Current Zoology 59 (2): 210–220, 2013

Female mate choice based on pheromone content may inhibit reproductive isolation between distinct populations of Iberian wall lizards

Marianne GABIROT[1,2*], Pilar LÓPEZ[2], José MARTÍN[2]

[1] Département Ecologie Comportementale, (U.M.R. 5175), CEFE-CNRS, 1919 Route de Mende, F34293 Montpellier, Cedex 5, France

[2] Departamento de Ecología Evolutiva, Museo Nacional de Ciencias Naturales, C.S.I.C., José Gutiérrez Abascal 2, 28006 Madrid, Spain

Abstract The Iberian wall lizard *Podarcis hispanica* forms part of a species complex with several morphologically and genetically distinct types and populations, which may or may not be reproductively isolated. We analyzed whether female mate choice based on males' chemical signals may contribute to a current pre-mating reproductive isolation between two distinct populations of *P. hispanica* from central Spain. We experimentally examined whether females choose to establish territories on areas scent-marked by males of their own population, versus areas marked by males of the other population. Results showed that females did not prefer scent-marks of males from their own population. In contrast, females seemed to attend mostly to among-individual variation in males' pheromones that did not differ between populations. Finally, to test for strong premating reproductive isolation, we staged intersexual encounters between males and females. The population of origin of males and females did not affect the probability nor the duration of copulations. We suggest that the different environmental conditions in each population might be selecting for different morphologies and different chemical signals of males that maximize efficiency of communication in each environment. However, females in both populations based mate choice on a similar condition-dependent signal of males. Thus, male signals and female mate choice criteria could be precluding premating reproductive isolation between these phenotypically "distinct" populations [*Current Zoology* 59 (2): 210–220, 2013].

Keywords *Podarcis hispanica,* Chemical communication, Mate preferences, Mate recognition, Species recognition, Conflict

Mate choice often requires that individuals engage in both species recognition and mate-quality recognition (Sherman et al., 1997). Because the types of traits used for each form of recognition may be similar, a conflict may often exist between species and mate recognition (Gerhardt, 1982; Ryan and Rand, 1993; Pfennig, 2000; Rosenthal and Ryan, 2011). Females may potentially face this conflict whenever there is overlap in mating signals between conspecifics and heterospecifics (Ryan and Keddy-Hector, 1992; Andersson, 1994; Rosenthal and Ryan, 2011). The problem is particularly acute when extreme traits serve as signals of direct and indirect benefits to females and/or offspring (Møller, 1990; Reynolds and Gross, 1992; Andersson, 1994; Petrie, 1994). In such cases, females that engage primarily in species recognition risk mating with relatively low-quality males, while females that attend to extreme secondary sexual charactersrisk heterospecific matings (Ryan and Rand, 1993; Pfennig, 1998, 2000; Rosenthal and Ryan, 2011).

Chemical signals, or pheromones, are important for species recognition but also for acquiring information about dominance, territoriality and during mate choice (Wyatt, 2003; Brennan and Kendrick, 2006; Johansson and Jones, 2007). The composition and relative proportion of pheromone components are highly species-specific (Bradbury and Vehrencamp, 1998; Wyatt, 2003). In many lizards, intraspecific communication is based on chemical cues from femoral gland secretions of males, which are epidermal structures on the ventral surface of the thigh connected to glands that produce copious amounts of holocrine secretion during the mating season (Mason, 1992; Alberts, 1993; Mason and Parker, 2010; Martín and López, 2011). Femoral gland secretions of males deposited on substrates can convey information about social status and competitive ability (Aragón et al., 2001; López and Martín, 2002; Carazo et al., 2007; Martín and López, 2007; Martín et al., 2007a),

---

Received Oct. 2, 2012; accepted Feb. 15, 2013.

* Corresponding author. E-mail: marianne.gabirot@cefe.cnrs.fr

and may transmit information about a male's health state or genetic compatibility (Martín and López, 2000, 2006c, e; Olsson et al., 2003). For example, the proportions of a steroid (cholesta-5,7-dien-3-ol, or provitamin D3) in femoral secretions of male Iberian wall lizards, *Podarcis hispanica*, is correlated with male immune function (López and Martín, 2005a; Lopez et al., 2008), and might therefore serve as a predictor of direct or indirect benefits of mating (Lopez et al., 2008Female mate choice decisions are at least partly based on interindividual variation in the proportion of cholesta-5,7-dien-3-ol in males' femoral secretions (López and Martín, 2005a). However, the chemical composition of femoral gland secretions of male *P. hispanica* also differs among populations (Martín and López, 2006a; Gabirot et al., 2010b, 2012). Interpopulational differences in chemical signals of male *P. hispanica*, and/or in female mate preferences, could be leading to reproductive isolation between distinct populations of this lizard species.

Here we address the possible conflict between female mate choice and potential speciation processes requiring conspecific (or population) recognition in the Iberian wall lizard *P. hispanica*. Molecular and morphological studies suggest that *P. hispanica* forms part of a species complex with at least five monophyletic lineages and some now well-recognized species (Guillaume, 1987; Harris and Sá-Sousa, 2001, 2002; Sá-Sousa et al., 2002; Pinho et al., 2007, 2008; Carretero, 2008; Kaliontzopoulou et al., 2011). Also, there are many clearly different populations of this lizard that are highly variable in size, shape, scale and color patterns, which suggest the existence of further cryptic speciation processes within taxa previously considered to be conspecifics. These small common lizards have well-developed chemical recognition abilities, and are able to use chemical cues alone to discriminate between conspecifics and heterospecifics (Cooper and Pérez-Mellado, 2002; Barbosa et al., 2005, 2006; Gabirot et al., 2010a), and between sexes (Gomez et al., 1993; López and Martín, 2001; López et al., 2002). We studied two distinct populations of *P. hispanica* from the Guadarrama Mountains (central Spain), inhabiting different altitudes and environmental conditions (Martín and López, 2006a,b). Previous studies indicated that males can discriminate by chemical cues alone between males of their own and the other population (Martín and López, 2006a, b). In contrast, females detected scent of males, but did not show differences in chemosensory exploration between scents of males from these two populations (Martín and López, 2006a, b). These results suggested that female mate recognition and female mate preferences might be based on some characteristics of chemical cues shared by males from different populations. In this study, we first examined genetic, morphological, and chemical differences between these two lizard populations. The failure to detect interpopulation discrimination by females (Martín and López, 2006a, b) does not entirely reject the hypothesis that female *P. hispanica* may still prefer to establish in areas scent-marked by males of their own population, thus increasing their opportunities to mate with these males (Martín and López, 2012). Then, we tested in the laboratory whether females chose to establish on areas scent-marked by males of their own population versus areas scent-marked by males of the other population, or whether females selected scent-marked areas based on the concentration of some chemical compounds in males' scent independent of the male's population of origin. Finally, to evaluate the strength of premating reproductive isolation between populations, we staged intersexual encounters between males and females of the same or different populations and analyzed differences in mating behavior and whether copulations occurred. We hypothesized that female mating preferences for certain features of a chemical signal that characterized males of their own population would promote divergence between populations. However, if females based their mate choice on characteristics of the chemical signal shared by males of the two populations, this lack of population discrimination might be precluding an effective reproductive isolation, even if other selective pressures (i.e., different environmental conditions) were promoting phenotypic divergence between populations.

