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# Sexual Dimorphism in the Volume of Song Control Nuclei in European Starlings: Assessment by a Nissl Stain and Autoradiography for Muscarinic Cholinergic Receptors

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ABSTRACT

Previous studies have found that the volume of several song control nuclei is larger in male songbirds than in female songbirds. The degree of this volumetric sex difference within a given species appears to be systematically related to the degree of the behavioral sex difference. The largest volumetric differences have been reported in species in which the male sings and the female sings little, if at all, and the smallest sex differences in volume have been reported in species in which males and females both sing in nearly equal amounts. We compared the volume of three song control nuclei in male and female European starlings (*Sturnus vulgaris*), a species in which females are known to sing, though at a much lower rate than males. We investigated the volume of hyperstriatum ventrale, pars caudale, nucleus robustus archistriatalis, and area X of the lobus parolfactorius as defined with the use of a Nissl stain. In addition, we measured the volume of area X as defined by the density of muscarinic cholinergic receptors visualized by in vitro receptor autoradiographic methods. The volumes of all three of the song nuclei, as defined by Nissl staining, are significantly larger in males than in females. For area X, Nissl staining and receptor autoradiography indicate the same significant volumetric sex difference. The three nuclei are approximately one and one half to two times larger in males than in females, a degree of dimorphism that is intermediate to those reported for other species. Previous investigations of sex differences in the avian vocal control system have used only Nissl stains to define nuclear volumes. We demonstrate in this paper that receptor autoradiography can be used to assess dimorphisms in nuclear volume. Broad application of this approach to a number of neurotransmitter receptor systems will better characterize the dimorphisms in the song system, and therefore will provide greater insight into the neuroanatomical and neurochemical control of birdsong. 

**Key words: acetylcholine, sexual differentiation, scopolamine, songbird, vocal behavior**

Sexually dimorphic behaviors often occur in the context of reproduction (Arnold, '80; Kelley '88). The investigation of the mechanisms underlying these differences provides an excellent opportunity to elucidate the cellular and molecular substrates of complex behaviors. A circuit of discrete nuclei has been implicated in the learning and production of song in passerine birds (Nottebohm et al., '76, '82; Bottjer et al., '84; Sohrabji et al., '90; Scharff and Nottebohm, '91). Many aspects of this circuit are sexually dimorphic (see Arnold et al., '86 for a review). For example, in most species studied to date, the volume of many of these nuclei is several times larger in males than in females (Nottebohm and Arnold, '76; Baker et al., '84; Kirn et al., '89; see Arnold et al., '86 for a review). This neuroanatomical sex difference parallels observed behavioral dimorphisms. In most temperate zone species, males sing to secure breeding territories and attract mates, whereas females sing less frequently, if at all. In contrast, in certain tropical species, males and females engage in complex vocal duets in which females have song repertoires roughly equivalent in size to those of their mates. In these cases, the song control nuclei are significantly less dimorphic than has been observed for temperate zone birds (Brenowitz et al., '85; Arnold et al.,

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'86; Brenowitz and Arnold, '86). In fact, male and female bay wrens (*Thryothorus nigricapillus*) have the same sized repertoires, and there are no volumetric dimorphisms in the song system (Brenowitz et al., '85). Thus, there seems to be a correlation between the extent of behavioral dimorphism and the degree to which the song control nuclei differ in volume.

An important issue to consider when investigating neural sex differences pertains to the methods used to investigate volumetric dimorphisms. All of the studies of sex differences in the volume of song control nuclei have used standard histological techniques (i.e., Nissl stains). However, Nissl stains, like other markers of cellular morphology, are limited in the information they provide. These stains selectively label basophilic acidic protein (Weiss and Greep, '77; Raine, '89). The rough endoplasmic reticulum and free ribosomes are highly basophilic, and hence stain darkly. Nissl stains, therefore, provide one widely used marker of neural cell groups that one can relate to behavior. However, the information derived from Nissl stains is subject to a variety of interpretations, as is the case for any marker of neural morphology. For example, differences in the volume of cell groups labelled with these stains may indicate differences in the activation of cells rather than differences in cell types. Thus, Nissl stains may highlight actual differences in structural characteristics of populations of cells or they may indicate differential activation in otherwise similar cells.

Additional information can be gained with the use of independent cytoarchitectural markers to establish the nature of sex differences in nuclear volume. The usefulness of this approach is demonstrated by the following examples. First, male canaries (*Serinus canaria*) sing often in the spring and much less frequently in the fall. One of the nuclei involved in song production, the hyperstriatum ventrale, pars caudale (HVc, sometimes also referred to as the high vocal center), was found to decrease in volume by 50% from the breeding to nonbreeding condition (Nottebohm, '81). Thus, just as Nissl stains indicate sexual dimorphisms in many song control nuclei, they also suggest a pronounced reduction in the volume of HVc based on the season in male canaries. Recent studies have shown that HVc in male and female canaries can also be characterized by immunocytochemistry for estrogen receptors (Gahr et al., '87; Gahr, '90). A comparison of HVc volume as defined by estrogen receptor immunoreactive cells in breeding and nonbreeding canaries does not indicate any seasonal change in volume of the nucleus (Gahr, '90).

*Abbreviations*

| | |
|---|---|
| AVT | area ventralis (Tsai) |
| HA | hyperstriatum accessorium |
| HV | hyperstriatum ventrale |
| HVc | hyperstriatum ventrale, pars caudale (sometimes referred to as the high vocal center) |
| ICo | nucleus intercollicularis |
| mMAN | nucleus magnocellularis neostriatalis anterioris, medial part |
| lMAN | nucleus magnocellularis neostriatalis anterioris, lateral part |
| LMD | lamina medullaris dorsalis |
| LPO | lobus parolfactorius |
| N | neostriatum |
| nXIIts | nucleus nervi hypoglossi, tracheosyringeal division |
| POA | nucleus preopticus anterioris |
| RA | nucleus robustus archistriatalis |
| Rt | nucleus rotundus |
| X | area X of the lobus parolfactorius |

Second, in canaries Nissl stains indicate a lateral and medial portion of the song control region nucleus magnocellularis of the anterior neostriatum (MAN). Nissl stains define only a lateral portion in zebra finches (*Taeniopygia guttata*). However, both a lateral and medial portion of MAN are defined by enkephalin-like immunoreactivity in zebra finches (Ryan et al., '81). Third, the pars compacta of the sexually dimorphic area in the preoptic area of Mongolian gerbils (*Meriones unguiculatus*) is defined by Nissl stains in males, but is rarely visible in females. When the vasopressinergic innervation of the sexually dimorphic area is characterized by immunocytochemistry, the pars compacta is defined by a dense fiber plexus in males and females, although the area in females is smaller (Crenshaw et al., '92).

These examples illustrate that the sole reliance on Nissl stains may at times lead to inaccurate, or at least incomplete, conclusions regarding supposedly structurally distinct populations of cells and demonstrate the usefulness of the employment of independent neurochemical markers to characterize the sexual dimorphisms reported in the song system. A variety of chemical neuroanatomical studies have characterized the innervation of various neurotransmitter and neuropeptidergic systems in many of the song control nuclei (see Ball, '90, for a review). These studies have shown that specific neurochemical markers clearly label the boundaries of the nuclei. This clear definition of the borders makes it possible to reconstruct nuclear volumes, yet few studies have used these markers for this purpose (cf. Commins and Yahr, '84; Gahr, '90).

Quantitative in vitro receptor autoradiography has been used to localize a variety of neurotransmitter receptors in the song control nuclei. While this technique does not permit fine anatomical localization of receptors at the cellular level, it does provide an indication of receptor density and in many cases clearly defines the borders of many of the song control nuclei. For example, autoradiography for muscarinic cholinergic receptors indicates a higher density of receptors in area X than in the surrounding lobus parolfactorius (LPO) in several songbird species (Ball et al., '90; see also Ryan and Arnold, '81). This differential density in muscarinic cholinergic receptors appears to define clearly the borders of area X and thus could potentially be used to reconstruct the volume of area X. This approach is constructive for at least two reasons. First, by employing independent neurochemical markers we can better characterize the dimorphisms as indicated by Nissl staining. Second, we can also identify cellular characteristics that may not differ between the sexes and therefore we can gain greater insight into the cellular and molecular components necessary for song.

