General and Comparative Endocrinology 282 (2019) 113207

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

Effects of fasting on the central expression of appetite-regulating and reproductive hormones in wild-type and Casper zebrafish (*Danio rerio*)

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ARTICLE INFO

Keywords:
Zebrafish
Reproduction
Appetite
mRNA expression
Endocrine regulation
Melanocortin

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 kisspeptin and GnIH modulate this action. Kisspeptin secretion is further controlled by neurokinin B (NKB) and dynorphin A (Dyn). To better understand the mechanisms regulating appetite and reproduction in fish, we examined the effects of fasting, 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, we used both wild-type and transparent Casper zebrafish. In female wild-type zebrafish, fasting increased the expression of all hormones investigated, with the exception of Kiss2. Only NPY and Kiss2 were increased in male wild-type zebrafish during fasting. In Casper zebrafish, only GnIH and NKB in males were affected by fasting, suggesting that Casper fish may be more resistant to fasting than wild fish. Fasting increased expressions of orexin, GnRH2, Kiss1, GnIH and NKB in wild-type females with more eggs or larger eggs relative to body weight, compared to those with fewer or smaller eggs, suggesting that more mature females are more affected by fasting. No significant interactions of fasting and reproductive stage were noted in female Casper fish. To investigate whether differences between Casper and wild-type fish were due to genes involved in pigmentation, we compared the brain mRNA expressions 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, our 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 in part responsible for these differences between strains.

1. Introduction

In all vertebrates, reproduction and appetite are largely controlled 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) (Parhar et al., 2016; Hu et al., 2014a). Appetite regulators are categorized as orexigenic (stimulate food intake, such as orexin and neuropeptide Y-NPY) or anorexigenic (inhibit food intake) (Volkoff, 2016; Ronnestad et al., 2017).

Gonadotropin-releasing hormone (GnRH) is a critical component of the hypothalamus-pituitary–gonadal (HPG) axis as it stimulates the release of gonadotropins [follicle-stimulating hormone (FSH) and luteinizing hormone (LH)] by pituitary gonadotrope cells (Zohar et al., 2010; Shahjahan et al., 2014). Gonadotropins then stimulate gonad development and the release of sex steroids, leading to gametogenesis and sexual behavior. Fish can have up to three forms of GnRH and four GnRH receptor types (Carolsfeld et al., 2000; Roch et al., 2014; Strandabø et al., 2016). Zebrafish (*Danio rerio*) have two forms of GnRH: GnRH2 and GnRH3. GnRH3 is the main hypophysiotropic GnRH in zebrafish, and both forms are thought to be involved in the regulation of reproductive behavior (Lee et al., 2018).

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) (Suppl. Fig. 1). In mammals, kisspeptin is a potent GnRH secretagogue (Ohtaki et al., 2001; Roa

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https://doi.org/10.1016/j.ygcen.2019.06.011
Received 13 December 2018; Received in revised form 12 June 2019; Accepted 12 June 2019
Available online 13 June 2019
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et al., 2008). In teleost fish, including zebrafish, two kisspeptin genes (*kiss1* and *kiss2*) are present. Kisspeptin regulates the HPG axis (Parhar et al., 2016; Ogawa et al., 2012; Kitahashi et al., 2009) and is involved in the initiation of sexual maturation (Kitahashi et al., 2009; Shahjahan et al., 2010). However, it has been suggested that kisspeptin may not be critical for reproduction in zebrafish, as fish lacking *kiss1* and *kiss2* genes display normal reproductive functions (Liu et al, 2017).

GnIH was originally discovered in Japanese quail (*Coturnix japonica*) and later identified in almost all vertebrate classes (Tsutsui et al., 2018). In mammals and birds, GnIH prevents gonadotropin release by inhibiting GnRH and kisspeptin neurons (Tsutsui et al., 2000; Ubuka et al., 2016; Kriegsfeld et al., 2006). The physiological functions of GnIH in fish are still unclear. For example, injections of GnIH in immature female goldfish (*Carassius auratus*) decrease GnRH, FSH, and LH mRNA levels (Qi et al., 2013), but administration of GnIH to pituitary cell cultures in mature female Nile tilapia (*Oreochromis niloticus*) increases LH and FSH release (Biran et al., 2014).

In mammals, GnRH secretion is pulsatile and this rhythm is regulated by two neuropeptides, neurokinin B (NKB, encoded by the tachykinin gene *Tac3*) and dynorphin A (Dyn, encoded by the prodynorphin *pdyn* gene) (Grachev et al., 2014; Herbison, 2018), which are co-localized with kisspeptin in neuronal populations (referred to as KNDy neurons) and stimulate and inhibit kisspeptin release, respectively (Suppl. Fig. 1), thus generating a synchronized kisspeptin release (Grachev et al., 2014; Tng, 2015; Herbison, 2018). Zebrafish express two *tac3* genes, *tac3a* and *tac3b* (both expressed in brain and ovary), which produce NKBa and NKBb, respectively (Biran et al., 2012) and one *pdyn* gene, which produces two Dyn peptides (DynA and DynB) (Gonzalez-Nuñez et al., 2007). There is no evidence of NKB and kisspeptin co-expression in zebrafish, suggesting that "KNDy neurons" might not exist in fish (Hu et al., 2014a; Ogawa et al., 2012). In zebrafish, injections of NKB stimulate LH secretion (Biran et al., 2012) and an increase in whole brain *tac3a* mRNA expression is seen during maturation along with an increase in kisspeptin, suggesting that, as in mammals, kisspeptin plays a role in the neuroendocrine control of reproduction.

In fish, orexin and NPY are two major regulators of feeding. Orexin treatment has been shown to increase feeding is several fish [goldfish (Nakamachi et al., 2006), zebrafish (Yokobori et al., 2011), cavefish (*Astyanax mexicanus*) (Penney and Volkoff, 2014)] and its brain expression increases following fasting [*e.g.* winter flounder (*Pseudopleuronectes americanus*) (Buckley et al., 2010), red-bellied piranha (*Pygocentrus nattereri*) (Volkoff, 2014), dourado (*Salminus brasiliensis*) (Volkoff et al., 2016)]. In goldfish, orexin has also been implicated in the control of sleep/locomotion (Volkoff et al., 1999; Nakamachi et al., 2014). Similar to orexin, NPY promotes food intake in fish [*e.g.* goldfish (Narnaware and Peter, 2001); zebrafish (Jeong et al., 2018)], and its expression affected by fasting [*e.g.* goldfish (Narnaware and Peter, 2001).

In fish (Volkoff and London, 2018), as in mammals (Shahjahan et al., 2014), feeding and reproduction are linked processes, as successful reproduction requires adequate resources in order to sustain the high-energy demands for the production of gametes and sexual behaviors. Any state of negative energy balance thus affects not only central appetite regulating systems but often also reproductive pathways and reproductive performance (Izquierdo et al., 2001). For example, fasting decreases GnRH2 mRNA expression in Ya fish (*Schizothorax prenanti*) (Wang et al., 2014) and winter flounder (*Pseudopleuronectes americanus*) (Tuziak and Volkoff, 2013), GnRH2 mRNA levels are higher in zebrafish fed excess food than those in fish fed a normal ration (Nishiguchi et al., 2012), and in sea bass (*Dicentrarchus labrax*), fasting increases kisspeptin expression in the male brain (Escobar et al., 2016). Additionally, close interactions exist between appetite-regulating pathways and the reproductive system (Shahjahan et al., 2014). For example, in goldfish, orexin inhibits spawning by decreasing GnRH brain mRNA expression (Hoskins et al., 2008), NPY treatment increases spawning by stimulating GnRH production (Peng et al., 1993), and GnRH2 injections inhibit feeding via the down-regulation of orexin (Hoskins et al., 2008).

