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Evaluation of Australian commercial honey bees for hygienic behaviour, a critical character for tolerance to chalkbrood

B. P. Oldroyd

School of Genetics and Human Variation, La Trobe University, Bundoora, Vic. 3068, Australia.
Present address: School of Biological Sciences A12, University of Sydney, NSW 2006, Australia.

Summary. Chalkbrood of honey bees (*Apis mellifera*) is caused by *Ascosphaera apis*, and is new to Australia. As yet, no treatment or prophylaxis is available for this disease. The best prospects for control are likely to come from the use of 'hygienic' bees, those with a strong genetic tendency to uncap and remove dead pupae, together with good beekeeping practice. Ten strains of Australian commercial honey bee were evaluated for hygienic behaviour. Dead pupae were inserted into the colonies and checked after 3, 5 and 7 days for the number of pupae removed. Most colonies (80%) were non-hygienic and hence likely to be susceptible to chalkbrood. However, 2 strains provided good overall performance in the test and comprised 1 or 2 colonies that were highly hygienic. Colonies were evaluated 3 times, and the good performance of these colonies was repeatable across trials. These data suggest that hygienic behavioural morphs exist in Australia's commercial bee strains, and it is unnecessary to obtain breeding stock from overseas for this reason alone. Selective breeding, with relatively simple techniques which can be used by beekeepers and queen breeders, should produce suitable genotypes.

Introduction

Chalkbrood of honey bees (*Apis mellifera*) is caused by the heterothallic fungus *Ascosphaera apis* (Gilliam and Vandenberg 1990). Only older larvae (>3 days) are susceptible to infection. Infection occurs via ingestion of the fungus, if it is fed to larvae by contaminated nurse bees (Heath 1982; Gilliam and Vandenberg 1990). Larvae which die are usually killed in the prepupal stage. If they are not subsequently removed by the bees, they dry out and take on a characteristic mummified appearance. These mummies are grey or black if the fungus sporulates, but are otherwise white. Nurse bees remove the mummies, which can often be found on the ground outside infected colonies (Heath 1982; Gilliam 1990).

The fungus is new to Australia (Matheson 1993; White 1993), and its initial impact on the honey industry is likely to be relatively severe due to beekeepers' inexperience with the disease and a high proportion of susceptible honey bee genotypes. Adverse affects are likely to be greatest in the queen production sector due to the high susceptibility of small mating nuclei to the disease and to quarantine restrictions placed on the sale of queens.

Ascosphaera apis is extremely infectious (Mehr *et al.* 1976), and has spread from the initial site of introduction in Queensland in 1993 (White 1993) to Victoria in less than 2 years (M. A. Z. Hornitzky pers. comm.). Rapid spread was probably facilitated by fungal spores on drifting bees and contamination of shared water sources (Koenig *et al.* 1987), the long survival time of spores (>12 months) (Hale and Menapace 1980), and the migratory nature of the industry. However, individual larvae and colonies may remain unaffected despite exposure to the pathogen. This phenomenon has led Gilliam (1986, 1990) to propose that the severity of the disease is related to environmental factors such as nutrition, temperature and humidity.

Chalkbrood cannot be effectively controlled with chemotherapy or comb sterilisation (Gilliam 1978; Heath 1982). The best prospects for control are the use of resistant bees and good beekeeping practice (Heath 1982).

Resistance to chalkbrood is probably not related to physiological tolerance, although this exists (de Jong 1977), but to good nest cleaning behaviour (Gilliam *et al.* 1983; Taber and Gilliam 1987). Young bees of most colonies clean up hive debris and dead bees or brood and remove them from the hive. This behaviour is usually termed 'nest cleaning' or 'cell cleaning'. Rothenbuhler (1964*a*, 1964*b*) demonstrated that cell cleaning behaviour is strongly influenced by 2 unlinked genes which control uncapping and removing respectively of dead or diseased pupae. This model has been challenged (Moritz 1988), but it is generally agreed that a small number of genes have a large effect on nest

cleaning behaviour. Bees which uncap cells and remove dead pupae in less than 72 h are defined as 'hygienic' (Spivak and Gilliam 1993), while those that tolerate dead brood in the colony for extended periods (>5–10 days) are termed 'non-hygienic' (Taber 1982; Taber and Gilliam 1987).

