



Research article

Temperature dependent virulence of obligate and facultative fungal pathogens of honeybee brood

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ABSTRACT

Chalkbrood (*Ascospaera apis*) and stonebrood (*Aspergillus flavus*) are well known fungal brood diseases of honeybees (*Apis mellifera*), but they have hardly been systematically studied because the difficulty of rearing larvae *in vitro* has precluded controlled experimentation. Chalkbrood is a chronic honeybee-specific disease that can persist in colonies for years, reducing both brood and honey production, whereas stonebrood is a rare facultative pathogen that also affects hosts other than honeybees and can likely survive outside insect hosts. Hive infection trials have indicated that accidental drops in comb temperature increase the prevalence of chalkbrood, but it has remained unclear whether virulence is directly temperature-dependent. We used a newly established *in vitro* rearing technique for honeybee larvae to test whether there are systematic temperature effects on mortality induced by controlled infections, and whether such effects differed between the two fungal pathogens. We found that increasing spore dosage at infection had a more dramatic effect on mortality from stonebrood compared to chalkbrood. In addition, a 24 h cooling period after inoculation increased larval mortality from chalkbrood infection, whereas such a cooling period decreased mortality after stonebrood infection. These results raise interesting questions about honeybee defenses against obligate and facultative pathogens and about the extent to which stress factors in the host (dis)favor pathogens with lesser degrees of specialization.

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1. Introduction

Social insects express multiple resistance responses to diseases such as innate immunity, social fever, and hygienic behavior (Cremer et al., 2007; Wilson-Rich et al., 2009), but many diseases still persist as chronic infections that can be managed when colonies are in good condition, but not eliminated. Chalkbrood is such a chronic disease caused by honeybee larvae consuming spores of *Ascospaera apis* (Ascomycota). Low hive temperatures and high humidity apparently increase the risk of chalkbrood outbreaks (Flores et al., 1996), similar to fluctuating

temperature influencing other host–pathogen interactions in bees (James, 2005) and other insects (e.g., Blanford et al., 2003; Stacy et al., 2003; Vojvodic and McCreadie, 2007). More particularly, a temperature drop in the 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 *A. apis* resembles normal hive temperatures of 33–36 °C (Bamford and Heath, 1989; Jones et al., 2004).

While obligate pathogens such as chalkbrood are likely to have adapted to exploiting honeybee hosts, this is less obvious for inadvertent Ascomycete *Aspergillus* pathogens. Although natural *Aspergillus* infections are rare, their mycelia are known to kill honeybees in all stages of development (Gilliam and Vandenberg, 1997). Most well known are *Aspergillus flavus* (stonebrood) infections, which

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turn honeybee larvae into hard mummies, but this disease is relatively rare, suggesting that spores are only infective when hives experience an unusual combination of stress factors, which would explain why experimental *A. flavus* infections of honeybee colonies have been unsuccessful (Bailey, 1963).

Because host mortality from both pathogens is correlated with spore concentration and subsequent hyphal mass, they are good models to study the growth dynamics of infections (Ebert and Weisser, 1997). We exploited a recently developed *in vitro* rearing procedure for honeybee larvae to establish fully controlled experimental infections with chalkbrood (Jensen et al., 2009) and extended this protocol to stonebrood. We used these experiments to obtain dosage–response mortality curves for both diseases across a range of spore concentrations. Finally, we tested the degree to which infection-induced mortality rates changed after lowering the ambient temperature during a critical period of larval development.

2. Materials and methods

2.1. Maintenance of cultures and inoculum preparation

Multiple strains of *A. apis* (USDA-ARS Collection of Entomopathogenic Fungal Cultures in Ithaca, New York, USA, ARSEF 7405 and ARSEF 7406) were isolated from *Apis mellifera* collected in Weslaco, TX, USA. Stock cultures were maintained on Sabouraud Dextrose Agar (SDA) at 25 °C with monthly transfer to new plates. Since *A. apis* is a heterothallic fungus, each strain was maintained on an individual plate. Hyphal subcultures from each of the two mating types were transferred onto one plate three weeks prior to the experiment to allow sexual reproduction and spore formation. Spores were removed from the plate with a small sterile spatula and placed into a sterile glass grinder with 20 µl of sterile deionized water. Following the grinding, 50 µl of sterile deionized water was added to the spore suspension. Large particles in the suspension were allowed to settle for 20 min, after which a sample of approximately 50 µl was taken from the middle of the suspension. Spore concentration in the resulting suspension was determined with a hemocytometer (Tiefe Depth Profondeur, Marienfeld, Germany) to achieve dosage suspensions of 2.0×10^6 , 1.0×10^6 , 5.0×10^5 , and 1.0×10^5 spores/ml. New spore solutions were made prior to each experiment.

