**Chemical characterization of the lipids in femoral gland
secretions of wild male tegu lizards, *Salvator merianae*
(Squamata, Teiidae) in comparison with captive-bred males**

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Abstract

Although chemical interactions play an essential role in lizard social behavior, the chemical composition of the femoral gland secretions that many lizards use for communication is known for only a few species, mainly European Lacertids. The tegu lizard, *Salvator merianae*, is the only species of the Teiidae family for which there is available information on lipids in femoral secretions, but only for captive bred males from Argentina. Here, based on mass spectra obtained by GC-MS, we found 69 lipophilic compounds in femoral gland secretions of wild males *S. merianae* from Brazil, including cholesterol and high amounts of saturated fatty acids (mainly hexadecanoic and octadecanoic). We found contrasting differences between wild and captive-bred males, which lack cholesterol but present high amount of 9,12-octadecadienoic acid. These within-species differences between wild and captive lizards strongly suggest the important influence of different diets on the chemical composition of the femoral gland secretion and suggest caution when interpreting results from captive animals, even in the same species.

Keywords: chemical communication, intraspecific variation, semiochemicals.

Introduction

Chemical interactions play an essential role in many social contexts of lizards and are mediated by different chemical compounds (semiochemicals) [1], especially from the femoral and precloacal gland secretion [2]. These glands are holocrine, their activity is higher during the mating season [2, 3] and the compounds secreted seem to be used in self- and familiar recognition [4, 5] and to signal health-status [6], size/age [7, 8] and/or fighting ability [9].

The lipophilic chemical profiles of femoral gland secretions vary widely but consistently between species [10] depending on diet [11, 12], climatic conditions [13, 14], or genetic differences [15]. However, these lipids have been characterized for relatively few lizard species, mainly European lacertids [10, 14], while for other families of South American lizards there are only studies with some *Liolaemus* species [16–18] and *Salvator merianae* [19].

The tegu, *S. merianae* is a large teiid lizard with a wide distribution along most cisandean South America [20]. In the southern part of its distribution, it spends the cold season hidden inside burrows [21]. Males and females have 41-52 femoral pores [22], but only males show femoral secretion [23], which merge as a solid cylinder with keratin layers and surrounded by epithelial cells [23]. There are no data about the role of chemical communication in reproduction of *S. merianae*, but it is likely important as males are actively involved in scent-marking territories [23] and use tongue-flicking during courtship [24].

The lipids in femoral gland secretion of male *S. merianae* were previously characterized based on samples collected from captive bred animals in Argentina during

the mating season [19]. This study showed a discrepant chemical profile when compared it with those of most species of lacertids, with *S. merianae* having very high amounts of fatty acids of 16 and 18 carbons and a total absence of cholesterol [19]. Cholesterol is, however, the major compound of the femoral gland secretion of many species of lizards [10]. To explain the absence of cholesterol, it was suggested that the keratin sheets and epithelial layers that surround the secretions of *S. merianae* [23] might have a role similar to cholesterol in protecting semiochemicals in substrate scent-marks [19]. However, there is no information on lipids in secretions of wild *S. merianae* animals. This is important because, as explained above, there are many factors that can affect the chemical composition of the femoral secretions in lizards and, captivity condition could mask the “true” composition of secretions in the wild.

In this study, we generated new information about the chemical composition of femoral secretions of wild range male *S. merianae* from natural habitats of Brazil. We aimed to understand whether the data from captive-bred animals can be a “reliable representation” of the chemical profiles of a lizard species. This is especially important for studies that could use chemical data of captive animals to understand the evolution of lizard diversity. We found some important differences with respect to the previously described chemical profile of captive bred lizards [19] and discuss how diet or other variables could explain these differences.

Material and Methods

We collected femoral gland secretions from four adult male *S. merianae*, captured using a polyvinyl chloride (PVC) funnel trap (15 cm x 1 m), during January and February

2016 at Fernando de Noronha, Pernambuco, Brazil (3°50'S, 32°25'W) (SISBIO 41682-6), and from one male captured in a pitfall trap during March 2017 in Parque Nacional da Serra da Capivara, Piauí, Brazil (8°25'S, 42°20'W) (SISBIO 54599-3). This low sample size is explained because *S. merianae* is a widely foraging large lizard, with large home ranges, low population densities and general high wariness, which makes difficult to find and capture adult specimens in natural conditions [25]. This is also the reason why an important part of our knowledge about the species comes from captive individuals [24]. The sampling period coincided with the middle of the activity period [21], but there are no specific data on the occurrence of the mating season in our capture sites.

