SEXUAL SELECTION AND POPULATION DIVERGENCE II. DIVERGENCE IN DIFFERENT SEXUAL TRAITS AND SIGNAL MODALITIES IN FIELD CRICKETS (*TELEOGRYLLUS OCEANICUS*)

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Running Title: Divergence in Multiple Sexual Trait Modalities

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Abstract

Sexual selection can target many different types of traits. However, the relative influence of different sexually-selected traits during evolutionary divergence is poorly understood. We used the field cricket *Teleogryllus oceanicus* to quantify and compare how five traits from each of three sexual signal modalities and components diverge among allopatric populations: male advertisement song, cuticular hydrocarbon (CHC) profiles and forewing morphology. Population divergence was unexpectedly consistent: we estimated the among-population (genetic) variance-covariance matrix, **D**, for all 15 traits, and $\mathbf{D}_{max}$ explained nearly two-thirds of its variation. CHC and wing traits were most tightly integrated, whereas song varied more independently. We modelled the dependence of among-population trait divergence on genetic distance estimated from neutral markers to test for signatures of selection vs. neutral divergence. For all three sexual trait types, phenotypic variation among populations was largely explained by a neutral model of divergence. Our findings illustrate how phenotypic integration across different types of sexual traits might impose constraints on the evolution of mating isolation and divergence via sexual selection.

KEY WORDS: acoustic communication, cuticular hydrocarbons, eigendecomposition, geometric morphometrics, multimodal signalling, sexual selection

Introduction

The role of sexual selection in evolutionary diversification has been the subject of research scrutiny, because it is predicted to increase the evolutionary rate of traits that cause reproductive isolation such as sexual signals and mating preferences (Lande 1981; West-Eberhard 1983; Ritchie 2007; Kraaijeveld et al. 2011). If sexual selection causes rapid evolution of such traits in isolated populations, mismatches in sexual communication arising from genetic drift, ecological selection, or other processes will become amplified, and may ultimately decrease the likelihood of gene flow upon secondary contact. Such patterns can then be exacerbated by reinforcement, when genetic incompatibilities between lineages in secondary contact reinforce existing patterns of selection on mate recognition. Sexual selection therefore has the potential to play a two-part role in evolutionary diversification: first, by accelerating the elaboration of sexual signals, and second, by being the causal mechanism by which signal mismatches create mating barriers between taxa. Two critical parameters for empirically testing these ideas are therefore the amount of sexual trait divergence among populations, and the rate at which it evolves relative to other traits (Rodríguez et al. 2013, Wilkins et al. 2016).

Studies examining the relationship between sexual selection and divergence frequently test how strongly genetic divergence correlates with divergence in male sexual trait values, or, less commonly, female preferences (e.g. Gage et al. 2002; Masta and Maddison 2002; Huang and Rabosky 2014; Hudson and Price 2014). Although drift can independently influence both genetic structure and phenotypic divergence, the rationale of such

67 approaches is that divergence in sexual traits should correlate with reproductive isolation
68 among populations or higher taxonomic groupings (e.g. Mendelson and Shaw, 2005). This
69 implies a possible role for sexual selection to elaborate sexual trait divergence above and
70 beyond what is expected by neutral processes (Ritchie 2007); a prediction that follows is
71 that phenotypic divergence is expected to be greater for sexual traits with a greater
72 influence on reproductive isolation (Rodríguez et al. 2013). Secondly, if sexual traits evolve
73 more rapidly due to coevolutionary feedback dynamics of sexual selection (Lande 1981),
74 these phenotypes should show greater divergence than those not subject to such selection
75 (Funk et al. 2009). However, few studies evaluate patterns of divergence among different
76 traits that might be targets of sexual selection, despite ample evidence that sexual selection
77 acts on traits in more than one modality within a species, for example olfactory, acoustic,
78 visual or tactile signals (Møller and Pomiankowski 1993, Hebets and Papaj 2005, Uetz et al.
79 2009, Girard et al. 2011). In addition, sexual selection can act upon different components of
80 complex or multicomponent signalling traits, for example morphologies and behaviours
81 which together generate a conspicuous acoustic or visual signal (Pomiankowski and Iwasa
82 1993; Rowe 1999). Given the potential multivariate, complex nature of sexual traits,
83 evaluating which are most likely to be targeted by sexual selection during evolutionary
84 elaboration or divergence remains challenging.
85
86 Testing for signatures of selection and drift in more than one sexual trait simultaneously can
87 illuminate constraints on the evolution of reproductive isolation via signal divergence. Here
88 we address this in a field cricket system (*Teleogryllus oceanicus*) by testing the
89 correspondence among patterns of phenotypic divergence in different male sexual traits—
90 acoustic advertisement signals, cuticular hydrocarbons, and morphology of sound-producing

wing structures—among allopatric populations, and by using this data with estimates of putatively neutral genetic divergence to subsequently test for signals of selection vs. neutral processes. Our key interest is the correspondence, or not, of population divergence among different sexual traits: Is population divergence of a similar magnitude across trait types, and do selection or other neutral processes similarly exaggerate different trait types? Do individual traits tend to be more integrated within each modality or component than they are between them, or are processes affecting divergence in one modality or component likely to constrain evolutionary responses in another?

T. oceanicus is found in northern and eastern Australia and Oceania (Otte and Alexander 1983). As with most grylline crickets, males produce conspicuous acoustic signals which function in mate recognition, mate location, close-range courtship, and aggression (Figure 1a) (Alexander 1967). The genus *Teleogryllus* has been a popular system for examining sexual selection on male song traits and the role of song in establishing reproductive barriers (e.g. Hoy et al. 1973, Simmons et al. 2001, Brooks et al. 2005). However, field crickets also express cuticular hydrocarbons (CHCs). CHCs are common in arthropods, and consist of long-chain waxy molecules thought to have evolved under selection for desiccation resistance (Figure 1b). Crickets can discriminate subtle variations in CHCs, the sexes express different CHC profiles, and there is evidence that both males and females discriminate among potential mates and thereby exert sexual selection on the composition of CHC blends (Tregenza and Wedell 1997, Thomas and Simmons 2009, 2010, Steiger et al. 2013, Capodeanu-Nägler et al. 2014, Simmons et al. 2014). Finally, acoustical properties of cricket songs are determined not only by variation in behaviours that produce temporal patterns of chirps such as wing closure rate, but also by structural features of the forewing

115 resonators that produce acoustic signals (Figure 1c) (Alexander 1962, Simmons and Ritchie
116 1996, Bennet-Clark 2003, Bailey et al. 2007, Moradian and Walker 2008). The male
117 forewings of *T. oceanicus* contain derived sound-producing structures, including two
118 oscillating membranes bounded by thickened, modified wing veins (Ragge 1955). These
119 morphological structures are also expected to be targets of sexual selection, although the
120 shape and intensity of that selection may differ from that on song, owing to the additional
121 behavioural motor patterns that combine to produce song phenotypes (Klingenberg et al.
122 2010).
123
124 This study combines previously-reported (Pascoal et al. 2016) and new data from allopatric
125 populations of *T. oceanicus* to examine male calling song traits, CHC profiles, and forewing
126 morphometrics measured in common garden laboratory conditions. Patterns of phenotypic
127 divergence were then compared with population genetic divergence. Our analyses tested
128 several hierarchical predictions. First, we predicted, and confirmed, that phenotypic trait
129 values vary across populations. The second prediction was that the three trait types show
130 corresponding patterns of phenotypic divergence among populations. The third was that
131 comparing this divergence to expectations under a neutral processes model derived from
132 neutral genetic markers would reveal a role for sexual selection in promoting variation
133 among populations in all three trait types. We report ample evidence for population
134 divergence within each modality and trait component, and unexpected phenotypic
135 integration (i.e. phenotypic correlation) across all three. However, phenotypic divergence
136 was largely consistent with expectations under neutral processes, and patterns of genetic
137 variation were less consistent with a stepping-stone model of island colonisation than they
138 were with simple isolation-by-distance. We discuss the evolutionary implications of

phenotypic integration and patterns of divergence across these three sexual traits.