1 Material and Methods

1.1 Study animals

We captured adult male and female *P. hispanica* lizards at two geographically close localities in the Guadarrama Mountains (Madrid Province, Spain) by noosing during March, before the start of their mating season. For all experiments, we used 43 females and 25 males captured from rock-cliffs at the edge of a pine forest ('Fuenfría', 40°47' N, 4°03' W, 1750 m elevation), and we caught 45 females and 25 males from rocky outcrops inside a large oak forest ('Golondrina', 40°44' N, 4°02' W, 1250 m elevation). These two areas are only 6 km apart, but the intermediate area is not occupied by stable populations of *P. hispanica* (Martín and López, 2006a, b). These two sites have similar rocky microhabitats that are adequate for *P. hispanica,* but the elevational difference results in different microclimates. For example, long-term records of meteorological stations located close to each lizard population indicate that from March to August 2007 (i.e., during the main activity season of lizards) average temperatures were considerably higher in Golondrina (17.3 ± 2.8 °C) than in Fuenfría (9.6 ± 2.5 °C), whereas average relative humidity was lower in Golondrina (49.1 ±3.4 %) than in Fuenfría (66.7 ± 6.5 %). Within each population, we captured lizards in different places over large areas (10 km²) to minimize the likelihood that individuals had not had previous interactions, which may affect their responses (López and Martín, 2002).

Lizards were individually housed at "El Ventorrillo" Field Station, about 5 km from both capture sites, in outdoor 80 × 50 cm PVC terraria containing sand substratum, rock for cover and water *ad libitum*. They were fed mealworm larvae *Tenebrio molitor* every day and kept on natural photoperiod and ambient temperature. Lizards were held in captivity at least one week before testing to allow acclimation to laboratory conditions. Terraria of males and females were in different places to avoid contact between them before the experiments. All animals were healthy, did not show adverse behavioral or physiological changes during the tests, and were returned to their capture sites at the end of trials.

1.2 Population genetic structure

We extracted DNA from lizards' tail samples (20 from each population) preserved in ethanol using the DNeasy Tissue extraction kit (Qiagen). Microsatellite procedure and PCR methods were described in Runemark et al. (2008).

We analyzed the genetic structure of the two populations and the possible gene flow between these populations. Tests for significant genetic heterogeneity between samples were performed using exact tests of allele and genotype frequencies and via the departure of measures of genetic differentiation (F_{ST}) from zero, using the software program FSTAT (Goudet, 1995). Population genetic structure was calculated using GENEPOP software (Raymond and Rousset, 1995). We followed Wright (1978) to estimate the degree of genetic difference (F_{ST}) between populations. We assessed gene flow between populations from the estimates of the number of migrants per generation [$Nm=(1-F_{ST})/4F_{ST}$; where N is the total effective number of lizards and m is the migration rate] (Wright, 1978). We used the microsatellite data and the STRUCTURE 2.1 software (Pritchard et al., 2000) to show the degree of genetic population structure and to infer the number of genetically independent populations (or "clusters"; K) that we could find support for in our data set. We used the method of Evanno et al. (2005) to infer the true value of number of populations. The likelihood of data [Ln$P(D)$] stabilized within 20,000 iterations for all tested values of K, and thus a run length of 100,000 was deemed as sufficient.

1.3 Morphological characteristics of males

We measured males' body weight with a digital balance to the nearest 0.01 g, and the snout-to-vent length (SVL) with a ruler to the nearest 1 mm. We also made morphological measurements of the head of males using digital calipers (to the nearest 0.05 mm). Head length was the distance between the tip of the snout and the posterior side of the parietal scales. Head width was the greatest distance between the external sides of the parietal scales. Head depth was the greatest distance from the highest portion of the head to the bottom of the lower jaw.

We counted under a magnifying glass the number of femoral pores on the right and left hind limbs of males and calculated an average number for both sides. Finally, we noted the number of small but distinctive and conspicuous blue ocelli that runs along each of the body sides on the outer margin of the belly, and calculated an average number for both sides. The presence, and the experimental manipulation, of blue ocelli elicit aggressiveness during agonistic encounters between male lacertid lizards, suggesting that they are used in sex recognition and intrasexual social relationships (López et al., 2004).

1.4 Analysis of femoral gland secretions of males

The femoral gland secretions of males were extracted by gently pressing with forceps around the femoral pores immediately after capture. We collected secretion in glass vials with Teflon-lined stoppers. Vials were stored at -20 °C until analyses were performed. We used the same procedure without collecting secretion in order to have blank control vials. Before the analyses, we added 250 µl of n-hexane to each vial. Samples were analyzed in a Finnigan-ThermoQuest Trace 2000 gas chromatograph-mass spectrometer (GC-MS) equipped with a 30 m Supelco, Equity-5 column, and temperature programmed from 50–280 °C at 5 °C/min and kept at 280 °C for 30 min (see López and Martín, 2005b, Martín and López, 2006a for details of similar analyses). Identification of compounds was done by comparison of mass spectra in the NIST/EPA/NIH 1998 library, and later confirmed with authentic standards. The relative amount of each compound was determined as the percentage of the total ion current (TIC). The relative areas of the peaks were transformed following Aitchison's formula: $Zij=\ln(Yij/g(Yj))$, where Zij is the standardized peak area i for the individual j, Yij is the peak area i for individual j and $g(Yj)$ is the geometric mean of all peaks for individual j (Aitchison, 1986; Dietemann et al., 2003; López et al., 2006).

