Evolutionary ecology of fungal parasites in honey bees

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In this thesis I used honey bee larvae as a model organism to study host-parasite interactions with fungal parasites: *Ascosphaera apis*, other *Ascosphaera* species and *Aspergillus flavus*. Below I begin by introducing the fungal pathogens, from the taxonomy to epidemiology. Next I introduce the honey bee host, focusing in particular on the physiology of worker larvae that makes them a target to fungal parasites. Finally, I discuss how variation in temperature, the presence of additional parasites, and genetic variation in both parasite and host, affect disease dynamics in this system.

**Fungal Parasites**

*Ascosphaera apis* (Chalkbrood)

*Ascosphaera* spp. are only found in nature associated with social and solitary bees. Some of the species are saprophytes (Skou, 1972; Bissett, 1988) whereas others are parasites. *Ascosphaera apis* (Maassen ex Claussen) Olive & Spiltoir is an economically important obligate parasite and the causative agent of chalkbrood disease in the honey bee *Apis mellifera* L. (Maassen, 1913) and a few solitary bee species (Gilliam and Vandenberg, 1997). An obligate parasite, by definition, has a narrow host range and requires a living host for survival and replication (Scheffer, 1991). Although *Asc. apis* is distributed worldwide and can cause severe infections, the disease has often been referred to as being ephemeral. The current taxonomic classification of *Asc. apis* is: Ascomycota; Pezizomycotina; Eurotiomycetes; Eurotiomycetidae; Onygenales; Ascosphaeraceae; *Ascosphaera* (Lumbsch and Huhndorf, 2007). Ascosphaerales have a unique morphology due to the spore–cyst (ascoma) developed from a single cell (Fig 1A) (Splitor 1955; McManus and Youssef, 1984). Within the spore–cyst there are numerous sexually produced spores (ascospores) (Fig 1B, C) (Bissett, 1988). Upon disintegration of the spore-ball membrane, the mucilaginous or granular coating remains that are believed to give the spores adhesive properties that can promote their dispersion (Bissett, 1988).
Symptoms and diagnosis. The infection is initiated when honey bee larvae consume the spores, which then germinate in the larval gut (Spiltoir, 1955; Hitchcock and Christensen, 1972; Rose et al., 1984). Roussy (1962) suggested that infection can also take place through the cuticle, but today it is generally accepted that *Asc. apis* spores does not invade the host from the larval cuticle (Maurizio, 1934; De Jong, 1976; Heath, 1982a; Puerta et al., 1994; Flores et al., 1996). The growing mycelia spread throughout the larva, eventually killing it due to the physical damage to the larval tissue. Larvae that have died from chalkbrood are usually diagnosed by the presence of the mycelia on the surface of the body. The host invasion is fairly quick, once the mycelium penetrates the larval gut. Under laboratory conditions, the first signs of infection are recorded 3 day post inoculation.

Heath (1982a,b) and Puerta et al. (1994) suggested that the presence of spores in the larval gut is by itself not sufficient to induce the infection and Flores et al. (1996) suggested lower temperatures in the brood area and high relative humidity as a predisposing condition for *Asc. apis* outbreaks. Similar fluctuations in temperature have been shown to influence host-parasite interactions in bees (James, 2005), and other insect hosts (James et al., 1998; Elliot et al., 2002; Blanford et al., 2003; Thomas and Blanford, 2003; Stacy et al. 2003). In particular, a temperature drop in the honey bee brood area one day prior to pupation appears to increase the number of infected larvae (Bailey, 1967), which is puzzling as the optimal vegetative growth temperature for *Asc. apis* (Chapter 1). Honey bee brood temperature below optimal induces higher larval mortality from chalkbrood infections, but lower temperature is not a limiting factor for the chalkbrood infections (Chapter1). For the most recent review on chalkbrood disease in honey bee see Aronstein and Murray (2010).

