Research article

Dynamics of multiple-mating in the bumble bee *Bombus hypnorum*

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Summary. The mating systems of social insects range from monandry, through low levels of polyandry to extremely high levels of polyandry. While the distribution of these mating systems across taxa is increasingly well-known, little is known about the proximate reasons behind the number of times social insect queens mate. Here we examined the role of mating duration and mating partner in predicting multiple mating in the naturally polyandrous bumble bee, *Bombus hypnorum*. We found that, as mating duration decreased, the likelihood of re-mating increased. Weak evidence suggested that male colony-of-origin may also influence re-mating. Dissections showed that males transferred a mating plug to queens. The plug does not last as long as in the congener *B. terrestris*, and males of different colonies varied in the allocation of resources to plug production. These results provide insight into the proximate factors behind multiple mating in bumble bees.

Key words: Polyandry, mating duration, multiple-mating, *Bombus hypnorum*, mating plug.

Introduction

Mating systems in animals reflect the conflicting interests of the male and female participants. According to sex-specific differences in investment into offspring, males, who typically invest little per offspring, are generally selected to maximise their number of matings and to monopolise receptive females, whilst females, who typically invest much more per offspring produced, are generally expected to mate with few, but with the best males (Halliday, 1983; Andersson, 1994). Whether females mate singly (monandry) or multiply (polyandry) is a particular instance of the evolutionary outcome of this intrinsic conflict. Consequently, the mating traits associated with single or multiple mating have recently been the focus of considerable attention (Yasui, 1998; Arnqvist and Nilsson, 2000; Jennions and Petrie, 2000).

In invertebrates, polyandry appears to be common, perhaps driven by direct benefits to the female, despite possible associated detrimental effects such as a decline in female lifespan (Thornhill and Alcock, 1983; Arnqvist and Nilsson, 2000). In contrast, most social Hymenoptera species appear to be monandrous, or to exhibit only very low levels of polyandry [the exceptions are the honey bees, leaf-cutting ants, Vespine wasps and *Pogonomyrmex* ants (Boomsma and Ratnieks, 1996; Strassmann, 2001)]. While numerous hypotheses for the existence of high levels of polyandry in social Hymenoptera have been suggested (reviewed by Crozier and Fjerdingstad, 2001), much less attention has been paid towards explaining the more anomalous widespread monandry (Strassmann, 2001). Bumble bees, which are annual eusocial insects, provide a model system for investigating social Hymenopteran mating systems. Unlike most social Hymenoptera they are easily mated in the laboratory, allowing the measurement of important features of the mating system. Recent work on a common European monandrous species, *Bombus terrestris*, has shown that males appear to impose monandry on queens by inserting a mating plug during copulation (Duvoisin et al., 1999; Baer et al., 2001; Sauter et al., 2001). In contrast, whether a virgin queen chooses to mate in the first place is likely to be under female control (Sauter and Brown, 2001). However, the imposition of single mating by males may be in the short-term interest of both sexes, as queen fitness with respect to multiple mating follows a U-shaped curve, being high for singly-mated queens, lower for queens with expected mating frequencies between 1 and 2.5, and higher again for queens with an expected mating frequency of 2.5 to 3 males (Baer and Schmid-Hempel, 2001). However, this convergence of interests holds only if queens are unable to shift directly from monandry to a relatively large number of matings.

While most *Bombus* species examined so far using molecular tools appear to be monandrous (Estoup et al., 1995; Schmid-Hempel and Schmid-Hempel, 2000), one exception is the European species *B. hypnorum*, which can mate up to 6 times (although with an average of about 2 matings) (Estoup

et al., 1995; Schmid-Hempel and Schmid-Hempel, 2000; Paxton et al., 2001). In this paper, we present data on the mating behaviour of this polyandrous species. Specifically, we examine the dynamics of mating, concentrating on mating duration – the length of time a copulation takes – and the mating plug, in an attempt to understand the proximate reasons behind polyandry in this species.

Methods

All animals used were the sexual offspring of laboratory-reared colonies of *B. hypnorum*. The mother queens of these colonies came from two populations, Switzerland and Sweden. For all mating experiments described below, young queens were placed in clear plastic boxes ($12.5 \times 10 \times 7$ cm) with two brother males from an unrelated colony. To ascertain the temporal dynamics of mating, we recorded the time spent mating ("mating duration"). The initiation and completion of a mating can be unambiguously scored as the linking and unlinking, respectively, of male and female external genital regions.

