The Ecology and Evolution of *Aspergillus* spp. Fungal Parasites in Honey Bees

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The candidate confirms that the work submitted is his/her own, except where work which has formed part of jointly-authored publications has been included. The contribution of the candidate and the other authors to this work has been explicitly indicated after each chapter. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others.

Chapter 2

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The candidate, Kirsten Foley, designed the experiments, carried out the experimental work, analysed the data and wrote the manuscript. GF assisted with the experimental work, specifically the collection of bee larvae. ABJ assisted with the design of the experiments and provided training. WOHH supervised the work, assisted with the design of the experiments, the data analysis and drafting of the manuscript.

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Abstract

Evolution of virulence in parasites has profound effects on both host-parasite co-evolution and ecology and is influenced by environmental factors and the genotypes involved. Many parasite infections consist of multiple strains or species that are predicted to result in the evolution of more virulent strains that exploit the host less prudently. In opportunistic parasites, the dynamics and evolution of virulence are poorly understood as traditional epidemiological models do not adequately describe parasites capable of persisting outside of the host. In addition, as microbial pathogenicity may shift from opportunistic to obligatory strategies with time, knowledge of the evolutionary dynamics of opportunistic pathogens is crucial for predicting and understanding disease emergence. *Aspergillus* species of fungi have a ubiquitous distribution and are the etiological agents of stonebrood disease in honey bees. It is generally considered that stonebrood occurs rarely in honey bees, but the epidemiology and predisposing conditions for the disease are almost entirely unknown. In this study, I examine the occurrence, pathogenicity and competitive ability of *Aspergillus* spp. when infecting honey bees, as well as the effect of nutrition on host susceptibility and the adaptation of the fungi over the course of experimental evolution. A high prevalence and diversity of *Aspergillus* spp. isolates were identified following the screening of an apiary, and the pathogenicity of three species (*A. flavus, A. nomius* and *A. phoenicis*) was established. Further, in laboratory-reared larvae a nutritionally limited diet increased susceptibility to *A. fumigatus.* In a series of single-generation interspecific competition experiments between *Aspergillus* spp. and the obligate pathogen chalkbrood (*Ascophaera apis*), the virulence and fitness of dual infections were influenced by complex within-host interactions depending on the species involved, which ranged from synergistic to inhibitory effects. Finally, following serial passage of *A. flavus* and *A. phoenicis* in the honey bee larval hosts to determine the evolution of virulence and fitness, no evidence of host adaptation was observed, revealing the unpredictability of these asexually reproducing opportunistic pathogens. These findings illustrate a complex relationship between *Aspergillus* spp. and honey bees and emphasises the significant influence these ubiquitous organisms can have on the ecology and evolution of honey bees.

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Chapter One

General Introduction

Parasites play a critical role in the regulation and coevolution of their host populations (Anderson and May 1979, Hamilton 1980), by influencing host life-history and the processes that structure local species assemblages, through negative effects on host fitness (Dobson and Hudson 1986, Minchella and Scott 1991, Moret and Schmid-Hempel 2004.) Parasitism may be responsible for speciation, sexual dimorphism and the maintenance and generation of genetic diversity within populations (Haldane 1949, Clarke 1979, Keymer and Read 1990). Coevolution is particularly important in host-parasite systems because of the close association, and thus the process of reciprocal adaptive genetic change, between interacting species (Thompson 1994). The reciprocal traits involved will usually be the infectivity of the parasite versus the resistance of the host forming an antagonistic interaction. Antagonistic coevolution can lead to the maintenance of genetic polymorphism through negative-frequency dependent selection (Hamilton 1993, Peters and Lively 1999, Berenos *et al.* 2011). This occurs as parasites are selected to overcome host immune defences and hosts are under selective pressure to resist parasites. Because a rare genotype which is favoured by selection becomes more frequent and triggers gene frequency changes in the coevolving species, cyclical gene-frequency dynamics will occur otherwise known as the Red Queen hypothesis (Bell 1982). The antagonistic coevolution model has been confirmed to explain the immense diversity found in vertebrate MHC genes as well as the maintenance of disease causing alleles (Kubinak 2012). The outcome of the host-parasite interaction will ultimately depend on the combination of host and pathogen genotypes. Theoretical models of host-parasite coevolution include 'matching genotype', 'gene-for-gene' and 'matching allele' models (Flor 1971, Anderson and May 1991, Lively and Apanius 1995, Gandon and Michalakis 2000). These predict a range of outcomes including dynamic polymorphisms, stable polymorphisms, instable cycles and selective sweeps leading to the fixation of alleles (Woolhouse 2002).

In addition to the genetic context of host-parasite coevolution, environmental factors will also affect infection outcomes. Here, environment is considered to be anything external to a given individual e.g. nutrition, temperature or the presence of other organisms. Phenotypic plasticity is the ability of a single genotype to express different phenotypic states in various environments. The range of phenotypes expressed by a single genotype is known as the reaction norm (Stearns 1992). When a genotype-environment interaction occurs, the relative resistance of hosts to specific

parasite strains will be environmentally dependent. Consequently, if there is a trade-off in adaptation to varying environmental conditions genetic variation will be maintained in a population (Byers 2005). Strong evidence of host genotype x parasite genotype x environment interactions is based on invertebrate or plant-pathogen systems (Wolinska and King 2009). Out of 91 experimental studies reviewed by Wolinska and King (2009), the environment significantly altered the specificity of selection in 31 cases when the three-way interaction was examined indicating that in some host–pathogen systems there are a range of optimum genotypes depending on the environment.

1.2 The evolution of virulence and multiple infections

The impact that parasites have on the ecology and evolution of host populations will depend on the virulence of infection. Virulence, broadly defined as the detrimental consequence of infection (loss of fitness) experienced by the host, is a major driver in host-parasite coevolution and may be viewed either as a property of the parasite, or as a property of the host-parasite interaction (Read 1994, Ebert and Herre 1996, Brown *et al.* 2002). The finite resources available in the system are allocated towards growth and fitness in the host or parasite. Virulence evolution theories are based on the main idea that parasites face a trade-off between growth and transmission and the assumption that a positive correlation exists between virulence and transmission success (Ewald and De Leo 2002). Support for the trade-off hypothesis is evident in Anderson and May's (1982) analysis of the Myxoma virus epidemic which showed how the highly virulent virus evolved to an intermediate level of virulence and maximum fitness (Fenner *et al.* 1957). Experimental data using mouse-malaria model also found that the more virulent clones had higher transmissibility and more persistent infections (MacKinnon and Read 1999a, 1999b, 2003, Fergusson *et al.* 2003). Serial passage experiments, which involve the serial transmission of parasite lines through genetically defined host populations, often result in the evolution of increased virulence in the derived parasites (Ebert 2000). Here, transmission is achieved by the experimenter, the parasite is no longer constrained by the cost of virulence, and it hence evolves greater virulence through maximum host exploitation.

Parasite infections are often genetically diverse consisting of multiple species or strains that compete for resources in the host (Read and Taylor 2001). Multiple infections may impose a significant impact on host populations through their potential for increased virulence and transmission (Nowak and May 1994). Competition among parasite genotypes within the host is hypothesised to be a major driving force in the

evolution of increased within-host growth rate and virulence (Nowak and May 1994, van Baalen and Sabelis 1995). The nature of the competitive interactions that take place within the host will vary depending on the traits of the parasites involved. The outcome of multiple infections will depend on a range of other factors including parasite dose, the temporal spacing of each inoculation, parasite relatedness and host immune responses (Read and Taylor 2001). Previous studies have yielded highly variable results with multiple infections resulting in higher, lower or similar levels of virulence when compared to single control infections (Taylor *et al.* 97, Imhoof and Schmid-Hempel 98, Read and Taylor 2001, Hughes and Boomsma 2004, de Rhoode *et al.* 2005). The overall virulence in the multiple infections is the result of interactions between the co-infecting parasites, mediated by the hosts' immune response. This observed virulence will not often predict how virulence evolves as the within-host growth and between-host transmission dynamics may differ due to epidemiological feedbacks (Alizon *et al.* 2013). For example, if a double infection becomes frequent in a population, higher virulence is favoured due to within-host competition, but when more virulent pathogens are predominant the force of infection (the rate at which the susceptible individuals become infected) will decrease favouring lower virulence (van Baalen and Sabelis 1995). Consequently, the impact of multiple infections on the evolution of parasite virulence and transmission remains poorly understood. Alizon *et al.* (2013) suggest that both within-host and between-host levels must be analysed concurrently to accurately predict how multiple infections affect virulence evolution.

1.3 Current concerns in honey bee health

Honey bee health has been a major cause for concern in recent years since the enigmatic colony declines in 2006 in the United States termed colony collapse disorder (CCD) (vanEngelsdorp *et al.* 2009, Cox-Foster *et al.* 2007). These CCD colonies share three main characteristics; (1) the sudden loss of adult worker bees with a high brood to adult bee population ratio remaining; (2) lack of dead worker bees in the vicinity of the affected hive; and (3) a delay in subsequent kleptoparasitism from neighbouring colonies and invasion of hive pests such as wax moths and small hive beetle (vanEngelsdorp *et al.* 2010). The cause of CCD has attracted many possible explanations and it has become increasingly apparent that it is a multifactorial syndrome which has greatly increased awareness of honey bee pathology (vanEngelsdorp *et al.* 2010, Oldroyd 2007). Among the explanations are nutritional and energetic stressors due to lack of foraging resources, leading to immunocompromised individuals that may be more susceptible to emerging and existing diseases (Naug and

Mayack 2009). Large scale epizootological surveillance of CCD affected and non-affected colonies by van Engelsdorp *et al.* (2010) detected more diverse coinfections with higher pathogen loads in CCD affected colonies. CCD colonies were also more likely to be neighbours indicating a contagious or common environmental element to the condition (vanEngelsdorp *et al.* 2010). Pesticide studies suggest a role for sublethal exposure to agricultural chemicals and in-hive chemicals used by apiarists (Chauzat *et al.* 2009, Johnson *et al.* 2009). So far there has been no clear explanation and further research, in particular with regard to honey bee parasites is required.

1.4 Characteristics of social insect colonies: potential for disease

Social insect colonies are expected to be susceptible to pathogen invasion due to the high density of individuals in colonies, low intracolonial genetic diversity with a high potential for disease transmission through frequent interactions. This is evident in honey bees which form large colonies of up to 50,000 workers and are susceptible to many parasites (Morse and Flottum 1997). Such high host densities can increase opportunities for horizontal transmission which may in turn lead to selection for higher virulence in parasites (Bull 1994). However, certain characteristics of advanced social organization reduce the impact of pathogens by making invasion of the colony more complex. The division of labour in social insect colonies comprised of morphological castes and age-related polyethism creates a heterogenous environment reducing parasite transmission. The parallel arrangements of worker castes create barriers preventing the spread of infection throughout the colony (Naug and Camazine 2002). High worker turnover where worker schedules progress from young workers that tend the queen, then brood nest, and finally to foraging outside the colony (Schmid-Hempel 1998), help prevent contamination from invading pathogens.

1.5 Honey bee immune defense

Honey bees are challenged by a broad range of parasites including viruses, bacteria, microsporidian, fungi, mites and beetles, for some of which their impacts on colony health remain elusive (Genersch 2010). Defences against disease in social insects involves both individual and colony level defences. The honey bee genome contains comparatively fewer immune genes than solitary insects (Weinstock *et al.* 2006) thus, sociality may reduce the cost of maintaining individual pathogen resistance (Moret and Schmid-Hempel 2000). Colony level immune responses comprise a number of behaviours, including selfgrooming, allogrooming and undertaking, collectively termed

as 'social immunity' (Cremer *et al.* 2007). In honey bees, hygienic behaviour is defined as a collective response by adult bees to the presence of diseased and parasitised brood and is an important defense against larval diseases such as American foulbrood *Paenibacillus larvae*, chalkbrood *Ascosphaera apis* and *Varroa destructor* (in *Apis ceranae*)(Evans and Spivak 2010, Gilliam *et al.* 1988). It has been described as a two-step process, firstly uncapping and secondly the removal of affected brood from the cell (Rothembuhler 1964). There is a strong consensus that hygienic behaviour has a genetic basis whereby a small number of loci affect the expression of hygiene levels of workers (Oldroyd and Thompson 2007). In a study using bees selected for hygienic and non-hygienic behaviour, bees from non-hygienic lines were less able to distinguish between diseased and non-diseased brood odours implying an olfactory stimulus for hygienic behaviour in bees (Masterman *et al.* 2000). Behavioural fever, a form of thermoregulation performed by upregulating nest temperature to combat disease threat, is generated as a colony-level response to prevent chalkbrood caused by the heat-sensitive fungus *Ascosphaera apis* (Starks *et al.* 2000). Interactions between colony members can function to prevent the spread of pathogens within the colony. A positive relationship between the effect of sociality on resistance to the fungal pathogen *Metarhizium anisopliae* has been observed in *Acromyrmex echinatior* ants relating to allogrooming and the secretions of antibiotic compounds (Hughes *et al.* 2002). However, parasites can also adapt to exploit interactions among individuals of the colony. Trophallaxis, the oral exchange of food between individuals, is an important transmission route for pathogens from adults to larvae via contaminated food, for example American foulbrood bacteria and chalkbrood spores (Schmid-Hempel 1998, Bailey 1968). Nest hygiene is another defence mechanism against microbes that increases sanitation of individuals and the nest (Wilson-Rich *et al.* 2009). Larvae are fed royal jelly and stored honey, while plant resins are collected and mixed with wax then used in the colony as propolis, all of these possess antimicrobial properties (Morse and Flottum 1997, Bancova *et al.* 2000).

1.6 Genetic diversity and disease resistance

The relationship between genetic diversity and disease resistance has been proposed to explain the evolution of polyandry in social Hymenoptera (Sherman *et al.* 1998). Empirical support for this hypothesis has been found in leaf-cutting ants (Hughes and Boomsma 2004), bumble bees (Baer and Schmid-Hempel 1999) and honey bees (Invernizzi *et al.* 2009, Tarpy and Seeley 2006, Palmer and Oldroyd 2003, Tarpy 2003) where colonies with greater genetic diversity, through multiply mated queens, show

greater resistance to infections. One investigation into intracolonial genetic diversity involving multiple infections implied that the genetic basis for resistance differed according to parasite (Invernizzi *et al.* 2009). Patrilines differ in their susceptibility to a pathogen irrespective of queen mating frequency which also suggests a strong genetic component to disease resistance. Furthermore, gene expression assays on honey bees from a single colony have shown a high level of variation in transcript abundance for the anti-microbial peptide abaecin that may reflect allelic variation between colony members (Evans 2004). In hymenopterans, sex is determined by a haplodiploid mechanism where unfertilized eggs produce haploid males and fertilized eggs produce diploid females (Borgia 1980). This means that on disease resistance loci males are homozygous. The haploid susceptibility hypothesis suggests that haploid males are more susceptible to disease than their diploid female conspecifics that possess greater immunity due to a heterozygote advantage (Wilson-Rich *et al.* 2009). This hypothesis has been suggested as a driver in the evolution of sociality in haplodiploid insects (O'Donnell and Beshers 2004). Support for this hypothesis in the form of reports of lower male immunocompetence in a number of social insects is often, however, confounded by factors offering alternative explanations as males in social insects often differ from females in their size, development time, location in brood nest and behaviour (Santillan-Galicia *et al.* 2002). Furthermore, when the hypothesis was tested on *Bombus terrestris* males and workers and their resistance to *Crithidia bombi* infections, the susceptibility and levels of infection in haploid males did not differ when compared to diploid workers. In fact a trend towards lower infection levels was observed (Ruiz-Gonzalez and Brown 2006).

1.7 The genus *Aspergillus*

Phylum: Ascomycota
Order: Eurotiales
Family: Trichocomaceae
Genus: *Aspergillus*

The genus *Aspergillus* is a group of diverse filamentous fungi containing over 250 species (Machida and Gomi 2010). They consist of anamorphic (asexual) species with known or presumed telomorphic (sexual) forms in the family Trichocomaceae (Verweij and Brandt 2007). Although at least 40 species of *Aspergillus* are known opportunistic

pathogens, the genus follows a predominantly saprophytic lifestyle (Varga and Samson 2008, Verweij and Brandt 2007). They have been isolated from soil and litter in all of the major biomes and are major players in ecosystems, involved in the degradation of organic substrates (Klich 2002). As a consequence of the role of these fungi in the degradation of complex dead organic matter one of their properties is to secrete a large diversity of extracellular enzymes and compounds that are widely used in biotechnology, medicine and foods (Po 1994).

The life cycles of fungi are characteristically pleomorphic whereby alternating anamorphic and teleomorphic phases of the same species occur that vary either temporally or spatially in their distribution owing to the different environmental requirements of each phase (Burnett 2003). Consequently, with many species it is difficult to determine the complete life cycle involving both phases in the field. The species *A. fumigatus, A. flavus* and *A. niger* are now all known to be heterothallic (Wadman *et al.* 2009, Scazzoccio 2006, Paoletti *et al.* 2005). Yet only recently have sexual reproductive cycles been discovered in *A. fumigatus* (O'Gorman *et al.* 2009) and *A. flavus* (Horn *et al.* 2009). In comparison to the complex sexual chromosomes of higher eukaryotes, fungal genomes contain short sequences at the MAT locus that dictate the mating processes. In heterothallic fungi the mating types are determined by the genes found on the MAT locus containing one of either MAT1-1 or MAT1-2 idiomorphs (abbreviated to MAT-1 and MAT-2) (Turgeon and Yoder 2000). MAT-1 idiomorphs contain a MAT gene encoding an α-domain protein, whereas MAT-2 idiomorphs contain a MAT gene encoding for high-mobility group (HMG) -domain protein (Paoletti *et al.* 2005). Gene deletion studies have shown that MAT-1 and MAT-2 genes are essential in the production of sexually produced ascospores (Paoletti *et al.* 2007). There is also evidence to suggest that MAT genes play a role in regulation of the pheromone signalling system in heterothallic species that aids in the detection of mating partners (Paoletti *et al.* 2007, Coppin *et al.* 2005). Previous genomic studies on *A. fumigatus* detected 215 genes associated with sexual development including a high-mobility group (HMG)-domain at the MAT locus, thought to be ancestral sex determinants in fungi (Idnurum *et al.* 2008). Although this genetic evidence implies a sexual reproductive cycle in *A. fumigatus*, the difficulty in inducing reproduction between opposite mating types experimentally was due to the highly specific conditions required. Mature cleistothecia of the teleomorph *Neosartorya fumigata* were produced only after MAT-1 and MAT-2 strains were kept on parafilm-sealed oatmeal agar plates which had been held in the dark at 30°C for six months (O'Gorman *et al.* 2009). A similar combination of these specialised conditions has been found to induce

sexual reproduction in the aflatoxoin producing species, *A. flavus,* the teleomorph of which has been named *Petromyces flavus* (Horn *et al*. 2009). An interesting observation in this study was that sexual reproduction occurred between compatible mating types belonging to different vegetative compatible groups (VGC) (Horn *et al*. 2009). These groups, comprising vegetatively compatible individuals, possess identical alleles on the *het* loci that allow hyphal fusions to occur (Horn *et al.* 2009). *Aspergillus niger* is also heterothallic and possesses all the genetic material required for sexual reproduction, however, a sexual cycle has not yet been observed (Wadman *et al.* 2009). Given recent developments, it appears likely that the species does reproduce sexually and identification of a teleomorph could possibly be achieved by revising the environmental conditions used to induce crossing between compatible mating types. The discovery of sexuality and recombination processes in a group of supposedly asexual species has the potential to contribute significantly to genomic studies on the expression of important characteristics associated with these fungi.

