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The role of pollen in chalkbrood disease in *Apis mellifera*: transmission and predisposing conditions

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Abstract: Chalkbrood in honeybees (*Apis mellifera* L. Himenoptera: Apidae) is a fungal disease caused by *Ascosphaera apis* (Maassen ex Claussen) Olive and Spiltoir. This disease requires the presence of fungal spores and a predisposing condition in the susceptible brood for the disease to develop. In this study we examined the role of pollen in the development of chalkbrood disease under two experimental conditions: (i) pollen combs were transferred from infected to uninfected beehives and (ii) colonies were deprived of adequate pollen supplies to feed the brood. The results of both treatments confirmed that pollen is an element that should be taken into account when controlling this honeybee disease.

Key words: *Ascosphaera apis,* fungal disease, epidemiology, honeybee

INTRODUCTION

Chalkbrood disease in honeybees (*Apis mellifera* L.) is a fungal infection produced by *Ascosphaera apis* (Maassen ex Claussen) Olive and Spiltoir that 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. Furthermore this disease requires a predisposing condition in the susceptible brood for it to develop (reviewed by Heath 1982). Larvae in the fifth stage, before and some hours after sealing, are most susceptible to the possible stress that triggers the disease (Bayley 1967, Puerta et al 1994, Flores et al 1996).

Due to the lack of effective treatments and the need to preserve the wholesome and natural characteristics of beekeeping products (Puerta et al 1995, Flores et al 2001), alternative methods must be developed to control chalkbrood. To determine the best control measures, it is important to know more about how the disease is transmitted and the possible predisposing conditions that trigger the disease. However, until techniques were developed that allowed the experimental expression of clinical symptoms in a controlled way while maintaining the natural conditions of the beehives as far as possible, the various hypotheses that had been put forward regarding this disease could not be confirmed (Puerta et al 1994, Flores et al 1996).

In previous research (Puerta et al 1994) we experimentally induced clinical symptoms of chalkbrood by chilling the susceptible brood, the starting point for further research on the disease. Later, using this same technique, we studied other possible predisposing conditions, such as excess dampness (Flores et al 1996). Furthermore we confirmed the lack of effective products to control the disease (Flores et al 2001). Nevertheless, further research was required on other topics, namely the role of pollen in the transmission of fungal spores and whether insufficient pollen supply could be a predisposing condition.

Pollen is harvested by the honeybee as a source of protein and is consumed by adults so they can feed the brood, making it a key factor in the development of the colonies. Pollen combs may be agents of transmission of *A. apis* spores and other possible bee diseases because beekeepers frequently use pollen combs from other beehives or weakened, ill or dead colonies to feed other colonies (reviewed by Heath 1982, Puerta et al 2001).

In addition pollen is harvested by beekeepers by placing a pollen trap at the entrance to the beehives. The overuse of pollen traps may lead to an inadequate pollen supply in the beehives and has been proposed as another possible predisposing condition in the development of brood diseases such as chalkbrood (Cardenal et al 1990).

Thus pollen may play an important role in this disease of the bee brood. In this study we used chilled brood to examine the role of pollen combs from infected colonies as a source and agent of transmission of chalkbrood, as well as insufficient pollen supply as a predisposing condition in the development of the infection.

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TABLE I. Number of investigated cells in colonies receiving three different treatments: colonies with chalkbrood (A); initially healthy colonies in which pollen combs from infected colonies were introduced (B); and healthy colonies that did not receive infected pollen (C). The table shows the number of colonies/evaluation, total investigated cells/evaluation and mean ± SE number of evaluated cells/colony/evaluation

	Evaluations	0	1	2	3	4
A	N° of colonies	3	3	3	3	3
	Total cells	512	495	542	450	494
	Mean ± SE	170.67 ± 7.17	165.00 ± 9.45	180.67 ± 9.21	150.00 ± 2.52	164.67 ± 8.97
B	N° of colonies	4	4	3	3	3
	Total cells	582	581	490	466	516
	Mean ± SE	145.50 ± 11.65	145.25 ± 21.38	163.33 ± 13.54	155.33 ± 6.69	172.00 ± 5.03
C	N° of colonies	4	3	3	4	3
	Total cells	617	489	516	586	374
	Mean ± SE	154.25 ± 14.91	163.00 ± 1.53	172.00 ± 13.53	146.50 ± 13.29	124.67 ± 28.61

MATERIAL AND METHODS

Experiment 1: Pollen combs as agents of transmission of chalkbrood.—The experiment was performed in the Andalusian Center for Organic Beekeeping (Córdoba, Spain) May–Jul 2002, coinciding with the beekeeping season. Trials were carried out in three groups of four Langstroth hives.

