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Seasonal plasticity in the song nuclei of wild rufous-sided towhees

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Abstract

Seasonal changes in the brain nuclei that control song behavior in songbirds are among the most striking examples of plasticity in the adult vertebrate brain. Although seasonal changes in the size of these brain nuclei have been found in several species in captivity, results on seasonal changes in the song nuclei of wild songbirds have been equivocal. In the present study, I measured plasma testosterone (T) concentrations and the size of song nuclei across seasons in wild male rufous-sided towhees (*Pipilo erythrophthalmus*). I found seasonal changes in both T concentrations and the size of song nuclei that were as large as or larger than those observed in this species in captivity. These results demonstrate that seasonal plasticity of the song nuclei occur in wild, as well as captive, songbirds.

Keywords: Seasonal; Androgen; Neural plasticity; Songbird

1. Introduction

The avian song control system is an excellent model for studying the relationship between neural and behavioral plasticity and the role of gonadal steroids in mediating such plasticity. The brain nuclei that control song in songbirds undergo morphological changes during development that are related to the development of song behavior (reviewed in [6,8,14,20,23,25]). In many songbird species, song nuclei have different developmental trajectories in males and females; and the resulting sex differences in neural structures are correlated with sex differences in vocal behavior [7,26]. In addition, song nuclei undergo pronounced seasonal changes in morphology in adult songbirds of several species. These seasonal changes in the song nuclei may be related to seasonal changes in the quality or quantity of song production and may serve as a substrate for seasonal modifications of song in species that change their song from year to year [24,27,33]. Several attributes of song nuclei change seasonally: (1) size of song nuclei [2,9,19,24,30,33]; (2) size, density, and number of neurons [9,18,33]; (3) dendritic and synaptic morphology [10,12,16]; and (4) incorporation and survival of new neurons [1,28].

The size of several song nuclei, including the higher vocal center (HVC), the robust nucleus of the archistriatum (RA), Area X of the parolfactory lobe, and the tracheosyringeal portion of the hypoglossal nucleus (nXIIts), changes seasonally. Seasonal changes in the size of one or more of these brain regions have been reported in several species in captivity: canaries (*Serinus canaria*), red-winged blackbirds (*Agelaius phoeniceus*), orange bishops (*Euplectes franciscanus*), rufous-sided towhees (*Pipilo erythropthalmus*), house sparrows (*Passer domesticus*), and Gambel's white-crowned sparrows (*Zonotrichia leucophrys gambelii*) [2,9,19,24,30,33]. In these studies, photoperiod and/or testosterone (T) were manipulated in captive birds to mimic naturally occurring seasonal environmental and hormonal changes.

Although captive studies of seasonal plasticity of the song control nuclei allow experimenters to control for factors such as the age, rearing conditions, social experience, and physical environment between subjects in different treatment groups, they have the disadvantage of depriving animals of natural environmental and social cues that may be important in mediating seasonal neural and behavioral plasticity. For example, white-crowned sparrows and song sparrows (*Melospiza melodia*) exposed to long-day photoperiods in captivity have lower plasma T concentrations than their wild breeding counterparts [33,37]. This difference may result either from captivity-induced stress or from the lack of appropriate environmental and social cues in captivity [37]. It is therefore important to conduct field studies in addition to laboratory studies to ensure that

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changes observed in captivity are representative of those observed in wild animals living under natural conditions.

The results of one study that examined seasonal changes in the song nuclei of wild songbirds were equivocal. Although captive male red-winged blackbirds housed on long days had significantly larger HVC, Area X, and nXII than captive males housed on short days, only nXII was larger in wild spring male red-winged blackbirds than in wild fall males [19]. One possible reason that this study did not observe seasonal changes in the forebrain song nuclei of wild male red-winged blackbirds is that the fall birds in this study were captured before migration [19]. It is possible that the song nuclei had not yet regressed in these birds, but would have regressed later in the fall. Alternatively, seasonal changes observed by manipulating photoperiod or testosterone in captive birds may be an artifact of captivity and may not occur in wild songbirds.