A. flavus was isolated from a single infected honeybee larva that appeared in another *in vivo* experiment at the University of Copenhagen. The identity of this fungal species was confirmed with PCR-ITS sequence analysis and by secondary metabolite production using high performance liquid chromatography (HPLC: Courtesy of J. Frisvad, Danish Technical Univ., Denmark). Stock cultures of *A. flavus* were maintained on SDA agar with monthly transfer to fresh plates. Spores used for inoculation of the honeybee larvae were taken from three week old subcultures. Spores were removed from the plates with a Drigalski spatula and suspended in deionized water, after which spore suspensions were shaken and mycelia fragments were allowed to settle for 15–20 min. Spore

concentration was determined with a hemocytometer to achieve dosage suspensions of 2.0×10^6 , 1.0×10^6 , 5.0×10^5 , and 1.0×10^5 spores/ml. New spore solutions were made prior to each experiment.

2.2. Growth of fungal pathogen cultures and spore viability

A temperature curve for vegetative growth of *A. apis* and *A. flavus* was determined by measuring the rate of increase in diameter of fungal colonies kept at constant temperatures (20, 27, 34 and 38 °C in the dark). Plugs of each fungus were removed from an established culture with a 7.5 mm diameter cork borer and transferred to the center of 9 cm diameter Petri dishes containing SDA for *A. apis* and Czapek Yeast Extract (CYA) for *A. flavus* cultures. Four replicated plates were set up for each temperature/pathogen combination. Petri dishes were sealed with parafilm and kept inverted for the duration of the experiment. Vegetative growth was measured every day starting on day 2 and ending on day 7. Two diametrically opposite lines were drawn across the bottom of each Petri dish crossing over the inoculum plug. The colony diameter was measured along these lines, with the mean covered distance along the two lines giving an estimate of colony size. Colony diameter was plotted against the incubation period to calculate the average growth rate (mm growth per day) over a period of 5 days at each temperature.

Spore viability for *A. apis* was tested following the protocol of James and Buckner (2004) with a few modifications. Spore suspensions were made with 2.0×10^7 spores/ml mixed with 150 µl GLEN, a liquid medium suitable for germination and *in vitro* growth of insect pathogenic fungi (Beauvais and Latgé, 1988; Jensen et al., 2009). Droplets of 10 µl of this mixture were placed on each of three spots of a sterile six-well Teflon coated slide, which was then placed in a sterile Petri dish lined with wet filter paper. Each Petri dish was subsequently placed in an airtight container and flushed with CO₂ to ensure spore activation and germination as suggested by Heath and Gaze (1987). The containers were incubated for 24 h at 34 °C, after which the sterile Teflon coated slide was covered with cover-slip and the germination percentage was determined using differential interference contrast microscopy at 400× magnification. One hundred spores were evaluated for enlargement or germ tube formation in three different randomly chosen fields of view. The spore germination rate for *A. apis* ranged from 70 to 100%. The spore viability protocol for *A. flavus* conidial germination was adapted from Nielsen et al. (2006) and produced germination rates of 94–100%.

2.3. Larval rearing and inoculation

Honeybee larvae were obtained from an apiary located at the University of Copenhagen, Denmark. The colonies were checked regularly and were free of any noticeable brood and adult bee diseases. For each experiment larvae were transferred from the three hives and reared *in vitro* following the protocol of Aupinel et al. (2005) and Jensen et al. (2009) with a few modifications. Larval age was estimated by size (Brødsgaard et al., 1998), and larvae

slightly older than 24 h were taken from the comb using a Swiss grafting tool (Swienty, Sønderborg, Denmark). Directly after removal each larva was placed in an individual cell of a 48-well tissue culture plate with 10 μ l 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 diet had been made prior to the experiment when it was mixed and frozen in smaller aliquots that could be pre-heated to 34 °C just before being used for feeding. The larvae were fed once a day with 20 μ l diet on the first three days, and 40 μ l on day four. With this feeding regime no additional transfer of the larvae was necessary, so that mortality due to injuries was avoided. Tissue culture plates with the larvae were stored in a humid chamber (to avoid larval dehydration) at 34 °C in constant darkness. For the experiments where the effect of a day with low temperature was tested, the incubation temperature was reduced to 27 °C for 24 h on the second day after inoculation, after which plates were returned to the 34 °C incubators for the remaining experiment. When the larvae started to defecate (after molting to the 5th instar) the wells were gently cleaned using cotton wool.

