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ARTICLE

Patterns of sperm use in two populations of Red-sided Garter Snake (*Thamnophis sirtalis parietalis*) with long-term female sperm storage

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Abstract: Long-term sperm storage may contribute to postcopulatory sexual selection because it enhances the commingling of sperm from different males within the female reproductive tract, which is the prerequisite for sperm competition. Long-term sperm storage and multiple paternity has been documented in snakes, but the identity of the last potential father is usually unknown in studies demonstrating multiple paternity. Here we present the first study in Red-sided Garter Snakes (*Thamnophis sirtalis parietalis* (Say in James, 1832)) to use experimental population crosses to assess stored sperm usage, mate-order effects, and the potential for interpopulational gametic isolation. We found a high rate of multiple paternity indicative of ubiquitous long-term sperm storage in this system, and observed last-male sperm precedence in all families ($n = 66$). Postzygotic isolation was absent, and we observed only a weak asymmetry in pattern of sperm precedence in our population crosses.

Key words: sperm competition, sperm storage, postcopulatory sexual selection, squamate, mating system, parentage, Red-sided Garter Snake, *Thamnophis sirtalis parietalis*.

Résumé : Le stockage de sperme à long terme pourrait participer à la sélection sexuelle post-copulatoire parce qu'il accroît le mélange de spermes de différents mâles dans le système reproducteur de la femelle, une condition nécessaire à la concurrence spermatique. Le stockage de sperme à long terme et la paternité multiple ont été documentés chez les serpents, mais l'identité du dernier père potentiel est habituellement inconnue dans les études qui démontrent la présence de paternité multiple. Nous présentons la première étude de serpents (*Thamnophis sirtalis parietalis* (Say in James, 1832)) faisant appel au croisement expérimental de populations pour évaluer l'utilisation de sperme stocké, les effets de l'ordre de copulation et le potentiel d'isolement gamétique entre populations. Nous avons constaté un taux élevé de paternité multiple, qui indique l'ubiquité du stockage de sperme à long terme dans ce système, et observé la préséance du sperme du dernier mâle dans toutes les familles ($n = 66$). L'isolement post-zygotique était absent, et nous n'avons observé qu'une faible asymétrie de la préséance des spermes dans les populations croisées. [Traduit par la Rédaction]

Mots-clés : concurrence spermatique, stockage de sperme, sélection sexuelle post-copulatoire, squamates, système d'accouplement, parenté, couleuvre rayée à flancs rouges, *Thamnophis sirtalis parietalis*.

Introduction

Female sperm storage of days to weeks is common in many taxa (Birkhead and Møller 1993, 1998; Simmons 2001) and can promote postcopulatory selection on ejaculate quality (Birkhead et al. 1999; García-González and Simmons 2005; Alonzo and Pizzari 2013) and the coevolution of male and female reproductive traits (Eberhard 1996; Miller and Pitnick 2002; Arnqvist and Rowe 2005; Pitnick et al. 2009). Long-term sperm storage (months to years) may intensify postcopulatory selection, as it prolongs interactions between the ejaculates of rival males within the female's reproductive tract (Birkhead and Møller 1993; Adams et al. 2005; Orr and Zuk 2013). However, with the exception of social insects (Hölldobler and Wilson 1990; Boomsma et al. 2005) and bats (Orr and Zuk 2013), the effect of long-term sperm storage on postcopulatory selection has not been well assessed (Uller and Olsson 2008; Uller et al. 2010; Orr and Zuk 2013). Characterizing the use of sperm stored for prolonged periods in a diversity of systems is therefore essential to our understanding of how postcopulatory sexual selection affects the evolution of mating systems (Shuster and Wade 2003; Parker and Birkhead 2013; Shuster et al. 2013).

In many nonavian sauropsids, sperm production, mating, and fertilization are often temporally disjunct (Crews and Moore 1986; Uller et al. 2010). Consequently, sperm must be stored within the male and (or) female reproductive tracts for weeks to months and in some cases years prior to fertilization (Birkhead and Møller 1993; Olsson and Madsen 1998; Sever and Hamlett 2002; Uller and Olsson 2008; Uller et al. 2010). Long-term sperm storage is particularly well documented in snakes (Uller and Olsson 2008). However, the evolutionary causes and implications of long-term sperm storage remain the subject of speculation due, in part, to a paucity of studies that evaluate the proportional use of sperm stored for long periods (Uller and Olsson 2008; Uller et al. 2010; Orr and Zuk 2013). For example, knowing the mean number of offspring a male is likely to father via long-term sperm storage is useful for estimating the strength of selection on male traits such as sperm longevity.

