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SUPPRESSION OF OVARY DEVELOPMENT OF WORKER HONEYBEES BY ASSOCIATION WITH WORKERS TREATED WITH CARBON DIOXIDE[1]

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Summary

Newly emerged worker honeybees (*Apis mellifera* L.) aged less than 24 h were placed into queenless cages and given a section of empty drone comb and unlimited supplies of honey, water and pollen. Cages were kept in an incubator at 34 ± 1°C. Workers treated with a 15-min exposure to CO_2 at 0-3 days of age produced significantly fewer eggs than controls that received no CO_2. Workers in queenless cages for 4–6 days before CO_2 narcosis had egg production equal to that of controls. Thus CO_2 had no apparent effect after the workers had been queenless for 4 days. In a second study, 5 untreated workers shared cages with 25 sisters that had been treated with three 15–min doses of CO_2. Association with the treated workers retarded ovary development of the untreated cagemates when compared to sister bees in identical cages that contained no bees given CO_2. Cages with treated workers consumed only 17% as much pollen as controls. So low pollen consumption may have reduced the total amount of protein that was available and shared within the caged population. Since oogenesis requires protein, a lack of protein may have retarded ovary development.

Introduction

Both worker and queen honeybees (*Apis mellifera* L.) are capable of producing eggs. Under normal conditions, the queen lays all the eggs in a colony, and the ovaries of the workers remain undeveloped. The development of worker ovaries is inhibited by the presence of a queen or the presence of brood (Jay, 1968, 1970, 1972). When the queen and brood are removed, many workers begin to develop their ovaries and lay eggs. Hess (1942) reported that 80–90% of the worker bees develop their ovaries in a queenless colony.

Although both worker and queen bees can lay eggs, their reproductive systems respond differently to carbon dioxide. Exposures to CO_2 (1–10 min) and placement into honeybee colonies stimulate egg-laying in young, unmated queens (Mackensen, 1947; Engels et al., 1976). In contrast, ovary development of worker bees is inhibited by exposure to CO_2 (Fyg, 1950; Biedermann, 1964; Kropacova et al., 1968).

Although worker bees can produce eggs, chosen worker honeybees are almost never used in honeybee breeding. Heretofore, egg production from selected worker bees has been demonstrated only with the Cape bee (*Apis mellifera capensis)* (Moritz & Klepsch, 1985; Velthuis & van der Kerk, 1988; Hillesheim et al., 1989). However, the Cape bee is unique in that 15–20% of the workers have developed ovaries in the presence of a queen (Anderson, 1963), and the unmated workers produce diploid (female) rather than haploid (male) eggs (Verma & Ruttner, 1983).

In this study, we explored the use of CO_2 narcosis to control oviposition within groups of worker bees. Our objectices were (1) to determine worker age at which CO_2 has the greatest inhibitory effect and (2) to use CO_2 to selectively inhibit ovary development in queenless groups of worker honeybees so that all of the eggs would be produced by one or more untreated workers in the group.

Our immediate goal is to produce eggs from selected worker bees. By producing drones (and sperm) from these eggs, we hope to expand bee breeding to include the direct selection of worker bees. Although some of the most important qualities of honeybees are group traits such as honey production and defence behaviour, which need to be measured at the colony level, many important traits such as disease resistance, longevity, and development time are measurable using single worker bees. Breeding from workers would not displace breeding

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from queens, but it would give a bee breeder more options, and it may provide the precision needed to detect and propagate genetic combinations that occur at very low frequencies.

Materials and Methods

General procedures

Groups of newly emerged bees (< 24 h old) were placed into cages in an incubator (34 ± 1°C and *c.* 50% RH). Each cage has a two-sided section of empty drone comb (5 × 8 cm) suspended in the centre of the cage, and a supply of honey, water, and pollen. Honey and water were supplied using inverted vials with holes drilled in their plastic caps. Pollen was supplied in a vial cap placed on the floor of each cage. The pollen has been trapped from three healthy colonies during the spring of 1989 and was kept frozen in a sealed container. The cages used in these experiments were like those used by Kulincevic and Rothenbuhler (1973) and measured 7·5 × 11 × 12·5 cm.

Carbon dioxide gas was administered at room temperature (*c.* 23°C) by placing the cages in a clear plastic bag (66 × 77 cm when flattened; non-pleated edges), and filling the bag with CO_2 at a flow rate of 10·5 l/min for 3–5 min. Timing of the 15-min exposure began when the first bees fell to the bottom of their cage. After the bag was fully expanded, a flow of *c.* 0·3 l/min was maintained to keep positive pressure during the 15-min treatment period.

