Genes, Brain and Behavior
Official publication of the International Behavioural and Neural Genetics Society

Genes, Brain and Behavior (2011) **10:** 557–564 doi: 10.1111/j.1601-183X.2011.00693.x

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

Received 1 February 2011, revised 16 March 2011, accepted for publication 30 March 2011

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 gonadotropins 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 aviaries at one time at (1) the beginning of the breeding season, after nest box acquisition, (2) during the middle of the breeding season, when egg 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).

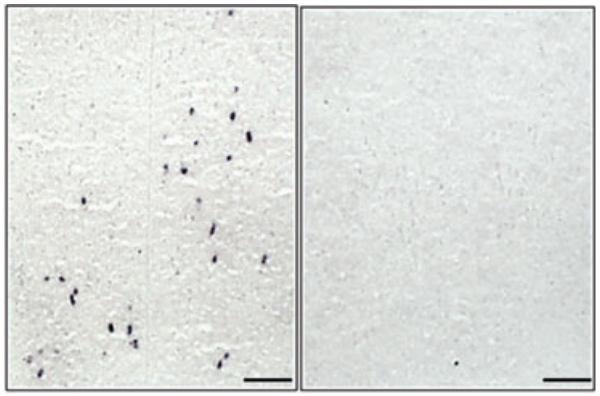

Figure 1: Representative photographs of GnIH mRNA positive cells (left) and negative controls (right) in the paraventricular nucleus of the hypothalamus demonstrate that non-specific binding did not occur. Scale bars are set at 100 μm.

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 ~0900–1300 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

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

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 starling 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 5-bromo-4-chloro-3-indolyl phosphate, toluidine salt). We controlled the specificity of the ISH using a DIG-labeled sense RNA probe, the sequence of which was complementary to that of the antisense probe (Fig. 1).

Immunocytochemistry

To better understand the dynamics of the GnIH system on the reproductive axis at the level of the brain, we conducted ICC to visualize cells labeled for GnRH-I peptide (GnRH-I peptide cell abundance). Sections were fixed in 4% paraformaldehyde for 1 h. Sections were then washed three times in phosphate buffered saline (PBS, 0.1 M) and treated with 0.01% hydrogen peroxide in PBS for 10 min to reduce background immunoreactivity. Sections were again washed three times with PBS and then submerged in 2% normal goat serum in 0.2% PBS + Triton X-100 (PBS–T) for 1 h to block background immunoreactivity. Gonadotropin-releasing hormone primary antibody (code HU60, generous gift from Henryk Urbanski, Portland, OR, USA) was used to incubate sections for 48 h at a concentration of 1:5000 in 0.2% PBS–T. Three more washes in 0.2% PBS–T were followed by 1 h of incubation in biotinylated 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; Vectastain 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 (Vectastain 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).

Quantification of GnIH and GnRH-I content

All hypothalamic GnIH peptide- and mRNA-immunoreactive (ir) and GnRH-I peptide-ir cells were counted using a Zeiss Axio Imager A1 microscope in a double-blind fashion in which an arbitrary number was assigned to each sample. We measured GnIH in three ways: (1) we counted the number of hypothalamic cells containing GnIH mRNA, indicating the number of cells producing the hormone (GnIH mRNA cell abundance), (2) we counted the number of hypothalamic cells immunoreactive for the GnIH peptide, indicating the number of cells containing the hormone (GnIH peptide cell abundance) and (3) we determined the optical density of the cells containing GnIH mRNA to measure the upregulation of the gene for the GnIH precursor (GnIH cell optical density). We assumed that increased optical density signified increased transcription and translation of the gene, but with the caveat that there was no way of telling whether all of the mRNA was translated into mature polypeptide and then cleaved to mature peptide. Gonadotropin inhibitory hormone cell optical density was measured using ImageJ software (U. S. National Institutes of Health, Bethesda, MD, USA) (Rasband, 1997–2009). An average pixelation score was taken from the 20 most darkly labeled cells per individual and subtracted from an average of equal background measurements taken from tissue surrounding cell areas.

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 their 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 $P < 0.050$ and report statistically significant relationships at $P < 0.050$.

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 = 0.650, $F_{6,10} = 3.101$, $P = 0.050$). *Post hoc* univariate F tests revealed that this outcome was due to a difference between GnIH peptide cell abundance between treatments ($F_{1,15} = 11.617$, $P < 0.050$; 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 ($P < 0.01$).

