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Short communication

Differential social regulation of two pituitary gonadotropin-releasing hormone receptors

Teresa M. Au [a], Anna K. Greenwood [b], Russell D. Fernald [b,c,*]

[a] *Program in Human Biology, Stanford University, Stanford, CA 94305, United States*
[b] *Neuroscience Program, Stanford University, Stanford, CA 94305, United States*
[c] *Department of Biological Sciences, Stanford University, Stanford, CA 94305, United States*

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Abstract

In many vertebrates, social interactions regulate reproductive capacity by altering the activity of the hypothalamic–pituitary–gonadal (HPG) axis. To better understand the mechanisms underlying social regulation of reproduction, we investigated the relationship between social status and one main component of the HPG axis: expression levels of gonadotropin-releasing hormone receptor (GnRH-R). Social interactions dictate reproductive capacity in the cichlid fish *Astatotilapia burtoni*. Reproductively active territory holders suppress the HPG axis of non-territorial males through repeated aggressive encounters. To determine whether the expression of GnRH-R is socially regulated, we quantified mRNA levels of two GnRH-R variants in the pituitaries and brains of territorial (T) and non-territorial (NT) *A. burtoni* males. We found that T males had significantly higher levels of pituitary GnRH-R1 mRNA than NT males. In contrast, GnRH-R2 mRNA levels in the pituitary did not vary with social status. Pituitaries from both T and NT males expressed significantly higher mRNA levels of GnRH-R1 than GnRH-R2. GnRH mRNA levels in the brain correlated positively with GnRH-R1 mRNA levels in the pituitary but did not correlate with pituitary GnRH-R2. Measurements of GnRH-R1 and GnRH-R2 mRNA levels across the whole brain revealed no social status differences. These results show that, in addition to the known effects of social status on other levels of the HPG axis, GnRH receptor in the pituitary is also a target of social regulation.

Keywords: GnRH; Receptor; Social regulation; Reproductive regulation; Teleost

1. Introduction

Social environment plays a critical role in shaping the reproductive physiology of many species. In species with social hierarchies, for example, dominant animals frequently suppress the reproductive function of non-dominant conspecifics through social interactions (e.g. [1–3]). To affect reproductive capacity, social influences must ultimately act on the hypothalamic–pituitary–gonadal (HPG) axis, which controls reproduction in all vertebrates. At the apex of the HPG axis are hypothalamic neurons that produce gonadotropin-releasing hormone (GnRH1). These neurons release GnRH1 into the pituitary where it binds to GnRH receptors (GnRH-R), stimulating the release of gonadotropins into the bloodstream. Gonadotropins subsequently act on the gonads to induce sexual maturation and secretion of sex steroids. Previous work has demonstrated that social interactions regulate the HPG axis at multiple levels, including GnRH1 mRNA abundance, GnRH1 peptide levels, amounts of circulating gonadotropins, and sex steroid levels [4–8]. GnRH receptors in the pituitary could also act as a target of social regulation, but this has not been previously studied.

In the present study, we investigated the social control of GnRH-R expression levels in the cichlid fish *Astatotilapia burtoni*. *A. burtoni* has proven useful for studying social regulation of the HPG axis because males exist in two socially controlled, reversible phenotypes: reproductively active territorial (T) males and reproductively incompetent non-territorial (NT) males [5,6]. T males display bright coloration, aggressively defend territories, and court females, whereas NT males display dull gray coloration, blend in with the females, and typically limit their behavior to schooling and fleeing [6]. Compared to NT males, T males possess larger GnRH-containing neurons in the hypothalamus, higher GnRH1 mRNA and peptide levels, and larger gonads containing mature sperm [4,5,7,8]. Manipulating the

* Corresponding author. Tel.: +1 650 736 8005; fax: +1 650 723 6132.
E-mail address: rfernald@stanford.edu (R.D. Fernald).

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social environment can cause T males to descend to NT status or NT males to ascend to T status, with corresponding changes in behavior and reproductive physiology [5,7].

Here, we assessed whether T and NT males differed in pituitary GnRH-R mRNA abundance. To date, two types of GnRH-R have been found in *A. burtoni*: GnRH-R1 and GnRH-R2 [9] (nomenclature follows Lethimonier et al. [10]). Many other vertebrates also express multiple GnRH-R variants [11], but little is known about their functional differences [10]. Since social interactions could act differentially on disparate GnRH-R types, we were interested in comparing the expression of each GnRH-R type as a function of social status. Quantitative real-time PCR was used to evaluate mRNA levels of GnRH-R1 and GnRH-R2 in pituitaries taken from T and NT males.

