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ORIGINAL ARTICLE

Differences in males' chemical signals between genetic lineages of the lizard *Psammodromus algirus* promote male intrasexual recognition and aggression but not female mate preferences

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Abstract Interpopulational variation in sexual signals may lead to premating reproductive isolation and eventually may result in speciation. We explored the role of chemical cues secreted by the femoral glands of male lizards *Psammdoromus algirus* in chemosensory recognition between two distinct genetic lineages from Central Spain. We hypothesized that if there were differences in chemical sexual signals between lineages, these may result in differential recognition and mate preferences. This might lead to reproductive isolation, which would allow the observed morphological and genetic differences between lineages. Our results showed that males of each lineage secreted a singular mixture of compounds in their femoral secretions. However, females were apparently not able to discriminate the lineage of males by chemosensory cues or, alternatively, this discrimination may not be important for females. Moreover, females did not select or reject areas scent marked by males of their own vs. the other lineage. However, previous studies suggest that females might prefer scent of males with particular chemical characteristics that show interindividual variability but do not vary between lineages. Similarly, males did not discriminate between the scents of females of the two lineages, although they had greater chemosensory responses to scents of larger females. In contrast, males clearly discriminated the lineage of other males based on their scents alone, showing chemosensory and aggressive responses that were higher to scents of males of the other lineage. If males of the opposite lineage were more prone to be detected and excluded from a male territory due to their differences in chemical signals, this may probably impede the access of males of one lineage to females of the opposite lineage. This might result in reproductive isolation between lineages. We suggest that the current genetic divergence observed between lineages of *P. algirus* lizards may be mediated by intrasexual relationships among males, but not by female mate preferences. Significance statement Sexual signals often vary geographically to maximize their efficiency in communication under local conditions. Such variation may, however, affect recognition between individuals of different populations, resulting in reproductive isolation and speciation. We studied two populations (lineages) of a lizard with genetic and morphological differences. We found clear inter-lineage variation in chemical profiles of sexual signals of males. However, females did not recognize these differences by chemosensory cues and did not prefer or reject areas scent-marked by males of the two lineages. In contrast, males recognized and responded more aggressively toward scent of males of the opposite lineage. This might impede access of males of one lineage to females of the other. We suggest that the observed differences between lineages may result from partial reproductive isolation, which can be mediated by agonistic interactions between males rather than by female mate preferences.

Keywords Mate choice · Rival recognition · Interpopulational recognition · Lizards · Pheromones · Femoral glands · Speciation

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Introduction

Chemical signals released during the reproductive season are important for mate and species recognition and may provide the basis for premating reproductive isolation and speciation in many animals (Smadja and Butlin 2009; Wyatt 2014). Differences in chemical sexual signals may preclude interspecific mating between related sympatric species (e.g., Cooper and Vitt 1986; Shine et al. 2002; Mas and Jallon 2005; Barbosa et al. 2006; Gabirot et al. 2010a,b, 2012). These differences may be a consequence of random genetic drift, differences in diet or physiological parameters, or they may reveal that different environments select for different signals that maximize their efficiency for communication (Alberts 1992; Boughman 2002; Symonds and Elgar 2008; Martín et al. 2015). Differences in sexual signals can be later amplified by sexual selection leading to differences in mating preferences (Boughman 2001, 2002; Panhuis et al. 2001; Richie 2007). Therefore, if chemical signals differed between populations of the same species, they could preclude mating between these populations (e.g., LeMaster and Mason 2003; Martín and López 2006a,b; Runemark et al. 2011; Heathcote et al. 2016), and, if the degree of reproductive isolation is high, it may finally result in speciation (Smadja and Butlin 2009).

In many lizards, intraspecific communication is partly based on chemical cues secreted by the femoral glands of males, which produce copious amounts of holocrine secretion during the mating season (Mason 1992; Mason and Parker 2010; Martín and López 2011, 2014). Femoral gland secretions deposited on substrate can convey reliable information about dominance status and competitive ability of a male, which may be important in intrasexual relationships (Aragón et al. 2001; López and Martín 2002, 2011; Carazo et al. 2007, 2008; Martín and López 2007; Martín et al. 2007b). Substrate scent marks can also contain honest information on a male's quality, which females may use to select potential mates (Martín and López 2000, 2006c,d, 2012, 2015; López et al. 2006; Olsson et al. 2003). Thus, because chemical signals secreted by femoral glands are involved in sexual selection, variations between populations in chemical profiles may affect interpopulation recognition and be relevant in speciation processes.

The large psammodromus (*Psammodromus algirus*) is a medium-sized (53–90 mm snout-vent length) lacertid lizard that inhabits shrub and woodlands habitats of the western Mediterranean (Salvador 2014). It is a widely distributed species that within the Iberian Peninsula contains two divergent eastern and western mtDNA clades (Carranza et al. 2006). An analysis of mtDNA revealed an ancient split between a western lineage, subdivided into south- and north-western haplogroups, and an eastern lineage with central, south-eastern and eastern haplogroups. In contrast, nuclear markers showed a postglacial admixture of central and western haplogroups, with the central haplogroup apparently isolated from the rest of its clade (Díaz et al. 2016). The distribution ranges of these lineages, which diverged as early as in the Pliocene, overlap along a large contact zone in central Spain, in which only a few hybrid populations have been found (Carranza et al. 2006; Verdú-Ricoy et al. 2010; Verdú-Ricoy 2013; Díaz et al. 2016). This suggests that there is some degree of reproductive isolation between the two lineages, which is also suggested by the clear phenotypic differentiation observed among lineages (e.g., in color pattern or reproductive parameters; Díaz 1993; Carretero 2002; Díaz et al. 2012, 2016; Verdú-Ricoy 2013; Verdú-Ricoy et al. 2014).

Courtship and matings of *P. algirus* occur between April and June. Early in the season, large females often mate sequentially with two males. However, late in the season, small females tend to mate with only one male, suggesting that male-male competition is stronger for larger females at the beginning of the mating season. Male pairing success is influenced by morphological traits; larger, older and more ornamented males pair with more females. However, sneaker younger subadult males (smaller and without nuptial coloration) also obtain some matings (Salvador et al. 1996, 1997; Martín and Forsman 1999; Salvador and Veiga 2001).