Transmission. While adult bees are not susceptible to this parasite, they can transmit the disease via food sharing (Gilliam and Vandenberg, 1997). New spores are formed on the surface of the dead host, and these can then be transmitted to the new larvae by contaminated wax and worker bees (Koenig et al., 1986; Flores et al., 2005), or inadvertently by bee keepers (Gilliam and Vandenberg, 1997). Furthermore, the need for sexual mating of different strains in order to produce spore-cysts may limit the spread of the parasite. Combs have been found with dead ("mummified") larvae covered with white mycelium and without the presence of dark spore-cysts. Consequently some have suggested infection by the consumption of the mycelium (Maurizio, 1934; Davis and Ward,
INTRODUCTION

2003), which has not been observed. Christiansen and Gilliam (1983) have shown that both mating strains can be present in the infected larvae, even though the larva appears to be white and without cysts present. This has been explained by variation in fungal growth rate between different strains (Bailey and Ball, 1991).

Isolation and Cultivation. The whole *A. apis* mummy can contain different strains of the same parasite as well as different species of microbes (Johnson et al., 2005). Prior to the isolation of an *A. apis* strain, the infected larvae should be surface sterilized in 10% sodium hypochlorite for 10 minutes followed by 2 minutes water washing (Reynaldi et al., 2003). Rinsed larvae can be cut into several pieces and placed on Sabouraud Dextrose Agar (SDA) growth medium at 34°C. After several days the *A. apis* mycelia grows on the agar plates. Single hyphal tips can be removed (isolated) with a sterile scalpel under a dissecting microscope, placed on to a new Petri dish with SDA growth medium, and incubated at 34°C. Cultures can be stored at 25 ºC for several weeks, or for longer term storage, mycelia can be placed in 20% glycerol at -80°C (Jensen et al., 2009). Cultures of *A. apis* were maintained on SDA at 25 ºC with monthly transfer to new plates. For spore production, hyphal subcultures of two mating types were transferred into one plate three weeks prior to the experiment to allow sexual reproduction and the maturation of the spores. Once spores formed and matured they were removed from the plate with a small sterile spatula and placed into a sterile glass grinder with sterile deionized water. Following the grinding, sterile deionized water was added to the spore suspension and large particles in the suspension were allowed to settle on the bottom for 20 min before the spore suspension was obtained.
Figure 1. A) *Ascosphaera apis* spore cyst surrounded by fungal mycelia; B) inside the spore cyst there are numerous spore-balls; C) each spore-ball has millions of oval shaped spores. Scanning electron microscope photos taken by José Bresciani.
Other *Ascosphaera* spp. Disease similar to chalkbrood has been found in solitary bees e.g., *Osmia* and *Megachile* spp., which are caused by different species of *Ascosphaera* (Vandenberg and Stephen, 1983; Skou and King, 1984). Flowers that that are visited by both solitary bees and honey bees can serve as a reservoir for parasite exchange, in which case honey bees could also be exposed to other *Ascosphaera* species that are parasites of solitary bees. Two species, *Ascosphaera atra* and *Ascosphaera proliperda*, were investigated in this thesis using mixed infections with *Asc. apis* (Chapter 2). Both of these species are homothallic (second mating type is not required for sexual reproduction) and they are most often found in association with solitary bees. *Ascosphaera proliperda* and *Asc. atra* separate in their own phylogenetic clades from *Asc. apis* (Fig. 2). Relatedness among parasites has been shown to be of importance for pathogen virulence, since genetically related species are predicted to have lower virulence due to reduced competition (Frank, 1996; Brown, 1999). *Ascosphaera atra* was originally isolated from the solitary bee *Megachile rotundata* (Skou and Hackett, 1979). *Ascosphaera atra* is often described as a pollen fungus associated with the pollen provisions of the solitary bee *M. rotundata* (Bissett, 1988) and honey bees (Anderson and Gibson, 1998). *Ascosphaera proliperda* can be distinguished from other *Ascosphaera* species by having relatively larger spores. This species was originally isolated from dead *Megachile centuncularis* and is a potential parasite of larvae *M. rotundata* (Vandenberg and Stephen, 1983).
**INTRODUCTION**

**Figure 2.** Phylogenetic tree showing the relationship of *Ascospaera* species (from Anderson et al., 1998). Boxes show the species studied in this thesis.

