

Evidence for a female sex pheromone mediating male trailing behavior in the red-sided garter snake, *Thamnophis sirtalis parietalis*

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Summary. Male garter snakes locate females during the breeding season utilizing conspecific trailing behavior. It has been hypothesized that the female-derived chemical cue responsible for mediating male reproductive trailing behavior is the sexual attractiveness pheromone, a previously characterized contact pheromone responsible for releasing male courtship behavior. To examine this hypothesis, we tested the response of male red-sided garter snakes, *Thamnophis sirtalis parietalis*, to pheromone trails produced by males, females and 'she-males'. She-males are a small subset of males in the natural population that are morphologically and behaviorally similar to other males except that they produce and express the sexual attractiveness pheromone during the breeding season. When tested on a Y-maze, males were found to detect and follow the skin lipid trails of females and she-males, but displayed no behavioral responses to male trails. In addition, males were unable to discriminate between she-male and female trails when given a choice. These results are consistent with the hypothesis that the sexual attractiveness pheromone is the chemical cue primarily utilized by males to mediate reproductive trailing behavior.

Key words. *Thamnophis sirtalis parietalis* – red-sided garter snake – trailing behavior – sexual attractiveness pheromone – methyl ketones

Taxa. Class: Reptilia – Order: Squamata – Family: Colubridae – Genus: *Thamnophis* – Species: *Sirtalis* – Subspecies: *Parietalis*

Introduction

Snakes of the genus *Thamnophis* (garter snakes) utilize pheromone trails extensively for mediating reproductive activity (reviewed in Ford 1986; Mason 1992). For example, female garter snakes produce pheromone trails that guide males to their exact location during the breeding season (e.g. Ford 1981; Ford & Low 1983;

Ford & O'Brien 1986; LeMaster *et al.* 2001). Pheromone trails have been found to not only communicate the sex and travel direction of a trail-producing individual to male garter snakes, but also contain information concerning the species identification and sexual attractiveness of the trail-producer (Ford 1978, 1982; Ford & Schofield 1984). Though there is clear behavioral evidence for a trailing pheromone produced and expressed by females, the chemical structure of such a pheromone has yet to be identified.

Here we report a study designed to evaluate the role of the sexual attractiveness pheromone, a previously characterized sex pheromone produced by female garter snakes, in mediating male reproductive trailing behavior. Composed of a homologous series of long-chain saturated and monounsaturated methyl ketones, this pheromone is contained within the skin lipids of attractive females during the breeding season (Mason *et al.* 1989, 1990). When a male comes in contact with a female expressing this pheromone, the male initiates courtship behavior characterized by increased tongue-flick rate, chin-rubbing along the dorsum of the female and alignment of cloacal openings (Noble 1937). In addition to its role in eliciting male courtship behavior, it has been hypothesized that the sexual attractiveness pheromone, when laid down with skin lipids on the substrate as a female passes, is the chemical cue utilized by males to follow the trails of attractive females (Noble 1937; Garstka & Crews 1981; Mason 1992).

The red-sided garter snake, *Thamnophis sirtalis parietalis*, provides an ideal system with which to test this hypothesis. A small subset of males, termed 'she-males', exist in several Manitoba, Canada populations of this species (Mason & Crews 1985; Shine *et al.* 2000). She-males are morphologically and anatomically indistinguishable from other males in the population except that they express the sexual attractiveness pheromone on their dorsal surface during the breeding season (Mason 1993). As a result, other males actively court these she-males as if they were females (Mason & Crews 1985). It is not known, however, whether males are capable of trailing she-males during the breeding season. We predict that if the sexual attractiveness pheromone represents the chemical cue utilized by male garter snakes for seeking out potential mates, then male

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red-sided garter snakes should detect and follow she-male trails to the same extent they do female trails during the breeding season while not responding to trails produced by other males.

Materials and methods

Study population and site

All research was conducted at a study site in the Interlake region of Manitoba, Canada (Narcisse Wildlife Management Area – 50°44'N, 97°34'W). Populations of red-sided garter snakes inhabiting this region over-winter in large, communal dens and mate immediately upon emergence in the spring (Gregory 1974). The Narcisse Wildlife Management Area contains three dens in close proximity to one another, with each den possessing in excess of twenty thousand red-sided garter snakes during the winter months (R. T. Mason, unpublished data).

The animals used in this study were captured over a one-week period during the breeding season in May of 1998. Adult males ($N = 91$) were randomly collected from the den site. Only those males which did not elicit courtship from other males in the field were included in this group. Adult females ($N = 27$) and she-males ($N = 24$) were collected by randomly selecting mating groups and locating the source of male courtship behavior (Mason & Crews 1985). To reduce the error in identifying she-males, we moved a suspected she-male to a new location at the den site and released it. If the release resulted in the immediate formation of a new mating group centered on the suspected she-male, then we classified the male as a she-male for use in the study.

