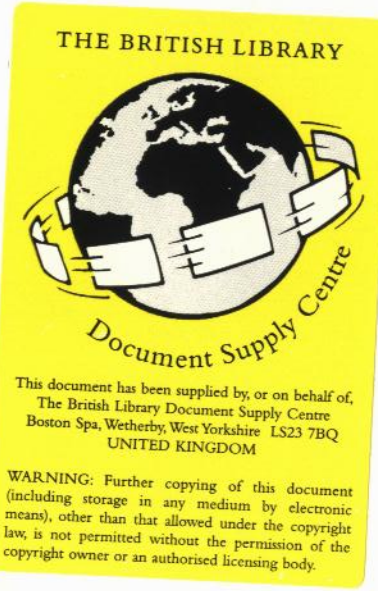

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Queen substitutes for small pollination colonies of the honey bee, *Apis mellifera* (Hymenoptera: Apidae)

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Honey bee, package bees, queen rearing inhibition, mandibular gland, queen pheromone, retinue response, pollination unit

Abstract. Ethanol extracts of egg-laying queens, extracts of unmated queens, and a mixture of four synthetic components of the queen mandibular pheromone were tested as queen substitutes. Worker bees were attracted to the extracts of fertile queens and to the synthetic mixture, but complete inhibition of queen rearing occurred with the extracts. However, the extracts of reproducing queens and the synthetic mixture and, with slightly lower efficiency, also the extract of virgin queens proved to be suitable queen substitutes in trials with queenless bee colonies, used for pollination in greenhouses.

INTRODUCTION

Formation of artificial but stable packages of honey bee workers with sustained foraging activity requires novel beekeeping techniques. In particular, an effective substitute for the queen is needed to maintain the queenless package in good condition for a sufficiently long period. The queen is known to regulate bees' physiology and behaviour by means of a variety of pheromones. Their effects on workers include community coherence, working activity regulation, inhibition of ovarian development, and suppression of queen-cell building (Free, 1987). These effects had been recognized prior to the discovery of the "queen substance" by Butler (1954) and Pain (1954). Several types of pheromone producing glands and the chemical structure of their products have been identified since then, but causal links between the pheromonal effects and the individual pheromonal components have not been elucidated fully.

Maintenance of economically exploitable workerbee packages should be possible with a suitable pheromone mixture replacing the pheromone-emanating queen. According to Slessor et al. (1988), the queen's retinue forms in response to the mandibular gland pheromone, in which the following compounds are regarded as crucial: 9-keto-2(E)-decenoic acid, 9-hydroxy-2(E)-decenoic(S+,R–)acid, 4-hydroxy-3-methoxy-phenylethanol, and methyl p-hydroxybenzoate. Various effects of pheromones from the tergal glands were described by Vierling & Renner (1977) and verified by Hazan et al. (1989), while the chemical nature of these pheromones was examined by Espeile et al. (1990). Products of the Koshevnikov's gland (Butler & Simpson, 1965) and of the tarsal glands (Lensky et al., 1984) were also implicated in queen's effect on the bee colony. Existence of other sources of effective substances in the queen is anticipated, in particular in the integument (Espeile et al., 1990).

Several laboratory assays have been developed to test the pheromone effects. Attractiveness for the worker bees is assessed as the retinue response (Slessor et al., 1988), while the over-all reaction of bees to volatiles can be assayed either by measuring changes in

oxygen consumption (Moritz & Southwick, 1986) or by electroantennography (Skirkevichiené, 1988). More specific responses, such as inhibition of queen rearing and suppression of ovarian activation, can be studied in cage tests and field trials (Willis et al., 1990).

Queen substitution by pheromone lures has been examined not only in various modifications of pollination units, but also with the aim to provide coherence and stability to packaged bees during transportation (Naumann et al., 1990). An attempt to substitute the queen in a small queenless colony was made by Showers (1967) but disposable pollination units for commercial use included live queens (Thorp et al., 1974). Youngs and Burgett (1982) tested the effect of synthetic 9-oxo-decenoic acid on the pollinating activity of queenless workers but these tests did not represent a possible practical application. However, a successful substitution of the queen by an ethanolic queen extract in the pollination units has been demonstrated (Krieg, 1987).

