



ARTICLES

Male red-sided garter snakes, *Thamnophis sirtalis parietalis*, determine female mating status from pheromone trails

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Recently mated females of some species experience a refractory period following mating during which a physiological change or a mating plug prevents remating. Males that determine female mating status from a distance will minimize time spent searching for mates by avoiding unavailable females. Female red-sided garter snakes experience a refractory period after mating due to a mating plug. Female garter snakes leave pheromone trails, which males detect and follow to find potential mates. We investigated the ability of male garter snakes to determine the mating status of females based solely on these pheromone trails. Males were given a choice between following two trails on a Y maze to examine whether they discriminate between mated and unmated females, whether this discrimination is based on changes in the sex-attractiveness pheromone or the copulatory fluids, and whether they continue to discriminate after the mated females have lost their mating plug. We found that male garter snakes discriminate between the trails of mated and unmated females. This discrimination is based on the presence of a copulatory pheromone, rather than changes in the sex-attractiveness pheromone. The duration of the copulatory pheromone coincides with the duration of the mating plug. Thus, male garter snakes are able to minimize time spent searching for unavailable females by determining female mating status from a distance.

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In choosing a mate, an individual must evaluate a variety of criteria. A potential mate minimally must be of the appropriate species, gender and age class. Once these basics are determined, other criteria may be used to determine the quality of one potential mate in comparison to others.

One potentially critical criterion in choosing among potential mates is whether they have recently mated (Wittenberger & Tilson 1980; Parker et al. 1997). Recently mated females of some species experience a refractory period after mating, during which they are unwilling or incapable of remating. This refractory period may be due to a physiological change (e.g. Nilson & Andrén 1982; Baer et al. 2001) or a physical change such as a mating

plug (e.g. Devine 1977; Ross & Crews 1977, 1978; Orr & Rutowski 1991; Groot & Smid 2000; Shine et al. 2000b). Males that continue to court females during the refractory period will suffer the cost of searching and courtship without the benefit of mating. This cost may be manifested as exposure to predators, energetics of locomotion, or missed mating opportunities with other individuals. Thus, it is advantageous for individuals to determine the mating status of potential partners from a distance, thereby minimizing the cost of searching. Males that determine whether females have recently mated will be able to avoid refractory females and direct courtship efforts elsewhere, where they may be more effective.

In some species in which females experience a refractory period, males are able to discriminate between mated and unmated females from a distance. In the mustard white butterfly, *Pieris napi*, females only remate after 4–6 days. During mating, males transmit an antiaphrodisiac pheromone to females, which makes other courting males quickly abandon them (Andersson et al. 2000). In the orange tip butterfly, *Anthocharis cardamines*, females reject

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additional courtship after mating by elevating their abdomen. Males court virgin females more intensely than mated females, although it is unclear how they discriminate (Wiklund & Forsberg 1985). In the Australian swallowtail, *Cressida cressida*, males deposit a large mating plug that is visible from a distance and signals female mating status to subsequent males (Orr & Rutowski 1991). In the common green capsid, *Lygocoris pabulinus*, a mirid bug, males also occlude female reproductive tracts with a mating plug. Male green capsids in a Y maze olfactometer do not prefer the odour of recently mated females to a blank (Groot & Smid 2000). Examples of male vertebrates determining from a distance whether females are in a postmating refractory period are limited to the brown lemming, *Lemmus sibiricus* = *trimucronatus*, and a variety of lizards. In the brown lemming, females become aggressive to conspecifics after mating, and do not accept further courtship. Males in a Y maze olfactometer prefer the odour of unmated to mated females (Huck et al. 1984). In some lizards, changes in throat colour indicate when a female is gravid. In most cases, these colour changes have been interpreted as aids in sex recognition, reducing male aggression towards gravid females that might otherwise be mistaken as intruding males, and as cues that serve as a visual signal of courtship rejection by gravid females (Watkins 1997; reviewed in Cooper & Greenberg 1992).

In the garter snakes *Thamnophis butleri*, *T. radix* and *T. sirtalis*, males deposit mating plugs in females that prevent them from remating for 2–4 days. These recently mated females are courted significantly less than unmated females (Devine 1977; Ross & Crews 1977, 1978). However, it is not known whether males can detect recently mated females from a distance.

