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Title: EFFECTS OF SYNTHETIC 9-OXODEC-TRANS-2-ENOIC ACID ON THE FORAGING ACTIVITIES OF HONEY BEES (APIS MELLIFERA L.)

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An experimental formulation of 9-oxodec-trans-2-enoic acid, the major component of honey bee (Apis mellifera L.) queen pheromone, was tested for its effect on worker foraging behavior in the pollination of sweet cherries (Prunus avium L.). The test formulation consisted of a solid beeswax cylinder impregnated with a measured quantity of pheromone. The experiment was conducted in two parts: the first, to test several concentrations of pheromone (3 mg, 9 mg and 15 mg of 9-oxo); the second, to compare the chosen concentration (15 mg 9-oxo) with queenright and queenless hives. Percentages of nectar and pollen foragers, effects on worker oögenesis, inhibition of queen rearing activity and drone attraction were tests of the efficacy of the wax-pheromone formulation.

The results of the experiments showed that while the presence of the pheromone initially stimulated pollen foraging to a greater

extent than in the queenless hives, the effect was negated by the development of laying worker brood in the queenless colonies and the subsequent increase in pollen foraging in those units. The queenright colonies collected significantly more pollen and had a significantly greater total number of foraging bees than either the queenless or pheromone treated hives. There was no significant difference between the queenless or the pheromone treated hives in any type of foraging activity. The percentage of bees surviving after three weeks was significantly greater in the pheromone treated hives than in the queenless units. Worker ovaries were inhibited to some extent in the pheromone treated hives, but queen rearing activities were not. Drones were attracted to all concentrations and formulations of 9-oxo used in the experiments.

Effects of Synthetic 9-Oxodec-Trans-2-Enoic Acid on the Foraging Activities of Honey Bees (*Apis mellifera* L.)

by

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INTRODUCTION

The pollination of self-sterile crops can be affected both by the numbers of honey bees (Apis mellifera L.) present and their distribution. The sweet cherry (Prunus avium L.) is one such crop in which the presence of honey bees has a positive effect on yields (Gardner, 1913; Wellington, 1923; Claypool et al., 1932). Traditionally, the recommended concentration for satisfactory pollination is one colony per acre (Schuster, 1925). However, Mommers (1951) found a positive correlation between colony numbers and cherry yields. He has recommended as many as five colonies per acre, as has Brown (1968). Saturation pollination has been endorsed primarily due to the cool weather which is common in some areas during the bloom period of sweet cherries (April-May), when relatively more bees are required to achieve a satisfactory degree of pollination.

Poor distribution of the honey bee population within the orchard can limit the number of flowers visited. Nevkryta (1957) recorded an inverse relationship between visitation rate per sweet cherry blossom and the distance of the tree from the apiary. Cool temperatures have a pronounced effect on foraging distance.

Nevkryta reported that flight activity was restricted to within 25 meters of the hive at temperatures between 12°-15°C. Butler, Jeffrie and Kalmus (1943) observed that bees furthest from the hive were more easily discouraged from foraging with a reduction in temperature. The minimum temperature at which bees will fly has been recorded at 12°-14°C for the month of April by Lundie (1925), working in Maryland. Overcast days require a 2° increase in temperature for commencement of flight activity.

Queenright overwintered colonies have proven to be the best pollination units. However, it may not always be possible for the grower to obtain a sufficient number of colonies when they are most needed. The disposable pollination unit (DPU) offers an alternative means of insuring a well distributed population of honey bees. These units consist of a package of bees (artificial swarm), usually queenright, which are hived in a disposable container and maintained in the orchard for the period of pollination, then destroyed. DPUs can be distributed singly throughout the orchard for maximum coverage.

The limiting factor in the production of package bees is the rearing of a queen. Packages can be readily shaken from existing hives, but the development of a mated queen requires three to four weeks. If a substitute queen could be developed using queen

pheromone, the DPU could be produced more efficiently and in less time. The following experiments were used to test the effect of just such a queen substitute on foraging activity in package bees.

Researchers have discovered in the past 20 years that queen honey bees produce chemical compounds, pheromones, which influence the behavior of worker bees (Voogd, 1955; Butler, 1957; De Groot and Voogd, 1959). The principle component of the queen pheromone complex is 9-oxodec-trans-2-enoic acid (Butler et al., 1961). This compound has been synthesized and biological activity demonstrated for the synthetic in inhibition of queen cell construction (Butler et al., 1951); inhibition of worker oogenesis (Butler and Fairey, 1963); and as a sex attractant to drone honey bees (Butler and Fairey, 1964). It is also slightly attractive to worker bees (Butler et al., 1973). (See Literature Review for complete discussion of queen pheromones and 9-oxo.) Showers (1967) and Jaycox (1970a) have demonstrated that synthetic 9-oxo can to some degree substitute for the queen in maintaining the foraging activity of a queenless colony.

Synthetic 9-oxo, impregnated in beeswax, was the basis for the artificial queen to be tested in these experiments. The beeswax-pheromone formulation was used to provide a slow release package that would not require maintenance. It could be installed at the time the DPUs are placed in the orchard and left undisturbed until their

removal. It has been suggested (Butler, 1969; Boch et al., 1975) that lipids may act as "fixatives" for the pheromone, reducing the rate of evaporation. Boch et al. (1975) reported that adding paraffin oil to a solution of synthetic 9-oxo extended its activity in attracting drones to equal that of queen extracts. Thus, incorporation of the pheromone in beeswax may prolong its effectiveness throughout the pollination period. Since beeswax is a natural product of the hive, its long-term acceptance as a substrate for the pheromone would be greater than if a foreign object were used. Bees tend either to remove or cover with propolis any foreign object encountered in the hive (Morse, 1972).

A two-part experiment was designed to be executed during the bloom period of sweet cherries in 1975 and 1976. In 1975, three concentrations of pheromone were tested to determine which evidenced the greatest effect on honey bee activity. In 1976, the selected 9-oxo concentration was tested against queenright and queenless packages. To assess the foraging behavior of each treatment, the foraging profile of each experimental unit was examined. The foraging profile consisted of a series of samples of incoming bees taken at regular intervals during the day. The samples were divided into foraging and nonforaging bees and foraging bees further subdivided into nectar and pollen foragers. Comparison between foraging profiles of each treatment was used to determine which treatment

demonstrated the greatest activity in any category of foraging behavior.

Several other bioassays were used to characterize the activity of the 9-oxo-beeswax formulation. Its capacity to suppress queen rearing and the development of worker ovaries, to promote the stability of the colony and the retention of bees in the hive, and to attract drone bees was measured. The results of these experiments, together with the foraging profile, were used to determine the efficacy of the synthetic queen in duplicating the effect of the presence of a live queen.

LITERATURE REVIEW

Disposable Pollination Units

The use of package bees, the basis of the DPU is not new to orchard pollination. Hutson (1928), Farrar (1931), Woodrow (1932, 1933, 1934) and Vansell (1942) compared the foraging ability of package bees with overwintered colonies in a number of pollination situations. Woodrow (1934) and Root (1938, cited in Kauffeld et al., 1970) reported on the placement of packages directly into the orchard in their shipping containers, essentially early DPUs.

Woodrow (1934) evaluated the performance of package bees and concluded that in units of equal population, overwintered colonies had greater total flight activity, while package bees gave more uniform numbers of foraging bees per colony. The lack of brood was cited as the major limitation of the package unit. The presence of brood stimulates pollen foraging (Free, 1967) and provides replacement foragers. Populations in package units decline until the first brood emerges, a minimum of 21 days after the release of the queen.

Recently, a number of authors (Kauffeld et al., 1970; Cantwell et al., 1972; Thorp et al., 1973; Erickson et al., 1974) have re-examined the use of package bees in DPUs. Thorp et al. compared two sizes of DPUs (3 lb and 6 lb) to overwintered colonies of relatively the same strength. Once again, the authors reported stronger

activity from overwintered colonies but more uniform flight from DPUs. DPUs with caged queens collected less pollen than those whose queens were released and laying. More pollen was collected in the overwintered colonies than in the DPUs.

Kauffeld et al. (1970) attempted to discover the optimum size for a DPU and the effects of the queen or a queen substitute on foraging activity. The authors reported that flights per thousand bees were relatively the same in units ranging from 2,000 to 21,000 bees. Thus, larger units do not have greater flight efficiency. DPUs with mated laying queens, caged or free, collected more pollen and nectar than those with caged virgins, no queens or queen pheromone. For the first three weeks of the experiment, flight activity and pollen collection was similar in DPUs with mated laying queens and CO_2 treated queens (CO_2 treatment reduces the period before a virgin will begin to lay haploid drone eggs (Mackensen, 1947)). This may be evidence for the stimulatory effect of the brood, worker or drone, on foraging activity.

Erickson et al. (1974) conducted field tests of several containers for use in DPUs. Styrofoam chests were ranked highest in flight activity, reduction of absconding, maintenance of favorable internal temperatures and reduction of mortality. No experimental container was superior to a wooden hive body with drawn comb.

Styrofoam chests were used by Cantwell et al. (1971, 1972) in their investigations of air dropping DPUs for cranberry pollination. Unlike previous researchers, the authors used queenless packages and reported a high rate of survival and active foraging and comb building.

Honey Bee Foraging Activity--Queen Effects

Research into the effect of the presence of the queen on the foraging behavior of the colony has been contradictory. Dreischer (1956) and Genrikh (1957) working with small observation hives and larger field units respectively, found a general decline in all foraging activity when the queen was removed. In contrast, Loffler (1961) reported no reduction in visits per hour to a feeder by bees from an observation hive after the removal of the queen. Kashkovskii (1957) reported a weight gain of 97 percent in queenless colonies over queen-right during a strong nectar flow. Louveaux (1954, 1958) found no apparent reduction in pollen collection after the loss of the queen until capped brood appeared. This observation once again introduces the problem of separating queen effects from those of the brood. The above mentioned authors examined colonies containing brood which had become queenless under experimental conditions.

Free (1967) investigated the relationship between the presence of the queen, with or without brood, and the foraging behavior of the

colony. He discovered that the absence of the queen, with or without brood, did not reduce the total number of foragers, but increased the ratio of nectar to pollen foragers. This substantiates to some degree the observation of Kashkovskii (1957) on the greater storage of nectar in queenless colonies.

The effects of queen pheromone, in particular 9-oxo, were examined by Showers (1967) and Jaycox (1970a). Showers compared queenright, queenless and 9-oxo treated packages in gain of new stores and retention of bees. Queenright colonies ranked the highest in both categories with 9-oxo treated units intermediate. Laying workers were observed in the 9-oxo treated units before the end of the third week of the test period.

Jaycox (1970a) studied in greater detail, the relationship of queen pheromones and nectar and pollen foraging activity of the workers. Comparisons were made between queenright, queenless, 9-oxo treated and whole queen extract treated colonies. No brood was present in any treatment. All queens were caged. The experiment was conducted both with small groups of bees (ca. 600) and field strength colonies. In the small units, queenright and 9-oxo treated bees carried nearly equal volumes of nectar, while queen extracted treated and queenless units carried significantly less. In field tests, queenright and 9-oxo treated colonies gained more weight than queenless colonies or colonies supplied whole queen extracts.

Cells of stored pollen did not differ significantly between treatments. Differences in response to queen extracts and 9-oxo could be explained by the low concentration of 9-oxo in the queen extract used in the experiment (10-20 μg of 9-oxo in the volume of queen extract compared to 100 μg of synthetic 9-oxo given as a separate treatment). Jaycox (1970a) concluded that queen pheromones and 9-oxo in particular, had a greater effect on nectar than on pollen foraging.

Honey Bee Foraging Activity--Brood Effects

The stimulation of pollen foraging by the presence of brood had been noted by several researchers. Filmer (1932) reported that in package bees used for pollination, brood area was a more important factor influencing foraging activity than colony size. Weight of pollen collected in pollen traps was significantly correlated to the number of eggs laid by the queen (Cale, 1968). Al-Tikrity et al. (1972) and Todd and Reed (1970) demonstrated a significant correlation between square inches of brood and weight of pollen collected in traps. Todd and Reed further noted that there was an upper limit to the significant increase of pollen collection with increasing brood area. Thus, smaller colonies gather relatively more pollen per unit area of brood than do larger colonies.