In addition, we examined GnRH-R1 and GnRH-R2 in the brain for potential social status differences. Studies of other species have shown that GnRH-containing neurons in the hypothalamus express GnRH-R and respond to autocrine feedback by modulating GnRH release [12,13]. In *A. burtoni*, GnRH1-containing neurons in the hypothalamus express GnRH-R2 [14]. Thus, social regulation of GnRH-R could occur not only in the pituitary but also in the brain. We therefore compared T and NT expression of each GnRH-R type in the brain as well.

2. Materials and methods

2.1. Animals

Laboratory-bred *Astatotilapia* (*Haplochromis*) *burtoni* derived from wild-caught stock were maintained in aquaria simulating natural conditions: pH 7.8–8.2, 29 °C water temperature, and 12-h light/12-h dark cycles [6]. Terra cotta pot shards in the aquaria served as territories. Fish were fed once daily (Aquadine, Healdsburg, CA). Plastic tags, secured near the dorsal fin of each male, identified individual fish. All work was performed in compliance with Stanford University animal care and use guidelines.

2.2. Behavioral observations

Fish in community tanks were observed three times weekly over 4 weeks. T males ($n = 10$) were selected based on their bright blue or yellow coloration, dark lachrymal stripe (eyebar), and demonstration of territorial, aggressive, and courtship behaviors. NT males ($n = 11$) were chosen for their dull gray coloration and for their submissive schooling and fleeing behavior. All fish maintained their social status for at least 4 weeks before they were killed. Three sets of detailed focal observations were also conducted one week before death. During each focal observation period, each male was observed for 3 min and the frequency of different behaviors was recorded. An average dominance index (DI) was calculated for each fish by subtracting the number of submissive behaviors from the number of aggressive acts during each focal observation session [5]. Standard length and weight were recorded for each fish at time of death (standard length, T males: 7.13 ± 0.13 cm, $n = 10$; NT males: 6.02 ± 0.16 cm, $n = 11$; body weight, T males: 9.16 ± 0.48 g, $n = 10$; NT males: 5.81 ± 0.48 g, $n = 11$).

2.3. Tissue preparation

Fish were killed by rapid cervical transection. Brains and pituitaries were rapidly removed, flash frozen, and stored at −80 °C until use. Gonads were weighed and used to calculate the gonadosomatic index (GSI) for each fish: GSI = (gonad weight/body weight) × 100.

Total brain and pituitary RNA were isolated (Ultraspec-II RNA Isolation System, Biotecx Laboratories, Houston, TX). Using an anchored poly T primer ($dVdT_{24}$) and SuperScript II enzyme (Invitrogen, Carlsbad, CA), total pituitary RNA (0.75 μg) and total brain RNA (2.5 μg) were reverse transcribed following the First Strand cDNA Synthesis protocol (Invitrogen).

2.4. Quantitative real-time PCR reactions

Real-time PCR was used to quantify mRNA levels of GnRH-R1 and GnRH-R2 in the pituitary and to measure mRNA levels of GnRH1, GnRH-R1, and GnRH-R2 in the brain. Primers specific to GnRH1, GnRH-R1, and GnRH-R2 were designed from full-length sequences (Genbank accession numbers, GnRH1: U31865, GnRH-R1: AY705931, GnRH-R2: AY028476). Oligo 6 software (Molecular Biology Insights, Cascade, CO) was used to construct primers with 50–60% GC content, amplifying products between 75 and 250 bp. Upper and lower primers were designed to have similar melting temperatures that were also higher than the melting temperatures of any secondary structures. Secondary structures were analyzed using a web-based program [15]. All primers were synthesized commercially as follows: GnRH1 upper primer 5′-CAG ACA CAC TGG GCA ATA TG-3′ and lower primer 5′-GGC CAC ACT CGC AAG A-3′ (128 bp product); GnRH-R1 upper primer 5′-TCA GTA CAG CGG CGA AAG-3′ and lower primer 5′-GCA TCT ACG GGC ATC ACG AT-3′ (187 bp product); GnRH-R2 upper primer 5′-GGC TGC TCA GTT CCG AGT T-3′ and lower primer 5′-CGC ATC ACC ACC ATA CCA CT-3′ (220 bp product). To control for differences in loading and cDNA synthesis, primers for the reference gene glyceraldehyde 3-phosphodehydrogenase (G3PDH, Genbank accession number AF123727) were also synthesized: upper primer 5′-CAC ACA AGC CCA ACC CAT AGT CAT-3′ and lower primer 5′-AAA CAC ACT GCT GCT GCC TAC ATA-3′ (78 bp product).

