

# Seasonal Plasticity and Sexual Dimorphism in the Avian Song Control System: Stereological Measurement of Neuron Density and Number

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## ABSTRACT

Differences in neuron density and number are associated with seasonal plasticity and sexual dimorphism in the avian song control system. In previous studies, neuron density and number in this system have been quantified primarily through nonstereological approaches in thick tissue sections by using the nucleolus as the unit of count. The reported differences between seasons and sexes may be inaccurate due to biases introduced by neuron splitting during sectioning. We used the unbiased optical disector technique on tissue from three previous studies (two investigations of seasonal plasticity and one investigation of sexual dimorphism in avian song nuclei) to assess seasonal and sex differences in neuron density and number. In two song nuclei, HVC and the robust nucleus of the archistriatum (RA), the optical disector yielded intergroup differences in neuron density and number that coincided well with the three previous reports.

We also estimated neuron number and density with a random, systematic, nonstereological counting protocol that used the neuronal nucleolus as the unit of count. We compared this method directly to the optical disector. In all cases, the two neuron-counting methods produced similar estimates of neuron number and density; the differences between treatment groups were equally discernible regardless of the counting method used. This study confirms previously reported seasonal and sex differences in the HVC and the RA by use of stereology and indicates that a random, systematic, nonstereological neuron-counting protocol is accurate and is well suited to the study of these phenomena in the avian song control system. *J. Comp. Neurol.* 396:186–192, 1998. © 1998 Wiley-Liss, Inc.

**Indexing terms:** birdsong; optical disector; stereology; bird

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Song behavior in songbirds is controlled by a discrete network of interconnected brain nuclei (Nottebohm et al., 1976; Bottjer et al., 1984; Sohrabji et al., 1990; Scharff and Nottebohm, 1991; for review, see Brenowitz and Kroodsm, 1996). Differences in singing behavior between individuals are correlated with differences in several anatomical attributes of the song control nuclei (Nottebohm, 1981; Canady et al., 1984; Brenowitz and Arnold, 1986; DeVoogd et al., 1993). In most species of songbirds, males typically sing much more than females, and males have larger song nuclei (Nottebohm and Arnold, 1976; Konishi and Akutagawa, 1985; Arnold et al., 1986). There are also seasonal differences in the morphology of the song nuclei that are correlated with changes in song behavior in seasonally breeding bird species. The song nuclei are largest during the breeding season, when birds sing most frequently and

with the greatest stereotypy (Nottebohm, 1981; Nottebohm et al., 1986; Arai et al., 1989; Kirn et al., 1989; Brenowitz et al., 1991; Rucker and Cassone, 1991; Bernard and Ball, 1995; Smith et al., 1997a–c). The observations of pronounced sex and seasonal differences in the song system were of seminal importance in motivating the investigation of comparable morphological patterns in the

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brains of other vertebrate groups (Konishi et al., 1989; Breedlove, 1992).

The gross anatomical differences in the song nuclei between the sexes and seasons result from changes in cell number, cell size, and/or cell spacing. Previous studies of the song system have almost universally used nonstereological cell sampling methods to quantify these sex and seasonal differences. Nonstereological sampling techniques may lead to biased estimates, however, because large objects, such as neuronal somata, may be split between histological sections (Coggeshall, 1992; West, 1993a; Coggeshall and Lekan, 1996). A neuron that is split between two serial sections leaves a "cap" that may be counted incorrectly as an entire neuron. Conversely, this cell might not be sampled if the cap is lost from the edge of the section during tissue processing. Either of these two scenarios can lead to significant errors in estimates of neuron density and number. Furthermore, if neuron size and/or shape differ significantly between two experimental treatment groups, then counting biases may be asymmetric across groups, thus obscuring or accentuating potential intergroup differences. These sorts of methodological biases can be addressed by the use of unbiased stereological cell counting techniques. For example, stereology has been used to clarify uncertainties regarding age-related neuron loss in the human hippocampus (West, 1993b).

