Biochemical Systematics and Ecology 61 (2015) 286–292

Contents lists available at ScienceDirect

Biochemical Systematics and Ecology

journal homepage: www.elsevier.com/locate/biochemsyseco

Lipophilic compounds in femoral secretions of males and females of the El Hierro giant lizard *Gallotia simonyi* (Lacertidae)

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ARTICLE INFO

Article history:
Received 27 February 2015
Received in revised form 25 June 2015
Accepted 26 June 2015
Available online xxx

Keywords:
Lizards
Gallotia simonyi
Femoral secretions
Intersexual variations
Canary Islands

ABSTRACT

Many lizards use femoral gland secretions in reliable intraspecific communication. Based on mass spectra, obtained by GC–MS, we found 57 lipophilic compounds in femoral secretions of males and females of El Hierro giant lizards, *Gallotia simonyi* (fam. Lacertidae). Compounds included steroids (mainly cholesterol) and fatty acids ranging between n-C_{16} and n-C_{22} (mainly hexadecanoic and octadecanoic acids), followed by aldehydes, alcohols, ketones, squalene and waxy esters. There were important intersexual differences in the presence, abundance and number of compounds (more numerous in males). Males had higher proportions of the most odoriferous compounds (fatty acids and aldehydes), while females had higher proportions of more stable compounds (steroids, waxy alcohols, waxy esters and terpenoids). This suggests sexual differences in function of femoral secretions. In addition, some compounds could reflect the physiological state, allowing monitoring health of lizards from secretions samples, which is especially important given the critical conservation status of this lizard.

1. Introduction

Chemical signals are important for intraspecific communication of many animals (Müller-Schwarze, 2006; Wyatt, 2014). For example, lizards produce femoral or precloacal gland secretions with chemical compounds that are used in communication and social organization and may have consequences for reproduction (Mason, 1992; Alberts, 1993; Mason and Parker, 2010; Martín and López, 2011, 2014). In many lizard species, males use femoral secretions to scent-mark territories, and lipophilic compounds in these scent-marks may provide information on the characteristics, dominance status and quality of the territory owner to other males (López and Martín, 2002, 2011; Labra, 2006; Carazo et al., 2007; Martín and López, 2007; Martín et al., 2007b; Khannoon et al., 2011b) and to females (Martín and López, 2000, 2006a,b; 2012; Olsson et al., 2003; López and Martín, 2005a, 2012). This information is reliable because the amount of some compounds that can be allocated to secretions is dependent on conflicting physiological requirements, such as the maintenance of the immune system,

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http://dx.doi.org/10.1016/j.bse.2015.06.027
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and it also depends on the diet and the endocrine and health state of males (Alberts et al., 1992a; López et al., 2006, 2009; Martín et al., 2007a; Kopena et al., 2011, 2014; Heathcote et al., 2014; see review in Martín and López, 2015). Therefore, to understand the role of chemical signals in reproduction and sexual selection of a lizard species, we need first to characterize the compounds secreted by the femoral glands and their variation.

Femoral secretions of lizards are composed of proteins and many lipophilic compounds, such as steroids, fatty acids, alcohols, etc (reviewed in Weldon et al., 2008; Martín and López, 2011, 2014). However, the specific chemical compounds found in each species and their relative proportions are only known for a few species, which are mainly lacertids (López and Martín, 2005b,c, 2006; 2009; Martín and López, 2006c,d; 2010; Gabirot et al., 2008, 2010, 2012b; Kopena et al., 2009; Khannoon et al., 2011a; Martín et al., 2013a) and several South American tropidurids of the genus *Liolaemus* (Escobar et al., 2001, 2003), although there is also information on an African cordylid (Louw et al., 2007), an American teid (Martín et al., 2011), two iguanids (Weldon et al., 1990; Alberts et al., 1992a,b; Martín et al., 2013b), three agamids (Chauhan, 1986; Martín et al., 2012, 2013c) and a few gekkonids (Chauhan, 1986; Khannoon, 2012). These studies show consistent interspecific variation in composition (Weldon et al., 2008; Martín and López, 2011, 2014), which probably reflects phylogenetic differences *per se*, although environmental conditions might also contribute to explain why some compounds have been selected in a given species (Martín and López, 2006c, 2013). Also, because allocation of compounds to secretions may depend on the physiological state of an individual (e.g. Martín et al., 2007a; Martín and López, 2015), examining interindividual variation in chemical compounds may also be useful in other applied contexts, such as in monitoring of populations.

