Journal of Invertebrate Pathology 103 (2010) S20–S29

Contents lists available at ScienceDirect

Journal of Invertebrate Pathology

journal homepage: www.elsevier.com/locate/jip

Chalkbrood disease in honey bees

K.A. Aronstein *, K.D. Murray

Honey Bee Research Unit, USDA-ARS, Weslaco, TX 78596, USA

ARTICLE INFO

Article history:
Received 28 May 2009
Accepted 30 June 2009
Available online 11 November 2009

Keywords:
Ascosphaera apis
Honey bee
Chalkbrood disease

ABSTRACT

Chalkbrood is a fungal disease of honey bee brood caused by *Ascosphaera apis*. This disease is now found throughout the world, and there are indications that chalkbrood incidence may be on the rise. In this review we consolidate both historic knowledge and recent scientific findings. We document the worldwide spread of the fungus, which is aided by increased global travel and the migratory nature of many beekeeping operations. We discuss the current taxonomic classification in light of the recent complete reworking of fungal systematics brought on by application of molecular methods. In addition, we discuss epidemiology and pathogenesis of the disease, as well as pathogen biology, morphology and reproduction. New attempts at disease control methods and management tactics are reviewed. We report on research tools developed for identification and monitoring, and also include recent findings on genomic and molecular studies not covered by previous reviews, including sequencing of the *A. apis* genome and identification of the mating type locus.

Published by Elsevier Inc.

1. Introduction

Chalkbrood is an invasive mycosis in honey bees (*Apis mellifera* L.) produced by *Ascosphaera apis* (Maassen ex Claussen) Olive and Spiltoir (Spiltoir, 1955; Spiltoir and Olive, 1955) that exclusively affects bee brood. Although fatal to individual larvae, the disease does not usually destroy an entire bee colony. However, it can cause significant losses in terms of both bee numbers and colony productivity (Bailey, 1963; Wood, 1998), with reductions in honey production of 5–37% reported (Heath, 1982a; Yacobson et al., 1991; Zaghloul et al., 2005). Chalkbrood is now found in honey bee colonies around the world, and there are some indications that the incidence of chalkbrood has increased in recent years (Heath, 1985; Kluser and Peduzzi, 2007). Aizen et al. (2009) have shown that human activities related to increased food demand have direct and indirect effects that could be at least in part responsible for this trend.

In this review we consolidate historic knowledge and recent scientific findings focusing on pathogen biology, disease pathogenesis, and control methods. Significantly, since previous reviews were published, the entire genome of *A. apis* has been sequenced—the first fungal entomopathogen genome to be sequenced (Qin et al., 2006). Therefore, recent findings will include genomic and molecular studies not covered by previous reviews, including identification of the mating type locus (Aronstein et al., 2007). In the future, genomic research is expected to yield a better understanding of mechanisms controlling reproduction and pathogenesis in *A. apis*, and these discoveries will hopefully translate into increasingly effective disease control strategies.

2. Historical incidence and geographical distribution

Chalkbrood disease in honey bees was recognized in the early 1900s (Maassen, 1913). Until the latter half of the 20th century, chalkbrood was not widely seen outside of Europe. It has long been known in Germany (Dreher, 1938), Scandinavia, Russia (Betts, 1932), and Great Britain (Heath, 1982a, 1985). By 1977, chalkbrood was recognized as the most serious infectious bee disease in Norway (Heath, 1985). One of the earliest reports of the fungus outside of Europe was in New Zealand in 1957 (Seal, 1957; Palmer-Jones, 1964). By the mid-1980s, the disease was reported in commercial hives (Reid, 1988), and by 1987 it had spread throughout most of the country. The disease was observed in several different areas in Argentina by 1978, but was still considered only a minor problem up until 1988. By that time, high apiary infection levels were occurring throughout a large number of provinces in the country (Reid, 1993). Chalkbrood was found to be widespread in Mexico (Wilson et al., 1984), and has now been detected in most beekeeping areas of Chile, Central America, Japan, and the Philippines, (Heath, 1985; Reynaldi et al., 2003).

By 1987, several Mediterranean countries had no reports of chalkbrood (Bradbear, 1988), although it is likely to be present. *A. apis* was first reported in Israel in 1984, and was present at a very low level until 1990 when there was a substantial increase in the rate of infection (Yacobson et al., 1991). Surveys in the spring

* Corresponding author. Address: USDA-ARS 2413 E. Hwy. 83, Weslaco, TX 78596, USA.
E-mail address: Kate.Aronstein@ars.usda.gov (K.A. Aronstein).

0022-2011/$ - see front matter Published by Elsevier Inc.
doi:10.1016/j.jip.2009.06.018

of 1988 showed that chalkbrood was widespread in many honey producing regions of Turkey. The primary source of this infestation was contaminated bee wax imported from several different countries between 1986 and 1988 (Tutkun et al., 1993).

In North America, chalkbrood was found in the mid-1960s, and by 1971 had begun to have an economic impact (Hitchcock and Christensen, 1972). The earliest reports of chalkbrood in the United States were from Utah in 1965 (Baker and Torchio, 1968) and then California in 1968 (Hitchcock and Christensen, 1972), although it might have been in the US much earlier (Gilliam et al., 1997). From 1969 to 1975, it spread to Arizona, Nebraska, Wyoming Montana, Minnesota, North Dakota, Wisconsin, Illinois, Texas, and Florida (Hitchcock and Christensen, 1972; Gilliam et al., 1978). About the same time, it was found to be prevalent in the mid-western and western regions of Canada (Hitchcock and Christensen, 1972). Since then, chalkbrood has spread throughout the United States, including to Alaska and Hawaii.

More recently, in Australia, chalkbrood was first identified in Queensland in 1993, but in just a few years it had spread to all Australian states (Hornitzky, 2001). The migratory nature of commercial beekeeping in North America and Australia is probably the most important factor contributing to the rapid spread of chalkbrood disease within these two continents.

3. Taxonomic classification

Most members of the genus *Ascosphaera* are associated with social and solitary bees. Some of them are saprophytes (Skou, 1972, 1988; Bissett, 1988; Anderson and Gibson, 1998), but several species are pathogens. *A. apis* was originally known as *Pericystis apis* (Maassen, 1913), but was reclassified as *Ascosphaera apis* by Spiltoir (1955).

The taxonomic classification of the genus *Ascosphaera* has been the focus of much debate. For a long time the Ascomycota were grouped into six classes: Hemiascomycetes, Plectomycetes, Pyrenomycetes, Discomycetes, Laboulbeniomycetes, and Loculoascomycetes. The *Ascosphaera* species were placed with class Plectomycetes, based on the cleistothecial type of the fruiting bodies (ascomata) (Skou, 1972). This traditional system of classification has been criticized due to its artificial nature because some phenotypic characteristics are unstable and dependent on environmental conditions (Cain, 1972; Malloch, 1981). Considering that in many cases some characters of ascoma can converge, Hawksworth et al. (1983) proposed to classify according to order in phylum Ascomycota without a class-level organization. However, Skou (1988) favored giving Ascosphaerales the rank of class. It became obvious that fungal taxonomy required changes in nomenclature.

Indeed, the application of new DNA-based methods in fungal systematics has recently resulted in a complete reworking of mycological systematics (James et al., 2006; Hibbett et al., 2007). However, in some cases the significance of DNA-based data is still unclear and conclusions drawn from such studies may not clarify classifications. For example, using PCR amplification and sequencing of rDNA, Berbee and Taylor (1992) and Anderson et al. (1998) concluded that genus *Ascosphaera* should be left in class Plectomycetes, where it was in the traditional classification. This conclusion, however, has not been widely accepted and the term Plectomycetes has been abandoned in recent years. The current taxonomic lineage of the chalkbrood fungus is: Ascomycota; Pezizomycotina; Eurotiomycetes; Eurotiomycetidae; Onygenales; Ascosphaeraceae; *Ascosphaera apis* (Lumbsch and Huhndorf, 2007).

4. Disease epidemiology

Chalkbrood disease is typically most prevalent during the spring, given that fungal growth is enhanced in cool and humid (poorly ventilated) beehives (Mehr et al., 1976; Gilliam et al., 1978; Puerta et al., 1994; Flores et al., 1996; Borum and Ulgen, 2008). In addition to environmental conditions, interactions between biotic factors such as differences in fungal strains and the genetic background of the bees may affect the incidence and severity of the disease. Concerning fungal strains, Glinski (1982) reported that various strains of *A. apis* showed up to a 20-fold difference in the level of virulence. A high concentration of fungal spores in the colony substantially increases chances of infection (Koenig et al., 1987; Gilliam et al., 1988b; Flores et al., 2005a,b), so the rate of disease incidence is likely dependent on a particular fungal strain's level of ascospore production, the rate of spore germination, and the efficiency of spore dispersal.

The genetic background of bees, general health status, and stress could also be important factors affecting incidence and severity of the disease. Over the past several decades, much research has focused on improving honey bee lines by selecting for resistance to infectious diseases (Gilliam et al., 1983; Gilliam et al., 1988b; Spivak and Reuter, 1998a, 1998b, 2001; Spivak and Downey, 1998). The most significant successes are described in Chapter 10 of this issue (Evans and Spivak, 2010). Furthermore, pre-existing stressors, both biotic and abiotic, could weaken the bee's innate immune responses, making them more vulnerable to diseases (Mehr et al., 1976; Hale and Menapace, 1980; Bailey, 1981; Heath, 1982a; Flores et al., 1996). A better understanding of chalkbrood epidemiology will lead to improved management tactics of this highly prevalent disease.

