

# Control of Attractivity and Receptivity in Female Red-Sided Garter Snakes

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Received October 20, 2000; revised March 15, 2001; accepted March 20, 2001

Female red-sided garter snakes emerge from their hibernacula in the spring attractive and receptive to males. Attractivity is communicated by a pheromone released through the female's skin and is a consequence of ovarian recrudescence the previous summer. Receptivity, on the other hand, is stimulated by ovarian estrogen secretion during emergence itself. Mating renders females both unattractive and unreceptive. Another "mating" pheromone of male origin is important in making females unattractive after mating. To investigate the role of cloacal stimulation in the loss of attractivity and receptivity we injected a local anesthetic (lidocaine or tetracaine) in the cloacal region of females before mating. This does not prevent mating, although it blocks neural transmission of copulatory sensory stimuli. The time course of transition from attractive and receptive states was then observed. Females treated with local anesthetic as well as control females were unattractive within 15 min of mating. However, when retested 2–3 and 24 h after mating, a significantly higher proportion of treated females regained their attractivity, while mated control females remained unattractive. This restorative effect was transient, though, as treated females retested 48 h after mating were as unattractive as the controls. Both anesthetized and control females were unreceptive when tested following mating and did not regain receptivity with time. Last, the mating-induced surge in circulating concentrations of prostaglandin was diminished in females that received a local anesthetic prior to mating. Taken together these results indicate that the loss of attractivity and receptivity following mating in the red-sided garter snake is due to combined effects of a mating pheromone and a physiological, neurally mediated

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**Key Words:** mating; receptivity; attractivity; reptiles; snakes.

Beach (1976) distinguished between attractivity, receptivity, and proceptivity as components of sexual behavior in females, emphasizing that the physiological mechanisms, as well as the functional outcomes, of each differ. Female red-sided garter snakes (*Thamnophis sirtalis parietalis*) are both attractive and receptive in the spring as they emerge from their hibernacula in the Interlake region of Manitoba, Canada. The females possess a pheromone, deposited in the lipids of the dorsal and lateral skin, that make them attractive to males (Gartska and Crews, 1981). This pheromone has been characterized chemically as a novel series of saturated and monounsaturated methyl ketones (Mason, Jones, Fales, Panell, Chinn, and Crews, 1989; Mason, Jones, Fales, Panell, and Crews, 1990). Responding to this pheromone, many males will court each female as they emerge, resulting in mating balls of 10–100 snakes (Aleksiuk and Gregory, 1974).

After mating the female becomes both unattractive and unreceptive (Gregory, 1974; Ross and Crews, 1977, 1978; Whittier, Mason, and Crews, 1985; Whittier and Crews, 1986a; Shine, Olsson, and Mason, 2000), moving rapidly in a zigzag fashion and frequently barrel-rolling to prevent further courtship (Crews, 1976; Mendonça and Crews, 1996), and leave the den area (Gregory, 1974). Recently mated females, whether in the laboratory or in the field, will almost never remate. Males cannot force a mating; a female must gape her cloaca, an action that allows intromission by a single male (Blanchard and Blanchard, 1942; Carpen-

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ter, 1977). Mating typically lasts for 10–15 min (Crews, 1976; Whittier and Crews, 1986a). Initially, both snakes remain stationary upon or soon after intromission; during this time, the male transfers sperm and a copulatory plug forms that blocks the cloacal opening of the female (Devine, 1975, 1977; Ross and Crews, 1977, 1978; Shine *et al.*, 2000). Sperm transfer occupies the majority of copulation, with the mating plug being produced and transferred at the end of copulation (Shine *et al.*, 2000). Female red-sided garter snakes occasionally are courted after mating by a small percentage of males, but it is rare for a female to remate within 24 h of a previous mating (Whittier and Crews, 1986a; Shine *et al.*, 2000).

