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# Mixed infections reveal virulence differences between host-specific bee pathogens

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ABSTRACT

Dynamics of host–pathogen interactions are complex, often influencing the ecology, evolution and behavior of both the host and pathogen. In the natural world, infections with multiple pathogens are common, yet due to their complexity, interactions can be difficult to predict and study. Mathematical models help facilitate our understanding of these evolutionary processes, but empirical data are needed to test model assumptions and predictions. We used two common theoretical models regarding mixed infections (superinfection and co-infection) to determine which model assumptions best described a group of fungal pathogens closely associated with bees. We tested three fungal species, *Ascosphaera apis*, *Ascosphaera aggregata* and *Ascosphaera larvis*, in two bee hosts (*Apis mellifera* and *Megachile rotundata*). Bee survival was not significantly different in mixed infections vs. solo infections with the most virulent pathogen for either host, but fungal growth within the host was significantly altered by mixed infections. In the host *A. mellifera*, only the most virulent pathogen was present in the host post-infection (indicating superinfective properties). In *M. rotundata*, the most virulent pathogen co-existed with the lesser-virulent one (indicating co-infective properties). We demonstrated that the competitive outcomes of mixed infections were host-specific, indicating strong host specificity among these fungal bee pathogens.


## 1. Introduction

Pathogens are detrimental to the fitness of their hosts, and changes in pathogen virulence are fueled by various evolutionary pressures (Read, 1994). Theoretical models and empirical studies used to quantify the pressures affecting pathogen virulence show a trade-off between virulence and the ability of the pathogen to be transmitted to future susceptible hosts (Anderson and May, 1982, 1979; de Roode et al., 2008; Doumayrou et al., 2013; May and Anderson, 1979). Models based on this trade-off predict that, over evolutionary time, host and pathogen populations persist when pathogen virulence is intermediate and pathogen transmission is high (Frank, 1996; Alizon et al., 2009). However, the presence of a second pathogen in the same host can alter a pathogen's ability to overcome the host's defenses (Woolhouse et al., 2002). Models that describe the evolution of pathogen virulence based on competition among multiple pathogens infecting the same host generally make predictions based on assumptions of one of two frameworks, superinfection and co-infection (May and Nowak, 1995; Nowak and May, 1994).

In the superinfective framework, one of the pathogens outcompetes the other and the virulence and transmission levels reflect those of only the most competitive pathogen, usually disturbing the balance predicted by the virulence/transmission trade-off theory (Alizon, 2013; Levin and Pimentel, 1981; Mosquera and Adler, 1998; Nowak and May, 1994). While superinfective properties have been observed in some studies (Hughes and Boomsma, 2004; Ben-Ami et al., 2008; Bashey et al., 2011), co-existence of more than one strain of the same pathogen can also occur. In a co-infection, pathogens co-exist until the host dies or recovers, and the resulting virulence and pathogen transmission will reflect a combination of both pathogens (Martcheva and Pilyugin, 2006;

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May and Nowak, 1995; van Baalen and Sabelis, 1995). The evolutionary basis for coexistence is more difficult to rationalize than super-infections, as it is not apparently selfish (Alizon et al., 2013). Low genetic diversity among multiple pathogens in a host has been suggested to increase the ability of two pathogens to co-infect the host (Frank, 1996; Buckling and Brockhurst, 2008; Rumbaugh et al., 2012); potentially creating an evolutionarily stable relationship if the pathogens share goods (*e.g.*, digestive enzymes) during the infection process.

Species-specific empirical data on mixed-infections is needed to advance our understanding of pathogen virulence models (Alizon et al., 2013; Brockhurst and Koskella, 2013). In this study, we chose the fungal genus *Ascosphaera* to quantify parameters associated with within-host, multi-pathogen dynamics in two bee host species. The *Ascosphaera* are always associated with social and solitary bees, and several species cause a disease known as chalkbrood. Of the 28 described *Ascosphaera* species, some are commensal pollen saprophytes found in bee nests, some are facultatively pathogenic to bees, and others are obligate pathogens only found in infected bees (Anderson and Gibson, 1998; Wynns, 2012). Chalkbrood is a disease common to megachilid bees (such as the alfalfa leafcutting bee, *Megachile rotundata* [Megachilidae]), but also afflicts the honey bee, *Apis mellifera* (Apidae). Chalkbrood only infects bee larvae, infecting *per os* after the larvae ingest pollen-based food provisions contaminated with fungal spores. After the spores germinate in the larval gut, the hyphae invade the hemocoel, grow throughout the larval body, and eventually emerge through the integument of moribund larvae, or for some *Ascosphaera* species, the hyphae sporulate just under the larval cuticle (McManus and Youssef, 1984). Only after host death do the hyphae produce spores *in vivo*, which then get disseminated to future larval food provisions by emerging adult bees, whereby the pathogen gets transmitted.