Methods

CRICKET SAMPLING AND MAINTENANCE

Previously-published data analysed here include microsatellite-based population genetic data, male calling song recordings, and CHC profiles (Pascoal et al. 2016). These are archived on the Dryad Digital Repository (doi:10.5061/dryad.tb552). The calling song parameters from Daintree and Townsville, Australia, that we analyse here were additionally reported in Bailey and Macleod (2014). Detailed methodological descriptions for microsatellite, calling song and CHC analyses are provided in Pascoal et al. (2016), so we briefly summarise the procedures below. To these data we have added a morphometric analysis of male forewing resonating structures.

We sampled seven *T. oceanicus* populations distributed across eastern Australia and the Pacific. Stock populations were maintained in the lab at approximately 25 °C on a 12:12 light:dark cycle in a temperature-controlled chamber. Crickets were kept in 16 L plastic containers and fed Excel Junior and Dwarf rabbit pellets, provisioned with cardboard egg cartons for shelter and moistened cotton wool. Maintenance was carried out twice weekly. When experiments required crickets to be isolated, they were placed into small 118 mL plastic cups provisioned and maintained as above.

POPULATION GENETICS

Twenty-four wild-caught individuals from each population were screened using a panel of

10 polymorphic microsatellite loci (Beveridge and Simmons 2005, Pascoal et al. 2016). DNA extraction details, primer sequences and PCR conditions are provided in Pascoal et al. (2016), and samples were run on an ABI 3730 sequencer at Edinburgh Genomics. We calculated estimates of F_{ST} and F'_{ST} (Peakall and Smouse 2012) and constructed population-pairwise genetic distance matrices for subsequent analyses using GenePop v.4.0.10 (Raymond and Rousset 1995; Rousset 2008), FSTAT v.1.2 (Goudet 1995) and the Microsoft Excel add-in GenAIEx v.6.5 (Peakall and Smouse 2012; Verity and Nichols 2014).

TRAIT QUANTIFICATION

Calling Song

We previously reared crickets in a common garden environment in the lab and recorded the calling songs of between 18-21 adult males per population (Bailey and Macleod 2014; Pascoal et al. 2016). Stock populations experienced at least two generations of lab rearing, thereby reducing the potential for maternal effects arising from field conditions. Recordings were made using a Sennheiser ME66 microphone under red light between 23 – 27 °C during the crickets' dark cycle, and we only analysed males from which we could obtain ten complete song phrases. We used Sony Sound Forge 7.0a to quantify 15 song traits.

Cuticular Hydrocarbons

We previously analysed the CHC profiles of 768 adult male crickets between the ages of 7 – 10 days post-eclosion (Pascoal et al. 2016). Frozen crickets were thawed and immersed in 4 mL of HPLC-grade hexane (Fisher Scientific) for five minutes. 2 µL samples of a 100 µL aliquot reconstituted in hexane with a 10ppm pentadecane standard were processed in an Agilent 7890 gas chromatographer and an Agilent 5975B mass spectrometer (GC-MS) on a

30 m x 0.25 mm internal diameter DB-WAX column with helium as a carrier gas. GC-MS conditions are described fully in Pascoal et al. (2016). We estimated the relative abundance of 26 CHC peaks using MSD CHEMSTATION v.E.02.00.493 (Agilent). Ion 57 was the target and we corrected peak abundances by dividing each by the abundance of the pentadecane standard. Log_{10} transformed relative peak abundances were used in subsequent statistical analyses.

Forewing Morphometrics

Shape and relative placement of sound-producing structures on male forewings were measured using landmark-based geometric morphometrics (Webster and Sheets 2010). We removed the right forewings from crickets that were used for the CHC analyses above (Pascoal et al. 2016) and mounted them between two microscope slides (n = 13 exclusions for torn or mislabelled wings). Wings were photographed using a Leica DFC295 digital camera attached to a Leica M60 dissecting microscope, and a 1 mm grid scale was included in photographs to facilitate later measurement. Using the program tpsDIG v.2.16 (Rohlf 2005), 11 landmarks were placed at prominent vein junctions defining the harp, scraper and mirror of the male forewing (Ragge 1955). Figure 1 illustrates the landmarks, which are modelled after those used in a morphometric study of a closely-related cricket, *Gryllus firmus* (Klingenberg et al. 2010). Several programs from the Integrated Morphometrics Package were used to superimpose landmark data from all samples and quantify shape variation using Procrustes distances (Zelditch 2012). Landmark data was combined from all individuals into a common dataset, and the program CoordGen6f (Zelditch 2012) was used to produce Procrustes distances. From this, we calculated principal components and scores describing the shape of resonating structures for each individual using PCAgen6l (Rohlf and

210 Slice 1990, Zelditch 2012).

211

212 Harp and mirror surface areas were calculated by measuring the area of the polygon
213 enclosing each wing structure (Figure 1). This technique was adopted for convenience, and
214 we validated it in a randomly-chosen subset of 50 wings for which the exact outlines of the
215 harp and mirror were drawn manually and the surface areas calculated. The validation
216 showed a strong positive correlation between the two measurement techniques (see
217 Supplemental Figure S1), so analysis proceeded using the original polygon-based
218 measurements. A further validation was performed on the same set of 50 wings, in which
219 we placed landmarks on the original photos a second time, and re-calculated harp and
220 mirror surface area. The results of this validation (see Supplemental Figure S1) similarly
221 indicated confidence in the precision of our protocol. Landmark placement and
222 measurement for the validation were performed blind to sample identity.

223

224 **ANALYSES**

225 *Population Variation in Sexual Traits*

226 We focused on a subset of five key sub-traits from each modality and component to
227 facilitate statistical modelling of divergence across populations, and to test how such
228 patterns of divergence did or did not correspond among the three types of traits. Wing (n =
229 755) and CHC (n = 768) traits were quantified from the same individuals in the previously
230 described experiment, which examined social environment effects, while calling song traits
231 were quantified from a different set of individuals (n = 137) (Pascoal et al. 2016). The five
232 calling song traits were: number of long chirps, number of short chirps, carrier frequency,
233 long chirp-short chirp interval, and inter-song interval. We chose these traits because they

were found to be the main targets of selection in a multivariate selection analysis of calling song in the closely-related sister species *T. commodus* (Brooks et al. 2005). The five CHC traits comprised the first 5 PCs based on the same extraction implemented in Pascoal et al. (2016), which cumulatively explained 71.9% of variation in CHC profiles (PC1 = 38.4%, PC2 = 16.5%, PC3 = 7.3%, PC4 = 5.1%, PC5 = 4.6%). Landmark-based morphometric data captured information about the shape and relative placement of key wing vein junctions independent of the absolute size of the surrounding features. However, harp and mirror surface area also have an important influence on male carrier frequency (Alexander 1962, Simmons and Ritchie 1996, Bennet-Clark 2003, Bailey et al. 2007, Moradian and Walker 2008), so our five wing morphology traits included absolute measures of both harps and mirrors, plus the first three relative warps which cumulatively explained just over 50% of the variation in forewing shape, independent of size (variance explained by relative warps for wing landmarks: RW1 = 25.1%, RW2 = 15.0%, RW3 = 10.2%).

