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Variation in the Gonadotrophin-releasing hormone-1 and the song control system in the tropical breeding rufous-collared sparrow (*Zonotrichia capensis*) is dependent on sex and reproductive state

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Abstract

Seasonal breeding in temperate zone vertebrates is characterised by pronounced variation in both central and peripheral reproductive physiology as well as behaviour. In contrast, many tropical species have a comparatively longer and less of a seasonal pattern of breeding than their temperate zone counterparts. These extended, more “flexible” reproductive periods may be associated with a lesser degree of annual variation in reproductive physiology. Here we investigated variation in the neuroendocrine control of reproduction in relation to the changes in the neural song control system in a tropical breeding songbird the rufous-collared sparrows (*Zonotrichia capensis*). Using *in situ* hybridization, we show that the optical density of GnRH1 mRNA expression is relatively constant across pre-breeding and breeding states. However, males were found to have significantly greater expression compared to females regardless of breeding state. Both males and females showed marked variation in measures of peripheral reproductive physiology with greater gonadal volumes and concentrations of sex steroids in the blood (i.e. testosterone in males; estrogen in females) during the breeding season as compared to the pre-breeding season. These findings suggest that the environmental cues regulating breeding in a tropical breeding bird ultimately exert their effects on physiology at the level of the median eminence and regulate the release of GnRH1. In addition, histological analysis of the song control system HVC, RA and Area X revealed that breeding males had significantly larger volumes of these brain nuclei as compared to non-breeding males, breeding females, and non-breeding females. Females did not exhibit a significant difference in the size of song control regions across breeding states. Together, these data show a marked sex difference in the extent to which there is breeding-associated variation in reproductive physiology and brain plasticity that is dependent on the reproductive state in a tropical breeding songbird.

Keywords

LHRH; GnRH1; median eminence; reproduction; songbird; HPG-axis; HVC

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INTRODUCTION

Seasonally breeding animals at mid to high latitudes rely on a number of environmental cues to successfully time reproduction (1-3). In most temperate zone species, the annual change in photoperiod can be viewed as an initial predictive cue that drives changes in the neuroendocrine system needed to regulate reproduction (4-8). Following initial predictive cues, supplementary cues, such as rainfall, temperature, and food availability are integrated to fine-tune the timing of breeding to match variation in the local environment (9). In environments where day length does not accurately predict the onset of good breeding conditions, for example in much of the tropics, organisms must rely on other cues that predict variation in local conditions to coordinate the activation of reproductive physiology and behaviour (10-13). The neural integration of local environment cues that signal suitable breeding periods in the tropics is not well understood.

A key neuropeptide that governs peripheral reproductive endocrine physiology is the gonadotrophin-releasing hormone 1 (GnRH1) in the preoptic area (POA) in avian species (see 14 for a review). GnRH1 acts on the anterior pituitary to increase the production of the gonadotrophins follicle-stimulating hormone (FSH) and luteinising hormone (LH) which in turn stimulate gonadal recrudescence and the synthesis of gonadal steroids (e.g. testosterone and estradiol), respectively (15, 16). In many avian species, hypothalamic GnRH1 content is far greater during the breeding season than during the non-breeding season (14). The recent cloning of complementary DNA for GnRH1 in songbirds revealed that GnRH1 mRNA also exhibits marked changes in expression across the reproductive cycle (17, 18). Phylogenetic comparisons of avian species suggest that plasticity in the GnRH1 system has evolved to facilitate different degrees of flexibility in timing reproduction in response to environmental cues (19,20). In highly seasonal species, variation in photoperiodic state is associated with marked seasonal changes in brain GnRH1 while in flexible species GnRH1 seems to be present in high concentrations throughout the year (21). Avian species of the genus *Zonotrichia* provide a valuable opportunity to study the variation in the neuroendocrine control of reproductive physiology and behavior due in part to the large degree of latitudinal distribution and reproductive phenology (22). In captive white-crowned sparrows (*Zonotrichia leucophrys*) the photoperiodic control of reproduction appears to regulate the release of GnRH1 but not the variation in the amount of GnRH1 protein (23). However, rufous-collared sparrows inhabiting the tropics were observed to have significantly larger GnRH1 cell sizes, but not the number of cells, during the breeding season compared to the non-breeding season (24). To date the amount of *gnrh1* mRNA in *Zonotrichia* has not been examined and given the increase in the size of immunoreactive GnRH1 cells in rufous-collared sparrows suggests that *gnrh1* mRNA expression may vary in association with local environmental cues.

