



Chemical differences in femoral gland secretions of arboreal and terrestrial tropical lizards from western Mexico

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Abstract. Chemical signals in animals stimulate different links and relationships. In lizards, chemical signals play an essential role in reproductive and social communication. Although lizard species inhabit tropical ecosystems in great diversity, the chemical composition of the femoral gland secretions that tropical lizards use for communication is poorly known. In this work, we used gas chromatography-mass spectrometry (GC-MS) to identify and compare the main compounds contained in the femoral gland secretions of the males of three species of arboreal lizards, *Hemidactylus frenatus*, *Sceloporus melanorhinus*, and *Urosaurus bicarinatus*; and of two species of terrestrial lizards, *Aspidoscelis lineatissimus* and *Sceloporus pyrocephalus*. We identified twelve lipophilic compounds contained, corresponding to fatty acid esters (~58%), carboxylic fatty acids (~25%), and other compounds (~17%). Carboxylic acids, mainly with esters content, revealed significant differences between lizard species. The potential role of these chemical compounds to social and sexual behaviours is discussed.

Key words. Chemical signals, femoral glands, interspecific variation, Squamata, tropical lizards.

Introduction

Chemical signals are pivotal information resources for communication in several animal groups (MÜLLER-SCHWARZE 2006, WYATT 2014). The use of chemical signals in both inter- and intraspecific interactions are of major interest to the study of animal behaviour (MARTÍN & LÓPEZ 2014, WYATT 2017). In reptiles, for example, some of these semiochemicals contribute to the control of social and reproductive behaviours (HOUCK 2009, MASON & PARKER 2010, MARTÍN & LÓPEZ 2011).

In recent years, lizards have become a popular model group to study the composition and function of vertebrate chemical signals (e.g., BAECKENS 2019). Lizards produce and release several chemical compounds through the skin or specialized body glands (WELDON et al. 2008). For example, many species have femoral or precloacal glands (MARTÍN & LÓPEZ 2011, KHANNOON 2012, VALDECANTOS et al. 2014). These glands produce holocrine secretions that are released through epidermal pores, and are constituted mainly of lipids and proteins (MARTÍN & LÓPEZ 2015, MANGIACOTTI et al. 2019). Both male and female lizards have femoral (or the analogous precloacal) pores, but in many species, secretions are only observed in males, which

have more abundant secretions during the mating season (MARTÍN & LÓPEZ 2015).

Although tropical ecosystems are home to a great diversity of lizard species (PIANKA & VITT 2003), the semiochemicals that many lizards use for communication are understudied compared to lacertid species from Mediterranean environments (GARCÍA-ROA et al. 2016, BAECKENS et al. 2018).

In the present study, we identify the lipophilic fraction of chemical compounds in the femoral secretions of males of three arboreal and two terrestrial lizard species from a tropical area of western Mexico, and evaluate the existence of interspecific differences in the relative abundance of each group of chemical compounds. We discuss our results with previously reported chemical compounds in other species from similar or different environments.

Materials and methods

Study area and lizard species

During fieldwork carried out between May and June 2017, coinciding with lizards' mating seasons (RAMÍREZ-BAUTISTA et al. 1995, 2000, 2006, RAMÍREZ-BAUTISTA &

OLVERA-BECERRIL 2004, DÍAZ et al. 2017), we captured by loop adult males of five species in the vicinity of Maruata Bay in the Michoacán State on the west coast of Mexico (18.271944° N, -103.355556° W). This area is characterized by a dry tropical climate with an average annual temperature of 28°C and a long annual dry season from November through June (ALVARADO et al. 1995). The dominant vegetation is dry deciduous forest (RZEDOWSKI 1978, ALVARADO et al. 1995). There are also patches of xerophilic vegetation, herbaceous communities in coastal dunes, and stands of introduced palm species and banana trees. Ten common house geckos, *Hemidactylus frenatus* DUMÉRIL & BIBRON, 1836, with snout-vent lengths (SVL) = 53.1 ± 1.2 mm, weight = 4.2 ± 0.2 g, and seven black-nosed lizards, *Sceloporus melanorhinus* BOCOURT, 1876, SVL = 73 ± 3.6 mm, weight = 18.6 ± 2.7 g, were captured on coconut palms (*Cocos nucifera*) and oil palms (*Elaeis guineensis*); five tropical tree lizards, *Urosaurus bicarinatus* DUMÉRIL, 1856, SVL = 44.2 ± 1.3 mm, weight = 3.5 ± 0.1 g, were captured on guanacaste trees (*Enterolobium cyclocarpum*) and fallen trunks. Fifteen terrestrial many-lined whiptail lizards, *Aspidoscelis lineattissimus* COPE, 1878, SVL = 90.7 ± 1.5 mm, weight = 22.5 ± 1.2 g, were captured in open areas of tropical deciduous forest and xerophilic vegetation where *Bursera* spp., *Cercidium* spp., *Prosopis* spp., *Acacia* spp., *Opuntia* spp. and *Stenocereus* spp. were the predominant plants. Additionally, nine terrestrial red-headed spiny lizards, *Sceloporus pyrocephalus* COPE, 1864, SVL = 66.6 ± 1.5 mm, weight = 14.8 ± 0.8 g, were captured in saxicolous habitats and around rock quarries with little or no xerophilic vegetation present (Fig. 1).

