A nonspecific fatty acid within the bumblebee mating plug prevents females from remating

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The best mating strategy for males differs from that of females, because females gain from mating with several males (polyandry), but males gain from monopolizing the females. As a consequence, males have evolved a variety of methods, such as the transfer of inhibitory substances from their accessory glands, to ensure exclusive paternity of the female's offspring, generally with detrimental effects on female fitness. Inhibitory substances have been identified as peptides or other specific molecules. Unfortunately, in social insects male-mating traits are investigated only poorly, although male social insects might have the same fundamental influence on female-mating behavior as found in other species. A recently developed technique for the artificial insemination of bumblebee queens allowed us to investigate which chemical compound in the mating plug of male bumblebees, *Bombus terrestris* L., prevents females (queens) from further mating. Surprisingly, we found that the active substance is linoleic acid, a ubiquitous and rather unspecific fatty acid. Contrary to mating plugs in other insect species, the bumblebee mating plug is highly efficient and allows the males to determine queen-mating frequencies.

linoleic acid | multiple mating | male-mating strategy | female monopolization

Why females mate multiply is not understood clearly given the potential costs of polyandry such as an increased risk of getting parasitized or predated, or the inevitable loss of time or energy. Recent work has concentrated to explain mainly the possible benefits of polyandry for the females (1, 2), whereas less attention has been given to an important counterpart: the males. In fact, male strategies might be important to understand observed variation in female-mating frequencies among or even within species. During copulation, males of many species transfer not only sperm but also additional substances into the female's sexual tract (3, 4), which cause a variety of changes in the female's reproductive behavior that are to the male's advantage (5–7). In some cases, female lifespan is reduced (8, 9), or males are able to monopolize females by transferring chemical substances to the female reducing their willingness to remate (3, 10). Such changes in female-mating behavior typically are induced by specific proteins (3, 10, 11), although in most cases the active substance is unknown (3). An interesting case is provided by males of the butterfly, *Pieris napi*, where females are "marked" with a volatile antiaphrodisiac that repels other males (12).

In social insects, mating strategies are of special interest, because female (queen)- mating frequency determines average among-worker relatedness and therefore influences the kin structure of the colony with important ramifications for the levels of cooperation, or conflicts, for example, over offspring sex ratios (13). The haplo-diploid sex determination system of *Hymenoptera* additionally selects for males to prevent females from multiple mating. With split sex ratios among colonies, singly but not multiply mated colonies should produce relatively more daughters that carry a male's genes (sons have no fathers; refs. 14 and 15). Although female multiple mating currently is discussed broadly in social insects, male-mating strategies and their potential influence on females are poorly investigated, even in intensively studied species of bees and ants.

The study system used here, *Bombus terrestris* L., is an annual social insect. Queens emerge from hibernation in the spring, and found colonies and emerging workers help the queen to establish a colony. In midsummer, sexual reproduction takes place, but only the young and inseminated queens hibernate and perform another colony cycle the following year. Similar to other insect-mating systems, females of the bumblebee *B. terrestris* benefit from multiple mating, whereas males should be selected to secure exclusive paternity over offspring (16). Queens of *B. terrestris* are mated singly in Central Europe (17), indicating that males might be very efficient in monopolizing queens. In fact, males transfer a sticky substance from their accessory glands, the "mating plug" (18), which prevents females from remating (A. Sauter, M. O. F. Brown, B.B., and P.S.-H., unpublished data).

Chemical analyses have shown that the plug consists of a unique dipeptide, cycloprolylproline, apparently not found anywhere else in insects and a mixture of fatty acids (oleic, linoleic, palmitic, and stearic acids; ref. 19). Because of the unique occurrence of cycloprolylproline, we hypothesized that this peptide is the active substance of the plug. This hypothesis was tested in two experimental series by transferring various chemical compounds into the female's sexual tract and testing for the probability of mating. The transfer was done with methods for the artificial insemination of bumblebee queens (20) mimicking the natural transfer of the plug by males. The queens prepared in this way then were given the opportunity to mate with males in experimental flight cages. All tests for treatment effects were matched to queens injected with Ringer's solution to control for possible background variation in the overall readiness of females to mate. As our experiments showed, the initial hypothesis was rejected, and another substance proved to be effective to prevent female remating.

Materials and Methods

Bumblebee colonies were reared from *B. terrestris* queens caught in the field during spring 2000 around Zurich, Switzerland. Colonies were kept in climate chambers under standardized conditions in red light at 28°C and 60% humidity. All test animals were removed from their natal nests as callows and were kept in sister/brother groups in plastic boxes (14 × 18 × 10 cm) 5–14 days before experiments and were fed ad libitum with pollen and sugar water. To test whether a substance affects queen-mating behavior, we artificially introduced the substance into the bursa copulatrices of queens as described (18, 20). Afterward, we compared copulation behavior in treated vs. control queens. Control queens received an injection equivalent of insect Ringer's solution (Merck) but were otherwise treated identically. After every transfer, the equipment was thoroughly cleaned with ethanol to avoid contamination of chemicals across treatments.