The seasonal change in the neuroendocrine control of breeding is essential for the accompanied changes in singing behaviour and associated neural plasticity (25-32). Songbirds have a series of discrete interconnected nuclei that are collectively referred to as the song control system (SCS; 25, 33-35). HVC (an acronym used as a proper name) is the primary sensorimotor nucleus that sends afferent projections to the premotor nucleus RA (robust nucleus of the arcopallium). HVC also projects to the nucleus Area X that plays a role during the learning and maintenance of song structure (33, 36, 37). In many temperate songbird species, several song control nuclei exhibit dramatic changes in volume that are associated with breeding states (27, 28). Male rufous-collared sparrows engage in singing behaviour at high rates during the breeding season but such songs are conspicuously absent during the non-breeding season (38). The SCS in male rufous-collared sparrows show extensive plasticity that is tied to breeding state that varies as a function of the local environment the birds occupy (38). There are no reports of females engaging in singing

behaviour in this species. Several studies have shown that the SCS in females of some songbird species exhibit a seasonal change in volume (39-43) while the SCS in females of other species do not show changes (44, 45). The change in SCS volume is generally assumed to coincide with the variation in sing rates; however, some female's exhibit marked changes in SCS volumes that are not associated with song production rates (39-43). It is currently unknown whether the SCS in female rufous-collared sparrows changes in association with breeding state.

This paper examined the *gnrh1* mRNA expression and SCS in male and female rufous-collared sparrows. In order to investigate whether the change in gonadal state associated with local environmental cues is reflected in the amount of *gnrh1* mRNA expression, we collected birds during pre-breeding and breeding periods. Furthermore, given that some female *Zonotrichia* show marked SCS plasticity, we sought to determine whether female rufous-collared sparrows exhibit an increase in SCS volume during the breeding period similar to their male counterparts. The data presented herein highlight the importance of considering sex differences when investigating the neural integration of environmental cues (46).

METHODS

Subjects

The rufous-collared sparrow is a common species found between sea level and 4000 m from southern Mexico to Tierra del Fuego (47). We caught adult male and female sparrows from the population in and around Papallacta, Napo Province, Ecuador (0°22.3'S, 78°8.2'W, 3,300 m elevation) during the prebreeding season (9-11 July, 2009; n= 5 males and 5 females) and the breeding season (18 September – 4 October, 2009; n= 6 males and 6 females). The timing of breeding in Rufous-crowned sparrows is dependent on the local environmental conditions and different population's exhibit asynchronous breeding periods (48). However, the GnRH1 system has been reported to be synchronous with regard to reproductive state, and therefore only one study population is necessary to study the specific nature of the change, if any, in the GnRH1 system (24). The terms "prebreeding" and "breeding" states were selected based on similar physiological and behavioral characteristics observed in photosensitive and photostimulated temperate songbirds (6, 14). The criteria used to classify breeding state included gonadal development, time of year and singing behaviors. Prebreeding birds had regressed gonads typical of the time of year caught (48) and males were not observed to sing and females did not exhibit any evidence of a brood patch. Breeding birds were observed to have developed gonads; males engaged in singing behavior and females had developed brood patches.

Capture and Assessment of Peripheral Reproductive Physiology

Birds were captured passively in mist nets at dawn. Within 3 min of capture a 250 µl blood sample was obtained from a wing vein and stored on ice until processing. Within 5 min of capture the birds were terminally anaesthetised with an intramuscular injection of 7.5 mg sodium pentobarbital and perfused transcardially with 0.9% heparinised saline (150 IU/10 ml) followed by 10% neutral buffered formalin. Birds were weighed and the length of the cloacal protuberance (CP) was measured after administration of sodium pentobarbital but prior to perfusion. A longer CP provides a reliable indication of prolonged exposure to elevated levels of testosterone (49) and CP size is correlated positively with testes size in this species (48). After perfusion, the brains and gonads were extracted within 10 minutes. The diameter of the largest follicle on the ovaries and the testis diameter and length were measured to 0.01mm using digital calipers. Brains were post-fixed in 10% formalin, and were stored under refrigeration until delivery to Johns Hopkins University. Within 5 hours

of collection, blood samples were centrifuged and the plasma separated and frozen. Average testis volume was calculated for each male using the formula for an ellipsoid and the average diameter and average length of both testes.