Analyses of lizard femoral gland secretions

Immediately after capture, femoral secretions were obtained by gently squeezing the femoral pores with forceps. Forceps were washed with alcohol and distilled water between extractions. Femoral secretions were collected with glass inserts and immediately placed inside sterilized glass vials that were closed with Teflon-lined stoppers. New glass inserts were used for every sample. Vials were stored at -20°C until analysis. We also used the same procedure, but without collecting secretions, to obtain blank control vials that were treated with the same procedure to compare with the femoral secretion samples, and to be able to exclude contaminants from the handling procedure or from the environment, and for further examining impurities in the solvent.

Solvent extraction of the solid samples was performed adding 200 µl of dichloromethane (Sigma). Samples were placed in a vortex for 5 min, then centrifuged for 5 min for particle-precipitated elimination (insoluble portion) and then stored at -20°C. The solvent-extract phase was collected and transferred to appropriate vials for GC-MS analysis.

The samples were analysed by GC-FID and GC-MS (GC; Agilent 7890A, equipped with MSD 5975C and FID detectors; Agilent Technologies), fitted with a fused-silica cap-

illary column (Zebron ZB-FFAP, 30 m length X 0.25 mm ID, 0.25-µm film thickness; Phenomenex) with helium as the carrier gas. 2 µl of each sample dissolved in dichloromethane were injected in splitless mode. Sample injection was performed at splitless temperature of 280°C and an FID detector temperature of 300°C. Oven temperature was programmed to start at 50°C, maintained in isothermal for 5 min, then increased to 280°C at a rate of 10°C/min, and then isothermal 280°C for 5 min. To analyse using both the MS and FID detectors, at the exit of the capillary column, a continuous-flow particle separation (50:50) was installed. The mass spectrometer was operated at an ionization voltage of 70 eV and with scanning between m/z 30–500 at 3.9 scans/s. Quantification was performed using the relative values of the peak areas in the chromatograms obtained by using an FID detector. Compounds were identified by comparison with the mass spectral library NIST/EPA/NIH (ChemStation, Agilent Technologies, rev. D.04.00 2014). The synthesized compounds used for GC-MS identification consisted of fatty acids (palmitic and stearic acids) and esters (n-butyl acetate) acquired from Sigma-Aldrich. Other synthesized compounds such as the esters isobutyl acetate, methyl laurate, isopropyl myristate, methyl palmitate, methyl palmitoleate, and methyl oleate were obtained by chemical synthesis in our laboratory using the respective chemical precursors. Precursor compounds were acetic acid, methanol, ethanol, isopropanol, isobutanol, lauric acid, myristic acid, palmitic acid, palmitoleic acid, oleic acid, and stearic acid (all from Sigma-Aldrich, Merck, or T.J. Baker). Briefly, esters synthesis was carried out by esterification of their respective alcohols with fatty acids treated with H₂SO₄ and KMnO₄ at 130°C for 30 min (CAMPOS-GARCÍA et al. 2018). All the ester compounds were extracted with dichloromethane solvent, evaporated, weighed, and dissolved in dichloromethane for GC-MS analysis. Additionally, FAME Mix C4-C24 (catalogue no. 18919, Supelco) were used as standards.

