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The Use of Bee-Borne Attractants for Pollination of Non-Rewarding Flowers: Model System of Male-Sterile Tomato Flowers

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**BARD Final Scientific Report
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Abstract

The use of bee natural product for enhancing pollination is especially valuable in problematic crops that are generally avoided by bees. In the present research we attempted to enhance bee visitation to Male Sterile (M-S) tomato flowers generally used in the production of hybrid seeds. These flowers that lack both pollen and nectar are unattractive to bees that learn rapidly to avoid them. The specific objects were to elucidate the chemical composition of the exocrine products of two bumble bee species the North American *Bombus impatiens* and the Israeli *B. terrestris*. Of these, to isolate and identify a bee attractant which when sprayed on M-S tomato flowers will enhance bee visitation, and to provide a procedure of the pheromone application regime. During the research we realized that our knowledge of *B. impatiens* is too little and we narrowed the objective to learning the basic social behavior of the bees and the pattern of foraging in a flight chamber and how it is affected by biogenic amines.

Colonies of *B. impatiens* are characterized by a high number of workers and a relatively small number of queens. Size differences between queens and workers are pronounced and the queen seems to have full control over egg laying. Only about 9% of the workers in mature colonies had mature oocytes, and there were no signs of a "competition phase" as we know in *B. terrestris*. Queens and workers differ in their exocrine bouquet. Queen's Dufour's gland possesses a series of linear, saturated and unsaturated hydrocarbons whereas that of workers contains in addition a series of wax-type esters.

Bees were trained to either visit or avoid artificially scented electronic flowers in a flight chamber. Since bee also learned to avoid scented non-rewarding flowers we attempted to interfere with this learning. We tested the effect of octopamine, a biogenic amine affecting bee behavior, on the choice behavior of free-flying bumblebees. Our results show that octopamine had no significant effect on the bees' equilibrium choice or on the overall rate of the behavioral change in response to the change in reward. Rather, octopamine significantly affected the time interval between the change in reward status and the initiation of behavioral change in the bee.

In *B. terrestris* we studied the foraging pattern of the bees on tomato flowers in a semi commercial greenhouse in Yad Mordechai. Bee learned very quickly to avoid the non-rewarding M-S flowers, irrespective of their arrangement in the plot, i.e., their mixing with normal, pollen bearing flowers. However, bees seem to "forget" this information during the night since the foraging pattern repeats itself the next morning. Several exocrine products were tested as visitation enhancers. Among these, tarsal gland extracts are the most attractive. The compounds identified in the tarsal gland extract are mostly linear saturated hydrocarbons with small amounts of unsaturated ones. Application was performed every second day on leaves in selected inflorescences. Bee visitation increased significantly in the treated inflorescences as compared to the control, solvent treated. Treatment of the anthers cone was more effective than on the flower petals or the surrounding leaves. Methanol proved to be a non-flower-destructive solvent.

We have shown that bumble bees (*B. terrestris*) can be manipulated by bee-borne attractants to visit non-rewarding flowers. We have further demonstrated that the bees learning ability can be manipulated by applying exogenously octopamine. Both methods can be additively applied in enhancing pollination of desired crops. Such manipulation will be especially useful in tomato cultivation for hybrid seed production.

Achievements

The major objective of this study was to achieve bee-directed pollination of tomato flowers for hybrid seed production. Since one of the tomato varieties involved had to be male-sterile (e.g., pollenless), and since tomato flowers lack nectar, this variety became rewardless for potential bee-pollinators. The bees normally avoid these flowers in favor of the rewarding variety. Using bumble bees as the pollinator, *Bombus terrestris* in Israel and *B. impatiens* in the US, we search for the proper conditions whereby the bees will visit at least a few times in the non-rewarding flowers. These included agrotechnical arrangement of the plants in the greenhouse, but mostly finding the proper chemical attractants that may render the non-rewarding variety attractive to the bees. The hypothesis was that bee-borne pheromones that are usually attractive to bees can be used as such attractants. Two sources were tested. Queen-derived chemicals, in accordance with the hypothesis that workers, which are generally attracted to the queen pheromones, will be attracted to these chemically treated flowers. The second source was the use of worker-derived chemicals, in accordance with the hypothesis that foraging workers leave scent marks that are attractive to other workers.

