Absence Makes the Heart Grow Fonder: Isolation Enhances the Frequency of Mating in *Coleomegilla maculata* (Coleoptera: Coccinellidae)

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Abstract Mating behavior can be a dynamic process that depends upon the insects' environment and condition. We performed a series of experiments to see if isolating individual ladybeetles changed the frequency of mating compared to when they were kept in mixed-sex groups. Our results indicate that individuals isolated for only 1 day were 26 times more likely to mate than individuals kept in a mixed-sex group. Isolation of either sex will increase the propensity to mate, but isolating males had a stronger effect than isolating females. We further demonstrate how isolating could be used as a technique for studying some aspects of mating behavior by showing that there is large variation in the frequency of remating amongst maternal lines.

Keywords Coccinellid · density-dependent behavior · mating behavior · polyandry

Introduction

Polyandry and polygyny are common in insects but the frequency of multiple mating is influenced by many factors (Thornhill and Alcock 1983). Such factors may make the benefits of polyandry context-specific and contribute to its variability (Torres-Vila et al. 2004). The local density of individuals is one such factor that can influence the incidence of remating. Increasing local density of *Drosophila* spp. increased the frequency of initial mating (Spiess and Spiess 1969; Eckstrand and Seiger 1975) and remating both in the laboratory (Harshman et al. 1988; Singh and Singh 2001) and in the field (Marks et al. 1988). Remating

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frequency also has decreased with increasing density (Gromko and Gerhart 1984), been unaffected by density (Harshman et al. 1988), or increased and then decreased as the density of flies increased (Turner 1986). Little, however, appears to be known about the effect of isolation, a situation of very low local density.

Multiple matings are likely to occur when the benefits outweigh the costs (Thornhill and Alcock 1983). For males, motivation for additional matings is high, because it can increase the chance of siring more offspring, leading to a direct increase in fitness. Local density may influence male motivation in that males become more motivated when sperm competition can be avoided or when they expect fewer future mating opportunities. Local density may also alter female receptivity to multiple matings if it alters the benefits they receive from material resources (Arnqvist and Nilsson 2000), the genetic benefits received from genetically variable males (Jennions and Petrie 2000; Srivastava and Omkar 2005b; Maklakov and Lubin 2006), or if isolation affects the associated costs of multiple matings (Majerus 1994a; Omkar and Mishra 2005 and references therein).

Coleomegilla maculata DeGeer (Coleoptera: Coccinellidae) can be quite common in habitats such as the maize agroecosystem (Schellhorn 1998; Wold et al. 2001), yet even at their highest density we have rarely observed multiple individuals on the same maize plant unless they are mating. Other ladybirds aggregate quite strongly to prey at a fine spatial scale (Schellhorn and Andow 2005), but *C. maculata* aggregates at a larger spatial scale (Schellhorn and Andow 2005) resulting in much less contact between individuals. When field-collected individuals are placed in mixed-sex groups, there is a tremendous number of matings; 50–95% of all adults mate during the first 4 h after being placed together (J.P.H., unpublished data). These observations suggest that individuals in the field are extremely likely to mate when given the chance. In the lab, however, mating frequency quickly declines over time when they are kept in mixed-sex groups; 20% after 24 h, and only 5% after 48 h (J.P. H., unpublished data). Mating will continue in confined mixed-sex groups in the laboratory, but at a diminished rate (1–3% per day).

We set out to experimentally investigate this apparent density-related pattern by determining whether isolation influences the frequency of remating in *C. maculata*. We further explored the relative effect of male and female isolation and whether isolation affects the frequency or temporal pattern of remating. We then investigated potential maternal or genetic contributions to mating frequency, by establishing maternal lines and testing progeny.

Materials and Methods

Effect of Isolation on Mating Frequency

To experimentally evaluate whether isolating *C. maculata* adults had any effect on their propensity to mate, we collected adults and pupae from mid August to early September in maize fields at the University of Minnesota Agricultural Experiment Station, St. Paul, MN, USA. All beetles were kept together in a controlled environment chamber (25°C, ~65% humidity, 16:8 light/dark cycle) and fed ad libitum on egg masses of the European corn borer, *Ostrinia nubilalis* Hübner

(Lepidoptera: Crambidae) and artificial diet (modified from diet 7, Atallah and Newsom 1966). Each individual was used in the experiment only once and experiments were performed within two weeks of capture. Most beetles mated soon after capture, but all had ample opportunity to mate during their confinement. We therefore believe that almost all of the matings observed in our experiments were rematings, but cannot rule out that some individuals may not have mated before being tested. Before each experiment, we sexed adults by observing the ventral aspect of their abdomens under a stereomicroscope, a technique we have verified with dissections to be >95% accurate.