We extracted the secretion cylinders from the femoral pores using clean forceps and stored them at -20 °C until analyses in glass vials closed with Teflon-lined stoppers. We also used the same procedure, but without collecting secretion, to obtain blank control to exclude potential contaminants from the handling procedure and impurities from the solvent. The snout-vent length (SVL) was measured and the lizards were released in perfect health conditions in their capture sites a few minutes after the extraction of the secretions. The SVL when sexual maturity is reached can be different for different populations of *S. merianae*, but it seems to be around 24-27 cm [26, 27].

We analyzed the samples with a ThermoQuest (Austin, TX, USA) Trace 2000 gas chromatograph fitted with a poly (5% diphenyl/95% dimethylsiloxane) column (Trace TR-5, 30 m in length, 0.25 mm ID, 0.25 µm film thickness; ThermoFisher Scientific Inc., Waltham, MA, USA) and a ThermoQuest Trace 2000 mass spectrometer as detector. We added 50 µL of hexane in each vial and 2 µL of the samples were injected in the splitless mode with an inlet temperature of 280°C. The gas

chromatograph was programmed so that the oven temperature was kept at 50 °C for 3 min, increased to a final temperature of 300 °C at a rate of 5 °C/min, and kept at this temperature for 15 min. The carrier gas was helium at 30 cm/s. Ionization by electron impact (70 eV) was carried out at 220 °C. Impurities identified in the solvent and/or in the control vial samples are not reported. A tentative identification of secretion components was performed by comparing the sample mass spectra with those in the NIST 14 computerized mass spectral library. The relative quantity of each chemical compound was determined as the percentage of the total ion current (TIC) [28].

We made a qualitative comparison between our data from wild males and those previously published from captive-bred males from Argentina [19]. Giving the striking differences between them (see results below), we summarized the data in a graph, comparing the main chemical compounds of captive males with those of the three wild male individuals with higher amounts of fatty acids in secretions.

Results

A total of 69 lipophilic compounds were tentatively identified in femoral gland secretions of wild male *S. merianae* (Table 1). Pooling all samples, we found 19 sterols (44.11% of TIC area), 11 fatty acids (35.82%), 10 esters of fatty acids (6.21%), 16 aldehydes (5.25%), four amides (4.72%), squalene (1.76%), four forms of tocopherol (0.8%), 4,8,12,16-tetramethyl-heptadecan-4-olide (0.72%), one alcohol (0.36%) and two ketones (0.25%). The proportions of the major chemical classes seemed to vary between body size/age classes; the two smaller males (SVL < 31 cm) showed as main components sterols (TIC > 50.92%) and esters of fatty acids ranging between *n*-C₁₆ and *n*-C₂₀ (TIC > 5.73%) (Figure 1). On the opposite, three large (older) males (SVL > 34

cm) showed as main components fatty acids ranging between n -C₁₀ and n -C₂₀ (between 37.84% and 76.39%) and sterols (between 18.14% and 45.54%) (Figure 1). Cholesterol was one of the most abundant compounds in all individuals (between 8.19% and 41.88%) (Figure 2). The fatty acids, hexadecanoic (between 8.47% and 17.3%), 9,12-octadecadienoic (2.09%-5.19%) and octadecanoic (21.23%-59.08%) were only found in the males with SVL larger than 34 cm (Figure 2). The 9-octadecenoic acid was found in three males in low quantities (< 3%).

In captive-bred males, 49 lipophilic compounds were identified [19], 31 of which were shared with those found in wild males in the current study. The 18 compounds that were found exclusively in captive-bred males were mainly some unidentified sterols, but cholesterol was not found in any captive individual. In contrast, cholesterol was relatively abundant in all wild males (Figure 3). The octadecanoic acid was abundant in captive males (mean = 57.8%) and in the three larger wild males (Figure 3). However, captive-bred males showed much higher amounts of 9,12-octadecadienoic acid (mean = 26.3%) and lower amounts of hexadecanoic acid (5.2%) than wild males (Figure 3).

Discussion

The analysis of femoral gland secretions of wild male *S. merianae* showed contrasting differences from those previously found in captive bred males [19]. The differences, however are not related to different analytical procedures, as the equipment and the methods used were very similar in both studies.