In many fish species, including zebrafish, differences in feeding [*e.g.* Oswald and Robison, 2008] and metabolic responses to fasting [*e.g.* glucose levels and body weight (Meyer et al., 2013)] and reproductive pathways [*e.g.* expression of genes associated with sex steroid production and reproduction (Wong et al., 2014)] have been observed between different strains. However, there is little information available on the possible involvement of appetite-regulating hormones and reproductive hormones in the generation of these differences. A number of zebrafish strains are available commercially including wild-type and Casper zebrafish. The Casper zebrafish (*nacre*$^{w2/w2}$; *roy*$^{a9/a9}$) is a transgenic transparent fish that lacks both melanophores and iridophores, due to mutations in the *mitfa* (microphthalmia-associated transcription) and *mpv17* (a mitochondrial protein) genes, respectively (White et al., 2008). Skin melanophores produce the pigment melanin by conversion of tyrosine into L-DOPA and then melanin (catalyzed by tyrosinase) (Braasch et al., 2007). Melanin production is in part regulated by the melanocortin system, which includes peptides derived from the melanocortin precursor proopiomelanocortin (POMC). These 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 interactions 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 examine the effects of fasting on the transcript expressions of appetite-regulating (orexin and NPY) and reproductive (GnRH, kisspeptin, GnIH, and NKB) hormones in wild-type and Casper zebrafish, and assess potential differences in the response to fasting between genders, reproductive stages and strains. We also compared the brain mRNA levels of enzymes involved in melanin synthesis (*i.e.* tyrosinase and TH) between the two strains, and examined the effects of fasting on the brain expression of POMC and melanocortin receptors (MC3R and MC4R) in the two strains.

2. Materials and methods

2.1. Experimental animals

AB wild-type and Casper zebrafish (*Danio rerio*) were obtained from the Zebrafish Core Facility at Dalhousie University (Halifax, NS, Canada). Both strains had similar sizes (average total length of 3.07 ± 0.23 cm and average weight of 0.34 ± 0.10 g for wild-type zebrafish; average of 3.27 ± 0.23 cm in length and 0.33 ± 0.07 g in weight for Casper zebrafish). A mixture of females and males from each strain were divided and acclimated into 4 separate 60L tanks. Fish were maintained at a water temperature of 28 °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, QC, 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.

2.2. Fasting experiments

Fifty zebrafish of each strain (25 females and 25 males for each of

Table 1
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 inhibiting 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 (tac3b)	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′	

AB wild-type and Casper fish) were separated into 4 tanks (8 tanks total, see Suppl. Table 1) and acclimated to these conditions for one week. Sex ratios were initially set at approximately 1 in each tank (for both Casper and wild-type). However, Casper fish displayed high mortality rates during the experiment (whereas no mortality was seen in wild-type fish), and this rate was higher in female fish (10) compared to males (3), which unfortunately biased the sex ratios.

Fish within one strain belonged to a same cohort and had similar sizes and ages. Zebrafish mature around 60-120dpf (around 80-100dpf for Casper (Jones and Lessman, 2014) and 60dpf for wild-type (Lawrence et al., 2012)), which corresponds to approximately 20 mm (or 2 cm) (Wang et al., 2015). All our fish were around 3 cm of length, which would correspond to around 6 month post-fertilization (Wang et al., 2015). Thus, all fish were mature and approximately of the same age. Based on the time of maturity and the age of the fish, we assume that they had had previous reproductive events.

After the acclimation period, two tanks continued to be fed once daily to satiation and two tanks were fasted for one week. On the day of sampling, both fed tanks were fed, and all fish were sacrificed 30 min post-feeding by immersion in 0.05% tricaine methanesulfonate (MS 222, Syndel Laboratories, Vancouver, BC, Canada) followed by spinal section. Gender was determined based on abdomen shape, coloration, 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 also recorded. Whole brains were collected and preserved in RNA*later* (Qiagen, Mississauga, ON, Canada) at −20 °C. RNA extraction and cDNA synthesis were performed, and brain mRNA expression levels were assessed using quantitative PCR (qPCR), as described below.

2.3. Reproductive stage analysis

The same fed and fasted female zebrafish from the above experiments were used for the analyses. Thus the same qPCR results were used but the data was analyzed differently. Female fish were separated into two categories based on their gonadosomatic index (GSI) (Suppl. Table 2). GSI was calculated as the proportion of gonadal mass relative to the total body mass $\left(i.\ e.\ \frac{weight\ of\ eggs\ (g)}{total\ body\ weight\ (g)} \times 100\%\right)$ and was used as an indicator of reproductive stage (West, 1990). As the fish were very small, we were unable to determine GSI for some females. We arbitrarily chose a value of 5% to separate small and large GSI, in order to have enough animals in all experimental groups. In addition, we were not able to collect male gonads (*i.e.* testes) during dissection, therefore only female fish were included.

2.4. Expression of genes involved in coloration

The expression levels of genes involved in pigmentation and 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).

2.5. RNA extraction and cDNA synthesis

RNA extractions were performed using a FastRNA Green Kit (MP Bio, Solon, OH, 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, DE, USA). All samples had absorbance ratios at 260 and 280 nm between 1.6 and 2.0. RNA integrity was also assessed by electrophoresis on a denaturing agarose gel.

200 ng of RNA from each brain tissue was reverse transcribed using a Verso cDNA Synthesis Kit (Thermo Fisher Scientific, Lafayette, CO, USA) following the manufacturer's protocol with 0.5 µl anchored oligo-dT and 0.5 µl random hexamers. All cDNAs were stored at −20 °C until further use.

2.6. 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/) (see Table 1 for primers used in fasting and reproductive stage experiments and Table 2 for primers used for genes involved in coloration). 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 °C). The amplicon size for each primer set was between 250 and 300 base pairs. Only primers with the highest efficiency and linearity were used in experimentation. Duplicate reactions were prepared using a mix containing 0.2 µl 10 mM forward primer, 0.2 µl 10 mM reverse primer,

Table 2
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′	

2.6 µl water, 5 µl SYBR FAST qPCR Master Mix (Kapa Biosystems, Boston, MA, 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, ON, Canada). The following cycling conditions were performed using a MasterCycler® Realplex 2S thermocycler (Eppendorf): 40 cycles; 1) Denaturation (94 °C for 30 s), 2) Annealing (57 °C for 45 s), and 3) Elongation (72 °C for 60 s). Efficiencies, R^2 values, and optimal annealing temperatures were determined for all primer pairs. Efficiencies ranged from 90 to 100% for all reactions. A melting curve analysis was also 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.

Gene expression levels were measured and quantified using Realplex 1.5 software (Eppendorf). Expression levels were compared using the ΔΔCT method; briefly, the average CT of the reference gene (*i.e.* EF) was subtracted from the average CT of the gene of interest to determine the ΔCT for each sample. The ΔCT of the calibrator (fed males, fed low GSI females or wild-type zebrafish) was then subtracted from the ΔCT of each of the samples to determine the ΔΔCT. This number was then used to determine the amount of mRNA relative to the calibrator and normalized by the reference gene (Livak and Schmittgen, 2001). Data are provided as the fold changes in expression relative to the reference gene and compared to a calibrator sample from the control group, which was arbitrarily set at 1. The average fold of all the control samples was taken and set at 100%. The experimental groups were normalized relative to the control group and the percentage values (100% for the control and other % for the other groups) were then compared statistically.

2.7. Statistics

Statistical analysis was then performed using Prism7 GraphPad Instat (GraphPad Software Inc., San Diego, CA, USA). Significance was considered at $p < 0.05$. Data are expressed as mean ± SEM. All samples are expressed as ratios of specific target gene to the reference gene and normalized as a percentage of mRNA levels of fish in the control groups. Shapiro-Wilk normality tests were used to determine that all data followed a normal distribution. Student's t tests and two-way ANOVAs followed by Tukey's post tests were used to compare two experimental groups and four experimental groups, respectively. Two-way ANOVAs, established the significance of the main effects (gender and fasting, GSI and fasting, strain and fasting) and of the interactions between them. ANOVA statistics in text are provided as "F (DFn, DFd), p" [F = ratio of the mean-square value for source of variation to the residual mean square; DFn = degree of freedom from between columns; DFd: degree of freedom from within columns].

3. Results

3.1. Effects of fasting on gene expression

3.1.1. Wild-type zebrafish

In wild-type zebrafish, fasting or sex alone had no significant effect on orexin mRNA expression, but there was a significant interaction between the two factors [F (1, 19) = 11.96, p = 0.0026; Fig. 1A], with an increase in orexin mRNA expression seen in females (p = 0.0172), but not in males. There was a significant effect of fasting [F (1, 14) = 14.4, p = 0.0021] for NPY expression, with an increase in males (p = 0.0254) but not females. There was no significant effect of gender in either orexin or NPY, but an interaction between gender and fasting was seen for orexin [F (1, 19) = 12.5, p = 0.0022] (Fig. 1A).

