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

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6 Ellen G. Klinger ^{1,2}, Svjetlana Vojvodic ^{3,4}, Gloria DeGrandi-Hoffman⁵, Dennis L. Welker², Rosalind R.
7 James^{1,6}

8 ¹USDA-ARS Pollinating Insect Research Unit; 1410 North 800 East; Logan, UT 84341

9 ²Utah State University; 5305 Old Main Hill; Logan, UT 84322

10 ³University of Arizona; Center for Insect Science; 1041 E. Lowell St.; Tucson, AZ 85721

11 ⁴Present address: Department of Biological Sciences; Rowan University; 201 Mullica Hill Road;
12 Glassboro, NJ 08028

13 ⁵USDA-ARS Carl Hayden Bee Research Center; 2000 East Allen Road; Tucson, AZ 85721

14 ⁶ Present address: USDA National Programs Office; 5601 Sunnyside Ave; Beltsville, MD 20705
15
16

17 Corresponding Author:
18 Ellen G. Klinger
19 USDA Pollinating Insect Research Unit
20 1410 North 800 East
21 Logan, UT 84341
22 Ph: 435-797-8011
23 Fax: 435-797-0196
24 E-mail: ellen.klinger@ars.usda.gov
25
26
27
28

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.

Keywords: alfalfa leafcutting bee, *Ascosphaera*, chalkbrood, honey bee, multiple infections

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 & 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; 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 & 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, *Apis mellifera* (the European honey bee) and *Megachile rotundata* (the alfalfa leafcutting bee), respectively (Aronstein and Murray, 2010; James and Pitts-Singer, 2013). *Ascosphaera 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 μ l of a 2×10^5 pathogen spores/ml diet mixture, resulting in a dose of 1000 spores per larva. For multiple pathogen treatments, larvae were fed 2.5 μ l of 4×10^5 spores/ml of each pathogen (1000 spores), resulting in a total dose of 5 μ l 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, and 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 minutes. The sample was allowed to settle by gravity for 20-45 minutes, 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). *Ascosphaera 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 CO₂ 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 hours, 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 hours 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 μ l 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 *et al.*, 2005). The diet was freshly prepared at the beginning of the experiment, partitioned into 2 ml tubes and frozen at -80°C until use, at which time it was thawed and gently heated at approximately 32°C. Two days post-grafting, larvae were fed 40 μ l of diet, followed by 80 μ l of diet on the third day for a total of 160 μ l over the 4 day feeding period. The larvae were incubated in the dark at 34°C and approximately 95% humidity. Feces from the larvae were gently cleaned from the wells with a sterile cotton swab.

Spore inocula for the treatments were prepared on the second day after larval grafting. For treatment, larvae were fed 5 μ l of spore suspension combined with 5 μ l of diet. After a larva consumed the entire diet + spore treatment (approximately 2 h), it was provided another 35 μ l of diet without spores. Control insects received 5 μ l of sterile deionized water combined with 5 μ l of diet as a treatment.

The complete bioassay was set up three times, and each time, 25-45 larvae from each of three hives were given one of the seven treatments. Thus, for the entire bioassay, a total of 75-135 larvae per treatment were used. Larvae that died from handling prior to the spore treatments were removed from

the experiment and not included in the data analyses. During daily assessment of larval mortality, bioassay plates were kept warm with a heating pad and ambient moisture was maintained via wet towels.

2.4 Alfalfa leafcutting bee bioassay

Alfalfa leafcutting bee nests were field collected from an alfalfa seed field in Corinne, Utah. Nests were brought back to the lab, cut open, and the nest cells removed. The nest cells consist of alfalfa leaf pieces cut by the mother bee and fashioned into a small cup-shape which contains a pollen and nectar provision and an egg (or developing larva). The eggs were removed from the nest cells, leaving the pollen provisions remaining. These pollen provisions, still in the nest cells, were sterilized using gamma irradiation at a dose of 28 kGy (Xu and James, 2009). Sterile nest cells were then dosed on the surface of the pollen with one of the seven treatments, using the same volume of spore material as in the honey bee assay. The provision was dried under a laminar flow hood for one hour. Using newly collected nests from the field, fresh eggs were removed, washed in a sterile disinfectant for 1 minute followed by three 1-minute rinses in a sterile saline solution (Xu and James, 2009). These surface-sterilized eggs were placed on the sterilized provisions, and then each of these nest cells was placed in a 96-well tissue culture plate and incubated at 29°C and 75% humidity (BD Biosciences, San Jose, CA).

