

Song and the song control pathway in the brain can develop independently of exposure to song in the sedge warbler

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Previous studies have shown that female sedge warblers choose to mate with males that have more complex songs, and sexual selection has driven the evolution of both song complexity and the size of the major song control area (HVC) in the brain. In songbirds, learning from conspecifics plays a major role in song development and this study investigates the effects of isolation and exposure to song on song structure and the underlying song control system. Sibling pairs of hand-reared nestling sedge warblers were reared to sexual maturity under two conditions. Siblings in one group were reared individually in acoustic isolation in separate soundproof chambers. In the other group, siblings were reared together in an aviary with playback of recorded songs. The following spring, analysis of songs revealed that siblings reared in acoustic isolation produced normal song structures, including larger syllable repertoires than those exposed to song. We found no significant differences in the volumes of HVC, nucleus robustus archistriatalis, the lateral portion of the magnocellular nucleus and the density of dendritic spines between the two groups. Males exceeded females in all these measures, and also had a larger telencephalon. Our experiments show that complex song, sexual dimorphism in brain structure, and the size of song nuclei can all develop independently of exposure to song. These findings have important implications for how sexual selection can operate upon a complex male trait such as song and how it may also shape the more general evolution of brain structure in songbirds.

Keywords: sedge warbler; song learning; brain; HVC; sexual selection

1. INTRODUCTION

The complex song of the European sedge warbler (*Acrocephalus schoenobaenus*) has become a classic example of a male trait that has been sexually selected (reviewed in Catchpole 2000). Songs are constructed from simpler acoustic units called syllables, and the number of different syllable types is used to measure song complexity. This measure is called repertoire size and is used as a standard measure of song complexity in this, and earlier published, studies. In the field, females select males with larger repertoires (Catchpole 1980; Buchanan & Catchpole 1997); and under controlled conditions in the laboratory, females display more when repertoire size is artificially increased (Catchpole *et al.* 1984). This and other evidence from *Acrocephalus* species (reviewed in Catchpole 2000) now form a considerable body of published evidence that implicates sexual selection by female choice as the major driving force behind the evolution of complex songs in this group.

It is well established that learning plays an important role in the development of the complex songs of oscine songbirds (Catchpole & Slater 1995; Kroodsma & Miller 1996). Yet, if song complexity is a sexually selected male

trait, then repertoire size should have some genetic basis. As song is a behavioural trait, the basis for this must lie in the underlying neural pathways that control song production. The song control system contains two major fore-brain nuclei that are involved in the production of learned vocal patterns, the HVC (High Vocal Centre) and the RA (nucleus robustus archistriatalis) (Nottebohm *et al.* 1976; Vu *et al.* 1994; Yu & Margoliash 1996; reviewed in DeVogd & Lauay 2001; Brainard & Doupe 2002). Studies, both between and within species, have now shown that the volume of HVC is positively correlated with increasing song complexity (e.g. Nottebohm *et al.* 1981; Kroodsma & Canady 1985; DeVogd *et al.* 1993; Airey & DeVogd 2000). By contrast, the volume of the lateral portion of the magnocellular nucleus (IMAN), a song system nucleus important for song acquisition but not production, is not significantly correlated with song repertoire size (Szekely *et al.* 1996).

In *Acrocephalus* warblers, a comparative study by Szekely *et al.* (1996) has shown that as repertoire size increased during evolution, so did the volume of HVC. A single-species study on the sedge warbler (Airey *et al.* 2000a) has now reported a strong positive correlation between repertoire size and the volume of HVC. We used sibling pairs of hand-reared sedge warblers to explore the effects of acoustic isolation and exposure to song upon both the development of song and song control nuclei, including HVC.