I asked whether the size of song nuclei of wild male rufous-sided towhees changes seasonally. Male rufous-sided towhees sing repertoires of 4–28 song types and do not learn new songs as adults [9,22,29]. Song behavior in this species is seasonally modulated. Males sing during the breeding season, but not in fall or winter [11]. In captivity, males of this species undergo seasonal changes in the size of several song nuclei as well as seasonal changes in neuron number, size, and density in the song nuclei [9]. Because rufous-sided towhees are present year-round in western Washington, I was able to collect wild birds from the same population during both the breeding and non-breeding seasons to ask if neural attributes of their song nuclei also change seasonally.

2. Materials and methods

I captured male rufous-sided towhees at field sites in western Washington state between 20 December 1991 and 7 January 1992 (winter, $n = 6$) and between 9 March and 28 May 1992 (spring, $n = 5$). In order to measure plasma concentrations of T, I punctured wing veins with a 26 gauge needle and collected blood samples into heparinized microhematocrit tubes. Blood samples were stored on ice and returned to the laboratory within 4 h. The blood samples were centrifuged, and the plasma was withdrawn and stored at −20°C.

2.1. Hormone assay

I measured plasma concentrations of T by radioimmunoassay (RIA) [4,35]. Plasma samples were equilibrated with 2000 c.p.m. of [^{3}H]T overnight (4°C) to determine percentage of T recovered from extraction and column chromatography. Steroids were extracted from plasma with methylene chloride for 4 h. Organic extracts were dried over nitrogen at 40°C and reconstituted in isooctane. T was separated from other steroids on columns containing a celite/glycol mixture. Steroids of increasing polarity were eluted from the columns with increasing concentrations of ethyl acetate in isooctane. The fraction containing T was dried and reconstituted in 550 μl phosphate buffered saline with gelatin (PBSG). A 100 μl aliquot was added to scintillant and counted on a Beckman liquid scintillation counter to determine the percentage of T recovered. I processed duplicate 200 μl aliquots by RIA by adding [^{3}H]T (10^4 c.p.m. in 100 μl PBSG) and T antiserum (Wien Laboratories). A series of tubes containing known concentrations of T was processed in parallel with the samples to establish a standard curve for the RIA. After overnight incubation (4°C), bound and free T were separated by adding dextran-coated charcoal and centrifuging (4°C, 2000 rpm, 10 min). Supernatants were decanted, added to scintillant, and counted on a liquid scintillation counter. The minimum detectable concentration of T varied between 0.07 and 0.10 ng/ml plasma, depending on plasma sample volume and percentage of T recovered from extraction and column chromatography. The intra-assay variation was 10.7%.

2.2. Histology and neural measures

The towhees were perfused on the day of capture with heparinized avian saline and 10% neutral buffered formalin (NBF). Brains and testes were removed and stored in 10% NBF.

Brains were embedded in gelatin and cryoprotected for 3–4 days in 10% NBF containing 20% sucrose. They were then frozen on dry ice and sectioned at 50 μm on a sliding microtome. Sections were mounted on slides, stained in thionin, dehydrated in ethanol, cleared in xylene, and coverslipped in DPX mountant (BDH Laboratory Supply, Poole, UK).

All neural measures were scored blind to the treatment group of each bird. Sections were viewed with a microprojector at a final magnification of 46× (brain nuclei) or 5.2× (entire telencephalon). I traced the Nissl-defined borders of HVC; RA; Area X; nXIIts; the lateral portion of the magnocellular nucleus of the anterior neostriatum (LMAN); the visual thalamic nucleus Pt; and the entire telencephalon, excluding the hippocampal complex, in every other (brain nuclei, sampling interval = 100 μm) or every sixth (telencephalon, sampling interval = 300 μm) section. The Nissl-defined borders of HVC in white-crowned sparrows and starlings coincide with the borders of this nucleus defined by other cytological markers [5,32]. HVC tracings included the caudomedial extension of the nucleus (paraHVC of Johnson Bottjer [18]) and therefore correspond with the 'inclusive' measure of HVC of Kirn et al. [19], and with measures of HVC used in studies of captive towhees [9] and Gambel's white-crowned sparrows [33].

