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Males of social insects can prevent queens from multiple mating

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During copulation, males of *Bombus terrestris* fill the queen's sexual tract with a mating plug after transferring their sperm. The sticky secretion is produced by the male's accessory glands and disappears within a couple of days. Experiments now show that the primary function of the plug is to reduce the subsequent mating probability of the queen. The plug is not efficient in preventing sperm migration into the spermatheca. Due to its low energetic value, the plug is also unlikely to serve as a nuptial gift. This type of male interference with female mating propensity has so far not been found in social insects. This finding could, at least tentatively, explain why females of *B. terrestris* may not be able to take advantage of the demonstrated benefits of multiple mating. Furthermore, such male interference could be a more general phenomenon in social insects, with obvious ramifications for the evolution of polyandry in this group.

Keywords: multiple mating; sexual conflict; mating plug; *Bombus terrestris*

1. INTRODUCTION

Why females should mate with more than one male (polyandry) is a matter of ongoing controversy (Birkhead & Parker 1996; Kraus & Page 1998; Sherman *et al*. 1998; Yasui 1998; Jennions & Petrie 2000). This is especially true for the social insects, where polyandry has been taken to extreme levels: for example, female honeybees may mate 50 or more times (Moritz *et al*. 1995). In fact, social insects present a major puzzle, since multiple mating appears to provide substantial fitness benefits (Baer & Schmid-Hempel 1999; Cole & Wiernasz 1999) while, at the same time, extensive polyandry seems to be restricted to a few taxa, such as honeybees and leaf-cutter ants (Boomsma & Ratnieks 1996; Fjerdingstad & Boomsma 1998). The reasons why such super-maters are not more commonly found have remained elusive (Boomsma & Ratnieks 1996; Fjerdingstad & Boomsma 1998; Fuchs & Moritz 1998).

Attention has therefore focused on how males can affect multiple mating in an attempt to assert their paternity (Boomsma 1996). Male interests have long been recognized as a key factor in shaping mating patterns and in generating sperm competition (Birkhead & Parker 1996). However, this aspect has, so far, been neglected in the biology of social insects (but see Boomsma 1996). This is primarily because several characteristics appear to erect formidable barriers to males influencing paternity or resource allocation to offspring. For example, at least in the social hymenoptera (wasps, ants and bees), fathers are always physically absent during the period of parental care (although their genes reside in the workers). Furthermore, females only engage in mating during a single episode, and sperm are thereafter retained for a long time (e.g. queen ants can live for many decades and still use sperm from a single mating; Wilson 1971). Thus, female control over sperm use may be easier in social insects than in many other taxa (but see Sundström & Boomsma 2000). To date, empirical evidence also suggests an apparent lack of sperm competition (e.g. Crozier & Brückner 1981; Haberl & Tautz 1998), thus lending only weak support to possible post-copulatory male–male competition. Finally, the social life in a colony gives workers the power to control offspring production (Sundström 1994). Here, we provide evidence that males of a social insect, contrary to previous views, may, nevertheless, affect queen mating behaviour and thereby ensure their interests, at least under some conditions.

The common European bumble-bee, *Bombus terrestris* L., has been used as a model system to address the question of what benefits multiple mating may confer on the females of social insects. In several independent studies of this species, one of the major consequences of polyandry, increased genotypic diversity among colony workers, was experimentally manipulated. In all cases, lower infection levels by parasites (Shykoff & Schmid-Hempel 1991) and a higher reproductive output under field conditions were observed (Liersch & Schmid-Hempel 1998; Baer & Schmid-Hempel 1999; see also Cole & Wiernasz (1999) in ants). In parasite-infested habitats, females of *B. terrestris* should thus have every incentive to mate multiply. Recent empirical evidence has complicated but not invalidated this picture: it was found that small numbers of multiple matings actually reduced colony reproductive output (for as yet unknown reasons, perhaps resulting from intra-colony conflicts). Nevertheless, when queens are inseminated with sperm from more than two males, substantial fitness benefits rapidly accumulate (Baer & Schmid-Hempel 2001), thus restoring the postulated advantage to polyandry. Several species of *Bombus* do, indeed, mate multiply. Yet recent empirical evidence indicates that *B. terrestris*, despite the potential benefits, should be considered as a singly-mating species (Estoup *et al*. 1995; Schmid-Hempel & Schmid-Hempel 2000), although it is possible that females may mate more than once in parts of the geographical range (Röseler 1973). This raises the question: why do not, or cannot, females of *B. terrestris* take advantage of multiple mating?

