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Queen Substance Dispersal by Messenger Workers in Honeybee Colonies

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Summary. 1. Worker honeybees contacting a queen can transport the queen's inhibitory signal, queen substance, to other workers unable to contact the queen. Airborne dispersal of queen substance is at most a minor mechanism for queen substance transmission.

2. This worker transport of queen substance is an important supplement to queen substance dispersal by direct queen–worker contacts. For although colonies lose their inhibition against queen rearing within 10 h of queen loss, a queen contacts only approximately 35% of the broodnest workers in 10 h.

3. The queen facilitates queen substance dispersal by frequently standing stationary, at which times workers can thoroughly contact her, and by occasionally making a major shift in her position within the nest.

4. Queen attendance by workers is strongly age-dependent, with 3–9 days being the age range for intense contact with the queen.

5. Workers that have made extensive (>30 s) queen contact appear to behave as 'messengers' dispersing queen substance. They walk more rapidly, antennate nestmates and receive inspections more frequently, and perform fewer labor acts in the 30 min following queen contact than do randomly chosen broodnest workers of the same age (control bees).

6. The following observations support the surface transport model over the food exchange model for queen substance transmission by workers: (1) the higher frequency of antennations with nestmates and of inspections by nestmates for messenger bees relative to control bees, (2) the close correlation ($r=0.76$) for messenger bees between duration of queen contact and number of inspections by nestmates, and (3) the low frequency of food donations ($\bar{x}=1.8$) compared with nestmate antennations ($\bar{x}=56.4$) by messenger bees in the 30 min following queen contact.

7. There are no messenger bee specialists cycling rapidly between contacts with the queen and workers.

8. Messenger bees were analyzed by gas chromatography for (E)-9-oxodec-2-enoic acid. As little as 0.1 ng ($=3.3 \times 10^{11}$ molecules) of the acid per messenger bee could have been detected, but none was found.

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9. The evolution of messenger behavior by workers and the significance of the findings to understanding the timing of colony reproduction are discussed.

Introduction

Observation of *identifiable individuals* is a powerful tool for analyzing the mechanisms integrating the behaviors of individuals within insect societies. This works because steady observations on particular individuals can reveal patterns in colony operations which are otherwise hidden amid a confusing tangle of colony members. Notable successes achieved with this technique include Lindauer's (1952) discovery of 'patrolling' behavior whereby workers apparently assess their colony's labor needs, and his (1955) elucidation of the decision-making process of scout bees selecting a future nest site. I now report studies based heavily upon observations of identifiable individuals in which I investigated the mechanisms of queen substance dispersal in honeybee colonies.

The queen in a honeybee colony profoundly affects the reproductive activities of her worker nestmates by inhibiting both the development of their ovaries and their rearing of additional queens. The principal channel for this queen–worker communication is chemical, and the main source of the inhibitory pheromone(s) is the queen's mandibular glands. The most abundant component of the mandibular gland secretion with pheromonal activity is (E)-9-oxodec-2-enoic acid (9-ODA). Other unidentified compounds apparently also serve in the queen's inhibition of the workers, perhaps as queen recognition scents which help coordinate collection and distribution of the inhibitory 9-ODA by workers (for reviews of this research, see Allen, 1965; Boch and Lensky, 1976; Butler, 1970; Gary, 1970, 1974; and Vierling and Renner, 1977). One lingering mystery in this system of queen–worker communication is the dispersal mechanism for the queen's pheromone(s) which inhibits the reproductive activities of workers, hereafter referred to simply as 'queen substance.'

Several properties of the dispersal process are known which help define the puzzle. First, workers must contact the queen for colony-wide worker inhibition, thus airborne dispersal of queen substance is at most a minor mechanism (Huber, 1814; Müssbichler, 1952; Butler, 1954; Groot and Voogd, 1954; Voogd, 1955; Verheijen-Voogd, 1959). Second, queen substance transmission is rapid and extensive. Within 24 h of removing a queen from a colony the nurse bees will have started rearing a replacement queen (Huber, 1814; Butler, 1954). Thus the interval between queen signals to individual nurse bees is apparently under 24 h. And because a typical honeybee colony contains many thousands of young bees whose queen-rearing activities and ovarial development are largely inhibited by the queen (Perepelova, 1929; Verheijen-Voogd, 1959), the transmission mechanism must achieve widespread signalling.

Given this information, there are two conceivable mechanisms for queen substance dispersal among the workers in a colony. First is by *direct queen–workers contacts*. A queen moves about her colony's broodnest, several workers can contact the queen simultaneously, and the turnover rate of bees contacting the queen is high (Allen, 1960). Thus large numbers of workers will contact the queen within the 24 or so hour lag period which colonies exhibit before initiating queen replacement. The second possible mechanism is for *worker collection and distribution* of the queen substance to supplement pheromone dispersal by direct queen–worker contacts. Verheijen-Voogd (1959) and Velthuis (1972) have demonstrated that workers in contact with a queen can transport an inhibitory queen signal to workers not in contact with the queen. However, their experiments used whole queens and only very small groups of workers (60–65 individuals). Thus their findings can be only tentatively applied to the workings of full-scale colonies. Butler (1954) attempted to test for worker transport of queen substance by using larger scale experimental units (3000–4000 bees). His technique involved transferring bees from a queenright colony to a queenless one, but the lack of control for the effects of colony disturbance when introducing bees into the test colonies makes these experiments inconclusive.

The studies reported here address the mystery of queen substance dispersal by considering, in order, the following three questions.

1. Does worker transport of queen substance exist?
2. How important is worker dispersal of queen substance relative to direct distribution by queen–worker contacts?
3. What, if any, queen and worker behaviors contribute to the dispersal of the queen substance?

Throughout these studies I worked with full-scale colonies or at least large nucleus colonies. In this way I hoped to simplify the generalization of my findings to understanding the inner workings of natural honeybee colonies.

Materials and Methods

1. Test of Worker Transport of Queen Substance

The design of this experiment resembles that of Verheijen-Voogd (1959) and Velthuis (1972) in testing the ability of workers with a queen to transmit the queen's inhibitory pheromone(s) through a screen to other workers. However, instead of using little Liebefeld cages (see Verheijen-Voogd, 1959) which hold only about 60 bees, I used small hives which held approximately 8000 bees.

Hive Design. Each hive (20 cm wide × 49 cm long × 26 cm high; interior dimensions) was divided into two separate chambers by either a single or double screen of 8-mesh hardware cloth (square apertures, 2.4 mm on a side). In hives with a double screen partition, the two screens were spaced 11 mm apart to prevent workers from contacting each other through the screens. Only one chamber in each hive had an entrance opening. Each chamber's lid contained an opening in which a feeder bottle could be inserted, and each chamber held two frames (Langstroth full-depth) of comb. As is shown in Fig. 2, there were four internal arrangements with the hives: single screen partition with or without a queen, and double screen partition with or without a queen.

Bee Preparation. The chamber with an entrance opening in each hive was given two frames of comb containing brood in all stages of development, honey and pollen, and which were covered with about 4000 worker bees total, mostly young nurse bees. Thus this chamber possessed the young brood, young workers, and food it would need to start rearing new queens if it did not receive an inhibitory signal from a queen. Into the adjacent chamber in each hive I shook about 4000 bees off two broodnest combs from the same colony as supplied the contents of the first chamber, and inserted two frames of empty combs for the bees to cluster upon. In hives of types I and III (see Fig. 2) the chamber without an entrance opening also contained a queen. She was prevented from contacting the partition screen by being tethered between the two frames on a 10-cm leash of nylon monofilament tied to a peg centered in one frame. The leash encircled the queen's petiole. This limited the range of the queen's movements but otherwise appeared to create little interference. The queen walked about smoothly, laid eggs in the empty cells she could reach, and received apparently normal retinues of workers.

