Androgen Control of Vocal Control Region Volumes in a Wild Migratory Songbird (*Junco hyemalis*) Is Region and Possibly Age Dependent

Cynthia C. Gulledge, Pierre Deviche

Institute of Arctic Biology, University of Alaska Fairbanks, P.O. Box 757000, Fairbanks, Alaska 99775-7000

Received 15 July 1996; accepted 6 November 1996

ABSTRACT: Previous laboratory studies have shown that photoperiodic adult songbirds experience seasonal variations in singing frequency that correlate with plasma androgen levels, as well as changes in the brain regions that control singing (vocal control regions). The present study investigates naturally occurring seasonal changes in the sizes of these regions in a wild migratory species (dark-eyed junco, *Junco hyemalis*), with samples from adolescence to post-breeding fall migration. In adult males, the volumes of the vocal control regions area X and the higher vocal center (HVC) were large during the breeding season when birds were singing and androgen levels were high, and decreased in size after the breeding season when singing had stopped and androgen levels were low. HVC volume in adolescent males caught in the fall (no singing), when plasma androgen levels were low, was smaller than in breeding adults, thereby following the seasonal pattern of change in plasma androgen levels. In adolescent males, however, area X volume was the same as in breeding adults. Thus, area X size in adolescent male juncos may be testosterone independent. The seasonal pattern of robust nucleus of the archistriatum volume was similar to that of the HVC. The volumes of neither the magnocellular nucleus of the anterior neostriatum nor the nucleus rotundus, a control region, differed seasonally. Castration of breeding adult males caused both area X and HVC volumes to decrease compared to castrated controls with testosterone replacement, indicating that maintenance of these two region volumes is testosterone dependent in adults. © 1997 John Wiley & Sons, Inc. *J Neurobiol* 32: 391–402, 1997.

Keywords: **seasonality; testosterone; song; storage phase; junco**

INTRODUCTION

In oscines, both song learning and expression are controlled by an interconnected set of brain regions called the vocal control system (Nottebohm et al., 1976; reviewed in Konishi, 1994). The best-studied vocal control regions (VCRs) are the higher vocal center (HVC), robust nucleus of the archistriatum (RA), lateral magnocellular nucleus of the anterior neostriatum (lMAN), and area X. The HVC and RA are essential for song expression (Nottebohm et al., 1976). Area X and lMAN are necessary for song development but not song production (Nottebohm et al., 1976; Bottjer et al., 1984; Sohrabji et al., 1990; Scharff and Nottebohm, 1991).

Songbirds learn to sing in stages (reviewed by Marler, 1991), the timing of which varies among species. Young birds memorize the song of a tutor (sensory phase), then store that information until they begin to practice the memorized song in a form known as ''plastic song'' (sensory-motor phase), which sounds similar to the song produced by adults but is more variable and usually not as loud. Once the bird develops the plastic song into a stable, stereotyped form, the song is referred to as ''crystal-

Correspondence to: C. Gulledge

Contract grant sponsor: National Science Foundation; contract grant number: BNS-9121258

 CCC 0022-3034/97/040391-12

lized'' (motor phase). Once song is crystallized, area X and lMAN appear not to play any further role in song expression (Bottjer et al., 1984; Nottebohm et al., 1976). Even so, area X occupies a large portion of the surrounding lobus parolfactorius throughout adulthood (Nottebohm et al., 1976, 1986).

During ontogeny and, in some species, between breeding seasons, VCRs grow rapidly as a result of both new neuron incorporation and increases in the sizes of existing cells (Nordeen et al., 1989; Brenowitz et al., 1991; Alvarez-Buylla et al., 1992; Smith et al., 1995). Once VCRs attain maximal size, their volumes are maintained by extending cell survival and by neuronal replacement (Alvarez-Buylla et al., 1992). Maintaining large HVC, RA and area X may be necessary for producing crystallized song or for storing learned song, respectively. In many seasonally breeding species, large VCR volumes are maintained during the breeding (singing) season but not afterward, when plasma concentrations of gonadal steroids decrease (Nottebohm et al., 1986; Smith, 1996; Brenowitz et al., 1996). This observation suggests that sex steroids play a role in maintaining region sizes. One study found that testosterone (T) administration increases RA volume in female canaries (*Serinus canaria*), and that subsequent removal of exogenous T reverses that increase in RA size, suggesting that chronically elevated levels of T are necessary to maintain the enlarged volume (Brown and Bottjer, 1993). No study, however, has directly investigated the role of steroid hormones in maintenance of naturally large VCR volumes. To address this issue, the present study includes a castration and T-replacement experiment using breeding male dark-eyed juncos (*Junco hyemalis*) to determine whether breeding season concentrations of plasma T are necessary to maintain VCR volumes.

Laboratory studies on male photoperiodic songbirds have found that in adults, seasonal patterns in song production correlate with plasma androgen levels, as well as with changes in VCR volumes (Nottebohm, 1981; Brenowitz et al., 1991; Smith et al., 1995). Little is known, however, about the relationship between gonadal steroids and VCR volumes during the developmental period between song memorization in juveniles and their first breeding season as adults, a stage referred to as ''adolescence'' (Nordeen and Nordeen, 1989). A few studies have investigated seasonal and developmental aspects of the vocal control system during adolescence (swamp sparrow, *Melospiza melodia:* Nordeen et al., 1989; Marler et al., 1987, 1988; canary: Nottebohm et al., 1986; white-crowned sparrow, *Zonotrichia leucophrys nuttalli:* Whaling et al., 1995). None of these studies, however, simultaneously measured VCR volumes and circulating androgen concentrations. Thus, whereas it is clear that VCR volumes in captive adult males correlate with plasma androgen levels (Bernard and Ball, 1995; Brenowitz et al., 1991), this relationship in adolescent birds remains unclear.

Also unclear is whether free-living birds experience the same seasonal changes in VCR volumes as those seen in captive birds exposed to breeding and nonbreeding photoperiods. A previous study investigated seasonal VCR changes in free-living red-winged blackbirds (*Agelaius phoeniceus*) and found that in males, only the tracheosyringeal portion of the hypoglossal nucleus (which innervates the sound-producing organ, the syrinx) (Konishi, 1994) was larger in the breeding season than in the following fall, although volume changes were detected in other VCRs of captive blackbirds exposed to long versus short photoperiods (Kirn et al., 1989). Investigators, however, have recently reported seasonal changes in VCR volumes of wild adult males (rufous-sided towhees, *Pipilo erythrophthalmus:* Smith, 1996 ; Nuttall's white-crowned sparrows: Brenowitz et al., 1996).

We investigated the role of androgens in regulating VCR volumes in a wild population of male dark-eyed juncos, a photoperiodic, seasonally breeding species (Deviche, 1995). For this, we determined whether VCR volumes and plasma androgen levels correlate seasonally in the wild and whether this relationship is similar in adult and adolescent males. In addition, we determined whether VCR volume maintenance in male adults is T dependent.

