First male sperm precedence in multiply-mated females of the cooperative spider *Anelosimus studiosus* (Araneae, Theridiidae)

Thomas C. Jones: Department of Biological Sciences, East Tennessee State University, Johnson City, Tennessee 37614, USA. E-mail: jonestc@etsu.edu

Patricia G. Parker: Department of Biology, University of Missouri at St. Louis, St. Louis, Missouri 63121, USA

Abstract.   Patterns of sperm usage in multiply-mated females have profound fitness consequences for males, and create strong selective pressure on male behavior. In the cooperative theridiid spider *Anelosimus studiosus* Hentz 1850 adult males are tolerated in females' webs, and females have been observed to mate multiply with different males. In this experiment, virgin females were mated with two different males on consecutive days under controlled conditions to determine paternity patterns and behavioral responses of males to non-virgin females. The paternity of broods was analyzed using randomly amplified polymorphic DNA (RAPDs). Fifteen broods were analyzed and complete first male sperm precedence was found. Mating behavior differed between first and second males with the first males attempting fewer intromissions, but having a longer total time of intromission. This suggests that the second males are either prevented from normal copulation, or are reacting to the different condition of the females. The sperm precedence pattern is discussed with respect to its ramifications for male behavior, juvenile inclusive fitness, and the evolution of cooperative behavior.

Keywords: Social spiders, mating behavior, RAPDs, sexual selection

When females mate multiply, sperm precedence patterns can affect the fitness of all individuals involved. For males who have both mated with the same female, the fitness consequences are clear and directly related to the proportion of her brood they have fertilized (Trivers 1972). Because of this, males often compete with each other for access to females, or to be chosen as mates by females (Andersson 1994). Males may also compete for fertilizations after copulation through such avenues as mate guarding or copulatory plugs, the necessity or efficacy of which is affected by sperm precedence (Parker 1984). Patterns of sperm precedence may affect the female's fitness by influencing the genetic variability of her brood, or the proportions of her brood fertilized by males of differing quality. There is also accumulating evidence of females manipulating fertilization patterns of their broods in response to male attributes (Eberhard 1996). Examples of this in spiders include selective sperm storage in response to copulation duration (Bukowski & Christenson 1997a & b), and the fact that, with paired spermathecae, spiders may be pre-adapted for paternity manipulation (Snow & Andrade 2005).

Patterns of sperm precedence will also affect the composition of full and half-sibs within broods of polyandrous females. In social species, the relatedness among brood-mates can have profound effects on their fitness (Hamilton 1964). The relatedness among group members is therefore important for a complete understanding of the selective costs and benefits to group living (for review see Caraco & Giraldeau 1991).

Mating systems are particularly important to the evolution of sociality in spiders. Social spider colonies are generally inbred (Riechert & Roeloffs 1993; Johannesen et al. 2002). In fact, genetic analysis of *Anelosimus eximius* Keyserling 1884 colonies suggests that there is no gene flow at all among colonies (Smith & Hagen 1996). It is likely that the cooperative behaviors and female-biased sex ratios of cooperatively social spider species are maintained by interdemic selection, fostered by the high levels of relatedness among colony members (Avilés 1997). Isolated local populations of asocial or subsocial spiders will become inbred through genetic drift, which could then promote the evolution of cooperative behaviors. The rate at which a population loses genetic diversity (i.e., the effective population size) is affected by its mating system in that monogamous populations lose diversity faster than promiscuous populations (Parker & Waite 1997). This inbreeding is likely an important factor in the evolutionary transition from subsociality to permanent sociality in spiders (Bilde et al. 2005).

