Sexual differences in behavioural response to femoral gland pheromones of *Acanthodactylus boskianus*

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Femoral glands of lizards are holocrine structures that produce compounds used by lizards in intraspecific communication. Here we show that the femoral gland secretions of a lacertid lizard, *Acanthodactylus boskianus*, contain chemicals that elicit sex-specific responses. These glands are found in both males and female *A. boskianus*. We used the secretions of both sexes as stimuli presented on cotton swabs. Tongue flicks as well as other behavioural responses of both male and female lizards were recorded over a 60 second interval. Both males and females increased their tongue-flick rate towards male secretions, and some males bit swabs containing male secretions. Only a small proportion of females (25%) showed a similarly aggressive response. Female secretions only elicited elevated tongue-flick frequencies in males and did not elicit any aggressive behaviour. As a result of male–male competition and mate choice, the secretions appear to play a role in male territorial behaviour and in sex recognition, which could, for example, reduce the cost of male–male aggression when competing for females. This work opens future opportunities to test the role of chemical cues in mate choice and dominance hierarchies in lizards.

Key words: aggression, chemical communication, Lacertidae, lizard, pheromones, sexual behaviour, Squamata

INTRODUCTION

Squamate reptiles use their chemical senses to obtain information about conspecific mates and competitors as well as about predators and prey (Cooper, 1994). They are equipped with the most highly developed vomeronasal system of all vertebrates (Halpern, 1992; Cooper, 1994) and use tongue flicking to detect conspecific odours. In lizards, pheromones are emitted during territory marking (Alberts, 1992) and used by conspecifics for sex recognition (Cooper et al., 1996), mate choice (Martin & Lopez, 2000), dominance recognition (Lopez et al., 2002), discrimination between familiar and unfamiliar individuals (Cooper, 1996) and self-recognition (Graves & Halpern, 1991).

Femoral pores are epidermal structures located on the ventral surface of the thigh of many squamates. They are openings to glands that produce holocrine secretions (Mason, 1992; Alberts, 1993). The secretory activity of these glands varies seasonally and increases during the reproductive season. In some lizard species, males have larger glands than females, and in other species only males have these glands (Van Wyk, 1990; Alberts, 1993; Khannoon, 2004). Glandular secretions consist of both lipids and proteins (Mason & Gutzke, 1990); the major compounds involved in chemical communication are lipids (Lemaster & Mason, 2001). Some studies have investigated the chemical composition of femoral glands (Escobar et al., 2001; Lopez & Martin, 2005; Louw et al., 2007), but few focused on their behavioural effects (Martin & Lopez, 2007). Here we provide data on the behavioural responses of both male and female *Acanthodactylus boskianus* to femoral gland secretions of both sexes.

A. boskianus is a medium to large sized lacertid species that inhabits deserts and semi-deserts in Egypt. Initial chemical analysis of the epidermal gland secretions of *A. boskianus* showed that the lipid part of the gland consists of semi-volatile compounds: alcohols, steroids, carboxylic acids, alkanes, amides, aldehydes, carboxylic acid esters and squalene (Khannoon, 2009). This study characterized behavioural responses to gland secretions, which were found to be quantitatively different between the sexes and between age groups. The study aimed to examine whether male and female *A. boskianus* display sex-specific responses and whether they can distinguish between femoral gland secretions of the same and the opposite sex.

MATERIALS AND METHODS

Adult *A. boskianus* up to 79 mm snout–vent length (SVL) were collected in Balteem on the northern coast of Egypt in March 2007, before the beginning of the breeding season, and transported to the Sensory and Chemical Ecology Laboratory at Hull University, United Kingdom. The lizards were individually housed in lidless transparent plastic cages (56 cm long × 39 cm wide × 28.5 cm high). Each terrarium was furnished with both sand and gravel substrate areas, and provided with rocks and tree branches for shelter and exercise and an ad libitum supply of water. Temperature ranged from 22 ºC (night) to 32 ºC (noon). Gradual temperature change was established by using a timer-controlled heating and ventilation system. A natural photoperiod of 12:12 hrs was simulated using fluorescent lights controlled by timers. UV lamps were used to compensate for a lack of exposure to sunlight.

