



Chastity belts in gartersnakes: the functional significance of mating plugs

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Male red-sided gartersnakes (*Thamnophis sirtalis parietalis*) deposit a thick gelatinous plug that occludes the female cloaca after copulation. Previous workers have interpreted the plug as a sexually-selected adaptation to (1) physically prevent re-mating by the female, and/or (2) provide pheromonal cues to discourage courtship by rival males or to decrease receptivity by females. Our data support the former hypothesis, but not the latter. Plugs serve as effective physical barriers to additional copulation for <72 h, but this is long enough for most females to become unreceptive, and/or disperse from the mating aggregation. Experimental removal of plugs immediately after copulation results in some re-mating by females, but plug removal several hours later does not rekindle sexual receptivity. Contrary to previous work, our experiments show that fluids associated with copulation (rather than the plug *per se*) are responsible for the rapid decline of male interest in mated females. Thus, the plug's primary function is to physically prevent matings rather than as a source of pheromonal cues to manipulate the behaviour of females or rival males. Plug mass is determined not only by a male's body size, but by his prior mating history (plug mass decreases with repeated mating) and by the size of his partner (males allocate larger plugs to larger females). Gartersnakes are unusual not only in their production of mating plugs, but also in their brief duration of copulation compared to other snakes. Mating plugs may have evolved in gartersnakes to reduce mating times, because of the extremely high 'opportunity cost' of prolonged mating to a male gartersnake in a mating aggregation.

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INTRODUCTION

The battle among rival males for reproductive success does not end with copulation, because females of many species will re-mate with other males (e.g. Parker, 1970b; Smith, 1984; Birkhead & Moller, 1998). In many circumstances, a male's chances of fathering a female's offspring may be increased if he delays or prevents her from re-mating (e.g. Birkhead & Moller, 1998). For example, re-mating may be precluded by mate-guarding, prolonged copulation, or physical removal of the female from the mating arena (e.g. Parker, 1970a; Andersson, 1994; Birkhead & Moller, 1998). One effective male tactic is to occlude the female's reproductive tract after copulation (i.e. produce a 'chastity belt'), such that she is physically incapable of additional copulation for some period of time. Such structures have evolved independently in many lineages, including nematodes (Barker, 1994), spiders (Masumoto, 1993), crustaceans (Diesel, 1990), and several types of mammals (e.g. squirrels, bats, primates: Koprowski, 1992; Tidemann, 1993; Dixson & Mundy, 1994). The exact form of the 'chastity belt' varies among lineages: for example, male damselflies leave part of their penis in place after intromission, whereas some mammals produce gelatinous mating plugs.

Although reptiles have attracted less research than several other kinds of animals in this respect (Andersson, 1994; Olsson & Madsen, 1998), there is good evidence that female reptiles often mate with many partners (e.g. Madsen *et al.*, 1992) and that various attributes of reptilian mating systems reflect adaptations of males to delay or prevent re-mating by their partner (Olsson & Madsen, 1998). Among squamate reptiles (lizards and snakes), there are many cases of prolonged copulation and mate-guarding (e.g. Luiselli, 1995). Mating plugs appear to be less common, but have been reported in natricine snakes (Devine, 1977), colubrine snakes (Vitt, cited in Devine, 1984) and lacertid lizards (de Bosch, 1994). In other reptilian lineages, the induction of oviductal muscular contraction by semen may function as a mating plug (Nilson & Andren, 1982), possibly to retard sperm leakage rather than to prevent multiple copulation (Stille, Madsen & Niklasson, 1986; Almeida-Santos & Salomao, 1997).

Most research on mating plugs in reptiles has focused on a single population of a single species. Male red-sided gartersnakes produce a very large gelatinous plug that completely occludes (and often, substantially distends) the female's cloaca after mating (Fig. 1). The plug has been interpreted as a sexually-selected adaptation to reduce sperm competition by preventing re-mating of copulated females (e.g. Devine, 1977, 1984; Ross & Crews, 1977, 1978), but authors have disagreed as to the mechanism involved. Either the plug may function as a physical block, or it may serve as a source of pheromonal cues that reduce female attractivity and receptivity



Figure 1. The cloacal region of a female gartersnake (*Thamnophis sirtalis parietalis*) soon after copulation. The female's cloaca is distended by two mating plugs, the ends of which are visible in the photograph. Thus, this female has mated at least twice.