MATERIALS AND METHODS

Seasonal Study

Animal Collection. Male dark-eyed juncos migrate to the same territory each breeding season. They arrive in interior Alaska in early May, breed in June, molt in August, and begin emigrating in September (Deviche, unpublished data). Males were collected near Fairbanks, Alaska (64°N), using mist nets or Potter traps during three different life stages: adolescents in September (no singing, migrating, approximately 2–3 months of age, light/dark cycle approximately 12:12, $n = 9$); adults in June (singing and breeding, on breeding territories, light/dark cycle approximately 21:3, $n = 6$); and adults in October (rarely singing, postbreeding and postmolt, migrating, light/dark cycle approximately 10:14, $n = 5$). Birds collected in June were second-year males, meaning

that they hatched the year before and were experiencing their first breeding season. The alternate plumage of such males differs from that of older males during the breeding season, but not after molt into basic plumage (Pyle et al., 1987). The age of adult males caught in October therefore could not be determined, but these males could be differentiated from juveniles based on plumage, eye color, and amount of skull pneumatization (Pyle et al., 1987). All necessary permits were obtained prior to the collection of the birds used in this study. A blood sample (approximately 250 μL) was collected from a wing vein immediately after capture and kept on ice until processed later the same day.

Brain Processing. Within hours of capture, birds received an overdose of anesthetic (ketamine–xylacine), then an intracardial injection of heparin [0.3 mL; 1000 IU/mL in 0.1 *M* phosphate buffer (PB) solution], followed by room temperature 0.1 *M* PB (20 mL) and cold 4% paraformaldehyde solution in 0.1 *M* PB (25 mL). The brains were postfixed in 4% paraformaldehyde solution overnight, dissected from skulls, and transferred to PB containing 0.1% Na azide for 3 days. Brains were then transferred to 30% sucrose solution in PB with Na azide for 10 days, after which they were blotted dry and weighed. Brains were coated with embedding matrix (M-1; Lipshaw, Pittsburgh, PA) and frozen in powdered dry ice, then stored at −70°C until processed further. They were coronally sectioned (50 μm) onto gelatin-coated slides in a cryostat at −15°C. The sections were dessicated overnight, then Nissl-stained with thionin.

VCR Volume Measurement. Vocal control regions (area X, lateral and medial MAN, HVC, RA) and a control region not involved in vocal behavior control, the nucleus rotundus (Rt), were identified using the canary stereotaxic atlas (Stokes et al., 1974; Nottebohm et al., 1976). The ''inclusive'' boundaries of HVC were used to measure that region (Kirn et al., 1989). Lateral and medial MAN were measured together because the boundary between them was difficult to distinguish in most cases. Regions were measured using the M1 MCID image analysis system (Imaging Research, St. Catherines, Canada), which calculates region volume by multiplying section thickness by region area. Areas were measured by using a computer mouse to trace the borders of each region projected on a monitor. Alternate sections were measured (left and right sides separately), the volumes of all measured sections in a brain were totaled and doubled, and values obtained for the left and right hemispheres were summed for each region. Previous studies indicate that volumes from right and left sides are not different (Gulledge and Deviche, unpublished data). Telencephalon width was also measured as a control for overall brain size. For this, all brain sections with the anterior commissure (CoA) present (usually three) were measured at the widest point, and the widths were averaged for each brain. Right and left hemispheres were measured separately, then summed. All data were collected without knowledge of bird identity. Total volumes for each VCR and Rt were analyzed separately using a one-way analysis of variance (ANOVA) followed by Student–Newman–Keuls multiple comparisons tests. All region data sets met ANOVA assumptions of normality and equal variance. We also determined whether differences in HVC and area X volumes across comparable groups of birds were due to differences in the length (rostrocaudal axis) and/or the width and height (mediolateral or dorsoventral axis) of these areas. For this, we counted the number of alternate brain sections containing a region (length) and measured the largest cross-sectional area of a region on any one section (width and height). For analysis of the number of sections, a nonparametric Kruskal–Wallis one-way ANOVA was used, followed by Dunn's method tests for multiple comparisons. Measures of the largest cross-sectional area for each region (largest of right and left hemispheres averaged) were analyzed using a one-way ANOVA, followed by Student–Newman–Keuls multiple-comparison tests. Telencephalon width, brain weight, and HVC cross-sectional area data sets did not comply with equal variance assumptions, so they were ranked before analysis.

T-Replacement Study

Animal Collection and Handling. Adult, male, dark-eyed juncos were captured near Fairbanks, Alaska, in May 1994, when males were singing frequently and defending territories. Birds were kept in individual cages on long photoperiod (light/dark cycle: 20:4) for 1–3 days, and they received food and water ad libitum. Because the gonads were initially large and heavily vascularized, birds were transferred to 8:16 light/dark cycles for 9–14 days to induce partial testicular regression and were then bilaterally gonadectomized under complete anesthesia. The birds remained on an 8:16 light/dark cycle for the remainder of the experiment. One day following castration, birds received subcutaneous Silastic capsules (3 cm; 1.45 mm internal diameter, 1.93 mm outer diameter; Dow Corning) either filled with T (Cx + T, $n = 6$) or empty (Cx, $n = 6$). We refer to the 12th day after transfer to the 8:16 light/dark cycle as day zero (DO, median day of gonadectomies). Another group of birds (R, $n = 5$) was perfused (see protocol above) on D1 to provide a reference for VCR volumes of the other two groups at the time of castration. Blood samples were taken from a wing vein of all birds at capture and on day minus 2 (D − 2). Additional blood samples were taken from Cx and Cx+T groups on D22 and D47. On D47, both groups were perfused as described above.

Brain Processing. Brains from the three groups were processed as in the seasonal study, except that Cx and Cx+T brains were kept in Na azide for 9 days before being weighed and transferred to 30% sucrose for 21 days, while R brains were kept in Na azide for 4 days

before transfer to 30% sucrose for 10 days. They were sectioned and measured without knowledge of the individual bird identity. Volume and brain size data were analyzed using a one-way ANOVA for each region, followed by Student–Newman–Keuls multiple-comparison tests. All of these data sets met ANOVA assumptions of normality and equal variance. As for the seasonal study, the number of alternate sections per region were analyzed using a Kruskal–Wallis one-way ANOVA, followed by Dunn's method tests for multiple comparisons. Cross-sectional area measures for area X did not pass equal variance testing, so data were ranked before analysis.

