Chemical communication, sexual selection, and introgression in wall lizards

Hannah E A MacGregor[1,2], Rachel A M Lewandowsky[1,2], Patrizia d'Ettorre[3], Chloe Leroy[3], Noel W Davies[4], Geoffrey M While[1,2] & Tobias Uller[2,5]

[1] School of Biological Sciences, University of Tasmania, Hobart 7001, Australia

[2] Department of Zoology, Edward Grey Institute, University of Oxford, OX1 3PS, Oxford, UK

[3] Laboratory of Experimental and Comparative Ethology, Sorbonne Paris Cite, University of Paris 13, Villetaneuse, France

[4] Central Science Laboratory, University of Tasmania, Hobart 7001, Australia

[5] Department of Biology, Lund University, Sölvegatan 37, 223 62 Lund, Sweden

Abstract

Divergence in communication systems should influence the likelihood that individuals from different lineages interbreed, and consequently shape the direction and rate of hybridization. Here, we studied the role of chemical communication in hybridization, and its contribution to asymmetric and sexually selected introgression, between two lineages of the common wall lizard (*Podarcis muralis*). Males of the two lineages differed in the chemical composition of their femoral secretions. Chemical profiles provided information regarding male secondary sexual characters, but the associations were variable and inconsistent between lineages. In experimental contact zones, chemical composition was weakly associated with male reproductive success, and did not predict the likelihood of hybridization. Consistent with these results, introgression of chemical profiles in a natural hybrid zone resembled that of neutral nuclear genetic markers overall, but one compound in particular (tocopherol methyl ether) matched closely the introgression of visual sexual characters. These results imply that associations between male chemical profiles, sexual characters and reproductive success largely reflect transient and environmentally driven effects, and that genetic divergence in chemical composition is largely neutral. We therefore suggest that femoral secretions in wall lizards primarily provide information about residency and individual identity rather than function as sexual signals.

Key words: femoral pores, hybrid zone, hybridization, pheromones, olfaction,

Introduction

Population divergence in sexual characters used in communication shapes interactions upon secondary contact, with potential evolutionary consequences (West-Eberhard 1983; Panhuis et al. 2001). For instance, where one lineage has evolved exaggerated sexual characters favoured by sexual selection, this can result in asymmetric patterns of introgression (e.g. Parsons et al. 1993; Stein and Uy 2006; Baldassarre and Webster 2013). The majority of research on sexually selected introgression has focussed on the role of traits attributed to inter-sexual selection (e.g., female choice; Ryan and Wagner 1987). However, allopatric divergence in traits that primarily function in intra-sexual communication, including colours and morphological features used in competition between males, can also contribute to hybridization and introgression (see Moore 1987; Loehr et al. 2008 as examples). For example, if aggression towards divergent male phenotypes is biased or relaxed in males of one or both lineages (e.g. Pauers et al. 2008), certain male phenotypes could have an advantage in accessing high quality resources and females. Alternatively, differences in signals may be used to discriminate and avoid males of the other lineage (e.g. Simeonovska-Nikolova 2006). In both cases, divergence in male communication should mediate spatial organisation within hybrid zones and, as a consequence, encounter rates between males and females of different lineages.

Studies of vertebrates demonstrate that an evolutionary history of strong intra-sexual selection can cause males of one lineage to be consistently dominant over males of the other lineage, contributing to asymmetric genetic and phenotypic introgression (Pearson and Rohwer 2000; McDonald et al. 2001; Owen-Ashley and Butler 2004; Teeter et al. 2008; While et al. 2015). Male-male contests are often resolved through

60 communication (Searcy and Nowicki 2005) hence divergence in signals or cues
61 associated with dominance and resource holding potential could reinforce or mitigate
62 asymmetric introgression. Within this context, the literature on sexually selected
63 introgression has thus far focussed largely on visual and vocal characters. This is
64 unsurprising given that colours and song are considered reliable signals with well-
65 established roles in male-male competition as well as female choice (e.g. Alonso-Alvarez
66 2004; Abrahams et al. 2005; Zeil et al. 2006; Hamilton et al. 2013). In contrast, the role
67 of chemical communication in mediating patterns of introgression is less clear, despite
68 that chemical communication is taxonomically wide spread and functionally important
69 in reproductive behaviour (Wyatt 2014).

70 Chemical communication is particularly prevalent in lizards. In many species,
71 males deposit femoral secretions over their home range (Mason and Parker 2010).
72 These secretions are chemically complex and their composition may mediate social
73 interactions, territoriality, and reproduction (e.g. López and Martín 2002; Carazo et al.
74 2007), and ultimately play a key role in determining mating success (Mayerl et al.
75 2015). Furthermore, it is widely believed that the composition or prevalence of
76 particular compounds have evolved robust associations with other phenotypic
77 characters and hence serve as signals of male health and competitive ability, i.e.,
78 function as badges of status (Martín et al. 2007; Lopez et al. 2009). In Lacertid lizards,
79 for example, the proportions of cholesterol and campesterol have been shown to
80 correlate positively with body size (Lopez et al. 2006; Martín and Lopez 2007), and
81 higher proportions of cholesta-5,7-dien-3-ol, ergosterol and waxy esters have been
82 associated with lower parasite loads and higher immune responses (Lopez et al. 2006;
83 Martín et al. 2008). This has led to the suggestion that divergence in chemical

84 composition is functional, and may contribute to reduced or biased hybridization upon
85 secondary contact (Gabirot et al. 2012; Garcia-Roa et al. 2016). However, direct
86 evidence for this hypothesis is limited. A role for chemical communication in
87 hybridization and introgression has also been inferred from behavioural experiments
88 suggesting that males discriminate between con- and hetero-specifics based on
89 chemical cues (e.g. Cooper and Garstka 1987; Martín and López 2006; Gabirot et al.
90 2010), and the observation that hybridization between chemically divergent but
91 sympatric species is rare under natural conditions (Carretero 2008).

92 We studied the role of chemical communication in male dominance, spatial
93 organisation and hybridization between two lineages of the common wall lizard,
94 *Podarcis muralis* (Laurenti, 1768). These lineages are native to north-central Italy and
95 Western Europe, and have come together in several zones of secondary contact as a
96 result of natural and human-mediated range expansion (While et al. 2015). Phenotypic
97 divergence between the lineages is indicative of differences in the strength of sexual
98 selection on morphology, colouration, and behaviour (Heathcote et al. 2016; MacGregor
99 et al. 2017). Hybridization is asymmetric, with evidence for adaptive introgression of
100 visual sexual characters from the dominant Italian lineage into the Western European
101 lineage (While et al. 2015). If chemical communication is also sexually selected then we
102 predict (i) divergence in chemical characters between the lineages, (ii) consistent
103 associations between chemical composition and male secondary sexual characters and
104 reproductive success, especially in the Italian lineage where sexual selection has been
105 more intense, and (iii) clines in chemical profiles across the contact zone that resemble
106 other sexually selected traits.

To test these predictions we first established the extent of divergence in chemical profiles between lineages and associations with other male phenotypic traits. Secondly, we tested experimentally if the compositions of femoral secretions are associated with spatial organisation, reproductive success and hybridization in experimentally replicated zones of secondary contact. Finally, we examined the pattern of introgression of chemical profiles across a zone of secondary contact and tested if they corresponded to the patterns of sexually selected introgression previously demonstrated for morphology and colouration (While et al. 2015).

Methods

Study species

Common wall lizards, *P. muralis*, are small (45-75 mm snout-vent length), diurnal lizards that inhabit a range of natural and human-modified habitats across southern and central Europe. Intraspecific diversity is high with several genetically and geographically distinct mitochondrial clades (Giovannotti et al. 2010; Schulte et al. 2012; Salvi et al. 2013). The lineages in this study represent two major mitochondrial clades which diverged in glacial refugia approximately 2 million years ago (Gassert et al. 2013; Salvi et al. 2013). Here we refer to them as the Italian (ITA, here specifically corresponding to the Tuscan subclade *sensu* Schulte et al. 2012) and the Western European (WEUR) lineages. As well as being genetically differentiated, the populations of the Italian and Western European lineages studied here differ substantially in morphology and colouration, in particular in male secondary sexual characters (e.g. relative head size, bite force, testes mass, outer ventral scale UV-blue reflectance, While et al. 2015; MacGregor et al. 2017). Ventral colour polymorphism (red/yellow/white) is

present in some *P. muralis* populations, however, the influence of colour polymorphism does not represent a target for our analyses because colour morphs are at low frequencies in the Western European lineage and absent in Italian lineage (Uller et al. unpublished data).