Given the importance of the song system as a model for sex and seasonal differences in the brain, it is important to verify the existence of these phenomena in song nuclei by using stereological methods. We did this in three species of birds by using the optical disector method (Sterio, 1984). As a second goal, we wished to determine whether the differences between sexes and seasons measured in the song nuclei with a stereological method differed in magnitude from those obtained with the nonstereological methods typically employed in earlier studies. We compared neuron density and number in the song nuclei of male white-crowned sparrows (*Zonotrichia leucophrys gambelii*) that were killed after exposure to different photoperiods and hormone treatments, and male song sparrows (*Melospiza melodia morphna*) that were killed in different seasons. Previous studies have reported pronounced seasonal changes in the song systems of both species (Nottebohm, 1981; Brenowitz et al., 1991; Smith et al., 1997a,b). We also compared cell counts in the robust nucleus of the archistriatum (RA) of male and female western marsh wrens (*Cistothorus palustris*), a species reported to have sexually dimorphic song nuclei (Brenowitz et al., 1994). The results of these previous studies were confirmed by using the unbiased optical disector. In addition, we found no differences between cell counts made with a nonstereological, random, systematic protocol and those made with the optical disector.

## MATERIALS AND METHODS

### Histology

The brains that we analyzed had been used in previous studies. White-crowned sparrow brains were from castrated males exposed in the laboratory to photoperiod and hormonal conditions typical of either the breeding or nonbreeding seasons (see Smith et al., 1997b). Song Sparrow brains were collected from wild birds in the spring and fall (Smith et al., 1997a). Marsh wren brains were collected from males and females during the breeding season

(Brenowitz et al., 1994). All birds were deeply anesthetized with methoxyflurane and killed by transcardial perfusion with avian saline followed by 10% neutral-buffered formalin. After perfusion, the brains were removed and stored in fixative. The protocols used in this experiment were approved by the University of Washington Animal Care Committee and were in accordance with the National Institutes for Health Guide for the Care and Use of Laboratory Animals.

After at least 2 weeks of postfixation, the brains were embedded in gelatin and immersed in a 20% sucrose/formalin solution for 48 hours. Transverse frozen sections were cut at a thickness of 50  $\mu\text{m}$  on an accurately calibrated microtome and collected into avian saline. Every other section was mounted, dehydrated, and stained with thionin.

### Song nucleus volume measurements

By using a microprojector, we projected an image of each mounted section containing either the neostriatal nucleus HVc or RA at a magnification of 46 $\times$ . We traced the Nissl-defined outline of the song nucleus from each section onto paper, scanned these tracings into a microcomputer, and calculated the area of each song nucleus profile. The Nissl-defined borders of HVc coincide with the borders delineated by the distribution of retrogradely filled Area X projecting neurons, the distribution of acetylcholinesterase-positive neuropil, and the distribution of androgen receptor immunolabeling (Smith et al., 1997c; Soma et al., 1997).

We estimated nucleus volume in the white-crowned sparrows and song sparrows by using the formula for a cone frustum over each measured area (Smith et al., 1995, 1997c). In the marsh wrens, nucleus volumes were obtained by multiplying each section area by the sampling interval of 100  $\mu\text{m}$  and summing these individual volumes (Brenowitz et al., 1994). HVc and RA are bilateral, so all volume data are presented as the sum of the right and left nuclei. HVc volume measurements included the caudomedial portion of the nucleus (para HVc of Johnson and Bottjer, 1995). The individuals who measured song nuclei volumes were blind to the treatment group of all birds.

### Sampling strategy

In the HVc and RA, we sampled a minimum of 140 and 100 cells, respectively, per bird. A previous analysis indicated that these sample sizes were sufficient to encompass the full range of variability in neuron density in these nuclei (Brenowitz et al., 1995). Our post-hoc analysis confirmed that these sample sizes were large enough to produce reliable density estimates in each song nucleus (see Results).

