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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 associate 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.

1. Introduction

Seasonally breeding animals at mid to high latitudes rely on a number of environmental cues to successfully time reproduction [1,12,14]. 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 [56,2,15,19,48]. 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 [57]. 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 [22,53,21,23]. 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 [5] 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 [45,44]. In many avian species, hypothalamic GnRH1 content is far greater during the breeding season than during the non-breeding season [5]. The recent cloning of complementary DNA for GnRH1 in songbirds revealed that GnRH1 mRNA also exhibits marked changes in expression across the reproductive cycle [49,52]. Phylogenetic comparisons of avian

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species suggest that plasticity in the GnRH1 system has evolved to facilitate different degrees of flexibility in timing reproduction in response to environmental cues [28,30]. 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 [41] 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 [35]. 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 GnRH1protein [32]. 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 [38]. 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 [39,3,51,7,4,8,10,11]. Songbirds have a series of discrete interconnected nuclei that are collectively referred to as the song control system (SCS; [39,40,42,33]). 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 acropallium). HVC also projects to the nucleus Area X that plays a role during the learning and maintenance of song structure [40,17,54]. In many temperate songbird species, several song control nuclei exhibit dramatic changes in volume that are associated with breeding states [51,7]. 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 [36]. 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 [36]. 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 [16,29,27,13,26] while the SCS in females of other species do not show changes [1,25]. 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 [16,29,27,13,26]. 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 [6].

2. Methods

2.1. Subjects

The rufous-collared sparrow is a common species found between sea level and 4000 m from southern Mexico to Tierra del Fuego [43]. 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, 3300 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 [37]. 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 [38]. The terms "prebreeding" and "breeding" states were selected based on similar physiological and behavioral characteristics observed in photosensitive and photostimulated temperate songbirds [15,5]. 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 [37] 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.

2.2. 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 [24] and CP size is correlated positively with testes size in this species [37]. After perfusion, the brains and gonads were extracted within 10 min. The diameter of the largest follicle on the ovaries and the testis diameter and length were measured to 0.01 mm using digital calipers. Brains were post-fixed in 10% formalin, and were stored under refrigeration until delivery to Johns Hopkins University. Within 5 h 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.

2.3. Hormone assay

Concentrations of testosterone and estradiol were measured by standard radioimmunoassay techniques following extraction and chromatographic separation [34,55]. For individual extraction efficiency determination, we equilibrated each sample overnight with 2000 cpm of tritiated steroid. Each sample was extracted with 5 μl 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 μl 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 μl of the sample while 200 μl of the sample was allocated to