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Differences in chemical signals may explain species recognition between an island lizard, *Podarcis atrata*, and related mainland lizards, *P. hispanica*

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ABSTRACT

Chemical signals can be the basis of interspecific recognition and speciation in many animals. The Columbretes Islands wall lizard, *Podarcis atrata* is very close genetically to the mainland Iberian wall lizard *Podarcis hispanica*. However, a previous study suggested that chemosensory interspecific recognition would avoid reproductive interactions and hybridization between these two species. These results suggested that chemicals used in intraspecific communication might differ in composition and/or proportions between these two species. In this paper, we used gas chromatography–mass spectrometry (GC–MS) to characterize the chemical composition of the lipophilic fraction from femoral gland secretions of male *P. atrata* and *P. hispanica*. The analysis showed that chemicals found in femoral secretions varied in composition and proportions between species and between populations. Seven steroids and two unidentified waxy esters, were exclusive of *P. atrata* lizards from the islands. In contrast, nine steroids and other six compounds were only found in mainland *P. hispanica*. There were also differences in proportions of shared compounds between species. Moreover, all these differences were higher between *P. atrata* and *P. hispanica* than between any population of *P. hispanica*. Chemical differences might be consequence of genetic differences, but they could also be explained by adaptation to different habitats with different climatic conditions or diet resources. Compounds that are specific of each species, or differences in the pattern of compounds, could explain species recognition. Therefore, these results of chemical composition and previous studies of chemosensory recognition reinforce the fact that the genetic differences between *P. hispanica* and *P. atrata* may result in an effective reproductive isolation between these two taxa.

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1. Introduction

Signals favored by sexual selection may play an important role in species recognition, reproductive isolation and speciation (Andersson, 1994; Boughman, 2001; Panhuis et al., 2001). Species recognition mechanisms based on behavior, visual, olfactory, auditory and tactile cues may prevent interspecific mating between sympatric related taxa (Cooper and Vitt, 1986; Shine et al., 2002). Lizards and snakes have a well-developed chemosensory system (Mason, 1992) and chemical stimulus can be the basis of interspecific recognition and speciation in reptiles and many other animals (reviewed in Smadja and Butlin, 2009), such as in sympatric sea snakes species (Shine et al., 2002), or in different populations of the same species of red-

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garter snakes (LeMaster and Mason, 2003) and in closely related species or populations of lizards (Cooper and Vitt, 1986; Barbosa et al., 2005, 2006; Martín and López, 2006a,b).

Many lizards have epidermal structures on the ventral surface of the thigh (femoral pores) connected to holocrine femoral glands that secrete chemical cues. These secretions are especially abundant in males during the reproductive season (Mason, 1992; Alberts, 1993), and chemicals secreted are thought to be important compounds involved in communication and sexual selection (Mason, 1992; Martín and López, 2006c). Femoral gland secretions of males could advertise residence in a home range and/or inform other males on a male's status and competitive ability (Aragón et al., 2001; Aragon et al., 2006; Carazo et al., 2007; Martín et al., 2007). They may also transmit information about a male's quality, which females may use to select potential mates (Olsson et al., 2003; Martín and López, 2006c). Finally, femoral secretions can also be used in the context of interspecific recognition (Cooper and Vitt, 1986; Cooper and Pérez-Mellado, 2002; Gabirot et al., 2010), and potentially lead to reproductive isolation and speciation processes. For example, in *Podarcis* lizards, chemosensory recognition is well-developed. These lizards can discriminate between conspecifics and more genetically distant heterospecifics (*Podarcis hispanica* vs. *Podarcis bocagei* or *Podarcis carbonelli* vs. *P. bocagei*), and between sexes by chemical cues alone (López and Martín, 2001; Cooper and Pérez-Mellado, 2002; López et al., 2002; Barbosa et al., 2005, 2006).

Podarcis lizards are highly variable in size, shape, escalation and color pattern, not only between currently recognized species (Arnold and Ovenden, 2002), but also between populations and individuals. Recently molecular and morphological studies suggest that the Iberian wall lizard, *P. hispanica*, is paraphyletic, and forms a "species complex" with at least five monophyletic lineages. This suggests the existence of ongoing speciation processes within taxa previously considered to be conspecific (Harris and Sá-Sousa, 2001, 2002; Pinho et al., 2007). The genetic divergence's analyses in this "species complex" help to distinguish the island lizard *Podarcis atrata* from the mainland *P. hispanica* (Castilla et al., 1998b).

The Columbretes wall lizard, *P. atrata*, is an endangered and endemic lacertid lizard from the Columbretes archipelago (Mediterranean, Spain) (Castilla et al., 1998a,b). This is the European lizard with the smallest (19 Ha) distribution area, and has a small population size (total of ca.12000 individuals) (Castilla and Bauwens, 1991). In contrast, in the Iberian Peninsula, the Iberian wall lizard, *P. hispanica,* is common and widely distributed (Barbadillo et al., 1999). *P. atrata* is genetically distinct (Castilla et al., 1998b), but much more closely related to some populations of *P. hispanica* than previously thought (Harris and Sá-Sousa, 2002) and the risk of possible hybridization of the endemic island *P. atrata* with introduced mainland *P. hispanica* has been suggested (Castilla et al., 1998a; Gabirot et al., 2010).