Androgen Radioimmunoassay (RIA)

Blood samples were centrifuged and plasma was removed and stored at −20°C. Blood samples from both studies were assayed for androgen by direct RIA using a protocol modified from Barnes et al. (1988). Steroids were extracted from plasma (40–100 μL) using 5 mL of freshly distilled dichloromethane (DCMA), after ^{3}H-T (approximately 4000 cpm; NEN, Boston, MA) was added to each sample for calculation of steroid extraction recovery. All extraction tubes were vortexed for at least 15 s after adding DCMA to the plasma sample, then were allowed to sit for 2 h before transferring the bottom (organic) phase to a dry tube. Samples were dried with N_2 in a warm bath, then resuspended in phosphate-buffered saline with gelatin (PBSG) and kept at 4°C overnight. The next day, an aliquot of each sample was counted for T recovery. Duplicate standards were made using unlabeled T (Sigma) solutions of known concentrations. All samples were divided in half, T antiserum (diluted 1:20,000; provided by Dr. Niswender, Ft. Collins, CO) was added to all but total counts and background tubes, and ^{3}H-T (approximately 17,000 cpm) was added to all tubes, which were then kept at 4°C overnight. Dextran-coated charcoal was added to all but the total-count tubes, and after 10 min, tubes were centrifuged and the supernatant was decanted into vials and counted for radioactivity. The T antiserum has a 69% cross-reactivity with dihydrotestosterone, so the results are reported as plasma concentrations of androgen, rather than of T. Antiserum cross-reactivity with estradiol is 0.3%. Samples from the seasonal study were assayed in a single series, which had an intra-assay coefficient of variation (CV) of 16.5%. The castration experiment samples were divided randomly between two assays that had an interassay CV of 13.3%. Average extraction recoveries for both experiments exceeded 87% and the final values for each sample were individually corrected for losses during extraction. The assay sensitivity was approximately 0.3 ng androgen/mL.

Plasma androgen concentrations in the three groups of birds from the seasonal study were compared using a one-way ANOVA on ranked data, followed by Student–Newman–Keuls multiple-comparison tests. Plasma androgen concentrations from the T-replacement study were analyzed using two-way repeated measures ANOVAs, followed by Student–Newman–Keuls tests for multiple comparisons. Because blood samples from the R group were available only from capture date and D − 2, one

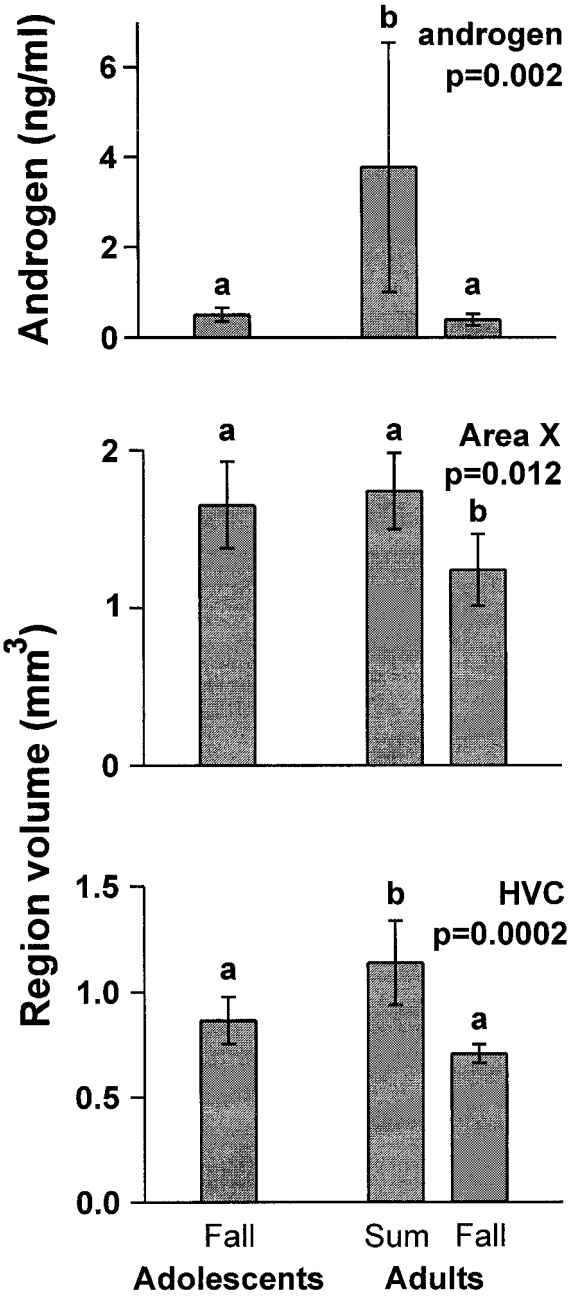


Figure 1 Plasma androgen concentrations (means ± SD; ng/mL) and volumes (means ± SD; mm^3) of area X and HVC in male dark-eyed juncos captured from a wild population during three times of the year: September (adolescents, nonsinging, $n = 9$); June (second-year adults, singing and breeding, $n = 6$); and October (fall adults, nonsinging, $n = 5$). p Values refer to one-way ANOVA results. Differing superscripts indicate significant differences between groups ($p < 0.05$) for each region.