The loss of attractivity following mating appears to depend upon pheromones associated with mating. In the red-sided garter snake it is the copulatory fluids secreted by the male at the onset of mating that are responsible for the loss of attractivity (Shine *et al.*, 2000). In both the plains garter snake, *T. radix* (Ross and Crews, 1977, 1978) and the red-sided garter snake removal of the copulatory plug immediately after the termination of copulation causes females to regain their attractivity, but not their receptivity and a significant proportion will remate within 24 h; this does not occur if the mating plug remains in place for several hours before its removal (Ross and Crews, 1977, 1978; Shine *et al.*, 2000; Whittier and Crews, 1986a).

Mating engenders both physiological and behavioral responses in the female red-sided garter snake. In addition to the loss of attractivity and receptivity, mating also initiates a neuroendocrine reflex. Circulating levels of plasma prostaglandin- $F_{2\alpha}$  (PG- $F_{2\alpha}$ ) are significantly elevated immediately after mating and remain elevated for at least 24 h (Whittier and Crews, 1989). Administration of PG- $F_{2\alpha}$  to unmated female red-sided garter snakes renders them unattractive, but the effectiveness of this treatment has a slower time course (hours) than the natural loss of attractivity after mating (minutes) (Whittier and Crews, 1986b). The sensation of copulatory stimuli also results in a surge in plasma estrogen 4–12 h after mating (Gartska, Camazine, and Crews, 1982; Whittier and Crews, 1986a; Mendonça and Crews, 1990). Finally, there are associated changes in neural activity with mating as measured by the accumulation of 2-deoxyglucose; there is a significant increase in uptake of 2-deoxyglucose in the preoptic area and a corresponding significant decrease in the ventromedial hypothalamus with mating (M. Mendonça and D. Crews, unpublished data).

This purpose of the present study was to test further the role of cloacal stimulation in the change in attrac-

tivity and receptivity of female red-sided garter snakes after mating.

## MATERIALS AND METHODS

### Animals

Male and female red-sided snakes were captured at hibernacula located in the Interlake region of Manitoba, Canada, at various time points, depending on the subsequent experiment. For Experiment 1, females and males were captured in the fall of 1988 and returned to the laboratory, where they were separated by sex and housed in 29-gal. aquaria. Snakes were kept at approximately 25°C and a 12:12 LD cycle when not in hibernation. Animals were given water *ad lib*, and fed ground fish (frozen and canned) supplemented with vitamins twice a week. To simulate hibernation, all snakes were exposed to 4°C for 17 weeks from October to February, conditions that mimic hibernation and result in successful mating (Gartska *et al.*, 1982). Females were then tested upon emergence for their attractivity and receptivity to stimulus males. For Experiments 2 and 3 animals were captured in the spring of 1989 and 1990, respectively, when they were emerging from natural hibernacula in Canada. In the field experiments animals were manipulated and tested within a day of capture and then released.

### Behavior Testing

In the laboratory, attractivity tests were conducted by placing a single, recently emerged (within 48 h), female in an aquarium with 10 males known to exhibit vigorous courtship behavior. The males' interaction with the female was observed for 10 min and the maximum number of males that actively courted the female was recorded. Females eliciting courtship from 6–10 males were classified as "attractive" and were used in all experiments.

In the field, a similar protocol was used. A female was placed in one of six outdoor nylon arenas (1 × 1 × 1 m) with 10 males known to exhibit vigorous courtship. The same criteria as above were used to score attractivity. We tested experimental and control animals simultaneously. Since ambient conditions are constantly changing in the field (e.g., under windy conditions some males are distracted and will not court), a daily standard of attractivity was developed. This consisted of initially testing very recently emerged females each day to compare the number of

males that would court. Test days in which too few males were courting because of ambient conditions were excluded from the analysis.

