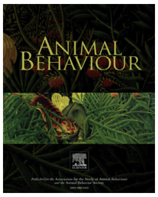
Animal Behaviour 107 (2015) 221–231

Contents lists available at ScienceDirect

Animal Behaviour

journal homepage: www.elsevier.com/locate/anbehav



Song divergence between subspecies of reed bunting is more pronounced in singing styles under sexual selection

Luís de Oliveira Gordinho [a], Eloisa Matheu [b], Dennis Hasselquist [c], Júlio Manuel Neto [a, c, *]

[a] CIBIO/UP—Centro de Investigação em Biodiversidade e Recursos Genéticos, Universidade do Porto, Campus Agrário de Vairão, Vairão, Portugal
[b] ALOSA—Sonidos de la Naturaleza, Barcelona, Spain
[c] Molecular Ecology & Evolution Lab, Department of Biology, University of Lund, Lund, Sweden

ARTICLE INFO

Article history:
Received 20 April 2015
Initial acceptance 19 May 2015
Final acceptance 10 June 2015
Published online
MS. number: 15-00324R

Keywords:
birdsong
geographical variation
sexual selection
singing modes
song categories
speciation

Song divergence between populations of a species can lead to reproductive isolation and speciation. However, birds may have different singing styles used in distinct social contexts, and songs of each style may change at different rates over time and space. Here, we tested whether song divergence between subspecies of reed bunting, *Emberiza schoeniclus*, differs with singing style, by comparing song traits of its three singing styles among three subspecies breeding in northern and western Europe. We show that the two singing styles under sexual selection (dawn and fast songs, related to obtaining extrapair and social mates, respectively) diverged significantly more than the slow songs (used as an all-clear signal to nest-attending social females). Multiple song traits differed significantly between the subspecies in all singing styles, with *E. s. lusitanica* generally being intermediate between *E. s. schoeniclus* and *E. s. witherbyi*, and the pattern of song complexity opposing the expected latitudinal gradient (of increasing complexity with increasing latitude). Cluster analyses of populations indicate that sexually selected singing styles are better for discriminating subspecies, describing a scenario of a major split in song features between the migratory, northern *E. s. schoeniclus* and the two resident, southern subspecies, rather than a clinal variation. The greater song divergence in fast and dawn singing styles suggests that sexual selection may be playing an important role in the incipient speciation of reed buntings.

Song is an important premating isolation barrier between passerine species (Catchpole & Slater, 2008; Marler & Slabbekoorn, 2004), and song divergence between populations of a species can lead to reproductive isolation and speciation (Martens, 1996; Price, 2008). Song divergence often follows morphological divergence (e.g. in bill or body size) resulting from a shift in ecology (e.g. in diet or feeding actions; Christensen, Kleindorfer, & Robertson, 2006; Grenier & Greenberg, 2005; Huber & Podos, 2006; Ratcliffe & Grant, 1985), or adaptation to the local acoustic environment (Cardoso & Price, 2010; Kirschel et al., 2009). However, it could also result from random cultural evolution and/or drift, especially in small, isolated populations (Lynch, 1996; Williams, Levin, Norris, Newman, & Wheelwright, 2013; Xing, Alström, Yang, & Lei, 2013).

In many birds and some marine mammals, there can be differences in the patterns of within-species spatial variation from one type of vocalization to another (Baker, 2011). Among passerines, some species have several singing styles: functionally nonequivalent song types used in specific contexts (Bradbury & Vehrencamp, 1998; Brunner & Pasinelli, 2010; Ewin, 1976; Hasselquist & Bensch, 1991; Nemeth, 1996). The songs used in each singing style may show distinct geographical patterns (Byers, 1996; Kroodsma, 1981) and change over time at different rates (Byers, Belinsky, & Bentley, 2010), suggesting that divergence between populations may be more pronounced in certain singing styles. Thus, as some social contexts are more relevant to reproductive isolation, certain singing styles could be of greater importance for speciation.

