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# Fungal diseases of the honeybee (*Apis mellifera* L.)

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## Introduction

Most fungi associated with colonies of the honeybee *Apis mellifera* are not a problem for beekeepers. A large number of yeasts can usually be isolated from the intestines of healthy brood or adult bees and none of them seem to be pathogenic. Stored pollen sometimes goes mouldy with the growth of *Bettsia alvei*, and a small number of bee larvae can be affected by several fungi of the genus *Aspergillus*, producing a disease named stonebrood. Nevertheless, this disease does not usually appear unless the colony is seriously weakened by other factors.

The only fungal disease causing a problem in honeybee colonies is named chalkbrood or ascosphaeriosis, and is caused by the fungus *Ascosphaera apis.*

## Definition of the disease

Chalkbrood disease in honeybees (*Apis mellifera* L.) is an invasive mycosis produced by the fungus *Ascosphaera apis*, affecting stretched larvae. At first, dead larvae inside recently capped cells, are covered by a fluffy white mould, and later they dry and become black or white mummies (Figs 1 and 2). At the peak of the disease, mummies are easily detected at the entrance to the hive as nurse bees remove them from their cells (Fig. 3).

## General epidemiology

The fungus *Ascosphaera apis* was first recognized by Maassen (1913). This author proposed the name *Pericystis apis*, which was retained by Claussen (1921) who made a short description of the fungus. The life cycle was first described by Spiltoir (1955). Spiltoir and Olive (1955) introduced the name of the genus, Ascosphaera.

Chalkbrood probably occurs in all the countries of the Mediterranean, although it has not been reported to be present in Portugal, Algeria, Albania and Morocco (Bradbear, 1988). Until 1968, it was considered to be a primarily European disease, but in 1971 it became recognised as economically important in the USA (Hitchcock and Christensen, 1972). It can be assumed that the disease is distributed world wide.

*Ascosphaera apis* rarely kills a colony, but the loss of larvae leads to a reduction in the adult bee population and so the production of honey and pollen and pollination efficiency, declines. Occasionally, severe cases of chalkbrood have been reported to kill colonies (Anderson, 1938; Roussy, 1962), but this is unusual.

## Etiology

### Pathogenic agent

#### Classification, morphology, biology and multiplication

The fungi *Ascosphaera apis*, *Ascosphaera major* and *Arrhenosphaera cranei* have been isolated

from diseased larvae, but only larvae fed *A. apis* have subsequently developed chalkbrood symptoms (De Jong, 1976; Gilliam *et al.*, 1988; Puerta *et al.,* 1994; Flores *et al.,* 1996). The latter is the fungus readily isolated from chalkbrood mummies and is assumed to be the cause of chalkbrood disease in *A. mellifera*.

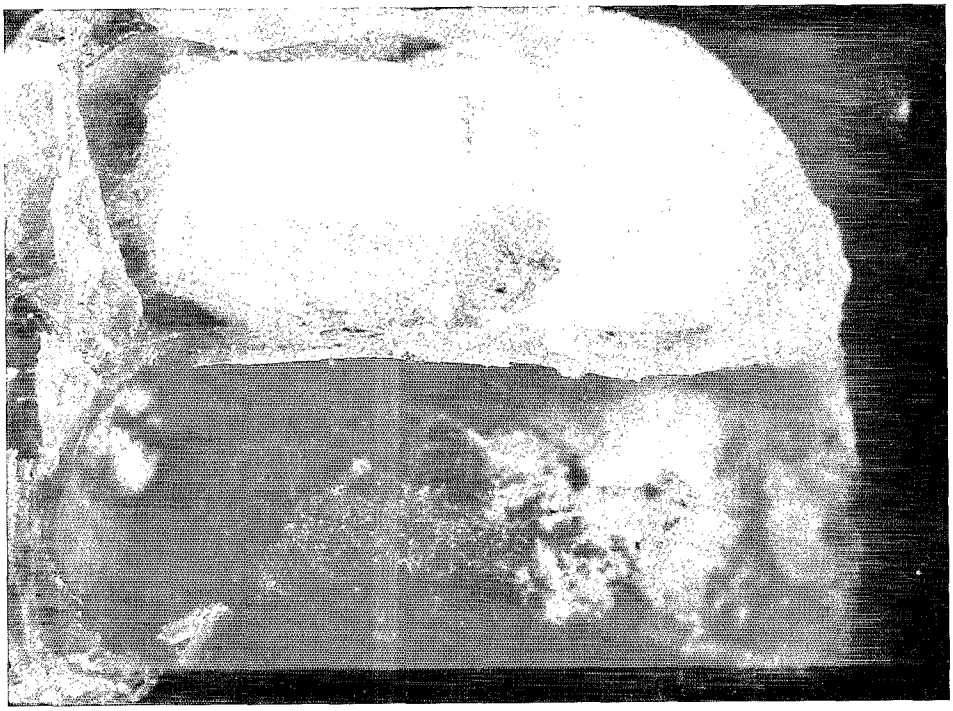

Fig. 1. White (upper) and black (lower) mummies produced by *Ascosphaera apis,* inside sealed cells.

