Sex and Seasonal Differences in Neurogenesis and Volume of the Song-Control System Are Associated With Song in Brood-Parasitic and Non-Brood-Parasitic Icterid Songbirds

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ABSTRACT: The song-control system in the brain of songbirds is important for the production and acquisition of song and exhibits both remarkable seasonal plasticity and some of the largest neural sex differences observed in vertebrates. We measured sex and seasonal differences in two nuclei of the song-control system of brood-parasitic brown-headed cowbirds (*Molothrus ater*) and closely-related non-parasitic red-winged blackbirds (*Agelaius phoeniceus*). These species differ in both the development and function of song. Brown-headed cowbirds have a larger sex difference in song than red-winged blackbirds. Female cowbirds never sing, whereas female blackbirds do though much less than males. In cowbirds, song primarily functions in mate choice and males modify their song as they approach sexual maturity and interact with females. In red-winged blackbirds, song is used primarily in territorial defence and is crystalized earlier in life. We found that the HVC was more likely to be discernable in breeding female blackbirds than in breeding female cowbirds. Compared to males, females had a smaller HVC and a smaller robust nucleus of the arcopallium (RA). However, females had higher doublecortin immunoreactivity (DCX+) in HVC, a measure of neurogenesis. Consistent with sex differences in song, the sex difference in RA volume was greater in cowbirds than in blackbirds. Males of both species had a smaller HVC with higher DCX+ in post-breeding condition than in breeding condition when song is more plastic. Sex and seasonal differences in the song-control system were closely related to variation in song in these two icterid songbirds. © 2016 Wiley Periodicals, Inc. Develop Neurobiol 76: 1226–1240, 2016

Keywords: brown-headed cowbird; doublecortin; NeuN; red-winged blackbird; sex differences; seasonal differencesintroduction

INTRODUCTION

The song-control system in the brain of songbirds is comprised of interconnected brain regions that control song acquisition and production. The song-control system, which receives input from the auditory system, is comprised of two pathways: the posterior descending pathway and the anterior forebrain pathway (Nottebohm, 2005). The posterior descending pathway, which is critical for song acquisition

and production, begins with the nucleus HVC, which sends projections to the robust nucleus of the arcopallium (RA), which in turn innervates the tracheosyringeal portion of the hypoglossal nucleus, followed by the syrinx, the song production organ (Nottebohm, 2005). The RA also sends projections to respiratory brain regions (Schmidt et al., 2012). The anterior forebrain pathway, which is critical for song acquisition and auditory feedback, also begins with HVC, which sends projections to area X, which in turn innervates the dorsolateral nucleus of the anterior thalamus (DLM). DLM then innervates the lateral magnocellular nucleus of the anterior nidopallium (LMAN), which innervates RA (Scharff and Nottebohm, 1991; Nottebohm, 2005). The HVC→RA neuronal projection from the posterior descending pathway carries a learned song, whereas the LMAN→RA projection from the anterior forebrain pathway allows for variability in motor output necessary for song imitation (Nottebohm, 2005). Silencing or removing LMAN neurons resulted in HVC→RA neurons firing in a stereotyped pattern, producing a stereotyped song, whereas song is more variable when LMAN→RA neurons are firing (Nottebohm, 2005).

Two distinct and consistent observations regarding HVC and RA have been reported across studies and in a variety of species. First, males, who generally sing more than females, have a larger HVC and RA, and the sex differences in HVC and RA volumes positively correlate with the sex differences in the rates of singing and song complexity (Brenowitz and Arnold, 1986; MacDougall-Shackleton and Ball, 1999; Hall et al., 2010). Second, HVC and RA increase drastically in size in association with breeding, and the increased volume is associated with increased singing rate and song stereotypy in many species (Tramontin and Brenowitz, 2000). For example, the volume of HVC can be two to three times larger in breeding condition than in post-breeding condition (Smith, 1996), and a smaller HVC in the non-breeding season is often associated with reduced song stereotypy (Smith et al., 1995; Smith, 1996; Smith et al., 1997) and/or increased song plasticity associated with learning new song elements (Nottebohm et al., 1986).

An important mechanism behind seasonal changes in HVC volume is neurogenesis. The number of mature neurons in HVC changes seasonally along with HVC volume in a variety of species (Smith et al., 1995; Smith et al., 1997; Tramontin et al., 1998; Tramontin et al., 2000). As circulating testosterone and estrogen increase after the winter solstice, the survival of new neurons and the total number of neurons in HVC increase, whereas cell division decreases (Rasika et al., 1994; Hidalgo et al., 1995; Tramontin

and Brenowitz, 1999). In addition to circulating steroids, the avian brain can produce steroids *de novo* (London et al., 2006). Neuron division is at its peak in the autumn when there is a reduction in song stereotypy and song rate, although song can be functional at this time of year in many species (Kirn et al., 1994; Tramontin and Brenowitz, 1999, 2000). In contrast to HVC, seasonal increases in RA volume do not involve neurogenesis and cell numbers in this nucleus do not change (Tramontin and Brenowitz, 2000). Rather, volume changes of RA are associated with axon and dendrite growth, along with changes in cell soma size and spacing (DeVoogd and Nottebohm, 1981). In sum, there are seasonal changes in neuron number and neurogenesis in HVC, but none in RA.

Because the sex differences and seasonal changes in the song-control regions are so extreme and are clearly linked to behavioral outcomes, they have become an important model in systems neuroscience. However, sex differences and seasonal changes are not often addressed together, which limits our ability to determine if the processes underlying seasonal neural plasticity are the same in both sexes. For example, few studies have examined sex differences in neurogenesis in the song-control system. Two studies reported sex differences in doublecortin immunoreactivity (DCX+; Balthazart et al., 2008; Hall and MacDougall-Shackleton, 2012). Doublecortin (DCX) is a microtubule-associated protein that has recently been used as an endogenous marker of neurogenesis (Balthazart et al., 2008; Hall and MacDougall-Shackleton, 2012; Balthazart and Ball, 2014a,b). With DCX, two immature cell types can be identified: round cells, which are interpreted to be immature differentiating neurons that have reached their final destination, and fusiform cells, which are interpreted to be in the process of migrating to their final destination (Balthazart and Ball, 2014a,b). In canaries (*Serinus canaria*) females had fewer fusiform cells than males in HVC and in the surrounding nidopallium (Balthazart et al., 2008), but in European starlings (*Sturnus vulgaris*) females had more fusiform and round cells in the HVC relative to the adjacent nidopallium than males (Hall and MacDougall-Shackleton, 2012), even though females of both species sing less than males (reviewed in Hall et al., 2010). Thus, there is no clear pattern of sex differences in DCX+, as sex differences vary according to species and likely also with season. Regardless, songbird males across studies sing less in post-breeding condition and have higher levels of cell division than breeding males (see above). In addition, male canaries that were housed with a female sang less and had more

Table 1   Number of brains collected for each experimental group for % discernable HVC (total number of brains in parentheses), volume of HVC and RA determined from NeuN-labelled sections, and neurogenesis (HVC Only), visualized with DCX+

Breeding condition	Brown-headed cowbird		Red-winged blackbird	
	Female	Male	Female	Male
HVC discernability (total)				
Breeding	40% (15)	100% (16)	100% (8)	100% (15)
Post-breeding	25% (8)	88% (8)	38% (8)	88% (8)
HVC volume				
Breeding	6	16	8	15
Post-breeding	2	7	3	7
HVC neurogenesis				
Breeding	6	16	8	16
Post-breeding	2	6	3	7
RA volume				
Breeding	15	16	8	16
Post-breeding	8	8	8	8

Brains were collected the day after the birds were captured in the field in breeding (March-May) and post-breeding (September-November) conditions.