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Table 1. Observed mating behavior of females injected with test and control substances (Ringer's solution)

	Test		Control	
Test substances	Mating	No mating	Mating	No mating
Sperm*	14	16	11	19
Cycloprolylproline†	15	15	17	13
Fatty acids‡§	1	45	20	26

Entries are number of queens with respective behavior.

*Pairwise posthoc test of sperm vs. control. Wald $\chi^2 = 0.61$, df = 1, $N = 30$, $P = 0.43$. Univariate, $\chi^2 = 0.617$, $P = 0.4$.

†Pairwise posthoc test of cycloprolylproline vs. control. Wald $\chi^2 = 0.27$, df = 1, $N = 30$, $P = 0.61$. Univariate, $\chi^2 = 0.268$, $P = 0.6$.

‡A mixture of oleic, linoleic, palmitic, and stearic acids in the proportions as in natural plugs.

§Pairwise posthoc test of fatty acids vs. control. Wald $\chi^2 = 11.313$, df = 1, $N = 46$, $P < 0.001$. Univariate, $\chi^2 = 22.275$, $P < 0.0001$.

Table 2. Mating behavior of females injected with test substances and a control substance (Ringer's solution)

	Test		Control	
Test substances	Mating	No mating	Mating	No mating
Oleic acid*	11	19	13	17
Linoleic acid†‡	3	27	22	8
Reduced plug§¶	12	18	14	16

Entries are number of queens with respective behavior.

*Pairwise posthoc test of oleic acid vs. control. Wald $\chi^2 = 0.277$, df = 1, $N = 30$, $P = 0.59$. Univariate, $\chi^2 = 0.278$; $P = 0.6$.

†Pairwise posthoc test of linoleic acid vs. control. Wald $\chi^2 = 19.039$, df = 1, $N = 30$, $P < 0.0001$. Univariate, $\chi^2 = 24.754$; $P < 0.0001$.

‡For unknown reasons, controls mated unusually often in this experiment. However, the result is robust, because (*i*) assuming controls had mated only with a probability of 49.4% (the average for all controls in Tables 1 and 2), the test is: $\chi^2 = 11.429$, $P < 0.001$, and (*ii*) assuming controls had mated with the lowest observed probability of 36.3% (as the controls in Table 1, "Sperm"), the test is $\chi^2 = 5.963$, $P = 0.02$.

§A mixture of oleic, palmitic, and stearic acids in the proportions as in natural plugs.

¶Pairwise posthoc test of reduced plug vs. control. Wald $\chi^2 = 0.271$, df = 1, $N = 30$, $P = 0.60$. Univariate, $\chi^2 = 0.271$, $P = 0.6$.

Queens were chilled on ice for 30 min before the insemination procedure. Afterward, they were assigned randomly to one of the groups, receiving either 1.5 μl of the test substance or 1.5 μl of Ringer's solution. At 30 min after insemination, each queen was placed in a flight cage (50 × 50 × 50 cm) containing a single male.

Occurrence and time until the onset of copulation were recorded over a subsequent period of 90 min. Experiments for substances were repeated at least 30 times. Queens and males inside a flight cage were unrelated to each other, and all animals were used only once for the tests. Within any one experiment, the two queens (test substance vs. control) were always matched to be sisters of the same age; males in the two tests were always brothers. In the first experimental series, the queens' bursa copulatrices were inseminated with sperm, injected with cycloprolylproline, or injected with a mixture of the four fatty acids. Sperm was collected from males of a stock colony unrelated to all experimental animals. Cycloprolylproline and the four fatty acids were purchased from a commercial dealer and stored at −20°C before use. All substances were introduced to treatment queens in similar quantities and mixtures as known from natural plugs. Cycloprolylproline was dissolved in Ringer's solution. The mixture of fatty acids forms the typical sticky secretion of a natural plug (19). This mixture was liquefied at ≈35°C before introduction into the queen's tract. Afterward, the mixture converted back into a sticky mass forming an artificial plug within the bursa copulatrix of the queen. In a second series of experiments, we identified the active substance among the fatty acids, testing the two liquid fatty acids, linoleic and oleic acid, first. Because we found no effect of oleic acid, we dissolved the two remaining solid fatty acids (stearic and palmitic acids) in oleic acid to produce a semisolid mass and tested this "reduced plug." Within this reduced plug we replaced the amount of linoleic acid with oleic acid to maintain the physical attributes (the stickiness) of the plug. For statistical analysis, copulation probability was analyzed by using logistic regression with copulation occurrence as the dependent variable. Treatment was coded as categorical value with indicator (dummy) coding. All statistics were done with SPSS 6.1 for Macintosh.