Chemosensory recognition is well developed in *P. algirus* lizards; chemical compounds secreted by the femoral glands of males are important in rival recognition during male-male interactions (López et al. 2003). Also, differential responses of females to scent of different individual males suggest that female mate choice decisions might be, at least partially, based on characteristics of chemical signals of males (Martín et al. 2007a). The role of femoral secretions as signals to conspecifics is explained because the type and proportion of compounds in secretions are related to, for example, the age (Martín and López 2006e) and health state of a male (Martín et al. 2007a). These results raise the possibility that this lizard can also use chemical signals to discriminate between genetic lineages, which might maintain reproductive isolation and explain the genetic and morphological differences observed between these two lineages.

In this study, we explored the potential role of chemical communication in interpopulational recognition and premating reproductive isolation (Smadja and Butlin 2009). To address this, we used as a model the two distinct lineages of *P. algirus* lizards from Central Spain. We first analyzed (1) whether there was variation between lineages (western vs. eastern) in the composition and proportions of chemical compounds in femoral gland secretions of males. We hypothesized that if there was variation between lineages in male chemical signals, this could allow chemosensory recognition between lineages. Thus, (2) we designed a chemosensory recognition experiment where we tested the responses of males and females to the scent of lizards (males and females) from their own or the other lineage. We also aimed to understand whether chemosensory recognition affected female mate preferences and lead to premating barriers. Thus, (3) we made an experiment where females could choose to establish in areas with substrates scent marked by males from the two lineages. The selection of a particular area may increase the probability of mating with the territorial male that has scent marked that area and, therefore, may have the same evolutionary consequences that direct mate choice decisions (Martín and López 2012). We expected that if there were differences between lineages in chemical signals of males, chemosensory mate and/or rival recognition, and female mate preferences, this could clarify the existence of reproductive isolation and possible ongoing cryptic speciation between these *P. algirus* lineages.

Materials and methods

Study populations

During April–May 2014, we captured by noosing adult male and female *P. algirus* lizards at two representative localities in Central Spain. Individuals (12 males and 12 females) from the 'western lineage' (haplogroup W2) were captured in "El Pardo" (Madrid Province; 40° 30′ N, 03° 45′ W; 658 m altitude), while individuals (12 males and 12 females) from the 'eastern lineage' (haplogroup E2) were captured in "Torrejoncillo del Rey" (Cuenca province; 40° 02′ N, 02° 35′ W; 925 m altitude). Identification of the genetic lineage found in each population had been made previously (Carranza et al. 2006; Verdu-Ricoy et al. 2010; Verdu-Ricoy 2013). Microsatellite analyses have not found gene flow between these two haplogroups (Verdu-Ricoy 2013; Díaz et al. 2016), in spite that there is a large contact zone without current geographical barriers between the areas occupied by each lineage. Both localities have a forest of holm oaks (*Quercus rotundifolia*) with a high cover of subarboreal perennial bushes (*Cistus*, *Lavandula*, *Cytisus*, etc).

All lizards were individually housed at "El Ventorrillo" Field Station (Cercedilla, Madrid), in outdoor 80 × 40 cm PVC terraria containing a coconut fiber substratum and a plywood refuge for cover. Every day, lizards were fed mealworm larvae (*Tenebrio molitor*) and crickets (*Acheta domesticus*) dusted with multivitamin powder for reptiles, and water was provided ad libitum. Lizards were returned to their exact capture sites with good health condition at the end of experiments.

Analyses of femoral gland secretions

The day after capture, we gently pressed around the femoral pores of males to extract femoral gland secretion, which was collected directly in glass vials with glass inserts. Vials were closed with Teflon-lined stoppers and stored at −20 °C until analyses. We also used the same procedure but without collecting secretion, to obtain blank control vials that were analyzed to compare with lizard samples to exclude potential contaminants. We analyzed lipophilic compounds in samples using a Finnigan-ThermoQuest Trace 2000 gas chromatograph (GC) fitted with a poly (5 % diphenyl/95 % dimethylsiloxane) column (Thermo Fisher Scientific Inc., Waltham, MA, USA, Trace TR-5, 30 m length × 0.25 mm ID, 0.25-mm film thickness) and a Finnigan-ThermoQuest Trace mass spectrometer (MS) as detector. The oven temperature program was as follows: 50 °C isothermal for 3 min, then increased to 300 °C at a rate of 5 °C/min, and finally isothermal (300 °C) for 15 min.

Initial tentative identification of compounds in secretion was done by comparison of mass spectra in the NIST/EPA/NIH 2002 computerized mass spectral library. Identifications were confirmed, when possible, by comparison of spectra and retention times with those of authentic standards from Sigma-Aldrich Chemical Co (Milwaukee, WI, USA). For unidentified or unconfirmed compounds, we used their characteristic *m*/*z* ratios together with retention times (RT) to confirm whether these compounds were present in a given individual.

For the statistical analyses of chemical profiles, the relative amount of each compound was determined as the percent of the total ion current (TIC). To correct the problem of non-independence of proportions, we transformed the proportion data by taking the Ln(proportion/(1–proportion)) (Aebischer et al. 1993). Then, we calculated Euclidean distances between every pair of individual samples to produce a resemblance matrix that formed the basis of further analyses. To analyze whether the composition of the femoral secretions differed between the two lizards' lineages, we used a single factor permutational multivariate analysis of variance test (PERMANOVA, Anderson 2001; McArdle and Anderson 2001) based on the Euclidean resemblance matrix using 999 permutations. Differences between lineages were also investigated using a canonical analysis of principal coordinates (CAP, Anderson and Willis 2003). Statistical analyses were made with the software PRIMER V6.1.13 (Clarke and Gorley 2006) with the PERMANOVA+ V1.0.3 add-on package (Anderson et al. 2008).

