

Mating frequency positively associates with fitness in *Ophraella communa*

ZHONG-SHI ZHOU,¹ SERGIO RASMANN,² HAI-YAN ZHENG,^{1,3} ALAN WATSON,⁴ JIAN-YING GUO,¹ JIAN-GUO WANG³ and FANG-HAO WAN¹ ¹State Key Laboratory for Biology of Plant Diseases and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing, China, ²Department of Ecology and Evolutionary Biology, University of California, Irvine, California, U.S.A., ³Department of Plant Protection, Agricultural College, Jiangxi Agricultural University, Nanchang, China and ⁴Department of Plant Science, McGill University, Quebec, Canada

Abstract. 1. Why animals mate multiple times, owing to the lack of immediate fitness benefits, presents an intriguing problem for evolutionary biologists. Yet, the profusion of this behaviour suggests it must be maintained by natural selection via increased performance.

2. The possible benefits of multiple mating using the leaf beetles *Ophraella communa* LeSage, the biological control agent of the invasive common ragweed *Ambrosia artemisiifolia* L., were studied and the fitness consequences of single, twice, three, four, and unrestricted mating events were assessed.

3. Overall, it was observed that the number of copulation events was positively associated with fitness parameters of the insects. Insects performed the best under unrestricted mating regimes, with average increases of 48% in longevity, 75% in fecundity, and 55% in egg hatch rate. In addition, females that experienced unrestricted access to mates maintained very high viability over their entire reproductive lives. Nevertheless, insects also performed better when allowed to mate four times compared with once or twice.

4. The present findings thus support the hypothesis that multiple-mating behaviour is maintained owing to increased fitness benefits in the current and the next generation. Selection for re-mating is, therefore, expected in field populations, which is likely to happen naturally owing to the aggregate lifestyle of *O. communa*.

Key words. Common ragweed, fecundity, fitness, longevity, multiple mating, oviposition.

Introduction

The role of multiple mating in insects has always captured wide interest by ecologists and biologists (Thornhill & Alcock, 1983; Drummond, 1984; Foster & Ayers, 1996; Hiroyoshi & Mitsuhashi, 1999; Orsetti & Rutowski, 2003; Srivastava & Omkar, 2005; Taylor *et al.*, 2008; Flay *et al.*, 2010; Hoffer *et al.*, 2012). Although many insects mate only once, multiple matings within the reproductive season are ubiquitous across species (Thornhill & Alcock, 1983; Ridley, 1988; Tregenza & Wedell, 1998, 2000, 2002; Arnqvist & Nilsson, 2000; Jennions & Petrie,

2000; Zeh & Zeh, 2006; Hadrill *et al.*, 2008; House *et al.*, 2009; Harley *et al.*, 2010; Ronkainen *et al.*, 2010; Simmons & Beveridge, 2011). Several studies have shown that increasing mating frequency increases female fitness (Arnqvist & Nilsson, 2000; Fox & Rauter, 2003; Avila *et al.*, 2011), and positively influences adult longevity and fecundity of numerous insect species (Wasserman, 1986; Savalli & Fox, 1999).

Based on studies addressing benefits of multiple mating, several hypotheses have been proposed for why insects should benefit from mating several times (Arnqvist & Nilsson, 2000; Drnevich *et al.*, 2001; Omkar & Sahu, 2012). First, the reproductive success of adult male insects depends on their ability to achieve multiple copulations (James & Jaenike, 1992) or fertilisations (Eady, 1991), in which the production of offspring may be limited by the availability of sperm to fertilise the eggs.

