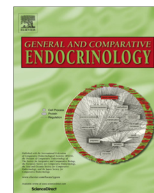
General and Comparative Endocrinology 228 (2016) 17–23

Contents lists available at ScienceDirect

General and Comparative Endocrinology

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



Estradiol and testosterone modulate the tissue-specific expression of *ghrelin*, *ghs-r*, *goat* and *nucb2* in goldfish

Juan Ignacio Bertucci [a], Ayelén Melisa Blanco [b], Luis Fabián Canosa [a,*], Suraj Unniappan [c,*]

[a] Instituto de Investigaciones Biotecnológicas-Instituto Tecnológico Chascomús (IIB-INTECH), Intendente Marino Km 8.2, B7130IWA Chascomús, Buenos Aires, Argentina CC 164 (7130), Argentina

[b] Departamento de Fisiología (Fisiología Animal II), Facultad de Biología, Universidad Complutense de Madrid, 28040 Madrid, Spain

[c] Laboratory of Integrative Neuroendocrinology, Department of Veterinary Biomedical Sciences, Western College of Veterinary Medicine, Saskatoon, Saskatchewan S7N 5B4, Canada

ARTICLE INFO

Article history:
Received 12 September 2015
Revised 5 January 2016
Accepted 6 January 2016
Available online 7 January 2016

Keywords:
Ghrelin
Nesfatin-1
Steroids
Food intake
Reproduction
Estradiol
Testosterone

ABSTRACT

Ghrelin, and nesfatin-1 (encoded by nucleobindin2/*nucb2*) are two metabolic peptides with multiple biological effects in vertebrates. While sex steroids are known to regulate endogenous ghrelin and *NUCB2* in mammals, such actions by steroids in fish remain unknown. This study aimed to determine whether estradiol (E2) and testosterone (T) affects the expression of *preproghrelin*, ghrelin/growth hormone secretagogue receptor (*GHS-R*), ghrelin O-acyl transferase (*GOAT*) and *NUCB2* in goldfish (*Carassius auratus*). First, a dose–response assay was performed in which fish were intraperitoneally (ip) implanted with pellets containing 25, 50 or 100 μg/g body weight (BW) of E2 or T. It was found that sex steroids (100 μg/g BW) administered for 2.5 days achieved the highest E2 or T in circulation. In a second experiment, fish were ip implanted with pellets containing 100 μg/g BW of E2, T or without hormone (control). RT-qPCR analyses at 2.5 days post-administration show that gut *preproghrelin* and *GOAT* expression was upregulated by both E2 and T treatments, while the same effect was observed for *GHS-R* only in the pituitary. Both treatments also reduced hypothalamic *preproghrelin* mRNA expression. *NUCB2* expression was increased in the forebrain of T treated group and reduced in the gut and pituitary under both treatments. These results show for the first time a modulation of *preproghrelin* and *nucb2*/nesfatin-1 by sex steroids in fish. The interaction between sex steroids and genes implicated in both metabolism and reproduction might help meeting the reproduction dependent energy demands in fish.

© 2016 Elsevier Inc. All rights reserved.

1. Introduction

The hormonal integration of metabolism and reproduction involves complex interactions between multiple hypothalamic neuropeptides, metabolic hormones and sex steroids (Volkoff et al., 2005, 2009). Two metabolic peptides that were recently added to the growing list of endocrine regulators of reproductive functions are ghrelin and nesfatin-1. Ghrelin is a natural ligand of the growth hormone (GH) secretagogue receptor (GHS-R) (Kojima et al., 1999). *Preproghrelin* mRNA expression has been found in the telencephalon, hypothalamus, gut, spleen and gill of goldfish, with most abundance in the gut (Unniappan et al., 2002; Unniappan and Peter, 2005). This hormone undergoes a unique post-translational modification with attachment of a medium-chain fatty acid, typically octanoic acid, to the third serine amino acid residue. This modification is enabled by ghrelin O-acyl transferase (GOAT) (Gutierrez et al., 2008; Hatef et al., 2015; Shlimun and Unniappan, 2011; Yang et al., 2008) and is necessary to activate its receptor (Jönsson, 2013). Ghrelin has been involved in a wide variety of physiological actions, and stimulates GH release and food intake (Kaiya et al., 2008). Ghrelin is now considered a multifunctional hormone in fish. Compared to ghrelin, nesfatin-1 is a relatively new peptide.

Nesfatin-1 is produced by the cleavage of N-terminal of its precursor nucleobindin-2 (NUCB2), encoded by *nucb2* gene (Oh-I et al., 2006). In rodents, wide distribution of *nucb2*/nesfatin-1 with prominent expression in hypothalamic nuclei, several brainstem areas, autonomic centers (Goebel-Stengel et al., 2011), gut (Stengel et al., 2010; Mohan et al., 2014; Ramesh et al., 2015) and pancreas (Gonzalez et al., 2011; Mohan and Unniappan, 2012) was found. *Nucb2* is abundant in many tissues including the hepatopancreas, brain, gonads, pituitary and gastrointestinal tract (Gonzalez et al., 2010). Nesfatin-1 reduces food intake after central or peripheral administration in mice (Shimizu et al.,

* Corresponding authors.
E-mail addresses: jbertucci@intech.gov.ar (J.I. Bertucci), ayelenmelisablanco@ucm.es (A.M. Blanco), lcanosa@intech.gov.ar (L.F. Canosa), suraj.unniappan@usask.ca (S. Unniappan).

http://dx.doi.org/10.1016/j.ygcen.2016.01.006
0016-6480/© 2016 Elsevier Inc. All rights reserved.

2009), rats (Gonzalez et al., 2011; Mortazavi et al., 2015; Oh-I et al., 2006; Sugino et al., 2004; Tang-Christensen et al., 2004; Shimizu et al., 2009; Shinsuke et al., 2006; García-Galiano et al., 2010b; Goebel et al., 2011) and fish (Gonzalez et al., 2010; Kerbel and Unniappan, 2012), which supports an anorexigenic role for this hormone. Ghrelin and NUCB2/nesfatin-1 were found co-localized in the anterior intestine and in the posterior part of the lateral tuberal nucleus of goldfish hypothalamus (Kerbel and Unniappan, 2012), suggesting a functional relationship between both peptides. Indeed, intracerebroventricular (i.c.v.) administration of nesfatin-1 decreases *preproghrelin* and *ghs-r* mRNA expression in brain. Meanwhile, icv administration of ghrelin suppresses *nucb2*, indicating opposing effects of these two peptides in regulating energy balance (Kerbel and Unniappan, 2012).