The red-sided garter snake, *Thamnophis sirtalis parietalis*, is a model species for the study of vertebrate reproductive behaviour and pheromonal communication (Mason 1993). In Canada, individuals of this species hibernate in limestone sinkholes for 6–8 months of the year (Gregory 1977b). These populations mate in the vicinity of these limestone 'dens' upon emergence in late April and May. The mating season lasts only 6 weeks (Gregory 1974; Shine et al. 2001a), and predation near the dens is high, making the search for mates costly (Aleksiuk 1977; Gregory 1977b; Shine et al. 2001b). On average, male snakes emerge before females and remain in the vicinity of the den for 7 days, whereas females emerge over the entire breeding season and leave the vicinity of the den after only 1–3 days (Gregory 1974, 1984; Shine et al. 2001a). This pattern of activity results in strongly male-biased sex ratios at the den, where males may outnumber females by more than 15 to one (Gregory 1984; Shine et al. 2001a).

Female garter snake skin lipids containing a sex-attractiveness pheromone are passively deposited as snakes move over the substrate, and these pheromone trails are detected and followed by males in search of females (Ford 1981; Ford & Low 1984; Ford & O'Bleness 1986; Mason 1992; LeMaster et al. 2001). Upon finding a female, a male competes with an average of three other males (but as many as 100) for the opportunity to mate (Joy & Crews 1985; Shine et al. 2001a). After a female mates, her cloaca is blocked by a gelatinous mating plug that physically

prevents mating, and the area around her cloaca is coated in copulatory fluids that contain the copulatory pheromone (Shine et al. 2000b). Males determine the mating status of females by tongue flicking in the vicinity of the cloaca and quickly depart if they detect the copulatory pheromone (Devine 1977; Ross & Crews 1977, 1978; Shine et al. 2000b). The plug disintegrates approximately 2 days after mating (Shine et al. 2000b), and females are then likely to remate. Rates of remating in the wild are not known for this population, but in outdoor enclosures 50% of females remate (J. Pasteris & R. T. Mason, unpublished data). In other populations up to 75% of litters are multiply sired (McCracken et al. 1999).

In this system it is important for males to discriminate between mated and unmated females. Courtship of a recently mated female is futile due to the presence of a mating plug. The cost of searching for females can be high, but is reduced by the use of pheromone trails that help males find females more efficiently. However, it is unknown whether males can also use these pheromone trails to discriminate between mated and unmated females, thus avoiding the potentially costly search for unavailable females.

We set out to answer the following three questions concerning male garter snakes' abilities to discriminate between the pheromone trails of females. First, can male red-sided garter snakes discriminate between the trails of mated and unmated females? Second, is the ability to discriminate between mated and unmated females based on a pheromone in the copulatory fluids or changes in the sex-attractiveness pheromone? Finally, is this ability to discriminate between mated and unmated females due to a long-term signal, or does this signal deteriorate after a few days when the mating plug disintegrates?

METHODS

We collected 157 male and 80 unmated female garter snakes from a hibernaculum near Inwood, Manitoba, Canada (50°31.58'N, 97°29.71'W) on 11 and 12 May 2002, at the peak of the breeding season. Female mating status was determined by examining newly emerged females for a pronounced mating plug. All collected females were reproductively mature (>50 cm snout-to-vent length, Shine et al. 2000a). Males and stimulus females were housed separately during the experiment in outdoor arenas (1 × 1 × 1 m). Males were allowed to court, but not mate with, females that were not included in this study. Males were housed with females in a sex ratio equivalent to that seen in the field. All animals were released within 10 days to the den site from which they were collected.

We tested male trailing preferences using a Y maze. The maze was constructed of wood and consisted of a base arm (45 × 13.5 cm) and two diverging arms (100 × 13.5 cm) at a 135° angle to the base arm. Each arm had two rows of pegs (6 cm tall) to provide push points for the snake. The rows were 6.5 cm apart, and the pegs within each row were separated by 7.3 cm. The surface of the maze was covered with butcher paper and the pegs were covered

with sections of plastic drinking straws, all of which were replaced after each trial to remove any pheromones. The maze was elevated 90 cm off the floor to discourage subjects from leaving the maze. The maze was built without walls because these snakes are strongly thigmotactic (Ford 1986; Costanzo 1989). Trials were conducted between 0900 and 1600 hours, which coincided with the peak of daily mating activity. Each male was tested only once and then released at the point of capture.

