


Phylogeographic structure without pre-mating barriers: Do habitat fragmentation and low mobility preserve song and chorus diversity in a European bushcricket?

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Abstract When animal species have a strong phylogeographic structure questions arise on the origin, maintenance and future evolutionary trajectory of that structure. One prediction is that phenotypic differences among populations serve as pre-mating barriers should secondary contact occur. Post-mating barriers may also function and ensure further separation of the populations. We tested these predictions in an acoustic insect, the European bushcricket *Ephippiger diurnus*, that is geographically distributed in separated, genetically isolated populations distinguished by pronounced differences in male songs and chorusing and female preferences for song. We staged mixed-population choruses to examine how males sing when in the company of other populations, and we released females in these choruses to assess their preference for or aversion toward males of their own population versus a different one. We also paired males and females of the same and different populations to test mating success, spermatophore transfer, and oviposition in the various pairings. In most cases males sang as effectively when accompanied by males from another population as when in a single-population chorus, and females overall expressed little aversion toward males from a different population. Inter-population pairs did not mate less frequently, transfer smaller spermatophores, or deposit fewer or smaller eggs than pairs of males and females from the same population. We infer that pre-mating barriers play little role in maintaining phylogeographic structure despite the acoustic differences between populations. Rather, the structure probably reflects the fragmented distribution of suitable habitat and the low mobility of both juveniles and adults. Thus, if secondary contact does occur, and we predict that it will in several key locations owing to ongoing

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environmental change, coalescence of populations and reduction of genetic and acoustic diversity may follow.

Keywords Acoustic adaptation · Global warming · Habitat fragmentation · Magic traits · Reproductive isolation · Sexual selection

Introduction

Animal species comprised of geographically separated populations that are differentiated genetically and phenotypically pose a fundamental problem in evolutionary ecology (Avise et al. 2016). The separated populations may simply reflect fragmentation of suitable habitat and low mobility of individuals, with differences between the populations arising from genetic drift and/or dissimilar ecological factors among the habitat fragments. But the distinctive phenotypic characteristics of the various populations may also act as a pre-mating barrier should populations contact one another secondarily (Nosil 2012), thereby sustaining the species' unusual phylogeographic structure. A focus on secondary contacts between populations indicates that diverse outcomes are possible and shows how pre-mating barriers may influence the course of the species' future evolution (Harrison 1993; Coyne and Orr 2004). Examples of such evolutionary trajectories include the following: (1) Where barriers are weak, previously isolated populations may mix freely upon contact. In the extreme case wherein habitat fragmentation diminishes markedly over time, the species eventually becomes a single, panmictic population lacking phylogeographic structure. This specific outcome has major implications for conservation biology, which we treat in the Discussion. (2) If barriers are intermediate in strength, some introgression of genes may occur in one or both directions, and an inter-population hybrid zone may persist near the contact area (e.g. Butlin and Hewitt 1985; also see Shapiro 2001; Bridle et al. 2001; Larson et al. 2013). (3) In the event that barriers are relatively strong, previously isolated populations may remain distinct with little admixture after meeting at a contact point. Here, pre- as well as post-mating barriers to inter-population mating may eventually increase, possibly via reinforcement (Liou and Price 1994; Ortiz-Barrientos et al. 2004). Thus, the various populations can represent incipient species (e.g. Schwander et al. 2013), and the developing phylogeographic structure can play an important role in speciation given the appropriate circumstances. Strong barriers may also indicate that secondary contact had already occurred in the past, followed by receding of the populations.

We addressed the special problem that phylogeographic structure poses in acoustic species by studying *Ephippiger diurnus* (formerly *E. ephippiger*; Orthoptera: Tettigoniidae: Bradyporinae), a European bushcricket distributed in separated populations that are differentiated genetically and by male song and chorusing (Fig. 1) and female preferences for song. In particular, we asked whether the distinctive songs, chorusing and preferences can serve as pre-mating barriers between the populations. Would differences in acoustic behavior be likely to maintain the phylogeographic structure should secondary contact occur or would cross-mating and admixture follow? Our main approach to the problem was testing male song and female preference in mixed-population choruses that we staged in the laboratory and examining the outcomes of inter-population pairing. Based on the pronounced acoustic variation among populations we expected to find pre- and post-mating barriers that could deter admixture and maintain or strengthen the current structure. Pre-mating barriers would be indicated if a male broadcast inferior song when amidst singers from another population and if females preferred males from their own population over a

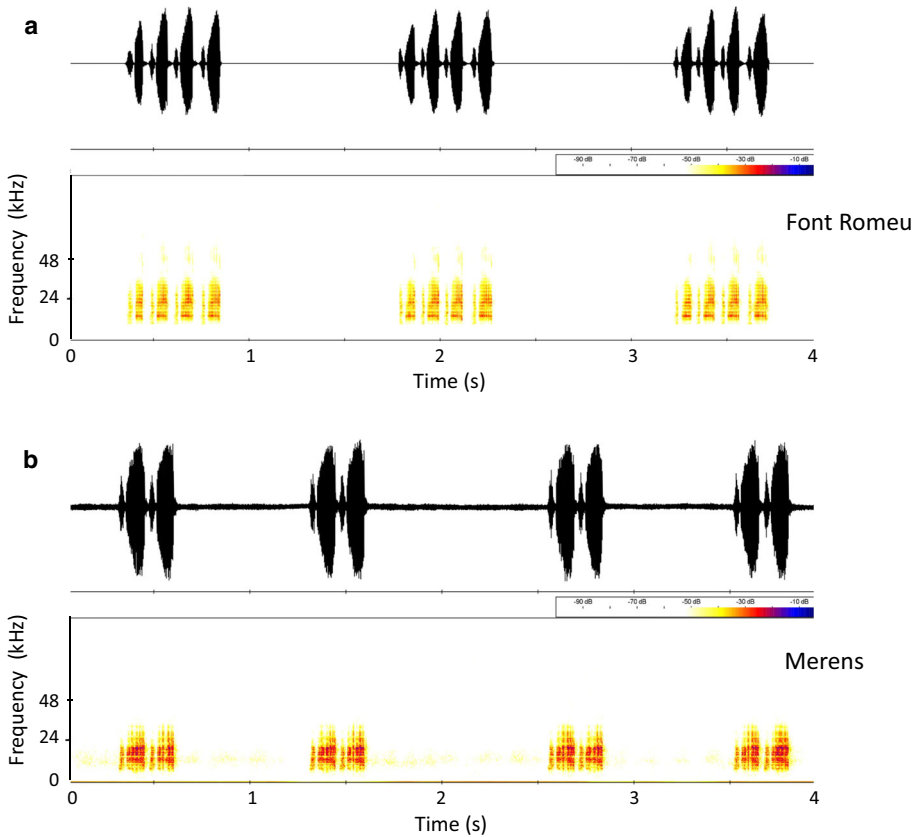


Fig. 1 Calling songs in *E. diurnus* males. **a** Oscillogram (upper trace) and spectrogram (lower trace) of 4-syllable calls in the Font Romeu population (clade 1). **b** Oscillogram and spectrogram of 2-syllable calls in the Merens-les-Vals population (clade 2). See Marin-Cudraz and Greenfield (2016) for recording methods and Table 1 for further information on the populations

male from elsewhere. Reduced oviposition by females in hetero-population pairs would indicate a post-mating barrier. On the other hand, an absence of these findings on male song, female preference, and oviposition would indicate that the observed phylogenetic structure likely results from habitat fragmentation and low mobility.

