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Resistance to Chalkbrood Disease in *Apis mellifera* L. (Hymenoptera: Apidae) Colonies with Different Hygienic Behaviour

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Abstract

Chalkbrood disease affects the larvae of honeybees *Apis mellifera* L. and is caused by the fungus *Ascosphaera apis*. Infected larvae die when they are stretched in the cap cell and suffer a gradual hardening that ends in a very hard structure (mummie). Several studies have demonstrated that colonies that express an efficient hygienic behaviour (uncapping of cell and subsequent removal of dead brood) exhibit a higher resistance to the disease. However, it remains unclear whether the advantage of hygienic colonies over less hygienic ones lies in the ability to remove mummies or in the early detection of infected larvae and its cannibalization before they harden. To elucidate this aspect, the hygienic behaviour of 24 colonies, which were subsequently provided with pollen cakes containig *A. apis,* was evaluated. The number of mummies and the number of partially cannibalized and whole larvae in uncapped cells were recorded. The most hygienic colonies controlled the disease better. These colonies also had a higher tendency to uncap cells that contained infected larvae and cannibalize them. The presence of *A. apis* in partially cannibalized and whole larvae in uncapped cells indicate that the advantage of hygienic colonies over less hygienic ones lies in the early detection of infected larvae death and their quick removal from the cell before they become mummies.

Introduction

Chalkbrood is a disease of honeybee (*Apis mellifera* L.) larvae caused by the heterothallic fungus *Ascosphaera apis*. The fungus spores enter the larvae by food and germinate in the hind end of the gut around the time when the cells are sealed (8-9 days of the cycle). Mycelia rapidly expand, cross the peritrophic membrane, reach the larva surface three days later, and continue their aerial growth. Most of the larvae die when they are stretched in the cell. Larvae killed by chalkbrood suffer a progressive change in their appearance. When white mycelium appears on the larva surface it gives the larva a fluffy appearance and, as it expands, the larva takes the hexagonal form of the cell. Subsequently, the dead larva begins to dry up, it separates from the cell walls and finally turns into a hard structure similar to a piece of chalk (mummy). If fruiting bodies are formed, the mummy becomes gray or black, otherwise it remains white (Bailey & Ball 1991, Gilliam & Vandenberg 1997).

Chalkbrood can be defined as a factorial disease since chilling of the larvae around the time the cells are capped, though only for a few hours, appears to be a requirement for the disease to express (Puerta *et al*

1994, Flores *et al* 1996).

Given the lack of effective fungicides, attempts to control chalkbrood disease by selecting colonies that express an efficient hygienic behaviour (uncapping of the cells and subsequent removal of the affected brood) are being made. This behaviour was widely studied in the 1960s in order to obtain colonies that were resistant to the American foulbrood, a brood disease caused by the bacteria *Paenibacillus larvae* (Rothenbuhler 1964a,b, Thompson 1964, Momot & Rothenbuhler 1971).

Several studies have shown that hygienic colonies reduce or eliminate the chalkbrood symptoms (Gilliam *et al* 1988, Spivak & Gilliam 1993, Spivak & Reuter 1998, Invernizzi 2001). Nevertheless, the review of previous investigations shows that, in general, they were based on the concept that hygienic bees are distinguished from the less hygienic ones by their ability to remove the affected larvae from the cells when they have already began the mummification process, especially when they are already dry and hard, form in which they can be found at the floor of the hive. Thus, the possibility of bees intervening from the moment the larva dies, before the mycelia spread throughout its body, for example by cannibalizing the larva, is not considered. In colonies resistant to the American foulbrood, bees were able to recognize the dead larva and to remove it from the cell before bacteria could form spores and spread inside the nest (Woodrow & Holst 1942).

In this study, it is sought to determine whether the hygienic behaviour of bees is an efficient mechanism of resistance to chalkbrood, considering the possibility that bees could detect and remove larvae killed by *A. apis* before the mummification process advances.