Gilliam *et al.* (1983) demonstrated that colonies that display a high level of hygienic behaviour have lower levels of chalkbrood. M. Spivak (pers. comm.) claims that strongly hygienic colonies remain permanently free of chalkbrood symptoms despite exposure to the pathogen. There is a good correlation between uncapping and removal of freeze-killed brood and resistance to chalkbrood (Milne 1983*a*; Gilliam *et al.* 1988). Since hygienic behaviour is advantageous in eliminating chalkbrood, it is surprising that hygienic behaviour is not fixed in all honey bee populations—many bee colonies tolerate high levels of diseased brood. However, it may be that for some other bee diseases, hygienic behaviour is actually disadvantageous (Spivak and Gilliam 1993). Nurse bees that clean out infected brood cells can spread disease when they subsequently feed larvae. Spivak and Gilliam (1993) observed that in some non-*Apis* bee species, the usual response to diseased pupae is to seal off the infected cell.

Since there is a need for the Australian honey industry to identify strains of bees resistant to chalkbrood, this paper evaluates 10 commercially available stains for this character.

Materials and methods

Queen bees for this study were obtained from 10 different sources. They were purchased from queen producers and honey producers in New South Wales, Victoria, South Australia and Tasmania. The sample included strains stated by the vendors to be derived from the 3 races of bees (*Apis mellifera*) used commercially in Australia (*ligustica*, *caucasica* and *carnica*). In selecting queen vendors, a representative sample of the genetic material available to Australian beekeepers in the spring of 1994 was obtained. Precise details of queen origins cannot be provided, as it was a condition of the funding body that supported this work that details on individual queen producers be kept confidential.

Each strain purchased comprised 10 replicate open-mated sister queens. Queens were received by mail and introduced to 100 populous 8-frame colonies which had been standardised with respect to population size and food reserves on 11 November using standard methods (Laidlaw and Eckert 1962). Queens were randomly allocated to colonies, and colonies randomly distributed in an apiary at La Trobe University in Melbourne. At the time of introduction, each queen was uniquely identified by clipping her wing and gluing a numbered coloured disk (Opalithplättchen Graze, Germany) to her thorax. Experiments commenced when worker bees from the original colonies had died of old age (about 5 weeks). Some introductions failed, and some queens died during the test. As a result, most strains were evaluated with less than 10 colonies.

To test for hygienic behaviour, a modification of methods described by Taber (1982), Gilliam *et al.* (1983) and Spivak and Gilliam (1991, 1993) was used. Combs of sealed brood (i.e. pupae) were obtained from populous colonies unrelated to the colonies under test. Small pieces (5 cm^2 and containing about 100 pupae per side) were cut from these combs using a sharp knife and a template. Comb sections were wrapped in absorbent paper and placed in a freezer at –20°C until required. Freezing killed the pupae.

Three pseudo-replicate experiments were then conducted commencing on 3 January, 16 February and 3 March 1995 (trials 1, 2 and 3 respectively). On each occasion, half of the colonies were challenged with dead brood on day 1 of the trial. The second half of the colonies were challenged the next day (this protocol was necessary for logistical reasons). Using a template, a section of comb was cut from the colony to be challenged, and a section of freeze-killed brood carefully inserted. A piece of overhead transparency acetate sheet was then placed over the test section and held in place with drawing pins. The precise location of every sealed brood cell was then marked with indelible ink on the acetate sheet which was then removed and stored.

On the third day after the colonies were challenged with the dead brood (about 48 h), the acetate sheets were again pinned over the test area in precisely the same location as they were on the first day of the test. Using a different coloured pen, all cells from which pupae had been removed were recorded on the sheets. Data were again recorded after 5 and 7 days.

For each colony and time, data were recorded as the proportion of dead pupae that had been removed by the bees. For tests of significance, these data were arcsine-transformed which helps normalise data of this type (i.e. proportions) (Steel and Torrie 1980). Data were tested for homogeneity of variance among strains, and no significant heterogeneity was detected. Within trials, transformed data were analysed as repeated measures analyses of variance.

The (untransformed) mean proportion of brood removed on each of the 3 days of each trial was calculated and the ranks of the strains obtained. Spearman rank correlations (Steel and Torrie 1980) were then computed from the ranks to determine if the performance of strains in removing dead brood was consistent over the 3 trials.

