RESEARCH ON CHALKBROOD DISEASE OF HONEY BEES

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Introduction

Chalkbrood disease of honey bees, *Apis mellifera* L., is thought to be caused by the heterothalic fungus, *Ascosphaera apis* (Maassen ex Claussen) Olive & Spiltoir. Spores are formed within fruiting bodies only when mycelia of opposite sex come together. Diseased larvae become mummified. The mummies are white due to the mycelium of the fungus. However, if fruiting bodies are formed, the mummies are dark gray or black.

Unfortunately, little is known of the epidemiology and pathogenesis of chalkbrood disease. According to BAILEY (1967), honey bee larvae are most susceptible to chalkbrood disease if they ingest spores of *A. apis* when they are 3—4 days old and are then chilled 2 days later after they are sealed in their cells to pupate. The spores then germinate in the gut of the larvae. Initially the dead larvae are covered with a white fluffy growth of mycelia and are swollen to the size of the cell. Later they dry into a hard, shrunken chalklike lump which may become gray to black if fruiting bodies are formed. The remains of larvae can be found in sealed or unsealed cells.

BARTHEL (1971) and MATUS and SARBAK (1974) stated that natural infection of *A. apis* could occur either by ingestion of spores with food or via the body surface from spores on combs and cell walls. ROUSSY (1962) found that the spores germinated on the surface of larvae, whereas MAURIZIO (1934) thought that infection was through the mouth and not through the cuticle or spiracles.

Chalkbrood disease has been reported from Europe for many years. However, it was not reported from the United States until BAKER and TORCHIO (1968) found *A. apis* associated with a leaf cutter bee, *Megachile inermis* Provancher and a soil nesting bee, *Anthophora pacifica* Cresson. Late THOMAS and LUCE (1972) reported chalkbrood from honey bee in California, and HITCHCOCK and CHRISTENSEN (1972) found the disease in honey bee larvae from Nebraska and Wyoming. We first found the disease in bees from Arizona in May, 1972 (GILLIAM and TABER, 1973). The disease now appears to be widespread in bees in the United States.

De JONG and MORSE (1976) noted the difficulty that researchers have experienced in inducing chalkbrood infections in bees for study.

Since 1972, we have been conducting experiments on the transmission of *A. apis*. Specifically, we have been attempting to infect brood artificially. Such infections would give information on the transmission and pathogenesis of the disease and would provide a reproducible bioassay. They would also allow us to perform controlled experiments on methods of treatment such as the use of mold inhibitors (TABER *et al.*, 1975). In this paper, we report the work that we have conducted on full-size colonies in the apiary and on brood maintained in an incubator.

Attempts to infect apiary colonies with *A. apis*

Many attempts were made to infect brood in apiary colonies with *Ascosphaera apis* under a variety of conditions affecting both the pathogen and the host colonies. These included the use of inocula prepared from artificial cultures of *A. apis* vs. suspensions from naturally infected larvae, application of the inocula in syrup sprays and/or in pollen cakes, use of mummies with sporulated vs. vegetative growth of the pathogen, alteration of the ratio of adult worker bees to brood by removal of adult bees or frames of brood prior to application of the inoculum, and application of ground mummies in dusts or in pollen.

The most severe infections were produced in colonies that had been fed uncontaminated pollen to promote brood rearing and from which varying numbers of adult bees were removed before a suspension of 3 black (sporulated) mummies in 90 ml of 5% sucrose in water. When this treatment was applied 3 times a day on the first, third, and fifth days of the experiment, it yielded heavy infections (10% of the brood) by the sixth day. Hwever, even this treatment was less effective in other colonies that were treated at later periods. Thus variation in susceptibility may be an important factor in expression of disease.

Attempts to infect brood in an incubator with *A. apis*

Because of the difficulty of inducing infections in apiary colonies, we devised techniques for removal of brood from combs, surface sterilization of brood, maintenance of brood in an incubator, and inoculation of brood with *A. apis*. No differences in survival were noted in larvae in the incubator fed 25% sucrose solution, royal jelly, or nothing. Thus, suspensions of *A. apis* for inocula were prepared by homogenizing 5 black or spore-bearing mummies (mated strain) or 5 white chalky mummies (unmated strain) in 5 ml of sterile distilled water. These suspensions were plated to confirm viability and were also sprayed on combs in apiary colonies. Some mummies were found in sprayed combs after 5 days. The dead *A. apis* suspensions used for controls were obtained by autoclaving the homogenate.

Attempts to infect small and large larvae from uncapped cells with *A. apis*

First we sought to determine whether *A. apis* causes chalkbrood disease or is simply a secondary invader of larvae killed or injured by other events. Evidence for the latter might be supported by growth of the fungus on dead larvae. We also wished to determine whether mummification arises from infection by both vegetative and sporulated strains and whether *A. apis* can invade larvae through the cuticle and/or *per os*. In addition, we wished to determine whether young and old larvae are equally susceptible to infection.