Ghrelin and nesfatin-1 also shares another common function, which is the modulation of reproduction. Shepperd et al. (2012) found a direct effect for ghrelin in regulating oocyte maturation in zebrafish. Nesfatin-1 is also implicated in reproductive functions in mice (García-Galiano and Tena-Sempere, 2013; Tena-Sempere, 2005; García-Galiano et al., 2012). Nesfatin-1 elicits a suppressive effect on the hypothalamo-pituitary–gonadal (HPG) axis of goldfish, and reduces serum LH levels and oocyte maturation (Gonzalez et al., 2012b). The broad distribution of NUCB2/nesfatin-1, as well as its role in regulating energy balance and HPG axis suggest a role for this peptide in integrating metabolism and reproduction (García-Galiano et al., 2010a; Gonzalez et al., 2012a). Collectively, these data indicate that ghrelin and nesfatin-1 are important physiological regulators in fish.

Sex steroids regulate endogenous ghrelin and nesfatin-1 in rodents (Matsubara et al., 2004; Park et al., 2013). The number of stomach ghrelin cells, plasma ghrelin and stomach *preproghrelin* mRNA expression significantly increase after ovariectomy in rats. These effects were all reversed by estradiol replacement (Matsubara et al., 2004). Supporting these results, Clegg et al. (2007) observed that ghrelin increases food intake in males and ovariectomized females, but not in intact females. Estradiol inhibits the orexigenic action of ghrelin. This suggests a functional interaction between estradiol and the ghrelinergic system in regulating food intake. Sex steroids also modulate *nucb2*. In the pituitary gland of mice, *nucb2* was dramatically decreased after ovariectomy, and it was rapidly increased after the injection of progesterone and estradiol (Chung et al., 2014). The same study reported that estradiol significantly increases *nucb2* expression in cultured pituitary tissue, while the opposite effect was observed for progesterone. In addition, Senin et al. (2015) found a correlation between testosterone changes associated with the initiation of puberty and levels of nesfatin-1 produced by stomach and adipose tissue in rats. They concluded that this fluctuation might regulate energy homeostasis during different developmental stages. Overall, these studies indicate that gonadal steroids are major regulators of endogenous nesfatin-1 in mammals.

Ghrelin and nesfatin-1 are now well characterized in fish. Although steroid hormones were found to regulate various endocrine factors in goldfish (Canosa et al., 2002; Lin et al., 2014; Manning et al., 2008; Peng et al., 1994; Trudeau et al., 1993; Volkoff et al., 2009; Wang et al., 2013), such effects on ghrelin and nesfatin-1 are unknown. Elucidating the role of gonadal steroids on endogenous ghrelin and nesfatin-1 is very important to better understand the endocrine milieu in a seasonal fish. The aim of this study was to determine the influence of sex steroids on ghrelin and nesfatin-1/NUCB2 in a teleost, goldfish. We studied whether continuous intraperitoneal (ip) administration of testosterone or estradiol regulates the expression of mRNAs encoding *preproghrelin* and *nucb2*, as well as the circulating levels of nesfatin-1 *in vivo*.

2. Materials and methods

2.1. Fish

Adult female goldfish (*Carassius auratus*) (comet variety, 10–13 cm long, body weight = 24.6 ± 0.38 g) undergoing sexually regressed or recrudescent reproductive phase, were acquired from a commercial supplier (Aquatic Imports, Calgary, AB, Canada) and the stock was maintained in 300 L tanks at 24 °C and a 12 h:12 h dark-light cycle photoperiod. For experiments, fish were kept in 10 L aerated aquaria (2 fish/aquarium), which received a constant flow of temperature controlled water (24 °C) and constant aeration. Fish underwent a weeklong acclimation period in these tanks and were fed *ad libitum* once daily on a commercial diet for goldfish (Martin Profishent, Ontario, Canada). All studies using fish strictly followed the Canadian Council of Animal Care guidelines, and protocols were approved by the Animal Research Ethics Board of the University of Saskatchewan (Protocol Number 2012-0082).

2.2. Reagents

Estradiol and testosterone were purchased from Sigma Chemical Co. (St. Louis, Mo., USA). Dimethyl silicone elastomer (Applied Silicone Corp., Ventura, Calif., USA) was prepared according to the manufacturer's instructions. Solid silicone pellets containing estradiol, testosterone or no steroid (blank) were manufactured according to previously established protocols (Pankhurst et al., 1986), washed in saline solution and implanted intraperitoneally as previously described (Trudeau et al., 1991). This method ensures a stable hormone supply for at least 20 days (Pankhurst et al., 1986).

2.3. In vivo studies

First, a dose–response study was conducted to determine the sex steroid hormone dose and duration of administration that would bring serum estradiol or testosterone significantly elevated compared with normal physiological values which are 2–7 ng/ml for estradiol (Zadmajid et al., 2012) and 0.4–2.5 ng/ml for testosterone (Trudeau et al., 1991). Fish were implanted with pellets containing either estradiol or testosterone at doses of 0 (control/placebo), 25, 50 and 100 μg/g BW. After 2.5 and 5 days of implantation, blood samples from early recrudescent females were collected, allowed to clot on ice, and centrifuged (7000 rpm, 9 min at 4 °C) to collect serum, which was stored at −80 °C until hormone analysis.

Based on the results from the dose–response experiment we used a dose of 100 μg/g BW and a duration of 2.5 days post pellet implantation to perform the second study aiming to determine the effects of estradiol and testosterone on the expression of mRNAs encoding *preproghrelin*, *goat*, its receptor *ghs-r and nucb2* in goldfish. After 2.5 days, fish were anesthetized by immersion in 0.05% tricaine methane sulfonate (Syndel Laboratories, Vancouver, British Columbia, Canada), euthanized by spinal transection and dissected. Forebrain, hindbrain, hypothalamus, pituitary, anterior intestine (J-loop region, anatomical equivalent of the mammalian stomach; Gonzalez and Unniappan, 2010) and serum were collected from female fish. All tissues were stored at −80 °C until RNA extraction.

2.4. RNA extraction and real-time quantitative PCR (RT-qPCR) analysis

Tissues were disrupted using a Mixer Mill MM 400 (RETSCH, USA) and total RNA was extracted using the Ambion® TRIzol®

Table 1
List of forward (F) and reverse (R) primers based on *Carassius auratus* mRNA sequences used for RT-qPCR analysis.