In order to determine if my definitions of the borders of the song nuclei were consistent with those of the previous study of captive towhees [9], I also measured the volumes

of HVC, RA, and X in six of the captive towhees used in that study (3 each LD and SD). My volume estimates for each nucleus differed by less than 5% from those of the previous study (mean ± S.E. = 2.55 ± 0.34%).

The hypoglossal nucleus consists of two portions: the lingual portion (nXII1), which contains motor neurons innervating the tongue, and the tracheosyringeal portion portion (nXIIts), which contains the motor neurons innervating the syrinx. Only the tracheosynringeal portion of nXII is sexually dimorphic in size and contains androgen receptors [3,13]. I therefore used the criteria of DeVoogd et al. [13] to measure only nXIIts. I also measured nXIIts in the brains from the previous study of captive towhees [9] to compare its volume with that of the wild towhees.

I digitized tracings with a flatbed scanner (BDH Microcomputer, Mountain View, CA). Areas of tracings were

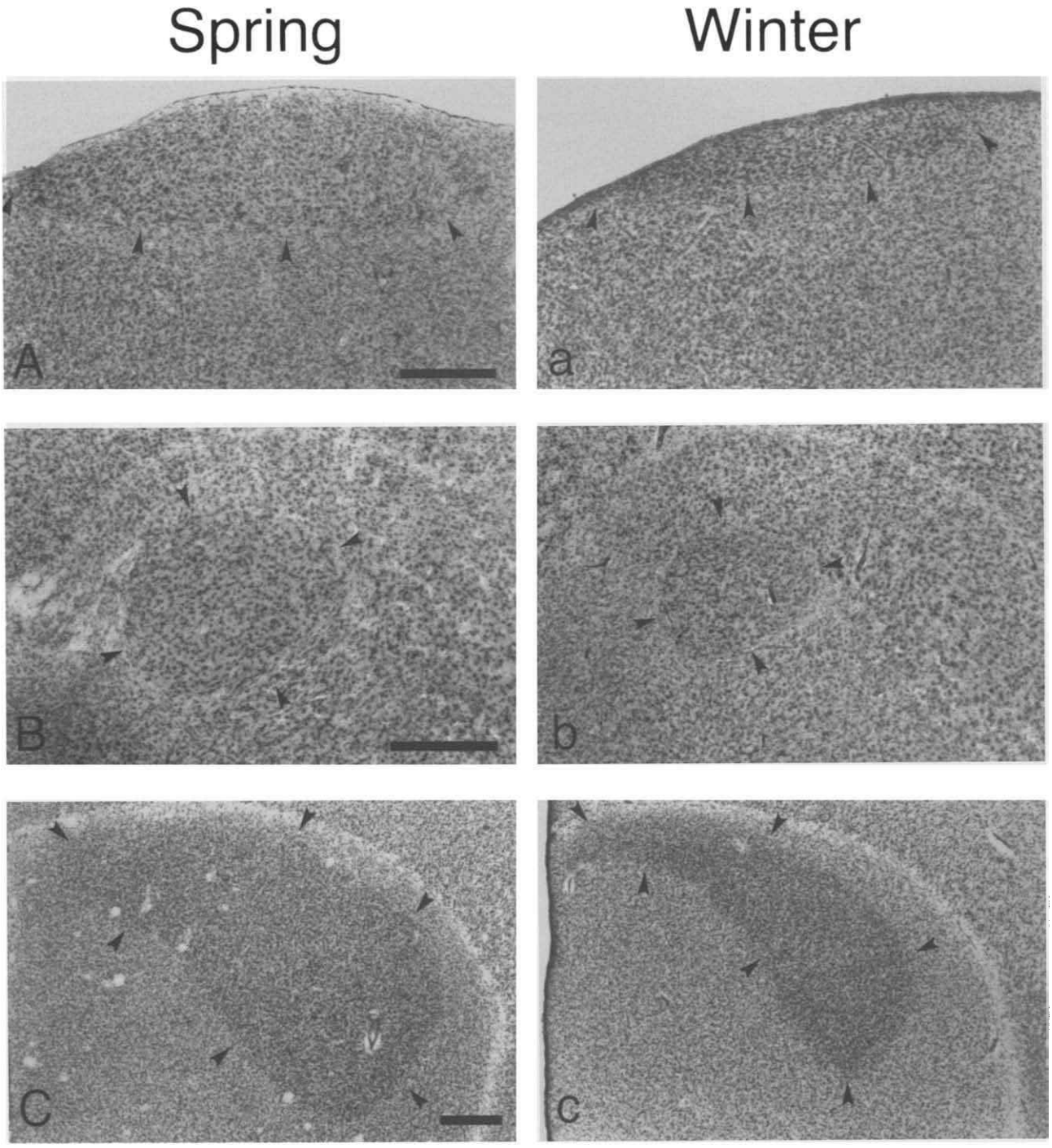

Fig. 1. Thionin-stained transverse sections of HVC (A,a); RA (B,b); and Area X (C,c) in spring (A,B,C) and winter (a,b,c) male rufous-sided towhees. All sections are from the central portion along the rostral–caudal axis of each nucleus. The hippocampal complex has been reflected in A,a. Arrowheads indicate borders of song nuclei. Bars = 0.5 mm.

Table 1
Plasma testosterone concentrations in wild and captive towhees

Season/treatment	Plasma T concentration (ng/ml) Median (interquartile range)	
	Wild	Captive [a]
Winter/short day	N.D. (N.D., 0.08)	0.31 (0.16, 0.42)
Spring/long day	6.93 (4.57, 11.98)	3.23 (2.34, 6.61)

[a] From Brenowitz et al. [9].
N.D. = not detectable.

measured using NIH Image (version 1.56) on a Macintosh computer. Several previous studies of seasonal plasticity of the song nuclei have used the cylinder method (i.e., multiplying the cross-sectional area of a nucleus in each section by the interval between sampled sections) to reconstruct volumes of song nuclei [9,19,24]. The volumes of brain nuclei reported in the present study were reconstructed using the formula for a cone frustum [21,33]. The cone frustum method of reconstruction is more accurate than the cylinder method because it accounts for gradual tapering of brain regions between sampled sections rather than assuming, as the cylinder method does, that the cross-sectional area of the nucleus remains constant between sampled sections. In order to determine the effect of using cone frustum vs. cylinder methods, I reconstructed the volumes of HVC, RA, X, and nXIIts in the towhees in this study using both cone frustum and cylinder methods. The cone frustum method yielded volume estimates that were 0.70–3.76% lower than those using the cylinder method. Using cone frustum vs. cylinder methods had no effect, however, on the statistical significance of any of the comparisons made in this study, including those between captive and wild towhees. I found no difference in the size of brain nuclei between sides of the brain, and therefore used total (left + right) volumes of brain nuclei for all further analyses.

2.3. Statistics

Volumes of brain nuclei were compared between winter and spring male towhees using Student's *t*-tests. Because plasma T concentrations in many winter males fell below the limits of detection of the RIA, these data did not approximate a normal distribution. I therefore compared the median plasma T concentrations between wild spring and winter males and between the wild winter males in this study and captive short-day (SD) males in a previous study [9] using a Mann–Whitney *U*-test, which does not assume a normal distribution. Data from these comparisons are reported as the median and interquartile range. Plasma T concentrations in breeding males did approximate a normal distribution, and I therefore used the more powerful Student's *t*-test to compare plasma T concentrations between wild spring males and captive long-day (LD) males of the previous study [9]. An alpha level of 0.05 was used for all tests.

Table 2
Volumes of brain regions (mean ± S.E.)

