

Females remate more frequently when mated with sperm-deficient males

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1 **Females remate more frequently when mated with sperm-deficient males**

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21 **Abstract**

22 Polyandry is a source of sexual conflict and males often try to limit female promiscuity.
23 Consequently, male manipulation of receptivity via antiaphrodisiacs and copulatory plugs that
24 prevent female remating can be a source of sexual conflict. This sexual conflict may be
25 intensified when females must remate for fertility insurance. Male red-sided garter snakes
26 produce a large, gelatinous copulatory plug that has been proposed to 1) physically prevent
27 remating and 2) contain an antiaphrodisiac that reduces female receptivity. These males may
28 become sperm depleted because of their dissociated reproductive pattern. If a female mates with
29 a sperm deficient male and is also rendered unreceptive to further mating, then this represents a
30 serious conflict. We tested whether female remating frequency is affected when females are
31 mated with a male that produces a sperm-less copulatory plug. We show that females are
32 significantly more likely to remate after mating with vasectomized males than intact males, even
33 though vasectomized males still produce a copulatory plug. These results suggest that the
34 ejaculate material of the plug does not contain an antiaphrodisiac. Instead, females may use
35 sperm as a cue for post-copulatory mate assessment and seek to remate for the direct benefit of
36 fertility insurance if they have mated with sperm-depleted males.

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42 **Keywords**

43 Sperm limitation, sexual conflict, cryptic female choice, postcopulatory sexual selection, garter
44 snake

45 Introduction

46 Explaining the occurrence of polyandry is one of the outstanding problems in the study of
47 postcopulatory sexual selection (Arnqvist & Nilsson, 2000; Jennions & Petrie, 2000; Parker &
48 Birkhead, 2013; Pizzari & Wedell, 2013; Simmons, 2005). Female promiscuity is proposed to
49 occur when females receive indirect benefits (genetic; good genes, and bet hedging etc. reviewed
50 in Jennions & Petrie, 2000), and/or direct benefits (e.g., extra paternal care, obscuring paternity
51 in social groups etc. reviewed in Arnqvist & Nilsson, 2000). However, mating can be costly, and
52 therefore the benefits must outweigh the costs of promiscuity (e.g., increased predation risk,
53 physical harm, and disease exposure [Daly, 1978; Rowe, 1994; Thrall, Antonovics & Dobson,
54 2000 respectively, but see Halliday and Arnold 1987]). Because of the potential costs of
55 promiscuity, females may choose whether to remate based on cues she receives from her mate.

56 Fertilization insurance, where females remate to ensure sufficient sperm to fertilize their
57 ova, is a direct benefit of female promiscuity and can occur in situations where sperm is limited.
58 For example, female common viviparous lizards (*Lacerta vivipara*) that mate more frequently
59 lay fewer infertile eggs (Uller & Olsson, 2005) and multiple mating increases fecundity in the
60 water skink (*Eulamprus quoyii*) (Noble, Keogh & Whiting, 2013), which suggests a single
61 mating may not supply sufficient sperm to fertilize all of a female's eggs. There may be
62 fundamental limitations on the production of sperm and other ejaculate components because
63 ejaculates may represent a substantial energetic expense (Dewsbury, 1982; Friesen, Powers,
64 Copenhaver & Mason, in review; Olsson, Madsen & Shine, 1997; Parker & Pizzari, 2010). In
65 anole lizards, males become sperm depleted if they do not alternate hemipene use (*Anolis sagrei*,
66 Tokarz & Slowinski, 1990), presumably because their two hemipenes and their associated sperm
67 ducts are functionally independent (Conner & Crews, 1980; Fox, 1977). Thus, male anoles
68 alternate which hemipene they use to allow time to replenish sperm in the ipsilateral sperm duct

69 during multiple matings (Tokarz, 1988; Tokarz & Kirkpatrick, 1991). A compressed breeding
70 period may impose an additional sperm-limitation. This is because males have restricted storage
71 capacity and the time to replenish sperm stores is abridged (Wedell, Gage & Parker, 2002);
72 females, then, should evolve mechanisms to mitigate the costs of mating with sperm depleted
73 males, such as remating to ensure fertility after initially mating with a sperm depleted male.

74 The red-sided garter snake (*Thamnophis sirtalis parietalis*) is an ideal model to study the
75 cues females use to make mating decisions based on fertilization insurance because males exhibit
76 a dissociated reproductive pattern (Olsson & Madsen, 1998). In this species, spermatogenesis
77 occurs during late summer and ceases as the testes regress in the fall; males then store sperm
78 over winter for use in the spring mating season (Crews, 1984; Crews, Camazine, Diamond,
79 Mason, Tokarz & Garstka, 1984; Krohmer, Grassman & Crews, 1987). Therefore, males have a
80 limited amount of sperm for use during the spring and may mate enough to become depleted of
81 sperm (there is a 5 fold decrease in sperm numbers from the first to second mating Friesen,
82 Squire & Mason, 2014b).

