

AN ABSTRACT OF THE THESIS OF

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Sperm competition is the post-copulatory analog of male-male combat, wherein sperm from more than one male coincide in a female's reproductive tract and compete with one another for fertilization of a limited number of ova. Post-copulatory sexual selection, such as sperm competition, can be a powerful driving force for the evolution of many reproductive traits that can lead to rapid divergence. I investigated the effect of sperm depletion on sperm competition in a model polygynandrous mating system of the Red-sided garter snake, *Thamnophis sirtalis parietalis*. The difference in P_1 (the proportion of offspring sired by the first male to mate) between a male's first and second matings was not statistically significant ($P = 0.314$). However, pooling the data to compare P_1 , P_2 , and P_{SS} did show a statistically significant difference between P_{SS} and P_1 ($P < 0.05$), but not between P_1 and P_2 or P_2 and P_{SS} ($P > 0.05$). Female and male copulation durations, male masses, and interval between matings did not have any significant affect on the paternity of the offspring (All $P > 0.05$). Genetic bet hedging, sexual conflict, and sperm degradation/extrusions are all ideas that may help to explain our results.

Key Words: red-sided garter snake, *Thamnophis sirtalis parietalis*, sperm depletion, sperm competition, sperm precedence, multiple paternity

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Sperm Depletion and Sperm Competition in the Red-sided Garter Snake,

Thamnophis sirtalis parietalis

by

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I understand that my project will become part of the permanent collection of Oregon State University, University Honors College. My signature below authorizes release of my project to any reader upon request.

Amelia R. Kerns, Author

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TABLE OF CONTENTS

	<u>Page</u>
INTRODUCTION	1
Theory	1
The Study System.....	4
The Study	7
 MATERIALS AND METHODS.....	 8
Specimen Collection and Captive Maintenance.....	8
Mating	8
DNA Extraction, PCR, and Genotyping.....	9
Stored Sperm Analysis	10
Statistical Analysis	10
 RESULTS	 11
Female Copulation Durations.....	11
Male Copulation Durations	11
Male Mass	11
P ₁ after 1 st mating vs. P ₁ after 2 nd mating	11
P ₁ vs. P ₂ vs. P _{SS}	12
Stored Sperm Analysis	12
 DISCUSSION.....	 15
 BIBLIOGRAPHY	 19

LIST OF FIGURES & TABLES

<u>Figure</u>	<u>Page</u>
1. Sperm precedence for 2007 <i>T.s.p.</i>	13
2. Sperm precedence after male's 1 st mating	13
3. Sperm precedence after male's 2 nd mating	14

<u>Table</u>	<u>Page</u>
1. Table of values for P_{SS} , minimum number of males representing stored sperm, and minimum number of total males mated for each family.....	14

Sperm Depletion and Sperm Competition in the Red-sided Garter Snake,

Thamnophis sirtalis parietalis

INTRODUCTION

Theory

Sexual selection, which arises from differences in mating success, is a powerful driving force for evolutionary diversification (Darwin 1871; Andersson 1994; Møller, Birkhead et al. 1998). Sexual selection has driven the evolution of sexually dimorphic traits. Variation in mating success is usually greatest in males, and thus sexual selection is thought to have the most profound effect on males (Bateman 1948; Arnold and Duvall 1994). This explains why it is often the males of a species that have elaborate displays or plumage and/or fight to secure mates. There are two main modes of sexual selection: male-male competition and female choice. Male-male competition occurs when males directly compete with one another in order to acquire mates, such as when bucks engage in combat to secure a group of females, and as a result, bucks have evolved antlers and larger body size (Darwin 1871; Andersson 1994; Birkhead and Møller 1998). Female choice occurs when a female shows a mating preference for particular males based on any number of characteristics, such as body plumage or habitat decoration (Darwin 1871; Andersson 1994). Female preferences can then drive the elaboration of these traits, even despite potential costs.