The goals of the investigation described in this paper were twofold. First, we measured the degree of difference in the volume of three song control nuclei between male and female European starlings (*Sturnus vulgaris*). The nuclei selected for study were HVc, area X, and the nucleus robustus archistriatalis (RA). These nuclei have been shown to be dimorphic in several songbird species (Arnold et al., '86). Male starlings sing acoustically complex songs, which sometimes contain mimicries of other bird species or sounds in the environment (e.g., Adret-Hausberger and Jenkins, '88; Eens et al., '91). Female starlings have been observed to sing in the spring and the autumn months and can be induced to sing with testosterone implants (e.g., Bullough and Carrick, '40; Feare, '84; Hausberger and Black, '91).

Studies of song development in starlings suggest that both males and females learn their songs and are able to do so beyond the first year of life (e.g., Adret-Hausberger, '89; Böhner et al., '90; Hausberger and Black, '91; Eens et al., '92). Firm conclusions about the acoustic structure and behavioral function of female song in starlings are difficult to make because few studies have been completed that concentrate on females and those that do consider females often have small sample sizes. However, it does seem clear that female starling song is not as complex as male song, but that female starlings do seem to sing with greater frequency than all of the other nonduetting female songbirds in which the dimorphism of the song control system has been investigated. Given their "intermediate" level of behavioral dimorphism, one might expect an intermediate level of brain dimorphism. We believe this makes starlings a good species in which to measure the dimorphism of the song control nuclei. Second, we tested the merits of using in vitro receptor autoradiography to reconstruct the volumes of song control nuclei and to characterize sex differences in cellular morphology. We therefore assessed the volumetric dimorphism of area X as defined by Nissl stains and autoradiography for muscarinic cholinergic receptors. The results show that autoradiographic methods can be used to define volumetric sexual dimorphisms in the adult vertebrate brain.

## MATERIALS AND METHODS

### Subjects and tissue preparation

Ten male and eight female European starlings were trapped at the Johns Hopkins University Farm in Parkton, Maryland in March, 1992. Although it is difficult to determine the precise age of wild-caught starlings, the length of iridescence on the hackles of the fore-neck is sometimes used to distinguish first-year and adult (beyond first year) birds (Kessel, '51). According to this criterion, two of the females and four of the males may have been first-year birds, and the remainder were adults. Age, however, did not correlate with any of the variables measured, and is therefore not considered further (see Nottebohm et al., '81, for similar results in canaries). The animals were housed in an aviary at Johns Hopkins University in Baltimore, Maryland under a photoperiod of 11L:13D. In the field, starlings have already broken their photorefractory state by March but are not yet photostimulated (Ball and Wingfield, '87). Under an artificial photoperiod of 11L, starlings will maintain a photosensitive state and will never go refractory (see Nicholls et al., '88, for a review). Thus, the birds used in this study were photosensitive and moderately photostimulated at the time of sacrifice. This condition was confirmed by gonadal measurements during necropsy.

Birds were killed by rapid decapitation. The brains were removed and immediately frozen on dry ice. Brains were stored at −70°C until sectioned. Coronal sections were cut on a cryostat at −15°C from a region just rostral to area X in LPO through the entire brain until caudal to RA in the archistriatum. Alternate sections were required for Nissl staining and receptor autoradiography; therefore, the following protocol was used: six 16 μm sections were cut and thaw-mounted on three gelatin-coated slides (two per slide), followed by one 40 μm section thaw-mounted on a gelatin-coated slide, followed by six 16 μm sections, and so on. Sections for autoradiography (16 μm) were air-dried and then quickly stored at −30°C until assayed.

### Nissl stained sections

The 40 μm sections were stained for Nissl substance with cresyl violet. The volumes of three song nuclei (HVc, area X, and RA) and one control nucleus, rotundus (Rt), were measured. Slides containing the four nuclei of interest were identified with the aid of the canary atlas (Stokes et al., '74). Slides were placed on a Chroma Pro 45 light box (Byers Photographic Equipment Company, Portland, OR) that projected an image of the section to a video camera (Sony Model XC-77, Japan) interfaced via a Data Translation Quick Capture digitizing board (Model DT2255, Marlboro, MA) to an Apple Macintosh IIci microcomputer (Cupertino, CA). The digitized image was measured and analyzed with the Image version 1.41 image analysis software package (program written by Wayne Rasband, NIH, Bethesda, MD). Area measures were obtained bilaterally from the rostral to caudal extent of the nucleus of interest by tracing the perimeter of the nucleus. A ruler was placed on the light box before analyses commenced and a known distance was measured to scale pixels to millimeters. Thus, area measures were in accurate units of square millimeters.

To derive an estimate of volume, each area measurement was multiplied by the distance between the section from which it was measured and the next measured section. In most cases, this was 136 μm (40 μm plus six 16 μm sections); however, there were instances in which 16 μm sections were lost between collections of 40 μm sections. In these cases, the thickness of the lost sections was added to the standard 136 μm sampling interval. The area by distance products were summed to arrive at an estimate of volume. Each nucleus was measured by two researchers.

Owing to technical problems during tissue sectioning and staining, not all of the nuclei could be measured in all of the subjects. The number of animals used to derive each volume estimate is indicated in the results section.

### Receptor autoradiography

Muscarinic cholinergic receptors were labelled by the in vitro receptor autoradiographic method described by Ball et al. ('90). Briefly, on the basis of the location of area X in Nissl stained sections, the corresponding thin section (16 μm) slides were removed from the deep freezer and fan-dried for approximately 30 minutes. Because of the number of slides involved (3 slides/Nissl stained slide), it was not possible to use all of the slides containing area X in the assay. However, at least 2 slides/Nissl stained slide were run through the procedure.

Once the slides had dried completely, they were placed in plastic slide mailers containing 8 ml of the incubation medium, consisting of 50 mM potassium phosphate buffer (pH 7.4) and 1 nM [$^3$H] N-methyl-scopolamine (NMS, 81.5 Ci/mmol, New England Nuclear, Boston, MA). NMS is a nonselective muscarinic antagonist. The 1 nM concentration was determined by Ball et al. ('90) to be two times the $K_d$ for this receptor in the starling brain. Nonspecific binding of the ligand was established by the addition of 5 μM atropine to the incubation medium for a subset of the slides. Two slides per bird were dedicated to the assessment of nonspecific binding. As established previously by Ball et al. ('90), [$^3$H]NMS binds with high specificity, and nonspecific binding for this ligand in the starling brain is equal to film background.

Slides were placed in the mailers containing the incubation medium for 60 minutes at room temperature. The

incubation medium was then poured off, and the slides were washed in ice-cold buffer for 10 minutes. To remove excess buffer salts, slides were dipped in ice-cold bi-distilled water before being placed out to dry under a fan for 45 minutes. Once dried, the slides were placed in stainless-steel X-ray cassettes along with tritium standards (American Radiolabeled Chemicals Inc., St. Louis, MO). Tritium-sensitive film (Hyperfilm, Amersham Corp., Arlington Heights, IL) was apposed to the slides, and the cassettes were sealed for 7 days.

The film was developed in Kodak D-19 developer at 18–20°C for 5 minutes, followed by a brief dip in stop bath (40 ml glacial acetic acid in 1 liter of bi-distilled water). Next, the film was placed in Kodak rapid fixer for 2 minutes, and, finally, rinsed for 5 minutes in tap water before air-drying.

The autoradiograms were analyzed with two objectives in mind: 1) to reconstruct the volume of area X; and 2) to measure the density of muscarinic receptors in area X and the LPO in males and females. The volume analysis was similar to that reported for the Nissl stained sections. Autoradiograms were placed on the light box and the video images were digitized for analysis. The perimeter of area X as indicated by the higher density of muscarinic receptors in comparison to the surrounding LPO was traced bilaterally from the first section on every other slide on the autoradiograms. The boundaries of area X were not clearly defined in all instances. In these cases, the second section on the slide was measured. The area measures were multiplied by the interval between measured sections, and these products were summed to derive a volume measure. The interval varied as a result of poor resolution in some cases and due to 40 μm sections (for Nissl) occurring between measured sections. However, on average the interval was 130 μm (range 32–168 μm). In all subjects, the full rostral to caudal extent of the nucleus was present on the film.

For purposes of receptor density measures, the tritium standards that were co-exposed with the assayed sections were used to calibrate optical density units into fmol of [$^3$H]NMS specifically bound per mg. A standard curve was generated from the various known tritium values. The curve was linear for the range of values in which area X and LPO density occurred. The standards gave values in terms of specifically bound ligand per mg wet weight. Ball et al. ('90) determined mean protein content in starling brain to be approximately 10% of tissue wet weight; therefore, the density measures were multiplied by 10 to arrive at final density units of fmol per mg protein. Specific binding was defined as binding with [$^3$H]NMS alone minus binding with [$^3$H]NMS and atropine together. [$^3$H]NMS binding was specific, as slides exposed to atropine failed to produce visible images on the film. Density measures from area X were obtained when the nucleus was traced for purposes of volume reconstruction. A measurement was also taken from the ventromedial portion of LPO adjacent to area X. The reported density measures are based on the average density measured from the rostral to caudal extent of area X. All measurements were made by two experimenters.