83 Male red-sided garter snakes deposit a large gelatinous copulatory plug that occludes the
84 female's cloaca after mating. In arena trials, evidence suggests that the copulatory plug reduces
85 female receptivity either by physically stretching the cloaca and/or by substances released by the
86 plug, such as antiaphrodisiacs (Mendonça & Crews, 2001; Ross & Crews, 1978; Ross & Crews,
87 1977; Shine, Olsson & Mason, 2000b; Whittier & O'Connor, 1991). In essence, these studies
88 showed that removing the copulatory plug soon after mating significantly increased female
89 receptivity as measured by remating frequency. The copulatory plug is a spermatophore
90 containing most of the sperm in the ejaculate that are released as the plug dissolves within the
91 vaginal pouch of the female's cloaca (Friesen, Shine, Krohmer & Mason, 2013b; Friesen et al.,

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 (Friesen et al., 2013b; Krohmer, 2004), 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, i.e. 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, Friesen et al., 2013b; Olsson, Gullberg & Tegelström, 1994) 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 (1977 & 1978).

Methods

Red-sided garter snakes are small (adult males average 45 cm snout-vent length (svl), and females 68 cm svl), non-venomous 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, Langkilde, Wall & Mason, 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: $t_{46} =$

115 0.653, $P = 0.747$; snout-to-vent length, $t_{46} = 1.734$, $P = 0.084$). In squamate reptiles (e.g., lizards,
116 snakes), each testis has a separate duct conveying sperm to one of two hemipenes (Fox, 1977;
117 Olsson et al., 1994). Likewise, each kidney has a separate duct carrying secretions produced by
118 the renal sexual segment (RSS), which produces the copulatory plug in garter snakes (Friesen et
119 al., 2013b; Krohmer, 2004). The Vx treatment simulated females mating with a sperm-depleted
120 male, such that a female would receive RSS secretions and a copulatory plug but not sperm. This
121 technique was first used in sand lizards (*Lacerta agilis*) to test the role of RSS secretions in
122 sperm competition trials (Olsson et al., 1994) and is uniquely applicable to squamate reptiles due
123 to their reproductive anatomy (Olsson & Madsen, 1998). We have shown previously that Vx
124 male red-sided garter snakes show no deficits: they court vigorously and mate within 24 h of
125 surgery and produce copulatory plugs at the same rate as either sham-surgery or unmanipulated,
126 intact control males (Friesen et al., 2013b) and show no difference in copulation duration ($F_{1,49} =$
127 0.168 , $P = 0.684$). Therefore, we felt it appropriate to utilize unmanipulated, intact control males
128 in addition to the vasectomized males in mating trials. We performed vasectomies following
129 Friesen et al (2013b). All animals survived surgery and engaged in courtship within 24 hours
130 after recovery. After mating trials, all vasectomized males were euthanized for whole-body
131 parasite counts for a separate experiment (Uhrig et al. in prep.). During the dissections, males
132 were checked to ensure the ligations were still intact after mating.

133

134 *Vx mating trials*

135

136 Two small circular arenas (45 cm dia. x 75 cm tall) were set up indoors at the Chatfield Research
137 Station with each placed under a 250W heat lamp 1m above the animals (Friesen et al., 2014b).

138 We placed the Vx males in one arena and the intact control males in the other. A single newly
139 emerged female was placed into each arena. A sex ratio of 20-40 males to one female is common
140 in and around the dens (Shine, Elphick, Harlow, Moore, LeMaster & Mason, 2001; Shine et al.,
141 2006) and a male-skewed sex ratio facilitates vigorous male courtship behavior (Joy & Crews,
142 1985). After copulation was initiated and had lasted one minute, the male and female pair was
143 gently removed to a separate, empty, circular arena so that they could copulate without
144 interference from the other males; this separation also allowed easy observation of the
145 termination of copulations (Friesen et al. 2014b). To keep the number of males constant during
146 the mating trials (to ensure females experienced similar courtship intensities), mated males were
147 replaced with an actively courting male that had tape placed over his cloaca to prevent him from
148 mating.

149 Forty-three newly emerged seasonal virgin females mated with either an intact control
150 or Vx male (83% of both the control and Vx males mated over two days, 5/4-5/5 2012). Three of
151 these matings to Vx males did not produce plugs and the females were removed from the
152 experiment. There were 40 mated females that received copulatory plugs available for the second
153 mating trials (Vx, N = 20; Ctrl, N = 20). Females in these groups did not differ in size (SVL: t_{20}
154 = 0.615, P = 0.680; mass: t_{20} = 0.570, P = 0.572). Each plug was marked with green food
155 coloring (FD&C Green No. 3) to identify it as the original plug in subsequent unobserved
156 matings (the food coloring stains the plug until it fully dissolves within the female's cloaca
157 ((Friesen pers. obs. 2012) and does not affect sperm viability (Johnson & Welch, 1999)). All
158 females were then placed in a natural outdoor enclosure (3.65m diameter x 1m, 11.5m²). The
159 floor of the enclosure was grass and brush like that of the aspen grove surrounding the Inwood
160 den site. In this type of substrate, females and males form small mating balls after females mate

161 in the den proper (Shine et al., 2001). Water was provided *ad libitum* in a 0.3m diameter hole
162 dug in the floor of the enclosure and lined with a clean sheet of plastic. One hundred vigorously
163 courting males from the nearby den (< 50m away) were placed in the arena with the mated
164 females. Females were collected and checked for new plugs every day for six days (a freshly
165 deposited plug will dissolve in two days (Shine et al., 2000b)). A fresh (not green colored) plug
166 was clear evidence that a female had remated.

