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Sharp acoustic boundaries across an altitudinal avian hybrid zone despite asymmetric introgression

W. HALFWERK*†, C. DINGLE‡§, D. M. BRINKHUIZEN*¶, J. W. POELSTRA*¶, J. KOMDEUR** & H. SLABBEKOORN*

*Behavioural Biology, Institute of Biology (IBL), Leiden University, Leiden, the Netherlands
†Animal Ecology, Department of Ecological Science, VU University Amsterdam, Amsterdam, the Netherlands
‡Behavioural Ecology Group, Zoology Department, Cambridge University, Cambridge, UK
§Department of Earth Sciences, The University of Hong Kong, Hong Kong, Hong Kong
¶Animal Ecology Group, Centre for Ecological and Evolutionary Studies, Groningen University, Haren, the Netherlands
**Behavioural and Physiological Ecology Group, Groningen Institute for Evolutionary Life Sciences, Groningen University, Groningen, the Netherlands

Abstract

Birdsong is a sexually selected trait that could play an important evolutionary role when related taxa come into secondary contact. Many songbird species, however, learn their songs through copying one or more tutors, which complicates the evolutionary outcome of such contact. Two subspecies of a presumed vocal learner, the grey-breasted wood-wren (*Henicorhina leucophrys*), replace each other altitudinally across the western slope of the Ecuadorian Andes. These subspecies are morphologically very similar, but show striking differences in their song. We examined variation in acoustic traits and genetic composition across the altitudinal range covered by both subspecies and between two allopatric populations. The acoustic boundary between the subspecies was found to be highly abrupt across a narrow elevational range with virtually no evidence of song convergence. Mixed singing and use of hetero-subspecific song occurred in the contact zone and was biased towards the use of *leucophrys* song types. Hetero-subspecific song copying by *hilaris* and not by *leucophrys* reflected a previously found asymmetric pattern of response to song playback. Using amplified fragment length polymorphisms (AFLP) markers, we detected hybridization in the contact zone and asymmetric introgression in parapatric populations, with more *leucophrys* alleles present in *hilaris* populations than vice versa. This pattern may be a trail of introgression due to upslope displacement of *leucophrys* by *hilaris*. Our data suggest that song learning may impact speciation and hybridization in contrasting ways at different spatial scales: although learning may speed up population divergence in songs, thereby enhancing assortative mating and reducing gene flow, it may at a local level also lead to the copying of heterospecific songs, therefore allowing some level of hybridization and introgression.

Introduction

Acoustic signals are important sexual traits which are often found to be divergent among closely related species or different populations of the same species (Miller, 1996; Irwin *et al.*, 2001). Such variation in sexual signals

Correspondence: Wouter Halfwerk, De Boelelaan 1085, 1081 HV Amsterdam, The Netherlands. Tel.: +31 20 5987492; fax: +31 20 5987123; e-mail: w.h.halfwerk@vu.nl

can play a critical role in evolutionary processes such as speciation and hybridization (Price, 2008; Hoskin & Higgie, 2010). Divergent signals could, for example, act as behavioural barriers to gene flow, preventing adjacent populations from merging (West-Eberhard, 1983; Endler, 1992). Bird song is a well-known sexual signal assumed to play an important role in speciation and hybridization (ten Cate, 2004; den Hartog & ten Cate, 2006; Verzijden *et al.*, 2012). Closely related taxa often show striking differences in song behaviour, and many

case studies have shown that individuals use these differences to discriminate against heterospecifics (e.g. Matessi *et al.*, 2001; Patten *et al.*, 2004; McEntee, 2014).

Many bird species learn to sing and/or recognize songs, which is thought to have important consequences for the rate of signal divergence, as well as for the evolution of behavioural barriers (Irwin & Price, 1999; Ellers & Slabbekoorn, 2003; Lachlan & Servedio, 2004). Typically, a young bird develops its song by listening to one or several local tutors and by copying song elements or whole song types into its own repertoire. Young birds, however, make occasional errors when copying songs from others, which results in the evolution of novel song types (Slabbekoorn & Smith, 2002). These novel song types may rapidly spread within a population and thereby speed up the rate of signal divergence between populations (Lachlan *et al.*, 2013). Some species may also imprint on the song(s) of their parents, which facilitates song discrimination and further enhances the evolution of a behavioural barrier between nearby populations (Irwin & Price, 1999; Verzijden & ten Cate, 2007).

Song learning could in contrast also lead to acoustic convergence, as individuals of two closely related taxa that come into contact can copy songs from each other (Grant & Grant, 1997; Qvarnstrom *et al.*, 2006). Such heterospecific song copying can result in individuals displaying the acoustic phenotypes of both taxa ('mixed singers') or individuals displaying an intermediate phenotype (Bensch *et al.*, 2002; Secondi *et al.*, 2003). Song learning in contact areas may thus lead to trait convergence and potentially aid hybridization (Grant & Grant, 1997; Qvarnstrom *et al.*, 2006; Tobias & Seddon, 2009).

The complex role of song learning in speciation and hybridization becomes clear when comparing studies on different songbird hybrid zones. For example, two species of *Hippolais* warblers come into contact in Western Europe and show a pattern of song convergence that is concurrent with the width of the hybrid zone (Secondi *et al.*, 2003). Two subspecies of song sparrow (*Melospiza melodia*) on the other hand show clear acoustic differences that remain distinct throughout most of the hybrid zone (Patten *et al.*, 2004). Furthermore, a study on two closely related *Phylloscopus* warblers showed that songs remain distinct in sympatry. However, genotypes and phenotypes did not always match in sympatry, as individuals displayed songs from their own or from the other species and these individuals had either a 'pure' parental or hybrid genotype (Bensch *et al.*, 2002). Clearly, these field studies demonstrate that the role of learning in song divergence and species isolation is far from understood. Importantly, we need more data on the link between genotype and phenotype from individuals in contact zones, preferably at the detailed microgeographical scale of individual territories.