Brain region	Winter volume (mm^3)	Spring volume (mm^3)
HVC	0.865 ± 0.100	2.495 ± 0.352 * * *
RA	0.403 ± 0.036	0.946 ± 0.088 * * *
Area X	3.024 ± 0.313	4.840 ± 0.780 *
nXIIts	0.119 ± 0.012	0.166 ± 0.008 * *
LMAN	0.253 ± 0.030	0.335 ± 0.035
Pt	0.135 ± 0.015	0.140 ± 0.007
Telencephalon	951.7 ± 7.2	980.4 ± 19.7

Student's *t*-test: * $P < 0.05$; * * $P < 0.01$; * * * $P < 0.001$.

3. Results

3.1. Plasma testosterone concentrations

Plasma concentrations of T changed seasonally. Wild male rufous-sided towhees captured in the spring had median plasma T concentrations that were greater than those of males captured in the winter (Table 1, Mann–Whitney U, $P = 0.005$). Four of the six winter males had plasma T concentrations below the detection limit of the hormone assay.

3.2. Neural measures

The size of several song nuclei changed seasonally (Table 2, Fig. 1). HVC was 188% larger in spring than winter males ($t_9 = 4.84$, $P < 0.001$). RA was 135% larger in spring than winter males ($t_9 = 6.13$, $P < 0.001$). Area X

Table 3
Comparison of seasonal volume changes of song nuclei in captive versus wild male towhees

Nucleus	Volume (mm^3, mean ± S.E.)			
	Wild winter	Captive short day	Wild spring	Captive long day
HVC	0.865 ± 0.100	1.194 ± 0.092 [a] *	2.495 ± 0.352	2.006 ± 0.127 [a]
RA	0.403 ± 0.036	0.602 ± 0.028 [a] *	0.946 ± 0.088	0.929 ± 0.057 [a]
Area X	3.024 ± 0.313	2.940 ± 0.237 [a]	4.840 ± 0.780	4.763 ± 0.267 [a]
nXIIts	0.119 ± 0.012	0.123 ± 0.008 [b]	0.166 ± 0.008	0.197 ± 0.016 [b]

* Student's *t*-test, wild winter vs. captive short day, $P < 0.05$.
[a] From Brenowitz et al. [9].
[b] Measured in present study from tissue of Brenowitz et al. [9] study (see text).

and nXIIts were respectively 60% and 40% larger in spring than winter males ($t_9 = 2.32$ and $t_7 = 3.52$, $P = 0.046$ and 0.001, respectively). The size of the song nucleus LMAN did not differ between seasons ($t_8 = 1.78$, $P = 0.11$).

These seasonal changes in volume were specific to the song nuclei. There were no differences between winter and spring males in the volumes of the Pt or of the entire telencephalon (Table 2, $t_9 = 0.30$ and 1.23, $P = 0.77$ and 0.26, respectively).

4. Discussion

I found that wild male rufous-sided towhees had greater plasma T concentrations and larger song nuclei in spring than in winter. These results may be compared with those of a study of seasonal changes in T concentrations and the size of song nuclei in captive male rufous-sided towhees [9].

As in the wild towhees in the present study, plasma T concentrations changed seasonally in captive towhees. Plasma T concentrations of the wild spring towhees in this study did not differ significantly from those of the captive male towhees exposed to a breeding photoperiod (Table 1, $t_{13} = -1.64$, $P = 0.12$). The low power ($1 - \beta < 50\%$) of the t-test comparing these samples should be considered, however, when interpreting this comparison. In contrast, the median plasma T concentration in the wild winter males in this study was significantly lower than that in the captive SD males (Table 1, Mann–Whitney U-test, $P = 0.02$). The significance of the difference in T concentrations between wild winter and captive SD males will be discussed below.