Initial research and debate focused on sexual selection in terms of pre-copulatory mechanisms, such as those described above (Andersson 1994). In 1970, Geoff Parker introduced the idea that sexual selection may continue after copulation via sperm

competition (Parker 1970). A decade later, Randy Thornhill proposed that female choice can also occur after copulation (Thornhill 1983; Eberhard 1996). Thus, post-copulatory sexual selection - selection that occurs after copulation - is analogous to pre-copulatory sexual selection in that both male-male combat and female choice may continue after copulation. Like precopulatory selection, post-copulatory sexual selection is a driving force for the evolution of many male and female reproductive traits that can lead to rapid divergence (Birkhead and Pizzari 2002). In polyandrous and polygynandrous mating systems, females mate with multiple males, and females may evolve mechanisms to control which male(s) fertilize their ova. Meanwhile, males may evolve better fertilization efficiency in a co-evolutionary arms race (Birkhead and Pizzari 2002).

Post-copulatory female choice is called cryptic female choice, and is a bias in the fertilization success of the males that copulate with a particular female due to *her* phenotype (Eberhard 1996; Patrick and Brown 2000; Simmons 2005). Sperm selection is one mechanism by which females can exercise post-copulatory cryptic female choice. Although cryptic female choice is difficult to study and identify, and as of yet there are few convincing studies, there are several potential mechanisms that females could evolve to bias sperm storage and use in favor of certain males. For example, in many taxa, female sperm storage organs are complex, highly differentiated structurally and functionally, and rapidly co-evolve with sperm or ejaculate traits. This indicates that they may allow females some control over fertilization and sperm use (Thornhill 1983; Eberhard 1996).

Sperm competition is a post-copulatory equivalent of male-male competition wherein there is competition among males for a limited number of unfertilized ova when

the ejaculates of more than one male overlap in a female's reproductive tract (Parker 1998). For example, when sperm from one male cannot fertilize an ovum because it has already been fertilized by another male, sperm competition is occurring (Birkhead and Møller 1998). There are many factors that have potential effects on male sperm competitive ability. One of these potential factors is copulation duration, which has been thought to be indicative of sperm expenditure (longer copulation duration can mean more sperm deposited) (Simmons 2001). A male that is able to deposit more sperm than his rivals may have an increased chance of fertilization of ova (Parker 1990). Another factor is male size, wherein larger males may potentially have larger sperm stores.

In polyandrous mating systems, theory suggests that a male's fertilization success will increase proportionately with the number of his sperm relative to those of rival males, and males should evolve increased sperm expenditure (Parker 1990). Thus sperm competition can lead to evolutionary adaptations for producing more sperm or "better" sperm, such as larger testes or increased sperm viability or speed. Sperm depletion, or the exhaustion of sperm stores after mating, may affect the proportion of offspring a male will sire in future matings in a female's litter (Parker 1990). However, a male's ability to produce unlimited numbers of sperm may be constrained by energetic costs (Olsson, Madsen et al. 1997).

If sperm depletion is a factor in situations with sperm competition, then virtually all male reproductive resources should be used for sperm production. If a male remates with another female before his sperm supply is replenished, he will not be able to deposit as many sperm during that mating. Depositing fewer sperm may reduce his ability to compete with sperm from other males who have also mated with that female. Therefore,

theory suggests that males would be selected to allocate sperm to females strategically (Birkhead and Pizzari 2002). This may negate the effect of Bateman's principal, which argues that a male's reproductive success should be limited only by the number of females with which he can copulate, while a female's reproductive success should be largely independent of the number of males by which she is inseminated (Bateman 1948; Arnold and Duvall 1994; Lorch et al 2002).

The Study System

For many reptile species, females mate with more than one male and may produce clutches of offspring that have multiple paternities, meaning that offspring within the same clutch are sired by different males. Evidence for multiple paternity in reptiles was first observed in captive specimens of *Thamnophis*, and has been substantiated many times since within *Thamnophis* as well as in many other sauropsid taxa (Blanchard and Blanchard 1941; Blanchard 1943; Gibson and Falls 1975; Schwartz, McCracken et al. 1989; McCracken, Burghardt et al. 1999; Garner and Larsen 2005; Olsson and Madsen 1998).). Multiple paternity has also been observed specifically in females from the Inwood, Manitoba population of *Thamnophis sirtalis parietalis* (Friesen et al. unpublished data). Sperm competition likely occurs frequently in this species and thus must be an important factor in the evolution of the *T. s. parietalis* mating system.