Anelosimus studiosus Hentz 1850 is a relatively small (about 8 mm long) theridiid spider which ranges from Argentina to New England (Agnarsson 2006; Agnarsson et al. 2007). This species is common in the southeastern USA and can be found in extremely high densities along waterways (Jones et al. 2007). This species is described as subsocial (Wilson 1971) and, specifically, "prolonged subsocial" (Rayor & Taylor 2006), in that juveniles and adult males are tolerated in an adult female's web, but other adult females usually are not (Brach 1977; but see Furey 1998; Jones et al. 2007). Previous experiments have demonstrated that, under controlled laboratory conditions, colony prey capture increases with the age and number of juveniles in the colony (though resources per individual decline with colony size), and variation in prey mass decreases with the number of juveniles present (Jones & Parker 2000). Females can produce up to at least three broods over their lives usually with several weeks between broods (Jones, unpubl. data). It has also been shown that in semi-natural conditions, delayed juvenile dispersal benefits juvenile survivorship and development as well as the mother's ability to produce future broods (Jones & Parker 2002). While it is clear that individual juveniles are better off in their natal group than on their own, the exact relationship of individual fitness to group size is not yet known. Whatever this relationship, a juvenile's fitness is likely to be affected by its relatedness to its brood-mates. We have observed that females will mate

multiply if presented with more than one male, so broods may be composed of either full-sibs, or a mixture of full and half-sibs.

Members of the family Theridiidae are "entelegyne" spiders, in that the female reproductive tract has a conduit morphology, with sperm leaving the storage organ to fertilize eggs through a different opening than that into which they were deposited (Foelix 1996). It has been suggested that this morphology will put the first mate's sperm closest to the point of fertilization, and thus lead to first mate sperm precedence (Austad 1984). However, studies of sperm precedence in entelegyne spiders have yielded estimates of proportions of first male from 0.95 to only 0.37 (reviewed by Elgar 1998). In the theridiid Australian redback spider the mean first male precedence was 0.44, but ranged from 0.0 to 1.0 (Andrade 1996). In this study we seek to determine sperm precedence in *A. studiosus* by sequentially pairing females with two males, recording mating behavior, and using the animals' DNA and RAPD analysis to determine parentage.

METHODS

Collection and Rearing.—*Anelosimus studiosus* colonies containing juveniles were collected in southern Louisiana in September of 1999 from bayous in Tickfaw State Park (30°22′N, 90°37′W) and Fontainebleau State Park (30°20′N, 90°02′W). The bayous were accessed by pirogue and the webs were mostly collected from low-hanging cypress branches (voucher specimens are deposited in the Museum of Biological Diversity at The Ohio State University, Columbus, Ohio). We maintained the colonies in plastic containers ($18 \times 14 \times 6$ cm) that were laced with sewing thread to provide substrate for web building. The colonies were kept at buffered ambient temperatures (20–28° C) under natural light conditions, were fed *Drosophila melanogaster* and *D. virilis* ad libidum, and misted with distilled water three times a week. We kept the colonies in a greenhouse under natural lighting conditions. In order to ensure the virginity of experimental animals, as the juveniles approached maturity, females were isolated in new containers, and the penultimate males were grouped together (by natal colony) in another set of containers.

Experimental Matings.—Females that had undergone their final molt in isolation were selected for this experiment. Twenty-five females from eight different colonies, but no more than four females from any one colony, were used. We released males from two different colonies (other than the colony of their prospective mate) into a female's container one at a time, on consecutive days. Matings were videotaped with a Sony digital 8 camera for behavioral analysis. The resolution of the video did not allow fine details of palpal insertions, such as the extension of the embolus, to be observed. We estimated copulation as periods when the male's palps were resting stationary against the female's genital opening. The onset of copulation was recorded as when the palp would ease against the genital opening and stop, and the end of copulation was recorded when the male pushed against the female and broke loose with a conspicuous jerk. We quantified the number and timing of copulations and copulation attempts. After mating, the males were frozen for later DNA extraction. Mating usually commenced within a few minutes after the introduction of the males. We removed the males after they had broken

copulation, moved off from the female, and for ten minutes showed no further attempt at copulation. On four occasions the males had not attempted copulation after 15 min. These males were removed from the container and the process was restarted with new males. Abstinent males were not reused. After the females had mated the second time, we returned them to the greenhouse rearing conditions. Females that produced broods were allowed to rear them through the third instar, after which the female and juveniles were frozen for DNA extraction.

