

## Chalkbrood development in honeybee brood under controlled conditions

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**Summary** — Third instar larvae from a honeybee colony were fed with high doses of spores of *Ascosphaera apis*, the causative agent of chalkbrood disease. Optimal survival of spores was detected during a short period after sealing the cell and before larval spinning by culture of the gut contents removed from 4 stages of brood development. The inocula ( $5 \times 10^5$  spores/larva) did not induce the disease and were not present in the digestive tract before pupation. In a second experiment, third instar larvae, fed with the same amounts of spores as before, received a cooling stress ( $22 \pm 2^\circ\text{C}$ , for 24 h). When chilling was applied 24 h before or after operculation, mummification occurred in the majority of larvae (59.6 and 65.5%, respectively). Chilling of older brood (spinning larvae or pupa) produced a much lower incidence of chalkbrood. This confirms the need for predisposing conditions over a short period of brood development for the development of this disease.

**chalkbrood / *Apis mellifera* / *Ascosphaera apis* / predisposing conditions / stress / brood chilling**

### INTRODUCTION

The fungus *Ascosphaera apis* Maassen ex Claussen (Olive et Spiltoir) is the causative agent of chalkbrood disease in the honeybee *Apis mellifera* L. The infection seems to start inside the brood, once larvae have ingested spores. Most chalkbrood mummies are stretched larvae; younger brood seem to be more resistant. There is some controversy about the reason for this. In his review, Heath (1982), pointed this out and emphasized the requirement for predis-

posing conditions to lead to the development of chalkbrood. According to Heath, development of infection seems to be mainly dependent upon the physiological and environmental conditions of the larvae. From this viewpoint, *A. apis* might be considered an opportunistic pathogen whose process of infection in honeybee brood can be seen mainly in prepupae.

In order to improve management measures for the control of this disease, it would be useful to detect whether there is a critical period in brood development for the action

of these predisposing conditions when larvae are more susceptible to chalkbrood. A related question is whether the fungus incites the disease without any stress factors, if the amount of infectious inoculum ingested by the brood is high enough.

In the first experiment, we tested the course of spore survival throughout the development of the susceptible brood, feeding young larvae with *A apis*, and recovering the inocula from different brood stages.

In a second experiment, other groups of young uncapped larvae, also reared under controlled environmental conditions and fed with a known dose of *A apis* spores, were stressed with 1 of the most widely assumed factors in chalkbrood disease: chilling at different points in the larval and prepupal stages. The incidence of chalkbrood (mummies) was then registered. The objective was to detect the periods during brood development when chilling can induce the infection process in larvae.

## MATERIALS AND METHODS

### *Inocula*

A homogeneous suspension of free spores in sterile water was obtained from 15 black mummies (Gilliam *et al*, 1988), mixed with honey (50% w/v) and fed to larvae. Each larva received a single dose of  $5 \times 10^5$  spores in 5  $\mu$ l food, placed in the larval mouthparts. Consumption was confirmed by direct observation. Concentration of spores was checked with a haemocytometer.

### *Inoculation, recovery and culture of A apis*

Pieces of comb containing third instar larvae were collected from colonies of *A mellifera*. The experiment involved 80 third-instar larvae and was repeated. These 80 larvae were divided into 4 groups, each of which was sampled at a different

stage of development: (L3) third instar larvae 6 hours after being fed with the mixture; (SL) fifth instar spinning larvae; (PP) prepupae; (WP) white pupae (Rembold *et al*, 1980). Each of these 4 groups involved 20 larvae, 10 of which were controls that did not receive spores when fed. A fifth group (DI), was composed of 10 plates, inoculated directly with the suspension of spores, without being previously ingested by the larvae.

After feeding, larvae which subsequently become groups SL, PP and WP were reintroduced into the hive until the sealing of their cells. They were then kept in an incubator at 30°C and 70–80% relative humidity, until extraction from their cells. The alimentary canal of each larva or pupa was then surgically removed (Dade, 1977). Aseptic techniques were used throughout the procedure.

The gut content of each larva or pupa was inoculated onto a Petri dish (9 cm) containing MY20 medium (Takatori and Tanaka, 1982) and kept in a incubator under appropriate conditions for spore development (12% CO<sub>2</sub>, 30°C, 80% relative humidity) (Heath and Gaze, 1987). Cultures were examined daily for fungal growth. No new growth was detected after 120 h culture. This technique was also used to test spore survival of the inocula after several periods in the brood.