An effect of fasting was seen for all reproductive genes examined, *i.e.* GnRH2 [F (1, 19) = 4.671, p = 0.0436], GnRH3 [F (1, 19) = 4.959, p = 0.0382], Kiss1 [F (1, 18) = 16.09, p = 0.0008], Kiss 2 [F (1, 15) = 11.71, p = 0.0038], GnIH [F (1, 18) = 18.03, p = 0.0005] and NKB [F (1, 18) = 12.07, p = 0.0027] (Fig. 2).

An effect of gender was seen for Kiss1 [F (1, 18) = 12.95, p = 0.0021], GnIH [F (1, 18) = 4.792, p = 0.0420] and NKB [F (1, 18) = 12.07, p = 0.0027]. Interactions between fasting and gender were seen for GnRH2 [F (1, 20) = 4.766, P = 0.0411], GnRH3 [F (1, 19) = 4.959, P = 0.0382], Kiss1 [F (1, 18) = 6.282, p = 0.0220], Kiss2 [F (1, 14) = 36.21, p < 0.0001], and GnIH [F (1, 18) = 10.27, p = 0.0049] (Fig. 2).

GnRH2, GnRH3, Kiss1, GnIH and NKB increased with fasting in females, while Kiss2 was significantly increased in male fish only (Fig. 2).

3.1.2. Casper zebrafish

In Casper zebrafish, there was no significant effect of either fasting or gender on either NPY or orexin expressions (Fig. 1B). An effect of fasting was seen for GnIH [F (1, 17) = 8.005, P = 0.0116] and an interaction was seen for NKB [F (1, 19) = 9.142, p = 0.0070] (Fig. 4). Fasting increased GnIH and NKB expressions in males and NKB expression was higher in fasted males compared to fasted females (Fig. 3).

3.2. Effects of reproductive stage on response to fasting in females

3.2.1. Wild-type zebrafish

In fed wild-type females, an effect of fasting was seen for orexin [F (1, 16) = 13.06, p = 0.0023] and GSI affected NPY [F (1, 16) = 11.48, p = 0.0038]. Orexin expression was increased by fasting in females with large, but not small, GSIs and NPY mRNA expressions were higher in females with higher GSIs than in females with small GSIs (Fig. 4). Interactions between fasting and GSI were seen for both orexin [F (1, 16) = 5.345, p = 0.0344] and NPY [F (1, 16) = 10.28, p = 0.0055].

An effect of fasting was seen for GnRH2 [F (1, 15) = 11.67, p = 0.0038], Kiss1 [F (1, 13) = 6.803, p = 0.0217], GnIH [F (1, 15) = 31.15, p < 0.0001] and NKB [F (1, 12) = 4.798, p = 0.0490]. An effect of GSI was seen for GnRH2 [F (1, 15) = 10.49, p = 0.0055], GnRH3 [F (1, 14) = 21.97, p = 0.0003] and Kiss1 [F (1, 13) = 5.679, p = 0.0331]. Interactions were detected for Kiss1 [F (1, 13) = 12.16, p = 0.0040] and GnIH [F (1, 15) = 11.41, =0.0041]. There were no significant effects or interaction for Kiss2 (Fig. 4).

In females with high GSIs, fasting increased the expressions of Kiss1, GnRH2, GnIH and NKB but did not affect GnRH3, or Kiss2 (Fig. 4). In females with small GSIs, fasting did not affect the expression of any of the hormones examined (Fig. 4).

In fed females, GnRH2 and GnRH3 expression levels were higher in females with large GSIs compared to females with small GSIs. In fasted females, GnRH2, GnRH3, Kiss1 and GnIH expression levels were higher

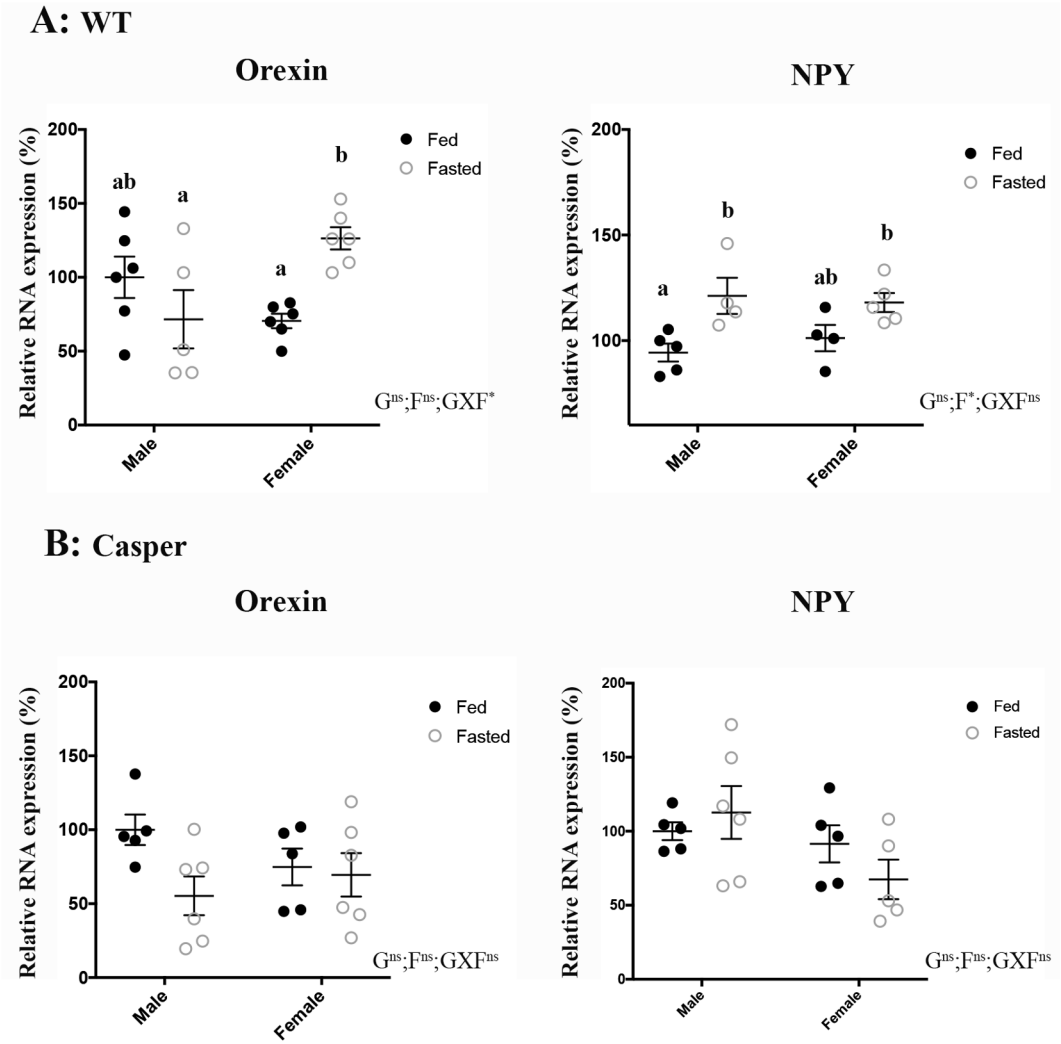

Fig. 1. Relative brain mRNA expression of orexin and NPY in fed and fasted wild-type (A: WT) and Casper (B: Casper). Zebrafish in males and females. Data are represented as mean ± SEM. Different superscripts (2-way ANOVA, Tukey post-test) indicate significant differences ($p < 0.05$) between groups. Effects of gender (G) and fasting (F) as well as interactions between them (G × F) are indicated in lower panels as being non-significant (ns, $p > 0.05$) or significant (*, $p < 0.05$) (n = 4–6 per group).

in females with large GSIs compared to females with small GSIs (Fig. 4).

3.2.2. Casper zebrafish

In Casper fish, there were no significant effects or interactions for orexin, NPY, GnRH2, GnRH3, Kiss1, Kiss2, GnIH or NKB (Fig. 5).

3.3. Strain-specific differences in enzymes involved in melanin synthesis and the melanocortin system

The brain mRNA expressions of enzymes involved in melanin synthesis (TH1, and TH2 tyrosinase) were significantly lower in Casper zebrafish compared to wild-type zebrafish (Fig. 6A).

