

Effects of Testosterone and Photoperiodic Condition on Song Production and Vocal Control Region Volumes in Adult Male Dark-Eyed Juncos (*Junco hyemalis*)

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In seasonally breeding male oscines, song learning and expression are controlled by brain regions (vocal control regions, VCRs) that exhibit seasonal neural plasticity in adulthood. Several VCRs contain androgen receptors, and gonadal androgens play important roles in the control of seasonal structural and functional changes of VCRs. Recent studies also found that adult VCRs are influenced by factors other than gonadal hormones, including photoperiod, but the relative importance of these factors and their mechanisms of action are poorly understood. To address this issue, we investigated the contributions of photoperiod and testicular androgens to the regulation of VCR volumes and to the control of song expression in adult dark-eyed juncos, *Junco hyemalis*. Exposing castrated (CX) photosensitive males to long days (LD) enhanced their high vocal center (HVC) volumes compared to those of males held on short days (SD). These volumes were not further increased by concurrent testosterone (T) treatment, revealing a marked and gonadal androgen-independent stimulatory influence of photoperiod on the size of this brain region. HVC sizes were smaller in LD-exposed photorefractory than photosensitive males irrespective of whether birds were intact or had been castrated before photoperiodic manipulations, but HVC sizes increased in response to T treatment in intact photorefractory males. Thus, LD exposure can increase HVC volumes in the absence of gonadal T, but large volume induction in photorefractory males requires elevated plasma T levels. Testosterone treatment of SD-exposed photosensitive males increased HVC, but not Area X, MAN, or RA volumes. Only T-treated males sang and this treatment given to cas-

trates was equally effective behaviorally when administered to photosensitive, photostimulated, or photorefractory juncos. This result indicates that the stimulating influence of LD exposure on HVC volumes is insufficient to induce song in the absence of elevated plasma T levels. © 2001 Academic Press

Key Words: androgen; neural plasticity; oscine; photosensitive; photostimulated; photorefractory; reproduction; song system.

In most bird species breeding at middle and high latitudes, timing of reproduction is regulated by seasonal changes in photoperiod. Long days (LD; > approximately 12 h of light per day) in the spring cause photosensitive birds to become photostimulated, thereby initiating gonadal recrudescence and a resulting increase in circulating gonadal steroid levels (Wingfield and Farner, 1980; Farner, 1986). At the end of the breeding season, when days are still longer than the threshold necessary to stimulate the reproductive system in spring, birds become photorefractory, at which time secretion of gonadal steroids decreases and the reproductive system is no longer responsive to LD (Nicholls, Goldsmith, and Dawson, 1988). Finally, the short days of early winter (SD; < approximately 12 h of light per day) terminate the photorefractory period, thereby restoring photosensitivity in preparation for the next breeding season (Nicholls *et al.*, 1988; Wilson, 1992). The physiological changes taking place during the reproductive period are associated with profound behavioral modifications. Most male oscines sing at a high rate during the breeding season, when they are photostimulated and plasma

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testosterone (T) levels are high, and singing decreases or stops when plasma T levels decline after the breeding season (Marler, Peters, and Wingfield, 1987; Nottebohm, Nottebohm, Crane, and Wingfield, 1987). In several species, singing is diminished or eliminated by castration and is reinstated by subsequent T treatment (Arnold, 1975; Heid, Guttinger, and Prove, 1985; Harding, Walters, Collado, and Sheridan, 1988).

In oscines, song learning and production are controlled by an interconnected set of brain regions (vocal control regions, VCRs) collectively called the vocal control system (Nottebohm, Stokes, and Leonard, 1976; reviewed by Konishi, 1994). This system includes the high vocal center (HVC), Area X of the parolfactory lobe, the magnocellular nucleus of the anterior neostriatum (MAN), and the robust nucleus of the archistriatum (RA). Area X and MAN are essential for song learning (Nottebohm *et al.*, 1976; Bottjer, Meisner, and Arnold, 1984; Sohrabji, Nordeen, and Nordeen, 1990; Scharff and Nottebohm, 1991), whereas HVC and RA are necessary for song expression (Nottebohm *et al.*, 1976).

The vocal control system exhibits neuronal plasticity throughout adulthood in many species (Nottebohm, Nottebohm, and Crane, 1986; Smith, 1996; Brenowitz, Nalls, Wingfield, and Kroodsmas, 1991; Gullledge and Deviche, 1997). In seasonally breeding adult songbirds, VCR volumes are larger during than after the breeding season (Smith, 1996; Smith, Brenowitz, Beecher, and Wingfield, 1997a; Gullledge and Deviche, 1997; Deviche and Gullledge, 2000). Changes similar to those observed in free-living birds occur in captive birds exposed to breeding versus nonbreeding photoperiods or T concentrations (Nottebohm, 1981; Brenowitz *et al.*, 1991; Smith, Brenowitz, Wingfield, and Baptista, 1995; Gullledge and Deviche, 1997). The effects of T on VCR volumes and singing are presumably mediated by androgen receptors located in HVC, RA, and MAN (Arnold, Nottebohm, and Pfaff, 1976; Smith, Brenowitz, and Prins, 1996). Area X does not contain androgen receptors but receives projections from HVC, suggesting that the effects of T on this region are mediated by projections from HVC (Arnold, 1980; Gahr, 1990). MAN also projects to Area X and may play a role in the effects of T on this region (Nixdorf-Bergweiler, Lips, and Heinemann, 1995; Vates and Nottebohm, 1995).

