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Mating periodicity and post-mating refractory period in the zoophytophagous plant bug *Macrolophus caliginosus* (Heteroptera: Miridae)

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Abstract. The zoophytophagous mirid bug *Macrolophus caliginosus* is an important biocontrol agent of whiteflies in the Mediterranean region. Periods of low productivity in commercial breeding units and unsuccessful establishment in greenhouses prompted this study of mating behaviour. Here we describe copulation behaviour, the diel mating periodicity and post-copulatory refractory period. A natural plant substrate needs to be provided if one wants to observe the copulatory behaviour of *M. caliginosus* in the laboratory. There was no apparent directional orientation in the approach of the two sexes, instead males pursued females after "accidentally" contacting them. Males mounted females from above, very rapidly, and without any obvious courtship behaviour, and copulation duration was very consistent (286.33 ± 4.23 s, mean ± SE). Observations over a 24-h period showed that mating was most frequent in the 8-h scotophase and first half of the 16-h photophase than in the second half of the photophase. Mated females became strongly unreceptive to new male mounting attempts, shaking their abdomen and leaving the plant if harassed. To determine the duration of the post-mating refractory period mating receptivity of females that had mated 1 or 2 weeks earlier was compared with that of virgin females of similar age. Mated females remained unreceptive even 2 weeks after mating, whereas half of the virgin females of equivalent age mated. Mated females were more likely to abandon a plant than virgin females when harassed by a male. Most males remated a few minutes after mating for the first time. *M. caliginosus* is atypical among mirids in that females apparently mate only once.

INTRODUCTION

The zoophytophagous plant bug *Macrolophus caliginosus* Wagner (Heteroptera: Miridae) is a relatively small and frail bright-green insect. It occurs naturally in the Mediterranean region where it colonizes greenhouse and open field crops when no broad-spectrum insecticides are applied (Alomar et al., 2002; Castañé et al., 2004). *M. caliginosus* is currently produced commercially in Europe for the biological control of whiteflies [*Trialeurodes vaporariorum* Westwood and *Bemisia tabaci* Gennadius (Sternorrhyncha: Aleyrodidae)] in tomato greenhouses (Gabarra & Besri, 1999). It is reared in large colonies and low fecundity (up to 30% of the females do not lay eggs, personal observation) is sometimes recorded. When released in greenhouses, population growth and establishment is unpredictable. Detailed knowledge of the mating behaviour of *M. caliginosus* could help determine the causes of the low fecundity in commercial production and poor establishment in greenhouses. The mating behaviour of *M. caliginosus* is largely unknown except for a few scattered observations (Fauvel et al., 1987; Constant, 1994). In the present paper new aspects of the mating behaviour of *M. caliginosus* are explored.

In most mirids there is a sexual maturation period of several days after adult emergence (Groot 2000; Wheeler, 2001). In contrast to most heteropterans, female mirids release a volatile sex pheromone to attract males (Aldrich, 1988, 1995; Zhang & Aldrich, 2003; Innocenzi et al., 2005). At close-range contact pheromones may also play a role in sexual communication (Groot et al., 1998; Drijfhout et al., 2003). Courtship in mirids can be relatively elaborate, including wing flapping, antennal patting and abdominal vibration, but in many species precopulatory sequences are simple or almost absent (Wheeler, 2001). Males normally mount females from the right side, because mirid genitalia are asymmetrical (Wheeler, 2001). Mating lasts from a few seconds or minutes to several hours, disengagement is often abrupt and post-copulatory genital cleaning is sometimes observed (Wheeler, 2001).

In many insects mating occurs at discrete periods of the day (Thornhill & Alcock, 1983). Knowledge of the period of mating activity is important when planning mating tests. Several mirid species mate readily during the day (Groot et al., 1998; Wheeler, 2001), but this does not imply that mating in these species is restricted to the daylight hours. *Lygus hesperus* (Knight) is probably diurnal because 94% of the 458 males collected in female-baited traps were captured between after-daybreak and before darkness (Strong et al., 1970). There are few observations of mirid mating at night so the exact periodicity of mating in most species is unknown. *Phytocoris difficilis* Knight and *Campylomma verbasci* (Meyer) are nocturnal and crepuscular, according to male captures in pheromone and female-baited traps, respectively (Thistlewood et al., 1989; Zhang & Aldrich, 2003). The period of mating activity of *M. caliginosus* is unknown, and mating

pairs are rarely seen during daytime (pers. observ.), which suggests that they mate mainly at night.