Three replicates of each of seven treatments were conducted, each replicate being initiated on different days. Each replicate a treatment started with 36-48 eggs, however, the washing process can damage eggs, thus any eggs or first instars that collapsed prior to larval feeding were removed from the experiment and were not used in the data analyses.

2.5 Quantification of fungal material from bioassays

Approximately one month after the bioassays were concluded, ten bee cadavers were chosen via number assignment and random number generation from each of the seven treatments, for both bee species. Each *Ascosphaera* fungus present in the cadavers was quantified using quantitative real-time PCR (qRT-PCR). Total DNA from each cadaver was extracted using the MoBio Ultra Clean Plant DNA extraction kit with some modifications (MoBio, Solina, CA). The modifications were: (1) an in-house lysis buffer was used (0.2 N NaOH, 1% SDS), (2) to break fungal walls, spore material and buffer were placed in a beadbeating tube (Sarstedt, Germany) with 0.1 mm zirconia/silica beads (Biospec Inc., Bartlesville, OK), (3) the tubes were beat on a MP Fast-Prep at a speed of 6.0 meters/second for 90 seconds total (MP Biomedicals, Solon, OH), (4) entire volumes of supernatant were used throughout this extraction process (not the fractions required by the kit protocol), (5) two filter tubes were used for each sample to maximize DNA recovery and (6) DNA from each filter tube was eluted with a shared volume of 50 µl TE buffer.

Each fungal species was quantified using species-specific primers (Table 2) (James and Skinner, 2005), without multiplexing. For each qRT-PCR run (each time a set of reactions was placed in the thermocycler), a series of standards were used to quantify the fungal DNA in the samples. These quantification standards were produced from a series of spore dilutions (for each *Ascosphaera* species) equal to 1×10^6 , 1×10^5 and 1×10^4 spores/reaction. The initial concentration of the spores was determined using a hemocytometer. Each quantification standard was extracted using the same methods as for the samples. Quantification standards were used to report the fungal material in “nuclear equivalents,” as each spore contains one nucleus, but the hyphae contain a variable number of nuclei. qRT-PCR reactions were 10 µl each (5 µl SYBR green Supermix, 3.6 µl molecular biology grade water, 0.2 µl of each 20 µM primer, and 1 µl sample DNA), and run using the following protocol: 3 minutes at 95°C

followed by 30 cycles of 15 seconds at 94°C, 15 seconds at 56°C, and 15 seconds at 72°C, with an optical plate read followed by a final melting curve between 55°C and 90°C to test for product purity.

Results were quantified using the Bio-Rad Opticon Monitor v. 3.0 system (Bio-Rad Laboratories, Inc.; Hercules, CA). Each sample was run twice. If both runs resulted in a variance less than 0.2% (of the log₁₀ number of nuclei), no additional samples were run. For a few samples, the variance was greater than 0.2%, so a third sample was run. All the third samples had values within 0.2% of one of the previous samples, so those two values were averaged and used as the sample value.

2.6 Statistical Analyses

Larval survivorship was evaluated using Kaplan-Meier survivorship analysis. We used a log-rank test to determine if survival functions were equal between fungal treatments, and used a post-hoc Tukey's test to compare individual treatment survival functions (PROC LIFETEST, SAS ver. 9.3).

A two-way linear mixed model was used to determine the effect of each pathogen species when it was applied alone versus in combination with the other (for each host species separately). The dependent variables were the final mortality of bees or the proportion of cadavers that produced visible fungal material (separate analyses), and the main effects were each pathogen species and the interactive term between the species. The time-replication of the assays (and the between-colony variation, in the case of the honey bee bioassays) were set as random effects (PROC MIXED, SAS ver. 9.3). The proportion values were first transformed using an arcsine-square root transformation to normalize the distribution and variance. For production of fungal material, Tukey's post-hoc tests were used to determine significant differences between specific treatments.

