Facultative expression of hygienic behaviour of honey bees in relation to disease resistance¹

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SUMMARY

Four experiments were conducted to examine factors that influence the expression of hygienic and non-hygienic behaviour in honey bees, *Apis mellifera*, and to examine the correlation between this behaviour and resistance to chalkbrood, *Ascosphaera apis*. Colonies were headed by instrumentally inseminated queens selected on the basis of uncapping and removal behaviour expressed by their progeny. In the first experiment, colony strength was altered by transferring hygienic and non-hygienic colonies from 10-frame field hives to 2-frame observation hives. This treatment significantly reduced the hygienic response of the hygienic bees but did not affect the response of the non-hygienic bees. In the second experiment, hygienic and non-hygienic bees displayed different responses to freeze-killed and live brood which had been partially or entirely uncapped. Both lines of bees recapped both partially and entirely uncapped live brood, but non-hygienic bees also recapped partially uncapped freeze-killed brood, suggesting that non-hygienic bees either could not detect dead or diseased brood or avoided it by sealing it within a comb cell. The third experiment tested whether the degree of hygienic behaviour could be increased by adding hygienic bees to non-hygienic colonies. Adding 20–30% young hygienic bees to non-hygienic colonies did not increase the degree of hygienic behaviour, but adding young non-hygienic bees to hygienic colonies suppressed the behaviour. The results suggest that although hygienic behavior is genetically determined, its expression depends on colony strength and composition of workers within the colony. In the fourth experiment, the hygienic and non-hygienic colonies were fed with pollen patties containing *A. apis* spores. The weak correspondence that was observed between removal behaviour and physiological resistance to chalkbrood suggested that few colonies are both highly hygienic and physiologically resistant to chalkbrood. Selection against uncapping and removing diseased brood might occur if this behaviour also promotes the spread of disease through the colony. This possibility is discussed in relation to avoidance behaviour of other social insects toward pathogens.

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INTRODUCTION

A classic study in animal behaviour involved nest-cleaning, or hygienic behaviour, by workers of the honey bee, *Apis mellifera* (Rothenbuhler, 1964a). Hygienic behaviour is considered the primary mechanism of resistance to at least two diseases deleterious to larval honey bees, American foulbrood (AFB) caused by the bacterium *Bacillus larvae* (Rothenbuhler, 1964a), and chalkbrood caused by the fungus *Ascosphaera apis* (Gilliam *et al.*, 1988). This behaviour is believed to be controlled by two independently assorting, recessive genes: one for uncapping and one for removing diseased brood from the nest (Rothenbuhler, 1964a; but see Moritz, 1988). In the case of AFB, resistance is conferred by hygienic bees detecting, uncapping, and removing diseased brood from the nest before the causative organism reaches the infectious sporulating stage (Woodrow & Holst, 1942; Woodrow & States, 1943). Secondary mechanisms of resistance to AFB include the ability of some adult bees to physiologically filter ingested *B. larvae* spores from the proventriculus (Sturtevant & Revell, 1953) and the presence of bacterial inhibitors in the brood food fed to larvae by adult bees (Thompson & Rothenbuhler, 1957; Rose & Briggs, 1969). In addition to hygienic behaviour, a secondary mechanism of resistance to chalkbrood currently being investigated involves naturally occurring antimycotic substances, antagonistic to the chalkbrood fungus, that are found in microorganisms from stored pollen (bee bread) and from the guts of nurse bees (Gilliam *et al.*, 1988; M Gilliam and S Taber, unpublished data).

An interesting aspect of hygienic behaviour is that it provides a rare example of a behavioural mechanism of disease resistance. Most theories on the evolution of pathogen (and parasite) resistance involve hypotheses on the coevolutionary race between specific strains of disease organisms and physiologically resistant hosts (Hamilton, 1980, 1982; Tooby, 1982; Rice, 1983). These hypotheses have been linked to the evolution of sex (e.g. Hamilton, 1980; Tooby, 1982) and, in the social Hymenoptera, to the evolution of polyandry (Sherman *et al.*, 1988). Polyandry increases genetic variation within colonies of social insects, which may reduce the likelihood that pathogens or parasites will negatively impact the colony's survival (Sherman *et al.*, 1988; but see also Crozier & Page, 1985; Page, 1986). Hygienic behaviour may provide a generalized behavioural response to remove pathogens and parasites from the nest, minimizing the adaptation of specific pathogens to the genotypes of individual workers.

