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SUMMARY

Determining relations between brain structure and function is a principal focus of evolutionary neurobiology. Here we investigate covariation between singing behaviour and the neuroanatomy in eight species of sylviid warblers from the closely related *Acrocephalus* and *Locustella* genera. We found a significant positive correlation between repertoire size and the volume of the higher vocal centre after controlling for variation in brain size and phylogenetic relatedness across species. This group is of particular interest, as earlier work has shown that an increase in male song complexity (as measured by syllable repertoire size) is caused by sexual selection pressure acting through female choice. Thus, in males of *Acrocephalus* species (which have complex songs), sexual selection appears to have led to increases in both syllable repertoire size and the relative volume of the higher vocal centre. In contrast, *Locustella* species have very simple songs, and repertoire size and the relative volume of the higher vocal centre remain small in males of these species. These results indicate that sexual selection may have shaped the evolution of a particular behavioural trait (song) by altering the relevant controlling area of the brain (higher vocal centre).

1. INTRODUCTION

The brain pathway which controls avian song production and learning has been the subject of intensive study by neurobiologists in recent years (reviewed by Arnold 1992; Nottebohm 1993; Ball *et al.* 1994; DeVoogd 1994; Catchpole & Slater 1995). The song system is complex and consists of many interlinked nuclei whose precise functions are just beginning to be unravelled. The more caudal nuclei such as the higher vocal centre (HVC) and robustus archistriatalis (RA) are essential for song production, whereas the more rostral nuclei such as Area X and l-MAN are involved in early song learning and do not appear to be directly involved in adult production. There is considerable variation in the volumes of these structures relative to total telencephalon volume, both within and between species. Several studies have related this variation in anatomy to variation in behaviour. For example, Nottebohm *et al.* (1981) found that HVC and RA were larger in male canaries *Serinus canaria* with larger

syllable repertoires than in males with smaller repertoires. Across several wren species, the volumes of HVC and RA in females are larger in those species that sing elaborate songs than in those that do not (Brenowitz & Arnold 1986).

Recent advances in comparative analysis (Felsenstein 1985; Harvey & Pagel 1991) make it possible to compare associations between neuroanatomy and behaviour across species in a rigorous, unbiased fashion. One prior study has applied these techniques to the song system. Using the method of phylogenetically independent contrasts, DeVoogd *et al.* (1993) compared song repertoire size and neuroanatomy across more than 40 species of songbird from eight families. They found a significant positive correlation between repertoire size and the relative volume of HVC. However, it is difficult to obtain equivalent measures of repertoire size or song complexity across such widely disparate groups. It is also possible that the nature of the functions carried out by particular nuclei within the song system may have shifted over the very long time since all the species in such a study had common ancestors, thereby making it difficult to interpret variation in song system

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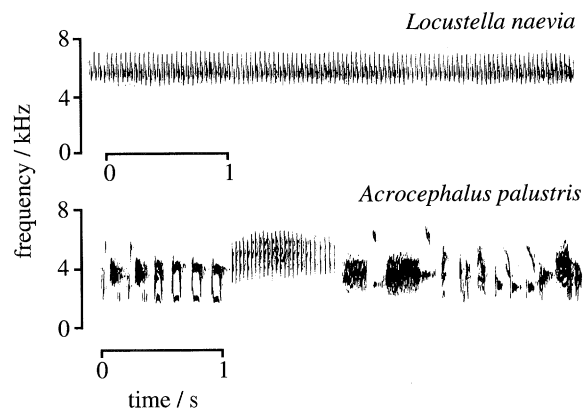
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607

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Figure 1. Sonagrams illustrating the basic differences in song structure between the genera *Locustella* and *Acrocephalus*. (*a*) The grasshopper warbler *Locustella naevia* has a remarkably simple song structure consisting of the repetition of a single syllable type. (*b*) The marsh warbler *Acrocephalus palustris* has a much more complex song structure and individual males can have up to a hundred different syllable types in their repertoire.

anatomy. These sources of ambiguity would be greatly reduced by using consistent, standard measures of song in a comparative study of closely related species which also share a more recent common ancestor.