Material and Methods

Twenty four colonies of highly hybridized honeybees, originated from crossings of European bees (*A. mellifera mellifera*) mainly with africanized bees (*A. m. scutellata*) (Diniz *et al* 2003), were used. Colonies were in Langstroth hives and had enough place for storage of nectar and honey. None of the colonies presented symptoms of chalkbrood at the begining of the study.

The hygienic behaviour of the colonies was evaluated four (17 colonies) or three (7 colonies) times in October and November. On each test, approximately 130 prepupae (10-12 days old) were killed by piercing with an entomological pin through the cell cap (pin-kill test, Newton & Ostasiewski 1986). Twenty four hours later the number of cells uncapped by the bees was counted, determining the Uncapping Rate (UR) of the colonies. Only the uncapped cells were counted following the criterion suggested by Invernizzi (2000) based on the finding that colonies differ in their ability to recognize the dead brood and to uncap the cells, and not in the speed with which they remove the dead brood. During the period the tests were done, adult bee population covered the brood chamber completely, and the average breeding area varied between eight and 17 honeycomb sides (x ± DE: 11.7 ± 2.5).

On November 28th all colonies received polen cakes containing *A. apis*. Cakes were made by mixing commercial polen with sugar and water syrup at 1:1 (weight:volume), in which 300 mummies without spores (white mummies) and 300 with spores (black mummies) had been liquified (modified from Gilliam *et al* 1988). Each colony was provided with a quantity of cake proportional to its subadult population, so as to receive two mummies per side of honeycomb with brood.

The brood was inspected 8, 14, 19, 25 and 31 days after the contamination of the colonies. Visible mummies as well as partially cannibalized and whole 10-11 day-old larvae without mummification in bee uncapped cells were recorded. In every inspection, the cells included in a 31 x 14 cm grid were checked. The grid was always placed at the same site on both sides of the three central honeycombs of the brood chamber (approximately 10,400 cells). The day after each of the first four inspections, each colony, regardless of their brood population, received pollen cakes of equal size, prepared as previously described, but containing only six mummies. This procedure ensured that a complete breeding cycle (22 days) took place in all cells and, regardless of when they were occupied with larvae, the colony always had food contaminated with *A. apis*. In this way, it was not necessary to take into account brood age distribution (and therefore neither the number of larvae old enough to get sick) during the entire period of observation.

After the records ended, two slightly hygienic colonies (UR < 80%) and two highly hygienic ones (UR > 90%) were contaminated again using the technique earlier described. From these colonies, seven types of brood were collected: 1) 10-11 day-old cannibalized larvae from bee uncapped cells , 2) 10-11 day-old whole larvae from bee uncapped cells, 3) 10-11 day-old larvae, 4) 12-day-old larvae, 5) 13-day-old pupae, 6) 14-day-old pupae, and 7) 15-day-old pupae. Brood`s age determination was based on descriptions made by Jay (1964). Mycological analyses were made for all samples to determine the presence of *A. apis* by inoculating eight larvae or pupae on agar malta medium supplemented with 20% sucrose, and cultivating at 28ºC with 12h photoperiod. The growth of *A. apis* was checked three days later.

Results

The evaluation of the hygienic behaviour of the 24 colonies showed important differences in the average UR. Many colonies exhibited a high variability in the hygienic response at the different evaluations, being the most hygienic colonies the least variable ones (Table 1).

Table 1 Records of brood population (honeycomb sides), uncapping rate and standard deviation (UR ± SD), coeficient of variation (CV), number of mummies (M) and number of partially cannibalized and whole larvae (PCL-WL) during the period of study.