We originally designed the present study to investigate methods of controlling chalkbrood. We wished to test whether the addition of hygienic bees to non-hygienic colonies in observation hives would increase the degree of hygienic behaviour of the

colony, and then to determine whether chalkbrood could be controlled by introducing hygienic bees to a colony infected with chalkbrood in the field. Previous studies of AFB with colonies composed of 50% hygienic bees in observation hives demonstrated that diseased or cyanide-killed brood was removed from the nest but only when the hygienic bees were young or, if they were over four weeks old, only during a nectar flow (Thompson, 1964; Trump *et al.*, 1967; Momot & Rothenbuhler, 1971). A similar study on chalkbrood has not been conducted. We also aimed to investigate further the correlation between hygienic behaviour and resistance to the chalkbrood pathogen, to gain insight into the question of why some colonies display only a weak correlation between hygienic behaviour and resistance to chalkbrood.

While setting up the experiments, we made observations which prompted additional questions and experiments related to the nature and expression of both hygienic and non-hygienic behaviour. Although hygienic behaviour is genetically controlled, the expression of the trait is dependent on environmental and colony conditions. It is known that resource conditions affect the expression of hygienic behaviour (Borchers, 1964; Mourer, 1964; Momot & Rothenbuhler, 1971), but how does colony strength affect its expression? Secondly, are there costs to hygienic behaviour in some contexts that lead to selection against uncapping and removing diseased brood? One explanation suggests that the cost of hygienic behaviour could exceed its benefit if the bees tend to remove healthy brood from the nest along with the diseased brood (Seeley, 1985). Another explanation, however, may be that uncapping and removing behaviours are not always appropriate responses to the presence of diseased brood in the nest, particularly if the bees perform the behaviours after the pathogen in the brood has reached the infectious sporulating stage. In some cases, an alternative response might entail avoidance of diseased brood rather than contact with it.

To address these questions, we conducted the following four experiments. First, we tested whether the expression of hygienic behaviour was altered by reducing colony strength. Strong, populous colonies are generally under less environmental stress than weak colonies and therefore would be expected to display rapid hygienic behaviour in a higher frequency. Second, we tested whether hygienic and non-hygienic bees displayed different responses to freeze-killed and live brood which had been partially or entirely uncapped. If hygienic bees tend to remove both healthy and diseased brood, they would be expected to remove both live and dead uncapped brood more frequently than non-hygienic bees. If non-hygienic behaviour is a mechanism to avoid dead or diseased brood or if non-hygienic bees are not able to recognize dead or diseased brood, they should respond to uncapped dead

brood by recapping it. In the third experiment, we tested whether the degree of hygienic behaviour (and ultimately the resistance to chalkbrood) could be increased by adding hygienic bees to non-hygienic colonies. In the fourth experiment, we challenged the hygienic and non-hygienic colonies with the chalkbrood pathogen to examine the correlation between hygienic behaviour and disease resistance.

MATERIALS AND METHODS

A quick assay for testing the hygienic behaviour of honey bees has been developed and refined to study resistance to chalkbrood (Cosenza & Silva, 1972; Taber, 1982; Gilliam *et al.*, 1983). This assay differs from those used in previous studies of AFB (e.g. Rothenbuhler, 1964a, 1964b) because freeze-killed brood is presented to the bees rather than larvae inoculated with the pathogen. In studies of the relation between hygienic behaviour and chalkbrood, most, but not all, colonies showed a good correlation between uncapping and removal of freeze-killed brood and resistance to the fungus (Milne, 1983a; Gilliam *et al.*, 1988). Hygienic behaviour is quantified by recording the time required for the bees to uncapped and remove the freeze-killed brood. Sections of comb containing approximately 200 cells of sealed brood are frozen at -20°C for 24 h and then inserted into the brood nest of the colony to be tested. For each colony, the freeze-killed brood inserts are placed in the centre of the brood nest. Bees from a naturally mated queen that remove the freeze-killed brood within 48 h are considered hygienic; bees that take over a week to remove the dead brood are considered non-hygienic (Taber & Gilliam, 1987).

The experiments were conducted at the USDA-ARS Carl Hayden Bee Research Center, Arizona, USA, from June to September 1991. To establish hygienic lines of bees, daughter queens were reared in March and April 1991 from two of the colonies that showed rapid hygienic behaviour in the freeze-killed brood assay in a preliminary survey (Spivak & Gilliam, 1991). Each virgin queen was instrumentally inseminated with 3–4 μl of mixed semen from drones from three different drone mother colonies that also displayed rapid hygienic behaviour. The same procedure was used to develop the non-hygienic lines: daughter queens were reared from two of the colonies that did not display hygienic behaviour (the freeze-killed brood was not removed after seven days) in the preliminary survey. The queens were inseminated with 3–4 μl of semen from drones from three different non-hygienic drone mother colonies.