Some unidentified sterols were exclusively found in captive *S. merianae* are, but in general steroids were little represented (3.64 %) [19], whereas these compounds are

the most abundant in many lizard species [10], and also in wild male *S. merianae*. It was surprising to find cholesterol in all the wild males' samples, since this sterol was absent in captive bred males [19], suggesting that this absence is not a natural feature of the species in the wild. The morphology of the femoral plug is the same in captive and wild males, suggesting that cholesterol may have another function than just be an unreactive matrix to the true semiochemicals [17]. In some lacertid lizards, cholesterol is related with fighting ability [7, 9] and age [29], which use to be related to body size [30]. However, in wild male *S. merianae* the proportion of cholesterol was not related to size/age, and cholesterol was not found in the bigger captive-bred animals analyzed [19].

In wild male *S. merianae*, individuals with SVL lower than 34 cm did not show high amounts of fatty acids (sum of TIC < 2.5%) but showed high amounts of steroids (> 50%). In contrast, older (bigger) wild males showed higher amounts of fatty acids (> 37%) and lower amounts of steroids (< 46%). This suggests that an ontogenetic increase in the amount of fatty acids, which are volatile and odoriferous compounds [31], could be related with a decrease in the amounts of steroids, which are more stable compounds [17]. Similarly, in green iguanas, males in the breeding season show femoral secretions with more unsaturated fatty acids, which are highly volatile, than during the nonbreeding season [32], which is related with changes in androgen levels and dominance [33].

The diet is an important factor that may contribute to quantitative and/or qualitative differences in the chemical signals of many animals [34]. In lizards, although diet does not seem to explain interspecific differences in chemical composition of the femoral secretion in lacertid lizards [11], at intraspecific level, the diet can significantly modify femoral gland secretions [12, 35 – 37]. For example, in male rock lizards,

Iberolacerta cyreni, vitamin E was only found in the femoral secretions of individuals that had received an experimental supplementary diet with this specific compound [12]. Similarly, in green lizards, *Lacerta viridis* and *L. schreiberi*, a supplementary diet with vitamin E increased the proportions of this vitamin, which was already naturally found in secretions [37, 38]. While wild male *S. merianae* in natural conditions, eat fruits, invertebrates and small vertebrates [39, 40], captive animals were fed a special diet consisting of poultry by-products (chicken head and legs) complemented with meat meal, soybean meal, and a supplement of vitamins, minerals and antioxidants [41]. These different diets could be a possible explanation for, at least, some of the contrasting differences in femoral secretions between wild and captive lizards.

Wild male *S. merianae* showed four different forms of tocopherol, including vitamin E, although in low proportions ($< 0.55\%$), while captive-bred males show only two forms of tocopherol and in higher proportions ($< 2\%$). Tocopherol is only synthesized by plants and microorganisms and must be sequestered from the diet [42]. The higher diversity of tocopherol forms in wild males could be a consequence of eating plants in natural conditions, while the higher amounts of α -tocopherol (vitamin E) in captive-bred males could be explained by the supplement of vitamins and antioxidants, but it also could come from the fat of their chicken meat-based diet. In an extensive work examining the role of the diet in shaping the chemical design in lacertids, Baeckens et al. [11] did not find a covariation at interspecific level between diet and chemical signals profiles. However, species that eat plants, at least occasionally, produce secretions of a greater chemical richness than species with a strictly arthropod-based diet. Similarly, our results showed that wild *S. merianae*, which frequently eat fruits and other plants items, have secretion with a higher diversity of

chemical compounds than captive animals that only eat meat. Because the diet of captive males had higher amounts of cholesterol than its natural diet in the wild, we could expect higher amounts of cholesterol in captive-bred males, but we found the opposite result. Not all lipids in the lizards' secretions need to be sequestered from the diet, but some of them may also be biosynthesized by the animal [11] and this biosynthesis processes might also differ between wild and captive condition. Future studies should test whether the diet could be an important factor affecting the chemical secretions of *S. merianae*.

Nevertheless, the diet is not the only factor that could affect the chemical composition of lizards' femoral secretions. Thus, for example, oviparous and viviparous populations of common lizards, *Lacerta vivipara*, showed differences in the relative proportions of some compounds, which may be explained by small genetic differences or by different microclimatic conditions in the original populations [43]. The effect of climatic differences on the efficacy (detectability and persistence) of scent marks may also explain interpopulational differences in secretions of Iberian wall lizards [13] and even may explain interspecific differences among several lacertid lizard species [14]. Furthermore, the chemical composition of femoral secretions may be an important key to reveal the cryptic genetic diversity that remains uncovered in many lizards' groups [15]. Therefore, further studies should also consider the potential variations between the climatic conditions and the genetic differences among *S. merianae* populations.