Name	Sequence	Tm (°C)	Gene
g-BActin-F	5′-CTACTGGTATTGTGATGGACT-3′	60	*β-Actin*
g-BActin-R	5′-TCCAGACAGAGTATTTGCGCT-3′	60	*β-Actin*
gf.18s.702-F	5′- GGATGCCCTTAACTGGGTGT - 3′	60	*18s ribosomal*
gf.18s.907-R	5′-CTAGCGGCGCAATACGAATG-3′	60	*18s ribosomal*
gf-NUCB2-F	5′-AGTCTCCCCAGAATGTGGAC-3′	60	*NUCB2*
gf-NUCB2-R	5′-CATCCAGCTTGGTTCTCACA-3′	60	*NUCB2*
gf-Ghrelin-F	5′-ATTCAGAGTGTTGTCGTA-3′	56.6	*Preproghrelin*
gf-Ghrelin-R	5′-AGGAAAGAGCACATAAGA-3′	56.6	*Preproghrelin*
gf-GHS-R-1a-F	5′-ATTCGAGCACCCGGTCAACA-3′	60	*GHS-R 1a*
gf-GHS-R-1a-R	5′-TCCAGGGGCATGCAGAGAAA-3′	60	*GHS-R 1a*
gf-GOAT-F	5′-ATTGCTGTTCTTCAGTGCCG-3′	60	*GOAT*
gf-GOAT-R	5′-TGTACAAGTGCCAGACGGTT-3′	60	*GOAT*

Reagent (Life Technologies, Canada) according to the protocol provided by the manufacturer. RNA purity was validated by optical density (OD) absorption ratio (OD 260 nm/280 nm) using a NanoDrop 2000c (Thermo, Vantaa, Finland) and only samples with a ratio between 1.8 and 2.0 were used for further procedure. Then, 1 µg of total RNA was reversely transcribed into cDNA using iScript™ Reverse Transcription Supermix for RT-qPCR (Bio-Rad, Canada).

Expression of genes was measured by RT-qPCR with iQ™ SYBR Green Supermix, (Bio-Rad, Canada) on a Thermal Cycler CFX Connect™ Real Time System (Bio-Rad, Canada), using *β-actin* (forebrain, hindbrain and hypothalamus) and *18S ribosomal* (gut and pituitary) as reference genes. All primers used for gene expression analysis are shown in Table 1. Each sample was run in duplicate and PCR reaction, without the addition of template, was used as negative control. Also, samples of RNA (RT-) were included to ensure there is no genomic. The RT-qPCR profiles contained an initial denaturation step at 95 °C for 5 min, followed by 35 cycles: 30 s at 95 °C, 30 s at the specific primer pair annealing temperature and extension for 30 s at 73 °C. After the amplification phase, a melt curve of 0.5 °C increments from 65 °C to 95 °C was performed, enabling confirmation of the amplification of a single product by each primer pair. The efficiency of the amplification for all genes studied was 95–100%. Gene expression levels were calculated by the $2^{-\Delta\Delta Ct}$ comparative threshold cycle (Ct) method (where $\Delta Ct = \Delta Ct$ sample − ΔCt; Livak and Schmittgen, 2001). The expression in the control group was considered as 1, and is indicated by a broken line in all graphs where mRNA expression is provided. The expression in other groups was normalized to the controls and was presented as a fold increase/decrease.

2.5. Serum analysis

Serum levels of steroid sex hormones were measured using estradiol and testosterone (Human) ELISA kits (Eagle Biosciences Inc., USA). The limit of assay sensitivity was 8.68 pg/mL and 0.07 ng/mL for estradiol and testosterone kit, respectively. Nesfatin-1 serum levels were determined using the nesfatin-1 (1-82) (Rat) ELISA Kit (Phoenix Pharmaceuticals Inc., California). The limit of assay sensitivity was 1.2 ng/mL. The amount of immunoreactive material was determined using a non-linear regression curve-fit. All ELISAs were performed following the manufacturer instructions. Considering that the nesfatin-1 ELISA (previously validated for use in goldfish; Gonzalez et al., 2010) detects both nesfatin-1 and the precursor NUCB2, "nesfatin-1/NUCB2-like immunoreactivity" was used to refer to the results of this assay.

2.6. Statistical analysis

Data were analyzed by one-way or two-way ANOVA, followed by post hoc Student–Newman–Keuls or Tukey tests, and significance was considered at $P < 0.05$. Data that failed to pass homogeneity tests were log-transformed and re-tested. All tests were performed using SigmaPlot Version 12.0 and Infostat Version 2008 software.

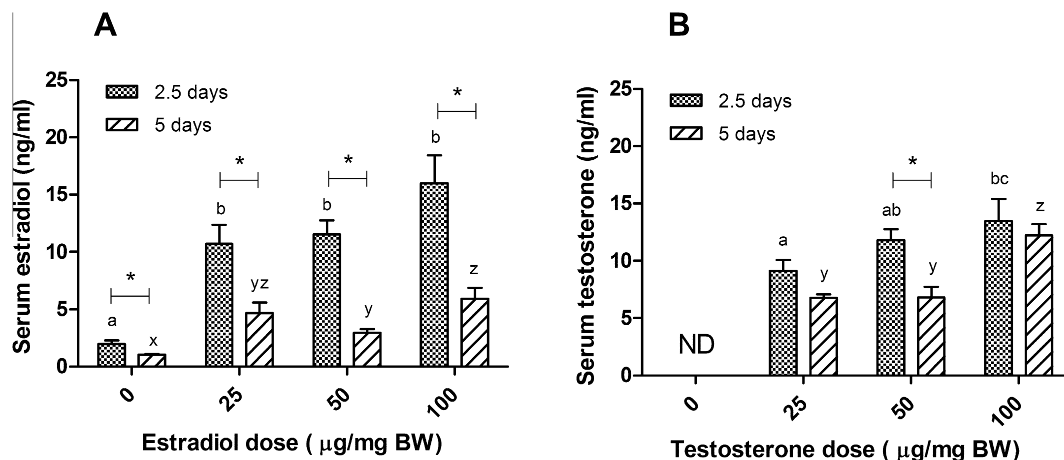


Fig. 1. Serum estradiol and testosterone levels after the dose–response and time course studies. Estradiol and testosterone serum levels in response to estradiol treatment (A) or testosterone (B) treatment, respectively, after 2.5 and 5 days. n = 9 fish/group. Statistical analyses were conducted by two way repeated measures ANOVA, followed by Student–Newman–Keuls test. In ANOVA, dose and time were considered as fixed factors, while estradiol or testosterone were the independent factors. Letters represent significant differences between doses, while asterisks indicate significant differences between various durations of treatment within the same dose group. ND: Not detected.

