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Title: Etiology and pathogenesis of chalkbrood in the alfalfa leafcutting bee, Megachile rotundata.

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Professor W. P. Stephen

Chalkbrood is a mycosis of larvae of the alfalfa leafcutting bee, Megachile rotundata (Fabricius). It is a serious threat to alfalfa seed growers in western North America who use populations of this bee for pollination. Basic studies were conducted to determine the etiology of this disease and the course of pathogenesis in afflicted larvae.

Spores of Ascosphaera aggregata Skou were found to cause chalkbrood in one- to nine-day-old larvae of M. rotundata. A distinctive syndrome accompanied infection of older larvae. The host hemolymph became cloudy with fungal mycelium at the time of death. A change from normal cream color to pink or tan occurred in larvae soon thereafter. Sporulation beneath the host cuticle took place within 2 weeks after death. Younger larvae did not undergo a color change but remained cream colored and the fungus did not sporulate.

Ascosphaera aggregata spores germinated in the midgut and penetrated the hemocoel of fourth instar larvae within 2 days after inoculation. Invasion of epidermis, tracheae and muscles followed within 3

more days. The entire hemocoel was nearly filled with mycelium at the time of death, 3 - 7 days after inoculation.

Conditions of reduced redox potential existed in the midguts of fourth instar larvae. A. aggregata spores germinated in vitro under conditions of reduced potential but mycelium proliferated under the oxidized conditions of an open petri dish. Events in vivo reflected preferences shown in vitro: germination occurred in the reduced midgut and mycelium proliferation occurred in the more oxidized tissues of the hemocoel.

Other Ascosphaera species were able to infect M. rotundata larvae. Spores of both A. apis (Maassen ex Claussen) Olive and Spiltoir and A. proliperda Skou caused a syndrome distinct from that caused by A. aggregata. No color changes were evident following infection by A. apis or A. proliperda. White mycelia erupted through the cuticle within 2 days after death. Sporulation outside the host cuticle followed within 1-2 weeks. The time to death was significantly shorter for larvae infected with A. proliperda than for those infected with A. apis. Spores taken from cadavers grew less readily on the pollen bee diet and appeared to be more virulent than spores produced in vitro. Ascosphaera atra Skou and Hackett and A. major (Prökschl and Zobl) Skou did not infect larvae but grew saprophytically on the pollen diet.

Etiology and Pathogenesis of Chalkbrood in the Alfalfa Leafcutting Bee, *Megachile rotundata*

by

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ETIOLOGY AND PATHOGENESIS OF CHALKBOOD IN THE ALFALFA LEAFCUTTING BEE, *MEGACHILE ROTUNDATA*

I. INTRODUCTION

History

Chalkbrood was first described as a mycosis of larvae of the honey bee, *Apis mellifera* L., by Maassen (1913), but Claussen (1921) was the first to give a detailed description of the presumed causative agent. Later studies by Maurizio (1934) confirmed the infectious nature of *Ascosphaera* (= *Pericystis*) *apis* (Maassen ex Claussen) Olive and Spiltoir. Spiltoir and Olive (1955) reviewed the early descriptive literature concerning fungal taxonomy and established the genus *Ascosphaera*. Spiltoir (1955) described the life cycle of *A. apis* in vitro. Bailey (1963, 1968) reviewed diseases of the honey bee including chalkbrood. Hitchcock (1972) provided a comprehensive review of the early reports of chalkbrood from Europe and New Zealand. Gilliam (1978a, b) has thoroughly reviewed several fungus/honey bee interactions including both chalkbrood and pollen mold (caused by a related fungus, *Bettsia alvei* (Betts) Skou).

While chalkbrood of honey bees has been known in Europe for many years, it was first reported in North America in 1970 (Hitchcock and Christensen 1972, Thomas and Luce 1972). It has spread through most of North America within the past ten years (DeJong and Morse 1976, Gochnauer and Hughes 1976, Menapace and Wilson 1976) and has caused problems to commercial apiarists.

The first record of chalkbrood of a solitary bee (Megachile sp.) was made by Melville and Dade (1944). Baker and Torchio (1968) isolated a fungus identified as A. apis from pollen in a cell of Megachile inermis Prov. in Utah, but the prepupa within the same cell was alive and later emerged as an adult. This fungus was probably not A. apis but another Ascosphaera species, perhaps A. atra Skou and Hackett. Thomas and Poinar (1973) noted a mycosis of M. rotundata (Fabricius) from California and diagnosed the causative agent as A. apis. However, Stephen and Undurraga (1978) regarded A. proliperda Skou as the likely agent. Stephen et al. (1981) corrected this diagnosis and now consider A. aggregata Skou as the causative agent of chalkbrood in this bee.

Skou (1972, 1975, Skou and Hackett 1979) has revised the Ascosphaerales and added new species. He cited megachilids as hosts for five of six species in the genus Ascosphaera. He discounted earlier reports of A. apis on solitary bees (Baker and Torchio 1968, Melville and Dade 1944) and suggested the fungi involved may belong to other Ascosphaera species. He reported M. rotundata as a host for A. aggregata and A. atra. Stephen et al. (1981) have reviewed the Ascosphaera species and have provided their own observations of several taxonomic characters.

Chalkbrood Research

Recently many workers in North America and elsewhere have pursued the chalkbrood problem in honey bees. Studies have been concentrated in the areas of the culture of A. apis, experimental infections of colonies (DeJong 1976, Gilliam et al. 1978, Gochnauer and Margetts 1979, Herbert et al. 1977, Mehr et al. 1976, Samsinakova et al. 1977, Thomas and Luce

1972), and control of A. apis with fungicidal compounds incorporated into supplemental food (Gochnauer and Margetts 1980, Moeller and Williams 1976, Samsinakova et al. 1977, Thomas and Luce 1972). Chalkbrood is not considered a serious threat to healthy hives, but can cause damage to honey bee populations under stress (Gilliam 1978a).

Megachile chalkbrood research has been more limited because of its relatively recent recognition as a serious threat to the alfalfa seed industry (Stephen and Undurraga 1978), and to the regional nature of commercial use of this bee. Laboratories in California (Hackett 1980), Idaho (Kish 1980), and Oregon (Fichter et al. 1981, Stephen et al. 1981, 1982, Vandenberg et al. 1980) are currently exploring the problem.

Hackett (1980) described several larval disease syndromes of M. rotundata and tried to demonstrate viral etiologies for some. Furthermore, he suggested a link between chalkbrood etiology and virus infection of the bee larvae. Although he was able to infect only early stage larvae with A. aggregata, Hackett suggested that this fungus is a primary pathogen but that "ubiquitous" virus particles (p. 420) stress the larvae (p. 454) and allow chalkbrood to develop. However, Hackett did not rear larvae aseptically and so worked with hosts which may not have been disease-free. Fichter et al. (1981) developed a technique for aseptic rearing of alfalfa leafcutting bee larvae which has permitted me to conduct controlled studies of the infectivity of Ascosphaera species for larvae of M. rotundata.

Kish (1980) reported a technique for germinating spores of A. aggregata in vitro. We have employed a modification of his method in order to test potential sporicides against A. aggregata (Stephen et al.

1982). Other work in our laboratory included a study of A. aggregata spore load on chalkbrood cadavers and on adults of M. rotundata emerging from populations with high disease incidence (Vandenberg et al. 1980). We later followed the fate of spores in the field and demonstrated their presence on older adults, in field domiciles, and in the air around domiciles (Stephen et al. 1981).

Chalkbrood Etiology

The causative agent of chalkbrood in honey bees is A. apis (Bailey 1967, Maurizio 1934), although its route of entry into the insect is open to question (Gilliam 1978b). Maurizio (1934) found the fungus in the intestines of larvae from infected colonies, and Bailey (1967) contended that spores need the reduced oxygen tension in the gut of early stage larvae in order to germinate. Subsequent hemocoelic invasion does not take place unless the larvae are chilled, thereby allowing oxygen to diffuse into the gut and stimulate mycelial growth. Gilliam et al. (1978), however, were able to infect larvae of three size classes and prepupae with A. apis inocula.

Holm and Skou (1972) described diseases of greenhouse and field populations of M. centuncularis L. in Denmark. Bees in the greenhouse population suffered from a chalkbrood-like disease apparently caused by A. proliperda. Grayish white mycelia were visible in and on larvae inside their cells. This fungus was found only in the greenhouse population of this bee. A population of M. rotundata inhabiting the same greenhouse was unaffected by the disease. In contrast, few larvae of M. centuncularis in the field population died of chalkbrood. The

pollen and leaves of inner cells supported growth of A. major (Prökschl and Zobl) Skou.

Skou (1975) later described A. aggregata from larval collections of M. rotundata from Spain and the U.S., and of M. centuncularis and Osmia rufa L. from Denmark. His preliminary studies indicated that spores of A. aggregata caused disease in larvae of O. rufa. Mycelia were not seen on the surface of infected larvae, but dusty gray spore cysts formed beneath the cuticle giving afflicted larvae a swollen ragged appearance.

Only first and second instar larvae of M. rotundata succumbed to infection by A. aggregata in tests by Hackett (1980), leading him to suggest that larvae may be susceptible to infection only when young. Like young honey bees, early instar leafcutting bee larvae have a blind gut, i.e. there is no opening to the anus. Consequently, conditions of reduced oxygen tension are expected and these conditions may change when the larvae begin defecation after the molt to the fourth instar.

Early attempts to obtain isolates from A. aggregata spores were successful only when inoculum on nutrient agar medium was covered with an additional layer of medium. Skou (1975) reported no similar difficulty with this fungus stating that ascospores germinate on agar medium and produce a non-sporulating mycelium. However, Bailey (1967) found A. apis spore germination was best when the inoculum was incubated beneath the surface of semi-solid agar. Kish (1980) was able to germinate a high proportion of A. aggregata spores on a nutrient medium only in the presence of high CO_2 concentrations. Other anoxic and anaerobic incubation systems do not promote germination of spores of this fungus (Stephen et al., unpublished). These data suggest that

oxygen tension per se may not be important, but that CO_2 concentration or the oxidation-reduction potential of the medium surrounding the spore both in vivo and in vitro may determine germinability (W. C. Denison and M. E. Martignoni, personal communications).

Sporulation of A. aggregata has not been achieved in vitro. Kish (personal communication) has tried many simple and complex media without success. Spore germination and colony formation occur routinely, but completion of the life cycle has not been observed. My own experiments with media for culture maintenance (Appendix IV) also did not yield sporulation by A. aggregata. However, I was able to obtain spores by infecting larvae reared by the method of Fichter et al. (1981). Spores produced in this way were subsequently tested for their ability to infect disease-free larvae of M. rotundata.

The purpose of my investigation was to determine whether Ascosphaera aggregata, found on bees or isolated from them, caused chalkbrood in larvae of Megachile rotundata. Larvae of different ages were tested for susceptibility. The route of fungus invasion and tissue pathogenesis was followed in diseased larvae. I have attempted to relate spore germination of A. aggregata in vitro to events in vivo by estimating the pH and redox potential of germination media and gut contents. Finally, I have established that other Ascosphaera species are pathogenic for alfalfa leafcutting bee larvae.