Chemosensory recognition

Many lizards respond to different chemical stimuli with increased and differential rates of tongue extrusions. This characteristic chemosensory exploratory behavior allows using tongue-flick (TF) rate as a quantitative bioassay of detection of chemical cues (Cooper and Burghardt 1990; Cooper 1994, 1998). Thus, to test for differential responses to different scents, we made comparisons of TF rate by lizards (males and females) in response to chemical stimuli arising from cotton applicators impregnated with femoral gland secretions of males or with cloacal secretions of females. We prepared stimuli dipping the cotton tip (1 cm) of a wooden applicator attached to a long stick (50 cm) in deionized water. Femoral secretions consisted of a waxy substance, which was easily extracted by gently pressing with forceps around the femoral pores, and collected directly on cotton tips of applicators. Cloacal secretions of females were liquid and easily collected on cotton tips. A new swab was used in each trial.

Three types of trials were made. First, females (11 western and 12 eastern) were exposed to scents from males of each of the two lineages. Then, we studied the responses of males (12 from each lineage) to scent of males from the two lineages. Finally, we tested the responses of males (12 from each lineage) to scent from females of each lineage. In the three trials, we also tested responses to deionized water (odorless control), which was used to gauge baseline TF's rates in the experimental situation (Cooper and Burghardt 1990). Every lizard was exposed to each stimulus and order of presentation was counterbalanced. One trial was conducted per day for each animal. Trials were conducted in outdoor conditions in May, which coincided with the mating season of lizards in their original natural populations (Díaz et al. 1994, 2012), and between 11:00 and 13:00 (GMT) when lizards were fully active.

In each trial, the same experimenter in al tests (PL) slowly approached the terrarium and slowly moved the cotton swab to a position 1 cm anterior to the lizards' snout. The swab tester was blind respect to the scent stimuli tested. Lizards usually explored the swab repeatedly by tongue-flicking or ignored it after the firsts TFs. The numbers of TFs directed at the swab were recorded for 60 s beginning with the first TF. Latency to the first TF was computed as the period elapsed between presentation of the cotton swab to the first TF directed at the swab. We also recorded the number of bites directed to the swabs (only observed in the male-male tests) as a measure of a defensive or aggressive response to the chemical stimuli.

To examine differences in number of TFs directed at the swab and latency to first TF among treatments, we used general linear models (GLMs) examining the effects of scent stimuli (repeated measures factor: own lineage vs. other lineage vs. water) and the lineage of the responding lizard (fixed factor: western lineage vs. eastern lineage). We included the interaction in the models to analyze whether responses to the different scents differed as a function of the lineage of the responding lizard. Analyses were made separately for the three different chemosensory trials. Data were log-transformed to ensure normality. Tests of homogeneity of variances (Levene's test) showed that in all cases variances were not significantly heterogeneous after transformation. We used Tukey's honestly significant difference (HSD) tests (Sokal and Rohlf 1995) for post hoc pairwise comparisons.

Females' choice of substrates scent-marked by males

We performed this experiment at the end of May, coinciding with the mating season of this lizard (Díaz et al. 1994, 2012). We ensured that lizards were in reproductive state because females had developed ovarian egg follicles detectable by abdominal palpation (the onset of sexual activity) but had not yet mated (i.e., mating scars were absent), and males had developed orange or yellow breeding coloration and had abundant femoral secretions. We had previously placed in the males' terraria several absorbent paper strips (35 × 10 cm) fixed to the floor, and left them there for 1 week to allow males to scent-mark the substrates. Female cages were virtually divided in three areas of equal surface (40 × 27 cm each). At the beginning of experiments (09:00 h, GMT; when females where still inactive) we fixed, wearing fresh gloves, on the center of the area located in one side of each female cage one paper strip from one individual male from her own lineage, and on the center of the other side a paper from another individual male from the other lineage, leaving a neutral intermediate zone. Different papers from each male were used in different choice tests with different females. Each female was tested four times, once a day, with papers from four different pairs of males (to avoid possible interindividual differences among males). Each trial lasted 3.5 h (from 8:30 h GMT, shortly after females appeared from refuges and until 12:00 h GMT). Females were monitored each 10 min from a hidden point (i.e., a total of 22 scans). If a female was located active on an area containing a paper strip, she was designated as having chosen temporarily that particular area, whereas if she was located in the intermediate neutral area without papers, she was designated as having made no choice (Martín and López 2000, 2006c; Olsson et al. 2003). We also noted those scans where the female was not seen active on any of the areas but she was hidden in the refuge. We calculated the number of times that females were observed active on each of the areas containing scent marked papers and in the non-choice area. At the end of the trials, we moved the female to another clean cage, removed the papers, and thoroughly rinsed the experimental cage with clean water and let it to dry outdoor before using it in another test. We did not use detergent to avoid contamination of the cage with artificial scents.

We compared the number of observations (squared root-transformed) of each female in the different sides of the terrarium (own lineage vs. neutral area vs. other lineage). We used GLMs to test for differences among the 4 days of the experiment and among the three sides of the terrarium, both as within-subject factors, and with the lineage of the responding female as a fixed factor.

Results

Chemical compounds in femoral secretions of males

Considering together all individual male *P. algirus* lizards from the two lineages, we found 59 lipophilic compounds in femoral gland secretion (Table 1), which is mainly a mixture of steroids (52.9 % of TIC area; the two lineages pooled), and carboxylic acids ranged between n-C_{16} and n-C_{18} and their esters (35.7 %), but we found also 11 aldehydes between n-C_7 and n-C_{20} (3.6 %), seven waxy esters (2.8 %), squalene (2.4 %), seven alcohols between n-C_{10} and n-C_{19} (1.8 %), and two amides (0.9 %). On average, the six most abundant chemicals were campesterol (17.2 % of TIC), hexadecanoic acid (13.3 %), ergosterol (11.3 %), octadecanoic acid (9.4 %), 9,12-octadecadienoic acid (9.3 %) and cholesterol (8.3 %).

There were 35 chemical compounds shared by lizards from both lineages, the proportions of which comprised 91.5 % of the overall TIC area, but we found differences between lineages in the presence/absence of 24 minor compounds and in the relative proportions of some major compounds (Table 1). The PERMANOVA based on the resemblance matrix comparing the chemical profiles of the two lineages was highly significant (pseudo $F_{1,22} = 15.37$, $P < 0.001$). The CAP analysis classified 100 % of the chemical profiles into the correct population using leave-one-out cross-validation ($\delta_1^2 = 0.96$, $P = 0.001$, $m = 2$ axes). Therefore, there were clear differences between lineages in chemical composition of femoral gland secretions.

