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Determinants of sperm release and storage in a spiny orbweaving spider

TODD C. BUKOWSKI & TERRY E. CHRISTENSON
Department of Psychology, Tulane University

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Abstract. Sperm release and sperm storage were examined in the spiny orbweaver, *Micrathena gracilis*. This species shares characteristics with other spiders that show a first-male advantage for egg fertilization. Male *M. gracilis* differentially released sperm to virgin females. Sperm release was not related to female age or copulatory duration. In this species, a complete mating between a pair requires two copulations that are separated by a dismount. The second copulation is twice as long as the first. After manipulating the duration of the second copulation, it was found that sperm were released early in the copulation. When a female copulated twice, storage of sperm in the female's first side was influenced by the prolonged second copulation. Considering that males often do not obtain two copulations, and that a female may alternate copulations when two males are present, it was experimentally determined that the copulatory stimulation provided by a second male does facilitate the storage of the first copulating male's sperm. It is concluded that sperm release and sperm storage are two separate processes. Copulation does not necessarily result in sperm release by the male. Not all sperm released are stored within the female, and the number stored is influenced by copulatory duration.

The reproductive consequences of a male's copulatory behaviour are determined in part by the number of his sperm that are released and taken up or stored by the female in relation to those of other males (Parker 1970, 1984; Parker et al. 1990). When one of several males that mate with a female has the advantage in fertilizing her eggs (Smith 1984; Birkhead 1988; Birkhead & Hunter 1990), males could benefit by responding differentially according to female mating history (Watson 1990, 1991a; Keller & Passera 1992; Lorch et al. 1993; references in Simmons et al. 1994; Lewis & Iannini 1995), especially when sperm are limited (Dewsbury 1988; Verrell 1990). For example, when there is a first-male advantage based upon some feature other than number of sperm released, males might be expected to avoid expending gametes on females that have previously mated. Discrimination on the basis of female mating history can occur before (Wicklund & Forsberg 1985; Lewis & Iannini 1995; Riechert & Singer 1995; Watson 1991a) or during copulation (Cordero & Miller 1990; Suter 1990; Lorch et al. 1993; Cigliano 1995).

In many species, the advantage in fertilization goes to the male with the greatest number of sperm within the female's reproductive tract (Martin et al. 1974; Woodhead 1985; Dickinson 1986). Males can adjust their ejaculate size in response to greater risk of cuckoldry (Baker & Bellis 1989; Gage 1991; Gage & Baker 1991) or to maximize the number of females they can inseminate (Dewsbury 1982; Pitnick & Markow 1994). This can be done by lengthening the duration of copulation, since sperm release can be time-dependent (Thornhill 1980; Dickinson 1986; Siva-Jothy & Tsubaki 1989; Keller & Passera 1992).

A male would also benefit reproductively by facilitating the storage of his own gametes once they are released. Eberhard (1985, 1991, 1994) noted that copulation may serve an 'internal courtship' function. Males with particular genitalic configurations might be better able to stimulate the female and facilitate the storage and usage of their sperm. Dewsbury (1988) suggested that tactile stimulation during copulation may affect female neuroendocrine processes that influence

Correspondence: T. C. Bukowski, Department of Psychology, Tulane University, New Orleans, LA 70118, U.S.A.

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male reproductive efforts. If the male is not able to deposit sperm directly into sperm storage sacs or onto the eggs, such stimulation of the female may prove critical for sperm movement to storage areas inaccessible to the male (Villavaso 1975; Miller 1987; Eberhard 1991; Ward 1993). Stimulation might, in part, be time- or duration-related (Cohn 1990; Ward 1993). More sperm might be stored from males that copulate for longer durations (Cohn 1990) or have greater intromission rates (Watson 1991b). Given the high energetic costs of copulation for the male and the positive relationship between male copulatory behaviour and general metabolic competence, males that copulate longer or more vigorously may be more fit (Watson & Lighton 1994). It has been suggested that this may be advantageous to the female.

We examined factors that might influence sperm release and storage in the spiny orbweaving spider, *Micrathena gracilis*. Spiders are a particularly good model for examining the gametic consequences of copulatory behaviour. The bilaterally paired organs that store sperm in both sexes are discrete and are readily accessible to the experimenter. Male spiders might be expected to discriminate between females because sperm numbers may be limited for the male (Christenson 1989; M. M. Wall, R. Sarojini, & T. E. Christenson, unpublished data) and because a first-male advantage in fertilization is common among spiders (Jackson 1980; Vollrath 1980; Austad 1982; Christenson & Cohn 1988; Watson 1991a, b; Masumoto 1993). Some evidence suggests that males discriminate on the basis of female mating history before (Riechert & Singer 1995) and during copulation (Suter 1990). We have suggested elsewhere that *M. gracilis* will also show a first-male advantage, and we have shown that although females are continuously sexually receptive, males discriminate between virgin and mated females after palpal insertion (T. C. Bukowski & T. E. Christenson, unpublished data). Males copulate with the non-virgin only once briefly before departing the web (T. C. Bukowski & T. E. Christenson, unpublished data). One purpose of the present study was to determine whether male *M. gracilis* differentially release sperm to virgin or non-virgin females. We also examined the effect of the often covarying factor of female age (Boorman & Parker 1976; Simmons et al. 1994) on sperm release and storage.

The relationship between copulation and release of sperm in spiders appears complex. In some species, males copulate without releasing sperm (Helsdingen 1965) or for durations longer than that necessary for the release of sperm (Austad 1982; Christenson & Cohn 1988; Willey Robertson & Adler 1994; Huber 1995). Such observations suggest that copulation may serve functions beyond that of sperm release. Spider sperm are non-motile at the time of copulation (Baccetti et al. 1970), and the length and width of the insemination duct appear to preclude the male from directly depositing sperm into the storage sacs (Watson 1991a; Huber 1993). It is likely that transport of sperm to the storage sacs requires some action by the female. Before and after males have released sperm, they might influence the female to store their sperm by copulating for longer durations.

