Sperm precedence in monarch butterflies (Danaus plexippus)

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We characterized sperm precedence in monarch butterflies (Danaus plexippus), using a series of experiments in which we manipulated male mating histories to vary spermatophore size and the number of sperm transferred. Several factors affected the outcome of sperm competition. There was a pattern of second-male sperm precedence, but second-male precedence was rarely complete, and several other factors had significant effects on paternity patterns. Larger males outcompeted smaller males when they were not matched for size. Phosphoglucose isomerase (PGI) genotype affected the outcome of sperm competition under very hot conditions. When sperm from the same pair of males competed in different females, males fared better when they transferred more sperm. These results demonstrate that sperm precedence within a species can be affected by many factors, including the circumstances under which it is measured. Key words: monarch butterflies, PGI genotype, sperm competition, sperm transfer. [Behav Ecol]

In polyandrous species in which females store sperm, selection should favor males that gain sperm precedence over other males. Factors that might affect the relative number of fertilizations gained by a male include the number of sperm transferred (Parker 1970), sperm motility, longevity, or ability to penetrate the ovum (e.g., Lanier et al. 1979; Gomendio and Roldan 1991; Briskie and Montgomerye 1992; Birkhead et al. 1995; Radwan 1996; LaMunyon and Ward 1998; García-González and Simmonse 2007), and cryptic female choice during genital coupling, intromission, insemination, or fertilization (e.g., Walker 1980; Simmons 1987; Eberhard 1991, 1996, 1998; Birkhead and Mölère 1993; LaMunyon and Eisner 1994; Ward 1998, 2000; Simmons et al. 1999; Bloch Qazi 2003; Bussière et al. 2006; Fedina and Lewis 2006; Luck et al. 2007).

Sperm precedence in insects ranges from complete first-male to complete last-male precedence (e.g., Simmons and Siva-Jothy 1998; Simmonse 2001). Although last-male sperm precedence is most common in Lepidoptera, first-male precedence and mixed paternity also occur (reviews in Drummond 1984; Silberglied et al. 1984; Simmons and Siva-Jothy 1998). The preponderance of last-male precedence in the Lepidoptera probably results from displacement of the first male’s sperm in the elongate spermatheca; after a pair separates, sperm move from the bursa copulatrix, through the ductus seminalis and into the spermatheca, where they are stored until being released to fertilize eggs. Because the opening from which sperm are released is the same opening into which sperm move, sperm from the last male are likely to comprise most of the fertilization set from which sperm are drawn (Parker 1970). However, some sperm from previous males may remain in the fertilization set, and mixing of sperm is likely to occur.

To better understand potential factors affecting the outcome of sperm competition in Lepidoptera, we studied patterns of sperm precedence in monarch butterflies (Danaus plexippus) in a series of three experiments. The first experiment was designed to determine how male mating history affects sperm precedence in this polygynandrous species. Subsequent experiments tested potential mechanisms for our initial results, with explicit tests of how relative sperm numbers affected sperm precedence patterns. We also analyzed the effects of relative male size and our genetic marker on sperm precedence patterns.

GENERAL METHODS

Rearing techniques and manipulation of mating history

Experimental butterflies were the second-fourth generations of lab colonies derived from wild adults. We reared larvae in screen cages (0.5 m × 0.3 m × 0.6 m) on milkweed (potted Asclepias curassavica and cut stems of Asclepias syriaca). Larvae developed at room temperature (~24 °C) under natural light conditions during the summer in either Minnesota or Ohio (~18L:6D). Five to nine hours after eclosion, we placed adults in glassine envelopes, and in experiment 1 only, weighed them to the nearest 0.01 mg 24 h after eclosion. We checked them for Ophryocystis elektroscirrhosa spores (a neogregarine protozoan parasite, Altizer et al. 2000) and euthanized infected individuals by freezing them. We labeled all healthy butterflies with a unique number on the hind wing discal cell using a fine-point permanent marker and measured both forewings from the point of attachment to distal wing tip.