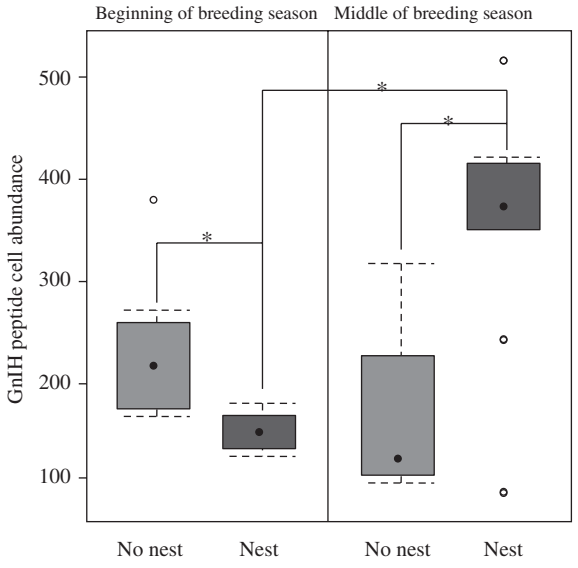

Figure 2: Nest box treatment and GnIH peptide cell abundance at the beginning and middle of the breeding season. All bird pairs with nests were laying or incubating eggs during the middle of the breeding season, except one pair, seen here as the two bottom outliers.

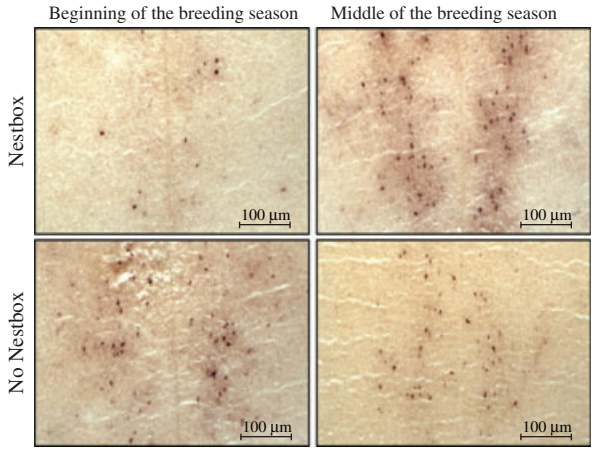

Figure 3: Gonadotropin inhibitory hormone peptide cell abundance and nest box treatment during the breeding season. Cells were visualized using Vector VIP, resulting in a brownish-purple label. Scale bars are set at 100 µm.

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

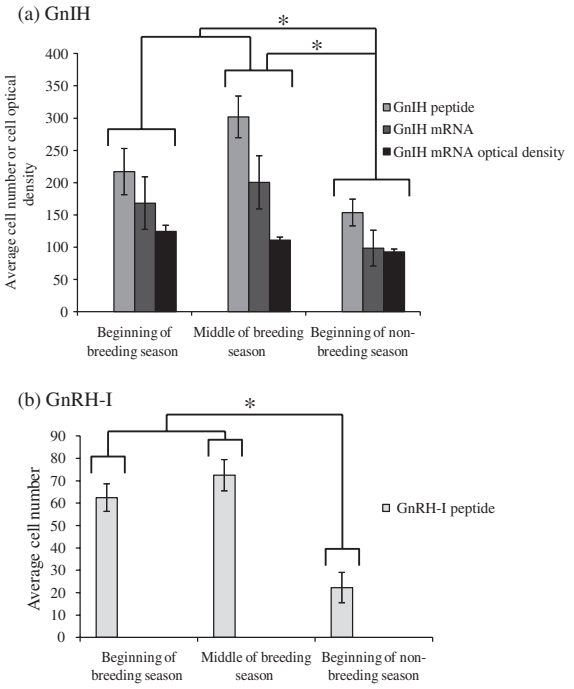

Figure 4: Seasonal distribution of (a) GnIH peptide and GnIH mRNA cell number and GnIH cell optical density and (b) GnRH-I peptide cell number. The average cell number and optical density and the standard error of all birds per treatment were plotted. Male and female cell numbers and optical density 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.

cell abundance ($P = 0.54$) or GnRH-I peptide cell abundance ($P = 0.33$). Gonadotropin inhibitory hormone peptide and 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 ~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, $F_{7,19} = 4.366$, $P < 0.01$). 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

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

	Beginning of the breeding season		Middle of the breeding season		Beginning of the non-breeding season
	Nest	No nest	Nest	No nest	No nest preferance
Testosterone					
M	0.48 ± 0.01	0.87 ± 0.45	2.12 ± 1.08	1.22 ± 0.00	0.19 ± 0.20
F	1.20 ± 1.24	No females	0.33 ± 0.17	0.21 ± 0.17	0.16 ± 0.10
Corticosterone					
M	Not collected		4.27 ± 2.02	4.63 ± 0.00	1.27 ± 0.55
F			4.83 ± 3.89	3.73 ± 2.31	3.00 ± 1.25

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 been 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 & Rissman 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

Adkins-Regan, E. (2005) *Hormones and Animal Social Behavior*. Princeton University Press, Princeton, New Jersey, USA.