The reed bunting, *Emberiza schoeniclus*, is a Palearctic passerine with several subspecies described on the basis of morphology (Byers, Olsson, & Curson, 1995; Cramp & Perrins, 1994). It has three singing styles, two of which are used for female attraction: fast song, the only style used by unpaired males, has been related to the attraction of a social mate (Ewin, 1976; Nemeth, 1996), and dawn song, which is sung by paired males and is associated with obtaining extrapair paternity (Suter, Ermacora, Rielle, & Meyer, 2009). In contrast, slow songs are used by paired males apparently as an all-clear signal to their nest-attending females (Wingelmaier, Winkler, & Nemeth, 2007) and do not influence extrapair paternity or the number of fertilizations (Bouwman, Dijk, Wijmenga, & Komdeur, 2007). In

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* Correspondence: J. M. Neto, Molecular Ecology & Evolution Lab, Department of Biology, University of Lund, Ecology Building, Sölvegatan 37, SE-22362 Lund, Sweden.
E-mail address: julio.m.neto@gmail.com (J. M. Neto).

http://dx.doi.org/10.1016/j.anbehav.2015.06.024
0003-3472/

addition, all three singing styles are probably important in the context of territory defence (Brunner & Pasinelli, 2010; Ghiot, 1976). Individual males have repertoires of 10–30 different syllables that are used to build the songs of the three singing styles (Cramp & Perrins, 1994; Ewin, 1978; Suter et al., 2009). Dawn and fast singing are stereotyped styles because of the stricter rules of syllable and song arrangement (Brunner & Pasinelli, 2010; Suter et al., 2009). Concomitantly, compared to the slow singing style, the fast style has greater syllable sequence predictability and greater syllable repetition (Ewin, 1978). Song sharing, matched countersinging and local dialects have been recorded at the syllable level, but not at the song level (Ehrengruber, Pasinelli, & Egli, 2006; Ewin, 1976, 1978).

Previous work has shown that the fast songs of nonmigratory, thick-billed central and western Mediterranean birds (*E. s. intermedia* and *E. s. witherbyi*) differ from those of the largely migratory, thin-billed central European *E. s. schoeniclus* (Matessi, Dabelsteen et al., 2000, 2001). However, the divergence in other singing styles and differential divergence across styles have never been studied. The differences in fast song seem to be recognized by the birds, as males react differently to the playback of their own and foreign fast songs (Gordinho, Hasselquist, & Neto, n.d.; Matessi, Pilastro et al., 2000, 2001).

In this study, we analysed quantitative properties of songs of the three singing styles in three subspecies of reed bunting breeding in northern and western Europe: *E. s. schoeniclus* (hereafter *schoeniclus*), widespread from France and the U.K. north and eastwards to beyond the Western Palearctic; *E. s. lusitanica* (hereafter *lusitanica*), endemic to northwestern Iberia, for which there was no previous quantitative study of song (for qualitative notes see Martínez & Romay, 2012); and *E. s. witherbyi* (hereafter *witherbyi*), from eastern Iberia, Balearics, southern France and, at least formerly, North Africa and Sardinia (Atienza, 2006; Cramp & Perrins, 1994; Fig. 1). These three subspecies probably constitute the closest link between thin- and thick-billed groups of subspecies, as *lusitanica* is intermediate in bill traits and bill thickness increases towards the east among the thick-billed subspecies; it is thus particularly interesting to study speciation in this system (Neto et al., 2013).

Our aims were (1) to compare quantitative properties of songs from each of the three singing styles between the three subspecies, (2) to quantify the extent to which males can be assigned to the correct subspecies on the basis of song properties in each singing style and (3) to evaluate whether the geographical pattern of song differentiation conforms to the subspecies distributions. Considering that (1) vocal signals with different functions can exhibit different geographical patterns (Baker, 2011), (2) singing styles used for female attraction can act as a behavioural isolating mechanism (Kroodsma, 1981) and (3) stronger sexual selection results in faster evolution/divergence (Kraaijeveld, Kraaijeveld-Smit, & Maan, 2011; Seddon et al., 2013), we hypothesized that fast and dawn singing styles (those used for female attraction) diverged faster and more extensively between reed bunting subspecies than the slow style.