One aspect of *M. gracilis* copulation is particularly important in regard to duration. Complete insemination of the female's reproductive tracts requires two copulations, separated by a dismount from the female. A male's second copulation with a given virgin female is always relatively long, on average more than twice the duration of the first (T. C. Bukowski & T. E. Christenson, unpublished data). Such differences in copulatory lengths have been reported in other spiders (Bristowe 1929; Huber 1993, 1995; Sasaki & Iwahashi 1995). The prolonged second copulation may serve a guarding function (T. C. Bukowski & T. E. Christenson, unpublished data). However, gametic implications of the longer second copulation have remained unexplored. In the present study, we determined the effects of the length of the second copulation on sperm release and storage.

During the course of this study, we found that a male's second copulation with a female facilitates the storage of sperm released during the first copulation. Given that different males often alternate copulations with a female (T. C. Bukowski & T. E. Christenson, unpublished data), it seems possible that if the first male copulates with only one of the female's two reproductive tracts and a second copulates with the remaining virgin tract, the second male could release sperm and also increase the number of the first male's sperm that are stored. We determined whether a male can facilitate the storage of another male's sperm.

GENERAL METHODS

Study Site

Observations were conducted at the F. Edward Hebert Center of Tulane University, 20 km south of New Orleans, Louisiana. The studies were conducted on a 30 × 40-m plot in a hardwood, bottomland forest. *Micrathena gracilis* occur there in relatively large numbers. The study area, located next to a lagoon, is frequently flooded during the mating season.

Procedures for Staged Encounters

Staged matings were conducted between 15 June and 15 August 1991 and between 1 July and 1 August 1993. To ensure they were virgin, females approaching the final instar were collected and held in 250-ml plastic collection vials until they moulted. Adult males were collected from the webs of penultimate-instar females, brought into the lab, inspected for bodily damage and weighed on a Mettler analytical balance. The reproductive histories of the males were not known. Some matings were conducted on webs with completed viscid spirals and some on webs consisting of single strands of silk. Web architecture does not influence copulatory duration (T. C. Bukowski & T. E. Christenson, unpublished data). When an encounter was staged on a web with a completed viscid spiral, a male was placed on the upper frame thread of the viscid spiral. When an encounter was staged on single strands of silk, the male was placed at the end of the single strand of silk opposite the female. If a male became disconnected between copulations and the pair was scheduled to copulate twice, the male was placed back onto the female's web. Duration of copulation, defined as the interval between insertion and dismount, as well as the male and female side (left or right) inserted were recorded. The intercopulatory interval refers to the amount of time between an animal's first and second copulation. The re-mating interval refers to the amount of time, where two copulations occurred, between the end of a female's mating encounter with one male and the first insertion of a second male.

Sperm Quantification

The methods for preparing the reproductive organs for the counting of sperm were based on those by Cohn (1990). Palps were removed by cutting through the palpal femur under a dissecting microscope. The spermathecae were then removed by cutting the sperm uptake duct at its intersection with the storage organ. A male's palps and a female's spermathecae were kept separate, and the side of the animal that the organ came from was noted. Each organ was then placed in a 1.5-ml polypropylene centrifuge tube (Brinkman) with 100 µl of a solution of 1 ml of 0.9% saline and 10 µl of 10% triton-x detergent drawn from a common stock. Sperm tended to aggregate, so treatment was required to facilitate a homogeneous distribution within a sample. Each organ was thoroughly ground with metal forceps for approximately 90 s and then vortexed for about 90 s. The tubes were then centrifuged for 25 min at 1000 *g*. The tubes were removed and the grinding, vortexing and centrifuging were repeated two more times. After the final vortexing, two 10-µl samples were removed and placed on a Nebauer improved double-chamber haemacytometer. The sperm were then counted under a light microscope at × 400. The mean of the two sampling fields was used for data analysis. All of the values reported are estimates of total numbers of sperm. The mean percentage difference between the two palps for each male was calculated by dividing the number of sperm in the palp with the least amount of sperm by the number of sperm in the palp with the greatest amount and subtracting 1 for each male.

Sampling with a haemacytometer requires that the cells be randomly distributed throughout the medium and across the sampling field. Clumping of the sperm cells can affect their distribution and the reliability of the estimate. When sperm clumps occurred, the total number of sperm in the clump was estimated and recorded. The two samples for each palp were grouped into those with no clumping, clumping in one sample or clumping in both samples. A correlation was then calculated between the two samples as an index of reliability. While the reliability of the sampling method was uniformly high, the correspondence of sperm in the two samples was slightly influenced by the degree of clumping: no clumping ($r=0.995$; $N=188$, $P<0.0001$), one sample clumped ($r=0.979$; $N=77$, $P<0.0001$) and both samples clumped ($r=0.93$; $N=84$, $P<0.0001$). Samples from three animals had clumps larger than an estimated 100 sperm, and they were excluded from all analyses.

The counting of spider sperm has presented certain challenges. Spider sperm tend to bind to the inside of glass and certain plastic containers. Sperm stored in females are particularly likely to be aggregated and are difficult to separate. Such aggregation may be common among spiders (Cohn 1988; Uhl 1993a, b). For these reasons, we modified the methods for counting sperm during the course of this study. We reduced the amount of scratching and abrasion inside the plastic sample vials to reduce mechanical binding. We also used detergent and ultrasonication to break up sperm aggregations. These procedures significantly reduced the amount of clumping and increased sperm counts both in males and females. These difficulties, however, do not invalidate our experimental effects, because all groups within a given experiment or within particular comparisons received the same sperm-handling treatment. Any reduction in sperm counts due to earlier methods would be constant across groups. Moreover, the effects of the influence of copulatory duration on sperm storage were replicated using the modified procedures.