Six to eight weeks after the daughter queens began laying eggs (when all the progeny was from the new queens), the hygienic behaviour was tested and quantified for each of the daughter colonies by again calculating the percentage of freeze-killed brood removed at 48 h and at seven days after the comb

insert was placed in the colony. Each colony was hived in one standard 10-frame box that contained 5–7 frames of brood and 3–5 frames of nectar and pollen stores. Of the 26 total daughter colonies, seven of the most hygienic and eight of the most non-hygienic colonies were chosen for the experiments. In addition, one feral colony (with a naturally mated queen) that displayed rapid hygienic behaviour was used as one of the selected hygienic colonies, bringing the sample size of hygienic colonies to eight. The feral colony was obtained from a swarm captured in April 1991 in an area north of Tucson (Tunnel Ranch) that was relatively isolated from managed colonies of honey bees.

Experiment 1

Sets of two hygienic and two non-hygienic colonies were tested at a time, first in full-size colonies in the field and next in observation hives. The first set was tested beginning on 15 June, the second on 3 July, the third on 25 August, and the last on 2 September. The bees brought in little nectar and pollen during the time of the tests, although all colonies had ample stores of nectar and pollen from the mesquite (*Prosopis velutina*) and wild flower blooms in April and May (see Moffett *et al.*, 1981; O'Neal & Waller, 1984). The hygienic behaviour was quantified using the freeze-killed brood assay.

After the colonies were tested in the field, one brood frame containing mostly unsealed brood and pollen, one frame containing nectar and honey, the queen, and the adhering bees (presumably of all ages) were removed from each colony and transferred to individual two-frame observation hives. The brood frame contained mostly eggs and larvae so that few if any young bees would emerge during the experiment. The observation hives were placed in an airconditioned greenhouse kept at 25°C . The entrances of the observation hives opened to the outside so the bees could forage freely. The walls of the hives were constructed of Plexiglas fitted with numerous 6.5-cm circular doors that could be opened to observe the bees with minimal disturbance. The number of bees in the observation hives was estimated to be approximately 3 000–3 500 bees by averaging the number of bees under the doors, measuring the area occupied by the bees in the observation hive, and extrapolating how many bees occupied the total area.

Two days after establishing the observation hives, the hygienic behaviour of the colonies was quantified using the freeze-killed brood assay. For each colony, the freeze-killed brood inserts were placed in the centre of the brood comb at 07.00 h, and the number of cells that were uncapped and had the cell contents removed were recorded at 10.00, 13.00, and 16.00 h for two days and at 07.00 h on the morning of the third day (48 h after the comb was

inserted). The percentage of uncapped cells was calculated by dividing the number of cells uncapped in the comb insert by the total number of capped cells remaining at each observation. The percentage of freeze-killed brood that had been removed at each observation was calculated by dividing the number of empty cells at each observation by the total number of sealed cells in the original insert.

The percentage of cells from which the freeze-killed brood was removed at 48 h by the eight hygienic and eight non-hygienic colonies in full-size hives versus observation hives was arcsine transformed and analysed using paired comparison *t*-tests (Wilkinson, 1989).

Experiment 2

Following experiment 1, the freeze-killed brood inserts were removed, and two sections of comb (comb inserts) containing approximately 100 cells each were then presented simultaneously to each hygienic and non-hygienic colony in an observation hive. One insert contained live sealed brood (removed from an unrelated colony of disease-free bees within one hour before performing the experiment), and the other contained freeze-killed brood of the same age from a different, unrelated colony.

In the first trial, holes were made in the wax capping of each cell using the end of a paper-clip to partially expose the live and dead brood. Care was taken not to pierce the live or freeze-killed brood under the capping. The inserts were placed adjacent to each other in the centre brood comb of the colonies and left for 24 h. The numbers of cells containing live or freeze-killed brood that had been recapped or removed were counted after 24 h.

In the second trial, conducted two days after the end of the first trial, a second set of comb sections was prepared. In these, the entire wax capping was removed from each cell in the inserts to expose completely the live or freeze-killed brood. The same procedure was followed to count the number of cells that had been recapped or removed after 24 h.

The results of the experiments were analysed separately using split-plot, nested analyses of variance, and the means were separated by least significant differences (G V Richardson, personal communication).

