Utah State University

DigitalCommons@USU

All Graduate Theses and Dissertations

Graduate Studies

8-2015

Virulence Evolution of Fungal Pathogens in Social and Solitary Bees with an Emphasis on Multiple Infections

Ellen G. Klinger
Utah State University

Follow this and additional works at: https://digitalcommons.usu.edu/etd

Part of the Biology Commons

UtahStateUniversity
MERRILL-CAZIER LIBRARY

VIRULENCE EVOLUTION OF FUNGAL PATHOGENS IN SOCIAL AND SOLITARY BEES

WITH AN EMPHASIS ON MULTIPLE INFECTIONS

by

Ellen G. Klinger

A dissertation submitted in partial fulfillment

of the requirements for the degree

of

DOCTOR OF PHILOSOPHY

in

Biology

Approved:

_____   _____
Dennis L. Welker   Rosalind R. James
Major Professor   Project Advisor

_____   _____
Donald W. Roberts   John R. Stevens
Committee Member   Committee Member

_____   _____
Carol D. von Dohlen   Mark R. McLellan
Committee Member   Vice President for Research and
                                                               Dean of the School of Graduate Studies

UTAH STATE UNIVERSITY
Logan, Utah

2015

Disclaimer regarding products used in the dissertation:

This paper reports the results of research only. The mention of a proprietary product does not constitute an endorsement or a recommendation by the USDA for its use.

Disclaimer about copyrights:

The dissertation was prepared by a USDA employee as part of his/her official duties. Copyright protection under U.S. copyright law is not available for such works. Accordingly, there is no copyright to transfer. The fact that the private publication in which some of this article appears is itself copyrighted does not affect the material of the U.S. Government, which can be freely reproduced by the public.

ABSTRACT

Virulence Evolution of Fungal Pathogens in Social and

Solitary Bees with an Emphasis

on Multiple Infections

by

Ellen G. Klinger, Doctor of Philosophy

Utah State University, 2015

Major Professor: Dr. Dennis L. Welker
Project Advisor: Dr. Rosalind R. James
Department: Biology

Pathogens can infect susceptible hosts by virtue of favorable combinations of

environments, phenotypes of both host and pathogen, and genotypes of hosts and pathogens. A

pathogen's virulence, or harm to the host, generally evolves to maximize pathogen reproduction

with variable results on host survival. Although pathogen reproduction is a primary driver of

virulence evolution, many other variables make pathogen virulence evolution complex to model,

primarily the occurrence of other pathogens infecting a host at the same time.

We investigated the relationships between fungal pathogens in the genus *Ascosphaera*

and the larvae of two of their hosts, *Apis mellifera*, the honey bee, and *Megachile rotundata*, the

alfalfa leafcutting bee. Of primary emphasis in our study were the phylogenetic relationships of

pathogens within the genus, host specificity of the pathogens, and pathogen expression in infections composed of more than one *Ascosphaera* species in the same bee.

We constructed a phylogeny using loci from multiple genes that presented an improvement on our previous understanding of the relationships in the *Ascosphaera*. Pathogens from social bees and solitary bees did not reside in sister clades. Also the genus may be paraphylectic, as shown with the bee pathogen *Ascosphaera torchioi*.

Pathogens in our experiments were mostly host specific, with a honey bee pathogen (*Ascosphaera apis*) showing exclusion of other pathogens during spore production, while an alfalfa leafcutting bee pathogen (*Ascosphaera aggregata*) did not exclude spore production by other species. A more detailed study in mixed infections in the alfalfa leafcutting bee showed that a less virulent pathogen (*Ascosphaera proliperda*) increased its reproductive fitness when in mixed infections with *A. aggregata*, and that host immune responses may account for greater larval survival when fungal doses are staggered. Finally, we conducted a study that showed no derived behaviors of the alfalfa leafcutting bee in the presence of *A. aggregata*, which would indicate an evolution of avoidance or hygienic behavior similar to those observed in the social honey bee.

(154 pages)

PUBLIC ABSTRACT

Virulence Evolution of Fungal Pathogens in Social and

Solitary Bees with an Emphasis

on Multiple Infections

Ellen G. Klinger

The health of pollinators, especially bees, is of the utmost importance to success of many agricultural ecosystems. Microorganisms can cause diseases in bees; such microbes are pathogenic. The ability of a pathogen to cause harm to its host (such as a bee) is termed its virulence. Studying the evolution of different levels of virulence can lead researchers to a better understanding of pathogens, and potentially predict how much harm a pathogen can cause in the future. We studied the evolution of virulence levels for a fungal disease of bees. This group of fungi is composed of 28 species, and some cause a disease in bees called chalkbrood while others do not. Using what we know about virulence evolution we wanted to see if the pathogens could infect all bees, if the pathogens varied in virulence when infecting at the same time as another pathogen, and if solitary bees had any behavioral adaptations that might increase or decrease chalkbrood infection.

By using DNA sequences, the relationship between the genetic structures of each of the fungal species was studied, and we found that pathogens of solitary bees grouped together while pathogens of social bees (honey bees) were not part of this group. We then found that a solitary bee pathogen did not infect honey bees very well, and vice versa. The nuances of the relationship between two solitary bee pathogens were examined more closely to determine how the two

pathogens interact in this bee. In this case, under varying conditions of infection, one pathogen always maintained a similar level of virulence and spore production, while the other pathogen varied in these measures. In addition, when doses of these fungi were fed to bee larvae at different times, more bees survived than when the doses were given at the same time, suggesting that bee immune responses are very important. Finally, we found no evidence of any specific behaviors of solitary bees exposed to infective spores that would suggest these bees have behaviors that are evolved to alter chalkbrood levels in populations.

ACKNOWLEDGMENTS

This research would not have been possible without the guidance and mentorship of

Rosalind James and Dennis Welker. I wish to thank Rosalind for sharing her knowledge and

emphasizing meticulousness in experiments. I appreciate her sharing her experience as an insect

pathologist and her thoughtful comments on my work and manuscript. Her guidance has forced

me to always think about the big picture. I would also like to thank Dennis Welker. He

performed all the sequencing of the *Ascosphaera* species for Chapter 2. Not only did he provide

valuable data, he reviewed every word I wrote and poured over it with a fine tooth comb, for

which I am very grateful. His guidance and encouragement allowed me to finish my dissertation.

I am very thankful for the members of my committee, Donald Roberts, John Stevens and

Carol vonDohlen. I feel honored to have scientists on my committee who I feel exemplify values

I wish to foster in my future scientific career. I appreciate the time they have taken to review and

comment on my work.

Lana Vojdovic assisted me with honey bee experiments and taught me a finicky larval

rearing technique. Without her assistance I would not have been able to use honey bee larvae in

my experiments, and I would also like to thank her for her mentorship. She was able to point out

some flaws in original experimental designs and was able to guide me to a place where my

manuscript was much improved. I would like to thank Nabil Youssef for helping conceive the

idea of the *Ascosphaera* phylogeny and providing thoughtful advice and encouragement to a

graduate student.

I thank the scientists and technical staff at the Carl Hayden Bee Research Laboratory in

Tucson, Arizona, especially my co-author Gloria De-Grandi Hoffman, scientist Kirk Anderson

CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

Microorganisms occupy almost every type of habitat conceivable on our planet. While this extended habitat means that microbes interact intimately with numerous organisms, some microbes have evolved to utilize the niche of another living host for their exclusive benefit. These microbes are known as pathogens, and are typically identified by the disease symptoms they elicit in their hosts. Pathogenicity is the ability of a pathogen to infect a host, while the "virulence" of the pathogen is a measure of the amount of harm it can do to its host, generally measured as speed and occurrence of host mortality (Shapiro-Ilan *et al.*, 2005). While no one specific character makes a microorganism pathogenic, a pathogen in a favorable environment with the right host species will show pathogenicity to the host (Figure 1.1; Méthot and Alizon, 2015).

The evolution of pathogenicity and virulence in microorganisms does not always follow a predictable pattern and is the subject of much debate amongst virulence researchers *(Alizon et al.*, 2009). However, understanding virulence evolution in pathogens is critical in order to predict and be prepared for new, emerging pathogens, as well as to manage diseases caused by existing pathogens. Fungi in the genus *Ascosphaera* are pathogens of managed and wild bees and studies of virulence within this group can produce practical and timely information critical to the preservation of both wild and managed bee populations.
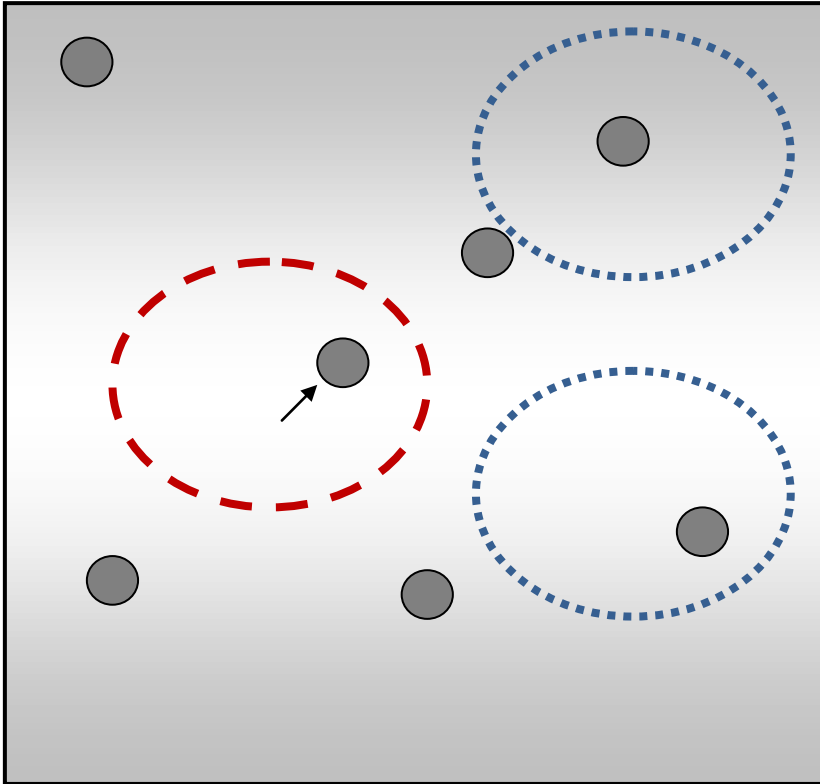
Figure 1.1. Diagram representing the interplay between host environment, pathogen biology and host biology that confers pathogenicity for some pathogen species. In an environment (large box) abiotic factors (temperature, humidity) can enhance pathogenicity (white areas) or be antagonistic to pathogenicity (grey areas). Susceptible hosts (red long-dashed circle) are found in the environment along with non-susceptible hosts (blue short -dashed circle). When susceptible hosts occur in favorable environments and meet pathogenic organisms (grey circles) diseases are elicited (arrow).

Some *Ascosphaera* are obligately pathogenic to bees and are only found parasitizing bee larvae; some are facultatively pathogenic, with the ability to grow saprophytically on nesting materials or pollen; and some are strictly saprophytes, growing in close association with bees, but not known to cause infection or disease (Table 1.1). The current taxonomic classification of the *Ascosphaera* is Ascomycota; Pezizomycotina; Eurotiomycetes; Eurotiomycetidae; Onygenales; *Ascosphaeraceae*; *Ascosphaera* (Wynns, 2012).

The larva is the only stage of bee susceptible to infection by *Ascosphaera* spores. Chalkbrood infections result when adult bees transfer spores from a diseased larva coated with spores to pollen provisions fed to bee brood. In social bees, contamination of adult bees usually results when worker bees remove diseased larvae from the hive (Aronstein and Murray, 2010). In solitary bees, this contamination can occur when healthy nest mates of a diseased bee attempt to leave the nest (Vandenberg *et al.*, 1980). When a contaminated adult bee is involved in feeding the next generation, whether it is through shared labor in a social hive or due to a solitary bee provisioning her nest with pollen contaminated with infective spores from the outside of her body, transmission can occur. Bees managed by humans for pollination have additional risk of chalkbrood exposure due to contamination of equipment and inadvertent transfer of spores (James, 2005; James and Pitts-Singer, 2005; Aronstein and Murray, 2010) .

Once placed in larval food and consumed, *Ascosphaera* spores germinate in the larval gut, most likely using nutrients from the gut contents (Vandenberg and Stephen, 1983; McManus and Youssef, 1984). Penetration through the midgut is a common mode of entry for insect pathogens that invade through the gut, as this is the one section of the digestive tract that is not lined with protective cuticle and the fungal hyphae eventually penetrate the midgut lining, invading the insect hemocoel.

Table 1.1. Known lifestyles of the 28 species in the *Ascosphaera* genus.   Pathogenic species in bold are considered obligate pathogens of their hosts and require their hosts for fungal reproduction. Adapted from Wynns (2012).

Saprophytic	Pathogenic	Unknown
A. atra	*A. acerosa*	*A. asterophora*
A. callicarpa	***A. aggregata***	*A. celerrima*
A. duoformis	***A. apis***	*A. cinnamomea*
A. fimicola	*A. larvis*	*A. flava*
A. flava	*A. osmophila*	*A. fusiformis*
A. naganensis	*A. proliperda*	*A. parasitica*
A. pollenicola	*A. saccaria*	*A. solina*
A. subglobosa	*A. subcuticularis*	*A. variegata*
A. tenax	***A. torchioi***	*A. xerophila*
A. verrucosa		

Ascosphaera hyphae will then rapidly invade the larval tissue, eventually killing the larvae (McManus and Youssef, 1984). Due to the resulting compact mass of fungal hyphae, the larval body turns hard and brittle like a piece of chalk, a symptom which gave this disease its common name. Depending upon the *Ascosphaera* species, infective spores can be produced either on the outside of the insect body or just underneath the cuticle (Figure 1.2).

Figure 1.2. Examples of chalkbrood cadavers. Alfalfa leafcutting bee (*Megachile rotundata*), left, and honey bee (*Apis mellifera*), right. Grey coloration is indicative of spore production. *Apis mellifera* photo by USDA-ARS.

Ascosphaera have been occasionally found in wild, solitary bee populations, and chalkbrood is a significant factor affecting the health of three managed bee species, the European honey bee (*Apis mellifera)*, the alfalfa leafcutting bee (*Megachile rotundata*), and the blue orchard bee (*Osmia lignaria*) (Batra and Bohart, 1969; Hitchcock and Christensen, 1972; Skou, 1972; Christensen, 1983; Vandenberg and Stephen, 1983; Torchioi, 1992). It is known to cause minor to severe losses in honey bee colonies and alfalfa leafcutting bee populations (Aronstein and Murray, 2010; Pitts-Singer and Cane, 2011).   Of the 28 known species of *Ascosphaera*, *Ascosphaera apis* is the main causative agent of chalkbrood in honey bees, *Ascosphaera torchioi* causes chalkbrood in the blue orchard bee, and *Ascosphaera aggregata* is the primary agent of disease in leafcutting bees, although several other species can cause disease in leafcutting bees

(*Ascosphaera proliperda, Ascosphaera larvis, Ascosphaera acerosa*, and *Ascosphaera asterophora;* Skou, 1982; Youssef *et al.*, 1984; Bissett *et al.*, 1996; Wynns, 2012).

Host-pathogen interactions

Pathogens are not the only components in the evolution of virulence, the host species that interact with pathogens are just as important as the pathogens themselves. Host-pathogen interactions are rife with evolutionary pressures for both organisms. For example, pathogens must first evolve the ability to invade a host and cause disease. The host will usually evolve some ability to resist this invasion (immune responses, behavioral avoidance of pathogen, etc.), but this resistance will then be overcome by a pathogen. The interplay between the host and pathogen can result in co-evolution of matching mutations or adaptations (Dybdahl and Storfer, 2003; Lively, 2010). The *Ascosphaera* are exclusively associated with bees, and relationships exist between the *Ascosphaera* and two of the most frequently used agricultural pollinators in North America, the European Honey bee, *Apis mellifera,* and the alfalfa leafcutting bee, *Megachile rotundata*.

Apis mellifera

The European honey bee is one of the most recognized insect pollinators in North America. Total numbers of honey bee colonies worldwide are approximately 78.2 million of which 2.49 million are in the United States (FAO, 2015). Honey bees are responsible for pollinating 37.07 million ha of pollinator dependent crops in the United States, and for 52% of the 115 global food commodity crops (Klein *et al.*, 2007; Calderone, 2012).   Honey bees are not the most effective pollinator choice for every crop, but are desirable bees for agricultural use,

especially monocultures, because they can forage year round, a colony may contain thousands of foragers, the populations can be easily increased with input from humans, and they nest in standardized equipment (vanEngelsdorp and Meixner, 2010). Globally, honey bee numbers are increasing, but in the United States the period between 1961 and 2008 was characterized with decreases in colony numbers (vanEngelsdorp and Meixner, 2010; Calderone, 2012).

Honey bees are classified as eusocial insects. Eusocial, or "social" insects live together in colonies with division of labor, including reproductive labor. Honey bees are divided into three castes: queen, worker, and drone. The queen is the sole reproductive female in the colony, she will lay unfertilized eggs to produce male drone bees and fertilized eggs to produce female worker bees. After egg laying, eggs hatch in 3-4 days, after which the larvae enters into several instar stages, feeding on food provided by worker bees and molting as they increase in size. Larvae are fed a combination of glandular secretions, honey, enzymes and water by adult worker bees. At the final stage, the larvae are sealed inside their honey comb cell and pupate. Pupation takes 8-9 days, after which a new adult emerges from the sealed cell.

Honey bees have many diseases and parasites that can affect the larvae, including bacteria, viruses, fungi and mites. To protect themselves from diseases, honey bees have mechanical barriers to prevent pathogen entry, including a hard chitinous exoskeleton, a mostly chitin lined digestive tract, and a gut environment that is inhospitable to most non-commensal microorganisisms. If a fungal pathogen does enter the insect body, antimicrobial proteins are synthesized to combat microorganisms in the insect hemocoel. Hemolymph can also be a source of protection, melanizing to encapsulate invading organisms. Bees, regardless of sociality, have similar number of immunity related genes, but less genes than when compared with *Drosophila* and *Anopholes* flies (Evans *et al.*, 2006; Xu and James, 2009; Barribeau *et al.*, 2015). Sociality of bees may, however, be a factor in selection pressure for immunity related genes (Barribeau *et al.*, 2015).

Megachile rotundata

The bee genus *Megachile* includes 1478 described species (Pitts-Singer and Cane, 2011). The solitary-nesting species *Megachile rotundata*, commonly called the alfalfa leafcutting bee (ALCB), is an introduced species, originating in Eurasia; however, once introduced to North America and managed, this bee was found to be a very effective pollinator of alfalfa. The ALCB is the most highly managed solitary bee species in the world. Two-thirds the world alfalfa seed production is attributed to the use of ALCB for pollination (Pitts-Singer and Cane, 2011).

Fidelity of solitary bees to nesting areas remains the major problem in employing these bees for managed use in agriculture. Unlike the social honey bee, each female solitary bee is responsible for provisioning nests and laying eggs. Agricultural systems for ALCB nesting include constructed domiciles in a field for shelter, within which polystyrene or wooden boards with 5-7mm diameter holes are placed (Figure 1.3). ALCB are cavity nesters and will nest in these boards.

In the western United States, ALCB populations are active for only approximately 2 months (July-August). Adult bees emerge from nests in early July. Female bees then mate with males, and begin the provisioning of their nests. To provision a nest, a female bee constructs an outer nest casing from approximately 15 pieces of leaves that have been cut with the bee's mandibles. The bee chews the edges of these leaf pieces in order to adhere them to each other and construct the nest. The female bee then forages for pollen and nectar to create the provision upon which her egg is laid. The ALCB forage at a distance up to 500 meters from her nest (Gathmann and Tscharntke, 2002; Greenleaf and Kremen, 2006). After laying an egg, the female closes the nest cell with leaf pieces and then begins a new nest cell. In optimal conditions, females build an average of 57 nest cells in their 7-week lifetime (Figure 1.4) (Maeta and Adachi, 2005).
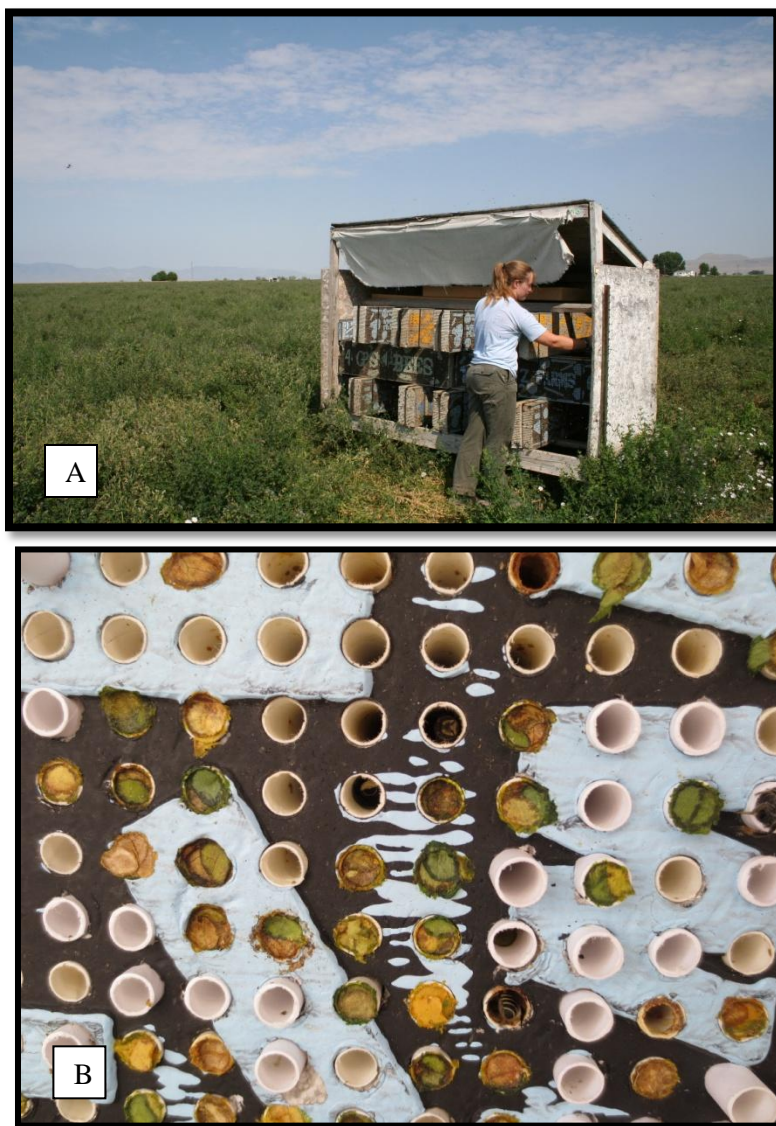
Figure 1.3. A. Alfalfa leafcutting bee shelter used for protecting polystyrene nesting boards.
Photo by T. Pitts-Singer. B. Close up of polystyrene nesting board for alfalfa leafcutting bee
nesting. Paper straws are placed for easy removal of nests for research purposes.

Figure 1.4. Nest cells from an alfalfa leafcutting bee nest and x-ray of a similar nest illustrating bee larvae developing within cells.

A *M. rotundata* egg will hatch into a small first instar larva that remains inside the egg chorion and does not feed. This instar will molt into a larger, second instar, which begins consuming the nectar and pollen provision. After several molts, the provision is completely eaten and the larva will either remain as a large larva (also called prepupae) during the winter months, or, in some environments, the larva will pupate and develop into an adult which will emerge and nest in the same season as its mother. This bivoltine scenario is commonly called "second generation," and exact causes of why some bees do it is unknown. In addition, sometimes eggs laid in nest cells fail or eggs are never laid on a pollen provision. This condition is known as "pollen ball" (Pitts-Singer, 2004).

ALCB are attacked by several groups of insect predators and parasitoids. The main disease in managed populations is chalkbrood, unlike in honeybees where there are numerous

other diseases. ALCB have very similar immune defenses as honeybees; however, there are

certain signaling pathways differ in the ALCB when compared to honey bee gene expression (Xu

and James, 2009).

The study of virulence evolution

To study the evolution of virulence in pathogens and hosts, researchers can take several

different approaches. These approaches include comparing virulence using phylogenies, studying

the host specificity of pathogens, studying the level of mortality and transmission of the pathogen

in a host, investigating the competitive ability a pathogen has with other pathogens in the

environment, and studying the variation of host behaviors when exposed to a pathogen.

Phylogenies

A common approach is to study the relationships between pathogens within a certain

taxonomic group, looking for species that are similar and also exhibit pathogenicity to a host.

With the increased utilization of molecular methods, phylogenies based on the genetic codes of

organisms are becoming the foundation on which to base hypotheses about pathogenicity in

groups. Phylogenies allow researchers the ability to separate organisms on genetic differences

and make testable assumptions based upon those hypothesized differences. For pathogens, study

of phylogenies, along with empirically testable levels of virulence, help researchers identify

points at which virulence may have evolved within groups.

A detailed and accurate phylogeny is essential to proper study of virulence evolution.

The *Ascosphaera* have been studied by a limited population of researchers, and most research has

focused on solving problems occurring from the disease in economically important bee

populations. Unlike pathogens that provide a good mechanism for studies of rapid evolution due to their short generation time (such as bacteria and viruses), the *Ascosphaera* have larger genome sizes and more complex life cycles than simpler pathogens, and phylogenetic studies are limited. The most recent molecular phylogeny of this genus was constructed more than 17 years ago, using only one genetic locus, that of the internal transcribed spacer region (ITS) of the ribosomal RNA, while more recent phylogenetic classifications of fungi utilize more than one locus with much success (Anderson *et al.*, 1998; James *et al.*, 2006). In addition, since that phylogeny, several new species of *Ascosphaera* have been added to the genus (Youssef and McManus, 2001; Wynns *et al.*, 2011).

Host specificity and cross-infectivity

Virulence researchers can also design empirical tests of pathogen ability to infect multiple hosts. Ability of a pathogen to infect more than one species of host can indicate that pathogenicity mechanisms are generalized for hosts, a trait that may have come from either convergent evolution events over a broad range of hosts, or from one ancestral event and divergence of pathogenicity through lineages. In addition, tests of pathogen host specificity can indicate potential areas in which host jumping may occur.

Exposure of pathogens to new hosts is a primary factor in host-switching of pathogens, and bees, both managed and wild populations, overlap with frequency (Greenleaf and Kremen, 2006). This overlap leads to concerns of disease transfer between various bee species (Fürst *et al.*, 2014; Ravoet *et al.*, 2014). There is evidence of some cross infectivity of *Ascosphaera* species to the blue orchard bee (*Osmia lignaria*), but no study has completed an extensive bioassay to determine host specificity to the ALCB or honey bee (Youssef *et al.*, 1984).

Pathogen success within a host

Much can be learned from studying pathogen success when invading a host. To parameterize this, researchers have generally used what is known as the "trade-off theory" of virulence evolution, in which pathogen virulence would be contained by the mortality of the host. A single infecting pathogen will experience selective pressures from the host that alter pathogen virulence. Models of these dynamics predicted that the ideal level of pathogen virulence would maximize pathogen transmission to new, susceptible hosts but would also maximize overall fitness of the affected host population. If a pathogen was too debilitating, it could kill a host before the pathogen was able to synthesize infective propagules, or it could eliminate a host population completely. If a pathogen was not virulent enough, a host would be more likely to overcome an infection (May and Anderson, 1979). A general mathematical model that describes a single infection on a single host is:

$$\frac{dY_i}{dt} = Y_i(\beta_i(1 - Y_i) - u - v_i) \quad \text{(Eq. 1) (May and Nowak, 1995)}.$$

where: Y_i = number of hosts available for infection, β_i = transmissibility coefficient for pathogen *i*, u = general host death (not pathogen related) and v_i = death of hosts due to pathogen *i* (virulence).

The "trade-off" hypothesis is a constraint to this model in that the parameters of v_i and β_i are dependent on each other and that an increase in the pathogen induced death rate results in a reduction of the transmission of a pathogen to future hosts (reviewed in Alizon *et al.*, 2009). By introducing this level of dependency, researchers can begin to infer evolutionary processes of pathogen virulence (Alizon *et al.*, 2009). However, this model is simplistic and limited in that

virulence and transmission of pathogens to hosts are complex and cannot always be distilled to single parameters.

Pathogen success during competition with other pathogens

One of the major complexities assimilated into the trade-off hypothesis is the additional parameter of another pathogen infecting the same host. Single infections are the exception in nature and it is more likely that an organism will be infected with more than one pathogen at a given time (Wynns *et al.*, 2011; Bordes and Morand, 2011; Griffiths *et al.*, 2011). Multiple infections add complexity to the mathematical model above (Eq. 1). Instead of a single pathogen *i*, we need to estimate parameters for all pathogens in our system ($i \ldots \infty$), account for all host deaths due to each pathogen ($v_i \ldots v_i \infty$), calculate a probability that a host might be infected by any one pathogen at a given time (a new parameter) and understand all pathogens' transmissibility coefficients ($\beta_i \ldots \beta_{i\infty}$) (Read and Taylor, 2001). In addition, any direct competition between the pathogen species would need to be assessed. Not only would we need to amend the model for these complex additional parameters, but the effects of multiple pathogens are not necessarily additive, and researchers need to understand how the success of one pathogen affects the success or failure of another while in the same host.

When two pathogens occupy the same host, there is ultimately a degree of competition between the two species for the finite resources of the host (Schjørring and Koella, 2003). The mechanisms of competition between the two pathogens for resources can determine virulence differences between pathogens. Models related to multiple infections have described two extremes in the relationships between competing pathogens, superinfection and co-infection ((May and Nowak, 1995; vanBaalen and Sabelis, 1995; Chao *et al.*, 2000; Brown *et al.*, 2002; Alizon, 2008; López-Villavicencio *et al.*, 2010; Alizon *et al.*, 2013).
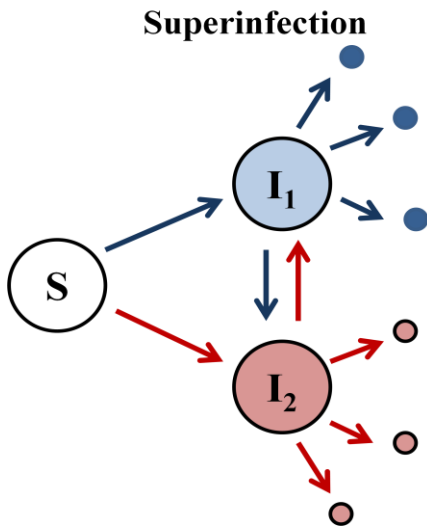
Superinfection

Figure 1.5. Diagram illustrating superinfection. In a superinfective scenario, a susceptible host (S) becomes an infected host (I). Depending upon which pathogen is most virulent (pathogen 1 or 2), the host may express the virulence level (e.g., host mortality level) and infective propagules of pathogen 1 (I_1, in blue), or of pathogen 2 (I_2, in red). Adapted from Alizon *et al.* (2013).

In a superinfection scenario, high pathogen virulence is always evolutionarily favored. A host can be infected by one pathogen, but if another, more virulent pathogen is introduced to the system, then the less virulent pathogen will be "replaced" by the more virulent pathogen, an effect that will show in the expression of the disease in the host (Figure 1.5) (Nowak and May, 1994). This scenario does not require much modification of our original single pathogen model (Eq. 1); we simply replace the transmissibility coefficient and virulence factor of the first pathogen with that of the second, more virulent pathogen.