II. ETIOLOGY AND SYMPTOMATOLOGY

Introduction

The alfalfa leafcutting bee, Megachile rotundata, is a commercially important domesticated pollinator of alfalfa in western North America. Bee populations are maintained in or near alfalfa fields in domiciles provided with drilled boards, boxes of paper soda straws, or other nesting materials (Stephen and Every 1970). Female bees construct linear series of cells within each tunnel. Each cell is lined with leaves of alfalfa and/or other plants, provisioned with alfalfa pollen and nectar, and topped by a single egg before being capped with circular leaf pieces. Usually all cells within a given tunnel contain siblings.

Since 1974, when it was first reported from Nevada, chalkbrood has become a devastating larval disease in commerical populations of M. rotundata. Bee losses exceeding 50% have occurred in many areas of the west (Stephen and Undurraga 1978; Stephen et al. 1981). While the presumed causative agent of chalkbrood is the fungus, Ascosphaera aggregata (Skou 1975), virus infections have also been implicated in this pathology (Hackett 1980).

Transmission of the disease is thought to occur as a result of a series of events beginning with the emerging adults chewing through sibling larval cadavers covered with spores (Vandenberg et al. 1980); contamination of their mates, eggs, and/or pollen provisions; germination of spores in the guts of infected larvae; and host death and fungal sporulation at or before the prepupal stage. Many aspects of this model need to be tested.

Materials and Methods

Chalkbrood cadavers used in this study were obtained by infecting aseptically reared larvae. The cadavers were surface sterilized in 0.4% Hyamine 10-X (Rohm & Haas, methyl benzethonium chloride) and rinsed twice in sterile distilled water (SDW) (Martignoni and Milstead 1960). The cuticle was removed, the spores were carefully scraped from the cadavers' outer surface, and stored in sterile vials at 2°C until used. These spores were either used dry or suspended in buffer (pH 7.1) composed of 0.02 M BES (N,N-bis(2-hydroxyethyl)-2-aminoethane sulfonic acid), 0.15 M NaCl, and 0.0075 g/l DOSS (dioctyl sulfosuccinate, sodium) (M. E. Martignoni, personal communication). Spore cysts, spore balls, ascospores, and mycelia were used to identify the fungus as Ascosphaera aggregata Skou (1975).

Subsamples of spores were germinated on a vegetable juice agar medium (Kish, 1980), and the resulting mycelium was isolated on the same medium. Since sporulation has not been achieved in vitro, the mycelial cultures that resulted from this isolation procedure were used as inocula.

Spore suspensions used as inocula were tested for contaminants as follows: duplicate plates or tubes of vegetable juice agar, Sabouraud dextrose agar, nutrient agar (Difco), and Bacto-AC Medium (Difco) were inoculated with 0.1 ml of an A. aggregata spore suspension at a concentration of ca. 10^7 spores/ml; plates and tubes were incubated at 30°C for 3-5 days and examined for bacterial or fungal colony growth. These tests were routinely negative.

M. rotundata eggs were collected from populations near Corning, California, and Ontario, Oregon. They were pre-rinsed for 10 minutes in SDW to remove attached pollen, surface sterilized by immersion for 5 minutes in an aqueous solution containing 0.05% Tween 20 and 0.5% Dry Chlorine (Georgia-Pacific, sodium dichloro-s-triazinetrione dihydrate), and rinsed again for 5 minutes in SDW. The chlorine compound is known to kill spores of A. aggregata in vitro (Stephen et al. 1982). Eggs were then transferred to an artificial diet in polystyrene titration plates for rearing. Procedures for rearing larvae aseptically on an autoclaved pollen-based diet have been described by Fichter et al. (1981).

One to 9 day old larvae were transferred to fresh sterile diet inoculated with 4 mm^2 blocks of mycelium or with dry or suspended spores. The bees were incubated with the inoculated diet in darkness at 30°C in high (> 80%) relative humidity. Larvae were monitored daily for survival and for the appearance of chalkbrood symptoms. It was not determined whether larvae ingested all of the inoculum, but since the inoculum was placed on the upper surface of the diet, all larvae consumed most of it. Bees that died within 48 hours of transfer were discarded as handling losses. Tissue smears were made of portions of cadavers suspected of having chalkbrood to determine the presence of fungal mycelium. To confirm the diagnosis, cadavers were surface sterilized in 0.4% Hyamine 10-X and portions of the body were used to inoculate the vegetable juice agar medium and establish mycelial cultures.

Results and Discussion

Diagnostic criteria were established for distinguishing healthy and diseased larvae. Living, healthy larvae were characterized by the presence of a heartbeat, clear hemolymph, movemement of mouthparts, and a translucent white to cream colored fat body. Chalkbrood signs first became apparent after cessation of body movement, usually during the fifth instar. Within 24 hours, the larval hemolymph appeared milky because of the presence of fungal mycelium. This was followed by the development of a pink, tan, or gray cast internally in one body area (e.g. head or abdominal dorsum) which spread throughout the host within another 24-48 hours. Diseased larvae remained tan or changed to a chalk- white color and finally assumed a mottled appearance as dark fungal cysts formed beneath the cuticle. Usually the cuticle remained intact, but occasionally the fungus erupted and grew to a limited extent on the residual pollen diet.

Of the 14 1-day-old larvae inoculated with spores, 4 died of chalkbrood before, and 5 shortly after, reaching the fourth instar. In each of these 9, chalkbrood symptomatology was different from that observed in larvae inoculated at a later age. No internal color change to pink, tan, or gray was observed. Within 24 hour after cessation of movement, the hemolymph became cloudy and a cream colored mycelium was visible within the host hemocoel. The opaque cream color was retained as the cadaver hardened within a few days. Only non-sporulating mycelial cultures were isolated from these cadavers. Thus, the symptomatology of A. aggregata infection in young larvae is distinctly different from that

in older larvae. Both sets of signs have been observed in M. rotundata larvae collected from field populations.

None of 9 mycelial-fed larvae died of chalkbrood. These 9 as well as all 8 agar-fed controls reached the prepupal stage. Thus the infective stage appears to be the fungal spore. The localized area of internal color change in the freshly dead host (described above) may represent the site of invasion of the fungus into the hemocoel from the gut. Spore germination in the gut and subsequent hemocoelic penetration have been observed and will be treated in section III.

The results of several infectivity experiments are presented in Table 1. Chalkbrood incidence in groups of eggs not surface sterilized prior to transfer to pollen diet (36%) was similar to that in field populations from which these eggs were collected (ca. 30%). All re-isolations of fungi from cadavers yielded only non-sporulating cultures. Mycelia have a creamy tan color, similar in appearance to that observable in the host, and typically darken the surrounding agar medium (c.f. Skou 1975).

Eighteen larvae from the 222 egg transfers died of causes not ascertained (Table 1, "Pct. other"). This undiagnosed mortality may be attributed to several factors. Although dead and dying larvae were discarded within 48 hours of handling, some injuries resulting from treatment may have gone unnoticed for longer periods. The nutritional suitability of the pollen-based diet has not been tested extensively, and the diet may be lacking in certain growth factors. No measure was taken of the proportion of surviving prepupae that successfully pupated and emerged as adults. Finally, the presence of other infectious micro-

organisms within the egg has been postulated (Hackett 1980), but none of the larvae which died from unknown causes exhibited symptoms or signs that would indicate an infectious disease.

Linear regressions of dose, the natural logarithm of dose, and host age at the time of inoculation on the time to death were not significant Analysis of variance did show significant differences in the average time to death for larvae inoculated at different ages (Table 2). Attempts to correlate larval age and the aerobic state of the gut with early events in the infection process will be reported in section III.

The inability to induce sporulation of A. aggregata in pure culture does not necessarily preclude definitive statements about chalkbrood etiology. Aseptically reared larvae have been inoculated with A. aggregata spores and sporulating cadavers have been obtained. This next generation of spores was then tested for possible contaminants (none were found) and used to infect aseptically reared larvae. Attempts are underway to separate spores from contaminants not detected by our tests by differential centrifugation and washing in bactericidal and virucidal solutions (Appendix I). Experiments on the pathogenicity of other Ascosphaera species that do sporulate in pure culture have been conducted and are reported in section IV. While some species appear to be pathogenic, typical chalkbrood symptoms and signs have not been observed. From the studies reported here, chalkbrood is a mycosis that develops following ingestion of A. aggregata spores by M. rotundata larvae.

Table 1. Response of Megachile rotundata larvae to inoculation with Ascosphaera aggregata spores.

Age[a]	Treatment[b]	No. treated	Percent surviving[c]	Percent chalkbrood	Percent other[d]
1	Control	11	82	0	18
	Fresh spores	14	0	100	0
4	Untreated	7	43	14	43
	Control	26	85	0	15
	Suspension	38	8	87	5
5	Control	9	89	0	11
	Suspension	9	0	89	11
6	Untreated	19	84	16	0
	Control	8	88	0	12
	Suspension	17	12	82	6
	Fresh spores	9	0	89	11
7	Untreated	10	10	90	0
	Control	11	82	0	18
	Suspension	22	9	91	0
9	Control	6	100	0	0
	Fresh spores	6	0	100	0

[a]Age of host in days at time of treatment. Fourth instar reached at 5-6 days.

[b]Untreated larvae reared from eggs not surface sterilized. Control larvae inoculated with buffer only. Suspended spores all at a dose of 10^4-10^5 spores/larva. Inoculum volume 5-10 µl. Fresh spores all at dose of 10^5-10^6 spores/larva.

[c]Survival past the fourth instar, or the stage at which chalkbrood may be diagnosed.

[d]Undiagnosed mortality.

Table 2. Effect of larval age at inoculation on time to death.

Age[a]	No. inoculated[b]	No. chalkbrood	Time to death[c]
1	14	14	8.5 i
4	38	33	9.8 i
5	9	8	4.2 k
6	26	22	6.4 j
7	22	20	5.6 jk
9	6	6	5.7 jk

[a]Age of host in days at time of treatment. Fourth instar reached at 5 to 6 days.

[b]Inoculated with Ascosphaera aggregata spores.

[c]Average, in days. Means followed by the same letter are not significantly different by analysis of variance, Student-Newman-Keuls' test, $p < 0.05$.

III. PATHOGENESIS

Introduction

Spores of Ascosphaera aggregata cling to emerging adults of Megachile rotundata as they chew through spore-laden chalkbrood cadavers (Vandenberg et al. 1980). Some adults carry spores throughout their lives (Stephen et al. 1981) and may contaminate their mates, eggs and pollen provisions. Developing larvae are invaded by spores germinating in the gut. This paper describes the course of infection from ingestion of spores until host death.

Ascosphaera aggregata spores may be germinated in vitro on a nutrient medium in the presence of high CO_2 concentrations (Kish 1980). When N_2 or reduced pressure was substituted for CO_2, spores did not germinate (Stephen et al., unpublished). Apparently oxygen tension alone is not responsible for germination, but CO_2 or oxidation-reduction potential may play a role in spore germination. Therefore the pH and redox conditions in guts of normal larvae were studied and an attempt made to relate in vitro phenomena with in vivo events.