We have found that bees are very sensitive to the plants and flower shape. It is therefore desirable to provide two cultivars that have similar phenotypic characteristics. It is especially important that the M-S plant will be as similar to the normal one as possible. Among the flower features, it is important that the M-S has as complete as possible anthers. This is because the bees have difficulties in hanging on to deformed anthers and this fact adds an additional repelling factor.

Plant arrangement in the greenhouse is also important for proper bee action. During foraging the bees seem to have a characteristic path along the line and only after avoiding several consecutive flowers move to another row, not necessarily the adjacent row. It is therefore important to have mixed rows of fertile-sterile plant, preferably in 3M-F+1M-S pattern along the row. Having too many consecutive sterile plants in a row results in the bees abandoning the row completely.

All the known exocrine glands of the queen and the worker were tested. Of these only worker tarsal glands have improved considerably the visitation of the bees on the M-S flowers. These experiments were first conducted in a small green house, second in a tunnel containing about 200 flowers in 5 rows and finally in a large greenhouse containing twice as much flowers. The results were reproduced under all conditions. The percent of visitations in the M-F flowers reached about 50% as compared to between 0-4% in the non-treated M-S

flowers. In contrast percent visitation M-S flowers treated with methanol solution of tarsal gland secretion was as high as that on the M-F flowers. The effect is rather specific because extracts of the other exocrine glands (Dufour's or head extract) were non-attractive. We also tested phenyl acetic acid, a known floral odor, and it was devoid of activity.

Chemical analysis of the tarsal glands revealed that it contains mostly hydrocarbons including alkanes, alkenes and alkadienes. Another major product was oleic acid and to a lesser extent stearic acid. Minor amounts of the corresponding methyl and ethyl esters were also detected. Preliminary experiments in a small net house revealed that the complete tarsal gland extract was far more active than either the hydrocarbons or acids or their esters singly or combined.

The knowledge regarding the foraging behavior of the North American species was less known and required more basic studies regarding the effect of odors on the learning behavior of the bees. In contrast to what was agreed when writing up the grant we were not able to perform experiment in tomato greenhouse. We therefore confined our studies to a flight chamber equipped with artificial flowers. The first task was to assess the bees learning ability. Assuming that bees have innate preference for pollen odors we first tried to train them to visit artificial flowers scented with pollen extract, or to avoid those flower and visit the unscented type. We found that both tasks are easy to achieve. Next we investigated the effect of several parameters such as frequency of flower refill, volume of sugar water and sugar concentration. Learning rate of the bees is relatively constant under several experimental setups (different spatial arrangement, number of feeders and magnitude of differences in profitability between flowers types). Visitation frequency on differently rewarding types of flowers was significantly affected by: 1. The magnitude of differences in profitability - the larger the difference between flower types, the larger the proportion of visits to the more rewarding type. 2. The number of flowers in the system – the more flowers in the system the higher the proportion of visits to the more rewarding type. 3. The spatial distribution of the flowers – the more rewarding type got higher proportion of visits when distributed in grid than in circle.

Learning process in the brain are controlled and modulated by several types of chemical compounds, one of them is the biogenic amine. Changing the level of biogenic amines in the brain may be a promising way to interfere with learning. We selected to study the effect of octopamine because of its well-known involvement in bees' associative learning by enhancing the responsiveness of the bee to a conditioned stimulus. In this study, we tested the effect of octopamine on choice behavior of free flying bumblebees using two-phase

experiment in an array of artificial flowers. During the first phase of the experiment a bee was allowed to collect octopamine laden sugar water from two types (yellow and blue) of equally rewarding “flowers”. In the second phase, one type of “flowers” was set to be less rewarding and the response of the bee (proportion of visits to the less rewarding flowers) was assessed using a sigmoid regression. Our results show that octopamine has no significant effect on bees’ choice or on the rate of the behavioral response to the change in the reward. Rather, it significantly affects the time interval between the change in reward and the initiation of the behavioral response by the bee.

The major achievement of this study was the demonstration that bumble bee foraging decision may be manipulated both by applying external cues (odor attractant) and by internal factors, manipulating the bee’s neurobiology. Using two or more manipulative agents will undoubtedly enhance our ability to direct the bees’ behavior to our target crop. Although the study was conducted specifically with tomatoes the results obtained may be applicable to different crop, whether for hybrid seed production or for more conventional pollination.