## Analyses

The measures of the two experimenters were highly correlated for all variables ($R^2 > 0.87$); therefore, only one experimenter's measurements are reported in all cases. No asymmetries were found; thus the reported data reflect means of the two hemispheres. Homoscedasticity was tested with the use of an F-test. The variances in the male and female samples were not significantly different for any of the comparisons. Parametric unpaired t-tests were thus used to compare males and females on the volume measures. Between-within analyses of variance were used to compare the area X volumes as defined by Nissl staining and NMS autoradiography, and to compare receptor densities in area X and LPO in males and females. All significant results are reported with respect to $\alpha = 0.05$.

# RESULTS

## Nissl staining

The volumes of the three song control nuclei as defined by Nissl staining are sexually dimorphic (see Figs. 1 and 2). Figure 1 shows representative photomicrographs of the three nuclei (area X, HVc, and RA) stained with cresyl violet in male and female starlings. The figure illustrates a larger area for all of the nuclei in males. It is also true that the nuclei in males are present for a longer extent in the rostral and caudal directions. Thus, these two factors probably contribute to the larger volumes seen in males. Mean volumes of the three song nuclei and Rt in males and females are presented in Figure 2. The volumes of HVc, RA, and area X for males and females are respectively, 2.182 and 1.33 mm$^3$, 0.9165 and 0.552 mm$^3$, and 5.377 and 2.757 mm$^3$. Thus, HVc, RA, and area X are 1.64, 1.66, and 1.95 times larger in males than in females. These differences are significant ($t$ values $> 3.15$, $P$ values $< 0.01$). There is no significant sex difference in the volume of Rt ($P > 0.05$). As mentioned above, not all the nuclei were measured in all of the birds; the number of subjects used to derive each volume measure is indicated in Figure 2.

We attempted to measure the volume of lMAN in males and females; however, interobserver reliability for these measurements was low. This is because the boundaries of lMAN could not be reliably identified in all animals. Therefore, we have not included these data.

The absolute volume measurements in both male and female starlings are considerably larger than have been reported for any songbird species to date. Starlings are also the largest songbirds in which these measures have been made, and the difference may be entirely allometric.

## Muscarinic receptor autoradiography

The volume of area X as defined by the density of muscarinic receptors is significantly larger in males than females (see Figs. 3–5). Figures 3A and 4A illustrate representative autoradiograms of sections containing area X as defined by [$^3$H]NMS binding in a male and female starling. The boundaries of the nucleus can be clearly discerned, and, as is the case for Nissl stained sections, the area of the nucleus is clearly larger in males. Figures 3B and 4B show the Nissl stained sections adjacent to the autoradiographic images presented. The boundaries of area X as defined by the two independent markers appear identical. Figure 5 illustrates the volume of area X as defined by the two markers in males and females. Both procedures indicate significant sex differences [1.95 and 1.90 times larger in males as defined by Nissl staining and NMS autoradiography; $F(1,16) = 40.452$]. However, within sexes, both measures indicated the same volume. Given the correspondence between the boundaries of area X as defined by the two methods, it appears as though Nissl staining and NMS

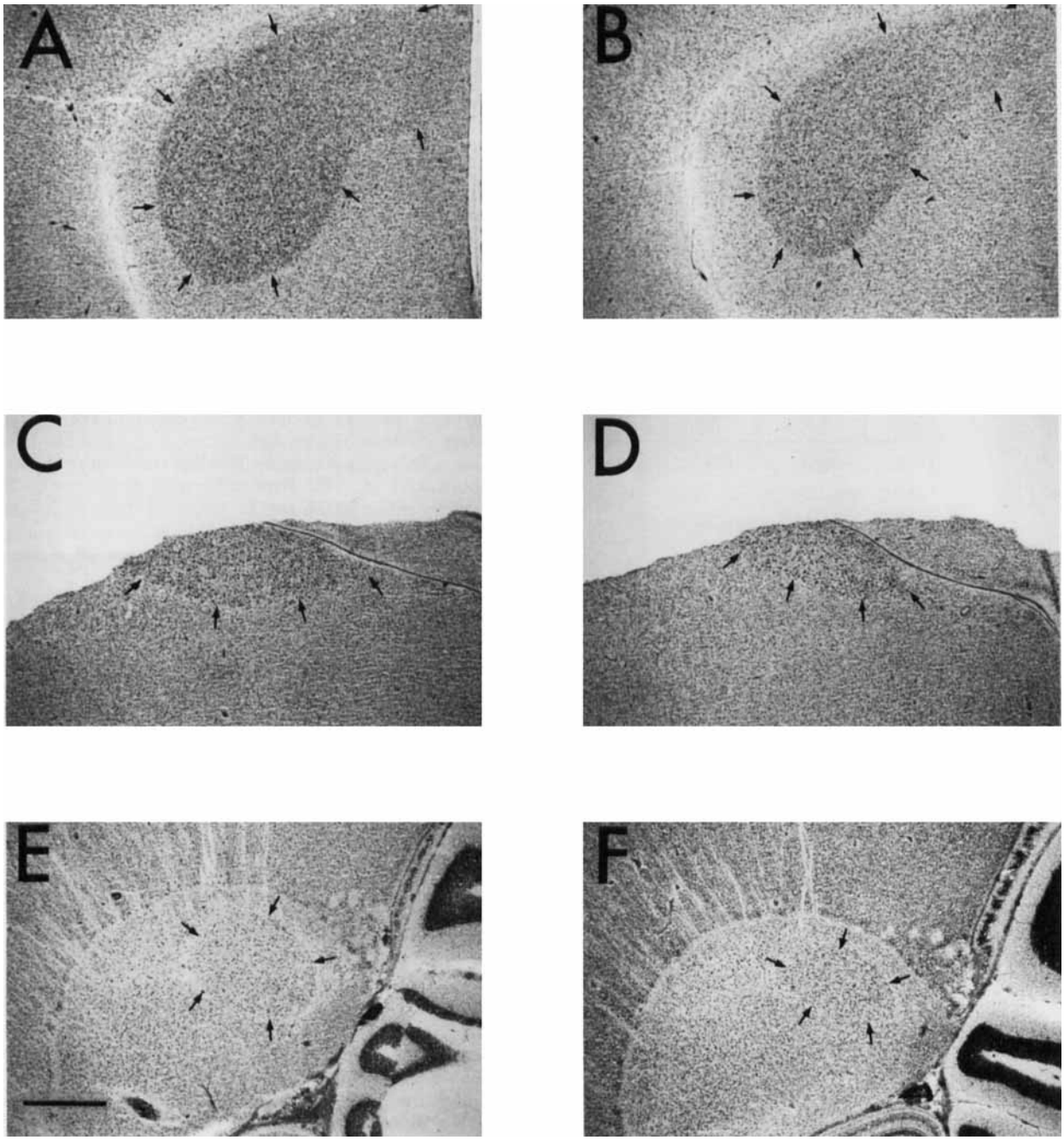


Fig. 1. Photomicrographs of Nissl stained sections illustrate three song control nuclei in male and female European starlings. In every case the boundaries of the song control nucleus are outlined with arrows. Male starlings are illustrated in the left panels, and females are illustrated in the right panels. Area X (**A**), HVc (**C**), and RA (**E**) in males. Area X (**B**), HVc (**D**), and RA (**F**) in females. Scale bar = 1 mm.

autoradiography demarcate the volumetric dimorphism in area X in the same way.

A comparison of the density of muscarinic receptors in area X and the LPO in males and females is depicted in Figure 6. For both sexes, the density of receptors is significantly higher in area X than in the surrounding LPO [$F(1,16) = 143.74$]; however, within the two regions there is no sex difference in receptor density. This pattern is consistent with previous results (Ball et al., '90). The densities of muscarinic receptors in area X do not differ between males and females, but the volumes of area X as defined by [$^3$H]NMS binding do differ. Therefore, the absolute number of receptors in area X must be greater in male than female starlings. The overall receptor density is slightly higher in the present study than that reported by Ball et al. ('90). The same procedure was run in both cases; therefore the reason for this discrepancy is unclear. However, the semiquantitative nature of the procedure precludes any definitive conclusions regarding the significance of this difference.

