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*Eraqi R. Khannoon, Afaf El-Gendy & Jörg
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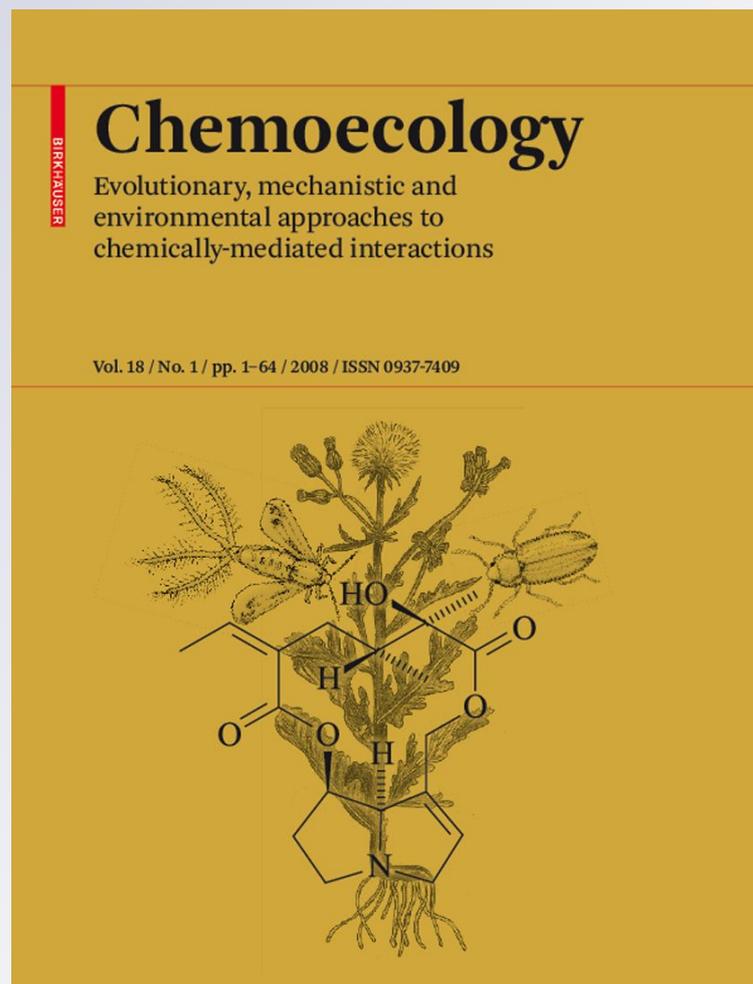
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Scent marking pheromones in lizards: cholesterol and long chain alcohols elicit avoidance and aggression in male *Acanthodactylus boskianus* (Squamata: Lacertidae)

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Abstract Femoral gland secretions are believed to play an important role in chemical communication and social organization of lizards. In spite of this, few studies have investigated the chemical composition and the behavioural roles of these secretions. The lacertid lizard *Acanthodactylus boskianus* is a good example, having these well-developed glands in both sexes. We used GC–MS chemical analysis of gland secretions and y-maze choice test bioassays to investigate the ability of the lizards to detect and respond to different synthetic blends made from compounds identified in the gland secretions. Based upon the GC–MS quantification data, we selected representatives of the main chemical groups (steroids, alcohols, acids, alkanes) detected in the lizard secretions and used these in a behavioural bioassay against controls. Males showed significant avoidance behaviour for cholesterol and alcohol blends, combined with agonistic behaviour towards these stimuli. Females did not show any significant selection to particular odour combinations. The data support the hypotheses that lizards can potentially use femoral gland secretions in chemical odour trails and utilize scent to mark territories and potentially also to establish dominance hierarchies. Cholesterol and long chain alcohols are suggested as potential candidates functioning as scent marking pheromones in *A. boskianus*.