The experiment described in Pascoal et al. (2016) examined the effects of a social environment manipulation on CHC expression. However, this effect was not of direct interest here and sample sizes were balanced across treatments in the experiment, so for the CHC and wing morphometric data we did not model the social environment (or incubator, for which we found no significant effect in the previous study (Pascoal et al. (2016)). Each trait was divided by its standard deviation (across all populations), giving a standard unit variance, to ensure that they all entered models scale-independent.

We used canonical variates analyses (CVA) implemented in SPSS v.21 to visualise patterns of population variation in song, CHC, and wing traits. This was only done for purposes of

illustrating overall patterns of phenotypic differentiation among populations, as the five individual traits selected for each trait type included existing latent variables extracted from PC analyses. CVA maximises variation among pre-defined groups and it is a useful tool for visualising differences among such groups. We therefore modelled "population" as a factor, and plotted scores from the first two canonical variates axes for each trait type. In addition, we used CVAgen v.6l to visualise the main sources of variation in wing landmark data across populations. The latter analysis used all relative warps from the landmark-based morphometric approach described above, and wing landmark variation was regressed on the first significant canonical variate axis to produce a Procrustes deformation grid and vectors describing the relative magnitude and direction of landmark displacement among populations. The scaling factor was set to 0.2.

Comparison of Phenotypic Divergence in Different Traits

We used REML linear models to formally evaluate among population differences within each trait type, and facilitate subsequent comparison against population divergence in individual traits. We first fit three multivariate linear models using REML, one for each modality (song, CHC, wing morphology). In each case, the five observed traits (in standard deviation units) were treated as response variables with population as a predictor (i.e. analogous to a classical MANOVA analysis). Given evidence of population effects on each modality (see Results), univariate REML models were used to test the significance of population effects on individual traits.

We then estimated the among-population (genetic) variance covariance matrix (**D**) for the complete set of 15 traits. Although **D** is defined as the among–trait covariance matrix of

population specific means, we chose to re-estimate these parameters using MCMC rather than REML to better carry statistical uncertainty forward to subsequent analytical steps. Thus, we re-estimated population specific trait means using a multivariate (15 trait) linear model fitted in MCMCglmm, with a single (fixed) factor of Population specified for each trait. The model was run with default priors for 20,000 iterations with a burn-in of 5,000 iterations and a thinning interval of 10. Model convergence was checked visually and by comparison of posterior means for each parameter to the REML estimates (which were very similar in all cases). **D** was then determined as the among-trait covariance matrix of the trait means. We defined credible intervals (CIs) as the 95% highest posterior density interval of the posterior for each element of **D**, and consider off-diagonal elements (i.e. covariances) to be significant at $P < 0.05$ if the CI did not span zero. We note that CIs for diagonal elements (i.e. variances) are constrained to positive space so cannot be used for inference, but among-population variance was already tested in the REML analysis. To better interpret the covariance structure of **D** matrix, we subjected it to eigendecomposition and also rescaled to the correlation matrix $\mathbf{D}_{cor}$. We also calculated the traces (with CI) of the 5x5 submatrices of **D** corresponding to each trait type to test whether among-population divergence was different between the three trait types.

Selection Versus Neutral Divergence of Phenotypes

To determine whether patterns of among-population divergence in song, CHC and wing traits were consistent with a neutral model we used several complementary approaches. First, using the point estimates of the multivariate phenotypic mean (from the MCMC model described above), we calculated the phenotypic distance matrix (as the Euclidean distance in 15 dimensional trait space) among populations and tested whether this was correlated

306 with the microsatellite-based F_{ST} and F'_{ST} distance matrices (where F'_{ST} scales from 0 to 1).
307 Second, we used Mantel tests to check for correlation of the phenotypic distance matrix
308 (and the microsatellite distance matrices) with geographic distance. Geographic distances
309 among all population pairs were calculated using the Great Circle Mapper
310 (www.gcmap.com), under two putative models of cricket dispersal and colonisation. The
311 first calculated point-to-point distances between population pairs assuming direct,
312 unimpeded movement from one location to the other, whereas the second calculated
313 pairwise distances assuming an island-hopping model in which crickets migrated from
314 coastal/mainland populations in Australia across successive Pacific islands. Patterns of allelic
315 diversity in this species are consistent with serial bottlenecks experienced by founding
316 propagules of crickets that dispersed from west to east across Oceania (Tinghitella et al.
317 2011). The second geographic distance model accounted for the different geographic
318 structure expected under such a scenario by assuming free movement of crickets among the
319 three mainland Australian populations, while constraining distance calculations involving
320 island populations to the following sequence: mainland → Fiji → Mangaia → Tahiti →
321 Hawaii. Such a sequence might be expected if crickets accompanied humans during early
322 migrations across Oceania, or where range expansion occurred in a stepping-stone fashion.
323
324 Finally, we followed the mixed-model approach described in Pascoal et al. (2016) to test
325 whether there was more among-population variance than expected under a neutral model.
326 For each trait, we fitted a mixed model using REML in which the phenotype was predicted
327 by a single fixed effect of the mean and a random effect of population. We assumed
328 populations have diverged neutrally (i.e., under neutral processes alone), such that levels of
329 the random effects are drawn from a normal distribution with mean 0 and variance, to be

estimated, of $V_{POP(neutral)}$. Provided the microsatellite data provide an unbiased expectation of neutral divergence, then the expected covariance between a pair of observations, one on an individual in population i and one on an individual in population j, is equal to (1-F'_{STij})*$V_{POP(neutral)}$. For each trait this model was then compared to one in which a second random effect of population was added to account for additional among-population variance above that expected under neutrality ($V_{POP(sel)}$). We assumed that twice the difference in model log-likelihoods (LnL) is distributed as a 50:50 mix of χ^2_1 and χ^2_0 (following Visscher 2006), with a significant improvement in fit being indicative of selection contributing to total among-population variance. As also noted in Pascoal et al (2016), we stress that the asymptotic approximation of the test statistic to a χ^2 distribution may not give reliable results with only seven levels (i.e. populations) for each random effect. Thus, while *P* values are provided they should be interpreted cautiously.