SPERM NUMBERS IN THE PALPS OF FREE-RANGING MALES

Methods

We determined the number of sperm a male might have prior to copulation by examining free-ranging individuals. We collected 21 males from the webs of penultimate-instar females at regular intervals throughout the 1991 season. Within 2 days of being collected, males were killed by hypothermia. Palps were removed and sperm were immediately quantified. We recorded lengths of male tibia–patella I and II, cephalothorax and cephalothorax–abdomen, as well as wet weight.

Results

Free-ranging males were collected throughout the season from webs of penultimate-instar females, and all of their palps contained sperm. Males ($N=21$) had a mean $\pm$ SD total (both palps) of $37\,755 \pm 15\,011$ sperm (range=20 889–61 000) and a mean $\pm$ SD difference of $18.5 \pm 11.9\%$ between the two palps. Positive relationships were found between male body size measurements and sperm number; only the relationship between cephalothorax length and sperm number was statistically significant ($r=0.50$, $P=0.02$).

MALE SPERM RELEASE IN RELATION TO FEMALE MATING HISTORY

Methods

We determined whether the number of sperm released by the male is influenced by the mating history of the female. We compared the numbers of sperm released by males that had mated with virgin females ($N=7$, Both Sides group; see Sperm Release and Storage in Relation to Copulatory Number and Duration, below) and males ($N=21$) collected from the webs of penultimate-instar females and with males ($N=19$) mated with non-virgin females. Seven males were added to the webs of females that had mated no more than 25 min before on both tracts (range=6–25 min, Short Re-mating Interval). Another 12 males were given females that had moulted and mated on both tracts 24 h earlier (Long Re-mating Interval). Males were collected after departing from the female's web and before they could induct sperm. Males were then placed on ice, brought into the laboratory and their sperm quantified. The durations of the mated females' first two copulations were not recorded; we did note that all mated on both sides. The females were free-ranging after their first encounter and therefore could have mated with additional males without our knowledge. Sperm content of these females was not quantified. The animals in this study were part of an investigation of factors influencing copulatory frequency and duration (T. C. Bukowski & T. E. Christenson, unpublished data).

Results

Males do discriminate between virgin and non-virgin females as indicated by copulatory frequency and duration (T. C. Bukowski & T. E. Christenson, unpublished data). We compared the palpal sperm content of males that were presented to virgin females with those given to non-virgin females. The non-virgin females had previously mated on both sides for the first time 24 h or more ($N=12$, Long Re-mating Interval) or 25 min or

less ($N=7$, Short Re-mating Interval). When the female's re-mating interval was longer, males copulated only once. When the re-mating interval was shorter, five of seven males copulated twice.

Males released few or no sperm to non-virgin compared with virgin females, as indicated by the larger number of sperm remaining in the palps after mating with non-virgins ($F_{2,23}=8.51$, $P=0.0017$). Neuman–Keuls post hoc tests show that both groups of males with non-virgin females had significantly greater numbers of sperm remaining in the palps than males with virgin females ($P<0.01$). The mean total ± SD amount of sperm remaining in the males' palps after copulating with females with short (37 311 ± 19 500) and long (41 800 ± 19 211) re-mating intervals was similar to that found in free-ranging males (37 755 ± 15 011; $F_{2,37}=0.25$, $P=0.78$).

The recency of a female's re-mating did not influence the total number of sperm released, as indicated by the number of sperm remaining in the male's palps ($F_{1,17}=0.87$, $P=0.37$). There also was no overall difference in the number of sperm remaining in the first versus second palp used ($F_{1,17}=3.75$, $P=0.07$). There was an interaction, however, between the female's re-mating interval and the number of sperm found in the first and second palps used ($F_{1,17}=7.89$, $P=0.012$). When the female's re-mating interval was short, males apparently did release some sperm during the first copulation (12 756 ± 8122) but not the second (20 556 ± 12 400). When the female's re-mating interval was long, males left the mating encounter with about an equal amount of sperm in the used (21 633 ± 10 033) and unused (20 178 ± 10 356) palps.

SPERM RELEASE AND STORAGE IN RELATION TO COPULATION NUMBER AND DURATION

Methods

We determined whether the pattern of copulatory behaviour affects sperm release by the male and sperm storage by the female. We varied the number and duration of copulation in mated pairs and assessed effects on sperm release and storage. Eighteen females were given one male and allowed to copulate on both sides (Both Sides group). All females and the first seven males were collected and their sperm quantified. Seventeen additional males (One Side group) were placed individually with virgin, newly moulted females but were collected after the first copulation. Twelve other males (Interrupted group) were placed individually with virgin, newly moulted females and allowed to copulate, but the second copulation was terminated at a time equal to the duration of that pair's first copulation. Termination was accomplished by taking hold of the male with forceps and plucking him free. When subjects were collected after a staged encounter, they were placed in vials and males were immediately placed on ice to inhibit sperm induction (the filling of palps with sperm). Subjects were then transported to the laboratory and males were kept in a refrigerator. One male was dropped and lost in the Interrupted group, and spermathecae from two females in that group were lost during removal. Males were killed within 2 days of mating and their sperm were quantified. Females were killed within 4–7 days of mating and sperm were then quantified.