Keywords Agonistic behaviour · Aggression · Scent pheromones · Femoral glands · *Acanthodactylus boskianus*

Introduction

Vertebrates are known to use chemical signals that stem from urine (Dehnhard et al. 2001) and glandular secretions (Quay 1972). Squamates (lizards and snakes) have the most highly developed vomeronasal system of all vertebrates (Halpern 1992; Cooper et al. 1994), and they respond to chemical stimuli by increased tongue flicking (Cooper et al. 1996). Squamate reptiles sense chemical stimuli carrying information about several biological properties of the sender (Cooper et al. 1994). Despite the fact that sex recognition is often based on colour signals, such pheromone communication occurs in many species of lizards (Mason 1992; Cooper et al. 1996; Martin and López 2000). In lizards, pheromones are important for discrimination of familiar and unfamiliar individuals (Cooper 1996), territory marking (Alberts 1992), sexual recognition (Cooper et al. 1996; Khannoon et al. 2010), and self-recognition (Graves and Halpern 1991).

Many squamate reptiles possess epidermal structures on the ventral surface of the thigh or the precloacal abdominal area. These femoral glands produce waxy secretions (Mason 1992; Alberts 1993). The secretory activity of these glands varies within the season and increases during the time of the year in which lizards are sexually active. In some families of squamates only males have these glands (Van Wyk 1990; Alberts 1993). Femoral gland secretions of reptiles can have pheromone properties (Cooper et al. 1994; López et al. 1997) and might play an important role in sexual signalling and territorial scent marking (Mason

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1992; Alberts 1993; Cooper and Vitt 1984; Aragón et al. 2001). Chemical signals are used in intraspecific communication between lizards, e. g. in the desert iguana (Alberts 1992; Mason 1992; Halpern 1992).

Studies have shown that femoral gland secretions are composed of both lipids and proteins (Mason and Gutzke 1990) giving rise to the theory that lipids are involved in chemical communication (Lemaster and Mason 2001). On the other hand, the involvement of proteins cannot be discounted, given their importance in amphibians (Toyoda et al. 2004). Few studies have examined the lipidic part of epidermal gland secretions (Weldon et al. 2008). In *Liolaemus* lizards, *n*-alkanes, long chain carboxylic acids, and steroids were found, and differences in the proportions of some compounds between populations have been reported (Escobar et al. 2001, 2003). In the lacertid lizard *Acanthodactylus erythrurus*, alcohols, steroids, carboxylic acids, esters, lactones, ketones, squalene, and α -tocopherol have been recorded (López and Martin 2005a). The same classes of compounds were found in another lacertid lizard, *Lacerta monticola cyreni* (López and Martin 2005b). In the girdled lizard or sun-gazer, *Cordylus giganteus*, carboxylic acids, alcohols, ketones, esters, and steroids occurred in both males and females (Louw et al. 2007).

In lizards (Cooper and Vitt 1986) and snakes (Chiszar et al. 1986; Burger 1991), pheromone communication was demonstrated in controlled laboratory experiments on trailing of conspecifics. Most of the studies on the role of femoral gland secretions used the tongue flicking as the major criteria of examining bioactivity (Aragón et al. 2001; Martin and López 2007). None of these studies used the choice behaviour to evaluate the ability of lizards to choose between odour trails. In this study, we wish to address the question whether *Acanthodactylus boskianus* lizards are able to use their sensitive tongue to discriminate between the femoral gland fractions and a control.

Acanthodactylus boskianus (Lacertilia: Lacertidae) is a medium to large sized lizard. Like other lacertids, this species has an array of epidermal glands in both, males and females. Behavioural experiments examining the femoral secretions of *A. boskianus* showed a sexual difference in the tongue flicking responses towards secretions (Khannoon et al. 2010). The composition of the femoral gland secretions of *A. boskianus* is quantitatively different between sexes and between different male ages (Khannoon 2009; Khannoon et al. 2011). These secretions compose a characteristic compound bouquet, typical for *A. boskianus*. The chemical analysis of these secretions showed that they consist of alcohols, steroids, acids, alkanes, glycerolmonoethers and monoglycerides as the most abundant groups of compounds (Khannoon et al. 2011). The chemical

content differences could play important roles in mate choice and/or the establishment of dominance hierarchies, or in the territorial marking. Here we tested the hypothesis that lizards can utilize odours to detect rivals. Male–male aggressive interactions in vertebrates often involve chemical signalling (Wyatt 2006), and we, therefore, also investigated this potential effect of the femoral gland secretions.