Results

POPULATION VARIATION IN SEXUAL TRAITS

Table 1 shows the results of multivariate fixed effect models and the univariate fixed effect models for each of the 15 traits. The multivariate model showed a clear difference in song traits across populations and the univariate models confirm that all traits contribute significantly to this overall multivariate effect (Table 1). There were also significant differences in the CHC profiles of males across populations in the multivariate model, and each of the five vectors describing CHC expression contributed to this overall multivariate effect (Table 1). Similarly, multivariate analysis showed that wing morphology varied significantly across populations (Table 1). Univariate analyses confirmed that the geometric

353 shape of the wings (Rw1-3), as well as mirror and harp area, significantly contributed to this
354 overall multivariate effect (Table 1). Supplemental Table S1 reports details of the canonical
355 variates analyses implemented to visualise population variation in each trait.
356
357 **POPULATION DIVERGENCE IN DIFFERENT TRAIT TYPES**
358 Table 2 presents the among-population variance-covariance matrix, **D**, for the five traits
359 contributing to each modality. The among-population variances in each modality are
360 provided along the diagonal of this matrix and the sum of these estimates within each
361 modality (the trace) provides an estimate of the total amount of divergence of traits in each
362 modality. The estimated amount of divergence was greatest in wing morphology (1.311,
363 95% CIs: 1.187, 1.501), followed by song traits (1.281, 95% CIs: 1.203, 1.950) and then CHC
364 traits (1.139, 95% CIs: 1.029, 1.316). However, overlapping credible intervals indicate there
365 were no significant differences in the amount of divergence between the three trait types.
366 The mean magnitude of correlations calculated using point estimates from Table 2 was
367 0.477 within types, and 0.507 between types. However, these were statistically
368 indistinguishable using an anti-conservative *t*-test (2-tailed *t*-test: $t = -0.528$, $P = 0.599$). The
369 magnitudes of within-type trait correlations were also similar when disaggregated by trait
370 type: they were 0.369 for song traits, 0.590 for CHCs and 0.472 for wings, and again
371 indistinguishable in an anti-conservative test (one-way ANOVA: $F_{2,27} = 1.264$, $P = 0.299$).
372
373 Table 3 presents the eigendecomposition of **D**. We retained the first six vectors from this
374 decomposition for interpretation, which collectively explained >99.9% of the variation in **D**.
375 The dominant vector ($\mathbf{D_{max}}$) explained 63.5% of this variance and was significantly loaded to
376 all CHC traits and four out of five wing morphology traits. In contrast, for song traits only the

number of long chirps and the number of short chirps were significantly loaded to $\mathbf{D_{max}}$ (Table 3).

TESTING FOR A SIGNAL OF SEXUAL SELECTION

Using Mantel tests, we compared the multivariate divergence in trait means across types to geographic distance matrices to determine if mean phenotypic divergence could be explained by the degree of geographical isolation. We used two different geographic distance matrices: the first was based on the shortest physical distance between population pairs, while the second was based on the hypothetical west-east island hopping colonization route proposed by Tinghitella *et al.* (2011). In both cases, mean trait divergence was significantly correlated with geographic distance (physical distance: $r = 0.738$, $P = 0.010$; island hopping: $r = 0.554$, $P = 0.010$), although the correlation was weaker in the latter scenario.

Univariate mixed models comparing the among population divergence expected under neutral divergence (based on the F'_{ST} matrix across populations) to a model that allows additional among population divergence (i.e. implicating a role for selection) are presented in Supplemental Table S2. Significance of these models could be taken as evidence that neutral processes alone are insufficient to explain divergence between populations for a given trait. However, for all traits, the neutral model adequately explained population divergence. Collectively, these analyses suggest that drift coupled to restricted gene flow is the likeliest explanation for most divergence in traits across populations. In support of this argument, a comparison of the multivariate divergence in trait means to the F'_{ST} matrix showed that these matrices were significantly positively correlated ($r = 0.764$, $P = 0.010$).

401

402 *Discussion*

403 Causally linking the process of sexual selection with patterns of phenotypic differentiation is
404 a fundamental challenge in evolutionary and behavioural research. Key to this is
405 understanding the form and features of total sexual selection; that is, the combined effects
406 of episodes of sexual selection arising from discrete mechanisms such as male-male
407 competition and female choice, or episodes of sexual selection occurring at different
408 timescales or through different sexual traits (Hunt et al. 2009). On a trait-by-trait basis, the
409 shape of sexual selection might be expected to differ among modalities and among trait
410 components, owing to variable constraints imposed by other sources of selection and
411 genetic architectures, and thus provoke disjointed evolutionary responses (Greig et al.
412 2015). Our results clearly indicate that *T. oceanicus* populations show phenotypic
413 divergence in sexually-selected traits. In addition, the three trait types—male calling song,
414 CHCs and wing morphology—show evidence of phenotypic divergence at roughly equal
415 levels. Populations diverge in a fully multivariate way, with the major axis of overall
416 differentiation in **D** loading on all three trait types.
417
418 The fact that a signal of selection was undetectable for all three sexual traits was
419 unexpected, particularly in view of the finding that female preferences for male calling song
420 vary across other populations of the same species (Simmons et al. 2001). Numerous studies
421 have documented mate choice for all three types of traits in field crickets; their use as
422 exemplars in sexual selection research is well-established. A potential explanation may lie in
423 the fact that most studies infer the action of sexual selection (a) within populations (b) using

mate choice experiments and (c) while keeping constant other potential sources of selection such as fecundity or ecological selection. Studies that demonstrate causal links between sexual selection, an evolutionary response to that selection, and patterns of phenotypic diversification are surprisingly uncommon, given theoretical expectations about the rapid rate of evolution by sexual selection (Svensson and Gosden 2007). Thus, while there is an abundance of evidence that sexual selection operates on a wide variety of traits in a multitude of organisms, extending that insight to demonstrate its causal role in promoting diversification is a challenge that has largely remained unmet. A recent meta-analysis highlights the importance of this conceptual distinction, finding that absolute phenotypic divergence in female preferences for male secondary sexual traits best predicts patterns of diversification of those traits, rather than the intensity of selection operating on the traits (Rodríguez et al. 2013).

Research on multimodal and multicomponent sexual selection is still relatively underdeveloped (Coleman 2009, Prokop and Drobniak 2016), but several recent studies have examined the form and intensity of sexual selection on different types of signalling traits within a single population or species. For instance, a population of the lark bunting *Calamospiza melanocorys* experienced highly variable sexual selection pressures on multiple size and plumage colouration traits across different years (Chaine and Lyon 2008). Other studies have examined different targets of sexual selection in more than one population. For example, closely-related forms of the flycatcher *Monarcha castaneiventris* in the Solomon Islands behaviourally discriminate male plumage and song characters, and both contribute to premating isolation (Uy et al. 2008). In a similar study, Veltsos et al. (2011) simultaneously estimated sexual selection on male calling song and olfactory profiles in the

448 fruit fly *Drosophila montana*. Both traits were targets of sexual selection, but the form of
449 selection differed between them, and also between two populations (Veltsos et al. 2011). A
450 recent study tested the relationship between acoustic signals in a sister species of field
451 cricket, *Teleogryllus commodus*, and morphological features of male forewings that
452 contribute to their resonant properties (Pitchers et al. 2014). Pitchers et al. (2014) found
453 that wing morphology and acoustic signal properties covaried with differing strength in
454 different populations of this species, but that overall covariance was minimal and appeared
455 unrelated to patterns of population divergence. Such a pattern may be influenced by a
456 greater degree of lability in behavioural traits compared to morphological traits which are
457 fixed during development (Pitchers et al. 2014, Ower et al. 2016).
458
459 In this context, we would have predicted that behaviour associated with the production of
460 calling song in *T. oceanicus*, i.e. the temporal dynamics of wing opening and closure, could
461 play a more important role in responses to sexual selection than the structural wing
462 features determining carrier frequency of male song. Although the overall magnitude of
463 population divergence in each sexual trait was similar, the observation that song traits
464 showed the lowest level of phenotypic integration, i.e. did not load as strongly or
465 significantly onto $\mathbf{D_{max}}$ as wing or CHC traits, supports this idea. A potential explanation is
466 that the development of male wing structures may be less susceptible to the influence of
467 environmental noise compared to motor neurons, central pattern generators and sensory
468 apparatus involved in the behavioural production of song, and for CHCs, the direction of
469 evolutionary change might be more heavily influenced by stabilising natural selection on
470 CHC composition, which plays an important role in desiccation resistance (Foley and Telonis-
471 Scott 2011). Apart from these differences, male *T. oceanicus* traits generally covaried within

and between modalities in a consistent manner in our study, suggesting that unconstrained axes of variation capable of independently responding to selection might be relatively minor.

