

Photoperiod-Independent Changes in Immunoreactive Brain Gonadotropin-Releasing Hormone (GnRH) in a Free-Living, Tropical Bird

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Key Words

Zonotrichia · Birds · GnRH · Reproduction · Tropics

Abstract

Timing of seasonal reproduction in high latitude vertebrates is generally regulated by photoperiodic cues. Increasing day length in the spring is associated with changes in the brain that are responsible for mediating reproductive activities. A primary example of this is the increased content of gonadotropin-releasing hormone (GnRH) in the preoptic area of the hypothalamus in birds as they enter the spring breeding season. Increased GnRH activity stimulates the release of luteinizing hormone and follicle-stimulating hormone from the anterior pituitary. These gonadotropins induce growth of the gonads and release of sex steroids which act on the brain to mediate reproductive behaviors. By contrast, seasonal breeding in the tropics can occur in the absence of significant changes in photoperiod. To our knowledge, no studies have investigated whether seasonal breeding in free-living tropical vertebrates is associated with seasonal changes in the GnRH system. We studied two populations of rufous-collared sparrows (*Zonotrichia capensis*) at the equator, separated by only 25 km, but with

asynchronous reproductive phenologies associated with local climate and independent of photoperiodic cues. We collected brains and measured GnRH immunoreactivity (GnRH-ir) during each population's breeding and non-breeding periods. Breeding males had larger, but not more, GnRH-ir cells than non-breeding birds. The plasticity of the GnRH system was associated with local climate, such that the two populations exhibited asynchronous changes in GnRH-ir despite experiencing identical photoperiod conditions. Our results demonstrate that tropical birds can exhibit neural changes similar to those exhibited in higher latitude birds. However, these tropical populations appear to be using supplementary cues (e.g., rainfall, temperature, food availability) in a similar way to higher latitude species using an initial predictive cue (photoperiod). These results raise questions about the evolution of reliance upon photoperiodism and the strength of reproductive responses to other environmental cues in congeners from higher latitudes. The ability to respond to a multitude of environmental cues might be part of the ancestral condition, and the subsequent obligate photoperiodism in high-latitude congeners could reflect a loss of flexibility in response to environmental signals.

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Introduction

Photoperiod has long been known to be crucial to the timing of seasonal reproduction in temperate-zone bird species [Rowan, 1925; Dawson et al., 2001; Gwinner, 2003]. In these mid- to high-latitude species, the ultimately predictable change in photoperiod is the initial predictive cue used to provide the annual template for timing of reproduction. Supplementary cues, such as food availability, temperature, and rainfall, are then used to fine tune the timing of breeding to the local environment [Wingfield and Kenagy, 1991; Wingfield and Farner, 1993; Hahn et al., 2005]. Initial predictive cues are crucial predictors of future environmental conditions because gonads take time to recrudescence and the suitable periods for reproduction can be brief [Hahn et al., 1995; Hunt et al., 1995]. In avian species breeding at temperate latitudes, plasma concentrations of testosterone in males, as well as estradiol and progesterone in females, are elevated during the breeding season and basal during non-breeding. The increase in sex steroid levels immediately preceding and during breeding results from the activation of the hypothalamic-pituitary-gonadal (HPG) axis [for review: Ball and Bentley, 2000].

Expression of seasonally appropriate reproductive behaviors, such as breeding and associated territoriality, are typically mediated by seasonal changes in the neuroendocrine system [Dawson et al., 2001]. Seasonally breeding high latitude birds are in a condition termed photosensitive in the winter months, in anticipation of increasing day lengths in the spring. When the ambient day lengths increase, birds transition into a photostimulated state that is maintained through the breeding season. The increase in day length in the spring stimulates neurons in the hypothalamus to release gonadotropin-releasing hormone (GnRH) into the pituitary-portal blood system, which triggers the release of the gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the anterior pituitary into the systemic circulatory system [Sharpe and Ciccone, 2005]. In turn, these pituitary gonadotropins cause gonadal growth and an increase in plasma sex steroid concentrations. At the termination of the breeding season, birds enter into a photorefractory state where their reproductive system regresses and is sustained in an inactive state by long photoperiods. Only when short photoperiods are experienced once more is photorefractoriness dissipated and the birds become photosensitive again. As birds progress from a photorefractory to a photosensitive and then photostimulated state, GnRH immunoreactivity (GnRH-ir) in the hypothala-

mus can increase dramatically. This hypothalamic GnRH-ir increase has been documented by radioimmunoassay [Dawson et al., 2002] as well as in neuron size, number, and staining intensity [for review see Ball and Bentley, 2000].