Ephippiger diurnus populations and behavior

Ephippiger diurnus are found in southern France and northeastern Spain, where they occur in garrigue (chaparral) and other open, natural habitats with gravelly soil (Duijm 1990). Their fragmented distribution largely reflects specific habitat preferences and the general patchiness of these habitats. *E. diurnus* are flightless and move relatively little as adults as well as juveniles, factors that probably contribute to the genetic differences observed between current populations.

Recent phylogeographic studies of *E. diurnus* show that populations west of the Rhone River in France can be grouped in two main clades (Party et al. 2015). Clade 1 includes populations along the Mediterranean littoral south of Narbonne, France and west on the

Table 1 *Ephippiger diurnus* populations studied, their geographic coordinates and characteristics, and parameters of male call and female preference for male call

Population	Geography			Male song ^a		Female preference ^a	
	Latitude, longitude	Elevation (m)	Phylogenetic clade	Mean call syllable number	Call rhythm (calls min ⁻¹)	Preferred call syllable number (peak)	Limits of preference for leading call (ms) ^b
Col de Chioula	42°45'N, 1°50'E	1430	2	1.98	40.85	6.87	50–600
Col de Mantet	42°29'N, 2°18'E	1700	1	4.52	22.09	9.84	50–900
Font Romeu	42°29'N, 2°00'E	1620	1	3.80	32.45	8.44	50–900
Leucate	42°55'N, 3°02'E	50	1	5.88	14.65 ^c	9.38 ^c	50–2000 ^d
Mérens-les-Vals	42°38'N, 1°49'E	1090	2	1.86	36.42	7.96	50–900
Mireval	43°30'N, 3°46'E	80	2	1.00	44.97	1.93	50–900

^a Data on male song and female preference taken from Barbosa et al. (2016b) and Greenfield et al. (2016)

^b Limits of preference for leading call are the beginning and ending of the delay interval, measured from the onset of a focal male's call, during which a female will ignore a neighboring male's call starting at that time; see Fig. 3 for illustration

^c Data from a neighboring population, Peyriac de Mer, used in the absence of measurements on Leucate males

^d Data from a neighboring population, Feuilla, used in the absence of measurements on Leucate females

southern slopes of the Pyrénées Mountains; clade 2 comprises populations along the Mediterranean north of Narbonne and west on the northern slopes of the Pyrénées and north into the Massif Central (Fig. 2). Finer analyses indicate that some branches within the clades represent populations along different valleys ascending toward higher elevations in the Pyrenees. For examples, distinct sub-clades can be identified that correspond with populations in the Cerdagne valley (clade 1) and in the valleys of the Ariège River and the upper Garonne River (clade 2) (Greenfield et al. 2016; cf. Ritchie et al. 2001; Spooner and Ritchie 2006). This overall phylogeographic structure may result from *E. diurnus* having been restricted to two separated refuges along the Mediterranean during the Pleistocene glaciation (ca. 20 KYA), followed by dispersal to higher elevations, and inland and northward, as the climate warmed afterward (Ritchie et al. 2001; Spooner and Ritchie 2006; cf. Hewitt 1996, 1999, 2000, 2004; Taberlet et al. 1998; Lovette 2005 for similar trends in other species). However, molecular clock analyses indicate a bifurcation date for the two clades that is much older than the Pleistocene (ca. 5–7 MYA; Spooner and Ritchie 2006). Laboratory tests have shown that crosses are possible between some *E. diurnus* populations (Ritchie 1996), but the extent of such crosses and the viability of hybrid offspring is completely unknown.

The most striking phenotypic difference between the two clades is the male calling song, and specifically it is the number of 'syllables' per call (Ritchie 1991, 1992; Barbosa et al. 2016b). Populations in clade 1 are characterized by a mean of 3–7 syllables per call,

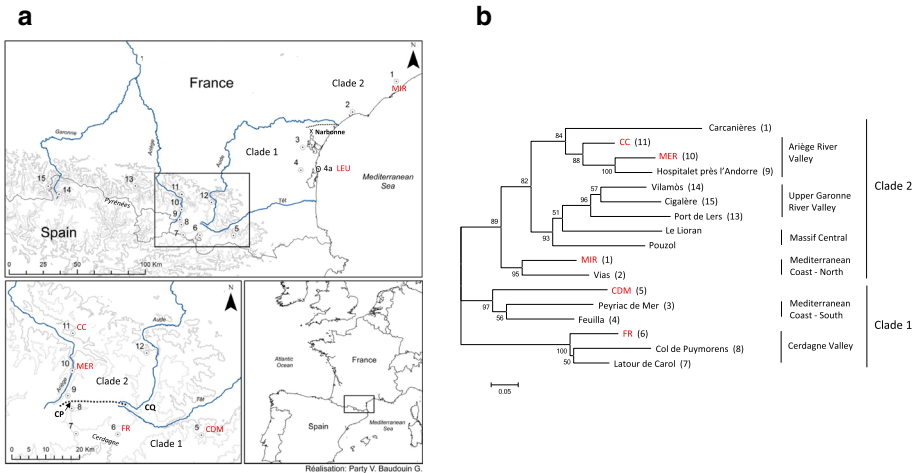


Fig. 2 **a** Map showing the locations of 16 *E. diurnus* populations studied in southern France and northern Spain [adapted from Party et al. (2015) and Greenfield et al. (2016); original map was generated with ArcGIS version 10.0 for desktop, ESRI (<http://www.esri.com/software/arcgis/arcgis-for-desktop>)]. Locations are indicated by circles with adjacent numerals. Locations which also have adjacent site abbreviations, in red, are those populations tested in the current study: Clade 1: 4A, LEU; 5, CDM; 6, FR. Clade 2: 1, MIR; 10, MER; 11, CC. Dotted lines show approximate boundary between the clades where known. Two mountain passes in the Pyrénées are shown in the inset: CQ (Col de Quillane), separating the FR population (6; clade 1) to the south from the Carcanières population (12; clade 2) to the north and CP (Col de Puymorens), separating the Col de Puymorens population (8; clade 1) to the south from the Hospitalet près l’Andorre population (9; clade 2) to the north. Contours represent 1200 and 1800 m elevations. **b** Phylogeny of 17 *E. diurnus* populations studied in southern France and northern Spain (see **a**; adapted from Greenfield et al. 2016). Numerals within parentheses adjacent to each branch refer to numbered locations on the map in **a**. Diagram shows an unrooted neighbor-joining tree generated from 16 microsatellite loci (see Esquer-Garrigos et al. 2016). Scale at bottom left indicates 0.05 nucleotide substitutions per site; values over branches represent posterior probabilities. Geographical groups and clades are indicated by vertical lines to the right of the tree. Populations tested in the current study are shown in red; the LEU population was not included in this phylogeny but is known to be very similar to Feuilla. The two Massif Central populations, Le Lioran and Pouzol, are far to the north of the others and are not shown on the map in **a**. (Color figure online)