Results

One copulation

To determine how many sperm are released during a single copulation with a virgin, newly moulted female, the palps of One Side males ($N=17$) were examined for presence of sperm. Used palps contained very few sperm compared to unused palps ($F_{1,16}=63.73$, $P<0.0001$; Table I). Males released an estimated 85.5% of the sperm from the palp used for the first copulation. The mean amount of sperm stored by the female (Table I) amounted to an estimated 20% of that released. No sperm were found in the unmated spermatheca.

Two copulations

Second-side copulations were significantly longer than first-side copulations ($F_{1,17}=21.56$, $P=0.0002$). The first and second spermathecae of females that mated on both sides ($N=18$; Both Sides group) contained about the same number of sperm (Table I; $F_{1,17}=0.28$, $P=0.61$). After mating but before sperm induction could occur, the males' ($N=7$) two palps also contained about an equal number of sperm ($F_{1,6}=0.002$, $P=0.99$). Palps of males that had mated with virgin females contained significantly fewer sperm than did

Table I. Sperm release and storage for pairs allowed one or two uninterrupted copulations and for pairs in which the second copulation was interrupted

	First side			Second side		
	N	Mean	SD	*N*	Mean	SD
One copulation						
Sperm in female	17	4055	4533	—	—	—
Sperm in male	17	3336	4527	17	23 248*	11 364
Duration	17	891†	904	—	—	—
Interrupted second copulation						
Sperm in female	9	7858	4627	9	6333	4275
Sperm in male	11	4189	2185	11	3855	2269
Duration	9	579	112	9	574	116
Two copulations						
Sperm in female	18	10 049	7289	18	9389	7958
Sperm in male	7	4421	3259	7	4436	3203
Duration	18	675	303	18	1998	1280

*Sperm counts of unused palp.
†One male could not remove palp until 4098 s; with this removed, the mean ± SD is reduced to 690 ± 380.
Also included are data on copulatory duration (in s).

the palps of free-ranging males ($F_{1,26}=24.26$, $P<0.0001$).

Interrupted second copulation

We determined whether sperm are released early or late during the second copulation and examined sperm storage in a shortened second copulation. The second coupling of each male ($N=12$) was terminated at a time matching the duration of the male's first copulation (first versus second copulation, $F_{1,11}=1.94$; $P=0.19$). At the time of interruption, the hematodocha was still inflated. There was no significant difference (Table I; $F_{1,10}=0.13$, $P=0.73$) in number of sperm remaining in the first (uninterrupted) and second palp (interrupted copulation). This result suggests that sperm are released early in the second copulation. There was also no significant difference ($F_{1,8}=0.44$, $P=0.53$) in number of sperm found in the first and second spermathecae inseminated (Table I).

Overall, the number of sperm stored in the first spermatheca inseminated was influenced by the second copulation. When the numbers of sperm stored in the first spermathecae for all groups are compared, interrupted copulations resulted in an amount ($\bar{X}=7858$) intermediate to that stored when only one copulation was allowed ($\bar{X}=4055$) and that stored when two normal copulations were allowed ($\bar{X}=10\,049$; $F_{2,41}=4.65$, $P=0.015$; Table I). Post hoc tests showed that only the Both Sides and One Side groups significantly differed from one another (Neuman–Keuls $P=0.03$) in numbers of sperm stored. Males that copulated twice had 59.6 and 21.8% more sperm stored in the first spermathecae than One Side and Interrupted males, respectively. There were 36.9% more total sperm (first and second sides combined) stored in the Both Sides females compared with the Interrupted females (Table I). The three groups of females did not differ ($F_{2,44}=1.22$, $P=0.31$) in terms of duration of first copulation or in the number of sperm released, as indicated by the number of sperm remaining in the male's palps after the first copulation ($F_{2,32}=0.66$, $P=0.53$; Table I). Group differences in numbers of sperm stored are therefore related to the degree of stimulation accompanying the second copulation.

SPERM RELEASE AND STORAGE IN RELATION TO FEMALE AGE

Methods

In 1993, we examined whether female age (i.e. the interval between the female's final moult and first copulation) influences the number of sperm that are released and stored. Day 1 females

Table II. Sperm remaining in male palps after copulation, sperm stored in females and copulatory durations (in s) for matings staged on the first or fifth day after the female's moult to adulthood

	First side			Second side		
	N	Mean	SD	*N*	Mean	SD
Day 1						
One copulation						
Sperm in female	11	22 967	11 822	—	—	—
Sperm in male	11	4022	1378	11	39 000*	12 467
Duration	11	632	290	—	—	—
Two copulations						
Sperm in female	11	35 689	10 455	11	32 667	11 189
Sperm in male	11	5067	2611	11	4600	3133
Duration	11	551	147	11	1471	918
Day 5						
One copulation						
Sperm in female	10	29 044	3289	—	—	—
Sperm in male	9†	4778	2978	9	40 122*	7311
Duration	10	771	315	—	—	—
Two copulations						
Sperm in female	11	39 267	4755	11	36 044	7089
Sperm in male	11	4233	1511	11	4844	2311
Duration	11	678	307	11	1343	474

*Sperm counts for unused palps.
†One male was lost after dismounting the female and falling to the ground.
Pairs were allowed to copulate once (i.e. on one side only) or twice (i.e. on both sides).

($N=22$) were mated within 24 h of moulting to adulthood, and Day 5 females ($N=21$) were mated 5 days after the final moult. We chose 5 days because the female's sperm uptake ducts and storage organs appear to have hardened by this time. About half of the animals in each group were allowed to copulate on only one side, while the remaining animals were allowed to copulate twice, once on each side. All matings in this study were conducted on a single strand of silk. After copulation, all subjects were placed in vials and males on ice. They were then brought into the laboratory and their sperm quantified.