Seasonal reproduction in tropical birds has not been as well studied, from a mechanistic perspective, as in temperate-zone species [Hau, 2001]. Tropical species often experience little seasonal change in photoperiod, yet observational studies, both in the Old and New World tropics, have described seasonal reproduction [reviewed in Stutchbury and Morton, 2001]. Other seasonally breeding tropical bird populations are thought to rely on different cues that vary locally, such as food availability, rainfall, and/or temperature [Stutchbury and Morton, 2001]. It is not known how tropical birds detect and translate their apparently important local cues into neuroendocrine signals that govern reproduction [Moore, 2005]. However, it is worth noting that even small changes in photoperiod can be used to time reproduction for some species such as the spotted antbird (*Hylophylax naevioides*) in Panama [Hau et al., 1998; Beebe et al., 2005].

The rufous-collared sparrow, *Zonotrichia capensis*, is a small emberizine sparrow with a species range from Southern Mexico to Tierra del Fuego. Populations of *Z. capensis* in Ecuador are ideal for investigations of non-photoperiodic reproduction because they exist near the equator where seasonal changes in photoperiod are minimal. In addition, this species is a congener of a temperate-zone species (the white-crowned sparrow, *Z. leucophrys*) whose reproductive neuroendocrinology has been studied in great detail [e.g., Wingfield and Farner, 1978a, b] and thus permits the use of established techniques and limits the phylogenetic confounds often associated with cross-species comparisons. Furthermore, *Z. capensis* is basal to other *Zonotrichia* species, which breed north of the tropics [Zink and Blackwell, 1996]. Thus, comparison of brain plasticity between congeners at different latitudes, and thus exposed to different environmental factors, should provide us with insight regarding the evolution of neuroendocrine control of seasonal reproduction.

We investigated two populations of *Z. capensis* that are year-round residents in the Ecuadorian Andes. The two sites (Papallacta: 0°21.7'S, 78°9.0'W, 3,300 m elevation; Pintag: 0°22.6'S, 78°22.5'W, 2900 m elevation) are approximately 25 km apart, but the populations are geographically separated by a high elevation mountain pass (~4,000 m). These populations experience annual changes in day length of less than 4 min (United States Naval

Observatory, Astronomical Applications Department: on-line day length calculator). Despite their proximity, these sites experience dramatically different climate patterns, particularly rainfall, as a result of Papallacta's position on the east slope of the Andes and Pintag being in an inter-Andean valley [for climate data see Bendix and Rafiqpoor, 2001; Moore et al., 2005]. The two populations of *Z. capensis* exhibit opposed reproductive patterns and are genetically isolated from one another [Moore et al., 2005]. The Papallacta population generally breeds from mid-August to December, and the Pintag population breeds from mid-November through April. Males of this species only sing during the breeding season, and do not sing during molt or during the non-breeding period [Moore et al., 2004b]. The telencephalic song control system shows seasonal growth and regression paralleling seasonal changes in song behavior. Thus this seasonal neuroplasticity is also out of phase between the populations [Moore et al., 2004b]. During the breeding period males have elevated plasma levels of testosterone that are as high as those observed in closely related northern species [Moore et al., 2002], with seasonal peaks that are also asynchronous between the two populations [Moore et al., 2004b] but not related to territorial aggression [Moore et al., 2004a]. The purpose of our study was to determine if asynchronous seasonal changes in reproductive hormones and the song control system in these two populations are correlated with neuroplasticity in the GnRH system by measuring the number and size of GnRH-ir neurons during the breeding and non-breeding seasons.