One of the requirements of this model is primarily that the pathogens must have some type of clear difference in virulence, so a "winner" can be established (Nowak and May, 1994). Superinfection theory predicts that evolution will favor increased virulence in pathogens (Nowak and May, 1994; Alizon and Michalakis, 2011). A pathogen that can infect and cause host death quickly while still passing on its infective propagules will be favored over its slower acting cohorts. As the pathogen population increases in overall virulence, hosts will be killed at a high and fast rate, eventually leading to a limitation of hosts for the more virulent pathogens. Limited hosts will lead to a reduction in the highly virulent pathogen levels, and a chance for less virulent pathogens to infect hosts. The cycle would then begin again. However, in superinfective models, virulence evolution depends upon the population structure of the pathogen population (vanBaalen and Sabelis, 1995). The selection pressure for a pathogen to increase its virulence and steal the hosts from another pathogen is only valid when the competition of other pathogens with a significantly higher virulence is present (Levin and Pimentel, 1981; vanBaalen and Sabelis, 1995).

In a co-infection scenario, more than one pathogen can infect a host at a given time (Figure 1.6). Co-infection is more of a "scramble process," sometimes the most virulent pathogen wins, sometimes the less virulent pathogen wins; however, groups of pathogens in a co-infection will tend to have similar virulence levels (Nowak and May, 1994; May and Nowak, 1995) . Depending on the co-infecting pathogen identities, relatedness and life histories, pathogens may engage in direct competition with each other or may exhibit "cooperative" behavior (Woolhouse *et al.*, 2002). Cooperative behavior could take the form of 'prudence' in virulence evolution where multiple pathogens have selective pressures for restricted virulence because both pathogens infect at the same time and each enhances the reproductive potential of the other. This is hypothesized to be more common in systems with closely related pathogens where the cooperative behavior is rewarded by the passing on of similar genes, regardless of

which pathogen infects to a greater degree (Buckling and Brockhurst, 2008). Cooperation can also take the form of "shared goods production" when production of extracellular chemicals required for infection are shared between two pathogens in a host, or when initial immune suppression on the behalf of one pathogen allows a second, less virulent pathogen to infect (Hughes and Boomsma, 2006; Buckling and Brockhurst, 2008; Barrett *et al.*, 2011). However, changes in virulence can vary further when a pathogen evolves to exploit features inherent to a specific host-pathogen interaction rather than that of an uninfected host, and these virulence
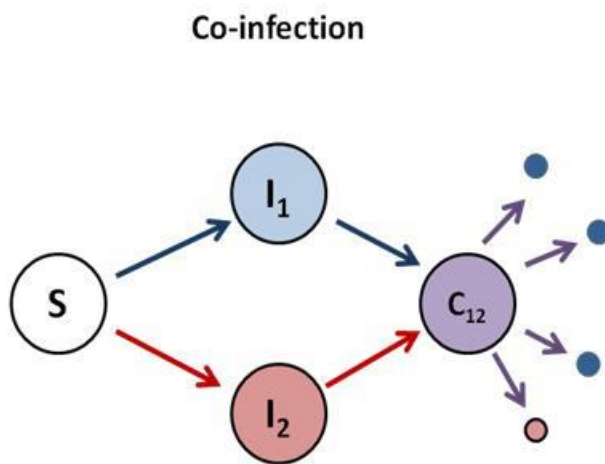
Figure 1.6. Diagram illustrating co-infection. In a co-infective environment, a susceptible host can become infected with pathogen 1 (blue), pathogen 2 (red) or a combination of both pathogens (C_{12}, in purple). When co-infected, the virulence (host mortality) may not exactly match that of pathogen 1 or pathogen 2, and the infective propagules produced are a mixture of both pathogens, although this mixture is not necessarily equal. Adapted from Alizon *et al.* (2013).

changes may also depend upon whether a pathogen requires host death for transmission (Ewald, 1993; Schjørring and Koella, 2003).

Variation in host behaviors

Variation in hosts and host populations, especially in their behavior, movement, and resistance to disease can be co-adapted and affect pathogen virulence. For example, social insects, living in close proximity to each other can increase chances of infection, but, at the same time, there is a component of social immune responses (such as social grooming) that simultaneously reduces infection risks (Naug and Camazine, 2002; Hughes and Boomsma, 2006; Evans and Spivak, 2010).

In addition, many pathogens can cause direct changes in host behavior to increase transmission of the pathogen (Roy *et al.*, 2006). Distribution of susceptible host populations in an environment will influence host specificity of pathogens, with fragmented populations of hosts selecting for a decrease in pathogen host specificity (Farrell *et al.*, 2015). Host mediated avoidance of pathogen dense environments can reduce transmission potential of a pathogen (Villani *et al.*, 1994). Varying levels of host resistance can alter responses to pathogens, and immune responses can also reduce the fitness of the host eliciting the response (Bonsall and Raymond, 2008; Graham *et al.*, 2011).

Virulence evolution and *Ascosphaera*

While these various approaches have been used in studies to examine virulence in the *Ascosphaera*, this dissertation takes each aspect presented above and uses the interaction of these

variables to describe virulence of the *Ascosphaera* in our two selected hosts, the ALCB and the honey bee.

First, I present an improvement in the current phylogeny, incorporating additional species of *Ascosphaera*, as well as genetic information from multiple loci. This phylogeny then becomes an improved base for the hypotheses that drive the rest of the research in the dissertation (Fig. 1.7). I then examine the host specificity of three *Ascosphaera* pathogens between the two host insects, not only measuring mortality of bees in response to the pathogens, but also, more importantly, measuring the quantity of fungal material produced after infection, a measure of the pathogen's transmission, and ultimately evolutionary success. In the same study, I also combine pathogens in infective doses to determine if pathogen levels are inhibited or enhanced by competition. I then delve more deeply into the competitive ability of *Ascosphaera* pathogens, but within the environment of only one host, the ALCB. This deeper look into competitive abilities allows for exploration as to the mechanisms of infection and competition taking place between two closely related pathogens. Finally, I determine if the ALCB's behavior is modified by the presence of *Ascosphaera* pathogens in the environment. Ultimately, by containing multiple levels of research within the parameters conjectured by the improved *Ascosphaera* phylogeny (Fig. 1.7), I can associate influences between these components in the different hosts.
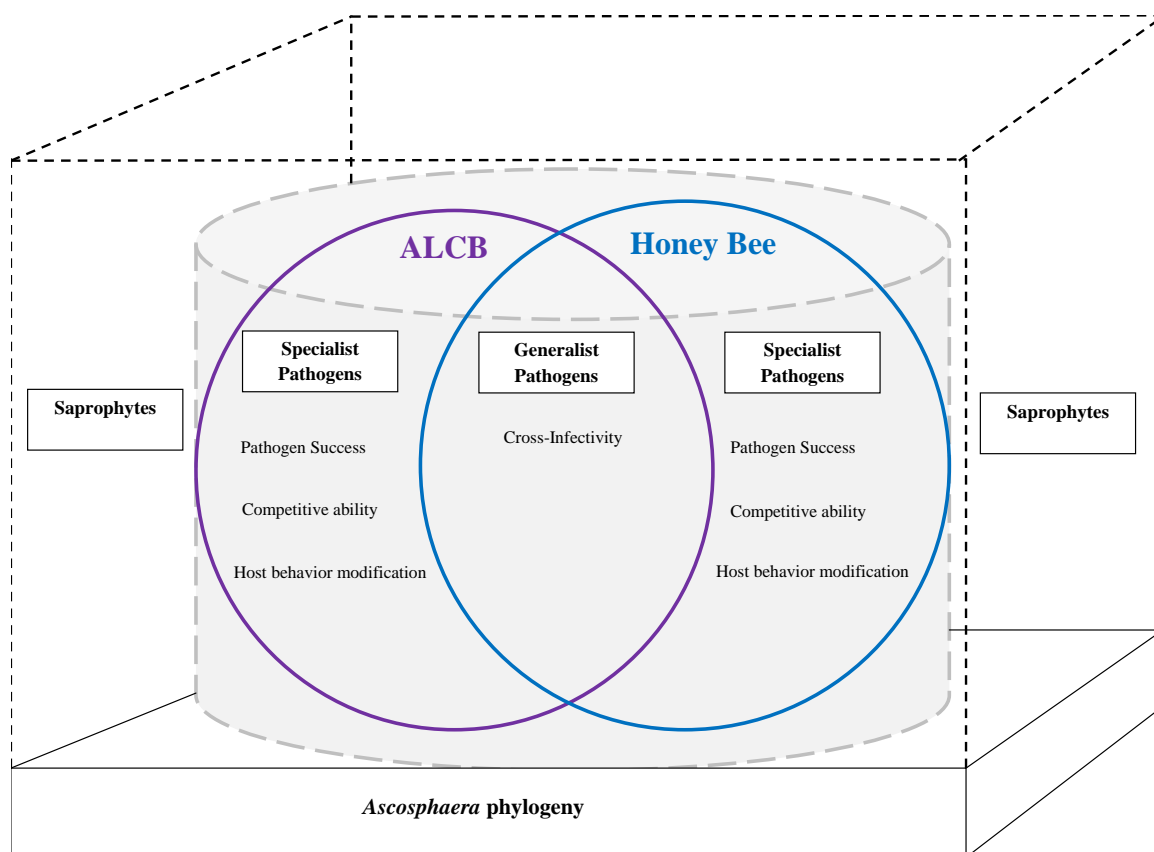
Figure 1.7. Diagram representing the structure of this dissertation. An improved phylogeny of the *Ascosphaera* (lower block) will provide a conjectured evolutionary space of these fungi (dotted box). By noting the presence of the pathogens in the phylogeny (grey cylinder) and adding susceptible hosts within the pathogen space (purple circle for ALCB, blue circle for honey bee), we can then give structure to the various components of virulence research (cross-infectivity, pathogen success, competitive ability, and host behavior modification).

References

Alizon, S., 2008. Decreased overall virulence in coinfected hosts leads to the persistence of virulent parasites. Am. Nat. 172, E67–E79.

Alizon, S., de Roode, J.C., Michalakis, Y., 2013. Multiple infections and the evolution of virulence. Ecol. Lett. 16, 556–567.

Alizon, S., Hurford, A., Mideo, N., van Baalen, M., 2009. Virulence evolution and the trade-off hypothesis: history, current state of affairs and the future. J. Evol. Biol. 22, 245–259.

Alizon, S., Michalakis, Y., 2011. The transmission-virulence trade-off and superinfection: comments to Smith. Evolution 65, 3633–3638.

Anderson, D., Gibbs, A., Gibson, N., 1998. Identification and phylogeny of spore-cyst fungi (*Ascosphaera* spp.) using ribosomal DNA sequences. Mycol. Res. 102, 541–547.

Aronstein, K.A., Murray, K.D., 2010. Chalkbrood disease in honey bees. J. Invertebr. Pathol. 103, S20–S29.

Barrett, L., Bell, T., Dwyer, G., Bergelson, J., 2011. Cheating, trade-offs and the evolution of aggressiveness in a natural pathogen population. Ecol. Lett. 14, 1149–1157.

Barribeau, S.M., Sadd, B.M., duPlessis, L., Brown, M.J.F., Buechel, S.D., Cappelle, K., Carolan, J.C., Christiaens, O., Colgan, T.J., Erler, S., Evans, J., Helbing, S., Karais, E., Lattorff, H.M.G., Marxer, M., Meeus, I., Näpflin, K., Niu, J., Schmid-Hempel, R., Smagghe, G., Waterhouse, R.M., Yu, N., Zdobnov, E.M., Schmid-Hempel, P., 2015. A depauperate immune repertoire precedes evolution of sociality in bees. Genome Biol. 16, 83.

Batra, S.W., Bohart, G.E., 1969. Alkali bees: response of adults to pathogenic fungi in brood cells. Science 165, 607.

Bissett, J., Duke, G., Goettel, M., 1996. *Ascosphaera acerosa* sp. nov. isolated from the alfalfa leafcutting bee, with a key to the species of *Ascosphaera*. Mycologia 88, 797–803.

Bonsall, M.B., Raymond, B., 2008. Lethal pathogens, non-lethal synergists and the evolutionary ecology of resistance. J. Theor. Biol. 254, 339–349.

Bordes, F., Morand, S., 2011. The impact of multiple infections on wild animal hosts: a review. Infect. Ecol. Epidemiol. 1, 1–10.

Brown, S.P., Hochberg, M.E., Grenfell, B.T., 2002. Does multiple infection select for raised virulence? Trends Microbiol. 10, 401–405.

Buckling, A., Brockhurst, M., 2008. Kin selection and the evolution of virulence. Heredity 100, 484–488.

Calderone, N.W., 2012. Insect pollinated crops, insect pollinators and US agriculture: trend analysis of aggregate data for the period 1992-2009. PLoS One 7, e37235.

Chao, L., Hanley, K., Burch, C., Dahlberg, C., 2000. Kin selection and parasite evolution: higher and lower virulence with hard and soft selection. Q. Rev. Biol. 75, 261–275.

Christensen, M., 1983. Notes on the *Ascosphaera* species inciting chalkbrood in honey bees. Apidologie 14, 291–297.

Dybdahl, M., Storfer, A., 2003. Parasite local adaptation: Red Queen versus Suicide King. Trends Ecol. Evol. 18, 523–530.

Evans, J.D., Aronstein, K., Chen, Y.P., Hetru, C., Imler, J.-L., Jiang, H., Kanost, M., Thompson, G.J., Zou, Z., Hultmark, D., 2006. Immune pathways and defence mechanisms in honey bees *Apis mellifera.* Insect Mol. Biol. 15, 645–656.

Evans, J.D., Spivak, M., 2010. Socialized medicine: individual and communal disease barriers in honey bees. J. Invertebr. Pathol. 103, S62–S72.

Ewald, P.W., 1993. The evolution of virulence. Sci. Am. 268, 86–93.

Farrell, M.J., Stephens, P.R., Berrang-Ford, L., Gittleman, J.L., Davies, T.J., 2015. The path to host extinction can lead to loss of generalist parasites. J. Anim. Ecol. *In Press*.

Food and Agriculture Organization of the United Nations (FAO); Statistics Division. 2015. Beehives: Production-live animals [Data file]. Retrieved from faostat3.fao.org.

Fürst, M. A., McMahon, D.P., Osborne, J.L., Paxton, R.J., Brown, M.J.F., 2014. Disease associations between honeybees and bumblebees as a threat to wild pollinators. Nature 506, 364–366.

Gathmann, A., Tscharntke, T., 2002. Foraging ranges of solitary bees. J. Anim. Ecol. 71, 757–764.

Graham, A.L., Allen, J.E., Read, A.F., Graham, L., 2011. Evolutionary causes and consequences of immunopathology. Annu. Rev. Ecol. Evol. Syst. 36, 373–397.

Greenleaf, S.S., Kremen, C., 2006. Wild bees enhance pollination by honey bees. Proc. Natl. Acad. Sci. 103, 13890–13895.

Griffiths, E.C., Pedersen, A.B., Fenton, A., Petchey, O.L., 2011. The nature and consequences of coinfection in humans. J. Infect. 620, 200–206.

Hitchcock, J., Christensen, M., 1972. Occurrence of chalk brood (*Ascosphaera apis*) in honey bees in the United States. Mycologia 64, 1193–1198.

Hughes, W.O.H., Boomsma, J.J., 2006. Does genetic diversity hinder parasite evolution in social insect colonies? J. Evol. Biol. 19, 132–143.

James, R.R., 2005. Impact of disinfecting nesting boards on chalkbrood control in the alfalfa leafcutting bee. J. Econ. Entomol. 98, 1094–1100.

James, R.R., Pitts-Singer, T.L., 2005. *Ascosphaera aggregata* contamination on alfalfa leafcutting bees in a loose cell incubation system. J. Invertebr. Pathol. 89, 176–178.

James, T.Y., Kauff, F., Schoch, C., Matheny, P.B., Hofstetter, V., Cox, C.J., Celio, G., Geuidan, C., Fraker, E., Miadlikowska, J., Lumbsch, T.H., Rauhut, A., Reeb, V., Arnold, A.E., Wynns, A.A., Stajich, J., Hosaka, K., Sung, G., Johnson, D., O'Rourke, B., Crockett, M., Binder, M., Curtis, J.M., Slot, J., Wang, Z., Wilson, A., Schüßler, A., Longcore, J.E., O'Donnell, K., Mozley-Standridge, S., Porter, D., Letcher, P.M., Powell, M.J., Taylor, J.W., White, M.M., Griffith, G.W., Davies, D.R., Humber, R.A., Morton, J.B., Sugiyama, J., Rossman, A., Rogers, J.D., Pfister, D.H., Hewitt, D., Hansen, K., Hambleton, S., Shoemaker, R.A., Kohlmeyer, J., VolkmannKohlmeyer, B., Spotts, R.A., Serdani, M., Crous, P.W., Hughes, K.W., Matsuura, K., Langer, E.,Langer, G., Untereiner, W., Lücking, R., Büdel, B., Geiser, D.M., Aptroot, A., Diederich, P., Schmitt, I., Schultz, M., Yahr, R., Hibbett, D., Lutzoni, F., McLaughlin, D.J., Spatafora, J.W.,Vilgalys, R., 2006. Reconstructing the early evolution of fungi using a six-gene phylogeny. Nature 443, 818–822.

Klein, A.-M., Vaissière, B.E., Cane, J.H., Steffan-Dewenter, I., Cunningham, S., Kremen, C., Tscharntke, T., 2007. Importance of pollinators in changing landscapes for world crops. Proc. Biol. Sci. 274, 303–313.

Levin, S., Pimentel, D., 1981. Selection of intermediate rates of increase in parasite-host systems. Am. Nat. 117, 308–315.

Lively, C.M., 2010. A review of Red Queen models for the persistence of obligate sexual reproduction. J. Hered. 101, S13–S20.

López-Villavicencio, M., Courjol, F., Gibson, A.K., Hood, M.E., Jonot, O., Shykoff, J., Giraud, T., 2010. Competition, cooperation among kin, and virulence in multiple infections. Evolution 65, 1357–1366.

Maeta, Y., Adachi, K., 2005. Nesting behaviors of the alfalfa leaf-cutting bee, *Megachile* (Eutricharaea) *rotundata* (Fabricius)(Hymenoptera, Megachilidae). Chugoku Kontyu 18, 5–21.

May, R.M., Anderson, R.M., 1979. Population biology of infectious diseases: Part II. Nature 280, 455.

May, R.M., Nowak, M., 1995. Coinfection and the evolution of parasite virulence. Proc. Biol. Sci. 261, 209–215.

McManus, W.R., Youssef, N.N., 1984. Life cycle of the chalk brood fungus, *Ascosphaera aggregata*, in the alfalfa leafcutting bee, *Megachile rotundata*, and its associated symptomatology. Mycologia 76, 830–842.

Méthot, P.O., Alizon, S., 2015. What is a pathogen? Toward a process view of host-parasite interactions. Virulence 5, 775–785.

Naug, D., Camazine, S., 2002. The role of colony organization on pathogen transmission in social insects. J. Theor. Biol. 215, 427–439.

Nowak, M., May, R., 1994. Superinfection and the evolution of parasite virulence. Proc. R. Soc. B. 255, 81–89.

Pitts-Singer, T.L., 2004. Examination of 'pollen balls' in nests of the alfalfa leafcutting bee, *Megachile rotundata*. J. Apic. Res. 43, 40–46.

Pitts-Singer, T.L., Cane, J.H., 2011. The alfalfa leafcutting bee, *Megachile rotundata*: the world's most intensively managed solitary bee. Annu. Rev. Entomol. 56, 221–237.

Ravoet, J., De Smet, L., Meeus, I., Smagghe, G., Wenseleers, T., de Graaf, D.C., 2014. Widespread occurrence of honey bee pathogens in solitary bees. J. Invertebr. Pathol. 122, 55–58.

Read, F., Taylor, L.H., 2001. The ecology of genetically diverse infections. Science 292, 1099–1102.

Rigaud, T., Perrot-Minnot, M.-J., Brown, M.J.F., 2010. Parasite and host assemblages: embracing the reality will improve our knowledge of parasite transmission and virulence. Proc. Biol. Sci. 277, 3693–3702.

Roy, H.E., Steinkraus, D.C., Eilenberg, J., Hajek, A. E., Pell, J.K., 2006. Bizarre interactions and endgames: entomopathogenic fungi and their arthropod hosts. Annu. Rev. Entomol. 51, 331–357.

Schjørring, S., Koella, J.C., 2003. Sub-lethal effects of pathogens can lead to the evolution of lower virulence in multiple infections. Proc. Biol. Sci. 270, 189–193.

Shapiro-Ilan, D.I., Fuxa, J.R., Lacey, L. Onstad, D.W., Kaya, H.K., 2005. Definitions of pathogenicity and virulence in invertebrate pathology. J. Invertebr. Pathol. 88, 1–7.

Skou, J., 1972. Ascosphaerales. Friesia 10, 24.

Skou, J., 1982. *Ascosphaera asterophora* species nova. Mycotaxon 14, 149–159.

Torchioi, P.F., 1992. Effects of spore dosage and temperature on pathogenic expressions of chalkbrood syndrome caused by *Ascosphaera torchioi* within larvae of *Osmia lignaria* propinqua (Hymenoptera: Megachilidae). Environ. Entomol. 21, 1086–1091.

vanBaalen, M., Sabelis, M.W., 1995. The dynamics of multiple infection and the evolution of virulence. Am. Nat. 146, 881–910.

Vandenberg, J., Stephen, W.P., 1983. Pathogenesis of chalkbrood in the alfalfa leafcutting bee, *Megachile rotundata*. Apidologie 14, 333–341.

Vandenberg, J.D., Fichter, B.L., Stephen, W.P., 1980. Spore load of *Ascosphaera* species on emerging adults of the alfalfa leafcutting bee, *Megachile rotundata*. Appl. Environ. Microbiol. 39, 650–655.

vanEngelsdorp, D., Meixner, M.D., 2010. A historical review of managed honey bee populations in Europe and the United States and the factors that may affect them. J. Invertebr. Pathol. 103 Suppl , S80–S95.

Villani, M.G., Krueger, S.R., Schroeder, P.C., Consolie, F., Consolie, N.H., Preston-Wilsey, L., Roberts, D.W., 1994. Soil application effects of *Metarhizium anisopliae* on Japanese beetle (Coleoptera: Scarabaeidae) behavior and survival in turfgrass microcosms. Environ. Entomol. 23, 502–513.

Woolhouse, M.E.J., Webster, J.P., Domingo, E., Charlesworth, B., Levin, B.R., 2002. Biological and biomedical implications of the co-evolution of pathogens and their hosts. Nat. Genet. 32, 569–577.

Wynns, A.A., 2012. The bee specialist fungus family Ascosphaeraceae and its allies: systematics, ecology and co-evolution with solitary bees. PhD dissertation. University of Denmark, Copenhagen.

Wynns, A.A., Jensen, A.B., James, R., Eilenberg, J., 2011. *Ascosphaera subglobosa*, a new spore cyst fungus from North America associated with the solitary bee *Megachile rotundata.* Mycologia 104, 108–114.

Xu, J., James, R., 2009. Genes related to immunity, as expressed in the alfalfa leafcutting bee, *Megachile rotundata*, during pathogen challenge. Insect Mol. Biol. 18, 785–794.

Youssef, N.N., McManus, W.R., 2001. *Ascosphaera torchioi* sp. nov., a pathogen of *Osmia lignaria propinqua* Cresson (Hymenoptera). Mycotaxon 127, 7–13.

Youssef, N.N., McManus, W.R., Torchio, A.F., 1984. Cross-infectivity potential of *Ascosphaera* spp. (Ascomycetes: Ascosphaera) on the bee, *Osmia lignaria* propinqua Cresson (Megachilidae: Osmia). J. Econ. Entomol. 78, 227–231.

CHAPTER 2

A MULTI-GENE PHYLOGENY PROVIDES INSIGHT INTO THE RELATIONSHIPS

AMONG *ASCOSPHAERA* SPECIES[1,2,3]

Abstract

Ascosphaera fungi are highly associated with social and solitary bees, with some species being pathogenic to bees (causing chalkbrood) while others are not, and proper identification within this genus is important. Unfortunately, morphological characterizations can be difficult, and molecular characterizations have only used one genetic region. We evaluated multiple phylogenies of the *Ascosphaera* using up to six loci: the Internal Transcribed Spacer (ITS) region, 18S rRNA, 28S rRNA, Elongation Factor-1α (EF-1α) and the RNA polymerase II largest subunit (RPB1), and the second largest subunit (RPB2). The ITS sequence alone produced an inadequate phylogeny, and the addition of both the 18S and 28S rRNA loci to the ITS sequence produced a phylogeny similar to that based on all six genetic regions. For all phylogenies, *Ascosphaera torchioi* was in a separate clade that was the most basal, with a strong genetic similarity to *Eremascus albus*, introducing the possibility of paraphyly within *Ascosphaera*. Also, based on this new phylogeny, we now suggest that the *Apis mellifera* (honey bee) pathogens arose within a group of saprophytes, and the Megachile (leafcutting bees) pathogens arose separately.

Introduction

Several, but not all, species in the genus *Ascosphaera* cause chalkbrood, a widespread fungal disease affecting numerous species of solitary and social bees. *Ascosphaera apis* causes chalkbrood in honey bee (*Apis mellifera*) larvae, and *Ascosphaera aggregata* is one of the main agents causing the disease in larvae of the second most economically important bee, the alfalfa leafcutting bee (*Megachile rotundata*) (Aronstein and Murray, 2010; Pitts-Singer and Cane, 2011). Several other species of *Ascosphaera* with varying amounts of virulence have been isolated from social and solitary bees, bee nests and nesting materials (Spiltor and Olive, 1955; Skou, 1975; Skou and King, 1984; Bissett *et al.*, 1996; Goettel *et al.,*1997; Youssef and McManus, 2001; Wynns *et al.*, 2011).

Ascosphaera develop a unique cyst-like double walled ascoma in which the ascospores mature, clustered in spore balls (Skou, 1982; Bissett, 1988). *Ascosphaera* species differ in morphological characteristics of the hyphae, spores, spore balls and ascomata (Skou, 1988). However, visual identification can be difficult because these morphological characteristics are not always discrete between species, and some species are not easily cultured in the laboratory, so all life stages and growth habits may not be available for laboratory observation. Molecular approaches circumvent these problems and may allow identifications of species in cases where morphological data are missing. Researchers have distinguished *Ascosphaera* species using isozyme analyses (Maghrabi and Kish, 1985a, 1985b, 1987), RAPD analyses (Lu *et al.*, 1996), and species-specific PCR primers based on the Internal Transcribed Spacer (ITS) region (ITS1–5.8S–ITS2) found between the 18S and 28S ribosomal RNA loci (James and Skinner, 2005; Murray, 2005).

The only molecular phylogeny constructed for the *Ascosphaera* was based solely on the ITS region (Anderson *et al.*, 1998). Phylogenetic analyses can be improved by the inclusion of

more than one genetic region (Gontcharov *et al.*, 2004; Delsuc *et al*, 2005; James *et al.*, 2006; Spatafora *et al.*, 2006; Rehner *et al.*, 2011). Species of *Ascosphaera* vary in their ecological niches, ranging from strictly saprophytic to highly virulent to bees, which makes this genus interesting with respect to the evolution of pathogenicity. Information from multiple loci could not only provide a robust phylogeny with which to make inferences about the evolution of these fungi, but may aid in the development of methods for more accurate molecular identification of *Ascosphaera*. We created phylogenies based on the ITS region, as well as the loci for 18S rRNA, 28S rRNA, Elongation Factor-1α (EF-1α) and RNA polymerase II subunits, RPB1 and RPB2. Our phylogenies include up to 23 fungal species and 39 isolates, including one species not used in previous phylogenies of this genus, *Ascosphaera torchioi* (Youssef and McManus, 2001) and the recently discovered *Ascosphaera subglobosa* (Wynns *et al.*, 2011).

Materials and methods

Fungal cultures

Genomic DNA was prepared from 25 isolates of *Ascosphaera* and an isolate of *Eremascus albus*, an osmophilic fungus in the same order (Onygenales) (Table 2.1) (Berbee and Taylor, 1992). Similar to *Ascosphaera*, *Eremascus* is considered an anomaly within the class Eurotiomycetes due to the absence of a fruiting body. Although *E. albus* is morphologically different, its 18S rRNA sequence is more similar to that of *Ascosphaera* than most other Ascomycetes (Berbee and Taylor, 1992). *A. torchioi* cannot be cultured; therefore, spores were sampled directly from diseased *Osmia lignaria* cadavers collected in California, Washington, and Utah. Each sample from an infected bee was considered a different isolate, although they were

Table 2.1. Fungal species used in phylogenetic analyses, including repository source and culture media.

Species	Isolate	Source [a]	Media [b]
Ascosphaera acerosa	201316	ATCC	SDA
Ascosphaera aggregata	690	ARSEF	V8
Ascosphaera aggregata	WILD1	USDA PIRU	V8
Ascosphaera apis	7405	ARSEF	PDA
Ascosphaera apis	7406	ARSEF	PDA
Ascosphaera apis	13786	ATCC	PDA
Ascosphaera apis	13785	ATCC	PDA
Ascosphaera asterophora	152.8	CBS	NC
Ascosphaera atra	693	ARSEF	SDA
Ascosphaera atra	5147	ARSEF	SDA
Ascosphaera celerrima	390.87	CBS	NC
Ascosphaera duoformis	5141	ARSEF	NC
Ascosphaera flava	5144	ARSEF	SDA
Ascosphaera fusiformis	373.87	CBS	NC
Ascosphaera larvis	62708	ATCC	SDA
Ascosphaera larvis	7945	ARSEF	SDA
Ascosphaera larvis	7946	ARSEF	SDA
Ascosphaera cf. larvis[c]	64254	ATCC	SDA
Ascosphaera major	694	ARSEF	SDA
Ascosphaera naganensis	374.87	CBS	NC
Ascosphaera osmophila	64269	ATCC	SDA
Ascosphaera pollenicola	62712	ATCC	SDA
Ascosphaera proliperda	28358	ATCC	SDA
Ascosphaera proliperda	WILD2	USDA PIRU	SDA
Ascosphaera proliperda	696	ARSEF	SDA
Ascosphaera solina	5146	ARSEF	NC
Ascosphaera subcuticulata	5145	ARSEF	NC
Ascosphaera subglobosa	AW-2011	A. A. Wynns	NC
Ascosphaera torchioi	WILD3	USDA PIRU	SDA
Ascosphaera torchioi	WILD4	USDA PIRU	SDA
Ascosphaera torchioi	WILD5	USDA PIRU	SDA
Ascosphaera torchioi	WILD6	USDA PIRU	SDA
Ascosphaera variegata[d]	160.87	CBS	NC
Ascosphaera variegata[d]	62710	ATCC	SDA
Ascosphaera variegata	194577	CCFC	NC
Ascosphaera xerophila	376.87	CBS	NC
Eremascus albus	UCB50-026	J. W. Taylor	ATCC347
Paracoccidioides brasiliensis	PB01	Broad Institute	NC
Paracoccidioides brasiliensis	PB03	Broad Institute	NC

never directly isolated in culture. All others were isolated in culture and maintained at 25°C and on various media (Table 2.1).

DNA extraction, PCR and sequencing

We extracted genomic DNA from fungal hyphae and spores using UltraClean® plant DNA isolation kits (Mo Bio Laboratories, Solana Beach, CA) following a modification of the manufacturer's suggested protocol: we increased centrifugation times after cell lysis, after protein precipitation and after the final ethanol wash step from the recommended 30-60 seconds to two minutes. DNA was stored at -20°C until use.