The methods for sperm quantification were modified for this study to reduce clumping and to increase the accuracy of the sperm counts. Female reproductive organs were removed one day after mating. The reproductive organs were ground, then centrifuged once at 1000 *g* for 20 min. Within 5 min, the samples were ultrasonicated using a Virsonic sonicator with a 3.2 mm probe at a low level (approximately 2% of total power output) for about 30 s. Within 1 min, the preparations were vortexed for approximately 60 s and a sample was immediately drawn and placed on a haemacytometer. One sample per palp or spermatheca was counted.

Results

Female age (the first mating at day 1 or day 5 after the final moult) did not significantly influence the total copulatory duration ($F_{1,20}=0.00002$, $P=0.996$; Table II). Overall, first copulations were significantly shorter than second copulations ($F_{1,20}=33.04$, $P<0.0001$). The durations of the first and second copulations did not vary as a function of female age ($F_{1,20}=0.86$, $P=0.37$). Female age also did not significantly influence the number of sperm released by males as indicated by the number of sperm remaining in the palps. When males mated on both sides of the female, female age did not influence the total number of sperm remaining in the palps ($F_{1,20}=0.10$, $P=0.76$). There was no significant overall difference in the number of sperm in the first and second palps used ($F_{1,20}=0.03$, $P=0.87$). The interaction between female age and the

number of sperm in the first and second palps used was also not significant ($F_{1,20}=1.47$, $P=0.24$). Males that copulated once with Day 1 and Day 5 virgin females released approximately 89.7 and 88.1% of the sperm in the used palp, respectively. No significant difference was found in the total number of sperm stored as a function of female age (Table II) when females copulated once (Day 1 versus Day 5; $F_{1,19}=2.46$, $P=0.13$). When females copulated twice, female age did not influence the total number of sperm stored ($F_{1,20}=1.08$, $P=0.31$). There was no overall difference in the number of sperm stored in the first and second side ($F_{1,20}=3.63$, $P=0.07$). The interaction between female age and the numbers of sperm stored in the first and second sides inseminated was also not significant ($F_{1,20}=0.004$, $P=0.95$).

When Day 1 and Day 5 data are collapsed, females that copulated on both sides had about 31% more sperm in the first storage sac than did females that copulated only once ($F_{1,41}=19.32$, $P<0.0001$). There was no significant difference, however, in the durations of the first copulation ($F_{1,41}=1.06$, $P=0.31$). These results support those reported in the previous study of sperm release and storage in relation to copulatory number and duration.

SPERM RELEASE AND STORAGE IN RELATION TO MALE AND FEMALE SIZE

Methods

The relationships between male ($N=16$) and female ($N=16$) size and the numbers of sperm released and stored were examined. Subjects were from animals that mated on both sides in the female age study using eight animals each from the two age groups. The modified sperm counting methods were used.

Results

The number of sperm remaining in the males' palps after release was significantly related to the males' total body length ($r=0.50$, $P=0.033$), but not to the lengths of the cephalothorax ($r=0.17$), tibia–patella I ($r=0.29$) or tibia–patella II ($r=0.31$) or to wet weight ($r=0.35$). The number of sperm stored in the female was significantly correlated with male cephalothorax length ($r=0.50$, $P=0.048$) but not with length of body ($r=0.49$, $P=0.054$), tibia–patella I ($r=0.21$) or tibia–patella II ($r=0.27$). Female body size was not significantly related to the number of sperm stored (all correlations $r<0.06$).

INFLUENCE OF A SECOND MALE ON THE STORAGE OF THE FIRST MALE'S SPERM

Methods

Two pieces of evidence would indicate that a second male's copulation facilitates the storage of the first copulating male's sperm: (1) if female A, which has copulated only once, has fewer sperm in the first spermatheca than has female B, which has copulated with two males (one on each side); (2) if female B, which has copulated with two males, shows sperm quantities in each spermatheca similar to those found in females that have copulated on both sides with a single male.

Pairs of females (one each from groups A and B, $N=8$ per group) were each given a male (group A, $N=8$; group B, $N=8$) at about the same time and allowed to copulate. When both males had copulated once and had used the same palp (right or left), male B was collected from female B after dismount and placed on ice. Then male A was transferred from female A to female B for a second copulation. Consequently, group B females received two copulations (one on each side) of normal durations, but they were performed with different males (first copulation with male B and second copulation with male A). Similarly, group A males obtained two copulations of normal durations, but they were with different females. As a non-switched control, females were given a male and allowed to mate on either one ($N=10$) or both sides ($N=10$). These control animals constituted the Day 1 group from the study of sperm release and storage in relation to female age noted above.

All of the females used in this study were mated within 24 h of moulting to adulthood. Two females were mated nearly simultaneously by starting the second mating immediately after the first male and female had begun copulating. The inter-copulatory intervals were recorded for females that were allowed two copulations. Male size was not controlled, and first and second males

Table III. Evidence that a second male can influence the storage of the first male's sperm

	First side			Second side		
	N	Mean	SD	*N*	Mean	SD
Group A females						
Number of sperm stored	8	25 889	7567	—	—	—
Duration	8	535	131	—	—	—
Group B females						
Number of sperm stored	8	33 917	3542	8	36 708*	10 048
Duration	8	590	149	8	1119	398
Group A males						
Number of sperm stored	8	25 889	7567	8	36 708	10 048
Number sperm in palps	8	4806	2891	8	5542	4034
Duration	8	543	138	8	1119	398
Group B males						
Number of sperm stored	8	33 917	3542	—	—	—
Number sperm in palps	8	5347	2585	8	37 014	11 037
Duration	8	590	149	—	—	—
Non-switched controls						
Number sperm in female	11	35 689	10 455	11	32 667	11 189
Number sperm in palps	11	5067	2611	11	4600	3133
Duration	11	551	147	11	1471	918

