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Adjustment of copula duration and ejaculate size according to the risk of sperm competition in the golden egg bug (*Phyllomorpha laciniata*)

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Several hypotheses have been proposed to explain the adaptive significance of prolonged copulations in insects, which include mate guarding and sperm loading functions. We have explored the adaptive significance of the prolonged copulations in the golden egg bug (copulations up to 50 h) and the effect of an increased risk of sperm competition on ejaculate investment. Our data support predictions derived from sperm competition theory, which posits that males are expected to increase ejaculate expenditure in response to an increased risk of sperm competition. Results show a combined response by males that has not been previously described: males in the presence of rivals increase copulation duration and the rate of sperm transfer. No relationship was found between male or female size and copulation duration or ejaculate size. Golden egg bug males transfer sperm slowly and gradually throughout copulation; thus an increase in the amount of sperm transferred and the corresponding increase in the male's numerical representation in the female's storage organs could be particularly important in a system in which so few sperm are transferred and in which so few sperm are stored by females. In addition, copulation duration may not only serve to increase the total amount of sperm transferred, but it may also increase the chances that the female will lay an egg soon after copulation has ended. This could explain why males tend to accept eggs after copulation, since they could be maximizing the chances that such eggs are fathered by them, and in this way they would substantially increase the survival rates of their offspring because eggs laid on plants suffer high mortality rates. *Key words:* copulation, ejaculate size, golden egg bug, *Phyllomorpha laciniata*, sperm competition, sperm loading hypothesis. *[Behav Ecol 15:23–30 (2004)]*

Sexual selection has favored the evolution of behavioral, physiological, and morphological traits in males that increase reproductive success in the face of male-male competition (Andersson, 1994). When females tend to copulate with several males and there is a temporal and spatial overlap of ejaculates from two or more males, a specific type of male-male competition takes place, which is known as sperm competition (Parker, 1970). Sperm competition has been a major selective force shaping many aspects of sexual reproduction, including mate guarding, frequency and duration of copulation, genitalia morphology, testes size, sperm numbers, ejaculate quality, and sperm size (Birkhead and Møller, 1998; Parker, 1970; Simmons, 2001; Smith, 1984).

In the context of sperm competition, copulation may serve other functions apart from the obvious of sperm transfer (Simmons, 2001; Thornhill and Alcock, 1983). Although, in general, sperm transfer is accomplished within a few seconds or minutes, some species remain in copula for longer periods. Prolonged copulations are assumed to be costly because they can be energetically expensive, may increase the risk of predation, may increase the probability of disease transmission, and may decrease time devoted to other activities (Daly, 1978). Despite such costs, prolonged copulations occur in many orders of insects (Alcock, 1994; Smith, 1984; Thornhill and Alcock, 1983), and several hypotheses have been put forward to explain the adaptive significance of prolonged copulation. First, the sperm removal hypothesis suggests that copula duration could reduce competition with previous ejaculates if males spend more time removing their rivals' sperm (Siva-Jothy, 1987; Siva-Jothy and Tsubaki, 1989). Second, Eberhard (1996) suggested that copula duration may be under female control and that the benefits for females have to do with facilitating cryptic female-choice mechanisms. Third, the in-copula guarding hypothesis suggests that remaining in copula may function as an extreme form of mate guarding if it prevents the female from remating before oviposition (Alcock, 1994), thus possibly reducing or avoiding sperm competition with future ejaculates. Finally, the sperm loading hypothesis proposes that copula duration may determine the amount of sperm transferred by the male (Dickinson, 1986; Parker et al., 1990), and thus prolonged copulation may enhance the competitive ability of the ejaculate by increasing sperm numbers (Parker, 1982, 1984, 1993).

High levels of sperm competition have been associated with longer copulations (Alcock, 1994; Alonso-Pimentel and Papaj, 1996; Clark, 1988; McLain, 1989; Sillén-Tullberg, 1981), and such prolonged copulations have generally been interpreted as a mate-guarding strategy. However, an increasing number of studies have shown that longer copulations may also imply the transfer of greater sperm numbers (Arnqvist and Danielsson, 1999; Birkhead et al., 1995; Dickinson, 1986; Parker et al., 1990). The hypothesis of sperm loading to explain prolonged copulations under increasing levels of sperm competition is plausible when sperm compete numerically because in this case the most obvious adaptation to sperm competition is selection on males for increased sperm numbers (Eady, 1995; Parker, 1982, 1984, 1993; Wedell and Cook, 1998).