The water removal technique of GARY et al. (1961) was employed without removing any cappings from the cells. Thus, the larvae were washed from a comb onto sterile absorbent paper. Then each larvae was individually picked up on the polished end of a glass tube attached to a water suction device. While held to the glass tube, each was washed 3 times with sterile distilled water from a plastic squeeze bottle.

The larvae were divided into two age groups, small and large, on the basis of size. The small larvae were 3—4 days old (average weight 54 mg), and the large larvae were 4.5—5.5 days old (average weight 134 mg). The treatment groups of larvae are shown in Table 1.

Larvae in the "alive" treatment groups were individually placed in sterile vial caps in sterile glass petri dishes that contained a piece of filter paper kept moist with sterile distilled water. Eight-10 larvae receiving the same treatment were placed in the same petri dish. Larvae in the "dead" treatment groups were placed in sterile vial caps and petri dishes and were killed with dry ice.

A drop of the appropriate homogenate was placed either on the mouthparts or on the dorsal side of the larvae. We confirmed consumption of the homogenate by direct observation. The dishes were placed in a 25°C incubator. Larvae were observed daily for 10 days with a dissecting microscope for movement, fungal growth, discoloration, and mummification.

The results are shown in Table 2. Eleven mummies were formed in control treatment groups receiving dead *A. apis*. These larvae may have had natural infections which were inapparent until the larvae were incubated. However, none of the colonies from which larvae were obtained contained any larvae that were visibly infected with *A. apis*. Nor were any mummies found at the entrance or on the bottoms of these colonies. Therefore, some bee colonies or larvae may harbor *A. apis* without showing signs of disease. Small larvae that were naturally infected required 5—7 days of incubation to mummify ; the large larvae required 6—8 days.

Table 1

TREATMENT GROUPS OF SMALL AND LARGE LARVAE FROM UNCAPPED CELLS USED TO DETERMINE THE PATHOGENIC AND SAPROPHYTIC PROPERTIES OF *ASCOSPHAERA APIS*

Treatment	*A apis*. strain	Site of inoculation
Large larvae [a]		
"Alive" larvae and live *A. apis*	Mated	Mouthparts
— do —	Unmated	— do —
— do —	Mated	Back
— do —	Unmated	— do —
"Alive" larvae and dead *A. apis*	Mated	Mouthparts
— do —	Unmated	— do —
— do —	Mated	Back
— do —	Unmated	— do —
"Dead" larvae and live *A. apis*	Mated	Mouthparts
— do —	Unmated	— do —
— do —	Mated	Back
— do —	Unmated	— do —
"Dead" larvae and dead *A. apis*	Mated	Mouthparts
— do —	Unmated	— do —
— do —	Mated	Back
— do —	Unmated	— do —
Small larvae [b]		
"Alive" larvae and live *A. apis*	Mated	Mouthparts
— do —	Unmated	— do —
— do —	Mated	Back
— do —	Unmated	— do —
"Alive" larvae and dead *A. apis*	Mated	Mouthparts
— do —	Unmated	— do —
— do —	Mated	Back
— do —	Unmated	— do —
"Dead" larvae and live *A. apis*	Mated	Mouthparts
— do —	Unmated	— do —
— do —	Mated	Back
— do —	Unmated	— do —
"Dead" larvae and dead *A. apis*	Mated	Mouthparts
— do —	Unmated	— do —
— do —	Mated	Back
— do —	Unmated	— do —

a 20 larvae per test
b 10 larvae per test

No mummies were formed as a result of the inoculation of dead larvae with *A. apis*. However, *A. apis* did grow on 33 of these larvae. Thus, it appears that *A. apis* can grow and in some instances sporulate on dead larvae but will not mummify them.

Forty-three mummies were formed as a result of inoculation of larvae with live *A. apis*. Both the small and large larvae required 5—7

days after inoculation for mummy formation. Twenty-six of the mummies were produced by inoculation of mouthparts, and 17 by inoculation on the back. Thus, infection may occur *per os* and through the cuticle. We observed the fungus growing from the mouth, the anus, and through the cuticle of larvae inoculated on the mouthparts. The fungus appeared to grow first aerially at the point of inoculation on larvae inoculated on the back. It then grew throughout the entire body, and the larvae became mummified. All experimentally produced mummies were much flatter than those generally found in bee colonies.