Chemical sampling and analysis

Chemical sampling and phenotypic measurements

We captured 172 sexually mature males during their first seasonal reproductive episode (April-May) across three consecutive years (2013 to 2015). Sixty four males were captured for use in our enclosure experiment (hereafter referred to as experimental males) from allopatric populations in Italy and Western Europe (to avoid the confounding effects of introgression; Table S1). One hundred and eight males were captured from sixteen populations in northern Italy (Figure 1, Table S1) to test for patterns of chemical introgression (hereafter referred to as cline males). The sixteen populations form a cline across a natural hybrid zone with an mtDNA centre near Pisa in Tuscany (While et al. 2015).

We collected secretions from the femoral glands of all males by gently pressing around their femoral pores with sterilized forceps. For each male, secretions were collected directly into a glass vial (1.5 mL screw thread vials, Sigma Aldrich). All samples from cline males were collected in the field immediately following capture. For experimental males, we collected two secretion samples from each individual to assess within-individual repeatability in chemical composition. The first sample was collected following their capture (April: half of individuals immediately upon capture, and the

remaining prior to the release of males into the enclosures, see below), and the second between 49 and 75 days later (in June), immediately upon capture following the enclosure experiment. The secretion samples were stored cold while in the field and then at -20 °C until chemical extraction. In addition to femoral secretions we also recorded a number of morphometric measurements and obtained tissue samples from each lizard for genetic analyses by removing the tip of the tail, which was preserved in 90% ethanol. Morphometric measurements included snout-vent length ((SVL), measured with a ruler to the nearest mm), body mass (measured to the nearest 0.01 g using digital scales), head length and head width (recorded to the nearest 0.1 mm with callipers), ventral blackness and dorsal greenness. From the experimental males we additionally measured testes mass, outer ventral scale colour (OVS blue area, OVS hue and OVS UV chroma), and a performance trait (maximum bite force) in the laboratory (see While et al. 2015; MacGregor et al. 2017 for full methods regarding morphology data).

Chemical extraction and identification

All secretion samples were dissolved in pentane and analysed by Gas Chromatography Mass Spectrometry (GC-MS) with an Agilent Technologies 7890A gas chromatograph equipped an Agilent HP-5MS capillary column (30m × 0.25 mm × 0.25 μm) with helium as carrier gas at 1mL/min. The oven temperature was programmed at 50 °C for 1 min, increased to 180 °C at 30°C/min, then to 250 °C at 10 °C/min and finally to 320 °C at 5 °C/min and kept at 320 °C for 30 min (total run time per sample = 33.3 minutes). The GC was coupled with an Agilent 5975 C mass spectrometer (MS) with 70eV electron impact ionization.

177 Where possible we identified chemical compounds within the samples on the
178 basis of their mass spectra (MS) and retention times, which we verified using a
179 computerized MS library (National Institute for Standards and Technology, 2008), and
180 the assistance of an analytical chemist (author ND). Relative retention times were also
181 used to assist in compound identification. When the identity of a compound was
182 uncertain, we added the MS to an 'in house' database for recognition across samples. As
183 in previous reports on lizard secretions, including for *P. muralis* (Pellitteri-Rosa et al.
184 2014), many steroids could not be specifically identified and are reported by their
185 characteristic retention times and ions. In total, we characterised 67 compounds in the
186 femoral pore secretions of the males (Table S2).

187 To quantify the abundance of each compound we integrated peak areas using MS
188 Data Analysis software (Hewlett-Packard Chemstation Version C.00.07) with fixed
189 integration parameters (Initial Threshold: 16, Initial Peak Width: 0.1, Initial Area Reject:
190 1.0). Several compounds had similar retention times, and thus co-eluted. To overcome
191 this, we quantified the abundance of fourteen compounds based on individual
192 diagnostic ions (following McLean et al. 2012, the diagnostic quantitative ions used are
193 reported in Table S2).

194 To reduce the number of variables to be used in multivariate statistical analyses
195 and due to issues associated with accommodating large numbers of zero values (Martin
196 and Drijfhout 2009; Ranganathan and Borges 2011), only compounds that were
197 consistently detected across secretion samples were selected for our analyses. Thus, the
198 number of compounds to be used in the enclosures and cline analyses was reduced to
199 the 21 compounds that were detectable in >98% of samples (Table 1). None of the
200 initial 67 compounds were specific to either lineage; therefore our removal of

compounds from the enclosures and cline analyses that had low detectability was unlikely to exclude potential targets for sexually selected introgression.

For both the experimental and cline males we generated a relative measure of abundance for each compound by log-normal transforming the peak area according to the formula: $Z_{i,j} = \ln[Y_{i,j}/g(Y_j)]$, where $Z_{i,j}$ is the standardized peak area i for male j, $Y_{i,j}$ is the peak area i for male j, and $g(Y_j)$ is the geometric mean of all peaks for male j (Aitchison 1986). To apply the transformation formula on profiles with non-detectable compounds, we replaced zero values (n = 10 within enclosure male samples and n = 1 within cline male samples) with the proportion of the total ion current that represented the minimum percentage that was detected for a single compound within a sample considering all samples. Secretion samples showing signs of contamination were excluded (n = 6 enclosure male samples).

Repeatability and divergence in chemical composition

From the experimental males, we estimated within-individual repeatability in the relative abundances of the 21 chemical compounds between the first and second secretion sample. Intra-class correlation coefficients (ICC values) and their confidence intervals were calculated using the anova-based method (i.e. Lessells and Boag 1987) implemented in R package, ICC (Wolak et al. 2012).

We assessed the extent of divergence in chemical profiles between the Italian and Western European lineages using a permutational MANOVA (adonis function, 'vegan' package, Oksanen et al. 2007) with lineage as a fixed effect and a Euclidean distance matrix of the relative abundances of all 21 compounds as a response. This was run on the experimental males only to avoid confounding effects of introgression (see

224 above). To confirm that there were consistent differences between the lineages
225 independent of sampling date during the breeding season, we performed the MANOVAs
226 separately for the relative abundances of compounds in the first and second secretion
227 sample of the males. Because half of the first samples were collected in the lab, we
228 included a fixed factor (captive/non-captive) in the former analysis to account for the
229 effects of conditions experienced in captivity. Chemical variation within and between
230 the lineages was visualised by principal coordinates analysis of the Euclidean distance
231 matrix.

232

233 **Associations with sexual morphology, spatial organisation and reproductive**
234 **success**

235 Using semi-natural enclosures, we tested experimentally if the relative chemical
236 composition of femoral secretions could function as sexual signals, via co-variance with
237 male phenotype, dominance, and within or between-lineage reproductive success. In
238 April 2013, we transported 128 sexually mature lizards (the 64 experimental males and
239 64 females) captured from the allopatric Italian and Western European localities (Table
240 S1) to laboratory facilities at the Department of Zoology, University of Oxford, UK. The
241 lizards were transported from the field in cloth bags (kept below 10 °C) and, once in the
242 lab, they were housed in plastic terraria (590 × 390 × 415 mm) under a 12:12 light/dark
243 cycle, and provided with six hours of UV lighting per day prior to the experiment.

244 *Semi-Natural Enclosures Set-up*

245 In May 2013 we simulated the initial stage of secondary contact between the
246 Italian and Western European lineages by releasing lizards into eight (~ 7 × 7m)

247 experimental enclosures at the John Krebs Field Station, University of Oxford. Full
248 details of the experiment are described elsewhere (MacGregor et al. 2017). In brief, we
249 released male lizards into one of eight enclosures such that there were four Italian and
250 four Western European males per enclosure. The males were allowed at least nine days
251 to establish territories prior to the release of four Italian and four Western European
252 females per enclosure. We monitored the eight enclosures during the lizards' second
253 seasonal reproductive episode (May and June 2013) to collect positional and social
254 interaction data (based on a previously published ethogram, Heathcote et al. 2016). To
255 distinguish territorial interactions from non-territorial male-male behaviour, we only
256 classified interactions as male-male competition if they also included a submissive
257 behaviour (i.e. a retreat) by one male in the presence of another. Submissive behaviour
258 determined which male was recorded as the winner of the encounter, and these data
259 were used to generate within-enclosure dominance scores for each male (David 1988;
260 Gammell et al. 2003).