These two lizard species that are genetically very closed could, however, diverge in chemical signals or in communication, thus allowing species recognition and avoiding hybridization. In a previous experiment, differential tongue-flick rates of lizards in response to different scents indicated that *P. hispanica* and *P. atrata* can discriminate between species based on chemical cues alone (Gabirot et al., 2010). Lizards of both species and sexes have more "interest" for exploring the scent of conspecific lizards than the scent from lizards of the other species (Gabirot et al., 2010). This indicates the occurrence of a clear interspecific chemosensory discrimination and suggests that chemicals of *P. hispanica* and *P. atrata* used in intraspecific communication might be different in composition and/or proportions. Other studies have shown that chemical secretions of lizards of different taxa differ in composition and proportion of compounds, even when the species are sympatric and closely related, which would allow species recognition (Martín and López, 2006a). In this paper, we aimed to explore whether there were differences between femoral secretions of male *P. atrata* from Columbretes islands and male *P. hispanica* from different mainland populations. For this, we used gas chromatography–mass spectrometry (GC–MS) to analyze and compare the chemical composition of the lipophilic fraction of femoral gland secretions of males of both species.

2. Material and methods

2.1. Study area and species

The Columbretes wall lizard *P. atrata*, is a small lizard (mean adult snout–vent length of 50–70 mm) endemic from the Columbretes archipelago (39°55′ N, 0°40′ E, Mediterranean Sea, Castellón, Spain), which inhabits three small islands characterized by high aridity and vegetation dominated by perennial shrubs, herbs and grasses (Castilla and Bauwens, 1991, 2000; Castilla, 2000). We collected by noosing 15 adult males in the main island, Columbrete Grande (13 ha), during the reproductive season in April 2008.

The Iberian wall lizard, *P. hispanica*, is a small lizard (mean adult snout–vent length of 49–61 mm) with a widespread distribution in Spain and the south east coast of France (Barbadillo et al., 1999). We captured by noosing adult male *P. hispanica* lizards at five localities within the Madrid Province (Central Spain) during the reproductive season in May–June 2008. Three of these were located in the mountain northern area ('Fuenfría', 'Cercedilla' and 'Pedrezuela'), and the other two were located in the plain southern area ('Belmonte' and 'Aranjuez'). In the north, we captured lizards from a population occupying granite rock-cliffs at the edge of a pine forest in the upper part of 'Fuenfría' Valley ('Ph1') (40°47′ N, 4°03′W; 1750 m altitude; $n = 21$ males), and from old stone walls near to cultivated fields in the 'Pedrezuela' village ('Ph2') (40°44′ N, 3°36′ W; 800 m altitude; $n = 19$ males). Finally, we caught lizards on granite rocky outcrops in a large oak forest ('Golondrina', 'Ph3') near Cercedilla village (40°44′ N, 4°02′ W; 1250 m altitude; $n = 29$ males). In the south, we captured lizards on human constructions in a public garden in the 'Belmonte del Tajo' village ('Ph4') (40°8′ N, 3°20′ W; 735 m altitude; $n = 22$ males), and on chalk and gypsum rocks in deforested bushy hills near 'Aranjuez' ('Ph5') (40°2′ N, 3°37′ W; 494 m altitude; $n = 21$ males).

2.2. Analyses of femoral gland secretions

Immediately after capture in the field, we extracted femoral gland secretion of males by gently pressing with forceps around the femoral pores, and collected secretion directly in glass vials with Teflon-lined stoppers. Vials were stored at −20 °C until analyses. We also used the same procedure on each sampling occasion but without collecting secretion, to obtain blank control vials that were treated in the same manner to compare with the lizard samples. Before the analyses we added 250 μl of *n*-hexane (Sigma, capillary GC grade) to each vial, We analyzed lipophilic compounds in samples by using a Finnigan–ThermoQuest Trace 2000 gas chromatograph (GC) fitted with a poly (5% diphenyl/95% dimethylsiloxane) column (Thermo Fisher, 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. Sample injections (2 μl of each sample dissolved in *n*-hexane) were performed in splitless mode using helium as the carrier gas at 30 cm/s, with injector temperature at 250 °C. The oven temperature program was as follows: 50 °C isothermal for 5 min, then increased to 270 °C at a rate of 10 °C/min, isothermal for 1 min, then increased to 315 °C at a rate of 15 °C/min, and finally isothermal (315 °C) for 10 min. Ionization by electron impact (70 eV) was carried out at 250 °C. Mass spectral fragments below $m/z = 39$ were not recorded. Impurities identified in the solvent and/or the control vial samples are not reported. Initial identification of secretion components was done by comparison of mass spectra in the NIST/EPA/NIH 1998 computerized mass spectral library. Identifications were confirmed by comparison of spectra and retention times with those of authentic standards from Sigma–Aldrich Chemical Co. For unidentified or unconfirmed compounds we report here their characteristic ions, which we used together with retention times and characteristic *m/z* ratios to confirm whether these compounds were present in a given individual.