Mating duration

The first set of mating experiments was designed to investigate the patterns associated with multiple mating and used queens and males from five Swiss colonies of *B. hypnorum* in 1997. On day one, we presented individual queens with two males (see above). Once copulation began, we removed the second non-copulating male from the box. On the following day (day two) we presented those queens that mated on the first day with two males (from a different colony to that used the previous day) and allowed them to mate again. We recorded if and for how long a second mating occurred. Opportunities for matings were provided on consecutive days for logistical reasons.

Mating plug dynamics

Preliminary dissections showed that, like *B. terrestris* (Duvoisin et al., 1999), males of *B. hypnorum* insert a mating plug into the queen during copulation (B. Baer, unpublished data). The second set of mating experiments was therefore designed to determine how long this mating plug remains in the bursa copulatrix (the organ where sperm is placed by males before its transfer into the spermatheca) of females. For this experiment we used queens and males from four colonies of Swedish *B. hypnorum* in 2000. We set up animals for mating as described above, but queens were only mated once. Males and queens used in these experiments had an average ($\pm$SD) age of 5 ($\pm$4.1) and 4 ($\pm$3.4) days respectively. We assigned mated queens to one of five treatment groups. Queens in these groups were frozen at $-80\,^{\circ}$C at intervals of 0, 12, 24, 36 and 48 hours post-mating; these time intervals were based on those used for *B. terrestris* by Duvoisin et al. (1999), to allow for a direct comparison between this monandrous species and the polyandrous *B. hypnorum*. Queens were then dissected blind to check for the presence of a mating plug and filling of the spermatheca. Based on results from these dissections (see Results), we mated a further set of queens and froze them at intervals of 6, 12, 18 and 24 hours after mating. Dissections were carried out as before.

In addition, we conducted a preliminary analysis of male investment into the mating plug. The mating plug in bumble bees comes from the accessory glands (Duvoisin et al., 1999). We examined the size of the accessory glands in males from two colonies of *B. hypnorum*. We made the reasonable assumption that gland size correlates with the volume of plug substances that can be produced and stored, or, at least, reflects male investment into the plug. Given that the mating plug is transferred rapidly and at the onset of copulation (Duvoisin et al., 1999), we assume that the plug is not synthesised *de novo* during a single copulation event, and thus its size is limited by the storage capacity of the accessory glands. Males were removed as callows from two colonies of *B. hypnorum* (Swedish) and allowed to mature for 7 days. We then dissected out the accessory glands of 14 males (seven from each colony) and laid them flat on a cover slip. Gland mass was measured to the nearest nanogram using a Mettler-Toledo UMT2 scale. To control for differences in absolute gland mass due to differences in male size, we measured the length of the radial cell of the right wing (a correlate of body size) under a dissecting scope at 25X magnification, and divided gland mass by radial cell length to produce a relative measure of mass.

Statistical analyses

To determine whether mating duration differed among samples we used *t*-tests for samples with equal or unequal variances (equality of variances was tested using Levene's test) and corrected the results for multiple tests using step-up sequential Bonferroni correction (Hochberg, 1988). All *P*-values are given in their uncorrected form, with significance determined by Bonferroni testing. To determine what factors might contribute to multiple mating we used a logistic regression following the method suggested by Hosmer and Lemeshow (1989). Independent variables were queen colony (deviation coding), 1st male colony (i.e., the colony of the male with whom the queen had already mated) (deviation coding), and duration of 1st mating (continuous variable), with occurrence of a 2nd mating as the dependent categorical variable. All possible interactions were tested to see whether they improved the model fit.

We examined mating plug persistence using G-tests with Williams' correction. Finally, accessory gland size was analyzed using a *t*-test for samples with equal variance. All statistics were done using SPSS 10 for the Macintosh.

