Original Article

Males responding to sperm competition cues have higher fertilization success in a soldier fly

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Sperm competition has been demonstrated to be an important force shaping male behavior in a number of species. For example, males may prolong copulation duration when they perceive sperm competition to be high. Although male behavioral responses to sperm competition have been shown in several species, their effects on reproductive success have rarely been demonstrated. In the soldier fly *Merosargus cingulatus*, males prolong copulations when sperm competition is high and when mating with more fecund females. Here, I tested the hypothesis that this behavioral response results in higher reproductive success for males. I exposed males to different simulated levels of sperm competition (high or low male density at the oviposition site) then introduced a female. I allowed the pair to mate and the female to oviposit. I determined the percentage of offspring sired by the male using amplified fragment length polymorphism profiles. Sperm competition did not affect clutch size, but it did affect fertilization success: males under higher simulated sperm competition increased copulation duration and fertilized a higher percentage of a female's egg clutch than did males under lower sperm competition. *Key words:* clutch size, fertilization success, postcopulatory sexual selection, soldier fly, sperm competition. *[Behav Ecol]*

INTRODUCTION

Sperm competition plays an important role in shaping male morphology and behavior (Simmons 2001). Whenever sperm is a limited resource, males are expected to selectively allocate sperm in a way that can increase their reproductive success (Gage 1991; Cook and Gage 1995; Gage and Barnard 1996; Marconato and Shapiro 1996; Simmons and Kvarnemo 1997; Fuller 1998; Parker et al. 1999). Sperm competition theory makes different predictions about male reproductive investment depending on the intensity and risk of sperm competition. Sperm competition intensity relates to the number of ejaculates from different males competing within a female's genital tract, whereas sperm competition risk relates to the probability that a female has mated before or will mate again with a different male. On the one hand, males are expected to transfer less ejaculate when sperm competition intensity is high because, after a certain point, males will obtain diminished returns from their increased investment in ejaculates. On the other hand, males are expected to transfer more ejaculate when there is a high risk of sperm competition: if there is a high probability that sperm competition will occur, the more sperm transferred to a female, the higher fertilization success will be, assuming all else is equal (Parker et al. 1996, 1997, reviewed by Wedell et al. 2002; Parker and Pizzari 2010). Because the models make opposite predictions, it is important that experimental studies distinguish between measurements of intensity and risk (Engqvist and Reinhold 2005). This is no easy task because, for example, varying the density of rivals a male is exposed to can potentially signal both risk and intensity to that male.

Males of several species have been shown to respond to sperm competition by changing their behavior. This includes changing courtship intensity and overall copulation duration (Lorch et al. 1993; Andrés and Cordero-Rivera 2000), as well as providing females with different quality nuptial gifts (Sakaluk 1985; Fox et al. 1995; Oberhauser 1998; Vahed 1998; Wagner 2005). For example, in *Drosophila melanogaster*, males court females more vigorously when sperm competition intensity is lower (Tompkins and Hall 1981; Friberg 2006). In this case, they assess sperm competition intensity by detecting female mating status through the female cuticular hydrocarbon profile. In other species, males attempt to assess sperm competition risk by assessing the operational sex ratio before and during mating. Although there are a large number of examples showing male behavioral responses to sperm competition in the literature, only one study has investigated its effects on male fitness (Bretman et al. 2009, reviewed in Bretman et al. 2011).

Males are also expected to selectively allocate resources (such as ejaculate size and nuptial gifts) when mating with females of different qualities, if doing so can increase their reproductive success. This process is known as cryptic male choice (Bonduriansky 2001). Males have been shown to selectively allocate more resources to females with traits that relate to high fecundity in a number of species (Gage and Barnard 1996; Yasui 1996; Gage 1998; Wedell 1998; Parker et al. 1999).

Males of the soldier fly *Merosargus cingulatus* respond to sperm competition by prolonging copulations when rival density at the oviposition site is high. They also increase copulation duration when mating with larger more fecund females (Barbosa 2011). In this species, mating occurs at the oviposition sites, where males try to capture and mate with any female that approaches. Both males and females mate multiply and frequently. Males do not court females before mating, but they perform copulatory courtship: during the entire duration of copulation, they alternate bouts of tapping the female abdomen and waving their hind legs in the air. This copulatory courtship induces the female to lay eggs immediately

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after copulation (Barbosa 2009). Females oviposit multiple egg clutches in the same day. They often return to the same oviposition site and lay additional clutches after having already oviposited in that area (Barbosa F, personal observation).