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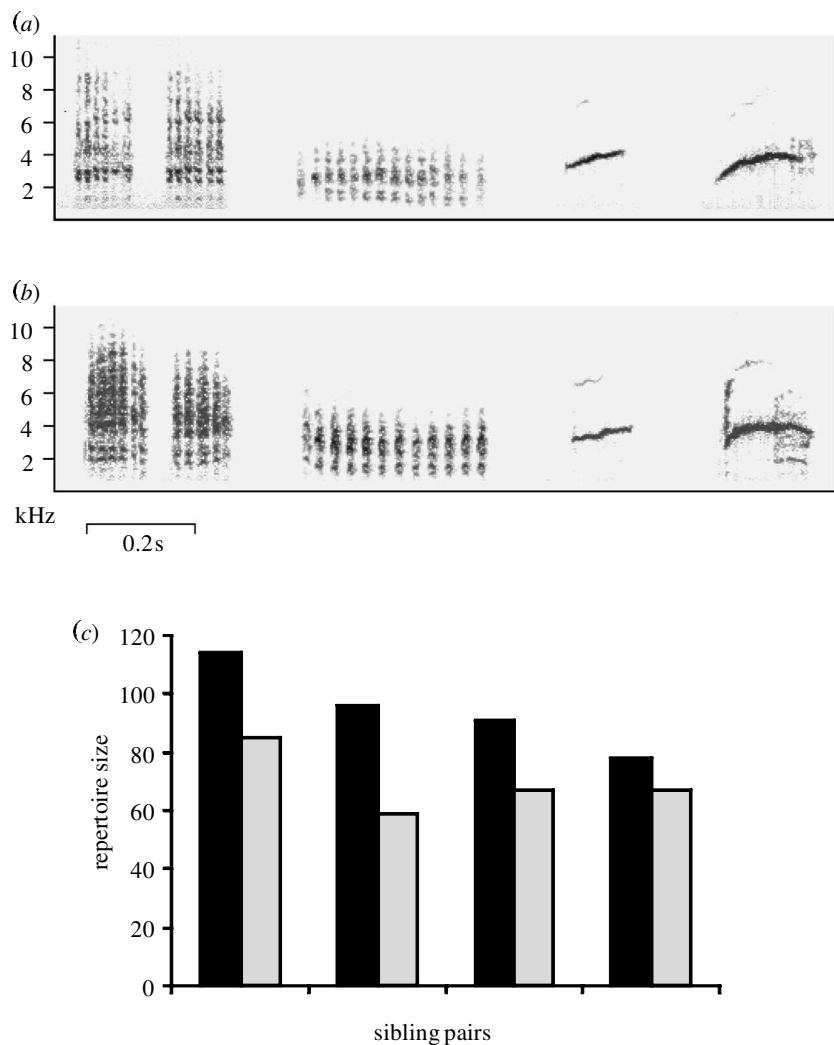


Figure 1. (a) Normal song structure of a male reared with exposure to playback of song, and (b) his sibling reared in acoustic isolation. (c) Repertoire sizes of males reared in acoustic isolation and their siblings reared with exposure to playback of song (black bars: males in acoustic isolation, grey bars: males with exposure of song playback).

2. MATERIAL AND METHODS

(a) *Experimental conditions*

In June 1997, broods of nestling sedge warblers were collected at age 5–8 days from Neusiedlersee in Austria and imported, under licence, to the Vogelwarte Radolfzell in Germany for hand rearing. The young were sexed using the W-chromosome molecular sexing technique (Griffiths *et al.* 1998) before being allocated to an experimental group. Nestlings to be reared individually in acoustic isolation were kept in standard cages within individual soundproof chambers. These chambers were hand-built to a high specification by the Max Planck Institute and housed underground to ensure complete acoustic isolation. Nestlings to be reared with song playback were housed together in a separate room. All birds were kept under the same conditions of temperature, lighting and standard diet. The light schedule followed natural photoperiod. Wherever possible, siblings were divided to minimize genetic variation between groups. In this way, four males and four females were allocated to individual soundproof chambers, and four males and four females were allocated to the room with playback. Four playback tapes were used, each containing songs recorded from different individuals, selected to encompass a wide range of natural repertoire sizes (49–85 different syllables). In the wild, sedge warblers sing