*Sperm counts of unused palp.
Sperm release and storage and copulatory duration (in s) when females (group A) were allowed one copulation (with group A males) or two copulations (group B females), each by a different male (groups B and A). Some males (group B) were allowed to copulate once and some males (group A) with two different females (groups A and B). Non-switched controls were single male–female pairs that copulated twice (see text). Number sperm stored = number of sperm stored within the female(s). Number sperm in palps = number of sperm remaining in the males' palps after mating, but before sperm induction.

did not differ in size in terms of tibia–patella length (mm) of the first leg ($\bar{X} \pm \text{SD} = 1.15 \pm 0.07$ and 1.10 ± 0.04, respectively; $F_{1,14} = 3.04$, $P = 0.10$). The inter-copulatory interval for females given two males (group B females) did not significantly differ from the inter-copulatory interval of females (day 1) mating twice with one male (138.2 ± 44.6 and 181.6 ± 181.8 s, respectively; $F_{1,17} = 0.43$, $P = 0.52$).

When staged matings were terminated, females were collected and kept at room temperature until sperm were quantified. Males were placed in vials and put on ice for transportation to the laboratory refrigerator. All animals were examined for sperm content within 24 h of collection using the revised sperm counting methods.

Results

We determined whether a female's second-side copulation by a second male can increase the storage of sperm released by the first male with whom she copulated on her first side. We compared sperm stored by two groups of females: group A females copulated on one side only and group B females copulated twice (i.e. once on both sides), once with each of two males (groups B and A males; Table III). The group B females' second copulation by group A males (on the second reproductive tract) was associated with the storage of the first males' (group B) sperm in the first spermatheca (sperm number in the first side of A versus B females, $F_{1,14} = 6.31$, $P = 0.0245$; see Table III). Furthermore, females that received two normal copulations (group B), one each from two different males (groups B and A), had about the same number of sperm stored in each spermatheca as did non-switched females copulating with one male on both sides ($F_{1,18} = 2.76$, $P = 0.11$; see Table III). Copulatory stimulation provided by the second male apparently facilitated storage of the first male's sperm.

Group A males that copulated once with each of two females (groups A and B) had significantly more sperm stored within the second female (group B) than within the first female (group A),

who received no second copulation ($F_{1,7}=5.92$, $P=0.045$; see Table III). In comparison, after a non-switched male copulated on both sides of a single female, the first spermathecae contained about the same number of sperm as the second ($F_{1,10}=2.85$, $P=0.12$; see Table III).

The sperm storage patterns reported here cannot be attributed to abnormal copulatory durations, because our experimental design provided a situation in which males obtained two copulations of normal durations (with two females) and females received two copulations of normal durations (with two males). The copulatory durations of males (group A) mating with two different females did not differ from those of a non-switched male and female pair (Table III); there was no difference in the total copulatory duration ($F_{1,17}=0.85$, $P=0.37$); first copulations were, as expected, shorter than second copulations ($F_{1,17}=22.63$, $P=0.0002$); and the durations of the two copulations did not differ as a function of number of females the males mated with ($F_{1,17}=1.19$, $P=0.29$). Similarly, the copulatory durations of females (group B) did not differ from those observed with non-switched females mating twice with one male ($F_{1,17}=0.69$, $P=0.41$; Table III). Overall, first copulations were shorter than second copulations ($F_{1,17}=18.94$, $P=0.0004$), and the interaction between the duration of copulation (first and second) and the number of males the females mated with (one or two) was not significant ($F_{1,17}=1.4$, $P=0.26$).

DISCUSSION

Sperm Release by the Male

The number of sperm released by the male was influenced by female mating history and related to one index of male body size, but was not related to duration of copulation (within or between copulations), or female size or age. With a first-male advantage in fertilization, one might expect males to discriminate between virgin and mated females. If the first-male advantage pattern is not based upon numbers of sperm released, that is, if a first-male advantage occurs even when the two males release equal numbers of sperm, then mated females would be of lower reproductive value. We have suggested elsewhere (T. C. Bukowski & T. E. Christenson, unpublished data) that a first-male advantage will be the common pattern in *M. gracilis*. Male *M. gracilis* discriminate between virgin and mated females in terms of overt copulatory behaviour (T. C. Bukowski & T. E. Christenson, unpublished data) and in the more hidden processes of sperm release. Prior to insertion, *M. gracilis* males apparently receive little information about the female's mating history (T. C. Bukowski & T. E. Christenson, unpublished data). Once copulation was initiated, males mating with virgin females released most of the sperm in their palps. Males mating with non-virgin females released relatively few or no sperm depending upon the recency of the female's previous mating.

The effects of female re-mating interval on sperm release suggest that copulation initiates a change in the female nearly immediately, and that up to 24 h are required for maximal inhibition of sperm release at subsequent copulation, at least with females that previously mated on both sides. The nature of this change in the female is not clear. It could be mechanical or structural in nature, for example, feedback from a filled spermatheca, gross changes in the degree of sclerotization of the female's reproductive tracts (Higgins 1989) or a plug (Kaston 1970; Levi 1975; Jackson 1980; Austad 1984; Masumoto 1993). It is also possible that a narrowing of the sperm uptake duct occurs with copulatory stimulation and that this narrowing serves functionally as a plug. The palps of some male *Micrathena* do stick inside the female, making withdrawal difficult. This difficulty in palp removal could be due, however, to other mechanical difficulties in disengaging from the female. Alternative to mechanical considerations is a male-induced change in the female that affects her signalling of virgin status (Suter 1990; Watson 1990).

