



The roles of geography, climate and sexual selection in driving divergence among insect populations on mountaintops

Joaquina Pato  | Juan Carlos Illera | José Ramón Obeso | Paola Laiolo

Research Unit of Biodiversity (UO, CSIC, PA), Oviedo University, Mieres, Spain

Correspondence

Joaquina Pato, Research Unit of Biodiversity (UO, CSIC, PA), Oviedo University, Mieres, Spain.
Email: joaquinapato@gmail.com

Funding information

Fundación para el Fomento en Asturias de la Investigación Científica Aplicada y la Tecnología, Grant/Award Number: CN-13-058; British Ecological Society, Grant/Award Number: 4278; Ministerio de Economía y Competitividad, Grant/Award Number: CGL2011-28177, CGL2014-53899-P and CGL2017-85191-P; edp-HC Energía, Grant/Award Number: CN-13-058

Editor: Dr. Pablo Vargas

Abstract

Aim: Analysing the drivers of intraspecific variation and how reproductive barriers arise is an essential step to infer the mechanisms of biogeographic differentiation. In populations of a specialized alpine species, we explore the role of geography and climate in the divergence of genetic, morphological and acoustic characters, and analyse the functional consequences of variation on mate choice.

Taxon: *Chorthippus cazurroi* (Orthoptera: Caelifera, Acrididae, Gomphocerinae).

Location: The entire distribution of the species (23 populations from six massifs of the Cantabrian Mountains, NW Spain).

Methods: First, we analysed the extent of intraspecific spatial divergence and the covariation among climatic niche, genetic (mtDNA), acoustic (song structure) and morphological (body size) traits. Then, we analysed the consequences of phenotypic variation by means of a crossing experiment among populations from different elevations. This served to test for differences in sexual selection among body size-divergent populations and for the relationship between male traits, female preference and reproduction.

Results: Genetic, morphologic and acoustic divergence increased with geographic distance. Female morphology was also affected by climate variation, while male one tightly covaried with the song differentiation. Females more closely approached males investing more time in song activities, but weakly responded to the rest of acoustic features and morphological variation. They also distanced themselves slightly more from males from different populations, although this behaviour did not lead to clear differences in reproductive parameters.

Main conclusions: The process of colonization of mountain massifs has led to significant genetic and phenotypic changes in *C. cazurroi*. Phenotypic divergence does not constitute a strong intrinsic barrier to reproduction and is largely unpaired from female preference, overall suggesting that sexual selection is a minor actor in the process of differentiation as compared, for instance, to drift. This does not exclude that traits associated with individual condition are under strong selection and, therefore, do not vary so extensively. This study dismisses the idea that alpine specialists with narrow distributions lack genetic and phenotypic variability, and highlights the importance of synthesizing biogeographic and experimental approaches to obtain stronger and deeper inferences about the dynamics and mechanisms of biological differentiation.

KEYWORDS

acoustic signals, Cantabrian Mountains, crossing experiment, intraspecific variation, isolation by adaptation, isolation by distance, mate choice

1 | INTRODUCTION

Geographical patterns of intraspecific genetic and phenotypic variation reflect diverse evolutionary forces triggered by environment fluctuations, including climate-related ones, through time (Grant & Grant, 2017; Yannic et al., 2014). Because of the implications in speciation, understanding these processes is a cornerstone in evolutionary biogeography (Salomon, 2001). Both genetic and phenotypic (e.g. morphology and behaviour) variations among animal populations can result from natural selection (Schluter, 2001), and sexual selection is often decisive in the maintenance of differentiation via female choice (Panhuis, Butlin, Zuk, & Tregenza, 2001; Wells & Henry, 1998). Random processes, such as founder effects, drift and mutation, also promote phenotypic and genetic differentiation (Armstrong et al., 2018; Hoeck, Bollmer, Parker, & Keller, 2010; Irwin, Thimgan, & Irwin, 2008). The action of deterministic and stochastic forces becomes especially overt in populations of patchy or heterogeneous habitats, where adaptations to local conditions may be favoured by a reduction of gene flow or where reproduction and survival are more prone to undergo strong chance effects (Nosil, Egan, & Funk, 2008; Shafer & Wolf, 2013). The study of intraspecific geographical variation in phenotype and genetic markers in populations isolated in mountaintops or oceanic islands may then be decisive for inferring the forces involved (Illera, Spurgin, Rodriguez-Exposito, Nogales, & Rando, 2016). In a scenario of isolation by colonization, spatial patterns of genetic structure might be determined by founder effects, and thus be strongly related to the colonization history of each taxon (Pierce et al., 2014; Spurgin, Illera, Jorgensen, Dawson, & Richardson, 2014). In an isolation by distance scenario, genetic differentiation among populations increases with increasing geographical distance as a consequence of dispersion limitations (Jenkins et al., 2010). In an isolation by adaptation scenario, gene flow among ecologically different populations may decrease as a result of local genetic adaptation (Shafer & Wolf, 2013). The same framework can be used to address the spatial patterns of phenotypic divergence (Illera et al., 2014; Spurgin et al., 2014; Wilkins, Seddon, & Safran, 2013), including sexual trait variation (Lehtonen et al., 2009; Tregenza, Pritchard, & Butlin, 2000a,b; Wilkins et al., 2013).

Geographic variation in selection pressures may result in the divergence of nearby populations especially if sexual selection also varies (Andersson & Simmons, 2006). Yet, spatial variation in the targets and intensity of sexual selection has been largely overlooked (Kwiatkowski & Sullivan, 2002). Among the targets of sexual selection, the acoustic signals produced by males play an important role in mate choice in many and diverse animal taxa. Given that producing sounds is costly from the point of view of energy consumption and exposure to predators (Hoback & Wagner, 1997; Prestwich, 1994; Zuk & Kolluru, 1998), acoustic signals are often honest indicators of specific attributes that increase female fitness. In particular, they are often sensitive to the overall condition of the individual, or covary with body size (Cotton, Fowler, & Pomiankowski, 2004; Scheuber, Jacot, & Brinkhof, 2003), being the latter another key

traits undergoing sexual selection in animal species (Andersson & Simmons, 2006; Peig & Green, 2010). Sexual selection on these traits has crucial implications in the dynamics of diversification (Kaneshiro, 1989; Panhuis et al., 2001). As an effective pre-zygotic barrier promoting reproductive isolation, traits involved in mate recognition are known to accelerate phenotypic differentiation among sympatric or parapatric populations (Wilkins et al., 2013). During founder events and range expansion, instead, it is a relaxation of sexual selection that fuels the divergence of traits otherwise constrained by female preference (Tregenza et al., 2000a,b).

To build up a picture of the general importance of different drivers on biogeographic differentiation, it is crucial to combine analyses of trait variation with experimental evidence on female preferences. In this study, we assess the potential role of geography, environment (specifically climate) and female preference in modelling spatial patterns of genetic and phenotypic (morphological and acoustic) divergence in *Chorthippus cazurroi* (Bolívar, 1898), an annual alpine grasshopper endemic to the Cantabrian Mountains, north-western Spain. Previous studies have shown that isolation and habitat determined genetic and phenotypic differentiation in grasshoppers (Hernández-Teixidor et al., 2014; Nogueras, Cordero, & Ortego, 2016; Ortego, Aguirre, & Cordero, 2012; Sathyan, Engelbrecht, & Couldridge, 2017). Orthopterans represent also an ideal model to address sexual selection issues (Greenfield, 1997; Robinson & Hall, 2002), since female uses male song and morphology both for species recognition and selection between conspecific males (Klappert & Reinhold, 2003; Saldamando et al., 2005). Moreover, grasshopper body size is strongly influenced by climate, and gene-environment covariation in development has been observed along climate gradients (Laiolo, Illera, & Obeso, 2013; Laiolo & Obeso, 2015; Laiolo & Obeso, 2017).

