Social and breeding status are associated with the expression of GnIH

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Discoveries of how social behavior can influence the plasticity of gonadotropin-releasing hormone (GnRH) have revolutionized the field of behavioral neuroendocrinology by providing new insights into the neural mechanisms controlling behavior. In 2000, the neuropeptide gonadotropin inhibitory hormone (GnIH) was discovered and is changing the way we view how the brain mediates reproduction and associated behaviors. GnIH acts as a reproductive ‘pause button’, momentarily inhibiting the activity of the reproductive system. However, how GnIH fluctuates naturally in response to social environment is unknown. We examine how the outcome of competition for limited resources needed for reproduction is associated with GnIH. We experimentally manipulated nesting opportunities for pairs of European starlings (Sturnus vulgaris) and examined brain GnIH mRNA and peptide content, as well as GnRH content and plasma testosterone and corticosterone. By limiting the number of nest boxes per enclosure and thus the number of social pairing and nesting opportunities, we observed that birds which outcompeted others for nest boxes (‘winners’) had significantly fewer numbers of GnIH peptide-producing cells than those without nest boxes (‘losers’) and this relationship changed with breeding stage. GnRH content, testosterone and corticosterone did not vary with nest box ownership. Thus, while birds appeared reproductively capable across treatments, our data indicate that GnIH may serve as a modulator of reproductive behaviors in response to social environment. Additionally, we provide some evidence of the adaptive value of this mechanism.

Keywords: Breeding status, corticosterone, European starling (Sturnus vulgaris), GnIH, GnRH, reproduction, social status, testosterone

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Differences in social status can have profound effects on circulating hormones (reviews: Adkins-Regan 2005; Creel 2001), but the effects of social environment on the brain are less understood (Kabelik et al. 2010; Pradhan et al. 2010; Remage-Healey 2010; Soma et al. 2008). For all vertebrates studied, the gonadotropin-releasing hormone (GnRH) family of peptides is a key regulator of the reproductive, or hypothalamic–pituitary–gonadal (HPG) axis. Gonadotropin-releasing hormone released from the hypothalamus signals the anterior pituitary gland to release the gonadotropic hormones luteinizing hormone (LH) and follicle-stimulating hormone (FSH) into the bloodstream. Luteinizing hormone and FSH then promote testicular spermatogenesis, follicular growth and estradiol and testosterone release which feedback to and modulate all levels of the HPG axis. Several studies on how social behavior can influence the plasticity of the GnRH system have revolutionized the field of behavioral and reproductive neuroendocrinology by expanding our understanding of how social interactions can influence neural mechanisms associated with reproduction (Burmeister 2005; Fox et al. 1997; Francis et al. 1993). The discovery of the novel neurohormone gonadotropin inhibitory hormone (GnIH; Tsutsui et al.) in 2000 is changing the way we view how reproduction and sexual behavior are regulated by the brain.

Found in the area of the brain responsible for reproduction, the hypothalamus, GnIH inhibits reproduction and sexual behavior. Gonadotropin inhibitory hormone decreases the activity of GnRH neurons in addition to reducing synthesis and release of the gonadotrophins LH and, in some cases, FSH from the pituitary gland. Gonadotropin inhibitory hormone also reduces testosterone release from the gonads. Central administration of GnIH can decrease copulation solicitations in birds and sexual behaviors in rodents, but how GnIH fluctuates naturally in response to social environment is unknown (reviewed: Bentley et al. 2009; Kriegsfeld et al. 2006; Ubuka et al. 2010).

European starlings (Sturnus vulgaris) are a socially monogamous obligate cavity-dwelling species and can experience limited and unpredictable nesting site availability from year to year (Aitken & Martin 2004; Newton 1994). We simulated the natural environment of social instability and nesting site competition, forcing our population to divide into two separate breeding cohorts – breeders (or ‘winners’) and non-breeders (or ‘losers’) – and compared GnIH content accordingly. To understand better the dynamics of GnIH and the reproductive axis as a whole, we measured GnRH-I peptide cell abundance and plasma testosterone concentrations. Also, we measured concentrations of the adrenal hormone corticosterone, a known indicator of the stress response, to control for any confounding effects.
capture stress may have had on data analyses. Finally, we conducted a parentage analysis to confirm whether social parents were genetic parents and thus true reproductive ‘winners’ in terms of fitness. Because GnIH presence and function are conserved throughout all vertebrates studied, including humans, studies of how social status regulates GnIH in the brains of other vertebrates can be far reaching, with implications for reproductive physiology and behavior across vertebrate classes.