In the mating system of *T. s. parietalis* from the Interlake region of Manitoba Canada, males and females emerge from large overwintering dens at the beginning of spring, and males begin pheromonally-mediated courtship and mating with females (Gregory 1974; Mason 1993). Typical male courtship behavior includes rapid tongue-flicking, chin rubbing, alignment of the male body with the female, caudo-cephalic

waves, and attempted or successful intromission (Whittier, Mason, and Crews 1985). The operational sex ratio, or the ratio of receptive females per male (Emlen and Oring, 1977), is highly skewed toward males (Shine, O'Connor, et al. 2001). Thus, upon emergence, females attract intense courtship from up to one hundred males, forming what is referred to as a mating ball (Gregory 1974, Shine, O'Connor, et al. 2001). This intense courtship can come with costs for the females, because it can become difficult for females to disperse from the den, and thus increase their vulnerability to predation (Shine, O'Connor, et al. 2001), and has been interpreted as an example of sexual conflict (Arnqvist and Rowe 2005). After copulation with a female, the male will deposit a copulatory plug into the female's cloaca that serves to prevent the female from remating, further limiting her choice to mate until the plug dissolves (Shine, Olsson, et al. 2000). In addition, the formation of the mating ball may also limit the female's initial mate choice. With so many males potentially trying to court with one female, it is unlikely that females are able to make any pre-copulatory decision about which male to mate with. These limitations on pre-copulatory female choice have also been interpreted as a form of sexual conflict (Arnqvist and Rowe 2005; Shine and Wall et al. 2005). Therefore, females may mate again to facilitate sperm competition or cryptic female sperm selection. Once mated, females can store sperm for long periods of time, sometimes up to two years or longer, and thus may mate once in the spring and again in the summer, fall, or following spring if she does not give birth that summer (Blanchard and Blanchard 1941; Blanchard 1943).

Sperm competition in the *T. s. parietalis* mating system, as evidenced in other reptilian mating systems, is likely to be beneficial for the viability and quality of

offspring produced from multiple matings (Birkhead and Møller 1998). Females that can utilize cryptic female choice can potentially choose the sperm from the “best” male to fertilize her offspring, thus producing offspring of higher quality or greater fitness. It is also by this mechanism that females may avoid using genetically incompatible sperm from males that would produce non-viable offspring. Genetic “bet hedging” is the idea that females may mate multiple times in order to prevent all of her ova from being fertilized by a poor-quality or incompatible male (Jennions and Petrie 2000). Evidence from studies on *Vipera berus* and *Lacerta agilis* have supported the genetic benefits of multiple matings – broods that have multiple sires have higher embryonic survival, fewer deformities, and in *Lacerta agilis*, the offspring are heavier and survive better during the first year of life (Madsen et al 1992; Olsson et al 1994). Thus there are many potential benefits to females mating multiply and making it likely that males must be successful in sperm competition to ensure his paternity.

In the *T.s.p.* mating system, female snakes will mate multiple times, and thus the sperm from the rival males will need to compete with each other for fertilization of that female’s ova. Females can store sperm in their reproductive tracts for up to two years, which may also compete with newer sperm and possibly reduce a recently mated male’s fertilization success. Mate order, or being the first male to mate rather than the second, for example, may also have an influence on sperm competition. Different mating systems have been shown to have skewed sperm precedence, some toward the first male to mate, as with 13-lined ground squirrels and spiders, and most toward the last male to mate, as seen in several rodent species (Dewsbury and Hartung 1980, Oglesby et al. 1981). Additionally, a male having depleted sperm stores may not be able to deposit as

many sperm in subsequent matings, thus reducing his ability to compete with sperm of rival males.