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Sperm competition theory suggests that males may respond to current information on sperm competition risk (the probability that a given male will be in direct competition for fertilizations). A male is expected to increase ejaculate expenditure in response to an increased risk of sperm competition (Ball and Parker, 1998; Parker, 1990a, 1998; Parker et al., 1997; Wedell et al., 2002). Such strategies of ejaculate allocation evolve because the costs of ejaculate production are not trivial (Dewsbury, 1982; Nakatsuru and Kramer, 1982; Olsson et al., 1997), and males are expected to partition their ejaculates optimally (Parker, 1982, 1990a, b; Simmons and Siva-Jothy, 1998). Several intraspecific studies have provided evidence in support of this prediction (Cook and Gage, 1995; Gage, 1995; Gage and Barnard, 1996; Oppliger et al., 1998; Schaus and Sakaluk, 2001; Simmons and Kvarnemo, 1997; Wedell and Cook, 1999a,b), although some have found that males do not react as predicted to an increase in the risk of sperm competition (Birkhead and Fletcher, 1995; Schaus and Sakaluk, 2001; Wedell, 1992). When males adjust sperm transfer to the perceived risk of sperm competition, they may do so by copulating more frequently, by increasing the amount of time spent engaged in sperm transfer, or by increasing the amount of sperm transferred per time; however, additive effects among these different mechanisms remain largely unexplored.

Phyllomorpha laciniata is a heteropteran species characterized by an atypical behavior among insects. Females exhibit a very flexible pattern of oviposition behavior: they can lay their eggs on host plants (*Paronychia argentea*), where they develop unattended, or on the body of conspecific males and females, where they are carried until hatching, when the nymphs start an independent life (Gomendio and Reguera, 2001; Kaitala, 1996; Reguera, 1999). Most of the eggs carried by conspecifics are carried by males, and there is an ongoing controversy concerning the evolutionary significance of male egg carrying in this insect. Some authors believe that egg carrying by males is likely to be the result of intraspecific parasitism (Kaitala et al., 2001). However, other authors have argued that, because the costs of carrying eggs are high, males should only accept eggs if by doing so they substantially increase the survival of some of their offspring (Gomendio and Reguera, 2001). Copulation duration is very long in *P. laciniata*, lasting around 20–30 h on average (Kaitala, 1998; Reguera, 1999) and sometimes lasting as long as 48 h. However, no studies have been conducted on the adaptive significance of the prolonged copulations in this insect, although this information would improve our understanding of this intriguing system. In natural populations, individuals are likely to experience sperm competition because females mate promiscuously and store sperm in a spermatheca. In this study we examine the response of males to an increased risk of sperm competition.

METHODS

Animals and general experimental conditions

Individuals for the experiments were collected in the field. For the experiments on copulation duration we collected 194 individuals of *P. laciniata* (91 males and 103 females) in Colmenar del Arroyo and Robledo de Chavela (Madrid, central Spain), and in El Espinar (Segovia, central Spain) on five different dates from 8 April to 10 May 1999. For the experiments on sperm transfer, 175 individuals (88 males, 87 females) were collected in Aldea del Fresno and Robledo de Chavela (Madrid, central Spain), and El Espinar (maximum distance among these localities is 40 km) on five different days from 25 April to the 16 May 2000. Individuals were transported in individual plastic vials to the laboratory in Madrid, placed in small Petri dishes (5.5 cm diameter), and kept at constant conditions (25°C, lights on from 0800 to 2100 h). The experiment on copulation duration was carried out from 16 April to 26 May 1999, and the experiment on sperm transfer was carried out from 3 May to 26 May 2000.

So far, all attempts to rear this insect in captivity have been unsuccessful. Although we have managed to obtain virgin females from advanced nymphs captured in the field, these adults do not mate until the following year (García-González, 2002). For this reason, we carried out experiments for which virgin females were needed using virgin females collected from the field. Virgin females can be found among females collected at the beginning of the reproductive season, though they are present in small numbers. We considered a female as a virgin if (1) she did not lay eggs during at least 5 days since she was captured in the field, (2) she had a normal abdomen (gravid females are recognized by a distended abdomen), and (2) as a indirect measure of the reproductive state of the local population, she did not carry eggs in her back. In a series of preliminary studies, these three requirements supported the "virgin-state assumption" for females collected at the beginning of the reproductive season: females fulfilling these requirements were dissected to find sperm in the spermatheca, and none of them carried sperm.

Throughout all the experimental period individuals were provided daily with ad libitum fresh branches of the host plant *Paronychia argentea* in both sets of experiments. Before the experiments, we removed eggs from carrying males as well as from carrying females and weighed individuals to the nearest 10^{-4} g with a Sartorius BP 110 S balance (Sartorius AG, Goettingen, Germany).

