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HONEYBEE (*Apis mellifera* L.) QUEEN FECES: SOURCE OF A PHEROMONE THAT REPELS WORKER BEES

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Abstract—When placed in a small observation arena with workers, most young virgin honeybee queens released fecal (hindgut) material during agonistic interactions with workers and with each other. On release of this material, workers moved to the sides of the arena and groomed themselves. Bioassays of virgin queen fecal material demonstrated that it contains pheromone that repels workers and stimulates grooming behavior. Pheromone was present only in the feces of virgin queens that were more than 24 hr old and less than 2 weeks old. Feces of 2- to 4-day-old workers and virgin queens more than 2 weeks old did not elicit an avoidance response by workers. Moreover, the feces of young virgin queens had a strong fragrance, while that of older queens had a rancid odor and that of young workers had no detectable odor.

Key Words—Honeybee, *Apis mellifera*, Hymenoptera, Apidae, feces, queen pheromone, queen acceptance, repellent.

INTRODUCTION

Queen honeybees have long been known to produce a variety of pheromones that are involved in integrating colony behavior. The mandibular glands produce substances (primarily 9-oxodec-*trans*-2-enoic acid) that suppress ovariole development and queen cell construction by workers (see reviews by Butler, 1967; Gary, 1974; Michener, 1974). Secondary queen substances are produced

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by epidermal glands located on the abdominal tergites (Velthuis, 1970). In addition, pheromone produced by Koschewnikow's glands located near the sting base (Butler, 1967), as well as the mandibular (Gary, 1961; Butler et al., 1973; Simpson, 1979) and tergal glands (Vierling and Renner, 1977), probably have a role in the attraction of workers to queens and in queen recognition. While the existence and function of pheromones in young virgin queens is not well known, little 9-oxodec-*trans*-2-enoic acid is produced by queens less than 3 days old (Butler and Paton, 1962; Velthuis, 1970), and the chemical composition of their mandibular secretion is very similar to that of workers (Crewe, 1982).

Honeybee colonies raise several new virgin queens in response to stimuli that vary with the age and physiological condition of the extant queen and in response to seasonal stimuli associated with the reproductive cycle. When newly emerged virgin queens encounter each other in the hive, they fight until all except one are stung and killed. By observing interactions between 2- to 4-day-old virgin queens in small arenas containing workers, Page and Erickson (1986) frequently found that during agonistic interactions one or both of the queens defecated. On release of the feces, the workers immediately moved away from the queens and clustered near the top of the arena, suggesting that a volatile substance was released. In this paper we describe laboratory studies of interactions between young virgin queens and workers that are confined in small observation arenas. We also present the results of bioassays of the responses of workers to material contained in the hindgut of queens.

METHODS AND MATERIALS

Rearing of Test Bees. Virgin queens and workers used for observations of virgin queen interactions were obtained from eight different colonies, while virgin queens used for the fecal bioassays came from four of the eight colonies. The queen of each colony was in her first season of egg laying during the virgin queen interaction studies (summer of 1984) and in her second season of egg laying during the bioassays of hindgut material (summer of 1985). Each queen of each colony was mated by instrumental insemination to two different unrelated drones. Thus each queen produced worker and virgin queen daughters belonging to two different subfamilies. Queen mothers were supersisters of each other (coefficient of relationship = 0.75).

Virgin queens were produced by grafting young larvae into queen cell cups that were placed in a queenright cell builder colony that was unrelated to the eight source colonies (see Laidlaw, 1979). Once the queen cells were provisioned and sealed by workers, they were placed into individual glass vials (4 dram) and incubated at 34°C until the queens emerged. The wooden base of each queen cell served as a lid for each vial.

Newly emerged queens were subjected to one of the following four treatments:

1. Provided with royal jelly and sugar candy. A small amount of sugar candy was placed in each glass vial, and the queen cells that contained some residual royal jelly were left in place over the tops of the vials. The vials containing queens were returned to the incubator for an additional 2–4 days. In order to determine when the fecal pheromone is produced by queens, some queens were sacrificed at various ages ranging from emergence to less than 48 hr old.

2. Provided with sugar candy only. Queens were treated as described above, except that queen cells were replaced with wooden tops to eliminate the source of royal jelly.

