted. The differences seen between *Casper* and wild-type zebrafish strongly indicate that the gene mutations underlying the *Casper* phenotype leads to strain-specific mechanisms, possibly mediated by the melanocortin system. Although more research needs to be conducted to fully understand why this transgenic model is responding so differently, my data suggests that the melanocortin precursor (pomc) and the melanin pathways may somehow be involved. In future studies, it would be interesting to

analyze the dopamine levels in wild-type and *Casper* zebrafish to evaluate the role dopamine is playing in the variation between these strains.

Due to the complexity of reproduction and appetite, the actions and interactions of the hormones thought to be involved in these two processes remain widely unknown. Though there is some evidence suggesting a relationship between the two, most of the literature focuses on mammals and birds, with very little research involving other vertebrate species, such as fish. The high diversity among fish species, especially teleost fish, creates substantial difficulties when attempting to compare and generalize physiological concepts. Zebrafish are commonly used as a scientific model; however, the *Casper* zebrafish is a relatively new strain and has never been used in a reproductive study such as this one. My data provides novel insights into the endocrine mechanisms that regulate feeding and reproduction. Continuing research is needed to fully explore this topic, considering the incredible diversity of fish species. However, the present study allows us to better illustrate the underlying interactions among several life processes, not only in fish models but in other animals.

Chapter 4: General Conclusion

In the second chapter, I investigated certain reproductive and appetite-regulating hormones in the glass catfish (*Kryptopterus vitreolus*). I was able to successfully isolate genes for several hormones, including GnRH1, GnRH2, NPY, orexin, and CART. I found a relationship between nutritional status and the expression of these isolated genes. GnRH1, but not GnRH2, was negatively affected by fasting, while all three appetite-regulators (orexin, NPY, and CART) were up-regulated during fasting. Unfortunately, despite the transparency of the fish, I was not able to distinguish between genders or reproductive stages, likely because the fish had very small gonads contained in their narrow abdominal cavities. Although more research is needed to fully explore the mechanisms, this provides novel data showing that there is a link between feeding and reproduction in the glass catfish.

In chapter 3, I compared the expression of reproductive (GnRH, kisspeptin, GnIH, and NKB) and appetite-regulating hormone (orexin and NPY) in two strains of zebrafish (*Danio rerio*) subjected to various conditions. I assessed the effects of nutritional status, gender, reproductive stage, and strain on the brain mRNA expression of these hormones. My results show that the response to fasting in wild-type zebrafish fish differed from what has been reported in mammals and other fish models, with females being more affected than males. I found evidence of reproductive stage-specific regulations of these hormones, specifically GnRH and NPY. The *Casper* zebrafish, however, seemed resistant to the effects of fasting, which was

highly unexpected. I found that very few hormones were affected by nutritional status and reproductive stage, unlike the wild-type fish. In order to assess if changes in pigmentation could be responsible for the difference between *Casper* and wild fish, I investigated certain peptides involved in the melanocortin system (i.e. Mc3r, Mc4r, tyrosinase, POMC, TH1, and TH2) and compared their expression levels betwee*n* the two strains. My results suggest that these strain-specific differences may be mediated by the melanocortin system, though future studies are needed to fully understand how the *Casper* mutation is affecting these fish.

Overall, I was able to show a clear relationship between appetite and reproduction in both zebrafish (*Danio rerio*) and glass catfish (*Kryptopterus vitreolus*). The regulation of appetite and reproduction is very complex, and involves a vast number of peptides, only a few of which were investigated in this study. As discussed, fish (especially teleosts) are incredibly diverse in their habitats, feeding behaviour, life cycle and reproductive strategies. This creates substantial difficulties when attempting to compare and generalize between mammals and fish, and even among fish species. My results suggest some deviations from what has been previously discovered in mammals, however these data provide new insights on the endocrine mechanisms regulating feeding and reproduction in fish. These studies contribute to the vastly growing body of knowledge on fish endocrine physiology.

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