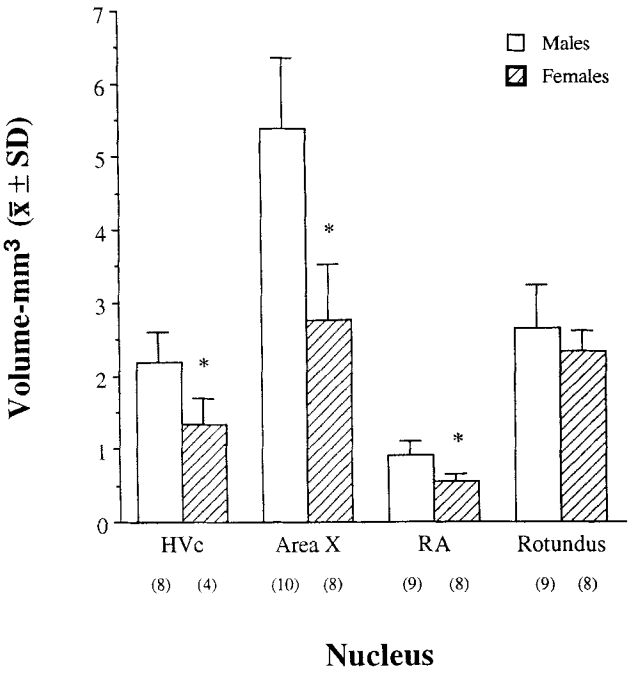


Fig. 2. Frequency histograms illustrate the means and standard deviations of the volumetric measurements for HVc, area X, RA, and Rt in male and female European starlings. The volumes represent the mean of measurements taken from the left and right hemispheres. The numbers in parentheses under each histogram refer to the number of birds that contributed to the generation of the mean in each case. An asterisk indicates a significant sex difference.

## DISCUSSION

### Dimorphisms in the song system

Previous work has suggested that the degree of behavioral dimorphism and the extent of volumetric differences in the song nuclei are correlated (see Arnold et al., '86). However, this correlation must be interpreted somewhat cautiously. There are approximately 4,500 living species of songbirds (Bock and Farrand, '80), yet the song system has been studied in only a handful. In the species that have been investigated, the behavioral sex dimorphisms have been either large (e.g., canaries and zebra finches; Nottebohm and Arnold, '76) or small (e.g., bay wrens and buff-breasted wrens, *T. leucotis;* Brenowitz et al., '85), and the extent of dimorphism in the song system has paralleled the behavioral differences. However, these species represent the ends of a continuum in terms of behavioral dimorphism. To confirm the apparent correlation, species with "intermediate" behavioral dimorphisms must be investigated. If they demonstrate intermediate level volumetric dimorphisms, there will be more support for the putative correlation.

In the present report, we investigated the sex dimorphism in three song nuclei in European starlings. As discussed in the introduction, starlings appear to exhibit an intermediate level sex difference in singing behavior relative to the species that have been studied to date. The volumes of the HVc, RA, and area X are all significantly larger in males than in females. The volume of Rt does not differ between the sexes, suggesting that the dimorphisms in the song nuclei reflect a specialized sexually differentiated system and not a general sex difference in brain size. The male to female ratios in volume ranged from 1.64 for HVc to 1.95 for area X. Thus, the extent of dimorphism is not as dramatic as has been reported for zebra finches and canaries, but is clearly more pronounced than that found in any duetting species studied to date, with the single exception of white-browed robin chats (*Cossypha heuglini*). In this species, males have much larger song repertoires than do females (Brenowitz et al., '85). Why the extent of the volumetric sex difference in starlings and robin chats is so similar is unclear. While female starlings and robin chats most certainly sing in different contexts, it is possible that the extent of their singing relative to male conspecifics is similar. This is only one interpretation, but it may be equally likely that the similarity in brain dimorphism may be coincidental, demonstrating the potential pitfalls of comparing noncongeneric species.

Ideally, the best interspecific comparisons are between congeneric species that differ in the extent of sex differences in song. However, given difficulties in accessibility to ideal subjects, this type of comparison has rarely been made (see Brenowitz et al., '85; Brenowitz and Arnold, '86). Still, important information can be garnered through investigations of more readily available subjects that vary along the continuum of sex difference in song behavior. This is true in the present case, in which starlings appear to have an intermediate level of dimorphism in the song system that parallels their apparent intermediate behavioral dimorphism.

Another possible factor influencing our results concerns the season during which the animals were collected. The animals were all sacrificed when they were in photosensitive to moderately photostimulated reproductive condition. Based on older reports (e.g., Bullough and Carrick, '40), female starlings are often heard singing in the autumn when they are photorefractory to long day lengths. However, a recent report has recorded and analyzed in detail female song in two female starlings from March to April when they would be moderately photostimulated and in breeding condition (Hausberger and Black, '91). It would be useful to know if the degree of dimorphism is the same in different seasons. A comparison of male and female starlings both within a given season and between different seasons (i.e., in conditions in which the birds may express their highest song outputs) could reveal different degrees of volumetric dimorphism. Preliminary data from our laboratory suggest that the volumetric dimorphism persists in males and females housed in two different photoperiods, although the extent of dimorphism may vary as a function of these different conditions (Bernard and Ball, unpublished data).

### Volume reconstruction using autoradiographic techniques

One of the novel results of this report is the demonstration that in vitro autoradiographic techniques can be used to reconstruct nuclear volumes. We found that the density of muscarinic cholinergic receptors and the Nissl stain defined the volumetric dimorphism in area X identically. These results contrast with the lack of correspondence between Nissl stains and immunocytochemistry for estrogen receptors in the way they characterize the volume of HVc in male canaries in different seasonal conditions (Gahr, '90). In vivo autoradiography has been used to assess the distribution of androgen receptors in HVc in

