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ORIGINAL ARTICLE

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Sperm competition and evidence of sperm fertilization patterns in the carrion ball-roller beetle *Canthon cyanellus cyanellus* LeConte (Scarabaeidae: Scarabaeinae)

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Abstract Field observation and laboratory experiments were conducted to investigate sperm competition and patterns of sperm fertilization under different experimental conditions in the carrion ball-roller beetle *Canthon cyanellus cyanellus*. Males in nature can mate with females whose spermathecae contains fertile sperm from other males. Sperm precedence was investigated using a visible genetic marker. The progeny of red (homozygous recessive) virgin females mated once with a red male and afterward, once with a green beetle (homozygous dominant) and vice versa, revealed that regardless of its color**,** the last male to mate fertilized c.a. 50% of the eggs, suggesting strong sperm competition. Males were able to achieve higher levels of paternity (more than 80%) when mated *ad libitum* with previously mated females, although large amount of variance in paternity does not exclude the possibility of first male sperm precedence or female cryptic choice. These results suggest that repeated mating and sperm replacement are the mechanisms by which last males achieve sperm precedence.

Keywords *Canthon cyanellus* · Scarabaeidae · Sperm competition · Genetic marker · Fertilization patterns

Introduction

Sperm competition, a special form of sexual selection, has been defined by Parker (1970) as competition among the ejaculations of two or more males for the fertilization of a female's ova. Multiple matings and sperm storage in insects have favored the mixing and preservation of sperm, promoting sperm competition (Parker 1984; Harvey and May 1989). Sperm used in successful fertilization in insects range from first male to last male sperm precedence, with examples of some degree of sperm mixing, yet frequently the sperm of the last male achieves more than 50% of fertilization (Thornhill and Alcock 1983; Simmons and Siva-Jothy 1998). Thus, how a male assures his own paternity has been one of the most analyzed and discussed questions since Parker's review was published. In species with high paternal investment, males should have evolved adaptations to reduce sperm competition (Trivers 1972), but that would not eliminate the possibility that a female might choose sperm from one male regardless of mating sequence (Eberhard 1996).

Paternity assurance is also related to the mating system (Whittingham et al. 1992). Monogamy is uncommon in insects, but male-female nesting pairs are the rule in ball roller scarabs of the subfamily Scarabaeinae (Halffter and Edmonds 1982). Males invest a considerable amount of time and energy in offspring, playing an active roll in searching for food, cutting it, and rolling a brood ball to the nest site. Males fight fiercely for a ball and a female, suggesting a substantial male parental investment. In contrast, females of most species have a passive role during these activities, and are transported on the ball to the nest by the male (but see Sato and Imamori 1987). However, in several species females that are transported to the nest by males have been found with their spermathecae filled with viable sperm, presumably from other males (Edwards and Aschenborn 1988; Favila and Díaz 1996; Halffter 1997). This suggests that sperm competition can occur in ball roller beetles, affecting other aspects of their reproductive behaviour.

In this paper we present field evidence of potential sperm competition and laboratory data on paternity after double and *ad libitum* mating in *Canthon cyanllus cyanellus*, a subsocial ball roller beetle in which the male participates with the female in brood care (Halffter et al. 1983; Favila 1993, 2001a). To our knowledge, this is the first examination

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of sperm competition in ball roller beetles (Scarabaeinae: Scarabaeini).

Methods

Females previously mated in the field

Field work was done in the tropical rain forest of UNAM's "Los Tuxtlas" biological research station in southern Veracruz, Mexico (95°04′ W, 18°34′ N). Fish pieces were laid on the ground to capture individual scarabs. Each captured female was placed in a plastic box (9 × 9 × 8 cm, called a nesting box) containing 2.5 cm of moist soil and a piece of fish suitable for reproduction (ca. 5 g). Nesting boxes were maintained in a rearing room at 27 ± 1°C, 70 ± 10% RH, and a photoperiod of 12:12 (L:D) h. Females were fed fish every other day for a period of 70 days, daily checks determined whether or not brood balls had been constructed. Brood balls made by each female were separated into plastic boxes with moist soil, and the adults that emerged from them were counted and sexed. Females that did not begin to make brood balls after 70 days were dissected to check spermathecal contents and ovarian development under a microscope. We only separated females on the basis of presence/absence of motile sperm, however, when we did find sperm, it was abundant. Ovarian development was classified in 3 categories according the Tyndale-Biscoe criterion (1984): immature, mature, and old. Ovarian stage was ranked in each of these categories considering: presence-absence of oocytes in vitellogenesis, length and width of the basal ova, and presence of *corpora lutae* (op. cit.). The presence of stored fertile sperm in females was considered if an adult had eclosed from its brood ball or if motile sperm was present in the spermatheca (Müller and Eggert 1989).

