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ORIGINAL ARTICLE

Multiple micro-organisms in chalkbrood mummies: evidence and implications

Rebecca N Johnson,[1,2]* M Tauheed Zaman,[1] Meredith M Decelle,[3] Adam J Siegel,[1] David R Tarpy,[4] Eli C Siegel[1] and Philip T Starks[1]

[1]Department of Biology, Tufts University, Medford, MA 02155, USA

[2]Current address: Evolutionary Biology Unit, The Australian Museum, 6 College Street, Sydney 2000, Australia

[3]University of Massachusetts, Lowell, MA 01854, USA

[4]Department of Entomology, Campus Box 7613, North Carolina State University, Raleigh, NC 27695-7613, USA

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SUMMARY

The pathogenic fungus *Ascosphaera apis* is the causative agent of the brood disease chalkbrood in honey bee (*Apis mellifera*) larvae. Infected, hardened larvae – in a powdered form – have been used to inoculate experimental honey bee colonies. While attempting to grow a pure isolate of *A. apis* from chalkbrood infected larvae, we discovered (via Gram staining and sequencing of the 5.8S rDNA region) that the inoculum also harbours many species of bacteria and additional species of fungi. Some of these fungi have been identified previously as antagonists of *A. apis*. It is not surprising that bacteria and fungi grow on dead bees; however, our data indicate that previous experiments intended to study the effects of *A. apis* in honey bees have rather studied the response of honey bees to a cocktail of fungal and bacterial agents. Accordingly, our results indicate that previous research on the *A. apis*-honey bee disease system was not specific to *A. apis* and raise the possibility that the observed honey bee response may have been muted, magnified, or both.

Keywords: chalkbrood, *Ascosphaera apis*, honey bees, *Apis mellifera*, brood diseases

INTRODUCTION

Chalkbrood is a brood disease of honey bees (*Apis mellifera*) and is common everywhere honey bees are managed. It is caused by the heterothallic spore-cyst fungus *Ascosphaera apis*, a member of a fungal genus only found in association with bees from the subfamily Apinae (Bailey & Ball, 1991). This fungus germinates in the larval gut either pre- or post-capping when colony temperature falls below the optimal temperature of 32–35 °C for a prolonged period (>2 h) (Bailey & Ball, 1991). No chemical treatment is presently available for the control of chalkbrood, and the most common practice for dealing with infected colonies is to requeen from different genetic stock and dispose of heavily infected equipment (Nelson & Gochnauer, 1982; Koenig *et al.*, 1986). While sometimes considered a fairly innocuous honey bee disease, chalkbrood remains common in many areas and a colony heavily infected suffers significant losses in both bee numbers and colony productivity (Wood, 1998), thus causing millions of dollars worth of damage per year through reduced honey production (e.g. Heath, 1982; Yacobson *et al.*, 1991).

Honey bee disease studies have focused on chalkbrood for years (e.g. Gilliam *et al.*, 1983; Heath, 1985; Gilliam *et al.*, 1988b; Spivak & Reuter, 1998b), particularly regarding the efficiency of hygienic bees in responding to infestations of *A. apis* (e.g. Gilliam *et al.*, 1983). Hygienic behaviour has long been thought to be under the simple genetic control of two separate, unlinked loci (Rothernbuhler, 1964; but see Moritz, 1988; Lapidge *et al.*, 2002). Following this, Gilliam *et al.* (1988b) found that hygienic behaviour was correlated with resistance to chalkbrood and, in addition to being hygienic, resistant colonies also showed reduced *A. apis* contamination in other areas of the colony such as stored pollen, honey and in the gut of nurse bees. Bees that exhibit poor hygienic behaviour also have *A. apis* in a wider variety of hive substrates such as stored food and wax comb (Gilliam *et al.*, 1983). These findings, combined with the propensity of beekeepers to requeen infected colonies with different genetic stock, suggest a strong genetic component to disease response in honey bees.

Recent work by Tarpy (2003) examined the relationship between genetic diversity within a honey bee colony and the prevalence of chalkbrood infection. This study demonstrated that disease prevalence (calculated as the number of chalkbrood-infected brood per unit area) was significantly less variable in genetically diverse colonies than in genetically similar colonies. These results suggest that the extreme polyandry (multiple mating) observed in *A. mellifera* may have an important function in reducing the likelihood of a catastrophic infection within a colony (Sherman *et al.*, 1988; Sherman *et al.*, 1998; reviewed by Schmid-Hempel, 2000).