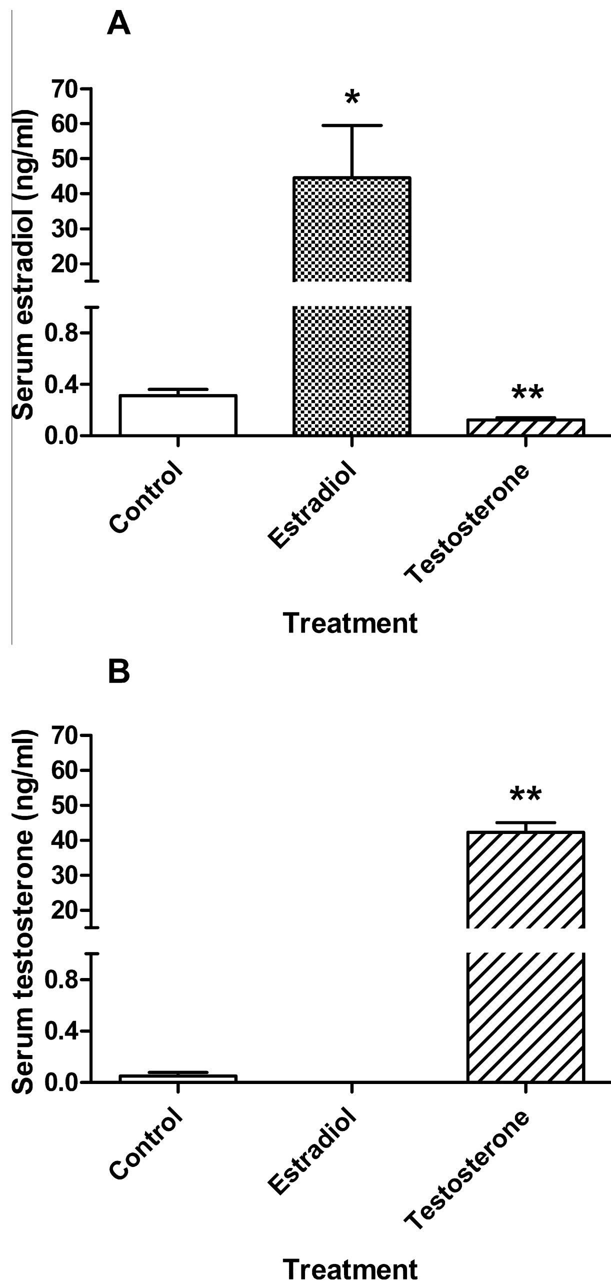


Fig. 2. Serum estradiol and testosterone were significantly increased by the administration of 100 μg/g BW of the specific hormone. Sex hormone serum concentrations for placebo control and estradiol (A) and testosterone (B) treatments after 2.5 days are shown. Asterisks represent significant differences between treatments and control ($^{*}p < 0.05$, $^{**}p < 0.01$). One-way ANOVA, Student–Newman–Keuls Method. ($n = 7$ fish/group).

3. Results

3.1. Estradiol and testosterone in serum increased after pellet implantation

On days 2.5 and 5 post-treatment, both estradiol (Fig. 1A) and testosterone (Fig. 1B) in serum were significantly elevated in the treated group at all three doses tested and two time points sampled, compared to the non-treated control group. In the case of estradiol we found that within the same dose the levels of hormone were highest at 2.5 days rather than 5 days of treatment. For testosterone assay, the same result was observed only with a dose of 50 μg/mg BW. In this case the major effect of steroid hormone administration on level was more affected by the dose rather than the time and was achieved with the maximum dose (100 μg/mg BW). Considering that period of time is prevalent for estradiol treatment and dose for testosterone treatment, we decided that the administration of 100 μg/g BW for 2.5 days will provide the most increase in circulating levels of steroids (Fig. 1A and B).

3.2. Effect of ip implants containing 100 μg/g BW estradiol or testosterone for 2.5 days on serum sex steroid levels

A significant increase ($p < 0.001$) in serum levels of estradiol (44.53 ± 15.02 ng/mL vs 0.3147 ± 0.04 ng/mL) (Fig. 2A) and testosterone (42.35 ± 2.72 ng/mL vs 0.04 ± 0.02 ng/mL) (Fig. 2B) was observed for estradiol and testosterone treatment, respectively. In addition, there was no increase in serum testosterone in the estradiol treated group, and vice versa (Fig. 2A and B).

3.3. IP implants containing 100 μg/g BW estradiol and testosterone modulate preproghrelin, goat, ghs-r and nucb2 mRNA levels

Gut *preproghrelin* mRNA expression was upregulated by both estradiol and testosterone (Fig. 3A). In the hypothalamus, both sex steroids decreased *preproghrelin* mRNA expression (Fig. 3A). No changes in *preproghrelin* mRNA was found in response to sex steroid hormones in other tissues studied (Fig. 3A). *Goat* mRNA expression was increased in the gut of fish treated with either estradiol or testosterone, whereas no changes were observed in other tissues compared with the control group (Fig. 3B). Meanwhile, *ghs-r* mRNA levels showed a significant increase only in the pituitary gland of fish treated with both sex steroids compared to controls (Fig. 3C). A down regulation of *nucb2* expression by both treatments was observed in the gut and pituitary (Fig. 3D). In addition, testosterone, but not estradiol, increased NUCB2 mRNA expression in the forebrain (Fig. 3D).

3.4. Estradiol and testosterone has opposing effects on circulating levels of nesfatin-1

Estradiol (100 μg/g BW) significantly increased nesfatin-1 serum levels compared to control group at 2.5 days post-implantation, while testosterone at the same dose and duration tested significantly decreased nesfatin-1 (Fig. 4).

4. Discussion

Many appetite-regulating hormones, including ghrelin and nesfatin-1, have been described to have an effect on reproduction (Fernández-Fernández et al., 2004; Garcia-Galiano et al., 2010; Unniappan, 2010). Whether gonadal steroids modulate metabolic hormones in fish remains poorly understood. Here, we provide the first set of evidence for the steroid hormone regulation of ghrelin and nesfatin-1 in fish. First, we determined that 100 μg/g BW of steroids for 2.5 days is effective in elevating serum estradiol or testosterone to a pharmacological range. This result is in agreement with previous research findings on steroid administration in goldfish (Canosa et al., 2002; Trudeau et al., 1991). Both estradiol and testosterone circulating levels were measured after each hormone treatment. This crossover measurement was carried out to ensure that the application of one steroid hormone does not affect the serum concentration of the other. Next, the above-mentioned dose of estradiol and testosterone was used to determine their effects on the ghrelinergic system and NUCB2/nesfatin-1 mRNA expression. Here again, the steroid hormone treatment resulted in pharmacological levels of E and T in circulation. The differences

