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Male Bias in the Song Control System despite Female Bias in Song Rate in Streak-Backed Orioles *(Icterus pustulatus)*

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Key Words
Area X · High vocal center · Songbird · Song control system · Streak-backed oriole

Abstract

The song control system is a group of discrete interconnected nuclei found in the brains of all songbirds (suborder Passeri). Previous studies have reported a positive relationship between sex differences in song nucleus volumes and sex differences in song behavior across numerous songbird species, with species exhibiting greater sex differences in behavior also exhibiting greater sex differences in the brain. This body of comparative research, however, has failed to incorporate data from a bird species in which females sing more than males. In this study, we examine song nucleus volumes in both sexes of the streak-backed oriole *(Icterus pustulatus)*, a New World blackbird with a female bias in song rate and similar song complexity between the sexes. Results from this neuroanatomical analysis are contrary to what was to be expected from previous research: despite the female bias in song rate, males have a significantly larger HVC and area X song nucleus volumes. Specifically, male HVC was 75% larger than that of females, and male area X was 64% larger than that of females. There was no significant sex difference in the size of the nucleus robustus arcopallialis. The lack of a positive relationship between song nuclei and singing behavior in these orioles demonstrates that our current understanding of song modulation via the song control system may be overly reliant on basic measures such as total volumes.

Introduction

The circuit of anatomically discrete, interconnected nuclei in the brains of songbirds, collectively called the song control system, has long been an ideal animal model for studying the relationships between the vertebrate brain and complex behaviors. Aptly named, this neural circuit has been functionally implicated in the learning, production and modification of birdsong [Tramontin and Brenowitz, 2000]. One of the unique features of this song control system across songbird species is a relationship between sex differences in the volumes of song nuclei and sex differences in singing behaviors. Specifically, the degree of sex difference in song nucleus volumes has been

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reported to reflect the magnitude of sex differences in song rate and complexity across numerous songbird species [Brenowitz et al., 1985; MacDougall-Shackleton and Ball, 1999].

Within the song control system, the correlation between anatomical morphology and singing behavior has been reported most commonly in HVC (sometimes called the high vocal center), a brain region that integrates sensorimotor information and projects to all major pathways implicated in birdsong production [Tramontin and Brenowitz, 2000]. Specifically, sex differences in HVC volume are largest in species exhibiting the most extreme sex differences in song behavior, such as in zebra finches *(Taeniopygia guttata)*, in which male-exclusive singing is reflected in a male HVC volume that is 7–10 times larger than that of females [Nottebohm and Arnold, 1976]. At the other extreme, songbird species in which both sexes sing with equal rate and complexity, often in antiphonal duets [Hall, 2004], show minimal sex differences in HVC [Brenowitz et al., 1985; Brenowitz and Arnold, 1986]. Between these extremes, bird species with an intermediate level of sex difference in singing were found to show a corresponding, intermediate level of sex difference in HVC volume [MacDougall-Shackleton and Ball, 1999]. In addition to the HVC, a similar, albeit less consistent, pattern has been reported in the song nucleus area X and nucleus robustus arcopallialis (RA), brain regions important for song learning and production, respectively [reviewed by Brenowitz et al., 1997]. Although this connection between song nucleus neuroanatomy and sex differences in singing has been shown to persist across species when phylogenetic relationships are accounted for [MacDougall-Shackleton and Ball, 1999], previous work on sex differences has failed to incorporate data from songbird species in which there is a female bias in song rate and/or complexity.

The streak-backed oriole *(Icterus pustulatus)* is a socially monogamous, tropical species that maintains a single territory year round [Murphy et al., 2009]. Nest building and incubation are performed exclusively by females and both sexes provision young. Although the species does not perform antiphonal duets, both sexes have been previously reported to sing solo songs [Howell and Webb, 1995; Jaramillo and Burke, 1999]. In a recent investigation into sex differences in vocal behavior during the breeding season (11th May to 1st June) and the period following breeding and nestling/fledgling (August 4–28), we found that females sing 4–6 times more often than males (songs per minute: breeding season = 0.78 ± 0.11 in females vs. 0.22 ± 0.05 in males; immediately following breeding = 0.12 ± 0.04 in females vs. 0.02 ± 0.01 in males [Price et al., 2008]). Females also produce an agonistic chatter vocalization more often than males during the breeding season (chatters per minute: 0.86 ± 0.10 in females vs. 0.64 ± 0.13 in males). Furthermore, we also showed that the overall acoustic structure of female songs is as complex as that of male songs (measured as duration, frequency range, number of syllables, syllable types and syllable diversity [Price et al., 2008]), indicating that overall song output, measured as a sum of song rate and complexity, was far greater in females than males. Thus, the streak-backed oriole is an ideal species to investigate the relationship between sex differences in the volumes of song control nuclei and singing behaviors of a songbird demonstrating female-biased sex differences in song.

