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Not just a chastity belt: the functional significance of mating plugs in garter snakes, revisited

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During the spring emergence of red-sided garter snakes (*Thamnophis sirtalis parietalis*) in Manitoba, Canada, the operational sex ratio is strongly skewed towards males, who scramble to locate and court newly emerged females. A high frequency of multiple paternity litters suggests that the females are promiscuous; the gelatinous copulatory plugs (CPs) deposited by males may confer fitness benefits via passive mate guarding. Because precopulatory female choice is limited in large mating aggregations, sexual conflict may place a premium on preventing females from ejecting male sperm. In snakes, sperm are produced in the testes and delivered through the ductus deferens, and the CP is thought to be produced by the renal sexual segment and conveyed through the ureter. We manipulated the delivery of the two fluids separately by surgically ligating the ducts. Ureter-ligated males did not produce a CP, causing their sperm to leak out of the female's cloaca immediately after copulation. Contrary to previous suggestions, histology revealed sperm distributed throughout the CP. Thus, the CP may function as a spermatophore: the protein matrix contains the sperm, which are liberated gradually as the plug dissolves. The likelihood of a male depositing a CP fell significantly after his second mating, perhaps limiting his reproductive success. These results challenge the hypothesis that passive mate guarding is the primary function of the CP in *T. sirtalis parietalis*. © 2013 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2013, **109**, 893–907.

ADDITIONAL KEYWORDS: copulatory plug – cryptic female choice – mating plug – mating system – reptile – sexual selection – sperm competition.

INTRODUCTION

Female sexual promiscuity is phylogenetically widespread (Smith, 1984; Birkhead & Møller, 1998). When a female mates with multiple males, the sperm of these males may co-mingle within her reproductive tract and compete for the fertilization of her ova: a phenomenon known as sperm competition (Parker, 1970). Males can gain an advantage in sperm competition by increasing the number of sperm they inseminate relative to their rivals (Parker, 1990); however, an alternative strategy is to reduce the risk of sperm competition altogether by preventing a mate from remating with another male. Males can limit remating opportunities by guarding their mates and/or by prolonging copulations, and thus reducing the risk of sperm competition (Birkhead & Møller, 1998; Simmons, 2001). A male may also passively guard a female by depositing substances that occlude the opening to her reproductive tract (known as copulatory, mating, or vaginal plugs; Birkhead & Møller, 1998; Simmons, 2001). Just as female sexual promiscuity is taxonomically widespread, so too are copulatory plugs (CPs; Voss, 1979; Devine, 1984; Birkhead

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& Møller, 1998; Shine, Olsson & Mason, 2000a; Simmons, 2001; Jia *et al*., 2002; Poiani, 2006; Althaus *et al*., 2010; Uhl, Nessler & Schneider, 2010).

Although the passive mate-guarding or 'chastity-enforcement' hypothesis has received some support (e.g. Shine, Olsson & Mason, 2000a; Simmons, 2001), the efficacy of CPs as a paternity guard has also been called into question (e.g. Michener, 1984; Moreira & Birkhead, 2004). This controversy has led researchers to revisit alternative explanations for the evolution of CPs. Voss (1979) succinctly summarized several of these alternative hypotheses for plug function: '(1) permit a gradual release of spermatozoa within the female tract as they disintegrate (Asdell, 1946), (2) prevent leakage of spermatozoa from the vagina (Leuckart, 1847), (3) induce pseudopregnancy in the female (Long [and Evans 1922]), (4) transport sperm through the cervix (Blandau, 1945), (5) prevent subsequent insemination of females by other males (Martan & Shepherd, 1976), or (6) act as an antiaphrodisiac (Happ, 1969).' Tests of these alternative hypotheses are sparse. Furthermore, these functional hypotheses are not mutually exclusive. For example, in scorpions the CP performs both mate-guarding and sperm-storage functions (*Euscorpius italicus*, Althaus *et al*., 2010), and in ground beetles the mate-guarding and anti-aphrodisiac hypotheses have found support (*Leptocarabus procerulus*, Takami *et al*., 2008). In some mammals, the passive mate-guarding hypothesis has been rejected in favour of the sperm-leakage hypothesis because paternity was not assured by the first male to mate (e.g. ground squirrels, *Spermophilus beecheyi*, Michener, 1984; Koprowski, 1992; deer mice, *Peromyscus* spp., Dewsbury, 1988; civets, *Paguma larvata*, Jia *et al*., 2002). However, sperm leakage in the absence of plug deposition was not experimentally tested in these studies, and confirmation of one hypothesis does not falsify or support the others. Each functional hypothesis needs to be tested independently (Uhl & Busch, 2009).

One species in which multiple hypotheses of plug function have been experimentally evaluated is the red-sided garter snake (*Thamnophis sirtalis parietalis*). The CP of *T. sirtalis parietalis* is the largest plug among reptiles (Olsson & Madsen, 1998), and can represent more than 0.4% of a male's mass (Shine *et al*., 2000a; C. R. Friesen & R. T. Mason, unpubl. data); this is the equivalent of a 90-kg human male producing a 360-mL ejaculate (based on a density of 1 g mL^{-1} for human semen; Matson *et al*., 2010). Given such an enormous investment, one would expect a CP to provide significant fitness benefits to the male who produces it (Dewsbury, 1982).

In Manitoba (in Canada's Interlake Region), *T. sirtalis parietalis* emerge en masse each spring from communal hibernacula (Gregory, 1974). Males emerge first and remain around the den for 2–4 weeks before migrating to summer feeding grounds, whereas females emerge throughout the 4–6 weeks of spring emergence, but stay an average of 4 days before migrating (Gregory, 1974; Shine *et al*., 2001, 2006a). This pattern of emergence generates an operational sex ratio (OSR) that is highly skewed towards males, generating intense competition among males to scramble for mates (Emlen & Oring, 1977). Mating aggregations can contain up to 62 males to one female (Shine *et al*., 2001). Two hypotheses for CP function have been explored in this system: the anti-aphrodisiac and the mate-guarding hypotheses. Both of these hypotheses propose that the CP functions to reduce the likelihood of females remating.

ANTI-APHRODISIAC HYPOTHESIS

Mated female plains garter snakes (*Thamnophis radix*) and *T. sirtalis* with CPs are less sexually attractive and receptive than unmated females, suggesting that the plug may discourage courtship (i.e. have an anti-aphrodisiac function: *T. radix*, Ross & Crews, 1977, 1978; *T. sirtalis sirtalis*, Devine, 1977, 1984; Whittier, Mason & Crews, 1985; Shine *et al*., 2000a). However, this reduction in female receptivity may result from the prostaglandins in the seminal fluid rather than from the plug itself (Whittier & Crews, 1986; Shine *et al*., 2000a).

