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Fertility and paternity in the Eucalyptus snout-beetle *Gonipterus scutellatus*: females might benefit from sperm mixing

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Mating in *Gonipterus scutellatus* is very long and lasts on average 6.98 ± 0.49 hr. This could be interpreted as a mechanism of male guarding, but males neither increase copulation duration nor ejaculate volume when sperm competition risk increases. We studied paternity patterns using RAPDs, and the influence of multiple matings on fertility, fecundity and sperm supply. Using twice-mated females, we compared the percentage of polymorphic bands shared between first male/offspring, second male/offspring, mother/offspring, unrelated adults (males). In agreement with a previous study on paternity with the sterile-male technique, we found that sperm mixing seems the prevalent sperm competition mechanism. In a second experiment, adults were randomly assigned to three treatments: females mated only once; females allowed to mate ad libitum with one male; and females that could mate with seven different males. Females of the last group showed a significant increase in fertility. Additionally, we compared the stored sperm volumes among four groups: female whose copulation was interrupted after 15 min, those that mated once, twice or three times. This experiment indicates that sperm supply increases with the number of matings, excluding the possibility of sperm removal. These facts and the occurrence of sperm mixing might explain the lack of male response to increased male density in previous studies.

KEY WORDS: Curculionidae, fecundity, multiple matings, P_2, RAPD, sperm competition.

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INTRODUCTION

Most female insects experience multiple matings during their reproductive cycle and store sperm masses in special organs, creating the opportunity for inter-male ejaculate competition (PARKER 1970). Consequently, sexual selection does not end after mating. Since PARKER's (1970) influential paper on the evolutionary consequences of sperm competition in insects, many studies have shown that sexual selection has favoured the evolution of complex mechanisms that increase last-male paternity success (usually measured as the proportion of eggs fertilized by the last of two males or P_2). Two recent reviews of sperm competition studies in insects (SIMMONS & SIVA-JOTHY 1998, SIMMONS 2001) indicate that last-male sperm precedence is common in many species, but variance among males in paternity success is usually very large. This fact has led some authors to propose that part of the variation in paternity success might be mediated by females through cryptic female choice (THORNHILL 1984, EBERHARD 1996).

Nevertheless, the existence of cryptic female choice has generated an intense debate (BIRKHEAD 1998, 2000; EBERHARD 1998, 2000). Of special significance is the possibility of sperm selection by female's genitalic environment (MILLER & PITNICK 2002), but many other behavioural mechanisms might allow females to bias the paternity success of their mates (EBERHARD 1996, WARD 2000, NILSSON et al. 2003).

Part of this debate is due to the difficulty of correctly separating male and female roles in postcopulatory mechanisms (ANDRÉS & ARNQVIST 2001). The reproductive behaviour of the *Eucalyptus* snout-beetle *Gonipterus scutellatus* (Coleoptera Curculionidae), a worldwide eucalypt pest (SANTOLAMAZZA CARBONE 2002), is a clear example of the difficulties of differentiating between sperm competition and cryptic female choice mechanisms. This species has a long-lasting mating association, on average 6.98 ± 0.49 hr (n = 75), that can last up to 55 hr (see SANTOLAMAZZA CARBONE 2002 for details). In other insects it has been shown that mating duration and sperm transfer vary in accordance with sperm competition theory (MARTIN & HOSKEN 2002). Although the *G. scutellatus* female fits all the requisites for sperm competition to occur, male population density (and consequently sperm competition risk) did not affect copulation duration or ejaculate volume, and longer matings did not result in greater ejaculate transmission (SANTOLAMAZZA CARBONE & CORDERO RIVERA 1998). Using the sterile male technique (males were exposed to gamma rays) with reciprocal matings, we found that the proportion of offspring fathered by the second male to mate (BOORMAN & PARKER 1976) shows a high variability, suggesting sperm mixing and a role for cryptic female choice (SANTOLAMAZZA CARBONE & CORDERO RIVERA 1998). Nevertheless, the sperm of sterilized males could be less competitive than that of normal males, so that a more powerful test of the sperm competition hypothesis is needed. To this end we studied paternity in this species by using the RAPD fingerprinting technique. The random amplified polymorphic DNA (RAPD) technique has been used to address issues of paternity, especially in Odonates (HADRYS et al. 1993, HADRYS 1994, SIVA-JOTHY & HOOPER 1995, HOOPER & SIVA-JOTHY 1996). The main advantages of the RAPD technology include the possibility to work on unknown genomes, the applicability to nanogram quantities of DNA, efficiency and low cost (HADRYS et al. 1992). Furthermore, we investigated whether females might benefit from multiple matings (ARNQVIST & NILSSON 2000), either because sperm might be limiting for this long-lived insect (SANTOLAMAZZA CARBONE 2002), or because genetic diversity increases fecundity or fertility.