Materials and Methods

Sample Preparation for Microscopy: Larvae were reared on an autoclaved, pollen-based diet in individual wells of polystyrene titration plates (Fichter et al. 1981). Fresh diet was inoculated with spores of A. aggregata at a dose of 10^5 spores per larva as described by Vandenberg and Stephen (1982). Disease-free larvae were transferred to this inoculated diet and allowed to feed at 30°C. Beginning at 24 hours

post-inoculation and at 12 to 24 hour intervals for 8 days, larvae were fixed for histological processing in alcoholic Bouin's fluid or buffered formalin (Humason 1972). Samples were dehydrated in ethanol and cleared in toluene. Alternatively, dioxane was used for both the dehydration and clearing. Paraplast®-plus (Sherwood Medical, St. Louis) was used as the embedding medium. Sections were made at 8-10 µm, affixed with Mayer's albumin to glass slides, and stained with hematoxylin and eosin (H and E), Mallory's triple stain (MTS), periodic acid-Schiff reagent (PAS) or Gomori's methenamine silver nitrate (GMS) (Humason 1972). Sections were examined for the presence of fungal growth within the host and photographed. Other cleared and dehydrated sections were examined by scanning electron microscopy.

Measurement of Redox Conditions: Rearing containers with 0.1 ml fresh diet were each treated with 10 µl of one of five filter-sterilized redox dyes at 0.1%. The indicators are shown in Table 3 with their characteristic colors. Ten third and fourth instar larvae per dye were transferred to treated diet. When enough colored diet had been consumed so that it appeared in the frass (2 days at 30°C), larvae were dissected in the following way. The cuticle was punctured and the pH of the hemolymph was measured to the nearest 0.2 units using narrow range pH paper (Hydrion, Micro Essential Laboratories, Brooklyn). The intact gut was then quickly removed following decapitation and removal of the last few abdominal segments. The color of each of four regions (anterior, central, and posterior midgut, and the hindgut) was estimated immediately. Then the peritrophic lining was cut and the pH of each region was measured with paper. Colorless regions were observed to

regain their color after several seconds of exposure to air and colored regions were made to lose their color following addition of a few crystals of sodium dithionite. The entire process, from initial rupture of the cuticle to estimation of redox color and pH of the gut regions, took approximately 45 seconds per larva.

The color of each region was recorded as: no detectable color, partial color, or full color. Full color was assigned to gut regions showing the color of oxidized diet and frass. The observed potential (Eh) for partial color was considered equal to the Eo (the redox potential of the dye when 50% reduced, corrected for pH). When no color was detected, Eh was considered less than Eo minus 30 mV (less than 10% oxidized). Similarly the Eh in the presence of full color was greater than Eo plus 30 mV (greater than 90% oxidized) (Hewitt 1950).

To estimate the redox potential of our in vitro spore germination system (Stephen et al. 1982), 10 µl of dye solution (0.1%) was added to duplicate samples of vegetable juice agar (pH 7.0) and bee diet (pH 5.8). Color was judged after 24 hours incubation at 30°C.

Results

Photomicrographs of paraffin sections are presented in Figures 1-4. Uninfected M. rotundata larvae are shown in Figure 1. Germ tube formation by A. aggregata within the host midgut and penetration of the peritrophic lining occurred within 1-3 days after inoculation of early fourth instar larvae (Figure 2). Within 2-4 days, fungus hyphae were detected in the host hemocoel invading the epidermis and tracheae (Figure 3) and muscles (Figure 4a). The entire hemocoel was filled with fungus tissue within 5-6 days (Figure 4b). The midgut and silk glands

were the last tissues to be invaded by the fungus, usually after host death.

The pH of each gut region varied little among larvae (Table 4). More basic conditions were found in the posterior midgut (pH 7.6), but were near neutral elsewhere.

All redox dye colors were easily discerned in the pollen diet at the concentrations used. A range of redox conditions existed among test larvae as shown by these dyes (Tables 5-8). The most reduced conditions were found in the central and posterior portions of the midgut (Tables 6-7). A wider range of potentials was evident in the anterior midgut and the hindgut (Tables 5 and 8).

All 30 larvae treated with toluylene blue, thionine and methylene blue showed no color in the central midgut (Eh < -38 mV) (Table 6). Toluylene blue remained colored (in the oxidized state) in all 10 anterior midguts (Eh = 125 mV) (Table 5), and in 8 of 10 posterior midguts (Eh = 90 mV) (Table 7). Most larvae (19 of 20) treated with thionine and methylene blue also showed colored (oxidized) anterior midguts (Eh = 11-74 mV) (Table 5), but 12 of 20 were colorless in the posterior midgut (Eh < 13 mV) (Table 7). Most larvae (23 of 30) treated with these 3 dyes showed partial color in the hindgut (Eh > 0 mV); the other 7 were colorless (Table 8). Treatment with nile blue left all 10 anterior and posterior midguts showing partial or full color (Eh > -175 mV) (Tables 5 and 7). Three central midguts were colorless (Eh < - 172 mV) (Table 6). Only 2 hindguts showed partial color, and the other 8 were completely oxidized (Eh > -159 mV) (Table 8). Phenosafranine was in the oxidized state (full color) throughout the guts of all test

larvae (Tables 5-8) except for the central midgut of 2 specimens which showed partial color (Eh = -260 mV) (Table 6).

A separate analysis of redox conditions for each gut region may indicate that the regions are independent of one another within a larva. The fact that this is not the case may be illustrated by a few further observations. All 50 larvae showed color in the anterior midgut. Thirty-seven showed some reduction in color between this region and the central midgut. Thirty-three of these 37 showed colorless midguts; the other 17 with color in the central midgut had been treated with nile blue and phenosafranine, the dyes with the lowest Eo. Twenty-one of 33 larvae with colorless midguts also had colorless posterior midguts, and 7 of these also had colorless hindguts. Fifteen of 42 larvae showing no color or partial color in the central midgut showed an increase in color (increased oxidation) in the posterior midgut. All 30 larvae that showed partial or full color in the posterior midgut also showed it in the hindgut (i.e. no further reduction). In addition, 21 larvae showed an increase in color between the posterior midgut and the hindgut. Twenty-nine showed no change between these two regions, but 10 of these were treated with phenosafranine and were fully oxidized in both regions.

The redox potential in the A. aggregata spore germination system in vitro is approximately 60 to 100 mV (Table 9). On the vegetable juice agar, all dyes showed color although the two with the highest Eo, toluylene blue and thionine, showed some reduction. Mixed with the bee diet at pH 5.8, the toluylene blue became colorless and the thionine underwent partial reduction.

Discussion

Chalkbrood pathogenesis depends upon A. aggregata spore germination in the midguts of M. rotundata larvae and subsequent invasion of the host hemocoel. Spores germinate in vivo under conditions of reduced redox potential within 1-3 days after they are ingested. The mechanisms by which germ tubes penetrate the peritrophic lining have not been studied but may involve extracellular enzyme production or mechanical disruption of this barrier. After invasion of the hemocoel, hyphae can be found within the epidermis, tracheae, muscles and fat body within 1-5 days.

Observations on redox conditions were made of larvae fed a pollen-based autoclaved diet. Pollen provisions collected by adult female M. rotundata for their offspring are more acidic and most likely differ in other respects from the laboratory diet. The gut floral composition may be influenced by this bee diet, pH, and perhaps by the presence of redox dyes (Jacob 1970). Alteration of the microorganisms within the gut as a result of ingestion of laboratory diet or dyes are possibilities that have not been explored.

Dyes have been observed in larvae not suffering from chalkbrood. Conditions in the guts of infected larvae may differ. Perhaps the redox potential is further reduced in the presence of germinating A. aggregata spores. The influence of inoculum concentration on chalkbrood pathogenesis is unknown.

Conditions in the midguts of M. rotundata larvae are more reduced than those required for A. aggregata spore germination in vitro. The optimum Eh range for spore germination is not known. Bailey (1967)

suggested that although chilled honey bee larvae are more susceptible to chalkbrood than are unchilled larvae, the temperature change is not critical. Rather, the increased oxygen levels in the gut at lower temperatures allow renewed growth of germ tubes and mycelia of A. apis. Oxidation-reduction potentials are very low in parts of the midgut of fourth instar M. rotundata larvae, regions in which spores of A. aggregata germinate and successfully penetrate the host hemocoel. Thus it appears that mechanisms associated with the induction of chalkbrood in this host differ from those proposed for honey bee larvae.

There are a number of problems associated with the use of dyes for the estimation of redox potentials (Hewitt 1950, Jacob 1970). 1) The dye in its oxidized form may itself oxidize the system and remain colored. In this study, 10 µl of a 0.1% dye was added to 0.1 ml diet. Thus the final concentration of the dye was 0.01%, relatively low compared to the 1 to 10% solutions used in studies of termite digestive systems (Eutick et al. 1976, Veivers et al. 1980). However, diet treated with nile blue was quite deeply colored. This dye may have overpoised the treated guts. Furthermore, the hindgut is a narrow tube in M. rotundata, and concentrations of all dyes may have been increased in this region through selective absorption of water and nutrients from the diet. 2) Dyes may also catalyze certain reactions or poison microorganisms in the system. This was not investigated, but the final dye concentration (0.01%) should have been low enough to prevent both catalysis and toxicity. 3) The reduction of the dye may be incomplete at the time of observation. In this study all larvae showed colored (oxidized) dye in the anterior midgut. Thus the dyes may not have been

fully reduced in this region closest to the point of ingestion. Conclusions drawn about the redox potential in this region must be considered with caution.

Following A. aggregata spore germination in vitro in high CO_2 concentrations (Kish 1980), mycelial cultures isolated from these germ tubes thrive on the surface of agar exposed to air. Skou (1975) reported no similar CO_2 requirement in order to isolate this fungus. Mycelia isolated in vitro resemble those evident within infected hosts (Vandenberg and Stephen 1982). Preliminary studies indicate that a small proportion of spores do germinate in vitro when incubated in air instead of CO_2 (Appendix V). The possible requirement for CO_2 or reduced redox potential by spores germinating in vivo has not been fully explored. Redox potentials in host organs other than those reported in this study have not been measured and it may be that mycelia normally proliferate in the presence of possibly elevated redox potentials within the host hemocoel.

Figure 1. Head and thorax (A) and epidermis and cuticle (B) of uninfected Megachile rotundata larvae. A) MTS. Bar = 120 μm. B) PAS. Bar = 30 μm. B = brain, E = epidermis, F = fat body, L = lumen of the gut, M = mandible, S = silk gland.