Feeding. Each chamber received a 1-liter feeder bottle containing 20% sucrose in distilled water, except the chambers lacking an entrance in hives of types I and II. These chambers received just distilled water. This forced the bees in one chamber to feed the bees in the adjoining chamber and so, I hoped, fostered close contact between these groups through the single screen.

Test Comparisons and Controls. The comparisons drawn in each test were of the number of emergency queen cells present after 24 h in hive type I vs. II, and in type III vs. type IV. The comparison between types I and II measured passage of the queen's inhibitory pheromones through a single screen (both odor and contact-requiring signals could pass through), and the type III vs. IV comparison measured such passage through a double screen (only odor signals could pass through). To reduce the variation in the queen cell counts besides that reflecting the presence or absence of queen substance transmission, the following measures were taken: (1) the bees and brood in each hive pair (I and II, or III and IV) came from the same colony, (2) the bees and brood in each hive pair were matched as closely as possible, (3) the paired hives were run simultaneously, and (4) the internal arrangements of the four experimental hives were rotated between trials. The statistical evaluation used Student's *t*-test of the difference between paired samples.

2. Estimation of Time Required for Colony Loss of Queen Inhibition

I estimated the time required by a colony to lose its inhibition against queen rearing by timing the appearance of emergency queen cells in recently dequeened colonies. The test colonies occupied Langstroth 8-frame hives and so matched in terms of colony size the observation hive colonies described below. Twelve colonies were divided into two groups of six experimental and six control colonies. Experimental and control colonies were run in pairs simultaneously. The experimental colonies had their queen removed; control colonies received a sham queen removal (like the experimental colonies, they were smoked, opened, and inspected frame by frame for their queens but without removing any). At 10 and 24 h following true or sham queen removal I inspected the colonies for emergency queen cells. Fortunately, such cells are quite distinctive even in their earliest stages (see description in Butler, 1974). The statistical evaluation used Student's *t*-test for paired observations.

3. Observation Hive Studies

Hive Design. Much of the research reported here was based upon a colony of bees in a large observation hive. Because I planned to study the spatial patterns of queen and worker movements, it was essential that the colony occupy an approximately normal-sized nest. Moreover, I wanted to perform several measurements on the sociology of queen control which would only be meaningful if made on a colony with a near normal-sized population. Therefore I constructed an observation hive (see Fig. 1) with interior dimensions 96.5 cm tall × 90 cm wide × 4.3 cm deep, which gave a nest cavity volume of 37.4 l. This approximates the modal volume for natural honeybee nests (Seeley and Morse, 1976), but is somewhat smaller than the median volume of about 45 l. The hive's waxen combs were arranged in one vertical plane and were supported in four wooden frames. Plate glass provided window-walls for the two principal sides of the hive. These were gridded with 5-cm-sided squares and numbered along their margins to provide a coordinate system for recording positions of bees on the hive's combs. Also, other glass sheets were sometimes placed over the gridded window-walls. By tracing on these with a wax pencil, I could record the movement patterns of individual bees.

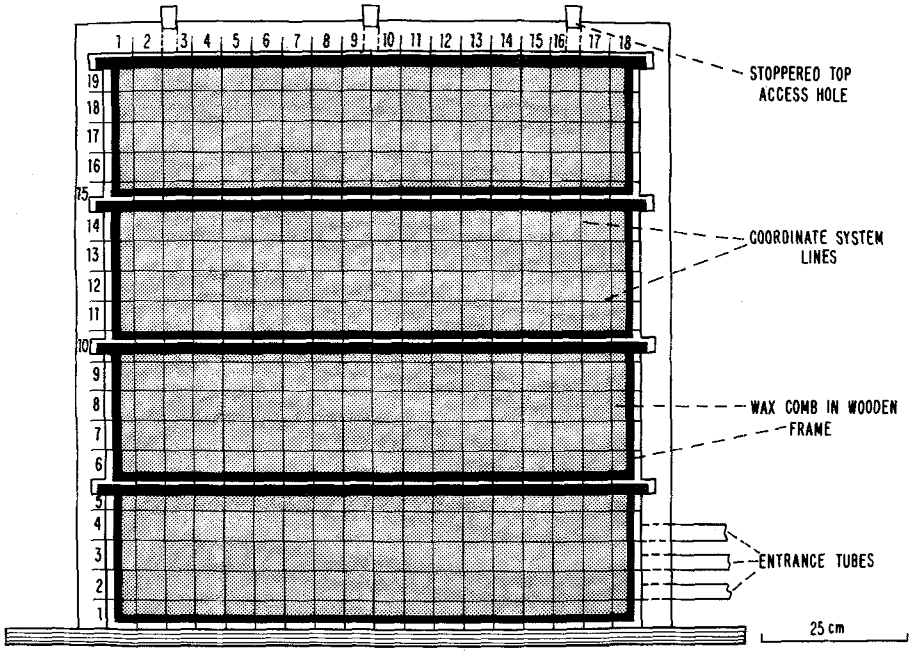

Fig. 1. Design of the large observation hive used with labelled workers in studying honeybee queen and worker behaviors related to queen substance dispersal

Colony Life. This hive was stocked with about 15,000 workers and a queen in early June 1977 and 1978. Both summers the colony thrived, rearing brood extensively, stockpiling about 10 kg of honey in 3 months and, apparently, generally maintaining itself as if it were a normal colony. However, the hive contained no drone comb and thus could not rear any drones. Although strong, with a population of about 17,500 during the periods of observation, the colony showed no signs of swarming preparations during the observation periods.

Individually Marked Workers. Long-term observations of individual workers were made possible by the introduction of 2000 distinctively marked bees into the hive once each summer. The marking system for these bees combined a colored and numbered plastic disk on the thorax (500 color-number combinations) (Opalithplättchen, Chr. Graze, KG, Endersbach, West Germany) with a dot on the abdomen in one of four colors of enamel paint. All 2000 bees were marked within a 48-h period, and all were marked within 12 h of emergence. Thus these bees provided a cohort whose ages spanned at most a 60-h range. Newly emerged bees were obtained by placing combs of sealed brood in an incubator.

To determine the number of marked bees in the hive for each day throughout the 15-day observation period following their introduction in 1978, I counted the number of dead, marked bees which were carried out each day and subtracted this from the previous day's estimate of the marked bee population. This calculation assumes that all of the marked bees which died during the 17-day study period (2 days of marking plus 15 days of observing) died inside the hive. I feel this is a sound assumption since workers of age 17 days or younger spend the vast majority of their time inside the nest. Measurements of daily mortality among the marked bees were made by using a Gary dead bee trap (Gary, 1960) mounted over the hive's entrance tubes. To calibrate the trap's recovery rate, I introduced into the hive each day 30 dead bees marked as calibration bees. The daily recovery rate, averaged over the 17-day study period, was 27.8 bees (range 26–30).

Population Estimation. Colony size was estimated by counting at night the bees inside 10 grid squares on one side of the hive's combs, and then multiplying this by 68.4 (684 grid squares total on the two hive sides). Repeating this five times provided a measure of the variability of the population estimation.

Observation Techniques. Close observations of individual bees were made with magnifying lenses mounted in a headset (Magni-Focuser, Model 105, Bausch and Lomb Co., Rochester, New York). Further details of observational techniques will be provided with the descriptions of separate experiments.

Statistical Tests. Unless stated otherwise, statistical evaluations were based upon Student's *t*-test and χ^2-test.

4. *(E)-9-oxodec-2-enoic acid (9-ODA) Studies*

Synthesis. 9-ODA was synthesized starting from cycloheptanone (Barbier et al., 1960; Barbier and Hügel, 1961). The product, mp 53.5–54.5° C, matched in its IR spectrum and in its retention time in gas chromatography (methyl ester) on Chromosorb 101 and silicone rubber SE-30 with synthetic 9-ODA (Glaxo Laboratories, Greenford, England; kindly supplied by Dr. Roger A. Morse).