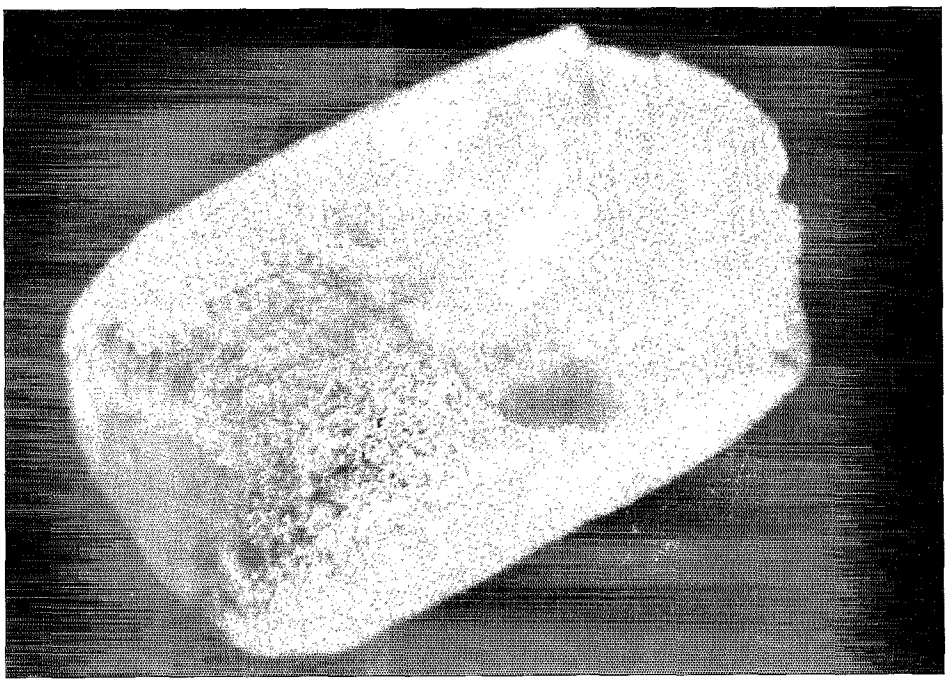

Fig. 2. A chalkbrood mummy covered by ascocysts (black) and mycelium (white).

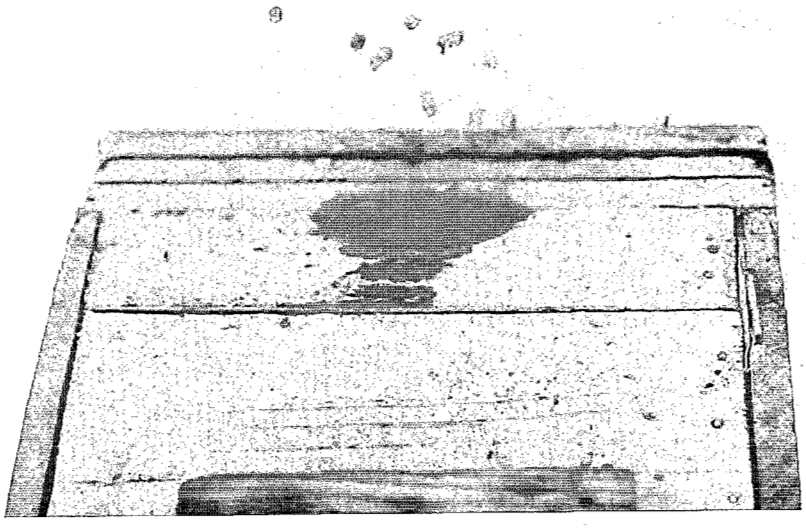

Fig. 3. Chalkbrood mummies at the entrance of a hive.

The taxonomic position of *Ascosphaera* spp. is given by Skou (1972):

Subdivision: Ascomycotina
Class: Plectomycetes
Family: Ascosphaeraceae
Order: Ascosphaerales

*Ascosphaera apis* has white and septate hyphae. No conidial state seems to be present and the fungus is heterothallic, the strains being morphologically distinct only during reproduction (Claussen, 1921). When + and – strains are grown in close proximity, sexual reproduction with the formation of spore cysts occurs.