Aspergillus spp. are major producers of mycotoxins including aflatoxins, ochratoxin and patulin (Varga and Samson 2008). *Aspergillus flavus*, the main source of aflatoxin contamination in food, is associated mainly with peanut, corn and cotton yields (Hedayati *et al.* 2007). Aflatoxins are extremely potent carcinogens, primarily targeting the liver, with teratogenic and immunosuppressant effects (Eaton and Groopman 1994). Acute aflatoxicosis can cause death (Bennett and Klich 2003). *Aspergillus flavus* strains consist of two morphologically distinct variants determined by sclerotial size and which differ in their likelihood to produce mycotoxins. These are termed the L-strain (average diameter >400μm) and the S-strain (average diameter <400μm) (Cotty and Bhatnagar 1994). When cultured on growth media in the dark, S-strain isolates produce elevated levels of aflatoxin, fewer conidia and more sclerotia than L-strains. Moreover, atoxigenic S-strain isolates are considered rare in natural environments (Orum *et al.* 1997) whereas up to 40% of L-strain isolates were found to be atoxigenic in the United States (Horn and Dorner 1998). A high level of variation in the production of mycotoxins (aflatoxin and cyclopiazonic acid) exists in populations of *A. flavus* (Horn and Dorner 1998). The occurrence of sexual reproduction between sexually compatible strains of *A. flavus* belonging to different vegetative compatibility groups (VCG) may indicate that crosses involve strains that differ in mycotoxin production capacity. As the ability to produce mycotoxins is linked to the presence of specific gene clusters, sexual recombination may explain this variation in mycotoxin production in *A. flavus* (Horn *et al.* 2009). *Aspergillus ochraceus* and *A. niger* belong to *Aspergillus* section Nigri, and are also among the most common fungi causing food

spoilage. Both are major sources of ochratoxin A (OTA) contamination (Abarca *et al.* 2004). OTA is nephrotoxic and a human renal carcinogen, like aflatoxin it is also teratogenic and has immunosuppressant properties (Al-anati and Petzinger 2006, Reijula and Tuomi 2003). It can occur in cereals, coffee, dried fruit, wine and grape juice (Magnoli *et al.* 2007, Varga and Kozakiewicz 2006, Chulze *et al.* 2006, Dalcero *et al.* 2002) with cereals being an important source of OTA in Europe (Juan *et al.* 2008).

Aspergillus spp. are ubiquitous fungi, with their airborne conidia (asexual spores) being extremely common in the environment. A number of pathogenic species cause disease in domesticated and wild animal species (Alvarez-Perez 2010, Tell 2005, Joseph 2000, Kunkle and Rimler 1996, Savage and Isa 1951). In humans, pulmonary infections acquired by inhalation of airborne conidia, cause a spectrum of clinical syndromes in immunocompromised hosts, particularly as a nosocomial disease in patients receiving immunosuppressant therapies (Zmeili and Soubani 2007, Latgé 1999). It is estimated that >200,000 cases of pulmonary aspergillosis occur globally each year with 30-95% mortality rates in infected populations (Brown 2012). Sea fan corals in the Caribbean have suffered declines due to the emerging pathogen *A. sydowii*, which as a species with high salt tolerance, is particularly suited as a marine opportunist (Parekh and Chatpar 1986). Thus it is apparent that as extreme generalists many *Aspergilllus* spp. can become pathogenic when presented with the opportunity.

1.8 Stonebrood

Due to their economic and public health importance there are many comprehensive studies on *Aspergillus*. In contrast, relatively few studies have focused on *Aspergillus* species as the etiological agent of stonebrood in honey bee colonies. A number of species cause stonebrood disease with *A. fumigatus*, *A. flavus* and *A. niger* being most commonly associated with honey bees (Gilliam and Vanderberg 1988). Stonebrood is generally not considered to be of major importance as a pathogen in honey bee colonies (Gilliam and Vanderberg 1988, Bailey 1968). *Aspergillus* spp. are relatively unspecialised moulds occurring saprophytically on combs and other hive substrates (Gilliam *et al.* 1988, Batra *et al.* 1973. It is believed that stonebrood does not usually appear unless the colony's defences are weakened by other factors (Puerta *et al.* 1988). However the specific optimal conditions required for pathogenicity to occur remain unknown. One of the properties of *Aspergillus* spp. is that they are thermotolerant and can survive in temperatures up to 75°C, which has no doubt contributed to their success as wide-ranging opportunistic pathogens (Bhabhra and Askew 2005, Beffa *et al.* 1998). Environmental conditions could potentially facilitate the build-up of

Aspergillus populations leading to increased larval mortalities and the risk of mycotoxin contamination in bee products. It is possible that a high number of stonebrood cases go undetected and infected brood could be removed from cells by workers before evidence of the disease can be seen.

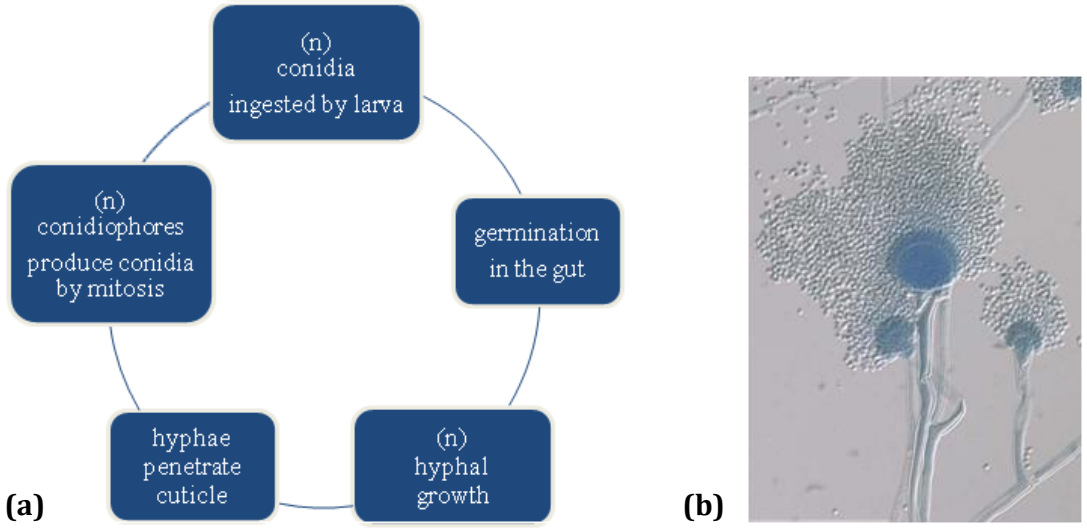

Figure 1: The life-cycle of the stonebrood pathogen showing (a) asexual reproduction in *Aspergillus* spp. (anamorphs) and (b) the production of conidia by the conidiophores in *A. fumigatus* (Courtesy of Deanna A. Sutton (2006) www.doctorfungus.org)

1.9 Chalkbrood

Subdivision: Ascomycotinia
Class: Plectomycetes
Family: Ascospaeraceae
Order: Ascosphaerales

Chalkbrood, caused by the fungus *Ascosphaera apis* is a brood disease of honey bees. The disease was not widely recognized outside Europe until the latter half of the 20th century. Although the earliest reports of the pathogen in the United States were not until 1965, it is believed to have been present up to 100 years before this (Gilliam et al 1997). More recently, since reports of chalkbrood in Queensland in 1993, it has spread rapidly to all states across Australia (Hornitzky 1989). Probably facilitated by large

scale commercial beekeeping practices, it has since become a widespread problem and can be associated with serious losses in brood and honey production (Zaghloul *et al.* 2005).

Chalkbrood is reported to be more prevalent in Spring when cool, humid conditions prevail (Flores *et al.* 2005, 1996). It is a multifactorial disease, requiring a combination of predisposing conditions and the presence of sexually produced spores for the persistence of the disease. The optimal temperature for brood development is 30-35°C (Winston 1987). The chilling of brood just prior to or after cell capping induces chalkbrood symptoms (Flores *et al.* 2005, 1996). High relative humidity in the colony has also been associated with infection (Flores *et al.* 1996). The apparent higher frequency of infection in drone larvae when compared to worker larvae may be due to their situation on the periphery of the brood chamber where chilling is most likely (Bailey 1968). High numbers of fungal spores on colony substrates will increase the likelihood of infection (Flores *et al.* 2005, Gilliam *et al.* 1988). The genetic background, either relating to hygienic behaviour by bees or intrinsic immune factors, can influence susceptibility to disease (Tarpy and Seeley 2006, Tarpy 2003, Gilliam *et al.* 1983). Ineffective uncapping and removal of mummies will increase the chances of larval exposure to disease whilst more varied hive substrates, contaminated with more viable spores, have been associated with colonies that display poor hygienic behaviour (Gilliam *et al.* 1983).

For infection to occur, *A. apis* spores must be consumed by the larvae at 3-4 days old when they are most susceptible (Jensen *et al.* 2009). Adult bees are not susceptible but play a role in disease transmission by feeding brood with food contaminated with spores from sporulating mummies or hive substrates e.g. honey, wax and pollen (Flores *et al.* 2005). Transmission between colonies in apiaries can occur via contaminated materials such as pollen combs and wax foundation that acts as a reservoir containing spores that can remain viable for up to 15 years (Aronstein and Murray 2010). Ingested spores become activated by carbon dioxide in the larval mid-gut and begin germination (Fig. 2) (Heath and Gaze 1987). The hyphae begin to penetrate the periotrophic membrane 48 hrs post ingestion. After 72 hrs the hyphae penetrate the gut wall, grow through the basal membrane and infiltrate the haemocoel, fat body and continue to colonise the visceral cavity (Chorbiñski 2004). Chitinolytic enzymes such as N-acetyl-β-glucosaminidase combined with the internal pressure exerted by the hyphae enable the fungus to pierce through the cuticle (Alonso *et al.* 1993). The larvae then become covered in fluffy white mycelium and once the invasion is complete become mummified and chalky in texture, and either white or dark-brown

to black due to the presence of ascoma (Gilliam *et al.* 1978). These are fruiting bodies that contain spore balls, or asci,that themselves contain ascospores (sexually produced spores). *A. apis* is a heterothallic fungus and sexually dimorphic microscopically (Spiltoir 1955), so to reproduce sexually it requires the presence two opposite mating types. Asexual reproduction is not known in *A. apis* (Aronstein and Murray 2010). It has been suggested that rather than white mummies representing infections with mycelia of a single mating type it is more likely that they are younger mummies that will turn black later once the ascospores develop (Aronstein and Murray 2010).

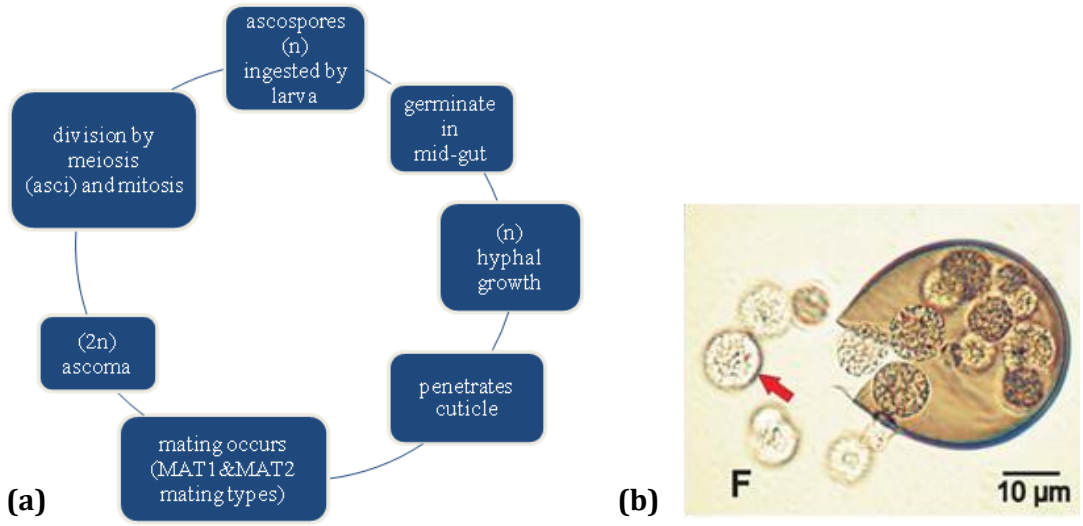

Figure 2: The life-cycle of the chalkbrood pathogen showing (a) sexual reproduction in *Ascosphaera apis* (teleomorph) and (b) a ruptured Ascoma containing asci (red arrow), each made up of many ascospores (Aronstein and Murray 2010).

1.10 Studying the stonebrood and chalkbrood system

It is probable that chalkbrood and *Aspergillus* spp. quite often co-occur in honey bee colonies. Microbiological studies have isolated *Aspergillus* spp. and other moulds including *Rhizopus* sp., *Mucorales* spp. and *Penicillium* spp. from pollen and bee products which produce enzymes and antimycotic substances that perform biochemical and anti-microbial roles (Gilliam *et al.* 1989, Gilliam *et al.* 1988). These were more frequently present in bee bread samples from resistant (hygienic) than susceptible honey bee colonies (Gilliam *et al.* 1988). When isolated from honey, pollen, forager guts and bee bread, *A. flavus* and *A. niger* had inhibitory effects on *A. apis*

growth implying a beneficial effect of these fungi in honey bee colonies (Gilliam *et al.* 1988). This also provides evidence of competitive interactions between *A. apis* and *Aspergillus* spp. occurring in nature. The factors influencing the outcome of these interactions which, for *Aspergillus*, range from beneficial to pathogenic effects, or the onset of chalkbrood disease warrants further investigation. Given that stonebrood appears, by comparison, to be a relatively avirulent disease at the colony level, the inhibitory effects observed experimentally on *A. apis* raise interesting questions on the relationship between virulence and competitive ability of these and other pathogens in general. The chalkbrood and honey bee system in itself has become a highly useful model for studying disease and disease resistance in honey bees (Jensen *et al.* 2009, Tarpy 2003, Starks *et al.* 2000, Sherman *et al.* 1998, Gilliam *et al.* 1988). Johnson *et al.* (2005) pointed out that studies involving inocula sourced from ground chalkbrood mummies may have included other microorganisms associated with larvae that induce antagonistic or immune system effects (Gilliam 1997, Gilliam *et al.* 1988). This would potentially alter the growth and pathogenicity of subsequent controlled *A. apis* infections. With this in mind it would appear that opportunistic *Aspergillus* spp. would be likely candidates to incorporate into a model system to investigate the dynamics of these diverse infections.

The likely existence of a natural chalkbrood versus stonebrood system makes it a potentially useful system for gaining insight into general host-parasite evolutionary ecology. The similarities between the two fungal infections make it ideal for investigating interspecific within-host competition. They both infect honey bees in the larval stage of development with infections occurring within days after hatching making synchronization of larval susceptible periods easy to achieve (Gilliam and Vandenberg 1988). They are semelparous, fungal pathogens so parameters are straight forward to quantify with death as a measure of virulence and sporulation rate a measure of fitness. The ease with which both fungi may be cultured *in vitro* under the same conditions can also make comparisons straightforward and competitive interactions at both the macroscopic (Poulsen and Boomsma 2005) and microscopic level easy to assess. Methods developed recently by Jensen *et al.* (2009) have been used to establish the susceptibility of honey bee brood to *A. apis* under controlled conditions whilst excluding social immunity.

Apicultural practices such as swarm control, drifting and sharing bee comb are predicted to impact parasite virulence by increasing intracolony horizontal transmission resulting in the evolution of higher virulence (Fries and Camazine 2001). Understanding the variation in host susceptibility and parasite virulence evolution has

significant practical applications for devising management strategies and disease control in honey bee populations. Chalkbrood has a worldwide distribution with control efforts now focusing on improved genetic stock, management practices and natural compounds due to the inefficacy of synthetic treatments (Aronstein *et al.* 2010). However, weakened immune systems due to the stresses imposed on managed bees may lead to an increase in disease incidence. Another missing element is the role of *Aspergillus* spp. in the epidemiology of stonebrood, in both single and multiple infections, and the predisposing factors that lead to pathogenicity in this ubiquitous microorganism. There is an overarching need for research into the impact of genetically diverse infections on the evolution of parasites in general. This is vital for a greater understanding of the current status of honey bee health as they are known to carry multiple infections.

1.11 Thesis aims

In this thesis I use a series of laboratory experiments to investigate key aspects in the ecology and evolution of the *Aspergillus* and honey bee system. In Chapter 2 I establish some basic parameters regarding the epidemiology of *Aspergillus* spp. fungi in honey bees. By conducting a screening in our experimental apiary I determine the natural prevalence and diversity of *Aspergillus* spp. fungi. I then carry out a series of controlled dose-response tests to determine the relative pathogenicity of a range of isolates in larvae and adult bees.

Aspergillosis is generally considered to occur in immunocompromised organisms (Tell 2005, Latgé 1999). In Chapter 3 I investigate the effect of nutritional limitation on the susceptibility of honey bee larvae to *A. fumigatus*, *A. flavus* and *A. phoenicis.* This study comprises two experiments using laboratory-reared larvae fed on different diets and then exposed to three *Aspergillus* species. In the first experiment I test the effect of diet quality on the susceptibility of larvae to *Aspergillus* spp. and in the second I examine the beneficial effects of different pollen species in the diet when faced with a combination of nutritional limitation and *Aspergillus* spp. exposure stressors.

Single generation competition experiments are a useful tool for identifying patterns in particular types of within-host competitive interactions (Alizon *et al.* 2013). These can be useful either for characterising specific coinfection combinations and their potential for producing synergistic, additive, inhibitory or antagonistic effects on parasite virulence, or be used as a basis for further investigations into the evolution of virulence in multiple infections. Microbiological studies indicate that the obligate chalkbrood pathogen *A. apis* and *Aspergillus* spp. fungi are likely to co-occur naturally

in honey bee larvae (Gilliam 1997, Gilliam *et al.* 1978). In Chapter 4 I therefore investigate the interspecific within-host competition of *A. apis*, *A. flavus* and *A. phoenicis* fungi in dual infections using single species infections as a reference. Here the three parameters will be: larval mortality rate, as a measure of overall virulence; within-host growth rate, as a measure of within-host virulence; and sporulation/conidiation rate, as a measure of parasite fitness and hence between-host transmission potential. This measure of fitness will be based on the assumption that the number of propagules produced on cadavers does not significantly affect the overall effect on fitness.

As virulence is known to vary between strains of parasites, the evolution of changes in virulence are conceivable outcomes (Ebert 1998). With the short generation times of parasites relative to the host it is possible to observe the evolution of virulence in diseases and examine their progression using experimental systems. Serial passage experiments (SPE's) are a useful tool for investigating parasite evolution where parasites are passaged through sequential hosts from genetically defined lines under controlled conditions (Ebert 2000). In my final experiment I serially passage infective doses of *A. flavus* and *A. phoenicis* through honey bee larvae of either high or low genetic diversity. The evolution of virulence and fitness in the derived parasite lines is then assessed by comparing mortality and conidiation rates with ancestral lines in a final post selection assessment.

CHAPTER 2

Prevalence of *Aspergillus* spp. opportunistic fungal parasites in an apiary and their pathogenicity to honey bees

2.1 Abstract

Stonebrood is a disease of honey bee larvae caused by fungi from the genus *Aspergillus.* As very few studies have focused on the epidemiological aspects of stonebrood and diseased brood may be rapidly discarded by worker bees, it is possible that a high number of cases go undetected. *Aspergillus* spp. fungi are ubiquitous and associated with disease in many insects, plants, animals and man. They are regarded as opportunistic pathogens that require immunocompromised hosts to establish infection. Microbiological studies have shown high prevalences of *Aspergillus* spp. in apiaries which occur saprophytically on hive substrates. However, the specific conditions required for pathogenicity to develop remain unknown. In this study, an apiary was screened to determine the prevalence and diversity of *Aspergillus* spp. fungi. A series of dose-response tests were then conducted using laboratory reared larvae to determine the pathogenicity and virulence of frequently occurring isolates. The susceptibility of adult worker bees to *A. flavus* was also tested. The high prevalences of *Aspergillus* spp. that were observed in adult bees and larvae comprised 10 species; these are considered to be conservative estimates with actual prevalence and diversity likely to be higher. Three (*A. flavus, A nomius* and *A. phoenicis*) of the ten isolates identified were pathogenic to honey bee larvae. Moreover, adult honey bees were also confirmed to be highly susceptible to *A. flavus* infection when they ingested conidia. Neither of the two *A. fumigatus* strains used in the dose-response tests induced mortality in larvae and were the least pathogenic of the isolates tested. These results confirm the ubiquity of *Aspergillus* spp. in the apiary environment and highlight their potential to infect both larvae and adult bees.

2.2 Introduction

The increasing demand upon honey bees for pollination services and their recent unexplained colony losses has lead to a surge of public and scientific interest in honey bee *Apis mellifera* pathology (Evans and Schwarz 2011, Aizen and Harder 2009). Honey bees are faced with multiple stressors such as poor nutrition, pesticides and exposure to pathogens, which combined may have a significant impact on colony survival (vanEngelsdorp *et al.* 2010). Thus, identifying the roles of cryptic, as well as more obvious, stressors is important for understanding their possible interactions with other threats. Honey bees are susceptible to high diversity of parasites of which certain newly emerged groups e.g. viruses, *Varroa destructor, Nosema ceranae* (Amdam *et al.* 2004, Fries 2010, Genersch and Aubert 2010) have attracted much attention. However, the impacts of other less studied parasites on colony health as well as their interactions with other stressors may also deserve more consideration.