The change induced in the female has a nearly immediate effect on the release of sperm by subsequent males and supports our suggestion that the prolonged second copulation with virgin females may serve a guarding function (T. C. Bukowski & T. E. Christenson, unpublished data). Perhaps males copulate for much longer during the second coupling so that they occupy the female until the change that prevents other males from releasing sperm is in effect. An alternative explanation related to guarding is that the latency or degree to which the change is affected depends on the duration of copulation. Just as a male might prolong the second copulation to

facilitate the storage of sperm on both sides of the female, a male might prolong the second copulation to occupy the female or facilitate changes that prevent subsequent males from releasing sperm on both sides of the female. The guarding function is likely to be of greater reproductive significance to the male, given that he might effectively block the release of sperm by subsequent males.

That longer copulations may serve a guarding function is corroborated by the behaviour of males copulating in the presence of competing males (T. C. Bukowski & T. E. Christenson, unpublished data). When two or more males are present and simultaneously courting a virgin female, the female will copulate with one male and respond to the courtship of another. The female will show the acceptance posture to the courting male, and he will contact the copulating male. Copulating males are not dislodged; rather, they increase the duration of copulation (T. C. Bukowski & T. E. Christenson, unpublished data). A male might prolong copulation until the change in the female is affected and the risk of losing fertilizations to other males is reduced. Males in other animal groups prolong copulation until female non-receptivity is induced (Riemann et al. 1967), to lengthen the female's non-receptive period (Drummond 1984) or until the female oviposits (Sillen-Tullberg 1981).

When assessing the effects of female mating history on male sperm release, female age should also be examined, because a mated female that a male encounters is likely to be older than a virgin female. Second males failed to release sperm to non-virgin females at least 1 day after the females' moult and first mating. This failure to release sperm is not due to female ageing, because males released their gametes to older virgin females, 5 days after the females' final moult. This result is somewhat surprising because the female reproductive tract is hardening during this time (T. C. Bukowski & T. E. Christenson, personal observation). Spermathecae were more rigid and easier to remove 5 days after moulting compared with 1 day post-moult. In the orbweaver *Nephila clavipes*, a similar hardening occurs (Higgins 1989), copulation is more difficult for the male to achieve (Christenson & Goist 1979) and relatively few sperm are released and stored within the female (Cohn 1990). Female ageing does not appear to have this effect in *M. gracilis*, at least over the first 5 days after the final moult. We can offer no explanation other than to note that 5 days might not be sufficient for complete hardening of the reproductive tract to occur. Comparative histological study of effects of female ageing on sperm release and storage is required.

There is probably a maximum number of sperm that a male *M. gracilis* can release to a female, and this number appears to be related to the male's body size. The number of sperm contained in the male's palps was significantly correlated with male cephalothorax length and was positively correlated with all measures of body size. Perhaps cephalothorax length is a more reliable measure of size than weight or overall body length, because the latter measures could be influenced by feeding and drinking. These data, and Cohn's (1990) on another spider, indicate that larger males have more sperm to release than smaller males. Larger males probably have more gonadal tissue to produce more sperm and larger palps in which to store more sperm (Cohn 1990). The relationship between body size and sperm number also suggests that the total number of sperm a male produces over a lifetime, as well as the number contained in the palps at any given time, might be influenced by foraging success as a juvenile, because spider body size as an adult is related to the foraging success as a juvenile (Vollrath 1987). Body size would limit the number of sperm a male could deliver to any one female, unless males mate and immediately induct sperm and re-mate with that female, which male *M. gracilis* do not.

Male *M. gracilis* released most (85–90%) but not all sperm in each palp to virgin females. Pitnick & Markow (1994) suggested that sperm retention might be a male sperm apportionment strategy. Retaining a portion of the sperm might be advantageous to the male *M. gracilis*, because he might fail to obtain a second copulation with a female. A male that fails to obtain a second copulation might have one full and one nearly empty palp. The behaviour patterns involved in sperm induction are complex and highly stereotyped (T. C. Bukowski & T. E. Christenson, unpublished data), and a male might not be able to preferentially fill only one palp. The male could use the full palp for the first copulation and the nearly empty palp for the second copulation with the next female it encounters. Perhaps the small number of sperm retained by that male is enough to inseminate one tract of another female and

induce changes in the female that prevent subsequent males from releasing sperm. Sperm retention would be particularly valuable if a male spider has a limited number of sperm, as has been suggested (Christenson 1989; M. M. Wall, R. Sarojini, T. E. Christenson, unpublished data).

The duration of copulation was not directly related to the number of sperm released. Early termination of the second copulation revealed that sperm are released early in the copulation, as others have noted (Austad 1982; Christenson & Cohn 1988; Willey Robertson & Adler 1994; Huber 1995). This would indicate that copulatory behaviour serves functions other than sperm release.

Sperm Storage in the Female

Sperm storage was influenced by the numbers and duration of copulation. Females that copulated twice stored a total number of sperm about three times that of females that copulated once, even though males released an equal number of sperm during the left and right copulations. The prolonged second copulation facilitates storage of sperm on the female's first side. Facilitation of storage apparently occurs throughout the second copulation, as indicated by the intermediate number of sperm stored when the second copulation was interrupted. The prolonged second copulation may facilitate sperm transport from the insemination duct to the spermatheca. As noted earlier, spider sperm are non-motile at the time of insemination (Baccetti et al. 1970), so another means of transport is necessary, assuming that males do not release sperm directly into the spermatheca. Anatomical evidence suggests that (at least with entelegynes, i.e. those with heavily sclerotized and conduit-shaped spermathecae) males are unable to release directly into the females' sperm storage organs because of intromittent organ size considerations (Watson 1991a; Huber 1993; but see Andrade 1996). An alternative is that stimulation from the second copulation reduces leakage from the spermathecae (Parker 1970). Little is known about sperm leakage and this merits further study. We are testing these hypotheses by histological analysis of position of the sperm at various times during copulation.