METHODS

Ethical Note

Field procedures used to obtain the sound recordings in which this study was based comply with the current laws of the countries where they were obtained (Portugal, Spain, France, Switzerland and Sweden).

Fieldwork

Most of the recordings were obtained using a 570 mm Telinga parabola with a Twin Science microphone and a Marantz PMD660 solid-state recorder (settings: 48 kHz sampling frequency, WAV 16-bit format) by L.G. (Fig. 1, Table 1). Additional recordings were obtained by E.M. using a similar Telinga parabola with a Stereo mic and a DA-P1 Tascam recorder (in 2004 and earlier), by the ‘Sound Approach to Birding’ team, and by Jean Roché (Fig. 1, Table 1). Further recordings were obtained at three Swiss lakes in 2006 by Patrick Brunner (part of which were published in Brunner & Pasinelli, 2010) and in 2009–2010 by Gilberto Pasinelli (Fig. 1, Table 1).

Given the rate of cultural evolution in birdsong (Byers et al. 2010), large temporal gaps in data may be a potential source of bias. However, in our data set, the overall distribution of recording years (Mann–Whitney U test: $P > 0.05$) and the median year of recording (median test: $P > 0.05$) did not differ between subspecies.

Sound Processing

We screened an initial pool of 239 recordings (116 *witherbyi*, 76 *lusitanica*, 47 *schoeniclus*) and visually attributed each of them to one of the three singing styles (114 fast, 80 slow, 45 dawn). Dawn song is highly distinctive because it is sung ‘continuously’, with intervals between songs of similar magnitude to intervals between syllables within songs (Brunner & Pasinelli, 2010; Suter et al., 2009). Fast and slow songs were distinguished based on the length of the intervals between the first two syllables (>0.3 s suggesting slow song) and the length of the interval between songs (if shorter than the length of songs, being suggestive of slow song; Nemeth, 1996; Brunner & Pasinelli, 2010). We only analysed songs from individual males that could be unambiguously identified based on the location of song posts and, especially, on the introductory syllables used by each bird, which are individual specific (Nemeth, 1996). We therefore excluded recordings that could possibly be of the same individuals, resulting in a sample size of 143 different males (Table 1). From each male, we analysed songs of just one singing style, digitized 100 consecutive syllables and classified them into syllable types, using syllable type catalogues for each area created for this work (following Suter et al., 2009). For dawn songs, the frequency of occurrence of syllables and short pauses between song bouts were used to identify introductory syllables (Brunner & Pasinelli, 2010). Those introductory syllables were then used to define songs within the continuous song bouts. We digitized up to 20 songs per individual male (following Brunner & Pasinelli, 2010), but in some cases this was not possible due to recording length. Overall, on average 18 ± 4 SD (range 6–20) songs per male were used.

Songs were screened and digitized in Raven Pro 1.3 (www.birds.cornell.edu/brp/raven) using default spectrogram settings, resulting in a time resolution of 2.67 ms and a frequency resolution of 187.5 Hz. In each song, seven traits were measured from the spectrogram (following Suter et al., 2009): maximum frequency (MaxF); minimum frequency (MinF); song length (SL), the duration of each song; first interval (FI), the duration of the interval between the first two syllables; song interval (SI), the interval between two consecutive songs; number of syllables (NS); and number of different syllables (NDS). Although extracting frequency measures from spectrograms can be problematic (Zollinger, Podos, Nemeth, Goller, & Brumm, 2012), it nevertheless produces good results (Cardoso & Atwell, 2012) and, importantly, does not introduce biases to the subspecies and style comparisons.

Statistical Analysis

The quantitative properties of song were compared between the three subspecies with general linear mixed models (GLMMs), in which the male identity was included as a random (subject) effect and subspecies and singing style were fixed factors (Grafen & Hails, 2002). The song variables were approximately normally distributed