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RAPID COMMUNICATION

Females Remate More Frequently When Mated With Sperm-Deficient Males

CHRISTOPHER R. FRIESEN[1,2]*, EMILY J. UHRIG[1], AND ROBERT T. MASON[1]

[1]School of Biological Sciences, University of Sydney, Sydney, Australia
[2]Department of Integrative Biology, Oregon State University, Corvallis, Oregon

ABSTRACT   Polyandry is a source of sexual conflict and males often try to limit female promiscuity. Consequently, male manipulation of receptivity via antiaphrodisiacs and copulatory plugs that prevent female remating can be a source of sexual conflict. This sexual conflict may be intensified when females must remate for fertility insurance. Male red-sided garter snakes produce a large, gelatinous copulatory plug that has been proposed to 1) physically prevent remating and 2) contain an antiaphrodisiac that reduces female receptivity. These males may become sperm depleted because of their dissociated reproductive pattern. If a female mates with a sperm deficient male and is also rendered unreceptive to further mating, then this represents a serious conflict. We tested whether female remating frequency is affected when females are mated with a male that produces a sperm-less copulatory plug. We show that females are significantly more likely to remate after mating with vasectomized males than intact males, even though vasectomized males still produce a copulatory plug. These results suggest that the ejaculate material of the plug does not contain an antiaphrodisiac. Instead, females may use sperm as a cue for post-copulatory mate assessment and seek to remate for the direct benefit of fertility insurance if they have mated with sperm-depleted males. *J. Exp. Zool. 321A:603–609, 2014.* © 2014 Wiley Periodicals, Inc.

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Explaining the occurrence of polyandry is one of the outstanding problems in the study of postcopulatory sexual selection (Arnqvist and Nilsson, 2000; Jennions and Petrie, 2000; Simmons, 2005; Parker and Birkhead, 2013; Pizzari and Wedell, 2013). Female promiscuity is proposed to occur when females receive indirect benefits (genetic; good genes, and bet hedging etc. reviewed in Jennions and Petrie, 2000), and/or direct benefits (e.g., extra paternal care, obscuring paternity in social groups etc. reviewed in Arnqvist and Nilsson, 2000). However, mating can be costly, and therefore the benefits must outweigh the costs of promiscuity (e.g., increased predation risk, physical harm, and disease exposure [Daly, '78; Rowe, '94; Thrall et al., 2000], respectively, but see Halliday and Arnold ['87]). Because of the potential costs of promiscuity, females may choose whether to remate based on cues she receives from her mate.

Fertilization insurance, where females remate to ensure sufficient sperm to fertilize their ova, is a direct benefit of female promiscuity and can occur in situations where sperm is limited. For example, female common viviparous lizards (*Lacerta vivipara*) that mate more frequently lay fewer infertile eggs (Uller and Olsson, 2005) and multiple mating increases fecundity in the water skink (*Eulamprus quoyii*, Noble et al., 2013), which suggests a single mating may not supply sufficient sperm to

fertilize all of a female's eggs. There may be fundamental limitations on the production of sperm and other ejaculate components because ejaculates may represent a substantial energetic expense (Dewsbury, '82; Olsson et al., '97; Parker and Pizzari, 2010; Friesen et al., in press). In anole lizards, males become sperm deleted if they do not alternate hemipene use (*Anolis sagrei*, Tokarz and Slowinski, '90), presumably because their two hemipenes and their associated sperm ducts are functionally independent (Fox, '77; Conner and Crews, '80). Thus, male anoles alternate which hemipene they use to allow time to replenish sperm in the ipsilateral sperm duct during multiple matings (Tokarz, '88; Tokarz and Kirkpatrick, '91). A compressed breeding period may impose an additional sperm-limitation. This is because males have restricted storage capacity and the time to replenish sperm stores is abridged (Wedell et al., 2002); females, then, should evolve mechanisms to mitigate the costs of mating with sperm depleted males, such as remating to ensure fertility after initially mating with a sperm depleted male.

The red-sided garter snake (*Thamnophis sirtalis parietalis*) is an ideal model to study the cues females use to make mating decisions based on fertilization insurance because males exhibit a dissociated reproductive pattern (Olsson and Madsen, '98). In this species, spermatogenesis occurs during late summer and ceases as the testes regress in the fall; males then store sperm over winter for use in the spring mating season (Crews, '84; Crews et al., '84; Krohmer et al., '87). Therefore, males have a limited amount of sperm for use during the spring and may mate enough to become depleted of sperm (there is a fivefold decrease in sperm numbers from the first to second mating [Friesen et al., 2014b]).