whereas clade 2 populations mostly have only 1–2 syllables per call (Fig. 1). Moreover, the call syllable numbers within a given population generally fall within a limited range, and syllable number is significantly repeatable within individual males (Barbosa et al. 2016b). In clade 1 populations females prefer a call syllable number that is considerably higher than the mean in their population, but they will accept a wide range of syllable numbers above a threshold value (Barbosa et al. 2016b). On the other hand, clade 2 females prefer a syllable number that is equivalent to or only moderately higher than the population mean, and they tend to reject calls with syllable numbers much higher than the preferred value. Males in clade 1 populations sing with a slower call rhythm and produce shorter syllables than males in clade 2 (Fig. 1). Choice tests show that females in some *E. diurnus* populations prefer males that sing with a faster rhythm (Party et al. 2014), but the general importance of this parameter in mate choice has not been analyzed. More importantly, we do not know how females evaluate songs that differ substantially in multiple parameters, as might occur when males from several populations call together in a secondary contact.

Ephippiger diurnus males are usually found in clusters of 4–10 individuals in the landscape, and collectively the cluster broadcasts a chorus characterized by alternation as

well as synchrony between the calls of neighboring individuals (Greenfield et al. 2016). Alternation and synchrony are ultimately generated by a particular aspect of mate choice in *E. diurnus*, female ignoring of male calls that follow a neighbor's call by a brief delay and a concomitant preference for calls that lead a neighbor's call (Greenfield et al. 2004). Preference for relative timing of male calls is a psychoacoustic feature that may represent an ancestral perceptual bias, and it is not clear whether females benefit in any tangible way from this choice: Males who produce many leading calls are not distinguished by other behavioral or morphological traits, and the incidence of leading calls is not repeatable within males (Party et al. 2014). But regardless of the function of female preference for leading calls, a comparative phylogenetic analysis of *E. diurnus* populations demonstrated that this aspect of female choice has selected for phase-delay adjustments of song rhythm by which males reduce their production of ineffective, following calls (Greenfield et al. 2016). And when all males in a local cluster call with similar rhythms and adjustments, a temporally-structured chorus in which a focal male alternates with his neighbor but by default synchronizes with his neighbor's neighbor emerges. Any male who does not alternate as such and who emits some following calls will be significantly less attractive to females visiting the cluster (Greenfield and Roizen 1993; Greenfield 2005, 2015; Hartbauer et al. 2014; Hartbauer and Römer 2016).

Materials and methods

Experimental choruses

We staged interactions between *E. diurnus* populations by selecting pairs of populations A/B to test and then forming four types of 4-male choruses comprised (1) entirely of A males, (2) of three A males and one B male, (3) of one A male and three B males, and (4) entirely of B males. The two mixed choruses, types 2 and 3, were designed to represent an interaction between an 'invader', expected to be rare, with the 'resident' population. We used a 3:1 ratio because a male typically interacts acoustically with approximately three neighbors (Greenfield and Snedden 2003). The choruses of pure composition, types 1 and 4, served as resident controls with which to compare a population when it was in the invader role. Our experimental choruses showed us how regularly and effectively a male sings when in a situation representing a secondary contact. We also released females in the experimental mixed choruses to test their mate preferences in secondary contact situations.

Our goal was to select population pairs from localities currently studied (Greenfield et al. 2016; Fig. 2a) that would represent encounters between the 2 major clades, between populations differing in syllable number, and from the same geographic region such that an actual meeting in the field of the 2 populations or similar ones would be conceivable (see Figs. 1, 2). Additionally, the 2 populations of a pair would have to be phenologically aligned such that the insects would all be approximately the same age, and they should be reasonably abundant for sufficient replicates to be tested. These stipulations narrowed our possibilities to one population pair, Font Romeu (FR; clade 1; mean call syllable number = 3.8) and Mérens-les-Vals (MER; clade 2; mean call syllable number = 2.0). FR and MER are both situated above 1000 m in elevation, abundant each year, and separated by less than 20 km. But to augment our analysis we also tested 2 other population pairs where some of the stipulations were not met: Col de Mantet (CDM; clade 1; mean call syllable number = 4.52) and Leucate (LEU; clade 1; call syllable number = 5.88); Col de Mantet

and Mireval (MIR; clade 2; call syllable number = 1.0). CDM is situated at 1750 m elevation, whereas LEU and MIR are close to sea level; in both pairs the 2 populations are separated by more than 60 km (Table 1). In 3 of the 5 populations test insects were collected in the field during spring or summer 2015, mostly as late-instar nymphs in order to ensure a standard, unmated status among all individuals. However, at LEU the males were collected as young adults, and for CDM we used laboratory-reared insects hatched from eggs deposited by females collected during a previous year. We tested 10–12 males in pure and mixed choruses in each of the 5 populations.

All tested insects were kept in individual, clear plastic cages (10-cm diameter, 15-cm height; mesh screen cover) in an environmental room maintained at 25 °C and under a 16:8 L:D photoperiod. Illumination included several hours of exposure to 160-W sunlamps (Solar Glo PT2193, Rolf C. Hagen Inc., Mansfield, Massachusetts) each day. The insects were fed cabbage, fish flakes, and pollen ad libitum and misted with water daily. We moved males to a separate laboratory room shortly after the adult molt so that females would not be exposed to them or their songs prior to testing. Males heard one another in this room, albeit at reduced intensity because of attenuation of sound by the plastic cages. This acoustic exposure would mimic the acoustic environment in which insects would develop when in a secondary contact situation in the field.

We tested choruses in a 1.5-m diam. arena within an open laboratory room exposed to natural sunlight. Room temperature ranged between 20 and 25 °C. The arena was surrounded by a 50-cm barrier of acoustic insulation foam to attenuate exterior sound and echoes that would otherwise rebound from the walls of the room. We placed each of the 4 males comprising a given chorus in individual screen cages (10-cm diameter, 15-cm height) evenly spaced along a 75-cm diam. circle centered within the arena. When a given male was used to form another chorus on a succeeding day, we generally placed him at a different position along the circle in the arena. We illuminated the males with sunlamps during the chorus trials to stimulate regular singing.

Choruses were composed of unmated, acoustically active males between 15 and 35 d old to ensure a standardized state of reproductive maturity and regular singing. We did not observe a change in singing over this age range in the populations studied. Each chorus, defined as a unique set of 4 males, was allowed up to 15 min to attain singing by all individuals. We generally tested a given male in at least three different choruses, a pure chorus of his population and the 2 mixed choruses in which his population was the majority (resident) and the minority (invader), respectively. But the testing of a significant sample of males in the minority role necessitated that many males were used in more than three choruses to compose the majority population in mixed choruses. This factor, combined with the limited numbers of individuals available from the various populations and the need to test them in defined combinations and at appropriate ages, precluded an experimental design regulating the sequence in which a male was tested in the three chorus types and the identities of the males with whom he sang in successive trials. To address these issues, we used short trials (<15 min), normally separated a given male's successive trials by ≥ 2 d, and changed approximately 50% of the individuals that a male sang with in each trial. We tested the choruses between 08:30 and 14:00 h to coincide with the daily activity peak of *E. diurnus* in the field.