For the statistical analyses of secretions, the relative amount of each component was determined as the percent of the total ion current (TIC). Then, relative areas of the peaks were transformed following Aitchison's formula: $[Z_{ij} = \ln(Y_{ij}/g(Y_j))]$, where *Zij* is the standardized peak area *i* for individual *j*, Y_{ij} is the peak area *i* for individual *j*, and $g(Y_j)$ is the geometric mean of all peaks for individual *j* (Aitchison, 1986; Dietemann et al., 2003). The homogeneity of variance of these variables was tested with Levene's test, and Bonferroni's correction was applied. The transformed areas were used as variables in a principal component analysis. The eight principal components (PC) extracted (with eigenvalues >1; which explained 82.26% of variance) were used as independent variables in a multivariate analysis of variance (MANOVA) to determine whether the five populations of male *P. hispanica* (mainland) and *P. atrata* (island) differed in the relative proportion of compounds. In addition, we used these extracted principal components (all 8 PCs) as covariates in a subsequent discriminant analysis. We used this test to verify whether chemical compounds in femoral secretions could be used to predict the population of origin of a male lizard.

In a further similar analysis, we selected the 28 compounds that were presented in all populations, re-standardized their TIC areas to 100% and used a MANOVA with transformed areas to test for differences between populations in the relative proportions of shared compounds.

3. Results

We found 47 lipophilic compounds in femoral glands secretions of male *P. atrata* (see Table 1). These secretions were a mixture of steroids (78.47% of TIC), carboxylic acids, ranging between *n*-C_{14} and *n*-C_{20} as a series of bishomologues, and their esters (9.89%), six waxy esters (8.30%), four alcohols between *n*-C_{16} and *n*-C_{22} (2.75%), squalene (0.35%), a ketone (0.13%), and an unidentified terpenoid (0.11%). On average, the five most abundant chemicals were cholesterol (63.38% of TIC), an unidentified waxy ester of the hexadecanoic acid (3.61%), octadecanoic acid (3.19%), hexadecanoic acid (2.98%), and campesterol (2.66%) (see Table 1).

The femoral gland secretions of male *P. hispanica* (all five populations pooled) were composed by 53 lipophilic compounds, which were a mixture of steroids (83.64% of TIC), and carboxylic acids ranged between *n*-C_{14} and *n*-C_{22} and their esters (10.30%), but we found also five alcohols between *n*-C_{16} and *n*-C_{24} (1.26%), four waxy esters (3.53%), squalene (0.60%), two terpenoids (0.40%), a ketone (0.18%), and a furanone (0.09%) (see Table 1). On average, the five most abundant chemicals were cholesterol (63.24% of TIC), cholesta-5,7-dien-3-ol (5.16%), hexadecanoic acid (3.73%), campesterol (3.66%) and octadecenoic acid (2.46%) (Table 1). However, when considered separately, there were some clear differences between populations of *P. hispanica* (see below).

There were differences between *P. hispanica* and *P. atrata* in the presence/absence of several compounds in femoral secretions. Male *P. atrata* from Columbretes islands had nine chemical compounds (seven steroids and two waxy esters) that were not found in mainland *P. hispanica* males (see Table 1). On the other hand, secretions from mainland *P. hispanica* (all populations pooled) had 15 compounds (nine steroids, three carboxylic acids, one alcohol, one furanone and one terpenoid) that were not found in island *P. atrata* lizards (see Table 1).

Also, proportion of the main classes of chemical compounds in secretions varied between species. In comparison with *P. hispanica, P. atrata* had significantly higher overall proportions of alcohols (ANOVA: $F_{1,119} = 44.22$ $p < 0.0001$) and waxy esters ($F_{1,119} = 38.53$, $p < 0.0001$), and significantly lower proportions of low molecular weight ($C_{14–18}$) fatty acids ($F_{1,119} = 28.39$, $p < 0.0001$), high molecular weight (C_{20}–C_{22}) fatty acids ($F_{1,119} = 6.75$, $p = 0.01$), and steroids ($F_{1,119} = 23.07$, $p < 0.0001$).

Multivariate analyses on the 8 PCs for all the 62 compounds found in femoral secretions showed that there were significant differences in the relative proportions of some compounds between species and/or populations (MANOVA, *Wilk's* $\lambda = 0.0001$, $F_{40,473} = 904.56$, $p < 0.0001$). Moreover, the discriminant and the canonical analyses showed that PCs scores of chemical

Table 1
Lipophilic compounds found in femoral gland secretions of male lizards, *P. hispanica* from five distinct populations of the mainland, and *P. atrata* from Columbretes Islands.