To understand how isolation influences mating, we performed a two by two factorial experiment that determined the frequency of mating between males that had been either kept in isolation or in a mixed-sex control group and females that had been either isolated or kept in a mixed-sex group. Isolated individuals were removed from a common, large, mixed-sex colony and placed in a Petri dish (5.5 cm diameter, 1.5 cm high). The mixed-sex control group contained 20 males and 20 females from the same colony that were placed together in a mason jar (8 cm diameter, 7 cm high) with a screen top. Individuals were provided with European corn borer egg masses and water ad libitum, and they were maintained in one of these two treatments for 24 h. Preliminary experiments indicated that the effects of isolation were similar for conditioning periods lasting 1 to 3 days. After the conditioning period, males and females were combined into a new mason jar and we recorded the number of matings that occurred over 3 h. Between 17 and 20 individuals of each sex were placed into the experimental arena (always equal numbers of males and females), and we used two arenas for each of the four treatment combinations. For one experimental arena of each treatment we also recorded when coupling was initiated. We removed pairs in copula to avoid having a single individual mate multiple times. Pairs were only removed after we observed them mating for at least 20 min to insure that we removed mating pairs and not attempted matings. Preliminary continuous observations indicated that all undisturbed matings lasted well over 20 min, with most lasting hours (as in Obata 1988a). We conducted all experiments at ~27°C, in florescent light between, 0800 and 1200 hours when beetles were at their most active. We analyzed the propensity to mate in each treatment using a log-linear contingency table analysis in Proc Catmod (SAS Institute Inc. 1989) with a categorical response variable indicating whether each potential mating pair of individuals had mated or not mated. Since we always used equal numbers of males and females, this response variable is the same as determining whether each individual female had mated or not mated or whether each individual male had mated or not mated. We looked for differences between treatments for when coupling was initiated using ANOVA.

Differentiating Between Accumulated and Enhanced Mating

Our first experiment indicated that putting males in isolation increased the number of observed matings (see "Results"). Our second experiment attempted to determine if isolation actually increases the beetles' propensity to mate or if isolation merely changes the timing of mating. The latter mechanism would produce results similar to what we observed if all of the matings that would have taken place during the

conditioning period (had they been allowed to mate) were aggregated and occurred during the observational period. If this alternative explanation is true, the number of matings between isolated individuals during the observational period should be equal to the total number of matings observed in the mixed-sex group over the 24-h conditioning period plus the subsequent 3-h observational period.

To determine if isolation increases the frequency of matings or simply changes when it takes place, we recorded the number of matings during the 24-h conditioning period for another mixed-sex group of 20 pairs. Before the experiment, we observed several hundred beetles periodically through scotophase and observed no matings. Hence, we confined our experimental observations to the photophase of a 24-h period. While grouped individuals were being observed, additional males were isolated as in the previous experiment. After the conditioning period, the isolated males were placed with equal numbers of females that had been kept in an unobserved mixed-sex group. All beetles from the observed mixed-sex group were moved to a new experimental arena to control for potential handling effects. All males and females from both the isolation and mixed-sex treatment groups were originally from a mixed-sex colony kept as above. Like the previous experiment, we recorded the number of matings observed over 3 h for both treatments, and removed mating pairs to avoid counting the same individual twice. We replicated the experiment five times and analyzed data with contingency table analysis again using the categorical response variable of whether each potential mating pair had mated or not mated.

Variation in Enhanced Mating Across Families

We conducted an experiment to determine if there is variation in the increased frequency of mating after isolation that may be attributed to genetic or maternal effects. The experimental methodology of isolation used above allows us to observe a large number of matings in a short time period, and this experiment demonstrates its utility for studying some aspects of mating behavior. We established nine maternal lines of C. maculata from field collected individuals. Most females mated with just one male, however, when egg production diminished, a new male was placed with the female such that most individuals within a line were full sibs but some were half sibs. We reared offspring as in Harmon (2003) and after sexing, gave each individual a unique identification marking with non-toxic enamel paint on its pronotum.