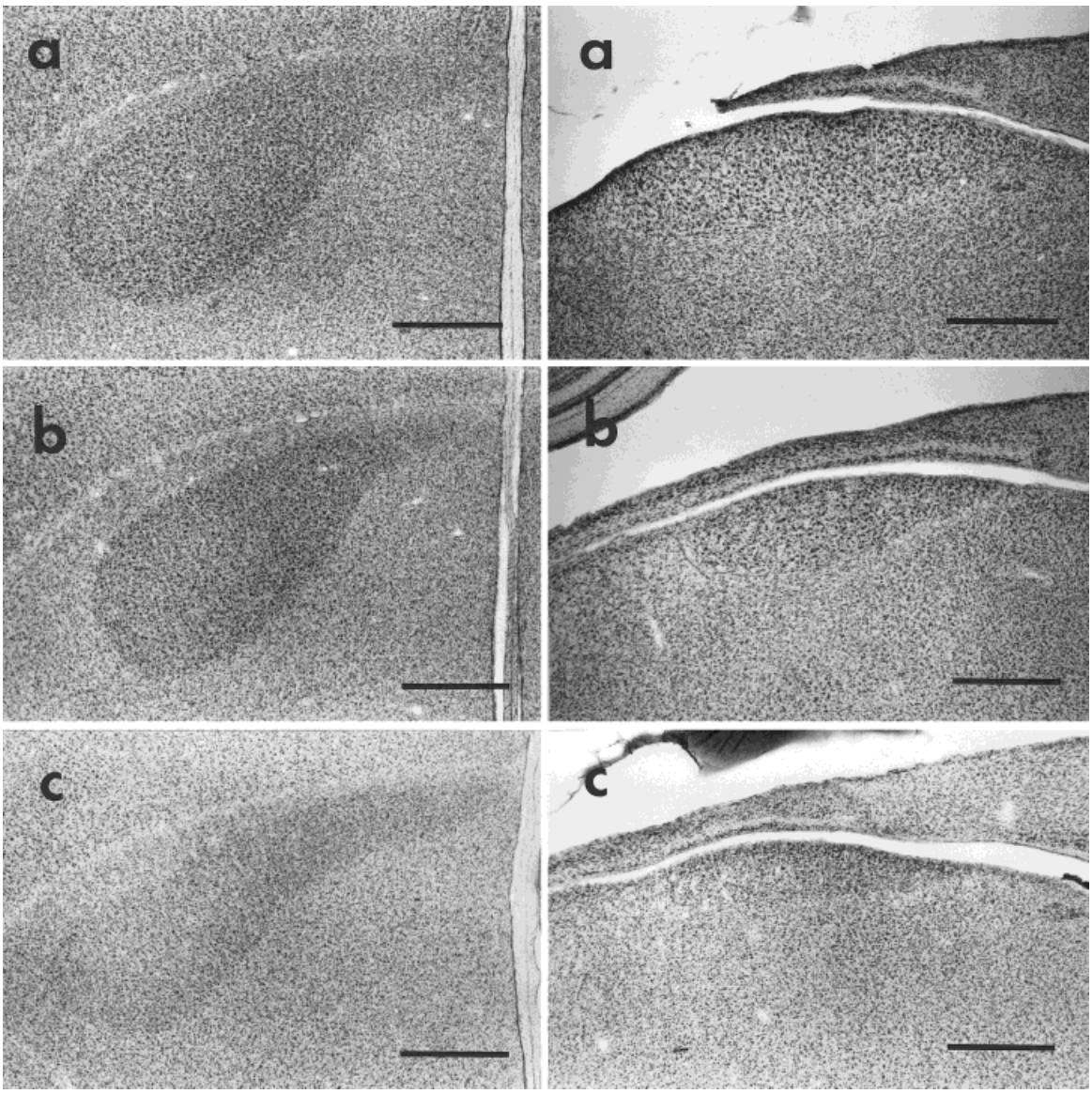


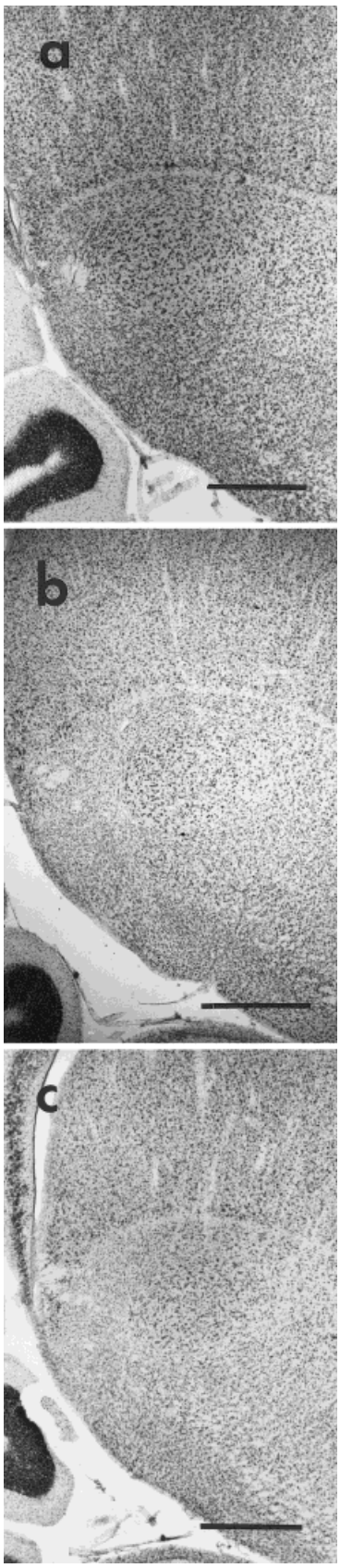
Figure 2 Nissl-stained coronal sections (50 μm thick) of area X (left) and HVC (right) in (a) intact breeding adult male, (b) intact postbreeding adult male, and (c) gonadectomized adult male junco 47 days after castration. The brain with the region volume closest to the mean for the group is represented, and the section with the largest cross-sectional area for that brain is shown. Right is medial and top is dorsal. Calibration bar = 0.5 mm.

ANOVA with all three treatment groups included was used for those dates and a separate ANOVA was used for Cx and Cx+T blood samples from all dates.

RESULTS

Seasonal Study

Of the regions studied, only area X and HVC exhibited significant seasonal volume changes (Figs. 1 and 2 and Table 1). The pattern of seasonal change for RA volume was similar to that of HVC, but was not quite significant ($p = 0.054$) (Table 1 and Fig. 3). In adults, the pattern of change followed that of plasma androgen concentrations, which were higher during than outside the breeding season (Fig. 1). Neither plasma androgen levels nor HVC volumes differed between fall adolescents and fall adults. Fall adolescents had smaller HVC volumes and lower plasma androgen concentrations than those of breeding adults. Adolescent area X volumes, however, were similar to those of breeding adults. A



comparison of the largest cross-sectional area and number of sections containing area X and HVC indicated that differences in area X volume among groups resulted from changes in the cross-sectional area rather than the length of this region. Changes in HVC volume between adolescence and the breeding season were due to changes in the length of this region; differences in HVC volume between the breeding season and postbreeding reflected changes in the cross-sectional area (Table 1 and Fig. 2). The volumes of neither MAN nor Rt differed significantly across seasons (Table 1). Male adolescent brains were significantly heavier than those of adult males, but brain weights in the two adult groups did not differ (Table 1). Average telencephalon width followed the same seasonal pattern, with adolescent brains being larger than those of adult males (Table 1). Brain weight and telencephalon width were positively correlated ($r^2 = 0.91$; $p < 0.0001$), with no overlap between adolescent and adult samples (Fig. 4), confirming that overall brain size is larger in adolescent than in adult males.

T-Replacement Study

Castration reduced plasma androgen levels to below detection limits (<0.3 ng/mL), and T replacement restored plasma androgen to the levels present in free-living males at the time of capture (Table 2). Keeping the birds on short photoperiod before DO reduced plasma androgen levels (Table 2).

Removal of circulating T by castration resulted in a decrease in both area X and HVC volumes (Fig. 5), which in Cx birds were 31% and 36% smaller, respectively, than those of Cx+T males. T administration did not, however, increase the volume of these regions beyond the initial volumes at D1 (group R). A comparison of the largest cross-sectional area and number of sections containing area X and HVC indicated that differences in area X volume among groups mainly resulted from changes in the length rather than cross-sectional area, although the trend in the cross-sectional area was for Cx area to be smallest (Table 3). Changes in HVC volume, however, were in both planes, with the most striking difference in the cross-sectional

Figure 3 Nissl-stained coronal sections (50 μm thick) of RA. Left is medial and top is dorsal. See legend for Figure 2. Calibration bar = 0.5 mm.