Mating normally renders female insects unreceptive for a time that ranges from immediate remating to no-further mating (Alcock, 1994; Ringo, 1996). Number of matings can have profound effects on fertility and fecundity (Ridley, 1988), and therefore it is of interest when trying to determine the causes of low productivity of stock cultures. If *M. caliginosus* females need multiple matings to fertilize all their eggs, as in the case of the seedbug *Nysius huttoni* White (Wang & Davis, 2006) or the stinkbug *Podisus nigrispinus* (Dallas) (Torres & Zanuncio, 2001), then a low number of matings could result in low fecundity. Few controlled remating experiments have been done with mirids, but the perception is that most mirids mate more than once (Groot, 2000; Wheeler, 2001). The number of times that females of *M. caliginosus* mate is unknown.

In the present study we describe the events from sexual encounter to copula termination in *M. caliginosus*. Observations were carried out over a 24-h period to determine the probability of mating at different times of the day. We observed that recently mated females became very unreceptive to males, and so performed remating trials to determine the duration of the post-mating refractory period of both males and females.

MATERIAL AND METHODS

Insects

A stock culture of *M. caliginosus* was maintained at 25 ± 1°C, 70 ± 10% RH, and a 16L : 8D photoperiod. Since 1994 stock insects were reared on tobacco plants (*Nicotiana tabacum* L.) with *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) eggs as prey. Insects from local tomato fields (Cabrils, Barcelona, Spain) were added to the stock culture periodically. Adults were separated daily from nymphs in the rearing cages and placed in groups of ca. 20 in two 0.5 l well-ventilated cages, one for each sex, and provided with green beans and a cotton wool-plugged water vial. Frozen eggs of *E. kuehniella* were added every other day. Nymphs and adults were handled using mouth aspirator.

Experimental arena

Initially we tried to film mating behaviour in a small arena (2 cm diameter × 0.5 cm deep plastic-bottle cap covered with a microscope slide), but females ran away from males around the perimeter of the cage resulting in few matings. Then we tried to make direct observations in a 9-cm-diameter Petri dish but the mating frequency was extremely low, because the encounter rate was low and most encounters resulted in females running away from the males. The addition of leaf disks, food (frozen *E. kuehniella* eggs) and water to the Petri dish did not increase mating frequency. From this experience it became clear that we needed an arena that allowed females to run away from males but that at the same time did not decrease the probability of mating. We observed that, on a plant, a running insect would stop shortly after moving to the other side of the leaf. Based on this observation a setup was developed that proved satisfactory and was used in this study.

A small potted tobacco plant was stripped of all its leaves but one, which was then cut down to an area of ca. 15 cm^2. This allowed females to run away from males, but ensued a reasonably high rate of reencounter between the sexes and allowed us to observe matings. To aid observation, the leaf was held vertical with the help of a metal paper clip inserted in the soil. Females were placed on the plant the day before the experiment because they were more receptive to males if given some time to accustom to the plant. Eggs of *E. kuehniella* were brushed onto the leaf surface to provide food for the insects. In preliminary trials one male and one female per plant were used, but this number of insects resulted in a very low mating rate. To increase the probability of mating four females and four males were released on each plant. Because *M. caliginosus* flies readily if disturbed, we covered the plant with a 15 cm diameter × 20 cm tall transparent plastic cylinder. The top of the cylinder (hereafter referred to as "cage") was covered with a white and removable polyester-organza fabric lid. The cage not only prevented insects from escaping, but also prevented air disturbances due to our own breathing during close-up observations. Both plant pot and cylinder rested on a plastic plate, which, during the observations, was turned very slowly and carefully so that insects that had walked outside the field of view could be continuously observed.

Diel pattern of mating

Four 6 to 8 days old females (sexually receptive age, Castañé et al., 2006) were placed on the plant. The next day four males of the same age range as the females were put in a plastic test tube (6 × 1.5 cm), plugged with cotton wool, and 30–45 min later the lid was removed from the plastic cylinder and the test tube was tapped gently right above the plant so that the males landed on it. The observation period started when the four males had left the tube and ended 30 min later. Observations were performed in a walk-in environmental chamber maintained at 23 ± 1°C with a 16L : 8D photoregime, with lights switched on at 8:00 AM.