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Garter Snakes (genus *Thamnophis* Fitzinger, 1943; Collins and Taggart 2002) make excellent subjects for the study of long-term sperm storage for several reasons. Multiple paternity is widespread among Garter Snakes (Wusterbarth et al. 2010); thus, postcopulatory selection is likely to be an important aspect of their mating systems. Furthermore, female Garter Snakes possess sperm storage crypts (Fox 1956; Hoffman and Wimsatt 1972; Halpert et al. 1982; Devine 1984; Sever and Hamlett 2002) and sperm remains viable for long periods within the female reproductive tract (8–9 months: Blanchard and Blanchard 1941; Blanchard 1943; years: Stewart 1972). However, no study has employed an experimental approach in which mate order is known; therefore, neither prevalence of stored sperm usage nor mate-order effects on sperm precedence could be established. Although studying mating system evolution can be difficult in secretive animals such as snakes (Seigel 1987; Duvall et al. 1993), this problem is obviated in studies of Red-sided Garter Snake (*Thamnophis sirtalis parietalis* (Say in James, 1832); Collins and Taggart 2002) populations from Manitoba, Canada. Males of this system display robust courtship in controlled mating trials (e.g., Whittier et al. 1985; Shine et al. 2000*b*; LeMaster and Mason 2002; Friesen et al. 2013) such that the last male to mate is easily identified in sperm precedence studies and mated females from these trials can be easily transported to laboratory facilities to give birth so that offspring can be collected and maternity is certain.

Mating system of *T. s. parietalis*

In central Manitoba, Canada, large numbers of *T. s. parietalis* overwinter in limestone sinkholes from which they emerge in late April and to which most of them faithfully return each fall (Gregory 1974; Gregory and Stewart 1975; Macmillan 1995). All Manitoba den sites share a number of characteristics. Mating has been observed during the fall migration period (Aleksiuk and Gregory 1974), but the most intense mating activity occurs at spring emergence. In early spring, males emerge at the surface from underground retreats before females and remain concentrated at the den for 4–5 weeks (Gregory 1974; Shine et al. 2001). In contrast, females emerge later and remain around the den, on average, for only 3–4 days before migrating to summer feeding grounds (Shine et al. 2001). The sexual difference in spatial and temporal distribution generates extremely male-biased operational sex ratios (OSRs) (Emlen and Oring 1977). The polygynandrous mating system of Manitoba *T. s. parietalis* is thus characterized by scramble competition (Thornhill and Alcock 1983; Duvall et al. 1993).

Population- and quantitative-genetic studies suggest a role for selection in our study system. Although den philopatry in snakes may lead to a high degree of population genetic structuring (Lougheed et al. 1999; Gibbs and Weatherhead 2001), Common Garter Snakes (*Thamnophis sirtalis* (L., 1758)) in general, and Manitoba populations in particular, show little differentiation at neutral markers (Bittner and King 2003; Westphal 2007; DiLeo et al. 2010). This pattern may owe itself to *T. s. parietalis* having recolonized Manitoba less than 12 000 years ago, following the last glacial retreat (Rye 2000; Dyke 2004; Placyk et al. 2007). During this time, however, *T. s. parietalis* within Interlake Manitoba have experienced a discontinuous spatial population structure based on den-site availability and philopatry (Gregory 1974; Gregory and Stewart 1975; Macmillan 1995; LeMaster and Mason 2003; Mooi et al. 2011), and the Interlake (including Inwood) and Snake Island populations of *T. s. parietalis* have diverged substantially in body size and coloration (Westphal 2007; Mooi et al. 2011). In the absence of comparable differentiation at neutral genetic markers, these phenotypic differences can be attributed to population-specific patterns of directional selection (Lande 1992; Spitze 1993), perhaps related to differences between mainland and island lifestyles of these animals.

Lastly, variation in the physical size of Manitoba den sites generates variation in den population sizes, which in turn can lead to differing rates of evolution in such populations. Sexual coevolution in small populations is predicted to occur rapidly along a line of equilibrium due to drift (Lande 1981; Gavrilets 2000; Uyeda et al. 2009). In contrast, large populations are predicted (Gavrilets 2000) and demonstrated (Martin and Hosken 2003; Gay et al. 2010) to be more responsive to selection promoted by sexual conflict.

Here we present data on paternity from experimental crosses of two natural populations of *T. s. parietalis*—Inwood and Snake Island—that represent the two extremes of population size and mating aggregation density observed in this system. We first evaluated the prevalence of long-term sperm storage to the genetic mating system of Manitoba *T. sirtalis*. Second, given that stored sperm were prevalent, we assessed mate-order effects on sperm precedence. Third, we assessed prezygotic reproductive isolation by testing for homotypic sperm precedence between experimental crosses.