The Study

Sperm depletion is an important factor in post-copulatory sexual selection because sperm depleted males may have reduced competitive ability for fertilization of ova. We investigated the effect of sperm depletion on sperm competition in the model polygynandrous mating system of the Red-sided garter snake, *Thamnophis sirtalis parietalis*, in Manitoba Canada. We assigned paternity to males who had mated multiply with females who had also mated multiply to assess the importance of sperm depletion on male fertilization success when faced with sperm competition. If males that mate multiply are affected by sperm depletion, we predict lower proportions of paternity for their second matings than for their first mating, barring any other possible contributing factors. If sperm depletion does not occur in multiply mating males, there will be no significant difference in proportion of offspring a male will sire between the first and second mating.

MATERIALS AND METHODS

Specimen Collection and Captive Maintenance

Male and female Red-sided Garter Snakes (*Thamnophis sirtalis parietalis*) were collected in the Spring of 2007 from overwintering dens in Inwood, Manitoba before they had a chance to mate, and brought back to the Chatfield field station for mating. Mated female garter snakes were transported to Oregon State University in Corvallis, Oregon and maintained in 10 gallon glass aquariums in a microprocessor controlled environmental chamber. During the summer months, garter snakes were fed an alternating diet of trout and earthworms each week and were kept at 24°C during the day and 14°C at night, with 40-watt incandescent bulbs providing a thermal gradient within each aquarium. The females gave birth from August 22nd to September 11th 2007. From October to April, snakes were maintained at 4°C to simulate natural hibernation conditions.

Mating

Twenty four males were set up in one meter by one meter “arenas” and were allowed to mate repeatedly. The mated females were then assisted in mating with another male once the copulatory plug from the first mating had dissolved. In total, there were 52 matings spread between 24 males, with an average of 2.17 matings per male. All but one male mated once, one male had four matings, and two males had five matings.

Of the 52 females that were remated with other males, about 60 percent gave birth in late August to early September. For this study we used an opportunistic sample of females and their litters. We selected pairs of females that had mated with the same male (n = 6 males). One of the females in the pair had mated with a particular male on his first

mating; the other of the females in each pair had mated with the same male on his second mating ($n = 12$ females). Each of these matings was presumed to be the first mating for each female as they were collected as they emerged from the ground at the Inwood den site. Then, each of these females was mated to a second randomly selected male for the female's second mating ($n = 12$ males for a female's 2nd mating). The interval between matings ranged from 5-16 days. Thus, there were 6 pairs of litters that could be used to compare the effect of sperm depletion on male fitness as measured via proportion of offspring sired P_2 . The twelve families for this study ranged from 8 to 25 offspring, for a total of 225 individuals.

DNA Extraction, PCR, and Genotyping

Tail tips were collected as tissue samples for DNA extraction. Tissue samples were collected from each male in the field in Manitoba during the spring of 2007, and from each female and her offspring once she gave birth. DNA was extracted using the Chelex method; incubation of 0.05 g of tissue, 0.01 g Chelex resin (Sigma # C7901), and 2 μ L Proteinase K (Qiagen # 19131) in 0.2 mL of sterile water at 56 °C for 2 hours followed by 8 minutes at 100°C. Extracted DNA was used in several Polymerase Chain Reactions (PCRs) in order to genotype every individual. We used 4 published, highly polymorphic microsatellite markers in the same conditions as published; Ts1 (McCracken, Burghardt et al. 1999), Ns μ 2, Ns μ 3, and Ns μ 9 (Prosser, Gibbs et al. 1999). PCR products were sent to CORE labs at Oregon State University for genotyping with an ABI 3100 capillary DNA sequencer. Offspring genotypes were compared to the known possible males using one or more published, highly polymorphic microsatellite

loci, in order to assign paternity unambiguously. Offspring that could not be assigned to our known male genotypes were attributed to stored sperm.

Stored Sperm Analysis

In order to determine the minimum number of males representing the paternity from stored sperm, we separated the individuals for which paternity could not be assigned to either of our focal males and ran their genotypes through the paternity analysis program Gerud 1.0. Gerud determines the minimum number of males that represent the genotypes for the unassigned males and ranks the possible paternal genotypes in order of most likely to least likely.

Statistical Analysis

Once paternity was assigned, P_1 , the proportion of offspring sired by the first male to mate (in this case our focal males), was compared using a paired t-test to assess the effect of sperm depletion. The interval between matings, male mass and snout to vent length (SVL), and copulation duration were tested using ANOVA and/or multiple regression models.