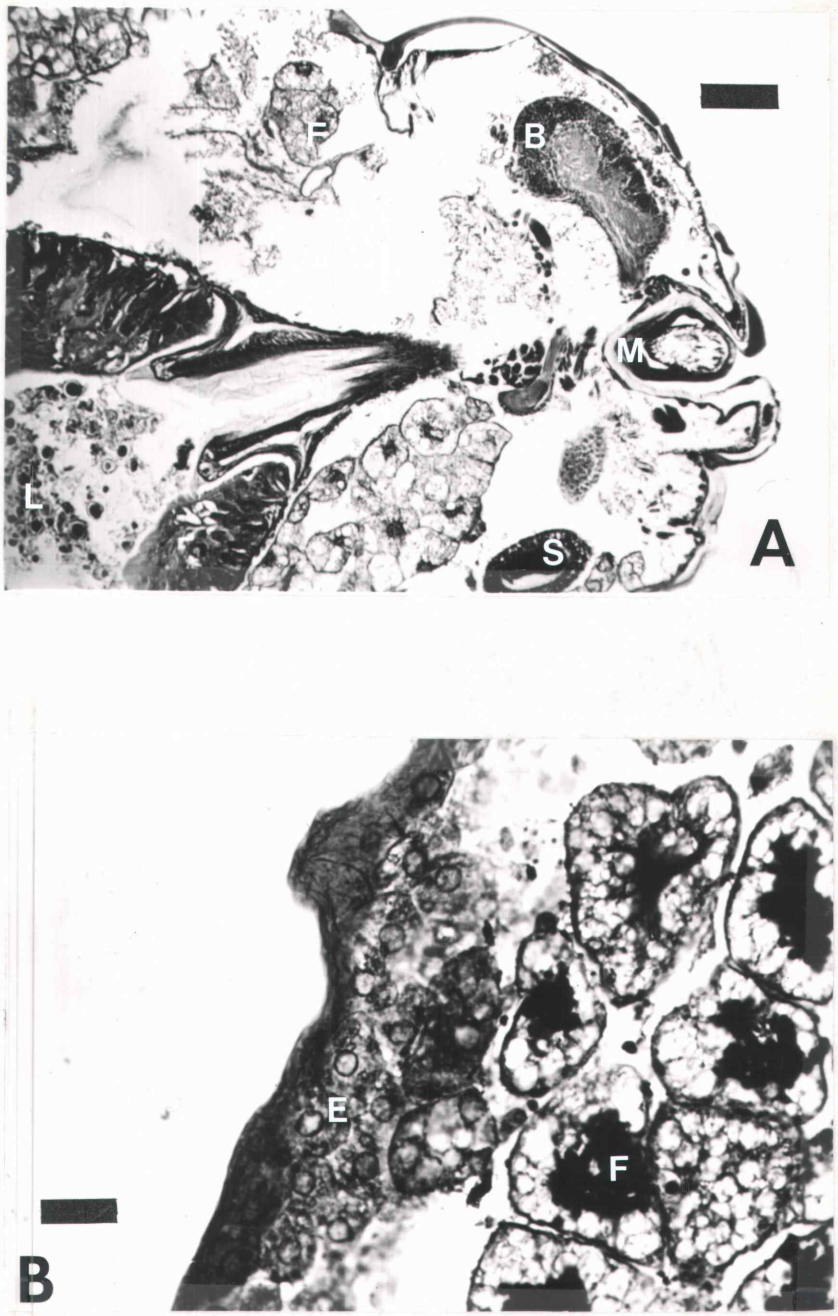

Figure 1

Figure 2. Germ tubes of Ascosphaera aggregata in the midgut of Megachile rotundata larvae, near the proventriculus (A), and the peritrophic lining (B). A) H and E. Bar = 30 µm. B) MTS. Bar = 20 µm. arrows = germ tubes, H = hemocoel, L = lumen of the gut, Pe = peritrophic lining, Po = pollen, Pr = proventriculus.

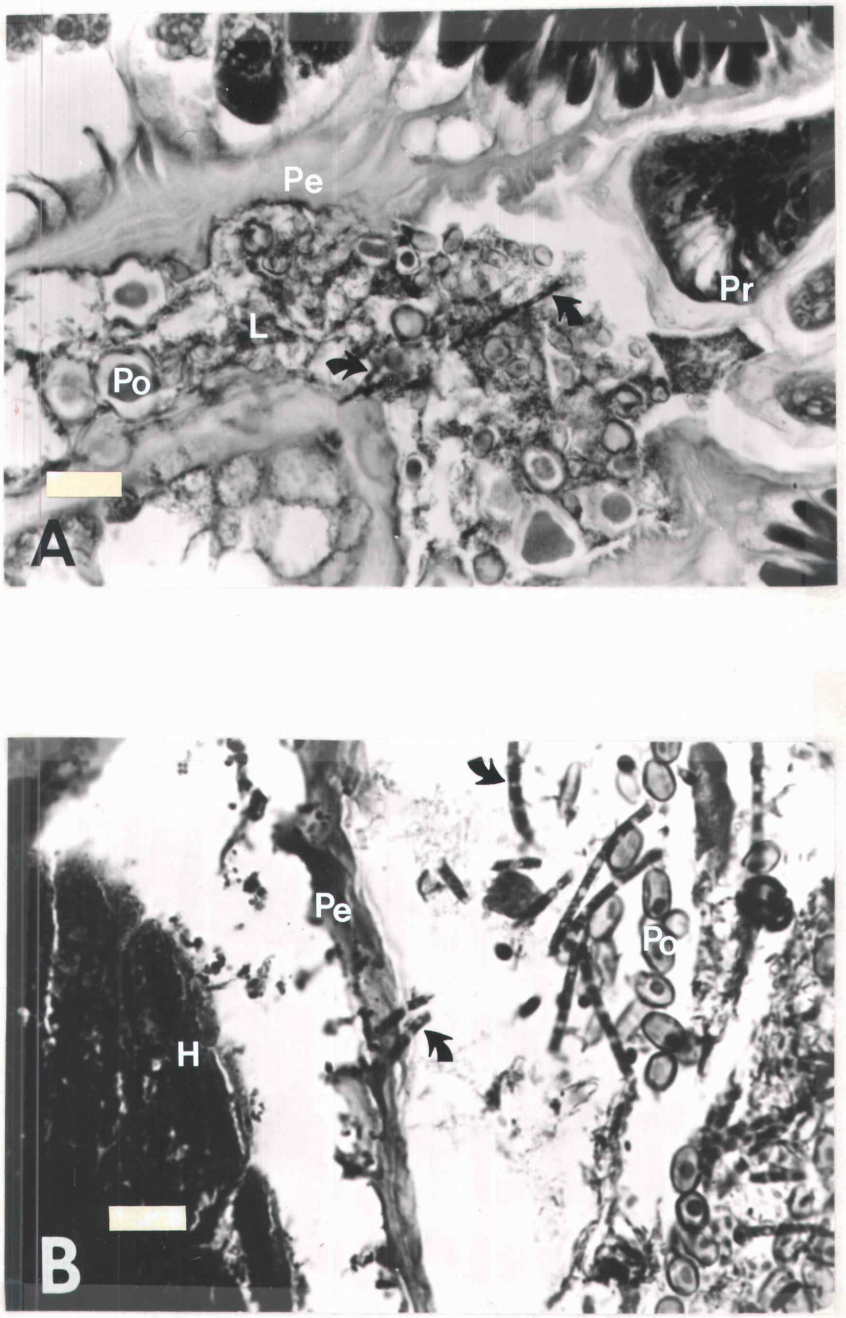

Figure 2

Figure 3. Ascosphaera aggregata mycelia in the epidermis (A) and trachea (B) of Megachile rotundata larvae. A) PAS. Bar = 30 µm. B) MTS. Bar = 30 µm. arrows = hyphae, E= epidermis, F = fat body, T = trachea.

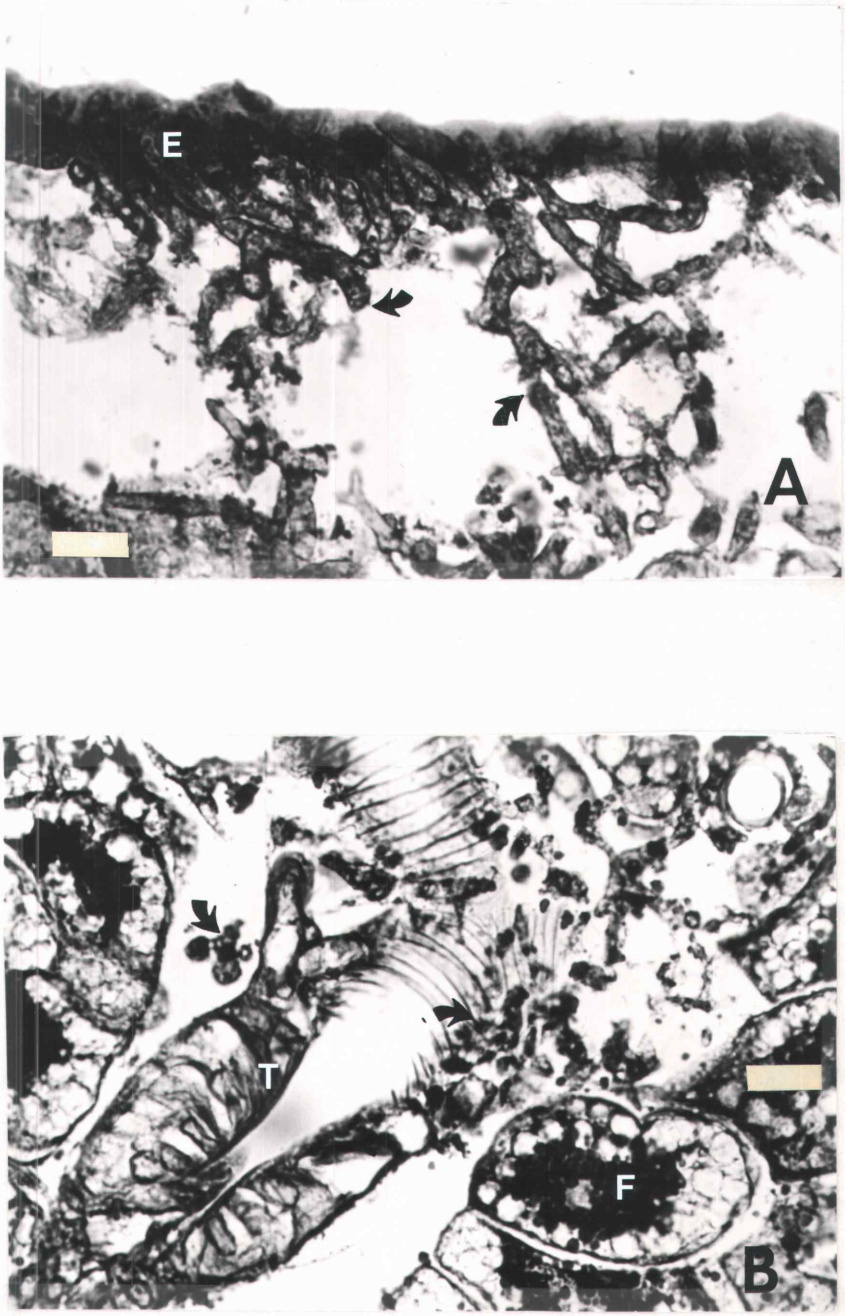

Figure 3

Figure 4. Ascosphaera aggregata mycelia in muscle and epidermis (A), and in the hemocoel (B) of Megachile rotundata larvae. A) SEM. Bar = 10 μm. B) GMS. Bar = 40 μm. arrows = hyphae, C = cuticle, F = fat body, M = muscle.

