Hygienic behaviour of honey bees and its application for control of brood diseases and varroa

Part II. Studies on hygienic behaviour since the Rothenbuhler era

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Part I of this review summarized the initial research on hygienic behaviour of honey bees, *Apis mellifera*. This early work that concerned hygienic behaviour as a mechanism of resistance to American foulbrood (AFB) has been the foundation for all subsequent research on hygienic behaviour. In Part II, research on hygienic behaviour in relation to other bee diseases and to *Varroa jacobsoni* and in *Apis* species and subspecies is reviewed. In addition, techniques to screen bee colonies for the behaviour are detailed, and practical applications of breeding bees for hygienic behaviour are given. A section on neuroethology demonstrates how modern neurobiological techniques are being used to detect the reasons for differences in responses of hygienic and non-hygienic bees to abnormal brood.

Hygienic behaviour as a defence mechanism against chalkbrood

Chalkbrood, a fungal disease of honey bee brood caused by *Ascosphaera apis*, results in mummified larvae. It has been known in Europe for many years prior to 1968 when it was discovered in the USA in honey bees from California[94]. In only 12 years after this initial report, chalkbrood was found throughout North America. The high incidence and rapid spread were indicative of a pathogen new to an area where the host had little natural resistance.

The resurgence of interest in hygienic behaviour and in its application for control of microbial brood diseases and mite pests of honey bees has been largely due to the efforts of Steve Taber. He has written numerous popular articles[81,82,83,84,85,86,87,88,89,90,91] and has spoken on the subject at many apicultural meetings. The authors of the present paper consider themselves fortunate to have worked with him and attribute, directly (MG) or indirectly (MS), their interest in

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hygienic behaviour to his influential guidance. Taber's contributions began when he and Gilliam found chalkbrood in Arizona and initiated efforts to understand the pathology of the disease and to devise control methods[20,22].

The observation that adult bees from some colonies removed larvae infected with chalkbrood from their cells within 24 hours[20] prompted studies on the mechanisms of tolerance or resistance of honey bee colonies to chalkbrood. Gilliam and coworkers[21] demonstrated that it is possible to select and breed honey bees for resistance to the disease. Increased resistance was evidenced by elevated hygienic behaviour on the part of nurse bees (uncapping and removal of dead and dying brood that are carried outside the hive and can be seen at the hive entrance); by decreased longevity of the fungal spores of *A. apis*; and by reduced pathogen contamination of bees, brood, and stored food. Hygienic behaviour was shown to be the primary mechanism of resistance to chalkbrood[23], although resistance involves other factors as well[23,53,77].

Simple tests using comb inserts of frozen brood for testing hygienic behaviour of bee colonies were devised[21,82] and refined[91]. Colonies of bees from a naturally mated queen that remove freeze-killed brood within 48 hours are considered hygienic; those that take over a week to remove dead brood are considered non-hygienic. In studies on the relation between hygienic behaviour and chalkbrood most, but not all, colonies showed good correlation between uncapping and removal of freeze-killed brood and resistance to chalkbrood[21,22,53]. Taber[83] noted similar exceptions and stated that research is required to determine different mechanisms of resistance to chalkbrood. The underlying genetic mechanisms may be more complex than originally thought[57].

Research has defined some secondary mechanisms of resistance to chalkbrood, and no doubt others exist. These include faster filtering of *A. apis* spores by the proventriculus of bees from resistant colonies than from susceptible colonies and increased resistance to *A. apis* spores by larvae from hygienic colonies[65]. Also, during pollen collection and storage in comb cells, bees add antagonistic moulds and bacteria (primarily *Bacillus* spp.) that inhibit *A. apis*[19,23]. Bee colonies that are resistant or tolerant have more of these antagonists. Bee bread and the guts of worker bees, the major sources of the pathogen, were the primary sources of the antagonistic micro-organisms[19,23]. Antimycotic substances active against *A. apis* were not produced by bees, larvae, bee bread, or honey[23]. Thus, antimycotic substances were produced by micro-organisms that originated in worker bee intestines. The most promising of these antagonistic micro-organisms are being tested as potential chalkbrood controls in field tests utilizing bee colonies selected for susceptibility to the disease (Gilliam & Taber, unpublished).

Most bee colonies tested display intermediate levels of hygienic behaviour[76]. Physiological resistance to chalkbrood may exist, but results have suggested that few colonies are both highly hygienic and physiologically resistant to chalkbrood[77]. Non-hygienic colonies, at least those without physiological resistance, may cope with chalkbrood by sealing larvae in their cells to avoid further spread of the pathogen by nurse bees to other larvae during feeding[77].

The practical results and recommendations from the research of Gilliam and Taber that can be used by beekeepers include the following:

- A technique for testing hygienic behaviour of bee colonies.

- Extension of this technique by feeding *A. apis* as homogenized chalkbrood mummies in a pollen diet to test colonies for chalkbrood resistance[91].
- The necessity of queen replacement in colonies that are highly susceptible to chalkbrood.
- The recognition that susceptible strains of bees can be eliminated by routine requeening from stock that has little or no chalkbrood.
- The importance of eliminating stress, reducing pathogen load through mummy removal, and ensuring that colonies receive optimal nutrition[18,19].
- The recommendation that since pollen is the major source of *A. apis*, it should not be collected from colonies contaminated with *A. apis*[23].