3. Fed by workers for 3–4 days. Queens were removed from the vials immediately after emergence and placed individually in 8-mesh screen cages, which were then inserted into an unrelated colony. The resident queen in the queen-storage colony was separated from the frames holding the young virgin queens by a queen excluder.

4. Fed by workers for 2–3 weeks. Queens were treated as described in (3) above, except they remained in the queen-storage colony for 2–3 weeks. The queens were then placed in individual glass vials, provisioned with sugar candy, and incubated for an additional two days.

Individual workers to be used as a source of feces were collected as they emerged from the brood comb, placed individually into glass vials provisioned with a small amount of sugar candy, and then subjected to one of the following treatments.

1. Provided with sugar candy and royal jelly. The queen cell cups from which queens emerged in treatments 2–4 above were placed over the top of each vial, providing a source of royal jelly for each worker. Each worker was incubated at 35°C for 2–4 days.

2. Provided with sugar candy only. The workers were treated as described in (1) above, except that vials were covered with wooden caps.

All sugar candy used in provisioning the glass vials was made from confectioner's sugar and came from the same batch.

Observations of Queen Interactions. Groups of ten supersister workers (members of subfamilies were phenotypically marked, see Page and Erickson, 1986) were collected at random from individual colonies by vacuuming them from the top bars of the brood chambers into 135-ml cardboard cups (arenas) with wire screen tops. Ten cups of bees were collected at a time and taken directly into the laboratory where behavioral observations began within 15 min after the collection of the last cup of bees. A single virgin queen from treatment 1 was introduced into each arena (cup of 10 bees) by tilting the arena on its side and inverting the vial containing the virgin queen over a hole in the side. Queens were observed individually for 5 min after introduction. Within 30 min to 6 hr after introduction, a virgin queen was removed from one arena and introduced into another that contained a previously introduced queen. Behav-

ioral interactions between the two queens and the workers were then observed for 5 min. Queens and workers were used just once, then discarded.

Procedures for Bioassays of Feces. Groups of 10 workers were collected in 235-ml cups as described above for the observations of queen interactions. Prior to the collection of bees, a 6.0-cm-diameter piece of filter paper with a 1.8-cm-diameter circle marked in the center was taped to the bottom of each cup for use during the bioassays. Cups were taken into the laboratory, and all bioassays with each group of 10 bees were completed within 1 hr.

Prior to each replicate, the feces of a test bee (queen or worker) was collected by immobilizing the bee by crushing its thorax. The terminal abdominal sternite and tergite were spread apart and a micropipet was placed over the anus. The abdomen was gently squeezed until all of the hindgut contents flowed into the pipet.

For each replicate, a test and a control observational arena (cup) were paired. The behavior of the workers was observed for 60 sec before and then for 60 sec after the introduction of feces in the test arena and for similar time periods before and following the introduction of 10–30 μl of distilled water (about equivalent to the volume of feces) in the control arena. The feces and distilled water were introduced into their respective arenas by using a rubber bulb to squeeze each substance out of a micropipet and into the circle on the floor of each arena. Three behavioral patterns of the workers were quantified during each control and test: (1) the number of workers that walked through the 1.8-cm-diameter circle on the bottom of the arena during each 60-sec observation period, (2) the number of bees that groomed their legs or antennae, and (3) the number of workers on the bottom of the arena at the end of each 60-sec observation period.

The responses of the workers to the tests and controls before and after the introduction of feces or distilled water, respectively, were summed for each treatment and analyzed using a χ^2 test of independence. All replicates in which more than five of the worker bees scent fanned were eliminated from the analysis because scent-fanning behavior (with exposure of the Nasonov gland) was interpreted as a consequence of external disturbances and the new surroundings of the bees.

RESULTS

Observations of Queen Interactions. Upon introduction into an arena of workers, 2- to 3-day-old virgin queens typically extended and bowed their bodies, lifted their mesothoracic pair of legs, and expressed a droplet of clear liquid from between open mandibles ($N = 312$ replicates). Workers usually imbibed the liquid, groomed the queen with their proboscis, and offered the queen a reciprocal droplet of clear liquid. Occasionally, one or more workers behaved

agonistically and grasped a body part of the queen (usually a leg or a wing) with their mandibles. When this occurred, queens often released a volume of liquid hindgut (fecal) material that was light yellowish-brown in color with a strong floral fragrance. Workers usually moved away from this substance to the top and sides of the arena and groomed themselves. Occasionally, queens released a very minute quantity of this hindgut material. Workers appeared to be highly attracted to small amounts.