Two days before the start of each experiment approximately 150 larvae were removed from each of the three hives and reared *in vitro* as described above. After 48 h acclimatization period 24 healthy larvae were fed 5 μ l of a designated spore suspension of each pathogen or distilled water as controls. For each treatment group (i.e. 2.0×10^6 , 1.0×10^6 , 5.0×10^5 , 1.0×10^5 spore/ml and distilled water) three groups of bees were used (one from each of the three hives), giving a total of 1080 larvae examined (i.e. 24 larvae \times 3 replicates \times 3 hives \times 5 dosages). Within a period of one day, the larvae ate all the diet including the spores, so that no spores remained in the wells that could have given later infection. During the daily microscopic examination larvae were removed from the incubator and kept on a pre-heated plate at which time the numbers of diseased, surviving, and infected larvae were recorded. Larvae were assessed as dead when they had ceased respiration, had lost body elasticity, or had changed color to gray or brown. Larvae were assessed as dead because of infection when fungal hyphae were observed on the cuticle. Died larvae without any visual presence of hyphae was examined again the following day, and if hyphae were then observed protruding through the host cuticle, these larvae were later added to the category of dead from disease. Susceptibility of honeybee larvae to *A. apis* has been shown to occur during the larval and prepupal stages (Gilliam et al., 1978; Bailey, 1967), but not in the pupal stage, so we terminated our experiments after Day 6, i.e. just before pupation.

2.4. Statistical analysis

Fungal *in vitro* growth rates were estimated by taking the mean daily growth for the period of Day 2 to Day 6. The relationship between culture growth and temperature was evaluated using a generalized linear model (Proc GLM, SAS ver. 9.1). To assess differences within each temperature

treatment, pair-wise *t*-tests (proc GLM) were done using the associated least squares means.

The effect of dose and colony of origin was analyzed using a Kaplan–Meier survivorship analysis (PROC LIFEST, SAS ver. 9.1) to estimate the effect of pathogen dosage on virulence and infection rate, as done previously by Jensen et al. (2009). Wilcoxon statistics was as used to compare the survivorship curves for all pairs of dosages. Finally, mean times to death were compared among pathogen species, temperature regimes, spore dosages, and host colonies, the first three as fixed factors and colony as random factor. Two-way interaction terms were analyzed as well, except for “colony” which appeared not to have an effect on mortality.

3. Results

3.1. *In vitro* hyphal growth

Temperature had a significant overall effect on the hyphal growth in agar medium ($F_{1,28} = 15.45$, $P = 0.0005$), but the effect differed between the two species ($F_{1,28} = 4.8$, $P = 0.0369$). Growth rates peaked at 34 °C for both pathogens (Fig. 1). The non-significant interaction between temperature and species ($F_{1,27} = 0.38$, $P = 0.5439$) indicated that the shape of the temperature–growth relationship was essentially the same for the two species (Fig. 1). *T*-test showed that differences in growth of *A. apis* cultures were significant for all four temperature treatments (all $P < 0.0275$). The same was true for growth of *A. flavus* cultures (all $P < 0.0001$), except for the middle two temperatures (27 °C and 34 °C) for which growth rate differences were only marginally significant ($P = 0.0589$).

3.2. Dosage respond and larval mortality

Larval mortality covaried with both temperature regime and pathogen dose, although the significance of the mortality curves varied somewhat for each combination of pathogen and temperature regime (Fig. 2). Even

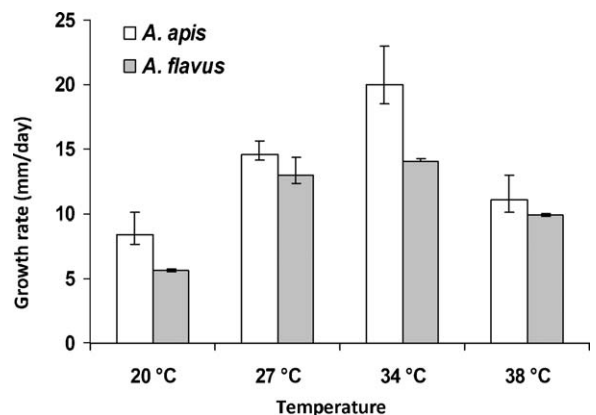


Fig. 1. Hyphal growth rates (averaged over 6 days \pm standard error) on agar plates for *Ascosphaera apis* (white bars) and *Aspergillus flavus* (grey bars) at different temperatures.