Two subspecies of the grey-breasted wood-wren (*Henicorhina leucophrys*) provide a good opportunity to study the role of song variation and the importance of song learning in contact zones. One subspecies, *H. l. hilaris* (hereafter referred to as *hilaris*), replaces *H. l. leucophrys* (hereafter referred to as *leucophrys*) at lower elevations along an environmental gradient on the western slope of the Ecuadorian Andes. The subspecies are genetically distinct (6.9–8.2% mtDNA divergence), morphologically similar and show a striking difference in acoustic structure of their songs (Dingle *et al.*, 2006, 2008). Both males and females can sing multiple song types that are presumably learned from their parents or territorial neighbours (Kroodsma & Brewer, 2005; Dingle *et al.*, 2008). Playback experiments have shown that *hilaris* males respond strongly to song playbacks of both their own and the other subspecies, whereas *leucophrys* males only respond strongly to songs of their own subspecies. This asymmetric response behaviour could result in an asymmetric pattern of introgression or territorial displacement (Dingle *et al.*, 2010). Alternatively, the ability to discriminate by one subspecies provides the basis for a behavioural barrier to gene flow (Dingle *et al.*, 2010). Our previous studies compared two parapatric populations separated by 10 km of continuous habitat, but did not include individuals from the exact zone of contact between the two subspecies. It is therefore still unclear whether songs remain distinct in contact and whether and to what extent the (asymmetric) song discrimination prevents hybridization among the two subspecies (Dingle *et al.*, 2010).

We studied the two subspecies of wood-wren in an area with continuous suitable habitat and located the altitude at which both subspecies co-occurred. We sampled random genomic markers and acoustic behaviour across most of the altitudinal range of the two subspecies to examine: (i) to what extent the subspecies hybridize; (ii) whether genetic introgression into adjacent populations occurs; (iii) whether acoustic differences remain distinct in contact; (iv) whether there are mixed singers in the contact zone and whether they sing discrete or intermediate song types; and (v) to what extent genotypes and phenotypes show assortative pairing.

Materials and methods

Sample sites and data collection

Previous work has suggested that *hilaris* and *leucophrys* come into contact in a narrow zone on the northwestern slope of the Ecuadorian Andes (Dingle *et al.*, 2006, 2008). In September 2006, the exact zone of contact, an area where both acoustic phenotypes occur, was located just above the Mindo valley in two areas with primary forest habitat (Fig. 1). We sampled territories at two separate sites in this contact zone (2 km apart, separated by a large river valley), four parapatric populations located 2 and 6 km away from the contact zone on
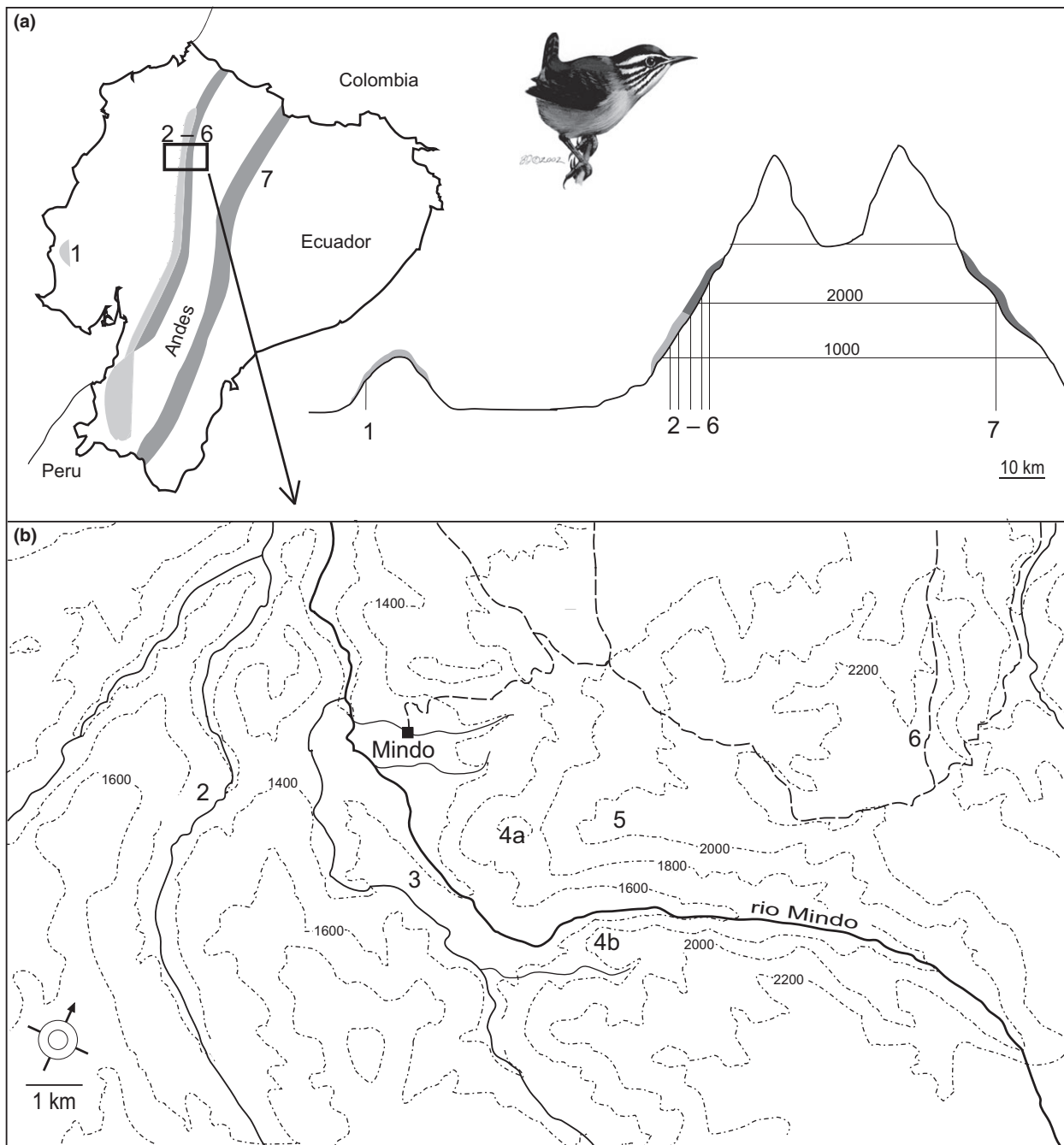
Fig. 1 Geographical distribution and location of sampling sites. (a) Overview map showing the distribution and altitudinal range of *H. l. hilaris*, indicated in light shading and *H. l. leucophrys*, in dark shading. Two allopatric populations (sites 1 and 7) and sampling sites across the area of contact (sites 2–6) are indicated. (b) The area of contact is characterized by continuous cloud forest. Subspecies co-occur at the top of the mountain ridge (site 4).

either side) and two distant allopatric populations (see Table S1 for further details of sampling sites). Samples were collected from September to December 2006 from a total of 19 territories in the contact zone, 18 territories in parapatry and 29 territories in allopatry.