We made trails on the paper by gently holding stimulus female snakes at the neck and just behind the cloaca, and wiping the ventral surface of the stimulus animal up one side of the base arm and across to the opposite arm of the Y maze (Fig. 1). Females were selected at random from the pool of 80 females. Assignment of the trails to the arms was randomized by coin flip. The trails crossed at the junction of the arms so that the subject snake encountered each trail before proceeding up an arm. The stimulus females were size-matched in all trials involving a choice between two females because males prefer larger females (Shine et al. 2001c) and can judge female size on the basis of skin lipid pheromones alone (LeMaster & Mason 2002; Shine et al. 2003). Females were size-matched by selecting the female nearest in length and mass to the first, randomly selected female.

We began each trial by randomly selecting a male and placing him into a box (31 × 18 × 9 cm) at the start of the Y maze. Test males were allowed to leave the box of their own accord through a small hole. Males typically proceeded up the base arm of the maze and down the left or right arm. Trials were not scored when the male failed to

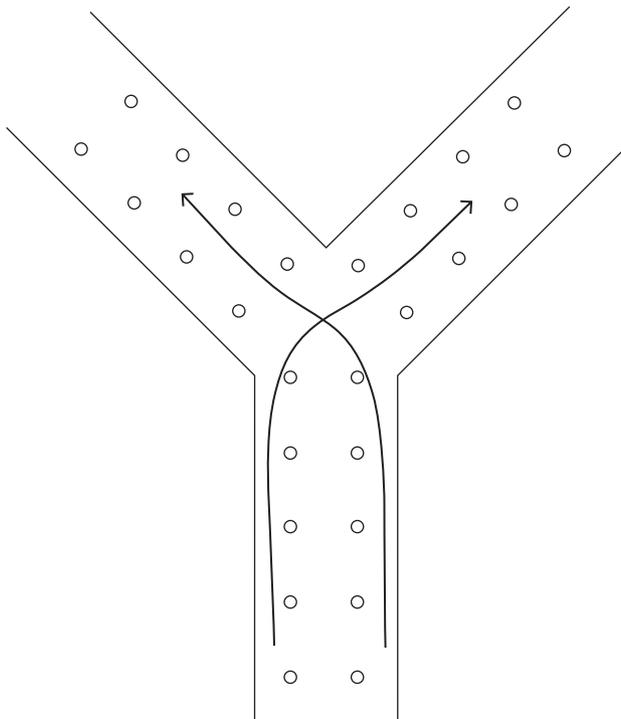


Figure 1. Overhead view of the Y maze. Note that the trails cross at the junction so that the subject male encounters both trails. Modified from LeMaster & Mason (2001).

show a pronounced trail contact response (a pause, lowering of the head, and tongue-flicks directed at the substrate; Brown & MacLean 1983; LeMaster & Mason 2001). For a trial to be scored, the snake's entire head had to pass a mark 30 cm up either of the arms. All decisions on whether to score a trial were made by a second observer who was blind to the treatments. Statistical significance in all experiments was determined with a two-tailed binomial test. Chi-squared tests were used to compare proportions between selected pairs of trials.

To test for a bias in the Y maze, 10 males were offered the choice between two blank arms. Trail contact responses were not required because no trails were present.

Experiment 1: Can Males Discriminate between the Trails of Unmated and Recently Mated Females?

Our first experiment investigated whether males prefer to follow trails of unmated females to those of recently mated females. We compared male preferences for the trail of an unmated female to a blank arm, a mated female to a blank arm, and an unmated female to a mated female. All mated females had mated with males that were not used in this study less than 60 min before the laying of the trail. All female cloacae were occluded with recently deposited mating plugs. Twenty-one unique males were used in each set of comparisons for a total of 63 males.

Experiment 2: Source of the Signal

Our second experiment examined whether the change in female attractiveness after mating was due to the copulatory pheromone in the copulatory fluids or to changes in the chemistry of the sex-attractiveness pheromone in the skin lipids. Males were given a choice between following a trail left by the midbody of a recently mated female versus one left by her cloacal region. These females had mated less than 60 min before the trial. The midbody trail was laid using the ventral surface of the female from the neck to just anterior to the cloaca. The cloacal region trail was laid using an equivalent length of the ventral surface that included the cloacal region. To control for potential differences in preference between the two regions not due to the copulatory fluids, we also compared male preferences for the midbody versus cloacal regions of mated females that had lost the mating plug. Twenty-one unique males were used in each set of comparisons for a total of 42 males.