Gene specific PCR products were prepared using the genomic DNA as the template and sets of gene-specific primers (Table 2.2). Initial primers for *A. apis* were designed using a partial *A. apis* genomic sequence (http://www.hgsc.bcm.tmc.edu [Last accessed 26 May 2015]). The primers were then improved using information from the gene alignments on the Fungal Tree of Life website (http://aftol.org [Last accessed 26 May 2015]), from sequences available on GenBank® (Benson *et al.*, 2011), and from sequences generated in this work. Liu *et al.* (1999) identified conserved RPB2 sequences which were used as guides for generating the initial RPB2 primers. Each PCR reaction contained 1–2 μl of DNA stock, 100–200 nM primers, 8 μl 2.5X master PCR mix (5 Prime, Gaithersburg, MD), 1μl $MgCl_2$ (25 mM), and 7–9 μl distilled H_2O. PCR consisted of: 92 °C for 2 minutes, and 40 cycles of 92 °C for 45 seconds, 45–60 °C for 45 seconds, and 68–70 °C for 3–5 minutes. PCR products were visualized on 0.8% agarose gels. Appropriately-sized PCR products were recovered from gel slices. DNA sequencing reactions were performed using an ABI prism 3730 DNA analyzer and Taq FS Terminator Chemistry. Nucleotide sequences were determined using the PCR products and sequence information was

Table 2.2. Sequences of major primers used to amplify loci in this study.

Primer	Sequence	Locus Amplified	Attachment Site
A220F	TGGTAATTCTAGAGCTAATAC	18S	Near the 5' end of the 18S ribosomal RNA gene
A221R	ARCYAYTCAATYGGTAGTAG	18S	Near the 3' end of the 18S ribosomal RNA gene
A107F	GTTTCCGTAGGTGAACCTG	ITS1-5.8S-ITS2	Near the 3' end of the 18S ribosomal RNA gene
A108R	TATGCTTAAGTTCAGCGGGT	ITS1-5.8S-ITS2	Near the 5' end of the 28S ribosomal RNA gene
A110R	AATGTGCGTTCAAAGATTC	18S-ITS1	In the 5.8S gene
A155F	GACATCGATGAAGAACGCAG	ITS2-28S	In the 5.8S gene
A222F	GAGGAAAAGAAACCAACAGGG	28S	Near the 5' end of the 28S ribosomal RNA gene
A225R	GATGGTAGCTTCGCGGCACTG	28S	Near the 3' end of the 28S ribosomal RNA gene
A1F	GTCGTTATCGGCCACGTCGATTCC	EF-1α	Near the 5' end of the EF-1α gene
A174F	GTYGTYATCGGNCACGTC	EF-1α	Near the 5' end of the EF-1α gene
A114R	TTAYTTCTTRSYRGCCTT	EF-1α	Near the 3' end of the EF-1α gene
A131R	TTAYTTCTTRSYRGCCTTCTG	EF-1α	Near the 3' end of the EF-1α gene
A15F	CTTGCGCACCATCAAGGA	RPB1	Near the 5' end of the RPB1 gene
A202F	GGNCAYTTTGGNCAYATNGA	RPB1	Near the 5' end of the RPB1 gene
A24R	TTACGTTGGACTGTATGTTGGAGAM	RPB1	Near the 3' end of the RPB1 gene
A293R	GGYGACGTRGGNGAGTA	RPB1	Near the 3' end of the RPB1 gene
A3F	ATGGCTGAGCCATACGAAGA	RPB2	Near the 5' end of the RPB2 gene
A58F	ATCAATATGRMGACGAATACT	RPB2	Near the 5' end of the RPB2 gene
A187F	ATCACNNCNGARGAYTGYTGG	RPB2	Near the 5' end of the RPB2 gene
A158R	CAATCWCGYTCCATYTCWCC	RPB2	Near the 3' end of the RPB2 gene
A159R	ACGTTGGTAGTAYGTRGG	RPB2	Near the 3' end of the RPB2 gene

assembled using the Genetics Computer Group's (Tampa, Florida) Wisconsin package software and Geneious™ software (Drummond *et al.*, 2011). Accession numbers for the sequences generated in this work (GQ and JX sets) are given in Table 2.3.

In addition to our sequences, we obtained ITS sequences for 11 *Ascosphaera* isolates from GenBank® (Table 2.3). Sequences for *Paracoccidioides brasiliensis*, a species that is sister to the clade that contains *Ascosphaera* and *Eremascus*, were obtained from the *Paracoccidioides brasiliensis* genome database (www.broadinstitute.org [Last accessed 26 May 2015]) and used as the outgroup for phylogenetic analyses (Geiser *et al.*, 2006).

Phylogenetic analyses

Introns were removed (Table 2.4) and sequences were aligned using Geneious™ software with a global alignment (65% cost similarity, gap opening penalty of 12 and gap extension penalty of 3). Alignments were deposited in TreeBase, submission #12325 (www.treebase.org). Sequences for all isolates were first aligned for each locus separately. For the ITS based phylogeny, an alignment was constructed for the 26 sequences generated in this study, 11 sequences obtained from GenBank® and sequence from the *P. brasiliensis* genome. Concatenated datasets were created using combinations of loci partitioned to allow variable rates of change for each gene.

Partitions were analyzed using jModelTest, from which a general time time-reversible nucleotide substitution model with gamma rate variation among sites (GTR+Γ) was chosen for all partitions except the ITS and subunit regions, for which a proportion of invariant sites were assumed for the same model (GTR+I+Γ) (Guindon and Gascuel, 2003; Posada, 2008).

Table 2.3.   GenBank® accession numbers for each genetic region sequenced from fungal species in this study [GQ set] and from other

Ascosphaera accessions [HQ, U sets]

Species	Isolate	18S	28S	ITS	EF-1α	RPB1	RPB2
Ascosphaera acerosa	201316	GQ867793	GQ867793	GQ867793	GQ867846	GQ867807	GQ867820
Ascosphaera aggregata	690	GQ867781	JX268536	GQ867781	GQ867823	JX401213	JX401208
Ascosphaera aggregata	WILD1	GQ867784	GQ867784	GQ867784	GQ867824	GQ867797	GQ867810
Ascosphaera apis	7405	GQ867785	GQ867785	GQ867785	GQ867826	GQ867798	GQ867811
Ascosphaera apis	7406	GQ867786	GQ867786	GQ867786	GQ867827	GQ867799	GQ867812
Ascosphaera apis	13786			GQ867765	GQ867825		
Ascosphaera apis	13785			GQ867764			
Ascosphaera asterophora	152.8			U68322.1			
Ascosphaera atra	693	GQ867794	GQ867794	GQ867794	GQ867831	GQ867800	GQ867813
Ascosphaera atra	5147			GQ867769			
Ascosphaera celerrima	390.87			U68325.1			
Ascosphaera duoformis	5141			U68316.1			
Ascosphaera flava	5144	GQ867788	GQ867788	GQ867788	GQ867835	GQ867802	GQ867815
Ascosphaera fusiformis	373.87			U68324.1			
Ascosphaera larvis	7945			GQ867774	GQ867836		
Ascosphaera larvis	7946	JX268535	JX268535	GQ867775	GQ867837	JX401215	JX401210
Ascosphaera larvis	62708			GQ867773			
Ascosphaera cf.larvis[a]	64254	JX268537	JX268537	GQ867776	GQ867839	JX401214	JX401209
Ascosphaera major	694	GQ867789	GQ867789	GQ867789	GQ867838	GQ867803	GQ867816
Ascosphaera naganensis	374.87			U68327.1			
Ascosphaera osmophila	64269	GQ867790	GQ867790	GQ867790	GQ867840	GQ867804	GQ867817
Ascosphaera pollenicola	62712	GQ867791	GQ867791	GQ867791	GQ867841	GQ867805	GQ867818
Ascosphaera proliperda	28358	GQ867792	GQ867792	GQ867792	GQ867842	GQ867806	GQ867819
Ascosphaera proliperda	WILD2			GQ867779	GQ867844		
Ascosphaera proliperda	696			GQ867777			
Ascosphaera solina	5146			U68328.1			
Ascosphaera subcuticulata	5145			U68331.1			
Ascosphaera subglobosa	MB519168			HQ540523.1			
Ascosphaera torchioi	WILD3	GQ867782	GQ867782	GQ867782	GQ867845	GQ867795	GQ867808
Ascosphaera torchioi	WILD4			GQ867780	GQ867821		
Ascosphaera torchioi	WILD5			GQ867763			
Ascosphaera torchioi	WILD6	JX268539	JX268539	JX268539	JX645710	JX401216	JX401211
Ascosphaera variegata[b]	160.87			U68320.1			
Ascosphaera variegata[b]	62710	JX268538	JX268538	GQ867772	GQ867833	JX401217	JX401212
Ascosphaera variegata	194577			U68319.1			
Ascosphaera xerophila	376.87			U68326.1			
Eremascus albus	UCB50-026	GQ867787	GQ867787	GQ867787	GQ867834	GQ867801	GQ867814
Paracoccidioides brasiliensis	PB01				4:828033–829054	14:437860–442722	20:450409–453690
Paracoccidioides brasiliensis	PB03	15:556564–558399	15:558998–562010	15:558410–558913			

[a]deposited as *A. obsidiana*

[b]deposited as *A. colubrine*

Table 2.4. Intron positions within the EF-1α and RPB1 loci. Columns are presented in 5' to 3' order relative to the open reading frames for the genes. Introns in the same column occur at homologous positions in the gene. Values correspond to nucleotide positions in the individual GenBank accession files.

[a]The 5' intron in the *A. apis* EF-1α gene occurs in a region of the gene for which sequences are not available for the other species.

[b]For the RPB1 locus, the region containing the 5' intron in most species was not available for analysis in *A. torchioi* and *E. albus*.

Species	Isolate	EF-1α introns			RPB1 introns		
Ascosphaera acerosa	201316			218-442	77-194	352-413	485-589
Ascosphaera aggregata	690			218-299	77-199	357-440	512-612
Ascosphaera aggregata	WILD1			218-299	72-194	352-435	507-607
Ascosphaera apis[a]	7405	39-130		400-479	77-172	330-403	475-563
Ascosphaera apis[a]	7406	60-151		421-500	77-172	330-403	475-563
Ascosphaera atra	693 gene A		79-315	455-508	77-155	313-382	454-541
Ascosphaera atra	693 gene B		79-302	442-497	77-155	313-382	454-541
Ascosphaera flava	5144			218-282	77-142	300-359	431-526
Ascosphaera larvis	7946			218-282	77-143	301-360	432-526
Ascosphaera cf .larvis	64254			224-288	77-143	301-360	432-526
Ascosphaera major	694			218-282	77-144	302-361	433-527
Ascosphaera osmophila	64269			224-311	77-246	404-528	600-730
Ascosphaera pollenicola	62712			218-281	77-143	301-360	432-527
Ascosphaera proliperda	28358			218-280	77-231	389-508	580-700
Ascosphaera torchioi[b]	WILD3	20-79	139-283	423-474		36-98	170-244
Ascosphaera torchioi[b]	WILD6	20-79	139-283	423-474		36-98	170-244
Ascosphaera variegata	62710			224-288	77-143	301-360	432-527
Eremascus albus[b]	UCB50-026	20-74	134-289	429-485		35-101	173-240

34

Phylogenetic trees were inferred from alignments in both MrBayes and Geneious™ (Ronquist and Huelsenbeck, 2003; Drummond *et al.*, 2011). Trees were constructed using Bayesian analysis (MrBayes) and $1x10^7$ generations, with a sample every 1000 generations. The first 1000 generations were discarded, and posterior clade probabilities were used to determine branch support. Trees were inferred for each locus separately and, to further test how the inclusion of data affected the structure of the phylogenetic trees that were produced, other trees were constructed using our concatenated datasets. A consensus tree was generated in Geneious™ using the same datasets as in MrBayes. Trees were also created using maximum likelihood analysis (ML) with the HKY genetic distance model and the Neighbor-Joining method, re-sampling with 100 bootstrap replications.

Results

We generated sequences for the ITS region and for the 18S rRNA, 28S rRNA, EF-1α, RPB1, and RPB2 loci for multiple *Ascosphaera* isolates and *E. albus* (Table 2.3). The sequences were consistent with haploidy for all species except *Ascosphaera atra*. Two alleles were observed for each of the three protein-coding genes from *A. atra,* which is consistent with this species being diploid. Where multiple isolates of the same species were sequenced, several instances of sequence variation, particularly in introns, were detected. Introns were trimmed from the EF-1α dataset. Most species had one intron, but *A. atra* had two, and *A. torchioi* and *E. albus* had three (Table 2.4). An additional intron was also identified in the *A. apis* EF-1α genes; however, this intron is in a 5' region from which sequence data was not available from the other *Ascosphaera* species. The presence of an additional intron in this position in the other *Ascosphaera* species and *E. albus* remains an open question. The RPB1 dataset was also trimmed of several introns, three for most *Ascosphaera* species, but two for *A. torchioi* and *E. albus* because the sequence for these

two species is missing a 5' region available for the other species in which one of the introns is located (Table 2.4).

We present phylogenetic trees from three datasets: ITS, 18S+ITS+28S, and the dataset including all six loci because they proved the most useful. The ITS-only dataset contained the most species and strains in our study (38 including 36 *Ascosphaera* isolates plus those of the two outgroups, one for *E. albus* and one for *P. brasiliensis*) with 503 characters (nucleic acids) and a pairwise identity of 83.0%; this is the region used in prior genetic and phylogenic identifications. Bayesian methods generated a slightly different tree topology than maximum likelihood (ML) methods; here we are only presenting the Bayesian tree. The ITS tree identified five main clades of related species in the Bayesian phylogeny (Figure 2.1). *A. torchioi* originates early in this phylogeny and has an unusually long branch length relative to the rest of the *Ascosphaera* (posterior probability=0.94). *Ascosphaera naganensis*, a species from Japan, also segregates from the rest of the *Ascosphaera* (posterior probability= 0.7). *Ascosphaera duoformis*, *A. atra* and *A. subglobosa* form the next most basal clade with well-supported splits between these species (Figure 2.1). The remaining two clades include the remainder of the *Ascosphaera* divided into a clade containing *A. aggregata* and a clade containing *A. apis*, and are generally well-supported, with the lowest support found in branches leading to single species (*Ascosphaera solina*, *Ascosphaera fusiformis*, and *Ascosphaera xerophila;* posterior probabilities 0.6, 0.52 and 0.65 respectively) (Figure 2.1).

The 18S+ITS+28S dataset contained 18 isolates (including 16 *Ascosphaera* isolates plus those of the two outgroups, *E. albus* and *P. brasiliensis*) with 5350 characters and a pairwise identity of 95.7%. The Bayesian tree from this alignment has four main *Ascosphaera* clades (Figure 2.2). The ML tree had a very similar topology, with the exception of the placement of *Ascosphaera acerosa*. In the ML tree, *A. acerosa* separates before the split between clades 3 and 4, but *A. torchioi* still arises as the most basal species. *A. atra* composes the second clade, and the
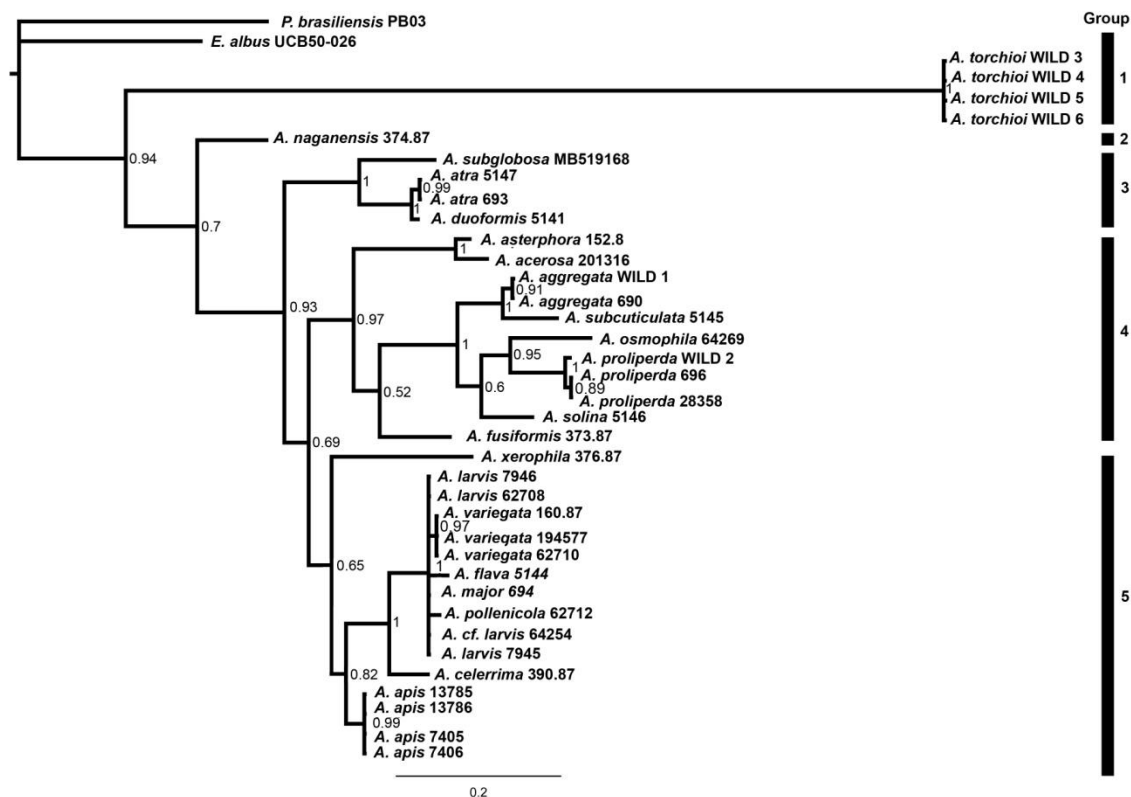
Figure 2.1. Phylogeny of 36 isolates of *Ascosphaera*, one isolate of *E. albus*, and one isolate of *P. brasiliensis* using sequences from the Internal Transcribed Spacer (**ITS1–5.8S–ITS2**) region of ribosomal RNA. Support values are posterior probabilities and the scale is number of expected changes per site as calculated in Bayesian analysis. Five clades of interest are indicated by vertical lines.
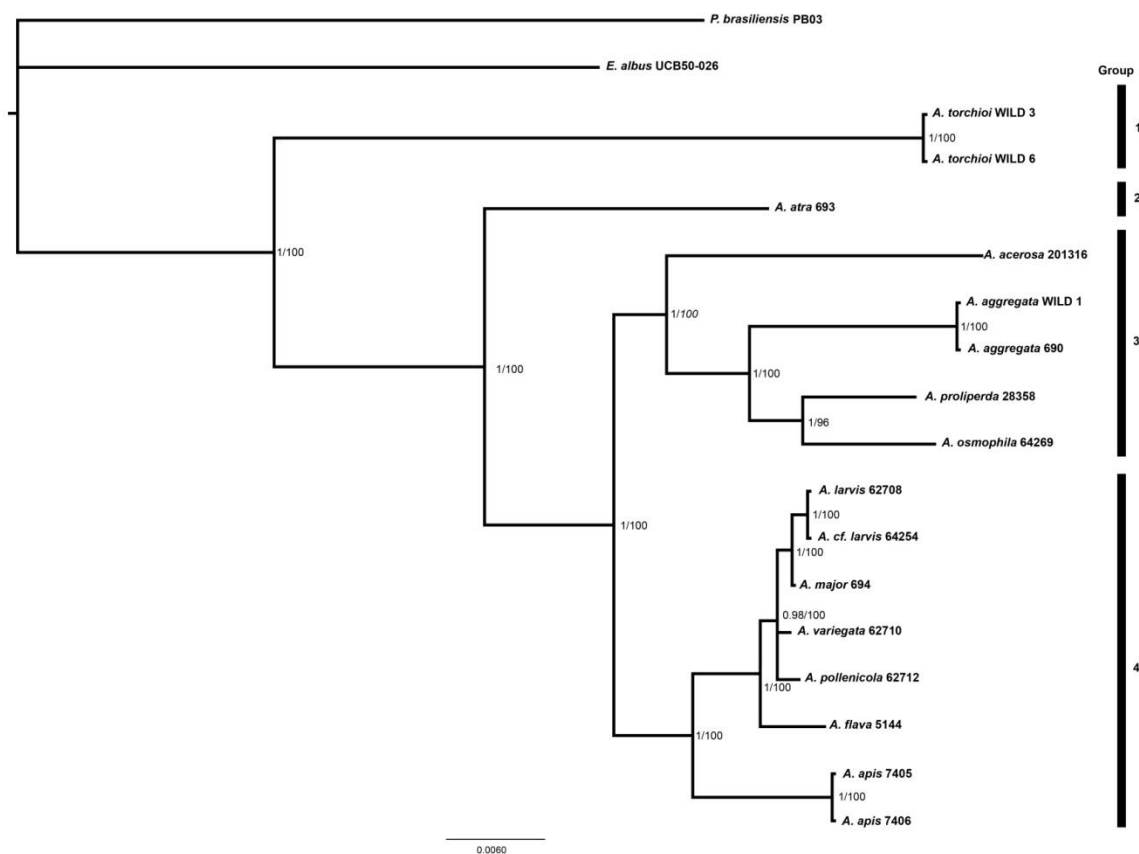
Figure 2.2. Phylogeny of 16 isolates of *Ascosphaera*, one isolate of *E. albus*, and one isolate of *P. brasiliensis* using concatenated sequences from the Internal Transcribed Spacer (**ITS1**–**5.8S**–**ITS2**) region of ribosomal RNA and the genes for the small and large subunits (**18S**, **28S**) of ribosomal RNA. Support values are posterior probabilities and bootstrap values (given as posterior probability/bootstrap value) and the scale is number of expected changes per site as calculated in Bayesian analysis. Bootstrap values in italics indicate that the topology for that node varies between the Bayesian and maximum likelihood trees, with the Bayesian topology presented in the figure. Four clades of interest are indicated by vertical lines.

Figure 2.3. Phylogeny of 16 isolates of *Ascosphaera*, one isolate of *E. albus*, and two isolates of *P. brasiliensis* using concatenated sequences from six loci: the Internal Transcribed Spacer (**ITS1**−**5.8S**−**ITS2**) region of ribosomal RNA, the genes for the small and large subunits (**18S**, **28S**) of ribosomal RNA, and the protein coding genes for Elongation Factor-1α (**EF-1α**) and RNA polymerase II subunits (**RPB1** and **RPB2**). Support values are posterior probabilities and bootstrap values (given as posterior probability/bootstrap value) and the scale is number of expected changes per site as calculated in Bayesian analysis. Four clades of interest are indicated by vertical lines. Relative spore sizes and spore length:width ratios of each species are given from published literature (Harrold, 1950; Anderson and Gibson, 1998; Youssef and McManus, 2001).
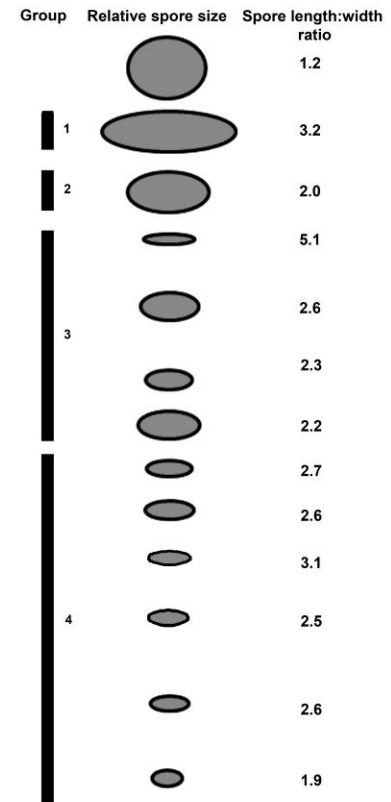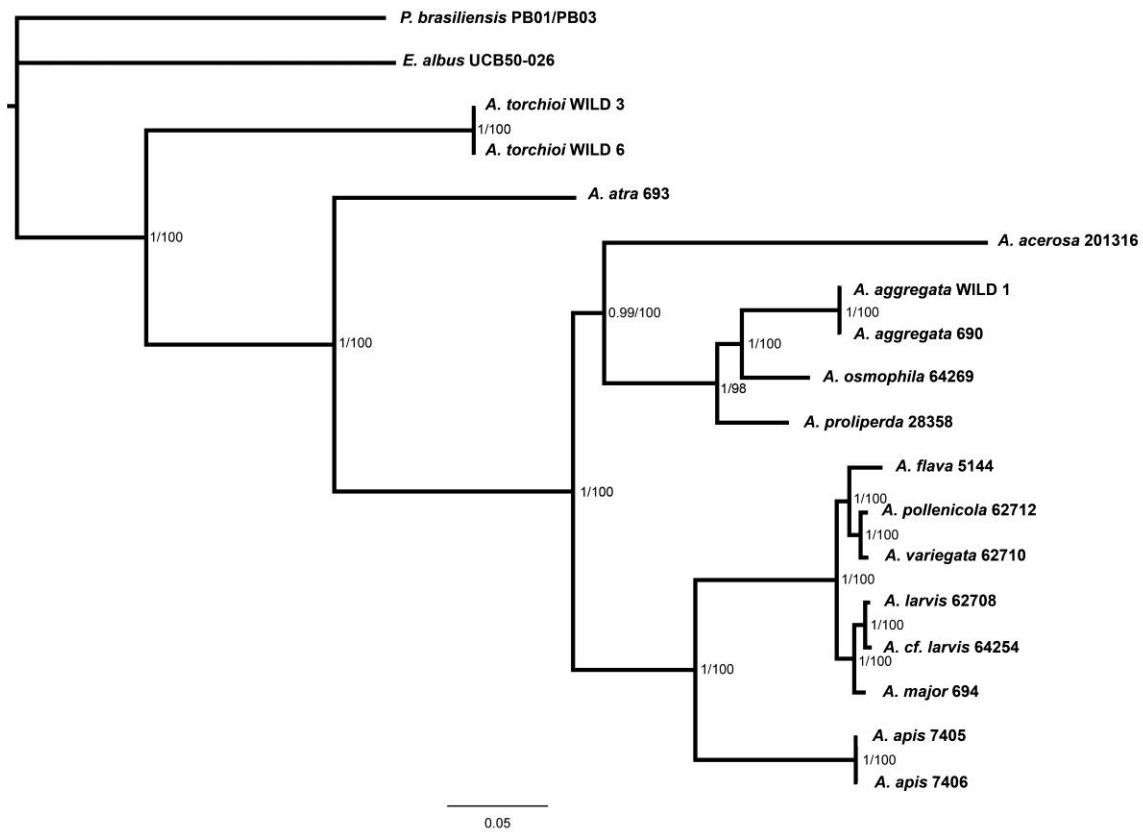
remaining two groups contain the same species as the clades from the ITS only phylogeny. Most posterior probability values are 1 in this concatenated tree and bootstrap values ranged from 96-100. There is discernible resolution between the species *Ascosphaera larvis* and *Ascosphaera major*, unlike in the ITS tree.

The six loci dataset contained 19 isolates (including 16 *Ascosphaera* isolates plus one for *E. albus* and two for *P. brasiliensis*) with 15051 total characters and a pairwise identity of 83.2% (Figure 2.3). Topologies for both the Bayesian tree and the ML tree were the same, both trees had high support values (lowest posterior probability was 0.99 and lowest bootstrap value was 98), and four main clades of *Ascosphaera* were identified. Species positioning was very similar to the 18S+ITS+28S dataset trees.

Discussion

Using sequence information from multiple loci helped clarify phylogenetic relationships within the *Ascosphaera*. For example, we were unable to differentiate between two species (*A. larvis* and *A. major*) using sequence data from only the ITS region, but adding more gene sequences to the analysis produced a phylogeny that discriminated between these two species. The ITS region alone could be insufficient for the identification of some new species, although it can be useful in other cases (e.g. Anderson *et al.*, 1998; Wynns *et al.*, 2011). Some phylogenetic relationships in our study were similar to those presented in Anderson *et al.* (1998), such as the grouping of *A. apis* in a clade with *A. major*, *Ascosphaera variegata*, *Ascosphaera flava*, *Ascosphaera pollenicola* and *A. larvis*, as well as several species pairing together in clades (*A. aggregata* and *Ascosphaera subcuticulata* and *A. atra* and *Ascosphaera duoformis*) (Figure 2.1). However, we consistently found *A. atra* in a more basal position than *A. acerosa*, where the

previous study found the opposite. The basal position of *A. atra* was found not just in an ITS phylogeny, but for most phylogenies produced in this study.

It is not proven that adding additional loci to a phylogeny makes it inherently superior to an analysis using fewer loci (Gontcharov *et al.*, 2004). Identifying patterns in morphological or biological characteristics can add to the confidence we place in our phylogenetic topology. Differences in ascospore size among the different species support the six gene phylogeny. For example, *A. torchioi* has very large, ellipsoid spores, *A. atra* has large short-ellipsoid spores, and the spores of *A. acerosa* are long and narrow-ellipsoid. These three species with unusual spores had the three most basal positions in the six-loci phylogeny (Figure 2.3). The remaining clades of species have spores that are much more similar to each other in size and shape (Anderson and Gibson, 1998). The six-loci dataset produced a phylogeny with a well supported topology, but it is more difficult to design primers to amplify protein coding genetic regions, and of course, much more work to sequence a large number of genes. Thus, it is desirable to identify the least number of genes necessary to produce a reliable phylogeny. Of the various phylogenies we constructed using single loci and combinations of loci, the combination of the 18S+ITS+28S datasets produced a tree that had topology very similar to that using all six loci, and we recommend that researchers consider these regions first when choosing additional loci to sequence in the *Ascosphaera*.

In all of our multi-loci phylogenies, *A. torchioi* holds the most basal position within in the *Ascosphaera*, usually followed in succession by *A. atra* and *A. acerosa*. These three species have unique morphological and/or molecular characteristics in addition to the previously mentioned spore size when compared to the remainder of the genus. *A. acerosa* is characterized by an inner spore-cyst lining unique among the *Ascosphaera* (Bissett *et al.*, 1996). The number of spores in developing spore balls (3−35) is very few in *A. atra* as compared to most of the *Ascosphaera*, and appears to be a diploid (Bissett, 1988; Kish *et al.*, 1988). *A. torchioi* does develop spores within a

cyst like ascoma, fitting one of the current defining characteristics of *Ascosphaera*, and it is also a

pathogen of bees, specifically the blue orchard bee, *Osmia lignaria*. However, for the EF-1α

locus, the intron positions between *E. albus* and *A. torchioi* were identical (Table 2.4). Further

studies utilizing additional closely related fungal species need to be done to determine whether *A.*

torchioi may comprise a separate genus than the rest of the *Ascosphaera*.

Anderson *et al.* (1998) found that those *Ascosphaera* species known to be pathogenic

grouped together phylogenetically, separate from those thought to be saprophytic. In our study

we also consistently found *A. aggregata, A. proliperda* and *A. osmophila* to group together. All

three of these are pathogens of *Megachile* bees. *Ascosphaera apis*, the primary pathogen of *Apis*

mellifera, grouped together with *A. larvis, A. major, A. flava, A. pollenicola,* and *A. variegata.*

The latter three species are most frequently isolated from nesting material and pollen provisions

and are saprophytic, although they may be possible opportunistic pathogens. An *A. apis*

infection, when present in a hive manifests differently when compared to the mortality and spore

producing ability of the *Megachile* pathogens, usually with fewer sporulated cadavers and less

overall level of disease. This discrepancy may be a result of its ability to infect honey bees, a

hygienic social bee as opposed to solitary bee species, or may be due to a phylogenetic

relationship with those *Ascosphaera* that are saprophytic. *A. torchioi*, the most basal

Ascosphaera member in our phylogeny is a pathogen of solitary bees. Pathogenicity is variable

in the remainder of the *Ascosphaera*, with several non-pathogenic species originating between *A.*

torchioi and the remainder of known pathogens such as *A. aggregata* and *A. apis*. This work

gives further information about the *Ascosphaera*, that pathogenicity in this genus may be a

retained characteristic or that the ability of fungal species to infect bees may have arisen multiple

times in this genus. The node depicting the split between the *A. aggregata* and *A. apis* clades may

hold a clue as to when pathogen virulence, host specificity or obligate pathogenicity arose most

recently in the *Ascosphaera*. This information can be utilized when designing studies to test

theories on the origins of pathogenicity in the *Ascosphaera*.