CHASTITY-BELT HYPOTHESIS

Supporting earlier speculation by Devine (1975), Shine *et al*. (2000a) found support for the hypothesis that the CP acts to discourage the remating of females in garter snakes red-sided garter snakes. Mated females that had the plugs removed shortly after mating (< 5 min) re-mated at a higher rate than did females with plugs removed a few hours after mating, or females in which the plug dissolved naturally after about 2 days. These results were interpreted as support for the mate-guarding hypothesis, but it seems that the possible effect on female receptivity was not controlled for.

Unfortunately for the 'chastity-belt' hypothesis, some inconvenient observations raise questions about the plug functioning as a form of mate guarding, even in this well-studied example (Uller, Stuart-Fox & Olsson, 2010). First, some females are able to re-mate even while plugs occlude their cloacas (Whittier & Crews, 1986; Shine *et al*., 2000a; C. R. Friesen, pers. observ.). Importantly, the plugs form only a temporary barrier to subsequent mating. Although most mating in this population occurs at the den during the spring emergence, the garter snake breeding season is protracted, and is much longer in duration than the period in which a CP remains in a female's reproductive tract. The CP does not even last the average duration that a female remains at the den, where males are constantly courting her: it only takes 2–3 days for the plug to dissolve completely, and the plug can be easily displaced after only 1.5 days if the temperature is high enough (Shine *et al*., 2000a). On average, female garter snakes leave the den to migrate to feeding grounds about 4 days after they emerge (Shine *et al*., 2001, 2006a). Thus, there is abundant opportunity for females to remate before migrating. Reinforcing the ineffectiveness of the CP as a chastity belt, multiple paternity of litters is ubiquitous in the genus *Thamnophis* (Schwartz, McCracken & Burghardt, 1989; McCracken, Burghardt & Houts, 1999; Garner *et al*., 2002, 2004; Wusterbarth *et al*., 2010). Although long-term sperm storage may account for some multiple paternity (Uller & Olsson, 2008; Uller *et al*., 2010), it is clear that the plug does not *necessarily* prevent multiple mating.

Many alternative hypotheses for the CP remain to be explored in this system. For example, the CP may function to keep a male's sperm in the female reproductive tract. If sperm leaks from the female after mating in the absence of plug deposition, then mate guarding, anti-aphrodisiacs, and the reduction in female attractiveness are secondary functions; however, it is possible that sperm move up the reproductive tract during copulation, and thus do not leak from the female's reproductive tract. In this case the previously supported hypotheses, such as mate guarding, may fully explain the adaptive significance of CPs in this system (e.g. Shine *et al*., 2000a). Unfortunately, the fate of sperm has not been directly examined in studies of CP function in this system. In garter snakes, a previous study reported abundant sperm in the oviducts of recently mated females (Devine, 1975). If this were the case more generally, then the sperm-leakage hypothesis would seem less likely; however, subsequent work found that 6 h after mating, very few sperm had entered the lumen of the posterior oviduct, and it took 24 h for detectable quantities of sperm to reach these posterior storage sites (Halpert, Garstka & Crews, 1982). Furthermore, in preliminary studies aimed at the collection of sperm to evaluate variation in ejaculate quality, sperm was not found in the oviducts immediately after copulation (C. R. Friesen & R. T. Mason, pers. observ.), supporting the hypothesis that one of the principal functions of the plug is to reduce or eliminate sperm leakage.

To test the hypothesis of sperm leakage, one must be able to prevent a plug from being deposited while at the same time allowing males to transfer sperm as usual. Squamate sauropsids are uniquely suited to this type of study because the sperm and plug are delivered via completely different tracts. In many squamates (lizards, snakes, and worm lizards), the distal portion of the nephron preceding the collection ducts of the kidneys is seasonally hypertrophied or regressed in males, but not in females (for a review in natricine snakes, see Krohmer, 2004). This sexual dimorphism suggests that this portion of the kidney may serve a sexual function; hence this portion of the kidney has come to be called the renal sexual segment (RSS; Burtner, Floyd & Longley, 1965). Seasonal regression and recrudescence of the RSS is also influenced by testosterone, which further supports its sexual function in males but not in females (Bishop, 1959; Krohmer, 2004).

Volsøe (1944) proposed that the CP was produced by the RSS. Olsson & Madsen (1998) suggested that squamate sauropsids would provide a perfect model to test the fate of sperm in the absence of plug formation, because the sperm and plug are delivered separately via two different duct systems, and could be manipulated separately (e.g. Olsson, Gullberg & Tegelström, 1994). Therefore, to experimentally test whether the CP prevents sperm leakage, we sought to determine whether the RSS is in fact the source of the CP material. If plugs were not deposited then we could test the sperm-leakage hypothesis by observing the fate of sperm in females. The 'plug as a spermatophore' hypothesis of CP function could be examined using histological methods. If the sperm were distributed homogeneously throughout the CP, the plug may actually be a spermatophore, rather than a mating plug. We also obtained sperm counts from the CP after dissolving the plug material, and compared them with sperm counts from flushings of the cranial pouch of the cloacal urodaeum (henceforth vaginal pouch; Blackburn, 1998; Siegel *et al*., 2011) and posterior oviducts. If most of the sperm were in the plug rather than within the female's reproductive tract, this would also support the role of the plug as a spermatophore. Finally, we addressed the ecological relevance of the prevention of sperm leakage by investigating whether males are limited in the number of plugs that they can produce (Shine *et al*., 2000a).

MATERIAL AND METHODS

ANIMAL COLLECTION

Twenty-nine male *T. sirtalis parietalis* were collected from the Inwood Quarry in Manitoba, Canada (Inwood Den: 50°31.58′N, 97°29.71′W) on 10 May 2008. They were placed in 1 m × 1 m × 1 m seminatural enclosures located at the Chatfield Research Station until surgery was performed 1–3 days after collection. Water was provided *ad libi-*

tum, but food was not (snakes are aphagous at this time; O'Donnell, Shine & Mason, 2004). Body mass and snout-to-vent length (SVL) were recorded.