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Spot lamps were provided for basking. Lizards were fed with calcium-enriched mealworms and occasionally with crickets.

We used 30 adult individuals (14 males and 16 females) in the experiments. Males were matched for size (SVL ±1 mm) and weight (±0.4 g). All individuals used in the experiment were isolated in cages 10–15 days prior to commencement of the experiments. Only the experimenter (E.K.) had access to the animals and animals quickly became accustomed to his presence. To collect secretions from femoral glands, males and females were placed ventral side up and all pores were pressed gently with forceps, resulting in the extrusion of glandular secretions. The semisolid secretions (8 mg) from each individual were dissolved in 500 µl dichloromethane (DCM) in glass vials if they were used on the same day. Alternatively, they were sealed and kept in a freezer at −18 ˚C until use. Dissolving in dichloromethane served to extract the lipids from the secretions, and the volume was enough to cover and dissolve all the secretions in the vials and to be spread over an adequate surface of the cotton to be detected by the animal.

For the experimental trials the extract of dissolved secretions (chemical stimuli) was spread over the cotton tip (1.5 cm) of a wooden applicator (40 cm). The solvent was then evaporated, in all trials including the control, using nitrogen gas flow. We used a new swab for each trial. The trials were conducted in May and June 2007 (breeding season), between 0900 and 1400, a time when the lizards are generally active in the field as well as in captivity. When the lizard was active and moving in its cage, the experimenter approached and presented the swab in a position 1.5–2 cm in front of the lizard's snout. Once the first tongue extrusion (tongue flick, TF) was observed, the TFs towards the cotton swab were counted for a period of 60 seconds, starting with the first TF. All trials were video recorded using a digital camera (Sony, DCR-TRV480E) in a fixed position so as to not disturb the animals.

DCM (used as the solvent for the secretions) served as a control to gauge baseline TF rates. Responses of both sexes were recorded. Each male was subjected to three trials: 1) DCM, 2) stimulus from a different male and 3) stimulus from a female. Each female was subjected to three trials: 1) DCM, 2) stimulus from a different female and 3) stimulus from a male. If the animal perceived a threat and ran away the trial was cancelled immediately and the animal was not tested again that day. The trials were conducted in a random order and each animal was tested only once a day using a random stimulus. Since lizards were kept in isolated cages (see above) these lizards were not familiar with each other.

Analysis of behavioural data
Videotapes were analysed by recording, during each trial, the following events:

1) Number of TFs toward the cotton tip.

2) Biting behaviour towards the cotton swab or the wooden applicator as a measure of aggressive response to the secretions.

Tongue-flick rates of males and females to control stimuli and to female or male secretions were analysed as paired data, using paired *t*-tests or non-parametric Wilcoxon signed-rank tests. In general, we used non-parametric statistics where tongue-flick data were not normally distributed ($P<0.05$; Shapiro–Wilk tests). Differences in tongue-flick rates between the sexes were analysed as unpaired data, using non-parametric Mann–Whitney U tests. Categorical data on biting behaviour, as number of biting animals, were tested using Fisher's exact test.

The ethics committee of the University of Hull approved the collection procedures for lizards undertaken in March 2007. During the experiment each animal was kept in its own cage and there was no need to mark test subjects.

RESULTS

Male responses
All 14 males tested immediately approached and tongue flicked the cotton swab carrying femoral secretions or the control solvent. Tongue-flick rates of males (Fig. 1) to male secretions were significantly elevated compared to control stimuli ($P<0.001$, paired *t*-test). Nine males bit the cotton swab aggressively when confronted with male secretions (Table 1), whereas no biting occurred when exposed to the control swabs ($P=0.0006$, Fisher exact test). Tongue flicking also increased significantly over the baseline control level when males were exposed to female secretions ($P=0.002$, paired *t*-test). However, no bites were directed towards the swabs bearing female secretions. This was also the case for response towards control swabs ($P=1.0$, Fisher exact tests). Males showed no difference in tongue flicking frequency between male stimuli and female stimuli ($P=0.37$, Mann–Whitney U-test).