Experiment 3

The four hygienic (which included the feral colony) and four non-hygienic colonies that had been placed in observation hives in June and July were tested in this experiment. Immediately after the second experiment (eight days after placing them in observation hives), frames of sealed brood that remained in the full-size hygienic and non-hygienic hives in the field

were placed in an environmentally controlled room held at 32–34°C and 75% RH while new bees emerged. The teneral bees were marked with a dot of enamel paint on the thorax to indicate whether they were from a hygienic or non-hygienic line and were introduced over a period of four to five days to an observation hive containing a colony of the opposite line. From 800 to 1 200 bees of the opposite line were added to each hygienic and non-hygienic observation hive (approximately 20–30% of the original total population). As little or no brood was emerging in the observation hives, the introduced bees comprised the majority of (if not the only) young bees in the colony from two to seven days old and were therefore of the age known to perform cell cleaning behaviour (Seeley, 1985; Winston, 1987).

When the youngest introduced bee was 48 h old, another assay was performed with freeze-killed brood as in experiment 1. In addition, at each observation counts were made of the number of marked and unmarked bees under a circular door that covered the insert containing dead brood, and of the number of these bees under a different door adjacent to the insert that covered a patch of healthy, sealed brood. From these counts, the percentage of marked bees observed on the insert and on healthy brood at each observation period was calculated. When this part of the experiment was completed, the bees and combs were returned to their original hives in the field.

The percentage of cells (arcsine transformed) from which the freeze-killed brood was uncapped and removed in the four hygienic and four non-hygienic colonies in the observation hives in experiment 1 was compared to the percentage of brood uncapped and removed in the same four colonies of each line when marked bees of the opposite line were added, using two-way ANOVAs for each observation time (Wilkinson, 1989). The means and 95% confidence intervals of the percentage of marked bees found on the insert and on healthy brood at each observation were compared using binomial confidence intervals (Sokal & Rolf, 1981).

Experiment 4

To examine the correlation between hygienic behaviour and disease resistance, the eight hygienic and eight non-hygienic full-size colonies in the field were inoculated with the chalkbrood pathogen, *A. apis*, in pollen patties beginning on 16 September. A 5-lb (2.3 kg) pollen cake containing approximately 50% pollen, 25% sucrose, 25% glucose, and five black and five white homogenized chalkbrood mummies was prepared in the manner described by Gilliam *et al.* (1988). Pieces of the diet selected from throughout the large patty were plated one week later to check for viability and uniform distribution

of *A. apis* (Gilliam *et al.*, 1988). After checking the centre comb to be certain that no chalkbrood mummies were present, a 1/4-lb (113g) piece of the pollen cake mixture was placed over the top bars of the combs of each test hive. The centre comb in each hive was examined for chalkbrood mummies on days 3, 5, 7, 9, 12, and 17 after inoculation. Dead bee traps (Atkins *et al.*, 1970) were placed under the colonies to capture any mummies that were removed by the bees. The number of mummies in dead bee traps was counted daily.

RESULTS

Experiment 1

The eight hygienic colonies in full-size hives removed $94.7 \pm 10.0\%$ (mean $\pm$ s.d.) of the freeze-killed brood at 48 h. When the same eight colonies were tested in observation hives, the proportion removed was significantly reduced to $75.6 \pm 17.74\%$ ($t = 3.34$; d.f. = 7; $P = 0.01$). However, in the eight non-hygienic colonies the proportion of freeze-killed brood removed in full-size hives ($33.9 \pm 13.76\%$) did not differ significantly from that removed in observation hives ($26.7 \pm 8.52\%$; $t = -1.62$; d.f. = 7; $P = 0.15$).

Despite the reduction in hygienic behaviour between full-size hives and observation hives, hygienic bees uncapped and removed significantly more freeze-killed brood than non-hygienic bees within 48 h after the insert was placed in the full-size hives ($t = 7.70$; d.f. = 7; $P = 0.00$) and observation hives ($t = 4.19$; d.f. = 7; $P = 0.004$).

Experiment 2

When the cell cappings were partially removed, there was a significant interaction between the response of the hygienic and non-hygienic bees toward live and freeze-killed brood ($F = 23.83$; d.f. = 1,14; $P = 0.0002$; fig. 1a). When the comb insert contained live brood, both hygienic and non-hygienic bees recapped the partially opened cells ($85.6 \pm 6.48\%$ vs. $90.3 \pm 7.33\%$, respectively; LSD = 10.51; $P > 0.05$). When the comb insert contained freeze-killed brood, hygienic bees continued to uncapped and remove the dead brood ($2.0 \pm 2.85\%$ recapped), whereas non-hygienic bees recapped $48.0 \pm 16.74\%$ of the dead brood (LSD = 12.82; $P \leq 0.01$).