These spore cysts measure a mean of 80 µ in diameter, but this mean size may be dependent on the temperature of cultivation and possibly on the substrate used, but the size of the spores within remains the same. The colour of the spore cysts is olive green or brown and the form is globose (Fig. 4). They occur in discrete areas or spread all over the agar when the fungus is cultured, and cover the surface of the mummy (except the head region) (Skou, 1972, 1986). The spore cyst contains many spore balls (11-17 µ in size) (Fig. 5) and the ascospores are hyaline, smooth and measure 1-2 x 2.5 µ. *Ascosphaera apis* differs from *A. major* and *A. proliperda* (a fungus causing chalkbrood in the leafcutter bee, *Megachile rotundata*) in the size of the cysts, sporeballs and ascospores and in the appearance of the spore cyst membrane.

Many media supporting the growth of *A. apis* have been described (reviewed by Heath, 1982). As far as the authors are aware, the most frequently used are Potato Dextrose Agar (Skou, 1972) and MY20 (Takatori and Tanaka, 1982). The latter is composed of 5 g peptone, 3 g yeast extract, 3 g malt extract, 200 g glucose, 20 g agar and 1 l of water. The culture plates must be maintained at 35°C under aerobic conditions for the development of the mycelium, but for the germination of spores a carbon dioxide source is needed [10% $CO_2$ in air (Heath and Gaze, 1987)].

## Spread and transmission

In nature, fungal spores are present in the honey and pollen stored in the hive. The fungus has also been isolated from the surface of combs, water sources, and from the digestive tract of adult bees. The continuous food sharing in a colony provides a mechanism by which spores can be spread amongst adults bees, including those feeding brood. In this way, the spores can easily reach the digestive tract of bee larvae; from stored food, contaminated surfaces or nurse bees.

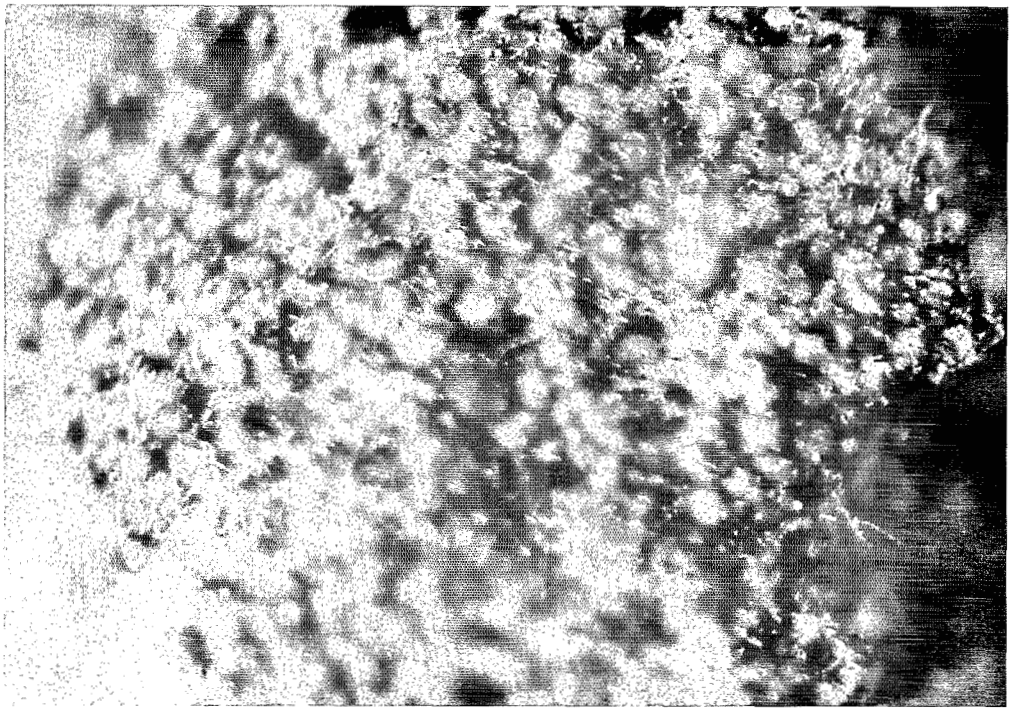

Fig. 4. Green-brown ascocysts of *A. apis* (×40).