Both males and females of the grey-breasted wood-wren have a repertoire of different song types that they either sing as solos or combine into duets (Dingle *et al.*, 2008; Hall, 2009). The sexes can be told apart on the basis of song structure, as female songs

typically increase in frequency towards the end of their song (Dingle, 2009). Male solos are the most commonly heard songs. Singing activity is low after the first hour of daylight and we therefore collected songs by playing back an individual male's own solo song every 10 min and recording the response. This was continued for one hour during which time an average of 5.6 ± 3.2 songs per male were recorded. All songs were recorded with a Sennheiser ME67 directional microphone in combination with a digital Sharp MD-MT190H(S) minidisk recorder or digital M-Audio Micro Track 24/96 recorder. Playback was done using a Radio Shack mini amplifier (Fort Worth, TX, USA).

After songs were recorded, the male (and the female when possible) was captured using mist nets to collect a small blood sample or a tail feather for genetic analysis. In the field, both blood and feathers were collected from all captured individuals. Samples were stored in a blood buffer (100 mM Tris, 100 mM EDTA and 2% SDS) to prevent DNA degradation in the field.

Genetic analysis

DNA was extracted from blood samples collected in the field using a standard phenol–chloroform protocol (Sambrook & Russell, 2001). DNA extractions from feather bases were performed using a DNeasy™ Tissue Kit (Qiagen, Valencia, CA, USA). Individual samples were first sexed using a molecular technique which detects a constant size difference in the CHD1 gene on the W and Z chromosomes in birds (modified from Fridolfsson & Ellegren, 1999).

To assess the level of hybridization between *leucophrys* and *hilaris*, amplified fragment length polymorphisms (AFLPs) were screened from all seven populations. This technique provides presence–absence data for specific markers, but does not allow identification of heterozygous individuals. DNA was quantified by running samples for several hours on an agarose gel and comparing bands to a standard ladder. This estimate of DNA quantity was used to determine how much of each sample to use for the ligation step. The protocol described in den Hartog *et al.* (2010) was used, modified from Vos *et al.* (1995). Total genomic DNA was restricted with 2.5 units each of the restriction enzymes EcoRI and MseI (New England Biolabs, Ipswich, MA, USA), in a total volume of 20 μL containing a $10\times$ ligase buffer (New England Biolabs) and 1 μg of BSA. After digestion at 37 °C for 1 h, 5 μL of ligation mix was added and incubation continued for another 3 h. The ligation mix contained 5.5 μM of M-E adaptor (sequences as in Bensch *et al.*, 2002) and 0.5 Weiss units of T4 DNA ligase (New England Biolabs, Westburg). The digested DNA with ligated adaptors was diluted 10 times in milli Q (Millipore, Billerica, MA, USA) H_2O and stored at -20 °C.

A preselective amplification was performed in volumes of 20 μL, containing 10 μL of the adaptor-prepared DNA, 0.3 μM of the E-primer with one additional T or A depending on the primer sequence used in the selective amplification, 0.3 μM of the M-primer with an additional C, 0.2 mM dNTPs, 2.5 mM $MgCl_2$, $1\times$ polymerase chain reaction (PCR) buffer and 0.4 units of *Taq* DNA polymerase (Qiagen). The temperature profile for the preselective PCR started with 94 °C for 2 min. followed by 20 cycles of 94 °C for 30 s and 72 °C for 60 s, and a terminal step at 72 °C for 10 min. The preselective amplification product was diluted 10 times in milli Q H_2O and stored at -20 °C. Selective amplification was performed in total volumes of 10 μL, containing 2.5 μL of the diluted preselective PCR product, 0.6 μM each of the E- and M-primer, 0.2 mM dNTPs, 2.5 mM $MgCl_2$, $1\times$ PCR buffer and 0.4 units of *Taq* DNA polymerase (Qiagen). The E-primer was labelled 5′ with fluorescein (Fam, Joe or NED). A touchdown temperature profile (94 °C for 2 min followed by 12 cycles of 94 °C for 30 s, 65 °C–0.7 °C/cycle for 30 s and 72 °C for 60 s, followed by 23 cycles of 94 °C for 30 s, 56 °C for 30 s and 72 °C for 60 s, and a terminal step at 72 °C for 10 min) was used to increase the specificity of the amplification.

End products were run on a megaBACE 1000 sequencing system (Amersham Biosciences, Little Chalfont, UK) with an internal size standard Rox 550. Spectral peak data were analysed in megaBASE Fragment Profiler version 1.2 (Amersham Biosciences, Little Chalfont, UK). For each primer pair combination, an automatic peak filter was customized to identify fragments and fragment size ranges. Electropherograms were visually inspected for ambiguous peaks. Eighteen randomly chosen individuals were reanalysed from whole blood samples to assure repeatability (which was 100%) of the AFLP results.

First, 37 individuals from both allopatric populations (20 *leucophrys* and 17 *hilaris*) were screened for variation using 12 combinations of the seven primers. To select robust and repeatable polymorphic markers for screening all individuals across the hybrid zone, AFLP bands were chosen if they occurred either (i) in >70% of the individuals of one allopatric population and in less than 30% in the other or (ii) in >40% of individuals in one allopatric population and not at all in the other. For each individual, a band was scored as present, absent or, in case of ambiguity, as a missing data point. Five of the primer pairs generated informative bands regarding subspecies identity. We used a subset of bands as diagnostic markers. These markers provide alleles for which all allopatric individuals of one subspecies display the absence allele (i.e. these individuals are negative homozygotes) and most of the other subspecies display the presence allele. Therefore, if an individual of the subspecies that should carry the absence allele carries the presence allele in the hybrid zone,

then it is assumed to be of heterospecific origin. We used diagnostic markers to assess the level of introgression across sites (c.f. Secondi *et al.*, 2006).