When the cell cappings were removed entirely, both hygienic and non-hygienic bees capped approximately half of the opened cells containing live brood ($43.8 \pm 21.82\%$ vs. $62.1 \pm 20.36\%$, respectively) but removed all of the freeze-killed brood from the cells (0.0% vs. $0.2 \pm 0.67\%$; fig. 1b). A Student's *t*-test, rather than an analysis of variance, was used to compare the response of the hygienic and non-hygienic lines to live brood because of the lack of variation in the response of the lines toward freeze-

FIG. 1. (a) Percentage of live and freeze-killed brood that was recapped 24 h after holes were poked in the cell cappings in eight hygienic and eight non-hygienic colonies in observation hives. (b) Percentage of live and freeze-killed brood that was recapped 24 h after cell cappings were entirely removed.

killed brood. There was no significant difference between the lines in their response to uncapped live brood ($t = -1.73$; d.f. = 14; $P = 0.11$).

Experiment 3

The results of the two-way ANOVAs at each observation period revealed significant differences between the four hygienic and four non-hygienic colonies in the amount of freeze-killed brood that was uncapped at 48 h ($F = 6.23$; d.f. = 1,12; $P = 0.03$; fig. 2a) and in the amount of brood removed from the cells at 30 h ($F = 5.07$; d.f. = 1,12; $P = 0.04$) and 48 h ($F = 8.74$; d.f. = 1,12; $P = 0.01$; fig. 3a). Adding 20–30% young (two to seven days old) hygienic bees to the non-hygienic colonies did not result in an increase in the amount of freeze-killed

TABLE 1. The mean percentage ($\pm$ 95% confidence interval) of introduced, marked bees on the freeze-killed brood insert and on a patch of healthy, live brood adjacent to the insert at various times over a 48-h period after the freeze-killed brood was placed in the observation hives. Marked hygienic bees were introduced into non-hygienic colonies, and marked non-hygienic bees were introduced into hygienic colonies. The number of marked bees introduced comprised approximately 20–30% of the total population of the colonies. Significant differences between the percentage of marked bees on the insert and on healthy brood within the hygienic and non-hygienic colonies at each observation period are indicated by an asterisk.

Hours	Introduced non-hygienic bees in hygienic colonies (%)		Introduced hygienic bees in non-hygienic colonies (%)	
	On freeze-killed brood	On healthy brood	On freeze-killed brood	On healthy brood
1	13.2 $\pm$ 5.0	21.4 $\pm$ 6.7	8.53 $\pm$ 4.8 *	29.0 $\pm$ 8.0
3	12.2 $\pm$ 5.0	19.4 $\pm$ 5.5	10.6 $\pm$ 4.5 *	32.2 $\pm$ 6.3
6	15.2 $\pm$ 4.5	19.9 $\pm$ 5.1	14.6 $\pm$ 4.7 *	28.6 $\pm$ 6.0
9	15.3 $\pm$ 4.9	22.1 $\pm$ 5.9	15.3 $\pm$ 5.1 *	26.0 $\pm$ 6.0
24	19.2 $\pm$ 5.1	21.1 $\pm$ 5.3	24.9 $\pm$ 6.1	28.4 $\pm$ 6.0
27	17.4 $\pm$ 5.0	20.5 $\pm$ 5.3	20.7 $\pm$ 5.2	25.0 $\pm$ 5.7
30	20.1 $\pm$ 5.5	23.3 $\pm$ 5.8	20.7 $\pm$ 5.6	21.8 $\pm$ 5.5
48	22.7 $\pm$ 5.4	20.3 $\pm$ 5.2	22.5 $\pm$ 5.5	30.1 $\pm$ 6.2

brood uncapped (fig. 2b) or removed (fig. 3b) in the non-hygienic colonies. However, when 20–30% young non-hygienic bees were added to the hygienic colonies, the amount of brood removed in the hygienic colonies was significantly reduced at 48 h ($F = 8.76$; d.f. = 1,12; $P = 0.01$); the proportion uncapped did not change (figs 2b and 3b).

In the non-hygienic colonies, there were significantly fewer introduced hygienic bees on the freeze-killed brood insert than on the healthy patches of brood during the first nine hours after the freeze-killed brood was presented to the colonies (table 1). From 24 h to 48 h, the percentages of introduced bees on the insert and on healthy brood patches were not different. In the hygienic colonies, there were no significant differences between the percentages of introduced non-hygienic bees found on the insert and on healthy brood at any time period.

Experiment 4

The first sign of chalkbrood infection in the full-size colonies was observed on day 3 after inoculation, which corresponds to observations by Gilliam *et al.* (1988). The peak period of infection was on days 5–9; after that the number of chalkbrood mummies in the colonies and in the dead bee traps either diminished in most hygienic colonies or remained the same in most non-hygienic colonies. The correspondence between the freeze-killed brood assay for the eight hygienic and eight non-hygienic full-size colonies in the field and the cumulative number of mummies in the centre comb on days 5, 7 and 9 after infection is shown in figure 4a. Figure 4b shows the correspondence between the results of the

freeze-killed brood assay and the cumulative number of mummies found in the dead bee traps on days 5–9.