Results

Mating duration – single vs. multiply mating queens and cross-population comparisons

On the first day of the double-mating experiment, 72 queens mated. Only 16 of these queens (22%) chose to remate when given the opportunity on the 2nd day of the experiment. The average duration of first matings was shorter (but not significantly so after Bonferroni correction for multiple tests) for queens that mated again than for those that did not (19.7 vs. 28.6 min;. $t_{62}=2.220$, $P=0.03$, N.S.; Table 1). The second mat-

Table 1. Summary data for mating duration in *B. hypnorum* queens. *N* is the number of queens observed

Experimental set	Mean duration $\pm$S.E. (min)	*N*	Range (min)
Set 1 – Mating duration queens			
all 1st matings[a]	26.3 ± 1.78	64	$6-75$
Singly-mated queens			
B. hypnorum[a]	28.6 ± 2.09	48	$7.6-75$
Doubly-mated queens			
1st matings[a]	19.7 ± 2.87	16	$6-43.9$
2nd matings[a]	16.0 ± 2.28	16	$2-31.3$
total mating[a]	35.7 ± 3.54	16	$13.8-62.2$
Set 2 – Mating plug queens			
1st matings[b]	31.6 ± 1.51	82	$10-86$

[a] Swiss population.
[b] Swedish population.

Table 2. The best-fit logistic regression model for the likelihood of mating a second time in *B. hypnorum*. For statistical details, see text

Variable	B[a]	S.E.[b]	Wald[c]	D.F.	P-value	R[d]	Exp(B)[e]
Duration of 1st mating	−0.0668	0.0297	5.0560	1	0.0245	−0.2069	0.9354
Male colony			6.0173	3	0.1108	0.0156	
colony 1	1.6640	9.1822	0.0328	1	0.8562	<0.001	5.2803
colony 2	3.4150	9.2264	0.1370	1	0.7113	<0.001	30.4177
colony 3	−5.5369	27.4958	0.0406	1	0.8404	<0.001	0.0039
Constant	−0.4295	9.2005	0.0022	1	0.9628		

[a] The estimated logistic regression coefficient.
[b] The standard error of B.
[c] The Wald statistic, which tests whether the coefficient B is different from zero.
[d] The R statistic examines the partial correlation between each independent variable and the dependent variable; negative values indicate that as the variable increases in value, the probability of a 2nd mating occurring decreases; small values indicate that the variable has only a small partial contribution to the model.
[e] The log odds for the occurrence of a second mating.

ings of doubly mated queens were not significantly shorter than their own first matings (16.0 vs.19.7 min; paired *t*-test: $t_{15}=0.87$, $P=0.397$, Table 1), but were significantly shorter than the matings of queens that mated once only (16.0 vs. 28.6 min $t_{62}=3.241$, $P=0.002$; Table 1). There was no correlation between the time a doubly-mated queen spent in copulation for its first and second matings (Spearman rank correlation: $r_S=-0.42$, $N=16$, $P=0.105$). Finally, the total time spent *in copula* was not significantly longer for those queens that mated twice than for those that chose to mate once only (35.7 vs. 28.6 min; $t_{62}=1.724$, $P=0.09$; Table 1).

Using data from the mating plug experiments (see below) we were able to compare mating duration across two populations, Swiss and Swedish, of *B. hypnorum*. We found a trend for Swiss *B. hypnorum* queens (all first matings) to have shorter copulations than Swedish queens, but this was not significant after Bonferroni correction for multiple tests (26.3 vs. 31.6 min; $t_{144}=2.25$, $P=0.026$, N.S.; Table 1).

Factors predicting multiple mating

Whether a queen remated was related to two factors, the duration of the first mating and the colony identity of the male involved in this first mating. The best-fit logistic regression model included duration of first mating and male colony (while not significant within the model, the presence of colony identity in the model significantly improved the model fit), and correctly classified 77.78% of the observations (43 out of 47 cases where the queen did not mate again, and six out of 16 cases where the queen did remate). Within the model, duration of the first mating was a significant predictor of remating (Logistic regression: Wald statistic=5.056, DF=1, P=0.0245; Table 2). As the duration of first mating increased, the likelihood of remating decreased exponentially by about 7% for each additional minute of mating (Exp(B)=0.9354; Table 2) and as shown in Fig. 1.
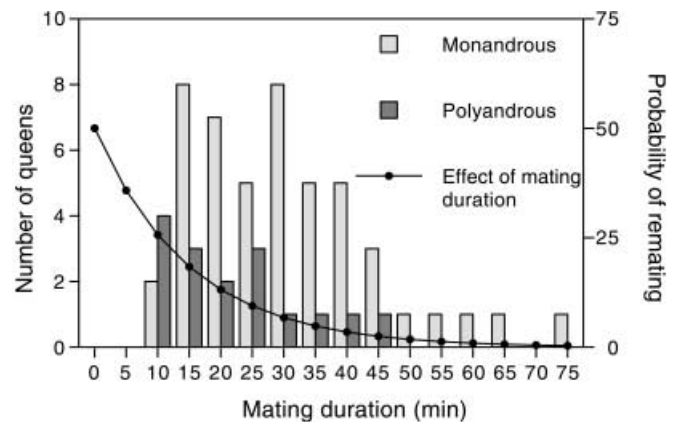