Materials and methods

Study populations

Thamnophis sirtalis parietalis were collected from two sites: Inwood (I), a large communal den in central southern Manitoba (50°31.58′N, 97°29.71′W) that houses approximately 35 000 snakes (Shine et al. 2006) and where mating aggregations range in size from 3 to 62 males courting a single female (Shine et al. 2001), and Snake Island (S), a small (2 km × 0.5 km) island in Lake Winnipegosis, Manitoba (51°38.53′N, 99°49.42′W), in which small mating aggregations (1–20 males per female) are distributed along a low limestone ridge home to 100–500 snakes (Mason et al. 1991). Four combinations of population pairings were used in mating trials: I × I and S × S (homotypic pairings), and I × S and S × I (heterotypic pairings). In the abbreviation of each cross, the female's population is listed first.

Animal collection

Unmated females were collected as they reached the ground surface of the den from underground retreats during the spring of 2004. It is unlikely that females mate while underground, as winter body temperatures are extremely low (2–3 °C; Lutterschmidt et al. 2006). Emerging females can therefore be considered "seasonal virgins", although they may carry sperm in their reproductive tracts stored from previous seasons. Males were collected from courting or basking groups of animals and placed into nylon sacks until mating trials.

Mating trials

Mating trials were conducted at the Chatfield Research Station, Manitoba, Canada (50°46′34.65″N, 97°32′46.69″W), within a few days of animal capture. In each mating trial, a female was placed in a 1 m × 1 m × 1 m nylon outdoor arena with 10 males from a particular population. Thus, the OSR for the mating trials was held at a moderate and realistic level for all matings (Shine et al. 2001). Once a female was placed in the arena, she was observed until mating occurred. The pair was then carefully removed from the arena before copulation terminated and placed in a smaller arena; the identity of the focal male was thus known with certainty. Males were allowed to mate once. In total, there were 110 matings (I × I: $n = 39$; I × S: $n = 22$; S × S: $n = 25$; S × I: $n = 24$). After copulation ended, the animals were weighed and measured, and small (<10 mm) tissue samples were taken from the tail tips of all mated animals. Tissue was placed into plastic (Nunc) tubes filled with calcium sulfate (Drierite) desiccant and stored at –20 °C until DNA extraction. The ventral scales of each female were clipped with unique markings to identify them through the next 12 months of captivity (Blanchard and Finster 1933). Males were released at the point of capture after tissue samples were taken.

Husbandry

Females were transported to Oregon State University, housed (14–25 °C to mimic natural conditions; 12 h light: 12 h dark) in 38 L

aquaria, given water ad libitum, and fed live worms (*Lumbricus terrestris* L., 1758) or frozen fish (Chinook salmon, *Oncorhynchus tshawytscha* (Walbaum, 1792)) weekly. Females gave birth in the laboratory from mid-August through early September. Neonates were weighed, measured, and tissue was collected in the same manner as the adults.

Molecular methods

DNA was extracted from tail-tip tissue (Garner et al. 2004) in 200 µL 5% chelex – 1% ProK solution incubated at 56 °C for 2 h and then 8 min at 100 °C. We used three microsatellite loci to exclude the focal male from paternity: *Ts1* (McCracken et al. 1999) and *Nsµ2* and *Nsµ3* (Prosser et al. 1999). All three loci were multiplexed in a single 12 µL polymerase chain reaction (PCR) (1 µL template; 6.25 µL Multiplex mix (Qiagen catalogue No. 206143); 1.25 µL of 2 µmol/L of each primer (Invitrogen); 3.5 µL molecular grade H_2O). PCRs were carried out in a Biorad thermal cycler (C1000) with amplification conditions consisting of 15 min at 95 °C, followed by 35 cycles of 30 s at 95 °C, 90 s at 58 °C, and 90 s at 72 °C, followed by a 10 min elongation phase at 72 °C. Reaction products were separated using an ABI 3100 genetic analyzer and the alleles were visualized using ABI Genotyper software. Peaks were assigned manually, and each offspring was checked against the mother's genotype. Samples from offspring that lacked a maternal allele were reanalyzed along with the mother's sample and a random subset of siblings to check for errors (DeWoody et al. 2006).

Parentage assignment

We used an exclusion-based protocol for paternity assignment that involved selecting a random sample of 28 adults from each population (*n* = 56) to estimate mean exclusion probability and test for Hardy–Weinberg equilibrium (HWE) using CERVUS version 3.0.3 (Kalinowski et al. 2007). A conservative estimate of the minimum number of fathers per litter was calculated by dividing the number of paternal alleles by 2 after assigning alleles to the known male. We then added one male to this number to account for the known male. Any paternity that we could not assign to our focal male was most likely due to stored sperm and thus necessarily from within-population (homotypic) matings.