Here, I tested the hypothesis that male behavioral response to sperm competition results in higher reproductive success for males. There are several nonmutually exclusive ways this behavior could increase a male's reproductive success. One possibility is that males stimulate females to lay a larger number of eggs through prolonged copulations or courtship (because copulatory courtship occurs during the entire copulation, copulation and courtship duration are equivalent in this species). Another possibility is that males that prolong copulations fertilize a larger proportion of a female's eggs, either by transferring more sperm or accessory gland secretions or through cryptic female choice for longer copulations, courtships, or more secretions. I tested both of these possibilities by submitting males to different treatments that simulated different amounts of sperm competition (high and low male density at the oviposition site). Although we do not know if and how males detect sperm competition in this species, it is possible that they use density at the oviposition site as a cue. This possible indicator of sperm competition will hereafter be referred to as "simulated sperm competition" or "sperm competition" for simplicity. In addition, female size plays a role in copulation duration in this species: males prolong copulations when mating with larger more fecund females. Because of that, I also tested if male reproductive success under different amounts of sperm competition differed between males that were given either large or small females as mates.

MATERIALS AND METHODS

This work was conducted at the Smithsonian Tropical Research Institute field station in Gamboa, Panama, between July and September 2009. All experiments were conducted in a $1.8 \times 1.8 \times 1.8$ m mesh enclosure containing an area of 80×40 cm covered with fruit peels to create suitable oviposition sites. This enclosure was located in an open area adjacent to small patches of forest in a residential area. All individuals used were field caught. To attract individuals, piles of fruit peels were set up in the field. Females were caught as they arrived at the sites, before they had a chance to oviposit. When individuals were not introduced to the experimental enclosure as soon as they were captured, they were temporarily housed individually in 50 ml plastic jars. They were not housed in the plastic jars for longer than 30 min. All individuals used were weighed.

Sperm competition and clutch size

Males were randomly assigned to a treatment that simulated either low or high sperm competition and were mated to either small or large females, resulting in a 2×2 design. To create the groups with different simulated amounts of sperm competition, males were assigned to different density treatments. In the low-density treatment, they were introduced into the enclosure alone; in the high-density treatment, they were introduced with 4 other males. The amount of oviposition substrate was the same for both treatments. Natural male density at oviposition sites in the field varies considerably, and the 2 treatments used are within the natural range (Barbosa 2011). For both treatments, males had an acclimation period of 1 h, during which they established territories on the oviposition substrate. After acclimation, the 4 extra males in the high-density treatment were removed. Following the density treatments, I introduced a female from 1 of 2 female size categories, small (<42 mg) or large (>42 mg). These size categories correspond to the top and bottom halves of a female size distribu-

tion that I obtained from previous measurements of field-caught individuals (Barbosa F, unpublished data). Females were introduced into the enclosure with an entomological net. I allowed the pair to mate and the female to oviposit. Females were allowed to terminate oviposition naturally and therefore were not captured until they left the oviposition substrate. I video recorded all copulations. This experimental design resulted in 4 groups: males that mated under conditions mimicking high sperm competition with large females ($n = 12$) or small females ($n = 8$) and males that mated under conditions mimicking low sperm competition with large females ($n = 10$) or small females ($n = 10$).

I measured 2 aspects of female oviposition behavior that may be affected by copulation duration: clutch size and the percentage of available mature eggs laid by the female. I focused on the percentage of mature eggs laid because larger *M. cingulatus* females are more fecund than small ones (Barbosa 2011), so female size likely affects clutch size. To determine clutch size, I counted the number of eggs laid by the female after mating. To determine the percentage of eggs laid, I dissected the females and counted the mature eggs that were left in their ovaries after they oviposited. I performed 2×2 analyses of variance (ANOVAs) with density (low, high) and female size (large, small) as between-subjects factors to compare clutch size, percentage of eggs laid, and copulation duration between treatments. Data were transformed if necessary to achieve normal distributions (arcsine for percentages, log, and square root).