Experiment 1

This experiment measured the effect of CO_2 when young worker bees had been queenless for different lengths of time. Experiment 1*a* began 27 April 1989 and ended 6 May 1989. Experiment 1*b* began 16 June 1989 and ended 30 June 1989.

We obtained honeybee workers by allowing them to emerge in an incubator from brood combs removed from a single colony. Their mother was a naturally mated queen. Twenty five of these workers (< 24 h old) were placed into each of 40 cages which were randomly assigned to one of four treatments in a completely randomized design. In experiment 1*a*, treatments were CO_2 when the bees were ≤ 1 day old, 2–3 days old, 4–5 days old, or no CO_2; in 1*b*, treatments were CO_2 when bees were 1–2 days old, 3–4 days old, 5–6 days old, or no CO_2. Each treatment contained 10 replicates.

The treatment groups were compared in terms of number of eggs produced and start of egg laying. Both egg production and worker mortality were compared among treatments using a Kruskal–Wallis non-parametric analysis of variance for ranks (Kruskal & Wallis, 1952). This non–parametric comparison was used to accommodate the frequent number of zeros that violated restrictions necessary for parametric analysis of variance. The Kruskal–Wallis Test was corrected for tied ranks. A non-parametric Tukey-type multiple comparison (Wilcoxon & Wilcox, 1964) was used for mean separation analysis after significant differences were indicated by the Kruskal–Wallis Test.

Experiment 2

The experiment attempted to produce eggs from selected workers by inhibiting the ovary development of other workers in cages. All workers emerged in an incubator from brood combs removed from a colony headed by a naturally mated queen. On 7 April 1989, twenty five bees (< 24 h old) were placed into each of 33 incubator cages. Thirty of these cages were provided a section of drone comb and a continuous supply of honey and water but no pollen during the initial three days when they were treated with CO_2. Bees in the remaining 3 cages differed from those above only in that the bees were provided with pollen and were marked on the thorax with paint.

The 30 cages were divided into three treatments: no CO_2, a single 15-min exposure to CO_2, or a series of three 15-min exposures to CO_2 (10 cages/treatment). Workers in the triple narcosis group received a 15-min CO_2 narcosis at 24-h intervals, starting immediately after the cages where stocked with bees. Workers in the single CO_2 narcosis group were given a 15-min exposure on the third day.

On the third day (after the treated workers had recovered from their last narcosis), a m[illegible] worker from each of the three pollen-fed cages was put into each of the 30 treatment [illegible] Thus, each cage contained 3 marked workers and 25 unmarked workers. Pollen was th[illegible]

supplied to all experimental cages, and the cages were maintained for 17 more days. Egg production and mortality were recorded at 24-h intervals. On the final day, all workers were frozen and remained so until their ovary development was evaluated.

The ovaries of all marked workers and a sample of 5 unmarked workers were examined from each cage. The extent of ovary development was evaluated using a grading system described by Velthuis (1970): class I ovaries were undeveloped with undifferentiated ovarioles, class II ovaries were slightly developed with rounded to bean-shaped eggs, and class III ovaries were fully developed with sausage-shaped eggs.

Treatments were compared in terms of the ovary development of the unmarked workers, and each unmarked worker was assigned the value of its greatest developed ovariole (I, II, III). Cage scores (the sum of 5 workers) were compared between treatments using the Kruskal-Wallis non-parametric analysis of variance. A non-parametric Tukey-type multiple comparison was used for mean separation analysis.

The different treatments were also compared in terms of the number of ovarioles per bee for marked and unmarked workers. The average number of ovarioles per worker was analyzed using a hierarchial analysis of variance (Proc GLM; *SAS Institute*, 1985) with the cage factor nested within treatment.

The treatment groups were also compared in terms of the numbers of dead marked and unmarked workers. The total numbers of dead workers were compared among treatments using a non-parametric analysis of variance and a non-parametric Tukey-type mean separation.

Experiment 3

This experiment tested the effects of CO_2 narcoses on 25 treated and 5 untreated bees within a cage. All worker bees came from the colony used in experiment 2. Cages were given bees that received no CO_2 (15 cages) or three, 15-min doses of CO_2 at 24-h intervals beginning immediately after the cages were stocked (25 cages). Cages were established as in experiment 2. No pollen was provided until the marked workers were added. However, the marked workers were three days younger than the unmarked workers and were added to the cages after the unmarked bees had recovered from their last CO_2 treatment.