167 **Results**

168 Of the 20 females that mated with Vx males, 14 (70%) remated. However, during
169 dissections of the Vx males, we found that four of them had unilateral vasectomies (i.e., only one
170 of the two ductus deferens was securely ligated at the time of dissection). The females that mated
171 with the unilateral Vx males were removed from the analysis; thus, 11 of 16 (68.8%) females
172 mated to Vx males remated. Of the 20 females that mated with intact control males, 4 of 20
173 (20%) remated. Significantly more Vx mated females remated than females mated with controls
174 (XLSTAT; Yates continuity correction (YCC) $\chi^2_{df 1} = 6.801$, $P = 0.009$). If we are extremely
175 conservative and assume the unilateral Vx males delivered sperm during mating and were, in
176 essence, sham controls, adding the females they mated with to the control group (11/16
177 rematings Vx; 7/17 rematings controls) still yields a significant difference in remating rate (YCC
178 $\chi^2_{df 1} = 4.583$, $P = 0.032$).

179 **Discussion**

180 **Proximate mechanisms**

181 There are at least two non-mutually exclusive, mechanistic hypotheses that may account
182 for the increased remating rates in our study: 1) the fluid derived from the ductus deferens may
183 contain an antiaphrodisiac and/or 2) females have a form of proprioception or sperm receptor
184 mediated mechanism allowing them to sense the sperm within their oviducts. We feel that

185 consideration of the reproductive anatomy and physiology of squamates lends support to the
186 second hypothesis, but we discuss both hypotheses below.

187 *An antiaphrodisiac: the chemical signal hypothesis*

188 In the garter snake, as sperm are liberated from the dissolving copulatory plug, other
189 substances may be released at the same time. Ross and Crews (1978; 1977) had suggested that
190 RSS secretions contain an antiaphrodisiac rendering females relatively unreceptive for 48 hours
191 in arena trials. In *Drosophila*, accessory gland proteins (Acps) (Pitnick, Wolfner & Suarez, 2009;
192 Wolfner, 2009) reduce female receptivity, but such proteins are unknown in garter snakes.
193 However, the copulatory plug of red-sided garter snakes, like the seminal fluid of many animals
194 (Poiani, 2006), contains measureable levels of prostaglandins, such as $\text{PGF}_{2\alpha}$ (Whittier &
195 O'Connor, 1991). Prostaglandins are known to affect reproductive processes and behaviors in
196 most vertebrates (Nelson, 2011; Norris, 2007) and female red-sided garter snakes exhibit a surge
197 in Prostaglandin $\text{F}_{2\alpha}$ ($\text{PGF}_{2\alpha}$) after mating, and injections of exogenous $\text{PGF}_{2\alpha}$ reduce female
198 receptivity (Whittier & Crews, 1986a; Whittier & Crews, 1989). The increase in $\text{PGF}_{2\alpha}$ in the
199 female 6-24 hours post-mating (Whittier & Crews, 1989) is consistent with liberation from the
200 plug as it dissolves (Shine et al., 2000b). If the level of prostaglandins (PGs) accurately reflects
201 the number of sperm inseminated, then they may be an honest cue that females use to make
202 mating decisions. However, if males are the source of PGs that reduce a female's receptivity
203 when she otherwise might mate again, then this might be a source of sexual conflict, in which the
204 interests of the sexes diverge (Arnqvist & Rowe, 2005; Gowaty, 1997).

205 In red-sided garter snakes, copulation is a period of conflict between males and females
206 with females trying to reduce copulation duration and copulatory plug size (Friesen, Uhrig,
207 Squire, Mason & Brennan, 2014c). Smaller copulatory plugs probably have less PGs and the

208 effect of exogenous PG is dose dependent (Whittier & Crews, 1986a), with low doses having
209 marginal effects on female receptivity. Therefore, if females limit the size of the copulatory plug,
210 they would also limit the amount of male derived PGs. However, the $\text{PGF}_{2\alpha}$ found in the
211 copulatory plug is not high enough to account for the increased plasma levels (Whittier &
212 O'Connor, 1991). Since PG is produced by many vertebrate tissues including the oviducts and
213 ovaries, this evidence strongly suggests that females, rather than copulatory plugs or sperm,
214 contribute to PG levels after mating.

215 Shine et al. (2000b) demonstrated that sperm, not the copulatory plug per se, was the cue
216 that made mated female garter snakes less attractive than unmated females. Furthermore, our
217 work corroborates findings of Mendonça and Crews (2001) in which the sensation of copulation
218 did not influence receptivity, a result that contrasts with species such as anole lizards (Crews,
219 1973) and golden hamsters (Buntin, Ciaccio & Lisk, 1981). In red-sided garter snakes, the
220 removal of the copulatory plug shortly after mating preserves female receptivity (Shine et al.,
221 2000b). This result makes sense if females use sperm to assess their mate post copulation,
222 because it takes time for sperm to be released as the spermatophore dissolves (Friesen et al.,
223 2013b). This is similar to changes in female receptivity after spermatophore removal in
224 plethodontid salamanders (e.g., Verrell, 1991) and some insects (Simmons, 2001).