Results

In the first experimental series, neither sperm nor cycloprolylproline affected queen-mating probabilities compared with the matched Ringer's solution-injected control queens (Table 1). However, the fatty acids mixture significantly reduced queen-mating probability. Therefore, contrary to our initial hypothesis, one of the four fatty acids must be the active substance and not cycloprolylproline.

In a second series of experiments, queens were injected with oleic or linoleic acid or with a reduced plug containing oleic, palmitic, and stearic acids. Compared with the matched Ringer's solution-injected control queens, only linoleic acid reduced the remating probability of the female (Table 2). The reduced plug, without linoleic acid, did not reduce mating probability as compared with controls.

In all experiments, males intensively attempted to copulate with queens, indicating that they do not discriminate against queens that have either one of the five plug compounds or sperm present in their sexual tracts. No obvious difference in rejection behavior by queens in the different treatments was observed. In fact, previous studies have shown that precopulatory behavior, for example the rate of rejections by the queen or the rate of approaches by males, is a poor predictor of the likelihood of copulation (21). As expected, the mating probabilities of Ringer's solution control queens varied between experiments, although not significantly ($\chi^2 = 7.93$, df = 5, $P = 0.16$). Such variation, particularly the unwillingness of queens to mate at all, appears in our experience to be related to factors such as season, times of day, etc. Interestingly, we found that queens that were injected with sperm mated significantly earlier than their matched Ringer's solution controls (ANOVA, $F_{1,24} = 4.58$, $P = 0.043$). For all remaining treatments, no difference in copulation latency between queens injected with test substances and matched Ringer's solution controls was detected. This observation resulted from the fact that either sample sizes were too small for statistical analysis (queens injected with fatty acids or linoleic acid) or no significant difference was found between the substances tested and their corresponding Ringer's solution controls (queens injected with cycloprolylproline (ANOVA, $F_{1,31} = 1.96$, $P = 0.171$, not significant), oleic acid, (ANOVA, $F_{1,24} = 0.26$, $P = 0.61$, not significant) and the reduced plug (ANOVA, $F_{1,25} = 1.49$, $P = 0.234$).

Discussion

Linoleic acid is the only substance found in the mating plug of bumblebees that decreased female remating behavior. Linoleic acid is an extremely common compound in living organisms, and many different functions have been ascribed to it. In insects it is found in the fat body (22) but is also a component of insect cuticular lipids (23). In social insects, linoleic acid has been found to attract ants (24) and to be a predator (ant) repellent (25).

However, to date, linoleic acid never has been associated with reproductive behavior.

The effect of linoleic acid is clearly in the male's interest, because females are effectively prevented from mating even when they did not receive sperm at the same time (as in our experiments). Our finding is surprising, because we expect females to have evolved counteracting mechanisms to secure their own interests in similar ways as known from other insects (3). For linoleic acid, this mechanism would appear easy, as a simple oxidation of one of the two double bonds within the molecules would degrade the substance. Currently, it is not known how long the effect of linoleic acid lasts, nor can we speculate on the actual physiological mechanisms that underlie the effect of this substance. However, mated queens typically do not copulate again, even up to a week after the first copulation (unpublished data). In addition, the plug remains in queens for up to 3 days after mating (18). Queens that received a reduced plug without linoleic acid did not show any reduction in queen-mating frequency. This observation indicates that the physical attributes of the plug within the queen's sexual tract, that is, its size or the degree of filling, are not effective in avoiding or delaying further copulations. However, the presence of sperm in the sexual tract of queens obviously stimulated these queens, because they were mating earlier compared with the corresponding Ringer's solution control queens. At any rate, the plug and its major active compound, linoleic acid, seem to be very efficient in preventing remating. This effect is in marked contrast to the generally imperfect ways in which males of most insect species attempt to monopolize females (3).

Obviously, males of *B. terrestris* use a range of mechanisms to manipulate females in different ways, including behavioral traits such as mate guarding (18). Although we did not detect any effect of the remaining chemical substances of the bumblebee mating plug (three fatty acids as well as the peptide), this finding does not mean that they have no effect on queens. Further experiments are needed to test these chemicals for other male effects. Given the data presented here, we can already conclude that the copulation event in bumblebees is a much more complex behavior than generally assumed. Because mating plugs as well as the transfer of accessory-gland compounds to females are known from other social insects as well (3, 26), the use of ubiquitous substances in similar ways such as those found here may be common in other social insects. Therefore, male-mating traits may finally help to explain why extreme mating frequencies such as those found in honeybees or leaf-cutter ants are surprisingly rare among social insects (13).

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