5. Disease pathogenesis

Sexually produced *A. apis* spores (ascospores) are historically considered the primary source of brood infection (Spiltoir, 1955; Hitchcock and Christensen, 1972; Rose et al., 1984). Early studies suggested that two avenues of infection by ascospores are possible: through the cuticle (Roussy, 1962), and by ingestion (Matus and Sarbak, 1974 Gilliam et al., 1978). It is now widely accepted that *A. apis* spores cannot germinate on the larval cuticle, therefore they must be consumed to infect larvae (Maurizio, 1934; De Jong, 1976; Heath, 1982a; Puerta et al., 1994; Flores et al., 1996). *A. apis* can infect brood of any caste (workers, drones, or queens). According to Bailey (1963, 1981), larvae are most susceptible at 3–4 days of age, while others report that 1 and 2 day-old larvae are highly susceptible as well (Maurizio, 1934; De Jong, 1976; Gilliam et al., 1978).

While adult bees are not susceptible to this pathogen, they can transmit the disease within and between beehives. Transmission of infectious materials between adult bees within the colony appears to be via food sharing (Gilliam and Vandenberg, 1997). Fungal spores can be carried by foraging bees and passed onto larvae by nurse bees feeding them with contaminated food. Transmission between managed colonies is mostly beekeeper assisted due to contaminated materials (Gilliam and Vandenberg, 1997). Because spores can accumulate on all parts of the beehive and in all beehive products (e.g., foundation wax, stored pollen and honey) and remain viable for at least 15 years, any hive material contaminated with fungal spores will serve as a long-lasting source of infection (Toumanoff, 1951; Gilliam, 1986; Gilliam and Taber, 1991; Anderson et al., 1997; Flores et al., 2005a, b).

Spores consumed by the honey bee larvae germinate in the lumen of the gut, probably activated by CO_2 (Heath and Gaze, 1987; Bailey and Ball, 1991). Infected larvae rapidly reduce food consumption, and then stop eating altogether. Theantana and Chantawannakul (2008) recently identified several enzymes produced by *A. apis*, some of them implicated in assisting the pathogen in penetration of the peritrophic membrane of the bee larval midgut.

After penetrating the gut wall, the fungal mycelium grows inside of the body cavity, eventually breaking out through the posterior end of the larva (Maurizio, 1934; Nelson and Gochnauer, 1982; Koenig et al., 1987) (Fig. 1A–C). Death occurs as a result of mechanical and enzymatic damage, disruption of haemolymph circulation and general toxicoses (Glinski and Buczek, 2003). *A. apis* vegetative growth extends from the posterior end to the anterior end of the larva, eventually covering the entire larva with a thick layer of white mycelium (Fig. 1D). Later, fungal growth is mottled with brown or black spots, due to production of ascomata that may vary in size and color.

Honey bee cadavers are usually found stretched out in the cells in an upright condition, swollen to the size of the cell (Flores et al., 2004). Eventually cadavers dry and form so-called chalkbrood mummies (Fig. 2) which may be white or black, depending on whether or not ascospores are present. Each black mummy contains about 10^8–10^9 ascospores (Hornitzky, 2001). Microscopic examination of white mummies revealed primarily cellular debris, mycelial fragments, but no detectable ascomata or ascospores (Gochnauer and Hughes, 1976; Aronstein, unpublished).

It has been suggested that white mummies are due to infection with mycelia of a single mating type (Davis and Ward, 2003). This seems questionable given that it has never been shown that mycelia are infective. Nor has it been shown that a single mating type is capable of producing asexual spores. In addition, both mating type idiomorphs were routinely isolated from white mummies (Rose et al., 1984), and fungal cultures originating from single white mummies and grown separately consistently produced ascospores in culture (Aronstein, unpublished). Others hypothesized that one of the *A. apis* mating types may inhibit growth of the other or they may not be equally distributed in the environment (Christensen and Gilliam, 1983). It may be true that there is an inhibition of one mating type by the other, but that does not seem to occur when opposite mating types are grown together in the laboratory, although their behavior may be different in the natural environment. What seems more likely is that younger mummies are white, and given enough time and the proper conditions, they will become black due to the eventual development of ascospores.

Fig. 2. Chalkbrood mummies. Chalkbrood mummies are white, brown, or black. White mummies contain few or no ascospores.

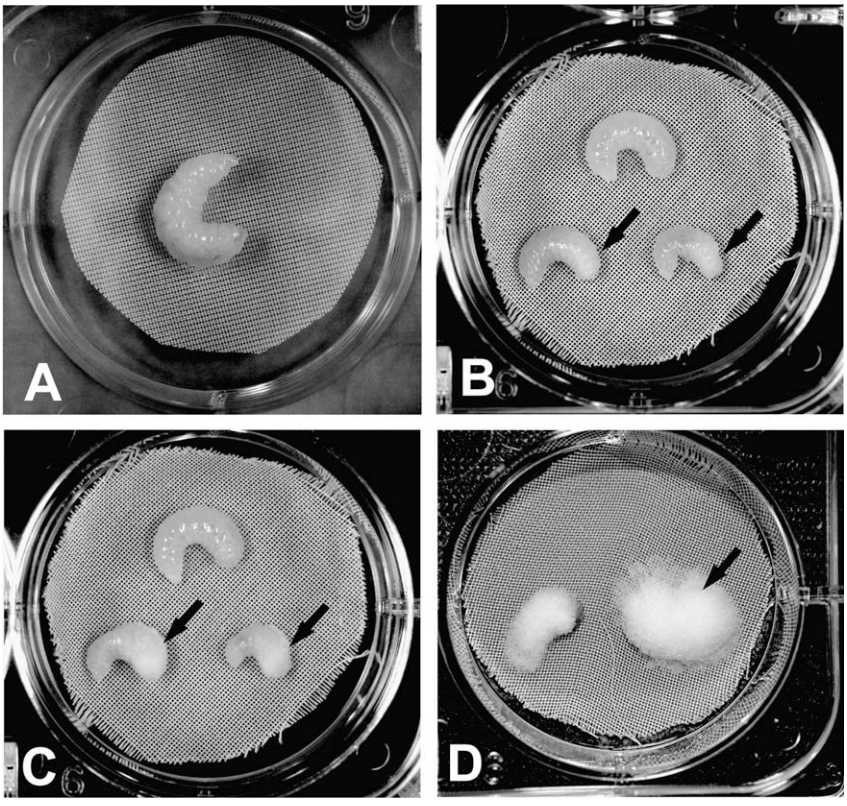

Fig. 1. In vivo bioassay.Three-day-old honey bee larvae were infected by feeding with spore-containing diet. (A) Control bee larva. (B) Bee larvae 72 h post-infection. Arrows denote white-colored mass formed by fungal mycelium growing under the skin. (C) Bee larvae 78 h post-infection showing clinical signs of chalkbrood (arrows). (D) Bee larvae 6 days post-infection. Cadaver is fully covered with fungal mycelium (arrow).

6. Morphology and reproduction

On solid culture media, *A. apis* grows as a dense white mycelium containing aerial, surface, and subsurface hyphae. The hyphae are septate, 2.5–8 μm in diameter and show pronounced dichotomous branching (Spiltoir, 1955; Skou, 1988). If a hypha is ruptured, the septum gives it some physical rigidity and limits loss of cytoplasm. Each septum has a pore that allows movement of cytoplasm and small nuclei between vegetative cells (Spiltoir, 1955). Scanning electron microscopy (SEM) revealed mitochondria and numerous ribosomes in the cytoplasm of the *A. apis* mycelium (Anderson and Gibson, 1998).

Sexual reproduction of this and closely related Ascosphaera species has been well studied (Spiltoir, 1955; Gilliam, 1978; McManus and Youssef, 1984; Heath and Gaze, 1987; Shimanuki and Knox, 1990; Gilliam and Taber, 1991; Hornitzky, 2001; Chorbinski and Rypula, 2003). *A. apis* belongs to the heterothallic Ascomycota, where sexual reproduction typically occurs between morphologically identical haploid partners, distinguished only by their mating type (MAT) locus (Bissett, 1988; Anderson and Gibson, 1998; Poggeler, 2001). According to Heath (1982a), cultures of the two *A. apis* mating types are indistinguishable from one another, with neither pigmentation nor the colony size/growth rate being different. On the other hand, Spiltoir (1955) described the two *A. apis* idiomorphs as sexually dimorphic at the microscopic level, where hyphae of opposite mating types produce specialized reproductive structures (Fig. 3A–E).

SEM revealed that the wall of an ascoma is double-layered with a smooth outer surface (Bissett, 1988; Skou, 1988; Anderson and Gibson, 1998; Chorbinski and Rypula, 2003). A mature ascoma measures in the range of 47–140 μm in diameter (Christensen and Gilliam, 1983; Chorbinski and Rypula, 2003) and contains numerous spore balls (asci) (Fig. 3F). The asci measure in the range of 7–18 μm in diameter and contain no visible outer membrane. SEM observations of *A. apis* specimens revealed that mature ascospores are tightly packed inside the ascus. They have a thick spore wall, a spore membrane with many depressions, and sporoplasm containing numerous ribosomes and mitochondria (Bissett, 1988). The size of the individual ascospore is in the range of 2.7–3.5 × 1.4–1.8 μm (Skou, 1972; Gochnauer and Hughes, 1976; Bissett, 1988; Bailey and Ball, 1991; Anderson and Gibson, 1998; Chorbinski and Rypula, 2003) (Fig. 4A). Ascospores are resistant to extreme temperatures and can survive for years in the environment.