Real-time PCR was performed in 30 μl reactions containing 1X IQ Supermix (Bio-Rad Laboratories, Hercules, CA), 0.1X SYBR Green I (Molecular Probes, Eugene OR), 10 nM Fluorescein (Bio-Rad Laboratories), 0.5 μM upper primer, 0.5 μM lower primer, and sample cDNA (for brain PCR reactions: 5 ng reverse transcribed total brain RNA, for pituitary PCR reactions: 3.75 ng reverse transcribed total pituitary RNA). PCR reactions were run on an iCycler (Bio-Rad Laboratories) using the following protocol: denaturation at 95 °C for 1 min, followed by 40 amplification and quantification cycles (95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s), and finishing with a melt curve analysis to assess primer–dimer formation and the number of amplified products.

All standards and samples were run in triplicate. Efficiencies for each primer pair were calculated using PCR Miner software, which uses the kinetics of individual reactions to objectively determine efficiency [16]. Melt curve analysis and gel electrophoresis performed on the PCR products confirmed that each primer pair amplified only a single product of the desired length. Brain cDNA produced the following efficiencies: $E_{\text{GnRH1}} = 0.93$, $E_{\text{GnRH-R1}} = 0.89$, $E_{\text{GnRH-R2}} = 0.93$, $E_{\text{G3PDH}} = 0.96$. Pituitary cDNA efficiencies were as follows: $E_{\text{GnRH-R1}} = 0.94$, $E_{\text{GnRH-R2}} = 0.91$, $E_{\text{G3PDH}} = 0.91$.

For each gene, PCR Miner set the threshold fluorescence level at the second derivative maximum of the amplification curve [16]. PCR Miner was then used to determine the fractional number of cycles (CT) needed to reach the threshold within each reaction's exponential phase [16]. CTs from triplicates were averaged for each sample. The following equation was then used to calculate relative gene expression levels (as a percentage of G3PDH expression levels) for each sample: $[(1 + E_{\text{G3PDH}})^{\text{CT}_{\text{G3PDH}}}]/[(1 + E_{\text{gene}})^{\text{CT}_{\text{gene}}}] \times 100$ [16,17]. Across all samples, the average coefficient of variation (CV) for CTs was $0.28\% \pm 0.02$.

2.5. Data analysis

Data are presented as means ± S.E.M. For GSI, DI, and pituitary GnRH-R1 mRNA expression, 10 T males and 11 NT males were used. Due to inadequate quantities of brain material for two subjects, real-time PCR was performed on brain cDNA from only 9 T males and 10 NT males. For the pituitary GnRH-R2 analysis, one outlier was excluded for exhibiting extraordinarily high levels of GnRH-R2 (greater than 4 S.D. above the mean). This decision was made on the basis that an extreme outlier can exert undue influence on the explanatory model that best fits the rest of the data, even when using nonparametric tests [18]. Excluding an extreme outlier can therefore produce a more accurate estimate of the actual population parameters [19]. Moreover, overall significance levels remained the same ($P > 0.05$), regardless of whether the outlier was included

or excluded in the analysis. Since not all data followed a normal distribution, Mann Whitney *U*-tests (two-tailed, SPSS) were used to assess all differences in relative gene expression levels. Correlations were assessed using Spearman rank tests. The significance level for all tests was set at $P < 0.05$.

3. Results

T males behaved significantly more aggressively than NT males, as reflected by their high dominance index scores (DI, T males: 9.93 ± 1.03, $n = 10$; NT males: -1.67 ± 0.47, $n = 11$; $U = 0$, $P < 0.001$). Also, T males had significantly higher gonad to body weight ratios than NT males (GSI, T males: $0.45 \pm 0.07\%$, $n = 10$; NT males: $0.29 \pm 0.04\%$, $n = 11$; $U = 23$, $P = 0.024$). These data are consistent with prior work [5,7] and verify that the T/NT categories reflect significant differences in behavioral and reproductive phenotypes.