Female responses
All 16 females tested immediately approached and tongue flicked the cotton swab carrying the femoral secretions or control stimuli (Fig. 2). The tongue-flick rate was

Table 1. Behavioural response (biting) of male and female *A. boskianus* towards cotton swabs carrying femoral gland extract of conspecific male and female individuals. Fisher's exact test *P* values are shown. DCM is the control.

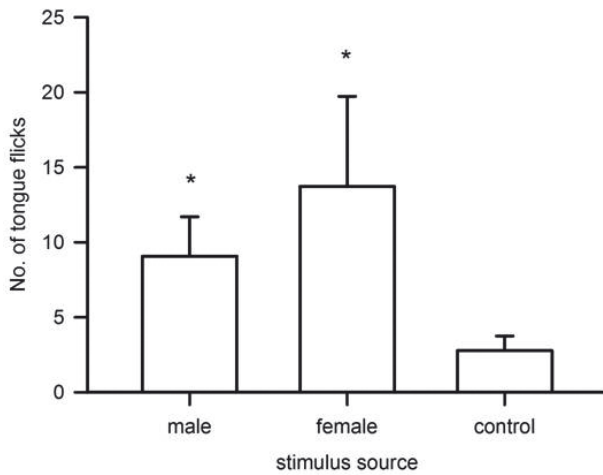|	Biting towards stimulus	Biting towards control	Significance level
Response of male towards male	9/14	0/14	$P=0.0006$
Response of male towards female	0/14	0/14	$P=1.0$
Response of female towards male	4/16	0/16	$P=0.101$
Response of female towards female	0/16	0/16	$P=1.0$

Fig. 1. Mean numbers of tongue flicks (± SEM) directed by male *A. boskianus* over 60 seconds towards cotton swabs carrying femoral gland secretion extracts of conspecific males and females, and towards a DCM control swab. Asterisks indicate significant differences (*P*<0.05) compared to control stimuli.
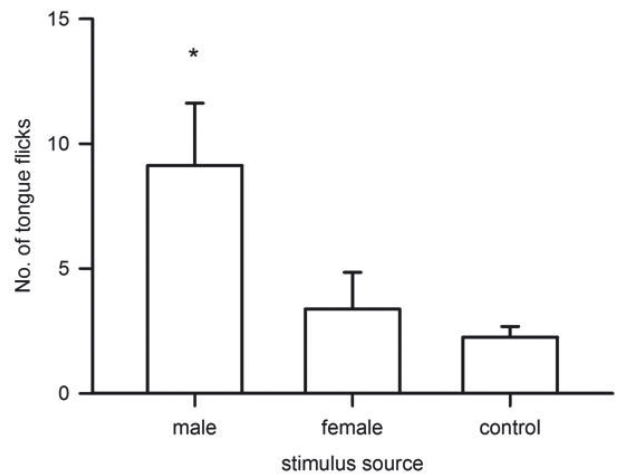
Fig. 2. Mean numbers of tongue flicks (± SEM) directed by female *A. boskianus* over 60 seconds towards cotton swabs carrying femoral gland secretion extracts of conspecific males and females, and towards a DCM control swab. The asterisk indicates a significant difference (*P*<0.05) compared to control stimuli.

significantly elevated towards the male secretions when compared to the control (*P*<0.001, Wilcoxon signed-rank test). However, aggression was not significantly different between the male stimulus and the control stimulus (*P*=0.10, Fisher exact test). Four females bit the cotton swab carrying the secretions, but 12 females only responded with tongue flicks. Tongue-flicking rate was not elevated towards the female secretions when compared to control stimuli (*P*=0.49, Wilcoxon signed-rank test), and there was no evidence of biting directed towards female secretions or control stimuli (*P*=1.0, Fisher exact test). Females displayed significantly higher tongue-flick rates to male stimuli than to female stimuli (*P*<0.001, Mann–Whitney U-test).