The El Hierro Giant Lizard (*Gallotia simonyi*), is a large sized lacertid species which was believed to be extinct in the 1930s but that was rediscovered in 1974 (Böhme and Bings, 1975; Barahona et al., 2000). It is included in the genus *Gallotia*, endemic from the Canary Islands (Spain) (Arnold, 1973). This species is restricted to El Hierro Island (Canary Islands) where it is now confined to a few small areas of a high cliff ("Risco de Tibataje") with sparse vegetation, although new populations have been recently reintroduced in other areas. The population in the wild is now about 300 animals (including re-introduced populations) (Rodríguez-Domínguez et al., 2014). The conservation status of *G. simonyi* is classified as "critical" by the International Union for Conservation of Nature (IUCN) guidelines (Pleguezuelos et al., 2002). Thus, this lizard is strictly protected by national and international laws and is object of a recovery plan, that includes a captivity breeding program and a regular monitoring of physiological health state of lizards.

Several articles have examined physiological and clinical aspects of *G. simonyi*, such as hematology (Martínez-Silvestre et al., 2004a, 2005), microbiology (Martínez-Silvestre et al., 2004b; Herrera-León et al., 2005), reproduction (Rodríguez-Domínguez and Molina-Borja, 1998), diseases (Martínez-Silvestre and Orós, 2002) and parasitology (Roca et al., 1999), as well as some ethological aspects (Cejudo et al., 1997). To date, nothing is known on the role of chemical ecology in social behavior and sexual selection of Canarian lizards of the genus *Gallotia*, or of *G. simonyi* in particular.

We report here the results of an analysis by gas chromatography-mass spectrometry (GC–MS) of the lipophilic fraction of femoral secretions of adult males and females of El Hierro giant lizards, *G. simonyi*. We specifically describe and compare between sexes the composition and relative proportions of compounds in femoral secretions of lizards.

2. Material and methods

2.1. Study animals

The lizards studied here (22 males and 9 females) came from the Recovery Centre for the Giant Lizard of El Hierro (El Hierro, Canary Islands, 14° 00′ W; 27° 45′N), located in the same area where natural populations of this lizard can be found. These animals are part of captive breeding programme, which originally used 12 wild animals captured between 20 and 30 years ago. New introduced populations have been established using lizards from this captive stock. The existence of relatively few adult examples for the breeding programme is due to the scarcity of wild adults compatible with a maximum genetic variability that would allow the viability of the species (Arano et al., 1997).

In this recovery centre, lizards were kept in pairs, in large outdoor seminatural enclosures under the same temperature and light conditions that they experienced in the wild. During the study period, temperature range was between 23 and 35 °C (night–day) and photoperiod was 14 h light and 10 h dark. Lizards were fed regularly (three times at week), on an omnivorous diet of fruit, vegetables and endemic plants, supplemented with crickets (*Grillus bimaculatus*) and giant mealworms (*Zophoba morio*). This diet resembles the natural diet of lizards in the wild (Pérez-Mellado et al., 1999). The lizards were all eating, drinking and behaving normally at the time of the study.

Lizards sampled were aged between four and twelve years. The study was performed in summer 2014, and most animals still maintained abundant active femoral secretions. However, females had inactive ovaries, and abdominal palpation of each one confirmed that they had no oviductal eggs.

2.2. Chemical analyses of femoral gland secretions

Samples of femoral secretions were collected in an active period of lizards (10.00–13.00 h) after they had finished their morning basking periods. We extracted secretions from femoral glands of lizards by gently pressing with forceps around the femoral pores. The secretion was directly collected into glass inserts contained in glass vials, which were later closed with Teflon-lined stoppers. Vials were stored at −20 °C until analyses. We also made the same procedure but without collecting secretion, to obtain blank control vials that were used to exclude contaminants from the handling procedure or from the environment, and for examining potential impurities in the solvent or laboratory equipment.

