Sperm competition and characteristics affecting fight outcome in precopulatory guarding crab spider males.

An Honors Thesis (HONRS 499)

by

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Abstract. A previous study of the crab spider <u>Misumenoides</u> <u>formosipes</u> showed that males guard immature females that are close to their adult molt. The limited number of immature females relative to the number of searching males, results in male-male conflicts to gain access to mates. Game theory, as applied to animal conflicts, predicts that opponents will settle contests without further escalation by assessing asymmetries in resource holding power. Size asymmetries are considered to be a likely cue for such assessments. In staged contests on flowers between differently-sized opponents, large males displaced smaller guarding males in 80% of the trials, with little or no escalation. Males sometimes used leg autotomization as a means of escape from opponents. In staged contests between equally-sized males, one if which was missing a raptorial front leg while the other had its legs intact, the two types of males won with equal probability. Analyses of <u>M.</u> <u>formosipes</u> behavior have been based on the assumption that they exhibit first-male sperm priority. I describe preliminary work using a unique application of DNA fingerprinting technology to test that assumption.

In the field of animal behavior, it is an obvious and well-documented phenomenon that males are more likely than females to compete for access to mates. Recently, analyses of this male-male competition have been expanded following the emergence of sperm competition theory. This theory holds that competition between males does not stop at the level of the whole individual, but extends to the actual gametes that each male contributes. Whenever gametes from more than one male are present within a female, there is the possibility for competition among those gametes to fertilize her eggs. This situation is found in species where females are able to store sperm from consecutive matings. If females use the sperm from multiple matings in a predictable pattern, then male mating behavior should exploit that predictability.

In 1970, G. A. Parker hypothesized that when sperm from the last male to mate with a female is consistently used to fertilize the majority of the female's eggs (last-male sperm priority), selection should favor post-copulatory mate guarding. In this type of mating system, males would be expected to guard the female after a mating in order to prevent other males from mating with her and displacing the original male's sperm. This behavior is well known among insects, where last-male sperm priority is the rule (Thornhill and Alcock 1983).

Reproductive morphology, specifically morphology of the sperm storage organ (spermatheca), is thought to play a major role in determining the sperm priority pattern of a species. Insect spermathecae have a single duct for insemination and fertilization,

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forming a sac-like structure in which the last sperm to enter is the first to exit. The female reproductive morphology of the majority of spider species (the entelegyne species) includes a more complex spermatheca with separate insemination and fertilization ducts (Foelix 1982). It was suggested by Austad (1984) that the "flow-through" morphology of the entelegyne spermatheca would favor a first-male sperm priority pattern, where the first sperm in would be the first sperm out and, consequently, fertilize the majority of the eggs. All previous sperm competition studies for entelegyne species have found first-male sperm priority patterns (Jackson 1980; Vollrath 1980; Austad 1982; Christenson & Cohn 1988; Watson 1991; Eberhard et al., manuscript).

Given first-male sperm priority, virgin females should be an extremely valuable resource for which males compete. Spider life cycles consist of a series of immature growth stages separated by a molt to the next stage. The female genitalia are not functional until after the final molt to the adult stage. Males therefore would be expected to find and guard immature females that were close to this final molt (precopulatory guarding) so as to have first access to a newly-molted adult. Austad (1984) predicted that entelegyne male spiders should be found cohabiting with immature females but rarely with mature females. Dodson and Beck (1993) found support for this prediction through experiments and field observations with the crab spider Misumenoides formosipes.

Competition among males for a limited number of virgin females leads to conflicts. In M. formosipes, conflicts occur when a male

spider intrudes onto a flower which is already occupied by an immature female and a guarding male. As is common in most conflicts between conspecifics, these contests are resolved before a death occurs. Game theory, as applied to animal conflicts, predicts that certain rules will be used to settle contests rather than the opponents fighting to the death. Specifically, contests are expected to be settled on the basis of assessment of asymmetries in resource holding power. Resource holding power (RHP) is a term used to describe all the characteristics of an individual with the potential to influence the outcome of a fight. Game theory suggests that assessment of RHP allows a contest to be settled without the death of one opponent because a lesser opponent will retreat without escalation. Escalation of a fight should only occur when the RHP of both opponents is equal, or so nearly so that the assessment is inconclusive. We used staged conflicts to test the predictions of game theory regarding male-male interactions of the crab spider species M. formosipes. We examined the effect of male size (commonly considered a reasonable measure of RHP) on the outcome of male-male fights by determining if large males displace small males cohabiting with an immature female.

