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VARIATION IN A FEMALE SEXUAL ATTRACTIVENESS PHEROMONE CONTROLS MALE MATE CHOICE IN GARTER SNAKES

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Abstract—Male red-sided garter snakes (*Thamnophis sirtalis parietalis*) display a courtship preference for larger females during the breeding season. Utilizing behavioral experiments and chemical analyses, we tested the hypothesis that males can discriminate among females of varying size solely by means of the sexual attractiveness pheromone, a previously characterized sex pheromone composed of a homologous series of long-chain saturated and ω-9 *cis*-unsaturated methyl ketones contained in the skin lipids of females. When presented with skin lipid extracts from large and small females, a greater proportion of males displayed courtship behaviors to large female extracts. This demonstrates that there is an intrinsic property of the female skin lipids that allows males to differentiate among large and small females. Analysis of the sexual attractiveness pheromone revealed that the necessary variation exists for this pheromone to function as a reliable indicator to males of female body size. Specifically, we observed a strong correlation between female snout–vent length and the relative concentration of saturated and ω-9 *cis*-unsaturated methyl ketones composing the pheromone; smaller females expressed pheromone profiles higher in saturated methyl ketones, while larger females expressed pheromone profiles dominated by unsaturated methyl ketones. The results of this study suggest that male red-sided garter snakes utilize compositional variation in the female sexual attractiveness pheromone to differentiate among potential mates of varying size.

Key Words—Mate choice, sexual attractiveness pheromone, qualitative variation, methyl ketones, red-sided garter snake, *Thamnophis sirtalis parietalis*.

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INTRODUCTION

In many vertebrate species, individuals of one or both sexes actively select among potential mates during the breeding season in an effort to optimize their reproductive success (Andersson, 1994). Individuals often discriminate among potential mates based on variation in the expression of a particular morphological trait. For example, females of some bird species differentiate among males based on variation in visual traits [e.g., color intensity (Hill, 1990), tail length (Møller, 1988)], whereas female frogs may utilize variation in auditory traits [e.g., call rate (Sullivan, 1983), call pitch (Ryan, 1980)]. Chemical traits, such as pheromones (Karlson and Lüscher, 1959), represent an additional type of morphological trait by which potential mates might be evaluated.

The occurrence of chemically mediated mate choice is relatively common in insects, where chemical communication is considered the dominant modality for controlling reproductive behavior in many species (Bell and Cardé, 1984). Unlike insects, however, few studies have documented chemically mediated mate choice in vertebrates (e.g., Verrell, 1985; Reece-Engel, 1988; Martín and López, 2000). This paucity of studies is primarily due to the multicomponent sensory nature of vertebrates where a combination of sensory inputs (e.g., visual, tactile, chemical) is often responsible for mediating a particular behavior (Albone, 1984). Such diversification makes it difficult to establish effective bioassays for measuring behavioral responses of individuals to isolated chemical cues to determine to what extent such cues are utilized.

Snakes offer an excellent model system in which to investigate the chemical mediation of mate choice in vertebrates. More than most vertebrates, snakes rely extensively upon the production and perception of specific sex pheromones for the coordination of reproductive behavior (reviewed in Mason, 1992; Mason et al., 1998). Hence, there is an increased probability that mechanisms evolved to select among potential mates will be chemical in nature [mate choice in snakes (Hawley and Aleksiuk, 1976; Luiselli, 1996)]. In addition, snakes are much like insects in that they respond to isolated chemical cues with stereotypical, robust behaviors (Brown and MacLean, 1983; Mason and Crews, 1985). Thus, behavioral bioassays can be constructed with relative ease to test the responses of snakes to chemical cues from potential mates when presented in isolation.

Annual aggregations of red-sided garter snakes (*Thamnophis sirtalis parietalis*) at underground hibernacula in Manitoba, Canada, are unique natural phenomena, representing the highest concentrations of snakes in the world (Gregory, 1984). Mating in these populations occurs directly at the hibernacula following spring emergence, with virtually all females [>95% (Garstka et al., 1982)] mating within one day of emerging from winter dormancy. During this period, it is not uncommon to observe 10–100 males courting a newly emerged female, forming what are termed "mating balls" (Gregory, 1974, 1977). Males do not randomly associate

with females, but instead display a courtship preference for larger females (Hawley and Aleksiuk, 1976; Shine et al., 2001).