SURGERY

Twenty-nine males were randomly assigned to one of three treatments: ureter ligation (Ux), vasectomy (Vx), or sham surgery ($N = 5$ Ux males; $N = 12$ sham males; $N = 12$ Vx males). For anaesthesia, 15 mg kg^{-1} (0.003 mL of 0.5% methohexital sodium g^{-1} body mass) was administered subcutaneously at the juncture between the dorsal and ventral scales, approximately 20 cm from the head of the snake (Preston, Mosley & Mason, 2010). An incision was made in the skin between the first and second lateral scale rows, directly dorsal to the fifth ventral scale (counting from the cloacal scale). The surgical window traversed no more than four scales, and the incision was made from the posterior to the anterior drection with corneoscleral scissors that were sterilized in a dry-bead sterilizer at 260 °C for 15 s. The peritoneal membrane was then cut using the same scissors and made wider than the skin window to allow for easier access to the ureter. The ureters (Ux males) or ductus deferens (Vx males) were isolated from the viscera and ligated using sterile suture (4-0 G silk). The surgical window was then sutured using sterile 4-0 G silk. In sham surgeries, the mesentery enclosing the ureters and ductus deferens was isolated and exposed, but not ligated. Subsequently, the incision site was rinsed with Ringer's solution (reptile physiological saline), then swabbed with 100% ethanol. The snakes were then placed in an aquarium with a heating pad (30 °C) and monitored every 10 min for the first hour after surgery until the return of the righting reflex. Males of this species regain their drive to court very soon after surgery, and show no deficit in this ability (e.g. Nelson *et al*., 1987). All animals survived surgery and engaged in courtship within 24 h of recovery.

MATING TRIALS

Males were randomly assigned to one of three arenas (45-cm diameter × 75-cm tall) with up to two females at a time, and were allowed to court and mate with the females. This density of males is common in and around the dens (Joy & Crews, 1985; Shine, Langkilde & Mason, 2004). Each indoor arena was heated by a 250-W heat lamp placed 1 m above the animals: we measured external body temperatures with a laser thermometer, and adjusted the output of each lamp using dimmer switches to maintain the optimal body temperature (29–30 °C: Kitchell, 1969; Hawley & Aleksiuk, 1975). Courtship was observed closely so that we could begin timing copulation duration. One minute after copulation commenced, the pair was removed to a separate circular arena to prevent interference from other snakes. Less than 30 s after copulation terminated, each female was inspected for plug deposition. In addition, a sample of fluid from outside the cloaca of females that mated with Ux males was collected by wiping the area with a slide to check for the presence of sperm. For comparison, we also checked five naturally plugged females for the presence of sperm outside the cloaca using the same method. The slides were viewed with a compound microscope (10× objective) and photos were taken to document sperm leakage (Fig. 1). The Ux males were then killed and the ureters were inspected to ensure that the ligation was effective and

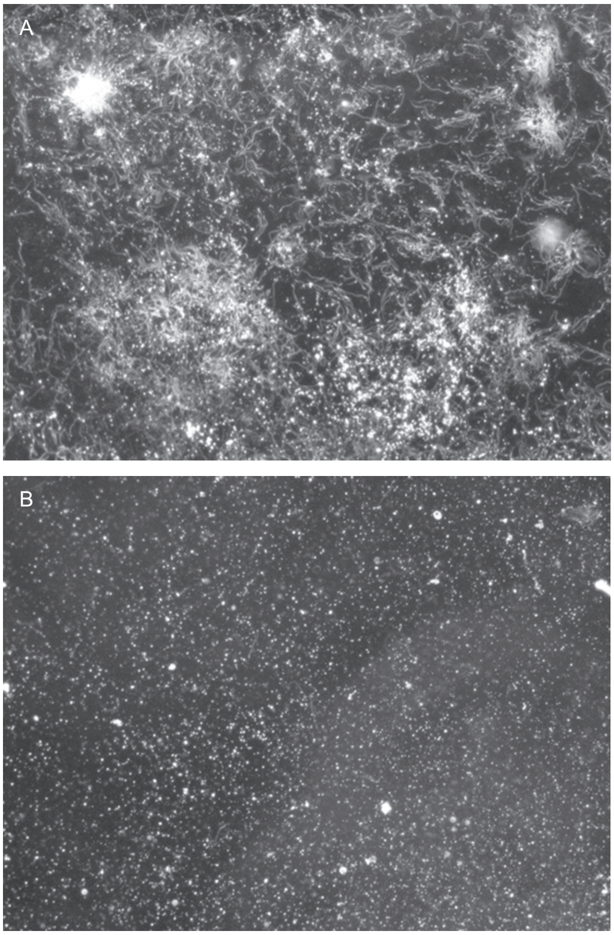


Figure 1. Two representative photographs of slides wiped across the cloaca of a female garter snake (*Thamnophis sirtalis parietalis*) within 30 s of the termination of copulation (at 20× magnification): A, female mated with a ureter-ligated male that did not produce a copulatory plug; B, female mated with a sham-operated male that did produce a copulatory plug. Note that there are no sperm in (B) and abundant sperm in photo (A).

to note the condition of the ureters after copulation. All ligations appeared effective, and we observed what was apparently plug material accumulated within the ureters anterior to the ligation.

CONDOM STUDY

In the mid-afternoon (1400–1600 h) of 15 May 2004, we allowed 14 females to mate in outdoor semi-natural arenas (1 m × 1 m × 1 m). Seven control females were kept in identical enclosures, but without males. We checked enclosures regularly for mating, and within 10 min of the cessation of copulation we collected the females (plus equivalent numbers of control females), and took them to the adjacent field laboratory where we manually removed the recently deposited CPs from half of the mated females. The plugs were placed in 70% alcohol for later analysis of sperm counts. On all females, we used tape to attach a balloon to the body above the cloaca (i.e. enclosing the cloaca and tail), such that any materials leaking from the cloaca would be collected in the balloon. The females were kept overnight in an enclosure that was large enough (1 m × 1 m × 1 m) to allow them to move around. The following morning (0800–1000 h) we removed the balloons, flushed out their contents, and preserved them in 70% alcohol. Those flushings were later examined with a haemocytometer to count sperm.

THE EFFECT OF MULTIPLE MATING ON PLUG DEPOSITION

To estimate the likelihood of plug deposition with successive matings, we analysed data collected from matings of the same Vx and sham males as described above. The Vx and sham males were given access to females for 8 hours a day over the course of 6 days to test the effect of multiple matings on plug deposition. The females were collected as they emerged the day before each day's mating trials were conducted, and were kept in cloth bags until the start of the mating trials. The mating trials were conducted indoors in the same three arenas (45-cm diameter × 75-cm tall) using the same protocols as described in the 'Mating trials' section above. Males were held in 1 m × 1 m × 1 m seminatural enclosures when not engaged in mating trials, and were given water *ad libitum*.