Collaboration between the two laboratories was constant both in the programming of the research and analyses of the data. A former student of the Israeli PI spent two years postdoc in the lab of the American PI and helped establish a bumble bee research in this honeybee oriented USDA lab.

The project resulted in two published papers and three additional are in preparation.

1. Cnaani J, Schmid-Hempel R, Schmidt JO (2002) Colony development, larval development and worker reproduction in *Bombus impatiens* Cresson. *Insectes Soc* 49:164-170.
2. Cnaani J, Schmidt JO and Papaj DR (2003) The effect of octopamine on behavioral responses of free-foraging bumblebees to a change in food source profitability *Naturwissenschaften* 90: 185-188
3. Cnaani J., Schmidt J.O, Hefetz A., and Papaj D.R. Switching by bumblebees in an artificial flower system: the effect of reward schedule, density, spatial distribution, and stimulus type. (In preparation)
4. Hefetz A., and Ionescu, A. The use of bee-borne odors for attracting foraging bumble bees to non- rewarding tomato flowers. (In preparation)
5. Cnaani J., and Hefetz, A. Dufour’s gland composition of queens and workers bumble bee *Bombus impatiens* (in preparation)

Appendix

Included are the major findings of the manuscript that are in preparations

Cnaani J, Schmidt JO, Hefetz A, and Papaj DR Switching by bumblebees in an artificial flower system: the effect of reward schedule, density, spatial distribution, and stimulus type.

In this paper we describe how 4 different properties of an artificial flower system affect learning rate and choice of free foraging bumblebee when the profitability of two flower types is changed. These properties are: reward availability, number of flowers, spatial distribution of the flowers and presence or absence of scent in relation to spatial distribution.

Bombus impatiens were obtained from a commercial source and kept in a flight room. Under these conditions the bee forage freely on the artificial flowers present in the flight room. Reward presented by the artificial flowers was computer controlled so that flowers were filled with sugar water (10 µl) in sequence with a fixed interval time. Flower refilling was also controlled and occurred at specific Time Interval between Refilling (TIR).

To assess how bees learn and make choices in the artificial flower array, a two-phase experiment was conducted. The flowers were divided into two groups marked by yellow or blue Plexiglas disks on top of the Petri dish for easy discrimination by the bee. During phase 1 (training) a naive bee was allowed to make 150 visits to the flowers. During this training phase, TIR was 60 sec for both flower types and each flower offered 4µl of 30% sugar water upon refilling. In phase 2 (the test) the TIR in each type of flower was changed (the blue flowers always set to be more rewarding than the yellow ones), and the bee was allowed to perform 420 more visits. To analyze learning performance and flower choice, flower-visits in each phase (and for each individual bee) were divided into sets of 30 visits, and the proportion of visits to the less rewarding group was calculated for each set. Changes in these proportions with time were analyzed using a four parameter sigmoid model of non-linear regression (Equation below); where “a” is the amplitude, the distance between the two asymptotes; “b” is the slope, the rate of transition between the two asymptotes; X_0 is the value of the inflection point, where 50% of the transition between the two asymptotes has occurred; and Y_0 is the value of second asymptote.

$$Y = Y_0 + \frac{a}{1 + e^{-\left(\frac{X - X_0}{b}\right)}}$$

Figure 1 shows the effect of TIR on flower visitation. In the first 150 visits (number -150 to 0), the two types of flowers were equally rewarding having TIR of 60 seconds. At the second phase of the experiment (visit numbers 0 to 420), TIR was change to be 30 sec for the blue flowers and 210, 420, 720 or never for the yellow. The proportion of visit of each bee to the yellow flowers was calculated for each set of 30 visits, and was analyzed by 4 parameters sigmoid regression. The curves were constructed from the average parameters for each treatment, and the mean value of each parameter and its standard error are represented by the symbols (Y_0 on the right, $a-Y_0$ on the left, X_0 , the inflection point in the middle). Shortly after TIR was changed (the beginning of phase 2), the proportion of visits to the yellow flowers (the less rewarding) declined, eventually reaching a new equilibrium (the Y_0 parameter in the sigmoid regression equation) at $42.5 \pm 1.3\%$ for the “210/30” schedule; $40.0 \pm 1.5\%$ for the “420/30” schedule; $29.2 \pm 3.1\%$ for the “720/30” schedule and $2.0 \pm 1.1\%$ for the “Never/30” schedule (ANOVA, $F_{3,14}=128$, $p<0.0001$). These values are significantly different from each other, except for the Y_0 values of “210/30” versus “420/30” schedules ($p=0.4$). The amplitude of the behavioral response (parameter ‘a’) was also significantly affected by the different TIRs (ANOVA, $F_{3,14}=71.4$, $p<0.0001$).