Materials and methods

To better understand the dynamics of GnIH, we used multiple measurements to serve as proxies for GnIH activity, including (1) cells expressing the GnIH peptide (GnIH peptide cell abundance), (2) cells expressing GnIH mRNA (GnIH mRNA cell abundance) and (3) cells producing GnIH mRNA via an optical density measurement (GnIH mRNA cell optical density). We sampled whole avian cerebri at one time at (1) the beginning of the breeding season, after nest box acquisition, (2) during the middle of the breeding season, when eggs laying and incubation were occurring and (3) the non-breeding season (post molt), when the activity of the reproductive axis is attenuated (breakdown of sex and number of individuals per nest box treatment reported in Table 1).

Housing and nest box manipulation

Thirty-nine (22 males and 17 females) European starlings were caught as juveniles and randomly assigned to large, naturalistic outdoor aviaries (Fig. 1). Aviaries shared wire fencing, and birds in separate aviaries came into contact (sight, song and possible touch) with birds in adjoining aviaries. As a result of this semi-natural setup, birds exhibited a full range of natural breeding behaviors, including singing, copulation solicitations, breeding, social monogamy, nest construction and defense and incubation (Calisi & Bentley 2009). A limited number of nest boxes were placed in all three aviaries at the beginning of the breeding season during the first week of February. There were four aviaries, one sampled in early breeding, two sampled in mid-breeding and one sampled in late breeding (note: Aviary 2, collected during the middle of the breeding season, combined data from two neighboring aviaries). Different birds were sampled at each collecting point; the same individuals could not be sampled repeatedly because of the need to sacrifice the animals to examine patterns of GnIH and GnRH-I content (umbrella terms for measurements taken, particularly for GnIH, in which three measurements were taken – peptide cell abundance, mRNA cell abundance and mRNA cell optical density).

Behavioral observations

Behavioral observations were taken during the first 5 days after nest boxes were placed in aviaries and the last 5 days before sampling; additional observations were taken periodically in between those time points. Observations were taken at ~900–1300 h. We used focal sampling of nest boxes and noted which birds came in direct contact with the nest box (perching on roof, on perch or entering nest box). During the beginning and middle of the breeding season, the same male and female pairs would visit a particular nest box over 95% of the time, and thus were assigned nest box ownership or ‘winner’ status. Birds showed no preference for a nest box at the beginning of the non-breeding season.

Brain and blood sampling

Aviary-housed birds were collected by mist net and hand net and sacrificed immediately by decapitation following rapid terminal anesthesia using isoflurane. Immediately following decapitation, brains were extracted and frozen on dry ice and then stored at ~80°C until sectioning. Approximately 1–2 ml of trunk blood was collected, centrifuged and frozen immediately for parentage analysis and radioimmunoassay (RIA) of plasma corticosterone and testosterone. Brains were sectioned coronally at 20 μm using a cryostat and mounted directly onto silane coated slides. Every eighth section

Table 1: Breakdown of sex and number of individuals per nest box treatment during each collection period

<table>
<thead>
<tr>
<th>Sampled</th>
<th>Aviary 1 Beginning of the breeding season (18 February 2008)</th>
<th>Aviary 2 Middle of the breeding season (24 April 2008)</th>
<th>Aviary 3 Non-breeding season (24 September 2008)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males</td>
<td>7</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>Females</td>
<td>3</td>
<td>10</td>
<td>4</td>
</tr>
<tr>
<td>Total</td>
<td>10</td>
<td>17</td>
<td>12</td>
</tr>
<tr>
<td>Number of nest boxes/aviary</td>
<td>3</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>Obtained nest box</td>
<td>5</td>
<td>12</td>
<td>No preference</td>
</tr>
<tr>
<td>Did not obtain nest box</td>
<td>5</td>
<td>5</td>
<td>No preference</td>
</tr>
<tr>
<td>Pairs incubating eggs</td>
<td>None laid</td>
<td></td>
<td>No longer breeding</td>
</tr>
</tbody>
</table>

Aviary 2 consisted of two neighboring aviaries: the first having three males, five females and three nest boxes, and the second having four males, five females and three nest boxes.
Throughout the hypothalamus was collected for GnIH and GnRH-I content. Content was measured via (1) immunocytochemistry (ICC) to visualize cells labeled for the GnIH and GnRH-I peptides (GnIH/GnRH-I peptide cell abundance) and (2) in situ hybridization (ISH) to visualize cells labeled for GnIH mRNA (GnIH mRNA cell abundance). The density of the latter label was used to estimate the amount of GnIH mRNA being produced (GnIH cell optical density) (see quantification of GnIH and GnRH-I content and statistical analysis). All procedures were approved by and in compliance with the University of California Office of Lab Animal Care and federal regulations.