References

Anderson, D.L., Gibson, N.L., Gibbs, A.J., 1998. Identification and phylogeny of spore-cyst fungi (*Ascosphaera spp*.) using ribosomal DNA sequences. Mycol. Res. 102, 541–547.

Anderson, D.L., Gibson, N.L., 1998. New species and isolates of spore-cyst fungi (Plectomycetes: Ascosphaerales) from Australia. Aust. Syst. Bot. 11, 53–72.

Aronstein, K.A., Murray, K.D., 2010. Chalkbrood disease in honey bees. J. Invertebr. Pathol. 103, S20–S29.

Benson, D.A., Karsch-Mizrachi, I., Lipman, D.J., Ostell, J., Sayers, E.W., 2011. GenBank. Nucleic Acids Res. 39, D32–D37.

Berbee, M.L., Taylor, J.W., 1992. Two ascomycete classes based on fruiting-body characters and ribosomal DNA sequence. Mol. Biol. Evol. 9, 278–284.

Bissett, J., 1988. Contribution toward a monograph of the genus *Ascosphaera*. Can. J. Bot. 66, 2541–2560.

Bissett, J.D., Duke, G.M., Goettel, M.S., 1996. *Ascosphaera acerosa* sp. nov. isolated from the alfalfa leafcutting bee, with a key to the species of *Ascosphaera*. Mycologia 88, 797–803.

Delsuc, F., Brinkmann, H., Philippe, H., 2005. Phylogenomics and the reconstruction of the tree of life. Nat. Rev. Genet. 6, 361–375.

Drummond, A.J., Ashton, B., Buxton, S., Cheung, M., Cooper, A., Duran, C., Field, M., Heled, J., Kearse, M., Markowitz, S., Moir, R., Stones-Havas, S., Sturrock, S., Thierer, T., Wilson, A., 2011. Geneious v5.4, Available from http://www.geneious.com/.

Geiser, D.M., Gueidan, C., Miadikowska, J., Lutzoni, F., Kauff, F., Hostetter, V., Fraker, E., Schoch, C.L., Tibell, L., Untereiner, W.A., Aptroot, A., 2006. Eurotiomycetes: Eurotiomycetidae and Chaetothyriomycetidae. Mycologia 98, 1053–1064.

Goettel, M.S., Duke, G.M., Goerzen, D.W., 1997. Pathogenicity of *Ascosphaera larvis* to larvae of the alfalfa leafcutting bee, *Megachile rotundata*. Can. Entomol. 129, 1059–1065.

Gontcharov, A.A., Marin, B., Melkonian, M., 2004. Are combined analyses better than single gene phylogenies? A case study using SSU rDNA and *rbc*L sequence comparisons in the Zygnemaophyceae (Streptophyta). Mol. Biol. Evol. 21, 612–624.

Guindon, S., Gascuel, O., 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst Biol. 52, 696–704.

Harrold, C.E., 1950. Studies in the Genus *Eremascus*: I. The rediscovery of *Eremascus albus* Eidam and some new observations concerning its life-history and cytology. Ann. Bot. 14, 127–148.

James, R.R., Buckner, J.S., 2004. Lipids stimulate spore germination in the entomopathogenic ascomycete *Ascosphaera aggregata*. Mycopathologia 158, 293–302.

James, R.R., Skinner, J.S., 2005. PCR diagnostic methods for *Ascosphaera* infections in bees. J. Invertebr. Pathol. 90, 98–103.

James, T.Y., Kauff, F., Schoch, C., Matheny, P.B., Hofstetter, V., Cox, C.J., Celio, G., Geuidan, C., Fraker, E., Miadlikowska, J., Lumbsch, T.H., Rauhut, A., Reeb, V., Arnold, A.E., Wynns, A.A., Stajich, J., Hosaka, K., Sung, G., Johnson, D., O'Rourke, B., Crockett, M., Binder, M., Curtis, J.M., Slot, J., Wang, Z., Wilson, A., Schüßler, A., Longcore, J.E., O'Donnell, K., Mozley-Standridge, S., Porter, D., Letcher, P.M., Powell, M.J., Taylor, J.W., White, M.M.,Griffith, G.W., Davies, D.R., Humber, R.A., Morton, J.B., Sugiyama, J., Rossman, A., Rogers, J.D., Pfister, D.H., Hewitt, D., Hansen, K., Hambleton, S., Shoemaker, R.A., Kohlmeyer, J., VolkmannKohlmeyer, B., Spotts, R.A., Serdani, M., Crous, P.W., Hughes, K.W., Matsuura, K., Langer, E.,Langer, G., Untereiner, W., Lücking, R., Büdel, B., Geiser, D.M., Aptroot, A., Diederich, P., Schmitt, I., Schultz, M., Yahr, R., Hibbett, D., Lutzoni, F., McLaughlin, D.J., Spatafora, J.W.,Vilgalys, R., 2006. Reconstructing the early evolution of fungi using a six-gene phylogeny. Nature 443, 818–822.

Kish, L.P., Bowers, N.A., Benny, G.L., Kimbrough, J.W., 1988. Cytological development of *Ascosphaera atra*. Mycologica 80, 312–319.

Liu, Y.J., Whelen, S., Hall, B.D., 1999. Phylogenetic relationships among Ascomycetes: evidence from an RNA polymerase II subunit. Mol. Biol. Evol. 16, 1799–1808.

Lu, R., Goerzen, D.W., Rank, G.H., 1996. Use of RAPD analysis for in situ identification of *Ascosphaera aggregata* and *Ascosphaera larvis* in larval cadavers of the alfalfa leafcutting bee, *Megachile rotundata*. J. Invertebr. Pathol. 68, 78–83.

Maghrabi, H.A., Kish, L.P., 1985a. Isozyme characterization of Ascosphaerales associated with bees. I. *Ascosphaera apis, Ascosphaera proliperda*, and *Ascosphaera aggregata*. Mycologia 77, 358–365.

Maghrabi, H.A., Kish, L.P., 1985b. Isozyme characterization of Ascosphaerales associated with bees. II. *Ascosphaera major*, *Ascosphaera atra*, and *Ascosphaera asterophora*. Mycologia 77, 366–372.

Maghrabi, H.A., Kish, L.P., 1987. Isozyme characterization of Ascosphaerales associated with bees. IV. Analyses. Mycologia 79, 519–523.

Murray, K.D., 2005. A molecular diagnostic method for selected *Ascosphaera* species using PCR amplification of internal transcribed spacer regions of rDNA. J. Apic. Res. 44, 61–64.

Pitts-Singer, T., Cane, J., 2011. The alfalfa leafcutting bee, *Megachile rotundata*: the world's most intensively managed solitary bee. Annu. Rev. Entomol. 56, 221–237.

Posada, D., 2008. jModelTest: phylogenetic model averaging. Mol. Biol. Evol. 25, 1253–1256.

Rehner, S.A., Minnis, A.M., Sung, G., Luangsa-ard, J.J., Devotto, L., Humber, R.A., 2011. Phylogeny and systematics of the anamorphic, entomopathogenic genus *Beauveria.* Mycologia 103, 1055–1073.

Ronquist, F., Huelsenbeck , J.P., 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19, 1572–1574.

Skou, J.P., 1975. Two new species of *Ascosphaera* and notes on the conidial state of *Bettsia alvei*. Fresia 11, 62–74.

Skou, J.P., 1982.   Ascosphaerales and their unique ascomata. Mycotaxon 15, 487–499.

Skou, J.P., 1988. More details in support of the class Ascosphaeromycetes. Mycotaxon 31, 191–198.

Skou, J.P., King, J., 1984. *Ascosphaera osmophila* sp. nov., an Australian spore-cyst fungus. Australian Journal of Botany 32, 225–231.

Spatafora, J.W., Sung, G.H., Johnson, D., Hesse, C., O'Rourke, B., Serdani, M., Spotts, R., Lutzoni, F., Hofstetter, V., Miadlikowska, J., Reeb, V., Gueidan, C., Fraker, E., Lumbsch, T., Lucking, R., Schmitt, I., Hosaka, K., Aptroot, A., Roux, C., Miller, A.N., Geiser, D.M., Hafellner, J., Hestmark, G., Arnold, A.E., Budel, B., Rauhut, A., Hewitt, D., Untereiner, W.A., Cole, M.S., Scheidegger, C., Schultz, M., Sipman, H., Schoch, C.L., 2006. A five-gene phylogeny of Pezizomycotina. Mycologia 98, 1018–1028.

Spiltor, C.F., Olive, L.S., 1955. A reclassification of the genus *Pericystis Betts*. Mycologia 47, 238–244.

Wynns, A.A., Jensen, A.B., James, R., Eilenberg, J., 2011. *Ascosphaera subglobosa*, a new spore cyst fungus from North America associated with the solitary bee *Megachile rotundata.* Mycologia 104, 108–114.

Youssef, N.N., McManus, W.R., 2001. *Ascosphaera torchioi* sp. nov., a pathogen of *Osmia lignaria propinqua* Cresson (Hymenoptera). Mycotaxon 127, 7–13.

CHAPTER 3

MIXED INFECTIONS REVEAL VIRULENCE DIFFERENCES BETWEEN HOST-SPECIFIC

PATHOGENS [4,5,6]

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.

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, 1979, 1982; May and Anderson, 1979; de Roode *et al.*, 2008; Doumayrou *et al.*, 2013). 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 (Nowak and May, 1994; May and Nowak, 1995).

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 (Levin and Pimentel, 1981; Mosquera and Adler, 1998; Nowak and May, 1994; Alizon, 2013). 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 (May and Nowak, 1995; van Baalen and Sabelis, 1995; Martcheva and Pilyugin, 2006). 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 and Goettel, 1995; Goettel *et al.*, 1997; Gilliam, 2000; Vojvodic *et al.* 2011b), but Vojvodic *et al.* (2012) found honey bee mortality was significantly increased when saprophytic *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 (Bissett, 1988; Goettel *et al.*, 1997). It is not reported to occur in honey bee hives.

Methods

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 3.1). 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 $\times 10^5$

Table 3.1. Summary of fungal dosages: 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.

Number of spores in infective dose

Treatment	*A. aggregata*	*A. apis*	*A. larvis*	Total dose	Host	Number of replicates	Total number of larvae in experiment
Control	0	0	0	0	*A. mellifera*	3	131
					M. rotundata	3	139
A. apis	0	1000	0	1000	*A. mellifera*	3	76
					M. rotundata	3	101
A. aggregata	1000	0	0	1000	*A. mellifera*	3	75
					M. rotundata	3	104
A. larvis	0	0	1000	1000	*A. mellifera*	3	76
					M. rotundata	3	100
A. apis + *A. larvis*	0	1000	1000	2000	*A. mellifera*	3	72
					M. rotundata	3	68
A. aggregata + *A. larvis*	1000	0	1000	2000	*A. mellifera*	3	72
					M. rotundata	3	71
A. apis + *A. aggregata*	1000	1000	0	2000	*A. mellifera*	3	72
					M. rotundata	3	100

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 Raymond *et al.,* 2007; Chouvenc *et al*., 2012; 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 host the cuticle), and presence or absence of spore production were recorded.

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.

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 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).

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 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 *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 µl of diet, followed by 80 µl of diet on the third day

for a total of 160 µl 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 μl of spore suspension combined with 5 μl of diet. After a larva consumed the entire diet + spore treatment (approximately 2 hours), it was provided another 35 μl of diet without spores. Control insects received 5 μl of sterile deionized water combined with 5 μl 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 75-131 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.

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 cells removed. The 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 cells, leaving the pollen provisions remaining. These pollen provisions, still in the cells, were sterilized using gamma irradiation at a dose of 28 kGy (Xu and James, 2009). Sterile 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 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 of 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.

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. The fungus in each cadaver was quantified using quantitative real-time PCR (qRT-PCR). Total DNA from each cadaver was extracted using the MoBio UltraClean® 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 3.2) (James and Skinner, 2005), without multiplexing. For each qRT-PCR run (each time a set of reactions was

Table 3.2. Primer sequences used to identify and quantify each species of *Ascosphaera* fungus in this study. Adapted from James and Skinner, 2005.

Species	Forward Primer (5'-3')	Reverse Primer (5'-3')
A. aggregata	GCACTCCCACCCTTGTCTA	CTCGTCGAGGGTCTTTTCC
A. apis	GCACTCCCACCCTTGTCTA	CAGGCTCGCGAGAACCC
A. larvis	CGAACCAACTATTATTTTTCTGTGG	ATATGCTTAAGTTCAGCGGGT

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 $\log_{10}$ 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.

Statistical analyses

Larval survivorship was evaluated using Kaplan-Meyer 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. The interaction terms between the species were also included in the model. 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).

Results

Honey bees

For honey bees, the survivorship functions significantly differed between treatments (χ^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. 3.1A, 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 3.3). 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. 3.1A, 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. 3.2A, 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. 3.2A, 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 (χ^2_1=0.0285, *P*=0.8658), nor between *A. apis* solo and *A. apis* + *A. larvis* mixed infections (χ^2_1=1.2948, *P*=0.2552) (Fig. 3.3A). *A. aggregata* fungal material in the host did not significantly differ between *A. aggregata* solo infections and mixed infections with *A. larvis* (χ^2_1=3.0223, *P*=0.0821), but the amount of *A. aggregata* was significantly reduced when paired with *A. apis* (χ^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* (χ^2_1=5.1491, P=0.0233), or *A. aggregata* (χ^2_1=9.9619, P=0.0016).

Alfalfa leafcutting bees

In the alfalfa leafcutting bee, different treatments significantly affected larval survivorship (χ^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. 3.1B, Table A4). Solo treatments of *A. larvis* had significantly lower survival than the control treatment, but
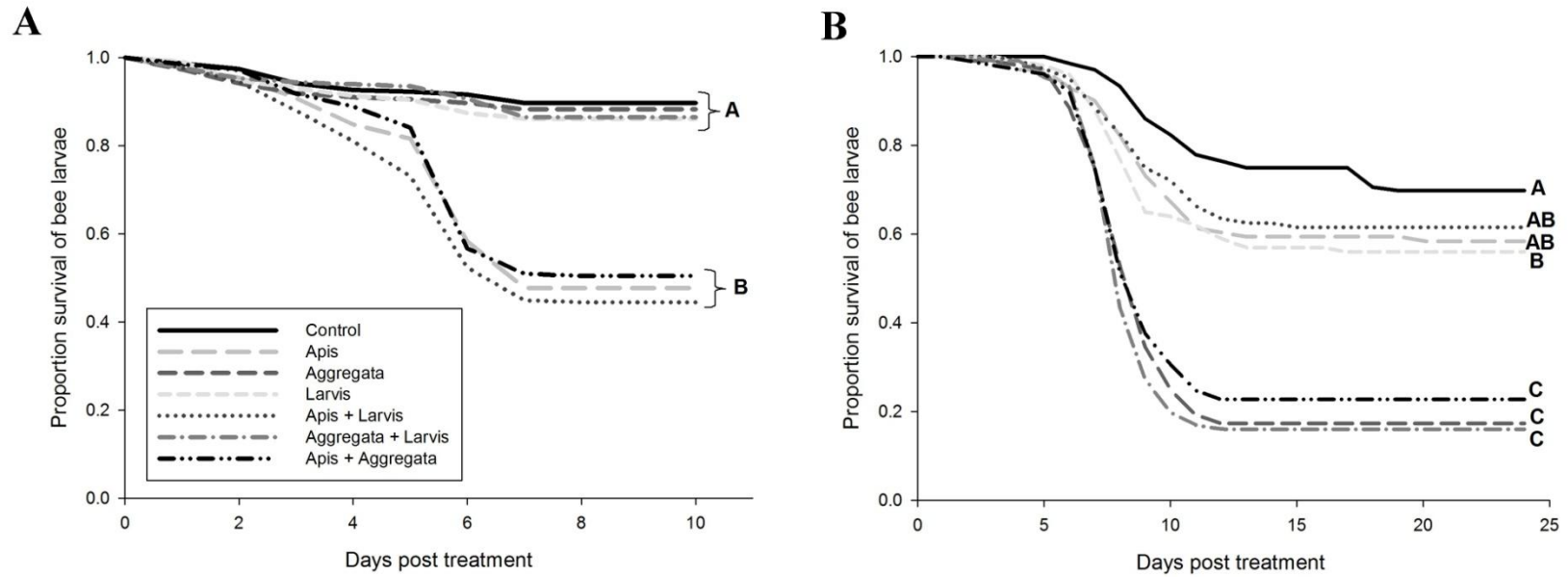
Figure 3.1 Survival of honey bee (A; *Apis mellifera*) and alfalfa leafcutting bee (B; *Megachile rotundata*) larvae challenged with one of 7

treatments. Letters represent significant differences in rate of survivorship as based from post-hoc Tukey's comparisons at an alpha level of 0.05.
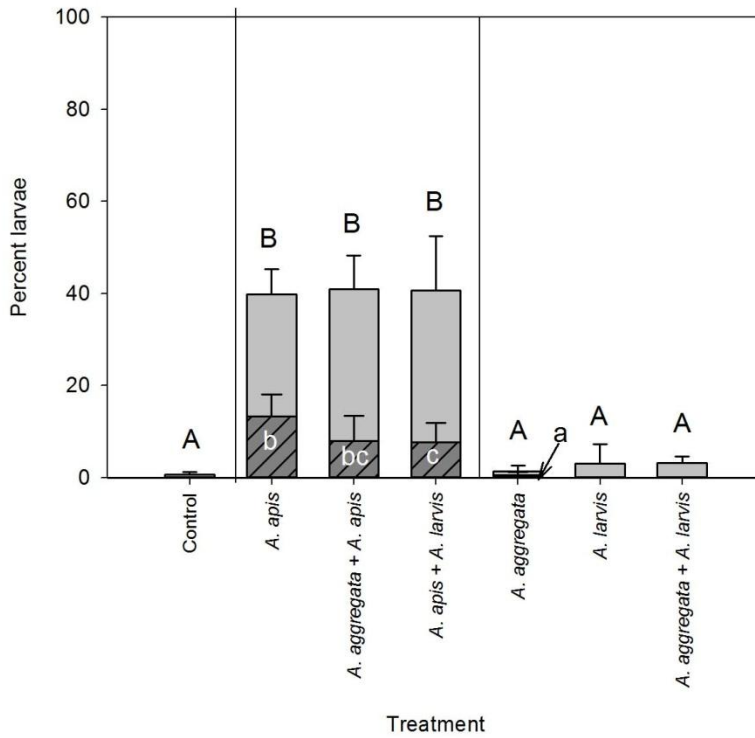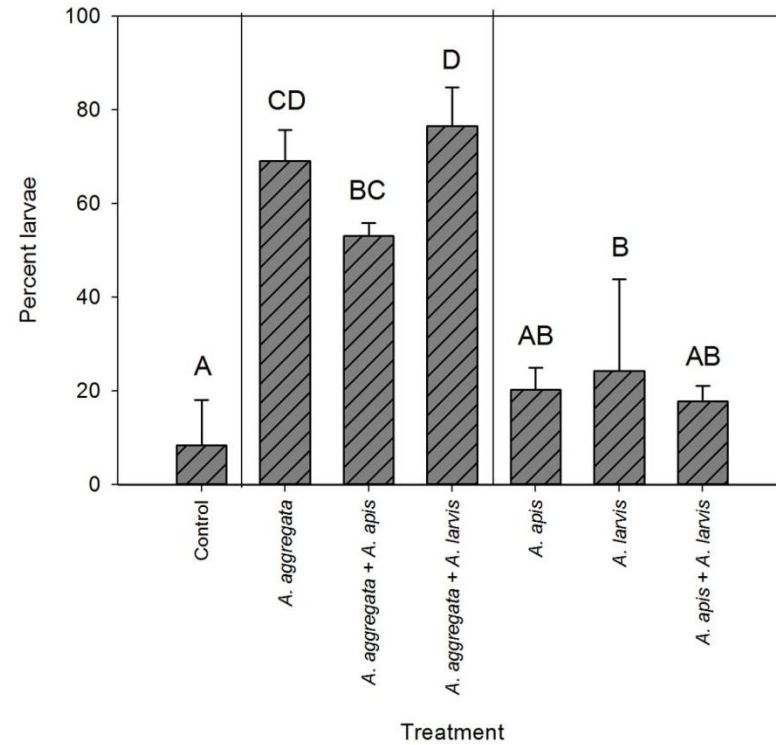
Figure 3.2. Proportion of total honey bee (A) and alfalfa leafcutting bee (B) larvae that showed external hyphal growth (solid grey bar) and spore development (hatched grey bar) after death. Letters represent significant differences in rate of hyphal or spore development as based from post-hoc Tukey's comparisons at an alpha of 0.05.
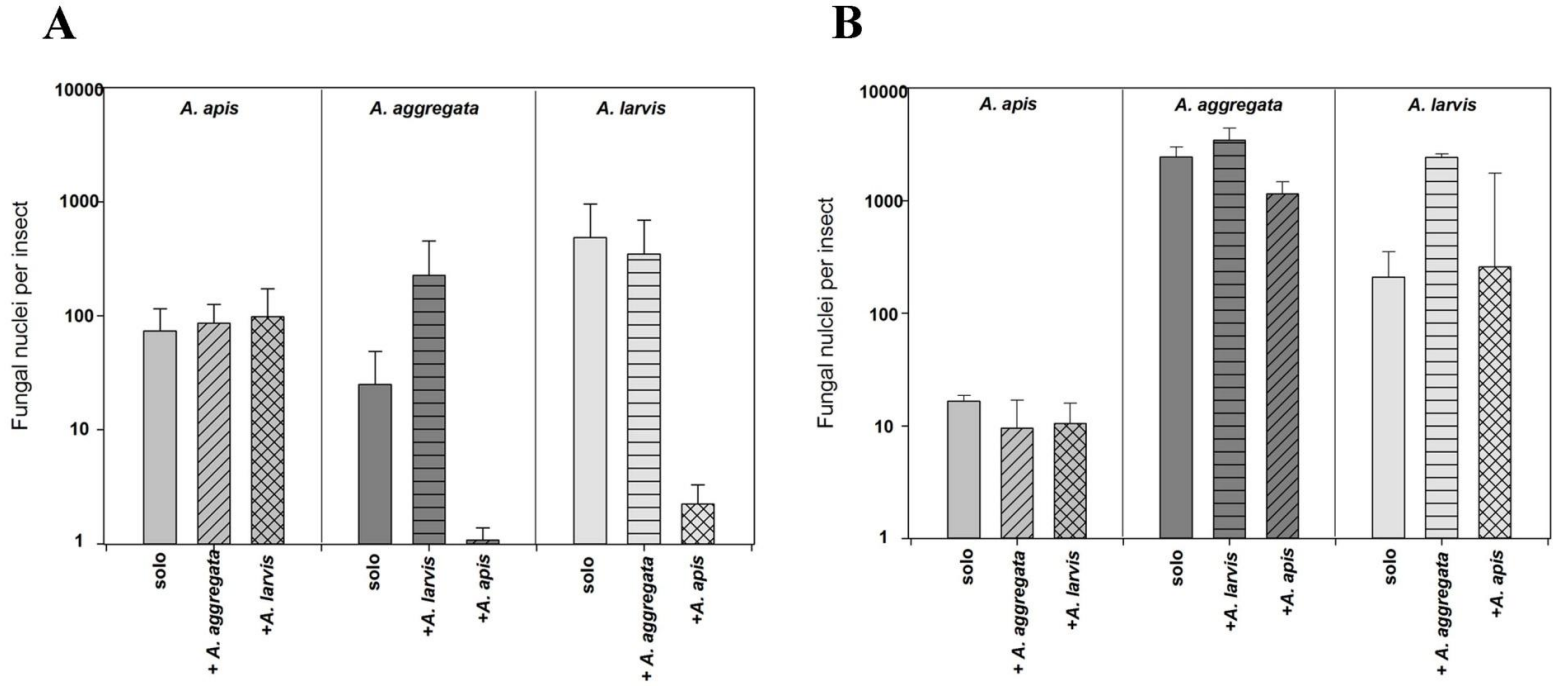
Figure 3.3. Relative amount of nuclear material identified in honey bee (A) and alfalfa leafcutting bee (B) larvae to a fungal species (top axis label) when combined with either no other species (solo) or one of the other species in this study (x-axis). Relative amounts based from quantifications taken from extractions of spores.

Table 3.3. Results of mixed model analysis for occurrence of mortality, hyphae and spores in honey bee larvae after exposure to one of six infective fungal treatments.

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

were not significantly different than survival in treatments with *A. apis* solo, or *A. apis* + *A. larvis* (Fig. 3.1B, Table A4). For the alfalfa leafcutting bee, it was only the obligate pathogen *A. aggregata* that significantly affected mortality (Table 3.4). 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 3.4). 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. 3.2B, Table A5). *A. apis* and *A. apis* + *A. larvis* treatments did not affect the proportion of larvae with spores, as compared to the

Table 3.4. Results of mixed model analysis for occurrence of mortality and spores in ALCB larvae after exposure to one of six infective fungal treatments.

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

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* (χ^2_1=0.9552, *P*=0.3284); nor when combined with *A. apis* (χ^2_1=2.9669, *P*=0.0850) (Fig. 3.3B). 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 (χ^2_1=2.9760, *P*=0.0845), and the *A. apis* + *A. larvis* (χ^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 (χ^2_1=1.2471, *P*=0.2641), nor *A. larvis* + *A. apis* treatments (χ^2_1=3.2272, *P*=0.0724) (Fig. 3.3B).

A small percentage of our control larvae were infected with *A. aggregata* (8.36±9.6%); these larvae died during the experiment and produced spores that were confirmed by PCR to be *A. aggregata* (Fig. 3.2B). 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**.**

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.

Each obligate pathogen displayed different competitive dynamics when fed to larvae with a congeneric pathogen. When *A. apis* was fed to honey bee larvae with either *A. larvis* or *A. aggregata*, it was highly competitive against these other pathogens. Not only did host mortality and fungal production levels remain unchanged with the addition of these other pathogens, but

also the fungal material found in the cadavers was primarily that of *A. apis*. Vojvodic *et al.* (2012) found honey bee mortality increased when *A. apis* was present in a mixed inoculation with *A. atra*, as compared to *A. apis* alone, which illustrates that specific characteristics of the co-infecting pathogen may be an important factor in the expression of *A. apis* within-host competiveness. However, in field surveys of chalkbrood-killed honey bee larvae, *Ascosphaera* species other than *A. apis* are not typically found on the larvae, although other bacterial and fungal species are present (Johnson *et al.*, 2005). Based on the evidence of *A. apis* as the only *Ascosphaera* species present post-larval death in both of our studies and other studies, and based on predictions from superinfective models (Nowak and May, 1994; Mosquera and Adler, 1998), virulence evolution of *A. apis* in honey bees may be driven by selective pressures to outcompete other non-specific *Ascosphaera* pathogens. We have shown in this study that the presence of *A. aggregata* or *A. larvis* does not alter the host/pathogen dynamics between *A. apis* and honey bees.

Although high virulence can make a pathogen very competitive against other pathogens, extremely high virulence will cause host populations to decline rapidly, reducing the long term ability of the pathogen to persist. Thus, superinfective pathogens are expected to ultimately maintain a competitive, but self-sustaining, level of virulence (Alizon, 2008; Vojvodic *et al.*, 2011a). And true to this expectation, *A. apis* infections in honey bees are widespread, but the disease occurs at low to moderate levels in affected honey bee colonies, especially when compared to other diseases of bees (Gilliam, 2000; Vojvodic *et al.*, 2012). Models of superinfection also predict that, within a superinfective pathogen species, many strains will occur with a high diversity of virulence levels, as superinfective competition will also take 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 (Thomas *et al.*, 2003; Harrison *et al.*, 2006; Lohr *et al.*, 2010; Vojvodic *et al.*, 2012). In our study, though, mixed pathogen inoculates did not significantly increase or decrease mortality as compared to solo pathogen inoculates. Chouvenc *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* (See Chapter 2), 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.

References

Alizon, S., 2008. Decreased overall virulence in coinfected hosts leads to the persistence of virulent parasites. Am. Nat. 172, E67–E79.

Alizon, S., 2013. Co-infection and super-infection models in evolutionary epidemiology. Interface Focus 3, 20130031.

Alizon, S., de Roode, J.C., Michalakis, Y., 2013. Multiple infections and the evolution of virulence. Ecol. Lett. 16, 556–567.

Alizon, S., Hurford, A, Mideo, N., Van Baalen, M., 2009. Virulence evolution and the trade-off hypothesis: history, current state of affairs and the future. J. Evol. Biol. 22, 245–259.

Anderson, D.L., Gibson, N., 1998. New species and isolates of spore-cyst fungi (Plectomycetes: Ascosphaerales) from Australia. Aust. Syst. Bot. 11, 53–72.

Anderson, R., May, R., 1982. Coevolution of hosts and parasites. Parasitology 55, 411–426.

Anderson, R.M., May, R.M., 1979. Population biology of infectious diseases: Part I. Nature 280, 361–367.

Aronstein, K.A., Murray, K.D., 2010. Chalkbrood disease in honey bees. J. Invertebr. Pathol. 103, S20–S29.

Aupinel, P.A., Fortini, D., Doufour, H., Tasei, J., Michaud, B., Odoux, J.-F., Pham-Delegue, M., 2005. Improvement of artificial feeding in a standard in vitro method for rearing *Apis mellifera* larvae. Bull. Insectol 58, 107–111.

Bashey, F., Reynolds, C., Sarin, T., Young, S.K., 2011. Virulence and competitive ability in an obligately killing parasite. Oikos 120, 1539–1545.

Ben-Ami, F., Mouton, L., Ebert, D., 2008. The effects of multiple infections on the expression and evolution of virulence in a *Daphnia*-endoparasite system. Evolution 62, 1700–1711.

Bissett, J., 1988. Contribution toward a monograph of the genus *Ascophaera*. Can. J. Bot. 66, 2541–2560.

Brant, S.V., Loker, E.S., 2005. Can specialized pathogens colonize distantly related hosts? Schistosome evolution as a case study. PLoS Pathog. 1, 167–169.

Brockhurst, M.A., Koskella, B., 2013. Experimental coevolution of species interactions. Trends Ecol. Evol. 20, 1–9.

Buckling, A., Brockhurst, M., 2008. Kin selection and the evolution of virulence. Heredity 100, 484–488.

Chao, L., Hanley, K., Burch, C., Dahlberg, C., 2000. Kin selection and parasite evolution: higher and lower virulence with hard and soft selection. Q. Rev. Biol. 75, 261–275.

Chouvenc, T., Efstathion, C.A, Elliott, M.L., Su, N.-Y., 2012. Resource competition between two fungal parasites in subterranean termites. Naturwissenschaften 99, 949–958.

de Roode, J.C., Yates, A.J., Altizer, S., 2008. Virulence-transmission trade-offs and population divergence in virulence in a naturally occurring butterfly parasite. Proc. Natl. Acad. Sci. U. S. A. 105, 7489–7494.

Doumayrou, J., Avellan, A., Froissart, R., Michalakis, Y., 2013. An experimental test of the transmission-virulence trade-off hypothesis in a plant virus. Evolution 67, 477–486.

Frank, S.A., 1994. Kin selection and virulence in the evolution of protocells and parasites. Proc. Biol. Sci. 258, 153–161.