Table 1 Measurements from Seasonal Study

Measure	Adolescent	Breeding Adult	Postbreeding Adult	ANOVA
Area X volume (mm^3)	1.65 ± 0.28^1	1.74 ± 0.24^1	1.24 ± 0.23^2	$p = 0.012$
HVC volume (mm^3)	0.90 ± 0.11^1	1.14 ± 0.20^2	0.71 ± 0.04^1	$p < 0.001$
RA volume (mm^3)	0.36 ± 0.09	0.45 ± 0.085	0.33 ± 0.04	$p = 0.054$
MAN volume (mm^3)	0.21 ± 0.07	0.18 ± 0.05	0.18 ± 0.05	$p = 0.42$
Rt volume (mm^3)	2.24 ± 0.29	2.16 ± 0.25	2.16 ± 0.19	$p = 0.78$
Largest area X cross-sectional area (mm^2)	1.26 ± 0.15^1	$1.16 \pm 0.18^{1,2}$	1.01 ± 0.08^2	$p = 0.02$
No. of alternate sections containing area X	12 (10, 12)	11 (10, 13.5)	11 (9.75, 11.5)	$p = 0.75$
Largest HVC cross-sectional area (mm^2)	0.76 ± 0.07^1	0.74 ± 0.15^1	0.53 ± 0.05^2	$p = 0.002$
No. of alternate sections containing HVC	11^1 (10, 11)	13^2 (12, 13.5)	$11^{1,2}$ (11, 12)	$p < 0.001$
Brain weight (mg)	935 ± 58^1	785 ± 20^2	772 ± 8^2	$p < 0.001$
Telencephalon width (mm)	14.7 ± 0.4^1	13.5 ± 0.1^2	13.6 ± 0.2^2	$p < 0.001$
n	9	6	5	

Characteristics (means ± SD, except for number of sections, which is the median and interquartile interval) of vocal control regions (area X, HVC, RA, MAN) and one nonvocal control region (Rt), as well as total brain size, in male dark-eyed juncos obtained from a wild population at three stages of their life cycle. Differing superscripts indicate significant differences ($p < 0.05$) within a given row.

area (the mean of the largest area in the Cx group was almost half that of R or Cx+T groups) (Table 3 and Fig. 2). MAN volume was larger in group R than in castrated birds, but did not differ between Cx and Cx+T groups (Table 3). Rt and RA volumes did not differ significantly among groups (Table 3). Brain weights and telencephalon widths also did not differ significantly among groups (Table 3) and were similar to those of adults in the seasonal study (Table 1).

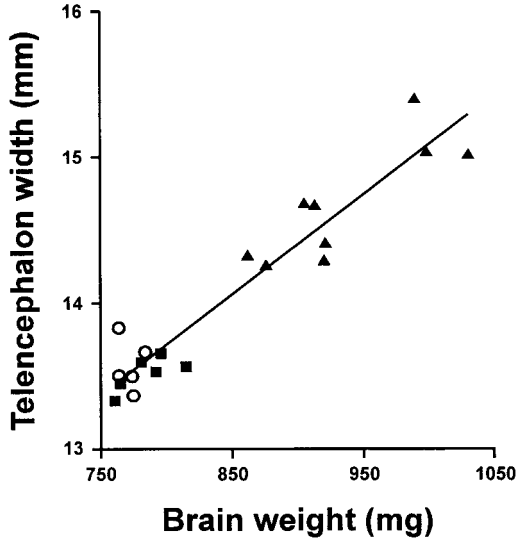


Figure 4 Correlation ($r^2 = 0.91$) between brain weight and telencephalon width in the seasonal study. There is no overlap in brain size between adolescents and adults. Closed triangles = adolescent males; open circles = breeding adult males; closed squares = post-breeding adult males.

Birds used for the T-replacement study were captured in mid-May and for the seasonal study in mid-June, so birds may have been experiencing different phases of the breeding season in the two studies (e.g., mate selection vs. breeding). This difference may explain why androgen levels of birds captured during the breeding season were elevated in both studies, but absolute concentrations differed (Fig. 1 and Table 2) (Wingfield and Farner, 1978).

DISCUSSION

This investigation confirms previous studies showing that VCR sizes are influenced by plasma concentrations of androgens in male birds. In addition, it reveals that the role of androgen in maintaining VCR size may be age and region selective. Both area X and HVC volumes are T dependent in adults during the breeding season, but this relationship is not apparent in area X in adolescent males. We found that area X, HVC, and possibly RA underwent seasonal volume changes in wild male juncos. These changes were VCR specific, rather than re-

Table 2 Plasma Androgen Concentrations (means ± SD; ng/mL) of Adult Male Juncos That Were Intact (R), Castrated (Cx), or Castrated with T Replacement (Cx+T)

Group	Capture	D − 2	D22	D47	*n*
R	7.1 ± 2.6^{1}	0.5 ± 0.2^{2}			5
Cx+T	8.1 ± 3.1^{1}	1.6 ± 0.8^{2}	13.4 ± 4.2^{1}	9.1 ± 3.8^{1}	6
Cx	7.2 ± 4.1^{1}	0.7 ± 0.1^{2}	ND	ND	6

R = intact (killed on D1); Cx = castrated on D0; Cx+T = castrated on D0 with T replacement on D1. ND = nondetectable. Differing superscripts indicate significant differences ($p < 0.05$) within a given row.

sulting from changes in brain size as a whole. Brain size (as measured by brain weight and telencephalon width) did not change in adults between the breeding season and the following fall (Table 1), yet VCR volumes decreased during this period. Further, area X volume was maintained and HVC volume increased from adolescence to the breeding season, even though overall brain size decreased during this time (Table 1 and Fig. 4).

The pattern of seasonal change in RA volume was nearly significant ($p = 0.054$) and similar to that of HVC through the year. In other studies RA volume varied in captive and wild birds exposed to long photoperiod or exogenous T (Nottebohm, 1981; Smith et al., 1995; Smith, 1996; Brenowitz et al., 1996). The changes in RA volume have typically been of lower magnitude than those in HVC (Nottebohm et al., 1986; Brenowitz et al., 1996), which may explain why they did not reach statistical significance in the present study. In addition, because MAN is relatively small, we may not have been able to detect volume differences across groups for this region while measuring alternate sections.

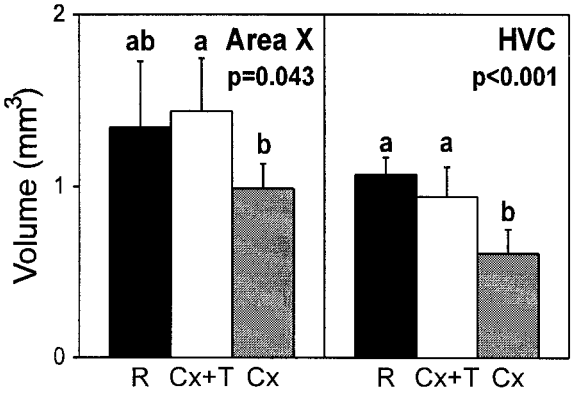


Figure 5 Volumes (means + SD: mm^3) of two vocal control regions of intact adult male juncos (group **R**, $n = 5$), and 47 days after castration (**Cx**, $n = 6$) or castration and testosterone replacement (**Cx+T**, $n = 6$). *p* Values refer to one-way ANOVA results. Differing superscripts indicate significant differences between groups ($p < 0.05$; Student–Newman–Keuls multiple-comparison tests) for each region.