Materials and methods

Samples

Acanthodactylus boskianus individuals were captured from Balteem, Northern coast of Egypt, during April which coincides with the activity and mating season of these lizards. The animals were transferred to Hull University, UK, and kept for experiments. For the whole work we selected 18 adult males and 23 adult females that had the highest snout-vent length (SVL) within a narrow range of SVL differences (SVL \pm 3 mm). The lizards were individually housed in lidless transparent plastic cages (56 \times 39 \times 28.5 cm; length, width, and height). Each terrarium was provided with rocks for shelter, sand and gravels as substrate, small tree branches, and water ad libitum. Temperature ranged from 22°C (night) to 32°C (noon). Gradual temperature change was established by using a timer controlled heating and ventilation system. Natural photoperiod 12:12 h was simulated using fluorescent lights on timers, and UV lamps were used for compensation of the sunlight. Spot lamps were used for basking of the lizards. Lizards were fed with calcium-enriched mealworms and occasionally with crickets. The lizards were kept separately in cages for around 10 days prior to commencement of experiments. Only the experimenter had access to the animals so that they quickly grew accustomed to his presence.

Chemistry

Previous work (Khannoon et al. 2011) using GC–MS analysis showed that the lipid part of the secretions consists of alcohols, steroids, carboxylic acids, alkanes, amides, aldehydes, carboxylic acid esters, squalene, glycerolmonoethers and monoglycerides. Most of these compounds showed sexual and age variations in their abundance. Alcohols, steroids, and carboxylic acids were the most abundant groups of compounds in the secretions. Some compounds of these groups showed sexual variability. On the other hand, despite alkanes representing only low percent of the total ion current (TIC), they showed higher abundance in females than in males.