All cages were held in an incubator for 10 days after introduction of the marked workers; egg production and mortality were recorded at 24-h intervals. Pollen consumption was measured each time fresh pollen was given to a cage. On the final day, all marked workers were removed from the cages and frozen until their ovary development could be evaluated. The unmarked workers were maintained in the incubator for an additional 24h to detect egg production by them.

The two treatment groups were compared in terms of the ovary development of marked workers. Marked workers were assigned the value of their most developed ovary (I, II or III), and the cage scores (the sum of 5 workers) were compared using a Wilcoxon-Mann-Whitney two-sample test (Mann & Whitney, 1947). Treatments were also compared in terms of the number of ovarioles per marked worker using a hierarchial ANOVA test as in experiment 2.

The two treatments were also compared in terms of the number of cages that produced eggs before and after removal of the marked workers. A Chi-squared value and Fisher's exact probability were used to analyse the 2×2 contingency tables (Proc FREQ; *SAS Institute*, 1985) formed by considering the two treatments of unmarked workers (no CO_2; 3 doses of CO_2) as rows and the classifications, 'eggs' versus 'no eggs', as columns. 'Eggs' and 'no eggs' denoted cages producing at least 1 egg and cages producing no eggs, respectively.

Treatments were also compared in terms of marked and unmarked worker mortalities. The numbers of dead marked or unmarked workers per cage were compared for the cages in which workers had died. The comparison was made using Student's *t*-Test (Proc TTEST; *SAS Institute*, 1985). The number of cages with dead workers and the number of cages with no dead workers were compared between the two treatments using a Chi-squared value and a Fisher's exact probability for the 2×2 contingency tables.

Pollen consumption was also compared between cages from the two treatments. The amount of pollen consumed between inspection periods was determined by subtraction from the initial amount given to a cage. Each cage was fitted with a card beneath the wire mesh floor, and this card served to catch pollen dropped by the bees. Any pollen recovered from the cards was added back to the vial cap before weighing. Total pollen consumed through 10 days was compared using Student's *t*-Test.

TABLE 1. Effect of CO_2 narcosis on worker bees in an incubator for 10 days (Experiment 1*a*) or 14 days (Experiment 1*b*). Twenty-five bees aged 0–1 day were put into each of 40 cages and given one, 15-min CO_2 narcosis immediately or up to 6 days later (controls received none).

Age at narcosis (days)	*No. of cages having eggs*	*Onset of egg-laying (days)*[1]	*No. of eggs (rank sum)*[1]	*No. of live bees at end*[2] *(rank sum)*
		Experiment 1*a*		
0–1	2	10 ± 0	8 ± 9 *b* (85·5)	24·7 ± 0·5 *a* (246·5)
2–3	6	9 ± 1	9 ± 9 *b* (131·5)	24·9 ± 0·3 *a* (275·5)
4–5	10	8 ± 1	62 ± 30 *a* (289·5)	23·7 ± 1·7 *a,b* (186·5)
controls	10	8 ± 1	72 ± 42 *a* (313·5)	22·2 ± 2·4 *b* (111·5)
		Experiment 1*b*		
1—2 d	1	13	67 *c* (83)	24·8 ± 0·4 (252)
3–4	9	12 ± 2	15 ± 19 *b,c* (165)	24·4 ± 1·1 (221·5)
5–6	9	10 ± 1	102 ± 101 *a,b* (266)	24·6 ± 0·5 (219)
controls	10	9 ± 2	107 ± 76 *a* (306)	22·8 ± 2·4 (127·5)

[1] Means (± SD) were calculated only from cages that produced eggs; rank sums were based on all 40 cages in each experiment.
[2] Data in a column having the same letter do not differ significantly at the $\alpha = 0{\cdot}05$ level (based on rank sums analysis and a non-parametric Tukey-type multiple comparison; 10 cages/treatment).