For chemical analyses of samples, we used a Finnigan-ThermoQuest Trace 2000 gas chromatograph (GC) fitted with a poly(5% diphenyl/95% dimethylsiloxane) column (Supelco, Equity-5, 30 m length × 0.25 mm ID, 0.25-μm film thickness) and a Finnigan-ThermoQuest Trace mass spectrometer (MS) as detector. We injected 2 μl of each sample dissolved in *n*-hexane (Sigma, capillary GC grade, 99.9% purity), using the splitless mode and helium as the carrier gas at 30 cm/s. The temperature of the injector was set at 300 °C. The oven temperature program started at 50 °C, was maintained isothermal for 3 min, then increased to 300 °C at a rate of 5 °C/min, and then isothermal (300 °C) for 15 min. Ionization by electron impact (70 eV) was carried out at 280 °C. Mass spectral fragments below $m/z = 40$ were not recorded. Impurities identified in the control vial samples are not reported. We made initial identification of secretions components by comparison of mass spectra in the NIST/EPA/NIH (NIST 02) computerized mass spectral library. Identifications were confirmed by comparison of spectra and retention times with those of authentic standards (from Sigma–Aldrich Chemical Co) when these were available.

2.3. Data analyses

We determined the relative amount of each compound in the secretions as the percent of the total ion current (TIC). To correct the problem of nonindependence of proportions, we transformed data using the compositional analysis (Ln (proportion)/(1 − proportion)) (Aebischer et al., 1993). Then, to test whether the chemical profiles differed between males and females, we used a single factor permutational multivariate analysis of variance test (PERMANOVA) using 999 permutations, (Anderson, 2001; McArdle and Anderson, 2001) based on a resemblance matrix produced by calculating Euclidean distances between every pair of individual samples. To calculate whether a chemical profile could be assigned to a sex based on its characteristics, we used a canonical analysis of principal coordinates (CAP, Anderson and Willis, 2003) and a permutation test. We used for these analyses the software PRIMER V6.1.13 (Clarke and Gorley, 2006) with the PERMANOVA V1.0.3 add-on package (Anderson et al., 2008).

3. Results

We found 57 lipophilic compounds in femoral gland secretions of male *G. simonyi* (Table 1). The main components were 22 steroids (43.1% of TIC) and nine carboxylic acids or their esters ranged between n-C_{16} and n-C_{22} (41.5%), but we also found ten aldehydes (6.9%), six alcohols between C_{15} and C_{22} (5.9%), squalene (1.3%), three ketones between C_{17} and C_{21} (0.7%) and five waxy esters (0.6%). On average, the five most abundant chemicals in males were cholesterol (24.9% of TIC) and octadecanoic acid (22.1%), followed by eicosanoic acid (6.7%), sitosterol (6.1%) and hexadecanol (4.9%).

In contrast, in females we only found 27 lipophilic compounds (Table 1). The main components were six steroids (50.3% of TIC), but we also found six alcohols between C_{15} and C_{24} (22.3%), squalene and other unidentified terpenoid (12.4%), six waxy esters (9.7%), four aldehydes (3.5%), two esters of carboxylic acids (1.4%), and one ketone (0.4%). On average, the most abundant chemicals in females were cholesterol (36.9% of TIC) and tetracosanol (14.8%), followed by squalene (12.3%), cholest-4-en-3-one (5.4%) and hexadecanol (4.7%).

Only 21 of the 63 identified compounds (33.3%) were shared by both sexes, which comprised 43.1% of TIC areas of males. Males had 36 exclusive compounds (63.2% of compounds) that comprised 56.9% of the TIC areas of males, while females had only six exclusive compounds (22.2%) that comprised 22.3% of their TIC areas. There were significant differences in the multivariate comparison of the chemical profiles of femoral secretions between sexes (PERMANOVA based on the resemblance matrix, pseudo $F_{1,28} = 43.30$, $p = 0.001$), and 100% of the chemical profiles could be classified into the correct sex (CAP analysis using leave-one-out cross-validation, $\delta_1^2 = 0.91$, $p = 0.001$, $m = 2$ axes).