Compared to NT males, T males expressed 1.9-fold higher levels of GnRH1 transcript in the brain (Fig. 1; $U = 9$, $P = 0.002$). Brains from T and NT males did not differ significantly in mRNA levels of either GnRH-R1 or GnRH-R2 (GnRH-R1, T males: $0.81 \pm 0.11\%$ G3PDH, $n = 9$; NT males: $0.72 \pm 0.07\%$ G3PDH, $n = 10$; $U = 35$, $P = 0.447$; GnRH-R2, T males: $0.26 \pm 0.03\%$ G3PDH, $n = 9$; NT males: $0.28 \pm 0.02\%$ G3PDH, $n = 10$; $U = 41$, $P = 0.780$). For both T and NT males, mRNA levels of GnRH-R1 were higher than mRNA levels of GnRH-R2 in the brain (GnRH-R1, $0.76 \pm 0.06\%$ G3PDH, $n = 19$; GnRH-R2, $0.27 \pm 0.02\%$ G3PDH, $n = 19$; $U = 6$, $P < 0.001$).

Pituitaries from T males contained significantly higher levels of GnRH-R1 mRNA than pituitaries from NT males, by a factor of 1.7 (Fig. 2A; $U = 17$, $P = 0.006$). In contrast, NT males expressed slightly higher levels of GnRH-R2 mRNA than T males, but this difference was not statistically significant (Fig. 2B; $U = 28$, $P = 0.105$). Overall GnRH-R1 mRNA levels were more than 50-fold higher than GnRH-R2 mRNA levels in the pituitaries of both T and NT males (GnRH-R1, $98.77 \pm 9.90\%$ G3PDH, $n = 21$; GnRH-R2, $1.87 \pm 0.24\%$

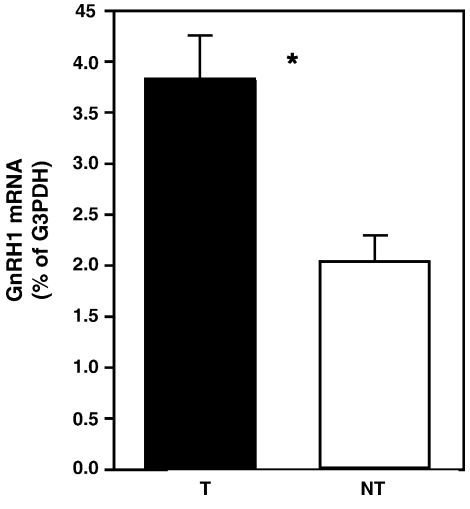

Fig. 1. GnRH1 mRNA levels in the brain as a function of social status. For each group, mean expression of GnRH1 mRNA is shown as a percentage of the reference gene (G3PDH) expression ± S.E.M. (T males: $n = 9$, NT males: $n = 10$). Asterisk indicates $P = 0.002$.

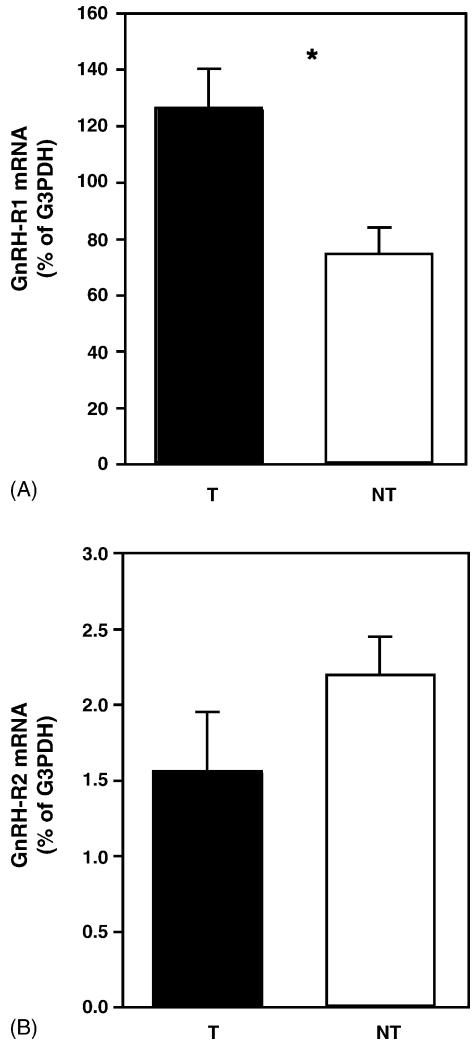

Fig. 2. GnRH-R expression in the pituitary as a function of social status. Mean expression for each group is shown as a percentage of the reference gene (G3PDH) expression ± S.E.M. (A) GnRH-R1 (T males: $n = 10$, NT males: $n = 11$); (B) GnRH-R2 (T males: $n = 10$, NT males: $n = 10$). Asterisk indicates $P = 0.006$.