We also found other differences in chemical composition of femoral secretions; wild males showed a higher diversity of aldehydes than captive males, although these compounds did not represent a significant proportion of the femoral gland secretion (sum of TIC < 9%). Further, high amounts of octadecanoic (stearic) acid in femoral

secretions seems to be a characteristic of captive-bred male *S. merianae*, although it was also abundant in the larger wild males. The stearic acid was identified in many lizard species [10], and, in some lacertid lizards males with lower parasite loads and greater immune responses had secretions with lower proportions of stearic acid [6, 44].

However, because captive *S. merianae* animals had a special diet and lived in a controlled habitat with veterinary care, the observed higher amounts of stearic acid probably should not be related with a worse health state. On the other hand, in the lacertid *Acanthodactylus boskianus*, males showed higher amounts of stearic acid than females [7] and in *Iberolacerta monticola*, more dominant males had femoral secretions with lower proportions of stearic acid [45]. More studies are needed to test if the stearic acid can be related with dominance status or sexual identification in *S. merianae*.

Wild male *S. merianae* showed much less amounts of the 9,12-octadecadienoic (linoleic) acid than captive males. This fatty acid has been identified in secretions of other lizards [10]. Being an unsaturated fatty acid, it may enhance the volatility and detectability of the scent marks [32]. The linoleic acid is one essential polyunsaturated fatty acids that is required for various biological processes and cannot be synthesized by animals [46]. It was proposed that its higher amounts in femoral secretion of males of *S. merianae* might be related with the quality of a male or of its territory, since only males with an adequate dietary supply could allocate it to secretions [19]. Thus, differences in diet may also explain the higher amounts of linoleic acid in captive than in wild animals, which, with a natural diet, would be unable to acquire and secrete in such a large amount. Another aspect that might affect the percentage of linoleic acid is the reproductive status [32, 33], if there were differences in the moment of the reproductive cycle when secretions were collected in captive and wild males.

Our results revealed the complexity of the chemical composition of the femoral gland secretions of adult male *S. merianae*. The within-species differences between wild and captive lizards suggest caution when interpreting results from captive bred animals, even in the same species. This is an important point to consider in future studies about the chemical composition of femoral gland secretions of lizards, especially for studies that use the chemical information for a better understanding of phylogenetic relationships or for studies based on samples from captive animals.

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Table 1. Lipophilic compounds found in femoral gland secretions of adult wild male tegu lizard, *Salvator merianae*. The relative content of each compound was determined as the percentage of the total ion current (TIC) and is reported as the average ($\pm$ SE) for five individuals. RT = retention time in minutes.

RT	Compound	Content (%)
4.0	Hexanal	1.55 $\pm$ 0.94
4.2	Hexanol	0.37 $\pm$ 0.30
9.1	2,4-Nonadienal	0.03 $\pm$ 0.03
12.4	Nonenal	0.03 $\pm$ 0.01
13.2	Nonanal	0.01 $\pm$ 0.01
16.2	Decanal	0.02 $\pm$ 0.01
18.7	2,4-Decadienal	0.03 $\pm$ 0.02
19.6	2-Dodecenal	0.02 $\pm$ 0.01
20.2	Decanoic acid	0.01 $\pm$ 0.01
20.8	Unidentified aldehyde	0.04 $\pm$ 0.04
26.6	Dodecanal	0.07 $\pm$ 0.07
27.1	Tetradecanal	0.12 $\pm$ 0.06
28.6	Unidentified fatty acid	0.03 $\pm$ 0.03
29.2	Pentadecanal	0.15 $\pm$ 0.05
30.0	Tetradecanoic acid	0.01 $\pm$ 0.01
31.0	Hexadecanal	1.31 $\pm$ 0.34
32.4	6,10,14-Trimethyl-2-pentadecanone	0.24 $\pm$ 0.05
32.5	14-Methyl-8-hexadecenal	0.10 $\pm$ 0.05
32.8	Pentadecanoic acid	0.02 $\pm$ 0.01
33.6	3-Heptadecanone	0.01 $\pm$ 0.01
34.1	Heptadecanal	0.12 $\pm$ 0.04
34.2	Hexadecanoic acid, methyl ester	0.30 $\pm$ 0.17
35.2	Hexadecanoic acid	7.24 $\pm$ 3.29
35.5	Hexadecanoic acid, ethyl ester	1.36 $\pm$ 0.79
36.1	Octadecanal	1.59 $\pm$ 0.47
36.9	Heptadecanoic acid	0.26 $\pm$ 0.15
37.3	Heptadecanoic acid, ethyl ester	0.03 $\pm$ 0.02
38.2	Octadecanoic acid, methyl ester	0.18 $\pm$ 0.10
38.5	9,12-Octadecadienoic acid	2.08 $\pm$ 0.98
38.6	9-Octadecenoic acid	1.51 $\pm$ 1.62
39.0	Octadecanoic acid	24.57 $\pm$ 11.6
39.2	9,12-Octadecadienoic acid, ethyl ester	0.48 $\pm$ 0.48
39.3	9-Octadecenoic acid, ethyl ester	2.15 $\pm$ 2.10
39.4	Hexadecanamide	0.07 $\pm$ 0.04