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**ASPERGILUS FLAVUS (STONEBROOD)**

*Aspergillus flavus* is a cosmopolitan filamentous fungus that occurs mostly in soil, but can be found thriving saprobiically as well as infecting numerous living hosts, including plants, insects and mammals. The current classification for *Asp. flavus* is Ascomycota; Eurotiomycetes; Eurotiales; *Aspergillus* (Bennett, 2010). The main characteristic of the genus *Aspergillus* is the aspergillum, spore-bearing structure (Machida and Gomi, 2010). The size and arrangement of the conidial heads as well as the color of the spores are important identifying characteristics (Bennett, 2010). *Aspergillus flavus* and several other species from the same genus are known as a facultative parasite that can kill adult as well as brood honey bees, causing the disease known as “stonebrood” (Gilliam and Vandenberg, 1997). Facultative parasites can colonize and produce disease in a range of hosts and they are also capable of growing and proliferating outside the host (Scheffer, 1991).
Symptoms and diagnosis. A germinating spore of *Asp. flavus* can penetrate the host larva through the gut lumen after ingestion or through the cuticle on the larval surface. This is a different mode of infection from chalkbrood, which can only invade the host once it is ingested (Fig. 3). Larvae infected with *Asp. flavus* rapidly turn into hard “mummies” covered with yellow-green conidia. Aflatoxins (which are mutagenic and carcinogenic even for humans) are produced by some *Aspergillus* species including *Asp. flavus*. Honey bee larvae are believed to be killed by aflatoxins in combination with physical damage caused by the growing mycelia (Burnside, 1930). Preliminary tests show that strains of *Aspergillus* that do not produce aflatoxins are equally capable of inducing infections in honey bees (Vojvodic et al., unpub. data) (Fig. 4). Due to the rarity of natural *Aspergillus* infections in honey bees and the fact that *Aspergillus* species are commonly found in soil, Bailey (1963) suggested that spores may induce disease only in circumstances when honey bees are stressed. Previous attempts to induce *Aspergillus* infections within a bee colony have been unsuccessful (Bailey, 1963), resulting in limited information on stonebrood disease etiology. Lowering the brood temperature for a day has resulted in reduced larval mortality from stonebrood infections, suggesting that *Aspergillus* uses different strategies to induce infections in the honey bee host compared to *Asc. apis* (Chapter 1). Still lacking is knowledge about factors that might play a role in the establishment of *Aspergillus* infections in honey bees.
Figure 4. Preliminary study showing the number of infected honey bee larvae exposed to five *Aspergillus* species / strains (n=35 larvae per treatment). Two strains of *Aspergillus flavus* induced most of the larvae mortality, whereas *Asp. bombicus* caused only a few infections. (*aflatoxin producing species/strains).

**Transmission.** The method of natural spread of the disease in honey bees is not known. Conidia of *Aspergillus* species are readily dispersed by the wind and other insects (Lillehoj et al., 1980; Holtmeyer and Wallin, 1981). The mycotoxins produced by these fungi have been isolated in small amounts from honey bee pollen grains (Gonzalez et al., 2005), suggesting a possible transmission mode.

**Isolation and cultivation.** *Aspergillus flavus* can be maintained on SDA agar but Czapek–Dox medium, which contains sucrose as the carbon source and nitrate as the nitrogen source (Raper and Fennell, 1965), is more suitable. Frequent transferring of mycelia to new plates can lead to reduced conidial production as observed previously for *Asp. flavus* (Scully and Bidochka, 2006; Vojvodic et al., unpub. data); therefore dry freezing some of the conidia might be a better choice for a longer-term storage.