Comparing the main classes of compounds between lineages, there were no significant differences in relative proportions of steroids, carboxylic acids, aldehydes, waxy esters or alcohols (Table 2), but lizards of the western lineage had significantly less squalene and more amides than lizards of the eastern lineage (Table 2). When comparing the relative proportions of the six most abundant shared compounds, lizards from the western lineage had significantly more 9,12-octadecadienoic acid, ergosterol and campesterol, but less cholesterol than lizards from the eastern lineage (Table 2). In contrast, there were no significant differences between lineages in relative proportions of hexadecanoic acid or octadecanoic acid (Table 2).

Chemosensory recognition

Responses of females to scent of males

All females directed TFs to the swab in all conditions. Mean latency to first TF differed significantly among scent stimuli (GLM, scent treatment: $F_{2,42} = 78.96$, $P < 0.0001$; Fig. 1a), but there was no a significant difference between the overall responses of both lineages of females (lineage: $F_{1,21} = 2.89$, $P = 0.10$), who responded in a similar way to the different stimuli (interaction: $F_{2,42} = 1.26$, $P = 0.29$). Females responded to either male's scents sooner than to water (Tukey's tests: $P < 0.0001$ in all cases), but there were no significant differences between latency times to scent of males of the own or the other lineage ($P = 0.86$).

There were significant differences among scent stimuli in numbers of TFs directed by females (GLM, scent treatment: $F_{2,42} = 102.24$, $P < 0.0001$; Fig. 1b), but there were no significant differences between the overall response of the two lineage of females ($F_{1,21} = 1.36$, $P = 0.26$), who responded in a similar way to the different stimuli (interaction: $F_{2,42} = 0.15$, $P = 0.86$). Females discriminated between scents of either type of male and water (Tukey's tests: $P < 0.0001$ in all cases), but did not discriminate between the two lineages of males ($P = 0.91$).

Responses of males to scent of other males

All males directed TFs to the swab in all conditions. Mean latency to first TF differed significantly among treatments (GLM: $F_{2,44} = 134.95$, $P < 0.0001$; Fig. 2a), but not in the overall responses of both lineages of males ($F_{1,22} = 0.01$, $P = 0.96$), which responded in a similar way to the different stimuli (interaction: $F_{2,44} = 0.40$, $P = 0.67$). Males responded to either type of male's scents significantly sooner than to water (Tukey's tests: $P < 0.0001$ in all cases), but there were no significant differences between latency times to scent of males of the own or the other lineage ($P = 0.54$).

There were significant differences among treatments in numbers of TFs directed by males (GLM: $F_{2,44} = 257.45$, $P < 0.0001$; Fig. 2b), and males from the eastern lineage had significantly higher overall TF responses than males from the western lineage ($F_{1,22} = 9.13$, $P = 0.006$), but all males responded in a similar way to the different stimuli (interaction: $F_{2,44} = 0.23$, $P = 0.80$). Males discriminated between scents of either type of male and water (Tukey's tests: $P < 0.001$ in all cases), and between scents of the two lineages of males ($P = 0.012$), responding with significantly higher TF responses to males of the other lineage than to males of their own lineage.

Nine males behaved aggressively and bit on 19 occasions the swab bearing scent of a male of the other lineage, seven bit on nine occasions the swab with scent of a male of their own lineage, and one bit the swab with water on one occasion. Based on the null hypothesis that the likelihood of biting was equal in all three conditions, the binomial probability that 19 of the 29 bites would be directed to scent of a male of the other lineage was $P = 0.0002$. Nevertheless, considering the lineages separately, the test remained significant for responding males of the eastern linage (14 of 19 bites, $P = 0.0002$), but not for responding males of the western lineage (5 of 10 bites, $P = 0.30$).

Table 1 Lipophilic compounds found in femoral gland secretions of male lizards, *P. algirus* from two different genetic lineages. The relative amount of each component was determined as the percent of the total ion current (TIC) and reported as the average (±1SD). Characteristics ions (m/z: mass-to-charge ratio) are reported for some unidentified (Unid.) compounds

