Nesfatin-1 suppresses fish reproductive axis and gonadal steroidogenesis

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Abstract

Nesfatin-1 is a naturally occurring orphan ligand in fish and mammals. Research in our lab resulted in the identification of an inhibitory role for nesfatin-1 on pituitary hormones (goldfish) and oocyte maturation (zebrafish). The present study is an extension of these original findings and aimed to determine whether nesfatin-1 has any additional effects on HPG genes in male and female goldfish. We found that a single i.p. injection of synthetic nesfatin-1 (50 ng/g body weight) downregulated the expression of salmon gonadotropin-releasing hormone (*sgnrh*), chicken *gnrh-II* (*cgnrh-II*), kisspeptin receptor (*gpr54a*) and brain aromatase (*cyp19a1b*) mRNAs in the hypothalamus of both male and female goldfish at 15 min post-administration. In the pituitary of both males and females, nesfatin-1 reduced luteinizing hormone beta (*lhβ*) and follicle stimulating hormone beta (*fshβ*) mRNA expression at 60 min and *gpr54a* mRNA at 15 min. Similarly, the gonadotropin receptors *lhr* and *fshr* were downregulated in the gonads. Meanwhile, gonadotropin inhibiting hormone (*gnih*), *gnih* receptor, kisspeptin 1 (*kiss1*) and *gpr54a* mRNA expression in the gonads were increased post-nesfatin-1 treatment. Nesfatin-1 negatively influences the *star*, cytochrome P450 family 11 subfamily A member 1, anti-mullerian hormone and aromatase mRNAs. In agreement with these results, nesfatin-1 reduced plasma estradiol and testosterone in female and male goldfish circulation at 60 min post-injection. The information generated through this research further solidified nesfatin-1 as an inhibitor of reproductive hormones in fish. Targeting nesfatin-1 and related peptides could yield beneficial effects in fish reproduction and aquaculture.

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Introduction

Vertebrate reproduction is heavily regulated by hormones primarily produced from the hypothalamo-pituitary-gonadal (HPG) axis (Unniappan 2010). Along with the HPG hormones and releasing factors, multiple non-HPG hormones and neuropeptides are also involved in the regulation of reproduction in vertebrates. As reproduction is a highly energy-dependent process, food supply and adequate body energy stores are crucial factors for the proper action of reproductive hormones and reproductive success (Schneider 2004, Scaramuzzi *et al.* 2006, Hill *et al.* 2008, Evans & Anderson 2012). The interplay between energy status and reproduction is primarily controlled by the endocrine regulatory centers in the brain, mainly through neuroendocrine signals. It was reported that many metabolic hormones can alter the release of the gonadotropins from the anterior pituitary either by directly acting on the pituitary or indirectly through its actions on the neuroendocrine centers of the brain, which includes the hypothalamus (Gonzalez *et al.* 2012a). There are many hormone-like factors that are emerging as regulators of both metabolism and

reproduction. One such example is nesfatin-1, encoded in the precursor nucleobindin-2 (Nucb2).

Nucb2 is a calcium and DNA binding precursor peptide that is proposed to be cleaved by prohormone convertases (PCs) to produce three shorter peptides, nesfatin-1, 2 and 3 (Oh-I *et al.* 2006). Of these, only nesfatin-1 was found to have an anorexigenic action (Oh-I *et al.* 2006). Multiple studies reported the importance of nesfatin-1 in energy homeostasis in mammals (Oh-I *et al.* 2006, Gonzalez *et al.* 2012b, Wernecke *et al.* 2014, Mortazavi *et al.* 2015). Nucb2 mRNA and protein increases during pubertal transition, and there is an increase in LH release in female rats post-nesfatin-1 administration. Morpholino-mediated gene knockdown resulted in a reduction in circulating LH levels in female pubertal rats (García-Galiano *et al.* 2010). The same year, Gonzalez and colleagues reported a significant decrease in food intake in goldfish after either i.p. (50 ng/g body weight) or intracerebroventricular (ICV, 5 ng/g body weight) injection of nesfatin-1 (Gonzalez *et al.* 2010). Studies in zebrafish suggest that nutrient status modulates the expression of *nucb2* in fish (Hatef *et al.* 2015). There is a decrease in *nucb2/nesfatin-1* in the brain and gut of zebrafish fasted for 7 days, which provides

