

# Seasonal Changes in Brain GnRH Immunoreactivity and Song-Control Nuclei Volumes in an Opportunistically Breeding Songbird

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## Key Words

Birds · White-winged crossbill · *Loxia leucoptera* · Seasonal plasticity · Song-control system · HVC · RA · Area X · GnRH · Photoperiodism

## Abstract

White-winged crossbills (*Loxia leucoptera*) are opportunistic breeders that can nest at almost any time of year if there is sufficient food. Other cardueline finches that have been shown to breed on a strictly seasonal schedule become absolutely refractory to the stimulatory effects of long-day photoperiod, dramatically down-regulate hypothalamic gonadotropin-releasing hormone (GnRH), and reduce the volume of several song-control nuclei in autumn. This study examined whether changes in photoperiod modify the GnRH and song-control systems in white-winged crossbills. Adult male and female crossbills were captured and held on a naturally changing photoperiod. Brains of male and female birds were collected in May, October, and January. GnRH content was assessed by immunocytochemistry and the volumes of Nissl-defined song-control nuclei (HVC, Area X, and the robust nucleus of the archistriatum) were reconstructed. In contrast to other cardueline finches, GnRH immunoreactivity was relatively stable across the year, exhibiting only modest seasonal variation. The song control system, on the other hand, exhibited large sea-

sonal changes as well as sex differences. Thus, crossbills appear to maintain hypothalamic GnRH content year round, perhaps to facilitate a rapid response to favorable breeding conditions, even on short days. However, song control nuclei are dramatically affected by photoperiod. Future work should examine these systems in crossbills breeding on short days to compare photoperiod-dependent and -independent effects on neural plasticity.

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## Introduction

Animals living at mid to high latitudes typically coordinate reproduction with favorable environmental conditions [Lack, 1968]. Such coordination requires neuroendocrine and endocrine integration of several types of environmental cues [Wingfield and Kenagy, 1991; Ball, 1993; Wingfield and Farner, 1993]. Long-term predictive cues such as changing photoperiod [i.e. *initial predictive cues*; Wingfield, 1980, 1983] permit anticipation of seasonally reliable events, such as amelioration of conditions in spring, well in advance of the onset of those events [Farner and Gwinner, 1980; Wingfield, 1983; Follett, 1984]. Short-term predictive cues [a combination of the *supplementary cues* and *synchronizing and integrating cues*; Wingfield, 1980, 1983], such as temperature, food supply, and behavioral interactions with conspecifics, accelerate or delay reproductive

development appropriately with respect to local conditions [Marshall, 1959, 1970; Wingfield, 1983; Wingfield and Kenagy, 1991]. Depending on the degree of long-term predictability of resources and conditions that are important to a particular species' reproduction, the relative importance of long- and short-term predictive cues can vary among species [Wingfield et al., 1992, 1993]. Thus, seasonal changes in the neural mechanisms that transduce these diverse cues into reproductive physiological and behavioral responses could also vary.

In this study, we examined the effect of changing photoperiod on both the septo-infundibular-preoptic gonadotropin-releasing hormone (GnRH) system and the song-control system in white-winged crossbills (*Loxia leucoptera*), oscine songbirds with flexible breeding schedules. The GnRH system controls pituitary release of gonadotropins and thereby gonadal gametogenic and steroidogenic activity in all vertebrates studied [Silverman et al., 1994]. The interconnected group of brain regions called the song-control system mediates learning and production of complex courtship and territorial vocalizations in oscine songbirds [Brenowitz et al., 1997]. Both of these systems undergo seasonal anatomical and physiological changes in strictly seasonally breeding songbirds [reviewed in Ball, 1993; Ball and Hahn, 1997; Tramontin and Brenowitz, 2000]. Further, they affect one another: activity of the GnRH system stimulates the production of sex steroid hormones that organize and activate the song-control system [e.g., Arnold, 1992], and song can influence reproductive physiology [Morton et al., 1985; Bentley et al., 2000; MacDougall-Shackleton et al., 2001]. Most studies of annual variation in these two systems have focused on species that breed only on a regular seasonal schedule and rely predominantly on photoperiod to coordinate changes in reproductive physiology and behavior. In addition, almost no studies have looked at both systems concurrently in the same individuals [but see Marsh et al., 1999]. White-winged crossbills feed primarily on conifer seeds, which have an unpredictable geographic production pattern in any given year [Fowells, 1965]. Consequently, these birds wander nomadically in search of large conifer seed crops and can potentially locate sufficient seeds to support breeding at almost any time of year. At least at mid-latitudes, white-winged crossbills regularly breed on all naturally-occurring photoperiods, though not necessarily in all months of the year [Benkman, 1990, 1992]. Thus, our study provides an interesting comparison with studies of seasonal breeders and permits evaluation of the generality of findings based on those species.

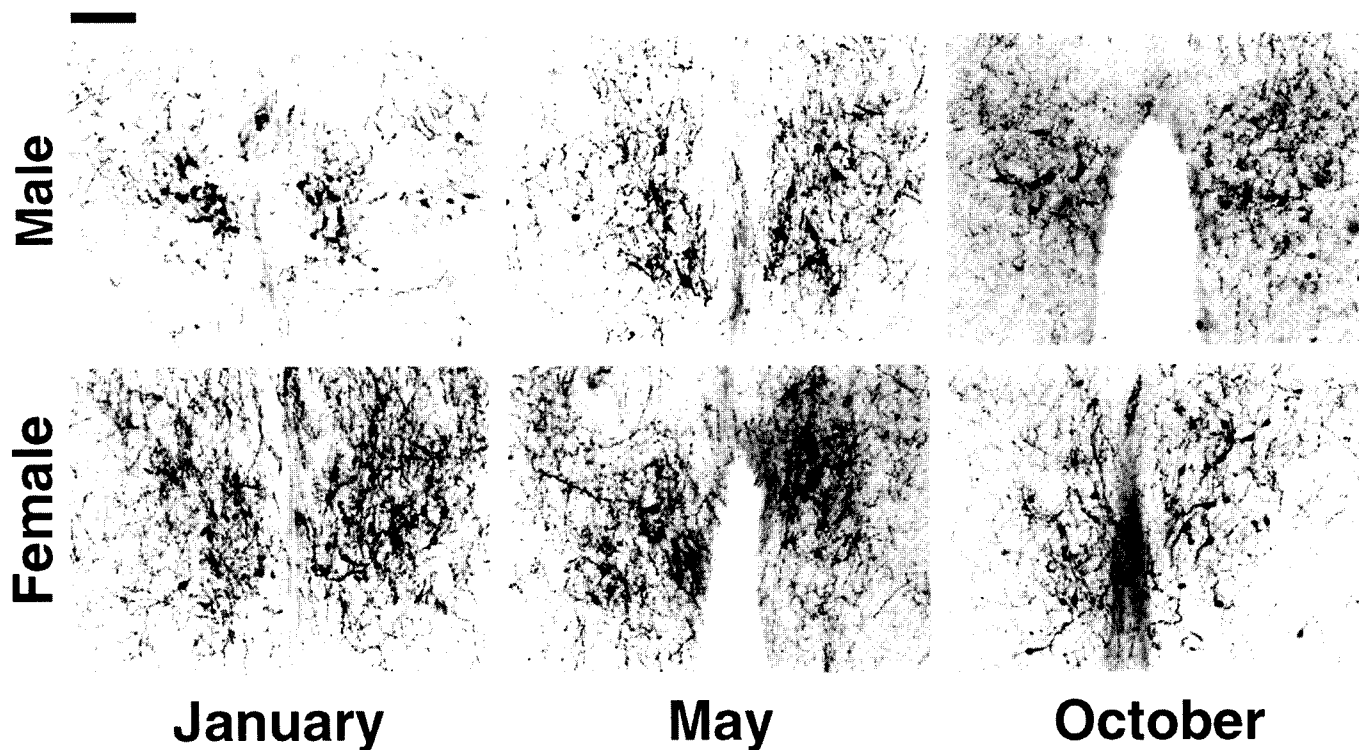
This study addresses two broad questions. First, do songbirds that retain reproductive responsiveness to environ-

