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Potential Chemosignals Associated with Male Identity in the Amphisbaenian *Blanus cinereus*

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Abstract

Pheromone-based chemosensory sex discrimination occurs in many reptiles, but the specific chemosignals responsible for this discrimination have been rarely identified. Chemoreception is especially important for amphisbaenians, a group of fossorial, almost blind, reptiles. We analyzed the role of semiochemicals produced by precloacal glands in intraspecific communication and chemosensory sex recognition of the amphisbaenian *Blanus cinereus*. We expected that sexual discrimination in amphisbaenians would be based on those chemicals that show intersexual differences in precloacal secretions, with squalene being the chemical that shows the greatest difference in relative abundance between sexes. Tongue-flick assays and behavioral responses to the scent of conspecifics confirmed that amphisbaenians are capable of detecting and discriminating between scent of conspecific males and females by using chemosensory cues alone. Differential responses of amphisbaenians to chemical compounds that are naturally found in precloacal secretions indicated that males can readily discriminate between different chemicals. Squalene, in particular, elicited in male amphisbaenians' chemosensory and aggressive responses that were similar to those elicited by precloacal secretions. This result suggests that squalene alone allows male discrimination by male amphisbaenians. Furthermore, squalene might also signal dominance status or aggressiveness of a male amphisbaenian because higher concentrations of squalene elicited higher levels of aggression by males.

Key words: amphisbaenians, *Blanus cinereus*, chemosensory recognition, precloacal secretions, sex discrimination, squalene

Introduction

Many reptiles from different taxonomic groups can discriminate between the scent of male and female conspecifics (e.g., Mason and Gutzke 1990; Cooper et al. 1994; LeMaster and Mason 2001; López and Martín 2001b; Cooper and Pérez-Mellado 2002). In these studies, differential tongue-flick (TF) rates in response to male and female scents, as well as other differential behavioral responses related to reproduction (such as aggressive, courtship, or trailing behaviors), are considered clear indications of sexual discrimination. However, few studies have yet documented which chemicals are the bases for this pheromonal recognition (Wyatt 2003; Martín and López 2008, forthcoming). Moreover, only a few studies have examined the chemical compounds found in glandular secretions of reptiles (reviewed in Weldon et al. 2008) and whether lizards can discriminate between these different chemicals (Cooper and Pérez-Mellado 2001; Cooper et al. 2002a, 2002b; Martín and López 2006a, 2006b, 2008).

Chemoreception may be the only means of recognizing the presence of conspecifics and discriminating between sexes for fossorial blind reptiles. Amphisbaenians are a group of reptiles that are morphologically and functionally adapted to fossorial life (Gans 1978). Morphological adaptations to burrowing include trunk elongation, head modification, reduced vision, and loss of limbs in most species (Gans 1978). These adaptations constrain amphisbaenians to meet ecological demands with a suite of responses that differ from those of epigean reptiles (e.g., Martín et al. 1990, 1991; López et al. 1998). The amphisbaenian *Blanus cinereus* is a fossorial species endemic to the Iberian Peninsula (Salvador 1998). This species is usually found under rocks in sandy soils with abundant leaf litter (Martín et al. 1991). Studies of TF behavior showed that male *B. cinereus* use their vomeronasal system to detect and discriminate between chemical cues of males and females (Cooper et al. 1994) and between their own scent and the scents of other males (López et al. 1997). Amphisbaenians also detect odors of prey (López and Salvador 1992, 1994) and potential predators (López and Martín 1994, 2001a).

Amphisbaenians have several precloacal pores connected to precloacal glands that produce a copious holocrine secretions, especially during the breeding season (Gabe and

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Saint-Girons 1965; Whiting 1967; Antoniazzi et al. 1993, 1994). Morphological and microscopical examination of secretions suggested that, as an amphisbaenian moves inside tunnels, the secretion plugs are abraded against the substrate, releasing a secretion trail (Jared et al. 1999). This trail might contain pheromones that may be important in intraspecific communication and home range recognition inside tunnels (Cooper et al. 1994; López et al. 2000). Moreover, the ability to discriminate between sexes has been shown to be greater in response to precloacal gland secretions than from skin chemicals (Cooper et al. 1994). This differential discrimination suggests that chemicals secreted from precloacal glands allow chemosensory sex recognition in amphisbaenians.