We assigned subspecies or hybrid identity using two different methods. First we ran a nonnetric multidimensional scaling (NMDS) analysis, a multivariate analysis similar to a PCA that is designed to handle binary and missing data, using CANOCO version 4.5 (terBraak & Smilauer, 2002). The first and second scores of the NMDS analysis were extracted for each individual and used to identify parental and first-generation hybrid groups. We used a Jaccard similarity coefficient for our analysis and calculated stress levels to assess how well the coefficient fitted our data (<0.1 being a good fit). Secondly, we used the program AFLPOP 1.1 (Duchesne & Bernatchez, 2002) to assign subspecies or hybrid identity to individuals. This program uses the known allele frequencies of allopatric populations to calculate the log-likelihood score for each individual of belonging to a parental, first- (F1) or second-generation (F2 or backcrosses) hybrid group. The following analysis parameters were used in analysis: zero replacement value = 0.001, artificial genotypes = 1000, $P = 0.001$ and minimum log-likelihood difference = 0.2.

Song analysis

We only used male solo song for our quantitative acoustic analyses. Song of the two subspecies can be easily distinguished in the field, with *leucophrys* singing a slow song with a few broadband high-pitched notes and *hilaris* singing about twice as fast with twice as many notes per song that lack high frequencies (Dingle *et al.*, 2008). In this study, recordings from each individual were classified into song types, and three sample songs per song type per male were selected as a representative set of songs for quantitative analysis (we recorded a total of 492 song types from 67 individuals and an average of 16.8 ± 9.7 SD songs per individual were analysed). All song analyses were carried out using LUSCINIA 1.0 (Lachlan, 2007).

Twenty-two different spectral and temporal characteristics of songs were analysed. For each song, the following measurements were taken: number of notes, maximum and minimum frequency, maximum note bandwidth (note with highest difference between minimum and maximum frequency), song duration, sound density (% of time within a song when sound is produced) and delivery rate (number of notes per second). Peak frequency (frequency within a note with loudest amplitude), frequency modulation (change in frequency per second), note duration and note bandwidth were measured for each note and averaged over songs. Additionally, maximum, minimum and peak frequency, note duration and frequency modulation were measured for every first and last note, and the number of

notes in a song that had a bandwidth over 2 kHz was counted. We averaged acoustic measurements over all songs of an individual (for the principal component analysis, see below) or over song types per individual (for the discriminant function analysis, see below).

We ran a principal component analysis (PCA) using SPSS v.17 (SPSS Inc., Chicago, IL, USA) to assess how acoustic phenotypes of the two subspecies changed in the contact zone. All 22 acoustic variables were used in the analyses to extract principal component scores for each individual. These principal component scores allowed us to compare song phenotypes of genetically pure *hilaris*, pure *leucophrys* and F1 hybrid individuals in the contact zone, as well as in relation to parapatry and allopatry.

We encountered a few individuals that displayed song types of both subspecies (referred to as *mixed singers*). Inspection of sonograms suggested that these birds sang the acoustic phenotype of one subspecies most of the time and only switched to one or two song types from the other subspecies at the end of the sampling period when they were expected to be more agitated as a result of the playbacks of their own song.

We ran a discriminant function analysis (DFA) with the 22 acoustic variables averaged over song types to assess whether song types recorded in the contact zone, including song types from mixed singers and hybrids, were similar to allopatric or parapatric populations of either *hilaris* or *leucophrys*. The DFA was set up to discriminate between four populations (allopatric or parapatric) of both subspecies, and a linear function from the analysis was used to assign each song type recorded in the contact zone to one of the four groups based on calculated probabilities. Song types were assigned to populations at a probability of >0.8. Song types that could not be assigned to a population were assigned to subspecies when probabilities for the allopatric and parapatric population of that subspecies were >0.8. Song types that could not be assigned to subspecies were regarded as intermediate between *hilaris* and *leucophrys*.

Distribution of phenotypes and genotypes in the contact zone

We mapped territories at the sympatric sites and scored the phenotypes of singing males and females as either pure *hilaris*, pure *leucophrys* or *mixed singer* in the field. In addition, while recording songs from one territory, we noted whether the neighbouring territories displayed *hilaris* songs or *leucophrys* songs. Both PCA and DFA confirmed the male field identifications except for one mixed singer that displayed two intermediate song types. Female identifications were confirmed on the basis of spectrographic readings. After genetic analysis, we compared whether males and females were paired

assortatively on the basis of their genotype and/or phenotype.

Results

Hybridization

Amplified fragment length polymorphisms analysis was based on blood samples collected from 67 individuals (males and females) across the contact zone and 37 individuals from the allopatric populations. The five primer pairs chosen for analysis of the hybrid zone yielded 21 informative markers, of which 16 were diagnostic and allowed the detection of introgression of heterospecific alleles (Table S2). NMDS analysis separated the two parental groups on the basis of the first score (accounting for 80.2% of variation in the data) and confirmed intermediate genotypes for six F1 (or F2) hybrids in the contact zone (Fig. 2a). The NMDS had a stress value of 0.05, indicating that the binary data are represented well by the new axes.

All allopatric individuals were correctly assigned to their parental group based on AFLP fragments using AFLPOP. In the contact zone, 32% of screened individuals were identified as pure *hilaris*, 22.6% as pure *leucophrys,* 19.4% as F1-hybrids (same individuals as in the NMDS analysis), 12.9% as second-generation backcrosses with *hilaris* and a further 12.9% could not be appropriately assigned to a group. In the parapatric *hilaris* populations, 40% of individuals were identified as parental *hilaris* and 33.3% of individuals were identified as second-generation backcrosses with *hilaris*. We could not assign 26.7% to any of the groups. In the parapatric *leucophrys* populations, we assigned 85% of individuals to the parental *leucophrys* group. Second-generation backcrosses with *leucophrys* were identified only in one parapatric population (site 5) and account for 15% of the total parapatric *leucophrys* individuals.