(see above references). The only empirical support for plug function comes from the observation that female attractivity declines after mating, and that removal of the plug can reduce the magnitude of this decline (Devine, 1977; Ross & Crews, 1977, 1978). Because female attractivity and receptivity in this species show different patterns, and are determined in different ways (Ross & Crews, 1978), it may be unwise to assume that a decline in attractivity will reduce a female's probability of re-mating. Also, previous experimental work has not clearly distinguished pheromonal effects of the plug itself, from that of the fluids associated with copulation (Devine, 1984). Thus, we gathered data to clarify the effect of the mating plug on the incidence of re-mating by females, as well as on their attractiveness to males. We also took the opportunity to quantify other aspects, such as the determinants of male investment into mating plugs.

MATERIAL AND METHODS

Study area and species

Red-sided gartersnakes (*Thamnophis sirtalis parietalis*) are small (adult males average 45 cm snout-vent length [SVL]) nonvenomous colubrids that are widely distributed

across North America (e.g. Rossman, Ford & Seigel, 1996). In one part of this extensive range, in southern Manitoba, the snakes overwinter in huge communal hibernacula (Gregory, 1974, 1977; Gregory & Stewart, 1975). Mating occurs immediately after emergence in spring, with most females mating and then dispersing soon after they emerge. In contrast, males remain near the den and continue to court receptive females (Gregory, 1974, 1977; Gregory & Stewart, 1975). We studied the snakes at these communal dens near Chatfield, Manitoba (50°44'N 97°34'W; see Mason & Crews, 1985 for details of locality and dens) in May of 1997, 1998 and 1999.

Influences on plug production

To clarify factors that might influence the production of plugs by mating males, we examined females immediately after the termination of copulation to determine whether or not a plug had been deposited. Plugs were removed from mated females by gentle manual eversion. Data on the presence and size (mass) of plugs were available from two sources:

- (i) mated pairs found *in copula* in the field (each pair was kept separately in a cloth bag until mating had concluded); and
- (ii) pairs that mated in outdoor arenas (1 × 1 × 0.8 m in size) as part of our experimental studies on the determinants of male mating success (Shine *et al.*, 1999a).

Because a male's prior mating history could plausibly affect plug production, we carried out trials to assess this possibility. Each male was placed separately in an outdoor arena with an unmated female (her status was determined by the lack of a mating plug). If mating did not occur within 60 min, the female was removed and replaced with another unmated female. Each mated female was taken from the arena as soon as the pair had separated after copulation, and her mating plug (if present) was removed and weighed. Another unmated female was then added to the arena, and this procedure was continued over 3 days.

Duration of retention of plugs

We kept females after they were found mating (either in the field or in laboratory arenas), and checked them regularly for the presence of mating plugs. Each check consisted of partial eversion of the cloaca and, if a plug was present, gentle pressure was applied laterally to see if it was still attached to the cloacal walls. The firmness of attachment was tested quantitatively in some of these trials, by pushing against the emergent face of the plug with a circular plastic probe 4 mm in diameter (the tip of a syringe-needle cover). The plug was pushed downwards (i.e. into the cloaca) to simulate a male's hemipenis; the force applied each time was 300 g (as measured on a spring balance attached to the probe). We recorded if the plug was detached by this procedure. We carried out these trials on two sets of females: one group kept in outdoor arenas containing males each day (simulating warm weather and frequent courtship) and one where the females were kept in cloth bags at approximately 15°C (simulating cool weather with little snake activity).

Effects of plug removal

In order to clarify the function of the plug, we examined sexual receptivity of mated females from which plugs had been removed, compared to those in which the plug remained in place. Two sets of these experiments were carried out, differing in the duration of time after copulation at which the plugs were removed.

- (a) Immediate removal. We took the recently-mated females from the 'repeated male mating' experiment (above), and placed each one in an arena with a group of 20 adult male snakes. The males were collected from courting groups within the den on the day of the trial. At 15-min intervals after the female was introduced, we scored the numbers of males courting these females (to measure attractiveness). To measure receptivity, the incidence of re-mating in these females was compared to that of other females that had been left with their mating plugs intact after copulation.
- (b) Delayed removal. The second set of experiments involved plug removal several hours after copulation, instead of <5 min after copulation as in the trials above. We used pairs of snakes that we collected when they were mating in the field around midmorning (0930 to 1210 h). The pairs were placed in cloth bags until the evening (1900 h), when mating plugs were removed from half of the females. The two groups of mated females (those with plugs intact, and those with plugs removed) were then placed in outdoor arenas with 20 reproductive males every day thereafter for 20 days. Each evening, we inspected the animals for old and new plugs, to quantify the timing and frequency of re-mating by females in the two groups.

