Integrative and Comparative Biology Advance Access published June 14, 2015

Integrative and Comparative Biology

Integrative and Comparative Biology, pp. 1–11
doi:10.1093/icb/icv063

Society for Integrative and Comparative Biology

SYMPOSIUM

Measurements of Neuronal Soma Size and Estimated Peptide Concentrations in Addition to Cell Abundance Offer a Higher Resolution of Seasonal and Reproductive Influences of GnRH-I and GnIH in European Starlings

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From the symposium "Neurohormones, Brain, and Behavior: A Comparative Approach to Understand Rapid Neuroendocrine Function" presented at the annual meeting of the Society for Integrative and Comparative Biology, January 3–7, 2015 at West Palm Beach, Florida.

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Synopsis Hypothalamic neuropeptides involved in vertebrate reproduction, gonadotropin releasing hormone (GnRH-I) and gonadotropin-inhibitory hormone (GnIH), can vary in the abundance of immunoreactive cells as a function of the reproductive status and nest box occupation of European starlings (*Sturnus vulgaris*). While using the abundance of cells as an indicator of the activity of neurohormones is informative, incorporating information on cell size (readily observed using immunohistochemistry) can offer a more detailed understanding of environmentally-mediated changes in hormonal dynamics. In this study, we tested the hypothesis that the size of cells' somas and the estimated concentration of peptides in cells immunoreactive (ir) for GnRH-I and GnIH would vary throughout the breeding season and as a function of nest-box status (resident or not). In the absence of a direct assay of protein, we estimated an index of the concentration of hypothalamic peptides via the relative optical density (i.e., the difference between the mean optical density and the optical density of background staining). In support of our hypothesis, we found that GnRH-I- and GnIH-ir soma size and peptide concentration changed both in males and females throughout the breeding season. Somas were largest and estimated peptide concentration was highest mid-season when compared with earlier in the season or to the non-breeding period. For nest-box residents, GnIH-ir soma size and peptide concentration were higher during the middle of the breeding season than earlier in the breeding season, although residence in the nest box was not related to GnRH-I-ir variables. Our results confirm that previously reported changes in cell abundance mimic changes we see in GnRH-I and GnIH-ir soma size and our proxy for peptide concentration. However, investigating changes in the soma of GnRH-I-ir cells revealed a peak in size during the middle of the breeding season, a change not evident when solely examining data on the abundance of cells. We also report that GnRH-I- and GnIH-ir soma size and our proxy for peptide concentration positively co-varied with each other and, in males, were positively correlated with testosterone. In summary, we offer a higher resolution of understanding of the function of GnRH-I and GnIH during the breeding period of European starlings.

Introduction

A symphony of endocrine events must occur to mediate reproduction at a time that best increases the chances of the offspring surviving. Predictable annual changes in resources create selection pressures for organisms to engage in reproductive activities when resources are most abundant. One powerful environmental cue for seasonally breeding animals in temperate zones is day length.

European starlings (*Sturnus vulgaris*) are photoperiodic, seasonally breeding songbirds that time their reproductive activity in accordance with day length. Winter in the northern hemisphere is characterized by short periods of daylight during which European

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starlings experience a state of ''photosensitivity''. At this time, their hypothalamic–pituitary–gonadal (HPG) axis is primed for activation by an increase in day length. As spring approaches and day length increases, a threshold is reached that activates the HPG axis, resulting in birds becoming ''photostimulated''. The preoptic neurons in the hypothalamus produce and secrete gonadotropin-releasing hormone (GnRH) to the median eminence, causing the pituitary gland to secrete gonadotropins, luteinizing hormone (LH), and follicle stimulating hormone (FSH). LH and FSH travel through the bloodstream and act on the gonads, stimulating gametogenesis and the secretion of sex steroids, such as testosterone (T). The production of sex steroids then acts on the HPG axis to create a feedback system that facilitates sexual behavior and reproduction. Late in the summer, when day length is still long, the HPG axis stops responding to long photoperiods and becomes ''photorefractory''. These photoperiod-dependent changes are seen as an adaptive response, because as autumn and winter approach fewer resources will be available compared with the spring, such that producing offspring at this time may put European starling chicks and their parents at a survival disadvantage (McGuire et al. 2010). After birds experience a certain amount of time in a photorefractory state, they become photosensitive again, their HPG axis primed for activation by the increasing day length of the following spring.

In 2000, Tsutsui et al. discovered gonadotropin-inhibitory hormone (GnIH) in the hypothalamus of quail and reported that it inhibited the release of LH from the pituitary gland. Since its initial discovery, GnIH has been reported to have an inhibitory effect on GnRH, LH secretion, and sexual behavior in numerous vertebrates (Calisi 2014). However, its precise role in reproduction, or the inhibition of it, remains unclear. For example, despite its inhibitory effect, the number of cells that contain, or are immunoreactive for, the GnIH protein (GnIH-ir) increases along with the number of GnRH-I-ir cells during the breeding season of European starlings (Calisi et al. 2011). This phenomenon suggests that GnIH potentially serves as a regulatory, rather than purely inhibitory, mechanism on reproduction and associated behaviors (Calisi et al. 2008, 2011; Calisi 2014).