SPERM COUNTS FROM COPULATORY PLUGS

Our initial attempts to collect whole ejaculates from the female's vaginal pouch and oviducts seemed to yield very few sperm, suggesting that most of the sperm may be contained within the CP itself. To address this question: (1) we examined five CPs removed from females, using histological methods; and (2) conducted sperm counts from plugs, and from the vaginal pouch and oviducts of 15 females that mated at the Inwood Den. Less than 30 s after copulation terminated, each of these 20 females was inspected for a CP. Each plug was removed by gently running a blunt probe around the plug to separate it from the walls of the vaginal pouch (Shine *et al*., 2000a). The plugs used for sperm counts ($N = 15$) were placed in a 1.5-mL microcentrifuge tube in 1 mL of Modified Ham's F-10 medium and 10 μg mL^{-1} of the antibiotic gentamicin sulfate (cat. no. 99175; Irvine Scientific; e.g. Mattson *et al*., 2007). The vaginal pouches of the females were lavaged with the same Ham's F-10 medium using a 20-ga. intubation needle affixed to a 1-mL syringe. The fluid from the vaginal wash should contain any sperm not embedded within the plug, and was placed in a separate 1.5-mL tube. We also recorded whether the oviductal sphincter was easily penetrated by the intubation needle during the flushing. We considered the sphincter to be 'open' if it was penetrated with little pressure, and closed if an effort was needed to pass the intubation needle into the oviduct. The tubes containing the plugs were placed in a refrigerator for 2 days and were gently agitated three times daily to aid the liberation of sperm embedded within the plug. The dissolution of the plug was evidenced by a dense 'cloud' of sperm above the plug. When most of the sperm were liberated, a small piece of the posterior portion of the plug remained. The approximate sperm concentration of the samples (1 μL) was estimated with a microscope and a counting chamber, and then diluted to facilitate accurate sperm counts (~100 sperm within the counting grid). After dilution, photos of triplicate samples spread over a Petroff–Hausser sperm cell counter (cat. no. 3900; Hausser Scientific) were taken with an Olympus DP-5 digital camera mounted on an Olympus CX31 phase-contrast compound microscope using the 4× objective. The images were captured using CELL-SENSE software from Olympus, and the sperm counts were conducted using the same software in the lab at Oregon State University. Sperm counts from the vaginal/oviductal washes were conducted in the same manner.

SECTIONING OF THE PLUG

The plugs that were to be examined using histological methods were fixed in 4% paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline (PBS) upon collection. Plugs were dehydrated in progressive alcohol, cleared in toluene, embedded in paraffin, and cut on a rotary microtome at a thickness of 10–15 μm. The sections were collected on gelatin-coated slides and allowed to dry. We used two staining methods: trichrome (haematoxylin, Biebrich scarlet-orange, and fast green) and regressive H&E (haematoxylin and eosin), which yields blue nuclei and pink cytoplasm, but leaves most proteins unstained, excepting collagen. After staining, we dehydrated the samples in progressive alcohols and xylene, and covered the sections with Permount (Fisher) and a coverslip (Fig. 1).

STATISTICAL METHODS

All statistical analyses were conducted in SIGMA PLOT 11.0 and/or XLSTAT Pro. Non-parametric tests were used if normality and equal variances could not be obtained by log transformations. Logistic regressions used a logit link function and binomial distribution in both SIGMA PLOT and XLSTAT. Maximum-likelihood estimates of P values in XLSTAT used the Newton–Raphson algorithm (100 iterations and a convergence threshold set to 0.0000001) for multiple-comparison tests in logistic regression (Venzon & Moolgavkar, 1988). XLSTAT tests of independence using Monte Carlo simulations (5000) were performed, which generate contingency tables that have the same marginal sums as the observed table. The chi-square statistic was computed for each of the simulated tables. The P value was then determined by using the distribution obtained from the simulations.

RESULTS

URETER LIGATION PREVENTS PLUG DEPOSITION

All five of the Ux males, ten of 12 Vx males, and ten of 12 sham males mated; however, none of the five Ux males deposited plugs, compared with nine of te ten sham (S) males and ten of the ten Vx males. Expected counts lower than five make a chi-square test of independence unreliable, so we used a Monte Carlo method to test for independence in XLSTAT (based on 5000 simulations). The probability of plug deposition differed among treatments ($\chi^2 = 20.07$, 1 d.f., $P < 0.001$).

SPERM RETENTION WITHOUT A COPULATORY PLUG

Mating by males that did not produce plugs resulted in visible sperm masses in the posterior portion of the female's cloaca. Within 30 s post-copulation, a considerable quantity of sperm had leaked from the cloaca of females that did not receive a plug, compared with females that did receive a plug and for which no sperm were apparent in the post-cloacal smear (Fig. 1).