To characterize the frequency of mating after isolation, we used the previous methodology with isolated male offspring and then recombined them with females from the same family that had been kept in a mixed-sex group. The principle difference in this experiment is that the propensity to mate after isolation was measured in the same individuals over four successive isolation periods, each 2–4 days apart. To maintain a group size of twenty pairs for all observational periods, new individuals from the same family replaced dead or missing individuals. Only individuals that completed all four trails were used in the data analysis. Data were analyzed using a log-linear contingency table analysis with the null hypothesis that mating propensity was equal across maternal lines. In addition, we weighed a subset of individuals ($n=81$) to determine if size affected the propensity to mate and analyzed the data using a nominal logistic model (SAS Institute Inc. 2000).

Results

Effects of Isolation on Mating

Isolating individuals for 1 day significantly changed the frequency of matings ($\chi^2_3 = 333.35$, $p<0.001$), with matings more frequent after *C. maculata* were isolated compared to those kept in mixed-sex groups. Out of the 39 potential pairs of males and females that had both been kept in mixed-sex groups, only one mating occurred during the observation period. We observed the largest increase in the frequency of mating if males were isolated from females for 1 day (males isolated and females in group: 27 out of 40 pairs; both sexes isolated 27 out of 39 pairs). When females were isolated and males kept in mixed-sex group, there was an intermediate response to isolation (eight out of 37 pairs mated). Isolating females may therefore have some effect on the frequency of mating, but its effect appears weaker than that of isolating males. Moreover, the effects of males and females did not seem additive since the response to both sexes being isolated was equivalent to that observed when only males were isolated.

Isolation may have also affected when matings occurred during the experiment. The one mating that occurred when both sexes were kept in the mixed-sex groups occurred after 150 min whereas 65% of the matings in the other treatments were initiated within 15 min of the experiment starting. There did appear to be differences in the average time a mating was initiated between the three treatments with multiple matings (average±SEM: males isolated and females in group 29±17 min, males and females isolated 57±18 min; females isolated and males in group 86±31 min). Yet these differences were not statistically significant ($F_{2,32}=1.65$, $p=0.21$), likely due to the fact that most matings in each of the these treatments occurred early on and the differences in averages were driven by when the remaining matings took place.

Differentiating Between Accumulated and Enhanced Mating

When males were isolated individually for 24 h, matings during the 3-h observation period were once again more common than they were for males and females that had been kept in mixed-sex groups (62 out of 94 vs. two out of 107; $\chi^2_1 = 55.01$ $p<0.001$). Matings did occur in the mixed-sex group during the 24-h treatment period (13 out of 107). However, the total number of matings in the mixed-sex group over the combined conditioning and observational periods (15 out of 107) was still much less than the matings observed with isolated males (62 out of 92) ($\chi^2_1 = 49.04$, $p<0.001$; Fig. 1). Therefore the observed increase in the frequency of matings after isolating males is not just the accumulation of matings that would have occurred if males had been allowed to mate during the conditioning period. Instead, isolating males appears to increase the propensity of mating compared to if males have continuous access to females.

Variation in Enhanced Mating Across Families

The propensity to mate after isolation varied with maternal lines ranging from as low as an average of one mating out of four attempts to over three matings