Worker Bee Collection. For assays of 9-ODA on workers that had contacted their queen, I collected in batches of 250 two kinds of workers: bees from the queen's retinue and, for comparison, randomly chosen bees from the broodnest of a colony. Retinue bees were collected by gently lifting the frame bearing the queen from a colony, carrying it to a slightly darkened laboratory room, and plucking bees at about 1-min intervals from the queen's retinue. During this process the queen appeared to behave normally, inspecting cells, laying eggs, and frequently standing stationary on the comb. When selecting a retinue bee, I chose only those having extremely close and fairly lengthy (>10 s) queen contact. After collecting 25 retinue bees, I would return the queen-bearing frame to its hive and then collect off a different broodnest comb 25 randomly chosen workers. Both kinds of workers were stored in ethanol.

Extraction. Batches of 250 workers were extracted for 10 h in 150 ml of ethanol in a Soxhlet extractor. Each ethanol extract was taken to near dryness on a rotary evaporator at 30° C and 6 cm Hg pressure, and the residue taken up as far as possible in six 25-ml washes of diethyl ether. The ether-insoluble residue was discarded, and the combined ether extracts were reduced to 25 ml under a stream of nitrogen. This concentrated ether extract was extracted with saturated aqueous sodium bicarbonate (3×25 ml). The combined bicarbonate extracts were acidified with hydrochloric acid and then extracted with ether (4×50 ml). The combined ether extracts were dried over anhydrous magnesium sulfate, taken to dryness under a nitrogen stream in a small tube, and finally redissolved in 200 µl of 10% methanol in ether in preparation for methylation and gas chromatographic analysis.

Analytic Methods. Methylations of synthetic 9-ODA and the worker extracts were carried out using diazomethane (Schlenk and Gellerman, 1960) generated from Diazald (Aldrich Chemical Co., Milwaukee, Wisconsin). The resulting methyl esters were analyzed using a Hewlett-Packard Model 402 gas chromatograph, with a flame ionization detector, and dual 1.2-m × 3-mm ID glass columns containing 10% silicone rubber SE-30 on 100–120 mesh Chromosorb WAW-DMCS operated at 160° C. Quantitation was achieved by preparing a logarithmic series of standard solutions of synthetic 9-ODA ranging in concentration from 0.1 to 100 ng/µl.

Results

1. Test of Worker Transport of Queen Substance

The results of this experiment, presented in Fig. 2, appear conclusive. Significantly fewer emergency queen cells were constructed in the single screen hive with a queen than in the identical hive without a queen, but there was no significant difference between double screen hives with and without a queen. Thus the queen's inhibitory pheromone(s) does not operate on a colony-wide scale as an odor, but can be transported by workers from a queen, around a comb, and through a screen to workers on the other side.

It is probably also significant that some emergency queen cells were found even in the type I hives. This suggests that although some queen substance was being received by the workers separated from the queen, this signal was not as strong as normal. Thus, apparently, either the queen's being tethered or the screen partitioning the hive interfered with the production or distribution of the queen substance.

2. The Importance of Worker Dispersal of Queen Substance

Given that workers can transport queen substance, the question arises of the importance of this indirect mode of signal transmission compared with direct signalling from the queen to workers contacting her. Answering this question involved two stages. First, I estimated the time required for a colony to lose inhibition against queen rearing following the loss of its queen. This sets an upper limit on the interval between queen signals to individual broodnest workers which cannot be exceeded, on average, for the colony to be inhibited from queen rearing. I am assuming here that colonies show inhibition in queen rearing

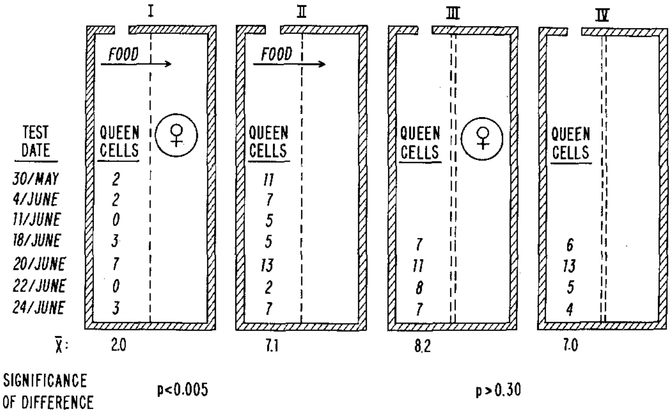

Fig. 2. Internal arrangements of experimental hives and results in the test for worker transport of queen substance from a tethered queen to queenless workers. *Female sign enclosed in a circle* denotes a tethered queen. *Numbers* denote number of queen cells after 24 h

so long as a large majority of the broodnest bees, the group responsible for replacing a queen following her loss, receive signals from the queen within a certain time interval.

And then secondly I measured the fraction of the broodnest workers contacting the queen within this estimated time limit for sequential queen signals. Insofar as the assumption stated above is correct, a small fraction contacted indicates a great importance for worker dispersal of queen substance.

a) Estimation of Time Lag to Loss of Queen Inhibition

The results of the queen removal experiments were that significantly more emergency cells were found in the experimental (*E*) than in the control (*C*) colonies after both 10 h (E: $\bar{x}=2.0$, SD$=1.7$, range 0–4; C: $\bar{x}=0$; $P<0.02$) and 24 h (E: $\bar{x}=6.3$, SD$=5.7$, range 0–16; C: $\bar{x}=0$; $P<0.02$). Thus 10 h stands as an upper limit to the time required by these colonies to lose their inhibition in queen rearing. And because the emergency queen cells observed after 10 h were apparently in the earliest stages of formation – 72% were recognized only by their larva floating in a pool of royal jelly half filling the cell – it appears that 10 h is close to the minimum interval needed to detect a colony's loss of its queen inhibition using this assay system.

b) Fraction of Broodnest Workers Contacting Queen in 10 Hours

Here I followed the queen in the observation hive steadily for 15 h noting the identity of every marked bee contacting the queen with her antennae. This included all or quite nearly all the marked bees which were at anytime within 1–2 cm of the queen. Most workers this close to the queen orient to her and at least briefly touch her with their antennae. My oral records of contacts were tape recorded and later transcribed for analysis. This experiment was performed when the marked workers were 3–5 days old and so (see Sect. 3a of Results) were at their maximum in attentiveness to the queen. And they were too young to venture much outside the hive. By knowing the number of marked bees still alive in the hive at the start of the experiment, I could calculate the fraction of these bees having one or more queen contacts within various time intervals during the experiment. And this provides an approximate measure for the fraction of all the broodnest bees contacting the queen in these intervals. Because the observations used bees at their peak in queen attentiveness, the calculated proportions of contacted bees are really upper limits to the value of this variable for the broodnest bees in general.

The results, presented in Table 1, lead to two striking conclusions. First, the proportions of bees receiving direct queen contact are quite high. In 5 and 10 h the queen contacted about 20% and 35%, respectively, of the marked bees. Thus a significant portion of queen substance dispersal occurs through direct queen–worker contacts. But secondly, the queen–worker contacts fall far short of totality for the broodnest bees. Even after 15 h the queen contacted

Table 1. Percentages of broodnest bees contacting the queen within various time intervals. Measurements are based upon 1710 individually marked bees 3–5 days old. Date of observations: 5 July 1978. Total colony population: 17,459 ± 316 ($\bar{x} \pm$ SE)

Time interval (h)	Time period	%Bees contacted	$\bar{x} \pm$ SD
5	0.8.00–13.00	23.3	21.3 ± 2.4
5	13.00–18.00	18.6	
5	18.00–23.00	22.1	
10	08.00–18.00	36.0	35.6 ± 0.6
10	13.00–23.00	35.2	
15	08.00–23.00	48.6	

fewer than half of the marked bees. Thus, insofar as a large majority of the broodnest bees must receive a queen signal within 10 h for the colony as a whole to be inhibited from queen rearing, worker transport plays a large role in queen substance dispersal.