There may be several obvious answers to this question. For example, males could be hard to find, or the cost of

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mating in terms of time and energy could be prohibitive. Mating costs are, in fact, notoriously difficult to assess, and remain largely unknown in social insects. For honeybees, *Apis mellifera*, a mortality risk of 4%–6% per mating flight (each of which results in many matings) has been estimated (Ratnieks 1990; Tarpy & Page 2000). The corresponding mating costs in *B. terrestris* are not known. However, field populations typically show an extremely male-biased operating sex ratio (Bourke 1998) and the large bumble-bee queens may have few enemies (Alford 1975), suggesting that mating costs could be low. Against this background, it has recently been found that males transfer a sticky secretion (the 'mating plug') from their accessory glands into the *bursa copulatrix* (where sperm is deposited) of the female (Duvoisin *et al*. 1999). Although the plug is transient and disappears within one or two days, the transfer and subsequent mate guarding by the male in copula takes *ca*. 40 min. This at least imposes a time cost of mating, but it is also suspected that the plug itself may have a long-term adverse effect on the female.

In other insect species, products of the males' accessory glands are known to affect the post-mating behaviour of the female (Alcock 1994; Gillott 1996; Simmons & Siva-Jothy 1998). Structures comparable to mating plugs are known to occur in the closest relatives of bumble-bees, the stingless bees (Meliponinae, where a mating sign and the torn-off male genitalia stick with the female; Roubik 1989), and in the honeybees (Apinae, where a similar process occurs; e.g. Koeniger 1991). At least in the honeybee, the function of the mating sign appears to be the prevention of a flow back of sperm (e.g. Woyciechowski *et al*. 1994). It does not, however, prevent multiple mating, and may even enhance it (Koeniger 1991). The function of plugs in other apid bees is not well understood (but see Da Silva *et al*. 1972). We have experimentally investigated the possible function of the mating plug in the bumble-bee, *B. terrestris*, using the methodology developed by Baer & Schmid-Hempel (2000) for artificial insemination of queens. In particular, we carried out two experiments to test whether the plug reduces the propensity of queens to mate. We discuss the results in relation to control of female mating behaviour.

2. MATERIAL AND METHODS

Bumble-bee colonies were reared from queens collected in the field in the spring of 1999 in areas around Zurich, Switzerland, and from queens of laboratory stock. Colonies were kept in climate chambers (continuous darkness, 26–29 °C, 60% relative humidity). Newly emerged queens and males were collected daily and kept, separated by sex, in nest-mate groups (typically 10–20 queens or 20–30 males per box (14 cm × 18 cm × 10 cm)) and fed *ad libitum* with sugar water and pollen. At the time of the experiment, queens were between 5 and 12 days old and males were between 4 and 20 days old. These are the most responsive age classes for mating (Duchateau 1985; Gretenkord 1997; Tasei *et al*. 1998). Experiments were carried out every day between 10.00 and 13.00. In a single trial, animals of the same sex were the same age and came from the same nest. All animals were tested only once. Treatment classes were randomly assigned to the flight cages (50 cm × 50 cm × 55 cm) where the experiments took place. The observer did not interfere with the behaviour of the bees in any trial. Behavioural observations will be described briefly here to elucidate our results, but will be reported fully elsewhere (A. Sauter, M. J. F. Brown and P. Schmid-Hempel, unpublished data).

In preparation for the treatments, virgin queens were immobilized by chilling (5 min at −20 °C followed by 30 min on ice). This protocol was the same for all queens. At room temperature, queens recover from chilling and resume normal behaviour within 5 min. The queens were then randomly assigned to one of four treatments. In treatment 'untreated', the queens were left untreated. In treatment 'Ringer', the queens were injected with Ringer's solution to fill the *bursa copulatrix*. In treatment 'plug', the queens were injected with a male's plug material (dissected out and contained in Ringer's solution) in the same way as for the 'Ringer' treatment. Injection protocols followed the methods developed for artificial insemination of *Bombus* queens (for details see Baer & Schmid-Hempel 2000). In the fourth treatment, 'mated', queens were allowed to mate before being tested. For this, the queens were placed with three males in plastic boxes (10 cm × 15 cm × 19 cm) for 30 min. To ensure that these queens mated only once before the tests (ensuring homogeneity within this control treatment) all males were removed immediately after copulation.

To collect the plug material, the reproductive system was removed from a virgin male (see Duvoisin *et al*. 1999, fig. 2; Baer & Schmid-Hempel 2000). The ducts between the accessory glands and the accessory testes were cut to prevent sperm from entering the plug material. The ejaculatory duct was then severed, enabling the accessory-gland products to flow out. These were picked up with a glass capillary (tip opening 0.5–0.7 mm in diameter) filled with Ringer's solution and mounted on a syringe. The material was then injected into the queen. To check that sperm were not transferred with the plug material, 18 queens were plugged in this way, kept alive for one week and their spermathecae checked for the presence of sperm under a microscope at 200 × magnification. None contained sperm. We therefore assumed that our procedure transferred only plug material.