In spite of these premises, however, it is also worth saying that alpine populations have often low genetic diversity as a result of the founder effects experienced during the recolonization of high elevations during the Pleistocene glacial phases (Ehinger, Fontanillas, Petit, & Perrin, 2002; Oromi, Richter-Boix, Sanuy, & Fibla, 2012). A study in this specific biogeographic context can, therefore, help shedding light on this aspect, as a way to test the evolutionary potential of alpine organisms. First, we analysed the spatial structure of genetic and phenotypic variability. We addressed the spatial extent of genetic (mtDNA), morphologic and acoustic variation, partitioning variation among massifs and populations, and tested the relationships between genetic and phenotypic differentiation versus geography and climate. We compared pairwise levels of genetic differentiation (F_{ST}) with pairwise geographic and climatic distances between populations (Barrowclough & Zink, 2009). We also tested if morphological and acoustic distance matrices were correlated with genetic, geographic or climatic ones to evaluate if, similarly to genetic data, phenotypic variation responds to geographical and/or climatic differentiation among populations. Under an isolation by distance scenario, the main differentiation force is expected to be drift, where geographic distance is expected to be the main driver

(Wilkins et al., 2013). Under an isolation by adaptation scenario, the environment will determine genetic and phenotypic spatial structure. In the lack of clear clines, however, multiple and overlapping causes may be evoked to explain the spatial structure of variation, being sexual selection one of these. To test for the effects of sexual selection, we performed an inter- and intrapopulation crossing experiment to investigate the relationship between male phenotypic traits (body size, song spectrographic features and singing rate) on female preference (distance with mate), and between the latter and reproductive parameters (occurrence of reproduction, number of eggs and emerged adults). The song is a key trait in sexual selection and population divergence in the *Chorthippus* genus: song patterns represent the main isolating barrier, being other types of pre-mating and post-mating barriers weak, as opposite to bush crickets in which this trait is highly conserved (Ciplak, Heller, & Willems, 2009; Heller, 2005). A number of features of the calls of Orthoptera influence female choice including the structure of calls, call loudness and call rate (reviewed in Robinson & Hall, 2002; *Chorthippus biguttulus*: Klappert & Reinhold, 2003). Some song characteristics, such as song duration or pause duration among syllables, often vary with male body size (Stange & Ronacher, 2012), and this trait may undergo sexual selection as well (Wagner, 2011). A simultaneous change in these traits and female preference would suggest that pre-zygotic isolation among populations is maintaining

differentiation, while female selection for different traits in different populations would indicate divergence in the patterns of sexual selection (Panhuis et al., 2001).

2 | MATERIALS AND METHODS

Chorthippus cazurroi is a flightless grasshopper that inhabits the upper portions of eastern Cantabrian Mountains, from 1,400 to 2,600 m a.s.l. The species distribution, covering 380 km² ca, is scattered in a matrix of unfavourable forested lowland habitats (Laiolo & Obeso, 2015; Laiolo et al., 2013). We sampled individuals at 23 sites (hereafter populations) located in the Picos de Europa National Park, and the Ponga and Redes Natural Parks, and distributed into six massifs (Figure 1, see Appendix S1, Table S1.1). This area reasonably represents the whole range distribution of the species as it was not found in another 166 sampling sites throughout the Cantabrian Mountains (Laiolo, Pato, & Obeso, 2018). Grasshoppers were collected by hand or using sweep nets mainly in July–November 2011–2013, with a few populations (5) sampled in 2015–2017. Individuals used for genetics and biometry were stored in 50-ml centrifuge tubes and frozen upon returning to the laboratory, whereas grasshoppers used for song recordings and experiments were reared in the laboratory (see below).

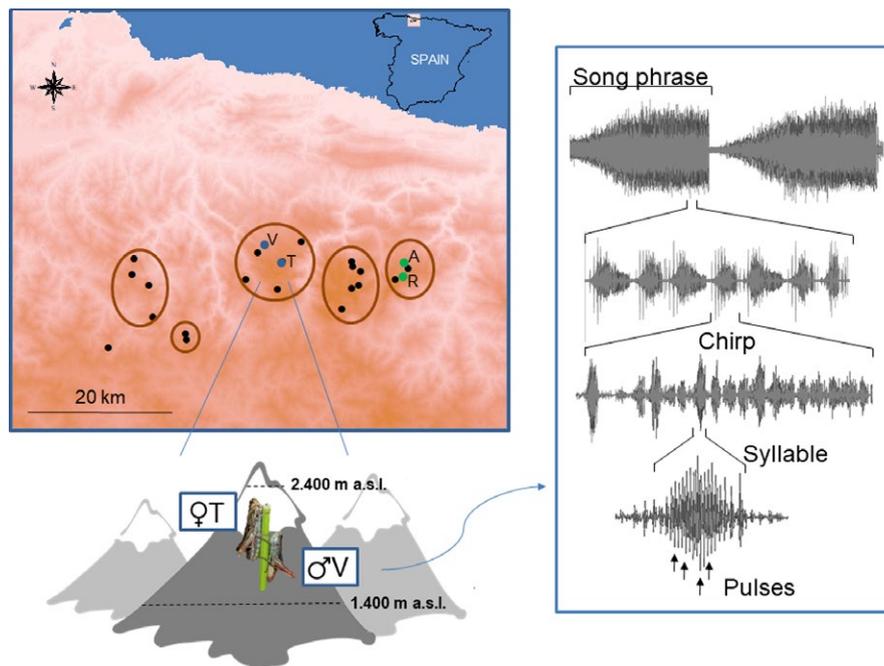


FIGURE 1 Map showing the 23 locations distributed in the Cantabrian Mountains where *Chorthippus cazurroi* was sampled (orange scale darker towards mountaintops). Populations were grouped in circles according to their massif (from left to right): Ponga massif, Colláu Zorru massif, Western massif, Central massif and Eastern massif. The westernmost population belongs to a sixth massif in which the species only occurred in one site. The four populations used in the crossing experiment are highlighted in blue (V: Vegarredonda, T: Traviesos) and green (A: Andara casetón, R: Rasa.). Below, a diagram indicating the origin of populations used for crossing experiments in the laboratory, in this case showing a cross between a male from Vegarredonda (lower elevation) and a female from Traviesos (higher altitude). On the right, oscillograms of the male calling song of *Chorthippus cazurroi* are shown, together with the terminology used to define song portions [Colour figure can be viewed at wileyonlinelibrary.com]

2.1 | Molecular procedures

A total of 326 individuals (6–19 per population) were used for molecular analyses. DNA was extracted from the hind femur of frozen specimens using the Qiagen Dneasy kit according to the manufacturer's instructions. A region of the mitochondrial cytochrome oxidase subunit I (COI) was amplified using the primers LCO1490 and HCO2198 (Folmer, Black, Hoeh, Lutz, & Vrijenhoek, 1994). Polymerase chain reactions (PCR) were performed in a 10 μ l volume containing 5.0 μ l of Gotaq Green Master Mix (Promega, Madison, WI), 1.5 μ l of DNA (around 50 ng of template) and 0.5 μ l of each primer (10 mM), following conditions used by Laiolo et al. (2013). Sequencing reactions were performed using the Perkin Elmer BigDye 3.1 (Applied Biosystems, Carlsbad, CA) terminator reaction mix in a volume of 10 μ l using 1 μ l of PCR product. PCR conditions were as follows: initial denaturation at 94°C for 2 min followed by 25 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s and extension at 60°C for 2 min, followed by a final extension at 60°C for 1 min. The final product was purified and sequenced on an ABI PRISM® 3130xl Genetic Analyzer. Unique COI sequences have been deposited in the National Center for Biotechnology Information (NCBI) gene bank database under the accession numbers MH271324–MH271356. The sequences were aligned by eye using BioEdit 7.0.9 (Hall, 1999) and Chromas Lite 2.1.1 (<http://chromas-lite.software.informer.com/2.1>).

2.2 | Morphometry

By means of a stereoscopic microscope LEICA M125 fitted with an ocular micrometre (accuracy 0.1 mm), we measured four morphological traits as proxies for body size from adult males and females of the 23 populations: (a) length of the left hind femur (hereafter femur length), (b) mid-dorsal pronotal length, (c) total body length (from the head to the last abdominal tergite, excluding supra-anal and subgenital plates, and (d) length of the left wing (Table S1.2) (Laiolo et al., 2013). In a few individuals (<10), the left hind femur was missing and the right one was measured. Mean values of morphological variables per population were standardized prior to analyses, and Euclidean pairwise distances between populations were calculated. Given a certain degree of sexual size dimorphism (Laiolo et al., 2013), separate matrices were constructed for males and females.