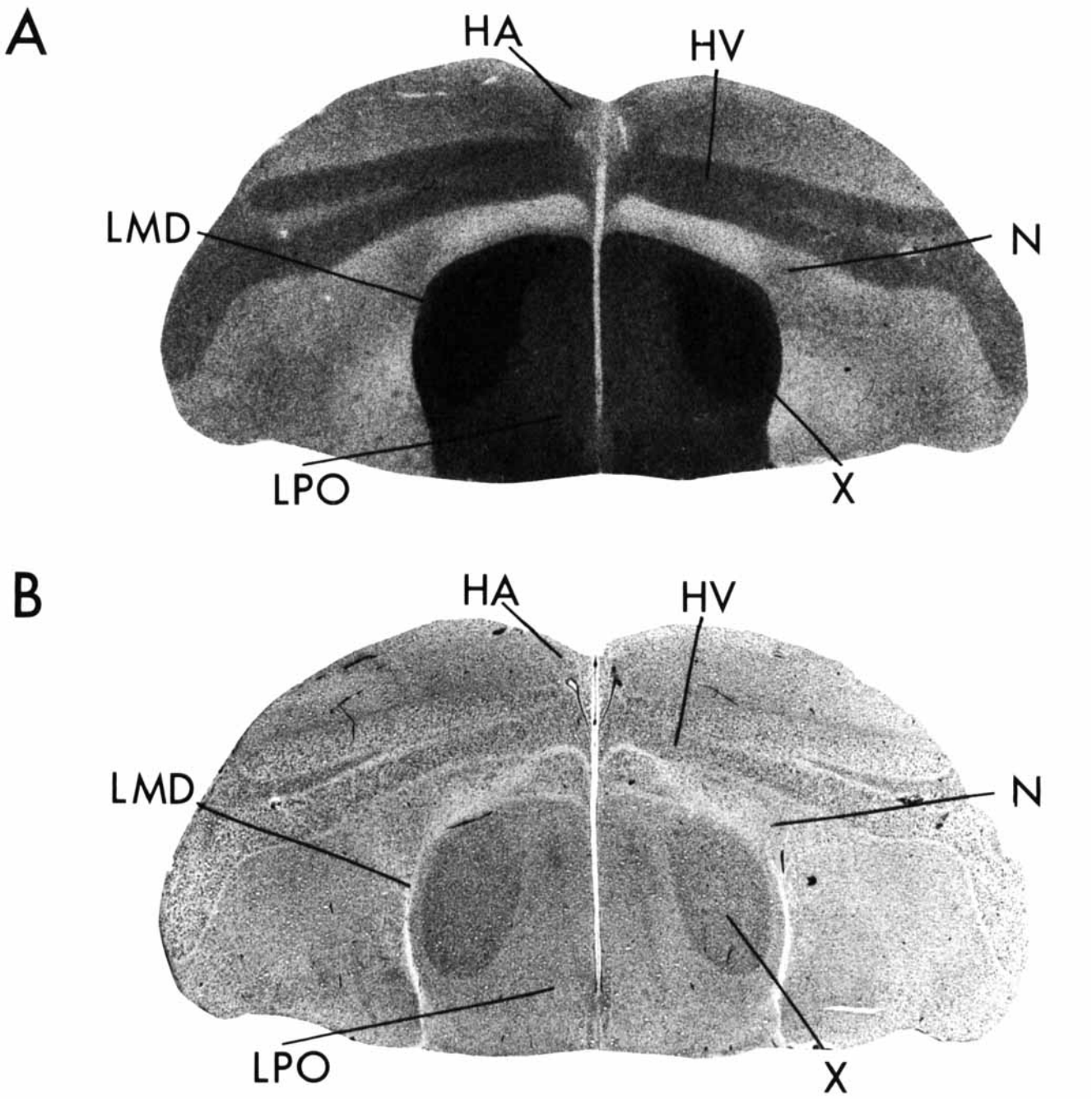


Fig. 3. Area X in a male starling in adjacent coronal sections defined by the high density of muscarinic cholinergic receptors as illustrated by the autoradiogram for [$^3$H]N-methyl scopolamine (NMS) binding (**A**) and by the Nissl stained section (**B**). The shape of the nucleus is similar in the two cases. A similar finding for females is shown in Figure 4, although the overall size of area X is smaller. HA, hyperstriatum accessorium; HV, hyperstriatum ventrale; LMD, lamina medullaris dorsalis; LPO, lobus parolfactorius; N, neostriatum; X, area X.

gonadectomized, short-day male canaries treated with testosterone or vehicle alone (Johnson and Bottjer, '93). In this case, both autoradiography and Nissl staining indicated the same boundaries of the nucleus regardless of the hormonal condition of the birds. Nordeen et al. ('92) studied the development of lMAN in male and female zebra finches where they defined the nucleus with both a Nissl stain and with the retrograde tracer fast blue that identified all the cells that project from lMAN to RA. Previous ontogenetic studies showing decreases in the apparent volume of lMAN as measured from Nissl stained sections had suggested that male zebra finches lose nearly 50% of their lMAN neurons during the juvenile period (e.g., Bottjer et al., '85; Burek et al., '91). However, no such developmental cell loss in males was detected when Nordeen et al. ('92) defined lMAN based on the cells that project to RA. Thus, there are instances in which Nissl stains and independent neurochemical markers label cell populations similarly, and

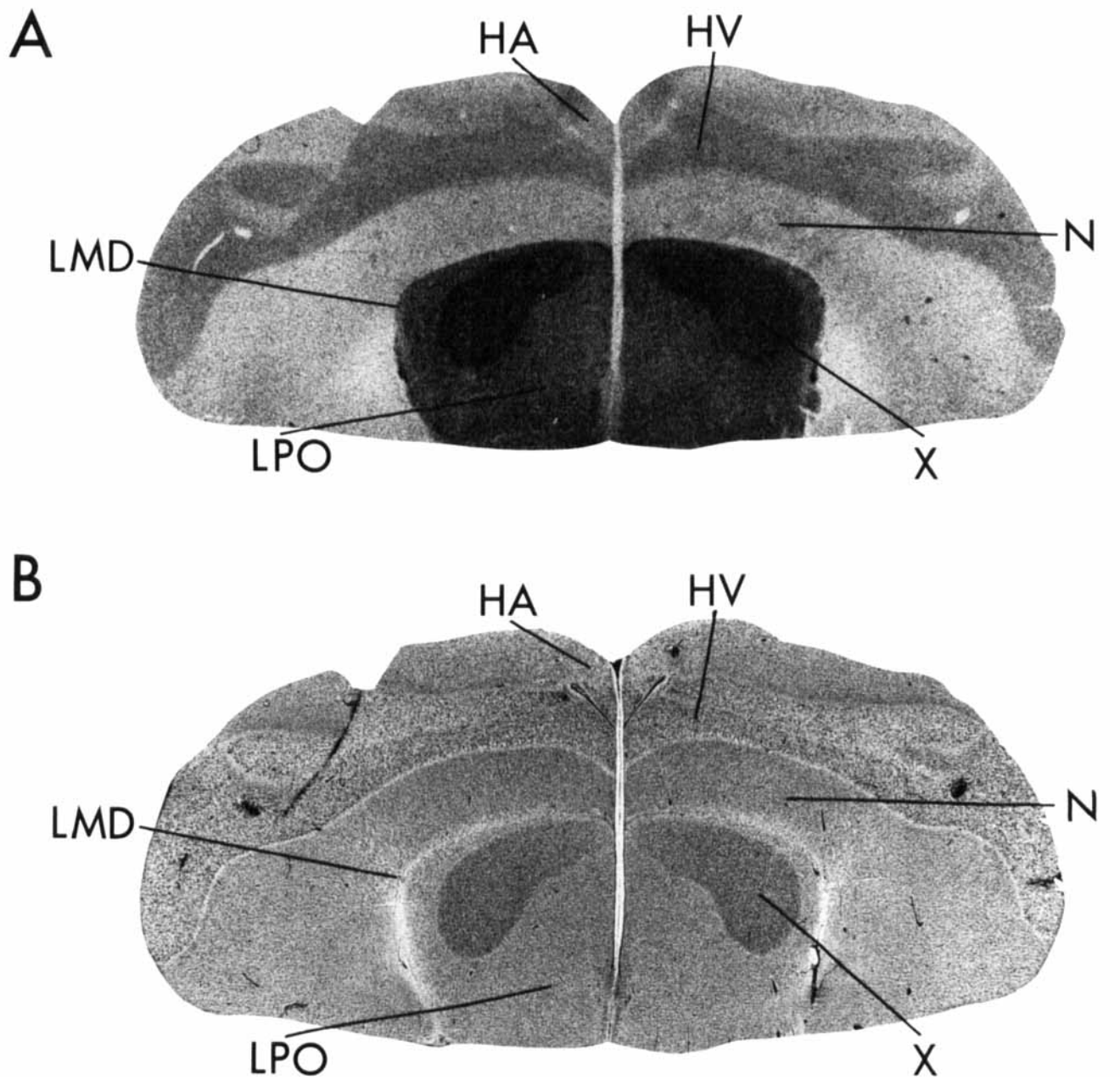


Fig. 4. Area X in a female starling in adjacent coronal sections defined by the high density of muscarinic cholinergic receptors as illustrated by the autoradiogram for [$^3$H]NMS binding (**A**) and by the Nissl stained section (**B**). The shape of the nucleus is similar in the two cases. A similar finding for males is shown in Figure 3, although the overall size of area X is larger.

there are instances in which they highlight potentially important differences.

This differential labeling of neuroanatomical structures suggests that it is useful to apply independent markers whenever a nucleus might be expected to exhibit plasticity, whether this is based on seasonal or ontogenetic variation, or when species or sex differences in size have been identified. Clearly, Nissl stains may not always highlight structural homogeneities in cell populations. Until recently, however, no other techniques had been applied to reconstruct nuclear volumes. Immunocytochemistry can be very effective (e.g., Gahr, '90) and can provide high anatomical resolution, but the appropriate antibodies are not always available. In vitro autoradiographic techniques are useful for indicating receptor densities, but have not been used for volume reconstructions because of their apparently poor resolution. We have shown, however, that the method can be used for this purpose.

In the present case, only the volume of area X was assessed by the two methods because resolution of this nucleus as defined by [$^3$H]NMS binding is better than for any of the other song nuclei. However, other ligands used to define densities of other neurotransmitter receptors clearly delineate the boundaries of several of the song nuclei. For

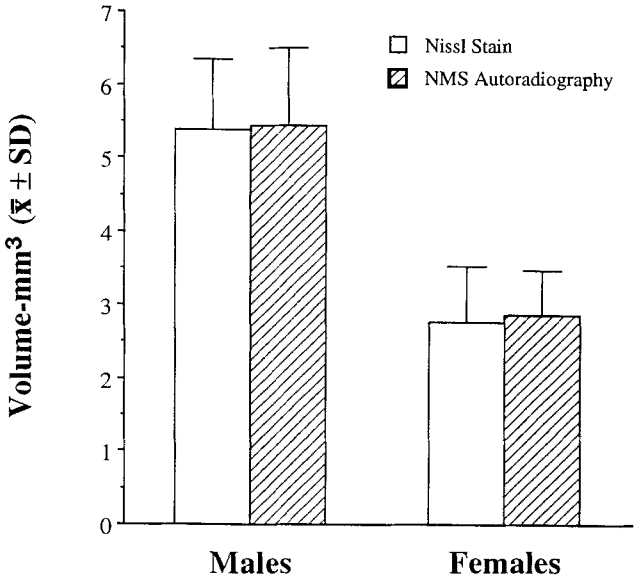


Fig. 5. Histograms compare the volume of area X in male and female starlings as defined by both the high density of [$^3$H]NMS binding in area X as compared with the surrounding LPO and by a Nissl stain. Both markers indicate a significant sex difference in the volume of area X, but the two markers define the volume similarly within each sex.