Compounds Characteristic ions observed (m/z)	RT (min)	*Podarcis hispanica*					*P. atrata*
		Ph1	Ph2	Ph3	Ph4	Ph5	
Steroids							
Un.steroid 1 (145,213,248,353,368,387)	29.92	0.01 ± 0.01	–	0.17 ± 0.05	1.49 ± 0.56	–	–
Cholesta-2,4-diene	30.58	0.68 ± 0.11	2.66 ± 0.44	0.44 ± 0.08	2.59 ± 0.46	0.96 ± 0.35	–
Un.steroid 2 (141,156,209,281,350,365)	30.64	–	–	–	–	–	0.44 ± 0.06
Un.steroid 3 (143,253,350,367)	30.74	–	–	–	–	–	0.09 ± 0.01
Cholesta-3,5-diene	30.81	0.42 ± 0.10	0.23 ± 0.04	0.30 ± 0.07	0.13 ± 0.03	0.25 ± 0.07	0.06 ± 0.01
Un.steroid 4 (155,197,251,350,365)	30.96	1.32 ± 0.16	1.00 ± 0.14	0.55 ± 0	0.45 ± 0.06	0.45 ± 0.17	0.79 ± 0.10
Cholesta-5,7,9(11)-trien-3β-ol	31.06	1.62 ± 0.18	1.07 ± 0.24	0.94 ± 0.11	0.65 ± 0.11	0.29 ± 0.07	0.94 ± 0.18
Un.steroid 5 (207,251,350,365)	31.13	0.40 ± 0.08	0.16 ± 0.02	0.18 ± 0.02	0.18 ± 0.07	0.08 ± 0.04	0.18 ± 0.04
Un.steroid 6 (143,195,207,351,366)	31.20	0.19 ± 0.02	0.08 ± 0.01	0.15 ± 0.04	0.18 ± 0.05	0.22 ± 0.06	0.35 ± 0.17
Un.steroid 7 (141,156,209,350,365)	31.37	0.37 ± 0.05	0.03 ± 0.01	0.30 ± 0.06	2.47 ± 0.42	–	0.53 ± 0.12
Un.steroid 8 (155,197,251,365,379)	31.64	0.06 ± 0.01	0.21 ± 0.02	0.08 ± 0.02	0.43 ± 0.07	0.45 ± 0.18	0.31 ± 0.08
Un.steroid 9 (195,209,251,365,379)	31.84	–	0.07 ± 0.01	0.27 ± 0.07	0.51 ± 0.08	0.32 ± 0.12	0.34 ± 0.22
Cholesterol	32.43	59.74 ± 2.79	62.33 ± 1.68	66.61 ± 2.00	53.03 ± 2.51	74.51 ± 2.04	63.38 ± 2.26
Cholestan-3β-ol	32.47	1.40 ± 0.14	0.53 ± 0.08	0.90 ± 0.11	0.60 ± 0.06	0.55 ± 0.12	1.33 ± 0.18
Cholesta-5,7-dien-3β-ol.	32.65	13.41 ± 1.85	2.68 ± 0.54	8.02 ± 1.33	1.16 ± 0.19	0.54 ± 0.17	0.62 ± 0.30
4-Methyl-cholestan-3β-ol	32.73	–	–	–	–	–	0.12 ± 0.03
Un.steroid 10 (213,255,353,368,386,415)	32.75	0.02 ± 0.01	0.03 ± 0.02	0.35 ± 0.11	0.09 ± 0.03	0.39 ± 0.16	–
Ergosterol	33.00	–	0.05 ± 0.02	–	0.17 ± 0.11	–	0.09 ± 0.02
Campesterol	33.17	1.61 ± 0.22	3.76 ± 0.28	3.27 ± 0.36	5.46 ± 0.28	4.22 ± 0.57	2.66 ± 0.37
Cholest-4-en-3-one	33.41	0.17 ± 0.03	0.53 ± 0.17	0.19 ± 0.05	0.20 ± 0.02	0.92 ± 0.38	1.33 ± 0.42
Un.steroid 11 (215,374,384,400,416)	33.45	–	–	–	–	–	2.02 ± 0.33
Ergosta-5,8-dien-3β-ol	33.50	2.43 ± 0.30	1.58 ± 0.22	2.38 ± 0.37	1.31 ± 0.24	0.56 ± 0.14	–
Cholesta-4,6-dien-3-one	33.69	0.24 ± 0.06	0.53 ± 0.08	0.29 ± 0.06	0.40 ± 0.06	–	
Un.steroid 12 (267,339,366,383)	33.72	–	–	–	–	–	0.48 ± 0.09
Sitosterol	33.92	0.65 ± 0.10	0.74 ± 0.16	0.94 ± 0.15	1.18 ± 0.11	1.13 ± 0.23	1.25 ± 0.17
Ergostanol	34.02	0.07 ± 0.01	0.08 ± 0.03	0.10 ± 0.02	0.11 ± 0.02	0.33 ± 0.11	0.13 ± 0.03
Stigmasterol	34.13	0.31 ± 0.06	0.27 ± 0.13	0.28 ± 0.04	1.22 ± 0.22	0.44 ± 0.26	–
Un.steroid 13 (267,366,381)	34.17	–	–	–	–	–	0.37 ± 0.04