	Genetic lineages		
RT	Compound	Western ($N = 12$)	Eastern ($N = 12$)
6.7	Heptanal	0.07 ± 0.11	0.19 ± 0.23
11.0	Octenal	0.33 ± 0.50	–
15.2	Decanol	0.22 ± 0.18	0.03 ± 0.04
20.7	Dodecanol	0.22 ± 0.20	0.18 ± 0.17
25.5	Tetradecanol	0.28 ± 0.23	0.14 ± 0.13
26.0	Tetradecanal	0.66 ± 0.48	0.30 ± 0.16
27.7	Hexadecenal	0.21 ± 0.20	0.04 ± 0.07
28.3	Pentadecanal	0.69 ± 0.46	0.58 ± 0.28
29.7	Octadecenal	0.13 ± 0.25	0.04 ± 0.06
30.4	Hexadecanal	0.41 ± 0.49	0.58 ± 0.40
31.7	Hexadecenol	–	0.42 ± 0.37
31.9	Hexadecanol	0.37 ± 0.14	0.45 ± 0.11
32.5	Heptadecanal	0.24 ± 0.31	0.20 ± 0.17
33.4	Hexadecanoic acid	12.47 ± 3.02	14.53 ± 9.80
34.4	Octadecanal	0.75 ± 0.54	0.46 ± 0.46
34.4	Hexadecanoic acid, 1-methylethyl ester	0.34 ± 0.30	–
35.9	Octadecanol	0.40 ± 0.50	0.98 ± 0.87
36.3	Nonadecanal	0.22 ± 0.44	1.05 ± 0.47
36.6	9,12-Octadecadienoic acid	14.89 ± 2.53	1.91 ± 2.91
37.0	9-Octadecenoic acid	0.89 ± 1.79	5.84 ± 6.59
37.0	9,12-Octadecadienoic acid, ethyl ester	0.88 ± 1.05	0.05 ± 0.09
37.1	Octadecanoic acid	9.68 ± 1.23	8.93 ± 4.67
37.7	Nonadecanol	–	0.09 ± 0.15
38.0	Eicosanal	–	0.04 ± 0.08
40.5	Octadecenamide	0.71 ± 0.49	0.29 ± 0.25
46.7	13-Docosenamide	0.73 ± 0.93	
47.3	Squalene	0.91 ± 0.30	4.35 ± 2.80
48.4	Cholesta-3,5-diene	–	0.13 ± 0.22
49.4	Unid. Steroid (197,251,362,377)	0.88 ± 0.40	0.68 ± 0.32
49.8	Unid. Steroid (250,361,377)	0.05 ± 0.09	–
49.9	Unid. Steroid (195,237,374,355,362,383)	0.07 ± 0.15	–
50.2	Unid. Steroid (197,251,364,379)	0.13 ± 0.16	0.75 ± 0.59
50.9	Cholesta-5,22-dien-3-ol	–	4.56 ± 4.22
51.0	Unid. Steroid (145,159,213,239,255,367,381,397)	–	0.06 ± 0.11
51.2	Unid. Steroid (255,300,355,368,383)	0.20 ± 0.39	0.36 ± 0.62
51.4	Unid. Steroid (197,251,378,393)	2.25 ± 1.55	0.53 ± 0.91
51.7	Cholesterol	6.20 ± 2.64	11.02 ± 2.23
51.8	Unid. Steroid (183,185,210,253,379,395)	0.83 ± 0.83	1.12 ± 0.53
52.1	Ergosta-7,22-dien-3-ol	1.13 ± 2.27	1.65 ± 1.23
52.4	Unid. Steroid (241,309,351,379,396)	1.49 ± 2.97	–
52.8	Ergosterol	12.87 ± 5.81	9.11 ± 3.92
52.7	Ergosta-7,22-dien-3-ol, derviative?	–	1.56 ± 1.39
53.0	Campesterol	20.44 ± 3.86	12.94 ± 2.03
53.3	Ergosta-5,7-dien-3-ol	–	0.98 ± 1.34
53.3	Ergostenol (=Ergost-7-en-3-ol)	0.83 ± 0.75	1.77 ± 1.54
53.6	Unid. Steroid (145,213,253,353,368,378,410)	–	0.91 ± 0.79
54.0	Sitosterol	1.08 ± 1.50	1.68 ± 1.48
54.1	Hexadecyl hexadecanoate	–	0.33 ± 0.28

Table 1 (continued)

	Genetic lineages		
54.3	20-Methyl-pregn-20-en-3-ol	–	0.58 ± 1.01
54.5	2,2-Dimethyl-cholest-7-en-3-ol	0.39 ± 0.78	–
54.7	Stigmast-7-en-3-ol	0.37 ± 0.74	3.70 ± 3.20
54.7	Unidentified ester of hexadecanoic acid	0.76 ± 1.52	–
54.8	Unid. Steroid (255,268,314,355,399,414)	–	0.79 ± 0.72
55.0	Sitosterol, methyl derivative?	1.90 ± 2.55	0.53 ± 0.92
56.2	Octadecyl 9-hexadecenoate	–	1.52 ± 2.63
56.3	Unidentified ester of octadecanoic acid	0.28 ± 0.35	0.28 ± 0.48
57.2	Octadecyl hexadecanoate	–	0.48 ± 0.54
57.9	Unidentified ester of hexadecanoic acid	0.71 ± 0.84	–
62.6	Unidentified ester of octadecanoic acid	1.19 ± 0.81	–

Responses of males to scent of females

All males directed TFs to the swab in all conditions. Mean latency to first TF differed significantly among treatments (GLM: $F_{2,44} = 196.20$, $P < 0.0001$; Fig. 3a), but there were no significant differences between the overall responses of both lineages of males ($F_{1,22} = 0.01$, $P = 0.93$), which responded in a similar way to the different stimuli (interaction: $F_{2,44} = 0.85$, $P = 0.44$). Males responded to either female's scents significantly sooner than to water (Tukey's tests: $P < 0.0001$ in all cases), but there were not significant differences between latency times to scent of females of the own or the other lineage ($P = 0.27$).

There were significant differences among treatments in numbers of TFs directed by males (GLM: $F_{2,44} = 224.91$, $P < 0.0001$; Fig. 3b), there were no significant differences between the response of the two lineages of males ($F_{1,22} = 1.76$, $P = 0.20$), which tended, although non significantly, to respond in slightly different ways to the different stimuli (interaction: $F_{2,44} = 2.72$, $P = 0.08$). Males

Table 2 Percentages (mean ± SE) of each type of compound and of the six major compounds in femoral gland secretions of male lizards, *P. algirus* from two different genetic lineages. Results of GLMs (*F*, *P*) comparing transformed proportions between lineages are shown. Significant results are marked in italics

	Western	Eastern	$F_{1,22}$	*P*
Steroids	51.1 ± 2.1	55.4 ± 4.2	0.83	0.37
Carboxylic acids	38.8 ± 1.3	31.6 ± 5.7	1.51	0.23
Aldehydes:	3.3 ± 0.5	3.3 ± 0.1	0.01	0.97
Waxy esters	2.9 ± 0.8	2.6 ± 0.8	0.09	0.76
Alcohols	1.5 ± 0.1	2.3 ± 0.4	3.90	0.06
Squalene	0.9 ± 0.1	4.3 ± 0.7	24.62	*<0.0001*
Amides	1.4 ± 0.3	0.3 ± 0.1	10.84	*0.003*
Hexadecanoic acid	12.5 ± 0.8	14.5 ± 2.5	0.63	0.43
9,12-Octadecadienoic acid	14.9 ± 0.6	1.9 ± 0.7	177.99	*<0.0001*
Octadecanoic acid	9.7 ± 0.3	8.9 ± 1.1	0.39	0.54
Ergosterol	12.9 ± 1.5	9.1 ± 0.9	4.37	*0.048*
Campesterol	20.4 ± 1.0	12.9 ± 0.5	44.47	*<0.0001*
Cholesterol	6.2 ± 0.7	11.0 ± 0.5	29.89	*<0.0001*