**Other Aspergillus spp.** Less commonly, *Aspergillus fumigates* can also cause stonebrood disease, in which case larvae are covered with gray–green conidia (Bailey and Ball, 1991).
THE HONEY BEE – a brief overview

Natural history. The common honey bee *Apis mellifera* (Hymenoptera, Apidae), also known the European honey bee, has attracted special attention from mankind due to their honey production and crop pollination. Even though colonies readily survive independently in nature, honey bees are often referred to as domesticated insects. Their closest relatives are the orchid bees (Euglossini), the bumble bees (Bombini) and the stingless bees (Meliponinae) (Winston and Michener 1977; Kimsey, 1982). The genus *Apis* is comprised of 10 species, 9 of which are found in Asia (Arias and Sheppard, 2005), and with more than 24 geographically and morphologically distinct subspecies of *A. mellifera* (Ruttner et al., 1978; Whitfield et al., 2006). The natural range of *A. mellifera* ranges from South Africa, Asia, and from the Mediterranean to Scandinavia in Europe, but as a result of beekeeping, *A. mellifera* can be found all over the world, which has resulted in numerous subspecies (Louveaux, 1966). Honey bees, together with stingless bees, bumble bees, ants, and some wasps (all order Hymenoptera) and termites (order Isoptera) are described as being “eusocial” insects, meaning that individuals live together in colonies with cooperative care of the brood, overlapping generations, and a reproductive division of labor (Wilson, 1971).

Hymenopteran eusocial colonies are composed of reproductive individuals (queen and drones) and non-reproductive females (workers), which are often full-sisters that are on average more closely related to each other than to their own offspring. Eusociality appears to be particularly common in the haplodiploid Hymenoptera and high relatedness among females caused by haplodiploidy has been proposed to have led to favorable conditions for the evolution and maintenance of female helping behavior through kin selection (Hamilton, 1964). However most authors now focus on other ways that haplodiploidy may favor the evolution of eusociality (reviewed by Linksvayer and Wade, 2005).

Honey bee colonies are composed of several thousands of workers headed by a single queen. Virgin honey bee queens stand out among the social insect due to a strikingly high mating frequency (7-41 mates, reviewed by Tarpy and Nielsen, 2002). While a high level of polyandry results in low relatedness among workers and potentially leads to within-colony
conflicts over resource allocation, colonies with increased within-colony genetic diversity tend to have higher overall colony performance (Mattila and Seeley, 2007). Many hypotheses have been suggested to explain benefits of increased genetic variation that results from polyandry. Greater intracolonial genetic diversity has been hypothesized to improve division of labor (Mattila and Seeley, 2007), and make colonies more resistance to parasites (Boomsma and Ratnieks, 1996; Liersch and Schmid-Hempel, 1998; Tarpy, 2003; Palmer and Oldroyd, 2003; Seeley and Tarpy, 2007).

All caste members of hymenopteran societies go through holometabolous development that includes four major developmental stages: egg, larva, pupa, and adult (Fig. 5). Honey bee queens lay fertilized eggs that develop into females (workers or queens) or unfertilised eggs that develop into drones. After approximately 3 to 4 days the egg membrane dissolves and the embryo is exposed. This process of egg membrane dissolving has only been observed in honey bees, whereas other insects hatch by rupturing the egg membrane (Du Praw, 1961). The size and the incubation time of eggs usually depend on the race of the honey bee (Collins, 2004). At this point there are no known parasites found attacking honey bee eggs.