Correspondence: Fang-Hao Wan, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, #2 West Road, Yuan-Ming-Yuan, Beijing 100193, China. E-mail: wanfanghao@caas.cn

As male insects may not be able to supply sufficient sperm when copulating only once with a female, it has been postulated that multiple mating behaviour may be needed to remedy low sperm viability (Walker, 1980; Harley *et al.*, 2010). Second, by mating several times, females increase their fertilisation success against the odds of an infertile first mating event (Daly, 1978; Walker, 1980; Keller & Reeve, 1995; Dickinson, 1997; Savalli & Fox, 1999). Third, in many insect species, mating initiates behavioural and physiological changes in the females, triggering responses in several processes related to fertility (Gillott, 2003; Avila *et al.*, 2011). For instance, seminal fluid proteins (SFPs), produced in the reproductive tract tissues of males, have been shown to induce numerous physiological and behavioural post-mating changes in females, including increased egg production, ovulation, and/or egg-laying rates (Gillott, 2003; Avila *et al.*, 2011). Finally, it has been shown that fertilisation efficiency is correlated with genetically superior males, therefore, multiple mating can serve as a mechanism for females to obtain the best genes (Keller & Reeve, 1995).

In spite of the several examples of how multiple mating or increased mating frequencies increases animal fitness, several other studies have shown that multiple mating can impose costs for both males and females (Thornhill & Alcock, 1983; Rowe, 1994; Chapman *et al.*, 1995; Crudgington & Siva-Jothy, 2000). For instance, the longevity of multiple-mated females decreased in *Drosophila* species as the number of matings increased (Wigby & Chapman, 2005; Avila *et al.*, 2011). Male longevity and reproductive investment decreased with increasing mating frequency (Taylor *et al.*, 1998; Papadopoulos *et al.*, 2010; McNamara *et al.*, 2012). Although there appear to be several benefits of multiple mating, it is not clear whether these benefits arise from improved fecundity, fertility or longevity. In the same context, if costs in fecundity, fertility or longevity outweigh the overall benefits, then multiple mating should decrease overall fitness.

Ophraella communa LeSage (Coleoptera: Chrysomelidae) is native to North America (LeSage, 1986; Futuyama, 1990), and has been successfully used for biological control of the invasive common ragweed, *Ambrosia artemisiifolia* L. (Asterales: Asteraceae), in Canada and China (Teshler *et al.*, 2002; Zhou *et al.*, 2009, 2010; Guo *et al.*, 2011). In recent years, the reproductive behaviour and biology of the beetle have been explored to facilitate the management of *A. artemisiifolia*. *Ophraella communa* adults have been observed to mate several times throughout the day and during their lifespan (Meng & Li, 2006; Zheng *et al.*, 2013).

We, therefore, hypothesised that a multiple-mating regime is under positive selection because *O. communa* beetles with higher mating frequencies should have higher fitness, which outweigh the possible negative direct effects of several consecutive copulations. To test this hypothesis, we measured adult longevity, female fecundity, and egg hatch rates across a range of mating regimes.

Materials and methods

Beetles were reared on common ragweed plants. Seeds were collected in Miluo, Hunan Province, China (28°49.089'N,

113°03.876'E) during September 2010, and stored at 4 °C, before being germinated in loamy clay soil in an unheated greenhouse at 14–15 h daylight. Plants were used for experiments when they were 15–16 cm tall. At the same sites, around 800 *O. communa* pupae from 40 randomly selected common ragweed plants were collected, and stored in clean transparent plastic boxes (19 × 12 × 6 cm³) covered with organdy mesh fabric (100 pupae per box), in the laboratory, at 28 °C, 70% RH, and 14 h daylight. To remove maternal effects on beetle performance, we reared three generations in the laboratory. In each generation, we randomly selected 200 males and 200 females to produce the next generation. In the third generation, newly emerged virgin males ($n = 800$) and females ($n = 800$) were randomly collected and kept separately on ragweed plants and served as the experimental stock population.