Materials and Methods

Field Study

Male birds were captured during a single year from each of the two populations (Papallacta and Pintag) during their respective breeding and non-breeding periods (Papallacta, breeding: $n = 6$, 16–24 September, 2002; non-breeding: $n = 7$, 25–30 April, 2002. Pintag, breeding: $n = 7$, 20–24 February, 2003; non-breeding: $n = 6$, 28–31 August, 2002). In each of the sampling periods, behavioral observations confirmed that all adult individuals in the populations were either breeding or not breeding. Birds were captured passively (no playback, models or decoys) at dawn using mist nets. Rapidly after capture (<10 min) a 250 μ l blood sample was obtained from a wing vein and stored on ice until return from the field. The blood samples were subsequently centrifuged and the plasma separated and frozen. The same day as capture, the birds were terminally anesthetized with an intramuscular injection of 7.5 mg sodium pentobarbital and perfused transcardially with 0.9% heparinized saline (150 IU/10 ml) followed by 10% neutral buffered formalin. Brains were extracted and post-fixed, under refrigeration, until return to the University of Washington. At that time, the

brains were cryoprotected in 30% sucrose until they sank, then frozen on dry ice and stored at -80°C . All work was approved by the University of Washington IACUC. Hormone data and attributes of the neural song control system were previously reported from these same birds [Moore et al., 2004b].

Immunocytochemistry

Each brain was sectioned in the coronal plane at 40 μ m on a cryostat. Every third section throughout the brain was collected into phosphate buffered saline (PBS) and immunocytochemistry for GnRH was performed. Sections were washed three times in PBS, background immunoreactivity blocked for one hour using 2% normal goat serum (NGS) in 0.2% PBS-T (PBS + Triton X-100), and then incubated in primary antibody (rabbit anti-chicken GnRH, code HU60H; a generous gift from Prof. H. Urbanski) at a concentration of 1:10,000 in 0.2% PBS-T. This antibody recognizes both cGnRH-I and -II. The neuronal populations containing the two GnRHs are found in very discrete areas in the avian brain [for review see Ball and Bentley, 2000] and can also be distinguished from one another by their distinctive morphology. Three subsequent washes in 0.2% PBS-T were followed by incubation for 1 hour in biotinylated goat anti-rabbit IgG (1:250 in 0.2% PBS-T) and another set of washes. Sections were then incubated for 1 hour in avidin-biotin complex (ABC; Vectastain Elite Kit, Vector Labs). The resulting complex was visualized using 0.03% 3,3'-diaminobenzidine (DAB) intensified with 0.15% nickel sulfate.

Quantification of GnRH Immunoreactivity and Photomicroscopy

Quantification of GnRH labeling was achieved by measuring the size of GnRH-ir cell bodies and by counting the total number of GnRH-ir cells throughout the medial preoptic area (MPO) of each brain; based on this location, all GnRH-ir cells analyzed were considered cGnRH-I neurons. Previous studies suggest that changes in GnRH-ir parallel total hypothalamic content of GnRH-I but not GnRH-II [Dawson and Goldsmith, 1997; Dawson et al., 2002]. Analyses were performed by an observer, blind to both period of sampling and population, using a Zeiss Axioskop microscope with bright field illumination. Mean cell size was measured on all cells that were visible (range: 29–141 cells per brain) and was determined by first digitizing images of labeled areas at 400 \times magnification with the use of an AxioCam color video camera (Carl Zeiss, Göttingen, Germany) connected to a computer. Image analysis software (AxioVision 4, Carl Zeiss) was then used to determine two-dimensional cell areas after tracing the perimeters of all cells that were in focus and had clearly identifiable boundaries using a Wacom pen tablet (Wacom, Vancouver, WA). Fibers sprouting from the cell body were excluded by continuing the tracing in an arc defined by the perimeter of the cell body (i.e. continuing as if the fiber was not present and the cell body was uniform in shape at the fiber's origin). Cell areas were averaged for each brain, and these values were used to determine the mean cell size for each group. Total cell number for each brain was determined by counting all labeled cells in the MPO at 400 \times magnification; mean cell number for each group was then calculated.