It has been hypothesized that the cue utilized by male red-sided garter snakes to differentiate among potential mates is the sexual attractiveness pheromone (Garstka et al., 1982; Gregory, 1984; Mason, 1992). Composed of a homologous series of saturated and ω-9 *cis*-unsaturated methyl ketones (Mason et al., 1989, 1990), this pheromone is sequestered in the skin lipids of females during the breeding season and is primarily responsible for eliciting male courtship behavior (Noble, 1937; Garstka et al., 1982). Indeed, if a male does not detect this pheromone on the dorsal surface of a female, then courtship behavior will not be initiated and mating will not occur (Mason, 1993). Thus, variation in the expression of the sexual attractiveness pheromone by females of varying size may directly influence male courtship behavior, leading to the mating preference observed in this species.

Here, we report a study designed to investigate the role of the female sexual attractiveness pheromone in mediating male mate choice in the red-sided garter snake. Our two major aims in this study were: (1) to determine whether males can discriminate among females of varying size based solely on chemical cues in the skin lipids, and (2) to evaluate whether the sexual attractiveness pheromone contains the necessary variation to function in male mate choice. To this end, we conducted behavioral experiments testing the courtship response of males to large and small females and skin lipid extracts from large and small females. Further, we collected pheromone samples from females of varying size and examined the quantity and quality of pheromone expressed by individual females.

METHODS AND MATERIALS

Study Population

Red-sided garter snakes utilized for this study were captured at a field site near the community of Inwood in the Interlake region of Manitoba, Canada (50°31.58′N; 97°29.71′W) during May 2000. This site is located at an abandoned gravel quarry containing a single overwintering hibernaculum possessing in excess of 10,000 red-sided garter snakes during the winter months (R. T. Mason, unpublished data). Adult females in this population attain an average snout–vent length (SVL) of 55–60 cm and a mean mass of 70–85 g, while adult males are much smaller, attaining an average SVL of 45–50 cm and a mean mass of 35–40 g (Shine et al., 1999).

Behavioral Experiments

Experimental Animals. Unmated large female adult red-sided garter snakes (SVL > 60.0 cm; $N = 35$) and small female adult red-sided garter snakes

(SVL < 50.0 cm; $N = 35$) were collected at the hibernaculum immediately upon emergence. We established the SVL criteria for large and small females based on the results of Hawley and Aleksiuk (1976), who found that female red-sided garter snakes with SVL > 60 cm were twice as likely to be courted by males than females with SVL < 50 cm. Adult males ($N = 380$; SVL $\pm$ SD $= 52.4 \pm 2.54$) were collected from naturally occurring mating balls located around the hibernaculum. We focused our collection on larger males (SVL > 50.0 cm), as larger males show an increased ability to differentiate between females based on size compared to smaller males in this species (Shine et al., 2001). Animals were segregated by sex and size in cloth bags and held at ambient temperatures until testing, after which the animals were returned to the hibernaculum and released.

Testing Conditions. Behavioral experiments were carried out in outdoor arenas measuring 1 × 1 × 1 m and constructed of nylon fabric attached to metal posts inserted in the ground. Arenas of this type do not appear to alter male reproductive behaviors compared to the behaviors observed in the wild, and as a result such arenas have been used extensively in the study of snake reproductive ecology (e.g., Mason and Crews, 1985; Shine et al., 1999; Moore et al., 2000). To avoid any confounding effects due to weather conditions, an array of four arenas was established, allowing for groups of trials to be carried out simultaneously. Testing days and times of day (10:00 hr to 16:00 hr) were chosen so that all experiments were conducted under similar ambient conditions (mostly sunny skies with light winds and temperatures of 15–20°C), which correspond with conditions when red-sided garter snakes are most active (M. P. LeMaster, personal observation).

Experiment 1. To verify that males in our study population exhibited a courtship preference for larger females, we first tested the behavioral response of males to large and small females using a simultaneous choice test design (e.g., Mason and Crews, 1985). Briefly, we introduced 10 randomly chosen males into an arena and allowed them to acclimatize for 5 min. We then placed into each arena simultaneously a large and small female. The snakes were allowed to interact undisturbed for 5 min, after which time the number of males actively courting each female size was recorded. A total of 16 tests were performed with unique male and female snakes used in each trial.

Male courtship behavior was assessed by using an ethogram of male garter snake mating behavior (Table 1). Similar ethograms are routinely used in field and laboratory studies with garter snakes (e.g., Crews et al., 1984; Moore et al., 2000). To prevent females from mating during the trials, a condition that drastically reduces further male courtship advances (Garstka et al., 1982), we placed adhesive tape across the female cloaca. The tape was then removed immediately upon completion of each trial.