Adults

Area X, HVC, and RA volumes in wild adult male juncos followed the seasonal pattern previously established in captive birds (canaries: Nottebohm, 1981; Nottebohm et al., 1986; red-winged blackbirds: Kirn et al., 1989; rufous-sided towhees: Brenowitz et al., 1991; white-crowned sparrows: Smith et al., 1995): They were large when circulating androgen levels were high and/or the photoperiod was long, and small when androgen levels were low and the day length was short. This suggests that androgen and/or photoperiod plays a causal role in seasonal VCR volume regulation.

Several studies have shown that in adults, increased plasma T levels can enlarge some VCRs. These studies involved birds with initially small VCR volumes and used T administration (and/or exposure to long photoperiod) to induce VCR growth (Smith et al., 1995; Rasika et al., 1994; Brown and Bottjer, 1993; Brenowitz et al., 1991). In addition, Bernard and Ball (1995) showed that elevated T is more important than a long photoperiod in increasing HVC volume in the European starling (*Sturnus vulgaris*). No previous study, however, has directly explored the role of plasma T in the maintenance of naturally large VCR volumes, although one study on female canaries found that RA volume that had increased following T administration decreased after the exogenous T was removed (Brown and Bottjer, 1993). Our T-replacement study confirms a causal relationship between breeding T levels and the maintenance of both area X and HVC volumes in adulthood, since removing circulating T by castration decreased the size of these regions and maintaining these levels by exogenous T administration prevented the effects of castration (Fig. 5). It is not clear whether maintenance of RA volume is T dependent in adult males, since

Table 3 Measurements from T-Replacement Study

Region	R	Cx+T	Cx	ANOVA
Area X volume (mm^3)	$1.34 \pm 0.38^{1,2}$	1.44 ± 0.31^{1}	0.99 ± 0.14^{2}	$p = 0.043$
HVC volume (mm^3)	1.07 ± 0.10^{1}	0.94 ± 0.17^{1}	0.61 ± 0.14^{2}	$p < 0.001$
RA volume (mm^3)	0.34 ± 0.03	0.30 ± 0.01	0.26 ± 0.02	$p = 0.091$
MAN volume (mm^3)	0.17 ± 0.023^{1}	0.14 ± 0.024^{2}	0.12 ± 0.015^{2}	$p = 0.003$
Rt volume (mm^3)	2.20 ± 0.10	2.26 ± 0.27	2.31 ± 0.18	$p = 0.63$
Largest area X cross-sectional area (mm^2)	1.08 ± 0.21	1.13 ± 0.13	0.95 ± 0.10	$p = 0.16$
No. of alternate sections containing area X	$11^{1,2}$ (9.75, 11.25)	11.5^{1} (10, 12)	9.5^{2} (9, 10)	$p = 0.03$
Largest HVC cross-sectional area (mm^2)	0.72 ± 0.07^{1}	0.70 ± 0.11^{1}	0.47 ± 0.11^{2}	$p = 0.001$
No. of alternate sections containing HVC	12^{1} (12, 12.5)	$11.5^{1,2}$ (10, 12)	10^{2} (9, 11)	$p = 0.007$
Brain weight (mg)	873 ± 42	830 ± 72	824 ± 38	$p = 0.3$
Telencephalon width (mm)	13.3 ± 0.25	13.4 ± 0.32	13.4 ± 0.33	$p = 0.578$
n	5	6	6	

Characteristics (means ± SD, except for number of sections, which is the median and interquartile interval) of vocal control regions (area X, HVC, RA, MAN) and one nonvocal control region (Rt), as well as total brain size, in male dark-eyed juncos in intact adult male juncos (group R), and 47 days after castration (Cx) or castration and testosterone replacement (Cx+T). Differing superscripts indicate significant differences ($p < 0.05$) within a given row.

the differences among groups in the castration study were not quite significant ($p = 0.091$). The VCR volumes of Cx birds may not have been fully reduced, however. Johnson and Bottjer (1993) found that administering antisteroid drugs (flutamide and 1,4,6-androstratriene-3,17-dione) to castrated male canaries decreased HVC volume below that of castration alone, possibly because Cx birds still had nongonadal sex steroids present, the actions of which were blocked by the antisteroid treatment.

Comparison of the nature of the HVC and area X volume changes in the two experiments revealed similarities beyond a decrease in total volume. Specifically, the decrease in HVC volume after castration and, in intact males, between the breeding and nonbreeding seasons, resulted from changes in cross-sectional area without concurrent change in region length. The length of area X was smaller in castrated males than in T-treated castrates, although there was no difference in length between adults caught in summer and fall. Cross-sectional area of area X did not differ across groups in either study. Additional investigations are needed to identify the specific cellular alterations (e.g., neuronal size, density, and/or number) that resulted in the observed changes in region cross-sectional area (HVC) and length (area X). The decrease in VCR volumes measured in Cx birds used in this study was quantitatively similar to that seen in adult birds after the breeding season, when their reproductive systems were inactive and circulating T levels plummeted. Thus, while changes in photoperiod may contribute to the regulation of seasonal VCR volumes, our T-replacement study indicates that plasma androgen level manipulations (independent of photoperiod) in adult males are sufficient to produce VCR volume differences that are similar to those found in intact adult birds across seasons. The previously detected correlation between VCR volumes and T levels in captive adult males therefore probably reflects a causal relationship, as is the case in subjects obtained directly from a free-living population.

Adolescents

As mentioned previously, a relationship between circulating androgen levels and VCR volumes in adolescents has not been established. We found that this relationship may depend on the region being considered. Plasma androgen concentrations were much lower in adolescent juncos than in breeding adults. The volume of HVC was also smaller in adolescents than in breeding adults. Further, neither circulating androgen levels nor HVC volumes differed in adolescent and adult males in the fall. Androgen levels therefore appear to control HVC volume in both adolescent and adult males.