mental cues for most of the year maintain relatively stable hypothalamic levels of GnRH year-round, in contrast to strictly seasonal breeders that become absolutely refractory to environmental stimulation [as hypothesized by Ball and Hahn, 1997; Hahn et al., 1997]? Second, do birds capable of breeding on a wide range of photoperiods still use day length as a cue to modify neural areas that control song? To address these questions, we brought male and female white-winged crossbills into captivity and maintained them on a natural photoperiod while holding other environmental conditions (food, temperature) constant. We collected brains at three times of year (January, May, and October) and quantified seasonal changes in both the GnRH and the song-control systems. These three sampling points correspond to times when seasonally-breeding songbirds would be photosensitive (non-reproductive on short days, but responsive to long days), photostimulated (in breeding condition on long days), and photorefractory (non-reproductive and reproductively unresponsive to any photoperiod), respectively [see Nicholls et al., 1988]. It remains unclear whether crossbills become absolutely photorefractory as do most other songbirds. However, crossbills usually have regressed gonads and are relatively unlikely to breed in autumn, can potentially develop their gonads and breed flexibly on short days in winter contingent on food availability, and are usually in breeding condition when days are long in late spring and early summer [Berthold and Gwinner, 1978; Coombs-Hahn, 1993; Deviche, 1997; Hahn, 1995, 1998; Hahn et al., 1995; Deviche and Sharp, 2001; see also Hahn et al., 1997]. Thus, the three sampling points in this study provide a useful comparison between flexibly breeding crossbills and the strictly seasonally breeding songbirds that have already been studied.

## Materials and Methods

Twelve male and 12 female white-winged crossbills were captured between 3 April and 31 May 1994 near Fairbanks, Alaska (64°50'N, 147°50'W). The research reported here was carried out following the guidelines of the University of Alaska, Fairbanks, animal care and use committee. Birds were moved indoors into individual cages and were provided with food (ground Purina finch chow) and vitamin-supplemented water ad libitum. Temperature throughout the experiment was maintained at approximately 20°C. Photoperiod during the experiment was changed weekly to match the ambient changes in photoperiod for the latitude of Fairbanks. This ranged from a photoperiod 4 L: 20 D at the winter solstice to 20 L: 4 D at the summer solstice. Although the particular birds and photoperiod used in this study were from a high latitude, the birds were from a large nomadic population that most likely disperses and breeds at lower latitudes as well [Benkman, 1992].

At three times of year (10 October 1994, 19 January 1995, 29 May 1995) we collected the brains of four males and four females. Birds



**Fig. 1.** Representative photomicrographs of immunoreactive GnRH cells in coronal brain sections of male and female white-winged crossbills collected at three times of year. Scale bar = 0.1 mm.

were anesthetized and perfused transcardially with 4% paraformaldehyde as described previously [Gulledge and Deviche, 1998]. After perfusion, brains were postfixed in situ overnight, dissected from the cranium, stored in sodium azide-containing buffer, and shipped to Johns Hopkins University. There the brains were cryoprotected in 30% sucrose, frozen on pulverized dry ice, and stored at  $-80^{\circ}\text{C}$  until further processing. As part of a parallel study [Crain, 1998], gonad masses were measured at the time of brain collection.

Brains were processed in groups of three, each group containing a brain from each of the three collection dates all of the same sex. We processed all brains from male birds before proceeding to the female brains. Brains were weighed to the nearest 0.1 mg then sectioned in the coronal plane at  $40\ \mu\text{m}$  in a cryostat at  $-17^{\circ}\text{C}$ . Sections containing areas of interest were collected into tissue wells containing 0.1 M PBS at pH 7.5. We collected every second section for GnRH immunocytochemistry, beginning at the division of the tractus septomesencephalicus (TSM) and extending caudally through to the choroid plexus. We collected sections for Nissl staining of the song-control nuclei; these included every second section spanning the nuclei HVc [sometimes called high vocal center; Brenowitz et al., 1997], Area X, and the robust nucleus of the archistriatum (RA). In addition, we collected every second section spanning the dorsal part of the nucleus mesencephalicus lateralis (MLd) as a control nucleus. Anatomical locations were determined with reference to a brain atlas for canaries [Stokes et al., 1974].

#### *Immunocytochemistry*

We used an immunocytochemistry protocol identical to that described in detail for other songbird species [Hahn and Ball, 1995; Cho et al., 1998]. The primary antibody used is specific to several forms of the intact GnRH decapeptide but does not react with GnRH fragments [Urbanski et al., 1990; Urbanski, 1992]. Primary antibody binding was localized using a biotinylated secondary antibody, and visualized by an avidin-biotin horseradish peroxidase reaction (Vectastain Elite ABC solution, 1:200 dilution). Stained sections were mounted on gelatin-coated microscope slides and protected by cover slips affixed with Permount.

#### *Immunoreactivity Quantification*

Slides were examined using bright-field light microscopy. Numbers of immunopositive GnRH cell bodies were counted directly in all sections beginning at the division of the TSM caudally until there were no cells observed in 5 consecutive sections (usually around the anterior commissure). This protocol resulted in cells being counted in about 11 to 19 of the alternately collected sections. Cell counts were not corrected for possible over sampling, therefore caution should be used in comparing our counts with cell counts obtained in other systems with other sampling methods. Cell sizes, cell body staining intensity, and the amount of axonal fiber staining were all assessed using images digitized using a CCD video camera connecting the microscope to a microcomputer. Digitized images were captured and analyzed using NIH Image 1.40. Details of the image analysis protocols used here have been described in detail previously [Hahn and Ball, 1995; Cho et

al., 1998]. Cell size was measured as the mean cross-sectional area of 20 randomly selected immunopositive cells per bird. Relative cell staining intensity was calculated in each bird for 20 randomly selected cells by dividing the optical density of each cell by the optical density of nearby background tissue. Size and optical density were measured for the same 20 randomly selected cells. Selection of these cells was performed by randomly choosing 4 brain sections per bird, and measuring 5 random clearly defined cells per section. Mean fiber staining was measured for each bird by calculating the mean cross-sectional area of a field of view occupied by stained fibers. That is, for each bird ten cell-body-free fields of view in the ventral preoptic area were selected, and the area within each view occupied by stained fibers calculated [see Hahn and Ball, 1995; Cho et al., 1998].

#### Quantification of Song-Control System

Sections containing song-control nuclei were mounted on gelatin-coated microscope slides, stained with thionin and dehydrated in ethanol, then protected by cover slips affixed with Permount. Brain regions of interest were digitized with a CCD camera connected to a microcomputer, and cross-sectional areas measured using NIH Image 1.40. Volume of each nucleus was reconstructed by summing the area of each section multiplied by the sampling interval (80  $\mu\text{m}$ ). Poor staining prevented volume reconstruction of HVC, Area X and RA in three male brains, and of MLD in two male brains.