METHODS

RAPD analyses of paternity

The experiment was performed with 43 virgin females and 40 virgin males of *G. scutellatus*, 1-month old, reared in laboratory from egg capsules collected in the field. The adults were individually marked on the elytra with permanent ink using a number code and a colour spot to distinguish more easily males from females. Sexes were initially separated in two different plastic boxes (1 litre), and provided with fresh *Eucalyptus globulus* leaves. To avoid any possible confusion during the observations, we introduced only a few females (5-6) each day in the box containing the males. When the pairs were naturally formed, we gently removed the leaf where they were perched, and introduced each pair in a separated small box (100 ml). Copulating pairs were checked every 30-60 min (in a few cases also during the night) until the two adults separated (SANTOLAMAZZA CARBONE & CORDERO RIVERA 1998). At the end of the first copulation, we noted male's and female's codes, but we kept away temporarily the mated males from the pool of males inside the box, to avoid the possibility that their mates could copulate twice with them. When the first group of females obtained two matings, we reintroduced all the males in the pool box. In some cases the same male mated with two different females. During night hours, all the males and females which were not copulating, were separated until the next day. In this way we were sure that each female was mated only by two different males.

We obtained 15 double matings, using 25 males and 15 females, but due to bad amplification in two cases, only 13 families (1 female + 2 males + 5 offspring) were used in the analysis. After mating every female was kept alone for 5 days in a plastic box (100 ml) with fresh *E. globulus* leaves to favour oviposition. The egg capsules were opened and the eggs placed in a Petri dish over a wet filter paper. For each female we randomly selected 50 eggs (10 every day) that developed at room temperature (20 °C, 70% relative humidity) for 6 days, to increase the amount of DNA. The eggs were then individually preserved in a marked sterile vial at – 20 °C. From each female we analyzed 5 eggs, one from each day of oviposition. Males and females were killed by freezing and stored at – 20 °C.

Fresh tissue samples consisted of thoracic muscle from putative fathers and known mothers, as well as the embryos in the eggs. DNA extraction and purification followed a procedure for DNA extraction for Odonates (H. HADRYS personal communication) to which some modifications have been added until a satisfactory result was obtainded with *G. scutellatus* adults and eggs. The DNA lysis buffer contained: 100 mM Tris HCl pH 8, 10 mM EDTA, 100 mM NaCl, 0.5% SDS, 50 mM DTT, 0.5 ml RNAse and 25 ml Proteinase K. Tubes were maintained for 2 hr at 40 °C in a bath. Purification was carried out firstly with the standard phenol/chloroform procedure (SAMBROOK et al. 1989), followed by a chloroform/2% isoamylalcohol procedure.

DNA concentrations were measured in a 1% agarose mini-gel stained with ethidium bromide, under a UV illumination, by comparison with a standard concentration of Fago-lambda DNA (Sigma-Aldrich Inc.) (SAMBROOK et al. 1989) and finally standardized to 50 ng/ml aqueous solution.