In contrast, adolescent area X volumes were as large as those of breeding adult males, even though adolescent androgen concentrations were low. This observation suggests that area X volume is maintained in adolescent males without high plasma T

concentrations. It is not clear, however, if area X volume maintenance is completely T independent in adolescent males, as plasma androgen levels were above detection limits (0.5 ± 0.2 ng/mL). Potentially, therefore, area X may be highly T sensitive, such that very low plasma T levels maintain the region volume. There is some evidence against this hypothesis. Androgen-concentrating cells have been found in HVC, MAN, and RA (reviewed in Arnold, 1992; Johnson and Bottjer, 1993), but most researchers agree that area X does not have steroid receptors (Gahr, 1990b; but see Walters et al., 1988). Any steroid effect on area X, therefore, is presumably indirect, possibly mediated through projections from HVC (Johnson and Bottjer, 1993; Gahr, 1990a; Gahr et al., 1993; Arnold, 1992), which in adolescent males has not yet reached spring adult size. In addition, a previous study on castrated adolescent swamp sparrows found that song development continued normally up to the point of song crystallization, even though plasma T concentrations were below detection (Marler et al., 1988). Since song development depends on area X (Scharff and Nottebohm, 1991; Sohrabji et al., 1990), this result indicates that area X functions normally in adolescent swamp sparrows without detectable levels of circulating T. Thus, maintenance of area X volume is probably T independent at this stage. Even though area X volume maintenance in adolescents may not be T dependent, it may depend on estrogen acting on the HVC. Estrogen receptors are present in HVC neurons that project to area X (Gahr, 1990a; Gahr et al., 1993), and nongonadal estrogen was detected in the plasma of castrated adolescent swamp sparrows (Marler et al., 1988).

Functional Implications

The different seasonal patterns of area X and HVC volume changes through a bird's first year may be related to the roles of these regions. Area X is necessary for song learning but not for production of crystallized song (Sohrabji et al., 1990; Scharff and Nottebohm, 1991; Nottebohm et al., 1976). Lesioning this area during song learning results in abnormal song development (Sohrabji et al., 1990; Scharff and Nottebohm, 1991). In some species, area X may also be important for modifying song repertoires between breeding seasons (Doupe, 1994; Nottebohm et al., 1990). Once a bird has crystallized its song, however, lesioning area X does not immediately affect song quality (Nottebohm et al., 1976). Area X, however, may be important for storing memorized song until it is crystallized. This might explain why area X would stay large from the time of song memorization through the storage phase. In addition, area X may aid in recognition of neighboring conspecific songs (DeVoogd et al., 1995; Doupe and Konishi, 1991). This role has not been conclusively proven, but if valid, it may also explain why area X stays large through the breeding season, then shrinks after conspecifics have ceased singing.

Previous studies on VCRs in photoperiodic birds have provided conflicting results regarding the seasonal pattern of volume change, in part due to species differences, but also because of differences in the methods employed to define region boundaries. A study that defined canary HVC boundaries by backfilling with fluoro-gold injected into RA found that HVC volume reaches breeding size by 4 months after hatching (Alvarez-Buylla et al., 1992). Another investigation on the same species found that both HVC and RA grow until about 8 months posthatching when measured using Nissl stain (Nottebohm et al., 1986). Recent work using VCR volume reconstruction based on cytologic markers (adrenergic receptors, *met*-enkephalin immunoreactivity) revealed differences in these volumes that matched those using Nissl stain (Ball et al., 1995; Bernard and Ball, 1995; Smith et al., 1994), indicating that seasonal volume changes have some functional significance besides the amount of Nissl substance present.

Conclusions

Adolescent juncos maintain large area X volumes until the breeding season despite low plasma T levels. There are many reasons for seasonal migratory species to avoid high plasma androgen levels outside of the breeding season. Chronically elevated androgen levels prevent premigratory fattening and molt (Deviche, 1995; reviewed by Ketterson et al., 1996) and decrease overwinter survival (Ketterson et al., 1996). Abnormally high T levels in adolescent white-crowned sparrows induced premature song crystallization, and the resulting song was abnormal (Whaling et al., 1995). Thus, in species with a prolonged storage phase between song memorization and practice, such as the junco (Marler et al., 1962), high T levels during adolescence may disrupt normal VCR development.

If T does not maintain area X volumes in adolescent males, one or several alternative chemicals may do so. Several neurochemicals and receptors have been found in VCRs of seasonally breeding birds, including opioid peptides and receptors (Gulledge

and Deviche, 1995; Ball et al., 1988), vasoactive intestinal peptide (Ball et al., 1988), adrenergic receptors (Bernard and Ball, 1995), and estrogen receptors (Gahr, 1990a, 1990b; Gahr et al., 1993). Most studies on the neuroanatomic localization of neurochemicals within the vocal control system involved adults, and the presence of these chemicals in VCRs of adolescents belonging to seasonally breeding species has not been reported.

Our results demonstrate that the role of androgen in maintaining VCR size may be age and region selective. Both area X and HVC volumes are T dependent in adults during the breeding season, but this relationship is not apparent for area X in adolescent males. Thus, area X volume in adolescent males may be controlled by some neurochemical that has been identified in adult VCRs, rather than by gonadal androgens.

The authors thank Martine Labelle for assistance with bird collection and histology, and Eliot Brenowitz for helpful comments on the manuscript. This work was supported by NSF Award BNS-9121258 to P.D.

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.

ALVAREZ-BUYLLA, A., LING, C.-Y., and NOTTEBOHM, F. (1992). High Vocal Center growth and its relation to neurogenesis, neuronal replacement and song acquisition in juvenile canaries. *J. Neurobiol.* **23:**396–406.

BALL, G. F., ABSIL, P., and BALTHAZART, J. (1995). Assessment of volumetric sex differences in the song control nuclei HVC and RA in zebra finches by immunocytochemistry for methionine enkephalin and vasoactive intestinal polypeptide. *Brain Res.* **699:**83–96.

BALL, G. F., FARIS, P. L., HARTMAN, B. K., and WINGFIELD, J. C. (1988). Immunohistochemical localization of neuropeptides in the vocal control regions of two songbird species. *J. Comp. Neurol.* **268:**171–180.

BARNES, B. M., KRETZMANN, M., ZUCKER, I., and LICHT, P. (1988). Plasma androgen and gonadotropin levels during hibernation and testicular maturation in golden-mantled ground squirrels. *Biol. Reprod.* **38:**616–622.

BERNARD, D. J., and BALL, G. F. (1995). Two histological markers reveal a similar photoperiodic difference in the volume of the high vocal center in male European starlings. *J. Comp. Neurol.* **360:**726–734.

BOTTJER, S. W., MIESNER, E., and ARNOLD, A. P. (1984). Forebrain lesions disrupt development but not maintenance of song in passerine birds. *Science* **224:**901–903.

BRENOWITZ, E., BAPTISTA, L., LENT, K., and WINGFIELD, J. (1996). Seasonal plasticity of the song system in wild Nuttall's white-crowned sparrows. *Abstr. Soc. Neurosci.* **22:**1401.

BRENOWITZ, E. A., NALLS, B., WINGFIELD, J. C., and KROODSMA, D. E. (1991). Seasonal changes in avian song nuclei without seasonal changes in song repertoire. *J. Neurosci.* **11:**1367–1374.

BROWN, S. D., and BOTTJER, S. W. (1993). Testosterone-induced changes in adult canary brain are reversible. *J. Neurobiol.* **24:**627–640.

DEVICHE, P. (1995). Control of avian premigratory hyperphagia and fattening: From ecophysiology to neuroendocrinology. *Amer. Zool.* **35:**234–245.

