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Spores of *Ascosphaera apis* contained in wax foundation can infect honeybee brood

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Abstract

Chalkbrood disease in honeybees (*Apis mellifera* L.) is caused by an infection with *Ascosphaera apis*. Disease expression requires the consumption of fungal spores and a predisposing condition in the susceptible brood. *A. apis* spores within sheets of wax foundation could be a source of inoculum leading to chalkbrood, but it is also possible that these spores remain confined in the wax and do not contribute to disease. We have resolved this topic by chilling susceptible brood within wax combs built on contaminated foundation (using treatments of spores from 1 mummy and spores from 10 mummies) versus uncontaminated foundation. We found significantly higher levels of chalkbrood in brood exposed to the higher dosage. Our results demonstrate that foundation wax contaminated with spores of *A. apis* spores may be a source of chalkbrood in honeybee colonies.

Keywords: Honeybee; *Apis mellifera*; *Ascosphaera apis*; Chalkbrood; Spores; Disease; Wax foundation

1. Introduction

Chalkbrood disease in honeybees (*Apis mellifera* L.) is caused by an infection with *Ascosphaera apis* (Olive and Spiltoir) which affects the developing brood. Larvae ingest the fungal spores when feeding, permitting the disease to develop in the stretched larvae after sealing. The stretched larvae are killed and later dry, leaving a mummified cadaver reminiscent of a small piece of chalk, which become dark if fruiting bodies of the fungi are formed (sporulated mummies). This disease requires a predisposing condition in the susceptible brood for it to develop (reviewed by Heath, 1982). Larvae in the fifth stage, prior to and some hours after sealing, are most susceptible to the disease (Bailey, 1967; Puerta et al., 1994; Flores et al., 1996).

To preserve the wholesome and natural characteristics of honey and other beekeeping products, alternative methods must be developed to control chalkbrood, to avoid the use of fungicides, which risk contaminating hive products. An important precautionary measure that beekeepers can take is to avoid transferring wax combs between colonies that contain chalkbrood infection and spores, because the occur-

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rence of the disease is proportional to the quantity of circulating spores (Puerta et al., 1990).

Any material with *A. apis* spores that has contact with bees can result in disease transmission (reviewed by Heath, 1982; Gilliam, 1990). It has been speculated that spores within the wax foundation (the wax base used in beekeeping on which bees draw out wax cells for brood development or honey and pollen storage) are a source of infection. However, this conjecture could not be confirmed until techniques were developed to allow the experimental expression of clinical symptoms in a controlled way while maintaining the natural conditions of the beehives as far as possible (Puerta et al., 1994; Flores et al., 1996). Now, by chilling susceptible brood to predispose them to infection (Puerta et al., 1994; Flores et al., 1996), we have been able to demonstrate the role of these spores in the expression of chalkbrood disease.

2. Materials and methods

Sporulated mummies were ground to obtain spores of chalkbrood. The number of spores per mummy was quantified using a Neubauer counting chamber and a compound microscope (400×). We obtained a mean value of 80,000 spores/mummy.

Sheets of foundation were manufactured starting from commercial wax. The wax was melted in a microwave. We added the spores of the fungus when temperatures were below 80 °C. The sheets of foundation were made with 140 g of wax per sheet, pouring the wax in a manual wax foundation mould (Thomas®). The sheets cooled quickly and consequently, the time which the spores were maintained at this temperature was brief, minimizing the possibility of any exposure to this temperature affecting spore survival (Anderson et al., 1997).

Treatments consisted of foundation without spores (control) (A), foundation contaminated with spores from one mummy (approximately 571 spores/g wax) (B), and foundation contaminated with spores from 10 mummies (approximately 5710 spores/g wax) (C).

The experiment was performed in the Andalusian Center for Organic Beekeeping (Córdoba, Spain) during spring and early summer (2002), coinciding with the beekeeping season. Trials were carried out in three healthy Langstroth hives. Each colony was used to test all three treatments. Sheets of foundation were introduced progressively in the beehives. First, a sheet of control foundation was introduced into each colony. When the wax cells were drawn, and there were fifth instar bee brood in the cells, we introduced foundation with spores from one mummy and later, in the same way, we introduced foundation with spores from 10 mummies. The process was repeated three times per colony (therefore each treatment was repeated three times per colony). We began the research in spring, when there were large numbers of eggs and young larvae in the combs. The interval between introducing two successive treatments was 12–14 days. Also, we alternated the treatments in three consecutives repetitions in each colony (1°: control, 2°: one mummy, 3°: 10 mummies; 4°: control, 5°: one mummy, 9°: 10 mummies), and we repeated the experiment in three colonies; thus, we reduced the effect of climatic conditions.