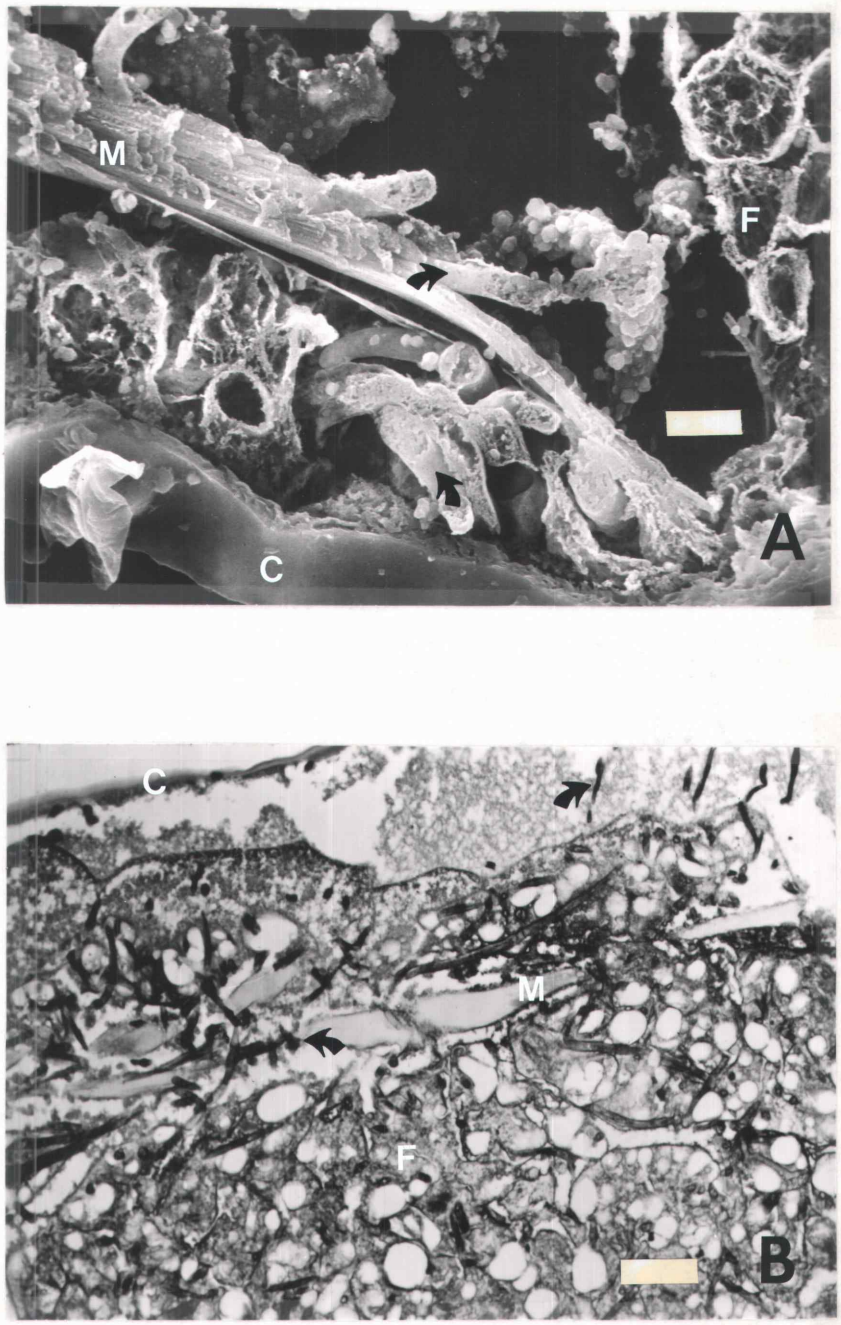

Figure 4

Table 3. Redox indicators employed, their E'o[a], and the colors associated with their oxidation state.

Dye	E'o	Color		
		Oxidized	Partial	Reduced
Toluylene Blue	+115 mV	R[b]	P	C
Thionine	+ 62 mV	G	LG	C
Methylene Blue	+ 11 mV	G	LG	C
Nile Blue	-142 mV	B	LB	C
Phenosafranine	-252 mV	R	P	C

[a]Potential of a 50% reduced dye at pH 7.0 and 30°C.

[b]B = blue, C = colorless, G = green, LB = light blue, LG = light green, P = pink, R = red.

Table 4. The pH of gut contents and hemolymph of fourth instar larvae of Megachile rotundata[a].

	Midgut			Hindgut	Hemolymph
	Anterior	Central	Posterior		
Mean[b]	6.8	7.2	7.6	7.0	6.9
Range	6.6-7.0	7.0-7.6	7.4-7.8	6.8-7.4	6.8-7.2

[a]Measured with narrow range pH paper.

[b]N = 50.

Table 5. Range of Eh[a] values obtained from dyes in the anterior midgut of fourth instar Megachile rotundata larvae.

Dye	Eo[b]	No. with partial color	Eh range (mV) max	Eh range (mV) min	No. with full or no color	Eh range (mV) max	Eh range (mV) min
Toluylene Blue	+123 mV	10	+132	+123	0	--	--
Thionine	+ 68 mV	10	+ 74	+ 62	0	--	--
Methylene Blue	+ 17 mV	9	+ 24	+ 11	1(full)	> + 47	--
Nile Blue	-133 mV	6	-133	-142	4(full)	> - 94	> -103
Phenosafranine	-238 mV	0	--	--	10(full)	> -194	> -222

[a]Eh = observed potential

[b]Eo = potential of a 50% reduced dye corrected for pH at 30°C. The pH in the anterior midgut averages 6.8.

Table 6. Range of Eh[a] values obtained from dyes in the central midgut of fourth instar Megachile rotundata larvae.

Dye	Eo[b]	No. with partial color	Eh range (mV) max	Eh range (mV) min	No. with full or no color	Eh range (mV) max	Eh range (mV) min
Toluylene Blue	+108 mV	0	--	--	10(no)	< + 85	< + 64
Thionine	+ 56 mV	0	--	--	10(no)	< + 32	< + 13
Methylene Blue	+ 4 mV	0	--	--	10(no)	< - 19	< - 38
Nile Blue	-151 mV	7	-142	-151	3(no)	< -172	< -181
Phenosafranine	-266 mV	2	-252	-266	8(full)	> -222	> -263

[a] Eh = observed potential

[b] Eo = potential of 50% reduced dye corrected for pH at 30°C. The pH in the anterior midgut averages 7.2.

Table 7. Range of Eh[a] values obtained from dyes in the posterior midgut of fourth instar Megachile rotundata.

Dye	Eo[b]	No. with partial color	Eh range (mV) max	Eh range (mV) min	No. with full or no color	Eh range (mV) max	Eh range (mV) min
Toluylene Blue	+ 94 mV	2	+ 94	+ 88	8(no)	< + 71	< + 58
Thionine	+ 43 mV	5	+ 50	+ 43	5(no)	< + 13	< 0
Methylene Blue	- 8 mV	3	- 8	- 14	7(no)	< - 32	< - 44
Nile Blue	-167 mV	10	-167	-175	0	--	--
Phenosafranine	-293 mV	0	--	--	10(full)	> -263	> -275

[a]Eh = observed potential

[b]Eo = potential of a 50% reduced dye corrected for pH at 30°C. The pH in the anterior midgut averages 7.6

Table 8. Range of Eh[a] values obtained from dyes in the hindgut of fourth instar Megachile rotundata.

Dye	Eo[b]	No. with partial color	Eh range (mV) max	Eh range (mV) min	No. with full or no color	Eh range (mV) max	Eh range (mV) min
Toluylene Blue	+115 mV	6	+123	+108	4(no)	< + 85	< + 78
Thionine	+ 62 mV	9	+ 68	+ 56	1(no)	< + 32	--
Methylene Blue	+ 11 mV	8	+ 17	+ 4	2(no)	< - 13	< - 26
Nile Blue	-142 mV	2	-133	-133	8(full)	> -133	> -159
Phenosafranine	-252 mV	0	--	--	10(full)	> -208	> -250

[a]Eh = observed potential

[b]Eo = potential of a 50% reduced dye corrected for pH at 30°C. The pH in the anterior midgut averages 7.0.

Table 9. Colors obtained following addition of redox dyes to bee diet and fungus spore germination medium in high CO_2 concentration (see text).

Dye	Eo[a] (mV)	Color on bee diet[a]	Eo[b] (mV)	Color on agar medium[b]
Toluylene Blue	+173	no color	+115	partial color
Thionine	+102	partial color	+ 62	partial color
Methylene Blue	+ 55	partial color	+ 11	full color
Nile Blue	- 79	full color	-142	full color
Phenosafranine	-174	full color	-252	full color

[a] pH 5.8

[b] pH 7.0

IV. PATHOGENICITY OF ASCOSPHAERA SPECIES

Introduction

In recent taxonomic studies of the Ascosphaerales (Skou 1972, 1975; Skou and Hackett 1979), several species of megachilids have been cited as possible hosts for the six species of Ascosphaera. In an earlier experiment (Stephen et al. 1981) preliminary evidence indicated that M. rotundata was susceptible to infection by other Ascosphaera species. The pathogenicity of five Ascosphaera species for larvae of M. rotundata is reported here. The symptoms and signs induced by those pathogenic fungi are described.

Materials and Methods

Spores of A. aggregata were obtained from aseptically-reared larvae (Fichter et al. 1981) infected in the laboratory (Vandenberg and Stephen 1982). The cadavers were surface-disinfected in 0.4% Hyamine 10-X (Rohm and Haas, methyl benzethonium chloride; Martignoni and Milstead 1960) and rinsed twice in sterile distilled water (SDW). The cuticle was removed, the spores carefully scraped into a sterile glass vial and stored at 2°C until used.

Sporulating cultures of Ascosphaera apis, A. atra, A. major, and A. proliperda were produced and maintained on Sabouraud dextrose agar (SDA, Difco) at 24°C. A. apis was isolated from Apis mellifera cadavers collected in August 1978 near Corvallis, Oregon, USA. A. atra was isolated from pollen in M. rotundata cells collected in August 1979 near Ontario, Oregon. Isolates of A. major and A. proliperda were obtained from J. Rose and M. Christensen (University of Wyoming, Laramie). These

fungi were subcultured monthly on SDA. Following initial experiments (Tables 10), spores of all five species produced on cadavers or on the pollen diet were used as inocula for later experiments without subculturing (Tables 11 and 12). For the first experiment (Table 10), fresh spores from subcultures were placed directly on the diet. In subsequent experiments, spore cysts were crushed gently between two sterile glass slides and the spores were suspended in sterile buffered saline (pH 7.1) composed of 0.02 M BES (N, N-bis (2-hydroxyethyl)-2-aminoethane sulfonic acid), 0.154 M NaCl, and 0.0075 g/l DOSS (dioctyl sulfosuccinate, sodium) (M. E. Martignoni, personal communication).

M. rotundata eggs were collected from field populations near Corning, California, and Ontario, Oregon, in 1980 and 1981. The eggs were rinsed in SDW to remove pollen, disinfected in 0.5% Dry Chlorine (Georgia-Pacific, sodium dichloro-s-triazinetrione dihydrate) plus 0.05% Tween 20, and rinsed again in SDW. Eggs were then transferred to an autoclaved pollen-based diet in polystyrene titration plates. The larvae were reared aseptically in individual cells in these plates (Fichter et al. 1981).