Subsequent work by Spivak[74] demonstrated that in experiments conducted in 1994, no chalkbrood mummies were found in four hygienic colonies; three of four non-hygienic colonies had mummies, and two of these three contained large numbers. Also, daughters of hygienic breeder (inseminated) stock retained the hygienic trait when they were allowed to mate naturally with unselected drones[78]. In all tests, hygienic colonies removed significantly more brood than unselected commercial colonies. Chalkbrood mummies were found in 8% of the hygienic colonies and 27% of the commercial colonies ($P = 0.02$). Comparison of colonies headed by hygienic queens ($n = 49$) that were naturally mated and unselected commercial queens ($n = 46$) that were reared and mated in the same location revealed that the hygienic colonies had significantly lower levels of chalkbrood and produced significantly more honey.

Work conducted by other researchers on hygienic behaviour and chalkbrood include that of Milne[53] who concluded that hygienic behaviour as well as other unknown mechanisms confer resistance to chalkbrood. To test hygienic behaviour, he used 100 newly-emerged worker bees in laboratory test cages supplied with sugar syrup, water, and 40 cells of freeze-killed brood in comb sections. Separate tests were done for uncapping and for removal behaviours. A visible hole in the capping was considered uncapping behaviour. Cells were manually uncapped for tests of removal behaviour. Removal behaviour but not uncapping behaviour in cages was significantly correlated with colony resistance to chalkbrood after inoculation of colonies by feeding *A. apis* spores in pollen cake. These results may have been influenced by the abnormal test conditions that included lack of a queen, lack of pollen, manual uncapping of cells, and consideration of only a hole in the capping as removal behaviour. As pointed out by Newton and Michl[58], bees may use dead brood as a protein source if pollen is unavailable, and this could affect the amount of brood that is removed. Also, uncapping is necessary before workers can remove dead brood from sealed cells, and worker bees in colonies often re-seal cells that have only a hole in the capping. Indeed, not all colonies have shown good correlation between hygienic behaviour results from field tests and from laboratory cages[68]. Harbo[30] attempted to measure the relationship between chalkbrood and hygienic behaviour in 27 colonies each headed by a queen mated to one drone. He used the freeze-killed brood test but measured natural infections of chalkbrood in the colonies by counting mummies. His analyses showed no significant correlations between hygienic behaviour and chalkbrood. It would have been better to inoculate each of the test colonies with the same dose of *A. apis* rather than depend on variable natural infections that would affect the results.

Hygienic behaviour as a defence against European foulbrood

Little work has been done in this area. Message and Gonçalves[51] published a paper on behavioural genetics with European foulbrood (EFB) in the title, but the reported work did not concern EFB nor were selected lines challenged with *Melissococcus pluton*, the causative agent of EFB. This report appeared to be a prelude to future studies with EFB.

Milne[54] used freeze-killed brood and determined the time required by bees in laboratory cages to uncap (a visible hole in the capping), enlarge a hole in a capping (made by a puncture with a pin), and remove freeze-killed larvae from cells that had the cappings removed with tweezers. He concluded that hygienic behaviour does not confer resistance to EFB because the course of the disease in 17 inoculated colonies was not significantly correlated with test results of uncapping and removal in laboratory cages. His definitions of uncapping and removal behaviours, and his use of laboratory cage tests are problematic. Since it is well known that most larvae with EFB die in the coiled stage in uncapped cells, uncapping might not be a necessary element in resistance to EFB, but removal behaviour would. Also, scales of EFB are much easier for the bees to remove than scales of American foulbrood (AFB). Thus, a better procedure to test for the relation between hygienic behaviour and EFB would be to screen colonies using frozen uncapped brood and then inoculate these colonies with *M. pluton* to obtain high incidences of EFB (12 out of 17 of Milne's colonies had fewer than 8 cells of EFB over the 2-month course of the experiment). Milne hypothesized that since *M. pluton* does not form spores as do the pathogens of AFB and chalkbrood, the cell contents with EFB would be infective whether removed quickly or slowly because the vegetative stage of *M. pluton* is infective. He thought that this reasoning strengthened the explanation of why hygienic behaviour is part of the colony control of AFB and chalkbrood but not EFB. An alternative explanation is that *M. pluton* lacks the survivability of the AFB and chalkbrood pathogens because no spores are formed. Researchers experienced in diagnoses of dead larvae for EFB are well aware that *M. pluton* is quickly overgrown by secondary bacterial invaders and is seldom seen in or isolated from larvae with EFB unless they are obtained almost as soon as they are infected. That is why the secondary invader *Paenibacillus alvei* is used as an indicator of EFB in dead larvae. Thus, it may be unnecessary for bees to remove larvae with EFB to prevent the spread of infection since the pathogen does not survive. In any case, it is clear that much more research is required to determine the resistance mechanism(s) of bee colonies to EFB.

Hygienic behaviour in subspecies of *Apis mellifera, A. cerana* and Africanized honey bees

Differences have been reported in the relative hygienic behaviour of subspecies of *Apis mellifera, A. cerana*, and Africanized honey bees. For example, Kefuss[42] found that *A. m. intermissa* colonies from Tunisia had the highest level of hygienic behaviour of several subspecies of *A. mellifera* (*A. m. mellifera, A. m. ligustica, A. m. carnica, A. m. caucasica*) that he tested from France, Tunisia and Chile. However, Rodriguez *et al.*[68] found no significant differences in hygienic behaviour between *A. m. mellifera, A. m. carnica*, and 'Buckfast' colonies. Good and poor hygienic colonies were found in each. Boecking *et al.*[9] reported that *A. cerana* is more effective than *A. mellifera* in removing mite-infested sealed worker brood.