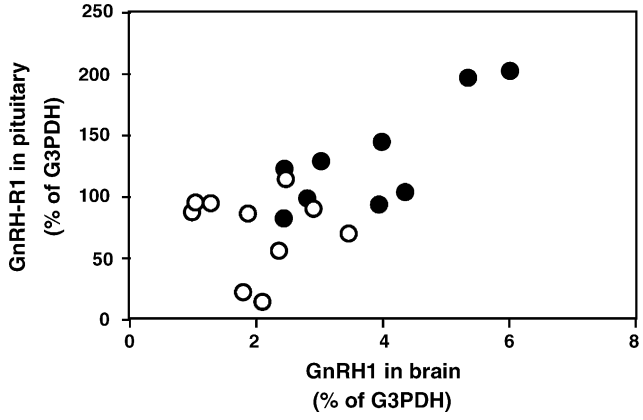

Fig. 3. Correlation between GnRH1 mRNA levels in the brain and GnRH-R1 mRNA levels in the pituitary. NT males are shown in unfilled circles and T males in filled circles. Spearman $\rho = 0.60$, $P = 0.006$, $n = 19$.

G3PDH, $n = 20$; $U = 0$, $P < 0.001$). GnRH1 mRNA levels in the brain correlated positively with GnRH-R1 mRNA levels in the pituitary (Fig. 3; Spearman $\rho = 0.60$, $P = 0.006$). However, when T and NT males were analyzed separately, this correlation was significant only for T males (T males: Spearman $\rho = 0.73$, $P = 0.025$; NT males: Spearman $\rho = -0.14$, $P = 0.701$). GnRH1 mRNA levels did not correlate significantly with pituitary GnRH-R2 mRNA abundance (Spearman $\rho = -0.30$, $P = 0.226$). GnRH1 mRNA levels also did not correlate significantly with expression of either GnRH-R type in the brain. There were no significant correlations between mRNA levels of the two GnRH-R types in the brain or pituitary.

4. Discussion

Previous work has shown that social interactions can act on the HPG axis at the level of GnRH1 in the hypothalamus and sperm maturation in the gonads [4,5,7,8]. Here, we show that social status is also linked to another component of the HPG axis: GnRH receptor in the pituitary. Using quantitative real-time PCR, we found that only one of the two types of GnRH-R transcripts is socially regulated in the pituitary. T males expressed significantly higher levels of GnRH-R1 mRNA in the pituitary than NT males, whereas GnRH-R2 mRNA expression in the pituitary did not vary as a function of social status. Replicating results from previous work [5], T males also exhibited higher GnRH1 mRNA levels in the brain and a higher gonad to body weight ratio. Social status is therefore associated with changes at all levels of the HPG axis in *A. burtoni*: GnRH1 in the brain, GnRH-R1 in the pituitary, and gonad size (GSI).

How does social status regulate GnRH-R1? One possibility is that socially regulated GnRH1 levels directly regulate GnRH-R1 expression. The significant positive correlation between GnRH1 and GnRH-R1 found in this study is consistent with this model. Interestingly, this correlation was significant only for T males, suggesting that higher amounts of GnRH1 may be needed to regulate pituitary mRNA levels of GnRH-R1. Both in vivo and in vitro studies in rats and fish have also shown that GnRH can regulate its own receptor mRNA and protein levels [20–22]. Other socially regulated components of the HPG axis, such as gonadal sex steroids, could also regulate GnRH-R [2,3,23]. While some studies suggest that gonadal sex steroids merely regulate GnRH-R indirectly through GnRH [20], others have shown that gonadal hormones can also directly regulate GnRH-R independently of GnRH [24]. Social status could also regulate GnRH-R1 through intermediaries outside of the reproductive axis. Glucocorticoids, for example, are subject to social control [25] and can directly regulate GnRH-R transcription in the pituitary [26]. Further work is needed to examine the relative contributions of social status, GnRH, and other factors to GnRH-R1 regulation in *A. burtoni*.