Hormone Assay

Concentrations of testosterone and estradiol were measured by standard radioimmunoassay techniques following extraction and chromatographic separation (50, 51). For individual extraction efficiency determination, we equilibrated each sample overnight with 2,000 cpm of tritiated steroid. Each sample was extracted with 5ul of distilled dichloromethane with the dichloromethane phase removed and dried in a warm water bath, under a stream of nitrogen gas, and resuspended in 10% ethyl acetate in isooctane. To remove neutral lipids and to isolate testosterone and estradiol, all samples were transferred to diatomaceous earth (Celite, Sigma) columns for chromatographic separation. Neutral lipids and other steroids were eluted with increasing concentrations of ethyl acetate in isooctane. After appropriate fractions were collected they were dried in a 40° C water bath under nitrogen gas, resuspended in 600 ul phosphate buffered saline, and maintained overnight at 4° C. Individual extraction efficiency for each steroid (mean recoveries were 79% for testosterone and 64% for estradiol) was determined from 100 ul of the sample while 200 ul of the sample was allocated to each of two duplicates for the assay. Serial dilutions for the standard curves were performed in triplicate (range of curves: testosterone and estradiol, 500 – 1 pg). All samples were then incubated overnight with 100 ul of antiserum (testosterone: T-3003, Wien Laboratories, Succasunna, NJ 07876) and 100 ul of tritiated steroid. Unbound steroid was separated using dextran-coated charcoal and the bound steroid decanted into scintillation vials. Samples were counted on a liquid scintillation counter and final concentrations corrected for individual extraction efficiency. Average intra-assay coefficients of variation (CV) were 5% for testosterone and 3% for estradiol.

In situ hybridization

Once the brains arrived at Johns Hopkins University they were saturated in 30% sucrose solution before freezing on dry ice. Brains were subsequently sectioned at 30µm into 3 series. For the song control nuclei all sections were placed in cryoprotectant. For GnRH1, two series were placed in 4% paraformaldehyde for *in situ* hybridization and the third series was placed in cryoprotectant. A plasmid containing starling GnRH1 cDNA (GenBank Acc. #FJ178434) corresponding to the decapeptide, GnRH1 associated peptide (GAP), and the 3' UTR (approx. 350 bp) was linearised with *SacI* (sense; Promega) or *NcoI* (antisense; Promega) and purified using GENE-CLEAN (Qbiogene, Vista, CA). S³⁵ labeled cRNA probes (PerkinElmer Life and Analytical Sciences, Boston, MA) were transcribed by SP6 (antisense) or T7 (sense) RNA polymerases using a MAXIScript kit (Ambion, Austin, TX). Unincorporated nucleotides were separated with NucAway spin columns (Ambion). Probe quality was verified by gel electrophoresis and probe specific activity was determined by a scintillation counter.

Free-floating sections were washed in glycine (0.79g/100ml 0.1M phosphate buffer saline; PBS; Ambion) twice for 3 minutes followed by a single wash in 0.1M PBS for 15 minutes at room temperature (RT). Next, sections were incubated in Proteinase K (1µl/mL; Invitrogen) for 30 minutes at 37°C. The tissue was neutralised in acetic anhydride solution (50mL H₂O; 650 µl triethanolamine [TEA; Sigma, St. Louis, MO] pH 8.0; 160 µl glacial acetic acid; 125 µl acetic anhydride [Sigma]) for 10 minutes at RT. The sections were then washed twice for 15 minutes each in 2X sodium chloride-sodium citrate (SSC; Ambion) at RT. Sections were incubated in radio-labeled riboprobe solution [2.5×10^5 cpm/ml diluted in 1X hybridization buffer (Sigma) with 0.01M dithiothreitol (Sigma)], overnight in humidified chambers at 65°C. The next morning, the sections were washed twice in 4X SSC for thirty minutes at