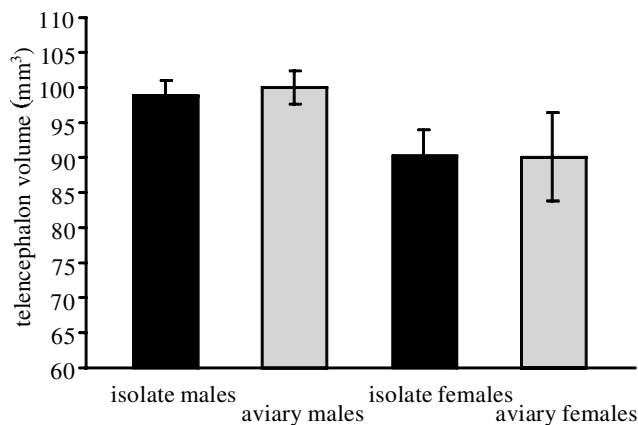


Figure 2. Telencephalon volume (mm³) shows significant differences between the sexes, but no differences between treatments.

a pronounced dawn and dusk chorus. Therefore, the songs were played to these birds for 30 min after lights went on, and for 30 min before lights went out. Natural vegetation in the room helped to minimize reverberation, and the amplitude was adjusted to a normal level using a sound-level meter. The pro-