DCX+ than males that were housed either alone or with another male (Balthazart et al., 2008; Alward et al., 2014). It seems that higher neurogenesis in HVC may be associated with less singing.

In the current study, we examined both sex and seasonal differences in HVC and RA volumes and DCX+ in HVC in brown-headed cowbirds (hereafter "cowbirds"; *Molothrus ater*) and red-winged blackbirds (hereafter "blackbirds"; *Agelaius phoeniceus*), two closely-related icterid songbirds. Both species are open-ended learners (Marler et al., 1972; Yasukawa et al., 1980; King and West, 1988; Brenowitz and Beecher, 2005), but their song development and sex differences in song differ. Cowbirds are obligate brood parasites, therefore cowbird nestlings are not exposed to a tutor of their own species. Isolated males develop a song that is innately preferred by females in captivity (Lowther 1993). In the wild, young males develop their songs in winter roost flocks (King and West, 1988), but also during their second year (Brenowitz and Beecher, 2005). Male cowbirds modify their song in response to behavioral feedback from females (King and West, 1988; Hamilton et al., 1997). Male song likely plays a strong role in male fitness because females observed in the wild only mated with their partner after being courted by up to 14 males (Yokel, 1986; Yokel and Rothstein, 1991). Thus, female choice in cowbirds is likely a strong feature of such sexual selection. In contrast to cowbirds, red-winged blackbird nestlings are exposed to a tutor of their own species and male blackbirds that are acoustically isolated develop abnormal songs (Marler et al., 1972). Although male blackbirds

stabilize their initial song in their first summer, song learning is partially open-ended because males can learn new song types into adulthood and add them to their repertoires (Marler et al., 1972; Yasukawa et al., 1980). Song in male blackbirds likely evolved in response to male-male competition as opposed to female choice (Marler et al., 1972; Yasukawa et al., 1980). In addition to differences in song development, these species also differ in the degree of sex difference in singing. Female cowbirds do not sing at all (King and West, 1990; Hamilton et al., 1997) whereas female blackbirds sing, although infrequently and with less complex songs compared to male blackbirds (Nero, 1956; Beletsky, 1983; Kirn et al., 1989; Garamszegi et al., 2005; Price et al., 2009; reviewed by Hall et al., 2010). In sum, there are several developmental and sex-related differences between blackbirds and cowbirds.

The goal of the present study was to investigate sex, species, and seasonal differences in the volumes of HVC and RA and neurogenesis in HVC, and to compare these differences to differences in song. Because female blackbirds sing, but female cowbirds do not, we predicted that the sex differences in HVC and RA would be more pronounced in cowbirds but seasonal differences would be more pronounced in blackbirds. Next, we predicted that seasonal differences in volume would be greater in males than in females, with larger HVC and RA volumes in breeding condition when singing rate peaks. Finally, because plasticity in HVC is associated with reduced singing rates and song stereotypy in some birds, we predicted that HVC DCX+ would be higher in

females than in males and higher in post-breeding condition than in breeding condition.

METHODS

Subjects

We collected cowbirds and blackbirds of both sexes in breeding and post-breeding conditions (Table 1). Birds were the same as those used in a related study on the hippocampus (Guigueno et al., in press). Birds from the breeding group were collected between mid-March and mid-May 2013 and birds from the post-breeding group were collected between mid-September and mid-November 2013. We captured all birds using ground traps and mist nets at various sites near Port Rowan, Ontario, Canada. Mean ($\pm$ SE) body weights were as follows: 39.62 ($\pm$ 0.63) g (female cowbirds; $n = 22$), 50.33 ($\pm$ 0.97) g (male cowbirds; $n = 23$), 42.49 ($\pm$ 0.66) g (female blackbirds; $n = 16$), and 65.29 ($\pm$ 0.98) g (male blackbirds; $n = 23$). Sample sizes for body weights are not the same as those indicated in Table 1 because the weight of three individuals used in the volume analyses were not taken in the field. After capture, we transported the birds to the Advanced Facility for Avian Research at the University of Western Ontario in London, Ontario, where they were housed overnight in individual cages with food and water.

Blood Sampling and Androgen Assays

We collected blood samples in the field to confirm breeding condition. Blood sampling methods and androgen assays are described in detail in Guigueno et al. (2010). Briefly, blood was collected immediately following capture in the field, and the plasma assayed for total androgens using a commercial EIA kit (Washburn et al., 2002).

Brain Collection

We collected the brains the day after capture. Being housed in cages likely increased circulating corticosterone concentrations in the birds, which may reduce circulating testosterone concentrations (Lynn et al., 2010). Decreases in testosterone may in turn reduce the volume of HVC and RA, but in order for an effect to be generated in the time span between capture and brain collection, an acute withdrawal of testosterone would have had to occur (Thompson et al., 2007). Thus, a potential decrease in testosterone due to stress from overnight housing would likely produce minimal effects. More importantly, all birds underwent the same treatment and were compared to each other.