Experiment 3: Duration of the Signal

Our third experiment investigated whether males continued to recognize mated females after the females had lost their mating plug. We compared male preferences for the trails of unmated females to those of mated females that had lost the mating plug and were able to remate. All females that had lost their plug did so between 2 and 4 days of mating. In addition, we compared male

preferences for the trail of a recently mated female to that of a mated female that had lost the mating plug. Recently mated females had mated less than 60 min before the trial. Twenty-one unique males were used in each set of comparisons for a total of 42 males.

RESULTS

When given the choice between two blank arms, six of 10 males chose the left arm, and four chose the right. There was no preference for either arm of the Y maze ($P = 0.75$).

Experiment 1: Can Males Discriminate between the Trails of Unmated and Recently Mated Females?

Males preferred the trail of unmated females to blank arms and to trails of recently mated females (Fig. 2). Almost all males followed the trail of an unmated female over a blank arm ($P = 0.0002$). The majority of males followed the trail of an unmated female over a mated female ($P = 0.007$). Although not reaching the level of significance, there was a tendency for males to avoid mated females. The majority of males followed the blank arm over the trail of a recently mated female ($P = 0.078$). This was significantly different from their response to the unmated female ($\chi^2_1 = 16.70, P < 0.0001$).

Experiment 2: Source of the Signal

The majority of males preferred a trail left by the midbody of a recently mated female over a trail from the same female's cloacal region ($P = 0.027$; Fig. 3). This preference for midbody over cloacal regions was no longer significant after the mating plug had disintegrated. Fewer

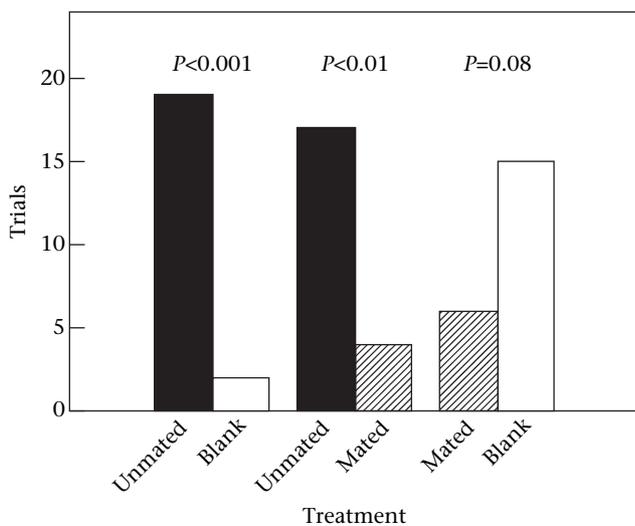


Figure 2. Number of trials in which males ($N = 21$) chose to follow the trail of an unmated female versus a blank arm, an unmated female versus a mated female, and a mated female versus a blank arm.

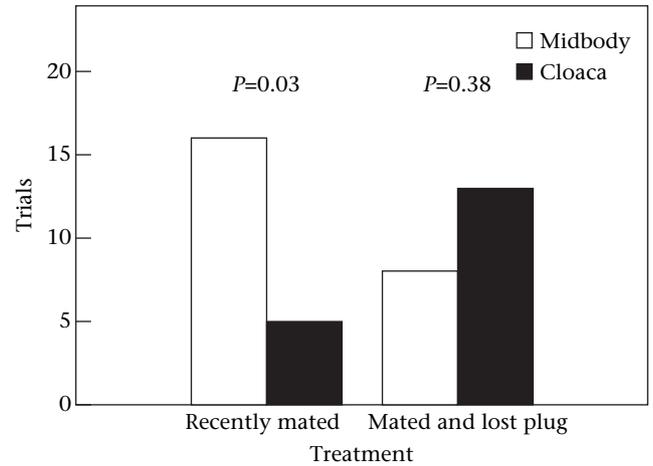


Figure 3. Number of trials in which males ($N = 21$) chose to follow trails from the midbody versus the cloacal region of a recently mated female, and trails from the midbody versus cloacal region of a female that had mated but had subsequently lost the copulatory plug.

than half of the males followed a trail left by the midbody of a mated female that had lost her mating plug over a trail from the same female's cloacal region ($P = 0.38$). These proportions were significantly different ($\chi^2_1 = 6.22, P = 0.013$).