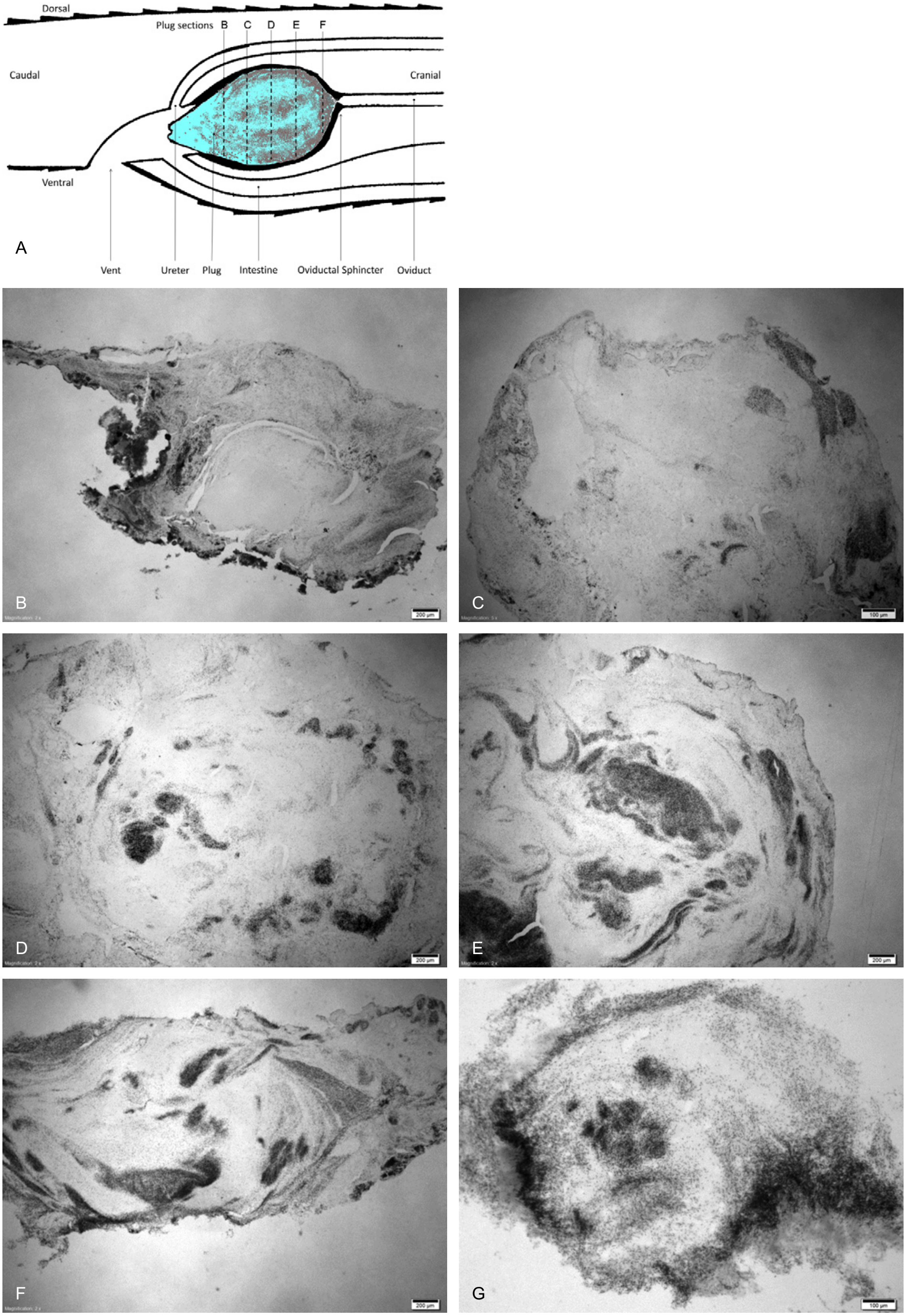
QUALITATIVE DESCRIPTION OF THE PLUG AND POST-MATING CLOACAL FLUIDS

Histological sectioning showed increased sperm numbers towards the anterior end of the plug (Fig. 2). With the trichrome stain the plug matrix stained pink, indicating a high concentration of sperm. The nuclei did not stain well with this method, but blue/green indicates that the matrix is largely proteinaceous (as suggested by Devine, 1975). The H&E stain highlights the nuclei well and is thus a good indicator of sperm number (Fig. 2).

SPERM COUNTS IN THE COPULATORY PLUG AND CLOACAL FLUIDS

An average number of 50.99×10^6 (SE = 10.17×10^6) sperm was obtained from CPs versus an average of 2.78×10^5 (SE = 1.20×10^5) sperm collected from the fluid taken from the vaginal pouch of the cloaca and the oviducts after the plug was removed (hereafter 'flushings'). Flushings contained significantly fewer sperm relative to those liberated from the CP (Signed rank test, W = 120.00, $P < 0.001$; Fig. 3). Eight of 15 (53%) flushings contained no sperm, and the average number of sperm in the remaining seven flushes was 5.96×10^5 (SE = 2.01×10^5). In the flushings that contained any sperm at all ($N = 7$), there was a median of 52.12 times more sperm in the CP than the flushing (with a range of 9.57–1388.33 times more). The flushings from females with 'open' sphincters had significantly more sperm than those with 'closed' sphincters (Mann–Whitney rank sum test, $U = 6.00$, d.f. = 1, $P = 0.009$). Sperm collected from the dissolved CPs and the flushings were motile. We conclude from these observations that most of the sperm are incorporated into the CP matrix itself, and are released as the plug dissolves within the female's vaginal pouch.

Figure 2. A, depiction of how the copulatory plug (CP) is situated in the cloaca of the female garter snake (*Thamnophis sirtalis parietalis*), showing the distribution of sperm within the CP and the approximate location of the photographed sections (B–G below; redrawn after Devine, 1975). B–G, cross sections of a CP stained with regressive H&E. The dark stains are masses of sperm heads and the unstained area within the plug represents proteinaceous RSS secretions. The bulk of the sperm is aggregated, but sperm are numerous throughout the plug. For example, section B is the most caudal, and shows masses of sperm to the top and right-hand side of the picture. The photos of sections B–F were all taken using the 4× objective (scale bar: 0.2 mm), and the photo of section G (the most cranial) was taken using the 10× objective (scale bar: 0.1 mm). The total length of sperm cells in this species is 100–110 μm. Photos were taken with an Olympus DP-5 digital camera mounted on an Olympus CX31 compound microscope, with the filter set to bright field. The images were captured and the scale bars added with Cell-Sense software from Olympus.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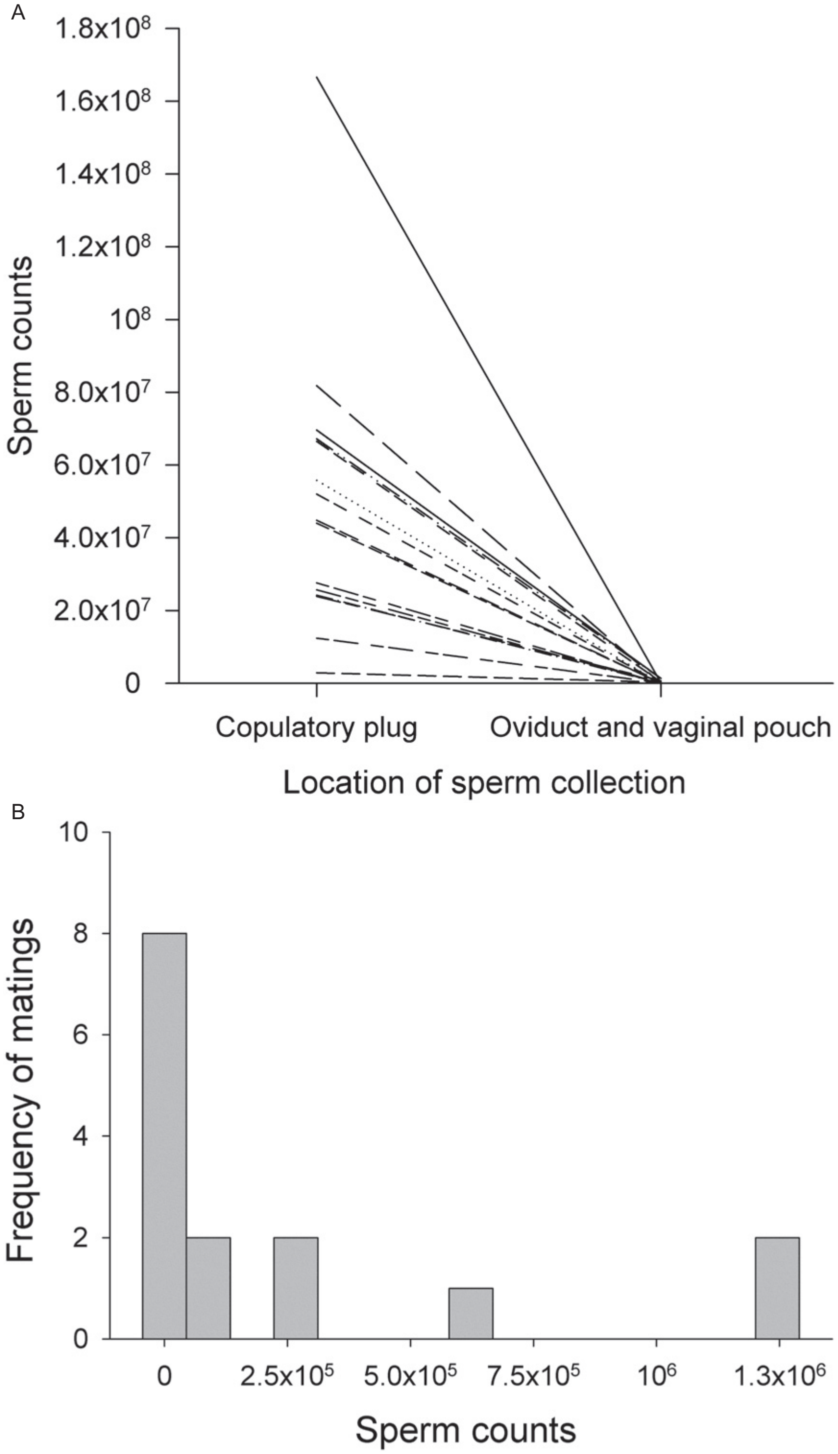