1.5 Female choice of males' scents

We placed in males' terraria several absorbent paper strips (35×10 cm) fixed to the floor, and left them there for three weeks to obtain the scents from males. Mate choice experiments were performed at the end of April, coinciding with the mating season of this lizard species. Females' terraria had two basking platforms (two identical flat tiles) placed symmetrically at each end of the terrarium, and rocks for cover in the center. At the beginning of experiments (09.00 h, GMT; when females where still inactive) we fixed, wearing fresh gloves, one paper strip from one male of one population on one tile, and a paper from a male of the other population on the other tile. Different papers from each male were used in four choice tests against the papers of other four males from the other population. Testing order and paper position were randomized. Each female was tested twice, once a day, with papers from two different pairs of males (own vs. other population). Each trial lasted 5 h (from 11.00 h GMT, shortly after females appeared from refuges and until 16.00 h GMT when females hid again). Females were monitored each 15 min from a raised point concealed by vegetation. If a female was located on a tile with paper strip, she was designated as having temporarily chosen that particular paper, whereas, if she located outside of the tiles, she was designated as having made no choice (Martín and López, 2000, 2006c; Olsson et al., 2003). At the end of the trials the papers were removed and the terrarium was thoroughly rinsed with clean water and let to dry for a whole day in the open.

We counted the number of times that each female was observed on each particular stimulus paper in each trial, and used a repeated measures three-way ANOVA to test for differences between the two days of the trial and between types of males (male from her own population vs. male from other population), both as within-subject factors. The population of origin of the female ('Fuenfría' vs. 'Golondrina') was included as a between-subject factor to test whether responses varied between populations (Sokal and Rohlf, 1995). Also, because a previous study suggested that females from 'Golondrina' prefer scents of males with relatively higher amounts of cholesta-5,7-dien-3-ol (López and Martín, 2005a), we performed a similar three-way ANOVA with the same data but classifying the two males within each pair according to the relative abundance of this lipid in their secretions (higher vs. lower) independent of the population of origin.

In addition, individual males were also classified according to the attractiveness of their scent; the paper on which a female spent greater than 50 % of her time (excluding time in the no choice area) was designated as the preferred paper in the trial (Martín and López, 2000). Each individual male was assigned an attractiveness index, calculated as the proportion of females that preferred a paper with his scent. Then, we used the morphological variables or the transformed areas of chemical compounds in femoral secretions of males (see above) as independent variables in forward stepwise general regression models (GRM) with male attractiveness index as the dependent variable.

1.6 Mating behavior

We staged encounters between male and female lizards from the same or from different populations to study whether successful copulations occurred and whether copulatory behavior different depending on the population of origin of the male and the female. Each male encountered two females, once per day over two days. Half of males were presented first with a female from their own population and the day after with a female from the other population, and the converse for the other half of males. Each female was used only once with a single male. The individual males and females used in each encounter were chosen at random. In each trial, we gently took a female from her terrarium and placed her in a male's terrarium. From a blind we observed whether or not a copulation occurred, and recorded the duration of the copulation (i.e., since the first cloacal contact with hemipenis intrusion until the moment that cloacae of individuals were physically separated). The female was removed from the male's terrarium immediately after the copulation finished. If copulation did not occur, trials were terminated after 30 min.

We used General Linear Mixed Models (GLMM) in SAS (SAS, 1989–1996 Institute Inc., Cary, NC, USA) to test the dependent variable with normal distribution (i.e., duration of copulation; Kolmogorov-Smirnoff's test: $P > 0.10$) or Generalized Linear Mixed Models (GLIMMIX) when the dependent variable was binomial (i.e., whether or not a copulation occurred). In this analysis, each male was used twice so we used male identity as a random factor. We also included the population of the male and the female and the order of presentation as categorical predictors.

2 Results

2.1 Population genetic structure

The comparison of the genetic structures of the two *P. hispanica* populations showed that there was low genetic divergence between the Fuenfría and Golondrina populations (F_{ST} = 0.0007). In line with this result, the estimation of the number of migrants per generation between the two populations was high (*Nm* = 6.12).

The analyses of the microsatellite data demonstrated that the clusters defined by the STRUCTURE software corresponded to one sampling locality or one group of sampling localities of the same subpopulation, as shown by the log-likelihood-values reaching a plateau at *K* = 1. Therefore, the hypothesis of one subpopulation (Golondrina and Fuenfría together) was more probable than that of two subpopulations.

2.2 Morphology of male lizards

Males from the two populations differed significantly with respect to morphological characteristics (weight, SVL, head size, number of femoral pores and number of lateral blue ocelli, all log-transformed) (MANOVA: Wilk's λ = 0.16, $F_{7, 12}$ = 8.77, P = 0.0006; Table 1). Males from Fuenfría were significantly larger and heavier, and had significantly larger heads than males from Golondrina, even controlling for body length (ANCOVAs, head length: $F_{1, 17}$ = 20.78, P = 0.0003; head width: $F_{1, 17}$ = 8.25, P = 0.01; head depth: $F_{1, 17}$ = 16.51, P = 0.0008). However, males of the two populations did not differ significantly in the number of femoral pores or blue ocelli (Table 1).