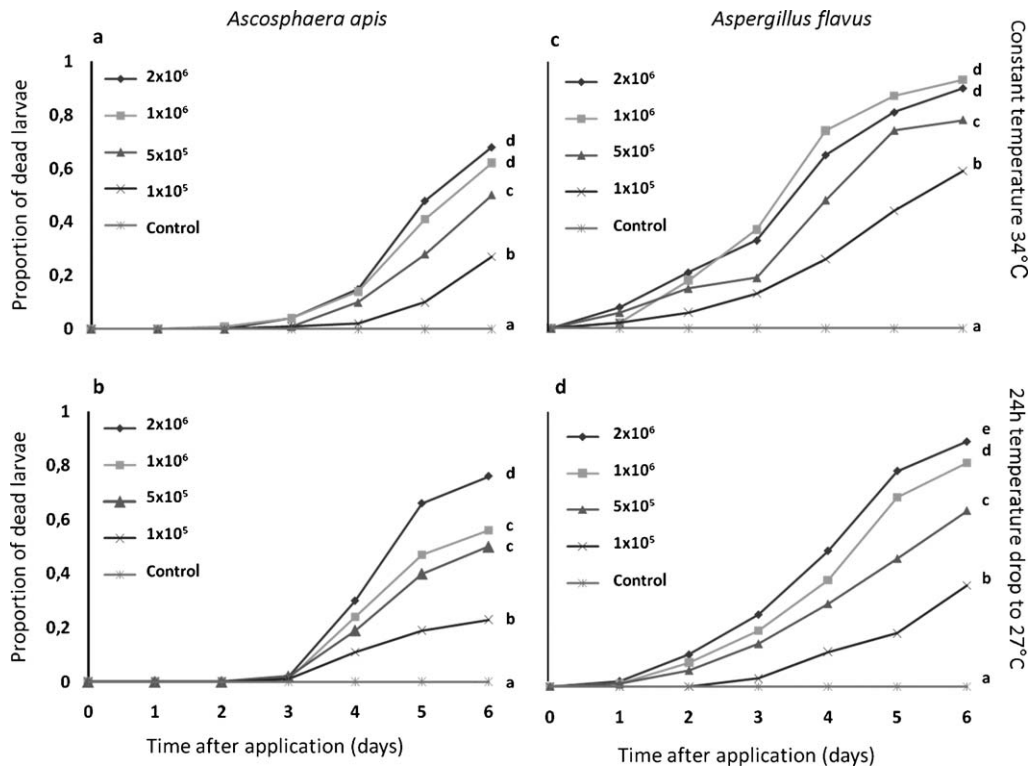


Fig. 2. Proportion of honeybee larvae dead from infection with different dosages of spores during the six days of the experiment: (a) *A. apis* at a constant temperature of 34 °C; (b) *A. apis* after a drop in temperature to 27 °C for 24 h on day 2; (c) *A. flavus* at constant temperature of 34 °C, and (d) *A. flavus* after a drop in temperature to 27 °C for 24 h on day 2. Data for control larvae treated with distilled water are also shown. Different letters indicate dosages whose survival distribution differed significantly at $P < 0.05$ (PROC LIFETEST).

after infection with the two highest spore dosages ca. 10% of the larvae infected with *A. flavus* never became diseased, whereas this figure was ca. 30% for *A. apis*. Similar differences between the two pathogens were found for the lower dosages, where an increasing number of larvae did not become diseased after infection. Although there was a clear tendency for *A. apis* infected larvae to die faster when experiencing a 24 h cooling period to 27 °C on Day 2, this difference was only significant at the highest spore dosage (Wilcoxon test $\chi^2 = 11.82$; d.f. = 1; $P < 0.0006$) when analyses were done separately for each dose. However, larvae inoculated with *A. flavus*, displayed significant differences in mortality between the two

Table 1

Results of a four-way ANOVA comparing the mean time to death of honeybee larvae infected with four spore concentrations (dosage) of *A. apis* or *A. flavus* (pathogen), under constant temperature (34 °C) or with a 24 h cooling period to 27 °C (temperature regime).