261 The core home range area of each lizard was estimated from positional data in
262 Ranges 8 (Kenward et al. 2008). We deemed the area of the 50% isopleth, generated
263 using a fixed-kernel contour analysis with a fixed smoothing parameter of 0.75 (a
264 balance between under and over smoothing; see Kie 2013), to represent a lizard's core
265 home range. For each male, we calculated the sum of his percentage core home range
266 overlap with the core home ranges of within-lineage males, other lineage males, within-
267 lineage females, and other lineage females. These overlap scores were used as
268 predictors in tests for associations between male chemical profiles and spatial overlap.

269 At the end of female gestation we recaptured all the experimental lizards bar two
270 males (ITA and WEUR) and four females (two ITA, two WEUR) that presumably died

during the experiment and one female (ITA) that we could not recapture until after the breeding season. The remaining females were housed in terraria until they laid, at which point we removed the clutches and incubated them at a constant 28 °C and humidity (5:1 vermiculite:water volume) until hatching. At hatching, we obtained tail tissue samples from all juveniles for paternity analysis. We isolated DNA from 203 offspring (hatchlings: 191, embryos: 12) and 128 adults using the DNeasy 96 Blood & Tissue Kit (Qiagen), following manufacturer's instructions (with overnight lysis). Given the limited number of potential fathers (eight per enclosure), we genotyped individuals at six microsatellite loci (Heathcote et al. 2015) and assigned offspring paternity in Cervus 3.0 (Marshall et al. 1998). This resulted in the retainment of 183 offspring for further analyses (see MacGregor et al. 2017 for further details).

Associations of chemical profiles with morphology, behaviour and reproductive success

Since ICC values were highly variable among compounds (see below), we based the descriptions of the chemical composition of secretions and the enclosure analyses on the relative abundances of the 21 compounds in the second secretion samples from males, collected immediately following the enclosures experiment (n = 57 after the removal of contaminated samples and accounting for two males that were not recaptured). To enable tests for associations between chemical profiles, male morphology, behaviour and reproductive success, and to assess the putative function of secretions as sexual signals, we performed principal components analyses on the relative abundances of the compounds separately by lineage. For each lineage, PC1 to PC5 were retained for further analyses (Table S3).

We ran several models to examine the extent to which these chemical profiles predicted male morphology, behaviour and reproductive success. First, to establish the

295 extent to which chemical profiles could function as signals of dominance status and
296 their association with phenotypic traits linked to male competitive ability, we assessed
297 the strength of correlations between within-lineage chemical PC scores (Table S3) and
298 male dominance scores and morphological trait values (all variables standardised
299 within-lineage: mean = 0, SD = 1). To test for statistical associations between chemical
300 profiles and dominance status, we ran a linear mixed model (LMM) for each lineage
301 with male dominance score as the response variable and PC1 to PC5 as predictors. Since
302 dominance depends upon social environment we controlled for enclosure as a random
303 effect. Second, to examine whether chemical profiles predicted male-male and male-
304 female spatial overlap, we generated candidate LMMs within each lineage, with all
305 possible linear combinations of PC1 to PC5 as putative predictors of overlap (owing to a
306 lack of a priori hypotheses), and enclosure as a random effect. Pairwise interactions
307 between components were not included due to difficulties in their interpretation. We
308 ran and evaluated all candidate models based on second-order Akaike Information
309 Criterion (AICc). In addition to the top supported models ($\Delta AICc \leq 2$), we report model-
310 averaged parameter estimates generated from full-model averaging due to the absence
311 of strongly supported best performing models (e.g. AICcWt > 90%, Symonds and
312 Moussalli 2011). Multimodal inferences were applied using the R package 'glmulti'
313 (Calcagno and de Mazancourt 2010). Finally, following the same method, we examined
314 associations between chemical profiles and relative within-lineage and between-lineage
315 fertilization success (the latter for Italian males only owing to differences in the
316 incidence of hybridization). Relative fertilization success was calculated by dividing the
317 absolute number of sired offspring for a male by the mean of all males within his
318 enclosure. We also tested if the associations of chemical profiles with morphology,
319 behaviour and reproductive success remained consistent when considering only those

compounds with moderate to high repeatability within-individuals (see below and Table S4).

Patterns of chemical profile introgression across a zone of secondary contact

Cline Analyses

We tested predictions regarding the direction of chemical introgression across our sixteen populations in northern Italy using a geographic cline approach (e.g. Szymura and Barton 1986; Gay et al. 2008). We first performed a principal component analysis on transformed relative abundances of the 21 compounds in the 108 cline samples. We retained the first six principal components for further analyses (accounting for 76% of the total variance, Table S5). To test the extent to which geographic variation among populations was a function of isolation-by-distance we performed a Mantel test between a matrix of chemical distances and geographic distances (based on 10,000 permutations). Chemical distances were defined as the mean Euclidean distances among populations based on PC1 to PC6 and geographic distances as linear terrestrial distances. In addition, we examined the correlation between a chemical index score (see below) and a hybrid index score (available for n = 66 individuals, generated based on neutral nuclear microsatellite marker for a previous study, While et al. 2015).

Compounds that differ significantly in their relative abundance between lineages and are repeatable within individuals are the most likely targets for selective divergence or selective introgression following secondary contact. Consequently, we analysed patterns of chemical introgression for the 12 compounds that met this criteria (see Table 1). To test for patterns of overall chemical introgression we generated a chemical index from PC1 to PC3 (accounting for 73% of the total variance, Table S6)

according to the formula: $S = (1 + (D_{ITA}/D_{WEUR}))-1$, where D_{ITA} is the Euclidean distance of PCs from an origin defined by the mean PCs of reference Italian individuals (populations VE and PE, Table S1), and D_{WEUR} is the Euclidean distance from an origin defined by the mean PCs of reference Western European individuals (populations LO, NL and VA, Table S1), such that $S > 0.5$ reflects more Italian-like profiles and $S < 0.5$ reflects more Western European-like profiles. Clines were fitted for the chemical index, the 12 individual compounds, mtDNA haplotype frequencies (for comparison with mitochondrial genetic background, data generated by While et al. 2015), a hybrid index (for comparison with neutral expectation, While et al. 2015), and male dorsal greenness (for comparison of patterns of selected introgression, While et al. 2015) using the Metropolis-Hastings Markov chain Monte Carlo algorithm implemented in the package HZAR in R version 3.1.2 (Derryberry et al. 2014). We excluded population LO from our analyses of patterns of chemical introgression due to low sample size. For the phenotypic characters we evaluated five candidate models (fitted tails (none, left, right, mirror, or both) all with estimated trait mean and variance (right, left, and centre)), and for mtDNA frequencies and the hybrid index we evaluated ten candidate models (all possible combinations of fitted tails (none, left, right, mirror, or both) and scaling (fixed or free) (Derryberry et al. 2014). Estimated cline-centre and width are reported from the best-fitting models based on AICc (see Table S7). The coincidence of cline centres for the chemical index *vs.* the hybrid index, the chemical index *vs.* greenness, and for the individual compounds *vs.* the hybrid index and greenness were assessed using the maximum-likelihood derived confidence intervals.

Results

Chemical composition and consistency

The lipophilic chemical composition of the femoral secretions (Table S2) was consistent with that previously reported for this species (Martín and Lopez 2006a; Martín et al. 2008; Pellitteri-Rosa et al. 2014). Considering those 21 compounds selected from the samples on the basis of their common occurrence (Table 1), the lizard secretions consisted primarily of steroids (85.7%), but also contained waxy esters (3.7%), tocopherols (7.6%), terpenoids (2.1%), alkenes (0.7%) and ketones (0.3%). On average the five most abundant compounds across both lineages were cholesterol (59.6%), cholesta-5,7-dien-3-ol (10.3%), alpha-tochopherol (5.8%), unidentified steroid_24.48 (3.9%) and ergosta-5,8-dien-3-ol (3.9%). However, the relative quantities of the 21 compounds varied considerably in their within-individual repeatability (range of intra-class correlation coefficients: 0 – 0.95; Table 1).

