

Male remating and its influences on queen colony foundation success in the bumblebee, *Bombus terrestris*

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Abstract – In most bumblebee species, queens mate with only one male, but males can mate more than once. However, the effects of male remating on colony foundation success of queens remain poorly understood. To test for effects of male remating, we assigned *Bombus terrestris* queens randomly to one of two groups: virgin males or non-virgin males that have mated once before. When males were allowed to mate for a second time with virgin queens, 58.57 % successfully remated. There was no significant difference in queen hibernation survival between groups, but queens mated with non-virgin males were more successful in founding a colony, and produced more workers and males than queens mated with virgin males. The mean copulation duration for the second mating of males (48.02 ± 14.36 min) was substantially longer than for the first mating (32.44 ± 8.74 min). Results also showed that male weight influenced both male remating success and copulation duration.

Bombus terrestris / mating behavior / copulation duration / male weight / colony development

1. INTRODUCTION

In the wild, monandrous *Bombus terrestris* queens emerge from hibernation in spring and found colonies. Towards the end of the colony cycle in late summer, sexuals (virgin queens and males) are produced. Young queens that mate with only one male hibernate and survive to produce the next generation (Alford 1969; Schmid-Hempel and Schmid-Hempel 2000).

In contrast to honeybees, *B. terrestris* queens and males can be mated in flight cages which allow mating behavior to be observed easily. Furthermore, *B. terrestris* males do not die after copulation and can remate (Amin et al. 2012). Males store about 600,000 sperm in their accessory testes, which is enough to inseminate more

than one queen. Between 40,000 and 60,000 sperm cells can be found in the spermatheca of once-mated *B. terrestris* queens (Tasei et al. 1998), and these numbers are similar in queens that have copulated with previously-mated males (Tasei et al. 1998; Duchateau and Marien 1995). In a study of *B. terrestris* queens artificially inseminated with sperm from one or several males, Baer and Schmid-Hempel (2005) reported that multiply insemination affect the performance of queens. There is also a positive correlation between male body size and sperm length in *B. terrestris* (Baer et al. 2003).

In many insect species, males transfer both sperm and additional substances to female sexual tracts. These male substances regulate physiological and behavioral changes in females (Chapman et al. 1995; Gillot 2003). Baer et al. (2001) reported that fatty acids in mating plugs transferred by males prevent the further mating of *B. terrestris* queens. Males thus determine not only mating frequency (only once) of queens but also copulation duration (Baer 2003), which normally lasts

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20–40 min, even though most sperm is transferred within the first 2 min (Duvoisin et al. 1999; Amin et al. 2007, 2009). Variation in male body size also affects mating behavior, with lighter males starting to mate later and copulate longer than heavier males (Amin et al. 2012). Further, traits such as age and size (Amin et al. 2012), volatile pheromones secreted from the labial glands (Bergstrom 1982) and environmental conditions such as temperature, humidity (Amin et al. 2010), photoperiod (Kwon et al. 2006), and nest material (Imran et al. 2015) also have effects on male mating success.

Studies have shown that male remating increases male fitness in some insect species, such as *Drosophila ananassae* (Singh and Singh 2000) and *Phthoricea striata* (Latha and Krishna 2014). It has also been reported that a positive correlation exists between remating success and male size in *Drosophila bipunctinata* (Prasad et al. 2008). In the last decade of bumblebee research, a number of reviews have examined male sexual selection (Baer 2003), male mating behavior (Paxton 2005), and the evolutionary significance of long copulation duration (Brown and Bear 2005). However, there remains a knowledge gap surrounding the effects of male remating on colony foundation success of queens. In the present study, we randomly allocated queens to one of two mating groups—virgin males or non-virgin males—and tested whether male remating influences the hibernation and colony foundation success of queens and various colony development traits in *B. terrestris*. We also compared mating success, copulation duration, and weight of males in their first and second mating.

2. MATERIALS AND METHODS

2.1. Obtaining young queens and males

B. terrestris queens and males were obtained from laboratory-reared colonies that started male and queen production. At the end of the colony life, newly emerged males from 15 colonies and newly emerged queens from 15 other colonies were collected daily in order to avoid inbreeding. One-day-old queens and males were put into separate storing boxes with 50 individuals in each box. They were kept in a climate-

controlled room at 27–28 °C and 50 % RH with ad libitum fresh thawed pollen and sugar solution (50 Brix) until sexual maturity, which occurs after 7 days for queens and 12 days for males (Duchateau and Marien 1995; Duvoisin et al. 1999).