Statistical analyses

The proportion of each peak identified in the GC chromatograms was identified by calculating the relative proportion of each compound (area under the peak) with respect to the total peak area for all chemicals (RAYA-GARCÍA et al. 2020). Then, relative areas of the peaks were transformed following Aitchison's formula: $[Z_{ij} = \ln(Y_{ij}/g(Y_j))]$, where Z_{ij} 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). Then, we calculated Euclidean distances between every pair of individual samples to produce a resemblance matrix that formed the basis of the analyses. We used a single factor permutational multivariate analysis of variance test PERMANOVA (ANDERSON 2001, MCARDLE & ANDERSON 2001) based on the Euclidean resemblance matrix using 999 permutations to analyse whether the composition of the femoral secretions varied between

the five species. All unidentified and identified compounds were included in the analysis. Differences between species were further investigated using canonical analysis of principal coordinates (CAP) (ANDERSON & WILLIS 2003). The software PRIMER V6.1.13 (CLARKE & GORLEY 2006) with the PERMANOVA V1.0.3 add-on package (ANDERSON et al. 2008) was used to investigate differences between chemical profiles.

In addition, we assessed if there were statistical differences in the relative amount (area under the peak) of each compound class among species by fitting negative binomial GLM analysis (GARDNER et al. 1995). Pairwise comparisons were each performed with a Tukey HSD test. All statistical analyses were performed with the interface Rstudio in R software version 3.3.2 (R Development Core Team 2013).