Effects of the plug and associated fluids on female attractivity

This set of experiments was designed to clarify whether female attractivity was affected by the plug itself, *versus* the fluids (semen, etc.) present in the female's cloaca after copulation. To evaluate these possibilities, we first placed each unmated female with ten males, and recorded the number of males in active courtship (i.e. with bodies aligned to the female, and displaying caudocephalic waves: see Whittier, Mason & Crews, 1985). We then retested the same females 1 h later after (i) no treatment (controls); (ii) allowing the female to mate; (iii) no mating, but with a recently-deposited (<2 h) mating plug from another female rubbed vigorously over the test female's dorsum; and (iv) no mating, but with the fluids from a mating pair rubbed over the test female's dorsum. The latter procedure involved taking a pair of snakes *in copula* and rubbing their joined cloacas against the test female. Different males were used for each test.

RESULTS

Incidence of multiple-mating in field-collected animals

Most female snakes appeared to mate only once, at least while they remained in the vicinity of the den. Twelve of 14 radio-tracked females dispersed less than 2

days after emerging from the den (Shine *et al.*, in prep.), and females maintained in captivity rarely re-mated over this period. The only definite evidence of multiple mating under field conditions involves females that contained more than one mating plug (see Fig. 1). This situation was recorded in three of 44 females examined in 1997, and four of 52 examined in 1998. Two of these animals contained at least three plugs when captured. Thus, we can be confident that at least seven of 96 females (7%) had mated twice in rapid succession. Many others may well have done so, but at intervals (>2 days?) great enough that they contained only a single mating plug when examined.

Influences on plug production

Frequency of plug production

Most but not all copulations resulted in deposition of mating plugs within the female's cloaca. This was true for matings in the field (plugs recorded in 45 of 49 females collected *in copulo*, =92%) as well as in the arenas (excluding repeat copulations by the same male, plugs from 78 of 86 matings, =91%). However, the 'repeated matings' experiment suggested that the proportion of matings that resulted in plug formation may have been affected by the male's prior mating history. Plugs were produced in 39 of 42 first-matings (=93%), but only 10 of 13 subsequent matings by the same males (77%). The difference between the two data sets does not reach statistical significance (Fisher's Exact Test, $P=0.14$), but warrants further study.

Mass of mating plugs

Data from mated pairs collected in the field show that larger males produced larger plugs (male SVL *versus* plug mass, $r=0.41$, $n=43$, $P<0.01$; male mass *versus* plug mass, $r=0.47$, $n=43$, $P<0.0002$), and larger females contained larger plugs (female SVL *versus* plug mass; $r=0.58$, $n=43$, $P<0.0001$; female mass *versus* plug mass; $r=0.62$, $n=43$, $P<0.0001$; see Fig. 2). A significant trend for larger males to mate with larger females (see Joy & Crews, 1988; Shine *et al.*, 1999a) confounds interpretation of these data. A correlation between plug mass and the size of one sex (either male or female) might indirectly generate a significant correlation between plug mass and body size in the other sex, even if there was no functional relationship in the latter case. However, partial correlation analysis (with male size held constant) reveals a significant correlation between female size and plug mass, independent of male body size (for SVL measures, $r=0.51$, $n=43$, $P<0.01$; for mass, $r=0.56$, $n=43$, $P<0.01$). The correlation between plug mass and male body mass also remained significant when female mass was held constant ($r=0.36$, $n=43$, $P<0.05$), although the pattern was weaker if SVL rather than mass was used as a measure of body size ($r=0.26$, $n=43$, $P>0.05$). Thus, female body size appears to be the main determinant of plug mass, but larger males also tend to produce larger mating plugs.

