Annual Cycle of the Black-Capped Chickadee: Seasonality of Singing Rates and Vocal-Control Brain Regions

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ABSTRACT: **Black-capped chickadees have a rich vocal repertoire including learned calls and the learned *fee-bee* song. However, the neural regions underlying these vocalizations, such as HVC, area X, and RA (robust nucleus of arcopallium), remain understudied. Here, we document seasonal changes in *fee-bee* song production and show a marked peak in singing rate during March through May. Despite this, we found only minimal seasonal plasticity in vocal control regions of the brain in males. There was no significant effect of time of year on the size of HVC, X, or RA in birds collected in January, April, July, and October. We then pooled birds into two groups, those with large testes (breeding condition) and those with small testes (nonbreeding), regardless of time of year. Breeding birds had slightly larger RA, but not HVC or X, than nonbreeding birds. Breeding birds had slightly larger HVC and RA, but not X, as a proportion of telencephalon volume than did nonbreeding birds. Birds collected in July had heavier brains than birds at other times of year, and had the greatest loss in brain mass during cryoprotection. The absence of any overall seasonal change in the vocal-control regions of chickadees likely results from a combination of individual differences in the timing of breeding phenology and demands on the vocal-control regions to produce learned calls year-round.** J Neurobiol 66: 1002–1010, 2006

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Across the annual cycle animals perform different behaviors, often depending on resources available at specific times of year. Behaviors associated with breeding, such as courtship singing in birds, are timed so that hatching occurs when food for nestlings is most abundant. In many species of songbirds, song production peaks in early spring just prior to the production of young. Food-storing behavior, on the other hand, tends to occur at a different time of year: when food supply is declining and less predictable in autumn. The timing of seasonal behaviors is often regulated by the change in photoperiod, or day length, although it can be modulated by other factors such as temperature (Dawson et al., 2001). Seasonal changes in brain regions associated with seasonal behaviors, such as the vocal-control system for song (Brenowitz, 2004) and the hypothalamic gonadotropin-hormone releasing system for reproduction (Ball and Hahn, 1997) are likewise regulated by the annual change in photoperiod. While photoperiod can be shown to affect food-storing behavior, it is not clear how it may directly affect changes in the hippocampus (Krebs et al., 1995; Shettleworth et al., 1995; MacDougall-Shackleton et al., 2003b; Karpouzos et al., 2005).

Many seasonal changes in song and the neural system that produces it (the vocal-control system) are driven by annual changes in testosterone, which in

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turn are driven by annual changes in photoperiod (Tramontin and Brenowitz, 2000). In many species, song production increases in spring, when days are becoming longer and birds are photostimulated and enter breeding condition. The dawn chorus is typically heard only during the breeding season. Song production decreases again in late summer when birds are no longer breeding. Another feature of song that changes seasonally is song stereotypy. Male song tends to be highly stereotyped in breeding season, but less so at other times of year. For example, both Gambel's white-crowned sparrows (*Zonotrichia leucophrys gambelli*) and song sparrows (*Melospiza melodia*) show more stereotypy in song production in the spring than in autumn (Smith et al., 1997).

Songbirds have neural regions that are specialized for production of learned vocalizations, such as HVC, area X, and RA (robust nucleus of the arcopallium). Although this neural system is often called the song-control system, it is also critical for the production of learned calls (Vicario et al., 2002). These specialized regions show several kinds of seasonal changes. In most seasonally breeding birds, volumes of these regions are larger in birds in breeding condition than in nonbreeding birds (Tramontin and Brenowitz, 2000), and this increase in size may precede the production of offspring (Tramontin et al., 2001; Caro et al., 2005). This change can be extreme. In white-crowned sparrows, for example, HVC and RA are twice the volume in breeding than in nonbreeding birds (Tramontin and Brenowitz, 1999). Another seasonal change in HVC is variation in neuronal recruitment, which results in variation in neuron number. Canaries (*Serinus canaria*), for example, show peaks of neuronal recruitment to HVC in spring and again in autumn (Kirn et al., 1994). In RA, neuron number does not change seasonally but neurons are larger and spaced further apart in breeding than in nonbreeding birds (Tramontin and Brenowitz, 1999). Finally, in area X, neuronal soma size is larger and neuronal density is greater in breeding than in nonbreeding birds, but the rate of neuronal recruitment does not change seasonally (Thompson and Brenowitz, 2005). In many species these changes in the vocal-control regions can be observed in birds captured in the wild at different times of year and can be produced by manipulating photoperiod in the laboratory (Tramontin and Brenowitz, 1999).

For black-capped chickadees (*Poecile atricapillus*), most studies of seasonal changes in brain and behavior have focused on food-storing and the hippocampus. Much less is known about seasonal changes in song production and associated brain regions for chickadees. The *fee-bee* song, produced mostly by chickadee males, is most frequently heard in spring, when birds are in breeding condition, though it is produced infrequently at other times of year as well (Dixon and Stefanski, 1970; Ficken et al., 1978). Black-capped chickadees held in captivity and exposed to long days after being on short days showed an increase in size of HVC and area X, but not RA, compared to birds kept on short days (MacDougall-Shackleton et al., 2003a). Two related European species of titmice (free-living blue tits, *Parus caeruleus,* and great tits, *Parus major*) have also been reported to exhibit seasonal changes in HVC size (Absil et al., 2001; Caro et al., 2005). Despite the wide spread study of singing behavior in chickadees and titmice, seasonal plasticity of vocal control regions is understudied compared to other songbird taxa.