Parentage analysis
European starlings are socially but not always genetically monogamous. To determine whether a pair that obtained a nest box and incubated eggs was in fact the genetic parents (or true winners), we conducted a parentage analysis using five microsatellite loci previously developed for *Sturnus unicolor* (Celis et al. 2006) in Cervus 3.0 (Field Genetics, London, UK) (Kalinowski et al. 2007), a computer program that uses codominant genotypic data to test among multiple candidate parents of a given offspring. We considered a parent pair to be the genetic parents when offspring were assigned at the 'strict' confidence level (95%) and had no more than one allelic mismatch with the parental pair.

Radioimmunoassay
Testosterone and corticosterone can vary with stress and social status (Creel 2001; Goymann & Wingfield 2004; Rubenstein 2007; Wingfield et al. 1990). To better understand the GnIH and reproductive axis dynamic, and to determine if the time it took to capture and sacrifice each individual (capture time) or social status affected corticosterone and testosterone and their association with GnIH and GnRH-I content, we measured plasma testosterone and corticosterone in an RIA using the methods of Wingfield and Farner (1975), modified by Ball and Wingfield (1987). We were not able to assay for corticosterone during the beginning of the breeding season due to the low quantity of blood collected. Samples were assessed in duplicate and measured in a single RIA to avoid inter-assay variation.

In situ hybridization
To visualize cells labeled for GnIH mRNA (GnIH mRNA cell abundance), we performed ISH according to Ubuka et al. (2008). We produced a DIG-labeled antisense RNA probe using a standard RNA labeling kit (Roche Diagnostics) and used partial starting GnIH precursor cDNA as a template. After hybridization, brain sections were incubated with alkaline phosphatase-labeled sheep anti-DIG antibody (Roche Diagnostics, Indianapolis, IN, USA), and the immunoreactive product was visualized by immersing the sections in a substrate solution (nitro blue tetrazolium chloride and 4′,5′-diaminobenzidine as the chromogen). We conducted ICC to visualize cells labeled for the GnIH peptide (GnIH peptide cell abundance). Sections were washed five times in 0.2% PBS–T. Goat anti-rabbit affinity-purified GnIH primary antibody (code: PAC 123/124, Bentley, Berkeley, CA, USA) was used to incubate sections for 48 h at a concentration of 1:5000 in 0.2% PBS–T. Three subsequent washes in 0.2% PBS–T were followed by incubation in ABC (Vectorstain Elite Kit, Vector Labs, Burlingame, CA, USA) for 1 h and visualized using 0.03% 3,3′-diaminobenzidine as the chromogen.

Statistical analyses
Statistical analyses were performed using PASW v18 (formerly SPSS, Chicago, IL, USA). All data were tested for deviations from normality using a Shapiro–Wilk test and transformed when necessary to achieve normality at $P < 0.05$. We modeled the relationship between capture time and corticosterone concentrations using linear regression analysis. Because of the dependent nature of the variables studied, we conducted multivariate analysis of variance (MANOVA) to examine (1) the effect of capture time and corticosterone concentrations on GnIH and GnRH content, and testosterone concentrations and (2) the effect of the three seasonal collection times on GnIH and GnRH-I content. Rather than conduct a repeated measures design (because different birds were sampled at each collecting point and not the same birds throughout the study) and because of the multiple dependent variables involved, we conducted a MANOVA to examine the effect of nest box treatment on GnIH and GnRH-I peptide cell abundance, GnIH mRNA cell abundance, GnIH cell optical density, testosterone and corticosterone concentrations. Although our correlational analysis revealed that only some of the variables were correlated during the time of sampling (see Results section), we know all our variables of reproductive function are biologically correlated (as discussed in the Introduction section) and we thus chose to err on the side of caution and to conduct conservative MANOVAs to examine data and interactions.

