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Behavioral evidence of pheromonal signaling in desert grassland scorpions *Paruroctonus utahensis*

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Abstract. Behavioral evidence suggests that, in some scorpion species, females deposit a pheromone that attracts mates. To date, however, no pheromone has been identified. The goal of our study was to isolate a pheromone from female desert grassland scorpions, *Paruroctonus utahensis* (Williams, 1968) (Scorpiones:Vaejovidae). We took in situ cuticular washes from female *P. utahensis* in a chloroform-methanol solution; the extract stratified into aqueous and organic layers. In controlled laboratory experiments, most males exposed to female extract (aqueous and organic fractions combined) exhibited pre-courtship behavior, whereas those exposed to the solvent control (2:1 chloroform-methanol) showed no change in behavior. When extract fractions were separately tested, males initiated pre-courtship behavior when exposed to the organic fraction but not when exposed to the aqueous fraction. These data are the first experimental evidence of a female pheromone in this species and are important early steps toward characterizing any scorpion pheromone.

Keywords: Pheromone, ground-directed chemical signaling, pectines, arachnid, arthropod

The mechanisms mediating ground-directed chemical signaling have received little empirical attention compared with mate tracking via airborne chemical cues. However, some animals, such as snakes, insects and arachnids, detect nonvolatile sex pheromones while moving along the ground. Male red-sided garter snakes follow the trail of female skin rubbings, allowing males to locate females over long distances (LeMaster & Mason 2001). Similarly, male parasitoid wasps (*Aphelinus asychis*) and male minute pirate bugs (*Orius sauteri*) detect and respond to conspecific female deposits on leaves, but they show no response to volatile female odors (Fauvergue et al. 1995; Nakashima & Hirose 1999).

In addition, both chemical and behavioral evidence suggests that spiders follow pheromone trails to find mates. Recent studies have isolated pheromones from spider silks and indicate a wide variety of chemical signals—from nonpolar fatty acids in the agenelid spider *Tegenaria atrica* (C.L. Koch 1843) to small, polar compounds like dimethyl citrate in the wandering spider (Papke et al. 2000; Trabalon et al. 2005; Jerhot et al. 2010). Further, male wandering spiders *Cupiennius salei* (Keyserling 1877) display courtship behavior upon contact with a silk dragline treated with a small concentration of female pheromone (Barth 1993; Tichy et al. 2001).

Several studies suggest chemical cues are important in scorpion mate-tracking and courtship behavior. In a Y-maze test, male desert hairy scorpions *Hadrurus arizonensis* (Ewing 1928) preferred substrate previously walked on by females but showed no preference for substrate previously walked on by males (Melville et al. 2003). Additionally, male dune scorpions *Smeringurus mesaensis* (Stahnke 1957) initiate pre-courtship behavior when they encounter chemical washes of conspecific female epicuticles (Gaffin & Brownell 1992).

Elaborate, sexually dimorphic organs provide further evidence that chemical communication guides male scorpion mate-searching behavior. All scorpions have movable, ground-directed organs called pectines on their mid-ventral abdomen (Cloudsley-Thompson 1955; Foelix & Müller-Vorholt 1983; Hjelle 1990). Pectines are likely important in mate tracking; upon contact with substrate previously walked on by females, pectinal sweeping increases in *S. mesaensis* (Gaffin & Brownell 1992). Additionally, in pectine-ablated *S. mesaensis* males, conspecific female chemical washes did not release pre-courtship behavior (Gaffin & Brownell 2001). In most scorpions, male pectines are longer and contain more chemosensory peg sensilla than do female pectines (Polis & Farley 1979; Swoveland 1978). A sexual dimorphism in peg chemosensitivity might also be present in some scorpion species: single peg stimulations of *P. utahensis* with citric acid evoked a higher response in male sensilla than in female sensilla (Knowlton & Gaffin 2011). Natural selection might have favored larger pectines with selective chemical sensitivity in males if these chemosensory organs help males track female sex pheromones.

In this paper, we present the first steps toward our goal of isolating and characterizing a scorpion sex pheromone. We collected female *Paruroctonus utahensis* (Williams 1968) scorpions during the peak of their mating season and immediately extracted cuticle-associated chemicals. We then exposed conspecific males to the female extract in laboratory experiments. Initial exposures evoked pre-courtship behaviors similar to those described in *S. mesaensis* (Gaffin & Brownell 1992). Subsequent tests on aqueous and organic fractions revealed strong behavioral responses to the organic fraction. Our evidence suggests that the pheromone has low polarity and is highly stable.