The aim of this study was to document changes in singing rates and vocal-control regions in free-living black-capped chickadees. We recorded dawn singing rates throughout the year. We also captured adult males at four times of year, at 3-month intervals (January, April, July, and October). For these males we measured testis size, and the size of the vocal-control regions HVC, area X, and RA as assessed by two histological markers (Nissl and NeuN immunoreactivity). As a secondary aim we compared estimates of HVC and telencephalon size using these two histological techniques. These data provide a profile of the annual cycle of song and the vocal-control system in free-living black-capped chickadees.

METHODS

Song Census

To assess seasonal changes in singing behavior, we performed standardized song censuses of dawn singing throughout the year, for a population of chickadees at the University of Western Ontario. For each census an observer slowly walked along a 2-km trail through a riparian mixed forest and counted the number of *fee-bee* songs heard and the number of different chickadees singing. This transect passed through approximately 12 individual breeding territories during spring/summer and at about 4 to 5 winter flock territories during autumn/winter. Each census was 1 h in duration, beginning 30 min prior to sunrise. Two observers performed the censuses. Each observer performed censuses across the entire year, and in several cases where both observers performed the census there was complete agreement in song counts and estimates of numbers of singing birds.

Subjects for Neuroanatomical Study

A total of 24 male adult black-capped chickadees were captured at four times of year: 15 January–15 February 2003

($n = 6$), 15 April–15 May 2003 ($n = 6$), 15 July–15 August (2002, $n = 3$; 2003, $n = 4$), 15 October–15 November 2002 ($n = 5$). Birds were captured from a banded population near Elginfield, Ontario, Canada (43.02°N, 81.15°W) approximately 30 km from the University of Western Ontario. Sex was determined in the field by body size and later confirmed by examination of the gonads during dissection. Age was determined through previous banding records and only adult (minimum, 10 months old) birds were used in this study. Birds were housed in cages in a rooftop enclosure to maintain exposure to natural photoperiod and weather conditions. Birds were given free access to food (ground sunflower seeds and Mazuri small bird maintenance diet supplemented with whole sunflower seed) and water when in the home cage, except prior to food storing sessions (see below).

As part of another study, birds were given injections of bromodeoxyuridine (BrdU; 75 μg/g) on the second day of captivity and were observed in food-storing and retrieval tests from the third to sixth day of captivity. Birds were sacrificed on the sixth day in captivity.

Perfusion and Histology

On the sixth day following capture, birds were killed by an overdose of ketamine and xylazine and transcardially perfused with heparinized phosphate-buffered saline (PBS, pH 7.5) followed by 4% paraformaldehyde. The brain was removed from the skull and weighed to the nearest 0.1 mg, then left in 4% paraformaldehyde for 24 h, weighed again, and then cyroprotected in 30% sucrose solution (in PBS) until saturated (approximately 24 h). The brains were then weighed a third time and then frozen on pulverized dry ice, and stored at −70° C until further processing. After perfusion, the left testis of each male was removed, the length and width measured to the nearest 0.1 mm with dial calipers, and testis volume calculated using the formula for an ellipsoid ($4/3\pi a^2 b$; where a = width/2 and b = length/2).

Histology

Brains were sectioned at 30-μm thickness in the coronal plane using a cryostat. We collected two series of sections for this study, one for thionin staining of Nissl and one for immunocytochemical staining of NeuN. Both sets were collected at 90 μm intervals (every third section) throughout the telencephalon, and each section in the Nissl set was adjacent to a section in the NeuN set. For the Nissl set, sections were collected into PBS then mounted on gelatin-coated slides, stained with thionin, serially dehydrated, and coverslipped using Permount (Fisher Scientific).

For the NeuN set, sections were initially collected into cryoprotectant solution (30% sucrose and 30% ethylene glycol in buffer) and stored at −20°C. For immunocytochemistry, sections were transferred from the cryoprotectant into PBS and then washed twice more in PBS. Sections were then incubated in 0.5% H_2O_2 for 30 min, and washed twice in PBS. Sections were incubated in 10% Normal Goat Serum in PBS with 0.3% Triton-X (Sigma, PBS-T) for 1 h, and then in primary antibody (Mouse-Anti NeuN, Chemicon) overnight (1:2000 in 0.3% PBS-T) at room temperature. Sections were washed in 0.1% PBS-T, then incubated in secondary antibody (1:250 goat anti-mouse in 0.3% PBS-T) for 1 h, then washed again in 0.1% PBS-T. Sections were then incubated in avidin–biotin horseradish peroxidase complex (Vectastain ABC, Elite Kit) 1:250 in 0.3% PBS-T for 1 h, then washed twice in 0.1% PBS-T and once in PBS. Finally, sections were incubated in diaminobenzidine, mounted on gelatin-coated slides, and coverslipped using Permount (Fisher Scientific).