Comparing the relative abundance (% of TIC) of the different type of compounds between sexes, males had significantly higher proportions of fatty acids (GLM, $F_{1,28} = 64.71$, $p < 0.0001$) and aldehydes ($F_{1,28} = 6.05$, $p = 0.02$) than females. In contrast, females had significantly more steroids ($F_{1,28} = 6.50$, $p = 0.017$), alcohols ($F_{1,28} = 31.89$, $p < 0.0001$), waxy esters ($F_{1,28} = 105.00$, $p < 0.0001$) and terpenoids ($F_{1,28} = 25.16$, $p < 0.0001$) than males.

4. Discussion

The lipophilic fraction of femoral secretions of *G. simonyi* is formed mainly by steroids and fatty acids, with cholesterol being the main compound, as it is usual in other lacertid lizards (Weldon et al., 2008). Also, there were minor amounts of other types of compounds, such as alcohols, aldehydes, terpenoids, etc that are also usually found in other related lizards. In addition, there were important intersexual differences in presence and relative proportions of compounds.

With respect to steroids, the large amount of cholesterol, as in other lizards, may form an unreactive apolar matrix to stabilize secretions and protect other compounds with a signaling function (Escobar et al., 2003). This would be especially important in the hot and dry habitats with high evaporation rates, where *G. simonyi* lives (Alberts, 1992; Gabirot et al., 2012a). Other steroids with a relative high abundance are sitosterol and campesterol, steroids of vegetal origin (i.e., phytosterols) that have to be obtained from the diet and diverted from their potentially beneficial physiological functions (Choudhary and Tran, 2011; Genser et al., 2012) to be allocated to secretions. Thus, the presence and relative amounts of these compounds in

Table 1
Lipophilic compounds found in femoral secretions of male and female El Hierro Giant lizards, *Gallotia simonyi*.