What physical mechanisms could underlie the facilitation of sperm storage on the first or contralateral side? Males do not contact the first copulatory pore after switching to the second side, and there are no internal ducts connecting the two insemination tracts (T. C. Bukowski & T. E. Christenson, personal observations; Figure 65 in Levi 1978). External stimulation in the area of the copulatory pore is a possibility (but see Huber 1993). Some female spiders have abdominal sensory receptors concentrated in the epigynal area (Barth 1985). Slit-sensillia (lyriform organs) respond to stretching or deformation of the integument and sensory hairs respond when bent. Expansion of the hematodocha against the protruding epigynal scape (T. C. Bukowski & T. E. Christenson, unpublished data) may stimulate sensory receptors. This structure is apparently broken off after copulation in some *Micrathena* species, but not *M. gracilis* (Levi 1985), and may prevent other males from insertion or facilitating the storage of their sperm.

About half of all females have more than one male present when they sexually mature, and the females can alternate copulations with them (T. C. Bukowski & T. E. Christenson, unpublished data). A second male to mate with a given female can facilitate the storage of sperm released by the first male. What are the reproductive implications for the two males that each copulate once in sequence? Both males will have about an equal number of their sperm stored. If the sperm are released from both spermathecae in equal numbers, then sperm of both males will be available for fertilization. The two males would then probably fertilize an equal number of eggs. By facilitating the uptake of the first male's sperm, the second male would not be at a disadvantage. To our knowledge, no direct evidence is available as to whether female spiders release sperm differentially during oviposition. Watson (1991a) suggested that for a linyphiid spider, *Neriene litigiosa*, females exercise choice by storing, or refusing to store sperm of latter males and not by differential use at oviposition. The morphology of the female's reproductive organs may allow her greater control of sperm uptake. It appears that *N. litigiosa* females do not preferentially use sperm from one spermatheca over the other at oviposition so the priority pattern is, in part, based on the relative number of sperm within the spermathecae. Discrimination at copulation or oviposition remains a central question for the interpretation of strategies of both male and female spiders. We are examining patterns of

sperm release by female *M. gracilis* during oviposition.

Numbers of sperm stored correlated significantly with male cephalothorax length. Given that this measure is also correlated with the number of sperm a male might have in its palps before mating, larger males store more sperm in the female than smaller males, probably because they have more sperm. Female body size was not related to the number of sperm stored. It is unclear how female body and spermathecal size are related in spiders. The current results suggest, however, that a single male cannot fill a female's spermathecae. If spermathecal size were related to body size, then body size should have accounted for some of the variance in the numbers of sperm stored, which it did not. We know little about female sperm capacity in spiders, a critical consideration in understanding the mechanisms underlying sperm priority patterns (Dickinson 1986; Parker et al. 1990; Pitnick & Markow 1994).

Conclusions

In summary, males and females accrue different costs and benefits from this particular copulatory pattern of two separate copulations with the second longer in duration. The requirement that the male dismount between copulations would be costly to the male, because the dismount offers another male the opportunity to copulate with that female (T. C. Bukowski & T. E. Christenson, unpublished data). From the female's perspective, it would be advantageous to require the male to dismount between insertions, because the first male may not be the best she will encounter. Benefits to polyandry, such as bet hedging, have been suggested (Watson 1991b). The female *M. gracilis* might benefit by not allowing a male that is less skilled or slower in switching sides to copulate again and allowing a second male to mate. Once a female has allowed a male access to both reproductive tracts, she is unlikely to obtain additional sperm from the numerous, and possibly better, males that will visit and copulate before oviposition (T. C. Bukowski & T. E. Christenson, unpublished data). Prolonging the second copulation increases the numbers of the male's sperm that are stored and might serve a mate guarding function as well (T. C. Bukowski & T. E. Christenson, unpublished data). The female also benefits from the prolonged copulation because more sperm are stored and are available for fertilization, and because duration or vigour of copulation might be one measure of male fitness. Copulation requires energy output, and perhaps copulatory vigour reflects an energetically healthy male (Watson & Lighton '1994).

Knowledge of female mating history is critical in understanding the gametic consequences of copulation. The failure to release sperm to a non-virgin female despite the overt appearance of a normal copulation is likely to be widespread, particularly among species with a first-male advantage. Much of the reported variance in copulatory behaviour might be explained by the mating history of the female (Elgar 1995), although more detailed data are required before general comparative statements can be made. We agree with Eberhard (1994) that descriptions of discrete copulatory behaviours have often been insufficient in detail to draw generalizations about the relationships between copulatory behaviour and the fate of the male's gametes.

To understand the role of copulation in sperm release and storage, direct examination of gametes is required. Since copulation can have courtship and information-gathering functions, copulation does not always mean release, storage or use of sperm (Eberhard 1985, 1991, 1994). One cannot assume that sperm are released during copulation even if the male previously inducted sperm and hematodochal inflation has occurred. Unless one knows how many sperm a male has before and after mating, failures to release sperm will be missed. Moreover, release of sperm does not necessarily imply sperm storage by the female. A large percentage of the sperm released by *M. gracilis* males was not found inside the females. Species could differ in this percentage, which could correlate with well described differences in overall copulatory pattern (e.g. Robinson & Robinson 1980). Quantification of sperm would also help determine whether priority patterns are based on the relative numbers of sperm or differential sperm placement within the female. Such quantification is only now beginning in the study of spider reproduction.

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