2.3 Chemicals in femoral gland secretions of males

The lipophilic fraction of femoral secretions of male *P. hispanica* consisted of several fatty acids, alcohols, waxy esters, squalene and steroids (Table 1). The most

Table 1 Morphological measurements (mean ± *SE*) and lipophilic chemical compounds (mean±*SE* of the percentage of the total ion current, TIC, area from the chromatogram) found in femoral gland secretions of male *Podarcis hispanica* from two populations (Fuenfría and Golondrina)

	Fuenfría	Golondrina	$F_{1,18}$	P
Morphology				
Weight (g)	5.1 ± 0.2	4.1 ± 0.2	16.55	**0.0007**
SVL (mm)	63 ± 1	57 ± 1	13.36	**0.0018**
Head length (mm)	14.90 ± 0.18	13.18 ± 0.15	51.69	**<0.0001**
Head width (mm)	9.29 ± 0.25	8.12 ± 0.12	17.25	**0.0006**
Head depth (mm)	6.28 ± 0.10	5.66 ± 0.06	24.91	**<0.0001**
Femoral pores	18.4 ± 0.4	18.7 ± 0.5	0.09	0.76
Blue ocelli	3 ± 1	4 ± 1	0.01	0.99
Compounds in femoral secretions				
Fatty acids (C_{12}-C_{15})	1.58 ± 0.22	2.91 ± 0.41	12.23	**0.0026**
Fatty acids (C_{16}-C_{18})	7.44 ± 1.87	8.50 ± 0.97	5.43	**0.03**
Fatty acids (C_{20}-C_{24})	1.49 ± 0.38	0.73 ± 0.09	3.70	0.07
Alcohols	2.25 ± 0.36	0.87 ± 0.05	14.60	**0.0012**
Waxy esters	2.01 ± 0.67	0.62 ± 0.28	3.62	0.07
Squalene	0.45 ± 0.12	0.42 ± 0.04	3.02	0.10
Cholesterol	52.14 ± 2.24	63.88 ± 1.22	6.55	**0.02**
Cholesta-5,7-dien-3-ol	19.34 ± 0.97	13.12 ± 1.14	0.66	0.43
Ergosta-5,8-dien-3-ol	3.37 ± 0.14	2.43 ± 0.08	0.09	0.77
4,4-Dimethyl-cholesta-5,7-dien-3-ol	2.04 ± 0.17	1.01 ± 0.20	3.19	0.09
Campesterol	0.33 ± 0.07	0.48 ± 0.06	4.53	**0.04**
Cholesta-4,6-dien-3-one	0.83 ± 0.15	0.16 ± 0.05	12.18	**0.003**
Cholestanol	0.46 ± 0.31	0.61 ± 0.25	1.54	0.23
Other minor steroids	6.21 ± 0.64	4.24 ± 0.38	0.01	0.93

Results (*F*, *P*) from protected one-way ANOVAs on transformed data (see methods) are shown. The bold values indicate that *P* is significant at the 0.05 level.

abundant compounds were cholesterol and cholesta-5,7-dien-3-ol. The other compounds were found in minor quantities. There were significant overall differences between populations in the relative proportion of compounds in femoral secretions of males (MANOVA: Wilks' $\lambda = 0.046$, $F_{14, 5} = 28.54$, $P < 0.001$; Table 1). Univariate protected ANOVAs showed that males from Fuenfría had significantly lower proportions of low molecular weight (C_{12}-C_{18}) fatty acids, cholesterol and campesterol, and significantly greater proportions of alcohols and cholesta-4,6-dien-3-one than males from Golondrina (Table 1). Males from Fuenfría also tended, although not significantly, to have greater proportions of fatty acids of high molecular weight (C_{20}-C_{24}) and of waxy esters than males from Golondrina (Table 1). For the other compounds there were no significant differences between populations.

2.4 Choice of males' scent by females

There were no significant differences between time spent by females (log-transformed number of observations) on paper strips scent-marked by a male of their own population or by a male from the other population (repeated measures three-way ANOVA: $F_{1, 38} = 0.08$, $P = 0.77$). The population of origin of the female had no significant effect on these responses ($F_{1, 38} = 0.08$, $P = 0.77$), and the interaction between population of male and female population was not significant ($F_{1, 38} = 0.07$, $P = 0.79$) (Fig. 1a). However, the overall number of observations of females on any of the papers varied significantly between the two days of the trial ($F_{1, 38} = 5.09$, $P = 0.03$). The interaction between day and female population was significant ($F_{1, 38} = 33.91$, $P < 0.0001$), but this variation between days did not affect the female choice of paper strips (male population x day: $F_{1, 38} = 0.01$, $P = 0.94$; three way interaction: $F_{1, 38} = 1.64$, $P = 0.21$).

There was no significant relationship between none of the male morphological variables (weigh, size, condition, head size, etc) and the attractiveness indexes of their scent (i.e., no variable entered the GRM model with significance). A similar lack of relationship was found when we analyzed each population separately. However, when we analyzed the possible relationships between male attractiveness and the major chemical compounds in femoral secretions, we found a significant positive relationship between the attractiveness index of a male and the relative proportions of cholesta-5,7-dien-3-ol in male secretions (GRM: $R^2 = 0.45$, $F_{1, 17} = 13.75$, $P = 0.0017$; Fig. 2). The relationship was similar when we calculated the attractiveness indexes of males from the responses of females of each of the two populations separately (GRM, Fuenfría females: $R^2 = 0.27$, $F_{1, 16} = 5.92$, $P = 0.027$; Golondrina females: $R^2 = 0.33$, $F_{1, 17} = 8.23$, $P = 0.011$). In addition, attractiveness residuals were significantly higher for males from Fuenfría (GLM, $F_{1, 17} = 4.67$, $P = 0.045$), suggesting that males from the Fuenfría population were more successful at transform-

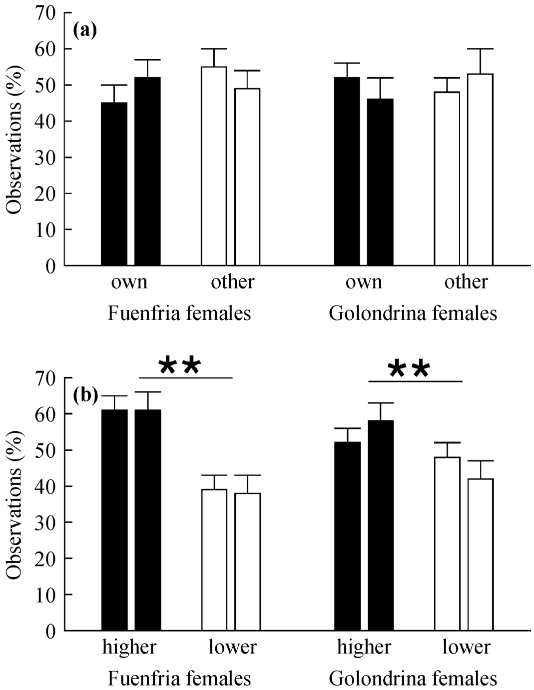

Fig. 1 Proportion of times (mean+*SE*), during each of two scent choice trials, that female *P. hispanica* from two populations (Fuenfría or Golondrina) were observed on paper strips scent marked by (A) males from their own or from the other population, or (B) by the male within each pair with relatively higher or lower proportions of cholesta-5,7-dien-3-ol in its femoral secretions, independent of the population of origin (= significant difference *P* < 0.005)**