Experiment 3: Duration of the Signal

There were no preferences for unmated females over mated females that had lost their mating plug (Fig. 4). About half of the males followed the trail of an unmated

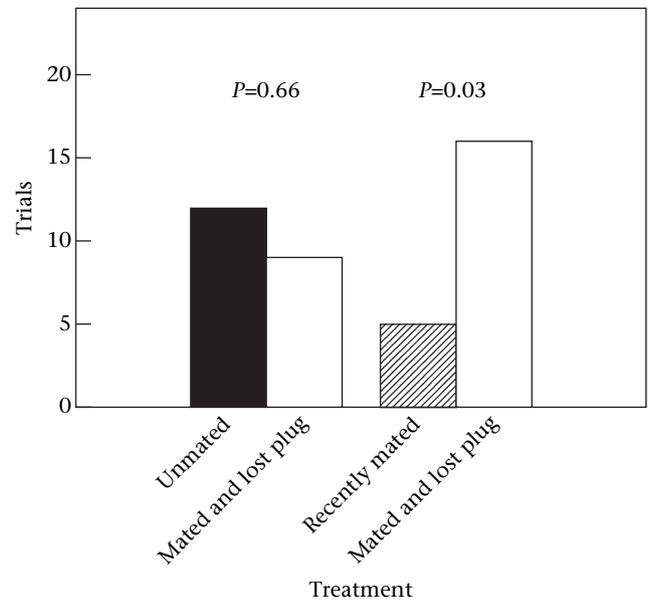


Figure 4. Number of trials in which males ($N = 21$) chose to follow trails from an unmated female versus trails from a female that had mated but had subsequently lost the copulatory plug, and a recently mated female versus a female that had mated but had subsequently lost the copulatory plug.

female over that of a mated female that had lost her mating plug ($P = 0.66$). In addition, males preferred to follow the trail of mated females that had lost their mating plug over recently mated, plugged females. The majority of males followed the trail of the mated female that had lost her mating plug over a recently mated, plugged female ($P = 0.027$). These proportions were significantly different ($\chi_1^2 = 4.84$, $P = 0.027$).

DISCUSSION

Male garter snakes are known to discriminate between mated and unmated females after they have initiated courtship. By tongue flicking in the vicinity of the cloaca, males can detect the copulatory pheromone and will quickly depart if the female has recently mated (Devine 1977; Ross & Crews 1977, 1978; Shine et al. 2000b). However, by the time the male is able to investigate a female's cloaca, he may have already invested considerable time and energy in searching for her. The present research is the first evidence that male garter snakes use pheromone trails to determine female mating status from a distance, and thus avoid a potentially costly search for a mated female.

Our results show that males are able to discriminate between the trails of mated and unmated females. Males follow the trails of unmated females and tend to avoid the trails of recently mated females. The status of a recently mated female is determined via the copulatory pheromone, not changes in the skin lipids. In addition, males do not discriminate between unmated females and mated females that have lost their mating plug.

Ross & Crews (1977) found that exposure to mated females reduced courtship behaviour of males, implying a courtship-inhibiting function of the copulatory pheromone. Devine (1977) reported that unsuccessful male *T. sirtalis* and *T. butleri* in a mating ball quickly disperse, implying a repellent nature of the copulatory pheromone. Interestingly, males in the present study tended to avoid the trails of recently mated females, although not significantly so. However, male responses to the trails of mated and unmated females were significantly different. We expected that males would follow mated females over a blank arm because there is an 8–9% chance that a plug will not be deposited during mating (Shine et al. 2000b). However, the fact that males tended to avoid the copulatory pheromone may indicate that the pheromone functions as more than just an inhibitor of courtship behaviour. Rather, the copulatory pheromone may actually repel males. Further research is needed to confirm the repellent nature of the copulatory pheromone.

The loss in attractiveness of female garter snakes after mating is due at least primarily, if not exclusively, to the copulatory pheromone. Male garter snakes preferred to follow the trail left by the midbody of a recently mated female over a trail from her cloacal region. This is not an artefact of a general preference for midbody trails because after the mating plug had disintegrated, this preference was no longer seen. In addition, there are no differences between the composition of skin lipids before and after

copulation (R. T. Mason, unpublished data). These results corroborate the earlier finding that the decline in attractiveness associated with mating is due to a copulatory pheromone contained in the copulatory fluids, and not due to changes in skin lipids (Shine et al. 2000b).