In nature, development and growth of *A. apis* strictly depends on the nutrients obtained from honey bee larvae. Germination of spores requires very specific conditions that are found in the larval gut environment (Bignell and Heath, 1985; Winston, 1991). The temperature and pH in the larval gut may have a major effect on the viability and germination of fungal spores (Bamford and Heath, 1989). In the laboratory, spores germinate best at 35 °C in a nearly anaerobic environment, producing a single germ tube (Fig. 4B). By contrast, mycelia require an aerobic environment for growth (Bailey, 1967), with a temperature optimum of about 30 °C.

Over the years, many different *A. apis* specimens were identified and deposited into different culture collections (Table 1).

Recently, two opposite *A. apis* MAT strains (ARSEF 7405, 7406) were isolated by Murray et al. (2005) and the genome of the ARSEF 7406 isolate (genome size is approximately 24 Mb) has been sequenced (Qin et al., 2006) and deposited at DDBJ/EMBL/GENBANK (Project accession number AARE00000000). Based on genome sequence analysis and MAT-specific PCR, these two isolates (ARSEF 7405 and 7406) were assigned MAT1-1 and MAT1-2, respectively (Aronstein et al., 2007), using the nomenclature originally proposed by Turgeon and Yoder (2000).

Asexual reproduction has never been described in *A. apis*, though it is a common mode of propagation in the Ascomycota. In fact, some pathogenic Ascomycota—the mitosporic Ascomycota

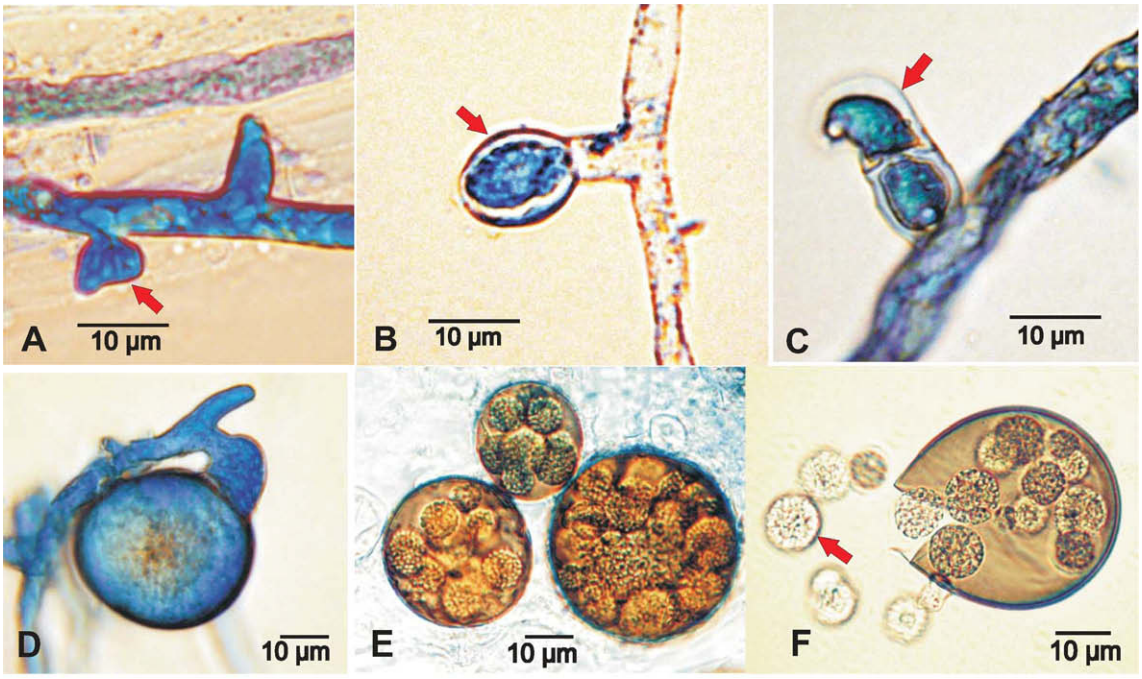

Fig. 3. *A. apis* ascogonial development. Slides were made from a mixed culture of two opposite mating type idiomorphs (ARSEF 7405 and 7406) plated on YGPSA culture medium and incubated at 35 °C under 6% CO_2. Slides were stained with the Lactophenol Cotton Blue Stain (LPCB) and photographed using Nikon optics. (A) Ascogonial primordia (arrow). (B) The nutriocyte begins to swell during or soon after contact with the opposite mating type hypha. (C) Development of a crozier (arrow). (D) Developing ascoma stained blue with LPCB dye. (E) Mature ascoma have a dark-brown appearance, as they do not absorb LPCB dye. The transparency of the ascoma walls allows observation of a number of small (~10 μm) spherical shaped asci. (F) When ascoma is ruptured, as seen in this figure, asci containing fungal ascospores are released (arrow). Scale bars (A–F) 10 μm.

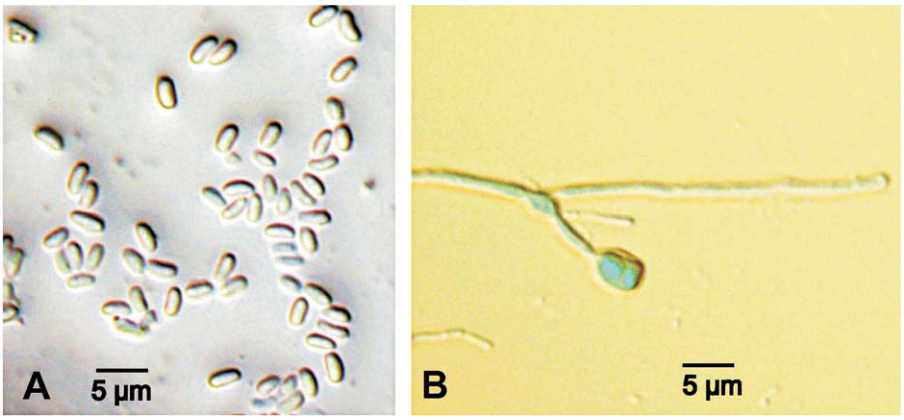

Fig. 4. *A. apis* ascospores. The microscopic slides were stained with LPCB and photographed using Nikon optics and Nomarsky Differential Interference Contrast (DIC) technique. (A) *A. apis* ascospores produced in a mixed culture of two mating type idiomorphs and purified using a 5.0 µm filter unit (MILLEX®-SV, Millipore). (B) Germinating ascospore. Upon germination, the ascospore swells, producing a single germ tube.

Table 1
List of *A. apis* specimens deposited into different culture collections and available to the public.

Culture collection name/number	Depositors
ATCC 13785, 13786	Olive, L.S., 1955.
ATCC 15020, 15021	ATCC cross
ATCC 388506	Samsinakova, A., 1977
ARSEF 691, 692	Vandenberg, J.D., 1979, 1981
ATCC 52320	Takatori, K., 1982
ATCC 56292, 56293	Christensen, M., 1983
ARSEF 5142; CSIRO 101	Anderson, D.L., 1993.
ATCC MYA-4450; ARSEF 7405, 7406	Murray, K.D., 2003

(fungi imperfecti or anamorphic fungi), have no known sexual phase in their life cycle. Future studies are needed to fill the gaps in our understanding of the biology and reproduction of this fungus.

7. Honey bee defenses against pathogenic fungi

Pathogenic fungi infect insects by breaching their primary physical barriers—cuticles and peritrophic membranes (Barr and Shope, 1975; Orihel, 1975; Gliński and Jarosz, 2001). However, the chemical components of the cuticle, such as waxes and unsaturated fatty acids, have a potent antifungal activity (Gliński and Jarosz, 2001). In addition, the biochemical environment of the midgut provides some defense against fungal food-borne pathogens. On the other hand, septic injury rapidly triggers activation of proteolytic cascades at the site of injury that leads to localized blood coagulation and formation of a melanin clot, thereby preventing loss of hemolymph though the wound. The by-product of melanin production is a release of reactive oxygen species that have cytotoxic antimicrobial properties (Davis et al., 2008).

When the outer physical barriers are breached, the invasive fungi encounter a variety of physiological immune defenses by activation of cellular and humoral immune reactions (Gliński and Jarosz, 2001; Glinski and Buczek, 2003). Cellular immune responses begin immediately after an invasion is detected in the haemolymph, while antimicrobial peptides (AMPs) typically appear in the haemolymph some hours after infection (Govind, 2008; Strand, 2008; Stanley et al., 2009). Phagocytosis and encapsulation are the most common defense mechanisms in bees against entomopathogenic fungi (Glinski and Buczek, 2003). Haemocytes can directly kill fungal spores and destroy other small foreign molecules by the phagocytic mechanism (Strand, 2008; Stroschein-Stevenson et al., 2009).