2.2. Mating experiment and hibernation of queens

Queens and males were mated in mating boxes (30 × 30 × 50 cm) in an illuminated mating room at 23 ± 1 °C and 50 ± 5 % RH. One hundred queens were randomly selected from storing boxes and placed in five separate mating cages with 20 queens in each cage. Thirty randomly selected males were then simultaneously added to each mating cage. Immediately after the initiation of copulation, the time was noted and each mating pair was separately transferred to a transparent plastic box. As soon as copulation was terminated, the copulation duration was recorded, after which males were individually weighted on an electronic balance (sensitivity ±0.001 g). After weighing, each male was kept alone in a separate box and fed with sugar solution for 3 h. To study male remating, 100 new queens were placed in the mating cages (20 queens for each cage). At the end of the feeding period, each male was individually placed in a mating cage and his remating behavior was observed. Copulation duration was calculated as mentioned above. If mating did not occur within 1 h, males were discarded and recorded as not having mated. Queens that mated with virgin and non-virgin males were individually placed in plastic tubes (Ø40 × 60 mm) and put into artificial hibernation at 2.5 °C for 2 months. At the end of the hibernation period, surviving and non-surviving queens were recorded to determine the effect of insemination by virgin and non-virgin males on queen hibernation success.

2.3. Colony rearing and observations

A total of 85 hibernated queens (46 mated with virgin males and 39 mated with non-virgin males) were placed in starting boxes and allowed to found colonies in a climate-controlled room (27–28 °C and 50 % RH). Queens and their colonies were fed ad libitum with freshly thawed pollen and sugar solution (50 Brix). To stimulate egg-laying, one callow *B. terrestris* worker was added to each queen. These callow workers were replaced by young individuals every week until the first

offspring emerged (Gurel and Gosterit 2008). When the colony reached about 10 workers, nests were transferred to larger boxes. From then on, nests were checked twice a week and developmental traits were recorded including: date of egg-laying of the queen (colony initiation), number of egg cells in first brood, timing of first worker emergence at the beginning of the social phase (Duchateau and Velthuis 1988), timing of gyne production and switch point where the queen lays the first haploid egg (Bourke 1997; Duchateau et al. 2004), and total number of workers, males, and gynes produced by each colony. Queens that produced more than 10 workers were considered to have successfully founded colonies, and colonies that reached 50 or more workers were considered to have reached sufficient size for providing normal pollination services, so we used the same threshold for separating large and small colonies. While the date of gyne production and switch point were determined relative to the beginning of the social phase, other dates were recorded from the time that the queens were put into their starting boxes. To calculate gyne production time, the development time of an average offspring queen (gyne; 30 days) was subtracted from the date of emergence of the first offspring queen. The timing of the switch point was calculated by subtracting the male developmental time (25 days) from the date of emergence of the first male (Duchateau and Velthuis 1988).

2.4. Data analysis

Descriptive statistics of mating and colony development traits were calculated. We used a square root transformation to approximate normality and two sample *t* tests were run to determine the effects of male remating on some colony development traits in Minitab Statistical Software. The percentage of queens laying eggs, the percentage of queens producing 10 and 50 workers, the remating ratio of males, and the survival ratio of queens in hibernation were compared using two proportions *z* tests. Additionally, we calculated Pearson correlation coefficients between traits such as male weight, copulation duration, and total number of diploid individuals (workers and gynes) produced in colonies. Weights of males in their first mating were used for data analysis.

3. RESULTS

A total of 70 virgin males mated at their first opportunity. Of the non-virgin males, 41

individuals (58.57 %) remated while 29 did not. During the 2-month hibernation period, survival rate of queens in the two groups was not significantly different: 61 of queens mated with virgin males (87.14 %) and 39 of queens mated with non-virgin males (95.12 %) survived ($Z = -1.36$; $P > 0.05$).

Copulation duration of 41 males during their first and second mating, and weights of 41 remated and 29 unremated males in their first mating (Table I) differed significantly ($P < 0.01$). Copulation duration increased when males mated for a second time (48.02 ± 14.36 min versus 32.44 ± 8.74 min) and these remated males were heavier (0.317 ± 0.044 gram) than unremated males (0.268 ± 0.046 gram).