Conclusion

Despite progress documenting the action of sexual selection in multimodal and multicomponent signals modalities across taxa (Candolin 2003), it remains challenging to test whether different sexually selected traits diverge among populations in a uniform versus inconsistent manner. Such data can provide an important step towards establishing the relative contributions of different sexual traits to evolutionary diversification in species where selection potentially targets more than one sexual signal. Our results suggest that phenotypic integration across multiple sexual traits can act as a significant evolutionary constraint. Traits least constrained by genetic correlation and countervailing natural selection might be behaviours that can be flexibly adjusted, such as wing movements associated with acoustic signals in *T. oceanicus*, but we did not find evidence that selection acting on these has contributed to patterns of phenotypic divergence among allopatric populations. Instead, neutral processes such as drift appear to have played a dominant role in generating population differences in the phenotypic values of all three sexual traits.

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TABLES

Table 1. Analysis of divergence in songs, CHCs and wing morphology across populations in *T. oceanicus*. We started the analysis of each trait type by running a multivariate linear model including each of the 5 sub-traits per type (described in the main text) as the response variables. Each multivariate model was then followed by separate univariate linear models for each sub-trait to determine how these individual traits contribute to the overall multivariate difference between populations.

	Trait	***df*[1]**	***F***	***P***
calling song	Multivariate	30,321.5	7.07	<0.0001
	Univariate			
	LONG CHIRPS	6,130	5.73	<0.0001
	SHORT CHIRPS	6,130	19.20	<0.0001
	FREQUENCY	6,129	3.50	<0.0001
	LC-SC INTERVAL	6,130	6.40	<0.0001
	INTER-SONG INTERVAL	6,130	3.56	<0.0001
cuticular hydrocarbons	Multivariate	30,2004.4	58.53	<0.0001
	Univariate			
	CHC1	6,761	36.08	<0.0001
	CHC2	6,761	25.47	<0.0001
	CHC3	6,761	68.33	<0.0001
	CHC4	6,761	18.37	<0.0001
	CHC5	6,761	13.72	<0.0001
wing morphology	Multivariate	30,1969.8	33.30	<0.0001
	Univariate			
	RWA1	6,748	11.85	<0.0001
	RWA2	6,748	67.63	<0.0001
	RWA3	6,748	24.34	0.0030
	MIRROR	6,748	55.87	<0.0001
	HARP	6,748	35.23	0.0027

[1] (numerator,denominator)

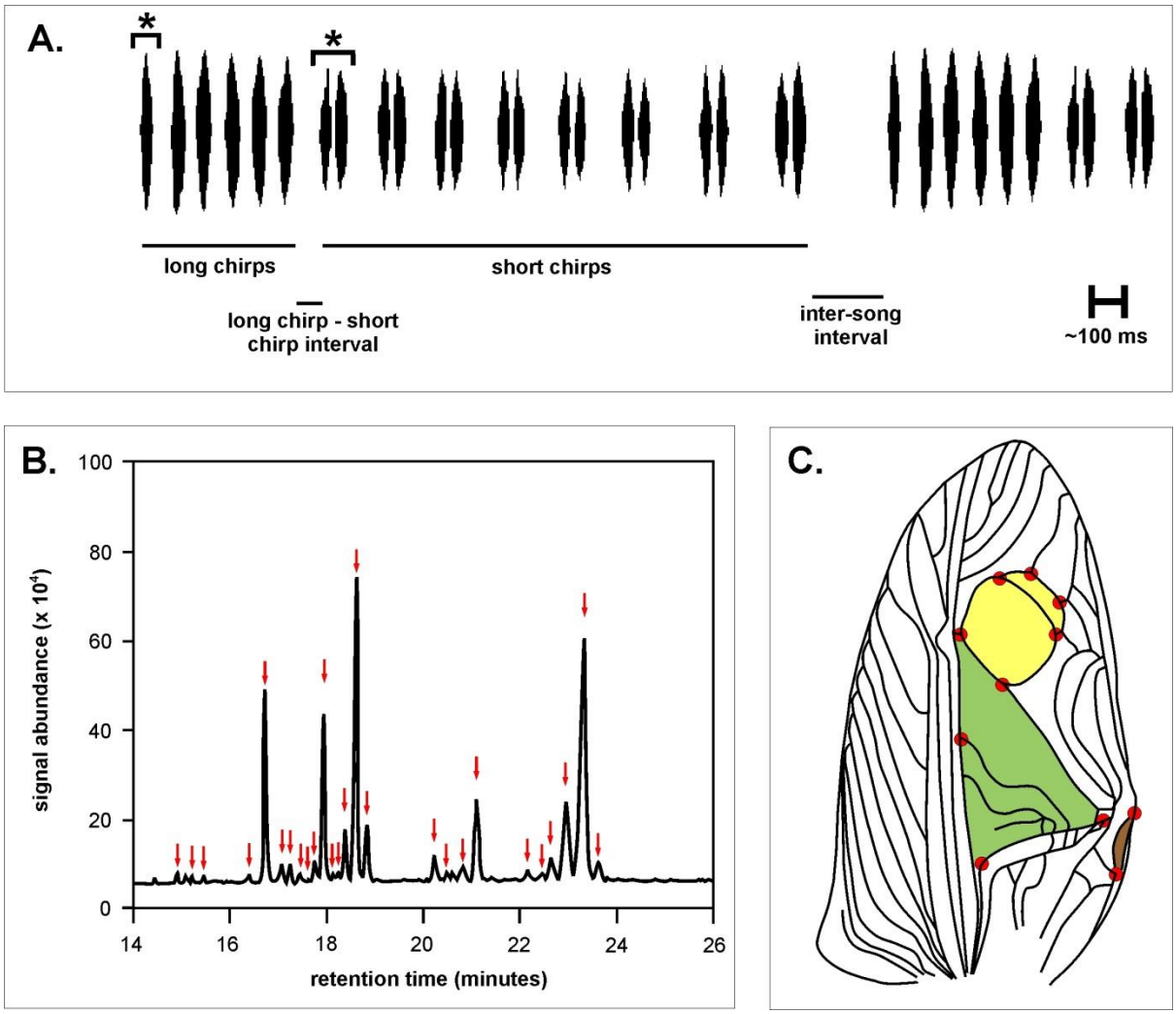
Table 2: The among-population variance-covariance matrix (**D**) among trait means for song, CHC and wing morphology traits showing among-population variances (shaded diagonal) and covariances (above diagonal), as well as corresponding correlations (below diagonal). 95% CIs are provided in brackets and bold font denotes statistically significant parameters (based on 95% CIs not overlapping zero).