Sperm precedence after double mating

A visible genetic marker was used to investigate sperm precedence in this species. The cuticular color of the genetic marker is red, and it behaves as a homozygous recessive character with regard to the wild green color (Favila et al. 2000). Thus, when a female of recessive genotype mates with males of different genotypes, the offspring will exhibit the father's phenotype (see Bartlett et al. 1968; Müller and Eggert 1989; Trumbo and Fiore 1991). Both red and green beetles were raised simultaneously in the rearing room. The rearing method was the same as that of Favila (1993). Emerged adults were maintained in plastic containers (40 × 25 × 20 cm) with soil and food until the beginning of the experiments. The age of the experimental beetles ranged from 15 to 25 days old, the optimal reproductive age in this species (Favila 1993). Only homozygous insects were used because results are easier to interpret than those of experiments that also involve heterozygous parents (de Jong et al. 1993).

We used the reciprocal double mating method to determine sperm competition between two males (Boorman and Parker 1976). Virgin red females were mated once, either to a red male (Mr) or to a green wild-type male (Mg), and later, once to a male of the alternate genotype. In both cases, male-female pairs were randomly selected and each one was placed in an observation arena (10 × 2 cm Petri dish with filter paper as floor support and a 2 g fish flesh ball). Daily observations were made continually from 9:00 to 18:00 h, since the beetles are diurnal (Favila and Diaz 1996). Mating time (in minutes) was registered as the total intromission time (from the moment the male introduced the aedeagus into the female tract until he extruded it). Time until mating was measured as the elapsed time (in days) between the pair's placement in the arena and the beginning of mating. Unmated pairs were separated and each beetle was maintained in a plastic box with food; the following day, unmated male-female pairs were placed in observation arenas and their food ball was replaced. This protocol was repeated until mating occurred. Over the course of 10 days, unmated females were removed from the experiment. Once the first mating had concluded, each female was placed in a nesting box with 2 g of food and the next day placed with the second male in an observation arena; mating was recorded as described above. Brood balls made by each female between the first and the second mating were separated and placed in a box. Females mated by the second male were immediately placed in other nesting boxes to avoid confusion with brood balls from the first mating. These females were fed daily and brood ball production was followed for 40 days, the most active period in the reproductive life of a female under laboratory conditions. After this period, females tend to produce brood balls sporadically and offspring survival is low (Favila 1993). Each adult that emerged from a brood ball was sexed and assigned to the corresponding first or last male by its cuticular color.

The males used in this and all subsequent experiments were remated with virgin females of their own phenotype (green or red) to check their fertility and whether they were homozygous or heterozygous. Each pair was maintained together over the female's life span; no heterozygous specimens were found, and all the males were reproductively successful.

Ad libitum mating by second males

As there were no significant differences between the fertilization ability of the two morphs (see results), we made one sequence to analyze the paternity of the last male when mated *ad libitum* to a previously mated female. Using the previously mentioned method, the first mating was obtained between a red female and a red male, after which the female was mated with a green male. After the second mating, each female was placed in a nesting box with the second male. Male-female pairs were fed 3 g of fish every other day and brood ball production was followed for 40 days, although males were removed from the nesting box after 10 days. As in the last assay, each adult emerged was sexed and its color determined.

Male and female behaviour during nesting

To investigate male behaviour with a previously mated female during the nesting period, we repeated the last protocol described herein. After the second mating, the pair was put into a plastic container (15 cm diameter × 5 cm high) filled 3/4 with sterilized damp soil and 4 g of fish flesh. Each container was put inside a plastic box (40 × 30 × 14 cm), covered with transparent plexiglass. This device allows us to observe male and female behaviour during cutting, rolling and brood ball construction without disturbing their behaviour (see Favila 2001a). A mating takes nearly 50 minutes in this species (Favila 2001a). Thus, to determine the frequency of all mating during the observation period, every 20 min we scanned the behaviour of each pair until the construction of the third brood ball (120 to 168 h). Night observations were made using red light to avoid disturbance reactions of the male-female pair. A one factor repeated measures ANOVA was used, considering the number of matings before the construction of each brood ball as the within subject or repeated measured, and brood ball construction as the repeated factor. Data were transformed to ranks.