225 Garter snakes, like all other squamate reptiles, do not have distinct sex glands such as
226 those found in mammals (e.g., prostate or seminal vesicles) or insects (e.g., accessory sex
227 glands). Instead, the renal sexual segment, which is homologous with the prostate and seminal
228 vesicles of mammals (Crews, 1980; Fox, 1977; Romer & Parsons, 1986), is integrated within the
229 kidney (reviewed in Aldridge, Jellen, Siegel & Wisniewski, 2011). In humans and other
230 mammals the seminal vesicles are the principle source of PGs (Kelly, 1981, Bendvold et al.,

231 1985). Prostaglandins are not found in high concentrations in the vas (aka ductus) deferens
232 (Bendvold, Svanborg, Bygdeman & Noren, 1985; Kelly, 1981) except at the low levels required
233 for the simulation of smooth muscle contraction during ejaculation and signaling of
234 spermatogenesis (Ruan, Zhou & Chan, 2011). This evidence suggests that the ductus deferens is
235 not a major source of prostaglandins. Thus, our results suggest that sperm or sperm surface
236 protein(s) are the signal females use to assess the fertility of their mate.

237 *Oviductal sperm “proprioception”: sperm as a signal hypothesis*

238 The presence of sperm within the oviduct has been documented to elicit physiological
239 and behavioral changes in females of many species. Within the reproductive tract of female
240 *Drosophila*, the presence of sperm stimulates the nervous system and the regulation of genes
241 which induce changes within the female in preparation for oviposition, and reduces female
242 receptivity (Heifetz & Wolfner, 2004; McGraw, Gibson, Clark & Wolfner, 2004). Females of
243 some moth species, only become unreceptive if their spermatheca are full of sperm
244 (Giebultowicz, Raina, Uebel & Ridgway, 1991; Karube & Kobayashi, 1999) which is activated
245 by innervated stretch receptors in bursa copulatrix (Sugawara, 1979) and/or of the setae (Lum &
246 Arbogast, 1980; reviewed in Wedell, 2005). Matings without sperm transfer also increase female
247 remating in spiders and fruit flies (Aisenberg & Costa, 2005 respectively; Kraaijeveld &
248 Chapman, 2004).

249 To our knowledge, there are no studies documenting the presence of mechanoreceptors in
250 the oviducts of snakes and this aspect is worthy of investigation. However, cilia, structures
251 known to exhibit mechanoreception in vertebrates (Bloodgood, 2010; Rupik, 2013; Takeda &
252 Narita, 2012), are abundant in the posterior regions of the oviduct (Fox, 1956; Halpert, Garstka
253 & Crews, 1982; Hoffman & Wimsatt, 1972; Siegel, Miralles, Chabarria & Aldridge, 2011).

254 There are also sperm storage crypts in the posterior oviduct where sperm aggregate (Halpert et
255 al., 1982) while the plug dissolves (Friesen et al., 2013b; Shine et al., 2000b); sperm later move
256 to anterior sperm storage crypts close to where fertilization occurs. The cilia in the posterior
257 oviduct may allow females to sense sperm stores within the reproductive tract and use this
258 information to evaluate whether their recent mate transferred sperm. Thus, females in this
259 species may use signals generated within posterior sperm storage organs for post-copulatory
260 mate assessment.

261

262 **Evolutionary implications**

263 Female red-sided garter snakes are more likely to remate if they do not receive sperm
264 from the ductus deferens regardless of whether they receive a renal sexual segment (RSS)-
265 derived copulatory plug as the major seminal fluid component. This suggests that females may
266 assess their mate post-copulation and that fertility insurance is an important driver of female
267 promiscuity in this system, although sexual conflict may also be important (Friesen et al., 2013b;
268 Friesen et al., 2014c; Shine, O'Connor & Mason, 2000a). Female red-sided garter snakes emerge
269 during the spring mating season slightly later than males (Gregory, 1974; Shine et al., 2006).
270 Thus, females may not know the mating history of their mates and, consequently, risk mating
271 with sperm-depleted males.

272 The risk and costs of mating with infertile males have been proposed to select for late
273 emergence in female sand lizards (Olsson & Madsen, 1996). Sand lizards undergo
274 spermatogenesis during the spring, thus, males need time to become fertile. However, female
275 red-sided garter snakes face a different problem. Given their dissociated reproductive pattern
276 males may become depleted of sperm (Friesen et al., 2014b). For females who mate with such

277 sperm-depleted males, remating during the spring is likely an adaptation to mitigate sperm
278 limitation. Additionally, matings during the fall season (Mendonca & Crews, 1989; Friesen pers.
279 obs. 2008; Whittier & Crews, 1986b) may be another opportunity to mitigate mating with a
280 sperm depleted male in the spring. However, sperm from fall matings may suffer considerable
281 attrition, as only 25% of offspring are fathered by sperm stored overwinter (Friesen, Mason,
282 Arnold & Estes, 2013a).