To run the PCR reaction we used the PCR kit Beads Ready-to-go (Amersham Pharmacia Biotech Inc.) adding 25 ml of a solution composed of 50 ng target DNA, 5 pmol primer (5 pmol/ml) (Operon Technologies Inc., primers OPA01, OPA10 or OPA11, see Table 1) and 23 ml distilled sterilized water. All the samples within a PCR run were maintained for 5 min at 94 °C, followed by 45 cycles of 1 min at 94 °C, 1 min at 35 °C and 2 min at 72 °C on a Eppendorf thermal cycler. The process concluded with a period of 7 min at 72 °C.

The PCR products were analyzed by electrophoresis (50 v, for 6 hr at room temperature) in 1.5% agarose gel stained with ethidium bromide and covered with TAE 1 ×. Size standards (PCR Marker, Sigma-Aldrich Inc.) were run in the peripheral lane. Finally, the stained gel was exposed to UV illuminator and photographed with a Polaroid GelCam 0.4 ×.

Because RAPD markers usually segregate as dominant Mendelian alleles (PELLISSIER SCOTT & WILLIAMS 1998) the presence of a given marker in the band pattern of two individuals

Table 1.

Primers used in the experiment and band patterns.

Primer	Sequence	Band patterns	Polymorphic markers
OPA01	5'-CAGGCCCTTC-3'	13	5
OPA10	5'-GTGATCGCAG-3'	16	10
OPA11	5'-CAATCGCCGT-3'	16	11
Total		45	26

indicates that both individuals share the allele at that locus. Moreover, the absence of a band represents the recessive phenotype at that locus, and should therefore also be scored in the analysis of genetic similarity. For each family/primer, we ran two independent repetitions of the PCR-RAPD reaction. Only bands that amplified in both repetitions were used in the analyses. The proportion of matches, M (the shared presence and the shared absence of a band) is estimated from: M = N*ab*/Nt, where N*ab* is the total number of matches in individuals *a* and *b*, and Nt is the total number of bands scored for each primer (HOOPER & SIVA-JOTHY 1996). Individuals are identical when M = 1 (i.e. they have identical banding pattern), while they do not share any bands when M = 0. The presence/absence of bands was scored on the photographs of the gels.

We compared the following groups: first male/offspring, second male/offspring, mother/offspring, unrelated adults (all the males). For each individual the number of polymorphic markers was increased by using the polymorphic bands obtained from all the primers used. Analyses were done with GenStat 6.0 (GENSTAT 2000) and xlStat 4.3 (www.xlstat.com).

Fecundity and fertility

To investigate if females benefit from multiple matings we used virgin laboratory-reared individuals of *G. scutellatus*. Adults were randomly assigned to three treatments (n = 20 males and 20 females per treatment). In treatment A, females were mated with one male that they had previously chosen in a box containing 20 males, and that was removed immediately after copulation (sperm- and genetic variability-limited females). In treatment B, females were allowed to mate with the male that they had chosen, but that in this case was maintained with his mate throughout the 30 days of duration of the experiment (genetic variability-limited females). Finally, treatment C females had the opportunity to mate with seven different males through the experiment (sperm- and genetic variability-unlimited females). These females received only one different male every 3 days. The same 20 males were rotated between females, but they visited each female only once.

All the experimental pairs were maintained in 100 ml plastic boxes and received fresh leaves of *E. globulus* as food and oviposition substrate. Sample sizes were reduced to 12, 18 and 15 females because some individuals died or laid less than 31 eggs during the experiment, and were excluded from the analysis.

Treatment A females were continuously observed in their first mating (including during the night hours), and the male removed immediately after copulation. Females of treatment B and C were observed twice daily and twice during the night and the number of copulations recorded. All adults were measured to the nearest 0.01 mm with a digital caliper before the start of the experiment. Females had the opportunity to lay eggs during 1 month. Eggs were introduced individually in a marked sterile vial (1 × 6 cm) and incubated at room temperature (23 °C, 70% relative humidity). They were checked daily during 2 weeks, and the number of larvae that emerged was noted. Afterward, egg masses were dissected, recording the number of sterile eggs and dead larvae. Fecundity was calculated as the number of eggs produced (including sterile eggs). Fully developed larvae that died inside the egg mass were counted as fertile

eggs, because this was likely to be due to the laboratory environment (the egg mass could experience a loss of humidity that could difficult larval eclosion).