60°C. The sections were then incubated in 1:1 mixture of 4X SSC and formamide (Sigma) for thirty minutes at 60°C. Sections were washed twice for ten minutes in an RNase buffer solution at RT before incubating in an RNase solution for thirty minutes at 37°C. The sections were then washed twice for twenty minutes each in 2X, 0.5X and 0.1X SSC at 60°C. To visualise hybridization signal, slides were placed in light-proof cassettes and exposed to Kodak Biomax MR film for 72 h at room temperature. The film was developed using D19 developer and Kodak Fixer (VWR Scientific).

Quantification of GnRH1 mRNA expression

The intensity of GnRH1 mRNA signal in autoradiograms was evaluated by measuring the optical density (OD). The distribution of GnRH1 mRNA in the songbird brain shows a dorsal-caudal progression in the POA (17, 52) and two anatomical landmarks (i.e. tractus septomesencephalicus [TSM] and the anterior commissure) flank the rostral and caudal boundaries. With the use of these two anatomical landmarks we can reliably delineate the distribution of GnRH1 cells. Digital images were captured from autoradiograms that ranged rostrally from the TSM split moving caudally until the posterior portion of the anterior commissure using a high resolution CCD Sierra Scientific camera and digitised with Alchemy TV DVR 2.5.1. A standard curve was computed using ImageJ (NIH) software from known amounts of C^{14} standards and the mean grey value for each control section was generated. The OD was determined for each section collected throughout the POA. For each region of interest, the hybridization signal was clearly discernible and consisted of an increase in OD several times greater than the background. The signal was circled and the mean grey value determined for each hemisphere. The OD value was corrected for background by subtracting the optical density from the mesopallium regions in the same section. Then, the OD for each area was estimated from the standard curve and summed across the two hemispheres and this value provided the value for OD intensity. Finally, the total OD was calculated multiplying each section OD value by the GnRH1 signal area and summed across all brain sections.

Statistical Analysis

All statistical analyses were conducted using SigmaStat 11.0 and graphs plotted with SigmaPlot. For measures of body mass we conducted a one-way ANOVA. Cloacal protuberance, testosterone, oestradiol, testicular volume and follicle length were analyzed with Student's T-test. Due to the large variability in testosterone and oestradiol concentrations, we conducted a log transformation on the raw values so as not to violate the assumption of homoscedasticity. The GnRH1 mRNA OD and the SCS were analyzed with two factor (sex by breeding state) ANOVA and statistical significance was evaluated at $p < 0.05$.

RESULTS

Assessment of peripheral physiology

There was no significant main effect of sex ($F(1,17) = 0.05$, $p = 0.48$) or breeding state ($F(1,17) = 1.06$, $p = 0.32$) or interaction ($F(1,17) = 0.25$, $p = 0.62$) for body mass (Figure 1A). A t-test revealed that breeding male sparrows had significantly greater CP lengths compared to pre-breeding males ($t(9) = 5.90$, $p < 0.001$; Figure 1B). There was a statistically non-significant trend for breeding females to have greater CP length compared to pre-breeding females ($t(8) = 0.22$, $p = 0.07$). We found that breeding male sparrows had significantly greater testosterone concentrations compared to pre-breeding males ($t(9) = 2.87$, $p < 0.05$; Figure 1C). There was a non-significant trend for breeding females to have greater estradiol concentrations compared to pre-breeding female sparrows ($t(9) = 1.84$, $p = 0.09$; Figure 1C). Breeding males had significantly greater testicular volumes compared to

pre-breeding males ($t(9) = 9.94$, $p < 0.001$; Figure 1D). Furthermore, breeding females had significantly greater follicles compared to pre-breeding females ($t(9) = 2.33$, $p < 0.05$; Figure 1D)

Assessment of GnRH1 mRNA expression

A two-way ANOVA revealed a non-significant trend for males to have greater GnRH1 mRNA OD intensity levels compared to females ($F(1,17) = 3.43$, $p < 0.08$; Figure 2B). There was no overall significant difference between breeding states ($F(1,17) = 0.01$, $p = 0.92$), nor a significant interaction ($F(1,17) = 0.39$, $p = 0.54$). We did find a significant difference in total GnRH1 mRNA OD between males and females ($F(1,17) = 5.09$, $p < 0.05$; Figure 2C). But there was no significant difference between breeding states ($F(1,17) = 0.04$, $p = 0.83$) nor a significant interaction ($F(1,17) = 1.23$, $p = 0.28$).