Colony	Brood	UR ± SD (%)	CV (%)	6/12		12/12		17/12		23/12		29/12		Total	
				M	PCL-WL	M	PCL-WL	M	PCL-WL	M	PCL-WL	M	PCL-WL	M	PCL-WL
1	12.0	95.6 ± 3.61	3.8	89	10	0	0	0	6	0	17	0	8	89	41
2	8.5	58.5 ± 19.25	33.0	27	0	3	26	3	14	1	5	2	15	36	60
3	10.0	42.0 ± 9.18	21.9	39	10	11	8	18	8	30	2	36	3	134	31
6	9.3	79.1 ± 15.27	19.3	48	0	16	3	18	5	13	7	22	3	117	18
7	11.8	95.7 ± 1.24	1.3	67	24	50	12	0	11	0	4	0	4	117	55
8	9.0	93.6 ± 5.93	6.3	0	10	0	11	0	8	0	4	0	10	0	43
9	9.0	93.4 ± 3.37	3.6	0	8	0	16	0	1	0	4	0	4	0	33
10	15.0	92.8 ± 6.80	7.3	24	10	0	4	0	4	0	2	0	0	24	20
11	11.3	73.8 ± 13.02	17.6	15	9	19	8	1	4	3	0	30	10	68	31
12	11.5	83.7 ± 13.28	15.9	0	15	0	1	0	0	0	11	0	0	0	27
14	11.3	73.2 ± 12.24	16.7	26	3	2	14	3	6	0	10	4	6	35	39
15	13.0	39.7 ± 15.82	39.8	38	2	11	4	4	1	12	3	13	1	78	11
16	8.0	72.4 ± 17.04	23.5	213	9	252	7	234	2	178	10	116	6	993	34
17	14.0	97.6 ± 2.03	2.0	0	8	0	0	0	1	0	1	0	0	0	10
18	10.5	77.3 ± 20.24	26.1	0	6	0	2	0	0	0	2	0	0	0	10
20	14.0	85.0 ± 11.91	14.0	156	1	158	5	40	5	9	23	34	14	397	48
21	10.0	51.2 ± 19.05	37.3	30	4	17	5	41	8	53	8	7	0	148	25
23	12.0	75.3 ± 12.39	16.5	0	3	0	0	0	0	0	2	0	1	0	6
24	12.0	68.3 ± 15.72	23.0	70	7	106	7	17	6	7	3	38	5	238	28
25	16.0	79.8 ± 19.96	25.1	5	3	0	7	0	3	0	9	0	8	5	30
26	8.3	79.9 ± 12.88	16.1	0	26	0	3	0	0	0	4	0	0	0	33
27	17.5	99.7 ± 0.58	0.6	0	5	0	2	0	1	0	3	0	0	0	11
28	14.0	95.5 ± 6.38	6.7	23	60	16	12	6	7	0	4	17	28	62	111
29	12.5	94.3 ± 3.97	4.2	13	24	0	0	0	1	0	0	0	1	13	26
Total				883	257	661	157	385	102	306	138	319	127	2554	781
Diseased colonies/healthy colonies				16/8		12/12		11/13		9/15		11/13			

Differences in the hygienic behaviour of the colonies did not appear associated with brood population (r = 0.35; P > 0.05; n = 24).

As a consequence of massive contamination of the colonies with *A. apis*, several colonies showed clinical symptoms of the disease in the successive inspections. However, the extent to which the disease manifested in the apiary was not constant during the period of study, with a larger number of mummies, average mummies and number of sick colonies in the first inspection than in the following ones. From the second inspection henceforth, the total number of mummies markedly decreased and the number of sick colonies stabilized. There were eight colonies that never showed symptoms of chalkbrood (colonies no 8, 9, 12, 17, 18, 23, 26, and 27) (Table 1).

The total number of mummies in the colonies was independent of the UR (r = - 0.19; P > 0.05; n = 24). However, when the analysis excluded colonies 16 and 20, which respectively presented 10 and four times more mummies that the average of the remaining sick colonies, colonies that presented higher UR had smaller total number of mummies (r = - 0.46; P < 0.001; n = 22) (Fig 1a). Considering the number of inspections in which colonies showed clinical symptoms, the most hygienic colonies were sick fewer times (r = - 0.66; P < 0.05; n = 24) (Fig 1b).