Results

Experiment 1

A 15-min treatment with carbon dioxide reduced ovary development of newly emerged worker bees only when it was given during the first four days (Table 1). In experiment 1*a*, the Kruskal–Wallis Test indicated significant differences in egg production ($H_c = 29{\cdot}0$; $df = 3$; $P < 0{\cdot}001$) between the treatment groups (note: the H_c value is analogous to a parametric ANOVA F value). Experiment 1*b* produced similar results ($H_c = 22{\cdot}7$; $df = 3$; $P < 0{\cdot}001$). The critical values of the Tukey-type non-parametric comparisons of rank sums between treatment groups were the same for both experiments (SE = 37·0; q [$\alpha = 0{\cdot}05$; $df = \infty,4$] = 3·633). A minimum difference of 131·4 between rank sums indicated significant differences at the $\alpha = 0{\cdot}05$ level.

The treatment groups also differed in terms of the number of live bees at the end of the experiment (Table 1). In experiment 1*a*, $H_c = 14{\cdot}3$ ($df = 3$; $0{\cdot}001 < P < 0{\cdot}005$). In experiment 1*b*, $H_c = 8{\cdot}2$ ($df = 3$; $0{\cdot}025 < P < 0{\cdot}05$); however, the differences were not significant enough to be separated using the Tukey-type multiple comparison.

Experiment 2

Carbon dioxide narcosis reduced ovary development in treated workers. Ovaries in workers given either one or three doses of CO_2 were significantly less developed than the ovaries in workers receiving none ($H_c = 21{\cdot}33$; $df = 2$; $P < 0{\cdot}005$) (Table 2). Although six of the workers that received a single narcosis developed their ovaries, and none of the workers sampled from the triple narcosis group had developed ovaries, the two groups were not significantly different (SE = 27·84; $q[\alpha = 0{\cdot}05$; $df = \infty,3] = 3{\cdot}314$). Treatments were not statistically compared in terms of the ovary development of the marked workers because their

TABLE 2. The effect of CO_2 on 25 narcotized bees and 3 untreated bees that shared the same cage in an incubator for 20 days (Experiment 2). Treated bees were given a single, 15-min dose or a series of three, 15-min doses of CO_2 (controls received none).

Treatment of the 25 workers[1]	*Cage scores (rank sums)*[2]	*Treated Workers, number of bees in each class*[3]			*Untreated Workers*[4]*, number of ovaries in each class*[3]		
		I	*II*	*III*	*I*	*II*	*III*
controls	8·1 ± 1·5 *a* (248·5)	27	15	8	18	5	2
1 dose of CO_2	5·6 ± 0·7 *b* (136·5)	44	6	0	13	3	3
3 doses of CO_2	5·0 ± 0·0 *b* (80·0)	50	0	0	20	1	1

[1] Each treatment had 10 cages.
[2] The means (mean ± SD) for cage scores (sum of the 5 untreated worker scores) with the same letter were not significantly different at the $\alpha = 0{\cdot}05$ level (based on rank sums analysis and a non-parametric Tukey-type multiple comparison).
[3] Class I = undeveloped ovaries; class II = ovaries with rounded to bean-shaped eggs; and class III = fully developed ovaries with mature eggs. Classification of an ovary was based on the most developed ovariole.
[4] Untreated workers were not statistically compared because of small and unequal sample sizes among cages.

numbers were small (≤ 30), and some died during the experiment. The numbers of marked bees in each of the three classes of development for the three treatments are tabulated in Table 2. Fewer marked bees from the triple narcosis group had developed ovaries (class II or III), and this result is comparable to experiment 3.

Egg production was also influenced by CO_2. No eggs were produced by untreated or treated workers in cages with workers that received any CO_2. Seven of the 10 control cages produced an average of 27 ± 17 eggs within 20 days. They began producing eggs after 13 ± 2·4 days (mean ± SD).

Treatment with CO_2 increased the mortality of the workers ($H_c = 11{\cdot}96$; $df = 2$; $0{\cdot}001 < P < 0{\cdot}005$). Workers given three doses of CO_2 had significantly higher mortality per cage (10·5 ± 5·2, $n = 10$) than workers given one dose of CO_2 (4·3 ± 3·1, $n = 10$) or untreated workers from control cages (3·0 ± 1·6, $n = 10$) (SE = 27·84; q [$\alpha = 0{\cdot}05$; $df = \infty$, 3] = 3·314). The latter two groups were not different. Mortality of marked workers was not reduced by association with workers that had been treated with CO_2 ($H_c = 2{\cdot}54$; $df = 2$; $P > 0{\cdot}25$).