Experiments took place in mesh cages (flight cages = 2 m³, oviposition cages = 0.7 m³) in a greenhouse with natural summer light conditions or outdoors. Butterflies had continuous access to milkweed and sponges saturated with honey water. We released males into the cages within 1–3 days of eclosion, and 4–7 days before their first experimental mating and females on the morning of their first assigned mating. Males and females were held separately except on assigned mating days. After their first mating, we kept experimental females in individual oviposition cages with potted A. curassavica plants, and their second mates were introduced to these cages on the assigned day (see details below). In experiment 1, cages were on a residential lawn; in experiment 2, on a fifth floor roof patio; and in experiment 3, in a temperature-controlled greenhouse (~24 to 28 °C).

Monarchs usually remain coupled overnight (Oberhauser 1988; Solensky and Oberhauser 2009) and do not initiate...
mating after dark, so all matings can be recorded by checking cages once at dusk. In some cases, we hand-paired males that had not yet mated by 1700h; we held their wings together above their bodies, then rubbed the end of the male’s abdomen near the genital opening at the ventral end of the female’s abdomen until he coupled with the female.

The mating history of the two males mated to each experimental female, and thus the size of the ejaculates transferred (Oberhauser 1988; Solensky and Oberhauser 2009), were independent variables in all experiments. We manipulated male mating histories to generate three categories of experimental males: mated 1 day prior to experimental mating that transferred small spermatophores (7–10 mg: S), mated 4 days prior to experimental mating that transfer large spermatophores (22–25 mg: L), and virgins that transfer large spermatophores (mean mass increases with age, > ~24 mg: V). After mating, we maintained females in oviposition cages, collecting up to 40 eggs/female/day for genetic analysis, until they had laid no eggs for a week or died.

**Paternity assessment**

We used variation at the phosphoglucone isomerase (PGI) locus to determine egg paternity. We used standard electrophoretic methods (Hebert and Beaton 1993) to determine genotype and assigned matings in the prior generation to obtain individuals homozygous for medium (m) and fast (f) alleles in experiments 1 and 2 and an additional allele (slow [s]) in experiment 3 (names based on electrophoretic mobility). Within each experiment, all females were homozygous for the same allele.

We sampled adult genotypes by drawing ~5 µl of hemolymph from a small incision in the abdomen that healed quickly (personal observation). We mixed the hemolymph into two drops of deionized water and centrifuged the solution before applying the sample to a Helena cellulose acetate gel. We stored eggs in liquid nitrogen (196°C) until analysis, crushed them with a fine-point forceps into 10 µl of Tris Borate buffer (McClellan 1982) held in a Helena well plate, and applied to a gel without further dilution. We ran gels at 300 V for 25 min.

To ensure that eggs were fertile, we allowed them to develop for 3–4 days before freezing. In experiment 1, we did not have enough f/males to assign each female two males of opposite genotypes, so some first and second mates had genotypes of mm and mf or mf and mm, respectively. To assign paternity in these cases, we assumed that heterozygous males produced equal numbers of sperm with m and f alleles and that these two genotypes had equal fertilization success.

After determining the number of eggs fertilized by each male, we calculated 95% confidence intervals for the proportion of eggs fertilized by the second male (P2) based on the binomial distribution. We concluded that sperm precedence, or nonrandom fertilization, had occurred if these intervals did not include 0.5. Our use of heterozygous males in experiment 1 introduced error into our assignments of paternity, because the two males shared an allele. To account for this error, we determined the confidence interval for P2/2, based on the actual number of eggs that contained the unique allele. We then doubled the endpoints of this interval to obtain the confidence interval for P2 itself.

**Individual experiment methods and results**

**Experiment 1: effects of male size and mating history on sperm precedence**

**Methods:** Males had either mated 1 day prior to their experimental mating and delivered small (S) spermatophores or were virgins (V). Virgin males either had no prior access to females or had been in cages with females but had not mated. The first experimental matings took place in outdoor flight cages, after which we randomly selected 24 females that had mated to S males and 24 females that had mated to V males. We then randomly assigned half of each group of females to either a V or S second mating, generating four mating treatments designated by the order and type of spermatophores each received: SS, SV, VS, and VV. No males were used for more than one experimental mating, and male genotypes were equally divided among treatments and mating order.