Behavioral trials

We utilized a standard Y-maze to test the trailing response of male red-sided garter snakes (e.g. Ford 1982; Burger 1989; Fig. 1a). The maze was constructed of wood and consisted of an initial base arm 1 m in length and 14 cm in width. Joined to the base arm at a Y-junction were two arms of similar dimensions to the base arm separated by a 45° angle. Two rows of vertical pegs were inserted in the arms of the maze to supply traction for the snakes. We covered the surface of the maze with butcher paper and the vertical pegs with drinking straws, all of which were replaced after each trial. To discourage snakes from exiting the maze, we elevated the maze 1 m off the ground to eliminate the potential for thigmotactic (wall-following) behavior sometimes observed in snakes during similar trailing experiments (e.g. Ford 1986; Constanzo 1989).

We performed four experiments testing the ability of male red-sided garter snakes to follow skin lipid trails on the Y-maze. The first two experiments tested the ability of males to follow male trails versus no trails ($N = 15$ trials) and female trails versus no trails ($N = 15$ trials). The final two experiments examined the response of male red-sided garter snakes to she-male trails. We tested the response of males to she-male trails versus no trails ($N = 12$ trials) and the response of males to she-male trails versus female trails ($N = 12$ trials). The last experiment (she-male trail vs. female trail) was performed to determine whether males could discriminate between the two trail types. We also performed a control experiment ($N = 10$ trials) at the onset of the study to verify that there was no bias in arm choice by males when no stimuli were present (both arms of the maze blank). Snakes collected in the field were randomly assigned to each experimental group and were used only once before being returned to the site of capture. Trials were conducted daily between the hours of 1000 to 1500, when the snakes were normally active in the field (M. P. LeMaster, personal observation) and environmental conditions (e.g. temperature, lighting) remained constant throughout the experimental period.

Trails were produced by randomly selecting a stimulus snake (male, female or she-male depending on the experiment) and rubbing the dorsal surface of the snake on the paper and the lower half of the straws covering the maze and pegs. Trails were laid down in this manner from the start of the base arm and continued up the maze to

the Y-junction and then out the entire length of a randomly chosen arm. The cloacal openings of the stimulus animals were covered with tape so that contamination from cloacal gland secretions did not occur. For the experiment testing the ability of males to discriminate between she-male trails and the female trails, trails were placed side-by-side on the base arm and then crossed over each other at the Y-junction before exiting out separate, randomly chosen arms (Fig. 1b). Trails were crossed at the Y-junction so that a male would have the opportunity to encounter both trails before exiting out one of the arms.

To begin a trial, we placed a male test snake into an opaque box at the start of the base arm and allowed it to acclimate for five minutes. The opening onto the maze was then uncovered and the test snake was allowed to enter the maze of its own accord. Trials ended when the test snake completely entered one exit arm of the Y-maze. Trials in which the test snake failed to exit the hide box after ten minutes or trials in which the test snake fell from the maze were eliminated (< 5% of trials performed). For all successful trials, we recorded the arm chosen by the test snake. We also noted whether the test snake displayed a trail contact response (TCR), an overt response characterized by a snake placing its chin in contact with the substrate and investigating with short, rapid tongue-flicks followed by subsequent movement along the pre-existing trail (Brown & MacLean 1983).

Statistics

Statistical significance in the trailing response of males for each experiment was examined through Chi-squared tests with a continuity

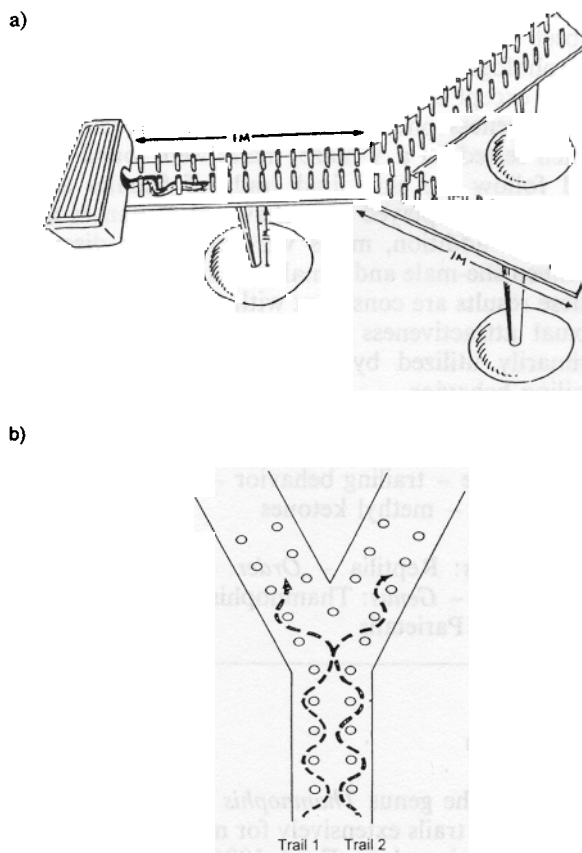


Fig. 1 Diagram of experimental Y-maze used for trailing experiments (a). When two trails were laid down simultaneously on the Y-maze, they were crossed over one another on the base arm near the Y-junction (b). This allowed for males following one trail to have an opportunity to encounter the second trail before having to choose at the Y-junction