The three loci chosen for this study were all highly polymorphic. The mean exclusion probability, based on the genotypes from 56 randomly chosen adults in a preliminary analysis, for each locus was as follows—*Ts1*: 1 – 0.1160 = 0.884 exclusion probability (second parent), 26 alleles, H_O = 0.9643; *Nsµ2*: 1 – 0.1654 = 0.8346 exclusion probability (second parent), 20 alleles, H_O = 0.5357; *Nsµ3*: 1 – 0.2508 = 0.7492 exclusion probability (second parent), 12 alleles, H_O = 0.8929. The combined mean exclusion probability using all three alleles is 1 – 0.0048 = 0.9952 and any pair of loci yields greater than 0.95 confidence of correctly excluding our focal male. In many cases, we relied on only two loci because one of the three loci was uninformative (e.g., mother and focal male shared a genotype at that locus). The single locus exclusion probability of *Ts1* calculated using all the adults from this study was 0.98, provided the maternal and focal male's genotypes did not match. Loci *Ts1* and *Nsµ3* were in HWE (*Ts1*: $\chi^2_{[325]}$ = 298.9, *P* = 0.847; *Nsµ3*: $\chi^2_{[66]}$ = 50.386, *P* = 0.923); however, locus *Nsµ2* displayed excess homozygosity (*Nsµ2*: H_O = 0.536, H_E = 0.926, $\chi^2_{[190]}$ = 503.5, *P* < 0.001). Excess homozygosity at the *Nsµ2* locus was found in both populations (Snake Island: H_O = 0.630, H_E = 0.915; Inwood: H_O = 0.448, H_E = 0.913). Excess homozygosity can arise from genotyping errors produced by null alleles, large allele dropout, or miscalling stutter as homozygotes; however, we compared progeny genotypes with maternal genotypes and found no evidence of deviation from expected Mendelian ratios (DeWoody et al. 2006).

Statistical analyses

Statistical analyses were conducted using SigmaPlot version 11.0 (χ^2, ANOVA, and Student's *t* tests), XLSTAT (ANCOVA, nested mixed models), or PASW version 17.0 (generalized linear regression). Proportion of offspring fathered was arcsine square root transformed and male snout–vent length (SVL) was ln-transformed to equalize variance among crosses for regression analyses. Body condition index (BCI) was calculated as the residual deviation from the quadratic regression of female mass on female SVL fitted in SigmaPlot (adjusted R^2 = 0.918), which provided a superior fit compared with a linear model both on untransformed data (adjusted R^2 = 0.827) and ln–ln transformed data (adjusted R^2 = 0.825). Differential offspring mortality before birth can skew results in studies of sperm precedence (Gilchrist and Partridge 1997; Zeh and Zeh 1997). To assess this possibility, we tested for differences between homotypic and heterotypic crosses in litter size, number of stillbirths, and incidence of parity after controlling for female size, which has a known, direct effect on litter size (Gregory and Larsen 1993). We tested for differences in homotypic sperm precedence by conducting a parametric test of the proportion of offspring fathered by the known male on the I × I vs. I × S crosses. However, the variance in the proportion of offspring fathered was not equal between S × S and S × I crosses, so we conducted a nonparametric test for this comparison.

Results

Population comparisons

Of the females that gave birth, Snake Island females were significantly longer than Inwood females, but not significantly heavier (Table 1). Accordingly, Inwood females had higher BCI than Snake Island females. Of the potential fathers, Snake Island males were significantly longer and heavier than Inwood males. However, Inwood males were in better condition than Snake Island males.

Postzygotic isolation

Birth rate

Seventy-one of the 110 mated females (64.5%) gave birth. Female Garter Snakes are capital breeders and most likely give birth every other year (Gregory 2006; R.T. Mason, personal observation), so this percentage is close to the expected 50%. Larger females were more likely to give birth (logistic regression; LRT = 33.203, *P* < 0.001). Female body condition differed significantly with parity and between populations (two-way ANOVA; female population: $F_{[1,106]}$ = 22.01, *P* < 0.001; gave birth: $F_{[1,106]}$ = 10.23, *P* = 0.002; interaction: $F_{[1,106]}$ = 0.429, *P* = 0.514; Table 1) such that Inwood females giving birth had higher BCI (8.4 ± 2.2; mean ± SE) than those that did not give birth (–0.62 ± 1.69), but the same relationship did not exist for Snake Island females (gave birth: –4.12 ± 2.10; did not give birth: –10.06 ± 1.82) (Holm–Šidák method for multiple comparisons; Inwood: *t* = 3.03, unadjusted *P* = 0.003; Snake Island: *t* = 1.65, unadjusted *P* = 0.103).

To test whether females were less likely to give birth when mated with a heterotypic male, we analyzed incidence of parity among crosses (for number of families per cross see Table 1). The proportion of females that gave birth did not differ significantly among crosses (χ^2 test; $\chi^2_{[1]}$ = 3.355, *P* = 0.340) or between populations (χ^2 test; $\chi^2_{[1]}$ = 1.327, *P* = 0.249). We also conducted pairwise comparisons of homotypic and heterotypic matings, but still found no differences in parity (χ^2 test; I × I vs. I × S: $\chi^2_{[1]}$ = 1.195, *P* = 0.274; χ^2 test; S × S vs. S × I: $\chi^2_{[1]}$ = 0.294, *P* = 0.588).