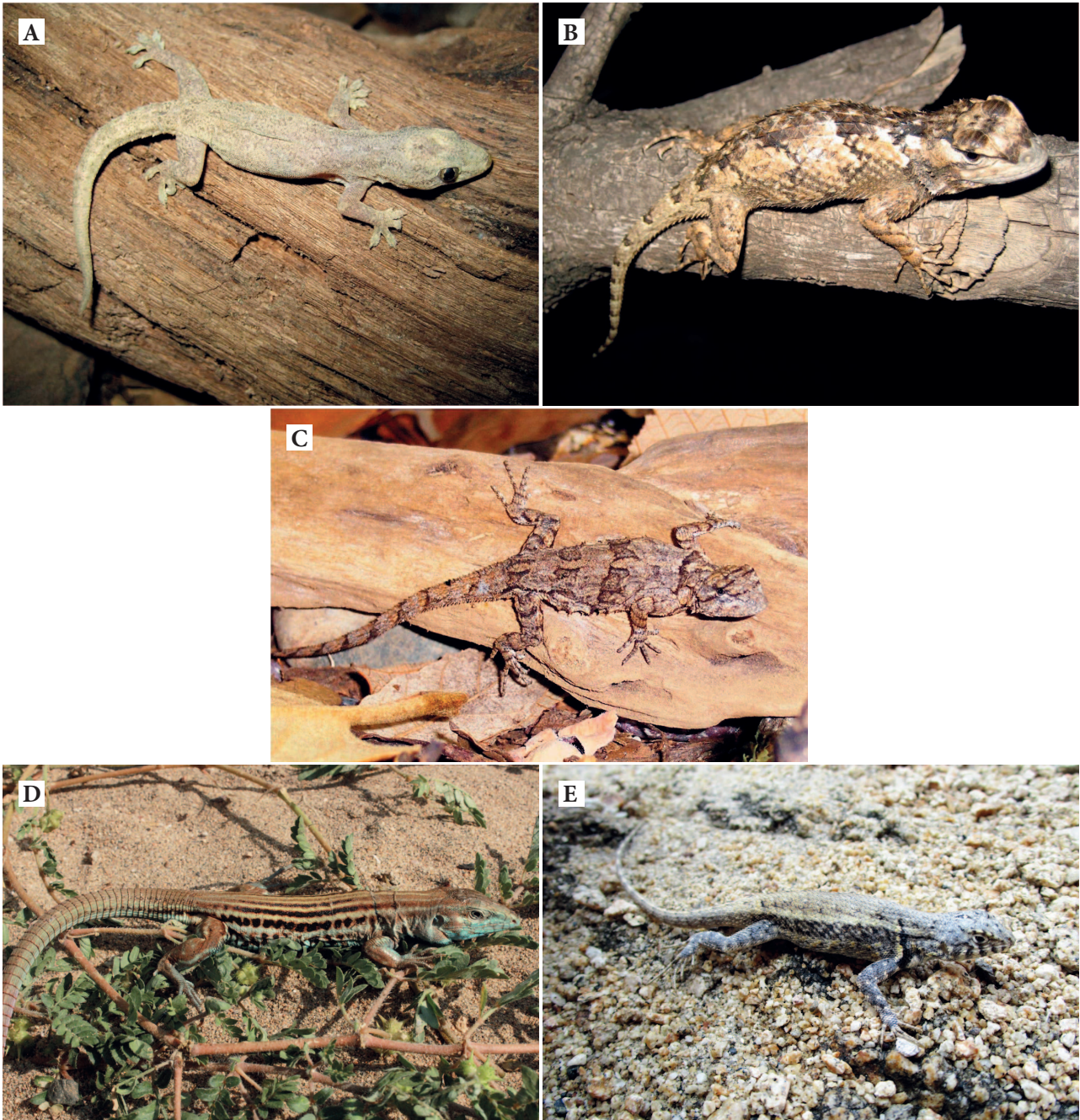


Figure 1. Species of lizards in their habitats in the tropical Maruata Bay: (A) *Hemidactylus frenatus*, (B) *Sceloporus melanorhinus*, (C) *Urosaurus bicarinatus*, (D) *Aspidoscelis lineattissimus*, and (E) *Sceloporus pyrocephalus*.

Table 1. Chemical compounds identified in femoral gland secretions of arboreal and terrestrial lizard species: Relative proportions (mean \pm SE of the percentage of the total peak areas from FID detector). nd = not detected. RT = retention time and retention index. Compounds without standard confirmation (*).

RT (min)	Compound name	R index	Arboreal lizards			Terrestrial lizards	
			<i>H. frenatus</i>	<i>S. melanorhinus</i>	<i>U. bicarinatus</i>	<i>A. lineattissimus</i>	<i>S. pyrocephalus</i>
Esters							
5.2	Isobutyl acetate	1012	0.55 \pm 1.32	7.01 \pm 9.78	13.27 \pm 4.69	9.61 \pm 1.65	nd
6.4	n-Butyl acetate	1074	nd	nd	0.22 \pm 3.70	0.15 \pm 1.05	0.24 \pm 5.51
19.3	Methyl laurate	1804	1.46 \pm 1.43	31.39 \pm 6.33	1.52 \pm 5.00	1.28 \pm 0.74	1.32 \pm 5.00
22.3	Isopropyl myristate	2027	0.21 \pm 2.32	0.20 \pm 6.19	0.42 \pm 1.40	0.36 \pm 2.13	0.80 \pm 17.77
24.5	Methyl palmitate	2208	nd	0.23 \pm 3.59	0.97 \pm 2.14	0.56 \pm 1.03	nd
24.7	Methyl palmitoleate	2240	2.19 \pm 2.69	2.02 \pm 8.32	1.23 \pm 8.54	1.12 \pm 0.65	nd
27.0	Methyl oleate	2434	nd	nd	nd	25.64 \pm 1.2	nd
Carboxylic acids							
14.1	Acetic acid	1449	1.35 \pm 1.17	0.64 \pm 3.70	1.52 \pm 7.67	1.18 \pm 0.22	4.96 \pm 1.59
31.4	Palmitic acid	2931	39.95 \pm 3.04	25.44 \pm 6.44	42.62 \pm 8.83	25.98 \pm 2.50	92.68 \pm 5.50
33.6	Stearic acid	3134	19.27 \pm 2.46	11.35 \pm 7.13	0.21 \pm 1.10	10.89 \pm 0.90	nd
Others							
33.1	t-Squalene*	2865	35.02 \pm 3.17	21.72 \pm 7.00	38.02 \pm 8.56	22.93 \pm 2.10	nd
41.3	Dihydro-lanosterol*	3823	nd	nd	nd	0.29 \pm 2.15	nd

Results

Chemical profiles of lizard species

We found eight lipophilic compounds in femoral gland secretions of male lizards *H. frenatus* (Table 1), which were constituted mainly of fatty acid esters corresponding to 4.4% of total area from all peaks found, three fatty acids (acetic, palmitic, and stearic) corresponding to 60.6%, and 35% of squalene. The three most abundant chemicals, which together comprised > 90% of the total peak areas, were palmitic and stearic acid and squalene (Table 1).