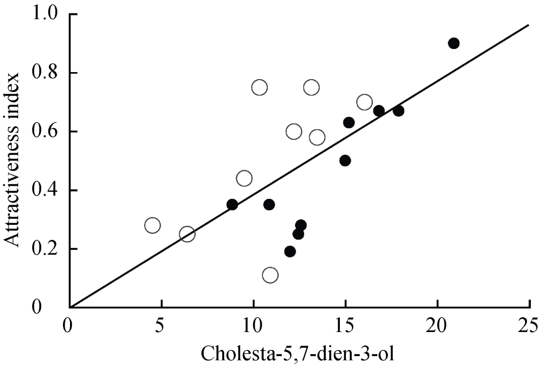

Fig. 2 Relationship between relative proportions of cholesta-5,7-dien-3-ol in femoral gland secretions and the attractiveness index scores of male lizards *P. hispanica* from two populations, Fuenfría (o) or Golondrina (●)

ing signaling intensity to attractiveness.

Moreover, in the trials of choice of males' scent by females, when we classified the two males within each pair according to the relative abundance of cholesta-5,7-dien-3-ol in their secretions (higher vs. lower) independent of the population of origin of the male, females spent significantly more time on paper strips scent-marked by the male, within each pair, with the higher proportion of cholesta-5,7-dien-3-ol (repeated measures three-way ANOVA: $F_{1,38} = 14.58$, $P = 0.0005$; Fig. 1b). This effect was similar independent of the population of origin of the female ($F_{1,38} = 0.09$, $P = 0.77$), and there were no significant differences between days ($F_{1,38} = 2.17$, $P = 0.15$), although the interaction between female population and day of the trial was significant ($F_{1,38} = 35.40$, $P < 0.0001$). All other interactions were not significant ($P > 0.16$ in all cases).

2.5 Mating behavior

The overall proportion of successful copulations in this experiment was 0.58 (29 copulations from 50 staged encounters) (Fig. 3). The probability of occurrence of a copulation was not significantly dependent of the population of the male (GLIMMIX: $F_{1,19} = 1.25$, $P = 0.27$) nor of the population of the female ($F_{1,19} = 0.21$, $P = 0.65$), and the interaction was not significant ($F_{1,19} = 0.09$, $P = 0.76$). The probability of copulation was significantly higher in the first trial than in the second one (order effect: $F_{1,19} = 9.06$, $P = 0.007$), but this effect was independent of the population of the male or of the female ($P > 0.20$ for all interactions of order with all other effects) (Fig. 3).

The duration of successful copulations did not significantly differ depending of the population of the male (GLMM, $F_{1,26} = 3.60$, $P = 0.06$) or of the female ($F_{1,26} = 1.84$, $P = 0.18$) and the interaction between male and female populations was not significant ($F_{1,26} = 2.71$, $P = 0.11$). Moreover, the duration of copulation was not significantly affected by the order of female presentation ($F_{1,26} = 0.39$, $P = 0.54$). All other interactions were not significant ($P > 0.40$ in all cases).

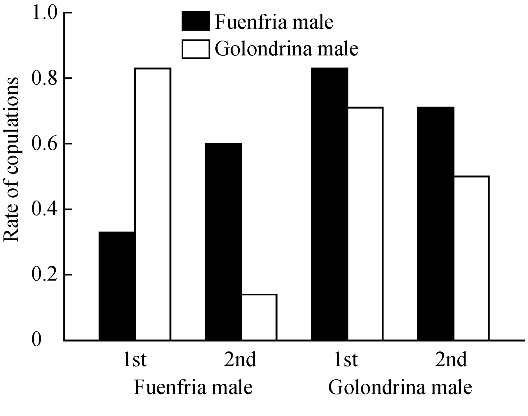

Fig. 3 Rate of successful copulations between male lizards *P. hispanica* from two populations (Fuenfría or Golondrina) with females from their own or from the other population, in two successive copulation trials of the same male with different individual females

Males that had higher mating success did not significantly differ in proportions of cholesta-5,7-dien-3-ol in their secretions with respect to males with intermediate or lower mating success (GLM, overall: $F_{2,24} = 1.05$, $P = 0.36$; with females from the same population: $F_{1,25} = 0.28$, $P = 0.60$; with females from the other population: $F_{1,25} = 0.35$, $P = 0.56$). The duration of copulation was not associated with the proportion of cholesta-5,7-dien-3-ol (GLM, overall: $F_{1,22} = 0.06$, $P = 0.81$).

3 Discussion

Our results showed that female Iberian wall lizards base their mate choice decisions on some characteristics of males' pheromones that vary between individuals, but not between populations, despite these two populations differing in other aspects of the chemical signals and in morphology. However, in spite of these clear and distinct inter-populational differences, genetic differences between populations are small, suggesting that they belong to the same population. Therefore, this mate choice criterion may inhibit rather than promote reproductive isolation and genetic divergence between these phenotypically distinct populations.

The clear differences between populations in morphology and chemical secretions of males might have arisen because natural selection may have favored individuals with characters better adapted to the different environments and climatic conditions in each population. Differences in body size may be simply due to different growth rates or other life history parameters promoted by altitude-related differences in thermal opportunities, food availability, predation risk, etc (e.g., Sears, 2005; Iraeta et al., 2006). Similarly, differences in proportion of chemicals in femoral secretions might be related to different microclimatic conditions, reflecting selection for the persistency and efficiency of chemical signals in different environments (Alberts, 1992; Endler, 1992; Bradbury and Vehrencamp, 1998; Endler and Basolo, 1998) less volatile and more stable compounds (e.g. fatty acids of high molecular weight, cholesterol), may be favored in lizards inhabiting more humid climatic conditions (Alberts, 1992; Martín and López, 2006a; Gabirot et al., 2012).