To manipulate the number of copulations during a beetle's lifetime, virgin males and females were randomly selected from the stock population described above and paired. Each pair of adults was placed on a common ragweed seedling that was inserted into plastic bottle (3 cm in diameter and 5 cm in height) filled with water in a transparent plastic box (19 × 12 × 6 cm³) covered with organdy mesh fabric, and observed continuously between 07.00 and 23.00 hours. As *O. communa* adults always mate more than four times between 07.00 and 23.00 hours, treatments consisted of separating males and females after they had copulated once, twice, three times, four times, or kept in an unrestricted copulation regime until the end of the experiment at 83 days ($n = 22$ pairs per treatment). For the unrestricted mating treatment, if the male died earlier, another newly emerged male was added. At the end of each treatment, except the unrestricted mating treatment, mated males and females were transferred separately into another new transparent plastic box (19 × 12 × 6 cm³) covered with organdy mesh fabric (a female or a male per box), and allowed to feed on common ragweed seedlings until death. Longevity (time until death) was scored for males and females, and fecundity was recorded for females. For the unrestricted mating treatment, only the longevity of the first male was recorded. Fresh seedlings were changed daily, and the number of eggs laid per female *O. communa* was counted. Observations ended, and longevity recorded when the female and the male adults died. We next scored the hatch rate of eggs that originated from females experiencing different mating regimes. Twigs with fresh eggs from the mating treatments were inserted into plastic bottles filled with water in order to avoid twigs wilting before eggs hatched. Eggs were checked daily, and the number of eggs hatched was recorded in all treatments until all eggs hatched. Daily egg hatch rate and average egg hatch rate per female were calculated in this experiment.

Finally, we performed an additional experiment to address the fact that in the continual mating treatment the beetles were continually housed with a conspecific, whereas in the restricted mating treatments, the females were left living individually after mating, which would lead to spurious conclusions when comparing the unrestricted mating versus the restricted mating regimes. The experiment was done as described above, but longevities of female and male *O. communa* adults were assessed using three different treatments; (i) virgin males and females were paired, and then separated when they had copulated once; (ii) a virgin

female and another virgin female, or a virgin male and another virgin male were paired, and allowed to live together for their entire lifespan; and (iii) virgin females or males were raised separately.

A two-way ANOVA was conducted to analyse the effect of mating treatment, sex, and their interaction on adult longevity. One-way ANOVAs were performed to assess the effect of mating treatment on female fecundity and hatch rates of eggs. Repeated measure ANOVAs were conducted to test the effect of mating frequency on female fecundity and egg hatch rate over time. All analyses were done using JMP, SAS Institute, 2004. The time-series analysis were done by incorporating zeros where data were missing as repeated measure ANOVA is robust with respect to outliers and does not assume sphericity or equal variances for every time period (Zar, 1999). For the last experiment, the effect of treatment on longevities was analysed separately for males and females using one-way ANOVAs. Longevity and fecundity data were $\log(x + 1)$ -transformed, and the hatch rates were arcsine square-root-transformed to meet homoscedasticity assumptions.

Results

Both female and male beetles had the longest longevity, 82 and 80 days respectively, in the unrestricted mating regime. Overall, females lived significantly longer than males independent of the mating regime treatment (Fig. 1a, sex effect; $F_{1,210} = 15.34$, $P < 0.0001$; and mating treatment by sex interaction; $F_{1,210} = 0.15$, $P = 0.96$). Longevity of adult beetles increased on average between 40% and 55% when they were allowed unrestricted mating compared with 1–4 mating events (Fig. 1a; mating treatment effect; $F_{4,210} = 492.86$, $P < 0.0001$), and both males and females lived 5–8% longer when allowed mating four times compared with 1–3 times.

Fecundity of *O. communa* females showed a monotonical increase with increasing mating frequency (Fig. 1b; mating treatment effect, $F_{4,105} = 5.09$; $P = 0.003$). The highest fecundity (1874.1 eggs per female) occurred in the unrestricted mating regime, and female fecundity was significantly higher in the four matings regime than in either the one or two matings regimes (Fig. 1b). Through time, daily fecundity was strongly affected by the number of mating events (Fig. 1c; time effect; $F_{93,13} = 26.19$, $P < 0.0001$, and time \times mating treatment interaction, Wilk's Lambda test, $F_{372,54.84} = 2.05$, $P = 0.0008$). Maximum daily fecundity was observed during the early ovipositional periods (i.e. the first 20 days) in all mating treatments, but whereas in the restricted mating regimes females stopped laying eggs after about 50 days, in the unrestricted mating treatment, egg laying stopped only after about 80 days (Fig. 1c).