European starlings have demonstrated changes in the abundance of GnIH-ir cells during the breeding season that are associated with social and ecological changes. Calisi et al. (2011) found that at the beginning of the breeding season, males and females that had acquired nest boxes and, thus, had a chance to breed (confirmed by analyses of genetic paternity) had no change in the number of GnRH-I-ir cells compared with those individuals that were outcompeted for nests. However, the winners of nest boxes had fewer GnIH-ir cells than did those without nest boxes (Calisi et al. 2011). Less inhibition by GnIH on the reproductive axis would potentially be adaptive for birds that have acquired the resources necessary to breed successfully, unlike those that remain without a nest box. This situation mimics natural conditions, in which starlings are obligate cavity dwellers and require a pre-made cavity to nest. During the middle of the breeding season, when those birds with nest boxes had begun to incubate eggs, both males and females exhibited a significantly increased abundance of GnIH-ir cells (Calisi et al. 2011). This, too, may be an adaptive response if the increase in GnIH plays a role in decreasing testosterone and in particular sexual behaviors that can interfere with parental care. The number of GnRH-I-ir cells, although more abundant during the breeding period than during the non-breeding period, was not associated with nest-box status (whether a bird had acquired a nest box and thus a chance to breed or not) or incubation status (Calisi et al. 2011).

While the quantity of GnRH-I-ir and GnIH-ir cells has been characterized over key periods of the European starling's breeding season (Calisi et al. 2011), it remains unclear whether cell counts can be equated with the amount of GnRH-I and GnIH being produced. Further immunohistochemistry analysis enables quantification of other indicators of neuronal activation, function, and response to the environment, including the actual size of the cells producing the substrate of interest (hereafter referred to as soma size) and peptide concentration (Davis and Fernald 1990; White and Fernald 1993; Charlier et al. 2008). Evaluating soma size and peptide concentration alongside the numbers of cells (Calisi et al. 2011) may provide a more robust understanding of how hormones such as GnRH-I and GnIH function during reproductive activity. In this study, we improve our understanding of the dynamics of GnRH-I and GnIH and their association with reproductive activities by examining seasonal changes in GnIH-ir and GnRH-I-ir soma size and peptide concentration as a function of nest-box status as well as in association with circulating testosterone. We undertake analyses on European starlings during the photostimulated period (early and mid-breeding season) and photorefractory period (non-breeding season).

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Materials and methods

Housing and the manipulation of nest boxes

The data collected for this study were derived from animals sampled in a previous study (Calisi et al. 2011), in which birds were examined for changes in the abundance of GnRH-I-ir and GnIH-ir cells. To summarize, 39 juvenile European starlings (22 males and 17 females) were caught in the wild in areas surrounding Lodi, CA. These juveniles were randomly assigned to one of four large outdoor aviaries at the University of California, Berkeley's Field Station for the Study of Behavior, Ecology, and Reproduction. The large outdoor aviaries ($12 \times 6 \times 3.5$ m) exposed European starlings to natural light and weather. The aviaries' natural ground was probed by starlings for invertebrates, with chick feed supplemented *ad libitum*. The birds displayed a wide range of behaviors naturally associated with changes in seasonality, such as singing, territorial disputes, nest building, incubating, breeding, and molting (Calisi and Bentley 2009). In all four aviaries, a set number of nest boxes—fewer than the number of pairs in order to create competition—were put in place at the beginning of the breeding season (first week of February). The sample sizes reported in Table 1 of Calisi et al. (2011) are as follows: Beginning of the breeding season (three nest boxes available): 7M, 3F, 5 obtained nest boxes, 5 did not. Middle of the breeding season (six nest boxes available): 7M, 10F, 12 obtained nest boxes, 5 did not, 5 of the pairs (of the six possible pairs) were incubating eggs at this time. The female of the sixth pair had yolky follicles, indicating she was about to lay. Nonbreeding season (three nest boxes available): 8M, 4F, birds showed no preference for a nest box at this time.

Behavioral observations

Behavioral observations were conducted between 0900 h and 1300 h for the 5 days immediately after the nest boxes were placed in the aviaries, and for the 5 days prior to sampling to determine the inhabitants of nest boxes. In addition, periodic observations were conducted between these time points. During the beginning and middle of the breeding season, resident status at a nest box was assigned to the male–female pair that visited a nest box more than 95% of the time (Calisi et al. 2011). Birds that obtained nest boxes at the beginning of the breeding season maintained them through the middle of the season (those that did not obtain a nest box early did not subsequently obtain one). Birds sampled at the beginning of the photorefractory, non-breeding period had no preference for particular nest boxes.

Sampling of birds

A detailed description of animal care, the sampling of birds, and sampling and processing of tissues can be found in Calisi et al. (2011). In brief, the birds in whole aviaries were sampled at one time to control for possible confounding factors, such as day length and effects from changes in social structure. Sampling occurred in the four aviaries at different times of the breeding season: (i) sampling was carried out in one aviary at the beginning of the breeding season (the first week of February as birds begin to acquire nests); (ii) in two adjacent aviaries at the middle of the breeding season (toward the end of April when laying and egg incubation occur); and (iii) and in one aviary during the photorefractory, non-breeding period (post-molt at the end of September when the reproductive axis becomes attenuated). This sampling design was necessary, as individual birds could not be repeatedly sampled due to the need to sacrifice the animal to extract the brain for further processing and analysis.