DNA Extraction and PCR.—To the 1.5 ml tubes containing the frozen spiders, 200 µl of CTAB and 1.5 µl Proteinase K (100 µg/ml) were added. The spiders were thoroughly ground with a pestle in the tube and incubated at 60° C for 1 h. One extraction with 100 µl of phenol and 100 µl of CIA (24:1 chloroform:isoamyl alcohol), and one extraction with 200 µl of CIA were performed. The samples were ethanol precipitated, resuspended in 100 µl of distilled water, and stored at 4° C. The concentration of the samples was estimated by gel comparison with concentration standards.

Randomly amplified polymorphic DNA (RAPDs) uses single relatively non-specific ten base pair primers (synthesized at OSU) to amplify regions of the genome that contain complementary primer annealing sites. The regions that are amplified are arbitrary but heritable, and therefore, useful (Williams et al. 1990). Under similarly controlled mating conditions, RAPDs were used to assign sperm precedence in a beetle (Carbone & Rivera 2003). The reactants for an individual 14 µl reaction consisted of: 9.9 µl, UV irradiated distilled water; 1.5 µl of 1 µM dNTPs; 1.5 µl reaction buffer (10 mM Tris HCl, pH 8.3; 50 mM KCl; 2 mM MgCl); 0.8 µl of 10 µM primer; 0.1 µl Taq DNA polymerase (5 U/µl); 1.2 µl template DNA (approx. 25 ng/µl). The reactions were run through four initial "touch down" cycles (94° C for 1 min; 35° C for 1 min; 0.3 slope to 72° C for 2 min), and then 32 amplification cycles (94° C for 10 s; 35° C for 30 s; 72° C for 30 s). The finished reactions were held at 4°C until they were visualized. For visualization, the amplified products were run out on a 1.2% agarose gel (80–120 V), stained with ethidium bromide, then visualized and photographed under UV light.

Paternity analysis.—RAPDs are dominant markers, and band presence/absence is particularly sensitive to reaction conditions because of the short length of the primers. Therefore, repeatability of RAPD markers has been problematic, making them not as robust in parentage analyses as some other molecular techniques (e.g., microsatellites or multilocus minisatellite DNA fingerprinting; for review see Parker et al. 1998). In this experiment, however, RAPDs were useful to assess paternity of broods because the pool of potential fathers is limited and known, and because repeatability was confirmed. A unique bands analysis was used to assign the father of each brood member. On the gels, the mother and two potential fathers were run as triads twice, flanking the offspring lanes. Bands that were observed in the lanes of one of the males, but not in the lanes of the other male or mother, were scored for their presence in the offspring lanes. Multiple primers were screened for the families until a total of at least two diagnostic bands were found for each juvenile. Such an analysis is simple and robust since no inference is made from band absence, and the

repeatability of each diagnostic band is confirmed by amplifying the triads of adults twice, and running them on the flanking lanes on both sides of the gels.

RESULTS

Mating behavior.—When the males were placed in the containers near the females, they would typically remain motionless for up to 1 min. They would then begin to move around in the web while rapidly drumming their first pair of legs on the silk. The movement of the males appeared undirected until the females moved within the web, at which point the males would begin to move toward the females while still drumming. As the males approached, the females would typically bounce in the web apparently signaling sexual receptivity because the males would move more quickly toward them afterwards. The males continued drumming even as they made contact with the females. The males would orient themselves to face the same direction as the female, with their ventral surfaces adjacent, but with no consistent absolute orientation. As the males moved into position, the amplitude of their drumming eased to a stop, which was taken to be the onset of copulation. After copulation the spiders separated with a conspicuous jerk, followed by the males moving a short distance from the females (1–2 cm). If only one copulation had taken place, the males would resume drumming and repeat the courtship, but would typically move in more quickly and insert on the other side. The females in this experiment, in all cases, appeared receptive to both males. Also, no occurrences or apparent attempts of sexual cannibalism were observed.