Methods

Birds

We collected 10 male and 11 female wild streak-backed orioles captured in southern Morelos, Mexico, adjacent to the Reserve of the Biosfera de la Sierra de Huautla (18°26′21′′N, 99°01′27′′W). All birds were collected May 10–14, 2009, which was approximately 2 weeks before clutch initiation in the local population. Note that these birds were collected at the same location and during the same time of the breeding season that we studied vocal behavior in our earlier work [Price et al., 2008]. Most birds were caught with mist nets using conspecific vocalizations to attract the birds to the nets. Birds caught in nets were sacrificed by thoracic compression and a few birds (n = 4) were collected by shooting. Brains were extracted within 1 h of collection, and were immediately preserved in 10% formalin. Sex was determined based on coloration of tail plumage [Murphy et al., 2009], and was subsequently confirmed by gonadal inspection (100% agreement). Age class (adult: >2nd year bird and immature: 2nd year bird) was determined based on feather wear of rectrices and on coloration of secondary coverts [Jaramillo and Burke, 1999]. Two female birds were identified as immature and excluded from the analyses. Birds were collected under permit of the Secreteria de Medio Ambiente y Recursos Naturales of Mexico and imported under the permission of the Canadian Food Inspection Agency and Canadian Wildlife Service.

Histological Procedures

Following fixation, all tissue was transported to the University of Western Ontario where brains were removed from formalin, cryoprotected in 30% sucrose in phosphate-buffered saline (PBS), frozen on dry ice and stored at –70°C until further processing.

All brains were sectioned coronally (section thickness = 40 μm) using a freezing cryostat (–18°C; 12° blade angle). Every second section of HVC, area X, RA and nucleus rotundus (Rt) and every 10th section of the telencephalon was collected in 0.1 M PBS (pH 7.5). Tissue sections were then mounted on Superfrost Plus microscope slides (VWR), Nissl stained with thionine, serially dehydrated, cleared and cover-slipped with Permount (Fisher).

Neuroanatomical Measurements

Estimates of song control nuclei and telencephalon volumes were made by summing frustrum volumes of adjacent sections. For HVC, area X, RA and Rt, images were captured with a Leica DFC420 C camera mounted on a Leica DM5500 B microscope using a ×20 objective lens. For each image, the perimeter of the nucleus of interest was traced by an experimenter blinded to the bird's sex using Leica Application Suite to yield cross-sectional areas. The volume of the region between sections was calculated using the formula for a frustrum (truncated cone), accounting for sampling interval (80 μm), and then serial volumes were summed to estimate total volume. If processing or tissue damage prevented the delineation of a nucleus, its area was estimated using the average of the cross-sectional areas of the sections before and after it and this estimated value was used in volume calculations. Volumetric analysis of Rt is included in this paper as a control, as this is a visual nucleus uninvolved in birdsong – Rt is not expected to differ in volume between sexes. Telencephalon images were captured using a high resolution (2,400 dpi) flatbed scanner. The perimeter of every 10th telencephalon image was traced using NIH ImageJ software to calculate cross-sectional areas; telencephalon volume was computed by calculating the frustrum volume between sections, accounting for sampling interval (0.8 mm), and summing. If tissue damage or loss of tissue during processing prevented measurement of a 10th telencephalon section, the next suitable section was used and the sampling interval was adjusted accordingly for volume calculations.

Statistical Analyses

To check for anatomical lateralization, a paired t test was used to compare the volumes of the right and left hemispheres in all birds studied. This test was used for HVC, area X, RA, Rt and telencephalon values. If no lateralization was found, left and right hemispheric volumes for the region of interest were summed to yield a total brain region volume. Sex differences in HVC, area X, RA and Rt were investigated using an independent two-sample t test between male and female values. This test was also used for telencephalon volume to examine sex differences in forebrain volume.

Results

For a few birds, tissue damage and/or poor staining precluded the identification of left from right hemispheres. These birds were excluded from calculations concerning volume differences in brain structures between hemispheres but included in statistics using total volumes. Final sample sizes for each comparison are indicated below.