Being inseminated by a non-virgin male increased the percentage of queens producing at least 10 workers ($Z = -2.23$; $P < 0.05$) and the percentage of queens producing at least 50 workers ($Z = -3.31$; $P < 0.01$) but not the percentage of queens laying eggs. In queens mated with virgin males ($n = 46$), 29 produced at least 10 workers and 19 produced colonies with at least 50 workers; in queens mated with non-virgin males ($n = 39$), 33 produced more than 10 workers and 30 produced more than 50 workers ($P < 0.05$ in both cases; Figure 1).

Queens mated with non-virgin males not only produced more workers ($P < 0.05$), but also more males ($P < 0.01$) than queens mated with virgin males. However, male remating did not influence the total number of new virgin queens. Gyne and male production in colonies founded by queens mated with virgin males started earlier but this difference was not significant (Table II).

Finally, we found a significantly negative correlations between male weight and copulation duration ($r_{111} = -0.390$, $P < 0.001$). However, the total number of diploid individuals (workers and gynes) produced in colonies was not significantly correlated with male mating weight ($r_{48} = -0.098$, $P > 0.05$) and copulation duration ($r_{48} = 0.057$, $P > 0.05$).

4. DISCUSSION

Our experiments showed that being inseminated by a non-virgin male does not influence queen

Table . Copulation durations of males in their first and second mating, and weights of remated and unremated males in their first mating.

Copulation duration (min)		Weight of males (g)	
Mating number	Mean \pm S.D	Mating success	Mean \pm S.D
First mating ($n = 41$)	32.44 \pm 8.74	Remated ($n = 41$)	0.317 \pm 0.044
Second mating ($n = 41$)	48.02 \pm 14.36	Unremated ($n = 29$)	0.268 \pm 0.046
P value	<0.001	P value	<0.001

hibernation survival in the bumblebee *B. terrestris*. Previous studies have indicated that queen survival during diapause is strongly determined by the weight of the queen and the duration of her diapauses (Beekman et al. 1998; Gosterit and Gurel 2009). Although the actual mechanism remains unknown, male genotype also influences queen survival (Korner and Schmid-Hempel 2003). Greeff and Schmid-Hempel (2008) reported that both queens and the males obviously benefit from prolonged survival of the hibernating queen because any sexual conflict is unlikely to occur during this period. Baer and Schmid-Hempel (2005) found that despite receiving approximately the same total amount of sperm, queens inseminated with a mixture of sperm from two males had a decreased hibernation success compared with queens inseminated with sperm

from a single male. However, data from the present study indicate that mating with non-virgin males (which have mated once before) did not affect queen hibernation survival.

Comparison of the copulation duration between groups in this study revealed that non-virgin males copulated for significantly longer periods than virgin males. Similarly, Amin et al. (2007) found a positive correlation between male mating number and copulation duration in *B. terrestris*. According to Brown and Bear (2005), possible reasons for this relationship might be that males invest more in mate-guarding behavior with each copulation or that it takes longer to transfer sperm and mating plug material with each copulation. Previous studies have also demonstrated a strong positive correlation between copulation duration

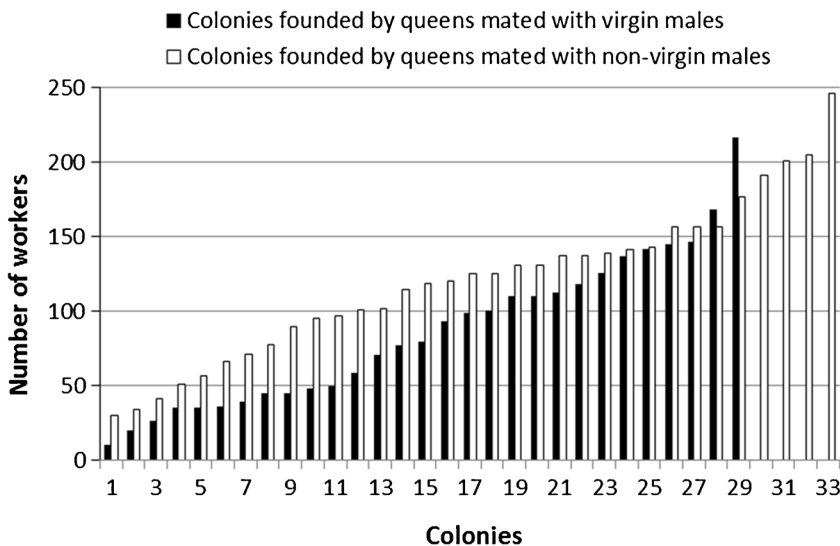
**Figure 1.** Number of workers produced in all colonies founded by queens mated with virgin and non-virgin males.