RESULTS

Female Copulation Durations

There were no significant differences between the copulation durations for a female's first and second matings (paired t-test; $P = 0.168$). One of the 12 females in this study was omitted from this analysis, as there were no data for her second copulation duration. An additional statistical analysis including copulation durations for a larger sample size ($n = 50$) from the Inwood, Manitoba *T.s.p.* population confirmed that there was no statistically significant difference between a female's first and second copulation durations (Wilcoxon Signed Rank Test; $P = 0.338$). The normality test failed for the paired t-test in this case so a Wilcoxon Signed Rank Test was performed instead. In addition, there was no statistically significant difference between the masses of males that mated first versus males that mated second (paired t-test; $P = 0.289$).

Male Copulation Durations

For the focal males who were the first males to mate, there was no significant difference between the copulation durations for a male's first and second copulations (One Way RM ANOVA; $P = 0.425$). For one male in this analysis these were his 3rd and 5th matings rather than his 1st and 2nd matings.

P_1 after 1st mating vs. P_1 after 2nd mating

There was no significant difference in the values of P_1 after a male's first mating and after his second mating (One Way Repeated Measures ANOVA, Power = 0.068, $P = 0.314$). The mean of the values for P_1 after the first mating was 0.259 and the mean of the values for P_1 after the second mating was 0.057. A repeated measures design was

used for this analysis because we were comparing the P_1 values of the same males after successive matings. This test was performed to determine if there was a difference in the proportion of offspring the first male to mate sires after his 1st and 2nd mating (essentially testing for sperm depletion). However, the low power in this analysis was due to the very small sample size and relatively high variance, and thus we may not have detected a true difference.

P_1 vs. P_2 vs. P_{SS}

Given that there was no significant difference in the P_1 after the 1st and 2nd matings, we pooled those data for the first male's two matings to see if there were any significant differences between P_1 , P_2 , and P_{SS} (P_{SS} is the proportion of offspring sired by stored sperm). The normality test failed for a One Way ANOVA, so a nonparametric test was used to compare P_1 , P_2 , and P_{SS} . Log transformation was not an option to normalize these data because several of the proportion values were equal to zero. This test showed a significant difference in the median values of "P". A multiple comparison analysis showed that P_{SS} was significantly different from P_1 , but P_1 was not significantly different from P_2 , nor was P_2 from P_{SS} (Fig. 1, 2, and 3, One-Way ANOVA, P_1 vs. P_{SS} , $P < 0.05$; P_1 vs P_2 , $P > 0.05$; P_2 vs. P_{SS} , $P > 0.05$).

Stored Sperm Analysis

An average minimum number of two males were responsible for the proportion of stored sperm in families with offspring of unidentifiable paternity. The range was from a minimum of zero males representing stored sperm to a minimum of three males representing stored sperm (Table 1).