Statistical Analysis

For statistical analysis, we performed a multiple analysis of variance (MANOVA) with population (Papallacta, Pintag), reproductive state (breeding, non-breeding), and population by reproductive

Table 1. Comparisons of GnRH expression between the two populations sampled during respective breeding and non-breeding periods

	F	d.f.	p value
MANOVA			
Intercept	530.60	2, 20	<0.001*
Population	4.04	2, 20	0.03*
Reproductive state	3.50	2, 20	<0.05*
Population X reproductive state	0.80	2, 20	0.46
ANOVAs			
Cell size			
Population	4.45	1, 21	0.047*
Reproductive state	6.71	1, 21	0.02*
Population X reproductive state	0.51	1, 21	0.48
Cell number			
Population	4.78	1, 21	0.04*
Reproductive state	1.06	1, 21	0.31
Population X reproductive state	1.30	1, 21	0.27

Effects of population, reproductive state, and the interaction between the two factors on reproductive factors and brain structures. Asterisks denote a statistically significant result. Significance level for univariate tests on population were 0.0253 (cell size), 0.05 (cell number), and 0.05 for reproductive state on cell size. These are based on sequential Bonferroni adjustments by the Dunn-Sidak method [Sokal and Rohlf, 1995].

state as explanatory variables, and GnRH-ir cell size and number as response variables. Subsequently, individual ANOVAs were conducted on the two response variables using the same explanatory variables. Significance levels for the univariate tests were adjusted based on sequential Bonferroni adjustments by the Dunn-Sidak method [Sokal and Rohlf, 1995].

Results

The MANOVA showed an overall significant effect of population ($F_{2,20} = 4.04$, $p = 0.03$) and reproductive state ($F_{2,20} = 3.50$, $p < 0.05$) but not the interaction between population and reproductive state. The individual ANOVA on GnRH-ir cell size showed a significant effect of population ($F_{1,21} = 4.45$, $p = 0.047$) and reproductive state ($F_{1,21} = 6.71$, $p = 0.02$). The individual ANOVA on GnRH-ir cell number showed a significant effect of population ($F_{1,21} = 4.78$, $p = 0.04$). See table 1 for complete statistical results, figure 1 for cell size and cell number averages and standard errors, and figure 2 for representative photomicrographs.

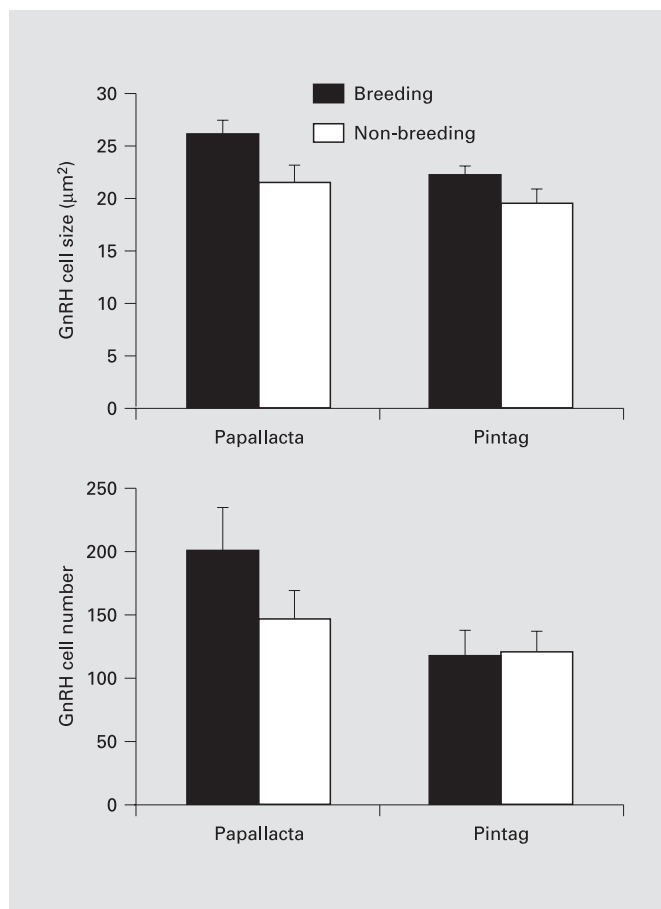


Fig. 1. A comparison of GnRH-ir cell size and cell number (mean \pm SE) of breeding and non-breeding male rufous-collared sparrows between the Papallacta and Pintag populations.