Copula duration

We tested variation in copulation duration depending on sex ratio conditions in two experiments. In the first experiment we used virgin females, whereas in the second experiment we used nonvirgin females. We randomly assigned individuals to different treatments. In the experiment with virgin females (experiment 1), 22 groups were established: 10 groups with 1 male and 1 female in each container and 12 groups with 2 males and 1 female. In the experiment with nonvirgin females (experiment 2), 20 groups were established: 10 groups with 1 male and 1 female and 10 groups with 3 males and 1 female. Individuals in groups were kept in plastic containers (16.5 cm × 16.5 cm × 10.5 cm). Because some individuals died during the experiment, the final number of replicates was slightly lower than initially designed.

We placed males in all groups in the container 2 days before the female to allow them to perceive the presence/absence of rivals and therefore to assess the risk of sperm competition. Males in groups with sex ratio 2:1 and 3:1 were marked in only one of the multiple lower abdominal chitinous extensions with a little green spot of typists' correction fluid to allow for identification. This facilitated identifying the mating male and monitoring the copula to check that the female did not remate with another male from the group. We included only the first mating of each female in the analyses.

We checked for copulations at least four times each day at 0900, 1300, 1700, and 2100 h. Copulation in this insect lasts on average for more than 12 h (23 h on average: Kaitala, 1998; 32.5 h on average: Reguera, 1999; 11 h minimum: Mineo, 1984), so it is unlikely that copulations went unnoticed. In those instances in which the start or the end of a copula was observed, we registered the exact time. Otherwise, to calculate the start and end of each copulation, we used the middle point between two intervals.

We carried out ANCOVAs and a mixed-model ANOVA. We entered male weight as a covariate in the analyses to examine the relationship between male weight and copulation duration. The dependent variable (copulation duration) was Box-Cox transformed to fulfill parametric assumptions (Sokal and Rohlf, 1981). We confirmed homogeneity of variances by using Levene's test or Hartley *F*-max statistic, Cochran *C* statistic, and the Bartlett chi-square test (Statsoft, 1996) in the mixed model of ANOVA.

Sperm transfer-ejaculate size

We identified 37 virgin females among all the females collected. The experiment consisted of placing a virgin female in a group of either one male or three males and determining how many sperm were inseminated after 1, 6, or 12 h after the beginning of the first copulation. We randomly assigned virgin females to the different treatments. In groups with sex ratio 1:1 females were distributed among three different experimental groups depending on the time at which copulations were experimentally terminated: 1, 6, and 12 h. In groups with sex ratio 3:1 females were distributed in groups in which copulations were terminated after 1 and 12 h.

All males were kept individually, or together in sets of three, depending on the experimental group, at least 3 days before the introduction of females to perceive the risk of sperm competition. In total, males were kept separate from females at least 7 days. We assume this time is sufficient to replenish sperm reserves in case they had mated in the field (see, e.g., Arnqvist and Danielsson, 1999).

Males in groups of sex ratio 3:1 were grouped in triads of individuals presenting similar weight. We entered data on male size as a covariate in the analyses (see below) to examine the relationship between male size and ejaculate size.

In studies on the effect of operational sex ratio (OSR; the ratio of the sexually active males in a population to receptive females; Emlen and Oring, 1977) over variation in traits affected by risk of sperm competition, sex ratio has been usually manipulated by changing the density of just one sex. This could cause OSR to be confounded with the density of that sex (Alonso-Pimentel and Papaj, 1996). To avoid OSR confounded with the absolute male density, we performed this experiment in Petri dishes of 5-cm diameter for groups of sex ratio 1:1 and dishes of 9-cm diameter for groups of sex ratio 3:1. This results in similar density of males under different OSR: 0.042 males/cm^2 in 1:1 and 0.047 males/cm^2 in 3:1.

All groups were set up in the morning and recorded all day with maximum intervals of 1 h, except in groups in which the duration of the copulation was designed as 1 h, which were monitored continually. We monitored copulations that started during the day and continued until night from 2100 h onward with the aid of red light. In all cases copulations were experimentally interrupted. Four pairs separated themselves before the desired experimental duration, and these were excluded from the analyses.