The day was divided into three 8-h periods: scotophase, first half of photophase (PH1) and second half of photophase (PH2). Twenty replications (each replication consisted of a 30 min observation of 4 males and 4 females on a plant in a cage) were made in each of the 3 periods. Most replications were made in the 2nd to the 7th h of each 8 h period (Table 1). Sampling near lights-off and lights-on was avoided because a sudden change of light intensity could affect the behaviour, at least momentarily. During the photophase illumination was provided by two 36 Watt fluorescent-light tubes, one white and the other red, placed directly above the cages, and during the scotophase the white fluorescent-light tube was turned off and the red one left on so that behaviour of the insects could be observed. Insects used in a replication were not used again. Individuals that left the plant

TABLE 1. Number of replications (i.e., cages) in each hour of the three 8-h periods (scotophase, first half of the photophase or PH1, and second half of the photophase or PH2) of a 24-h photocycle during which the mating behaviour of *M. caliginosus* was observed. Four males and 4 females were put in each cage and observed continuously for 30 min.

Hour of observation in each 8 h period	Number of replications		
	Scotophase	PH1	PH2
1	0	1	0
2	4	2	2
3	6	2	5
4	5	3	6
5	3	2	3
6	2	7	2
7	0	2	2
8	0	1	0

did not encounter each other, and so the only matings in the cage were on the plant.

As dependent variables we used (a) the total number of matings per cage (i.e., replication), (b) the number of contacts of any kind between males and females between the time the males were released and when the first mating occurred, (c) the time between male release and the first mating, and (d) copulation duration. Since both males and females sometimes left and then returned to the plant, and this varied in the 3 periods, we also recorded the maximum number of males and females that were on the plant for at least 2/3 of the observation period (maximum 4 per sex), and this variable was used to compare the 3 time periods in which each day was divided. Comparisons among treatments were performed using a general linear model (SAS, 2000) and transformed data (log [x + 1]), with the only independent variable being the period of the day (scotophase, PH1, PH2).

Remating

In the previous experiment we observed that recently mated females vigorously rejected attempts by males to mate. Preliminary observations indicated that this rejection continued for 2 or 3 days (data not shown), so we carried out a controlled experiment to determine whether mated females would remate 1 or 2 weeks after the first mating. Because age was a variable in this experiment we included a control, which consisted of virgin females of the same age as the mated females. In the previous experiment we also observed that males remated soon after a previous mating (up to four times in one hour in one case). A test was also carried out to determine the percentage of males that remated after their first mating. In this case no age control was necessary because, unlike females, males remated within an hour. Based on the results of the diel periodicity test, the remating experiment was performed during the first half of the photophase, and also with 6 to 8 days old adults. Observations were carried out on a bench of the laboratory with a mixture of natural reflected light from a nearby window and artificial light from a fluorescent tube attached to the ceiling of the room.

Female remating

A virgin 6 to 8 days old female was placed on the plant and the next day 4 males of the same age were introduced into the cage. In this experiment we used a new release method that resulted in more males staying on the plant. Three males were aspirated into a glass tube (7 cm long × 5 mm internal diameter), and 30 minutes later the end of the tube was placed at the base of the plant by inserting it through a hole in the cylinder wall, and the males were encouraged to leave the tube by pushing them out very slowly with a plunger. If a female did not mate within 30 min she was discarded. As soon as a female finished mating she was placed in a cage with *E. kuehniella* eggs and a tobacco plant where she had access to oviposition sites and food. The female remained on the tobacco plant for 1 or 2 weeks and then was tested for remating with 4 virgin males. The experiment was replicated 19 times (1 week) and 9 times (2 weeks). As controls, 2 and 3 weeks old (respectively) virgin females that had been kept on green beans and fed *E. kuehniella* eggs, were tested for mating just once. These two controls were replicated 18 and 17 times, respectively. The percentage of mated females that remated was compared with the percentage of control virgin females of the same age that mated using the test of two percentages (Lehner, 1996).

Male remating

To determine the percentage of males that remated four females were placed on the plant and a single male was introduced into the cylinder the next day as described above. If the male did not mate in 30 min it was discarded. When mating finished, the male was sucked into an aspirator for 20–25 min, after which it was introduced into a new cylinder with another 4 virgin females of the same age as the ones of the previous mating, and observed until mating took place or for a maximum of 30 min. The experiment was replicated 18 times with different males and females. The percentage of males that mated a second time was compared with the percentage of males that did not mate (G test).