Results

Female sperm storage in the field

Of the 115 females captured, 105 (ca. 90%) made at least one brood ball. Of the 10 females opened, five were mature and had no sperm, while the other five had abundant motile sperm. From the latter, four were classified as mature according to ova stage and one as old. If we add the four mature females to those that made brood balls, 94% of all females had viable sperm in their spermathecae. Thus, it appears that a male has a good chance of beginning to nest with a female that has viable sperm stored in her spermatheca. Females made 738 brood balls (median 7 brood balls per female, range 1–13 brood balls), and overall offspring survival was 72.36%. Females began to make brood balls 3 days after being placed in the nesting boxes and continued to do so for a period of 64 days (median 5 days). During the first 5 days, 68.6% of the females began to make brood balls; 90% were still doing so 15 day after being left in the nesting boxes. The last 10% of the females began to nest 16 to 64 days after placement in the nesting boxes, keeping the sperm alive during this entire time.

Sperm precedence after double mating

In the MrMg mating sequence, 19 females from 25 pairs were double-mated. These females made 38 brood balls between the first and the second mating, and 29 red specimens emerged (76.3% survival). For the MgMr mating sequence, 18 females from 25 pairs were double-mated and made 44 brood balls between the first and the second mating, eclosing 44 green heterozygous specimens (75.0% survival). From the second mating for the MrMg sequence, 132 brood balls were made and 107 scarabs emerged (81.0% survival). In the case of the MgMr mating sequence, after the second mating, females made 146 brood balls and 112 individuals emerged (76.7% survival). P_2 values were obtained for each female from the relative frequencies of genotypes in F_1 progeny. Whether the first male to mate was red or green had no significant effect on P_2, indicating that the two morphs have similar fertilizing capabilities. (P_2 of MrMg mating = 55.25±37.59%, P_2 of MgMr mating = 43.35±31.09%, Mann-Whitney $U_{(19,18)}=149$, $P=0.52$). Considering the two sequences, the second male fertilized 49.46±36.01% of the eggs. It is clear that the paternity obtained after one mating with a second male is no greater than that of the first male. However, there was considerable individual variation, and 15.62% of all double matings resulted in 100% sperm precedence by the last male (Fig. 1).

Using only successful first insemination with successful remating for comparison, the two mating times were not different for the MrMg sequence (mating Mr: median = 58 min, range 34–65 min; mating Mg: median=62 min, range 30–71 min; Wilcoxon matched-pairs signed-ranks test, $T=51$, $n=19$, $P=0.08$), the same being true for the MgMr sequence (mating Mg: median=59 min, range 35-70 min; mating Mr: median=56 min, range 22–70 min; Wilcoxon matched-pairs signed-ranks test, $T=80$, $n=18$, $P=0.55$). Furthermore, no significant differences were found in mating times between last males in both experiments ($U_{(19,18)}=145$, $P=0.43$).

Time until mating was not significantly different between successful matings of the MrMg sequence (mating Mr: median=3 days, range 1–7; mating Mg: median=3 days, range 1–9 days; Wilcoxon matched-pairs signed-ranks test, $T=28.5$, $n=19$, $P=0.69$) or between successful matings of the MgMr sequence (mating Mg: median=4 days, range 2–7; mating Mr: median=4 days, range=1–7 days; Wilcoxon matched-pairs signed-ranks test, $T=30$, $n=18$, $P=0.48$). There were no differences in time until mating for either red or green last males ($U_{(19,18)}=159$, $P=0.62$).