The three full-size colonies that displayed the most rapid hygienic behaviour in the freeze-killed brood assay (colonies N, O, and P) also showed physiological resistance to the disease, since they had few or no chalkbrood mummies in the comb (2, 0 and 1 mummies, respectively) and had very few mummies in the dead bee traps (2, 4 and 1, respectively). Colony N was the feral colony. The remaining hygienic colonies displayed differing degrees of hygienic behaviour and physiological resistance. Colony J showed behavioural resistance but not physiological resistance. It contracted a relatively severe infection of chalkbrood but responded to it by removing mummies from the nest (8 mummies in the comb, 74 mummies recovered in the trap) thus giving the appearance that the colony was resistant. Hygienic colonies I, K and L showed susceptibility to the disease but did not remove the mummies rapidly, leaving 30, 28 and 30 mummies in the combs, and 37, 11 and 37 mummies in the traps, respectively.

The non-hygienic colonies A, B, and H displayed some physiological resistance to the pathogen. They had fewer chalkbrood mummies in the combs (9, 12 and 11, respectively) and in the trap (10, 16 and 2) than other non-hygienic (and some hygienic) colonies. Colonies D and E appeared to be both non-hygienic and susceptible; they had relatively large numbers of mummies in the comb (32 and 33) and few or no mummies in the trap (0 and 4).

FIG. 2. (a) Percentage freeze-killed brood uncapped at intervals up to 48 h by four hygienic and four non-hygienic colonies in observation hives. **(b)** Percentage of freeze-killed brood uncapped by the same four hygienic and four non-hygienic colonies when 20–30% marked bees of the opposite line were introduced.

DISCUSSION

The results of the first experiment demonstrated that the expression of hygienic behaviour is dependent on colony strength. Placing approximately 15% of the bees and brood of a field hive into an observation hive significantly reduced the hygienic response. The expression of hygienic behaviour was also influenced by the composition of the colony. In the third experiment, the addition of 20–30% young hygienic bees to non-hygienic colonies did not increase the degree of hygienic behaviour of the colonies in the observation hives. However, adding young non-hygienic bees to hygienic colonies suppressed the behaviour, even though the majority of

FIG. 3. (a) Percentage of freeze-killed brood removed at intervals up to 48 h by four hygienic and four non-hygienic colonies in observation hives. **(b)** Percentage of freeze-killed brood removed by the same four hygienic and four non-hygienic colonies when 20–30% marked bees of the opposite line were introduced.

the workers were hygienic. Observations on the location of the introduced, marked bees within the hive indicated that significantly fewer introduced hygienic bees were on the freeze-killed brood during the first day but were found in relatively equal proportions between the insert and healthy brood the following day. The introduced non-hygienic bees were distributed evenly over the comb during the 48 h of the test.

These results are not readily explained. In the first experiment, the reduction in hygienic behaviour could have been due to the loss of field bees when the colony was placed in the observation hive. The disruption of the age structure of the colonies may

FIG. 4. (a) Correspondence between the percentage of freeze-killed brood removed by the eight hygienic and eight non-hygienic colonies in full-size hives and the cumulative number of chalkbrood mummies found in the centre comb of the colonies on days 5, 7, and 9 after inoculation with *Ascosphaera apis* in pollen patties. (b) Correspondence between the percentage of freeze-killed brood removed and the cumulative number of mummies found in the dead bee traps on days 5–9 after inoculation. O = hygienic colonies; ● = non-hygienic colonies. Hygienic colony ‘N’ was the feral colony.

have confounded the results. However, in other experiments, colonies maintained in observation hives for over a month so that the age distribution of the bees was equilibrated still did not perform hygienic behaviour as rapidly as larger colonies in the field when tested simultaneously (M Spivak, unpublished observations).