Under both testing conditions the distinction between attractivity and receptivity was clear. Attractivity is defined by the behavior of the males directed toward the female; for example, male courtship behavior is scored on a scale from 0 to 3, or ranging from the female being ignored by males (0) to the male repeatedly and rapidly traversing her length (2.0), eventually aligning his body and exhibiting caudocephalic waves, attempting cloacal apposition (2.5). If the male achieves intromission he is given a score of 3.0. Receptivity, on the other hand, is distinguished by the female's response to courting males. It is clear that males cannot force a female to mate; that is, intromission requires the female to gape her cloaca, enabling the male to insert one of two hemipenes. Further, females may also try to escape the male by exhibiting sudden darting movements to distance themselves from males or, if that is not possible, by barrel rolls.

Anesthetized females show all components of sexual receptivity: they remain stationary, as do receptive, untreated females, and gape their cloaca. However, they do not have active motor control of this latter facet of behavior. This, coupled with the fact that the local administration causes their tail to become limp, enables a courting male to achieve intromission much more rapidly than in untreated females. Experiments in the field indicate that tetracaine-treated females mate significantly sooner (average of 8.4 vs 28.6 min;  $P = 0.002$ ) and copulation lasts significantly longer than that with untreated females (31.6 vs 17.8 min;  $P = 0.001$ ). In the laboratory experiment, mating plugs were either removed or left in place. The manipulation caused no difference in the restoration of female attractivity at 24 h (Mann-Whitney  $U$  test,  $Z = -0.15$ ;  $P = 0.87$ ).

### *Prostaglandin Radioimmunoassay*

Blood samples were taken immediately following the end of intromission. Plasma was analyzed for PG- $F_{2\alpha}$  levels using silicic acid chromatographic purification followed by a radioimmunoassay using a modified kit (Travenol Genetech Diagnostics); see Whittier and Crews (1989) for details regarding validation with snake plasma.

### *Statistics*

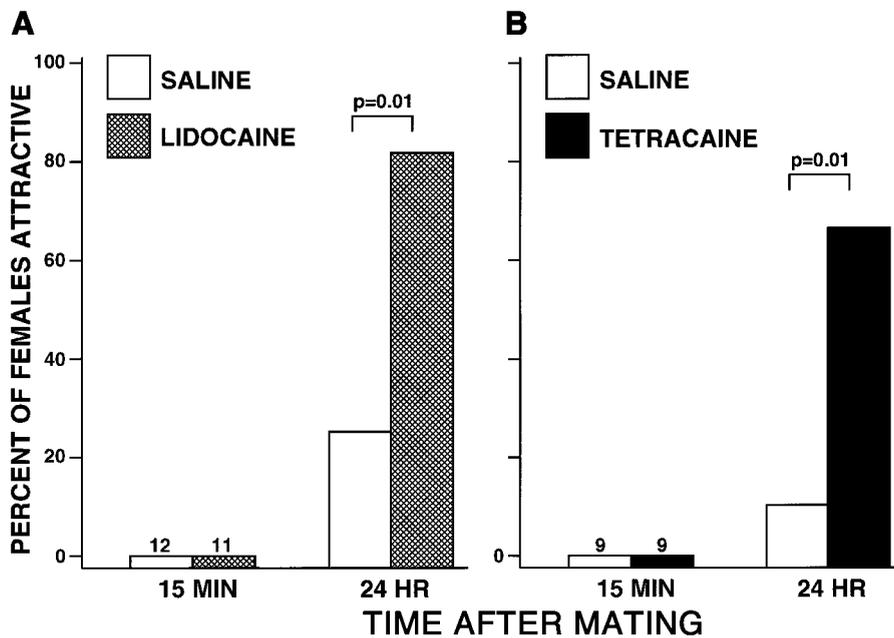
Differences between the frequencies of females exhibiting the different responses were analyzed using a

Fisher's Exact or  $\chi^2$  test (Sokal and Rohlf, 1981). Differences in PG- $F_{2\alpha}$  levels were tested using a Student's unpaired  $t$  test.