We recorded each chorus with a multi-track digital tape recorder (Tascam DR-680, Teac Corporation) and 4 microphones (Avisoft UltraSoundGate CM16/CPMA, ± 3 dB from 10 to 150 kHz; Avisoft Bioacoustics, Glienicke, Germany) positioned via tripods directly over the 4 males (see Party et al. 2014; see Fig. 3 for chorus example). Recordings were saved to a secure digital (SD) card and analyzed later via acoustic signal processing

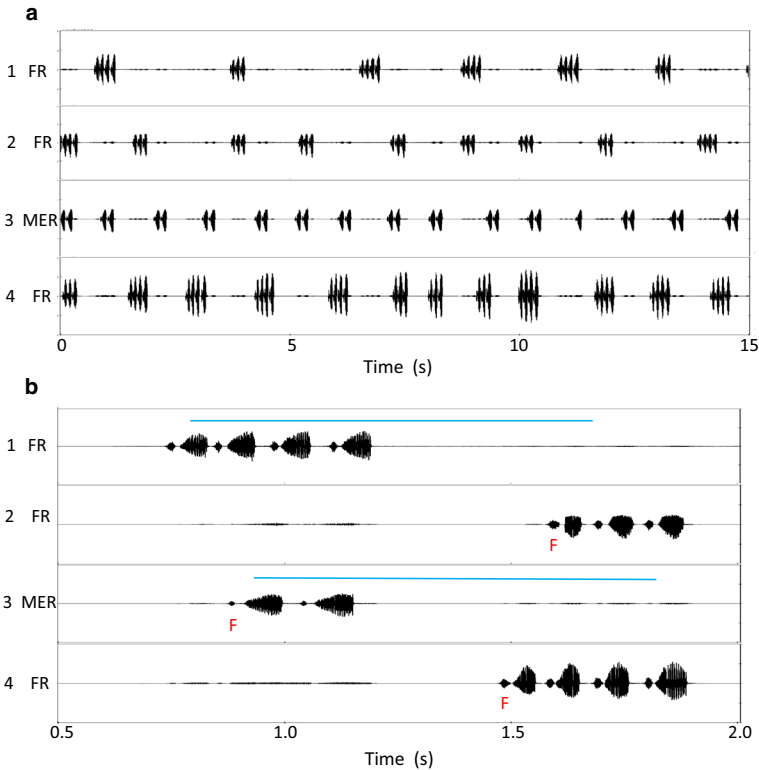


Fig. 3 **a** Multi-channel oscillogram showing timing of calls by 3 Font Romeu (FR) and 1 Mérens-les-Vals (MER) males in a mixed-population chorus. **b** 1.5-s segment of multi-channel oscillogram in **a**. Blue lines in channels 1 and 3 show the delay intervals for which calls initiated by any neighboring male would be designated as ‘following’ the focal male in the respective channel (see text and Table 1). An ‘F’ at the beginning of a call indicates that it followed one or more other males in the chorus; male 1 is followed by males 2, 3 and 4 and male 3 is followed by males 2 and 4. (Color figure online)

software (BatSound Pro, Pettersson Elektronik AB, Uppsala, Sweden; Audacity version 2.1.0, <https://sourceforge.net/projects/audacity/files/audacity/2.1.0/>). From each chorus we selected a 1-min segment during which all 4 males sang and measured the number of calls, mean call length, and mean call syllable number of each male. We employed repeated-measures ANOVA (a linear model with chorus type as a fixed factor and male identity as a random factor) to compare the call rate, length, and syllable number of individual males when they sang in a pure chorus, in the majority role within a mixed chorus, and in the minority role within a mixed chorus. Where a male was tested multiple times as a member of the majority population in a mixed chorus, we only used his song characteristics during the initial trial in later analysis.

We also examined each male’s relative call timing during the selected segment, focusing on the incidence of calls that ‘followed’ any neighbor’s call by a brief delay. The limits of the delay qualifying a call as ‘following’ are population-specific (Table 1) and were determined from female responses: In a population where females exhibit a significant preference for a focal male’s call whose onset leads a neighbor’s call onset by at least x ms but not more than y ms (Fig. 3b; Table 1; cf. Marin-Cudraz and Greenfield

2016), calls that begin between x – y ms after a focal male's call are designated as following. For examining relative male call timing in mixed choruses, we used the 'female preference' delay found in the majority population and applied it to males in that majority population as well as to the male from the minority population. In this latter case we evaluated how attractive an invader's calls would be with respect to the responses and preferences of the resident females. We calculated a male's incidence of following by tallying the number of calls timed as such among the first 30 calls he broadcast beginning at the onset of the selected 1-min segment. We then compared his incidence of following calls when he sang in the minority role within a mixed chorus with his incidence in a pure chorus.

Based on previous studies of calling within the various *E. diurnus* populations, we predicted that the acoustic environment in which a male sang would not influence his call syllable number (Rebar et al. 2016) but his call rate and incidence of following would be affected: Upon hearing a neighbor, a male's call cycle is inhibited and delayed, and these events will be more frequent for a male singing with a relatively slow call rate among faster neighbors (Greenfield 2005). And to offset deceleration of his call rate, a slow singer may forgo inhibition by a neighbor and broadcast a following call (Marin-Cudraz and Greenfield 2016). Consequently, we expected males in relatively slow call rate clade 1 populations, such as CDM or FR, to slow their singing further and to produce a higher incidence of following calls when in the minority (invader) role in a mixed chorus with a fast call rate clade 2 population, such as MIR or MER. Similarly, we expected males from LEU, a slow call rate clade 1 population, to decelerate and produce more following calls when in the minority role in a mixed chorus with another clade 1 population, e.g. CDM, singing at a relatively faster rate.

To gain a more precise estimate of the potential attractiveness of residents and invaders, we tested the actual preferences of females for the males singing in mixed choruses. Whereas a male from a fast call rate population from clade 2 may maintain his normal rhythm and broadcast relatively few following calls in a mixed chorus that includes males from a slow call rate population from clade 1, the clade 1 male calls have a higher mean syllable number. All three call parameters may jointly influence a male's attractiveness to females (Party et al. 2014), and there is no substitute for examining female choice in a mixed chorus to learn whether admixture and introgression might occur in the event of secondary contact between populations. To that end, in approximately 50% of the experimental mixed choruses that were staged, we released individual females from the majority or minority population in the chorus. Females were placed on a 20-cm wooden post in the center of the arena and allowed 15 min to descend, move toward a male cage, and remain in the vicinity of that cage for a minimum 30 s (cf. Party et al. 2014). Releases were only made when all 4 males were singing regularly. To ensure standard physiological condition, all tested females were unmated and 15–35 d old. Whereas unmated females in some orthopterans may exhibit less selectivity of mates than previously mated females do (Bateman et al. 2001), we note that unmated females in *E. diurnus* show considerable preference for the syllable number, call rhythm, and relative call timing in male song (Barbosa et al. 2016b; Greenfield et al. 2016). We tested each female twice, once when her population was the majority and once when the minority. As with males, we were unable to implement a formal experimental design regulating the sequence of a female's 2 trials, but we allowed a minimum 2 d to elapse between the trials to reduce the potential influence of prior experience. In each population approximately 50% of the females were first tested in a mixed chorus where her population was the majority one. In our analysis we asked whether females from a given population in a chorus showed a

preference for a male from their own population, the other population, or were indifferent. We tested 9–12 females in the various populations.