Discussion

Most environments are seasonal. At northern and southern latitudes, the change in day length is largely responsible for the change in seasons. However, even tropical latitudes exhibit seasonal changes in the environment, often notably in terms of wet and dry seasons. Animals must be able to respond to changes in their environment to survive and reproduce. Timing of reproduction is especially important as the energetic demands of raising offspring require that reproduction be coordinated with peaks in food availability [Lack, 1968]. Although we know a great deal about the mechanisms that temperate-zone species use to time reproduction [Ball and Bentley, 2000; Dawson et al., 2001], we know much less about tropical species [Hau, 2001]. To our knowledge, this is the first study to investigate seasonal changes

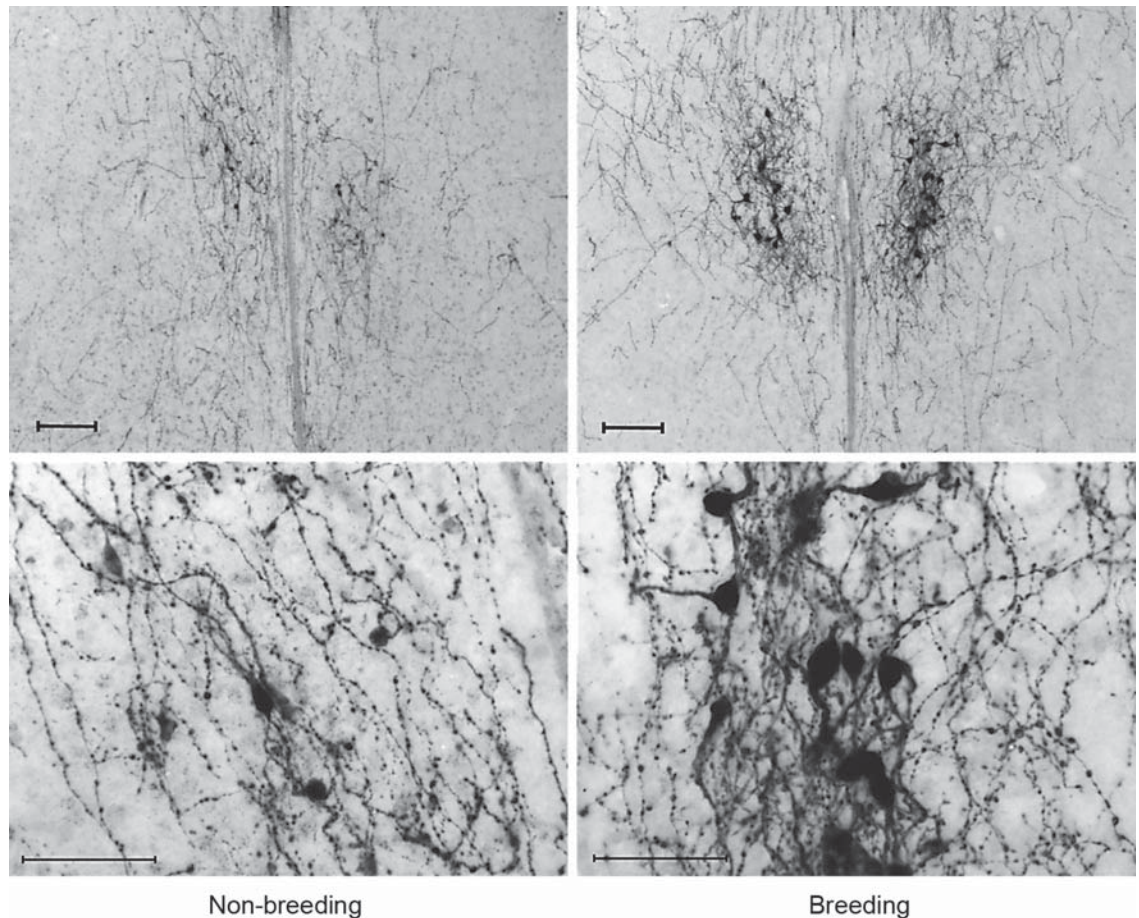


Fig. 2. Representative photomicrographs of GnRH-ir cells in the preoptic area of the hypothalamus in breeding and non-breeding male rufous-collared sparrows. Images of the same section were taken at magnifications of 50 \times (top) and 400 \times (bottom). The breeding bird is from the Papallacta population and the non-breeding bird is from the Pintag population. Scale bar is 100 μ m.

in the GnRH system, which is responsible for mediating reproduction, in a free-living tropical vertebrate.