A male's mating history also influences the size of the plug that he produces. In the 'repeated matings' experiment, a comparison of plug mass between first and second matings by the same male reveals that plug mass declined with successive matings in six males, stayed the same in two, and did not increase in any of them. Even if we omit three additional cases in which a male produced a plug in his first

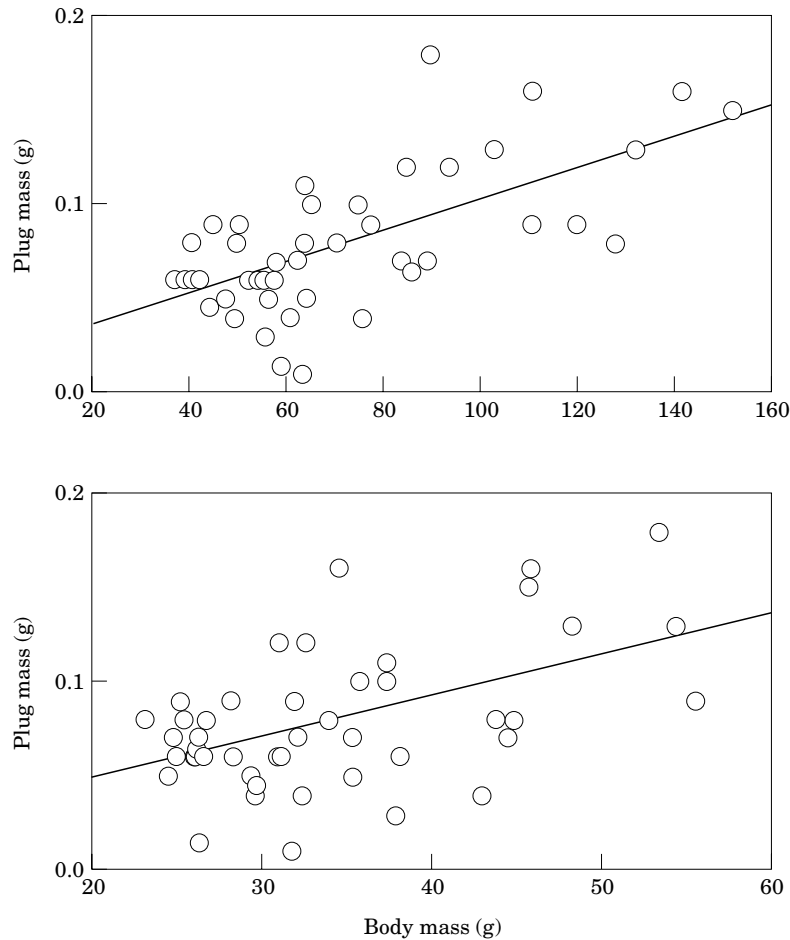


Figure 2. The mass of the mating plug is related both to the mass of the female gartersnake containing the plug (upper graph) and the male snake producing it (lower graph). See text for statistical analysis of these data.

mating but not his second, this outcome enables rejection of the null hypothesis of an equal probability of an increase or decrease in plug mass in a second mating (6 versus 0: binomial test, $P < 0.05$).

In another paper, we document bilateral asymmetry both in hemipenis size and in plug mass (right-hand-side hemipenes are larger, and deposit larger plugs: Shine *et al.*, 1999b). However, our extensive data from arena trials did not reveal any other significant correlates of plug mass. For example, the mass of the mating plug was not significantly associated with the temperatures of mating snakes, the duration of courtship prior to copulation, the numbers of rival males attempting to copulate with the female, or the duration of copulation prior to male-female separation (correlation analyses, $P > 0.05$ for all comparisons).

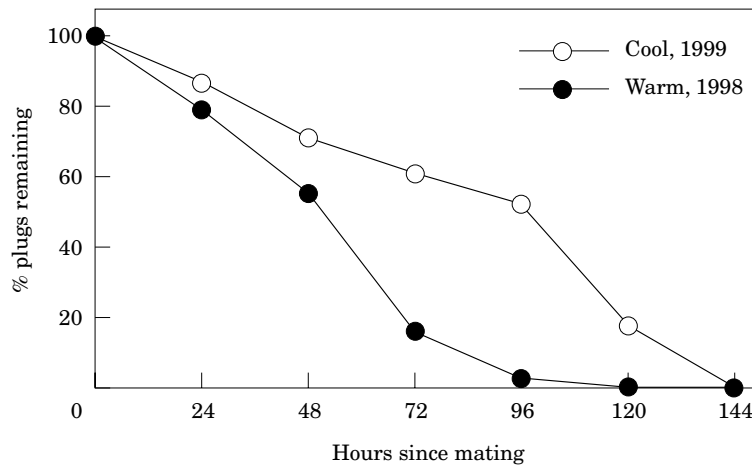


Figure 3. Duration of retention of copulatory plugs in female gartersnakes, as a function of the time since the plug was deposited. One group of females was kept in outdoor arenas with courting males ('warm' treatment, $n=46$ females) whereas the others were kept in cloth bags at 15°C ('cool' treatment, $n=31$ females).