Adkins-Regan, E. (2008) Do hormonal systems produce evolutionary inertia? *Philos Trans R Soc B Biol Sci* **363**, 1599–1609.

Aitken, K.E.H. & Martin, K. (2004) Nest cavity availability and selection in aspen–conifer groves in a grassland landscape. *Can J For Res* **34**, 2099–2109.

Ball, G.F. & Hahn, T.P. (1997) GnRH neuronal systems in birds and their relation to the control of seasonal reproduction. In Parhar, I.S. & Sakuma, Y. (eds), *GnRH Neurons: Gene to Behavior*. Shuppan Publishers, Tokyo, pp. 325–342.

Ball, G.F. & Wingfield, J.C. (1987) Changes in plasma levels of luteinizing hormone and sex steroid hormones in relation to multiple broodness and nest site density in male starlings. *Physiol Zoo* **60**, 191–199.

Bentley, G.E., Ubuka, T., McGuire, N.L., Calisi, R.M., Perfito, N., Kriegsfeld, L.J., Wingfield, J.C. & Tsutsui, K. (2009) Gonadotropin inhibitory hormone: a multifunctional neuropeptide. *J Neuroendocrinol* **21**, 276–281.

Burmeister, S.S., Jarvis, E.D. & Fernald, R.D. (2005) Rapid behavioral and genomic responses to social opportunity. *PLoS Biol* **3**, 1996–2004.

Calisi, R.M. & Bentley, G.E. (2009) Lab and field experiments: are they the same animal? *Horm Behav* **56**, 1–10.

Celis, P., Gil, D. & Graves, J.A. (2006) Isolation and characterization of polymorphic microsatellites isolated from the spotless starling (*Sturnus unicolor*) and cross-species amplification in the European starling (*Sturnus vulgaris*). *Mol Ecol Notes* **7**, 251–253.

Creel, S. (2001) Social dominance and stress hormones. *Trends Ecol Evol* **16**, 491–497.

Fox, H.E., White, S.A., Kao, M.H.F. & Fernald, R.D. (1997) Stress and dominance in a social fish. *J Neurosci* **17**, 6463–6469.

Francis, R.C., Soma, K.K. & Fernald, R.D. (1993) Social regulation of the brain-pituitary-gonadal axis. *Proc Natl Acad Sci U S A* **90**, 7794–7798.

Goymann, W. & Wingfield, J.C. (2004) Allostatic load, social status and stress hormones: the costs of social status matter. *Anim Behav* **67**, 591–602.

Kabelik, D., Morrison, J.A. & Goodson, J.L. (2010) Cryptic regulation of vasotocin neuronal activity but not anatomy by sex steroids and social stimuli in opportunistic desert finches. *Brain Behav Evol* **75**, 71–84.

Kalinowski, S.T., Taper, M.L. & Marshall, T.C. (2007) Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. *Mol Ecol* **16**, 1099–1006.

Kauffman, A.S. & Rissman, E.F. (2004) A critical role for the evolutionarily conservative gonadotropin-releasing hormone II: mediation of energy status and female sexual behavior. *Endocrinology* **145**, 3639–3646.

Ketterson, E.D., Nolan, V. Jr. Cawthorn, M.J., Parker, P.G. & Ziegenfus, C. (1996) Phenotypic engineering: using hormones to explore the mechanistic and functional bases of phenotypic variation in nature. *Ibis* **138**, 70–86.

Kriegsfeld, L.J., Mei, D.F., Bentley, G.E., Ubuka, T., Mason, A.A., Inoue, K., Ukena, K., Tsutsui, K. & Silver, R. (2006) Identification and characterization of a gonadotropin-inhibitory system in the brain of mammals. *Proc Natl Acad Sci U S A* **103**, 2410–2415.

Loyau, A., Moureau, B., Richard, M., Christe, P., Heeb, P. & Sorci, G. (2005) Cross-amplification of polymorphic microsatellites reveals extra-pair paternity and brood parasitism in *Sturnus vulgaris*. *Mol Ecol Notes* **5**, 135–139.

MacDougall-Shackleton, S.A., Stevenson, T.J., Watts, H.E., Pereyra, M.E. & Hahn, T.P. (2009) The evolution of photoperiod response systems and seasonal GnRH plasticity in birds. *Integr Comp Biol* **49**, 580–589.