We studied the role of semiochemicals produced by precloacal glands in intraspecific communication and chemosensory sex recognition of this amphisbaenian. Both male and female amphisbaenians *B. cinereus* produce copious amount of secretion from the precloacal glands. Chemical analyses by gas chromatography (GC)–mass spectrometry indicated that amphisbaenians' secretions contain 29 major lipophilic compounds, including several steroids (mainly cholesterol and cholesteryl methyl ether), carboxylic acids, and waxy esters, along with squalene (López and Martín 2005). In many vertebrates, intersexual differences in relative abundance of a compound in secretions may be indicative of putative pheromones (Wyatt 2003; Zhang et al. 2007; Zhang, Liu, et al. 2008). In the precloacal secretions of amphisbaenians, there are clear differences between males and females in the presence/absence of sex-specific compounds and in the relative proportions of some compounds expressed in both sexes. In particular, squalene is much more abundant in males (8.5%) than in females (0.6%) (López and Martín 2005; see Figure 1). This sex difference in squalene production is also found in the skin of garter snakes (Mason et al. 1989). Only male garter snakes present squalene in the skin, and courtship of females by males is partially inhibited if squalene is experimentally added to female skin (Mason et al. 1989). Thus, we hypothesized that squalene may be part of the male sex recognition system in amphisbaenians too.

In this paper, we used TF assays to study chemosensory responses of the amphisbaenian *B. cinereus*. We predicted that if amphisbaenians are able to detect and discriminate by chemosensory cues alone between scent of male and female precloacal secretions, amphisbaenians should also be able to discriminate squalene from other chemicals found in precloacal secretions. Moreover responses to the whole-precloacal secretions should be similar to responses to the "relevant" chemicals presented alone. However, males and females might differ in the magnitude of their chemosensory responses to the different chemicals found in secretions. This is because different chemicals might convey different messages for males and females. Thus, if squalene "signals" the presence of a male, males, but not females, should respond aggressively toward squalene stimuli, whereas neutrally toward other chemicals. Furthermore, if the concentration of squalene in secretions was related to aggressiveness level or dominance status of a male, as occurs with other chemicals in some lizards (Martín and López 2007; Martín et al. 2007), male, but not female, amphisbaenians should show greater chemosensory responses and higher levels of aggression to higher concentrations of squalene.

Materials and methods

Study animals

We captured by lifting stones 9 male and 10 female adult *B. cinereus* during April 2008, which coincided with the mating season of amphisbaenians, in different places over a large

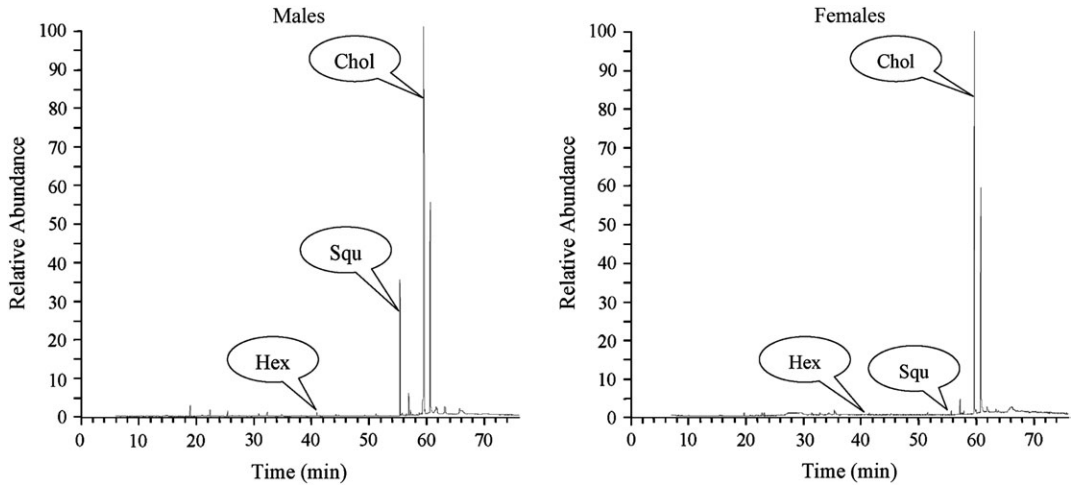

Figure 1 Representative GC profiles of hexane extracts of precloacal gland secretions of male and female amphisbaenians. The peaks that correspond to cholesteryl methyl ether (Chol), hexadecanoic acid (Hex), and squalene (Squ) are indicated. See López and Martín (2005) for GC conditions.