Stonebrood is considered to be a pathogen of low virulence in honey bee colonies yet very little is known regarding the stonebrood and honey bee host-parasite system (Gilliam and Vanderberg 1988, Peurta 1988, Bailey 1968). A number of species from the genus *Aspergillus* are facultative parasites and have been reported as agents of stonebrood in Europe (Tomac 1983), United States (Gilliam and Vanderberg 1988), Egypt (Shoreit and Bagy 1995) and Australia (Hornitzky 1998). In colonies showing the symptoms of stonebrood, hard mummified larvae that have been transformed by the fungus are visible in the brood cells (Bailey 1968). On rarer occasions infections in adult bees have been reported (Burnside 1928, Batra 1973). *Aspergillus flavus* has been cited as the primary species responsible, with *A. fumigatus* occurring occasionally (Gilliam and Vanderberg 1988). *Aspergillus niger* has also been identified as a dominant species in affected colonies, however the contribution that each of these species play in causing overt stonebrood symptoms has not been determined (Shoreit and Bagy 1995).

Aspergillus spp. may also play a beneficial role in the hive. Many species of fungi are found in association with honey bee colonies where they exist on honey, pollen, bee bread and within the bees and larvae themselves (Gilliam 1997, Gilliam *et al.* 1989, Gilliam and Prest 1987, Gilliam and Prest 1972). These associations may be parasitic, commensal, or mutualistic by nature. Moulds including *Aspergillus* spp., *Rhizopus* spp., *Mucorales* spp. and *Pennicillium* spp. from pollen and bee products produce enzymes and antimycotic substances have biochemical and anti-microbial roles (Gilliam *et al.* 1989). When isolated from honey, pollen, bee bread and forager

bee guts, *A. flavus* and *A. niger* had inhibitory effects on *in vitro* growth of the chalkbrood fungus *Ascosphaera apis* (Gilliam 1997). Though it is generally accepted that pathogenicity occurs only in colonies weakened by other factors, the specific conditions predisposing the onset of disease are not fully understood (Bailey 1968, Shoreit and Bagy 1995). In experimental infections, nutritional status has been shown to effect the survival of larvae following *A. fumigatus* exposure (Foley *et al.* 2012). In addition, *A. flavus* was highly virulent when larvae were maintained at 34°C and showed decreased virulence following a 24 hr cooling period at 27°C (Vojvodic *et al.* 2011), so clearly, it is a multifactorial disease with a combination of host and environmental components required for overt disease to occur.

Due to their economic and public health importance there are many comprehensive studies on *Aspergillus* spp. fungi (Goldman and Osmani 2008, Varga and Samson 2008). They are primarily saprophytic fungi, occurring commonly in soils and on other organic and inorganic substrates. The genus consists of over 250 species of which 40 are known to be opportunistic pathogens (Varga and Samson 2008). As a consequence of the role of these fungi in the degradation of complex dead organic matter, one of their properties is to secrete a high diversity of extracellular enzymes and metabolites which are widely used in biotechnology, medicine and foods (Po 1994). At the same time they produce mycotoxins, including aflatoxins and ochratoxins which are extremely potent carcinogens with teratogenic and immunosuppressant effects in a number of different animals including man (Fung and Clark 2002). The conidia (asexual spores) are hydrophobic and readily airborne with the capacity to germinate in a wide range of conditions. They are thermotolerant, and capable of growth in temperatures ranging from 12°C to over 50°C (Bhabhra and Askew 2005) which has contributed to their success as wide ranging opportunistic pathogens in vertebrates (Casadevall 2005). Aspergillosis is known to occur in all domesticated and in many wild animal species (Tell 2005). In particular birds are susceptible to infection, with turkeys (Kunkle and Rimler 1996), ducks (Savage and Isa 1951), raptors (Joseph 2000) and penguins (Alvarez-Perez *et al.* 2010) being routinely infected. In humans, *Aspergillus* causes a range of disease including allergic bronchopulmonary disease (ABPV), aspergilloma and various forms of invasive aspergillosis (Goldman and Osmani 2008). Medical developments in immunosuppressive therapies have resulted in a new ecological niche for *Aspergillus* spp. as an important nosocomial disease in people with impaired immune systems causing serious and often fatal mycoses (Latgé 1999). *Aspergillus fumigatus* is the primary cause of infections among human pathogenic species followed by *A. flavus*, *A. terreus* and *A. niger* with the severity of the disease

highly dependent upon the immune status of the host (Dagenais 2009, Denning 1998). In agriculture, *A. flavus* is an important opportunistic pathogen of developing seeds such as corn, peanuts and cottonseed, when colonization by the fungus results in food spoilage due to aflatoxin production (Amaike and Keller 2011, Klich 2007). In developing countries where detection and decontamination is not feasible, aflatoxin is a serious food safety issue (Osmani and Goldman 2008).

Given that *Aspergillus* spp. occur in such close association to bees within the colony and their potential to cause serious disease in organisms with weakened immune systems, this host-parasite relationship deserves more detailed enquiry. In this study I determined the diversity and relative abundance of isolates of *Aspergillus* spp. isolates in an apiary, and then used controlled dosing of individual larvae to determine the pathogenicity and virulence of the most common species identified from the screening. The susceptibility of adult bees to aspergillosis was also examined by controlled dosing of individual worker bees with an *A. flavus* isolate.

2.3 Materials and Methods

2.3.1 Aspergillus prevalence

The apiary was located in West Yorkshire, UK, and contained 39 colonies of *Apis mellifera carnica* which were screened for *Aspergillus* spp. fungi in 2010. To assess colony-level prevalence, 10 larvae and 10 live adult worker bees were collected from each colony in April, as well as 22 soil samples from a) immediately outside hive entrances and b) 5 m from the hives. To assess within-colony prevalence, 20 nurse bees, which were identified as adult bees that were observed feeding larvae on the brood frames, 20 large larvae (approximately 4-8 days old) and 20 small larvae (approximately 1-3 days old) were collected from three colonies in August. In addition, airborne fungal conidia were sampled in these colonies using sterile agar plates covered in gauze to prevent contact with bees and attached to the top and middle of a central frame in the brood box, while an uncovered sterile agar plate was placed on the floor of each colony to collect fungal conidia carried by the bees. The selective *Aspergillus flavus* and *parasiticus* agar (AFPA), containing 0.1% chloramphenicol and 0.05% streptomycin, was used, which is suitable for the enumeration, growth and identification of *Aspergillus* spp. (Pitt *et al.* 1983).

Adult worker bees were dissected 24-48 h after collection. The guts (caecum, ventriculus, oesophagus and crop) of adult bees, or whole larvae, were placed in sterile

water and homogenised using a sterile pestle. For the colony-level prevalence samples, the guts from all 10 bees were pooled in 500µl of sterile water and all 10 larvae from each colony similarly pooled in 500µl of sterile water. Each sample was then divided in half and spread onto two 90 mm AFPA plates. For the within-colony prevalence samples, each of the nurse bee guts or whole larvae were individually homogenised in 200µl of water and spread on to individual AFPA plates. The plates were incubated at 30°C and checked daily for the presence of fungal colonies.

Soil samples were collected with a 2.5 x 10 cm core from immediately in front of, and 5 m away from, the entrances of every fifth hive (n = 11). A 1g amount of each sample was added to 9 ml of 0.05% Triton-X suspension and vortexed for 1 min to form a suspension. 200µl of each suspension was then pipetted onto an AFPA media plate which was then incubated at 30°C and monitored daily for the presence of fungal colonies.

2.3.2 *Fungal identification and quantification*

The growth media plates were examined daily for the presence of fungal colonies. As AFPA agar is not strictly selective Aspergillus spp. fungi species were differentiated according to common morphological characteristics as being from section Fumigati, Flavi, Nigri or 'other' depending on the conidial colour. Pure cultures of *Aspergillus* isolates grown on malt extract agar (MEA), were prepared for molecular identification to confirm morphological findings and identify other species from the genus that were present. To determine an estimate of relative fungal spore intensity per sample in larvae and adult bees, colony forming units (CFU's) were counted on each of the plates for each colony. Assuming that each colony represented a single conidial inoculum from these quantities, the mean number of viable conidia per colony and/or individual was calculated.

To extract the fungal DNA approximately 0.05 g of the fungal conidia were collected from freshly conidiating plates and added to 200 µl 5% Chelex suspension (in 10 mM Tris buffer) and 0.05 g of 0.1 mm Zircona/Silica beads and placed in a QIAGEN Tissue Lyser beadbeater for 4 min at 50 oscillations/s. Samples were then incubated in a 90°C water bath for 20 min then centrifuged for 30 min at 8°C. The supernatant was cleaned with OneStep-96 PCR Inhibitor Removal Kit (Zymo Research) prior to PCR. Species identification was carried out by sequencing 547 and 459 bp long fragments from the ribosomal internal transcribed spacer regions 1 and 2 respectively (Henry *et al.* , 2000) and then BLASTn searches carried out. For isolates where BLASTn searches produced *A. niger* spp. matches where it was not possible to distinguish to species

level, a 649 bp long fragment of the calmodulin gene was sequenced and a BLASTn search carried out to identify the species (O'Donnell *et al.* 2000).

2.3.3 *Aspergillus virulence in honey bee larvae*

Six *Aspergillus* isolates were included in 3 different dose-response experiments on larvae (Experiments 1a, 1b and 1c; see Table 1) where each experiment tested isolates using a different dose range. In each case honey bee larvae were sampled from 4 colonies, asymptomatic of pests and disease and, headed by one year old queens. One to two day old larvae were grafted onto 48 well sterile tissue culture plates. Larvae were grafted using a Swiss grafting tool and placed the same side up onto a 50µl drop of royal jelly mix at the base off each well that had been warmed to approximately 30˚C. The royal jelly mix consisted of 50% of fresh frozen royal jelly (v/v) (Apitherapy, UK), 6% D-glucose (w/v), 6% D-fructose (w/v) and sterile distilled water and was used to feed larvae according to the Aupinel *et al.* (2005) protocol. Larvae were kept under conditions of 34˚C at 90% relative humidity in an incubator (Sanyo Environmental Test Chamber).

Conidia suspensions from six different isolates were used to inoculate the honey bee larvae. These were F2 fungi that had been isolated and identified previously in the season. The conidia were harvested from MEA plates and suspended in 1 ml of sterile water and vortexed for 15 seconds. The conidia were counted with a 0.001ml Neubauer haemocytometer and the conidia concentrations per ml calculated. This suspension was adjusted to make an approximate 2 x 10^6 conidia/ ml concentration.

Table 1: Fungal species/ strains and spore (conidia) concentrations used in honey bee larvae dose response experiments.

Experiment 1: Larval dose-response tests			
Experiment	***Aspergillus* species (isolate code)**	**Isolate source**	**Treatment doses (conidia/larvae)**
1a	*A. fumigatus* (G23) *A. flavus* (G49) *A. niger* (L29)	adult bee guts adult bee guts larvae	5, 50, 500 and 5,000
1b	*A. fumigatus* (N30.04) *A. phoenicis* (N38.02)	nurse bee gut nurse bee gut	50, 500, 5000 and 25000
1c	*A. nomius* (G7) *A. flavus* (G49)	adult bee guts adult bee guts	2, 5, 50 and 500

To test for conidial viability 40ul of the suspension was added to 200ul GLEN, a liquid media suitable for the germination of entomopathogenic fungi (Beauvais and Latgé 1988), and vortexed for 5 seconds. 15ul of this suspension was micropippetted into six of the 6mm spots on sterile Teflon coated slides and placed into sterile petri dishes with moist filter paper. One slide was prepared per species. These were incubated for 18-24 hours at 30˚C after which time the proportion of germinated conidia per species was calculated. Once the spore suspensions had been adjusted to account for the percentage of non-viable conidia the suspensions were diluted to the desired concentrations. Three separate dose response experiments were carried out each testing a different range of conidia doses (Table 1). The suspensions were diluted to the required concentrations and placed in the incubator at 34˚C for an hour prior to dosing. A standard volume of 5µl of each conidia concentration was given per dose. The inoculum was pippetted onto the royal jelly food near the mouth parts of the larva to encourage ingestion of the conidia. The larvae were monitored daily for 7 days post-exposure to the fungi under microscope for mortality and signs of stonebrood infection. This was recognised by the presence of hyphae and conidiophores on the cuticle.

2.3.4 *Aspergillus flavus virulence against adult honey bees*

For the adult dose-response test, frames of sealed brood were obtained from colonies that were asymptomatic of pests and diseases, and kept at 34°C and 60% RH for the collection of newly emerged workers. The bees were confined in cages in groups of 15

and fed a sterile sucrose suspension (50% w/w in deionised water). Three-four days following eclosion the bees were starved for 1.5 h and 30 bees per colony, per dose, were each fed 5 µl of sucrose suspension containing either 50, 500, 5000 or 25000 *A. flavus* conidia or a pure sucrose suspension for the controls. Prior to dosing, bees were anaesthetised by cooling at 4°C until they were motionless (approximately 5mins). Once returned to room temperature the suspension was micropipetted onto the mouthparts when the bees started to awaken ensuring complete ingestion of the dose. The bees were then caged according to dose and colony of origin. For 9 days post inoculation the bees were monitored and dead bees were counted and removed daily.

2.3.5 *Statistical analysis*

Differences in the numbers of CFU between honey bee life stages (young larvae, old larvae, adults) were analysed in R 2.1.14.1 using a generalized linear model, with a negative binomial distribution and a log link function, with colony included as a factor in the within-colony analyses. The effects of fungal isolates and dose on the survival of larvae and adult bees were analysed with Kaplain-Meier models, using the *survreg* function with death defined as the event and non-constant hazard (Weibull) error distribution. Isolate, dose, colony and interactions between these terms were removed stepwise to obtain the minimum adequate model. For the adult dose-response test data, the *frailty* function was included in order to fit colony as a random term as (Therneau 2012). Pairwise comparisons of fungal doses were conducted using multiple comparisons from the means of the model using Tukey contrasts in the *multcomp* package.

2.4 Results

2.4.1 *Colony level prevalence*

There was a significant effect of host life stage on the number of fungal CFU's within samples of pooled adult bee guts and larvae ($\chi^2_{1,76} = 88.82$, $P < 0.001$), with CFU's generally found to be higher in larvae than in adults. CFUs also showed a highly aggregated distribution with significant differences in intensities between colonies ($\chi^2_{1,38} = 2142.37$, $P = 0.02$, Fig. 3). Cultures from the soil samples showed that there was a significant effect of proximity to colonies on the number of CFU's in soil ($\chi^2_{10,10} = 22.68$, $P = 0.02$), with samples taken from close to the hive entrance having

significantly more CFU's than corresponding samples taken 5 m from each hive ($\chi^2_{1, 20}$ = 43.7, P < 0.001).

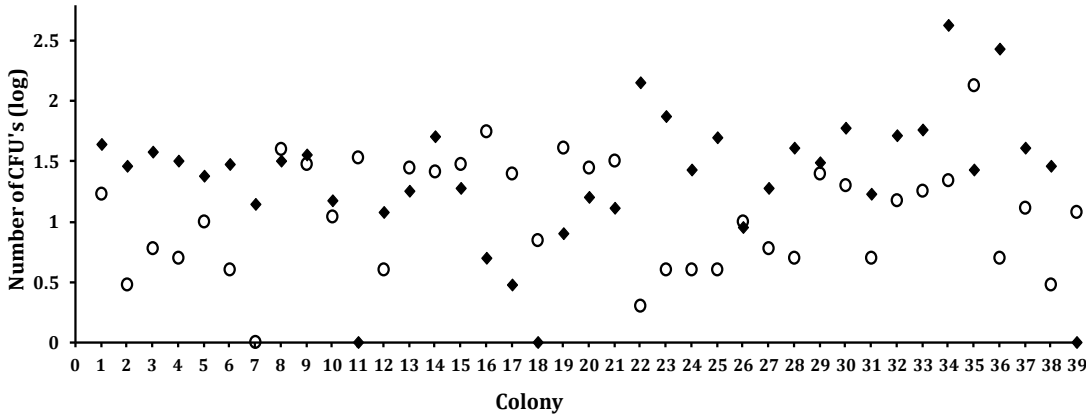

Figure 3: Colony-level prevalence of viable CFU's in honey bees. The total number of colony forming units found in either a pooled sample of 10 adult bee guts (open circles), or a pooled sample of 10 larvae (black diamonds), for each of 39 colonies of *A. mellifera carnica* honey bees.

2.4.2 *Within-colony prevalence*

The conidia intensities observed in the individual samples of three colonies differed significantly between life stages ($\chi^2_{2, 177}$ = 252.09, P< 0.001), and this effect of life stage also varied significantly between colonies ($\chi^2_{2, 175}$ = 236.84, P < 0.001). There was a significant interaction between the two factors ($\chi^2_{4, 177}$ = 183.56, P < 0.001). Intensities in small larvae were significantly lower than in large larvae (P < 0.01) and there were no differences between adult nurse bee and large larvae. Viable conidia were least prevalent in small larvae in which 41.6% carried viable conidia whereas 88.3 and 81.6% of nurse bees and large larvae respectively carried viable fungal conidia.

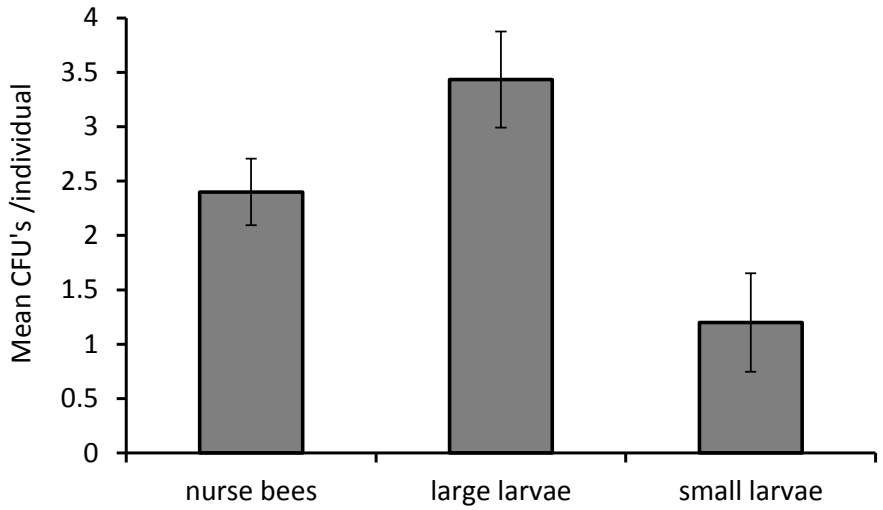

Figure 4: The mean number of fungal CFU's (+/- se) per individual larva or bee gut sample for each of the three age groups (n = 60) sampled from 3 colonies.

The mean airborne viable fungal spore counts of the gauze covered media plates positioned at the top and middle of 3 colonies were 250 (se +/- 70.9) and 245 (se +/- 179.3) CFU's respectively. No viable fungal colonies appeared on two of the uncovered plates which were positioned at the bottom of each hive with only 10 CFU's counted on the third.

2.4.3 *Aspergillus spp. diversity*

The apiary screenings yielded a total of 378 *Aspergillus* isolates from larvae, adult bees, airborne and soil substrates which were identified by gross morphology (Table 2). This may be interpreted as a highly conservative estimate of *Aspergillus* prevalence as it was not possible to identify non-sporulating colonies. Of these 46 were sequenced and identified using BLASTn with a minimum 97% threshold. Out of the 15 isolates sequenced from section Fumigati all 15 were confirmed to be *A. fumigatus*. Five isolates were sequenced from section Flavi in which *A. flavus* (2/5), *A. oryzae* (2/5) and *A. nomius* (1/5) were identified. The 20 isolates sequenced from section Nigri comprised *A. phoenicis* (12/20), *A. niger* (5/20), *A. tubingensis* (2/20) and *A. ochraceus* (1/20) were identified. Other *Aspergillus* isolates identified were *A. versicolour* (4) and *A. sclerotiorum* (2) (see Table 3).

Table 2: The percentage of cultures in which *Aspergillus* isolates from each group were identified by gross morphology, including the pooled (10 adult bee guts or larvae per colony), individual (20 nurse bee gut, large larvae or small larvae individuals from 3 colonies) and soil cultures (hive entrance and 5m from 11 hives).