The effects of T on song production and VCR volumes are modulated by photoperiodic condition. Nowicki and Ball (1989) showed that the song rate of photosensitive T-treated male song sparrows, *Melospiza melodia*, increased following transfer from

SD to LD, even though this transfer did not increase plasma T levels. In the same study, the authors concluded that T treatment is equally effective in inducing song in photorefractory or photosensitive birds exposed to LD. Bernard and Ball (1997) found HVC volume to be larger in T-treated photostimulated than in intact photosensitive or T-treated photorefractory adult male European starlings, *Sturnus vulgaris*. T-treated adult castrated and photostimulated male dark-eyed juncos also have larger HVC and Area X volumes than untreated males, indicating that large VCR volume maintenance in these birds depends on gonadal steroids (Gullledge and Deviche, 1997).

In addition to modulating the effects of T, photoperiod itself exerts gonadal androgen-independent effects on VCRs. The HVC, Area X, and RA volumes of American tree sparrows, *Spizella arborea*, that were castrated prior to photostimulation increased in response to LD exposure (Bernard, Wilson, and Ball, 1997). In Gambel's white-crowned sparrows, *Zonotrichia leucophrys gambelii*, Smith, Brenowitz, and Wingfield (1997b) found a small but significant steroid-independent stimulatory effect of photostimulation on the volume of HVC and on the size of RA neurons. In adolescent photorefractory male juncos, exposure to LD increased the volumes of Area X, HVC, and RA despite low plasma T concentrations (Gullledge and Deviche, 1998). Finally, seasonal changes in photoperiod regulate neuron death rate in adult male canaries, *Serinus canaria*, independent of changes in gonadal steroid levels (Kirn and Schwabl, 1997).

No study has compared the effects of T treatment on song production and VCR volumes among SD-photosensitive, photostimulated (LD-photosensitive), and photorefractory males concurrently. In the present work, we independently manipulated photoperiodic condition and T treatment in adult male dark-eyed juncos, a photoperiodic, high-latitude breeder used in previous song system research (Gullledge and Deviche, 1997, 1998; Deviche and Gullledge, 2000). We investigated whether (a) photostimulation increases song production and VCR volumes independent of elevated plasma T levels; (b) the effects of T treatment on song rates and VCR volumes are modulated by photoperiodic condition; (c) individual differences in song rates do in fact correlate with differences in HVC volumes, and (d) the VCR volumes of photosensitive males that are castrated prior to transfer from SD to LD do not increase in response to photostimulation or decrease when birds become photorefractory, due to the absence of gonadal T.

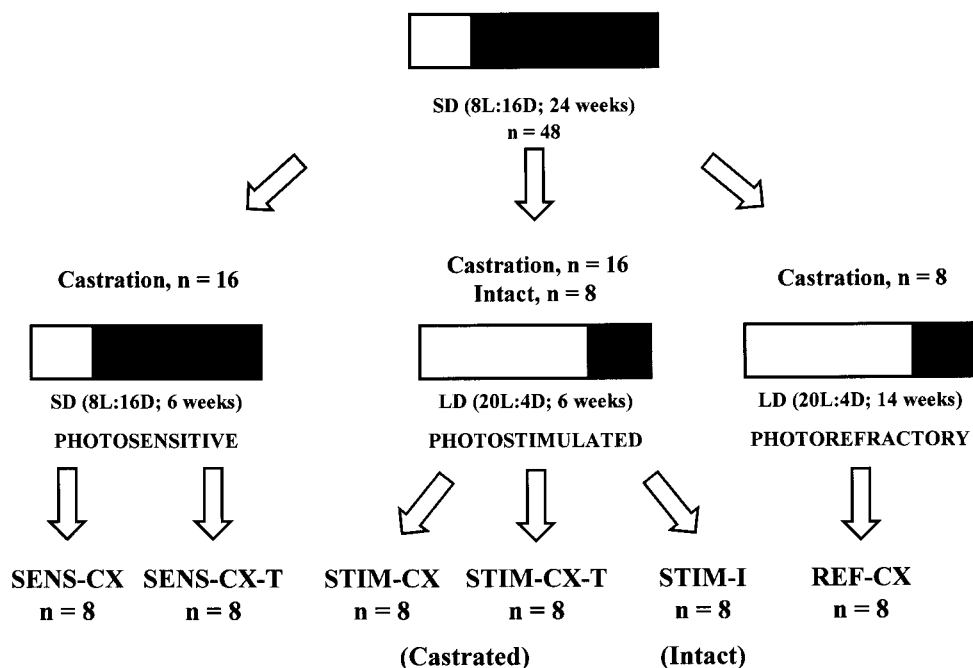


FIG. 1. Design of Experiment 1. Male dark-eyed juncos were treated as indicated and for the specified durations. Abbreviations: SD, short days; LD, long days; *n*, sample size; SENS-CX, photosensitive castrates; SENS-CX-T, photosensitive castrates and testosterone-treated; STIM-CX, photostimulated castrates; STIM-CX-T, photostimulated castrates and testosterone-treated; STIM-I, intact photostimulated; REF-CX, photorefractory castrates.