Sperm competition and paternity

I used amplified fragment length polymorphism (AFLP) profiles to determine what percentage of the eggs laid by the female after mating in the previous experiment was fertilized by the experimental male. After oviposition, I collected the mating pair and the eggs laid. I allowed the eggs to develop to larvae and randomly selected 25 larvae from each family for analysis. For pairs with less than 25 offspring, all larvae were analyzed. DNA extraction, AFLPs, and analysis were done as in Barbosa (2009). For 4 of the 40 pairs, it was not possible to obtain genotype data for one of the parents because the polymerase chain reaction (PCR) failed. Because data from both parents are necessary to determine paternity, those families were not analyzed. Some of the offspring of the other families were excluded from the analyses because of PCR failure.

Barbosa (2011) showed that copulations are longer when sperm competition is high, so I tested if there was an effect of simulated sperm competition on copulation duration and on copulatory courtship. I quantified different components of copulatory courtship (rate of taps, rate of waves, duration of tap bouts, and duration of wave bouts) by measuring them from the video recordings and comparing them between the treatment groups.

I performed 2×2 ANOVAs with density (low, high) and female size (large, small) as between-subjects factors to compare clutch size, percentage of eggs laid, and copulation duration between treatments. I also performed linear regression analyses to test the effects of male and female size, sperm competition, and the interactions between these variables on copulation duration. I initially tested a model with all the interactions then removed the statistically nonsignificant interactions to arrive at a reduced model with higher power. Data were transformed if necessary (arcsine for percentages, log, and square root). I used dummy coding to code for sperm competition (0 = high sperm competition, 1 = low sperm competition).
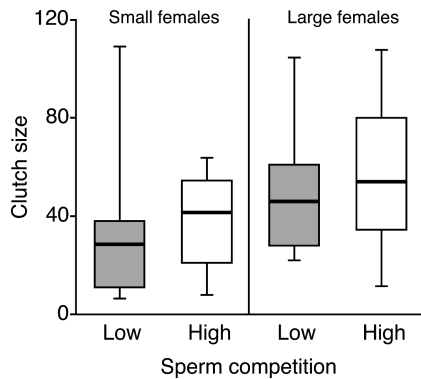
Figure 1
Clutch size was not affected when females (small or large) mated with males under high or low simulated sperm competition. Box plots show median (horizontal bars), upper, and lower quartiles (borders of the box). Whiskers extend from the 10th to the 90th percentiles.

RESULTS

Sperm competition and clutch size

Simulated sperm competition did not affect clutch size (Figure 1). A 2 × 2 ANOVA on square-root transformed clutch size showed no effect of density ($F_{1,36} = 0.121$, $P = 0.730$) or female size ($F_{1,36} = 2.991$, $P = 0.092$), and there was no interaction between density and female size ($F_{1,36} = 0.001$, $P = 0.972$). Simulated sperm competition also did not affect the percentage of eggs laid (Figure 2). A 2 × 2 ANOVA on arcsine transformed percentage of eggs showed no effect of density ($F_{1,36} = 0.548$, $P = 0.464$). There was an effect of female size on percentage of eggs laid with smaller females tending to lay a larger percentage of their available eggs ($F_{1,36} = 5.287$, $P = 0.027$). There was no interaction between density and female size ($F_{1,36} = 0.474$, $P = 0.469$). Female size averaged 29.31 ± 8.98 mg for the small female group and 61.09 ± 14.84 mg ($\bar{x}$ ± standard deviation [SD]) for the large female group. Male size averaged 44.66 ± 20.09 mg ($\bar{x}$ ± SD).