The question that arises is whether these differences, which could reflect local selection for the efficacy component of the sexual signals, are so great as to affect differences in preference and promote reproductive isolation (Boughman, 2002). In fact, differences in chemical compounds seem relevant for population recognition based on previous experiments that showed that male *P. hispanica* of these two populations can discriminate between scents from males from their own or from other population (Martín and López, 2006a, b), which can have consequences for rival recognition in intrasexual contests (López and Martín, 2002; Carazo et al., 2007). In contrast to males, females do not seem to discriminate between male populations based on chemical cues, although females clearly detect scent of males from a baseline odor (Martin and Lopez, 2006a,b), and discriminate them from heterospecific lizards (Cooper and Pérez-Mellado, 2002; Gabirot et al., 2010a). Moreover, the results of the current experiment further showed that females did not prefer, nor reject, areas scent marked by males based on the criterion of the population of origin of the male. Furthermore, the results of staged mating encounters showed that males and females were equally likely to copulate, and copulation duration was similar, independent of their population of origin, although also independent of male chemical attractiveness. Therefore, our results indicate that there is a lack of pre-mating and mating reproductive isolation between these two populations, in spite of marked differences in male morphology and chemical sexual signals. Although it remains to be analyzed whether interpopulation mating results in the same reproductive success and whether offspring from interpopulational and intrapopulational pairs have similar fitness, our data suggest that reproductive isolation between these two populations is absent.

This lack of reproductive isolation might be firstly explained by an incapacity of female *P. hispanica* to discriminate between populations of males, or perhaps simply by a lack of female mate choice. Females might select to establish in areas scent marked by any male, and accept mating with any male with enough genetic relatedness. This would explain hybridization between populations which are not clearly distinct species within *Podarcis,* as evidenced by laboratory crosses (e.g., *P. bocagei* × *P. carbonelli*, Galán, 2002) and genetic analyses of natural populations (Capula, 2002; Pinho et al., 2007). However, our study showed that female *P. hispanica* did select scent of some specific individual males using as a criterion the proportion of chemicals in femoral gland secretions, independent of the population of origin of the male. Females preferred males with relatively higher amounts of cholesta-5,7-dien-3-ol (=provitamin D_3) in femoral secretions. Previous studies showed that female *P. hispanica* can actually detect changes in concentration of this lipid (Martín and López, 2006d). This mate choice criterion may be explained by the positive relationship between the amount of cholesta-5,7-dien-3-ol in secretions and the robustness of the immune response of a male observed in this species (López and Martín, 2005a; López et al., 2008) and in other lacertid lizard species (Martín and López, 2006c). Cholesta-5,7-dien-3-ol is a precursor for vitamin D_3 and, in humans and other mammals, there is considerable scientific evidence that the active form of vitamin D is a potent immune system modulator with a variety of effects on immune system function that may enhance immunity (Griffin et al., 2003; Hayes et al*.,* 2003). In this and other lacertid lizard species, experimental dietary supplementations and challenge of the immune system suggest that there may be a trade-off between physiological regulation of the immune system and the allocation of essential nutrients (provitamins) to sexual ornaments (Martin and Lopez, 2006e; Martín et al., 2007b; López et al., 2008), which may allow males to honestly signal health state via chemical cues.

Interestingly, average amounts of cholesta-5,7-dien-3-ol in males' secretions did not vary between our study populations, although interindividual variability was high in both populations. Therefore, despite females using this chemical signal to select areas scent marked by particular males, the population of origin would not affect to the mate selection, which would rather depend on the individual characteristics of each male. This mate choice based on specific characteristics of chemical signals of males that not differ between populations would lead to a lack of reproductive isolation. In fact, our results confirmed that successful copulation with similar characteristics can occur between males and females of both populations. Such conflicting preferences within females between sexual selection and species recognition may be widespread. For example, within individual female pygmy swordtails *Xiphophorus pygmaeus*, directional preferences for conspicuous ornamentation are inversely associated with discrimination against the sympatric heterospecific, *X. cortezi* (Rosenthal and Ryan, 2011). Similarly, there is a trade-off between avoiding heterospecific matings and choosing attractive males in spadefoot toads (Pfennig, 2000).

In summary, we have found that despite clear differences in morphology and chemical signals between two populations of *P. hispanica* lizards, genetic differences were small. This could be a consequence of gene flow between populations unimpeded by interpopulational discrimination based on chemical recognition and pre-mating reproductive isolation. While a previous study showed that males can discriminate between these populations by chemical cues (Martín and López, 2006a, b), females did not seem to discriminate between male chemical cues, or, alternatively, did not show preferences for the scent of males from their own population, and the probability of a successful mating did not depend on the population of origin. However, females seemed to base their mate choice on pheromone traits shared by males of both populations. These results support that, even in phenotypically distinct populations, sexual selection via mate choice may retard rather than promote population divergence.

Acknowledgments We thank G. Rosenthal and two anonymous reviewers for helpful comments and "El Ventorrillo" MNCN Field Station for use of their facilities. Financial support was provided by the project MICIIN-CGL2011-24150/BOS, and by an “El Ventorrillo” CSIC grant to MG. Capture of lizards and experiments were performed under license from the “Comunidad de Madrid” Environmental Agency.

References

Aitchison J, 1986. The Statistical Analysis of Compositional Data: Monographs in Statistics and Applied Probability. London: Chapman and Hall.

Alberts AC, 1992. Constraints on the design of chemical communication systems in terrestrial vertebrates. American Naturalist 139: 62–89.

Alberts AC, 1993. Chemical and behavioral studies of femoral gland secretions in iguanid lizards. Brain Behavior and Evolution 41: 255–260.

Andersson M, 1994. Sexual selection. Princeton, New Jersey: Princeton University Press.

Aragón P, López P, Martín J, 2001. Discrimination of femoral gland secretions from familiar and unfamiliar conspecifics by male Iberian rock-lizards *Lacerta monticola*. Journal of Herpetology 35: 346–350.

Barbosa D, Desfilis E, Carretero MA, Font E, 2005. Chemical stimuli mediate species recognition in *Podarcis* wall lizards. Amphibia-Reptilia 26: 257–263.