Litter size and stillbirths per cross

To assess whether genetic incompatibility and embryo reabsorption skewed paternity patterns, we tested for differences in litter size and the number of stillbirths among crosses. Without controlling for female size, litter size was not different among crosses ($F_{[3,71]}$ = 0.577, *P* = 0.632; Table 1). As noted earlier, females differ in size between populations and female mass predicts litter size (adjusted R^2 = 0.471, $F_{[1,71]}$ = 67.168, *P* < 0.001). We therefore compared residuals of litter size given female mass between

Table 1. Population differences in Red-sided Garter Snakes (*Thamnophis sirtalis parietalis*) based on those females that gave birth and fathers used in the paternity analysis.

	Population or cross	n	df	Mean (SE)	t	p	No. of females that gave birth/no. of mated pairs (%)
Female							
Mass (g)	Inwood (I)	32	64	106.9 (5.26)	1.69	0.094	
	Snake Island (S)	34		121.6 (6.57)			
SVL (cm)	I	32	64	65.3 (1.15)	3.67	**<0.001**	
	S	34		71.2 (1.12)			
BCI	I	32	64	5.81 (2.20)	3.68	**<0.001**	
	S	34		−5.63 (2.2)			
Male							
Mass (g)	I	35	64	30.4 (1.25)	3.44	**0.001**	
	S	31		38.7 (2.17)			
SVL (cm)	I	35	64	45.7 (0.87)	6.46	**<0.001**	
	S	31		52.2 (0.95)			
BCI	I	35	64	1.05 (0.52)	2.59	**0.012**	
	S	31		−1.22 (0.72)			
Litter size × cross	I × I	18	3, 61	21.2 (2.08)	0.64	0.591	
	I × S	14		23.9 (1.39)			
	S × I	17		20.8 (1.81)			
	S × S	17		20.4 (2.07)			
Birth rate × cross	I × I						21/39 (53)
	I × S						15/22 (68.2)
	S × I						18/24 (75)
	S × S						17/25 (68)
Proportion of still births × cross	I × I			0.064 (0.022)			
	I × S			0.129 (0.083)			
	S × I			0.020 (0.008)			
	S × S			0.093 (0.055)			
Proportion of multiple paternity × cross	I × I			0.89 (0.076)			
	I × S			0.93 (0.071)			
	S × I			0.88 (0.081)			
	S × S			0.69 (0.120)			

Note: The t statistics and p values were generated from ln-transformed data (p values in boldface type are significant at $p < 0.05$). BCI, body condition index; SVL, snout–vent length.

homotypic and heterotypic crosses and found no differences (one-tailed Student's t test; I × I vs. I × S: $t_{[34]} = 0.005$, $P = 0.498$; S × S vs. S × I: $t_{[35]} = 0.134$, $P = 0.447$). The number of stillbirths per cross yielded no evidence for postzygotic sexual isolation. Across all females, the mean proportion of stillbirth offspring was 0.073 (95% confidence interval (CI) of mean = 0.023). Median proportion of stillbirth offspring was not affected by cross (Kruskal–Wallis one-factor ANOVA; $H_{[3]} = 1.844$, $P = 0.605$; Table 1) and comparisons between homotypic and heterotypic crosses showed no significant differences (χ^2 test of independence; I × I vs. I × S: $\chi^2_{[1]} = 2.057$, $P = 0.151$; S × S vs. S × I: $\chi^2_{[1]} = 0.011$, $P = 0.915$). Focusing on litters in which we were able to assign paternity (below), stillbirth offspring were no more likely to have been fathered by heterotypic males (χ^2 test; I × I vs. I × S: $\chi^2_{[1]} = 1.637$, $P = 0.201$; S × S vs. S × I: $\chi^2_{[1]} = 0.2.057$, $P = 0.151$).

Postcopulatory–prezygotic isolation

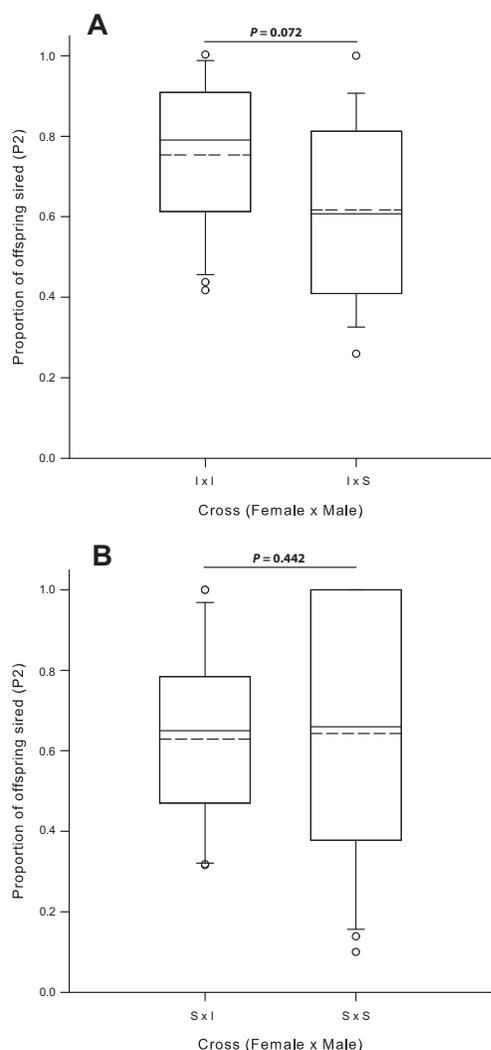#### Minimum number of fathers per litter

