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Sexual selection and population divergence III: Interspecific and intraspecific variation in mating signals

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Abstract

A major challenge for studying the role of sexual selection in divergence and speciation is understanding the relative influence of different sexually selected signals on those processes in both intra- and interspecific contexts. Different signals may be more or less susceptible to co-option for species identification depending on the balance of sexual and ecological selection acting upon them. To examine this, we tested three predictions to explain geographic variation in long- versus short-range sexual signals across a 3,500 + km transect of two related Australian field cricket species (*Teleogryllus* spp.): (a) selection for species recognition, (b) environmental adaptation and (c) stochastic divergence. We measured male calling song and male and female cuticular hydrocarbons (CHCs) in offspring derived from wild populations, reared under common garden conditions. Song clearly differentiated the species, and no hybrids were observed suggesting that hybridization is rare or absent. Spatial variation in song was not predicted by geography, genetics or climatic factors in either species. In contrast, CHC divergence was strongly associated with an environmental gradient supporting the idea that the climatic environment selects more directly upon these chemical signals. In light of recently advocated models of diversification via ecological selection on secondary sexual traits, the different environmental associations we found for song and CHCs suggest that the impact of ecological selection on population divergence, and how that influences speciation, might be different for acoustic versus chemical signals.

KEYWORDS

acoustic signalling, character displacement, chemical signalling, ecological speciation, environmental selection, multi-modal signalling, sexual selection, *Teleogryllus*

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1 | INTRODUCTION

Animals detect, recognize and respond to potential mating partners using a wide range of preferences and communication signals: their mate recognition system (MRS) (Greenfield, 2002). Divergence of MRSs is often associated with population divergence and can be a major cause of sexual isolation between species (Coyne & Orr, 2004; Paterson, 1985). Measuring intra- and interspecific variability in MRSs across species ranges can help distinguish the contribution of sexual selection from other sources of selection. For example, sexual signals might vary due to different balances of sexual selection and various forms of natural selection (Blows, 2002; Endler, 1992; Kirkpatrick, 1982; Lande, 1981; Wilkins, Seddon, & Safran, 2013). The former has received much interest, and drift can enhance sexual selection's role in divergence by causing the MRSs of isolated populations to stochastically diverge to different trait optima (Kirkpatrick, 1982; Lande, 1981; Pascoal, Mendrok, Wilson, Hunt, & Bailey, 2017; Uyeda, Arnold, Hohenlohe, & Mead, 2009). Nevertheless, MRS divergence may be constrained due to countervailing natural selection, and variable natural selection across habitats or environmental gradients may result in predictable geographic patterns of MRS variation (Boughman, 2001; Seehausen et al., 2008). Species interactions may also constrain or enhance divergence in MRSs through gene flow and reproductive character displacement (Gerhardt, 2013; Greig, Baldassarre, & Webster, 2015; Haavie et al., 2004). In general, population divergence driven primarily by sexual selection does not make an a priori prediction about the direction of change in an MRS, whereas ecological selection (or other forms of natural selection) superimposed upon this does predict strong associations with geographic or environmental variables (Ritchie, 2007).

Multi-modal signal components involved in sexual behaviour can operate over different spatiotemporal scales (Candolin, 2008; Leonard & Hedrick, 2009; Rowe, 1999), and thus, the strength and form of selection imposed may differ. For example, long-range signals may experience stronger selection to enhance assortative mating if they are earlier acting and therefore have the greatest potential to reduce gene flow, or if they reduce signal interference compared to those that operate over short distances. This leads to the prediction that long-range signals should be more likely to show character displacement in sympatry compared to short-range signals. In addition, sexual signals that are under both natural and sexual selection should exhibit enhanced population divergence compared to those that are primarily under sexual selection, and that divergence should co-vary with relevant environmental or ecological selection pressures. These predictions assume that selection acts primarily on the signal rather than the receiver, which may not always be the case (Latour et al., 2014; Rundle & Scluter, 1998; Servedio, 2001). We tested these predictions for how both long- and short-range sexual signals diverge within and between species using a classic system, the Australian field cricket genus *Teleogryllus*.

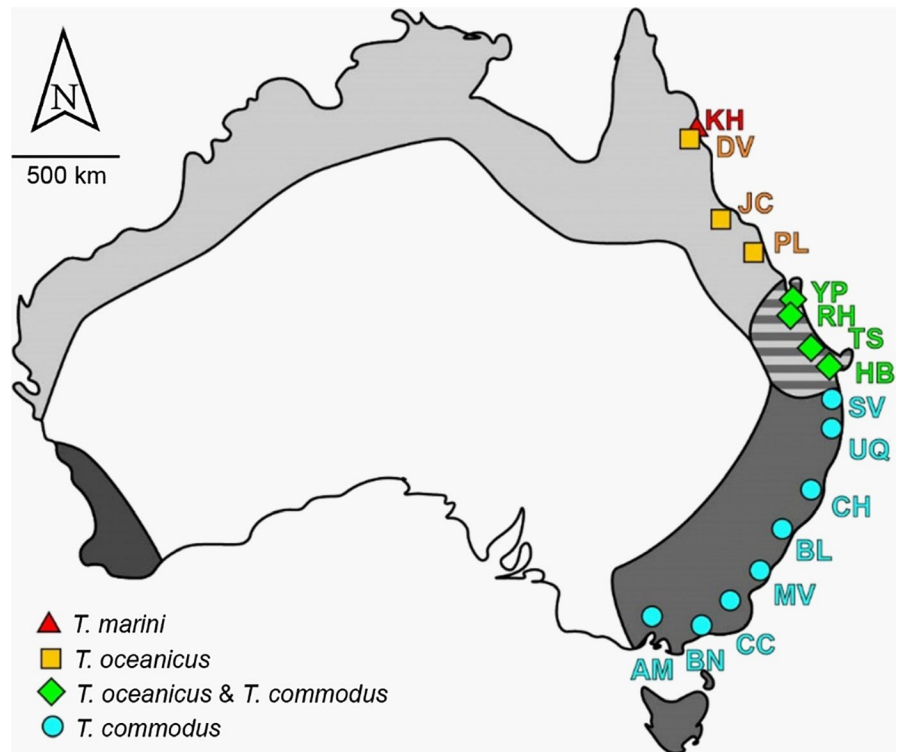
The closely related species *T. commodus* and *T. oceanicus* occupy coastal regions of Australia and overlap across approximately

400 km of the central eastern seaboard (Figure 1). Both are ecological generalists and are often found in close proximity with no obvious differences in resource use or habitat preferences (Otte & Alexander, 1983). They readily hybridize in the laboratory. Hybrid females are sterile, but hybrid males are fertile and capable of mating which suggests that hybridization and reinforcement could occur in sympatry (Hogan, 1971; Hogan & Fontana, 1973; Moran, Ritchie, & Bailey, 2017). Little is known about these species' interactions in sympatry, because research has focused almost exclusively on allopatric populations (e.g. Bailey & Macleod, 2013; Moran, Hunt, Mitchell, Ritchie, & Bailey, 2019; Pitchers et al., 2013; Simmons, Zuk, & Rotenberry, 2001). However, there is no evidence for contemporary gene flow (Moran et al., 2018) which could be due to successful reinforcement or reproductive isolation prior to secondary contact. Here, we quantified long-range acoustic signals and short-range chemical signals, both of which are known to be under sexual selection, across a >3,500-km transect traversing allopatric and sympatric populations of both species (Figure 1).

Male calling song plays a critical role in maintaining the boundary between *T. commodus* and *T. oceanicus* (Bailey & Macleod, 2013; Hill, Loftus-Hills, & Gartside, 1972; Moran et al., 2019). In both species, calling song is a nondirectional, long-range signal that transmits across distances orders of magnitude further than those involved in physical interactions (i.e. metres versus millimetres). In Australian *Teleogryllus* species, it consists of an initial trill-like cluster of pulses followed by groups of shorter-duration pulses (Figure 2). In *T. commodus*, song carrier frequency is lower (ca. 4 kHz) and the short-duration pulses are grouped into long clusters. In *T. oceanicus*, carrier frequency is higher (ca. 5 kHz) and the short-duration pulses tend to occur in pairs. For females of both species, frequency and pulse rate have been shown to play an important role in mate choice and species discrimination (e.g. Bailey, Moran, & Hennig, 2017; Brooks et al., 2005; Hennig & Weber, 1997). A third putative species occupying coastal regions of northern Queensland, *T. marini*, was described once in the 1980s (Otte & Alexander, 1983) but remained unstudied until we re-located it in sympatry with one of our sampled *T. oceanicus* populations (Moran et al., 2018). We were unable to rear this species in the laboratory but recorded male calling song in the field. It also consists of two echemes, or stereotyped polysyllabic chirp elements (Broughton, 1976); a higher amplitude longer-pulse trill followed by a lengthy array of shorter-duration pulses typically clustered into groups of three or four (Figure 2c). The carrier frequency is low for a gryllid (ca. 3 kHz).