Experiment 2. This experiment utilized behavioral trials to determine whether chemical cues contained within the skin lipids of females are sufficient for male mate discrimination. For each trial, we introduced 10 randomly chosen males into

TABLE 1. ETHOGRAM OF COURTSHIP BEHAVIOR FOR MALE RED-SIDED GARTER SNAKES, *Thamnophis sirtalis parietalis*[a]

Courtship score	Description of behavior
1.0	Male investigates female with increasing tongue-flick rate
2.0	Male chin-rubs dorsal surface of female and aligns body with female
3.0	Male actively tail searches and attempts cloacal apposition with female
4.0	Male copulates with female

[a] Modified from Crews et al. (1984). A behavioral score of 2.0 or greater only occurs in a reproductive context and is, therefore, indicative of sexual behavior for males of this species.

an arena and allowed them to acclimatize for 5 min. We then randomly placed in with the males one of four treatments: (1) a small female, (2) a large female, (3) filter paper containing a small female skin lipid sample, or (4) filter paper containing a large female skin lipid sample. Male snakes were allowed to interact with the female or filter paper undisturbed for 5 min, after which the number of males actively courting the treatment was recorded. For filter paper treatments, a male had to be rapidly tongue-flicking and chin-rubbing the filter paper for it to be considered actively courting. A total of 38 trials were performed using small ($N = 9$) and large ($N = 9$) female snakes and skin lipid samples from small ($N = 10$) and large ($N = 10$) female snakes. Unique males and females were used in each behavioral trial.

Skin lipid samples were collected from large and small females by rubbing down the dorsal surface of the donor snake with filter paper wetted with 100% hexane. Care was taken to prevent contamination from cloacal gland secretions (Mason et al., 1989). For each skin lipid sample collected, a unique female was used that was independent of females used in the behavioral trials. To control for surface area differences in the two groups of females, we rubbed down the length of the large females once with the filter paper and rubbed down the length of the small females three times. We established this protocol based on prior knowledge that hexane extracts from large females yield three times the amount of skin lipids compared to small females and that multiple rubbing of a female does not affect male behavior towards the subsequent skin lipids extracted (M. P. LeMaster, unpublished data). To verify that similar quantities of skin lipids were laid down for each female size, we compared the average skin lipid amount laid down on the filter papers for each female size upon completion of the trials [skin lipids (milligrams) = weight of treated filter paper − weight of blank filter paper].

Chemical Analysis

Pheromone Collection. Adult, sexually attractive female red-sided garter snakes of varying size (SVL range 48.0–74.2 cm; $N = 20$) were collected

immediately upon spring emergence from the hibernaculum. The size range of females collected corresponded with the size range of females used in our behavioral experiments. The animals were killed with an overdose of brevital sodium. Each snake was then placed dorsal side down in a 500-ml glass beaker and covered with 25–50 ml of 100% hexane (C_6H_{12}) for 12 hr (Mason et al., 1989, 1990). Care was taken to keep the head and cloaca out of the hexane to avoid possible contamination by internal body fluids. After removal of the animals, the excess solvent was removed under reduced pressure by rotoevaporation at 35°C. The resulting residues were weighed on a digital scale (Mettler AT400), resuspended in fresh hexane (1–2 ml), and sealed in 9-ml glass vials with polyethylene-lined caps for storage at −20°C.

To isolate the methyl ketones composing the sexual attractiveness pheromone, we fractionated the skin lipid extracts using column chromatography as described by Mason et al. (1989). Briefly, we loaded the skin lipid extracts onto glass columns (350 mm long × 22 mm ID) packed with alumina (activity III) and eluted the columns with hexane and ethyl ether ($C_4H_{10}O$) solutions of increasing polarity. For each sample, the fraction containing the appropriate methyl ketones [fraction 5 (Mason et al., 1989)] was collected, and the excess solvent was removed by rotoevaporation (35°C). The resulting methyl ketone residues were weighed on a digital scale (Mettler AT400) and resuspended in 1 ml fresh hexane. Samples were then placed in 9-ml glass vials with polyethylene-lined caps and stored at −20°C until further analysis.

Pheromone Analysis. Multicomponent pheromones, such as the sexual attractiveness pheromone of the red-sided garter snake, can show variation in both the quantity and quality of pheromone expressed. To examine variation in the quantity of pheromone expressed by female snakes of varying size, we calculated the amount of methyl ketones expressed per unit skin surface area (micrograms per square centimeter) for individual females. This was accomplished by dividing the weight of the isolated methyl ketone residues extracted from a female by the total skin surface area of the female. A general index of skin surface area for each female was determined by multiplying the snout–vent length of a female by its circumference at mid-body (Mason et al., 1990).