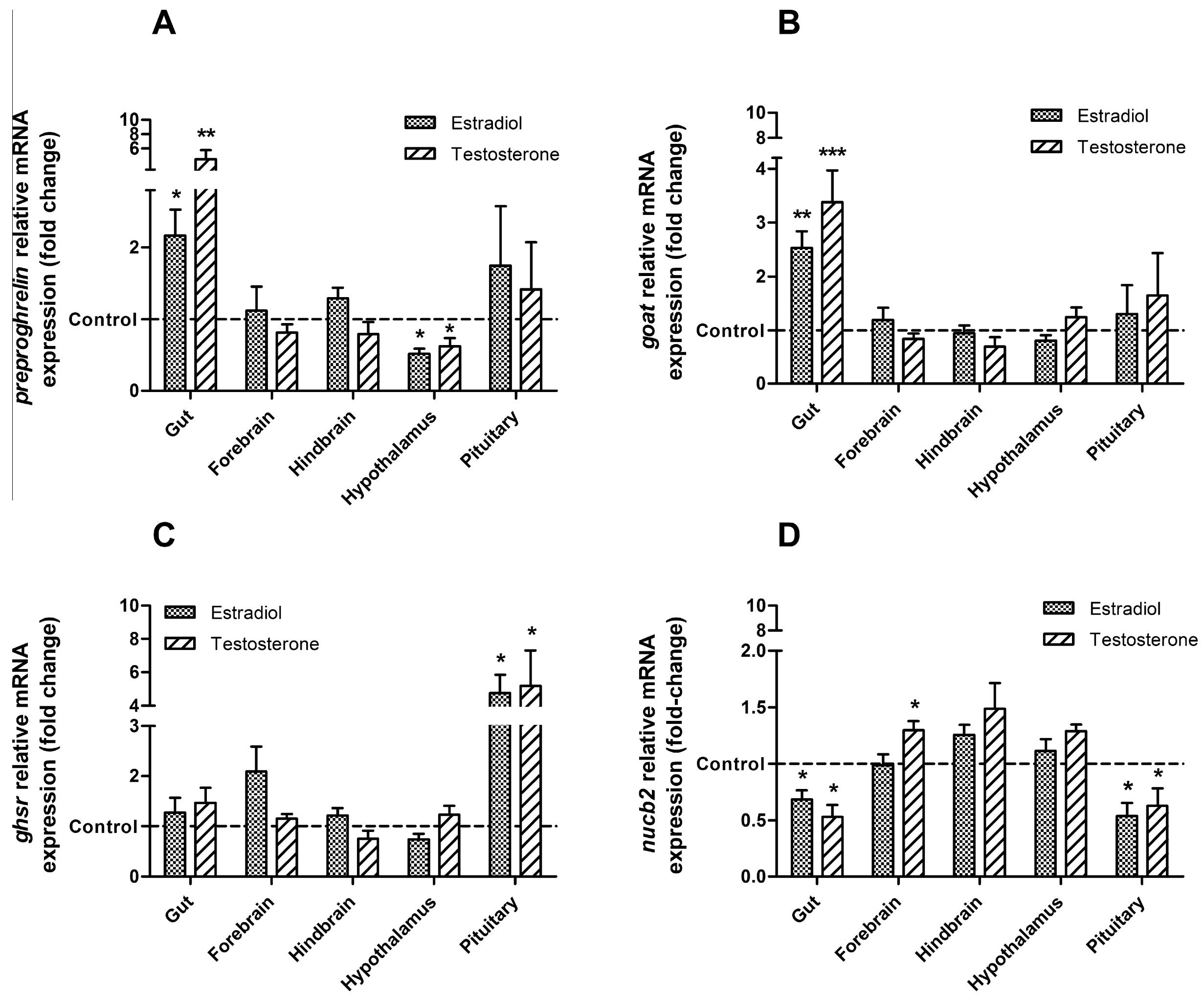


Fig. 3. Administration of estradiol and testosterone modulates the expression of mRNAs encoding ghrelin, ghrelin O-acyl transferase (GOAT), ghrelin receptor and nesfatin-1 in different tissues. Expression of *preproghrelin* (A), *goat* (B), *ghs-r* (C) and *nucb2* (D) in gut, forebrain, hindbrain, hypothalamus and pituitary after 2.5 days of treatment with estradiol or testosterone relative to the placebo-treated control group. Expression of mRNAs in the control group is shown as 1, and is indicated by a broken line. Bars below the control line indicate a downregulation and bars above an upregulation compared to the control. Asterisks represent significant differences between treatments and control ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$). One-way ANOVA, Student–Newman–Keuls Method. ($n = 7$ fish/group).

in fold-increase of E and T between the two experiments (studies 1 and 2) is likely due to individual differences in basal hormone levels, tissue uptake and hormone clearance. Similarly, these are likely reasons for why we did not see any dose response differences in E tested in study 1. Despite this variation in absolute levels, our objective to determine a dose that elevates E and T to pharmacological levels was successful. It was found that both sex steroid hormones exert a stimulatory effect on *preproghrelin* expression in goldfish gut. Similarly, *goat* was also upregulated in the gut by both estradiol and testosterone. In addition, enhanced *ghs-r* mRNA expression in goldfish pituitary was found. A limitation of this study is that we only measured the tissue specific expression of mRNAs, not proteins. These results only suggest that steroids could increase transcription of the ghrelinergic system in goldfish.

While ghrelin, *goat* and GHS-R are found to be upregulated in the gut and pituitary, an inhibitory effect of sex steroid hormones on *preproghrelin* expression was observed in the hypothalamus. Estradiol has an anorectic effect in mammals (Butera, 2010; Blaustein and Wade, 1976; Butera et al., 2014) and in some teleost fishes including sea bass (Leal et al., 2009) and Eurasian perch (Mandiki et al., 2005). The tissue specific effects of gonadal steroids on endogenous ghrelin, nesfatin-1 and other metabolic peptides might contribute to feeding variations seen in different reproductive stages. Estrogen replacement therapy in hysterectomized postmenopausal women led to an increase in active plasma ghrelin levels (Kellokoski et al., 2005). Frazao et al. (2014) found that higher levels of circulating estradiol increased the expression of *ghs-r* mRNA selectively in the arcuate nucleus. A similar relationship between plasma ghrelin and testosterone was also found in men (Pagotto et al., 2003) and postmenopausal women (Greenman et al., 2009). In general, it appears that estradiol and testosterone increases circulating ghrelin in humans. Similar to this, *nucb2* expression in the pituitary gland decreased after ovariectomy and increased with exogenous estradiol in female mouse (Chung et al., 2014). Additionally, the same research confirms an increase in *nucb2* mRNA expression with estradiol. Our results indicate that both estradiol and testosterone has a positive effect on *nucb2* mRNA expression in the brain, while these steroids have an inhibitory effect on *nucb2* mRNA in the gut and pituitary. These findings further highlight the tissue- and species-specificity of gonadal steroids in regulating metabolic hormones.

In agreement with the inhibitory effect of testosterone on *nucb2* mRNA expression in gut and pituitary, serum nesfatin-1 was significantly decreased after testosterone treatment. While *nucb2* expression was downregulated in those tissues after estradiol treatment, an increase in nesfatin-1 in circulation was observed.