(a) *Mating probability with no choice*

We first tested whether queens differed in their mating probability across treatments. A mating was considered to be established if the pair was observed in copula. Queens from all four treatment classes were tested individually at the same time of day in four separate flight cages. In each trial, one queen was put with one male. Copulations that occurred within 20 min were recorded. The males used were not related to the males used for plug extraction nor to those used for the natural matings, and were unrelated to the queens used in the experiment.

(b) *Mating probability when given a choice*

In a second experiment, we tested whether males avoid plugged queens when given a choice between a Ringer-injected queen and an experimentally plugged queen (i.e. treatments 'Ringer' and 'plug'), following the same procedures as in § 2(a). To distinguish the two queens, the bees were individually marked before the tests. In addition to these choice tests, samples of 18 queens each, matched by age and mother colony, were assigned to treatments 'untreated', 'plug' and 'Ringer' as before. These queens were again tested individually (with no choice) and simultaneously (with choice). This additional sample thus served as an independent control for overall mating propensity. During these experiments, a further 18 queens were experimentally plugged, kept individually in plastic boxes, fed *ad libitum* and dissected 3–4 h later. This sample served to check

whether the transfer of the plug was successful and whether the plug persisted for the duration of the experiment.

(c) *Sperm transfer*

Queens of the 'untreated', 'Ringer' and 'plug' treatments from both experiments that had mated in the flight cage received sperm from their males and were also naturally plugged. We used these queens to check whether the plug (which was also experimentally injected in the 'plug' treatment) is capable of preventing sperm (received in the natural mating) from entering the spermatheca. For this purpose, the mated queens were kept alive for 24 h after copulation, fed *ad libitum*, then freeze-killed at −80 °C and dissected later to check for sperm and the presence of a plug. According to Duvoisin *et al.* (1999), sperm reach the spermatheca 30–80 min after the onset of copulation (sperm are transferred before the plug). In their study, the plug was still present in 70% of the queens one day later. Therefore, our experimentally injected plug should still have been present in most of our queens and the sperm should not have entered the spermatheca if the plug had acted as a physical barrier. For the sperm count, the queen's spermatheca was carefully removed in Ringer's solution under a stereoscopic microscope with 6–25× magnification. It was then placed in the well of a degreased microscope slide and all tissue except for the inner layer of the spermatheca was removed. The spermatheca was then transferred to another well containing 1.5 µl of Ringer's solution. Using one pair of degreased forceps, the spermatheca was opened, transferred to a glass V-vial (Infochroma AG, Zug, Switzerland) containing 100 µl of Ringer's solution and crushed with the same forceps for 5 min. We then added 300 µl of Ringer's solution and the mixture was sonicated for 1 min and vortexed. Five replicates of 10 µl were placed on a super-Teflon slide, air dried and fixed for 1 min in ethanol–acetic acid (3:1 by volume), and incubated for 20 min in a 5 µg ml^{-1} DAPI–Ringer's solution (method similar to that described in Bressac & Hauschteck-Jungen (1996)). From the slides, the sperm number was counted using a fluorescence microscope (Nikon Eclipse with DAPI-filter; Nikon Corporation, Tokyo, Japan). We used the average counts of the five replicates as our data points.

(d) *Persistence of plug*

To test whether the experimentally injected plug persisted, a further sample of 18 queens were plugged, kept individually in plastic boxes and fed *ad libitum*. We checked the queen's sexual tract for the presence of the plug one day after the operation.

(e) *Energetic value of the plug*

The energetic value of the plug was estimated based on the chemical analysis of the material (Baer *et al.* 2000). The values for complete oxidation of one mole of substance to CO_2 and H_2O were taken from Weast *et al.* (1986). The value for the oxidation of linoleic acid (10.943×10^6 J mol^{-1}) was estimated from the value for oleic acid, which also has 18 carbon atoms, taking into account the additional double bond (E. D. Morgan, personal communication).

(f) *Statistical analysis*

Mating probability was analysed using logistic regression with copulation (yes or no) as the dependent variable. Treatments were coded as categorical values with indicator (dummy) coding. Male avoidance of plugged queens (yes or no) was analysed with a binomial test, testing against $f = 0.5$. All statistics were performed using SPSS 6.1 for Macintosh.