Evidence for divergence between the lineages

The overall chemical profile of secretions differed between the lineages at both sampling times (First secretion samples – Lineage: $F_{1,55} = 30.53$, $p < 0.001$, $R^2 = 0.35$, Captivity: $F_{1,55} = 1.89$, $p = 0.10$, $R^2 = 0.02$; Second secretion samples – Lineage: $F_{1,56} = 44.95$, $p < 0.001$, $R^2 = 0.45$). The lineages differed in the relative abundance of all six chemical classes represented by the 21 compounds, specifically, in tocopherols and a terpenoid, which were higher in abundance in the Italian lineage, and steroids and a waxy ester, alkene and ketone, which was higher in abundance in the secretions of Western European males (Table 1, Table S8). Consequently, principal coordinates analysis resulted in clear clustering by lineage (Figure 2). Of the 21 compounds, 14 showed significant differences in their relative abundance between Italian and Western European secretion samples, and of these, 12 compounds had moderate to high

391 repeatability within individuals (defined as ICC values with confidence intervals
392 excluding zero, Table 1).

393 **Associations with sexual characters, spatial organisation and reproductive**
394 **success**

395 We examined the associations between chemicals and morphological and colour
396 characters that have established (i.e. outer ventral scale ornamentation) and putative
397 (i.e. dorsal greenness and ventral blackness) signal function. Within-lineage chemical
398 variation was correlated with dominance, body size, and colour characters in the Italian
399 and Western European lineages, however, the significance of these correlations
400 dropped out after correcting for false discovery rate (Table S9). From a LMM, chemical
401 variation captured by PC4 was marginally significantly associated with dominance in
402 the Italian lineage; however, there were no significant associations between chemical
403 profiles and dominance in either lineage when considering only the repeatable
404 compounds (Table S10).

405 The core territories of Italian males overlapped significantly more with the core
406 territories of both Italian and Western European females than did the core territories of
407 Western European males. Italian males also overlapped less with males of their own
408 lineage than did Western European males with males of their own lineage (Figure S1).
409 For Italian males, PC3, PC4 and PC5 predicted overlap with same lineage males (Table
410 2). Chemical components also occurred within the best-performing models predicting
411 overlap between Italian males and Western European males and male-female spatial
412 overlap, however, the null model was the top supported model in all cases (Table 2). For
413 Western European males, the null model occurred within the best-performing models
414 predicting male overlap with same lineage and other lineage males and with females of

the same lineage. However, PC1, PC3, PC4, and PC5 predicted overlap with Italian females and the null model was not equally well supported (Table 3).

Hybridization was highly asymmetric and occurred mostly between Italian males and Western European females (35% of Western European female offspring sired by an Italian father vs 6% in the opposite direction, detailed results reported in MacGregor et al. 2017). We found limited support for a relationship between chemical profiles and reproductive success in males of the Italian lineage. Specifically, while there was some evidence for chemical associations with within-lineage and between-lineage (i.e. hybridization) reproductive success, the null models were equally well supported in both cases (Table 4). In contrast, for Western European males, a single model for within-lineage reproductive success was supported with PC1 as a negative predictor. We were unable to examine the corresponding effects on between-lineage reproductive success in Western European males due to a lack of incidence of hybridization. When we re-ran the above analyses of the predictors of spatial overlap and reproductive success using principal components generated from only compounds that were repeatable (n = 14, Table S4) the null model occurred within the best-performing models in all cases (see Tables S11 to S13).

Patterns of chemical introgression across a zone of secondary contact

Geographic distance between pairs of populations was positively correlated with chemical distance (Mantel Test (10,000 perm): $r = 0.52$, $p < 0.001$, Figure S2). Chemical index score was highly correlated with a hybrid index score based on nuclear microsatellite markers generated by While et al. 2015 ($r = 0.74$, Figure S3). Consistent with this, cline-fitting of the chemical index suggested geographic patterns of chemical variation across the contact zone are similar to that of introgressed microsatellite

markers (Figure 3, Figure S4, and Table S14). However, from the clines fitted individually to the 12 compounds, we found that three compounds, unidentified steroid_RT20.76, tochopherol methyl ether and cholesterol, did not support the patterns of neutral introgression, and instead suggested a geographic pattern of introgression similar to dorsal greenness (Figure S5, Table S14).

Discussion

Under sexual selection, divergence in chemical signals should mediate patterns of hybridization during secondary contact and lead to asymmetric patterns of introgression. In this study, we identified characteristics of chemical profiles in two lineages of the common wall lizard. We found that the chemical profiles of wall lizards were variable between lineages, but were only weakly associated with male secondary sexual characters with no consistent sexual selection on individual compounds. Furthermore, we found limited evidence for selective introgression of chemical profiles across a natural contact zone where sexually-selected introgression of colour and morphology has previously been documented (While et al. 2015). Nevertheless, three candidate compounds were candidates for asymmetric introgression via direct selection or genetic linkage with visual or behavioural characters. Combined, our results suggest that divergence in the chemical composition of femoral secretions in wall lizards is largely neutral, and that associations with male phenotypes and reproductive success may be transient or environment-dependent and play a minor role in the evolution of reproductive isolation and introgression. This implies that the likely function of wall lizard scent marks may be to mediate individual recognition and territory residency rather than to convey physical or behavioural attributes.

The causes of divergence in the chemical composition of lizard secretions are contentious (Font et al. 2012). Divergence may be driven by differences in the direction and intensity of intra- or inter-sexual selection on males (López and Martín 2004), local adaptation through, for instance, selection for transmission efficiency under differing climates (Alberts 1992; Martín et al. 2015); or through stochastic change (e.g. Runemark et al. 2011). Our study goes some way towards testing the sexual selection hypothesis. The two lineages used here have evolved distinct differences in morphology and visual traits that function in male-male competition, which give a competitive advantage to Italian males. This drives the asymmetric introgression of suites of sexually selected characters from the Italian lineage into the Western European lineage (While et al. 2015). If male chemical profiles have similarly diverged under sexual selection, we would predict that some chemical characteristics (i) associate with male secondary sexual characters; (ii) influence success in male-male competition for territory and fertilizations; (iii) predict reproductive success and hybridization; and (iv) show evidence of adaptive introgression from the Italian to the Western European lineage. In this study, we found evidence for some but not all of these predictions.

The relative abundances of several compounds were associated with sexual characters, spatial overlap and reproductive success. However, these associations were not always consistent between the lineages and in some instances were even reversed. One potential explanation for this is that different chemical characters function in intra-sexual *vs.* inter-sexual communication. Indeed, behavioural studies of closely related species suggest females can discriminate between males based on the composition of their femoral secretions (e.g. Lopez et al. 2003; Martín and Lopez 2006b). However, a role for inter-sexual selection in shaping the chemosensory traits of wall lizards is not

empirically supported by the literature (Font et al. 2012), and, overall, our results are consistent with the conclusion. For example, we found little evidence that females associate with males with a particular chemical composition. Furthermore previous work suggests that neither Italian nor Western European females discriminate based on male quantitative traits or lineage (Heathcote et al. 2016).

Based on the overall weakness of associations between chemical profiles and within- and between-lineage fertilization success we infer that heritable chemical characters that have diverged between the Italian and Western European lineages are unlikely to be under consistent ongoing sexual selection (although PC1 negatively associated with within-lineage reproductive success in Western-European males). In support of this, and in contrast to morphological and visual traits, the pattern of introgression of the overall chemical profiles followed the pattern from microsatellite markers, and hence, conformed to neutral expectations in the presence of asymmetries in hybridization. This result is consistent with the limited evidence for a relationship between chemical profiles and hybridization success in Italian males. Combined this suggests that divergence in chemical profiles has played a limited role in mediating the asymmetric introgression observed in zones of secondary contact (While et al. 2015). The clear exception to this is tocopherol methyl ether, whose geographic cline closely resembled geographic variation in dorsal greenness across the contact zone. Interestingly, this compound was negatively associated with Italian male dominance, and dominance was the best predictor of Italian male reproductive success in the enclosures (MacGregor et al. 2017). Thus, this does not suggest that direct selection would cause the introgression of tocopherol methyl ether but instead supports that genetic linkage with genomic regions contributing to sexually selected colour and morphology is a more likely

explanation. Thus, we believe the data is consistent with the interpretation that overall chemical variation across the contact zone is largely driven by neutral processes.