Un.steroid 14 (221,253,281,355,380,430)	34.30	2.23 ± 0.32	0.70 ± 0.18	1.01 ± 0.16	–	–	–
Un.steroid 15 (143,253,354,380,395,413)	34.31	–	–	–	–	–	0.30 ± 0.07
Cholest-5-en-3-one	34.38	–	–	–	1.33 ± 0.24	0.91 ± 0.28	–
Ergosta-5,22-dien-3β-ol	34.47	–	0.13 ± 0.07	0.12 ± 0.03	0.15 ± 0.04	–	0.36 ± 0.08
Un.steroid 16 (214,267,395)	35.30	0.12 ± 0.04	0.21 ± 0.11	–	0.56 ± 0.44	0.22 ± 0.09	–
Carboxylic acids and their esters							
Tetradecanoic acid	20.64	0.16 ± 0.04	0.38 ± 0.13	0.22 ± 0.06	0.24 ± 0.05	0.85 ± 0.55	0.21 ± 0.05
Pentadecanoic acid	21.68	0.13 ± 0.02	0.15 ± 0.12	0.10 ± 0.03	0.18 ± 0.05	0.41 ± 0.19	0.25 ± 0.08
Hexadecanoic acid. methyl ester	22.33	–	0.05 ± 0.02	–	0.09 ± 0.02	0.25 ± 0.08	0.11 ± 0.04
Hexadecenoic acid	22.54	0.16 ± 0.02	0.40 ± 0.20	0.25 ± 0.07	0.57 ± 0.33	0.28 ± 0.09	0.17 ± 0.05
Hexadecanoic acid	22.76	3.68 ± 0.32	4.36 ± 0.65	3.11 ± 0.35	5.98 ± 0.51	1.54 ± 0.23	2.98 ± 0.20
Hexadecanoic acid, ethyl ester	22.98	–	0.37 ± 0.11	–	0.19 ± 0.06	0.40 ± 0.17	–
9,12-Octadecadienoic acid	24.35	0.10 ± 0.01	0.11 ± 0.02	0.12 ± 0.02	0.27 ± 0.08	0.06 ± 0.02	0.18 ± 0.04
Octadecenoic acid	24.43	1.99 ± 0.18	1.76 ± 0.20	2.76 ± 0.57	4.82 ± 1.41	1.01 ± 0.21	2.24 ± 0.21
Octadecanoic acid	24.60	1.39 ± 0.12	2.52 ± 0.34	1.41 ± 0.13	2.55 ± 0.23	0.99 ± 0.18	3.19 ± 0.22
Octadecanoic acid, ethyl ester	24.82	–	0.51 ± 0.23	–	0.14 ± 0.04	0.55 ± 0.23	–
Eicosanoic acid	26.31	0.46 ± 0.09	0.63 ± 0.15	0.76 ± 0.11	0.59 ± 0.17	0.64 ± 0.18	0.53 ± 0.21
Docosanoic acid	28.00	–	0.01 ± 0.01	–	0.01 ± 0.01	–	0.03 ± 0.01
Docosanoic acid, ethyl ester	28.21	–	0.45 ± 0.12	–	0.21 ± 0.05	0.23 ± 0.12	–
Alcohols							
Hexadecanol	21.02	0.23 ± 0.05	–	0.19 ± 0.07	0.16 ± 0.04	0.16 ± 0.05	0.26 ± 0.06
Octadecanol	23.87	0.26 ± 0.05	0.69 ± 0.16	0.19 ± 0.06	0.29 ± 0.08	–	1.58 ± 0.45
Eicosanol	25.67	0.17 ± 0.03	0.55 ± 0.13	0.28 ± 0.08	0.21 ± 0.05	0.81 ± 0.28	0.59 ± 0.10
Docosanol	27.33	0.23 ± 0.05	0.52 ± 0.15	0.23 ± 0.04	0.23 ± 0.04	0.73 ± 0.26	0.32 ± 0.10
Tetracosanol	29.80	0.03 ± 0.01	0.07 ± 0.01	0.02 ± 0.01	0.07 ± 0.02	0.01 ± 0.01	–
Waxy esters							
Un.waxy ester of hexadecanoic acid 1	28.47	–	–	–	–	–	0.08 ± 0.04
Un.waxy ester of octadecanoic acid 2	29.45	0.28 ± 0.08	0.98 ± 0.20	–	1.37 ± 0.47	0.75 ± 0.23	0.74 ± 0.16
Un.waxy ester of hexadecanoic acid 3	35.00	–	–	–	–	–	0.03 ± 0.01
Un.waxy ester of hexadecanoic acid 4	35.57	0.58 ± 0.10	2.61 ± 0.60	0.42 ± 0.08	2.84 ± 0.45	0.69 ± 0.26	3.61 ± 0.69
Un.waxy ester of octadecenoic acid 5	38.06	0.23 ± 0.06	0.29 ± 0.06	0.20 ± 0.05	0.09 ± 0.03	0.37 ± 0.23	1.47 ± 0.26
Un.waxy ester of octadecanoic acid 6	38.27	0.63 ± 0.11	1.78 ± 0.26	0.47 ± 0.10	2.26 ± 0.30	0.82 ± 0.16	2.37 ± 0.29