Mating frequency also significantly affected the hatch rate of *O. communa* eggs (Fig. 2a; mating treatment effect, $F_{4,105} = 245.79$, $P < 0.0001$). The hatch rate remained at very high levels (around 80%) until the end of the experiment in the unrestricted mating regime, whereas, for the 1–4 time of mating events treatments the hatch rate drastically declined between 10 and 40 days after first oviposition (Fig. 2b; time

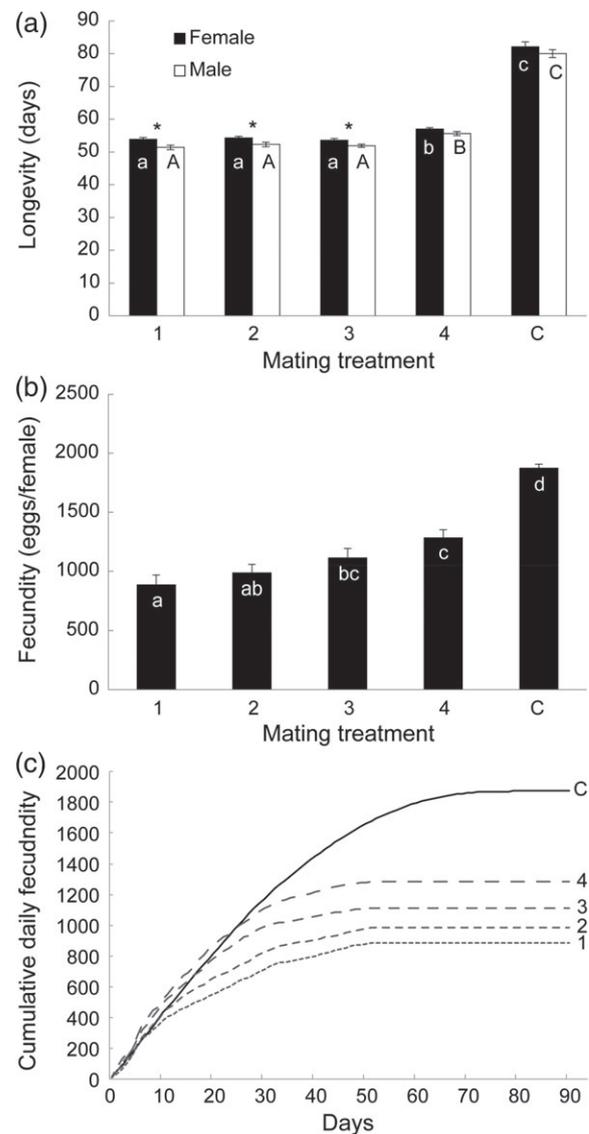


Fig. 1. *Ophraella communa* adult longevity and fecundity after different mating events. Virgin males and females were paired, and then separated when they had copulated once (1), twice (2), three (3), four (4) times, or allowed unrestricted mating (C). Shown is (a) female (black bars) and males (open bars) adult longevity, (b) female average fecundity, and (c) average daily egg oviposition per female across the different treatments. Bars are averages (± 1 SE). Different letters in bars show significant differences ($P < 0.05$, HSD Tukey). Asterisks indicate significant difference between males and females within a mating treatment.

effect; $F_{82,23} = 29.21$, $P < 0.0001$, and time \times mating treatment interaction, Wilk's Lambda test, $F_{328,94.78} = 1.62$, $P = 0.003$).

Finally, we found that longevities of female and male beetles increased on average between 130% and 190% when they were allowed to mate once compared with individuals that did not mate (Figure S1; female: $F_{2,63} = 275.92$, $P < 0.0001$; male: $F_{2,63} = 143.99$, $P < 0.0001$), but longevity did not differ significantly between individuals raised with a conspecific and those raised with no access to conspecifics (Figure S1).