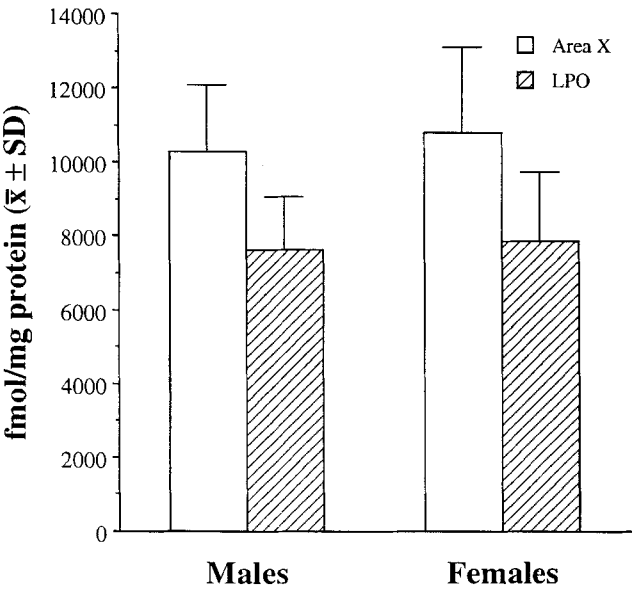


Fig. 6. Histograms illustrate the density of [$^3$H]NMS binding in area X and LPO of male and female European starlings. Area X has a higher density than LPO in both sexes, but there is no sex difference in receptor density in either brain area.

example, [$^3$H]p-aminoclonidine (PAC), an $\alpha_2$-adrenergic agonist, clearly defines the boundaries of area X, HVc, MAN, and RA in several songbird species (see Ball, '90, for a review). Similarly, the distribution of β-adrenergic receptors delineates the boundaries of RA and HVc in starlings and zebra finches (Casto et al., '92), and the density of N-methyl-D-aspartate (NMDA) excitatory amino acid receptors as defined by [$^3$H]MK-801 binding clearly labels HVc and RA in starlings (Ball and Casto, '91). Watson et al. ('88) indicate that the density of nicotinic cholinergic receptors in HVc of zebra finches allows one to precisely define the borders of this nucleus in males and in females. Thus, autoradiography for several different receptor systems can potentially be used to reconstruct nuclear volumes in situations in which cellular changes may be expected, but may not be indicated by Nissl stains. Similarly, autoradiographic assessment of neurotransmitter receptor defined borders may not show the same changes indicated by Nissl staining. Thus, the same limitations that we have highlighted with respect to a strict reliance on Nissl staining may also apply to the technique we employ. The important issue that derives from this point is that only through application of a broad variety of markers will we arrive at a thorough understanding of plasticity in the song system.

In area X there are more muscarinic receptors in male than in female starlings, yet there may be neurotransmitter receptor systems that neither allow you to define sex differences nor to discern clearly the boundaries of the nucleus from the surrounding structures. For example, Watson et al. ('88) found that nicotinic cholinergic receptors were present in area X of male zebra finches but that the density of receptors in this nucleus did not allow one to distinguish area X from the surrounding LPO. By employing the approach described in this report, we can investigate a variety of receptor subtypes and possibly distinguish among those attributes of the song system that are similar and different between males and females and thus gain some insight into the cytoarchitectural and neurochemical bases of bird song. The approach is not limited to sex differences, however. It can be applied to any situation in which there are clear behavioral differences and the neural substrate of the behavior in question has been identified. For the case of bird song, this approach will be important for understanding the basis of seasonal changes in song production and development of song, as well as for understanding sex differences in song.

In order to gain a thorough functional understanding of the role played by muscarinic cholinergic receptors in song learning and production, it will be necessary to map the distributions of the various muscarinic receptor subtypes. A variety of muscarinic cholinergic receptor subtypes have been identified in mammals based on pharmacological and molecular studies. Five different molecular forms of the muscarinic receptor have now been cloned, and pharmacological studies clearly delineate three different types (Goyal, '89; Hulme et al., '90). In general the three receptor subtypes as defined by pharmacological criteria appear to correspond to the M1 to M3 subtypes that have been identified in the cloning studies. In the present case, it should be noted that the use of [$^3$H]NMS precludes any discussion of potential sex differences in density of muscarinic receptor subtypes. [$^3$H]NMS is a nonselective antagonist that appears to bind to most, if not all of the muscarinic receptor subtypes. However, the few studies that have been completed on muscarinic receptors in birds suggest that there may be taxonomic differences in the characterization of these different subtypes. For example, studies in mammals have found that the M1 receptor subtype shows a high affinity for the muscarinic antagonist pirenzepine, while the M2 subtype shows a low affinity for pirenzepine but a high affinity for agonists such as carbachol (e.g., Cortès and Palacios, '86; Hammer et al., '80). Therefore, autoradiographic investigations of muscarinic receptors in rats that employ [$^3$H]NMS as the ligand reveal a negative correlation

between the amount of [$^3$H]NMS binding that is displaced by pirenzepine and carbachol in distinct areas of the brain, suggesting that there are two receptor subtypes that are differently distributed throughout the rat brain (Dietl et al., '88). A similar analysis in the pigeon brain (Dietl et al., '88) revealed a positive correlation between the amount of displaced [$^3$H]NMS binding by pirenzepine and carbachol, suggesting either that these two receptors subtypes are not present in the avian brain or that they show a close neuroanatomical localization (see Ball et al., '90 for discussion). Thus, it is likely that if starlings possess any of the five muscarinic receptor subtypes, the sex difference reported herein probably reflects a difference in more than one subtype. However, this issue clearly requires further investigation.

## Gonadal sex steroids and the dimorphic innervation of area X

We have shown that the innervation of area X by the cholinergic system is sexually dimorphic. It is likely that the cause of this difference stems from the action of gonadal steroid hormones acting either perinatally, or in adulthood, or at both times. Treatment of female zebra finches with estradiol or aromatizable androgens early in development leads to masculinization of the song system (e.g., Gurney and Konishi, '80; Gurney, '81, '82). Androgen treatment in these masculinized females causes singing in adulthood (e.g., Pohl-Apel and Sossinka, '84). Adult female canaries experience masculinization of the song system when treated with testosterone (e.g., Nottebohm, '80; De Voogd and Nottebohm, '81). In addition, several cellular characteristics are masculinized by hormone administration, including an increase in the number of cells that concentrate androgens in zebra finches (K.W. Nordeen et al., '86; E.J. Nordeen et al., '87), increases in soma diameter and cell number in HVc and RA and decreases in cell density in RA of canaries (Bottjer and Dignan, '88), and increases in the size of synaptic profiles and number of synaptic vesicles in RA of canaries (De Voogd et al., '85). Thus, steroid hormones are known to modulate many different aspects of the song system that are different between the sexes.

A variety of steroid effects on cholinergic function have been reported in adult animals. The density of muscarinic receptors as defined by [$^3$H]quinuclidinyl benzilate (QNB) increases in the medial basal hypothalamus of ovariectomized (OVX) rats (*Rattus norvegicus*) following systemic injections of estradiol benzoate (EB; Dohanich et al., '82). Similarly, muscarinic receptor density in the preoptic area (POA) is highest during proestrus in intact cycling female rats, and treatment of OVX females with EB increases [$^3$H]QNB binding in POA (Olsen et al., '88). Gonadectomy in male and female Japanese quail (*Coturnix japonica*) reduces the density of muscarinic receptors in a rostral subregion of a vocal control area, the nucleus intercollicularis (ICo; Ball and Balthazart, '90). In zebra finches and canaries it has been established that castration reduces the activity of both acetylcholinesterase (AChE) and choline acetyltransferase (ChAT) at the neuromuscular junction of the vocal production organ, the syrinx (Luine et al., '80). In zebra finches castration and testosterone replacement influences the number of syringeal acetylcholine receptors (Bleisch et al., '84). Testosterone replacement enhances the activity of AChE, but not ChAT, at the syrinx (Luine et al., '80). This enhancement of AChE at the syrinx appears to require the metabolism of testosterone to 5$\alpha$-dihydrotestosterone (Luine et al., '83). Steroids have also been shown to modulate the activity of these enzymes in some of the song control nuclei (Gardner and Harding, '90).

