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A comparison of methods to experimentally induce chalk brood disease in honey bees

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Abstract

Chalk brood in honey bee is a fungal brood disease caused by *Ascosphaera apis*. It is very difficult to reproduce chalk brood in a homogeneous way in a large number of colonies, therefore appropriate methods to experimentally induce and evaluate the disease would be useful for research. We inoculated experimentally the colonies, using three treatments: spores sprayed over combs, spores mixed with pollen and sugar syrup with spores. The collection of mummies in traps in the hive entrance, bottom board and combs showed a lack of efficacy in the quantification of the disease. A controlled chilling of the brood yielded very satisfactory results. Using this last technique, spores mixed with water sprayed over combs reached 90.63% of mummification, while colonies fed with spores mixed up with pollen reached 86.32%. The use of sugar syrup with spores (60.13% of mummification) proved to be less effective.

Key words: *Apis mellifera, Ascosphaera apis,* fungal disease, inoculations tests, chilling.

Resumen

Evaluación de métodos para la inducción de la ascosferiosis en la abeja de la miel

La ascosferiosis en la abeja de la miel es una enfermedad de la cría causada por el hongo *Ascosphaera apis*. Es difícil reproducir esta enfermedad de forma homogénea en un alto número de colmenas, por ello sería útil para la investigación hallar un método apropiado para inducir el proceso de forma controlada. Inoculamos experimentalmente las colmenas utilizando tres tratamientos: esporas fumigadas sobre los panales, esporas mezcladas con polen y esporas suministradas en jarabe azucarado. La recolección de cadáveres de la cría afectada en trampas situadas en las entradas de las colmenas, los fondos y los panales carece de eficacia si se pretende cuantificar la enfermedad. El enfriamiento controlado de la cría ofrece resultados satisfactorios. Usando esta última técnica, las esporas del hongo fumigadas sobre los panales alcanzaron un 90,63% de eficacia, mientras que las esporas mezcladas con polen mostraron un 86,32% de eficacia. Por el contrario, cuando las esporas fueron suministradas en un jarabe azucarado, la efectividad se redujo al 60,13%.

Palabras clave: *Apis mellifera, Ascosphaera apis,* enfermedad fúngica, ensayos de inoculación, pollo escayolado.

Introduction

Chalk brood in honey bee is a fungal disease affecting streatched larvae. These infected larvae then become black or white mummies. The name of the disease refers to the appearance of dead larvae, resembling a piece of chalk (reviewed by Moeller and Williams, 1976; Heath, 1982; Puerta *et al.*, 1989; Gilliam, 1990).

The increase in importance of the varroa mite and the decline of frecuency of chalk brood have relegated this fungal disease from the foreground of bee pathology (Puerta *et al.*, 2001), but it is still a sanitary problem for beekeepers. Research is needed as long as a large number of questions about the disease and its control still are waiting for solutions.

Spores of *Ascosphaera apis* (Maasen ex Claussen) Olive and Spiltoir are the only material for the transmission of chalk brood. The spores germinate in the midgut of larvae, and the mycellium penetrates the larval cuticle when the larva is capped but before the pupal stage. Besides of the presence of spores, a set of predisposing conditions, some of them yet unknown, seems to be necessary for the disease to develop. These unknown conditions have obstructed the research on chalk brood, as the disease is not likely to be induced in a uniform way. Thus, many questions about pathogenicity, treatment and prevention of the

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disease are yet to be answered (reviewed by Moeller and Williams, 1976; Heath, 1982; Puerta *et al.*, 1989; Gilliam, 1990).

Several predisposing conditions have been pointed out. Only some of them appear to be effective: chilling of larvae susceptible to be infected around capping period (Bailey, 1967; Puerta *et al.*, 1994; Flores *et al.*, 1996), a low brood-to-nurse bees ratio or the introduction of empty combs or honeycombs in the middle of the brood area (De Jong, 1976; Koenig *et al.*, 1987; Befus-Nogel *et al.*, 1992). On the other hand, several methods for introducing spores of *A. apis* inside the colonies have been used, revealing no uniform results (De Jong, 1976; Mehr *et al.*, 1976; Gilliam *et al.*, 1978; Moffett and Wilson, 1978; Carrera *et al.*, 1987; Spivak and Gilliam, 1993).