additional support for its anorexigenic property. The first evidence for the regulatory role of Nucleobiliary/nesfatin-1 on reproduction in a non-mammalian vertebrate was reported in goldfish (Gonzalez et al. 2012a). I.p. injection of goldfish nesfatin-1 significantly reduced the expression of *gnrh*, *lh* and *fsH*, and serum Lh in goldfish. In addition, incubation of ovarian follicles with nesfatin-1 significantly reduced germinal vesicle breakdown and maturation of oocyte in zebrafish. Nesfatin-1 appears to be another linker of energy homeostasis and reproduction in fish. However, studies in female rainbow trout did not show any significant change in circulating nesfatin-1 during long-term fasting, and no positive correlation between circulating nesfatin-1 levels and seasonal maturation was found (Caldwell et al. 2014). This suggests that nesfatin-1 possibly has species- and sex-specific regulatory effects on reproduction and warrants further research on nesfatin-1 and reproduction.

While most of the aforementioned research in mammals and fish focused on females, Gao and colleagues reported a reduction in the expression of many mRNAs and proteins involved in the reproductive axis of adult male rats post-nesfatin-1 administration (Gao et al. 2016). This includes *Gnrh*, *Kiss1*, *Lhb*, *Fshb* and *Star*, 3 β -hydroxysteroid dehydrogenase (3 β -*hsd*), 17 β -hydroxysteroid dehydrogenase (17 β -*hsd*), and cytochrome P450 cleavage (*p450sc*), mRNA transcripts and serum levels of LH, FSH and testosterone. This finding further suggests a sex-specific role for nesfatin-1 on reproduction in vertebrates. We hypothesized that nesfatin-1 suppresses the expression of HPG and non-HPG genes involved in reproduction and circulating levels of gonadal hormones in a sex-specific manner in fish. This research is a logical extension of our own studies that discovered a role for nesfatin-1 in fish reproduction (Gonzalez et al. 2012a). It determined how exogenously administered nesfatin-1 influences critical genes involved in goldfish reproduction (HPG and non-HPG axis regulators of reproduction). Further, we tested whether there are any effects for nesfatin-1 on gonadal steroids in goldfish circulation.

Materials and methods

Animals

Mature male and female goldfish (*Carassius auratus*) of the common variety, 20 $\pm$ 3 g, were obtained from a local vendor through the institutional animal order desk. Fish were housed in freshwater aquaria (60 L capacity) at 23 $\pm$ 2°C, under a simulated 12 h light: 12 h darkness photoperiod (lights on at 0700 h). Once daily, at a scheduled feeding time (1100 h), fish were fed with commercial slow sinking pellets (Aqueon, Franklin, USA). All studies using fish complied with the Canadian Council for Animal Care regulations and were approved by the Animal Research Ethics Board (Animal Use Protocol # 2012-0082) of the University of Saskatchewan.

Experimental design and sampling

All experiments were completed between late spring and early summer (May–June) when goldfish were reproductively active. Male and female goldfish were randomly selected (weight matched) and were acclimated to 10 L experimental aquaria ($n=2$ /aquaria), maintained in a constantly aerated, continuous flow, temperature (23 $\pm$ 2°C) controlled water current system. The objective of this research was to further determine the effects of nesfatin-1 on reproduction. Therefore, for the *in vivo* injection study, we used the same dose of nesfatin-1 and time-points that were previously found effective in our own research (Gonzalez et al. 2012a). Briefly, male and female goldfish were anesthetized by using tricaine methanesulfonate-222 (TMS, 0.5%, Syndel Laboratories, BC, Canada) and injected intraperitoneally with 100 μ L of saline containing (50 ng/g body weight) goldfish synthetic nesfatin-1. The goldfish nesfatin-1 (1–82) (gfnesfatin-1; VPISIDKTKVKLPETVKES PQNVDLTGLHYDRYLREVIDFLEKDQHFREKLHNTDMEDIK QGKLAKELDFVSHHVRTKLDEL) was custom synthesized (Genscript, Piscataway, NJ, USA) and the purity (>95%) of the synthetic peptide was confirmed by mass spectrometry and MALDI-TOF (Gonzalez et al. 2010). Although the circulating levels of nesfatin-1 varies due to physiological conditions, the dose used here is likely supraphysiological based on the serum NUCB2/nesfatin-1 concentrations reported in goldfish (Gonzalez et al. 2010). The control group was injected with sterile saline (0.9% NaCl; 100 μ L). Six male and six female fish from each of the groups (control and treatment) were anesthetized and euthanized at 15 min and 60 min post-nesfatin-1 administration. Tissues including hypothalamus, pituitary and gonads were collected and flash-frozen immediately in liquid nitrogen and stored at –80°C until further processing. Blood was collected from caudal vein and serum was separated and stored at –20°C for ELISA.