To examine variation in the quality of pheromone expressed by females, we determined the number of unique methyl ketones expressed by individual females and calculated the relative contributions of saturated *versus* monounsaturated methyl ketones to the individual pheromone profiles. The methyl ketones present in the pheromone extracts were identified utilizing a Hewlett Packard 5890 Series II gas chromatograph fitted with a split injector (280°C) and a Hewlett Packard 5971 Series mass selective detector. Aliquots (1 μl) of the methyl ketone fractions were injected onto a fused-silica capillary column (HP-1; 12 m × 0.22 mm ID; Hewlett Packard) with helium as the carrier gas (5 cm/sec). Oven temperature was initially held at 70°C for 1 min, increased to 210°C at 30°C/min, held at 210°C for 1 min,

increased to 310°C at 5°C/min, and finally held at 310°C for 5 min. Once the methyl ketones were identified, we calculated the relative concentrations of saturated and monounsaturated methyl ketones in each sample by using peak integration [e.g., percent relative concentration of saturated methyl ketones = (area under saturated methyl ketone peaks/total area of all methyl ketone peaks) × 100]. Identification of compounds and peak areas were determined utilizing ChemStation software (Version B.02.05; Hewlett Packard) interfaced with the gas chromatograph/mass spectrometer.

Statistics

Statistical analyses were performed using Jandel SigmaStat Version 2.0 software package (Jandel Corporation). The numbers of males courting large females compared to small females when presented simultaneously were examined by using χ^2 analyses. Differences in the proportion of males courting large and small females and large and small female skin lipid samples were initially analyzed by using a χ^2 test of proportions, with Tukey-type multiple comparison tests then used to perform pairwise comparisons among the four treatments (Zar, 1984). Finally, the relationship between the size of the female (SVL) and various quantitative and qualitative measures of pheromone expression were examined utilizing Pearson product moment correlation tests (Sokal and Rohlf, 1995). Level of significance for each test was set at $P < 0.05$.

RESULTS

Behavioral Experiments

Experiment 1. The average SVL ± SD of small females used in the simultaneous choice tests was 46.2 ± 2.70 cm, whereas the average SVL ± SD for large females was 63.0 ± 2.57 cm. This resulted in an average ± SD difference between small and large females presented to males during individual trials of 16.84 ± 3.93 cm. Over the 16 trials conducted, a greater proportion of males was observed courting the larger female over the smaller female following the 5-min interaction period ($\chi^2 = 56.47$, 1 *df*, $P < 0.001$; 64.3% of males courted large females vs. 11.9% of males courting small females) (Figure 1). We pooled the data for analysis after a heterogeneity χ^2 test showed no significant difference in male courtship preference among the individual trials conducted ($\chi^2 = 7.09$, 15 *df*, $P > 0.5$).

Experiment 2. When presented with the four treatments, male garter snakes responded with stereotypical courtship behaviors including increased tongue-flick rate, chin rubbing along the dorsum of the female (or filter paper), and body alignment with the female (or edge of filter paper). Overall, we observed significant variation in the proportion of males actively courting the various treatments

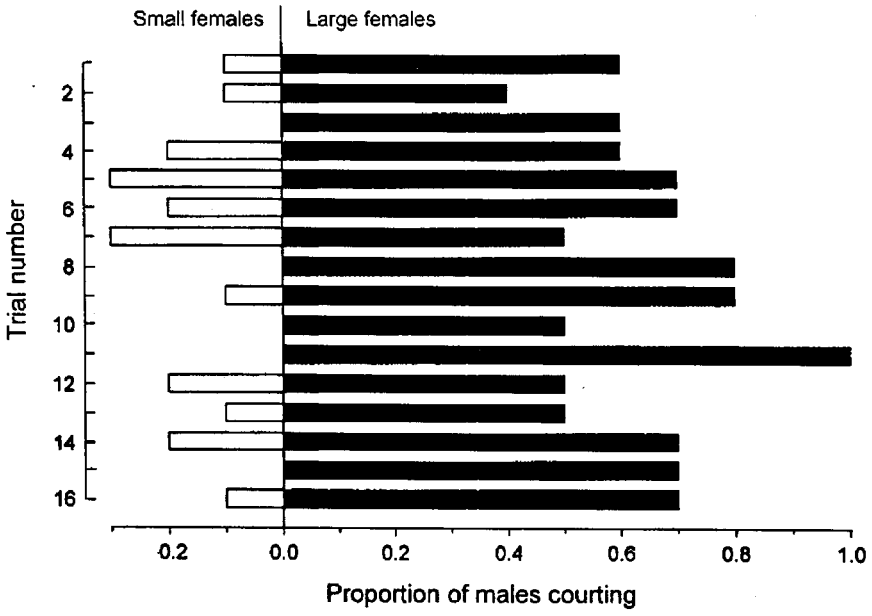

FIG. 1. Results of simultaneous choice tests in which a large and a small female red-sided garter snake (*Thamnophis sirtalis parietalis*) were placed in an outdoor arena with 10 courting males. After 5 min, the number of males that courted either female was recorded.