METHODS

Experimental Design

Experiment 1: Photosensitive males. We collected 48 adolescent male dark-eyed juncos from a wild population near Fairbanks (Alaska; 65°N, 148°W) in September 1997, using seed-baited Potter traps. Birds were brought into captivity and housed in groups of 8–12 in indoor flight cages. They were exposed to SD (8L: 16D; lights on at 0800 AM, AST) until March 11, 1998 (Fig. 1). At this time, birds were moved to individual cages in which they were visually, but not acoustically, isolated from each other. No birds other than those in this study were present in the same room at any time. Forty birds were bilaterally castrated under complete anesthesia via methoxyflurane inhalation (Metofane; Pitman-Moore Inc., Mundelersin, IL) between March 18 and 20. At this time, these males either remained exposed to SD ($n = 16$) or were transferred to a photostimulating light regime ($n = 24$). All SD birds were housed in one room, and all LD birds were housed in another room. Photostimulated birds were exposed to gradually increasing day length, by adding 1 h of light per day until 20 h of light

was reached (LD; 20L:4D; lights on at 0400 AM, AST; Fig. 1). The remaining 8 birds (STIM-I group) were laparotomized and did not receive Silastic capsules (see below). They were also transferred to LD to serve as a photostimulated intact group. On March 26, 8 SD (SENS-CX-T group) and 8 LD (STIM-CX-T group) males received two subcutaneous T-filled Silastic capsules. Testosterone treatment to STIM-CX-T juncos began 2 days after day length reached 12 h. This sequence reflects conditions experienced in the natural environment. Juncos breeding at high latitudes are normally exposed to a photoperiod exceeding 12L beginning in March, several weeks before they reach breeding grounds at the end of April–early May. In other migratory passerines, plasma T levels in males remain relatively low until birds reach their breeding area (Wingfield and Farner, 1978a,b). Each T capsule consisted of a 10-mm length of Silastic tubing (Konigsberg Instruments, Inc., Pasadena, CA; internal diameter, 1.5 mm; external diameter, 2 mm) filled with crystalline T (Sigma Chemical Co., St. Louis, MO) and sealed with silicone adhesive (Dow Corning, Midland, MI). Capsules were incubated in physiological saline solution at 37°C for 24 h prior to implantation to

initiate release of the steroid. Another 8 SD (SENS-CX group) and 16 LD (STIM-CX and REF-CX groups) castrates received empty, control capsules. Birds remained exposed to their respective photoperiods for the remainder of the experiment. All SD and 24 LD birds (STIM-CX, STIM-T, and STIM-I groups) were killed on May 6 or 7. REF-CX males were kept until they had become photorefractory, as determined by the onset of prebasic molt (Morton, King, and Farner, 1969; Dawson, 1997; Dawson and Sharp, 1998), and were killed on July 14 (approximately 2 weeks after the onset of molt). At the time of sacrifice, body cavities of all -CX males were inspected to ensure that castrations were complete. One SENS-CX male was not completely castrated and data for this bird were, therefore, not included in the analyses. Juncos received Mazuri parrot and small bird pelleted food (PMI Nutrition Int., St. Louis, MO) and Avi-Con vitamin-treated water (Vet-A-Mix Inc., Shenandoah, IA) *ad libitum* throughout the study.

Experiment 2: Photorefractory males. During the second half of June 1998, when birds are naturally exposed to constant light, we used mist nets and conspecific song playbacks to collect 20 adult male juncos. Birds were housed in individual cages, received food and water *ad libitum* as in the first experiment, and continued to be exposed to LD (20L:4D; lights on at 0500 AM, AST). They were checked periodically for the onset of molt as an indicator of photorefractoriness. All birds were molting by July 14. On July 21, 12 males received T capsules (REF-I-T) as described in the first study. The duration of T treatment was identical to that of Experiment 1. The remaining 8 males received empty capsules (REF-I). All birds were housed in the same room and were visually, but not acoustically, isolated from each other. Birds were kept on LD until they were killed on September 2 or 3.

Blood Samples and Testosterone Assays

During each study, blood samples were collected from the left alar wing vein 12 or 13 (D_{12}) and 32 or 33 (D_{32}) days after hormonal treatments began. Samples were immediately centrifuged and plasma was drawn off and stored at -20°C until assay. Aliquots of plasma ($25\ \mu\text{l}$ /assay tube) were assayed for total T using a direct, solid-phase commercial radioimmunoassay system (Diagnostic Products Corp., Los Angeles, CA). This assay has been used previously for measuring T in dark-eyed juncos (Gulledge and Deviche, 1998) and is sensitive (lower detection limit, 10 pg/tube) and specific (cross-reactivity, 3% with dihy-

drotestosterone, 0.02% with estradiol). All samples were assayed in duplicate in two series. The intra- and interassay coefficients of variation were 5.8 and 11.6%, respectively.

Morphological Measures

To assess the effectiveness of T capsules, we measured cloacal protuberance widths (CP; a T-sensitive secondary sex characteristic: Schwabl and Farner, 1989; Deviche, 1992) to the nearest 0.1 mm with digital calipers on D_{12} and D_{32} . Gonads of photorefractory birds in Experiment 2 were collected and weighed to the nearest milligram at the time of sacrifice.