In some studies, Africanized honey bees had better hygienic behaviour than European bees[13,45]; in one, there were no significant differences[44]; and in another, European colonies were found to be superior to Africanized colonies in this regard[14]. Cosenza and Silva[13] reported that Africanized (n = 4) and most Africanized–Caucasian hybrid colonies (n = 4) were significantly more hygienic than the Caucasian colonies (n = 4) tested. Lengler[44] compared three colonies headed by 'Starline' queens and three by Africanized colonies. The lack of significant differences between the Starline and Africanized colonies is interesting in view of the report by Kefuss[41] that Starlines displayed the best hygienic behaviour of all the different lines and races of bees that he had tested with the exception of *A. m. intermissa*.

Loper[45] found that 98.7% of the Africanized colonies (n = 12) he tested removed pierced brood within 48 hours; the response of European colonies (n = 17) was more variable, although some cleaned cells within 24 hours. Recently Aumeier *et al.*[1] found no race-specific differences in removal rates between *A. m. carnica* and Africanized honey bees after varroa or ants were introduced into capped brood cells.

Danka and Villa[14] compared European (Italian and Carniolan) colonies (n = 9) derived from queens obtained from Hawaii, USA, and Africanized colonies (n = 9) established from swarms caught in Costa Rica. In addition to hygienic behaviour tests using the pierced brood assay (see section on Assays), they inoculated larvae from eight colonies of each bee type with *Paenibacillus larvae* spores, the causative agent of AFB. Because larvae in Africanized colonies had significantly lower infection rates of AFB, some physiological resistance to the disease may exist in these bees. The authors noted that this difference between bee types might be exaggerated by the slightly shorter development period for Africanized eggs; Africanized larvae, if older when inoculated, could have been less susceptible to the disease.

It is clear that larger bee populations must be evaluated before general statements about the superior hygienic behaviour of a species, subspecies, or commercial variety of honey bees can be accepted; it is likely that good hygienic behaviour exists in at least some colonies of each of these.

Hygienic behaviour as a defence against varroa

Honey bee resistance to the parasitic mite *Varroa jacobsoni*, with reference to hygienic behaviour as one mode of defence, has been reviewed by Boecking and Ritter[8], Büchler[11], and more will be reviewed in depth in Boecking and Spivak[10]. Thus, only a brief summary follows.

While conducting experiments in China on the natural defences of the Asian honey bee, *A. cerana*, against the endemic parasitic mite, *V. jacobsoni*, Peng *et al.*[64] found that *A. cerana* was able to uncap and remove pupae that were infested with the mite. A significant correlation was found between the infestation level of observation hives containing *A. cerana* or *A. mellifera* and the degree of removal of infested pupae by the bees. Rath and Drescher[66] subsequently found that *A. cerana* detected and removed 98.8% of worker pupae experimentally infested with varroa (but see Rosenkranz *et al.*[69]). North American and European stocks of *A. mellifera* also removed varroa from infested capped cells of worker brood but to a limited extent compared to *A. cerana*[3,4,5,6,8,64,74].

The removal of infested pupae may theoretically limit the growth of the mite population in three ways:

- Immature mites which have begun to develop in brood cells are killed,

decreasing the average number of offspring per mother mite.

- The mother mites may be damaged during the removal process.
- The phoretic period (time spent on an adult bee) of a mother mite is extended if she escapes during the removal process[16].

When varroa was experimentally introduced into *A. m. carnica* colonies in Germany, Boecking and Drescher[5] found that the mean rate of removal by the Carniolan bees on day 10 after infestation was 29.3% when one mite per cell was introduced but 55.1% when two mites per cell were introduced. A positive correlation ($r = 0.74$) was found between the removal rates of mite-infested brood and the removal of freeze-killed brood, a commonly used assay to test for hygienic behaviour.

Spivak[74] used a similar approach in the USA to test *A. mellifera* colonies of Starline–Italian origin for their ability to remove infested pupae. The colonies tested had been bred *a priori* for two generations for hygienic behaviour with the goal of breeding colonies resistant to chalkbrood (see[21,23,77] and section on Breeding below). A freeze-killed brood assay was used to select colonies for hygienic behaviour (see procedure in Assay section). Daughter queens raised from the hygienic colonies were instrumentally inseminated with semen from drones from other hygienic colonies. A non-hygienic line of bees was also bred as a control. All inseminated queens were wintered in full-size colonies and included in experiments the following summer.

Following the methods of Boecking and Drescher[4,5], cells containing recently sealed fifth-instar larvae within the hygienic and non-hygienic colonies were inoculated with one varroa per cell. In the first year of the study (1994), the hygienic colonies removed significantly more infested pupae than the non-hygienic colonies 10 days after the cells were infested; in the second year (1995), there was no significant difference in the removal rate (fig. 1). However, when two mites per cell were introduced into the same colonies in 1995, the hygienic colonies removed significantly more infested pupae than the non-hygienic colonies[74]. The expression of hygienic behaviour is known to be influenced by resource conditions[55,95,97]. However, there was a strong nectar flow during the experiment in 1995. Thus, it was hypothesized that honey bees may have a threshold response to cues elicited by abnormal (mite-infested or diseased) brood (see section on Neuroethology). If the colony is highly infested, as they all were in 1995, the bees may cease to respond to the cues that trigger removal behaviour. In addition, removing large numbers of infested pupae may help reduce the growth of the mite population, but the advantages may be outweighed by the costs of reducing the number of eclosing (emerging) bees in the colony.