To analyze the results of this experiment we used a Generalized Linear Mixed Model (GLMM), with treatment as the fixed effect and female body length as a random variable. Male body length was not included in these models because group C included several males per female. The error structure was modeled as following a Poisson distribution in the case of fecundity, and as a binomial distribution in the case of number of sterile eggs (with the total number of eggs laid as the binomial denominator). The link function was logarithm for fecundity and logit for fertility, and the dispersion parameter was estimated from the data. These analyses were done with Genstat 6.0 (GENSTAT 2000).

Number of matings and sperm volume

We compared the volume of sperm stored in the spermatheca of 84 laboratory-reared females that were randomly assigned to four treatments: (1) once mated females (n = 21); (2) twice mated females (n = 21); (3) females that mated 3 times (n = 21); and (4) females whose first mating was interrupted after 15 min of intromission (n = 21). Some females failed to mate and this reduced sample size to 9, 11, 21 and 6. We predicted that if the last male to mate could remove rivals' sperm, or the spermatheca is filled with just one ejaculate, then in treatments 1, 2 and 3 we should found approximately the same volume of sperm in the spermatheca. Treatment 4 allowed us to assess (i) if males use the first minutes of copulation to inseminate or to remove rivals' sperm, (ii) if sperm is transferred immediately to the spermatheca, and (iii) if sperm fills it completely or partially.

All females were preserved in 70% ethanol until dissection. To estimate sperm volume we extracted the sperm mass from the spermatheca and measured the area of the mass compressed to a uniform thickness (see CORDERO & MILLER 1992 for details of the method).

Table 2.

Probability of sharing bands between males and the offspring of each family.

Family	1st male	2nd male
1	0.82	0.59
2	0.68	0.74
3	0.74	0.33
4	0.80	0.62
5	0.57	0.71
6	0.63	0.62
7	0.72	0.74
8	0.88	0.94
9	0.78	0.91
10	0.70	0.51
11	0.78	0.71
12	0.80	0.71
13	0.71	0.72
Mean	0.74	0.68
SE	0.02	0.04

Table 3.

Proportion of shared bands in the paternity experiment. The first column shows the range of probability of sharing bands. The remaining columns indicate the number of cases included in each range of shared bands.

Range	1st male/offspring	2nd male/offspring	Unrelated adults	Mother/ offspring
< 0.4	0	1	3	0
< 0.5	0	0	3	0
< 0.6	1	2	3	0
< 0.7	3	2	2	2
< 0.8	7	6	1	5
< 0.9	2	0	1	1
< 1.0	0	2	0	0

RESULTS

RAPD analyses of paternity

The proportion of shared bands between both males and the offspring is presented in Table 2. In some cases (i.e. families 1, 3, 4) the first male had greater success, while in other cases (families 5, 9) the last male apparently fertilized more eggs. However, there are no differences in the proportion of bands shared between both males and the offspring (ANOVA on arcsin-square root transformed data, $F_{1,24} = 0.891$, $P = 0.332$).

The proportion of shared bands in all families and among males is presented in Table 3. In agreement with the previous analysis, the first and second male shared a similar proportion of bands with the offspring (comparison of columns 2 and 3 in Table 3; $\chi^2_6 = 5.61$, $P = 0.468$). There was no difference between the proportion of bands shared by mother/offspring and males/offspring (comparison of the sum of the columns 2 and 3 with the column 5; $\chi^2_6 = 2.29$, $P = 0.892$). Nevertheless, unrelated adults (the males used for the experiment) shared less bands than males/offspring (comparison of the sum of the columns 2 and 3 with the column 4; $\chi^2_6 = 15.27$, $P = 0.018$).