DISCUSSION

Femoral gland secretions are deposited on the substrate either passively as lizards move through their home range, or actively as they sometimes drag or rub their thighs on the rocks. In squamates, differences in tongue-flick rates in response to different stimuli indicate the ability to discriminate odours using the vomeronasal organ (Halpern, 1992; Cooper, 1994). Our results show that female and male *A. boskianus* were able to differentiate between femoral secretions and the DCM control, suggesting that the femoral secretions contain chemical signals. The low tongue-flick rate towards control DCM stimuli appears to represent a general response of the lizard to a novel chemical stimulus. This low tongue-flick response to the control stimulus was similar in all lizards tested. This represents the normal tongue-flick activity of lizards when they are not stimulated. No significant difference was

recorded between the number of TFs made by males towards male or female secretions. However, the aggressive behaviour of males towards male secretions and the lack of any aggression towards female secretions demonstrate that males may use such compounds present in femoral gland secretions to discriminate between sexes. This can be explained by the fact that different concentrations and different bouquets of chemicals are characteristic of male and female excretions (Khannoon et al., submitted). Some alcohols (hexacosanol, tetracosanol and octacosanol), steroids (cholesterol and dehydrocholesterol) and acids (hexadecanoic, octadecanoic and octadecenoic acids) showed variability between males and females. Females also potentially have the ability to discriminate between the sexes using information conferred by the secretions, as indicated by the higher TF frequencies elicited by male secretions compared to female secretions. Our findings confirm the conclusions of earlier work suggesting that lizards are able to discriminate between the sexes in at least two different families: Gerrhosauridae (Cooper & Trauth, 1992) and Cordylidae (Cooper et al., 1996).

Lizards exhibit social dominance and use pheromones as social signals (Mason, 1992). Intra-sexual aggression in lizards is preceded by chemical recognition (Cooper & Vitt, 1987). In our study, males responded to male secretions with a high rate of TFs and with aggressive biting. Each male was separated for 10–14 days prior to being exposed to such signals. The aggression of males towards other males can be explained by the tendency of the resident male to defend its territory. In many organisms that exhibit social hierarchies, at first, visual estimation of size occurs. Between size-matched males, however, chemoreception may provide the crucial information about a

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rival's fighting ability (Martin & Lopez, 2007). Our study indeed revealed that some males showed no aggression (biting) towards the male stimulus, which might be explained by the fact that the receiver can chemically assess the strength and relative fighting ability of the donor through the odours (characteristic bouquet of chemicals), thus avoiding costly aggressive interaction.

Since the chemical stimulus alone was sufficient to trigger behavioural responses in the lizards we conclude that olfactory stimuli play a major role in social interactions in *A. boskianus*. The response of males towards female secretions is an indication that sex discrimination in *A. boskianus* may be based on chemical cues. The high TF frequencies and lack of aggression towards females also suggests that the interest of males in females is predominantly sexual in nature. The high frequency of tongue flicking by males indicates the interest of the sexually deprived male in the female odour. Whether the male simply detects the female's presence, or whether it responds to a female courtship signal that may be present in the secretions and potentially indicates female receptiveness, requires further study.

Female *A. boskianus* can discriminate between male and female femoral secretions. Unexpectedly, some females showed some aggression (albeit insignificant when compared to males) towards male femoral secretions, suggesting that females may not have been receptive for mating or that they may have perceived signal odours interpreted as being aggressive. Females showed no interest in female secretions. Discrimination of individual characteristics of conspecifics such as body size (Shine et al., 2003), reproductive history (Shine et al., 2000) and sex (Mason, 1992) may require few TFs. It could be argued that, compared to male–male and female–male relationships, female–female relationships elicit little aggression because there is little competition between females.

In conclusion, we show that the femoral gland secretions of *A. boskianus* trigger aggression of males towards other males and different responses by each sex. Because males can identify other males' secretions, leaving secretions on the ground can signal territory ownership. This might suggest an important role in territorial marking. Our data also suggest that both males and females are able to chemically discriminate between secretions of their own sex and the opposite sex. The results indicate that the secretions contain sex-specific components that are employed as reproductive signals. Further chemical characterization of these sex-specific odour fingerprints will aim to identify the chemical identity of the components that signal sex, receptivity and an aggressive state.

ACKNOWLEDGEMENTS

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