One day after their first mating, we put females into separate oviposition cages, and collected 10–30 eggs from each to test their fertility and genotype. Beginning on the third day, we put a male of the assigned genotype and mating history for the second mating into the cage with each female. We replaced the male if the pair did not mate on a given day. We removed 10 females that failed to remate within 7 days or lay eggs after the second mating. Four of the 38 successful second matings were the result of hand pairing, but we did not record which pairings required this intervention, so could not assess its effects.

**Results:** Females (n = 38) laid an average of 700 eggs (range 152–1179), and we analyzed the genotype of an average of 301 eggs (range 26–616) from each female. There was strong second-male precedence; 71% of the 38 females had P2 values significantly more than 0.5, 11% had values for which the 95% confidence interval included 0.5, and 18% had values significantly less than 0.5 (Figure 1a).

Male mass ranged from 437 to 629 mg, with mass differences between a female’s two mates ranging from 2 to 162 mg. In a linear regression of arcsine-transformed P2 values weighted by egg number, the mass difference between the males was a significant predictor of P2 (Table 1A, Figure 1b). The negative coefficient for M1–M2 indicates that larger males did better in sperm competition. The effect of being in the SV treatment had a marginally negative effect; virgin second males tended to fare poorly against previously mated first males (Figure 1c). Male genotypes, intermating interval (3–to7-day range), and total fecundity did not affect P2.

To further analyze the effect of male mating history, we did a post hoc comparison of P2 between S and V second males. There was a trend for an effect of treatment on rank, with higher P2 when the second male had mated 1 day prior (Mann–Whitney U = 120, n = 38, one-tailed P = 0.0519, Figure 1c).

**Experiment 2: effect of spermatophore size on sperm precedence**

**Rationale:** Experiment 2 explored mechanisms for the increased precedence enjoyed by second males mated 1 day prior (S) in experiment 1. The two male groups in the first experiment were not random subsets of our experimental population; some V males had not mated despite having access to females, whereas all S males had mated. Three hypotheses for the advantage of S males include 1) females preferentially used sperm from S males, perhaps using small spermatophores to indicate previous mating success (Oberhauser 1989), 2) females use a cue other than spermatophore size to bias sperm use in favor of males that are better maters, or 3) males that were more successful at mating transferred more competitive sperm. The first two hypotheses suggest cryptic female choice, whereas the third suggests that male competition determines sperm precedence patterns. We rejected a fourth possible hypothesis that spermatophores from the S males contained more sperm; S males transfer fewer eupyrene (nucleated) sperm than V males (Solensky and Oberhauser 2009).

To test the first three hypotheses, we set up situations in which sperm from the same two males competed in different
females. By manipulating male mating history, we could determine if males fared better when they had mated more recently. Although not part of the experimental design, conditions during the experiment led to a test of an interaction between PGI genotype and temperature.

Methods: We created 12 pairs of males, matched for wing length, to mate to a series of females. One male of the pair was always the first mate, with an equal number of ff and mm males assigned to this role. We manipulated male mating history so that males had either mated 1 (S) or 4 (L) days previously. Each female received two spermatophores—LS, SL, or SS—with 1 day between matings. Four male pairs mated to three females, seven mated to two females, and one mated to one female.

On the first 3 days of this study, temperatures on the concrete rooftop patio where cages were placed reached or exceeded 40°C each day (high daily temperatures near the concrete roof ranged from 40 to 42°C), resulting in some mortality or subsequent failure to mate or lay eggs. Males first experienced the high temperatures on the day of their first experimental mating (matings to manipulate spermatophore size occurred in the temperature-controlled greenhouse), so sperm received by the first female to which each male pair mated were only exposed to high temperatures for a few hours before mating. Subsequent females to which the male pairs mated received sperm from males that had been exposed to hot conditions for more than 1 day.