DEVOOGD, T. J., HOUTMAN, A. M., and FALLS, J. B. (1995). White-throated sparrow morphs that differ in song production rate also differ in the anatomy of some song-related brain areas. *J. Neurobiol.* **28:**202–213.

DOUPE, A. (1994). Songbirds and adult neurogenesis: a new role for hormones. *Proc. Natl. Acad. Sci. USA* **91:**7836–7838.

DOUPE, A. J., and KONISHI, M. (1991). Song-selective auditory circuits in the vocal control system of the zebra finch. *Proc. Natl. Acad. Sci. USA* **88:**11339–11343.

GAHR, M. (1990a). Delineation of a brain nucleus: comparisons of cytochemical, hodological, and cytoarchitectural views of the song control nucleus HVC of the adult canary. *J. Comp. Neurol.* **294:**30–36.

GAHR, M. (1990b). Localization of androgen receptors and estrogen receptors in the same cells of the songbird brain. *Proc. Natl. Acad. Sci. USA* **87:**9445–9448.

GAHR, M., GUTTINGER, H.-R., and KROODSMA, D. E. (1993). Estrogen receptors in the avian brain: survey reveals general distribution and forebrain areas unique to songbirds. *J. Comp. Neurol.* **327:**112–122.

GULLEDGE, C. C., and DEVICHE, P. (1995). Autoradiographic localization of opioid receptors in vocal control regions of a male passerine bird (*Junco hyemalis*). *J. Comp. Neurol.* **356:**408–417.

JOHNSON, F., and BOTTJER, S. W. (1993). Hormone-induced changes in identified cell populations of the higher vocal center in male canaries. *J. Neurobiol.* **24:**400–418.

KETTERSON, E. D., NOLAN, V., JR., CAWTHORN, M. J., PARKER, P. G., and ZIEFENFUS, C. (1996). Phenotypic engineering: using hormones to explore the mechanistic and functional bases of phenotypic variation in nature. *Ibis* **138:**70–86.

KIRN, J. R., CLOWER, R. P., KROODSMA, D. E., and DEVOOGD, T. J. (1989). Song-related brain regions in the red-winged blackbird are affected by sex and season but not repertoire size. *J. Neurobiol.* **20:**139–163.

KONISHI, M. (1994). An outline of recent advances in birdsong neurobiology. *Brain Behav. Evol.* **44:**279–285.

MARLER, P. (1991). Song-learning behavior: the inter-

face with neuroethology. *Trends Neurosci.* **14:**199–206.

MARLER, P., KREITH, M., and TAMURA, M. (1962). Song development in hand-raised oregon juncos. *Auk* **79:**12–30.

MARLER, P., PETERS, S., BALL, G. F., DUFFY, A. M., JR., and WINGFIELD, J. C. (1988). The role of sex steroids in the acquisition and production of birdsong. *Nature* **336:**770–772.

MARLER, P., PETERS, S., and WINGFIELD, J. C. (1987). Correlations between song acquisition, song production, and plasma levels of testosterone and estradiol in sparrows. *J. Neurobiol.* **18:**531–548.

NORDEEN, K. W., MARLER, P., and NORDEEN, E. J. (1989). Addition of song-related neurons in swamp sparrows coincides with memorization, not production of learned songs. *J. Neurobiol.* **20:**651–661.

NORDEEN, E. J., and NORDEEN, K. W. (1989). Estrogen stimulates the incorporation of new neurons into avian song nuclei during adolescence. *Dev. Brain Res.* **49:**27–32.

NOTTEBOHM, F. (1980). Testosterone triggers growth of brain vocal control nuclei in adult female canaries. *Brain Res.* **189:**429–436.

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., ALVAREZ-BUYLLA, A., CYNX, J., KIRN, J., LING, C.-Y., NOTTEBOHM, M., SUTER, R., TOLLES, A., and WILLIAMS, H. (1990). Song learning in birds: the relation between perception and production. *Phil. Trans. R. Soc. Lond. B* **329:**115–124.

NOTTEBOHM, F., NOTTEBOHM, M. E., and CRANE, L. (1986). Developmental and seasonal changes in canary song and their relation to changes in the anatomy of song-control nuclei. *Behav. Neural Biol.* **46:**445–471.

NOTTEBOHM, F., STOKES, T. M., and LEONARD, C. M. (1976). Central control of song in the canary, *Serinus canaria. J. Comp. Neurol.* **165:**457–486.

PYLE, P., HOWELL, S. N. G., YUNICK, R. P., and DESANTE, D. F. (1987). *Identification Guide to North American Passerines.* Slate Creek Press, California.

RASIKA, S., NOTTEBOHM, F., and ALVAREZ-BUYLLA, A. (1994). Testosterone increases the recruitment and/or survival of new high vocal center neurons in adult female canaries. *Proc. Natl. Acad. Sci. USA* **91:**7854–7858.

SCHARFF, C., and NOTTEBOHM, F. (1991). A comparative study of the behavioral deficits following lesions of various parts of the zebra finch song system: implications for vocal learning. *J. Neurosci.* **11:**2896–2913.

SMITH, G. T. (1996). Seasonal plasticity in the song nuclei of wild rufous-sided towhees. *Brain Res.* **734:** 79–85.

SMITH, G. T., BRENOWITZ, E. A., and WINGFIELD, J. C. (1994). Seasonal changes in the avian song nucleus HVC as defined by multiple labels. *Abstr. Soc. Neurosci.* **20:**163.

SMITH, G. T., BRENOWITZ, E. A., WINGFIELD, J. C., and BAPTISTA, L. F. (1995). Seasonal changes in song nuclei and song behavior in Gambel's white-crowned sparrows. *J. Neurobiol.* **28:**114–125.

SOHRABJI, F., NORDEEN, E. J., and NORDEEN, K. W. (1990). Selective impairment of song learning following lesions of a forebrain nucleus in juvenile zebra finches. *Neural Behav. Biol.* **53:**51–63.

STOKES, T. M., LEONARD, C. M., and NOTTEBOHM, F. (1974). The telencephalon, diencephalon, and mesencephalon of the canary, *Serinus canaria,* in stereotaxic coordinates. *J. Comp. Neurol.* **156:**337–374.

WALTERS, M. J., MCEWEN, B. S., and HARDING, C. F. (1988). Estrogen receptor levels in hypothalamic and vocal control nuclei in the male zebra finch. *Brain Res.* **459:**37–43.

WHALING, C. S., NELSON, D. A., and MARLER, P. (1995). Testosterone-induced shortening of the storage phase of song development in birds interferes with vocal learning. *Dev. Psychobiol.* **28:**367–376.

WINGFIELD, J. C., and FARNER, D. S. (1978). The annual cycle of plasma irLH and steroid hormones in feral populations of the white-crowned sparrow, *Zonotrichia leucophrys gambelii. Biol. Reprod.* **19:**1046–1056.