RT (min)	Compound	Males ($n = 22$) mean ± SE	Females ($n = 9$) mean ± SE
26.0	Tetradecanal	0.05 ± 0.03	–
27.7	Hexadecenal	2.06 ± 0.44	0.75 ± 0.04
28.2	Pentadecanal	0.21 ± 0.04	0.18 ± 0.18
29.9	Pentadecanol	0.09 ± 0.02	0.95 ± 0.62
30.4	Hexadecanal	1.42 ± 0.46	0.81 ± 0.24
30.9	Unidentified terpenoid	–	0.09 ± 0.09
31.4	Hexadecenol	0.01 ± 0.01	–
31.6	Hexadecenal	0.01 ± 0.01	–
31.9	Hexadecanol	4.95 ± 0.81	4.74 ± 0.84
32.1	2-Heptadecanone	0.22 ± 0.06	–
32.4	Heptadecanal	0.02 ± 0.02	–
32.6	14-Methyl-hexadecanoic acid, methyl ester	0.03 − 0.03	0.17 ± 0.17
33.1	9-Hexadecenoic acid	0.04 ± 0.03	–
33.3	Hexadecanoic acid	4.28 ± 0.27	–
33.9	Octadecenal	0.10 − 0.06	–
34.4	Octadecanal	2.38 ± 0.74	1.75 ± 0.41
34.4	1-Methylethyl ester-hexadecanoic acid	0.11 ± 0.09	1.28 ± 0.52
35.7	Octadecanol	0.58 ± 0.11	0.45 ± 0.45
35.9	2-Nonadecanone	0.40 ± 0.12	0.40 ± 0.40
36.0	5-Dodecyldihydro-2(3H)-furanone	0.14 ± 0.04	–
36.3	Nonadecanal	0.04 ± 0.04	–
36.7	9-Octadecenoic acid	1.48 ± 0.30	–
37.1	Octadecanoic acid	22.09 ± 1.77	–
37.9	6-Octadecenoic acid	4.37 ± 1.60	–
38.0	Eicosanal	0.62 ± 0.34	–
39.2	Eicosanol	0.14 ± 0.11	0.27 ± 0.27
39.4	2-Heneicosanone	0.05 ± 0.05	–
40.5	Eicosanoic acid	6.68 ± 2.87	–
41.5	Docosenoic acid	2.40 ± 1.69	–
42.6	Docosanol	0.09 ± 0.09	1.11 ± 1.11
47.3	Squalene	1.29 ± 0.79	12.29 ± 8.92
47.9	Cholesta-2,4-diene	0.24 ± 0.17	–
48.1	Cholesta-4,6-dien-3-ol	0.94 ± 0.22	–
48.2	Tetracosanol	–	14.78 ± 12.48
48.4	Cholesta-3,5-diene	0.70 ± 0.13	–
48.7	Unid.Steroid (197,251,355,364,387,400,414)	0.02 ± 0.02	–
49.6	Cholesta-4,6-dien-3-ol, methyl derivative?	0.23 ± 0.18	–
50.0	Unid.Steroid (215,253,376,393)	0.02 ± 0.02	–
50.6	Unid.Steroid (197,251,371,382,390,396)	0.13 ± 0.06	–
50.8	Cholesta-4,6-dien-3-ol, ethyl derivative?	0.51 ± 0.24	–
51.0	Cholesta-3,5-diene, ethyl derivative?	0.33 ± 0.13	–
51.4	Cholesterol	24.94 ± 2.96	36.90 ± 3.72
51.5	Cholestanol	–	3.64 ± 0.06
51.9	Cholestan-3-one, methyl derivative?	–	1.44 ± 0.46
52.0	Cholesta-5,24-dien-3-ol (=Desmosterol)	1.60 ± 0.20	–
52.8	Campesterol	2.41 ± 0.22	1.64 ± 0.08
53.0	Cholest-4-en-3-one	0.76 ± 0.22	5.41 ± 1.61
53.2	Stigmasterol	1.31 ± 0.18	–
53.3	Ergost-22-en-3-ol	0.02 ± 0.02	–
53.4	Cholesta-4,6-dien-3-one	0.74 ± 0.21	1.28 ± 1.28
53.7	Unid.Steroid (211,253,271,351,377,411)	0.06 ± 0.06	–
53.8	Hexadecyl 9-hexadecenoate	0.15 ± 0.15	1.58 ± 0.51
53.9	Sitosterol	6.11 ± 0.92	–
54.0	Hexadecyl hexadecanoate		2.33 ± 0.65
54.1	Stigmastanol	1.30 ± 0.21	–
54.4	9-Octadecenyl 9-hexadecenoate	0.05 ± 0.05	–
54.4	20-Methyl-pregn-20-en-3-ol	0.09 ± 0.06	–
54.8	Ergostenol, methyl derivative?	0.41 ± 0.11	–
54.9	Octadecyl 9-hexadecenoate	0.13 ± 0.13	0.92 ± 0.92
55.3	Nonadecyl 9-hexadecenoate		1.47 ± 0.71
56.3	Cholesta-4,6-dien-3-one, derivative?	0.21 ± 0.11	–
56.9	Eicosyl 9-hexadecenoate	0.16 ± 0.11	1.34 ± 1.34
57.2	Octadecyl hexadecanoate	0.08 ± 0.07	2.02 ± 0.78

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 compounds. RT: Retention time.

secretions might be directly related to the characteristics, and may be quality, of the diet of each individual lizard (Martín and López, 2015).

The fatty acids that appear in secretions are those usually found in other lizards (Weldon et al., 2008; Martín and López, 2014). However, the high relative proportion of octadecanoic acid in males is notorious, when comparing it with most of other lacertid lizard species, where hexadecanoic acid is more abundant (e.g. López and Martín, 2005b; Martín and López, 2006c). Nevertheless, this fatty acid is also the most abundant one in secretions of the lacertid lizard *Psammodromus algirus* (Martín and López, 2006d). *Psammodromus* is considered the genus most phylogenetically related to the genus *Gallotia*, both forming the subfamily Gallotiinae, which shows a great genetic divergence from the rest of lacertids (Arnold, 1973; Mayer and Pavlicev, 2007). This suggests that phylogenetic differences *per se* between lizard species might account for a high percentage of the observed interspecific variability in femoral secretion composition. Nevertheless, octadecanoic acid is not so especially abundant in other species of the genus *Gallotia* from others of the Canary Islands (J. Martín unpubl. data), suggesting that the environment also modulates the compounds found in lizards' secretions.