Activation of humoral immunity induces synthesis of AMPs and bacteriolytic enzymes, and activates the prophenoloxidase system. These act synergistically to inactivate or kill the invading microorganisms (Hedengren-Olcott et al., 2004; Stanley et al., 2009). Most of the current knowledge related to humoral insect immunity is based on the studies conducted in *Drosophila* and mosquito models. In *Drosophila*, the humoral response involves two major NF-κB (Toll and IMD) signaling pathways. Toll/Dif signaling is activated by fungal and Gram-positive bacterial infections, and the IMD/Relish signaling is mostly activated by Gram-negative and some Gram-positive bacterial pathogens (De Gregorio et al., 2002; Govind, 2008; Evans and Spivak, 2010). Activation of both pathways leads to synthesis of the AMPs by the fat body cells and haemocytes (Hoffmann, 1995). Most AMPs kill or inactivate invaders by pore formation, but other mechanisms of action have been also described. Furthermore, the NF-κB-like Relish mutant flies showed increased susceptibility to both fungal and bacterial infections, thereby implying regulatory cross-talk between the two major NF-κB signaling pathways (Hedengren-Olcott et al., 2004).

Most components of the honey bee NF-κB signaling pathways were recently identified by Evans et al. (2006). However, mechanisms for microbial recognition and signaling in bees are still largely unknown. Genome analysis revealed that immune related molecules were substantially reduced in the bee genome compared to the fly orthologues (Evans et al., 2006). This reduction in the repertoire of immune related molecules may have a profound effect on the ability of the individual bee to protect itself against microbial infections. However, some degree of protection is also provided by the social organization of the honey bee colony, the major part of which is hygienic behavior (Spivak and Gilliam, 1993; Evans and Spivak, 2010).

Currently, there are very few studies which have investigated the molecular mechanism of honey bee immune responses to chalkbrood (Aronstein and Saldivar, 2005; Aronstein et al., 2006). These studies investigated the expression profiles of one of the three honey bee lysozymes (XM_001120995) and several AMPs (defensin, abaecin, apidaecin, and hymenoptaecin) in 5-day-old larvae injected with *A. apis*, *Paenibacillus larvae* (Gram-positive) spores and *Escherichia coli* (Gram-negative) cells. Though all types of septic injections produced rapid induction of AMPs, none of the responses seemed pathogen-specific. This puzzling result could be interpreted in light of recent studies by Tanji et al. (2007), which determined that fungal septic injections (in contrast to the natural

route of infection) can activate components of both the Toll signaling pathway and the c-Jun N-terminal kinases (JNK) branch of the IMD signaling pathway (Davis et al., 2008), suggesting more complex cross-regulation of the two pathways (Evans and Spivak, 2010.

It is clear that further research is needed to better understand how *A. apis* and other fungal pathogens are recognized by the host immune system and what signaling pathways are activated in bees in response to mycoses.

8. Disease control

A broad range of chemotherapeutic compounds have been tested for their ability to control chalkbrood (Heath, 1982a; Liu, 1991; Glinski and Chmielewski, 1996; Davis and Ward, 2003). Hornitzky (2001) listed chemicals that seemed promising for controlling fungal growth either in culture or in bee colonies. Unfortunately none of the tested compounds achieved the level of control required to fight the disease (Hornitzky, 2001).

Over the years, a number of alternative strategies have been developed and implemented to control chalkbrood disease (Heath, 1982a; Hornitzky, 2001). We describe below some of those which are most effective and widely accepted by beekeepers. These methods include chalkbrood resistant bee lines, improved management and sanitation practices, and use of ecologically safe natural products. Considering that dependence on synthetic pesticides and antimicrobials could lead to general deterioration of the colony environment and bee health in general, it is advisable to minimize use of pesticides inside and outside of bee colonies (Bogdanov et al., 1998, 2004; Frazier et al., 2008).

8.1. Improving genetic stock

Hygienic behavior in bees is defined as the ability of bees to detect and remove diseased or parasitized brood. It is considered the primary mechanism of honey bee resistance to a variety of brood diseases (Rothernbuhler, 1964; Gilliam et al., 1983; Spivak and Gilliam, 1993; Spivak and Reuter, 2001; Lapidge et al., 2002). Therefore, replacement of a queen using good hygienic stock has become one of the most common practices for dealing with chalkbrood. There appears to be a strong genetic component to chalkbrood resistance in hygienic bees (Gilliam and Vandenberg, 1997). Over the past several decades, much research has been focused on enhancement of the hygienic behavior of bees through breeding (Gilliam et al., 1983; Spivak and Reuter, 1998a, 2001; Spivak and Downey, 1998). Colonies exhibiting significant hygienic behavior have reduced numbers of fungal spores in stored food and comb wax (Gilliam et al., 1983; Gilliam et al. 1988b). In most cases, hygienic bee colonies did not require any additional treatments for control of chalkbrood disease.

Recent studies showed that the genetic basis of hygienic behavior involves a number of genes whose products interact in a complex way, and demonstrated that increased genetic diversity in bees may have an important function in reducing the likelihood of outbreaks of the disease (Lapidge et al., 2002; Spivak et al., 2003; Tarpy, 2003; Goode et al., 2005; Evans and Spivak, 2010).

8.2. Management and sanitation

Fungal spores can be present on all surfaces within the beehive (Puerta et al., 1994, 1995), and can remain viable for many years, providing a continual source of infection. Spores can be found in bee-stored pollen (Heath, 1982a), in comb wax (Flores et al., 2005a), and in retail packs of honey (Anderson et al., 1997; Reynaldi et al., 2003). Viable spores have been isolated from honey after 2 years of storage at 20 °C and 30 °C (Gilliam and Vandenberg, 1997), and spores can remain viable for 15 years (Toumanoff, 1951).

Management and sanitation strategies are directed toward helping bees fend off infection or avoiding infection in the first place. These practices include supplemental feeding to improve the nutritional and health status of bees, keeping hives clean and well ventilated (Seal, 1957; Gochnauer et al., 1975), using clean equipment, replacing storage and brood combs annually, and avoiding transfer of combs between colonies (Betts, 1951; Nelson and Gochnauer, 1982; Koenig et al., 1986; Malonova and Titera, 1995; Flores et al., 2005a,b). An additional benefit to replacing old combs is that they often contain residual pesticides (Bogdanov et al., 1998, 2004).

Several different sterilization methods have been tested in attempts to reduce the fungal spore load in beehives. Fumigation of beehive equipment using various chemicals was performed by Gochnauer and Margetts (1980) and Faucon et al. (1982), but these were not widely accepted due to residuals found in both wood and wax. Gamma irradiation from a Cobalt-60 source was effectively used to sterilize contaminated beekeeping equipment, old frames, and honey bee combs (Hornitzky and Willis, 1983; Baggio et al., 2005). Irradiation was also tested to sterilize wax and honey (Gochnauer and Hamilton, 1970; Wooton et al., 1985). At the optimum level of radiation (10 kGray) there were no negative effects detected on wax composition; though some physico-chemical effects were observed in honey, including decreases in enzymatic activity, change of color, and leakage from frames (Baggio et al., 2005). However, the accessibility of radiation facilities is the limiting factor in utilizing this technology.

Sterilization of honey using heat showed good results, although this method had its limitations. Anderson et al. (1997) demonstrated that *A. apis* spores can be killed by incubation of honey for 8 h at 65 °C or at 70 °C for 2 h in water baths. Heating honey above 90 °C resulted in caramelization, and change of color. The heating also increases the level of the harmful chemical hydroxymethylfurfural (HMF) and considerably reduces beneficial enzymes (e.g. diastase) activity in honey. Therefore, current research efforts are focused on other alternative methods such as microwave radiation, infrared heating, ultra-sonication and ultra-filtration to preserve honey quality (Subramanian et al., 2007).

8.3. Control of chalkbrood with natural products and microorganisms

Considering the worldwide spread of chalkbrood disease and the lack of registered chemicals to fight it, there is a great interest in developing alternative control methods (Winston, 1995). Natural compounds for control of chalkbrood fungus would be a welcome alternative to synthetic fungicides. A broad range of compounds have been tested in honey bee colonies and on *A. apis* in culture in an attempt to control chalkbrood (Heath, 1982a). Some of them include natural plant-derived antimicrobial products (Hornitzky, 2001; Davis and Ward, 2003; Aronstein and Hayes, 2004; Mourad et al., 2005). The antifungal activity of many natural compounds has been tested. Essential oils containing citral, geraniol and citronellal were reported to have the best inhibiting effect on fungal growth in vitro (Calderone et al., 1994; Davis and Ward, 2003). These findings need to be tested in field studies to evaluate the efficacy of the most active products in bee hives.

A broad spectrum antimicrobial compound (lysozyme) was tested in field studies in Canada and showed promise for the control of chalkbrood in bee colonies (Van Haga et al., unpublished). Numerous microbes associated with honey bees, such as certain *Penicillum*, *Aspergillus*, *Bacillus* species, showed inhibiting effects on growth of *A. apis* in culture (Gilliam et al., 1988a; Wood, 1998).

9. Methods used in *A. apis* research

9.1. Fungal culture and storage conditions

Many techniques have been used to cultivate *A. apis* for research purposes (Maurizio, 1934; Skou, 1972; Christensen and Gilliam, 1983; Bailey and Ball, 1991; Anderson and Gibson, 1998; Ruffinengo et al., 2000). In culture, *A. apis* can grow on a wide variety of media in either aerobic (Spiltoir, 1955; Heath, 1982b; Rose et al., 1984; Bissett, 1988; Gilliam et al., 1988b) or anaerobic conditions (Thomas and Luce, 1972; Heath and Gaze, 1987; Anderson et al., 1997; Johnson et al., 2005). A list of 19 different media supporting the growth of *A. apis* can be found in Heath (1982b). Some of the most frequently used media are: Potato–Dextrose agar (PDA), Yeast–Glucose–Starch agar (YGPSA), and Sabouraud Dextrose agar (SDA) (Bailey 1981; Anderson and Gibson, 1998).