During closer range sexual encounters, crickets use their antennae to detect cuticular hydrocarbons (CHCs) (Tregenza & Wedell, 1997). CHCs are waxy molecules secreted on insect cuticles, and in *T. commodus* and *T. oceanicus*, they comprise a bouquet of numerous individual compounds (Figure 3). These are likely to have evolved under selection for desiccation resistance but have been co-opted for social communication functions (Balakrishnan & Pollack, 1997; Howard & Blomquist, 2005; Smadja & Butlin, 2009). CHC profiles in *T. oceanicus* are heritable (Thomas & Simmons, 2008a), sexually

FIGURE 1 Distribution and sampling of three Australian *Teleogryllus* species, adapted from Moran et al. (2018). Dark and light grey shading indicates the known distributional ranges of *T. commodus* and *T. oceanicus*, respectively. Striped shading shows the approximate area of sympatric overlap between *T. commodus* and *T. oceanicus*, and the single red triangle shows the location at which both *T. oceanicus* and *T. marini* were observed. Two-letter population codes correspond to the main text



dimorphic (Moran et al., 2019; Thomas & Simmons, 2008b) and under sexual selection through both male and female choice (Berson & Simmons, 2019; Thomas & Simmons, 2009, 2010). However, little is known about which specific hydrocarbons mediate mate choice and even less is known about the nature of CHCs in *T. commodus* and the role that chemical communication may play in mate recognition and reproductive isolation.

For both sexual signal traits, we tested if selection for species recognition contributed to signal divergence by examining whether individuals from sympatric populations exhibit accentuated phenotypic divergence and if the direction of divergence increases dissimilarity between species and is more consistent among sympatric populations than it is among allopatric populations. We predicted such a pattern would be more likely and stronger for calling song than for CHCs, because of calling song's known role as an early-acting mechanism of sexual isolation in this species group and greater potential for signal interference due to being broadcast over longer distances (Bailey & Macleod, 2013; Bailey et al., 2017; Hennig & Weber, 1997; Hoy & Paul, 1973; Simmons, 2004). In addition, potential cross-sex genetic correlations for CHC profiles combined with sexual selection in both sexes are likely to constrain this signal modality in a distinct manner compared to sex-limited, long-range male calling song. We then examined whether sexual signal divergence corresponded to environmental adaptation or random genetic drift by testing for associations between signal divergence and genetic, geographic and climatic distances. Climate is a rough proxy for the local environment of populations which may be predicted to impose selection on sexual signals by influencing both their development and transmission properties. As the populations sampled were linearly ordered along the transect (Figure 1), clear

geographic structuring with spatial and genetic distance predicting the extent but not direction of signal divergence would support genetic drift (i.e. isolation by distance), whereas geographic structuring of populations with climatic distance predicting both the extent and direction of signal divergence would support adaptation to an environmental gradient (isolation by environment). In the latter case, we anticipated that CHCs would show a stronger relationship reflecting adaptive divergence to a south–north temperature gradient given their known association with temperature and desiccation resistance in insects (Chung & Carroll, 2015; Foley & Telonis-Scott, 2011).

2 | METHODS

2.1 | Study populations

The crickets used in this study were the first generation derived from wild-caught individuals and reared in a common garden environment to minimize the effects of phenotypic plasticity (although we cannot rule out the potential for maternal effects). Progenitor adult male and female crickets (ca. 30 per population) were sampled from the field and kept together, free to breed, in small plastic boxes (ca. 8 litres) for ca. 3–5 days to establish laboratory colonies. Crickets were collected from 16 sites in 2013, encompassing an extensive latitudinal transect across eastern Australia that included allopatric and sympatric populations (Figure 1). Species identity could be assigned to males based on their calling song whereas females were indistinguishable. Areas of sympatry were located based on published studies (Hill

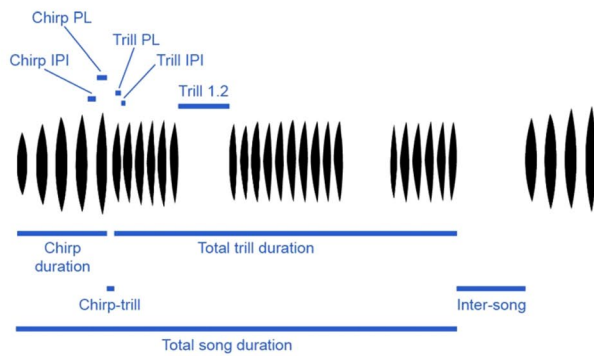
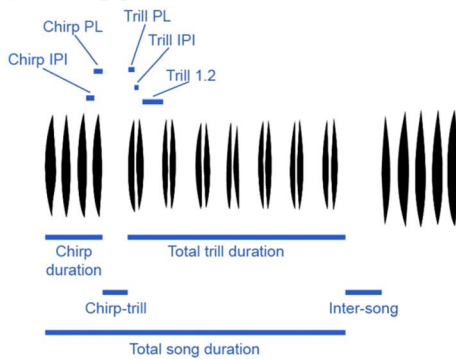
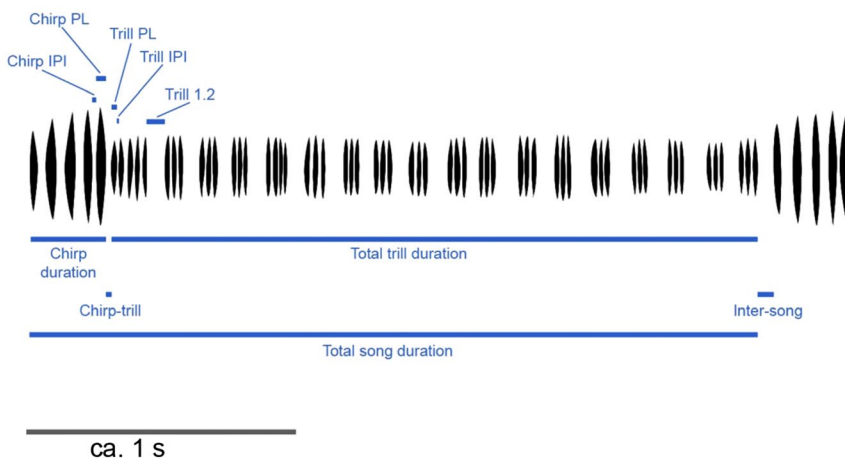
(a) *Teleogryllus commodus*(b) *Teleogryllus oceanicus*(c) *Teleogryllus marini*

FIGURE 2 Calling song schematics for Australian *Teleogryllus* species. Labels indicate the 13 shared song traits measured in this study of *T. commodus* and *T. oceanicus* (carrier frequency, chimp number, number of trills and average pulses per trill not indicated). An illustrative *T. marini* calling song is provided for comparison. Terminology for song parameters was coordinated to enable comparison between the two species and follows Moran et al. (2019)

et al., 1972; Otte & Alexander, 1983). We were able to directly verify that populations were sympatric because we found adult, singing males of both species in close physical proximity (<5 m) within the same habitat. In addition, genetic data from the progenitors later confirmed that males and females of both species were sampled from sympatry and that *T. oceanicus* outnumbered *T. commodus* almost 3:1 (Moran et al., 2018). However, *T. commodus* from sympatry failed to produce offspring when reared in the laboratory. Crickets were kept in 16-L plastic boxes of ca. 80 individuals in a 25°C temperature-controlled room on a 12-hr:12-hr light:dark cycle. Cardboard egg cartons were provided for shelter,

moistened cotton pads for water and ovipositing substrate, and ad libitum Burgess Excel Junior & Dwarf rabbit pellets for food.

Field observations confirmed the presence of a third putative species, *T. marini*, distinguished primarily by its calling song which consisted of a low carrier frequency (ca. 3 kHz) and triplet pulses in a proportionally longer trill (Figure 2c) in the north-eastern region around Daintree (Figure 1). A genomic analysis of three *Teleogryllus* spp. by Moran et al. (2018) confirmed *T. marini*'s distinct species identity. No *T. marini* individuals were used in laboratory analyses, but it is important to recognize that our population transect covers at least two areas of sympatry with *T. oceanicus*.