RT-qPCR based mRNA expression analysis

Total RNA extraction and cDNA synthesis were carried out as previously described (Rajeswari et al. 2019). Briefly, total RNA was extracted from tissue samples from goldfish including hypothalamus, pituitary and gonads using RiboZol RNA isolation reagent (aMReSCO, Canada) followed by DNase I (Thermo Fisher Scientific) treatment. Optical density (OD) absorption ratio (OD 260 nm/OD 280 nm) was measured using a Nanodrop 2000 (Thermo Fisher Scientific) for determining the quantity and quality of RNA extracted. iScript cDNA synthesis kit (Bio-Rad) was used for cDNA synthesis. Quantitative PCR (qPCR) was used as described earlier (Blanco et al. 2017, Rajeswari et al. 2019) for mRNA expression analysis employing the Universal SYBR Green Master Mix (Bio-Rad) and the CFX Connect Real-Time PCR Detection System (Bio-Rad). Primer validation and efficiency of primers were confirmed for each primer set as described earlier (Gonzalez et al. 2012a). Genes quantified in this study were all previously documented to have a role in goldfish reproduction. Details of primers and primer-specific annealing temperatures used are provided in Table 1. Rest of the qPCR conditions are as follows: denaturation/polymerase activation: 95°C (3 min); followed by 35 cycles

Table 1 List of primers and annealing temperatures used in PCR.

Gene	Accession no.	Primer sequence (5'–3')		Annealing temperature (°C)
		Forward	Reverse	
18SrRNA	FJ710819.1	GGATGCCCTTAAC TGGGTGT	CTAGCGGCGCAATACGAATG	60
β -actin	AF079831.1	CTACTGGTATTGTGATGGACT	TCCAGACAGAGTATTTGCGCT	60
sgnrh	AB020243.1	GGATGATGGATTCTGGTGATG	CTATGTGTATCGGTGAAAGC	59
cgrrh-II	U30386.1	TACGATTCCTCAGAGGTTTCAG	CATCCAGCAGTATTGTCTTCAG	59
kiss1	FJ465138.1	GACTCCAAGCAGCACTATC	CCCAAACGGGTTGTAGTT	61
gpr54a	FJ465139.1	CTGGTGCCCTTTTCTTCTC	GGGTAGAGGGTAGCAGTGAA	61
gnih	AB078976.1	CTCCACCATCCTGCGACTT	CTTTGCGGTAGGGTGGCTGA	57
gnihR	JN204438.1	CCAGCGTCAACCTATTATC	AGCAGCCGTATTCAAAGG	59
lh β	MH536845.1	GCTTGCCAGACTGTCCTC	GTCAGATGTGTCCATAGTGC	59
fsH β	MH536843.1	GTTGTTATGGTGATTCTGTTG	ACGGTGATGGAGATATTGG	60
fsHr	HM347775.1	CGTCCACAATCCTACCTTCG	TGAGAAACGGGTGATTAAGCGG	57
lhr	HM347776.1	CCTCTGCATCGGTGTGTATC	TAGACAGATGATTCGCCCCGCC	60
cyp11a1	JQ340311.1	TACATTGATCCAGACGCCCA	TTAAAGATGCCGTCCCAAGC	57
cyp19a1a	JN620808.1	GTGCTCAAGACAATGTGTGC	AAATGAACCTACCAACCCG	60
cyp19a1b	AB009335.1	AGGCGAGCGGGATGTAGAGT	CGTCCGATGTTCAAGATGAGG	59
StAR	AY877430.1	ATGGCTGGCAACTGAGATCGAGA	TCCATGTTATCCACCAGCTCCTCA	58
amh	KF640083.1	TCTCCGAAAGGAGAGGACGA	TTCATCCGCTCTGACTGCTC	57
ar	AY090897.1	TGGCTCGAACTTTTCCAAGG	TCTGTGGAAGTGCCTCTG	58
er	AY055725.1	ACAGGTCCAGTGTGTGTGT	ATGATAGCCGGACGCATAGT	58