In 1996 and 1997, the experiment was repeated, and in both years the hygienic colonies removed significantly more infested pupae than the non-hygienic colonies (fig. 1). Two of the hygienic queens in colonies tested in 1995 survived a second winter, and the colonies were re-tested in 1996 when the mite infestations in the colonies were low. The bees from the two colonies removed 5% and 44% of the infested pupae in 1995; in 1996, they removed 60% and 70%, respectively. These results lend support to the hypothesis that bees may have a threshold response to the cues associated with abnormal brood.

In all years, the bees removed infested pupae throughout the duration of the pupal period, indicating that they were responding to cues associated with the parasitized pupae

and not to the initial disturbance caused by the experimental procedure.

The infested brood in some cells may be opened by the bees, then resealed with wax by other bees without removing the brood[7,92]. During the removal process, the female mite that parasitized the larva may escape and re-enter a different brood cell. Experiments by Boecking[3] and Boecking and Drescher[6] revealed that most of the adult female varroa (*n* = 104) that escaped the brood cells after removal could invade other brood cells (mean = 61.3%). Some mites attached themselves to adult bees (14.6%), and a small percentage (10.9%) were killed by bees. If a cell containing an infested pupa is detected by the bees after the female mite has laid eggs, her offspring are killed by the bees during the removal of the pupa.

Hygienic behaviour may not be the main mechanism of resistance to varroa. Harbo[31] and Harbo and Hoopingarner[32] determined that non-reproduction of mites is the most significant factor related to the decrease in mite populations within tested colonies. The suppression of mite reproduction was found to be a heritable characteristic of the bees, and a selective breeding programme was initiated to increase its expression. Preliminary findings on the mechanism of suppression indicated that the bees may cause the production of non-functional male mites that do not fertilize their sisters or that the bees may cause mites to delay egg laying[33,34]. Hygienic behaviour, grooming behaviour, and post-capping duration also may be important mechanisms of resistance[15], but, as with suppression of mite reproduction, the traits may only be effective if expressed at a very high level in the bee colony.

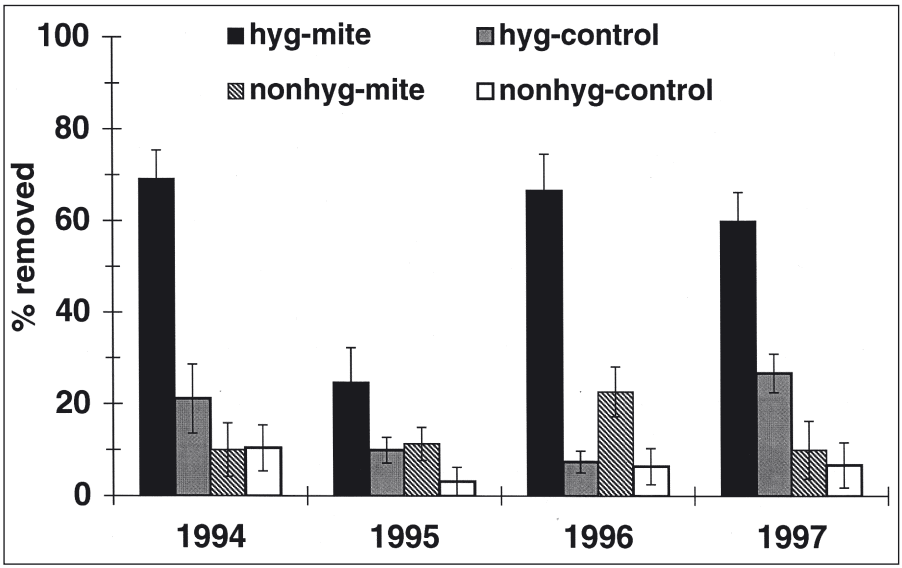

FIG. 1. The mean (± s.e.) percentage removal of mite-infested pupae by hygienic and non-hygienic colonies 10 days after introduction of the mites through cell bases (methods in Spivak[74]). In 1994, 1996, and 1997, the hygienic colonies (*n* = 4, 10, and 6 respectively) removed significantly more pupae infested with one mite per cell than did the non-hygienic colonies (*n* = 3, 6, and 6) (*P* < 0.01; split-plot 2-way ANOVA for each year). There also was a significant difference between the removal of infested pupae and controls (*P* < 0.05) in those years. Tests in 1995 (*n* = 7 hyg, 4 non-hyg) revealed a significant difference only when two mites per cell were introduced (treatment effect: *P* < 0.01).