		calling song					cuticular hydrocarbons					wing morphology				
		LONG CHIRPS	SHORT CHIRPS	FREQUENCY	LC-SC INTERVAL	INTER-SONG INTERVAL	CHC1	CHC2	CHC3	CHC4	CHC5	RWA1	RWA2	RWA3	MIRROR	HARP
calling song	LONG CHIRPS	**0.224** (0.118,0.445)	**-0.306** (-0.461,-0.182)	-0.074 (-0.184,0.033)	0.056 (-0.062,0.169)	0.046 (-0.044,0.183)	**-0.118** (-0.226,-0.05)	**0.106** (0.045,0.206)	**-0.186** (-0.295,-0.074)	-0.037 (-0.111,0.038)	**0.066** (0.012,0.153)	**-0.062** (-0.143,-0.009)	**-0.228** (-0.345,-0.133)	**0.089** (0.018,0.18)	**-0.171** (-0.285,-0.083)	**-0.19** (-0.276,-0.102)
	SHORT CHIRPS	**-0.896** (-0.967,-0.607)	**0.521** (0.368,0.777)	0.088 (-0.067,0.208)	-0.017 (-0.153,0.129)	-0.037 (-0.21,0.074)	**0.248** (0.171,0.339)	**-0.188** (-0.259,-0.102)	**0.404** (0.318,0.515)	**0.171** (0.097,0.248)	**-0.173** (-0.264,-0.118)	**0.136** (0.063,0.205)	**0.429** (0.342,0.538)	-0.01 (-0.081,0.078)	**0.362** (0.288,0.476)	**0.322** (0.238,0.409)
	FREQUENCY	-0.411 (-0.737,0.137)	0.321 (-0.179,0.629)	**0.145** (0.057,0.354)	-0.015 (-0.13,0.097)	-0.058 (-0.16,0.043)	**0.125** (0.036,0.22)	**-0.103** (-0.188,-0.018)	0.001 (-0.122,0.108)	-0.023 (-0.097,0.053)	-0.04 (-0.111,0.024)	0.047 (-0.013,0.116)	0.109 (-0.012,0.218)	**-0.081** (-0.16,-0.001)	0.102 (-0.011,0.205)	**0.11** (0.009,0.187)
	LC-SC INTERVAL	0.236 (-0.22,0.557)	-0.047 (-0.378,0.299)	-0.08 (-0.48,0.407)	**0.252** (0.132,0.478)	**0.17** (0.069,0.299)	-0.033 (-0.133, 0.05)	0.054 (-0.027,0.14)	0.046 (-0.055,0.159)	**0.095** (0.043,0.192)	**-0.088** (-0.165,-0.031)	**0.109** (0.033,0.16)	-0.048 (-0.147,0.075)	**0.082** (0.025,0.178)	-0.026 (-0.117,0.099)	-0.009 (-0.102,0.079)
	INTER-SONG INTERVAL	0.259 (-0.185,0.712)	-0.135 (-0.566,0.25)	-0.406 (-0.72,0.197)	**0.898** (0.468,0.977)	**0.143** (0.078,0.368)	**-0.087** (-0.186,-0.007)	**0.092** (0.022,0.188)	0.023 (-0.114,0.115)	0.059 (-0.022,0.124)	-0.036 (-0.095,0.035)	0.048 (-0.026,0.106)	**-0.082** (-0.229,-0.004)	0.058 (-0.02,0.142)	-0.071 (-0.204,0.008)	-0.05 (-0.158,0.022)
cuticular hydrocarbons	CHC1	**-0.503** (-0.736,-0.183)	**0.693** (0.491,0.806)	**0.659** (0.224,0.87)	-0.133 (-0.46,0.177)	**-0.464** (-0.811,-0.112)	**0.246** (0.199,0.335)	**-0.17** (-0.221,-0.126)	**0.197** (0.151,0.26)	**0.093** (0.041,0.135)	**-0.116** (-0.161,-0.073)	**0.076** (0.031,0.118)	**0.283** (0.243,0.353)	0.024 (-0.028,0.063)	**0.279** (0.234,0.335)	**0.217** (0.171,0.266)
	CHC2	**0.492** (0.221,0.78)	**-0.569** (-0.721,-0.34)	**-0.592** (-0.801,-0.11)	0.236 (-0.107,0.543)	**0.535** (0.159,0.824)	**-0.748** (-0.883,-0.614)	**0.208** (0.142,0.268)	**-0.085** (-0.145,-0.034)	-0.028 (-0.072,0.012)	**0.055** (0.013,0.099)	**-0.084** (-0.123,-0.04)	**-0.203** (-0.267,-0.157)	0.042 (-0.008,0.078)	**-0.167** (-0.23,-0.122)	**-0.13** (-0.175,-0.079)
	CHC3	**-0.613** (-0.794,-0.253)	**0.873** (0.767,0.952)	0.003 (-0.47,0.331)	0.143 (-0.145,0.463)	0.094 (-0.355,0.45)	**0.617** (0.477,0.725)	**-0.289** (-0.466,-0.106)	**0.412** (0.339,0.509)	**0.223** (0.161,0.27)	**-0.177** (-0.237,-0.135)	**0.102** (0.044,0.146)	**0.344** (0.297,0.411)	**0.117** (0.057,0.163)	**0.323** (0.271,0.384)	**0.252** (0.202,0.309)
	CHC4	-0.194 (-0.507,0.176)	**0.591** (0.359,0.773)	-0.153 (-0.568,0.273)	**0.472** (0.214,0.792)	0.391 (-0.111,0.66)	**0.469** (0.229,0.62)	-0.155 (-0.392,0.059)	**0.866** (0.731,0.942)	**0.161** (0.1,0.215)	**-0.115** (-0.152,-0.078)	**0.074** (0.029,0.104)	**0.155** (0.098,0.209)	**0.126** (0.081,0.163)	**0.162** (0.104,0.209)	**0.099** (0.05,0.142)
	CHC5	**0.419** (0.068,0.736)	**-0.718** (-0.885,-0.524)	-0.317 (-0.641,0.18)	**-0.523** (-0.782,-0.205)	-0.288 (-0.605,0.22)	**-0.704** (-0.83,-0.477)	**0.36** (0.1,0.587)	**-0.829** (-0.932,-0.684)	**-0.861** (-0.953,-0.686)	**0.111** (0.074,0.173)	**-0.086** (-0.12,-0.05)	**-0.158** (-0.221,-0.111)	**-0.06** (-0.105,-0.024)	**-0.16** (-0.207,-0.11)	**-0.13** (-0.174,-0.087)
wing morphology	RWA1	**-0.387** (-0.741,-0.103)	**0.559** (0.292,0.775)	0.366 (-0.092,0.679)	**0.645** (0.197,0.791)	0.379 (-0.178,0.678)	**0.457** (0.218,0.675)	**-0.544** (-0.739,-0.313)	**0.472** (0.247,0.679)	**0.546** (0.222,0.703)	**-0.768** (-0.906,-0.501)	**0.114** (0.068,0.172)	**0.107** (0.052,0.156)	0.01 (-0.038,0.04)	**0.091** (0.042,0.139)	**0.09** (0.042,0.13)
	RWA2	**-0.757** (-0.914,-0.483)	**0.931** (0.817,0.971)	0.451 (-0.024,0.729)	-0.149 (-0.406,0.2)	**-0.339** (-0.719,-0.02)	**0.894** (0.804,0.957)	**-0.698** (-0.821,-0.565)	**0.841** (0.766,0.908)	**0.607** (0.396,0.731)	**-0.746** (-0.868,-0.57)	**0.498** (0.284,0.699)	**0.406** (0.346,0.511)	0.024 (-0.029,0.082)	**0.369** (0.328,0.444)	**0.296** (0.247,0.36)
	RWA3	**0.445** (0.084,0.709)	-0.034 (-0.256,0.243)	**-0.504** (-0.764,-0.023)	**0.387** (0.13,0.725)	0.366 (-0.042,0.708)	0.116 (-0.13,0.271)	0.216 (-0.033,0.409)	**0.432** (0.219,0.571)	**0.747** (0.553,0.871)	**-0.429** (-0.636,-0.161)	0.071 (-0.256,0.289)	0.089 (-0.113,0.275)	**0.178** (0.127,0.246)	**0.065** (0.005,0.105)	-0.008 (-0.055,0.036)
	MIRROR	**-0.605** (-0.833,-0.315)	**0.839** (0.713,0.937)	0.445 (-0.018,0.728)	-0.085 (-0.372,0.252)	**-0.313** (-0.751,-0.015)	**0.939** (0.849,0.969)	**-0.613** (-0.768,-0.463)	**0.842** (0.763,0.909)	**0.673** (0.478,0.803)	**-0.802** (-0.899,-0.607)	**0.449** (0.187,0.627)	**0.968** (0.923,0.992)	**0.257** (0.022,0.394)	**0.358** (0.297,0.451)	**0.275** (0.224,0.332)
	HARP	**-0.793** (-0.947,-0.513)	**0.882** (0.749,0.963)	**0.572** (0.072,0.762)	-0.037 (-0.37,0.277)	-0.262 (-0.665,0.107)	**0.866** (0.76,0.937)	**-0.564** (-0.731,-0.403)	**0.776** (0.68,0.88)	**0.489** (0.278,0.666)	**-0.769** (-0.892,-0.583)	**0.525** (0.307,0.737)	**0.919** (0.834,0.963)	-0.039 (-0.246,0.164)	**0.909** (0.84,0.969)	**0.256** (0.181,0.319)