Femoral gland secretions of male *S. melanorhinus* contained a mix of nine compounds that could be identified by mass fragmentation profiles (Table 1). Overall, these compounds consisted mainly of esters at 40.9%. These were mainly of two types: esters of acetate, such as isobutyl acetate and esters from fatty acids, such as methyl laurate. The three most abundant chemicals, which together comprised > 70% of the total peak areas, were methyl laurate, palmitic acid and squalene (Table 1).

In the gland secretions of *U. bicarinatus* males, ten compounds including esters at 17.6%, three carboxylic acids at 43.4%, and others such as squalene at 38% were found. The three most abundant chemicals, which together comprised > 90% of the total peak areas, were isobutyl acetate, palmitic acid and squalene (Table 1).

Femoral gland secretions of *A. lineattissimus* males contained a mix of twelve compounds, including esters at 38%, carboxylic acids at 38%, and others such as squalene and dihydro-lanosterol at 24%. The five most abundant chemicals, which together comprised >90% of the total peak areas, were isobutyl acetate, methyl oleate, palmitic acid, stearic acid and squalene (Table 1).

We recorded the presence of five compounds in the femoral gland secretions of *S. pyrocephalus* males, including three esters (2.4%) and two carboxylic acids (97.6%). The most abundant chemical was palmitic acid (Table 1).

Chemical variation between species

The PERMANOVA analysis based on the resemblance matrix comparing individuals of each species exhibited significant differences in the proportions of compounds between the five evaluated species (pseudo $F_{4,41} = 7.35$, $p = 0.001$). The pairwise comparisons revealed significant differences in all cases (permutation tests, $2.06 < t < 3.60$, $p = 0.001$ for all). The CAP analysis allocated 100% of the chemical profiles to the correct species when using leave-one-out cross-validations and $m=6$ axes (permutation test, $\delta_1^2 = 0.982$, $p = 0.001$, Fig. 2).

With regard to the five species examined, carboxylic acids, fatty acid esters and squalene were the most abundant chemical types, with palmitic acid being the main and most abundant compound. We found significant differences in the relative proportions of carboxylic acids between our study species (GLM; $\chi^2 = 9.63$, $df = 4$, $p < 0.001$) but not in palmitic acid ($\chi^2 = 1.81$, $df = 4$, $p = 0.14$). Pairwise comparisons showed that *H. frenatus* and *S. pyrocephalus* have lower proportions of carboxylic acids than *A. lineattissimus* and *S. melanorhinus* (Tukey's test, $p < 0.01$ for all), but *U. bicarinatus* did not differ significantly from any other species ($p = 0.15$). Squalene, although absent in *S. pyrocephalus*, was the second most abundant compound (29%) in the other species. However, we found no significant differences in the relative proportions of squalene between species ($\chi^2 = 2.10$, $df = 3$, $p = 0.11$). Regarding es-

ters, we found significant differences between species ($\chi^2 = 9.79$, $df = 4$, $p < 0.001$), and comparisons revealed that *S. melanorhinus*, *U. bicarinatus* and *A. lineattissimus* have higher proportions of esters than *H. frenatus* and *S. pyrocephalus* ($p < 0.01$ for all), other comparisons did not yield significant differences.

We found some differences in the chemical profiles between arboreal and terrestrial species (Table 1). Arboreal lizards (*H. frenatus*, *S. melanorhinus*, and *U. bicarinatus*) shared eight compounds, whereas the terrestrial lizards (*A. lineattissimus* and *S. pyrocephalus*) shared five compounds. Exclusive compounds were found only in *A. lineattissimus* (methyl oleate and dihydro-lanosterol), while the remaining species did not have exclusive compounds. Both groups of species, arboreal and terrestrial, shared four compounds; two esters and two carboxylic acids (Table 1).

Discussion

Our study reports fatty acids, esters, and squalene as the main chemical compounds in the femoral gland secretions of five tropical lizard species (ALBERTS et al. 1992, MARTÍN et al. 2013a, b, IBÁÑEZ et al. 2017). This composition is similar to those recorded from other lizard's species. For example, hexadecanoic acid (palmitic acid) was the main fatty acid and the most abundant compound present in both terrestrial and arboreal lizards. Other long-chain carboxylic acids, such as octadecanoic acid (stearic acid), were found in all studied species except *S. pyrocephalus*; but palmitic acid was found in higher proportions than in other lizards. Fatty acids of long-chain and very-long-chain types have been reported from other lizards from arid and desert ecosystems (LÓPEZ & MARTÍN 2005a, MARTÍN et al. 2012) and have a functional role as territorial scent markers (KHANNOON 2016). It is probable that one or more carboxylic acids in the secretions of our study lizards facilitate the

development of similar social contexts, but this is as yet an information gap that needs to be addressed.