Results: Females laid an average of 177 eggs (range 40–353) over the 10 days after their second mating, and P2 values ranged from 0 to 1 (Figure 2a). Complete sperm precedence was common; only 44% of females laid eggs fertilized by both males. First- and second-male sperm precedences were equally common, and only one 95% confidence interval included 0.5.

Sperm precedence was influenced by male PGI genotype and an interaction between female number (first or subsequent) and genotype, but not by hand pairing, male mating history treatment, male or female wing length, male pair identity, or the difference in wing length between the two males (Table 1b). Second males that were mm fertilized more eggs than ff males, and this pattern was weaker for matings to first females (Table 1b [positive coefficient for first female × second male ff], Figure 2b).

Arcsine-transformed P2 values for the regression reported in Table 1b failed to meet assumptions of normality.

Table 1
Factors affecting P2 values

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Coefficient</th>
<th>Std error</th>
<th>P-value</th>
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<tbody>
<tr>
<td>a. Experiment 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Constant</td>
<td>0.8492</td>
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<td>b. Experiment 2</td>
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<td></td>
<td></td>
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<tr>
<td>Constant</td>
<td>1.145</td>
<td>0.904</td>
<td>0.131</td>
</tr>
<tr>
<td>Second male ff</td>
<td>-1.262</td>
<td>0.170</td>
<td>&lt;0.001</td>
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<td>First female × second male ff</td>
<td>0.564</td>
<td>0.168</td>
<td>0.003</td>
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<tr>
<td>c. Experiment 3</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Constant</td>
<td>0.663</td>
<td>0.713</td>
<td>0.355</td>
</tr>
<tr>
<td>Only first male hand paired</td>
<td>0.267</td>
<td>0.146</td>
<td>0.071</td>
</tr>
<tr>
<td>Second male ff</td>
<td>0.298</td>
<td>0.105</td>
<td>0.006</td>
</tr>
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</table>

Weighted (by number of eggs analyzed) stepwise linear regression models (backward elimination criterion: P > 0.10). P2 values were arcsin transformed; mating and genotype treatments were included in models as indicator variables. (a) Experiment 1 (n = 38, R2 = 0.2255). (b) Experiment 2 (n = 27, R2 = 0.698). The positive interaction term for first female × second male ff indicates that the advantage of mm males was weaker for first experimental matings. (c) Experiment 3 (n = 88, R2 = 0.123).

Figure 1
P2 in experiment 1. Significant sperm precedence is defined by a value whose binomial 95% confidence interval does not include 0.5. (a) P2 ranged from 0 to 1, with second-male precedence more common. (b) P2 was negatively correlated with the difference in mass between the two males (male 1– male 2). (c) P2 separated by mating treatment. Triangles are not significantly different from 0.5, and numbers indicate that more than one female had that value.
(Kolmogorov–Smirnov test: $P = 0.001$), so we verified significant parameters identified by regression analysis using the nonparametric Mann–Whitney $U$ test. Male genotype at the PGI locus influenced sperm precedence, with $mm$ males fertilizing more eggs than $ff$ males (Mann–Whitney $U = 161, n = 27, P < 0.001$). We tested the significance of the interaction between male genotype and female number using separate analyses for first and subsequent females. The positive effect of the $mm$ male genotype on $P_2$ was weaker in matings to the first female than to subsequent females (first female: Mann–Whitney $U = 18, n = 9, P = 0.020$; subsequent females: Mann–Whitney $U = 72, n = 18, P < 0.001$).

For six of the male pairs that mated with multiple females, 95% confidence intervals of $P_2$ values from all of their mates overlapped (data not shown). For the five that did not overlap, no mating history combination resulted in consistently higher $P_2$ values; in three cases, the male pairs had mated with three different females, and in two cases, they had mated with two different females. The mating history combinations and significant differences in $P_2$ values for these five pairs are as follows: SS $> LS = SL$, SS $> LS$, SS $> LS = SL$, SS $> LS$, and SL $> SS$. Thus, relative ejaculate size did not appear to affect the outcome of sperm competition in this experiment.