As measured by the number of copulation onsets and breaks, first males had fewer copulations (mean 2.2, range 2–4) than second males (mean 3.7, range 2–12; Mann-Whitney $U = 489$, $P < 0.001$, Fig. 1). Only three of the 25 first males had more than two copulations, and in those cases there were one or two short copulations followed by two long ones. The total time spent in copula was longer for first males (mean 44.3 min, range 34.7–52.4) than for second males (mean 15.9 min, range 1.5–42.1; $t = 11.3$, $P < 0.001$, Fig. 1). In six cases the second males had more than three copulations, but their total time of copulation (mean $= 11.0$ min) was significantly shorter than the second males that had three or fewer copulations (mean $= 19.6$; $t = 1.9$, $P = 0.04$). Considering individual females, their first mate's total time of copulation was not related to their second mate's total time (Fig. 2). The duration of the first male's first copulation was a strong predictor of the duration of his second copulation (in cases where there were more than two copulations, the initial apparent "false starts" [copulations lasting less than one minute] were excluded: Fig. 3).

Paternity analysis.—Of the 25 females in the mating experiment, 22 produced egg cases, of which 17 had juveniles emerge (which was considerably lower than the mean of 36 juveniles observed in nature; Brach 1977). The average number of juveniles per family was 11.6 (range 4–21). Two of the families were unusable because one of the males (in each family) could not be amplified by PCR. Of the fifteen remaining families, all 168 of the juveniles were assigned to the first male. While the assignments were based on the presence of at least two of the first male's unique bands, the lack of the second male's unique bands in juvenile lanes further confirmed the assignments.
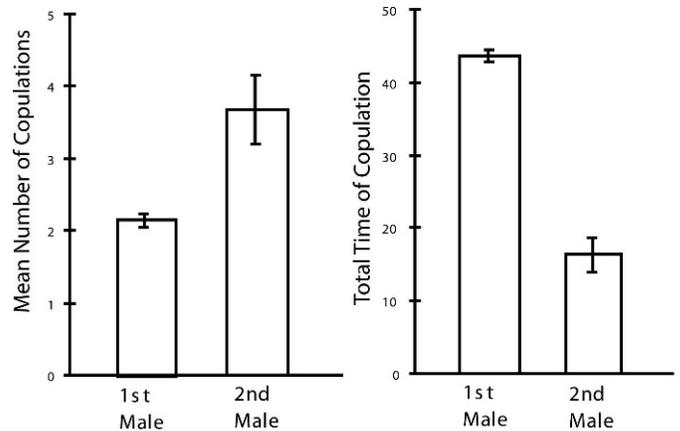

Figure 1.—Comparison of the mean number of copulations and total duration of copulation between first and second males in *Anelosimus studiosus*. Reported are means with standard error bars.

DISCUSSION

Using a direct DNA-based analysis of parentage, we found complete first male sperm precedence in *A. studiosus*. We also found significant differences in the mating behavior of first and second males. The patterns of sperm precedence, the increased frequency of starting and stopping copulation with less actual time spent in copula of second males, suggests that the second males could not successfully copulate.