Table II. Developmental traits of colonies founded by queens mated with virgin and non-virgin males.

Colony development traits	Founder queens: mated with				
	Virgin males		Non-virgin males		<i>P</i>
	<i>n</i>	Mean \pm S.D	<i>n</i>	Mean \pm S.D	
Colony initiation time (days)	41	10.90 \pm 3.42	37	10.43 \pm 1.38	0.439
Number of egg cells in first brood	39	5.95 \pm 2.29	37	6.86 \pm 2.63	0.109
First worker emergence (days)	29	33.41 \pm 5.33	33	31.40 \pm 2.37	0.054
Timing of gyne production (days)	25	7.88 \pm 11.43	23	12.26 \pm 7.44	0.126
Switch point (days)	24	-6.42 \pm 14.85	30	-1.40 \pm 13.57	0.201
Total number of workers	29	86.28 \pm 50.92	33	120.21 \pm 51.41	0.012
Total number of males	30	30.07 \pm 28.24	33	57.88 \pm 38.58	0.002
Total number of gyne	25	24.80 \pm 15.80	23	18.39 \pm 12.18	0.125
Total number of diploid individuals	25	106.60 \pm 48.50	23	123.50 \pm 43.60	0.211

and frequency of multiple mating in some North American bumblebee species (Foster 1992). This is in contrast to polyandrous *Bombus hypnorum*, in which the likelihood of remating in queens increases with decreasing copulation duration (Brown et al. 2002).

We also found that heavy males tended to remate more often, and copulate for less time, compared with lighter males. Similarly, Amin et al. (2007) suggested that copulation duration was negatively correlated with male body mass, but positively with queen body mass. Male body mass was a significant predictor of the speed of mating initiation and copulation duration, with large males mating more quickly (Amin et al. 2012). Consequently, males of *B. terrestris* seem to control both copulation duration and queen mating frequency (Baer 2003).

B. terrestris colonies show much variation in the number of workers, males and queens produced even if colonies are kept under the same laboratory conditions (Beekman and van Stratum 2000). Many factors such as split sex ratio, variable worker/larva ratios, food quality and quantity, diapause history of founder queen influence worker, male, and queen production (Duchateau and Velthuis 1988; Ribeiro et al. 1996; Duchateau et al. 2004). Duchateau et al. (2004) hypothesized that sex ratio is correlated both with the absolute timing of switch point and competition point and

with the time span between these two crucial switches. Colonies with an early switch point tend to have an early competition point and a male biased sex ratio. Our data show that the switch point occurred relatively early in the colony cycle in both groups.

In this study, all colonies were kept in identical climatic conditions, and were fed the same pollen and sugar water, and were headed by queens that did not differ in diapause history. Interestingly, however, we found that queens mated with non-virgin males were more successful in founding a colony, and produced more workers and males, but that the number of new queens produced by colonies appeared to be similar in both groups. Remated males transfer less sperm than virgin males in *Bombus atratus* (Garofalo et al. 1986). But *B. terrestris* males apparently transfer enough sperm to the bursa copulatrix of queen's spermatheca no matter whether they are virgin or not (Tasei et al. 1998). As all other factors were controlled for, this suggest that males that are able to mate twice either provide more viable sperm, or better quality genes in their sperm, so that queens mated with non-virgin males produce stronger colonies. This is interesting because numerical sex ratios in *B. terrestris* are often male biased, so that not many males may get the chance to mate multiple times.

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Accouplement multiple des mâles et son influence sur le succès de la fondation de la colonie par la reine, chez le bourdon *Bombus terrestris*

Bombidae / comportement d'accouplement / durée de l'accouplement / poids du mâle / développement de la colonie

Der Einfluss männlicher Mehrfachpaarung auf den Erfolg der Koloniegründung der Königin bei der Erdhummel *Bombus terrestris*

***Bombus terrestris* / Paarungsverhalten / Dauer der Paarung / Gewicht des Männchens / Volksentwicklung**

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