To determine the effects of treatments on amount of each pathogen produced per host, Mann-Whitney U-tests were used to compare between each single infection and mixed infection treatment groups, analyzed separately for each host species (PROC NPAR1WAY, SAS ver. 9.3).

3. Results

3.1 Honey bees

For honey bees, the survivorship functions significantly differed between treatments ($\chi^2_6=325.11$, $P<0.0001$). Those treatments with *A. aggregata* or *A. larvis* that did not also contain *A. apis*, had no significant effect on honey bee larval survival, as compared to the control (Fig. 1A, Table A1). The obligate pathogen *A. apis* was the only factor that significantly decreased survivorship, and reduced the occurrence of cadavers with emerging hyphae (Table 3). In addition, the proportion of larvae surviving was significantly lower than the control, but not significantly different from each other, for any treatments containing *A. apis*. In other words, the addition of either *A. aggregata* or *A. larvis* to *A. apis* spores did not affect larval survival over *A. apis* solo, although non-significant interaction terms suggest any relationship would be additive between all pathogens (Fig. 1A, Table A1).

Of the larvae that died after fungal exposure, the proportion with visible fungal growth was significantly greater in the *A. apis* treatments, as compared to treatments with the other two fungi (Fig. 2A, Table A2). Not all honey bee larvae that died in the fungal treatments had pathogen spores, but of those that did, only *A. apis* treatments resulted in a significantly higher proportion of cadavers with spores, as compared to any other treatment (Fig. 2A, Table A3).

The amount of *A. apis* fungal material (hyphae and spores combined) produced per infected host did not differ between the *A. apis* solo and *A. apis* + *A. aggregata* mixed infections ($\chi^2_1=0.0285$, $P=0.8658$), nor between *A. apis* solo and *A. apis* + *A. larvis* mixed infections ($\chi^2_1=1.2948$, $P=0.2552$) (Fig. 3A). *A.*

aggregata fungal material in the host did not significantly differ between *A. aggregata* solo infections and mixed infections with *A. larvis* ($\chi^2_1=3.0223$, $P=0.0821$), but the amount of *A. aggregata* was significantly reduced when paired with *A. apis* ($\chi^2_1=5.3719$, $P=0.0205$). More cadavers with spores were found in the solo dose of *A. larvis*, than when *A. larvis* was combined with *A. apis* ($\chi^2_1=5.1491$, $P=0.0233$), or *A. aggregata* ($\chi^2_1=9.9619$, $P=0.0016$).

3.2 Alfalfa leafcutting bee

In the alfalfa leafcutting bee, different treatments significantly affected larval survivorship ($\chi^2_6=189.13$, $P<0.0001$). Those treatments with *A. aggregata*, regardless of whether the other fungi were present or not, had resulted in a significantly lower survival of alfalfa leafcutting bees and more rapid time to death than any of the other treatments (Fig. 1B, Table A4). Solo treatments of *A. larvis* had significantly lower survival than the control treatment, but were not significantly different than survival in treatments with *A. apis* solo, or *A. apis* + *A. larvis* (Fig. 1B, Table A4). For the alfalfa leafcutting bee, it was only the obligate pathogen *A. aggregata* that significantly affected mortality (Table 4). However, *A. aggregata*, *A. aggregata* combined with *A. apis*, and *A. apis* combined with *A. larvis* were all significantly contributing to the variation in the percent of larvae with spores after death (Table 4). Of the larvae that died, the *A. aggregata* solo and *A. aggregata* + *A. larvis* treatments each had significantly more larvae with spores than any other treatment in the bioassay (Fig. 2B, Table A5). *A. apis* and *A. apis* + *A. larvis* treatments did not affect the proportion of larvae with spores, as compared to the control.