Determination of Song Rate

In each study, the average song rate of each bird in groups SENS-CX, SENS-CX-T, STIM-CX, STIM-CX-T, REF-I, REF-I-T, and STIM-I was quantified twice: Between 7 and 10 (D_{7-10}) and between 28 and 31 (D_{28-31}) days after the onset of T-filled or empty capsule administration. At both times, the same observer recorded the number of songs each bird produced during two 30-min periods. Time periods were randomly assigned to each bird, and all observations were made between 0600 and 1130 AM. The two counts of number of songs produced by each individual were then averaged at each time.

Brain Processing and VCR Volume Measurement

Males were anesthetized by an intramuscular injection (0.2 ml) of xylazine (0.032 mg; Loyd Laboratories, Shenandoah, IA) plus ketamine (1.6 mg; Phoenix Pharmaceutical Inc., St. Joseph, MO) dissolved in saline solution followed by methoxyflurane inhalation. Each bird then received an intracardiac injection of 0.3 ml heparin solution (1000 IU per 1.0 ml of 0.1 M phosphate buffer; Sigma Chemical Co., St Louis, MO) followed by 0.1 M phosphate buffer (20 ml) and 4% buffered paraformaldehyde (25 ml). Brains were stored *in situ* in 4% paraformaldehyde at 4°C for 24 h and then were dissected out, weighed, and stored in a sodium azide-containing buffer solution for 4 days, followed by a buffer solution containing 30% sucrose for 4 days (both at 4°C). At this time, they were frozen on powdered dry ice and stored at -70°C until further processed. Brains were sectioned coronally (section thickness, $35\ \mu\text{m}$) in a cryostat, and alternate sections were collected on gelatin-coated slides and stained for Nissl substance using thionin. We used the MCID

TABLE 1
Plasma Testosterone (T) Levels, Cloacal Protuberance (CP) Widths, and Song Rates^a

Experimental group ^c	Plasma T (ng/ml) ^b		CP width (mm) ^b		Songs/30 min ^b	
	Day 12	Day 32	Day 12	Day 32	Days 7–10	Days 28–31
SENS-CX (<i>n</i> = 7)	n.d.b	n.d.b	3.6 ± 0.1b	3.3 ± 0.1b	0b	0b
SENS-CX-T (<i>n</i> = 8)	11.50 ± 1.15a	12.19 ± 1.32a	4.6 ± 0.2a	4.7 ± 0.2a	24 ± 12a	8 ± 3a
STIM-CX (<i>n</i> = 8)	n.d.b	n.d.b	3.5 ± 0.1b	3.8 ± 0.1b	0b	0b
STIM-CX-T (<i>n</i> = 8)	13.95 ± 1.02a	13.97 ± 2.02a	5.0 ± 0.2a	5.1 ± 0.1a	21 ± 10a	18 ± 6a
REF-I (<i>n</i> = 8)	n.d.b	n.d.b	3.6 ± 0.1b	3.5 ± 0.1b	0b	0b
REF-I-T (<i>n</i> = 12)	12.49 ± 0.97a	12.73 ± 1.50a	4.6 ± 0.1a	5.0 ± 0.2a	17 ± 6a	24 ± 7a
STIM-I (<i>n</i> = 8)	1.06 ± 0.4	0.61 ± 0.23	4.0 ± 0.1	5.6 ± 0.3	5 ± 3	13 ± 5

Note. n.d., nondetectable. (a,b) Different letters indicate significant differences among groups within a column ($P < 0.05$, Student–Newman–Keuls tests). STIM-I males are not included in the multiple comparison tests. See Results for a detailed description of analyses.

^a Means ± standard errors.

^b Days indicate the number of days after the onset of testosterone treatment.

^c Numbers in parentheses indicate sample sizes.

image analysis system (Imaging Research, St. Catherine, Canada) as described in Gullledge and Deviche (1998) to measure the volumes of four VCRs (HVC, RA, MAN, and Area X), except that each region volume is reported as the average for the left and right hemispheres, rather than summed left and right hemispheres (Gullledge and Deviche, 1998). We also measured the volume of a control region not associated with the control of song (nucleus rotundus, Rt). Regions were identified using the canary stereotaxic atlas (Stokes, Leonard, and Nottebohm, 1974; Nottebohm *et al.*, 1976). We measured lateral and medial MAN together due to the difficulty of distinguishing the boundary between them and volumes of HVC using the inclusive boundaries for the nucleus as described by Kirn, Clower, Kroodsmas, and DeVogd (1989). Telencephalon width was measured to determine if overall brain sizes differed between groups. To do this, three sections with the anterior commissure present were chosen from each brain. The width of the telencephalon at the widest point on each section was then measured and averaged over the three sections.

All methods were approved by the Institutional Animal Care and Use Committee of the University of Alaska Fairbanks and met the standards of the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Statistical Analyses

Unless indicated otherwise, group comparisons were performed using one- and two-way analyses of variance (1- and 2-ANOVAs) followed by Student–

Newman–Keuls (SNK) multiple comparison tests as appropriate. Data sets that did not meet assumptions of normality and/or homoscedasticity were transformed to ranks before analysis (Conover and Iman, 1981). All results are presented as means ± standard errors and the statistical significance level of all tests was set at $\alpha = 0.05$.