***Aspergillus* group**	**Pooled cultures (n=39)**		**Individual cultures (n=60)**			**Soil (n=11)**	
	Adults	**Larvae**	**Nurse bees**	**Large larvae**	**Small larvae**	**Hive**	**Grass**
Fumigati	60	72	28	68	26	45	27
Nigri	33	31	45	7	7	27	18
Flavi	23	13	3	3	3	0	0

Table 3: The *Aspergillus* isolates sequenced from the between-colony (pooled cultures) and within-colony (individual cultures) and airborne samples and including subset of non-*Aspergillus* isolates which were also sequenced. Genbank ID numbers indicate the closest matches found in the BLASTn search.

Between colony samples (n=39)		
Sample code	**species**	**Genbank ID**
Adults		
G5	*A. phoenicis*	JF838353.1
G7	*A. nomius*	DQ467991.1
G15	*A. fumigatus*	KC411924.1
G20	*A. fumigatus*	KC237291.1
G23	*A. fumigatus*	KC411924.1
G30	*A. fumigatus*	KC411924.1
G49	*A. flavus*	GU172440.1
Larvae		
L27	*A. fumigatus*	JQ356539.1
L29	*A. niger*	JF838355.1
L29	*A. versicolour*	JX139733.1
L38	*A. oryzae*	EU409806.1
L41	*A. phoenicis*	JF838353.1
Within-colony samples (n=3)		
Nurse bees		
N30.02	*A. sclerotiorum*	JN851035.1
N30.04	*A. fumigatus*	AF138288
N30.04	*A. niger*	AM270081.1
N30.10	*A. sclerotiorum*	JN851004.1
N30.17	*A. fumigatus*	KC237291
N38.01	*A. fumigatus*	JQ356539
N38.02	*A. phoenicis*	FR751413.1
N38.09	*A. phoenicis*	JF838353.1
N38.11	*A. phoenicis*	JF838353.1
N38.14	*A. phoenicis*	JF838353.1
N38.16	*A. niger*	AM270081.1
N38.20	*A. tubingensis*	EF661152.1
N40.02	*A. phoenicis*	JF838353.1
N40.03	*A.phoenicis*	JF838353.1
N40.04	*A. phoenicis*	JF838353.1
N40.10	*A. niger*	AM270081.1
N40.11	*A. phoenicis*	JF838353.1
N40.15	*A. fumigatus*	KC237291
N40.15	*A. phoenicis*	JF838353.1
N40.16	*A. tubingensis*	JX024265.1
N40.17	*A. phoenicis*	JF838353.1
N40.18	*A. phoenicis*	JF838353.1

Table 3: continued

Sample code	species	Genbank ID
Larvae		
LL30.01	*A. fumigatus*	JN638776.1
LL30.08	*A. fumigatus*	KC237291
LL30.13	*A. versicolour*	JX139733
LL30.13	*A. niger*	AM270081.1
LL38.03	*A. fumigatus*	KC411924.1
LL38.15	*A. fumigatus*	KC237291
LL40.18	*A. fumigatus*	KC200122.1
LS30.02	*A. versicolor*	AM883156.1
LS30.02	*A. flavus*	JX292092
LS30.08	*A. versicolour*	AM883156.1
Airborne		
ABB38	*A. ochraceus*	EU805804.2
ABM30	*A. fumigatus*	DQ370002.1
ABM40	*A. oryzae*	GU385811.1
Non-*Aspergillus* isolates		
G11	*Penicillium glabrum*	KC009784.1
G13	*Purpureocillium lilacinum*	JX969622.1
L2	*Fusarium sp.*	JF803825.1
L3	*Fusarium oxysporum*	KC196121.1
L4	*Penicillium ilerdanum*	JN899311.1
L7	*Penicillium crustosum*	KC193255.1
L9	*Ascosphaera apis*	KC193255.1
L30	*Penicillium ilerdanum*	JN899311.1
N38.17	*Penicillium commune*	KC009833.1
LL30.11	*Penicillium spinulosum*	JN624898.1
4H	*Fusarium tricinctum*	JQ676180
45H	*Penicillium crustosum*	GU723443

*Source code letters: G = adult bee guts, L = larvae, N = nurse bee gut, LL = large larva, LS = small larva, ABB = bottom airborne, ABM = mid airborne, H = hive entrance soil. Numbers denote the colony or the colony and individual number.

2.4.4 *Dose-response tests*

The fungal treatments A. *flavus* (G49) , *A. fumigatus* (G23) and *A. phoenicis* (L29) in Experiment 1a differed significantly in their effect on the survival of larvae ($\chi^2_{3, 307}$ = =217.61, P < 0.001), and there was also a significant effect of colony ($\chi^2_{3, 300}$ = 16.25, P < 0.001). The effect of dose differed depending on the species, although this was not significant ($\chi^2_{4, 303}$ = 8.59, P = 0.07). Larvae treated with *A. flavus* (G49) showed significantly higher mortality than the controls in all doses (P < 0.001 in all cases; Fig. 5), whereas the survival of larvae was not affected significantly by any of the *A. fumigatus* (G23) (P > 0.05 in all cases) and *A. niger* (L29) treatments (P > 0.05 in all cases; Fig. 5). In Experiment 1b the effect of the fungal treatments *A. fumigatus* (N30.04) and *A. phoenicis* (N38.02) species on the survival of larvae was significant ($\chi^2_{2, 566}$ = 110.53, P < 0.001) which was affected by both dose ($\chi^2_{4, 562}$ = 27.24, P < 0.001) and colony ($\chi^2_{3, 559}$ = 54.48, P < 0.001). Higher mortalities were observed in larvae with increasing doses of *A. phoenicis* treatments showing a clear dose response effect (Fig. 5). All doses showed a significantly greater effect on mortality than the control treatments. In Experiment 1c there was a significant effect of parasite species ($\chi^2_{2, 567}$ = 88.13, P < 0.001) and dose ($\chi^2_{4, 563}$ = 24.93, P < 0.001) on the survival of larvae treated with *A. flavus* (G49) and *A. nomius* (G7). There was no significant effect of colony on the survival of larvae overall ($\chi^2_{3, 560}$ = 5.04, P = 0.17). Again, in all *A. flavus* (G49) treatments the mortality of larvae was significantly greater than the control treatment (P < 0.01 in all cases). However, colony was not a significant factor in larval mortalities exposed to *A. flavus* (G49) treatments in this experiment ($\chi^2_{3, 370}$ = 3.92, P = 0.27). There was a significant effect of dose ($\chi^2_{4, 373}$ = 21.09, P < 0.001) and colony ($\chi^2_{3, 370}$ = 34.06, P < 0.001) on the survival of larvae treated with *A. nomius* (G7) with the two highest doses (50 and 500 conidia) showing significantly higher mortality than the controls.

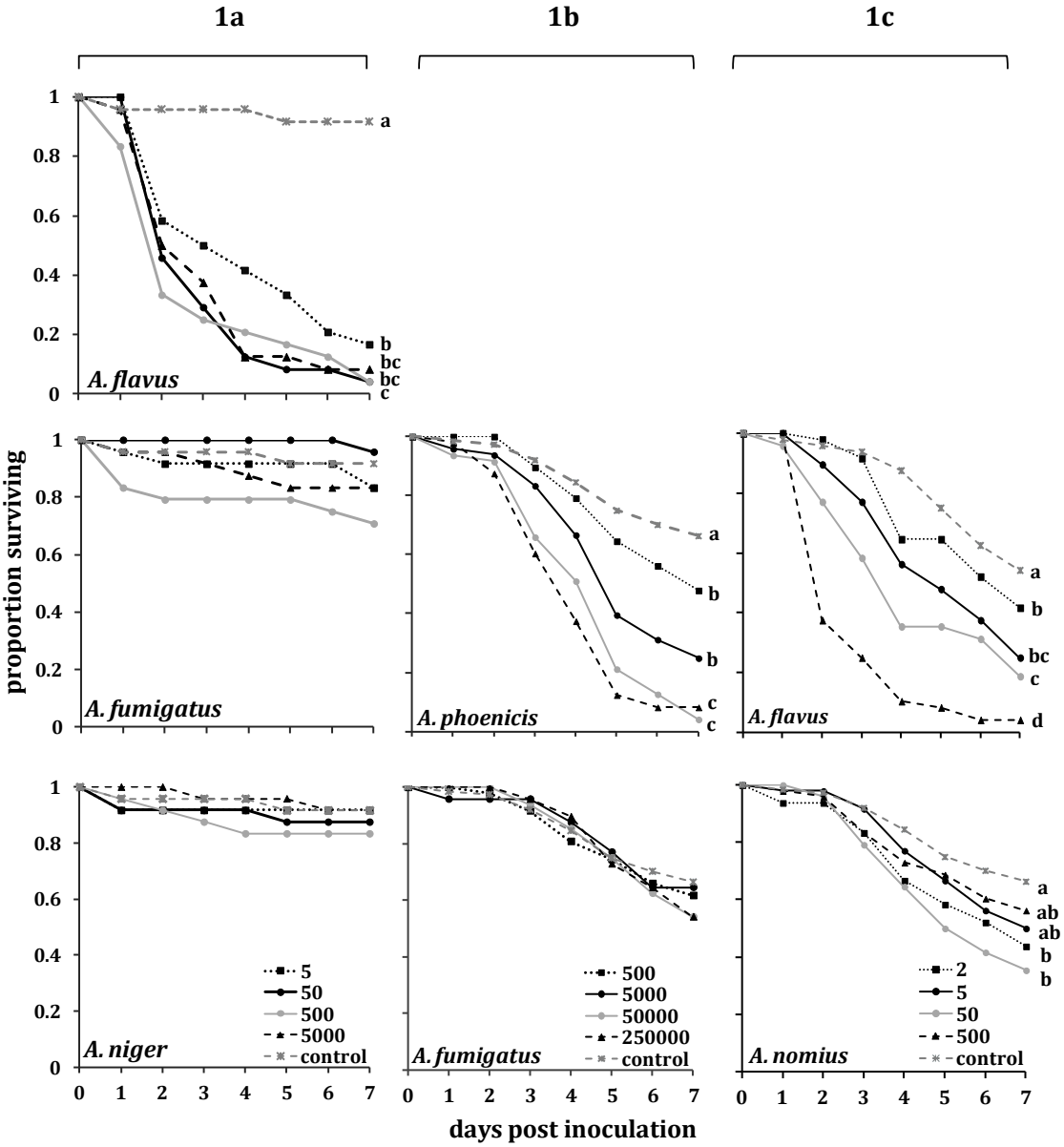

Figure 5: The survival rates of larvae over 7 days following fungal treatment in Experiment 1a (left column) for: *A. flavus* (G49), *A. fumigatus* (G23) and *A. niger* (L29), Experiment 1b (centre column): *A. phoenicis* (N38.02) and *A. fumigatus* (N30.04) and Experiment 1c (right column): *A. flavus* (G49) and *A. nomius* (G7) with a different dose range in each experiment. For each experiment, the dose legend is shown in the bottom graph. The different letters denote significant differences in pairwise comparisons between doses for each *Aspergillus* strain.

There was a significant effect of *A. flavus* treatment on the survival of adult bees ($\chi^2_{1,447}$ = 156.46, P < 0.001). The overall effect of dose was significant ($\chi^2_{4,443}$ = 103.23, P < 0.001) with all doses causing significantly higher mortality than the control treatments. There was also a significant frailty effect of unobserved heterogeneity between colonies ($\chi^2_{0.005,443}$ = 53.23, P < 0.001, Fig. 6).

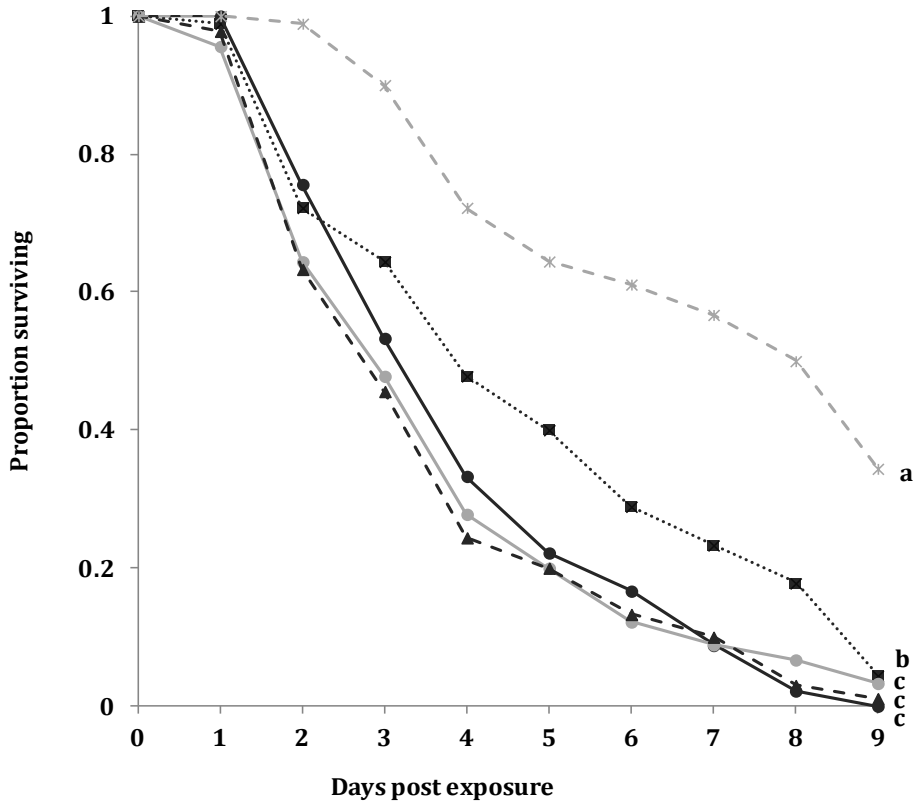

Figure 6: The survival rates of adult bees over 9 days following *A. flavus* (G49) treatment for each treatment of 25,000 conidia (black triangles), 5000 conidia (grey circles), 500 conidia (black circles), 50 conidia (black squares) and controls (grey crosses). The different letters denote significant differences in pair-wise comparisons between doses.

2.5 Discussion

Honey bees are exposed to a high diversity and abundance of fungal conidia in the hive environment. This was evident in the high CFU intensities found in larvae, adult honey bees and airborne samples collected in this study. High prevalences of *Aspergillus* spp. fungi were also observed, and we note that these results are a conservative measure and that actual prevalences are likely to be higher. Three (*A. flavus, A. nomius* and *A. phoenicis*) of the ten species identified were pathogenic to honey bee larvae. Moreover, adult honey bees were also confirmed to be highly susceptible to *A. flavus* infection when they ingested conidia.

Of the most frequently occurring isolates belonging to section Fumigati, all were confirmed as *A. fumigatus.* Neither of the two *A. fumigatus* isolates used in the dose-response tests induced mortality in larvae and were the least pathogenic of all the fungal isolates tested. The majority of isolates from the next most common section Nigri, were confirmed to be *A. phoenicis*. A dose-response test using an *A. phoenicis* (N38.02) isolate showed increased mortality in all doses and is the first report of this species showing pathogenicity in bees. The *A. niger* (L29) isolate was not pathogenic and illustrates how subtle phenotypic variation between cryptic *Aspergillus* spp. can significantly affect their virulence underscoring the importance of accurate microbial identification (Bathoorn *et al.* 2013). The dose-response tests using *A. flavus* and *A. nomius* strains from the least common section Flavi showed *A. flavus* to be pathogenic at all doses. This high virulence has also been reported by Vojvodic *et al.* (2011) using an *A. flavus* isolate sourced from honey bee larvae, which showed higher virulence than the obligate brood pathogen *Ascosphaera apis* in larvae reared under the same conditions at the same dose. This is also interesting as *A. flavus* conidiates more prolifically than *A. apis* produces infective ascospores, presumably releasing higher densities of infective propagules into the environment, yet appears to cause disease outbreaks far less frequently than *A. apis*. A clear dose-response with *A. nomius* treatment showed it to be pathogenic at doses of 50 conidia or more. *A. nomius* is commonly isolated from insects and is a known entomopathogen; it is also a producer of A and G-type aflatoxins (Poulsen *et al.* 2006, Kurtzman *et al.* 1987). The high virulence of *A. flavus* was also evident in the adult bee experiment where all doses were pathogenic. Similar mortalities were reported by Burnside (1930) using much higher and uncontrolled dosing methods. He also noted that, in free flying colonies, infected bees left the hive to die. These results are noteworthy as the importance of aspergillosis as an adult bee disease may be underestimated in comparison to its importance as a brood disease.

Viable conidia intensities showed highly aggregated distribution which may suggest variation in exposure to fungal conidia. Larvae generally carried higher conidia loads than the adult bee gut samples. This may be partly accounted for by the inclusion of cuticle surface conidia in the larval samples. Conidia acquired by larvae from nurse bees via feeding are retained in the gut until the first defecation event prior to pupation (Winston 1987). The larval CFU counts represent the accumulation of conidia ingested during development, reflected in the relatively low CFU counts in individual small larvae. One of the main sources of the fungi in the hive are most likely to be from pollen, as nectar is not thought to harbour large quantities of fungal conidia (Gonzalez *et al.* 2005). Conidia present in the air may also colonlise hive substrates saprophytically and produce conidia which may become airborne in the hive or transmitted via physical contact or food sharing in adult bees. Conidia may then in turn be fed to larvae in the cells. The *Varroa destructor* mite may also potentially act as vector of the fungal disease (Benoit *et al.* 2004). Our results show how the ingestion of conidia and infection route through the gut is successful in both larvae and adults with a number of species of *Aspergillus*. The presence of higher densities of conidia on colony substrates will increase the likelihood of infection. Environmental conditions could potentially facilitate the build-up of *Aspergillus* populations increasing the risk of infection.

Completion of the genome sequencing of nine *Aspergillus* species has facilitated numerous investigations into the genetic basis of pathogenicity characters in the genus (see http://www.aspergillus.org.uk/). *Aspergillus flavus* is the most common *Aspergillus* species reported to infect insects and second most common in human aspergillosis (Hedayati *et al.* 2007, St. Leger *et al.* 1997). Murine studies have shown *A. flavus* doses having 100-fold higher infectivity when compared to *A. fumigatus* (Mosquera *et al.* 2001). Damage to the host tissues during mycoses can result either from the mechanical action exerted by the mycelium during growth or chemically by enzymes and toxins secreted by the fungus (Rosengaus and Traniello 1997). The toxicity of aflatoxin to honey bees has been investigated in a few studies and suggests a high tolerance for the aflatoxin B1 compound due to P450-mediated metabolic detoxification (Niu *et al.* 2009). Propolis extracts and a honey diet also ameliorated toxicity effects in bees (Johnson *et al.* 2012, Niu *et al.* 09). These studies suggest that bees have a capacity to cope with mycotoxins they may be exposed to but the role mycotoxins play in the pathogenicity of *Aspergillus* spp. infections has yet to be determined.

Given the high mortality of larvae and adult bees in this study, it is apparent that individual immune responses are easily overcome by certain *Aspergillus* strains. This suggests that colony level defences play a vital role in the defences against aspergillosis under natural conditions. Hygienic behaviour is a specific response by adult bees to the presence of diseased or parasitised brood and is an important defence against larval diseases such as American foulbrood, chalkbrood and *V. destructor* mite infestations in *Apis ceranae* (Gilliam *et al.* 1988, Evans and Spivak 2010). For the behaviour to be effective the bees must detect and remove infected larvae before the infective transmission stage of the disease is reached. It is possible that this is routinely carried out in colonies in response to stonebrood and has gone undetected. The removal of larvae that were experimentally administered high doses of *A. flavus* conidia has been observed previously, however this mechanism still requires further investigation under controlled conditions to determine if it is characteristic of hygienic behaviour (Burnside 1930). Nest hygiene defined as behaviours that increase sanitation of an individual or the nest (Wilson-Rich *et al.* 2009) is another possible defence mechanism of high importance against stonebrood. Larvae are fed royal jelly and stored honey which both possess antimicrobial properties (Morse and Flottum 1997). Plant resins, complex plant secretions that have diverse antimicrobial properties, are collected and mixed with wax and used in the colony as propolis (Bancova *et al.* 2000). The efficacy of propolis as an antimicrobial against honey bee disease is evident with American foulbrood agent *Paenibacillus larvae* where studies have also shown that the antibacterial activity varies depending on the source of the plant resin (Bastos *et al.* 2008). Airborne conidia of *Aspergillus* spp. are ubiquitous in terrestrial habitats and pathogenic strains may gain access via foraging workers and hive entrances and potentially colonise hive substrates, the presence of propolis on all hive surfaces may act as a persistent barrier preventing the establishment of harmful fungi. The presence of resins in the nest is known to result in the down-regulation of immune gene expression in individual bees emphasizing a significant role for propolis in disease resistance and colony fitness (Simone *et al*. 2009). Bees have been shown to significantly increase their resin foraging rate in response to chalkbrood challenge, more so than to American foulbrood, indicating a specific response to this long-lasting fungal pathogen (Simone-Finstrome and Spivak 2012, Gilliam 1986).