Given the influence of steroids on masculinization of the song system and the modulatory effects of steroids on muscarinic receptor density, it is possible that differential exposure to steroid hormones during ontogeny (or in adulthood) may be responsible for the differential innervation of area X by the cholinergic system. The notion that the cholinergic system is modulated by early organizational effects of steroids in area X is supported by the fact that ChAT immunoreactive fibers do not demarcate the boundaries of area X in 30-day-old zebra finches, but they do at day 50 (Sakaguchi and Saito, '91). Thus, the cholinergic innervation of area X may be differentiated early in development when steroids appear to exert their masculinizing effects on the song system.

The exact mechanism through which steroids exert their masculinizing effect on area X volume is not understood at present. Immunocytochemical and autoradiographic studies of the song system have failed to localize any estrogen or androgen receptors in area X (e.g., Arnold et al., '76; Nordeen et al., '86; Gahr et al., '87; Gahr and Konishi, '88; Balthazart et al., '92; Gahr et al., '93). However, HVc has been shown to contain androgen receptors in all species studied to date, and estrogen receptors in some species (Arnold et al., '76; Balthazart et al., '92; Gahr et al., '93). HVc sends a major efferent projection to area X (Nottebohm et al., '76, '82). Disruption of the projection from HVc to area X blocks the masculinizing effects of steroids on area X volume in zebra finches (Herrmann and Arnold, '91). These results strongly suggest that steroids act through a transsynaptic mechanism in order to masculinize area X. A concomitant synergistic influence of steroids acting directly on area X via neuronal membrane effects is also possible (Schumacher, '90); however, such membrane effects of steroids have yet to be described in the song system.

HVc cell bodies stain for AChE; therefore the HVc–area X projection may be cholinergic (Ryan and Arnold, '81). However, ChAT immunoreactive cell bodies have been localized in LPO, but not in HVc, suggesting that the cholinergic input to area X may emerge from LPO or from some unknown source in the brainstem or elsewhere (Sakaguchi and Saito, '91).

In conclusion, the differential activation of the cholinergic system in males and females may lead to certain aspects of the behavioral differences in song. This is somewhat speculative, for it has not yet been established if the increased number of receptors in males relative to females represents a difference in the number of cells expressing the receptor, the number receptors expressed by each cell, or both. In any case, the differential innervation by the cholinergic system in area X may influence differences in the ability of males and females to learn song and later produce it in adulthood.

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## LITERATURE CITED


Adret-Hausberger, M. (1989) Song ontogenesis in starlings *Sturnus vulgaris:* Are song and subsong continuous? Bird Behav. *8:*8–13.

Adret-Hausberger, M., and P.F. Jenkins (1988) Complex organization of the warbling song in the European starling, *Sturnus vulgaris.* Behaviour *107:*138–156.

Arnold, A.P. (1980) Sexual differences in the brain. Am. Sci. *68:*165–173.

Arnold, A.P., F. Nottebohm, and D.W. Pfaff (1976) Hormone concentrating cells in vocal control and other areas of the brain of the zebra finch (*Poephila guttata*). J. Comp. Neurol. *165:*487–512.

Arnold, A.P., S.W. Bottjer, E.A. Brenowitz, E.J. Nordeen, and K.W. Nordeen (1986) Sexual dimorphism in the neural vocal control system in song birds: Ontogeny and phylogeny. Brain Behav. Res. *28:*22–31.

Baker, M.C., S.W. Bottjer, and A.P. Arnold (1984) Sexual dimorphism and lack of seasonal changes in vocal control regions of the white-crowned sparrow brain. Brain Res. *295:*85–89.

Ball, G.F. (1990) Chemical neuroanatomical studies of the steroid-sensitive songbird vocal control system: A comparative approach. In J. Balthazart (ed): Hormones, Brain, and Behavior in Vertebrates. I. Sexual Differentiation, Neuroanatomical Aspects, Neurotransmitters and Neuropeptides. Comp. Physiol. Basel: Karger, pp. 148–167.

Ball, G.F., and J.C. Wingfield (1987) Changes in plasma levels of luteinizing hormone and sex steroid hormones in relation to multiple-broodedness and nest-site density in male starlings. Physiol. Zool. *60:*191–199.

Ball, G.F., and J. Balthazart (1990) Steroid modulation of muscarinic cholinergic and $\alpha_2$-adrenergic receptor density in the nucleus intercollicularis of the Japanese quail. Eur. J. Neurosci. *2:*828–835.

Ball, G.F., and J.M. Casto (1991) Autoradiographic localization of NMDA receptors in the avian song control system using [$^3$H] MK-801. Soc. Neurosci. Abstr. *17:*1053.

Ball, G.F., B. Nock, J.C. Wingfield, B.S. McEwen, and J. Balthazart (1990) Muscarinic cholinergic receptors in the songbird and quail brain: A quantitative autoradiographic study. J. Comp. Neurol. *298:*431–442.

Balthazart, J., A. Foidart, E.M. Wilson, and G.F. Ball (1992) Immunocytochemical localization of androgen receptors in the male songbird and quail brain. J. Comp. Neurol. *317:*407–420.

Bleisch, W.V., V.N. Luine, and F. Nottebohm (1984) Modification of synapses in androgen-sensitive muscle. I. Hormonal regulation of acetylcholine receptor number in the songbird syrinx. J. Neurosci. *4:*786–792.

Bock, W.J., and J. Farrand, Jr. (1980) The number of species and genera of recent birds: A contribution to comparative systematics. Am. Museum Novitates *2703:*1–29.

Böhner, J., M.L. Chaiken, G.F. Ball, and P. Marler (1990) Song acquisition in photosensitive and photorefractory male European starlings. Horm. Behav. *24:*582–594.

Bottjer, S.W., and T.P. Dignan (1988) Joint hormonal and sensory stimulation modulate neuronal number in adult canary brains. J. Neurobiol. *19:*624–635.

Bottjer, S.W., E.A. Meisner, and A.P. Arnold (1984) Forebrain lesions disrupt development but not maintenance of song in passerine birds. Science *224:*901–903.

Bottjer, S.W., S.L. Glaessner, and A.P. Arnold (1985) Ontogeny of brain nuclei controlling song learning and behavior in zebra finches. J. Neurosci. *5:*1556–1562.

Brenowitz, E.A., and A.P. Arnold (1986) Interspecific comparisons of the size of neural song control regions and song complexity in duetting birds: Evolutionary implications. J. Neurosci. *6:*2875–2879.

Brenowitz, E.A., A.P. Arnold, and R.N. Levin (1985) Neural correlates of female song in tropical duetting birds. Brain Res. *343:*104–112.

Bullough, W.S., and R. Carrick (1940) Male behaviour of the female starling in autumn. Nature *145:*629.

Burek, M.J., K.W. Nordeen, and E.J. Nordeen (1991) Neuron loss and addition in developing zebra finch song nuclei are independent of auditory experience during song learning. J. Neurobiol. *22:*215–223.

Casto, J.M., P. Absil, J. Balthazart, and G.F. Ball (1992) Autoradiographic localization of beta-adrenergic receptors in the songbird vocal control system. Soc. Neurosci. Abstr. *18:*459.

Commins, D., and P. Yahr (1984) Acetylcholinesterase activity in the sexually dimorphic area of the gerbil brain: Sex differences and influences of adult gonadal steroids. J. Comp. Neurol. *224:*123–131.

Cortès, R., and J.M. Palacios (1986) Muscarinic cholinergic receptor subtypes in the rat brain. I. Quantitative autoradiographic studies. Brain Res. *362:*227–238.

Crenshaw, B.J., G.J. DeVries, and P. Yahr (1992) Vasopressin innervation of sexually dimorphic structures of the gerbil forebrain under various hormonal conditions. J. Comp. Neurol. *322:*589–598.

DeVoogd, T.J., and F. Nottebohm (1981) Gonadal hormones induce dendritic growth in the adult avian brain. Science *214:*202–204.