RESULTS

Comparison of Intact, Castrated, and T-Treated Castrated Photostimulated Males

The comparison of STIM-I, STIM-CX, and STIM-CX-T groups showed that the effects of T treatment given to castrated and photostimulated males were physiological. Plasma T levels differed among these three groups (2-ANOVA for repeated measures, $F(2, 21) = 78.6$, $P < 0.001$) and did not change between D₁₂ and D₃₂ (Table 1). They were highest in STIM-CX-T males and in these males were within the range of levels measured at the beginning of the breeding season in free-living birds (Deviche, Wingfield, and Sharp, 2000; Table 1). Levels were intermediate in STIM-I males and the steroid was undetectable in plasma from STIM-CX juncos. Cloacal protuberance sizes also differed among groups (2-ANOVA for repeated measures, $F(2, 21) = 63.2$, $P < 0.001$; Table 1), but there was a Group × Time interaction ($F(2, 21) = 9.3$, $P = 0.001$). As shown by SNK tests, CPs increased between D₁₂ and D₃₂ in STIM-I males, but not in the other groups. As a result CPs were smaller

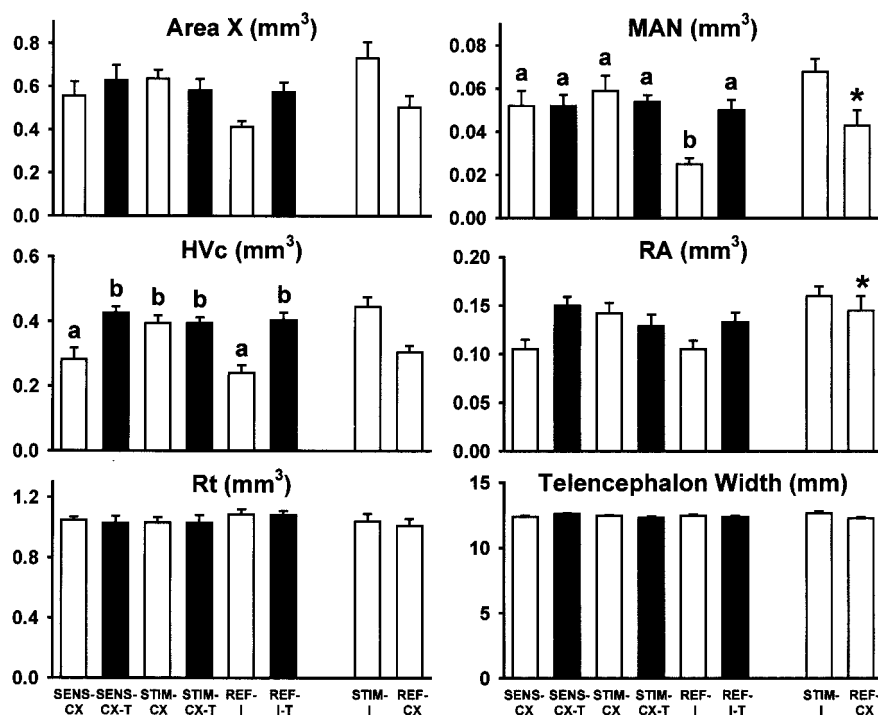


FIG. 2. Volumes (average values for left and right brain hemispheres) of vocal control (Area X, HVC, MAN, RA) and of a control (Rt) brain regions and telencephalon widths of photosensitive (SENS), photostimulated (STIM), and photorefractory (REF-I, $n = 8$; REF-I-T, $n = 12$) male dark-eyed juncos. Males were castrated (-CX) or sham-operated (-I) and were (-T; filled columns) or not (empty columns) treated with testosterone chronically. See Fig. 1 for additional information on group definitions, sample sizes, and experimental treatments. All data are presented as means \pm standard errors. For a given brain region, different letters above columns report significant differences among groups (Student–Newman–Keuls pairwise comparisons, $P < 0.05$; STIM-I and REF-CX males not included in comparisons). An asterisk indicates a significant difference between REF-CX and REF-I males (Student's t test).

in STIM-I than in STIM-CX-T males on D_{12} , but not on D_{32} .

Song rates were similar in STIM-CX-T and in STIM-I males and higher in either group than in STIM-CX males, which did not sing (2-ANOVA for repeated measures, $F(2, 21) = 11.83$, $P < 0.001$; Table 1). Rates did not vary between D_{7-10} and D_{28-31} . The three groups of males did not differ with respect either to the volumes of any brain region or to telencephalon widths (Fig. 2).

Comparison of Intact and Castrated Photorefractory Males

Potential methodological differences (time spent in captivity, surgery, etc.) between Experiments 1 and 2 did not appear to influence most of the physiological and morphological parameters under study.

No bird belonging to the REF-CX or REF-I group had detectable plasma T. Cloacal protuberance widths decreased between D_{12} and D_{32} in both groups (2-

ANOVA for repeated measures, $F(1, 14) = 12.7$, $P = 0.003$) and did not differ between REF-CX and REF-I males. The two groups were similar also with respect to other variables examined except that REF-CX males had larger MAN and RA volumes than REF-I males (Student's t tests, $P_s < 0.04$; Fig. 2).