A. aggregata was present in infected larvae, and the quantity of *A. aggregata* in the dead larvae did not change when combined with *A. larvis* ($\chi^2_1=0.9552$, $P=0.3284$); nor when combined with *A. apis* ($\chi^2_1=2.9669$, $P=0.0850$) (Fig. 3B). Mixed infections did not affect total fungal quantity in cadavers, either. For example, cadavers from the *A. apis* solo treatments had the same total fungal quantity as those in the *A. apis* + *A. aggregata* treatments ($\chi^2_1=2.9760$, $P=0.0845$), and the *A. apis* + *A. larvis* ($\chi^2_1=1.1468$,

$P=0.2842$) treatments. A similar response was seen with solo *A. larvis* treatments. That is, the amount of fungal material in cadavers treated with *A. larvis* solo was not significantly different than *A. larvis* + *A. aggregata* treatments ($\chi^2_1=1.2471$, $P=0.2641$), nor *A. larvis* + *A. apis* treatments ($\chi^2_1=3.2272$, $P=0.0724$) (Fig. 1F).

A small percentage of our control larvae were infected with *A. aggregata* ($8.36\pm9.6\%$); these larvae died during the experiment and produced spores that were confirmed by PCR to be *A. aggregata* (Fig. 2B). Sterilization of the pollen provisions and/or eggs was not complete, and thus some of the alfalfa leafcutting bees not treated with *A. aggregata* did have a very low level of *A. aggregata* exposure.

4. Discussion

We found that the two obligate pathogens in our study (*A. aggregata* and *A. apis*) caused greater host mortality and had higher pathogen reproduction in both solo and mixed infections when they infected their common hosts compared to when they infected a novel host. A close host-pathogen relationship in obligate pathogens often arises when a pathogen develops an ability to exploit one host, but this arises to the detriment of being able to exploit other hosts, thus leading to pathogen specialization (Kirchner and Roy, 2002). However, host-pathogen co-evolution does not necessarily eliminate the potential for host switching (Brant and Loker, 2005). Both of the obligate pathogens in our study were able to infect both bee species, but infection of the atypical host occurred infrequently. That is, *A. apis* had very low pathogenicity towards alfalfa leafcutting bee, and *A. aggregata* had very low pathogenicity towards the honey bee. Thus, these pathogens, at our manipulated level of infection, either lack the ability to parasitize other hosts readily (*e.g.*, lost the ability to be generalists), or these hosts developed specialized defenses to evade certain pathogens, or a combination of both. It is possible that, at higher or lower inocula levels, different host ages, or with various environmental stresses, virulence towards the atypical hosts would be greater, or lesser, than what we observed in our assays.

309 Each obligate pathogen displayed different competitive dynamics when fed to larvae with a congeneric
310 pathogen. When *A. apis* was fed to honey bee larvae with either *A. larvis* or *A. aggregata*, it was highly
311 competitive against these other pathogens. Not only did host mortality and fungal production levels
312 remain unchanged with the addition of these other pathogens, but also the fungal material found in the
313 cadavers was primarily that of *A. apis*. Vojvodic *et al.* (2012) found honey bee mortality increased when
314 *A. apis* was present in a mixed inoculation with *A. atra*, as compared to *A. apis* alone, which illustrates
315 that specific characteristics of the co-infecting pathogen may be an important factor in the expression of
316 *A. apis* within-host competitiveness. However, in field surveys of chalkbrood-killed honey bee larvae,
317 *Ascosphaera* species other than *A. apis* are not typically found on the larvae, although other bacterial
318 and fungal species are present (Johnson *et al.*, 2005). Based on the evidence of *A. apis* as the only
319 *Ascosphaera* species present post-larval death in both of our studies and other studies, and based on
320 predictions from superinfective models (Mosquera and Adler, 1998; Nowak and May, 1994), virulence
321 evolution of *A. apis* in honey bees may be driven by selective pressures to outcompete other non-
322 specific *Ascosphaera* pathogens. We have shown in this study that the presence of *A. aggregata* or *A.*
323 *larvis* do not alter the host/pathogen dynamics between *A. apis* and honey bees.