283 Our experiment using Vx-male mated females to examine remating rates establishes the
284 foundation for several lines of inquiry including answering the question, “Are females ever
285 sperm-limited?”. For some species, when females mate with sperm-depleted males, the answer is
286 yes (reviewed in Wedell *et al.* 2002). In arena trials, male garter snakes will mate multiply (4-5
287 times) if given the opportunity and some males are more successful than others (Friesen pers.
288 obs. 2007-9, Friesen, Kerns & Mason, 2014a; Friesen et al., in review). Male sperm stores are
289 fixed in early fall and, during spring matings, sperm counts drop significantly from a male’s first
290 mating to his next (Friesen et al., 2014b). If some males are systematically more successful, then
291 they may become sperm-exhausted, but females would also be more likely to mate with them
292 because of the male’s mating proficiency. In this case, a female may mate with, essentially, a
293 sterile male. A follow-up experiment could collect ejaculates over successive matings to
294 establish the rate of sperm depletion. In parallel, the effect of female sperm-limitation on female
295 reproductive success could be addressed by testing for an effect of male mate number on
296 fecundity.

297 *Conclusion and Future directions*

298 We feel the evidence is in favor of females using sperm as a cue to assess male quality
299 (i.e., sperm-depleted). The ductus deferens of snakes functions in sperm storage analogous to the

300 epididymis of the mammalian testes and as such exhibits secretory capacity (Trauth & Sever,
301 2011). In keeping with the primary function of the epididymis, these secretions undoubtedly
302 function to maintain sperm during long-term sperm storage over winter. However, it is possible,
303 though unlikely, that the ductus deferens contains substances, other than sperm, which inhibit
304 female receptivity. Thus, to fully remove any uncertainty between the “sperm sense” and
305 “antiaphrodisiac” hypotheses, artificial insemination techniques could be used in combination
306 with Vx male matings. Artificial insemination has been conducted in snakes (Mattson, Vries,
307 McGuire, Krebs, Louis & Loskutoff, 2007; Quinn, Blasedel & Platz Jr, 1989) including red-
308 sided garter snakes (Friesen unpublished data). Thus, it may be possible to inseminate females,
309 which received a plug (*sans* sperm) from mating with a Vx male, with washed and unwashed
310 sperm. Such an approach would address whether receptivity is affected by sperm *per se* or some
311 other component of the ejaculate. These experiments would allow us to unequivocally
312 differentiate between these hypotheses. Furthermore, histology and/or immunohistochemistry
313 could be used to identify the prerequisite afferent innervation and whether it is associated with
314 the sperm storage crypts and their response to the presence of sperm. The “sperm sense”
315 hypothesis suggests a form of cryptic female choice in which females remate if they mate with a
316 suboptimal (i.e., sperm-depleted) male. Sensing the presence of sperm could form the
317 mechanistic basis for the evolution of more elaborate mechanisms for sperm selection and/or to
318 inform female remating behavior (i.e., whether to seek out a second mate or not) in this species.

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327 **Animal Ethics**

328 Procedures performed on animals were approved by Oregon State University [IACUC 2009-11
329 ACUP-3738], and the research was conducted under permit from Manitoba Conservation
330 [WB1240].

331 **Data archiving**

332 All data is contained within the manuscript.

333

334 **Conflict of Interest**

335 We, the authors, declare no conflict of interest.

336

337 **References**

338

- 339 **Aisenberg A, Costa FG. 2005.** Females mated without sperm transfer maintain high sexual receptivity in
340 the wolf spider *Schizocosa malitiosa*. *Ethology* **111**: 545-558.
- 341 **Aldridge RD, Jellen BC, Siegel DS, Wisniewski SS. 2011.** The sexual segment of the kidney. In:
342 Aldridge RD and M. SD, eds. *Reproductive biology and phylogeny of snakes*. Enfield, NH; Boca
343 Raton, FL: Science Publishers ; Marketed and distributed by CRC Press. 477-509.
- 344 **Arnqvist G, Nilsson T. 2000.** The evolution of polyandry: multiple mating and female fitness in insects.
345 *Animal Behaviour* **60**: 145-164.
- 346 **Arnqvist G, Rowe L. 2005.** *Sexual Conflict*. Princeton University Press: Princeton, New Jersey.
- 347 **Bendvold E, Svanborg K, Bygdeman M, Noren S. 1985.** On the origin of prostaglandins in human
348 seminal fluid. *International Journal of Andrology* **8**: 37-43.
- 349 **Bloodgood RA. 2010.** Sensory reception is an attribute of both primary cilia and motile cilia. *Journal of*
350 *cell science* **123**: 505-509.
- 351 **Buntin JD, Ciaccio LA, Lisk RD. 1981.** Temporal aspects of mating-induced inhibition of sexual
352 receptivity and its recovery in the female golden hamster. *Behavioral and Neural Biology* **31**:
353 443-456.
- 354 **Conner J, Crews D. 1980.** Sperm transfer and storage in the lizard, *Anolis carolinensis*. *Journal of*
355 *Morphology* **163**: 331-348.
- 356 **Crews D. 1973.** Coition-induced inhibition of sexual receptivity in female lizards (*Anolis carolinensis*).
357 *Physiology & Behavior* **11**: 463-468.
- 358 **Crews D. 1980.** Studies in Squamate Sexuality. *Bioscience* **30**: 835-838.