METHODS

Research animal.—We collected *P. utahensis* during early September from sand dunes near Monahans, Texas (31°29′N, 102°39′W) at night using UV. We deposited a voucher specimen in the Sam Noble Oklahoma Museum of Natural History in Norman, Oklahoma. The mating season of *P. utahensis* runs from mid-August through early September (Bradley 1988). In many ways, the natural history and surface activity of *P. utahensis* is similar to the well-described ecology of *S. mesaensis* (formerly *P. mesaensis*; Polis & Farley 1979).

Animal care.—The scorpions were kept in the laboratory in 3.8 l glass jars partially filled with sand from the animals' desert environment. We used timed lighting to maintain a 14:10 h L:D cycle; temperature and humidity were kept within

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a consistent range (22°C, 55–60% RH). The scorpions were fed a wax worm once every 2–3 weeks and were provided water twice a week.

Extract preparation.—In these experiments, we tested male response to a female extract. To produce the female extract, we anesthetized 35 adult (at least 200 mg) female scorpions on ice within an hour of collection and then immediately submerged them in a 2:1 chloroform-methanol solvent. After 8 h, the extract was decanted. Two days later in the laboratory, we condensed the extract to a total volume of about 25 ml using a filter flask and a vacuum line. A few ml of water were added as the solution was drying. The resulting solution stratified to an aqueous layer on top and an organic (relatively nonpolar) layer on bottom. The solution was kept in an opaque glass container at room temperature.

Apparatus and trial preparation.—We used infrared cameras (two Defender SP301-C cameras and one Sony CCD-TRV16 camera) to record male scorpion behavior in contained glass arenas (Pyrex© 15 cm diameter, 7.5 cm deep). We divided each arena into four fictive quadrants with small pieces of tape adhered to the arena rim. The bottom of each arena was thinly coated with sand. Infrared light emitted from photodiodes on the cameras was the only light source during these experiments; scorpions are not sensitive to infrared wavelengths (Blass & Gaffin 2008).

We conducted two experiments to determine if the female extract would release pre-courtship behavior in male scorpions. To determine sample size, we conducted an a priori power analysis using data from unpublished research by Gaffin and from pilot studies ($dz = 2.06$; $\alpha = 0.05$; $1-\beta = 0.95$; paired). We concluded that we needed at least six legitimate trials per experiment to be confident in our results (see criteria for legitimacy below); we used extra scorpions in each experiment to ensure we met this minimum. Each trial started between 1–3 h after the beginning of the dark cycle, when scorpions are normally active in the field. The order of treatment exposure to each scorpion was random (as determined by a computer random number generator). The scorpion was placed in the quadrant opposite the test quadrant, initiating the trial. After each trial, we removed the sand, cleaned the arena with 70% ethanol, and placed fresh sand into the arena.

Experiment 1.—In the first experiment, conducted in October and November, we exposed 12 male scorpions to the female extract and to a control solution at separate times. The extract was shaken to create a homogeneous solution before application to the sand on the arena floor; 2:1 chloroform-methanol solvent was used as the control. The trial preparer deposited 100 µl of either the extract or the control solution near the arena wall of the randomly selected test quadrant (by computer random number generator). Scorpions rested at least 2 d between trials.

Experiment 2.—We conducted a second experiment a month after the first experiment ended. Ten of the 12 scorpions used in the first experiment were used for the second experiment. The other two scorpions died between Experiments 1 and 2. For the second experiment, extract fractions were separately tested. The trial preparer deposited 100 µl of either the organic fraction or the aqueous fraction near the arena wall in the randomly selected test quadrant. We placed the scorpion in the quadrant opposite the test quadrant, initiating the trial. Scorpions rested 6 d between trials.