324 Although high virulence can make a pathogen very competitive against other pathogens, extremely high
325 virulence will cause host populations to decline rapidly, reducing the long term ability of the pathogen
326 to persist. Thus, superinfective pathogens are expected to ultimately maintain a competitive, but self-
327 sustaining, level of virulence (Alizon, 2008; Vojvodic *et al.*, 2011a). And true to this expectation, *A. apis*
328 infections in honey bees are widespread, but the disease occurs at low to moderate levels in affected
329 honey bee colonies, especially when compared to other diseases of bees (Gilliam, 2000; Vojvodic *et al.*,
330 2012). Models of superinfection also predict that, within a superinfective pathogen species, many
331 strains will occur with a high diversity of virulence levels, as superinfective competition will also take
332 place within a species (Nowak and May, 1994). Indeed, variability in virulence of *A. apis* is observed (Lee

et al., 2013), as well as a variation in host susceptibility (Vojvodic et al., 2011b). In our study, we did not use a single isolate of *A. apis*, but mixtures of several isolates. Combining multiple within-species isolates in infective inoculations could mask the specificity of a pathogen to a host (Luijckx et al., 2011), but it also allowed us to remove any unknown strain-specific effects.

Ascosphaera aggregata showed very different competitive properties. While mortality of the typical host, the alfalfa leafcutting bee, was always high after *A. aggregata* exposure, we did not see the exclusion of other pathogens inside the host during mixed infections. Similar to this result, in field managed alfalfa leafcutting bee populations, co-infected individuals are often found (R. James, unpublished results). However, McFrederick et al. (2014) found that the presence of *A. aggregata* in the alfalfa leafcutting bee larval gut inhibits the growth of other fungi, and most bacteria, altering the microbial community structure in the gut and demonstrating that *A. aggregata* is competitive with other microbes.

Pathogens with low infection capabilities are sometimes successful by “tagging along” with a more infective pathogen (Hughes and Boomsma, 2004). In most studies, the combination of a second, weaker, parasite with a virulent parasite resulted in increased host mortality than when the virulent pathogen occurred alone (Harrison et al., 2006; Lohr et al., 2010; Thomas et al., 2003; Vojvodic et al., 2012). In our study, though, mixed pathogen inoculates did not significantly increase or decrease mortality as compared to solo pathogen inoculates. Chouvinc et al. (2012) saw a similar response to dual pathogen exposures, and proposed that suppression of the host immune reaction facilitated infection by the lesser virulent pathogen (*Aspergillus nomius*) when it co-existed in a termite host with *Metarhizium anisopliae*.

Buckling and Brockhurst (2008), Chao et al., (2000), Frank (1994), and Rumbaugh et al. (2012) all propose that pathogens are more likely to successfully co-exist in a host if they are closely related, and

that the evolution of such systems arises due to shared use of individually produced extracellular material that is beneficial to successful host infection, as well as reduced direct conflict between pathogen species that have a high genetic similarity. *Ascosphaera larvis* and *A. apis* are phylogenetically more similar than *A. larvis* and *A. aggregata* (Klinger *et al.*, 2013), but pathogen co-existence was more common in the latter. The virulence of *Ascosphaera* species in mixed infections appears to be influenced more by host specificity than genetic similarity among these pathogens.

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Table 1

Treatment	<u>Number of spores in infective dose</u>			Total dose	Host	Replicate number	Number of larvae per bioassay
	<i>A. aggregata</i>	<i>A. apis</i>	<i>A. larvis</i>				
Control	0	0	0	0	<i>A. mellifera</i>	3	131
					<i>M. rotundata</i>	3	139
<i>A. apis</i>	0	1000	0	1000	<i>A. mellifera</i>	3	76
					<i>M. rotundata</i>	3	101
<i>A. aggregata</i>	1000	0	0	1000	<i>A. mellifera</i>	3	75
					<i>M. rotundata</i>	3	104
<i>A. larvis</i>	0	0	1000	1000	<i>A. mellifera</i>	3	76
					<i>M. rotundata</i>	3	100
<i>A. apis</i> + <i>A. larvis</i>	0	1000	1000	2000	<i>A. mellifera</i>	3	72
					<i>M. rotundata</i>	3	68
<i>A. aggregata</i> + <i>A. larvis</i>	1000	0	1000	2000	<i>A. mellifera</i>	3	72
					<i>M. rotundata</i>	3	71
<i>A. apis</i> + <i>A. aggregata</i>	1000	1000	0	2000	<i>A. mellifera</i>	3	72
					<i>M. rotundata</i>	3	100