Inter-population pairing

Given the considerable proportion of inter-population attraction of females observed in experimental mixed choruses (see “Results” section), we conducted a series of trials to assess the likelihood that 2 populations would mate if paired. Thus, we tested males and females from Mérens-les-Vals (MER; clade 2) and Font Romeu (FR; clade 1) in their 4 permutations: female MER/male MER; female FR/male FR; female MER/male FR; female FR/male MER. To provide additional checks of intra-clade and inter-clade pairings, we also tested Col de Chioula (CC; clade 2) in its 4 permutations with Mérens-les-Vals and in its 4 permutations with Font Romeu. All insects were unmated and between 15 and 20 d old. We placed the pair to be tested in a plastic cage supplied with a perch and allowed 6 h for them to mate, which included the male’s transfer of a large spermatophore to the female. The spermatophore in *E. diurnus* represents male reproductive effort, although it remains uncertain whether the transfer is a nutritional investment and/or a device ensuring fertilization of the female’s eggs by the male’s sperm (Jarrige et al. 2013; Barbosa et al. 2016a). We recorded the latency to, and the duration of, copulation. We also determined the weight of the spermatophore transferred by weighing both male and female prior to and following mating, measuring the before-after weight differential in the male and the after-before differential in the female, and averaging the male and female weight differentials. We tested 7–10 pairs in each of the 9 male–female permutations of the 3 populations examined.

Following mating, we returned the female to an individual plastic cage with 4 cm sand on the bottom for oviposition. We sieved the deposited eggs from the sand 10 d after mating, inspected the eggs under a dissecting microscope, and counted them. When the count reached an asymptotic level, we assumed that the sieving process had extracted all eggs in the sample. Females were fed with the same regime as during earlier rearing throughout the 10-d interval allotted for egg laying. We used a 10-d interval because it was sufficiently long for a female to deposit a large complement of eggs but short enough that her survival would not be expected to be a confounding factor. *E. diurnus* eggs hatch after an obligatory 4-month diapause at 4–6 °C, and a significant proportion of eggs require 2 or more diapauses before hatching (Hockham et al. 2001). Consequently, determination of percent hatch and subsequent development of nymphs was beyond the scope of our study, and we made no attempt to measure offspring viability of the various pairings.

Results

Experimental choruses

In general, *E. diurnus* males sang as regularly and effectively in mixed-population choruses as they did in pure ones. We found no evidence that singing in the company of males from another population influenced a male’s call syllable number (cf. Rebar et al. 2016), but in one case a male’s call length was affected: Font Romeu (FR) males sang longer calls when they were the minority (invader) in a mixed chorus with Mérens-les-Vals (MER) males than when the majority (resident) or in a pure chorus comprised only of FR males.

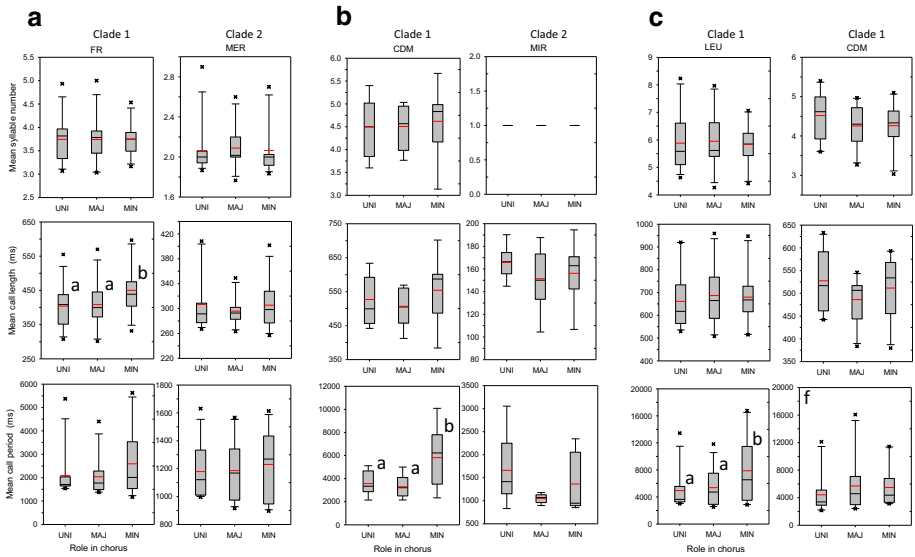


Fig. 4 Song parameters in experimental choruses. **a** Choruses comprised of Font Romeu (FR) and Mérens-Vals (MER) males. *Box plots* show mean syllable number, mean call length, and mean call period by male individuals in each population when in a uniform chorus comprised of their own population only (UNI), when they were the majority population within a mixed chorus (MAJ), and when they were the sole representative of their population within a mixed chorus (MIN). *Data* were only taken from individuals that sang at least 10 calls during the representative 1-min segment sampled in each of the 3 chorus types (UNI, MAJ, MIN). $n = 12$ FR males and 12 MER males tested in 31 choruses. **b** Choruses comprised of Col de Mantet (CDM) and Mireval (MIR) males. *Horizontal lines* for mean syllable number in MIR indicate that every male always sang with 1 syllable per call. $n = 10$ CDM males and 9 MIR males tested in 36 choruses. **c** Choruses comprised of Leucate (LEU) and Col de Mantet (CDM) males. $n = 12$ LEU males and 10 CDM males tested in 24 choruses. *Plots* indicate median (*black line*), mean (*red line*), 25–75% range (*box*), 10–90% range (*whiskers*), and outliers (*crosses*). *Boxes* followed by different letters indicate significant pairwise differences between the chorus roles shown below the panel (repeated measures ANOVA; post hoc multiple comparisons by Holm–Sidak method, $p < 0.05$). An ‘f’ in the *upper-left corner* of the panel indicates that unequal variances necessitated use of the Friedman test. (Color figure online)

Whereas call syllable number is a critical parameter in female preference in all *E. diurnus* populations (Party et al. 2014; Barbosa et al. 2016b), preferences for small increments in call length have not been examined and such variation in calling may have little influence on male attractiveness. We observed two changes in call rate in mixed choruses: As predicted, CDM males sang slower when they were the minority in a mixed chorus with MIR males, and LEU males sang slower when they were the minority in a mixed chorus with CDM males (Fig. 4). In both cases call rhythm was more than 50% slower in the mixed chorus. Call rhythm and call length were largely independent in our recordings, as we only found significant within-individual correlations (Pearson correlation, $p < 0.05$) between these parameters in 2 cases, LEU males singing in pure choruses and in the majority role in a mixed chorus with CDM. In both cases the correlations were negative.

