

PHEROMONALLY MEDIATED SEXUAL ISOLATION AMONG DENNING POPULATIONS OF RED-SIDED GARTER SNAKES, *Thamnophis sirtalis parietalis*

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Abstract—Utilizing behavioral experiments and chemical analyses, we examined whether pheromonally mediated sexual isolation exists between denning populations of red-sided garter snakes (*Thamnophis sirtalis parietalis*) in Manitoba, Canada. Simultaneous choice tests conducted during the breeding season revealed that adult males from a hibernaculum in central Manitoba displayed a strong courtship preference for females from their own population over females from a hibernaculum in western Manitoba, whereas males from the western Manitoba hibernaculum showed no such preference. In addition, trailing experiments testing the response of males from the two hibernacula to familiar and unfamiliar female trails showed similar results, demonstrating that the observed male preference is mediated through chemical cues. Subsequent chemical analysis of the female sexual attractiveness pheromone, a homologous series of long-chain saturated and ω -9 *cis*-unsaturated methyl ketones responsible for eliciting male courtship behavior and trailing behavior in garter snakes, showed significant variation in the composition of the pheromone between the two populations. Specifically, the two populations varied in the relative concentrations of individual unsaturated methyl ketones expressed by females. These results suggest that sexual isolation exists to a degree among denning populations of red-sided garter snakes due to variation in the expression of the female sexual attractiveness pheromone.

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

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INTRODUCTION

Studies utilizing insect models have dominated pheromone research in recent decades, resulting in the chemical identification and characterization of over a thousand pheromones (Abelson, 1985). Knowledge of the chemical structure of insect pheromones has allowed investigators to make significant advances in the understanding of how these chemical cues regulate insect behavior (Bell and Cardé, 1984; Eisner and Meinwald, 1995). In contrast to the abundance of pheromones identified in insects, few vertebrate pheromones have been isolated and identified to date. These few include pheromones for fish (e.g., goldfish—Stacey and Sorenson, 1986), amphibians (e.g., newts—Kikuyama et al., 1995; salamanders—Rollmann et al., 1999; frogs—Wabnitz et al., 1999), reptiles (e.g., snakes—Mason et al., 1989) and mammals (e.g., pig—Patterson, 1968; elephant—Rasmussen et al., 1996).

The lack of knowledge regarding the basic structure of vertebrate pheromones has hampered our efforts to understand vertebrate pheromone systems and has left many basic areas of research unexplored. For example, few studies have attempted to address intraspecific variation in the chemical composition of a vertebrate pheromone at the level of the population (e.g., Rollman et al., 2000). In insects, intraspecific variation in sex pheromone expression among geographically isolated populations is commonly observed (e.g., turnip moth—Löfstedt et al., 1986; Wu et al., 1999; corn borer—Huang et al., 1998). Such variation often results in the disruption of courtship and mating between the populations involved (e.g., pine beetle—Lanier et al., 1972; Miller et al., 1997). Similar occurrences of pheromonally mediated sexual isolation may exist within vertebrate species, but this has yet to be explored.

The red-sided garter snake (*Thamnophis sirtalis parietalis*) is the most northern living reptile in North America (Logier and Toner, 1961). Annual aggregations of red-sided garter snakes at underground hibernacula in Manitoba, Canada, are unparalleled in scope, representing the highest concentration of snakes in the world (Gregory, 1984). Marshes, shallow lakes, and areas of poor drainage offer good summer feeding grounds for the snakes, while limestone bedrock provides hibernaculum sites where the snakes are constrained to spend up to eight months of the year to avoid the harsh winters (Aleksiuk and Stewart, 1971). A scarcity of suitable hibernation sites, coupled with a high degree of den fidelity and mating occurring almost exclusively at the dens (Gregory, 1974, 1977), has effectively divided the red-sided garter snakes in this region into geographically isolated populations defined by particular hibernacula.

Mating of the red-sided garter snake occurs directly at the hibernaculum following spring emergence (Gregory, 1974). Like most snakes (reviewed in Mason, 1992), the regulation of reproductive behaviors in the red-sided garter snake depends primarily on the production and expression of specific sex pheromones

(Mason, 1993). Of particular importance is the sexual attractiveness pheromone. 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 and is responsible for eliciting male courtship behavior (Noble, 1937; Garstka et al., 1982). If a male does not detect the sexual attractiveness pheromone, then courtship will not be initiated and subsequent mating will not occur (Mason, 1993).