***Experiment 1. Effect of lidocaine and tetracaine on attractivity and receptivity in female red-sided garter snakes in the laboratory.*** Females that had recently emerged from hibernation in the laboratory (spring, 1989) were divided into two groups. One group was injected in the cloacal region with 20  $\mu$ l (10  $\mu$ l on each side of the cloaca) of a 1.0% solution of lidocaine ( $n = 11$ ) or in a similar manner with a saline solution ( $n = 12$ ). Another group of females was injected in the same fashion with either a 1.0% solution of tetracaine ( $n = 9$ ) or with saline ( $n = 9$ ). That lidocaine and tetracaine treatment effectively block the females' sensation of mating is indicated by the finding that anesthetic applied locally to the cloaca was as effective as spinal transection in preventing the mating-induced estrogen surge and ovarian recrudescence characteristic of female red-sided garter snakes (Mendonça and Crews, 1990). Approximately 10 min after injection (the time necessary to produce the numbing effect), females were individually placed in aquaria with 10 actively courting males, scored for attractivity, and allowed to mate. Within 15 min after mating, females were placed in other aquaria with new males, observed for 10 min, and again scored for attractivity. The next day (24 h later), these same females were again exposed to courting males, observed for 10 min, and scored for attractivity.

***Experiment 2. Effect of tetracaine on attractivity and receptivity in female red-sided garter snakes in the field.*** Recently emerged females in the field were injected with tetracaine ( $n = 16$ ) or saline ( $n = 17$ ) in the cloacal region as described for Experiment 1. Ten minutes after injection, females were placed in the arena and allowed to mate. These females were then tested for attractivity and receptivity with new males in another arena 15 min and 2–3, 24, and 48 h after mating. For each test period, females were exposed to males for at least 2 h, which is more than enough time to insure copulation (Whittier and Crews, 1986a). As a control for the introduction of the drug itself, additional females ( $n = 7$ ) were injected intramuscularly with the same dosage of tetracaine in a body region other than the cloaca.

***Experiment 3. Effect of tetracaine on circulating concentrations of prostaglandin- $F_{2\alpha}$  in female red-sided garter snakes in the field.*** To determine if blocking the sensation of mating can affect plasma levels of prostaglandin, we bled other female red-sided garter snakes that received local anesthetic ( $n = 18$ ) or saline ( $n = 14$ ) 24 h after mating in the field in spring, 1989.



**FIG. 1.** Effect of local anesthetic on attractivity in female red-sided garter snakes (*Thamnophis sirtalis parietalis*) tested in the laboratory after emergence from artificial hibernation. Illustrated in A are females injected with lidocaine or saline and, in B, females injected with tetracaine or saline that were attractive when tested with courting males 15 min and 24 h after mating. Numbers in histograms indicate sample size.

## RESULTS

### Experiment 1. Effect of Lidocaine and Tetracaine on Attractivity and Receptivity in Female Red-Sided Garter Snakes in the Laboratory

All mated females, regardless of treatment, were unattractive and unreceptive to new males 15 min after the termination of mating (Fig. 1). When tested 24 h after mating, significantly more females that had been treated with either lidocaine and tetracaine were attractive to males compared to their saline-treated counterparts (Fisher's Exact test,  $P = 0.01$  and  $P = 0.01$ , respectively; Fig. 1). A higher percentage of lidocaine-treated females were attractive than in the tetracaine-treated group but this was not significant ( $P = 0.40$ ). Saline-injected animals also did not differ between the two treatment groups (25% vs 11%, respectively,  $P = 0.41$ ; Fig. 1).

### Experiment 2. Effect of Tetracaine on Attractivity and Receptivity in Female Red-Sided Garter Snakes in the Field

None of the females injected with tetracaine or saline were attractive to males when tested 15 min after mating (Fig. 2). When tested 2–3 and 24 h after mating, a significant proportion of the tetracaine-treated females had regained their attractivity when compared

to control, saline-injected females ( $P = 0.003$ ), although the percentage of difference was not as great at 24 h as at 2–3 h following mating (70 vs 40%,  $P = 0.04$ ). At 48 h after mating, tetracaine-treated females still elicited courtship but this percentage did not differ statistically from that of the controls (Fig. 2).