The risk of becoming infected with chalkbrood disease was evaluated in newly capped brood (worker brood sealed within a period of 14 h) for each treatment comb. Unsealed fifth instar larvae (Rembold et al., 1980) were marked on plastic sheets. The brood combs were subsequently returned to the beehives and again removed after 14 h. Portions of combs with newly sealed brood were cut, removed and maintained in incubators at 25 °C and 60% relative humidity to chill the brood for 5 days. After that the cells were opened and the percentage of mummified larvae was determined (Flores et al., 1996).

In each test foundation we only used the first generation of bee brood, because cocoons from these larvae could confine the spores contained in the wax, being inaccessible to the following larval generations. When the combs were filled with honey or pollen, they were removed and replaced by a new sheet of foundation with the same treatment.

The data obtained were evaluated statistically using descriptive parameters (mean percentage of chalkbrood ± S.E.); analysis of variance (one-way ANOVA) and post hoc tests ('Tukey honest significant difference (HSD) test', $P < 0.05$). SPSS 8.0 for Windows.

3. Results and discussion

Results are shown in Table 1.

Table 1
Number of investigated cells (susceptible larvae) and mean percentage of chalkbrood ± S.E. in colonies receiving three different treatments: control foundation combs without spores (A), foundation combs contaminated with the spores from one mummy of chalkbrood (B) and foundation combs contaminated with the spores from 10 mummies (C)

	Treatment A control		Treatment B		Treatment C		One-way ANOVA among treatments
	Number of investigated cells	Chalkbrood (%)	Number of investigated cells	Chalkbrood (%)	Number of investigated cells	Chalkbrood (%)	
Colony 1	2176	0.76 ± 0.25	3106	2.46 ± 0.63	2108	4.44 ± 0.98	0.002
Colony 2	2184	2.12 ± 0.36	3153	2.81 ± 0.51	3398	3.42 ± 0.76	0.351
Colony 3	3178	2.85 ± 0.49	3347	3.52 ± 0.51	3018	4.02 ± 0.51	0.266
Total	7538	2.04 ± 0.26 a	9606	2.94 ± 0.32 ab	8524	3.84 ± 0.43 b	0.002

Overall means and S.E. followed by different letters (last row of table) are significantly different at $P < 0.05$ (Tukey, HSD).

The spores of *A. apis* contained within sheets of wax foundation could be the source of the chalkbrood for healthy bee colonies, but it is possible too that these spores remain confined in the wax, without being an important risk for the transmission of the disease. We have resolved this topic by chilling susceptible brood within wax combs built on contaminated foundation.

Statistical analysis showed that the high dose treatment had a significant effect on the incidence of chalkbrood symptoms (one-way ANOVA among treatments, $F = 6.746$, d.f. = 2, $P = 0.002$). In particular, Tukey HSD tests revealed significant differences were found between wax foundation without spores (treatment A controls) and wax foundation contaminated with the spores from 10 mummies (treatment C). We did not observe significant differences between treatment B (wax foundation contaminated with the spores of one mummy) and the controls or treatment C.

Nevertheless, 2.04% of the brood showed chalkbrood symptoms in the controls. It is possible that the commercial wax used for manufacture of foundation was contaminated. However, the results given in Table 1 show that colonies 2 and 3 had a statistically higher percentage of chalkbrood symptoms compared to colony 1 (one-way ANOVA among colonies within treatment A. $F = 6.329$, d.f. = 2, $P = 0.003$). We hypothesize that although the commercial wax was free of spores, the expression of chalkbrood was due to the circulating spores in each colony.

On the other hand, we did not find significant differences among colonies within treatments B (one-way ANOVA, $F = 0.969$, d.f. = 2, $P = 0.386$) or C (one-way ANOVA, $F = 0.434$, d.f. = 2, $P = 0.650$).

In contrast, when we analyzed the differences among treatments within each colony, we found significant differences among treatments for colony 1 (one-way ANOVA, $F = 7.386$, d.f. = 2, $P = 0.002$), but not for colony 2 ($F = 1.066$, d.f. = 2, $P = 0.351$) or colony 3 ($F = 1.355$, d.f. = 2, $P = 0.266$). However, we did observe higher disease levels for spore-contaminated combs in all colonies. Our interpretation is that colony 1 had less background contamination, and the infected wax foundation significantly increased the risk of larvae contracting chalkbrood. In contrast, colonies 2 and 3 had higher background quantities of spores, so any effect of the spores contained within the foundation was masked by the circulating spores.

We conclude that the spores of chalkbrood contained in foundation combs are a potential risk for the dispersion of the disease, and research should be conducted on preventives measure for the elimination of these spores.

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