We used a random, systematic sampling scheme to measure neuron density in both HVc and RA. For any sampled section containing either nucleus, we used NIH Image (Ver. 1.57; Wayne Rasband, National Institutes of Health, Bethesda, MD) to capture a low-magnification (1 $\times$  to 4 $\times$ ) video image of the song nucleus profile. We traced the perimeter of the nucleus on the computer screen and used a Cavalieri macro (Glen MacDonald, University of Washington) to overlay the tracing with a sampling grid. The area of each square on this grid (56.8  $\mu\text{m} \times 56.8 \mu\text{m} = 3,226 \mu\text{m}^2$ ) corresponded with the area of an ocular micrometer that we used as our cell-counting frame. This

produced a mapping grid of all of the visual fields that could be sampled over the song nucleus.

To choose which visual fields to sample from the mapping grid, we employed a random, systematic sampling scheme. This insured that every part of the nucleus had an equal probability of being sampled in both the dorsoventral and the mediolateral directions. Before beginning, we looked at several randomly chosen visual fields at 100× magnification to estimate the number of neurons we were likely to encounter in any given counting frame. This gave us an estimate of how many frames were required to achieve our minimum cell sample number. With this information, we estimated a systematic sampling number ( $k$ ).

We used a random number generator to produce a number ( $r$ ) between 1 and our systematic sampling number ( $k$ ). Starting at either the medial boundary or the lateral boundary of the nucleus (chosen at random and alternated thereafter), we counted  $r$  squares in the mediolateral direction to arrive at the first sample column on the map. In this column, we generated another random number ( $r^*$ ) between 1 and  $k$  and counted  $r^*$  grid squares in the dorsal-ventral direction. After marking this first sample square on the video screen map, we counted every  $k$ th square in that column. After completing the column, we repeated this procedure in every  $k$ th column until the entire nucleus was mapped for sampling.

We sampled the first two of every three mounted sections throughout RA and the first section of every three throughout HVC. The initial section of each region to be sampled was chosen randomly. These sampling schemes are different for the two nuclei, because RA is smaller than HVC. These schemes insured that each section throughout the rostrocaudal extent of each nucleus had an equal probability of being sampled. The sampling interval between tissue sections (e.g., 300  $\mu\text{m}$  in HVC) was chosen to coincide approximately with the distance between sampling columns in the mediolateral direction ( $k$  / sampling-grid width). Density measurements were performed on a Nikon Optiprot-2 light microscope (Tokyo, Japan) equipped with a Heidenhain Metro stage micrometer (Schaumburg, IL) and interfaced with a microcomputer running NIH Image.

### Counting methods

**Optical-disector count.** Upon completion of the mapping process, we placed the tissue section under a 100× oil-immersion objective and moved to the first square in the sampling map by using tissue landmarks such as blood vessels. Nucleoli that were bisected by either the left boundary or the upper boundary of the counting frame were counted, whereas those that were bisected by the right boundary or the lower boundary were not counted. Cells with one or two round nucleoli, a well defined nuclear envelope, nongranular cytoplasm, and/or an obvious axonal hillock were judged to be neurons. Neurons containing two nucleoli were always counted as one cell (see Discussion).

We measured the thickness of the tissue section by focusing on its top and bottom edges in every visual field sampled. We then counted nucleoli by using the optical disector method. We identified two optical planes within the section that were 10  $\mu\text{m}$  apart. Starting at the first plane (the reference plane), we focused through the section and counted all nucleoli that came into focus throughout

the 10- $\mu\text{m}$ , three-dimensional slab but that were not bisected by the second plane (the look-up plane).

We performed optical disector counts in two directions in every visual field sampled. We performed the first count while focusing up from the reference plane, which was below the look-up plane. The second count was performed while focusing down from the reference plane, which was now above the look-up plane. This was done to test the hypothesis that nucleoli were distributed evenly along the  $z$ -axis of our material. To account for sectioning artifacts at the tissue edge (e.g., lost nucleolus caps), we never included the top and bottom 3  $\mu\text{m}$  of the section in any of our counts.

**Nonstereological count.** Once the optical disector count was completed in any given counting frame, we counted the tops of neuronal nucleoli throughout the entire depth ( $z$ -axis) of the section that fell within the boundaries of the counting frame. Each counting frame represented a sampling volume of  $1.61 \times 10^5 \mu\text{m}^3$  (counting frame area = 3,226  $\mu\text{m}^2$ ;  $z$ -axis = 50  $\mu\text{m}$ ). This counting method is referred to as “nonstereological count” throughout the remainder of this paper. It is similar to the three-dimensional sampling performed with the disector but does not correct for nucleolus splitting between sections.