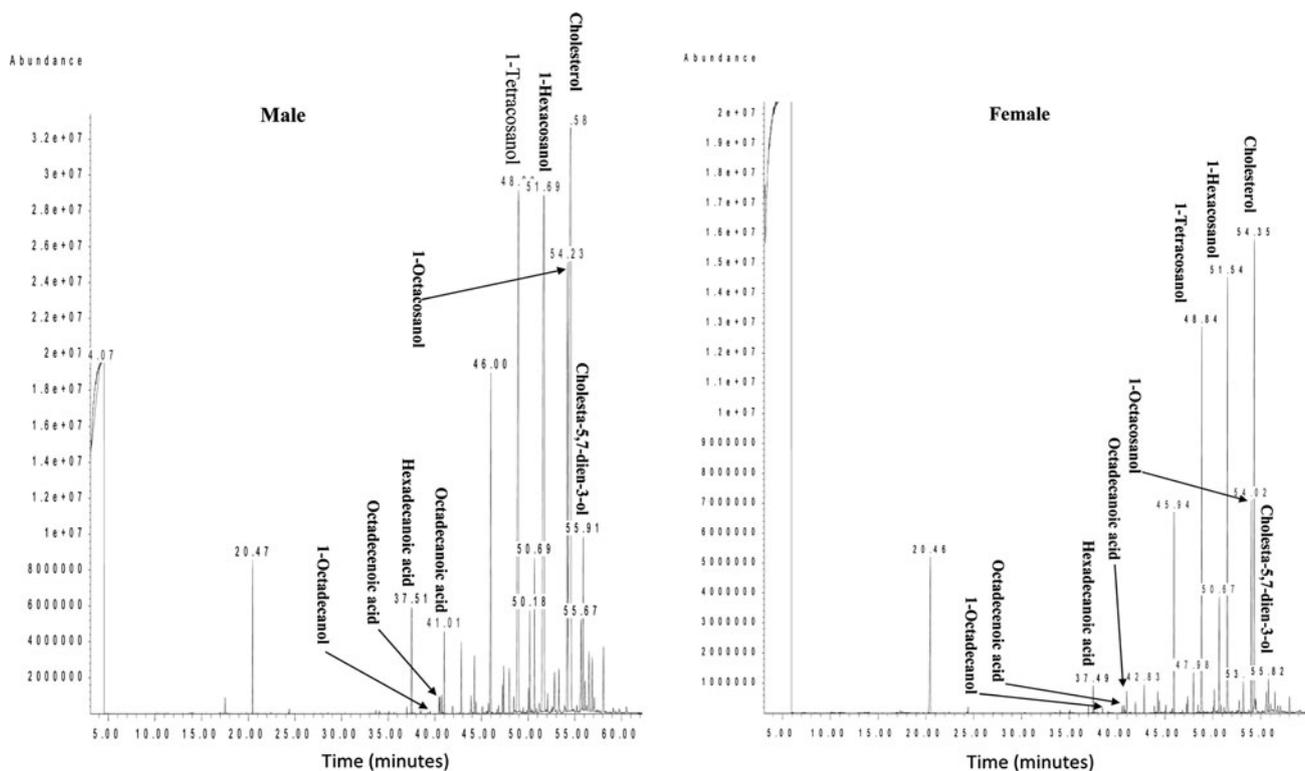


Fig. 1 GC traces of femoral gland secretions collected in dichloromethane from male and female lacertid lizard, *Acanthodactylus boskianus*. Compounds, detected as the respective trimethylsilylated, selected for the bioassay are shown

In the current study, we quantified the most important individual components (Fig. 1) which fulfilled two conditions: (1) to exist in both sexes in high abundance, in GC–MS analysis, relative to other chemicals; (2) to show sexual variations and/or male age variations. These chemicals were quantified in both males and females and we then used synthetic compounds representing these chemicals in the behavioural bioassay to investigate the behavioural active parts of the secretions. Secretions were collected by gently squeezing the plugs from the femoral pores of the lizards (14 males and 10 females) using forceps. 8 mg of secretion of each individual was collected directly into glass vials with Teflon-lined caps and then dissolved in 250 μL of dichloromethane (DCM) (Aldrich, GC grade). The collected samples were kept at -18°C until processing for analysis. Control samples with the solvent at the same conditions of collecting the secretion were used to exclude impurities. Quantification was conducted by using external standards of all the nine compounds selected for the bioassay (Table 1), and following the same procedures of derivatization and GC–MS protocol. On the other hand, alkanes were not quantified because of the low abundance. Based on our analytical results, mixtures of synthetic samples used for bioassays were prepared according to the qualitative and quantitative composition of the natural secretion.

Table 1 Mean amounts ($\mu\text{g}/\text{mg}$ secretions) \pm SE of the bioassay selected compounds from femoral gland secretions of *Acanthodactylus boskianus* lizards

Compounds	Mean \pm SE		P
	Males (N = 18)	Females (N = 23)	
Cholesterol	20.39 \pm 4.50	2.99 \pm 0.64	<0.001
Cholesta-5,7-diene-3-ol	1.03 \pm 0.36	0.04 \pm 0.005	0.001
Octadecanoic acid	3.07 \pm 0.81	0.09 \pm 0.02	<0.001
Octadecenoic acid	0.48 \pm 0.12	0.16 \pm 0.04	0.035
Hexadecanoic acid	2.69 \pm 0.80	0.54 \pm 0.06	0.005
1-Hexacosanol	0.47 \pm 0.14	0.04 \pm 0.01	0.001
1-Tetracosanol	14.41 \pm 3.12	1.53 \pm 0.41	<0.001
1-Octacosanol	7.63 \pm 2.31	0.39 \pm 0.13	<0.001
1-Octadecanol	3.96 \pm 1.17	0.40 \pm 0.09	0.004

Results of *t* test which compared males and females concentrations are shown as *P* values

Derivatization

50 μL of the liquid secretion was placed in a 2 ml vial and the solvent was removed in a gentle stream of nitrogen at 50°C . The residue was taken up in 10 μL dichloromethane and 50 μL *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) were added. The mixture was heated to 50°C for

30–60 min in a vial with a closed cap. Then the solvent and the remaining reagent were evaporated in a gentle stream of nitrogen at 50°C and the residue was taken up in 10 µL dichloromethane. Exactly 1.0 µL was injected into the gas chromatography–mass spectrometry (GC–MS) system.