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area in an oak forest of Navacerrada (Madrid, Central Spain). Amphisbaenians were weighed (body mass; males: mean ± standard error [SE] = 7.2 ± 0.6 g, range = 5.8–8.7 g; females: 7.8 ± 0.5 g, range, 6.5–9.8 g) and their snout–vent length was measured (males: mean ± SE = 191 ± 4 mm, range, 174–223; females: 203 ± 5, range, 186–228). They were transported to the laboratory at "El Ventorrillo" Field Station, 5 km by air from the capture site. The animals were housed individually in terraria containing sand substrate, leaf litter, and stones from the capture area. They were fed mealworm pupae (*Tenebrio molitor*), adult ants (*Pheidole pallidula*), and earthworms twice weekly to satiation. To standardize hunger levels, they were not fed for 2 days prior to the study. Humidity was raised daily with a spray. Amphisbaenians were held in captivity 2 weeks prior to testing to allow acclimation to laboratory conditions and experimenter's presence.

Behavioral assays

Reptiles such as lizards, snakes, and amphisbaenians react to a variety of chemical stimuli with increased and differential rates of tongue extrusions (Cooper and Burghardt 1990; Cooper 1998). TF rate can, therefore, be used as a quantitative bioassay of detection of chemical cues of conspecifics (e.g., Cooper et al. 1994; Cooper and Pérez-Mellado 2002).

In a first experiment, each of 19 amphisbaenians was tested with chemical scent stimuli from the precloacal pores of conspecific male or female amphisbaenians or with deionized water. Water served as a control for TF's rates in the experimental situation in the absence of odors or vomodors (Cooper and Burghardt 1990). Amphisbaenians were in reproductive condition and had abundant secretion from precloacal pores. We prepared stimuli dipping the cotton tip (1 cm) of a wooden applicator (10 cm) in deionized water (Cooper 1998). Other stimuli were added by rolling wetted swabs over the precloacal pores of males or females (Cooper et al. 1994). The 3 conditions (male odor, female odor, and control deionized water) were presented to all individuals in a partially counterbalanced sequence in a randomized blocks design, only 1 trial per day being conducted for a given individual. Swabs were used in the trials immediately after collection to avoid fading of the stimuli, and a new swab was used in each trial.

In a second experiment, we compared TF rate by amphisbaenians in response to stimuli arising from cotton applicators bearing scents of 1) cholesterol, 2) hexadecanoic acid, 3) squalene, or 4) dichloromethane (DCM). The rationale for testing these chemicals was that intersexual differences were found for 3 of these substances in precloacal secretions of amphisbaenians (Figure 1 and see López and Martín 2005). Thus, 1) cholesteryl methyl ether is the most abundant steroid in secretions and is relatively more abundant in males than in females (44.7% vs. 40.0%), 2) hexadecanoic acid is the most abundant fatty acid in secretions (0.48% vs. 0.25%), 3) squalene might be a putative chemical signal of male identity as its abundance in precloacal secretions of males is much greater than in females (8.5% vs. 0.6%), 4) DCM was used as a control to gage baseline TF rates under the experimental conditions. We prepared chemical stimuli the same day of the tests by dissolving each compound (authentic standards, GC grade, from Sigma-Aldrich Chemicals) in DCM (30 mg/ml) in glass vials with Teflon-lined stoppers. We shook the solution for 1 min using a vortex and kept the vials in a refrigerator.

In a third experiment, we presented amphisbaenians with cotton swabs bearing 2 different concentrations of squalene dissolved in DCM: "low" (8 mg/ml) and "high" (56 mg/ml) and prepared as above.

Trials were conducted at the beginning of May, which coincided with the reproductive season of these amphisbaenians, and between 1100–1300 h (GMT) when amphisbaenians were fully active. We simulated fossorial conditions during the experiments by placing amphisbaenians in transparent plastic tubes (30 × 1 cm) having sand in the lower half and closed with cotton plugs at each end (López and Salvador 1992; Cooper et al. 1994; López and Martín 1994, 2001a). A different tube was used for each individual. The laboratory was darkened during trials and observations were made using a 50-W red light. Room temperature during trials was maintained constant at 20 °C, which allowed amphisbaenians to achieve optimal body temperatures for activity (Martín et al. 1990). Amphisbaenians were placed in their tubes 5 h/day during the acclimation period. The day before the experiments were begun, the animals behaved normally and fed in the tubes. Each animal was placed in its tube 10 min before each of its trials.