2.3 | Acoustics

Adult males from 10 of the 23 populations (from 1 to 12 per population, Table S1.3) were maintained in the laboratory and fed with fresh grass provided *ad libitum* to record their calling songs (i.e. male call to attract females). Songs were recorded by means of a Marantz PMD661 recorder with a sampling frequency of 96.6 kHz and then analysed with the software Avisoft SASLab Pro 3.91 (Avisoft Bioacoustics, Glienicke, Germany). All recordings included spontaneous calling songs and were obtained from untethered intact animals at a temperature of c. 30°C. As in other gomphocerine grasshoppers,

song production in *C. cazurroi* occurs by rubbing the row of stridulatory pegs on the inner side of the hind femur against a modified wing vein. This behaviour is shared between gomphocerines, but the coordination of movements may differ largely between closely related species, thus also their songs (Elsner, 1974; Ragge & Reynolds, 1988). Typically, *C. cazurroi* produces four quick, loud syllables per chirp/echeme (Reynolds, 1987). However, as in other gomphocerine species, the number of syllables can increase when the two hind legs move slightly out of phase, masking gaps within the chirp (Elsner, 1974). Thus, the number of syllables in this case represents how out-of-phase males sang. Here, we considered four acoustic parameters, as depicted in Figure 1 following terminology in Elsner (1974). These were chirp duration, the number of syllables per chirp, syllable duration (the central syllable of the chirp was measured) and the number of pulses per syllable. These variables have been shown to play a role in sexual selection in other species of the *Chorthippus* genus (Gottsberger & Mayer, 2007; Klappert & Reinhold, 2003; Saldamando et al., 2005). In each song, we took measurements on five chirps at the end of the first song phase (corresponding to the sequence of first order in Elsner, 1974) and estimated the average value per individual of the above variables. To avoid observer effects, all measurements were made by the same researcher (JP). Among the above variables, the number of pulses was excluded from the analyses as it showed high levels of intra-individual variability (Table S1.4). The same morphological traits mentioned above were measured in these males upon death, and we also counted the number of stridulatory pegs and the overall length of the peg row. Mean values of acoustic variables per population were standardized prior to analyses, and Euclidean distances between pairs of populations were calculated.

2.4 | Geographic and ecological variables

Geographic distances were calculated from coordinates recorded in the field with Garmin Navigators and *log* transformed for analyses. As a proxy for the ecological characteristics of each population, we used a selection of variables associated with the thermal niche, given that the habitat (grasslands with high percentages of rock cover; Laiolo & Obeso, 2015) and the precipitation regime (Atlantic climate with abundant rainfall, Laiolo, Illera, Meléndez, Segura, & Obeso, 2015) changed little throughout its distribution. Four variables (average temperature, minimum temperature, maximum temperature and sunshine radiation) were extracted from the digital layers of the Climate Atlas of the Iberian Peninsula (Ninyerola, Pons, & Roure, 2005, description in Table S1.1) and standardized prior to analyses. Euclidean distances between pairs of populations were calculated for these variables as described above.

2.5 | Behaviour and reproduction

We carried out a crossing experiment to assess the relationships between male calling song and female behaviour, and between the latter and male reproductive output. We crossed two populations located in the Eastern Massif (Andara casetón, A, at 1,660 m a.s.l.,

and Rasa, R, at 2,280 m), and two populations located in the Western Massif (Vegarredonda, V, at 1,465 m and Traviesos, T, at 2,395 m) (Figure 1), which were known to show variation in body size (Laiolo & Obeso, 2015). These crosses permitted to test for the possible reproductive isolation of populations that display high level of morphological differentiation.

Early in the season of 2013 (July–August), we collected individuals in the first or second instar phase, and some adults. Individuals caught in the wild were kept under natural photoperiod and temperature (26–33°C day/19–23°C night) conditions with grass provided *ad libitum*. From 2–3 days after moult, they were assorted into pairs in individual plastic jars. Eight crossing types were established according to the origin population of both female and male (intra- and interpopulation crosses within each massif: AxA, hereafter AA, AR, RR, RA, TT, TV, VV and VT). Some intrapopulation crosses included females that were collected as adults and served solely for mate behavioural observations, while the rest of the pairs included a virgin female (moulting in the laboratory) and also served for studying male reproductive performance. We performed from four to six 5-min observation sessions per day, during the late morning. During the sessions, we recorded male calling songs (as described above), noted the singing rate (proportion of observations in which the male sang) and measured mate distance (in cm) with a ruler. The morphology of these individuals at death was measured as described above. We obtained the complete set of behaviour and morphological data from 103 pairs, but songs were recorded in only 30 paired males. In the 48 pairs with virgin females, we checked if female laid eggs (occurrence of reproduction, treated as a binary variable). We also measured the total number of eggs and hatched eggs (32 out of 48 paired females laid eggs) and the total number of emerged adults (24 pairs produced instars that successfully moulted to adults). These reproductive variables were measured by tracking the complete life cycle of these grasshoppers 6 months later. Egg pods were maintained at room temperature for 30 days, transferred to a refrigerator at 5°C for 4.5 months to break diapause, and then incubated until hatching (details in Laiolo & Obeso, 2015). All hatched nymphs were reared in the same conditions, fed with the same mixture provided to adults (plus wheat leaves grown in greenhouses) and exposed 4 hr a day to the light of 40W lamps at 33–38°C, 8 hr to natural light at 26–33°C and 12 hr in the dark at 19–23°C.

2.6 | Statistical analyses

2.6.1 | Partitioning of variance

We tested differentiation among populations calculating pairwise F_{ST} values using the program DNASP 5.10.01 (Librado & Rozas, 2009). In addition, we built a parsimony haplotype network using the program TCS 1.21 (Clement, Posada, & Crandall, 2000), with the aim of inferring genealogical relationships among mitochondrial sequences. We performed an analysis of molecular variance (AMOVA) using the program ARLEQUIN 3.5.1.2 (Excoffier & Lischer, 2010) to test the hypothesis that genetic differences are stronger among than within massifs, which could suggest a scenario of differentiation among mountains

with some genetic admixture within massifs. Variance was also partitioned among and within massifs by considering morphological and acoustic traits in univariate linear mixed models with massif and population nested into massif as random factors ('aov' and 'lmer' functions, R package 'lme4', Bates, Maechler, Bolker, & Walker, 2015). These analyses were performed with femur length, the number of syllables and syllable duration as dependent variables, given that the rest of morphological and acoustic traits were highly correlated with these variables (see Results). Five massifs were considered in partitioning analyses, as only one population was found in the sixth massif (Figure 1).

2.6.2 | Correlation among dissimilarity matrices

We tested if morphological, genetic (23 populations) and acoustic (10 populations) distances among populations were correlated among each other and with geographic or ecological distances by means of Mantel tests and partial Mantel tests with 9,999 random permutations ('mantel' function, R package 'vegan', Oksanen et al., 2012). Mantel tests calculate correlation coefficients between similarity/dissimilarity matrices whereas the partial Mantel test calculates the relationship between two matrices after controlling for covariation with a third matrix. The relationship between morphological and acoustic variables was also assessed by means of univariate tests at the level of the individual, by means of linear mixed models with population as a random factor with the 'lme' function (R package 'nlme', Pinheiro, Bates, DebRoy, & Sarkar, 2017).

2.6.3 | Experimental crossing

We first tested for differences in male femur length and song parameters among the four different populations, rather than among crossings, given that these features are not expected to change with respect to mate identity, as opposed to behavioural variables, likely influenced by motivation or mate choice. We used ANOVA and Tukey's post hoc test with male population (four levels) as a fixed factor for these latter analyses. To assess the potential of interbreeding between populations, we tested whether behavioural features (mate distance and male singing rate) and reproduction variables (occurrence of reproduction, number of eggs and emerged adults) changed among different types of crossing. This served to check if females (through approximation to the male and fertility) and males (through singing rate) behaved differently in intra- and interpopulation crossings, which can indicate possible pre-zygotic and/or post-zygotic barriers. For this purpose, we used statistical models with crossing type as a fixed factor. In the case of mate distance and male singing rate, we used a one-way ANOVA and Tukey's post hoc test to test for differences among crossing types within massifs. Since crosses among different populations showed similar values for mate distance and reproduction variables when the male or the female belonged to a given population (i.e. male A+female R = male R+female A, etc.; Figures S1.1 and S1.2), data from these crossings were summed so as to obtain three levels of the factor crossing type (AA, RR and AR+RA for the Eastern massif, and TT, VV and

VT+TV for Western massif). In the case of reproduction variables of virgin females, we only considered the Eastern massif data due to the low number of laying pairs of the Western massif. Generalized linear models were used for the latter analysis, with binomial (occurrence of reproduction, 'glm' function in R) or negative binomial distribution (number of eggs, hatched eggs and emerged adults, 'glm.nb' function), and controlling for female life span. Tukey's post hoc test was used to check differences among crossing types ('glht' function).