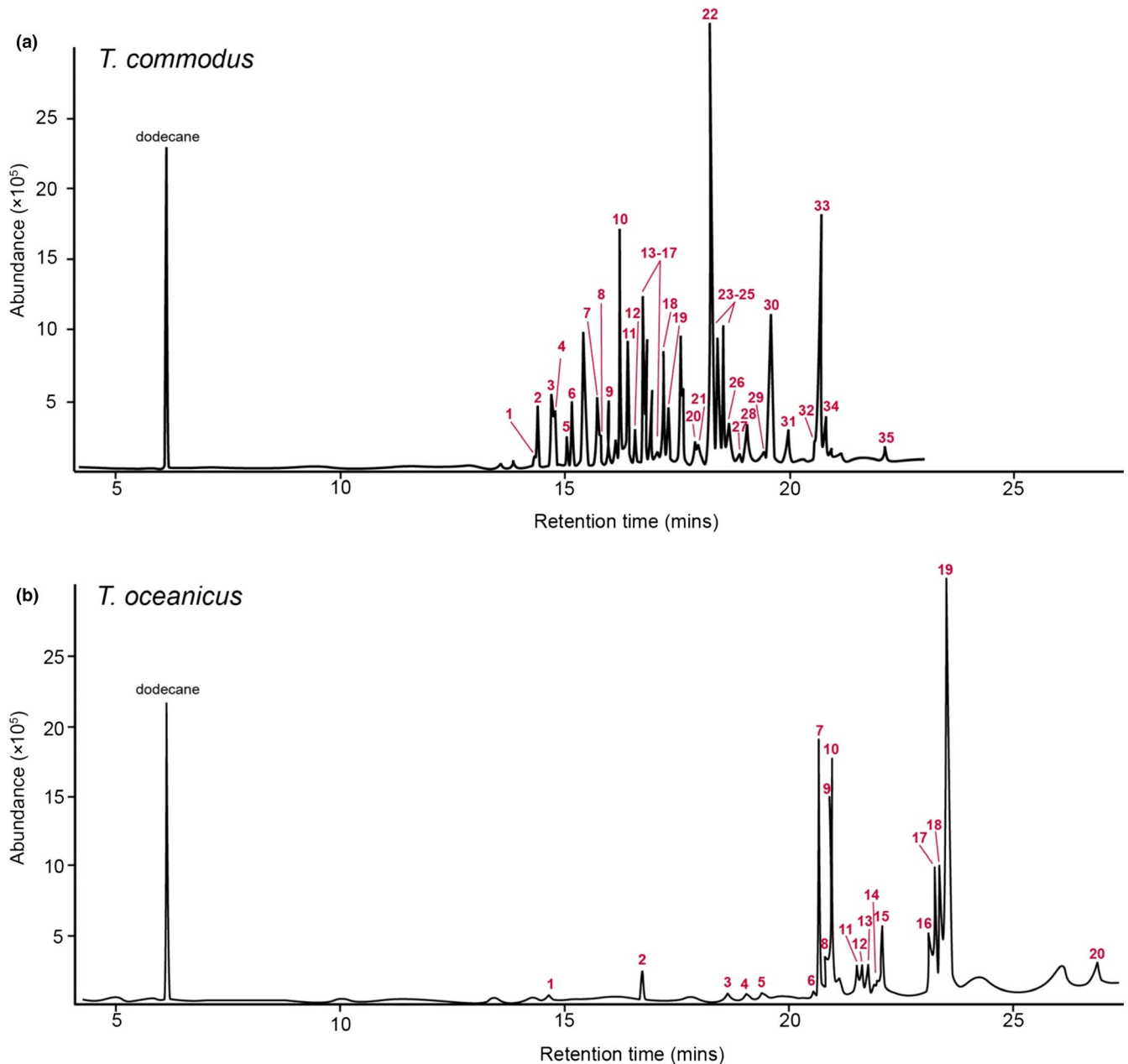


FIGURE 3 Re-drawn and labelled diagrams showing typical gas chromatography profiles for (a) *T. commodus* and (b) *T. oceanicus*. CHC peaks were assigned according to retention time, with the first peak the internal standard (10 ng/ μ l dodecane). Peak numbering is species-specific, and peaks that are not numbered were not included in the analysis due to high variability in presence versus absence across individuals. The 9 peaks shared between both species are provided in Table S5

2.2 | Song recording and analysis

We recorded male calling song from common garden reared offspring as described above. Recordings were made using a Sennheiser ME66 microphone and an Olympus LS-10 handheld recorder in a temperature-controlled room (mean \pm SD; 24.3°C \pm 0.7) under red light. Approximately 15 males ca. 10–20 days post-adult eclosion were recorded from each population (total $n = 258$). A minimum of one minute of calling song was recorded per individual, from which five complete songs (the basic repetitive unit comprising the chirp and trill echemes described above and labelled “Total song duration” in Figure 2) were

analysed. Eighteen parameters were measured for each song using Sony Sound Forge (V.7.0). To ensure valid inter- and intraspecific comparisons, we focused on 13 parameters which are shared between both species and assumed to be homologous (Figure 2). For each parameter, means of the five measurements per individual were used in subsequent analyses. Body size and temperature can influence song traits in insects, so we noted temperature, cricket weight and pronotum length and controlled for temperature variation between recordings for each species separately by using temperature-corrected residuals from analyses of covariance (Methods S1, Tables S1 and S2). All statistical analysis was conducted using R (version 3.4.1).

2.3 | Cuticular hydrocarbon profiling

CHCs were extracted from laboratory-reared males and females ca. 15–30 days after adult eclosion. Individuals were isolated for a minimum of 5 days in 118 ml plastic containers provisioned with food and water, then anesthetized by chilling, placed in 5-ml glass extract vials and stored at -20°C . Following Pascoal et al. (2015), samples were thawed at room temperature for ca. 10 min prior to CHC extraction. Four millilitre of HPLC-grade hexane (Fisher Scientific) was pipetted into each vial and left for 5 min before removing the cricket with clean forceps. 100 μl of this extract was pipetted into 0.3 ml fixed insert vials (Chromacol LTD, Item # 11573680) and evaporated under a fume hood overnight. After transport to the laboratory of J.H., CHC samples were reconstituted using 100 μl of hexane containing 10 ng/ μl dodecane as an internal standard. Samples were analysed using gas chromatography–mass spectrometry (GC-MS; an Agilent 7890A gas chromatograph coupled to a 5975B mass spectrometer) equipped with a HP-5 MS capillary column (30 m \times 0.25 mm ID \times 0.25 μm ; Agilent J&W). A 2 μl volume of each sample was injected into an inlet (4 mm ID splitless inlet liner (Agilent 5062-3587)) and held at 250°C in splitless mode for 1 min. The helium carrier gas flow was 1 ml/min. The initial oven temperature was held at 50°C for 1 min, then ramped at a rate of $20^{\circ}\text{C}/\text{min}$ to 250°C followed by a $4^{\circ}\text{C}/\text{min}$ ramp to 320°C and a 5 min hold at this temperature. Ionization was achieved by electron ionization (EI) at 70 eV. The quadrupole mass spectrometer was set to 3.2 scans/s, ranging from 40 to 500 Da.

The abundance of each CHC peak in chromatograms was estimated using MSD ChemStation software (version E.02.00.493; Agilent Technologies) by measuring the area under the peak. As *T. commodus* and *T. oceanicus* CHC profiles are divergent with few shared peaks (see Figure 3), two species-specific methods were used. Within each species, diagnostic ions were identified for each chemical peak allowing for the relative abundance of peaks across samples to be compared. The CHC peaks were measured blind to the individual's population identity. We assigned individuals to either species group based on an initial qualitative assessment of their chromatograms and analysed them using the corresponding species-specific method. A number of samples exhibited CHC profiles which did not correspond to either species profile and were not included in this analysis (see Moran et al., 2017). Five peaks among *T. commodus* samples were found to be highly variable in their presence and absence and were dropped from the analysis. In total, 35 peaks were used for *T. commodus* and 20 for *T. oceanicus* (Figure 3). To compare patterns of CHC variation across both species' ranges, 9 CHC peaks that were shared between both species and assumed to be homologous were selected for an additional analysis. Prior to analysis, data were standardized by dividing the abundance of each peak by the internal standard (10 ng/ μl dodecane) and normalized using a \log_{10} transformation.