To begin a trial, an experimenter slowly approached a tube, removed the cotton from one side, and slowly moved the cotton swab to a position 2 cm anterior to the amphisbaenian's snout and closed the tube. Total TFs and TFs directed to the cotton swabs for 60 s beginning with the first TF were recorded. Latency to the first TF was computed as the period elapsed between closing the tube and the first TF. Because many amphisbaenians bit the swabs (see Results) early in the trial and then maintained the swab bitten during most of the trial, without being able to do further TFs, we also calculated TF attack score for repeated measures designs (TFAS(R); Cooper and Burghardt 1990). This is considered the best indicator of overall response strength because it combines effects of number of TFs with the occurrence of and latency to bites (Cooper and Burghardt 1990). If an individual does not bite a swab, its TFAS(R) is the number of directed TFs in that trial. If it bites, TFAS(R) is the largest number of directed TFs performed by that individual in any 1 stimulus condition plus (60 – latency to bite in seconds). Bites are more heavily weighted than any number of TFs because a bite represents an aggressive attack, not simply chemosensory investigation. Bites at short latency are more heavily weighted than bites at longer latency because they indicate more rapid identification of the chemical stimuli (Cooper and Burghardt 1990).

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Statistical analyses

To examine differences in latency to first TF among chemical stimuli, we used repeated-measures 2-way analyses of variance (ANOVAs) examining the effects of scent stimuli (within factor) and sex of the responding amphisbaenian (between factor: "male" vs. "female"). We included the interaction in the model to analyze whether responses to the different scents differed as a function of the sex of the responding amphisbaenian. Data were log transformed to ensure normality. Tests of homogeneity of variances (Levene's test) showed that variances of latencies were not significantly heterogeneous after transformation. Pairwise comparisons were planned using Tukey's honestly significant difference (HSD) tests (Sokal and Rohlf 1995).

Tongue-flick attack scores (TFAS) showed heterogeneous variances, even after transformation. Thus, to examine differences in number of TFAS, we used nonparametric Friedman's 2-way ANOVA (for water and the 2 conspecific scent stimuli and for the 4 chemical compounds stimuli) or Wilcoxon matched-pairs signed-ranks tests (for the 2 concentrations of squalene) (Siegel and Castellan 1988). Pairwise comparisons followed nonparametric post hoc procedures described in Sokal and Rohlf (1995). Comparisons between TFAS responses of males and females were made with Mann–Whitney *U*-tests. Significance level was 0.05, and all tests were 2 tailed.

Results

Chemosensory sex recognition

All amphisbaenians directed TFs to the swab in all conditions. Mean latency to first TF differed significantly between scent stimuli (2-way repeated-measures ANOVA: $F_{2,34} = 27.43$, $P < 0.0001$) and between responding males and females ($F_{1,17} = 77.79$, $P < 0.0001$), but the interaction was significant ($F_{2,34} = 5.89$, $P = 0.006$) (Figure 2a). Males responded to water significantly later than to the scent of males (Tukey's test: $P = 0.007$) or to the scent of females ($P = 0.0001$) and later to the scent of males than to the scent of females ($P < 0.05$), whereas females responded to water significantly later than to the scent of males ($P = 0.001$) or to the scent of females ($P < 0.05$). However, latency times of females to respond to the scent of males and females did not differ ($P = 0.61$). Thus, males and females responded in a similar way to the scent of males ($P = 0.17$), but females responded significantly later than males to water ($P = 0.04$) and to the scent of females ($P = 0.00016$).

Four male amphisbaenians behaved aggressively (i.e., bit the swab) when presented swabs bearing scent of conspecific males. Males did not bite swabs bearing water or female scent. Based on the null hypothesis that the likelihood of biting was equal in all 3 conditions, the 2-tailed binomial probability that all the 4 bites would be in the male scent condition is 0.016. Females did not bite the swabs with any stimuli.

In males, TFAS differed significantly between scent stimuli (Friedman's ANOVA: $\chi^2_2 = 14.11$, $n = 9$, $P < 0.001$) (Figure 2b).