Magrath, M.J.L. & Komdeur, J. (2003) Is male care compromised by additional mating opportunity? *Trends Ecol Evol* **18**, 424–430.

Maney, D.L., Richardson, R.D. & Wingfield, J.C. (1997) Central administration of chicken gonadotropin-releasing hormone-II enhances courtship behavior in a female sparrow. *Horm Behav* **32**, 11–18.

Newton, I. (1994) The role of nest sites in limiting the numbers of hole-nesting birds: a review. *Biol Conserv* **70**, 265–276.

Pinxten, R., de Ridder, E., Arckens, L., Darras, V.M. & Eens, M. (2007) Plasma testosterone levels of male European starlings (*Sturnus vulgaris*) during the breeding cycle and in relation to song and paternal care. *Behaviour* **144**, 393–410.

Pradhan, D.S., Newman, A.E.M., Wacker, D.W., Wingfield, J.C., Schlinger, B.A. & Soma, K.K. (2010) Aggressive interactions rapidly increase androgen synthesis in the brain during the non-breeding season. *Horm Behav* **57**, 381–389.

Remage-Healey, L., London, S.E. & Schlinger, B.A. (2010) Birdsong and the neural production of steroids. *J Chem Neuroanat* **39**, 72–81.

Rich, E.L. & Romero, L.M. (2005) Exposure to chronic stress downregulated corticosterone responses to acute stressors. *Am J Physiol Regul Integr Comp Physiol* **288**, R1628–R1636.

Rubenstein, D.R. (2007) Stress hormones and sociality: integrating social and environmental stressors. *Proc R Soc B* **274**, 967–975.

Soma, K.K., Scotti, M.A.L., Newman, A.E.M., Charlier, T.D. & Demas, G.E. (2008) Novel mechanisms for neuroendocrine regulation of aggression. *Front Neuroendocrinol* **29**, 476–489.

Tsutsui, K., Saigoh, E., Ukena, K., Teranishi, H., Fujisawa, Y., Kikuchi, M., Ishii, S. & Sharp, P.J. (2000) A novel avian hypothalamic neuropeptide inhibiting gonadotropin release. *Biochem Biophys Res Commun* **275**, 661–667.

Tsutsui, K., Bentley, G.E., Bedecarrays, G., Osugi, T., Ubuka, T. & Kriegsfeld, L.J. (2010) Gonadotropin inhibitory hormone (GnIH) and its control of central and peripheral reproductive function. *Front Neuroendocrinol* **31**, 284–295.

Ubuka, T., Cadigan, P.A., Wang, A., Liu, J. & Bentley, G.E. (2009) Identification of European starling GnRH-I precursor mRNA and its seasonal regulation. *Gen Comp Endocr* **162**, 301–306.

Ubuka, T., Kim, S., Huang, Y., Reid, J., Jiang, J., Osugi, T., Chowdhury, V.S., Tsutsui, K. & Bentley, G.E. (2008) Gonadotropin-inhibitory hormone neurons interact directly with gonadotropin-releasing hormone-I and -II neurons in European starling brain. *Endocrinology* **149**, 268–278.

Ubuka, T., Morgan, K., Pawson, A.J., Osufi, T., Chowdhury, V.S., Minakata, H., Tsutsui, K., Millar, R.P. & Bentley, G.E. (2010) Identification of human GnIH homologs, RFRP-1 and RFRP-3, and the cognate receptor, GPR147 in the human hypothalamic pituitary axis. *PLoS One* **4**, e8400.

Wingfield, J.C. & Farner, D.S. (1975) Determination of 5 steroids in avian plasma by radioimmunoassay and competitive protein binding. *Steroids* **26**, 311–327.

Wingfield, J.C., Hegner, R.E., Dufty, A.M. & Ball, G.F. (1990) The ''Challenge Hypothesis'': theoretical implications for patterns of testosterone secretion, mating systems, and breeding strategies. *Am Nat* **136**, 829–846.

Acknowledgments

We thank L. Kriegsfeld, E. Lacey, P. Lopes, N. McGuire, I. Moore, N. Perfito, D.R. Rubenstein and T. Ubuka for their thoughtful insight and discussion of this work. We also thank all the wonderful people from the Berkeley Field Station for Behavioral Research for their hard work and patience. This work was funded by NSF IOS 0641188 (to G.E.B.), NSF DBI 1003112 (to R.M.C.) and Ford Foundation and UC Berkeley Fellowships (to R.M.C. and S.L.D.M.).