Birds that outcompete others for nest boxes (winners) gain a reproductive opportunity and this behavioral outcome has adaptive implications. Therefore, in order to look for what differences between winners and losers may be present to promote future studies, we conducted univariate $F$ tests on variables within models that had a $P < 0.05$ and report statistically significant relationships at $P < 0.05$. 

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Goat anti-rabbit immunoglobulin G (1:250 in 0.2% PBS–T), followed by three more washes of 0.2% PBS–T. Sections were incubated in avidin–biotin complex (ABC, Vectorstain Elite Kit, Vector Labs, Burlingame, CA, USA) for 1 h and visualized using 0.03% 3,3′-diaminobenzidine as the chromogen.

We conducted ICC to visualize cells labeled for the GnIH peptide (GnIH peptide cell abundance). Sections were washed five times in 0.2% PBS–T. Goat anti-rabbit affinity-purified GnIH primary antibody (code: PAC 123/124, Bentley, Berkeley, CA, USA) was used to incubate sections for 48 h at a concentration of 1:5000 in 0.2% PBS–T. Three subsequent washes in 0.2% PBS–T were followed by incubation in ABC (Vectorstain Elite Kit, Vector Labs) for 1 h and visualized in Vector VIP (Vector Labs). This protocol has been used successfully in previous studies on this and other species (Ubuka et al. 2008, 2010).
Results

**Beginning of the breeding season**

The model examining the effect of nest box treatment on cells immunoreactive for the GnIH and GnRH-I peptide and GnIH mRNA, GnIH mRNA optical density and circulating testosterone plasma concentrations was not significant (Pillai’s trace $= 0.637$, $F_{5,4} = 1.403$, $P > 0.050$). However, visual inspection of the data revealed an apparent difference between GnIH peptide cell abundances from the different nest box treatments (Fig. 2). A post hoc analysis of these data suggested a difference in GnIH peptide cell abundances between treatments ($F_{1,8} = 1.191$, $P < 0.050$; Figs 2 and 3) in which winners had fewer cells immunoreactive for the GnIH peptide than losers. Because our original model incorporated numerous non-statistically significant variables, this particular relationship may have been masked. However, we wish to remain conservative in our interpretation and make it clear that a relationship may exist between GnIH peptide and winner status during this time. We discuss the implications of such a relationship in the Discussion section and call for further examination.

**Middle of the breeding season**

The model examining the effect of nest box treatment on cells expressing the GnIH and GnRH-I peptide cell abundance, GnIH mRNA cell abundance, GnIH mRNA cell optical density and circulating testosterone and corticosterone plasma concentrations was significant (Pillai’s trace $= 0.557$, $F_{6,19} = 3.981$, $P < 0.05$; post hoc univariate $F$ tests: GnIH peptide cell abundance: $F_{1,24} = 13.919$, $P < 0.05$, GnIH mRNA cell abundance: $F_{1,24} = 10.158$, $P < 0.05$, GnIH mRNA cell optical density: $F_{1,24} = 1.410$, $P < 0.05$; GnRH-I: $F_{1,24} = 22.652$, $P < 0.001$; Fig. 4). Gonadotropin inhibitory hormone peptide cell abundance did not differ between the beginning and the middle of the breeding season ($P = 0.08$), nor did average GnIH mRNA abundance between GnIH peptide cell abundance between treatments ($F_{1,16} = 11.617$, $P < 0.050$; Figs 2 and 3), in which winners had a greater GnIH peptide cell abundance than losers. Comparison between sampling periods revealed that winners during this time had a 100.1% increase in GnIH peptide cell abundance over losers at the beginning of the breeding season ($P < 0.01$).

**Non-breeding season**

Birds did not show a preference for a nest box and thus comparisons of neuroendocrine activity as a function of nest box status could not be undertaken for this sampling period.

**Neuroendocrine seasonal profiles**

Data collected from all birds were combined to establish a seasonal GnIH and GnRH-I profile. Cells expressing the peptide for GnIH and GnRH-I and GnIH mRNA were present at all sampling points. Although treatment effects of winner vs. loser on GnIH peptide cell abundance were apparent during the beginning and middle of the breeding season, as reported above, the average GnIH peptide cell abundance did not differ between seasons ($P = 0.104$; beginning of the breeding season average GnIH cell abundance ± standard deviation): $217.10 ± 113.32$, middle of the breeding season: $301.94 ± 71.66$), thus justifying the combination of winner vs. loser treatment groups to examine the general seasonal pattern of GnIH.