While tetracaine-treated females were attractive 2–3 h after mating, eliciting vigorous courtship from males, most were not receptive. Only 2 of the 17 tetracaine-treated females continued to be receptive and mated again; this was not significantly different from the saline-injected animals ( $P = 0.26$ ). None of the females mated 24 and 48 h later despite vigorous courtship from males. Although the tetracaine-treated females did not mate, they did not exhibit overt nonreceptive behavior (e.g., barrel-rolling), as did the saline-injected females.

All seven females that were injected intramuscularly with tetracaine in other body regions prior to mating were as unattractive and unreceptive as saline-injected females.

### Experiment 3. Effect of Tetracaine on Circulating Concentrations of Prostaglandin- $F_{2\alpha}$ in Female Red-Sided Garter Snakes in the Field

Saline-injected females tended to have higher PG- $F_{2\alpha}$  levels than their locally anesthetized counterparts

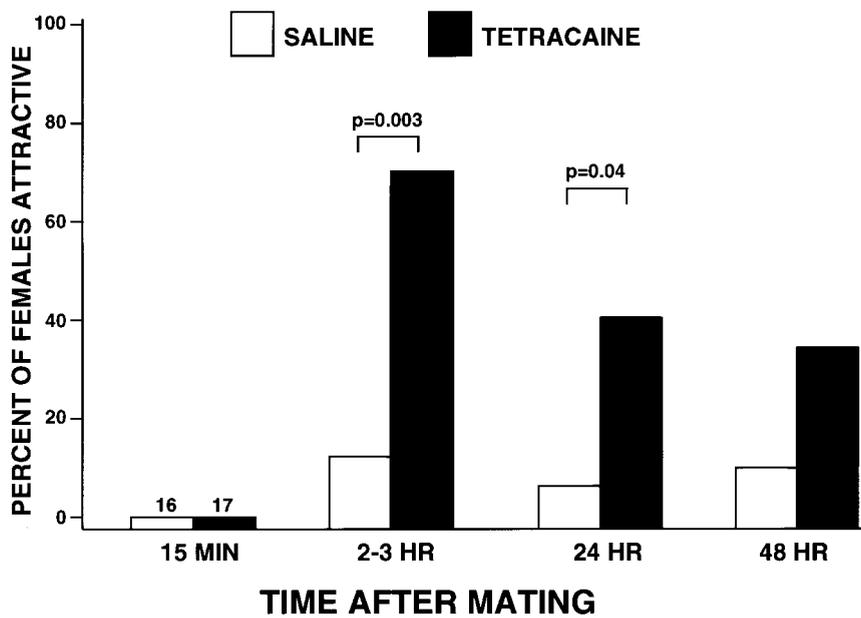


FIG. 2. Effect of local anesthetic on attractivity in female red-sided garter snakes (*T. s. parietalis*) tested in the field on emergence from natural hibernation. Illustrated are the percentages of females injected with tetracaine or saline that were attractive when tested with courting males 15 min and 2–3, 24, and 48 h after mating. Numbers in histograms indicate sample size.

(control vs anesthetized:  $x \pm 1 \text{ SE} = 4.6 \pm 0.97 \text{ ng/ml}$  vs  $2.5 \pm 0.56 \text{ ng/ml}$ ,  $t = -1.96$ ,  $P = 0.059$ ).

## DISCUSSION

Mating reduces further sexual receptivity in females in a variety of animal taxa (insects: Cordero, 1995; amphibians: Propper, 1992; reptiles: Whittier and Tokarz, 1992; Devine, 1984; mammals: Adler, 1978; Huck and Lisk, 1986; Villars, Erskine, Lambert, Jacobson, Weaver, and Baum, 1990). A concomitant reduction in attractivity with mating occurs in a variety of invertebrates (Cordero, 1995), but apparently is less well documented in vertebrates (Vandenbergh, 1994). In the present study, we observed that blocking the sensation of mating inhibited the transition to a nonattractive, but not to a nonreceptive, state. Examination of the time course of the response is revealing as to the possible contribution of the male vs female in the induction of this behavioral change.