The reliance of the red-sided garter snake on the sexual attractiveness pheromone to initiate reproductive behavior, coupled with its discontinuous population structure in Manitoba, offers a strong basis for investigating pheromonally mediated sexual isolation in this species. Here, we present a study initially designed to test the courtship preference of male red-sided garter snakes from two geographically isolated hibernacula to females from their own den versus females from the distant den. Because a courtship preference was detected for males from one of the dens, we set out to determine whether the observed preference was mediated through variation in the sexual attractiveness pheromone. This was accomplished by: (1) utilizing trailing experiments to determine whether the observed preference was mediated through chemical cues, and (2) performing chemical analyses to evaluate whether detectable variation existed in the female sexual attractiveness pheromone between the two hibernacula.

METHODS AND MATERIALS

Study Populations. Red-sided garter snakes used in this study were obtained from two hibernacula located approximately 240 km apart in Manitoba, Canada (Figure 1). The Narcisse den, located in the Interlake region of Manitoba (50°44'37"N, 97°31'26"W), houses in excess of 20,000 snakes during the winter months, whereas the Reeder den, located in western Manitoba (50°06'34"N, 101°06'32"W), shelters approximately 10,000 individuals (R.T. Mason, unpublished data). Snakes from both populations demonstrate similar temporal patterns; individuals emerge in late April, participate in a brief mating season at the hibernaculum, and then migrate to the summer feeding grounds before returning to the hibernacula in early September (Gregory, 1977). Adult females from the two populations attain an average snout-vent length (SVL) of 55–60 cm and a mean mass of 70–75 g. Adult males are smaller, attaining an average SVL of 45–50 cm and a mean mass of 35–40 g in the Narcisse population and an average SVL of 50–55 cm and a mean mass of 42–47 g in the Reeder population (Shine et al., 1999; M. P. LeMaster, unpublished data).

Behavioral Experiments

Experimental Animals. Unmated female red-sided garter snakes ($N = 11$ per hibernaculum) and sexually active adult male snakes ($N = 150$ per hibernaculum)

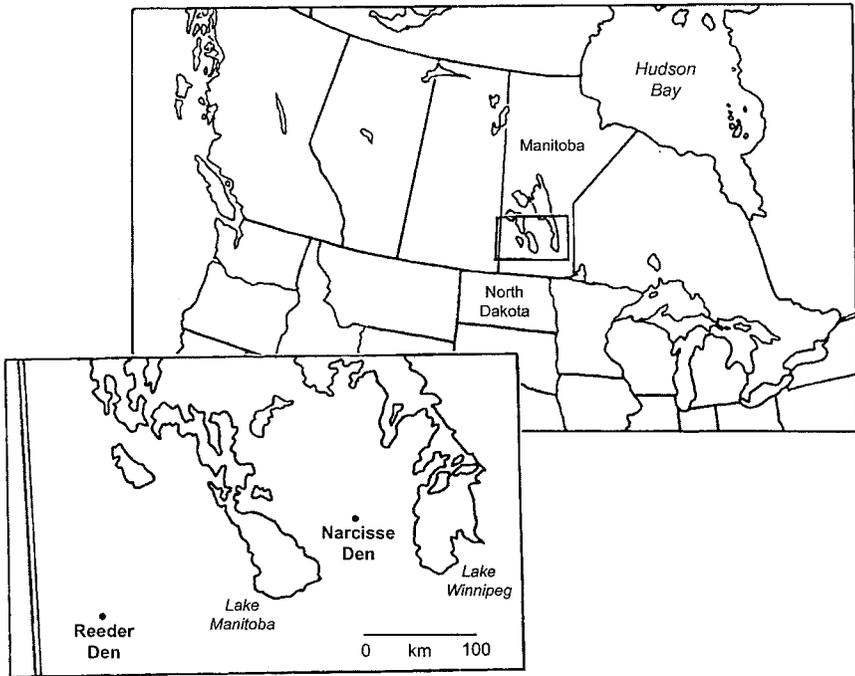


FIG. 1. Map of Manitoba, Canada, showing the location of the two populations of red-sided garter snakes (*Thamnophis sirtalis parietalis*) used in this study. The Narcisse den population overwinters in a limestone sinkhole; the Reeder den population shelters in a shale outcrop.

were collected at the two hibernacula immediately upon emergence in May of 1998. To avoid any confounding effects due to temporal variation in mating behavior during the breeding season, snakes were collected from the two hibernacula over a single 24-hr period. Animals were segregated by sex and hibernaculum in cloth bags and returned to the Chatfield Research Station (Chatfield, Manitoba) where they were held at ambient temperature for 2 days until testing was initiated. Following testing, the animals were returned to their respective hibernacula and released.