Effects of Testosterone Treatment across Photoperiodic Conditions

The effects of T administration on plasma levels of the steroid, CP widths, and song expression were not influenced by the photoperiodic condition of the birds. SENS-CX-T, STIM-CX-T, and REF-I-T males had elevated and similar plasma T levels, whereas corresponding controls (SENS-CX, STIM-CX, and REF-I groups) had undetectable plasma steroid concentrations (Tables 1 and 2). Likewise, CP widths were larger in T-treated than in control juncos and did not differ among T-treated groups (Tables 1 and 2). In Experiment 2, REF-I-T birds had heavier gonads

TABLE 2
Two-Way ANOVA Results for Analysis of the Effects of Testosterone Treatment across Photoperiodic Conditions

Parameter	Photoperiodic condition	Testosterone	P × T ^a
Plasma T			
D ₁₂	<i>F</i> (2, 45) = 1.04, <i>P</i> = 0.36	<i>F</i> (1, 45) = 347.2, <i>P</i> < 0.001	<i>F</i> (2, 45) = 1.04, <i>P</i> = 0.36
D ₃₃	<i>F</i> (2, 43) = 0.24, <i>P</i> = 0.79	<i>F</i> (1, 43) = 150.4, <i>P</i> < 0.001	<i>F</i> (2, 43) = 0.24, <i>P</i> = 0.79
CP width			
D ₁₂	<i>F</i> (2, 45) = 0.83, <i>P</i> = 0.44	<i>F</i> (1, 45) = 97.1, <i>P</i> < 0.001	<i>F</i> (2, 45) = 1.68, <i>P</i> = 0.20
D ₃₃	<i>F</i> (2, 44) = 3.14, <i>P</i> < 0.06	<i>F</i> (1, 44) = 114.9, <i>P</i> < 0.001	<i>F</i> (2, 44) = 0.11, <i>P</i> = 0.89
Song rate			
D ₁₂	<i>F</i> (2, 45) = 0.15, <i>P</i> = 0.86	<i>F</i> (1, 45) = 21.1, <i>P</i> < 0.001	<i>F</i> (2, 45) = 0.15, <i>P</i> = 0.86
D ₃₃	<i>F</i> (2, 45) = 0.22, <i>P</i> = 0.81	<i>F</i> (1, 45) = 42.2, <i>P</i> < 0.001	<i>F</i> (2, 45) = 0.22, <i>P</i> = 0.81
HVc volume	<i>F</i> (2, 45) = 4.63, <i>P</i> < 0.02	<i>F</i> (1, 45) = 26.9, <i>P</i> < 0.001	<i>F</i> (2, 45) = 6.55, <i>P</i> = 0.003
RA volume	<i>F</i> (2, 42) = 1.40, <i>P</i> = 0.26	<i>F</i> (1, 42) = 5.15, <i>P</i> < 0.03	<i>F</i> (2, 42) = 3.59, <i>P</i> < 0.04
Area X volume	<i>F</i> (2, 45) = 3.60, <i>P</i> < 0.04	<i>F</i> (1, 45) = 1.91, <i>P</i> = 0.17	<i>F</i> (2, 45) = 2.88, <i>P</i> = 0.07
MAN volume	<i>F</i> (2, 42) = 6.88, <i>P</i> = 0.003	<i>F</i> (1, 42) = 2.22, <i>P</i> = 0.14	<i>F</i> (2, 42) = 4.62, <i>P</i> < 0.02
Rt volume	<i>F</i> (2, 45) = 1.25, <i>P</i> = 0.30	<i>F</i> (1, 45) = 0.10, <i>P</i> = 0.75	<i>F</i> (2, 45) = 0.02, <i>P</i> = 0.98
Telencephalon width	<i>F</i> (2, 45) = 0.60, <i>P</i> = 0.56	<i>F</i> (1, 45) = 0, <i>P</i> = 1.00	<i>F</i> (2, 45) = 2.18, <i>P</i> = 0.13

^a P × T, Photoperiodic condition × testosterone interaction.

(mean ± standard error, 46 ± 19 mg) than REF-I birds (2 ± 0.2 mg; Mann-Whitney *U* test, *P* = 0.02). Molt progressed normally in REF-I males, but feather replacement stopped in REF-I-T males in response to T administration.

No SENS-CX, STIM-CX, or REF-I bird ever sang. All SENS-CX-T and STIM-CX-T males sang, but only 8 of 12 REF-I-T birds sang. Song rates did not, however, differ among T-treated groups (Tables 1 and 2). A comparison of the proportion of birds singing within the three T-treated groups using a two by three contingency table and the χ^2 statistic also revealed no group difference. Song rates of T-treated birds did not correlate with the plasma T levels (Spearman rank correlation, $r^2 = 0.052$, *P* = 0.79) or the HVc volumes ($r^2 = -0.206$, *P* = 0.29) of these birds.

In contrast, the effects of T treatment on VCR sizes were photoperiodic condition-dependent. This treatment was equally effective in increasing HVc volumes when administered to photosensitive males held on SD (SENS-CX vs SENS-CX-T groups) or to photorefractory males held on LD (REF-I vs REF-I-T groups). Transferring castrated males from SD to LD by itself enhanced HVc sizes (SENS-CX vs STIM-CX groups). This effect was not further enhanced by concurrent T administration (Table 2 and Fig. 2). RA volumes showed the same pattern of change as HVC, but multiple comparison tests revealed no significant differences among groups. REF-I birds had smaller MAN volumes than all other groups (SNK, *P* < 0.05), which did not differ, i.e., T treatment increased volumes of this region in photorefractory birds to the same size as

that of photosensitive and photostimulated birds. Photoperiodic condition influenced Area X volumes (Table 2; Fig. 2), but multiple pairwise comparisons tests revealed only one significant difference (STIM-CX > REF-I). Telencephalon widths and Rt volumes were similar in all groups.