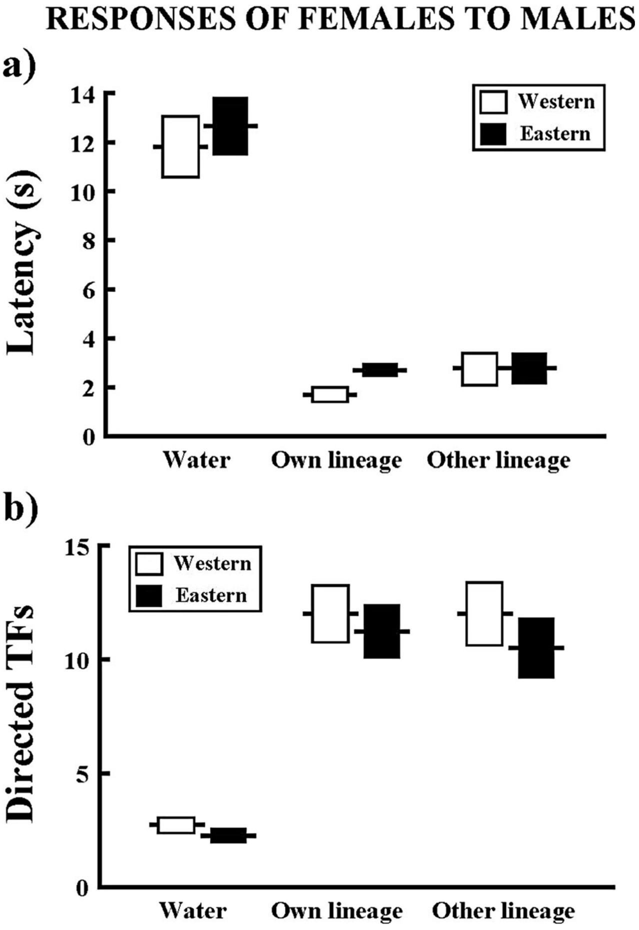

Fig. 1 Mean (±SE) **a** latency (s) to the first tongue-flick, and **b** number of tongue-flicks directed to the swab by female *P. algirus* of two lineages (*open boxes*: western; *black boxes*: eastern) in response to control deionized water, or scents from femoral gland secretions of males of their own or of different lineage presented for 60 s on cotton-tipped applicators

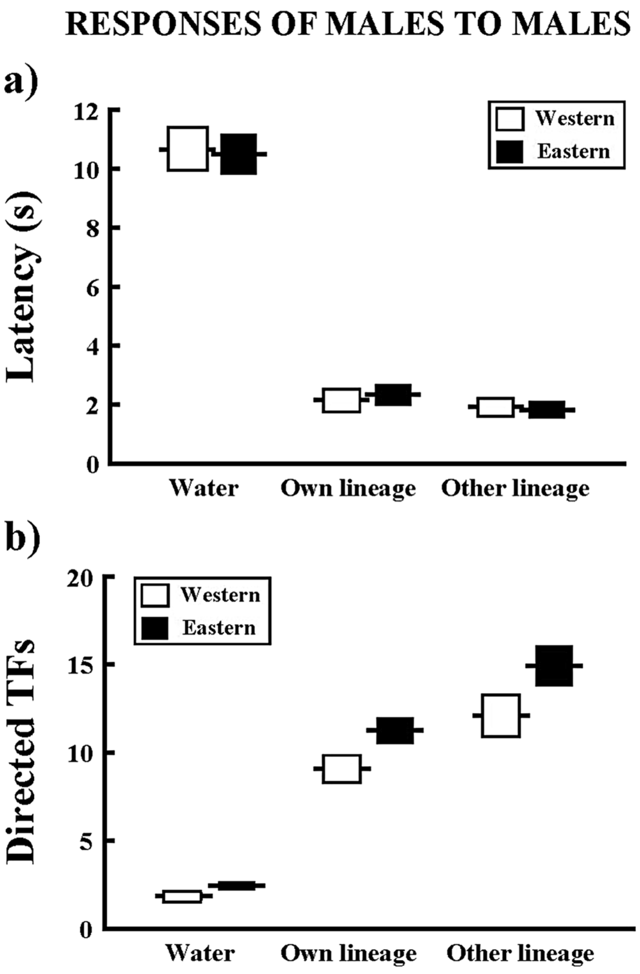
RESPONSES OF MALES TO MALES

Fig. 2 Mean (±SE) **a** latency (s) to the first tongue-flick, and **b** number of tongue-flicks directed to the swab by male *P. algirus* of two lineages (*open boxes*: western; *black boxes*: eastern) in response to control deionized water, or scents from femoral gland secretions of males of their own or of different lineage presented for 60 s on cotton-tipped applicators