### Conversion of nucleolar profile counts to estimates of neuron density and number

To estimate neuron density (in neurons/ $\text{mm}^3$ ) from our optical disector counts, we used the following equation:

$$\text{density} = \frac{n}{\sum_{i=1}^g (a \times p \times \frac{T}{t})} \times 10^9 \mu\text{m}^3/\text{mm}^3,$$

where  $n$  = the number of nucleoli sampled,  $a$  = grid square area (3,226  $\mu\text{m}^2$ ; coincides with the area of our ocular micrometer),  $p$  = thickness between optical planes,  $T$  = cutting thickness (50  $\mu\text{m}$ ),  $t$  = measured tissue thickness in each grid square, and  $g$  = the number of grid squares sampled. In the above equation, we divided the cutting thickness by the actual measured thickness of the tissue to account for tissue shrinkage during processing and mounting. Cell number in each nucleus was estimated by multiplying neuron density by the Nissl-defined volume of the nucleus. We used the following equation to estimate neuron density from our nonstereological counts:

$$\text{density} = \frac{n}{(a \times T)g} \times 10^9 \mu\text{m}^3/\text{mm}^3,$$

where  $n$  = the number of neuronal nucleoli sampled,  $a$  = grid square area,  $T$  = cutting thickness (50  $\mu\text{m}$ ), and  $g$  = the number of grid squares sampled.

### Statistics

Song nucleus volume differences between treatment groups were assessed with Student's unpaired  $t$ -tests. We used paired-samples  $t$ -tests to compare upward vs. downward nucleolar counts in the optical disector paradigm. These two measures never differed statistically; therefore, we averaged them to arrive at an optical disector density score for each HVC and RA. This number was then