There was a seasonal effect on GnIH and GnRH-I content (Pillai’s trace $= 0.557$, $F_{6,19} = 3.981$, $P < 0.05$; post hoc univariate $F$ tests: GnIH peptide cell abundance: $F_{1,24} = 13.919$, $P < 0.05$, GnIH mRNA cell abundance: $F_{1,24} = 10.158$, $P < 0.05$, GnIH mRNA cell optical density: $F_{1,24} = 1.410$, $P < 0.05$; GnRH-I: $F_{1,24} = 22.652$, $P < 0.001$; Fig. 4). Gonadotropin inhibitory hormone peptide cell abundance did not differ between the beginning and the middle of the breeding season ($P = 0.08$), nor did average GnIH mRNA...
GnIH mRNA optical density were not different at the beginning of the breeding season and during the non-breeding season; were more abundant or had a higher GnIH mRNA cell optical density than during the beginning of the non-breeding season; were combined to increase sample size. During both sample points in the breeding season, (a) cells immunoreactive for GnIH peptide, GnIH mRNA and the optical density of GnIH mRNA cells and (b) cells immunoreactive for GnRH-I peptide were not significantly different within treatments and thus data were combined to increase sample size. Male and female cell numbers and optical density were not significantly different within treatments and thus data were combined to increase sample size. During both sample points in the breeding season, (a) cells immunoreactive for GnIH peptide, GnIH mRNA and the optical density of GnIH mRNA cells and (b) cells immunoreactive for GnRH-I peptide were more abundant or had a higher GnIH mRNA cell optical density, than during the beginning of the non-breeding season; however, GnIH peptide and GnIH mRNA cell abundance and GnIH mRNA optical density were not different at the beginning of the breeding season as compared to the beginning of the non-breeding season.

Figure 4: Seasonal distribution of (a) GnIH peptide and GnIH mRNA cell number and GnIH mRNA cell optical density and (b) GnRH-I peptide cell number. The average cell number and optical density and the standard error of all birds per treatment were plotted. Male and female cell numbers and optical density were not significantly different within treatments and thus data were combined to increase sample size. During both sample points in the breeding season, (a) cells immunoreactive for GnIH peptide, GnIH mRNA and the optical density of GnIH mRNA cells and (b) cells immunoreactive for GnRH-I peptide were more abundant or had a higher GnIH mRNA cell optical density, than during the beginning of the non-breeding season; however, GnIH peptide and GnIH mRNA cell abundance and GnIH mRNA optical density were not different at the beginning of the breeding season as compared to the beginning of the non-breeding season.

Table 2: Average testosterone and corticosterone (ng/ml) per season, sex and nest box treatment

<table>
<thead>
<tr>
<th></th>
<th>Beginning of the breeding season</th>
<th>Middle of the breeding season</th>
<th>Beginning of the non-breeding season</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nest</td>
<td>No nest</td>
<td>Nest</td>
</tr>
<tr>
<td>Testosterone</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>0.48 ± 0.01</td>
<td>0.87 ± 0.45</td>
<td>2.12 ± 1.08</td>
</tr>
<tr>
<td>F</td>
<td>1.20 ± 1.24</td>
<td>No females</td>
<td>0.33 ± 0.17</td>
</tr>
<tr>
<td>Corticosterone</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>Not collected</td>
<td></td>
<td>4.27 ± 2.02</td>
</tr>
<tr>
<td>F</td>
<td>4.83 ± 3.89</td>
<td></td>
<td>3.73 ± 2.31</td>
</tr>
</tbody>
</table>

Social and breeding status associated with GnIH mRNA cell abundance did not differ between the beginning of the breeding season and during the non-breeding season ($P = 0.28$ and $0.17$, respectively).

Parentage analysis of nest eggs
There was no instance of extra-pair paternity or intra-specific brood parasitism.

Testosterone and corticosterone concentrations
Recoveries were 52.02–75.96% and intra-assay variation was 1.36–5.42% for testosterone and corticosterone, respectively, and the assay detection limit was $\sim 0.1$ ng/ml. The average testosterone and corticosterone concentrations per season, sex and nest box treatment are reported in Table 2.