2.4 | Statistical analysis

2.4.1 | Analysis Part 1: Interspecific variation in calling song and CHCs

First, we verified that calling song variation is predictably delimited by species identity. As hybrid calling song is intermediate between both species (Hoy, 1974; Hoy & Paul, 1973; Moran et al., 2019), we tested whether any hybrids were detectable among males from sympatric populations. CHC profiles in hybrids are highly variable and tend to show a stronger similarity with *T. commodus* (Moran et al., 2019), thus making CHCs less reliable for detection of hybrids. While we applied the same analysis pipeline to both sexual signals, we focused the interspecific analysis more heavily on calling song as most song parameters are shared between species (Figure 2). In contrast, CHC profiles are highly divergent between the species with few homologous peaks (Figure 3), thus limiting the power to examine CHC divergence across both species' ranges. We used principal component analysis on calling song and male and female CHC data (both sexes examined separately) implemented in the R package FactoMineR using a correlational matrix (Lê, Josse, & Husson, 2008). Principal components (PCs) with eigenvalues >1 were retained (song $n = 4$, male CHCs $n = 3$, female CHCs $n = 3$). Geographic differences between species and regions (allopatry or sympatry) were then evaluated using the scores from all retained PCs in a multivariate analysis of variance (MANOVA), with "species" and "geographic region" modelled as fixed effects. To test whether interspecific signal divergence was greater in sympatry, in line with reproductive character displacement, we analysed each signal (song or CHCs) for both species together using discriminant function analysis on PCs (DAPC) (described below). As multivariate approaches could potentially mask important differences between allopatric and sympatric populations, we also examined univariate signal components (13 song elements, 9 CHC peaks) across both species' ranges.

2.4.2 | Analysis Part 2: Intraspecific variation in male calling song and both sexes' CHCs

Given the high level of interspecific signal divergence, we used a species-specific approach to be able to detect intraspecific differences across populations and to avoid statistical problems due to a large amount of missing data. Calling songs and CHCs are complex and multivariate, and relatively little information is available, specifically for CHCs, about which components are under selection in either *Teleogryllus* species (Bailey et al., 2017; Brooks et al., 2005). Thus, it was appropriate to use multivariate statistical methods to reduce data dimensionality (Higgins & Waugaman, 2004). To examine and compare patterns of population differentiation for both signal traits, song data and CHC data were split into two species-specific subsets and analysed using principal component analysis (PCA) and discriminant analysis (DA). For song, sympatric

individuals were classified into either parental species group, informed by the results from the initial PCA on the total dataset (see above). For CHCs, individuals were assigned a priori to either species group based on visual inspection of their chromatograms and analysed following the corresponding species-specific method (see above). To test for population differentiation and sex differences (the latter in CHCs only), principal components with eigenvalues >1 were retained, the individual scores extracted and MANOVAs performed modelling “population”, “sex” and their interaction as fixed effects.

To test whether sympatric populations show accentuated signal divergence compared to allopatric populations, we performed DAPC with “population” as the prior grouping factor, using the R package *adegenet* (Jombart, Devillard, & Balloux, 2010). DAPC performs a PCA on the initial data and then identifies a linear combination of the variables (LDs) that maximizes between-group variance while minimizing within-group variance. Data were standardized using the Z-score transform prior to analysis. Group membership was assigned based on each individual's population identity. The optimal number of PCs to retain is an important consideration to ensure a fair trade-off between the power to discriminate and not over-fitting, so we used the cross-validation method in *adegenet* (*xvalDapc* function) to identify the correct number of PCs to retain. This procedure performs DAPC on a subset of the data (in our case 70% of observations from each population) and then finds the optimal number of PCs to retain that maximizes the prediction success of assigning the remaining 30% of individuals to their correct group. If species interactions contribute to signal divergence, then we expected population differentiation to be greatest in sympatry. Therefore, Tukey's pairwise population comparisons on the discriminant function scores were used to test whether sympatric populations were disproportionately differentiated using the R package *multcomp* (Hothorn, Bretz, & Westfall, 2008).

2.4.3 | Analysis Part 3: Spatial, environmental and genetic patterns of sexual signal divergence

First, Mantel tests were used to test whether geographic, climatic or genetic distance best predicted sexual signal variation among populations. Upon finding associations between climate and sexual signal divergence, we followed up with partial Mantel tests to examine whether these associations remained after controlling for genetic and geographic distances. Tests were performed in R using the *vegan* package (Oksanen et al., 2018) with 10,000 permutations. Song and CHC distance matrices were calculated using mean population discriminant function coordinates for LD1, and population differentiation in traits was measured as dissimilarity in their respective discriminant function scores. Geographic distances were estimated from each site's latitude and longitude coordinates (obtained from Google maps: <http://www.google.com/maps>).

Genetic distances among *T. commodus* and *T. oceanicus* populations were obtained from a previously published RADseq dataset of autosomal SNPs (26,447 and 34,010 SNPs, respectively; details provided in Moran et al., 2018). Pairwise population F_{ST} values were calculated in R using *Hierfstat* (Goudet, 2005), following the method of Weir and Cockerham (1984).

Climate data for each sampling location were extracted from the WorldClim database encompassing long-term observations (1950–2000) with a spatial resolution of ca. 4.5 km (2.5 min of a degree) (Hijmans, Cameron, Parra, Jones, & Jarvis, 2005). To account for annual and seasonal environmental variation, three descriptors of temperature (annual mean temperature, maximum temperature in the warmest month and minimum temperature in the coldest month) and precipitation (annual mean precipitation and precipitation in the wettest and driest months) were extracted. To summarize the major climatic trends, we used principal component analysis (PCA) and retained the individual scores from the first axis (PC1) for further analyses. For *T. commodus* populations, PC1 accounted for 72% of the variation and all six climatic variables were positively loaded on the first axis. Similarly, for *T. oceanicus* populations, PC1 accounted for 67% of the variation and contrasted the amount of precipitation in the driest month against the five other variables.

3 | RESULTS

3.1 | Results Part 1a: Song differentiates species with no evidence of recent hybridization

Calling song shows unambiguous interspecific variation in laboratory-recorded males. In a multivariate analysis of song, the first four PCs had eigenvalues >1 and cumulatively accounted for just over 85% of the variation (Table S1). Both species were distinguished primarily on PC1 (ANOVA: $F_{1,256} = 2,803.8$, $p < .001$) (Figure 4a), and PC1 loadings highlight the main species differences: *T. oceanicus* song has higher carrier frequency, a greater number of trills and longer duration of the chirp–trill interval (Figure 4b, Table S1). The absence of intermediate calling songs among our wild-derived laboratory populations (and also from populations recorded in the field, Figure S1) suggests contemporary hybridization is rare or absent. Unexpectedly, laboratory recordings of all males derived from sympatric populations clustered among the *T. oceanicus* call type (Figure 4a), despite having physically located calling males together in wild populations (see above) and genetic data confirming the two species' co-occurrence in sympatry (Moran et al., 2018). The absence of sympatric *T. commodus* samples in the laboratory therefore may represent differences between the species in their egg laying (or survival) during the establishment of laboratory stock colonies.

Separate PCAs on male and female CHCs for both species combined revealed clear interspecific differences (Figures 4a and 5b,c). For both sexes, the first three PCs had eigenvalues >1 and cumulatively accounted for just over 89% of the variation in males and 84% in females, with PC1 primarily differentiating the species (Figure 4a).