RESULTS

Copulatory behaviour

Females normally remained stationary on the plant when the males were introduced. Male-male contacts were frequent and normally resulted in males running away from each other. Males sometimes rested very close to females for several minutes without showing any reaction towards them and then walked away. Male encounters with females (as with other males) was apparently random. Most females at first ran away from males and only accepted mating after several contacts (data not shown) with the same or a different male. Males started to walk faster after encountering a female, actively pursuing her, which in turn resulted in further encounters with other individuals on the plant. Eventually a male was able to mount a female, sometimes flapping his wings very rapidly and then slid his abdomen downwards, always on her right side, inserted his genitalia into hers, and turned to the right through 180°C, so that they remained united in the same axis but facing in opposite directions. This process happened very rapidly (< 5 s) and they remained in copula for 286.33 ± 4.23 s (n = 107, mean ± SE, range 173–421 s). About 4 min after copulation started, females became restless and started to walk dragging the males and kicking them with their hindlegs. After separation males and females cleaned their genitalia with their legs and sometimes with their mouthparts. Mated females were very reluctant to mate immediately after the first mating, rejecting males by moving their abdomen from side to side and running away. If males persisted females jumped off the plant or walked down the stem to the soil.

Males sometimes attempted to copulate with a mating pair, which ran away, usually led by the female. Although attempts to copulate with a mating pair were aggressive they never resulted in the disengagement of the couple.

Period of mating

Mating was more frequent in the scotophase and first half of the photophase (PH1) than in the second half of the photophase (PH2) (df = 2,59, F = 5.25, P = 0.007, Fig. 1). The number of contacts between males and females before mating was much lower in the scotophase and PH1 than in PH2 (df = 2,52, F = 0.91, P < 0.001, Fig. 1). Similarly, there was a shorter time between male release into the cage and the first mating in the scotophase than in PH2 (df = 2, 53, F = 4.05, P = 0.023, Fig. 1). This indicates a higher female receptivity and probably higher male mate-seeking activity in the period of highest mating frequency. The duration of mating was similar in the 3 periods (df = 2, 106, F = 1.25, P = 0.292).

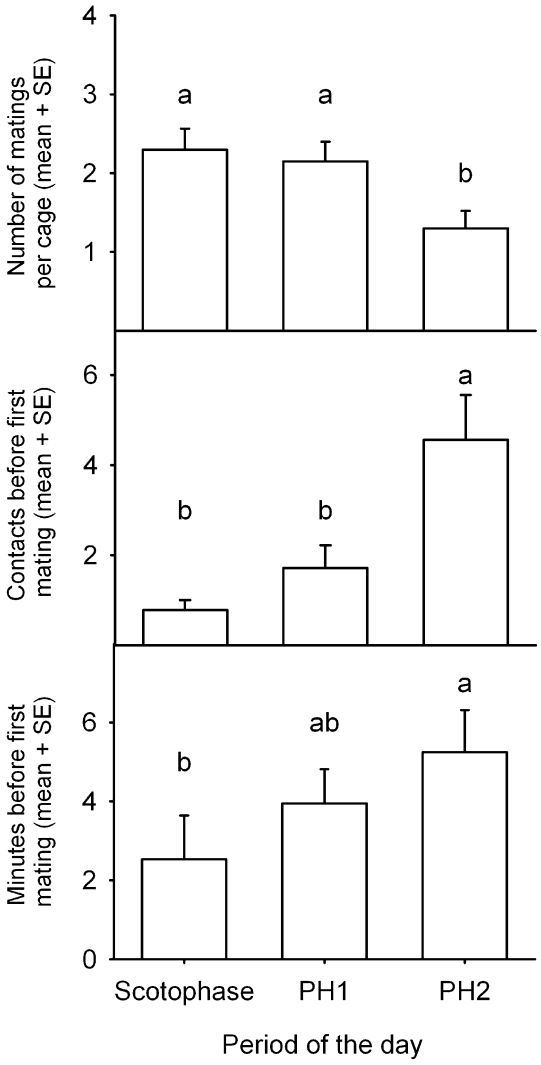

Fig. 1. Number of matings (top), contacts between males and females before first mating (middle), and minutes that elapsed before the first mating (bottom) during the scotophase, and first (PH1) and second (PH2) halves of the photophase. Four *Macrolophus caliginosus* males were released in cages containing 4 females and observed continuously for 30 min. The values are means per replication (i.e., cage; n = 20). Significant differences among periods are indicated by different letters ($P < 0.05$, Tukey multiple comparison test after ANOVA).