Free (1967) also found an increase in pollen collection with an increase in brood. In experiments where bees were denied direct

access to the brood, pollen collection was reduced. He concluded that while brood odor and contact with nurse bees stimulates pollen foraging, they cannot substitute for direct contact with the brood.

Jaycox (1970b) elaborated on the findings of Free. He compared the effects of brood and/or brood extracts in queenless and queenright colonies. The queen alone had a greater stimulation on nectar foraging than brood alone. Pollen foraging was greatest with both queen and brood extracts present, and significantly less with the queen alone. Pollen foraging was least in queenless colonies containing either brood or brood extract. However, when nectar was scarce and pollen abundant, pollen collection was greatest in colonies containing only larvae. There was no significant difference in pollen collection between queenright, broodless units and colonies containing larval extract (with or without a queen). Jaycox (1970b) theorized that two factors influenced pollen foraging: the chemical and social stimuli of the presence of the queen and brood; and the field behavior of the foraging bees in relation to the nectar supply. He concluded that while brood and queen both affect foraging activity, the mechanisms underlying the relationship are as yet undefined.

Queen Pheromone and 9-Oxodec-Trans-2-Enoic Acid

Karlson and Butenandt (1959) defined pheromones as chemical

substances "secreted to the outside by an individual and received by a second individual of the same species, in which they release a specific reaction, for example, a definite behavior or developmental process." Prior to the formal definition of the concept, a chemical basis for queen control of honey bee worker behavior and physiology was postulated independently by Butler (1954), De Groot and Voogd (1954) and Pain (1954). Butler coined the term "queen substance" for the compound which he believed to be responsible for suppression of worker oögenesis and queen rearing activity. Earlier, Hess (1942) observed that direct access to the queen was necessary for inhibition of oögenesis, suggesting the possibility of the exchange of some inhibitory substance through contact chemoreception. Experiments by De Groot and Voogd (1954), Voogd (1955), Butler (1957), and Verheijen-Voogd (1959) demonstrated inhibitory effects on worker ovary development and queen rearing activity of either dead queens or extracts of queen in organic solvents. Their findings supported the theory of pheromonal control.

Butler and Gibbons (1958) reported that queen extracts need not be associated with any queenlike object to be effective in inhibiting queen cell construction, given that a sufficient number of workers made contact with the source of the extract.

Butler and Simpson (1958) identified the site of production of "queen substance" as the mandibular glands located in the head of

the queen. Extracts of queen mandibular glands inhibited queen rearing. Worker ovary development was also inhibited by extracts of these glands (Butler, 1959). Chemical analysis of queen extracts soon followed. Barbier and Lederer (1960) and Callow and Johnson (1960) independently announced the isolation and synthesis of 9-oxo from honey bee queen extracts.

Although 9-oxo is the most abundant component of "queen substance" analysis by gas-liquid chromatography has separated approximately 30 different compounds, of which 12 have been identified (Callow, Chapman and Paton, 1964). In a companion study, Butler and Fairey (1964) were able to demonstrate biological activity for only 9-oxo and one other compound, 9-hydroxydec-trans-2-enoic acid.

Biological evaluations of 9-oxo effects on drone attraction, worker attraction, swarm stabilization, inhibition of queen rearing and worker oögenesis have generally resulted in a level of efficiency less than that of whole queen extracts or live queens. It is theorized that the other components of queen substance act with 9-oxo to modify or amplify its effects.

The degree to which 9-oxo can replace the queen varies with the concentration used, the size of the experimental population of bees and the particular effect under study. The inhibition of queen

rearing and worker oögenesis have served as the primary tests of biological activity in evaluations of queen pheromones.

Butler, Callow and Johnson (1961), Butler and Fairey (1963) and Velthius and Van Es (1964) recorded inhibition of worker oögenesis by synthetic 9-oxo in small groups of bees. In all cases, inhibition was significantly greater than in queenless controls but less than that obtained from live queen or queen extracts. Velthius (1972) demonstrated a direct relationship between the concentration of 9-oxo and the percent of bees with undeveloped ovaries.

Queen rearing is inhibited in small experimental honey bee populations partially, but not completely by 9-oxo in concentrations normally found in mated laying queens (Butler et al., 1961). Chaudhry and Johansen (1971) obtained complete inhibition in single story colonies given abnormally high concentrations of 9-oxo, as did Shaposhnikova et al. (1971). Boch and Lensky (1976) reaffirmed earlier results, that 9-oxo could only effect partial inhibition when supplied in concentrations occurring in single queen extracts. Queen extracts were found to be less effective than live queens in preventing queen rearing.

Gary (1962), Pain and Ruttner (1963) and Butler and Fairey (1964) investigated drone attraction to queen pheromones. Gary postulated and Butler and Fairey later confirmed, that 9-oxo is the

primary sex attractant in queen substance. This attraction occurs outside the hive only.

Butler et al. (1973) found some slight worker attraction to the odor of synthetic 9-oxo. Shapashnikova and Gavrilov (1973) observed retinue behavior of worker bees toward a wooden queen model impregnated with 9-oxo.

Velthius and Van Es (1964), Butler and Simpson (1967) and Morse and Boch (1971) demonstrated a slight stabilization effect of 9-oxo on queenless swarm clusters. Another component of queen substance, 9-hydroxy, has been credited with the major role in swarm stabilization. However, 9-oxo proved to be strongly attractive to queenless swarms in flight (Butler and Simpson, 1967).

Recent research has revealed that 9-oxo is present in extracts of queens of all four species of Apis (Butler, Calam and Callow, 1967; Shearer et al., 1970; Sannasis and Ranjulu, 1971). Amounts of 9-oxo found in extracts of queen heads of A. dorsata and A. cerana indica appear to be in the same range of concentration as A. mellifera (Shearer et al., 1970). Apis cerana indica drones and to a lesser extent, A. dorsata and A. florea drones were attracted to 9-oxo and extracts of A. mellifera queens. Extracts of queens of A. florea and A. cerana indica inhibited queen rearing and worker oögenesis in their own workers (Butler, Calam and Callow, 1967).

The mode of perception and transmission of 9-oxo is subject to debate. It is relatively nonvolatile, implying a need for a certain degree of contact chemoreception. Butler (1954) proposed that the queen spreads the mandibular gland secretions over her body, the attendant workers lick it from her, and transmit the pheromone to their nestmates through trophalaxis. Perception is through injestion of the compound. Velthius (1972) theorized that attendants carry pheromone adhering to their bodies from contact with the queen during grooming. The queen is constantly moving and her circle of attendants changing (Allen, 1965). Bees not in direct contact with the queen receive pheromone information from contact with the bodies of recent attendants. Perception is via the mouthparts or antennae. Injestion is not necessary. There is evidence for both theories, though none conclusive. The mode of transmission of 9-oxo and the other components of queen substance needs elucidation.

MATERIALS AND METHODS--1975

Foraging Profile

The experiment was conducted on a 1.6 acre (0.65 ha) sweet cherry block on the OSU, Lewis Brown Horticulture Farm near Corvallis, Oregon. Each experimental unit consisted of a queenless 1.4 kg package of bees (approximately 12,000 bees), housed in a five frame wooden hive body (referred to as a nuc) on four frames of a clean, drawn comb. The nucs were painted red, white or blue and the colors distributed so that no two adjacent nucs had the same color. The colonies were placed in a straight line along a row of trees with approximately five meters between colonies. All colonies faced east.

Treatments consisted of three concentrations of synthetic 9-oxodec-trans-2-enoic acid (9-oxo) with three replications. In addition, a queenless package was established as a control. The bees absconded within a week from the control hive.

The 9-oxo was delivered to the experimental units impregnated in a solid beeswax cylinder. Each cylinder weighed one gram and contained either 3 mg (Treatment A), 9 mg (Treatment B), or 15 mg (Treatment C) of 9-oxo. These levels corresponded to approximately 20, 60 and 100 times the average amount of 9-oxo recovered from single queen extracts.

The pheromone cylinders were formulated by heating the beeswax in a mineral oil bath just to the melting point, 65°C and stirring in the crystalline 9-oxo. 9-Oxo has a melting point near 55°C (Callow, Chapman and Paton, 1964). It quickly changed to liquid phase and combined with the melted wax. The mixture was immediately removed from heat and poured into 8 mm test tubes, which had been prewarmed in a hot water bath to prevent too rapid cooling and possible cracking of the wax. After slow cooling, the cylinders were frozen, freed from the test tubes and the excess wax trimmed until each weighed one gram. Each cylinder was approximately 2.5 cm in length. A slender wire was heated and passed through the cylinder. Each cylinder was suspended by the wire between the center two frames of the colony, about 10 cm from the top bars of the frames.

The experiment began on April 22, 1975. The cherry trees were in 10 percent open bloom. The packages had been installed in the nucs two days previously on the OSU Entomology Farm. On day one of the experiment, the nucs were moved to the cherry block, the queens removed and the 9-oxo cylinders installed. The low temperature at the time of installation severely limited foraging activity. Each nuc was provided with a feeder and 0.5 liters of 50 percent sugar syrup. The syrup was replaced once.

The foraging profile of each colony was determined following the procedure devised by Erickson, Whitefoot and Kissinger (1973). The hive entrances were temporarily closed and incoming foragers collected in a sweep net. Each sample of bees was immediately placed in crushed dry ice to immobilize and kill the bees without regurgitation of the contents of the honey stomach. Samples were sealed in plastic bags and returned to dry ice for field storage until they could be transported to the laboratory for analysis. All samples remained frozen at -3°C until analysis.

Samples of 10 bees were taken three times daily, at 1000 hr, 1200 hr and 1400 hr PDT. Duration of each sampling process was approximately one-half hour. Colonies were sampled in the same order each time. A randomized block design was selected to minimize the time effects between the first and the last colony sampled. Due to poor weather conditions which prevented flight activity or severely limited it, samples were taken on only seven days of the 28-day experiment. Temperatures on April 30, May 1, 7, 8, 9, 12, and 13 were sufficiently high for good activity throughout the daily sampling schedule. The experiment was terminated on May 20. (Total bloom period for Royal Ann, the main variety, was from April 20 to May 15.)

During the laboratory analysis, the sample bees were separated into nectar and pollen foragers on the basis of the presence

or absence of pollen grains in the corbicula. The size of the pollen load was not a factor. Pollen foragers were further subdivided into those foraging on cherry and those foraging on other sources. Mixed loads containing cherry pollen were counted as cherry foragers. Separation of the pollen foragers was based on microscopic examination of pollen grains in a random sample from each load.

The honey stomach of each sample bee was removed and blotted on number one filter paper. Examination of the subsequent stain indicated if the bees were nectar or water foragers. Water left no stain (Park, 1926). Bees without pollen and whose honey stomachs were empty were classified as nonforagers.

The data were subjected to analysis of variance as a split-split plot in time according to Steele and Torrie (1960). Treatments were whole units, days classified as subunits, and time of day as sub-subunits. Numbers of cherry pollen foragers, total pollen foragers, nectar foragers and total foragers (nectar and pollen combined) were treated as observations and analyzed separately. To determine if there were differences between treatments within days, each day was analyzed individually as a split plot.

At the end of the experiment, the hives were examined for accumulated stores. The cells of stored pollen were tabulated for each unit. Comparison of the mean number of cells of stored pollen

per treatment served as an additional check on pollen foraging, assuming that pollen consumption was similar in each hive.

Queen Rearing

At the end of the first week and subsequently at weekly intervals, the combs were examined for the presence of emergency queen cells. Emergency queen cells are typically enlargements of existing worker cells containing eggs or very young larvae. Since brood was absent from the experimental units, any enlargement of a worker cell was counted as a queen cell. The number of queen cells present at the end of the first week was recorded and subjected to analysis of variance as a randomized block.