Sperm competition and paternity

The fertilization success of the experimental male was influenced by simulated sperm competition: when sperm competition was higher, males fertilized a higher percentage of the
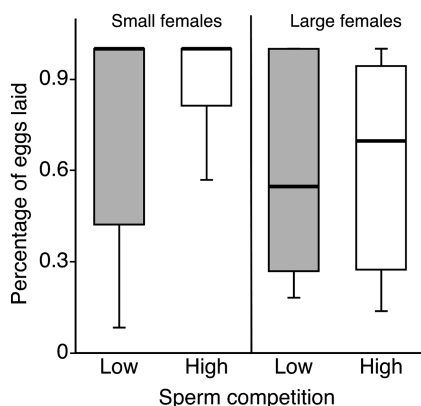
Figure 2
The percentage of mature eggs laid was not affected when females (small or large) mated with males under high or low simulated sperm competition. Box plots show median (horizontal bars), upper, and lower quartiles (borders of the box). Whiskers extend from the 10th to the 90th percentiles.
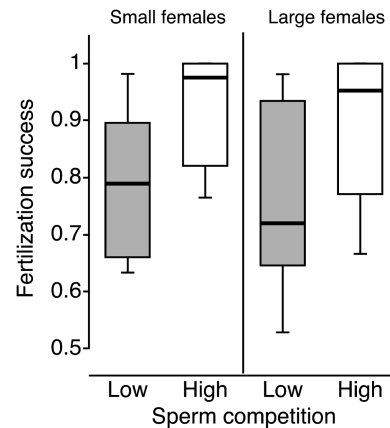
Figure 3
Male fertilization success increased significantly under higher simulated sperm competition. Box plots show median (horizontal bars), upper, and lower quartiles (borders of the box), and whiskers extend from the 10th to the 90th percentiles, of the percentage of eggs fertilized by the experimental male under low and high simulated sperm competition when mating with small and large females.

clutch than when sperm competition was low (for small females, low sperm competition: 79.21 ± 13.86%, high sperm competition: 91.78 ± 10.41%; for large females, low sperm competition: 77.21 ± 17.81%, high sperm competition: 87.57 ± 14.04%, (mean ± SD), Figure 3). A 2 × 2 ANOVA on arcsine transformed fertilization success revealed a main effect of sperm competition ($F_{1,31} = 5.758$, $P = 0.023$). There was no effect of female size ($F_{1,31} = 0.195$, $P = 0.662$), and there was no interaction between sperm competition and female size ($F_{1,31} = 0.067$, $P = 0.797$).

As expected, copulation duration was longer when simulated sperm competition was higher (Table 1). Female and male size also affected copulation duration: copulations were longer for smaller males and for larger females. A linear regression model containing male size, female size, simulated sperm competition, and the interaction between sperm competition and male size on copulation duration was statistically significant (Table 1).

A linear regression model with fertilization success as the dependent variable showed a statistically significant interaction between copulation duration, male size, and female size (Table 2). Males of a similar size needed to mate longer with a large female than with a small one to obtain the same fertilization success. In addition, for a given female body size,

Table 1

The effect of female size, male size, and sperm competition on copulation duration

Predictor	B	SE	P
Intercept	12.465	1.976	
Sperm competition	**−7.720**	**2.777**	**0.009**
Female size	**0.063**	**0.029**	**0.035**
Male size	**−0.114**	**0.047**	**0.020**
Male size × sperm competition	0.104	0.058	0.084

Regression analysis with copulation duration as the dependent variable, showing the unstandardized coefficients (B), standard errors of the unstandardized coefficients (SE), and significance values (P) of the individual predictors present in the final model. Bold values are statistically significant at P < 0.05. Full model: $R^2 = 0.401$, $P = 0.003$.

Table 2

The effect of copulation duration, female size, and male size on fertilization success

Predictor	B	SE	P
Intercept	0.213	0.336	0.532
Copulation duration	0.058	0.033	0.092
Male size	0.013	0.008	0.144
Female size	**0.020**	**0.008**	**0.014**
Female size × male size	$\mathbf{-4.925 \times 10^{-4}}$	**0.000**	**0.012**
Female size × copulation duration	**−0.002**	**0.001**	**0.009**
Male size × copulation duration	0.000	0.001	0.386
Male size × female size × copulation duration	$\mathbf{4.578 \times 10^{-5}}$	**0.000**	**0.020**

Regression analysis with fertilization success as the dependent variable. Table shows the unstandardized coefficients (B), standard errors of the unstandardized coefficients (SE), and significance values (P) of the individual predictors that were entered into the model. Bold values are statistically significant at P < 0.05. Full model: $R^2 = 0.342$, $P = 0.092$.

smaller males would need to mate for longer than larger males to obtain the same fertilization success.

Because copulatory courtship occurs for the entire duration of copulation, the duration of copulatory courtship was different in the groups of low and high sperm competition. However, sperm competition did not affect the composition of the courtship display. There were no statistically significant differences among the treatments in the rate and the average duration of bouts of taps or of leg waves (Mann–Whitney tests, $P = 0.81$, 0.54, 0.54, and 0.72, respectively).