In this study, we examine the relations between song complexity and song system anatomy within eight closely related species of European warblers (family *Sylviidae*) from the genera *Acrocephalus* and *Locustella*, common inhabitants of marshland. Songs of species in both genera are composed of a series of stereotyped sound units (syllables) produced in bouts that are readily quantifiable.

Acrocephalus warblers are renowned for the complexity of their songs (figure 1). Different *Acrocephalus* species also differ considerably in syllable repertoire size, ranging from about 25 to more than 90 syllables (table 1) and are thus an ideal group in which to investigate possible relations between song complexity and brain structure. Furthermore, field studies and laboratory experiments have shown that the evolution of extreme song complexity by *Acrocephalus* species has

been driven by sexual selection and female choice (Catchpole 1980; Catchpole *et al.* 1984; Catchpole 1987).

In contrast, *Locustella* warblers have some of the simplest songs of all songbirds, consisting of the repetition of a single or double pulse reminiscent of insect stridulation (figure 1). The extreme differences in song organization between these two related genera provide additional opportunities for investigating the evolution of brain–behaviour relations.

2. METHODS

Song complexity was estimated using the number of different syllable types a male produces (syllable repertoire size) in the eight species of warblers (table 1). Repertoire size ranges are based upon published data (Dowsett-Lemaire 1979; Catchpole 1980, 1987; Catchpole *et al.* 1984; Cramp 1992; Hasselquist 1994) augmented by sonagraphic analyses of *A. schoenobaenus*, *L. naevia* and *S. nisoria* (X. Buchanan & C. K. Catchpole, unpublished data). Log-transformed midpoints of repertoire ranges are used in statistical analyses.

A total of two adult males of each species were caught under license in Hungary. Males were caught in May, in 1993, 1994 or 1995. This brief time window ensured that the birds and their song system nuclei were in reproductive condition (Nottebohm *et al.* 1986; Kirn *et al.* 1989). They were anaesthetised and perfused transcardially with 0.8% saline followed by 10% formalin in saline. The brains were removed, embedded, frozen, sectioned in the traverse plane (40 μm) and stained with cresyl violet. Profiles of HVC, RA, Area X and l-MAN were traced using a camera lucida. The margins of the telencephalon were traced in alternated sections from digitized images. The areas of the song nuclei and the telencephalon profiles were determined using NIH Image (Wayne Rasband, NIH), and volumes of these regions were computed. All volumes were log-transformed before statistical analyses.

In the first phase of analysis, phylogenetically independent differences ('contrasts', Felsenstein 1985) were derived for repertoire size, and for volumes of song system nuclei and of telencephalon by using the CAIC statistical package (Purvis & Rambaut 1994). We used the phylogeny shown in figure 2, where the numbers refer to nodes used in the comparison. This phylogeny is based upon current taxonomy (Glutz von Blotzheim 1991) and supported by a recent molecular phylogeny of B. Leisler (unpublished data). *Sylvia nisoria* was

Table 1. *Sizes of repertoires and brain areas in eight species of European warblers*

(Mean volumes of nuclei and telencephalon are given in cubic millimetres. Nucleus volume is the mean of left plus right. Repertoire size ranges are based on published data (Dowsett-Lemaire 1979; Catchpole 1980; Catchpole *et al.* 1984; Catchpole 1987; Cramp 1992; Hasselquist 1994) augmented by sonagraphic analyses of *A. schoenobaenus*, *L. naevia* and *S. nisoria* (K. L. Buchanan & C. K. Catchpole, unpublished data).)