Figure 1. The relationship between mating duration, monandry and polyandry in *B. hypnorum*. The histograms (left *y*-axis) show the distribution of mating duration for monandrous (light bars; *N*=48) and polyandrous (1st mating)(dark bars; *N*=16) queens. The line (right *y*-axis) shows the predicted probability of re-mating as a function of the duration of the first mating, and as calculated from the logistic regression model (see Table 2 for statistics)

Mating plug dynamics

The initial analyses showed that, whilst nearly all queens were successfully inseminated (47 of 49 queens had a filled spermatheca; in three additional queens the spermatheca could not be found and thus was probably empty), most mating plugs in *B. hypnorum* had disappeared before 12 hours after mating (Fig. 2a). Data from the second experimental set showed that most plugs disappeared between 6 and 12 hours post-mating (Fig. 2b; *G*-test with Williams' correction: adjusted-G_3=16.5, $P<0.001$).

Males from the two colonies differed in the relative mass of their accessory glands (Absolute mass, colony 1: X±SD=0.7±0.17 ng; colony 2: X±SD=0.5±0.13 ng; t=3.039, DF=11, P=0.011), suggesting environmental or genetic differences in investment into mating plugs.
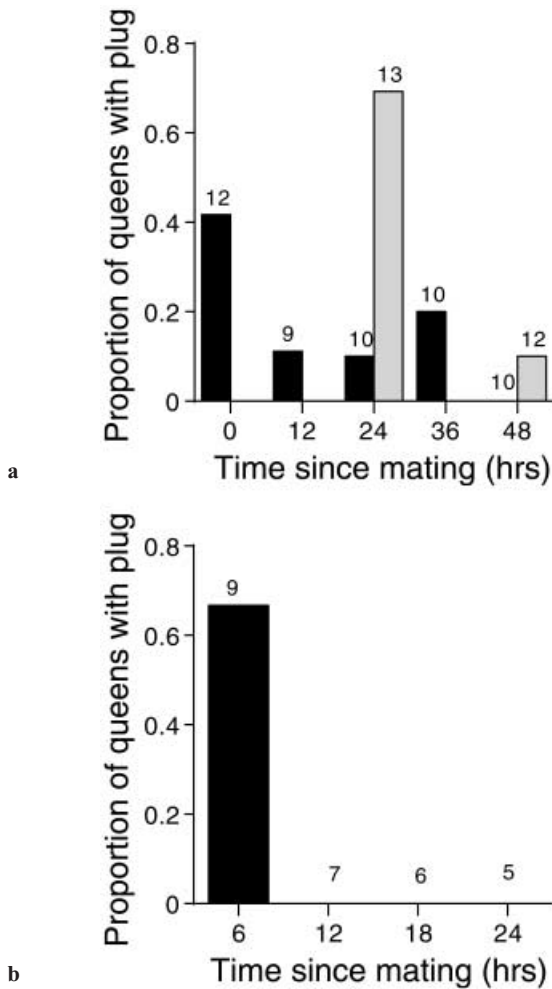
a

b

Figure 2. The dynamics of mating plug disappearance in *B. hypnorum*. a) shows data from the first set of experiments, where queens were frozen at 0, 12, 24, 36 and 48 hour intervals after mating (dark bars), as well as comparable data for *B. terrestris* (light bars; from Duvoisin et al. (1999)). b) shows data from the second set of experiments, where queens were frozen at 6, 12, 18 and 24 hour intervals after mating. Bars show the proportion of queens with a mating plug, and values above bars show the sample size for each group. Total sample size for *B. hypnorum* in a) is 51, as in one of the 12-hour queens the dissection failed. The differences between the two experimental runs are not statistically significant