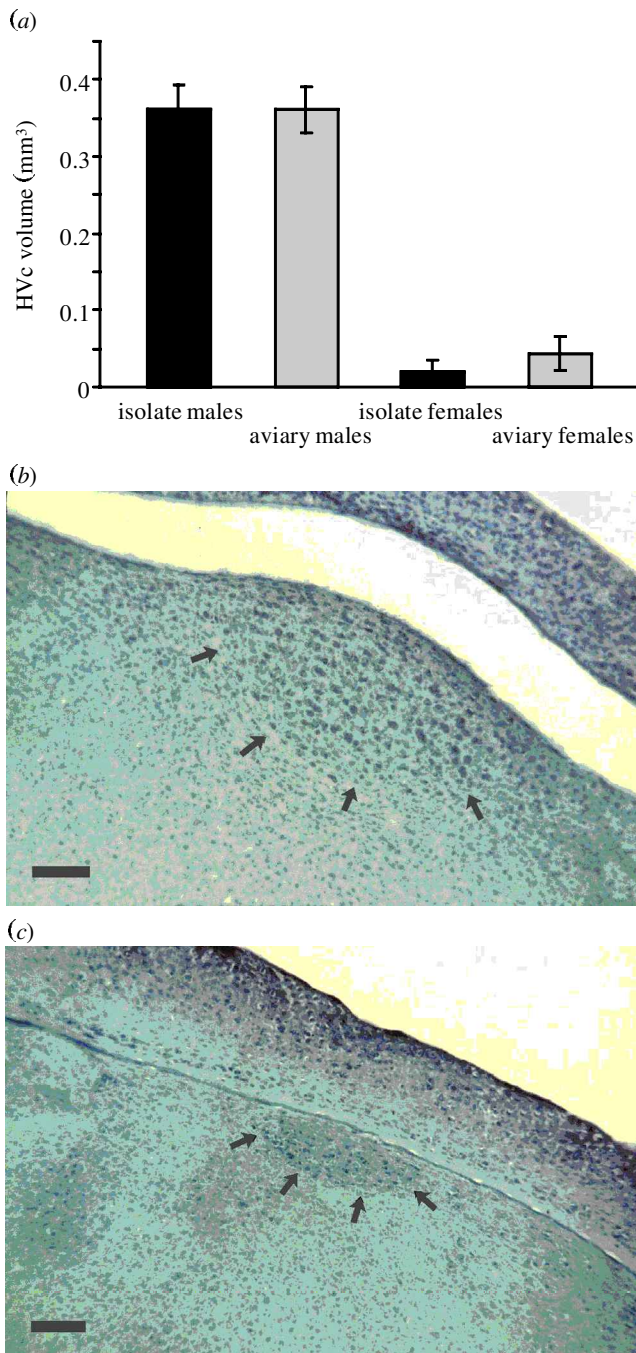


Figure 3. (a) HVC volume (mm^3) shows significant sexual dimorphism, but no differences between treatments. (b) HVC of a male and (c) HVC of a female. Arrows indicate the borders of HVC. Scale bar, 50 μm .

cedures were designed to replicate as closely as possible a natural situation in which songs would be regularly heard from four neighbouring territories.

(b) Song analysis

Songs were recorded and played using a Sony TC-D5PRO recorder fitted with a Sennheiser ME88 microphone. Recorded songs were analysed later on a PC using A_{VISOFT-SASLAB} PRO software. Sonographic analysis of song used standard procedures on a sample of 20 consecutive songs developed in earlier studies on sedge warbler song quality in relation to female choice (Catchpole 1980; Buchanan & Catchpole 1997). Syllables were identified, classified by type and stored as a library, on disc, for

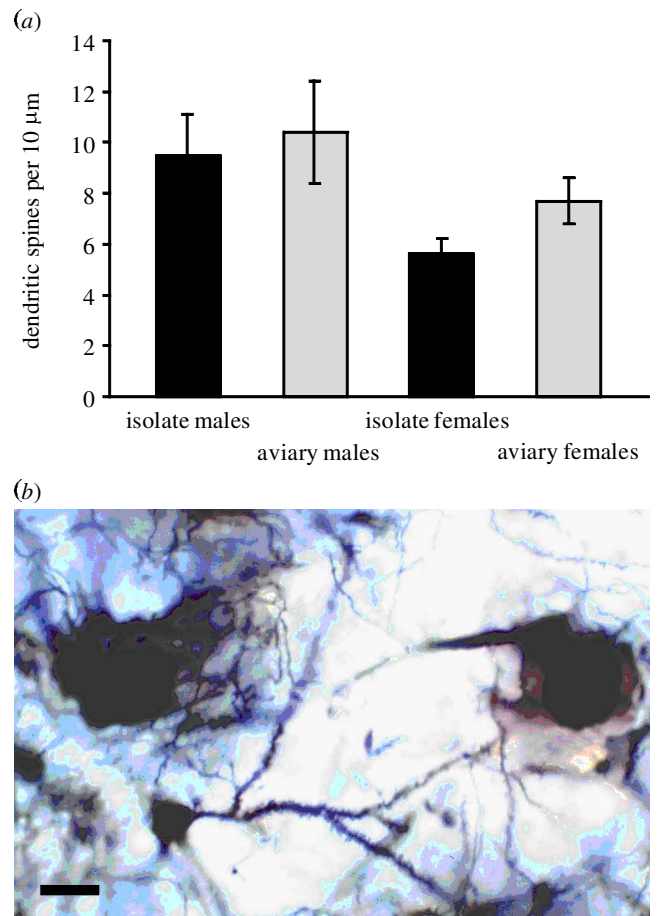


Figure 4. (a) Dendritic spine density within HVC, expressed as the number of spines per 10 μm dendrite, also shows a significant difference between sexes, but no difference between treatments. (b) Golgi-stained neurons with a spiny neuron in the centre. Scale bar, 15 μm .

each individual male. These were used to derive repertoire size (the number of different syllable types in the sample), within-song complexity (the mean number of different syllable types per song), the mean total number of syllables per song, mean song length in seconds and the mean number of syllables per second.