Effect of Photoperiodic Condition in Castrated Males

In addition to modulating HVc sizes in T-treated birds, photoperiodic condition influenced these sizes in castrates. HVc volumes differed between SENS-CX, STIM-CX, and REF-CX groups (1-ANOVA, *F*(2, 20) = 4.92, *P* < 0.02; Fig. 2). The previously noted increase in HVc volume in response to photostimulation dissipated as birds became photorefractory, as shown by the fact that REF-CX males did not differ from SENS-CX males. Other VCR volumes and telencephalon widths were similar among the three groups.

DISCUSSION

This study investigated the independent and synergistic effects of T and photoperiodic condition on song production and VCR volumes in dark-eyed juncos. Our results support previous studies in that we identified effects of T as well as photoperiodic condition on VCR volumes. In addition, we report for the first time that LD exposure of castrated photosensitive males increases HVc volume maximally. Unlike in white-

crowned sparrows (Smith *et al.*, 1997b), concurrent T administration does not further increase HVc volume in juncos. Also in contrast to other studies, song production in response to T treatment did not vary with photoperiodic condition. Further, a large HVc is not necessarily associated with song production, as photostimulated castrates never sang despite having large HVc volumes, and HVc volumes did not correlate with song output. Finally, we have shown that the HVc of castrated males shows plasticity in response to photoperiodic manipulations in the absence of detectable circulating T.

Regulation of Brain Plasticity by Testosterone and Photoperiodic Condition

One of our purposes was to determine the relative influences of T and photoperiodic condition on VCR volumes. Testosterone treatment increased HVc volumes whether administered to SD-exposed photosensitive or LD-exposed photorefractory males, and these volumes did not differ among T-treated birds regardless of their photoperiodic condition. However, exposure to LD was sufficient to increase HVc size in castrates and concurrent T treatment did not increase this size further. Long day exposure also increased HVc size in castrated male Gambel's white-crowned sparrow, another high-latitude breeder (Smith *et al.*, 1997b), but in this species T treatment induced an additional volume increase. Testosterone administration increased HVc volume in castrated photorefractory less than in SD photosensitive male European starlings (Bernard and Ball, 1997). However, that study did not include photorefractory birds that did not receive T, so the relative magnitude of T effects on VCR volumes in photostimulated compared to photorefractory starlings could not be determined. It is unlikely that the observed stimulatory effects of photoperiod on brain region volumes in juncos resulted from an interaction between day length and circulating gonadal hormones. No information on the clearance rate of T in small passerines is to our knowledge available. Plasma T in anseriformes is, however, rapidly metabolized and eliminated from the circulation (Pekin duck, *Anas platyrhynchos*: Jallageas and Assenmacher, 1973). Also, an injection of this steroid to castrated male quail, *Coturnix japonica*, resulted in supraphysiological plasma concentrations that fell rapidly and returned to baseline levels within 5 to 24 h (Adkins-Regan and Ottinger, 1988). Juncos in this study were castrated while exposed to SD, when they have undetectable plasma T levels (Gullledge and De-

viche, 1998). Further, castrates that were then photostimulated were not exposed to photoperiod equal to or exceeding 12L until several days after the surgery, at which time any gonadal steroid had presumably been eliminated from the circulation.

Melatonin may mediate gonadal steroid-independent increases in HVc volumes in response to LD exposure. The duration of the daily melatonin peak closely parallels that of the dark phase of the light-dark cycle (Dawson and King, 1994; Goldman and Nelson, 1993; Kumar and Follett, 1993). Specific VCRs, including those of juncos, contain melatonin binding sites (Gahr and Kosar, 1996; Whitfield-Rucker and Cassone, 1996; Bentley, Deviche, Sartor, Spar, and Ball, 2000). Further, melatonin treatment to male European starlings attenuated the LD-induced increase in HVc volumes and decreased the volume of Area X (Bentley, Van't Hof, and Ball, 1999). It should also be recognized that melatonin is unlikely to completely account for the changes in HVc volumes observed in castrates following LD exposure. Indeed, these volumes declined as birds became photorefractory although melatonin secretion presumably remained elevated.

An enlarged HVc volume in response to long vernal day length and in anticipation of seasonally elevated plasma T levels may be adaptive for birds breeding in regions where the period that is favorable to complete a reproductive cycle is very brief, as is generally the case at high latitudes. Juncos belonging to the interior Alaska population are migratory. These birds are naturally exposed to LD starting in March, i.e., 4 to 6 weeks before they reach breeding areas. In other migratory species, plasma T levels in males remain relatively low until birds reach these areas (Wingfield and Farner, 1978a,b). A large, possibly maximally developed HVc when birds complete their spring migration and initiate reproduction may facilitate the onset of song production in response to rapidly increasing T levels.