3. RESULTS

(a) *Behaviour of queens and males*

Males approach queens and typically attempt to copulate by mounting, placing the middle legs on the thorax and trying to insert the genitalia. Males may sometimes struggle over access to a female. A queen reacts to such approaches either by moving away or by repulsing the male by raising her middle legs and trying to push the male away. Alternatively, the queen sometimes shows no obvious reaction, which may then lead to eventual copulation. As will be reported in detail elsewhere (A. Sauter, M. J. F. Brown and P. Schmid-Hempel, unpublished data), the rate at which males approach a queen and the rate at which queens repulse males are both correlated with the propensity to eventually copulate. However, taken together, pre-copulatory behaviour (by the male and by the queen) was found to be a surprisingly poor predictor of the likelihood of copulation. Rather, queen status (i.e. 'untreated', 'mated', 'plug' or 'Ringer') is of overriding importance (A. Sauter, M. J. F. Brown and P. Schmid-Hempel, unpublished data). We therefore report here only our results for queen status.

(b) *Mating probability with no choice*

None of the 25 queens that were allowed to mate before the test ('mated') re-mated in our experiment (binomial test for $f = 0.5$, $p < 0.001$). This treatment class could not really be compared with the others because of the possible confounding influence of prior contact with males on queen behaviour. A total of 40 trials each were run for the remaining treatments, 'untreated', 'Ringer' and 'plug'. Across these treatments, mating probabilities were significantly different, with queens in the 'plug' treatment clearly being less likely to mate (figure 1). We found no difference between the 'plug' treatment and the 'mated' treatment (Wald $\chi^2 = 0.008$, d.f. = 1, $p = 0.98$); the other two treatments differed from the 'mated' treatment.

(c) *Mating probability when given a choice*

A total of 18 choice trials were run. Out of these, mating occurred in only five trials. In all cases, the males mated with the 'Ringer' queens. Although this bias is not significant (binomial test against $f = 0.5$, $p = 0.2$, $n = 5$), the result at least suggests that plugged queens do not remate in choice situations. Among the queens that were tested individually at the same time (control sample), the same pattern as in the first experiment emerged. Out of 18 queens tested for each treatment, the number of matings was seven in the 'untreated' group, six in the 'Ringer' group and one in the 'plug' group, and thus varied significantly with treatment (logistic regression: $G = 7.11$, d.f. = 2, $p = 0.029$). *Post-hoc* tests suggested no differences between the 'Ringer' and 'plug' groups (Wald $\chi^2 = 3.499$, d.f. = 1, $p = 0.061$) or between the 'Ringer' and 'untreated' groups (Wald $\chi^2 = 0.120$, d.f. = 1, $p = 0.73$), but the 'plug' group mated less than the 'untreated' group (Wald $\chi^2 = 4.3863$, d.f. = 1, $p = 0.036$).

(d) *Persistence of plug*

Our procedure of artificially applying the plug in queens is experimentally desirable but cannot perfectly copy the natural situation. As a likely consequence of this

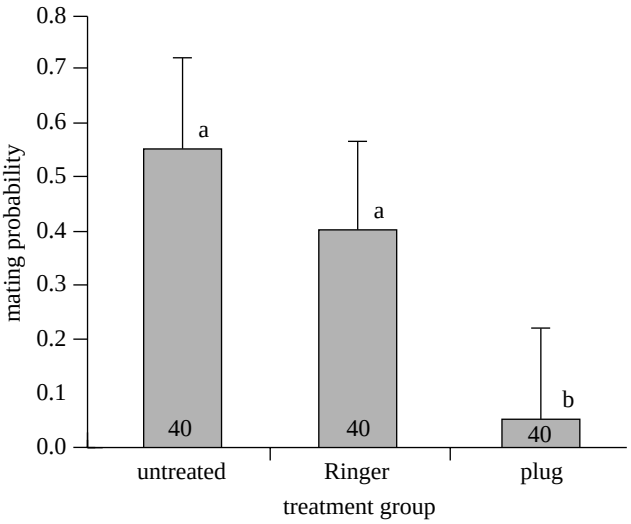

Figure 1. The mating probability of queens in the three treatments varied (log-likelihood ratio = 27.990, d.f. = 2, $p < 0.001$). Letters above the bars denote groups that were statistically the same in *post-hoc* tests ('untreated' versus 'plug': Wald $\chi^2 = 15.771$, d.f. = 1, $p < 0.001$; 'untreated' versus 'Ringer': Wald $\chi^2 = 1.791$, d.f. = 1, $p = 0.18$; 'Ringer' versus 'plug': Wald $\chi^2 = 10.224$, d.f. = 1, $p < 0.001$) (see § 2 for definition of treatments). Figures in the bars indicate the number of queens tested.