3. Queen and Worker Behaviors Contributing to Queen Substance Dispersal

a) Age Dependence of Queen Attendance by Workers

If there exists specialization among workers based upon age in the activities of queen substance collection and distribution, then this should be revealed by variation as a function of age in the workers' tendencies to approach and contact the queen. Rösch (1925, 1927) and Allen (1955) report age ranges of 0–27 and 0–36 days, respectively, for workers contacting the queen. But what is really needed here is knowledge of the frequency per individual of queen attendance as a function of age. Sakagami (1953) and Allen (1960) report that workers under 2–4 days old show disproportionately few participations in the queen's retinue relative to older broodnest bees, and they show that the rate of queen attendance declines steadily after a peak at 3–6 days. But, because in neither study was the frequency of queen contacts by bees of different ages adjusted for the group size of the age cohorts, neither study yielded the crucial information: rate per individual of queen attendance as a function of age.

Every 3 days starting 2 July 1978, the first day that all the marked bees were in the observation hive, I recorded for four 30-min periods all the labelled bees attending the queen. By 'attending' I mean approaching the queen and showing steady, stable (not jerky) behavior while contacting the queen for at least 10 s. These criteria excluded bees which briefly contacted the queen but then quickly withdrew as if repelled by the queen. If the same bee was recorded attending the queen more than once, she was nevertheless counted just once. All observations were made from 12.00 to 14.00 h, and were preceded by estimating the colony's population size. Because I also made a daily determination of the number of surviving marked bees in the colony, I could correct the observed rates of marked bee queen attendance for differences due to changes in the size of the pool of marked bees.

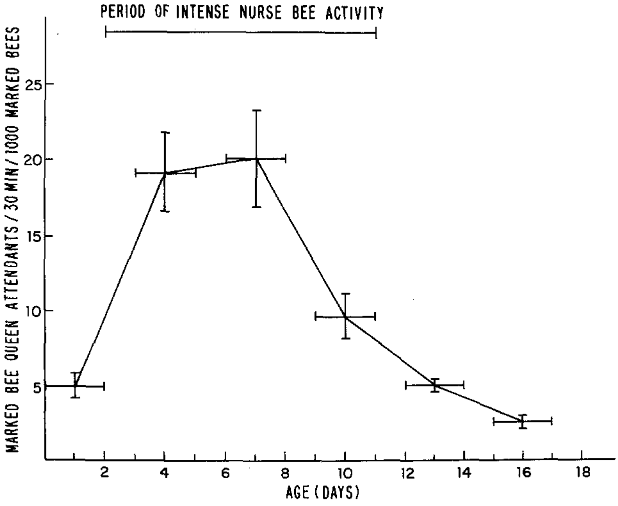

Fig. 3. Age dependence of queen attendance by worker honeybees. *Horizontal bars* denote age span of marked bees for each observation period; *vertical bars* indicate ±SE

The results, presented in Fig. 3, reveal a pronounced effect of age upon queen attendance. This behavior is concentrated heavily among workers 3–9 days old. The average rate of marked bee queen attendance/30 min/1 000 marked bees was significantly higher ($P < 0.01$) for bees about 4 and 7 days old ($\bar{x} = 19.6$, SD$=0.6$) than for bees about 1, 10, 13, and 16 days old ($\bar{x} = 5.6$, SD$=3.0$). It is undoubtedly significant that the age span of intense queen attendance matches the period of intense nurse bee activities. A tempting interpretation of this congruence is that because these bees can respond to the loss of their queen by rearing a replacement, they are also the bees most attentive to monitoring her presence.

The hive population showed no significant change between observation periods. Thus this variable could not have been an important source of the variation illustrated in Fig. 3.

b) Queen Behavior Related to Queen Substance Dispersal

Although it was demonstrated in Sect. 1 that workers can transport queen substance, and in Sect. 2 that this dispersal mode is important, there are good reasons not to relax attention from the queen in analyzing queen substance dispersal. First, the queen appears to play a significant role in queen substance dissemination by contacting about 35% of the broodnest bees within 10 h. And this contact rate may reflect certain behavioral patterns of the queen. Secondly, given that workers can transport queen substance, to do this they must first collect the pheromone(s), and this requires contacting the queen. The queen's behavior may facilitate collection of the pheromone(s) by her workers and thereby promote its (their) dispersal.

Description of Queen Behavior. Queen activities can be neatly divided into three nonoverlapping categories: travelling, laying, and standing stationary. Travelling is rapid walking by the queen which takes her from one part of the nest to another. It is easily distinguished from the movements made by a queen when laying, both by its high velocity and by the absence of cell inspections by the queen. The consequences of the travelling for queen substance dispersal appear to be twofold. First, although the queen's retinue of workers orienting to and achieving at least intermittent contact with her at any one moment is relatively small ($\bar{x}=4.2$, SD$=2.1$, $n=45$) while she is travelling, the durations of individuals' times when in contact with the queen are also small ($\bar{x}=1.7$ s, SD$=2.0$, $n=53$), so the turnover rate in workers making queen contact is high relative to when the queen is either laying or standing stationary. To compare these rates quantitatively, I watched the queen continuously for 3 h noting the category of the queen's behavior from moment to moment and contacts of the queen by the labelled workers. These observations were made by tape recording oral statements of observations and then transcribing the tapes with a stopwatch to make the time measurements. Marked workers which contacted the queen several times were counted just once, unless the contacts were separated by at least 10 min. The mean rates of marked worker contacts/min were 6.4 (SD$=1.6$, $n=13$), 1.9 (SD$=1.3$, $n=38$), and 1.1 (SD$=1.8$, $n=27$) for the travelling, laying, and stationary phases, respectively.

The second apparent contribution to queen substance dispersal from this queen travelling is its repositioning of the queen in different portions of the nest. This is illustrated in Fig. 6 and is further discussed below in describing the spatial pattern of the queen's movements.

When the queen is laying, she moves forward slowly, inspecting cell after cell by placing her head in cells, and periodically stopping to insert her abdomen into a cell to lay an egg. In contrast to when travelling, the laying queen generally remains in one small region of the nest. Also, her retinue of contacting workers is larger ($\bar{x}=10.9$, SD$=2.9$, $n=154$) and more stable with the mean duration of a worker's time of queen contact 17.3 s (SD$=25.0$, $n=165$). The thoroughness of workers' contacts with their queen is higher when she is laying than travelling, but this increases still further when the queen becomes stationary.