## Results

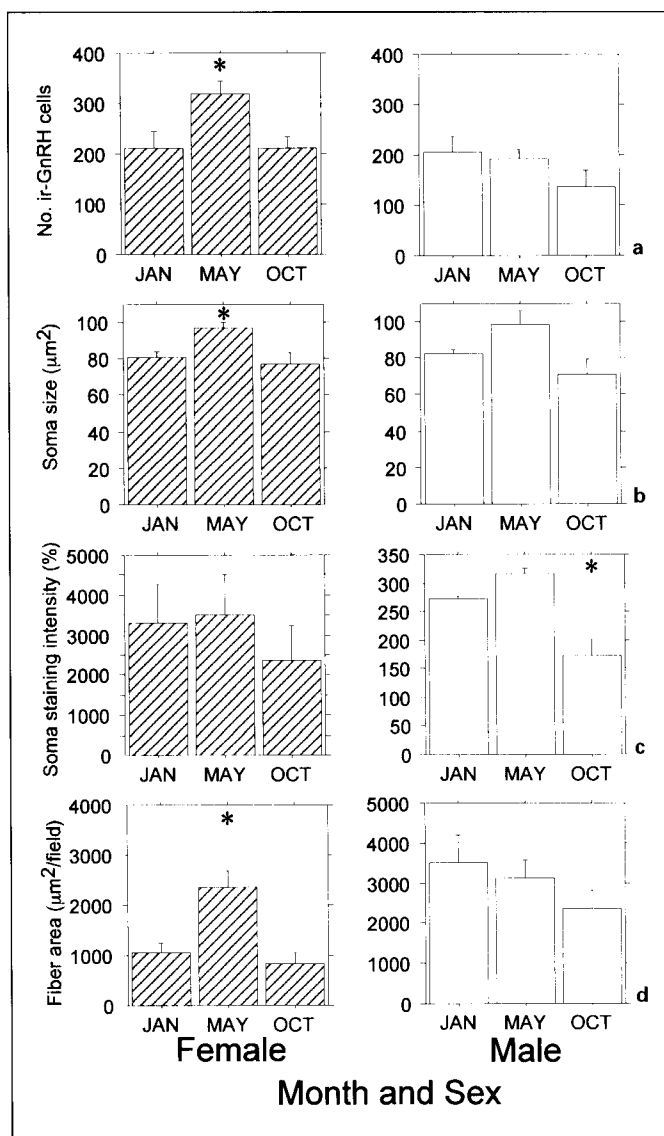
### Reproductive Condition

Testes of male birds were small in October and January, and large in May [paired testes mass: October: mean  $\pm$  SE =  $7.7 \pm 2.3$  mg; January: mean  $\pm$  SE =  $5.5 \pm 1.1$  mg; May: mean  $\pm$  SE =  $278.6 \pm 27.4$  mg; data from Crain, 1998]. Ovaries of female birds were relatively undeveloped (i.e. follicular hierarchies minimal, no sign of yolk deposition) at all three dates [Crain, 1998].

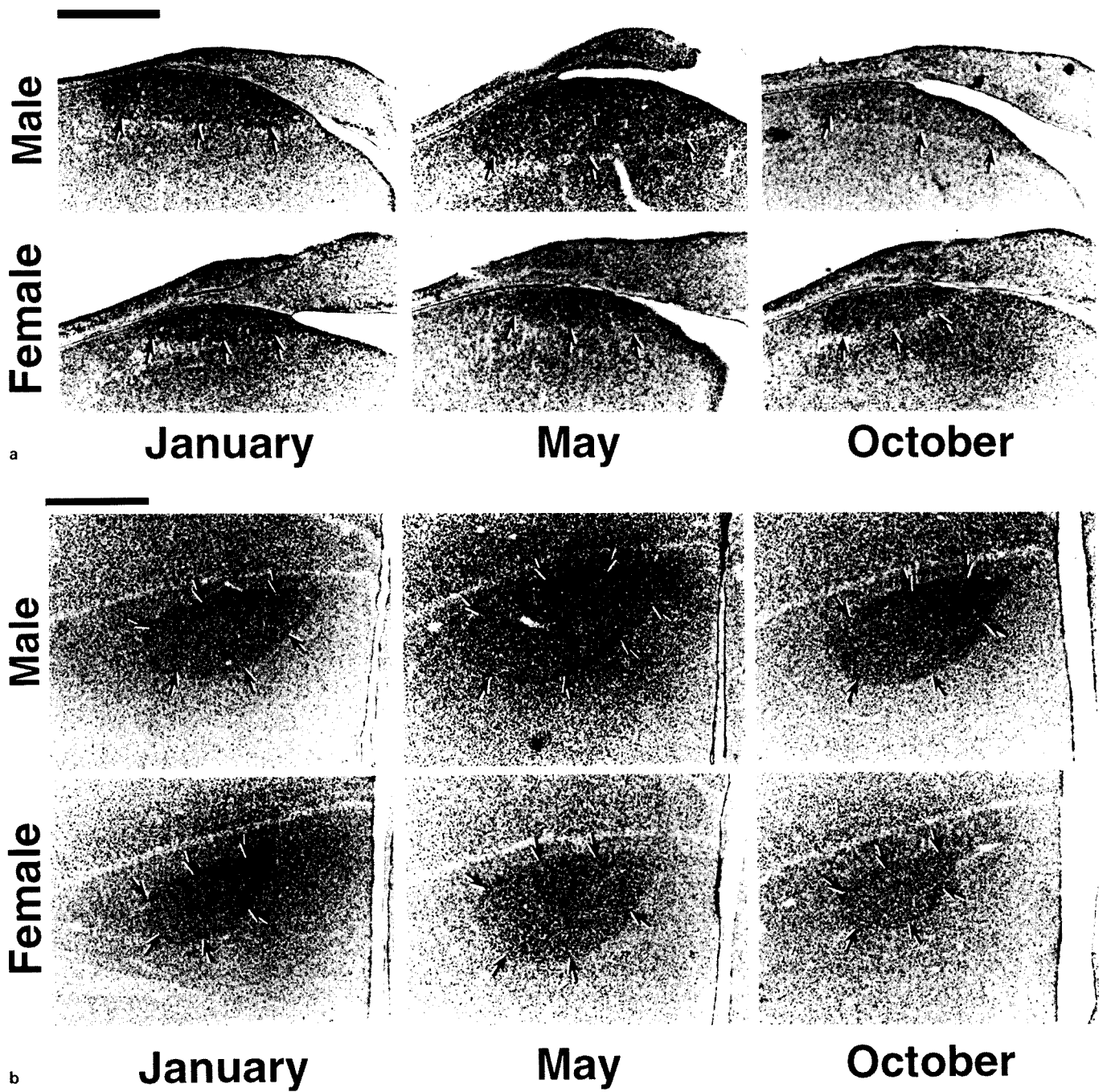
### GnRH Immunoreactivity

In general, white-winged crossbills exhibited relatively stable GnRH immunoreactivity year round. Immunoreactive cells and fibers were distributed throughout the preoptic area caudal to the septum at all sampling dates (fig. 1). Because male and female brains were processed in separate immunocytochemistry runs, we could not directly compare data obtained for the two sexes. Thus, results from males and females were analyzed separately.