In conclusion, our results indicate that *Aspergillus* fungi are ubiquitous in the environment of honey bees and may pose a significant stress on the health of their colonies. Under experimental conditions both adults and larvae from apparently healthy colonies, were found to be susceptible to infection by *Aspergillus* fungi isolated

from honey bees. It is clear that further research is required to determine how colonies coexist with these potentially harmful organisms and to identify the key defences involved in aspergillosis prevention.

2.6 Acknowledgements

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Chapter 3

Nutritional limitation and resistance to opportunistic *Aspergillus* parasites in honey bee larvae

3.1 Abstract

Honey bees are threatened by land use changes that reduce the availability and diversity of pollen and nectar resources. There is concern that poor nutrition may be involved in recent population declines, either directly or due to indirect effects on immunocompetence. The larval stage is likely to be the most vulnerable to a poor diet, but the effects of larval nutrition on disease susceptibility of bees are not well known. In this study laboratory-reared honey bee larvae were used to investigate the effects of diet quality on disease susceptibility to the opportunistic fungal parasites *Aspergillus flavus, A. phoenicis* and *A. fumigatus.* Larvae fed on a nutritionally poor diet were found to be significantly more susceptible to *A. fumigatus* than larval resistance to *A. fumigatus* was enhanced by feeding with a diet supplemented with either dandelion or polyfloral pollens. This indicates that dandelion and polyfloral pollens contain elements that enhance resistance to this fungal disease, illustrating an interaction between nutrition and parasitism and emphasising the benefit of diverse floral resources in the environment to maintain honey bee health.

3.2 Introduction

Honey bee (*Apis mellifera*) health is a major current concern because of the substantial colony losses experienced in some countries in recent years and the important role honey bees play in pollination and food production (Breeze *et al.* 2011, Evans and Schwarz 2011, vanEngelsdorp and Meixner 2010, Williams *et al.* 2010). Many hypotheses have been proposed to explain these serious population declines and it has become increasingly apparent that multiple factors, possibly acting in synergy, are involved (vanEngelsdorp *et al.* 2009). In particular, it has been suggested that nutritional limitation due to poor diet could result in immunocompromised individuals that are more susceptible when exposed to pathogens (Naug 2009). Modern intensive agricultural practices, characterised by monocultures and simplification in crop rotations, can provide abundant supplies of pollen and nectar when in bloom, but limited resources at other times due to a lack of continuity in the flowering phenology of crops (Decourtye *et al.* 2010). Moreover, habitat fragmentation and loss of buffer zones of wild and semi-wild habitats in intensively farmed areas not only leave bee colonies short of food resources during times of dearth, but also lack the natural forage diversity that may be required for optimum nutrition (Kremen *et al.* 2002).

Activation of the immune system is energetically costly and nutritionally limited organisms are less able to meet this cost, making them more susceptible to parasite infections (Coop and Kyriazakis 1999, Moret and Schmidt-Hempel 2000). In addition, parasites compete directly with the host for their own nutritional requirements and nutritionally deprived hosts may be less able to tolerate this added stress (Thompson and Redak 2008). The microsporidian parasite *Nosema ceranae* has been confirmed to impose an energetic stress on adult honey bees, increasing either hunger levels or susceptibility to disease in bees with a lower sugar intake (Mayak and Naug 2009). Diets may also provide particular elements which may be essential for immune responses and/or the detoxification of xenobiotics (Johnson *et al.* 2012). Excess nutrients and energy reserves are stored in the insect fat body and are mobilised in response to the energy demands of other tissues. This storage function is essential in larval stages to ensure survival during starvation periods and for development during metamorphosis (Arrese and Soulages 2010). During many types of immune response, the fat body cells release proteins including antimicrobial peptides (AMP's) and lysozymes into the haemolymph (Bulet *et al.* 1999, Cheon *et al.* 2006). Phagocytosis and encapsulation are the most well known immune defenses of bees against fungal pathogens (Gliński and Buczek 2003), but there can be upregulation of a variety of transcripts including a chitinase-like enzyme, serine protease, lysozymes and

AMP's abaecin and defensin (Aronstein and Spivak 2010). Immune responses to fungi and bacteria were also associated with a downregulation of major storage proteins vitellinogen and hexamerins, suggesting a trade-off between immune responses, and the biosynthesis and accumulation of these proteins (Aronstein and Spivak 2010, Laurenço *et al.* 2009). The immune effects of poor nutrition in honey bee larvae have not been well studied, yet it is this stage that may be most vulnerable. During temporary food shortages colonies tend to regulate brood rearing rather than rear malnourished pupae (Imdorf *et al.* 1998). However, under experimental conditions, impairments in development have been reported (Brodshneider *et al.* 2000, Matila and Otis 2006), and sub-lethal effects can persist into the adult stage (Hoover *et al.* 2006).

Pollen provides the main source of dietary proteins, lipids, sterols, vitamins and minerals for honey bees (Crailshem 1990). The pollen from different floral sources has different nutritional value for bees with most pollen analysis studies focusing on the protein content (DeGroot 1953, Roulston and Cane 2000). The nutritive value of different pollen types has been assessed using various physiological and productivity parameters such as brood rearing capacity (DeGrandi-Hoffman *et al.* 2008, Keller *et al.* 2005 Loper and Berdel, 1980; Matila and Otis, 2007), lifespan (Maurizio, 1954), hypopharyngeal gland development (DeGrandi-Hoffman *et al.*, 2010; Maurizio, 1954; Standifer 1967) and fat body growth and development (Maurizio 1954). Using colony reared larvae, Rinderer *et al.* (1974) reported decreased mortality caused by American foulbrood (*Paenibacillus larvae)* exposure when the diet was supplemented with bee collected pollen from a variety of floral sources. More recently, pollen and protein supplement fed caged, adult bees yielded lower Deformed Wing Virus titres in comparison to sugar syrup fed bees, indicating a vital role for dietary protein in immune responses of honey bees (DeGrandi-Hoffman 2010). Diet quality affected by pollen diversity, rather than protein content, has been shown to exert immunocompetence effects in adult honey bees (Alaux *et al.* 2010). Yet the significance of pollen diversity in the larval diet has not previously been studied.

Here, we investigated under controlled, laboratory conditions, whether nutritional limitation affects the susceptibility of honey bee brood to the Ascomycetous fungi from the genus *Aspergillus*, the causative agents of aspergillosis or stonebrood disease in bees. As our model parasites we used *Aspergillus flavus, A. phoenicis* and *A. fumigatus*. These fungi are ubiquitous, opportunistic pathogens that have been little studied in honey bees, but which, in other animal host species such as dogs, horses and birds, generally require immunocompromised individuals for successful infection (Tel 2005). We monitored the survival of exposed and unexposed larvae fed diets varying

in either (1) the amount of royal jelly and sugars or (2) the origin and diversity (monofloral versus polyfloral) of pollen to test how diet quality affects the ability of larvae to resist exposure to stonebrood parasites.

3.3 Materials and methods

3.3.1 *Larvae sampling*

We used larvae from a total of 9 colonies of honey bees *Apis mellifera carnica* that were headed by one year old unrelated queens and asymptomatic of pests and disease. One to two day old larvae (age estimated by size) were grafted into sterile 48-well tissue culture plates using a Swiss grafting tool. During the grafting process, plates and diet were warmed, approximately to hive temperature (32-34°C), and a moist tissue was placed between each filled plate and its lid to keep the larvae in a relatively high humid atmosphere. Plates were then transferred into an incubator at 34°C, 90% relative humidity and a 24 h dark cycle. The larvae were fed *ad libitum* with either a standard diet which consisted of 50% of royal jelly (RJ) (v/v) (Apitherapy, Norfolk, UK), 6% D-fructose (w/v), 6% D-glucose (w/v) and distilled water (Jensen *et al.* 2009), or with modified diets according to the experiment (see sections 3.4 and 3.5).

3.3.2 *Aspergillus species identification*

The *Aspergillus* isolates used were collected from naturally-exposed adult worker bees and larvae in our experimental apiary (West Yorkshire, UK) and cultured on malt extract agar (MEA) plates at 30ºC until the fungi produced conidia. The plates were then stored at 4°C until use. To extract the fungal DNA approximately 0.05g of the fungal conidia were added to 200µl 5% Chelex suspension (in 10mM Tris buffer) and 0.05g of 0.1mm Zircona/Silica beads and placed in a QIAGEN Tissue Lyser beadbeater for 4mins at 50 oscillations/s. Samples were then incubated in a 90°C water bath for 20mins then centrifuged for 30mins at 8°C. The supernatant was cleaned with OneStep-96 PCR Inhibitor Removal Kit (Zymo Research) prior to PCR. Species identification of the *Aspergillus flavus* (G49) and *A. fumigatus* (G23) isolates was carried out by sequencing 547 and 459 bp long fragments from the internal transcribed spacer regions 1 and 2 respectively (Henry *et al.* 2000). BLASTn searches produced a100% maximum identity match with *A. flavus* (GenBank ID: GU172440.1) and 96% maximum identity with *A. fumigatus* (GenBank ID:KC411924.1). *A. phoenicis* (N40.04) was identified by sequencing a 694 bp long fragment of the calmodulin gene (O'Donnell *et*

al. 2000) producing a 99% maximum identity match with *A. phoenicis* (GenBank ID: JF838353.1).

3.3.3 *Parasite treatments*

Conidia of *A. flavus, A. fumigatus* or *A. phoenicis* were harvested from MEA plates, suspended in 2 ml of sterile water and vortexed for 15 seconds. The conidia were counted with a 0.001ml Neubauer haemocytometer and the conidia concentrations adjusted to 2 x 10^6 conidia/ ml. To test for conidia viability, 40µl of the conidia suspension was added to 200µl GLEN liquid medium which is suitable for the germination of entomopathogenic fungi (Beauvais and Latgé 1988), and vortexed for 5 seconds. 15µl of this suspension was pipetted into six of the 6mm spots on sterile Teflon coated slides and placed into sterile Petri dishes with moist filter paper. One slide was prepared per species. These were incubated for 18-24 hours at 30˚C after which time the proportion of germinated conidia per species was calculated and the conidia suspensions, which contained >90% viable conidia, were considered as suitable for use. Once they had been adjusted to account for the percentage of non-viable conidia, *A. fumigatus* and *A. phoenicis* suspensions of 5x10^6conidia/ml and an *A. flavus* suspension of 1x10^3conidia/ml were prepared. These concentrations were previously determined according to a doses response procedure as LD_{50} doses (unpublished data). Larvae were exposed to 5 µl of each parasite treatment by pipetting the suspension onto the food near the mouthparts. Larvae were not fed again for another 24 h when all the food in the wells had been ingested to ensure thorough intake of the administered parasite treatment. Following exposure, larvae were maintained for 7 days and examined daily under binocular microscope for mortality (i.e. no movement) and signs of stonebrood infection (i.e. hyphae and conidia on the cuticle).

3.3.4 *Experiment 1- variation in royal jelly and sugars concentrations*

We used a total of 768 larvae, from four colonies. Four diets were tested: 1) standard diet: 50% royal jelly (v/v), 6% D-fructose (w/v), 6% D-glucose (w/v) and sterile water; 2) reduced RJ diet: 40% royal jelly (v/v), 6% D-fructose (w/v), 6% D-glucose (w/v) and sterile water; 3) reduced sugars diet: 50% royal jelly (v/v), 3% D-fructose (w/v), 3% D-glucose (w/v) and sterile water; 4) reduced RJ and reduced sugars diet: 40% royal jelly (v/v), 3% D-fructose (w/v), 3% D-glucose (w/v) and sterile water. Larvae were treated with either *A. flavus*, *A. phoenicis*, *A. fumigatus* or a sterile water control

(as described in section 3.3.3), in a full factorial design. Forty-eight larvae per colony were tested with each diet-treatment combination.

3.3.5 *Experiment 2- variation in pollen origin and diversity*

Pollen from three plant species were used; oilseed rape *Brassica napus*, dandelion *Taraxacum officianale* and hawthorn *Crataegus monogyna*, which are among the species most commonly collected by honey bees in the UK during springtime (Keller *et al.* 2005). All pollen was collected in a local apiary from honey bee colonies using pollen traps. Supplementation was 1.25% (w/v), which approximated the quantity of pollen larvae naturally ingest during their development (Aupinel *et al.* 2005, Babendreier *et al.* 2004). Five diets were tested. As our control, pollen-free diet, we used the reduced RJ diet from Experiment 1, because of its reasonably high control survival and interaction with *A. fumigatus* exposure. The three monofloral diets consisted of this reduced RJ diet supplemented with pollen from oilseed rape, dandelion or hawthorn, and the polyfloral diet consisted of a mix of all three in equal measure up to a total of 1.25% (w/v). A total of 960 larvae, from four colonies, were treated with either *A. fumigatus* or sterile water control, 24 larvae per colony were tested with each diet-treatment combination (as described in section 3.3.3).

3.3.6 *Statistical analysis*

The survival of larvae was analysed using Cox proportional-hazards regression models, with treatment, diet and colony of origin, and interactions between these factors included in the models. Terms were removed in a stepwise manner to obtain the minimum adequate model which contained all main effects. When the diet factor was significant, the different diets were subsequently compared pair-wise using Kaplan-Meier analyses with the Breslow statistic. All analyses were carried out in SPSS 16.0

3.4 Results

Daily observations showed only dead larvae had signs of infection and these were either hyphae and/or conidia growing from the cuticle. No signs of infection were observed on larvae in the control treatments.

3.4.1 Experiment 1- variation in royal jelly and sugars concentrations

There were no significant differences in the survival time of larvae between the experimental colonies, and no significant interactions between colony and either diet or parasite treatment ($P > 0.05$ in all cases). The survival of larvae was significantly different between parasite treatments (Wald = 39.2, df = 3, $P < 0.001$), being the highest for unexposed larvae (67.8%), the lowest for larvae exposed to *A. flavus* (37.0%), and intermediate for larvae exposed to *A. phoenicis* (49.5%) or *A. fumigatus* (55.2%) (Fig. 7). The survival of larvae was also significantly different between diets (Wald = 21.5, df = 3, $P < 0.001$), being generally higher for larvae fed the normal diet (62.0%) and lowest for larvae fed the diet with both reduced RJ and reduced sugars (38.0%), and intermediate for larvae fed the reduced RJ diet (49.0%). There was a significant effect of diet on larvae exposed to *A. fumigatus* (Wald = 12.9, df = 3, $P = 0.005$), most larvae survived when fed the normal diet (77.1%), but survival was lower when fed either the diet with reduced royal jelly (43.8%) or with reduced royal jelly and reduced sugars (37.5%) (Fig. 7). There was, in contrast, no effect of diet on the survival of larvae exposed to *A. flavus* (Wald = 2.17, df = 3, $P = 0.537$), with survival being low in all cases (Fig. 7). The effect of diet was also not statistically significant for unexposed larvae (Wald = 7.45, df = 3, $P = 0.059$) or for larvae exposed to *A. phoenicis* (Wald = 6.96, df = 3, $P = 0.073$), although in both these cases there was a trend for lower survival in larvae fed the diet with reduced royal jelly and reduced sugars (unexposed 52.1%; *A. phoenicis* 29.2%) suggesting that the diet induced increased larval mortality.

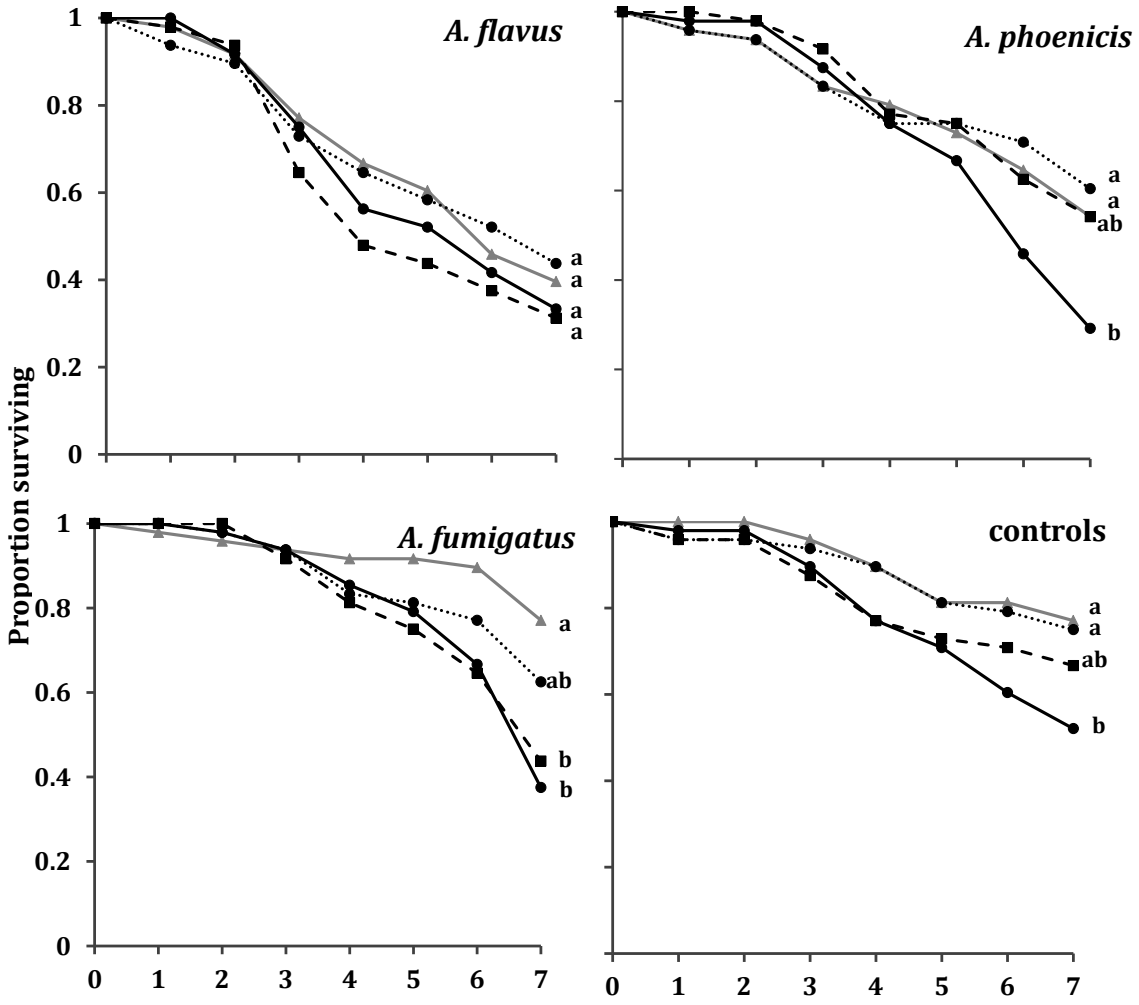

Figure 7: Survival of laboratory-reared larvae exposed to either, *Aspergillus flavus*, *A. phoenicis, A. fumigatus* or a control and fed with either the normal (grey triangles and solid lines), reduced royal jelly (black squares and dashed lines), reduced sugars (black circles and dotted lines) or reduced royal jelly and reduced sugars (black circles and solid lines) diets. Different letters indicate diets which resulted in significant differences in survival based on pairwise Kaplan-Meier tests ($P<0.05$).

3.4.2 *Experiment 2 - variation in pollen origin and diversity*

There was a significant interaction between diet and parasite treatment (Wald = 33.1, df = 4, $P < 0.001$; Fig. 8). The effect of *A. fumigatus* exposure on the survival of larvae varied depending on the diet, with more control larvae surviving than those that were exposed to *A. fumigatus* when fed the pollen-free diet (unexposed 59.4%; *A. fumigatus* 15.6%) (Wald = 33.8, df = 1, $P < 0.001$) and the hawthorn pollen diet (unexposed 41.7%; *A. fumigatus* 22.9%) (Wald = 31.3, df = 1, $P < 0.001$). Parasite treatment had no significant effect on the survival of larvae fed the dandelion (Wald = 0.6, df = 1, p = 0.440) and mixed pollen diets (Wald = 2.29, df = 1, $P = 0.130$). Larvae fed oilseed rape diet survived similarly poorly whether they were exposed to *A. fumigatus* or not (Wald = 2.75, df = 1, $P = 0.097$). There was no significant difference in survival between colonies (Wald = 5.3, df = 4, $P = 0.254$).

