

Short communication

Social status and GnRH soma size in female convict cichlids (*Amatitlania nigrofasciatus*)



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HIGHLIGHTS

- We examine social influences on gonadotropin-releasing hormone in the hypothalamus.
- Female social status affects GnRH neuron soma size in the preoptic area.
- Dominant females have larger GnRH neuron soma.
- We found no effects on number of GnRH neurons.

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ABSTRACT

Gonadotropin-releasing hormone (GnRH) neurons in the preoptic area (POA) of the hypothalamus play a key role in regulating reproductive function. These neurons in turn are modulated by environmental influences, including the social environment. In both the Old World cichlid *Astatotilapia burtoni* and the New World cichlid *Amatitlania nigrofasciatus*, the size of the soma of GnRH expressing neurons in the POA varies with social status in males and breeding state in females. Dominant males have larger GnRH-releasing cells than subordinate males, and spawning females have larger GnRH-releasing cells than brooding females. *A. nigrofasciatus* is monogamous and both sexes engage in similar levels of aggression and territorial defense. Here we test whether female *A. nigrofasciatus* display GnRH-releasing cell plasticity as a function of dominant status. We find that GnRH-releasing neuron soma sizes are larger in dominant females and that this difference is independent of differences in gonado-somatic index in *A. nigrofasciatus*.

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Among many cichlid fish, the right time to reproduce is determined by the social environment [1]. Males must either establish a dominant status (as in the African cichlid *Astatotilapia burtoni*) [2–4] or ownership over a more conventional territory (as in the Central American cichlid *Amatitlania nigrofasciatus* aka the convict cichlid [5,6]), and the females of these species must also regulate the timing of their reproduction according to the social and asocial environment.

The proximate pathway regulating reproduction is the hypothalamic-pituitary gonadal (HPG) axis, the head of which is

gonadotropin-releasing hormone (GnRH) neurons in the preoptic area (POA) of the hypothalamus [7–9]. GnRH stimulates the release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the anterior pituitary, resulting in sex steroid synthesis and gametogenesis at the gonads [8], thus enabling reproduction. This makes the POA the center of integration of environmental influences acting downstream on the POA [10].

Over 20 different forms of GnRH have been described [11], with teleost fish having three variants: sbGnRH (GnRH I), CGnRH-II (GnRH II), and sGnRH (GnRH III) [12,13]. Different forms of GnRH can have varying distributions and functions [13]; however, in many teleosts, GnRH I-expressing cells located in the POA are primarily responsible for gonadotropin release and show plasticity with respect to the social environment (reviewed by [14]). These gonadotropins increase gametogenesis and sex steroid synthesis, as well as the expression of sexual behavior.

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Experimental manipulations of male social status in both *A. burtoni* and *A. nigrofasciatus* have shown increases in GnRH-releasing neuron soma size, but not cell number, in the POA upon attaining a dominant status (reviewed by [7,15]). Despite the differences in breeding system at the upstream end of the HPG axis, dominant status seems to have the same physiological effect in males between the two cichlid species.

Experiments manipulating female breeding state show an association between reproductive state and POA GnRH-releasing neuron soma size in both *A. burtoni* and *A. nigrofasciatus*, with spawning females having larger soma sizes than brooding females [15–18]. Female *A. burtoni* and *A. nigrofasciatus* may be expected to differ more than males in both their social experiences and the relationship between social cues and regulation of breeding state. Only males aggressively defend their territory in *A. burtoni*, while both males and females engage in similar levels of aggressive territorial defense in *A. nigrofasciatus* [6,19].

This makes convict cichlids an ideal model to study the effects of social status manipulation on GnRH-releasing neuromorphology in females. Here, we test whether GnRH-releasing neuron soma size in female convict cichlids show the same plasticity with respect to social status manipulations as in males by experimentally manipulating female fish to have dominant or subordinate phenotypes.