Brain Morphometry

In Nissl-stained sections, the volumes of HVC, RA, and Area X were calculated by measuring the region's area in every mounted section (90-μm intervals) in which the region appeared; area estimates were then combined using the formula for a frustrum, or truncated cone (Sherry et al., 1989). We used the same method to calculate HVC volume in the NeuN-stained sections. For both Nissl- and NeuN-stained sections, we measured the area of the telencephalon in every second mounted section (180-μm intervals) and combined these using the frustrum formula. Area measurements were made using Spot Imaging software from digital images captured with a Spot Insight digital camera mounted on a Zeiss Axiophot microscope using a 5× objective for HVC, RA, and area X, and a 1.25× objective for the telencephalon.

RESULTS

Singing and Gonad Size

Figure 1 shows song rate throughout the year. There is a peak in song production in black-capped chickadees in spring at the start of breeding season. The number of birds singing increased from 0 in January to a peak of 10 to 12 birds singing in April. Figure 1 also shows testes volumes across the year. A one-way ANOVA on testes volume showed that testes of birds captured in April were significantly larger than birds captured at any other time of year [$F(3,20) = 15.08$, $p < 0.0001$; Fisher's PSLD, $p < 0.01$].

Brain Mass and Telencephalon Size

Figure 2 shows brain mass postperfusion, postfix, and postcryoprotection for birds collected at each time of year. A repeated measures ANOVA with time of year as a between-subjects factor and three measures of weight as a within-subjects factor showed no main effect of time of year [$F(3,18) = 1.38$, $p = 0.28$], but a significant effect of brain weight measure [$F(2,36) =$

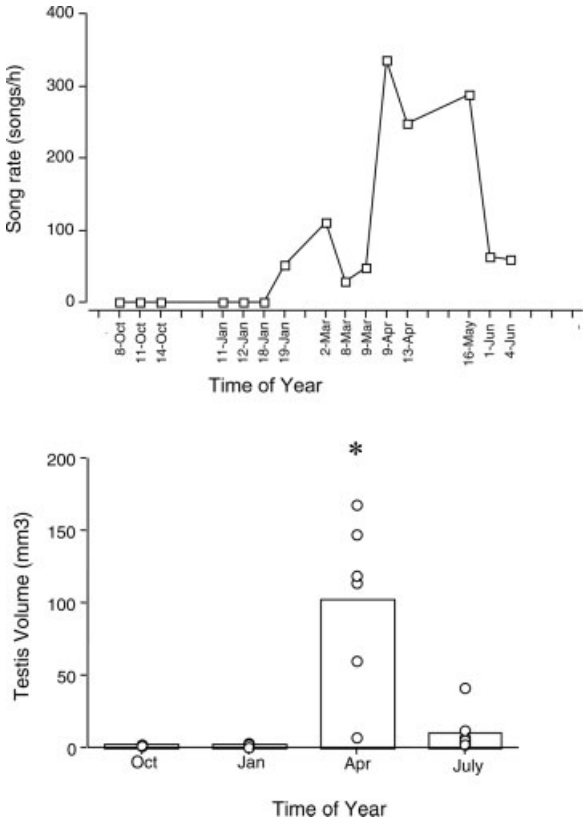

Figure 1 Seasonal changes in singing rates and testis size in black-capped chickadees. Upper panel depicts the number of songs heard during 1 h around dawn across the year. Lower panel depicts mean (bars) and individual data (circles) for left testis volume of birds collected at four times of year. Asterisk indicates that birds in April had larger testes than at other times of year.

162.40, $p < 0.0001$] and a significant interaction [$F(6, 36) = 5.52$, $p = 0.0004$]. Post-hoc tests (PLSD) showed that postfix brains were heaviest, postperfusion brains were intermediate weight, and postcryoprotection brains were lightest, with all groups significantly different from one another.

To further analyze the time of year × brain weight measure interaction, we performed separate one-way ANOVAs for each measure. On postperfusion brains, there was a significant effect of time of year [$F(3,18) = 6.42$, $p = 0.0038$]. Post-hoc analyses showed that July brains were significantly heavier than brains in all other months. On postfix brains, there was a near significant effect of time of year [$F(3,18) = 3.075$, $p = 0.054$], and on postcryoprotection brains there was no significant effect of time of year [$F(3,18) = 0.049$, $p = 0.9853$]. Therefore, brains were not different across season except for postperfusion weights in July, which were heavier than postperfusion brains in October, January, and April. A repeated measures test of July weights alone showed that all three brain weight measures were significantly different from each other.

We computed a measure of shrinkage as a percentage difference from postperfusion weight to postcryoprotection weight and found a main effect of time of year [$F(3,20) = 4.39$, $p = 0.016$]. Post-hoc tests showed that July had more shrinkage than all other months, which were not different from each other.