An additional behavioural mechanism to reduce the likelihood of chalkbrood infection has recently been identified: honey bees appear to recognize and react to infestation with *A. apis* by up-regulating brood-comb temperature (Starks *et al.*, 2000). It has been hypothesized that worker honey bees produce a colony 'fever' to reduce growth of temperature sensitive chalkbrood mycelia in larvae that are already infected and to prevent infection in healthy larvae (Starks *et al.*, 2000).

Given its usefulness in disease studies, there have been surprisingly few molecular analyses of *A. apis*. Previous studies have, however, characterized several *Ascosphaera* species, including *A. apis* for the internal transcribed spacer region of nuclear DNA (Anderson *et al.*, 1998). This study not only discovered that the sequence of *A. apis* recorded in the international database was actually *Ascosphaera atra*, a non-pathogenic fungal associate of *A. mellifera* (Anderson & Gibson, 1998), but also found that there was no sequence variation between *A. apis* samples from 23 widely distributed isolates (Anderson *et al.*, 1998).

While we have focused on a harmful pathogen, many microbes have been shown to live in symbiotic relationships with honey

*Corresponding author: rebeccaj@austmus.gov.au

bees (reviewed in Gilliam, 1997). These microbes are usually found in the intestines of worker bees and include Gram-variable pleomorphic bacteria, *Bacillus* spp., Enterobacteriaceae, as well as several moulds and yeasts (Gilliam *et al.*, 1988a). Certain micro-organisms play an important role in the biochemical and microbiological conversion of pollen to beebread and in assisting the preservation of honey. Some of these microbes are thought to be important for disease resistance in honey bees by acting as antagonists against certain honey bee pathogens (Gilliam *et al.*, 1988b; Gilliam, 1997). Therefore, if a dose of *A. apis* inadvertently included such micro-organisms, it would likely affect the level of, and response to, chalkbrood infection.

Many techniques have been used to cultivate *A. apis* for identification purposes (e.g., Bailey & Ball, 1991; Anderson & Gibson, 1998; Ruffinengo *et al.*, 2000). Despite previous success in cultivating *A. apis*, past studies of chalkbrood infection have typically used a 'whole ground mummy' inoculation protocol which involves grinding entire 'mummies' (hardened, dried remains of infected larvae), adding the particulate to a solution, and then collecting the spores released into the solution for inoculation purposes (Gilliam *et al.*, 1983; Gilliam *et al.*, 1988b; Starks *et al.*, 2000; Tarpy, 2003). While results from these experiments indicate that the colonies were infected with chalkbrood, it is possible (or even likely) given the lack of use of a pure isolate, that such inocula also contained a combination of other fungi, yeast and bacteria. These additional agents may have been in the larva while it was still alive, colonized it after death from chalkbrood infection, or both.

The chalkbrood–honey bee relationship serves as an ideal model system for the study of disease, disease resistance, and disease prevention in honey bees. Therefore proper identification of the chalkbrood inoculant is imperative. *A. apis* has been used in many studies of honey bee disease (Gilliam *et al.*, 1983; Gilliam *et al.*, 1988b; Starks *et al.*, 2000; Tarpy, 2003). In the process of researching fever in honey bee colonies in response to fungal infection (see Starks *et al.*, 2000), we attempted to produce a pure *A. apis* innoculant. Here we present the outcome of this attempt and identify non-*A. apis* agents associated with sporulating mummies.

MATERIALS AND METHODS

Specimen collection

Black (sporulating) and white (mycelia only) mummies were obtained from three separate colonies (NYS1, NYS2 and NYS3) from Ithaca, New York and from several colonies (AZMIX) from the USDA bee laboratories at Tucson, Arizona (kindly supplied by Mona Chambers and Gloria DeGrandi-Hoffman).

Fungus cultivation techniques

Cultivation of *A. apis* was done on Potato Dextrose Agar (Remel laboratories premix) + 0.4% yeast extract (ICN Biochemicals) (PDA-0.4%YE) plates, both with and without 100 iu/ml of penicillin G and 100 µg/ml of streptomycin to exclude growth of both Gram-positive and Gram-negative bacteria. Scrapings were taken from 40 individual mummies from each of the NYS1, NYS2 and AZMIX groups and 20 mummies from the NYS3 bee strain. From an individual mummy, we scraped the outer and inner surfaces with a sterile applicator stick, applied it to our growth medium plates, and incubated these plates at temperatures ranging from 22 °C to 30 °C. In addition to *A. apis*, these methods cultivated a number of additional fungal and bacterial agents.