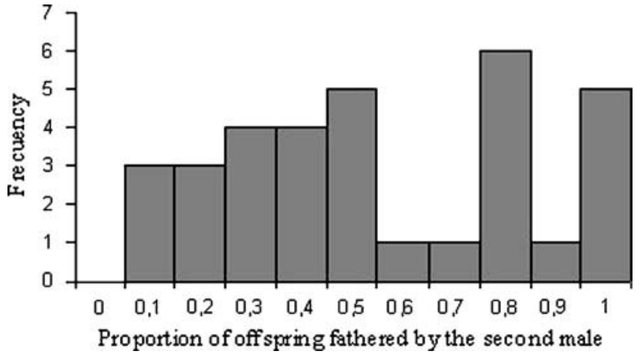

Fig. 1 Frequency of distribution of P_2 values (the proportion of eggs fertilized by the second male to mate). Pooled data from all treatments ($n=37$). Five females had no offspring from the second male

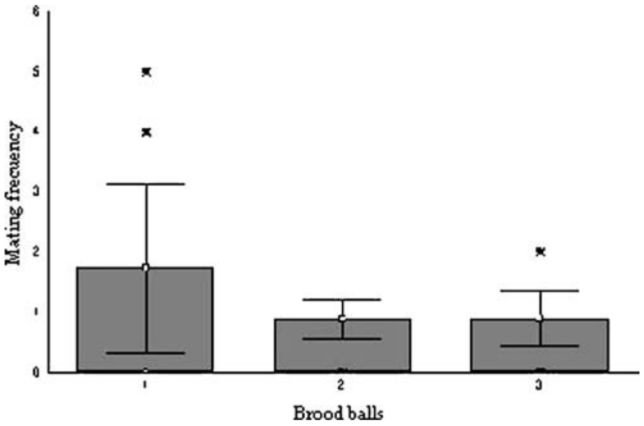

Fig. 2 Mating frequency prior to brood balls construction by a male-female pair in which the female was previously mated

Sperm precedence after *ad libitum* mating

From 25 observed pairs, 22 double-mated. From these pairs, 17 produced at least 2 fertile eggs. From a total of 112 brood balls, 97 beetles emerged (86.5% survival). The last males that mated *ad libitum* with a female were able to obtain a high paternity level (median=83%, range 50–100) which differed significantly from that obtained after a single mating ($U_{(19,17)}$=95.5, $P = 0.03$).

Male and female behaviour during nesting

During the entire observation period males mated 3.59±1.50 (range: 1 to 7) times with previously mated females. However, mating frequency was significantly different in relation to brood ball construction over time (rm ANOVA, brood balls: F=5.84, df=2, $P < 0.001$). Mating frequency prior to the construction of the first brood ball was significantly higher than prior to the fabrication of the next two brood balls (Tukey test, P=0.01, Fig. 2).

Discussion

Our results strongly suggest that sperm competition occurs in *C. c. cyanellus*. In nature, males have a high probability of beginning a nest with a previously mated female. Of course, there is no way to determine when females captured in the field were mated. However, if we consider the period between the capture date and the day when they began to make brood balls, it is clear that the competition to fertilize the egg of a previously mated female is stronger during the first 15 days. Additionally, it occurs sporadically with females mated with another male at least 60 days before.

The proportion of previously mated females varies from one reproductive season to the next. We collected females in July, when they had likely already made nests (Favila, unpublished data). After a period of hibernation that lasts from October to early May, beetles become active during the rainy season (mid-May through September). Thus, males are exposed to females in different physiological states and may be capable of recognizing their status and acting differently with a virgin female than with a mated one. It could be that the number of matings with a previously mated female is higher than that with a virgin female. Müller et al. (2003) found that *Nicrophorus vespilloides* females use chemical cues to discriminate between breeding partners and intruders, refusing the latter males. It is likewise possible that *C. cyanellus* males are able to recognize the female's status from chemical cues.

The double-mating experiments show that there are no differences in the competitive ability of the two phenotypes, a fundamental requisite for studies of sperm competition (Eady 1991). Thus, the visible genetic marker of *C. c. cyanellus* can be used for this type of study, as others have been used in similar studies of other insect species (Eady 1991; Gwynne 1984).

After double mating under controlled conditions, intermediate P_2 values indicate that sperm from successive males mixes within the female spermatheca**,** suggesting strong competition (Simons and Siva-Jothy 1998). Spheroid spermathecae are related to low sperm displacement and elongated or tubular sperm storage organs to high sperm displacement (Walker 1980). Thus, the C-shaped spermatheca in female *C. c. cyanellus* (López-Guerrero and Halffter 2000) could explain why after double mating, second male insemination had almost 1:1 precedence over first male insemination.