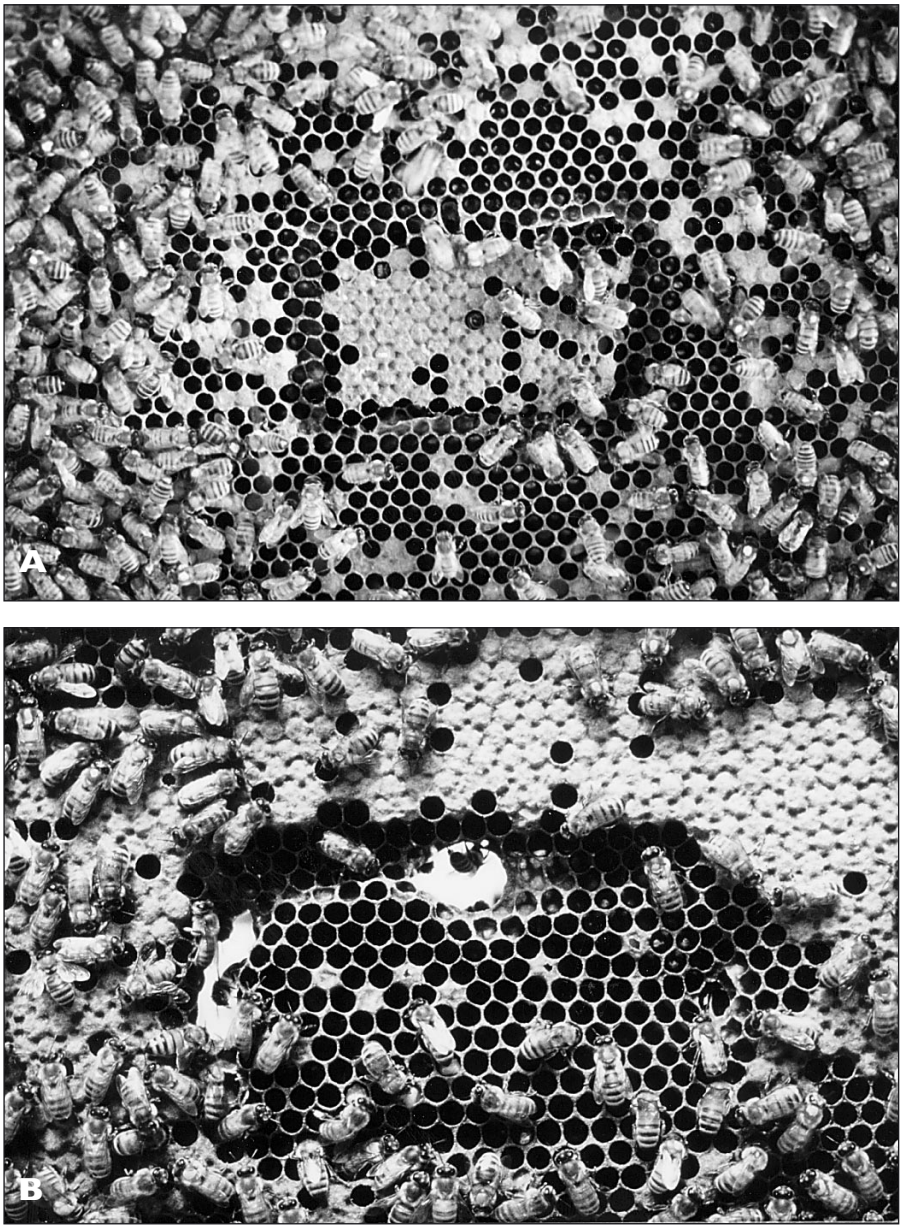

FIG. 2. A freeze-killed brood assay. (a), A 5 × 6 cm section of sealed brood that had been cut from the comb, frozen for 24 h, and then replaced within the comb in the centre of the brood nest. (b), The same comb section after 48 h. The hygienic bees uncapped and removed the majority of the freeze-killed brood.

Assays used to screen colonies for hygienic behaviour

Due to inherent problems in inoculating colonies with AFB spores, Jones and Rothenbuhler[40] determined that resistant colonies would remove cyanide-killed brood. The use of cyanide-killed instead of AFB-killed brood allowed for greater flexibility in experimental design in subsequent tests of hygienic behaviour such as assessment of genetic or environmental factors that might influence the propensity of bees to perform hygienic behaviour[55,77]. Test colonies were given a uniform section of dead brood which eliminated variation caused by larval response to AFB.

Comb sections containing sealed larvae of various ages were placed in a chamber of Cyanogas for several hours[40]. After aeration of combs, sections were placed into one Brown (AFB resistant) and one Van Scoy (AFB susceptible) colony. Each colony was tested three times. Although a colony would sometimes remove the dead brood from one side of the comb section faster than from the opposite side, there was no difference in the rate of removal of larvae and pupae between the tests. All Brown colonies removed all of the dead brood within 24 hours, whereas the Van Scoy colonies required 40–72 hours. In most countries, it is illegal or difficult to obtain Cyanogas to kill bee larvae. Thus, potentially less dangerous methods of killing brood were developed.

Researchers in Brazil were the first to use the freeze-killed brood assay. However, their publications in Portuguese appear in proceedings that are not readily available. The first that is accessible is by Cosenza and Silva[13] although Gonçalves and Kerr[24] (cited by Moretto[56]) and Cosenza[12] appear to have published earlier works using the method. Cosenza and Silva[13] used small pieces of comb (10 × 10 cm) frozen for 48 hours.

Newton *et al.*[60] were the first to use a freeze-killed brood assay to survey colonies in the USA for hygienic behaviour. In their study, an entire frame of sealed brood was frozen from 24–72 hours at –17°C and then placed in the test colony. The time required for the bee colonies to remove the frozen brood was recorded. Taber[82] and Taber and Gilliam[91] modified the assay by presenting the bees with frozen brood in a 5 × 6 cm comb section containing approximately 100 larvae and pupae per side and then recording the time required for the colonies to detect, uncap and remove the brood. These comb sections were cut from the comb of a frame within the brood nest of a colony, frozen at –20°C for 24 hours, and then placed in the brood nest of the test colony. Colonies that removed the freeze-killed brood from the comb section within 48 hours were considered hygienic; colonies that took longer than 6–7 days to remove the brood were considered non-hygienic[75,91].