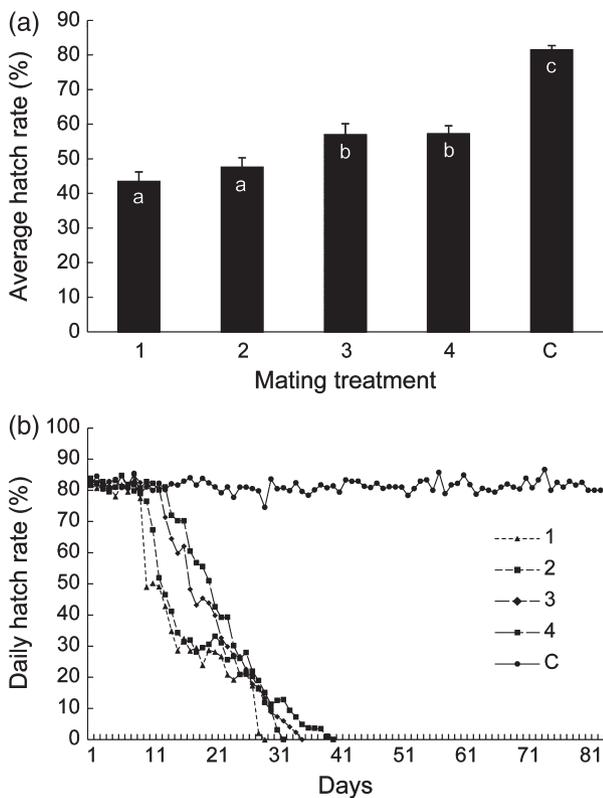


Fig. 2. Hatch rate of *Ophraella communa* eggs after parent beetles experienced different mating treatments. Virgin males and females were paired, and then separated when they had copulated once (1), twice (2), three (3), four (4) times, or allowed unrestricted mating (C). Shown is (a) average egg hatch rate and, (b) daily egg hatch rate across the different treatments. Bars are averages (± 1 SE). Different letters in bars show significant differences ($P < 0.05$, HSD Tukey).

Discussion

The present study showed that an increase in the number of matings of adult *O. communa* significantly increased longevity, survival, ovipositional period, and fecundity of female beetles, thus supporting previous evidence of the positive correlation between multiple mating and increased performance in insects (e.g. Southgate *et al.*, 1975; Wasserman, 1986; Ofuya, 1995; Savalli & Fox, 1999; Arnqvist & Nilsson, 2000; Eady *et al.*, 2000; Drnevich *et al.*, 2001; Taylor *et al.*, 2008).

Interestingly, the longevity of both males and females *O. communa* increased significantly in response to increasing mating frequency indicating that it is indeed mating, and not the company of a conspecific, affecting individual longevity. This implies that both sexes of *O. communa* similarly benefit from multiple mating. The increased longevity of females observed in this study could be explained by the fact that females may obtain nutritional resources from male investment and thus increase the females' physiological performance allowing them to produce more offspring (Ofuya, 1995; Chapman & Partridge, 1996; Taylor *et al.*, 2008). The fact that males also benefited from an unrestricted mating regime is more puzzling, as

classic theory predicts ecological and genetic costs; yet, no real benefits of multiple mating for males (Zeh & Zeh, 2001). However, multiple mating for males may also be favoured by natural selection via benefits such as higher probability of own offspring production with each new mate, or production of more genetically diverse offspring (reviewed in Dickinson, 1997). Alternatively, longevity and mating abilities may be positively correlated not because multiple mating confers a survival advantage, but because some quality of males makes them good at both surviving and mating (Dickinson, 1997). The present experiments, however, showed a general trend of multiple-mated males being more long lived, suggesting direct physiological benefits of multiple mating. The positive correlation between mating frequency and longevity could also be explained by changes in food intake. Multiple-mated males may increase food intake and thus compensate for mating costs, such as in *Adalia bipunctata* L. ladybirds where males increased feeding rates after spermatophore transfer thus compensating for spermatophore production (Perry & Tse, 2013). This suggests that additional food intake of multiple-mated *O. communa* males could favour individual life-time fitness, and this should be tested in future experiments.