There was an effect of fasting for MC3R [$F(1, 17) = 35.94$, $p < 0.0001$], and POMC [$F(1, 17) = 14.18$, $p = 0.0015$], but not MC4R. There was an effect of strain for MC4R [$F(1, 17) = 14.78$, $p = 0.0013$] and POMC [$F(1, 17) = 9.302$, $p = 0.0072$]. Interactions between strain and fasting were seen for MC3R [$F(1, 18) = 9.161$, $p = 0.0073$] and POMC [$F(1, 17) = 5.997$, $p = 0.0255$] (Fig. 6B).

Fasting induced an increase in MC3R expression in Casper but not wild-type fish, and a decrease in POMC in wild-type, but not Casper fish. No significant effect of fasting was seen in the brain expression of MC4R in either strain (Fig. 6B). In fed fish, MC3R, but not MC4R or POMC expressions were lower in Casper zebrafish compared to wild-type zebrafish (Fig. 6B). In fasted fish, MC4R expression was higher in Casper than wild-type fish (Fig. 6B).

4. Discussion

4.1. Wild-type zebrafish

4.1.1. Effects of fasting on appetite-regulating hormones

The expressions of the two appetite-regulating hormones investigated (NPY and orexin) increased during fasting (Suppl. Table 3). 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.* zebrafish (Novak et al., 2005; Yokobori et al., 2011), winter flounder (Buckley et al., 2010), dourado (Volkoff et al., 2016), and goldfish (Nakamachi et al., 2006)]. Orexin has also been implicated in sleep/wake cycles and locomotion in both mammals (Tyree et al., 2018) and fish. In zebrafish, overexpression of orexin results in increased locomotion (Prober et al., 2006), and orexin injections result in increased searching behavior in both goldfish (Volkoff et al., 1999) and cavefish (Penney and Volkoff, 2014). In our study, although this was not quantified, fasted fish appeared to be more active (*i.e.* swam more rapidly and in a more random pattern) than fed fish, suggesting that

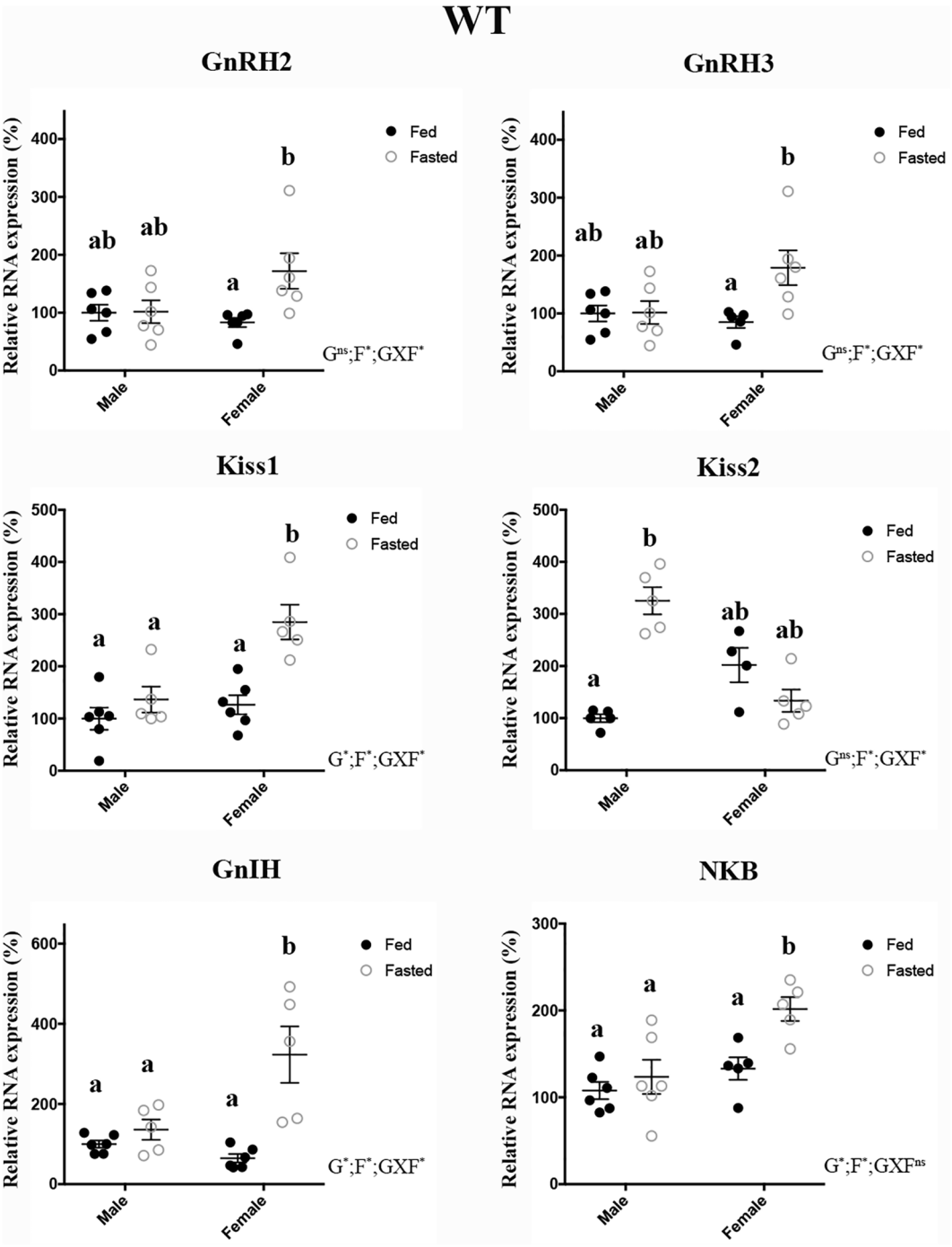

Fig. 2. Relative brain mRNA expression of GnRH2, GnRH3, Kiss1, Kiss2, GnIH, and NKB in male and female fed and fasted wild-type (WT) zebrafish. Data are represented as mean ± SEM. Different superscripts (2-way ANOVA, Tukey post-test) indicate significant differences ($p < 0.05$) between groups. Effects of gender (G) and fasting (F) as well as interactions between them (G × F) are indicated in lower panels as being non-significant (ns, $p > 0.05$) or significant (*, $p < 0.05$) (n = 5–6 per group).

they increased their locomotor activity in an attempt to find food.

The increase in NPY during fasting is also consistent with results in mammals (Marks et al., 1992) and fish, including zebrafish (Jeong et al., 2018), winter skate (*Raja ocellata*) (MacDonald and Volkoff, 2009a), channel catfish (*Ictalurus punctatus*) (Peterson et al., 2012), blunt snout bream (*Megalobrama amblycephala*) (Ji et al., 2015) and winter flounder (MacDonald and Volkoff, 2009b). In both goldfish and zebrafish, injections of NPY lead to an increase in feeding, further showing the orexigenic role of NPY in fish.

Interestingly, an increase in orexin was seen only in females, whereas NPY only increased in males. To our knowledge, there is no published data on gender-specific responses to fasting of these hormones in fish. However, our results are consistent with studies in rats showing that fasting increases the number of orexin neurons and the expression of the orexin receptors in females but not in males (Funabashi et al., 2009; Iwasa et al., 2015). Both orexin and NPY have been shown to interact with the hypothalamic-pituitary-gonad axis. For example, in goldfish, NPY stimulates GnRH release (Peng et al., 1993) whereas orexin decreases GnRH brain mRNA expression (Hoskins et al., 2008), thus likely affecting sex steroid levels. In the cichlid fish (*Cichlasoma dimerus*), injections of NPY increase gonadotropin levels in a sex-specific manner, with females requiring higher doses to elicit a