Barbosa D, Font E, Desfilis E, Carretero MA, 2006. Chemically mediated species recognition in closely related *Podarcis* wall lizards. Journal of Chemical Ecology 32: 1587–1598.

Boughman JW, 2002. How sensory drive can promote speciation. Trends in Ecology and Evolution 17: 571–577.

Bradbury JW, Vehrencamp SV, 1998. Principles of Animal Communication. Sunderland, Massachusetts: Sinauer Associates

Brennan PA, Kendrick KM, 2006. Mammalian social odours: Attraction and individual recognition. Philosophical Transactions of the Royal Society of London, Series B 361: 2061–2078.

Capula M, 2002. Genetic evidence of natural hybridization between *Podarcis sicula* and *Podarcis tiliguerta* (Reptilia: Lacertidae). Amphibia-Reptilia 23: 313–321.

Carazo P, Font E, Desfilis E, 2007. Chemosensory assessment of rival competitive ability and scent mark function in a lizard *Podarcis hispanica*. Animal Behaviour 74: 895–902.

Carretero MA, 2008. An integrated assessment of the specific status in a group with complex systematics: The Iberomaghrebian lizard genus *Podarcis* (Squamata, Lacertidae). Integrative Zoology 4: 247–266.

Cooper WE, Pérez-Mellado V, 2002. Pheromonal discrimination of sex, reproductive condition, and species by the lacertid lizard *Podarcis hispanica*. Journal of Experimental Zoology A 292: 523–527.

Dietemann V, Peeters C, Liebig J, Thivet V, Hölldobler B, 2003. Cuticular hydrocarbons mediate discrimination of reproductives and nonreproductives in the ant *Myrmecia gulosa*. Proceedings of the National Academy of Sciences of USA 100: 10341–10346.

Endler JA, 1992. Signals, signal conditions, and the direction of evolution. American Naturalist 139: S125–S153.

Endler JA, Basolo AL, 1998. Sensory ecology, receiver biases and sexual selection. Trends in Ecology and Evolution 13: 416–420.

Evanno G, Regnaut S, Goudet J, 2005. Detecting the number of clusters of individuals using the software STRUCTURE: A simulation study. Molecular Ecology 14: 2611–2620.

Gabirot M, Castilla AM, López P, Martín J, 2010a. Chemosensory species recognition may reduce the frequency of hybridization between native and introduced lizards. Canadian Journal of Zoology 88: 3–80.

Gabirot M, Castilla AM, López P, Martín J, 2010b. Differences in chemical signals may explain species recognition between an island lizard *Podarcis atrata* and related mainland lizards *P. hispanica*. Biochemical Systematic and Ecology 38: 521–528.

Gabirot M, López P, Martín J, 2012. Interpopulational variation in chemosensory responses to selected steroids from femoral secretions of male lizards *Podarcis hispanica* mirrors population differences in chemical signals. Chemoecology 22: 65–73.

Galán P, 2002. Hibridación en laboratorio de *Podarcis bocagei* y *Podarcis carbonelli*. Boletín de la *Asociación* Herpetológica Española 13: 28–31.

Gerhardt, HC. 1982. Sound pattern recognition in some North American treefrogs (Anura: Hylidae): Implication for mate choice. Am. Zool. 22: 581–595.

Gómez A, Font E, Desfilis E, 1993. Chemoreception in the Lacertidae: Exploration and conspecific discrimination in the Spanish wall lizard *Podarcis hispanica*. In: Valakos ED, Böhme W, Pérez-Mellado V, Maragoú P ed. Lacertids of the Mediterranean Region. Athens, Greece: Helenic Zoological Society, 213–230.

Goudet J, 2001. FSTAT, A program to estimate and test gene diversities and fixation indices. Version 2.9.3. Available from http://www.unil.ch/popgen/softwares/fstat.htm (accessed september 2011).

Griffin MD, Xing N, Kumar R, 2003. Vitamin D and its analogs as regulators of immune activation and antigen presentation. *Annual Review of Nutrition* 23: 117–145.

Guillaume CP, 1987. Les petits Lacertidés du Bassin Mediterranéen Occidental (Genera *Podarcis* et *Archeolacerta* essentiellement). PhD Thesis. Montpellier, France: University of Science and Techniques of Languedoc.

Harris DJ, Sá-Sousa P, 2001. Species distinction and relationships of the western Iberian *Podarcis* lizards (Reptilia, Lacertidae) based on morphology and mitochondrial DNA sequences. Herpetological Journal 11: 129–136.

Harris DJ, Sá-Sousa P, 2002. Molecular phylogenetics of Iberian wall lizards (*Podarcis*): Is *Podarcis hispanica* a species complex? Molecoluar Phylogeny and Evolution 23: 75–81.

Hayes CE, Nashold FE, Spach KM, Pedersen LB, 2003. The immunological functions of the vitamin D endocrine system. Cellular Molecular Biology. 49: 277–300.

Iraeta P, Monasterio C, Salvador A, Díaz JA, 2006. Mediterranean hatchling lizards grow faster at higher altitude: A reciprocal transplant experiment. Functional Ecology 20: 865–872.

Johansson BG, Jones TM, 2007. The role of chemical communication in mate choice. Biological Reviews 82: 265–289.

Kaliontzopoulou A, Pinho C, Harris DJ, Carretero MA, 2011. When cryptic diversity blurs the picture: A cautionary tale from Iberian and North African *Podarcis* wall lizards. Biological Journal of the Linnaean Society 103: 779–800.

López P, Amo L, Martín J, 2006. Reliable signaling by chemical cues of male traits and health state in male lizards *Lacerta monticola*. Journal of Chemical Ecology 32: 473–488.

López P, Gabirot M, Martín J, 2008. Immune activation affects chemical sexual ornaments of male Iberian wall lizards. Naturwissenschaften 96: 65–69.

López P, Martín J, 2001. Pheromonal recognition of females takes precedence over the chromatic cue in male Iberian wall lizards *Podarcis hispanica*. Ethology 107: 901–912.

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

López P, Martín J, 2005a. Female Iberian wall lizards prefer male scents that signal a better cell-mediated immune response. Biology Letters 1: 404–406.