So once mating starts, copulation duration is not affected by the period of the day or female receptiveness.

About 81% (3.2 out of 4 per cage) of the individuals remained on the plant for at least two thirds of the 30 min observation period in the three 8-h periods (mean ± SE): 3 ± 0.19, 3.41 ± 0.19 and 2.85 ± 0.18 males on plant (scotophase, PH1 and PH2 respectively; df = 2,47, F = 2.35, P = 0.106), and 3.35 ± 0.16, 3.70 ± 0.11 and 3.5 ± 0.15 females on plant (scotophase, PH1 and PH2, respectively; df = 2,46, F = 1.44, P = 1.24). Therefore a similar number of individuals were available for mating at the three periods of the day. An exact calculation of the percentage of females that mated per cage is not possible because the individuals that left the plant cannot be included in the total. However, using the previous values we estimated that the percentages of mating per cylinder for the scotophase, PH1 and PH2 were 71, 61 and 42%, respectively.

Remating

In the remating experiment only 1 of the 28 mated females remated, and this second mating was unusually short (less than 2 min). Two-week-old females had a higher probability of mating if they were virgin (11/18) than if they had mated 1 week earlier (1/19) ($Z = 3.63$, $P < 0.05$). Similarly, three-week-old females also had a higher probability of mating if they were virgin (9/17) than if they had mated 2 weeks earlier (0/9) ($Z = 2.63$, $P < 0.05$).

The strong rejection behaviour of mated females was maintained both in the first and second week after mating. Moreover, after repeated male mounting attempts most mated females left the plant, mainly by walking down the stem. Significantly more mated (24/28) than virgin (10/35) females abandoned the plant in response to continuous male harassment ($Z = 4.52$, $P < 0.05$). Of these a similar proportion of virgin and mated females subsequently returned to the plant (6/10 and 21/24, respectively; $Z = 1.81$, $P > 0.05$). This cycle of leaving and returning to the plant was repeated several times.

With regard to male remating, significantly more mated males remated (15/18) than did not (3/18) ($G = 8.73$, $P < 0.01$). One of the 3 males that did not remate also did not encounter any females. There were no differences between the first and second matings in a) time between male release and first contact (6 ± 1.33 and 5.71 ± 2.03 min, df = 13, t = 0.14, P = 0.89), b) time between male release and copulation (8.07 ± 1.74 and 7.73 ± 2.39 min, df = 14, t = 0.12, P = 0.9), c) number of contacts (touching or mounting attempts) before mating (2 ± 0.29 and 1.86 ± 0.31, df = 13, t = 0.35, P = 0.73), or d) mating duration (337.46 ± 21.22 and 315 ± 26.03 s, df = 12, t = 0.41, P = 0.62). All this suggest that the first mating did not affect the mating capability of males.

DISCUSSION

The few reports of nocturnal reproductive behaviour in mirids are based on trap captures (Zhang & Aldrich, 2003), and on the inference that mating is nocturnal because it is not observed during the day (Smith & Borden, 1991; Constant, 1994), and more rarely on direct nocturnal observations (Kakizaki & Sugie, 1997). Here we present data from a controlled experiment where virgin males and females were put together and observed continuously during both scotophase and photophase, to determine the period of mating. Under our test conditions *M. caliginosus* mated at any time during the photoregime, but clearly there was higher mating activity in the scotophase and first half of the photophase than in the second half of the photophase. High percentages of mating corresponded with fewer encounters and less time to first mating, indicating that at the periods when mating was most likely the males were more active and the females more receptive, whereas the opposite was true for the period when there was less mating.

The diel pattern of mating may have been slightly different if males and females were allowed to interact continuously (as probably happens under natural conditions) and not just in the discrete time periods used in our experiment. Continuous interaction between males and females could result in a narrower period of mating because those females that mate during the most receptive period would no longer be available for mating at other times. In addition, we did not sample the periods of changing light conditions, the crepuscular periods, which in some species are times of high sexual activity (*Campylomma verbasci* (Meyer), Thistlewood et al., 1989). Furthermore, we increased the likelihood of encounters by placing 8 adults on a 30 cm^2 leaf surface, which probably augmented the natural rate of mating. All this taken into consideration, our results suggest that *M. caliginosus* will mate at any time of the day, but mating is more likely in the scotophase and first half of the photophase. Therefore, studies of mating behaviour in this species should be made during these periods. In addition, providing experimental conditions similar to those we used may be necessary if observation of a large number of matings is required.