The number of uncapped cells containing partially cannibalized and whole larvae found in the colonies was not associated with hygienic behaviour (r = 0.18; P > 0.05; n = 24). Considering altogether, the number of partially cannibalized and whole larvae relative to the number of mummies at each inspection, the most hygienic colonies tended to uncap the cells and cannibalize the larvae before they mummified (r = 0.43; P < 0.001; n = 120) (Fig 1c).

Mycological analysis of the seven types of brood showed that from 77% to 90% of the partially cannibalized and the whole 10-11 day-old larvae found in uncapped cells devoloped *A. apis* colonies (Table 2). However, this amount decreased substantially for the 10-11-day-old larvae extracted from capped cells, especially in the highly hygienic colonies. Among the 12-day-old larvae and the pupae, the number of infected samples decreased to values even lower, at most 10%.

Discussion

The hygienic behaviour of honeybees constitutes a social mechanism for controlling brood diseases. The

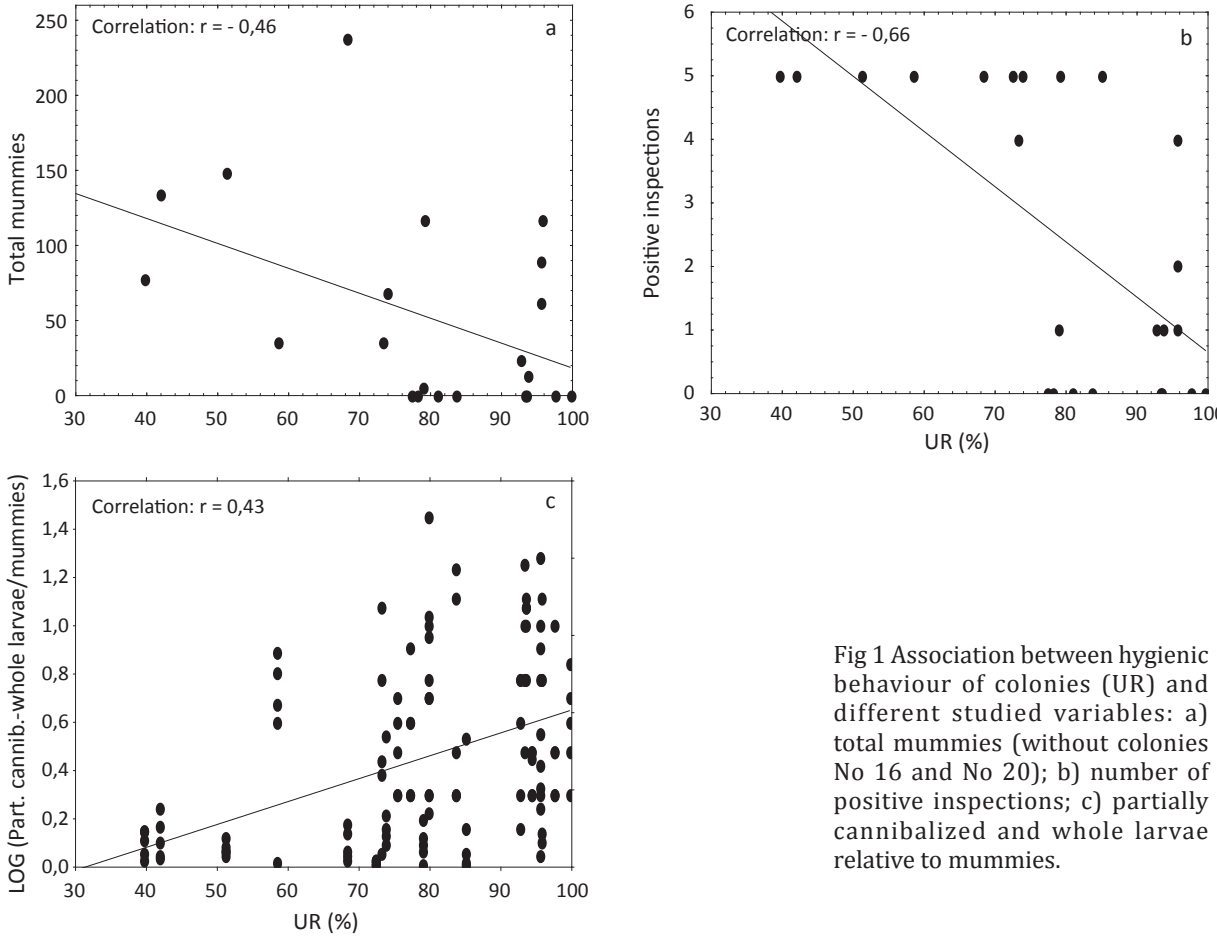

Fig 1 Association between hygienic behaviour of colonies (UR) and different studied variables: a) total mummies (without colonies No 16 and No 20); b) number of positive inspections; c) partially cannibalized and whole larvae relative to mummies.

Table 2 Mycological analysis of different types of larvae and pupae extracted from slightly hygienic colonies (UR < 80%) and highly hygienic colonies (UR > 90%) after providing them with pollen cakes containing *Ascosphaera apis*.

Type of brood	Slightly hygienic colonies			Highly hygienic colonies		
	No of larvae	No infected	%	No of larvae	No infected	%
10-11 days PCL	29	26	89.7	30	23	76.7
10-11 days WL	27	24	88.9	19	15	78.9
10-11-day-old	30	9	30	30	4	13.3
12-day-old	30	1	3.3	30	2	6.7
13-day-old	29	3	10.3	30	3	10
14-day-old	30	0	0	30	2	6.7
15-day-old	30	0	0	30	2	6.7

PCL: partially cannibalized larvae
WL: whole larvae in cells uncapped by the bees