One possible explanation for the lack of hygienic behaviour in the mixed colonies (third experiment) is that the hygienic bees tended to other needs or tasks within the colonies before removing dead brood. The fact that the hygienic bees were not found as frequently on the freeze-killed brood insert on the first day may lend support to this possibility. A second explanation for the lack of hygienic response is that the introduced hygienic bees were not of the correct age to perform hygienic behaviour. It is commonly held that the youngest bees in the colony clean cells (Seeley, 1985; Winston, 1987); however, bees that polish already emptied cells may not be of the same age as those that detect and clean out cells containing diseased brood. This explanation would also explain why fewer young, introduced hygienic bees were found on the comb insert on the first day when the freeze-killed brood was still sealed. Trump *et al.* (1967) observed rapid hygienic behaviour in colonies composed of 50% hygienic bees and slower hygienic behaviour in colonies composed of 13% hygienic bees. They found hygienic bees in higher numbers on the diseased brood than on healthy brood. However, the hygienic bees (Brown line) and non-hygienic bees (VanScoy line) were transferred into the observation hives simultaneously after emerging from their cells in an incubator. This procedure might have eliminated the experimental stress associated with introducing marked adults into the observation hives as in our procedure. It is not clear how old the bees were at the time of the tests in Trump *et al.* (1967); however, the same bees were tested twice over a two-month period and, therefore, were older than the bees in our experiment.

Even when hygienic behaviour is expressed, it does not necessarily follow that a colony will demonstrate resistance to diseases. In this case, adult bees may be hygienic but lack physiological mechanisms to reduce the spread of the disease when they remove from cells the larvae that contain infectious micro-organisms. Adult honey bees remove diseased brood by ingestion of the diseased larvae and subsequent defaecation of the spores outside the nest (in the case of AFB), or by extraction of the mummified larvae from the cell using the mandibles and legs (in the case of chalkbrood). Bees which have no mechanism of removal or neutralization of the pathogens from the gut and body hair may subsequently infect susceptible larvae when feeding them or may disseminate the spores to other adults during grooming. Thus, uncapping and removal behaviours may result in the spread of the disease throughout the nest. Simultaneously, the larvae that are fed by adult bees may be either resistant or susceptible depending on the particular pathogen and mode of transmission (see Rothenbuhler & Thompson, 1956; Thompson & Rothenbuhler, 1957) and their own physiological response. This is also the case in other insects. For example, the pathogenic microsporidium

Burenella dimorpha is disseminated by the fire ant, *Solenopsis geminata*, when the ant feeds infective spores to the developing brood (Jouvenaz *et al.*, 1981). Also, grooming between subterranean termites, *Reticulitermes*, has been documented to spread the pathogenic fungus *Metarhizium anisopliae* (Kramm *et al.*, 1982). Cannibalism, or the ingestion of infected hosts and cadavers, which in honey bees is an aspect of hygienic behaviour, has been shown to spread pathogens in the crane fly, *Tipula oleracea* (Carter, 1973).

The overall expression of both hygienic behaviour and resistance within a colony would, therefore, primarily depend on the particular disease, the proportion of bees (or subfamilies) displaying hygienic behaviour, and the degree of physiological resistance in both adults and larvae. The correlation between rapid hygienic behaviour and disease resistance is most apparent in colonies of bees which have been experimentally selected for the traits and inbred for many generations through instrumental insemination (e.g. Rothenbuhler, 1964a). In these cases, the dosage-infection regression slopes would be high (Watanabe, 1987). However, unselected colonies composed of multiple patrines would be expected to show differential expressions of hygienic behaviour and modes of resistance, resulting in low or curvilinear dosage-infection regression slopes (Watanabe, 1987).

These points were demonstrated in the present study when colonies were challenged with the chalkbrood pathogen. The colonies had been selected for hygienic behaviour only one generation before the experiment, and they were inseminated with 3–4 µl of semen from three drone mother colonies and therefore were not inbred. The eight hygienic and eight non-hygienic colonies were chosen because they demonstrated the most extreme responses to the freeze-killed brood assay. However, when inoculated with *A. apis*, some colonies became severely infected with chalkbrood despite their hygienic tendencies. Three colonies, one of which was a feral colony with a naturally mated queen, appeared to be both hygienic and physiologically resistant. The remaining five hygienic colonies displayed varying degrees of susceptibility to chalkbrood; one was highly susceptible but removed the large numbers of mummies from the nest. Three of the non-hygienic colonies showed some degree of resistance to chalkbrood. The remaining non-hygienic colonies showed increasing degrees of susceptibility. We speculate that among colonies not selected for resistance to a particular disease, most are physiologically susceptible to most diseases. Hygienic and non-hygienic behaviour is highly polymorphic in USA populations of bees. The minority of colonies appear to be physiologically resistant. Whether feral European colonies in general demonstrate a high frequency of rapid hygienic behaviour remains to be tested.