of denaturation: 95°C (10 s); and annealing: specific to each gene (30 s) (Table 1). A melting curve analysis was performed at 65°C to 95°C (5 s) to confirm a single amplicon or single PCR product generated. Livak method (Livak & Schmittgen 2001) was used for the qPCR data analysis, and the data were normalized to the housekeeping genes (18s rRNA and β -actin).

Serum testosterone and estradiol measurement using ELISA

Serum gonadal steroids were measured using testosterone (human) and estradiol (human) ELISA kits (Eagle Biosciences Inc., USA) previously validated for quantifying goldfish sex steroids by our lab (Bertucci et al. 2016). ELISAs were carried out according to the manufacturer's instructions.

Statistical analysis

Statistical analysis were performed using Student's *t*-test and the levels of significance were set at $P < 0.05$. PRISM version 5 (GraphPad Inc.) was used for generating graphs and statistical analysis. All data are presented as mean $\pm$ S.E.M.

Results

Nesfatin-1 administration downregulated the expression of gnrh and kisspeptin and brain aromatase in the hypothalamus of goldfish

We observed a significant reduction in the expression of salmon growth hormone-releasing hormone (*sgnrh*) and chicken growth hormone-releasing hormone-II (*cgrrh-II*) mRNA expression in the hypothalamus of male and female goldfish 15 min post-nesfatin-1 administration (Fig. 1A, B, C, D). A significant reduction in kisspeptin 1 receptor (*gpr54a*) mRNA expression was found in

both male and female treatment groups 15 min post-nesfatin-1 administration (Fig. 1G and H). Similarly, cytochrome P450, family 19, subfamily A, polypeptide 1b (*cyp19a1b*, also called brain aromatase) mRNAs were attenuated in both male and female treatment groups at 15 min post-injection (Fig. 1I and J). No significant changes in kisspeptin 1 mRNA expression were observed in the hypothalamus of male and female treatment groups compared to control (Fig. 1E and F). No significant changes in *gnih* and *gnih* receptor (*gnihR*) mRNA expression were detected in the hypothalamus of goldfish post-nesfatin-1 administration (data not shown).

Nesfatin-1 suppressed the expression of the gonadotropin β subunits and kisspeptin system in the pituitary of goldfish

Nesfatin-1 downregulated the expression of *lh β* , *fsH β* mRNA expression in the pituitary of both male and female goldfish groups 60 min post-injection (Fig. 2A, B, C, D). Nesfatin-1 caused a significant decrease in *gpr54a* mRNA expression in the pituitary of both treatment groups at 15 min post-injection (Fig. 2G and H). However, there were no significant changes in *kiss1* mRNA expression in male or female treatment groups post-nesfatin-1 administration (Fig. 2E and F).

Nesfatin-1 modulated the expression of gonadotropin receptor and sex steroid receptor mRNA expression in the gonads of goldfish

In the gonads of both male and female goldfish, we found a reduction in the expression of *lh* receptor (*lhr*) mRNA expression at 15 min post-nesfatin-1 administration (Fig. 3A and B). However, the *fsH* receptor (*fsHr*) mRNA

Figure 1 The mRNA expression profile of *sgnrh*, *cgnrh-II*, *gpr54a*, and *cyp19a1b* in the brain of male (left panel) and female (right panel) goldfish post-nesfatin-1 administration (A–J). Nesfatin-1 suppresses the mRNA expression of *sgnrh* (A, B), *cgnrh-II* (C, D), *gpr54a* (G, H), and *cyp19a1b* (I, J). No significant changes in *kiss1a* were observed in the brain of goldfish post-nesfatin-1 injection (E, F). Data obtained by RT-qPCR are represented as mean $\pm$ S.E.M. ($n=6$ fish/group). Student's *t*-test was used for statistical analysis, and the level of significance was set at $P<0.05$. Asterisks denote significant differences between control and treated groups.