expression of this behaviour in an efficient way allows the removal of diseased larvae from the hive before the pathogen can spread throughout the colony, for example by producing spores.

Differences in the hygienic behaviour presented by the honeybee colonies in this study were not associated with brood population. Usually, the latter accompanies the growth of the adult population. In here, the amount of adult bees was not recorded. Nevertheless, all the colonies had at least the breeding chamber full of bees. Invernizzi (1998) evaluated the hygienic behaviour of colonies during spring and summer, finding that it was expressed in a very variable way, although not affected by the natural increase and decrease of the bee population. Palacio (unpubl data) measured the hygienic behaviour of colonies with different amounts of adults and subadults in the population and found that none of these variables affected the expression of this behavior. Additionally, Gramacho (1995) studied the hygienic response of colonies of three different sizes without finding significant differences among them. However, Spivak & Gilliam (1993) found that hygienic behaviour decreases its expression when the size of colonies is artificially reduced.

The association between the efficiency of the hygienic behaviour and the lower presence of mummies found in the colonies along a breeding cycle indicates that behavioral resistance is an important mechanism of social control of chalkbrood. The option of providing the colonies with food containing a high concentration of *A. apis* at the beginning of the study and to continue the contamination with lower loads of the pathogen was conceived considering that part of the initial food supply would be stored as a reserve available for the following days, and that the presence of mummies with spores would cause reinfections in the brood. This phenomenon did not happen as expected, possibly because the reinfection was not widespread and because the amount of *A. apis* provided in the last four pollen cakes was not enough to infect the necessary number of larvae.