Duration of retention of plugs

The copulatory plug rapidly hardens after deposition; 6 h after the snakes mated, we could not displace the plug with 300 g pressure (31 plugs tested). We doubt that a 30-g male gartersnake could apply such pressure with his hemipenis. The plugs remained solidly attached in most females for another 2 days, especially when the snakes were kept inactive and cool (Fig. 3). After that time, most plugs were lying free within the cloaca and readily fell out when the female was handled. Plugs expelled on testing were much smaller than those originally deposited (means of 0.02 *versus* 0.08 g: $F_{1,44} = 12.21$, $P < 0.0012$), indicating that the plug had been broken down prior to expulsion.

Effects of plug removal

Experiments in which we removed mating plugs from some females but not others, clarify the effect of the plug on female re-mating. The numbers of courting males declined precipitously after a female copulated, even if the plug had been removed. The numbers of courting males per female decreased from a mean of >15 of 20 (75%) males in active courtship immediately prior to mating, to <1 (5%) 15 min later. The numbers of actively courting males then continued to fall progressively over the next few hours (Spearman Rank Correlation Coefficient between mean number of courting males *versus* number of 15-min intervals since copulation: $n=9$ intervals, $\rho = -0.72$, $P < 0.05$).

Despite this relatively low level of male interest, however, 5 of 24 females from which the plug was removed <5 min after the termination of copulation mated again within 24 h. Thus, the incidence of re-mating was significantly higher in this group than in females from which plugs were removed several hours after copulation,

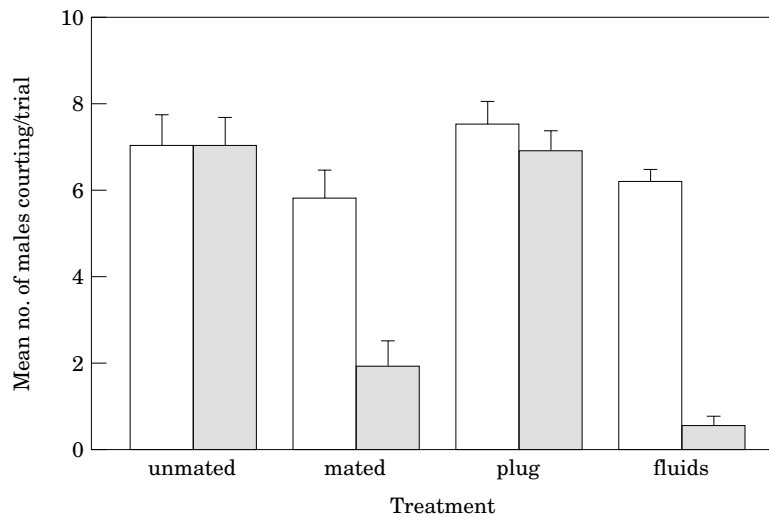


Figure 4. Effects of experimental treatments on the attractiveness of female gartersnakes, as evaluated by the number of males courting them in outdoor arenas. Each female was tested once, then retested after (1) no treatment (control); (2) mating; (3) not mating, but being smeared with a mating plug from another female; or (4) not mating, but being smeared with fluids from the cloacal regions of another pair of snakes *in copulo*. Female attractiveness was reduced by mating, and by the copulatory fluids, but was not reduced by the addition of plug material.

rather than immediately ($\chi^2 = 8.51$, 1 df, $P < 0.004$). Data on the latter group suggest that plug removal several hours after mating does not alter the rate of re-mating compared to that expected if the plug is left in place. Over a 20-day period following the initial copulation, re-mating was recorded in 3 of 46 of these 'delayed-removal' females, *versus* 5 of 45 females from which plugs were never removed ($\chi^2 = 0.16$, 1 df, $P = 0.69$). All re-matings within both groups occurred < 8 days after the initial mating.