When two young virgin queens encountered each other, one or both usually released their hindgut content on each other during biting and stinging behavior ($N = 81$ replicates). Workers responded by moving away from the queens and grooming. Many fights between virgin queens lasted for several minutes to several hours with intermittent short periods of biting and attempted stinging followed by longer periods of reduced activity. During periods of reduced activity, workers groomed and fed queens, although frequently queens with feces on their bodies were not cared for by workers or received less care than uncontaminated queens. Occasionally fights resulted in injury and eventual death to both queens.

Fecal Bioassays. At emergence as an adult, virgin queens did not have any hindgut material ($N = 10$). However, queens less than 24 hr old that emerged in glass vials provisioned with royal jelly and sugar candy had approximately 5–20 μl ($\bar{X} = 12.5$; $N = 8$) of material in their hindgut, while queens 24–48 hr old had about 10–30 μl ($\bar{X} = 22.1$; $N = 7$). All fecal material was light yellowish-brown in color and had a strong floral fragrance.

After the hindgut material of a queen from treatments 1 or 2 was introduced into the arena, worker bees moved away from the bottom, clustered on the side and top, and then groomed themselves. Significantly fewer workers walked through the circle and significantly fewer remained on the bottom of the arena after the introduction of feces from treatment 1, yet grooming was significantly more frequent after introduction of fecal material (Table 1). The results of bioassays using the hindgut content of virgin queens from treatment 2 were similar to those of treatment 1, except the decrease in the number of workers remaining on the bottom after 60 sec was not statistically significant. The cessation of walking and frequent grooming were evident 30 sec after the introduction of feces into the arena, and, generally, the workers turned away from the feces and/or clustered for as long as 2–3 min after its introduction.

The feces from 3- to 4-day-old queens fed by workers (treatment 3) elicited responses from workers similar to that of queens reared in glass vials (treatments 1 and 2) (Table 2). However, the feces of 2- to 3-week-old virgin queens fed by workers (treatment 4; Table 2) and 2- to 4-day-old workers (Table 3) did not elicit grooming behavior or repel the bees. There was no significant difference in the number of workers on the bottom of the arena or walking through the circle before and after the introduction of feces.

The feces of all the queens and workers less than 5 days old had a milky

TABLE 1. RESPONSES OF WORKER HONEYBEES TO FECES OF VIRGIN QUEENS REARED IN GLASS VIALS WITH ROYAL JELLY AND SUGAR CANDY (TREATMENT 1) OR WITH SUGAR CANDY ONLY (TREATMENT 2)[a]

	Before	After	χ^2	*P* value
Numbers of workers walking through circle				
Treatment 1				
Control	357	402	76.31	<0.001
Test	381	152		
Treatment 2				
Control	227	254	41.22	<0.001
Test	227	97		
Numbers of workers on bottom				
Treatment 1				
Control	26	38	6.80	0.009
Test	39	22		
Treatment 2				
Control	23	29	1.17	0.279
Test	17	12		
Numbers of workers grooming				
Treatment 1				
Control	25	34	18.70	<0.001
Test	21	124		
Treatment 2				
Control	13	22	6.76	0.009
Test	12	66		

[a] Data are the total numbers of workers walking through a 1.8-cm-diameter circle on the bottom of the arena during 60 sec before and after the presentation of the control (distilled water) and the test (feces), the total numbers of workers on the bottom of the arena, and the numbers grooming at the end of each 60-sec period. Data for the bioassays in treatment 1 and treatment 2 are based on 40 and 25 replicates, respectively, of each control and test.

yellowish-brown color, although the feces from workers had a slight greenish tint. In addition, the feces from young queens had a strong floral scent, while the feces of workers had no detectable odor. Conversely, the feces from 2- to 3-week-old queens fed by workers was clear with chunks of dark solid matter and had a rancid odor.