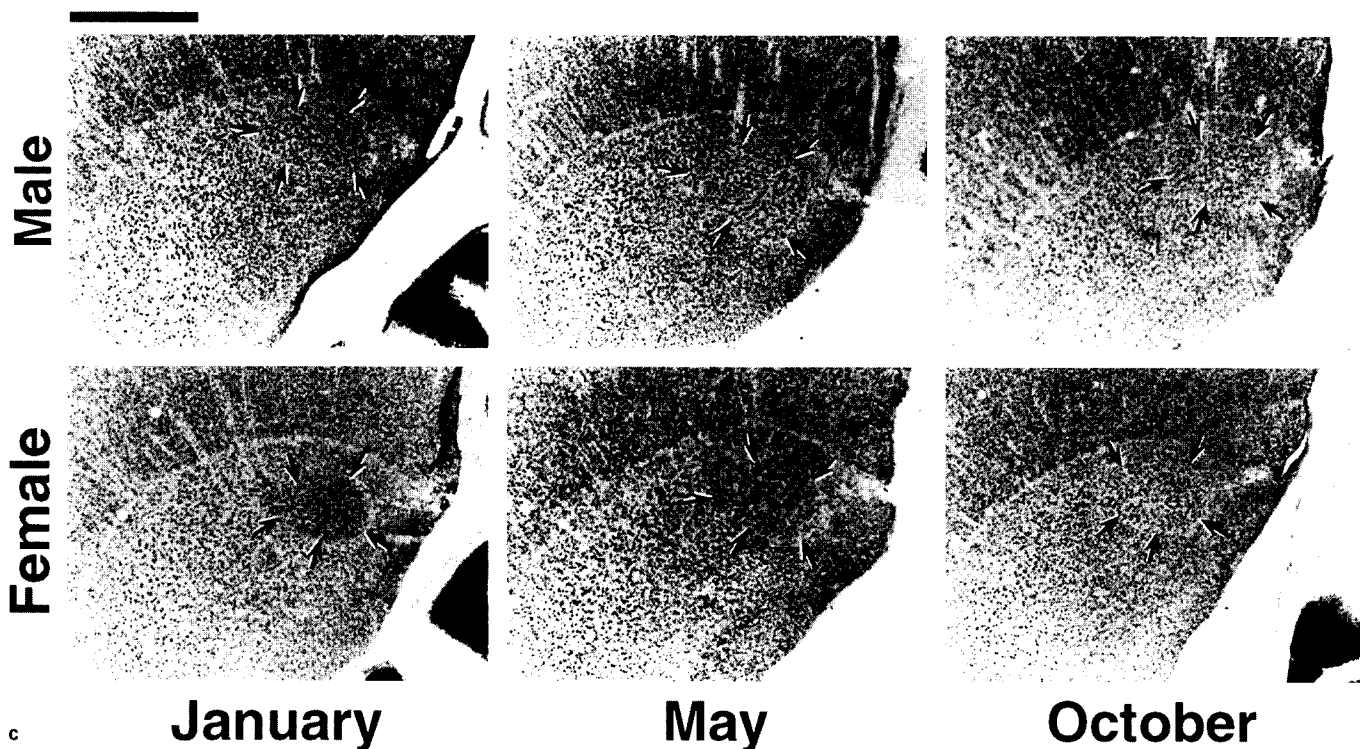
The male crossbills exhibited only limited changes in GnRH immunoreactivity (fig. 2). There was no statistically significant seasonal variation in the number of immunoreactive cells ( $F_{2,9} = 1.72$ ,  $p = 0.23$ ) or in immunoreactive fiber area ( $F_{2,9} = 1.18$ ,  $p = 0.35$ ). Staining intensity of immunoreactive cells varied seasonally ( $F_{2,9} = 17.68$ ,  $p < 0.001$ ). Post-hoc analyses (Fisher's protected least square differ-



**Fig. 2.** Quantification of GnRH immunoreactivity in female (hatched bars) and male (open bars) white-winged crossbills collected at three times of year: January (JAN), MAY, and October (OCT). Bars represent mean  $\pm$  SE of **a** number of immunoreactive (ir-GnRH) cells, **b** cross-sectional area of immunoreactive cell bodies, **c** cell staining density above background, and **d** area of fiber staining. See text for details of quantification methods and statistical differences. Asterisks indicate means that differ significantly ( $p < 0.05$ ). Vertical scales of cell staining density differ between males and females because optical density of background was an order of magnitude lower for females than for males.



**Fig. 3.** Representative photomicrographs of three song-control nuclei in coronal brain sections of male and female white-winged crossbills collected at three times of year. Arrows indicate nucleus borders. **a** nucleus HVc; **b** Area X; **c** RA. Scale bar = 1 mm.



ence, PLSD) indicated that brains collected in October had reduced staining compared to those collected in May ( $p = 0.0003$ ) or January ( $p = 0.003$ ). As well, there was a near-significant effect of season on the size of immunoreactive cells ( $F_{2,9} = 4.19$ ,  $p = 0.052$ ).

The female crossbills similarly showed only limited seasonal changes in GnRH immunoreactivity (fig. 2). There was no seasonal effect on staining intensity of immunoreactive cells ( $F_{2,9} = 0.43$ ,  $p = 0.43$ ). There was a significant effect of season on the number of immunoreactive cells ( $F_{2,9} = 5.20$ ,  $p = 0.03$ ), the size of these cells ( $F_{2,9} = 5.71$ ,  $p = 0.03$ ), and the immunoreactive fiber area ( $F_{2,9} = 9.72$ ,  $p < 0.01$ ). In all three cases, post-hoc analyses (Fisher's PLSD) indicated that brains collected in May differed significantly from those collected in October and January ( $p < 0.05$ ).

#### *Song-Control System*

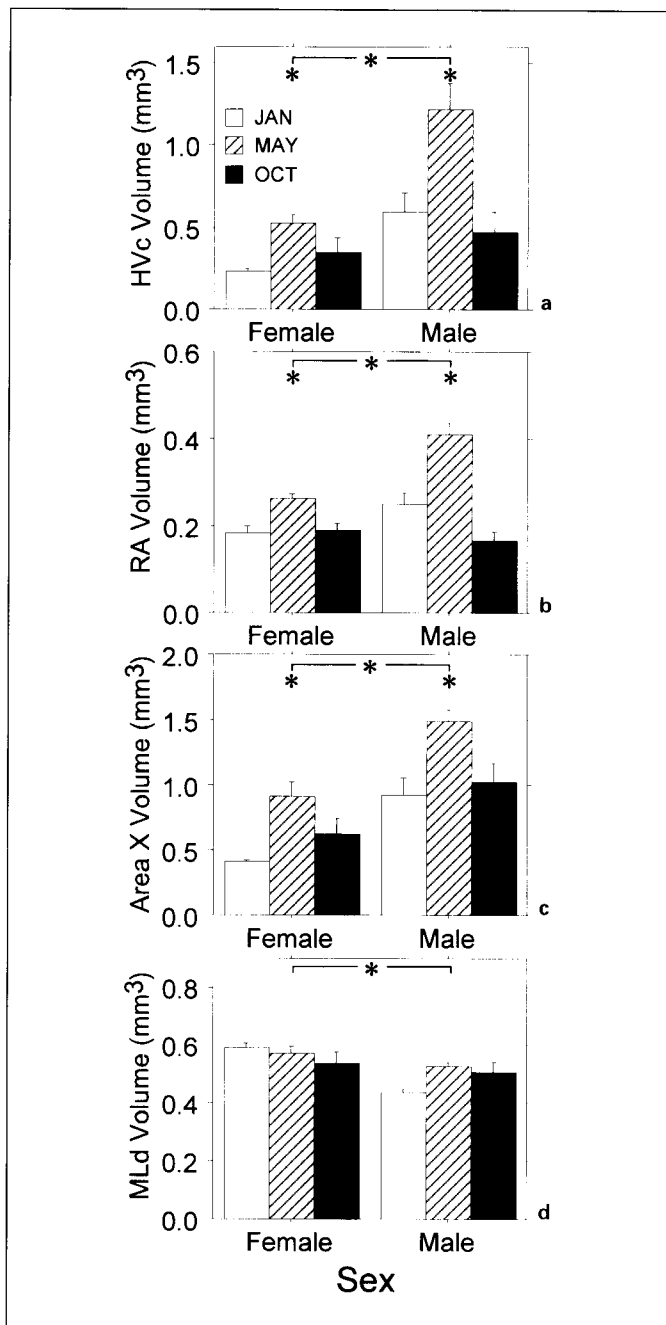
Both male and female crossbills exhibited seasonal changes in the song-control system, but neither in a control nucleus (MLd) nor in overall brain mass (fig. 3). Because there were no statistically significant hemispheric differences in the left and right volumes of the song-control nuclei, we used the average of the left and right volumes for each bird in the following analyses. HVC and RA volumes