Figure 3. A, most sperm were located in the copulatory plug compared with flushings taken from the vaginal pouch and oviducts. B, histogram of sperm counts from the flushings.

In the ‘condom study’, we found no sperm in vials containing the CP or in flushings from the condoms that had been attached to unmated females. Mated females with intact CPs yielded an average of 1000 sperm (sperm found in only one of seven females), whereas those with CPs removed yielded an average of 73 000 sperm (with sperm found in three of the seven females). Thus, mean sperm counts differed significantly between these two treatments (Kruskal–Wallis test, $H = 3.45$, $P < 0.001$).

EFFECT OF MULTIPLE MATINGS ON PLUG DEPOSITION

In our arena mating trials, 20 males mated once, and of those, 15 mated twice, and then ten mated a third time. Five of the thrice-mated males mated a fourth time, and one mated a fifth time. There was no significant difference between Sham and Vx treatments in terms of: (1) the total number of matings (matings, $t = 1.25$, d.f. = 22, $P = 0.22$); (2) the total number of plugs produced (plugs, $t = 1.32$, d.f. = 22, $P = 0.20$); or (3) plugs produced per mating (plugs/mating Mann–Whitney rank sum test, $U = 41.50$, d.f. = 1, $P = 0.52$). Thus, we excluded treatment (Vx versus Sham) from our analyses of the effect of multiple matings on plug deposition. The number of matings per treatment differed primarily for the number of third matings: eight of 12 Vx males mated three times (= 67%) versus two of 12 sham males that mated three times (= 17%), and this difference is significant ($\chi^2 = 4.29$, d.f. = 1, $P < 0.04$).

Nine males deposited one plug, six made two plugs, four made three plugs, and one made four plugs. The average number of plugs per male was 1.87, and the average number of plugs per mating across all matings was 0.78 plugs per mating over 6 days of mating trials. Plugs were produced in 19 of 20 first matings (95%), 12 of 15 second matings (80%), but only in four of ten third matings (40%) and one of five fourth matings (20%); no plug was found in the single fifth mating (0%). We tested for the effect of male mate number on plug deposition with several analyses. Given the binary response variable (plug or no plug), and the imbalance in the repeated design, we first ran a multiple logistic regression on a limited subset of the data using the ten males that copulated three times or more, and limited our analysis to their first three matings ($N = 30$ observations among ten males). In this analysis, male mating number (mate #) was a significant predictor of plug deposition, but male identity (ID) was not (full model, likelihood ratio test statistic = 12.52, $P = 0.002$; mate #, Wald statistic = 5.980, $P = 0.01$; ID, Wald statistic = 0.022, $P = 0.88$). Friedman's repeated measures rank-sum test confirmed these results on the same data set ($\chi^2 = 10.33$, d.f. = 2, $P = 0.006$). Next, we included the data from all matings (excluding male ID as a factor because of the previously mentioned analysis). We used maximum likelihood-based logistic regression to provide pairwise comparisons of rates of plug deposition with successive matings [model: –2log(likelihood) $\chi^2 = 17.88$, $P < 0.0001$]. See Figure 4 for a comparison of categories (male mate number on likelihood to produce a plug). All of our analyses show that the likelihood of plug deposition drops off significantly after the second mating, and that this result is robust to multiple methods of analysis.

NO EFFECT OF MALE AND FEMALE SIZE ON PLUG DEPOSITION

Neither female body size (SVL, Wald statistic = 1.706, $P = 0.192$; mass, Wald statistic = 1.960, $P = 0.162$) nor male body size (SVL, Wald statistic = 0.428, $P = 0.513$; mass, Wald statistic = 0.104, $P = 0.747$) was significantly associated with the probability of plug deposition. Lastly, the average copulation duration of matings in which plugs were produced (= 23.16 min) was significantly longer than those that produced no plugs = 18.38 min; $t = 2.148$, d.f. = 44, $P = 0.037$).

DISCUSSION

The copulatory plug (CP) of *T. sirtalis parietalis* performs multiple functions. As shown by previous studies, the CP of *T. sirtalis parietalis* can work as a chastity belt, reducing the probability of remating by the female (for at least a few days; Shine *et al.*, 2000a). However, our data suggest that mate guarding may not be the only, or indeed primary, adaptive function of the CP in this species. Our experimental studies show that the CP reduces the leakage of sperm from the female reproductive tract: sperm readily leaked from females after copulations in which no plug was deposited, or after we had removed recently deposited plugs. In contrast, sperm leakage was minimal from females after mating with males that deposited CPs. Also, our study suggests another, and perhaps equally significant function: the CP may act as a spermatophore (Mann, 1984), gradually releasing sperm into the vaginal pouch as the plug breaks down. Many other species of snakes show very prolonged copulations (sometimes lasting for days; Gillingham, 1987; Olsson & Madsen, 1998), which would fulfill all of the same functions that we have identified for CPs in *T. sirtalis parietalis*; however, the short mating season and availability of multiple receptive females imposes a high 'opportunity cost' to prolonged mating in this system, plausibly favouring a reduction in the duration of copulation (i.e. allowing a male to begin courting another potential mate; Shine *et al.*, 2000a). Our data suggest that male garter snakes can reap the same fitness benefits as conferred by prolonged copulation, but without the associated opportunity costs, by adopting the tactic of a brief mating followed by the production of a CP. Furthermore, the fact that the plug is a spermatophore may be the outcome of female resistance to plug deposition during copulation.