Figure 1: Change in the proportion of visits to the yellow flowers (the less rewarding) over 570 visits.

Figure 2. The effect of flowers spatial distribution and TIR on changes in the proportion of visits to the yellow (the less rewarding) flowers.

Adding more flowers to the foraging arena did not affect learning performance as reflected in the values of b and X_0 (unpaired t-test, $df=20$; $p=0.36$ and $p=0.5$ for b and X_0 respectively). Choice (Y_0) and the amplitude of behavioral change (a), however, were both significantly affected. Y_0 was higher when the bees foraged on 12 flowers ($42 \pm 1\%$) versus 6

($37.5 \pm 1\%$) (unpaired t-test, $df=20$; $p=0.01$). Amplitude (a) was higher when foraging on 6 flowers ($14.6\% \pm 1\%$) versus 12 flowers ($10.5\% \pm 1.5\%$) (unpaired t-test, $df=20$; $p=0.01$).

Spatial arrangement had a decisive effect of the bees' learning ability. When the flowers were arranged as a grid as opposed to circle arrangement, the bees showed clear preference to the yellow flowers, although both flower types were equally rewarding. The effect of TIR and spatial arrangement on learning is depicted in figure 2. These two parameters interacted to affect the learning of the bees. The inflexion point in the greed arrangement was greater in the long TIR. In contrast in the circle arrangement the inflexion point was greater in the short TIR.

Effects of scent (scent present versus absent) and spatial distribution (circle versus grid) never interacted significantly for any model parameter. Adding scent to flowers had a significant effect only on Y_0 (an estimate of the new choice made by the bees at equilibrium), preference for the better type being stronger in the presence of scent.

The result obtained show that learning rate of the bees is relatively constant under the several experimental setups. Visitation frequency on differently rewarding types of flowers was significantly affected by: 1. The magnitude of differences in profitability - the larger the difference between flower types, the larger the proportion of visits to the more rewarding type. 2. The number of flowers in the system - the more flowers in the system the higher the proportion of visits to the more rewarding type. 3. The spatial distribution of the flowers - the more rewarding type got higher proportion of visits when distributed in grid than in circle.

Cnaani J., and Hefetz, A. Dufour's gland composition of queens and workers bumble bee *Bombus impatiens* (in preparation)

Bees are endowed with exocrine glands, which in many social species produce caste specific pheromones. In the elaborately studied honeybee for example at least three major glands produce queen specific compounds. These manifest the presence of the queen in the hive promoting cooperation and harmony in the hive. The pheromone chemistry of bumble bees is less known but being social, although considered more primitively social compared to honey bees, one would predict to find caste specific pheromones. Although bumble bees are endowed with pheromone gland, the chemistry of only a few has been unraveled.

The labial gland secretion of male bumble bees was extensively studied, revealing a plethora of terpenoids and terpenoid esters that seems to serve as territorial pheromones. A comparative study of many European bumble bees demonstrated that Dufour's gland secretion contains species specific multicomponent mixtures covering a wide range of volatility. The function of this glandular secretion in *B. hypnorum* is to mark a trail leading from the nest entrance to the inner parts of the nest. Chemical analysis of the secretion in this species also showed interesting intra-nidal differences between workers that are correlated with size polymorphism. The chemical composition of queens bumble bees was recently studied, revealing complex mixtures that may contain up to 80 different substances.

In the present study we investigated the chemical composition of queens and worker *Bombus impatiens*.

Glands were dissected from queens and workers that were freshly killed by freezing. The excised glands were extracted in pentane and were subsequently analyzed by GC/MS.