We deeply anesthetized the birds using isoflurane. We transcardially perfused the birds with heparinized saline, followed by 4% paraformaldehyde. The brains were then carefully removed from the skull and placed in 4% paraformaldehyde for 24 h, followed by 30% sucrose for 48-72 h (until the brains sunk to the bottom of the vial). Finally, we froze the brains on
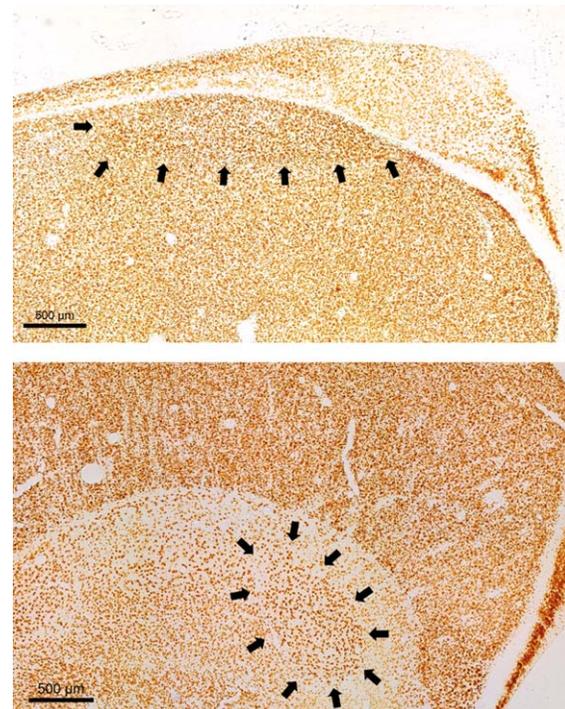

Figure 1 Examples of NeuN labeled brain sections with HVC (top) and robust nucleus of the arcopallium (RA) (bottom) indicated by arrows. [Color figure can be viewed at wileyonlinelibrary.com]

powdered dry ice and stored them in aluminium foil at $-80°C$ until the start of immunohistochemistry.

Immunohistochemistry

We sectioned the brains into 40 μm sections in the coronal plane using a cryostat. Two sets of brain sections, each set two sections apart throughout the HVC and RA, were collected for NeuN and DCX immunohistochemistry. NeuN is a protein expressed in most mature neurons (Mullen et al., 1992) and was used to delineate HVC and RA to calculate their volume (Newman et al., 2008). DCX is a protein expressed by migrating and immature differentiating neurons (Francis et al., 1999; Gleeson et al., 1999), and was used to quantify neurogenesis (Balthazart and Ball, 2014a,b). Each immunohistochemistry run consisted of two to eight randomly selected brains from different groups (Table 1).

Detailed methods of the immunohistochemistry protocol are described in detail in Guigueno et al. (in press). Briefly, free-floating sections were treated with 0.5% H_2O_2 to reduce endogenous peroxidases, blocked with normal serum then incubated with the primary antibody (catalogue numbers MAB377 [Millipore] for NeuN and sc-8067 [Santa Cruz Biotechnology] for DCX). Next, sections were incubated in a biotin-conjugated secondary antibody and then incubated with an avidin-biotin complex (Vectastain Elite kit, Vector) and visualized with diaminobenzidine before being mounted on microscope slides.
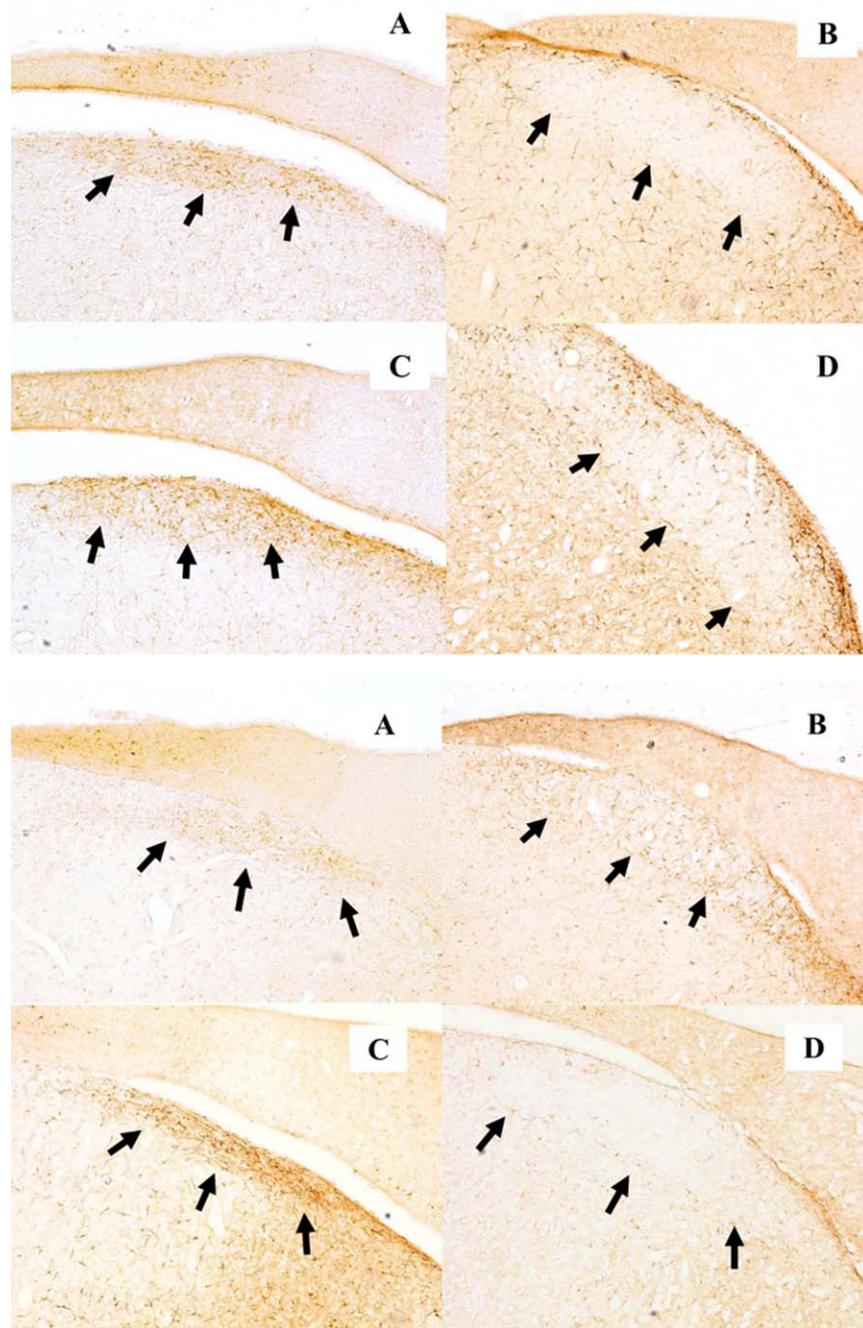
Figure 2 Doublecortin labelled brain sections with HVC indicated by arrows for breeding (top) and post-breeding conditions (bottom) in female (A) and male (B) brown-headed cowbirds (*Molothrus ater*) and female (C) and male (D) red-winged blackbirds (*Agelaius phoeniceus*). [Color figure can be viewed at wileyonlinelibrary.com]