Groups of six-day-old defecating larvae were transferred to cells containing fresh sterile diet inoculated with spore cysts of one of the Ascosphaera species listed above. The bees were incubated with the inoculated diet in darkness at 30°C in high (> 80%) relative humidity, and monitored daily for survival and/or sign of disease. Each larva received ca. 10^5 spores. All larvae were observed consuming some inoculum and most of the diet in each cell was consumed. Any

microorganisms evident upon or within cadavers or on the pollen diet were isolated on SDA or nutrient agar (Difco) at 24° or 30°C.

Observations of certain characteristics of the Ascosphaera species used in our studies have been published recently along with comparisons of our measurements with other published descriptions (Stephen et al. 1981). Subcultures of fungi used in these experiments have been deposited in the American Type Culture Collection (Rockville, Maryland, 20852, USA), and at the Insect Pathology Resource Center, Boyce Thompson Institute (Ithaca, New York, 14853, USA).

Results and Discussion

The results of spore feeding are presented in Tables 10-12. No disease occurred among larvae in the uninoculated control group nor among larvae inoculated with A. atra (cf. Skou and Hackett 1979) or A. major (cf. Holm and Skou 1972). Fifty-four of these 62 individuals survived to the prepupa. Twenty-seven of the 33 larvae infected with A. aggregata showed the typical chalkbrood syndrome described earlier (Vandenberg and Stephen 1982; Figure 5 a, c). Nine larvae inoculated with A. apis and 21 inoculated with A. proliperda were infected, but none exhibited those color changes associated with A. aggregata infection. Rather, the larvae retained their cream color. The hemolymph turned cloudy with multiplying mycelia within 12 hours before death. White mycelia erupted first through intersegmental membranes and later through other portions of the cuticle within 48 hours after death (Figure 5 b, c). Sporulation occurred on the surface of the cadaver and on the remaining pollen diet within 1-2 weeks. Mortality or survival

were not independent of the species used as inocula ($p < 0.001$, 3 x 2 independence test, Sokal and Rohlf 1969, p. 598).

From the onset of signs of disease until fungus sporulation, it was not possible to distinguish larvae infected with A. apis from those infected with A. proliperda. However, survival patterns for larvae inoculated with these fungi and with A. aggregata were compared using the Mantel-Haenszel equation, an improved rank-order procedure (M. E. Martignoni, personal communication). Significant differences in sample survival patterns were found between larvae infected by A. aggregata versus A. apis ($p < 0.001$) and A. apis versus A. proliperda ($p < 0.001$), but not A. aggregata versus A. proliperda ($p > 0.10$). The average time to death for larvae infected with A. aggregata was 7.1 days ($n = 27$, standard deviation, s.d. = 3.1 days). For larvae infected with A. apis it was 9.2 days ($n = 9$, s.d. = 1.9 days), and for those infected with A. proliperda it was only 4.5 days ($n = 21$, s.d. = 1.3 days). In an earlier study, an average time to death of 6.4 days for 6-day-old M. rotundata larvae inoculated with A. aggregata spores was obtained (Vandenberg and Stephen 1982). One- and four-day-old larvae survived significantly longer (8-10 days) while 5- to 9-day-old larvae died in 4 to 6 days. Gilliam et al. (1978b) observed A. mellifera mummy formation within 5-7 days after inoculation with A. apis.

A. apis, major, and proliperda proliferated on the pollen diet of 20 of the 90 specimens and overgrew the still-living larvae within their cells (No. Overgrown, Tables 10-12). It was not determined whether larval death in these specimens resulted from fungal growth in the host or from nutritional deprivation or other stresses as a consequence of

mycelial growth. A. atra grew and sporulated on the pollen diet of all cells inoculated with it. It did not appear to affect larval development, but further studies are necessary to determine its prevalence in M. rotundata populations and its possible effects on this host.

The undiagnosed mortality of 12 of the 157 specimens (No. Other, Tables 10-12) may be due to injuries resulting from handling, nutritional deficiencies in the diet, or other unknown factors. In no cases were these deaths associated with microbial contamination, nor did we isolate any bacteria on nutrient agar. The reason for a relatively high proportion of undiagnosed mortality (5 of 30) among larvae inoculated with A. apis cannot be explained.

With the exception of A. aggregata spores, spore inocula for initial experiments were taken from fungi that had been subcultured monthly at least 6 months (Table 10). Three of 16 (19%; Table 10) larvae inoculated from a subculture of A. apis became infected, but 6 of 14 (43%; Tables 11 and 12) became infected when inoculated with spores produced on cadavers. The proportion of infected larvae also increased when A. proliperda spores from cadavers were used as the inoculum, although the increase may not be significant: 61% (11 of 18; Table 10) with spores from a subculture, versus 71% (10 of 14; Tables 11 and 12) with spores from cadavers.

Nearly half of the larvae inoculated with subcultured A. apis (7 of 16, 44%; Table 10) were overgrown by the fungus proliferating on the pollen diet, while none of those (0 of 14; Tables 11 and 12) inoculated with A. apis spores from cadavers were overgrown. Similarly for A. proliperda, the proportion of overgrown cells decreased from 33% with

subcultured inoculum (6 of 18; Table 10) to 14% when cadaver inoculum was used (2 of 14; Tables 11 and 12). A 2 X 2 X 3 test for independence (Sokal and Rohlf 1969, p. 602) was employed to compare inoculum source (cadaver or subculture), species (A. apis and A. proliperda), and outcome (healthy, diseased, overgrown). Neither the 3 parameters together nor any pair of them were independent ($p < 0.001$). These data suggest that pathogenicity or virulence was altered with subculturing. Continued passage through a host might further increase the pathogenicity of these fungi. These tests included only a single dose level of all species. Higher doses may increase the proportion that become infected.

Four of the five Ascosphaera species tested (A. apis, atra, major, and proliperda) grew and sporulated on the diet provided M. rotundata larvae. Of these A. atra and A. major appear to be strict saprophytes whereas A. apis and A. proliperda act as facultative parasites. Neither A. major nor A. proliperda have been reported from North America (Gochnauer and Hughes 1976; Stephen et al. 1981). A. major is common in Europe and has been implicated as a causative agent of chalkbrood in honey bees. Holm and Skou (1972) found it to be a facultative parasite, commonly growing saprophytically in cells of healthy M. centuncularis larvae. A. proliperda, on the other hand, is only known from chalkbrood cadavers of greenhouse-reared M. centuncularis in Denmark.

Both A. apis and A. proliperda are capable of infecting M. rotundata larvae and both cause a similar syndrome, distinct from that caused by A. aggregata. A. aggregata and A. apis have been reported for the first time in North America within the last 10 years. The cross-

infectivity of A. apis, especially as it might relate to sudden epizootic of chalkbrood in M. rotundata in the western United States in the mid 1970's, is not understood. It is evident that the determination of the degree of host overlap among Ascosphaera species requires more extensive investigation. Such studies will help clarify the relationships among species of Ascosphaera.

Figure Caption

Figure 5. *Megachile rotundata* larvae infected with *Ascosphaera* species. a) *A. aggregata* infection showing chalkbrood symptoms including dark spore cysts forming beneath intact cuticle. Bar = 3 mm. b) *A. proliperda* infection showing white mycelium erupting through intersegmental membranes. Bar = 3 mm. c) Chalkbrood cadavers (top), *A. proliperda* cadavers (bottom) and healthy prepupa (center). Bar = 2 mm.

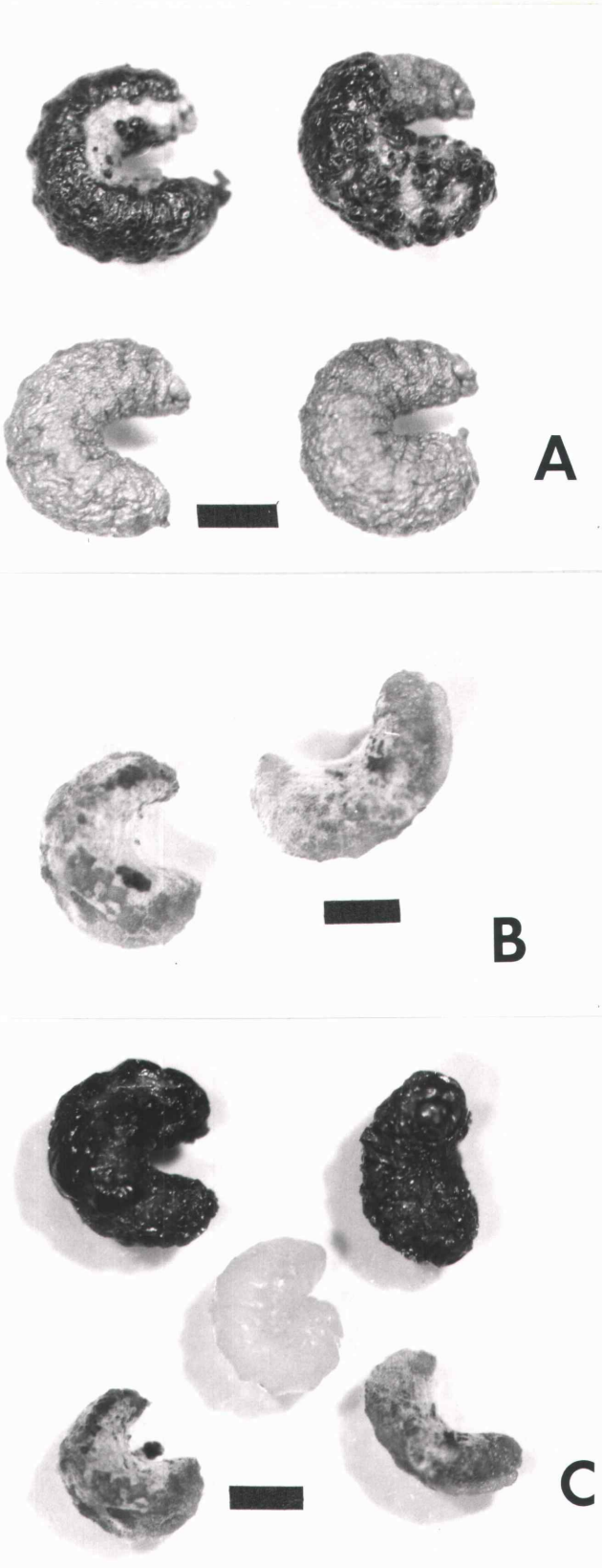

Figure 5

Table 10. Response of *Megachile rotundata* larvae to inoculation with *Ascosphaera* species.

Treatment[a]	Number Treated	Number Prepupae	Number Diseased	Number Overgrown[b]	Number Other[c]
Uninoculated control	8	7	0	0	1
Buffer control	7	7	0	0	0
A. aggregata test 1	9	0	8	0	1
test 2	9	3	6	0	0
A. apis test 1	9	1	1	4	3
test 2	7	1	2	3	1
A. atra	9	9	0	0	0
A. major	9	6	0	2	1
A. proliperda test 1	9	0	2	6	1
test 2	9	0	9	0	0

[a]Each larval cell received 10^5 spores. All inocula from subcultures except *A. aggregata* from cadavers.