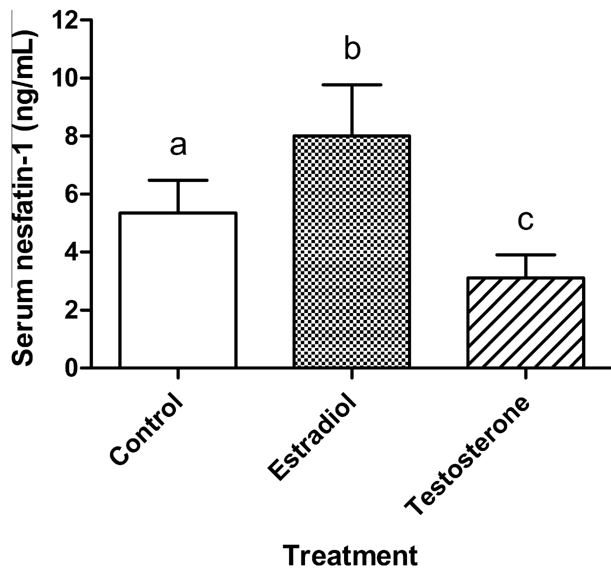


Fig. 4. Administration of estradiol and testosterone influences serum nesfatin-1 levels. Values of serum nesfatin-1 concentration in placebo treated control and both treatments groups (estradiol and testosterone) after 2.5 days are shown. Letters represent significant differences between treatments and control ($p < 0.05$). One-way ANOVA, Student–Newman–Keuls Method. ($n = 7$ fish/group).

These results suggest that estradiol has differential effects on nucb2 mRNA expression and secretion. While the reasons for this are unclear, we hypothesize a negative feedback mechanism. An increase in endogenous circulating nesfatin-1 by estradiol possibly signals the abundance of the peptide. This in turn causes a suppression of the nesfatin-1 synthesis machinery, where downregulation of gene expression is likely a first step to prevent additional production and secretion of nesfatin-1. Another important observation is the differential effects of E and T on ghrelin and nesfatin-1. Further studies on why and how E and T exert tissue- and peptide-specific effects on ghrelin and nesfatin-1 warrant further consideration.

The endocrine regulation of feeding and reproduction are multifactorial and redundant (Volkoff et al., 2005, 2009; Unniappan, 2010). Through this work, we provide new information on how two metabolic peptides and gonadal steroids interact in goldfish. Both E and T elicit a tissue-specific modulation of the ghrelinergic system, and NUCB2/nesfatin-1. A major limitation of this study is that mostly only mRNA expression was studied. However, these results provide insights on how endogenous ghrelin and nesfatin-1 are likely regulated in fish. The regulation of these two hormones was poorly understood in fish. For the first time in female fish, we show that gonadal steroids, which are dependent on the reproductive stage, regulate ghrelin, ghsr, goat and nucb2 gene expression. The orexigen ghrelin is implicated in the regulation of growth hormone (Unniappan and Peter, 2005; Grey and Chang, 2009), gonadotropins (Unniappan and Peter, 2005) and reproduction (Shepperd et al., 2012; Unniappan, 2010). In addition to its anorexigenic effects, nesfatin-1 regulates gonadotropin secretion and gonadal physiology in fish (Gonzalez et al., 2012b). The interactions identified in this research shed light into possible ways by which the endocrine system self-regulates to meet the reproductive season specific energy demands. Ghrelin and nesfatin-1 are likely two targets modulated by the changing gonadal steroid levels. This in turn might help regulate energy intake and utilization, as well reproductive hormones and gonadal functions. In fact, seasonal changes in circulating nesfatin-1 were observed in both *ad libitum* fed and feed restricted iteroparous female rainbow trout (Caldwell et al., 2014). Future studies should focus to elucidate how food intake and endogenous E and T, as well as ghrelin and nesfatin-1 help fish in maintaining energy balance under various reproductive and environmental conditions.

Acknowledgments

This study was supported by the Discovery Grant and Discovery Accelerator Supplement award from the Natural Sciences and Engineering Research Council (NSERC) of Canada, Establishment grant from Saskatchewan Health Research Foundation (SHRF), John Evans Leader's Fund from the Canada Foundation for Innovation to S. Unniappan, and by the Argentinian Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT, project: PICT 2010-1493) to L.F. Canosa. S. Unniappan is a recipient of the CIHR New Investigator Award. J. I. Bertucci is a pre-doctoral fellow from the ANPCyT, and is a recipient of the Emerging Leaders of the Americas Program (ELAP) scholarship from the Government of Canada. A. M. Blanco is a predoctoral fellow from the Spanish Ministerio de Educación y Ciencia (MEC).

References

Blaustein, J., Wade, G., 1976. Ovarian influences on the meal patterns of female rats. Physiol. Behav. 17, 201–208.

Butera, P.C., 2010. Estradiol and the control of food intake. Physiol. Behav. 99, 175–180.

Butera, P.C., Clough, S.J., Bungo, A., 2014. Cyclic estradiol treatment modulates the orexigenic effects of ghrelin in ovariectomized rats. Pharmacol. Biochem. Behav. 124, 356–360.

Caldwell, L.K., Pierce, A.L., Riley, L.G., Duncan, C.A., Nagler, J.J., 2014. Plasma nesfatin-1 is not affected by long-term food restriction and does not predict rematuration among iteroparous female rainbow trout (*Oncorhynchus mykiss*). PLoS One 9, e85700.

Canosa, L.F., Lin, X., Peter, R.E., 2002. Regulation of expression of somatostatin genes by sex steroid hormones in goldfish forebrain. Neuroendocrinology 76, 8–17.

Chung, Y., Kim, J., Im, E., Kim, H., Yang, H., 2014. Progesterone and 17β-estradiol regulate expression of nesfatin-1/NUCB2 in mouse pituitary gland. Peptides 63C, 4–9.

Clegg, D.J., Brown, L.M., Zigman, J.M., Kemp, C.J., April, D., Benoit, S.C., Geary, N., 2007. Estradiol-dependent decrease in the orexigenic potency of ghrelin in female rats. Clin. Rep. 1051, 1–11.

Fernández-Fernández, R., Tena-Sempere, M., Aguilar, E., Pinilla, L., 2004. Ghrelin effects on gonadotropin secretion in male and female rats. Neurosci. Lett. 362, 103–107.

Frazao, R., Dungan Lemko, H.M., da Silva, R.P., Ratra, D.V., Lee, C.E., Williams, K.W., Elias, C.F., 2014. Estradiol modulates Kiss1 neuronal response to ghrelin. Am. J. Physiol. Endocrinol. Metab. 306, E606–E614.

García-Galiano, D., Tena-Sempere, M., 2013. Emerging roles of NUCB2/nesfatin-1 in the metabolic control of reproduction. Curr. Pharm. Des. 19, 6966–6972.

García-Galiano, D., Navarro, V.M., Gaytan, F., Tena-Sempere, M., 2010a. Expanding roles of NUCB2/nesfatin-1 in neuroendocrine regulation. J. Mol. Endocrinol. 4, 281–290.