GC–MS analyses

Samples were analyzed using a Hewlett-Packard model 6890 gas chromatograph connected to a Hewlett-Packard model 5973 mass-selective detector equipped with a BPX-5 column: 25 m × 0.22 mm i.d., 0.25 mm film thickness (SGE). The temperature program was as follows: 50°C for 5 min, then with 5°C/min to 320°C, 30 min hold time. Helium was used as carrier gas with 1 ml/min in constant flow mode. Accelerating voltage of MS was 70 eV. Compounds were identified by comparison of mass spectra and retention indices of derivatized and underivatized samples with those of reference compounds.

Behavioural bioassay

According to chemistry results, the chemicals chosen for the bioassay (Fig. 1) were obtained as following: 1-octadecanol (≥99.0%, Fluka), 1-tetracosanol (≥99%, Sigma), 1-hexacosanol (≥97.0%, Fluka), 1-octacosanol (≥99%, Sigma), cholesterol (≥99%, Sigma), cholesta-5,7-diene-3-ol (dehydrocholesterol) (≥98.0%, Sigma), hexadecanoic acid (≥99.0%, Fluka), octadecanoic acid (≥99.5%, Fluka), octadecenoic acid (oleic acid) (~99%, Sigma), pentacosane (≥99.5%, Fluka), hexacosane (≥99.5%, Fluka), and heptacosane (≥99.5%, Fluka). The chemicals were dissolved in dichloromethane (DCM) in enough volumes to allow these to be spread over the gravels used in the bioassay. The chemicals were dissolved in a concentration mimicked that found in the adult males. The stimuli used were in the same dilution predetermined from previous studies (Khannoon et al. 2010), which showed that the whole secretions were functional and caused significant response at 8 mg secretions/500 µL DCM. Each chemical was prepared as equivalent to that found in 8 mg and dissolved in 500 µL DCM. The next step of the assay was to mix compounds of the same chemical group in one blend solution except for cholesterol and dehydrocholesterol. Cholesterol and dehydrocholesterol were tested separately because we wanted to test the suggested role of each one (López and Martín 2005c). Five stimuli were used in the bioassay; three blends of alcohols (1-octadecanol, 1-tetracosanol, 1-hexacosanol, and 1-octacosanol), acids (hexadecanoic acid, octadecanoic acid, and octadecenoic acid), and alkanes (pentacosane, hexacosane, and heptacosane), and two individual chemicals; cholesterol and dehydrocholesterol. The stimuli were prepared by

spreading them to be tested on 10 mm gravels, which were used as a simulation to the actual habitat, and then evaporating the DCM solvent using a gentle stream of nitrogen gas. DCM was used as a control. Each trial was undertaken by using one of the five stimuli in one side arm against a DCM control in the other side arm of the y-maze.

Y-maze

We chose a y-maze choice test to determine whether the lizard could avoid or choose to follow a trail of chemical compounds. The experiments were undertaken in a glass cage 39.5 × 98.5 × 39.5 cm, divided into two parts; entrance and y-maze partitions, separated by a mobile door (10.5 × 20 cm) which was controlled from the top by a long rope. This was used by the experimenter to open and close up and down, and was controlled from around 2 m distance. A video monitor showed the experiment that was recorded by a Sony handycam placed 40–50 cm over the top of the cage. The entrance partition (23.5 × 39.5 cm) was the part in which the lizard under test should stay for 5 min for relaxation before the door was opened to start the experiment towards the y-maze. The base arm of the y-maze was 17 cm long, 20 cm wide and 25.3 cm high, and at the end of the base arm, there were two side arms (60° angles from the base arm) of the same height and 10 cm wide and 54.5 cm length. The gravels carrying the stimuli and the control were placed on both sides of the base arm and continued until they diverged into the two side arms, one side carrying the stimulus and the other carrying the control. The lizard had the chance to flick towards both sides once it entered the base arm, and had the chance to select and detect the favourite side. Placing the stimuli and controls in the sides of the base arm was to give the lizards enough time for detection of the signals and to be able to detect the favourite side before going to the side arms. Presence of the base arm improves the design of the y-maze.