In previous fight trials, Dodson and Beck (1993) observed that males occasionally detach one or more of their legs (autotomy) to escape the grasp of another male. Autotomization is a common phenomenon across spider species. In the field, the incidence of disabled males (those missing at least one leg) increases as the season progresses (Dodson and Beck 1993). This may be due to

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increased competition for diminishing numbers of virgin females. In escalated fights, males use their long forelegs to grasp the other male and draw it close to its chelicerae (jaws). It is therefore reasonable to assume that the absence of a leg may be a disadvantage in fights. We staged fights between equally-sized males, one one of which was disabled while the other had its legs intact, to determine if the absence of a leg is a disadvantage in fights.

Because the crab spider **M. formosipes** is an entelegyne species, it has been assumed in studies of this species that they exhibit first-male sperm priority (see above). We are testing this assumption through a unique application of DNA fingerprinting technology. Following isolation of DNA from parents and offspring we plan to use the Polymerase Chain Reaction to amplify highly variable regions and obtain a fingerprint of the parents and offspring. We then plan to use these fingerprints to quantify the proportion of offspring fathered by each of two males mated consecutively to a female.

METHODS

Subjects and study site

Misumenoides formosipes is a sit-and-wait predator found on flowers such as black-eyed susan (Rudbeckia hirta), Queen Anne's lace (Daucus carota), and goldenrod (Solidago spp.). Field studies were conducted on the grounds of Ball State University's Cooper Woods in Muncie, Indiana. Adult males and immature females required for laboratory studies were collected and transported

individually in small plastic vials. Spiders were held on moist filter paper in petri dishes in an environmental chamber. Light and temperature were cycled on a 14 h day: 10 h night pattern. Daytime temperature was $26°C$, nighttime temperature was $18°C$, and humidity was maintained at 75%. Females were fed live house flies (Musca domestica) which had been reared in the lab. A new fly was offered no later than 2 days following the last successful feeding. Males were not offered prey because preliminary studies revealed that adult male feeding behavior was negligible (Dodson and Schwaab, unpublished data). Prey consumption and molts were recorded for each spider.

Male-male interaction experiments: Small Resident vs Large Intruder

The effect of size on the outcome of male-male interactions was examined by staging fights between differently sized males on flowers in the presence of immature females. The smaller male in each trial was allowed to establish residency with the female, and the larger male was released as an intruder. To control for statistical independence, each male was used in only one fight trial. The three variables used to estimate overall size were cephalothorax width at its widest point, abdominal length (from the dorso-anterior margin of the abdomen to its apex), and right tibial length. These measurements were made to the nearest 0.01 mm using a filar micrometer on a Unitron dissecting microscope. Dodson and Beck (1993) found that a 10% difference in cephalothorax width predicted fight outcome in this species. To increase the likelihood that opponents would perceive themselves as differing in

size, we used males that differed by at least 10% in all three variables. The mean differences for the three variables were: abdominal length 19.7% ± 1.48 S.E., right tibial length 21.1% ± 2.06 S.E., cephalothorax width 15.4% ± 0.96 S.E..

Previous staged contests had been conducted in petri dishes with no female present. Our trials were conducted in the lab on cuttings of Queen Anne's lace and black-eyed susans taken from the field, creating a controlled but natural substrate for the fights. An immature female was allowed to move out of its petri dish onto the inflorescence and given approximately 5 min to settle into the flowers. The smaller of the two males was then released onto the inflorescence and allowed to make contact with the female. After the smaller, resident male settled into one position, the larger, intruder male was released onto the inflorescence. We recorded times for the following events:

1. Release of small resident male onto the inflorescence.

2. Contact between resident male and female.

3. Release of large intruder male onto the inflorescence.

4. Initial contact between males.

5. Subsequent contact, grapples, and/or retreat of one or both males (see below).

6. Final retreat of one male.

Initial contact between males was followed by either the retreat of one or both males or a grapple. Grapples consisted of one or both males using their forelegs in an attempt to draw their opponent in towards their chelicerae. A single trial often

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consisted of more than one instance of contact, grapples, or retreats. The trial was terminated and a winner declared when one male left the inflorescence completely, or retreated to a position further from the female than the winning male. In a single trial, the losing male was killed.

Male-male interaction experiments: Disabled vs Intact

We examined the effect of leg loss on male-male interactions by staging fights between pairs of equally-sized males. Males were considered to be equally-sized if all three size variables (cephalothorax width, abdominal length, and right tibial length) showed less than a 10% difference between opponents (see above). The mean differences for the three variables were: abdominal length 1.46% $\pm$ 0.19 S.E., right tibial length 2.02% $\pm$ 0.49 S.E., cephalothorax width 0.97% $\pm$ 0.25 S.E.. One male from each pairing was induced to autotomize its right foreleg by pinching the foreleg with a forceps. This procedure was done 24 hours prior to the use of the male in a trial so as to: 1) reduce the chances of our disturbance influencing fight outcome, and 2) assure that the health of the individual was not compromised. All legs were left intact on the opposing male.