- 359 **Crews D. 1984.** Gamete production, sex hormone secretion, and mating behavior uncoupled. *Hormones*
 360 *and Behavior* **18**: 22-28.
- 361 **Crews D, Camazine B, Diamond M, Mason R, Tokarz RR, Garstka WR. 1984.** Hormonal
 362 independence of courtship behavior in the male garter snake. *Hormones and Behavior* **18**: 29-41.
- 363 **Daly M. 1978.** The Cost of Mating. *The American Naturalist* **112**: 771-774.
- 364 **Dewsbury DA. 1982.** Ejaculate cost and male choice. *The American Naturalist* **119**: 601-610.
- 365 **Fox H. 1977.** The urogenital system of reptiles. In: Gans C, ed. *Biology of the Reptilia*. London, UK:
 366 Academic Press. 1-157.
- 367 **Fox W. 1956.** Seminal receptacles of snakes. *The Anatomical record* **124**: 519-539.
- 368 **Friesen CR, Kerns A, Mason R. 2014a.** Factors influencing paternity in multiply mated female red-
 369 sided garter snakes and the persistent use of sperm stored over winter. *Behavioral Ecology and*
 370 *Sociobiology* **68**: 1419-1430.
- 371 **Friesen CR, Mason RT, Arnold SJ, Estes S. 2013a.** Patterns of sperm use in two populations of Red-
 372 sided Garter Snake (*Thamnophis sirtalis parietalis*) with long-term female sperm storage.
 373 *Canadian Journal of Zoology* **92**: 33-40.
- 374 **Friesen CR, Powers DR, Copenhaver PE, Mason RT. in review.** Energetic costs associated with
 375 courtship and mating. *The American Naturalist*.
- 376 **Friesen CR, Shine R, Krohmer RW, Mason RT. 2013b.** Not just a chastity belt: the functional
 377 significance of mating plugs in garter snakes, revisited. *Biological Journal of the Linnean Society*
 378 **109**: 893-907.
- 379 **Friesen CR, Squire MK, Mason RT. 2014b.** Intrapopulation variation of ejaculate traits and sperm
 380 depletion in red-sided garter snakes. *Journal of Zoology* **292**: 192-201.
- 381 **Friesen CR, Uhrig EJ, Squire MK, Mason RT, Brennan PLR. 2014c.** Sexual conflict over mating in
 382 red-sided garter snakes (*Thamnophis sirtalis*) as indicated by experimental manipulation of
 383 genitalia. *Proceedings of the Royal Society B: Biological Sciences* **281**: 20132694.
- 384 **Giebultowicz JM, Raina AK, Uebel EC, Ridgway RL. 1991.** Two-step regulation of sex-pheromone
 385 decline in mated gypsy moth females. *Archives of Insect Biochemistry and Physiology* **16**: 95-
 386 105.
- 387 **Gowaty P. 1997.** Sexual dialectics, sexual selection, and variation in reproductive behavior *Feminism*
 388 *and evolutionary biology: Boundaries, intersections and frontiers*. New York: Chapman & Hall.
 389 351-384.
- 390 **Gregory PT. 1974.** Patterns of spring emergence of the red-sided garter snake (*Thamnophis sirtalis*
 391 *parietalis*) in the Interlake region of Manitoba. *Canadian Journal of Zoology* **52**: 1063-1069.
- 392 **Halliday T, Arnold SJ. 1987.** Multiple mating by females: a perspective from quantitative genetics.
 393 *Animal Behaviour* **35**: 939-941.
- 394 **Halpert AP, Garstka WR, Crews D. 1982.** Sperm transport and storage and its relation to the annual
 395 sexual cycle of the female red-sided garter snake, *Thamnophis sirtalis parietalis*. *Journal of*
 396 *Morphology* **174**: 149-159.
- 397 **Heifetz Y, Wolfner MF. 2004.** Mating, seminal fluid components, and sperm cause changes in vesicle
 398 release in the *Drosophila* female reproductive tract. *Proceedings of the National Academy of*
 399 *Sciences of the United States of America* **101**: 6261-6266.
- 400 **Hoffman LH, Wimsatt WA. 1972.** Histochemical and electron microscopic observations on the sperm
 401 receptacles in the garter snake oviduct. *American Journal of Anatomy* **134**: 71-95.
- 402 **Jennions MD, Petrie M. 2000.** Why do females mate multiply? A review of the genetic benefits.
 403 *Biological Reviews* **75**: 21-64.
- 404 **Johnson L, Welch G. 1999.** Sex preselection: high-speed flow cytometric sorting of X and Y sperm for
 405 maximum efficiency. *Theriogenology* **52**: 1323-1341.
- 406 **Joy JE, Crews D. 1985.** Social dynamics of group courtship behavior in male red-sided garter snakes
 407 (*Thamnophis sirtalis parietalis*). *Journal of Comparative Psychology* **99**: 145-149.
- 408 **Karube F, Kobayashi M. 1999.** Combinative stimulation inactivates sex pheromone production in the
 409 silkworm moth, *Bombyx mori*. *Archives of Insect Biochemistry and Physiology* **42**: 111-118.