Male red-sided garter snakes deposit a large gelatinous copulatory plug that occludes the female's cloaca after mating. In arena trials, evidence suggests that the copulatory plug reduces female receptivity either by physically stretching the cloaca and/or by substances released by the plug, such as antiaphrodisiacs (Ross and Crews, '77; Ross and Crews, '78; Whittier and O'Connor, '91; Shine et al., 2000b; Mendonça and Crews, 2001). In essence, these studies showed that removing the copulatory plug soon after mating significantly increased female receptivity as measured by remating frequency. The copulatory plug is a spermatophore containing most of the sperm in the ejaculation that are released as the plug dissolves within the vaginal pouch of the female's cloaca (Friesen et al., 2013b, 2014b). Although males are significantly less likely to deposit more than two plugs over the course of two days (Friesen et al., 2013b), the plug material can be replenished because the renal sexual segment (Krohmer, 2004; Friesen et al., 2013b), unlike the testes, is hypertrophied and fully functional throughout the spring breeding season (late April–May). Therefore, a male with dwindling sperm stores may deposit a plug containing little or no sperm. Since males are able to produce plug material, but not sperm,

throughout the spring mating season, it is therefore possible that sperm released from the plug influences a female's receptivity, that is, it is the cue she uses to determine whether to remate or not.

In the current study, we tested whether female receptivity was affected by mating with a sperm-depleted male (i.e., vasectomized, Olsson et al., '94; Friesen et al., 2013b) compared with intact control males. This would indicate that females remate to receive a direct benefit from polyandry and test whether the copulatory plug material contains an antiaphrodisiac as previously proposed by Ross and Crews ('77, '78).

METHODS

Red-sided garter snakes are small (adult males average 45 cm snout-vent length [svl], and females 68 cm [svl]), nonvenomous natricine colubrids. Our study population, from which we collected 48 vigorously courting males and 48 newly emerged females on the same day in May 2012, is located near Inwood, Manitoba, Canada (50° 31.58′N 97° 29.71′W) and contains approximately 35,000 individuals (Shine et al., 2006).

Vasectomies

Males were randomly assigned to one of two treatment groups: vasectomy (Vx; $N = 24$) and intact controls ($N = 24$). Male size did not differ between these two groups (mass: $t_{46} = 0.653$, $P = 0.747$; snout-to-vent length, $t_{46} = 1.734$, $P = 0.084$). In squamate reptiles (e.g., lizards, snakes), each testis has a separate duct conveying sperm to one of two hemipenes (Fox, '77; Olsson et al., '94). Likewise, each kidney has a separate duct carrying secretions produced by the renal sexual segment (RSS), which produces the copulatory plug in garter snakes (Krohmer, 2004; Friesen et al., 2013b). The Vx treatment simulated females mating with a sperm-depleted male, such that a female would receive RSS secretions and a copulatory plug but not sperm. This technique was first used in sand lizards (*Lacerta agilis*) to test the role of RSS secretions in sperm competition trials (Olsson et al., '94) and is uniquely applicable to squamate reptiles due to their reproductive anatomy (Olsson and Madsen, '98). We have shown previously that Vx male red-sided garter snakes show no deficits: they court vigorously and mate within 24 hr of surgery and produce copulatory plugs at the same rate as either sham-surgery or unmanipulated, intact control males (Friesen et al., 2013b) and show no difference in copulation duration ($F_{1,49} = 0.168$, $P = 0.684$). Therefore, we felt it appropriate to utilize unmanipulated, intact control males in addition to the vasectomized males in mating trials. We performed vasectomies following Friesen et al. (2013b). All animals survived surgery and engaged in courtship within 24 hr after recovery. After mating trials, all vasectomized males were euthanized for whole-body parasite counts for a separate experiment (Uhrig et al., in prep.). During the dissections, males were checked to ensure the ligations were still intact after mating.