When in this behavior phase the queen may groom herself or be fed, but what is most striking is the large, tight enclosure of workers pressing in and contacting a stationary queen. The average size of this retinue is 17.2 bees (SD$=3.2$, $n=93$) and the mean duration of time workers spend contacting the queen is 116.8 s (SD$=122.3$, $n=59$). Given that the workers can transport the queen substance, and that this requires queen contact, it seems likely that the workers collect queen substance most heavily when the queen is stationary. For only when the queen is stationary does it appear possible for the workers to achieve close and lengthy contact with the queen. The nature of this queen–worker contact is described below in Sect. 3c of Results, but I will now draw a few comparisons which reinforce the idea that the queen's adoption of stationary behavior is an important contribution to the dispersal of her queen substance. First is her posture. When travelling and laying, the queen's long legs extend far from her body, but when stationary, she frequently

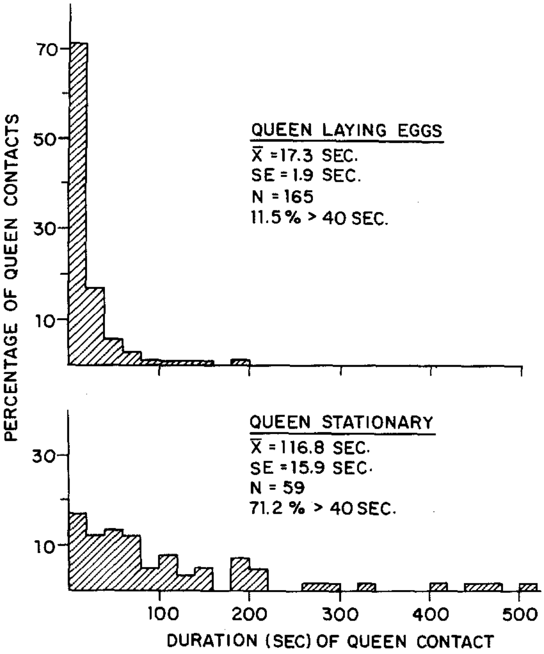

Fig. 4. Distributions of duration in queen contacts by workers for when the queen is either laying eggs or standing stationary

draws her legs tightly beneath herself, thus seeming to facilitate approach by workers. Second is the effect of queen behavior upon duration of queen contact. As is shown in Fig. 4, the distribution of queen contact durations differs markedly between a laying and a stationary queen. And third is the effect of queen behavior on the cause of turnover among the queen attendants. When the queen is stationary the break in contact between queen (Q) and worker (W) comes usually by the worker withdrawing from the queen, but proceeds oppositely when the queen is laying (queen stationary: 10 Q departures, 17 W departures; queen laying: 25 Q departures, 4 W departures). Thus it appears that workers can regularly become saturated with queen contact when she is standing still, but that this is significantly rarer ($P < 0.005$) between workers and a laying queen.

Temporal Pattern of Queen Behavior. In order to understand how a queen partitions her time among the three very different activities – travelling, laying, and standing stationary – I followed the queen in the observation hive steadily for 5 h making a continuous record of her activities. I noted at all times which of the three behavior forms she was showing, instances of egg laying and being fed, and whatever else that I felt was interesting.

The resultant temporal record, shown in Fig. 5, reveals several patterns in the queen's behavioral partitioning of her time. First, travelling occurs relatively rarely whereas both laying and being stationary were quite time consuming.

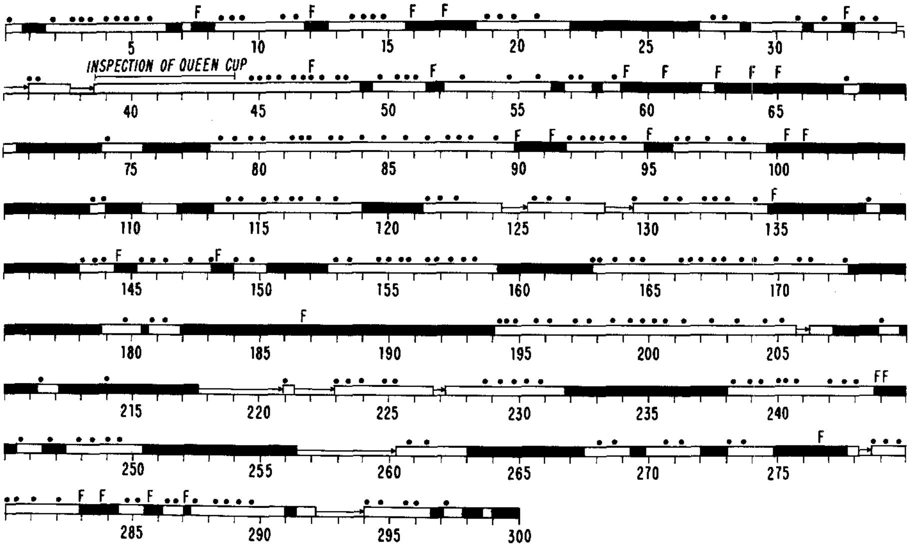

Fig. 5. Temporal pattern of queen behaviors. *Arrows* denote periods of travelling, *clear bars* indicate laying times, and *solid bars* represent stationary periods. *Dots* above the lines signify when eggs were laid and an *F* denotes the start of feeding of the queen. *Numbers* indicate time in minutes. Observations from 19.30 h, 2 July 1978 to 00.30 h, 3 July 1978

In the 300-min observation period, the queen travelled for 934 s (5.2% of the time), laid eggs for 9924 s (55.1%), and was stationary for 7142 s (39.7%). Also there was fairly rapid switching among the three activities. The observation period contained 11, 60, and 51 segments with mean durations of 84.9, 165.4, and 140.0 s for travelling, laying, and stationary behavior, respectively. Thus the periods between times when the queen was stationary were fairly brief and the workers were never deprived for long of thorough contact with the queen.

One question raised by these data is the function of the queen's large allocation of time to simply standing still. Queen feeding or lack of empty cells are probably minor reasons for standing still. As shown in Fig. 5, queen feeding was relatively rare, and often when the queen resumed laying she found cells suitable for egg laying within several seconds. The most reasonable explanation is that stationary periods permit: (1) queen resting in preparation for further egg laying, (2) reception of hygienic grooming by workers, and/or (3) collection of queen substance by workers.

Spatial Pattern of Queen Movements. Besides noting the sequence of activities during the 5 h of observations, I recorded the spatial pattern of the queen's movements by tracing her positions in the observation hive with a wax pencil on two sheets of glass laid over the glass window-walls of the hive. Similar recordings were made over 12 additional hours. The spatial patterns described below are representative of those found in all these observations.

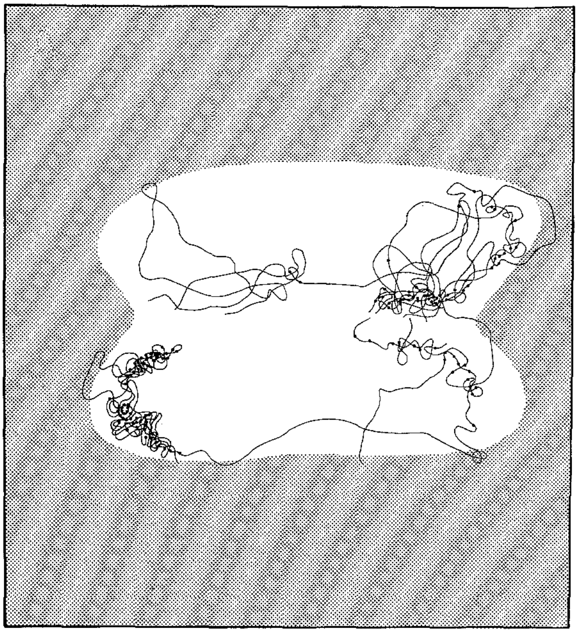

Fig. 6. Tracings of queen movements over one side of an observation hive over 5 h. *Dots* along lines denote locations of eggs laid. The queen's passage from one side of the hive to the other created the many stops and starts of the tracing lines. *Nonshaded area* denotes comb containing brood; *shaded area* represents comb containing food or empty comb. Dimensions: 96.5 cm tall × 90 cm wide

The striking feature of this spatial record, shown in Fig. 6, is the large extent of the queen's coverage of the broodnest within just 5 h. The obvious suggestion derived from this is that although the queen cannot make direct contact with all the broodnest workers in 5 h, she can facilitate the workers' further dispersal of her queen substance by frequent changes in position within the nest. And it did appear that the queen moved about more than was necessary to find cells for laying. For example, during the 5 h of observations the queen twice left and returned to the laying patch in the upper right region of the broodnest, and each of the three times she was in this area she laid extensively. However, this switching of laying patches could reflect temporary exhaustion of cells appropriate for laying as well as programmed dispersal by the queen. Careful experimentation could test these two motives for the queen's movements. Either way, the result is the same: the queen, the source of the inhibitory signal, moves extensively about the broodnest.

c) Worker Behavior Related to Queen Substance Dispersal

I limit this description of worker behavior to that which occurs during and following contact with a stationary queen. As described in the preceding section on queen behavior, it is during the periods of queen immobility that workers achieve their most thorough queen contact, and thus undoubtedly collect the queen substance most extensively.