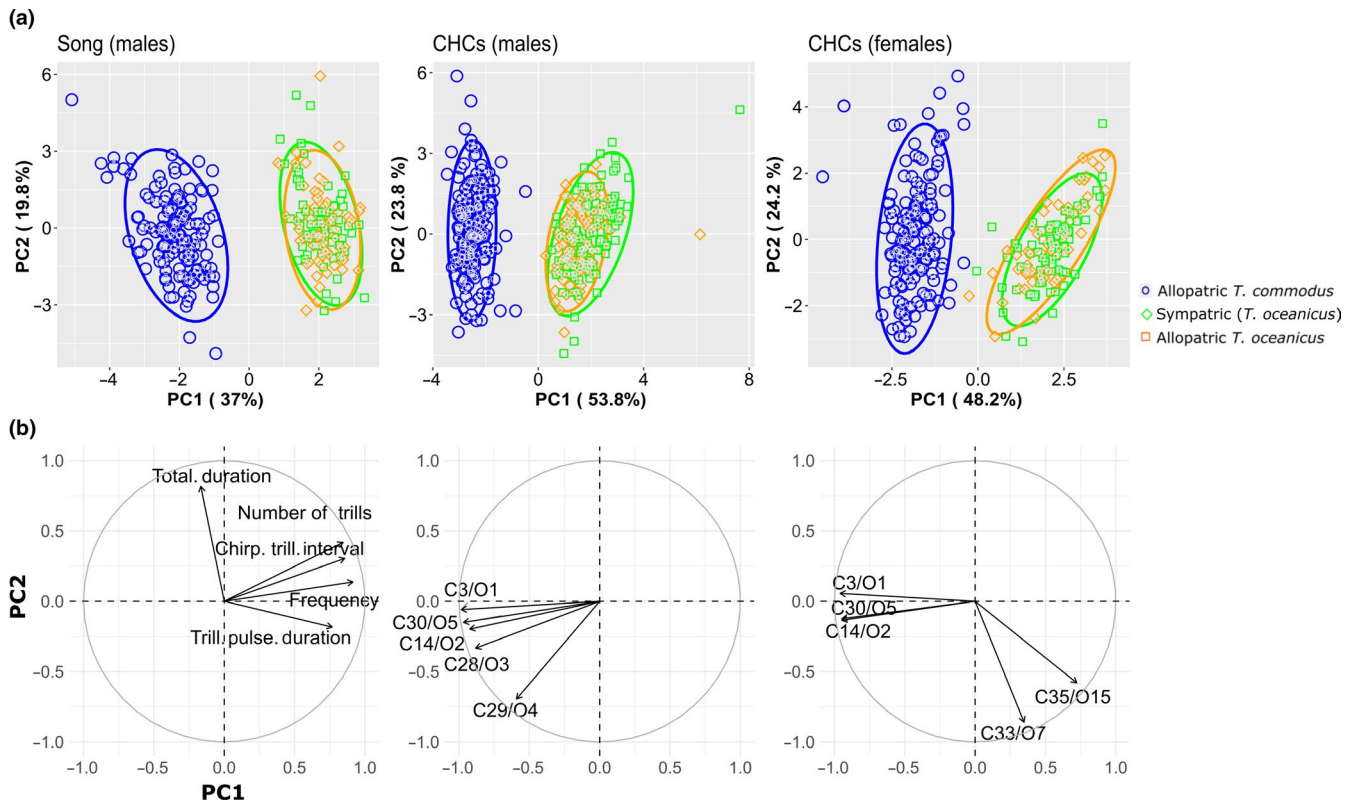


FIGURE 4 Interspecific variation in *Teleogryllus* spp. calling song and CHCs. (a) Principal component scores showing variation in song ($n = 258$), male CHCs ($n = 358$) and female CHCs ($n = 283$), with proportion of variance explained for PCs 1 and 2 in parentheses. Individuals are labelled based on species identity. Colours and shapes correspond to Figure 1, and ellipses delineate the 0.95 confidence intervals. (b) Variable factor map illustrating the top five parameters contributing to variation in PCs 1 and 2, indicated by bold lines and labels. For ease of visualization, the sign of PCs was reversed for male and female CHCs to ensure that the species were ordinated in the same direction for all three traits. PCA results on field recorded songs are also included in Figure S1

PC1 loadings highlight the main species differences, and the relative contribution of peaks was similar for both sexes (Figure 4b).

3.2 | Part 1b: Divergence in song and CHCs does not support reproductive character displacement

Multivariate analyses of calling song and CHCs for both species together indicated that neither sexual signal exhibits reproductive character displacement. Comparing calling song between allopatric and sympatric populations of *T. oceanicus*, we found no overall differences (MANOVA using the origin of individuals (i.e. allopatric or sympatric) as a factor: PCs, Wilks $\lambda = 0.99$, $F_{1,129} = 0.355$, $p = .84$). Univariate song analyses revealed no significant differences between allopatric and sympatric populations for any of the 13 song components (ANOVAs on individual song components, all $p > .05$). We were limited to examining only *T. oceanicus* in sympatry as no *T. commodus* were detected among the sympatric populations recorded in the laboratory (Figure 4a). Comparing CHCs between allopatry and sympatry for *T. oceanicus*, we found that males but not females differed in their CHC profiles (MANOVA PCs: male CHCs $F_{1,223} = 5.894$, $p < .001$; female CHCs $F_{1,130} = 0.481$, $p = .696$). However, the direction

of change was contrary to that expected under reproductive character displacement as male CHC divergence appeared to bring sympatric *T. oceanicus* populations closer to that of the heterospecific (Figure 5b). Univariate comparisons of CHCs between allopatric and sympatric populations of *T. oceanicus* revealed no differences among females but for males 7 peaks were significantly different (ANOVAs on individual CHC peaks $p < .05$). The two nonsignificant peaks were C32/O6 and C33/O7. Interestingly, among *T. commodus* populations CHC dissimilarity to heterospecifics increased in populations closer to sympatry (Figure 5b). However, the absence of sympatric *T. commodus* among our laboratory populations limited our ability to test directly whether CHC divergence is maximized in sympatry in line with reproductive character displacement.

3.3 | Results Part 2a: Song shows weak population differentiation

Separate PCAs for each species indicated significant population differences in calling song for *T. commodus* but not *T. oceanicus* (MANOVA using population as a factor on PCs; *T. commodus*: Wilks $\lambda = 0.589$, $F_{7,116} = 1.813$, $p = .004$; *T. oceanicus*: Wilks $\lambda = 0.74$, $F_{7,123} = 1.349$, $p = .113$). The significant population differences observed for song

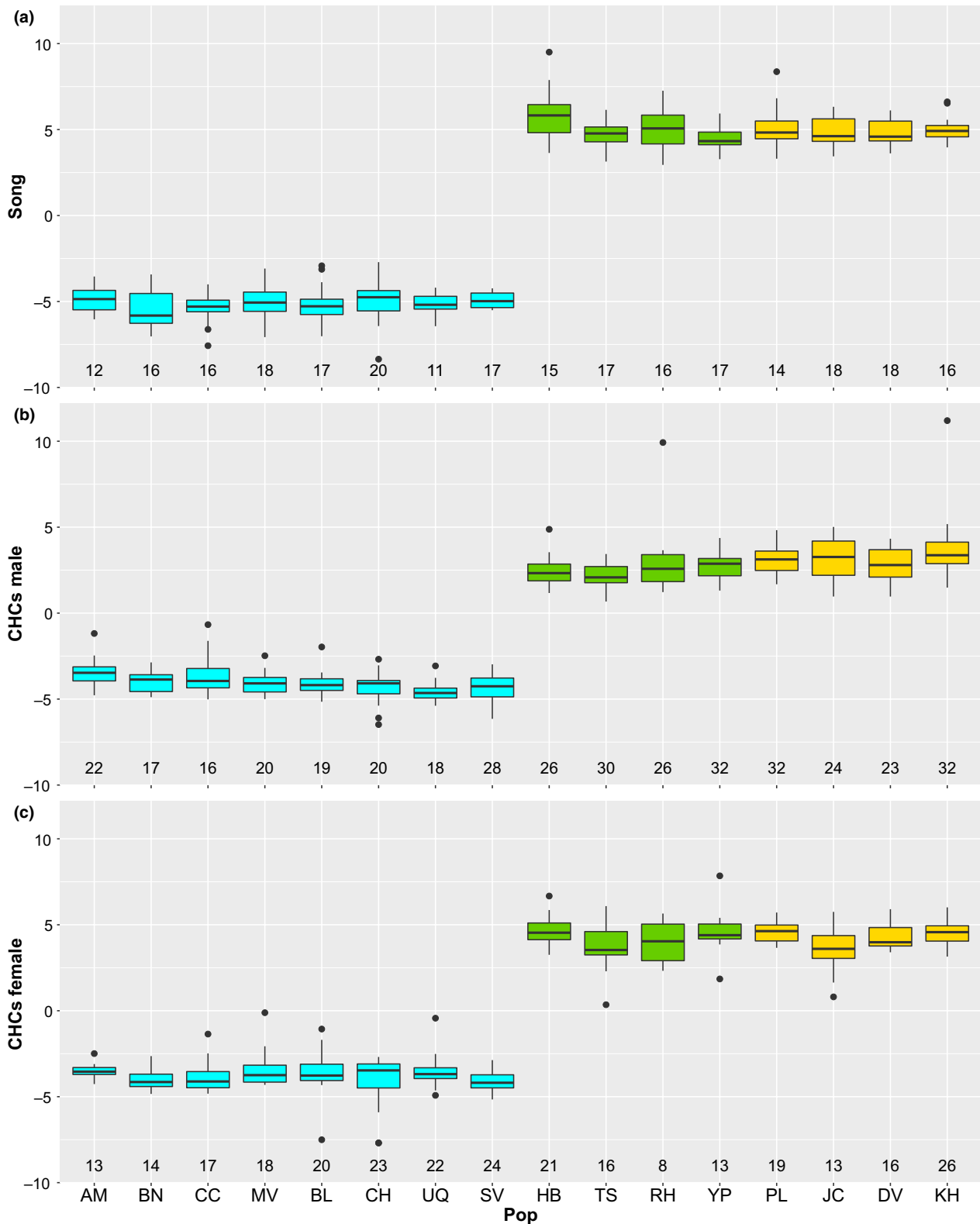


FIGURE 5 Discriminant analysis of principal components (DAPC) for both species combined for song (a) and male and females CHCs (b,c). The first discriminant component (LD1) is plotted on the Y-axis. Boxplots showing the median (horizontal lines), first and third quartile (boxes) and 95% confidence interval (bars) are grouped by population and arranged on the x-axis in geographic order from south (left) to north (right). Sympatric populations are coloured in green and allopatric populations in yellow and blue. KH and DV were sampled only several hundred metres from each other and, although allopatric in respect to *T. commodus*, are sympatric with the third putative species *T. marini*. Sample sizes for each population are included along the x-axis. For male CHCs, the difference between allopatry and sympatry remained after removing the two outlier samples from KH and RH

across *T. commodus* populations remained even after removing the most differentiated population, AM (Wilks $\lambda = 0.633$, $F_{6,105} = 1.641$, $p = .0196$) (Figure S2). Comparing calling song between allopatric and sympatric populations of *T. oceanicus*, we found no overall differences (MANOVA using the origin of individuals (i.e. allopatric or sympatric) as a factor: PCs, Wilks $\lambda = 0.988$, $F_{1,129} = 0.39$, $p = .815$). DAPC performed on both species song combined versus separately produced consistent results (Figure 5a; Figure S2). There was no evidence that populations close to the contact zone (e.g. UQ or SV for *T. commodus*) or in sympatry (*T. oceanicus* populations HB, TS, RH or YP) exhibited accentuated song divergence, which would have supported a role for species interactions contributing to song divergence (Figure 5a). Overall, our results indicate that populations are weakly differentiated in song and there is no evidence of reproductive character displacement.