The same set-up was used in these trials as with the small resident vs large intruder trials with one exception. Both males were placed onto the inflorescence containing the immature female at the same time in order to eliminate the influence of residency on the trials. Again, contact between males, grapples, and retreats were recorded with a winner being declared in the same

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manner as in the small resident vs large intruder fight trials.

Controlled mating of two males to a virgin female

Females were reared to maturity in the lab by following the previously described feeding schedule. This allowed us to control mating activity of the females. Known adult virgin females were allowed to mate with a male. All matings occurred on flowers in the lab. Two days later the same female was mated to a second male. Each male was mated with only female.

After matings, the feeding schedule resumed until the females laid their egg clutch. At this time the female, both males, and the egg clutch from each of the 17 trials were separately preserved in a solution of 50% ethanol and 50% phosphate buffered saline and placed in an ultra freezer at -70°C. Because the ultimate goal of this project was to analyze the DNA of these specimens, extreme care was taken when handling them in order to prevent cross contamination. Rubber gloves were worn and forceps were washed with ethanol and flamed between the handling of each specimen.

Polymerase Chain Reaction (PCR) experiments

The following steps were used to extract DNA from the specimens in preparation for PCR (Erlich 1989). The solution containing the specimens was brought to a total volume of 400 ul with 1X TE buffer (10mM Tris, 1mM EDTA) and gently boiled for 5 minutes. The specimen was then homogenized within the solution, by vortexing until the supernatant could be drawn off. Less than 1ul of RNAse A (5490 units/ml) was added to some samples which were incubated at 37°C for 30 minutes in order to remove the RNA. 7.5M

ammonium acetate (200 ul) and cold ethanol (1200 ul) was added and the DNA was precipitated overnight at 20°C. The next day the tubes were centrifuged (14,000 x g for 10 min) and the DNA pellet was resuspended in 100 ul of water. DNA was quantified spectrophotometrically in capillary tubes at 260 nm.

To minimize the possibility of contamination, rubber gloves, micropipetmen, and tips were exposed to ultraviolet light for about five minutes to degrade any DNA that may have been on them. All solutions needed for the PCR were kept on ice at all times once they had been removed from the freezer.

The preliminary reaction was performed using purchased ITS primers. The primers flank a highly variable internal transcribed spacer (ITS) region (600-700 bp) and bind to conserved sequences of transcribed-translated ribosomal genes (Szabo 1991). The reaction was run in a total volume of 50 ul containing Taq buffer (1X), magnesium chloride (8uM), dNTP's (200uM ea), primer (1uM), BSA (1ul), DNA (~100ng), Taq (2 units per reaction). The amplification was performed in a thermal cycler programmed for 45 cycles of 1 min at 94° C, 1 min at 45°C, and 2 min at°72 C. A hot start was used with 4 min at 94°C before the TAQ was added to insure denaturation of the DNA. Amplification products were analyzed in a 1.5% agarose gel and detected by staining with ethidium bromide. A 123 base pair ladder marker was run simultaneously with the DNA samples.

Subsequent PCR reactions were run using randomly amplified polymorphic DNA (RAPD) primers which were constructed at Ball State University on a DNA synthesizer. RAPD primers are short,

arbitrary nucleotide sequences which amplify random fragments of DNA (Williams et al. 1991, Chapco et al. 1992). Because DNA varies between organisms (even within the same species), different fragments will be amplified for different individuals.

This procedure generates DNA fragment patterns or fingerprints which, in relation to sperm competition, can be used to identify which male's sperm was used to fertilize an egg. The protocol for PCR with RAPD primers is the same as with the ITS primers (see above) except that the annealing temperature for RAPD primers is 36°C rather than 45°C.

RESULTS

Male-male interactions: Small Resident vs Large Intruder

In staged contests between a small resident male and a large intruder male, the large intruder won significantly more contests than the smaller resident male (Figure 1). Escalation (represented by grapples) occurred in 15% of the trials. In all but one contest, the losing male left the inflorescence completely (N=16), or retreated to a position further from the female than the winner (N=2). In the remaining trial, the loser was killed.

Male-male interactions: Disabled vs Intact

In staged contests between a disabled male and an intact male, there was no significant difference in the probability of winning for either type of male (Figure 2). Escalation occurred in 32% of the trials. All losing males left the inflorescence. No deaths occurred.