García-Galiano, D., Navarro, V.M., Roa, J., Ruiz-Pino, F., Sánchez-Garrido, M.A., Pineda, R., Castellano, J.M., Romero, M., Aguilar, E., Gaytán, F., Diéguez, C., Pinilla, L., Tena-Sempere, M., 2010b. The anorexigenic neuropeptide, nesfatin-1, is indispensable for normal puberty onset in the female rat. J. Neurosci. 30, 7783–7792.

García-Galiano, D., Pineda, R., Ilhan, T., Castellano, J.M., Ruiz-Pino, F., Sánchez-Garrido, M.A., Vazquez, M.J., Sangiao-Alvarellos, S., Romero-Ruiz, A., Pinilla, L., Diéguez, C., Gaytán, F., Tena-Sempere, M., 2012. Cellular distribution, regulated expression, and functional role of the anorexigenic peptide, NUCB2/nesfatin-1, in the testis. Endocrinology 153, 1959–1971.

Goebel, M., Stengel, A., Wang, L., Taché, Y., 2011. Central nesfatin-1 reduces the nocturnal food intake in mice by reducing meal size and increasing inter-meal intervals. Peptides 32, 36–43.

Goebel-Stengel, M., Wang, L., Stengel, A., Taché, Y., 2011. Localization of nesfatin-1 neurons in the mouse brain and functional implication. Brain Res. 1396C, 20–34.

Gonzalez, R., Unniappan, S., 2010. Molecular characterization, appetite regulatory effects and feeding related changes of peptide YY in goldfish. Gen. Comp. Endocrinol. 166, 273–279.

Gonzalez, R., Kerbel, B., Chun, A., Unniappan, S., 2010. Molecular, cellular and physiological evidences for the anorexigenic actions of nesfatin-1 in goldfish. PloS One 5, e15201.

Gonzalez, R., Perry, R.L., Gao, X., Gaidhu, M.P., Tsushima, R.G., Ceddia, R.B., Unniappan, S., 2011. Nutrient responsive nesfatin-1 regulates energy balance and induces glucose-stimulated insulin secretion in rats. Endocrinology 152, 3628–3637.