In the laboratory females tested within the first 15 min of being mated were invariably unattractive regardless of treatment. However, when tested again 2–3 h after mating, a significant proportion of both the lidocaine- and tetracaine-treated animals had regained their attractivity. This result and other studies suggest

that the initial and rapid loss of attractivity is not due to an endogenous change in the female in reaction to copulatory stimuli but rather attributable to male-secreted pheromone in the copulatory fluids; indeed, application of these male secretions will render unmated females unattractive to males (Ross and Crews, 1977, 1978; Shine *et al.*, 2000). While this initial loss of attractivity is due to the male-secreted pheromone, the large copulatory plug that is deposited in the female's cloaca initiates changes that curtail further attractivity and receptivity. If the copulatory plug is removed immediately after mating in both the plains garter snake and the red-sided garter snake, the female's attractivity is restored (Ross and Crews, 1977, 1978; Shine *et al.*, 2000). If the mating plug is removed several hours after mating, however, the female remains unattractive to males (Shine *et al.*, 2000).

The present study indicates that in both laboratory and field experiments injection of anesthetic into the region of the cloaca results in the female regaining her attractivity. Thus, blocking the sensation of mating apparently prevented some endogenous response by the female, thereby enabling the resumption of attractivity. It is interesting to note that unlike in the laboratory experiments, where the majority of the treated females were attractive 24 h after mating, in the field experiments the proportion of tetracaine-treated fe-

males that were still attractive was highest 2–3 h after mating, gradually declining thereafter. In garter snakes the copulatory plug tightly adheres to the walls of the cloaca (Devine, 1975, Devine, 1977; Halpert, Garstka, and Crews, 1982; Shine *et al.*, 2000). In mammals, loosening this seal reduces dramatically the likelihood of fertilization (Adler, 1978). It is possible that the anesthesia of the cloacal region somehow weakens the seal between the mating plug and the cloacal walls; that is, if the cloacal walls are flaccid, it is possible that a mating plug would not adhere as firmly and would be expelled prematurely. This is likely to occur sooner in the field than in the laboratory because the female must travel over many obstacles, including males, to escape the den area. However, since the effects of the anesthetic have dissipated by 30–40 min of administration, the possible effects the anesthetic on the plug would not contribute to the loss of attractivity and receptivity that were observed at the later time intervals.

Although tetracaine treatment modified the timing of the loss of attractivity, it was not effective in blocking the loss of receptivity. Virtually all of the tetracaine-treated females failed to remate. In nature it is uncommon for female red-sided garter snakes to remate. Most females leave the den area immediately; indeed, if females are transported from the area of the hibernaculum they will not return until the fall. The 2 females (of 17) that did mate again could be attributable to the average, low proportion of females that remate in the field (Whittier and Crews, 1986a). Although most of the tetracaine-treated females failed to mate again, they also did not exhibit overt, nonreceptive behavior (no quick zigzag movements nor barrel-rolling), as did many of the mated controls, perhaps indicating some lessened sensation or response to copulation.

Previous studies with the red-sided garter snake demonstrate that the administration of tetracaine or lidocaine to the cloaca of female garter snakes can block the neural transmission of copulatory stimuli and thus inhibit certain physiological responses (Mendonça and Crews, 1990); that is, females treated with local anesthetic to desensitize the cloaca do not exhibit the postmating estrogen surge observed in saline-injected females nor do they undergo subsequent ovarian recrudescence. Further, tetracaine-treated mated females also lack the significant increase in neural activity, as measured by the 2-deoxyglucose method in the preoptic area of the hypothalamus exhibited immediately after mating by control females (M. Mendonça and D. Crews, unpublished data).