Fecundity and fertility

Fig. 1 shows the number of eggs laid and percentage sterility by treatment in relation to female size. The minimum number of matings of females from treatments B and C was respectively 2.44 ± 0.39 (± SE) and 2.68 ± 0.32, but while the first group mated with only one male, the second group mated at least with an average of 2.26 ± 0.21 males. Preliminary analyses (Fig. 1) indicated a positive effect of female body length on fecundity and fertility. This variable was therefore included as a random term in the model. The mean number of eggs laid (± SE) during the experiment does not differ between treatments (A: 141.3 ± 9.4; B: 220.7 ± 23.6; C: 187.6 ± 27.4; GLMM, Wald test = 3.49, 2 df, $P = 0.175$).

The percentage of sterile eggs was 4.80 ± 1.09 (± SE) for treatment A, 4.58 ± 0.67 for treatment B and 3.99 ± 0.69 for treatment C. In this case the analysis indicates significant differences between treatments (Wald test = 7.57, 2 df, P = 0.023). Back-transformed means estimated with the GLMM procedure, after accounting for the effect of female body length, indicate a 2% decrease in sterility in treatment C (A: 5.68, B: 5.23, C: 3.16%).

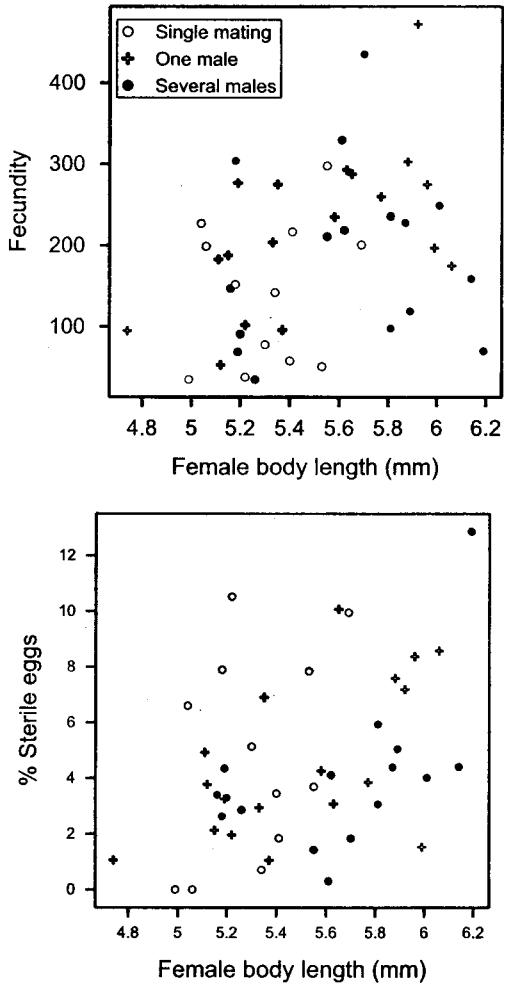

Fig. 1. — The relationship between female size, fecundity and fertility in the three treatments (A, single mating; B, one male = several matings with one male; C, several males). Fecundity does not differ between treatments (eggs laid: A: 141.3 ± 9.4; B: 220.7 ± 23.6; C: 187.6 ± 27.4; GLMM, Wald test = 3.49, 2 df, P = 0.175), but group C females are significantly more fertile (Wald test = 7.57, 2 df, P = 0.023).