The brain mass difference for birds collected in July was not a result of the July samples being collected in two years, as mean brain mass was almost identical across the two years. For example

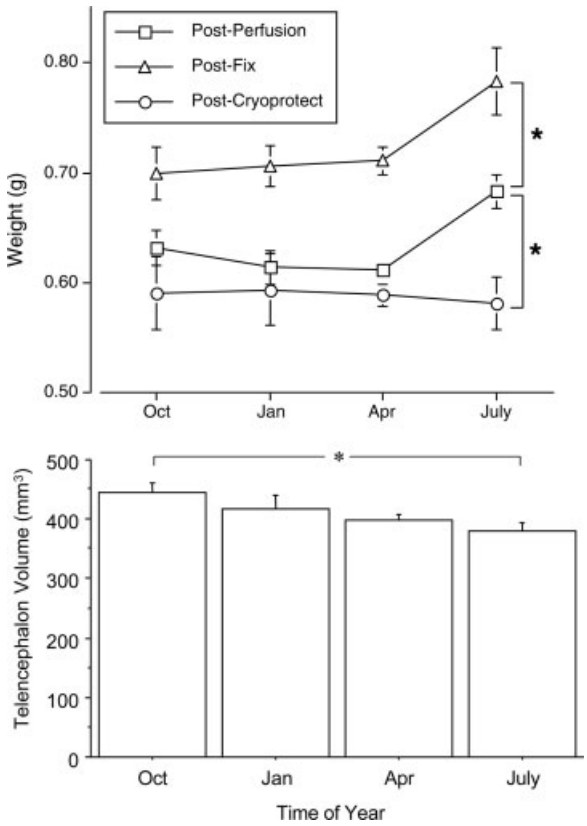

Figure 2 Seasonal changes in brain mass and telencephalon volume. Upper panel: Data are mean ± SE brain mass of birds collected at four times of year. Brain mass was measured immediately following perfusion, following 24 h immersion in fixative, and following 24 h immersion in cryoprotectant (30% sucrose in buffer). Asterisks indicate significant differences among the three weighing methods. There was no significant main effect of time of year, but a significant interaction (see text). Lower panel: Telencephalon volume (mean ± SE) measured from Nissl-stained brain sections in birds collected at four times of year. October birds had a significantly larger telencephalon than July birds, with no other significant differences between groups.

mean (+ SEM) brain mass postperfusion was 0.695 (± 0.016) g in 2002 and 0.693 (± 0.028) in 2003.

We also found significant variation across the year in measures of telencephalon volume [$F(3,20) = 3.1$, $p = 0.048$]. Fisher's post hoc tests revealed that birds collected in October telencephalon significantly larger than that in birds collected in July (Fig. 2). No other groups differed from each other. This significantly smaller telencephalon volume in July may have resulted from increased shrinkage during cryoprotection.

Song Control System: Nissl Stain

To test for potential relationships between vocal-control regions and overall brain size, we compared volume measurements for each region (HVC, RA, and Area X) to telencephalon volume. Calculations of Pearsons' correlation coefficient showed that the volumes of these regions were not correlated with telencephalon volume (HVC, $r = 0.005$, $p = 0.99$; RA, $r = -0.19$, $p = 0.46$; Area X, $r = 0.39$, $p = 0.12$).

One-way ANOVAs showed no significant changes in absolute volumes of vocal-control regions across season {Fig. 3; HVC, [$F(3,20) = 0.18$; $p = 0.91$); RA, $F(3,20) = 1.81$, $p = 0.18$; Area X, ($F(3,20) = 0.04$, $p = 0.99$]}. Qualitatively identical results were obtained when we compared the proportion of the telencephalon occupied by each brain region across the four seasons of the year.

Breeding phenology appeared to vary among males. Testis volume data had a bimodal distribution and were discontinuous at about 20 mm^3 (Fig. 1). One bird in the April sample appeared not to be in breeding condition and one bird in the July sample appeared still to be in breeding condition. We thus recategorized our sample with respect to breeding condition, based on testis volume. Six birds with testis volume greater than 20 mm^3 were considered to be in breeding condition [mean (± SD) testis size = 108.09 (± 49.3) mm^3], while 18 birds with testis volume less than 20 mm^3 were considered to be in nonbreeding condition [mean (± SD) testis size = 3.06 (± 2.7) mm^3]. We then compared volumes for each brain region between breeding and nonbreeding birds using *t* tests (Fig. 4). We found no statistically significant difference between breeding and nonbreeding birds in absolute volumes of HVC and area X [HVC, $t(22) = 1.83$, $p = 0.08$; Area X, $t(22) = 0.33$, $p = 0.74$], but RA was significantly larger in breeding than in nonbreeding birds [RA: $t(22) = 3.31$, $p = 0.003$]. The proportion of the telencephalon occupied by HVC and RA exhibited significant differences between breeding and nonbreeding males [Fig. 4; HVC, $t(22) = 2.06$, $p = 0.05$; RA, $t(22) = 3.46$, $p = 0.002$], but there was no difference in Area X [$t(22) = 0.94$, $p = 0.36$].