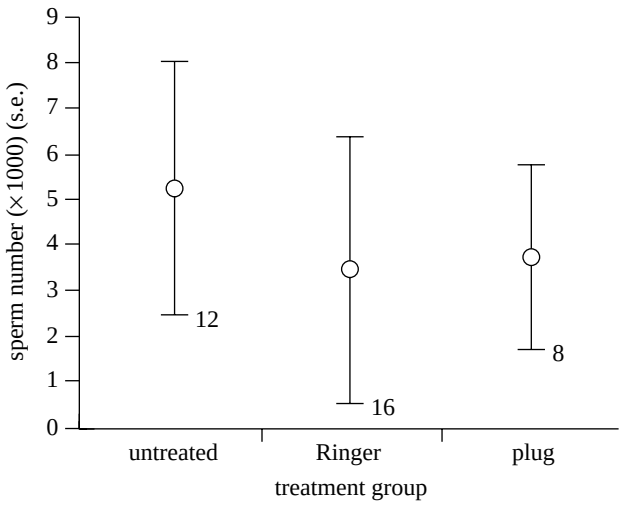

Figure 2. Sperm numbers in the spermatheca of queens 24 h after copulation according to treatment class. Although 'Ringer' and 'plug' queens had slightly less sperm, no statistical differences were found (one-way ANOVA, $F_{2,33} = 1.709$, $p = 0.20$). Figures below bars indicate the number of queens dissected.

experimental constraint, we found that the artificially applied plug vanished faster than a natural plug. When observed 3–4 h after plugging only 13 out of the 18 queens (72%) still showed a plug, a rate observed only after 1 day with natural matings (Duvoisin *et al*. 1999).

(e) *Sperm transfer*

As shown in figure 2, data from the queens that mated in the two experiments indicated that although the plug may lower the number of sperm present in the spermatheca, no significant effect was found. In the queens from the 'plug' treatments, we found that 1 day after application of the plug (i.e. when these queens were dissected) the experimentally set plug was present in only three out of 18 queens. This confirmed the relatively faster loss of the artificial plug from the control sample (see § 3(d)) dissected 3–4 h after plugging. The proportion of the artificial plugs remaining after 1 day is equivalent to that found only after 2 days in natural matings. Compared to the observations for the same time period, therefore, the experimentally set plug, not surprisingly, vanishes faster ($\chi^2 = 10.6$, d.f. = 1, $p < 0.01$) than the natural plug (Duvoisin *et al*. 1999).

(f) *Energetic value of the plug*

To assess the role of the mating plug as a nuptial gift, we reasoned that the energetic value is probably the most valuable component, since energy requirements for flight in bumble-bees are very high (Ellington *et al*. 1990) and queens must forage and fly to collect fat and glycogen stores to prepare for hibernation (Alford 1975). According to Baer *et al*. (2000), the chemical composition of an average-sized plug is 980 ng of palmitic acid (yielding 38.147×10^{-3} J per plug for complete oxidation), 5400 ng of linoleic acid (211.044×10^{-3} J per plug), 1700 ng of oleic acid (66.951×10^{-3} J per plug) and 440 ng of stearic acid (17.459×10^{-3} J per plug). The fifth component, the amino acid cycloprolylproline, is difficult to metabolize (E. D. Morgan, personal communication) and thus, presumably, has very little energetic value. In addition, each plug contains 500–800 ng of saccharose (with 1 mg sugar providing 16.7 J, and generously assuming *ca*. 1000 ng, this corresponds to an equivalent of 16.7×10^{-3} J per plug). Thus, a plug has an energetic equivalent of approximately 350×10^{-3} J. On the other hand, the mass-specific oxygen consumption of bumble-bees in forward flight is *ca*. $0.445\ \mathrm{J\,s^{-1}\,g^{-1}}$ (Wolf *et al*. 1999). The average body mass of newly hatched autumn queens of *B. terrestris* in the field (i.e. the young queens that mate) is mean ± s.e.m. = 0.336 ± 0.007 g ($n = 516$) (C. Müller, unpublished data). Therefore, an average queen requires *ca*. $0.151\ \mathrm{J\,s^{-1}}$ to keep flying. Given this estimate, a queen that could metabolize the plug would remain aloft for only 2.3 s for an average-sized plug. A similarly small utility would result if the plug material was used instead to increase the fat body of the queen. This estimate assumes perfect conversion of the substrate into usable energy. Hence, it is unlikely that the plug provides tangible resources for the queen, although we cannot rule out any other effect not, so far, envisioned.