Gonzalez, R., Mohan, H., Unniappan, S., 2012a. Nucleobindins: bioactive precursor proteins encoding putative endocrine factors? Gen. Comp. Endocrinol. 176, 341–346.
Gonzalez, R., Shepperd, E., Thiruppugazh, V., Lohan, S., Grey, C.L., Chang, J.P., Unniappan, S., 2012b. Nesfatin-1 regulates the hypothalamo-pituitary-ovarian axis of fish. Biol. Reprod. 87, 84.
Greenman, Y., Rouach, V., Limor, R., Gilad, S., Stern, N., 2009. Testosterone is a strong correlate of ghrelin levels in men and postmenopausal women. Neuroendocrinology 89, 79–85.
Grey, C.L., Chang, J.P., 2009. Ghrelin-induced growth hormone release from goldfish pituitary cells involves voltage-sensitive calcium channels. Gen. Comp. Endocrinol. 160, 148–157.
Gutierrez, J.A., Solenberg, P.J., Perkins, D.R., Willency, J.A., Knierman, M.D., Jin, Z., Hale, J.E., 2008. Ghrelin octanoylation mediated by an orphan lipid transferase. Proc. Nat. Acad. Sci. USA 105, 6320–6325.
Hatef, A., Yufa, R., Unniappan, S., 2015. Ghrelin O-acyl transferase in zebrafish is an evolutionarily conserved peptide upregulated during calorie restriction. Zebrafish. http://dx.doi.org/10.1089/zeb.2014.1062.
Jönsson, E., 2013. The role of ghrelin in energy balance regulation in fish. Gen. Comp. Endocrinol. 187, 79–85.
Kaiya, H., Miyazato, M., Kangawa, K., Peter, R.E., Unniappan, S., 2008. Ghrelin: a multifunctional hormone in non-mammalian vertebrates. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 149, 109–128.
Kellokoski, E., Pöykkö, S.M., Karjalainen, A.H., Ukkola, O., Heikkinen, J., Kesäniemi, Y. A., Hörkkö, S., 2005. Estrogen replacement therapy increases plasma ghrelin levels. J. Clin. Endocrinol. Metab. 90, 2954–2963.
Kerbel, B., Unniappan, S., 2012. Nesfatin-1 suppresses energy intake, co-localises ghrelin in the brain and gut, and alters ghrelin, cholecystokinin and orexin mRNA expression in goldfish. J. Neuroendocrinol. 24, 366–377.
Kojima, M., Hosoda, H., Date, Y., Nakazato, M., Matsuo, H., Kangawa, K., 1999. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature 402, 656–660.
Leal, E., Sánchez, E., Muriach, B., Cerdá-Reverter, J.M., 2009. Sex steroid-induced inhibition of food intake in sea bass (*Dicentrarchus Labrax*). J. Comp. Physiol. B Biochem. Syst. Environ. Physiol. 179, 77–86.
Lin, F., Zhou, C., Chen, H., Wu, H., Xin, Z., Liu, J., Li, Z., 2014. Molecular characterization, tissue distribution and feeding related changes of NUCB2A/nesfatin-1 in Ya-fish (*Schizothorax prenanti*). Gene 536 (2), 238–246.
Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods San Diego Calif. 25, 402–408.
Mandiki, S.N.M., Babiak, I., Bopopi, J.M., Leprieur, F., Kestemont, P., 2005. Effects of sex steroids and their inhibitors on endocrine parameters and gender growth differences in Eurasian perch (Perca Fluviatilis) juveniles. Steroids 70, 85–94.
Manning, A.J., Murray, H.M., Gallant, J.W., Matsuoka, M.P., Radford, E., Douglas, S.E., 2008. Ontogenetic and tissue-specific expression of preproghrelin in the Atlantic halibut, (*Hippoglossus hippoglossus L.*). J. Endocrinol. 196, 181–192.
Matsubara, M., Sakata, I., Wada, R., Yamazaki, M., Inoue, K., Sakai, T., 2004. Estrogen modulates ghrelin expression in the female rat stomach. Peptides 25, 289–297.
Mohan, H., Unniappan, S., 2012. Ontogenic pattern of nucleobindin-2/nesfatin-1 expression in the gastroenteropancreatic tissues and serum of Sprague Dawley rats. Regul. Pept. 175, 61–69.
Mohan, H., Ramesh, N., Mortazavi, S., Le, A., Iwakura, H., Unniappan, S., 2014. Nutrients differentially regulate nucleobindin-2/nesfatin-1 in vitro in cultured stomach ghrelinoma (MGN3-1) cells and in vivo in male mice. PLoS One 9, e115102.
Mortazavi, S., González, R., Ceddia, R., Unniappan, S., 2015. Long-term infusión of nesfatin-1 causes a sustained regulation of whole-body energy homeostasis of male Fischer 344 rats. Front. Cell Dev. Biol. 3, 22.
Oh-I, S., Shimizu, H., Satoh, T., Okada, S., Adachi, S., Inoue, K., Eguchi, H., Yamamoto, M., Imaki, T., Hashimoto, K., Tsuchiya, T., Monden, T., Horiguchi, K., Yamada, M., Mori, M., 2006. Identification of nesfatin-1 as a satiety molecule in the hypothalamus. Nature 443, 709–712.
Pagotto, U., Gambineri, A., Pelusi, C., Genghini, S., Cacciari, M., Otto, B., Castañeda, T., Tschöp, M., Pasquali, R., 2003. Testosterone replacement therapy restores normal ghrelin in hypogonadal men. J. Clin. Endocrinol. Metab. 88, 4139–4143.
Pankhurst, N.W., Stacey, N.E., Peter, R.E., 1986. An evaluation of techniques for the administration of 17ß-estradiol to teleost. Aquaculture 52, 145–155.
Park, J., Nam, Y., Moon, H., Kim, C., Yoon, H., Chung, Y., Kim, H., Kim, J., Yang, H., 2013. Expression of nesfatin-1/NUCB2 in pituitary gland is regulated by estradiol and progesterone. Dev. Reprod. 17, 67–71.
Peng, C., Gallin, W., Peter, R.E., Blomqvist, A.G., Larhammar, D., 1994. Neuropeptide-Y gene expression in the goldfish brain: distribution and regulation by ovarian steroids. Endocrinology 134, 1095–1103.
Ramesh, N., Mortazavi, S., Unniappan, S., 2015. Nesfatin-1 stimulates glucagon-like peptide-1 and glucose-dependent insulinotropic polypeptide secretion from STC-1 cells in vitro. Biochem. Biophys. Res. Commun. 462, 124–130.
Senin, L.L., Al-Massadi, O., Barja-Fernandez, S., Folgueira, C., Castelao, C., Tovar, S.A., Seoane, L.M., 2015. Regulation of NUCB2/nesfatin-1 production in rat's stomach and adipose tissue is dependent on age, testosterone levels and lactating status. Mol. Cell. Endocrinol. 411, 105–112.
Shepperd, E., Peng, C., Unniappan, S., 2012. Ghrelinergic system in fish ovaries and ghrelin inhibition of germinal vesicle breakdown in zebrafish oocytes. Gen. Comp. Endocrinol. 176, 426–431.
Shimizu, H., Oh-I, S., Hashimoto, K., Nakata, M., Yamamoto, S., Yoshida, N., Mori, M., 2009. Peripheral administration of nesfatin-1 reduces food intake in mice: the leptin-independent mechanism. Endocrinology 150, 662–671.
Shinsuke, Oh.-I., Shimizu, H., Satoh, T., Okada, S., Adachi, S., Inoue, K., Mori, M., 2006. Identification of nesfatin-1 as a satiety molecule in the hypothalamus. Nature 443, 709–712.
Shlimun, A., Unniappan, S., 2011. Ghrelin o-acyl transferase: bridging ghrelin and energy homeostasis. Int. J. Pept. 2011, 217957.
Stengel, A., Goebel, M., Wang, L., Taché, Y., 2010. Ghrelin, des-acyl ghrelin and nesfatin-1 in gastric X/A-like cells: role as regulators of food intake and body weight. Peptides 31, 357–369.
Sugino, T., Hasegawa, Y., Kurose, Y., Kojima, M., Kangawa, K., Terashima, Y., 2004. Effects of ghrelin on food intake and neuroendocrine function in sheep. Anim. Reprod. Sci. 82–83, 183–194.
Tang-Christensen, M., Vrang, N., Ortmann, S., Bidlingmaier, M., Horvath, T.L., Tschöp, M., 2004. Central administration of ghrelin and agouti-related protein (83–132) increases food intake and decreases spontaneous locomotor activity in rats. Endocrinology 145, 4645–4652.
Tena-Sempere, M., 2005. Ghrelin: novel regulator of gonadal function. J. Endocrinol. Invest. 28, 26–29.
Trudeau, V.L., Peter, R.E., Sloley, B.D., 1991. Testosterone and estradiol potentiate the serum gonadotropin response to gonadotropin-releasing hormone in goldfish. Biol. Reprod. 44, 951–960.
Trudeau, V.L., Sloley, B.D., Wong, A.O., Peter, R.E., 1993. Interactions of gonadal steroids with brain dopamine and gonadotropin-releasing hormone in the control of gonadotropin-II secretion in the goldfish. Gen. Comp. Endocrinol. 89, 39–50.
Unniappan, S., 2010. Ghrelin: an emerging player in the regulation of reproduction in non-mammalian vertebrates. Gen. Comp. Endocrinol. 167, 340–343.
Unniappan, S., Peter, R.E., 2005. Structure, distribution and physiological functions of ghrelin in fish. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 140, 396–408.
Unniappan, S., Lin, X., Cervini, L., Rivier, J., Kaiya, H., Kangawa, K., Peter, R.E., 2002. Goldfish ghrelin: molecular characterization of the complementary deoxyribonucleic acid, partial gene structure and evidence for its stimulatory role in food intake. Endocrinology 143, 4143–4146.
Volkoff, H., Canosa, L.F., Unniappan, S., Cerdá-Reverter, J.M., Bernier, N.J., Kelly, S.P., Peter, R.E., 2005. Neuropeptides and the control of food intake in fish. Gen. Comp. Endocrinol. 142, 3–19.
Volkoff, H., Unniappan, S., Kelly, S.P., 2009. The endocrine regulation of food intake. In: Bernier, N.J., Van der Kraak, G., Farrell, A.P., Brauner, C.J. (Eds.), Fish Neuroendocrinology. Elsevier, London, UK, pp. 421–465.
Wang, Q., Sham, K.W.Y., Ogawa, S., Li, S., Parhar, I.S., Cheng, C.H.K., Lin, H., 2013. Regulation of the two kiss promoters in goldfish (Carassius auratus) by estrogen via different ERα pathways. Mol. Cell. Endocrinol. 375, 130–139.
Yang, J., Brown, M.S., Liang, G., Grishin, N.V., Goldstein, J.L., 2008. Identification of the acyltransferase that octanoylates ghrelin, an appetite-stimulating peptide hormone. Cell 132, 387–396.
Zadmajid, V., Reza Imanpoor, M., Shabani, A., Baharlouei, A., 2012. Evaluation of egg production and sex steroid profiles in the goldfish *Carassius auratus* during four consecutive seasons. Global Veterinaria 9, 367–375.