Microscopy

We used the NeuN-labeled sections to measure the volumes of HVC and RA. We captured images of HVC and RA with a Spot Idea 5-megapixel digital camera (Diagnostics Instruments) mounted on a Zeiss Axiophot microscope using a 1.25× objective lens. Only a random bird ID was assigned to each photo, therefore the images were analyzed without reference to species, sex, or season. The perimeters of HVC and RA, when visible, were traced in ImageJ software (NIH) (Fig. 1). We summed the frusta (truncated cone) volumes between sections (80 μm) to estimate the total volumes of HVC and RA in both hemispheres. We used as a covariate for the HVC and RA volume analyses the same
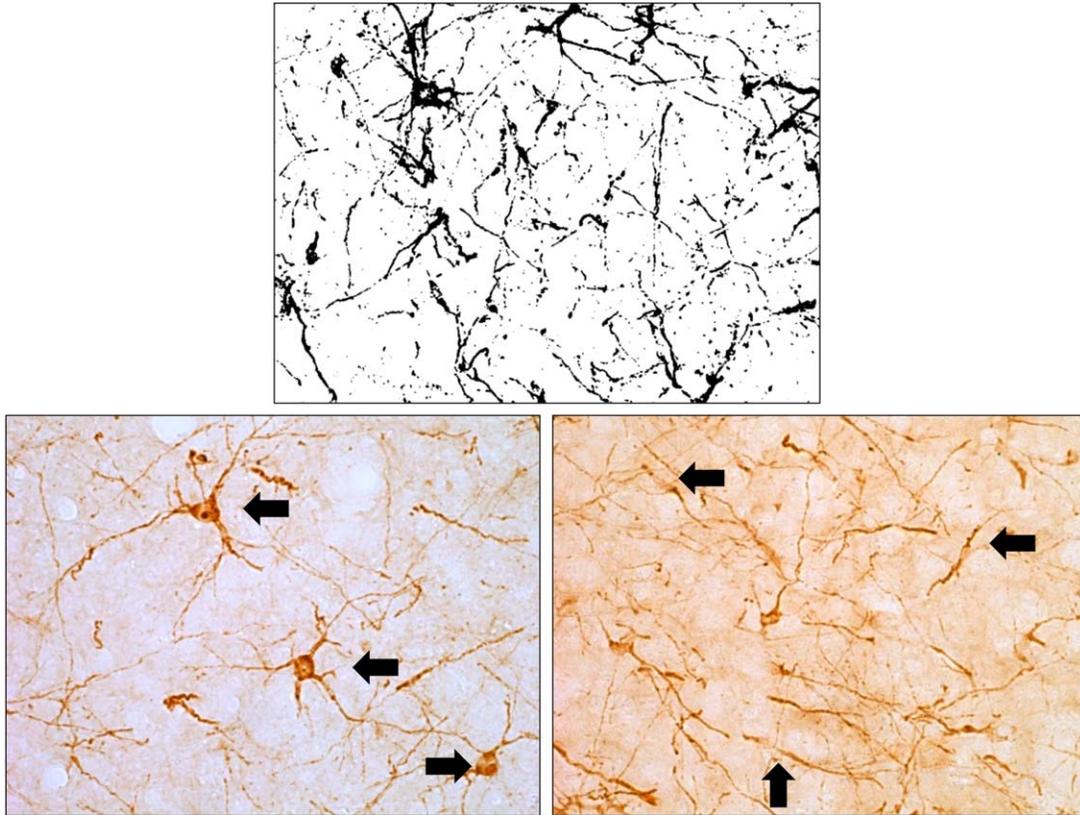
Figure 3 Fields of view in doublecortin-labeled sections, with an example of each type of measurement taken: thresholding to measure the % doublecortin immunoreactive cover (top), number of round cells, indicated by arrows (bottom left) and number of fusiform cells, indicated by arrows (bottom right). [Color figure can be viewed at wileyonlinelibrary.com]

telencephalon measurements as those used in the hippocampus companion article (Guigueno et al., in press). HVC and RA volumes used in the analyses for each bird were the average between hemispheres. We adjusted the sampling interval and used the next nearest section if a section was damaged or lost. In some groups HVC was indiscernible from the surrounding nidopallium, especially in female cowbirds and in post-breeding female blackbirds. For one male breeding blackbird, tissue was too damaged to measure its HVC volume. One brain was damaged during the sectioning and could not be used for any volume measurement, hence the total sample size for RA volume ($n = 87$) is one fewer than the total sample sizes for androgen measurements ($n = 88$). Final sample sizes for HVC and RA volumes according to experimental group are found in Table 1.

We used DCX-labelled sections to quantify neurogenesis in HVC (Fig. 2). We captured images to analyze % DCX+ cover (cells and projections) and the number of DCX+ round and fusiform cells (Fig. 3) with a Leica DFC 420C camera mounted on a Leica DM5500B microscope. We chose five sections 80 μm apart and centered on the largest cross-sectional area of HVC from the hemisphere that was most intact. We analyzed two fields of view per section. One field of view was positioned in the centre of the HVC, whereas the other was positioned just outside and ventral to

HVC (in the nidopallium; see Wada et al., 2014 for schematic drawing). We averaged values from all five sections for each location for further analyses. We did not analyze DCX+ in RA because there was little to no immunoreactivity in this song-control nucleus (as reported in Boseret et al., 2007; Balthazart et al., 2008; Wada et al., 2014). For each field of view, we captured z-stack images in 0.63 μm steps through the focal planes with a 40X objective lens. Following Hall et al. (2010), we compiled these images using the montage mode in Leica Application Suite software, which resulted in an image that displayed all DCX+ cells and projections in focus. We used the threshold feature in ImageJ to calculate the % coverage by DCX+ cells and projections. We counted and analyzed fusiform cells and round cells separately. We were not able to quantify neurogenesis in some birds due to poor staining, therefore sample sizes from the DCX analyses differed from those for the volume analyses (Table 1).