Results

When data for the 3 sampling days of each experiment were combined, significant overall differences in the rate of removal of dead brood were found among strains for

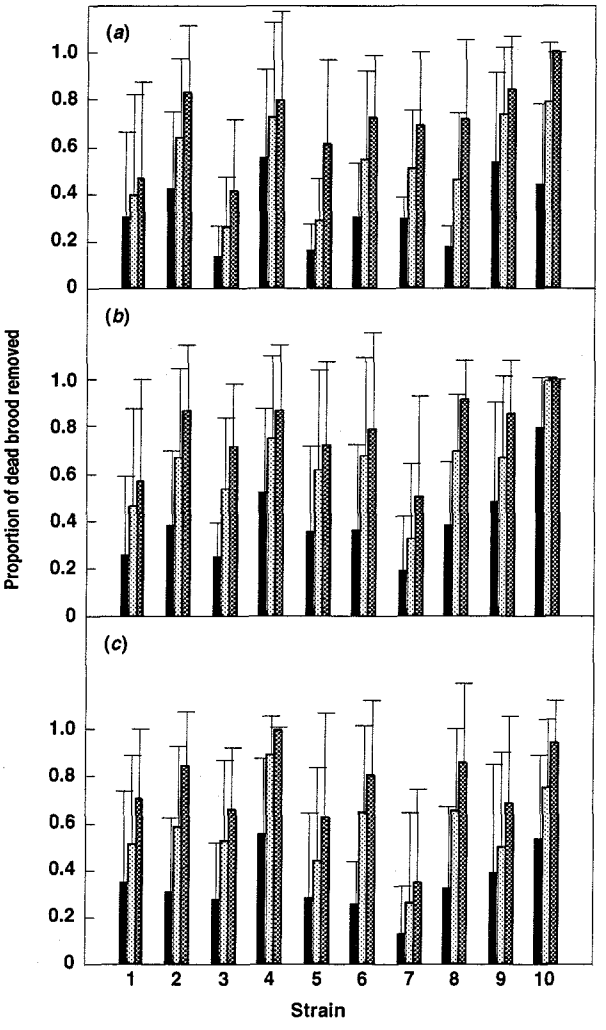

Figure 1. Mean proportion of dead brood removed by 10 strains of Australian commercial honey bees. Trials commenced on (*a*) 3 January 1995, (*b*) 16 February 1995, and (*c*) 3 March 1995. Brood were removed after 48 h (day 3) (solid bars), on day 5 (lightly stippled bars) and day 7 (heavily stippled bars). Error bars indicate s.e.

the first and second trials, while differences were less pronounced for trial 3 (Fig. 1). On the more critical measure of removal after 48 h (day 3), there were significant differences among strains for trials 1 and 2 (P = 0.051 and 0.015 respectively) but not for trial 3 (P = 0.43). In these analyses, there were no significant strain x day interactions (P = 0.31, 0.36 and 0.13 for trials 1–3 repectively). This suggests that using the amount of brood removed after 5 days may be a satisfactory measure of hygienic behaviour.

The mean proportion of dead pupae removed by each strain on day 3 was significantly correlated over the 3 trials, indicating that differences among strains were consistent in different months of the year (Table 1). The first trial was conducted during a period of abundant pollen supplies, while colonies were expanding in population. The second trial was conducted during a period of heavy nectar production, mainly from *Eucalyptus camaldulensis*. The third trial was conducted during a nectar dearth, when bee populations were contracting. However, despite the significant and consistent differences among strains, there was a great deal of within-strain variability (Table 1). All strains had some colonies that performed poorly, whereas the worst strains contained no individual colonies that performed well (Table 1).

Hygienic colonies are defined here as those that remove all dead pupae within 48 h, our earliest sampling period (day 3). By this definition, 15 colonies displayed sufficiently efficient nest cleaning behaviour on at least one occasion to be likely to be disease resistant. This suggests that about 20% of Australia's commercial bee colonies would show good hygienic behaviour and have considerable if not complete resistance to chalkbrood. Of these 15 colonies, one was hygienic in all 3 trials, 4 were hygienic on 2 occasions, and 10 once. Strains 1, 4 and 9 contained hygienic colonies in all trials (Table 1). Strains 4 and 10 showed consistently good average performance over all trials over most colonies (Fig. 1).