For morphological examination of fungal cultures, Spiltoir (1955) recommended using PDA media supplemented with 0.4% yeast extract that supports a strong vegetative growth and abundant sexual reproductive structures. Malt agar (2%) has been found better for microscopic observations on the basis that it limits growth of aerial hyphae. *A. apis* can grow in a wide range of temperatures from 22 °C to 37 °C with the optimum growth at 30 °C (Heath, 1982b; Anderson et al., 1997; Liang et al., 2000). According to Thomas and Luce (1972) and Bailey (1981), *A. apis* mycelium can grow only under aerobic conditions. Anaerobic conditions seem to facilitate germination of fungal spores (Bailey and Ball, 1991). Murray et al. (2005) reported that culturing *A. apis* on YGPSA (1% yeast extract, 0.2% glucose, 0.1 M KH_2PO_4, 1% soluble starch, 2% agar) solid culture medium at 35 °C under 6% CO_2 produce sufficient vegetative growth and abundant reproductive structures (Fig. 5A and B).

Storage conditions were tested using *A. apis* spores and hyphae (Jensen et al., 2009). Both freeze-dried and cryogenically stored *A. apis* spores preserved well. In contrast, *A. apis* hyphae preserved well only using cryopreservation in 10% glycerol, and remained highly viable (up to 98%) even after a year of storage at −80 °C.

9.2. Disease diagnostics

Diagnosis of the disease in a bee colony is generally made on the basis of white, black, or gray mummies at the hive entrance, on the bottom board, or in sealed and unsealed brood cells (Gilliam et al., 1978; Heath, 1982a; Shimanuki and Knox, 1990; Gilliam and Vandenberg, 1997). Mummies are then microscopically examined for presence of ascomata. If only white mummies are present, the preparation must be plated on the agar medium to detect reproductive structures. For identification purposes, a heat treatment of the inoculum is routinely performed prior to plating to kill non-spore forming microbes that are routinely found in chalkbrood mummies (Johnson et al., 2005). Fungal growth is typically visible on plates in 2–3 days (Fig. 5A). After 4–6 days of incubation, black specks of ascomata will appear on the mycelial lawn. When strains of different mating types are inoculated onto plates a slight distance apart, ascomata will appear as characteristic black lines where the mycelia intersect (Fig. 5B).

9.3. Microscopic examination

An *A. apis* culture can be observed by microscopic examination of slide preparations at 40× magnification (Anderson and Gibson, 1998). Staining of slides with Lactophenol Cotton Blue Stain (LPCB) can enhance contrast for better analysis of culture morphology. The presence of ascomata in samples is diagnostic for the disease (Fig. 3E and F).

9.4. Molecular Identification of A.apis

Very often, honey bee colonies contaminated with *A. apis* have no visible signs of the disease. Therefore, it is useful to have early detection techniques, such as biochemical assays or molecular diagnostics that will allow detection of the pathogen at sub-clinical levels. Gilliam and Lorenz (1993) and Chorbiński (2003) identified several enzymes present in *A. apis*. Among them, the enzyme valine aminopeptidase was produced only by unmated strains and was proposed for identification of the mycelial stage of the fungus. ß-galactosidase and α-mannosidase enzymes were suggested for identification of both mated and unmated strains because few other honey bee-associated fungi produce these enzymes.

In recent years, a number of different molecular methods have been developed for identification of Ascosphaera spp. using DNA-based tools. Most of these studies utilized rDNA and the internal transcribed spacers (ITS1, ITS2) that have proven especially useful for elucidating relationships among closely related species. This is now the most widely sequenced DNA region in fungi. In most of these studies, the authors used a somewhat labor intensive method of amplifying the ITS1–5.8S-ITS2 region, followed by cloning and sequencing of the entire fragment (Anderson et al., 1998; Chorbinski, 2004; Borum and Ulgen, 2008). A different approach, repetitive-sequence-based PCR (Rep-PCR) was utilized by Reynaldi et al. (2003) to characterize *A. apis* isolates.

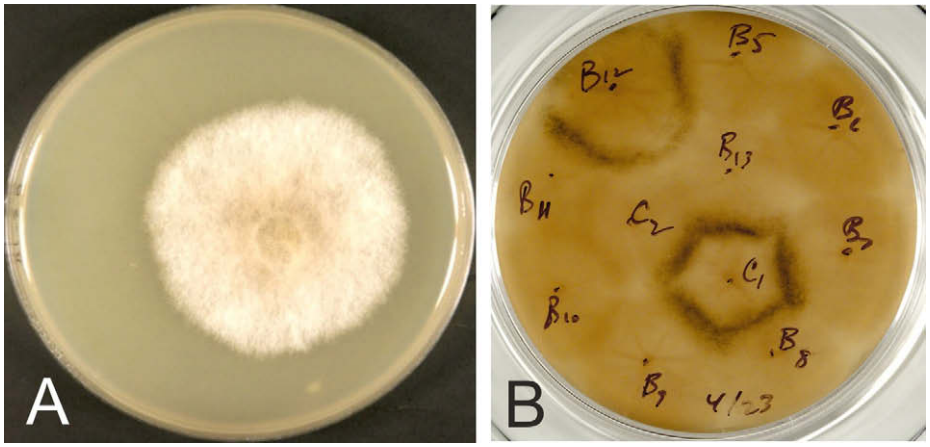

Fig. 5. *A. apis* culture. (A) Mixed *A. apis* idiomorphs cultured on solid YGPSA culture medium at 35 °C under 6% CO_2. (B) Mating type test. Characteristic black lines of ascomata formed at the interface of the opposite mating type colonies can be observed, when fungal colonies are plated at a distance.

Recently, two new species-specific methods were developed for identification of *A. apis* and other closely related fungi (Murray et al., 2005; James and Skinner, 2005). Both of these methods utilized species-specific PCR amplification of fungal rDNA. In both methods, the presence of a band from a PCR amplification using species-specific primers indicates the presence of DNA from that species.

9.5. Larval bioassay

Since 1972, researchers have been infecting honey bee larvae in vivo (De Jong, 1976; Gilliam et al., 1978; Vandenberg and Shimanuki, 1987; Puerta et al., 1994; Flores et al., 1996, 2004). The majority of the investigators used "whole chalkbrood mummy" inoculums (Gilliam et al., 1988b; Starks et al., 2000; Tarpy, 2003). Recently, Johnson et al. (2005) showed that in addition to *A. apis*, whole mummy inoculums contain various species of microbes, and therefore may produce unintended results in bioassays. One of the most common problems in conducting bioassays is damage to larvae during collection from cells and during transfer from an old to a new plate. Stress to larvae during collection can be reduced by using the warm water removal technique. Using this technique, bee larvae are quickly washed out of their brood cells by a gentle stream of warm water Also, to reduce injury to larvae, they can be manipulated using Chinese grafting tools commonly used in queen rearing, instead of forceps. A high efficiency bioassay incorporating these tactics that produced a reliable level of infection was described by Aronstein et al. (2006).

10. Conclusion

While chalkbrood is a mild annoyance for some beekeepers, for others it is an economic issue. With the increasing stresses on managed bees over the past couple of decades in the form of new parasites and pathogens, increased pesticide use, and increased long-distance transportation to pollination sites, the resulting weakened immune system may provide additional opportunity for chalkbrood infections. Requeening with hygienic queens and replacing combs do solve the problem, but that obviously does involve cost and labor. In addition, it would not be surprising if *A. apis* were to evolve mechanisms to persist even with hygienic bees as hosts. The fact that many chalkbrood mummies are white and are therefore not involved in fungus propagation suggests that it is advantageous for the fungus to limit its ascospore production. That is, a balance has evolved wherein both the fungus and the host survive. The practice of managed beekeeping perhaps upsets this natural host-pathogen balance because the survival and reproduction of the fungus and its host is no longer dependent on nature alone. Of course, natural balances are not necessarily compatible with economically-feasible beekeeping. A goal for future research is to manipulate the beehive environment in such a way that will keep reproduction of the fungus to a level below an economic threshold.

Conflicts of interest

There are no conflicts of interest to be declared.

Acknowledgments

The authors acknowledge the authors of extensive publications, reviews and book chapters, whose work has been quoted in this review.