The experiment started by transferring the animal under test into the experimental cage's entrance partition while the door was closed. The animal was allowed to accommodate for 5 min and then the door was opened using the rope, allowing the lizard to enter the y-maze partition. If the animal spent long time (over 15 min) after the door was opened without entering the y-maze, the trial was cancelled and the animal transferred back to its home cage. Each side arm was marked into three-thirds, if the animal chose one arm and passed the line of the second-third this was considered as a decision to choose that side. If the lizard returned back before the second line then this was not considered as a choice. Also the behaviour of the lizards toward the gravels carrying the stimuli was monitored. After each trial the experimental cage was cleaned, the gravels were replaced and the y-maze was cleaned for the

next trial. The location of the stimulus was swapped randomly. Each animal was tested randomly for any stimulus not more than once a day and the choice of sex used with the stimulus was also random.

Two reactions were recorded to evaluate the behaviour of the lizards: (1) the choice between the stimulus and the control; (2) the behaviour towards the stimulus-carrying gravels. The important response we aimed to record was any type of aggression. This was designed to examine which compounds could be responsible for such male aggression recorded from the response towards the femoral gland secretions (Khannoon et al. 2010).

Statistical analysis

An independent sample *t* test was used to analyze the differences in the quantities of compounds produced between males and females. *G* test was used to evaluate the response frequency in the y-maze. The test statistic *G* were corrected using a Williams' correction.

Results

Chemical composition of the secretion

The compounds detected in the total ion chromatogram of both male and female *A. boskianus* secretions were qualitatively similar, but they differed quantitatively between the sexes. A total of more than 120 chemicals were identified in both sexes. These compounds were identified using standard procedures. The extracts were analyzed after derivatization with MSTFA to form trimethylsilyl-derivatives, thus enabling the GC–MS analysis of the more polar compounds. The major compound groups, measured as relative percent to the TIC, present in the secretion were alcohols (50.2%), steroids (35.5%), carboxylic acids (9.3%), glycerol monoethers (2.1%), and monoglycerides (0.4%). The other classes occurred only in minor percents.

The concentrations ($\mu\text{g}/\text{mg}$ of the secretions) of the selected compounds were significantly different between males and females, particularly for cholesterol and alcohols (Table 1), and these concentrations were used for the bioassay.

Behavioural bioassay

Once the door separating the entrance partition from the y-maze was opened, lizards started to tongue flick and move towards the base arms of the y-maze. Towards cholesterol and alcohols, males showed significant avoidance and chose the control side arm ($N = 15$, $G_{\text{adj}} = 5.59$, $P < 0.05$ and $N = 16$, $G_{\text{adj}} = 4.06$,

Table 2 *G* test results evaluating the choice of both males and females from between the stimuli and dichloromethane control in the y-maze

Stimulus	Males			Females		
	<i>N</i>	G_{adj}	<i>P</i>	<i>N</i>	G_{adj}	<i>P</i>
Cholesterol	15	5.59	<0.05	15	3.28	>0.05
Cholesta-5,7-diene-3-ol	14	2.56	>0.05	17	0.05	>0.05
Alcohols	16	4.06	<0.05	15	0.06	>0.05
Acids	17	1.45	>0.05	21	1.17	>0.05
Alkanes	15	0.06	>0.05	15	0.06	>0.05

Number of animals (*N*), adjusted test statistic *G* (after Williams' correction), and significance *P* values are given

$P < 0.05$, respectively). Towards dehydrocholesterol, acids, and alkanes, males showed no significant choice (Table 2). Females showed no significant choice for any of the stimuli used, and only one female did bite once towards the alkanes. Male aggressive behaviour was observed through their biting towards the gravels carrying the stimuli of both the cholesterol and alcohols blends. Five males bite the cholesterol flavoured-gravels aggressively, four of them chose the control arm and only one chose the cholesterol arm. Three males did bite the gravels flavoured with alcohols, and all of them chose the control arm.