**Table 1.** F-statistics for two-way ANOVAs comparing effects of sex and time of year on volume of three song-control nuclei (HVC, Area X and RA), volume of a control nucleus (MLd), and total brain mass

Brain region	Effect		
	Sex	Time of year	Sex $\times$ time of year
HVC	$F_{1,16} = 24.4^{***}$	$F_{2,16} = 14.3^{***}$	$F_{2,16} = 4.02^*$
Area X	$F_{1,15} = 32.4^{***}$	$F_{2,15} = 12.9^{***}$	$F_{2,15} = 0.34$ (ns)
RA	$F_{1,15} = 16.8^{***}$	$F_{2,15} = 38.6^{***}$	$F_{2,15} = 10.1^{**}$
MLd	$F_{1,16} = 10.45^{**}$	$F_{2,16} = 0.88$ (ns)	$F_{2,16} = 2.43$ (ns)
Total brain mass	$F_{1,18} = 2.13$ (ns)	$F_{2,18} = 0.58$ (ns)	$F_{2,18} = 1.54$ (ns)

\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.0001$ ; ns = not statistically significant.

were sexually dimorphic and changed seasonally, and in both nuclei there was a significant sex by season interaction (table 1, fig. 4). Area X volumes also differed between males and females and among sampling dates but there was no significant interaction between these variables (table 1, fig. 4). Post-hoc analyses indicated that HVC, RA, and area X volumes in May were significantly larger than in January or October (Fisher's PLSD;  $p < 0.05$ ; fig. 4). MLd volumes



**Fig. 4.** Volumes of three song-control nuclei: **a** HVC; **b** RA, and a control nucleus; **c** Area X; **d** MLd from male and female white-winged crossbills collected at three times of year. Bars represent mean  $\pm$  SE. Asterisks indicate means that differ significantly ( $p < 0.05$ ).

did not change seasonally but were slightly larger in females than males (table 1, fig. 4). There was no significant sex by season interaction for this nucleus. Brain mass did not vary either seasonally or between the sexes (table 1).

To explore further the significant interaction between sex and season on HVC and RA volume, we ran separate one-way ANOVAs on the data from males and females. For males, there was a significant effect of season on both HVC ( $F_{2,6} = 12.7$ ,  $p = 0.007$ ) and RA ( $F_{2,6} = 25.4$ ,  $p = 0.001$ ). Post-hoc analyses again indicated that song control nuclei were significantly larger in May than either in January or in October (Fisher's PLSD;  $p < 0.05$ ). Similarly, for females there was a significant effect of season on both HVC ( $F_{2,9} = 5.6$ ,  $p = 0.03$ ) and RA ( $F_{2,9} = 9.5$ ,  $p = 0.006$ ) volume. Post-hoc analyses indicated that HVC was significantly larger in May females than in January or October females, but for RA only January and May nucleus volumes differed significantly (Fisher's PLSD;  $p < 0.05$ ). Taken together, we interpret the significant interaction terms as indicating that although there were significant seasonal changes in song-control nuclei volume in both sexes, these changes were of significantly larger magnitude in males than in females (see fig. 4).

## Discussion

The white-winged crossbills in this study showed modest seasonal changes in the condition of the GnRH system, but seasonal changes and sex differences in the sizes of song control nuclei. The relatively small changes in the GnRH system compared with other temperate zone songbirds are interesting because crossbills are opportunistic breeders, capable of nesting almost all year round, especially at mid-latitudes [Benkman, 1992]. The seasonal changes in the song-control system are interesting because large song nucleus volumes occurred only on long days (May), as observed in many seasonally-breeding birds, and not in January even though crossbills can breed this early at mid-latitudes [Benkman, 1992]. The sex differences in song-control nuclei volumes add white-winged crossbills to the list of songbirds with sexually dimorphic singing behavior that also have dimorphic song nuclei [reviewed in MacDougall-Shackleton and Ball, 1999].

### *Seasonal Changes in the GnRH System*

Most seasonally breeding birds studied thus far eventually become absolutely refractory to the stimulatory effects of long days [Nicholls et al., 1988; Wilson and Donham, 1988]. In these species, refractoriness typically correlates

with a dramatic decrease in the amount of GnRH in the hypothalamus, as compared to levels in photosensitive and/or photostimulated birds [reviewed in Ball and Hahn, 1997; Hahn et al., 1997]. This centrally-mediated down-regulation of reproductive physiology has been documented in European starlings [*Sturnus vulgaris*; Dawson et al., 1985; Foster et al., 1987], garden warblers [*Sylvia borin*; Bluhm et al., 1991], dark-eyed juncos [*Junco hyemalis*; Saldanha et al., 1994; Deviche et al., 2000], house sparrows [*Passer domesticus*; Hahn and Ball, 1995], American tree sparrows [*Spizella arborea*; Reinert and Wilson, 1996; Wilson and Reinert, 1996], house finches [*Carpodacus mexicanus*; Cho et al., 1998] and Gambel's white-crowned sparrows [*Zonotrichia leucophrys gambelii*; Wingfield and Farner, 1993; but see Meddle et al., 1999]. On the whole, these data suggest that the hypothalamo-pituitary-gonad axis might be switched off at the hypothalamic level during refractoriness in many species, either precluding or reducing reproductive responses to environmental cues.

White-winged crossbills represent an interesting case for cross-species comparisons owing to their opportunistic breeding tendencies. Crossbills are cardueline finches closely related to some of the other songbirds described above (particularly house finches, another cardueline), but they feed their young conifer seeds that they may discover in abundance on an unpredictable schedule. At middle latitudes, these seeds can be sufficiently abundant to support successful breeding during most of the year [Newton, 1972; Benkman, 1987, 1990]. Furthermore, neither white-winged [Deviche and Sharp, 2001] nor red [*Loxia curvirostra*; Hahn, 1995, 1998] crossbills appear to become absolutely refractory. Thus, crossbills are an excellent group in which to examine the relationship between seasonal changes in responsiveness to environmental cues (development and dissipation of refractoriness) and seasonal plasticity of the GnRH system [see Ball and Hahn, 1997; Hahn et al., 1997]. The patterns observed here are similar to those observed in Japanese quail (*Coturnix japonica*, a non-passerine bird). Japanese quail breed seasonally, but retain the physiological capacity to respond to long days throughout the year [Robinson and Follett, 1982]. Termination of breeding by quail in late summer occurs when they become relatively refractory. At this time they are less responsive to long days than they were in the spring, but very long days can maintain or reinstate reproductive competence [Robinson and Follett, 1982]. Also, Japanese quail show no decrease in hypothalamic GnRH when relatively refractory [Foster et al., 1988]. Our data suggest that white-winged crossbills could be more similar to Japanese quail than to the seasonally breeding songbirds studied thus far. Changes in the

GnRH system of crossbills were of lesser amplitude than observed in house finches, a closely related species that becomes absolutely refractory [Cho et al., 1998]. Prior research indicated that male house finches had about an order of magnitude greater number of GnRH-immunoreactive cells in May compared to October [Cho et al., 1998]. In this study, white-winged crossbills had only 1.4–1.5 times as many GnRH-immunoreactive cells in May compared to October. Likewise, changes in all other measures of GnRH system status were more pronounced in house finches than in crossbills. Although alternative explanations need to be considered (discussed below), crossbills clearly show less seasonal variation in GnRH than any other songbird yet studied.