A total of 66 families (1409 offspring) were successfully genotyped. Seven families were eventually excluded from analysis either because of apparent maternal null alleles ($n = 2$), difficulty amplifying maternal template DNA ($n = 4$), or a mismatch between a maternal genotype and that of her litter perhaps resulting from a novel mutation ($n = 1$). Eighty-five percent of litters exhibited multiple paternity, which means that only 15% of females showed no evidence of having used stored sperm, but females that exhibited single paternity may have had stored sperm that was not used. Crosses did not differ in proportion of litters with multiple

paternity ($\chi^2_{[3]} = 3.901$, $P = 0.272$; Table 1). For all crosses combined, a conservative estimate of the median minimum number of fathers per litter was 2.0 (1–3 fathers), which did not differ among crosses (Kruskal–Wallis one-factor ANOVA on ranks; $H_{[3]} = 0.823$, $P = 0.844$). With the caveat that our method may underestimate the number of fathers per litter, litter size was not larger in litters with more fathers: 3 fathers, 22 ± 2.6 offsprings (mean ± SE); 2 fathers, 22 ± 1.2 offsprings; 1 father, 17 ± 1.8 offsprings (one-factor ANOVA; $F_{[2,62]} = 1.983$, $P = 0.146$); and larger females did not have more fathers per litter (one-factor ANOVA; SVL: $F_{[2,62]} = 0.775$, $P = 0.465$).

Gametic isolation

Mean proportion of offspring fathered by the known (last) male across all crosses was 0.67 ± 0.06 (mean ± SE), which is significantly greater than the 0.50 expected under a model of equal weighting of paternity with two fathers (two-tailed Student's t test; $t_{[65]} = 5.595$, $P < 0.001$). Mean (±SE) proportion of offspring fathered by the known male within crosses were as follows: I × I = 0.754 ± 0.045, I × S = 0.617 ± 0.060, S × I = 0.629 ± 0.052, S × S = 0.643 ± 0.081 (Figs. 1A, 1B). Inwood females gave birth to slightly more offspring attributable to homotypic stored sperm when they mated with heterotypic males (two-tailed Student's t test; I × I vs. I × S: $t_{[30]} = 1.866$, $P = 0.072$). Snake island female crosses (S × S and S × I) did not have equal variance in proportion of offspring fathered (Levine's test; $F_{[1,31]} = 6.123$, $P = 0.019$); however, the proportion of offspring attributable to stored sperm did not differ between these hetero-

Fig. 1. (A, B) Box plots showing asymmetric gametic isolation in Red-sided Garter Snakes (*Thamnophis sirtalis parietalis*); more stored sperm from homotypic males was used when Inwood (I) females mate with Snake Island (S) males. The horizontal broken lines within the boxes are means, whereas the horizontal solid lines are medians. The boxes enclose 50% of the data, whereas the whiskers are 1.5 interquartile ranges. There is more variance within the S × S cross than the other crosses.
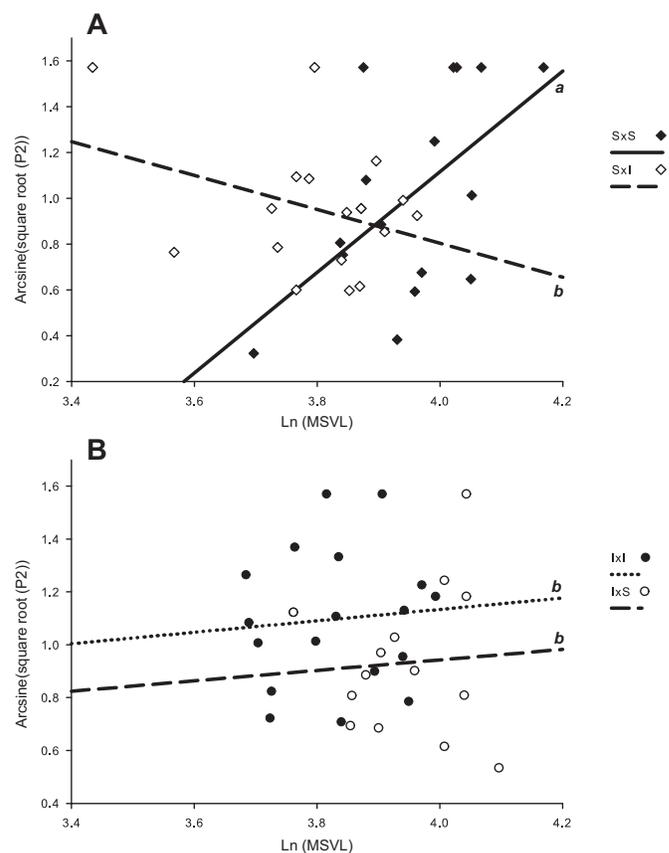