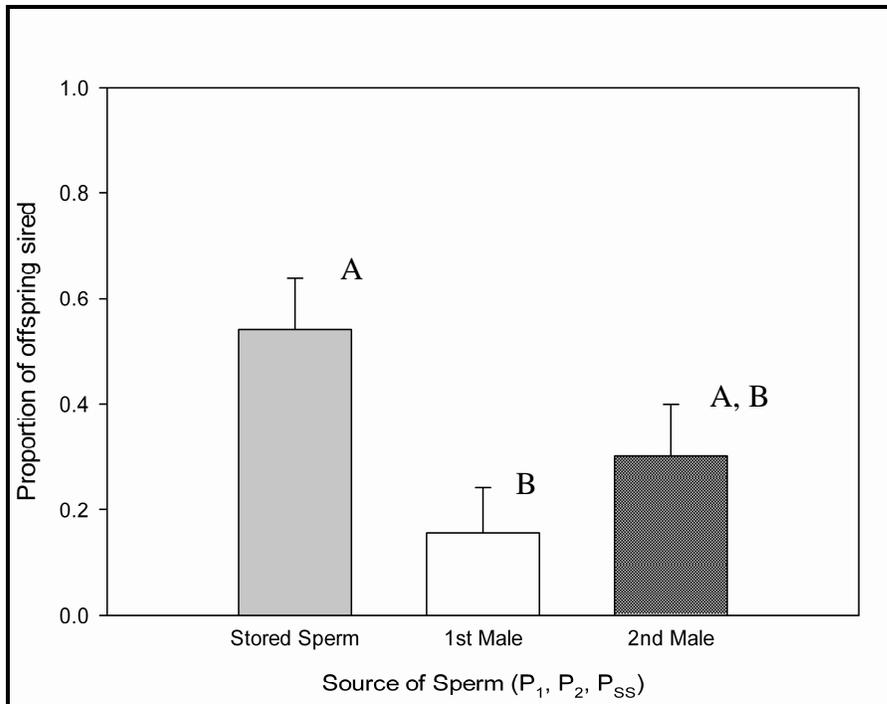


Figure 1: Sperm Precedence: P_1 vs. P_2 vs. P_{SS} for pooled data (mean P_1 , P_2 , and P_{SS} values). Stored sperm explains the majority of the paternity for the offspring in this experiment.

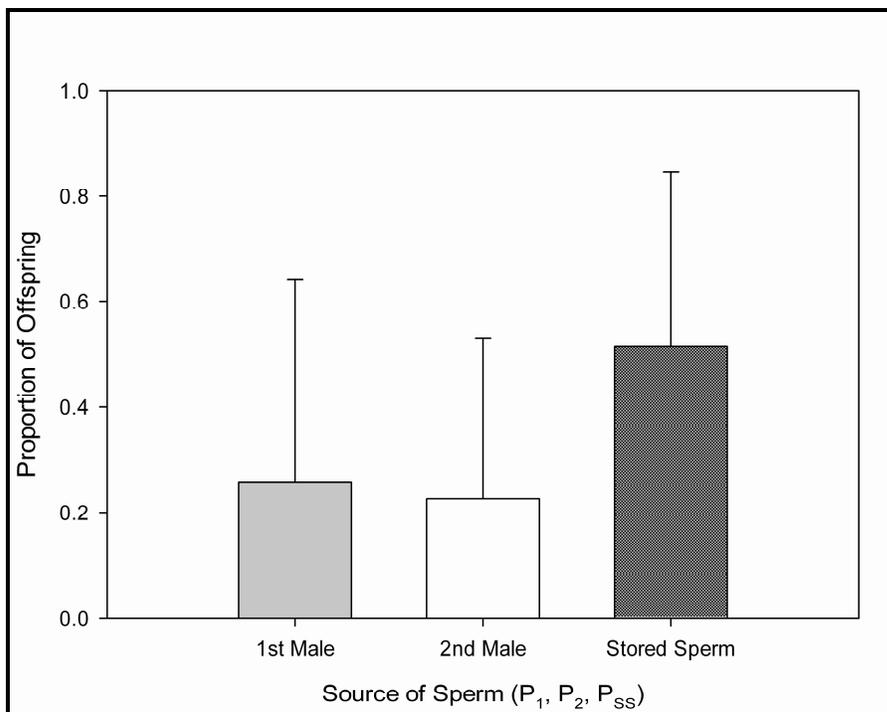


Figure 2: Sperm precedence after 1st mating. Stored sperm accounts for the highest proportion of offspring, followed by 1st male to mate and 2nd male to mate, which are close in value.