Trial viewing and scoring.—Each trial was recorded (Defender SN501 DVR) and viewed later. Each trial lasted for 45 min after we placed the scorpion in the arena. The trial scorer was blind to the treatment and test quadrant for each trial. Since the test quadrant could be inferred from scorpion placement in the arena, another researcher previewed each trial and recorded when the scorpion began moving; the scorer began viewing the trial 10 s after movement began. The reviewer viewed and scored trials every couple of days. Since the order of treatment exposure was randomized, the reviewer never knew what extract was being tested. The previewer recorded scorpion position relative to the test quadrant after 10 s of movement; analysis of these data suggest that scorpions had no bias toward any quadrant during these initial movements ($X^2 = 0.5$, $df = 2$, $P = 0.779$), keeping the test quadrant unknown to the scorer. Further, no scorpion exhibited courtship behaviors during the first 10 s of movement. The previewer also determined trial legitimacy; in a legitimate trial, the scorpion had to cross the test quadrant at least once.

We based our scoring criteria on pre-courtship behaviors documented in *S. mesaensis* males (Gaffin & Brownell 1992). Before starting experiment 1, we conducted pilot studies on *P. utahensis* to ascertain species-specific differences in pre-courtship behavior. Trials were assigned a score of 1–5 according to the following criteria. 1) No change in behavior. 2) Slight change in behavior, such as creeping (change of normal motion to shorter forward steps and frequent turning) and sudden stops throughout arena. 3) One occurrence of pedipalp-reaching or scrunching (drawing the pedipalps back and toward the body midline), back-up (an abrupt stop of forward motion followed by one or two steps backwards), or sidestepping. 4) Two three-level responses in the same quadrant. 5) Grasping the substrate with the pedipalps, tail wagging, juddering, or three three-level responses in the same quadrant.

Statistical analysis.—In both experiments, only scorpions with two legitimate trials were included in the statistical analysis. All 12 scorpions in Experiment 1 had two legitimate trials, whereas only six out of 10 scorpions in Experiment 2 had two legitimate trials. The final score assigned to each trial was the highest score observed in the test quadrant during that trial. We collected paired, nonparametric data in both experiments and used the Wilcoxon signed-rank test (SPSS software, release 12.0.0) to determine whether treatments within each experiment evoked statistically different behavioral responses.

RESULTS

In these experiments, we exposed *P. utahensis* males to washes from the cuticle of female conspecifics. Of the 22 pairs of trials conducted, 16 (73%) were legitimate. The behavior of male *P. utahensis* changed as they encountered female extracts deposited on the sand substrate of the arena. Back-ups and pedipalp-scrunches were the most common behaviors observed, and males displayed these behaviors to varying degrees. Males that received a score of 5 often continued to perform strong behaviors (sidesteps, back-ups, pedipalp-scrunches) in the test quadrant for several minutes.

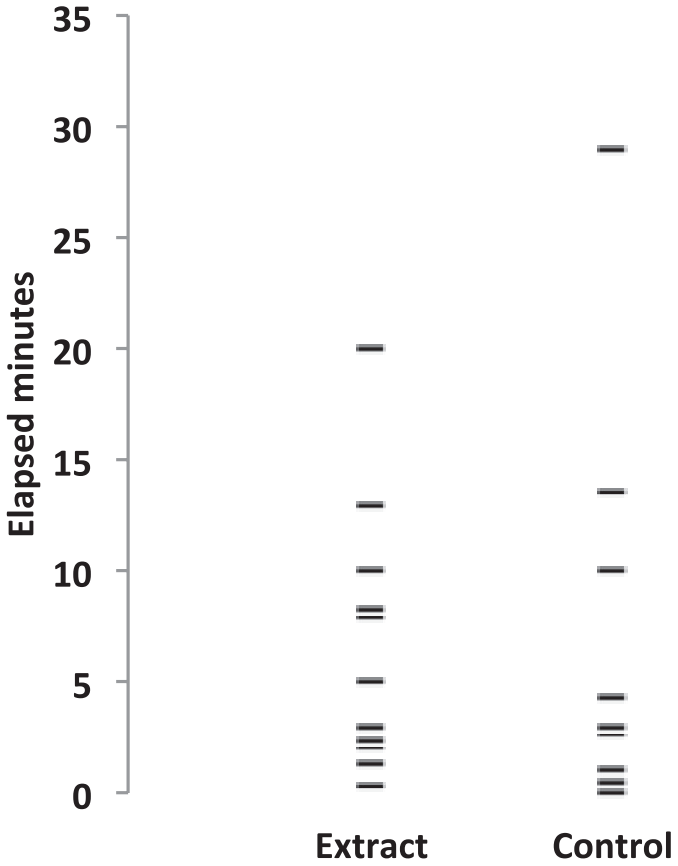

Figure 1.—Distribution of elapsed time before entering treatment quadrant. Shown are durations in minutes for extract and control trials in Experiment 1. Each bar represents the duration for one scorpion.