Courtship Trials. To examine whether red-sided garter snakes preferentially court females from their own hibernaculum, we tested the courtship response of males from the Narcisse and Reeder dens to resident (same den) and nonresident (distant den) females utilizing a simultaneous choice test design (Mason and Crews, 1985). Briefly, we introduced 10 randomly chosen males from a particular den into

an outdoor arena [$1 \times 1 \times 1$ m; constructed of nylon cloth (Moore et al., 2000)] and allowed them to acclimate for 5 min. We then placed into each arena simultaneously a female from the Narcisse den and a female from the Reeder den. The two females were matched according to SVL, as male red-sided garter snakes demonstrate a strong courtship preference for females of larger size (Shine et al., 2001). In addition, tape was placed across the cloaca of each female to prevent females from mating during the trials, an event that drastically reduces further male courtship behavior (Garstka et al., 1982). Following the introduction of the females, the snakes were allowed to interact undisturbed for 5 min after which time the number of males actively courting each female was recorded.

We performed two experiments utilizing the simultaneous courtship test design to examine the courtship response of Narcisse den males (experiment 1; $N = 9$ trials) and Reeder den males (experiment 2; $N = 9$ trials) to resident and nonresident females. For each experiment, test males were replaced with new males following each trial. In addition, female pairs of similar size were only used once per experiment. Male courtship behavior was assessed using an ethogram of male garter snake mating behavior (Crews et al., 1984). For a male to be considered actively displaying courtship behavior during the trials, it was necessary for us to observe chin-rubbing by the male along the dorsal surface of the female. This behavior is only observed in a reproductive context and is, therefore, indicative of sexual behavior for males of this species. To test for significant male responses in the two experiments, the difference in the frequency of males courting females from their own hibernaculum versus females from the distant hibernaculum were analyzed by using χ^2 analyses (Zar, 1984). Level of significance for each test was set at $P < 0.05$.

Trailing Experiments. To determine whether the male snake courtship preference observed is mediated through skin-derived chemical cues, we tested the trailing response of male red-sided garter snakes when presented with resident and nonresident females on a standard Y maze previously described by LeMaster and Mason (2001). We performed two experiments testing the trailing preference of Narcisse den males (experiment 1; $N = 11$ trials) and Reeder den males (experiment 2; $N = 11$ trials). We also performed a control experiment utilizing Narcisse den males ($N = 10$ 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). In each experiment, unique males were used for each trial. In addition, female pairs (the same nine pairs used in the courtship experiments plus an additional two pairs) were used only once per experiment. Trials were conducted daily from 10:00-16:00 hr, 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. Statistical significance in male trailing behavior was analyzed utilizing one-way

binomial tests (Sokal and Rohlf, 1995). Level of significance for each test was set at $P < 0.05$.

For each trial, we randomly selected a pair of stimulus females and rubbed the dorsal surface of each on the surface of the maze. 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. The cloacal openings of the donor females were covered during transfer of the skin lipids so that contamination from cloacal gland secretions did not occur. We then placed a male test snake (Narcisse or Reeder den male depending on experiment) into an opaque box at the start of the base arm and allowed it 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 10 mins, or trials in which the test snake fell from the maze were eliminated (<4% 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 preexisting trail (Brown and MacLean, 1983).

Chemical Analysis

Pheromone Collection. Adult, sexually attractive female snakes from the Narcisse den ($N = 10$) and the Reeder den ($N = 12$) were collected immediately upon spring emergence from the hibernacula in May of 1999. We focused our collection on females in a particular size range (SVL = 62.0–75.0 cm) as the sexual attractiveness pheromone of female red-sided garter snakes varies qualitatively in relation to female body length (LeMaster and Mason, 2002). The animals were killed with an overdose of brevitall sodium, and each snake was then placed dorsal side down in a 500-ml glass beaker and covered with 25–50 ml of 100% hexane (Mason et al., 1989, 1990). Following a 12-hr immersion period, the animals were removed from the hexane, and 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 × 22 mm ID) packed with alumina (activity III) and eluted the columns with hexane and ethyl ether 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 fresh hexane (1 ml). 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 expressed by female snakes from the two hibernacula, we calculated the amount (micrograms) of methyl ketones expressed per unit (square centimeter) skin surface 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. A general measure of skin surface area for each female was determined by multiplying the SVL of a female by its circumference at mid-body (Mason et al., 1990).