If in some cases hygienic behaviour may promote the spread of diseases, there may be selection against the trait. However, are there circumstances when it might benefit a colony to leave diseased brood under a capped cell? Non-hygienic behaviour in honey bees has been described as a 'lack of hygienic behaviour' the bees appear to 'ignore cells with dead pupae' (Alcock, 1979). It has also been conjectured that non-hygienic behaviour is due to a 'blockage or defect in some link of the chain which generates the (hygienic) behaviour pattern' (Gould, 1982). However in other insects, pathogens are avoided by behaviours which in honey bees are termed 'non-hygienic.' For example, subterranean termites avoid accumulations of dead colony members and wall them off with dirt within the nest (S Jones, personal communication). *Reticulitermes* do not cannibalize other termites infected with *M. anisopliae* as they would normally do in caste regulation (Kramm *et al.*, 1982). The avoidance of the fungus prevents the spread of the disease. Alkali bees, *Nomia melanderi*, and sweat bees, *Lasioglossum versatum*, use dirt to wall off brood cells infected with fungi and do not reuse those cells (Batra, 1968; Batra & Bohart, 1969). Another species of honey bee, *Apis cerana*, uncaps and removes worker larvae infested with the parasitic mite *Varroa jacobsoni*, but does not remove infested drone larvae (Koeniger, 1987; Rath & Drescher, 1990; Boecking & Drescher, 1992). *Apis mellifera* tends to seal off foreign objects (sticks, rocks and menthol crystals used as a treatment for tracheal mites, *Acarapis woodi*) with propolis (personal observation). One colony of honey bees in Tucson was observed to seal cells containing chalkbrood mummies with a thick covering of propolis (S Thoenes, personal communication).

Are non-hygienic honey bees simply unable to distinguish healthy from unhealthy brood, or do they avoid contact with dead and diseased brood? These questions have not been tested directly. Our second experiment showed that non-hygienic bees tended to recap partially uncapped cells containing dead brood, whereas hygienic bees never recapped those cells. However, when the cell cappings were removed entirely, non-hygienic bees did not recap the cells; instead they removed the dead brood. At this time we are unable to conclude that non-hygienic bees actually avoid dead brood. It could be that non-hygienic bees perceive an opening in a sealed cell as a cell which is in the process of being capped and thus continue to cap it. They may remove dead brood only if it is entirely exposed.

The results of the second experiment also indicated that neither hygienic nor non-hygienic bees removed significantly different proportions of uncapped live brood from the cells. Thus, non-hygienic bees appear to recognize brood that is healthy and recap it. These results indicate that it is unlikely that there is a consistent cost to hygienic behaviour because

the bees remove healthy larvae (see also Milne, 1983b).

Hygienic behaviour may be a generalized adaptation for cell reuse. Unlike other closely related social insects such as bumble bees and stingless bees (Sakagami & Michener, 1962; Michener, 1974), honey bees characteristically reuse the cells from which brood emerges rather than building new wax cells or nests which are energetically costly. Hygienic behaviour is more readily observed when the bees are bringing in large quantities of nectar (Borchers, 1964; Mourer, 1964). It has been demonstrated that hygienic honey bees over four weeks old will revert from foraging to nest cleaning (hygienic) behaviour when there is incoming nectar (Thompson, 1964; Momot & Rothenbuhler, 1971) and thus a need to clear out comb space. If cells containing diseased brood were not cleaned out and reused, the bees would have to rearrange the brood and honey within the nest, which may not be feasible given the volume of the nest cavity. Thus, there may be a trade-off in honey bees between risking infection by removing diseased brood from the cells and spending the energy and resources to construct new cells or establish a new nest site. If the pathogen is ultimately fatal to the colony, the best responses may be removing the diseased larvae quickly or abandoning the nest altogether. However, if the pathogen can survive within the bee colonies without causing overt disease (e.g. Gilliam, 1986), and the disease is stress-related, it may be a viable strategy to risk reinfection by allowing the pathogen to remain within a sealed cell until the period of stress is over. Then the infected brood could be removed from the colony and the cells cleaned and reused. Verification of this possibility awaits further research.

The results of these experiments reveal some aspects of hygienic behaviour which have not been investigated previously. First, although uncapping and removing behaviours are genetically determined, they are not always expressed. The expression of these behaviours appears to be facultative, depending on colony strength, composition of workers within the colony, cell space requirements, resource conditions, and factors yet unknown. Secondly, the weak correspondence between hygienic behaviour (as determined by a freeze-killed brood assay) and physiological resistance to the chalkbrood pathogen observed in this study suggests that colonies which are both highly hygienic and physiologically resistant to diseases may occur in low frequency. Finally, non-hygienic bees tend to recap brood which has been partially uncapped, suggesting that either these bees cannot detect dead or diseased brood, or they avoid it by sealing it off within a cell. Comparisons of non-hygienic behaviour in honey bees with behaviours of other social insects allude to outcomes of avoiding diseased brood which might be beneficial in some circumstances.

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