The identity of Dufour's gland components was first determined by gas chromatography coupled to mass spectrometry (VGM250Q) using a DB-5 fused silica capillary column (Temperature program: 60°C-270°C at 5°C/min; hold at 270°C for 30 min and then heated to 295°C at 20°C/min). The eluting compounds were identified by their fragmentation pattern.

Figure 1 and Table 1 show a comparative analysis of Dufour's gland secretion of queens and workers *B. impatiens*. There is clear caste specificity in the secretory composition. The queens' secretion is composed almost exclusively of hydrocarbons, among which tricosene, tricosane, pentacosene and pentacosane dominates. The dominance of alkenes is unusual in Dufour's gland secretion. The secretion of workers is also endowed with

hydrocarbons having the same major components as the queen does. However worker's secretion possess an additional series of wax-type esters that are completely missing from the queen's secretion.

Table 1. The compounds identified in Dufour's gland secretion of queens and workers of *Bombus impatiens*.

Substance	Workers	Queens	Substance	Workers	Queens
Heptadecene	++	--	Hexadecyloctanoate	++++	--
Decylisobutyrate	+	--	Hexacosene	--	+
Hexadecenal	++	--	Hexacosane	+++	+
Hexadecanal	+++	--	Heptacosene	++++	++
Nonadecane	++	+	Heptacosene	++++	+
Decyloctanoate	++	--	Heptacosane	++++	+
Dodecylhexanoate	++	--	Dodecyltetradecenoate	+++	--
Eicosane	+	--	Hexadecyldecanoate Tetradecyldodecanoate	+++	--
Octadecenal	+	--	Octacosane	++	+
Octadecanal	+	--	Nonacosene	++++	++
Heneicosene	++	+	Nonacosene	++++	++
Heneicosane	+++	++	Nonacosane	++++	+
Oleic acid	+	+	Dodecylhexadecenoate	++++	--
Docosene	+	+	Dodecylhexadecanoate+ Hexadecyldodecanoate	++++	--
Dodecyloctanoate	+++	--	Triacontane	++	+
Docosane	++	++	Hentriacontene	++++	++
Tricosene	++++	++++	Hentriacontane	+++	+
Tricosane	++++	++++	Dodecyloctadecenoate	++++	--
Tetracosene	+	++	Tritriacontene	+++	+
Dodecyldecanoate	++++	--	Tritriacontane	+	+
Tetracosane	+++	+	Hexadecenylhexadecenoate	+++	--
Pentacosene	++++	++++	Hexadecylhexadecenoate	++++	--
Pentacosene	++++	++++	Hexadecylhexadecanoate	+	--
Pentacosane	++++	++++	Hexadecenylotadecenoate	++	--
Hexadecenylotanoate	++	--	Hexadecyloctadecenoate	++++	--
Dodecyldodecanoate	++++	--	Octadecenylotadecenoate	+++	--

Hefetz, A., and Ionescu, A. The use of tarsal gland secretion for attracting foraging bumble bees to non- rewarding tomato flowers. (In preparation)

Bumble bees are considered as lone foragers that do not communicate about the spatial co-ordinate of the food. Each forager presumably makes its own foraging decision based on the information acquired during previous foraging trips. Notwithstanding, it was recently shown that the bees use pheromone communication to optimize their foraging efforts. Bees tend to mark rewarding flowers in which future visits may be profitable. Bees also convey the presence of good forage by emitting scent from their tergal glands while moving excitedly and fanning their wings. The use of pheromones for making foraging decision may be therefore utilize to manipulate the bees to certain flowers that the bees may have ignored otherwise.

The production of tomato hybrid for better fruit quality is well established. Since tomatoes have a large degree of self-compatibility it is mandatory that one of the line will be Male-Sterile (MS) so that all the fruits it bears will be from cross-pollination. To date pollination is achieved by hand, a process that is labor intensive and costly. The use of pollination agents to alleviate this labor-intensive process is therefore desirable, and among the potential pollinators bumble bees are the best candidates. They perform buzz-pollination and are therefore able to extract the pollen from the closed anthers peculiar to the Solanacea. Optimally, the bees should forage equally on both plant variety and therefore achieve effective cross-pollination. However tomatoes present a special problem since M-S flowers are pollenless as well as nectarless. The bees learn rapidly to recognize these non-rewarding flowers and avoid them in favor of the rewarding pollen bearing plants.