In groups with sex ratio 3:1, the mating male was quickly marked at the beginning of the copulation in one lower abdominal chitinous extension with a little green spot of typist's correction fluid (Tipp-ex GmbH and Co. KG) to ensure that there were no takeovers (although no takeovers of short duration have ever been seen). We carefully observed all matings to ensure penetration had taken place. We interrupted copulation at 1, 6, or 12 h from the start as designed depending on the groups. Copulation was instantaneously interrupted by quickly submerging the mating pair in ethylene glycol monoethylether (2-ethoxiethanol) at −80°C. Then the pair was maintained in 70% ethanol until the female was dissected. We carefully dissected the spermatheca and isolated it on a glass slide in a drop of distilled water, where it was crumbled with the aid of tweezers. Then we added 5 µl of phosphate-buffered saline without Ca^{2+} and Mg^{2+} and with 1% Triton and stirred spermathecal fragments for 2 min. After adding 5 µl of propidium iodide (PI; 0.05 mg/ml) fragments were again stirred for 1 min, and then the sample was spread over an area of 25 × 15 mm previously drawn in the slide. A few preliminary experiments showed that this method allows dispersal of agglutinated sperm and also showed that the numbers of sperm inseminated are usually low. Thus, we did not dilute the suspension, and we counted all the sperm in the sample. Preliminary experiments also showed that dilution and counting using a Neubauer hemocytometer gave unreliable estimates.

This method allowed for a good identification of individual spermatozoa. Occasionally, some sperm clumps were seen, but staining with the nuclear dye PI allowed counting of sperm heads. We counted the sperm using a fluorescence microscope Axiolab (C. Zeiss, Germany).

Because male size could be related to the number of sperm transferred, we statistically controlled this variable in the analyses. At the end of the experiment, we estimated male size from three length-measurements of the right hind tibia. These measurements were carried out capturing the tibia images (tibias were previously prepared on a glass slide) using a stereomicroscope Zeiss (Stemi SV6). Images were captured with a CCD camera (JVC TK-C1381) and tibias measured with NIH Image 1.60 software (National Institutes of Health). Repeatability of the three lengths reached $R = .999$ ($p < .001$).

We entered male size as a covariate in ANCOVAs to examine the relationship between male size and ejaculate size. The dependent variable (sperm transferred) was log transformed (Sokal and Rohlf, 1981). We confirmed homogeneity of variances by using Levene's test (Statsoft, 1996). Nonparametric test were used when parametric assumptions were not fulfilled.

RESULTS

Copulation duration

In experiment 1 copulations were significantly longer in the group with male-biased sex ratio ($F_{1,13} = 5.12$, $p = .041$; Table 1). Male weight was entered as covariate ($F_{1,13} = 5.86$, $p = .031$). There were no significant differences in male weight between groups differing in sex ratio. In addition, male weight was not correlated with copulation duration ($r = .41$, $p = .12$, $n = 16$).

There were no significant differences in female weight between groups in experiment 1 (*t* test; $t = -1.51$, df = 14, $p = .15$). Females did not show a consistent preference to copulate with the largest male in each group (two-tailed Fisher's Exact test, $p = .29$). In addition, in the groups where sex ratio was 2:1, mating males (Table 1; mean = 11.87 mg, SE = 0.35, min. = 10.33, max. = 13.15, $n = 9$) were not significantly larger than nonmating ones (mean = 11.49 mg, SE = 0.26, min. = 10.25, max. = 12.8, $n = 9$; $F_{1,16} = 0.76$; $p = .4$).

In experiment 2 copulations were also significantly longer in the male-biased sex ratio group ($F_{1,12} = 7.16$, $p = .02$; Table 1). Male weight was nonsignificant in the model ($F_{1,12} = 0.13$, $p = .72$). Male weight was not correlated with copulation duration in this experiment either ($r = .08$, $p = .78$, $n = 15$).

There were no significant differences in female weight between groups in experiment 2 (*t* test; $t = -1.7$, df = 13, $p = .11$). Largest males were not selected preferentially to mate (two-tailed Fisher's Exact test, $p = 1.0$). In addition, in the groups with sex ratio 3:1, mating males (Table 1; mean = 11.41 mg, SE = 0.55, min. = 9.1, max. = 13.7, $n = 7$) were not larger than nonmating ones (mean = 10.56 mg, SE = 0.45,

Table 1

Copulation duration and mating male weight in groups of virgin and nonvirgin females under different operational sex ratio (OSR) conditions

		Copula duration (h)				Male weight (mg)				
	OSR	Mean	SE	Min.	Max.	Mean	SE	Min.	Max.	*N*
Virgins	1:1	13.32	2.58	7.50	24.00	12.45	0.46	10.50	13.77	7
	2:1	18.67	2.67	8.00	32.00	11.87	0.35	10.33	13.15	9
Nonvirgins	1:1	13.61	2.10	4.00	22.00	10.85	0.41	9.00	12.90	8
	3:1	21.18	1.68	12.25	25.38	11.41	0.55	9.10	13.70	7

min. = 7.8, max. = 14, $n = 14$; $F_{1,6} = 2.61$; $p = .16$). In this case, differences were examined using a mixed model of variance analysis because it was necessary to control by female when analyzing differences in male size selection by females. We entered female identity as a random factor and male status (mating/nonmating) as a fixed factor (Statsoft, 1996).