Independent variables	F-value	d.f.	P
Pathogen	218.51	1	0.000
Dosage	49.94	3	0.000
Colony	1.87	2	0.170
Temperature regime	9.64	1	0.004
Pathogen × dosage	7.93	3	0.000
Pathogen × temperature regime	39.98	1	0.000
Dosage × temperature regime	1.22	3	0.318

Colony of origin ($n = 3$) was added as a random factor, but interactions with colony were not analyzed.

temperature regimes for all spore dosages (Wilcoxon test range $\chi^2 = 5.04$ to 36.28; for all tests included $P < 0.0247$) and the effect of temperature regime was opposite.

Essentially the same results could be obtained by focusing the analysis on time to death as response variable, which allowed a 4-way ANOVA (Table 1). This showed that colony of larval origin did not have a significant effect on the overall mortality of larvae ($F_{2,33} = 1.87$, $P = 0.170$), allowing us to ignore this factor and its possible interaction terms with the other factors. Spore dosage had a significant overall effect ($F_{3,33} = 49.94$, $P < 0.0001$) (Table 1) and the same was true for pathogen species ($F_{1,33} = 218.81$, $P < 0.0001$) and temperature regime ($F_{1,33} = 9.64$, $P = 0.004$). This indicates that mean time to death is significantly shorter for successful stonebrood infections compared to successful chalkbrood infections and that a 24 h temperature drop tended to delay pathogen reproduction in both cases. However, the biologically most interesting results emerged from the interaction terms of the analysis. The dosage × temperature regime interaction term was not significant ($F_{3,33} = 1.22$, $P = 0.318$), indicating that the dosages had comparable mortality effects across the two temperature regimes. However, the pathogen × dosage interaction was significant ($F_{3,33} = 7.93$, $P < 0.0001$) confirming that stonebrood death rates respond more clearly to dosage than chalkbrood death rates (see also Fig. 2). In addition, the pathogen × temperature regime interaction was highly significant

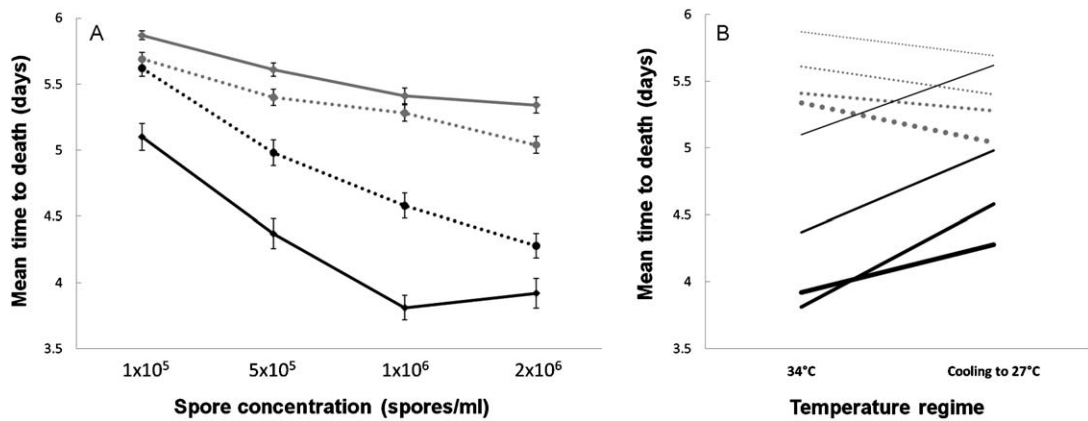


Fig. 3. Graphical illustration of the significant interaction terms in the ANOVA in time to death after infection (Table 1). (A) The pathogen × dosage interaction showing the *A. apis* curves at 34 °C (grey dots connected by solid lines) and after a 24 h temperature drop to 27 °C (grey dots connected by dotted lines), and the *A. flavus* curves at 34 °C (black dots connected by solid lines) and after a 24 h temperature drop to 27 °C (black dots connected by dotted lines). (B) The pathogen × temperature regime interaction showing the consistent decrease of time to death in *A. apis* (grey dots connected by dotted lines increasing in thickness with the four spore concentrations) and in *A. flavus* (black dots connected by solid lines increasing in thickness with the four spore concentrations). Bars are SEMs.

($F_{1,33} = 39.98$, $P < 0.0001$) indicating that this differential response of the two pathogens was much more important than the overall effect of temperature regime on time to death. The significance of the two interaction terms are illustrated in Fig. 3: the steeper response of stonebrood mortality to increasing spore dosage (Fig. 3a) and the opposite effects of the temperature cooling regime, reducing mean time to death after chalkbrood infection and increasing mean time to death after stonebrood infection (Fig. 3b).