To examine variation in the quality of pheromone expressed by females from the two populations, we determined the number of unique methyl ketones expressed by individuals and compared the relative concentrations of individual methyl ketones comprising the overall pheromone profiles for the two populations. The methyl ketones present in the pheromone extracts were identified by 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 μ l) of the methyl ketone fractions were injected onto a fused-silica capillary column (HP-1; 12 m \times 0.22 mm ID; Hewlett Packard) with helium as the carrier gas. 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 individual methyl ketones in each sample by using peak integration. Identification of compounds and peak areas were determined utilizing ChemStation software (Version B.02.05; Hewlett Packard) interfaced with the gas chromatograph-mass spectrometer.

Statistical significance in the amount of sexual attractiveness pheromone expressed on the dorsal surface of the female and the total number of unique methyl ketones expressed were examined using a Student *t* test and a Mann-Whitney rank sum test, respectively (Sokal and Rohlf, 1995). We used a randomization test (Manly, 1991) to test for a difference in the relative concentrations of individual methyl ketones composing the sexual attractiveness pheromone among the two dens. The test statistic was the Euclidean distance (*S*) between the average proportions of all individual methyl ketones observed. The randomization test generated a *P* value by comparing the observed test statistic to a simulated distribution based on 10,000 reshufflings of the data set. Level of significance for all tests was set at $P < 0.05$.

RESULTS

Behavioral Experiments

Courtship Experiments. The average SVL (\pm SD) of Narcisse females used in the simultaneous choice tests was 59.0 (\pm 8.9) cm, whereas the average SVL (\pm SD) for Reeder females was 60.1 (\pm 10.0) cm. When paired for individual trials, the average SVL difference (\pm SD) among females from the two hibernacula was 1.5 (\pm 1.2) cm. Narcisse den females were observed to be slightly larger in four of the nine trials for each experiment, while Reeder den females were slightly larger in the remaining five trials.

When presented with the females from the two hibernacula, male garter snakes responded with stereotypical courtship behaviors, including increased tongue-flick rate and chin rubbing along the dorsum of the females. Narcisse den males displayed a significant courtship preference for females from their own den over females from the Reeder den. Across the nine trials conducted, a greater proportion of males were observed courting the Narcisse den females over the Reeder den females following the 5-min interaction period ($\chi^2 = 8.80$, 1 *df*, $P < 0.01$; 43.3% of males courted Narcisse den females versus 17.8% of males courting Reeder den females; Figure 2). Interestingly, Reeder den males did not demonstrate a similar courtship preference to the Narcisse den males across the nine trials conducted ($\chi^2 = 0.02$, 1 *df*, $P > 0.50$; 26.7% of males courted Reeder den females versus 25.6% of males courting Narcisse den females; Figure 2). We pooled the data for analyses after heterogeneity χ^2 tests showed no significant differences in male courtship preference among the individual trials conducted for each experiment (Narcisse males: $\chi^2 = 6.62$, 8 *df*, $P > 0.50$; Reeder males: $\chi^2 = 6.84$, 8 *df*, $P > 0.50$).

Trailing Experiments. When presented with the control maze (both arms blank), male red-sided garter snakes showed no preference for one arm over the other (binomial test; $P = 0.205$; Table 1). When tested with the simultaneous trails, males in both experiments responded with trail contact responses upon first exiting the start box, regardless of which trail a male might have initially encountered. Overall, Narcisse den males displayed a strong trailing preference for females from their own hibernaculum over females from the Reeder hibernaculum, choosing the arm with the Narcisse den female trail in 9 of the 11 trials conducted (binomial test; $P = 0.027$; Table 1). Reeder den males, however, showed no such preference among the trail types, appearing to randomly choose among the trails at the Y junction (binomial test; $P = 0.226$; Table 1).