3.4 | Results Part 2b: CHCs are highly variable among populations and between the sexes

We found considerable variation in CHC profiles among *T. commodus* populations. Principal component analysis indicated that the relative abundance of CHC peaks differed between populations and sexes (MANOVA on the PC scores, using population as a factor: Wilks

$\lambda = 0.553$, $F_{7,295} = 4.37$, $p < .001$; sex: Wilks $\lambda = 0.458$, $F_{1,295} = 57.3$, $p < .001$; interaction between population and sex: Wilks $\lambda = 0.823$, $F_{7,295} = 1.374$, $p = .057$). Discriminant function analysis indicated a clear geographic pattern of CHC population differentiation. In both sexes, the first discriminant axis (LD1) exhibited a strong positive association with geographic distance (Figure 6a), but the major CHC peaks associated with LD1 differed between the sexes (Table S5).

CHC profiles also varied considerably among *T. oceanicus* populations and sexes (MANOVA on PCs, using population and sex as a factor: Wilks $\lambda = 0.553$, $F_{7,341} = 2.999$, $p < .001$; Wilks $\lambda = 0.341$, $F_{1,341} = 163.179$, $p < .001$). A "population \times sex" interaction showed that the sexual dimorphism in CHCs was not equivalent in different populations (MANOVA: Wilks $\lambda = 0.872$, $F_{7,341} = 1.684$, $p = .015$). CHC profiles differed between allopatric and sympatric *T. oceanicus* populations for males (MANOVA: Wilks $\lambda = 0.872$, $F_{1,223} = 6.437$, $p < .001$) but not females (MANOVA: Wilks $\lambda = 0.964$, $F_{1,130} = 0.947$, $p = .453$). Discriminant function analysis highlighted considerable variation in the extent and direction of differentiation among populations and between sexes (Figure 6b, Table S7). Interestingly, female *T. oceanicus* exhibited considerable variation in the direction of CHC divergence between sympatry and allopatry whereas the direction of divergence was more consistent among males (Figure 6b). Trait loadings for males and females are provided in Table S8.

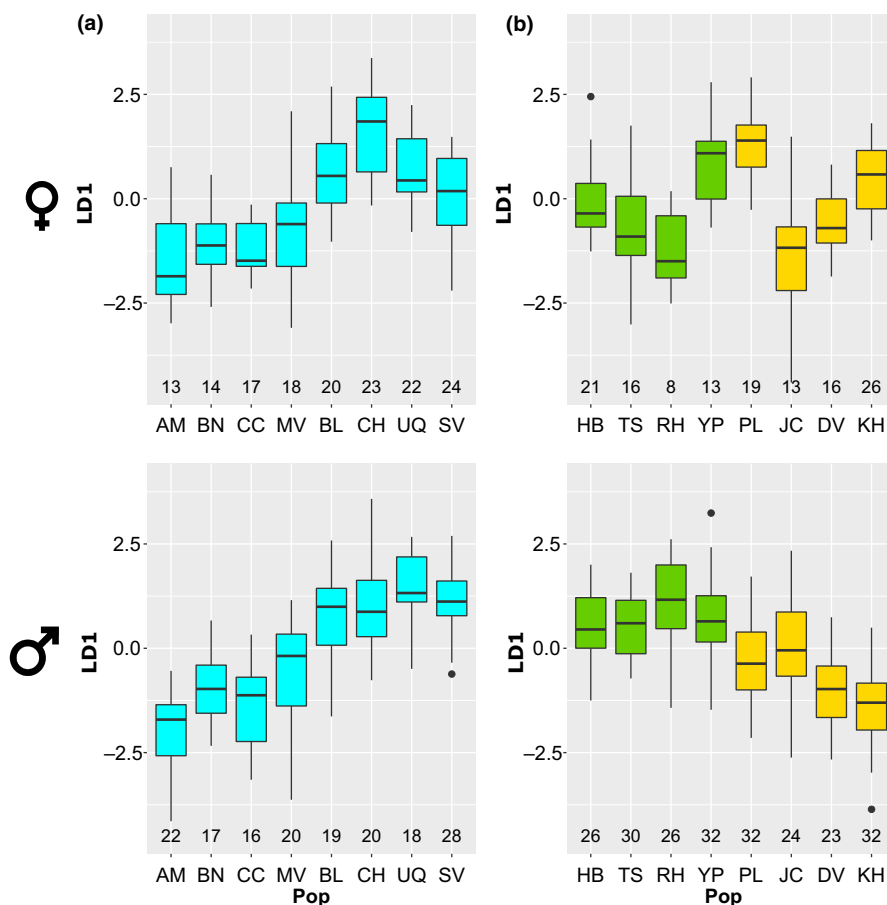


FIGURE 6 Intraspecific CHC variation, examined using discriminant analysis of principal components (DAPC) for each species separately, which prevents direct comparisons between the species. (a) Population-level differentiation in *T. commodus* CHCs, showing LD1 scores for females (top) and males (bottom). Boxplots showing the median (horizontal lines), first and third quartile (boxes) and 95% confidence interval (bars) are grouped by population and arranged on the x-axis in geographic order from south (left) to north (right). (b) Population-level differentiation in *T. oceanicus* CHCs, showing LD1 scores for females (top) and males (bottom). Sympatric populations are indicated by green and allopatric populations by yellow. Boxplots for each population are as above, arranged in geographic order from south (left) to north (right). Sample sizes for each population are included along the x-axis

3.5 | Results Part 3: Divergence in CHCs but not song is associated with a climate gradient

The geographic pattern of song divergence was not associated with spatial, climatic or genetic distance for either species (Figures 7 and 8). In contrast, variation in CHC composition was predicted by all three variables in both species. Moreover, there were clear sex differences between the species in the extent to which CHC divergence was associated with the above variables. Among *T. commodus* populations, CHC divergence for both males and females was strongly predicted by all three variables (Figure 7). In contrast, among *T. oceanicus* populations CHC composition (LD1) covaried with the above variables only for males (Figure 8). Geographic, climatic and genetic distance all covary which makes it difficult to disentangle their individual contributions (Figure S5). Partial Mantel tests indicated that the association

between climate and CHC divergence remained after controlling for both genetic (*T. oceanicus* male CHCs: $r = .728, p = .005$) and geographic distances (*T. oceanicus* male CHCs: $r = .466, p = .002$). Examining CHC loadings for both males and females indicated that the pattern of population differentiation was influenced by a large number of peaks, with very few peaks showing a major contribution (Tables S5 and S8). In addition, the peaks that contributed most to population differentiation among males were not the same as those in females, even though the overall geographic trend was similar (Tables S5 and S8).