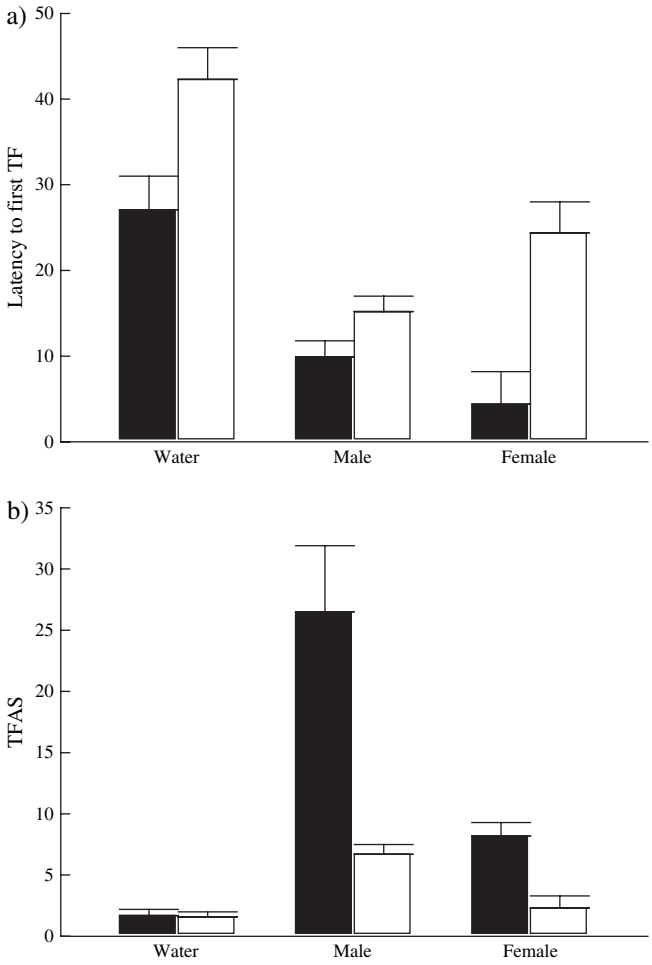

Figure 2 TF latency and TF attack scores of amphisbaenians responding to scents from a deionized water control (Water) or to precloacal gland secretions from conspecific males (Male) or females (Female). Scents were presented for 60 s on cotton-tipped applicators. **(a)** Mean latency (+SE) to the first TF. **(b)** TFAS. See text for definition of TFAS. Black boxes, males; open boxes, females.

TFAS of males were significantly higher in response to conspecific male and female scent than to water (nonparametric post hoc: $P < 0.01$ in both cases) and were significantly higher in response to male than to female scent ($P < 0.05$). In females, TFAS differed significantly between scent stimuli (Friedman's ANOVA: $\chi^2_2 = 8.43$, $n = 10$, $P = 0.015$) (Figure 2b). TFAS of females were significantly higher in response to male scent than to female scent (nonparametric post hoc: $P = 0.038$) and to water ($P = 0.008$), but TFAS did not significantly differ between water and female scent stimuli ($P = 0.29$). Males showed significantly higher TFAS than females in response to conspecific male (Mann–Whitney's *U*-test: $Z = 2.93$, $n = 9,10$, $P = 0.003$) and female scent ($Z = 3.36$, $n = 9,10$, $P = 0.0008$), but TFAS in response to water were similar in males and females ($Z = 0.04$, $n = 9,10$, $P = 0.97$).

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Chemosensory and aggressive responses to chemicals

All amphisbaenians directed TFs to the swab in all conditions. Mean latency to first TF differed significantly between chemical compound stimuli (2-way repeated measures ANOVA: $F_{3,51} = 13.15$, $P < 0.0001$) and between responding males and females ($F_{1,17} = 19.79$, $P = 0.0003$), but the interaction was significant ($F_{3,51} = 3.28$, $P = 0.028$) (Figure 3a). Males responded to squalene significantly earlier than to DCM (Tukey's test: $P = 0.00013$) to cholesteryl methyl ether ($P = 0.0003$) and to hexadecanoic acid ($P = 0.049$), but latency times to DCM, cholesteryl methyl ether, and hexadecanoic acid were not significantly different from each other ($P > 0.15$ in all cases). In contrast, latencies of females did not differ between chemical compounds ($P > 0.30$ in all cases).

Six male amphisbaenians behaved aggressively (i.e., bit the swab) when presented swabs bearing squalene, and 3 bit swabs bearing hexadecanoic acid. Males did not bite swabs bearing DCM or cholesteryl methyl ether. Based on the null hypothesis that the likelihood of biting was equal in all 4 conditions, the 2-tailed binomial probability that 6 of the 9 bites would be in the squalene condition is 0.020. Three females bit the swab bearing cholesteryl methyl ether (binomial: $P = 0.031$) but did not bite other stimuli.