Worker Ovary Development

At the time of installation of the 9-oxo cylinders and at weekly intervals, 10 bees were selected from the population within the hive. No attempt was made to obtain bees of a similar age. Sample bees were killed and stored at -3°C until analysis.

Worker ovaries were examined under 20 power binocular magnification. Bees were pinned, venter down, in a wax dissecting dish and immersed in 70 percent ethanol. Incisions on the laterals and the anterior of the abdomen allowed the dorsum to be removed.

The intestine was removed and the ovaries exposed. The ovaries were excised and placed on slides to be examined under 40 power magnification. Classification into stages of development followed the system used by Velthius (1970). Stage one represented resting and inactive ovaries, which were slender and filamentous. In stage two, the ovaries were swollen and eggs in an early stage of development were visible. In stage three, the eggs were elongated and ready for passage into the oviduct. For simplicity, ovaries in stage one were designated undeveloped and ovaries in stages two and three were combined and treated as developed. (Note that ovary development does not refer to embryological organ formation but to activation of a fully developed organ in a resting state.)

Data were analyzed as a split plot in time. Treatments were considered main effects and days classified as sub plots (Steele and Torrie, 1960). Observations consisted of the total number of developed ovaries for each sample.

During the weekly hive examination for queen cell construction, the combs were checked for eggs of laying workers. Upon termination of the experiment, the total number of eggs, larvae and capped brood was recorded for each unit.

MATERIALS AND METHODS--1976

Several modifications of the experimental procedure and design were made in 1976. Two additional bioassays of 9-oxo were added: inhibition of queen rearing when eggs and larvae were present; and drone attraction. The modifications are listed under each section of the experiment.

Foraging Profile

Despite color coding, some drifting of foragers occurred when the experimental units were placed in a straight line. A double V formation was substituted with approximately six meters between hives on a diagonal. Three nucs painted yellow accommodated the additional treatment.

Four treatments, each with three replications, were compared. The treatments were as follows: treatment A, queenless packages; treatment B, queenright packages; and treatments C and D both contained synthetic 9-oxo at 15 mg per gram of wax. Treatment C cylinders were formulated by melting the wax as described earlier. Treatment D pheromone cylinders were formulated by dissolving beeswax in ethyl ether and adding the pheromone to the resulting slurry. 9-Oxo is soluble in ether and its extraction in ether is a common technique. The ether was slowly evaporated from

the wax-pheromone slurry. Frequent stirrings prevented separation of the wax and the pheromone during evaporation. As 9-oxo is relatively nonvolatile, very little was lost in this process. Once the ether was evaporated, the solid wax-pheromone mixture was forced into 8 mm test tubes, frozen and freed from the tubes and the excess wax trimmed. The resulting one gram cylinders were approximately 2.8 cm in length.

The 1.4 kg packages of bees were installed in the nucs in the sweet cherry orchard on April 14. The queens remained caged. Each hive contained three frames of clean comb and one frame filled with 50 percent sugar syrup treated with oxytetracycline at the recommenced dosage for prevention of American Foul Brood (Johansen, 1974). On April 18, caged queens were removed from treatments A, C and D. Queens were released in treatment B. Pheromone cylinders were impaled on insect pins and fixed to the inner side of the inner combs (see Fig. 4). Boardman feeders with 0.5 liters of oxytetracycline treated sugar syrup were attached to the front of the nucs.

The April 21 hive check revealed an extra queen had been shaken into the package used in treatment D-1. The queen was removed and clean comb substituted for the comb containing eggs. A new pheromone cylinder was added and the old cylinder removed.

As in the previous year, cold, wet weather limited sampling to seven days; April 27, 29, 30 and May 1, 3, 7, and 8. The experiment was terminated on May 9. Royal Ann total bloom period for 1976 was April 12 to May 7.

The bees remaining in treatments A, C and D were killed and weighed. Cells of stored pollen were recorded. The combs in the queenright hives of treatment B were shaken free of bees of photographed to estimate pollen storage.

Queen Rearing

The hives were checked twice weekly for emergency queen cells.

An additional test of queen rearing inhibition in the presence of brood was conducted in August. Packages containing approximately 500 g of bees were shaken from the OSU apiary and established in five frame nucs on two frames of clean comb and one frame of eggs and young larvae. Treatments consisted of: treatment A, queenless packages; treatment B, packages with ether formulated pheromone cylinders at 15 mg 9-oxo per gram of wax; and treatment C, packages with heat formulated cylinders at the same concentration of pheromone. Each treatment was replicated four times. All nucs were supplied with 50 percent sugar syrup in Boardman feeders.

The experiment was conducted for one week. The total number of queen cells constructed was recorded for each treatment. Data were subjected to analysis of variance as a completely randomized design.

Ovary Development

Samples of 10 bees were obtained at the inception, twice a week during the experimental period, and upon termination of the experiment. Samples were stored and analyzed in the same manner as in 1975.

At the end of the experiment, the total number of eggs, larvae and capped brood (if any) from laying workers was recorded for treatments A, C and D. Brood in treatment B was estimated from the photographs.

Drone Attraction

To further assess the biological activity of the 9-oxo-wax formulation, each concentration and formulation of 9-oxo used in 1975 and 1976 was tested for drone attraction. Butler and Fairey (1964) have shown that 9-oxo is the primary sex attractant for drone honey bees. Caged, live queens and wax blanks served as checks. Each treatment was attached to a helium filled balloon and flown near the OSU apiary. To prevent 9-oxo contamination, fresh string

and balloons were used when wax blanks were tested. The experiment was repeated on two successive days during the peak of drone activity from 1400-1600 hr. Since the test took place in a flight lane rather than a drone congregation area, attractiveness of the test units was scored as positive if at least two drones were attracted. No effort was made to record the total number of drones attracted.

RESULTS--1975

Foraging Profile

The analysis of the data from the foraging profile samples encompassed only the three pheromone treatments. The data from the queenless hive were not included because the bees absconded from the colony very early in the sampling period.

In comparing the mean number of foraging bees in each category of activity, differences between the pheromone treatments were observed, although none were statistically significant ($P < 0.05$). The difference between mean numbers of nectar foragers for each treatment was nearly significant at the 5 percent level of probability (tabulated F for 2,4 degrees of freedom = 6.94; calculated F = 6.89). The column on the right of Tables 1a-1d shows the grand mean number of bees for each treatment in each category of foraging activity, averaged over all days, times of day and replicates. Individual means within the body of the table are averaged over time of day and replicate. The grand mean for nectar foraging increased with pheromone concentration, while the grand mean for pollen foraging decreased. The grand mean for the total number of foraging bees declined with increasing pheromone level.

When the daily means are examined for each foraging category (averaged over treatment and time), there were significant

Table 1a. Daily Treatment Means - 1975 - Nectar Foragers.

Treat.	Sample Dates							Treat. [a]
	4-30	5-1	5-7	5-8	5-9	5-12	5-13	$\bar{X}$
A	6.78	5.89	3.89	3.33	2.89	1.44	1.89	3.73
B	6.67	6.56	3.89	3.33	2.56	1.67	2.22	3.95
C	6.89	6.33	4.44	3.78	3.11	2.22	2.44	4.22
Day $\bar{X}$ [b]	6.78	6.26	4.07	3.48	2.85	1.85	2.48	

N = 10

[a] No statistical difference between treatment means.

[b] Treatment means statistically different. $LSD_{.05} = 0.52$

Treatment A = 3 mg 9-oxo
Treatment B = 9 mg 9-oxo
Treatment C = 15 mg 9-oxo

Table 1b. Daily Treatment Means - 1975 - Cherry Pollen Foragers.

Treat.	Sample Dates							Treat. [a]
	4-30	5-1	5-7	5-8	5-9	5-12	5-13	$\bar{X}$
A	0.22	0.56	0.78	1.00	0.67	0.78	0.44	0.67
B	0.56	0.44	0.44	0.67	1.00	0.89	0.33	0.63
C	0.11	0.33	0.44	0.67	0.56	0.33	0.11	0.37
Day $\bar{X}$ [b]	0.30	0.44	0.56	0.85	0.78	0.67	0.30	

N = 10

[a] No statistical difference between treatment means.

[b] Treatment means statistically different. $LSD_{.05} = 0.42$

Table 1c. Daily Treatment Means - 1975 - Total Pollen Foragers.

Treat.	Sample Dates							Treat.[a]
	4-30	5-1	5-7	5-8	5-9	5-12	5-13	$\bar{X}$
A	0.67	1.33	1.89	2.89	2.56	4.56	4.78	2.66
B	1.22	0.89	1.78	1.78	2.56	4.00	3.89	2.30
C	0.78	0.78	1.67	2.11	2.11	3.11	4.22	2.01
Day $\bar{X}$ [b]	0.89	1.00	1.78	2.26	2.41	3.67	4.30	N = 10

[a] No statistical difference between treatment means.

[b] Treatment means statistically different. $LSD_{.05} = 0.56$

Treatment A = 3 mg 9-oxo
Treatment B = 9 mg 9-oxo
Treatment C = 15 mg 9-oxo

Table 1d. Daily Treatment Means - 1975 - Total Foraging Bees.

Treat.	Sample Dates							Treat.[a]
	4-30	5-1	5-7	5-8	5-9	5-12	5-13	$\bar{X}$
A	7.45	7.22	5.78	6.22	5.45	6.00	6.67	6.39
B	7.89	7.45	5.67	5.11	5.12	5.67	6.11	6.25
C	7.67	7.11	6.11	5.89	5.22	5.33	6.66	6.22
Day $\bar{X}$ [b]	7.67	7.11	6.11	5.89	5.22	5.33	6.66	N = 10

[a] No statistical difference between treatment means.

[b] Treatment means statistically different. $LSD_{.05} = 0.53$

differences ($P < 0.05$) between sampling days for all foraging activities, with the exception of cherry pollen foraging. The mean number of nectar foragers for all treatments declined over the two week sampling period, while pollen foragers increased. Tables 1a-1d and Figures 1a-1d demonstrate the change in foraging activity over time for all categories of foraging behavior.

The mean number of cherry pollen foragers displayed the least amount of fluctuation over time. There was a slight rise in activity toward the middle of the sampling period, followed by a decline. The end of the foraging sample period was coincidental with the termination of the cherry bloom. The general reduction in available cherry pollen may explain the decline in cherry pollen foraging despite the rise in total pollen foraging demonstrated by all pheromone treatments as the sample period progressed. Table 2 shows the change in cherry pollen foraging activity, expressed as a percentage of total pollen foraging.

Examination of the foraging activity on individual days showed a significant difference between treatments on only one day, May 1, and in only one foraging category, pollen collection (treatment means: treatment A = 1.33; treatment B = 0.89; and treatment C = 0.78).