A. The first group comprised colonies showing chalkbrood symptoms.

B. The second group was composed of colonies showing no chalkbrood symptoms in which two pollen combs were replaced with pollen combs from infected beehives.

C. The third group included colonies showing no chalkbrood symptoms that did not receive pollen combs from infected beehives.

The risk of becoming infected with chalkbrood disease was evaluated in newly capped brood (worker brood sealed 14 h). Unsealed fifth instar larvae (Rembold et al 1980) were marked on plastic sheets. The brood combs subsequently were returned to the beehives and again removed after 14 h. Portions of combs with newly sealed brood were removed and maintained in incubators at 25 ± 1 C and 60% relative humidity. Five days after sealing, the cells were opened and the percentage of mummified larvae was checked (Flores et al 1996). The number of investigated cells are provided (TABLE I).

The risk of developing the disease was evaluated five times in all treatments. The first evaluation occurred before introducing the infected pollen combs. The remaining four evaluations were done biweekly, beginning 3 d after introducing the infected pollen combs in Group B (FIG. 1).

Experiment 2: Overharvesting of pollen as a predisposing condition for chalkbrood.—The experiment was carried out Oct-Dec 2002. Trials were carried out in 15 Langstroth hives. Each colony was inoculated weekly for a total of four times (7, 14, 21 and 28 Oct) with the ground-up spores of five sporulated mummies (the spores/mummy average was quantified with a Newbauer chamber and a microscope with a magnification range of 400×, giving a mean value of 80 000 spores/mummy). To inoculate the colonies, we extracted approximately 100 g of live adult bees/colony from different brood, pollen and honeycombs. The sporulated mummies were ground and powdered on the bees and returned to their respective beehives. Afterward a sample was taken of susceptible brood from each colony to induce the disease as described in Experiment 1 (4 Nov). After determining the rate of development of chalkbrood in the colonies, the colonies were grouped accordingly to administer three different treatments (five colonies/treatment) (12 Nov):

A. Pollen reserves were removed from the colonies in the first group.

B. Pollen reserves were removed from the colonies in the second group and a pollen trap was placed in such a way as to impede the entrance of fresh pollen.

C. The third group of colonies was used as a control. All the stored pollen remained in the beehives and no impediments were placed on harvesting fresh pollen.

Six days after applying the treatments, clinical symptoms of the disease were evaluated. Sections of susceptible brood combs were removed weekly from each colony and the disease was induced as described in Experiment 1, for a total of three times (18 and 25 Nov and 2 Dec). The availability of brood decreased after the third evaluation, making it necessary to conclude the experiment. The number of investigated cells are provided (TABLE II). The data obtained were evaluated statistically using descriptive parameters, analysis of variance (one-way ANOVA and univariate analysis of variance) and post-hoc tests (Duncan's multiple range test, $P < 0.05$) (SPSS 8.0).

RESULTS

Experiment 1.—In the initial evaluation, significant differences were found among groups of beehives regarding the incidence of chalkbrood symptoms (one-way ANOVA, $F = 33.632$; $df = 2$; $P = 0.000$),

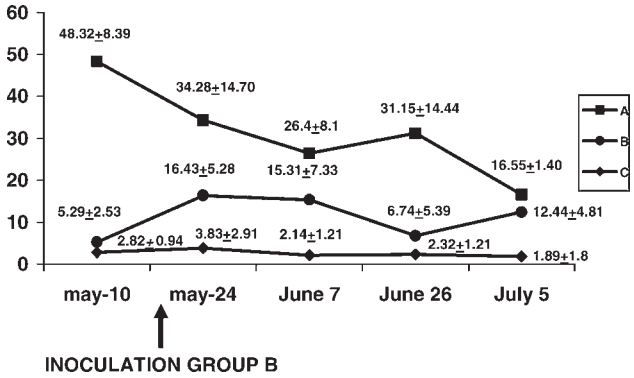

FIG. 1. Evolution of the risk of chalkbrood disease in colonies receiving three different treatments: colonies with chalkbrood (A); initially healthy colonies in which pollen combs from infected colonies were introduced (B); and healthy colonies that did not receive infected pollen (C). The results show the risk of disease before introducing infected pollen combs in group B (10 May) and at four times after the infected pollen was introduced (24 May, 7 and 26 Jun and 5 Jul). The results are expressed as the mean percentage of mummification ± SE.

in particular between the colonies that initially showed symptoms of chalkbrood and the other two groups of healthy colonies.

After introducing infected pollen combs in the initially healthy beehives (Group B), the disease was observed to increase. In contrast, the percentage of mummification in the initially sick colonies decreased. Finally the disease remained at a low level in the healthy colonies that had not received infected pollen combs (FIG. 1).