Condition	N	Arm treatment	Number of times selected	P (χ^2)
Control	10	Blank	6	0.655 (NS)
		Blank	4	
Males tested on male trail	15	Male skin rubbing	9	0.465 (NS)
		Blank	6	
Males tested on female trail	15	Female skin rubbing	15	<0.001
		Blank	0	
Males tested on she-male trail	12	She-male skin rubbing	11	<0.001
		Blank	1	
Males tested on she-male versus female trail	12	Female skin rubbing	5	0.683 (NS)
		She-male skin rubbing	7	

Table 1 Responses of male red-sided garter snakes, *Thamnophis sirtalis parietalis*, to male, female and she-male trails when tested on a Y-maze (NS = not significant)

correction (Sokal & Rohlf 1995). Level of significance was set at $P < 0.05$.

Results

When presented with the control maze (both arms blank), male red-sided garter snakes showed no preference for one arm over the other ($\chi^2 = 0.20$; $P = 0.655$; Table 1). Males responded significantly to female trails, choosing the arm with the female trail over the blank arm in all trials conducted ($\chi^2 = 26.13$; $P < 0.001$; Table 1). All test males also displayed trail contact responses (TCRs) immediately upon first exiting the start box when tested on female trails. Males did not, however, appear to recognize male trails ($\chi^2 = 0.53$; $P = 0.655$; Table 1), and no TCRs were observed during these trials.

Males responded significantly to she-male trails, exiting out the arm of the maze with the she-male trail in 11 of the 12 trials conducted ($\chi^2 = 13.50$; $P < 0.001$; Table 1). All test males displayed TCRs, with the lone male not exiting out the she-male trail arm appearing to lose the trail as he approached the Y-junction. Males did not, however, show a preference when presented with a she-male and female trail ($\chi^2 = 0.17$; $P = 0.683$; Table 1). All test males did display TCRs in these trials, demonstrating that they were actively trailing, but did not appear to differentiate between the she-male and female trails when having to choose at the Y-junction.

Discussion

Results from this study confirm our initial predictions regarding the response of male red-sided garter snakes to female, she-male and male trails during the breeding season. Male snakes were observed to follow female and she-male trails when presented on the Y-maze but did not show an overt response to male trails. In addition, males were unable to discriminate between female and she-male trails when presented in unison.

The sex-specific response of male red-sided garter snakes to female and male trails in this study is concordant with the hypothesis that the sexual attractiveness pheromone is responsible for mediating male trailing behavior in this species. Males responded to female skin

rubbing trails, which contain the sexual attractiveness pheromone, but did not respond to male skin rubbing trails, which do not contain the pheromone (Mason *et al.* 1989). These results confirm prior investigations of garter snake trailing behavior which demonstrate a similar sex-specific trailing response by male snakes (Noble 1937; Ford 1982; Ford & O'Brien 1986). The ability of male garter snakes to detect and follow female trails is believed to be an adaptation that allows males, limited by auditory and visual cues, to locate potential mates over long distances (Ford 1986).

The response of males when presented with she-male trails provides the more compelling evidence that the sexual attractiveness pheromone is the chemical cue utilized by males to follow female trails. The response of males to isolated she-male trails demonstrates that the trailing pheromone is sequestered on the skin surface of she-males, similar to the observations with females. Likewise, behavioral studies reveal that the sexual attractiveness pheromone is present on the dorsal surface of she-males and females during the breeding season (Mason & Crews 1985; Shine *et al.* 2000). Furthermore, the inability of males to discriminate between female and she-male trails when presented simultaneously suggests the presence of a pheromone of similar composition; otherwise we would expect males to possess the ability to discriminate among the two trail types owing to the strong selective pressure to locate females, and not she-males, during the breeding season. Chemical analyses of female and she-male skin lipid extracts collected during the breeding season reveal a similar pheromone profile, one that is not observed in the skin lipids of males collected during the same time period (Mason 1993).

Although the results of this study support the hypothesis that the sexual attractiveness pheromone mediates male reproductive trailing behavior in the red-sided garter snake, we acknowledge that alternative hypotheses do exist. For example, the pheromone responsible for eliciting male trailing behavior in this species may be a novel compound, or suite of compounds, contained in the skin lipids of females and she-males, but not males, which has yet to be identified. Future research is necessary to determine whether male garter snakes are capable of detecting and following trails consisting solely of the sexual attractiveness pheromone when isolated from the skin lipids of attractive females and she-males.

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