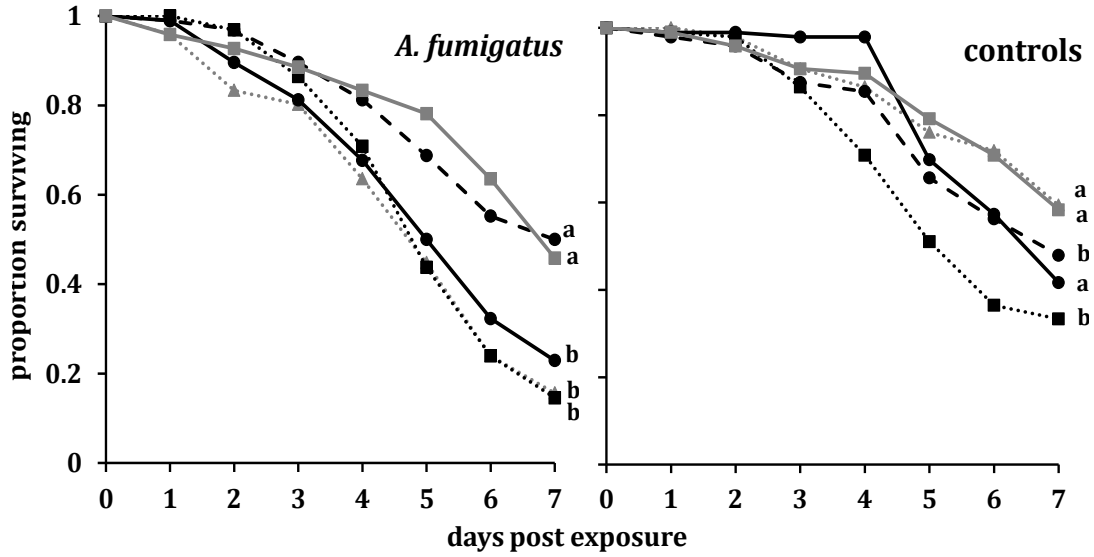

Figure 8: Survival of laboratory-reared larvae that were exposed to either *Aspergillus fumigatus* or a control suspension and fed either reduced royal jelly, pollen-free diet (grey triangles and dotted lines), or reduced royal jelly diet supplemented with pollen from oilseed rape (black squares and dotted lines), dandelion (black circles and dashed lines), hawthorn (black circles and solid lines) or all three (grey squares and solid lines). Different letters indicate diets which differed significantly from one another in pairwise Kaplan-Meier tests ($p < 0.05$).

5. Discussion

Our results showed that nutritional limitation can significantly increase the susceptibility of honey bee larvae to *A. fumigatus*, and that this effect was rectified if diet contained dandelion or polyfloral pollen. Pollen diversity is known to enhance the immunocompetence of adult bees (Alaux *et al.* 2010), and polyfloral pollen supplements have also been shown to confer resistance to *Paenibacillus larvae* infections in honey bee larvae (Rinderer *et al.* 1974). Our results show there are differences in the ameliorative potential of individual pollen species in nutritionally limited larvae on exposure to a ubiquitous, opportunistic fungal parasite.

The effect of diet on the survival rate of larvae depended on the parasite treatment. The most virulent parasite, *A. flavus*, caused high mortality irrespective of the diet on which larvae were fed. The apparent impact of nutritional limitation is therefore dependent upon the strength of the parasite challenge. In a wide variety of host-parasite systems a dose-dependent invasion threshold for successful parasite infection has been observed, which is related to parasite fitness and the effectiveness of the hosts defences (Ebert *et al.* 2000, Hughes *et al.* 2004, Regoes *et al.* 2002). *A. flavus* is the most frequent *Aspergillus* species reported to infect insects with its ability to produce pectinase and protease isoenzymes being implicated as important virulence factors (St. Leger *et al.* 2000). Moreover, *Aspergillus* spp. produce a variety of mycotoxins including aflatoxins and ochratoxins which not only vary between species and strain of the fungus but also according to substrate (Medina *et al.* 2004). It is not known what role these mycotoxins play in the virulence of stonebrood infections, but it is possible that larval mortalities were in part due to toxicity rather than fungal invasion.

Royal jelly is comprised of water (60-70%), protein (12-15%), carbohydrates (10-16%), lipids (3-7%), trace vitamins and mineral salts, and is known to contain potent antioxidants and antimicrobial compounds (Crane 1990, Lercker *et al.* 1993). A reduction of 20% of these royal jelly components in the artificial larval diet made larvae more susceptible to *A. fumigatus* exposure, indicating that the nutrients are important for resisting infection or for detoxification purposes. In larvae not exposed to the parasite, a reduction of sugars as well as royal jelly resulted in low survival, which may suggest a threshold level for deficiencies in dietary sugars or the effects of a macronutrient imbalance (Human *et al.* 2007).

In Experiment 2, the survival of larvae in both the *A. fumigatus* and control treatments showed different effects of each pollen species in the diet. The positive effects of dandelion and polyfloral pollen diets are evident by the increased survival

observed in larvae exposed to *A. fumigatus*. Whether the increased survival of larvae fed diet supplemented by polyfloral pollen is simply due to the presence of dandelion pollen, or due to the combination of pollens, is not clear. Roulston and Cane (2000) reported protein concentrations for 377 species of floral pollen, yet few complete analyses are available for the composition of many bee collected pollens. Honey bees collect pollen that ranges from 12% - 61% in protein, 0.8% - 31.7% in lipid, and 21% - 48% in carbohydrate (corbicular pollen) content (Evans *et al.* 1991; Roulston and Cane, 2000, Todd and Bretherick 1942). However, results may vary according to the extraction methods used. A total of 10 amino acids are regarded as essential in the diet of honey bees (DeGroot 1953) and it may be the presence of these that determine the nutritive quality of the pollen species rather than the total protein content. Dandelion has been reported to contain 19.2% protein and 15.1% lipids, oilseed rape 31.9% protein and 25.4% lipids and hawthorn 26.19% protein and 2.81% lipids (Evans *et al.* 1991, Roulston and Cane 2000, Zhang *et al.* 1999). Analyses have shown that relative to honey bee nutritional requirements, dandelion pollen possesses multiple amino acid deficiencies and is lacking tryptophan, phenylalanine and arginine (Loper and Cohen 1987). Yet, it is also considered to be rich in lipids, with a high diversity of fatty acids, antioxidants and vitamins (Guo *et al.* 2009, Standifer 1966). It has been noted that dandelion pollen is highly attractive to bees with high colony productivity observed in colonies situated in areas where dandelion blooms are abundant (Keller *et al.* 2005, Standifer 1966). Nevertheless, the brood rearing capacity of colonies fed solely on dandelion pollen is severely diminished or absent in comparison to pollen from other floral sources with higher protein content (Loper and Cohen 1987). This suggests that some other nutrient or element present in dandelion pollen is beneficial for brood rearing. Pollen fed naturally to honey bee larvae by nurse bees may be providing an important source of lipids, vitamins and antioxidants and could be providing essential components needed for general good health and disease resistance, as well as the macronutrients required for normal growth and development.

Oilseed rape is a widespread, intensively cultivated crop and provides an important pollen source for honey bees (Abrol 2007). The increased mortality rates of larvae fed the oilseed rape pollen diet in both the *A. fumigatus* and control treatments is surprising as the pollen is of apparently high nutritional value to honey bees. One possible explanation could be that the pollen contains pesticide residues which were toxic to the larvae, with a lethal dose being present in the oilseed rape single pollen diet but not in the mixed pollen diet. Larvae fed on the hawthorn pollen showed higher mortality when exposed to *A. fumigatus* than uninfected larvae suggesting that pollen

of this species may lack the properties involved in supporting resistance, as observed with larvae fed the dandelion and polyfloral diets. Larvae are typically fed pollen in the form of bee bread rather than the corbicular pollen used in this study. The pollen is fermented by lactic acid bacteria produced in the honey stomach of forager bees, which is thought may standardize the process (Vasquez and Olofsson 2009). Enzymes produced by other microorganisms present e.g. moulds, are involved in lipid, protein and carbohydrate metabolism which increase the digestibility and nutritive value of the pollen (Gilliam *et al.* 1989). However this process may not compensate for all the nutrients in which the raw pollen is lacking and honey bee colonies may not be able to compensate for an unbalanced diet in environments where foraging diversity is low leading ultimately to deficiencies in particular essential nutrients.

The results of this study show that rather than susceptibility of honey bee larvae to fungal parasites being affected by nutrition in a general way, different nutrients may be important for coping with different parasite species. It is possible that honey bee colonies may be able to compensate for food of limited quality by adjusting their brood production or by the fermentation of pollen into bee bread before feeding to larvae. However, the results nevertheless suggest that the impact of inadequate foraging resources in the environment on honey bee health can be significant and that nutritional limitation of larvae may be an important factor in colony losses. Schemes to enhance biodiversity on agricultural land include seed mixes to provide pollen resources for bees and further work would therefore be warranted to determine which pollen species will provide the best nutritional resources to help bees deal with the complexity of threats with which they are faced.

3.6 Acknowledgements

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CHAPTER 4

Interspecific within-host competition between specialist and opportunistic fungal parasites in honey bee larvae

4.1 Abstract

Parasite infections often consist of multiple parasite species or strains that compete within the host. These competitive interactions, combined with host resistance, are major drivers in the evolution of parasite strategies for survival, growth and fitness. The outcomes of these interactions are highly variable due to many factors including host immune responses, life stage, and the frequency, temporal spacing and order of each strain inoculation. Microbiological studies of honey bee hive substrates reveal high prevalences of opportunistic *Aspergillus* spp. yet little is known about how these fungi interact with other brood pathogens such as *Ascosphaera apis* and the potential impact of multiple infections on virulence evolution. In this study laboratory reared honey bee larvae were exposed to single and dual inoculations of *A. apis*, *Aspergillus flavus* and *A. phoenicis* to investigate how interspecific competitive interactions affect pathogen growth, fitness and virulence. Compared to single infections, *A. flavus*/*A. phoenicis* treatments showed a highly synergistic effect on larval mortality and conidiation rate. *Ascosphaera apis/Aspergillus* treatments revealed increased within-host growth yet with inhibitory effects on pathogen fitness. These results illustrate the significance of within-host competition in the epidemiology and evolution of pathogen infections and provide insight into the complexity of inter-pathogen interactions.

4.2 Introduction

Parasitism is one of the most successful modes of life in living organisms and acts as a major selective force through the negative effects inflicted on host fitness and survival (Poulin and Morand 2000, Ebert and Hamilton 1996, Anderson and May 1983). Infections often consist of multiple parasite strains or species that compete for resources in the host (Holmstadt *et al.* 2005). These competitive interactions, which may be broadly characterised as exploitative, interference or apparent (e.g. immune mediated) competition, are major drivers in the evolution of virulence in parasites (Alizon *et al.* 2008, Ewald 1994). Theory predicts that genetically diverse infections result in the evolution of more virulent strains (van Baalen and Sabelis 1995, Frank 1996, Gandon *et al.* 2001). In single infections, prudent exploitation of the host to maximise the production of transmission stages is optimal as opposed to mixed infections. Parasites that rapidly exploit the host will out-compete parasites that prudently exploit it so mixed infections select for greater virulence. Multiple infection outcomes are influenced by a range of complex factors including host immune responses, infection intensity, temporal spacing and order of each strain inoculation, as well as abiotic environmental factors (Duneau *et al.* 2011, Carillo *et al.* 2007, Harrison *et al.* 2006, Read and Taylor 2001). Infection outcomes are also influenced by the relatedness of the coinfecting parasites. According to kin selection theory, within-host competition between strains may restrict parasite virulence evolution, for example through parasite co-operation (Chao *et al.* 2010, Frank 1996). Conversely, a decrease in relatedness among coinfecting pathogens may potentially lead to lower co-operation resulting in greater exploitation rates and virulence (Alizon and van Baalen 2008a). Thus, parasite life-history and precise host-pathogen and inter-pathogen interactions will determine infection dynamics and the selection for optimal virulence in multiple infections (Staves and Knell 2010, Alizon and vanBaalen 2008b).

The complexity of inter-pathogen interactions is evident in the increasing body of empirical evidence available. Using rodent malaria *Plasmodium chabaudi* strains, competing clonal lines have shown higher virulence and transmission to mosquitos than single clones (De Rhoode 2005, Taylor 97). Multiple infections can be more harmful than the most virulent component parasite due to synergistic effects on host fitness (Read and Taylor 2001). Genetically diverse infections may also be just as harmful as the most virulent strains present (Hughes and Boomsma 2004) and parasites may reallocate resources from within-host growth to the production of transmission stage propagules (Taylor *et al.* 2002). Other scenarios may present cases

of decreased virulence due to the competitive exclusion of virulent strains by more competitive avirulent strains (Harrison *et al.* 2006, Berchieri and Barrow 1990).

Host genetic diversity can positively affect resistance to disease (Baer and Schmid-Hempel 2003, Reber *et al.* 2008, Seeley and Tarpy 2007, Tarpy and Seeley 2006, Hughes and Boomsma 2004, Tarpy 2000, Liersch and Schmid-Hempel 1998). The benefits of host genetic diversity will also depend on the diversity of the parasitic challenge (Ganz 2010) and heterogenous host populations may be more or less prone to infection depending on whether there are susceptible host genotypes present. Using single versus mixed clone trypanosome *Crithidia bombi* infections in the bumble bee *Bombus terrestris*, no differences were found in parasite prevalence, infectivity, duration and clearance rate. However, an effect of host colony, or genetic background, on parasite virulence was observed demonstrating the significance of host-parasite interactions on the expression of virulence (Imhoof and Schmid-Hempel 1998).

Multiple infections and within-host parasite interactions may be particularly significant in social animals such as honey bees. Colonies containing up to 50,000 bees may be exposed to a high diversity of parasites that are introduced via returning forager bees. Recently, honey bee health has been a major cause for concern due to severe colony losses in Europe and the United States (Neumann and Carreck 2010). These enigmatic declines have greatly increased awareness in honey bee pathology and attracted many possible explanations. Epidemiological surveys, including a metagenomic study, have shown that honey bees carry a high diversity of pathogens (vanEngelsdorp *et al.* 2009, Cox-Foster *et al.* 2007) yet studies showing how co-infecting parasites interact within the host are scarce. Long term observations have provided statistical evidence showing outbreaks of the chalkbrood fungus *Ascosphaera apis* were related to *Varroa destructor* and *Nosema ceranae* infections in apiaries, suggesting that complex host-parasite interactions between taxa can occur (Hedtke *et al.* 2011).

Chalkbrood is a disease of honey bee larvae caused by the obligate, fungal pathogen *Ascosphaera apis* and is responsible for significant brood mortality leading to reduced fitness and productivity in colonies (Zaghloul *et al.* 2005). Genetic background, either relating to hygienic behaviour or other intrinsic immune factors can influence susceptibility to the disease (Tarpy 2003, Gilliam *et al.* 1983). Transmission between colonies in apiaries can occur via contaminated materials such as pollen combs and wax foundation that act as reservoirs containing ascospores that can remain viable for up to 15 years (Flores *et al.* 2005, Toumanoff 1951). *Aspergillus* spp. are unspecialised soil fungi, a number of which are opportunistic pathogens and etiological agents of

stonebrood in honey bees. The fungi are commonly found on hive substrates such as in pollen, bee bread, honey and within the bees and larvae themselves (Gilliam 1997, Gilliam *et al.* 1989, Batra, *et al.* 1973, Gilliam and Prest 1972). It is probable that chalkbrood and *Aspergillus* spp. quite often co-occur in honey bee colonies. Microbiological studies have isolated *Aspergillus* and other moulds including *Rhizopus* spp., *Mucorales* spp. and *Penicillium* spp. from pollen and bee products that produce enzymes and antimycotic substances that perform biochemical and anti-microbial roles (Gilliam *et al.* 1989). These were more frequently present in bee bread samples from resistant (hygienic) than susceptible honey bee colonies. This may also indicate that competitive interactions between *A. apis* and *Aspergillus* spp. occur in nature. The factors influencing the outcome of these interactions that, for *Aspergillus* spp. could range from beneficial to pathogenic effects, or the onset of chalkbrood disease warrants further investigation.

Experimental data with mixed entomopathogen studies on individual insects have shown both synergistic and antagonistic effects between pathogens on the overall virulence of infections (Anderson 2011, Hughes and Boomsma 2004, Guzman-Franco *et al.* 2009). Recently a number of studies have used quantitative PCR (qPCR) protocols for *in vivo* measurements of fungal entomopathogen growth dynamics (Anderson 2011a, Guzman-Franco *et al.* 2011, Bell 2009). The number of sequence copies of a given parasite has shown how infection dynamics can be mediated by initial dose rate, the immune responses and by the presence of coinfecting pathogens (Anderson 2011a, Guzman-Franco *et al.* 2011).

The aims of this study were to determine how interspecific competitive interactions affect the growth, fitness and virulence of fungal pathogens in honey bee larvae. Honey bee larvae were exposed to single and dual species inoculations of the obligate chalkbrood fungus *A. apis* and opportunistic stonebrood fungi *A. flavus* and *A. phoenicis*, and then reared individually under controlled conditions. Infection parameters were quantified using sporulation rate (the rate at which cadavers produced spores/conidia as a measure of pathogen fitness and larval mortality rate as a measure of pathogen virulence. Using qPCR it was possible to evaluate differences in growth dynamics of different fungal species and determine if the growth rates of isolates in dual infections are indicative of the occurrence of within host competitive interactions.

4.3 Materials and Methods

4.3.1 *Sampling and in-vitro rearing of larvae*

We used larvae from three healthy colonies of honey bees *Apis mellifera carnica* that were asymtomatic of pests and disease and headed by one year old unrelated queens. One day old larvae (age estimated by size) were transferred into sterile 48-well tissue culture plates. Each larva was placed onto a 30 µl drop of standard artificial diet which consisted of 50% royal jelly (RJ) (v/v) (Apitherapy, Norfolk, UK), 6% D-fructose (w/v), 6% D-glucose (w/v) and distilled water (Jensen *et al.* 2009) and had been warmed, approximately to hive temperature (32-34°C). Plates were then transferred into an incubator at 34°C, 90% relative humidity in darkness. On days 1 and 2 each larva was fed 10µl of diet, 20µl on day 3, 30µl on day 4, 40µl on day 5 and 50µl on day 6 (Aupinel *et al.* 2010).

4.3.2 *Parasite treatments*

The isolates of *Aspergillus* spp. used were collected from naturally-exposed adult worker bees in our experimental apiary (West Yorkshire, UK) and cultured on malt extract agar plates at 30°C until the fungi produced conidia. The plates were then stored at 4°C until use (see Chapter 2). The isolates used were A. flavus (G49) and A. phoenicis (N40.04). The conidia were harvested from the malt extract agar plates, suspended in 2 ml of sterile water and vortexed for 15 seconds.

For the chalkbrood ascospore suspensions, subcultures from strains of each mating type of the heterothallic *Ascosphaera apis* strains (KVL 06-123; KVL 06-132, Copenhagen) were transferred on to Sabouraud Dextrose Agar (SDA) plates and maintained at 30°C for one month for mating to occur and for the fungus to produce ascospores. Mature ascomata were removed from the mating plates with a sterile spatula and placed in to a sterile glass tissue grinder with 500 µl of sterile deionized water. Once ground, another 1000 µl of water was added and then left to settle for 20 mins to allow any debris to separate from the spores in the suspension. 1000 µl was collected from the centre of the ascospore suspension. To test for the spore (conidia/ascospore) viability for the pathogen treatments, spore concentrations were determined using a 0.001 ml Neubauer haemocytometer and adjusted to 2 x 10^6 conidia/ ml. 40 µl of the spore suspension was added to 200 µl GLEN liquid medium (Beauvais and Latgé 1988), and vortexed for 5 s. 15 µl of this suspension was pipetted into six of the 6 mm spots on sterile Teflon coated slides and placed into sterile Petri

dishes with moist filter paper. One slide was prepared per species. These were incubated for 18-24 h at 30°C after which time the proportion of germinated spores per species was calculated and the spore suspensions, which contained >90% viable spores, were considered as suitable for use. Once they had been adjusted to account for the percentage of non-viable spores, an *A. phoenicis* (pho) suspension of 5 x 10^6spores/ml (for single doses), 1 x 10^7spores/ml (for dual doses), *A. flavus* (fla) suspension of 1 x 10^2 spores/ml (for single doses) and 5 x 10^3spores/ml (for dual doses) and chalkbrood (CB) suspensions of 3 x 10^5 (for single doses) and 6 x 10^5 (for dual doses) were prepared. These were concentrations for which the single dose response had previously been determined according to dose-response tests (Chapter 2). The dual doses each consisted of two full single doses of two parasite species administered within the same volume of 5 µl inoculum as the single doses. There were seven treatments in total, three single species treatments (CB, fla and pho), three dual species treatments (CB/fla, CB/pho and fla/pho) and a sterile water treatment as controls. Larvae were exposed to 5 µl of each treatment by pipetting the suspension onto the food near the mouthparts, they were not fed again for another 24 h until all the food in the wells had been ingested to ensure thorough intake of the administered inoculum. Following exposure to the treatments the larvae were maintained for 7 days and examined daily under a microscope for mortality (i.e. no movement and discolouration) and signs of stonebrood or chalkbrood infection. The day of death, appearance of hyphae and conidia and/or ascospores on the cuticle of the larvae were recorded. The species of spores produced was also identified and recorded. Samples for the qPCR assay were treated concurrently. On each day following exposure to the fungal treatments, nine larvae per treatment (3 per colony) were sampled randomly from each treatment group and stored in 96% ethanol suspension at -20°C until qPCR analysis was carried out.