### *Rearing inoculated larvae under cooling stress*

A single dose of  $5 \times 10^5$  spores was administered to third instar honeybee larvae. Pieces of comb containing groups of 22–50 individuals were kept in a commercial hive until sealing of cells, and then transferred to an incubator (EBT-100, Lab Navarra, Spain), under appropriate conditions for development of larvae (30°C, 70–80% relative humidity). This incubator has a range of temperature between 0 and 60°C, and was programmed to chill the inoculated brood as follows.

Groups of previously inoculated larvae received a chilling stress ( $22 \pm 2^\circ\text{C}$ ) for 24 h at 1 of 4 periods of development: 24 h before sealing of cell (24 h BS); 24 h after sealing (24 h AS); 48 h after sealing (48 h AS); and 72 h after sealing (72 h AS). A control group received spores without being chilled. The test was repeated 5 times. Cells were opened 10 d after sealing to quantify the number of chalkbrood mummies.

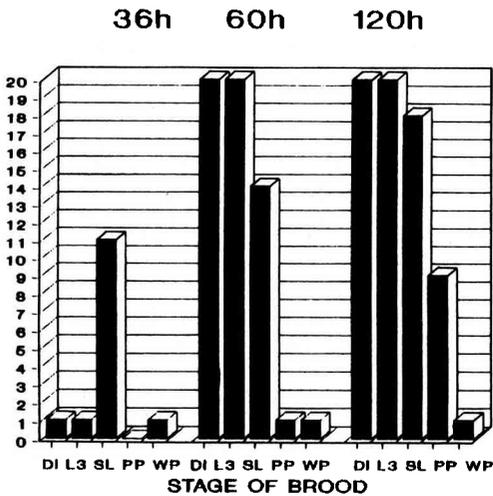
**Statistical analysis**

Data were analysed using a general linear models procedure (SAS Institute, 1982), which applied a 2-way ANOVA and a Tukey's studentised range test. Data of a group were considered to differ significantly from the others inside a 95% fiducial limit.

**RESULTS**

Figure 1 shows data concerning the feeding of larvae in order to subsequently recover the inocula.

Growth on group DI plates, whose spores were directly inoculated onto the media, was completed at 60 h of culture. This period is needed for spores of the inocula to develop



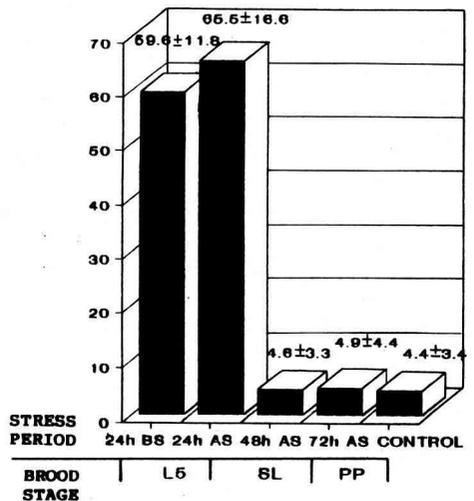
**Fig 1.** Plates with growth of *A. apis* after having been inoculated with spores removed from the digestive tract of honeybee brood. Spores of the pathogen were fed to the third instar larvae and removed from the gut content at 4 different times in brood development: L3: third instar larvae, 6 h after feeding; SL: spinning larvae; PP: prepupa; WP: white pupa. Group DI was directly inoculated onto the artificial medium, without being fed to the larvae. Each group involved 20 gut contents, which were individually inoculated onto the plates.

on artificial media. Only the spores from spinning larvae (SL), developed faster: 11 plates with growth in group SL; and only 1 in DI at 36 h of culture.

Spore survival at 120 h (no new fungal growth was detected after this time) for DI, third instar larvae (L3) and SL groups was 20, 20 and 18 plates, respectively. This differs significantly from groups of prepupa (PP) and pupa (WP) with 9 and 1 plates, respectively. A progressive decrease was observed from group L3 to group WP. Group PP also differs from WP in the final spore survival.

No mummies were found in any of the groups. Contamination of plates did not reach significant levels, with only 2 plates contaminated of the 80 used.