In males, TFAS differed significantly between chemical compounds (Friedman's ANOVA: $\chi^2_3 = 15.31$,, $n = 9$, $P = 0.0016$) (Figure 3b). TFAS of males were significantly higher in response to squalene and hexadecanoic acid than to DCM (nonparametric post hoc: $P = 0.008$ in both cases) and to cholesteryl methyl ether ($P < 0.05$ in both cases), TFAS were significantly higher in response to squalene than to hexadecanoic acid ($P < 0.05$). TFAS in response to cholesteryl methyl ether was significantly higher than to DCM ($P < 0.05$). However, in females, TFAS did not differ significantly between chemical compounds (Friedman's ANOVA: $\chi^2_3 = 1.58$, $n = 10$, $P = 0.66$) (Figure 3b). Males showed significantly higher TFAS than females in response to squalene (Mann–Whitney's U-test: $Z = 3.66$, $n = 9,10$, $P = 0.0002$) and hexadecanoic acid ($Z = 2.98$, $n = 9,10$, $P = 0.003$), but TFAS of males and females were similar in response to DCM ($Z = 1.60$, $n = 9,10$, $P = 0.11$) and cholesteryl methyl ether ($Z = 1.39$, $n = 9,10$, $P = 0.16$).

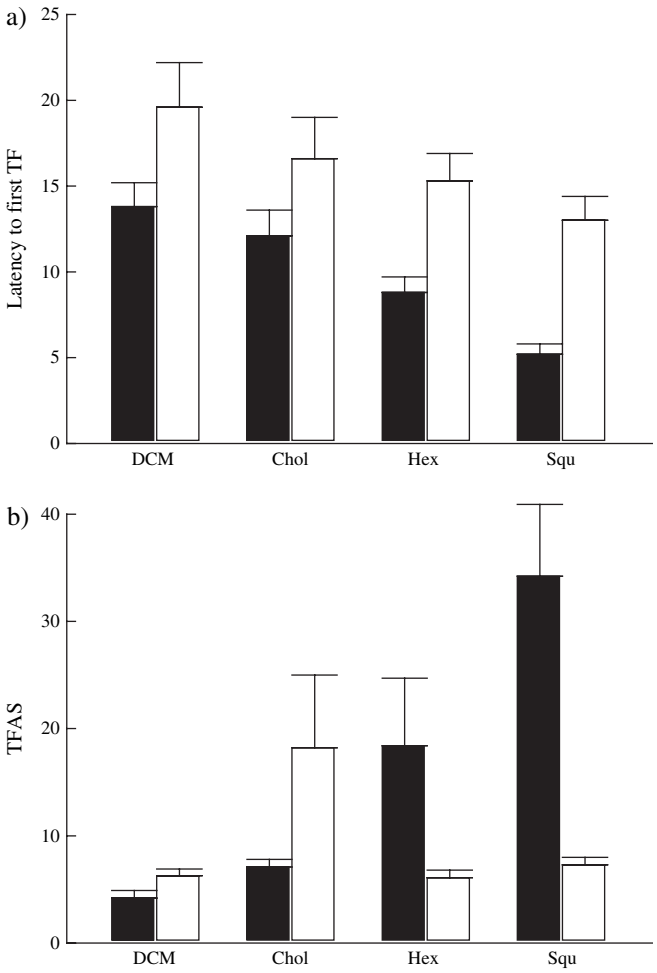

Figure 3 TF latency and TF attack scores of amphisbaenians responding to control DCM, cholesteryl methyl ether (Chol), hexadecanoic acid (Hex), or squalene (Squ), all dissolved in DCM. Chemicals were presented for 60 s on cotton-tipped applicators. **(a)** Mean latency (+SE) to the first TF. **(b)** TFAS. See text for definition of TFAS. Black boxes, males; open boxes, females.

Chemosensory and aggressive responses to different concentrations of squalene

All amphisbaenians directed TFs to the swab in all conditions. Mean latency to first TF was significantly longer for the low concentration of squalene (2-way repeated-measures ANOVA: $F_{2,34} = 17.17$, $P < 0.05$), males showed significantly shorter latencies than females ($F_{1,17} = 33.69$, $P < 0.0001$), and the interaction was not significant ($F_{2,34} = 1.15$, $P = 0.30$) (Figure 4a).

Six males bit the swab bearing high concentrations of squalene, but no male bit it in response to the low concentration of squalene (binomial test: $P = 0.031$). Females did not bite any swab. TFAS were significantly higher for the high concentration of squalene in males (Wilcoxon matched-pairs tests: $Z = 2.25$, $n = 9$, $P = 0.024$) but not in females ($Z = 0.17$, $n = 10$, $P = 0.87$) (Figure 4b). Males showed significantly higher TFAS than females in response to the high concentration of squalene (Mann–Whitney's U-test: $Z = 2.42$, $n = 9,10$, $P = 0.016$), but TFAS of males and females were similar in response to the low concentration of squalene ($Z = 0.66$, $n = 9,10$, $P = 0.51$).