On sampling days, birds in the aviaries were caught by mist net or by hand net, and immediately sacrificed by terminal anesthesia with isoflurane followed by immediate decapitation. Their brains were dissected out and frozen on dry ice before being transferred to a $-80\,^{\circ}$C freezer prior to sectioning. Subsequently, approximately 1–2 mL of blood from the trunk was collected and centrifuged and the plasma extracted and frozen for radioimmunoassay (RIA) of plasma testosterone (as reported previously by Calisi et al. 2011). Coronal sections of the brain (sliced at 20 µm thickness) were collected throughout the hypothalamus, using a cryostat, and mounted directly on silane-coated slides. Brains were cut in a series of eight and each eighth section stained via immunocytochemistry (Calisi et al. 2011). All procedures were performed in accordance with, and with the approval of, the University of California Office of Laboratory Animal Care and federal regulations.

Immunohistochemical staining

Sections of the brain were fixed in 4% paraformaldehyde for 1 h, followed by three washes in 0.1 M phosphate buffered saline (PBS) and subsequently treated in 0.01% hydrogen peroxide in 0.1 M PBS for 10 min to minimize background immunoreactivity. Next, sections were washed three times with 0.1 M PBS and placed in 2% normal goat serum in 0.2% PBS + Triton X-100 (PBS-T) for 1 h to block

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background immunoreactivity. Sections were incubated in GnRH primary antibody for 48 h at a concentration of 1:5000 in 0.2% PBS-T. The sections were then washed three times in 0.2% PBS-T followed by incubation in biotinylated goat anti-rabbit immunoglobulin G (1:250 in 0.2% PBS-T) for 1 h, followed by another three washes in 0.2% PBS-T. Sections were then incubated in avidin–biotin complex (ABC; Vectastain Elite Kit, Vector Labs, Burlingame, CA, USA) for 1 h. Sections were visualized using 0.03% 3,3′-diaminobenzidine.

To visualize cells labeled for GnRH-I and GnIH peptide, sections were washed five times in 0.2% PBS-T. Slices were incubated in goat anti-rabbit affinity-purified GnIH primary antibody for 48 h at a concentration of 0.2% PBS-T, followed by three washes in 0.2% PBS-T. Sections were then incubated in ABC (Vectastain Elite Kit, Vector Labs, Burlingame, CA, USA) for 1 h. Sections were visualized using Vector VIP (Vector Labs).

Measuring soma size of GnRH-I-ir and GnIH-ir cells

Image analysis of the hypothalamic cells containing GnRH-I-ir and GnIH-ir was conducted as a double-blind experiment, in which an arbitrary number was assigned to each sample. Cells were photographed at ×20 magnification using a Zeiss Axio Imager A1 microscope and Image Pro software (Media Cybernetics, Silver Springs, MD, USA). Images were stitched together to form a composite image of slices of the whole brain, using a photomerge function in Adobe Photoshop CS5 (Adobe Systems Incorporated, San Jose, CA, USA). These images where then analyzed for soma size and optical density in ImageJ.

The surface areas (in pixels) of all immunoreactive cells were measured manually using the magic wand tool in ImageJ. Similar software in METAVUE was used by Kelm et al. (2011) and Riters et al. (2013). The tolerance level was set to 30 to minimize the measurement of any background staining without compromising the measurement of soma size. To prevent measuring cells more than once, a black dot was placed next to each neuron measured. All measurements of surface area were saved automatically on an ImageJ spreadsheet along with the mean optical density.

The relative optical density was defined as the difference between the mean optical density and the optical density of the background staining (Charlier et al. 2008). A circle, 300-px in diameter, was created using the magic-wand tool to obtain an average of the measurements of the optical density of the entire area of the cell. Additionally, measurements of the background staining were collected on the right side of each slice, lateral to the PVN. To estimate the relative concentration of peptide within the hypothalamus, the difference between the cell of interest and background staining was multiplied by the size of the soma and the number of cells.

Measuring testosterone

This study used previously reported data collected from these same birds and reported by Calisi et al. (2011). To summarize, plasma testosterone was measured by RIA (Wingfield and Farner 1975; modified by Ball and Wingfield 1987). Samples were assayed in duplicate and measured in a single RIA to avoid inter-assay variation.

Statistical analyses

We performed all analyses using RStudio, version 3.1.3 (2014). We built analysis of variance (ANOVA) models to explore the predictor value of sex, season, and nest-box status, and their interactions, on (1) GnRH-I-ir soma size, (2) GnRH-I-ir estimated peptide concentration, (3) GnIH-ir soma size, and (4) GnIH-ir estimated peptide concentration. We set alpha at 0.05 and conducted Tukey's honest significant difference (HSD) tests on relationships below the threshold of significance.

We conducted Pearson's product-moment correlation (Pearson's r) analyses to determine whether GnRH-I and GnIH variables were correlated. They were, and we conducted independent linear regression analyses to examine whether male and female GnRH-I and GnIH variables are predicted by the concentrations of circulating testosterone. Because males circulate higher concentrations of testosterone than do females, we also examined the sexes separately.