Some lizards inhabiting high mountain areas with low temperatures and high humidity frequently have greater relative proportions of esters in their femoral gland secretions than lizards inhabiting low-altitude areas (GABIROT et al. 2012, MARTÍN et al. 2017). This ester variation is explained as an adaptation to maximize efficacy of substrate scent marks under different microclimatic conditions (MARTÍN et al. 2015, BAECKENS et al. 2018). In our study, lizards from tropical climates with high temperatures and humidity produced relatively higher amounts of esters (5–42%) than the high-mountain lizard *Psammotromus algirus* (5.5% of esters) from Mediterranean climates (MARTÍN et al. 2017). Possibly based on its chemical properties, esters are more volatile and prone to oxidation than carboxylic acids in environments with tropical climates. Therefore, it is possible, but has not as yet been conclusively demonstrated, that more humid habitats are conducive to higher proportions of esters due to accelerated hydrolysis.

Although in relatively low proportions, squalene is a common compound in femoral secretions of many lizard species (WELDON et al. 2008). This chemical is considered an antioxidant that will enhance the signalling function of other compounds (MARTÍN & LÓPEZ 2014). As squalene might stabilize other lipid compounds by limiting oxidation (GABIROT et al. 2008), its abundant presence in four of the five studied species may be an adaptation to the usually high humidity of their tropical environment. Squalene's role as a pheromone has only been tested in fossorial amphisbaenians, and it has been considered an important component in antagonistic and sex-discrimination behaviours (LÓPEZ & MARTÍN 2005b).

Although diverse steroids are frequently present in the femoral secretions of lizards (WELDON et al. 2008), in our study only one steroid, the lanost-8-en-3 β -ol (dihydro-lanosterol), was found in one terrestrial species (*A. lineattissimus*). Cholesterol and some of its derivatives are the most important steroids recorded in almost all lizard species (MARTÍN et al. 2015). However, there are other steroids of plant origin (phytosterols), one example of these is lanosterol, abundant in the femoral gland secretions of the green iguana, *Iguana iguana*, suggesting a possible relationship between diet and the chemical characteristics of gland secretions (ALBERTS et al. 1992, BAECKENS et al. 2017). Similar to lanosterol, dihydro-lanosterol, present in *A. lineattissimus*, is a phytosterol that could possibly have some relationship with the food spectrum of this species.

In conclusion, we identified a significant diversity of lipophilic compounds in the femoral gland secretions of arboreal and terrestrial species of tropical lizards and clear interspecific differences in the fatty acids and esters they contain. Some of the compounds reported in this study have been described as semiochemicals with signalling function (pheromones) involved in the sexual communication of some lizard species, but their behavioural role in tropical lizards remains to be confirmed through future research.

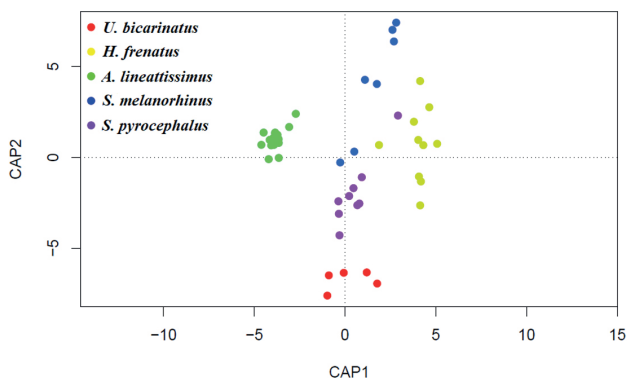


Figure 2. Canonical analysis of principal coordinates (CAP), showing classification of chemicals identified in femoral secretions of three arboreal *H. frenatus*, *S. melanorhinus*, and *U. bicarinatus*; and two terrestrial *A. lineattissimus* and *S. pyrocephalus* lizard species.

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