Discussion

The aim of this study was to determine if bee genotypes commonly used in Australia are likely to show resistance to chalkbrood via effective nest cleaning behaviour. The majority (about 80%) of colonies evaluated did not show adequate performance in the hygienic behaviour test, and would be likely to be susceptible to chalkbrood (Milne 1982; Taber 1982; Gilliam *et al.* 1983; Milne 1983*a*, 1983*b*; Spivak and Gilliam 1993). However, of the 10 strains evaluated, 2 (strains 4 and 10) provided good overall performance in the test (Fig. 1) and contained 1 or 2 colonies that were highly hygienic. The good performance of these superior strains and colonies was repeatable across trials in different seasons. These data suggest that hygienic behavioural morphs exist in Australia's commercial bee strains, and it is unnecessary to obtain breeding stock from overseas for this reason alone. Selective breeding, with relatively simple techniques which can be used by beekeepers and queen breeders, should produce suitable genotypes. If these genotypes can be identified and used, problems associated with chalkbrood should be largely alleviated. If no selection is undertaken by the industry, then the high proportion of susceptible colonies being used at this time is predicted to result in significant losses due to chalkbrood.

Honey bee queens mate on the wing with about 10–20 males (Page 1986; Estoup *et al.* 1994) drawn, presumably at random, from the local population (Loper *et al.* 1987; Pechhacker 1994, but see Koeniger *et al.*

Table 1. Proportion of dead brood removed by ten Australian strains of honey bees after 48 h

Rem., proportion of dead pupae remaining; Min., amount of dead brood removed in the worst colony; Max., amount of dead brood removed in the best colony; *n*, number of colonies in strain; Hygienic, number of colonies which removed all dead brood

Strain	Trial 1					Trial 2					Trial 3				
	Rem.	Min.	Max.	*n*	Hygienic	Rem.	Min.	Max.	*n*	Hygienic	Rem.	Min.	Max.	*n*	Hygienic
1	0.30	0.0	0.81	7	0	0.26	0.0	1	8	1	0.34	0.06	1	7	1
2	0.41	0.04	1.0	10	2	0.38	0.04	0.90	10	0	0.30	0.04	1	10	1
3	0.13	0.01	0.39	8	0	0.25	0.11	0.45	8	0	0.27	0.03	0.79	8	0
4	0.54	0.03	1.0	8	1	0.52	0.06	1	9	2	0.55	0.23	1	8	1
5	0.16	0.02	0.31	6	0	0.35	0.07	1	6	1	0.28	0.0	0.94	6	0
6	0.30	0.06	0.66	9	0	0.36	0.01	0.99	9	0	0.25	0.02	0.62	9	0
7	0.29	0.17	0.42	5	0	0.19	0.01	0.56	6	0	0.13	0.0	0.49	5	0
8	0.17	0.02	0.26	6	0	0.38	0.10	0.85	6	0	0.31	0.04	1	6	1
9	0.53	0.04	1.0	9	2	0.49	0.02	1	9	1	0.38	0.01	1	9	3
10	0.43	0.08	0.97	9	0	0.80	0.40	1	10	2	0.53	0.12	1	10	2
Mean	0.72					0.80					0.77				

Spearman rank correlations between mean strain performance over trials ($n = 10$ strains):

	Trial 1	Trial 2
Trial 2	0.77**	
Trial 3	0.74**	0.80**

**, significant correlation at $P < 0.01$.

1989). Because of this system of mating, most colonies used for honey production are comprised of workers whose maternity is known and selected, but whose paternity is unknown and unselected. Can hygienic colonies be provided to the industry given these biological and genetic constraints? Let us assume that Rothenbuhler's genetic model of hygienic behaviour is substantially correct, and that the major alleles conferring hygienic behaviour are recessive (Rothenbuhler 1964*a*, 1964*b*). Let us further assume that queens used as breeding stock to produce queens for sale can be mated only with males carrying hygienic alleles (this is a valid assumption since queens used for breeding stock are usually mated in isolation with a controlled population of males, or by artificial insemination). Under these assumptions, it should be perfectly possible for queen breeders to produce queens for sale which are homozygous for the alleles which confer hygienic behaviour. Trump *et al.* (1967) demonstrated that colonies comprised of 50% hygienic bees produced the hygienic phenotype. Therefore, provided that at least 50% of the males available for mating in queen production areas are of a hygienic genotype, it should be possible for queen producers to provide commercial queens whose daughter workers will have the hygienic phenotype.

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