Assessment of variation in the song control system

A two-way ANOVA revealed that males had significantly greater volumes of Area X compared to females ($F(1,17) = 21.27$, $p < 0.001$; Figure 3B) and there was a significant interaction with males having larger volumes during the breeding season ($F(1,17) = 8.98$, $p < 0.01$). There was no significant effect of breeding state ($F(1,17) = 2.94$, $p = 0.11$).

A two-way ANOVA revealed a significant difference in HVC volumes with greater volumes in males compared to females ($F(1,17) = 30.82$, $p < 0.001$; Figure 3C); there were greater volumes in the breeding compared to pre-breeding states ($F(1,17) = 6.74$, $p < 0.05$) and a significant interaction ($F(1,17) = 9.46$, $p < 0.01$).

A two-way ANOVA revealed a significant difference in RA volumes with great volumes in males compared to females ($F(1,17) = 17.48$, $p < 0.001$; Figure 3D). There was a non-significant trend for the factor for reproductive state ($F(1,17) = 4.25$, $p = 0.05$). There was no significant sex by reproductive state interaction ($F(1,17) = 2.51$, $p = 0.13$).

DISCUSSION

Here, we show that the seasonal change in breeding state in a tropical bird is characterised by marked variation in gonadal volume and gonadal steroids, but with no appreciable change in GnRH1 mRNA expression. Interestingly, we found that males exhibited greater GnRH1 mRNA expression compared to female sparrows. To our knowledge, these findings are the first to report a sex difference in the amount of GnRH1 mRNA in any avian species. This study also confirmed earlier findings that many brain regions involved in song production (i.e. HVC, Area X, and RA) show marked changes in male sparrows with larger volumes occurring during the breeding season. However, our new results show that females do not exhibit a change in SCS volumes associated with breeding state and were consistently observed to have significantly smaller volumes than males.

Environmental regulation of GnRH1

Several avian species in the order Passeriformes have evolved extensive plasticity in the GnRH1 system in response to photoperiodic cues (20, 53). Our findings of an absence of variation between prebreeding and breeding GnRH1 mRNA levels in a tropical *Zonotrichia* species, combined with an earlier study showing larger GnRH neuron size but not number (24), suggest that the integration of environmental cues that regulate reproduction act to control the release of GnRH1 from the median eminence. However, male rufous-collared sparrows were observed to have larger immunoreactive GnRH1 during in breeding compared to non-breeding birds (24). There are a number of factors that could be associated with the increase in GnRH1 cell size including greater metabolic demands, intracellular

trafficking and transcriptional/post-translational modifications. Indeed the present study does not have the resolution to determine whether individual GnRH1 mRNA expressing cells increase in size. Given that the amount of *gnrh1* mRNA appears to be constitutively expressed and is similar to previous reports on the GnRH1 protein content (48), suggest then that the change in GnRH1 cell sizes is most likely a result of other factors not associated with GnRH1 mRNA and/or protein synthesis. It remains possible that rufous-collared sparrows exhibit GnRH1 plasticity in response to supplementary cues, for example social cues, the timing of reproduction is more highly synchronised within monogamous rufous-collared sparrows than among the population as a whole (48). Given that GnRH1 cell numbers increase in males when paired with females in both European starlings (*Sturnus vulgaris*; 54) and ring dove (*Streptopelia risoria*; 55), it remains possible that the GnRH1 system in rufous-collared sparrows would respond to social cues with changes in GnRH1 mRNA or protein.