In the present study, a synthetic mixture of the complex mandibular pheromone is tested as an inexpensive queen substitute and its activity is compared with that of ethanolic extracts from virgin and fertile queens. Laboratory tests were complemented by trials with commercially available pollination packages of the worker bees.

MATERIAL AND METHODS

Tested materials

Crude pheromone extracts were prepared from virgin queens (VQ), which had been isolated in small compartments of the beehive's honey chamber for five days after emergence, and from fertile queens (FQ) that were more than one year old. Selected queens were immersed in 96% ethanol for about one year and then subjected to extraction in Soxhlet apparatus for 40 hours (Krieg, 1987) to obtain extracts, labelled VQ-S and FQ-S, respectively. A simple filtrate of the ethanol macerate of fertile queens was also used (FQ-E).

Queen mandibular pheromone (QP) was formulated using 9-oxo-2-(E)-decenoic acid, 9-hydroxy--2-(E)-decenoic (R–/+, S–/–) acid (both synthesized in the Institute of Organic Chemistry and Biochemistry, Prague), methyl p-hydroxybenzoate, and homovanillyl alcohol (both purchased from Aldrich, U.S.A.). The ratio of these components was in compliance with the composition of natural pheromone in the mandibular glands of mated queens (Slessor et al., 1988).

Queens used for the extracts, as well as living queens (Q) added to packaged bees in the control experiments, were reared according to a standard procedure (Škrobal & Krieg, 1985).

Laboratory assays

The assay of retinue response described by Slessor et al. (1988) was used to determine the reaction of worker bees to the test mixtures. Queen dummies ("pseudoqueens"), represented by polyethylene toothpicks (5 × 2.5 × 88 mm), were treated with 20 µl of test mixture 2–3 hrs before the trial. Dilutions corresponding to 10^{-3} queen equivalents (Qeq) were administered to small holes drilled in the toothpicks. Random samples of worker bees from regular colonies were kept for 2–3 hrs in cages before being used for the trials. After a mild anaesthesia with N_2O they were placed in Petri dishes (15 bees per dish of 150 mm in diameter) containing queen dummies. The number of bees contacting the dummy was recorded in the intervals of 30 secs for a period of 5 mins at room temperature. The final result represents an average of all replications.

The test for supression of queen rearing was conducted according to Škrobal (1974) with small groups of worker bees (30 g, i.e. about 250 bees) kept in cages at 28°C in the dark. Each cage contained food (rape honey and pollen paste), a vial enclosing 25 ml test medium and equipped with a wick to allow direct contact with the bees, and a piece of lightly coloured comb of 20 × 50 mm with the youngest brood (eggs and small larvae) that was attached to the cage ceiling. Worker cells were cut (shortened) on one

side of the comb to 1/3 of their original length. Test mixtures were diluted with ethanol to obtain 0.25 Qeq/25 ml. Assays were evaluated after four days and the substance considered ineffective when building of a queen cell was observed.

Field trials

Worker bees were collected in regular colonies and weighed to provide 1,000 g packages (about 8,000 bees) which were accommodated in pre-fabricated disposable containers (Krieg, 1987). Each container was supplied with standard bee candy and with 1 Qeq of a test sample in 140 ml ethanol. The sample vial equipped with a wick was suspended from the side wall at 2/3 of cage height and close to its rear wall. The control package did not contain the sample vial but was provided with a fertile queen. Established bee packages were kept for 3 days in cold and darkness in a cellar before being used for pollination of cucumbers. Each package was transfered to a separate plastic greenhouse of 180 m^2 at the time of cucumber flowering, usually in the 1st week of July. Changes in package weight, comb building and pollinating activity were evaluated at weekly intervals. The pollinating activity was assessed at 11:00 and 13:00 by counting the number of bees visiting flowers on an area of 10 m^2 during one minute. Greenhouse temperature was also recorded. Packages were used, for a maximum for 50 days and all experiments were performed within the first 30 days. Results obtained in each of the four experimental seasons were evaluated separately.