Pheromone Analysis

Pheromone Quantity. There was no difference in the average SVL [Narcisse den females = 68.3 (\pm 4.9) cm; Reeder den females = 69.6 (\pm 6.6) cm: Student's

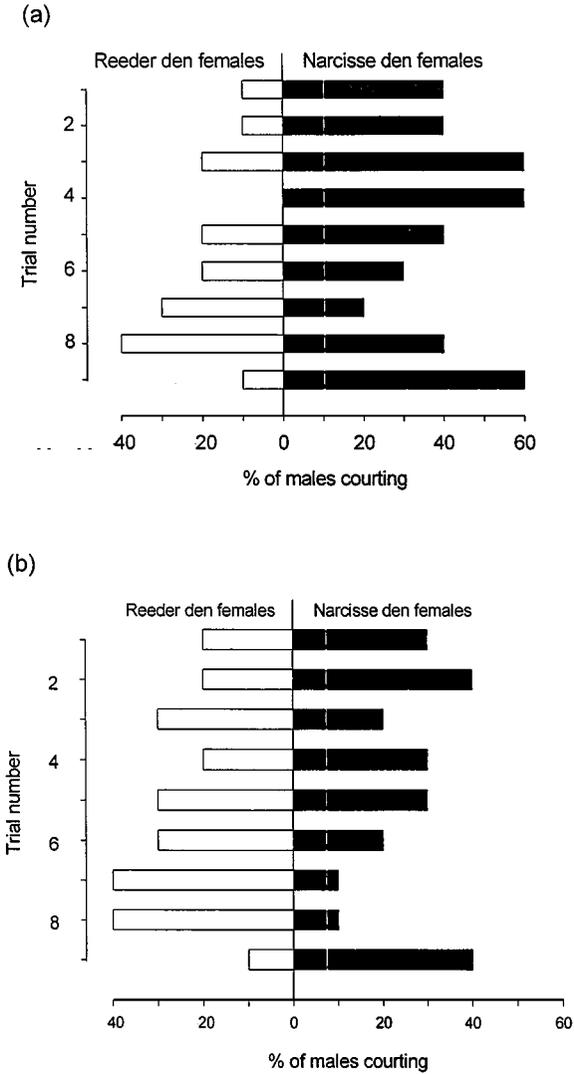


FIG. 2. Results of simultaneous choice tests in which a resident and a nonresident female red-sided garter snake (*Thamnophis sirtalis parietalis*) were placed in an outdoor arena with 10 courting males from (a) Narcisse den and (b) Reeder den. After 5 min the number of males courting each female was recorded.

TABLE 1. TRAILING RESPONSE OF MALE RED-SIDED GARTER SNAKES (*Thamnophis sirtalis parietalis*) FROM TWO HIBERNACULA TO FEMALES FROM THEIR OWN DEN VERSUS FEMALES FROM A DISTANT DEN WHEN PRESENTED SIMULTANEOUSLY ON A Y MAZE^a

Condition	N	Arm treatment	Number of times selected	P
Control	10	Blank	6	0.205
		Blank	4	
Narcisse males tested on Narcisse versus Reeder female trails	11	Narcisse female	9	0.027
		Reeder female	2	
Reeder males tested on Narcisse versus Reeder female trails	11	Narcisse female	5	0.226
		Reeder female	6	

^a The control experiment was performed utilizing Narcisse den males.

t test; $t = -0.522$, $P = 0.607$] or average mass [Narcisse den females = 114.7 (\pm 31.0) g; Reeder den females = 106.2 (\pm 28.7) g; Student's *t*-test; $t = -0.664$, $P = 0.514$] among the females sampled for pheromone analysis. The hexane extractions of individual females yielded an average (\pm SD) of 25.1 (\pm 8.8) mg of skin lipids per female from the Narcisse den and 22.8 (\pm 12.2) mg of skin lipids per female from the Reeder den. Subsequent fractionation of the lipids yielded an average (\pm SD) methyl ketone fraction of 4.8 (\pm 2.9) mg per Narcisse den female and 4.2 (\pm 2.4) mg per Reeder den female. Overall, the methyl ketones accounted for an average (\pm SD) of 20.0 (\pm 10.6)% of the skin lipids collected from the Narcisse den females and 19.5 (\pm 7.0)% of the skin lipids collected from the Reeder den females. After accounting for variation in skin surface area, we did not observe a significant difference between the females from the two hibernacula and the amount of methyl ketones extracted per unit of surface area (Student's *t* test; $t = 0.561$, $P = 0.581$; Table 2).