Results

We found that GnRH-I-ir and GnIH-ir soma size and estimated peptide concentrations were predicted by season (GnRH-I-ir soma size: $F_{2,31} = 182.810$, $P < 0.001$; GnIH-I soma size: $F_{2,31} = 13.170$, $P < 0.001$; GnRH-I-ir peptide concentration: $F_{2,31} = 14.886$, $P < 0.001$; GnIH-I peptide concentration: $F_{2,31} = 13.002$, $P < 0.001$). These results and those that were above the threshold of statistical significance are reported in Table 1. We discovered a significant interaction effect between season and nest-box status predicting GnRH-I-ir soma size ($F_{1,31} = 0.023$, $P = 0.010$) and GnIH-ir peptide concentration ($F_{1,31} = 8.938$, $P = 0.006$). Results from

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Table 1 The relationships of sex, season, and nest-box status, and their interactions, on GnRH-I-ir soma size and estimated peptide concentration (a) and GnIH-ir soma size and estimated peptide concentration (b) as tested by four analyses of variance and subsequent Tukey HSD *post-hoc* results

(a)

	Soma size			Peptide		
GnRH-I	***F***	**df**	***P***	***F***	**df**	***P***
ANOVA						
Sex	1.611	1, 31	0.214	0.864	1, 31	0.360
Season	182.81	2, 31	<**0.001**	14.886	2, 31	<**0.001**
Nest box status	0.023	1, 31	0.880	1.521	1, 31	0.227
Sex × season	2.99	2, 31	0.065	0.033	2, 31	0.967
Sex × nest box status	7.616	1, 31	0.881	0.002	1, 31	0.968
Season × nest box status	0.023	1, 31	**0.010**	0.325	1, 31	0.573
Tukey's HSD						
Season						
Early-mid			<**0.001**			0.076
Mid-nonbreeding			<**0.001**			<**0.001**
Nonbreeding-early			<**0.001**			**0.032**
Season × nest box status						
Early, nest box-mid, nest box			<**0.001**			
Early, no nest box-mid, no nest box			**0.005**			
Early, nest box-early, no nest box			0.947			
Mid, nest box-mid, no nest box			0.978			

(b)

	Soma size			Peptide		
GnIH	***F***	**df**	***P***	***F***	**df**	***P***
ANOVA						
Sex	0.279	1, 31	0.601	0.209	1, 31	0.651
Season	13.17	2, 31	<**0.001**	13.002	2, 31	<**0.001**
Nest box status	0.035	1, 31	0.854	4.609	1, 31	**0.040**
Sex × season	0.392	2, 31	0.679	0.884	2, 31	0.424
Sex × nest box status	2.447	1, 31	0.128	1.117	1, 31	0.299
Season × nest box status	0.073	1, 31	0.789	8.938	1, 31	**0.006**
Tukey's HSD						
Season						
Early-mid			**0.001**			**0.012**
Mid-nonbreeding			<**0.001**			<**0.001**
Nonbreeding-early			0.976			0.384
Nest box status						0.099
Season × nest box status						
Early, nest box-mid, nest box						**0.018**
Early, no nest box-mid, no nest box						0.999
Early, nest box-early, no nest box						0.997
Mid, nest box-mid, no nest box						**0.046**

Note: Bold values indicate statistically significant relationships.

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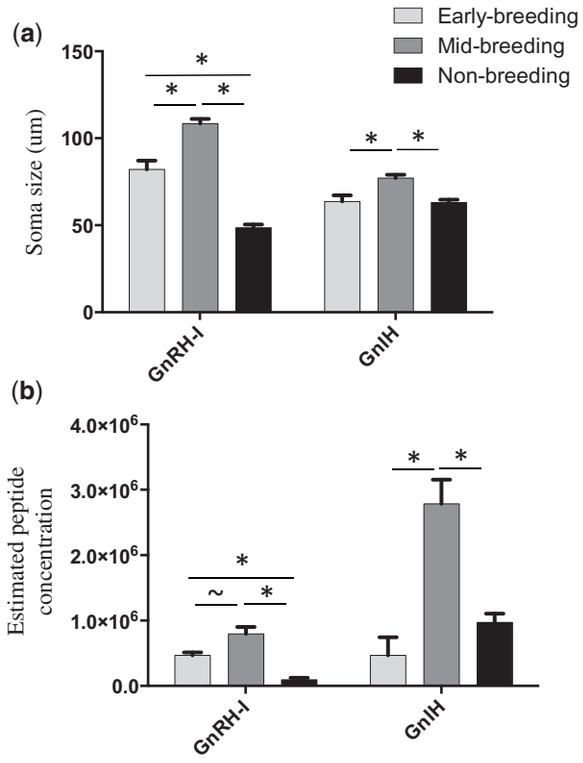

Fig. 1 GnRH-I-ir and GnIH-ir soma size (**a**) and estimated peptide concentration (**b**) across two breeding periods and one non-breeding period. Asterisks denote significant differences at alpha = 0.05, and a tilde denotes a relationship of 0.076; column bars represent standard errors of the mean.

our *post-hoc* tests for these variables are below. We found no differences between the sexes in GnRH-I-ir and GnIH-ir soma size and estimated peptide concentrations, and data were combined to visualize the relationships depicted in Figs. 1–3.