Data Analysis

We conducted all statistical analyses in SAS (version 9.3, SAS Institute Inc., Cary, NC). HVC was not always discernable in females (Table 1), therefore we ran Fisher exact
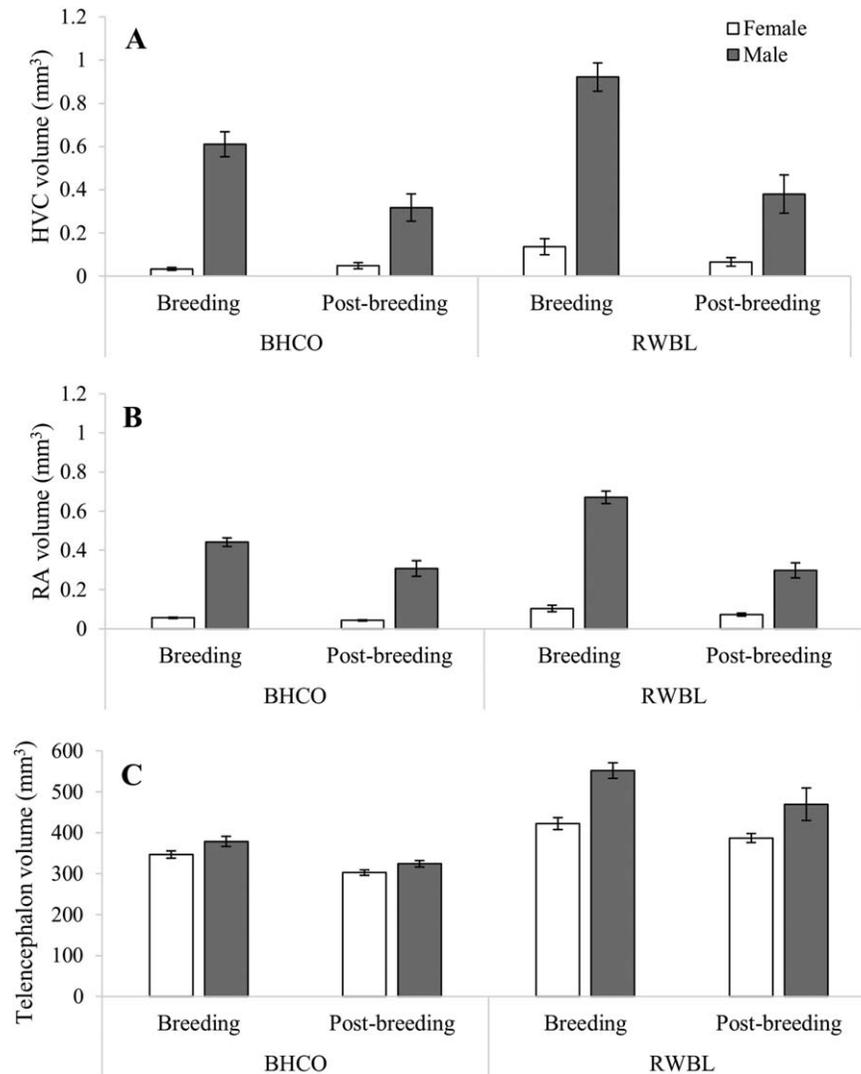
Figure 4 Mean ± SE volumes of HVC (A), RA (B), and telencephalon (C) in female and male brown-headed cowbirds (BHCO) and red-winged blackbirds (RWBL) in breeding and post-breeding conditions. Overall, males had larger HVC and RA relative to telencephalon volume than females. Telencephalon volume data from Guigueno et al. (in press).

tests to determine whether the proportion of females with discernable HVC differed between the breeding conditions for blackbirds and for cowbirds. For the volume analyses, we used general linear models (PROC GLM) with species, sex, breeding condition, and all interactions as explanatory variables, telencephalon volume (minus HVC or RA) as a covariate, and HVC and RA volumes as the dependent variables. To analyze the average %DCX+ cover, number of round cells, and number of fusiform cells inside the HVC, we used general linear models, with species, sex, breeding condition, and all interactions as explanatory variables, and the respective DCX+ measurements in the nidopallium as the covariate. To produce normally distributed residuals, we arcsine-transformed proportions from the %DCX+ cover data and log-transformed the remaining data if the residuals were not already normally distributed. Significant

interactions were further analyzed using predetermined Fisher's LSD post-hoc tests. Data are presented as means ± SE and results were considered significant if $p \leq 0.05$.

RESULTS

Androgens

Data are the same as those presented in Guigueno et al. (in press). There was a significant main effect of breeding condition, with higher androgen levels in breeding condition than in post-breeding condition ($F_{1,79} = 24.55$, $p < 0.0001$), confirming breeding condition in these birds. There was also a significant

Table 2 Summary of statistical effects of species, sex, breeding condition and their interactions on the volumes of the HVC and the RA

Factors	F	d.f.	p-value
HVC volume			
Species	0.71	1,55	0.40
Sex	42.36	1,55	**<0.0001**
Breeding condition	5.08	1,55	**0.03**
Species × Sex	0.15	1,55	0.70
Species × Breeding condition	1.85	1,55	0.18
Sex × Breeding condition	8.23	1,55	**0.006**
Species × Sex × Breeding condition	0.03	1,55	0.86
Telencephalon (covariate)	15.25	1,55	**0.0003**
RA volume			
Species	0.27	1,78	0.61
Sex	603.67	1,78	**<0.0001**
Breeding condition	21.20	1,78	**<0.0001**
Species × Sex	16.26	1,78	**0.0001**
Species × Breeding condition	5.13	1,78	**0.03**
Sex × Breeding condition	5.65	1,78	**0.02**
Species × Sex × Breeding condition	1,78	1,78	0.19
Telencephalon (covariate)	22.38	1,78	**<0.0001**

Results are from a general linear model. Significant effects are in bold.

main effect of sex, with males showing higher levels than females ($F_{1,79} = 3.85$, $p = 0.05$) (Supporting Information Fig. S1; Supporting Information Table S1). Species differences and all interactions were not significant (Supporting Information Table S1).

Discernable HVC in Females

A 4 X 2 Fisher exact test indicated the number of birds with an HVC discernable from background nidopallium was significantly different across breeding and post-breeding female blackbirds and cowbirds ($p = 0.008$; Table 1). We used 2 × 2 Fisher exact tests for pairwise comparisons. In breeding condition, female blackbirds had more discernable HVC than did female cowbirds ($p = 0.007$; Table 1). Female blackbirds were more likely to have a discernable HVC in breeding condition than in post-breeding condition ($p = 0.03$; Table 1). Finally, in female cowbirds, there was no significant difference in the number of birds with a discernable HVC between breeding condition and post-breeding condition ($p = 0.66$; Table 1). All further analyses below include data only from those birds that had a discernable HVC.

Volume

HVC Volume. As predicted, there were significant effects of sex and season on HVC volume, and there was also a significant interaction between sex and

season (Table 2). There was no significant difference between species in HVC size (Table 2). Males had a larger HVC than females in both cowbirds and blackbirds [Fig. 4(A)]. The significant sex by breeding
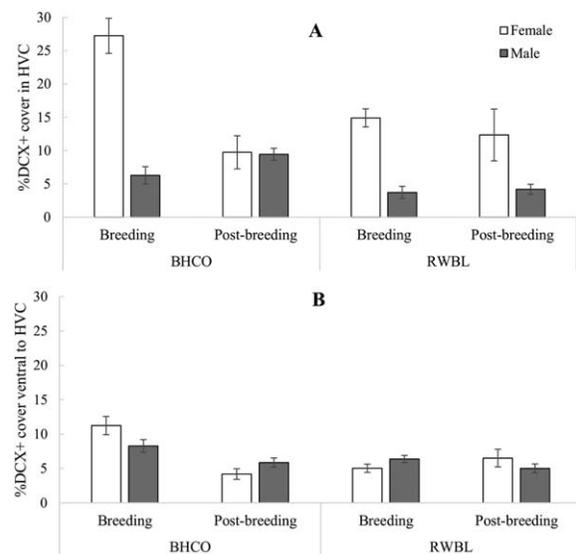

Figure 5 Mean ± SE %DCX+ cover in fields of view inside (A) and outside (B) the HVC in female and male brown-headed cowbirds (BHCO) and red-winged blackbirds (RWBL) in breeding and post-breeding conditions. Means were calculated from five fields of view inside the HVC and five fields of view outside the HVC over five coronal sections centered in the middle of the HVC. Overall, females had higher levels of doublecortin immunoreactivity inside the HVC relative to outside the HVC than males.