Discussion

Our results show that amphisbaenians can detect and discriminate between scent from precloacal secretions of conspecific males and females by using chemosensory cues alone. This sexual discrimination may be, at least partly, based on the intersexual differences in concentrations of

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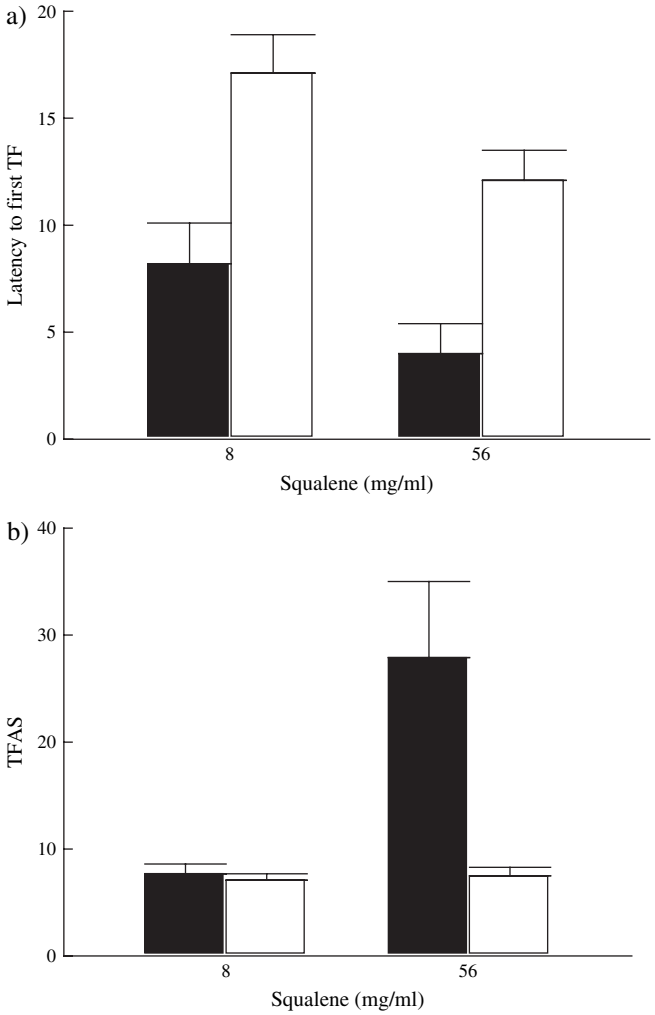

Figure 4 TF latency and TF attack scores of amphisbaenians responding to 2 different concentrations of squalene dissolved in DCM. Chemicals were presented for 60 s on cotton-tipped applicators. **(a)** Mean latency (+SE) to the first TF. **(b)** TFAS. See text for definition of TFAS. Black boxes, males; open boxes, females.

squalene in precloacal secretions. Moreover, the detection of chemicals "signalling" male identity elicits, only in males, aggressive responses similar to those observed in agonistic interactions between males in a reproductive context. This aggressive response reveals the importance of chemical signals in reproductive behavior of fossorial amphisbaenians.

The documentation of chemosensory responses to conspecific scents from precloacal secretions confirmed previous results for male amphisbaenians (Cooper et al. 1994) and also indicated that females were capable of sexual discrimination. Furthermore, our results indicated that responses of males and females were different, which suggests that precloacal secretions convey different messages for each sex, as it occurs in some lizards (Cooper and Steele 1997; Martín and López 2008). Thus, although male scent elicited aggressive responses in some males, females did not respond aggressively in any case. Also, female scent elicited greater chemosensory responses (i.e., higher TF rates) in males than in females, suggesting that the detection of female scent induces further chemosensory searching of the origin of the stimulus in males but not in females. These results support the hypothesis that chemical signals induce sex-specific reproductive-related behaviors in amphisbaenians. Similarly, male leopard geckos (*Eublepharis macularius*) perform aggressive behaviors toward male scent, but not to female scent, and tail vibrations (a courtship behavior) in response to female, but not to male, scent (Cooper and Steele 1997).