| species	repertoire range	HVC	Area X	l-MAN	RA	Telencephalon
Acrocephalus palustris	80–100	0.840	0.746	0.042	0.150	205.8
Acrocephalus scirpaceus	70–90	0.754	0.539	0.048	0.142	204.8
Acrocephalus melanopogon	60–80	0.420	0.522	0.031	0.063	220.0
Acrocephalus schoenobaenus	35–75	0.343	0.306	0.033	0.082	170.4
Acrocephalus arundinaceus	25–45	0.808	1.008	0.076	0.179	412.9
Locustella fluviatilis	2–4	0.351	0.644	0.062	0.126	242.4
Locustella naevia	1–2	0.082	0.243	0.041	0.078	216.4
Locustella luscinioides	1–2	0.305	1.019	0.125	0.164	258.5
Sylvia nisoria	71–77	0.783	0.710	0.087	0.207	387.0
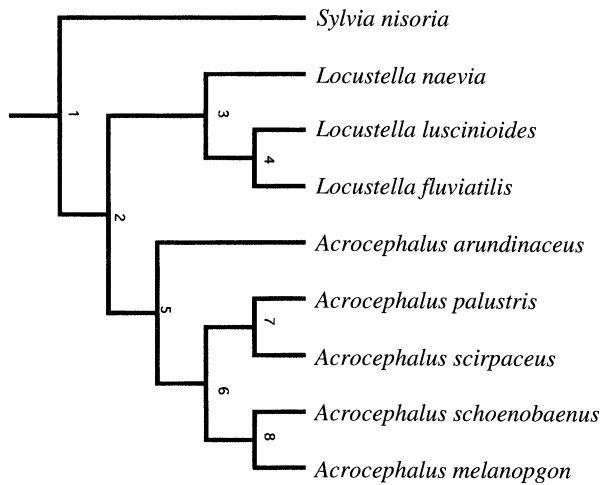
Figure 2. Reconstructed phylogeny of *Acrocephalus* and *Locustella* warblers based on current taxonomy (Glutz von Blotzheim 1991) which is supported by a recent molecular phylogeny (B. Leisler, unpublished data). The numbers refer to the eight nodes used in the analysis.
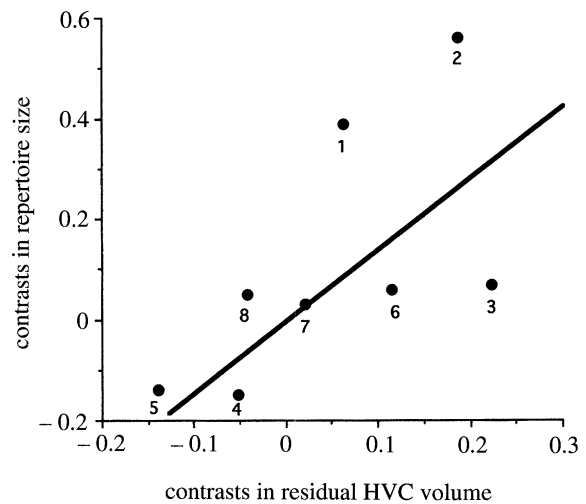

Figure 3. Correlation between syllable repertoire size and residual volume of HVC ($r = 0.685$, $p < 0.05$, $n = 8$). The phylogenetically independent contrasts were based upon the phylogeny in figure 2 and the numbers refer to the nodes used in the analysis.

selected as an outgroup, as *Sylvia* warblers belong to the same family as *Acrocephalus* and *Locustella* warblers (Monroe & Sibley 1993). Branch lengths were set to unity because estimates of the amount of divergence between species and groups are not yet available.

In the second phase of the analysis we controlled for the influence of brain size by taking residuals from the contrasts in volumes of song system nuclei (dependent variable) and the contrasts in telencephalon volume. Telencephalon volumes did not include the volume of song system nuclei being assessed. These residuals (deviations in contrasts in volumes of song system nuclei) were therefore independent of telencephalon size. Finally, we investigated the linear association between contrasts in repertoire size (dependent variable) and residual contrasts in volumes of song system nuclei. In these analyses all regressions were forced through the origin (Harvey & Pagel 1991; Garland *et al.* 1992). Pearson correlation coefficients and two-tailed probabilities are given. In univariate analyses $n = 8$ (from the eight possible contrasts in this phylogeny), and for the multiple regression analysis $F(df_{regression}, df_{residual})$ is given.