**Experiment 3: effect of spermatophore size on sperm precedence (controlled temperatures)**

**Rationale:** Because the effect of genotype, likely exacerbated by extreme heat, outweighed all other effects in experiment 2, we conducted a third experiment in which we kept adults in a temperature-controlled greenhouse ($-24$ to $28^\circ C$) throughout the experiment.

**Methods:** We used 60 male pairs, each of which consisted of one $mm$ and one $ff$ male, and 32 experimental females. Each male pair was scheduled to mate with two females, with preassigned mating history treatments: 1) both females, SS; 2) first female, SL; second female, SS; or 3) first female, LS; second female, LL. Female intermating intervals were all 1 day. Of the 60 male pairs, 28 mated to two females as planned, and 32 mated to one female.

**Results:** Females laid an average of 187 eggs in the 10 days after their second mating (range 22–450). $P_2$ values ranged from 0 to 1, with mixed paternity (82%) and second-male precedence (70%) common (Figure 3a). $P_2$ was higher when the second male was $ff$ (Figure 3b), and was not influenced by male mating history, male or female wing length, hand pairing or the difference in male size (Table 1c).

We used a paired $t$-test to compare the outcome of sperm competition when the same two males competed in two different females with different mating sequences (SL and SS or LL and LS) in the two females. In these cases, the second but not the first male’s mating history changed from one female to the next. $P_2$ values tended to be higher when the second male delivered a large spermatophore than when he delivered a small spermatophore (mean difference $= 0.14, t = 1.71, df = 22, P = 0.0504$ [$t$-test done on arcsine-transformed values]). Figure 3c compares the difference in $P_2$ values between the two females mated to the same pair of males for all mating treatments. The values illustrated in the first two columns of Figure 3c represent the mean difference between $P_2$ values when the second male is $S$ versus when he is $L$, respectively; positive values indicate that the second male does better when he delivers a large spermatophore, regardless of the mating history of the first male. When the mating history combinations were the same in both females (third column, shown for comparison), the mean difference between the two values was smaller.

**DISCUSSION**

We observed a general pattern of second-male sperm precedence, except in one experiment in which the effect of genotype outweighed any other effects. This pattern was affected by the mating histories and sizes of the two males, and genotype by environment interactions. Clearly, an understanding of sexual selection in polyandrous species requires knowledge of events that affect sperm use by females, and these events are likely to be complicated and variable.

**Overall sperm precedence patterns**

Second-male sperm precedence was more common than first-male or no sperm precedence in monarch butterflies in two of the three experiments reported here. The advantage of second males suggests that sperm in the elongate spermatheca are pushed back by incoming sperm, resulting in stratification of sperm from different males. The finding that second-male
sperm precedence is rarely complete indicates that sperm from the first male remain in the fertilization set (Parker et al. 1990). Spermatophores delivered by the first male may serve as a barrier for subsequent males, because the spermatophore must align correctly with the ductus seminalis for sperm to move into the spermatheca (Drummond 1984). Variation in the ability to generate or overcome this barrier could cause the bimodal distribution in sperm precedence: 5%, 33%, and 9% of females exhibited complete first-male precedence in experiments 1, 2, and 3, respectively.

Male size

In experiment 1, when males were not matched for size, larger males tended to fare better in sperm competition (Figure 1b). This effect of male size on the outcome of sperm competition is common in insects (Lewis and Austad 1990; Simmons and Parker 1992; LaMunyon and Eisner 1993, 1994; Gwynne and Smelden 1995; Simmons et al. 1996; Bissoondath and Wiklund 1997; Wedell and Cook 1998; Arnaquist and Danielson 1999) and could be a numerical effect if larger males transfer more sperm, as they do in the dung fly *Scatophaga stercoraria* (Simmons and Parker 1992; Simmons et al. 1996). Female choice could also mediate a numerical effect. For example, male field crickets (*Gyllus bimaculatus*) allow longer spermatophore attachments and multiple inseminations from larger males (Simmons 1986), resulting in the transfer of more sperm and higher success in sperm competition.