Effects of the plug and associated fluids on female attractivity

Repeated-measures ANOVA confirmed that our experimental treatments modified the amount of courtship directed to females, and did so in different ways ($F_{3,34} = 21.95$, $P < 0.0001$; see Fig. 4). At the time of retesting, attractivity was not reduced in unmated (control) snakes, but declined dramatically in mated females (Fig. 4). Unmated females smeared with the copulatory plug remained attractive, whereas unmated females smeared with copulatory fluids no longer attracted significant courtship (Fig. 4). Post-hoc tests (Bonferroni–Dunn) showed that these differences among post-treatment groups were highly significant ($P < 0.0001$ for all above comparisons). Thus, the plug itself is not the source of pheromonal cues that discourage male courtship. Instead, these cues emanate from the fluids (semen, etc.) associated with copulation.

DISCUSSION

The mating plug of the red-sided gartersnake appears to be the largest such object produced by any reptile (Olsson & Madsen, 1998). Our experiments directly address only the current function of this structure, but may nonetheless clarify the selective forces responsible for the evolution of the plug. The recency of speciation within *Thamnophis*, and the fact that mating plugs are produced by some but not all species within the genus (Ford, in Rossman *et al.*, 1996), suggest that current utility may reflect evolutionary origins. Our study indicates that male investment into the plug is determined by several factors, that the plug functions as a 'chastity belt', and that this effect is due to physical blockage rather than pheromonal effects.

The mating plug in gartersnakes is basically a simple gelatinous blob, produced by the renal sex segment (Devine, 1977). Hence, absolute mass should provide a reasonable measure of male investment. It is not surprising to discover that plug mass is related to male body size. Larger males have larger kidneys and hemipenes (Shine *et al.*, 1999a) and hence are better able to produce the plug-forming material, and transmit it to the female during copulation. Similarly, the lack of correlation between plug mass and time *in copula* is consistent with our observations of the time course of transfer of plug material during copulation. We were able to observe this process closely on one occasion, when a male inserted his hemipenis between the female's body wall and transparent foodwrap that we had taped to her body. We were able to observe the flow of semen and plug granules (easily recognisable because they were more dense and opaque than the semen) from the hemipenis. The plug material was produced in a short period (2 min) at the conclusion of copulation, after a long (8-min) period of semen transfer.

The decrease in plug mass in copulations occurring in quick succession suggests that males may be unable to synthesise or transfer plug material rapidly for use in multiple matings in quick succession. This interpretation is consistent with the observations that some (but not all) male gartersnakes are reluctant to re-mate for several hours after a copulation (Ross & Crews, 1978; Whittier *et al.*, 1985) and that when they do re-mate, males generally alternate hemipene use (Shine *et al.*, 1999b). The other correlate of plug mass identified by our data is the size of the female with whom the male mates. There is no obvious physical reason for this relationship, and it may represent an 'investment' decision by the male. Larger females may warrant larger plugs for two reasons: their larger cloacas need a larger plug if the physical barrier to copulation is to be effective; and the higher litter sizes of larger females (e.g. Gregory, 1977; D. Lerner, pers. comm.) may provide an additional fitness benefit to any male that succeeds in delaying or preventing re-mating by that female. Male moths and fishes may similarly adjust ejaculate volume to the size of their partner (Gage, 1998).

The duration of retention of mating plugs within female garter snakes has been reported as ranging from 2 days to 2 weeks, depending on ambient temperatures (Devine, 1984). Our study suggests that the plug serves as an effective physical barrier for about 2 days. Female snakes kept in outdoor arenas lost their plugs sooner than those maintained inactive, under cooler conditions (Fig. 3).

Does the mating plug of *T. s. parietalis* function as a 'chastity belt'? This hypothesis has been suggested by others, but without any demonstration that the plug reduces the incidence of re-mating by females. Instead, this function of the mating plug has been inferred from the observation that mated females rapidly become less attractive

to males, and that this drop in attractivity is at least partly due to the presence of the plug or associated fluids (Ross & Crews, 1977, 1978; Devine, 1977). Our data support these earlier studies, in confirming the rapid drop in female attractiveness after mating. In our studies, this decrease occurred even in females from which plugs had been removed (contra Ross & Crews, 1977, 1978), but the decrease in both attractiveness and receptivity was not absolute. Thus, we recorded a significant (albeit low) incidence of re-mating in females from which the plug was removed immediately after copulation had finished. In contrast, removing the plug some hours after copulation had no such effect; females treated in this way were refractory to courtship regardless of whether or not the mating plug remained *in situ*.