4. DISCUSSION

Our experiments show that the plug transferred during mating by males of *B. terrestris* to queens lowers the subsequent mating probability of these queens, similar to findings in various other non-social insect species (e.g. Simmons & Siva-Jothy 1998). This effect was most obvious when queens were tested individually with no choice for the male (figure 1). On the other hand, the experimentally injected plug did not effectively prevent sperm from entering the spermatheca in the case of a subsequent mating (figure 2). Our experimental

procedure of artificially applying the plug in queens is crucial for these tests as it removes confounding factors, such as variation in queen and male quality, that may affect the likelihood of a queen to mate. On the other hand, the experimental plugs disappeared relatively fast, an experimental shortcoming that may help to explain the sperm-transfer result. An accidental observation may shed further light on this issue. One plugged queen used in the second experiment was mistakenly not separated from the male after the mating trial. She subsequently mated again with the same male (she was the only naturally mated queen that ever mated again) and was frozen at −80 °C immediately after the end of the second copulation (this queen was excluded from the analyses). The dissection revealed that, during his second copulation, the male had been able to push his first plug towards the oviducts and had placed a second sperm package just in front of the opening of the duct that leads to the spermatheca (where sperm packages are typically placed). The artificially set plug was no longer visible in this particular case. Re-matings are exceedingly rare, so this incidence provides limited scope from which to infer a general role for such male–male interference within the female. The small energetic value makes it very unlikely that the plug serves as a nuptial gift. Therefore, it appears that the primary function of the plug in *B. terrestris* is to prevent the female from further matings and not to prevent sperm transfer, contrary to the suggestions of Duvoisin *et al*. (1999).

The exact mechanism by which the plug reduces mating probability remains unclear. On the one hand, it may affect the queen's behaviour. For example, queens might be able to assess the filling of the *bursa copulatrix* (as in the butterfly, *Pieris rapae crucivora*; Sugawara 1979). This might explain why the mating probability of 'Ringer' queens (whose *bursa copulatrix* was filled with Ringer's solution) was slightly lower than that of 'untreated' queens (which had an empty *bursa copulatrix*). However, it appears that the chemical compounds forming the plug directly alter queen behaviour (Baer *et al*. 2001). On the other hand, male behaviour may be affected. For example, the plug may contain compounds that repel competing males (e.g. Andersson *et al*. 2000). At this point, it becomes necessary to distinguish two levels of control over the mating process. On an immediate behavioural level, the queen appears to be in control of the onset of copulation, since the male must insert his genitalia into the female tract, which he cannot do without the female actively giving access, for example, by moving away her sting. The male then appears to be in control of the duration of copulation, since his claspers hold the female's genitalia in place (see Duvoisin *et al*. 1999). However, pre-copulatory behaviour, by males or queens, is not a good predictor of the likelihood of copulation (A. Sauter, M. J. F. Brown and P. Schmid-Hempel, unpublished data). Therefore, even with this immediate behavioural control in place, queen status, especially the presence or absence of a plug, is the decisive factor. This then defines a second level of control over the mating process, which is not correlated with pre-copulatory behaviour. Our results show that the male exerts control at this level, since the plug renders the queen refractory to mating through unknown (but not necessarily behavioural) processes. We emphasize that it was not our aim to investigate these mechanisms, but rather to address the question of whether males interfere with the propensity of females to re-mate.

Viewed in the context of a possible conflict of interests between queen and male over mating frequency in *B. terrestris*, our results pose a number of questions. There is firm evidence that a queen that mated with several males would benefit from the resulting increase in genotypic diversity among her workers, because, in field situations, colony parasite loads are demonstrably smaller and the reproductive output is significantly larger (in fact, almost double) than that of a single-mated colony queen (Liersch & Schmid-Hempel 1998; Baer & Schmid-Hempel 1999). This increase in colony fitness would primarily benefit the queen, because any fitness advantage has to be shared among the queen's mates. For example, a two-fold advantage in fitness achieved by multiple mating (as in Baer & Schmid-Hempel 1999) must be shared among the males. If two males participated, then the expected fitness per male is at best equal to what he could achieve if he prevented the female from re-mating (and drops below this when more matings are added if the colony fitness does not increase correspondingly). Additional benefits to the male from a single mating could accumulate through the effects of split sex ratios (for the argument and discussion, see Sundström 1994; Boomsma 1996). However, recent empirical evidence indicates that when the genotypic diversity of the colony is only slightly elevated over that corresponding to single mating, the colony fitness actually decreases, and only increases again when more matings are added (Baer & Schmid-Hempel 2001). Therefore, for low expected mating frequencies the interests of the queen and the male may be convergent but may start to diverge when multiple mating becomes more frequent. This depression phenomenon could help to explain why, in *B. terrestris*, males appear to have evolved the 'plug' as a successful tool to reduce re-mating by females: the females could not resist this manipulation, at least for low mating frequencies.