Pattern of introgression

Heterospecific alleles were found in a total of 21 individuals across the hybrid zone: six F-1 hybrids and 15 other individuals (Fig. 2b). Heterospecific alleles were found in all sympatric and parapatric sites, except for site 6 (Fig. 2b), which was the *leucophrys* population most distant from the sympatric zone. Thirteen individuals carrying heterospecific alleles were assigned to a backcross or unknown group in the AFLPOP assignment. However, all but two of these individuals also clearly grouped into one of the parental species in the NMDS analysis. It is thus likely that most of these individuals were not second, but third- or fourth-generation backcross. Furthermore, several of these individuals showed ambiguous bands for at least one marker and it is unclear how this influenced their assignment. Given this uncertainty, we decided to use
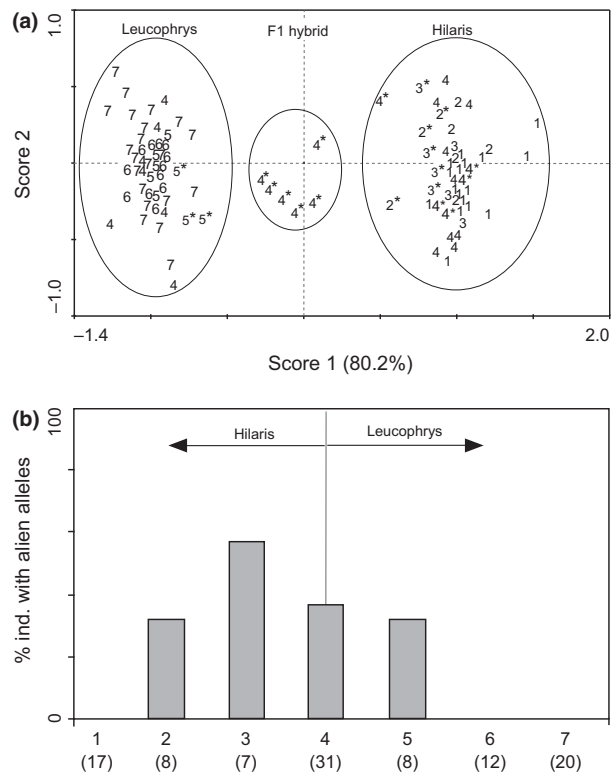

Fig. 2 Amplified fragment length polymorphisms markers reveal hybridization between two subspecies of *H. leucophrys* and introgression of heterospecific alleles. (a) The first and second scores of a nonmetric multidimensional scaling (NMDS) based on 21 random markers represent 80.2% and 7.4% of total genetic variation, respectively. Numbers correspond to sampling site as depicted in Fig. 1. Individuals on the left side were identified as leucophrys parental genotype, on the right side as hilaris parental genotype and the six individuals in between as F1 or F2 hybrids. Individuals that carried at least one heterospecific allele are marked with an asterisk. (b) Heterospecific alleles are present in all populations of the contact zone, except at site 6, showing introgression across the hybrid zone, with a maximum at site 3. No heterospecific alleles are present in either allopatric population (sites 1 and 7) by definition of reference. Numbers under the *x*-axis represent sampling site, and numbers in parentheses represent sample sizes.

the NMDS results for the acoustic analyses. We therefore genetically grouped individuals as parental *hilaris*, *leucophrys* or hybrids from the F1 generation.

Acoustic phenotypes remain distinct in the contact zone

Acoustic analyses, based on the 22 acoustic measurements derived from 492 song types from 67 males, showed clear differences between *hilaris* and *leucophrys* songs. A PCA collapsed the measurements (averaged over all song types of an individual) into 5 principal components which explained 84.1% of acoustic

variation among individuals (Table S3). PC1 explained 54.1% of variation and confirmed that the subspecies-specific acoustic phenotypes are readily distinguishable
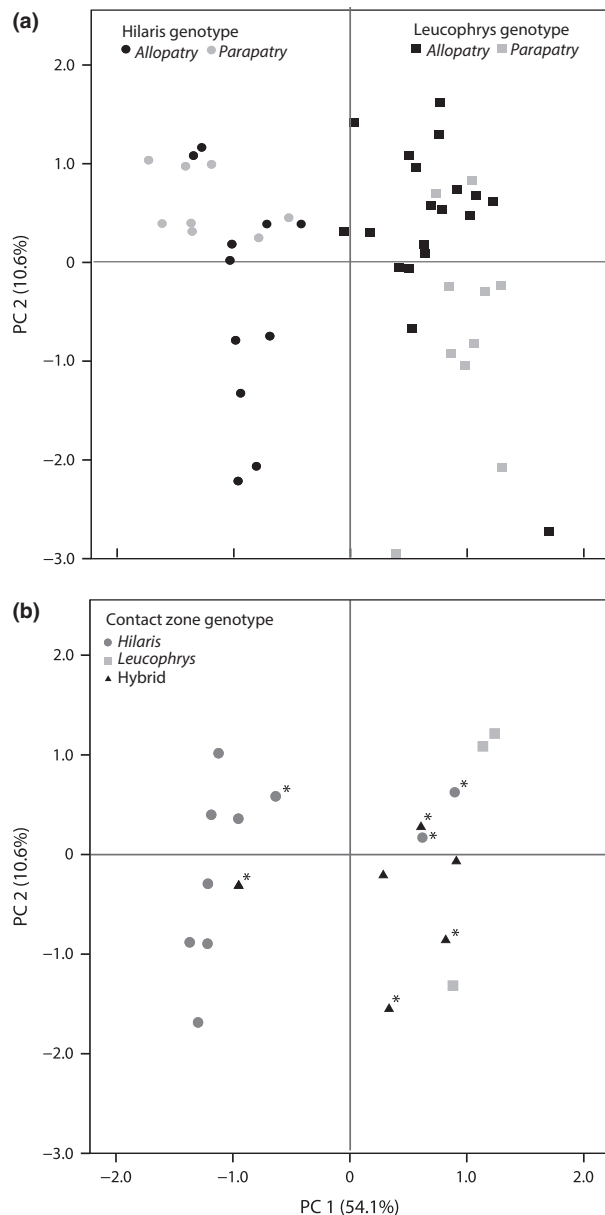

Fig. 3 Principal components analysis on quantitative song characteristics discriminates acoustic phenotypes of hilaris and leucophrys. The first and second components explain 54.1% and 10.6% of total acoustic variation. (a) Individual phenotypes can be separated on basis of PC1 between subspecies in both allopatry (black symbols) and parapatry (grey symbols). Differences between subspecies in parapatry show a divergent pattern compared with allopatry. (b) Differences in PC1 values between subspecies-specific acoustic phenotypes remain in the contact zone. Hybrid individuals display average phenotype of one or the other subspecies. Note that two hilaris individuals display acoustic phenotype of leucophrys. Individuals identified as mixed singers are indicated with an asterisk.