shows a differential expression pattern. In the testis of goldfish, a significant decrease in *fshr* was observed at 15 min (Fig. 3C) post-injection. In the ovary of goldfish, we found a significant reduction in *fshr* mRNA

Figure 2 Expression levels of mRNAs encoding gonadotropin β subunits and kisspeptin system in the pituitary of male (left panel) and female (right panel) goldfish post-nesfatin-1 administration (A–H). Nesfatin-1 suppresses mRNA expression of *lhβ* (A, B) and *fshβ* (C, D) in the pituitary of both male and female goldfish 60 min post-injection. We observed a decrease in *gpr54a* (G, H) mRNA expression 15 min post-nesfatin-1 administration in both sex groups. However, we did not observe any significant changes in the expression of *kiss1* (E, F) in the pituitary of both sex groups. Data obtained by RT-qPCR are represented as mean $\pm$ S.E.M. ($n=6$ fish/group). Student's *t*-test was used for statistical analysis, and the level of significance was set at $P<0.05$. Asterisks denote significant differences between control and treated groups.

expression only at 60 min post-nesfatin-1 administration (Fig. 3D). Androgen receptor (*ar*) mRNA expression was significantly upregulated in the testis (at 15 min) and downregulated in the ovary of goldfish (at 15 min) post-nesfatin-1 administration (Fig. 3E and F). However, we did not observe any significant changes in estrogen receptor (*er*) mRNA expression in the gonads of goldfish post-nesfatin-1 administration (Fig. 3G and H).

Figure 3 The mRNA expression profile of gonadotropin and sex steroid receptor mRNAs in the testis (left panel) and ovary (right panel) of goldfish post-nesfatin-1 administration (A–H). In the gonads of goldfish, a significant decrease in *lhr* (A, B) and *fsh* (C, D) mRNA expression was observed post-nesfatin-1 administration. In the testis of goldfish, a significant increase in *ar* mRNA expression was observed at 15 min post-nesfatin-1 injection (E). However, in the ovary, a significant decrease in *ar* mRNA expression was observed 15 min post-injection (F). We did not observe any significant changes in the expression of *estrr* (G, H) mRNA expression in the gonads of both sex groups post-nesfatin-1 injection. Data obtained by RT-qPCR are represented as mean $\pm$ S.E.M. ($n=6$ fish/group). Student's *t*-test was used for statistical analysis, and the level of significance was set at $P<0.05$. Asterisks denote significant differences between control and treated groups.

Nesfatin-1 modulated the expression of *gnih*, *gnih*, *kiss1* and *gpr54a* mRNA expression in the gonads of goldfish

A significant increase in *gnih* mRNA expression was observed in the testis (only at 60 min) and ovary (both 15 and 60 min) post-nesfatin-1 injection (Fig. 4A and B). In addition, *gnih* mRNA expression was also upregulated

Figure 4 Expression profile of *gnih* and *kisspeptin* system mRNAs in the testis (left panel) and ovary (right panel) of goldfish post-nesfatin-1 administration (A–H). A significant increase in *gnih* mRNA expression was observed in the testis (only at 15 min) (A) and ovary (both 15 and 60 min) (B) post-nesfatin-1 administration. Nesfatin-1 elevates the expression of *gnih* mRNA expression at both 15- and 60-min time points in the testis and ovary of goldfish (C, D). The mRNA expression of both *kisspeptin* and *gpr54a* mRNAs were significantly increased in the gonads of both male and female goldfish (E–H). Data obtained by RT-qPCR are represented as mean $\pm$ S.E.M. ($n=6$ fish/group). Student's *t*-test was used for statistical analysis, and the level of significance was set at $P<0.05$. Asterisks denote significant differences between control and treated groups.

at both 15 and 60 min post-nesfatin-1 injection (Fig. 4C and D). We found a significant increase in *kiss1* (Fig. 4E and F) (at 15 min) and *gpr54a* (60 min in male and 15 min in female) (Fig. 4G and H) mRNA expression in the gonads of both treatment groups.