after the 5-min interaction period ($\chi^2 = 66.48$, 3 *df*, $P < 0.001$; Figure 2). Pairwise analysis revealed that a significantly greater proportion of males directed courtship behavior to large females and filter paper containing large female skin lipids than to small females and filter paper containing small female skin lipids (multiple comparison tests; $q = 10.4$–19.4; $P < 0.001$ for all tests). However, little difference was observed in the proportion of males displaying courtship behavior to large females and large female skin lipid extracts (multiple comparison test; $q = 3.23$; $P > 0.10$) and to small females and small female skin lipid extracts (Multiple comparison test; $q = 2.59$; $P > 0.20$).

When we examined the amount of skin lipids placed on the treated filter papers, we found no difference between filter papers containing large female skin lipid samples and filter papers containing small female skin lipid samples (large female average $\pm$ SD $= 3.0 \pm 2.4$ mg, small female average $\pm$ SD $= 3.2 \pm 2.0$ mg; ANOVA, $F_{1,18} = 0.041$, $P = 0.842$). In addition, there was no difference in SVL $\pm$ SD between large females presented to males and large female skin lipid donors (test female $= 66.1 \pm 3.63$ cm, donor female $= 66.7 \pm 4.66$ cm; t test: $t = 0.308$, $P = 0.762$) or between small females and small female skin lipid

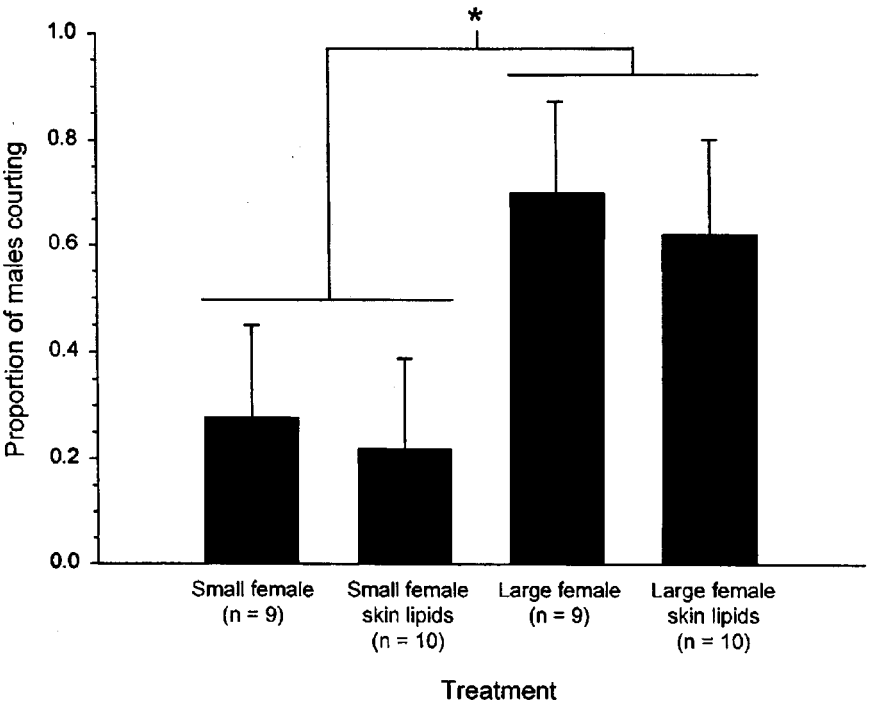

FIG. 2. Proportion (mean ± SD) of male red-sided garter snakes (*Thamnophis sirtalis parietalis*) displaying courtship behavior to large and small females and skin lipid samples from large and small females.

donors (test female = 46.7 ± 4.60 cm, donor female = 44.2 ± 3.16 cm; t test: $t = 1.379$, $P = 0.186$).

Pheromone Analysis

Pheromone Quantity. The hexane extractions of individual females yielded an average ± SD of 21.3 ± 7.5 mg of skin lipids per female. Subsequent fractionation of the lipids yielded an average ± SD methyl ketone fraction of 1.4 ± 1.1 mg/female. Overall, the methyl ketones accounted for an average ± SD of 6.1 ± 3.4% of the skin lipids collected from the female snakes. After accounting for variation in skin surface area, individual females were found to vary widely in the quantity of methyl ketones expressed on their dorsal surface (range = 1.8–16.4 $\mu g/cm^2$; Table 2). There was not, however, a significant relationship between the female SVL and the amount of methyl ketones extracted per unit of surface area (Pearson product-moment correlation: $r = 0.169$, $N = 20$, $P = 0.476$).