No significant volume differences were found between the right and left HVC (d.f. = 13, t = 0.27, p = 0.79, n = 9 males and 6 females), area X (d.f. = 12, t = 1.05, p = 0.31, n = 8 males and 6 females) and RA (d.f. = 13, t = 1.06, p = 0.30, n = 8 males and 7 females). Although there was no significant lateralization, the telencephalon (d.f. = 15, t = 1.97, p = 0.07, n = 10 males and 7 females/right hemisphere = 323.60 ± 7.27 mm^3, left hemisphere = 321.40 ± 7.27 mm^3) and Rt (d.f. = 8, t = 2.02, p = 0.07, n = 6 males and 4 females/right Rt = 1.03 ± 0.21 mm^3; left Rt = 1.00 ± 0.20 mm^3) showed non-significant trends towards being larger on the right side of the brain. Because neither the telencephalon nor any of the song nuclei showed any significant lateralization in volume, all further calculations were performed on the total volumes, calculated by summing the volumes of the left and right structure within the same brain.

No sex difference in telencephalon volume was found (d.f. = 17, t = 0.17, p = 0.87, n = 10 males and 9 females). However, there was a significant male-biased sex difference in both total HVC volume (d.f. = 15, t = 4.52, p = 0.0004, n = 9 males and 8 females) and area X volume (d.f. = 14, t = 4.491, p = 0.001, n = 8 males and 8 females), with female/male volume ratios of 0.57–0.61, respectively (fig. 1, 2). Unlike HVC and area X, RA and Rt volumes showed no significant sex difference (RA: d.f. = 16, t = 0.97, p = 0.35, n = 9 males and 9 females; Rt: d.f. = 10, t = 0.26, p = 0.80, n = 6 males and 6 females). All calculations made to investigate potential sex differences in HVC, area X, RA and Rt were made with absolute volumes. We additionally corrected for brain volume by dividing each song nucleus volume by telencephalon volume, and re-

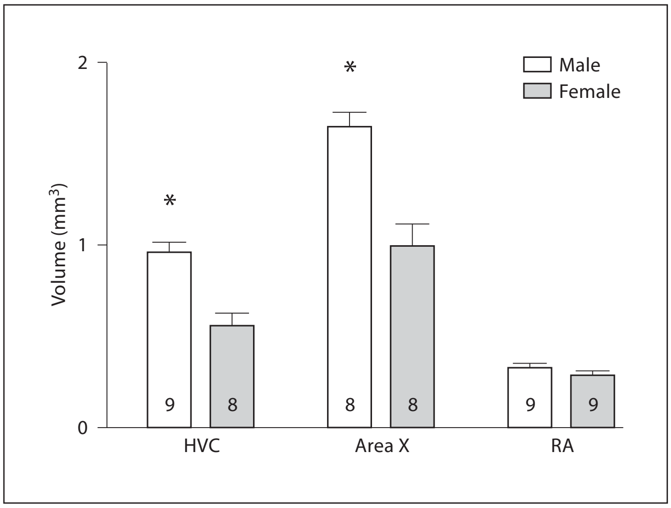

Fig. 1. Volumes of the major song control nuclei HVC, area X and RA in the brains of male and female streak-backed orioles captured in spring. Bars represent mean nuclear volumes ± SE. Sample sizes for each calculation are indicated by the numbers within the bars. Refer to the text for specific methods of quantification and statistical analysis. * $p < 0.05$ males vs. females.