Identification techniques: bacteria

Bacterial isolates were streaked onto LB plates (1% tryptone, 0.5% yeast, 1% NaCl, 1.5% agar) so that single colonies were obtained. The number of bacterial types was estimated morphologically by eye and further identification done on bacteria using a Gram stain. *Escherichia coli* and *Staphylococcus aureus* were used as Gram-positive and Gram-negative controls respectively.

TABLE 1. Number of morphologically distinct isolates (including *Ascosphaera apis*) observed in growth from different bee strains, and percentage of plates with *A. apis* growth.

Bee strain	No. of morphologically distinct isolates observed	Plates with A. apis growth (%, A. apis/total no. plates × 100)
NYS1	10	17.5% (7/40)
NYS2	9	20% (8/40)
NYS3	4+	5% (1/20)
AZMIX	13	72.5% (29/40)

Morphological and molecular identification of fungal isolates

Fungi were identified morphologically at × 10 and × 40 magnification using reference images in Anderson & Gibson (1998). Molecular identification was carried out by PCR and sequencing of the ITS1 region of 5.8S ribosomal DNA. All genetic analysis was conducted in the ISIRF (International Social Insect Research Facility) genetics laboratory in Dana Hall at Tufts University, Medford, MA, USA. Suspensions from each isolate were made by placing spores and/or mycelia with a sterile applicator stick into 500 µl sterile double distilled water followed by incubation at room temperature overnight. Suspensions were vortexed and centrifuged for 2 min at 13 000 rpm before use in PCR. Briefly, PCR (30 cycles of: 1 min at 94 °C, 1.5 min at 54–55 °C and 2 min at 72 °C) was carried out using an Applied BioSystems Gene Amp PCR System 2700 thermocycler in 25 µl reactions using universal 5.8SrDNA primers TW81 (5'-GTTTCCGTAGGTGAACCTGC-3') and AB28 (5'-ATATGCTTAAGTTCAGCGGGT-3') (Anderson *et al.*, 1998): 4 µl DNA suspension, 1x reaction buffer (Promega), 15 mM $MgCl_2$, 200 µM each dNTP, 600 nM each primer, and 1.25 units Taq polymerase (Promega). Fragment visualization was carried out on 1.5% agarose gel. PCR products were purified using Montage™ PCR centrifugal filters (Millipore) before cycle sequencing with a ThermoSequenase cycle sequencing kit (USB) with TW81 and AB28 IRD800 labelled primers. DNA sequences were visualised on 6.5% denaturing polyacrylamide gels using a LI-COR single channel 4200 NEN Global Edition IR^2 DNA Analyzer.

Some of the more commonly observed isolates were sequenced for the 5.8SrDNA region and then used to query the blastn program in the NCBI database (http://www.ncbi.nlm.nih.gov) to scan for sequence similarities. The BLAST search is able to find the DNA sequence in the database which is most similar to the query sequence. The BLAST output arranges these sequences according to their similarity to the query sequence. The output also includes a statistic for each sequence showing how likely it is that an unrelated random sequence would be as similar to the query (hence the closer to zero, the less likely to by similar by chance).

RESULTS

Bee cadavers from each bee sample grew *A. apis* (table 1). However, the proportion of *A. apis* growth in relation to other fungal growths varied considerably between bee strains (table 1). Bee strain NYS3 appears to have grown the lowest proportion of *A. apis* (5% of all plates). The AZMIX bee strains produced the highest proportion of *A. apis* (73%). All plates produced multiple morphologically distinct isolates of fungi in addition to *A. apis*.

There were five commonly observed types of growth among the non-*A. apis* strains. These were all identified morphologically as mould or yeast and were given the working names: GBL1, GBL2, GBD, WP, OWM. These commonly observed isolates were sequenced for the 5.8SrDNA region and then used to query the blastn program in the NCBI database (http://www.ncbi.nlm.nih.gov) to assist with identification. Sequences revealed several different genera of fungi, including basidiomycetous, dematiaceous, filamentous, and ascomycetous fungi (table 2).

Several bacterial species were isolated from our cultures. Gram staining from single colonies revealed three morphologically distinct isolates to be Gram-positive rods, one to be Gram-positive cocci and one to be composed of Gram-variable rods. These preliminary identifications were consistent with previously published work identifying a large number of non-pathogenic microbes in honeybee colonies (Gilliam, 1997). The Gram-variable rods may be an artefact (if a culture is a mixture of live and dead cells, the live will stain Gram-positive and the dead Gram-negative) or may be from known Gram-variable genera (e.g., *Gardnerella* and *Nocardia*). Further identification of these bacteria was not carried out in this investigation.