Figure 5 mRNA expression profile of genes involved in the sex steroidogenic pathway in the testis (left panel) and ovary (right panel) of goldfish post-nesfatin-1 administration (A–H). Nesfatin-1 suppresses the expression of *star* mRNA in the testis (A) and elevates it in the ovary (B) of goldfish. A significant decrease in *cyp11a1* (at 15 min) and *cyp19a1a* (at 60 min) was found in the testis (C & E) and ovary (D & F) of goldfish. A significant increase in the mRNA expression of *amh* was observed in the testis (at 60 min) and ovary (at 15 min) of goldfish post-nesfatin-1 administration (G, H). Data obtained by RT-qPCR are represented as mean $\pm$ S.E.M. ($n=6$ fish/group). Student's *t*-test was used for statistical analysis, and the level of significance was set at $P<0.05$. Asterisks denote significant differences between control and treated groups.

Nesfatin-1 influenced the expression of genes involved in the sex steroidogenesis in the gonads of goldfish

In the testis, a significant reduction in *star* mRNA expression was observed (Fig. 5A) 15 min post nesfatin-1 injection. However, in the ovary, a significant increase in mRNA expression was observed at 15 min post-nesfatin-1 administration (Fig. 5B). Cytochrome P450 family 11 subfamily A member 1 (*cyp11a1*), also called

cholesterol side-chain cleavage enzyme (*p450ssc*), expression was significantly downregulated in both testis and ovary of goldfish at 15 min post-nesfatin-1 administration (Fig. 5C and D). Cytochrome P450 family 19 subfamily A member 1 (aromatase, *cyp19a1a*) mRNA expression was significantly downregulated at 60 min post-nesfatin-1 administration in the gonads of goldfish (Fig. 5E and F). In the testis, we observed a significant increase in *amh* mRNA expression at 60 min post-injection (Fig. 5G). However, in the ovary, *amh* mRNA expression was upregulated only during the 15-min post-nesfatin-1-injection (Fig. 5H) time point.

Nesfatin-1 downregulated the circulating levels of sex steroids in both male and female goldfish

We found a significant decrease in circulating testosterone (T) levels in the male treatment group at 60 min post-nesfatin-1 administration (Fig. 6A). Similarly, a significant decrease in plasma estradiol (E) levels in females was detected at 60 min post-nesfatin-1 administration (Fig. 6B).

Discussion

Nesfatin-1 was originally identified as a metabolic regulator involved in feed intake regulation and energy homeostasis (Oh-I *et al.* 2006, Gonzalez *et al.* 2010). Recent evidences indicate that nesfatin-1 also has sex-specific regulatory roles in metabolism and reproduction (Bloem *et al.* 2012, Hofmann *et al.* 2015, Schalla & Stengel 2018). As an extension of our own previous findings (Gonzalez *et al.* 2012a) on nesfatin-1 and reproduction, this research explored how nesfatin-1 affects goldfish HPG axis. In goldfish, two GnRH isoforms are expressed in the brain, namely *sGnRH* and *cGnRH-II*, and both are shown to stimulate gonadotropin release in goldfish (Peter *et al.* 1985, Kim *et al.* 1995). We found that nesfatin-1 suppresses the expression of

Figure 6 Serum testosterone and estradiol profiles in male and female goldfish post-nesfatin-1 administration. A significant decrease in serum levels of testosterone was observed in male fish group 60 min post-nesfatin-1 administration (A). A significant decrease in serum estradiol was observed in female fish groups at 60 min post-nesfatin-1 administration (B). Data are represented as mean $\pm$ S.E.M. ($n=6$ fish/group). Student's *t*-test was used for statistical analysis, and the level of significance was set at $P<0.05$. Asterisks denote significant differences between control and treated groups.