(c) Histology

In June 1998, the birds were killed by injecting a lethal dose of barbiturate. Birds were weighed and perfused with 0.8% saline, followed by 10% formalin solution. Fixed brains were dissected and their weight was recorded. The brains were hemisected and the hemispheres were postfixed in 10% formalin solution. One hemisphere was then moved to 10%, after 24 h to 30% sucrose in phosphate buffer until sectioning. Brains were sectioned sagittally on a freezing microtome at 40 μm . Alternate sections were mounted onto gelatin-coated slides. After drying overnight, slides were Nissl-stained with thionin. Slides were dehydrated, immersed in xylene and coverslipped. Brain regions HVC, RA, IMAN and telencephalon were measured on the sections using digitized video images obtained from a light microscope (Leitz Orthoplan) and an image analysis program (META MORPH, Visi-tron Systems, Germany). Area size was determined on the screen using the built-in cursor and measurement tools. Volumes were calculated by summing the area measurements, and

multiplying by two (sampling interval) and by 40 μm (section thickness).

The remaining hemispheres were stained according to a modified version of the Golgi protocol of Glaser & Van der Loos (1981). Briefly, brains were exposed to Golgi-Cox solution (5% potassium dichromate, 5% mercuric chloride and 5% potassium chromate) for at least six weeks, with a change of solution after one week. The brain halves were dehydrated, embedded in 12% Celloidon (i.e. Cedukol in ether/ethanol) and mounted on a wooden block. The embedded brains were sectioned at 90 μm on a sliding microtome. Sections were then exposed to ammonia to reduce the Golgi precipitate, followed by *p*-phenylenediamine, before they were counterstained with methylene blue/cresyl violet and dehydrated. Free-floating sections in xylene were mounted and coverslipped with Merckoglas.

Slides were investigated under bright-field illumination with a light microscope to delineate the borders of HVc. Under high magnification ($\times 1250$), neurons for determination of dendritic spine density were selected. We considered only spiny neurons (Nixdorf *et al.* 1989; Airey *et al.* 2000b) and measured up to three dendrites per neuron, by projecting them with a camera lucida. In addition, spine density was calculated at 20 μm intervals from the soma to the tip of the dendrites. Spine density generally varies over a dendritic tree with respect to such factors as distance to the soma, dendritic orientation and neuronal position within HVc (e.g. Benton *et al.* 1998). To standardize the measurements, we determined the peak spine density within each dendrite and calculated means for each animal. All analyses were done blind to the type of condition.

(d) Statistics

To control for individual variation in brain and body characteristics, we measured HVc, RA and IMAN size relative to telencephalon size for the calculations, but show absolute HVc size in the figures. Statistical analysis was performed using one-way ANOVA for overall comparisons between treatments and sexes, and two-way ANOVAs followed by post-hoc tests (www.graphpad.com) for comparisons within treatments and sexes. All statistical tests are two-tailed and values shown are mean \pm s.d.

3. RESULTS

(a) The songs

The songs of the isolate males turned out to be surprisingly normal in terms of their detailed structure (figure 1*a,b*). There were no significant group differences in our measurements of number of syllable types per song, number of syllables per song, song length or song rate. The only significant difference to emerge was with repertoire size. As shown in figure 1*c*, in each case the isolate male had a larger syllable repertoire than his song-exposed sibling. The repertoire size for the isolate males was 95 ± 13 (range 78–114) compared with 69 ± 10 (range 59–85) for the song-exposed males, and the overall difference between the two groups was significant ($t = 4.631$, $p < 0.02$). The song-exposed males had an average repertoire size of 69, remarkably close to a control sample of wild first-year males with an average repertoire size of 70. The isolate males' larger repertoires were clearly not obtainable by imitation and can only have arisen by either inheritance or improvisation. The former seems unlikely as siblings shared only between 0 and 13% of syllable types. Instead,

each bird appeared to develop its own large and distinct repertoire, presumably by improvisation.