DISCUSSION

The aim of this study was to produce a pure isolate of *A. apis* and to identify, to the level of genus, the micro-organisms present in larvae that had died from chalkbrood disease. Our attempt at culturing *A. apis* by scraping spores from the surface of chalkbrood infected 'mummies' yielded a veritable 'cocktail' of moulds, yeasts and bacteria (see tables 1 and 2). These results were surprising, as our larvae apparently died from chalkbrood, and we had assumed (possibly naively) these mummies had been solely infected with mycelia and spores from *A. apis*. Nevertheless, we were successful at culturing *A. apis* from chalkbrood-infected mummies even without the recommended levels of CO_2. However, our success was variable with the AZMIX bee strains being the most likely to produce *A. apis* growth and the NYS3 bee strain yielding only one *A. apis* colony from 20 culture plates (table 1). It is possible that we did not get 100% growth from chalkbrood cadavers because *A. apis* grows best in CO_2-rich environments. However, by reducing *A. apis* growth, it is likely that our methods facilitated the detection of previously undetected micro-organisms associated with chalkbrood cadavers (see tables 1 and 2).

Impact on previous research

While our data do not overturn the findings of previous studies (e.g. Gilliam *et al.*, 1988b; Starks *et al.*, 2000; Tarpy, 2003), it seems likely that the inoculant used in these studies contained microbes in addition to *A. apis*. Importantly, we identified at least one known antagonist to *A. apis* growth (*Aureobasidium* sp.: Gilliam *et al.*, 1988b) and micro-organisms reported to be beneficial or even essential to honey bees (e.g., *Cladosporium* spp. and *Torulopsis* spp.: Gilliam, 1997). However, some of the moulds known to be common in honey bee colonies – *Aspergillus* spp. and *Penicillium* spp. (Gilliam, 1997) – were not found in our investigation. In addition, preliminary results from some dual cultures (i.e. *Aureobasidium* + *A. apis* on the same agar plate) imply a strong antagonistic effect by certain fungal species that could have been in the original 'ground mummy inoculant.'

Given that the inocula used in previous studies likely included a suite of agents, our data raise the interesting possibility that the previous results are indicative of a more generalized response to infection, and not just to *A. apis*. Indeed, given that the inoculant used in previous studies likely included known *A. apis* antagonists (Gilliam *et al.*, 1988b), previous results, such as honey bee fever (Starks *et al.*, 2000), were perhaps muted relative to that of a 'pure' infection. Similarly, the additional microbes may have affected the immune systems of infected individuals to a greater degree than *A. apis* would have in isolation, potentially affecting levels of infection. With this said, we do not assume that the other microbes given in the original inoculants are all pathogens. However, our major point is that we do not know exactly what was in these original inoculants, nor do we believe that we have been able to grow the full spectrum of microbes present in them. Nonetheless it is likely that additional microbes have made it into the process and have the potential to alter the growth and effect of the *A. apis* fungus.

Conclusions

The chalkbrood–honey bee relationship is emerging as an ideal model system for disease and disease resistance in honey bees. Current evidence has highlighted bee behaviour and genetic diversity as partial regulators of chalkbrood disease (Gilliam *et al.*, 1983; Spivak & Gilliam, 1993; Spivak & Downey, 1998; Spivak & Reuter, 1998a; Spivak & Reuter, 1998b; Tarpy, 2003). In light of our results, however, these data are no longer clearly specific to *A. apis*. As such, researchers should be aware that when using cadavers as a source of spores, the spores are likely contaminated with other micro-organisms. Workers in this field should consider using pure isolates or, alternatively, appropriate identification techniques to confirm the agents in question.

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TABLE 2. Molecular identification of some common isolates.

Working name	Genus based on blastn search	Description	Pathogen or *A. apis* antagonist [ref]
AA	*Ascosphaera apis*	spore-cyst mould	pathogen[1]
GBL1	no amplification	filamentous mould	?
GBL2	*Aureobasidium pullulans*	yeast-like mould	antagonist[3]
GBD	*Cladosporium* spp.	filamentous mould	potential antagonist[2]
WP	*Rhodotorula minuta*	yeast	antagonist[3]
OWM	*Laccaria fraternal*	basidiomycetous mould	?

[1]Bailey (1991), [2]Gilliam (1997),[3] Gilliam (1988b)

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