Virulence studies of *Ascosphaera* and other fungal infections in honey bees have been mostly limited to describing single pathogen infections (Vandenberg & Goettel, 1995; Goettel et al., 1997; Gilliam, 2000; Vojvodic et al., 2011), but Vojvodic et al. (2012) found honey bee mortality was significantly increased when *Ascosphaera atra* was combined with an obligate pathogen, *Ascosphaera apis.* Thus, mixed infections of *Ascosphaera* species may be more detrimental to their bee hosts than single infections, but to what extent is that the case throughout the remainder of the *Ascosphaera* pathogens is hard to predict. We selected three pathogens, *A. apis*, *Ascosphaera aggregata*, and *Ascosphaera larvis* to determine if mixed infections with these pathogens in bees favor the assumptions of the superinfective or co-infective framework. Both *A. apis* and *A. aggregata* are the most prevalent obligate pathogens of two economically important bee species, *A. mellifera* (the European honey bee) and *Megachile rotundata* (the alfalfa leafcutting bee), respectively (Aronstein and Murray, 2010; James and Pitts-Singer, 2013). *A. larvis* is pathogenic to the alfalfa leafcutting bee, but it also grows saprophytically on the pollen provisions (Goettel et al., 1997; Bissett, 1988). It is not reported to occur in honey bee hives.

## 2. Methods

### 2.1. General bioassay methods

To quantify the competition dynamics of mixed *Ascosphaera* spp. infections in the bees, we compared host survival and fungal production (post host death) in bees fed combinations of pathogens. Bioassays were conducted using three pathogens (*A. apis*, *A. larvis*, and *A. aggregata*) and two hosts (honey bee and alfalfa leafcutting bee). For each treatment replicate, 36–60 bee larvae were given one of seven treatments (252–420 larvae per replicate) with various combinations of one or two pathogens mixed in their diet (Table 1). In the single pathogen treatments, larvae were fed 5 μl of a $2 \times 10^5$ pathogen spores/ml diet mixture, resulting in a dose of 1000 spores per larva. For multiple pathogen treatments, larvae were fed 2.5 μl of $4 \times 10^5$ spores/ml of each pathogen (1000 spores), resulting in a total dose of 5 μl and 2000 spores per larva, providing a two-way multivariate experimental design to test the effects of co-infections as compared to single infections (for similar designs, see Chouvenc et al., 2012; Raymond et al., 2007; Vojvodic et al., 2012). After treatment, larval survival was checked every day until all surviving larvae had either pupated (for the honey bee, up to 10 days) or spun a prepupal cocoon (for the alfalfa leafcutting bee, up to 24 days). Larvae were considered deceased when feeding movement could not be observed and larval body structure appeared rigid. After death, the cadavers were monitored for evidence of fungal growth. Time to death (in days), presence or absence of external hyphae (for honey bees only because *A. aggregata* does not emerge from the host cuticle), and presence or absence of spore production were recorded.

### 2.2. Fungal source cultures

The spores of *A. aggregata* and *A. apis* used in the experiments came from dead, infected honey bee or alfalfa leafcutter bee larvae found in the field in the general vicinity of Logan, Utah between July and August 2011. These dead larvae with sporulating infections were stored for approximately one year at 4 °C until experimental use. For each replicate, spores were collected from three alfalfa leafcutting bee larvae killed by *A. aggregata* and three honey bee larvae killed by *A. apis*. The purity and identification of the spores was verified using PCR (James and Skinner, 2005). All the spores from one host species were placed in a sterile glass tube and ground with a small glass tissue grinder (Radnoti Glass Company, Monrovia, CA) to break apart the spore balls and separate the spores. Sterile water (1 ml) was added, the mixture further homogenized, then transferred to a 1.5 ml microcentrifuge tube and mixed on a vortex mixer for 20 min. The sample was allowed to settle by gravity for 20–45 min, and then spores were removed from the middle of the suspension with a sterile pipet. Spore concentration was determined using a hemocytometer and adjusted to concentrations for use in the experiment (Table 1). *A. larvis* spores were obtained from the American Type Culture Collection (ATCC® 62708™; Manassas, VA) and were originally isolated from an alfalfa leafcutting bee cadaver (Bissett, 1988). We maintained *A. larvis* on Sabouraud dextrose agar. Spore viability for all three *Ascosphaera* species was verified for each experiment following a $CO_2$ rich, liquid germination protocol in the dark at either 29 °C (for *A. apis*) or 34 °C (for *A. aggregata*) (James and Buckner, 2004).

### 2.3. Honey bee bioassay

Honey bee larvae were obtained from three queenright and visibly disease-free nucleus colonies located at the USDA-ARS Carl Hayden Bee Research Center in Tucson, Arizona. To collect larvae of a uniform age, a clean frame of honey comb was placed in the center of each colony and the queen was restricted to part of the comb using a metal cage. The queen was caged for approximately 48 h, after which most of the comb available to her was filled with one egg per cell. Three days after cage removal, larvae younger than 24 h old were grafted (removed) from the frame and placed into a warmed, sterile, 48-well plate (BD Biosciences, San Jose, CA). Each well contained one egg and 40 μl of honey bee larval diet, consisting of 50% fresh frozen royal jelly (Stakich, Royal Oak, MI) and 50% (v/v) of an aqueous solution containing sterile deionized water, 12% glucose, 12% fructose and 2% yeast extract (Aupinel



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