![Figure 5. Four major stages of honey bee development from left to right: egg, larva, pupae, adult (Adapted from Winston, 1991).](image)

During the first 4 molts, the cells with the larvae are open and larvae are continuously fed by nurse bees. The secretions produced by nurse hypopharyngeal and mandibular glands is mixed with honey, digestive enzymes, and water, and fed to the larvae. During this period larvae grow to be 900 times the weight on an egg. In the last days of larval development the cells are sealed and the feeding stops (Winston, 1991). Larvae are affected by numerous parasites such as viruses (Chen et al., 2006; Ribièr et al., 2008; Chen and Seide, 2007), fungi...
(Aronstein and Murray, 2010; Fries 2010), bacteria (Genersch et al., 2005; Forsgren et al., 2005; Forsgren, 2010; Genersch, 2010) and mites (Anderson and Trueman, 2000; Anderson and Morgan, 2007). Most of the larval body cavity is occupied by the midgut, which is not connected to the hindgut, resulting in the entire waste collection at the posterior end of the midgut (Fig. 6). Parasites usually invade larvae after they are ingested. Furthermore the midgut wall lacks ectodermal lining and it is easily penetrated by parasites (Schmidt-Hempel, 1998). The developing larvae molt six times, on average every 24 hours.

![Diagram of honey bee larva anatomy]

**Figure 6.** External and internal anatomy of honey bee larvae. The midgut and the hindgut are not connected during the larval stage and all the waste is expelled prior to pupation (Adapted from Winston, 1991).

Just prior to cocoon construction the larvae defecate and after the last molt they metamorphose into pupae. The pupal stage for the worker larvae lasts between 8-9 days, during which time the pupal cuticle becomes darker, and these color changes can be used to determine pupal age (Jay, 1962). Generally for the European honey bee it takes 21 days from egg laying until adult emergence, with the life span for an adult worker bee ranging from a few weeks to a year, depending on seasonal factors, food availability, function and race of the bee (Fukuda and Sekiguchi, 1966). Adult worker bees obtain their nutrition mostly from honey, or nectar in some cases. Protein in the form of pollen has also been shown to be required during the first 8-10 days of adult life (Maurizio, 1950) and the type of pollen seems to be of importance, as well as the diversity of protein nutrition that has a
positive impact on honey bee immunity (Alaux et al., 2010). Worker bees perform different tasks during their life time, usually depending on their age (i.e. age polyethism Seeley, 1985).

Queens differ markedly from workers in morphological and physiological features and have contrasting reproductive capabilities, and very different behavioral repertoires. Honey bee queens generally survive 1-3 years, compared to workers living for several weeks to several months depending on the season (Winston 1991). Genetically similar female larvae develop into workers and queens, depending on the quantity and quality of nutrition provided by nurse workers, which affects larval endocrine response and patterns of gene expression (Wheeler, 1986; Kucharski, et al., 2008). Characteristics of the colony such as the relatedness among workers, division of labor, and worker longevity are important factors when considering the spread of parasites in the colony as well as host resistance.

**In vitro rearing of honey bee larvae.** Due to the difficulties of rearing honey bee larvae in *vitro*, in the past four decades *Asc. apis* epidemiology has been mostly studied at the hive level, an indirect approach that has led to somewhat ambiguous results (e.g. Gilliam et al., 1978; Vandenberg and Shimanuki, 1987; Puerta et al., 1994; Flores et al., 2004). One of the most common problems in conducting *in vitro* bioassays is damage to the larvae during collection from cells and during transfer from an old to a new plate. The larvae used in this thesis were removed from the hive when they were 24 to 36 hours old. During removal it was ensured that the environment surrounding the larvae was moist by performing the larval removal in a humid environment or by placing damp cloth over the frames. Directly after removal, each larva was placed in an individual cell of a 48-well tissue culture plate with 10 μl of larval diet, consisting of 50% freshly frozen royal jelly (v/v) (Sonnentracht Imkerei GmbH, Bremen, Germany), 6% D-glucose (w/v), 6% D-fructose (w/v), 1% (w/v) yeast extract and sterile deionized water. The larvae were fed once a day with 20 μl diet on the first day, 30 μl on days 2-3, and 40 μl on day four after grafting. With this feeding regime no additional transfer of the larvae was necessary, so that mortality due to injuries was avoided. When the larvae started to defecate (after molting to the 5th instar) the wells were gently cleaned using cotton wool. Tissue culture plates with the larvae were stored in a humid chamber with saturated potassium-sulfate solution at 34 °C in constant darkness.
INTRODUCTION