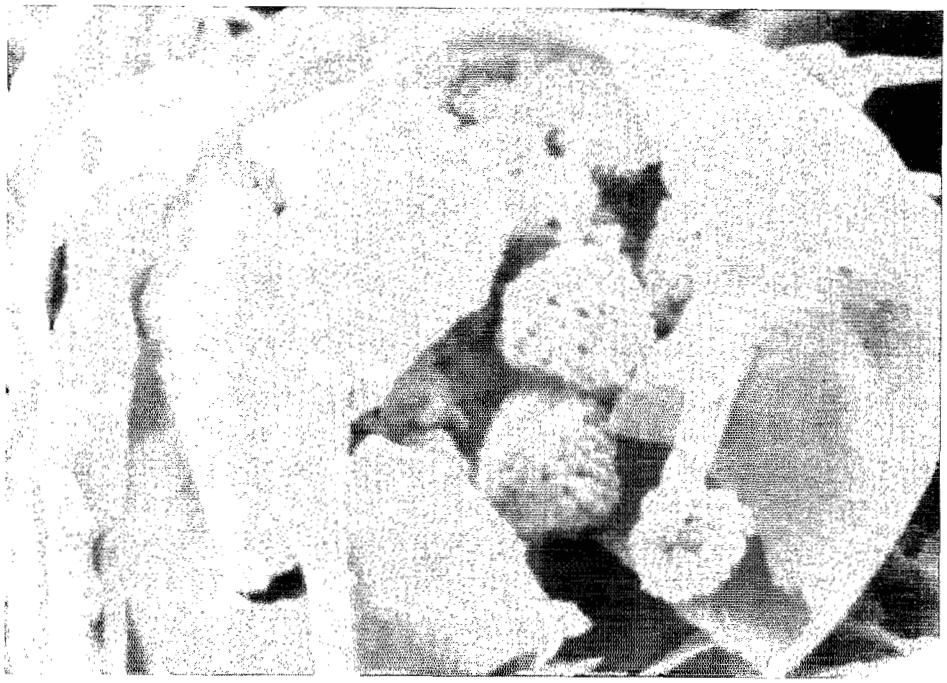

Fig. 5. Detail of an ascocyst partially opened, containing sporeballs (SEM image).

Beekeepers are one of the most important means of transmission of infection between colonies by the transfer of contaminated combs. Drifting or robbing bees and drones may also make a minor contribution to the spread of infection.

As a mummified larva may produce approximately 100 million to one billion of ascospores (Nelson and Gochnauer, 1982), and these appear to be long lived (Heath, 1982), the presence of large numbers of fungal spores is almost assured inside each bee colony.

## Factors affecting disease outbreaks and seriousness

Honeybee larvae may be fed *A. apis* and not develop the disease (Puerta *et al.*, 1994; Flores *et al.*, 1996). Apparently uninfected colonies developed the disease when larvae were chilled by removing the comb briefly from the brood nest (Maurizio, 1934). As mentioned previously, exposure to this widespread pathogen is the usual situation for larvae, but it also seems that the presence of predisposing conditions is necessary for the development of the disease. Heath (1982) reviewed these factors: dampness, chilling of the brood, weakening of the colony by other diseases such as viruses, *Varroa jacobsoni* or foulbrood, manipulations which decreased the brood to adult bee ratio, poor hygienic behaviour of nurse bees, etc.

It has been demonstrated that in colonies with less than 12% infection, chalkbrood is not detected by the routine inspections of beekeepers (De Jong, 1976). Linking this with the need for predisposing conditions, which are not well defined, it seems that outbreaks of the disease can readily appear unexpectedly. Chalkbrood may be considered as an insidious disease, when a small and variable proportion of the brood is affected, or as a seriously debilitating infection when there is a sharp increase in the number of larvae killed by the fungus, which reduces the strength of the colony.

## Pathogenesis

Several histological studies have been made on larvae infected with chalkbrood (Maurizio, 1934; Carrera *et al.*, 1987; Bamford and Heath, 1989; Puerta *et al.*, 1994). Nevertheless, there is still controversy about the route of invasion of the fungus into larvae.

The most widely accepted theory is that spores are ingested by the uncapped larvae and germinate inside the gut lumen, eventually growing through the gut wall. Although the fungus lacks many of the lytic enzymes usually found in other fungal pathogens (Gochnauer and Margetts, 1979), *A. apis* can emerge through the larval cuticle by a combination of mechanical (pressure of hyphae produced from the invaded larval tissues) and enzymatic action (proteolytic, lipolytic and N-acetyl-glucosaminidase). Mechanical pressure would not be present in the case of surface infections (Alonso *et al.*, 1993).