Frank, S.A., 1996. Models of parasite virulence. Q. Rev. Biol. 71, 37–78.

Gilliam, M., 2000. Infectivity and survival of the chalkbrood pathogen, *Ascosphaera apis.* Apidologie 17, 93–100.

Goettel, M.S., Duke, G.M., Goerzen, D.W., 1997. Pathogenicity of *Ascosphaera larvis* to larvae of the alfalfa leafcutting bee, *Megachile rotundata.* Can. Entomol. 129, 1059–1065.

Harrison, F., Browning, L.E., Vos, M., Buckling, A., 2006. Cooperation and virulence in acute *Pseudomonas aeruginosa* infections. BMC Biol. 4, 21.

Hughes, W.O.H., Boomsma, J.J., 2004. Let your enemy do the work: within-host interactions between two fungal parasites of leaf-cutting ants. Proc. Biol. Sci. 271, S104–S106.

James, R.R., Buckner, J.S., 2004. Lipids stimulate spore germination in the entomopathogenic ascomycete *Ascosphaera aggregata*. Mycopathologia 158, 293–302.

James, R.R., Pitts-Singer, T.L., 2013. Health status of alfalfa leafcutting bee larvae (Hymenoptera: Megachilidae) in United States alfalfa seed fields. Environ. Entomol. 1166–1173.

James, R.R., Skinner, J.S., 2005. PCR diagnostic methods for *Ascosphaera* infections in bees. J. Invertebr. Pathol. 90, 98–103.

Johnson, R., Zaman, M., Decele, M., 2005. Multiple microorganisms in chalkbrood mummies: evidence and implications. J. Apic. Res. 44, 29–32.

Kirchner, J., Roy, B., 2002. Evolutionary implications of host-pathogen specificity: fitness consequences of pathogen virulence traits. Evol. Ecol. Res. 27–48.

Lee, G.M., McGee, P.A., Oldroyd, B.P., 2013. Variable virulence among isolates of *Ascosphaera apis*: testing the parasite-pathogen hypothesis for the evolution of polyandry in social insects. Naturwissenschaften 100, 229–234.

Levin, S., Pimentel, D., 1981. Selection of intermediate rates of increase in parasite-host systems. Am. Nat. 117, 308–315.

Lohr, J.N., Yin, M., Wolinska, J., 2010. Prior residency does not always pay off–co-infections in *Daphnia*. Parasitology 137, 1493–1500.

Luijckx, P., Ben-Ami, F., Mouton, L., Du Pasquier, L., Ebert, D., 2011. Cloning of the unculturable parasite *Pasteuria ramosa* and its *Daphnia* host reveals extreme genotype-genotype interactions. Ecol. Lett. 14, 125–131.

Martcheva, M., Pilyugin, S.S., 2006. The role of coinfection in multidisease dynamics. SIAM J. Appl. Math. 66, 843–872.

May, R.M., Anderson, R.M., 1979. Population biology of infectious diseases: Part II. Nature 280, 455–461.

May, R.M., Nowak, M.A., 1995. Coinfection and the evolution of parasite virulence. Proc. Biol. Sci. 261, 209–215.

McFrederick, Q.S., Mueller, U.G., James, R.R., 2014. Interactions between fungi and bacteria influence microbial community structure in the *Megachile rotundata* larval gut. Proc. R. Soc. B. 281. 20132653.

McManus, W.R., Youssef, N.N., 1984. Life cycle of the chalk brood fungus, *Ascosphaera aggregata*, in the alfalfa leafcutting bee, *Megachile rotundata*, and its associated symptomatology. Mycologia 76, 830–842.

Mosquera, J., Adler, F.R., 1998. Evolution of virulence: a unified framework for coinfection and superinfection. J. Theor. Biol. 195, 293–313.

Nowak, M., May, R., 1994. Superinfection and the evolution of parasite virulence. Proc. R. Soc. B. 255, 81–89.

Raymond, B., Davis, D., Bonsall, M.B., 2007. Competition and reproduction in mixed infections of pathogenic and non-pathogenic *Bacillus* spp. J. Invertebr. Pathol. 96, 151–155.

Read, A.F., 1994. The evolution of virulence. Trends Microbiol. 2, 73–76.

Rumbaugh, K.P., Trivedi, U., Watters, C., Burton-Chellew, M.N., Diggle, S.P., West, S.A., 2012. Kin selection, quorum sensing and virulence in pathogenic bacteria. Proc. Biol. Sci. 279, 3584–3588.

Thomas, M., Watson, E., Valverde-Garcia, P., 2003. Mixed infections and insect–pathogen interactions. Ecol. Lett. 6, 183–188.

van Baalen, M., Sabelis, M.W., 1995. The dynamics of multiple infection and the evolution of virulence. Am. Nat. 146, 881–910.

Vandenberg, J.D., Goettel, M.S., 1995. Chalkbrood susceptibility among alfalfa leafcutting bee (Hymenoptera: Megachilidae) larvae reared at different temperatures. J. Econ. Entomol. 88, 810–814.

Vojvodic, S., Boomsma, J.J., Eilenberg, J., Jensen, A.B., 2012. Virulence of mixed fungal infections in honey bee brood. Front. Zool. 9, 5.

Vojvodic, S., Jensen, A.B., James, R.R., Boomsma, J.J., Eilenberg, J., 2011a. Temperature dependent virulence of obligate and facultative fungal pathogens of honeybee brood. Vet. Microbiol. 149, 200–205.

Vojvodic, S., Jensen, A.B., Markussen, B., Eilenberg, J., Boomsma, J.J., 2011b. Genetic variation in virulence among chalkbrood strains infecting honeybees. PLoS One, e25035.

Woolhouse, M.E.J., Webster, J.P., Domingo, E., Charlesworth, B., Levin, B.R., 2002. Biological and biomedical implications of the co-evolution of pathogens and their hosts. Nat. Genet. 32, 569–577.

Wynns, A.A., 2012. The bee specialist fungus family Ascosphaeraceae and its allies: systematics, ecology and co-evolution with solitary bees. PhD dissertation. University of Denmark, Copenhagen.

Xu, J., James, R., 2009. Genes related to immunity, as expressed in the alfalfa leafcutting bee, *Megachile rotundata*, during pathogen challenge. Insect Mol. Biol. 18, 785–794.

CHAPTER 4

COMPETITION BETWEEN TWO CO-INFECTING FUNGAL PATHOGENS OF THE

ALFALFA LEAFCUTTING BEE, *MEGACHILE ROTUNDATA*[7]

Abstract

Infection of insect hosts with more than one pathogen is a common occurrence, and continued study of competing pathogens advances our understanding of virulence evolution. We studied a co-infection that occurs readily between *Ascosphaera aggregata* and *Ascosphaera proliperda*, two fungal pathogens in the alfalfa leafcutting bee. Growth of the fungi, bee larval mortality, and fungal spore production were studied in treatments with each fungal species solo, and in combinations of both species together. We found that *A. aggregata* was highly virulent to the bees, in solo and in mixed infections, although this fungus was limited to specific environments for growth *in vitro*. *A. proliperda* was able to utilize a wide range of *in vitro* growth environments, but had low virulence to bees, although its virulence levels increased in mixed infections with *A. aggregata*. Sequential infections yielded higher larval survival than simultaneous infections. No chemical inhibition was found between these species that could account for reduced larval mortality in the sequential infections, and increased larval survival most likely depends upon immune response variations in the host.

[7] This chapter is co-authored by D.L. Welker and R.R. James.

Introduction

Many species of microbes are pathogenic to other host organisms; however, there is no one characteristic that confers pathogenicity. The ability of a pathogen to infect a host comes from the convergence of a favorable environment, susceptible host genotype and phenotype, and an infective pathogen genotype and phenotype (Méthot and Alizon, 2015). Despite these constraints, host species are generally infected with multiple species or strains of a pathogen, as opposed to infection by a single pathogen (Rigaud *et al.*, 2010).

The fungal genus *Ascosphaera* is specific to bee nesting habitats and contains several species that exhibit a high level of pathogenicity to various bee species (manifesting as a disease known as chalkbrood). However this genus also contains species that are less frequently pathogenic or apathogenic (Anderson and Gibson, 1998). One species, *Ascosphaera aggregata*, is the major species infecting the solitary nesting bee, *Megachile rotundata*, also known as the alfalfa leafcutting bee (McManus and Youssef, 1984). The alfalfa leafcutting bee (ALCB) is an economically important pollinator species whose services are essential to the successful production of alfalfa seed in the northwestern United States and western Canada (Pitts-Singer and Cane, 2011). ALCBs are solitary nesting bees where each female bee prepares and provisions her own nest cells with pollen, nectar, and eggs. Management of diseases in this bee can be difficult due to the closed nature of nest cells (James and Pitts-Singer, 2005). Chalkbrood rates in this bee can exceed 20% of larval populations (James and Pitts-Singer, 2013). It is important to understand diseases and the dynamics between infecting microbes in this bee in order for researchers to determine the best practices to reduce larval mortality in this system.

A recent study of diseased ALCB larvae in the United States and Canada showed that co-infections composed of more than one *Ascosphaera* species were not rare, and that the most common co-infecting *Ascosphaera* species with *A. aggregata* was *Ascosphaera proliperda* (R.

James, unpublished data). We studied the dynamics of growth and virulence between these two pathogens, as co-infections can influence pathogen virulence (Alizon, 2013). We first characterized the *in vitro* growth of each pathogen to evaluate the range of environments and nutrients the pathogen can potentially utilize, as well as to evaluate whether such growth correlated with overall fungal competitive ability. We compared the growth rates obtained to those of a closely related saprophytic species. Next, we quantified larval mortality, and the quantity and identity of spores produced in infections with each pathogen in a solo infection, as well as in simultaneous co-infections with both pathogens, and in sequential infections with both pathogens. Finally, in an effort to determine if the pathogens directly compete via chemical inhibition, we attempted to grow each pathogen in the presence of filtrates of liquid broth that had been used to grow itself or the other pathogen.

Methods

Growth experiment fungal sources and media

A growth comparison experiment was carried out using three fungal species, *A. aggregata*, *A. proliperda* and a closely related saprophytic species, *Chrysosporium farinicola*. *C. farinicola* is found growing on bee pollen and associated with bee habitats and is the anamorph to *Eremascus albus*, which is closely related to the *Ascosphaera* (Pitt *et al.*, 2013).

Single spore isolates of *A. aggregata* and *A. proliperda* were taken from ALCB cadavers collected in the vicinity of Logan, Utah. Isolate identity was confirmed via PCR (James and Skinner, 2005). A culture of *C. farinicola* was obtained from the American Type Culture Collection (ATCC® 18053™; Manassas, VA; Pitt, 1966). Cultures of *A. aggregata* were maintained on modified V8 agar (James, 2005), *A. proliperda* was maintained on Sabourad

Dextrose Agar (SDA) and *C. farinicola* was maintained on a high glucose medium (HG), which was composed of 41.37g glucose, 4.1g yeast extract, 6 g of malt extract, 0.68 g peptone, and 2 g agarose per 100 ml of reverse osmosis water. All fungal cultures were maintained in the dark at 25 °C.

Growth experiment design

Cultures were grown for 10 days on appropriate media (see above), and at the time of experiment, 6 mm diameter plugs were cut 3 mm from the margins of the growing hyphal culture with a sterilized metal cork borer. These plugs were placed in the center of petri dishes containing 20 ml of fresh agar. Plates were sealed with parafilm and perpendicular axes were drawn on the top of each Petri dish so that the two lines intersected in the center of the plug. Each of the three species of fungi (*A. aggregata, A. proliperda* and *C. farinicola*) was placed on all three types of fresh agar media (V8, SDA and HG). After sealing, each plate was placed at one of four temperatures (20, 25, 30 or 35 °C). For each replicate, six plates were made for each fungal species for each combination of media and each combination of temperature, for a total of 54 plates per temperature and 216 plates per replicate. The experiment was replicated three times (648 total plates).

Fungal growth was recorded as distance (mm) along each axis every 3 days for the first 9 days, after which growth was measured every 6 days. Average daily growth was calculated. To determine differences in growth rate between each set of variables, a 4 x 3 x 3 factorial ANOVA was used to determine significant effects between the main variables of temperature, media and fungal species, as well as all levels of interactions between the variables.

Larval bioassay

ALCB larvae were exposed to one of six fungal pathogen treatments. To expose larvae in a lab setting, ALCB nests were collected from a commercial field (Corrine, UT). These nests were dissected and the egg in each nest cell was removed, so that only the pollen provision and the outer leaf cell material remained. These dissected, eggless cells were sterilized via gamma irradiation at a dose 28 kGy (Xu and James, 2009). After sterilization, the pollen provision within the cells was coated with either a layer of infective or non-infective spore material. To coat the pollen provisions, the leaf cells were placed in a 96 well plate so that the leaf cells sat upright with access to the pollen provisions via the top. Either 2.5 or 5 µl of liquid material (depending upon the treatment, see below) was pipeted directly on the surface of the pollen provision. The inoculated leaf cells were placed in a laminar flow hood for one hour to allow the excess moisture to evaporate, leaving any infective spores. 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, then the 96-well tissue culture plates containing the treatments were incubated at 29°C and 75% humidity (BD Biosciences, San Jose, CA). Larval mortality was checked every day until all surviving larvae had spun a prepupal cocoon (24 days maximum). Larvae were considered deceased when feeding movement was no longer observed and larval body structure appeared rigid. After death, the cadavers were monitored for evidence of fungal growth. Time to death (in days) and presence or absence of spores was recorded.

Treatment fungal sources and dosage calculations

Bioassays were conducted using two *Ascosphaera* pathogens (*A. aggregata* and *A. proliperda*) in the ALCB. Initial *A. aggregata* inocula were field collected from dead, infected bee larvae found in the vicinity of Logan, UT, between July and August 2010 and 2011. Spores were prepared in an identical manner as Chapter 3.   *A. proliperda* spores were obtained from cultures isolated and maintained identically as in our previous growth experiment. For each experiment, spore viability for the *Ascosphaera* species was verified following a CO_2 rich, liquid germination protocol for *A. aggregata* and a liquid SD broth germination for *A. proliperda* (James, 2005).

In the single pathogen treatments, larvae were fed 5 μl of a 5 x10^5 spores/ml mixture, resulting in a dose of 2500 spores per larva. For multiple pathogen treatments, larvae were fed 2.5 μl of 1x10^6 spores/ml of each pathogen (2500 spores), resulting in a total dose of 5 μl and 5000 spores per larva, providing a two-way multivariate experimental design to test the effects of co-infections as compared to single infections.

Three replicates of each of six treatments were conducted, each replicate being initiated on different days. Each treatment started with 36 eggs; however, the washing process can damage eggs, and eggs or first instars that collapsed prior to larval feeding were removed from the experiment and were not used in the data analyses. The experiment was conducted over two years and replicated 3-4 times each year for a total of 146-329 larvae in each treatment.

Quantification of fungal material from bioassays

Approximately one month after the bioassay, 10 bee cadavers were chosen via random number selection from each of the five infective treatments (not the control treatment). Spore

material on the outside of the larval body was separated from the dense inner hyphal material for each larvae in order to quantify the spore material separately from the vegetative hyphal material. Total DNA from each cadaver was extracted using the MoBio UltraClean® Plant DNA extraction kit with some modifications (MoBio, Solina, CA; see Chapter 3). Each fungal sample was quantified using quantitative real-time PCR (qRT-PCR) using the Bio-Rad Opticon Monitor v. 3.0 system (Bio-Rad Laboratories, Inc., see Chapter 2). Fungal quantities are reported as "number of nuclei", as the standards for PCR quantification were created from fungal spores, which contain one nucleus each (hyphae can contain multiple nuclei).

Statistical analyses

Larval survivorship was statistically evaluated using Kaplan Meyer 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. 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 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 treatments. A generalized linear model with post hoc Tukey's tests was used to determine the sources of variation in the amount of fungal material produced in each treatment, including the identity of the fungal material as well as whether it was reproductive spores or vegetative hyphae.

Inhibition

The ability of extracts from the broth of growing *Ascosphaera* hyphae to inhibit the growth of new hyphae was tested through radial growth tests. Five 9 mm diameter agar plugs were cut 3 mm from the growing margins of either an *A. aggregata* or *A. proliperda* culture that was 10 days old. The five plugs were placed into a 100 ml flask containing the ideal liquid broth for each species (V-8 broth for *A. aggregata* and SD broth for *A. proliperda*). Flasks were incubated at 29 °C with gentle agitation at 50 rpm for four days. After 4 days, the hyphae were filtered from the broth. For *A. aggregata*, hyphae were separated from broth first through gravity filtration through four layers of miracloth (Merck Millipore, Bilerica, MA), then vacuum filtered through glass fiber filters (Grade G6, Fisher Scientific, Pittsburgh, PA), and finally filtered through cellulose nitrate syringe membrane filters (0.45 um pore size, Whatman, Dassel, Germany). The same steps were followed for *A. proliperda* hyphae except the glass filtration step was removed. Flasks of broth only (no hyphal plugs) were incubated the same as those with hyphae and filtered in the same fashion, these are hereafter referred to as "blank filtrate." Various amounts of filtrate from hyphal cultures were then combined with amounts of the blank filtrate as well as various amounts of new, sterile V8 or SD agar to create a range of concentrations of filtrate in agar (Table 4.1). Five 20 ml plates were poured from each of the 100 ml total agar solutions. New agar plugs (9 mm diameter) were cut from either an *A. aggregata* or *A. proliperda* culture plate. These plugs were placed hyphae side down in the centers of the experimental agar plates. Plates were incubated at 29°C and fungal growth was checked daily, as in our first growth experiment. The experiment was replicated twice.

Inhibitory ability of the broth extracts from *Ascosphaera* germinated spores was tested on newly germinating spores. Spores were collected from dead ALCB cadavers (*A. aggregata*) and culture plates (*A. proliperda*), in the same manner as for the larval bioassay portion of our

study (see above). A 1 x 10^6 spore/ml mixture was made in sterile water. Sterile broth (either

900 μl of V8 or SD broth) was placed in wells of a 24 well plate (Falcon®) and 100 μl of the 1 x

10^6 spore/ml solution was added to each well. The 24 well plate was placed on a shaker at 29 °C

and incubated for 48 hours. The 24 well plate containing *A. aggregata* was also incubated in the

same manner with the addition of 20% CO_2.

Table 4.1. Experimental design of hyphae-hyphae competition experiment. Broth extract from

growing hyphal cultures was filtered and added (filtrate) to amounts of blank filtered broth (blank

filtrate). This combined liquid was then added to an amount of an agar-based media and poured

into petri dishes (20 ml per petri dish).

Treatment	Amount of filtrate (ml)	Amount of blank filtrate (ml)	Amount of agar (ml)
1	0	20	80
2	5	15	80
3	10	10	80
4	20	0	80

After incubation, fungal filtrate was recovered (as above). To test the effect of these filtrates on the germination of new spores, we added a total volume of 200 µl of experimental treatment to 800 µl of new broth (Table 4.2). New spores were added to the experimental broth combinations, as a volume of 100 µl of 1 x 10^6 spores/ml. The plates were incubated with gentle shaking at 29 °C and at 20% CO_2. Each day, for 5 days, the optical density of the wells was calculated using a microplate reader (PowerWave XS2; Bio-Tek; Winooski, VT). Two replicates of the experiment were conducted.   Average fungal growth per day was analyzed within a generalized linear model framework with level of inhibitor as a continuous dependent variable.

Table 4.2. Experimental design of spore-spore competition experiment. Broth extract from germinating spores was filtered and added (filtrate) to amounts of blank filtered broth (blank filtrate). This combined liquid was then added to broth media and re-inoculated with new spore material.

Treatment	Amount of filtrate (µl)	Amount of blank filtrate (µl)	Amount of broth (µl)
1	0	200	800
2	50	150	800
3	100	100	800
4	200	0	800

Results

Growth experiment

There were highly significant differences between fungal growth rates (F [2, 22]=1034.79, $P<0.0001$), with *A. proliperda* having the fastest average growth rate (8.30 ± 2.61 mm/day), *A. aggregata* the next fastest rate (1.38 ± 1.50 mm/day) and *C. farinicola* the slowest growth rate (0.30 ± 0.51 mm/day). Media and temperature were both highly significant for all three fungal species, as was the interaction between the two factors (Table 4.3). *C. farinicola* grew best on HG medium between 20 and 25 °C (Figure 4.1) and had much reduced growth on the V-8 and SDA media. Conversely, *A. aggregata* was not able to grow on the HG medium, but grew well on both the V-8 and SDA media between 25-30 °C (Figure 4.1). *A. proliperda* was able to utilize all three types of media, with similar growth rates at 30°C (Figure 4.1).

Larval bioassay

In the alfalfa leafcutting bee, different treatments significantly affected larval survivorship (χ^2_5=348.21, $P<0.0001$). All fungal treatments significantly reduced larval survival over the mortality experienced by the control larvae (approximately 22%; Figure 4.2). *A. aggregata* in a solo dose reduced larval survival as compared to the *A. proliperda* solo dose (χ^2_1=105.4, $P<0.001$; Figure 4.2). When *A. aggregata* and *A. proliperda* were combined and given to the larvae simultaneously, larval survival did not significantly decrease from the survival seen with *A. aggregata* solo (χ^2_1=2.036, P=0.71; Figure 4.2). However, when the combined dose of *A. aggregata* and *A. proliperda* was staggered, larval survival increased over that observed with either the solo *A. aggregata* or the simultaneous dose. The order of sequential dosage did not significantly affect the resulting larval survival (χ^2_1=0.150, P=0.9989; Table B1).

Table 4.3. Analysis of variance table describing the effect of fungal species (*A. aggregata*, *A. proliperda* and *C. farinicola*), media type (V8, SDA, and HG) and temperature (20, 25, 30, and 35°C) on average fungal growth.

Treatment	DF	F Value	Pr>F
Mortality			
Rep	2	2.01	0.1417
Temperature	3	22.63	<0.0001
Fungal species	2	1034.79	<0.0001
Media type	2	28.94	<0.0001
Fungal species*Temperature	6	20.65	<0.0001
Temperature*Media type	6	5.91	<0.0001
Fungal Species*Media type	4	19.04	<0.0001
Fungal Species*Media type*Temperature	12	5.33	<0.0001

Figure 4.1.   Average growth rate (mm/day) of three fungal species, *A. aggregata* (top), *A. proliperda* (middle) and *C. farinicola* (bottom) grown at different temperatures (20, 25, 30 and 30°C) on three different types of media (V-8, SDA and HG).   Data points within a box are not significantly different from each other, based on Tukey's multiple comparisons at a significance level of 0.05.

A. aggregata

A. proliperda

C. farinicola

Survival of ALCB larvae treated with *Ascospahera* spores
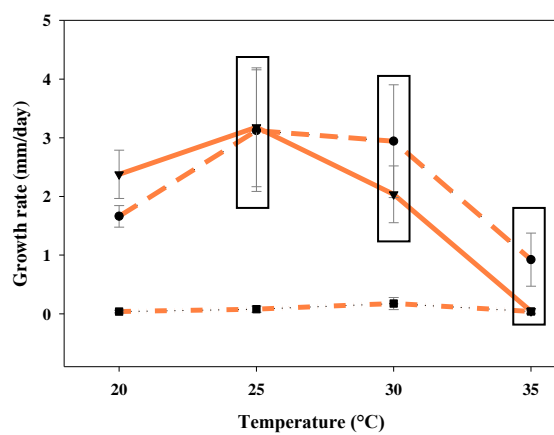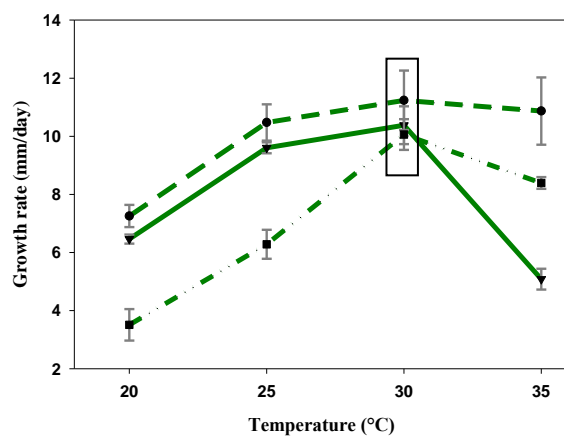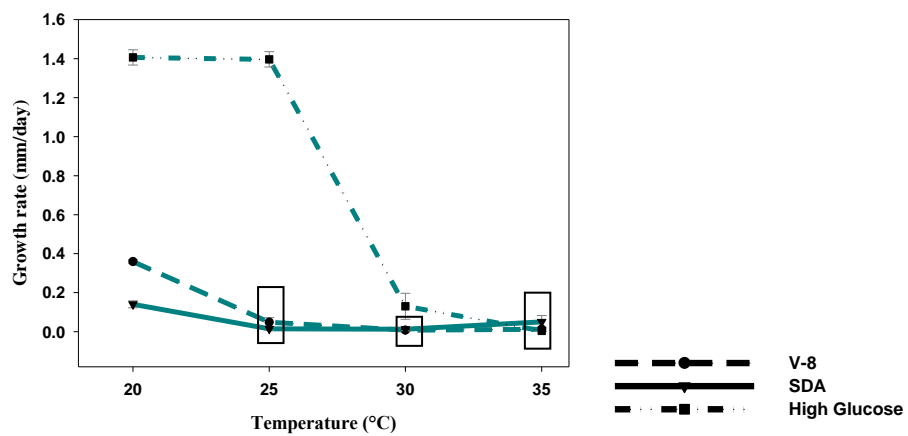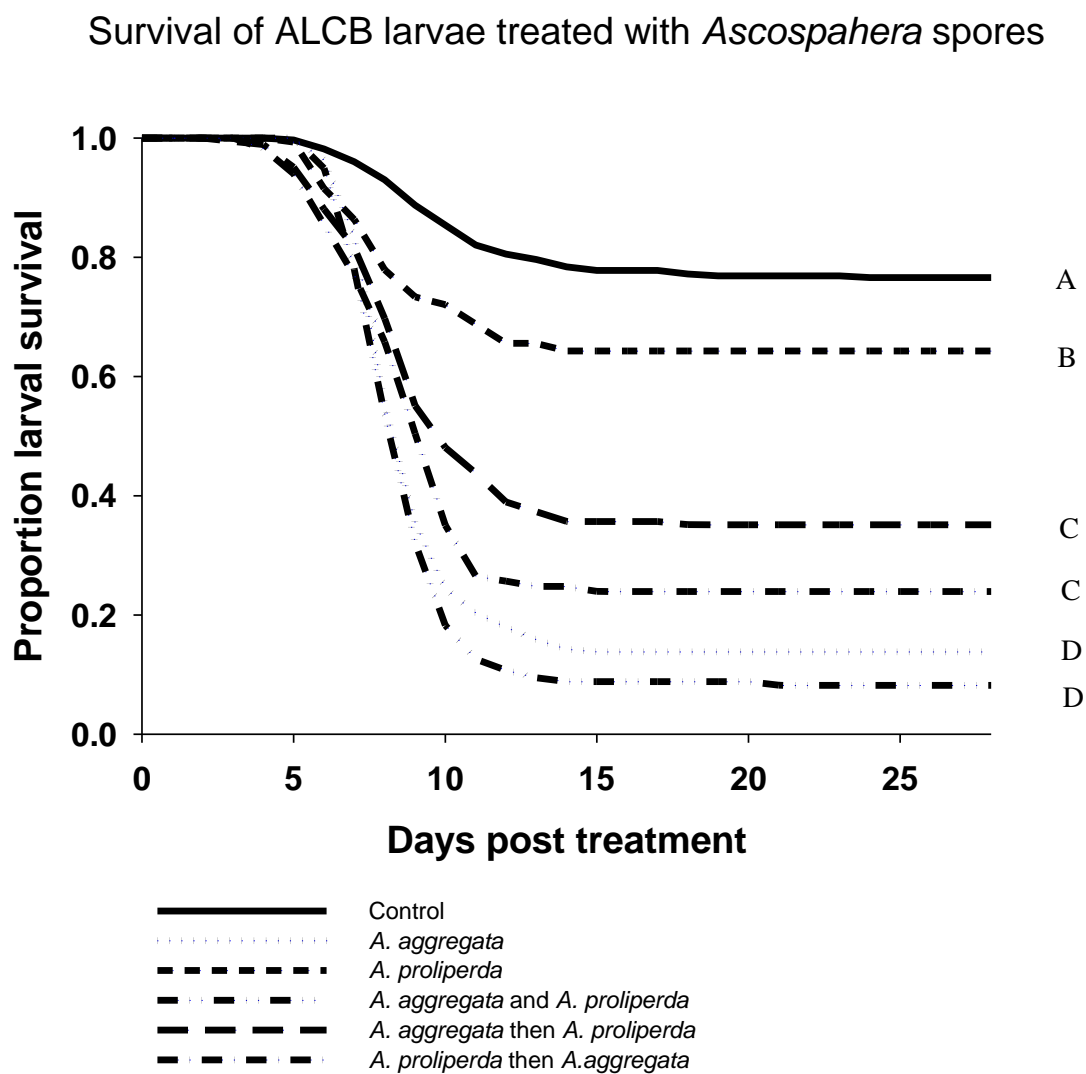
Figure 4.2. Survival of alfalfa leafcutting bee larvae challenged with one of six treatments. Letters represent significant differences in rate of survivorship as based from post-hoc Tukey's comparisons at an alpha level of 0.05.

Treatment was a significant factor in the proportion of cadavers that produced spores (F [5, 45]=16.79, p<0.001). Larvae killed by *A. proliperda* alone did not produce significantly more sporulated cadavers than the control treatment (there was a small fraction of control larvae that died due to incomplete sterilization of the eggs or pollen provisions; Figure 4.3). All other treatments produced significantly more sporulating cadavers than the control treatment, but at proportions that did not differ significantly from each other (Figure 4.3). The simultaneous *A. aggregata* and *A. proliperda* treatment produced spores the fastest, averaging 16.6 ± 2.5 days (Table 4.4). This was significantly faster than spores were produced in the treatment with a staggered dose of *A. aggregata* first, followed by *A. proliperda* dose. However, it was not significantly faster than the reverse sequential treatment or than the solo *A. aggregata* treatment (Table 4.4).

Table 4.4. Average (±S.E.) days until spores were visible on dead ALCB larvae that were given one of six treatments. Letters correspond to significant differences between mean times based on Tukey's multiple comparisons at a significance level of 0.05.