Table 3 Summary of statistical effects of species, sex, breeding condition and their interactions on the doublecortin immunoreactivity (DCX+) in the HVC of female and male brown-headed cowbirds and red-winged blackbirds in breeding and post-breeding conditions

Factors	F	d.f.	p-value
%DCX+ cover			
Species	3.76	1,55	0.06
Sex	71.40	1,55	**<0.0001**
Breeding condition	0.01	1,55	0.92
Species × Sex	0.51	1,55	0.48
Species × Breeding condition	0.60	1,55	0.44
Sex × Breeding condition	19.52	1,55	**<0.0001**
Species × Sex × Breeding condition	1.02	1,55	0.32
Telencephalon (covariate)	42.81	1,55	**<0.0001**
Round cells			
Species	0.71	1,55	0.40
Sex	30.62	1,55	**<0.0001**
Breeding condition	3.08	1,55	0.08
Species × Sex	3.32	1,55	0.07
Species × Breeding condition	2.37	1,55	0.13
Sex × Breeding condition	4.13	1,55	**0.05**
Species × Sex × Breeding condition	0.08	1,55	0.78
Telencephalon (covariate)	11.17	1,55	**0.002**
Fusiform cells			
Species	0.03	1,55	0.86
Sex	5.10	1,55	**0.03**
Breeding condition	1.33	1,55	0.25
Species × Sex	10.89	1,55	**0.002**
Species × Breeding condition	4.64	1,55	**0.04**
Sex × Breeding condition	7.90	1,55	**0.007**
Species × Sex × Breeding condition	3.32	1,55	0.07
Telencephalon (covariate)	28.32	1,55	**<0.0001**

DCX+ is a measure of neurogenesis. Results are from a general linear model. Significant effects are in bold.

condition interaction resulted from the effect that males had a larger HVC in breeding condition than in post-breeding condition ($t_{43} = 4.53$, $p < 0.0001$), but females did not [$t_{17} = 0.27$, $p = 0.78$; Table 2, Fig. 4(A)].

RA Volume. Similar to HVC volume, for RA there was a significant main effect of sex and season (Table 2). All three interactions between sex, season and species were also significant (Table 2). These interactions appear to result from the following effects. First, males had a larger RA in breeding condition than in post-breeding condition [$t_{46} = 5.08$, $p < 0.0001$, Fig. 4(B)], with no significant seasonal change in females ($t_{37} = 1.84$, $p = 0.07$). Second, the sex difference in RA volume of cowbirds (Cohen's $d = 5.75$; $t_{45} = 23.15$, $p < 0.0001$) was greater than the sex difference in blackbirds [Cohen's $d = 4.34$; $t_{38} = 14.25$, $p < 0.0001$; Fig. 4(B)]. Finally, there was a greater effect of breeding condition in blackbirds (Cohen's $d = 1.42$; $t_{38} = 4.81$, $p < 0.0001$), than in

cowbirds [Cohen's $d = 0.55$; $t_{45} = 2.00$, $p = 0.05$; Table 2, Fig. 4(B)]. The main effect of species and the three-way interaction between species, sex, and breeding condition were not statistically significant (Table 2).

Doublecortin

Percentage Immunoreactivity. In HVC, females had higher %DCX+ than males and a significant sex by breeding condition interaction resulted from the effect that %DCX+ levels were higher in males in post-breeding condition ($t_{43} = 4.14$, $p = 0.0001$), but higher in females in breeding condition ($t_{17} = 1.46$, $p = 0.02$) (Table 3, Fig. 5). There were no significant main effects of species and breeding condition and all other interactions were not significant (Table 3). Thus, although males had a larger HVC, females had a greater percentage DCX+ than males, and seasonal changes in DCX+ were in the opposite directions for males and females.
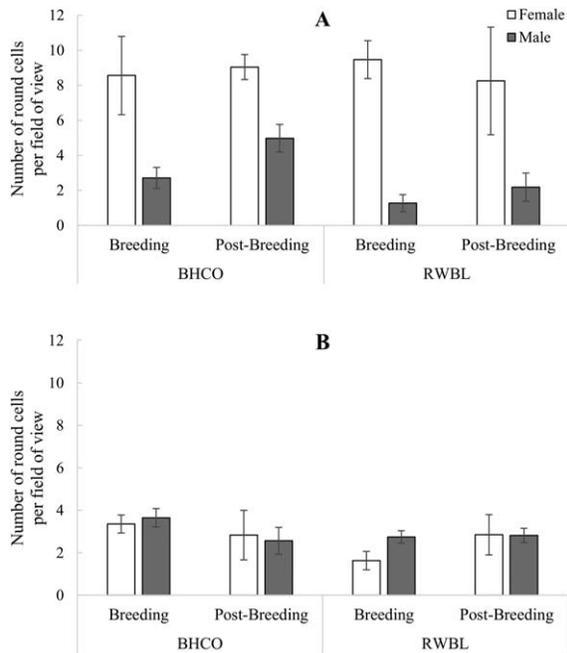
Figure 6 Mean ± SE number of round cells per field of view inside (A) and outisde (B) the HVC in female and male brown-headed cowbirds (BHCO) and red-winged blackbirds (RWBL) in breeding and post-breeding conditions. Means were calculated from five fields of view inside the HVC and five fields of view outside the HVC over five coronal sections centered in the middle of the HVC. Overall, females had more round cells per field of view inside the HVC relative to outside the HVC than males.

Number of Round Cells. In HVC, females had more round cells per field of view than males and a significant sex by breeding condition interaction resulted from the effect that males had more round cells per field of view in post-breeding condition than in breeding condition ($t_{43} = 3.56$, $p = 0.0008$), whereas there was no seasonal effect in females ($t_{17} = 0.17$, $p = 0.86$) (Table 3, Fig. 6). Species, breeding condition, and all other interactions were not significant (Table 3, Fig. 6).