Table 1 (*continued*)

Compounds Characteristic ions observed (m/z)	RT (min)	*Podarcis hispanica*					*P. atrata*
		Ph1	Ph2	Ph3	Ph4	Ph5	
Others							
Tetradecanone	22.11	0.20 ± 0.05	0.27 ± 0.11	0.13 ± 0.03	0.15 ± 0.03	0.18 ± 0.06	0.13 ± 0.03
Un.furanone (55,69,85,97,111,192,249)	24.19	0.12 ± 0.02	0.10 ± 0.03	0.06 ± 0.01	–	–	–
Squalene	30.07	0.93 ± 0.26	0.70 ± 0.10	0.66 ± 0.20	0.35 ± 0.04	0.40 ± 0.19	0.35 ± 0.08
Un. terpenoid 1 (55,69,81,95,135,207)	30.83	0.09 ± 0.03	0.07 ± 0.03	0.08 ± 0.02	0.03 ± 0.01	0.13 ± 0.07	–
Un.terpenoid 2 (69,81,95,107,121,135,147)	31.94	0.48 ± 0.09	–	0.48 ± 0.12	0.05 ± 0.01	–	0.11 ± 0.05

The relative amount of each component was determined as the percent of the total ion current (TIC) and reported as the average (± 1SE). Characteristic ions (*m/z*) are reported for unidentified (Un.) compounds. RT: Retention time.

compounds in femoral secretions of male *P. atrata* clearly allowed differentiating them from males from any of the five mainland populations of *P. hispanica* (Fig. 1). This analysis classified correctly 100% of individual *P. atrata* as belonging to this species. Moreover, the squared Mahalanobis distances between groups showed that all *P. hispanica* individuals had significantly shorter distances (based on chemical components) to individuals from any *P. hispanica* population than to *P. atrata* individuals ($F_{5,535} = 632.92$, $p < 0.0001$).

According to the correlations of relative proportions of all compounds found in femoral secretions with the PCs, the PC-1 scores were related negatively to the proportion of tetracosanol, cholesta-2,4-diene, ergosta-5,8-dien-3-ol, cholesta-4,6-dien-3-one and stigmasterol, and were related positively with the proportions of the unidentified waxy esters 1 and 3, 4-methyl-cholestan-3-ol and six unidentified steroids (2, 3, 11, 12, 13 and 15). PC-1 scores varied significantly between populations (ANOVA, $F_{5,115} = 2092.48$, $p > 0.0001$) (see Table 2). Island *P. atrata* positive and significant different PC-1 scores than all *P. hispanica* populations, which had negative values (Tukey's tests: $p < 0.0002$ for all). PC-1 scores of Ph1 and Ph3 were not significantly different ($p > 0.60$), but both differed significantly from values of Ph4 and Ph5 ($p < 0.0002$); Ph4 and Ph5 were significantly different ($p = 0.00012$).

PC-2 scores correlated positively with the proportion of tetracosanol, cholesterol, cholestanol, cholesta-3,5-diene and campesterol, and negatively with the proportions of ergosterol. There were significant differences between populations ($F_{5,115} = 144.64$; $p < 0.0001$) (Table 2). Males from Ph1, Ph2 and Ph5 had positive values whereas Ph2, Ph4 and *P. atrata* had negative values. PC-2 scores of Ph1 and Ph5 ($p = 0.11$) and those of *P. atrata* and Ph2 ($p = 0.31$) were not significantly different, whereas all the rest of comparisons were significantly different ($p < 0.00011$ for all).

PC-3 scores described negatively the relative proportions of hexadecanoic acid ethyl and methyl esters, octadecanoic acid ethyl ester, docosanoic acid ethyl ester, and cholest-5-en-3-one and positively the proportions of octadecanol, the unidentified steroid 7, and the unidentified terpenoid 2. PC-3 values differed significantly between populations ($F_{5,115} = 610.65$, $p < 0.0001$) (Table 2). Males from Ph1 and Ph2 had similar positive values ($P = 0.99$), while other populations had negative values. All other comparisons of PC-3 scores between populations were significant ($P < 0.0002$ for all).

PC-4 scores were negatively related to the proportions of the unidentified steroid 16 and positively with the proportions of the unidentified steroid 9. PC-4 scores differed significantly between populations ($F_{5,115} = 205.39$, $p = 0.0006$) (Table 2). Males from Ph1 had positive PC-4 scores, while the rest of populations had PC-4 scores around zero and negative. The comparisons

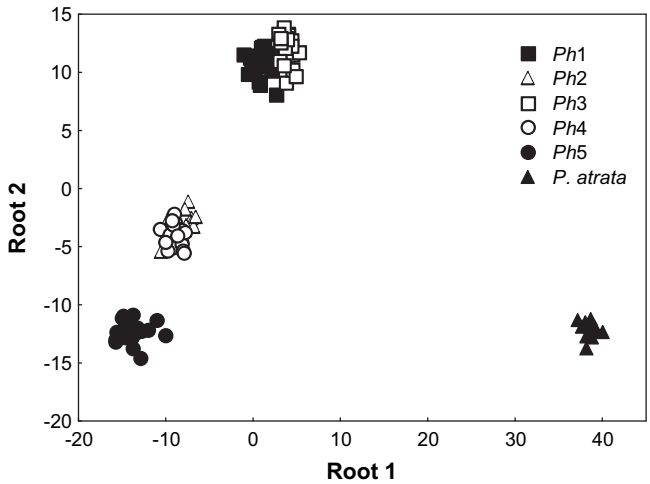

Fig. 1. Separation of the principal components scores (PCs) describing chemicals from femoral secretions of male lizards in a discriminant analysis based on population of origin.