As no differences were found in copulation duration depending on whether females were virgins (sex ratio 1:1, *t* test, $t = -0.10$, df = 13, $p = .92$; exp. 1, $n = 7$, exp. 2, $n = 8$), we pooled the data from the two experiments. When all females are analyzed together, the results show that copulations were significantly longer in the groups with male-biased sex ratios ($F_{2,27} = 4.11$, $p = .028$; sex ratio 1:1, $n = 15$, sex ratio 2:1, $n = 9$, sex ratio 3:1, $n = 7$; Figure 1 and Table 1). The mean copulation duration for all groups was 16.72 h (SE = 1.25, min. = 4 h, max. = 32 h). Male weight was nonsignificant in the model ($F_{1,27} = 1.35$, $p = .25$). Overall, neither male body weight ($r = .20$, $p = .287$, $n = 31$) nor female body weight were correlated with copulation duration ($r = .29$, $p = .11$, $n = 31$).

Sperm transfer

In some experiments there was no sperm transfer. These cases were removed from analyses because absence of sperm transfer occurred with similar frequency between treatments (mean percentage of copulations involving no sperm transfer = 30%).

In groups in which sex ratio was 1:1, the numbers of sperm inseminated increased with the duration of copulation ($F_{2,7} = 5.03$, $p = .044$; Figure 2 and Table 2). There were significant differences between the numbers of sperm transferred in copulations lasting 1 h and copulations lasting 12 h (Duncan's multiple range test, $p = .032$). Male size (hind tibia length) was entered as a covariate in the model ($F_{1,7} = 6.24$, $p = .041$; Table 2). There was no association between mating-male size and numbers of sperm transferred ($r = .44$, $p = .18$, $n = 11$).

In groups where sex ratio was 3:1, the mean number of sperm transferred in copulations lasting 12 h was greater than in copulations lasting 1 h, but the difference did not reach statistical significance, probably due to the small sample sizes and the large variation in sperm transfer observed in copulations lasting 12 h ($F_{1,4} = 0.73$, $p = .44$; Table 2). Male size was nonsignificant in the model ($F_{1,4} = 0.34$, $p = .59$). There was no association between mating-male size and ejaculate size ($r = -.55$, $p = .2$, $n = 7$).

A general model including sperm transfer in groups with sex ratio 1:1 and 3:1 was carried out. In this model, we excluded data from copulations lasting 6 h because they were only examined under equal sex ratio. The numbers of sperm inseminated in copulations lasting 12 h were significantly greater than those inseminated in copulations lasting 1 h ($F_{1,10} = 6.85$, $p = .026$). In addition, males inseminated more sperm in male-biased conditions (1:1 vs. 3:1; $F_{1,10} = 7.18$, $p = .023$), as shown in Table 2 and Figure 3. The interaction between copulation duration and sex ratio was nonsignificant ($F_{1,10} = 0.02$, $p = .89$). Male size was nonsignificant ($F_{1,10} = 0.37$, $p = .85$). There was no correlation between male size and number of sperm transferred ($r = -0.27$, $p = .33$, $n = 15$) or between female body weight and sperm transferred ($r = .0$, $p = .98$, $n = 14$).

Five females dissected in the peak of the reproductive period (assumed to have mated multiply in nature) contained an average of 476.6 sperm in the spermatheca (SE = 158.1, min. = 71, max. = 947). There were no differences between this number and the sperm in the spermatheca of females mated for 12 h in the groups with male-biased sex ratio (Mann-Whitney *U* test, $U = 7$, $p = .88$).

There were no significant differences in female weight between time treatments or in the groups of sex ratio 3:1 ($F_{1,5} = 2.87$, $p = .15$), or when all data were analyzed together (time effect, $F_{1,16} = 2.60$, $p = .13$; sex ratio effect, $F_{1,16} = 2.93$, $p = .11$; time × sex ratio, $F_{1,16} = 1.43$, $p = .25$).