Presuming that the chemical profile of a male wall lizard's femoral gland secretions is not under consistent inter- or intra- sexual selection, what then is the function of chemical communication? One possibility is that the chemical profiles primarily function as a signature mixture, a variable set of compounds which is learnt by other males, allowing them to distinguish individuals (Wyatt 2010, 2014). Indeed, due to their chemical complexity, femoral gland secretions may be better suited than any other cue for use in individual recognition because a very high level of specificity is possible. This explanation is consistent with our observation of only weak associations between male chemical profiles and fertilization success, and is supported by a wealth of empirical studies on lizards demonstrating differential male behavioural responses to the scents of familiar and unfamiliar individuals (e.g. Aragón et al. 2001; Font and Desfilis 2002), and even recognition of individual identity based on chemical cues (Carazo et al. 2008). If primarily functioning as signature mixtures, the correlations between chemical characters and male sexual characters presented here more likely reflect transient associations with weakly heritable chemical traits; associations that may easily break down during hybridization, thereby leading to effectively neutral pattern of introgression.

Conclusions

Combined with previous studies (While et al. 2015; MacGregor et al. 2017), our experimental and field data highlight the potentially differing functions for visual and chemical communication systems in lizards, with consequences for patterns of character introgression between two lineages (see Greig et al. 2015 for similar

discordant patterns between plumage colour and song in birds). In contrast to comparative evidence invoking intra-sexual selection as a mechanism for the evolution of visual traits used for communication in Lacertid lizards (Pérez i de Lanuza et al. 2013), our study suggests that chemical traits may not be subjected to the same selection pressures. In fact, we suggest that the chemical profiles of femoral gland secretions in wall lizards may not reliably function as sexual signals as is commonly assumed. Instead, the utility of chemical profiles may be because they allow recognition of competitors based on experience, thereby playing little role in the evolution of reproductive isolation or adaptive introgression.

References

Abrahams, M. V., T. L. Robb, and J. F. Hare. 2005. Effect of hypoxia on opercular displays: evidence for an honest signal? Anim Behav 70:427-432.

Aitchison, J. 1986. The statistical analysis of compositional data. Chapman and Hall, London.

Alberts, A. C. 1992. Constraints on the Design of Chemical Communication Systems in Terrestrial Vertebrates. American Naturalist 139:S62-S89.

Alonso-Alvarez, C. 2004. Ultraviolet reflectance affects male-male interactions in the blue tit (*Parus caeruleus ultramarinus*). Behavioral Ecology 15:805-809.

Aragón, P., P. López, and J. Martín. 2001. Chemosensory discrimination of familiar and unfamiliar conspecifics by lizards: implications of field spatial relationships between males. Behavioral Ecology and Sociobiology 50:128-133.

557 Baldassarre, D. T. and M. S. Webster. 2013. Experimental evidence that extra-pair
558 mating drives asymmetrical introgression of a sexual trait. Proceedings of the Royal
559 Society B: Biological Sciences 280:20132175.

560 Boake, C. R. B. 1989. Repeatability: Its role in evolutionary studies of mating behavior.
561 Evol Ecol 3:173-182.

562 Calcagno, V. and C. de Mazancourt. 2010. glmulti: an R package for easy automated
563 model selection with (generalized) linear models. Journal of Statistical Software 34:1-
564 29.

565 Carazo, P., E. Font, and E. Desfilis. 2007. Chemosensory assessment of rival competitive
566 ability and scent-mark function in a lizard, *Podarcis hispanica*. Anim Behav 74:895-902.

567 Carazo, P., E. Font, and E. Desfilis. 2008. Beyond 'nasty neighbours' and 'dear enemies'?
568 Individual recognition by scent marks in a lizard (*Podarcis hispanica*). Anim Behav
569 76:1953-1963.

570 Carretero, M. A. 2008. An integrated assessment of a group with complex systematics:
571 the Iberomaghrebian lizard genus *Podarcis* (Squamata, Lacertidae). Integr Zool 3:247-
572 266.

573 Cooper, W. E. and W. R. Garstka. 1987. Discrimination of male conspecific from male
574 heterospecific odors by male scincid lizards (*Eumeces laticeps*). J Exp Zool 241:253-256.

575 David, H. A. 1988. The Method of Paired Comparisons. London: C. Griffin.

576 Derryberry, E. P., G. E. Derryberry, J. M. Maley, and R. T. Brumfield. 2014. hzar: hybrid
577 zone analysis using an R software package. Mol Ecol Resour 14:652-663.

578 Font, E., D. Barbosa, C. Sampedro, and P. Carazo. 2012. Social behavior, chemical
579 communication, and adult neurogenesis: Studies of scent mark function in *Podarcis* wall
580 lizards. Gen Comp Endocr 177:9-17.

581 Font, E. and E. Desfilis. 2002. Chemosensory recognition of familiar and unfamiliar
582 conspecifics by juveniles of the Iberian wall lizard *Podarcis hispanica*. Ethology 108:319-
583 330.

584 Gabirot, M., A. M. Castilla, P. Lopez, and J. Martín. 2010. Chemosensory species
585 recognition may reduce the frequency of hybridization between native and introduced
586 lizards. Can J Zool 88:73-80.

587 Gabirot, M., A. M. Castilla, P. Lopez, and J. Martín. 2010. Differences in chemical signals
588 may explain species recognition between an island lizard, *Podarcis atrata*, and related
589 mainland lizards, P hispanica. Biochem Syst Ecol 38:521-528.

590 Gammell, M. P., H. d. Vries, D. J. Jennings, C. o. M. Carlin, and T. J. Hayden. 2003. David's
591 score: a more appropriate dominance ranking method than Clutton-Brock et al.'s index.
592 Anim Behav 66:601-605.

593 Garcia-Roa, R., C. Cabido, P. Lopez, and J. Martín. 2016. Interspecific differences in
594 chemical composition of femoral gland secretions between two closely related wall
595 lizard species, *Podarcis bocagei* and *Podarcis carbonelli*. Biochem Syst Ecol 64:105-110.

596 Gassert, F., U. Schulte, M. Husemann, W. Ulrich, D. Rödder, A. Hochkirch, E. Engel, J.
597 Meyer, and J. C. Habel. 2013. From southern refugia to the northern range margin:
598 genetic population structure of the common wall lizard, *Podarcis muralis*. J Biogeogr
599 40:1475-1489.

600 Gay, L., P.-A. Crochet, D. A. Bell, and T. Lenormand. 2008. Comparing clines on molecular
601 and phenotypic traits in hybrid zones: a windom on tension zone models. Evolution
602 62:2789-2806.

603 Giovannotti, M., P. Nisi-Cerioni, and V. Caputo. 2010. Mitochondrial DNA sequence
604 analysis reveals multiple Pleistocene glacial refugia for *Podarcis muralis* (Laurenti,
605 1768) in the Italian Peninsula. Ital J Zool 77:277-288.

606 Hamilton, D. G., M. J. Whiting, and S. R. Pryke. 2013. Fiery frills: carotenoid-based
607 coloration predicts contest success in frillneck lizards. Behavioral Ecology 24:1138-
608 1149.

609 Heathcote, R. J. P., G. M. While, H. E. A. MacGregor, J. Sciberras, C. Leroy, P. D'Ettorre, and
610 T. Uller. 2016. Male behaviour drives assortative reproduction during the initial stage of
611 secondary contact. J Evolution Biol:1003–1015.

612 Heathcote, R. P., D. Dawson, and T. Uller. 2015. Characterisation of nine European wall
613 lizard (Podarcis muralis) microsatellite loci of utility across sub-species. Conserv Genet
614 Resour 7:85-87.

615 Kenward, R. E., S. S. Walls, A. B. South, and N. Casey. 2008. Ranges8 : For the analysis of
616 tracking and location data. Anatrack Ltd, Wareham , UK

617 Kie, J. 2013. A rule-based ad hoc method for selecting a bandwidth in kernel home-range
618 analyses. Anim Biotelemetry 1:1-12.

619 Lessells, C. M. and P. T. Boag. 1987. Unrepeatable repeatabilities: a common mistake.

620 Loehr, J., J. Carey, H. Ylönen, and J. Suhonen. 2008. Coat darkness is associated with
621 social dominance and mating behaviour in a mountain sheep hybrid lineage. Animal
622 Behaviour 76:1545-1553.

623 Lopez, P, and J. Martín. 2004. Sexual selection and chemoreception in Lacertid lizards
624 119-137. In Pérez-Mellado, V., N. Riera, and A. Perera. The biology of Lacertid lizards:
625 evolutionary and ecological perspectives. Institut Menorquí d'Estudis.