4 | DISCUSSION

It is debated whether some sexually selected signal features or modalities are more predisposed than others to influence sexual

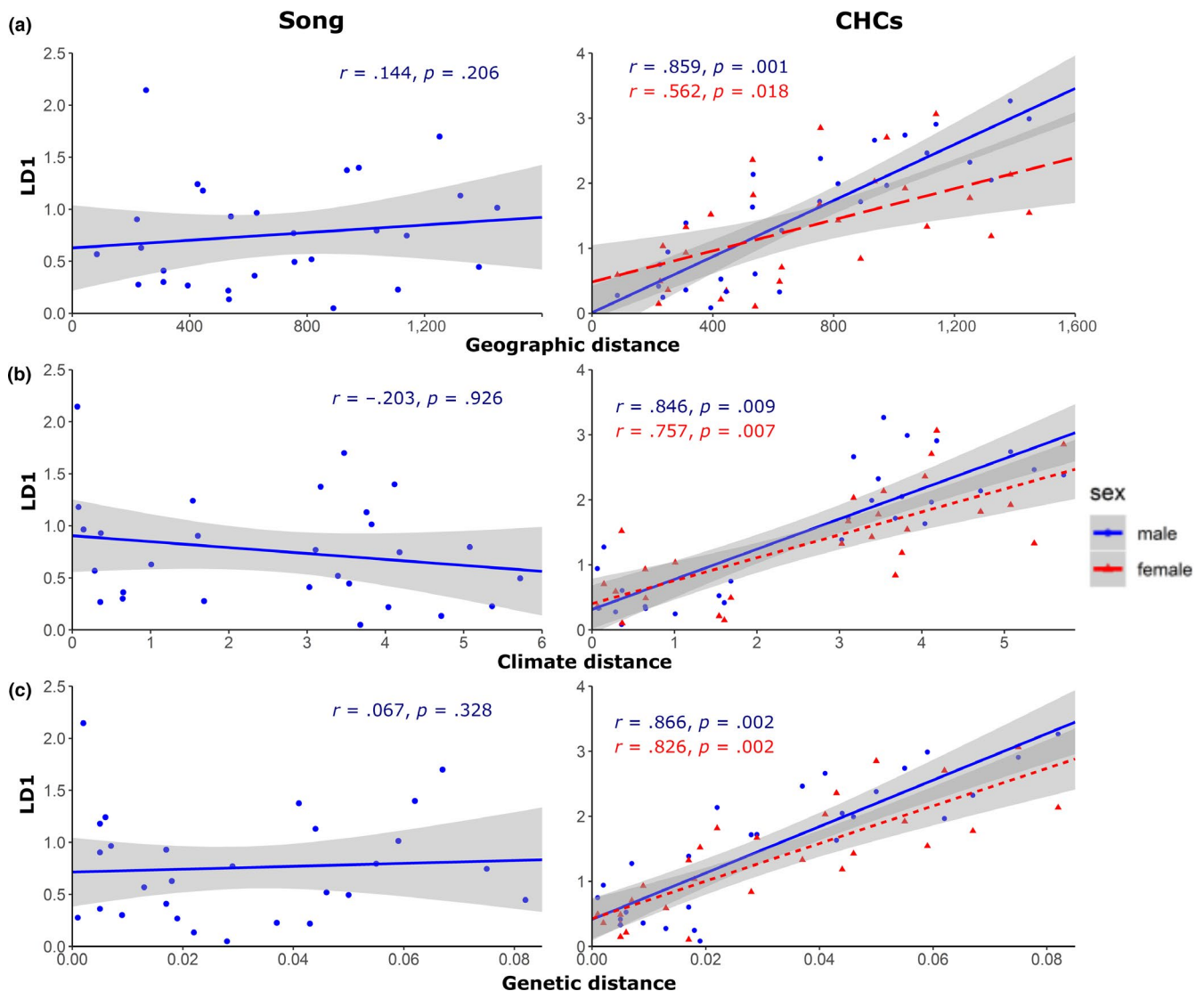


FIGURE 7 Relationships between sexual signal divergence (calling song on the left and CHCs on the right) and geographic distance (a), climatic distance (b) and genetic distance (c) in *T. commodus*. Discriminant analysis of principal components (DAPC) was performed on each species separately. For CHCs, the sexes were examined separately. Red triangles and dashed lines indicate females, and blue circles and solid lines indicate males. Mantel test correlation coefficients with p -values are shown, and grey areas around the regression lines indicate 95% confidence intervals

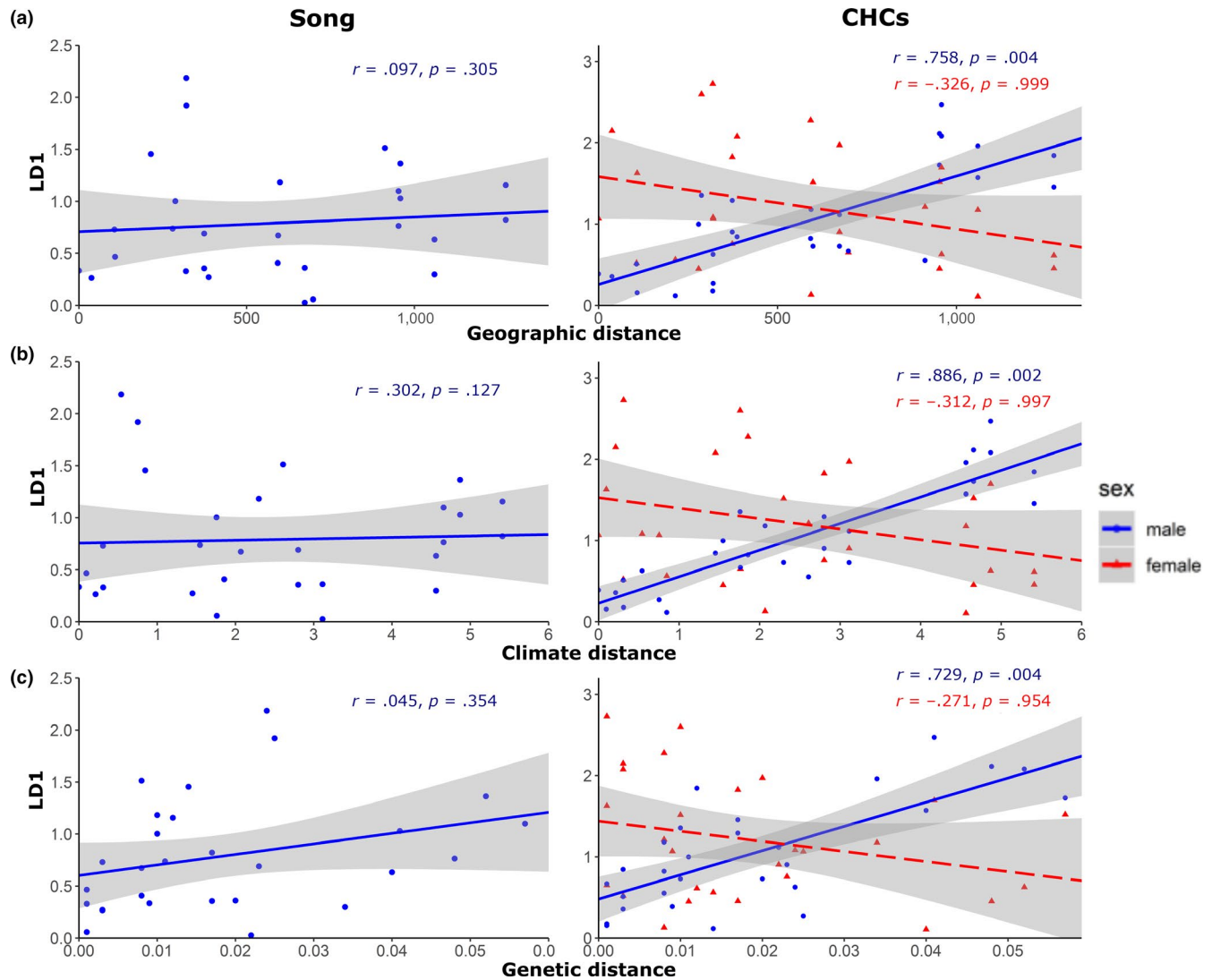


FIGURE 8 Relationship between sexual signal divergence (calling song on the left and CHCs on the right) with geographic distance (a), climatic distance (b) and genetic distance (c) in *T. oceanicus*. Discriminant analysis of principal components (DAPC) was performed on each species separately. For CHCs, the sexes were examined separately. Red triangles and dashed lines indicate females, and blue circles and solid lines indicate males. Mantel test correlation coefficients with p -values are shown, and grey areas around the regression lines indicate 95% confidence interval

isolation, particularly those acting over long versus short ranges (e.g. Gray, 2005; Pfennig, 1998; Zuk, Rebar, & Scott, 2008). The absence of intermediate calling songs among our wild-derived laboratory populations (and also from populations recorded in the field, Figure S1) suggests contemporary hybridization is rare or absent, as F1 hybrid songs are known to be intermediate (Hoy, 1974; Moran et al., 2019). This is in line with genetic data which found no evidence for recent hybridization (Moran et al., 2018). In the absence of hybridization, divergence in signal elements may occur to minimize signal interference (Gerhardt, 2013). Long-range signals may be particularly prone to such interference as a result of being broadcast over long distances, and they may be subject to greater selection to reduce mismatings as earlier acting barriers have greater potential to limit gene flow. It was therefore unexpected that we found short-range CHCs exhibited a stronger pattern of divergence between allopatric and sympatric populations of Australian *Teleogryllus* field crickets than long-range

male calling song. The discordance between the signal traits may highlight a more important role than previously appreciated for close-range signals such as CHCs in mediating species isolation (Veen, Faulks, Tyler, Lloyd, & Tregenza, 2013). Further data on CHC preferences would be useful to interpret the ultimate impact of different patterns of divergence for short- and long-range sexual traits on species isolation, as long- and short-range signals such as calling song and CHCs can interact to influence mating outcomes (Bailey, 2011; Leonard & Hedrick, 2011). Another significant question is whether CHC divergence in allopatric versus sympatric populations has been driven by species interactions in sympatry or whether it represents the outcome of a clinal gradient in some other environmental factor.