DISCUSSION

This study shows that male behavioral response to variation in sperm competition results in higher fertilization success. Male soldier flies prolong copulations when mating under high simulated sperm competition, and under those conditions, males fertilized on average 14.4% more of a female's clutch than males under low simulated sperm competition. This result is important because it demonstrates not only that males are detecting and responding to variation in sperm competition but also that this behavioral response affects male fitness. To the best of my knowledge, this is the only species in which increased fitness has been demonstrated other than *D. melanogaster* (Bretman et al. 2009); this is also the first time this phenomenon has been demonstrated under field conditions. The fact that males prolong copulations under high simulated sperm competition is consistent with previous work in this species (Barbosa 2011).

The relationship between copulation duration and fertilization success is complex because the effect of copulation duration depended on both male and female size. There was a negative relationship between copulation duration and male size but a positive relationship between copulation duration and female size. Female body size is known to play a role in copulation duration in this species (Barbosa 2011). Body size, copulation duration, and interactions between these factors all play a role in determining fertilization success in this species (see Table 2). For a given female body size, a small male would need to mate for a longer time than would a large male to obtain the same fertilization success. Likewise, males of similar size need to mate for a longer time with a large female than with a small one to obtain the same fertilization success.

The sperm competition treatments used in this study, where males were exposed to different densities of rivals, can potentially signal both risk and intensity to that male. Therefore, it is unclear whether soldier fly males were responding to risk or intensity of sperm competition. The results found here are comparable to those found by Bretman et al. (2009) in *D. melanogaster*. In that study, males prolonged copulation duration under high risk of sperm competition, but, as expected, copulations were shorter under high sperm competition intensity. Soldier flies are highly polygamous; both males and females mate multiply (Barbosa 2009), so sperm competition is likely to play an important role in this species. The levels of both risk and intensity of sperm competition are likely to be high for this species, but it is unknown whether males assess and respond to these components differently.

The mechanism that leads to higher fertilization success after longer copulations in this species is unknown. One possibility is cryptic female choice: females may prefer longer copulations (or longer courtships) and bias fertilization toward these preferred males. For example, females might control sperm transfer and storage, biasing fertilization toward males that perform more intense copulatory courtship as in the cucumber beetle (Tallamy et al. 2002, 2003) and flour beetle (Edvardsson and Arnqvist 2000, 2005; Fedina and Lewis 2004). Cryptic female choice occurs in this species by female control of whether oviposition occurs immediately after copulation (Barbosa 2009), but it is unknown if females can also control sperm transfer, sperm storage, and fertilization.

Another possibility is that longer copulations result in increased transfer of sperm or of other seminal products, which in turn leads to higher fertilization success. Previous studies in a variety of species have shown that males adjust ejaculate size in response to sperm competition (Gage 1991; Wedell and Cook 1999; Engqvist 2007; Ramm and Stockley 2007). Other studies have shown that males had higher fertilization success when they increased ejaculate transfer (Simmons 1987; Simmons et al. 1996; Arnqvist and Danielsson 1999b). The relationship between copulation duration and sperm transfer is unknown for soldier flies, so this hypothesis also remains to be explored.

Finally, there is no evidence that the changes in male behavior caused by increased simulated sperm competition affected female egg production in the soldier fly. This contrasts with the results of Bretman et al. (2006) in *D. melanogaster*, where increased sperm competition risk resulted in larger clutch sizes. In that study, it was hypothesized that larger clutch sizes were caused by an increase in seminal fluid transfer, which can affect female egg production behavior. In other species, females have been shown to increase clutch size, as well as the amount of resources allocated to eggs, as a result of cryptic female choice (Thornhill 1983; Wedell 1996; Arnqvist and Danielsson 1999a, 1999b; Bretman et al. 2004; Aquiloni and Gherardi 2008). Changes in clutch size do not seem to occur in this context in *M. cingulatus*.

In summary, these results demonstrate that sperm competition affect male copulatory behavior in soldier flies, and this in turn results in increased reproductive success. Under high

simulated sperm competition, males prolong copulations and obtain higher fertilization success, which could be due to higher sperm transfer or cryptic female choice.

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