Treatment	Average days to sporulation	
Control	23.3 ± 1.4	A
A. aggregata	16.9 ± 2.3	BD
A. proliperda	23.8 ± 0.3	A
A. aggregata and *A. proliperda*	16.6 ± 2.5	BC
A. aggregata then *A. proliperda*	20.0 ± 2.7	AD
A. proliperda then *A. aggregata*	18.7 ± 3.8	CD
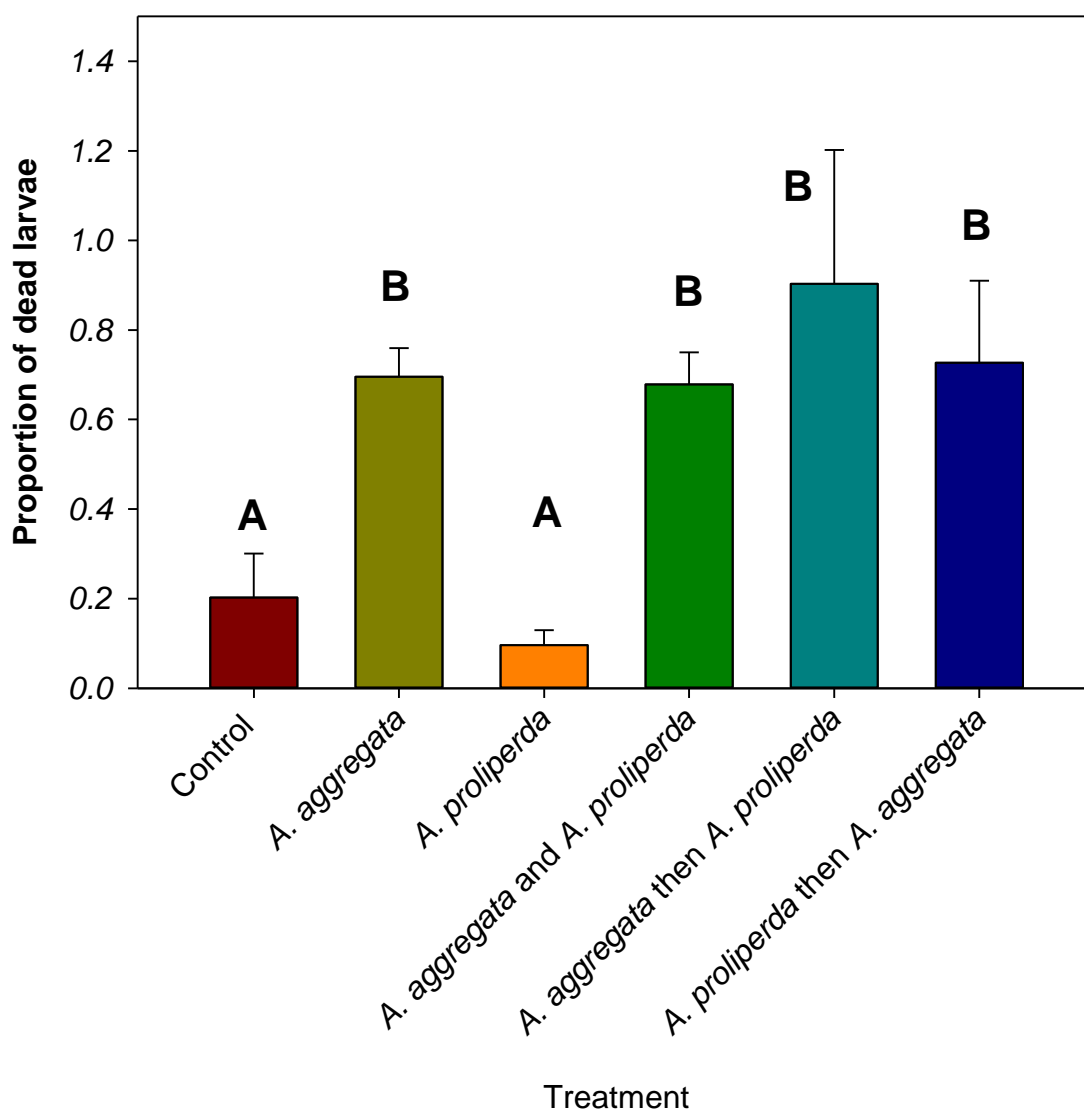
Figure 4.3. Proportion of total alfalfa leafcutting bee larvae that showed spore development after death. Letters represent significant differences in rate of hyphal or spore development as based from post-hoc Tukey's comparisons at an alpha of 0.05.

Both species were identified from dead larvae via PCR (Figure 4.4). For *A. aggregata*, treatment did not significantly alter the number of spores found in an infected cadaver, but in all cases, spores outnumbered the hyphae (as counted by number of nuclei) throughout all treatments (Table 4.5, Figure 4.4). For *A. proliperda*, the treatment was significant in determining the amounts of spores produced (Table 4.5); the interaction term between treatment and fungal stage (spores or hyphae) was significant, indicating that the part that holds the majority of nuclei (spores or hyphae) changes with changing treatment.

Inhibition

Only *A. proliperda* hyphal extracts had a significant effect on the growth rate of growing hyphae of this species, and *A. aggregata* extracts did not affect any hyphal growth rates (Figure 4.5, Figure 4.6; F [1, 19]=8.89, p=0.0080). Only *A. proliperda* spore extract significantly affected the success of *A. aggregata* spore germination (Figure 4.7; F [3, 291]=2.87, p=0.0369).

Discussion

A. aggregata and *A. proliperda* are two pathogens that differ in virulence to the ALCB as well as in their ability to grow outside the host; however, they are pathogens that have evolved to be mutually successful when occupying the same host, at the same time. Maintaining co-infections within ALCB populations ensures success of each pathogen in the host environment. Much like we would expect from an obligate host specific pathogen, *A. aggregata* has a relatively high and consistent level of virulence to the ALCB, while showing high growth restriction on substrates and temperatures that are not similar to its host organism. *A. aggregata* successfully

Table 4.5. Analysis of variance table for the amount and location of spore production on dead ALCB larvae. Average number of fungal nuclei was evaluated based on one of six treatments, as well as the location of the fungal material, as well as whether the fungal material was spore or hyphae.

Source	df	F value	Pr>F
A. aggregata			
Treatment	3	2.29	0.0858
Location of material	1	32.36	<0.0001
Treatment*Location	3	2.20	0.0956
A. proliperda			
Treatment	3	5.49	0.0019
Location of material	1	2.13	0.1489
Treatment*Location	3	10.91	<0.0001
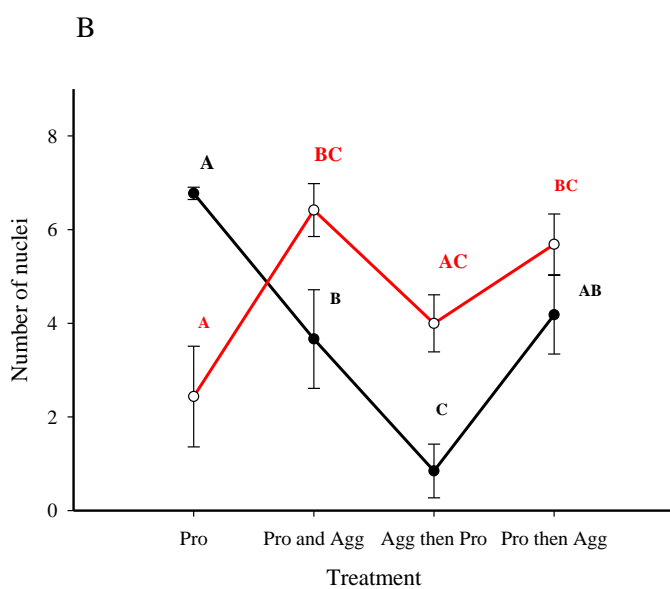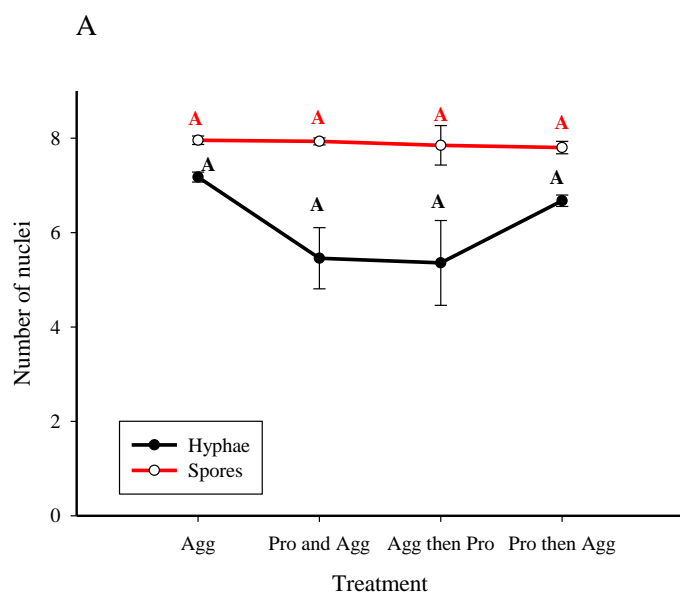
Figure 4.4. Amount of nuclear material identified as *A. aggregata* (A) or *A. proliperda* (B) in alfalfa leafcutting bee larvae after exposure to one of four infective treatments. Letters correspond to Tukey's multiple comparisons at a significance level of 0.05, and spore data (red line, open symbols) and hyphal data (black line, closed symbols) were analyzed separately.
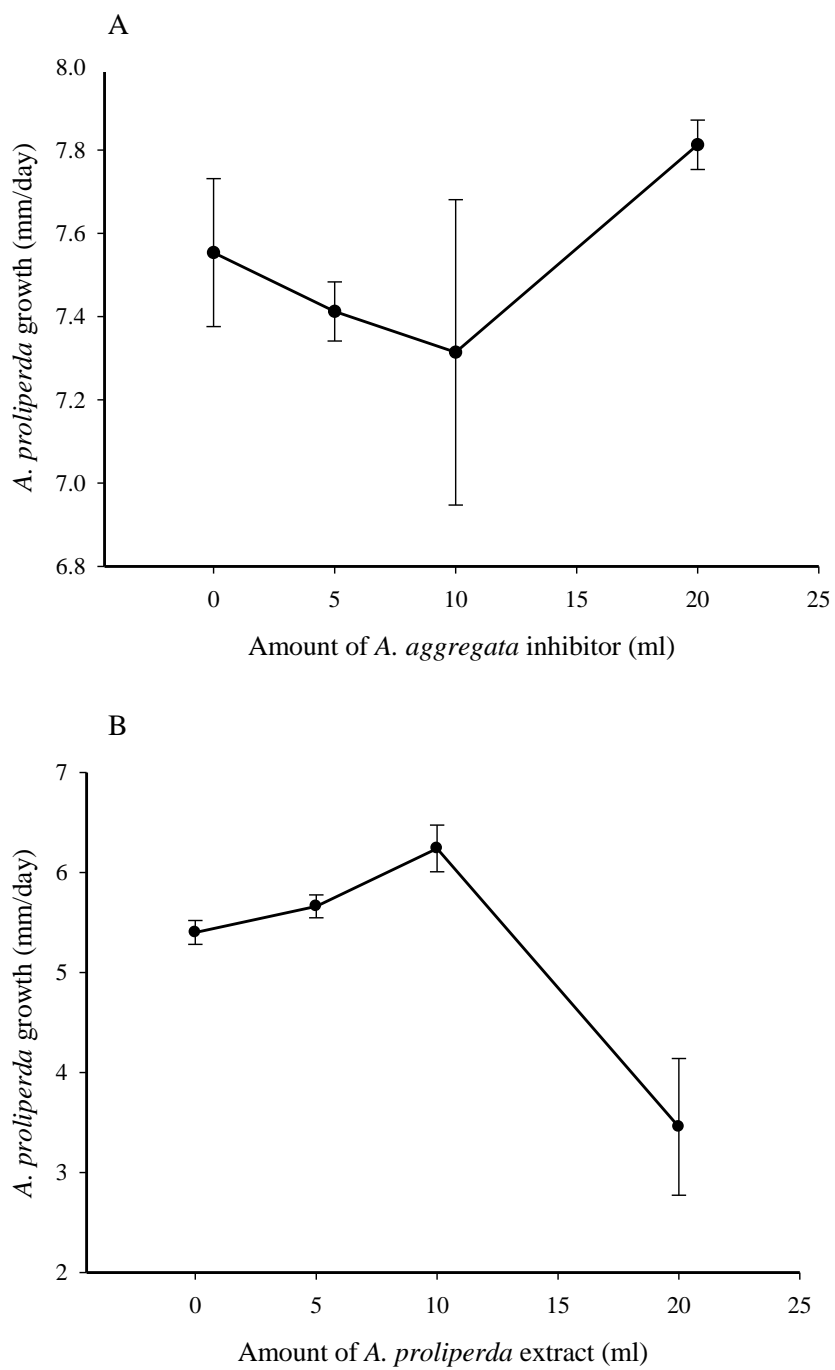
Figure 4.5. Inhibition of *A. aggregata* (A) and *A. proliperda* (B) extracts on hyphal growth of *A. proliperda*.
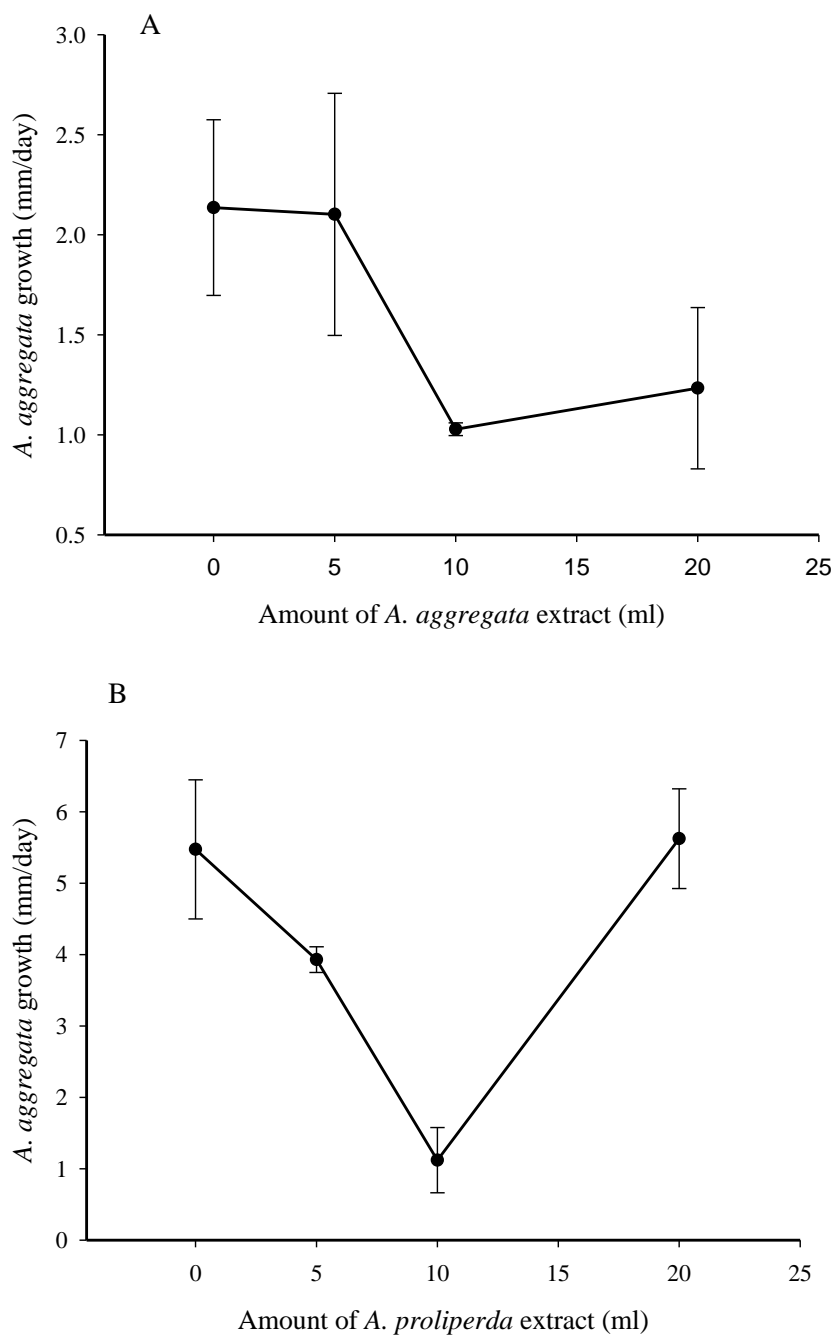
Figure 4.6. Inhibition of *A. aggregata* (A) and *A. proliperda* (B) extracts on hyphal growth of *A. aggregata*.
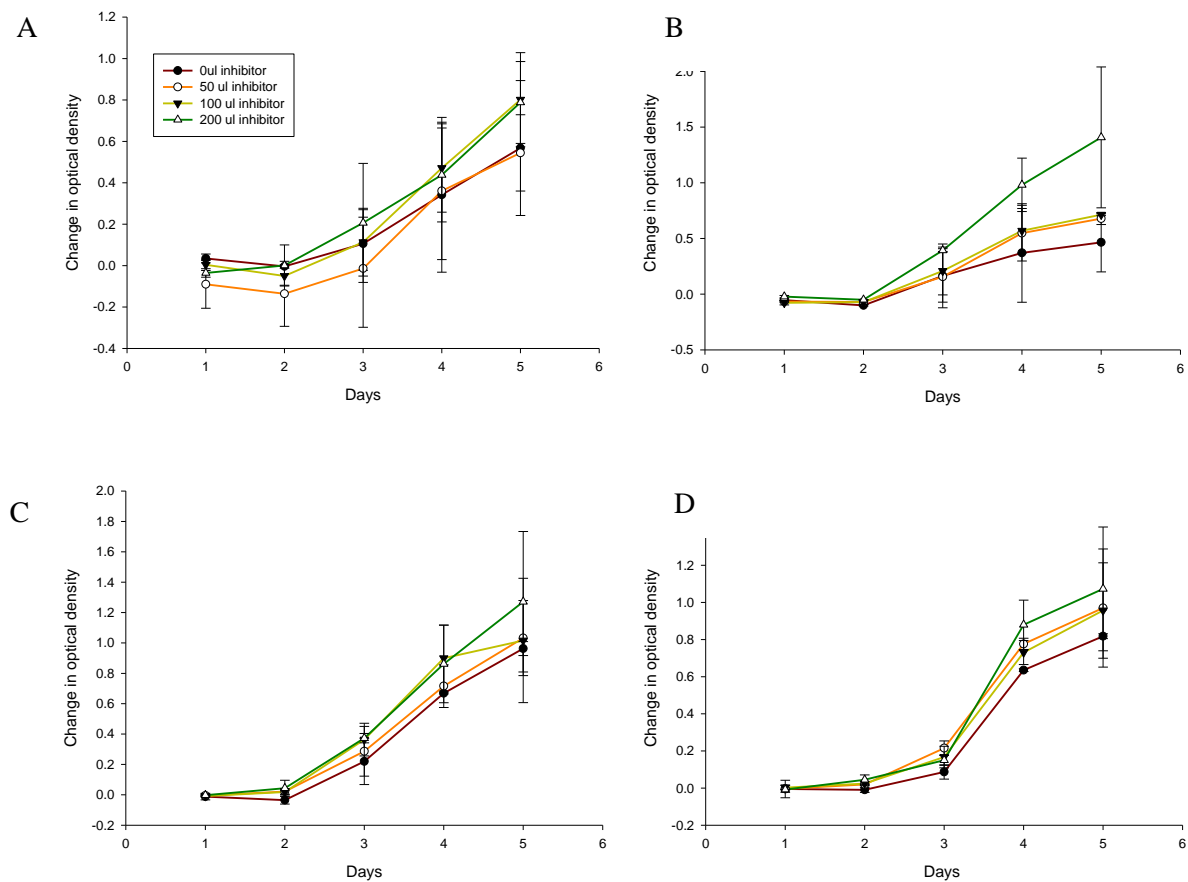
Figure 4.7. Effect of fungal filtrates on germinating spores of *A. aggregata* and *A. proliperda*. Optical density of *A. aggregata* cultures subjected to filtrates of *A. aggregata* spores (A) or *A. proliperda* spores (B). Optical density of growth in *A. proliperda* cultures subjected to filtrates of *A. aggregata* spores (C) or *A. proliperda* filtrate (D).

infects a host, and will also produce numerous spores to ensure transmission of the pathogen to other hosts. The uniformity of *A. aggregata* in disease manifestation of its host may point to selection over time between strains of variable virulence to fewer strains of more uniform virulence (Evison *et al.*, 2015).

Unlike *A. aggregata*, *A. proliperda* shows very low levels of mortality and spore production when infecting the ALCB without a co-infecting pathogen. Host mortality is required for chalkbrood transmission, so low host mortality is highly non-adaptive to pathogen success for an obligately killing pathogen (Ebert and Weisser, 1997; Méthot and Alizon, 2015).   However, *A. proliperda* is much more successful at utilizing a wide range of nutrients and temperatures for rapid hyphal growth. *A. proliperda* will most likely be able to utilize pollen provisions within a nest for vegetative growth (as evidenced by *A. proliperda* growth rates that are superior to *C. farinicola* on high sugar media), and, based on past research, can most likely reproduce on pollen provisions as well (Stephen *et al.*, 1981).

It is only in co-infections that we can observe pathogen adaptations to each other. In simultaneous doses of both *A. aggregata* and *A. proliperda,* host mortality and spore production do not indicate any variation from *A. aggregata* solo doses; however, the mixed infection drastically increased the amount of spores that *A. proliperda* produced after host death, as compared a solo dose of *A. proliperda*. If we define pathogen virulence as a combination of both host mortality and the production of reproductive spores (Alizon, 2008), then presence of *A. aggregata* increases the virulence of *A. proliperda*. Pathogens with low virulence producing more reproductive propagules when co-infecting with a high virulence pathogen than when infecting in a solo dose has been noted for a variety of hosts and pathogens, including parasitic trematodes and snails and fungal entomopathogens and termites (Hughes and Boomsma, 2004; Gower and Webster, 2005; Chouvenc *et al.*, 2012).

Sequential doses of the two pathogens did decrease overall host mortality as compared to simultaneous doses. Variability in sequential as opposed to simultaneous doses has been observed in many studies (Thomas *et al.*, 2003; Lohr *et al.*, 2010; Hoverman *et al.*, 2013; Doublet *et al.*, 2014; Natsopoulou *et al.*, 2014); however, our simultaneous infections were not asymmetric in their competitive interaction (meaning the order of the doses did not make a significant difference in host mortality or spore production). One of the explanations for this reduced mortality would be direct competition between an established pathogen and a new, introduced pathogen. We did not, however, find evidence of chemical inhibition between the two fungal species, either in the spore or the hyphal components. This suggests that strong chemical inhibition is not taking place between these species.

A limitation of host resources also does not explain the variability of spore production in our mixed infections. Independent of treatment, *A. aggregata* consistently produced the same amount of reproductive spores, and in a mixed infection, *A. proliperda* increased spore production, leading to an increase in total spores produced. The most likely cause for variability in sequential infections lies with the host immune response.

Generally, an elicitation of the immune response by the first of a pair of sequential doses can lessen the impact of the second pathogen dose in invertebrates; this is known as "immune priming" (Sadd and Schmid-Hempel, 2006). Even if the ALCB immune system was primed with the introduction of the first pathogen, we would still expect to see similar mortality and sporulation between our sequential treatment where *A. aggregata* was the first dose and our solo *A. aggregata* dose, but the sequential dose yielded higher larval survival. There is some component of *A. proliperda* introduction that alters the disease dynamics in our experiment. Perhaps a strong immune response is elicited in the beginning of an infection, and the secondary infection hours later results in an overall stronger immune response than the solo dose, and hence, higher larval survival. We are just beginning to understand the ALCB immune response, and

work is needed in this area (Xu and James, 2009). It does not seem as if *A. aggregata* and *A. proliperda* differ in their ability to elicit the host immune response, as sequential infections were similar, independent of which species infected first, indicating similar immune responses.

References

Alizon, S., 2008. Decreased overall virulence in coinfected hosts leads to the persistence of virulent parasites. Am. Nat. 172, E67–E79.

Alizon, S., 2013. Co-infection and super-infection models in evolutionary epidemiology. Interface Focus 3, 20130031.

Anderson, D.L., Gibson, N., 1998. New species and isolates of spore-cyst fungi (Plectomycetes: Ascosphaerales) from Australia. Aust. Syst. Bot. 11, 53–72.

Chouvenc, T., Efstathion, C.A., Elliott, M.L., Su, N.Y., 2012. Resource competition between two fungal parasites in subterranean termites. Naturwissenschaften 99, 949–958.

Doublet, V., Natsopoulou, M.E., Zschiesche, L., Paxton, R.J., 2014. Within-host competition among the honey bee pathogens *Nosema ceranae* and deformed wing virus is asymmetric and to the disadvantage of the virus. J. Invertebr. Pathol. 124, 31–34.

Ebert, D., Weisser, W.W., 1997. Optimal killing for obligate killers: the evolution of life histories and virulence of semelparous parasites. Proc. Biol. Sci. 264, 985–991.

Evison, S.E.F., Foley, K., Jensen, A.B., Hughes, W.O.H., 2015. Genetic diversity, virulence and fitness evolution in an obligate fungal parasite of bees. J. Evol. Biol. 1–10.

Gower, C.M., Webster, J.P., 2005. Intraspecific competition and the evolution of virulence in a parasitic trematode. Evolution 59, 544–553.

Hoverman, J.T., Hoye, B.J., Johnson, P.T.J., 2013. Does timing matter? How priority effects influence the outcome of parasite interactions within hosts. Oecologia 173, 1471–1480.

Hughes, W.O.H., Boomsma, J.J., 2004. Let your enemy do the work: within-host interactions between two fungal parasites of leaf-cutting ants. Proc. Biol. Sci. 271, S104–S106.

James, R., 2005. Temperature and chalkbrood development in the alfalfa leafcutting bee, *Megachile rotundata*. Apidologie 36, 15–23.

James, R.R., Skinner, J.S., 2005. PCR diagnostic methods for *Ascosphaera* infections in bees. J. Invertebr. Pathol. 90, 98–103.

James, R.R., Pitts-Singer, T.L., 2005. *Ascosphaera aggregata* contamination on alfalfa leafcutting bees in a loose cell incubation system. J. Invertebr. Pathol. 89, 176–178.

James, R.R., Pitts-Singer, T.L., 2013. Health status of alfalfa leafcutting bee larvae (Hymenoptera: Megachilidae) in United States alfalfa seed fields. Environ. Entomol. 1166–1173.

Lohr, J.N., Yin, M., Wolinska, J., 2010. Prior residency does not always pay off–co-infections in *Daphnia*. Parasitology 137, 1493–1500.

McManus, W.R., Youssef, N.N., 1984. Life cycle of the chalk brood fungus, *Ascosphaera aggregata*, in the alfalfa leafcutting bee, *Megachile rotundata*, and its associated symptomatology. Mycologia 76, 830–842.

Méthot, P.O., Alizon, S., 2015. What is a pathogen? Toward a process view of host-parasite interactions. Virulence 5, 775–785.

Natsopoulou, M.E., McMahon, D.P., Doublet, V., Bryden, J., Paxton, R.J., 2014. Interspecific competition in honeybee intracellular gut parasites is asymmetric and favours the spread of an emerging infectious disease. Proc. R. Soc. B 282, 20141896.

Pitt, J.I., 1966. Two new species of Chrysosporium. Trans. Brit. Myco. Soc. 49, 467–470.

Pitt, J.I., Lantz, H., Pettersson, O.V., Leong, S.L.L., 2013. *Xerochrysium gen*. nov. and *Bettsia*, genera encompassing xerophilic species of *Chrysosporium*. IMA Fungus 4, 229–241.

Pitts-Singer, T.L., Cane, J.H., 2011. The alfalfa leafcutting bee, *Megachile rotundata*: the world's most intensively managed solitary bee. Annu. Rev. Entomol. 56, 221–237.

Rigaud, T., Perrot-Minnot, M.-J., Brown, M.J.F., 2010. Parasite and host assemblages: embracing the reality will improve our knowledge of parasite transmission and virulence. Proc. Biol. Sci. 277, 3693–3370.

Sadd, B.M., Schmid-Hempel, P., 2006. Insect immunity shows specificity in protection upon secondary pathogen exposure. Curr. Biol. 16, 1206–1210.

Stephen, W., Vandenberg, J., Fichter, B.L., 1981. Etiology and epizootiology of chalkbrood in the leafcutting bee, with notes on *Ascosphaera* species. Corvallis, OR.: Agricultural Experiment Station, Oregon State University.

Thomas, M., Watson, E., Valverde-Garcia, P., 2003. Mixed infections and insect–pathogen interactions. Ecol. Lett. 6, 183–188.

Xu, J., James, R., 2009. Genes related to immunity, as expressed in the alfalfa leafcutting bee, *Megachile rotundata*, during pathogen challenge. Insect Mol. Biol. 18, 785–794.

CHAPTER 5

PRESENCE OF PATHOGEN KILLED LARVAE MAY ENHANCE NESTING BEHAVIOR

OF THE ALFALFA LEAFCUTTING BEE, *MEGACHILE ROTUNDATA*[8]

Abstract

The alfalfa leafcutting bee is a commercial pollinator of alfalfa for seed production, and these bees are susceptible to disease via ingested fungal spores. Many diseases of insects are known to cause behavioral changes in their hosts, but there are no known adaptive behaviors of alfalfa leafcutting bees to infection. Therefore, we conducted field studies to determine if bees in pathogen-dense environments altered their nesting patterns, specifically if bees exposed to chalkbrood spores produced higher proportions of nest cells that failed as eggs or small larvae (a state known as "pollen ball"). We found that non-exposed bees had the highest proportion of pollen ball cells, and exposed bees produced higher numbers of nest cells overall, implying that there may be an underlying behavior of exposed bees that reduces the proportion of failed nest cells.

[8] This chapter is co-authored by D.L. Welker and R.R. James.

Introduction

We investigated the effect of pathogen exposure on the nesting behavior of the alfalfa leafcutting bee (ALCB), *Megachile rotunda* (F.), a solitary nesting bee used as a commercial pollinator for alfalfa (*Medicago sativa* (L.)) seed production in the United States and Canada (Pitts-Singer and Cane 2011). Fungal pathogens in the genus *Ascosphaera* (Maassen ex Claussen) cause a disease known as chalkbrood in several bee species, including the ALCB. *Ascosphaera* can infect bee larvae through the gut, and once killed, the larvae can be covered with over a million spores (Vandenberg *et al.* 1980).

Alfalfa leafcutting bee (ALCB) females construct nests in linear cavities (Figure 5.1D). Nests are composed of a series of nest cells, all cells in a cavity constitute a nest, and each cell is lined with leaf pieces and partially filled with a mass provision of pollen and nectar. The female lays a single egg on the provision, places a cap on the cell, and commences production of a new nest cell directly anterior to the previous cell in the nest. If healthy larvae are in cells posterior to a chalkbrood-infected larvae, then the adults must physically chew through the spore covered cadaver to exit the nest, inadvertently coating their integument with infective spores (Vandenberg *et al.* 1980; Tepedino and Frohlich 1984). Spore contaminated female bees unknowingly place spores in the mass provisions they construct for their offspring, perpetuating the disease in populations.

Entomopathogens can cause behavioral changes in their hosts, both directly and indirectly (Roy *et al.* 2006). Behavioral adaptations to pathogens are readily observed in social bee species (Fefferman *et al.* 2006). In contrast, solitary bees have few known behavioral defenses against pathogens, although there is some evidence of behaviors observed in specific host-pathogen interactions. Alkali bees (*Nomia melandri* (Cockerell)) will open diseased brood

cells in their underground nests and pack the nest with dirt, which may reduce spread of contaminating fungal spores (Batra and Bohart 1969). *Trachusa byssina* (Panzer) females line their nest with antimicrobial tree resin which may prevent disease (Cane *et al.* 1983), and specialized host plant selection and dietary prophalxys may reduce diseases the solitary bee *Chelostoma florisomme* (L.) (Wynns 2012).

In ALCB nest cells, some eggs fail early in larval development, or an egg is never laid on the provision. This condition is known as "pollen ball" and constitutes an uneaten pollen provision. High ambient temperature can explain some pollen ball occurrence, but bee nesting behavior may also be an important factor (Pitts-Singer and James 2008). We conducted field studies to determine if pollen ball production is higher in disease heavy environments and specifically if there were a higher proportion of pollen balls in nesting treatments with infective chalkbrood spores.

Methods

Field experiments were conducted in Mt. Sterling, UT (41° 37' 27.70'' N, 111° 52' 58.92'' W), in an unmanaged location that had flowering patches of alfalfa, sweet clover (*Melilotus officinalis* (L.)) and balsamroot (*Balsamorhiza sagittata* (Pursh)) and were adjacent to fields with alfalfa growing for hay production. No ALCB were kept commercially in this area, and the absence of the bee was confirmed with observation surveys prior to the first field season (Table 5.1).