[b]The fungus proliferated on the pollen diet.

[c]Undiagnosed mortality.

Table 11. Response of *Megachile rotundata* to inoculation with *Ascosphaera* species taken from cadavers and cells.

Treatment[a]	Number Treated	Number Prepupae	Number Diseased	Number Overgrown[b]	Number Other[c]
Control	8	8	0	0	0
A. aggregata	8	0	7	0	1
A. apis	8	4	3	0	1
A. atra	8	8	0	0	0
A. major	6	2	0	3	1
A. proliperda	7	1	5	0	1

[a]Each larval cell received 10^5 spores from cadavers or cells represented in Table 10. Controls received sterile buffered saline only.

[b]The fungus proliferated on the pollen diet.

[c]Undiagnosed mortality.

Table 12. Response of Megachile rotundata to inoculation with Ascosphaera species taken from cadavers and bee cells.

Treatment	Number Treated	Number Prepupae	Number Diseased	Number Overgrown[b]	Number Other[c]
Control	7	7	0	0	0
A. aggregata	7	1	6	0	0
A. apis	6	3	3	0	0
A. proliperda	7	0	5	2	0

[a]Each larval cell received 5 x 10^4 spores from cadavers or cells represented in Table 10. Controls untreated.

[b]The fungus proliferated on the pollen diet.

[c]Undiagnosed mortality.

V. SUMMARY AND CONCLUSIONS

Ascosphaera aggregata is the primary pathogen of chalkbrood in the alfalfa leafcutting bee, Megachile rotundata. One- to nine-day old larvae are susceptible to infection per os by spores obtained from laboratory-infected cadavers. The fungus does not sporulate in vitro. Spores germinate in the host midgut, penetrate the peritrophic lining and invade tissues within the hemocoel. Larvae die within 3 to 10 days after inoculation. Those inoculated at an earlier age have a significantly longer average time to death.

The first signs of disease appear at the time of death. Larval hemolymph becomes cloudy as the cadaver is filled with white mycelium. Larvae inoculated when young do not support fungus sporulation and remain white or cream colored following death. Older larvae undergo characteristic color changes after death. A pink or tan cast develops in one body area and spreads throughout the entire cadaver within 1 day. The color observed in older infected larvae may be due to pigment produced by the fungus at a focus of development. The reason younger infected larvae do not exhibit a color change is unknown.

Sporulation typically takes place beneath the host cuticle within 2 weeks of death, but the fungus does not sporulate in all infected larvae. The conditions required for sporulation may depend upon the host age at inoculation, its nutritional or developmental state, and abiotic factors such as temperature and relatively humidity.

Conditions of highly reduced oxidation-reduction potential exist in the midguts of M. rotundata larvae. Ascosphaera aggregata spores may germinate in response to this reduced potential and later penetrate the

hemocoel of their host. Early instar larvae have a blind gut with no opening to the anus. Conditions of reduced redox potential might be expected in these larvae. The studies reported here indicate that portions of the midgut of fourth instar larvae also have very low redox potential. The potential in the guts of younger larvae may not be low enough (or may be too low) to allow germination. This may explain why younger larvae have a longer average time to death.

At least three Ascosphaera species are able to infect larvae of M. rotundata. Ascosphaera aggregata causes the chalkbrood syndrome described above and has been reported from populations of this bee worldwide (Skou 1975, Stephen and Undurraga 1978). Ascosphaera apis is the etiologic agent of chalkbrood in honey bees and causes a similar syndrome in larvae of M. rotundata. White mycelium erupts through the cuticle and sporulates outside the host. The signs of M. rotundata infection by A. proliperda are identical to those produced by A. apis except that the time to death for the latter is significantly longer. Spores of these two species taken from cadavers grow less readily on the pollen diet and appear to be more virulent than spores taken from sub-cultures. Ascosphaera proliperda has only been reported from a greenhouse population of M. centuncularis in which it caused a chalk-brood-like disease in larvae (Holm and Skou 1972). Ascosphaera atra and A. major have both been reported as saprobes in leafcutting bee cells (Holm and Skou 1972, Skou and Hackett 1979). These two species are unable to cause infection of M. rotundata larvae but they do proliferate on the pollen-based bee diet.

Both A. apis and A. proliperda are able to infect leafcutting bee larvae although spores of the latter do not require CO_2 in order to germinate (Appendix V). Perhaps spores of A. proliperda germinate on the surface of their hosts and penetrate the outer cuticle. By following the course of infection of these fungi it may be possible to elucidate interspecific variability in the modes of pathogenicity exhibited.

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VII. APPENDICES

Appendix I

Since Ascosphaera aggregata spores cannot yet be produced in vitro, they must be obtained from cadavers. These cadavers are often contaminated with other microorganisms including A. atra, a saprophyte in leafcutting bee cells. Therefore a method has been undertaken by which A. aggregata spores may be separated from A. atra spores and washed in bactericidal and virucidal solutions. This method is outlined below and a tabular accounting of results follows (Tables 13-16). Good separation of these two spores types is possible on sucrose gradients following brief centrifugation. Merthiolate and formalin solutions are innocuous to A. aggregata spores (Stephen et al. 1982) and were employed to limit bacterial and viral contamination. Comprehensive tests for possible contaminants have not been conducted but are required for further studies. In Appendix II are displayed some infectivity data obtained using centrifuged spore inocula (Table 17).

Protocol for Spore Centrifugation:

1. Prepare spore suspensions of A. aggregata and A. atra in 30% (w/w) sucrose plus merthiolate tincture (0.01%, v/v).
2. Estimate spore concentration by duplicate hemacytometer counts.
3. Make 10 ml continuous centrifuge gradients with 35 to 40% sucrose in 15 ml cellulose nitrate tubes.
4. Carefully load 0.5 ml spore suspension sample in each tube and centrifuge 2 minutes at 1500 x g on a clinical bench top centrifuge with a swinging bucket rotor.
5. Collect three 3 ml fractions and one 1 ml fraction in sterile tubes from the punctured tube bottom by dropping paraffin oil on top.

6. Estimate spore concentrations in each fraction by hemacytometer counts.

Data obtained by this method are shown in Tables 13-16. The rest of the procedure was used to obtain inocula for the experiments reported in Table 17.

7. Collect fraction 3 and add it to 10 ml 1% buffered formalin in 15 ml centrifuge tubes.

8. Centrifuge 20 minutes at 1500 x g.

9. Pour off supernatant, resuspend spore pellet in bufferd saline (0.15 M, pH 7.0, see section IV for recipe).

10. Centrifuge 20 mintues at 1500 x g.

11. Repeat 9 and 10.

12. Pour off supernatant, resuspend in 1 ml buffer.

13. Estimate concentration by hemacytometer counts.

14. Dilute to desired concentration.

Table 13. Number of Ascosphaera aggregata spores in sucrose gradients following 60 second centrifugation at 1500 x g.

Fraction[a]	Tube Number 1	2	3
1	0[b]	0	7
2	15	11	31
3	380	336	312
4	79	18	64
Total	474	365	414
Hemacytometer Est.	475	475	475

[a]Fraction 1 at tube bottom. Fractions 1 to 3 are 3 ml each, fraction 4 is 1 ml.

[b]Number of spores per fraction x 10^4.

Table 14. Number of Ascosphaera aggregata spores in sucrose gradients following 120 second centrifugation at 1500 x g.

Fraction[a]	Tube Number 1	2	3	4	5	6	7	8
1	16[b]	25	18	8	14	0	1	1
2	88	139	159	33	29	29	24	34
3	263	273	255	186	213	234	166	162
4	84	59	62	64	75	28	37	30
Total	451	496	494	291	331	291	228	227
Hemacytometer Est.	475	475	475	352	352	269	269	269

[a]Fraction 1 at tube bottom. Fractions 1 to 3 are 3 ml each, fraction 4 is 1 ml.

[b]Number of spores per fraction x 10^4.

Table 15. Number of Ascosphaera atra spores in sucrose gradients following 60 second centrifugation at 1500 x g.

Fraction[a]	Tube Number 1	2	3
1	1[b]	3	11
2	28	43	41
3	35	26	15
4	12	17	12
Total	76	89	79
Hemacytometer Est.	80	80	80

[a]Fraction 1 at tube bottom. Fractions 1 to 3 are 3 ml each, fraction 4 is 1 ml.

[b]Number of spores per fraction x 10^4.

Table 16. Number of Ascosphaera atra spores in sucrose gradients following 120 second centrifugation at 1500 x g.

Fraction[a]	Tube Number 1	2	3	4	5	6
1	40[b]	31	26	33	18	17
2	17	24	10	18	19	20
3	7	7	7	3	2	14
4	8	3	5	6	5	3
Total	72	65	48	60	44	54
Hemacytometer Est.	80	80	80	75	75	59

[a]Fraction 1 at tube bottom. Fractions 1 to 3 are 3 ml each, fraction 4 is 1 ml.

[b]Number of spores per fraction x 10^4.

Appendix II

The following data supplement those in the etiology section. Table 17 presents the results of infectivity experiments conducted using centrifuged spore inocula. Table 18 shows results of a preliminary bioassay using inoculum prepared from laboratory-infected cadavers (methods described in section II). In both sets of experiments, some larvae exposed to a dose of less than 10^4 spores survived. Lower doses will also be employed when bioassay experiments planned for 1982 are conducted.

Table 17. Response of Megachile rotundata larvae to inoculation with Ascosphaera aggregata spores separated from contaminants by centrifugation.

Age[a]	Treatment[b]	Treated	Number Surviving[c]	Chalkbrood	Other[d]
4	Control	16	13	0	3
	Suspension[e]	18	1	17	0
4	Control	7	5	0	2
	Suspension[f]	6	4	2	0
5	Control	9	9	0	0
	Suspension[g]	9	0	9[h]	0
7	Control	5	5	0	0
	Suspension[f]	8	2	6	0

[a]Age of host in days at time of treatment. Fourth instar reached at 5-6 days.

[b]Spores suspended in saline buffer (0.15 M, pH 7.0). Controls treated with buffer only. Inoculum volume 5-10 µl.

[c]Survival past the fourth instar, or the stage at which chalkbrood may be diagnosed.

[d]Undiagnosed mortality.

[e]3×10^4 spores per larva.

[f]7×10^3 spores per larva.

[g]5×10^4 spores per larva.

[h]Bacterial contamination of all 9 cadavers.

Table 18. Response of Megachile rotundata to inoculation with different doses of Ascosphaera aggregata spores.

Age[a]	Treatment[b]	Treated	Number Surviving[c]	Chalkbrood	Other[d]
4	Control	5	4	0	1
	10^3 [e]	4	2	2	0
	10^4	5	0	5	0
	10^5	4	0	3	1
7	Control	6	5	0	1
	10^3	5	2	3	0
	10^4	6	0	4	2
	10^5	6	0	6	0

[a]Age of host in days at time of treatment. Fourth instar reached at 5-6 days.

[b]Spores suspended in saline buffer (0.15 M, pH 7.0). Controls treated with buffer only. Inoculum volume 5-10 µl.