Finally, we scrutinized the role of male song on female behaviour. First, we analysed the relationship between mate distance and male singing rate with linear models in which we also entered male femur length, female population and the interaction between song rate and population (to highlight potential differences in female preferences among populations). In addition, we tested for the relationship between behavioural features and acoustic variables, centring on crossings within the same population but excluding crossings from different populations. We used linear mixed models ('lme' function) entering crossing type as a random factor and again controlling for male femur length. We then tested for the relationship between the behavioural features and reproductive output, centring on crossings with virgin females. Reproduction variables could not be directly related to acoustic variables as the number of singing males successfully paired with virgin females was too low for analyses (only 6 were successful). Generalized linear mixed models were used, with binomial distribution (occurrence of reproduction) or negative binomial distribution (number of eggs, hatched eggs and emerged adults) with the 'glmmadmb' function in the R package 'glmmADMB' (Fournier et al., 2012) and controlling for the effects of female and male size on fecundity (e.g. Akman & Whitman, 2008).

When necessary, we dealt with collinearity by dropping covariates based on the variance inflation factor (VIF <3; Zuur, Ieno, & Elphick, 2010). We performed *log* transformations when model residuals were not normally distributed in the case of linear and linear mixed models.

3 | RESULTS

3.1 | Genotypic and phenotypic divergence among populations

Genetic data were obtained from 326 individuals from 23 populations, morphological traits were measured in 168 males and 271 females from 23 populations, and songs were analysed from 60 males of 10 populations (Table S1.2 and S1.3). A total of 33 unique haplotypes were found. Molecular variance analyses provided a significant level of differentiation among massifs ($\Phi_{ST} = 0.98$, $p < 0.0001$) and among populations within massifs ($\Phi_{SC} = 0.66$, $p < 0.0001$), but we did not find a significant differentiation within populations ($\Phi_{SC} = 0.95$, $p = 0.1180$) (Table S1.5). The haplotype network showed an incipient level of differentiation among massifs, despite some massifs shared common haplotypes (Figure S1.3). Variation among massifs in femur length (41%–45%) and syllable number (46%) was also significant, although a high

proportion of variance was also found among populations within massifs (13%–36%; Table S1.5).

Male and female morphological ($p < 0.001$), acoustic ($p = 0.003$) and genetic ($p = 0.003$) distances were all positively related to geographic distances (Figure 2). Genetic distances were not, however, related to the sets of phenotypic distances (Table S1.6). Pairwise acoustic distances increased significantly with morphological distances ($p = 0.008$), and this relationship remained significant when controlling for geographical or ecological distance ($p = 0.033$, $p = 0.003$, respectively). Finally, the sole phenotypic dissimilarities that were conditioned by ecological distances were those associated with female morphology ($p = 0.019$). When analysing variation at the individual rather than population level, larger males (longer femurs) with more stridulatory pegs produced longer chirps and more syllables per chirp (Table S1.7).

3.2 | Behavioural and reproductive data

3.2.1 | Intra- versus interpopulation crosses

We measured morphology, singing rate and female distance in 103 pairs, analysed songs in 30 paired males and checked reproductive variables in 48 pairs. Confirming previous evidence (Laiolo & Obeso, 2015), male femur length differed among populations and was always smaller in high elevation populations ($F_{3,97} = 6.558$, $p < 0.001$, Figure 3a). Acoustic characteristics also tended to vary significantly among populations (Figure 4, Table S1.8). Conversely, male singing rate did not differ (among Eastern massif crossings: $F_{2,47} = 0.312$, $p = 0.734$; among Western massif crossings: $F_{2,48} = 1.662$, $p = 0.200$; all pairwise comparisons n.s.). Mate distance varied slightly among crossing types and tended to be shorter for intrapopulation crossings, with significant results in the Eastern massif ($F_{2,47} = 6.971$, $p = 0.002$) and marginally significant in the Western massif ($F_{2,48} = 2.883$, $p = 0.066$). However, when scrutinizing pairwise comparisons, only females from one population (R) showed differential mate approximation in inter- and intrapopulation crossings (Figure 3b). Results of reproductive parameters also did not point to marked differences (Table S1.9; Figure S1.2). The number of eggs, after controlling for female life span, was larger in the lowland crossing AA with respect to RR and AR, but this difference did not persist at the phase of adult emergence.

3.2.2 | Intersexual selection: male song effects on female behaviour

We found that mate distance was related to male singing rate ($F_{1,92} = 15.490$, $p < 0.001$, Figure 5) with no difference among populations in this relationship (interaction between singing rate and female population, $F_{3,92} = 0.625$; $p = 0.600$) and no effect of male femur length ($F_{1,92} = 3.365$, $p = 0.07$). This result suggests that females tended to approach males with high singing propensity rather than large ones, and this occurred in all populations. Females also more closely approached males producing more syllables per chirp

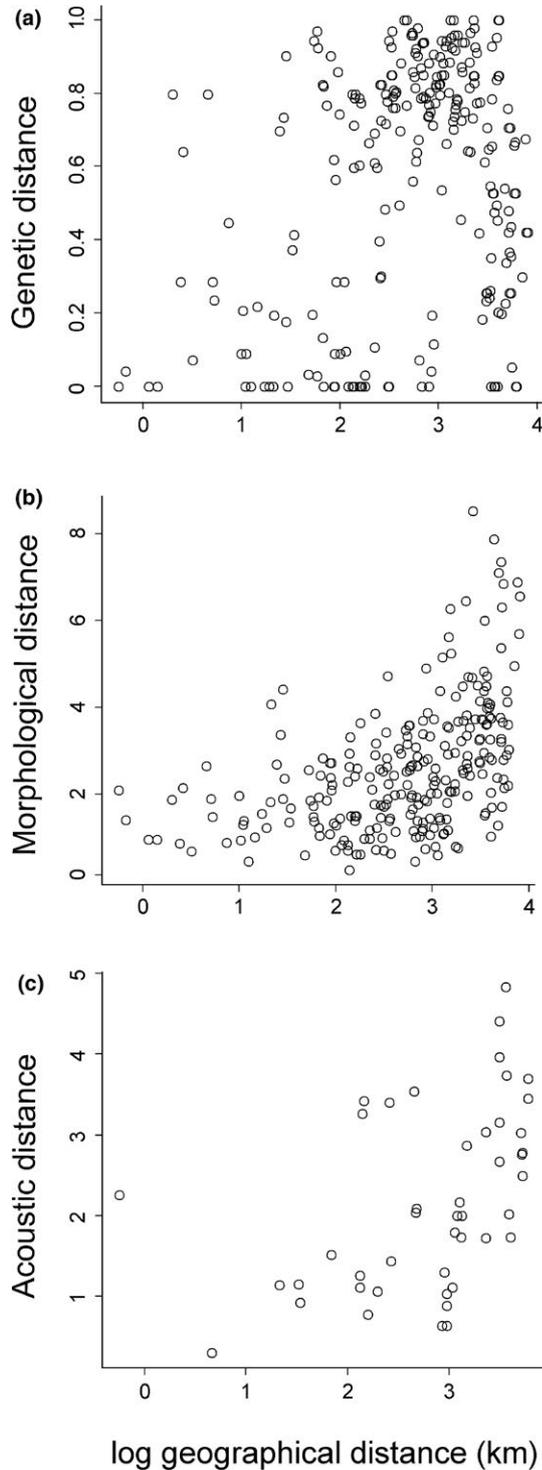


FIGURE 2 Influence of pairwise geographical distance (log transformed) on (a) genetic, (b) morphological (males) and (c) acoustic distance for *Chorthippus cazurroi* in the Cantabrian Mountains

(Table 1). Female preferences expressed through mate distance were not related to any reproduction variable when controlling for female life span and the size of mates (Table 2). Conversely, male singing rate (a variable that did not vary among populations and crossings) almost significantly conditioned the number of eggs, hatched eggs and emerged adults (Table 2).