Both mated and unmated strains of *A. apis* caused mummy formation. Of the mummies produced from the material prepared from black mummies, 11 were black and 11 were white. However, of the mummies produced by inoculation of material prepared from white mummies, 18 were black and only 3 were white. Thus, by selecting 5 mummies for production of the inoculum, we obviously mixed + and — strains to produce the mated strains that infected the larvae. However, it is interesting that an equal number of black and white mummies were formed from material prepared from black mummies. A separation of +/— material could have occurred or, perhaps, sporulation was in some way inhibited.

Table 2

LARVAE EXPERIMENTALLY INFECTED WITH *ASCOSPHAERA APIS*

Larvae	Inoculation Site	*A. apis* Strain	Mummies Produced		Larvae With A. apis Growth	
			Black	White	Mycelium	Spores
Small-alive	Mouthparts	Live-mated	1	2		
— do —	— do —	Live-unmated	5			5
— do —	Back	Live-mated	1			
— do —	— do —	Live-unmated	3		1	2
— do —	Mouthparts	Dead-mated	2			
— do —	Back	— do —	2	5		
Small dead	— do —	Live-unmated			1	
Large-alive	Mouthparts	Live-mated	7	2	2	1
— do —	— do —	Live-unmated	6	3	4	
— do —	Back	Live-mated	2	7		
— do —	— do —	Live-unmated	4			2
— do —	Mouthparts	Dead-unmated		3		
— do —	Back	Dead-mated		3		
Large-dead	Mouthparts	Live-unmated			1	14
— do —	Back	— do —			6	7

Small and large larvae were equally susceptible to infection since we used twice as many large larvae as small ones. Thirty mummies were produced among large larvae and 12 among small larvae. Thus, 3—4

day-old and 4.5—5.5 day-old larvae appear to be susceptible to chalkbrood disease. This conjecture is reinforced by the fact that 5 of the small and 6 of the large larvae apparently had a natural infection of chalkbrood.

Next we developed a method of surface sterilization of larvae with a germicide to eliminate comb contaminants (*Aspergillus* spp. and *Penicillium* spp.) without harming the larvae.

After the larvae were washed from a comb onto a sterile Massilin^R towel, they were individually picked up with the suction apparatus, washed with a 1 : 1250 solution of Roccal^R from a plastic squeeze bottle, and rinsed twice with sterile distilled water from a squeeze bottle. They were placed on sterile paper to blot them dry and were then put into sterile vial caps in petri dishes by releasing the suction.

Attempts to infect eggs, larvae and pupae with *A. apis*

Then we designed an experiment to determine whether *A. apis* would grow on or cause mummification of eggs ; small, medium, and large larvae ; prepupae ; pupae with no eye color ; and pupae with eye color.

The treatment groups are shown in Table 3. Eggs were individually removed from their cells with special forceps (TABER, 1961), rinsed with Roccal, and washed twice with sterile distilled water. Then they were blotted and placed against the side of a sterile vial cap to simulate the position occupied in the cell of the comb. Small and medium larvae were washed from uncapped combs. Large larvae, prepupae, and pupae were washed from combs after uncapping the cells. All larvae were sterilized as described before. Pupae were washed with Roccal on the sterile towel on which they were collected. They were then individually held with forceps and rinsed twice with sterile distilled water. Each insect was placed in a sterile vial cap and treated as shown in Table 3. Since we noted that the medium and large larvae, prepupae, and pupae required less humidity in the petri dishes than the eggs and small larvae, we added less water to the filter papers in dishes containing these stages. The mummified larvae and prepupae looked more like those from natural hive infections, possibly because of the lower humidity.

All brood was incubated at 25°C and observed for 15 days for growth of *A. apis* and mummification. No growth occurred on any eggs or pupae. After incubation for 7 days, several with eye color had developed wings, legs, antennae, and hair.

As shown on Table 4, 26 mummies were formed. However, 12 of these were apparently the result of natural infections of *A. apis* since they were in the control groups inoculated with dead *A. apis*. Of these

Table 3

TREATMENT GROUPS OF EGGS, LARVAE, AND PUPAE INOCULATED WITH *ASCOSPHAERA APIS*[a]