TABLE 2. QUANTITATIVE AND QUALITATIVE VARIATION IN EXPRESSION OF SEXUAL ATTRACTIVENESS PHEROMONE AMONG INDIVIDUAL FEMALE RED-SIDED GARTER SNAKES, *Thamnophis sirtalis parietalis*[a]

Individual female	Snout-vent length (cm)	Methyl ketone expression ($\mu g/cm^2$)	Unique methyl ketones observed	Relative (%) methyl ketone concentrations	
				Saturated	Unsaturated
1	48.0	5.6	17	75.1	24.9
2	50.4	2.5	12	74.8	25.2
3	54.0	2.2	17	70.3	29.7
4	55.5	2.3	18	58.5	41.5
5	55.6	4.3	18	53.0	47.0
6	56.0	2.1	15	55.9	44.1
7	57.5	16.4	18	49.1	51.9
8	58.5	2.0	16	70.8	29.2
9	59.2	7.0	17	71.5	28.5
10	59.8	1.8	10	17.5	82.5
11	60.4	1.9	15	57.4	42.6
12	61.3	3.4	14	11.1	88.9
13	61.8	3.4	15	60.3	39.7
14	62.0	3.6	18	54.1	45.9
15	66.6	5.4	14	26.3	73.7
16	68.5	5.4	13	13.7	86.3
17	69.1	4.9	10	11.6	88.4
18	69.6	2.5	13	7.4	92.6
19	71.5	10.2	15	20.4	79.6
20	74.2	5.3	16	25.2	74.8

[a] Female snakes are arranged according to snout-vent length (small to large).

Pheromone Quality. Complete GC-MS analysis of the methyl ketone fractions revealed the presence of 18 unique long-chained methyl ketones (Figure 3). Nine of these were identified as long-chain saturated methyl ketones, while the remaining nine had mass spectra in accord with long-chain ω-9 *cis*-unsaturated methyl ketones (Mason et al., 1990). The saturated methyl ketones ranged in size from 394 mass units to 506 mass units, whereas the unsaturated methyl ketones ranged in size from 420 mass units to 532 mass units. Individual females were found to vary in the number of methyl ketones expressed in their skin lipids, ranging from pheromone profiles composed of 10 unique methyl ketones to pheromone profiles composed of all 18 unique methyl ketones (Table 2). However, when we plotted the number of unique methyl ketones present against the SVL of the females, a significant relationship was not observed (Pearson product-moment correlation: $r = -0.338$, $N = 20$, $P = 0.144$).

The relative contribution of the saturated and unsaturated methyl ketones to the overall pheromone profiles varied extensively among females (Table 2).

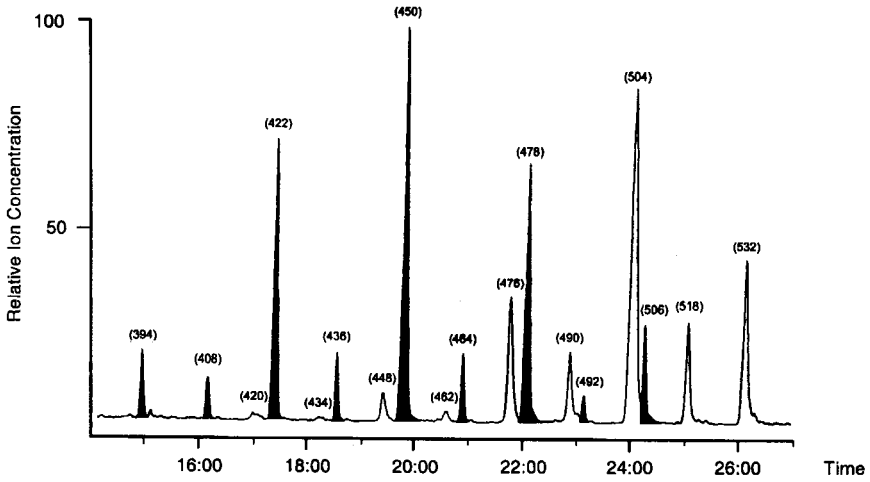

FIG. 3. Gas chromatogram of the female sexual attractiveness pheromone profile for the red-sided garter snake, *Thamnophis sirtalis parietalis*. Pheromone profiles are composed of saturated (shaded peaks) and unsaturated (open peaks) methyl ketones. The numbers over the peaks represent the molecular weights of individual components.