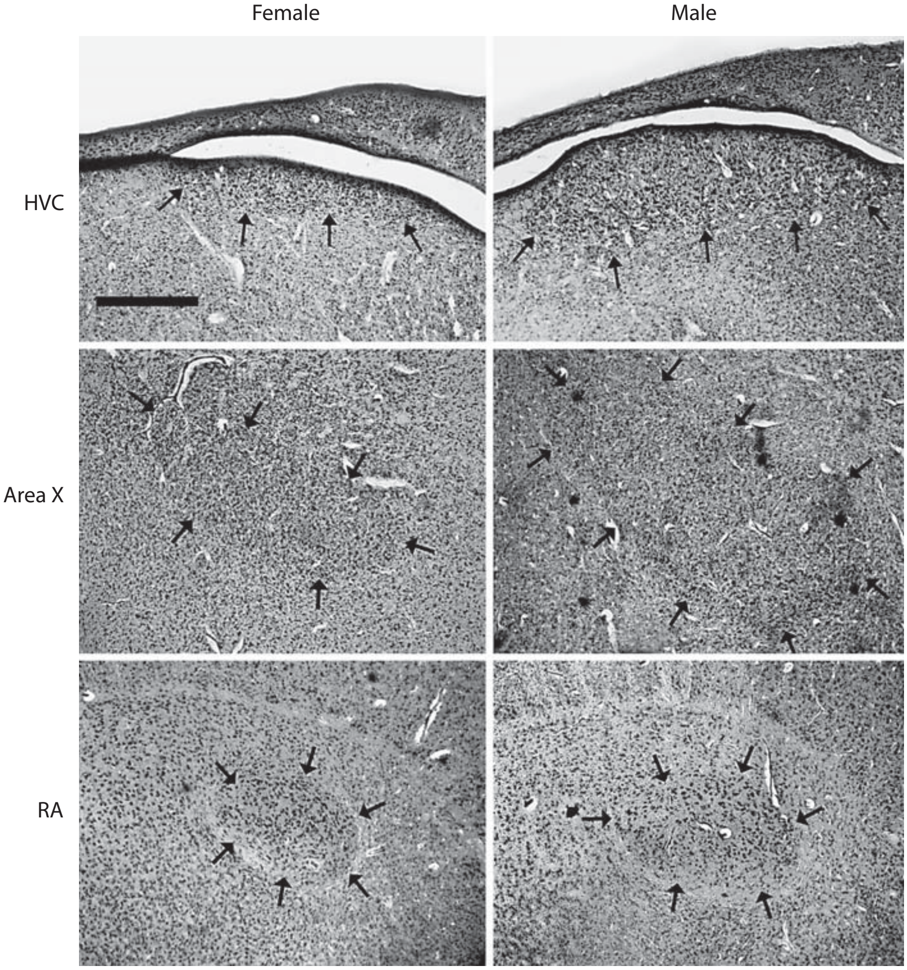

Fig. 2. Photomicrographs of cross-sections of the three major song nuclei HVC, area X and RA in the brains of male and female streak-backed orioles. Arrows indicate nuclear borders with surrounding neural substrate. Bar = 500 μm (applies for all images).

sults from analyses using these brain-size-corrected volumes were not qualitatively different from absolute volumes. All absolute neuroanatomical values discussed are summarized in table 2.

Discussion

We report the first evidence of sex differences in the song control system of a songbird species that shows a female-biased song rate. Volumes of the song nuclei HVC and area X are larger in male streak-backed orioles when compared to females. This finding was unexpected compared to the sex difference predicted by a priori brain-behavior relationships studied in numerous songbird species. Previous knowledge suggests that the sex exhibiting a greater song complexity and/or rate will have larger song nuclei; however, despite the strong female bias in song rate in this species and the sexual similarity in song complexity, the song nucleus HVC and area X exhibit a male bias in volume. Interestingly, incorporating these data with previous studies (table 1) shows that HVC volume is consistently larger in males in all songbird species studied thus far.

Previous comparative studies have proposed a positive correlation between sex differences in song control structures and sex differences in singing behavior, namely song rate [Ball et al., 1994; Brenowitz, 1998]. Despite early criticism [Gahr et al., 1998], a re-analysis of these comparative data incorporating phylogenetic relationships between species has confirmed this positive, but weak, correlation [MacDougall-Shackleton and Ball, 1999]. Species in which males sing exclusively show the greatest male-biased sex differences in the volume of HVC, whereas species in which males and females sing with similar complexity and/or frequency, sex differences are small or

Table 1. Relative female HVC size (female/male HVC volume) among songbird species with a variety of sex differences in singing behavior