Table 2
Principal components (PCs) scores (mean + SE) from a PCA for relative proportions of compounds in femoral secretions of *P. hispanica* populations (Ph1–5) and *P. atrata*.

PCs	*Eigenvalue*	*Podarcis hispanica*					*P. atrata*
		Ph 1	*Ph 2*	*Ph 3*	*Ph 4*	*Ph 5*	
PC-1	16.59	-0.33 ± 0.02^{b}	-0.36 ± 0.02^{b}	-0.31 ± 0.02^{b}	-0.59 ± 0.02^{a}	-0.06 ± 0.02^{c}	2.81 ± 0.02^{d}
PC-2	15.33	0.79 ± 0.07^{a}	-0.71 ± 0.08^{b}	0.4 ± 0.07^{c}	-1.48 ± 0.08^{b}	1.09 ± 0.08^{a}	-0.43 ± 0.10^{c}
PC-3	7.67	0.96 ± 0.03^{a}	-0.05 ± 0.04^{a}	0.97 ± 0.03^{c}	-0.18 ± 0.04^{d}	$-1.79 \pm 0.04e$	0.20 ± 0.05^{b}
PC-4	3.88	1.37 ± 0.06^{a}	0.28 ± 0.07^{b}	-1.57 ± 0.06^{d}	0.15 ± 0.06^{bc}	-0.20 ± 0.07^{c}	0.03 ± 0.08^{bc}
PC-5	2.84	-0.12 ± 0.04^{c}	2.06 ± 0.05^{a}	0.10 ± 0.04^{b}	-1.19 ± 0.05^{d}	$-0.47 \pm 0.05e$	-0.08 ± 0.06^{bc}
PC-6	1.83	0.30 ± 0.20	-0.46 ± 0.23	0.17 ± 0.20	-0.19 ± 0.21	0.04 ± 0.22	0.002 ± 0.27
PC-7	1.67	-0.35 ± 0.20	-0.01 ± 0.23	0.27 ± 0.20	0.35 ± 0.20	-0.21 ± 0.22	-0.09 ± 0.27
PC-8	1.16	-0.11 ± 0.20	0.02 ± 0.23	0.11 ± 0.20	0.21 ± 0.21	0.31 ± 0.22	0.08 ± 0.27

Same small letters after means indicated lack of significant posthoc differences (Tukey's tests) between populations within each PC.

between populations were all significant ($p < 0.0002$), except between Ph2 and Ph4 ($p = 0.84$) or between *P. atrata* and Ph2 ($p = 0.31$), Ph5 ($p = 0.34$) or Ph4 ($p = 0.89$).

Finally, the proportion of hexadecanol in secretions (i.e., PC-7 scores) differed with the population of origin ($F_{5,115} = 386.05$, $p < 0.0001$) (Table 2). Males from Ph2 showed positive PC-5 scores whereas all other populations had negative values. All comparisons of PC-5 scores between populations were significant ($p < 0.0001$), excepted between *P. atrata* and Ph1 ($p = 0.99$) and Ph2 ($p = 0.18$).

There were not significant differences between populations for PC-6 ($F_{5,115} = 1.59$, $p = 0.16$), PC-7 ($F_{5,115} = 1.75$, $p = 0.12$) or PC-8 ($F_{5,115} = 0.76$, $p = 0.57$) (Table 2).

In an additional analysis, when we selected only the 28 compounds shared (i.e. presented) between all populations, the relative proportion of these shared chemicals also varied significantly with the population of origin (MANOVA, *Wilks'* $\lambda = 0.03$, $F_{125,452} = 8.29$, $p < 0.0001$).

4. Discussion

The analyses of chemical composition showed that similarly to other lacertid lizards, femoral gland secretions of *P. hispanica* and *P. atrata* have steroids and carboxylic acids as predominant components (reviewed in Weldon et al., 2008). Among steroids, in both species, cholesterol was the main steroid, which was also presented in abundance in secretions of many other lizard species (Weldon et al., 2008). More interestingly, chemicals found in femoral gland secretions of males differed in composition and proportion between the two species, and secondarily between populations of *P. hispanica*. The chemical differences between island *P. atrata* and mainland *P. hispanica* were higher than between continental populations of *P. hispanica* alone. For some chemicals, there were clear differences in presence/absence between species. Some steroids and waxy esters were presented only in secretions of *P. atrata*, whereas other chemicals such as tetracosanol or cholesta-2,4-diene, ergosta-5,8-dien-3-ol or stigmasterol, were found only in secretions of *P. hispanica*. Also, the proportions of the main classes of compound varied between species. For example lizards from the islands had secretions with higher proportions of alcohols and waxy esters. In addition, the proportions of shared chemicals also varied between *P. atrata* and *P. hispanica*.