GnRH-I-ir changes with season but not with nest-box status

GnRH-I-ir soma size was largest and peptide concentration was highest during the middle of the breeding season when compared with the non-breeding season ($P<0.001$; Fig. 1). Both values were also larger earlier in the breeding season when compared with the non-breeding season (soma size: $P<0.001$, peptide concentration: $P=0.032$). GnRH-I-ir soma size was larger in the middle of the breeding season when compared with earlier that season ($P<0.001$) although we did not find such a strong relationship when examining GnRH-I-ir peptide concentration at these time points ($P=0.076$).

The combined effect of season and nest-box status on GnRH-I-ir revealed that birds that obtained nest boxes had larger neuronal somas during the middle of the breeding season when compared with earlier ($P<0.001$; Fig. 2). However, birds without nest boxes at this time also experienced an increase in the size of neuronal somas during the middle of the breeding season when compared with earlier as well ($P=0.005$), thereby negating the effect of nest-box status on GnRH-ir soma size.

Changes in GnIH-ir with season and with nest-box status

Like GnRH-I-ir soma size and peptide concentration, GnIH-ir soma size and peptide concentration were largest/highest during the middle of the breeding season when compared with the non-breeding season ($P<0.001$; Fig. 1). Both values also increased in the middle of the breeding season when compared with earlier that season (GnIH-ir soma size: $P=0.001$, peptide concentration: $P=0.012$).

The combined effect of season and nest-box status on GnIH-ir revealed that birds that obtained nest boxes had higher peptide concentrations during the middle of the breeding season when compared with earlier ($P=0.018$; Fig. 2). Birds with nest boxes during the middle of the breeding season also had higher peptide concentrations than those without nest boxes collected at the same time ($P=0.046$).

GnRH-I-ir and GnIH-ir are positively correlated

GnRH-I-ir and GnIH-ir soma sizes and estimated peptide concentrations were positively correlated (Table 2, Fig. 3). Soma size and estimated peptide concentrations were positively correlated (GnRH-I-ir: $r=0.713$, $P<0.001$; GnIH-ir: $r=0.605$, $P<0.001$). GnRH-I-ir and GnIH-ir soma sizes and GnRH-I-ir and GnIH-ir estimated peptide concentrations were also positively correlated ($r=0.653$, $P<0.001$, $r=0.406$, $P=0.011$).

GnRH-I-ir and GnIH-ir are positively related to testosterone

Circulating concentrations of testosterone were related to GnRH-I-ir and GnIH-ir soma sizes and peptide concentrations (Table 3). Testosterone data were reported previously in Table 2 of Calisi et al. (2011), and assays had a recovery rate of 52.02%, an intra-assay variation of 1.36%, and a detection limit of approximately 0.1 ng/mL. Testosterone was positively related to GnRH-I-ir soma size ($R^2=0.202$, $P=0.005$) and peptide concentration ($R^2=0.113$, $P=0.039$; Table 3(a)). Testosterone was also positively related to GnIH-ir soma size ($R^2=0.108$, $P=0.040$) and estimated peptide concentration

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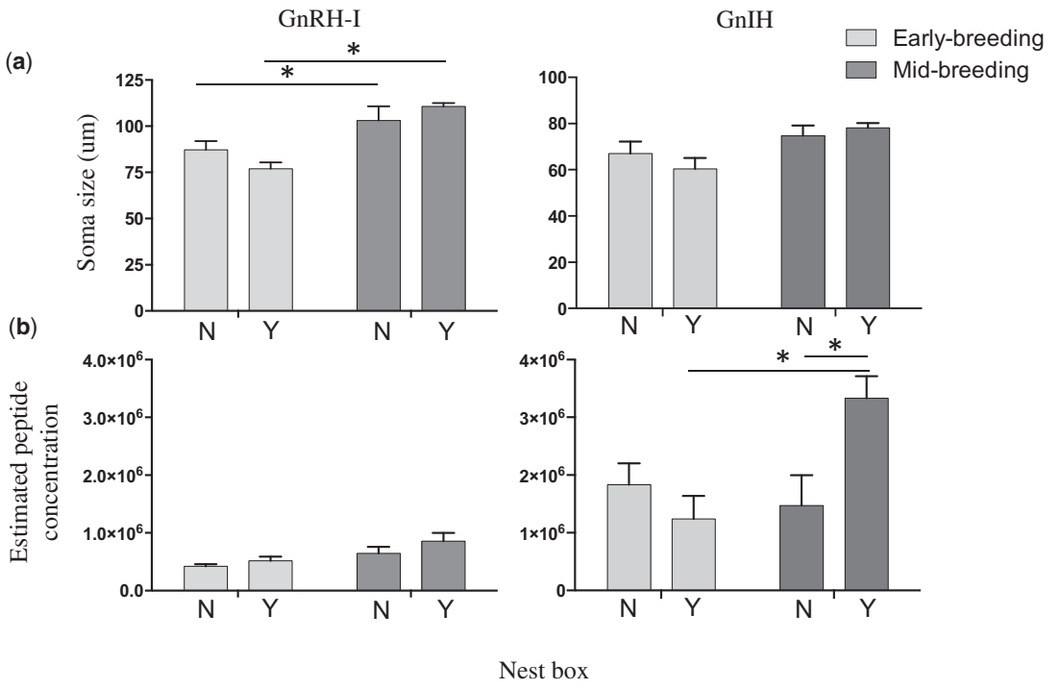

Fig. 2 GnRH-I-ir (left) and GnIH-ir (right) soma size (**a**) and estimated peptide concentration (**b**) in relation to the successful acquisition of a nest box (N = Not acquired, Y = Yes, acquired). Asterisks denote significant relationships; column bars represent standard errors of the mean.