Three bee shelters were constructed using a modified form of the "bee mail shelter" (Cane 2006). Our shelters were supported by two metal fence posts securely attached to a mail tote via custom metal brackets. Shelters were approximately 4 ft off the ground and each shelter was positioned so that it was located 800 ft from other shelters, well beyond the 165 ft distance

Table 5.1. Survey of bees observed at nesting site. Surveys were a result of three 10-minute observations of flowering plants within a 20 ft radius of each of the three shelters (nine observations total).

Shelter	Apidae	# obs.	Halictidae	#obs.	Megachilidae	# obs.
1	*A. mellifera*	10	*Halictus* sp.	2	*Megachile* sp.	1
	Nomada sp.	1				
2	*A. mellifera*	50	*Lasioglossum* sp.	2	*Megachile* sp.	3
	Ceratina sp.	1			*Osmia* sp.	4
3	*A. mellifera*	12	*Halictus* sp.	1	*Osmia* sp.	4
			Lasioglossum sp.	2		

that is considered to be the acceptable isolation distance to prevent bee mediated transfer of genetically modified pollen (Fitzpatrick *et al.* 2003). The treatment assigned to each shelter was rotated each year. A polystyrene board with cavities (15 x 50 holes; Beaver Plastics, Acheson, Alberta, Canada) was securely placed along the back wall of each shelter. Paper tubes (0.218 x 3.73 inch; Johnson Tube Company, New Port Richey, FL; Phoenix Tube Company, Dayton, OH) were placed in each hole to facilitate removal of the nests. Environmental data was collected from the Utah Climate Center (Logan, UT) for a weather station 2.16 mi from the field site.

Chalkbrood cadavers of ALCB larvae were collected in the vicinity of Logan, Utah and incubated at 4 °C for approximately 10 mo before treatments. Some cadavers were sterilized via autoclave at 121 °C for 20 min. We prepared 100 straws per treatment each year (900 straws total) in the following way: a small hole was cut in the middle side of each paper straw, and using this hole, either an autoclaved or non-autoclaved infective chalkbrood cadaver was adhered to the inside of the paper straw using non-toxic glue, and the hole in the straw was sealed. Control treatment straws were not altered.

Alfalfa leafcutting bee cocoons were purchased from a commercial broker (JWM Leafcutters, Inc; Nampa, ID) and incubated at 29 °C, ambient humidity (20%) and a no light-dark cycle. Bees were incubated for approximately 18 d, until male bee emergence (Frank 2003). At this time, males were collected each day, fed and housed in plexiglass cages. After two days of male emergence, males were placed at dusk in the field nesting boxes.

Each year, 100 cocoons selected after male emergence (approximately 22 d post incubation) were assigned to each treatment (control, autoclaved cadaver or infective cadaver). These cocoons were gently placed in the back of the prepared paper straws and were inserted into each nesting board in holes determined through random number generation. Boards were placed in the field, and bees were free to emerge and nest for approximately one month. During this time, a 20 min observation was made on the number of actively nesting females seen at each block approximately 1.5 wk post block placement.

After the nesting period, paper straws were removed from the polystyrene boards and x-rayed. Straws were kept at ambient temperatures until late October at which point the bees were placed at 4 °C for storage. After the 2013 nesting only, bees were incubated at 29 °C in early June 2014 to allow bees to develop and obtain the sex ratio of the offspring (only adults can be easily sexed).

The experiment was designed to study chalkbrood treatments in a 3 x 3 Latin square design (3 years x 3 shelters). For all count data (number of cells per treatment, number of cells in a straw) a generalized linear model using the Poisson distribution was used to determine significant differences between groups (PROC GENMOD, SAS Institute 2011). A generalized linear model was used to determine differences in weather variables between years, sex ratio of the offspring in 2013, and the proportion of pollen balls, healthy larvae and chalkbrood infected larvae between treatments (PROC GLM, SAS Institute 2011). For all proportion data, the

proportions were transformed using an arcsine square root transformation to ensure normal

distribution.

Results

During the three year study, 6,513 total cells were built by the 900 female alfalfa

leafcutting bees released. Significantly more cells were produced in 2012 (3707 cells) as

compared to 2011 (1438 cells; Z=-31.29; P<0.001) and 2013 (1368 cells; Z=31.44; P<0.001).

The number of cells produced in 2011 and 2013 did not differ significantly (Z=0.19; P=0.9801).

No significant variation occurred between years for the average minimum temperature (13.2 ±

2.67 °C), (F[2, 127]=0.15, P=0.8649), average maximum temperature (31.47 ± 2.40 °C),

(F=3.06; df=2, 127; P=0.0504), average precipitation (0.36 ± 2.71 in), (F=1.28; df=2, 127;

P=0.2821), and evapotranspiration (5.94 ± 0.72 mm/day), (F=2.82, df=2, 127; P=0.0635). Thus,

the differences in nesting among years were probably not due to environmental effects.

Over the 3-year period, fewer cells were produced in the control shelters (1859 nests)

than in either the infective cadaver treatments (2210 nests; Z=5.62; P<0.001) or the autoclaved

cadaver treatments (2444 nests; Z=8.85; P<0.001; Table 2). The autoclaved cadaver treatment

had significantly more cells than the control treatment and the regular cadaver treatment (Z=3.26;

P=0.032). There was no significant difference between the total number of cells in a nest straw

between treatments ($\chi2$=0.59; P=0.7448). The largest nest size in our study had 11 cells but since

there were only a total of 9 cells constructed at the 11th position for the entire experiment, data

from the 11th cell position was not used.

We used x-ray imagery to quantify the number of larvae that were healthy, had

chalkbrood, were pollen ball, or were parasitized, and the rest were classified as unknown death.

Parasites infected less than 3% of all larvae, and unknown deaths accounted for less than 1% of

larvae; these categories were not investigated further. The proportion of cells in each health category was calculated from the total number of cells constructed for each treatment, for each year. The control and autoclaved cadaver treatments had a higher proportion of healthy cells than did the infective cadaver treatment (F=3.26; df=2,81; P=0.0436 (Figure 5.1A)). The cumulative proportion of chalkbrood cells significantly varied by treatment over increasing cell position (F=78.18; df=2,81; P<0.0001; Figure 5.1B), as did the average cumulative proportion of pollen ball cells (F=10.58, df=2,81; P<0.0001; Figure 5.1C).

In 2013, 182 total nests were produced, with an average cell count of 6.80±2.51 cells per nest. For each nest, the proportion of females per nest was calculated and this proportion was averaged by treatment to obtain the average sex ratio. There was no significant difference in the sex ratio in each treatment for the 2013 trial (F=2.22; df=2,167; P=0.119) (Table 5.2).

Discussion

The highest number of, and overall healthiest, nest cells were produced from the autoclaved cadaver treatment, as compared to the control and the infective cadaver treatment. In both treatments that provided a physical barrier to emergence (autoclaved and infective cadaver) greater numbers of cells were produced. Spring emerging female bees have been documented to wait behind a non-emerging nest mate for up to 6 days before ultimately chewing through the nest mate to exit the straw (Tepedino and Frohlich 1984). Perhaps an additional barrier to exiting the straw serves as an enticement to immediately nest upon exit, as opposed to searching for a new nesting area.

The highest proportion of nests resulting in pollen balls was not in the infective cadaver treatment, but in the control treatments. Pollen ball incidence is known to correlate with extreme

Figure 5.1. Health of larvae produced during treatment. Cumulative proportions of: **A.** Cells with healthy larvae, **B.** Cells with chalkbrood and **C.** Cells with pollen balls as seen over progressive nest building where position 1 is the first cell made by the mother bee and cell 10 was the last cell made. Letters represent significant differences via Tukey's multiple comparison procedures at an alpha of 0.05. **D.** X-ray image of an alfalfa leafcutting bee nest depicting 10 cells and a final leaf plug to the right of the final, tenth cell.

A
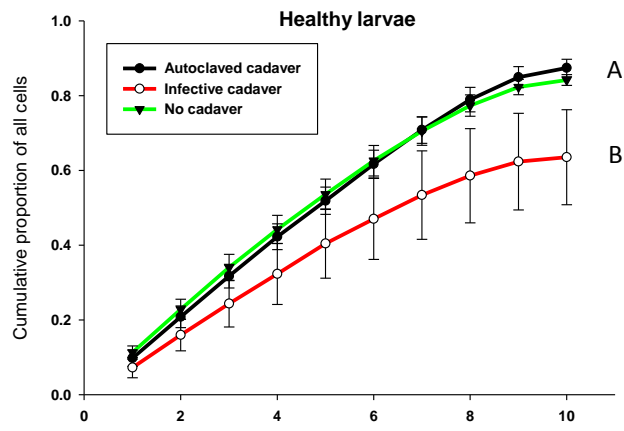
B
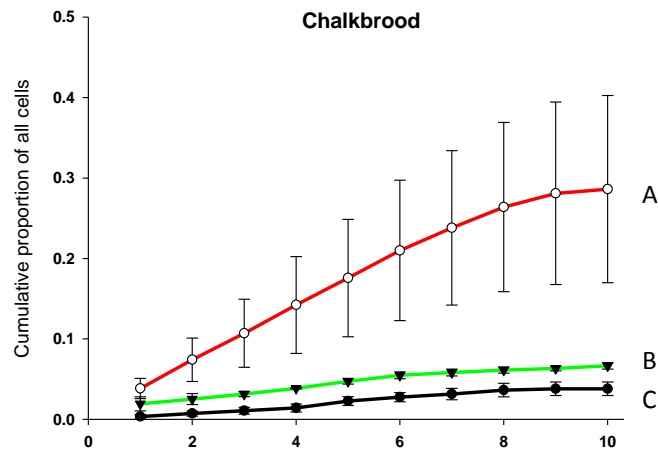
C

D
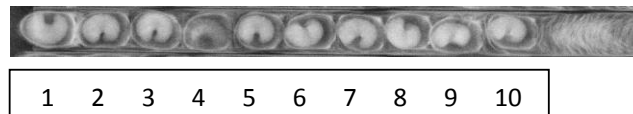
Table 5.2. Number of nest cells for each treatment for each year, the percentage ± S.D. of females emerged from 2013 treatments, and a summary of bee observation counts of nes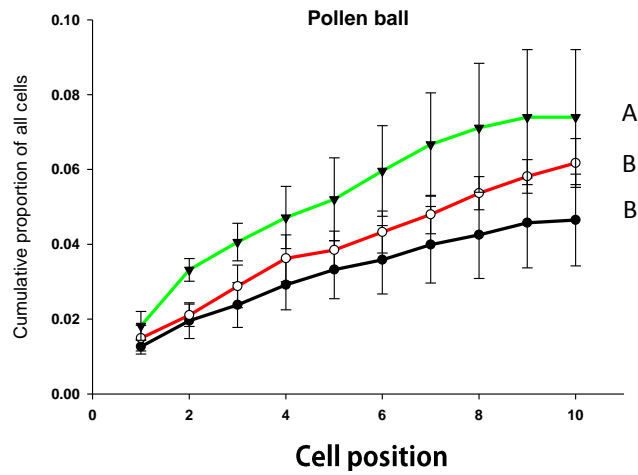ting bees present at each shelter.   Number of nesting bees are from 20 minute observations.   Letters represent significant differences based on Tukey's multiple comparisons.

	Control	Autoclaved	Infective
Number of cells produced			
2011 # Cells produced	489	554	395
2012 # Cells produced	1152	1213	1342
2013 # Cells produced	218	677	473
Total cells produced	1859 *A*	2444 *B*	2210 *C*
Average percent female			
2013 Sex ratio (% ♀)	43.75 ± 29.02% *A*	51.12 ± 28.29% *A*	40.42 ± 33.92% *A*
Number of nesting bees per 20 minute observation			
2011 Day count (#bees/20 min)	88	84	31
2012 Day count (#bees/20 min)	28	37	28
2013 Day count (#bees/20 min)	8	14	8
Total bees counted	124 *A*	135 *A*	67 *A*

temperatures (Pitts-Singer and James 2008), but all our treatments experienced similar temperature conditions throughout the three years. Female bees must ingest a pollen meal before successfully laying eggs (Richards 1994). The cadavers in our experiment may be providing sources of protein to bees as they emerge from the nest, reducing the need to feed on alternate pollen sources before nesting. This enhanced nutritional intake could have led to more successful nest cells.

The lower proportions of pollen ball in cadaver treatments may be explained by increased self-grooming of adult bees in response to spore contamination. Solitary bees are known to self-groom and increased grooming may reduce levels of other microbes that may lead to pollen ball formation. While we did not directly observe bee grooming behaviors, the decrease in chalkbrood incidence in the autoclaved cadaver treatment supports this. The highest proportion of chalkbrood larvae occurred in the infective cadaver treatment, but the lowest levels occurred in the autoclaved treatments (commercial bee cocoons are known to have incidental amounts of chalkbrood contamination (James and Pitts-Singer 2005)). Importantly, significantly lower chalkbrood levels, as well as pollen balls, occurred in the autoclaved cadaver treatment than occured in the control. Increased grooming in the autoclaved cadaver treatment can account for this difference.

References

Batra, S. W., and G. E. Bohart. **1969**. Alkali bees: response of adults to pathogenic fungi in brood cells. Science 165: 607.

Cane, J. H. 2006. The Logan BeeMail shelter: A practical, portable unit for managing cavity-nesting agricultural pollinators. Am. Bee J. 146: 611–613.

Cane, J.H., S. Gerdin, and G.Wife. 1983. Mandibular gland secretions of solitary bees (Hymenoptera: Apoidea): potential for nest cell disinfection. J. Kansas Entomol. Soc. 56: 199–204.

Fefferman, N. H., J. F. A. Traniello, R. B. Rosengaus, and D. V. Calleri. **2006**. Disease prevention and resistance in social insects: modeling the survival consequences of immunity, hygienic behavior, and colony organization. Behav. Ecol. Sociobiol. 61: 565–577.

Fitzpatrick, S., P. Reisen, and M. McCaslin. **2003**. Pollen-mediated gene flow in alfalfa: A three-year summary of field research. *In* Proceedings of the 2003 Central Alfalfa Improvement Conference, virtual meeting: 21-25.

Frank, G. 2003. Alfalfa seed and leafcutter bee production and marketing manual. Irrigated Alfalfa Seed Producers Association, Brooks, Alberta.

James, R. R., and T. L. Pitts-Singer. **2005**. *Ascosphaera aggregata* contamination on alfalfa leafcutting bees in a loose cell incubation system. J. Invertebr. Pathol. 89: 176–178.

Pitts-Singer, T. L., and R. R. James. **2008**. Do weather conditions correlate with findings in failed, provision-filled nest cells of *Megachile rotundata* (Hymenoptera: Megachilidae) in western North America? J. Econ. Entomol. 101: 674–685.

Pitts-Singer, T. L., and J. H. Cane. **2011**. The alfalfa leafcutting bee, *Megachile rotundata*: the world's most intensively managed solitary bee. Annu. Rev. Entomol. 56: 221–237.

Richards, K. 1994. Ovarian development in the alfalfa leafcutter bee, *Megachile rotundata*. J. Apic. Res. 33: 199–203.

Roy, H. E., D. C. Steinkraus, J. Eilenberg, A. E. Hajek, and J. K. Pell. **2006**. Bizarre interactions and endgames: entomopathogenic fungi and their arthropod hosts. Annu. Rev. Entomol. 51: 331–357.

SAS Institute. 2011. PROC user's manual, version 6th ed. SAS Institute, Cary, NC.

Tepedino, V. J., and D. R. Frohlich. **1984**. Fratricide in *Megachile rotundata*, a non-social megachilid bee: impartial treatment of sibs and non-sibs. Behav. Ecol. Sociobiol. 15: 19–23.

Vandenberg, J. D., B. L. Fichter, and W. P. Stephen. **1980**. Spore load of *Ascosphaera* species on emerging adults of the alfalfa leafcutting bee, *Megachile rotundata*. Appl. Environ. Microbiol. 39: 650–655.

Wynns, A. A. 2012. The bee specialist fungus family Ascosphaeraceae and its allies: systematics, ecology and co-evolution with solitary bees. PhD dissertation, University of Denmark, Copenhagen.

CHAPTER 6

CONCLUSION

Evolutionary patterns of pathogen virulence are complex, making them difficult to

define, describe and predict. However, understanding how and why a pathogen causes specific

levels of harm to their host advances our understanding of pathogen biology, in addition to

providing essential information to predict the evolutionary pressures that can lead to novel,

emerging pathogens or to host jumps by already existing pathogens (Woolhouse *et al.*, 2005). In

addition, studies of pathogen dynamics in bee hosts can lead to improved methods and practices

that ultimately preserve pollinator health. This study was an attempt to take multiple approaches

commonly used to evaluate the evolution of pathogen virulence and combine these approaches

under a unified framework. This unified framework could then illustrate this specific genus of

bee pathogens in a way no other study has before, emphasizing interactions between various

species of pathogens as well as multiple hosts.

The base of my framework is a molecular phylogeny of *Ascosphaera*. In Chapter 2, I

significantly improved this phylogeny, adding combinations of 5 additional loci to the already

utilized ITS region. Using multiple loci is critical in understanding the true relationships between

fungal pathogens, as several important groupings were resolved with additional loci (James *et al.*,

2006). Additional loci also provided data that showed similarities in intron positioning between

groups of *Ascosphaera*. While pathogenicity of a microbe cannot be attributed to one genetic

component (such as intron positions or genetic code variation), changes in the underlying genetic

code of microorganisms can show patterns that lead to the adaptability of pathogens that place

them in a favorable environment to utilize susceptible hosts (Weber and Agrawal, 2012; Méthot

and Alizon, 2015). For example, plant pathogens in the Ascomycota have evolved the ability to detoxify plant toxins, ensuring the plant pathogens are not harmed during the utilization of the plant hosts (Berbee, 2001).

Using this improved phylogeny I was able to make some assumptions regarding the groups of pathogenic *Ascosphaera*. For example, many pathogens associated with solitary bees group together in one clade, illustrating that the social bee pathogen *A. apis* does not share a recent ancestor with the main group of solitary bee pathogens, rather it is found in a sister clade with species that are generally apathogenic, or mildly pathogenic (Anderson *et al.*, 1998). I suggest that pathogenicity arose separately in the solitary and social bee pathogen species. In addition, the paraphylectic position of a third solitary bee pathogen, *A. torchioi*, gave further evidence that pathogenicity evolved up to three times in this group of fungi (Fig. 6.1). Multiple evolutionary events leading to pathogenicity is the suspected pattern of most plant and animal pathogens in the Ascomycota (Berbee, 2001), and pathogenicity arising multiple times in the *Ascosphaera* would be similar to this.

With three separate evolution events hypothesized between the main groups of pathogens (Fig 6.1), we would expect that cross-host infectivity would be limited (Woolhouse *et al.*, 2005). In Chapter 3, I took two host species, *M. rotundata* and *A. mellifera,* and determined if the *Ascosphaera* pathgoens were generally pathogenic to all bees, or if they held high host specificity. I found that *A. apis* is a highly competitive pathogen in the honey bee, basically out competing all other *Ascosphaera* pathogens. *A. aggregata* in the ALCB, when mixed with other pathogens, allowed non-self pathogens (*A. larvis* and *A. apis*) to produce spores after infections, even if larval mortality was not affected (Fig. 6.2) (Bonsall and Raymond, 2008). This co-infection is important to note because this pathogen, through the tolerance of mixed infections, can create environments with spore inocula of various species (Alizon *et al.*, 2013). This mixed
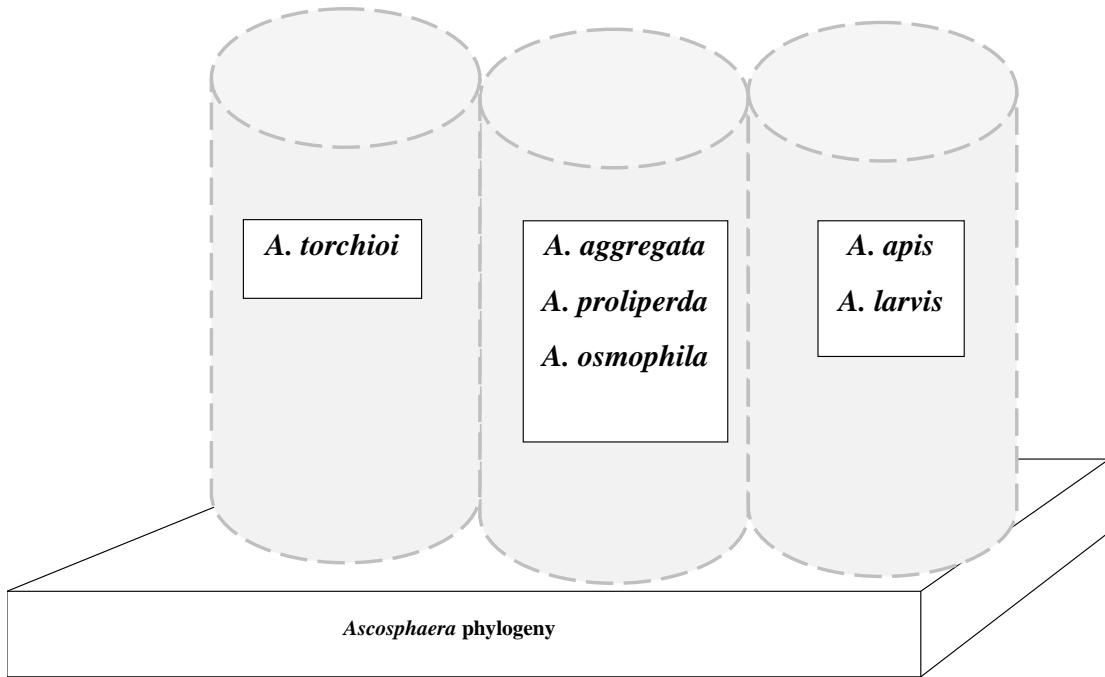
Fig. 6.1. Distinct groups of pathogens derived from separate virulence evolution events in the *Ascosphaera*. Utilizing a robust phylogeny (lower block) we can then project three groupings of pathogens within this genus, and make experimental assumptions based upon these groupings.
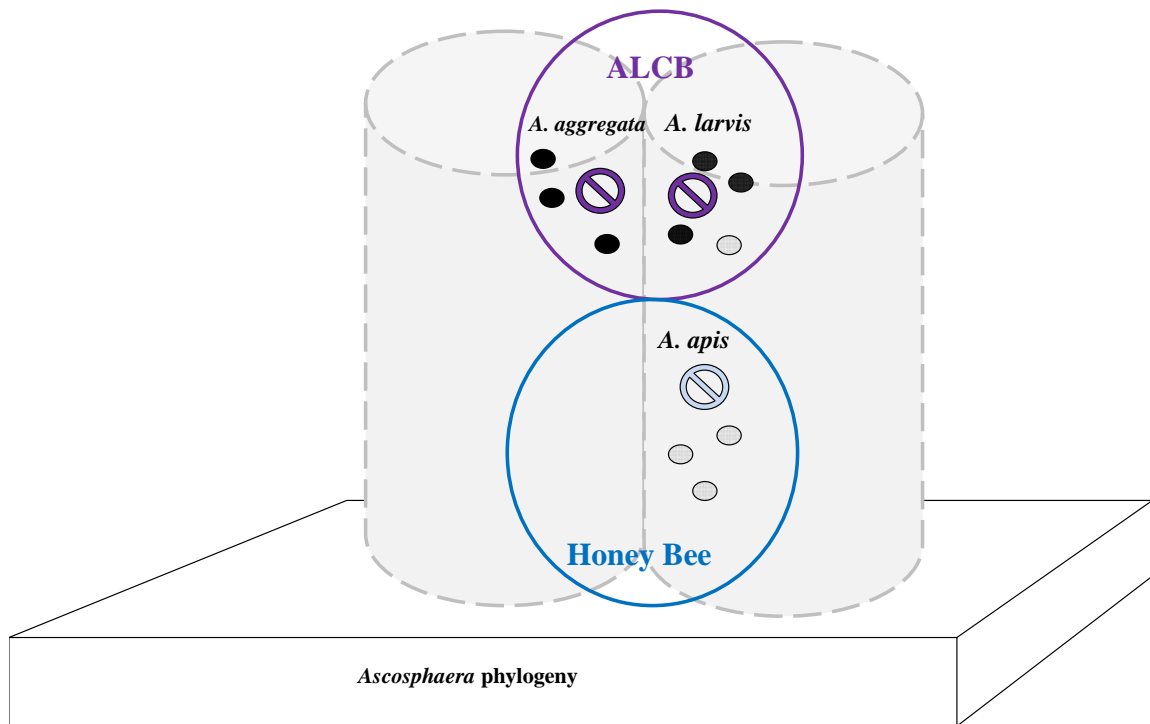
Fig. 6.2. Host specificity of *Ascosphaera* pathogens to the alfalfa leafcutting bee (purple circle) and the honey bee (blue circle). The only mortality caused in the honey bee (light blue cross-out) is that from *Ascosphaera apis*, the known obligate pathogen for that bee. Mortality to the alfalfa leafcutting bee (purple cross-out) was caused by pathogens from each pathogen clade (grey cylinders). Due to mixed infections, *A. apis* spores were found in dead alfalfa leafcutting bee cadavers (light dotted circles), along with *A. aggregata* spores (black circles) and *A. larvis* spores (dark dotted circles). The only spores found in honey bees were those of *A. apis*.

species inocula increases the opportunities for the evolution of pathogenicity of the *Ascosphaera* to new bee hosts, by virtue of increased contact between fungi and hosts. In addition, mixed infections can also serve as a source of inocula in environments for pathogens of other bees (Casadevall and Pirofski, 2007).

I did find that *A. aggregata* was more likely to co-infect with *Ascosphaera larvis* than was *A. apis* despite the fact that *A. apis* and *A. larvis* are in sister clades (Fig 6.2). Success of *A. larvis* in the ALCB indicates that, even if the two clades (*A. aggregata* and *A. apis + A. larvis*) had separate pathogen evolution events, methods of infection are probably similar between the two groups. Host specificity rather than genetic similarity distinguishes the social bee pathogens from the solitary bee pathogens, and host specificity most likely includes differences in the host's immune responses. The immune responses of solitary and social bees differ; however, work on solitary bee immune systems is in the early stages (Xu and James, 2009).

Host immune response was also predicted to be a major component in virulence differences in Chapter 4. In this chapter, I took a closer look at the dynamics between two pathogens that are phylogenetically very similar (see Chapter 2) and infect a common host with frequency. I found the high adaptation of one pathogen, *A. aggregata*, to the host, while the second pathogen, *A. proliperda* was less successful in parasitizing the ALCB, but retained an ability to utilize many substrates and temperatures for growth (Fig. 6.3). The ability of *A. proliperda* to be able to utilize environments outside of a host could alter virulence evolution in this fungal species, as it is not limited by host population numbers (Ebert and Weisser, 1997). Despite differences in virulence to the ALCB, *A. aggregata* and *A. proliperda* do share some adaptations, as mixed infections of both fungal species, resulted in improvements in *A. proliperda* evolutionary fitness, as this species produced more spores than in a solo dose, a direct measure of pathogen fitness (Ebert and Weisser, 1997; Alizon *et al.*, 2009).
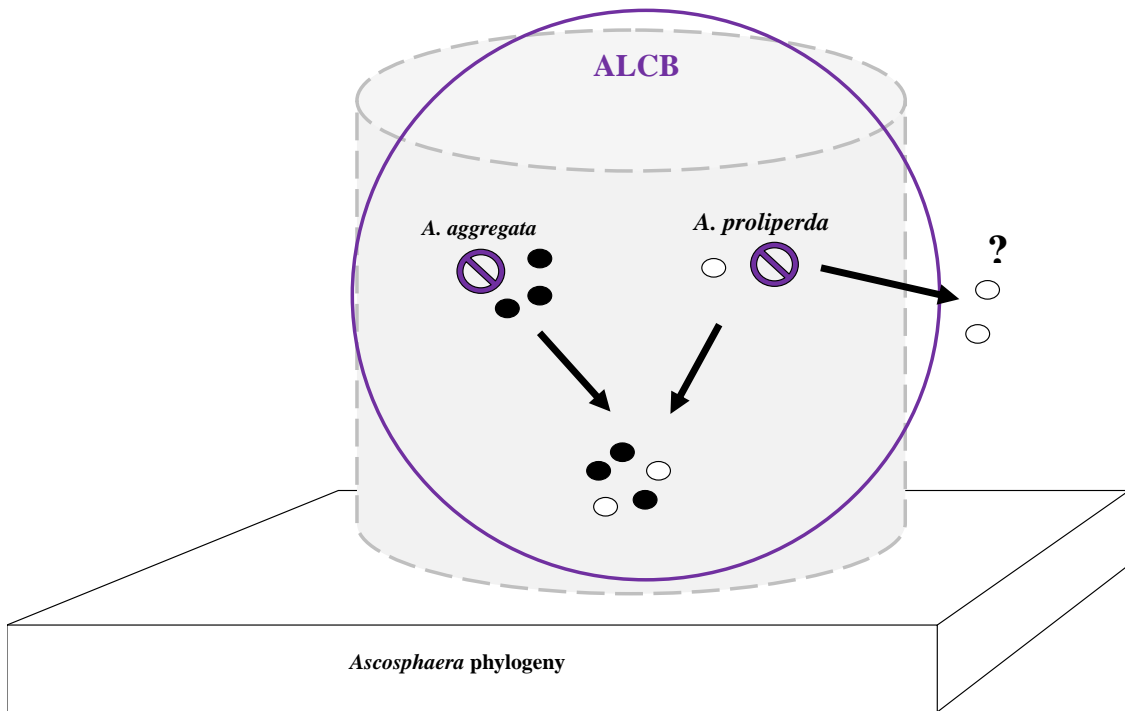
Fig. 6.3. Competition of *A. aggregata* and *A. proliperda* in the host, alfalfa leafcutting bee.

Alone, each pathogen can cause mortality (◎ symbol) and produce spores (● for *A. aggregata*

spores, ○ for *A. proliperda* spores). However, more spores of *A. proliperda* are generated in a

mixed infection than when the pathogen occurs solo, improving the fitness of this pathogen. In

addition, *A. proliperda* may be able to reproduce outside the host, affecting this pathogen's

virulence evolution.

Finally, in Chapter 5 I investigated hygienic behavior in response to pathogen inocula which is important for the amount of exposure the host has to a pathogen (Gilliam *et al*., 1983; Hart, 1990). The presence of *A. aggregata* infective cadavers did not elicit any specific nesting behaviors that would either increase or decrease the exposure of the pathogen in the population. However, bees exposed to fungal spores that were sterilized produced better quality nest cells. This could imply that there is a general behavioral adaptation of these bees to increased spores in the environment that eventually lead to higher numbers of successful nest cells than in those bees without spore exposure. While this behavior does not seem like a highly evolved adaptive behavior, it could help explain disease levels in chalkbrood infected fields, where increased nest production compensates for loss of nest cells due to disease.

While evidence that pathogenicity arose more than once among bee pathogens in the *Ascosphaera* is shown in this study, virulence of pathogens throughout the genus is variable, and not predictable based on phylogenetic placement. Two *Ascosphaera* pathogens are optimized to infect the two bee host species in this study, *A. aggregata* for the ALCB and *A. apis* for the honey bee. In both cases, host mortality remains constant with various dosages of these pathogens, even when another pathogen is given concurrently. In the honey bee, *A. apis* shows moderate virulence, but high competitive ability when other pathogens are present, ensuring that resulting spores from chalkbrood infection are only *A. apis*. However, *A. aggregata* infections in the ALCB allow for reproduction of spores other than *A. aggregata* and mixed infections can actually increase other *Ascosphaera* species' spore production levels.