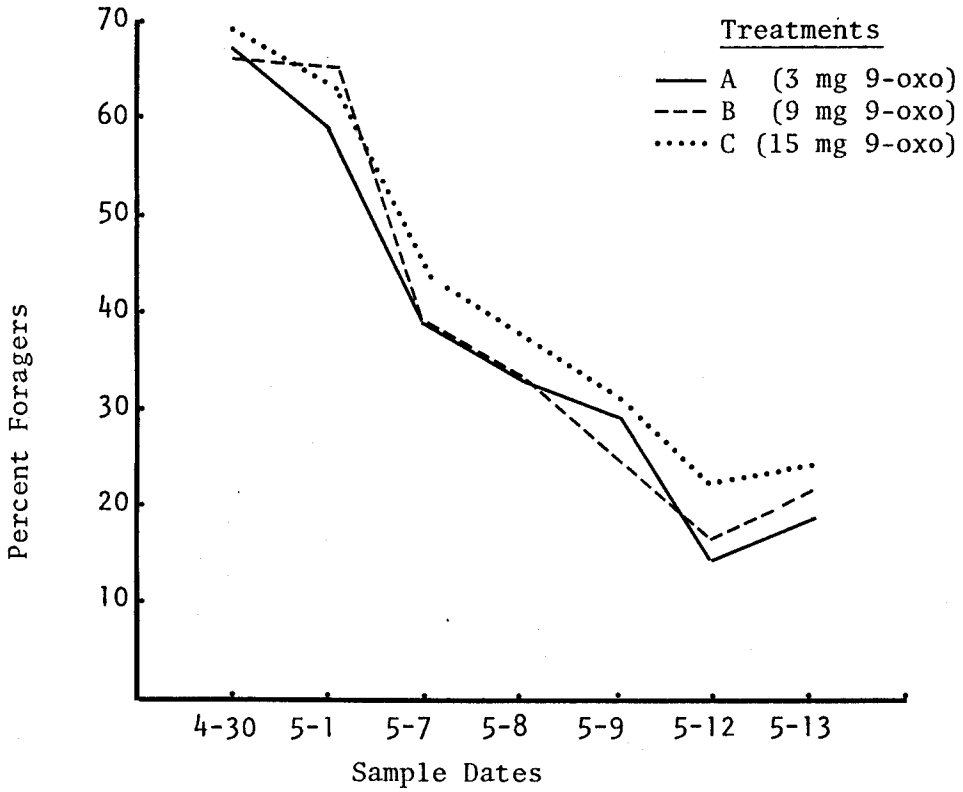

Figure 1a. 1975 daily treatment means - nectar foragers.

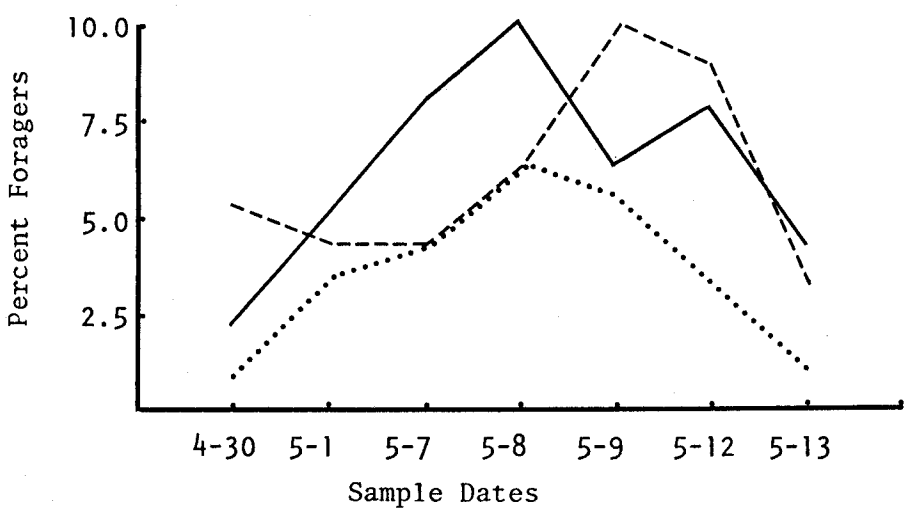

Figure 1b. 1975 daily treatment means - cherry pollen foragers.

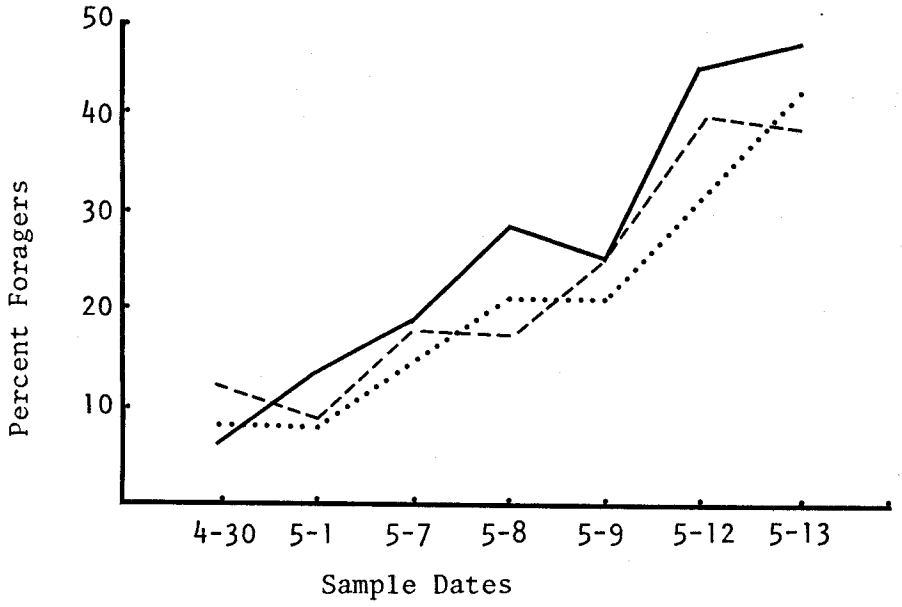

Figure 1c. 1975 daily treatment means - total pollen foragers.

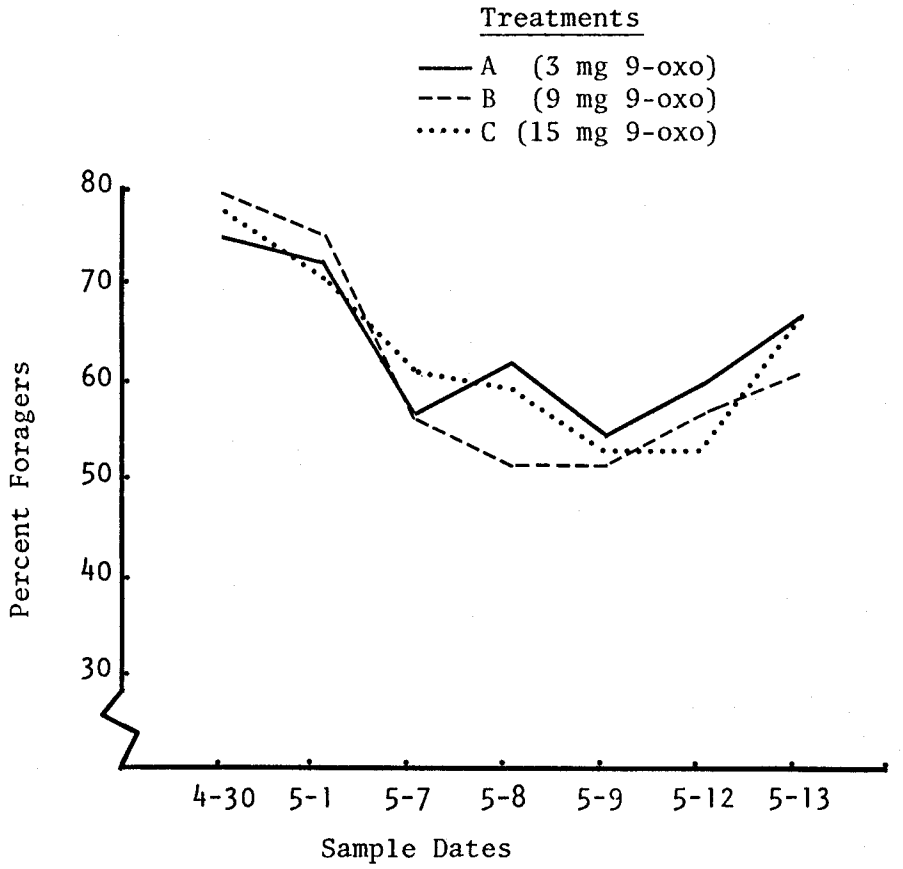

Figure 1d. 1975 daily treatment means - total foraging bees.

Table 2. Cherry pollen foragers expressed as a percent of total pollen foragers - 1975.

Treatment	Sample Dates 4-30	5-1	5-7	5-8	5-9	5-12	5-13
A (3 mg 9-oxo)	33	42	41	35	26	17	9
B (9 mg 9-oxo)	46	49	25	38	39	22	9
C (15 mg 9-oxo)	14	42	26	32	27	11	3
Day Grand Mean	33	44	31	38	32	18	7

The mean number of cells of stored pollen was highest for treatment C and lowest for treatment A. Since there was considerable variation in the amount and stage of development of the brood present in the different treatments, and since brood is a major consumer of pollen, the actual means cannot be taken as an accurate assessment of the total pollen collection for each treatment. For this reason, data were not subjected to analysis. As can be seen from Table 3, the differences between replicates are nearly as great as those between treatments.

Table 3. Cells of stored pollen - 1975.

Rep.	Treatments A	B	C
1	542	460	951
2	590	1408	917
3	466	533	1106
$\bar{X}$ Treat.	533	807	991

Treatment A = 3 mg 9-oxo
Treatment B = 9 mg 9-oxo
Treatment C = 15 mg 9-oxo

Queen Rearing

The April 28 hive examination, one week after the initiation of the experiment, revealed queen cells present in all hives. The mean number of queen cells per treatment was: treatment A (3 mg 9-oxo) = 3.67; treatment B (9 mg 9-oxo) = 3.00; and treatment C (15 mg 9-oxo) = 3.67. The queenless hive contained five cells. There was no significant difference between treatment means for the pheromone treatments (the queenless hive was excluded from the analysis).

One unit each from the 3 mg 9-oxo treatment and the 9 mg 9-oxo treatment had laying worker larvae present by April 28. In both hives two or three cells containing drone larvae from laying workers were enlarged and provisioned as queen cells.

Worker Ovary Development

The treatments did not differ significantly in number of bees with developed ovaries. Comparisons of daily means summed over treatments showed a significant increase in ovary development over time. Figure 2 illustrates the rise in the percent of sampled bees with developed ovaries for each treatment. Table 4 presents the daily treatment means expressed as a percent of the sample size. There was a slight decline in ovary development by the fourth week.

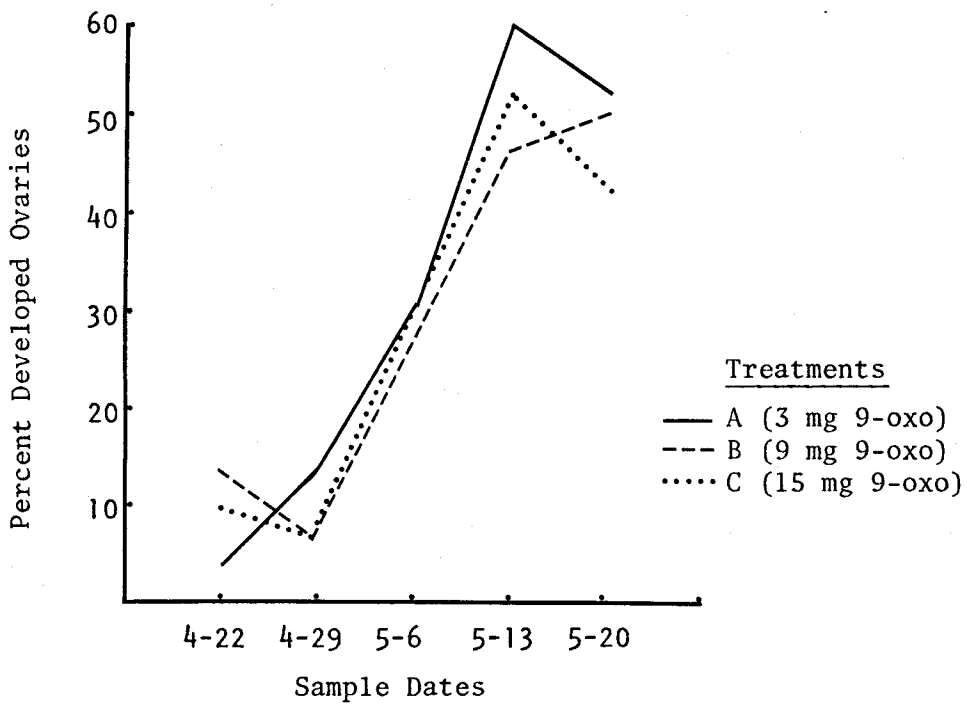

Figure 2. Percent ovarian development over time - 1975.