With regard to the larvae receiving a cooling stress (fig 2) statistical analysis shows 2 clearly differentiated blocks: those larvae



**Fig 2.** Percentage of chalkbrood mummies produced in honey bee larvae after being fed with  $5 \times 10^5$  *A. Apis* spores and receiving chilling in 1 of 4 periods of brood development. The control group received no chilling. Data at top of columns are expressed as the mean  $\pm$  sd of 5 tests. BS: before sealing of cells; AS: after sealing of cells. L5: fifth larval instar before sealing; SL: spinning larvae; PP: Prepupa.

which received chilling soon before or after operculation (groups 24 h BS and 24 h AS); and those stressed 48–72 h after sealing of the cells. The control group showed a slight percentage of mummification, which did not differ from values found for groups 48 h AS and 72 h AS. Nevertheless, when chilling was applied to groups 24 h BS and 24 h AS, it had a consistent influence on mummification rates, reaching  $59.6 \pm 11.8$  and  $65.5 \pm 16.6$ , respectively (represented as the mean  $\pm$  sd of the 5 tests).

## DISCUSSION

Although no mummies were found, data from the first test (fig 1) show some aspects of development of the disease. Spores ingested by the larvae were almost entirely voided from the digestive tract before pupation or died in the tract. A progressive loss of the inoculated infectious material is observed from the third instar to pupae. While all plates inoculated with gut contents from group L3 (third instar larvae) had completed growth at 120 h, the inocula administered seemed to be lost at the pupal stage (WP) (only 1 plate showed growth).

The consistent difference in spore survival between the 2 intermediate stages (spinning larvae SL and prepupa PP) shows discharge or death of infectious material to be basically completed in the prepupal period, while spinning larvae did not defecate and contained almost all the inocula (differences in the spore survival are not significant at 120 h between group SL, group L3 or DI). The faster growth of the spores removed from the spinning stage may show that this period of brood development is most conducive to pathogen survival.

Bamford and Heath (1989) fed spores of *A apis* to larvae and demonstrated that hyphae are seen on the exterior of larvae 3 d later. During brood feeding, besides the glandular secretion of workers, some of the

pollen and honey begins to be fed directly to the larvae from the third day of life onwards (Michener, 1974). Since spores of the fungus have been widely isolated from stored honey and pollen (Heath, 1982; Gilliam, 1986; Koenig *et al*, 1987a; Gilliam *et al*, 1988), larvae younger than fifth instar could be resistant, as was pointed out by Bailey (1967), because there was not enough time for most of the ingested spores to germinate and grow before being voided. Moreover, Bamford (1987) showed that royal jelly inhibited *A apis* spore germination *in vitro*, so the pure gland secretion diet of the young larvae may inhibit spore germination, although Gilliam *et al* (1978) reported that this substance did not appear to have any inhibitory effect *in vitro* on the viability or growth of the fungus.

Our data support Bailey (1967) as well as Heath (1984), who characterized *A apis* as an opportunistic pathogen that kills larvae only when they are subjected to a predisposing condition.

In the first part of this study, we maintained constant and appropriate conditions for bee development throughout all post-capping stages, avoiding predisposing factors, which are not easily controlled in a colony. Ingestion of infectious material was not sufficient to induce the disease. A large amount of spores fed to larvae ( $5 \times 10^5$  spores/larva), failed to produce any mummies. These data were confirmed by the results from the control group of chilled larvae in the second part of our study (fig 2).

Gilliam (1986) reported that only 2 periods of major infection occurred, even though *A apis* was sprayed on the combs for 4 months, and Koenig *et al* (1987b) detected considerable variation in chalkbrood levels within colonies given the same treatment. These 2 facts also support the incidence of predisposing conditions in chalkbrood.