Infection seems to be initiated by ascospores (Heath, 1982), although some authors suggest that infection is directly produced by invading hyphae (Gilliam *et al.*, 1978). Larvae can ingest the fungus at an early stage, but only stretched larvae, inside capped cells, present symptoms of the disease. Gilliam (1978) and Gilliam *et al.* (1978) demonstrated that eggs and pupae are not susceptible to laboratory infection.

## Clinical (field) symptoms and differential diagnosis

### Sample collection

Affected larvae die of chalkbrood after their cells have been capped. The fluffy white mould covering the stretched larvae can be seen inside the cells uncapped by the worker bees. After they dry, the mummies can retain their white colour or, if sporulation occurs, appear black (Figs 1 and 2). They can remain inside the brood cells for some time before being removed by the bees. Nevertheless, when the infection is prevalent, some mummies will appear at the entrance (Fig. 3) and at the bottom of the hive and when the brood combs are shaken, the mummies rattle inside their cells. Clinical symptoms are the presence of mummies in these places and a progressive weakening of the colony.

*Bettsia alvei*, a mould growing on stored pollen, can be mistaken for chalkbrood, but the substrate supporting the fungus is friable and does not have the appearance of a piece of chalk, as in the disease. *Aspergillus* spp., which can infect brood (stonebrood), can be easily differentiated from chalkbrood. The latter has globose ascocysts, while *Aspergillus* forms conidia.

The collection of mummies can be made from brood cells or the hive entrance. As cultivation of the fungus is not usually needed for differential diagnosis, no aseptic techniques in the collection and transport of mummies to the laboratory are necessary. Samples may also be collected by cutting out a small piece of comb containing affected larvae.

## Laboratory diagnosis

### Identification and isolation

The presence of globose, dark ascocysts on the surface of mummies is a pathological symptom of the disease. A magnification of ×30 is sufficient to detect these reproductive forms (Fig. 4).

### Cultivation

If only white mummies are available in the sample, ascocysts will not be visible. Diagnosis can then be confirmed by cultivation of the pathogen on MY20 medium. Mycelium from the surface of several white mummies must be taken aseptically and spread on to the surface of the agar medium. Plates should be incubated at 35°C with high (70-80%) R.H. Usually, growth of the mycelium fills the plate in one or two days, and sporulation (ascocysts) first appears in lines, at the points of contact between the growing zones of + and – mycelium. After a week, the surface of the plate is usually covered with ascocysts.

### Experimental inoculation

Experimental inoculation can be made on groups of bee larvae under controlled conditions in the laboratory (Puerta *et al.*, 1994; Flores *et al.*, 1996) or by inoculating the pathogen into colonies (Gilliam, 1986; Koening *et al.*, 1987). Both techniques are arduous and not recommended for diagnostic purposes. Routine diagnosis can best be made by detecting ascocysts on mummies and/or by culture on MY20 medium.

## Treatment and prophylaxis

### Treatments

A review of chemical treatments which, under laboratory (in culture), or field conditions (fed to colonies, sprayed over bees, etc.) have given some control of chalkbrood in *A. mellifera* is given by Heath (1982). As this author pointed out, it is not easy to develop a chemotherapy for the control of this disease that is cheap, effective, non-toxic for bees and which can be steadily released in the colony throughout the active season. However, an essential oil of Labiaceae (Savory: *Satureia montana*) has been used successfully in apiaries. This essential oil is mixed with sugar-candy (dilution 0.1%; dose 1 g per colony) and fed to the colony at the end of wintering (Colin *et al.*, 1989).

It is very difficult to induce chalkbrood infection at a homogeneous level in a group of 10-15 hives or more, by experimental inoculation. This is one of the primary problems that has inhibited the investigation of treatments or prevention. As far as the authors are aware, there is not an effective and widely-used treatment for chalkbrood in *A. mellifera*. However, some preventative measures can help to control the disease.

## Prevention

The use of clean combs annually is one of the most effective measures for preventing disease outbreaks. The reason for this is that the pathogen can remain infective as spores on the combs inside colonies for many years, and a single chalkbrood mummy can produce a huge amount of these spores.

Selection of bees with pronounced hygienic behaviour holds the most promise for the control of this disease. If exposure to the pathogen is the normal situation in hives, and outbreaks of the disease are dependent on many physiological and environmental factors, it seems very difficult for professional beekeepers to control the disease with periodical and systematic actions.

Suitable management of the colonies for disease prevention must avoid:

(i) The accumulation of spores inside the hive; periodically renew the brood combs and eliminate combs severely contaminated with mummies.

(ii) Manipulations that decrease the adult bee to brood ratio. When the brood nest temperature or the nutrition of larvae is inadequate, chalkbrood seems to appear.

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