Vx Mating Trials

Two small circular arenas (45 cm dia. × 75 cm tall) were set up indoors at the Chatfield Research Station with each placed under a 250 W heat lamp 1 m above the animals (Friesen et al., 2014b). We placed the Vx males in one arena and the intact control males in the other. A single newly emerged female was placed into each arena. A sex ratio of 20–40 males to one female is common in and around the dens (Shine et al., 2001, 2006) and a male-skewed sex ratio facilitates vigorous male courtship behavior (Joy and Crews, '85). After copulation was initiated and had lasted 1 min, the male and female pair was gently removed to a separate, empty, circular arena so that they could copulate without interference from the other males; this separation also allowed easy observation of the termination of copulations (Friesen et al., 2014b). To keep the number of males constant during the mating trials (to ensure females experienced similar courtship intensities), mated males were replaced with an actively courting male that had tape placed over his cloaca to prevent him from mating.

Forty-three newly emerged seasonal virgin females mated with either an intact control or Vx male (83% of both the control and Vx males mated over 2 days, May 4–5, 2012). Three of these matings to Vx males did not produce plugs and the females were removed from the experiment. There were 40 mated females that received copulatory plugs available for the second mating trials (Vx, $N = 20$; Ctrl, $N = 20$). Females in these groups did not differ in size (SVL: $t_{20} = 0.615$, $P = 0.680$; mass: $t_{20} = 0.570$, $P = 0.572$). Each plug was marked with green food coloring (FD&C Green No. 3) to identify it as the original plug in subsequent unobserved matings (the food coloring stains the plug until it fully dissolves within the female's cloaca ([Friesen pers. obs., 2012] and does not affect sperm viability [Johnson and Welch, '99]). All females were then placed in a natural outdoor enclosure (3.65 m diameter × 1 m, 11.5 m^2). The floor of the enclosure was grass and brush like that of the aspen grove surrounding the Inwood den site. In this type of substrate, females and males form small mating balls after females mate in the den proper (Shine et al., 2001). Water was provided *ad libitum* in a 0.3 m diameter hole dug in the floor of the enclosure and lined with a clean sheet of plastic. One hundred vigorously courting males from the nearby den (<50 m away) were placed in the arena with the mated females. Females were collected and checked for new plugs every day for six days (a freshly deposited plug will dissolve in two days [Shine et al., 2000b]). A fresh (not green colored) plug was clear evidence that a female had remated.

RESULTS

Of the 20 females that mated with Vx males, 14 (70%) remated. However, during dissections of the Vx males, we found that four of them had unilateral vasectomies (i.e., only one of the two ductus deferens was securely ligated at the time of dissection). The females that mated with the unilateral Vx males were removed from the analysis; thus, 11 of 16 (68.8%) females mated to Vx males remated. Of the 20 females that mated with intact control males, 4 of 20 (20%) remated. Significantly more Vx mated females remated than females mated with controls (XLSTAT; Yates continuity correction (YCC) $\chi^2_{df\,1} = 6.801$, $P = 0.009$). If we are extremely conservative and assume the unilateral Vx males delivered sperm during mating and were, in essence, sham controls, adding the females they mated with to the control group (11/16 rematings Vx; 7/17 rematings controls) still yields a significant difference in remating rate (YCC $\chi^2_{df\,1} = 4.583$, $P = 0.032$).

DISCUSSION

Proximate Mechanisms

There are at least two nonmutually exclusive, mechanistic hypotheses that may account for the increased remating rates in our study: 1) the fluid derived from the ductus deferens may contain an antiaphrodisiac and/or 2) females have a form of proprioception or sperm receptor mediated mechanism allowing them to sense the sperm within their oviducts. We feel that consideration of the reproductive anatomy and physiology of squamates lends support to the second hypothesis, but we discuss both hypotheses below.

An Antiaphrodisiac: The Chemical Signal Hypothesis. In the garter snake, as sperm are liberated from the dissolving copulatory plug, other substances may be released at the same time. Ross and Crews ('77, '78) had suggested that RSS secretions contain an antiaphrodisiac rendering females relatively unreceptive for 48 hr in arena trials. In Drosophila, accessory gland proteins (Acps, Pitnick et al., 2009; Wolfner, 2009) reduce female receptivity, but such proteins are unknown in garter snakes. However, the copulatory plug of red-sided garter snakes, like the seminal fluid of many animals (Poiani, 2006), contains measureable levels of prostaglandins, such as PGF$_{2\alpha}$ (Whittier and O'Connor, '91). Prostaglandins are known to affect reproductive processes and behaviors in most vertebrates (Norris, 2007; Nelson, 2011) and female red-sided garter snakes exhibit a surge in Prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$) after mating, and injections of exogenous PGF$_{2\alpha}$ reduce female receptivity (Whittier and Crews, '86a, '89). The increase in PGF$_{2\alpha}$ in the female 6–24 hr post-mating (Whittier and Crews, '89) is consistent with liberation from the plug as it dissolves (Shine et al., 2000b). If the level of prostaglandins (PGs) accurately reflects the number of sperm inseminated, then they may be an honest cue that females use to make mating decisions. However, if males are the source of PGs that reduce a female's receptivity when she otherwise might mate again, then this might be a source of sexual conflict, in which the interests of the sexes diverge (Gowaty, '97; Arnqvist and Rowe, 2005).