The differential responses of male amphisbaenians to chemical compounds found in precloacal secretions indicate that males can readily discriminate between the different chemicals tested in our experiment. However, responses of females did not differ among these chemicals, which might suggest that other chemical compounds not tested here may be used for chemosensory sex discrimination by females. Alternatively, the low number of TFs characteristic of amphisbaenians' chemosensory responses (see López and Salvador 1992, 1994) might have obscured a true differences between responses. The latter might explain why some females bit swabs with cholesteryl methyl ether but did not bite swabs with other chemicals, which suggests that at least this chemical may have been discriminated. Because this steroid is the most abundant compound in precloacal secretions, females might use it to detect conspecific scent trails in underground tunnels. Also, it is possible that precise sex discrimination by females required a particular combination of multiple chemical stimuli, as it naturally occurs in the whole-precloacal secretions rather than a single chemical. Similarly, in many vertebrates, pheromones are a mixture of several chemical compounds (Müller-Schwarze 2006) that may act all together, providing specific or individual "odor profiles" or "mosaics" (e.g., Johnston 2005). In contrast, male amphisbaenians might have evolved to respond after detecting a single chemical stimulus because, for example, the early detection of a competitor male should be more important for males than for females.

Studies have shown chemosensory sex discrimination in many species of reptiles, but the chemical bases were not identified (Mason 1992; Martín and López, forthcoming). We expected that sexual discrimination in amphisbaenians would be based on those chemicals that show intersexual differences in precloacal secretions (López and Martín 2005), with squalene being the chemical that shows the greatest difference in relative abundance between sexes (see Figure 1). Because the difference is so large and male amphisbaenians can discriminate it by chemosensory cues alone, squalene is a suitable substance for identification of sex. Our results indicated that squalene, at least, elicits in male amphisbaenians chemosensory and aggressive responses similar to those for the whole of precloacal secretions. This suggests that squalene alone allows sex discrimination by male amphisbaenians. Nevertheless, it is also likely that other chemicals that differ between sexes contribute by facilitating or

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confirming sexual discrimination when the whole-precloacal secretion is available for "investigation." Thus, other chemicals (e.g., hexadecanoic acid) were discriminated too and also elicited in males relatively higher chemosensory and aggressive responses. They, too, might be implicated in sex discrimination. Further experiments should examine responses of amphisbaenians to different mixtures of chemicals and with different concentrations of components.

Also, it remains to be analyzed whether female discrimination is based on the low proportions of squalene per se in secretions of females or in the additional presence of other chemicals found exclusively in precloacal secretions of females, such as tocopherol (López and Martín 2005). For example, in leopard geckos (*E. macularius*), there are distinct sex differences in lipid fractions of the skin, with some steroids being characteristics of males and long-chain methyl ketones being characteristics of females (Mason and Gutzke 1990). Male geckos court females, but when females are shedding the skin (and consequently lack these female chemicals), males respond aggressively to females, as if they were males (Mason and Gutzke 1990).

Interestingly, squalene has been identified as one component of the male recognition pheromone system of garter snakes (*Thamnophis sirtalis parietalis*) (Mason et al. 1989). During courtship in mating balls, male garter snakes are ignored by other males probably because their skin lipids include squalene, which is absent in female skin. Courtship can be experimentally inhibited by adding extracts of male skin lipids or squalene alone to the skin lipids of females (Mason et al. 1989). Also, male garter snakes that behave like females, the so-called "she males," have no squalene, which allows them to avoid aggression from other males (Mason et al. 1989). Moreover, squalene with activity of male pheromone has been identified in the anogenital gland secretion of giant pandas (*Ailuropoda melanoleuca*) (Zhang, Liu et al. 2008) and in the preputial gland of the rat (*Rattus norvegicus*) (Zhang et al. 2007; Zhang, Sun et al. 2008). The occurrence of pheromonal use of squalene in a snake, an amphisbaenian, and 2 mammals coupled with reliance on other compounds for similar pheromonal functions in some squamates and mammals suggests that biochemical convergence of pheromonal use of squalene may have occurred among distantly related taxa (Wyatt 2003).

Lizards and other reptiles often exhibit social dominance systems (Mason 1992), which may be based on pheromones (Martín and López 2007; Martín et al. 2007). Squalene might signal dominance status or aggressiveness threat level of a male amphisbaenian because higher concentrations of squalene elicited higher levels of aggression by males. Because squalene is the biochemical precursor of many steroids, including the steroid hormones such as testosterone, it is likely that there is some metabolic relationship between the circulating amounts of these 2 compounds. Also, it is possible that variations in testosterone levels are implicated in modulating lipid and steroid biochemistry, as it occurs in other reptiles (e.g., Lacy et al. 2002), which will in turn affect the amount of circulating squalene. This relationship would be reflected in the composition of the precloacal secretion because this is produced by holocrine glands. Experimental manipulation of testosterone levels is needed to investigate its effects on secretions and aggressiveness.

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