Behavior During Queen Contact. Many workers appear to contact the queen with hesitation, touching her lightly with their antennae and then rapidly withdrawing a few centimeters by walking backward. In contrast, other workers press right up to the queen and stay with her, contacting her closely and vigorously. Still other workers exhibit the full range of approaches between these extremes of hesitancy and forcefulness. Some of this variation probably reflects age differences among the workers. But even among the marked workers observed at any one time, there was considerable variation in the strength of queen contact. My observations concentrated upon workers which undertook extensive queen contact for it seemed reasonable to assume that these bees are responsible for the worker dispersal of queen substance.

The two principal modes of queen contact by workers are with the antennae and the tongue. Occasionally other body parts contact the queen, such as the forelegs, when workers climb onto the queen, and head surfaces which may bump against the queen. In terms of contact duration, antennation is the primary contact mode. To quantify this, I watched marked bees which had extensive queen contact (>20 s), and measured for each bee the total time spent either antennating or licking the queen. Forty workers were observed with alternate bees being clocked for antennation or licking times. The average times spent antennating or licking were 26.2 s (SD 15.2) and 9.2 s (SD 34.8), respectively ($P<0.05$).

The manner of antennal contact with the queen shows considerable variation. Frequently just the antennal tips are lightly tapped over the queen's body, and the antennae bend little, if any, under the force of contact. But when a worker antennates a queen very closely, her antennae move over the queen in a flurry and bend under the force applied to the queen. At these times a large portion of each antenna, back to the pedicel, is brushed over the queen. The antennae are frequently groomed with the forelegs when the bee steps back and pauses from contacting the queen. This antennal grooming with the forelegs can continue downward in the same episode into grooming of the tongue. Thus if workers acquire the queen substance during contact with her, it would be primarily on the workers' anterior surfaces, but given the behavior observed, dispersed among the antennae, tongue, and forelegs.

Behavior Following Queen Contact. When contact breaks between the queen and a worker which has achieved thorough queen contact, the worker often appears very excited, moving about with agitation as if searching for the queen and frequently grooming her antennae and mouthparts. As a first step in examining this worker behavior for apparent contributions to queen substance dispersal, I compared the movement patterns of retinue bees and randomly chosen bees of the same age as the retinue bees (control bees). Movements of each were recorded for 30 min as traces with a wax pencil on a glass sheet over each window side of the observation hive. Observations of retinue bees and control bees were made in pairs, with the control bee observations always immediately following those of the retinue bees. Only labelled bees were followed, and this ensured that paired retinue and control bees were matched in age within a 60-h range. Retinue bees were selected for observation by watching for a labelled worker to join the queen's retinue, and if it achieved at least 30 s of queen contact, then I continued timing its period of queen contact and upon its breaking queen contact, I traced its movements for 30 min. Besides the tracing, I orally noted and tape recorded the bee's location on the grid coordinate system. Control bees were observed in the same manner but were selected by randomly choosing a grid square within the colony's broodnest and following a labelled bee found within this square. Sometimes several grid squares had to be chosen before choosing one with a marked bee. If the square contained more than one labelled bee, I chose for observation the one whose marking colors most closely matched those of the preceding retinue bee.

The tracings, a random sample of which are shown in Fig. 7, revealed a striking difference in character between the movements of these two classes of bees. Whereas the control bees moved comparatively little in 30 min, the retinue bees undertook broad excursions throughout the broodnest. A quantitative comparison of these movements was made by counting the number of grid squares each bee passed through in 30 min. For retinue bees this averaged 59.0 squares (SD 20.0, $n = 14$), and for control bees 26.4 squares (SD 17.6, $n = 14$), thus showing a highly significant difference ($P = 0.001$).

Having detected this large difference in movement rate between retinue and control bees, I next compared the behaviors of these two classes of bees in finer detail. Selection of the bees for observation and recording of observations proceeded as in the prior experiment. To see the details of the workers' behaviors, observations were made using the headset of lenses. I concentrated upon comparing the frequencies of behaviors apparently involved in dispersal of queen substance. Thus I recorded frequencies of inspection on the subject bee by nestmates, where an 'inspection' was defined as a bee turning toward the subject bee and passing her antennae over the subject bee. 'Antennation' designates the subject bee and another bee contacting each other through their antennae. I counted only unmistakable antennations, those in which the two bees' antennae folded over each others' at least momentarily. Food exchanges were easily recognized, and the direction of food transfer readily determined because the tongue of the recipient bee clearly extends forward to the spread mouthparts of the food donor bee. Cell inspections were also quite distinctive in that they involve a bee's very brief (< 3 s) and not very deep insertion of her head in a cell. Whenever the observed bee crawled partway or deeply into a cell and stayed there a significant time (> 10 s), this was counted as one 'labor act.' The bee was either cleaning the cell or feeding a larva in the cell. Other labor acts included such things as bouts of fanning, transporting dead bees, removing ragged cappings from cells' rims, and shaping bits of comb.

These observations, summarized in Table 2, reveal several further differences between retinue and control bees. Frequency of inspection by nestmates and of antennation with nestmates are both higher for the retinue bees ($P < 0.01$ and $P < 0.001$) while the frequency of labor acts is lower for these bees relative

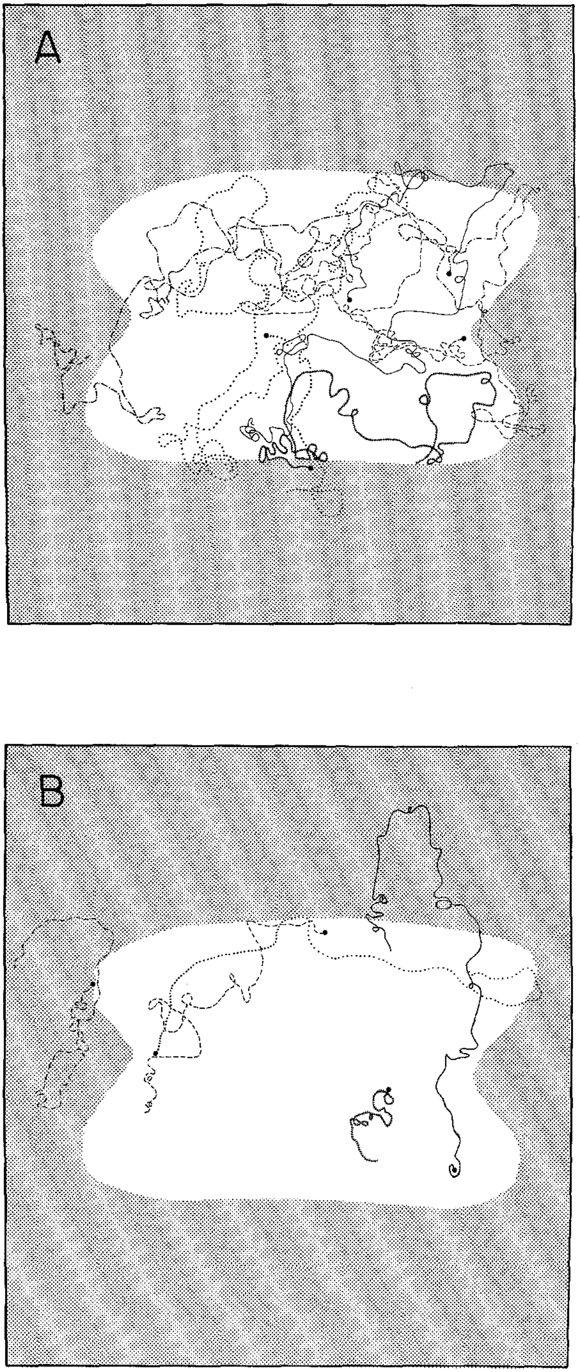

Fig. 7 A and B. Tracings of workers' movements for 30 min. *Dots* denote starting points. Tracings of movements on both sides of the hive have been combined in a single drawing. *Shading* and dimensions as in Fig. 6. **A** Tracings of retinue bees; **B** tracings of control bees (see text for details)