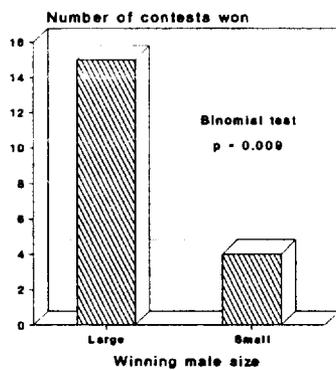Small resident versus large intruder
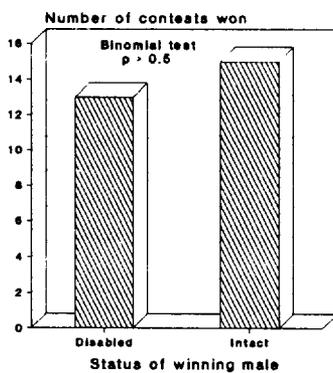

Figure 1

Disabled versus intact male

Figure 2

Polymerase Chain Reaction experiments

A preliminary reaction was conducted using the DNA extracted from a single male spider and ITS primers #4 and #5 which flank the ITS region (see above). A single band was viewed under ultraviolet light after gel electrophoresis, indicating successful amplification of the DNA.

Additional reactions were conducted using extracted male DNA and combinations of one or two 10-mer RAPD primers (see above). Gels from these reactions showed non-specific amplification of DNA or no amplification at all.

DISCUSSION

Male-male interactions: Small Resident vs Large Intruder

Game theory predicts that contests between conspecifics will be settled on the basis of assessment of asymmetries in resource holding power. Male size (considered a possible measure of RHP) has been documented to be an advantage in male-male interactions for several spider species (Vollrath 1980, Austad 1983, Suter and Keiley 1984, Wells 1988, Watson 1990, Jackson and Cooper 1991). Dodson and Beck (1993) conducted experiments in petri dishes with the crab spider M. formosipes and found cephalothorax width to be associated with fighting success. We conducted experiments with M. formosipes on flowers because the natural substrate introduces factors (uneven terrain, places to hide, etc.) which are not present in petri dishes. Natural conditions were also better approximated with the inclusion of a critical resource for the males: an immature female. In trials with a small resident male

and a large intruder male, the larger male displaced the smaller resident in a significant number of trials despite little overt aggression. These results support the prediction that size differences are used to settle male-male contests.

Male-male interactions: Disabled vs Intact

A method of escape for *M. formosipes* in male-male grapples is leg autotomy (Dodson and Beck 1993). However, autotomization might compromise a male's chances of winning subsequent fights. Dodson and Beck (1993) staged fights in petri dishes between equally-sized males (fully intact vs disabled) to test the prediction that absence of a leg was disadvantageous in fights. Their results (disabled males lost significantly more fights than intact males) appeared to support this prediction.

The disabled males used in those trials were collected in the field with one of their legs already missing. Because leg autotomy is an escape tactic, it is possible that these males used leg autotomy to escape from a victorious male in a previous fight. Otronen (1990) demonstrated that prior experience influenced the outcome of future fights in burying beetles. The use of disabled males that were already "losers" may have produced biased results in these trials. To eliminate this bias, our trials were conducted on flowers (see above) with males taken from the field, their legs fully intact, and later induced to autotomize a leg in the lab. Results of our trials revealed no disadvantage for disabled males fighting fully intact males of equal size. Given the aforementioned role of the legs in grapples, these results were

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somewhat unexpected, and this question will be investigated further.

Polymerase Chain Reaction experiments

The ITS primers were used in preliminary reactions to amplify a 600-700 bp region of DNA. The amplified DNA from any individual would be expected to be the same length (equal number of base pairs), and visible as a single band on a gel. Preliminary experiments amplifying DNA with the ITS primers were performed to insure that DNA could be easily extracted from individuals and amplified using PCR.

Our amplification reactions using RAPD primers produced DNA smears rather than fingerprints containing several distinguishable bands. We obtained this same result even after various modifications to eac of several factors in the reaction (annealing temperature, DNA concentration, primer concentration, magnesium chloride concentration, and BSA concentration). We then discovered that the RAPD primers were heterogeneous DNA fragments due to a bad seal on the DNA synthesizer. This could account for our inability to generate fingerprints. In future PCR reactions, fingerprints may be obtained either by using homogenous RAPD primers, or by performing restriction enzyme digests of DNA amplified by ITS primers to show restriction fragment polymorphisms. Once differing DNA fingerprints are obtained for two males mated consecutively to a female, DNA isolated from the entire eggsac mass may be scanned with a densitometer to quantify the proportion of offspring fathered by each male.

ACKNOWLEDGMENTS

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