Taxon	Females sing more than males	Males and females duet or sing equally	Males and females duet but males sing more	Males sing more than females	Females never sing
Malaconotinae		0.56 *Laniarius funebris* [Gahr et al., 1998]			
Muscicapinae			0.34 *Cossypha heuglini* [Brenowitz et al., 1985]		
Sturnini				0.63 *Sturnus vulgaris* [Bernard and Ball, 2004]	
Troglodytinae		0.77 *Thryothorus leucotis* [Brenowitz et al., 1985] 0.67 *Thryothorus nigricapillus* [Brenowitz and Arnold, 1986]	0.46 *Thryothorus rufalbus* [Brenowitz et al., 1985]		0.08 *Cistothorus palustris* [Canady et al., 1984] 0 *Thryothorus ludovicianus* [Nealen and Perkel, 2000]
Parinae				0.64 *Poecile atricapillus* [MacDougall-Shackleton et al., 2003]	
Estrildidae			0.67 *Uraeginthus bengalis* [Gahr and Güttinger, 1985]		0 *Lonchura striata* [Okanoya, 1997; Tobari et al., 2005] 0.14 *Taeniopygia guttata* [Nottebohm and Arnold, 1976]
Ploceinae		0.67 *Ploceus bicolor* [Gahr et al., 2008]			0 *Euplectes franciscanus* [Arai et al., 1989]
Fringillinae				0.32 *Carpodacus cassinii* [MacDougall-Shackleton et al., 2005] 0.43 *Loxia curvirostra* [MacDougall-Shackleton et al., 2000] 0.3 *Serinus canaria* [Nottebohm and Arnold, 1976]	
Emberizini				0.27 *Zonotrichia leucophrys* [Baker et al., 1984] 0.55 *Zonotrichia albicollis* [Devoogd et al., 1993]	0.26 *Junco hyemalis* [Deviche and Gulledge, 2000]
Icterini	0.57 *Icterus pustulatus* (this study)			0.31 *Agelaius phoeniceus* [Kirn et al., 2004] 0.39 *Molothrus badius* [Hauber et al., 1999]	0.39 *Molothrus rufoaxillaris* [Hauber et al., 1999] 0.14 *Molothrus bonariensis* [Hauber et al., 1999] 0.08 *Molothrus ater* [Latanzio, unpubl.]
Cardinalini		0.6 *Cardinalis cardinalis* [Jawor and MacDougall-Shackleton, 2008]			
Average:	0.58	0.65	0.49	0.43	0.12

Table 2. Brain region volumes measured in adult male and female streak-backed orioles

Brain region	Male volume, mm^3	Males n	Female volume, mm^3	Females n	p value
Telencephalon	644.12 ± 1.98	10	648.93 ± 1.98	9	0.87
HVC	0.96 ± 0.05	9	0.55 ± 0.07	8	<0.001
Area X	1.64 ± 0.08	8	1.00 ± 0.12	8	0.001
RA	0.32 ± 0.03	9	0.29 ± 0.02	9	0.35
Rt	2.02 ± 0.20	6	1.95 ± 0.13	6	0.80

All measures are presented as means ± SEM. p values were all determined using unpaired t tests.

absent. Lastly, in species where females sing, but with less frequency or complexity than males, there is an intermediate male-biased sex difference [reviewed in Brenowitz, 2008]. One problem with these analyses is that they fail to incorporate data from species in which males sing less than females. Our study is the first to test the relationship between sex differences in the brain and sex differences in behavior in a species in which females sing significantly more than males and with similar complexity.

To present this information in a comparative context, we have tabulated all previously reported female-to-male HVC volume ratios for a number of species, arranged by taxa and extent of sex differences in singing behavior (table 1). It should be noted that a recent study reported a male bias in HVC volume in the large-billed crow *(Corvus macrorhynchos)*, despite no previously reported sex differences in song [Wang et al., 2009]. These data were not included in table 1 as the differences between crow song and 'typical' birdsong [reviewed in Wang et al., 2009] may impair comparative analysis.

In table 1, two points emerge. First, sex differences in song control structures are ubiquitous and male biased (table 1). In fact, only in duetting *Thryothorus* wrens has there been no significant male bias in HVC detected. This lack of significance, however, may be due to a limiting sample size as the mean HVC volume is smaller for females in these studies [Brenowitz et al., 1985; Brenowitz and Arnold, 1986] (table 1). Furthermore, other studies on duetting birds have found significant sex differences in HVC size [Gahr et al., 1998]. Interestingly, this sex difference persists despite similarities in song complexity and production rates (table 1). This finding suggests the possibility that enhanced song control system telencephalic mass may be associated with a fundamental component of male birdsong, instead of specific song parameters. For example, larger regions in males may reflect the tendency for male songbirds to focus on producing courtship song, which is then perceived, processed and selected by female songbirds, whose telencephalic space may be more devoted to regions implicated in song perception [Ball et al., 2008]. This idea is supported by the findings that the brain nucleus Rt, unrelated to song behavior, shows no volumetric difference between sexes whilst both HVC and area X were found to have a male bias in the orioles studied here. A potential explanation of these sex differences is that sexual differentiation of the song control system is largely controlled by genes and not the organizational influences of hormones, as is true in mammals [reviewed in Arnold, 2004]. If selective pressure has acted on genes responsible for telencephalic allocation of the song control system to produce a male bias in song nucleus volumes, it is plausible that the streak-backed oriole's exceptional female bias in song may be overshadowed in the brain by a sex-related evolutionary artifact promoting song nucleus telencephalic allocation in males. This sort of evolutionary artifact could also explain the weak nature of the correlation between sex differences in HVC and song complexity/rate among songbirds.