The plug is not 100% effective. Re-mating by plugged females did occur, both in our experimental enclosures and in the wild (based on records of females with more than one mating plug *in situ*). However, re-mating was rare during the time period for which the plug remains in place. Because genetic data indicate that multiple mating is common in *T. sirtalis* (e.g. Schwartz, McCracken & Burghardt, 1989; Zweifel, 1998), the obvious inference is that females often re-mate after the initial plug has been lost. Much of this re-mating may occur after the females leave the vicinity of the den.

The mating plug may have multiple functions; for example, it may reduce leakage of sperm from the female tract for a day or so after mating. However, its primary effect is likely to be a reduction in the probability that a female will mate twice in quick succession. Our experiments provide the first direct evidence for this effect: re-mating was more frequent in females from which plugs were removed immediately post-mating. There are three different ways in which the plug could have such an effect: (i) direct physical blocking; (ii) pheromonal reduction in attractiveness of the female; and (iii) reduction in receptivity of the female to further mating. We can clarify the validity of each of these putative effects.

(i) Physical blockage.

This hypothesis is based on logic rather than any experimental data. Ross & Crews (1978) suggested that the plug in *T. radix* does not fully solidify for several hours, and thus may not be an effective barrier in that initial period. This is not the case in *T. sirtalis*; within 15 min of copulation, plugs in this species are so firm (and so well-attached to the cloacal walls) that it is difficult to imagine them being dislodged by a rival's hemipenis. Our failure to dislodge plugs with 300 g force suggests that courting males have little chance of displacing a recently-deposited plug.

(ii) Reduced attractivity of the female.

The evidence for this putative function (pheromonal discouragement of rivals) involves the attractiveness of females from which plugs were removed, and then smeared over the female's body (Ross & Crews, 1977, 1978). However, these trials did not separate the effects of the plug *per se* from that of seminal fluids associated with copulation (Devine, 1984). Our experiments support Devine's (1984) criticism, by showing that the pheromonal discouragement of other courting males arises from fluids associated with copulation rather than with the plug *per se*.

(iii) Induction of non-receptivity by the female.

This third putative function is supported by the higher re-mating frequency of females from which plugs were removed immediately after mating, as compared to those with plugs removed several hours later (see above). However, these data are

also consistent with the simpler notion of an endogenous decline in female receptivity immediately after mating. That is, the plug may function as a physical block (hypothesis [i] above) for long enough that female receptivity declines to the point that re-mating becomes unlikely. Further work is needed to clarify this issue.

We conclude that the mating plug of *T. s. parietalis* functions as a physical block to delay re-mating by the female. Although the plug persists only briefly, the rapid dispersal of females away from the den means that this delay may be long enough to preclude re-mating of many females. Natricine snakes display enormous diversity in many traits, including the sizes of den aggregations and the presence or absence of mating plugs (e.g. Gregory & Stewart, 1975; Larson, 1987; Rossman *et al.*, 1996). Thus, gartersnakes may provide ideal model systems in which to clarify the determinants of male mating tactics, and the circumstances in which male snakes may enhance their mating success by constraining the re-mating opportunities of their partners.

Why do gartersnakes have mating plugs whereas most other snakes do not? A recent review of the duration of copulation in reptiles indicates that copulation is briefer in gartersnakes than in most other snakes (Olsson & Madsen, 1998). Plausibly, this fact may be linked to the evolution of the mating plug (T. Madsen, pers. comm.). Prolonged copulation may enhance male reproductive success because it prevents the female from re-mating for a substantial period—perhaps until she has become sexually unreceptive. In species where males have an opportunity to re-mate rapidly, however, prolonged copulation might substantially reduce a male's reproductive success. Such 'opportunity costs' of prolonged copulation may be higher for male gartersnakes (which often mate in large aggregations, over a brief period) than for males of most other snake species. Indeed, male gartersnakes sometimes re-mate in quick succession (e.g. Blanchard & Blanchard, 1941). Thus, the evolution of the mating plug in natricine snakes may have permitted a simultaneous reduction in the duration of copulation, because the plug provides a 'chastity belt' for a period similar to that resulting from prolonged copulations by other snakes. Under this hypothesis, the evolution of the mating plug ultimately reflects the mating systems of natricine snakes, and in particular the way in which their mating aggregations provide opportunities for rapid re-mating by males.

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Humane Care and Use of Laboratory Animals' and the National Institutes of Health 'Guide to the Care and Use of Laboratory Animals'.

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