In our study, however, *ad libitum* mating in the nest yielded higher P_2 values. The male can mate with a female 3 to 5 times during its time in the nest, mating more frequently before the construction of the first brood ball than before the construction of the subsequent ones. We do not, however, know the mechanism involved in generating high P_2 values during the nesting period. Removal of sperm from previous males with specialized penis structures (Gage 1992; Siva-Jothy 1987; Siva-Jothy et al. 1996; Waage 1979) is not obvious in *C. cyanellus*, since its penis has no ornamentation (Halffter 1961). Furthermore, we were unable to find significant differences in mating duration and latency to remating between first and second males that might suggest anatomical displacement of rival sperm. Another way to obtain last-male sperm precedence is when the last male to mate deposits a large quantity of sperm, reducing the probability of fertilization from rival sperm (Simmons 1987, 2001; Simmons and Siva-Jothy 1998). We believe that in *Canthon cyanellus cyanellus*, repeated mating during nesting, especially before brood ball elaboration, gives the last male a numerical advantage of sperm, increasing its paternity significantly. Spermathecae from wild females and females mated in laboratory (unpublished data) were always full of sperm, suggesting that the male mechanism to reduce sperm competition is replacement.

There are, however, other mechanisms that could be involved in the reduction of sperm competition by the last male; for example, chemical or physical destruction of rival sperm during mating (Gack and Peschke 1994; Harshman and Prout 1994). *C.c. cyanellus* males make large spermatophores (Huerta 1977) full of substances that could cause rival sperm to die. It is also possible that chemical cues provoke muscular contractions of the spermatheca that expel rival sperm. Moreover, cryptic female choice may affect the level of sperm precedence as established by any of the previously commented mechanisms (Eberhard 1994, 1996). For example, boll weevil females use spermathecal muscle to limit the amount of sperm displacement by the second male (Villavaso 1975). The muscles that connect the apex of the C-shaped spermatheca to its base in *C. c. cyanellus* females (López-Guerrero and Halffter 2000) could be used in a similar way, permitting them to control sperm from different males over time. On the other hand, repeated mating could be a copulatory courtship that increases the probability that the last male sperm fertilizes the female's egg (see Eberhard 1996). The male mounts the female during mating and hits her pronotum with his foreleg tibias; this behaviour, as in *T. castaneum* (Edvardsson and Arnqvist 2000), could be a copulatory courtship that motivates the female to select sperm from the last male.

Male nest presence has been interpreted as cooperative behaviour in *C. c. cyanellus* (Halffter et al. 1983). According to these authors, the male cooperates with the female in nest care. However, because sperm competition occurs in this species, it seems likely that the male's presence in the nest is not only cooperative behaviour but also a way of guarding. Copulatory guarding can be advantageous when (1) unguarded females are receptive to new males after insemination, (2) takeover attempts and intrasexual competition are common, (3) the probability of finding an unmated female is low, and (4) males give some kind of resource to the female during copulation (Harari et al. 2003). Our results show that females are receptive during the nesting period, and several matings occur during that time. Fierce fights for food and females are common in *C. c. cyanellus* males but are also observed between females (Favila and Díaz, 1996; Favila 2001a). Males tend to make a nest with previously copulated females. During copulation, males give females two things: the resources necessary for making another brood ball and a large spermatophore filled with various substances that probably nourish her. Furthermore, *C. c. cyanellus* males produce 4-methoxyphenylacetic acid (Favila 2001b), which has antibiotic properties that prevent fungal growth on brood balls (Favila et al., unpublished data); it is not produced by females, so that they also benefit from being guarded. In *C. c. cyanellus* sperm competition could thus be a key factor favoring the male presence in the nest during brood ball elaboration by the female as a mechanism to increase its paternity.

We do not know how male paternity is ensured in other ball roller species in which sperm competition is suspected (Halffter 1997). Non-subsocial ball roller beetles make male-female associations during the rolling and burying of the brood ball, but the male only mates wit the female once and then leaves in search of another female. We encourage comparative analysis with other species of ball roller scarabs to understand such unknown aspects of the reproductive behaviour of this insect7 group.

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