The aim of this work was to evaluate appropiate techniques to experimentally encourage and quantify the disease under controlled conditions. For this purpose, we have compared different methods to introduce spores in the colony and to measure the incidence of chalk brood disease.

Material and Methods

The experiments were performed in the Andalusian Center for Ecological Beekeeping in Córdoba (Spain) from 24 April, 2001 (first inoculation) to 1 June, 2001 (final control of colonies), coinciding with the spring season. Trials were carried out in 12 Langstroth hives (5-7 brood combs and 8-10 combs covered of adult bees). Each one included a modified pollen trap (holes of 6 mm Ø, that allow pollen entry and keep the mummies in) placed in the entrance of the hive for collecting mummies.

Inoculation of spores into the colonies

Three inoculations were made on 24 April, 8 May and 22 May, and four treatments were applied (3 colonies/treatment):

— Treatment 1. In each colony all combs with adults bees were sprayed with 100 ml of a water suspension containing the spores of 15 black mummies (about $1{,}250 \times 10^6$ spores/hive). Spore concentration was quantified using an haemocytometer.

— Treatment 2. A similar amount of spores was supplied in 1 kg of glucose syrup (50% glucose in water). This syrup was put into a feeder located under the roof.

— Treatment 3. A similar amount of spores was supplied by crushing 15 black mummies in 150 g of pollen. This mixture was distributed on the top of combs.

— Treatment 4. Control. Colonies with no treatment whatever.

Quantification of the disease

We quantified the incidence of chalk brood using two methods:

— Method 1: Collecting mummies in pollen traps, combs and hive bottoms. Before the inoculations were carried out, colonies were checked for three days in pollen traps, combs and hive bottoms, and no mummies were detected. Mummies were collected in pollen traps each 3 days after the first inoculation (27 April), and up to the end of the experiment (1 June). Then, all the mummies from the bottom boards and the combs were collected. All sealed brood cells were opened for this purpose.

— Method 2: Inducing the disease by chilling any brood susceptible to infection. Two days after each inoculation of spores, a piece of comb containing unsealed fifth instar larvae (Rembold *et al.*, 1980) was taken away from the colony and chilled in a refrigerator (18ºC for 24 h). These larvae were returned back to the hive for 15 h to be sealed. After this period, the pieces of comb containing the brood were taken away from the colony again and were kept in an incubator at 25ºC for 5 days. Only sealed cells were checked out for mummification (Flores *et al.*, 1996).

For the statistical analysis of the results non parametric tests were used, Statistica/w 5.0 (STATSOFT, 1995). A Kruskal-Wallis ANOVA test was used in order to compare all treatments together and Mann-Whitney U test ($p < 0.05$) was used for inpaired comparisons of treatments.

Results

When comparing tests of inoculation with controls, using method 1 of quantification (collection of mummies in traps, bottom boards and combs), no significant differences were registered (Table 1). This is true if considering all collected mummies ($p = 0.1$), only

Table 1. Mummies of chalk brood produced by natural process and collected from colonies receiving spores of the pathogen using three different methods of inoculation and not inoculated control colonies (three colonies/treatment). Data are given as mean number of mummies ± standard error

Inoculation	Pollen traps	Combs	Bottom boards	Total
Glucose-water solution food	121.67 ± 113.23	0.00 ± 0.00	0.00 ± 0.00	121.67 ± 113.23
Feeding mixture with pollen	50.33 ± 48.37	90.67 ± 90.67	0.00 ± 0.00	141.00 ± 78.00
Sprayed in a water solution	9.50 ± 6.50	0.00 ± 0.00	0.00 ± 0.00	9.50 ± 6.50
Control	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

mummies collected in pollen traps ($p = 0.1$), only mummies collected in the bottom board ($p = 1$) and only mummies collected in the combs ($p = 1$).

When comparing tests of inoculation using method 2 of quantification (inducing the disease under controlled conditions) (Table 2), significant differences were registered between each treatment and the control, and among treatments themselves. Treatment 2 (spores in glucose syrup) showed less efficacy in inducing the disease if compared with spores sprayed in a water suspension or with spores mixed with pollen.