- 410 **Kelly R. 1981.** Prostaglandin synthesis in the male and female reproductive tract. *Journal of reproduction*
411 *and fertility* **62**: 293-304.
- 412 **Kraaijeveld K, Chapman T. 2004.** Effects of male sterility on female remating in the Mediterranean
413 fruitfly, *Ceratitis capitata*. *Proceedings of the Royal Society of London. Series B: Biological*
414 *Sciences* **271**: S209-S211.
- 415 **Krohmer RW. 2004.** The male red-sided garter snake (*Thamnophis sirtalis parietalis*): Reproductive
416 pattern and behavior. *Ilar Journal* **45**: 65-74.
- 417 **Krohmer RW, Grassman M, Crews D. 1987.** Annual reproductive cycle in the male red-sided garter
418 snake, *Thamnophis sirtalis parietalis*: field and laboratory studies. *General and Comparative*
419 *Endocrinology* **68**: 64-75.
- 420 **Lum P, Arbogast R. 1980.** Ultrastructure of setae in the spermathecal gland of *Plodia interpunctella*
421 (Hübner)(Lepidoptera: Pyralidae). *International Journal of Insect Morphology and Embryology*
422 **9**: 251-253.
- 423 **Mattson KJ, Vries AD, McGuire SM, Krebs J, Louis EE, Loskutoff NM. 2007.** Successful artificial
424 insemination in the corn snake, *Elaphe gutatta*, using fresh and cooled semen. *Zoo Biology* **26**:
425 363-369.
- 426 **McGraw LA, Gibson G, Clark AG, Wolfner MF. 2004.** Genes regulated by mating, sperm, or seminal
427 proteins in mated female *Drosophila melanogaster*. *Current Biology* **14**: 1509-1514.
- 428 **Mendonça M, Crews D. 2001.** Control of attractivity and receptivity in female red-sided garter snakes.
429 *Hormones and Behavior* **40**: 43-50.
- 430 **Mendonca MT, Crews D. 1989.** Effect of fall mating on ovarian development in the red-sided garter
431 snake. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*
432 **257**: R1548-R1550.
- 433 **Nelson RJ. 2011.** *An introduction to behavioral endocrinology*. Sinauer Assoc.: Sunderland, Mass.
- 434 **Noble DW, Keogh JS, Whiting MJ. 2013.** Multiple mating in a lizard increases fecundity but provides
435 no evidence for genetic benefits. *Behavioral Ecology* **24**: 1128-1137.
- 436 **Norris DO. 2007.** *Vertebrate endocrinology*. Elsevier Academic Press: Amsterdam; Boston.
- 437 **Olsson M, Gullberg A, Tegelström H. 1994.** Sperm competition in the sand lizard, *Lacerta agilis*.
438 *Animal Behaviour* **48**: 193-200.
- 439 **Olsson M, Madsen T. 1996.** Costs of mating with infertile males selects for late emergence in female
440 sand lizards (*Lacerta agilis* L.). *Copeia* **1996**: 462-464.
- 441 **Olsson M, Madsen T. 1998.** Sexual selection and sperm competition in reptiles. In: Birkhead TR and
442 Møller AP, eds. *Sperm competition and sexual selection*. San Diego: Academic Press. 503-578.
- 443 **Olsson M, Madsen T, Shine R. 1997.** Is sperm really so cheap? Costs of reproduction in male adders,
444 *Vipera berus*. *Proceedings of the Royal Society of London. Series B: Biological Sciences* **264**:
445 455-459.
- 446 **Parker GA, Birkhead TR. 2013.** Polyandry: the history of a revolution. *Philosophical Transactions of*
447 *the Royal Society B: Biological Sciences* **368**.
- 448 **Parker GA, Pizzari T. 2010.** Sperm competition and ejaculate economics. *Biological Reviews* **85**: 897-
449 934.
- 450 **Pitnick S, Wolfner MF, Suarez SS. 2009.** Ejaculate-female and sperm-female interactions. In: Birkhead
451 TR, Hosken DJ and Pitnick S, eds. *Sperm Biology: An Evolutionary Perspective*. London:
452 Elsevier/Academic Press. 247-304.
- 453 **Pizzari T, Wedell N. 2013.** The polyandry revolution. *Philosophical Transactions of the Royal Society*
454 *B: Biological Sciences* **368**.
- 455 **Poiani A. 2006.** Complexity of seminal fluid: a review. *Behavioral Ecology and Sociobiology* **60**: 289-
456 310.
- 457 **Quinn H, Blasedel T, Platz Jr CC. 1989.** Successful artificial insemination in the checkered garter snake
458 *Thamnophis marcianus*. *International Zoo Yearbook* **28**: 177-183.
- 459 **Romer AS, Parsons TS. 1986.** *The Vertebrate Body*. Saunders College Publishing: Philadelphia,
460 Pennsylvania USA.