There is evidence from other spider species that second males can be prevented from successfully copulating. Copulation "plugs" made by a hardening of seminal fluids in the female reproductive tract or by the tip of the male's intromittent organ breaking off, have been reported for several spider species *Phidippus johnsoni* Peckham & Peckham 1983 (Jackson 1980), *Agelena limbata* Thorell 1897 (Matsumoto 1993; Yoward & Oxford 1996; Schneider et al. 2005). However, the efficacy of these plugs in preventing subsequent
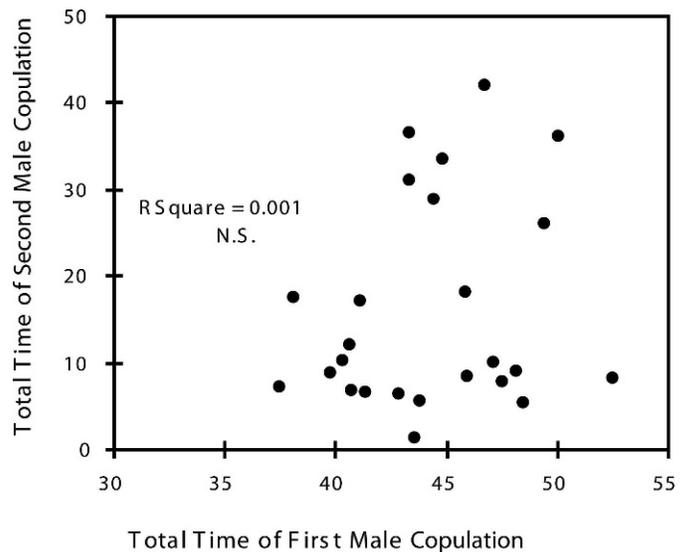

Figure 2.—Plot of a female's total time of copulation with the first male versus her total time of copulation with the second male in *Anelosimus studiosus*.
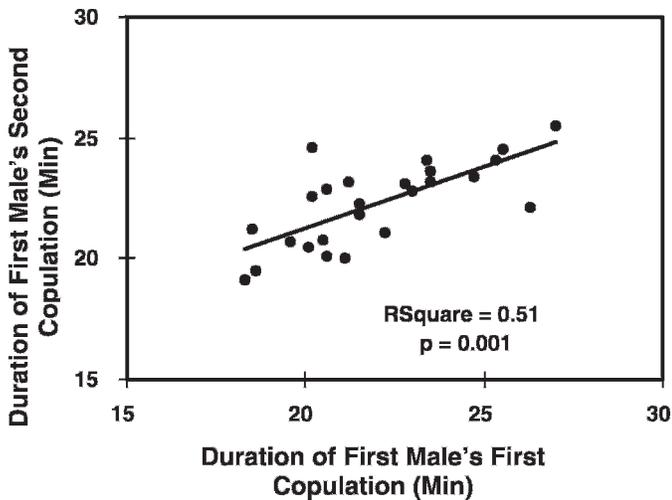
Figure 3.—Plot of the duration of the first male's first copulation versus the total time of his second copulation in *Anelosimus studiosus*.

fertilizations is mixed among taxa, and they may instead function to increase the sperm retention and survival (reviewed in Huber 2005). In another theridiid spider, fertilization patterns are affected by where the broken organ tip rests in the female reproductive tract (Snow et al. 2006). Across spider taxa, male genital mutilation appears to be an indicator of strong selection on paternity protection, being correlated with the evolution of male sacrifice behavior and size dimorphism (Miller 2007). We did not determine in this study whether or not the second male was in any way prevented from copulation. The fact that four of the second males' copulatory behavior was similar to the first males' (having two intromissions totaling > 30 min), suggests that copulatory plugs are not ubiquitous, or at least not completely effective. However, we have observed a second male repeatedly moving in and breaking from a female, apparently struggling to insert his palps.

Interestingly, first male total copulation time was only 16% that observed in a congener (Klein et al. 2005). The fact that second males spent less total time in copulation contradicts previous findings in an araneid spider (*Micrathena gracilis* Walckenaer 1805) in which second males copulated over twice as long as the first male (Bukowski & Christenson 1997b), but similar to patterns found in a tetragnathid spider (*Tetragnatha versicolor* Walckenaer 1842; Danielson-Francois & Bukowski 2004). In the latter case sperm release was equal between first and second males. A similar experiment in which males were introduced to females that had only been mated on one side of their tract suggested that males are responding specifically to the reproductive status of the female's reproductive tract, rather than the female's behavior or overall condition (Bukowski et al. 2001). Second males also copulated for shorter periods in a cellar spider, *Pholcus phalagioides* Fuesslin 1775 (Schafer & Uhl 2002). In this case, however, the second males had a higher proportion of reproductive success, apparently as a result of their ability to remove the first male's sperm.