Pheromone Quality. Complete GC-MS analysis of the methyl ketone fractions revealed the presence of 18 unique long-chained methyl ketones (Figure 3).

TABLE 2. QUANTITATIVE AND QUALITATIVE VARIATION IN EXPRESSION OF SEXUAL ATTRACTIVENESS PHEROMONE AMONG FEMALE RED-SIDED GARTER SNAKES (*Thamnophis sirtalis parietalis*) FROM TWO DENNING POPULATIONS IN MANITOBA, CANADA^a

Population	SVL (cm)	Mass (g)	Methyl ketone expression (μ g/cm ²)	Unique methyl ketones expressed
Narcisse den (<i>N</i> = 10)	68.3 (\pm 4.9)	114.7 (\pm 31.0)	13.1 (\pm 7.9)	16.9 (\pm 0.57)
Reeder den (<i>N</i> = 12)	69.6 (\pm 6.6)	106.2 (\pm 28.7)	11.5 (\pm 5.1)	17.4 (\pm 0.52)

^a Average (\pm SD) measurements are presented for each denning population.

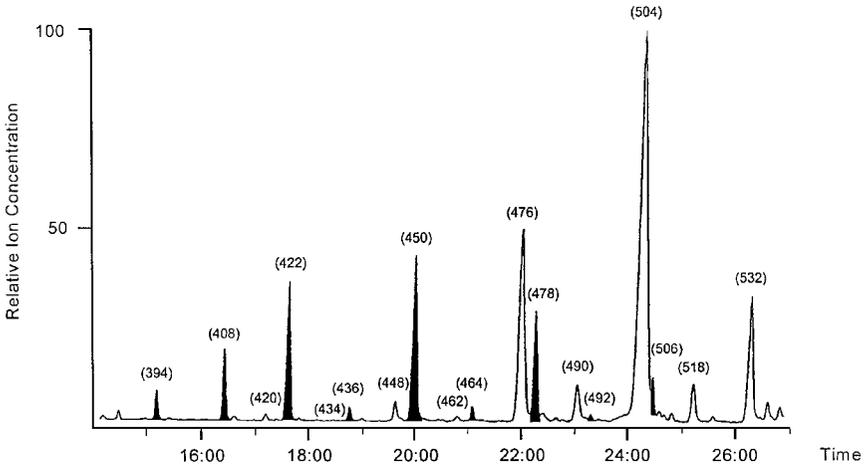


FIG. 3. Gas chromatogram of the female sexual attractiveness pheromone profile for the red-sided garter snake, *Thamnophis sirtalis parietalis*. Pheromone profiles are composed of saturated (shaded peaks) and unsaturated (open peaks) methyl ketones.

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 to 506 mass units, whereas the unsaturated methyl ketones ranged in size from 420 to 532 mass units. Females from the two hibernacula varied in the number of methyl ketones expressed in their skin lipids, ranging from pheromone profiles composed of 16 unique methyl ketones to pheromone profiles composed of all 18 unique methyl ketones. However, when we compared the average number of methyl ketones expressed by females from each hibernaculum, a significant relationship was not observed (Mann-Whitney rank sum test; $T = 92.5$, $P = 0.144$; Table 2).

Although the two hibernacula did not vary in the number of unique methyl ketones expressed, we did observe variation in the relative contribution of the individual methyl ketones composing the pheromone profiles for the two hibernacula (randomization test; S observed = 0.14, S generated = 0.07, $P = 0.038$; Figure 4a). Subsequent analyses showed that this difference was not due to variation between hibernacula in the relative contribution of total saturated versus unsaturated methyl ketones (t test; $t = 1.492$, $P = 0.151$). Instead, independent analyses of the two methyl ketone groups revealed that the two hibernacula varied in the relative concentration of individual unsaturated methyl ketones (randomization test; S observed = 0.14, S generated = 0.06, $P = 0.011$; Figure 4b), whereas the relative concentration of saturated methyl ketones remained stable among