[c]Survival past the fourth instar, or the stage at which chalkbrood may be diagnosed.

[d]Undiagnosed mortality.

[e]Number of spores per larva.

Appendix III

In order to determine the pH and redox conditions in guts of normal M. rotundata larvae, studies preliminary to those reported in section III were conducted. The results of these experiments are shown in Tables 19-21. They are consistent with those obtained in section III. Midgut pH is near neutral and the redox potential is quite reduced in the central midgut.

Table 19. Midgut pH of fourth instar Megachile rotundata larvae as shown by pH indicators.

Indicator[a]	Range	Colors[b]	Midgut Observations[c]
Bromocresol green	3.8 - 5.4	Y - BG	7/7 BG
Bromocresol purple	5.2 - 6.8	Y - P	8/8 P
Bromothymol blue	6.0 - 7.6	Y - B	9/9 Y[d]
Brilliant yellow	6.6 - 7.9	Y - RO	7/7 O
Cresol red	7.2 - 8.8	Y - R	5/8 R, 3/8Y
m-Cresol purple	7.4 - 9.0	Y - P	8/8 Y
Thymol blue	8.0 - 9.6	Y - B	8/8 Y
o-Cresolphthalein	8.2 - 9.8	C - R	8/8 C
Thymolphthalein	9.3 -10.5	C - B	9/9 C

[a]All dyes prepared according to Windholz et al. (1976) at 0.04% except brilliant yellow, 0.1% aq.

[b]B = blue, BG = blue-green, C = colorless, R = red, RO = red-orange, P = purple, Y = yellow.

[c]Third and fourth instar larvae were allowed to feed for 2 days on 0.1 ml bee diet treated with 10 µl dye solution. Number of observations and colors are given.

[d]A color change should have been detected in these larvae, perhaps the dye was too weak and masked by the diet color.

Table 20. Response of third instar Megachile rotundata larvae to redox indicator dyes in the midgut.

Indicator[a]	E'o[b]	Midgut Region Anterior	Central	Posterior
TB	+115	5/5 +[c]	5/5 -	5/5 -
Th	+ 62	3/3 +	3/3 -	1/3 +, 2/3 -
MB	+ 11	4/4 +	4/4 -	3/4 +, 1/4 -
NB	-142	2/4 ++, 2/4 +	4/4 -	4/4 +
Ph	-252	5/5 ++	1/5 ++, 4/5 +	5/5 ++

[a]10 µl of dye solution (0.1%) was added to 0.1 ml bee diet. Larvae fed for 2 days before observation. TB = toluylene blue, Th = thionine, MB = methylene blue, NB = nile blue, Ph = phenosafranine.

[b]Potential of a 50% reduced substance at pH 7.0, in mV.

[c]Proportion of larvae showing response. ++ = full color, + = partial color, - = no color.

Table 21. Response of fourth instar Megachile rotundata to redox indicators in the midgut.

Indicator[a]	E'o[b]	Midgut Region Anterior	Central	Posterior
TB	+115	2/2 +[c]	2/2 -	1/2 +, 1/2 -
Th	+ 62	2/4 +, 2/4 -	4/4 -	4/4 +
MB	+ 11	5/5 +	5/5 -	3/5 +, 2/5 -
NB	-142	3/5 ++, 2/5 +	2/5 +, 3/5 -	5/5 ++
Ph	-252	2/3 ++, 1/3 +	3/3 +	3/3 ++

[a]10 µl of dye solution (0.1%) was added to 0.1 ml bee diet. Larvae fed for 2 days before observation. TB = toluylene blue, Th = thionine, MB = methylene blue, NB = nile blue, Ph = phenosafranine.

[b]Potential of 50% reduced substance at pH 7.0 in mV.

[c]Proportion of larvae showing response. ++ = full color, + = partial color, - = no color.

Appendix IV

Mycelial growth experiments were conducted to determine media suitable for the growth and maintenance of Ascosphaera species in culture. Tables 22 and 23 show results of pH and temperature preference studies with vegetative mycelia of A. aggregata. Tables 24-27 display the growth of four Ascosphaera species on each of four media.

High pH inhibited mycelial growth of A. aggregata. The growth rate was higher at higher temperatures. A. aggregata grew most luxuriantly on vegetable juice agar but also grew well on SDA. Both PDA and MA supported growth but at a lower rate. The other three Ascosphaera species grew at similar rates on all four media.

Table 22. Colony diameters of Ascosphaera aggregata mycelia on vegetable juice agar[a] of different pH.

pH[b]	Days Post-Inoculation 0	6
6.4	10 ± 0[c]	48 ± 1
6.8	10 ± 0	46 ± 3
7.2	10 ± 0	49 ± 0
7.6	10 ± 0	34 ± 4
8.0	10 ± 0	28 ± 8

[a]For formula see Kish (1980).

[b]After autoclaving.

[c]Colony diameter in mm. Average of 2 replicates ± standard deviation. Petri dish diameter 50 mm. Incubation temperature 30°C, in darkness.

Table 23. Colony diameters of Ascosphaera aggregata mycelia on vegetable juice agar[a] at different incubation temperatures.

Temperature	Days Post-Inoculation 0	3	6	8	10	12	14
15°C	7 ± 0[b]	8 ± 0	8 ± 1	9 ± 0	10 ± 0	10 ± 0	11 ± 0
20	7 ± 0	10 ± 0	17 ± 1	22 ± 1	32 ± 1	41 ± 1	47 ± 1
25	7 ± 0	10 ± 1	21 ± 5	29 ± 6	44 ± 5	49 ± 1	50 ± 0
30	7 ± 0	14 ± 2	37 ± 9	49 ± 2	50 ± 0	--	--

[a]For formula see Kish (1980).

[b]Colony diameter in mm. Average of 3 replicates ± standard deviation. Petri dish diameter 50 mm. Incubated in darkness.

Table 24. Colony diameters of Ascosphaera aggregata mycelia on different media.

Medium[a]	Days Post-Inoculation 0	2	4	6	8	10	14
VJA	7 ± 0[b]	9 ± 0	16 ± 1	28 ± 1	46 ± 3	50 ± 0	--
SDA	7 ± 0	8 ± 0	18 ± 2	28 ± 0	43 ± 3	50 ± 0	--
PDA	7 ± 0	8 ± 0	15 ± 4	26 ± 4	36 ± 8	44 ± 8	46 ± 6
MA	7 ± 0	8 ± 0	12 ± 4	12 ± 4	18 ± 6	27 ± 6	33 ± 3

[a]VJA, vegetable juice agar, pH 7.0. SDA, Sabauroud dextrose agar, pH 5.6 PDA, potato dextrose agar, pH 5.6. MA, malt agar, pH 5.6.

[b]Colony diameter in mm, average of 2 replicates ± standard deviation. Petri dish diameter 50 mm. Incubation temperature 30°C, in darkness.

Table 25. Colony diameters of Ascosphaera atra mycelia on different media.

Medium[a]	Days Post-Inoculation 0	2	4	6	8	10
VJA	7 ± 0[b]	10 ± 4	42 ± 6	50 ± 0	--	--
SDA	7 ± 0	10 ± 4	32 ± 3	48 ± 0	50 ± 0	--
PDA	7 ± 0	8 ± 2	21 ± 4	32 ± 4	41 ± 1	42 ± 0
MA	7 ± 0	8 ± 1	18 ± 2	30 ± 4	41 ± 4	50 ± 0

[a]VJA, vegetable juice agar, pH 7.0. SDA, Sabauroud dextrose agar, pH 5.6. PDA, potato dextrose agar, pH 5.6. MA, malt agar, pH 5.6.

[b]Colony diameter in mm, average of 2 replicates ± standard deviation. Petri dish diameter 50 mm. Incubation temperature 30°C in darkness.

Table 26. Colony diameters of Ascosphaera major mycelia on different media.

Medium[a]	Days Post-Inoculation 0	2	4	6	8
VJA	7 ± 0[b]	17 ± 8	48 ± 3	50 ± 0	--
SDA	7 ± 0	18 ± 4	50 ± 0	--	--
PDA	7 ± 0	16 ± 5	44 ± 7	49 ± 1	50 ± 0
MA	7 ± 0	12 ± 5	34 ± 2	50 ± 0	--

[a]VJA, vegetable juice agar, pH 7.0. SDA, Sabauroud dextrose agar, pH 5.6. PDA, potato dextrose agar, pH 5.6. MA, malt agar, pH 5.6.

[b]Colony diameter in mm, average of 2 replicates ± standard deviation. Petri dish diameter 50 mm. Incubation temperature 30°C, in darkness.

Table 27. Colony diameters of Ascosphaera proliperda mycelia on different media.

Medium[a]	Days Post-Inoculation 0	2	4	6
VJA	7 ± 0[b]	21 ± 1	50 ± 0	--
SDA	7 ± 0	20 ± 3	49 ± 1	50 ± 0
PDA	7 ± 0	18 ± 2	46 ± 3	50 ± 0
MA	7 ± 0	15 ± 1	38 ± 0	50 ± 0

[a]VJA, vegetable juice agar, pH 7.0. SDA, Sabauroud dextrose agar, pH 5.6. PDA, potato dextrose agar, pH 5.6. MA, malt agar, pH 5.6.

[b]Colony diameter in mm, average of 2 replicates ± standard deviation. Petri dish diameter 50 mm. Incubation temperature 30°C, in darkness.

Appendix V

In order to assess the potential variability in modes of pathogenesis among Ascosphaera species, spore inocula were prepared and tested for germination using our in vitro system (Stephen et al. 1982). Results are shown in Table 28. They indicate that a small proportion of A. aggregata spores will germinate without CO_2 incubation. The isolate of A. apis appears to require CO_2 (or reduced redox conditions) in order to germinate. (An earlier study showed some A. apis spore germination when incubated in air, but bacterial contamination of the agar medium occurred.) Ascosphaera atra spores germinated best in CO_2 but also in air. The implications of this result compared with the apparent lack of pathogenicity of A. atra toward M. rotundata larvae are unknown. Only a very few A. major spores germinated in either incubation chamber. Ascosphaera proliperda germinated equally well in both.

Larvae inoculated with A. aggregata, A. apis and A. proliperda have been prepared for histologic examination. These samples may provide more information on the route by which these species invade leafcutting bee larvae.

Table 28. Germination of Ascosphaera species spores following incubation in air or CO_2[a].

Inoculum[b]	Germination[c] Air	CO_2
A. aggregata	12.6 ± 1.3[d]	35.6 ± 0.8
A. apis	0.0 ± 0.0	13.1 ± 1.2
A. atra	20.7 ± 3.3	47.5 ± 4.0
A. major	0.4 ± 0.0	2.4 ± 1.9
A. proliperda	74.0 ± 2.3	75.8 ± 3.2

[a]For details of inoculum preparation and incubation see Stephen et al. (1982).

[b]For inoculum sources see section IV.

[c]Incubation took place at 30°C, high relative humidity (> 95%), on Sabouraud dextrose agar for 2 days (A. aggregata, atra, proliperda) or 5 days (A. apis, major).

[d]Mean ± standard deviation, 2 replicates. Four microscope fields of 25-250 spores per field were counted for each replicate.