Upregulation of both GnRH1 and GnRH-R1 mRNA constitutes a dual way of increasing the signal to pituitary gonadotropes. Ultimately, this enhanced signal increases the release of gonadotropins, which stimulate gonadal maturity and the production of sex steroids critical to reproduction. Our finding that GnRH-R1 mRNA levels vary with social status, while GnRH-R2 levels do not, implies distinct functions for the two GnRH-R variants. Functional differences have also been suggested by recent work revealing distinct distributional patterns of GnRH-R1 and GnRH-R2 in the pituitary. GnRH-R1 in *A. burtoni* has been localized to ventral-anterior and posterior parts of the pituitary where gonadotropes are located, while GNRH-R2 has been found in the dorsal-anterior and posterior parts of the pituitary [14]. Coupled with these localization data, the upregulation of GnRH-R1 in reproductively active T males suggests that GnRH-R1 serves as the main GnRH-R implicated in the HPG axis. Lending further support to this notion, we show in this study that GnRH-R1 mRNA levels are much higher than GnRH-R2 mRNA levels in the pituitary. Studies of other fish species have also found high expression of GnRH-R1 in the pituitary, localization of GnRH-R1 to gonadotropes, and an association between GnRH-R1 mRNA abundance and sexual maturation [27,28]. Together, these lines of evidence suggest that GnRH-R1 operates principally in the HPG axis.

In contrast to the social status differences in GnRH-R1 mRNA levels, no clear differences in pituitary GnRH-R2 mRNA levels emerged between T and NT males. Reproductively suppressed NT males tended to express higher levels of GnRH-R2 mRNA in the pituitary than T males, but this difference did not reach statistical significance. Recent work suggests that GnRH-R2 may be involved in regulating somatic growth, based on its distribution pattern in the pituitary [14]. It will be interesting to determine the expression patterns of both GnRH-Rs in specific pituitary cell types and to identify the function of GnRH-R2.

The present study also assessed GnRH-R1 and GnRH-R2 mRNA levels in the brains of T and NT males and found no significant social status differences in either GnRH-R type. We interpret this lack of difference in the whole brain with caution, since measurements of expression across the whole brain may mask differences in specific brain regions. Different distribution patterns of GnRH-R1 and GnRH-R2 have been observed in the brain [14], indicating that a closer examination of specific brain regions will be important for further investigating GnRH-R1 or GnRH-R2 social regulation in the brain.

In summary, this work shows that pituitary GnRH-R in *A. burtoni* is an additional site of social regulation within the HPG axis. While pituitary GnRH-R1 mRNA levels reflect differences in socially controlled behavioral phenotypes, GnRH-R2 mRNA levels are not socially regulated. This differential regulation implies divergent functions for these two receptor types. Since GnRH-R plays such a crucial role in the HPG axis of all vertebrates, unraveling its complex relationship with environmental and endogenous cues is essential for forming a better understanding of the neural control of reproduction.