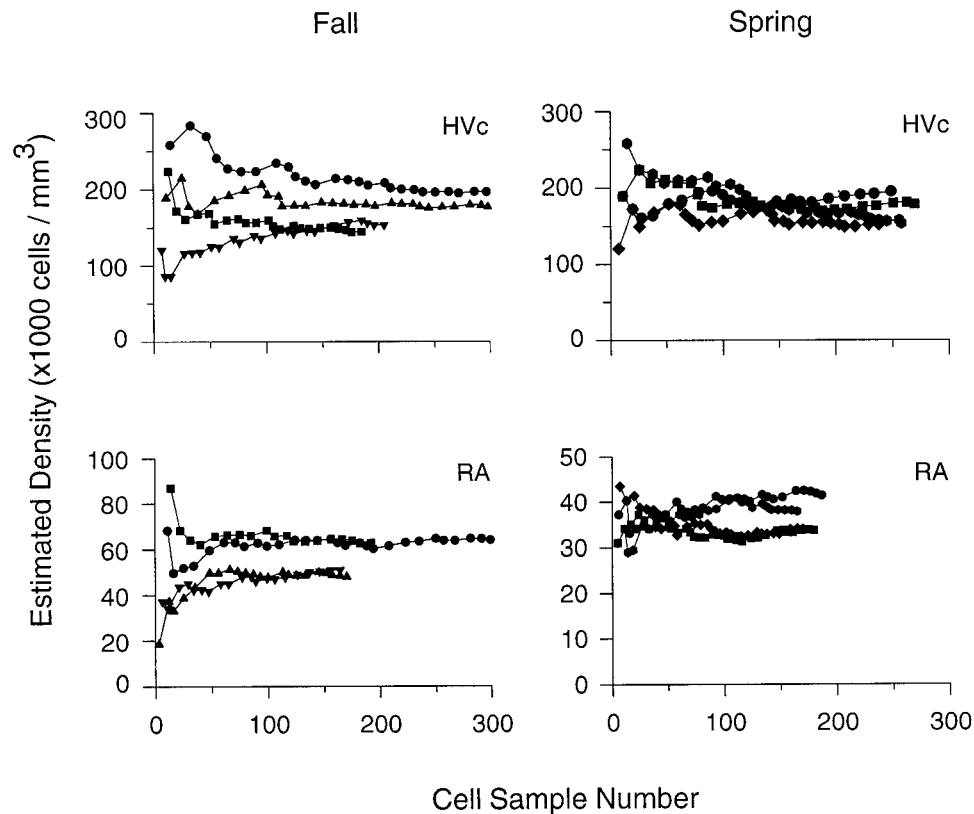


Fig. 1. For each bird, we sampled at least 140 neurons in HVC and 100 neurons in the robust nucleus of the archistriatum (RA). Shown are cumulative density estimates (determined from nonstereological counts) plotted against neuron sample number in song sparrows. Estimates of neuron density did not change when our minimum

sample numbers were exceeded. This was also true for the other species examined in this study (data not shown). Each symbol represents cumulative density estimates for HVC and RA from a single bird. Note the ordinate scale difference between fall and spring RA plots.

compared by using a paired t-test with the density estimated from our nonstereological counting method. We also compared estimated neuron number via paired t-tests, as described above. We performed sign tests to probe for consistent over- or under-estimations of neuron density or number by using our nonstereological cell counting method. We used a two-way, repeated-measures analysis of variance (treatment  $\times$  counting method) to assess treatment differences within each species investigated. For all tests, the level of significance was set at  $\alpha = 0.05$  (two-tailed).

## RESULTS

### Cell sample number

We counted at least 140 neurons in the HVC and 100 neurons in the RA for each bird. In each species, a post-hoc analysis revealed that density estimates did not change when these minimum sample sizes were exceeded (Fig. 1). This indicates that sampling variances were small compared to interanimal variance.

### Treatment effects

In all three species, there was a significant effect of treatment on both HVC and RA volume (Table 1). In white-crowned sparrows, neuron number in HVC was significantly greater in the long-day plus testosterone treatment group than in the short-day group without

TABLE 1. Song Nucleus Volume Estimates<sup>1</sup>

Treatment	Estimate	df*	P*
White-crowned sparrow			
HVC		6	0.004
Short day	0.600 $\pm$ 0.052		
Long day + T	1.191 $\pm$ 0.123		
RA		6	0.007
Short day	0.281 $\pm$ 0.009		
Long day + T	0.479 $\pm$ 0.049		
Song sparrow			
HVC		6	0.010
Fall	1.048 $\pm$ 0.134		
Spring	1.714 $\pm$ 0.142		
RA		6	0.003
Fall	0.430 $\pm$ 0.047		
Spring	0.748 $\pm$ 0.048		
Marsh wren			
RA		6	0.002
Females	0.063 $\pm$ 0.002		
Males	0.393 $\pm$ 0.034		

<sup>1</sup>Values are means  $\pm$  S.E.M. (n = 4 in all treatment groups). RA, robust nucleus of the archistriatum; T, testosterone.

\*Student's t-test effect of treatment (two-tailed).

testosterone ( $F[6,1] = 18.00$ ,  $P = 0.005$ ). There was no significant effect of counting method or interaction between treatment and counting method ( $F[6,1] = 0.19$ ,  $P = 0.68$ ;  $F[6,1] = 0.01$ ,  $P = 0.92$ , respectively; Table 2). HVC neuron number also differed between fall and spring song sparrows ( $F[6,1] = 9.25$ ,  $P = 0.02$ ), with no effect of counting method ( $F[6,1] = 1.94$ ,  $P = 0.21$ ), or interaction

TABLE 2. Estimates of Neuron Number Using Either Nonstereological Count or Optical Disector<sup>1</sup>

Treatment	Mean estimated neuron number		% Diff.	df**	P**
	Nonstereological count	Optical disector			
White-crowned sparrow					
Hvc*				7	0.65
Short day	108,647 ± 7,646	109,468 ± 7,859	0.8		
Long day + T	236,379 ± 28,798	236,895 ± 29,387	0.2		
RA				7	0.09
Short day	24,476 ± 446	24,970 ± 807	2.0		
Long day + T	25,459 ± 2,438	25,858 ± 2,583	1.6		
Song sparrow					
Hvc*				7	0.24
Fall	173,779 ± 24,744	174,117 ± 25,213	0.2		
Spring	290,310 ± 29,072	282,815 ± 25,760	-2.6		
RA				7	0.75
Fall	23,868 ± 1,808	23,984 ± 2,113	0.5		
Spring	27,794 ± 538	27,902 ± 479	0.4		
Marsh wren					
RA*				7	0.45
Females	3,041 ± 154	3,072 ± 177	1.0		
Males	15,172 ± 2,728	16,264 ± 1,864	7.2		