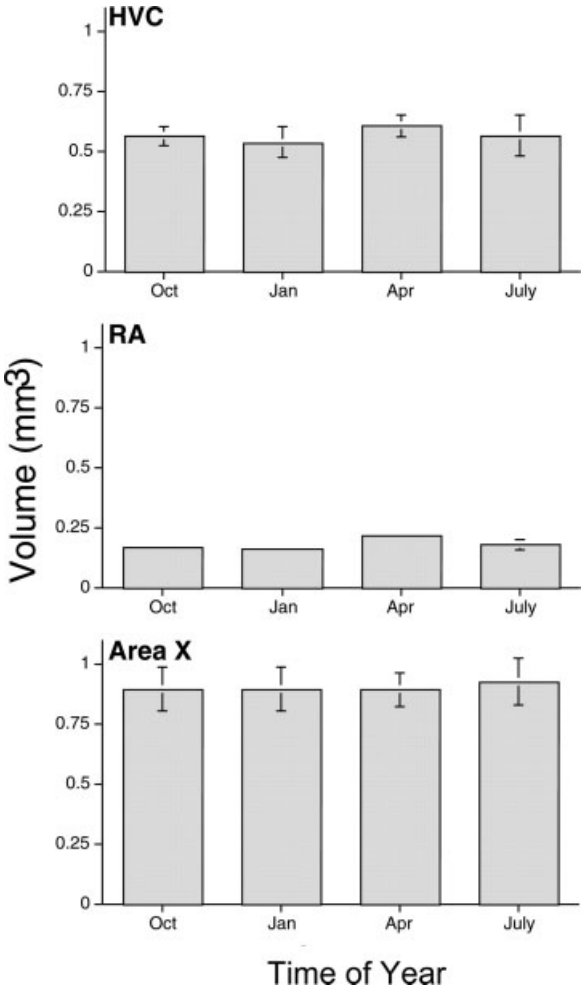

Figure 3 Mean ± SE volume of three vocal control regions (as assessed by Nissl stain) in black-capped chickadees collected at four times of year. Error bars are too small to be visible for some bars.

Comparison of Nissl and NeuN Delineation of HVC

We used two methods of tissue staining, thionin staining of Nissl and immunocytochemical labeling of the neuronal protein NeuN, and compared volume measurements of HVC and the telencephalon obtained with each technique. Nissl and NeuN measurements of HVC volume were highly correlated ($r = 0.80$, $p < 0.0001$). Nissl and NeuN measurements of telencephalon were also corrrelated ($r = 0.43$, $p < 0.03$). In NeuN-stained sections, HVC volume was

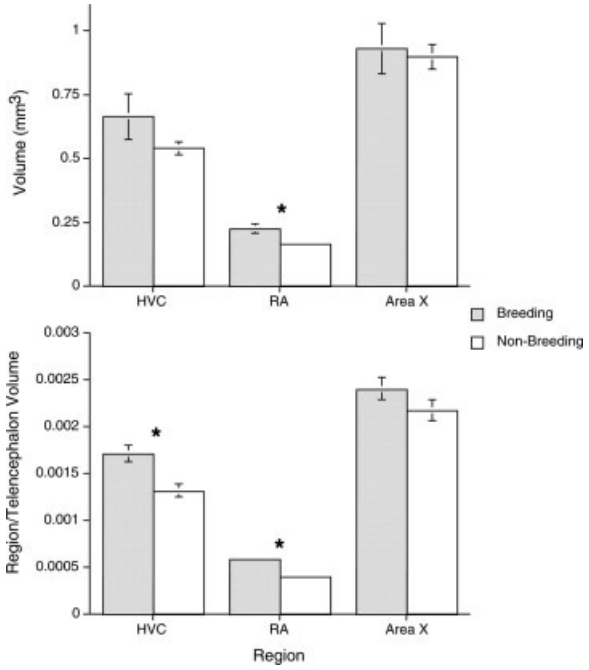

Figure 4 Volume of three vocal control regions (as assessed by Nissl stain) in black-capped chickadees, comparing birds with large testes (Breeding) to birds with small testes (Nonbreeding). Upper panel depicts mean ± SE volume. Lower panel depicts the mean ± SE proportion of the telencephalon occupied by each vocal region. Asterisks indicate cases where breeding birds differ significantly from nonbreeding birds.

correlated with telencephalon volume ($r = 0.51$, $p = 0.01$). However, in Nissl-stained sections HVC and telencephalon volumes were not correlated ($r = 0.005$, $p = 0.99$). Although Nissl and NeuN measures of the same structure were correlated, a correlation between HVC and telencephalon was apparent only for NeuN, not Nissl measurements.

A two-way ANOVA (time of year × staining method) on volumes of HVC showed no significant differences across time of year [$F(3,20) = 0.24$, $p = 0.87$], but a significant difference between methods [$F(1,20) = 5.57$, $p = 0.03$]. Nissl measurements of HVC were significantly larger than NeuN measurements of HVC (Fig. 5). To test whether this difference was because of overall tissue shrinkage during immunocytochemistry or because there was a fundamental difference in how the methods delineated HVC, we repeated the ANOVA using proportion of HVC relative to telencephalon volume. This analysis produced qualitatively similar results: there was no significant effect of time of year [$F(3,20) = 0.68$, $p = 0.58$], but a significant effect of method [$F(1,20) = 13.14$, $p = 0.002$]. Again, Nissl measurements were significantly larger than NeuN (Fig. 5).