Experiment 3

Egg production differed between the two treatments. Cages did not contain eggs if some of the workers had been treated with CO_2, but all control cages had eggs (Chi-squared = 40·0; $df = 1$; $P < 0{\cdot}001$; Fisher's exact $Pr = 2{\cdot}5 \times 10^{-11}$). Bees in the control cages began laying eggs after an average of 9·7 ± 0·7 days, and they had produced an average total of 90 ± 58 eggs after 13 days (mean ± SD).

Ovary development for the marked workers was higher when they were caged with untreated workers than when caged with workers treated with CO_2 ($U = 19{\cdot}5$; $U' = 265{\cdot}5$; $U_{\infty(2),\ 15,\ 19} = 200$; $P < 0{\cdot}001$)(Table 3). Each of six of the cages from the triple narcosis group lost one marked worker, hence, these cage scores were not used in the Wilcoxon-Mann-Whitney Test. The two treatments did not differ in worker mortality or ovariole number. Marked workers in control cages had 7·6 ± 2·9 ovarioles, and marked workers in cages with workers treated with CO_2 had 7·1 ± 2·0 ovarioles ($F = 1{\cdot}58$; $df = 1{\cdot}38$; $P = 0{\cdot}2158$).

Pollen consumption was significantly reduced in cages containing workers that had been given CO_2 (Student's $t = 9{\cdot}42$; $df = 15{\cdot}7$ [unequal variances]; $P = 0{\cdot}0001$). The bees in these cages ate 17% as much pollen as the controls (Table 3).

Discussion

To explain the reduction in the ovary development of untreated workers, the role of

TABLE 3. Ovary development of untreated bees that shared a cage with bees treated with CO_2. Each cage was kept in an incubator for 10 days and consisted of 25 treated and 5 untreated worker bees (Experiment 3).

Treatment of the 25 bees	*No. of cages*	*Cage scores (rank sums)*[1]	*Numbers of untreated bees in each class*			*Pollen consumed (g/cage)*[2]
			I	*II*	*III*	
no CO_2	15	9·1 ± 1·8 (385·5)	34	19	22	1·9 ± 0·6
3 doses of CO_2	19	5·7 ± 1·1 (209·5)	83	9	3	0·3 ± 0·2

[1]Mean (± SD) cage scores (sum of the 5 untreated workers per cage) were significantly different ($P < 0·05$; based on the Wilcoxon-Mann-Whitney Test).
[2] Pollen consumption was significantly different between the two treatment groups ($P = 0·0001$).

trophallaxis in worker oogenesis might be considered. Korst & Velthuis (1982; see also Velthuis, 1985) provided evidence that trophallactic and reproductive dominance are related in small groups of queenless workers: worker bees *receiving* the most food in trophallaxis have the least amount of pollen in the hindgut and the best developed ovaries, while worker bees *giving* the most food in trophallaxis often have hindguts swollen with pollen and the least developed ovaries. Because exposure to CO_2 significantly reduced the pollen consumption of workers, there was less protein available to be shared within the caged population. This low level of protein may have reduced ovary development in the 3 or 5 untreated, marked workers.

The data suggest that CO_2 does not inhibit worker ovaries that have already begun to develop. Young bees that were queenless for at least 5 days prior to receiving a 15-min narcosis produced as many eggs as untreated workers. Egg production was significantly lower when bees were queenless $\leq$ 3 days before narcosis. In our experiment, worker age and extent of ovary development were confounded because workers were made queenless on the day they emerged from the combs. So, was the effect of CO_2 limited to young workers or limited by the level of ovary development at the time of narcosis? Data from Fyg (1950) indicated that it was not limited to young workers. He showed that CO_2 narcosis inhibited ovary development of workers that were randomly chosen from field colonies (bees of all ages).

Therefore, CO^2 narcosis may yet be a useful tool in manipulating the oviposition of worker bees. Our data indicated that CO_2 narcosis reduced pollen feeding. Less pollen feeding may have caused a drop in the level of protein among the workers in a cage, and lack of protein may retard ovary development. It seems possible that if older bees were used as attendants (bees that were already protein rich and actively feeding a queen and larvae), CO_2 narcosis would inhibit the ovaries of such workers but a high level of protein may remain.

In our study, CO_2 was not a practical technique for manipulating the oviposition of a group of young worker bees. Other techniques have been more successful for us. These include (1) replacing attendants at 5-day intervals (Harris & Harbo, 1988); (2) using a slow-ovary-developing stock as attendants; and (3) allowing a select worker to develop her ovaries within a group and then transferring her to a cage with newly emerged bees (Harris, 1990).

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