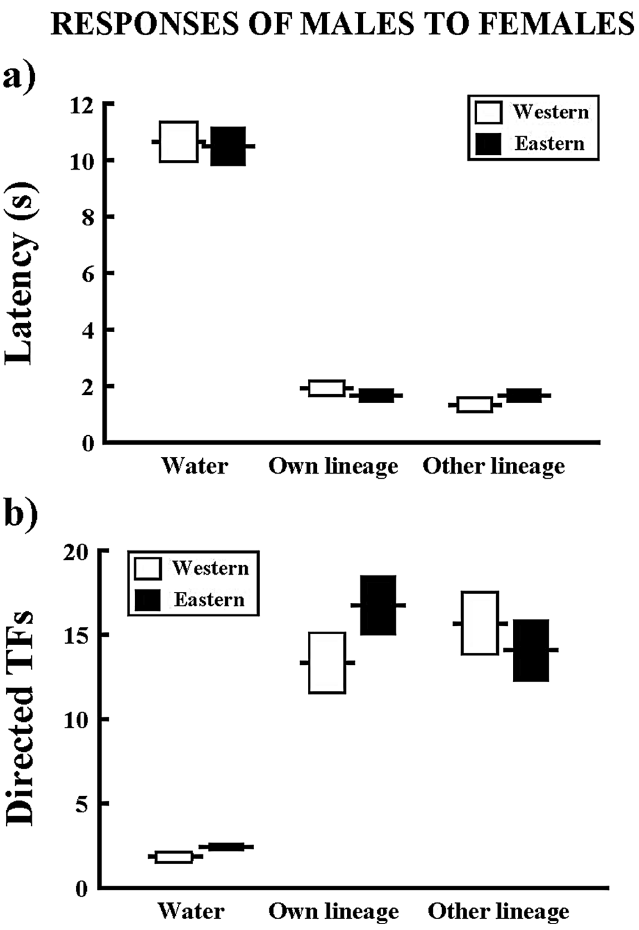
RESPONSES OF MALES TO FEMALES

Fig. 3 Mean (±SE) **a** latency (s) to the first tongue-flick, and **b** number of tongue-flicks directed to the swab by male *P. algirus* of two lineages (*open boxes*: western; *black boxes*: eastern) in response to control deionized water, or scents from cloacal secretions of females of their own or of different lineage presented for 60 s on cotton-tipped applicators

discriminated between scents of either type of female and water (Tukey's tests: $P < 0.001$ in all cases), but there were no significant differences between TF responses to scent of females of the own or the other lineage ($P = 0.99$).

Because we presented scent from the same females to different males in different days, we calculated the average number of TFs that the scent of each individual female, irrespective of her lineage, elicited in males; larger females elicited significantly higher TF responses by males ($r_s = 0.45$, $n = 23$, $P = 0.029$).

Females' choice of substrates scent-marked by males

The number of observations of females differed significantly among the three areas of the terarria (GLM, treatment: $F_{2,44} = 3.59$, $P = 0.036$; Fig. 4). Thus, females were seen more often on papers scent marked by either type of male than on the neutral side (Tukey's test: $P < 0.05$ in both cases), but there were no significant differences between the papers scent marked by the two lineages of males ($P = 0.93$). There were also significant differences in the responses of females among days (day: $F_{3,66} = 18.35$, $P < 0.0001$), reflecting that in the

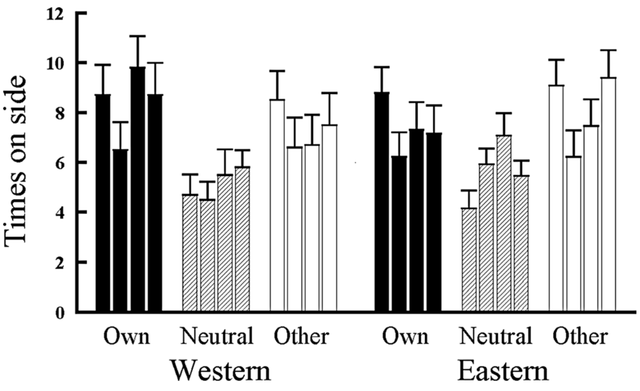

Fig. 4 Number of times (mean ± SE), during each of four scent's choice trials, that female lizards *P. algirus* from two genetic lineages (western vs. eastern) were observed on areas with paper strips scent marked by males of their own or of the other lineage or on a neutral area

second day females were observed active on the surface a lower number of times than in the rest of the days (Tukey's tests: $P < 0.001$ in all cases), which did not differ ($P > 0.85$ in all cases). However, females of both lineages did not significantly differ in their overall number of observations (lineage: $F_{1,22} = 0.01$, $P = 0.94$), and none of the interactions were significant (side x lineage: $F_{2,44} = 1.16$, $P = 0.32$; day x lineage: $F_{3,66} = 1.67$, $P = 0.18$; day x side: $F_{6,132} = 0.93$, $P = 0.48$).

Discussion

Our study showed that femoral gland secretions of male *P. algirus* lizards varied clearly in proportion of some chemical compounds between genetic lineages. Males of each lineage secreted a singular and characteristic mixture of compounds. However, females were apparently not able to discriminate the linage of males by chemosensory cues alone, or, alternatively, this discrimination was not important for females. Moreover, females did not select areas scent marked by males of their own vs. the other lineage. Neither did males discriminate between the scents of females of the two lineages. In contrast, males showed a clear chemosensory discrimination of the lineage of other males based on their scent alone, showing chemosensory and aggressive responses that were higher to scent of males of the other lineage. However, these results could be tentative because we have considered only one representative population from each lineage. Future studies should examine chemical signals and chemosensory responses among several different populations within each of the two lineages in comparison with several populations from the other lineage. Nevertheless, several traits, both morphological (such as striped versus unstriped dorsal coloration, head colors of males, etc) and physiological (such as temperature-adjusted incubation time) show much more variation between lineages than among populations of the same lineage (Díaz et al. 2012, 2016; Verdú-Ricoy et al. 2014).

Chemical profiles of femoral secretion of males of the two lineages are apparently similar in overall composition (in comparison with other related lizard species), but have some significant important differences that clearly allow assigning statistically a given secretion to the correct lineage. Moreover, these inter-lineage differences in chemical signals are also observed in other populations not considered here (JM unpubl. data). Differences are mainly based in the different relative proportions of some major compounds shared by both lineages, although there are also some minor compounds that are exclusive of each lineage. These differences might arise from random genetic drift, differences in sustrate, habitat structure or diet (e.g., Martín and López 2006d; Kopena et al. 2014), and/or from local adaptation or phenotypic plasticity aimed to maximize the efficiency of the chemical signal under the climatic conditions in areas where each lineage inhabits (Alberts 1992; Martín and López 2013; Martín et al. 2015). However, to test this latter hypothesis, further experiments are needed to examine the efficiency (durability and persistence) of each type of secretion in the climatic conditions of each population.