both *sgnrh* and *cgnrh-II* mRNAs in the brain of both male and female goldfish. This is in agreement with the earlier report by [Gonzalez et al. 2012a](#), and adds on to the original findings to indicate that, in both sexes, nesfatin-1 applies the same effect on GnRh. We observed a reduction in the expression of GnRh mRNAs within 15 min of nesfatin-1 administration, and this suggests that the action of nesfatin-1 in the brain after acute administration is very rapid. It was documented in mammalian studies that nesfatin-1 administered peripherally reach the brain roughly in 10 min and can cross the blood brain barrier in a non-saturable way ([Pan et al. 2007](#), [Price et al. 2007](#)). Our results suggest a similar kind of mechanism is in operation in fish as well. The doses and time-points chosen here were previously validated in goldfish and are known to affect feeding and reproductive hormones ([Gonzalez et al. 2010](#), [2012a](#)). The circulating levels of nesfatin-1 in goldfish is 2.52 ± 0.26 ng/mL ([Gonzalez et al. 2010](#)). Based on this, the dose tested here appears to be supraphysiological or pharmacological. A significant reduction in the expression of *gpr54a* (*kiss1* receptor) mRNA expression in the hypothalamus of both male and female treatment groups at 15 min post-injection was observed. The role of nesfatin-1 in the expression of the kisspeptin system is not addressed in any teleosts so far. Incubation of murine hypothalamic (GT1-7) and pituitary (LBT2) cell lines with nesfatin-1 increased the expression of *gpr54a* ([Hatef & Unniappan 2017](#)). This study points to the ability of nesfatin-1 to act directly on both hypothalamic neurons and gonadotrophs derived from mice. Direct action of nesfatin-1 on fish hypothalamic neurons and pituitary gonadotrophs is yet to be determined. In mammals, nesfatin-1 is generally considered as a positive regulator of reproduction ([García-Galiano et al. 2010, 2012](#)). However, intracerebroventricular (ICV) injection of nesfatin-1 decreases the expression of *gnrh* and kisspeptin in male rats ([Gao et al. 2016](#)). This suggests that the sex of the animal and mode of administration might affect the action of nesfatin-1. We also found that nesfatin-1 suppresses the expression of brain aromatase (*cyp19a1b*) mRNA in the hypothalamus of male and female goldfish. Aromatase plays an important role in the sexual behavior in both male and female goldfish ([Lord et al. 2009](#)). Reduction in brain aromatase transcript suggests possible role of nesfatin-1 in goldfish sexual behavior. Nesfatin-1 effects on feeding, anxiety and depression-like behaviors have not been documented in fish and mammals ([Lin et al. 2014](#), [Kühne et al. 2018](#)). A role for nesfatin-1 in sexual behavior has not yet been documented, but the results provided here raises a possible role for nesfatin-1 on sex behavior. In addition, it was reported that nesfatin-1 influence the genes involved in sex steroidogenesis in males rats ([Gao et al. 2016](#)), which suggest regulatory mechanism of nesfatin-1 in vertebrates conserved across species. Overall, our data provides additional

support to the notion that nesfatin-1 negatively influences hypothalamic hormones in fish.

In the pituitary, *fsh β* , *lh β* and *gpr54a* mRNA expression was decreased after nesfatin-1 administration. However, we did not observe any significant changes in *kiss1* mRNA expression, which is similar to what we observed in the hypothalamus. It was reported earlier by immunohistochemical analysis that nesfatin-1 is abundantly expressed in the pituitary ([Gonzalez et al. 2012a](#)), suggesting its possible regulatory role in pituitary functions. The suppression of gonadotropin mRNA expression, the key regulators of vertebrate reproduction ([Gharib et al. 1990](#), [Zhang et al. 2015](#)), is additional evidence for the negative effect of nesfatin-1 on goldfish reproduction. More evidence for the negative influence of nesfatin-1 was observed in the gonads of goldfish. In both sexes, *lh* and *fsh* receptor mRNA expressions were suppressed after nesfatin-1 administration. Contrary to what was observed in the hypothalamus and pituitary, we found an increase in both *kiss1* and *gpr54a* mRNA expression in both male and female gonads. Our results from gonadal mRNA expression suggest that nesfatin-1 has tissue-specific effects on goldfish reproduction. Further studies are needed to confirm whether elevated *kiss1* and *gpr54a* is a feedback response or not. While most of the stimulatory hormones were downregulated, an opposite (upregulation) effect was found in the case of inhibitory hormones. A significant increase in both *gnih* and *gnihR* mRNA expression, which is a potent inhibitor of reproduction in vertebrates ([Tsutsui et al. 2010](#)), was found in the gonads of goldfish. This possibly is an additional mechanism by which nesfatin-1 exerts its negative effects on goldfish reproduction. Together, these results suggest that nesfatin-1 influences all three components of the HPG axis to modulate reproductive hormones.