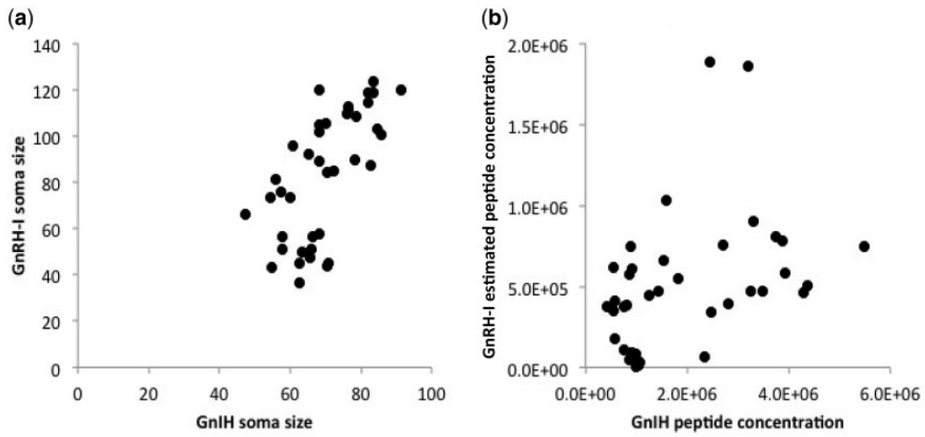

Fig. 3 Scatterplot depicting the relationship of GnRH-I-ir and GnIH-ir soma size (**a**) and GnRH-I-ir and GnIH-ir estimated peptide concentration (**b**).

($R^2 = 0.207$, $P = 0.004$; Table 3(a)). When we analyzed the sexes separately, we discovered that testosterone in males, but not in females (results for females in Table 3(c)), was related to GnRH-I-ir and GnIH-ir soma sizes and estimated peptide concentrations. Males' testosterone was positively related to GnRH-I-ir soma size ($R^2 = 0.556$, $P < 0.001$) and estimated peptide concentration ($R^2 = 0.282$, $P = 0.013$; Table 3(b), Fig. 4a). Testosterone was also positively related to GnIH-ir

Table 2 Correlation analyses examining whether GnRH-I-ir and GnIH-ir variables co-vary

	T	df	*r*	*P*
Pearson's *r* correlation				
GnRH-GnIH soma size	5.245	1, 37	0.653	<**0.001**
GnRH-GnIH peptide concentration	2.669	1, 37	0.406	**0.011**
GnRH soma-GnRH peptide	6.097	1, 37	0.713	<**0.001**
GnIH soma-GnIH peptide	4.618	1, 37	0.605	<**0.001**

Note: Bold values indicate statistically significant relationships.

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Table 3 Linear regression analyses examining the relationship of GnRH-I-ir and GnIH-ir with testosterone in males and females combined (a), in males only (b), and in females only (c)

(a)					
Testosterone—M&F	***F***	**df**	R^2	**Adjusted R^2**	***P***
Linear regression					
GnRH soma size	9.100	1, 37	0.202	0.180	**0.005**
GnRH peptide concentration	4.573	1, 37	0.113	0.088	**0.039**
GnIH soma size	4.465	1, 37	0.108	0.084	**0.040**
GnIH peptide concentration	9.667	1, 37	0.207	0.186	**0.004**
(b)					
Testosterone—males	***F***	**df**	R^2	**Adjusted R^2**	***P***
Linear regression					
GnRH soma size	23.830	1, 20	0.556	0.533	**<0.001**
GnRH peptide concentration	7.468	1, 20	0.282	0.244	**0.013**
GnIH soma size	15.600	1, 20	0.438	0.410	**<0.001**
GnIH peptide concentration	28.550	1, 20	0.588	0.568	**<0.001**
(c)					
Linear regression					
GnRH soma size	0.055	1, 15	0.004	−0.063	0.819
GnRH peptide concentration	0.478	1, 15	0.031	−0.034	0.500
GnIH soma size	0.089	1, 15	0.122	0.064	0.169
GnIH peptide concentration	0.010	1, 15	0.001	−0.067	0.921

Note: Bold values indicate statistically significant relationships.

soma size ($R^2 = 0.438$, $P < 0.001$) and estimated peptide concentration ($R^2 = 0.588$, $P < 0.001$; Table 3(b), Fig. 4b).