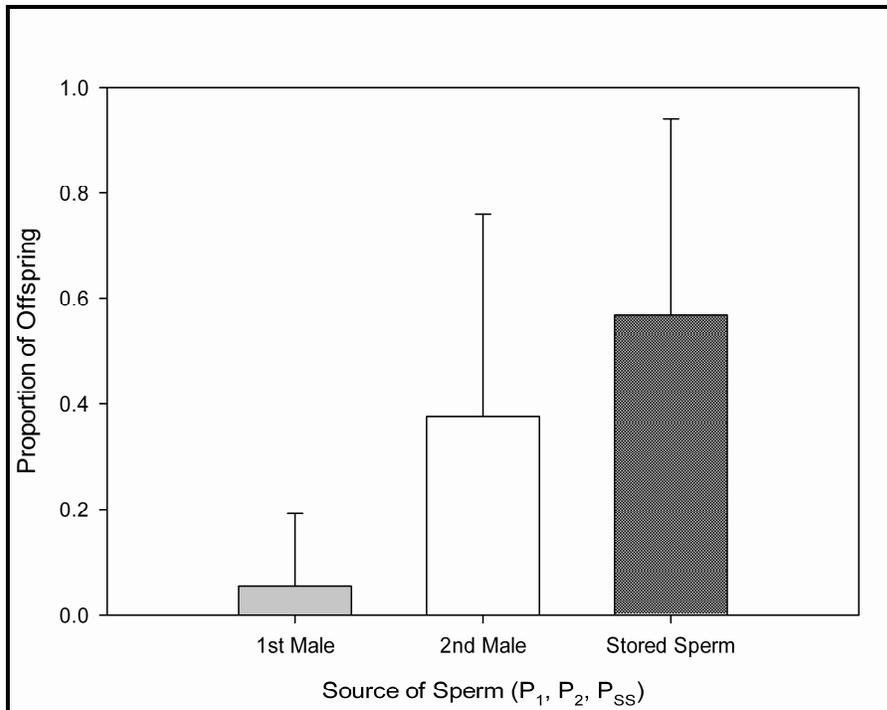


Figure 3: Sperm precedence after 2nd mating. Stored sperm accounts for the highest proportion of offspring, followed by the 2nd male to mate as the next highest, and 1st male to mate as the lowest.

Table 1: Table displaying P_1 , P_2 , and P_{SS} values, minimum number of males representing stored sperm, and minimum number of total males mated with for each female. Average number of males representing stored sperm (where stored sperm is present) = 2.

Family	P_1	P_2	P_{SS}	# males in SS	Total # males
712	0.00	0.63	0.38	2	4
732	1.00	0.00	0.00	0	2
744	0.00	0.13	0.87	3	5
760	0.05	0.60	0.35	2	4
764	0.23	0.00	0.77	2	4
768	0.27	0.00	0.73	2	4
772	0.33	0.17	0.50	2	4
808	0.00	0.50	0.50	1	3
812	0.00	0.65	0.35	1	3
852	0.00	0.00	1.00	3	5
868	0.00	0.94	0.06	1	3
884	0.00	0.00	1.00	3	5

DISCUSSION

Based on our results, we conclude that sperm depletion does not have an effect on sperm competition when a male mates twice. The male reproductive tract is bifurcated, including two separate hemipenes. It is possible that males have two complete ejaculates stored and ready for multiple copulations. However, the power for the One Way ANOVA used to analyze the values of P_1 after the 1st and 2nd matings (Power = 0.068) is much lower than the desired power of 0.80. The low power in this situation was due to the very small sample size and high variance in P_1 values. Although the difference in the means of P_1 after the 1st and 2nd matings (0.259 and 0.0556 respectively) was not statistically significant, the trend in the data, as depicted in Figure 1, indicates that sperm depletion may still be a significant factor in determining paternity in this *T.s.p.* mating system, which deserves future investigation, especially in those cases where males mate more than twice. Because male and female copulation durations, male masses, and interval between copulations were all ruled out as having any significant affect on P_1 , sperm depletion seems to be the likely candidate to explain the trend toward a difference in P_1 after the 1st and 2nd matings. It would be informative to repeat the study with a larger sample size to determine if these trends are consistent throughout the Inwood, Manitoba *T.s.p.* population. In addition, estimated allele frequencies in the *T.s.p.* for those four microsatellite loci that we used to determine paternity would be helpful for evaluating the confidence in our paternity analysis. We have 20 adult females and about 40 adults males with which we can make a more accurate measure of allelic frequencies of each locus to assign paternity exclusion probabilities to these data. This would allow us to assign confidence values to our paternity assignments. In addition, one modification to the

experimental design that would be helpful to investigate the effect of sperm depletion would be to mate individual males multiply with several females to track changes in paternity associated with many matings. The data from our investigation seem to show slight 2nd male precedence over the 1st male to mate (P_2 is larger than P_1 after both the 1st and 2nd matings), and the values for P_1 after both the 1st and 2nd matings are relatively low. These small values for P_1 make it somewhat difficult to determine how significant of a difference there is between the values of P_1 after the 1st and 2nd matings. Since the values of P_2 are larger, both after the 1st and 2nd matings, using some males that have mated multiply as the 2nd males to mate (possibly even 6 males, first unmated and then mated again with another female, as with the group of 1st males to mate) may make it easier to determine how significant a difference there is between P_2 after the 1st and 2nd matings, and thus we may be better able to estimate the affect that sperm depletion has on paternity.