References

Aizen, M.A., Garibaldi, L.A., Cunningham, S.A., Klein, A.M., 2009. How much does agriculture depend on pollinators? Lessons from long-term trends in crop production. Ann. Bot. 103 (9), 1579–1588.
Anderson, D.L., Gibson, N.L., 1998. New species and isolates of spore-cyst fungi (Plectomycetes: *Ascosphaerales*) from Australia. Aust. Syst. Bot. 11, 53–72.
Anderson, D.L., Giacon, H., Gibson, N., 1997. Detection and thermal destruction of the chalkbrood fungus (*Ascosphaera apis*) in honey. J. Apicult. Res. 36, 163–168.
Anderson, D.L., Gibbs, A.J., Gibson, N.L., 1998. Identification and phylogeny of sporecyst fungi (*Ascosphaera* spp.) using ribosomal DNA sequences. Mycol. Res. 102, 541–547.
Aronstein, K., Hayes, G., 2004. Antimicrobial activity of allicin against honeybee pathogens. J. Apicult. Res. 43, 57–59.
Aronstein, K., Saldivar, E., 2005. Characterization of a honeybee Toll related receptor gene Am18w and its potential involvement in antimicrobial immune defense. Apidologie 36, 3–14.
Aronstein, K.A., Pankiw, T., Saldivar, E., 2006. SID-1 is implicated in systemic gene silencing in the honey bee. J. Apicult. Res. 45, 20–24.
Aronstein, K.A., Murray, K.D., de Leon, J., Qin, X., Weinstock, G., 2007. High mobility group (HMG-box) genes in the honey bee fungal pathogen *Ascosphaera apis*. Mycologia 99 (4), 553–561.
Baggio, A., Gallina, A., Dainese, N., Manzinello, C., Mutinelli, F., Serra, G., Colombo, R., Carpana, E., Sabatini, A.G., Walner, K., Piro, R., Sangiorgi, E., 2005. Gamma radiation: a sanitating treatment of AFB-contaminated beekeeping equipment. Apiacta 40, 22–27.
Bailey, L., 1963. Infectious Diseases of the Honeybee. Land Books Ltd., London. p. 176.
Bailey, L., 1967. The effect of temperature on the pathogenicity of the fungus, *Ascosphaera apis*, for larvae of the honey bee. *Apis mellifera*. In: Van der Laan, P.A. (Ed.), Insect Pathology and Microbial Control. North Holland Publishing Co., Amsterdam, The Netherlands, pp. 162–167.
Bailey, L., 1981. Honey Bee Pathology. Academic Press, London, UK. pp. 40–44.
Bailey, L., Ball, B.V., 1991. Honey Bee Pathology. Academic Press, London, UK. pp. 53–62.
Baker, G.M., Torchio, G.M., 1968. New records of *Ascosphaera apis* from North America. Mycologia 60, 189–190.
Bamford, S., Heath, L.A.F., 1989. The effects of temperature and pH on the germination of spores of the chalkbrood fungus, *Ascosphaera apis*. J. Apicult. Res. 28 (1), 36–40.
Barr, A.R., Shope, R.E., 1975. The invertebrate gut as a barrier to invading parasites. In: Maramorosh, K., Shope, R.E. (Eds.), Invertebrate Immunity. Academic Press, New York, USA, pp. 113–114.
Berbee, M.L., Taylor, J.W., 1992. Two ascomycete classes based on fruiting-body characters and ribosomal DNA sequence. Mol. Biol. Evol. 9 (2), 278–284.
Betts, A.D., 1932. Fungus diseases of bees. Bee World 40, 156.
Betts, A.D., 1951. The Diseases of Bees: Their Signs, Causes and Treatment. Hickmott, Camberley, England. p. 69.
Bignell, D.E., Heath, L.A.F., 1985. Electropositive redox state of the fifth-instar larval gut of *Apis mellifera*. J. Apicult. Res. 24, 211–213.
Bissett, J., 1988. Contribution toward a monograph of the genus *Ascosphaera*. Can. J. Bot. 66, 2541–2560.
Bogdanov, S., Kilchenmann, V., Imdorf, A., 1998. Acaricide residues in some bee products. J. Apicult. Res. 37, 57–67.
Bogdanov, S., Kilchenmann, V., Seiler, K., Pfefferli, H., Frey, T.H., Roux, B., Wenk, P., Noser, J., 2004. Residues of para-dichlorobenzene in honey and beeswax. J. Apicult. Res. 43 (1), 14–16.
Borum, A.E., Ulgen, M., 2008. Chalkbrood (*Ascosphaera apis*) infection and fungal agents of honey bees in north-west Turkey. J. Apicult. Res. 47 (2), 170–171.
Bradbear, N., 1988. The world distribution of major honeybee diseases and pests. Bee World 69, 15–39.
Cain, R.F., 1972. Evolution of the fungi. Mycologia 64, 1–14.
Calderone, N.W., Shimanuki, H., Allen-Wardell, G., 1994. An in vitro evaluation of botanical compounds for the control of the honeybee pathogens *Bacillus larvae* and *Ascosphaera apis*, and the secondary invader *B. alvei*. J. Essent. Oil Res. 6, 279–287.
Chorbiński, P., 2003. Enzymatic activity of strains of *Ascosphaera apis*. Vet. Med. 59, 1019–1022.
Chorbinski, P., 2004. Identification of *Ascosphaera apis* strains using ribosomal DNA sequences. Med. Vet. 60 (2), 190–192.
Chorbinski, P., Rypula, K., 2003. Studies on the morphology of strains *Ascosphaera apis* isolated from chalkbrood disease of the honey bees. Vet. Med. 6 (2), 1–12.
Christensen, M., Gilliam, M., 1983. Note on the *Ascosphaera* species inciting chalkbrood in honey bees. Apidologie 14 (4), 291–297.
Davis, C., Ward, W., 2003. Control of chalkbrood disease with natural products: a report for the RIRDC. Publication No. 03/107, Kingston, ACT, AU, pp. 1–23.
Davis, M.M., Primrose, D.A., Hodgetts, R.B., 2008. A member of the p38 mitogen-activated protein kinase family is responsible for transcriptional induction of dopa decarboxylase in the epidermis of *Drosophila melanogaster* during the innate immune response. Mol. Cell Biol. 28 (15), 4883–4895.
De Gregorio, E., Spellman, P.T., Tzou, P., Rubin, G.M., Lemaitre, B., 2002. The Toll and Imd pathways are the major regulators of the immune response in *Drosophila*. EMBO J. 21 (11), 2568–2579.
De Jong, D., 1976. Experimental enhancement of chalkbrood infection. Bee World 57, 114–115.