Birds were collected by mist net and hand net within 13.077 ± 6.603 min of us entering the aviary. Over the course of the year, prior to collection, birds had been habituated to people entering their aviary on a daily to weekly basis. At the time of collection, the peak average corticosterone concentration was below 5 ng/ml, suggesting that these concentrations may be indicative of basal, or non-stressed, circulating corticosterone concentrations. Rich and Romero (2005) have reported basal corticosterone levels of captive European starlings at about 5 ng/ml, with a peak of 30–40 ng/ml after restraint stress.

Effects of sex on GnIH, GnRH-I, testosterone and corticosterone
The model examining the effects of sex on GnIH and GnRH-I content, testosterone and corticosterone was significant (Pillai’s trace = 0.617, $F_{1.19} = 4.366$, $P < 0.01$). Univariate F tests revealed testosterone concentrations only differed between the sexes during the middle of the breeding season ($F_{1.25} = 6.151$, $P < 0.05$), with males having higher concentrations. However, no differences in GnIH or GnRH content or corticosterone concentrations were evident between the sexes, and thus data from males and females were combined to increase sample size. In this combined data set, plasma corticosterone concentration was positively correlated with capture time ($r = 0.63$, $P < 0.01$), negatively correlated with GnIH peptide cell abundance at the middle of the breeding season ($r = −0.679$, $P < 0.05$) and positively correlated with capture time ($r = 0.708$, $P < 0.01$).
correlated with GnIH mRNA cell abundance at the middle of the breeding season ($r = 0.670$, $P < 0.05$). To control the effects of capture time, residuals were taken from these relationships and used for further examination of the effects of the nest box treatment.

**Discussion**

Our goal was to investigate how differences in social and breeding status were associated with the recently discovered GnIH system. Our data show effects of social status, as measured by obtainment of a nest box (winner vs. loser), and breeding status on GnIH expression. We interpret data in the following parsimonious fashion: presence of the peptide is indicative of cells containing GnIH. An increase in GnIH mRNA cell abundance or cell optical density is indicative of an increase in GnIH production. A decrease in GnIH mRNA cell abundance or optical density is indicative of inhibition or a reduction of synthesis of GnIH, and we discuss the implications of such activity, including alternate interpretations.

**Beginning of the breeding season**

Gonadotropin inhibitory hormone peptide cell abundance appeared to be lower in birds that outcompeted others for a nest box (winners) vs. those that did not (losers), although GnRH-I peptide cell abundance and testosterone concentrations did not differ. The model we ran to explore the interrelationships between all variables and nest box treatment was not statistically significant, but a post hoc test revealed that GnIH peptide cell abundance was lower in winners, implicating further study of effects of social status on the GnIH system.

Although the number of GnIH peptide-containing cells may have lower in winners, GnRH-I peptide cell abundance, testosterone and corticosterone concentrations did not differ. This lack of change in GnRH-I and testosterone could suggest that (1) fluctuations in concentrations were not apparent at the time of sampling, (2) changes in GnIH content could result in (or be the result of) changes in action within the brain to directly affect behavior and (3) the animals’ endocrine reproductive capabilities remained intact, no matter the reproductive opportunity. Having a constantly functioning reproductive axis could lessen the time between the loss of a resource and the obtainment of a replacement. This would permit optimization of reproductive output, as nesting sites can be lost to predators and need to be re-established throughout the breeding season. An adaptive response to obtaining a nest box and mate would be to have little to no inhibition of the reproductive axis to allow for initiation of breeding. In support of this, males and females that obtained a nest box had fewer cells containing the GnIH peptide than birds that did not obtain a nest box. However, we do not know whether these differences seen in GnIH cell abundance occurred prior to or after nest box obtainment. If the differences were prior, birds with fewer GnIH cells and less inhibition to their reproductive axis may have been better competitors for nest boxes and mates, yielding the same data. Alternatively, differences in GnIH cell abundance may not be due to social environment at all, but to the breeding physiology associated with having (or not having) a nest box and thus, a chance to breed. More studies are needed to elucidate the direction of causality.

**Middle of the breeding season**

Gonadotropin inhibitory hormone peptide cell abundance was higher in winners, who at the time were laying and incubating eggs vs. (1) losers and (2) all birds (winners and losers) from the beginning of the breeding season. Only one reproductive pair was not laying/incubating eggs during this sampling period, although the female of this pair had yolky follicles, indicating she was about to lay. This pair had the smallest number of GnIH peptide cells compared to the other pairs laying/incubating eggs (as seen in Fig. 2). Corticosterone plasma concentrations did vary with capture time, but GnRH-I peptide cell abundance, testosterone and corticosterone concentrations did not differ between nest box treatments, again suggesting endocrine reproductive capabilities remained intact, and again implying a possible important function of GnIH within the brain, rather than on the HPG axis here.