While mating might initiate a fairly rapid (within a few hours), neurally mediated female response, there likely are longer term or longer lasting signals present which exceed the duration of the local anesthetic and indicate to the female that she has been mated. For example, male-produced substances can be introduced into the female reproductive tract during mating (Craig, 1967; Mane *et al.*, 1983; Gromko *et al.*, 1984; Cordero, 1995). Prostaglandins have been measured in male ejaculate, but also arise from the mechanical stimulation of the reproductive tract (Kelley, 1981). Prostaglandins have been shown to have central nervous system effects (Rodríguez-Sierra and Komisaruk, 1982) and, depending on the organism, can have either stimulatory or inhibitory effects on receptivity (stimulatory effects: externally fertilizing fish, Stacey, 1987; rat, Rodríguez-Sierra and Komisaruk, 1977; hamster, Buntin and Lisk, 1979; inhibitory effects: guinea pig, Irving *et al.*, 1981; green anole lizard, Tokarz and Crews, 1981). Injection of PG-F<sub>2α</sub> significantly decreases attractivity and receptivity in unmated, female red-sided garter snakes (Whittier and Crews, 1986b); attractivity is not affected when injected females are tested 30 min after treatment, but is reduced when females are retested 24 h later. Therefore, unmated females injected with PG-F<sub>2α</sub> may not immediately lose their attractivity because they have not been exposed to the putative male mating pheromone.

Mating induces both a short-term and a long-term inhibition of attractivity that have different underlying mechanisms. Circulating concentrations of PG-F<sub>2α</sub> are significantly elevated in female red-sided garter snakes immediately after mating and remain high for at least 24 h (Whittier and Crews, 1989, and the present study). Thus, the finding that attractivity is retained for up to 24 h after mating in locally anesthetized females suggests that the mating-induced loss of attractivity may be due to the production of prostaglandin in response to the sensation of mating. The subsequent loss of attractivity that was observed 48 h after mating even in anesthetized females raises the possibility that the long-term response may be due to PG deposited by the male in the reproductive tract of the female rather than an endogenous response to mating.

Unlike its delayed effect on attractivity, injection of PG-F<sub>2α</sub> rapidly decreases receptivity in newly emergent female red-sided garter snakes (Whittier and Crews, 1986b). In the present study, mating rapidly and effectively inhibited further receptivity even in females whose cloaca area had been locally anesthetized, despite their continued attractivity. This sug-

gests that the cue that stimulates the change in the female's receptivity is perceived despite blocking the neural transmission of copulatory stimuli. Therefore, it appears that central sensation of mating, which is apparently crucial in initiating the postcopulatory estrogen surge (Mendonça and Crews, 1990), is not the major contributor in inhibiting receptivity. It may be that the level of PGs produced by mechanical stretching of the tract or contributed by male semen is sufficient to inhibit receptivity. Lower circulating concentrations of PGs may be effective in inhibiting receptivity, while higher levels are necessary to affect attractivity. As noted previously, mated, anesthetic-treated females did not exhibit the full range of unreceptive behaviors and it may be that neural stimuli are a necessary component for full expression of unreceptive behaviors. Taken together these results indicate that the loss of attractivity and receptivity following mating in the red-sided garter snake is due to combined effects of a mating pheromone and a physiological, neurally mediated response to the sensation of stimuli associated with the act of mating.

## ACKNOWLEDGMENTS

We thank Andrea Gore, Jon Sakata, and Rick Shine for their comments on the manuscript. We also thank Robert Mason, Alan Tousignant, and Joan Whittier for their assistance, companionship, and discussions while in the field. Joan Whittier graciously ran preliminary prostaglandin assays for us. We are also grateful to the Manitoba Department of Natural Resources for issuing us the necessary permits for this work. We especially thank Merlin Shoesmith and William Koonz of Manitoba Department of Natural Resources for all their able and much appreciated assistance in this research. This research was supported in part by NIMH NRSA 09831 (M.T.M.) and NIMH Training Grant MH18837 and Research Scientist Award 00135 (D.C.).

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