(Fig. 3). All of the birds sampled in allopatry and parapatry displayed acoustic phenotypes that matched their parental genotype based on the first principal component (Fig. 3a). In the contact zone, most male genotypes and phenotypes also matched (Fig. 3b), but there were two cases in which birds that were genetically identified as *hilaris* were mixed singers that primarily displayed the phenotype of *leucophrys*. These two males did not carry heterospecific alleles from *leucophrys*.

F1 hybrids mainly displayed a parental phenotype instead of an intermediate phenotype: one displayed a primarily *hilaris* phenotype, whereas five exclusively or primarily displayed the *leucophrys* phenotype (Fig. 3b). Four of these F1 hybrids also included occasional heterospecific songs and were thus classified as mixed singers (see below).

Discrete song types of mixed singers

We found several mixed-singing individuals that sang song types containing acoustic features of both subspecies. We ran a discriminant function analysis (DFA) on song type level to assess whether these mixed singers sang discrete song types (that could be assigned to a parental group), or intermediate song types containing elements from both subspecies (and could thus not be assigned). The first and second scores from the discriminant function analysis (DFA) explained 92.4% of the acoustic variation among allopatric and parapatric song types and distinguished between subspecies and population, respectively (Fig. 4; Table S4). The majority of song types could be classified and were assigned to either population (72.3%) or subspecies (26.9%). Seven individuals were classified as mixed singers, four of which were F1 hybrids. The three other mixed-singing individuals were from the *hilaris* genotype group and one of them carried alien alleles from *leucophrys*. Only 1.8% of recorded song types could not be assigned to one of the groups and were considered to be intermediate song types. Inspection of sonograms showed that some of these intermediate song types were a construction of *leucophrys* song type, followed by a short *hilaris* song type (Fig. 4: mid-panel on bottom row).

Match between phenotypes and genotypes

Whereas most genetically identified *hilaris* and *leucophrys* individuals in the contact zone produced the acoustic phenotype that matched their genotype, two genetically identified *hilaris* individuals primarily sang *leucophrys* songs (Fig. 3b), and one *hilaris* male was identified as mixed singer. Nevertheless, all 10 genetically identified *hilaris* males were paired with *hilaris* females (seven females genetically identified, three acoustically identified; Fig. 5). The three genetically identified *leucophrys* males also paired assortatively (one
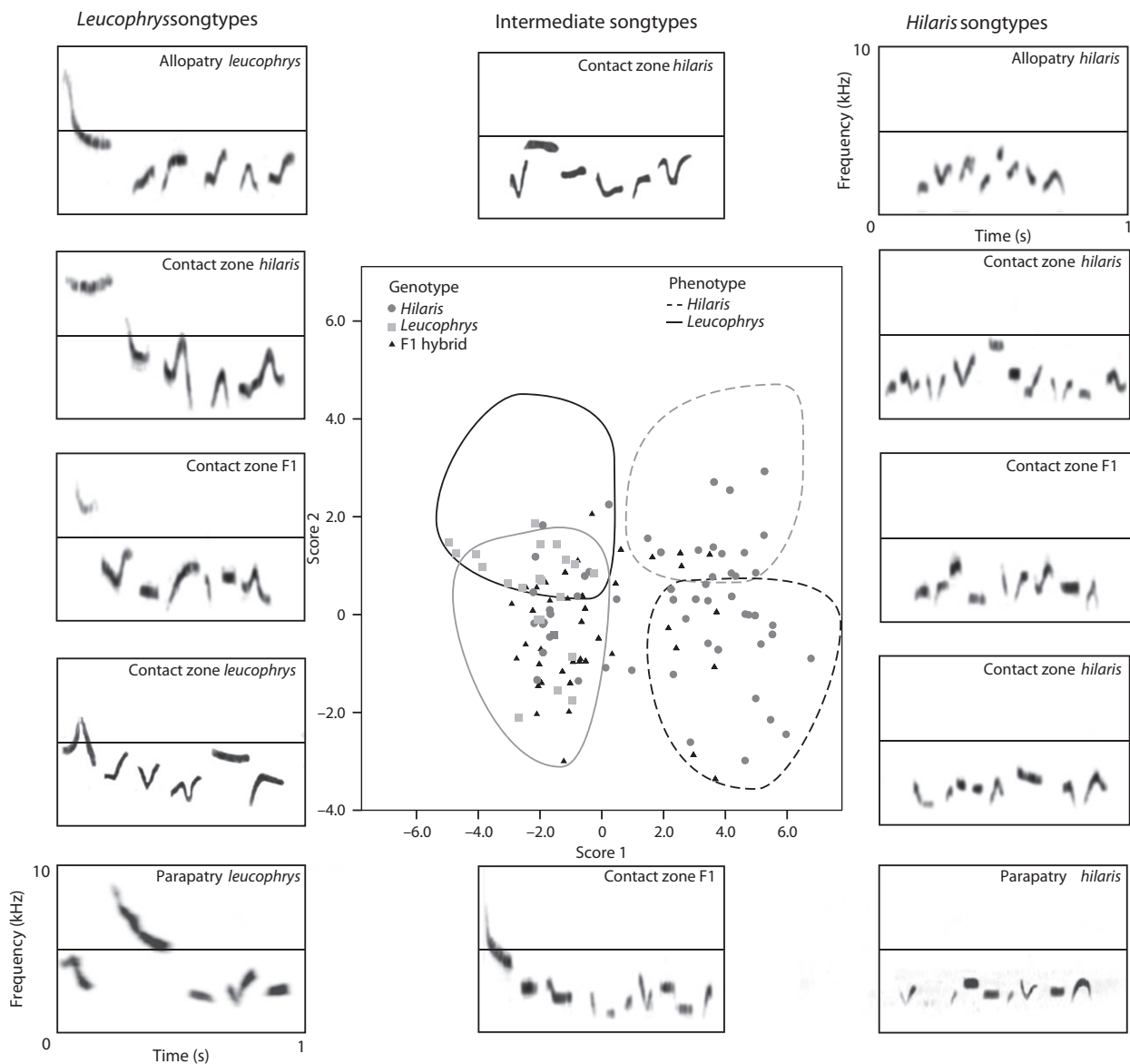
Fig. 4 Song types' classification into allopatric/parapatric populations of two subspecies of *H. leucophrys* using discriminant function analysis. The first and second scores explain 77.6% and 14.8%, respectively. Discriminant functions were based on song types recorded in allopatry (black lines) and parapatry (grey lines) for both subspecies and were used to classify song types recorded in the contact zone. Only sympatric song types are shown in the plot and grouped according to the genetic background. Genetically identified leucophrys individuals sing only leucophrys song types, whereas individuals with hilaris and hybrid genotype can sing both phenotypes. Six song types could not be classified according to the subspecies and were identified as intermediate song types. Sonographic examples of song types depict leucophrys phenotypes on the left side, hilaris types on the right side and intermediate types in between. The second and third panels from above show song types that were recorded from the same individuals, which were identified as mixed singers.