Some populations exhibited changes in relative call timing when in mixed choruses. Again as predicted, CDM and FR males increased their incidence of following calls when they were the minority in mixed choruses with MIR and MER males, respectively (Fig. 5). For the observed changes in call rate and relative call timing it was always the population

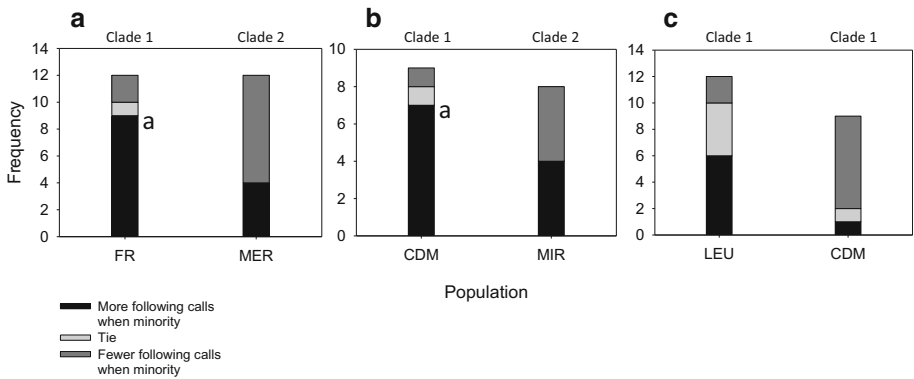


Fig. 5 Relative call timing in experimental mixed choruses. *Stacked bars* indicate the frequency of male individuals that exhibited a higher incidence of ‘following’ calls when in the minority role within a mixed chorus than when in a pure chorus (*black*), males that had a lower incidence when in the minority role (*dark grey*), and males whose incidence was equivalent in the two situations (*light grey*). A male’s incidence is defined as the number of following calls among his first 30 calls broadcast after the onset of the representative 1-min chorus segment sampled and analyzed for Fig. 4. **a** Font-Romeu (FR)/Mérens-les-Vals (MER) mixed choruses. **b** Col de Mantet (CDM)/Mireval (MIR) mixed choruses. **c** Leucate (LEU)/Col de Mantet (CDM) mixed choruses. *Data* represent all males analyzed for Fig. 4 that continued a singing rate ≥ 10 calls min^{-1} for the duration of their 30-call sample. *Bars* followed by ‘a’ indicate that a significant fraction of males produced a higher incidence of following calls when in the minority role in a mixed chorus than when in a pure chorus; 1-tailed binomial test, $p < 0.05$

with a higher call syllable number and a slower call rate in solo that modified its song when in a mixed chorus. In both CDM and FR a male’s proportion of following calls increased more than 30% in a mixed chorus as compared with a pure one.

Our main finding on mate choice in experimental mixed choruses was that females in the population singing with a faster call rate preferred a male from their own population when he represented a minority in the chorus (Fig. 6). We observed this result for MER females (clade 2) choosing among FR (clade 1) and MER males (Fig. 6a) and for MIR females (clade 2) choosing among CDM (clade 1) and MIR males (Fig. 6b). However, this preference was not found in CDM females choosing among CDM and LEU (clade 1) males (Fig. 6c). We did not find that females in the population singing with a slower call rate preferred—or avoided—a male from their own population when he represented a minority in the chorus. Owing to insufficient statistical power, we were unable to test whether females preferred or avoided a male from their own population when these males represented the majority in the chorus. Overall, released females descended from their post and responded to a caged male in approximately 2/3 of the trials. Males did not alter their singing when a female arrived in the vicinity of their cage.

Inter-population pairing

Approximately 75% of the males and females that we paired in cages eventually mated during the 6-h interval allotted, and we found no difference in the proportions that mated between intra-population pairs and any of the inter-population permutations (Fig. 7a). Similarly, there were no differences in latencies to mating between any of the within- and between-population pairs (Fig. 7b). Whereas we observed several differences between

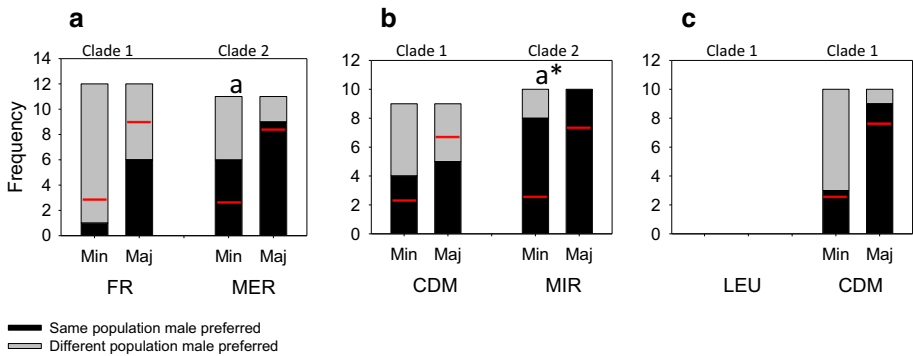


Fig. 6 Female choice in experimental mixed choruses. *Stacked bars* indicate the frequency of females from each of the 2 populations comprising a mixed chorus that chose a male from the same population (*black*) and from the other population (*light grey*) after released in the chorus center. *Min* males from the female’s population were in the minority role in the chorus, *Maj* males from the female’s population were in the majority role in the chorus. **a** Font-Romeu (FR)/Mérens-les-Vals (MER); $n = 22$ mixed choruses. **b** Col de Mantet (CDM)/Mireval (MIR); $n = 12$ mixed choruses. **c** Leucate (LEU)/Col de Mantet (CDM); $n = 14$ mixed choruses. A lack of LEU females of appropriate age prevented us from testing female choice in this population. *Bars* followed by ‘*a*’ indicate a female preference that differed significantly (2-tailed binomial test, $p < 0.05$) from the random expectation shown by the *red line*, which represents the proportion of males, from the given population, in the chorus; *asterisks* indicates $p < 0.5$ following sequential Bonferroni correction for multiple tests. In each population female individuals were tested twice, once in a *Min* mixed chorus and once in a *Maj* mixed chorus; the mixed choruses used represent a subset of those shown and analyzed in Fig. 4. (Color figure online)

pairs in spermatophore weight as a proportion of the male’s initial body weight, there were no indications that males transferred smaller spermatophores to females of another population or clade. The 2 lowest spermatophore values were in CC male × MER female (a within-clade combination) and in CC male × CC female pairs, and the 2 highest values were in FR male × MER female (a between-clade combination) and MER male × MER female pairs (Fig. 7c). From another perspective, the several populations differed in relative spermatophore weight as observed in within-population pairs, but males never transferred significantly smaller—or larger—spermatophores when paired with a female from another population in the same or a different clade (Fig. 7c). Nearly all deposited eggs were normal in size and shape, and we observed no differences in the number deposited between intra-population, inter-population and inter-clade pairs (Fig. 7d).