Discussion

The main problem for research in chalk brood is the difficulty to induce the disease in a controlled way (De Jong, 1976; Gilliam, 1978; Heath, 1982). Several authors have pointed out the technique of releasing a certain amount of spores into the colony, and letting the disease appear as a natural process. Nevertheless, in most cases chalk brood may appear at a low or an irregular rate. A replication of the experiment is then very difficult (Bailey, 1967; De Jong, 1976; Mehr *et al.*, 1976; Gilliam *et al.*, 1978; Gilliam, 1986; Befus-Nogel *et al.*, 1992; Herbert *et al.*, 1986).

In our research, when the disease was quantified by method 1, counting mummies in the hive (Table 1), none of the three methods used to inoculate spores proved to be effective: no significant differences were noticed when comparing our results with the control colonies, and a high variability was measured within each method of inoculation. This fact remarks the difficulty existing in the research of chalk brood disease directly in the hives, where a large amount of predisposing conditions proves the appearance of the process to be irregular.

When we induced chalk brood by chilling susceptible brood confirmed that spores were present to a high degree in all the inoculated colonies, in spite of the irregular rate of mummification showed by them when method 1 was applied. The convenience of encouraging the disease using a controlled stress may then be evident, if repetitive and uniform results are required (Table 2).

On the other hand, only around sealing brood is susceptible to chilling (Bailey, 1967; Puerta *et al.*, 1994; Flores *et al.*, 1996). When we study chalk brood directly on the hives, it is really difficult to control the distribution of the brood age. Due to this fact a same predisposing condition could induce different rates of mummification in the colonies, simply because a different amount of larvae susceptible to infection was present in each hive during the period of the experience. A similar fact may occur when results are given as a percentage of mummification with respect to

Table 2. Percentage of mummification: mummies of chalk brood in colonies using different methods to inoculate spores and not inoculated control colonies (3 colonies/method). Mummies were produced in pieces of combs containing brood susceptible to infection by a controlled chilling. In each colony, the treatment was repeated three times. Data are given as mean percentage of mummification ± standard error. Differences between methods of inoculation are showed by Mann-Whitney U test – non parametric statistics ($p < 0.05$)

Inoculation	Number of investigated cells	% of mummification
Glucose-water solution food	950	60.13 ± 6.74^{a}
Feeding mixture with pollen	871	86.32 ± 2.18^{b}
Sprayed in a water solution	1,084	90.63 ± 3.34^{b}
Control	1,018	5.92 ± 1.00^{c}

the total brood available in the hive, and the real number of susceptible larvae is not taken into account. It may be possible that this circumstance could account for any differences in susceptibility to chalk brood among colonies tested in some works (De Jong, 1976; Gilliam, 1978 and 1986; Herbert *et al.*, 1986; Spivak and Gilliam, 1993). In our research, such problems are avoided by the use of a specific number of larvae in a known stage of development.

The use of method 1 to quantify mummification led to some uncontrolled factors such as the hygienic behaviour of bees (Moeller and Williams, 1976). Colonies with a higher tendency to gnaw mummies or to remove any diseased larva before mummification may underestimate disease prevalence if compared with colonies that primarily removed dried hard mummies. The incubation of recently sealed larvae in an incubator (method 2) eliminates any problem of different removal behaviour of bees when evaluating the actual infectivity of the fungal spores.

A second matter refers to the most adequate method to introduce chalk brood spores in the hive. Several methods have been used by researchers. The methods mostly utilised are spraying a suspension of spores on the comb, supplying spores mixed in a sugar suspension made with honey, glucose or sucrose as part of a feeding syrup, feeding pollen mixed up with macerated mummies, or supplying spores mixed with a feeding mixture of pollen and sugars (De Jong, 1976; Mehr *et al.*, 1976; Gilliam *et al.*, 1978; Moffett and Wilson, 1978; Carrera *et al.*, 1987; Spivak and Gilliam, 1993). Our results showed that the use of spores in sugar syrup (method 1) was less effective in producing diseased larvae compared to methods 2 and 3: only 60.13% of the brood susceptible to the disease was affected. On the contrary, those fed with spores mixed with pollen (method 3) or sprayed with spores from a water solution (method 2), reached higher rates of mummification (86.32 % and 90.63%, respectively). A feeding mixture of spores with pollen seems to be the best choice as it is easier to carry out than spraying a spore water suspension on combs.

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