4. Discussion

Our study shows that *in vitro* experimental procedures for rearing honeybee larvae are suitable for testing virulence effects in both stonebrood and chalkbrood. While natural *A. flavus* infections are relatively unsuccessful, we found that artificial infections develop much faster and from a lower spore dosage than in *A. apis*. Finally, we obtained the interesting result that the two pathogens react in opposite manners to a 24 h cooling period, even though their virulence (the number of killed host larvae), was similarly dependent on pathogen concentration. As the proportion of diseased larvae was higher for *A. flavus* than *A. apis* for every spore dosage, this may imply that honeybee larvae may have evolved more resistance to infections with the obligate pathogen *A. apis* than to infections with the facultative pathogen *A. flavus*. However, the rarity of stonebrood probably also makes this pathogen less effective in entering hives and building up sufficient spore masses to be infective, underlining the likely importance of the different life histories of these pathogens.

4.1. *Ascosphaera apis*—a specialized obligate pathogen of honeybee brood

A. apis is lethal for honeybee larvae regardless of rearing temperature, demonstrating that this disease is not just an

opportunistic pathogen as suggested previously (Bailey, 1967; Heath, 1982; Puerta et al., 1994; Flores et al., 1996), but a real threat provided it obtains optimal growth conditions. In our study larvae became infected four days after exposure to chalkbrood spores, confirming observations by Gilliam et al. (1978). The first signs of infection could occasionally be later when prepupae were sealed in their cells, particularly after a short cooling period, as observed by Bailey (1967). These late infections probably reinforce spore production and dispersal within hives, increasing the disease pressure. We found that cool temperatures by themselves do not enhance mycelial growth of *A. apis* (Fig. 1), and that the optimal temperature for mycelial growth on agar was similar to the average temperature in a honeybee hive (~34 °C) (Bamford and Heath, 1989). This implies that the optimal temperature for successful infection and maximal mycelial growth are different.

4.2. *Aspergillus flavus*—an opportunistic, facultative, but deadly pathogen of honeybees

Before we embarked on our study, only a single stonebrood study was available investigating aflatoxin production by two *Aspergillus* species (Gunst et al., 1978) and lacking the pathogen etiology in honeybee host. We show that once infection has taken place in honeybee larvae, stonebrood actually has a high virulence and rapid development towards spore transmission, which makes it somewhat puzzling that this disease is not more common. In contrast to *A. apis*, *A. flavus* has the advantage of being able to infect hosts both *per os* via the larval gut and by penetrating the cuticle from the outside. However, the latter pathway did not play a role in our study, because we directly fed the spores to the larvae and the first signs of mycelia were always observed on the cuticle of the posterior dorsal region of the larvae, similar to chalkbrood infections. Host mortality and mycelial growth of *A. flavus* were reduced when larvae were chilled for a day, although

average mycelial growth on agar plates was not significantly reduced at 27 °C compared to 34 °C (this was also true for *A. apis*). The higher mortality of larvae at 34 °C is unlikely to be due to increased aflatoxin production since temperatures above 30 °C are known to result in low or no aflatoxin production (O'Brian et al., 2007).

4.3. Comparing life history adaptations in *Ascosphaera apis* and *Aspergillus flavus*

As the obligate pathogen *A. apis* is rarely confronted with competing stains of *A. flavus*, its reproductive strategy may not be adapted to competition, but primarily to securing as many host resources as possible even if this would mean killing the host somewhat later. It is interesting that spores of *A. apis* produced on agar plates require several weeks to mature and that spores younger than three weeks failed to kill honeybee larvae when fed to them in similar experiments as reported here (Vojvodic et al., unpublished data). Possibly, the innate immune system of honeybee larvae is able to mount variable amounts of resistance to *A. apis* infections, so that it takes longer to overwhelm these defenses than those against a rare opportunistic pathogen like *A. flavus*. Whatever limits the efficiency of natural *A. flavus* infections, a life history of rapid growth and fast host killing is what one would expect from a rare facultative pathogen that is unlikely to meet a specific adaptive immune response. It would be interesting to do simultaneous infection experiments with two obligate honeybee pathogens, for example combining chalkbrood with American foulbrood (*Paenibacillus* bacteria), particularly because genetic variation for resistance in honeybees has been shown to differ between these two diseases (Invernizzi et al., 2009).

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