Despite these controversies, our findings clearly show that males of social insects can affect the mating frequencies of queens in subtle ways. It is possible, although still speculative, that such male–female conflicts, which have so far been largely neglected in social insects, explain the generally low mating frequencies in this group (Boomsma & Ratnieks 1996). Evolution towards super-maters could occur when females can 'escape' the interference of males, which may happen in a few, taxonomically isolated, groups.

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REFERENCES

Alcock, J. 1994 Post-insemination associations between males and females in insects: the mate-guarding hypothesis. *A. Rev. Entomol.* **39**, 1–21.

Alford, D. V. 1975 *Bumblebees*. London: Davis-Poynter.

Andersson, J., Borg-Karlson, A.-K. & Wiklund, C. 2000 Sexual cooperation and conflict in butterflies: a male-transferred anti-aphrodisiac reduces harassment of recently mated females. *Proc. R. Soc. Lond.* B **267**, 1271–1275.

Baer, B. & Schmid-Hempel, P. 1999 Experimental variation in polyandry affects parasite loads and fitness in a bumblebee. *Nature* **397**, 151–154.

Baer, B. & Schmid-Hempel, P. 2000 The artificial insemination of *Bombus terrestris* queens. *Insectes Sociaux* **47**, 183–187.

Baer, B. & Schmid-Hempel, P. 2001 Unexpected consequences of polyandry for parasitism and fitness in the bumblebee *B. terrestris*. *Evolution*. (In the press.)

Baer, B., Maile, R., Schmid-Hempel, P., Morgan, E. D. & Jones, G. R. 2000 Chemistry of a mating plug in bumblebees. *J. Chem. Ecol.* **26**, 1869–1875.

Baer, B., Morgan, E. D. & Schmid-Hempel, P. 2001 A non-specific fatty acid within the bumblebee mating plug prevents females from re-mating. *Proc. Natl. Acad. Sci.* **98**, 3926–3928.

Birkhead, T. R. & Parker, G. A. 1996 Sperm competition and mating systems. In *Behavioural ecology—an evolutionary approach* (ed. J. R. Krebs & N. B. Davies), pp. 121–145. Oxford, UK: Blackwells.

Boomsma, J. J. 1996 Split sex ratios and queen–male conflict over sperm allocation. *Proc. R. Soc. Lond.* B **263**, 697–704.

Boomsma, J. J. & Ratnieks, F. L. W. 1996 Paternity in eusocial hymenoptera. *Phil. Trans. R. Soc. Lond.* B **351**, 947–975.

Bourke, A. F. G. 1998 Sex ratios in bumblebees. *Phil. Trans. R. Soc. Lond.* B **352**, 1921–1933.

Bressac, C. & Hauschteck-Jungen, E. 1996 *Drosophila obscura* females preferentially select long sperm for storage and use. *J. Insect Physiol.* **42**, 323–328.

Cole, B. J. & Wiernasz, D. C. 1999 The selective advantage of low relatedness. *Science* **285**, 891–893.

Crozier, R. H. & Brückner, D. 1981 Sperm clumping and the population genetics of hymenoptera. *Am. Nat.* **117**, 561–563.

Da Silva, D. L. N., Zucchi, R. & Kerr, W. E. 1972 Biological and behavioural aspects of the reproduction in some species of *Melipona* (Hymenoptera, Apidae, Meliponinae). *Anim. Behav.* **20**, 123–132.

Duchateau, M. J. 1985 Analyse einiger Methoden der Hummelzüchtung. *Apidologie* **16**, 225–227.

Duvoisin, N., Baer, B. & Schmid-Hempel, P. 1999 Sperm transfer and male competition in the bumblebee, *Bombus terrestris* L. *Anim. Behav.* **58**, 743–749.

Ellington, C. P., Machin, K. E. & Casey, T. M. 1990 Oxygen consumption of bumblebees in forward flight. *Nature* **347**, 472–473.

Estoup, A., Scholl, A. A. P. & Solignac, M. 1995 Monoandry and polyandry in bumble bees (Hymenoptera; Bombinae) as evidenced by highly variable microsatellites. *Mol. Ecol.* **4**, 89–93.

Fjerdingstad, E. & Boomsma, J. J. 1998 Multiple mating increases the sperm stores of *Atta colombica* leafcutter ants. *Behav. Ecol. Sociobiol.* **42**, 257–261.

Fuchs, S. A. & Moritz, R. F. 1998 Evolution of extreme polyandry in the honey bee, *Apis mellifera* L. *Behav. Ecol. Sociobiol.* **39**, 269–275.

Gillott, C. 1996 Male insect accessory glands: functions and control of secretory activity. *Invertebr. Reprod. Dev.* **30**, 199–205.