Sexually mature female convict cichlids were obtained from a local supplier and housed in a unisexual stock tank (75 cm × 31 cm × 41 cm) containing a 2 cm layer of sand and multiple terracotta pots. Fish were housed for 2 weeks prior to experimentation. Water temperature was maintained at $23 \pm 2^\circ\text{C}$ and lighting conditions were set at 12 h:12 h light:dark cycle. Fish were fed TetraMin fish flakes 5 days a week. Animal housing and experimental procedures adhered to the principles and guidelines of the Canadian Council on Animal Care, and the University of Alberta Biological Sciences Committee approved all protocols (Protocol # AUP00000055).

Twenty-eight females were assigned to dominant or subordinate treatment groups ($n = 14$ dominant, 14 subordinate). Prior to experimentation, the standard length (defined as the length from the anterior tip of the head to the anterior end of the caudal fin) was measured using a ruler (to the nearest 0.1 cm). Weights were determined using an electronic balance (to the nearest 0.01 g; Mettler). Social status manipulation was achieved by transferring fish to aquaria (50 cm × 25 cm × 30 cm) containing a 2 cm layer of sand and a single terracotta pot. Fourteen fish assigned to the dominant group were housed with three smaller female stimulus fish and 14 fish assigned to the subordinate group were housed with three larger female stimulus fish, of at least 15% weight difference. The fish remained in the altered social environment for 14 days. Upon termination of the experimental period, stimulus fish were returned to the unisexual stock tank. If the experimental fish did not display characteristics of dominant or subordinate social status after 14 days, experimental and stimuli fish were returned to the stock tank.

Social interactions in each aquarium were observed for 7 min at the end of 14 days. Females were considered dominant if they met the following criteria: (1) they resided in and defended the terracotta pot; (2) they chased other conspecifics; (3) they exhibit dark colored vertical bars on their bodies. Fish were considered subordinate if they met at least two of the following criteria: (1) they fled from larger conspecifics; (2) they exhibited light colored vertical bars; (3) they swam near the surface of the aquarium. Following removal from the experimental aquaria, fish were anesthetized in 0.002% 2-phenoxy ethanol. Gonads were dissected out and weighed, and fish were perfused with 4% paraformaldehyde and immediately decapitated. Brains were dissected out and weighed. Weights were determined using an electronic scale (to the nearest 0.001 g; Denver Instruments). Condition was calculated as the

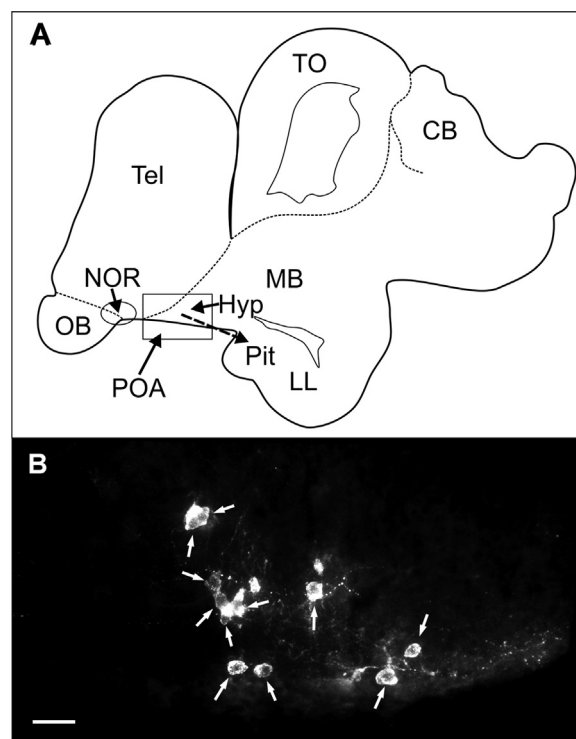


Fig. 1. Gonadotropin-releasing hormone (GnRH+) immunoreactive cells in the preoptic area (POA) of the convict cichlid (*Amatitlania nigrofasciatus*). (A) An example mid-sagittal section through the brain indicating the POA. Abbreviations: NOR, nucleus olfacto-retinalis; OB, olfactory bulb; Tel, telencephalon; TO, optic tectum; CB, cerebellum; MB, midbrain; LL, lateral lobe; Pit, pituitary; Hyp, hypothalamus; POA, preoptic area. The dashed arrow indicates the location of projections of GnRH+ immunoreactive neurons. (B) Photomicrograph showing a cluster of GnRH+ cells in the POA, with the white arrows indicating the labeled cell bodies. Scale bar: 25 μm .