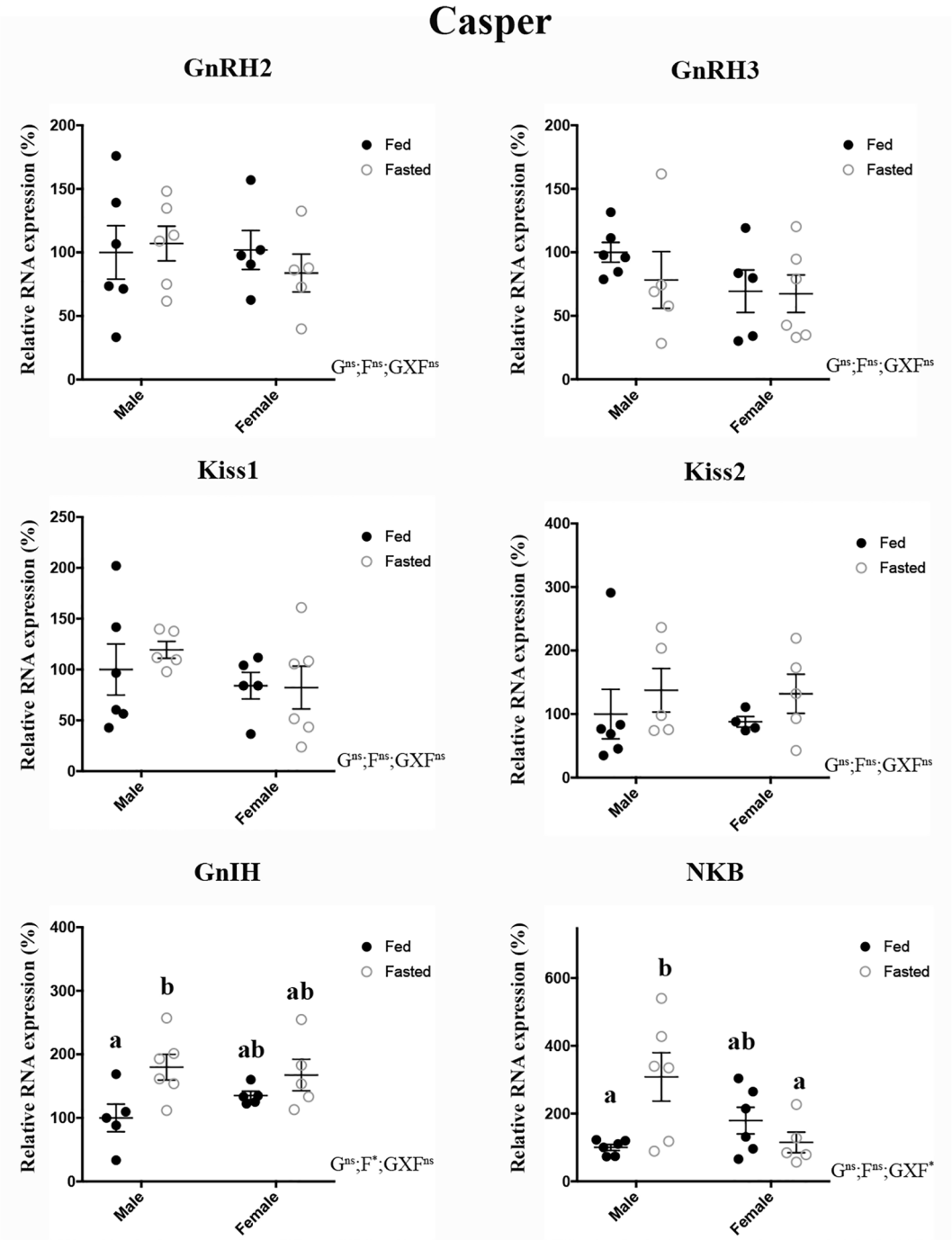

Fig. 3. Relative brain mRNA expression of GnRH2, GnRH3, Kiss1, Kiss2, GnIH, and NKB in in male and female fed and fasted Casper zebrafish. Data are represented as mean ± SEM. Different superscripts (2-way ANOVA, Tukey post-test) indicate significant differences ($p < 0.05$) between groups. Effects of gender (G) and fasting (F) as well as interactions between them (G × F) are indicated in lower panels as being non-significant (ns, $p > 0.05$) or significant (*, $p < 0.05$) (n = 4–6 per group).

response (Di Yorio et al., 2015). Conversely, sex steroids have been shown to affect the expression and secretions of orexin [*e.g.* in pigs (Kiezun et al., 2019) and rats (Silveyra et al., 2009)] and NPY [*e.g.* (Dhillon and Belsham, 2010)]. It is thus not surprising that gender-specific differences were observed in the response to fasting in our study.

4.1.1.1. Effects of fasting on reproductive hormones. In wild-type zebrafish, all of the reproductive hormones investigated were up-regulated during fasting, albeit in a gender-specific manner, with an increase in GnRH 2 and 3, Kiss1, GnIH and NKB in females and Kiss 2 in males (Suppl. Table 3).

The fasting-induced increases in GnRH2 and GnRH3 seen in females contrast with previous findings in mammals and fish. Indeed, fasting decreases the expression of GnRH in mammals (Gruenewald and Matsumoto, 1993; Martin et al., 2008; Parillo et al., 2014) and GnRH2 and GnRH3 in winter flounder (Tuziak and Volkoff, 2013). In zebrafish, although fasting has not been investigated directly, overfeeding fish results in higher expression levels of GnRH2 (Nishiguchi et al., 2012). Central injections of GnRH2 decrease food intake in both goldfish (Hoskins et al., 2008; Matsuda et al., 2008) and zebrafish (Nishiguchi et al., 2012), and decrease orexin brain expression in goldfish (Hoskins