Sex differences in GnRH1 expression

It is well established that environmental signals are integrated differently in males and female in avian species (e.g., 8, 46). Males can readily attain full reproductive development in response to photic cues, whereas females generally require additional supplementary cues (46). Here we report a sex difference in the amount of GnRH1 mRNA expression regardless of breeding state. In the opportunistically breeding white-winged crossbill, males do not show significant changes in GnRH1 protein content across the year whereas females show an increase during periods associated with increased food availability (44). Indeed females are generally more sensitive to supplementary factors compare to males (44). The lower levels of GnRH1 mRNA expression observed in female rufous-collared sparrows may be attributable to the time in which birds were collected, the absence/presence of appropriate environmental cues and/or sex differences in the acute response to capture.

Variation in SCS is dependent on breeding state

Variation in song control nuclei in temperate breeding bird species has been well described (see 26-32 for reviews). It is generally accepted that the increase in testosterone facilitates the photoinduced growth of many song control regions (32). It was originally shown that the non-migratory male Nuttall's white-crowned sparrows (*Z. leucophrys nuttalli*) failed to exhibit seasonal changes in song control regions (44). However, there were a number of methodological confounds (e.g. low testosterone concentrations) that may have contributed to the lack of neuroplasticity. Subsequent studies conducted in male Nuttall's and Gambel's white-crowned sparrows (*Z. l. gambelii*) revealed volumetric variation in song control brain regions that were associated with testosterone levels (56, 57). The findings presented here support those previously shown in male rufous-collared sparrows (24) and provide further evidence for marked variation in SCS volumes that are tied to circulating levels of testosterone in *Zonotrichia*. We also show that the volumes of many SCS in females are significantly smaller than males and unlike males do not exhibit significant variation in volumes across breeding states. Several species of songbirds show significant sex differences in the volume of SCS (58). Many seasonally breeding female birds exhibit marked changes in SCS volumes that do not appear to be related to song production rates (39-43). In the Baker et al., (44) study, female white-crowned sparrows were not found to show a significant change in SCS volume. In light of the data presented here, it appears that female birds in the genus *Zonotrichia* may not exhibit the changes in SCS characteristic of other seasonally breeding songbirds.

Conclusions

In summary, the present study provides data suggesting that the seasonal change in the neuroendocrine control of reproduction in a tropical species that breeds periodically is

primarily regulated at the level of the median eminence and in turn controls the release of GnRH1. This is in contrast to seasonally breeding temperate zone species that do exhibit marked changes in GnRH1 mRNA and protein content. Furthermore, we show that free-living females do not display the SCS neuroplasticity characteristic of the male SCS. Taken together, these data illustrate that a genetic marker (i.e. GnRH1 expression) of reproductive state in a tropical songbird may be constitutively expressed and presents an opportunity to determine how variation in expression between the sexes may relate to the integration of local supplementary cues leading to a change in the neuroendocrine control of reproduction.

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Highlights

1. Seasonal change in GnRH1 mRNA and song nuclei were examined in Rufous-collared sparrow
2. GnRH1 mRNA is higher in male compared to female sparrows
3. GnRH1 mRNA is constitutively expressed in the POA regardless of breeding state.
4. Female sparrows do not exhibit plasticity in HVC, RA, or Area X volumes.
5. Data highlight sex differences for the neural integration of environmental cues.

Figure 1.

Assessment of reproductive physiology in rufous-collared sparrows. A) body mass; B) CP height; C) testosterone concentrations in males; D) oestradiol concentration in females; E) testicular volume in males; and F) follicle length in females. Black and white bar graphs represent male and female data respectively. Letters indicate significant differences.

Figure 2. GnRH1 mRNA in male and female rufous-collared sparrows. A) representative photomicrographs from male and female sparrows collected during prebreeding and breeding conditions. B) GnRH1 mRNA OD intensity; and C) GnRH1 total OD. Letters indicate significant differences.

Figure 3.

The song control system in rufous-collared sparrows. A) representative photomicrographs of Nissl sections from a breeding male, prebreeding male and breeding female for Area X, HVC, and RA. Black arrows indicate nucleus boundaries. Nucleus volumes for B) Area X; C) HVC; and D) RA. Letters indicate significant differences.