residual from the mass–length regression [20]. The gonado-somatic index (GSI) was calculated as follows:

$$\frac{\text{gonad weight (g)}}{\text{body weight (g)}} \times 100$$

Brains were fixed in 4% paraformaldehyde (pH 7.4) and stored at -4°C for up to 4 weeks before processing. Brains were then cryoprotected in 30% sucrose in 0.1 M phosphate-buffered saline (PBS) for 24 h. Brains were frozen at -21°C and 40 μm sagittal sections were cut on a cryostat (Leica). Sagittal sections were cut to maintain a consistent plane throughout sections. Sections were mounted on electrostatic slides (Fisher Superfrost/Plus) and allowed to dry on a slide warmer (Fisher Scientific) set at 40°C for 2 h. The mounted tissue was stored at room temperature for up to 3 weeks, then rinsed 3 times with 0.1 M PBS before being incubated in donkey blocking serum (1:10 normal donkey serum, Jackson ImmunoResearch Laboratories) with 0.1 M PBS and 0.03% Triton X for 1 h. Tissue was then labeled with a polyclonal anti-GnRH primary antibody raised in rabbits against the mammalian form of GnRH (1:1625 anti-LHRH; Chemicon International) with 0.1 M PBS and 10% normal donkey serum for 24 h. Immunoreactive GnRH (hereafter referred to as GnRH+) neurons were identified using a fluorescent secondary antibody (1:200 Alexafluora 594 donkey anti-rabbit; Jackson ImmunoResearch Laboratories) for 2 h. Tissue was rinsed three times with 0.1 M PBS.

Previous research suggests that in *A. burtoni*, it is the soma size of GnRH+ neurons in the POA responding to changes in social status that are of particular significance [21]. Therefore, we measured all GnRH+ neurons located within the POA (Fig. 1). Areas of GnRH+ cells were viewed with a compound light microscope (Leica DMRE) with a green fluorescence filter (Fig. 1B). Images were obtained using the

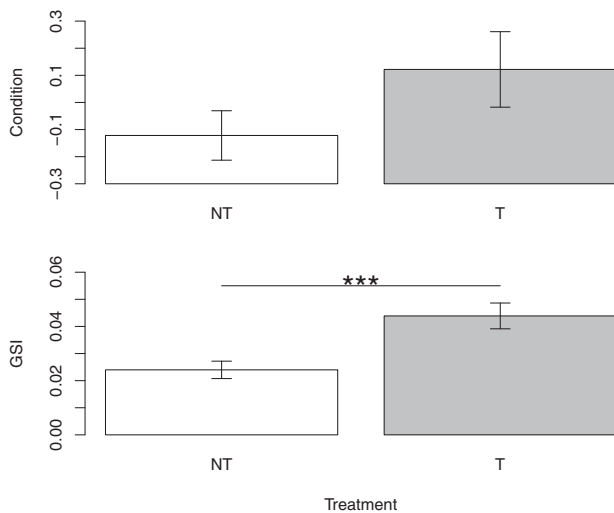


Fig. 2. Condition and gonado-somatic index in females as a function of treatment group. Panel A shows a non-significant ($p = 0.16$) trend towards differences in condition (the residuals from the mass–length regression line) between treatment groups. Panel B shows the significantly larger GSI in territorial females ($p = 0.009$).

OPEN-LAB imaging system (Improvision, Lexington, MA). Image-J software (National Institutes of Health, Maryland, USA) was used to measure the area of GnRH+ cells by tracing the entire area of the fluorescently labeled cell body. All labeled cells that had a clearly defined perimeter were measured (see Fig. 1B). The mean GnRH+ soma size was calculated for each individual fish. The number of GnRH+ cells in the POA was also counted. Two observers, one of whom was blind to the social status of the individual fish, performed measurements and cell counts. Mean cell areas between the two observers were strongly correlated ($r(26) = 0.80$, $p < 0.001$) indicating repeatability of GnRH+ soma size measurements.