In red-sided garter snakes, copulation is a period of conflict between males and females with females trying to reduce copulation duration and copulatory plug size (Friesen et al., 2014c). Smaller copulatory plugs probably have less PGs and the effect of exogenous PG is dose dependent (Whittier and Crews, '86a), with low doses having marginal effects on female receptivity. Therefore, if females limit the size of the copulatory plug, they would also limit the amount of male derived PGs. However, the $PGF_{2\alpha}$ found in the copulatory plug is not high enough to account for the increased plasma levels (Whittier and O'Connor, '91). Since PG is produced by many vertebrate tissues including the oviducts and ovaries, this evidence strongly suggests that females, rather than copulatory plugs or sperm, contribute to PG levels after mating.

Shine et al. (2000b) demonstrated that sperm, not the copulatory plug per se, was the cue that made mated female garter snakes less attractive than unmated females. Furthermore, our work corroborates findings of Mendonça and Crews (2001) in which the sensation of copulation did not influence receptivity, a result that contrasts with species such as anole lizards (Crews, '73) and golden hamsters (Buntin et al., '81). In red-sided garter snakes, the removal of the copulatory plug shortly after mating preserves female receptivity (Shine et al., 2000b). This result makes sense if females use sperm to assess their mate post copulation, because it takes time for sperm to be released as the spermatophore dissolves (Friesen et al., 2013b). This is similar to changes in female receptivity after spermatophore removal in plethodontid salamanders (e.g., Verrell, '91) and some insects (Simmons, 2001).

Garter snakes, like all other squamate reptiles, do not have distinct sex glands such as those found in mammals (e.g., prostate or seminal vesicles) or insects (e.g., accessory sex glands). Instead, the renal sexual segment, which is homologous with the prostate and seminal vesicles of mammals (Fox, '77; Crews, '80; Romer and Parsons, '86), is integrated within the kidney (reviewed in Aldridge et al., 2011). In humans and other mammals, the seminal vesicles are the principle source of PGs (Kelly, '81; Bendvold et al., '85). Prostaglandins are not found in high concentrations in the vas (aka ductus) deferens (Kelly, '81; Bendvold et al., '85) except at the low levels required for the simulation of smooth muscle contraction during ejaculation and signaling of spermatogenesis (Ruan et al., 2011). This evidence suggests that the ductus deferens is not a major source of prostaglandins. Thus, our results suggest that sperm or sperm surface protein(s) are the signal females use to assess the fertility of their mate.

Oviductal Sperm "Proprioception": Sperm as a Signal Hypothesis. The presence of sperm within the oviduct has been documented to elicit physiological and behavioral changes in females of many species. Within the reproductive tract of female *Drosophila*, the presence of sperm stimulates the nervous system and the regulation of genes which induce changes within the female in preparation for oviposition, and reduces female receptivity (Heifetz and Wolfner, 2004; McGraw et al., 2004). Females of some moth species, only become unreceptive if their spermatheca are full of sperm (Giebultowicz et al., '91; Karube and Kobayashi, '99) which is activated by innervated stretch receptors in bursa copulatrix (Sugawara, '79) and/or of the setae (Lum and Arbogast, '80; reviewed in Wedell, 2005). Matings without sperm transfer also increase female remating in spiders and fruit flies (Aisenberg and Costa, 2005), respectively (Kraaijeveld and Chapman, 2004).

To our knowledge, there are no studies documenting the presence of mechanoreceptors in the oviducts of snakes and this aspect is worthy of investigation. However, cilia, structures known to exhibit mechanoreception in vertebrates (Bloodgood, 2010; Takeda and Narita, 2012; Rupik, 2013), are abundant in the posterior regions of the oviduct (Fox, '56; Hoffman and Wimsatt, '72; Halpert et al., '82; Siegel et al., 2011). There are also sperm storage crypts in the posterior oviduct where sperm aggregate (Halpert et al., '82) while the plug dissolves (Shine et al., 2000b; Friesen et al., 2013b); sperm later move to anterior sperm storage crypts close to where fertilization occurs. The cilia in the posterior oviduct may allow females to sense sperm stores within the reproductive tract and use this information to evaluate whether their recent mate transferred sperm. Thus, females in this species may use signals generated within posterior sperm storage organs for post-copulatory mate assessment.