RESULTS

Retinue response test

Laboratory tests of retinue response were performed as 9 sets of experiments during three bee seasons of 1990–1992. All results were combined and are presented for each kind of tested material in a single figure (Table 1). Most frequent contacts of bees with a pseudoqueen, amounting in average to 27.2 encounters in 5 min, were found using FQ-S extract. A similar value (26.6) was established with the synthetic mixture QP. The filtered extract from reproducing queens, FQ-E, appeared considerably less active (19.5 contacts/5 min), the difference was not significant at 0.01 probability level. In contrast, the extract from virgin queens (VQ-S) was significantly less efficient (15.4 contacts/5 min). Ethanol control with 0.3 contacts per 5 min. revealed the effect of random bee movement in the pseudoqueen area.

TABLE 1. Reaction of worker bees to dummies treated with 10^{-3} queen equivalent of different queen extracts and with synthetic pheromone mixture.

Treatment	Number of bees contacting the pseudoqueen ($\bar{x} \pm$ S.D.)	Number of replications (5 min intervals)
Ethanol	0.3 ± 0.2^a	60
FQ-S	27.2 ± 12.4^b	133
FQ-E	19.5 ± 11.5^c	95
VQ-S	15.4 ± 7.8^c	75
QP	26.6 ± 12.0^b	11

a, b, c Values marked with different letters differ from one another at > 1% probability level.
FQ-S: Soxhlet extract from ovipositing queens stored for one year in 96% ethanol.
FQ-E: Cold filtrate of 96% ethanol extract of ovipositing queens.
VQ-S: Extract from 5-days old virgin queens prepared using the method as employed for FQ-S.
QP: Mixture of five synthetic components of queen mandibular pheromone after Slessor et al. (1988).

Inhibition of queen rearing

Trials were conducted in three seasons but, for un unknown reason, all of those performed in 1991 yielded negative results. Hence, only experiments made in 1990 and 1992

were considered in the final evaluation. Both kinds of extracts from mated queens were maximally effective in inhibiting queen rearing (Table 2). The extract from unmated queens exhibited in 21 replications an average efficiency of 76.2%. The synthetic mixture failed to inhibit the formation of queen cells only in one out of 8 experimental cages, exhibiting thus an average inhibition of 87.5%.

TABLE 2. Inhibition of queen rearing (building of queen cells) in cages.

Treatment *	Number of tests	Number of cages with one or more queen cells	Inhibition %
Ethanol	18	18	0
FQ-S	16	0	100
FQ-E	9	0	100
VQ-S	21	5	76.2
QP	8	1	87.5

* For explanations see Table 1: 0.25 Qeq of extracts and QP was used per cage.

Coherence of pollination packages

Stability of bee packages was assessed from the decrease of bees number after 10 and 30 days (Table 3). Highest coherence of packaged bees was established in 1990 and 1993, when the package size decreased in 10 days to 50–67%, and in 30 days to 30–45% of the original value, and these changes were similar in all experiments. In the two other seasons, the loss of bees was higher, particularly in the package treated with FQ-S in 1991: the average package size was reduced by 68% in 10 days and only 18% of bees were left after 30 days. In all seasons, differences in the loss of bees in packages exposed to various treatments were insignificant. The comb area built in 30 days varied between 5.4 and 1130 cm^2, in accordance with the package size.

TABLE 3. Coherence of bee packages examined 10 and 30 days after their use for cucumber pollination (for details see text).