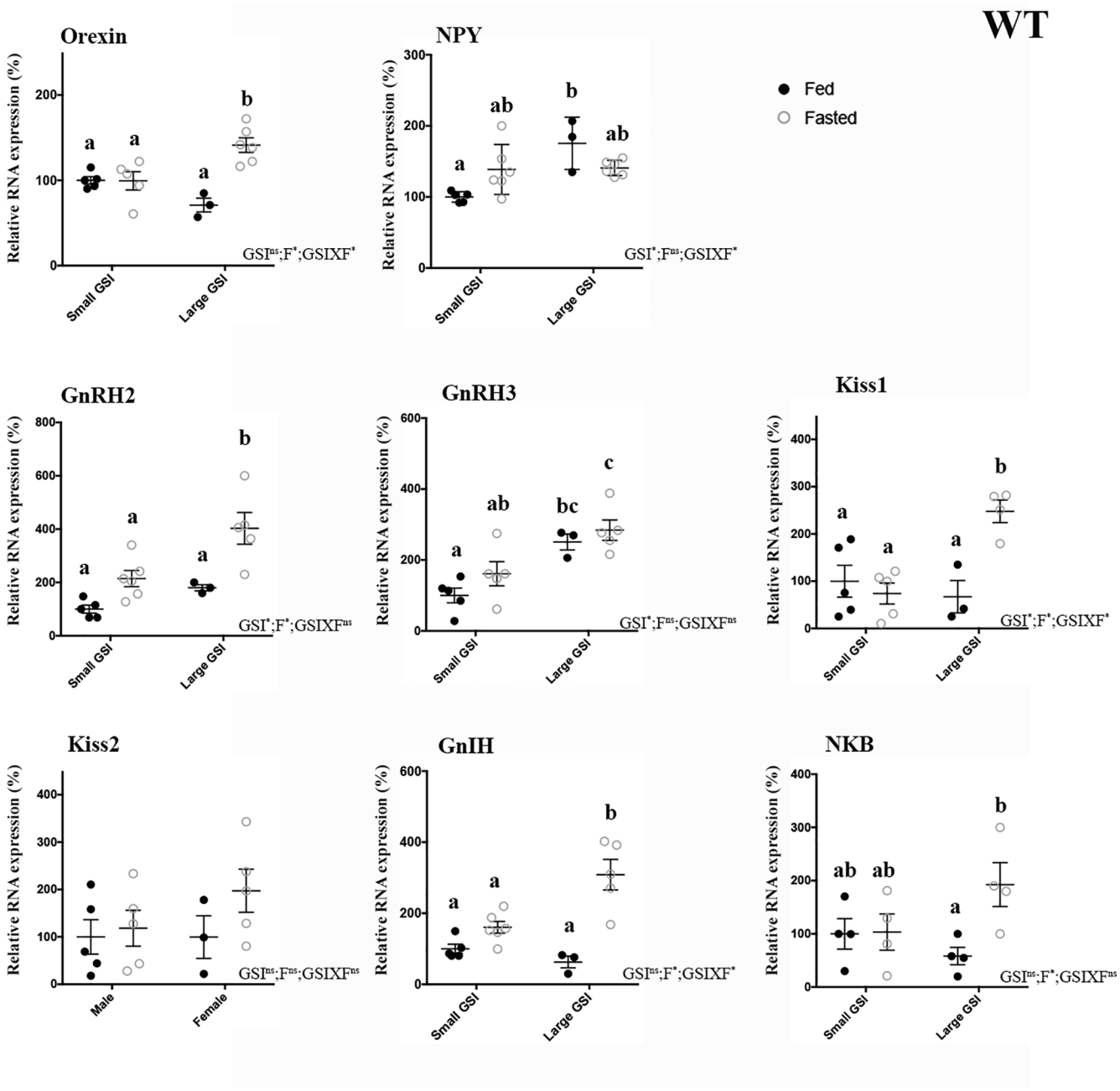

Fig. 4. Relative brain mRNA expression of orexin, NPY, GnRH2, GnRH3, Kiss1, Kiss2, GnIH, and NKB in fed and fasted female wild-type (WT) zebrafish with small (< 5%) and large (> 5%) GSI. Data are represented as mean ± SEM. Different superscripts (2-way ANOVA, Tukey post-test) indicate significant differences ($p < 0.05$) between groups. Effects of GSI and fasting (F) as well as interactions between them (GSI × F) are indicated in lower panels as being non-significant (ns, $p > 0.05$) or significant (*, $p < 0.05$) (n = 3–5 per group).

et al., 2008), suggesting that GnRH acts as an anorexigenic factor. However, in zebrafish, double GnRH2/GnRH3 knockouts exhibit down-regulation of agouti-related peptide 1 (agrp1), an orexigenic peptide (Jeong et al., 2018; Marvel et al., 2018), suggesting that the GnRH system might stimulate feeding.

The increase in GnIH expression in females is consistent with previous studies showing an orexigenic action of GnIH and fasting-induced increases in GnIH expression in mammals [rat (Johnson et al., 2007); sheep (Clarke et al., 2012)], and birds [chicken (Tachibana et al., 2005); duck (Fraley et al., 2013; McConn et al., 2016)]. In goldfish, GnIH lowers serum LH levels and decreases gonadotropin mRNA expression (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-Salmeron et al., 2016).

In our study, fasting increased the brain expressions of Kiss1 in females and Kiss2 in males. In agreement with our results, food restriction increases hypothalamic expression levels of Kiss1 and Kiss2 in both sea bass (Escobar et al., 2016) and Senegalese sole (*Solea senegalensis*) (Mechaly et al., 2011) and of Kissr2 receptor in pejerrey (*Odontesthes bonariensis*) (Mechaly et al., 2018). In contrast, fasting in mammals has been shown to decrease kisspeptin brain expression [rats (Ladyman and Woodside, 2014); lambs (Polkowska et al., 2015); monkeys (Shamas et al., 2015)]. Our results suggest that the different forms of kisspeptin might differentially regulate reproduction in male and female fish.

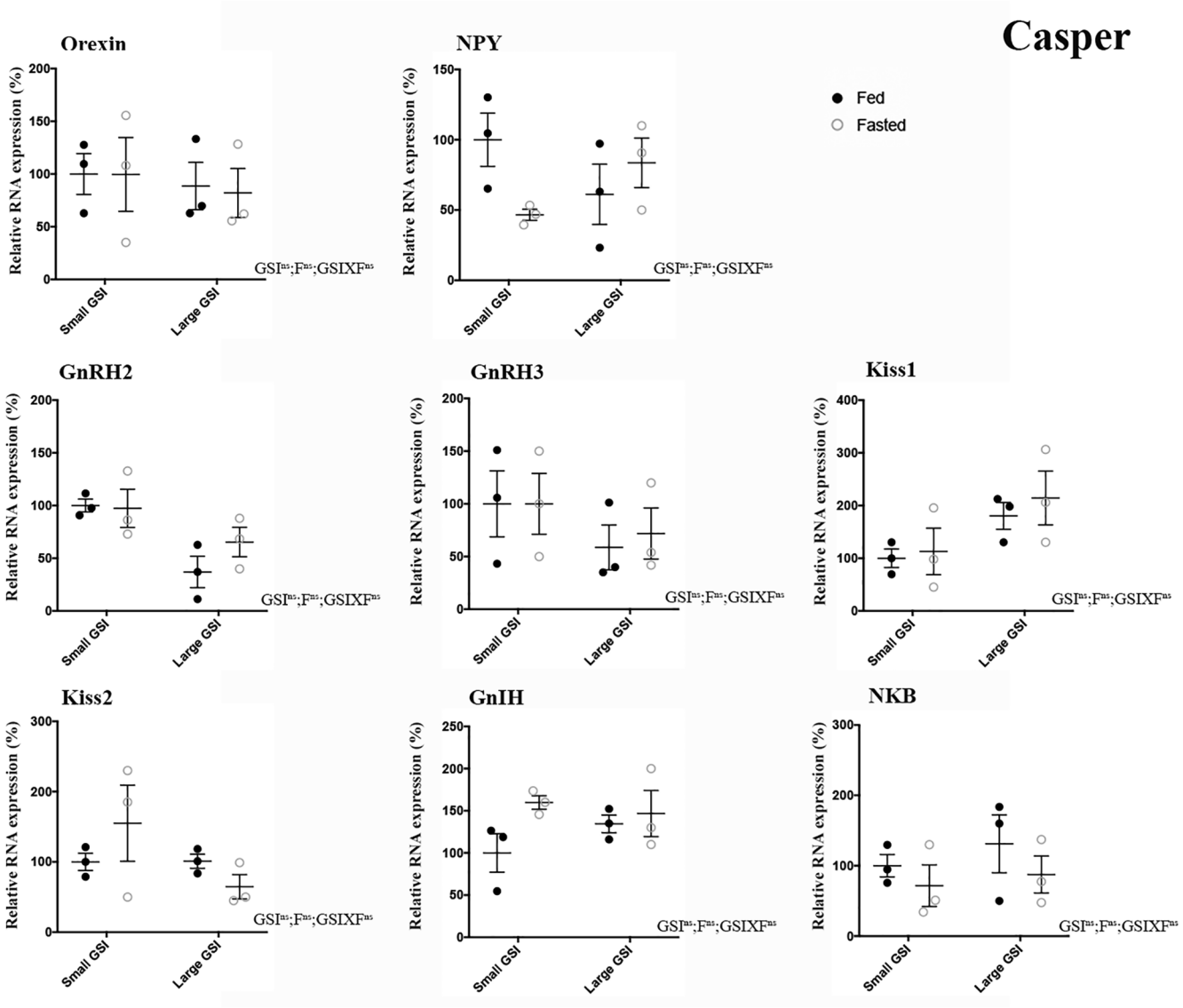

Fig. 5. Relative brain mRNA expression of orexin, NPY, GnRH2, GnRH3, Kiss1, Kiss2, GnIH, and NKB in fed and fasted female Casper zebrafish with small (< 5%) and large (> 5%) GSI. Data are represented as mean ± SEM. Different superscripts (2-way ANOVA, Tukey post-test) indicate significant differences ($p < 0.05$) between groups. Effects of GSI and fasting (F) as well as interactions between them (GSI × F) are indicated in lower panels as being non-significant (ns, $p > 0.05$) or significant (*, $p < 0.05$) (n = 3 per group).

Consistent with these results, fasting in sea bass increases mRNA levels of Kiss2 more than Kiss1 in males (Escobar et al., 2016). In castrated male seabass, testosterone treatment decreases hypothalamic expression of Kiss2 but not Kiss1, whereas in castrated females, estradiol treatment has no effect on either Kiss1 or Kiss2 (Alvarado et al., 2016). In medaka (*Oryzias latipes*), *kiss1*, but not *kiss2*, neurons are positively regulated by ovarian estrogens (Mitani et al., 2010).

In our study, fasting increased Tac3b brain expression in females, suggesting that the NKB system is affected by nutritional status and that NKBb might be involved in appetite regulation in wild type zebrafish. To our knowledge, there are no published studies on the effects of fasting on the NKB system in fish. In contrast to results in fish, in female rats, fasting decreases hypothalamic expressions of NKB and its receptor (Navarro et al., 2012). These results suggest that nutritional status affects the kisspeptin and NKB systems differently in mammals and fish. It is noteworthy that 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). These structural differences might indicate different physiological actions of NKB and kisspeptin in fish and in mammals, and perhaps explain in part these apparently contradictory results.