EVOLUTION OF VIRULENCE

Temperature and host-parasite interactions. Much work has been done on the effects of temperature on the physiology and behavior of animals, but how temperature affects the interactions between organisms has been somewhat neglected (Thomas and Blanford, 2003). Temperature can have an important role in the outcome of host-parasite interactions, as has been demonstrated in numerous studies particularly in applied research. For example, mortality of locust and grasshoppers when exposed to Beauveria bassiana and Metarhizium anisopliae var. acridium depends on temperature (Inglis et al., 1996; Blanford and Thomas, 2000), M. anisopliae efficiency to kill beetles Strophosoma spp. under laboratory and fluctuating field conditions (Nielsen et al., 2006), and thermal effects on parasite virulence have been recorded for Entomophaga maimaiga against larvae of gypsy moths (Hajek et al., 1993). The effect of temperature can be expected to be minimal if the host and the parasite share a similar temperature range, however if the optimal temperature for the host and the parasites differ the temperature effect can be expected to be greater (Fig. 7) (Blanford et al., 2003).

The characteristics and mechanisms of thermoregulation in honey bee colonies have been intensely studied (e.g. Jones et al., 2004; Jones et al., 2005; Jones and Oldroyd, 2007), and it has been shown that honey bees maintain brood nest temperature in a narrow range between 33 and 36 °C (Winston, 1991). Although temperature fluctuations within the colony are not common, the temperature can drop to extreme lows of 18 °C (Cooper, 1980); especially in small colonies (Seeley, 1974). Short periods of cooling of honey bee brood has been shown to increase the prevalence of chalkbrood infections (Chapter 1), possibly by reducing the survival of the host larvae due to delayed development, allowing extra time for Asc. apis spore germination prior to the return of warmer temperatures in the hive. For the facultative parasite Asp. flavus both host mortality and vegetative mycelial growth within the host were reduced when larvae were chilled for a day (Chapter 1). Rapid growth and fast host killing is what could be expected from a rare facultative parasite, such as Asp. flavus, that is unlikely to meet resistance from an adaptive immune response. In addition, cooler temperatures by themselves do not enhance mycelial growth of Asc. apis (Fig. 8), therefore, the optimal temperatures for successful infection and maximal mycelial growth
are different for *Asc. apis*. This phenomenon has also been observed for other entomopathogenic fungi, such as *Ascosphera aggregate* infecting the alfalfa leafcutting bee *M. rotundata* (James, 2005), *M. anisopliae* var *acrididum* infecting locusts (Ouedraogo et al., 1997), and *B. bassiana* infecting ladybird beetle larvae (James et al., 1998). One should keep in mind when studying temperature effects on host-parasite interactions that these interactions are complex and the outcome might not be immediately predictable. Parasites use different strategies when colonising the host and one set of conditions might not be optimal for all parasite species as has been observed for *Asp. flavus* and *Asc. apis* infections in honey bees.