Correlation coefficients were obtained using the Pearson product-moment correlation test. Analysis of covariance (ANCOVA) and Students *t*-tests were used to determine differences between treatment groups. All statistics were calculated using R 3.0.1 (R Core Development Team, Vienna, Austria), and all significance levels were set at 0.05.

We found that female body mass was strongly positively correlated with standard length ($r(26) = 0.96$, $p < 0.001$). An analysis of covariance found no significant effect of social status on the relationship between body mass and standard length ($F(1,24) = 2.57$, $p = 0.12$) or an interaction effect of social status ($F(1,24) = 0.03$, $p = 0.87$). We therefore used the residuals from this regression as our measure of condition, or the health of each individual fish, as in [15].

There was a positive correlation between GSI and condition across the experimental fish ($r(26) = 0.58$, $p = 0.0013$). Condition showed a non-significant relationship between condition and social status, with dominant fish having lower condition ($t(22.42) = 1.46$, $p = 0.16$; Fig. 2A), while GSI was significantly higher among dominant fish ($t(22.38) = 2.86$, $p = 0.0090$; Fig. 2B).

GnRH+ cell bodies and fibers were detected in the POA (Fig. 1). The number of GnRH+ cells did not correlate with standard length ($r(26) = 0.11$, $p = 0.56$), even after including social status as a cofactor ($F(1,24) = 0.22$, $p = 0.64$). Condition did not correlate with the number of GnRH+ cells ($r(26) = 0.007$, $p = 0.97$), even with social status as a cofactor ($F(1,24) = 0.004$, $p = 0.95$). A two-sample *t*-test showed no significant difference in number of GnRH+ cells between social status groups ($t(26) = 0.36$, $p = 0.73$).

A two-sample *t*-test showed a difference in mean size between T and NT females (length: $t(26) = 2.64$, $p = 0.01$, $d' = 1.0$; mass:

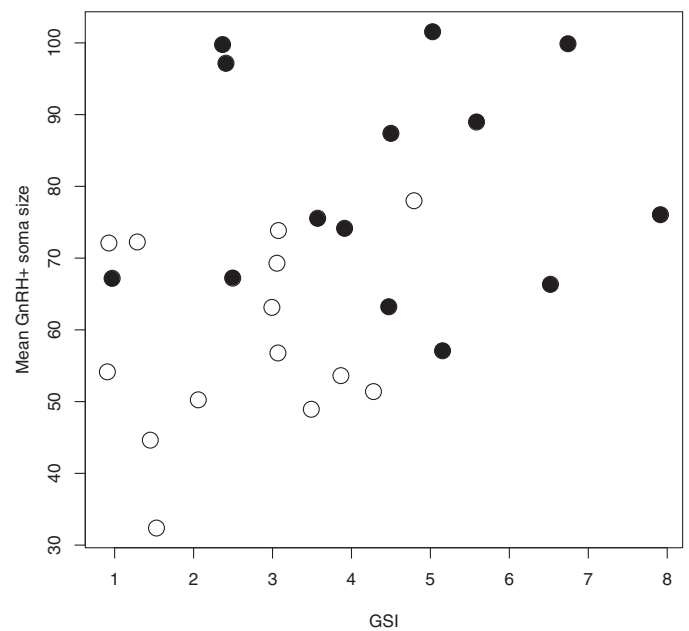


Fig. 3. Relationship between mean GnRH+ soma size and GSI in females as a function of social status group, with dominant females with solid dots and subordinate females with open dots. There is no significant correlation between GnRH+ soma size and GSI ($p = 0.058$), although dominant females have larger GnRH+ soma sizes than subordinate females.

$t(26) = 3.08$, $p < 0.01$, $d' = 1.2$). However, mean GnRH+ soma size was not correlated with length ($r(26) = 0.30$, $p = 0.12$), mass ($r(26) = 0.25$, $p = 0.21$), or condition ($r(26) = -0.17$, $p = 0.40$). Given the substantial overlap in size between the groups, and the lack of correlation between size or condition and GnRH+ soma size, we see no evidence that a size confound accounts for these results. There was a trend towards a positive correlation between GSI and mean GnRH+ soma size ($r(26) = 0.36$, $p = 0.058$; Fig. 3); a correlation was to be expected given that the T/NT treatment influenced both these traits. The relationship was not seen, however, when social status treatment was included as a cofactor ($F(1,25) = 0.20$, $p = 0.66$). An analysis of covariance showed that social status had a significant effect on GnRH+ soma size after correcting for GSI (dominant females have larger GnRH+ soma sizes than subordinate females; $F(1,25) = 10.2$, $p = 0.0038$; Figs. 3 and 4). While we found no significant effect of condition, previous work [15] suggested that it might be a potential confound, and so we conducted an ANCOVA on GnRH+ soma size that included both GSI and condition as cofactors. The effect of social status manipulation was even stronger in this case ($F(1,24) = 14.4$, $p < 0.001$).