All genes examined were affected by fasting in males or females, but the expressions of reproductive hormones in females were more affected by fasting than males. Gender-specific differences in energy allocation have been reported in other vertebrates [*e.g.* mice (Perrigo and Bronson, 1985), rats (Ray and Hansen, 2004), and rainbow trout (Øverli et al., 2006)], with females being more sensitive to energetic stress than males, likely because they require more energy for reproduction. Indeed, in almost all species, females invest considerable energy in gamete production and parental care (Hayward and Gillooly, 2011; Lode, 2012; Penn and Smith, 2007) whereas males typically spend less energy on reproductive behaviors (*e.g.* courting, chasing) (Andersson, 1994). This may explain why fasting predominantly affected the expressions of reproductive hormones (*i.e.* GnRH2, GnRH3, GnIH, and NKB) in females, but not males, in this study.

All of these studies, including the present one, support the idea that

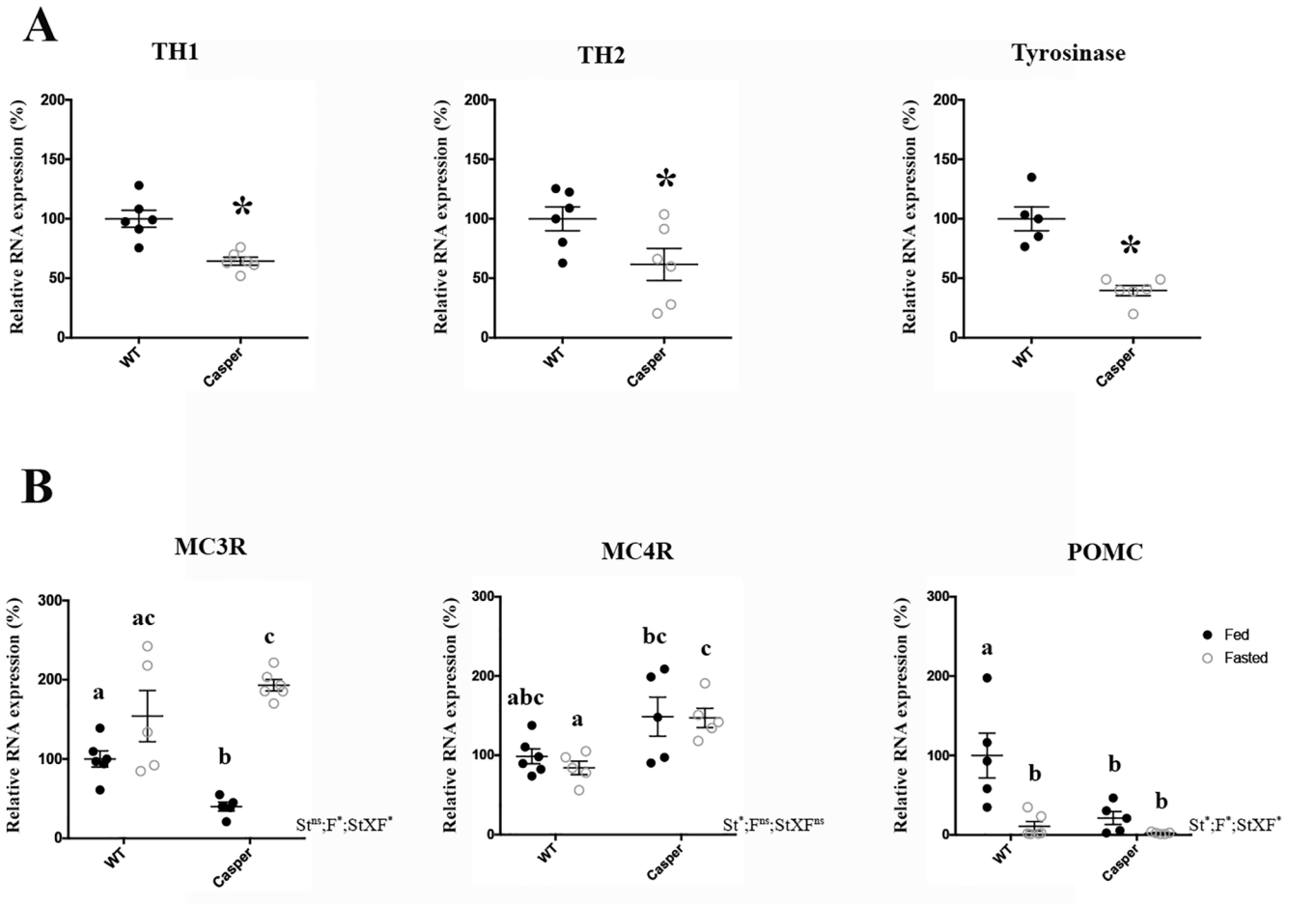

Fig. 6. (A) Relative brain mRNA expression of TH1, TH2 and Tyrosinase in fed wild-type and Casper zebrafish. Significant differences ($p < 0.05$) are indicated by stars (*) (Student's test). Data are represented as mean ± SEM. (B) Relative brain mRNA expression of MC3R, MC4R, and POMC in fed and fasted wild-type and Casper zebrafish. Data are represented as mean ± SEM. Different superscripts (2-way ANOVA, Tukey post-test) indicate significant differences ($p < 0.05$) between groups. Effects of strain (St) and fasting (F) as well as interactions between them (St × F) are indicated in lower panels as being non-significant (ns, $p > 0.05$) or significant (*, $p < 0.05$) (n = 5–6 per group.

reproduction and appetite are regulated differently depending on gender, which agrees with the differences in morphology, physiology, and energetic requirements between males and females.

4.1.2. Influence of reproductive stage on the response to fasting in females

In fed wild-type females, fasting induced increases in orexin, GnRH2, Kiss1, GnIH, and NKB, but not NPY, GnRH3 or Kiss2, mRNA expressions in females with large GSIs (more eggs or larger eggs relative to body weight) whereas no effects were seen in females with low GSIs.

The results in females with large GSIs are consistent (except for GnRH3) with those seen in females overall, suggesting that these females are "driving" the changes when all females are considered (Suppl. Table 3). These results show that females ready to spawn are more responsive to fasting, which is not surprising, as they might have increased energy demands for growth and maintenance of their eggs.

Differences in expression levels were seen between females with large GSIs and females with low GSIs (orexin, GnRH2, GnRH3, Kiss1, GnIH in fasted females; NPY in fed females).

To our knowledge, there are no studies on the effects of sexual stage on orexin in fish. In male rats, although the number of orexin neurons increase during maturation, it decreases and remain stable in adult individuals (Sawai et al., 2010), suggesting that orexin might play a more prominent role in development and maturation than in the regulation of adult reproductive cycles. However, in female rats, peripheral injections of orexin receptor antagonists decrease pro-oestrus gonadotropins and ova number (Silveyra et al., 2007).

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 with more egg might relate to its stimulating actions on GnRH neurons.

Consistent with our results, previous studies have shown increases in GnRH2 levels during sexual maturation in zebrafish (Kitahashi et al., 2009). An increase in GnRH3 expression levels in females with higher GSIs is not surprising as it has been suggested that in zebrafish, GnRH3 regulates pituitary functions and stimulates the release of gonadotropins, which in turn induce gonadal development and gamete production (Liu et al., 2017; Zohar et al., 2010). In addition, sexual maturation in zebrafish has been correlated to increases in GnRH3 and GnRH2 levels (Kitahashi et al., 2009).

Previous studies have shown increases in both *kiss1* and *kiss2* mRNA levels during sexual maturation in zebrafish (Kitahashi et al., 2009), but this refers to first maturation (immature to mature fish), not cycles in adult fish. Similarly, recent evidence shows that 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) and GnRH2/GnRH3 double knockout zebrafish display normal reproductive functions (Marvel et al., 2018). The lack of changes in GnRH2 and kisspeptin expression between females with different GSIs might indicate that these peptides are not required for normal gonadal maturation in zebrafish.