Treatment * (1 Qeg/package)	Year	Package weight (in g)**		Total comb construction (cm^2)	Number of packages
		10 days	30 days		
Q	1990	500	350	600	2
FQ-S		550	450	1130	3
FQ-E		550	430	1110	6
VQ-S		500	300	750	1
FQ-S	1991	320	180	890	4
VQ-S		500	300	940	5
FQ-S	1992	420	290	770	3
VQ-S		500	290	630	4
QP		350	220	540	3
FQ-S	1993	675	325	710	2
VQ-S		620	350	720	3
QP		650	385	640	4

* For explanation see Table 1; Q – living fertile queen. All differences between treatments are insignificant at 5% probability level.
** Initially 1,000g.

Pollinating activity of packaged bees

In 1990, packages supplied with a fertile queen were employed as standards, to which the packages treated with ethanolic queen extracts were compared. Packages subjected to different treatments exhibited similar pollinating activities at 11:00 and nearly twice as high, but again similar activities at 13:00 (Table 4). At the latter time, packages treated with the extract from virgin queens were significantly more efficient that those treated with the extract from fertile queens. Trials performed in 1991 included 9 packages treated with the extracts from either reproducing or virgin queens. There was no difference in the effect of these two extracts (Table 4). The pollinating activity considerably decreased between 11:00 and 13:00, probably due to the rise of ambient temperature to 38°C. Tests conducted in 1992 included treatments with the extracts as well as with the synthetic components of mandibular pheromone. Packages exposed to the synthetic mixture exhibited similar pollinating activity as those receiving the queen extracts. The number of bees found on the flowers decreased between 11:00 and 13:00 in all experiments, probably due to an increase in the temperature beyond 38°C. Pollinating activity established in 1993 was higher than in any of the preceeding seasons. Packages exposed to the extract from fertile queens or to the synthetic mixture performed clearly better than those treated with the extract from virgin queens; but the difference was significant at 5% level at 13:00 between FQ-S and VQ-S only (Table 4).

TABLE 4. Pollinating activity of worker bee packages (number of visits of cucumber flowers on 10 m^2 in 1 min).

Treatment *	Year	Visit at 11 a.m. ($\bar{x} \pm$ S.D.)	n	Average temperature	Visit at 1 p.m. ($\bar{x} \pm$ S.D.)	n	Average temperature
Q	1990	2.40±2.0	12	27°C	4.75 ± 1.3	10	30°C
FQ-S		2.10±2.2	12		3.62 ± 1.5[a]	10	
FQ-E		1.93±1.5	36		3.83 ± 1.6[a]	30	
VQ-S		2.25±1.7	12		5.40 ± 1.5[b]	10	
FQ-S	1991	1.92±1.2	13	29°C	1.26 ± 1.2	13	38°C
VQ-S		2.52±1.2	23		1.14 ± 1.2	20	
FQ-S	1992	3.77±2.9	13	32°C	1.50 ± 1.7	12	38°C
VQ-S		2.64±2.5	11		1.50 ± 1.2	12	
QP		3.00±2.8	13		1.16 ± 1.4	12	
FQ-S	1993	5.00±2.5	40	23°C	6.64±2.3[c]	14	27°C
VQ-S		3.93±1.8	28		4.85±1.7[d]	20	
QP		4.90±2.6	30		5.60±2.7	23	

* For explanation see Table 1 and 3. Values marked with different letters differ at 5% probability level.

DISCUSSION

Queen substitution is an important task of practical apiculture that requires experimental laboratory approach. Laboratory tests represent an indispensable tool for identifying techniques and compounds that deserve testing in the field. Retinue response, which was employed in this study to assay attractivity for the worker bees, revealed high potency of the extract from fertile queens; the dummies treated with this extract allured, in 10

standard assays performed during 3 seasons, 21 to 30 worker bees. Slessor et al. (1988) found a similar attractiveness (24.7±3.3 contacts) in mandibular gland extracts. They concluded that pheromonal products of the mandibular gland are responsible for the retinue response. However, none of the five major substances isolated from the gland, nor any combination of 2–4 of them, mimicked the action of full gland extract. A synthetic blend of these five components also elicited only a weak retinue response consisting of 17.1 encounters (Slessor et al., 1988). In contrast with this report, in our tests a similar synthetic mixture exerted nearly the same response as the most effective queen extract. We cannot explain this discrepancy between our results and those of Slessor et al. (1988).