Evolutionary Implications

Female red-sided garter snakes are more likely to remate if they do not receive sperm from the ductus deferens regardless of whether they receive a renal sexual segment (RSS)-derived copulatory plug as the major seminal fluid component. This suggests that females may assess their mate post-copulation and that fertility insurance is an important driver of female promiscuity in this system, although sexual conflict may also be important (Shine et al., 2000a; Friesen et al., 2013b, 2014c). Female red-sided garter snakes emerge during the spring mating season slightly later than males (Gregory, '74; Shine et al., 2006). Thus, females may not know the mating history of their mates and, consequently, risk mating with sperm-depleted males.

The risk and costs of mating with infertile males have been proposed to select for late emergence in female sand lizards (Olsson and Madsen, '96). Sand lizards undergo spermatogenesis during the spring, thus, males need time to become fertile. However, female red-sided garter snakes face a different problem. Given their dissociated reproductive pattern males may become depleted of sperm (Friesen et al., 2014b). For females who mate with such sperm-depleted males, remating during the spring is likely an adaptation to mitigate sperm limitation. Additionally, matings during the fall season (Whittier and Crews, '86b;

Mendonca and Crews, '89; Friesen pers. obs., 2008) may be another opportunity to mitigate mating with a sperm depleted male in the spring. However, sperm from fall matings may suffer considerable attrition, as only 25% of offspring are fathered by sperm stored overwinter (Friesen et al., 2013a).

Our experiment using Vx-male mated females to examine remating rates establishes the foundation for several lines of inquiry including answering the question, "Are females ever sperm-limited?" For some species, when females mate with sperm-depleted males, the answer is yes (reviewed in Wedell et al., 2002). In arena trials, male garter snakes will mate multiply (4–5 times) if given the opportunity and some males are more successful than others (Friesen pers. obs., 2007–2009, Friesen et al., 2014a, in press). Male sperm stores are fixed in early fall and, during spring matings, sperm counts drop significantly from a male's first mating to his next (Friesen et al., 2014b). If some males are systematically more successful, then they may become sperm-exhausted, but females would also be more likely to mate with them because of the male's mating proficiency. In this case, a female may mate with, essentially, a sterile male. A follow-up experiment could collect ejaculates over successive matings to establish the rate of sperm depletion. In parallel, the effect of female sperm-limitation on female reproductive success could be addressed by testing for an effect of male mate number on fecundity.

Conclusion and Future Directions. We feel the evidence is in favor of females using sperm as a cue to assess male quality (i.e., sperm-depleted). The ductus deferens of snakes functions in sperm storage analogous to the epididymis of the mammalian testes and as such exhibits secretory capacity (Trauth and Sever, 2011). In keeping with the primary function of the epididymis, these secretions undoubtedly function to maintain sperm during long-term sperm storage over winter. However, it is possible, though unlikely, that the ductus deferens contains substances, other than sperm, which inhibit female receptivity. Thus, to fully remove any uncertainty between the "sperm sense" and "antiaphrodisiac" hypotheses, artificial insemination techniques could be used in combination with Vx male matings. Artificial insemination has been conducted in snakes (Quinn et al., '89; Mattson et al., 2007) including red-sided garter snakes (Friesen, unpublished data). Thus, it may be possible to inseminate females, which received a plug (*sans* sperm) from mating with a Vx male, with washed and unwashed sperm. Such an approach would address whether receptivity is affected by sperm *per se* or some other component of the ejaculate. These experiments would allow us to unequivocally differentiate between these hypotheses. Furthermore, histology and/or immunohistochemistry could be used to identify the prerequisite afferent innervation and whether it is associated with the sperm storage crypts and their response to the presence of sperm. The "sperm sense" hypothesis suggests a form of cryptic female choice in which females remate if they mate with a suboptimal (i.e., sperm-depleted) male. Sensing the presence of sperm could form the mechanistic basis for the evolution of more elaborate mechanisms for sperm selection and/or to inform female remating behavior (i.e., whether to seek out a second mate or not) in this species.

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