The data reported here support a previously proposed hypothesis [Hahn et al., 1997; Ball and Hahn, 1997] linking species variation in seasonal GnRH plasticity with species variation in degree of photorefractoriness. Unlike seasonally breeding songbirds, white-winged crossbills could maintain GnRH production, though not necessarily secretion, year-round. This might allow them to develop reproductive competence whenever they discover favorable conditions. The modest reduction in GnRH observed here could reflect either a lack of photorefractoriness or only relative refractoriness. In contrast, species that become absolutely refractory down-regulate hypothalamic GnRH availability dramatically, which probably precludes induction of reproductive competence during this state. According to this explanation, then, the differences between this study and other studies of seasonally breeding songbirds reflect functional differences in GnRH plasticity, photorefractoriness mechanisms, and/or reproductive flexibility.

There are several potential alternative explanations for the relative lack of seasonal changes in GnRH observed here; we discuss three of these below. First, it is possible that the birds became absolutely refractory and had lower hypothalamic GnRH at some time between our sampling dates in May and October, or between October and January. We suggest this is unlikely given the regular breeding of both white-winged and red crossbills through the summer months and well into September/October [Benkman, 1990, 1992; Deviche, 1997, 2000; Hahn, 1998]. However, further studies with a more finely resolved time scale would be required to test this hypothesis conclusively.

A second alternative explanation for the difference in results between this study and those found in house finches [Cho et al., 1998] is that the house finches were free-living breeding and refractory individuals, whereas the crossbills studied here were held in captivity. Thus, we could have failed to detect large changes in GnRH because the birds



were not maximally stimulated in May by natural breeding conditions. This explanation also seems unlikely. In May, house finches and crossbills had similar numbers of immunoreactive GnRH cells (crossbills: mean  $\pm$  SE = 191.0  $\pm$  20.1; house finches: mean  $\pm$  SE = 236.3  $\pm$  30.1), so the wild house finches did not appear to be dramatically more stimulated than the captive crossbills. The species difference resulted from the fact that the house finches had markedly reduced numbers of immunoreactive cells in October (mean  $\pm$  SE = 24.3  $\pm$  0.2) whereas the crossbills did not (mean  $\pm$  SE = 137.3  $\pm$  31.1). Thus, crossbills maintained relatively stable high levels of GnRH, not relatively stable low levels.

A third alternative explanation is that the continuous ad libitum food provided to the captive crossbills in autumn induced stimulation that was absent in the wild house finches. The benign temperatures and unlimited food might have sustained year-round high levels of GnRH in the crossbills. When photorefractory, other songbirds housed in captivity and provided with ad libitum food show a large reduction of GnRH immunoreactivity [e.g., dark-eyed juncos; Saldanha et al., 1994; Deviche et al., 2000]. However, crossbills might differ from these seasonal breeders by having the ability to respond to cues such as abundant food even during autumn and winter. Comparison studies of GnRH plasticity in wild crossbills and/or captive crossbills given varying amounts of food will be undertaken in the future to resolve this issue.

#### *Sex Differences in the GnRH System*

Because we ran the ICC for each sex at different times, we did not evaluate potential sex differences in the GnRH system statistically. However it does appear that the patterns of seasonal change in the GnRH system could differ between the sexes. In particular, cell numbers and fiber staining intensities in males were similar in January and May, but in females were most similar in January and October. These differences warrant further exploration, as they might relate to differences in reproductive strategy between the sexes. The relatively greater number of immunoreactive cell bodies and more substantial fiber staining of January males could indicate that at this time these males have enhanced readiness compared to females to respond quickly to stimulatory cues such as increased food or behavioral stimulation from conspecifics. Consistent with this conclusion, plasma LH in free-living adult male white-winged crossbills increased between September–October and November when food was locally abundant, but in adult females did not change seasonally between September and June of the following year [Deviche and Sharp, 2001].

#### *Seasonal Changes in the Song-Control System*

Seasonal changes in the song-control system of passerine birds were first described for canaries (*Serinus canaria*), another species of cardueline finch [Nottebohm, 1981]. Since then, seasonal differences and/or photoperiod-induced changes in the song-control system have been documented in many other seasonally breeding songbirds [reviewed in Ball, 1999; Tramontin and Brenowitz, 2000]. Our data on crossbills are consistent with those from typical seasonal breeders: song systems of both sexes were enlarged only when days were long. The results are notable because, unlike most other temperate zone songbirds, white-winged crossbills frequently attain full reproductive activity as early as January under favorable conditions at middle latitudes [Benkman, 1990, 1993]. The fact that our captive birds were not in breeding condition in January probably accounts for the lack of song system development at that time. Thus, although crossbills can breed in winter they do not maintain fully enlarged song-control nuclei when they are not breeding during short days. This contrasts to the GnRH system, which showed little seasonal variation. These findings suggest that in crossbills, as in several seasonally breeding songbirds, the major hormone regulating seasonal variation in the song-control system is gonadal testosterone [e.g. Smith et al., 1997a; see Ball, 1999, for review]. Photoperiod-mediated and gonadal steroid-independent changes in the song-control system have been identified in some sparrow species and in starlings [e.g. Bernard et al., 1997; Smith et al., 1997b; Bentley et al., 1999; Deviche and Gullledge, 2000; Dloniak and Deviche, 2001]. Because crossbills can breed on both the shortest and longest days of the year, comparing the song-control system of winter- and summer-breeding individuals could be particularly useful for disentangling gonadal steroid-dependent and -independent effects of photoperiod.

#### *Sex Differences in the Song-Control System*

Sexual dimorphisms of the song system of behaviorally dimorphic songbirds have been well documented [Nottebohm and Arnold, 1976; reviewed in MacDougall-Shackleton and Ball, 1999]. The degree of song behavior dimorphism has not been quantified in crossbills. In red crossbills both sexes sing, but male breeding song is much louder and appears more stereotyped and more elaborate than female song [T.P. Hahn, unpubl. observ.]. The degree of sex dimorphism in the song-control nuclei of white-winged crossbills is intermediate between that observed in extremely dimorphic species in which females never sing, and monomorphic species in which females duet with males [reviewed in MacDougall-Shackleton and Ball, 1999]. In May, HVc and

RA in females were 0.43 and 0.64 times the volume of HVC and RA, respectively, in males. Thus, the degree of sexual dimorphism in crossbills is similar to that in other species in which females sing, but less often and with a smaller song repertoire than males [e.g. European starlings, *Sturnus vulgaris*, Bernard et al., 1993; rufous-and-white wren, *Thryothorus rufalbus*, Brenowitz and Arnold, 1986]. White-winged crossbills, then, likely conform to the pattern observed among species with respect to the degree of sex difference in singing behavior and in the song-control system.