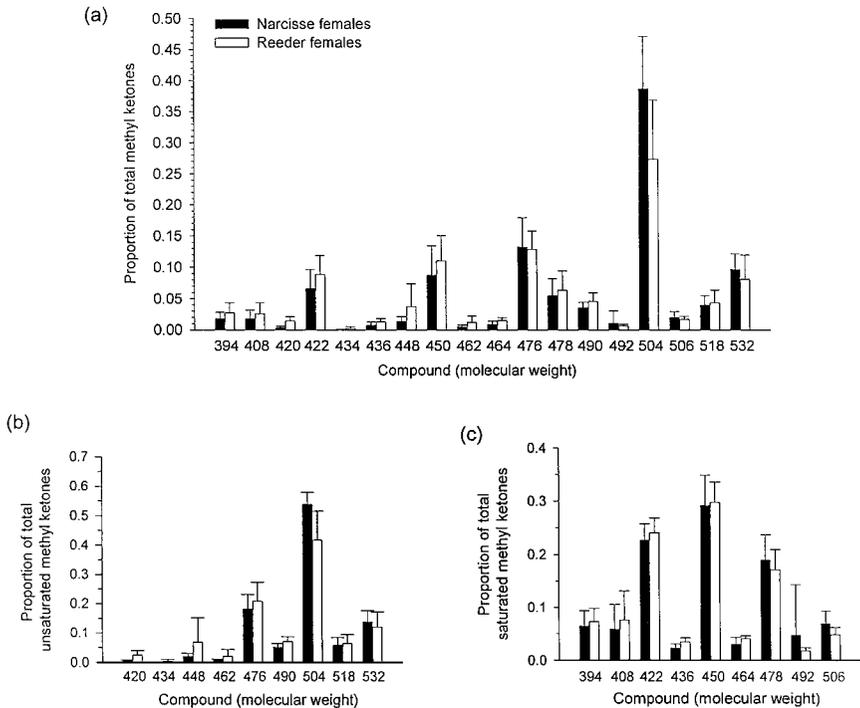


FIG. 4. Relative concentrations (\pm SD) of individual methyl ketones composing the sexual attractiveness pheromone of the red-sided garter snake (*Thamnophis sirtalis parietalis*) for females from the Narcisse den ($N = 10$) and the Reeder den ($N = 12$). Overall, there was significant variation in the pheromone profiles (a) with the majority of variation present in the relative concentration of individual unsaturated methyl ketones (b) and not in the individual saturated methyl ketones (c) (see text for statistical analyses).

dens (randomization test; $S_{\text{observed}} = 0.07$, $S_{\text{generated}} = 0.05$, $P = 0.195$; Figure 4c).

DISCUSSION

Intraspecific sexual isolation has been documented in a variety of vertebrates including fish (e.g., Ayvazian, 1993; Ziuganov, 1995), amphibians (e.g., Houck et al., 1988; Verrell and Arnold, 1989), and mammals (e.g., Pillay et al., 1995; Pillay, 2000). While multiple studies have utilized behavioral trials to explore the role of chemical cues in sexual isolation (e.g., Ovaska, 1989; Pillay et al., 1995), few studies have evaluated whether structural variation exists among populations for a particular chemical cue. For example, Rollman et al. (2000) observed biochemical

variation in a male courtship pheromone among populations of salamanders of the *Plethodon jordani-glutinosus* complex, suggesting that this pheromone might be responsible for the moderate levels of sexual isolation previously observed among populations of this species. However, behavioral trials testing the receptivity of females to pheromone extracts from various populations have yet to be performed. To our knowledge, the present study represents the first attempt to integrate behavioral experiments and chemical analyses towards understanding the role of chemical cues in sexual isolation within a vertebrate species.

The results of the courtship experiments demonstrate that sexual isolation exists to a degree among denning populations of red-sided garter snakes. Males from the Narcisse den displayed a courtship preference for females from their own den over those from the Reeder den. Interestingly, males from the Reeder den did not display a similar preference, indicating that male courtship preference in these two populations is asymmetrical. Similar instances of asymmetrical sexual isolation have been observed in other species (e.g., Krebs, 1990; Kobayashi et al., 2001), including vertebrates (e.g., Ayvazian, 1993; Pillay, 2000). Why these two denning populations of snakes show asymmetrical sexual isolation is not known. We suggest that this pattern might result from reproductive character displacement due to selective pressures to recognize conspecific mates; red-sided garter snakes in central Manitoba, unlike western Manitoba, share hibernacula with a second species of garter snake, the plains garter snake (*Thamnophis radix*). Thus, males in the Narcisse den may be more narrowly tuned to reproductive cues, allowing for discrimination among conspecific and heterospecific females, while the Reeder males, under no such pressure, are more broadly tuned. Alternatively, the pattern of sexual isolation observed in this study could represent a transient phenomenon, resulting from divergence in traits and mating preferences based on those traits through sexual selection acting within each population (Arnold et al., 1996).