<sup>1</sup>Values are means ± S.E.M. (n = 4 in all treatment groups). Neuron number estimate from optical disector is obtained by averaging "up" and "down" optical disector counts. % Diff. reflects the % difference between the two counting methods.

\*Repeated-measures analysis of variance effect of treatment:  $P < 0.05$  (two-tailed).

\*\*Nonstereological count vs. optical disector paired-samples t-test (two-tailed) performed without regard to treatment.

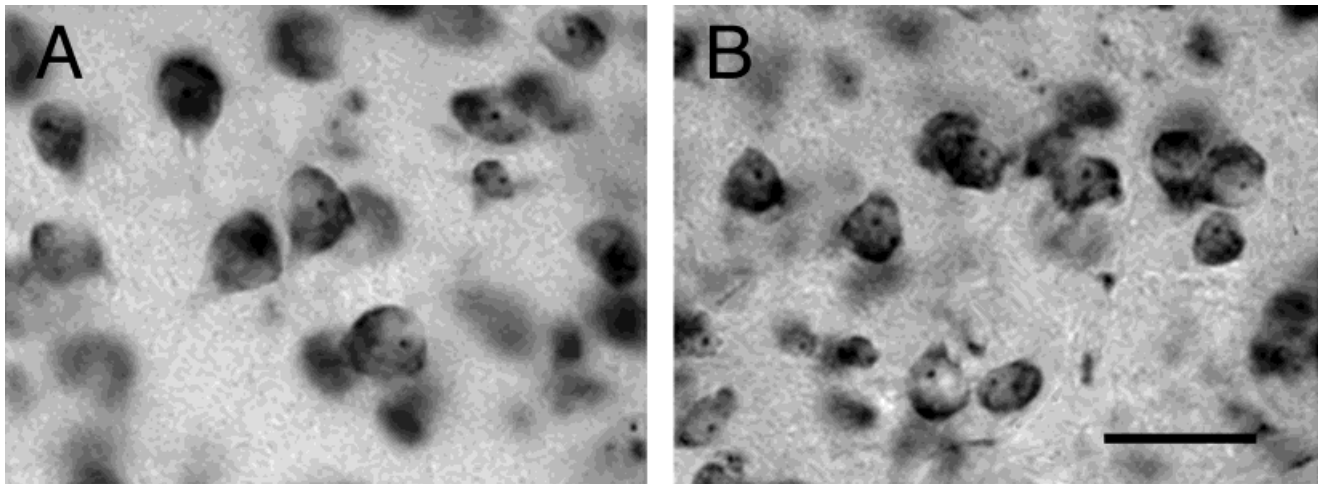


Fig. 2. Shown are video micrographs of representative Nissl-stained robust nucleus of the archistriatum (RA) neurons in white-crowned sparrows under long-day + testosterone (A) and short-day (B) conditions. Regardless of the counting method used, neuron density differed between treatment groups, whereas neuron number

did not. Images were captured by using an Olympus BH-2 microscope (Tokyo, Japan) equipped with a Sony DXC-950 video camera (Tokyo, Japan) yolked to a microcomputer. Brightness and contrast were adjusted to provide maximum resolution in the digitized image. Section thickness = 50  $\mu$ m. Scale bar = 30  $\mu$ m.

between treatment and counting method ( $F[6,1] = 2.33$ ,  $P = 0.18$ ).