Fig. 1 The total proportion of
individuals that mated during the
3-h observational period (*white*)
and the 24-h conditioning period
(*grey*). Individuals in the
grouped treatment were kept in a
mixed-sex group that had the
opportunity to mate during both
periods. Individuals in the iso-
lated treatment were isolated
during the conditioning period
and could therefore only mate
during the observational period.
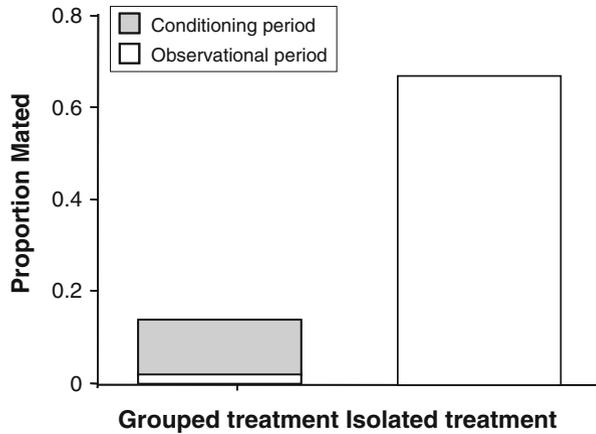
(Figure 2; $F_{8,122}=4.00$, $p<0.001$). Across all families, the frequency of matings
changed over time with fewer matings occurring during the first attempt (66/131)
than in the subsequent three (81/131, 89/131, 76/131; Cochran's Q test,
$\chi^2_3 = 11.12$, $p=0.011$). This effect could be a function of age since younger
beetles can be less likely to mate than middle-aged individuals (Obata 1988b;
Pervez et al. 2004; Srivastava and Omkar 2004; Omkar and Pervez 2005).
Alternatively, it could be some type of learning or association with the repeated
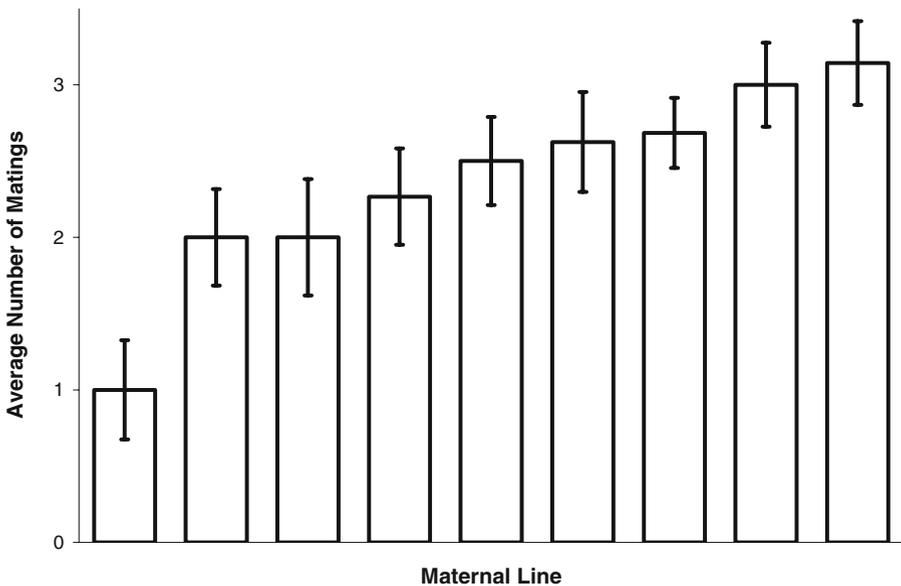isolation technique.

Fig. 2 The average number of rematings (±1 SE) after isolation (maximum=4) for individuals from nine
maternal lines.

These results may indicate a potential genetic or maternal component related to *C. maculata's* propensity to mate after isolation. The size of an organism can be influenced by genetic and maternal factors, and the size of an organism can influence its fertilization success (e.g. Ueno 1994). However, in this experiment, there was no evidence of an association between the size of an individual male (measured as weight) and its propensity to mate ($\chi^2_1 = 1.83$, $p=0.177$). Therefore, male body size may not be an important proximate mechanism for understand mating propensity in this system, and the observed differences among maternal lines is unlikely to be due to effects relating to body size.

Discussion

The propensity for males to remate increased immensely after a period of isolation. This difference was not due to a simple change in when matings took place. If isolated individuals simply "stored up" matings, the number of matings for isolated ladybirds during the observational period should have been equal to the total number of matings seen in the observation plus conditioning periods for ladybirds kept in mixed-sex groups. Instead, we saw almost three times as many matings when males were isolated (Fig. 1), indicating that isolation does actually enhance mating frequency. Moreover, by taking advantage of this technique, we determined that there is a maternal or genetic influence on the frequency of remating, or at the least on the magnitude of the effect of isolation (Fig. 2).