Discussion

The present results, together with the previous results on tongue flicking (Khannoon et al. 2010) show that *A. boskianus* lizards are able to detect femoral gland secretions. The relative differences in chemical concentration between male and female secretions support the potential role of these chemicals in communication as scent pheromones. Cholesterol and some alcohols showed concentration differences between the sexes, which makes them ideal candidate compounds for a potential role as chemosignals. Using the y-maze bioassay showed the ability of lizards to discriminate chemicals of conspecifics, similar to studies on snakes (Burger 1989, 1991). The significant responses of *A. boskianus* lizards (Table 2) were towards the most common and sexually and/or age variable components of the femoral gland secretions detected by GC–MS. *A. boskianus* is territorial, and dominant males advertise with a slow tail wave after each locomotion (Schleich et al. 1996). This leads to a high likelihood of leaving secretions from the femoral glands on the ground gravels during this movement. In addition, males drag their legs on the ground leaving potentially traces of these secretions.

The present data showed that the males were avoiding cholesterol. The later is the most abundant individual

component in the femoral gland secretions of *A. boskianus* (Khannoon et al. 2011). It showed sexual and males' age variability. This means that cholesterol abundance can signal for stronger lizards. In males, cholesterol levels may also provide information about the condition of the male such as the dominance status or its genetic quality (Martin and López 2000). When a territorial male *A. boskianus* moves around its territory it leaves marks showing its presence. When an intruder male comes into the territory the odour profiles of gland secretions containing cholesterol could allow quick assessment of a rival's fighting ability (Martin and López 2007). Some males bit the cholesterol stimulus, a behaviour which supports the hypothesis of the cholesterol role in expressing aggression. The lack of aggression for some males could be explained as the receiver probably assessing the strength of the odour's owner as superior to its own strength, thus avoiding costly aggressive interactions.

There was neither avoidance nor aggression towards dehydrocholesterol. It is feasible that dehydrocholesterol has no direct effect alone, but is an important component in the bouquets within the secretions. López and Martin (2005b) speculated that the relative abundance of dehydrocholesterol was related to the physical or health condition of the lizard and it, therefore, may play a role in female mate choice.

Alcohols could also form important chemosignals in the femoral gland secretions of *A. boskianus*, since this group of chemicals represents the highest abundant class of chemicals in the secretions (Khannoon et al. 2011). In the present experiment these alcohols were used in a stimulus blend, constituting long chain C₁₈, C₂₄, C₂₆, C₂₈ alcohols. Avoidance and aggression occurring in males indicate such possible role of alcohols specifically since we only tested a proportion of the 22 alcohols detected in the gland secretions.

Males did not show any significant choice towards acids or towards alkanes. Despite high abundance of acids in the secretions and significant sexual variation, they did not elicit any interest or avoidance by males. Acids represent substantial part of the lipids in the secretions and give the genus *Acanthodactylus* its specific secretion contents (López and Martin 2005a; Khannoon 2009), and further studies are required to clarify their role. Unlike males, the females did not show any significant responses towards any of the five stimuli. Initially this is surprising since cholesterol appears to indicate strong males, and that potentially makes them attractive to females. Cholesterol might be an announcement of aggression and strength towards males (competitors) but not used for mate choice, at least not as a single compound.

Overall, steroids and alcohols seem to be important chemosignals in *A. boskianus* lizards and could be used in

territorial marking and dominance hierarchies. Using scent marking to announce a dominant male's presence in its territory would save cost related to fighting between males. One benefit of scent markings is that they announce messages even when the animal is not present (Wyatt 2006). Scent marks provide information to intruding animals on the status of the resident through the intrinsic characteristics of the marks or give the identity of the owner (Wyatt 2006). Other reptiles are using cloacal gland secretions as marking signals. Prairie rattlesnakes show a rise in their heart rate when exposed to cloacal gland materials from conspecifics (Graves and Duvall 1988). In alligators, the cloacal glands are used to mark territories during the breeding season (Evans 1961). Other chemicals which did not elicit any significant response in the present study cannot be excluded entirely from being semiochemicals or at least sharing such roles when they are in mixtures with other secretion components. Many vertebrate semiochemicals consist of dozens of compounds, which can lose their signal function when separated into fractions (Duvall et al. 1986).

In conclusion, here we present linked behavioural and chemical data that support the theory that femoral gland secretions, in particular cholesterol and long chain alcohols, could play a role in territorial marking and in dominance hierarchies in lizards, thus increasing our understanding of communication in lizard communities. Future work is needed to examine other potential roles of femoral gland secretions such as in mate choice.

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