Gretenkord, C. 1997 Laborzucht der Dunklen Erdhummel (*Bombus terrestris* L.) (Hymenoptera, Apidae) und toxikologische Unterscuhungen unter Labor- und Halbfreilandbedingungen. PhD thesis, University of Bonn, Aachen, Germany: Shaker Verlag.

Haberl, M. & Tautz, D. 1998 Sperm usage in honey bees. *Behav. Ecol. Sociobiol.* **42**, 247–255.

Jennions, M. D. & Petrie, M. 2000 Why do females mate multiply? A review of the genetic benefits. *Biol. Rev.* **75**, 21–64.

Koeniger, G. 1991 Diversity in *Apis* mating systems. In *Diversity in the genus* Apis (ed. D. R. Smith), pp. 199–212. Boulder, CO: Westview Press.

Kraus, B. & Page, R. E. J. 1998 Parasites, pathogens, and polyandry in social insects. *Am. Nat.* **151**, 383–391.

Liersch, S. & Schmid-Hempel, P. 1998 Genetic variability within social insect colonies reduces parasite load. *Proc. R. Soc. Lond.* B **265**, 221–225.

Moritz, R. F. A., Kryger, P., Koeniger, G., Koeniger, N., Estoup, A. & Tingek, S. 1995 High degree of polyandry in *Apis dorsata* queens detected by DNA microsatellite variability. *Behav. Ecol. Sociobiol.* **37**, 357–363.

Ratnieks, F. L. W. 1990 The evolution of polyandry by queens in social Hymenoptera: the significance of the timing of removal of diploid males. *Behav. Ecol. Sociobiol.* **26**, 343–348.

Röseler, P.-F. 1973 Die Anzahl Spermien im Receptaculum seminis von Hummelköniginnen (Hymenoptera, Apidae, Bombinae). *Apidologie* **4**, 267–274.

Roubik, D. W. 1989 *Ecology and natural history of tropical bees*. Cambridge Tropical Biology Series. Cambridge University Press.

Schmid-Hempel, R. & Schmid-Hempel, P. 2000 Female mating frequencies in social insects: *Bombus* spp. from Central Europe. *Insectes Sociaux* **47**, 36–41.

Sherman, P. W., Seeley, T. D. & Reeve, H. K. 1998 Parasites, pathogens, and polyandry in honey bees. *Am. Nat.* **151**, 392–396.

Shykoff, J. A. & Schmid-Hempel, P. 1991 Parasites and the advantage of genetic variability within social insect colonies. *Proc. R. Soc. Lond.* B **243**, 55–58.

Simmons, L. W. & Siva-Jothy, M. T. 1998 Sperm competition in insects: mechanisms and the potential for selection. In *Sperm competition and sexual selection* (ed. T. R. Birkhead & A. P. Møller), pp. 341–434. London: Academic Press.

Sugawara, T. 1979 Stretch reception in the bursa copulatrix of the butterfly, *Pieris rapae crucivora*, and its role in behaviour. *J. Comp. Physiol.* B **130**, 191–199.

Sundström, L. 1994 Sex ratio bias, relatedness asymmetry and queen mating frequency in ants. *Nature* **367**, 266–268.

Sundström, L. & Boomsma, J. J. 2000 Reproductive alliances and posthumous fitness enhancement in ants. *Proc. R. Soc. Lond.* B **267**, 1439–1444.

Tarpy, D. R. & Page, R. E. 2000 No behavioral control over mating frequencies on queen honey bees (*Apis mellifera* L.): implications for the evolution of extreme polyandry. *Am. Nat.* **155**, 820–827.

Tasei, J. N., Moinard, C., Moreau, L., Himpens, B. & Guyonnaud, S. 1998 Relationship between aging, mating and sperm production in captive *Bombus terrestris*. *J. Agricultural Res.* **37**, 107–113.

Weast, R. C., Astle, M. J. & Beyer, W. H. 1986 *Handbook of chemistry and physics*. Boca Raton, FL: CRC Press.

Wilson, E. O. 1971 *The insect societies*. Cambridge, MA: Harvard University Press.

Wolf, T. J., Ellington, C. P. & Begley, I. S. 1999 Foraging costs in bumblebees: field conditions cause large individual differences. *Insectes Sociaux* **46**, 291–295.

Woyciechowski, M., Kabat, L. & Krol, E. 1994 The function of the mating sign in honey bees, *Apis mellifera* L.: new evidence. *Anim. Behav.* **47**, 733–735.

Yasui, Y. 1998 The 'genetic benefits' of female multiple mating reconsidered. *Trends Ecol. Evol.* **13**, 246–250.