39.6	Octadecanoic acid, ethyl ester	1.41 ± 0.49
39.7	Eicosanal	0.06 ± 0.06
40.0	Eicosanoic acid	0.01 ± 0.01
40.1	Hexadecanoic acid, oxironyl methyl ester	0.12 ± 0.09
40.7	8,11,14-Eicosatrienoic acid	0.08 ± 0.08
40.9	4,8,12,16-Tetramethyl-heptadecan-4-olide	0.72 ± 0.34
42.0	9-Octadecenamide	0.02 ± 0.01
42.2	Octadecanamide	0.07 ± 0.07
42.7	9-Octadecenoic acid, oxironyl methyl ester	0.04 ± 0.04
43.2	Octadecanoic acid, oxironyl methyl ester	0.13 ± 0.08
47.2	Unidentified derivate of cholest-5-en-3-ol?	0.85 ± 0.36
47.3	13-Docosenamide	4.56 ± 2.82
48.0	Squalene	1.76 ± 1.13
48.5	Unidentified derivate of cholesta-2,4-diene?	0.48 ± 0.20
48.7	Cholesta-4,6-dien-3-ol	3.13 ± 0.90
49.0	Cholesta-3,5-diene	3.04 ± 1.32
50.4	Unidentified derivate of ergost-5-en-3-ol?	0.52 ± 0.27
50.8	β -Tocopherol	0.01 ± 0.01
50.8	γ -Tocopherol	0.10 ± 0.10
51.4	Unidentified derivate of cholestan-3-ol?	0.18 ± 0.18
51.5	Unidentified form of tocopherol	0.16 ± 0.16
51.6	Stigmasta-3,5-diene	0.26 ± 0.18
52.0	Cholesterol	21.05 ± 6.38
52.1	α -Tocopherol	0.53 ± 0.52
52.3	Desmosterol	1.40 ± 0.81
52.5	Cholestan-3-one	0.89 ± 0.65
52.8	Ergosterol	0.05 ± 0.05
53.1	Cholesta-3,5-dien-7-one	1.77 ± 0.74
53.3	Campesterol	2.12 ± 1.26
53.6	Cholest-4-en-3-one	3.16 ± 1.66
54.0	Cholesta-4,6-dien-3-one	2.04 ± 2.24
54.5	γ -Sitosterol	1.78 ± 1.00
54.8	Lanosterol	0.30 ± 0.03
55.1	4-Campestene-3-one	0.60 ± 0.46
56.6	Stigmast-4-en-3-one	0.49 ± 0.49

Figure legends:

Figure 1. Sum of percentages of the total ion current (TIC) of fatty acids (black), esters of fatty acids (grey) and sterols (white) in five adult males of *Salvator merianae*.

Figure 2. The percentage of the total ion current (TIC) of hexadecanoic (black), 9,12-octadecadienoic (light grey) and octadecanoic (dark grey) acids and cholesterol (white) in five adult males of *Salvator merianae*.

Figure 3. The mean of the total ion current (TIC) of the four chemical compounds that showed striking differences between captive-bred (black) and wild (grey) males of *Salvator merianae*. For the wild males, the mean represents only the three males that showed high amounts of fatty acids in their secretions.

Fig 1

Fig. 2

Fig. 3