There are several difficulties inherent in the freeze-killed brood assay. First, the amount of freeze-killed brood removed within 48 hours by a particular colony may vary between assays[61,68]. Environmental conditions[52,55,95] and the age of the frozen brood can influence the test result. Freeze-killed brood recently sealed with a wax capping was removed more quickly than pupae that had been sealed for five days[68].

Another assay for hygienic behaviour was developed by Newton and Ostasiewski[59]. This assay involved inserting a fine (insect) pin through larvae or pupae covered by wax cappings and then recording the time required for colonies to remove the pierced brood. This assay is not as labour intensive as the freeze-killed brood test and does not cause as much damage to the combs. However, holes left in the wax cappings and the

exposed haemolymph from the pierced larvae or pupae may increase the rate of removal of the brood by the bees[75]. Newton and Ostasiewski[59] determined that there was a significant correlation between the freeze-killed brood assay and the pierced brood assay (r = 0.956). Unfortunately, this correlation was based solely on five colonies, all of which took over six days to remove freeze-killed brood and thus were non-hygienic.

Gramacho *et al*.[25] found that the average body temperature of pin-killed pupae was significantly lower than the temperature of live pupae in neighbouring comb cells. They thought that even small differences in body temperatures could be involved in the recognition of dead brood by house-cleaning bees.

Titera and Kokkoris[96] injected sealed brood with water, saline, ethanol, extract of varroa in saline, or extract of dead drone pupae in saline to test the hygienic reaction of honey bees. The varroa extract did not stimulate the cleaning behaviour of the bees, but the dead drone pupal extract elicited a strong reaction.

A recent study used sufficient numbers of bee colonies to evaluate factors inherent in the freeze-killed brood assay that might influence the consistency of the results and then compared results from the freeze-killed and pierced brood assays[75]. Colonies in the experiment displayed a wide range of removal rates and were grouped as hygienic, non-hygienic or intermediate. The results indicated that neither the age nor the source of the frozen brood had a significant effect on the removal rate by hygienic colonies (i.e. those colonies that consistently uncapped and removed freeze-killed brood within 48 hours), although the response of colonies in the intermediate and non-hygienic groupings varied depending on the age of the frozen brood. Only a weak correlation was found between the removal of young freeze-killed and pierced pupae, but a significant correlation existed between the removal of pre-eclosion freeze-killed and pierced pupae. It was concluded that the freeze-killed brood assay is a more conservative and reliable test of hygienic behaviour because five of 19 colonies removed all of the freeze-killed brood within 48 hours, while 13 of 19 colonies removed the pierced brood within the same time. If the pierced brood assay is used to screen colonies for hygienic behaviour, it is recommended that the colonies be inspected within 24 hours of treatment. Only one study to date has compared colony resistance to AFB with the rate of removal of pierced brood[14]. This study yielded unexpected results because colonies that removed pierced brood slowly were determined to be more resistant to *P. larvae* than those that removed the brood rapidly.

Since 1996, the freeze-killed brood assay has been simplified by using liquid nitrogen to freeze a section of comb directly within the frame[67,79]. The use of liquid nitrogen eliminates variability in handling and thawing the sections of comb and reduces the number of times a colony must be opened. A cylinder of thin metal, 6–8 cm in diameter, is formed (e.g. a cut and soldered clothes-dryer vent). The cylinder is twisted into comb containing brood sealed within 1–3 days, and 300 ml of liquid nitrogen is poured slowly into the cylinder (fig. 3). When the cylinder has thawed, it is removed, and the frame is marked to indicate its location within the brood nest. After 24–48 hours, the frame is removed, and the percentage of cells from which larvae have been uncapped and removed is determined.

Although the majority of hygienic colonies selected by the freeze-killed brood assay demonstrated resistance to the disease being studied, there has not been a perfect correlation between the removal of freeze-

killed brood and measures of disease resistance[21,23,77]. In research on the relation between hygienic behaviour and chalkbrood, it was noted that behavioural resistance was not necessarily linked to physiological resistance. A highly hygienic colony could be physiologically susceptible to the disease, and a non-hygienic colony could be physiologically resistant[77]. Thus, when developing hygienic breeder stock, it is essential that the hygienic colonies are challenged subsequently with the AFB or chalkbrood pathogen to ensure resistance.

Current studies on neural mechanisms of hygienic behaviour

Recently, studies were initiated by Spivak, in collaboration with neurobiologist K Mesce, on the neural mechanisms of hygienic behaviour[47,48,49]. The hypothesis being tested is that individual hygienic bees have a low stimulus response threshold for abnormal olfactory or chemical cues associated with dead, diseased or parasitized brood; whereas non-hygienic bees have a higher response threshold to those cues. This means that a hygienic worker bee responds to a stimulus (removes abnormal brood) when the intensity of that stimulus (odour concentration) is low, while a non-hygienic bee responds to the stimulus only when the intensity is higher. Thus, the expression of hygienic behaviour may be dependent on the perception of appropriate cues as well as on the response threshold of bees to attend to or remove those agents releasing such cues. Individual response thresholds, although genetically determined, are dynamic[93]; thus, the threshold of a hygienic bee may change with time and resource conditions. The dynamic nature of the response may explain the variability in the expression of the behaviour displayed in previous experiments[55,74,77,95]. This approach has not been taken previously because emphasis has been placed on the behavioural genetics of the trait rather than on its neuromodulation (how the trait is modulated by the nervous system).