Discussion

This study provides a deeper understanding of the dynamics of cells that produce GnRH-I- and GnIH in European starlings, complementing and augmenting early work by Calisi et al. (2011). Specifically, we examined how the size of these cells and the amount of peptide they may be producing relate to seasonality, an individuals' nest-box status (a resource necessary for successful breeding), and the level of circulating testosterone. We report that male and female GnRH-I-ir and GnIH-ir soma size and peptide concentration change with breeding season. In some cases, these changes were dependent on an individual's nest-box status. We also found that GnRH-I-ir and GnIH-ir soma size and estimated peptide concentration positively co-varied, and an increase in soma size and peptide concentration was related to an increase in the level of circulating testosterone. These findings, coupled with previous knowledge of changes in the abundance of these cells during the starlings' breeding season (Calisi et al. 2011) provide a more detailed understanding of the activity of GnRH-I and GnIH cells in this species.

Breeding season

GnRH-I-ir and GnIH-ir soma size and peptide concentration were associated with the stage of the breeding season in which birds were sampled. Specifically, soma size and peptide concentration of both variables were at their highest during the middle of the breeding season when compared with earlier in the season and to the non-breeding season. Calisi et al. (2011) also found that the number of GnIH-ir cells were more abundant at this time when compared with earlier in the breeding season and during the non-breeding season. However, they found that cells producing GnRH-I, while in greatest abundance during the breeding season, did not differ in number between the early and middle parts of the breeding season. Here, we discovered that a measurement of GnRH-I-ir does indeed change between these time points, and GnRH-I-ir soma sizes are larger during the middle of the breeding season when compared with earlier in this period.

GnRH-I is an important stimulator of reproduction and associated reproductive behaviors. Thus, the peak in the activity of this compound during the middle of the breeding season might reflect that individuals are in full breeding condition, whereas earlier they are just becoming photostimulated and commencing reproductive activity.

Calisi et al. (2011) reported that GnIH-ir cell abundance, mRNA expression, and the optical density of that expression, were higher during the breeding period than during the non-breeding period, and peaked during the middle of the breeding period. Here, we report that GnIH-ir soma size and peptide concentration follow the same pattern (higher during the breeding season than during the non-breeding period, peaking during the middle of the breeding period). While an increase in GnIH during heightened reproductive activity may at first seem counterintuitive, evidence suggests that GnIH may be an important modulator of reproductive function (Calisi 2014; Tsutsui et al. 2013; Ubuka et al. 2014). This conjecture would explain why we see GnRH-I and GnIH rise and fall together.

Previously, Parry et al. (1997) investigated the abundance of GnRH-I cells and their precursor (proGnRH-GAP cells) as well as soma size during

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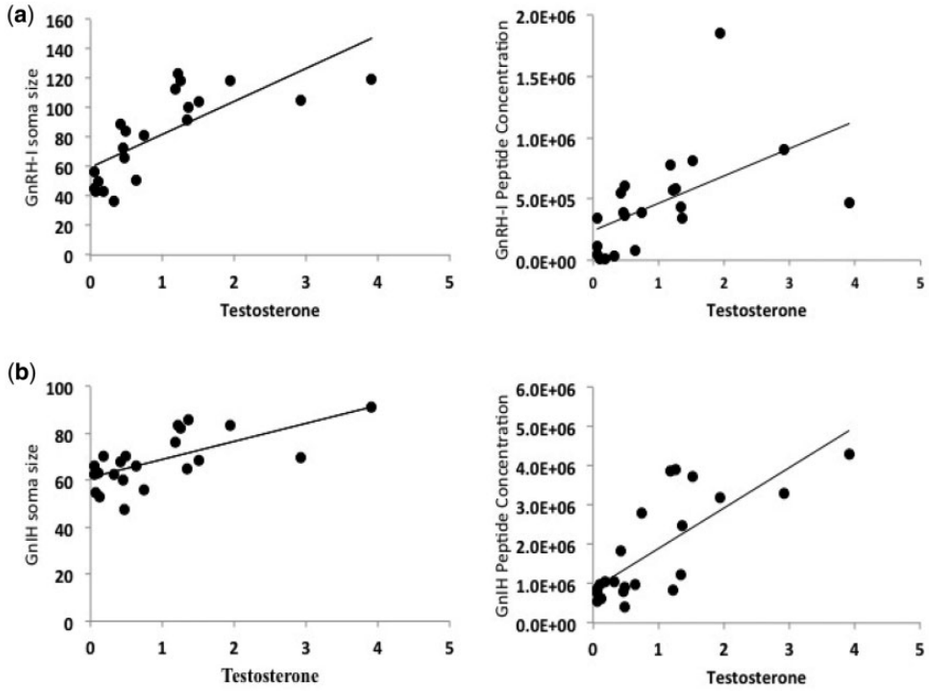

Fig. 4 The relationship of GnRH-I-ir soma size and peptide concentration (**a**) and GnIH-ir soma size and peptide concentration (**b**) with the concentration of circulating testosterone.

artificial, photoperiodically-induced stages of the reproductive cycle in captive, male European starlings. GnRH-I cells, as well as its precursor, increased in number and size during the induced photostimulated period, compared with the induced photorefractory period. In our study, and that of Calisi et al. (2011), both sexes were examined and housed outdoors under natural conditions of light, and similar results were obtained. This pairing of results from the laboratory (Parry et al. 1997) and from a semi-natural environment (this study and Calisi et al. 2011) are reinforcing, increasing our confidence in the relationships yielded and thus our understanding of the dynamics of the GnRH-I system in European starlings.