Number of Fusiform Cells. In HVC, females had more fusiform cells per field of view than males and a significant sex by breeding condition interaction resulted from the effect that males had more fusiform cells per field of view in post-breeding condition than in breeding condition ($t_{43} = 3.80$, $p = 0.0004$), whereas there was no seasonal effect in females ($t_{17} = 0.93$, $p = 0.36$; Table 3, Fig. 7). In addition, a significant sex by species interaction resulted from the effect that female blackbirds had significantly more fusiform cells per field of view than male blackbirds($t_{32} = 4.31$, $p < 0.0001$), but no sex difference

occurred in cowbirds ($t_{28} = 0.60$, $p = 0.55$) (Table 3, Fig. 7). Finally, there was a significant species by breeding condition interaction, with blackbirds having more fusiform cells per field of view in post-breeding condition than in breeding condition ($t_{32} = 2.70$, $p = 0.009$), whereas no seasonal effects were present in cowbirds ($t_{28} = 0.67$, $p = 0.51$) (Table 3, Fig. 7). Species, breeding condition and the three-way interaction were not significant (Table 3).

DISCUSSION

We found multiple effects of sex, season, and species in the volume of HVC and RA and in DCX immunoreactivity in HVC. The HVC of breeding condition female blackbirds was more likely to be discernable than the HVC of breeding condition female cowbirds (Table 1). As predicted, males, which sing more than females, had greater HVC and RA volumes than females and their HVC and RA were greater in volume in breeding condition, when rates of singing are highest [Table 2, Fig. 4(A,B)]. Consistent with our prediction, there was a greater sex difference in RA volume in cowbirds than in blackbirds, likely because
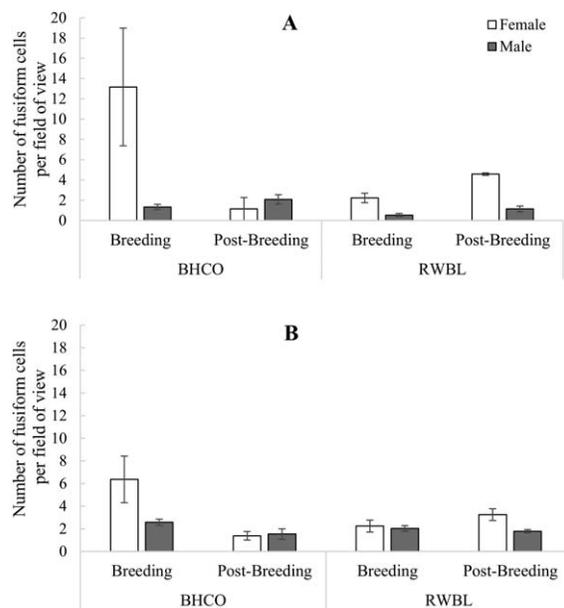

Figure 7 Mean ± SE number of fusiform cells per field of view inside (A) and outisde (B) the HVC in female and male brown-headed cowbirds (BHCO) and red-winged blackbirds (RWBL) in breeding and post-breeding conditions. Means were calculated from five fields of view inside the HVC and five fields of view outside the HVC over five coronal sections centered in the middle of the HVC. Overall, females had more fusiform cells per field of view inside the HVC relative to outside the HVC than males.

female cowbirds do not sing at all [Hamilton et al., 1997; Table 2, Fig. 4(B)]. Breeding condition had a greater influence on RA volume in blackbirds than in cowbirds, likely because both sexes in blackbirds sing [Table 2, Fig. 4(B)]. We predicted a lower singing rate would be associated with higher neurogenesis based in part on previous research with starlings and canaries (Balthazart et al., 2008; Hall and MacDougall-Shackleton, 2012). Indeed, females had higher neurogenesis in HVC than males as indicated by the density of DCX+ cells and fibres in HVC relative to the surrounding nidopallium. Males had higher levels of neurogenesis (DCX+) in post-breeding condition than in breeding condition (Table 3; Figs. 5–7). Female neurogenesis patterns were similar between breeding conditions, except for %DCX+ cover, which was higher in breeding condition than in post-breeding condition, a seasonal difference in an unexpected direction.

Discernibility and Volumes

Sex and seasonal differences in HVC and RA volume results were consistent with sex differences and seasonal changes in singing behaviour of these species. Female blackbirds sing less than males and song is most frequent in breeding condition (Nero, 1956; Beletsky, 1983; Kirn et al., 1989) whereas female cowbirds do not sing at all (King and West, 1990; Hamilton et al., 1997). Projections from HVC to RA are part of the posterior descending pathway of the song-control system required for song production (Nottebohm, 2005) and we found species differences in females for both of these nuclei. First, breeding female blackbirds had a higher proportion of brains with a discernable HVC than breeding female cowbirds and female blackbirds showed an increase in the proportion of brains with a discernable HVC from post-breeding to breeding condition (Table 1). In species in which females never sing, HVC is often not anatomically discernable (MacDougall-Shackleton and Ball, 1999). We found that was the case for some of the females in this study, and this varied between species and seasons. Second, for RA volume, the effect of breeding condition was greater in blackbirds, suggesting that both male and female blackbirds underwent an increase in RA volume whereas this effect of breeding condition was dampened in cowbirds because only male cowbirds underwent an increase in RA volume [Fig. 4(B)]. Finally, for RA volume, there was a greater sex difference in cowbirds than in blackbirds, which we expected because both sexes of blackbirds sing whereas only

male cowbirds sing (Table 2). Together, these discernibility and volumetric differences between species suggest that neuroanatomy of the song system reflects the fact that female blackbirds produce more song in breeding condition than in post-breeding condition and sing more than female cowbirds (Nero, 1956; Beletsky, 1983; Kirn et al., 1989; King and West, 1990).

Males had larger HVC and RA than females [Table 2, Figs. 4(A,B)], as previously reported in blackbirds (Kirn et al., 1989) and in cowbirds (Hamilton et al., 1997). Male blackbirds and cowbirds sing more than females and multiple studies have shown a positive correlation between sex differences in singing rate and sex differences in song-control nuclei (Brenowitz and Arnold 1986; Ball et al., 1994; Brenowitz, 1997), even after taking phylogenetic relationships into account (MacDougall-Shackleton and Ball, 1999). In addition, the female/male HVC size ratio increases as the female/male singing ratio increases from species in which females do not sing at all (i.e., cowbirds) to species in which females sing but males sing more (e.g., blackbirds) to duetting species (e.g., *Thryothorous* wrens; reviewed in Hall et al., 2010).