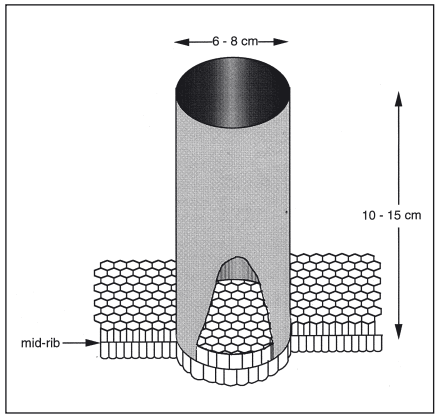

FIG. 3. Diagram of the hollow cylinder through which liquid nitrogen is poured to freeze a circular section of sealed brood. The cylinder is twisted into the comb down to the midrib. The cylinder should be 10–15 cm in length and 6–8 cm in diameter.

Hygienic behaviour appears to be a generalized response of honey bees to pathogens and parasites in the nest. Various kinds of cues may be used by honey bees to determine that a larva or pupa within a cell is abnormal. These include chemically mediated cues; the diseased, parasitized or dead brood may have a characteristic odour that is perceived by the bees to be abnormal[75,96]. A second class of cues may be mechanical; the diseased, parasitized or dead brood may have abnormal or no movements and vibrations within the cell[71]. A third class may be thermal; the bees may perceive a difference in heat generated by a healthy and an abnormal larva or pupa[25]. Other possible cues such as pheromones or metabolic products of healthy brood may be important (W Rath, personal communication).

Preliminary field and laboratory experiments, using proboscis-extension reflex (PER) conditioning (see methods in Bitterman *et al.*[2]), i.e. training bees to extend their tongues to a sucrose reward, indicated that non-hygienic bees have a higher response threshold than hygienic bees to olfactory stimuli associated with brood infected with chalkbrood[47,49]. These results support the hypothesis that the differences between the bee lines lie in their responsiveness to olfactory stimuli associated with abnormal brood.

In addition, it is speculated that the overall neural 'machinery' of hygienic and non-hygienic bees are comparable, but that such systems may be altered by the influence of endogenous neuromodulatory substances (chemical substances that influence nerve transmission), especially the biogenic amines (chemical substances that influence the transmission of the nervous signal across the synapse). Numerous studies have demonstrated that genetically determined behaviours are not necessarily regulated by fixed or 'hard-wired' individual neurons and their interconnections. Rather, behaviours are generated by highly flexible or polymorphic neural networks[17,35,72,73]. The biogenic amines have dramatic influences on the plasticity of individual neurons and networks underlying behaviour[36,46]. Hygienic behaviour may be influenced by the biogenic amine, octopamine. An injection of octopamine into the bee brain enhances the response of bees to olfactory stimuli[37,50]. Intracellular stimulation of neurons that contain octopamine (the VUMmx1 interneuron)[27] and local injection of octopamine in the brain[28] can substitute for the reward (unconditioned stimulus) in the PER assay[29].

Thus, because of its sensitizing effects on olfactory systems and its actions on the motivational state of the honey bee, octopamine might facilitate the detection and/or response of the bee to detect and respond to diseased or parasitized brood. Supportive of this idea are our preliminary studies in which neurons containing octopamine were identified in whole-mounted brains using fluorescence and confocal microscopy. The images of the brain indicated that the staining intensity of some octopamine-immunoreactive neurons differed between the brains of hygienic and non-hygienic bees[48]. These studies are ongoing.

Breeding for hygienic behaviour

Because hygienic colonies demonstrate resistance to AFB[70,98] and chalkbrood[21,23], and tend to remove brood infested with varroa[5,74], the trait is highly desirable and would be expected to be incorporated into honey bee stocks. Unfortunately, this is not a common practice among bee breeders in their selection criteria. However, there are examples of the applications and benefits of using hygienic bees.

Hygienic queens (designated DR) were commercially developed by Taber Apiaries in California in the 1980s. Scientific data from independent evaluation of these queens to reduce brood diseases in beekeeping operations that use them are lacking. Milne[54] tested hygienic behaviour of DR and randomly selected lines in cages and found no significant differences between the two. He erroneously stated that DR queens were used by Gilliam *et al.*[21], but these queens were not available at the time of their experiments and thus were not used. This is clear from their description of colonies randomly chosen for hygienic behaviour testing.

Beekeepers often make inadvertent selections for hygienic bees by re-queening diseased colonies in the expectation that progeny of the new queen will have fewer disease

problems than the current offspring. Routine re-queening in this way eliminates susceptible strains of bees. The best queens can then be used as breeders. Colonies within an apiary that consistently display little or no disease or mite infestation in contrast to adjacent colonies that have a moderate to high incidence are logical sources for tolerant or resistant stock.

In Denmark, lines of honey bees were tested for hygienic behaviour, and after four generations of selection and breeding, queens were offered to Danish beekeepers[38]. Colonies headed by these queens had 9.1% chalkbrood compared to 71.4% in the original stock of the beekeepers. A screening and breeding programme for hygienic behaviour has also been initiated in Chile[43]. In Australia, Oldroyd[61] evaluated 10 commercial honey bee strains for hygienic behaviour to locate hygienic stock that could withstand chalkbrood, a disease new to that country.

In Argentina, a selection and breeding programme for hygienic behaviour was begun in 1992[62,63]. This programme was instituted primarily because of problems with AFB and chalkbrood, and has wide support from national and provincial institutions, universities, and governments. A frozen brood assay is used for selections, and colonies are headed by naturally mated queens. Incidence of brood diseases (AFB, EFB, chalkbrood and sacbrood) in the selected hygienic colonies was 80% less than those in non-selected ones. Daughter queens from these selected colonies were sent to centres throughout the country for additional evaluation (M A Palacio, personal communication).