As in the study of captive towhees I measured the volumes of brain nuclei, including HVC, from Nissl-stained sections. Gahr [15] reported that although the Nissl-defined volume of HVC changed seasonally in male canaries, the volume of HVC remained constant across seasons if the borders of the nucleus were defined either by the distribution of neurons immunoreactive with the H222 antibody to the human estrogen receptor or by the distribution of neurons projecting to Area X. This result raised questions about the use of Nissl stains to define the borders of HVC. Recent studies, however, have reported seasonal or hormonally-induced changes in the volume of HVC when its borders are defined by cellular markers other than Nissl staining. In male starlings (*Sturnus vulgaris*), the size of HVC changed seasonally to the same extent when its borders were defined either by Nissl staining or by the distribution of α_2 adrenergic receptors [5]. Equivalent seasonal changes in the size of HVC in male Gambel's white-crowned sparrows were observed using three different labels: (1) Nissl staining, (2) the distribution of acetylcholinesterase-positive neuropil, or (3) the distribution of Area X-projecting neurons [32]. The borders of HVC as defined by the distribution of either androgen-accumulating or estrogen-accumulating cells or by the distribution of RA- or X-projecting cells coincided with the Nissl-defined borders in short-day male canaries that received either T or the anti-androgen flutamide [17,18]. These results indicate that seasonal changes in Nissl-defined boundaries of HVC accurately reflect changes observed using other physiologically relevant cellular markers for HVC.

The magnitude of the seasonal changes in some song nuclei of the wild towhees was greater than that of captive towhees (Table 3). Captive towhees underwent seasonal changes in HVC and RA volumes of 68% and 54%, respectively [9], compared with changes of 188% and 135% in wild towhees. These differences between captive and wild towhees were due primarily to differences in the absolute volumes of HVC and RA between captive SD males and wild winter males. Neither HVC nor RA were significantly larger in wild spring males compared to captive LD males in the previous study (Table 3, $t_{13} = 1.62$ and 0.18, $P = 0.13$ and 0.86, for HVC and RA, respectively). In contrast, the wild winter males had significantly smaller HVC and RA than the captive SD males (Table 3, $t_{14} = 2.32$ and 4.35, $P = 0.036$ and 0.0007, respectively). The smaller HVC and RA in the wild winter males may be related to the lower plasma T concentrations in these birds compared to the captive SD males (Table 1). Testosterone is known to be important in mediating seasonal plasticity in the size of these nuclei [31].

There are several possible explanations for the difference in the plasma T concentrations and the size of HVC and RA between the wild winter and captive SD males. One possibility is that there may be differences between populations or subspecies in plasma T concentrations or the size of song nuclei. The males in the captive study were collected from a migratory, eastern population of towhees (subspecies *erythrophthalmus*), while the towhees in the present study were collected from a nonmigratory, western population (subspecies *oregonus*). Potentially, these two subspecies may differ in the extent to which T declines in the winter or in the responsiveness of the song nuclei to the low T concentrations experienced in winter.

A second possibility is that environmental factors may account for the difference in plasma T concentrations and HVC and RA volumes between wild winter and captive SD males. The captive males were housed indoors and were given ad libitum access to food. In contrast, the wild winter males in their natural habitat were exposed to lower temperatures, more inclement weather, and a paucity of food relative to captive males. Temperature, weather conditions, and food abundance are all known to influence gonadal state in songbirds [34,36]. It is therefore possible that these environmental factors account for lower T concentrations and smaller song nuclei in the wild winter males.

The differences in plasma T concentrations and the size of HVC and RA between captive SD and wild winter birds

may also be due to differences in the social experience or rearing conditions of the birds in the two studies. The males in the captive study were collected as nestlings, hand-reared in capivity, tutored with tape-recorded songs, and housed either in isolation or in trios during tutoring. In contrast, the wild birds in the present study experienced a natural social environment during development.

Finally, it is also possible that captive SD and wild winter males were different ages. The captive SD towhees were all 2 years old when sacrificed. The ages of the towhees in the present study is not known. If the wild winter towhees were older or younger than the captive SD towhees, the differences in plasma T and the size of HVC and RA might be explained by this age difference.