Table 4. Mean number of bees with developed ovaries expressed as a percent of sample - 1975.

	Sample Dates					Treat.[a]
Treatment	4-22	4-29	5-6	5-13	5-20	$\bar{X}$
A (3 mg 9-oxo)	3.3	13.3	30.0	60.0	53.3	32.0
B (9 mg 9-oxo)	13.3	6.7	26.7	46.7	50.0	28.7
C (15 mg 9-oxo)	10.0	6.7	30.0	53.3	43.3	28.7
Daily $\bar{X}$ [b]	8.9	8.9	28.9	53.3	48.3	

[a] No statistical difference between treatment means.

[b] Treatment means statistically different. $LSD_{.05} = 8.5$

In the queenless hive, ovary development paralleled that of the 3 mg 9-oxo treatment, for the first week, before the bees abandoned the queenless unit.

Laying worker brood was found in all hives by the third week (May 12). Table 5 shows the total brood in all stages found in the experimental units at the end of the fourth week. The brood in treatment A was the most advanced and the brood in treatment C the least advanced. Brood in treatment B was intermediate in development, with most of the brood in the larval state. It appears that a delay in the production of laying worker brood is found with increasing pheromone concentration. Multiple eggs and multiple larvae were observed in the laying worker brood (Fig. 3).

Table 5. Laying Worker Brood - 1975.

Treatment	Eggs	Larvae	Capped	Total
3 mg 9-oxo				
A-1	35	739	195	969
A-2	450	578	1666	2694
A-3	222	1049	555	1826
$\bar{X}$	236	789	805	1830
9 mg 9-oxo				
B-1	261	1362	229	1852
B-2	87	711	269	1067
B-3	208	1036	785	2029
$\bar{X}$	185	1036	428	1649
15 mg 9-oxo				
C-1	1041	1080	272	2393
C-2	119	756	185	1054
C-3	457	749	449	1655
$\bar{X}$	539	960	302	1701

Figure 3. Comb containing worker eggs and larvae.

Figure 4. Worker bees inspecting pheromone cylinder affixed to comb.

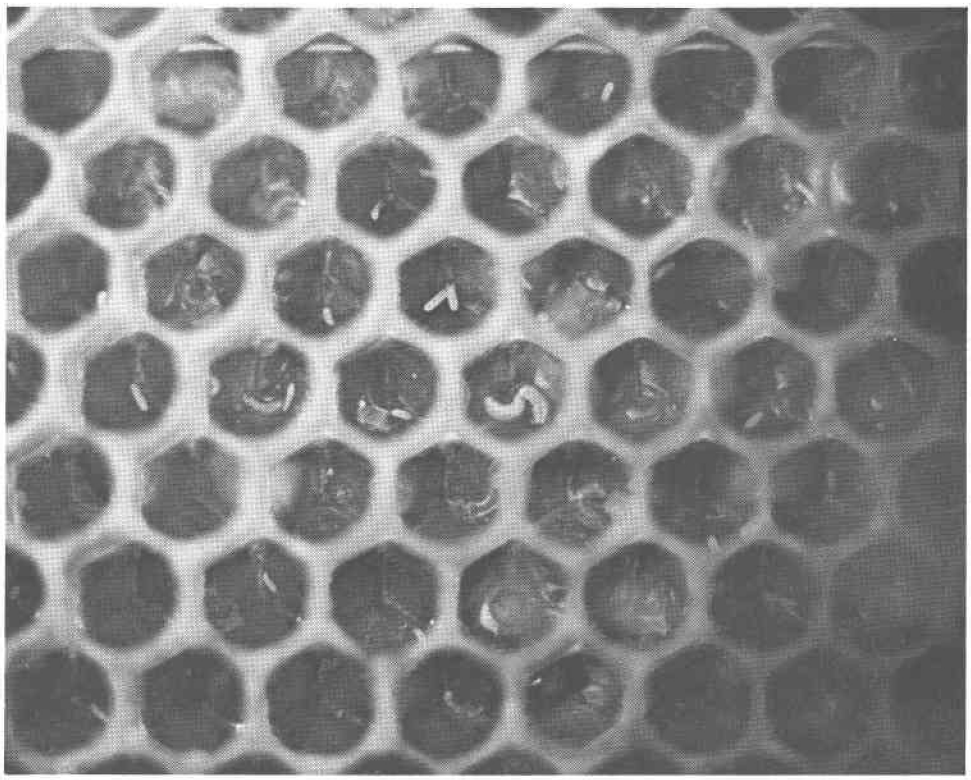

Figure 3.

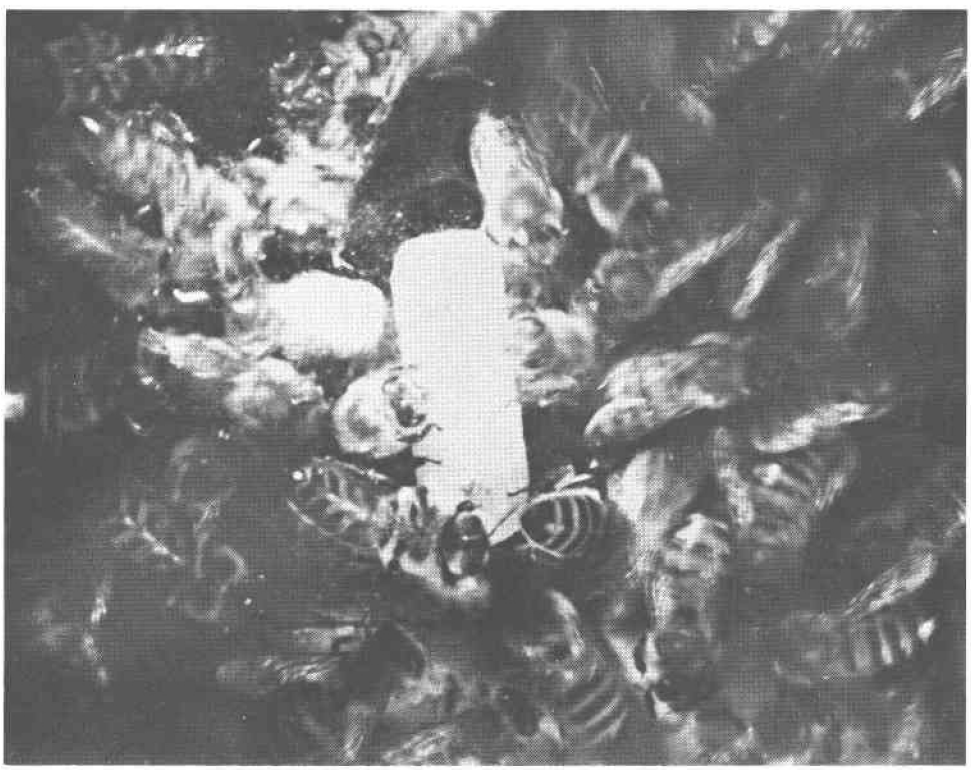

Figure 4.

RESULTS--1976

Foraging Profile

Data from the foraging profile were subjected to three separate analyses. To determine if there were differences between treatments over a period of time, all seven days were examined together as a split-split plot in time. Individual days were then analyzed separately to determine how the treatments varied from day to day. Since laying worker eggs were present on the fifth sampling day (two weeks after the initiation of the treatments), the last three days' data were discarded from the third analysis in order that the pheromone treatments could be compared with the queenless control before the presence of brood could affect foraging behavior.

In the first analysis, a significant difference between treatments ($P < 0.05$) occurred in all four categories of foraging activity. Tables 6a-6d present the daily means (averaged over time of day and replicate) and the grand mean (averaged over days, time of day and replicate) for each treatment. Treatment A (queenless) was designated the control against which treatment B (queenright) and treatments C and D (pheromone) were compared using the LSD at the 5 percent level of probability. LSD values for each analysis are included in Tables 6a-6d. Treatment B had significantly greater mean numbers of cherry pollen foragers, total pollen foragers, and

Table 6a. Daily Treatment Means - 1976 - Nectar Foragers.

Treat.	Sample Dates 4-27	4-29	4-30	5-1	5-3	5-7	5-8	Treat. [a] $\bar{X}$
A	2.00	2.89	4.33	3.22	3.22	4.67	5.22	3.65
B	2.11	2.78	2.56	2.67	2.33	3.11	3.44	2.71
C	2.33	3.67	3.67	3.67	3.00	4.44	5.22	3.71
D	2.67	3.78	3.00	3.76	3.88	3.22	5.11	3.63
Day $\bar{X}$ [b]	2.28	3.28	3.39	3.33	3.11	3.86	4.75	N = 10

[a] Treatment means statistically significant. $LSD_{.05} = 0.57$

[b] Treatment means statistically significant. $LSD_{.05} = 0.58$

Treatments

A (queenless)
B (queenright)
C (9-oxo, heat formulated)
D (9-oxo, ether formulated)

Table 6b. Daily Treatment Means - 1976 - Cherry Pollen Foragers.

Treat.	Sample Dates 4-27	4-29	4-30	5-1	5-3	5-7	5-8	Treat. [a] $\bar{X}$
A	1.56	1.00	0.89	0.33	0.78	0.00	0.00	0.65
B	4.11	2.55	2.56	1.78	2.33	1.44	0.78	2.22
C	1.67	1.33	1.33	0.89	0.22	0.00	0.00	0.78
D	2.00	1.00	1.44	0.78	0.22	0.33	0.11	0.84
Day $\bar{X}$ [b]	2.33	1.47	1.56	0.94	0.89	0.44	0.22	N = 10

[a] Treatment means statistically significant. $LSD_{.05} = 0.44$

[b] Treatment means statistically significant. $LSD_{.05} = 0.44$

Table 6c. Daily Treatment Means - 1976 - Total Pollen Foragers.

Treat.	Sample Dates 4-27	4-29	4-30	5-1	5-3	5-7	5-8	Treat. $\bar{X}$ [a]
A	2.56	2.00	1.44	0.67	2.00	1.78	2.33	1.83
B	5.44	3.56	4.56	3.56	4.89	4.00	4.33	4.33
C	2.78	1.78	1.89	2.00	1.33	1.56	1.33	1.81
D	2.78	1.33	2.11	2.22	1.00	2.22	1.67	1.90
Day $\bar{X}$ [b]	1.33	2.17	2.50	2.11	2.31	2.39	2.42	N = 10

[a] Treatment means statistically significant. $LSD_{.05} = 0.68$

[b] Treatment means statistically significant. $LSD_{.05} = 0.54$

Treatments

A (queenless)
B (queenright)
C (9-oxo, heat formulated)
D (9-oxo, ether formulated)

Table 6d. Daily Treatment Means - 1976 - Total Foraging Bees.

Treat.	Sample Dates 4-27	4-29	4-30	5-1	5-3	5-7	5-8	Treat. $\bar{X}$ [a]
A	4.78	4.89	5.78	3.89	5.22	6.44	7.56	5.51
B	7.56	6.33	7.11	6.22	7.22	7.11	7.78	7.05
C	5.11	5.44	5.56	5.67	4.33	6.00	6.56	5.52
D	5.44	5.11	5.11	6.00	4.89	5.44	6.78	5.54
Day $\bar{X}$ [b]	5.72	5.44	5.89	5.44	5.42	6.25	7.17	N = 10

[a] Treatment means statistically significant. $LSD_{.05} = 0.44$

[b] Treatment means statistically significant. $LSD_{.05} = 0.69$

total foraging bees than the queenless control. The mean number of nectar foragers was significantly less in treatment B compared with the control. Neither of the pheromone treatments differed significantly from the queenless hives in any category of activity.

Daily means (averaged over time and treatment) differed significantly for each set of observations. The mean number of nectar foragers increased over time. Pollen foraging decreased considerably between the first and second samples, then leveled out. The total number of foragers increased with time, reflecting the increased number of nectar foragers. Figure 5 depicts the change in daily means (treatments averaged together). Figures 6a-6d show the change in daily means for each treatment.