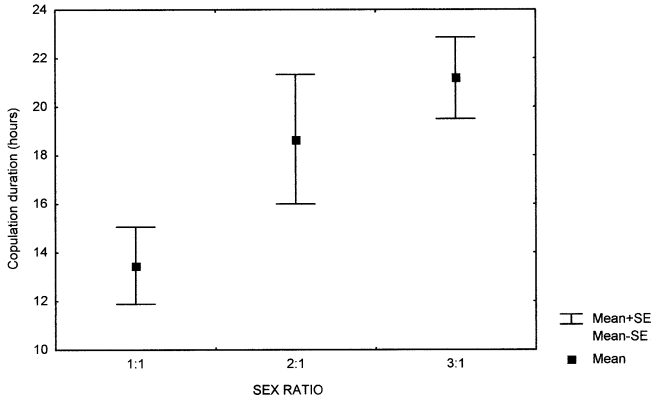

Figure 1
Mean copulation duration (and standard error) under different sex ratio conditions for experiment 1 (virgin females) and experiment 2 (nonvirgin females) together (see text for details).

DISCUSSION

Male golden egg bugs adjust copulation duration and rate of sperm transfer according to the perceived risk of sperm competition. Our results show that the presence of rivals prior and during mating lead to an increase in both (i) copulation duration and (ii) the number of sperm transferred per unit of time. To our knowledge this is the first study to show that males respond to an increase in the perceived risk of sperm competition by combining an increase in the rate of sperm transfer with an increase in the amount of time they spend engaged in sperm transfer. By using both mechanisms simultaneously males maximize the number of sperm transferred

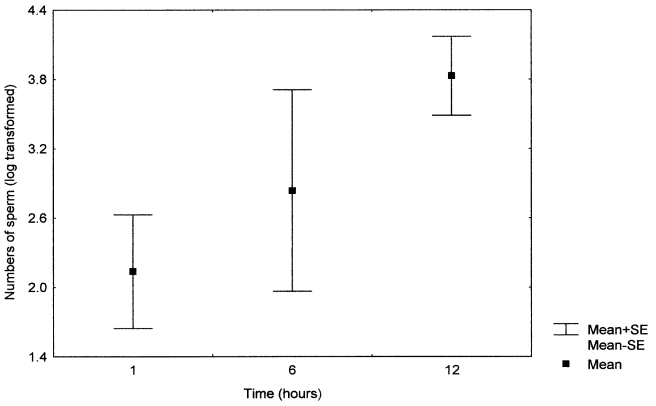

Figure 2
Numbers of sperm inseminated after copulations of 1, 6, or 12 h under a 1:1sex ratio.

and thus the possibilities of fertilization in a context in which sperm competition is likely. Thus, the results of this study support the prediction that males increase ejaculate expenditure when faced with a high risk of sperm competition and reveal a complex underlying mechanism, which has not been described previously.

Our results show that copulations are unusually long in this species; they last on average 16.7 h. Previous studies have found even longer copulations (mean = 23 h, range 3–53 h: Kaitala, 1998; mean = 32 h: Reguera, 1999). The experiments carried out in this study also show that copulations are longer when males are in the presence of other males (mean = 18.7 h in sex ratio 2:1; mean = 21.2 h in sex ratio 3:1) than when males are housed individually with a female (mean = 13.5 h). The fact that males tend to prolong copulations in the presence of rivals may explain the longer durations observed by Reguera (1999). Her experimental conditions included a male-biased sex ratio (2:1), and males were, in addition, previously housed in high-density conditions. Prolonged copulations may represent a mate-guarding strategy or may be a means by which males increase the amount of sperm transferred (see below); both hypotheses assume that copulation duration is under male control. Golden egg bug males try to force females to copulate, they have claspers with spines in the genitalia possibly having the function of female retention, and when pairs are in copula it is difficult to separate them because they remain firmly attached by the male genitalia. Thus, copulation duration seems to be mostly under male control, as in most heteropterans (Arnqvist, 1988; Arnqvist and Danielsson, 1999; Carroll, 1991; Sillén-Tullberg, 1981).

Our results show that longer copulations lead to an increase in the numbers of sperm inseminated. Contrary to other heteropterans (see below), sperm transfer occurs throughout copulation. However, males in the presence of rival males achieve an increase in the number of sperm inseminated not only by prolonging copulations, but also by increasing the rate of sperm transfer throughout copulation. While in-copula males inseminate a mean of 10 spermatozoa in the first hour when there is no risk of sperm competition, they inseminate a mean of 50 spermatozoa when other males are present. After 12 h, males transfer a mean of 54 sperm when housed with a female, whereas they transfer a mean of 856 sperm when housed with other males.