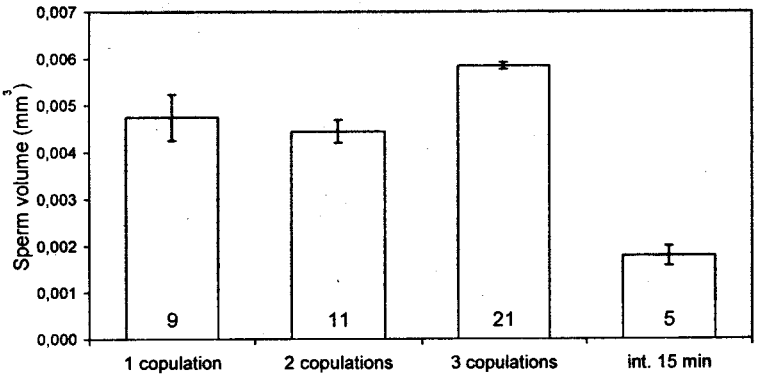

Fig. 2. — The volume of sperm of females mated 1-3 times and interrupted after 15 min of their first copulation. Numbers indicate sample size and lines are SE. Sperm volume increases with the number of matings (differences between treatments: $F_{2,38} = 12.28$, $P < 0.0001$), but there is no difference between sperm volume of the females interrupted and the females mated once (ANOVA, $F_{1,13} = 3.24$, $P = 0.095$), although this is close to significance.

Number of matings and sperm volume

There were differences in sperm volume between treatments (Fig. 2). We planned a priori the following comparisons: once mated females versus females interrupted after 15 min of copulation (ANOVA, $F_{1,13} = 3.24$, $P = 0.095$) and females mated 1-3 times ($F_{2,38} = 12.28$, $P < 0.0001$).

DISCUSSION

P_2 values are often used to infer the mechanisms that underlie the pattern of sperm use by the female and the mechanisms of sperm competition: intermediate values of P_2 usually indicate sperm mixing that lead to intense sperm competition, while high values of P_2 indicate last-male sperm precedence, sometimes due to sperm displacement by the second male (SIMMONS & SIVA-JOTHY 1998). The experiment with molecular markers suggests that both males had a similar success in egg fertilization. A previous experiment estimated P_2 using sterilized males of *G. scutellatus* and suggested that even if the second male seems to fertilize the largest amount of eggs, the female still has the possibility of using the first male sperm (SANTOLAMAZZA CARBONE & CORDERO RIVERA 1998). The main problem we found in the experiment of P_2 with genetic markers is the high percentage of shared bands among unrelated adults, which suggests that all the members of this introduced population are genetically very similar. This clearly diminished our ability to detect differences in paternity. The Galician population of *G. scutellatus* probably has been generated from a few founders introduced accidentally in 1991 (MANSILLA 1992). Nevertheless, the number of diagnostic bands has allowed us to obtain some

information about the reproductive success of the two males. The results agree with those of the previous irradiation experiment (SANTOLAMAZZA CARBONE & CORDERO RIVERA 1998), again suggesting a mixed paternity.

The alternate use of sperm masses of different origin is a frequent phenomenon in insects (EBERHARD 1996). An exhaustive review of experiments of P_2 (SIMMONS & SIVA-JOTHY 1998) based on 109 species belonging to 10 different orders of insects, has shown that in 45% of the cases the second male obtains some advantage in paternity, while only in 17% of the cases did he obtain complete paternity. The cases of a clear advantage for the first male are comparatively rare. It is clear that a high variance of P_2 is the norm and not the exception in insects.

The time available for sperm mixing depends on the interval between copulation and egg fertilization (SIMMONS 2001). If females oviposit immediately after mating, it is possible that a reduction in fertilization success of the last male does not occur, but if the time interval is large (which is the case of *G. scutellatus*), sperm mixing takes place, and optimal copulation duration should allow the male to inseminate a larger quantity of sperm.

There are three factors that can influence variance in P_2: the time lapsed between the two matings, between copulation and egg fertilization and the quantity of sperm transferred to the female. The time factor can be positive or negative for the increment of P_2. The sperm need some time to reach the spermatheca, and if the second mating takes place too soon, the first male's sperm will be diluted by the second male's sperm mass. In this sense any male's ability to retard future matings of the female (male guarding) is clearly favoured by sexual selection (ALCOCK 1994). In a broad sense, the reproductive behaviour of *G. scutellatus* is similar to other beetle species (EBERHARD 1993, EBERHARD & KAKIRO 1996, DICKINSON 1997). Nevertheless, male behaviour is especially interesting because some features suggest it is unlikely that the long copulation duration (up to 55 hr) is a case of guarding: (1) males neither increase copulation duration, nor sperm volume when the risk of sperm competition increases (SANTOLAMAZZA CARBONE & CORDERO RIVERA 1998), (2) females do not oviposit immediately after mating (SANTOLAMAZZA CARBONE 2002), and (3) the last male does not father the most offspring. It is known that most mating strategies are strongly dependent on population density (ANDERSSON 1994). *G. scutellatus* has reached a pest status in all the countries where it has been introduced, while in its country of origin it is a rare insect (TOOKE 1955). Consequently, it is possible that this species has not yet evolved those sexual selection strategies typical of high density populations. Moreover, at the moment we can not discuss any possible role for strategic ejaculation (MARTIN & HOSKEN 2002, WEDEL et al. 2002), because all the females employed in the experiment had the same age and the same mating status.