Breeding programmes using the frozen-brood test to select for hygienic behaviour have been initiated in the USA by researchers[39,78] and by a co-operative of beekeepers[26]. These programmes were begun primarily to combat varroa. The breeding programme by M Spivak at the University of Minnesota began in 1993 with two colonies of Starline bees that consistently removed 100% of the freeze-killed brood within 48 hours. Daughter queens from one colony were crossed to drones from the other colony, and vice versa, using instrumental insemination. To ensure adequate genetic variability, each year one or two new queens of Italian origin have been included in the breeding programme. The queens to be included are selected from among groups of queens that are purchased from various commercial queen producers throughout the USA. The colonies with these queens are evaluated over the course of a year for honey production, wintering success and hygienic behaviour. Daughters of the best performing, most hygienic queens are inseminated with a mixture of sperm of drones from the most hygienic colonies of the previous generation already in the breeding programme to create a new subline. As of the summer of 1998, there are nine sublines in the programme, a minimum of 10 colonies from each subline are maintained through the winter for continued evaluation, propagation and research purposes.

The performance of hygienic colonies from the breeding programme at the University of Minnesota was evaluated in commercial apiaries to determine whether colonies with naturally-mated queens from this hygienic line of Italian honey bees would have lower levels of varroa, less disease and produce a larger honey crop than commercial lines of Italian bees not selected for hygienic behaviour[78]. In previous studies on the relation between hygienic behaviour and resistance to diseases and mites, the test colonies contained instrumentally-inseminated queens[21,23,70,77]. This was the first study in the USA to evaluate hygienic stock in large field colonies with naturally-mated queens. The results of tests conducted in 1995 and 1996 revealed that the hygienic

colonies removed significantly more freeze-killed brood than the commercial colonies, had significantly less chalkbrood, had no AFB, and produced significantly more honey than the commercial colonies[78]. No treatments were applied to the colonies to control for mites for one year. Estimates of the number of varroa on adult bees at the end of one year indicated that the hygienic colonies had fewer mites than the commercial colonies in three of four apiaries, although the numbers of mites within all of the colonies were very low.

The experiment was repeated in 1997 comparing the hygienic colonies to a line of bees renowned for honey production (Starline bees). The same procedures were followed, but the colonies were left untreated for a longer period. Preliminary findings indicated that the hygienic colonies had significantly less chalkbrood, no AFB, and produced as much honey as the Starline colonies. Although the infestation levels of varroa were considerably higher after the first year than they were in the previous test, the hygienic colonies had significantly fewer mites than the Starline colonies across all apiaries[80].

These results demonstrated that colonies with naturally-mated queens from a selected line of hygienic bees have less disease and fewer mites than colonies not selected for the behaviour. The fact that the hygienic colonies produced as much, if not more, honey than the unselected colonies indicates it is possible to select for hygienic behaviour without compromising honey production. The reduction in diseases and parasite pressure may lead to increased colony populations and hence, to more foragers. No negative characteristics were apparent in the hygienic colonies; however, there could be negative fitness effects associated with removing diseased brood that have yet to be substantiated[77].

Conclusions

Hygienic behaviour of honey bees provides multiple benefits for beekeepers with no apparent negative characteristics that accompany the trait. Breeding stock can be selected for hygienic behaviour, and established methods of queen rearing can be used to produce large numbers of hygienic queens from a few breeder queens. Any race of bees can be bred for hygienic behaviour.

Most colonies headed by queens from commercial breeders have very low hygienic behaviour, and only a small percentage of managed colonies today express the behaviour. It will be necessary to have many queen breeders that will select for the behaviour among their own lines of bees to maintain genetic variability within and among bee lines and to increase the behaviour in the general population of honey bees. Commercially available lines of productive, hygienic bees would greatly benefit the beekeeping industry by ameliorating the effects of AFB, chalkbrood and varroa; reducing beekeeper dependence on chemical controls, and decreasing the contamination of bee products with pesticides and antibiotics. Selection for hygienic behaviour should be a routine component of bee breeding.

The assays used to determine hygienic behaviour are screening procedures. Subsequent challenge with a specific pathogen or parasite is necessary to assure that the colony is resistant to that particular malady. With microbial pathogens, this is most easily accomplished by introducing diseased larvae rather than cultures of micro-organisms into the colony. Chalkbrood mummies can be fed in pollen diets, and small comb sections with brood suffering from other diseases such as AFB can be used as inocula.

It must be emphasized that selection for resistance to diseases and parasites cannot

be done while colonies are being treated with chemicals since the chemical does not allow the pathogen or mite to act as a selective agent. Thus, natural resistance will be masked.

Research has clearly demonstrated the benefits of hygienic bees. Beekeepers should be using this information to improve bee stock.

Acknowledgements

We thank A Collins, G Hunt, and B Oldroyd, W Rath, and H Shimanuki for their helpful comments on both parts of the mansucript. Research mentioned in this review was funded (to MS) by the National Science Foundation IBN-9722416, Agricultural Utilization Research Institute PRO-130 and 310, and USDA-SARE 97-COOP-1-4132, with matching funds from beekeeping associations in MN, WI, ND, SD, IA, and CA. This is contribution #98-1170011 from the Minnesota Agriculture Experiment Station.

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