Sexual selection theory provides four different hypotheses to explain prolonged copulations in insects: (1) Sperm removal hypothesis (Siva-Jothy, 1987; Siva-Jothy and Tsubaki, 1989), (2) cryptic female-choice hypothesis (Eberhard, 1996), (3) in-copula guarding hypothesis (Alcock, 1994), and (4) sperm loading hypothesis (Dickinson, 1986; see Introduction for more details).

To our knowledge, sperm removal in Heteroptera has been never been documented. The sperm removal hypothesis seems unlikely in *P. laciniata* because longer copulations under high sperm competition risk also occur when males copulate with virgin females who have no sperm in the storage organs. Several indirect lines of evidence suggest that females have little control over the duration of copulation in this species: male genital morphology seems to indicate that males have the ability to retain females in copula, females show no preferences for males based on size (Reguera, 1999) or egg-carrying (Kaitala, 1998; Reguera, 1999), and there is no effect of male or female body weight on copulation duration.

Both mate guarding and sperm loading hypotheses predict longer copulations as sperm competition risk increases. Thus, to distinguish between these hypotheses, it is necessary to carry out detailed examinations of ejaculate transfer and/or sperm utilization and fertilization success (Simmons, 2001). A sole analysis of copulation duration in the golden egg bug would have supported the mate guarding hypotheses because copulations are longer when rivals are present, and most studies assume that such unusually long copulations are not needed to achieve high sperm transfer. Prolonged copulations are common in other species of Heteroptera (Carroll, 1991, 1993; Clark, 1988; McLain, 1980, 1989; Rubenstein, 1989; Sillén-Tullberg, 1981), and longer copulations in male biased sex ratios have been found in *Neacoryphus bicrucis*, *Lygaeus equestris*, *Jadera haematoloma*, *Gerris remigis*, and *Nezara viridula* (Carroll, 1991; Clark, 1988; McLain, 1980, 1989; Sillén-Tullberg, 1981). The mechanism of gradual sperm transfer observed in *P. laciniata* differs from that described in other heteropterans, where complete sperm transfer occurs only minutes after copulation is initiated (e.g., *L. equestris*: Sillén-Tullberg, 1981; *J. haematoloma*: Carroll, 1991). Prolonged copulations in these species thus seem to be a typical male postinsemination strategy to prevent subsequent matings by females, and the same occurs in *G. remigis* (Clark, 1988; but see below) and *N. bicrucis* (McLain, 1989). However, in *P. laciniata*, longer copulations lead to an increase in the number of sperm

Table 2
Ejaculate size along copulation duration in conditions of sex ratio 1:1 and 3:1

		Total number of sperm transferred				Male tibia size (10^{-4} m)				
	Time	Mean	SE	Min.	Max.	Mean	SE	Min.	Max.	*N*
Sex ratio 1:1	1 h	10.00	3.34	1	17	45.93	1.09	42.90	47.95	4
	6 h	27.33	12.99	2	45	43.82	1.57	40.68	45.58	3
	12 h	54.00	19.38	23	108	45.38	2.29	38.82	48.73	4
Sex ratio 3:1	1 h	50.00	13.83	29	88	45.96	2.55	41.70	52.71	4
	12 h	856.33	684.69	29	2215	40.26	1.67	36.95	42.30	3

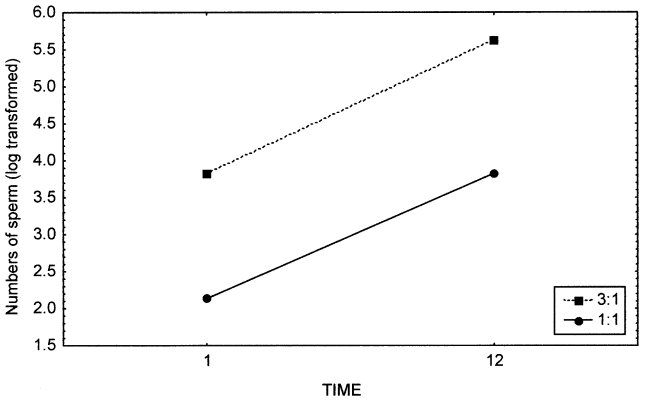

Figure 3
Mean numbers of sperm inseminated after copulations of 1 or 12 h under an equal or male biased (3:1) sex ratio.

inseminated because sperm transfer takes place throughout copulation, as has been shown to occur in *G. lateralis* (Arnqvist and Danielsson, 1999).