## References

- Arnold, A.P. (1992) Developmental plasticity in neural circuits controlling birdsong: sexual differentiation and the neural basis of learning. *J. Neurobiol.*, *23*: 1506–1528.
- Ball, G.F. (1993) The neural integration of environmental information by seasonally breeding birds. *Am. Zool.*, *33*: 185–199.
- Ball, G.F. (1999) The neuroendocrine basis of seasonal changes in vocal behavior among songbirds. In *The Design of Animal Communication* (ed. by M.D. Hauser and M. Konishi), MIT Press, Cambridge, Mass., pp. 213–253.
- Ball, G.F., and T.P. Hahn (1997) GnRH neuronal systems in birds and their relation to the control of seasonal reproduction. In *GnRH Neurons: Gene to Behavior* (ed. by I.S. Parhar and Y. Sakuma), Brain Shuppan, Tokyo, pp. 325–342.
- Benkman, C.W. (1987) Food profitability and the foraging ecology of crossbills. *Ecol. Monogr.*, *57*: 251–267.
- Benkman, C.W. (1990) Foraging rates and the timing of crossbill reproduction. *Auk*, *107*: 376–386.
- Benkman, C.W. (1992) White-winged Crossbill. In *The Birds of North America No. 27* (ed. by A. Poole, P. Stettenheim, and F. Gill), The Academy of Natural Sciences, Philadelphia, and The American Ornithologists Union, Washington, D.C.
- Benkman, C.W. (1993) Adaptation to single resources and the evolution of crossbill (*Loxia curvirostra*) diversity. *Ecol. Monogr.*, *63*: 305–323.
- Bentley, G.E., T. Van't Hof, and G.F. Ball (1999) Seasonal neuroplasticity in the songbird telencephalon: a novel role for melatonin. *Proc. Natl. Acad. Sci.*, *96*: 4674–4679.
- Bentley, G.E., J.C. Wingfield, M.L. Morton, and G.F. Ball (2000) Stimulatory effects on the reproductive axis in female songbirds by conspecific and heterospecific male song. *Horm. Behav.*, *37*: 179–189.
- Bernard, D.J., J.M. Casto, and G.F. Ball (1993) Sexual dimorphism in the volume of song control nuclei in European starlings: assessment by a Nissl stain and autoradiography for muscarinic cholinergic receptors. *J. Comp. Neurol.*, *334*: 559–570.
- Bernard, D.J., F.E. Wilson, and G.F. Ball (1997) Testis-dependent and -independent effects of photoperiod on volumes of song control nuclei in American tree sparrows (*Spizella arborea*). *Brain Res.*, *760*: 163–169.
- Berthold, P., and E. Gwinner (1978) Jahresperiodik der Gonadengröße beim Fichtenkreuzschnabel (*Loxia curvirostra*). *J. Ornithol.*, *119*: 338–339.
- Bluhm, C.K., H. Schwabl, I. Schwabl, A. Perera, B.K. Follett, A.R. Goldsmith, and E. Gwinner (1991) Variation in hypothalamic gonadotropin-releasing hormone content, plasma and pituitary LH, and in-vitro testosterone release in a long-distance migratory bird, the garden warbler (*Sylvia borin*), under constant photoperiods. *J. Endocrinol.*, *128*: 339–345.
- Brenowitz, E.A., and A.P. Arnold (1986) Interspecific comparisons of the size of neural song control regions and song complexity in duetting birds: evolutionary implications. *J. Neurosci.*, *6*: 2875–2879.
- Brenowitz, E.A., D. Margoliash, and K.W. Nordeen (1997) An introduction to birdsong and the avian song system. *J. Neurobiol.*, *33*: 495–500.
- Cho, R.N., T.P. Hahn, S.A. MacDougall-Shackleton, and G.F. Ball (1998) Seasonal variation in brain GnRH in free-living breeding and photorefractory house finches (*Carpodacus mexicanus*). *Gen. Comp. Endocrinol.*, *109*: 244–250.
- Coombs-Hahn, T.P. (1993) Integration of environmental cues to time reproduction in an opportunistic breeder, the red crossbill (*Loxia curvirostra*). PhD dissertation, University of Washington, Seattle, Washington.
- Crain, R.D. (1998) Environmental influence on reproduction in two passerine birds, the dark-eyed junco and the white-winged crossbill. Unpublished M.S. Thesis, University of Alaska Fairbanks, Fairbanks, Alaska.
- Dawson, A., B.K. Follett, A.R. Goldsmith, and T.J. Nicholls (1985) Hypothalamic gonadotropin-releasing hormone and pituitary and plasma FSH and prolactin during photostimulation and photorefractoriness in intact and thyroidectomized starlings (*Sturnus vulgaris*). *J. Endocrinol.*, *105*: 71–77.
- Deviche, P. (1997) Seasonal reproductive pattern of white-winged crossbills in interior Alaska. *J. Field Ornithol.*, *68*: 613–621.
- Deviche P. (2000) Timing, pattern, and extent of first prebasic molt of white-winged crossbills in Alaska. *J. Field Ornithol.*, *71*: 217–226.
- Deviche P., and C.C. Gullledge (2000) Vocal control region sizes of an adult female passerine songbird change seasonally in the absence of detectable circulating testosterone. *J. Neurobiol.*, *42*: 202–211.
- Deviche P., and P.J. Sharp (2001) Reproductive endocrinology of a free-living, opportunistically breeding passerine (white-winged crossbill, *Loxia leucoptera*). *Gen. Comp. Endocrinol.*, *123*: 268–279.
- Deviche P., C.J. Saldanha, and R. Silver (2000) Changes in brain gonadotropin-releasing hormone- and vasoactive intestinal polypeptide-like immunoreactivity accompanying re-establishment of photosensitivity in male dark-eyed juncos (*Junco hyemalis*). *Gen. Comp. Endocrinol.*, *117*: 8–19.
- Dloniak, S.M., and P. Deviche (2001) Effects of testosterone and photoperiodic condition on song production and vocal control region volumes in adult male dark-eyed juncos (*Junco hyemalis*). *Horm. Behav.*, *39*: 95–105.
- Farner, D.S., and E. Gwinner (1980) Photoperiodicity, circannual and reproductive cycles. In *Avian Endocrinology* (ed. by A. Epple and M.H. Stetson), Academic Press, New York, pp. 331–366.
- Follett, B.K. (1984) Birds. In 'Marshall's' *Physiology of Reproduction*, Vol. 1 (ed. by G.E. Lamming), Longman Green, Edinburgh, pp. 283–350.
- Foster, R., G. Plowman, A.R. Goldsmith, and B.K. Follett (1987) Immunohistochemical demonstration of marked changes in the luteinizing hormone-releasing hormone system of photosensitive and photorefractory European starlings. *J. Endocrinol.*, *115*: 211–220.
- Fowells, H.A. (1965) Silvics of forest trees in the United States. USDA Agricultural Handbook No. 271.
- Gullledge, C.C., and P. Deviche (1998) Photoperiod and testosterone independently affect vocal control region volumes in adolescent male songbirds. *J. Neurobiol.*, *36*: 550–558.

## Acknowledgments

We thank Cynthia Gullledge for logistic support. The primary antibody was kindly provided by Dr. Henryk Urbanski, Division of Neuroscience, Oregon Regional Primate Center, Beaverton, Oreg. Financial support was provided by NSF BNS-9121258 to PJD, NSF IBN 9905401 and NIH NS 35467 to GFB, NSF IBN 9808470 to TPH, and postdoctoral fellowships from NIMH (NRSA) and NSERC Canada to SAM-S.