Nest-box status

At the beginning of the breeding period, starlings compete for nest cavities and for mates. As nesting cavities are created by other organisms or events, cavities are considered to be a limiting resource for reproduction. Establishing these nest sites fuel breeding activities (Kessel 1957; Feare 1984; Gwinner et al. 2002). We have observed both male and female starlings engaging in aggressive behaviors over such resources. To recreate this competition in our semi-natural environments, we limited the number of nest boxes available in each aviary. Competition ensued with clear winners of nest boxes and, consequently winners of mates, being observed. This allowed us to examine the relationship of these events to the dynamics of GnRH-I and GnIH.

European starlings that occupy next boxes show dominant behaviors, such as displacement of other birds and increased singing (Riters et al. 2000), that are related to changes in the brain, such as increased immunolabeling of androgen receptors (Cordes et al. 2014). In several species of fish, GnRH-I soma size has been reported to change in response to social status (*Haplochromis burtoni*, Fernald 2002; *Astatotilapia burtoni*, Maruska and Fernald 2013 and Maruska 2014; *Amatitlania nigrofasciatus*, Nesjan et al. 2014). Hence, given these results for fish and the observations on starlings, we expected that the winners of nest boxes would have larger GnRH-I-ir somas. We found no support for this prediction. Indeed, like Calisi et al. (2011), we found no relationship between nest-box status and any measure used to assess GnRH-I activity. GnRH-I-ir soma size in the owners of nest boxes was higher during the middle of the breeding season than earlier in the season, but this was also true for birds without nest boxes. Thus, nest-box status in this species may not be a factor influencing GnRH-I-ir soma size.

Previously, Calisi et al. (2011) reported that GnIH-ir cells were less abundant in birds that had

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secured nest boxes at the beginning of the breeding season than in those that had not. They suggested that less inhibition to the HPG axis by GnIH at the time of acquiring a nest might facilitate reproductive behaviors in various ways (Calisi et al. 2011). In this study, we did not find a relationship between ownership of a next box and either GnIH-ir soma size or peptide concentration early in the breeding season. However, by mid-season when owners were in full breeding condition and incubating eggs, there was a significant increase in GnIH-ir estimated peptide concentration among the owners of next boxes when compared with owners earlier in the season and those without nest boxes collected at the same time. Calisi et al. (2011) reported remarkably similar changes in GnIH-ir abundance. As birds with nest boxes were incubating eggs at this time, it is possible that an increase in inhibition of the HPG axis could help facilitate a behavioral transition to parental care.

Testosterone

GnRH-I-ir and GnIH-ir soma size and peptide concentration were positively correlated with the level of circulating testosterone. However, our R^2 values were rather low (between 0.113 and 0.207). Although we did not observe differences between the sexes in GnRH-I-ir and GnIH-ir variables measured during different times or in relation to nest-box status, male European starlings in general have higher concentrations of circulating testosterone compared with females. Thus, we conducted separate linear regression analyses and discovered that only testosterone in males, but not in females, is positively related to GnRH-I-ir and GnIH-ir soma size and peptide concentration (R^2 values between 0.282 and 0.588).

An increase of GnRH-I activity may lead to, or result from, the activation of sex steroids, like testosterone. However, GnIH has been shown to inhibit such processes and associated behaviors (Bentley et al. 2009; Calisi 2014). As testosterone can facilitate male socio-sexual behaviors, it is perplexing that GnIH would exhibit a positive relationship with testosterone. It is possible that there is finer-scale regulation of this process, such that greater activity of GnRH-I and testosterone requires greater potential for inhibition by GnIH, resulting in the parallel rise and fall of both neural substrates. It is plausible that the direct activation and inhibition of reproductive function does not occur solely via these neurohormones. Indeed, studies are revealing that this system is far more complex than originally thought (Calisi 2014; Tsutsui and Ubuka 2014).

Summary

An important concern in the field of behavioral neuroendocrinology is whether the abundance of cells is an accurate representation of the activity of cells. The neuronal snapshot we can view by using the popular technique of immunohistochemistry permits not only a measurement of cells' abundance, but also measurements of cells' soma size and optical density. Here, we report that changes in the abundance of GnRH-I and GnIH cells throughout the breeding season (Calisi et al. 2011), in general, mirror changes in neuronal soma size and estimated peptide concentration. In this case, the use of the abundance of neuronal cells appears to be a dependable metric that we can use to measure changes in neuroendocrine activity in association with the environment. However, investigating changes in the soma of GnRH-I-ir cells revealed a peak in size during the middle of the breeding season, a change not evident when examining only data on the abundance of cells. The activation of neurohormones and the modulation of reproduction are complex, and an increased resolution characterizing neurohormones that mediate reproductive function is possible using immunohistochemistry. With this single assay, we can obtain multiple informative metrics of assessment that have the potential to enhance our understanding of environmentally-mediated changes in hormonal dynamics.

Acknowledgments

The authors thank George Bentley for use of his laboratory facilities and Sam Díaz-Muñoz for his thoughtful discussion of this work.

Funding

This work was supported by the University of California President's Postdoctoral Fellowship Program [to R.M.C].

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