Potentially, male red-sided garter snakes may utilize multiple cues to discriminate among females from various dens. Evidence suggests that both chemical and visual cues assist males of this species in selecting among potential mates during the breeding season (Shine and Mason, 2001). However, the results of the trailing experiments demonstrate that chemical cues alone are sufficient to allow males to discriminate among resident and nonresident females. When presented with the two female trails simultaneously, we observed males to display preferences similar to those observed in the courtship trials. In addition, the similarity in male preferences observed between the courtship and trailing experiments suggests that both behaviors are mediated by a similar chemical cue. In the case of the red-sided garter snake, that cue appears to be the female sexual attractiveness pheromone (Mason et al., 1989; LeMaster and Mason, 2001).

Subsequent analysis of the female sexual attractiveness pheromone from the two hibernacula demonstrated that it contains the necessary variation to function as a reliable indicator to males of a female's resident status. Specifically, females

were found to vary between dens in the relative concentration of individual unsaturated methyl ketones composing the sexual attractiveness pheromone. We were not surprised to observe that the variation was greatest with respect to the unsaturated methyl ketones. Although both unsaturated and saturated methyl ketones are necessary to elicit full male courtship, unsaturated methyl ketones appear to be the more biologically active of the two groups, eliciting a fivefold increase in male courtship behavior over saturated methyl ketones when presented in isolation (Mason et al., 1989). Thus, low levels of variation in the relative concentration of unsaturated methyl ketones between dens, as were observed in this study, are more likely to affect male courtship behavior than similar variation in saturated methyl ketones.

Why females from the two hibernacula vary in the relative concentration of individual methyl ketones is unclear. German cockroaches (*Blattella germanica*) produce a sex pheromone composed of methyl ketones (Schal et al., 1990), similar to those utilized by the red-sided garter snake. In the cockroach system, the pheromone derives its origins from fatty acid biosynthesis (reviewed in Tillman et al., 1999), suggesting that the sexual attractiveness pheromone of the red-sided garter snake is synthesized through a similar process. Thus, variation in the expression or activity levels of enzymes in the biosynthetic pathway for females from various hibernacula could result in the observed variation. In addition, the fact that males from the Narcisse den, but not the Reeder den, show a courtship preference for resident over nonresident females suggests that variation also exists among the two male detection systems. Such variation may be present at the level of the receptor neurons (e.g., Wu et al., 1999), located in the vomeronasal organ of the male snake (Halpern and Kubie, 1983) or may occur in higher processing centers, including the olfactory and accessory olfactory bulbs (Halpern, 1976). Future studies are necessary to resolve the underlying mechanism(s) responsible for variation of pheromone production and detection in this system.

The potential use of the female sexual attractiveness pheromone by male red-sided garter snakes to distinguish females from their own den over females from other dens suggests that this pheromone may represent a compound signal containing hierarchical mapping. Hierarchical mapping refers to signals in which differences in mean patterns are used to code for one question and different variants around that mean are then used to answer other questions (Bradbury and Vehrencamp, 1998). Thus, in the red-sided garter snake, the presence of saturated and unsaturated methyl ketones signifies the presence of a female, whereas variation in the relative concentration of individual unsaturated methyl ketones conveys the home locality. Other examples of compound signals containing hierarchical mapping in vertebrates can be found in electric fish (e.g., Crawford, 1992) and birds (e.g., Becker, 1982).

In summary, the results of this study demonstrate that pheromonally mediated sexual isolation exists to a degree among denning populations of red-sided

garter snakes. Male snakes from the Narcisse den displayed a courtship preference for females from their own den over females from the Reeder den, and this preference was maintained when males were presented with female trails. Further, we observed population-specific variation in the composition of the female sexual attractiveness pheromone, the pheromone responsible for mediating courtship and trailing behavior, strongly suggesting that this pheromone is responsible for the observed sexual isolation.

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