- Hahn, T.P. (1995) Integration of photoperiodic and food cues to time changes in reproductive physiology by an opportunistic breeder, the red crossbill, *Loxia curvirostra* (Aves: carduelinae). *J. Exp. Zool.*, 272: 213–226.
- Hahn T.P. (1998) Reproductive seasonality in an opportunistic breeder, the red crossbill, *Loxia curvirostra*. *Ecology*, 79: 2365–2375.
- Hahn, T.P., and G.F. Ball (1995) Changes in brain GnRH associated with photorefractoriness in house sparrows (*Passer domesticus*). *Gen. Comp. Endocrinol.*, 99: 349–363.
- Hahn, T.P., J.C. Wingfield, R. Mullen, and P.J. Devische (1995) Endocrine bases of spatial and temporal opportunism in arctic-breeding birds. *Am. Zool.*, 35: 259–273.
- Hahn, T.P., T. Boswell, J.C. Wingfield, and G.F. Ball (1997) Temporal flexibility in avian reproduction: patterns and mechanisms. *Curr. Ornithol.*, 14: 39–80.
- Lack, D. (1968) Ecological adaptations for breeding in birds. Methuen, London.
- MacDougall-Shackleton, S.A., and G.F. Ball (1999) Comparative studies of sex differences in the song-control system of songbirds. *Trends Neurosci.*, 22: 432–436.
- MacDougall-Shackleton, S.A., E.A. MacDougall-Shackleton, and T.P. Hahn (2001) Physiological and behavioural responses of female mountain white-crowned sparrows to natal- and foreign-dialect songs. *Can. J. Zool.*, 79: 325–333.
- Marsh, R.H., S.A. MacDougall-Shackleton, and T.P. Hahn (1999) Seasonal changes in brain GnRH and song control nuclei in a late-summer breeding songbird. *Soc. Neurosci. Abstracts*, 25: 864.
- Marshall, A.J. (1959) Internal and environmental control of breeding. *Ibis*, 101: 456–478.
- Marshall, A.J. (1970) Environmental factors other than light involved in the control of sexual cycles in birds and mammals. *In* La Photorégulation de la Reproduction chez les Oiseaux et les Mammifères (ed. by J. Benoit and I. Assenmacher), Editions du Centre National de la Recherche Scientifique, Paris, pp. 53–64.
- Meddle, S.L., D.L. Maney, and J.C. Wingfield (1999) Effects of N-methyl-D-aspartate on luteinizing hormone release and Fos-like immunoreactivity in the male white-crowned sparrow (*Zonotrichia leucophrys gambelii*). *Endocrinology*, 140: 5922–5928.
- Morton, M.L., M.E. Pereyra, and L.F. Baptista (1985) Photoperiodically induced ovarian growth in the white-crowned sparrow (*Zonotrichia leucophrys gambelii*) and its augmentation by song. *Comp. Biochem. Physiol.*, 80: 93–97.
- Newton, I. (1972) Finches. Taplinger, Engelwood Cliffs, N.J.
- Nicholls, T.J., A.R. Goldsmith, and A. Dawson (1988) Photorefractoriness in birds and comparison with mammals. *Physiol. Rev.*, 68: 133–176.
- Nottebohm, F. (1981) A brain for all seasons: cyclical anatomical changes in song control nuclei of the canary brain. *Science*, 214: 1368–1370.
- Nottebohm, F., and A.P. Arnold (1976) Sexual dimorphism in vocal control areas of the songbird brain. *Science*, 194: 211–213.
- Reinert, B.D., and F.E. Wilson (1996) The thyroid and the hypothalamus-pituitary-ovarian axis in American tree sparrows (*Spizella arborea*). *Gen. Comp. Endocrinol.*, 103: 60–70.
- Robinson, J.E., and B.K. Follett (1982) Photoperiodism in Japanese quail: the termination of seasonal breeding by photorefractoriness. *Proc. R. Soc. Lond. B*, 215: 95–116.
- Saldanha, C.J., P.J. Devische, and R. Silver (1994) Increased VIP and decreased GnRH expression in photorefractory dark-eyed juncos (*Junco hyemalis*). *Gen. Comp. Endocrinol.*, 93: 128–136.
- Silverman, A.-J., I. Livnev, and J.W. Witkin (1994) The gonadotropin-releasing hormone (GnRH), neuronal systems: immunocytochemistry and in situ hybridization. *In* The Physiology of Reproduction, ed. 2 (ed. by E. Knobil and J.D. Neill), Raven Press, New York, pp. 1683–1709.
- Smith, G.T., E.A. Brenowitz, and J.C. Wingfield (1997a) Seasonal changes in testosterone, neural attributes of song control nuclei, and song structure in wild songbirds. *J. Neurosci.*, 17: 6001–6010.
- Smith, G.T., E.A. Brenowitz, and J.C. Wingfield (1997b) Roles of photoperiod and testosterone in seasonal plasticity of the avian song control system. *J. Neurobiol.*, 32: 426–442.
- Stokes, T.M., C.M. Leonard, and F. Nottebohm (1974) The telencephalon, diencephalon and mesencephalon of the canary, *Serinus canarius*, in stereotaxic coordinates. *J. Comp. Neurol.*, 156: 337–374.
- Tramontin, A.D., and E.A. Brenowitz (2000) Seasonal plasticity in the adult brain. *Trends Neurosci.*, 23: 251–258.
- Urbanski, H.F. (1992) Photoperiod and modulation of luteinizing hormone secretion in orchidectomized Syrian hamsters and the influence of excitatory amino acids. *Endocrinology*, 131: 1665–1669.
- Urbanski, H.F., S.O. Kim, and M.L. Connolly (1990) Influence of photoperiod and 6-methoxybenzoxazolinone on the reproductive axis of inbred LSH/Ss Lak male hamsters. *J. Reprod. Fert.*, 90: 157–163.
- Wilson, F.E., and R.S. Donham (1988) Daylength and control of seasonal reproduction in male birds. *In* Processing of Environmental Information in Vertebrates (ed. by M.H. Stetson), Springer-Verlag, Berlin, pp. 101–120.
- Wilson, F.E., and B.D. Reinert (1996) The timing of thyroid-dependent programming in seasonally breeding male American tree sparrows (*Spizella arborea*). *Gen. Comp. Endocrinol.*, 103: 82–92.
- Wingfield, J.C. (1980) Fine temporal adjustments of reproductive function. *In* Avian Endocrinology (ed. by A. Epple and M.H. Stetson), Academic Press, New York, pp. 367–389.
- Wingfield, J.C. (1983) Environmental and endocrine control of reproduction: an ecological approach. *In* Avian Endocrinology: Environmental and Ecological Perspectives (ed. by S.I. Mikami, K. Homma and M. Wada), Japan Scientific Society Press, Springer-Verlag, Berlin, pp. 265–288.
- Wingfield, J.C., and D.S. Farner (1993) Endocrinology of reproduction in wild species. *Avian Biol.*, 9: 163–327.
- Wingfield, J.C., and G.J. Kenagy (1991) Natural regulation of reproductive cycles. *In* Vertebrate Endocrinology: Fundamentals and Biomedical Implications (ed. by M. Schreiber and R.E. Jones), Academic Press, New York, pp. 181–241.
- Wingfield, J.C., T.P. Hahn, and D. Doak (1993) Integration of environmental factors regulating transitions of physiological state, morphology and behaviour. *In* Avian Endocrinology (ed. by P.J. Sharp), J. Endo. Ltd., Bristol, UK, pp. 111–122.
- Wingfield, J.C., T.P. Hahn, R. Levin, and P. Honey (1992) Environmental predictability and control of gonadal cycles in birds. *J. Exp. Zool.*, 261: 214–231.