For example, one female expressed a pheromone profile composed of 75.1% saturated methyl ketones and 24.9% unsaturated methyl ketones, whereas a second female expressed a profile composed of 7.4% saturated methyl ketones and 92.6% unsaturated methyl ketones. Saturated methyl ketones composed (average $\pm$ SD) $44.2 \pm 24.5\%$ of the total methyl ketones in the pheromone profiles, and unsaturated methyl ketones composed $55.8 \pm 24.5\%$. When we plotted the SVL of the females against the relative contribution of unsaturated methyl ketones to the pheromone profiles, a significant correlation was observed (Pearson product moment correlation: $r = 0.788$, $N = 20$, $P < 0.001$; Figure 4). This relationship showed that smaller females express pheromone profiles comprised predominantly of saturated methyl ketones, while larger females express profiles composed predominantly of unsaturated methyl ketones.

DISCUSSION

The results of this study demonstrate that adult red-sided garter snakes are able to discriminate among females of various size based solely on chemical cues contained within the skin lipids of the female. Males displayed a courtship preference for large females over small females when presented both simultaneously and in isolation, and continued to demonstrate a similar preference when visual, tactile, and behavioral cues from the females were removed through the use of skin lipid

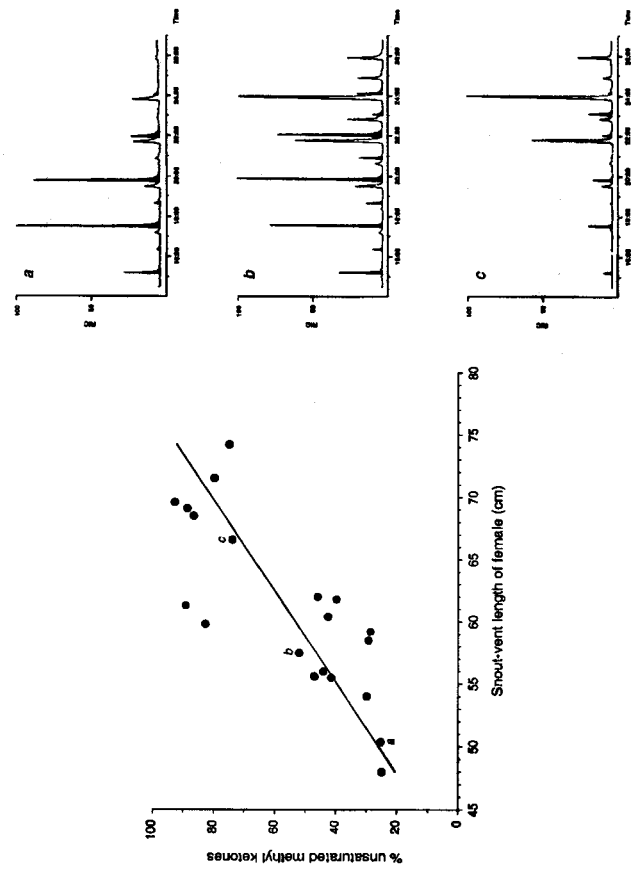

FIG. 4. Regression of the percent relative concentration of unsaturated methyl ketones composing the sexual attractiveness pheromone profile on snout-vent length for female red-sided garter snakes, *Thamnophis sirtalis parietalis*. Small females express pheromone profiles consisting predominantly of saturated methyl ketones (a), mid-size females express profiles consisting of equal proportions of saturated and unsaturated methyl ketones (b), and large females express profiles consisting predominately of unsaturated methyl ketones (c).

extracts. Furthermore, we observed size-specific variation in the chemical structure of the female sexual attractiveness pheromone, suggesting that this pheromone represents the skin lipid-borne cue responsible for mediating male mate choice in this species. Previous studies have documented chemically mediated mate choice in a variety of vertebrates [e.g., mammals: lemmings (Huck and Banks, 1982), rabbits (Reece-Engel, 1988), amphibians: salamanders (Verrell, 1985; Marco et al., 1998), reptiles: lizards (Martín and López, 2000)] but have focused exclusively on the behavioral responses of individuals to chemical cues. To our knowledge, this study represents the first attempt to provide empirical evidence towards the identification of a chemical cue responsible for mediating mate choice in a vertebrate species.