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References

[1] Abbott DH, Hodges JK, George LM. Social status controls LH secretion and ovulation in female marmoset monkeys (*Callithrix jacchus*). J Endocrinol 1988;117:329–39.
[2] Cardwell JR, Sorensen PW, Van der Kraak GJ, Liley NR. Effect of dominance status on sex hormone levels in laboratory and wild-spawning male trout. Gen Comp Endocrinol 1996;101:333–41.
[3] Faulkes CG, Abbott DH, Jarvis JU. Social suppression of ovarian cyclicity in captive and wild colonies of naked mole-rats, *Heterocephalus glaber*. J Reprod Fertil 1990;88:559–68.
[4] Robison RR. Social regulation of reproduction. Psychology and the committee on graduate studies. PhD Thesis, Stanford University; 2000.
[5] White SA, Nguyen T, Fernald RD. Social regulation of gonadotropin-releasing hormone. J Exp Biol 2002;205:2567–81.
[6] Fernald RD, Hirata NR. Field study of *Haplochromis burtoni*: quantitative behavioral observations. Anim Behav 1977;25:964–75.
[7] Francis RC, Soma K, Fernald RD. Social regulation of the brain–pituitary–gonadal axis. Proc Natl Acad Sci USA 1993;90: 7794–8.
[8] Fraley NB, Fernald RD. Social control of developmental rate in the African cichlid, Haplochromis burtoni. J Comp Ethol 1982;60:66–82.
[9] Robison RR, White RB, Illing N, et al. Gonadotropin-releasing hormone receptor in the teleost Haplochromis burtoni: structure, location, and function. Endocrinology 2001;142:1737–43.
[10] Lethimonier C, Madigou T, Munoz-Cueto JA, Lareyre JJ, Kah O. Evolutionary aspects of GnRHs, GnRH neuronal systems and GnRH receptors in teleost fish. Gen Comp Endocrinol 2004;135:1–16.
[11] Millar RP, Lu ZL, Pawson AJ, Flanagan CA, Morgan K, Maudsley SR. Gonadotropin-releasing hormone receptors. Endocr Rev 2004;25:235–75.
[12] Krsmanovic LZ, Martinez-Fuentes AJ, Arora KK, et al. Autocrine regulation of gonadotropin-releasing hormone secretion in cultured hypothalamic neurons. Endocrinology 1999;140:1423–31.
[13] Xu C, Xu XZ, Nunemaker CS, Moenter SM. Dose-dependent switch in response of gonadotropin-releasing hormone (GnRH) neurons to GnRH mediated through the type I GnRH receptor. Endocrinology 2004;145:728–35.
[14] Chen CC, Fernald RD. Distributions of two gonadotropin-releasing hormone receptor types in a cichlid fish suggest functional specialization. J Comp Neurol 2006;495:314–23.
[15] Zuker M. Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res 2003;31:3406–15.
[16] Zhao S, Fernald RD. Comprehensive algorithm for quantitative real-time polymerase chain reaction. J Comput Biol 2005;12:1045–62.
[17] Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 2001;29:e45.
[18] Zimmerman DW. A note on the influence of outliers on parametric and nonparametric tests. J Gen Psychol 1994;121:391–401.
[19] Judd CM, McClelland GH. Data analysis: a model comparison approach. San Diego: Harcourt Brace Jovanovich; 1989.
[20] Frager MS, Pieper DR, Tonetta SA, Duncan JA, Marshall JC. Pituitary gonadotropin-releasing hormone receptors. Effects of castration, steroid replacement, and the role of gonadotropin-releasing hormone in modulating receptors in the rat. J Clin Invest 1981;67:615–23.
[21] Kaiser UB, Jakubowiak A, Steinberger A, Chin WW. Regulation of rat pituitary gonadotropin-releasing hormone receptor mRNA levels in vivo and in vitro. Endocrinology 1993;133:931–4.
[22] Levavi-Sivan B, Safarian H, Rosenfeld H, Elizur A, Avitan A. Regulation of gonadotropin-releasing hormone (GnRH)-receptor gene expression in tilapia: effect of GnRH and dopamine. Biol Reprod 2004;70:1545–51.
[23] Parikh VN, Clement TS, Fernald RD. Androgen level and male social status in the African cichlid, *Astatotilapia burtoni*. Behav Brain Res 2005.
[24] Turzillo AM, DiGregorio GB, Nett TM. Messenger ribonucleic acid for gonadotropin-releasing hormone receptor and numbers of gonadotropin-releasing hormone receptors in ovariectomized ewes after hypothalamic-pituitary disconnection and treatment with estradiol. J Anim Sci 1995;73:1784–8.
[25] Fox HE, White SA, Kao MH, Fernald RD. Stress and dominance in a social fish. J Neurosci 1997;17:6463–9.
[26] Maya-Nunez G, Conn PM. Transcriptional regulation of the GnRH receptor gene by glucocorticoids. Mol Cell Endocrinol 2003;200:89–98.
[27] Alok D, Hassin S, Sampath Kumar R, Trant JM, Yu K, Zohar Y. Characterization of a pituitary GnRH-receptor from a perciform fish, Morone saxatilis: functional expression in a fish cell line. Mol Cell Endocrinol 2000;168:65–75.
[28] Gonzalez-Martinez D, Madigou T, Mananos E, et al. Cloning and expression of gonadotropin-releasing hormone receptor in the brain and pituitary of the European sea bass: an in situ hybridization study. Biol Reprod 2004;70:1380–91.