Brain Plasticity Associated with Photorefractoriness

The present research investigated whether the VCR volumes of castrated males change when birds become photorefractory. To our knowledge, this is the first study examining VCR volume plasticity in males that were castrated *prior to* photoperiodic manipulations. Bernard *et al.* (1997) found testis-dependent and -independent effects of photoperiod in American tree sparrows, but birds were castrated when already pho-

to refractory. Bentley *et al.* (1999) compared VCR volumes of castrated photorefractory starlings with those of photostimulated and photosensitive birds, but birds in that study were likewise castrated when photorefractory. Prior to the present work, whether the VCR volumes of birds castrated *before* transfer to LD change as these birds are photostimulated and then become photorefractory had not been determined. We found that castrated juncos have smaller HVc volumes when photorefractory than when photosensitive, although these birds were exposed to low (undetectable) plasma T levels throughout the study. Thus, LD-induced increase, and also subsequent decrease, in HVc volume in birds that were not exposed to elevated gonadal steroid levels as adults apparently does not require these steroids. In contrast, gonadal steroids mediate seasonal VCR volume changes in intact adults. Indeed, these volumes in adult male juncos that were in breeding condition decreased following castration, an effect that was prevented by T treatment to castrates (Gulledge and Deviche, 1997).

Hormonal Control of Song Production

Testosterone treatment induced similar song rates whether administered to males that were photosensitive but exposed to SD, photostimulated, or photorefractory. Thus, these males can respond to T treatment behaviorally irrespective of their photoperiodic condition. In contrast, photostimulation increased song rate independent of T in song sparrows (Nowicki and Ball, 1989). However, that study did not include untreated control birds. Further, the authors measured song rates after photostimulated sparrows had received exogenous T for 6–8 weeks, whereas photosensitive sparrows had been exposed to T for 1 to 4 weeks. Therefore, the increase in song rate measured in photostimulated sparrows possibly resulted from a difference in duration of hormonal treatment rather than in photoperiod (see Smith *et al.*, 1997b, for a detailed discussion of these issues). It should be observed that we measured only song rates and cannot exclude the possibility that the three groups of T-treated juncos differed with respect to other aspects of their vocal behavior. Song attributes in free-living male song sparrows vary across seasons (early and late spring, and early and late fall; Smith *et al.*, 1997a). Specifically, trill duration was longer and song note structure more stereotyped in the spring than in the fall. The rate at which new variations of song types were produced was greater in the fall than in the late spring. These changes paralleled changes in circulating T levels, but

the authors did not investigate whether photoperiodic condition *itself* plays a role in these seasonal changes.

Castrated and photostimulated juncos had large HVc volumes, but only males that concurrently received T sang, indicating that song expression requires elevated circulating levels of the steroid. Three mechanisms may account for this requirement. First, photoperiod and T may exert different cellular effects on HVc and possibly other VCRs. In support of this hypothesis, T treatment increased the area of HVc neurons more in SD- than LD-exposed male Gambel's white-crowned sparrows (Smith *et al.*, 1997b). This treatment increased the number of these neurons to the same extent in males exposed to either light regime and did not affect HVc neuronal densities. None of these parameters differed between SD- and LD-exposed sparrows, yet HVc sizes were larger in males exposed to the longer photoperiod. Second, song expression may require stimulation of peripheral in addition to brain tissues. Song production depends on appropriate development of the syringeal musculature, which in some species increases in response to androgen administration (Deviche and Schumacher, 1982; Luine, Nottebohm, Harding, and McEwen, 1980). Third, gonadal steroid-sensitive brain regions other than those within the song system may be involved in the activation or motivation of singing, regardless of large HVc volume. Riters and Ball (1999) found that lesioning the medial preoptic nucleus (POM) decreases song output in male European starlings. Aromatization of T into estradiol in the POM influences the expression of male courtship and sexual behavior prior to, and in anticipation of, copulation (Riters, Absil, and Balthazart, 1998; Balthazart, Absil, Gérard, Appeltants, and Ball, 1998).

Potential Modulation of Vocal Control Region Sizes by Social Factors

In a recent study, social cues (the presence of a female in reproductive condition) influenced VCR sizes in male white-crowned sparrows independently of plasma T levels (Tramontin, Wingfield, and Brenowitz, 1999). LD-exposed castrates in the present study were visually, but not acoustically, isolated from T-treated and intact males, which sang at a high rate. Therefore, VCR sizes in LD-exposed castrates increased possibly in response to auditory stimulation provided by singing T-treated males. Likewise, these sizes may have decreased in refractory males (REF-CX) as a result of singing (STIM-I and STIM-CX-T) males being removed at the time of sacrifice from the

room where REF-CX males were housed. We do not favor this hypothesis because SD-exposed castrates retained small VCR sizes, although they were also in acoustic contact with singing, T-treated males. Further, in Experiment 2 refractory control males (REF-I group) had smaller HVC volumes than singing REF-I-T males, although, as was the case in Experiment 1, birds belonging to the two groups were in auditory contact with each other throughout the study.

Hormonal Control of Area X Volume

In the present work, Area X and MAN were large in both control and T-treated SD photosensitive castrated males. Area X in adolescent male juncos is the same size as in breeding adult males, even though plasma T levels are low in adolescence and high during the breeding season (Gulledge and Deviche, 1997). Taken together, these results suggest that Area X volume increases in a plasma androgen-independent fashion in adolescents in the fall and remains large until early spring, when birds are still exposed to SD and have presumably low T levels. Gulledge and Deviche (1997) found that castrating adult male juncos during the breeding season caused Area X to shrink compared to that of T-treated castrated birds. This indicated that T is necessary to maintain large Area X volumes during the breeding season.

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