Table 2. Comparisons of behavior frequencies between bees with recent, intense queen contact (retinue bees) and same age, randomly chosen bees (control bees). See text for definitions of behavior categories

Date	Time	Age (days)	Queen contact (s)	Times inspected		Number of antennations		Grooming (s)		Food exchange				Cells inspected		Labor acts	
										Give		Receive					
				R	C	R	C	R	C	R	C	R	C	R	C	R	C
6 July 78	11.12–12.20	4–6	169	11	0	98	22	120	159	0	1	6	2	82	62	2	5
6 July 78	14.20–15.30	4–6	216	6	0	94	22	294	665	6	1	4	8	87	67	0	10
6 July 78	20.30–21.30	4–6	157	17	1	53	8	770	254	3	0	2	4	21	51	0	12
7 July 78	09.55–10.55	5–7	152	30	0	45	12	248	481	1	1	0	1	69	68	4	7
7 July 78	13.30–14.30	5–7	320	9	4	54	6	492	135	2	0	8	0	89	31	1	29
7 July 78	16.35–17.35	5–7	730	42	1	77	9	630	155	0	0	7	0	30	147	0	15
7 July 78	21.05–22.10	5–7	52	4	0	40	7	115	634	0	2	3	1	30	71	0	9
7 July 78	22.25–23.25	5–7	130	11	1	23	14	327	660	1	2	1	3	49	72	12	7
8 July 78	10.30–11.35	6–8	86	3	1	50	7	310	49	3	2	4	0	42	71	9	5
8 July 78	12.10–13.15	6–8	60	8	2	30	9	230	387	2	8	1	6	21	41	5	10
			$\bar{X}$:	14.1	1.0	56.4	11.6	354	358	1.8	1.7	3.6	2.5	52.0	68.1	3.3	10.9
Significance level of difference:				$P<0.01$		$P<0.001$		$P>0.90$		$P>0.90$		$P>0.40$		$P>0.30$		$P<0.04$	

R = Retinue; C = Control

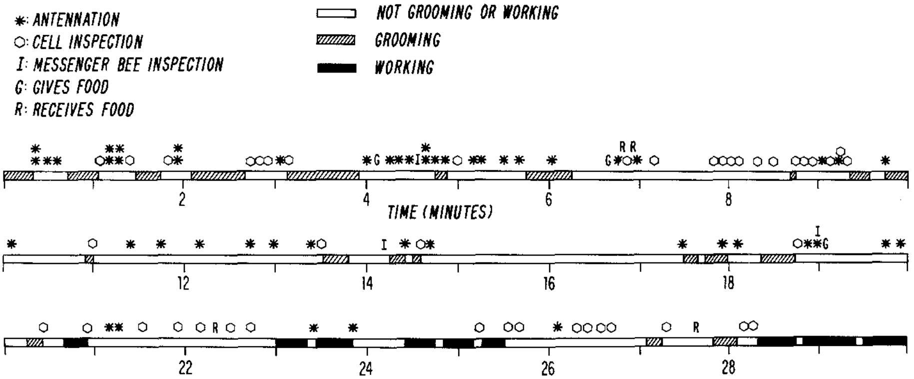

Fig. 8. Record of a messenger bee's behavior for 30 min following her separation from the queen. Bee observed from 10.30 to 11.00 h on 8 July 1978 (see Table 2)

to the control bees ($P<0.04$). Moreover, for the retinue bees, there was a significant ($P<0.01$) correlation between duration of queen contact and frequency of inspections by nestmates, $r=0.76$. These differences and correlation pattern are quite consistent with the hypothesis that workers which thoroughly contact the queen acquire queen substance on their anterior surfaces and then help disperse the queen's signal by moving extensively about the broodnest making active contact with nestmates.

I suggest that these workers which achieve extensive queen contact and then move widely about the broodnest be called 'messenger bees.' Their labor specialization as dispersers of queen substance lasts only briefly, apparently less than 30 min. This is shown in Fig. 8 which presents the temporal sequence of behaviors of a messenger bee. Antennations are clearly concentrated in the first half, while labor acts appear in the second half of the 30-min observation period. That this pattern is general among messenger bees was shown by comparing between the first and second halves of the 30-min observation periods the frequencies of antennations ($\bar{x}=39.5$ and 16.9, respectively, $P<0.01$) and of labor acts ($\bar{x}=0.5$ and 2.8, respectively, $P<0.07$) for the 10 messenger bees represented in Table 2. The same comparisons for the 10 control bees yielded no significant differences ($\bar{x}=5.8$ and 5.8, $P>0.90$, for antennations; and $\bar{x}=4.6$ and 5.7, $P>0.60$, for labor acts).

4. Test for Workers Specializing in Queen Substance Dispersal

Because the labor organization within honeybee colonies sometimes relies upon specialized behavior by a small number of workers, for example, scout bees which search for new food sources and nest sites, I considered it worthwhile to check for the existence of specialist messenger bees. I figured that if these bees exist, then they could be detected by their unusually high frequency of queen contacts as they would presumably operate by contacting the queen,

Table 3. Distribution of queen contact frequencies for individual workers over 5-h periods. Data were taken as a check for specialist messenger bees which would appear as bees with unexpectedly high frequencies of queen contact

Hours 1–5			Hours 6–10		
Number of queen contacts per worker	Number of workers with given number of contacts	Number of workers expected from Poisson distribution	Number of queen contacts per worker	Number of workers with given number of contacts	Number of workers expected from Poisson distribution
0	1311	1313.3	0	1391	1388.5
1	350	347.1	1	285	290.0
2	46	45.8	2	31	30.1
3	2	4.0	3	3	2.0
4	1	0.3	4	0	0.1
5	0	0.0	5	0	0.0
Probability that observed distribution deviates from the Poisson distribution by chance alone exceeds 0.50 ($\chi^2 = 1.03$, 2 *df*)			Probability that observed distribution deviates from the Poisson distribution by chance alone exceeds 0.75 ($\chi^2 = 0.50$, 2 *df*)		

picking up the queen substance, contacting workers, then recontacting the queen, and so on, over and over.

I determined the distribution of queen contacts per worker from data gathered while making 15 h of continuous observations on the labelled workers contacting the queen (see Sect. 2b of Results). Every marked worker which contacted the queen with her antennae during the 15 h was noted by tape recording my oral reports of queen–worker contacts. Later the tapes were transcribed and the time (to the nearest min) of each queen–worker contact was noted. Finally I counted for two 5-h periods, hours 1–5 and 6–10, the number of labelled bees having 0, 1, 2, 3, 4, or 5 queen contacts. Two or more queen contacts by the same worker within 10 min of each other were counted as just one distinct contact. This was done to prevent one long and intermittent contact of the queen by a worker from being counted as several separate queen contacts.