Dreher, K., 1938. Auftreten von Bienenkrankheiten in Niedersachsen und Braunschweig im Jahre 1937. Niedersachsische Imker 73 (12), 282–284.
Evans, J.D., Spivak, M., 2010. Socialized medicine: individual and communal disease barriers in honey bees. J. Invertebr. Pathol. 103, S62–S72.
Evans, J.D., Aronstein, K.A., Chen, Y.P., Hetru, C., Imler, J.-L., Jiang, H., Kanost, M., Thompson, G., Zou, Z., Hultmark, D., 2006. Immune pathways and defense mechanisms in honey bees, *Apis mellifera*. Insect Mol. Biol. 15, 645–656.
Faucon, J.P., Arvieu, J.C., Collin, M.E., 1982. Possibility of utilizing methyl bromide for disinfection of apicultural material. Med. Vet. 133, 207–210.
Flores, J.M., Ruiz, J.A., Ruz, J.M., Puerta, F., Bustos, M., Padilla, F., Campano, F., 1996. Effect of temperature and humidity of sealed brood on chalkbrood development under controlled conditions. Apidologie 27, 185–192.
Flores, J.M., Gutierrez, I., Puerta, F., 2004. A comparison of methods to experimentally induce chalkbrood disease in honey bees. Span. J. Agricult. Res. 2 (1), 79–83.
Flores, J., Spivak, M., Gutierrez, I., 2005a. Spores of *Ascosphaera apis* contained in wax foundation can infect honeybee brood. Vet. Microbiol. 108, 141–144.
Flores, J.M., Gutierrez, I., Espejo, R., 2005b. The role of pollen in chalkbrood disease in *Apis mellifera*: transmission and predisposing conditions. Mycologia 97, 1171–1176.
Frazier, M., Mullin, C., Frazier, J., Ashcraft, S., 2008. What have pesticides got to do with it? Am. Bee J. 148, 521–523.
Gilliam, M., 1978. Fungi. In: Morse, R.A. (Ed.), Honey Bee Pests, Predators, and Diseases. Cornell University Press, Ithaca, NY, pp. 78–101.
Gilliam, M., 1986. Infectivity and survival of the chalkbrood pathogen, *Ascosphaera apis*, in colonies of honey bees, *Apis mellifera*. Apidologie 17 (2), 93–100.
Gilliam, M., Lorenz, B.J., 1993. Enzymatic activity of strains of *Ascosphaera apis*, an entomopathogenic fungus of the honey bee, *Apis mellifera*. Apidologie 24, 19–23.
Gilliam, M., Taber, S., 1991. Diseases, pests, and normal microflora of honeybees, *Apis mellifera*, from feral colonies. J. Invertebr. Pathol. 58 (2), 286–289.
Gilliam, M., Vandenberg, J.D., 1997. Fungi. In: Morse, R., Flottum, K. (Eds.), Honey Bee Pests, Predators, and Diseases, third ed. AI Root, Ohio, OH, pp. 81–110.
Gilliam, M., Taber, S., Rose, J.B., 1978. Chalkbrood of honeybees, *Apis mellifera* L., a progress report. Apidologie 9 (1), 75–89.
Gilliam, M., Taber, S., Richardson, G.V., 1983. Hygienic behavior of honey bees in relation to chalkbrood disease. Apidologie 14, 29–39.
Gilliam, M., Lorenz, B.J., Richardson, G.V., 1988a. Digestive enzymes and microorganisms in honey bees, *Apis mellifera* – influence of streptomycin, age, season and pollen. Microbios 55, 95–114.
Gilliam, M., Taber, S., Lorenz, B.J., Prest, D.B., 1988b. Factors affecting development of chalkbrood disease in colonies of honey bee, *Apis mellifera*, fed pollen contaminated with *Ascosphaera apis*. J. Invertebr. Pathol. 52, 314–325.
Gilliam, M., Lorenz, J., Werner, A.M., Thorp, R.W., 1997. Occurrence and distribution of *Ascosphaera apis* in North America: chalkbrood in feral honey bee colonies that had been in isolation on Santa Cruz Island, California for over 110 years. Apidologie 28, 329–338.
Glinski, Z., 1982. Studies on pathogenicity of *Ascosphaera apis* for larvae of the honeybee *Apis mellifera* L. Part II. Relationships between biochemical types and virulence of *A. apis*. Annales Universitatis Mariae Curie Sklodowska Sectio DD. Med. Vet. 37 (8), 69–77.
Glinski, Z., Buczek, K., 2003. Response of the *Apoidea* to fungal infections. Apiacta 38, 183–189.
Glinski, Z., Chmielewski, M., 1996. Imidazole derivatives in control of the honey bee brood mycoses. Pszczeln. Zesz. Nauk. 40 (2), 165–173.
Gliński, Z., Jarosz, J., 2001. Infection and immunity in the honey bee *Apis mellifera*. Apiacta 36 (1), 12–24.
Gochnauer, T.A., Hamilton, H.A., 1970. Disinfection of honey bee combs by gamma radiation. J. Apicult. Res. 9, 87–94.
Gochnauer, T.A., Hughes, S.J., 1976. Detection of *Ascosphaera apis* in honey bee larvae (Hymenoptera: Apidae) from Eastern Canada. Can. Entomol. 108, 985–988.
Gochnauer, T.A., Margetts, V.J., 1980. Decontaminating effect of ethylene oxide on honeybee larvae previously killed by chalkbrood disease. J. Apicult. Res. 19, 261–264.
Gochnauer, T.A., Furgala, B., Shimamuki, H., 1975. Diseases and enemies of the honeybee. In: The Hive and the Honey Bee (revised ed.). Dadant Hamilton, Ill., USA, pp. 615–621.
Goode, K., Huber, Z., Mesce, K.A., Spivak, M., 2005. The relationships of honey bee (*Apis mellifera*) behaviors in the context of octopamine neuromodulation: hygienic behavior is independent of sucrose responsiveness and foraging ontogeny. Horm. Behav. 49, 391–397.
Govind, S., 2008. Innate immunity in *Drosophila*: pathogens and pathways. Insect Sci. 15, 29–43.
Hale, P.J., Menapace, D.M., 1980. Effect of time and temperature on the viability of *Ascosphaera apis*. J. Invert. Pathol. 36, 429–430.
Hawksworth, D.L., Sutton, B.C., Ainsworth, G.C., 1983. Ainsworth and Bisby's dictionary of the fungi. CAB International, Kew. pp. 30–31.
Heath, L.A.F., 1982a. Development of chalk brood in a honey bee colony; chalk brood pathogens: a review. Bee World 63 (3), 119–135.
Heath, L.A.F., 1982b. Chalk brood pathogens: a review. Bee World 63, 130–135.
Heath, L.A.F., 1985. Occurrence and distribution of chalk brood disease of honeybees. Bee World 66, 9–15.
Heath, L.A.F., Gaze, B.M., 1987. Carbon dioxide activation of spores of the chalkbrood fungus *Ascosphaera apis*. J. Apicult. Res. 26 (4), 243–246.
Hedengren-Olcott, M., Olcott, M.C., Mooney, D.T., Ekennnnnngren, S., Geller, B.L., Taylor, B.J., 2004. Differential Activation of the NF-κB-like factors relish and dif in *Drosophila melanogaster* by fungi and Gram-positive bacteria. J. Biol. Chem. 279 (20), 21121–21127.
Hibbett, D.S., Binder, M., Bischoff, J.F., Blackwell, M., Cannon, P.F., Eriksson, O.E., Huhndorf, S., James, T., Kirk, P.M., Lucking, R., Lumbsch, H.T., Lutzoni, F., Matheny, P.B., McLaughlin, D.J., Powell, M.J., Redhead, S., Schoch, C.L., Spatafora, J.W., Stalpers, J.A., Vilgalys, R., Aime, M.C., Aptroot, A., Bauer, R., Begerow, D., Benny, G.L., Castlebury, L.A., Crous, P.W., Dai, Y.C., Gams, W., Geiser, D.M., Griffith, G.W., Gueidan, C., Hawksworth, D.L., Hestmark, G., Hosaka, K., Humber, R.A., Hyde, K.D., Ironside, J.E., Koljalg, U., Kurtzman, C.P., Larsson, K.H., Lichtwardt, R., Longcore, J., Miadlikowska, J., Miller, A., Moncalvo, J.M., Mozley-Standridge, S., Oberwinkler, F., Parmasto, E., Reeb, V., Rogers, J.D., Roux, C., Ryvarden, L., Sampaio, J.P., Schussler, A., Sugiyama, J., Thorn, R.G., Tibell, L., Untereiner, W.A., Walker, C., Wang, Z., Weir, A., Weiss, M., White, M.M., Winka, K., Yao, Y.J., Zhang, N., 2007. A higher-level phylogenetic classification of the Fungi. Mycol. Res. 111, 509–547.
Hitchcock, J.D., Christensen, M., 1972. Occurrence of chalk brood (*Ascosphaera apis*) in honey bees in the United States. Mycologia 64 (5), 1193–1198.
Hoffmann, J.A., 1995. Innate immunity of insects. Curr. Opin. Immunol. 7 (1), 4–10.
Hornitzky, M., 2001. Literature review of chalkbrood. A report for the RIRDC. Publication No. 01/150, Kingston, ACT, AU.
Hornitzky, M.A.Z., Willis, P.A., 1983. Gamma radiation inactivation of bacillus larvae to control American foul brood. J. Apicult. Res. 22, 196–199.
James, R.R., Skinner, J.S., 2005. PCR diagnostic methods for *Ascosphaera* infections in bees. J. Invertebr. Pathol. 90 (2), 98–103.
James, T.J., Kauff, F., Schoch, C.L., Matheny, P.B., Hofstetter, V., Cox, C.J., Celio, J., Gueidan, C., Fraker, E., Miadlikowska, J., Lumbsch, H.T., Rauhut, A., Reeb, V., Arnold, A.E., Amtoft, A., Stajich, J.E., Hosaka, K., Sung, G., Johnson, D., O'Rourke, B., Crockett, M., Binder, M., Curtis, J.M., Slot, J.C., Wang, Z., Wilson, A.W., Schüßler, A., Longcore, J.E., O'Donnell, K., Mozley-Standridge, S., Porter, D., Letcher, P.M., Powell, M.J., Taylor, J.T., White, M.M., Griffith, G.W., Davies, D.R., Humber, R.A., Morton, J.B., Sugiyama, J., Rossman, A.R., Rogers, J.D., Pfister, D.H., Hewitt, D., Hansen, K., Hambleton, S., Shoemaker, R.A., Kohlmeyer, J., Volkmann-Kohlmeyer, B., Spotts, R.A., Serdani, M., Crous, P.W., Hughes, K.W., Matsuura, K., Langer, E., Langer, G., Untereiner, W.A., Lücking, R., Büdel, B., Geiser, D.M., Aptroot, A., Diederich, P., Schmitt, I., Schultz, M., Yahr, R., Hibbett, D.S., Lutzoni, F., McLaughlin, D.J., Spatafora, J.W., Vilgalys, V., 2006. Reconstructing the early evolution of fungi using a six-gene phylogeny. Nature 443, 818–822.
Jensen, A.B., James, R.R., Eilenberg, J., 2009. Long-term storage of *Ascosphaera aggregata* and *A. apis*, pathogens of the leafcutting bee (*Megachile rotundata*) and the honey bee (*Apis mellifera*). J. Invert. Pathol. 101 (2), 157–160.
Johnson, R.N., Zaman, M.T., Decelle, M.M., Siegel, A.J., Tarpy, D.R., Siegel, E.C., Starks, P.T., 2005. Multiple micro-organisms in chalkbrood mummies: evidence and implications. J. Apicult. Res. 44 (1), 29–32.
Kluser, S., Peduzzi, P., 2007. Global pollinator decline: a literature review. UNEP/GRID-Europe, pp. 1–12.
Koenig, J.P., Boush, G.M., Erickson, E.H., 1986. Effect of type of brood comb on chalk brood disease in honeybee colonies. J. Apicult. Res. 25, 58–62.
Koenig, J.P., Boush, G.M., Erickson, E.H., 1987. Effects of spore introduction and ratio of adult bees to brood on chalkbrood disease in honeybee colonies. J. Apicult. Res. 26, 191–195.
Lapidge, K., Oldroyd, B., Spivak, M., 2002. Seven suggestive quantitative trait loci influence hygienic behavior of honey bees. Naturwissenschaften 89, 565–568.
Liang, O., Chen, D., Wang, J., 2000. Effects of temperature, relative humidity and pH on germination of chalkbrood fungus, *Ascosphaera apis* spore. J. Appl. Ecol. 11 (6), 869–872.
Liu, T.P., 1991. Ultrastructural changes in the spore and mycelia of *Ascosphaera apis* after treatment with benomyl (Benlate 50 W). Mycopathologia 116, 23–28.
Lumbsch, H.T., Huhndorf, S.M., (Eds.), 2007. Outline of Ascomycota, vol. 13. Myconet, pp. 1–58.
Maassen, A., 1913. Weitere Mitteilungen uber der seuchenhaften Brutkrankheiten der Bienen [Further communication on the epidemic brood disease of bees]. Mitteilungen aus der Kaiserlichen Biologischen Anstalt fur Land- und Forstwirtscshaft 14, 48–58.
Malloch, D., 1981. The plectomycete centrum. In: Reynolds, D.R. (Ed.), Ascomycete Systematics: The Luttrellian Concept. Springer-Verlag, New York, pp. 73–91.
Malonova, D., Titera, D., 1995. Acrinathrin, an effective varroacide and its residues in stores, honey and wax. Apidologie 26, 321–322.
Matus, F., Sarbak, I., 1974. Occurrence of chalkbrood disease in Hungary. Magyar Allat. Lapja 29, 250–255.
Maurizio, A., 1934. Uber die Kaltbrut (Pericystis-Mykose) der Bienen. Archiv Bienenkunde 15, 165–193.
McManus, W.R., Youssef, N.N., 1984. Life cycle of the chalkbrood fungus, *Ascosphaera aggregata*, in the alfalfa leaf cutting bee, *Megachile rotundata*, and its associated symptomatology. Mycologia 76 (5), 830–842.
Mehr, Z., Menapace, D.M., Wilson, W.T., Sacket, R.R., 1976. Studies on the initiation and spread of chalkbrood within an apiary. Am. Bee J. 116, 266–268.
Mourad, A.K., Zaghloul, O.A., Kady, E.L., Nemat, F.M., Morsy, M.E., 2005. A novel approach for the management of the chalkbrood disease infesting honeybee *Apis mellifera* L. (Hymenoptera: Apidae) colonies in Egypt. Commun. Agric. Appl. Biol. Sci. 70 (4), 601–611.
Murray, K.D., Aronstein, K.A., Jones, W.A., 2005. A molecular diagnostic method for selected *Ascosphaera* species using PCR amplification of internal transcribed spacer regions of rDNA. J. Apicult. Res. 44 (2), 61–64.
Nelson, D.L., Gochnauer, T.A., 1982. Field and laboratory studies on chalkbrood disease of honey bees. Am. Bee J. 122, 29–34.