Discussion

Recordings of males in experimental choruses revealed relatively few differences between singing in the company of another population versus singing in a pure chorus. The main effect observed was that when a lone male from a population characterized by a relatively high call syllable number and a relatively low call rate sang amidst males from a population with a lower syllable number and a higher call rate, the male with the lower call rate sang even slower than normally (Fig. 4c), produced more following calls (Fig. 5), or both. These two changes, and especially the latter, are known to reduce a male’s attractiveness to females in most populations (Party et al. 2014, Greenfield et al. 2016). The changes

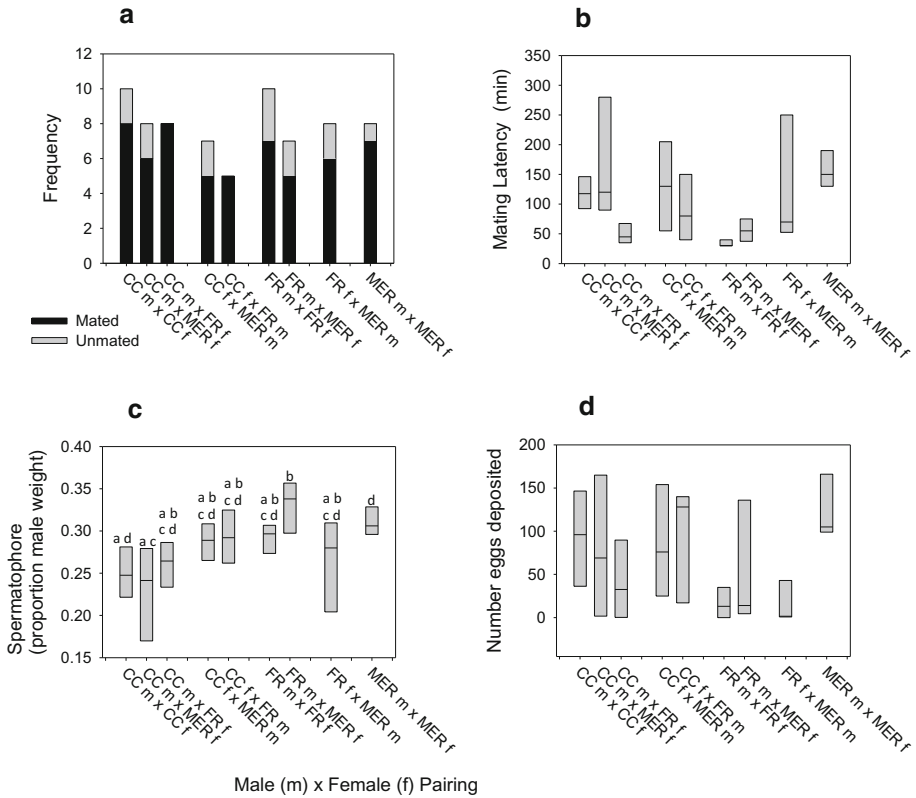


Fig. 7 Inter-population pairing. **a** Stacked bars indicate frequency of pairs that mated (black) and that remained unmated (light grey) for nine different crosses. Proportions of pairs that mated did not differ among crosses or between intra-clade and inter-clade crosses; Fisher’s exact tests, $p > 0.05$. **b** Latency to mating in pairs from nine different crosses. Plots indicate median (black line) and 25–75% range (box). Latencies did not differ among crosses (1-way ANOVA; post hoc multiple comparisons by Holm–Sidak method; $p > 0.05$) or between intra-clade and inter-clade crosses (nested ANOVA, GLM, Minitab v. 13; $p = 0.23$). **c** Spermatophore weight relative to male weight in pairs from nine different crosses. Crosses that are not followed by at least one letter in common differed significantly (1-way ANOVA; post hoc multiple comparisons by Holm–Sidak method; $p < 0.05$). Relative spermatophore weight did not differ between intra-clade and inter-clade crosses (nested ANOVA, GLM; $p = 0.55$). **d** Number of eggs deposited by females from nine different crosses. Egg number did not differ among crosses (1-way ANOVA; post hoc multiple comparisons by Holm–Sidak method; $p > 0.05$) or between intra-clade and inter-clade crosses (nested ANOVA, GLM; $p = 0.24$)

observed in high-syllable number males most likely originate in the phase-delay adjustments of rhythm that all *E. diurnus* populations make in response to neighbors’ calls. A reduced call rate would arise due to repeated inhibition by the much faster low-syllable number males (Greenfield et al. 1997). An increased production of following calls probably represents an ignoring of some calls by neighbors (Greenfield and Snedden 2003; Marin-Cudraz and Greenfield 2016; cf. Minckley et al. 1995; Snedden et al. 1998), which may be the only means for the high-syllable number male to maintain any level of regular singing, albeit with relatively ineffective broadcasts.

Observations of females released in mixed-population choruses showed that in most cases they neither preferred nor avoided males from their own population. The only exceptions to this generalization were the preferences of females in high-call rate, clade 2 populations for a same-population male when that population was the minority in the chorus. These preferences probably reflected the faster call rate, higher incidence of leading calls, and appropriate call syllable number broadcast by the male in the female's own population. Females in low-call rate, clade 1 populations did not prefer a same population male when that population was the minority, and we infer that cross-attraction in this combination was due to faster call rates and a reduced incidence of following calls by the clade 2 males, factors that offset the low syllable numbers of clade 2. When a female's population was the majority in the chorus, which occurred automatically whenever the other population was the minority, there were no cases where she preferred—or avoided—males of her own population. To be sure, low sample sizes and a $\frac{3}{4}$ probability of success reduce the power of tests in this latter situation such that statistical inference is not possible. Nonetheless, we have no overall indication that *E. diurnus* females in a majority population avoid cross-attraction toward a male from a minority population.

Inter-population barriers and secondary contact

Male singing and female preferences in our experimental mixed-population choruses indicate a strong potential for cross-attraction between the various *E. diurnus* clades and sub-clades. Except in the case of MIR (clade 2) and CDM (clade 1)—a population pair that is separated by over 100 km (Fig. 2a) and is not a candidate for secondary contact—females showed no strong preference for males from their own population (Fig. 6). And preferences that clade 2 females show for same population males when their population is a minority may not have an opportunity to be expressed if the density of an invading group is simply too low: almost all males that an invading clade 2 female meets would be from the resident clade 1 population. Importantly, our pairing tests in the laboratory demonstrate that cross-attraction would lead to mating and oviposition. Thus, distinctive male songs and chorusing and female preferences of the various populations do not appear to serve as potential barriers to cross-breeding should the opportunity arise in the field. In certain situations distinctive chorusing may actually promote cross-breeding, as when a clade 1 male decreases his call rate and/or incidence of leading calls when singing in the company of clade 2 males, influencing some clade 1 females to gravitate toward those clade 2 males.

Male choice of females at mating may serve as an additional pre-mating barrier between populations, and such choice is particularly likely in situations where males make major investments in mating or offspring, as via a large spermatophore (e.g. Bateman and Ferguson 2004). While male choice as such does occur in *E. diurnus* (Jarrige et al. 2013), our inter-population pairing trials did not reveal any aversion to hetero-population mating or a reduction in spermatophore size when mating with females from other clades or populations (Fig. 7).