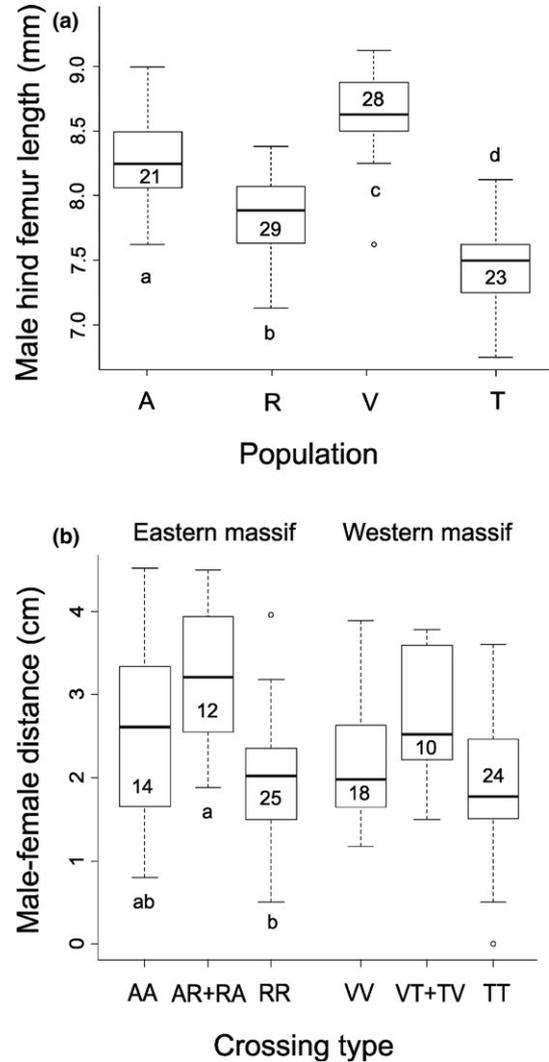


FIGURE 3 (a) Male hind femur length from the four populations of *Chorthippus cazurroi* used in the crossing experiment. (b) Distance between males and females in different crosses according to the original population of individuals of *Chorthippus cazurroi* in the Cantabrian Mountains (AA: Andara casetón female × Andara casetón male, AR: Andara casetón female × Rasa male, RA: Rasa female × Andara casetón male, RR: Rasa female × Rasa male, VV: Vegarredonda female × Vegarredonda male, VT: Vegarredonda female × Traviesos male; TV: Traviesos female × Vegarredonda male, TT: Traviesos female × Traviesos male). The number of pairs per crossing type is shown inside boxes. For the Eastern massif, differences between crossing types are shown with different letters; no differences were found in the Western massif

4 | DISCUSSION

In spite of its narrow distribution, *Chorthippus cazurroi* displays substantial genetic and phenotypic variability. Morphological and acoustic differences were compatible with an isolation by distance scenario in males, whereas a combined scenario of isolation by distance and adaptation (or phenotypic plasticity, matching climatic variation) explained morphological divergence in females. Slightly higher interindividual mate distances were observed in experimental crossing of phenotypically divergent populations, but this behaviour had no apparent

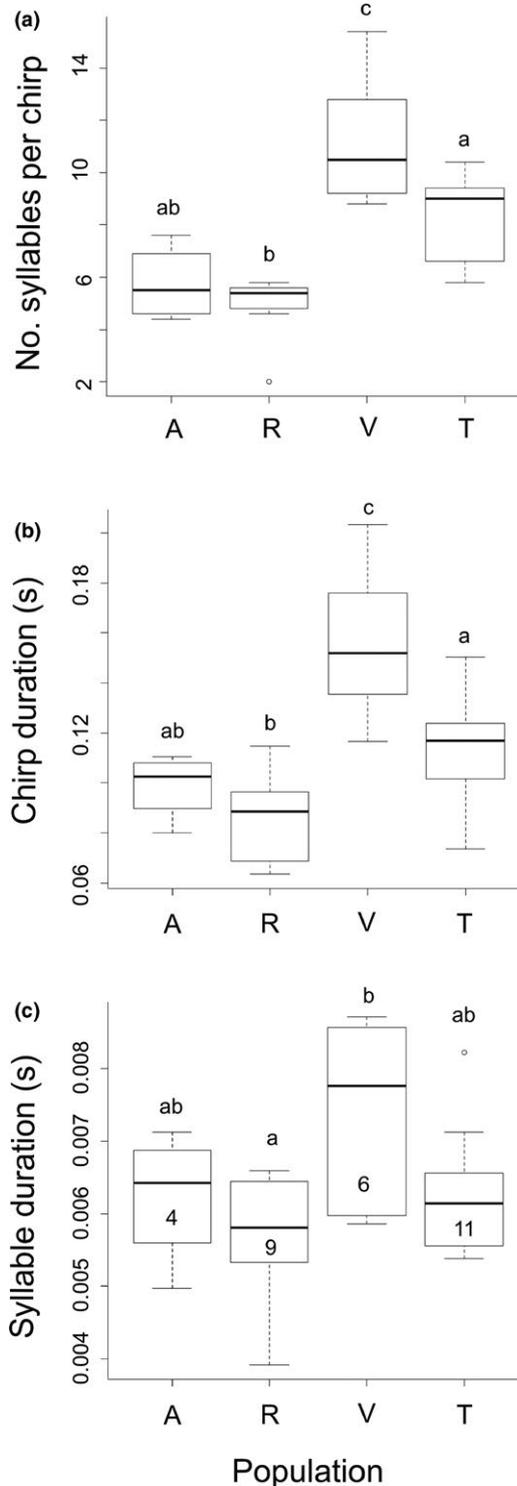


FIGURE 4 Number of syllables per chirp (a), chirp duration (b) and syllable duration (c) in recorded *Chorthippus cazurroi* males from the four different populations located in Cantabrian Mountains and used in the crossing experiment (A: Andara casetón, R: Rasa, V: Vegarredonda, T: Traviesos). Differences among populations are shown with different letters. The number of males per population is shown inside boxes in (c)

consequences on reproduction. Similarly, no change in the direction of sexual selection has been observed, as the same song parameters influenced female behaviour irrespective of population identity. In

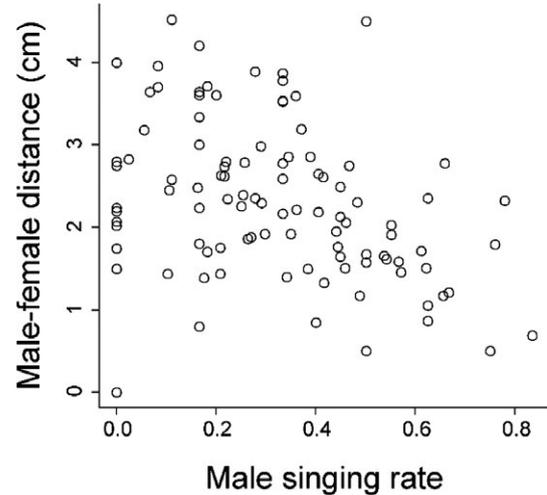


FIGURE 5 Relationship between the frequency of male singing rate and the distance between males and females during the pairing experiment with *Chorthippus cazurroi* from the Cantabrian Mountains

particular, high singing rates, dependent on body condition in organisms with stridulatory apparatus, appears to be under strong selection and almost significantly led to enhanced reproductive output.

4.1 | Genotypic, phenotypic and reproductive differences among populations

Our findings suggest that *C. cazurroi* exhibits a genetic divergence congruent with the geographical location of its different populations. The relationship between genetics and geography is probably the result of the isolation after colonization fuelled by the poor dispersal potential of this species, which likely determined that geographically distant populations have progressively become unconnected over time. These results are compatible with a scenario of incipient differentiation with some genetic admixture and/or uncompleted lineage sorting. The lack of climate effects on genetic differentiation, in turn, is not supporting the idea of isolation by adaptation, a result that should, however, be confirmed by tests with other molecular markers. Morphology and acoustic data display indeed higher levels of population variation, thus likely faster divergence than the molecular marker target of this study. It is also possible that the restricted range of the species limits the potential action of the environment, less variable than the gradient experienced by taxa with broad distributions (see Shafer & Wolf, 2013 and Sexton, Hangartner, & Hoffmann, 2014 for reviews). This general idea, however, may not apply to southern Europe alpine species, the genome of which has been moulded by several climate cycles in glacial and interglacial periods, with outcomes difficult to predict from present-day patterns (Hewitt, 2004).