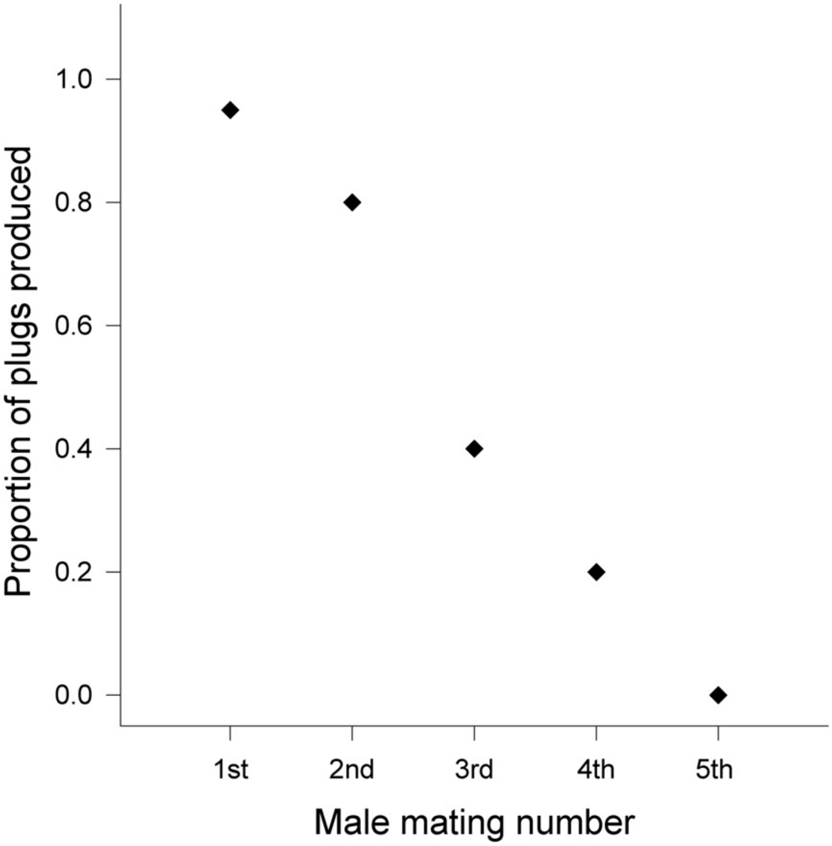

Figure 4. Proportion of copulatory plugs produced as a function of the number of times that a male garter snake (*Thamnophis sirtalis parietalis*) mates. The likelihood of producing a plug on successive matings decreased significantly after a male's second mating.

Our data confirm and extend previous analyses of this topic. For example, Shine *et al*. (2000a) reported that the incidence of copulations without CP production increased (albeit, non-significantly) if males engaged in multiple matings in quick succession. With larger sample sizes and a more extended period, this latter trend attains statistical significance in the current study. Additionally, we reveal an effect of copulation duration on plug production, supporting the hypothesis that the brief duration of copulation is functionally linked to plug production. CPs may be as important as sperm numbers in the context of sperm competition in this system, because sperm that leaks from the female's reproductive tract cannot contribute to male fitness. Male *T. sirtalis parietalis* are clearly limited in the number of plugs that they can produce, hinting that male snakes in this population may be under intense selection for rapid and repeated plug production.

URETER LIGATION

Although for decades circumstantial evidence has pointed to the RSS as the source of plug material (e.g. Bishop, 1959; Devine, 1975), our study provides the first conclusive support for this hypothesis in garter snakes: none of the ureter-ligated males produced a plug. This technique, along with vasectomy, can be used to further elucidate the role of RSS secretions in assuring paternity (e.g. Olsson, Gullberg & Tegelström, 1994), and also to disentangle the effect of plug material and seminal fluids on female mating behaviour.

TESTING THE SPERM LEAKAGE HYPOTHESIS

The technique of ureter ligation allowed us to test a specific hypothesis about plug function. Without the plug, sperm leaks from the female's cloaca. Neither Devine (1975) nor Shine *et al*. (2000a) completely disregarded the sperm leakage hypothesis, but they instead focused on the mate-guarding function. In the most comprehensive study on plug function in garter snakes to date, Shine *et al*. (2000a) concluded that passive mate guarding is the primary current function of the plug, and that seminal fluid, not the plug itself, is responsible for the reduced post-mating female attractiveness. Their work demonstrated that if the plug is removed, a female often re-mates quite soon afterwards. Nevertheless, the plug is not a totally effective chastity belt, because some females contain multiple plugs (Shine *et al.*, 2000a; C. R. Friesen & R. T. Mason, pers. observ.), and multiple paternity is common in garter snakes (reviewed in Wusterbarth *et al.*, 2010). Thus, the presence of a CP does not necessarily render a female unattractive or unreceptive (Shine *et al.*, 2000a; C. R. Friesen, pers. observ.). Cases of female *T. sirtalis parietalis* bearing multiple CPs calls into question the ecological relevance of reduced post-mating female attractiveness, when large numbers of males are searching and competing for matings (e.g. Devine, 1977; Ross & Crews, 1978; Shine *et al.*, 2000a). Even in these cases, the plug may not protect the first male's paternity. For example, male Iberian rock lizards produce a CP, but this can be displaced by subsequent males (Moreira & Birkhead, 2003); therefore, the plug does not ensure the first male's paternity (Moreira *et al.*, 2007).

THE COPULATORY PLUG TURNED SPERMATOPHORE

Spermatophores have been defined as a package or mass composed of male secretions in which the spermatozoa are encapsulated or embedded (Mann, 1984). According to this definition, the CP of *T. sirtalis parietalis* is a spermatophore; however, based on data from a single female, Devine (1975) suggested that most of the sperm lie within the oviduct and vaginal pouch. Our data on plug composition (especially the presence of abundant sperm within it), and the paucity of sperm in the cloaca and oviduct, contrast with Devine's (1975) description. First, in 53% of the matings, essentially all of the sperm were embedded within the CP: the flushings of the other 47% of matings contained a fraction of the total sperm (between nine and 1388 times fewer sperm than found in the CP). Second, in partial agreement with Devine, we observed that there are more sperm in the anterior sections than the posteriormost sections of the plug; however, sperm were distributed further posteriorly than is indicated by Devine's schematic drawing (Devine, 1975: fig. 1). Devine (1975) stated that 'a far more homogeneous distribution of sperm in the plug would be likely if the contents of the ductus deferens contributed significantly to the plug material.' Our observations reveal exactly this pattern: a more homogeneous distribution of sperm, consistent with material from the ductus deferens (i.e. sperm) being embedded within the plug. Furthermore, our results are consistent with observations of sperm transport in this species. Halpert *et al*. (1982) found that 6 hours after mating only slightly more sperm are found in the posterior oviduct of *T. sirtalis parietalis* than in that of unmated control females (although these authors did not count the sperm or conduct statistical analyses). Sperm moved from the CP to the posterior oviduct after 24 h (Halpert *et al.*, 1982: 152). Thus, Halpert *et al.*'s (1982) description of sperm transport also supports the hypothesis that sperm are gradually released from the CP as it dissolves within the female's cloaca.