3. RESULTS

We found that syllable repertoire size was positively correlated with the residual volume of HVC ($r = 0.685$, $p < 0.05$, figure 3). However, repertoire size was unrelated to the residual volumes of RA ($r = 0.199$, $p > 0.6$), Area X ($r = 0.112$, $p > 0.7$) and l-MAN ($r = -0.228$, $p > 0.5$). Because the residual volume of HVC was also related to the residual volumes of Area X ($r = 0.673$, $p < 0.05$) and RA ($r = 0.644$, $p = 0.06$), we also investigated the association between these three nuclei and repertoire size.

The positive relation between repertoire size and residual volume of HVC was confirmed by a multiple regression analysis in which repertoire size was the dependent variable and the residual volumes of HVC, Area X and RA were the independent variables ($r^2 = 0.749$, $F_{(3,5)} = 4.959$, $p = 0.06$). In this analysis, repertoire size remained positively related to residual volume of HVC (slope = 2.590, $p < 0.02$) and it

remained unrelated to the residual volumes of Area X (slope = -1.665, $p > 0.1$) and RA (slope = -1.178, $p > 0.3$).

4. DISCUSSION

This study supports the earlier findings of the broader comparative study of DeVoogd *et al.* (1993), that the relative volume of HVC is correlated with repertoire size. Such confirmation is important, as their wide-ranging study suffered from a number of comparability problems. First, their estimates of song repertoire size were taken from the literature, and as pointed out by Kroodsma (1982), researchers frequently differ in their definitions of song and their methodology in measuring it. Furthermore, many species do not have a repertoire of stereotyped song types, but construct their much longer and more complex songs from variable combinations of smaller units called syllables (Catchpole & Slater 1995). DeVoogd *et al.* (1993) suggested that syllable repertoire size may be a more appropriate measure of learning or song complexity, but these measures were often not available. However, in the present study we were able to use syllable repertoire as the standard unit of comparison. Second, in a diverse comparative study, the functions of particular brain nuclei may have diverged in parts of the phylogeny, limiting meaningful comparisons across the entire phylogeny.

As *Acrocephalus* and *Locustella* warblers were not included in the earlier study by DeVoogd *et al.* (1993), we now have two independent comparative studies which suggest that there is a positive correlation between the relative volume of HVC and song complexity, a correlation not shared by other principal song system nuclei. This general finding increases the strength of brain–behaviour comparisons based on a pair of species or populations. For example, Canady *et al.* (1984) found that marsh wrens *Cistothorus palustris*

from New York had smaller repertoires than those from California, and this was reflected in the relative sizes of HVC and RA. Kroodsma & Canady (1985) tutored experimental hand-raised males from both populations with recorded songs, and demonstrated that the New York birds ability to learn fewer songs was genetically constrained by their more limited brain space.

In the present study the positive correlation between repertoire size and HVC is particularly interesting from an evolutionary perspective. Previous studies have shown that the evolution of increasing repertoire size in *Acrocephalus* warblers is driven by sexual selection and female choice (Catchpole 1987). For example, in the sedge warbler *A. schoenobaenus* males with larger repertoires paired first in the wild (Catchpole 1980), and females in the laboratory displayed more to larger repertoires (Catchpole *et al.* 1984). In the great reed warbler *A. arundinaceus*, males with larger repertoires obtained more females and also produced more offspring (Catchpole 1986; Hasselquist 1994). Taken together, these results suggest that sexual selection pressure may have driven the evolution of song complexity by shaping the evolution of song system nuclei within the brain.

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