One of the most intriguing results is the precedence of stored sperm. Pooling the data for all matings and comparing P_1 , P_2 , and P_{SS} revealed a significant difference between the median values of P_1 and P_{SS} (0.000 and 0.500 respectively). Previous research suggested that degeneration and extrusion of stored sperm from deep regions of the oviduct, called the infundibulum, can potentially be stimulated by mating (Halpert et al. 1982). The extrusion of stored sperm from the female's oviduct may prevent sperm competition from occurring by limiting the overlap of ejaculates in a female's reproductive tract. In addition, the copulatory plug deposited by the male after copulation lasts approximately 48 hours after mating (Shine 1999). This would imply that by the time the copulatory plug has dissolved, some (or all) newly deposited sperm from

a mating would have reached the infundibulum, and would thus be in potential “danger” of being stimulated to degrade and be expelled from the oviduct by any subsequent matings. If this is the case, it is possible that the low values for P_1 , both after the 1st and 2nd matings may be explained by degradation and expulsion of the 1st male’s sperm due to the stimulus of the 2nd male mating. If the 2nd male to mate stimulates the extrusion of the 1st male’s sperm, this could explain why we see greater values for P_2 than for P_1 (although not statistically significant). This explanation, however, does not account for the large values we see for P_{SS} , after both the 1st and 2nd matings. The idea that mating stimulates the breakdown and extrusion of sperm from the oviduct does not appear to apply equally to older (pre-hibernation) stored sperm as it does to newly deposited sperm from spring matings. It is possible that some of the stored sperm (pre-hibernation) may be located in deep regions of the tube-like receptacles that branch off the main infundibular lumen. This might make these stored sperm more resistant to sloughing off, and thus may not be as readily expelled from the oviduct as newer sperm that has not had the time to reach the deeper parts of the infundibulum. This could help to explain why we see such large proportions of offspring being sired by stored sperm (large values of P_{SS}), but smaller values for P_2 and even smaller values for P_1 . It is also possible that the location within the infundibulum that the stored sperm are held in allows these stored sperm them to be the first to leave upon ovulation and thus gain the majority of the fertilizations (Simmons 2001).

Females in this *T.s.p.* system are typically courted en masse by interested males immediately upon emergence from the den and are coerced into mating. Some have interpreted this behavior as a form of sexual conflict, because it makes any kind of pre-

copulatory mate choice virtually impossible for the female. In this case, it may be in a female's best interest to mate a second time in conditions where she can make a choice about which male to mate. She may then be able to employ cryptic female choice to select the sperm from her second mating to fertilize most of her ova, or she even may be stimulated by the second mating to expel the sperm from the first male to mate.

However, if the extrusion of the 1st male's sperm is forced (not a "choice" made by the female), this may also be a form of sexual conflict. These ideas may help account for why we see larger average values of P_2 than for P_1 , which is what we would expect to see if sexual conflict is occurring and females were remating to resolve the conflict. This theory, however, does not explain why we see larger average values for P_{SS} than for both P_1 and P_2 . It could be that the stored sperm is from males that the female chose to mate with, and thus the female chooses to fertilize a large portion of her ova with the stored sperm. The fact that we see an average minimum of two males representing the stored sperm for these females, however, seems to indicate that females may mate multiple times in order to have a variety of sperm to choose from (using cryptic female choice), to force sperm into competition, and/or to ensure that she will have enough sperm to fertilize all of her ova. She may also engage in multiple matings in order to prevent fertilizing all of her ova with sperm from one low-quality or incompatible male (bet hedging).

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