The objectives of this study were: 1). To learn the foraging decisions made by the bees while visiting an array of rewarding and non-rewarding flowers. 2). To discover a bee-born odor that may attract the bees to the non-rewarding flowers and by that inducing 2-3 bee visits per flower. Preliminary studies revealed that such a visitation results in satisfactory seed production.

The experiments were performed either in a net-house where about 60 tomato plants were arranged at a ratio of 3:1 male fertile to male sterile, or in a green house were about 160 plants at the same M-F to M-S ratio. All M-S plants were regularly inspected and those that contained pollen (i.e., male fertility restored) were removed. The experimental plot received the same treatments as the larger greenhouse, according to the recommended procedures. When the tomato plants began blooming, a bumblebee (*Bombus terrestris*) colony was placed

in the experimental plot. The hive contained at least 20 workers and was replaced as soon as the first virgin queen emerged. Males were removed from the colony once a day. The hive was supplied with an unlimited amount of sugar-water and twice a week with pollen (non-Solanacea), to supplement the deficiency in pollen due to the relatively small number of plants in the experimental plot. The top of the hive was transparent to enable direct in-nest observations. In certain experiments the hive had two entrances, a regular entrance/exit hole to which a clear plastic (Tygone) tube was attached, and a top one-way entrance that allowed the bees to enter only. This arrangement enabled the release of a specific bee for foraging while diverting all other bees that exited the nest back to the nest via the top entrance. The bees were individually marked with a numbered, color plastic tag glued on their thorax. Experienced bees after a successful foraging trip were additionally marked with a bright color on the abdomen.

For obtaining the bees pheromone complement either glands or selective body parts were homogenized in chloroform methanol 2:1(respectively) for a total lipid extraction or alternatively in methylene chloride. For application, the extracts were either emulsified with Tween 20 and admixed with tap water, or evaporated to dryness and reconstituted in methanol. Plants or flower were sprayed individually with 1 bee equivalent using a small spray bottle.

Figure 1 depicts the foraging pattern of individual bees on an array of rewarding and non-rewarding flowers. In the first foraging trip the bees encountered both the M-F and M-S in accordance with their proportion in the net-house and the proportion of visits was similar for both types of flowers. In the consecutive trips however encounters on the M-F flowers became progressively higher than their proportion in the net house with a proportional decrease in encounters with the M-S flowers. Moreover while the percent visits in the M-F

flowers remained constant reaching about 90% that on the M-S flower progressively declined reaching less than 10% in the M-S flowers.

In the second set of experiments we tested the effect of various exocrine glands on the frequency of bee visitation on the M-S flowers. Of the five extracts that were tested, Dufour's, mandibular, labial, tarsal gland and cuticular washes, only the tarsal glands proved attractive. The results of treatment with tarsal glands are presented in Table 1. Application of the secretion on the whole flower augmented the visitation rates of the bees on the treated M-S from 7.3% for untreated or 2.3% for the methanol treated to 28.6% for the tarsal gland treated. During these observations we noted that the bees inspected the anther cones closely before landing. We therefore repeated the experiments but applied the secretion on either the cones or the flower petals. The results corroborated our preliminary observation. Visitation on the treated M-S cones rose to 53.6% that was not different from visitation on the M-F flowers. Visitation on plants in which only the flower petals were treated was similar to whole treated M-S flowers, amounting to 28%.

Table 1. The effect of tarsal gland secretory application on the tendency of the bees to visit M-S flowers.

Flower type	Application of tarsal gland secretion	Cumulative number		
		Encounters	Visits	% Visits
Male fertile	None	1077	511	47.4
Male sterile	None (control I)	165	12	7.3
Male sterile	Methanol (control II)	43	1	2.3
Male sterile	On whole flowers	56	16	28.6
Male sterile	On anthers cone	69	37	53.6
Male sterile	On flower petals	50	14	28

The results corroborate previous findings that bees leave tarsal gland secretion on rewarding flowers, inducing repeated visits by the bees on these flowers. We also clearly demonstrated that we may "fool the bees" and induce them to visit non-rewarding flowers.