Treatment	Site of Inoculation
Eggs	
Live *A. apis*-mated	Body
Live *A. apis*-unmated	Body
Dead *A. apis*-mated	Body
Dead *A. apis*-unmated	Body
Small larvae	
Live *A. apis*-mated	Back
Live *A. apis*-mated	Mouthparts
Live *A. apis*-unmated	Back
Live *A. apis*-unmated	Mouthparts
Dead *A. apis*-mated	Back
Dead *A. apis*-mated	Mouthparts
Dead *A. apis*-unmated	Back
Dead *A. apis*-unmated	Mouthparts
Medium larvae	
Live *A. apis*-mated	Back
Live *A. apis*-mated	Mouthparts
Live *A. apis*-unmated	Back
Live *A. apis*-unmated	Mouthparts
Dead *A. apis*-mated	Back
Dead *A. apis*-mated	Mouthparts
Dead *A. apis*-unmated	Back
Dead *A. apis*-unmated	Mouthparts
Large larvae	
Live *A. apis*-mated	Back
Live *A. apis*-mated	Mouthparts
Live *A. apis*-unmated	Back
Live *A. apis*-unmated	Mouthparts
Dead *A. apis*-mated	Back
Dead *A. apis*-mated	Mouthparts
Dead *A. apis*-unmated	Back
Dead *A. apis*-unmated	Mouthparts
Prepupae	
Live *A. apis*-mated	Back
Live *A. apis*-mated	Mouthparts
Live *A. apis*-unmated	Back
Live *A. apis*-unmated	Mouthparts
Dead *A. apis*-mated	Back
Dead *A. apis*-mated	Mouthparts
Dead *A. apis*-unmated	Back
Dead *A. apis*-unmated	Mouthparts
Pupae-No Eye Color	
Live *A. apis*-mated	Back
Live *A. apis*-mated	Mouthparts
Live *A. apis*-unmated	Back
Live *A. apis*-unmated	Mouthparts
Dead *A. apis*-mated	Back
Dead *A. apis*-mated	Mouthparts
Dead *A. apis*-unmated	Back
Dead *A. apis*-unmated	Mouthparts
Pupae-Eye Color	
Live *A. apis*-mated	Back
Live *A. apis*-mated	Mouthparts
Live *A. apis*-unmated	Back
Live *A. apis*-unmated	Mouthparts
Dead *A. apis*-mated	Back
Dead *A. apis*-mated	Mouthparts
Dead *A. apis*-unmated	Back
Dead *A. apis*-unmated	Mouthparts

a 5 insects per treatment

12, 5 were medium-sized larvae, 5 were large larvae, and 2 were prepupae. Therefore, by experimental inoculation, one mummy was produced in the medium-sized larvae, 8 in the large larvae, and 5 in the prepupae. Thus, more mummies were produced from large larvae though few mummies were produced in any larvae on prepaue. De JONG and MORSE (1976) also observed wide variation in infection rates of larvae fed *A. apis*. In the present test, all mummies found were white (unmated) to gray (probably a mixture of mated and unmated strains). Other than the mummies, only 1 small and 1 medium-sized larva inoculated with live *A. apis* showed any growth of *A. apis*. In these 2 larvae, *A. apis*

grew from the mouth, anus, and through the cuticle. None of the other inoculated insects yielded any growth of *A. apis*.

Table 4

MUMMIES AND GROWTH OF *ASCOSPHAERA APIS* PRODUCED BY INOCULATION OF EGGS, LARVAE, AND PUPAE WITH *ASCOSPHAERA APIS*

Stage	Site of Inoculation	*A. apis* Strain	Mummies Produced	Growth of *A. apis*	
				Mycelium	Spores
Small larvae	Mouthparts	Live-mated		1	
Medium larva	Mouthparts	Live-mated		1	
Medium larva	Back	Live-unmated	1		
Medium larva	Mouthparts	Dead-mated	1		
Medium larva	Mouthparts	Dead-unmated	2		
Medium larva	Back	Dead-mated	1		
Medium larva	Back	Dead-unmated	1		
Large larva	Mouthparts	Live-mated	1		
Large larva	Mouthparts	Live-unmated	1		
Large larva	Back	Live-mated	1		
Large larva	Back	Live-unmated	5		
Large larva	Mouthparts	Dead-mated	1	1	
Large larva	Back	Dead-mated	3		
Large larva	Back	Dead-unmated	1		
Prepupae	Back	Live-mated	1		
Prepupae	Mouthparts	Live-mated	1		
Prepupae	Back	Live-unmated	2		
Prepupae	Mouthparts	Dead-mated			1
Prepupae	Mouthparts	Dead-unmated	1		
Prepupae	Back	Dead-unmated	2		

Eggs, pupae, and the smallest larvae of the honey bee are not susceptible to laboratory infection with *A. apis*. Medium and large larvae and prepupae are susceptible, and large larvae are the most susceptible. Also, all three of these age groups are susceptible to natural infections. The colony from which these insects were obtained had no visible signs of chalkbrood disease. Perhaps, *A. apis* resides within bee colonies without causing the overt signs of disease until the proper conditions exist. Il chilling is a necessary prerequisite for infection with our strain(s) of *A. apis* as with European strains (BAILEY, 1967), this condition should be met by our procedures for removal of brood from the comb and surface sterilization. However, in Arizona we have noted heavy year-round infections of larvae, even when the average monthly temperatures are 29°C. Strains of *A. apis* found in Arizona may thus

differ from those reported from Europe. We are now investigating this question.

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