![Diagram](image)

**Figure 7.** Ectotherm performance (e.g. immunity, growth) curves of host and parasite with some degree of separation (e.g. host performance, solid line, is optimized at a higher temperature than that of the parasite, dotted line; Adapted from Thomas and Blanford, 2003).
Mixed infections. Clearly when studying host-parasite interactions, the environment (e.g., temperature) matters, as does the presence of other parasites. Often it has been shown that mixed infections of two or more parasites result in higher virulence than single infections (Brockhurst et al. 2003; Read and Taylor, 2001), possibly due to increased parasite densities (Choisy and de Roode, 2010), or due to the host immune system being less efficient in clearing up mixed compared to single infections (e.g. de Roode et al., 2003). Co-infecting parasites can act independently, synergistically, or antagonistically, but these interactions are often influenced by the environmental conditions, and the order of the infection, as shown by Thomas et al., (2003) and de Roode et al., (2005). The outcome of parasite interactions can result in under-exploitation of the host and a reduction in virulence, possible as a result of cooperation between parasites when benefits are shared (Chao et al., 2000; Schjørring and Koella, 2003). In some cases the virulence of the mixed infection follows the virulence of the most virulent strain or species (e.g. Thomas et al., 2003; Hughes et al., 2004, Wargo et al., 2007; André and van Baalen, 2007). Preliminary tests have shown that mixed infections of chalkbrood (Asc. apis) and stonebrood (Asp. flavus) follow the infection of the most virulent parasite, in this case the facultative parasite Asp. flavus (Fig. 9).
Figure 9. Preliminary results showing the proportion of infected honey bee larvae with chalkbrood parasite (*Asc. apis*), stonebrood parasite (*Asp. flavus*), equal combination of the two parasites (mixed) and the control (non-infected). Bars represent SEMs.

Virulence theory predicts that mixed infections with genetically closely related parasites result in lower virulence due to reduced competition; whereas genetically distant parasites are expected to compete for resources inside a host, causing rapid exploitation and development of higher virulence (Frank, 1996; Brown, 1999; Griffin et al., 2004). The intricate dynamics of mixed infections are not reserved to only parasites that harm their host (Chapter 2). *Ascosphaera proliperda* and *Asc. atra* were previously known as avirulent fungi, but in the mixed infection experiment with *Asc. apis* have lead to some interesting results. In the case of the avirulent pollen fungus *Asc. atra* host mortality was higher compared to single infections with *Asc. apis*, and the presence of the causative agent of chalkbrood in solitary bees, *Asc. proliperda*, had no effect on the virulence of the mixed infections as measured by honey bee host survivorship (Chapter 2).

Empirical studies have shown that honey bee colonies resistant to chalkbrood also have symbiotic microbes (e.g. yeast) in the bee bread (Gilliam et al., 1988) that can potently suppress chalkbrood outbreak. Previously *Aurobasidium pullulans* yeast has been isolated from the honey stomachs of three bee species (*Apis, Megachile* and *Osmia*) (Batra et al., 1973), and suggested to have an antagonistic effect on the chalkbrood parasite (Johnson et
Using *Aur. pullulans* in mixed infections with chalkbrood I have shown that even though *Aur. pullulans* yeast showed inhibitory properties against chalkbrood on the agar plates, the mixed and sequential infections with the *Asc. apis* showed no significant reduction in mortality of honey bee larvae when using the same amount of spores as the *Asc. apis* parasite (Wald = 3.1908; p = 0.202) (Vojvodic unpub. data).

DEFENCES AGAINST FUNGAL INFECTIONS

An exceptionally diverse assembly of parasites has indeed been described for honey bees suggesting that honey bee colonies face considerable risks of reduced productivity and colony failure (Morse and Flottum, 1997; Schmid-Hempel, 1998; Cox-Foster et al., 2007). Disease pressure on social insects has led to multiple resistance responses such as innate immunity, social fever, and extensive hygienic behavior (Cremer et al., 2007, Wilson-Rich et al., 2009). Fungal infections are usually followed by host defenses that result in upregulation of antimicrobial peptides, melanization, cellular phagocytosis and encapsulation (Gillespie et al., 2000), with the last two being the most common defense mechanisms against entomopathogenic fungi in bees (Glinski and Buczek, 2003). Parasites, such as *Asp. apis* that can generate novel strains through sexual reproduction might have an advantage over the host immune system, relative to less genetically variable pathogens. However obtaining optimal virulence can be inhibited by increasing the host genetic diversity for resistance, assuming that susceptibility to parasites is genetically variable (Boomsma and Ratnieks, 1996; Liersch and Schmid-Hempel, 1998).