RA neuron density was significantly affected by treatment in both sparrow species (Fig. 2, Table 3). Increased day length and circulating testosterone significantly decreased RA neuron density in white-crowned sparrows ( $F[6,1] = 36.35$ ,  $P = 0.001$ ), whereas neither the counting method nor the interaction between day length and counting method affected this measure ( $F[6,1] = 2.53$ ,  $P = 0.16$ ;  $F[6,1] = 0.35$ ,  $P = 0.57$ , respectively). Song sparrows captured in the spring had lower neuron densities in RA than did those captured in fall ( $F[6,1] = 17.66$ ,  $P = 0.006$ ). Again, RA neuron density was not affected by counting method nor was the interaction between season and counting method significant ( $F[6,1] = 0.03$ ,  $P = 0.88$ ;  $F[6,1] = 0.06$ ,  $P = 0.82$ , respectively).

Male marsh wrens had more neurons in RA than conspecific females ( $F[6,1] = 32.24$ ,  $P = 0.001$ ), regardless

of which neuron counting method was used. No differences arose from either counting method or from the interaction between sex and the counting method ( $F[6,1] = 0.61$ ,  $P = 0.46$ ;  $F[6,1] = 0.55$ ,  $P = 0.49$ , respectively). Neuron density in RA was lower in male marsh wrens than in females ( $F[6,1] = 9.121$ ,  $P = 0.023$ ). Neither the effect of counting method nor the interaction between sex and counting method were significant ( $F[6,1] = 0.94$ ,  $P = 0.37$ ;  $F[6,1] = 0.52$ ,  $P = 0.50$ , respectively).

### Nonstereological counts vs. optical disector counts

In none of the three species did we find differences between neuron numbers or densities determined by optical disector counts performed in the upward direction vs. those done in the downward direction (data and paired sample T-statistics not shown; all  $P$  values  $> 0.55$ ). We also

TABLE 3. Estimates of Neuron Density Using Either Nonstereological Count or Optical Disector<sup>1</sup>

Treatment	Mean estimated density		% Diff.	df**	P**
	Nonstereological count	Optical disector			
White-crowned sparrow					
HVc				7	0.60
Short day	182,943 ± 12,306	184,380 ± 12,966	0.8		
Long day + T	197,654 ± 9,917	198,055 ± 11,530	0.2		
RA*				7	0.14
Short day	87,370 ± 3,321	89,248 ± 4,973	2.1		
Long day + T	53,746 ± 3,697	54,602 ± 4,021	1.6		
Song sparrow					
HVc				7	0.30
Fall	165,820 ± 11,278	166,284 ± 12,004	0.3		
Spring	169,478 ± 10,441	165,161 ± 8,007	-2.5		
RA*				7	0.87
Fall	56,463 ± 4,156	56,410 ± 3,360	-0.1		
Spring	37,577 ± 2,122	37,846 ± 2,746	0.7		
Marsh wren					
RA*				7	0.35
Females	48,036 ± 1,140	48,481 ± 1,366	0.9		
Males	38,029 ± 4,265	41,029 ± 1,281	7.9		

<sup>1</sup>Values are means ± SEM in neurons/mm<sup>3</sup>; n = 4 in all treatment groups. Density estimate from optical disector is obtained by averaging "up" and "down" optical disector counts. % Diff. reflects the % difference between the two counting methods.

\*Repeated-measures analysis of variance effect of treatment:  $P < 0.05$  (two-tailed).

\*\*Nonstereological count vs. optical disector paired-samples t-test (two-tailed) performed without regard to treatment.

found no difference between neuron numbers or densities estimated from our nonstereological counts vs. those estimated from the optical disector counts (Tables 1 and 2). It should be noted that the nonstereological counts tended to give slightly lower estimates of mean neuron number (9 of 10 comparisons) and mean neuron density (8 of 10 comparisons), but these differences were not significant (all  $P$  values  $> 0.29$ ; sign test).

## DISCUSSION

We observed intergroup differences in all three species by using the optical disector counting method. Song sparrows captured during the breeding season had more neurons in HVc and less densely spaced neurons in RA than did birds collected in the fall. Similarly, a long photoperiod and testosterone administration caused an increase in HVc neuron number and RA neuron spacing in white-crowned sparrows. Finally, we observed a male-biased sex difference in RA neuron number and density in western marsh wrens. These results agree with those of the three studies that previously analyzed this same tissue (Brenowitz et al., 1994; Smith et al., 1997a,b). The use of unbiased stereological sampling methods provides a rigorous confirmation of the existence of sex and seasonal differences in the avian song control system.