In the second analysis, that of the individual days, data from all but April 29 and May 8 showed significant differences between treatments. The mean number of nectar foragers was significantly less in the queenright and the ether formulated 9-oxo treatment than in the queenless control on April 30. The treatment means for nectar foraging did not vary significantly on any other day. The mean number of cherry pollen foragers was significantly greater in the queenright colonies than in the queenless colonies on April 27, 30 and May 1 and 3. Both pheromone treatment means for cherry pollen foraging were significantly less than the queenless colonies on May 3. On all other days, the pheromone treatments did not differ from the

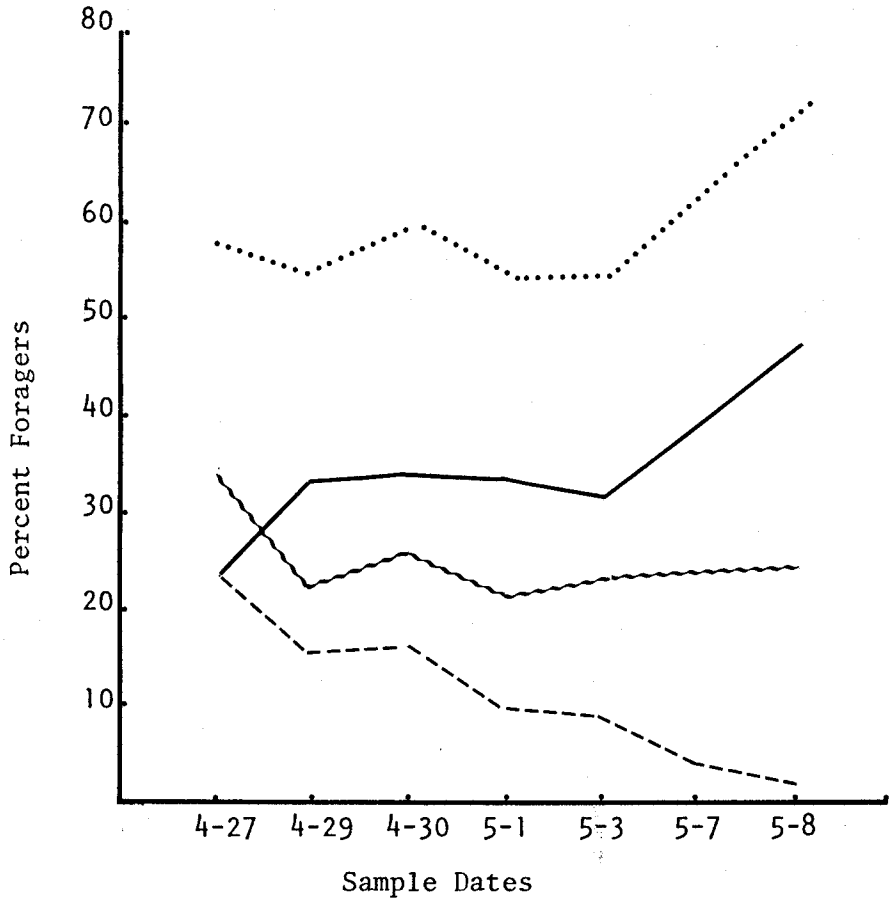

Figure 5. Daily foraging means, treatments combined - 1976.

—— Nectar Foragers
--- Cherry Pollen Foragers
~~~ Total Pollen Foragers
····· Total Foragers
~~~

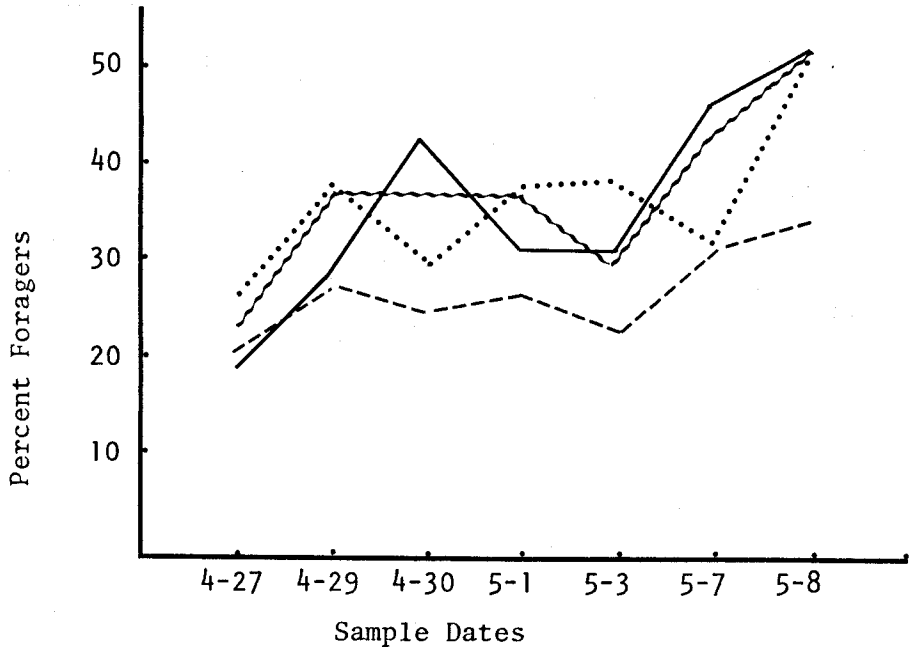

Figure 6a. 1976 daily treatment means - nectar foragers.