The premounting behaviour of *M. caliginosus* is similar to that of other mirids (including another *Macrolophus*) in which males simply approach females and jump onto them (Strong et al., 1970; Wheeler et al., 1979; Stork, 1981; Kakizaki & Sugie, 1997). As in *Calocoris angustatus* Lethiery, males aggressively pursue females, which sometimes repell the males with their hind legs (Hiremath & Viraktamath, 1992). As in other mirids, mating is from the right due to the asymmetrical configuration of the male genitalia (Wheeler, 2001). The duration of mating in *M. caliginosus* is relatively short compared with other mirids, up to 15 min in *Cyrtorhinus lividipennis* Reuter (Liquido & Nishida, 1985), up to 3 h in *Nesiodiocoris caesar* (Ballard) (Chatterjee, 1984), but only a little bit longer than in *L. pabulinus* L. (2 min, Groot et al., 1998), *L. hesperus* (2.23 min, Strong et al., 1970) and other species (Wheeler, 2001). What is interesting is the very low variation in mating duration in *M. caliginosus.*

The strength of the mating rejection behaviour of recently mated females of *M. caliginosus*, which takes the form of shaking the abdomen and leaving the plant, was not less 1 or 2 weeks after mating. Furthermore, almost no mated females mated a second time whereas about 50% of the virgin control females of equivalent age did, so clearly mating induced a depression in responsiveness. Given that the estimated adult female longevity of *M. caliginosus* is 41.07 ± 3.70 days (Castañé & Zapata, 2005) and that 3-week-old mated females do not remate, it is likely that females of this species mate only once in their life-time. The long, perhaps permanent, refractory period of female *M. caliginosus* is atypical of mirids (Wheeler, 2001). In contrast, *L. pabulinus* can copulate on consecutive days (Groot et al., 1998), and females of *L. hesperus* Knight can mate 3 times in their lifetime (Strong et al., 1970). Again, caution should be taken when extrapolating our results to the natural situation because several environmental factors such as food (Torres-Vila et al., 2005) and oviposition substrate (Harano et al., 2006) and genetic factors (Torres-Vila et al., 2002; Harano & Miyatake, 2005) affect remating probability.

The apparent monandry of *M. caliginosus* raises several questions. From the practical point of view of mass-rearing it means that the number of males in the colony could be reduced considerably without reducing the overall fecundity of the females. Productivity may then increase because less harassment of females by males would give the females more time to feed and lay eggs (Mendes et al., 2003). Before testing this possibility we need to know if male fertility decreases on successive matings, as happens in other species (Torres-Vila & Jennions, 2005; Marcotte et al., 2006). From a behavioral point of view we wonder to what extent monandry in *M. caliginosus* has resulted in sexual selected traits such as female choosiness or male-male competition (Thornhill & Alcock, 1983). Females of *Ozophora baranowskii* Slater & O'Donell (Heteroptera: Lygaeidae) tap males with their hind legs while mating, with more intense tapping resulting in shorter copulations (Rodriguez, 1998). This type of female choosiness could take another form in *M. caliginosus*, such as running away from some males and facilitating copulation with others, a possibility that remains to be tested. In addition, we did not observe any obvious sign of male-male antagonism, but males were very persistant in following and trying to copulate with females, even when they were already copulating with another male, which suggests that there is strong male-male competition for access to females in this species.

In summary, our study shows that *M. caliginosus* is more active and sexually receptive, and mates more frequently, in the scotophase and first half of the photohase than in the second half of the photophase. Courtship is short and copulation duration very consistent. We recommend that observations on mating behaviour in this species be made during the first half of the photophase in large arenas where the insects can walk freely on a leaf. Mated females reject males by shaking their abdomen vigorously and leaving the plant. The potential lifetime monogamy of *M. caliginosus* females contrasts with the apparent polyandry of many other mirids. Detailed experiments on the mating and remating behaviour of other mirid species will reveal if *M. caliginosus* is exceptional in its remating behaviour.

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