López P, Martín J, 2005b. Chemical compounds from femoral gland secretions of male Iberian rock lizards *Lacerta monticola cyreni*. Zeitschrift für Naturforschung C 60: 632–636.

López P, Martín J, Cuadrado M, 2002. Pheromone mediated intrasexual aggression in male lizards *Podarcis hispanicus*. Aggressive Behavior 28: 154–163.

López P, Martín J, Cuadrado M, 2004. The role of lateral blue spots in intrasexual relationships between male Iberian rock-lizards *Lacerta monticola*. Ethology 110: 543–561.

Martín J, López P, 2000. Chemoreception, symmetry and mate choice in lizards. Proceedings of the Royal Society of London, Series B, Biological Sciences 267: 1265–1269.

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

Martín J, López P, 2006b. Pre-mating mechanisms favoring or precluding speciation in a species complex: Chemical recognition and sexual selection between types in the lizard *Podarcis hispanica*. Evolutionary Ecology Research 8: 643–658.

Martín J, López P, 2006c. Links between male quality, male chemical signals, and female mate choice in Iberian rock lizards. Functional Ecology. 20: 1087–1096.

Martín J, López P, 2006d. Chemosensory responses by female Iberian wall lizards *Podarcis hispanica* to selected lipids found in femoral gland secretions of males. Journal of Herpetology 60: 556–561.

Martín J, López P, 2006e. Vitamin D supplementation increases the attractiveness of males' scent for female Iberian rock lizards. Proceedings of the Royal Society of London, Series B, Biological Sciences 273: 2619–2624.

Martín J, López P, 2007. Scent may signal fighting ability in male Iberian rock lizards. Biology Letters 3: 125–127.

Martín J, López P, 2011. Pheromones and reproduction in Reptiles. In: Norris DO, Lopez KH ed. Hormones and Reproduction of Vertebrates, Vol 3. Reptiles. San Diego, California: Academic Press, 141–167.

Martín J, López P, 2012. Supplementation of male pheromone on rock substrates attracts female rock lizards to the territories of males: A field experiment. PLoS ONE 7: e30108.

Martín J, López P, Gabirot M, Pilz KM, 2007b. Effects of testosterone supplementation on chemical signals of male Iberian wall lizards: Consequences for female mate choice. Behavioral Ecology and Sociobiology 61: 1275–1285.

Martín J, Moreira PL, López P, 2007a. Status-signaling chemical badges in male Iberian rock lizards. Functional Ecology 21: 568–576.

Mason RT, 1992. Reptilian pheromones. In: Gans C, Crews D ed. Biology of the Reptilia. Vol 18. Chicago: University of Chicago Press, 114–228.

Mason RT, Parker MR, 2010. Social behavior and pheromonal communication in reptiles. Journal of Comparative Physiology A 196: 729–749.

Møller, AP. 1990. Effects of a haematophagous mite on the barn swallow *Hirundo rustica*: A test of the Hamilton and Zuk hypothesis. Evolution 44: 771–784.

Olsson M, Madsen T, Nordby J, Wapstra E, Ujvari B, 2003. Major histocompatibility complex and mate choice in sand lizards. Proceedings of the Royal Society of London, Series B, Supplement 270: 254–S256.

Petrie M, 1994. Improved growth and survival of offspring of peacocks with more elaborate trains. Nature 371: 598–599.

Pfennig KS, 1998. The evolution of mate choice and the potential for conflict between species and mate-quality recognition. Proceedings of the Royal Society of London, Series B 265:1743–1748.

Pfennig KS, 2000. Female spadefoot toads compromise on mate quality to ensure conspecific matings. Behavioral Ecology 11: 220–227.

Pinho C, Harris DJ, Ferrand N, 2007. Comparing patterns of nuclear and mitochondrial divergence in a cryptic species complex: The case of Iberian and North African wall lizards (*Podarcis*, Lacertidae). Biological Journal of the Linnaean Society 91: 121–133.

Pinho C, Harris DJ, Ferrand N, 2008. Non-equilibrium estimates of gene flow inferred from nuclear genealogies suggest that Iberian and North African wall lizards (*Podarcis* spp.) are an assemblage of incipient species. BMC Evolutionary Biology 8: 63.

Pritchard JK, Stephens M, Donnelly P, 2000. Inference of population structure using multilocus genotype data. Genetics 155: 945–959.

Raymond M, Rousset F, 1995. GENEPOP (version 1.2): Population genetics software for exact tests and ecumenism. Journal of Heredity 86: 248.

Reynolds JD, Gross, MR, 1992. Female mate preference engances off-spring grouth and reproduction in a fish *Poelia reticulate*. Proceedings of the Royal Society of London, Series B 250:57–62.

Rosenthal GG, Ryan MJ, 2011. Conflicting preferences within females: Sexual selection versus species recognition. Biology Letters 7: 525–527.

Runemark A, Gabirot M, Bensch S, Svensson EI, Martin J et al., 2008. Cross-species testing of 27 pre-existing microsatellites in *Podarcis gaigeae* and *Podarcis hispanica* (Squamata: Lacertidae). Molecular Ecology Research 8: 1367–1370.

Ryan MJ, Keddy-Hector A, 1992. Directional patterns of female mate choice and the role of sensory biases. American Natualist 139: S4–S35.

Ryan MJ, Rand AS, 1993. Species recognition and sexual selection as a unitary problem in animal communication. Evolution 47:647–657.

Sá-Sousa P, Vicente L, Crespo EG, 2002. Morphological variability of *Podarcis hispanica* (Sauria: Lacertidae) in Portugal. Amphibia-Reptilia 23: 55–69.

Sears MW, 2005. Geographic variation in the life history of the sagebrush lizard: The role of thermal constraints on activity. Oecologia 143: 25–36.

Sherman PW, Reeve HK, Pfennig DW, 1997. Recognition systems. In: Kebs JR, Davies NB ed. Behavioural Ecology: An Evolutionary Approach. 4th edn. London: Blackwell, 69–96.

Sokal RR, Rohlf FJ, 1995. Biometry. New York: Freeman.

Wright S, 1978. Evolution and Genetics of Regulation. Vol. 4. Variability within and among Populations. Chicago: University of Chicago Press.

Wyatt TD, 2003. Pheromones and Animal Behaviour. Cambridge: Cambridge University Press.