4.3.3 *DNA extraction and real-time qPCR assay*

Small (day 1-3 days post-inoculation (DPI)), medium (day 3-5 DPI) and large larvae (day 6-7 DPI) were homogenized in 100, 300 and 600 µl of sterile, nuclease-free water respectively using 0.05 g of 0.1 mm Zircona/silicon beads and one 5 mm stainless steel bead with a Qiagen Tissue Lyser for 7 mins at 50 oscillations/s. Genomic DNA was extracted from the homogenate using E.Z.N.A Mollusc DNA Kit (Omega bio-tek) according to the manufacturer's instructions. For the pathogen treatments CB, fla and pho one set of primers were designed on the ITS gene (ITS-F: TGC CTG TCC GAG CGT CAT; ITS-R: ACA AAG CCC CAT ACG CTC G) and Molecular-Groove Binding Non-

fluorescence Quencher (MGBNFQ) probe for each pathogen; *Ascosphaera apis* (6 FAM-MGBNFQ-TGA CGG CGT CGT GTT), *Aspergillus flavus* (NED- MGBNFQ-TGT TGG GTC GTC GTC) and *A. phoenicis* (VIC- MGBNFQ-TGT GTT GGG TCG CCG T) were designed using Primer Express v3.0 (ABI) and the sequences (*Ascosphaera apis*: Genbank< U68313>; *Aspergillus flavus*: Genbank< AB573935.1>; *A. phoenicis*: Genbank< AB573935>). Primers for the *A. mellifera β-actin* gene as in Lourenco *et al.* (2008) was used with Taqman® with the (NED-MGBNFQ- AAT TAA GAT CAT CGC GCC AC) as in (Roberts *et al*. 2012).The probe standard curve analyses were run for each assay and the calculated efficiencies fell between the acceptable range of 98-103% over a 10000-fold range. The real-time PCR was carried out in uniplex reactions using 5µl of Mastermix ungII (Applied Biosystems), 2µl template DNA, 900nM of each primer, 0.25 µl of Taqman probe and 0.95µl DNase-free water. Reference DNA from each pathogen species and the host (calibrator) and negative control reactions containing water instead of DNA were also run on each plate. Each sample was performed at the same time in triplicate and results were reported as averages of the triplicates. PCR amplification and data acquisition were performed using a Step-One v2.2 thermal cycling machine. DNA levels were measured and calculated by the comparative $2^{-\Delta\Delta ct}$ method (Smittgen and Livak 2008) with the actin gene as the endogenous reference for sample normalisation. For each set of treatment samples taken at each given time point the mean of the $2^{-\Delta\Delta ct}$ values for a given pathogen gene was used as a measure of fold increase relative to the same DNA amplicon in the coinfection dual treatments.

4.3.4 *Statistical analysis*

All statistical analyses were carried out using R 1.14.1 software (R Development Core Team 2012). Survival analysis was performed using a parametric Kaplain-Meier model with the *survreg* function of the *survival* package. Differences in the survival and sporulation rates of larvae between treatments, were examined using models fitted with Weibull distribution and using a gamma *frailty* function to allow for unobserved heterogeneity due to colony differences (Therneau 2012). The hazard-ratio for each treatment was determined, where the hazard is equal to the slope of the survival curve using the control treatment as the reference category (Crawley 200??). Pair-wise comparisons of the treatments were carried out using ghlt function on the *multcomp* package. Differences in parasite DNA quantities between treatments were analysed using glm models incorporating day post inoculation (DPI) and colony as factors and with quasipoisson distribution. Non-significant interaction terms were dropped to produce the minimum adequate model.

4.4 Results

4.4.1 *Pathogen virulence*

All six fungal treatments were found to cause significant mortality in the exposed larvae and all dual inoculations had a significantly greater effect than the single inoculations . We found a significant effect of both pathogen treatment on the survival of larvae ($\chi^2_{6, 2008} = 993.9$, $P < 0.001$) and there was also a significant frailty effect of unobserved heterogeneity between colonies ($\chi^2_{1.01, 2007} = 51.78$, $P < 0.001$).

All single and dual pathogen treatments had a significant effect on the survival of larvae compared to the controls ($P < 0.001$ in all cases). In the single infections, *A. flavus* appeared to be the most virulent treatment, causing significantly greater mortality than *A. phoenicis*, and also greater mortality than chalkbrood (Fig. 9). There was a slightly higher mortality rate in larvae treated with chalkbrood than *A. phoenicis* but this was also non-significant. All dual treatments, which were in effect a double dose of parasite inoculum, caused significantly greater mortality of larvae than single pathogen treatments (Fig. 9). According to hazard ratio values the effect of the chalkbrood dual treatments on mortality rate (CB/fla and CB/pho) were slightly less than additive. In contrast the effect of fla/pho treatment on mortality was highly synergistic (Fig. 10).

Table 4: The coefficient values for the survival model factors for each treatment and including colony frailty heterogeneity after seven days post inoculation with single and dual fungal pathogen treatments in honey bee larvae.

Individual factors	Z value	*P*
A. apis	-12.10	>0.001
A. apis/A. flavus	-16.51	>0.001
A. apis/A. phoenicis	-15.52	>0.001
A. flavus	-13.67	>0.001
A. flavus/A. phoenicis	-25.93	>0.001
A. phoenicis	-11.23	>0.001
Colony 8	0.18	0.08
Colony 23	-0.04	0.09
Colony 30	-0.16	0.08

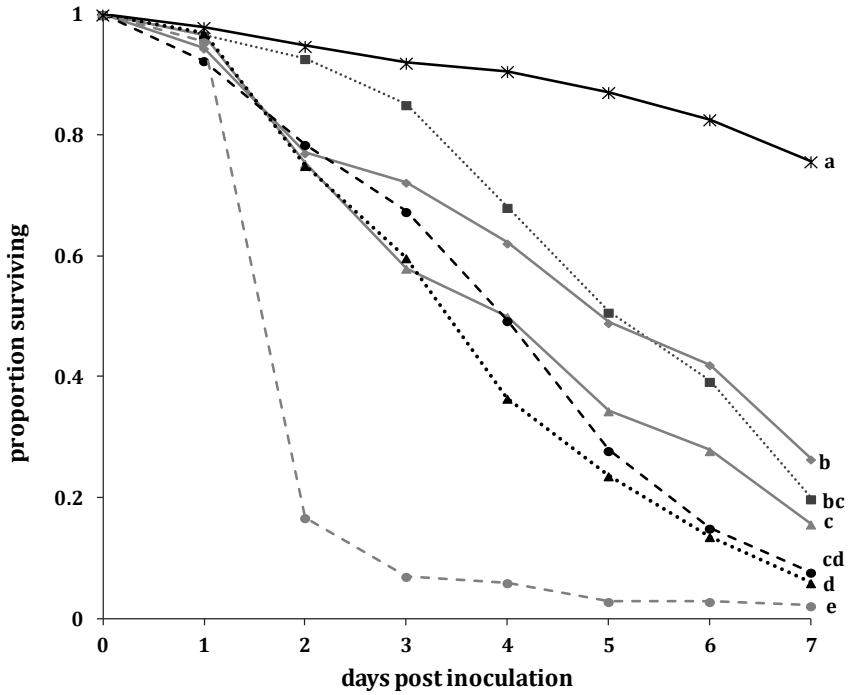

Figure 9: The survival of honey bee larvae reared individually under controlled conditions over seven days following exposure to the single fungal treatments *Ascosphaera apis* (CB; black squares), *Aspergillus flavus* (fla; grey triangles) or *Asp. phoenicus* (pho; grey diamonds) and dual treatments: CB/fla (black triangles), CB/pho (black circles), fla/pho (grey circles) or control suspension (black crosses; n = 288 per treatment). Different letters to the right of the survival lines denote significant differences between treatments.

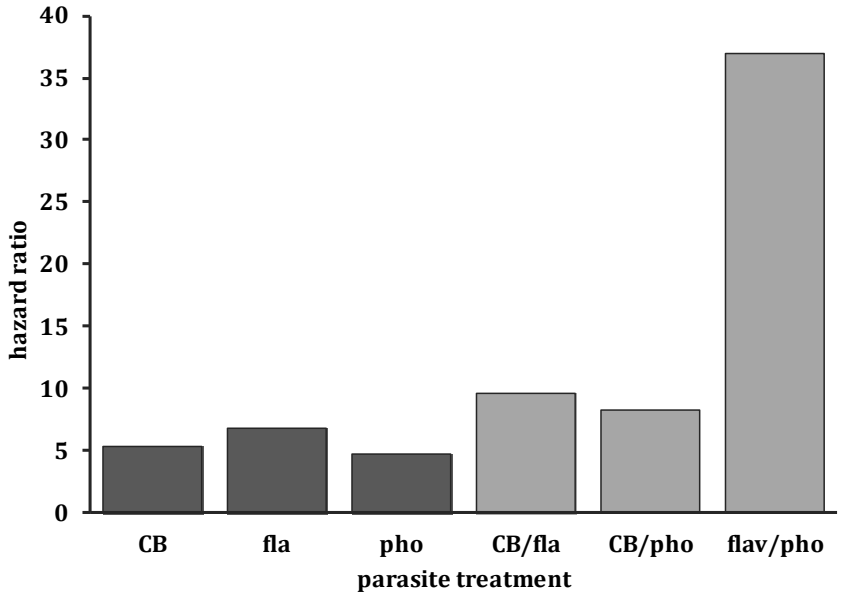

Figure 10: The hazard ratios for honey bee larvae reared individually under controlled conditions of exposure to either single (light grey) or dual (dark grey) inoculations of the fungal parasites *Ascosphaera apis* (CB), *Aspergillus flavus* (fla) or *A. phoenicus* (pho), compared to exposure to the control suspension.

4.4.2 *Pathogen fitness*

There was a significant effect of parasite treatment on the fungal sporulation rate of infected larvae ($\chi^2_{6, 2008} = 725.29$, $P < 0.001$) and there was also a significant frailty effect between colonies ($\chi^2_{0.27, 2007} = 25.99$, $P < 0.001$, Table ?). Of the three single parasite treatments, *A. flavus* sporulated the most rapidly and sporulation rates in *A. flavus* dual treatments were similar in both cases. Both dual chalkbrood treatments had a significantly faster sporulation rate than the single treatment. In both of these treatments the proportions of larvae producing *Aspergillus* spp. conidia was greater than those producing chalkbrood ascospores (Fig. 11b)

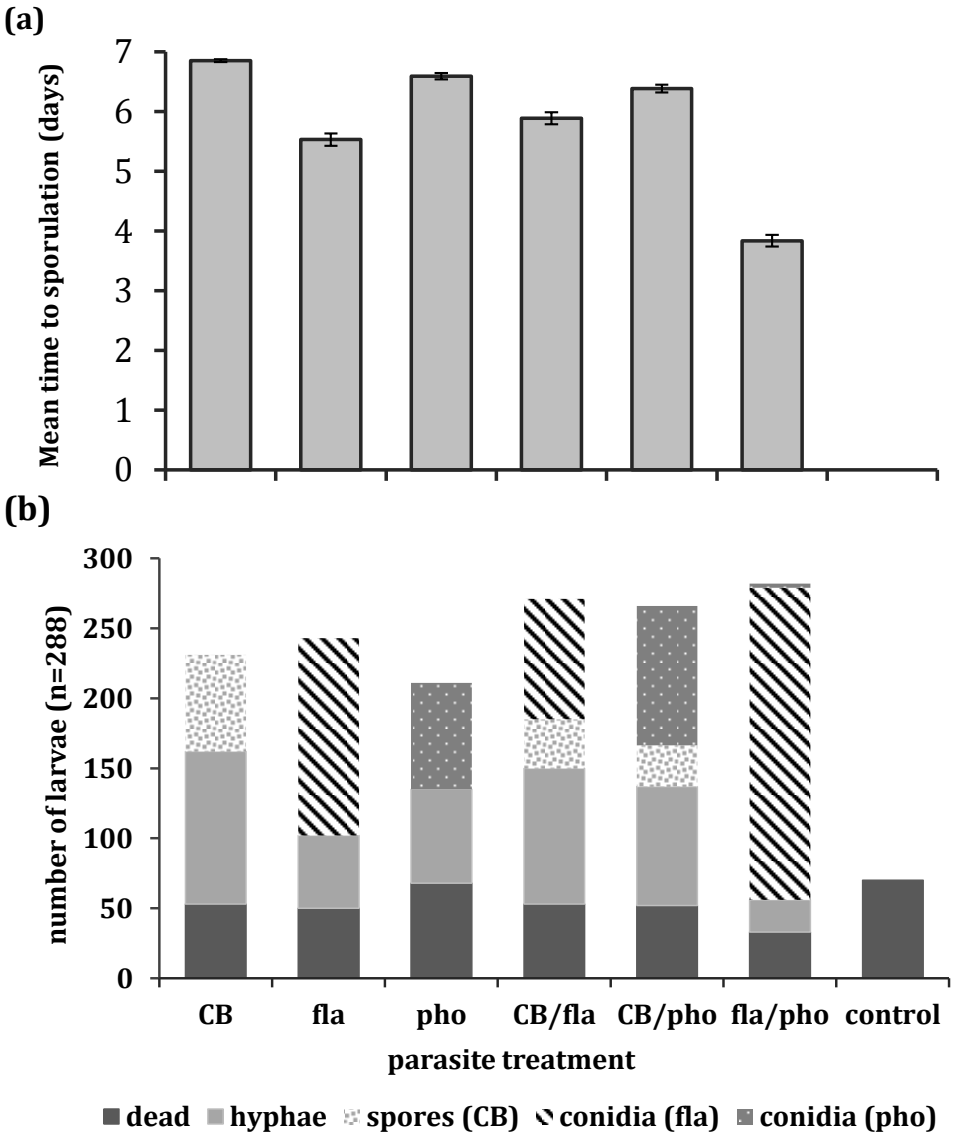

Figure 11: The sporulation patterns of each single and dual treatment of chalkbrood *Ascosphaera apis* (CB), *Aspergillus flavus* (fla) and *Asp. phoenicis* (pho) showing: (a) the mean time (+/- s.e.) for spores/conidia to be first observed on a cadaver, different letters denote significant differences between treatments following Kaplain-Meier pairwise comparisons and (b) numbers of dead larvae per treatment after seven days, showing the proportion producing hyphae and spores and the species of spores produced in each treatment.

Table 5: The coefficient values for the sporulation/conidia production model colony heterogeneity factors after seven days post inoculation with single and dual fungal pathogen treatments in honey bee larvae.

Individual factors	Z value	*P*
Colony 8	-6.65	0.5
Colony 23	2.46	0.01
Colony 30	-1.92	0.06

4.4.3 *Within-host growth*

There was an overall effect of colony (F_2= 13.43, P < 0.001) and the number of days post inoculation (DPI) on pathogen growth (F_1 = 13.09, P < 0.001). In the chalkbrood treatments the quantity of chalkbrood DNA varied significantly between treatments ($F_{2, 139}$ = 11.67, P < 0.001) though not amongst DPI ($F_{1, 138}$ = 3.22, P = 0.07). There was a significant effect of treatment on the amount of *A. flavus* DNA detected in the *A. flavus* treatments ($F_{2, 146}$ = 3.72, P =0.02) which varied depending on DPI ($F_{1, 145}$ = 21.1, P < 0.001). In the *A. phoenicis* treatments the *A. phoenicis* DNA quantities differed significantly between treatment ($F_{2, 155}$ = 39.71, P < 0.001) and also varied between DPI ($F_{1, 154}$ = 91.55, P < 0.001). There was also a significant effect of colony of origin ($\chi^2_{2, 153}$ = 30.01, P < 0.001) on the quantities of DNA detected in larvae between treatments. The within-host growth trends of the fungal treatments over the seven day period were variable (Fig. 12a and 12b), but in general an increase with DPI was observed with greater measures of fungal DNA were detected on days five, six and seven. The greatest increases in within–host growth were found in the chalkbrood treatments which maintained higher levels in both dual treatments. *A. flavus* within-host growth showed the least increase over time, particularly in the single infection. Higher within-host growth rates of *A. flavus* were detected however in both dual treatments.

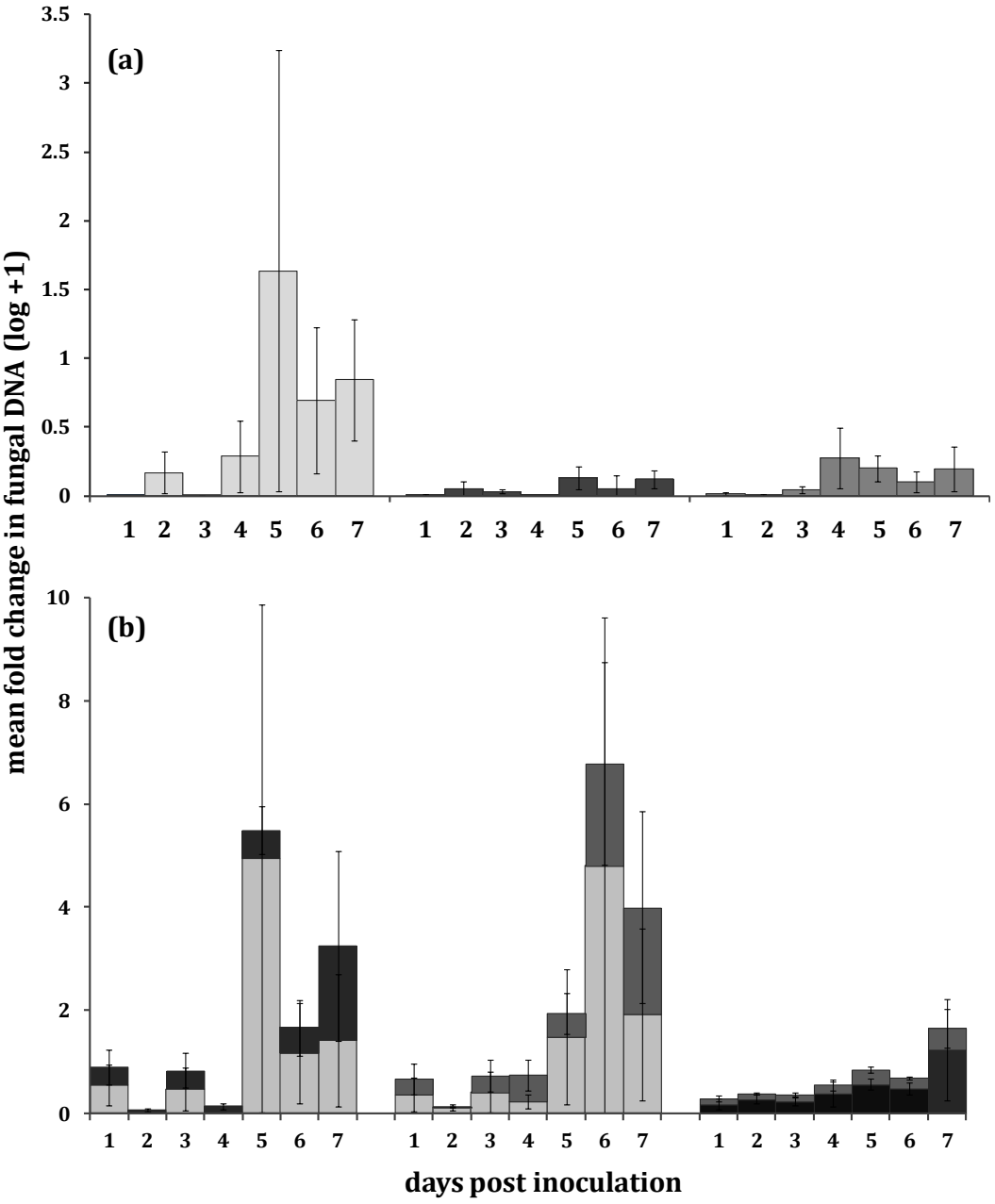

Figure 12: The within-host growth dynamics of each fungal treatment over seven days following inoculation. The mean ± s.e. pathogen intensity is the number of copies of the pathogen gene relative to the host control gene, and is the log+1 transformed fold change. Note the different scales on the y-axes for (a) single species treatments consisting of *Ascosphaera apis* (CB; light grey), *Aspergillus flavus* (fla; black) and *Aspergillus phoenicis* (pho; mid grey), and (b) dual treatments CB/fla, CB/pho and fla/pho with corresponding shading.