female acoustically identified; Fig. 5). We did not find any hybrid females and the six F1 hybrid males consequently did not mate assortatively. Consequently, the percentage of assortative-mated males was 68% in the contact zone. All six hybrids were paired to *leucophrys* females (three females identified genetically, three identified acoustically). Five of these hybrids

(primarily) sang *leucophrys* songs (Fig. 5). Interestingly, we also found one female (with a *hilaris* genotype) that displayed song types of both subspecies based on spectrographic comparisons. Finally, all mixed singers held territories at the sharp boundary between acoustic phenotypes (Fig. 5) and had neighbouring territories such that both phenotypes were within hearing distance. We
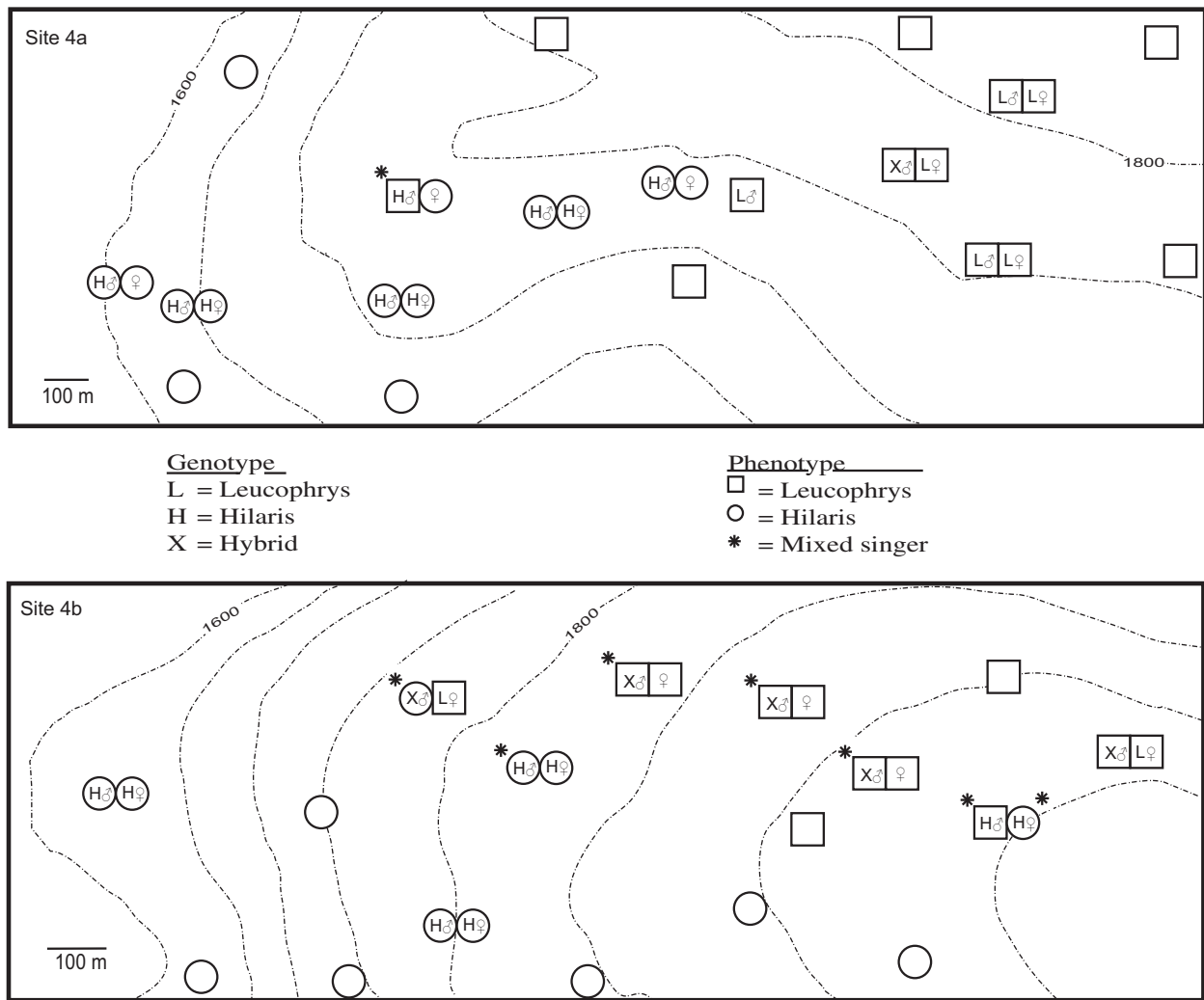
Fig. 5 The distribution of territories, genotype and acoustic phenotype of males and females are given for the two contact zone sites: 4a and 4b.

Discussion

Our study of acoustic phenotypes and genotypes in an avian contact zone revealed: (i) considerable hybridization in the contact zone with about 19.4% of individuals in the contact zone being hybrid and a further 35.8% carrying hetero-subspecific alleles; (ii) asymmetric genetic introgression in parapatry with more alleles from high-elevation *leucophrys* present in low-elevation *hilaris* populations than vice versa; (iii) acoustic differences between subspecies that remain distinct in contact; (iv) mixed-singing individuals who predominantly sang *leucophrys* songs, consisting of both *hilaris* and

did not find mixed pairs between genetic *leucophrys* and genetic *hilaris* wood-wrens.

hybrid genotypic birds; and (v) consistent pairing of hybrid males (either mixed singers or *leucophrys* phenotypes) with *leucophrys* females.