Others abruptly stopped when walking over the deposited extract, backed up, and then continued walking around the arena (a score of 3). Juddering and tail wagging were each observed in one trial. The strongest response observed was often during a male's first or second crossing of the test quadrant, but in some trials, the male's behavior did not change until he had encountered the deposited extract several times. When males detected no stimulant, they typically walked around the perimeter of the arena and/or attempted to climb the arena walls.

Volatile chemicals from the female extract apparently did not attract males to the treatment quadrant. The average elapsed time before entering the treatment quadrant was 6.7 min in extract trials and 5.9 min in control trials (Fig. 1). In addition, no change in behavior was noted before males entered the test quadrant, providing additional evidence that males are responding to a ground-based chemical.

Experiment 1.—Behavior changed in 10 out of 12 males exposed to homogenized female extracts. The trial scorer also noted changed behavior in two of the 12 control trials, which indicates a low level of experimental error. Males received significantly higher behavioral scores when exposed to female extract than when exposed to solvent control (Fig. 2: $Z = 2.68$, $p < 0.001$, two-tailed).

Experiment 2.—The trial-scorer recorded modified behavior in all six males exposed to the organic fraction of the female extract; four males (66%) received the highest behavioral score. No changed behavior was noted as males walked across the aqueous fraction of the female extract. Males received significantly higher behavioral scores in organic fraction trials than in aqueous fraction trials (Fig. 3: $Z = 2.26$, $p = 0.026$, two-tailed).

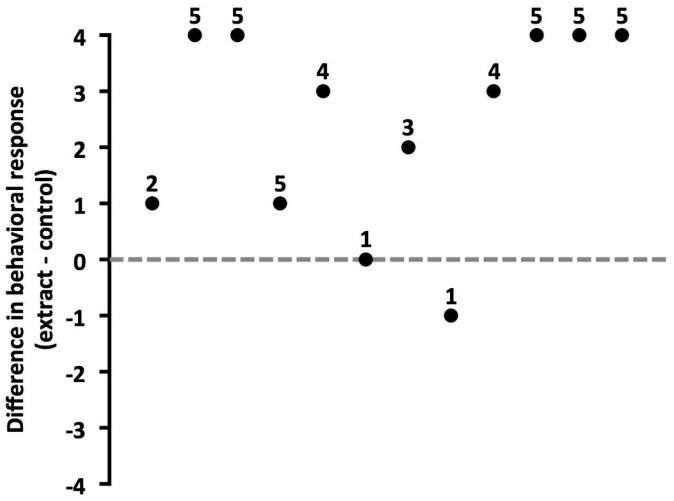

Figure 2.—Comparison of male behavioral responses to homogenized female extract or control fluid of chloroform/methanol. Plotted is each male's difference in behavioral response between trials (extract minus control). Numbers above each point indicate the score of the extract trial. Subtracting the y-axis value from the extract trial value gives the corresponding control trial value.

DISCUSSION

This study represents the first steps toward isolating a scorpion pheromone. Our first experiment revealed that male *P. utahensis* behavior changes when encountering a female chemical extract, which provides the first evidence for substrate-borne chemical signaling in this species. Our second

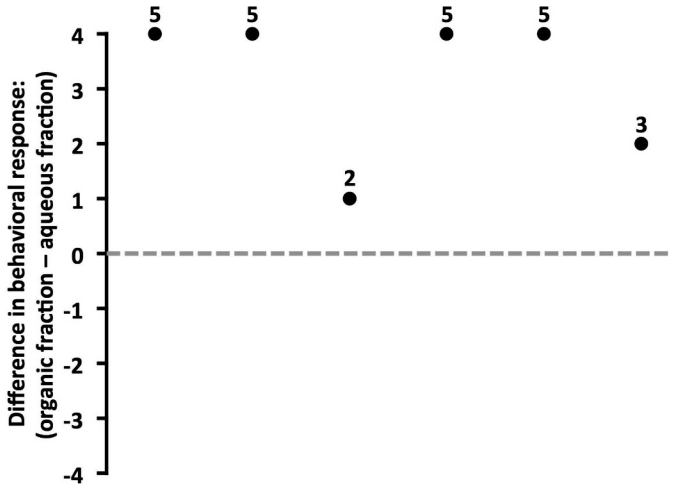

Figure 3.—Comparison of male behavioral response to fractioned female extracts. Plotted is each male's difference in behavioral response between trials (organic fraction minus aqueous fraction). Numbers above each point indicate the score of the organic trial. Subtracting the y-axis value from the organic trial value gives the corresponding aqueous trial value.