Treatments

—— A (queenless)
--- B (queenright)
~~~ C (9-oxo, heat form.)
····· D (9-oxo, ether form.)

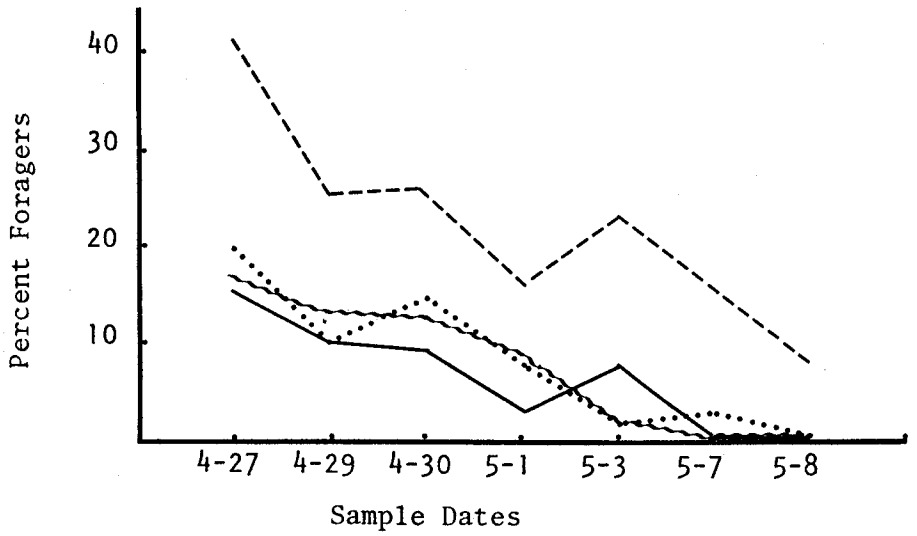

Figure 6b. 1976 daily treatment means - cherry pollen foragers.
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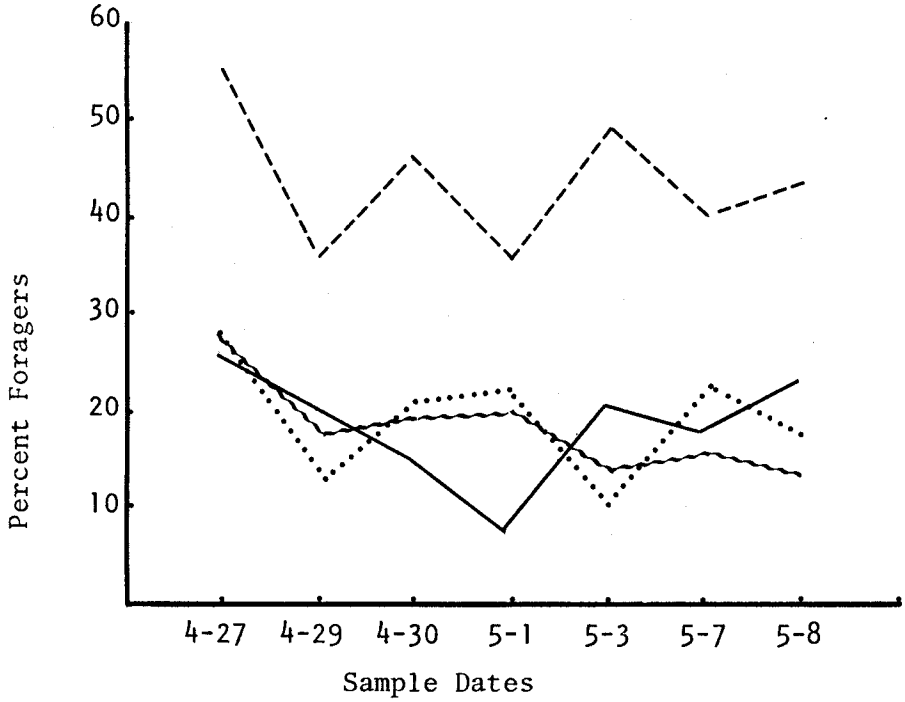

Figure 6c. 1976 daily treatment means - total pollen foragers.

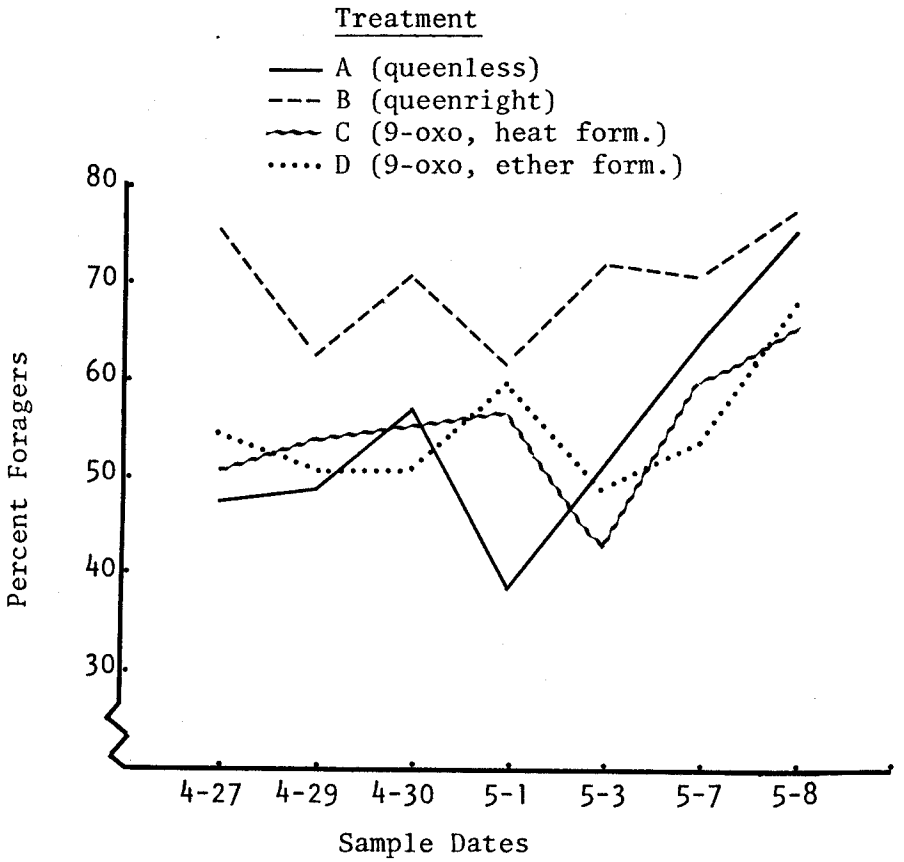

Figure 6d. 1976 daily treatment means - total foraging bees.

queenless control. Cherry pollen foraging was minimal in all hives on the last two days of sampling. At that time, there was little cherry pollen available in the orchard (see Table 7).

Table 7. Cherry pollen foragers expressed as a percent of total pollen foragers - 1976.

	Sample Dates						
Treatments	4-27	4-29	4-30	5-1	5-3	5-7	5-8
A (queenless)	61	50	62	50	39	0	0
B (queenright)	76	72	56	50	48	36	18
C (9-oxo, heat)	60	75	70	45	17	0	0
D (9-oxo, ether)	72	75	68	35	22	15	7
Daily mean	67	68	64	45	32	13	6

The mean number of pollen foragers showed the greatest daily fluctuation (Fig. 6c). Treatment B (queenright) means were significantly higher than the control on April 27, 30, and May 1, 3, and 7. There was no significant difference between the pheromone treatments and the control except on May 1 and 3. On May 1, both treatments C and D means were significantly greater than the control. On May 3, treatment D (ether formulated 9-oxo) was significantly less than the queenless treatment.

There was a significant F value for time of day (averaged across treatments) on April 27, in the categories of cherry pollen

and total pollen collection. The highest means occurred in the 1000 hr sample (1000 hr = 3.25, 1200 hr = 1.91, 1400 hr = 1.83 for cherry pollen foragers; and 4.42, 2.67 and 3.08 respectively, for total pollen foragers). In general, both pollen and nectar collection tended to be greatest in the morning, although the overall differences between times of day were not significant.

The results of the third analysis did not differ markedly from the analysis of all seven sampling days. The queenless treatment differed significantly from the control in all foraging activities, while the pheromone treatments did not. Table 8 compares the treatment means for each foraging activity for the first four days with those of all seven days averaged together. There is a slight rise in the mean number of pollen collectors in the pheromone treatments when only the first four days are considered. The queenless treatment means for pollen and nectar collection are lower in the first four days than in all seven days together.

The total number of pollen cells present in each treatment is given in Table 9. Some data were missing from treatment B due to the loss of the photographic record during the process of development. In both replicates, data from one side of one frame were lost. In addition, the pollen consumption by the brood developing in treatment B (queenright) makes assessment of pollen collection by tabulation of cells of stored pollen subject to considerable error. While the

Table 8. Treatment means for sample days 1-4 versus sample days 1-7, 1976.

Treatment	Nectar Foragers		Cherry Pollen Foragers	
	Days 1-4	1-7	Days 1-4	1-7
A (queenless)	3.11	3.65	0.94	0.65
B (queenright)	2.53	2.71	2.75	2.22
C (9-oxo, heat)	3.33	3.71	1.31	0.78
D (9-oxo, ether)	3.31	3.63	1.31	0.84
$LSD_{.05}$	0.57	0.57	0.57	0.44

	Total Pollen Foragers		Total Foragers	
	Days 1-4	1-7	Days 1-4	1-7
A (queenless)	1.67	1.83	4.83	5.51
B (queenright)	4.28	4.33	6.81	7.05
C (9-oxo, heat)	2.11	1.81	5.44	5.52
D (9-oxo, ether)	2.11	1.90	5.42	5.54
$LSD_{.05}$	0.71	0.54	0.93	0.69

Table 9. Cells of stored pollen - 1976.

Rep.	Treatment A (queenless)	B (queenright)	C (9-oxo, heat)	D (9-oxo, ether)
1	761	1313	1339	1226
2	621	625 [a/]	987	1061
3	635	580 [a/]	977	1084
Total Cells	2017	2518 [a/]	3303	3371

[a/] missing data from one side of one frame.

mean number of cells of pollen are lowest for the queenless units, laying worker brood was present in these units. Most of the brood found in the pheromone treatments was in the egg stage and could not appreciably reduce pollen stores. Therefore, due to the errors introduced by the presence of brood, the data were not analyzed for differences between treatments. A low mean number of cells of stored pollen could be interpreted as either brood consumption or reduced pollen foraging activity.

Both pheromone treatments had a significantly greater weight of bees remaining at the end of the experiment than did the queenless control. Comparisons were made between the mean weight of bees per treatment, taken immediately after the bees were killed. The extermination occurred early in the morning, before flight activity commenced. Treatment B (queenright) hives were excluded from the analysis because the egg laying activity of the queen supplied additional bees to the colony. The final weight of dead bees for each colony is presented in Table 10. The LSD value at the 5 percent level of probability for comparison between treatment means is included with the table.

Table 10. Weight of bees remaining in treatments A, C and D on May 9, 1976.

	Treatments	(Weight in grams)	
Replicate	A (queenless)	C (9-oxo, heat)	D (9-oxo, ether)
1	318.76	656.74	741.44
2	263.96	419.50	562.53
3	451.50	560.57	666.00
Treat. $\bar{X}$ a/	344.74	545.60	656.66

a/ Treatment means significantly different. $LSD_{.05}$ = 150.4 gm.

Queen Rearing

The first hive check occurred April 21, three days following the installation of the treatments. None of the queenright or queenless colonies contained queen cells. The ether formulated 9-oxo treatment had six queen cells and the heat formulated 9-oxo treatment had two queen cells. In both treatments, two out of three replicates contained queen cells. In replicate D-3, wax had been added to the pheromone cylinder to form the base of a queen cell. No data could be recorded from replicate D-1 because an extra queen had been inadvertently introduced into the package and was not removed at the time of treatment installation. The queen had begun laying and the workers had chewed down the pheromone cylinder.

The queen was removed, the egg contaminated frames replaced, and a new pheromone cylinder affixed to the comb.

By the April 25 hive check, treatment A had four queen cells, treatment C still had six cells and treatment D now contained six cells. In colony D-1, two queen cells were constructed from wax added to the pheromone cylinder. At the end of the experiment (May 9), treatment means were: treatment A (queenless), 5.33 queen cells; treatment B (queenright), 0; treatment C (heat formulated 9-oxo), 5.33; and treatment D (ether formulated 9-oxo), 4.33 queen cells. Only treatment B had significantly fewer queen cells than the queenless control.

In the test of the inhibition of queen rearing by synthetic 9-oxo in the presence of brood, differences between treatments were not significant. The mean number of queen cells built in the queenless colonies was 3.25; in the ether formulated 9-oxo, 5.00; and in the heat formulated pheromone treatment, 5.50. There was considerable variation between replicates.

Worker Ovary Development

There was a significant difference between treatments ($P < 0.05$) in the mean number of bees with developed ovaries averaged over time; between daily means, averaged across

treatments; and a significant Day X Treatment interaction. When all treatments were compared with the queenless hives, only queenright colonies had a significantly lower mean number of sampled bees with developed ovaries. Table 11 presents the daily and grand mean number of bees with developed ovaries (stages II and III) for each treatment. In Figure 7 the change in ovarian development over time is depicted for each treatment.

Ovaries in stage III of development (the last stage before oviposition commences) did not appear in the samples until the April 28 hive inspection. Samples from both pheromone treatments contained 3.3 percent of the bees with ovaries in stage III. By May 2, 6.7 percent of the bees sampled from the queenless treatment had ovaries in stage III. The hive examination on that date revealed laying worker eggs present in two out of three replicates in the queenless units. On May 6, all replicates in treatment A and one replicate in treatment C (heat formulated pheromone) contained laying worker eggs. By May 9, laying worker eggs had appeared in one replicate in treatment D (ether formulated 9-oxo). Table 12 presents the number of cells of brood present in treatments A, C and D at the end of the experiment on May 9.

While some degree of activation of worker ovaries appeared in the queenright treatment throughout the experiment, no samples contained bees with ovaries in stage III. At least one bee with ovaries

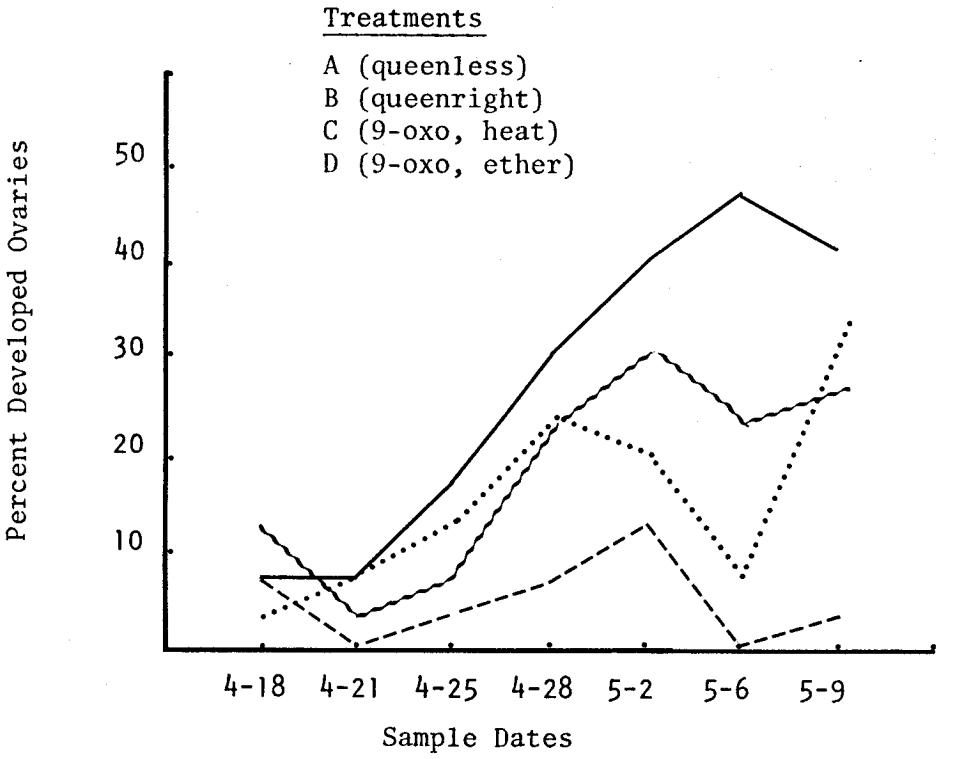

Figure 7. Percent ovarian development over time - 1976.

Table 11. Mean number of bees with developed ovaries expressed as a percent of sample - 1976.

Treat.	Sample Dates 4-18	4-21	4-25	4-28	5-2	5-6	5-9	Treat. $\bar{X}$ a/
A	6.7	6.7	16.7	30.0	40.0	46.7	40.0	26.7
B	6.7	0.0	3.3	6.7	13.3	0.0	3.3	4.8
C	13.3	3.3	6.7	23.3	30.0	23.3	26.7	18.1
D	3.3	6.7	13.3	23.3	20.0	6.7	33.3	15.2
Daily $\bar{X}$ b/	7.5	4.2	10.0	20.8	25.8	19.2	25.8	

a/ Treatment means statistically significant. $LSD_{.05}$ = 11.6

b/ Treatment means statistically significant. $LSD_{.05}$ = 7.8

Table 12. Laying Worker Brood - 1976.

Treatments	Eggs	Larvae	Capped Brood	Total Brood
Queenless				
A-1	1258	263	0	1521
A-2	913	361	28	1302
A-3	59	0	0	59
$\bar{X}$	743	208	9	961
9-oxo, heat				
C-1	2504	167	0	2671
C-2	0	0	0	0
C-3	0	0	0	0
$\bar{X}$	835	56	0	890
9-oxo, ether				
D-1	200	0	0	200
D-2	0	0	0	0
D-3	0	0	0	0
$\bar{X}$	67	0	0	67

in stage II was present in all treatments in the initial sample on April 18. This was despite the presence of a caged queen in all units sampled, since sampling occurred just prior to the initiation of the treatments and the removal of the queens from treatments A, C, and D.

Drone Attraction

Drones were attracted to live caged queens, and all pheromone cylinders, in all concentrations and formulations used in 1975 and 1976. Cylinders prepared in 1975 and 1976 and stored frozen and unused as well as cylinders recovered from the 1975 and 1976 experiments were tested. Since testing occurred in a flight lane rather than a congregation area, no more than five to six drones were attracted at one time. A blank wax cylinder flown as a control did not prove attractive. The test was repeated on the following day with the same results.

DISCUSSION AND CONCLUSION

Evaluation of the 1975 pheromone trials based on the results of the foraging profile was made difficult by the presence of laying worker brood. As noted earlier (Free, 1967; Jaycox, 1970b), brood has a stimulatory effect on foraging activity, especially pollen foragers. Both treatments A and B contained some laying worker brood by April 28, two days before the initiation of sampling for foraging behavior. By May 12, all colonies had laying worker brood in an early state of development, with some capped brood in treatments A (3 mg 9-oxo) and B (9 mg 9-oxo). This pattern of brood distribution may have affected the results of the foraging profile. While the total number of foraging bees per sample varied little between treatments, the proportion assigned to nectar and pollen foraging appeared to be influenced to a greater extent by the concentration of pheromone. However, the number of pollen foragers not only increased with decreasing pheromone concentration (and increasing amounts of brood), but increased over time. This strongly suggests that the development of laying worker brood affected the pattern of nectar and pollen collection. Thus, selection of a pheromone concentration to be used in further testing should be evaluated on the basis of the other bioassays incorporated in the experiment.

Unfortunately, no clear choice was offered by comparisons of treatment means for either the total number of queen cells constructed or the number of bees with developed ovaries. The differences between treatments rested with the degree to which each could substitute for the queen, as the experimental results demonstrated that the synthetic pheromone could not duplicate the effects of a live queen.

The decision to use the pheromone concentration of treatment C (15 mg 9-oxo per gram of wax) was based primarily on the results of the bioassay for inhibition of worker oögenesis. While treatment C did not inhibit the development of laying worker brood, the appearance of brood in that treatment was delayed in comparison with treatments A or B. As Table 5 illustrates, the greatest proportion of the brood in treatment C was in an early stage of development. Consideration was given to the observation that brood counts were taken on May 20, seven days after the last foraging sample. All eggs and a large proportion of the brood present on May 20 had not yet appeared during the foraging samples (egg to capped cell = 8-9 days). Thus, in the 15 mg 9-oxo treatment, there was less brood related stimulus for foraging during most of the sampling period.