In temperate zone species, seasonal breeding is often (but not always) correlated with changes in the hypothalamic content of GnRH, with breeding birds having more and larger GnRH-ir cells in the preoptic area of the hypothalamus [for review see Ball and Hahn, 1997; Ball and Bentley, 2000]. This has been well described among closely related cardueline finches where opportunistically breeding species exhibit less dramatic seasonal changes in GnRH-ir than more seasonal and less flexible species [MacDougall-Shackleton et al., 2005; Pereyra et al., 2005]. For example, opportunistically breeding male white-winged crossbills (*Loxia leucoptera*) showed nearly significant changes ($p = 0.052$) in cell size but no differences in cell number through the year [MacDougall-Shackleton

et al., 2001]. In contrast, seasonally breeding common redpolls (*Carduelis flammea*) and pine siskins (*Carduelis pinus*) show dramatic (~ 2 -fold in cell number) seasonal changes in those measures of GnRH-ir [Pereyra et al., 2005]. These species differences are thought to be associated with a reliance on photoperiodic cues to time seasonal processes [Hahn et al., 2004].

Changes in GnRH-ir are not necessarily consistent across species. For example, not all seasonally breeding animals exhibit strong seasonal changes in the GnRH-ir. Among mammals, neither Syrian nor Djungarian hamsters exhibit seasonal changes in GnRH-ir cell number despite large changes in gonad size and exhibiting seasonal reproduction [Urbanski et al., 1991; Yellon, 1994]. A congener to the bird used in the current study, the photoperiodic white-crowned sparrow (*Zonotrichia leucoph-*

rys gambelii), also does not exhibit a seasonal change in the number of GnRH-ir cells [Meddle et al., 1999]. Supposedly photoperiodic canaries, *Serinus canaria*, did not show any GnRH-ir responses to experimental manipulation of photoperiod [Bentley et al., 2003], although in the wild these birds can exhibit flexible breeding [Leitner et al., 2003]. Seasonally breeding house sparrows (*Passer domesticus*) exhibited ~2-fold differences in GnRH-ir cell numbers between those with regressed gonads and those with enlarged gonads [Hahn and Ball, 1995]. Additionally, Japanese quail (*Coturnix coturnix japonica*), which exhibit relative photorefractoriness, do not exhibit changes in GnRH-ir across the annual cycle [Foster et al., 1988]. Thus it appears that not all seasonally breeding animals exhibit plasticity of the GnRH system, as measured by immunocytochemistry, and some differences appear associated with degree of photoperiodicity whereas others remain unexplained [MacDougall-Shackleton et al., 2005].

In the current study, breeding male rufous-collared sparrows birds had significantly larger, but not more, GnRH-ir cells than non-breeding birds. This is most similar to the opportunistically breeding crossbills [MacDougall-Shackleton et al., 2001]. Interestingly, both species also exhibit seasonal changes in the song control system [MacDougall-Shackleton et al., 2001; Moore et al., 2004b] and flexible breeding schedules [Hahn, 1998; Moore et al., 2005]. The similarities in GnRH-ir among the current tropical species, opportunistically breeding species [MacDougall-Shackleton et al., 2001; Pereyra et al., 2005], and species that exhibit relative photorefractoriness [Foster et al., 1988] suggest that more robust plasticity of the GnRH system evolved with obligate photoperiodicity.

Interestingly, we did not see an effect of reproduction on GnRH-ir cell number but we did see significant effects of population on both GnRH-ir cell size and number. The lack of a cell number response associated with reproductive condition is not unusual, as the congener white-crowned sparrow shows no seasonal change in GnRH-ir [Meddle et al., 1999]. Our results of population effects are possibly associated with the fact that samples were obtained at different sub-stages of reproduction between the populations. Plasma testosterone levels can vary in these populations with breeding sub-stage [Moore et al., 2004a; Moore, 2005] and the breeding sample from the Papallacta population was obtained earlier in the breeding period than the Pintag population samples, which were obtained later in the breeding period. That said, a study using the same birds from this study [Moore et al., 2004b], found no population differences in either testosterone

levels or testis volume, making an effect of sub-stage of breeding unlikely. To investigate this, a future study could examine seasonal changes in GnRH-ir at a finer temporal scale (e.g., pre-breeding, breeding, nest incubation, nestling feeding, re-nesting, etc.).