In all three species analyzed, intergroup differences were equally discernible with either neuron counting method. When we compared the two counting methods directly, without regard to experimental treatment (by using paired samples t-tests), we found no differences in estimates of neuron number or density. Thus, the magnitude of sex and seasonal differences observed in the song nuclei using stereological methods is the same as that observed with a nonstereological method that was used in previous studies.

We used the nucleolus (not the cell body) as the unit of count in our nonstereological protocol. The nucleolus (1–2  $\mu$ m diameter) is small relative to the 50- $\mu$ m-section thickness (Smith et al., 1995). Nucleoli are therefore less likely to be split between two successive histological sections than a larger object, such as a nucleus or a cell body. Thus, miscounted or lost caps are less likely to bias our neuron

counts significantly. Counting small objects in thick sections does not guarantee that counts will be unbiased (Coggeshall and Lekan, 1996), but this study indicates that our protocol gives reliable cell counts in 50- $\mu$ m sections through the avian song system within the contexts of seasonal plasticity and sex differences. It should be stressed that the estimates obtained with the nonstereological counting method have only been validated in 50- $\mu$ m sections by using the nucleolus as the unit of count. Any cell counting method that uses different section thickness, a different unit of count, or different histological preparations will need to be revalidated by using a stereological counting method.

Some cells contain two nucleoli. A split nucleus could distribute its two nucleoli into two different physical (or optical) sections; such nucleolar splitting could result in counting some cells twice. In theory, this is a source of potential bias in both the stereological and the nonstereological neuron counting methods. In actuality, however, we do not consider this to be a problem in our tissue. Brenowitz et al. (1985) measured the occurrence of neurons with two nucleoli in the RAs of three songbird species. The greatest mean proportion of neurons with two nucleoli was 4% in bay wrens (*Thryothorus nigricapillus*). This proportion was only 1% in white-browed robin chats (*Cossypha heuglini*) and in buff-breasted wrens (*Thryothorus leucotis*). These percentages were calculated by counting nucleoli in profiles and might have been slightly different had they been calculated by reconstructing the nuclei in which the counts were performed. Nevertheless, the low incidence of neurons with two nucleoli indicates that nucleolar splitting cannot account for the magnitude of sexual and seasonal differences observed in the song system. A significantly larger proportion of cells with two nucleoli would be cause for concern.

The optical disector yields reliable neuron density estimates only if one is able to measure mounted section thickness accurately. Fifty-micron sections that are dehydrated and cleared in xylene can shrink as much as 65% in the z-axis (A.D. Tramontin, unpublished observations). Tissue shrinkage should not affect the accuracy of neuron counts in any single sampling volume (West, 1993a), but it is necessary to account for that shrinkage when estimating

cell density. Accounting for differential tissue shrinkage becomes especially important if one wishes to compare results from different studies or from studies performed in different laboratories. We found that the measurement of section thickness can be a source of uncontrolled variability in the optical disector protocol. The measurement of section thickness requires a judgment on the part of the investigator about the location of the top and the bottom of the mounted section. It is unlikely that this judgment would result in systematic biases, but measurement error could obscure subtle differences between groups. Slight inaccuracies in the measurement of section thickness can result in significant errors. For example, a 1- $\mu\text{m}$  misjudgment in the measurement of section thickness translates to a 3% error for a 30- $\mu\text{m}$  section.

In conclusion, we used the optical-disector protocol to investigate neuron density and number in three species of birds. We observed the same magnitude of seasonal and sex differences in neuron density and number that were reported previously in these species. We found no differences when we compared neuron counts generated either with the optical disector protocol or the nonstereological procedure. Both methods used the same random, systematic sampling scheme to give every cell in each nucleus an equal probability of being sampled. The two methods differed only in the use of a disector to account for the possible splitting of neuronal nucleoli. By using the avian song system as a model, we found that our nonstereological method produced estimates of neuron density and number that matched those obtained with the optical disector.

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