Table 3. Eigendecomposition of the **D** matrix. Only the first six vectors are retained for interpretation as they collectively explain >99.9% of the observed among-population (co)variance in song, CHC and wing morphology traits. 95% CIs are provided in brackets. Estimates of trait loadings are considered statistically significant (bold font) if 95% CIs do not overlap zero (note this is necessarily true for the eigenvalues themselves).

	Vector	**1**	**2**	**3**	**4**	**5**	**6**
	Eigenvalue	2.372 (2.184, 2.789)	0.680 (0.558, 0.987)	0.297 (0.266, 0.522)	0.269 (0.172, 0.360)	0.101 (0.066, 0.183)	0.016 (0.013, 0.080)
	Proportion of variance	0.635 (0.556, 0.659)	0.182 (0.148, 0.239)	0.080 (0.066, 0.123)	0.072 (0.045, 0.089)	0.027 (0.017, 0.044)	0.004 (0.002,0.019)
	Trait load						
calling song	LONG CHIRPS	**0.236** (0.142, 0.345)	-0.184 (-0.417, 0.054)	-0.188 (-0.647, 0.442)	0.461 (-0.163, 0.631)	0.036 (-0.369, 0.316)	-0.083 (-0.430, 0.530)
	SHORT CHIRPS	**-0.446** (-0.518, -0.364)	0.015 (-0.175, 0.169)	0.088 (-0.438, 0.470)	-0.402 (-0.557, 0.051)	0.174 (-0.347, 0.353)	-0.211 (-0.550, 0.304)
	FREQUENCY	-0.107 (-0.217, 0.027)	0.235 (-0.065, 0.475)	0.365 (-0.328, 0.725)	0.295 (-0.359, 0.645)	-0.373 (-0.669, 0.031)	-0.466 (-0.604, 0.179)
	LC-SC INTERVAL	0.008 (-0.117, 0.126)	**-0.503** (-0.704, -0.259)	0.501 (-0.129, 0.655)	0.136 (-0.497, 0.568)	-0.068 (-0.358, 0.332)	0.090 (-0.446, 0.375)
	INTER-SONG INTERVAL	0.054 (-0.032, 0.206)	**-0.399** (-0.582, -0.171)	0.259 (-0.243, 0.494)	-0.156 (-0.472, 0.348)	-0.036 (-0.477, 0.301)	-0.229 (-0.483, 0.506)
cuticular hydrocarbons	CHC1	**-0.280** (-0.327, -0.235)	0.116 (-0.022, 0.235)	-0.031 (-0.420, 0.428)	0.424 (-0.026, 0.500)	-0.147 (-0.406, 0.202)	0.099 (-0.255, 0.446)
	CHC2	**0.191** (0.133, 0.237)	**-0.240** (-0.332, -0.086)	-0.169 (-0.416, 0.325)	-0.309 (-0.571, 0.169)	**-0.692** (-0.772, -0.285)	-0.097 (-0.412, 0.479)
	CHC3	**-0.367** (-0.412, -0.314)	**-0.290** (-0.378, -0.135)	-0.250 (-0.421, 0.149)	-0.242 (-0.449, 0.214)	-0.028 (-0.283, 0.229)	0.113 (-0.267, 0.435)
	CHC4	**-0.172** (-0.214, -0.107)	**-0.345** (-0.400, -0.209)	-0.137 (-0.279, 0.131)	0.065 (-0.175, 0.271)	0.126 (-0.184, 0.300)	-0.316 (-0.493, 0.214)
	CHC5	**0.177** (0.123, 0.224)	**0.200** (0.096, 0.281)	-0.117 (-0.256, 0.129)	-0.122 (-0.295, 0.192)	0.124 (0.201, 0.297)	0.029 (-0.380, 0.383)
wing morphology	RWA1	**-0.127** (-0.174, -0.072)	-0.146 (-0.249, 0.004)	0.402 (-0.071, 0.459)	0.102 (-0.486, 0.500)	0.306 (-0.052, 0.600)	0.263 (-0.202, 0.590)
	RWA2	**-0.409** (-0.452, -0.366)	0.079 (-0.042, 0.159)	-0.089 (-0.214, 0.091)	0.047 (-0.137, 0.217)	0.081 (-0.125, 0.285)	-0.240 (-0.450, 0.193)
	RWA3	-0.031 (-0.079, 0.034)	**-0.391** (-0.497, -0.205)	-0.409 (-0.573, 0.236)	0.277 (-0.381, 0.567)	0.081 (-0.225, 0.452)	0.122 (-0.399, 0.385)
	MIRROR	**-0.373** (-0.413, -0.327)	0.002 (-0.112, 0.118)	-0.180 (-0.337, 0.212)	0.233 (-0.171, 0.351)	-0.174 (-0.361, 0.100)	-0.123 (-0.378, 0.283)
	HARP	**-0.310** (-0.348, -0.257)	0.064 (-0.049, 0.152)	0.102 (-0.104, 0.218)	0.002 (-0.232, 0.197)	-0.392 (-0.561, 0.049)	**0.617** (0.011, 0.723)

736 **FIGURES**



737
738 **Figure 1.** Male *T. oceanicus* traits subject to sexual selection. (A) Oscillogram of a typical
739 male calling song, indicating the temporal parameters measured in the present study
740 (modified from Bailey and Macleod (2014)). The brackets indicated with asterisks highlight a
741 single long chirp (one pulse) and a single short chirp (typically paired pulses). (B)
742 Diagrammatic illustration of a gas chromatograph of a male cuticular hydrocarbon profile.
743 Peaks analysed in the present study are indicated with red arrows. (C) Principal sound-
744 producing structures on the male forewing, adapted from Pascoal et al. (2014). Red circles
745 indicate the 11 landmarks used in this study, which define the harp (green shading), mirror
746 (yellow shading) and scraper (brown shading).
747