626 Lopez, P., L. Amo, and J. Martín. 2006. Reliable signaling by chemical cues of male traits
627 and health state in male lizards, Lacerta monticola. J Chem Ecol 32:473-488.

628 Lopez, P., P. Aragon, and J. Martín. 2003. Responses of female lizards, Lacerta monticola ,
629 to males' chemical cues reflect their mating preference for older males. Behavioral
630 Ecology and Sociobiology 55:73-79.

631 Lopez, P., M. Gabirot, and J. Martín. 2009. Immune activation affects chemical sexual
632 ornaments of male Iberian wall lizards. Naturwissenschaften 96:65-69.

633 López, P. and J. Martín. 2002. Chemical rival recognition decreases aggression levels in
634 male Iberian wall lizards, Podarcis hispanica. Behavioral Ecology and Sociobiology
635 51:461-465.

636 MacGregor, H. E. A., G. M. While, J. Barrett, G. Pérez i de Lanuza, P. Carazo, S. Michaelides,
637 and T. Uller. 2017. Experimental contact zones reveal causes and targets of sexual
638 selection in hybridizing lizards. Funct Ecol 31: 742-752.

639 Marshall, T. C., J. Slate, L. E. B. Kruuk, and J. M. Pemberton. 1998. Statistical confidence
640 for likelihood-based paternity inference in natural populations. Mol Ecol 7:639-655.

641 Martin, S. J. and F. P. Drijfhout. 2009. How reliable is the analysis of complex cuticular
642 hydrocarbon profiles by multivariate statistical methods? J Chem Ecol 35:375-382.

643 Martín, J., L. Amo, and P. Lopez. 2008. Parasites and health affect multiple sexual signals
644 in male common wall lizards, *Podarcis muralis*. Naturwissenschaften 95:293-300.

645 Martín, J., E. Civantos, L. Amo, and P. López. 2007. Chemical ornaments of male lizards
646 Psammodromus algirus may reveal their parasite load and health state to females.
647 Behav Ecol Sociobiol 62:173-179.

648 Martín, J. and P. Lopez. 2006. Vitamin D supplementation increases the attractiveness of
649 males' scent for female Iberian rock lizards. Proc Biol Sci 273:2619-2624.

650 Martín, J. and P. Lopez. 2007. Scent may signal fighting ability in male Iberian rock
651 lizards. Biol Lett 3:125-127.

652 Martín, J. and P. López. 2006. Interpopulational differences in chemical composition and
653 chemosensory recognition of femoral gland secretions of male lizards Podarcis
654 hispanica: implications for sexual isolation in a species complex. Chemoecology 16:31-
655 38.

656 Martín, J., J. Ortega, and P. Lopez. 2015. Interpopulational Variations in Sexual Chemical
657 Signals of Iberian Wall Lizards May Allow Maximizing Signal Efficiency under Different
658 Climatic Conditions. Plos One 10.

659 Mason, R. T. and M. R. Parker. 2010. Social behavior and pheromonal communication in
660 reptiles. J Comp Physiol A Neuroethol Sens Neural Behav Physiol 196:729-749.

661 McDonald, D. B., R. P. Clay, R. T. Brumfield, and M. J. Braun. 2001. Sexual selection on
662 plumage and behavior in an avian hybrid zone: experimental tests of male-male
663 interactions. Evolution 55:1443-1451.

664 McLean, S., N. W. Davies, and N. L. Wiggins. 2012. Scent chemicals of the brushtail
665 possum, *Trichosurus vulpecula*. J Chem Ecol 38:1318-1339.

666 Moore, W. S. 1987. Random mating in the northern flicker hybrid zone - implications for
667 the evolution of bright and constrasting plumage patterns in birds. Evolution 41:539-
668 546.

669 Nakagawa, S. and H. Schielzeth. 2010. Repeatability for Gaussian and non-Gaussian data:
670 a practical guide for biologists. Biol Rev Camb Philos Soc 85:935-956.

671 Oksanen, J., R. Kindt, P. Legendre, B. O'Hara, M. H. H. Stevens, M. J. Oksanen, and M.
672 Suggests. 2007. The vegan package. Community ecology package 10.

673 Owen-Ashley, N. T. and L. K. Butler. 2004. Androgens, interspecific competition and
674 species replacement in hybridizing warblers. Proc Biol Sci 271 Suppl 6:S498-500.

675 Panhuis, T. M., R. Butlin, M. Zuk, and T. Tregenza. 2001. Sexual selection and speciation.
676 Trends Ecol Evol 16:364-371.

677 Parsons, T. J., S. L. Olson, and M. J. Braun. 1993. Unidirectional spread of secondary
678 sexual plumage traits across an avian hybrid zone. Science 260:1643-1646.

679 Pauers, M. J., J. M. Kapfer, C. E. Fendos, and C. S. Berg. 2008. Aggressive biases towards
680 similarly coloured males in Lake Malawi cichlid fishes. Biol Lett 4:156-159.

681 Pearson, S. F. and S. Rohwer. 2000. Asymmetries in male aggression across an avian
682 hybrid zone. Behav Ecol 11:93-101.

683 Pellitteri-Rosa, D., J. Martín, P. Lopez, A. Bellati, R. Sacchi, M. Fasola, and P. Galeotti.
684 2014. Chemical polymorphism in male femoral gland secretions matches polymorphic
685 coloration in common wall lizards (*Podarcis muralis*). Chemoecology 24:67-78.

686 Pérez i de Lanuza, G., E. Font, and J. L. Monterde. 2013. Using visual modelling to study
687 the evolution of lizard coloration: sexual selection drives the evolution of sexual
688 dichromatism in lacertids. J Evolution Biol 26:1826-1835.

689 Ranganathan, Y. and R. M. Borges. 2011. To transform or not to transform: that is the
690 dilemma in the statistical analysis of plant volatiles. Plant signaling & behavior 6:113-
691 116.

692 Runemark, A., M. Gabirot, and E. I. Svensson. 2011. Population divergence in chemical
693 signals and the potential for premating isolation between islet- and mainland
694 populations of the Skyros wall lizard (*Podarcis gaigeae*). J Evolution Biol 24:795-809.

695 Salvi, D., D. Harris, A. Kaliontzopoulou, M. Carretero, and C. Pinho. 2013. Persistence
696 across Pleistocene ice ages in Mediterranean and extra-Mediterranean refugia:
697 phylogeographic insights from the common wall lizard. Bmc Evol Biol 13:1-18.

698 Schulte, U., A. Hochkirch, S. Lötters, D. Rödder, S. Schweiger, T. Weimann, and M. Veith.
699 2012. Cryptic niche conservatism among evolutionary lineages of an invasive lizard.
700 Glob Ecol Biogeogr 21:198-211.

701 Searcy, W. A. and S. Nowicki. 2005. The evolution of animal communication: reliability
702 and deception in signaling systems. Princeton University Press.

703 Simeonovska-Nikolova, D. M. 2006. Interspecific social interactions and behavioral
704 responses of *Apodemus agrarius* and *Apodemus flavicollis* to conspecific and
705 heterospecific odors. J Ethol 25:41-48.

706 Stein, A. C. and J. A. C. Uy. 2006. Unidirectional introgression of a sexually selected trait
707 across an avian hybrid zone: A role for female choice? Evolution 60:1476-1485.

708 Symonds, M. E. and A. Moussalli. 2011. A brief guide to model selection, multimodel
709 inference and model averaging in behavioural ecology using Akaike's information
710 criterion. Behav Ecol Sociobiol 65:13-21.

711 Szymura, J. M. and N. H. Barton. 1986. Genetic Analysis of a hybrid zone between the
712 fire-bellied toads, Bombina bombina and B. variegata, near Cracow in southern Poland.
713 Evolution 40:1141-1159.

714 Teeter, K. C., B. A. Payseur, L. W. Harris, M. A. Bakewell, L. M. Thibodeau, J. E. O'Brien, J.
715 G. Krenz, M. A. Sans-Fuentes, M. W. Nachman, and P. K. Tucker. 2008. Genome-wide
716 patterns of gene flow across a house mouse hybrid zone. Genome Res 18:67-76.