DeVoogd, T.J., B. Nixdorf, and F. Nottebohm (1985) Synaptogenesis and changes in synaptic morphology related to acquisition of a new behavior. Brain Res. *329:*304–308.

Dietl, M.M., R. Cortés, and J.M. Palacios (1988) Neurotransmitter receptors in the avian brain. II. Muscarinic cholinergic receptors. Brain Res. *439:*360–365.

Dohanich, G.P., J.A. Witcher, D.R. Weaver, and L.G. Clemens (1982) Alteration of muscarinic binding in specific brain areas following estrogen treatment. Brain Res. *241:*347–350.

Eens, M., R. Pinxten, and R.F. Verheyen (1991) Organization of song in the European starling: Species-specificity and individual differences. Belg. J. Zool. *121:*257–278.

Eens, M., R. Pinxten, and R.F. Verheyen (1992) Song learning in captive European starlings, *Sturnus vulgaris.* Anim. Behav. *44:*1131–1143.

Feare, C. (1984) The Starling. Oxford: Oxford University Press.

Gahr, M. (1990) Delineation of a brain nucleus: Comparisons of cytochemical, hodological, and cytoarchitectural views of the song control nucleus HVC of the adult canary. J. Comp. Neurol. *294:*30–36.

Gahr, M., and M. Konishi (1988) Developmental changes in estrogen-sensitive neurons in the forebrain of the zebra finch. Proc. Natl. Acad. Sci. USA *85:*7380–7383.

Gahr, M., G. Flügge, and H.-R. Güttinger (1987) Immunocytochemical localization of estrogen-binding neurons in the songbird brain. Brain Res. *402:*173–177.

Gahr, M., H.-R. Güttinger, and D.E. Kroodsma (1993) Estrogen receptors in the avian brain: Survey reveals general distribution and forebrain areas unique to songbirds. J. Comp. Neurol. *327:*112–122.

Gardner, A., and C.F. Harding (1990) Steroid modulation of choline acetyltransferase activity in the zebra finch brain. Soc. Neurosci. Abstr. *16:*922.

Goyal, R.K. (1989) Muscarinic receptor subtypes: Physiology and clinical implications. N. Engl. J. Med. *321:*1022–1029.

Gurney, M. (1981) Hormonal control of cell form and number in the zebra finch song system. J. Neurosci. *1:*658–673.

Gurney, M. (1982) Behavioral correlates of sexual differentiation in the zebra finch song system. Brain Res. *231:*153–172.

Gurney, M., and M. Konishi (1980) Hormone induced sexual differentiation of brain and behavior in zebra finches. Science *208:*1380–1383.

Hammer, R., C.P. Berrie, N.J.M. Birdsall, A.S.V. Burgen, and E.C. Hulme (1980) Pirenzepine distinguishes between different subclasses of muscarinic receptors. Nature *283:*90–92.

Hausberger, M., and J.M. Black (1991) Female song in European starlings: The case of noncompetitive song-matching. Ethol. Ecol. Evol. *3:*337–344.

Herrmann, K., and A.P. Arnold (1991) Lesions of HVc block the developmental masculinizing effects of estradiol in the female zebra finch song system. J. Neurobiol. *22:*29–39.

Hulme, E.C., N.J.M. Birdsall, and N.J. Buckley (1990) Muscarinic receptor subtypes. Annu. Rev. Pharm. Toxicol. *30:*633–673.

Johnson, F., and S.W. Bottjer (1993) Hormone-induced changes in identified cell populations of the higher vocal center in male canaries. J. Neurobiol. *24:*400–418.

Kelley, D.B. (1988) Sexually dimorphic behaviors. Annu. Rev. Neurosci. *11:*225–251.

Kessel, B. (1951) Criteria for sexing and aging European starlings (*Sturnus vulgaris*). Bird Banding *22:*16–23.

Kirn, J.R., R.P. Clower, D.E. Kroodsma, and T.J. DeVoogd (1989) Song-related brain regions in the red-winged blackbird are affected by sex and season but not repertoire size. J. Neurobiol. *20:*139–163.

Luine, V., F. Nottebohm, C. Harding, and B.S. McEwen (1980) Androgen affects cholinergic enzymes in syringeal motor neurons and muscle. Brain Res. *192:*89–107.

Luine, V.N., C.F. Harding, and W.V. Bleisch (1983) Specificity of gonadal hormone modulation of cholinergic enzymes in the avian syrinx. Brain Res. *279:*339–342.

Nicholls, T.J., A.R. Goldsmith, and A. Dawson (1988) Photorefractoriness in birds and comparison with mammals. Physiol. Rev. *68:*133–176.

Nordeen, E.J., K.W. Nordeen, and A.P. Arnold (1987) Sexual differentiation of androgen accumulation within the zebra finch brain through selective cell loss and addition. J. Comp. Neurol. *259:*393–399.

Nordeen, E.J., A. Grace, M.J. Burek, and K.W. Nordeen (1992) Sex-dependent loss of projection neurons involved in avian song learning. J. Neurobiol. *23:*671–679.

Nordeen, K.W., E.J. Nordeen, and A.P. Arnold (1986) Estrogen establishes sex differences in androgen accumulation in zebra finch brain. J. Neurosci. *6:*734–738.

Nottebohm, F. (1980) Testosterone triggers growth of brain vocal control nuclei in adult female canaries. Brain Res. *189:*429–436.

Nottebohm, F. (1981) A brain for all seasons: Cyclical anatomical changes in song control nuclei of the canary brain. Science *214:*1368–1370.

Nottebohm, F., and A.P. Arnold (1976) Sexual dimorphism in vocal control areas of the songbird brain. Science *194:*211–213.

Nottebohm, F., T.M. Stokes, and C.M. Leonard (1976) Central control of song in the canary, *Serinus canaria.* J. Comp. Neurol. *165:*457–486.

Nottebohm, F., S. Kasparian and C. Pandazis (1981) Brain space for a learned task. Brain Res. *213:*99–109.

Nottebohm, F., D.B. Kelley, and J.A. Paton (1982) Connections of vocal control nuclei in the canary telencephalon. J. Comp. Neurol. *207:*344–357.

Olsen, K.L., E. Edwards, N. Schecter, and R.E. Whalen (1988) Muscarinic receptors in preoptic area and hypothalamus: Effects of cyclicity, sex and estrogen treatment. Brain Res. *448:*223–229.

Pohl-Apel, G., and R. Sossinka (1984) Hormonal determination of song capacity in females of the zebra finch: Critical phase of treatment. Z. Tierpsychol. *64:*330–336.

Raine, C.S. (1989) Neurocellular anatomy. In G.J. Siegel, B.W. Agranoff, R.W. Albers, and P.B. Molinoff (eds): Basic Neurochemistry. 4th ed. New York: Raven Press, pp. 3–33.

Ryan, S.M., and A.P. Arnold (1981) Evidence for cholinergic participation in the control of bird song: Acetylcholinesterase distribution and muscarinic receptor autoradiography in the zebra finch brain. J. Comp. Neurol. *202:*211–219.

Ryan, S.M., A.P. Arnold, and R.P. Elde (1981) Enkephalin-like immunoreactivity in vocal control regions of the zebra finch brain. Brain Res. *229:*236–240.

Sakaguchi, H., and N. Saito (1991) Developmental change of cholinergic activity in the forebrain of the zebra finch during song learning. Dev. Brain Res. *62:*223–228.

Scharff, C., and F. Nottebohm (1991) A comparative study of the behavioral deficits following lesions of the various parts of the zebra finch song system: Implications for vocal learning. J. Neurosci. *11:*2896–2913.

Schumacher, M. (1990) Rapid membrane effects of steroid hormones: An emerging concept in neuroendocrinology. Trends Neurosci. *13:*359–362.

Sohrabji, F., E.J. Nordeen, and K.W. Nordeen (1990) Selective impairment of song learning following lesions of a forebrain nucleus in the juvenile zebra finch. Behav. Neural Biol. *53:*51–63.

Stokes, T.M., C.M. Leonard, and F. Nottebohm (1974) The telencephalon, diencephalon, and mesencephalon of the canary, *Serinus canaria,* in stereotaxic coordinates. J. Comp. Neurol. *156:*337–374.

Watson, J.T., E. Adkins-Regan, P. Whiting, J.M. Lindstrom, and T.R. Podleski (1988) Autoradiographic localization of nicotinic acetylcholine receptors in the brain of the zebra finch (*Poephila guttata*). J. Comp. Neurol. *274:*255–264.

Weiss, L., and R.O. Greep (1977) Histology. 4th ed. New York: McGraw-Hill.