The uncertainty of the ages of the wild towhees in this study should also be considered in interpreting the seasonal differences in plasma T concentrations and the size of the song nuclei in these birds. I cannot exclude the possibility that the wild towhees collected in different seasons represented different age cohorts. It is therefore possible that the differences in T concentrations and song nuclei observed between wild winter and spring birds may, at least in part, reflect age-related, rather than seasonal, differences. For two reasons, however, it seems unlikely that such an age effect entirely explains the difference between winter and spring birds: (1) similar seasonal changes occur in the size of song nuclei in captive towhees of known ages [9]; and (2) there was no overlap in the size of HVC or RA between winter and spring birds. If this difference were entirely due to age, it would suggest that the ages of the winter and spring birds did not overlap. This possibility seems unlikely, particularly because towhees were collected at the same field sites from a population that is resident and territorial year-round.

The seasonal change in the size of Area X in the wild towhees (60%) is comparable to that in captive towhees (62%, [9]). The size of Area X did not differ between captive LD males and wild spring males (Table 3, $t_{12} = 0.11$, $P = 0.91$) or between captive SD males and wild winter males (Table 3, $t_{14} = 0.22$, $P = 0.83$).

I observed a seasonal change in the volume of nXIIts in the wild towhees in the present study. In contrast, the volume of the entire nXII in captive male towhees did not change seasonally [9]. This difference may be explained by the fact that I measured only the tracheosyringeal portion of nXII, while all of nXII (tracheosyringeal and lingual portions combined) was measured in the captive towhee brains. To test this hypothesis, I measured only the tracheosyringeal portion of nXII from the brains in the study of captive towhees. As in the wild males, nXIIts differed significantly between captive LD and SD males (Table 3, $t_{13} = 3.48$, $P = 0.004$). Furthermore, the size of the nXIIts did not differ significantly between captive LD and wild spring males ($t_{12} = 1.33$, $P = 0.21$) or between captive SD and wild winter males ($t_8 = 0.27$, $P = 0.80$). This result suggests that seasonal changes occur in the size of the tracheosyringeal, but not the lingual, portion of nXII. The size of MAN did not differ between seasons in captive towhees [9]; and I did not find a seasonal change in the size of the lateral portion of this nucleus, LMAN, in wild towhees.

The present results may also be compared with those of a study of wild red-winged blackbirds [19]. The sizes of HVC, RA, and Area X of wild male red-winged blackbirds did not change seasonally, though the size of Area X and HVC did change seasonally in captive male red-winged blackbirds [19]. One likely explanation of these results is that the fall wild male red-winged blackbirds in this study were captured in October, before fall migration. It is possible that the decline in volumes of the song nuclei of these birds was not yet complete, but that they would regress further later in the fall. Further study of seasonal changes in the song nuclei of a nonmigratory population of this species is needed to test this hypothesis. Interestingly, the sizes of HVC and RA did change seasonally in wild female red-winged blackbirds [19].

In summary, plasma T concentrations and the volumes of several song nuclei differed significantly between wild male rufous-sided towhees captured in winter vs. spring. These seasonal changes are as large as or larger than those reported in captive males of the same species. This result suggests that seasonal plasticity observed in captive birds exposed to photoperiod and/or T manipulations reflects changes in brain regions that occur in wild populations of songbirds exposed to naturally occurring seasonal environmental and social cues.

The information provided by laboratory and field studies of seasonal neural plasticity in the avian song control system is complementary. The study of seasonal plasticty in the song nuclei of captive towhees demonstrated seasonal changes in neural attributes of song nuclei under conditions in which age, social experience, and physical environment were well-controlled [9]. Such factors are often difficult to precisely control in field studies. Furthermore, laboratory studies provide the opportunity to manipulate individual environmental factors (e.g. photoperiod, temperature, food availability), and therefore investigate the mechanisms by which seasonal environmental cues induce changes in the neural attributes of song nuclei [31]. In contrast, field studies allow one to study seasonal neural and behavioral plasticity in the context of naturally occurring seasonal environmental and social cues, many of which may be difficult to identify or reproduce in laboratory situations. By combining these approaches, it may be possible to better understand both the mechanisms underlying the neural and behavioral plasticity and the functional significance of this plasticity.

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