Simmons (2001) noted that in the studies of McLain (1980) and Rubenstein (1989) on *Nezara viridula* and *Gerris remigis*, respectively, prolonged copulations could also be interpreted as a way to increase the number of sperm transferred because copula duration is associated with fertilization success. In *N. viridula* prolonged copulation reduced the fertilization success of rival males that copulated subsequently, whereas in *G. remigis* the last male's fertilization success was dependent on his copulation duration (Simmons, 2001). The present study clearly reveals that in the golden egg bug, prolonged copulations are needed to increase the number of sperm transferred because sperm are inseminated continuously, and thus our results support the sperm-loading hypothesis.

This study shows that males adjust ejaculate expenditure to the risk of sperm competition (Ball and Parker, 1998; Parker, 1990a, 1998; Parker et al., 1997) and suggest that prolonged copulation and increased rate of sperm transfer in this insect have evolved by direct male-male competition for fertilizations. Other studies have shown that males respond to and increase in sperm competition risk adaptively (see Introduction; reviews by Parker et al., 1997; Simmons, 2001; Simmons and Siva-Jothy, 1998), but no other study has found a combined response of increasing the duration of copulation and sperm transfer rate. Production of high numbers of sperm is advantageous in sperm competition contexts when there is complete sperm mixing in the female's spermatheca, or when larger ejaculates are more effective at displacing sperm already stored by the female (Arnqvist and Danielsson, 1999; Dickinson, 1986; Eady, 1995; Parker, 1998; Parker and Simmons, 1991; Wedell and Cook, 1998). A knowledge of the mechanisms of sperm competition in the golden egg bug is needed to understand the adaptive significance of the variations in ejaculate expenditure. The fact that sperm are transferred at such a low rate seems to suggest that sperm displacement is unlikely because when it happens the last ejaculate needs to be large enough to physically displace previous sperm.

The golden egg bug spermatheca consists of a small, sac-shaped organ, the seminal receptacle or bulb, with a sclerotized channel and a spermathecal pump which separates the spermathecal duct from the sperm store. Multiply mated females store low sperm numbers in the spermatheca (around 477 spermatozoa), which is in accordance with the low numbers of sperm transferred by males. Thus, increased sperm transfer seems to be a male strategy to maximize fertilization success when sperm mixing takes place. Recent evidence shows that sperm precedence patterns support a mechanism of sperm mixing (García-González et al., 2003). Mean P_2 values (i.e., the proportion of eggs fathered by the second male to mate with a female) are around 0.5 in some heteropteran species (Economopoulus and Gordon, 1972; McLain, 1980, 1985). Other heteropterans show second-male advantage (Arnqvist, 1988; McLain, 1989; Sillén-Tullberg, 1981; Smith, 1979), although success of last males is highly variable in some of them (Carroll, 1991; Rubenstein, 1989; for a review, see Simmons and Siva-Jothy, 1998).

It is surprising that copulation duration is so long in a species with a cryptic phenotype, which has been most likely selected under strong predation pressure. Pairs in copula are likely to be detected more easily by predators and have impaired locomotory capacity (Kaitala and Axén, 2000; Reguera, 1999). The benefits of long copulations may be related to the slow rate at which sperm are transferred, which may require a long time to ensure that enough sperm are transferred to ensure fertilization. Males may also benefit from staying physically engaged with the female for long periods if in this way they maximize the chances of being close to the female when the next egg is laid (females lay one egg at a time several times a day throughout the breeding season) and, most important, they may maximize the chances that they father the next egg. Males often accept eggs after copulating with the females (García-González and Gomendio 2003), but they incur high costs because they are more vulnerable to predators when carrying eggs (Kaitala and Axén, 2000; Kaitala et al., 2000; Reguera and Gomendio, 1999). Long copulations may be the means by which males try to ensure paternity of the eggs they accept after copulating. It may be important for males to maximize the chances that the female lays the next egg soon after the end of copulation and that they are able to accept them on their backs because eggs laid on plants have very low chances of surviving (Reguera and Gomendio, 2002).

The fact that males respond to the presence of rivals by increasing both copulation duration and sperm transfer implies that sperm competition levels are not always high because otherwise males should maximize ejaculate expenditure on every occasion. It makes sense for males to increase ejaculate expenditure in the presence of other males if this situation is not the norm. This is in accordance with field data suggesting that population densities are low, and when males find females they are unlikely to be surrounded by other competitors. The fact that sperm are transferred slowly over long periods of time suggests that sperm mixing is taking place. The existence of this sperm competition mechanism implies that a greater proportion of sperm present in the sperm storage organs is the best way to maximize fertilization success, and this is precisely what males attempt to achieve by combining an increase in the rate of sperm transfer with the time devoted to it. A large numerical representation could be particularly important in determining fertilization success when so few sperm are stored by the female, as occurs in this species.

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