Discussion

What are the proximate cues that predict polyandry? We found that the likelihood of remating in *B. hypnorum* was significantly related to the duration of the previous mating. Two potential explanations suggest themselves, depending upon whether mating is viewed from the male or the queen perspective. First, in the congener *B. terrestris*, males prevent female re-mating with a mating plug (Sauter et al., 2001). We found that *hypnorum* males similarly deposit a mating plug in the female's reproductive tract, although it does not last as long as that of *terrestris* (G-test for plug presence 24-hours post-mating, adj-G, = 8.37, P < 0.005). While the mating plug is transferred rapidly at the start of mating in *B. terrestris* (Du-

voisin et al., 1999), and after the sperm has been transferred, males remain attached to queens long after this event (Duvoisin et al., 1999). If male attachment is necessary for proper plug function, perhaps by enabling the plug to set in the correct part of the reproductive tract, shorter mating in *B. hypnorum* may reflect less effective male control. An alternative explanation is that mating duration may impose a cost on the queen, in terms of energy or exposure to predation, and thus female choice to re-mate may depend upon previous time spent mating and the allocation of resources to mating activity. Our data cannot distinguish between these two hypotheses.

While a non-significant effect within the model, the presence of male colony in the final logistic regression model for polyandry (Table 2) suggests a role for colony-level male variation in determining re-mating. Intriguingly, we also found significant differences between colonies in male investment into accessory glands, the origin of the mating plug substance. Such colony variation in plug quality in *B. hypnorum* could explain the presence of the factor "male colony" in the final logistic regression model. If smaller plugs contain less of the active anaphrodisiac chemical (Baer et al., 2001), then males producing such plugs may be less able to prevent queens from re-mating. This could be tested explicitly by injecting plug material from different colonies into queens and then attempting to re-mate them under controlled conditions. Whether male accessory gland variation is due to environmental or genetic differences remains unknown, although it is likely that environment plays a major role. Colony-level variation in other mating-related traits, such as sperm-length (B. Baer, unpubl. data), suggests that such variation, hitherto relatively unexplored in social insect males, may play a significant role in mating interactions.

The mating plug in *B. terrestris* is highly efficient in preventing re-mating (Baer et al., 2001; Sauter et al., 2001), reflecting the generally monandrous status of wild queens (Schmid-Hempel and Schmid-Hempel, 2000). In contrast, as we have shown here, the mating plug in *B. hypnorum* is much less efficient. This may go some way towards explaining the high rates of multiple mating found in this latter species (Paxton et al., 2001). Other possible causal factors, e.g., rapid sequential mating before the plug can take effect, remain to be explored.

So far, the proximate factors behind polyandry have only been examined in two other species of social Hymenoptera. Reichardt and Wheeler (1996) suggested that the likelihood of re-mating in the polyandrous ant *Acromyrmex versicolor* is under queen control and determined by the number of previous matings, although with a sample size of four queens their suggestion was obviously tentative. In *Apis mellifera*, the highly polyandrous honey bee, variation in the degree of polyandry, rather than the likelihood of polyandry *per se*, has been studied, and the results are conflicting. Tarpy and Page (2000) found no predictors of multiple mating flights, whereas Schlüns et al. (2001) found that queens which chose to have a second mating flight had mated with fewer males than queens which chose to have only one flight.

Environmental factors, such as meteorological conditions, availability of males, and time available for mating, are clearly

important proximate factors in determining mating frequencies in social insects (e. g., Boomsma and van der Have 1998). Only by integrating information about these factors with the results of controlled behavioural studies will a clear picture of the proximate factors underlying mating frequencies in social insects be revealed.

Mating duration has been recorded in a number of bumble bee species, both European and North American. Duvoisin et al. (1999) recorded an average duration of 36.9 minutes in a Swiss population of *B. terrestris* (N=93), while van Honk et al. (1978) reported a duration of 60 minutes in *B. pratorum* (N=6). Foster (1992) recorded mating durations in four North American species of 42.2 minutes in *B. bifarius* (N=16), 44.7 minutes in *B. californicus* (N=51), 10.2 minutes in *B. frigidus* (N=16), and 9 minutes in *B. rufocinctus* (N=19). Of these six species, only the four North American species are thought to be naturally polyandrous, but whether mating duration also predicts the likelihood of re-mating in these four species remains unknown. Foster (1992) reported a positive correlation between mean mating duration and mean number of matings across the four North American species. In contrast, the two monandrous European species (*pratorum* and *terrestris*) have longer mean mating durations than the polyandrous *hypnorum*. Further work in both monandrous and polyandrous species should shed interesting light on the proximate factors that encourage polyandry in bumble bees.

Acknowledgements

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