It is critically important to include both measures of spore production and the effects of mixed infections in future studies of the *Ascosphaera*. Without either of these parameters, our results and conclusions would be drastically different. In addition, continued investigation and characterization of bee immune responses when exposed to *Ascosphaera* infection is important, especially in the context of mixed infections. While host response to chalkbrood has been studied

somewhat in both species of bees, and evidence currently points to genetic variability that can confer chalkbrood resistance in honey bees, no studies to date encompass multiple infections, especially non-sequential infections in either host (Jensen *et al*., 2009; Xu and James, 2009; Aronstein and Murray, 2010). This dissertation has also provided additional information about two potentially damaging pathogens, *A. torchioi* and *A. proliperda*. As *A. torchioi* is a pathogen of another commercially used bee, *Osmia lignaria*, this pathogen deserves further study. *A. proliperda*, if continued to be found in commercial *Megachile rotundata* populations, may cause future bee health problems, especially if virulence evolution in this pathogen is uncoupled from host population numbers.

References

Alizon, S., de Roode, J.C., Michalakis, Y., 2013. Multiple infections and the evolution of virulence. Ecol. Lett. 16, 556–567.

Alizon, S., Hurford, A., Mideo, N., Van Baalen, M., 2009. Virulence evolution and the trade-off hypothesis: history, current state of affairs and the future. J. Evol. Biol. 22, 245–259.

Anderson, D., Gibbs, A., Gibson, N., 1998. Identification and phylogeny of spore-cyst fungi (*Ascosphaera* spp.) using ribosomal DNA sequences. Mycol. Res. 102, 541–547.

Aronstein, K.A., Murray, K.D., 2010. Chalkbrood disease in honey bees. J. Invertebr. Pathol. 103, S20–S29.

Berbee, M.L., 2001. The phylogeny of plant and animal pathogens in the Ascomycota. Physiol. Mol. Plant Pathol. 59, 165–187.

Bonsall, M.B., Raymond, B., 2008. Lethal pathogens, non-lethal synergists and the evolutionary ecology of resistance. J. Theor. Biol. 254, 339–349.

Casadevall, A., Pirofski, L., 2007. Accidental virulence, cryptic pathogenesis, martians, lost hosts, and the pathogenicity of environmental microbes. Eukaryot. Cell 6, 2169–2174.

Ebert, D., Weisser, W.W., 1997. Optimal killing for obligate killers: the evolution of life histories and virulence of semelparous parasites. Proc. Biol. Sci. 264, 985–991.

Gilliam, M., Taber, S.I., Richardson, G. V., 1983. Hygienic behavior of honey bees in relation to chalkbrood disease. Apidologie 14, 29–39.

Hart, B.L., 1990. Behavioural adaptations to pathogens and parasites - 5 strategies. Neurosci. Biobehav. Rev. 14, 273–294.

James, T.Y., Kauff, F., Schoch, C., Matheny, P.B., Hofstetter, V., Cox, C.J., Celio, G., Geuidan, C., Fraker, E., Miadlikowska, J., Lumbsch, T.H., Rauhut, A., Reeb, V., Arnold, A.E., Wynns, A.A., Stajich, J., Hosaka, K., Sung, G., Johnson, D., O'Rourke, B., Crockett, M., Binder, M., Curtis, J.M., Slot, J., Wang, Z., Wilson, A., Schüßler, A., Longcore, J.E., O'Donnell,K., Mozley-Standridge, S., Porter, D., Letcher, P.M., Powell, M.J., Taylor, J.W., White, M.M.,Griffith, G.W., Davies, D.R., Humber, R.A., Morton, J.B., Sugiyama, J., Rossman, A., Rogers, J.D., Pfister, D.H., Hewitt, D., Hansen, K., Hambleton, S., Shoemaker, R.A., Kohlmeyer, J., VolkmannKohlmeyer, B., Spotts, R.A., Serdani, M., Crous, P.W., Hughes, K.W., Matsuura, K., Langer, E.,Langer, G., Untereiner, W., Lücking, R., Büdel, B., Geiser, D.M., Aptroot, A., Diederich, P., Schmitt, I., Schultz, M., Yahr, R., Hibbett, D., Lutzoni, F., McLaughlin, D.J., Spatafora, J.W.,Vilgalys, R., 2006. Reconstructing the early evolution of fungi using a six-gene phylogeny. Nature 443, 818–822.

Jensen, A.B., Pedersen, B.V., Eilenberg, J., 2009. Differential susceptibility across honey bee colonies in larval chalkbrood resistance. Apidologie 40, 524–534.

Méthot, P.O., Alizon, S., 2015. What is a pathogen? Toward a process view of host-parasite interactions. Virulence 5, 775–785.

Weber, M.G., Agrawal, A.A., 2012. Phylogeny, ecology, and the coupling of comparative and experimental approaches. Trends Ecol. Evol. 27, 1–10.

Woolhouse, M.E.J., Haydon, D.T., Antia, R., 2005. Emerging pathogens: the epidemiology and evolution of species jumps. Trends Ecol. Evol. 20, 238–244.

Xu, J., James, R., 2009. Genes related to immunity, as expressed in the alfalfa leafcutting bee, *Megachile rotundata*, during pathogen challenge. Insect Mol. Biol. 18, 785–794.

APPENDICES

APPENDIX A

Appendix A: Full multiple comparison statistics for bioassays described in Chapter 2.

Table A1. Log-rank test comparing the survival curves of six infective fungal treatments given to honey bee larvae. Results were adjusted with Tukey's multiple comparison adjustment.

Treatment Comparison		χ^2	p-value	
Treatment 1	Treatment 2		Raw	Tukey
Control	*A. apis*	104.2	<.0001	<.0001
Control	*A. aggregata*	2.4440	0.1180	0.7059
Control	*A. larvis*	4.9267	0.0264	0.2848
Control	*A. apis + A. larvis*	127.2	<.0001	<.0001
Control	*A. aggregata + A. larvis*	3.9500	0.0469	0.4226
Control	*A. apis + A. aggregata*	92.3790	<.0001	<.0001
A. apis	*A. aggregata*	89.1288	<.0001	<.0001
A. apis	*A. larvis*	73.9184	<.0001	<.0001
A. apis	*A. apis + A. larvis*	1.0406	0.3077	0.9496
A. apis	*A. aggregata + A. larvis*	81.0116	<.0001	<.0001
A. apis	*A. apis + A. aggregata*	0.5756	0.4480	0.9887
A. aggregata	*A. larvis*	0.5367	0.4638	0.9906
A. aggregata	*A. apis + A. larvis*	112.3	<.0001	<.0001
A. aggregata	*A. aggregata + A. larvis*	0.2107	0.6462	0.9993
A. aggregata	*A. apis + A. aggregata*	77.0260	<.0001	<.0001
A. larvis	*A. apis + A. larvis*	94.6706	<.0001	<.0001
A. larvis	*A. aggregata + A. larvis*	0.0785	0.7794	1.0000
A. larvis	*A. apis + A. aggregata*	62.9352	<.0001	<.0001
A. apis + A. larvis	*A. aggregata + A. larvis*	103.1	<.0001	<.0001
A. apis + A. larvis	*A. apis + A. aggregata*	3.2433	0.0717	0.5474
A. aggregata + A. larvis	*A. apis + A. aggregata*	69.3971	<.0001	<.0001

Table A2. Multiple comparison results for the transformed percentage of honey bee larvae

producing visible post infection fungal hyphae given either control or one of six infective

treatments. Results were adjusted with Tukey's multiple comparison adjustment.

Treatment Comparison		T-value	p-value	
Treatment 1	Treatment 2		Raw	Tukey
Control	*A. apis*	-10.52	<0.0001	<0.0001
Control	*A. aggregata*	-0.29	0.7742	0.9999
Control	*A. larvis*	-1.00	0.3213	0.9515
Control	*A. apis + A. larvis*	-10.65	<0.0001	<0.0001
Control	*A. aggregata + A. larvis*	-1.33	0.1888	0.8993
Control	*A. apis + A. aggregata*	-10.56	<0.0001	<0.0001
A. apis	*A. aggregata*	10.23	<0.0001	<0.0001
A. apis	*A. larvis*	9.52	<0.0001	<0.0001
A. apis	*A. apis + A. larvis*	-0.13	0.8996	1.0000
A. apis	*A. aggregata + A. larvis*	9.19	<0.0001	<0.0001
A. apis	*A. apis + A. aggregata*	-0.03	0.9732	1.0000
A. aggregata	*A. larvis*	-0.71	0.4791	0.9912
A. aggregata	*A. apis + A. larvis*	-10.36	<0.0001	<0.0001
A. aggregata	*A. aggregata + A. larvis*	-1.04	0.3017	0.9412
A. aggregata	*A. apis + A. aggregata*	-10.27	<0.0001	<0.0001
A. larvis	*A. apis + A. larvis*	-9.65	<0.0001	<0.0001
A. larvis	*A. aggregata + A. larvis*	-0.33	0.7424	0.9999
A. larvis	*A. apis + A. aggregata*	-9.55	<0.0001	<0.0001
A. apis + A. larvis	*A. aggregata + A. larvis*	9.32	<0.0001	<0.0001
A. apis + A. larvis	*A. apis + A. aggregata*	0.09	0.9262	1.0000
A. aggregata + A. larvis	*A. apis + A. aggregata*	-9.22	<0.0001	<0.0001

Table A3. Multiple comparison results for the proportion of honey bee larvae (arcsine square-root transformation) producing visible post-infection fungal spores after treatment with a control or one of six infective pathogen inoculated diets. Not all treatments resulted in fungal infections that produced spores. Results were adjusted with Tukey's multiple comparison adjustment.

Treatment Comparison		T-value	p-value	
Treatment 1	Treatment 2		Raw	Tukey
A. apis	*A. aggregata*	7.44	<0.0001	<0.0001
A. apis	*A. apis + A. larvis*	-0.42	0.6755	0.9995
A. apis	*A. apis + A. aggregata*	0.34	0.7333	0.9999
A. aggregata	*A. apis + A. larvis*	-7.86	<0.0001	<0.0001
A. aggregata	*A. apis + A. aggregata*	-7.10	<0.0001	<0.0001
A. apis + A. larvis	*A. apis + A. aggregata*	0.76	0.4486	0.9874

Table A4. Log-rank test comparing the survival curves of six infective fungal treatments given

to ALCB larvae. Results were adjusted with Tukey's multiple comparison adjustment.

Treatment Comparison		χ^2	p-value	
Treatment 1	Treatment 2		Raw	Tukey
Control	*A. apis*	7.5571	0.0060	0.0865
Control	*A. aggregata*	80.7464	<.0001	<.0001
Control	*A. larvis*	10.2396	0.0014	0.0233
Control	*A. apis + A. larvis*	5.2378	0.0221	0.2494
Control	*A. aggregata + A. larvis*	90.7326	<.0001	<.0001
Control	*A. apis + A. aggregata*	67.1092	<.0001	<.0001
A. apis	*A. aggregata*	40.3203	<.0001	<.0001
A. apis	*A. larvis*	0.1992	0.6554	0.9994
A. apis	*A. apis + A. larvis*	0.2066	0.6495	0.9993
A. apis	*A. aggregata + A. larvis*	47.4783	<.0001	<.0001
A. apis	*A. apis + A. aggregata*	30.4294	<.0001	<.0001
A. aggregata	*A. larvis*	35.1954	<.0001	<.0001
A. aggregata	*A. apis + A. larvis*	45.8476	<.0001	<.0001
A. aggregata	*A. aggregata + A. larvis*	0.2716	0.6023	0.9986
A. aggregata	*A. apis + A. aggregata*	0.8040	0.3699	0.9732
A. larvis	*A. apis + A. larvis*	0.8110	0.3678	0.9726
A. larvis	*A. aggregata + A. larvis*	41.9039	<.0001	<.0001
A. larvis	*A. apis + A. aggregata*	25.9165	<.0001	<.0001
A. apis + A. larvis	*A. aggregata + A. larvis*	53.4355	<.0001	<.0001
A. apis + A. larvis	*A. apis + A. aggregata*	35.3528	<.0001	<.0001
A. aggregata + A. larvis	*A. apis + A. aggregata*	2.0324	0.1540	0.7881

Table A5. Multiple comparison results for the proportion of alfalfa leafcutter bee larvae (arcsine square-root transformation) producing visible, post-infection, fungal spores after treatment with a control or one of six infective pathogen inoculated diets. Results were adjusted with Tukey's multiple comparison adjustment.

Treatment Comparison		T-value	p-value	
Treatment 1	Treatment 2		Raw	Tukey
Control	*A. apis*	-3.21	0.0074	0.0798
Control	*A. aggregata*	-10.78	<0.0001	<0.0001
Control	*A. larvis*	-3.59	0.0037	0.0434
Control	*A. apis* + *A. larvis*	-2.60	0.0233	0.2083
Control	*A. aggregata* + *A. larvis*	-12.07	<0.0001	<0.0001
Control	*A. apis* + *A. aggregata*	-8.09	<0.0001	<0.0001
A. apis	*A. aggregata*	-7.57	<0.0001	0.0001
A. apis	*A. larvis*	-0.37	0.7170	0.9997
A. apis	*A. apis* + *A. larvis*	0.62	0.5496	0.9949
A. apis	*A. aggregata* + *A. larvis*	-8.86	<0.0001	<0.0001
A. apis	*A. apis* + *A. aggregata*	-4.87	0.0004	0.0052
A. aggregata	*A. larvis*	7.19	<0.0001	0.0002
A. aggregata	*A. apis* + *A. larvis*	8.18	<0.0001	<0.0001
A. aggregata	*A. aggregata* + *A. larvis*	-1.29	0.2211	0.8438
A. aggregata	*A. apis* + *A. aggregata*	2.70	0.0195	0.1806
A. larvis	*A. apis* + *A. larvis*	0.99	0.3433	0.9477
A. larvis	*A. aggregata* + *A. larvis*	-8.49	<0.0001	<0.0001
A. larvis	*A. apis* + *A. aggregata*	-4.50	0.0007	0.0095
A. apis + *A. larvis*	*A. aggregata* + *A. larvis*	-9.47	<0.0001	<0.0001
A. apis + *A. larvis*	*A. apis* + *A. aggregata*	-5.49	0.0001	0.0020
A. aggregata + *A. larvis*	*A. apis* + *A. aggregata*	3.99	0.0018	0.0223

APPENDIX B

Appendix B. Multiple comparisons for treatments listed in Chapter 4.

Table B1. Log-rank test comparing the survivorship curves of six infective fungal treatments given to alfalfa leafcutting bee larvae. Results were adjusted with Tukey's multiple comparison adjustment.

Treatment Comparison		χ^2	p-value	
Treatment 1	Treatment 2		Raw	Tukey
Control	*A. aggregata*	222.1	<.0001	<.0001
Control	*A. proliperda*	40.16	<.0001	<.0001
Control	A. agg+A. pro (simul.)	238.0	<.0001	<.0001
Control	A. agg then A. pro.	123.5	<.0001	<.0001
Control	A. pro then A. agg	134.5	<.0001	<.0001
A. aggregata	*A. proliperda*	105.4	<.0001	<.0001
A. aggregata	A. agg+A. pro (simul.)	2.039	0.1533	0.7100
A. aggregata	A. agg then A. pro.	30.78	<.0001	<.0001
A. aggregata	A. pro then A. agg	27.88	<.0001	<.0001
A. proliperda	A. agg+A. pro (simul.)	102.3	<.0001	<.0001
A. proliperda	A. agg then A. pro.	28.35	<.0001	<.0001
A. proliperda	A. pro then A. agg	33.44	<.0001	<.0001
A. agg+A. pro (simul.)	A. agg then A. pro.	22.49	<.0001	<.0001
A. agg+A. pro (simul.)	A. pro then A. agg	19.55	<.0001	<.0001
A. agg then A. pro.	A. pro then A. agg	0.15	0.6990	0.9989

APPENDIX C

Appendix C. Non-committee co-author release forms

16 March 2015

Dr. Nabil Youssef
Utah State University
5305 Old Main Hill
Logan, UT 84322

Dear Dr. Youssef:

I am in the process of preparing my dissertation in the Biology Department at Utah State University. I plan to complete my degree in the summer semester of 2015.

I am requesting your permission to include our co-authored paper "A multi-gene phylogeny provides insight into the relationships among *Ascosphaera* species" as a chapter in my dissertation. I will include acknowledgements and appropriate citations to your role as co-author in the dissertation.
Please indicate your approval to release the paper to be published in my dissertation by signing in the space provided below. If you have any questions, please contact me.

Thank you for your cooperation,

Ellen G. Klinger

I hereby give permission to Ellen G. Klinger to release the publication "A multi-gene phylogeny provides insight into the relationships among *Ascosphaera* species", to be printed in her dissertation.

Signed _____ Date 3/21/15

16 March 2015

Dr. Svjetlana Vojvodic
Rowan University
201 Mullica Hill Rd.
Glassboro, NJ 08028

Dear Dr. Vojvodic:

I am in the process of preparing my dissertation in the Biology Department at Utah State University. I plan to complete my degree in the summer semester of 2015.

I am requesting your permission to include our co-authored paper "Mixed infections reveal virulence differences between host-specific bee pathogens " as a chapter in my dissertation. I will include acknowledgements and appropriate citations to your role as co-author in the dissertation.
Please indicate your approval to release the paper to be published in my dissertation by signing in the space provided below. If you have any questions, please contact me.

Thank you for your cooperation,

Ellen G. Klinger

Ellen G. Klinger

_____

I hereby give permission to Ellen G. Klinger to release the publication "Mixed infections reveal virulence differences between host-specific bee pathogens", to be printed in her dissertation.

Svjetlana Vojvodic

Signed _____ Date ___March 16, 2015___

19 February 2015

Dr. Gloria DeGrandi-Hoffman
USDA Carl Hayden Bee Research Center
200 East Allen Road
Tucson, AZ 85719

Dear Dr. DeGrandi-Hoffman:

I am in the process of preparing my dissertation in the Biology Department at Utah State University. I plan to complete my degree in the summer semester of 2015.

I am requesting your permission to include our co-authored paper "Mixed infections reveal virulence differences between host-specific bee pathogens " as a chapter in my dissertation. I will include acknowledgements and appropriate citations to your role as co-author in the dissertation.
Please indicate your approval to release the paper to be published in my dissertation by signing in the space provided below. If you have any questions, please contact me.
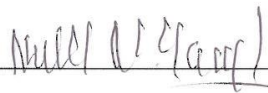
Thank you for your cooperation,

Ellen G. Klinger

_____

I hereby give permission to Ellen G. Klinger to release the publication "Mixed infections reveal virulence differences between host-specific bee pathogens", to be printed in her dissertation.

Signed _____ Date _2-19-15_

APPENDIX D

Appendix D. Permission to reprint published articles

Klinger, Ellen

From:	Permissions Helpdesk [permissionshelpdesk@elsevier.com]
Sent:	Tuesday, May 19, 2015 10:55 AM
To:	Klinger, Ellen
Subject:	RE: YJIPA 6675

Dear Ms. Klinger:

As an Elsevier journal author, you retain various rights including Inclusion of the article in a thesis or dissertation (provided that this is not to be published commercially) whether in part or *in toto*; see http://www.elsevier.com/about/policies/author-agreement/lightbox_scholarly-purposes for more information. As this is a retained right, no written permission is necessary provided that proper acknowledgement is given.

Can you confirm that the Elsevier journal article will have "published" (i.e. at minimum, a proof version will be available online) prior to your dissertation in both cases? If not, this should not be problematic, provided this information is fully disclosed to the editors; see e.g. http://www.elsevier.com/about/open-access/open-access-policies/article-posting-policy#pre-print:

In general, Elsevier is permissive with respect to authors and electronic preprints. If an electronic preprint of an article is placed on a public server prior to its submission to an Elsevier journal or where a paper was originally authored as a thesis or dissertation, this is not generally viewed by Elsevier as "prior publication" and therefore Elsevier will not require authors to remove electronic preprints of an article from public servers should the article be accepted for publication in an Elsevier journal.

If I may be of further assistance, please let me know.

Best of luck with your dissertation and best regards,
Hop

Hop Wechsler
Permissions Helpdesk Manager
Elsevier
1600 John F. Kennedy Boulevard
Suite 1800
Philadelphia, PA 19103-2899
Tel: +1-215-239-3520
Mobile: +1-215-900-5674
Fax: +1-215-239-3805
E-mail: h.wechsler@elsevier.com
Contact the Permissions Helpdesk:
☎ *+1-800-523-4069 x 3808* ✉ *permissionshelpdesk@elsevier.com*

Hi,

I have a manuscript accepted for publication in the Journal of Invertebrate Pathology (YJIPA 6675), and I am completing the copyright transfer. This manuscript is part of my dissertation work, and, since submission, I have defended and am making final edits to my dissertation. Since the timeline of this article's publication and my dissertation submission is

somewhat parallel, I just wanted to make sure that stating that the work "has not been published elsewhere" is acceptable, or if additional steps are needed.

In addition, I have an additional article, YJIPA_6371 that has been published in the same journal, and I need to request reprint permission for my dissertation (attached). Please note that, on that manuscript, 2 of the 4 authors (including myself) are US government employees.

Please contact me with any questions,
Thank you,

Ellen Klinger
Biological Science Technician
Logan Location Safety Coordinator
USDA-ARS Bee Biology and Systematics Laboratory
Utah State University
1410 North 800 East
North Logan, UT 84341
(435)797-8011
(435)797-0196 (Fax)

CURRICULUM VITAE

Ellen G. Klinger

ellen.klinger@ars.usda.gov

1410 North 800 East
USDA Pollinating Insect Research Unit
North Logan, UT 84341
(435)797-8011 (p)
(435)797-0196 (f)

Education:

Ph.D. in Biology   August 2015
Utah State University, Logan, UT 84322
Dissertation: Virulence evolution of fungal pathogens in social
and solitary bees with an emphasis on multiple infections.
Advisors: Dr. Dennis L. Welker; Dr. Rosalind R. James
GPA: 3.98 out of 4.00

M.S. in Ecology and Environmental Sciences   August 2003
University of Maine, Orono, ME 04469
Thesis: Susceptibility of adult Colorado potato beetles to the fungal
entomopathogen *Beauveria bassiana*.
Advisor: Dr. Eleanor Groden
GPA: 3.98 out of 4.00

B.S. in Biology   May 2000
Lycoming College, Williamsport, PA 17701
GPA: 3.96 out of 4.00
Summa cum laude, departmental honors

Professional Experience:

Biological Science Technician   January 2004- present
United States Department of Agriculture, Agricultural Research Service
Pollinating Insect Research Unit, Logan, UT 84341
Worked with diseases of solitary and social bees in pathology laboratory

Research Assistant   June 2003-September 2003
University of Maine; Orono, ME 04469
Led field surveys of European imported red ant (<u>Myrmica rubra</u>) in Acadia National Park.

Research Assistant   January 2001-September 2001;
University of Maine; Orono, ME 04469   January 2002- May 2003
Lab and field work on biological control of insect pests in potatoes and corn.

Peer-Reviewed Publications:

Klinger, E.G., S. Vojvodic, G. DeGrandi-Hoffman, D.L. Welker and R.R. James (in revision). Mixed infections reveal virulence differences between host-specific bee pathogens. Journal of Invertebrate Pathology.

Koch, J.B, B. Love, **E. Klinger** and J.P. Strange. 2014. The effect of photobleaching on bee (Hymenoptera: Apoidea) setae color and its implications for studying aging and behavior. Journal of Melittology 38: 1-9.

Klinger, E.G., R.R. James, N.N. Youssef, and D.L. Welker. 2013. A multi-gene phylogeny provides additional insight into the relationships between several *Ascosphaera* species. Journal of Invertebrate Pathology 112(1):41-48.

Klinger, E.G., E. Groden and F. Drummond. 2006. *Beauveria bassiana* infection between cadavers and adults of the Colorado potato beetle, *Leptinotarsa decemlineata* (Say). Environmental Entomology 35(4): 992-1000.

Published Abstracts and Scientific Presentations (Presenter name in bold):

Ellen Klinger, Dennis Welker and Rosalind James. Multiple fungal infections in the solitary bee, *Megachile rotundata.* 2014. Presented at the Entomological Society of America annual meeting, Portland OR.

Ellen Klinger, **Dennis Welker**, Svjetlana Vojvodic, Gloria Degrandi-Hoffman, and Rosalind James.   Pathogenicity of mixed infections of *Ascosphaera* in solitary and social bees. 2014. Presented at the Entomological Society of America Pacific Branch meeting, Tucson, AZ.

Ellen Klinger, Dennis Welker and Rosalind James. Pathogenicity of mixed infections of *Ascosphaera* in solitary and social bees. 2014. Presented at the Intermountain Graduate Research Symposium, Logan UT.

Ellen Klinger and Rosalind James. Multiple infections in bees: chalkbrood in managed *Megachile rotundata.* 2013. Poster presented at the 2013 International Conference on Pollinator Biology, Health and Policy, State College, PA.

Ellen Klinger, Svjetlana Vojvodic, and Rosalind James. Pathogenicity of mixed infections of *Ascosphaera* in solitary and social bees. 2013. Presented at the Society of Invertebrate Pathology Annual Meeting, Pittsburgh, PA.

Ellen Klinger, Rosalind James and Dennis Welker. Does pathogen relatedness affect the success of mixed fungal infections in bees? 2012. Presented at the Intermountain Graduate Research Symposium, Logan UT.

Ellen Klinger and **Rosalind James**. Competition between chalkbrood infections in alfalfa leafcutting bees. 2011. Poster presented at the Northwest Alfalfa Seed Growers Association annual meeting, Las Vegas, NV.

Ellen Klinger and Rosalind James. Chalkbrood co-infections of leafcutting bees *(Megachile rotundata)*: Understanding the arms race between obligate, facultative, and non-pathogenic fungal species. 2011. Presented at the Entomological Society of America annual meeting, Reno, NV.

Ellen Klinger, Rosalind James and Dennis Welker. Multi-gene phylogeny of *Ascosphaera* fungi, the causative agent of chalkbrood disease in bees. 2011. Presented at the Intermountain Graduate Student Symposium, Logan UT.

Ellen Klinger. Chalkbrood infection dynamics in the alfalfa leafcutting bee. 2011. Presented at the USDA Alfalfa Pollinator Workshop, Logan UT.

Rosalind James, Theresa Pitts-Singer and **Ellen Klinger**. Chalkbrood Distribution and transmission in U.S. populations of the alfalfa leafcutting bee. 2009. Paper presented at the Society of Invertebrate Pathology Annual Meeting, Park City UT.

Rosalind James, **Ellen Klinger** and Craig Huntzinger. Disease transmission in solitary bees- the case of chalkbrood in *Megachile rotundata*. 2009. Paper presented at the Entomological Society of America annual meeting, Indianapolis, IN.

Junhuan Xu, Rosalind James, Craig Huntzinger and **Ellen Klinger**. Development and characterizations of expressed sequence tags from the alfalfa leafcutting bee (*Megachile rotundata*). 2008. Paper presented at the Entomological Society of America annual meeting, Reno, NV.

Rosalind James, Craig Huntzinger, **Ellen Klinger** and Jeff Skinner. Fungal Diseases in Bees: a story of *Ascosphaera*. 2004. Paper presented at the Society of Invertebrate Pathology annual meeting, Helsinki, Finland.

Ellen Klinger and Eleanor Groden. Horizontal transmission of *Beauveria bassiana* between cadavers and adults of *Leptinotarsa decemlineata*. 2003. Poster presented at the Society for Invertebrate Pathology Annual meeting, Burlington, VT (Student Competition).

Ellen Klinger and Eleanor Groden. Susceptibility of adult Colorado potato beetle to the fungal entomopathogen *Beauveria bassiana*: 2002. Paper presented at the Acadian Entomological Society Annual meeting, Machias, ME (Student Competition).

Ellen Klinger, Eleanor Groden and Francis Drummond. Avoidance Behavior of adult Colorado potato beetle to the fungal entomopathogen *Beauveria bassiana* 2002. Paper presented at the University of Maine Graduate Research Exposition.

Ellen Klinger and Eleanor Groden. Susceptibility of adult Colorado potato beetle to the fungal entomopathogen *Beauveria bassiana*: 2002. Paper presented at the Entomological Society of America Eastern Branch meeting, Ocean City, MD (Student Competition).

Ellen Klinger and Eleanor Groden. Susceptibility of adult Colorado potato beetle to the fungal entomopathogen *Beauveria bassiana*: 2001. Poster presented at the National Entomological Society of America meeting, San Diego, CA.

Other Publications:
Arthropod Containment Guidelines, section written for the Agricultural Research Service's Biohazard Control/Laboratory Biosafety Manual. 2008.

Honors and Awards:
- Federal Employee Assistance Scholarship, September 2012
- Outstanding Presentation Abstract Award, Intermountain Graduate Research
     Symposium, April 2012
- Performance Based Award, USDA –Agricultural Research Service, June 2006, May
     2007, March 2008, September 2008, September 2009, September 2010.
- Society of Invertebrate Pathology Student Poster Competition, third place, July 2003.
- Maine Agricultural and Forestry Experiment Station Clements Award, December 2002.
- Acadian Entomological Society Student Paper Competition, first place, July 2002.
- Path-Lathrop Prize in Entomology, University of Maine, April 2002.
- Entomological Society of America Eastern Branch Student Paper Competition, third
     place, March 2002.

Scientific Society Office and Committee Assignments:
- Utah State University Biology Graduate Student Association, Secretary,
  November 2010-2011 (elected).
- University of Maine's Association of Graduate Students, Biological Sciences Student
  Representative, September 2002-May 2003 (elected).
- Chair, Graduate Student Committee for Entomological Society of America Eastern
  Branch- May 2001-May 2002 (elected).
- University of Maine's Biological Sciences Seminar Committee, 2001-2002 (volunteer).

Other Committee Assignments:
- Collateral Duty Safety Officer, USDA-ARS Northern Plains Area 2006-present
  (appointed).
- Location Safety Coordinator, USDA-ARS Pacific West Area 2014 (appointed).

Professional Society Memberships:

Society of Invertebrate Pathology	June 2003-June 2004; March 2011- present
Entomological Society of America	Dec. 2000- Oct. 2004; July 2010-present
American Society of Safety Engineers	June 2006- June 2007
Acadian Entomological Society	April 2002-April 2004

Other Memberships:

Utah State University Entomology Club	September 2007-present

Invited Presentations:
- Washington State University Beginning Beekeeping Course, Invited lecturer, June
  2014.
- ARS Seminar Series, Logan UT, December 2013.
- Determining susceptibility of insects to fungal pathogens, Symposium speaker, ESA
  Eastern Branch Annual meeting, March 2003.
- Master Gardner Basic Entomology Course, Guest Lecturer, Falmouth, ME- June 2002.
- Master Gardner Advanced Entomology Course, Guest Lecturer, Bangor and Gray, ME-
  May 2002.

Symposiums organized:
- DNA Technologies in Entomological Research, ESA eastern branch annual meeting
  2002, Ocean City, MD.

Teaching Experience:
-Graduate Teaching Assistant, Utah State University Biology 1610, Biology I Laboratory
Teaching Assistant, 2014.
-Teaching Assistant, University of Maine, Introduction to Entomology Lab September-
December 2000; September-December 2001.
- Biology Teaching Assistant, Lycoming College, General Biology Laboratory 1997-
2000.

Workshops:
- Logan Utah Farmer's Market Pollinator Day, June 2012; June 2014.
- Discovering the World of Bees - Pollinator Workshop, USDA Pollinating Insect
Research Unit- Bee Disease Workshop, prepared and assisted,   June 2010.