The increased benefits of multiple matings are, however, not always visible. In contrast to our study, in several insect species, the longevity of males and females were not affected by mating frequency (Yang *et al.*, 2011), and it has been shown that female fitness decreases when mating times are increased (e.g. Wigby & Chapman, 2005; Avila *et al.*, 2011; McNamara *et al.*, 2012). For instance, there was a significant decrease in female fecundities of the leaf beetles, *Leptinotarsa decemlineata* Say (Orsetti & Rutowski, 2003) and the seed bug, *Lygaeus equestris* L. (Shuker *et al.*, 2006) with an increase in mating events. Indeed, it is expected that multiple mating is intrinsically associated with additional direct costs of reproduction (Rowe, 1994; Chapman *et al.*, 1995; Crudginton & Siva-Jothy, 2000). For example, repeated copulations have been shown to damage the female's reproductive tract (Shuker *et al.*, 2006). This and other mechanisms explain the decrease in longevity of multiple-mated females (Taylor *et al.*, 1998; Wigby & Chapman, 2005; Avila *et al.*, 2011; McNamara *et al.*, 2012). Additionally, as stated above, we should expect sex-biased cost:benefit ratios of multiple mating. For instance, copulation decreases the longevity of males but not that of females in *Saltella sphondylii* Schrank. However, when females were allowed to lay eggs, mated females died earlier than virgin females (Martin & Hosken, 2004). This implies longevity costs for males, but reproduction costs for females in some insect species. Here, we hypothesise that females may benefit from male investment during copulation, and both direct and indirect benefits of multiple mating may provide females, and males, with substantial fitness returns in the process of copulation, which may outweigh the negative direct effects of multiple mating (Fox, 1993a, 1993b; Ofuya, 1995; Chapman & Partridge, 1996; Taylor *et al.*, 2008).

We also observed an increased number of eggs laid when females had continuous access to mates. Previous studies have demonstrated that *Callosobruchus maculatus* F. females could lay more large-sized eggs, and larval survival could be increased when they have performed multiple matings (Wasserman &

Asami, 1985; Fox, 1993b). In our present study, although the egg size of *O. communa* was not measured, the hatch rate of progeny *O. communa* eggs increased significantly with the number of copulations. In particular, the hatch rate of progeny eggs decreased significantly with female age in which the number of mating events was limited to one, two, three, or four. However, females with unrestricted access to mates maintained egg viability averaging >80% over their entire reproductive lifespan, whereas the hatch rate of progeny eggs laid by females declined to 0 at the 29th, 32th, 35th, and 40th day after oviposition in one, two, three or four matings regime, respectively. This implies that the eggs laid by copulation-constrained females were unfertilised in the late ovipositional period, and suggests that multiple matings can significantly increase the number of fertilised eggs in insects. However, as a counterexample, Orsetti and Rutowski (2003) found that the hatch rate of *L. decemlineata* beetles decreased significantly with increasing mating frequency, suggesting species-specific responses and variable benefits of multiple matings.

All material benefit hypotheses focus on limiting resources that can be supplied for females and increasing the number of mating events can increase the resource, which should translate into the production of more offspring (Orsetti & Rutowski, 2003). In the present study, increasing the number of mating events increased not only the longevities of female and male *O. communa*, but also increased fecundity and ovipositional periods of females, and the hatch rate of progeny eggs laid. This implies that multiple mating is pivotal to the evolution and development of *O. communa* populations.

Our results show that multiple mating has fitness benefits for both males and females *O. communa*. For this reason, we expect that re-mating occurs and is maintained in natural populations. Re-mating in the *O. communa* system may be facilitated because both adults and larvae are gregarious when feeding on leaves of the same *A. artemisiifolia* plant (Zhou et al., 2010). Such behaviour provides females with a greater chance to meet their mates in the field. Our experimental design, however, did not allow us to measure whether multiple mating with the same partner or of switching partners is more effective for increasing the beetles' fitness. Future work is needed to disentangle the effect of gregariousness on mating preference and the insects' fitness outcomes.

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Supporting Information

Additional Supporting Information may be found in the online version of this article under the DOI reference: 10.1111/een.12184

Figure S1. Longevities of female (a) and male (b) *Ophraella communa* adults in three different treatments. (i) Mating once: virgin males and females were paired, and then separated when they had copulated once; (ii) no mating-1: a virgin female and another virgin female/or a virgin male and another virgin male were paired, and allowed to live together without limit; (iii) no mating-2: virgin females/or virgin males were raised separately. Different letters in bars show significant differences ($P < 0.05$, HSD Tukey).

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