After the mating plug disintegrates, mated female garter snakes regain their attractiveness to a level that is not significantly different from that of unmated females. Males in the present study preferred to follow the trail of mated females that had lost their plug to that of recently mated, plugged females. This may simply be due to avoidance of recently mated females. However, it seems more likely that males are attracted to females that have lost their plug, because males showed no significant preference for unmated females over mated females that had lost their plug. Mated females that have lost their plugs are available to remate, and will likely do so (McCracken et al. 1999; J. Pasteris & R. T. Mason, unpublished data). There is a correlation between the duration of the plug and the duration of the copulatory pheromone. However, this correlation does not imply that the copulatory pheromone is contained in the plug. Rather, the copulatory pheromone is contained in the copulatory fluids (Shine et al. 2000b). The copulatory pheromone indicates recent mating and should only be detected as long as the plug remains effective. If copulatory pheromone levels decline, or the pheromone decays, below the threshold of detection before the plug becomes ineffective, selection would favour males with lower thresholds of detection. Conversely, if the copulatory pheromone lasts longer than the plug, selection would favour males with a higher threshold of detection. The available data support the hypothesis that males detect the copulatory pheromone about as long as the mating plug is effective. For example, after 36–48 h, 27% of mated females are courted again (Ross & Crews 1977). During the same period, 21–45% of mated females lose their mating plugs (Shine et al. 2000b). More work is needed to verify this correlation between the duration of the pheromone and the duration of the plug.

Males of some species avoid courting mated females to reduce the risk of sperm competition rather than to avoid a female refractory period (e.g. Happ 1969; Krames & Mastromatteo 1973). The duration of the copulatory pheromone implies that this is not the case in red-sided garter snakes. If the males were avoiding sperm competition, we would expect them to continue to discriminate against mated females after the mating plug disintegrates. Rather, males do not significantly discriminate against mated females once the females lose their mating plug. Thus, males are probably not discriminating against mated females strictly to avoid sperm competition. Males that can mate should do so, even if it means sharing paternity with another male. Only when the female is entirely unable to mate (while she is plugged) should the male not invest in courting her.

It is not clear from this study whether the copulatory pheromone is a product of the male or the female, or both. Ross & Crews (1978) found that vasectomizing male garter snakes before mating blocked production of the copulatory pheromone, but noted that this did not exclude the

possibility of contribution by the female. In this population, females benefit from the pheromone because it allows them to avoid further courtship after mating and to escape quickly to the summer feeding grounds, or at least to the aspen groves near the den where the concentration of males is significantly reduced (Shine et al. 2000a, 2001a). Although females will probably mate again, they may be more able to exercise mate choice in these situations. If females produce or contribute to the pheromone, then the decay of the signal after the plug decays is easily explained: females would not want to deter suitors from competing for them in the aspen groves. If the males produce the signal, then the decay may be due to a trade-off between volatility of the pheromone (reaching many males) and duration. In addition, it is difficult to explain why mating males would advertise the unavailability of the females with which they mated, even though the mating plug would prevent the females from remating. In the absence of evidence to the contrary, we might predict that mating males would try to draw their competitors towards mated females, distracting them from the remaining available females. Perhaps the copulatory pheromone is not adaptive to the signallers, and instead is an exaptation by the receivers (sensu Gould & Vrba 1982). The second males may simply be detecting the presence of sperm or seminal fluid left by the mating males. Further research is needed on the chemical identity of the copulatory pheromone.

If mating males do produce the copulatory pheromone to lower the chances that a female will remate, then they essentially sequester females with which they mate without having to miss opportunities to mate with other females (Devine 1984). This sequestering of females is expected to evolve in populations with strongly male-biased sex ratios (Dewsbury 1982). The lack of a mating plug in other snake species may be explained by the less male-biased sex ratios in those species, where competition for females is less intense. Only eight snake species are known to have mating plugs, and these species tend to mate in large aggregations, although the reproductive behaviour of most snake species is not known (Gregory 1977a; Devine 1984). A more thorough survey of snake species for the presence of mating plugs would add to our understanding of the evolution of this trait.

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