These chemical differences between species could result from the different habitats where each lizard species lives. A first possible explanation is that these differences in lipidic secretions might be the consequence of different diets (Symonds and Elgar, 2009), or different available food sources in the island and in the mainland. Interestingly, for example, the few existing studies on the diet of *P. atrata* indicates that this species includes in its diet marine isopods and poisonous scorpions, while this has never been reported for any mainland populations of *P. hispanica* (Castilla et al., 2008c,d; Castilla and Herrel, 2009). Although, to confirm this relationship we would need more studies of diet, and also experiments examining whether different diets lead to differences in femoral secretions.

Alternatively, or in addition, these variations in chemical composition and proportions in secretions might be correlated to the different microclimatic conditions of different geographical areas. Presumably, the variation of femoral chemical composition also would reflect the environment where each lizard species lives (Alberts, 1992; Escobar et al., 2001, 2003). Therefore, it is likely that selection for a better efficiency of chemical signals used in intraspecific communication had led to differences in composition of femoral secretions of lizards inhabiting different environments (see Alberts, 1992). The abiotic conditions in a small island and in the continent may be very different (e.g., altitude, temperature, wind, relative humidity) and this could have influenced the characteristics of chemical secretions of lizards. A study showed that femoral secretions of two populations of *P. hispanica* inhabiting areas with different microclimatic conditions differ in the abundance of more stable waxy esters and long chain fatty acids, which are more abundant in lizards from areas with higher humidity levels (Martín and López, 2006a). These less volatile compounds would confer more stability to secretions under conditions where high levels of humidity increase evaporation (Alberts, 1992). Similarly, our results indicate that waxy esters, which would confer more stability to secretions in the humid conditions of the island environment, are more abundant in *P. atrata*.

Finally, differences in chemicals in femoral secretions may simply result from genetic differences between populations and species. The Iberian wall lizard, *P. hispanica* is paraphyletic with respect to *P. bocagei*, *P. carbonelli*, and *P. atrata* and appears to form a species complex (Harris and Sá-Sousa, 2002; Pinho et al., 2007). Genetic divergence is high between the population of *P. atrata* from the Columbretes islands and a population of *P. hispanica* from the continent, which justified that it was described as a different species (Castilla et al., 1998a,b; Harris and Sá-Sousa, 2001, 2002). Chemical differences in femoral secretions may be the result of colonization and adaptation to different habitats; or just from random genetic divergence due to the geographical isolation, such that each species could have evolved in different ways and produce different chemical signals. Similarly, in some rodents, genetic differences within a species complex seem to explain differences in scent characteristics (Heth and Todrank, 2000; Heth et al., 2001; Talley et al., 2001).

The question that arises is whether these differences in chemical signals affect recognition systems and whether this may have had consequences for speciation. A previous experiment of chemosensory recognition by tongue-flick tests between *P. hispanica* and *P. atrata* lizards (Gabirot et al., 2010) and the results of the current study suggest that these differences in chemical signals could allow species recognition. This chemosensory species discrimination could have been an important requisite to preclude reproductive interactions between *P. hispanica* and *P. atrata*. This is something that has been already observed within other wall lizard species (Cooper and Pérez-Mellado, 2002; Barbosa et al., 2005, 2006; Martín and López, 2006a,b). Compounds that are specific for femoral secretions of each species could allow species recognition. Furthermore, chemical recognition between species could not be only based on differences in one or a few compounds alone, but also on the pattern of compounds found in each species. In vertebrates, pheromones are often a mixture of several chemical compounds with different properties (Müller-Schwarze, 2006). These multicomponent pheromones may have different functions, but may also act together providing specific "odor profiles", also named "gestalts", "patterns", or "mosaics" (Johnston, 2005). Not only the mixture of chemicals, but also the relative proportions or concentrations of these chemicals, are often needed to be biologically active as a pheromone. Thus, a vertebrate pheromone may be defined as a group of active compounds in a secretion that supply information to, or change behavior in, another conspecific (Müller-Schwarze, 2006), and that could also allow species recognition.

In summary, our study showed that chemical signals (femoral secretions) of *P. hispanica* and *P. atrata* lizards were different in composition and proportions of chemical compounds. Moreover, this difference is higher between these two species than between any distinct population of *P. hispanica*. All these chemical differences could be the consequence of random genetic variation, but could also be due to adaptation to different habitats, with differences in climatic conditions or diet resources. This chemical variation could be used by individuals to recognize lizards from their own species. Therefore, these results of chemical composition and previous studies of chemosensory recognition reinforce the fact that the genetic differences between *P. hispanica* and *P. atrata* may reflect reproductive isolation between these two taxa. Our results could be a preliminary cue of the high improbability of reproduction between *P. hispanica* and *P. atrata*. If individuals do recognize and discriminate between species, which may be based on differences in chemical compounds in femoral secretions, interspecific mating could not occur. However, further experiments of mate choice and staged encounters should be done to ensure that premating reproductive isolation may actually prevent hybridization between island and mainland lizards.

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