Interestingly, in the related *P. algirus*, individual males with lower blood parasite loads and greater immune responses (i.e. with a better health state) have femoral secretions with lower proportions of two carboxylic acids (octadecanoic and eicosanoic acids) and higher proportions of their correspondent alcohols (octadecanol and eicosanol) (Martín et al., 2007a). Moreover, female *P. algirus* discriminate these compounds and respond with higher chemosensory rates to secretions of healthier males, suggesting that the scents of these males are more attractive (Martín et al., 2007a). A similar, yet unexplored, signaling function of these compounds might occur in *G. simonyi*, which prompts for the design of future experiments to unravel the function of femoral secretions in this lizard. Also, data from femoral secretions composition might complement the already-known physiological parameters and be useful in future studies to estimate in an innocuous way the health state of lizards in both wild and captive populations of this highly threatened species.

In most species of lizards with femoral glands, females also possess femoral pores, but they are usually much smaller and with very little or without apparent secretion (Mason, 1992). Femoral secretion of female *G. simonyi* is also less abundant than that of males, but the large body size of this lizard allowed us to collect and analyze it. The chemical analysis showed important differences in composition, with females having a lower number of compounds than males and with only a few of them being exclusive of females. But intersexual differences did not arise just because the lack of some compounds in females, but also because some of the main shared compounds and the different major types of compounds had very different proportions. In contrast, in the other lacertid, *Acanthodactylus boskianus*, where secretions of females have been analyzed, the same compounds are found in both sexes, although there are some small differences in proportions of compounds (Khannoon et al., 2011a). Nevertheless, it is possible that the lack of many compounds in our sample of female *G. simonyi* might be explained by seasonal differences in the production of femoral secretions of females, as it has been observed in iguanian lizards (Alberts, 1990; Kelso and Martins, 2008), although the specific secreted compounds were not analyzed in these species.

Intersexual differences in *G. simonyi* allowed predicting statistically the sex of an individual based on its chemical profile, and probably also allow lizards to easily discriminate by chemosensory cues the sex of conspecifics. The occurrence of this chemosensory ability has not been experimentally tested in this species, although it is expected based on the ability of *G. simonyi* to discriminate chemical cues of its prey (Cooper and Pérez-Mellado, 2001) and on the well known sex discriminatory abilities of most lacertid lizard species (Mason and Parker, 2010; Martín and López, 2011). Sex discrimination based on chemical cues could be especially important in intraspecific relationships of this species where both sexes are similar in coloration, being both black with some yellow ocelli, only differing in average body size and relative head size (Rodríguez-Domínguez et al., 1998).

Among the most important intersexual differences, females had high proportions of compounds that would confer a high stability to the secretions, notably a high proportion of a waxy alcohol (i.e. tetracosanol), that occurred only in females, and more cholesterol, squalene and waxy esters than males. In contrast, females lacked or had lower proportions than males of the more odoriferous compounds, such as fatty acids and aldehydes, which could be used in intraspecific communication at short-range. This suggests an entirely different role of femoral secretions in males and females of this lizard that merits further studies.

The results of this study suggest that if compounds in secretions of this lizard could reflect their physiological health state, future studies should establish which reliable relationships could be inferred from the chemical study of femoral secretions. This could allow monitoring the health state of lizards from a simple sample of their femoral secretions, avoiding other more invasive techniques such as blood sampling, which is especially important given the highly threatened status of *G. simonyi*.

Acknowledgements

We thank Elena Fernández and Luis Cuadra for technical assistance with chemical analyses. Financial support was provided by the project MICIIN-CGL2011-24150/BOS. Lizards were handled and sampled thanks to special permits from Cabildo de El Hierro.

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