Extracts from virgin females proved to be relatively ineffective in the retinue response test and this is consistent with the finding that their mandibular glands contain only low amount of the pheromone (Slessor et al., 1988), but in contradiction to the report that attractiveness of 5–6 days old virgin queens is similar to that of fertile queens 16–18 months old (de Hazan et al., 1989). It cannot be excluded that some attractants released by virgin queens, such as secretion of the tergal glands (de Hazan et al., 1989), were missing in our extracts. No additional attractants were needed, however, when mandibular gland pheromone was present in sufficient quantity; our observations proved that the synthetic pheromonal mixture is equally efficient as whole-queen extracts.

Kaminski et al. (1990) established effective concentrations of individual components of the mandibular gland pheromone. They also examined effects of age and physiological state of bees on their response to the pheromone, and found that bee sensitivity is independent of their age but is influenced by the size and state of the bee colony from which the experimental bees are derived. In addition sensitivity to the pheromone fluctuates during season and varies among individual bee colonies. To eliminate these variations, we kept our experimental conditions as constant as possible and based all comparisons on parallel tests. Consistently, we used anaesthesia by N_2O instead of CO_2 which was shown to affect bee behaviour (Kaminski et al., 1990).

Extracts from fertile queens were highly effective in inhibiting queen rearing, while the extract from virgin queens failed in a quarter of the cases. Škrobal (1974) also reported that extracts from three days-old queens were insufficiently active in this test. Synthetic mixture surpassed in activity the extract from virgin queens but did not fully match the efficiency of the extract from fertile queens. The effect of synthetic mandibular gland pheromone on queen-cell building was examined by Winston et al. (1989). During a 10 day-long investigation period they found a partial suppression but not a complete inhibition of queen-cell construction. However, in another paper, Winston et al. (1990) demonstrated suppression of queen rearing within 6 days after exposing a colony to 1 Qeq of mandibular pheromone; synthetic pheromone mixture was applied on a glass slide that was replaced daily. The slide was in direct contact with the bees. Similarly, in the present tests as well as in the previous field experiments (Krieg, 1987), administered materials could be perceived olfactorially, perorally, or through the body surface, and their supply was continuous. Despite of this, synthetic pheromone mixture caused only an incomplete inhibition of queen rearing.

We interpret this observation and the report that the synthetic mixture of mandibular gland pheromone fails to block the activation of worker ovaries (Willis et al., 1990) as

indicating that mandibular gland pheromone is not the sole supressor of queen replacement in queenless colonies.

Trials with pollinating bee packages in plastic greenhouses confirmed that queens can be substituted with FQ-S extract (Krieg, 1987) and demonstrated that the extract from virgin queens is also suitable, in spite of its low activity in the laboratory tests. Five days old queens are readily available and the cost of their production is low. The extracts from such queens can also be used for collecting drones as suggested by Williams (1987) and for stabilizing bee packages during transport (Naumann et al., 1990). Their most important application, however, remains the queen substitution in incomplete and disposable packages designated for pollination in closed environments. It is important to note that the polinating activity of packages examined in this study was not strictly correlated with the package size and that considerable losses of bees were tolerated. Owing to extreme conditions inside plastic greenhouses (high temperature, limited flight space) packages suffered greatest loss of bees during the first two days. Subsequent decline was slow, and the polinating activity remained satisfactory, unless temperature rose to extreme values. In 1991 and 1992 trials, pollinating activity was clearly reduced when ambient temperature rose to 38°C.

Our data indicate that the queen can also be replaced by the synthetic mandibular gland pheromone. Such a treatment is highly reproducible and inexpensive and our results justify its practical exploatation.

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