GnIH remained stable across reproductive stages in female wild-type fish. Whereas GnIH inhibits reproduction in birds and mammals,

its role in fish remains controversial as both inhibitory or stimulatory effects have been reported, depending on factors such as species, reproductive strategies and doses used (Muñoz-Cueto et al., 2017).

Although the role of NKB in puberty has been established in mammals (Young et al., 2010), very few studies examine its variations during reproductive cycles. In human females, treatments with NKB antagonists during the follicular phase of the menstrual cycle results in reduced LH secretion and follicle growth and delayed ovulation (Skorupskaite et al., 2018). In fish, the actions of NKB appear to be form- and species-specific. In zebrafish, both NKBa (encoded by *Tac3a*) and NKBb (encoded by *Tac3b*) stimulate LH secretion but NKBa has no effect on LH in in grass carp (*Ctenopharyngodon idellus*) (Biran et al., 2012; Hu et al., 2014b). In goldfish, ovariectomy increases *Tac3a*, but not *Tac3b*, hypothalamic mRNA expression (Qi et al., 2015). In our study, we only assessed changes in expression of *Tac3b*, and it is possible that *Tca3a* might have displayed changes during the reproductive stages.

4.2. Casper zebrafish

In Casper zebrafish, with the exception of GnIH and NKB in males, none of the hormones examined were affected by fasting (Suppl. Table 3). The increases in NKB and GnIH in Casper males are not seen in wild-type zebrafish. These results are surprising as they are two strains of the same species that were subjected to the same experimental conditions. The mutation underlying the Casper phenotype is a knockout of two genes that results in the complete lack of melanophores and iridophores. To our knowledge, neither gene is directly involved in feeding or reproduction. Interestingly, when comparing fed fish, brain mRNA levels of all hormones, with the exception of GnRH2, are higher in Casper than in wild-type zebrafish (Suppl. Fig. 2). The reason for these differences is not clear, but high levels of expression in Casper might have masked any slight changes in mRNA expression between groups.

It is noteworthy that due to high mortality rates, the sample size was relatively low for Casper zebrafish (n = 3 per group) and caution should be used when interpreting the results. In addition, the sex ratio within the Casper experimental tanks was not 50:50. It is known that sex ratio can have an effect of sexual maturation of both sexes in fish (Lima, 2018; Örn et al., 2016). However, in our study, all the fish were sexually mature. It is also possible that sex ratio might have affected reproductive behavior. However, this is unlikely, as it has been shown that zebrafish kept at different sex ratios (1 male:1 female; 3 males:1 female; 1 male:3 females) show no difference in the total number of eggs or the number of fertilized eggs produced (Ruhl et al., 2009).

It is also possible that the lack of coloration of the Casper zebrafish might have influenced reproductive cycles and behavior. In many animals, including fish, coloration patterns can affect or drive reproductive behavior. For example, goldfish tend to associate with other similarly colored fish rather than fish with a different color or pattern (Breder and Halpern, 1946). Zebrafish often prefer fish with stripes to those without stripes (McCann et al., 1971), and female zebrafish favor horizontally striped over vertically striped males (Turnell et al., 2003). Therefore, it is possible that because the Casper zebrafish lack the typical striped pattern, their reproductive behavior, and consequently reproductive hormones, were negatively influenced.

However, in GloFish™, which are genetically engineered zebrafish that express red fluorescent protein (RFP) resulting in on overall red coloration under the dark longitudinal stripes, no differences in either shoaling or reproductive behavior compared to wild-type fish (Snekser et al., 2006). In addition, transgenic transparent medaka (who have other genes mutated) appear to be healthy and reproduce normally in the lab (Wakamatsu et al., 2001), and in the naturally transparent glass catfish, GnRH1 decreases with fasting (London and Volkoff, 2019).

Further studies using more Casper zebrafish are needed to better understand the mechanisms underlying these differences between Casper and wild-type zebrafish.

4.3. Strain-specific differences in melanin synthesis pathway and the melanocortin system

In order to assess if mutations of genes/cells involved in pigmentation might be responsible for differences in fasting response between Casper and wild-type zebrafish, we investigated enzymes involved in the melanin synthesis and peptides of the melanocortin system.

Our results show that fed Casper fish displayed lower mRNA levels of enzyme involved in melanin synthesis (tyrosinase, TH1, TH2) compared to wild-type fish. This down-regulation in Casper zebrafish indicates a decrease in melanin synthesis, which is not surprising, as these transgenic fish are fully transparent and lack pigmentation.

Melanin and melatonin share in part a synthesis pathway through BH4, an essential cofactor not only for the synthesis of peripheral eumelanin but also for the production of serotonin, which is converted into melatonin in the pineal gland (Leclercq et al., 2010). Although the pineal system is still poorly characterized in teleosts, it has been shown that melatonin, from the pineal gland, increases melanophore aggregation in teleost fish (Hafeez, 1970). In zebrafish, most of the brain reproductive genes (*gnrh2, gnrh3, kiss1, kiss2* and *gnrhr3*) display a daily rhythm of expression (Paredes et al., 2019), likely regulated in part by melatonin, which stimulates reproduction by activating the HPG axis (Falcón et al., 2007; Falcón et al., 2010). Therefore, it is possible that the lack of melanin synthesis could be disrupting the retinal and pineal melatoninergic system, leading to changes in the fish reproductive physiology and expression of reproductive genes.

Interestingly, fed Casper also had lower expression levels of MC3R and POMC than fed wild-type fish. In addition, fasted Casper fish had higher MC4R expression compared to fasted wild-type fish. These results suggest that changes in the melanin synthesis pathway in Casper also affect the melanocortin system.

When comparing the response to fasting of the melanocortin system peptides, fasting induced an increase in MC3R expression in Casper but not wild-type fish, and a decrease in POMC in wild-type, but not Casper fish, but no effect of fasting was seen in the brain expression of MC4R in the brain of either strain.

Tyrosinase also catalyzes the conversion of tyrosine into L-DOPA, which can be converted to dopamine by DOPA decarboxylase. It is possible that lower expression levels of TH might lead to decreased levels of dopamine in some brain cells. Dopamine has been shown to affect both feeding and reproduction in fish. In goldfish, fasting increases the brain mRNA expression of enzymes of the dopamine pathway (Mandic and Volkoff, 2018). In addition, in cavefish (Penney and Volkoff, 2014) and goldfish (Mandic and Volkoff, 2018), orexin injections induce an increase in brain expression of these enzymes, suggesting that dopamine might mediate some of the effects of appetite regulators. Dopamine has also been shown to be an inhibitor of reproduction in many fish species, including zebrafish (Fontaine et al., 2013). In rats, dopamine receptors are located on the POMC-immunopositive neurons (Romanova et al., 2018), suggesting that dopamine might affect the synthesis of POMC. It is possible that in our study, a disruption in the melanin synthesis pathway may alter the dopamine pathway and potentially explain the differences seen between the two strains. Unfortunately, we were unable to compare dopamine levels between the two strains to verify this hypothesis.

5. Conclusion

Overall, our results suggest a link between reproduction and nutritional status in zebrafish, though the relationship seems to differ from that of mammals in some aspects. Our study shows gender- and reproductive stage-specific differences in the mechanisms regulating these two processes. Fasting induced changes in the expression of several appetite and reproductive hormones in wild-type zebrafish, with females being more 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 the Casper model is responding so differently, our data suggests that the melanocortin precursor (POMC) and the melanin pathways may be involved.

Further experiments are needed to verify our hypotheses as this study presents some limitations. First, due to high mortality rates, the sample size was relatively low (n = 3) for Casper zebrafish. The high mortality rates in Casper warrant further investigation. The high mortality rates were seen mostly during the acclimation period and during the first days of fasting, and not in the last portion of the fasting period, suggesting the major strain differences (and gender differences within Casper) might be indicative of higher stress levels in Casper (in particular females) compared to wild type fish. Another caveat of the study is that it is based on single cohorts and the same gene expression data was used in our comparisons between fed and fasted and male and female fish. The experiments should be repeated with multiple cohorts in order to verify the results.

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. Although our data provides novel insights into the endocrine mechanisms that regulate feeding and reproduction, the high diversity among fish species creates substantial difficulties when attempting to compare and generalize physiological concepts.

Acknowledgements

This work was supported by Natural Sciences and Engineering Research Council (NSERC) Discovery (DG, grant number 261414-03) to HV.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2019.06.011.

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