Similar to genetic patterns of differentiation, both acoustic and morphological divergence were correlated with geographical distances. To explain these phenotypic patterns, it is again important to stress that divergence along geographic distance can also

TABLE 1 Results of linear mixed models testing the relationship between (A) mate distance and (B) male singing rate and song characteristics in *Chorthippus cazurroi* from the four different populations used in the crossing experiment, located in the Cantabrian Mountains. In the full model, dependent variables were modelled as a function of the covariates listed in the first column. In the reduced model, statistical values are presented for the model after collinearity has been removed. In both cases only one variable removal was necessary. The variance explained by variables with a significant effect (in bold) is shown. Population was entered as a random factor

	Full model				Reduced model			
	df	t	p	VIF	df	t	p	Variance (%)
(A) Mate distance								
Male femur length	22	1.821	0.082	1.598	24	1.670	0.108	
Chirp duration	22	-0.245	0.808	5.783				
Syllables per chirp	22	-1.532	0.140	3.254	24	-2.826	0.009	20.3
Syllable duration	22	0.527	0.603	2.003	24	0.567	0.575	
(B) Male singing rate								
Male femur length	22	-1.429	0.167	1.598	24	-0.618	0.543	
Chirp duration	22	1.632	0.117	5.783				
Syllables per chirp	22	-0.154	0.879	3.254	24	1.798	0.085	
Syllable duration	22	-0.574	0.572	2.003	24	0.340	0.737	

TABLE 2 Results of generalized mixed models testing the relationships between reproductive variables (occurrence of reproduction, total eggs, total hatched eggs and total emerged adult) and behavioural variables (mate distance and male singing rate) in *Chorthippus cazurroi* from the four different populations used in the crossing experiment, located in the Cantabrian Mountains. Crossing type was entered as a random factor. Statistically significant values ($p \leq 0.05$) are highlighted in bold

	Occurrence of reproduction		Egg no.		Hatched egg no.		Emerged adult no.	
	z	p	z	p	z	p	z	p
Female life span	2.93	0.003	4.72	<0.001	1.21	0.225	1.63	0.097
Female femur length	0.76	0.449	0.24	0.808	-0.90	0.368	0.08	0.946
Male femur length	0.15	0.883	0.97	0.330	1.63	0.103	0.31	0.750
Mate distance	1.27	0.202	0.62	0.533	-0.27	0.790	0.46	0.650
Male singing rate	-0.34	0.736	1.86	0.063	1.68	0.092	1.81	0.070

depend on the time span over which variation can arise. For example, some populations of the European meadow grasshopper *Chorthippus parallelus* were confined to glacial refuges during the Quaternary climatic oscillations, whereas others diverged during subsequent expansions events after post-glacial expansions. These contractions and expansions caused distinctive patterns of divergence in song and morphology in this grasshopper species, resulting in marked shifts in the number of syllables per chirp and the duration of each chirp in male songs (Tregenza et al., 2000a). In our study, we found a similar pattern of variation for these traits but at a much more reduced scale (380 km² vs. European continent), which would indicate that phenotypic differentiation may occur even when genetic exchange between neighbouring populations is feasible. Although part of the variation we observed in relation to climate, especially in females, may be the result of phenotypic plasticity rather than of local adaptations, it is worth stressing that genetic effects cannot be discarded in the species, given that the size of offspring reared in different thermal conditions remains highly correlated to parental body size (Laiolo & Obeso, 2015; this study).

4.2 | Trait variation and mating

Sexual selection is typically evoked to explain both the rapid divergence of phenotypic traits involved in reproduction and the lack of environmental clines in these variables (Panhuis et al., 2001). In line with this hypothesis, a substantial variation in male morphology and bioacoustics of *C. cazurroi* is found within massifs and these sets of features do not vary with climate. However, we also found no clear impediment to reproduction between populations diverging in these traits, which means that these features do not constitute a strong intrinsic barrier and are rather neutral from a sexual selection point of view. The pattern we observed is compatible with the action of stochastic forces highlighted by previous results, and is not comparable, for instance, to the magnitude of divergence in naturally hybridizing *Chorthippus brunneus* and *C. jacobsi*. In the latter case, variation in syllable length is associated with shifts in female preferences across a geographic cline of similar extent (Bridle, Saldamando, Koning, & Butlin, 2006; Saldamando et al., 2005). When scrutinizing the song traits selected by females, the sole male feature with an almost significant effect on reproduction in *C. cazurroi* is singing rate, which is

under selection in all populations and, notably, does not vary among experimental populations. By combining this evidence with that on biogeographic patterns and the history of the study area, our result seems compatible with a scenario in which populations have passed through founder events of low population density during which mate choice on phenotypic traits has relaxed, opening the way to stochastic and, to a minor extent, environmental driven differentiation.

We acknowledge that a choice experiment in which females simultaneously assess the quality of multiple males, as well as a test of importance of body size in male–male competition, would have helped obtaining more conclusive evidence about the reproductive consequences of the study traits. Yet, our results still provide useful information on the nature of traits under selection. In all experimental populations, we found that female approach more closely males singing more frequently and with more syllables per chirp after controlling for male size. In other words, females select males that strive for songs greater than those corresponding to their size, a result that also explains why we only found slight changes (in just one population) in distance among mates with significant differences in body size. If we take into account that the number of syllables per chirp represents a lack of synchrony in stridulation, that is reduced gap duration between syllables, a preference for males with more syllables per chirp may correspond to female preferences for gapless songs observed in other grasshopper species (Klappert & Reinhold, 2003). Moreover, in crickets song low pause to syllable ratios (Hoback & Wagner, 1997) and high song rate (Gray & Eckhardt, 2001; Holzer, Jacot, & Brinkhof, 2003; Wagner & Hoback, 1999) have been proved to be costly, and singing exposes males to predation or parasitism (Wagner, 2011). On the bases of this evidence, and on the fact that singing is, in any case, more costly than non-singing, it can be argued that the singing rate is a better indicator of male quality than larger body sizes per se, and that *C. cazurroi* females are selecting, through the continuous motivation to sing, males in better physical condition.

5 | CONCLUSIONS

The relationships between genetics, climate, geography, morphology and acoustics suggest that geographic patterns of divergence in *C. cazurroi* have been mainly generated through serial colonization from nearby populations and subsequent genetic drift, favoured by the limited dispersal capacity and restricted habitat of the species. Sexual selection tends to be relaxed on phenotypic traits, thus is likely a minor actor in the process of differentiation. This does not exclude that some song features associated with individual condition are under strong selection and, as predicted by sexual selection theory, they do not vary so extensively. All in all, this study dismisses the idea that alpine specialists inhabiting narrow distributions lack substantial genetic and phenotypic variability, and highlights the importance of synthesizing biogeographic and experimental approaches to obtain stronger and deeper inferences about the dynamics and mechanisms of biological differentiation.

ACKNOWLEDGEMENTS

We are grateful to M. Brunetti, A. Segura and L. Viesca for field and laboratory assistance, and to Sarah Young for editing the language. We also thank Picos de Europa National Park and Consejería de Medio Ambiente del Principado de Asturias for providing permissions to collect grasshoppers in protected areas. Funding for this study was provided by grant 4278 of the British Ecological Society, grants CGL2011-28177, CGL2014-53899-P and CGL2017-85191-P from the Spanish Ministry of Economy, Industry and Competitiveness, and grant LIQUENES 2014 from FICYT and edp-HC Energía.

ORCID

Joaquina Pato  <http://orcid.org/0000-0002-1919-5181>

REFERENCES

- Akman, O., & Whitman, D. (2008). Analysis of body size and fecundity in a grasshopper. *Journal of Orthoptera Research*, 17, 249–257. <https://doi.org/10.1665/1082-6467-17.2.249>
- Andersson, M., & Simmons, L. W. (2006). Sexual selection and mate choice. *Trends in Ecology & Evolution*, 21, 296–302. <https://doi.org/10.1016/j.tree.2006.03.015>
- Armstrong, C., Richardson, D. S., Hipperson, H., Horsburgh, G. J., Küpper, C., Percival-Alwyn, L., ... Spurgin, L. G. (2018). Genomic associations with bill length and disease reveal drift and selection across island bird populations. *Evolution Letters*, 2, 22–36. <https://doi.org/10.1002/evl3.38>
- Barrowclough, G. F., & Zink, R. M. (2009). Funds enough, and time: mtDNA, nuDNA and the discovery of divergence. *Molecular Ecology*, 18, 2934–2936. <https://doi.org/10.1111/j.1365-294X.2009.04271.x>
- Bates, D., Maechler, M., Bolker, B., & Walker, S. (2015). Fitting linear mixed-effects models using lme4. *Journal of Statistical Software*, 67, 1–48.
- Bridle, J. R., Saldamando, C. I., Koning, W., & Butlin, R. K. (2006). Assortative preferences and discrimination by females against hybrid male song in the grasshoppers *Chorthippus brunneus* and *Chorthippus jacobsi* (Orthoptera: Acrididae). *Journal of Evolutionary Biology*, 19, 1248–1256. <https://doi.org/10.1111/j.1420-9101.2006.01080.x>
- Ciplak, B., Heller, K. G., & Willemsse, F. (2009). Review of the genus Eupholidoptera (Orthoptera, Tettigoniidae): Different genitalia, uniform song. *Zootaxa*, 2156, 1–77.
- Clement, M., Posada, D. C. K. A., & Crandall, K. A. (2000). TCS: A computer program to estimate gene genealogies. *Molecular Ecology*, 9, 1657–1659. <https://doi.org/10.1046/j.1365-294x.2000.01020.x>
- Cotton, S., Fowler, K., & Pomiankowski, A. (2004). Do sexual ornaments demonstrate heightened condition-dependent expression as predicted by the handicap hypothesis? *Proceedings of the Royal Society B: Biological Sciences*, 271, 771. <https://doi.org/10.1098/rspb.2004.2688>
- Ehinger, M., Fontanillas, P., Petit, E., & Perrin, N. (2002). Mitochondrial DNA variation along an altitudinal gradient in the greater white-toothed shrew, *Crocidura russula*. *Molecular Ecology*, 11, 939–945. <https://doi.org/10.1046/j.1365-294X.2002.01487.x>
- Elsner, N. (1974). Neuroethology of sound production in gomphocerine grasshoppers (Orthoptera: Acrididae). *Journal of Comparative Physiology*, 88, 67–102. <https://doi.org/10.1007/BF00695923>
- Excoffier, L., & Lischer, H. E. (2010). Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux

- and Windows. *Molecular Ecology Resources*, 10, 564–567. <https://doi.org/10.1111/j.1755-0998.2010.02847.x>
- Folmer, O., Black, M., Hoeh, W., Lutz, R., & Vrijenhoek, R. (1994). DNA primers for amplification of mitochondrial cytochrome C oxidase subunit I from metazoan invertebrates. *Molecular Marine Biology and Biotechnology*, 3, 294–299.
- Fournier, D. A., Skaug, H. J., Ancheta, J., Iannelli, J., Magnusson, A., Maunder, M. N., ... Sibert, J. (2012). AD Model Builder: Using automatic differentiation for statistical inference of highly parameterized complex nonlinear models. *Optimization Methods and Software*, 27, 233–249. <https://doi.org/10.1080/10556788.2011.597854>
- Gottsberger, B., & Mayer, F. (2007). Behavioral sterility of hybrid males in acoustically communicating grasshoppers (Acrididae, Gomphocerinae). *Journal of Comparative Physiology A*, 193, 703–714. <https://doi.org/10.1007/s00359-007-0225-y>
- Grant, B. R., & Grant, P. R. (2017). Watching speciation in action. *Science*, 355, 910–911. <https://doi.org/10.1126/science.aam6411>
- Gray, D. A., & Eckhardt, G. (2001). Is cricket courtship song condition dependent? *Animal Behaviour*, 62, 871–877. <https://doi.org/10.1006/anbe.2001.1825>
- Greenfield, M. D. (1997). Acoustic communication in Orthoptera. In S. K. Gangwere, M. C. Muralirangan, & M. Muralirangan (Eds.), *The bionomics of grasshoppers, katydids, and their kin* (pp. 197–230). Wallingford, UK: CAB International.
- Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. In Nucleic acids symposium series (Vol. 41, No. 41, pp. 95–98).
- Heller, K. G. (2005). Song evolution and speciation in bushcrickets. In S. Drosopoulos & M. Claridge (Eds.), *Insect sounds and communication: Physiology, behaviour, ecology and evolution* (pp. 144–158). Boca Raton, FL: CRC Press.
- Hernández-Teixidor, D., Lopez, H., Nogales, M., Emerson, B. C., Juan, C., & Oromi, P. (2014). Genetic, morphological, and dietary changes associated with novel habitat colonisation in the Canary Island endemic grasshopper *Acrostira bellamyi*. *Ecological Entomology*, 39, 703–715. <https://doi.org/10.1111/een.12151>
- Hewitt, G. M. (2004). Genetic consequences of climatic oscillations in the Quaternary. *Philosophical Transactions of the Royal Society of London B: Biological Sciences*, 359, 183–195. <https://doi.org/10.1098/rstb.2003.1388>
- Hoback, W. W., & Wagner, W. E. Jr (1997). The energetic cost of calling in the variable field cricket, *Gryllus lineaticeps*. *Physiological Entomology*, 22, 286–290. <https://doi.org/10.1111/j.1365-3032.1997.tb01170.x>
- Hoeck, P. E., Bollmer, J. L., Parker, P. G., & Keller, L. F. (2010). Differentiation with drift: A spatio-temporal genetic analysis of Galapagos mockingbird populations (*Mimus spp.*). *Philosophical Transactions of the Royal Society B: Biological Sciences*, 365, 1127–1138.
- Holzer, B., Jacot, A., & Brinkhof, M. W. (2003). Condition-dependent signaling affects male sexual attractiveness in field crickets, *Gryllus campestris*. *Behavioral Ecology*, 14, 353–359.
- Illera, J. C., Palmero, A. M., Laiolo, P., Rodríguez, F., Moreno, Á. C., & Navascués, M. (2014). Genetic, morphological, and acoustic evidence reveals lack of diversification in the colonization process in an island bird. *Evolution*, 68, 2259–2274.
- Illera, J. C., Spurgin, L. G., Rodríguez-Exposito, E., Nogales, M., & Rando, J. C. (2016). What are we learning about speciation and extinction from the Canary Islands? *Ardeola*, 63, 15–33. <https://doi.org/10.13157/arla.63.1.2016.rp1>
- Irwin, D. E., Thimman, M. P., & Irwin, J. H. (2008). Call divergence is correlated with geographic and genetic distance in greenish warblers (*Phylloscopus trochiloides*): A strong role for stochasticity in signal evolution? *Journal of Evolutionary Biology*, 21, 435–448. <https://doi.org/10.1111/j.1420-9101.2007.01499.x>
- Jenkins, D. G., Carey, M., Czerniewska, J., Fletcher, J., Hether, T., Jones, A., & McGuire, M. (2010). A meta-analysis of isolation by distance: Relic or reference standard for landscape genetics? *Ecography*, 33, 315–320.
- Kaneshiro, K. Y. (1989). The dynamics of sexual selection and founder effects in species formation. In L. V. Giddings, K. Y. Kaneshiro, & W. W. Anderson (Eds.), *Genetics, speciation and the founder principle* (pp. 279–296). New York, NY: Oxford University Press.
- Klappert, K., & Reinhold, K. (2003). Acoustic preference functions and sexual selection on the male calling song in the grasshopper *Chorthippus biguttulus*. *Animal Behaviour*, 65, 225–233. <https://doi.org/10.1006/anbe.2002.2034>
- Kwiatkowski, M. A., & Sullivan, B. K. (2002). Geographic variation in sexual selection among populations of an iguanid lizard, *Sauromalus obesus* (= *ater*). *Evolution*, 56, 2039–2051. <https://doi.org/10.1111/j.0014-3820.2002.tb00130.x>
- Laiolo, P., Illera, J. C., Meléndez, L., Segura, A., & Obeso, J. R. (2015). Abiotic, biotic, and evolutionary control of the distribution of C and N isotopes in food webs. *The American Naturalist*, 185, 169–182.
- Laiolo, P., Illera, J. C., & Obeso, J. R. (2013). Local climate determines intra- and interspecific variation in sexual size dimorphism in mountain grasshopper communities. *Journal of Evolutionary Biology*, 26, 2171–2183. <https://doi.org/10.1111/jeb.12213>
- Laiolo, P., & Obeso, J. R. (2015). Plastic responses to temperature versus local adaptation at the cold extreme of the climate gradient. *Evolutionary Biology*, 42, 473–482. <https://doi.org/10.1007/s11692-015-9341-8>
- Laiolo, P., & Obeso, J. R. (2017). Life-history responses to the altitudinal gradient. In J. Catalan, J. Ninot, & M. Aniz (Eds.), *High mountain conservation in a changing world. Advances in global change research* (vol. 62, pp. 253–283). Cham, CH: Springer. <https://doi.org/10.1007/978-3-319-55982-7>
- Laiolo, P., Pato, J., & Obeso, J. R. (2018). Ecological and evolutionary drivers of the elevational gradient of diversity. *Ecology Letters* 21(7), 1022–1032. <https://doi.org/10.1111/ele.12967>
- Lehtonen, P. K., Laaksonen, T., Artemyev, A. V., Belskii, E., Both, C., Bureš, S., ... Nord, A. (2009). Geographic patterns of genetic differentiation and plumage colour variation are different in the pied flycatcher (*Ficedula hypoleuca*). *Molecular Ecology*, 18, 4463–4476. <https://doi.org/10.1111/j.1365-294X.2009.04364.x>
- Librado, P., & Rozas, J. (2009). DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. *Bioinformatics*, 25, 1451–1452. <https://doi.org/10.1093/bioinformatics/btp187>
- Ninyerola, M., Pons, X., & Roure, J. M. (2005). *Atlas Climático Digital de la Península Ibérica. Metodología y aplicaciones en bioclimatología y geobotánica*. Barcelona, Spain: UAB, Bellaterra.
- Noguerales, V., Cordero, P. J., & Ortego, J. (2016). Hierarchical genetic structure shaped by topography in a narrow-endemic montane grasshopper. *BMC Evolutionary Biology*, 16, 96. <https://doi.org/10.1186/s12862-016-0663-7>
- Nosil, P., Egan, S. P., & Funk, D. J. (2008). Heterogeneous genomic differentiation between walking-stick ecotypes: “isolation by adaptation” and multiple roles for divergent selection. *Evolution*, 62, 316–336.
- Oksanen, J., Blanchet, F. G., Kindt, R., Legendre, P., Minchin, P. R., & O’Hara, R. B. (2012). *vegan: Community Ecology Package*. R package version 2.1-16/r2187.
- Oromi, N., Richter-Boix, A., Sanuy, D., & Fibla, J. (2012). Genetic variability in geographic populations of the natterjack toad (*Bufo calamita*). *Ecology and Evolution*, 2, 2018–2026. <https://doi.org/10.1002/ece3.323>
- Ortego, J., Aguirre, M. P., & Cordero, P. J. (2012). Genetic and morphological divergence at different spatiotemporal scales in the grasshopper *Mioscirtus wagneri* (Orthoptera: Acrididae). *Journal of Insect Conservation*, 16, 103–110.
- Panhuis, T. M., Butlin, R., Zuk, M., & Tregenza, T. (2001). Sexual selection and speciation. *Trends in Ecology & Evolution*, 16, 364–371.
- Peig, J., & Green, A. J. (2010). The paradigm of body condition: A critical reappraisal of current methods based on mass and length. *Functional*