experiment showed that male behavior only changes upon encountering nonpolar chemicals from the female chemical extract. Aqueous chemicals from the female extract released no pre-courtship behavior in any of the males tested.

Males are not attracted to the female extract at a distance. In none of our studies did males initiate pre-courtship behavior outside of the test quadrant of the arena. In addition, males were not drawn to the test quadrant more quickly in trials with the female extract than in control trials in Experiment 1. We therefore conclude that the chemical (or mixture of chemicals) that males responded to in our studies was not volatile.

Although the pheromonal chemical(s) remain unknown, it is possible that male scorpions are reacting to a lipid in the female cuticle. Nonpolar chemicals, such as cuticular lipids, might be more stable in a hot, windy habitat than polar chemicals. In general, longer carbon chains have lower polarity and a higher boiling point than shorter carbon chains. Although we cannot be certain that the female *P. utahensis* chemical signal has long hydrocarbon chains, cuticular extracts from another scorpion, *Hadrurus arizonensis*, show a high proportion of compounds with chains exceeding 18 carbons (Trabalon & Bagnères 2010).

Males responded vigorously to the extract in Experiment 2, four months after initial extraction. In these scorpions' habitat, it is likely that heat, wind, and exposure to sunlight would break down chemicals faster than we observed in our laboratory. Still, winds may blow sand grains covered with female-deposited pheromone across the dunes. If the pheromone remained stable for even a few days, the pheromone could spread long distances from the female. During our collections in the mating season, we observed several males within 20 m of each female, an unusual grouping for *P. utahensis* at this field site. With the dispersing sands, it seems unlikely that males follow a female trail directly to her. Instead, it is more likely that males compare concentrations of female pheromone and move toward increasing concentration.

The stability of the female pheromone is noteworthy and may lead to some interesting follow-up studies. For example, we hope to test male response to fractions within the organic female extract. We have conducted some preliminary trials (two trials each on three different fractions) and did not see the vigorous male responses we observed with the entire organic extract. One scorpion exposed to the most nonpolar fraction stopped in the test quadrant during his first crossing, backed up, and moved forward four times, but no other male responded. The low response rate in these preliminary trials might suggest that male scorpions respond to combinations of female chemicals. However, these observations must be approached with caution, because of the small sample size. In addition, because reproduction is seasonal, male sensitivity might fluctuate throughout the year, influencing their responses to these fractions. It is also possible that the active compound or compounds had begun to break down before these trials, which were conducted eight months after the extraction was made.

Since it is likely that males use their pectines to detect female pheromones (Gaffin & Brownell 2001), future studies might focus on pectinal sweeping as animals move across extract-contaminated substrates. For example, Blass and Gaffin (2008) made circular tracks composed of a small Petri dish glued inside a larger Petri dish. In such a setup, the pectines can be filmed from below through a clear Plexiglas stage. Extract fractions could be blotted directly on the Petri dish surface or dried onto grains of sand sprinkled in the arena, and the number of pectinal sweeps could be tracked as the animal moved across various stimuli. It might also be possible to use thin-layer liquid chromatography (TLC) to separate the extract into bands and test the animal directly in a rectangular arena atop the TLC plate. In this case, the monitoring would be from the top, but an angled mirror could provide visual access to pectinal activity.

Identifying scorpion mate-tracking pheromones has several possible implications, from controlling populations of dangerous scorpion species (thousands of humans die of scorpion stings annually: Warrell 2007) to understanding the physiology and evolutionary significance of pectines. Given our ability to electrophysiologically record directly from scorpions' pectinal peg sensilla (Knowlton & Gaffin 2010), the identification of pheromone compounds presents a unique opportunity to relate proximate sensory mechanisms with the evolution of a ground-based sexual signaling system.

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