The increase in the number of GnIH peptide cells in birds laying and incubating eggs may be related to the neural inhibition of aggressive and sexual behaviors during incubation. Testosterone in many birds, including European starlings (Pinxten et al. 2007), can decrease during a time of parental care to facilitate parental behaviors (Ketterson et al. 1996; Magrath & Komdeur 2003). European starling testosterone peaks during nest building, remains high during the fertile and incubation period, particularly if nesting sites are close together, and then decreases after nestlings hatch (Ball & Wingfield 1987; Pinxten et al. 2007). As in Pinxten et al. (2007), testosterone concentrations from our study remained high during the incubation period. Thus, our data imply that the change in GnIH (and lack of change in testosterone) may be related to function/action within the brain that facilitates the switch from aggressive and sexual behaviors to incubation behaviors. In support of this hypothesis, receptors for GnIH are expressed on two avian hypothalamic populations of GnRH cells: GnRH-I and GnRH-II (Ubuka et al. 2008). Gonadotropin-releasing hormone II is thought to play a role in sexual behaviors (Bentley et al. 2009; Kauffman & Risman 2004; Maney et al. 1997), and thus GnIH may be having an effect on behaviors during this time by influencing GnRH-II.

**Parentage analysis**

The results of parentage analyses show no evidence for extra-pair paternity (EPP) or intra-specific brood parasitism (IBP). While rates of IBP and EPP in natural populations reported in the literature vary, these are generally relatively low (EPP: 9–17% of offspring and 29–45% of nests; IBP: 0–27% of chicks and 0–45% of nests) and it has been suggested that these may reflect local ecological conditions (Loyau et al. 2005). Thus, it is possible that the reduced space and increased food availability lowered the energetic...
costs of nest and mate guarding leading to the absence of EPP and IBP, at least in the nests that were examined. The parentage results provide additional evidence that the differences we observed in GnIH content may impact the fitness of starlings. Comparative analyses have shown that neuroendocrine mechanisms are under selection to facilitate the timing of reproduction (reviewed in Adkins-Regan 2008; MacDougall-Shackleton et al. 2009). Our results suggest, at an intra-specific level, that GnIH regulation may influence the fitness of individual starlings. This finding paves the way for future studies examining fitness differences among individuals that differentially regulate the physiological mechanisms of reproduction.

**Neuroendocrine seasonal profiles**

The average GnIH peptide, GnIH mRNA cell abundance and GnIH cell optical density of all birds were higher during the breeding season as compared to the beginning of the non-breeding season. This may at first glance seem counter-intuitive (a reproductive inhibitory hormone increasing during the breeding season), but these data are consistent with other studies on songbirds (Bentley et al. 2009) and mammals (Tsutsui et al. 2010). An increase in cells containing and producing GnIH may be beneficial during the breeding season not to stop, but to modulate or ‘pause’ the reproductive axis in response to environmental conditions. Furthermore in agreement with the current study, GnIH cell number decreased after the end of the breeding cycle. Once birds enter the non-breeding life-history stage, there is no need for further regulation, and cells containing and producing GnIH become downregulated during the non-breeding season.

As with other studies on European starlings, our data show greater GnRH peptide cell abundance during the breeding vs. non-breeding season (Ball & Hahn 1997; Ubuka et al. 2009).

**Conclusions**

In this paper we report for the first time that social and breeding status are associated with the recently discovered GnIH system. By experimentally manipulating nesting opportunities in both male and female European starlings, we observed that birds which outcompeted others for nest boxes (winners) had significantly different numbers of GnIH peptide-producing cells than those without nest boxes (losers), and this relationship changed when birds were exhibiting incubating behaviors. We posit GnIH may play a key role in the switch from mating and aggressive behaviors to those of parental care and call for further studies.

Results from the parentage analysis revealed the social parents to be the genetic parents, and thus true ‘winners’ from an evolutionary standpoint. This finding suggests that differences in GnIH content may impact reproductive fitness, paving the way for studies examining the adaptive value, and thus evolution, of mechanisms underlying physiological preparedness for breeding. As GnIH presence and function appear to be conserved throughout all vertebrates studied, including humans, these results may have greater implications for reproductive physiology and behavior across vertebrate classes.

**References**


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