Most of the sperm inseminated is contained in the CP, rather than in the oviducts, as was previously thought (Devine, 1975). An anatomical explanation for our observation is that oviductal sphincters close, or the muscles of the vaginal pouch constrict, during copulation, and thus prevent sperm from entering the oviducts, causing it instead to swirl into the cranial–medial portions of the plug matrix. Thus, there may not be an adaptive explanation of the CP acting as a spermatophore. Instead, the spermatophore may be the result of female resistance to plug deposition, i.e. sexual conflict. This hypothesis could be tested by manipulating the female's ability to constrict the vaginal pouch during copulation using local anaesthetics.

To the extent that the copulatory plug of *T. sirtalis parietalis* limits female remating, it is a form of sexual conflict (Devine, 1984; Stockley, 1997; Arnqvist & Rowe, 2005; Uhl *et al.*, 2010). Although the loss of sperm may seem like a disadvantage for the female, she might thereby be able to void sperm from unwanted suitors (as do feral fowl; Pizzari & Birkhead, 2000). Given that female *T. sirtalis parietalis* have little pre-copulatory mate choice (Shine, O'Connor & Mason, 2000b; Shine, Langkilde & Mason, 2003), voiding of 'unwanted' sperm post-copulation might be the only mechanism by which a female could influence the paternity of her offspring. A CP would prevent this form of cryptic female choice. In a related species, *Thamnophis marcianus*, after sperm transfer, females begin to roll during some copulations, thus terminating coitus and preventing plug deposition (Perry-Richardson, Schofield & Ford, 1990). Hence, copulation duration may represent sexual conflict in garter snakes (King *et al.*, 2009). From the male's perspective the plug may function as a time-release capsule to 'out-wait' the oviductal sphincters and/or vaginal pouch constriction, which otherwise would prevent sperm from advancing into the oviducts during copulation. Thus, the sperm leakage reduction function of the CP might be a partial resolution of sexual conflict in a system where females would otherwise eject sperm (perhaps because their pre-copulatory choice is limited; Gowaty, 1997; Arnqvist & Rowe, 2005). If there truly is sexual conflict over plug deposition, then the determinants of variation in copulation duration and plug deposition would be fertile ground for further research in this and other species with different mating systems and operational sex ratios.

Plug deposition

Ejaculates are costly for males to produce because they contain not only sperm, but also accessory proteins and other substances that keep sperm viable and protect them from the harsh environment within the female reproductive tract (Dewsbury, 1982; Ramm, Parker & Stockley, 2005; Poiani, 2006; Suarez & Pacey, 2006). The CP of *T. sirtalis parietalis* can represent more than 0.4% of a male's mass (Shine *et al*., 2000a; C. R. Friesen & R. T. Mason, unpubl. data), which represents a substantial energetic investment (C. R. Friesen, D. R. Powers & R. T. Mason, unpubl. data). Given the leakage of sperm after matings that do not involve a CP, males would gain little or no paternity without plug deposition. Thus, the prevention of sperm leakage may constitute a major benefit to plug production.

Effect of multiple matings on plug deposition

A male snake's prior mating history has a strong effect on plug deposition. Like Shine *et al*. (2000a), we found no difference in plug deposition between first and second matings. However, we found that the likelihood of plug deposition dropped off significantly after the second mating: only 40% of third matings and only 20% of fourth matings produced a plug. The RSS remains hypertrophied throughout the spring (Krohmer, 2004), so presumably males have the capacity to produce more plug material. Notably, males are aphagous during the spring breeding season, and may be limited by their energy stores (O'Donnell *et al*., 2004), as evidenced by a rapid loss of mass during this period (Shine & Mason, 2005). In addition to the energetic requirements of mate searching and courtship, a male must maintain and allocate resources for plug production. We were unable to distinguish the effect of the number of matings from the time between matings on plug deposition (e.g. Oku & Kitsunezuka, 2011), so we cannot assess whether or how quickly males might replenish their stores of plug material. If they are not able to replenish plug material when there are ample mating opportunities, we might expect males to exhibit mate choice. In fact, males in this system do prefer to court and follow trails left by larger, and therefore more fecund, females (LeMaster & Mason, 2002; Shine *et al*., 2006b; Shine, 2012).

The observation that males are limited in the number of CPs they can produce suggests multiple further studies. For example, do males reduce courtship activity when they are unable to produce a plug? We predict that males would curtail courtship until plug material is replenished and then resume intense courtship; however, males may not be able to assess their reservoir of plug material. Some males continued to court and mate without producing plugs (although our observations hint at a reduced mating frequency for such animals). As Vx males can mate without becoming sperm-depleted, it would be interesting to use them to more explicitly test whether sperm depletion was a cue for curtailing courtship and mating effort. Furthermore, sperm numbers are important for success in sperm competition (Parker, 1990), and in many organisms copulation duration correlates directly with sperm numbers and can co-vary with male size (e.g. Simmons & Parker, 1992). Studies on correlates of variation in sperm counts per ejaculate would clarify the targets of postcopulatory selection within the *T. sirtalis parietalis* mating system.

CONCLUSION

Contrary to previous studies, our experiments suggest that the CP produced by male *T. sirtalis parietalis* has a range of functions. It not only reduces the probability of the female remating, but also reduces sperm leakage after copulation, and works as a spermatophore. All of these functions fit well with the idea that specific features of the mating aggregations of *T. sirtalis parietalis* impose intense selection against a long copulatory duration, and hence place a selective premium on a male's ability to transfer sperm quickly (Shine *et al*., 2000a). These multiple advantages may explain why male snakes invest so many resources into such an ineffective 'chastity belt'. We do not know enough about the mating systems of related garter snake species, or even of other populations within our study species, to disentangle the evolutionary history of the CP. Plausibly, it initially evolved primarily to fulfill one of the functions that we have identified, and was then co-opted for the others. For example, passive mate guarding may be a spandrel (Gould & Lewontin, 1979) affixed to an adaptation to prevent sperm leakage. The presence of sperm in the plug may be a proximate consequence of selection on female ability to control sperm transfer, and ultimately reflect sexual conflict in these populations. Future work could usefully explore the fitness benefits that accrue to males that invest additional resources into larger and larger CPs, and could also investigate the possibility of cryptic female control over the use of sperm from within a CP. Renal sexual segment secretions may confer other advantages to males, such as sperm mobility (Cuellar *et al*., 1972) or utero-muscular twisting that may also act as a copulatory plug (Nilson & Andrén, 1982; Siegel & Sever, 2006). These possible functions should be explored in garter snakes. Moreover, comparative studies among snake species that include data on mating systems, copulation durations, and female resistance behaviour (e.g. body rolls; King *et al.*, 2009), and how these factors relate to CP size, and sperm transfer and distribution, should shed light on the evolutionary pathways that lead to CP production.

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