Gilliam (1986) and Koenig *et al* (1987b) have also reported on the incidence of availability of the pathogen in the colony. In both

studies, a major period of infection is detected after artificial inoculation of spores in the hives. Nevertheless, in both tests, temperature conditions in brood development could not be precisely controlled, and consequently it was difficult to establish whether an increase in ingested spores alone is enough to provoke chalkbrood, without the presence of predisposing conditions, such as a nutritional stress, chilling, *etc* (Heath, 1982). In his review, Heath (1982) indicated that the chilling of larvae was one of the most widely assumed factors for production of chalkbrood disease. The main controversy regarding this is based on the records of ascospaerosis in hot dry weather (Mehr *et al*, 1976) or for areas where average monthly temperature is around 30°C (Gilliam, 1978). Nevertheless, this average temperature does not guarantee the maintenance of uniform conditions during all the brood developmental stages. Cooper (1980) registered fluctuations in temperature in the brood-nest inside the range used in this work, reaching as low as 18°C. Moreover, applying low temperatures does not seem to be necessary during the entire period (24 h) used in the study to provoke mummification. A cooling stress of a few hours causes almost the same effect (Puerta *et al*, unpublished data).

As can be seen by the low numbers of mummified larvae in groups 48 h AS and 72 h AS (fig 2), the stress applied may have little or no effect on the colony unless a high percentage of this brood has been capped or is capped within 24 h of application of stress. The fact that chilling must take place during 24 specific hours in the course of capped brood development may hitherto have led to some confusion and controversy. We suggest that chilling is in fact an important predisposing factor for chalkbrood disease if and when it takes place during 24 h before or after the sealing of cells.

The spinning stage has a mean duration of 45 h (Rembold *et al*, 1980). As signifi-

cant differences are observed in the percentage of mummies between groups 24 h AS and 48 h AS, both inside the spinning stage, susceptibility to mummification during this phase seems to be restricted to the first half of this period.

Bailey (1967) suggested that the fungus inside the larva is reactivated by a chilling of 22°C immediately after cell sealing. Data from the present study show that this stress has the same effect if applied 24 h before capping.

The technique described in this study can avoid the problems linked with field trials. As chalkbrood can be incited under controlled conditions, complementary studies of etiology, epidemiology, predisposing conditions and chemical treatments may be developed.

## ACKNOWLEDGMENTS

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## Résumé — Développement du couvain plâtré chez *Apis mellifera* en conditions contrôlées.

Nous avons suivi le développement de spores d'*Ascospaera apis*, agent du couvain plâtré chez *Apis mellifera*, administrées par voie orale à des larves de 3<sup>e</sup> stade. Pour cela, nous avons cultivé sur un milieu approprié (MY20, Takatori et Tanaka, 1982) le contenu des tubes digestifs de lots de 20 larves à 4 phases de leur développement : i) (L3) larves de 3<sup>e</sup> stade 6 h après administration des spores ; ii) (SL) larve de 5<sup>e</sup> stade en train de filer ; iii) (PP) prénymphe ; iv) (WP) nymphe non pigmentée (Rembold *et al*, 1980). Malgré la dose élevée de spores administrées (5 x

$10^5$  spores/larve), aucune momie ne s'est formée. Les spores provenant des larves fileuses (phase SL) se sont développées le plus rapidement (fig 1). Le moment le plus favorable à l'activation des spores d'*A apis* après ingestion par la larve se situerait dans les heures suivant l'operculation de la cellule, avant que la larve ne s'étire à l'intérieur et commence le filage du cocon. Les facteurs prédisposant à la maladie, et semblant indispensables au déclenchement de celle-ci, pourraient agir sur la larve principalement durant les heures qui suivent l'operculation. Pour suivre le déroulement de l'infection nous avons appliqué, dans la seconde partie de cette étude, une nouvelle technique qui déclenche la maladie de façon contrôlée chez des larves maintenues dans des conditions déterminées. Des larves de 3<sup>e</sup> stade ont été alimentées avec des doses de  $5 \times 10^5$  spores par larve et maintenues ensuite dans la ruche jusqu'à l'operculation. Puis elles ont été prélevées et maintenues en étuve dans des conditions contrôlées d'humidité et de température. Les larves furent soumises à un stress thermique ( $22 \pm 2^\circ\text{C}$  durant 24 h) à chacun des 4 stades de développement suivants : 24 h avant operculation (24 h BS) ; 24 h après operculation (24 h AS) ; 48 h après operculation (48 h AS) et 72 h après operculation (72 h AS). Les groupes 24 h BS et 24 h AS ont atteint un très haut niveau de momification (59,6 et 65,5%) et se différencient significativement des autres groupes ( $p \leq 0,01$ ). Dans les lots témoins, ayant reçu la même dose de spores mais pas le stress, le taux de momification a été très réduit (fig 2). Toutes ces données confirment la nécessité de facteurs prédisposants pour qu'apparaisse la maladie, l'ingestion d'un nombre élevé de spores ne suffisant pas. Le moment critique de l'action du stress thermique se situe dans les 24 h précédant ou suivant l'operculation.