- 461 **Ross P, Crews D. 1978.** Stimuli Influencing Mating Behavior in the Garter Snake, *Thamnophis radix*.
462 *Behavioral Ecology and Sociobiology* **4**: 133-142.
- 463 **Ross P, Jr., Crews D. 1977.** Influence of the seminal plug on mating behaviour in the garter snake.
464 *Nature* **267**: 344-345.
- 465 **Rowe L. 1994.** The costs of mating and mate choice in water striders. *Animal Behaviour* **48**: 1049-1056.
- 466 **Ruan YC, Zhou W, Chan HC. 2011.** Regulation of smooth muscle contraction by the epithelium: role
467 of prostaglandins. *Physiology* **26**: 156-170.
- 468 **Rupik W. 2013.** Ultrastructural studies of cilia formation during thyroid gland differentiation in grass
469 snake embryos. *Micron* **44**: 228-237.
- 470 **Shine R, Elphick MJ, Harlow PS, Moore IT, LeMaster MP, Mason RT. 2001.** Movements, mating,
471 and dispersal of red-sided gartersnakes (*Thamnophis sirtalis parietalis*) from a communal den in
472 Manitoba. *Copeia* **2001**: 82-91.
- 473 **Shine R, Langkilde T, Wall M, Mason RT. 2006.** Temporal dynamics of emergence and dispersal of
474 garter snakes from a communal den in Manitoba. *Wildlife Research* **33**: 103-111.
- 475 **Shine R, O'Connor D, Mason RT. 2000a.** Sexual conflict in the snake den. *Behavioral Ecology and*
476 *Sociobiology* **48**: 392-401.
- 477 **Shine R, Olsson MM, Mason RT. 2000b.** Chastity belts in gartersnakes: The functional significance of
478 mating plugs. *Biological Journal of the Linnean Society* **70**: 377-390.
- 479 **Siegel DS, Miralles A, Chabarría RE, Aldridge RD. 2011.** Female reproductive anatomy: Cloaca,
480 oviduct, and sperm storage. In: Aldridge RD and M. SD, eds. *Reproductive biology and*
481 *phylogeny of snakes*. Enfield, NH; Boca Raton, FL: Science Publishers ; Marketed and distributed
482 by CRC Press. 347-409.
- 483 **Simmons LW. 2001.** *Sperm Competition and Its Evolutionary Consequences in the Insects*. Princeton
484 University Press: Princeton, New Jersey.
- 485 **Simmons LW. 2005.** The evolution of polyandry: Sperm competition, sperm selection, and offspring
486 viability. *Annual Review of Ecology, Evolution, and Systematics* **36**: 125-146.
- 487 **Sugawara T. 1979.** Stretch reception in the bursa copulatrix of the butterfly, *Pieris rapae crucivora*, and
488 its role in behaviour. *Journal of Comparative Physiology* **130**: 191-199.
- 489 **Takeda S, Narita K. 2012.** Structure and function of vertebrate cilia, towards a new taxonomy.
490 *Differentiation* **83**: S4-S11.
- 491 **Thrall PH, Antonovics J, Dobson AP. 2000.** Sexually transmitted diseases in polygynous mating
492 systems: prevalence and impact on reproductive success. *Proceedings of the Royal Society of*
493 *London. Series B: Biological Sciences* **267**: 1555-1563.
- 494 **Tokarz RR. 1988.** Copulatory behaviour of the lizard *Anolis sagrei*: alternation of hemipenis use. *Animal*
495 *Behaviour* **36**: 1518-1524.
- 496 **Tokarz RR, Kirkpatrick SJ. 1991.** Copulation frequency and pattern of hemipenis use in males of the
497 lizard *Anolis sagrei* in a semi-natural enclosure. *Animal Behaviour* **41**: 1039-1044.
- 498 **Tokarz RR, Slowinski JB. 1990.** Alternation of hemipenis use as a behavioural means of increasing
499 sperm transfer in the lizard *Anolis sagrei*. *Animal Behaviour* **40**: 374-379.
- 500 **Trauth SE, Sever DM. 2011.** Male urogenital ducts and cloacal anatomy. In: Aldridge RD and M. SD,
501 eds. *Reproductive biology and phylogeny of snakes*. Enfield, NH; Boca Raton, FL: Science
502 Publishers ; Marketed and distributed by CRC Press. 411-475.
- 503 **Uller T, Olsson M. 2005.** Multiple copulations in natural populations of lizards: evidence for the fertility
504 assurance hypothesis. *Behaviour* **142**: 45-56.
- 505 **Verrell PA. 1991.** Insemination temporarily inhibits sexual responsiveness in female salamanders
506 (*Desmognathus ochrophaeus*). *Behaviour*: 51-64.
- 507 **Wedell N. 2005.** Female receptivity in butterflies and moths. *Journal of Experimental Biology* **208**: 3433-
508 3440.
- 509 **Wedell N, Gage MJG, Parker GA. 2002.** Sperm competition, male prudence and sperm-limited
510 females. *Trends in Ecology & Evolution* **17**: 313-320.

- 511 **Whittier JM, Crews D. 1986a.** Effects of prostaglandin F2 alpha on sexual behavior and ovarian
512 function in female garter snakes (*Thamnophis sirtalis parietalis*). *Endocrinology* **119**: 787-792.
- 513 **Whittier JM, Crews D. 1986b.** Ovarian development in red-sided garter snakes, *Thamnophis sirtalis*
514 *parietalis*: relationship to mating. *General and Comparative Endocrinology* **61**: 5-12.
- 515 **Whittier JM, Crews D. 1989.** Mating increases plasma levels of prostaglandin F2 alpha in female garter
516 snakes. *Prostaglandins* **37**: 359-366.
- 517 **Whittier JM, O'Connor CS. 1991.** Prostaglandin F2 alpha, sexual behavior and ovarian estrogen
518 synthesis in garter snakes (*Thamnophis sirtalis parietalis*). *Comparative Biochemistry and*
519 *Physiology, Part A: Molecular & Integrative Physiology* **100**: 881-885.
- 520 **Wolfner MF. 2009.** Battle and ballet: molecular interactions between the sexes in *Drosophila*. *Journal of*
521 *Heredity* **100**: 399-410.
- 522