In addition to the overall negative effects of nesfatin-1 on HPG genes that promote reproduction, we observed the influence of nesfatin-1 on genes involved in sex steroidogenesis. A reduction in *star* (in testis only), *cyp11a1*, and *cyp19a1a* mRNA expression was found after nesfatin-1 administration. This suggests that nesfatin-1 effects on sex steroidogenesis is limited not only to mammals as previously described ([Gao et al. 2016](#)), but a similar regulatory pattern is in operation in fish as well. We also found an increase in the expression of *amh*, a potent inhibitor of sex steroidogenesis in vertebrates including fish ([Rodríguez-Marí et al. 2005](#), [Halm et al. 2007](#), [Pala et al. 2008](#), [Pfennig et al. 2015](#), [Lambeth et al. 2016](#), [Xu et al. 2019](#)). This furnishes additional support for the suppressive effect of nesfatin-1 on goldfish reproduction. A sex-specific expression pattern of *ar* was found in nesfatin-1 treated fish. In the final study, we determined whether the changes in mRNA expression would eventually result in alterations in hormone levels. In fact, a significant reduction in serum T and E levels was found at 60 min after nesfatin-1

injection. These results, for the first time, show that gonadal sex steroids are targets of nesfatin-1. Nesfatin-1 influences male and female sex steroidogenic enzymes in a time-dependant manner. However, it is unclear whether nesfatin-1 affects gonadal steroid synthesis, secretion and/or clearance, or all of these processes that determine circulating levels. A significant decrease in *star* and *cyp11a1* found in male goldfish possibly contributes to the reduction in circulating testosterone levels. It is possible that elevated *amh* is another factor affecting circulating testosterone levels in male fish treated with nesfatin-1. In the ovary, significant decreases found in both *cyp11a1* and *cyp19a1a* likely mediate the negative effects of nesfatin-1 on circulating estradiol levels. The increase in *star* mRNA at 15 min post-injection in the ovary is possibly a feedback response to induce cholesterol uptake and thereby increase substrate availability. The mechanism of action of nesfatin-1 in causing these steroidogenic responses requires additional studies. For example, we currently do not know the significance of nesfatin-1 suppression of gonadal *cyp19a1a* and *cyp19a1b* (brain aromatase) expression in male fish and its implications in male reproductive physiology. Since the circulating levels of estradiol in male fish was not measured, we are unsure to what extent the changes in aromatase (*cyp19a1a*) gene expression will reflect in circulating estradiol levels. During development, majority of fish species exhibit gonadal expression of aromatase in males (reduced expression) and females (elevated expression) (Guiguen *et al.* 2010). Additional studies to understand the physiological relevance of such patterns of gonadal aromatase expression is essential. In addition, we did not quantify *cyp17a1* expression in the gonads of goldfish due to the lack of sequence information (to design primers). Further studies are required to clearly understand the exact processes in steroid production and secretion that are influenced by nesfatin-1 in both male and female fish. Together, our results indicate nesfatin-1 influence all tissues of the HPG axis and influence many critical hormonal regulators of fish reproduction.

In conclusion, we reconfirmed some of the acute effects of nesfatin-1 on goldfish reproduction (Gonzalez *et al.* 2012a) in both males and females separately. In addition, we extended these results into additional brain and pituitary hormones including kisspeptin and *gnih* and gonadal steroids. Most of the *in vivo* effects of nesfatin-1 found in this study ceased within 60 min of administration. Additional research is necessary to determine the chronic effects of nesfatin-1 on fish reproductive hormones and reproduction. The novelty of this research lies in the findings of nesfatin-1 effects on kisspeptin, *gnih* and steroidogenic enzymes, and hormone receptors, as we all circulating sex steroids. Our results suggest sex- and tissue-specific effects for nesfatin-1 in goldfish. Nesfatin-1 in goldfish is a suppressor of pro-reproductive hormones, while it

promotes inhibitors of reproduction. Additional studies are required to understand the actions and mechanism of actions of nesfatin-1 on steroidogenic cells. While we only studied mRNA expression in tissues, the measurement of sex steroids provided a meaningful endpoint reflective of reproductive outcomes in fish. The results provided here enabled us to reach an overall conclusion that, by acting at all levels of the HPG axis, nesfatin-1 acts as a suppressor of reproduction in fish.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

JJR planned and conducted the studies, analyzed and interpreted data, prepared the manuscript draft and revised it for submission. SU provided the original idea and funding, helped in the planning, designing of experiments and assisted in tissue sampling, data analysis and interpretation, manuscript preparation and revisions.

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