Since second males generally attempted copulation, the differences in their mating behavior seems most likely because they are prevented from normal copulation. The possibility

remains however, that they could be altering their behavior in response to the previously mated condition of the female. There are examples of non-virgin spiders being less attractive to males. The presence of sex pheromones has been documented across a wide range of spider species and, in some cases, these pheromones are volatile (Shultz & Toft 1993; Miyashita & Hayashi 1996; Rovner 1996; Costa et al. 1997; Searcy et al. 1999), and in other cases are contact based (Trabalon et al. 1997, 1998). In one linyphiid spider (*Nereine litigiosa* Keys 1886) pheromones are incorporated in the female's web, and males destroy the web prior to mating, reducing the probability that a second male will find her (Watson 1986). Whether pheromones exist in this species is not known, but pheromone-like compounds have been extracted from the cuticle of its congener *A. eximius* (Bagneres et al. 1997). It is possible that, even though the female remains sexually receptive after mating, her production of pheromones decreases, thus making her less attractive to second males. The speed with which the first and second males begin drumming and searching for the female might give insight into pheromone levels, but such a measure would be confounded by the introduction of the males, which was not standardized in terms of their distance from the female.

This study found complete first male sperm precedence, accentuating the question why an *A. studiosus* female should mate multiply at all. There are many potential costs to mating such as loss of foraging opportunities, increased predation risk, and disease transmission (reviewed in Lewis 1987). Male spiders provide no parental care, and it is unlikely that any substances that males transfer along with sperm provide direct benefits to the females as has been observed in some insects (Gwynne 1984; Boggs 1990). With linyphiid spiders the male cohabitats in the female's web, eating prey, until he has mated with her; females apparently mate with these males to induce them to leave (Watson 1993). However, there is evidence from the same species that multiple mating has indirect benefits in terms of the size and growth rate of juveniles (Watson 1998). In the pisaurid species *Pisaura mirabilis* (Clerk 1757), in which females mate with multiple males, males present nuptial prey items (Drengsgaard & Toft 1999). Mating multiply allows the possibility of cryptic female choice in which she chooses the sperm of the male she prefers (Eberhard 1996). Again, given complete first male precedence, this seems unlikely to be occurring with this species. Perhaps the simplest benefit to a female from multiple mating would be to ensure that all her eggs get fertilized. It may also be that there is no selective benefit to mating with multiple males, and that *A. studiosus* females simply remain receptive from maturity until their abdomens are distended with eggs regardless of the number of times they have mated.

Previous work on this species has demonstrated that by delaying dispersal and remaining part of their natal colony, juveniles enhance their survival and development (Jones & Parker 2002). This can be extrapolated to suggest that juveniles' direct fitness benefits from delayed dispersal. This study finds complete first male sperm precedence within the broods of doubly-mated *A. studiosus* females. This suggests that if an individual juvenile's presence in the colony contributes to the survivorship of its brood-mates, its indirect fitness (sensu Hamilton 1964) would be maximized because

they are all full-sibs. In this species it is now documented that there are colonies that contain multiple adult females in North America (Furey 1998), the incidence of which increases with latitude (Jones et al. 2007). If these colonies develop by non-dispersal of juveniles, fertilization patterns could have profound effects on the genetic structure of these large colonies.

Finding complete first-male sperm precedence may be surprising, but these results should be taken with some caution given the highly controlled conditions. Factors such as the number and timing of matings, which may influence precedence (Eberhard 1996), were held constant. Currently studies are underway exploring relatedness within and among natural colonies using microsatellite loci.

ACKNOWLEDGMENTS

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