It is unknown which environmental cues, and the sensory mechanisms used to perceive them, are being used in these bird populations to time seasonal reproduction. Rainfall is the environmental cue that differs most prominently between the two sites; for example, July can be the wettest month in Papallacta and the driest in Pintag [Bendix and Rafiqpoor, 2001; Moore et al., 2005]. However, other cues, such as temperature and humidity, change as well. It is possible that the birds are not using environmental cues directly, but rather are relying on related changes in food availability [Hahn et al., 2005]. Changes in food availability have been previously shown to be important cues for timing growth of the gonads and reproduction in a number of species, but they are primarily supplementary cues [Komdeur, 1996; Hau et al., 2000; Leitner et al., 2003]. It is worth noting that one sex may be more sensitive to environmental cues than the other. Previously we have documented a close relationship between gonadal growth in males and females within monogamous pairs and independent of the rest of the population [Moore et al., 2005]. This raises the possibility that one sex monitors the environment (possibly the female, as their energetic investment in reproduction is typically greater) whereas the other sex cues in on its mate [Moore, 1982, 1983]. Finally, it is possible that these birds use an endogenous rhythm to time reproduction as described in the African stonechat, *Saxicola torquata axillaris*, another equatorial bird [Gwinner and Dittami, 1990]. For this to occur, the rhythm would have to be entrained by an environmental cue that is not in phase between the two populations.

The plasticity we document here in the GnRH system parallels the plasticity previously documented in both gonadal activity and the neural song control [Moore et al., 2004b]. In that study, we used tissue from the same birds as in the current study, and found significant effects of reproductive state on testis size, plasma testosterone levels, and the proportion of the telencephalon occupied by the song control nuclei HVC, RA and X. There were no population differences but there were significant effects of reproduction on testis volume and plasma testosterone levels [Moore et al., 2004b]. From those previous results we hypothesized that the GnRH system would also be seasonally plastic. Indeed, we see very similar seasonal patterns between the three factors: hypothalamic GnRH-

ir, gonadal activity, and telencephalic song control system. In temperate zone species these three factors are causally related [Ball et al., 2004; Brenowitz, 2004] and there is no reason to believe such a relationship does not exist in this species.

Seasonal changes in hypothalamic GnRH-ir have previously been associated with photoperiodic cues and a transition from a photorefractory to a photosensitive state [Ball and Bentley, 2000; Dawson et al., 2001]. In the current study we describe a seasonal change in GnRH-ir that occurs independently of photoperiod. This is most clearly shown by breeding samples obtained from the Papallacta population at almost the same time of year as non-breeding samples were obtained from the Pintag population (see Materials and Methods above). Previous studies have investigated photoperiodicity in tropical birds. Tropical spotted antbirds (*Hylophylax naevioides*) can be sensitive to even minimal photoperiodic cues but they do not exhibit absolute photorefractoriness [Hau et al., 1998; Wikelski et al., 2000; Beebe et al., 2005]. Previous studies of *Z. capensis* from Colombia have experimentally demonstrated individuals to be sensitive to photoperiodic cues [Miller, 1965], although females may be more sensitive than males. It is worth noting that based on mitochondrial DNA, *Z. capensis* is basal to *Z. leucophrys* [Zink and Blackwell, 1996]. Therefore, *Z. capensis* is closer to the ancestral form of the group, which might

have been able to respond to a variety of environmental cues to time reproduction advantageously. Indeed, this species ranges from southern Mexico to Tierra del Fuego and thus different populations experience very different seasonal environmental cues. It is not known if the basal form of *Z. capensis* was tropical or from more southern latitudes. With the invasion of higher latitude environments over evolutionary time, the importance of photoperiod cues predominated and, at least to the north where the range extends further, the sensitivity to photoperiodic cues dominated sensitivity to other cues. Thus, *Z. leucophrys* appears to be obligatorily photoperiodic whereas *Z. capensis* from the tropics is not. Future studies investigating seasonal reproduction of more southern populations of *Z. capensis* would shed further light upon the evolution of photoperiodicity.

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