Statistical analysis (univariate analysis of variance) showed that the applied treatments had a significant effect ($F = 12.146$; $df = 2$; $P = 0.000$), resulting in three statistically different groups (Duncan's multiple range test, $P < 0.05$): the initially diseased colonies with a larger percentage of mummified brood; a second group composed of the initially healthy colonies in which infected pollen combs had been introduced; and a third group comprising the healthy colonies that did not receive infected pollen combs and which had the smallest percentage of mummification. A significant effect was not observed among evaluations within each treatment ($F = 0.6487$; $df = 3$; $P = 0.591$).

Experiment 2.—On the first evaluation date (4 Nov) significant differences in percentage of mummification were not found among the three groups of beehives (one-way ANOVA, $F = 0.046$; $df = 2$; $P = 0.955$) (FIG. 2). The first evaluation after removing the stored pollen of groups A and B (18 Nov) showed that chalkbrood decreased in the control group (C), levels that then were maintained during the rest of the experiment. In the group of beehives from which the stored pollen was removed (Group A), the disease remained at high levels until the last evaluation (2 Dec), at which time it decreased. Finally, in the group of colonies from which the stored pollen was removed and in which a pollen trap was placed to prevent the entrance of fresh pollen (Group B), chalkbrood initially decreased, then rose to the highest level and then dropped of the experiment (FIG. 2).

The results of the evaluations after treatment were analyzed statistically (univariate analysis of variance), showing a significant effect ($F = 2.744$; $df = 2$; $P < 0.072$). Differences were detected among the control group and the other two groups (Duncan's multiple range test, $P < 0.05$) (FIG. 2). However significant differences were not observed across dates within each treatment ($F = 1.584$; $df = 2$; $P = 0.213$).

TABLE II. Number of investigated cells in colonies receiving three different treatments: colonies from which the stored pollen was removed (A); colonies from which the stored pollen was removed and the harvesting of fresh pollen was impeded (B); and control colonies with stored pollen and freshly harvested pollen (C). This table shows the number of colonies/evaluation, total investigated cells/evaluation and means ± SE of evaluated cells/colony/evaluation

Evaluations	0	1	2	3
Group A	N° of colonies	5	4	5
	Total cells	920	379	637
	Mean ± SE	184.00 ± 28.24	94.75 ± 26.81	127.40 ± 9.09
Group B	N° of colonies	5	4	4
	Total cells	742	401	322
	Mean ± SE	148.40 ± 23.76	100.25 ± 21.23	80.50 ± 10.74
Group C	N° of colonies	5	4	4
	Total cells	638	354	386
	Mean ± SE	127.60 ± 26.65	88.50 ± 12.20	96.50 ± 24.30

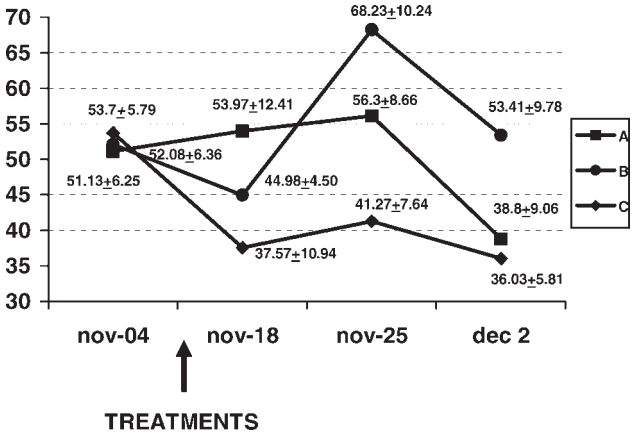

FIG. 2. Expression of chalkbrood disease in colonies artificially infected with *A. apis* spores that received three different treatments: colonies from which the stored pollen was removed (A); colonies from which the stored pollen was removed and the harvesting of fresh pollen was impeded (B); and control colonies with stored pollen and freshly harvested pollen (C). The results show the risk of disease before treatments A and B (4 Nov) and at three additional times after treatments (18 and 25 Nov and 2 Dec 2). Results are expressed as the mean percentage of mummification ± SE.

DISCUSSION

Initially any material that comes in contact with bees infected with *A. apis* spores, including pollen, can act as an agent for the transmission of chalkbrood (reviewed by Heath 1982, Gilliam 1990). Moeller and Williams (1976) and Nelson and Gochnauer (1982) suggested that bees could harvest pollen contaminated with *A. apis* spores from flowers after they found mycelial growth on this pollen in specific culture medium. Nevertheless it is possible for pollen to be contaminated by spores from forager bees (Koenig et al 1987). However we suggest that the small number of spores entering the beehives in this manner does not significantly increase the risk of infection because the amount of spores has a direct influence on the development of chalkbrood (Puerta et al 1990). It is likely therefore that the most important agent in the transmission of this disease is the transference of either live or inert contaminated material from infected hives (reviewed by Heath 1982, Gilliam 1990).