The effects of species interactions on the evolution of mate attracting signals will depend on localized properties of each population, such as the relative proportion of each species and the degree of ecological and behavioural overlap (Nosil, Crespi, & Sandoval, 2003;

Servedio & Noor, 2003; Taylor et al., 2006). Lemmon (2009) found that in two- and three-species assemblages of chorus frogs (*Pseudacris* spp.), populations of the rarer species diverged in different acoustic signal traits but dissimilarity with the heterospecific signal was nevertheless maximized in populations across the contact zone. In contrast, an experimental evolution study by Higgie, Chenoweth, and Blows (2000) on *Drosophila serrata* and *D. birchii* demonstrated that species interactions in the laboratory could drive allopatric populations to converge on the same cuticular hydrocarbon profiles as sympatric populations from the field. In our study, there was variation among sympatric populations in the direction of divergence for both sexual signal traits (Figure 6a; Figure S2). For CHCs, the combined analysis of both species indicated that for *T. oceanicus* the direction of CHC divergence provided no support for reproductive character displacement, as the direction of phenotypic change appears to bring sympatric populations closer to that of heterospecifics (Figure 5b). In contrast, for *T. commodus* the combined species analysis revealed that CHC dissimilarity to heterospecifics increased in the northern populations closer to sympatry. However, it is important to consider that our lack of sympatric *T. commodus* samples limited our ability to test reproductive character displacement for both sexual signal traits in the rarer species, *T. commodus*, where selection for species recognition is more likely to occur. Overall, calling song and CHCs are highly divergent between the species and this may have reduced the potential for selection for species recognition.

The comparatively weak level of population differentiation at the phenotypic level provides no evidence for rapid intraspecific divergence of song, and we observed greater population differentiation for CHCs. Differences in the form of sexual selection between male song (largely stabilizing) and male and female CHCs (different elements under disruptive, directional and stabilizing selection) may contribute to these patterns. Female preferences for calling song components in *T. commodus* have been found to be largely stabilizing (Bentsen, Hunt, Jennions, & Brooks, 2006; Brooks et al., 2005; Hunt, Blows, Zajitschek, Jennions, & Brooks, 2007) which may constrain song diversification across the species ranges. In contrast, Simmons et al. (2001), Simmons (2004) examined both geographic and genotypic variation in song (in particular the proportion of chirp) and associated female preferences among *T. oceanicus* populations and found no covariance between female preference and song traits, and a mismatch between female preferences and the mean population trait values. CHCs are liable to experience selection from both male and female choice as both sexes express them. Female preferences for male CHC profiles in *T. oceanicus* have been found to be largely under disruptive sexual selection (Thomas & Simmons, 2009), but some peaks are also under stabilizing selection (Simmons, Thomas, Simmons, & Zuk, 2013). In contrast, male preferences for female CHCs have been found to be under both stabilizing and directional selection gradients, and the intensity of male preferences acting on female CHCs is stronger than female preferences acting on male CHCs (Thomas & Simmons, 2010).

Distinguishing the relative contributions of sexual selection and ecological adaptation and how they interact to delimit species

boundaries is a serious challenge (Safran, Scordato, Symes, Rodríguez, & Mendelson, 2013; Scordato, Symes, Mendelson, & Safran, 2014). Many classic examples invoking sexual selection as an important force underlying species isolation also implicate a role for ecological adaptation which may interact to cause divergence in sexual traits (Arnegard & Kondrashov, 2004), and in some cases, ecological divergence appears to be the dominant factor driving reproductive isolation (Funk, Nosil, & Etges, 2006). Examples include male nuptial colour and opsin divergence in cichlids (Seehausen, 2006; Seehausen, Alphen, & Witte, 1999) and body size in sticklebacks (Head, Price, & Boughman, 2009; Nagel & Schluter, 1998). In our study, the clear discordance between song and CHCs in their relationships with geographic, genetic and climatic distances (Figures 7 and 8), which was particularly distinct among *T. commodus* populations, is consistent with the two traits experiencing a different balance of ecological and sexual selection pressures. Such interaction between ecological and sexual selection on signal traits may be antagonistic and constrain signal diversification and sexual isolation (Sharma, Hunt, & Hosken, 2011). The strength and direction of selection pressures on traits involved in sexual signalling may also differ between the sexes, leading to sex-specific patterns of trait divergence among populations and species. For example, environmental selection could contribute to CHC differences between males and females if they differ in their life history and are exposed to different environmental conditions. Sex differences can also occur due to differences in body size and physiology. In this study, the striking absence of an association with environmental and geographic distance for CHCs in *T. oceanicus* females compared to males is in line with female CHCs being under stronger sexual selection (Berson & Simmons, 2019; Thomas & Simmons, 2010), whereas male CHCs are potentially more affected by environmental selection. Little is known about CHC preferences in *T. commodus*, but previous close-range mating trials of both species suggest male choice based on female CHCs is stronger in *T. oceanicus* (Moran et al., 2019). Future work should examine CHC preferences in *T. commodus* and test whether the strength of sexual selection on both sexes differs between the species. Given the large variation in ages of crickets used in this study, we cannot rule out the potential for age effects to contribute to CHC variation and sex differences, though this is unlikely to have confounded our comparisons (Tregenza, Buckley, Pritchard, & Butlin, 2000). These results contrast with previous findings that *T. oceanicus* populations surveyed from relatively similar latitudes and climatic niches across north-east Queensland and oceanic islands of the south Pacific showed patterns of sexual trait variation consistent with neutral divergence (Pascoal et al., 2017). The strong south-north/latitudinal gradient in our current study is more reminiscent of geographic clines for CHCs that have been identified in some *Drosophila* species from this region; however, the factors that promote and maintain this variation have not been fully resolved (Coyne & Elwyn, 2006; Frentiu & Chenoweth, 2010). Environmental selection and in particular adaptation to desiccation resistance have been suggested to be a primary driver of CHC divergence. Long chained CHC compounds increase desiccation resistance (Foley & Telonis-Scott, 2011;

Rouault, Capy, & Jallon, 2001; Savarit & Ferveur, 2002). However, in *D. serrata* on the east coast of Australia, a cline of CHC compounds increasing in chain length is not consistent with adaptation to desiccation resistance, because the CHC chain lengths increase in northern populations which are considerably more humid than those in the south. In our study, the cline in CHCs among *T. commodus* males and females, encompassing a similar geographic region as *D. serrata*, is not associated with a clear change in CHC chain length (Table S5), suggesting it is also not primarily due to desiccation resistance.

5 | CONCLUSIONS

The complex interplay between different sexual signal modalities and geography is important for determining how mate recognition systems diverge during or after speciation. Our extensive transect of a classic Australian field cricket study system encompassed at least two contact zones with potential for interspecies interactions. Calling song clearly delimited the species boundary, and the lack of intermediate individuals suggests current hybridization is rare. However, song variation weakly differentiated populations, and there was no overall difference between allopatric or sympatric populations. In contrast, CHC profiles varied widely among both species' geographic ranges, with population-level variation predicted by climate, geography and genetic structure. This discrepancy between the ways in which song and CHCs vary with geography likely reflects an environmental association along a strong latitudinal gradient, rather than an increased importance of CHCs in mediating species interactions. Taken together, these patterns illustrate that the impact of sexual and ecological selection on population divergence and speciation might be different for acoustic versus chemical sexual signals: not all sexually selected signals are likely to have equivalent impacts on population divergence and speciation.

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AUTHOR CONTRIBUTIONS

P.A.M. conceived the study and designed experiments with M.G.R. and N.W.B. P.A.M. and N.W.B. performed field collections. P.A.M. collected behavioural data, C.M. collected GC-MS data, and P.A.M., J.H. and C.M. analysed cuticular hydrocarbon profiles. P.A.M., M.G.R. and N.W.B. wrote the manuscript. P.A.M. performed all other statistical analyses with guidance from M.G.R. and N.W.B.

DATA AVAILABILITY STATEMENT

Data deposited at dryad <https://doi.org/10.5061/dryad.wpzgmsbhr>

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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