The volumes of HVC and RA increased from post-breeding condition to breeding condition [Table 2, Figs. 4(A,B)] and that was especially true in males, as previously reported in blackbirds (Kirn et al., 1989), but not been previously investigated in cowbirds. Nottebohm (1981) was the first to show this dramatic seasonal increase in HVC and RA, which are due to changes in cell sizes, cell spacing and cell numbers (Tramontin and Brenowitz, 2000). We found that, from post-breeding to breeding males, HVC size increased by 196% in cowbirds and 243% in blackbirds whereas RA increased by 143% in cowbirds and 224% in blackbirds (Fig. 4). The increase in HVC volume in blackbirds is similar to the 288% increase in HVC size in spotted towhees (*Pipilo maculatus*), which is among the greatest volumetric increase reported in a song-control nucleus (Smith, 1996). Indeed, this seasonal brain plasticity in songbirds is among the most pronounced of any adult vertebrate and the volume of the song-control system and singing behaviour have been shown to be seasonally plastic in every seasonally breeding songbird studies so far (reviewed by Tramontin and Brenowitz, 2000). These seasonal changes in the song-system result primarily from photoperiod-driven changes in gonadal steroid hormones (reviewed in Tramontin and Brenowitz, 2000) potentially interacting with gonad-independent mechanisms (Ball et al., 2008; Robertson et al., 2014).

Neurogenesis in HVC

DCX has only recently been used extensively to measure neurogenesis in the adult avian brains. Even if some limitations have been expressed (Vellema et al., 2014), multiple arguments suggest that it is a reliable endogenous marker of neurogenesis (Balthazart and Ball, 2014a,b). Very few studies have simultaneously examined sex and seasonal changes in neurogenesis in general, or with DCX in particular. In the current study, we measured the density of DCX+ cells and fibres in fields of view within the HVC. However, without stereology looking at the entire HVC, we cannot conclude that the *total* number of new cells in the HVC, relative to outside the HVC, differs between groups.

Heightened levels of neurogenesis, as measured by DCX+, seemed to be associated with a lower rate of singing in blackbirds and cowbirds. Female blackbirds and cowbirds had higher levels of neurogenesis than males, based on all three DCX+ density measurements (%DCX+ cover, number of round cells, and number of fusiform cells per field of view; Table 3, Figs. 5–7), a female-biased sex difference that is also present in starlings (Hall and MacDougall-Shackleton, 2012). Because we did not measure the total number of cells in the HVC, we can only conclude that the density of DCX+ cells (i.e., % coverage or cells per field of view) was higher in females than in males. In addition, DCX+ in male blackbirds and cowbirds was higher in post-breeding condition than in breeding condition (Table 3, Figs. 5–7). A negative relationship between neurogenesis levels and song rate is consistent with female blackbirds and cowbirds singing less than males and post-breeding males singing less than breeding males (Nero, 1956; Beletsky, 1983; Kirn et al., 1989; King and West, 1990). A negative correlation between singing rate and DCX+ was also reported in male canaries that sing less in the presence of females, but show more DCX+ in HVC (Balthazart et al., 2008; Alward et al., 2014). However, Balthazart et al. (2008) also showed that castrated males exposed to testosterone and photosensitive and photostimulated birds, individuals that are expected to sing more, had higher levels of DCX+, indicating that the direction of the relationship between neurogenesis and singing is not always consistent. In sum, HVC volume in blackbirds and cowbirds was positively correlated with singing whereas neurogenesis was negatively correlated with singing.

Neurogenesis is likely modulated by hormone levels. Differences in DCX+ in breeding and post-breeding blackbirds and cowbirds paralleled differences in circulating androgen levels . Elevated testosterone and estrogen levels reduce the turnover rate of neurons in HVC and increase the survival of new neurons, thus increasing the number of total neurons in HVC in the breeding season (Rasika et al., 1994; Hidalgo et al., 1995; Tramontin and Brenowitz, 1999). Neurogenesis in HVC generates new RA-projecting neurons and interneurons, replacing old cells (Paton et al., 1985; Kirn and Nottebohm, 1993). Peak neuron turnover in the autumn coincides with a peak in song learning and a reduction in song stereotypy in the canary, an open-ended song learner (Kirn et al., 1994). However, this seasonal peak in neuron turnover and a drop in song stereotypy is also present in an age-limited learner that does not change its song in adulthood (Tramontin and Brenowitz, 1999), suggesting that peak neuron turnover may be more closely associated with song stereotypy only or that neuron turnover may be necessary for song learning, but not sufficient on its own (Tramontin and Brenowitz, 2000). Both cowbirds (King and West, 1988) and blackbirds (Marler et al., 1972; Yasukawa et al., 1980) are open-ended learners, so the post-breeding season may be a time during which song modification is at its maximum.

Species differences in neurogenesis may reflect differences in sexual selection. Male blackbirds had fewer migrating cells than females, with no sex difference in cowbirds (Table 3, Fig. 7). This sex by species interaction in the production of new neurons may be explained by cowbird males continuously modifying their song in response to stimulation by females (King and West, 1988; Hamilton et al., 1997). Male blackbirds are not known to modulate song learning in response female feedback as has been observed in cowbirds. In contrast, blackbirds, who establish breeding territories only during the breeding season, had more migrating cells in post-breeding condition than in breeding condition, whereas no seasonal effect was present in cowbirds (Table 3, Fig. 7). In sum, although neurogenesis peaked in post-breeding males of both species, it was more concentrated in post-breeding blackbirds, which may be due to different forms of sexual selection acting on these species.

In contrast to males, the density of differentiating and fusiform cells in females remained similar between breeding conditions, but %DCX+ cover was greater in breeding condition (Table 3, Figs. 5–7). This seasonal effect in %DCX+ cover in the HVC relative to outside the HVC was mainly driven by data from female blackbirds (Fig. 5). Female blackbirds sing two song types; one is for pair-bond maintenance and the other is apparently territorial (Beletsky, 1983). Unlike males that use their song to

attract mates and thus would require peak song performance established by the start of the breeding season, female blackbirds may need to modify their pair-bonding song during the breeding season based on the mate they choose for that year. Song modification in breeding female blackbirds may contribute to females having a higher density of migrating cells than males with no sex difference existing in cowbirds (Table 3, Fig. 7). In sum, neurogenesis in HVC peaks during opposite times of the year in female and male blackbirds possibly to accommodate differential timing in song modification, although this possibility would need to be investigated further and should be interpreted with caution as it is based on small samples sizes for post-breeding female cowbirds and blackbirds (Table 1).

In conclusion, we show that song differences between the sexes and seasons are related to differences in the volume and neurogenesis of the song-control system. Increases in song rate, whether it is males singing more than females or breeding birds singing more than non-breeding birds, were generally associated with increases in volume and decreases in neurogenesis. New neurons seem to disrupt song by interfering with memory for songs or interfering with stereotyped performance of songs. The arrival and incorporation of new neurons into HVC may be inhibited in breeding males, especially in blackbirds, to allow high song output. However, the processes underlying seasonal neural plasticity are not identical between the sexes because female blackbirds had more discernable HVC with more neurogenesis in breeding condition than in post-breeding condition, whereas neurogenesis peaked in post-breeding condition in males. In sum, sex and seasonal differences in the song-control system were closely related to variation in song in these two icterid songbirds.

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