The propensity to increase rematings with isolated males versus mixed-sex groups could be due to female choice and a potential benefit received from multiply mating. While it has been suggested that a single copulation is sufficient for full fertility of most coccinellid species (Hodek and Honěk 1996) and that females mate more frequently than necessary to maintain fertility (Majerus 1994b), it is clear that in many coccinellid species increasing mating does enhance fertility and fecundity (Semyanov 1970; Majerus 1994a; Omkar and Srivastava 2002; Omkar 2004; Omkar and Mishra 2005; Omkar and Pervez 2005; Srivastava and Omkar 2005b). For this to explain the pattern observed here, however, the benefits or perceived benefits from mating with an isolated male would have to be different than those received from a male kept in a group and females would have to prefer to mate with isolated males. Such a preference might increase mate diversity and avoid inbreeding, which would enhance the genetic benefits of multiple mating. In at least some coccinellids, females do not mate randomly (reviewed in Hodek and Ceryngier 2000; Srivastava and Omkar 2005a), and some exhibit active rejection of males (as in Obata 1988b; Omkar and Pervez 2005 and references therein). However, we did not observe any of these rejection behaviors here, so it seems more likely that the isolation effect occurs due to a decrease in the males' motivation to mate when maintained in mixed-sex groups.

Since mating as many times as possible is the most obvious way for a male to enhance fitness, it is unclear why males would curtail mating in mixed-sex groups, unless there is some cost of mating for the male (Simmons 2001). One potential cost could be sperm competition. Female ladybird beetles are slightly more likely to utilize sperm from the most recent mating, but there is tremendous variation in

sperm utilization ranging from using all of the first male's sperm to using all of the second male's sperm (reviewed in Simmons 2001). A group environment would likely have sperm competition because of the many possible male competitors, while in isolation there are no competitors. It may therefore be advantageous for a male to reduce mating motivation in a group environment to avoid sperm competition (Schwagmeyer and Parker 1990). This potential explanation for our observed pattern is analogous to work with dynamic state models that has explored how mating or egg-laying strategies can be conditional to the perceived environment (e.g. Mangel and Clark 1988; Luttbeg 2004).

There are, however, some problems with the idea that sperm competition is the cost to mating in this system. First, sperm competition models suggest that males change the amount of sperm transferred in response to competition (e.g. Parker et al. 1997; Ball and Parker 2007) as opposed to changing the frequency of mating as we observed. Secondly, we performed preliminary experiments suggesting that males kept in same-sex groups mated more frequently than males kept in mixed-sex groups (J.P.H., unpublished data). This result is inconsistent with the idea that males reduce their frequency of mating in response to the presence of more males so as to reduce sperm competition.

It is not clear what proximate mechanisms a male *C. maculata* could use to perceive a group environment and consequently decrease its matings, or how it may perceive isolation and consequently enhance its likeliness of mating. It does not seem like there is a short-term cue that comes from mating or the absence of mating. If that was the case, males from the mixed-sex group that did not mate during the conditioning period should have mated as frequently as males kept in isolation. However, in our continuous observations, only 17% of the males from mixed-sex groups that did not mate during the conditioning period were found to have mated during the observational period. This is compared to the 67% of males that had been isolated during the conditioning period which mated during the observation period. This cue may also be unlikely to stem from contact with other male ladybirds given the preliminary results discussed above. It seems more likely that males perceive their environment through contact with females. In another ladybird species, *Adalia bipunctata*, males respond to encounters with a contact pheromone present on or in a female's elytra (Hemptinne et al. 1996). Males could become more receptive to this sex pheromone when isolated from females or desensitized to its presence when kept in confinement with females. Either may change the male's propensity to mate and provide the proximate explanation for the observed pattern.

Multiple mating appears to be the norm for *C. maculata* and most ladybird species (Majerus 1994a; Hodek and Honěk 1996). Mating frequency can vary in response to age (Obata 1988b; Pervez et al. 2004; Srivastava and Omkar 2004; Omkar and Pervez 2005) and food availability (Obata 1988b), but studies have not shown how additional factors such as density may influence coccinellid mating behavior, even in cases where such factors are expected (as in Webberley et al. 2002). In addition to understanding sexual selection, this behavior may also have ecological implications. For example, *C. maculata* can be one of the most common ladybirds, but does not aggregate to its aphid prey as closely as other species (Schellhorn and Andow 2005). This study suggests that such aggregations may reduce the frequency of mating and its potential benefits. Such behavioral insights

could be crucial for understanding how to preserve this and other native species in the face of invasion from competitors (Harmon et al. 2007) and how to enhance their utility as biological control agents.

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