Second, it is interesting to examine the streak-backed oriole's place in this comparative field. Concerning the magnitude of HVC differences, these orioles agree with the postulated interspecific correlation between sex differences in song and HVC volume, showing an intermediate level of sex difference in HVC volume and sex difference in song production. The directionality of the sex difference in this species, however, is opposite to that predicted by the female bias in singing rates, with males having larger HVC and area X volumes. In a previous interspecific comparison [Brenowitz et al., 1997], it was suggested that by comparing sex differences in the song control system to sex differences in song behavior we can make inferences about song control structure function. For example, MacDougall-Shackleton and Ball [1999] reported a positive correlation between sex differences in

HVC volume and song repertoire size using interspecific comparative techniques, suggesting a potential role of this nucleus in song repertoire memory or production. The streak-backed oriole demonstrates a potential dissociation between HVC volume and song traits. Male orioles exhibited an HVC 75% larger than those of conspecific females despite a similar song complexity on all traits previously measured [Price et al., 2008], although song repertoire has not been investigated directly in this species. Likewise, sex differences in RA have been correlated interspecifically with sex differences in singing rates [MacDougall-Shackleton and Ball, 1999]; however, no significant RA difference was found in these orioles, despite a female bias in singing rates in both the breeding and nonbreeding season [Price et al., 2008], especially at the time when these birds were sampled. These discrepancies may be due to the tendency of researchers to focus on gross anatomical traits (i.e. song nucleus volume), which may oversimplify neuroanatomy by analyzing functionally distinct neuronal populations together as a single brain region, implying homogeneity in structure and function [Gahr et al., 1998]. Future studies examining sex differences in the song control system should use retrograde tracers or cytoarchitectonic analysis (immunohistochemistry to identify interneurons [Scotto-Lomassese et al., 2007]) to identify distinct neuron populations within larger song control structures to aid in brain-behavior correlations. Additionally, the analytical limitations of Nissl staining analysis (i.e. quantifying only total region volume) could be avoided using similar histological techniques that permit the quantification of specific, cytoarchitectonic characteristics known to influence nuclear volume, such as cell spacing and soma size [Tramontin and Brenowitz, 2000]. Last, the song control system is renowned for exhibiting extreme seasonal plasticity [Tramontin and Brenowtiz, 2000], which causes sex differences in these brain regions to fluctuate throughout the year [Kirn et al., 1994]. Although no reversal of sex differences has been reported due to this seasonality, future analyses should sample birds multiple times throughout the year instead of a single time as we have done.

The streak-backed oriole appears to show an extreme dissociation between song control system volumes and singing frequency. This male bias in HVC volume despite a female bias in song production confounds a popular, current view that HVC volume is reflective of vocal motor output. This concept is referred to as the performance-induced hypothesis of HVC volume and specifically suggests that song production itself may stimulate the hypertrophy observed in HVC and other song control nuclei during the breeding season [Li et al., 2000]. It would be interesting to investigate whether or not singing in streak-backed orioles can stimulate song nucleus growth in a fashion similar to singing in captive canaries by neurotrophin release in HVC, as previously reported [Li et al., 2000; Sartor and Ball, 2005]. Although this hypothesis does not explain sex differences in song nucleus volume in this species, it may still tenably explain seasonal differences in these nuclei and future investigation in this species should explore whether or not such seasonal plasticity exists in these orioles.

Songbird neuroanatomy has provided an invaluable model for studying the vertebrate brain's relationship to complex behaviors and environmental factors. The recent discovery of the female bias in singing behavior in the streak-backed oriole [Price et al., 2008] and other species (bellbird, *Anthornis melanura* [Brunton and Li, 2006]; stripe-headed sparrows, *Aimophila r. ruficauda* [Illes and Yunes-Jiménez, 2009]) presents a new facet for examining how telencephalic substrate allocation may be linked to sexually different behaviors. This study demonstrates that the neuroanatomical investigation of unique species such as the streak-backed oriole can provide important data that must be integrated into the current theory of brain-behavior relationships.

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