DISCUSSION

The rectal content of virgin queens less than 2 weeks old contains a pheromone that, under our test conditions, repels workers and stimulates grooming behavior. Production of this material is independent of the food source of virgin

TABLE 2. RESPONSES OF WORKER HONEYBEES TO FECES OF 3- to 4-DAY-OLD (TREATMENT 3) OR 2- to 3-WEEK-OLD (TREATMENT 4) VIRGIN QUEENS FED BY WORKERS[a]

	Before	After	χ^2	P value
Numbers of workers walking through circle				
Treatment 3				
Control	87	107	41.87	<0.001
Test	110	27		
Treatment 4				
Control	149	132	1.55	0.213
Test	96	107		
Numbers of workers on bottom				
Treatment 3				
Control	9	13	1.23	0.267
Test	10	7		
Treatment 4				
Control	11	12	0.32	0.569
Test	12	18		
Numbers of workers grooming				
Treatment 3				
Control	7	8	6.72	0.009
Test	3	24		
Treatment 4				
Control	1	0		
Test	4	5		

[a] The types of responses recorded are the same as in Table 1. The responses were recorded during 60-sec time periods. Data for treatment 3 and treatment 4 are based on 13 and 14 replicates, respectively, of each control and test.

queens. Although feces from young workers is somewhat similar in appearance to that of young queens and very different from that of old queens, our results show clearly that workers do not have the same material in their feces even when fed the same diet as young virgin queens. These results also corroborate the significance of the odor of the feces; only feces of young queens that had a strong floral fragrance elicited avoidance behavior by workers.

Renner and Baumann (1964) found that young virgin queens have a strong floral odor that apparently originates from tergal glands. This odor is found on the abdomens of virgin queens 4–18 days old, but not on newly emerged or very young laying queens (Boch et al., 1975). Although the function of this odor is not known, it does not appear to have a role in drone attraction during mating flights (Boch et al., 1975). Since our results indicate that a similar odor originates from material in the hindgut, it is possible that the odor detected in previous studies actually originated in the hindgut and spread over the abdomen.

TABLE 3. RESPONSES OF WORKER HONEYBEES TO FECES OF WORKERS REARED IN GLASS VIALS WITH ROYAL JELLY AND SUGAR CANDY (TREATMENT 1) OR WITH SUGAR CANDY ONLY (TREATMENT 2)[a]

	Before	After	χ^2	*P* value
Numbers of workers walking through circle				
Treatment 1				
Control	214	202	1.89	0.169
Test	209	162		
Treatment 2				
Control	274	299	0.11	0.741
Test	259	294		
Numbers of workers on bottom				
Treatment 1				
Control	15	23	1.21	0.271
Test	31	30		
Treatment 2				
Control	21	29	1.40	0.236
Test	19	42		
Numbers of workers grooming				
Treatment 1				
Control	8	9	0.07	0.791
Test	16	21		
Treatment 2				
Control	27	33	0.27	0.605
Test	19	42		

[a] The types of responses recorded are the same as in Table 1. The responses were recorded during 60-sec time periods. Data for treatment 1 and treatment 2 are based on 25 and 38 replicates, respectively, of each control and test.

The adaptive significance (if any) of the fecal pheromone of young virgin honeybee queens remains to be demonstrated. It may be important in inhibiting rejection of virgin queens by disrupting agonistic behavior of workers. In addition, it may provide a ritualistic element to the dominance fight of virgin queens and help ensure that a queen, and thus the colony, survives. Evenly matched virgin queens may inflict reciprocal damage on each other, reducing the potential for the colony to successfully produce a replacement queen. However, if workers use contamination of a queen with feces as a symbolic determinant of subordination or dominance, then they may aid in the final determination of colony succession by not feeding and grooming contaminated queens and thereby placing the contaminated queen at a disadvantage.

Pheromone in the feces may also provide labels used by workers in kin recognition. Page and Erickson (1986) demonstrated that virgin queens have

genetically determined labels and that workers learn these labels and respond differentially on the basis of genetic relationship. The location and nature of these labels are unknown.

Additional behavioral assays under less artificial test conditions are necessary to determine if queens and workers respond to the pheromone in the same way in a natural context and to determine the adaptive significance of the feces pheromone. Further studies also are needed to determine if the odor described by Renner and Baumann (1964) as emanating from the tergites actually originates from the hindgut, and, if it does not, then to determine the functional relationship of these two sources of pheromone. Identification and testing of specific chemical compounds from the fecal material to determine the active components would greatly facilitate investigations of the possible functional significance of this newly discovered honey bee queen pheromone.

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