Our results from the behavioral experiments confirm previous studies documenting a courtship preference by male red-sided garter snakes for larger females during the breeding season (Hawley and Aleksiuk, 1976; Garstka et al., 1982; Shine et al., 2001). Similar courtship preference by males for larger females has been observed in other reptiles including lizards (e.g., Olsson 1993; Cooper and Vitt, 1997; Whiting and Batemen, 1999) and snakes (e.g., Luiselli, 1996). One potential benefit incurred by male red-sided garter snakes for choosing a female of larger body size is an increase in the number of offspring sired. Fecundity has been shown to increase with body size in a variety of animals (reviewed in Andersson, 1994), and this appears true for red-sided garter snake populations in Manitoba, where larger females are observed producing larger clutches of young (Gregory, 1977; R. T. Mason, unpublished data).

We are confident that our bioassay results represent a true measure of male courtship preference for skin lipids from larger females. When male red-sided garter snakes were presented with skin lipid extracts from both large and small females, we observed courtship behavior closely mimicking what was observed with the actual females, including chin-rubbing and body alignment (with edge of filter paper). These behaviors are only observed in a reproductive context (Mason, 1993), confirming that we were measuring a courtship response and not some other male behavior (e.g., aggregation). Furthermore, analysis of the treated filter papers showed that similar amounts of skin lipids were transferred to the filter papers from the large and small snakes. Potentially, variation in the total amount of skin lipids transferred for the two female sizes could have driven the male courtship preference observed. Instead, our results suggest that there is some intrinsic property of the female skin lipids that allows for males to differentiate among large and small females.

The observed relationship between the SVL of females and the methyl ketone group ratios demonstrates that the sexual attractiveness pheromone can function as a reliable indicator to males of female body size. Indeed, the observed uniform shift in methyl ketone group ratios as females increased in size conforms well with the observed uniform change in male courtship behavior displayed to females of varying size when tested in the field (Hawley and Aleksiuk, 1976). In addition,

the pattern of variation we observed in pheromone composition is concordant with what would be expected for such variation to function in male mate selection. Although both saturated and unsaturated methyl ketones are required to elicit full male courtship behavior in red-sided garter snakes, unsaturated methyl ketones appear to be the more biologically active. When presented in isolation, unsaturated methyl ketones elicit a five-fold increase in male response over saturated methyl ketones (Mason et al., 1989). Accordingly, higher levels of unsaturated methyl ketones in the pheromone ratios of larger females, as observed in this study, are a likely explanation for why males find these females more attractive.

Why larger females express higher levels of unsaturated methyl ketones is unclear. The production and expression of this pheromone appears to be under hormonal control [estrogen (Crews, 1976; Crews et al., 1984)], suggesting that variation in the release rate of hormones and/or sensitivity of target cells to the hormones may explain the variation. For example, larger females produce a greater number of follicles in the fall [hence, greater numbers of offspring (Gregory, 1977)], which may translate into higher levels of estrogen circulating in the blood during the period of pheromone biosynthesis. Alternatively, females of varying size may differ in the organization of biosynthetic pathways involved in methyl ketone production. Future studies are necessary to resolve the underlying mechanism(s) responsible for the observed variation in methyl ketone production.

We might have expected to find variation in the quantity of sexual attractiveness pheromone expressed by female red-sided garter snakes of varying size or variation in the presence or absence of individual methyl ketones. Similar mechanisms of mate choice have been observed in insects (reviewed in Andersson, 1994). For example, female rattlebox moths (*Utetheisa ornatrix*) prefer to mate with males producing higher quantities of a courtship pheromone (Dussourd et al., 1991), whereas female Oriental moths (*Grapholita molesta*) preferentially court males expressing a food-related chemical in their pheromone blend (Löfstedt et al., 1989). However, we did not observe a correlation between female body size and either of the two measures in the expression of the sexual attractiveness pheromone, suggesting that such variation is not utilized by male red-sided garter snakes to differentiate among females.

Although the results from this study strongly suggest that male red-sided garter snakes differentiate among females of varying size by utilizing size-specific variation in the female sexual attractiveness pheromone, we can not rule out the possibility that additional cues may assist males in mate choice for this species (Shine et al., 2001). For example, male garter snakes searching for sexually attractive females in the field are initially attracted to movement (Joy and Crews, 1988; Holtzman, 2001). Thus, males might initially respond to perceived areas of greater movement (i.e., larger female moving through grass or action of larger mating ball) and then confirm their choice by sampling the skin lipid composition of the female once contact is made. In addition, tactile cues may provide additional

relevant information to the male. When a male initially comes in contact with a female, he proceeds to chin-rub up and down the female, turning back at the head and tail before finally coming to rest with his head resting behind the female's head (Noble, 1937). Males perform this behavior to detect quickly whether the female is mated by means of postcopulatory pheromones deposited near the cloaca (Shine et al., 2000), but males might also be able to judge the size of the female based on distance covered. Further studies are required to determine to what extent these additional cues may be utilized.

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