In spite of obvious differences in chemical profiles of males of the different lineages, females showed similar chemosensory responses to secretions of males of either lineage and did not prefer, nor avoid, scent-marks of males from their own lineage. This suggests that although females can clearly detect and discriminate a male's scent from a blank control, and prefer to establish on areas scent marked by males against unmarked areas, they do not recognize the lineage of a male from his scent alone. Several explanations are possible. The simplest one is a lack of ability of females to recognize the differences in proportions of major compounds or the differences in presence/absence of minor compounds. However, at least female *P. algirus* of the western lineage have been shown to be able to discriminate many compounds from males' secretions and to respond differentially to individual differences among males as well as to manipulated variations in these compounds (Martín et al. 2007a). In that previous study, females responded differently to scent of different individual males according to the parasite load and health of these males, traits that were related to the variability in the proportions of some lipids (e.g., octadecanol or octadecanoic acid) in secretions of males (Martín et al. 2007a). Thus, it is very likely that female *P. algirus* are able to detect male scent and, perhaps, may discriminate and select individual males based on their particular chemical characteristics (e.g., those compounds signaling health that show interindividual variability but that do not vary between lineages) but irrespective of the inter-lineage characteristics (i.e., other compounds that do vary in average proportion between lineages). Similarly, female lizards *Podarcis hispanica* of some populations do not seem to recognize by their scent males of two populations that differ in chemical profiles. However, female *P. hispanica* select individual males based on the variation in proportions of some compounds that do not differ between populations but show interindividual variability unrelated to the population of origin (Gabirot et al. 2013). Thus, female *P. hispanica* prefer males with relatively high amounts of cholesta-5,7-dien-3-ol (=provitamin D_3) in femoral secretions. This preference may be explained by the positive relationship between the amount of this steroid in secretions and the robustness of the immune response of a male (López and Martín 2005; López et al. 2009). Therefore, in both lizard species, the female preferences for scent of individual males of high quality, irrespective of their population of origin, and the fact that this "quality" may be signaled by compounds that do not vary between populations or lineages, may be precluding a female dependent premating isolation between genetic lineages.

Males were also apparently unable to discriminate between scents of females of the two lineages, although they clearly discriminated the female scent from a control. This could be firstly explained if females of the different lineages did not vary in chemical composition of their cloacal secretions (something that remains to be studied), or if males based their female identification on compounds that did not vary between lineages. Interestingly, males did show differential responses to females of different body size, irrespective of the female lineage, suggesting that the cloacal secretion characteristics change with body size, and that males can assess it. Either qualitative differences in chemical composition or changes in concentration of the scent related to body size might be the proximal basis of this discrimination. In any case, a higher chemosensory response of males to scent of larger females seems to reflect the preferences of males observed in the field for pairing with larger females (Salvador and Veiga 2001). This preference may be explained because large females lay more eggs (Salvador 2014). As in the case of female responses, the preference of males for females of high quality (i.e., larger) irrespective of their population of origin may be also precluding initially a male dependent premating isolation between lineages. Nevertheless, because in the natural populations there are some differences in overall body size between females of the two lineages (western females being larger on average), the males' preferences for large females might be favoring differential matings in the contact zones between lineages.

In contrast, responses of males to scent of other males were clearly dependent on the lineage of the responding and donor male. Males were able to discriminate between lineages of rival males based on their scent alone. The importance of chemical signals for rival recognition and rival assessment between males has already been shown in the western lineage of *P. algirus* (López et al. 2003) and in other lacertid lizards (López and Martín 2002, 2011; Carazo et al. 2007, 2008; Martín and López 2007; Martín et al. 2007b; Heathcote et al. 2016). Moreover, experimental manipulations showed that chemical cues may even have precedence over visual cues in male-male identification in *P. hispanica* (López et al. 2002). In the current study, males discriminated and often responded aggressively to the presentation of the swab with scent of other males. Interestingly, chemosensory responses of males were stronger and more aggressive (or defensive) when presented with scent of males of the other lineage, at least when responding males were in their own terraria. This suggest that the scent of male of the other lineage was considered as a greater threat, inducing stronger aggressive or defensive responses, than the scent of a male of the own lineage. This could probably be due to familiarity with the overall characteristics of the scent of local males ("dear enemy" recognition; e.g., López and Martín 2002, 2011; Carazo et al. 2008). Therefore, in potential encounters of males of the two lineages in the field (i.e., in the contact zones between lineages), vagrant males of one lineage entering the territory of males of the opposite lineage will receive a stronger aggressive response from resident males. If males of the opposite lineage were more prone to be detected and excluded from a male territory due to their differences in chemical signals, this may probably difficult the access of males of one lineage to females of the opposite lineage.

Interestingly, males of the eastern lineage showed higher overall chemosensory responses and higher aggression to scent of males of the other lineage than western males. This might suggest that chemical communication is more important, or more effective, for lizards in the eastern populations. In contrast, visual signals (= nuptial bright coloration) are more developed in males of the western lineage (Díaz 1993; Carretero 2002), suggesting that the relative importance of chemical and visual signals depends on the effectiveness of each type of communication in different environments. A similar environmental-dependent variation in the relative importance of alternative communication systems was also suggested for *Liolaemus* lizards (Fox and Shipman 2003).

A recent study with two divergent lineages of *Podarcis muralis* lizards showed that in staged encounters between individuals there was no evidence of differential female mate choice, but that assortative reproduction was driven by male mate preferences and male-male competition (Heathcote et al. 2016). Earlier studies of chemosensory discrimination in other lacertid lizards showed that only males, but not females, were apparently able to discriminate, or interested in discriminating, between closely related species (e.g., Barbosa et al. 2006; Martín and López 2006a). Therefore, our results and published data suggest that, in some lizards, processes of reproductive isolation and speciation may be mediated by male preferences and intrasexual relationships between males rather than by female mate choice. Further studies should examine the characteristics of "hybrid" individuals and the role of specific chemical signals in these processes, testing especially whether diet or physiological differences, and/or adaptations to maximize efficiency of these signals under the local environmental conditions may be driving inter-lineage differences in chemical signals and behavioral responses. Also, future experiments should examine the relative importance of direct male-male agonistic interactions in reproductive isolation.

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Compliance with ethical standards

Ethical approval Captures and observations were performed under license from the Environmental Agency of Madrid Government ("Consejería del Medio Ambiente y Ordenación del Territorio de la Comunidad de Madrid", Spain) and of Castilla la Mancha Government, and Patrimonio Nacional allowed access to El Pardo populations.

Conflict of interest The authors declare that they have no conflicts of interest.

Informed consent Informed consent was not required.

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