Fig. 2. Proportion of offspring fathered by the focal male (arcsine(square root (P_2)) Red-sided Garter Snake (*Thamnophis sirtalis parietalis*) as a function of male size (Ln (MSVL)) for each separate cross, where MSVL is male snout–vent length, I is Inwood, and S is Snake Island. (A) The solid diamonds represent the S♀ × S♂ cross, whereas the open diamonds represent the S♀ × I♂ cross. (B) The solid circles represent I♀ × I♂ cross, whereas the open circles represent the I♀ × S♂ cross. The ANCOVA analysis of differences in slopes is presented in supplementary Table S1[1] and the italic letters to the right of each regression line indicates which lines are significantly different from one another.

typic and homotypic crosses (one-tailed Mann–Whitney rank-sum test; S × S vs. S × I: $H = 0.148$, $P = 0.442$).

Effect of male size on paternity

Longer Snake Island males had significantly higher paternity when they mated with Snake Island females, but male size had no effect on paternity in any other cross (generalized linear regression in PASW; Wald χ^2 test; male SVL (MSVL): $\chi^2_{[1]} = 1.58$, $P = 0.209$; S × S: $\chi^2_{[3]} = 10.55$, $P = 0.001$; S × S × Ln (MSVL) interaction: $\chi^2_{[1]} = 10.44$, $P = 0.001$). ANCOVA corroborated this statistical result (full-model ANOVA; $F_{[7,63]} = 2.714$, $P = 0.017$; Ln (MSVL) × cross interaction; $P = 0.005$), which provided a comparison of slopes (Figs. 2A, 2B, supplementary Table S1[1]). We conducted separate regression analyses to illustrate the difference in slopes (Fig. 2A); these are the results from the individual regressions—S × S: ad-

justed $R^2 = 0.252$, $P = 0.023$; S × I: adjusted $R^2 = 0.000$, $P = 0.817$; I × I: adjusted $R^2 = 0.000$, $P = 0.749$; I × S: adjusted $R^2 = 0.040$, $P = 0.217$. We note that this result is robust even with the removal of the lowest point in the S × S cross (Fig. 2A). In contrast, female size had no effect on paternity (Wald χ^2 test; female SVL: $\chi^2_{[1]} = 0.392$, $P = 0.531$; S × S: $\chi^2_{[3]} = 2.458$, $P = 0.483$; interaction: $\chi^2_{[1]} = 2.580$, $P = 0.461$).

Discussion

This is the first study in snakes that addresses sperm precedence patterns in natural populations. We demonstrate last male sperm precedence and show that stored sperm from previous seasons sires a substantial proportion of offspring from any given litter.

Sperm storage in *T. s. parietalis*

Our data demonstrate that sperm stored from previous seasons fertilize ova and may therefore play an important role in post-copulatory selection (Uller and Olsson 2008; Uller et al. 2010). In our study system, as in most populations of Garter Snakes, limited mating occurs in the autumn prior to winter hibernation, as well

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as during the primary breeding season in spring (Blanchard and Blanchard 1941; Blanchard 1943; Aleksiuk and Gregory 1974; Whittier et al. 1985; C.R. Friesen, unpublished data, autumn 2008 and 2009). Because all females used in this study were collected immediately upon emergence and had no opportunity to mate during the spring except in our controlled mating trials, our study provides strong evidence for the effectiveness of autumnal mating and (or) mating during the previous spring. Blanchard and Blanchard (1941) found that autumn mating can produce viable offspring the following summer (over 10 months later). Although recently published examples of facultative parthenogenesis in snakes may call into question stored sperm usage across seasons (Booth et al. 2012), our study found none of the hallmarks of parthenogenesis; i.e., unusually small litters composed entirely of the homogametic sex (Booth et al. 2012).