717 West-Eberhard, M. J. 1983. Sexual Selection, Social Competition, and Speciation. Q Rev
718 Biol 58:155-183.

719 While, G. M., S. Michaelides, R. J. P. Heathcote, H. E. A. MacGregor, N. Zajac, J. Beninde, P.
720 Carazo, G. Pérez i de Lanuza, R. Sacchi, M. A. L. Zuffi, T. Horváthová, B. Fresnillo, U.
721 Schulte, M. Veith, A. Hochkirch, and T. Uller. 2015. Sexual selection drives asymmetric
722 introgression in wall lizards. Ecol Lett 18:1366-1375.

723 Wolak, M. E., D. J. Fairbairn, and Y. R. Paulsen. 2012. Guidelines for estimating
724 repeatability. Methods Ecol Evol 3:129-137.

725 Wyatt, T. D. 2010. Pheromones and signature mixtures: defining species-wide signals
726 and variable cues for identity in both invertebrates and vertebrates. J Comp Physiol A
727 196:685-700.

728 Wyatt, D. T. 2014. Pheromones and animal behaviour. 2nd ed. Chemical signals and
729 signatures. Cambridge University Press.

Table 1: Details of the mean relative abundances and repeatability for 21 chemical compounds in the secretions collected from the experimental males. Compounds are listed in order of their characteristic retention time in minutes (_RT), appended to each compound ID. Detection percentage, and across and within-lineage mean relative abundances (based on secretions collected in the second sampling period) are reported for each compound, as well as within-individual repeatability (ICC) estimates between the first and second sampling periods based on all males and separately by lineage. Negative ICC estimates are interpreted and reported as evidence for zero repeatability (Nakagawa and Schielzeth 2010). The 95% confidence intervals for values are given in brackets. Compounds in bold differed between the lineages in their relative abundance based on non-overlapping confidence intervals. Compounds highlighted in grey were repeatable based on ICC confidence intervals that did not include zero.

		Detection %	Mean relative abundance ($CI_{95\%}$)			ICC value ($CI_{95\%}$)		
	Compound Class	All Samples	All Males	ITA	WEUR	All Males	ITA	WEUR
Heptadecene_RT8.25	Alkene	100	-0.60 (-0.86,-0.34)	-1.26 (-1.51,-1.01)	0.13 (-0.16,0.41)	0.49 (0.26,0.67)	0.31 (-0.06,0.60)	0.31 (-0.09,0.63)
2-Heptadecanone_RT9.85	Ketone	100	-1.20 (-1.38,-1.01)	-1.45 (-1.64,-1.26)	-0.91 (-1.21,-0.61)	0.40 (0.15,0.60)	0.29 (-0.08,0.59)	0.45 (0.07,0.72)
Squalene_RT18.34	Terpenoid	100	0.34 (0.09, 0.59)	0.51 (0.09,0.93)	0.15 (-0.11,0.41)	0.22 (-0.05,0.46)	0.43 (0.07,0.68)	0.00 (-0.62,0.11)
Unidentified steroid_RT19.10	Steroid	99	-1.37 (-1.49, -1.25)	-1.56 (-1.72,-1.39)	-1.17 (-1.32,-1.01)	0.30 (0.03, 0.53)	0.29 (-0.08, 0.59)	0.00 (-0.45, 0.33)

Cholesta-3,5-diene_RT19.34	Steroid	98	-1.60 (-1.86,-1.35)	-0.22 (-0.50,0.06)	0.17 (0.00,0.35)	0.31 (0.05,0.54)	0.53 (0.21,0.75)	0.01 (-0.38,0.40)
Unidentified steroid_RT19.57	Steroid	99	-0.03 (-0.20,0.14)	-0.22 (-0.50,0.06)	0.17 (0.00,0.35)	0.16 (-0.11,0.41)	0.33 (-0.04,0.62)	0.00 (-0.40,0.39)
Unidentified steroid_RT19.75	Steroid	98	-0.87 (-0.98,-0.75)	-0.85 (-1.00,-0.70)	-0.89 (-1.08,-0.70)	0.37 (0.11,0.58)	0.60 (0.30,0.79)	0.25 (-0.15,0.59)
***Unidentified steroid_RT20.76**	Steroid	100	-1.06 (-1.19,-0.94)	-1.27 (-1.47,-1.07)	-0.83 (-0.93,-0.73)	0.31 (0.04,0.54)	0.00 (-0.38,0.34)	0.14 (-0.27,0.50)
***Tocopherol methyl ether_RT20.78**	Tocopherol	98	-0.75 (-1.28,-0.22)	0.92 (0.61,1.23)	-2.61 (-3.00,-2.23)	0.95 (0.92,0.97)	0.75 (0.53,0.87)	0.82 (0.62,0.92)
Unidentified steroid_RT20.99	Steroid	99	-1.73 (-1.89,-1.57)	-2.07 (-2.26,-1.89)	-1.34 (-1.53,-1.16)	0.40 (0.15,0.60)	0.21 (-0.17,0.53)	0.36 (-0.03,0.66)
***alpha-Tochopherol_RT21.95**	Tocopherol	100	0.50 (-0.02,1.02)	2.10 (1.70,2.51)	-1.28 (-1.61,-0.95)	0.81 (0.70,0.89)	0.34 (-0.03,0.63)	0.38 (-0.02,0.67)
***Cholesterol_RT21.95**	Steroid	100	4.19 (4.07,4.31)	3.87 (3.77,3.98)	4.54 (4.41,4.68)	0.48 (0.24,0.66)	0.00 (-0.38,0.35)	0.23 (-0.17,0.57)
Cholesta-5,7-dien-3-ol_RT22.27	Steroid	100	2.07 (1.83,2.32)	2.58 (2.36,2.79)	1.51 (1.15,1.88)	0.39 (0.13,0.60)	0.00 (-0.36,0.37)	0.08 (-0.32,0.46)
***Ergosterol (Ergosta-5,7,22-trien-3-ol)_RT22.79**	Steroid	100	-1.01 (-1.18,-0.84)	-0.71 (-0.91,-0.51)	-1.36 (-1.58,-1.11)	0.27 (0.00,0.50)	0.26 (-0.11,0.57)	0.00 (-0.51,0.26)
***Unidentified steroid_RT22.95**	Steroid	100	-0.69 (-0.91,-0.47)	-1.17 (-1.37,-0.96)	-0.15 (-0.46,0.15)	0.46 (0.22,0.65)	0.43 (0.08,0.69)	0.08 (-0.32,0.46)
***Campesterol (Ergost-5-en-3β-ol)_RT23.01**	Steroid	100	0.05 (-0.29,0.38)	-0.79 (-1.12,-0.46)	0.98 (0.61,1.34)	0.47 (0.22,0.65)	0.20 (-0.17,0.52)	0.00 (-0.43,0.35)
***Cholesta-4-en-3-one_RT23.26**	Steroid	100	-0.28 (-0.58,0.02)	-0.68 (-1.08,-0.27)	0.16 (-0.25,0.58)	0.47 (0.23,0.66)	0.40 (0.04,0.67)	0.38 (-0.01,0.67)

*Ergosta-5,8-dien-3-ol_RT23.48	Steroid	100	1.25 (1.04,1.46)	1.39 (1.19,1.60)	1.10 (0.72,1.47)	0.00 (-0.28,0.26)	0.07 (-0.30,0.42)	0.00 (-0.45,0.33)
*Unidentified steroid_RT24.24	Steroid	99	0.46 (0.32,0.59)	0.41 (0.27,0.54)	0.51 (0.25,0.76)	0.17 (-0.10,0.42)	0.25 (-0.12,0.57)	0.14 (-0.26,0.51)
*Unidentified steroid_RT24.48	Steroid	99	1.19 (0.92,1.46)	1.43 (1.21,1.64)	0.93 (0.41,1.45)	0.26 (-0.01,0.49)	0.35 (-0.02,0.63)	0.22 (-0.18,0.56)
Unidentified waxy ester_RT25.45	Waxy Ester	100	1.14 (0.91,1.37)	0.74 (0.42,1.05)	1.59 (1.31,1.87)	0.20 (-0.07,0.45)	0.00 (-0.37,0.35)	0.00 (-0.44,0.34)

* Compounds quantified based on the scaling of individual quantitative ions rather than the integration of compound peak areas

Table 2: The top supported models (<2 ΔAICc) from analyses to identify associations between chemical profiles (defined as PC1 to PC5, Table S3) and male-male and male-female overlap in the Italian lineage. For each model, the number of parameters (*k*), second order Aikaike information criterion (AICc), difference in AICc from the top performing model (ΔAICc), and the relative likelihood of the model (AICcWt) are reported. Regression coefficients (β) and 95% confidence intervals ($CI_{95\%}$) are presented for each predictor. Model-averaged parameter estimates (model-averaged β) generated via full-model averaging and unconditional 95% confidence intervals (Unconditional $CI_{95\%}$) are also reported, adjacent to predictors on their first appearance in the table. Predictors with 95% confidence intervals for effect sizes that do not include zero are in bold. When not included in the top performing models AICc values for the null model are presented in italics for comparison.