Pollen stored as beebread in infected beehives may be a source of chalkbrood spores (reviewed by Heath 1982). Nevertheless, due to the lack of appropriate techniques, it has not been possible to confirm this hypothesis. On the other hand research has been carried out along these lines with artificially infected pollen (Mehr et al 1976, Moffett et al 1978, Befus-Nogel et al 1992). In our research we used pollen combs from contaminated hives, approaching what could be considered common beekeeping practice.

Our results initially would seem to suggest that contaminated pollen has an immediate effect, leading to an increase in chalkbrood. Nevertheless this was possible in our experiment due to the fact that the susceptible brood was artificially chilled, immediately triggering the disease. However this does not normally occur in the apiaries of beekeepers, in which a delay in the performance of a possible predisposing condition may lead to the development of the disease at a later time. This gives rise to confusion of the beekeepers who do not associate the subsequent development of chalkbrood with poor management practices carried out at an earlier time.

In a second reading the results of our experiment have shown how the viability of spores increases the risk of chalkbrood. These findings agree with those by Hale and Menapace (1980) who found that the *A. apis* spores can remain viable at least 12 mo in beebread. When this is the case, the existence of a predisposing condition will trigger chalkbrood, producing new spores during the normal cycle of the disease. In contrast the absence of a predisposing condition will permit infected material to be eliminated gradually, thus decreasing the risk of chalkbrood. This is similar to what has been observed in the group of initially sick colonies, where the disease decreased, aided by the advantageous environmental conditions of the experiment (mild and stable temperatures and heavy nectar flow). It is possible that, if this experiment was carried out in fall, under worse climatic conditions and inferior nectar flow, the reduction of chalkbrood could be slower or the disease could even be increased. The same thing could happen in treatment B.

In addition 2.82% of the brood showed chalkbrood symptoms in treatment C (FIG. 1). We hypothesize that the expression of chalkbrood in this treatment was due to a small number of circulating spores in each colony.

Finally we cannot overlook the fact that pollen combs contaminated with *A. apis* spores constitute a reserve of infectious material in the beehive. Thus a measure of control in infected beehives should include either the substitution of infected pollen combs for pollen combs from healthy beehives or a pollen substitute.

On the other hand the deficit of proteins repeatedly has been considered a possible predisposing condition in chalkbrood (reviewed by Heath 1982, Gilliam 1990). It also has been suggested that the disease is aggravated as much by the supply of old pollen (reviewed by Heath 1982) as by the use of pollen traps (Cardenal et al 1990). These latter

authors, however, obtained contradictory results because pollen traps were found to be associated with the disease in a given beekeeping season, but not in the following one.

In our study we have examined two circumstances in which the deficit of pollen could act as a predisposing condition. In the first we removed the stored pollen, but let the bees harvest fresh pollen (Experiment 2; Group A), leading us to determine that this deficit has only an occasional impact on the development of the disease. In contrast, in the second, we removed the stored pollen and placed a pollen trap at the entrance to the beehive, thus achieving a long-term protein deficit (Experiment 2; Group B). In both cases, chalkbrood was aggravated compared to the control colonies, although the tendency seems to indicate that the risk of developing the disease remained higher in the colonies in which the pollen trap impeded the harvesting of fresh pollen. For this reason we recommend that beekeepers use pollen traps with caution, do not leave them on the beehives for long periods and closely supervise any pollen deficit. It is possible that, if this experiment was carried out in spring (mild and stable temperatures and heavy pollen entrance and nectar flow), the reduction of chalkbrood in treatments A and C could be quicker.

Finally the joint effect of both experiments must be considered. Although in Experiment B we placed a pollen trap on the beehive before removing the stored pollen, thereby causing a greater deficit of protein in the colonies, this would not occur in normal beekeeping practice. Beekeepers normally do not remove the pollen combs before using pollen traps, therefore letting the bees resort to this reserve supply to continue feeding the brood. This can cause the spores remaining in the beebread to recirculate and lead to two possible effects in the infected colonies. First, if the increased number of circulating spores does not coincide with a predisposing condition for chalkbrood to develop, it probably will have a beneficial effect by reducing the spores of the fungus in the colonies. On the contrary, if it coincides with a predisposing condition, the disease could increase in the colonies, producing more spores. This situation could be aggravated further by a persistent deficit of pollen, which then would act as a predisposing condition. Nonetheless further research must be carried out along these lines if we are to confirm these last suppositions.

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