***Apis mellifera* / *Ascospaera apis* / couvain platré / stress**

### **Zusammenfassung — Entwicklung der Kalkbrut bei der Brut von *Apis mellifera* L unter kontrollierten Bedingungen.**

Der Entwicklungsablauf von einer definierten Anzahl infektiöser Sporen des Kalkbruterregers *Ascospaera apis* wurde beobachtet. Die Sporen wurden peroral an Larven im dritten Larvenstadium verabreicht. Um den Vorgang zu verfolgen, wurde der Inhalt des Verdauungstraktes auf einen geeigneten Nährboden (MY20, Takatori und Tanaka, 1982) aufgebracht, der von je 20 Larven in 4 verschiedenen Entwicklungsphasen gewonnen wurde: a) L3, drittes Larvenstadium, 6 Stunden nach Verabreichung der Sporen; b) SL, spinnende Streckmaden im fünften Larvenstadium; c) PP, Praepuppen; d) WP, weiße Puppen (Rembold *et al*, 1980). Obwohl mit einer sehr hohen Dosis ( $5 \times 10^5$  Sporen pro Larve) infiziert wurde, entstand keine einzige Mumie. Die aus der SL Phase gewonnenen Sporen entwickelten sich am schnellsten und auf den meisten Nährböden (Abb 1). Der günstigste Zeitpunkt für die Aktivierung der Sporen von *A apis* nach der Aufnahme durch die Brut scheint einige Stunden nach der Verdeckelung der Zelle einzutreten, bevor die Larve sich streckt und beginnt sich einzuspinnen. Die prädisponierenden Faktoren dieser Krankheit, die wahrscheinlich absolut notwendig sind, um zum Ausbruch der Krankheit zu führen, könnten hauptsächlich kurz nach der Verdeckelung der Zelle auf die Larve wirksam werden. Um den Infektionsablauf zu verfolgen, wurde im zweiten Teil dieser Studie eine neue Technik angewandt, die die Krankheit in kontrollierbarer Form bei der Brut auslöst, die unter bekannten Bedingungen gehalten wird. Gruppen von Larven im dritten Larvenstadium wurden künstlich mit einer einmaligen Dosis von  $5 \times 10^5$  Sporen pro Larve gefüttert und bis zu ihrer Verdeckelung in ein Bienenvolk gehängt. Anschließend wurden sie erneut herausgenommen und bei kontrollierter Feuchtigkeit und Temperatur in einem Brutschrank gehalten. Die infizierten Larven

wurden in 4 verschiedenen Phasen ihrer Entwicklung einem thermischen Streß von  $22^{\circ}\text{C} \pm 2^{\circ}\text{C}$  für 24 Stunden ausgesetzt: a) 24 Stunden vor der Verdeckelung der Zellen (24 h BS); b) 24 Stunden nach der Verdeckelung der Zellen (24 h AS); c) 48 Stunden nach der Verdeckelung der Zellen (48 h AS); und d) 72 Stunden nach der Verdeckelung der Zellen (72 h AS). Die Mumifizierung in der Gruppe 24 h BS und 24 h AS erreichte mit 59,6% bzw 65,5% ein sehr hohes Niveau mit signifikantem Unterschied zu den beiden anderen Gruppen ( $P \leq 0,01$ ). Die Kontrollgruppen mit derselben Sporendosis ohne thermischen Streß erreichten nur geringe Prozentzahlen von Mumifikation (Abb 2). Die gesamten Daten bestätigen die Notwendigkeit von prädisponierenden Faktoren als Voraussetzung für den Ausbruch der Krankheit, da die alleinige Aufnahme einer erhöhten Sporendosis nicht ausreicht. Die Wirkung des thermischen Stresses hat ihren kritischen Zeitpunkt 24 Stunden vor bis 24 Stunden nach der Verdeckelung.

### ***Apis mellifera* / *Ascosphaera apis* / Kalkbrut / Streß**

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