Orihel, T.C., 1975. The peritrophic membrane: its role as a barrier to infection of the arthropod host. In: Maramorosch, K., Shope, R.E. (Eds.), Invertebrate Immunity. Academic Press, New York, USA, pp. 67–73.
Palmer-Jones, T., 1964. Diseases of honey bees in New Zealand. New Zeal. Entomol. 3, 41–44.
Poggeler, S., 2001. Mating-type genes for classical strain improvements of *Ascomycetes*. Appl. Microbiol. Biotechnol. 56, 589–601.
Puerta, F., Flores, J.M., Bustos, M., Padilla, F., Campano, F., 1994. Chalkbrood development in honeybee brood under controlled conditions. Apidologie 25, 540–546.
Puerta, F., Flores, J.M., Bustos, M., Padilla, F., 1995. Field and laboratory studies on chalkbrood disease of honeybees. Am. Bee J. 122, 29–34.
Qin, X., Evans, J.D., Aronstein, K.A., Murray, K.D., Weinstock, G.M., 2006. Genome sequences of the honey bee pathogens *Paenibacillus larvae* and *Ascosphaera apis*. Insect Mol. Biol. 15 (5), 715–718.
Reid, M., 1988. Diseases of honey bees in New Zealand. Surveillance 15, 15–17.
Reid, M., 1993. Chalkbrood disease: the NZ experience. Aust. Beekeeper, 497–508. June.
Reynaldi, F.J., Lopez, A.C., Albo, G.N., Alippi, A.M., 2003. Genomic fingerprinting. J. Apicult. Res. 42 (4), 68–76.
Rose, J.B., Christensen, M., Wilson, W.T., 1984. *Ascosphaera* species inciting chalkbrood in North America and taxonomic key. Mycotaxon XIX, 41–55.
Rothernbuhler, W.C., 1964. Behavior genetics of nest cleaning in honey bees. IV. Responses of F1 and backcross generations to disease-killed brood. Am. Zool. 4, 111–123.
Roussy, L., 1962. New contribution to the study of *Pericystis apis* (MN). La Gazette Apicole 63, 101–105.
Ruffinengo, S., Pena, N.I., Clemente, G., Palacio, M.A., Escande, A., 2000. Suitability of culture media for the production of ascospores and maintenance of *Ascosphaera apis*. J. Apicult. Res. 39, 143–148.
Seal, D.W.A., 1957. Chalk brood disease of bees. New Zealand. J. Agricult. 95, 562.
Shimanuki, H., Knox, D.A., 1990. Diagnosis of Honey Bee Diseases. Agriculture Handbook No. AH-690, US Department of Agriculture.
Skou, J.P., 1972. Ascosphaerales. Friesia 10 (1), 1–24.
Skou, J.P., 1988. More details in support of the class *Ascosphaeromycetes*. Mycotaxson XXXI (1), 191–198.
Spiltoir, C.F., 1955. Life cycle of *Ascosphaera apis*. Am. J. Bot. 42, 501–518.
Spiltoir, C.F., Olive, L.S., 1955. A reclassification of the genus *Pericystis betts*. Mycologia 47, 238–244.
Spivak, M., Downey, D.L., 1998. Field assays for hygienic behavior in honey bees (Hymenoptera: Apidae). J. Econ. Entomol. 91, 64–70.
Spivak, M., Gilliam, M., 1993. Facultative expression of hygienic behavior of honeybees in relation to disease resistance. J. Apicult. Res. 32, 147–157.
Spivak, M., Reuter, G.S., 1998a. Honey bee hygienic behavior. Am. Bee J. 138, 283–286.
Spivak, M., Reuter, G.S., 1998b. Performance of hygienic honey bee colonies in a commercial apiary. Apidologie 29, 285–302.
Spivak, M., Reuter, G.S., 2001. Resistance to American foulbrood disease by honey bee colonies, *Apis mellifera*, bred for hygienic behavior. Apidologie 32, 555–565.
Spivak, M., Masterman, R., Ross, R., Mesce, K.A., 2003. Hygienic behavior in the honey bee (*Apis mellifera* L.) and the modulatory role of octopamine. J. Neurobiol. 55, 341–354.
Stanley, D., Miller, J., Tunaz, H., 2009. Eicosanoid actions in insect immunity. J. Innate Immun. 1, 282–290.
Starks, P.T., Blackie, C.A., Seeley, T.D., 2000. Fever in honeybee colonies. Naturwissenschaften 87, 229–231.
Strand, M.R., 2008. Insect cellular immunity. Insect Sci. 15, 1–14.
Stroschein-Stevenson, S.L., Foley, E., O'Farrell, P.H., Johnson, A.D., 2009. Phagocytosis of *Candida albicans* by RNAi-treated *Drosophila* S2 cells. Methods Mol. Biol. 470, 347–358.
Subramanian, R., Hebbar, U.H., Rastogi, N.K., 2007. Processing of honey. A review. Int. J. Food Prop. 10, 127–143.
Tanji, T., Hu, X., Weber, A.N.R., Ip, Y.T., 2007. Toll and IMD pathways synergistically activate an innate immune response in *Drosophila melanogaster*. Mol. Cell Biol. 27 (12), 4578–4588.
Tarpy, D.R., 2003. Genetic diversity within honeybee colonies prevents severe infections and promotes colony growth. Proc. R. Soc. Lond. Ser. B 270, 99–103.
Theantana, T., Chantawannakul, P., 2008. Protease and ß-N acetylglucosaminidase of honey bee chalkbrood pathogen *Ascosphaera apis*. J. Apicult. Res. 47 (1), 68–76.
Thomas, G.M., Luce, A., 1972. An epizootic of chalkbrood, *Ascosphaera apis* (Maassen ex Clausen) Olive and Spiltoir in the honeybee, *Apis mellifera* L. in California. Am. Bee J. 112 (3), 88–90.
Toumanoff, C., 1951. Lea Maladies des Abeilles. Rev. Fr. Apic. Num. Spec. 68, 325.
Turgeon, B.G., Yoder, O.C., 2000. Proposed nomenclature for the mating type genes of filamentous Ascomysetes. Fungal Genet. Biol. 31, 1–5.
Tutkun, E., Maden, S., Inci, A., Yilmarz, B., 1993. General situation of chalkbrood disease in honeybees in Turkey. Turk. Entomol. Derg. 17 (2), 65–68.
Vandenberg, J.D., Shimanuki, H., 1987. Technique for rearing worker honey bees in the laboratory. J. Apicult. Res. 26, 90–97.
Wilson, W.T., Nunamaker, R.A., Maki, D., 1984. The occurrence of brood diseases and the absence of the *Varroa* mite in honeybees in Mexico. Am. Bee J. 124, 51–53.
Winston, M.L., 1991. The Biology of the Honey Bee. In: Development and nutrition. Harvard University Press, Cambridge, USA, p. 48.
Winston, M., 1995. We need alternatives. Pesticide resistance. Bee Culture 123 (7), 389–390.
Wood, M., 1998. Microbes help bees battle chalkbrood. J. Econ. Entomol. 46 (8), 16–17.
Wooton, M., Hornitzky, M.A.Z., Beneke, M., 1985. The effects of gamma-radiation from cobalt-60 on quality parameters of Australian honey. J. Apicult. Res. 24, 188–189.
Yacobson, B.A., Elad, D., Rosenthal, K., Kmer, I., Slovecky, I., Efrat, H., 1991. A recent chalkbrood outbreak in Israel: attempts at therapeutic intervention. Am. Bee J. 131, 786.
Zaghloul, O.A., Mourad, A.K., El Kady, M.B., Nemat, F.M., Morsy, M.E., 2005. Assessment of losses in honey yield due to the chalkbrood disease, with reference to the determination of its economic injury levels in Egypt. Commun. Agricult. Appl. Biol. Sci. 70 (4), 703–714.