- Ecology*, 24, 1323–1332. <https://doi.org/10.1111/j.1365-2435.2010.01751.x>
- Pierce, A. A., Zalucki, M. P., Bangura, M., Udawatta, M., Kronforst, M. R., Altizer, S., ... de Roode, J. C. (2014). Serial founder effects and genetic differentiation during worldwide range expansion of monarch butterflies. *Proceedings of the Royal Society of London B: Biological Sciences*, 281, 20142230. <https://doi.org/10.1098/rspb.2014.2230>
- Pinheiro, J., Bates, D., DebRoy, S., & Sarkar, D., & R Core Team. (2017). nlme: Linear and nonlinear mixed effects models. R package version 3.1-131.
- Prestwich, K. N. (1994). The energetics of acoustic signaling in anurans and insects. *American Zoologist*, 34, 625–643. <https://doi.org/10.1093/icb/34.6.625>
- Ragge, D. R., & Reynolds, J. (1988). The songs and taxonomy of the grasshoppers of the *Chorthippus biguttulus* group in the Iberian Peninsula (Orthoptera: Acrididae). *Journal of Natural History*, 22, 897–929. <https://doi.org/10.1080/00222938800770611>
- Reynolds, W. J. (1987). A description of the song of *Chorthippus cazurroi* (Orthoptera: Acrididae) with notes on its taxonomic position and distribution. *Journal of Natural History*, 21, 1087–1095. <https://doi.org/10.1080/00222938700770671>
- Robinson, D. J., & Hall, M. J. (2002). Sound signalling in Orthoptera. In P. Evans (Ed.), *Advances in insect physiology*, (vol. 29, pp. 151–278). London, UK: Elsevier.
- Saldamando, C. I., Miyaguchi, S., Tatsuta, H., Kishino, H., Bridle, J. R., & Butlin, R. K. (2005). Inheritance of song and stridulatory peg number divergence between *Chorthippus brunneus* and *C. jacobsi*, two naturally hybridizing grasshopper species (Orthoptera: Acrididae). *Journal of Evolutionary Biology*, 18, 703–712. <https://doi.org/10.1111/j.1420-9101.2004.00838.x>
- Salomon, M. (2001). Evolutionary biogeography and speciation: Essay on a synthesis. *Journal of Biogeography*, 28, 13–27.
- Sathyan, R., Engelbrecht, A., & Couldridge, V. C. (2017). Morphological, acoustic and genetic divergence in the bladder grasshopper *Bullacris unicolor*. *Ethology, Ecology & Evolution*, 29, 552–573. <https://doi.org/10.1080/03949370.2017.1287915>
- Scheuber, H., Jacot, A., & Brinkhof, M. W. (2003). Condition dependence of a multicomponent sexual signal in the field cricket *Gryllus campestris*. *Animal Behaviour*, 65, 721–727. <https://doi.org/10.1006/anbe.2003.2083>
- Schluter, D. (2001). Ecology and the origin of species. *Trends in Ecology & Evolution*, 16, 372–380.
- Sexton, J. P., Hangartner, S. B., & Hoffmann, A. A. (2014). Genetic isolation by environment or distance: Which pattern of gene flow is most common? *Evolution*, 68, 1–15. <https://doi.org/10.1111/evo.12258>
- Shafer, A., & Wolf, J. B. (2013). Widespread evidence for incipient ecological speciation: A meta-analysis of isolation-by-ecology. *Ecology Letters*, 16, 940–950. <https://doi.org/10.1111/ele.12120>
- Spurgin, L. G., Illera, J. C., Jorgensen, T. H., Dawson, D. A., & Richardson, D. S. (2014). Genetic and phenotypic divergence in an island bird: Isolation by distance, by colonization or by adaptation? *Molecular Ecology*, 23, 1028–1039.
- Stange, N., & Ronacher, B. (2012). Grasshopper calling songs convey information about condition and health of males. *Journal of Comparative Physiology A*, 198, 309–318. <https://doi.org/10.1007/s00359-012-0709-2>
- Tregenza, T., Pritchard, V. L., & Butlin, R. K. (2000a). Patterns of trait divergence between populations of the meadow grasshopper, *Chorthippus parallelus*. *Evolution*, 54, 574–585. <https://doi.org/10.1111/j.0014-3820.2000.tb00060.x>
- Tregenza, T., Pritchard, V. L., & Butlin, R. K. (2000b). The origins of premating reproductive isolation: Testing hypotheses in the grasshopper *Chorthippus parallelus*. *Evolution*, 54, 1687–1698. <https://doi.org/10.1111/j.0014-3820.2000.tb00713.x>
- Wagner, W. E. Jr (2011). Direct benefits and the evolution of female mating preferences: Conceptual problems, potential solutions, and a field cricket. In H. J. Brockmann, T. J. Roper, M. Naguib, J. C. Mitani, & L. W. Simmons (Eds.), *Advances in the study of behavior* (vol. 43, pp. 273–319). London, UK: Elsevier.
- Wagner, W. E. Jr, & Hoback, W. W. (1999). Nutritional effects on male calling behaviour in the variable field cricket. *Animal Behaviour*, 57, 89–95. <https://doi.org/10.1006/anbe.1998.0964>
- Wells, M. M., & Henry, C. S. (1998). Songs, reproductive isolation, and speciation in cryptic species of insects. In D. J. Howard & S. H. Berlocher (Eds.), *Endless forms: Species and speciation* (pp. 217–233). New York, Oxford: Oxford University Press.
- Wilkins, M. R., Seddon, N., & Safran, R. J. (2013). Evolutionary divergence in acoustic signals: Causes and consequences. *Trends in Ecology & Evolution*, 28, 156–166. <https://doi.org/10.1016/j.tree.2012.10.002>
- Yannic, G., Pellissier, L., Ortego, J., Lecomte, N., Couturier, S., Cuyler, C., ... Kolpashikov, L. (2014). Genetic diversity in caribou linked to past and future climate change. *Nature Climate Change*, 4, 132.
- Zuk, M., & Kolluru, G. R. (1998). Exploitation of sexual signals by predators and parasitoids. *The Quarterly Review of Biology*, 73, 415–438. <https://doi.org/10.1086/420412>
- Zuur, A. F., Ieno, E. N., & Elphick, C. S. (2010). A protocol for data exploration to avoid common statistical problems. *Methods in Ecology and Evolution*, 1, 3–14. <https://doi.org/10.1111/j.2041-210X.2009.00001.x>

BIOSKETCH

The authors are broadly interested in the mechanisms affecting the distribution and coexistence of animal species, the dynamics of alpine populations, and the evolution of behaviour and life histories (see <https://www.unioviado.es/UMIB/es/>).

Author contributions: P.L., J.P., J.R.O., J.C.I. designed the study; P.L. and J.R.O. performed field sampling; P.L., J.C.I. and J.P. performed laboratory work; J.P. and J.C.I. analysed data; J.P., P.L., J.C.I. wrote the manuscript with inputs from J.R.O. All authors read and approved the final manuscript.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Pato J, Illera JC, Obeso JR, Laiolo P.

The roles of geography, climate and sexual selection in driving divergence among insect populations in mountaintops. *J Biogeogr.* 2019;46:784–795. <https://doi.org/10.1111/jbi.13540>