Overlap	Category	Model	*k*	AICc	ΔAIC	AICcW	Predicto	β	$CI_{95\%}$	Model-averaged	Unconditional
Male-Male	Same Lineage	PC3 + PC5	5	300.2	0	0.27	**PC3**	**10.85**	**1.68, 20.38**	8.23	-3.46, 19.92
							PC5	**13.73**	**4.51, 22.99**	**13.27**	**2.97, 23.56**
		PC3 + PC4 +	6	300.5	0.39	0.22	PC3	10.66	1.94, 19.65		
							PC4	-6.88	-15.20, 1.33	-2.97	-11.13, 5.19
							PC5	14.20	5.45, 22.97		
		null	*3*	*307.56*	*7.36*	*0.01*					
	Other Lineage	null	3	326.9	0	0.22					
		PC1	4	327.7	0.81	0.15	PC1	-	-30.82, 5.73	-4.76	-19.39, 9.87
Male-Female	Same Lineage	null	3	356.6	0.00	0.28					
		PC4	4	358.2	1.63	0.12	PC4	15.53	-14.86,	4.48	-12.31, 21.27
	Other Lineage	null	3	341.5	0	0.28					
		PC3	4	343.4	1.82	0.11	PC3	10.29	-12.25,	2.78	-8.31, 13.86

Table 3: The top supported models (<2 ΔAICc) from analyses to identify associations between chemical profiles (defined as PC1 to PC5, Table S3) and male-male and male-female overlap in the Western European lineage. For each model, the number of parameters (*k*), second order Aikaike information criterion (AICc), difference in AICc from the top performing model (ΔAICc), and the relative likelihood of the model (AICcWt) are reported. Regression coefficients (β) and 95% confidence intervals ($CI_{95\%}$) are presented for each predictor. Model-averaged parameter estimates (model-averaged β) generated via full-model averaging and unconditional 95% confidence intervals (Unconditional $CI_{95\%}$) are also reported, adjacent to predictors on their first appearance in the table. Predictors with 95% confidence intervals for effect sizes that do not include zero are in bold. When not included in the top performing models AICc values for the null model are presented in italics for comparison.

Overlap	Category	Model	*k*	AICc	ΔAICc	AICcWt	Predictor	β	$CI_{95\%}$	Model-averaged β	Unconditional $CI_{95\%}$
Male-Male	Same Lineage	PC3	4	293.82	0.00	0.18	**PC3**	**16.56**	**0.90, 36.92**	10.54	-4.61, 25.68
		null	3	294.25	0.44	0.15					
		PC3 + PC5	5	294.77	0.96	0.11	**PC3**	**20.55**	**4.42, 40.53**		
							PC5	13.01	-4.48, 31.64	4.73	-17.6, 27.06
	Other Lineage	null	3	286.83	0.00	0.33					
Male-Female	Same Lineage	null	3	286.08	0.00	0.21					
		PC4	4	286.66	0.57	0.16	PC4	12.09	-4.20, 27.04	4.84	-8.93, 18.61
	Other Lineage	PC4 + PC5	5	295.16	0	0.16	**PC4**	**19.42**	**0.85, 37.33**	15.05	-8.77, 38.87
							PC5	**-21.58**	**-38.88, -4.81**	-11.69	-35.83, 12.44
		PC5	4	296.29	1.13	0.09	**PC5**	**-24.55**	**-43.81, -6.21**		
		PC1 + PC4	5	296.53	1.38	0.08	**PC1**	**-18.69**	**-35.88, -1.92**	-4.83	-20.39, 10.72
							PC4	**23.32**	**4.88, 49.96**		
		PC3 + PC4 + PC5	6	296.74	1.59	0.07	PC3	11.61	-1.15, 30.29	4.95	-10.14, 20.04
							PC4	**20.25**	**2.36, 37.79**		
							PC5	**-18.17**	**-35.77, -0.80**		
		PC1 + PC3 +PC4	6	297.11	1.95	0.06	PC1	-16.38	-33.08, 0.08		
							PC3	**14.29**	**2.41, 34.62**		

							PC4	**23.81**	**6.34, 41.26**
		null	*3*	*300.01*	*-4.85*	*0.01*			

Table 4: The top supported models (<2 ΔAICc) from analyses to identify associations between chemical profiles (defined as PC1 to PC5, Table S3) and relative fertilization success within-lineage or between-lineage (Italian males only). For each model, the number of parameters (*k*), second order Aikaike information criterion (AICc), difference in AICc from the top performing model (ΔAICc), and the relative likelihood of the model (AICcWt) are reported. Regression coefficients (β) and 95% confidence intervals ($CI_{95\%}$) are presented for each predictor. Regression coefficients (β) and 95% confidence intervals ($CI_{95\%}$) are presented for each predictor. Model-averaged parameter estimates (model-averaged β) generated via full-model averaging and unconditional 95% confidence intervals (Unconditional $CI_{95\%}$) are also reported, adjacent to predictors on their first appearance in the table. Predictors with 95% confidence intervals for effect sizes that do not include zero are in bold. When not included in the top performing models AICc values for the null model are presented in italics for comparison.

Lineage	Relative Fertilization Success	Model Predictors	k	AICc	ΔAICc	AICcWt	Predictor	β	$CI_{95\%}$	Model-averaged β	Unconditional $CI_{95\%}$
ITA	Within-Lineage	PC4	4	96.06	0.00	0.15	PC4	0.32	-0.07, 0.70	0.15	-0.24, 0.54
		null	3	96.08	0.02	0.15					
		PC3	4	97.43	1.37	0.08	PC3	0.23	-0.17, 0.62	0.07	-0.17, 0.31
		PC3 + PC4	5	97.50	1.44	0.07	PC3	0.23	-0.15, 0.60		
							PC4	0.32	-0.06, 0.69		
		PC5	4	97.77	1.71	0.06	PC5	0.20	-0.20, 0.59		
		PC4 + PC5	5	97.88	1.82	0.06	PC4	0.32	-0.06, 0.70		
							PC5	0.20	-0.18, 0.57	0.05	-0.15, 0.26
	Between-Lineage	null	3	99.67	0.00	0.20					
		PC3	4	100.03	0.36	0.16	PC3	-0.32	-0.73, 0.10	-0.14	-0.52, 0.24
		PC5	4	101.51	1.84	0.08	PC5	-0.19	-0.61, 0.23	-0.05	-0.25, 0.15
WEUR	Within-Lineage	PC1	4	76.79	0.00	0.35	**PC1**	**-0.46**	**-0.79, -0.12**	-0.40	-0.87, 0.07

		null	*3*	*80.80*	*4.01*	*0.05*	

Figure 1: Map of the natural contact zone in northern Italy to show the locations of the sixteen populations sampled for cline analyses. Green and brown dots indicate association to the Italian and Western European mitochondrial lineages, respectively (data from While et al. 2015). Populations VI and BT have a mix of Italian and Western European haplotypes, and approximate the centre of the contact zone.

Figure 2: Principal coordinates analysis plot to show separation of the lineages based on the relative abundances of 21 compounds in the femoral secretions of males. Filled triangles are Italian males and unfilled triangles are Western European males. Ellipses represent the 95% confidence for each lineage. Percentage variation explained by each coordinate is reported in brackets.

Figure 3: The maximum-likelihood cline and the 95% credible cline region for the best-fitting models (Table S14) for the chemical index, mtDNA haplotype frequencies, the hybrid index, and dorsal greenness (scored on a scale of 1–10 and log transformed to improve fit to model assumptions). Transect distance is the cumulative distance from the south-easternmost population Colle di Val D'Elsa (VE) in Tuscany with increasing distance westwards towards the westernmost population Loano (LO) in Liguria. The chemical index was calculated based on the 12 compounds that differed between the lineages in their relative abundance and were repeatable within individuals (see Table 1), and excludes data from population LO due to low sample size.