(b) The brain and song nuclei

There were no differences in body weight between either the treatments or the sexes, suggesting that the two treatments caused no physiological or morphological differences to develop during the experiment. Nor was there a significant difference in brain weight between the two treatments. Males had heavier brains than females ($F_{1,14} = 8.8$, $p < 0.02$), although these differences just failed to attain significance when comparing males and females within the two treatment groups ($F_{3,12} = 3.8$, $p < 0.06$).

Males have a larger telencephalon than females, whether rearing treatments are collapsed ($F_{1,14} = 18.6$, $p < 0.001$) or not ($F_{3,12} = 11.3$, $p < 0.01$). There are no significant differences in telencephalon volume between the two treatments. This pattern of results can be clearly seen in figure 2.

HVc volume is much greater in males than in females (figure 3) either when treatments are collapsed ($F_{1,14} = 410.2$, $p < 0.001$) or not ($F_{3,12} = 136.6$, $p < 0.001$). There are no significant differences in the volume of HVc when comparing the two treatments within either sex. RA and IMAN are also highly dimorphic, but their borders are too indistinct to detect and measure in females. There are no significant differences between the two male treatment groups in the volume of RA (isolate: $0.12 \pm 0.04 \text{ mm}^3$, aviary: $0.12 \pm 0.02 \text{ mm}^3$, $t = 0.13$, $p > 0.05$) or in the volume of IMAN (isolate: $0.05 \pm 0.01 \text{ mm}^3$, aviary: $0.06 \pm 0.01 \text{ mm}^3$, $t = 1.30$, $p > 0.05$).

Finally, we looked for any cellular differences in dendritic spine density within HVc using the Golgi-stained sections (figure 4). We found an overall significant difference between the sexes in spine densities ($F_{1,14} = 11.4$, $p < 0.01$). This did not remain when the sexes were compared within each treatment. As with the volume measurements, there were also no significant differences in spine densities between the two treatment groups ($F_{1,14} = 1.4$, $p > 0.05$).

In summary, although we have found consistent differences in neuroanatomy between the sexes, we could detect no differences at all between birds reared in acoustic isolation, and their siblings exposed to playback of recorded song. Such experience does not appear to influence either the size of HVc, RA and IMAN or the density of dendritic spines within HVc.

4. DISCUSSION

Perhaps the most striking finding from the experiment is that all the isolated males developed larger syllable repertoires than their song-exposed siblings. One possible interpretation is that artificial exposure to playback of recorded song produced males with abnormally small repertoires. However, this was not the case, as comparison with wild first-year males revealed an almost identical average repertoire size. This suggests that our isolate males inherit the potential for a much larger repertoire than they will eventually acquire if given the opportunity to learn from either tape or real tutors. Although at first this seems unusual, there are similar findings from other

studies of early song development. Marler & Peters (1982) found that young swamp sparrows *Melospiza georgiana* initially developed large repertoires, then lost syllables through a process they called selective attrition. Similar findings have been found in a range of North American emberizine songbirds (Nelson *et al.* 1996). However, very few studies have reared males in complete acoustic isolation, one exception being the study by Kroodsma *et al.* (1997) on the grey catbird *Dumetella carolinensis*. This study tutored some males with small or large repertoires, but also reared one in acoustic isolation. As in our study, the isolate bird produced quite normal songs. Catbirds produce enormous syllable repertoires, and the tutored males developed approximately 230–330 syllables. The isolate male improvised a larger repertoire of over 400 syllables. It seems that some of the most complex songs known may be produced initially by improvisation, and that selective attrition and later learning fine-tunes the final repertoire before crystallization. The parallel development of the underlying song control pathway in the brain holds the key to how this takes place.