Finally, to compare the two histological techniques we directly compared the estimates of overall telencephalon size using the two methods. Again, Nissl-based estimates of telencephalon volume were significantly larger than NeuN-based estimates [$t(23) = 78.5$, $p < 0.0001$]. Thus, the consistently smaller estimates of HVC volume reported above are likely due to a general shrinkage of tissue during immunocytochemistry processing rather than differences in HVC delineation.

As we did for Nissl-stained sections, we divided birds into the same breeding and nonbreeding groups based on testis size and repeated comparisons of both raw and proportional volumes of HVC using t tests. The NeuN analyses produced qualitatively similar results as we found for Nissl sections: there was no difference between breeding and nonbreeding birds in absolute volume of HVC [$t(22) = 1.40$, $p = 0.18$] but when we compared proportional volumes HVC was larger in breeding than nonbreeding birds [$t(22) = 2.06$, $p = 0.05$].

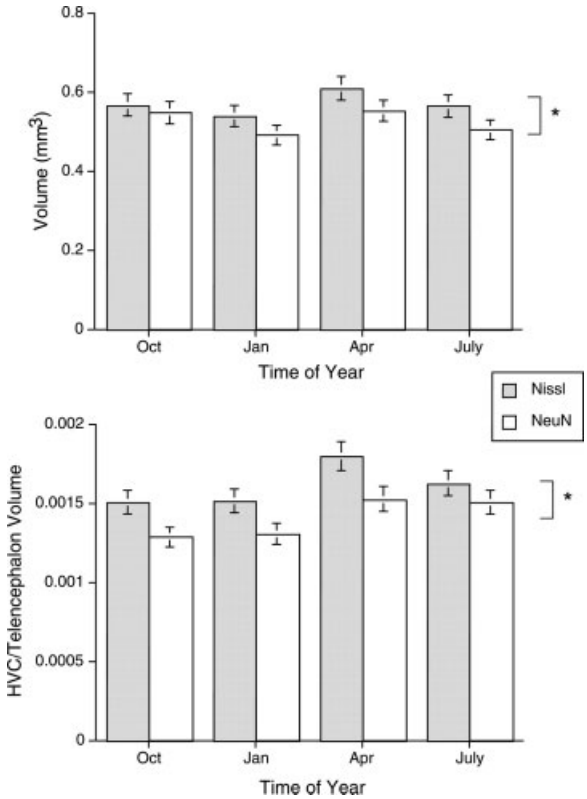

Figure 5 HVC size in male black-capped chickadees as assessed by Nissl stain and NeuN immunocytochemistry. Upper panel depicts mean ± SE volume. Lower panel depicts the mean ± SE proportion of the telencephalon occupied by HVC (HVC/Telencephalon). Asterisks indicate a significant difference between the two histological methods.

DISCUSSION

We found that while adult male black-capped chickadees displayed obvious plasticity in gonad size and behavior, there was minimal change, if any, in the vocal-control regions HVC, RA, and area X. Song production peaked during the breeding season in April; this coincided with a peak in testis volume, indicating birds were in breeding condition. We did not observe changes in size of HVC, RA, or area X across the annual cycle. When comparing birds in breeding condition versus nonbreeding birds (as determined by gonad size), RA and HVC as a proportion of telencephalon were larger in breeding condition birds. The magnitude of change in these regions was minimal when compared to the magnitude of change in other species where HVC may almost double in size (Tramontin and Brenowitz, 2000).

Plasticity of Vocal-Control Regions

We found that, when compared across the four time points of capture (January, April, July, and October) there were no changes in absolute volume of HVC, RA, or area X. When we categorized birds into breeding or nonbreeding condition based on testis size, we found that RA showed a larger volume in breeding birds. When we compared proportion of regions to telencephalon in breeding and nonbreeding birds, we found both HVC and RA were larger in breeding than in nonbreeding birds. Smulders et al. (2006) similarly captured chickadees across the year (October, December, February, April, June, and August) and found no seasonal variation in HVC, area X, or RA. Combined, these studies suggest minimal seasonal plasticity of vocal-control regions compared to other species.

In contrast to the two field studies described above, in a laboratory study MacDougall-Shackleton and colleagues (2003a) did find a photoperiod-induced change in HVC and area X volume in black-capped chickadees that had been held captive long-term. Birds that were photostimulated (switched from short to long days for 16 days) had HVC about 133% the size of HVC in birds held long-term on short days (photosensitive) or long-term on long days (photorefractory). Thus, experimentally induced breeding conditions resulted in increased HVC size in captive chickadees. Similar discrepancies between laboratory and field studies of HVC plasticity have been reported for canaries (*Serinus canaria*; Leitner et al., 2001) and a population of free-living European starlings (*Sturnus vulgaris*; Ball et al., 2004). For both species, photoperiod-induced changes in HVC have been observed in captive populations that have not been detected in free-living populations. Thus, despite the widespread evidence for seasonal HVC plasticity in many songbird species, these changes may not always be apparent in samples taken from the wild.