In summary, we found that our experimental treatment resulted in dominant females with larger GnRH+ cells in the POA and a larger GSI than subordinate females and that the magnitude of the social status manipulation on GnRH+ soma size was independent of the magnitude of the effect on GSI. This effect of status manipulation on GnRH+ soma size is the same as that seen in males in the same species [15].

While we found that social status manipulation in female convict cichlids was associated with both increased GSI and POA GnRH+ soma size in females manipulated to be dominant compared to subordinates, we found no significant correlation between GSI and GnRH+ soma size. In the case of the simple correlation, this is likely a type-II error, a stochastic effect of modest sample size ($p = 0.058$). The lack of a strong trend towards a correlation between these two traits after controlling for treatment could reflect individual differences in the time course of effects of GnRH on different targets.

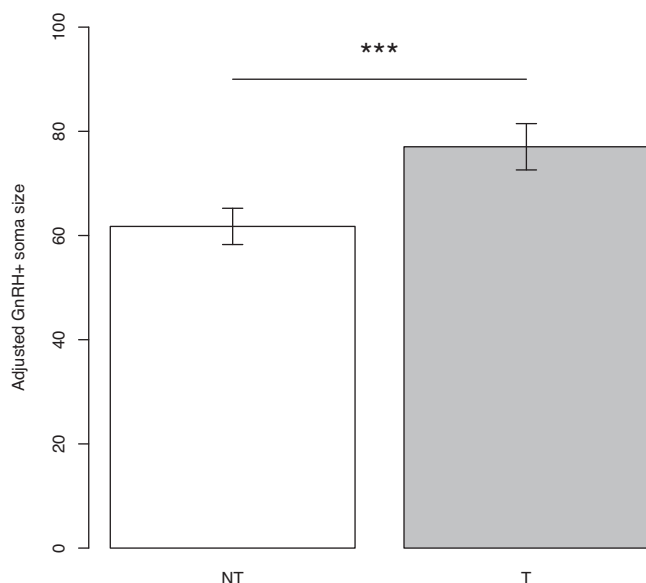


Fig. 4. Mean GnRH+ soma size in females as a function of social status group, after correcting for GSI. Females experimentally manipulated to be dominant had significantly larger GnRH+ soma sizes than subordinate females (see text for statistical details).

Differing DNA transcription effects are seen in the gonads from minute through hours and days after male *A. burtoni* begin to transition from subordinate to dominant [10]. It may be that females with biparental territorial defense mobilize resources to tissues in a more complex manner when faced with the opportunity to compete for a territory than species in which investment in egg production is a stronger priority [e.g. 22,23].

We did not find any differences in GnRH+ cell numbers between treatment groups, body length, or condition, in agreement with earlier work on males of this species [15]. There are different subsets of neurons in the POA, some with larger somas and some with smaller somas, which express GnRH under different levels of opportunity to ascend in a social hierarchy or different reproductive stages [24]. If a difference in GnRH+ cell numbers was found, this would indicate that GnRH+ soma size might reflect different cell populations expressing GnRH under different conditions. While we cannot directly rule out that differently sized GnRH+ neurons express GnRH in different social environments, it is generally agreed that these cells are plastic with respect to soma size in cichlids [e.g. 14].

This is the first study to compare GnRH+ neuronal soma size in female *A. nigrofasciatus* experimentally manipulated to display dominant and subordinate behaviors. Our results indicate that GnRH+ soma size and GSI vary independently and that GnRH+ cell number does not vary with female social status in this monogamous, biparental, aggressive cichlid fish.

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