Even though the colonies with high UR seemed to control the mummification of the brood, different colonies presented a great variation in the amount of mummies. For example, considering the colonies that showed clinical symptoms at the five inspections, the total amount of mummies ranged between 36 (colony No 2) and 993 (colony No 16) (Table 1). Several factors hardly controllable in field conditions may be affecting the number of larvae that get sick in each colony: 1) the use of the pollen cake depending on the adult population, the number of larvae to be fed, the amount of nectar and pollen that enters the colony, and the available pollen reserves; 2) the reinfection with spores from other mummies depending on the number of mummies with fruiting bodies; 3) the chilling of the brood; 4) the physiological resistance to the development of *A. apis*. Considering only the number of inspections in which the colonies exhibited clinical symptoms of chalkbrood, regardless of the magnitude of symptoms, the results also clearly showed that the most hygienic colonies kept a healthier condition.

The importance of hygienic behavior of honeybees as a mechanism of resistance to chalkbrood is supported by several studies in which the brood is artificially infected or the natural infection is analyzed (Gilliam *et al* 1988, Spivak & Gilliam 1993, Spivak & Reuter 1998, Invernizzi 2001). Recently, Tarpy (2003) provided a new perspective on the problem, reporting that genetically diverse colonies show lower variance in the expression of the hygienic behaviour and in the prevalence of chalkbrood than the least diverse ones. Thus, colonies with low genetic diversity, e.g. in cases where the queen mates few drones or are unevenly represented in the workers (low

effective number of matings), are more likely to acquire the disease.

The observation of cannibalism of 10-11 day-old larvae in cells partially or totally uncapped by bees, and the fact that most of them contained *A. apis* -infected larvae, suggest that the hygienic behaviour confer resistance to chalkbrood as infected specimens are eliminated before mummification. It is feasible to suppose that the early detection of larva death caused by the development of *A. apis* allows the bees to remove the larvae from cells before the fungus continues its growth and larvae mummify. In these cases the larvae with normal appearance are ingested by bees in a few hours (Invernizzi 2000). When the mummification process is advanced, it is very difficult for the bees to cannibalize larvae with much mycelium on the surface and they must wait to remove the whole larvae once the mummification process has ended and the larvae are not adhered to the cells. In highly infected colonies these larval forms are frequently found on the floor of the hive. Thus, the advantage of the most hygienic colonies in the control of chalkbrood would lie in the quick detection of larvae death and their early removal from cells before they become mummies and eventually form spores that will spread throughout the nest. This is the same process proposed by Woodrow and Holst (1942) to explain why hygienic bees are more resistant to American foulbrood.

Cases in which larvae collected from manually uncapped cells were positive to the presence of *A. apis* could correspond either to sick larvae that had not died yet or to physiological resistance to germination or growth of the fungus. Larvae present in cells uncapped by bees that were negative in the mycological analysis could have died because of other causes rather than chalkbrood, e.g. parasitized by the mite *Varroa destructor* or simply by chilling. The low proportion of prepupae and pupae that developed the fungus in the Petri dishes agrees with the results reported by Puerta *et al* (1994), and can be explained by the evacuation of the spores before the beginning of pupation.

It is curious how many beekeepers mistakenly assess as hygienic those colonies that present numerous mummies in the entrance floor when, in fact, they are colonies that were unable to disrupt the process of mummification of the larvae because they did not detect their death rapidly enough.

The way in which the bees detect brood death through the wax cap of cells constitutes a fundamental aspect to explain the differences in the hygienic behaviour. Masterman *et al* (2000, 2001) using the proboscis extension reflex (PER) conditioning, found that bees differ in their ability to recognize odours from mummies, and that hygienic bees have lower thresholds than the non-hygienic ones. Gramacho *et al* (2003), using the same technique, compared the response of bees that uncap cells against the ones that remove dead brood, and found that the former showed greater ability to recognize the mummies` odour.

In conclusion, we showed that honeybee colonies which express an efficient hygienic behaviour have a higher probability of controlling chalkbrood disease. The advantage of the hygienic colonies would lie in the quick detection of larvae death, which allows bees to cannibalize them before the mycelium expands and mummifies the larvae.

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