In light of our failure to identify acoustic pre-mating barriers between *E. diurnus* populations and clades, we ask what maintains the strong phylogeographic structure in this species? And, what are potential future trajectories for this structure in the face of ongoing environmental change? We emphasize that molecular genetic data from microsatellite markers sampled among *E. diurnus* populations do not indicate recent inter-clade, or even inter-subclade introgression in the field. Clades 1 and 2 are geographically close yet remain distributed in separated, non-overlapping ranges along the Mediterranean coast and at several mountain passes in the Pyrénées. At these points of approaching ranges and

potential migration and interbreeding, clade 2 populations do not show signs of recent introgression and admixture with clade 1 genes and vice versa (see Figure 6B in Greenfield et al. 2016; population pair 2 and 3 on the coast; population pair 6 and 12 and pair 8 and 9 in the Pyrénées). In the Pyrénées, populations on both sides of the Col de Quillane (1713 m) and the Col de Puymorens (1920 m) may remain separate because winter temperatures at the summits are too cold for eggs, which are deposited in the soil at the end of the summer, to survive or develop and hatch during a subsequent year (Kidd and Ritchie 2000). Moreover, these climatic conditions may have been in effect for a very long time, and probably they were colder in past millennia: Climate reconstruction indicates colder winter temperature means and anomalies in southwest Europe throughout the Holocene until the last 500–1000 years (Davis et al. 2003; cf. Terral and Mengual 1999). Thus, the present geography of *E. diurnus* clades in the Pyrénées may represent the long-term ascent up river valleys, e.g. the Ariège, Aude and Tet (Fig. 2), and mountain slopes since expanding from coastal refugia at the end of the Pleistocene glaciation (cf. Weiss and Ferrand 2007 for similar examples among the European biota). The species' current distribution in the mountains may culminate at its environmental limits, which happen to separate the two clades and some of the major subclades. At the Mediterranean coast the factor(s) maintaining separation of clades 1 and 2 is less clear; it may result from alluvial soil in the delta and valley of the lower Aude River being unsuitable for oviposition. In general, low mobility of both adults and juveniles would sustain the disjunct ranges, as the insects may not traverse a gap of unsuitable habitat greater than several km in width. Moreover, successful migration would demand that an entire gap be crossed in one season, and by either mated females or by insects that arrive early enough to mate with a partner in the new area. Consequently, what seem at first to be rather unimposing physical barriers have probably been sufficient for maintaining phylogeographic structure in *E. diurnus*.

Barriers that effectively separate populations and species today may not continue to do so in the face of environmental change. In *E. diurnus*, montane barriers that maintain the disjunct distributions of clades 1 and 2 as well as those of major subclades may cease to function in a climate with warmer winter temperatures. For example, at the Col de Puymorens clade 1 and clade 2 populations are currently separated by no more than 5 km, and the two populations are found at elevations only 100 m below the summit. We predict that an increase in mean winter temperature as little as 2 °C may render the summit suitable for oviposition and egg development, thereby generating a secondary contact. Based on our observations of experimental mixed choruses and pairing trials, we would not expect pre-mating barriers to intervene in secondary contact. The likely outcomes are (1) limited or uni-directional introgression and a resulting hybrid zone or (2) substantial admixture at the contact point followed by slow diffusion of genes in one or both directions. For example, individuals from a clade 2 population arrive as 'invaders' within a clade 1 population. While females of the invader prefer invader males, females of the resident would be equally attracted by resident and invader males. Alternatively, individuals from a clade 1 population arrive as invaders within a clade 2 population. Here, both resident and invader females may respond equally toward resident and invader males. In both situations above, admixture would continue in subsequent generations following the secondary contact.

We cannot predict the specific outcome with certainty at this point, but our pairing tests and earlier findings that *E. diurnus* populations will produce hybrids (Ritchie 1996) suggest that substantial admixture and gene diffusion is more likely than a narrow hybrid zone. Similarly, our characterization of clades and subclades by a set of 16 neutral microsatellite markers (Fig. 2b; see Esquer-Garrigós et al. 2016) limits our ability to define the extent of overall genomic changes following a secondary contact. However, the earlier findings on

hybrids reported males and females with intermediate acoustic characters, indicating that major phenotypic changes would be expected. Another complication in potential secondary contacts is that invader males might congregate and chorus among themselves because of very specific habitat preferences and/or mutual attraction to their population's song (cf. Muller et al. 1997; Muller 1998). We have little information on these possibilities save that the various populations do not have specific host plant affinities for either feeding, thermoregulation or defense, including camouflage. Additionally, the general absence of female preference for same population males that we observed implies that some admixture would be expected in choruses forming at a secondary contact.

We have no evidence for local adaptation of song (cf. Greenfield 2016) or camouflage in *E. diurnus* populations, and this factor may account for the lack of pre-mating barriers. The specific call syllable numbers of the songs in the various populations are not 'magic traits' (sensu Servedio et al. 2011) that indicate superior transmission in a particular environment, and there may be little selection against hybrids if they are produced. For example, in many populations females may 'tolerate' hybrid songs with intermediate acoustic characters (cf. Barbosa et al. 2016b), and cryptic coloration is highly variable within populations. Acoustic diversity in *E. diurnus* may largely reflect chance inherent in population founding and the sexual selection process. Pre-mating barriers may not exist in this species because habitat fragmentation and low mobility has prevented most secondary contact, which would have been necessary to impose selection pressure favoring those barriers. Moreover, where and when secondary contact had occurred, post-mating barriers may have been too weak to select for pre-mating barriers that otherwise reduce loss of time, energy, and gametes.

Genetic diversity in a changing world

One of the major concerns associated with current and predicted environmental change is the fragmentation of habitat and species ranges, which may have adverse consequences for some organisms (Fahrig 2003). Inbreeding may increase if habitat fragments are small and isolated (Anderson et al. 2004). Moreover, requisite behaviors in animal species with social structure may not have an opportunity to occur in small populations (Courchamp et al. 1999; Stephens et al. 1999; Taylor and Hastings 2005). Our study on *E. diurnus* demonstrates that the reverse—defragmentation, with consequent modification of genetic variation—is also possible and worthy of consideration. We have shown that the rich genetic and behavioral diversity of this acoustic insect, currently a complex of isolated populations with divergent male songs, chorusing, and female evaluation of song, is potentially endangered by coalescence of these groups in some key locations. In spite of the markedly different songs and female preferences in the various populations, our tests indicate that certain populations may not remain separate following secondary contact. An admixture of the populations appears most likely for the inter-clade situations we examined in the Pyrénées, although we are less certain about the direction of an invasion and the rapidity with which diffusion of genes might occur. These findings are interesting in their own right as a window into the dynamics of population genetic structure and how acoustic behavior may or may not intervene in such changes. But the study also serves as a valuable lesson for conservation biology by highlighting an underappreciated facet of environmental change that can imperil genetic diversity.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest pertaining to the research reported in this manuscript.

Human and animal right statement All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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