Distinct phenotypes and limits to gene flow

Divergent acoustic phenotypes may restrict gene flow through reduced settlement or mating success. Individuals may have to display a local song type when trying to attract mates or defend territories successfully, which may limit dispersal across acoustic boundaries (Slabbekoorn & Smith, 2002). The findings in the current study are partly in line with such a behavioural barrier, which may rapidly evolve due to song learning in birds (e.g. Ellers & Slabbekoorn, 2003; Yeh & Servedio, 2015). We found co-occurrence of both parental

subspecies of wood-wren exclusively in an area covering a very narrow altitudinal range (<300 m). The two subspecies are known to cover a much wider altitudinal range in areas of allopatry (Dingle *et al.*, 2008), which suggests that the range of parapatric populations is restricted by the presence of the other subspecies and not by environmental conditions. Our data are in line with individual restrictions on dispersal across acoustic boundaries: despite considerable hybridization at the contact zone and introgression in parapatry, we did not find any individual with pure heterospecific genotypes or phenotypes in parapatry. In other words, we do not find birds genetically identified as *hilaris* in parapatric *leucophrys* populations, and vice versa.

The findings of the current study also highlight the potentially undermining role of learned song in reproductive isolation and population divergence (c.f. Grant & Grant, 1997; Bensch *et al.*, 2002; Qvarnstrom *et al.*, 2006). It seems very likely that hybridization in wood-wrens is facilitated by learning heterospecific songs from males of different genotype in neighbouring territories, who predominantly sang *leucophrys* songs. Although we did not find mixed pairs (even mixed-singing *hilaris* males had paired assortatively with *hilaris* females), we found F1 hybrids between the subspecies only at the two sites where the subspecies come into direct contact, and not at other sites. Furthermore, all six F1 hybrids occupied territories within hearing distance of both subspecies and four of six F1 hybrids were *mixed singers*. We also consider it possible that we would have found all F1 hybrids to be *mixed singers* if we had sampled more songs through longer recording periods. Highly consistent song variants with abrupt geographical transitions, as well as the presence of *mixed singers* just at the transition zone, are also found in dialect studies on song variation among populations within species. These patterns have been widely accepted to be a consequence of song learning from nearby neighbours after dispersal (Baptista, 1977; Ellers & Slabbekoorn, 2003; Podos & Warren, 2007).

Mating patterns and asymmetric introgression

Most individuals in the contact zone were paired assortatively on the basis of their genotype. However, we found a bias among hybrids in being paired only with *leucophrys* females, suggesting a potential route for asymmetric introgression. We found most of these hybrids to be mixed singers, but displaying primarily songs of *leucophrys*. Furthermore, we detected several *hilaris* males in the contact zone to be mixed singers or to sing primarily heterospecific song types. An asymmetric pattern in song behaviour and mating pattern may be explained by male responsiveness to subspecific song variation, which also exhibits an asymmetric response pattern, both in allopatry and parapatry

(Dingle *et al.*, 2010). *Hilaris* males respond strongly to song playbacks of both their own and the other subspecies, whereas *leucophrys* males only respond strongly to songs of their own subspecies. This response pattern may lead *hilaris* males to copy *leucophrys* songs, but not vice versa. This in turn may allow *hilaris* males to attract *leucophrys* females or to outcompete *leucophrys* males in defending their territory or (extra-pair) paternity.

It is also possible that differences in aggression or dominance correlate with these asymmetries, since in several species pairs with asymmetric response patterns, it is the larger or socially dominant species that has been found to discriminate less or not at all between own and heterospecific song (Sorjonen 1986, Freeman 2016, Pearson & Rohwer 2000). Difference in territorial behaviour and aggression can cause one (sub)species to expand its range at the expense of the other (sub)species, which results in a moving hybrid zone (Pearson, 2000; Dingle *et al.*, 2010). It seems therefore likely that *hilaris* is currently moving towards higher altitudes at the expense of *leucophrys*, which may explain why we found a bias in the presence of heterospecific alleles in the parapatric *hilaris* populations. In fact, the highest proportion of alien alleles was not found in the current contact zone but at a downslope *hilaris* site. Such an asymmetric pattern of introgression has been associated previously with moving hybrid zones, as it typically 'leaves a ghost trail of alien alleles behind' (Pearson, 2000).

Conclusions

Overall, our data suggest that learned song can play a role at different levels in hybridization and speciation. At the population level, learning can lead to rapid song divergence, for example, through high level of copy errors (Lachlan *et al.*, 2013). Learning also allows the maintenance of distinct phenotypes, even in the presence of ongoing introgression, as song types can be culturally inherited independent of genotype. These processes promote song divergence and may thereby enhance assortative mating and reduce gene flow (Gill & Murray, 1972; Baker, 1991; Matessi *et al.*, 2001; Patten *et al.*, 2004; Greig & Webster, 2013). However, at the territorial level, song learning may allow copying of heterospecific songs, as clearly demonstrated by our results. Displaying a heterospecific phenotype can enhance the probability of hybridization and consequently result in introgression (Secondi *et al.*, 2003). Consequently, song learning may either enhance, or slow down speciation, depending on geographical scale. Whether song-learning taxa that come into contact remain distinct or collapse into single taxa may thus depend on the overlap in their geographical distributions and territory density in the contact zone. Our results demonstrate that hybrid zones seem particular

suitable to study the role of geography on these important evolutionary processes (Grant & Grant, 1997; Qvarnstrom *et al.*, 2006). Divergent and adjacent populations, such as the replicated system of grey-breasted wood-wrens along environmental gradients in Ecuador and Colombia (Dingle *et al.*, 2008; Dingle *et al.*, 2010; Caro *et al.*, 2013; Burbidge *et al.*, 2015), provide excellent natural laboratories to put the role of song learning in speciation to the test. Phylogenetically corrected song divergence could, for example, be related to the amount of introgression between these populations. Heterospecific song copying in areas of contact on the other hand can be related to the width of the hybrid zone, or the amount of hybridization.

Acknowledgments

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1. Location of sampling sites.

Table S2. Frequencies of informative AFLP markers for each of the five primer pair combinations.

Table S3. Factor loadings for the 22 acoustic variables used in a principal component analysis.

Table S4. Variable coefficients from the discriminant function analysis separating song types into an allopatric or parapatric population of either *hilaris* or *leucophrys*.