Individual variation in time of sampling and breeding phenology may mask within-individual changes in the vocal-control system. However, this is unlikely to fully explain the null results in the current study, or those of Smulders et al. (2006). The proportional volumes of HVC and RA in nonbreeding birds were about 75% of those of breeding birds. This is a small effect size compared to seasonal changes observed in other species in which nonbreeding birds have vocal control regions about half the size of breeding birds (Tramontin and Brenowitz, 2000). Thus, changes in the size of vocal control regions in chickadees appear to be attenuated compared to other songbirds.

Another reason for the lack of annual change observed in HVC and area X may be that chickadees display only minimal plasticity in these regions. Although the production of learned song in males does peak in spring during the breeding season (see Fig. 1), chickadees have a wide repertoire of vocalizations that they produce year-round. The *chick-a-dee* is a contact call that is learned during development (Hughes et al., 1998) and exhibits experience-dependent plasticity in adulthood (Nowicki, 1989). Similarly, the *gargle* is a short-distance aggressive call that is modified through experience (Ficken and Weise, 1984; Kroodsma et al., 1995). Given that production of learned calls depends on vocal-control regions such as HVC in other songbirds (Vicario, 2004), it is likely that the production of the *chick-a-dee* and *gargle* calls are HVC dependent as well. Thus, annual changes in HVC may be attenuated in chickadees in comparison to species that do not produce learned vocalizations year-round.

In other species with extreme seasonal plasticity in vocal-control regions, calls appear to develop in the absence of imitative learning, supporting the idea that reduced seasonal changes in HVC are associated with year-round production of learned vocalizations. Counter to this, however, are species that sing year-round but still exhibit large changes in HVC size (Smith et al., 1997). Determining why free-living chickadees have attenuated changes in HVC compared to other species will clearly require further study.

Brain Weight Variation

We found that brain mass prior to cryoprotection varied across the year, and the change in brain mass in

response to cryoprotection also changed across the year with July birds exhibiting a larger decrease in mass than brains collected at other times of year (Fig. 2). Smulders and coworkers (1995) similarly demonstrated that brains differentially changed mass during cryoprotection with brains from birds captured in June losing more mass than other times of year, and seasonal changes in brain mass occur in other species as well (Smulders, 2002). These effects indicate that caution is required in interpreting changes in absolute measures between brains collected from animals at different times of year or in different physiological conditions. It is unlikely in the present study that the seasonal changes in postfix brain mass and mass and shrinkage during cryoprotection affected our results. In particular, brain mass was consistent across three of the sampling points (October, January, April). As well, our correction for overall brain size was to use the size of a region relative to telencephalon size. This should correct for differences in brain shrinkage as a result of cryoprotection.

Comparison of Nissl and NeuN Measures of Volume

We used two methods to mark tissue for measurement of HVC and telencephalon volumes. The first method, thionin stain of Nissl bodies, is used most commonly to assess the size of brain regions. The second method used immunocytochemistry to mark for NeuN, a protein found only in neurons. We found that Nissl and NeuN measures of HVC and telencephalon volumes were correlated and produced the same results when volumes were compared across season. However, we also found that, overall, volume measures were smaller when using NeuN than when using Nissl. Thus, immunocytochemical processing shrank the tissue more than did Nissl staining. NeuN staining provides many potential advantages to Nissl by allowing definitive identification of neurons through cellular chemistry rather than cellular anatomy. Prior studies using Nissl stains have attempted to differentiate neurons from glia through features such as cell size, and comparison with NeuN stained tissue could provide verification.

CONCLUSIONS

In sum, we found that despite marked seasonal variation in gonad size and singing rates there were minimal changes in vocal-control regions such as HVC. This lack of change may result from a combination of (a) interindividual differences in breeding phenology, (b) a smaller seasonal change in HVC in chickadees compared to other species, and (c) potential functional requirements related to the year-round production of learned calls.

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REFERENCES

Absil PC, Balthazart J, Ball GF, Pinxten R, Eens M. 2001. Seasonal plasticity of the catecholaminergic innervation of song nuclei in blue tits. Soc Neurosci Abstr 27: 1708.

Ball GF, Auger CJ, Bernard DJ, Charlier TD, Sartor JJ, Riters LV, Balthazart J. 2004. Seasonal plasticity in the song control system: multiple brain sites of steroid hormone action and the importance of variation in song behavior. Ann N Y Acad Sci 1016:586–610.

Ball GF, Hahn TP. 1997. GnRH neuronal systems in birds and their relation to the control of seasonal reproduction. In: Parhar IS, Sakuma Y, editors. GnRH neurons: gene to behavior. Tokyo: Brain, Shuppan. p 325–342.

Brenowitz EA. 2004. Plasticity of the adult avian song control system. Ann N Y Acad Sci 1016:560–585.