In most songbird species, song is produced exclusively or primarily by the male and functions in both territorial defence and female attraction (Catchpole & Slater 1995). A pathway of discrete neural nuclei controls song production and learning, and a variety of studies have established considerable sexual dimorphism, with males having a much larger neural pathway (recent reviews by Brenowitz 1997; MacDougall-Shackleton & Ball 1999; Ball & MacDougall-Shackleton 2001; DeVoogd & Lauay 2001; but see Gahr *et al.* 1998). The majority of earlier studies have sampled males and females from captive or wild populations, but none have reared sibling pairs under conditions of isolation and exposure to song. In the sedge warbler, only the male sings and the size of the main nucleus HVC is seven times larger in males than in females. The nuclei RA and IMAN are smaller than HVC in males, and cannot be reliably measured in females. Our study has shown that the same pattern of sexual dimorphism develops under conditions of acoustic isolation and of exposure to playback of song. This suggests that sexual dimorphism develops independently of experience and has a strong genetic basis.

Our other main finding was that within each sex, the size of HVC, RA and IMAN did not differ between the isolates and those exposed to song. There are very few comparable experimental studies, except those on the American marsh wren *Cistothorus palustris* (Kroodsma & Canady 1985; Brenowitz *et al.* 1995). These used a slightly different technique of tutoring males with tapes of varying song complexity. Tutoring with either small or large repertoires of song types resulted in large differences in the number of song types produced by the birds as adults, but did not affect the volumes of HVC and RA. This also applied to the smaller HVC of female marsh wrens (Brenowitz *et al.* 1994).

Burek *et al.* (1991) looked for effects on song-system volumes of radically divergent auditory experience. They deafened juvenile male zebra finches (*Taeniopygia guttata castanotis*) and later found no differences in the volume of HVC between these birds and normal controls. Recently Airey *et al.* (2000c) reported on a study of heritability of the song control pathway in breeding experiments on the

zebra finch. They found that HVC has a moderate heritability (h^2) of 0.43. They also found that the brain weighed more and the telencephalon was larger in males than females. In our study of the sedge warbler, we find the same trend: males have a significantly larger telencephalon as well as HVC than females. Taken together, these findings may well have important implications for our understanding of how sexual selection affects the evolution of brain size and structure.

Very few studies have looked for the effects of rearing on neuronal morphology in song-system nuclei. Using hemispheres from the same marsh wrens described above, Airey *et al.* (2000b) found that males exposed to larger song repertoires had a greater density of dendritic spines in HVC. In the present study, using a similar technique, we find no differences in dendritic spine density between males reared in acoustic isolation and those exposed to song. Perhaps the critical difference between the results of the two studies is not in the amount of song exposure and opportunity for learning from a model, which are quite similar, but rather in what the two species do under these circumstances. The marsh wrens that were studied were exposed to 5 or 45 song types and ultimately produced repertoires that differed by 600%. Sedge warblers reared in isolation improvise so as to produce a repertoire just 30% greater than those exposed to song models. Thus, the similarity of neuronal structure in song production nucleus HVC in the two groups in the present study may reflect the similarity in the size of the song repertoires that they are able to produce.

In *Acrocephalus* warblers, we know that sexual selection and female choice drive the evolution of song complexity (Catchpole 2000). However, a central problem remains. How is song complexity maintained as an honest indicator of male quality? One possibility is that neural costs are incurred and that only the best males can afford to invest in a larger HVC. In a comparative study of *Acrocephalus* species (Szekely *et al.* 1996), it was shown that song complexity and the size of HVC increased during evolution. In a field study (Airey *et al.* 2000a), we were also able to show that male sedge warblers who paired successfully had larger repertoires than males that did not. Moreover, males with a larger repertoire also had a larger HVC. Our present experiments on the sedge warbler suggest that HVC volume and its consequent capacity to store song may well have a strong genetic component which would enable sexual selection to work.

The increase in size of HVC could also have led to the increase in size of the telencephalon. Airey *et al.* (1996) have shown that larger telencephalon size covaries with the size of the song control pathways. These findings suggest that the integration of greater song complexity into the developing brain may have hitherto unsuspected costs which go far beyond the simple allocation of neural space to HVC. Identifying and quantifying such costs must be the next step before we can understand how sexual selection may shape the evolution of the songbird brain.

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