Multiple paternity and opportunity for sperm competition

The prevalence of multiple paternity (Figs. 1A, 1B) clearly indicates the opportunity for postcopulatory selection within our study populations. The frequency of multiple paternity gauges the strength of postcopulatory selection; i.e., the risk that a male's sperm will compete with that of other males. Eighty-five percent of the litters in the current study showed multiple paternity. Our data are consistent with previous studies in *T. sirtalis* that found multiple paternity in 37.5%–100% of litters (McCracken et al. 1999; King et al. 2001; Garner et al. 2002; reviewed in Uller and Olsson 2008), as well as with studies of other Garter Snake species reporting multiple paternity in 50%–100% of litters (Garner and Larsen 2005; Wusterbarth et al. 2010). Our estimate of the incidence of multiple paternity probably underestimates the occurrence of female remating because some males may father all offspring even in the face of sperm competition—either by chance, because of superior ejaculate quality, or due to cryptic female choice.

Our study suggests that the risk of sperm competition is relatively high in these *T. sirtalis* populations. Indeed, multiple paternity was common even though females were only allowed to mate once in the spring, meaning that intermale sperm competition resulted only from sperm stored in previous seasons. The number of competitor ejaculates a male is expected to encounter provides another measure of the intensity of sperm competition. Our conservative approach found that as many as three fathers were required to explain paternity, with a mean of two detectable fathers per litter. These results indicate a moderate intensity of sperm competition (Parker 1998).

Mate-order effects

Sperm precedence is defined as the nonrandom utilization of sperm from one of several males to mate with a female (Simmons and Siva-Jothy 1998; Simmons 2001). Passive sperm loss (e.g., random sperm death) is a mechanism by which last-male precedence can occur (Parker 1998), but first-male precedence occurs in some species by other mechanisms (Birkhead and Møller 1998). Because females were allowed to mate only once in the spring, neither mate-order effects within the spring mating season nor the prevalence of multiple mating in the spring could be addressed in this study. Our data do, however, provide some insight into the effects of mate order on sperm precedence in the case of an extended interval between matings (i.e., over 7 months). Without considering the effect of cross, last-male precedence was moderate to high in litters of females allowed to mate only once in the spring (Figs. 2A, 2B). The last male to mate fathered, on average, 67% of the offspring, providing evidence for at least moderate last-male sperm precedence (Simmons 2001). Clearly, the spring males had an advantage over the fall males, but future work should address patterns of sperm precedence with multiple spring matings.

Weak asymmetric gametic isolation between populations of *T. s. parietalis*

Our analysis took advantage of the fact that stored sperm would necessarily come from homotypic males. We found weak evidence of homotypic sperm precedence within Inwood females (Figs. 1A, 1B). Given that homotypic sperm stored from autumnal mating would likely be at a disadvantage due to sperm attrition, this trend is remarkable and suggests that the observed asymmetry would be even more pronounced if there was parity in sperm numbers between potential fathers. Parity in sperm numbers can only be assured in artificial insemination trials, but these experiments reduce or eliminate precopulatory–copulatory cues that females might use to bias paternity via sperm usage or differential sperm transport (Eberhard 1996; Eberhard et al. 1998). Reduced paternity in between-population crosses is not necessarily the result of gametic isolation, but can result from postzygotic factors such as genetic incompatibility (Zeh and Zeh 1997; Stockley 1999; Tregenza and Wedell 2000; Birkhead and Brillard 2007); however, we found no evidence of postzygotic isolation. The proportion of stillbirths was not different for heterotypic vs. homotypic matings and the number of stillbirth offspring attributable to heterotypic males was no different than for homotypic males. Neither the proportion of females that gave birth nor litter size differed significantly after controlling for female size among population crosses. Thus, the hypothesis that offspring were aborted and reabsorbed before birth lacks support.

Large-male postcopulatory advantage in the Snake Island population

We found that larger Snake Island males had increased probability of paternity, but only when mated to females from their own population (Figs. 2A, 2B). Male body size is a predictor of mating success in other snake species (Blouin-Demers et al. 2005; Ursenbacher et al. 2009), such as the Northern Water Snake (*Nerodia sipedon* (L., 1758)), where OSR is male biased (3:1) and larger males have a mating advantage (Kissner et al. 2005). In Manitoba *T. s. parietalis*, however, mating was completely random with respect to male size in a den with large mating aggregations (up to 62 males per female; Shine et al. 2000b, 2006), but experimental mating trials with small aggregations similar to those of Snake Island (<20 males; Shine et al. 2000b) revealed a large male mating advantage. Because the male size advantage was nonexistent when Snake Island males were mated with Inwood females, our results are consistent with intersexual co-adaptation within the Snake Island population and (or) cryptic female choice (Eberhard 1996; c.f., Thornhill 1983; Evans et al. 2003).

Conclusions

The high frequency of multiple paternity discovered in this study, coupled with minimal precopulatory choice (Shine et al. 2000a; Shine 2004) and female sperm storage in *T. s. parietalis*, sets the stage for the evolution of cryptic female choice (Eberhard 1996) in these snakes (Devine 1984; Uller et al. 2010). More work is needed to identify mechanisms of postcopulatory selection in general and of cryptic female choice in particular. Garter Snakes are ideal candidates as models for such research given that long-term sperm storage is well documented.

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