In a previous experiment (SANTOLAMAZZA CARBONE & CORDERO RIVERA 1998) we found that mating behaviour exhibited passive phases (sensu PARKER 1970) and clear syn- and post-copulatory courtship (sensu EBERHARD 1994), such as the male's leg rubbing on the female elytra and male-thorax/female-elytra rubbing (SANTOLAMAZZA CARBONE & CORDERO RIVERA 1998), which calls for an interpretation of the male behaviour in the light of the cryptic female choice mechanism. Nevertheless, careful experiments are needed to disentangle this mechanism from sperm competition.

There are two independent mechanisms that could benefit multiple-mated females by elevating fertility or embryo survival: the "good sperm" hypothesis (YASUI 1997) and the genetic incompatibility hypothesis (ZEH & ZEH 1996). In the former hypothesis, vigorous males possessing "good genes" fertilize more successfully than non-vigorous rival males in sperm competition and father vigorous off-

spring. In the latter hypothesis, assuming that females have a mechanism that allows them to bias fertilization towards compatible mates, genetically compatible males within the mates of a polyandrous female fertilize a greater number of eggs and it results in a lower proportion of sterile eggs. In our study, sperm competition between different males could occur only in treatment C (multi-male treatment) and the problem of genetic incompatibility was possible in treatments A and B (single-male treatment). It is possible that using the same males for the group C females could have determined a temporal sperm depletion, as it occurs in other coleopterans (MBATA et al. 1997), but precisely to avoid this problem we left every male 3 days with his mate.

Our results indicate that female *G. scutellatus* might benefit from multiple matings and sperm mixing by increasing fertility and sperm volume but not fecundity. Increasing fertility should be especially relevant in a population where genetic variability seems very low. The difference in fertility is quite small (2%), and therefore it is difficult to judge its biological significance. Nevertheless, this effect was detected in a short experiment (1 month), and is likely to increase if females live longer (ARNQVIST & NILSSON 2000) (this species may live up to 2 years in the laboratory; MANSILLA 2001). Data in Fig. 1 also suggest that females of treatment C had lower variability in sterility (with one exception), a result consistent with the genetic compatibility hypothesis. Further experiments are needed to confirm the effect of multiple matings and genetic variability of sperm on female fitness, as well as the existence of possible costs of multiple matings (CRUDGINGTON & SIVA-JOTHY 2000, BLANCKENHORN et al. 2002).

The results of the experiment on sperm volume show that after 15 min from the beginning of copulation the sperm has almost filled the spermatheca, suggesting that males start insemination immediately. On the other hand, the increase in sperm volume shows that the last male can not remove, dilute or displace the first male's sperm, as occurs in other insects (SIMMONS 2001). This result was expected, considering that the anatomy of the genitalia excludes the possibility that the aedeagus of the male reaches inside the female's spermatheca (SANTOLAMAZZA CARBONE & CORDERO RIVERA 1998).

In conclusion, our study shows that sperm mixing seems to be the current strategy adopted by females, which indirectly benefit from multiple matings by increasing the proportion of fertile eggs. It would be interesting to know if the same behaviour is observed under lower densities in the country of origin (SE Australia), where *G. scutellatus* is a rare species (TOOKE 1955).

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