Table 2

Species	Forward Primer (5'-3')	Reverse Primer (5'-3')
<i>A. aggregata</i>	GCACTCCCACCCTTGTCTA	CTCGTCGAGGGTCTTTTCC
<i>A. apis</i>	GCACTCCCACCCTTGTCTA	CAGGCTCGCGAGAACCC
<i>A. larvis</i>	CGAACCAACTATTATTTTTTCTGTGG	ATATGCTTAAGTTCAGCGGGT

Table 3

Treatment	DF	F Value	Pr>F
Mortality			
<i>A. apis</i>	52	148.89	<0.0001
<i>A. aggregata</i>	52	0.13	0.7180
<i>A. larvis</i>	52	0.56	0.4596
<i>A. apis</i> * <i>A. larvis</i>	52	0.01	0.9257
<i>A. aggregata</i> * <i>A. larvis</i>	52	0.04	0.8348
<i>A. apis</i> * <i>A. aggregata</i>	52	0.04	0.8495
Hyphae			
<i>A. apis</i>	52	204.93	<0.0001
<i>A. aggregata</i>	52	0.07	0.7986
<i>A. larvis</i>	52	0.33	0.5665
<i>A. apis</i> * <i>A. larvis</i>	52	0.05	0.8240
<i>A. aggregata</i> * <i>A. larvis</i>	52	0.01	0.9351
<i>A. apis</i> * <i>A. aggregata</i>	52	0.00	0.9484
Spores			
<i>A. apis</i>	52	37.70	<0.0001
<i>A. aggregata</i>	52	2.53	0.1178
<i>A. larvis</i>	52	2.67	0.1081
<i>A. apis</i> * <i>A. larvis</i>	52	2.59	0.1133
<i>A. aggregata</i> * <i>A. larvis</i>	52	0.02	0.9020
<i>A. apis</i> * <i>A. aggregata</i>	52	3.51	0.0667

Table 4

Treatment	DF	F Value	Pr>F
Mortality			
A. apis	12	0.06	0.8084
A. aggregata	12	85.52	<0.0001
A. larvis	12	0.50	0.4936
A. apis * A. larvis	12	4.26	0.0612
A. aggregata * A. larvis	12	2.36	0.1505
A. apis * A. aggregata	12	4.09	0.0659
Spores			
A. apis	12	1.91	0.1919
A. aggregata	12	156.9	<0.0001
A. larvis	12	2.71	0.1258
A. apis * A. larvis	12	8.83	0.0117
A. aggregata * A. larvis	12	2.63	0.1306
A. apis * A. aggregata	12	17.46	0.0013

Captions

Table 1: Summary of fungal dosages, give in total number of infective spores (*Ascosphaera aggregata*, *Ascosphaera apis* or *Ascosphaera larvis*) given per treatment for each bee host (*Apis mellifera* or *Megachile rotundata*), as well as experimental design parameters.

Table 2: Primer sequences used to identify and quantify each species of *Ascosphaera* fungus in this study. Adapted from James and Skinner, 2005

Table 3: Results of mixed model analysis for occurrence of mortality, hyphae and spores in honey bee larvae after exposure to one of 6 infective fungal treatments.

Table 4: Results of mixed model analysis for occurrence of mortality and spores in ALCB larvae after exposure to one of 6 infective fungal treatments.

Figure 1

Figure 2

A

B

Figure 3

Figure Captions

Figure 1. Survival of honey bee (A; *Apis mellifera*) and alfalfa leafcutting bee (B; *Megachile rotundata*) larvae challenged with one of 7 treatments. Letters represent significant differences in rate of survivorship as based from post-hoc Tukey's comparisons at an alpha level of 0.05.

Figure 2. Proportion of total honey bee (A) and alfalfa leafcutting bee (B) larvae that showed external hyphal growth (solid grey bar) and spore development (hatched grey bar) after death. Letters represent significant differences in rate of hyphal or spore development as based from post-hoc Tukey's comparisons at an alpha of 0.05.

Figure 3. Relative amount of nuclear material identified in honey bee (A) and alfalfa leafcutting bee (B) larvae to a fungal species (top axis label) when combined with either no other species (solo) or one of the other species in this study (x-axis). Relative amounts based from quantifications taken from extractions of spores.