These counts, assembled in Table 3, reveal that the pattern of individuals' frequencies of distinct queen contacts is distributed in a manner indistinguishable from a Poisson distribution. Thus within 5-h periods, the queen's contacts with the labelled workers proceeded at random. The absence of workers showing unusually high frequencies of queen contacts indicates that none of the labelled workers were acting as messenger specialists with rapid cycling between contacts with the queen and workers.

5. Analysis of Messenger Workers for (E)-9-oxodec-2-enoic acid

To check the purely behavioral evidence that the messenger bees are the principal workers providing worker dispersal of queen substance, I assayed by gas chromatography groups of these bees for 9-ODA, the principal, if not sole, component of queen substance.

First I determined the yield of the extraction procedure by adding 1 mg of synthetic 9-ODA each to three groups of 250 workers taken at random from the broodnest of a colony and stored in 80 ml of ethanol. Extraction and analysis of these three samples, using techniques identical to those for the experimental bees, gave a mean yield of 86% (80%, 89%, 89%).

Analysis of two 250-bee samples each of messenger bees and randomly chosen broodnest bees gave no detectable 9-ODA. The minimum detectable 9-ODA was 0.1 ng/μl and 1 μl samples were injected into the gas chromatograph. Given these figures, the recovery yield of about 80%, and the final sample volume of 200 μl, it can be concluded that the mean content of 9-ODA per messenger bee was less than 0.1 ng. However, for 9-ODA, with a molecular weight of 184, 0.1 ng is 3.3×10^{11} molecules. With this large number as the lower limit to the number of detectable 9-ODA molecules on a messenger bee, it is perhaps not too surprising that none was detected. Obviously, more powerful analytic techniques or more elaborate sampling techniques are needed to conclusively test directly for 9-ODA transfer from the queen to the workers.

Discussion

1. Mechanism of Queen Substance Dispersal

The three questions posed in this paper's introduction can now be succinetly answered by stating that worker transport of queen substance exists, it is important in dispersing the queen's signal, and that behaviors of both the queen (standing stationary) and workers (messenger activities) aid the dispersal. Thus these findings confirm and extend those of Verheijen-Voogd (1959) and Velthuis (1972). The negative evidence for messenger specialists is also consistent with the conclusion of Butler and associates (1973) that workers do not travel from distant nest regions to join the queen's retinue. These authors, however, did not detect messenger behavior using time-lapse photography of retinue workers. Perhaps this reflects the difficulty of following unmarked bees in an observation hive.

One question not directly addressed in this study is the mechanism of queen substance transport by workers, for which there are two hypotheses. Butler (1954) proposed that workers lick queen substance off queens and then transmit it to other colony members via food exchange. Verheijen-Voogd (1959) advanced a second hypothesis by which workers contacting a queen pick up queen substance on their exterior surfaces and then function as 'substitute' queens, presumably being recognized as such by olfaction. The evidence surrounding these two hypotheses has been reviewed by Gary (1974) and Michener (1974), and although neither hypothesis has been disproven, most of the evidence supports the second, surface transport hypothesis (Butler, 1974). And the following circumstantial evidence from the present study also supports the surface transport hypothesis over the food exchange hypothesis: (1) the higher frequency of antennations with nestmates and of inspections by nestmates for messenger bees relative to control bees, (2) the close correlation ($r=0.76$) for messenger bees between duration of queen contact and number of inspections by nestmates, and (3) the low frequency of food donations ($\bar{x}=1.8$) compared with nestmate antennations ($\bar{x}=56.4$) by messenger bees in the 30 min following queen contact.

Throughout this paper I have considered that the signal transported by messenger bees is the chemical signal of the queen's inhibitory pheromone(s). In support of this, Butler (1970) reports detecting 9-ODA on workers, and there is a pleasing parsimony to the idea that the queen's inhibitory signal is the same for both direct and indirect queen–worker communication. Nevertheless, the messenger bees might merely be stimulated to action by the queen's pheromones and subsequently signal the queen's presence to nestmates by a nonchemical signal, such as a special pattern of antennal contacts. With this point in mind, I watched the messenger and control bees (Sect. 3c of Results), but detected no obvious difference between their techniques of nestmate antennation.

2. Evolution of Messenger Behavior

An evolutionary puzzle raised by the finding of workers acting as queen signal messengers is the level of selection which produced this behavior. Why should workers, which are generally, though not always, in conflict with their queen over the rights to lay male eggs (Trivers and Hare, 1976; Oster and Wilson, 1978) aid in the dispersal of the queen's signal that inhibits workers' ovaries? Perhaps this reflects colony level selection which has promoted worker behavior contributing to the tight integration, ergonomic efficiency, and so ultimately to the reproductive success of the colony as a whole. Or, at the level of selection among individual workers, it might reflect worker–worker conflict over male egg production. At this level, it may be advantageous for workers which have received a dose of the queen signal, and thus are rendered temporarily infertile, to try inhibiting the egg laying by her worker nestmates through dispersal of the queen's inhibitory pheromone(s). However, the observation that many workers appear to deliberately initiate contact with the queen by pressing toward her apparently contradicts this worker–worker conflict hypothesis. For by this scheme, workers should try to avoid thorough queen contact until they have accidentally contacted the queen and become 'contaminated' by her inhibitory signal. A further possibility is that there is a sufficiently large difference in the energetic cost per individual between worker-produced males and queen-produced males that the inclusive fitness of workers is maximized by the queen producing all the males (Oster and Wilson, 1978). And still another explanation is that workers will suspend their messenger behavior when conditions are right for male production and start competing with the queen over the rights to produce males. Because the observation hive lacked drone comb, the study colonies never reared any drones, thus my observations may have been limited to conditions which especially favored messenger behavior by workers. Observations of workers in colonies with or without drone rearing could test the last hypothesis and would provide a test of the concept of queen–worker conflict.

But arguing against this last hypothesis is the absence of solid evidence in the honeybee literature of workers ever laying eggs while a queen is present.

3. Timing of Colony Reproduction

The initial motivation for this study was the hope of clarifying the mechanism whereby honeybee colonies 'compute' the switching time for shifting from colony growth to colony reproduction. Understanding this process in detail would be both a major achievement in insect sociology and an important contribution to practical beekeeping. The pivotal event is the onset of queen rearing, which generally follows a colony's becoming crowded in its nest cavity. Because this queen rearing is usually inhibited by the current queen, the analysis of the switching mechanism logically focuses upon changes in queen–worker communication with crowding. Queen signal dilution does not appear important because small, crowded colonies will rear queens and swarm whereas large, but uncrowded colonies tend not to undergo reproduction (Simpson, 1973). There are three other conceivable points of change: signal production, signal transmission, and response to the signal. Butler (1960) has demonstrated that queens in swarms from crowded colonies contain no less queen substance than queens in nonswarming colonies. Assuming that the rate of release of queen substance by a queen is proportional to the amount of queen substance extractable from the queen, it thus appears that signal production does not change with increasing crowding. Butler (1960) also tested for a difference in inhibitory response to fixed amounts of queen substance between workers from swarming and nonswarming colonies and found no difference. This suggests that the inhibitory power of queen substance does not decline in crowded colonies, but more sophisticated experiments are needed before this can be concluded. One shortcoming of Butler's experiment is that by taking workers from swarming colonies out of their crowded nest environment, he may have separated them from the very environmental cue(s) (such as jostling with nestmates) which triggers the change in a worker's response to queen substance.

The simplest link between overcrowding and a drop in queen inhibition may involve the process of queen signal transmission. Crowding may block the extensive movements of queens and workers within broodnests needed for queen substance dispersal. This study constitutes the first phase in testing this final hypothesis by analyzing the mechanisms of queen substance dispersal, and by providing base line descriptions of the queen's and workers' behaviors in uncrowded, nonswarming colonies.

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