A numerical advantage is unlikely to explain the effect of male size in the outcome of sperm competition in monarch butterflies. Male size does not appear to affect the number of eupyrene or apyrene sperm transferred in monarchs (Solensky and Oberhauser 2009), and the large male advantage occurred across treatments in experiment 1, even when the larger male had mated previously and thus transferred fewer sperm (Solensky and Oberhauser 2009).

Other mechanisms could lead to a large male advantage in sperm competition. For example, female dung flies store sperm from larger males where they are probably more likely to be used to fertilize eggs (Ward 1998, 2000). A large male advantage could also be due to differential fertilizing capacity of different-sized sperm. In mammals and birds, longer sperm swim faster (Gomendio and Roldon 1991; Briskie and Montgomerie 1992), and faster sperm may be more likely to fertilize eggs (Birkhead et al. 1995). Male bulb mites *Rhizoglyphus robini* (Radwan 1996) and nematodes *Caenorhabditis elegans* (LaMunyon and Ward 1998) that transferred larger sperm fared better in sperm competition. However, in the dung beetle *Onthophagus taurus*, shorter sperm had higher fertilization success in a way that depended on the size and shape of the spermatheca (García-González and Simmons 2007). We are not aware of studies showing within-species correlations between male size and sperm size, but this correlation exists across butterfly species (Gage 1994).

Male mating history

Previously mated second males tended to fare better in sperm competition than virgins in experiment 1, even though they transferred smaller spermatophores and probably fewer sperm (Oberhauser 1988; Solensky and Oberhauser 2009). Experiments 2 and 3 were designed to determine if this advantage was due to mating history per se, perhaps because females used spermatophore size to indicate past mating success and biased sperm use in favor of these males. We hypothesized that spermatophore size is likely to be an honest indicator of mating history; large spermatophores result in longer delays before female remating (Oberhauser 1992), and males...
probably transfer the largest spermatophore allowed by their mating history and age (Oberhauser 1988). Alternatively, the advantage could have been due to something inherent in the males or their sperm, because previously mated males were not a random subset of our population. Experiment 2 was inconclusive because of the large genotype effect, but experiment 3 showed that females do not preferentially use sperm from males that transfer small spermatophores. When the second male transferred a larger spermatophore than he had in another competition with the same male, he did better, rather than worse. Results of experiment 1 are thus likely to be due to something inherent in this nonrandom subset of males, perhaps the result of cryptic female choice based on something other than spermatophore size or a correlation between male mating ability and sperm competitiveness. Experiment 3 does not allow us to distinguish unequivocally between these two hypotheses. However, when the same male pairs competed in different females, the outcome of sperm competition varied in ways predicted by the number of sperm transferred. This result does not rule out cryptic female choice, but consistent patterns between females in experiment 3 would provide a stronger argument for this mechanism.

PGI genotype

Male PGI genotype affected sperm precedence in experiments 2 and 3, but not experiment 1. In experiment 2, mm males fertilized significantly more eggs than ff males, and the reverse was true in experiment 3. Males and females were reared under similar laboratory conditions and were of similar ages at the start of the experiments. However, butterflies in experiment 2 were exposed to very hot conditions, whereas those in experiment 3 were kept in a temperature-controlled greenhouse.

PGI (an enzyme used in glycolysis, Watt 1992) genotypes affect fecundity and mate choice in Colias butterflies (Watt et al. 1986; Watt 1992) and monarch butterfly flight ability at low temperatures (Hughes and Zalucki 1993). In this study, PGI may have affected sperm function or transfer, and the mm allele appears less susceptible to denaturation or loss of activity at high temperatures. The extreme heat occurred only during the first matings, but the genotype effect was even stronger in subsequent matings (Figure 2b). The effect of temperature appears to be lasting and less severe after a short exposure. An alternative explanation stems from our use of a relatively small number of population founders. Matings that established the experimental population were designed to generate adults of known genotypes, but not maximize genetic diversity. PGI genotype may have been confounded with a heritable trait that we did not assess.

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