The performance of synthetic 9-oxo in 1976 varied from a position intermediate between the queenless and queenright packages, to one barely distinguishable from the queenless controls. In all

trials, there was very little difference between the two formulations of 9-oxo in the results observed. This indicates that the heating process used in the preparation of treatment C cylinders did not affect the potency of the pheromone.

The queenright colonies were clearly superior in foraging activity to the pheromone-treated and the queenless units, both in total number of foragers and percent of bees foraging for pollen. The higher proportion of nectar foragers evidenced by treatment A (queenless) in comparison with treatment B (queenright), corroborates the observation of Free (1967), that queenless colonies tend to forage more for nectar than pollen. The reduction in total number of foragers in the queenless treatment seems to contradict Free's assertion that there is no change in the total foraging population with the queenless condition. However, his experiments were conducted with queenless and queenright colonies containing brood. In the present experiment, brood did not appear in the queenless colonies until near the end of the sampling period, while brood was present in the queenright colonies before sampling began.

The similarity in foraging activity between the queenless and pheromone treated colonies is in contrast with the results obtained by Jaycox (1970a). In his experiments, he recorded the collection of comparable volumes of nectar by queenright and pheromone treated colonies. Queenless bees collected significantly less nectar.

He found no difference between treatments in pollen collection and concluded that queen pheromones affect nectar rather than pollen foraging. Jaycox's experiments dealt primarily with the immediate effects of queen pheromone on foraging behavior, especially nectar collection. Data were recorded for three days only following the application of the treatments. In the 1976 trials of the present experiment, data collection did not begin until more than a week after the initiation of treatment and continued for a 12 day period. There were differences as well in the concentration and formulation of 9-oxo used in the two experiments. Jaycox administered 100 μg of 9-oxo dissolved in alcohol to ca. 600 bees and 1000 μg of 9-oxo to small colonies of 10,000 to 11,000 bees. Although the 9-oxo concentration used in the present experiment was larger (15 mg), its incorporation into beeswax considerably reduced the amount available at any one time. These differences in the timing and duration of sampling for foraging activity and differences in 9-oxo formulation and concentration per bee, may explain why Jaycox was able to record greater activity in the pheromone treated in comparison with queenless colonies.

When a comparison is made between treatment means for the entire sampling period and those for the first four days (prior to the appearance of laying worker brood in queenless and pheromone treated colonies), the situation changes to some extent. As

Table 8 indicates, the means for pheromone treatments (C and D) are greater for sample days 1-4 than for days 1-7. Those of the controls (A) have decreased. This widening of the difference between queenless and 9-oxo treated colonies in the first four sample days, although not statistically significant, brings the results of the experiment more in line with those obtained by Showers (1967). In his experiments with small 0.45 kg colonies (ca. 3,500 bees), supplied daily with 1500 μg of 9-oxo, he recorded a gain of new stores (nectar and pollen) intermediate between queenless and queenright colonies of equal strength.

The significantly greater weight of bees remaining in the pheromone treated colonies compared to the queenless controls, gives further evidence of the biological activity of the 9-oxo-beeswax formulation. Both Jaycox (1970a) and Showers (1967) reported the retention of a greater number of bees in their pheromone treated colonies, than in the queenless units. Showers stated that pheromone treated bees remained well organized and evidenced less restlessness than those in queenless colonies. A similar pattern of behavior was observed in the queenless and pheromone treated colonies of this experiment.

The results of the tests for suppression of worker ovary development appear to be in variance with those of Butler et al.

(1961), Butler and Fairey (1964), Velthius and Van Es (1964), and Velthius (1972); all found significant differences between queenless and pheromone treated bees. However, each of the above authors dealt with small groups of bees (40-50) which were regularly supplied with 9-oxo in solution. Only Butler and Fairey (1964) reported on experiments where comparisons were also made with queenright groups of bees. In this experiment, 9-oxo treated units were intermediate between queenless and queenright, but with significantly less ovary development than queenless. Butler et al. (1961) also found that queen extract inhibited ovary development to a greater extent than 9-oxo.

The discrepancy in results between the present experiment and those of previous researchers may be due to differences in experimental design. In the experiments of the above authors, ovary development was measured from one sample taken at the end of two weeks (on an average). In the present experiment, ovary development over time was measured from a series of samples. Comparison between treatments was based on cumulative effects of the 9-oxo formulation over a period of three weeks. Once again, the differences in formulation and amount of 9-oxo available per bee are factors that can influence experimental results. It may well be that the slow release formulation used in the present experiment did not

supply sufficient 9-oxo per bee to prevent oögenesis in all members of the colony.

The amount of laying worker brood present in the 9-oxo treatments (C and D) compared to the queenless units (treatment A) indicates that the pheromone delayed the development of worker ovaries. Within two weeks, two out of three replicates in treatment A had laying worker brood. Evidence of laying worker brood did not appear in treatments C or D until almost a week later and then in only one replicate per treatment. This agrees with the results of the previous year, in which laying worker brood did not appear in the 15 mg 9-oxo treatment until about three weeks after the experiment began. Showers (1967) also reported the development of laying worker brood in two out of three pheromone treated colonies after the experiment was continued for three weeks. This was despite the daily supply of 1500 μg of 9-oxo, ten times the amount recovered from single queen extracts.

One possible explanation for the appearance of laying worker brood in only two out of the six pheromone treated colonies lies with the fate of wax cylinder inside the hive. Bees either removed wax from the cylinders or covered them with burr comb, making them the bases for worker and occasionally, queen cells. In both treatments containing laying worker brood, the wax cylinder was completely

covered with burr comb. This may have prevented bees from gaining access to pheromone impregnated wax. In the other pheromone treated replicates without brood, either wax was removed, or if burr comb was added, more than half of the cylinder remained free. In no case was the cylinder left untouched.

The role of 9-oxo, or for that matter, the queen herself, in the suppression of oögenesis in worker bees is not clearly defined. Peraplova (1928), Jay (1968, 1970), and Kropacova and Haslbachova (1969) have recorded workers with varying degrees of ovary development in queenright colonies. Samples of workers from the package bees used in the present experiments contained a small percent of workers with ovaries in a developed state, despite the presence of a caged queen. Treatment B (queenright) colonies still evidenced a few bees with slight ovary development after the queen was free and laying. Jay (1970, 1972) concluded from experiments with various combinations of queens and brood, that the inhibition of ovary development depended not only on the presence of the queen, but on the presence of brood. In addition, queenless colonies with unsealed brood had fewer bees with developed ovaries than queenright, broodless colonies.

The importance of both the concentration of 9-oxo and the absence of other chemical and behavioral stimuli from the presence of a live queen, is demonstrated by the contrasting results of the

tests for drone attraction and suppression of queen rearing. While drones were attracted by all concentrations and formulations of 9-oxo used in 1975 and 1976, not one treatment was able to prevent the construction of queen cells. Butler and Fairey (1964) and Boch et al. (1975) have shown that drone attraction to the queen is primarily a function of her 9-oxo content. While a few drones can be attracted by concentrations as low as 10 μg (Boch et al.), their numbers increase with increasing pheromone concentration. The amount of 9-oxo contained in each of the wax cylinders is well above the initial threshold of drone attraction.

Inhibition of queen rearing, although affected by 9-oxo concentration, requires additional substances for full achievement. Butler (1961) and Butler et al. (1961) obtained total inhibition of queen rearing only when 9-oxo was supplemented by the scent of a queen. A queen yields on an average 150 μg of 9-oxo (Butler and Paton, 1962; Shearer et al., 1970). Yet Chaudhry and Johansen (1971) reported complete inhibition only when colonies were supplied with 2,000-3,000 μg of 9-oxo per day. Boch and Lensky (1976) suggested that the queen produces highly potent substances that combine with 9-oxo to prevent queen rearing. Thus far, possibly due to extreme volatility or instability which makes extraction impossible, these compounds have not been identified.

Butler (1961), Chaudhry and Johansen (1971), and Boch and Lensky (1976) demonstrated partial inhibition of queen rearing with 9-oxo. This was not achieved in the present experiment. The failure of the wax formulation to duplicate this effect and indeed, its inability to prevent worker oögenesis could be due to inadequate distribution of the pheromone within the hive. Velthius (1972) has shown that queen substances are transmitted both by the queen and by her attendants. The movement of the queen through the hive and the constant replacement of her circle of attendants, facilitates the distribution of the pheromones. Each bee must receive some pheromone to be aware of the queenright condition. The stationary position of the wax cylinder may limit the number of bees in contact with the 9-oxo. Manipulation of the wax, its removal and reuse in other parts of the comb can aid in pheromone distribution. However, it is possible that despite the large concentration of 9-oxo used, not all bees received sufficient pheromone to respond to the stimulus.

The inability of the 9-oxo-beeswax formulation to significantly improve foraging over queenless colonies may not be solely the result of inadequate distribution or concentration of 9-oxo. Foraging is a complex activity which is affected by a number of factors both inside and outside the hive. The size of the colony, its present needs, the amount and stage of development of the brood, the

condition of the queen, as well as the availability and abundance of nectar and pollen sources all influence the pattern of foraging behavior. The queen does not produce one pheromone (9-oxo), but many. It has already been demonstrated that more than 9-oxo is required for complete inhibition of worker ovary development and queen cell construction. It is quite possible that these additional substances have subtle effects on foraging behavior as well.

From the results of these experiments it appears that the 9-oxo-beeswax formulation has its greatest effect in promoting the cohesiveness of the colony and in increasing the retention of bees within the hive. In this respect, it offers distinct advantages over the use of queenless DPUs. However, it cannot fully substitute for the queen nor compensate for the increased stimulus for pollen collection provided by the presence of brood.

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