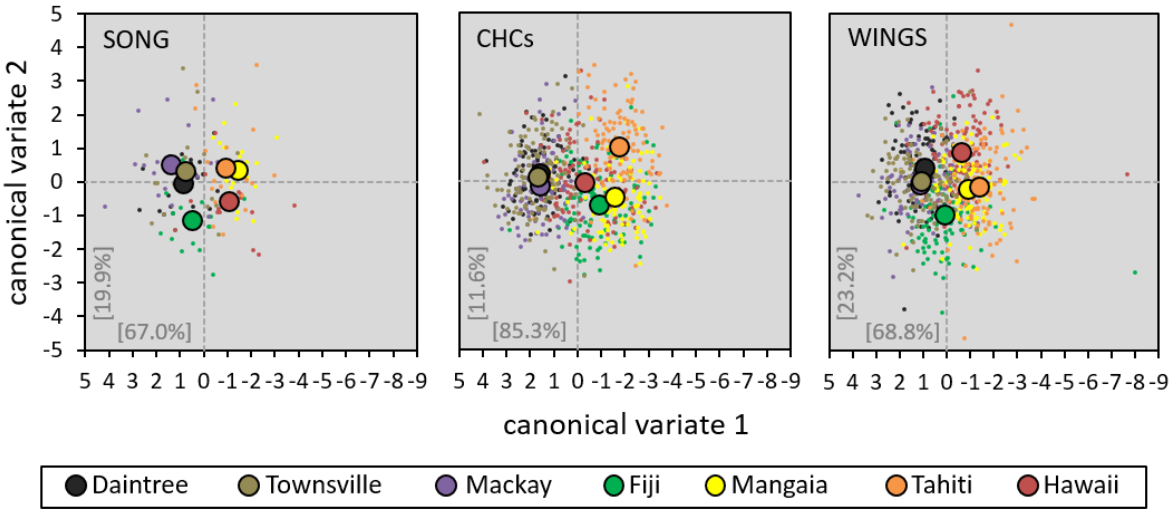


Figure 2. Population divergence in three sexually-selected male traits. Canonical variate analyses (CVAs) were used to visualise overall patterns of population divergence for calling song (n = 137), CHC profiles (n = 768), and forewing morphology (n = 755). All five individual traits for each sexual trait type were used in the respective CVAs. Data from the first two canonical variates components are plotted, and the proportion of variance explained by each axis is indicated by the grey text in brackets (see Table S1 for additional statistical details). Centroids for each population are depicted with larger dots. Colour-coding is indicated in the key. Some X-axes are reversed to maintain consistency with other figures.

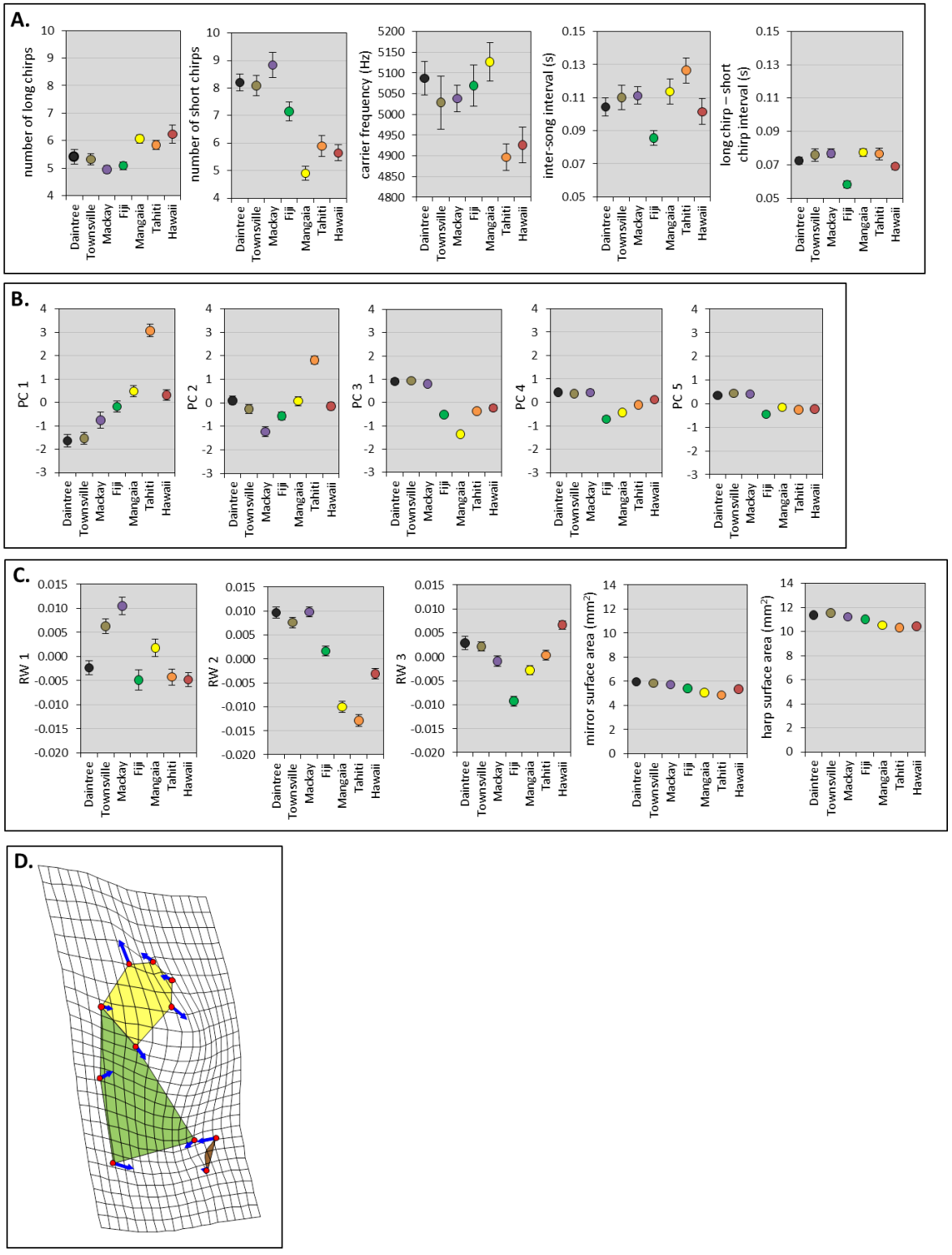


Figure 3. Population variation among the 5 individual traits measured for each modality in male *T. oceanicus*. Means and standard errors are indicated, and colour coding follows Figure 2. Where standard error bars are not visible, it is because they were obscured by the data points. (A) Calling song. The five traits examined in this study; data from Bailey and Macleod (2014) and Pascoal et al. (2016) are shown, and terminology follows Figure 1. (B) Cuticular hydrocarbons. The first five principal components describing relative abundances of 26 CHC peaks; data from Pascoal et al. (2016) are shown. (C) Wing venation. Population

768 variation in the first 3 relative warps describing variation in landmark placement on male
769 wings are depicted, as well as mean harp and mirror surface area in each population. (D)
770 Male forewing landmark deformation across all populations. The deformation grid
771 illustrates the main sources of variation in the shape of sound-producing structures among
772 populations, and the blue arrows are vectors showing the magnitude and direction of
773 landmark displacement. Highlighted structures are as in Figure 1C and demonstrate how
774 landmarks were joined to calculate mirror and harp surface area. Vectors were scaled using
775 a Procrustes deformation scaling factor of 0.2.