Differences in the genetic background of honey bee larvae within colonies due to polyandry can be expressed as variation in host immune system, potentially resulting in variation in susceptibility to infections, susceptibility to pathogen replication, or pathogen tolerance. While high levels of polyandry result in low relatedness among workers and potentially lead to within-colony conflicts over resource allocation, colonies with increased within-colony genetic diversity tend to have higher overall colony performance (Mattila and Seeley, 2007). In addition, host populations with high genetic diversity are predicted to have lower pathogen prevalence than populations with lower diversity (Schmid-Hempel, 1998), as has
been observed when exposing honey bees to both chalkbrood (Tarpy 2003) and American foulbrood (Rothenbuhler and Thompson, 1956; Palmer and Oldroyd, 2003; Seeley and Tarpy, 2007). In fact, honey bees infected with spores from dead larvae, potentially harboring numerous chalkbrood strains, showed patriline-specific resistance to chalkbrood infections (Invernizzi et al., 2009). Recently it has been shown that heterogeneous ant colonies are better in the performance of allogrooming and removal of larvae infected with fungal parasite than homogenous colonies, suggesting that host genetic diversity can play an important role in the social immunity of the colony (Ugelvig et al., 2010). The relevant genetic variation in the honey bee host for chalkbrood disease resistance exists (Chapter 3). The experimental protocols to measure disease resistance are available and could be combined with queen breeding programs in which mating is controlled either naturally or artificially.

Social insects have evolved specific behaviors that involve collective anti-parasite defenses such as hygienic behavior in honey bees (Cremer et al., 2007). Hygienic behavior is a type of general nest hygiene that was originally defined as the ability of honey bees to detect and remove brood infected by American foulbrood from the nest (Woodrow and Holst, 1942; Rothenbuhler, 1964). Hygienic behavior has been shown to be an important defense also against chalkbrood disease (Gilliam et al., 1988). Within the colony individual bees show variation in the detection threshold necessary for the performance of hygienic behavior, which has been shown to be influenced by a number of different loci (Oxley et al., 2010). Early detection of diseased brood is critical for resistance, and colonies that detect and remove infected brood within the first 24-48 hours, before the parasite sporulate, are considered 'hygienic' (Arathi et al., 2000; Arathi et al., 2006; Arathi and Spivak, 2001; Gramacho and Spivak, 2003). Detection of diseased larvae is based on olfactory stimuli (Spivak et al., 2003), with one of the compounds recently being isolated from chalkbrood infected larvae (Swanson et al., 2009). Similar studies could be performed for the olfactory cues that can elicit hygienic behavior in larvae parasitized other fungal diseases such as stonebrood.

Honey bees maintain a homeostatic environment within the colony (Seeley 1985), and can to actively elevate the brood temperature for accelerated larvae development (Milum, 1930). Elevated temperature in the brood area can potentially serves as a defense
mechanism against chalkbrood disease – the so-called social fever (Starks et al., 2000). Even a slight increase in temperature above the parasite optimum can result in drastically slower parasite growth (Starks et al., 2000). Social fever has been observed in other insect systems as a defense against fungal parasites (Kluger, 1991; Elliot et al., 2002). Elevated temperatures may also increase the host's immune system by phagocytosis (Ouedraogo et al., 2003). However, the higher temperatures are not detrimental for the chalkbrood or stonebrood parasites, and can only potentially slow the mycelial growth (Fig. 8). Given that honey bee larvae are not susceptible to Asc. apis infections after pupation, induced fever could be a very effective way to overcome the diseases, but furthered investigations are warranted.
REFERENCES


INTRODUCTION


INTRODUCTION


INTRODUCTION