Caro SP, Lambrechts MA, Balthazart JB. 2005. Early seasonal development of brain song control nuclei in male blue tits. Neurosci Lett 386:139–144.

Dawson A, King VM, Bentley GE, Ball GF. 2001. Photoperiodic control of seasonality in birds. J Biol Rhythms 16:365–380.

Dixon KL, Stefanski RA. 1970. An appraisal of the song of the black-capped chickadee. Wilson Bull 82:53–61.

Ficken MS, Ficken RW, Witkin R. 1978. Vocal repertoire of the black-capped chickadee. Auk 95:34–48.

Ficken MS, Weise CM. 1984. A complex call of the black-capped chickadee (*Parus atricapillus*). 1. Microgeographic variation. Auk 101:349–360.

Hughes M, Nowicki S, Lohr B. 1998. Call learning in black-capped chickadees (*Parus atricapillus*): the role of experience in the development of chick-a-dee calls. Ethology 104:232–249.

Karpouzos H, Hernandez AM, MacDougall-Shackleton EA, MacDougall-Shackleton SA. 2005. Effects of day-length and food availability on food caching, mass and fat reserves in black-capped chickadees (*Poecile atricapillus*). Physiol Behav 84:465–469.

Kirn J, Oloughlin B, Kasparian S, Nottebohm F. 1994. Cell death and neuronal recruitment in the high vocal center of adult male canaries are temporally related to changes in song. Proc Nat Acad Sci U S A 91:7844–7848.

Krebs JR, Clayton NS, Hampton RR, Shettleworth SJ. 1995. Effects of photoperiod on food-storing and the hippocampus in birds. Neuroreport 6:1701–1704.

Kroodsma DE, Albano DJ, Houlihan PW, Wells JA. 1995. Song development by black-capped chickadees (*Parus*

atricapillus) and Carolina chickadees (*P. carolinensis*). Auk 112:29–43.

Leitner S, Voigt C, Garcia-Segura LM, Van't Hof T, Gahr M. 2001. Seasonal activation and inactivation of song motor memories in wild canaries is not reflected in neuroanatomical changes of forebrain song areas. Hormones Behav 40:160–168.

MacDougall-Shackleton SA, Hernandez AM, Valyear KF, Clark AP. 2003a. Photostimulation induces rapid growth of song-control brain regions in male and female chickadees (*Poecile atricapilla*). Neurosci Lett 340:165–168.

MacDougall-Shackleton SA, Sherry DF, Clark AP, Pinkus R, Hernandez AM. 2003b. Photoperiodic regulation of food storing and hippocampus volume in black-capped chickadees, *Poecile atricapillus*. Animal Behav 65:805–812.

Nowicki S. 1989. Vocal plasticity in captive black-capped chickadees—the acoustic basis and rate of call convergence. Animal Behav 37:64–73.

Sherry DF, Vaccarino AL, Buckenham K, Herz RS. 1989. The hippocampal complex of food-storing birds. Brain Behav Evol 34:308–317.

Shettleworth SJ, Hampton RR, Westwood RP. 1995. Effects of season and photoperiod on food storing by black-capped chickadees, *Parus atricapillus*. Animal Behav 49:989–998.

Smith GT, Brenowitz EA, Beecher MD, Wingfield JC. 1997. Seasonal changes in testosterone, neural attributes of song control nuclei, and song structure in wild songbirds. J Neurosci 17:6001–6010.

Smulders TV. 2002. Natural breeding conditions and artificial increases in testosterone have opposite effects on the brains of adult male songbirds: a meta-analysis. Hormones Behav 41:156–169.

Smulders TV, Lisi MD, Tricomi E, Otter KA, Chruszcz B, Ratcliffe LM, DeVoogd TJ. 2006. Failure to detect seasonal changes in the song-system nuclei of the black-capped chickadee (Poecile atricapillus). J Neurobiol 66: 991–1001.

Smulders TV, Sasson AD, Devoogd TJ. 1995. Seasonal variation in hippocampal volume in a food-storing bird, the black-capped chickadee. J Neurobiol 27:15–25.

Thompson CK, Brenowitz EA. 2005. Seasonal change in neuron size and spacing but not neuronal recruitment in a basal ganglia nucleus in the avian song control system. J Comp Neurol 481:276–283.

Tramontin AD, Brenowitz EA. 1999. A field study of seasonal neuronal incorporation into the song control system of a songbird that lacks adult song learning. J Neurobiol 40:316–326.

Tramontin AD, Brenowitz EA. 2000. Seasonal plasticity in the adult brain. Trends Neurosci 23:251–258.

Tramontin AD, Perfito N, Wingfield JC, Brenowitz EA. 2001. Seasonal growth of song control nuclei precedes seasonal reproductive development in wild adult song sparrows. Gen Comp Endocrinol 122:1–9.

Vicario DS. 2004. Using learned calls to study sensorymotor integration in songbirds. Ann N Y Acad Sci 1016: 246–262.

Vicario DS, Raksin JN, Naqvi NH, Thande N, Simpson HB. 2002. The relationship between perception and production in songbird vocal imitation: what learned calls can teach us. J Comp Physiol A 188:897–908.