4.5 Discussion

Using parameters to measure the virulence, fitness and within-host growth dynamics of *A. flavus* and *A. phoenicis* and *A. apis* in honey bee larvae infections, we were able to reveal key interspecific pathogen interactions occurring in dual inoculations. *A. flavus* was the most virulent single treatment and when inoculated with *A. phoenicis* the effect on mortality was highly synergistic. This was accompanied by a marked increase in cadaver sporulation rate compared to all other treatments with almost all larvae producing *A. flavus* conidia. Simultaneously, a greater increase in the growth rate of *A. flavus* than *A. phoenicis* was evident in the within-host growth dynamics. *A. phoenicis* and *A. apis* showed similar levels of virulence in single treatments. When *A. apis* was co-inoculated with *Aspergillus* spp. (CB/fla and CB/ pho), a slightly less than additive effect on mortality was observed in both cases. The proportions of larvae producing spores for each species in these dual treatments was less than that of the corresponding single treatments yet with a greater proportion of larvae producing *Aspergillus* spp. conidia. The majority of larvae produced either *A. apis* or *Aspergillus* spores. However, these sporulation patterns did not correlate with within-host DNA quantities of coinfecting chalkbrood and *Aspergillus* spp., which showed *A. apis* to be the dominant competitor.

The synergistic effect of the fla/pho combination on virulence and the almost complete exclusion of *A. phoenicics* sporulation was similar in dynamics to a superinfection (Nowak and May 1994). This provides support for predictions that virulent parasites will be more competitive during a mixed infection (Gandon *et al.* 2001, Frank 1996, van Baalen and Sabelis 1995). The tendency for *A. flavus* to increase sporulation rate in the presence of a competitor however, is not restricted to coinfections with less virulent parasites. In *Acromyrmex* leaf-cutter ants when *A. flavus* was co-inoculated with a more virulent obligate pathogen *Metarhizium anisopliae* var. *anisopliae* although there was no effect on overall virulence, the majority of cadavers produced *A. flavus* conidia (Hughes and Boomsma 2004). Here, it was suggested that with the immune responses depleted by its more specialised mycotoxin producing competitor, a competitive advantage was conveyed to the less virulent *A. flavus*. Another explanation for an increased sporulation rate in *A. flavus* could be phenotypic plasticity as organisms may alter their chemistry, physiology, development or behaviour to maximise fitness in challenging environments (Agrawl 2001, Dudley and Schmitt 1996). *Aspergillus* spp. are primarily soil inhabitants where they interact with a high abundance of diverse microorganisms and environmental conditions (Klich 2002, Wicklow *et al.* 1998). *Aspergillus flavus* possesses a high competitive saprophytic

ability and can out-compete many other soil saprobes (Garrett 1970). It is also particularly well adapted at colonising seeds even though they represent <1% of soil flora (Horn 2005). *In-vitro* studies show that asexual sporulation and mycotoxin production in *Aspergillus* can be induced in response to stressful conditions such as nutrient deprivation, increased medium osmolarity and other conditions that inhibit growth (Han *et al.*, 2004, Lee and Adams 1995). It is possible that a dual infection could provide such conditions either through depletion of resources, or by the presence of allelochemical substances secreted by competitors, and induce a rapid sporulation response.

Both *A. apis*/*Aspergillus* dual treatments produced similar outcomes in that there were no differences between survival, sporulation rates and the proportions of larvae producing chalkbrood vs. *Aspergillus* spores in each treatment. This suggests that the nature of interspecific interactions within the host were likely to be similar. Within the seven day experimental observation period, the proportions of larvae producing spores or conidia were reduced for all species in both treatments. It is possible that the proportions of cadavers producing spores/conidia recorded represent a conservative estimate of the overall spore/conidia production given the high proportion of cadavers showing hyphal growth only and greater numbers of spores may be produced by certain species over a longer time period. This was particularly evident in the *A. apis/Aspergillus* spp. dual infections. The results observed however do suggest the presence of interspecific competitive interactions in dual infections. These may be mediated either directly via scramble competition or indirectly via interference (toxin production) or immune mediated competition (Read and Taylor 2001). Chalkbrood was better able to invade host tissues than *Aspergillus* and increased levels of chalkbrood DNA were detected in both dual chalkbrood treatments compared to the single treatment. This effect was also seen in entomopathogenic fungi by Guzman- Franco *et al.* (2011) who reported the accelerated growth rate of *Zoopthora radicans* in the presence of *Pandora blunkii* in diamondback moth larvae. Competition for limited resources between the fungi may also be mediated by nutrient resource partitioning (Giorni *et al.* 2009). Chalkbrood, the more specialised parasite, may be more adept at exploiting the specific resources available, yet generalist *Aspergillus* spp. remain highly competitive with the ability to assimilate a high diversity of nutrient resources (Bennett 2010). Analysis of compounds secreted by chalkbrood has shown them to have fungistatic properties (Gallardo *et al.* 2008), yet their efficacy against *Aspergillus* spp. has not been tested. Moreover, *in-vitro* assays have shown how *A. flavus* and *A. niger* had inhibitory effects on chalkbrood growth

indicating the presence of mutually antagonistic interactions between these fungi outside of the host (Gilliam *et al.* 1989). The possibility that direct antagonism between chalkbrood and *Aspergillus* spp. occurs due to antimicrobials is an area to be explored.

It is evident by the high host mortality rates observed that many honey bee larvae were unable to mount effective immune response against the model pathogens in this study. As a specialist pathogen in honey bee larvae, *A. apis* is presumably adapted to overcome host immune responses to exploit the host efficiently. The defences against mycosis in honey bees include phagocytosis and encapsulation (Glinski and Buczek 2003), yet these have not been well studied in larvae. Transcriptomic responses following chalkbrood challenge in larvae have revealed the upregulation of a variety of transcripts accompanied by a downregulation of major storage proteins demonstrating detrimental effects on several physiological pathways (Aronstein *et al.* 2010). Little is known about how immune responses to *Aspergillus* spp. in honey bees compare to the *A. apis* response. *Aspergillus flavus* spores appear to be able to resist digestion by haemocytes in other insects (St. Leger *et al.* 2000). The extent to which competitive interactions are modulated by the immune system remains unclear and further investigations into the immune responses of honey bees to *Aspergillus* spp. are required.

Our results show that influences on different parameters are complex which reveal different aspects of within-host competitive interactions in honey bee fungal pathogens. These interactions, which can produce synergistic effects on host mortality may potentially lead to greater disease transmission in colonies due to increased proportion of sporulating cadavers in concomitant infections. It is apparent that the outcome of multiple infections will rely on the specific traits and interactions of the pathogens involved underscoring the need for further investigation into multiple infections in honey bee colonies. Apicultural practices such as swarm control, drifting and sharing bee comb have been predicted to promote higher parasite virulence by increasing intercolony horizontal transmission (Fries and Camazine 2001). A clearer understanding of within-host microbial interactions and parasite virulence evolution has noteworthy practical applications for devising management strategies and disease control in honey bee populations.

4.6 Acknowledgements

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Chapter 5

The effects of Muller's ratchet and lack of host adaptation during the experimental evolution of opportunistic fungal pathogens in honey bees

5.1 Abstract

Serial passage experiments (SPE) are an invaluable tool for investigating the adaptation of parasite populations to the host. The majority of SPE's yield similar results, showing increased parasite virulence in the passaged hosts and attenuation of virulence in unfamiliar hosts. *Aspergillus* spp. fungi are the causative agents of stonebrood disease in honey bees and experimental infections have revealed that certain strains can be highly virulent. They are environmental fungi occurring ubiquitously as soil saprobes and are common on honey bee hive substrates. This close association could possibly lead to the emergence of more virulent strains with increased host specificity. However, as opportunists the evolution of virulence in these parasites presents a challenge to standard virulence evolution theory which is based only on obligate pathogens and host-parasite coevolution within closed systems. In this chapter two parasite populations of *A. flavus* and *A. phoenicis* were serially transmitted through laboratory-reared honey bee larvae to examine the evolution of virulence and fitness traits. Two host treatments were used to assess how host genetic diversity affects the evolution of these traits. In the post-selection assessment experiment larvae from the same colonies as used in the serial passages and also six new colonies, were inoculated with the ancestral and derived parasite lines to determine if there was any evidence for the evolution of host specialization. The overall effect of the derived treatments following four passages was loss of virulence and fitness with a high extinction rate. The lack of adaptation by both *Aspergillus* parasites show how the effect of Muller's ratchet can be substantial and how opportunistic parasites may show quite different evolutionary dynamics from those shown by the specialist pathogens that are more commonly studied.

5.2 Introduction

Explaining parasite virulence evolution is a fundamental question in evolutionary biology because of the profound effects it has on both host-parasite coevolution and ecology (Anderson and May 1982, Lenski and May 1994, Gandon *et al.* 2002). Theoretical studies predict that parasites evolve towards an optimal level of virulence (Anderson and May 1982, Ewald 1994). However, the evolution of virulence in opportunistic parasites presents a challenge to standard virulence evolution theory which is based only on obligate parasites and host-parasite coevolution within closed systems (Brown *et al.* 2012). Opportunistic parasites are commonly defined in medicine as infectious agents that cause disease when the host's resistance is low. Brown *et al.* (2012) defined them more broadly as non-obligate and/or non-specialist parasites of a focal host and proposed their classification as either commensal opportunists, environmental opportunists or parasite opportunists (or zoonoses). The trade-off hypothesis, which is central to the understanding of virulence evolution theory, states that parasites evolve towards an optimal level of virulence in order maximise transmission success (Anderson and May 1982, Ewald 1994, Frank 1996, Alizon 2009). However, opportunistic parasites are less likely to express optimal virulence in an alternative host environment as virulence factors are adapted to their primary niche and are not correlated with parasite success within the focal host (Brown *et al.* 2012).

Serial passage experiments (SPEs) are a useful tool for investigating parasite adaptation to hosts by measurement of parasite virulence and fitness where parasites are passaged through sequential hosts from genetically defined lines under controlled conditions (Ebert 2000, Ebert 1998). Experimental evolution studies are useful in the development of vaccines, studying resistance in antibiotics and for identifying the molecular sites of host-parasite interactions (Browne *et al.* 2001). The majority of SPE studies involving a variety of different hosts, parasites and conditions have yielded similar results in general, showing increased virulence in passaged hosts and attenuation of virulence in former hosts (Ebert 2000). Host-parasite adaptations in SPE's often lead to increased specificity in which the most successful parasite genotypes become more infective in the prevailing subset of host genotypes (Karron *et al.* 1988, Fry 1990, Gould 1979). Conversely, these parasite genotypes may be less successful in, and less adapted to, other host genotypes, known as the antagonistic pleitropy effect where there is a negative correlation of fitness in parasites infecting different hosts (Kubinak *et al.* 2012). This cyclical process fosters a perpetual arms race where parasites evolve to evade immune responses of the most common

genotypes, which in turn provides a selective advantage to less common genotypes (Haldane 1949).

Concern for honey bees in recent years has intensified due to unexplained mass colony losses and the implications of these for the vital pollination services provided by honey bees (Aizen and Harder 2009, vanEngelsdorp *et al.* 2009, Neuman and Carreck, 2010, Rogers and Williams 2007). Honey bees are faced with multiple stressors, including pesticides (Johnson *et al.* 2012), poor nutrition (Naug 2009) and emerging diseases (Fries 2010, Griffiths and Bowman 1981), and it appears that a combination of these may be responsible (Oldroyd 2007, vanEngelsdorp *et al.* 2009, Aufauvre *et al.* 2012). Such stressful conditions could present an ideal scenario for the emergence of opportunistic pathogen epidemics, but our understanding of these is still very limited.

Aspergillus spp. are opportunistic pathogens that are the causative agents of stonebrood disease in honey bees, and experimental infections have revealed that certain strains can be highly virulent (Foley *et al.* 2012, Vojvodic *et al.* 2012). *Aspergillus* spp. are environmental fungi occurring ubiquitously as soil saprobes on decaying matter, and common on honey bee hive substrates (Gilliam 1997, Gilliam *et al.* 1989, Gilliam and Prest 1987, Batra *et al.* 1973, Gilliam and Prest 1972, Chapter 2). This close association could possibly lead to the emergence of more virulent strains with increased host specificity. Owing to the broad spectrum of protein and polysaccharide-hydrolysing enzymes that *Aspergillus* spp. produce they are extreme generalists capable of colonising a wide range of substrates (St. Leger *et al.* 2000). They are also opportunistic pathogens of mammals, birds, reptiles, sea fan corals and humans and are pathogenic to many insects including honey bees (Kunkle and Rimler 1996, Geiser *et al.* 1998, Latgé *et al.* 1999, Tell 2005, Goldman and Osmani 2008, Rypiens 2010). *Aspergillus flavus* is the most common species infecting insects and infection usually involves conidial (asexual spore) germination on the insect cuticle (St. Leger *et al.* 2000). Previous experimental infections with the *A. flavus* and *A. phoenicis* isolates used in this study and other isolates from honey bees, have shown infections occur predominantly via the ingestion of conidia with food in both adult honey bees and larvae (Foley *et al.* 2012, Vojvodic *et al.* 2012, Chapter 2). Experimental evolution studies involving *A. flavus* infecting *Galleria mellonella* larvae showed no change in virulence in derived lines following five serial passages, though a decreased capacity to grow on an alternative agar media was reported (Scully and Bidochka 2005). Interestingly, one of the derived strains was a cysteine/methionine auxotroph and showed marked host restriction losing the ability to conidiate on agar medium whilst retaining the ability to infect and sporulate on various insects, indicating the potential

for *Aspergillus* spp. to evolve into a more specialised parasite of insects (Scully and Bidochka 2006).

In this study, the evolution of two populations of the opportunistic parasites *Aspergillus flavus* and *A. phoenicis* were studied when serially transmitted through laboratory-reared honey bee larvae. The aims were to examine how the virulence, measured by mortality rate, and fitness, measured by conidiation rate, of a virulent strain of *A. flavus* and a moderately virulent strain of *A*. *phoenicis* evolve following serial passage through honey bee larval hosts. To assess how host genetic diversity affects the evolution of these traits, two host treatments were used, one of low diversity in which the parasites were serially transmitted through larvae from the same colony, and one of high diversity in which parasites were transmitted through larvae sourced from different colonies for each passage. In the post-selection assessment experiment I inoculated with the derived lines, larvae from the same colonies as used in the serial passages and also six new colonies, to determine if there was any evidence of the evolution of host specialisation.

5.3 Materials and methods

5.3.1 *Serial passage experiment*

The *Aspergillus flavus* and *A. phoenicis* isolates were isolated from adult honey bee guts in 2010. The F4 isolates were maintained on malt extract agar at 30°C until the fungi produced conidia and then stored at 4°C, with subcultures being made every 6 months. Species identification of the *A. flavus* isolate was carried out by sequencing a 547 bp long fragment from the ITS regions 1 and 2 (Henry *et al.* 2000), with a BLASTn search producing a 100% maximum identity match with *A. flavus* (GenBank ID: GU172440.1). *Aspergillus phoenicis* was identified by sequencing a 694 bp long fragment of the calmodulin gene (O'Donnell *et al.* 2000), producing a 99% maximum identity match with *A. phoenicis* (GenBank ID: JF838353.1). Conidial suspensions were made by harvesting conidia from freshly sporulating agar plates into 1 ml of sterile water. Conidia concentrations were determined using a haemocytometer and diluted to 2 x 10^3 conidia/ml (10 conidia / 5 µl dose) for *A. flavus* and 5 x 10^5 conidia/ml (2500 conidia / 5 µl dose) for *A. phoenicus*, based on previous dose-response experiments (see section 2.3.3). The viability of the conidia was tested and found to be 95-98% (see section 2.3.3).

For the serial passage experiment, newly-hatched to 1 day old larvae were grafted from six colonies that were asymptomatic of pests and disease (Am3, 6, 9, 18, 29 and 40). To inoculate the larvae, 5µl of the conidia suspension was pipetted onto the food near the mouthparts. The larvae were not fed for another 24 hrs or until all the food in the wells was gone to ensure all of the treatment had been ingested. Four treatments and a sterile water control were tested, consisting of a low (L) and high (H) host diversity for each of the *A. flavus* (fla) and *A. phoenicis* (pho) pathogens. For each passage (parasite generation), 12 larvae were treated per line, with six replicate lines per treatment (one per colony), giving 24 replicate lines in total. At the end of each 7 day passage, conidia were harvested from the cuticles of the conidiating cadavers from each line and conidia suspensions were prepared (as above) at the same concentration and used to inoculate another set of 12 larvae per line in the following passage. For the low host diversity lines, the parasites were passaged through larvae from the same colony in all passages. For high host diversity lines, the parasites were passaged through larvae from a different colony in each passage. Four passages were carried out in total.

5.3.2 *Post-selection assessment*

To assess the effect of the serial passages on the evolution of the fungal parasites, the larval mortality (parasite virulence) and conidiation (parasite fitness) rates of the derived lines were compared to that of the ancestral isolates. We used larvae from four of the six colonies which were used in the passage experiments (Am3, 6, 9 and 18). To assess the virulence and fitness of the low host diversity treatments, the derived lines were used to treat larvae from the same colony as the parasite had been passaged through. To assess the virulence and fitness of the high host diversity treatment the derived lines were used to inoculate larvae from the colony which had been used in the first passage for each line. In addition, both derived and ancestral isolates were tested against larvae from six novel colonies (Am11, 13, 17, 23, 33 and 36) to assess the virulence and fitness of the parasites in a genotypically different set of hosts to that which the parasite had experienced during the serial passages. A total of 24 derived lines were tested in this assessment experiment, with seven larvae per colony being inoculated with conidia from each derived line, and the same total number of larvae per colony (i.e. 48) being treated with each of the ancestral *A. flavus* and *A. phoenicis* isolates. The larvae were maintained and monitored for seven days post inoculation as described previously.

5.3.3 *Statistical analysis*

All statistical analyses were carried out using SPSS (PASW 18 Statistics) software package. The mortality and conidiation rates of larvae were analysed using the Generalized Estimating Equation function fitted to a negative binomial model with a log link function, with line entered as the subject variable, individual larva as the within-subjects repeated measure, and day of death or day of conidiation as the dependent variable. Treatment colony type (SPE or new), and colony nested within colony type, were included as factors in the model, as well as the treatment by colony type interaction. Pairwise comparisons of the survival and conidiation rates of the different treatments were subsequently carried out using Kaplain-Meier analyses with the Breslow statistic.

5.4 Results

5.4.1 *Virulence*

For *A. flavus*, there was a significant effect of treatment and of colony on the survival of larvae following inoculation with the derived and ancestral lines (Wald = 447.8, df = 2, $P < 0.001$; Wald = 28.7, df = 8, P = 0.001, respectively), and there was also a significant interaction between treatment and colony type (Wald = 62.8, df = 2, $P < 0.001$). The derived lines passaged through a low diversity of hosts were less virulent than the lines passaged through a high diversity of hosts, causing similar mortality to the control treatment (Fig. 13a). Both ancestral treatments had the greatest impact on the survival of larvae with all (SPE colonies = 100%) or nearly all (new colonies = 98.2%) larvae succumbing to infection. For *A. phoenicus*, there was a significant difference in survival between treatments (Wald = 5111.9, df = 2, $P < 0.001$) and this effect was similar for serial passage and new colony types (Wald = 0.02, df = 1, P = 0.88). There were clear differences between the derived and the ancestral treatments in their effect on the survival of larvae(Fig. 13b). With both ancestral treatments, nearly all larvae died, and all the derived treatments had significantly higher survival rates ($P < 0.001$ in all cases), although mortality was still higher than for the control treatments.

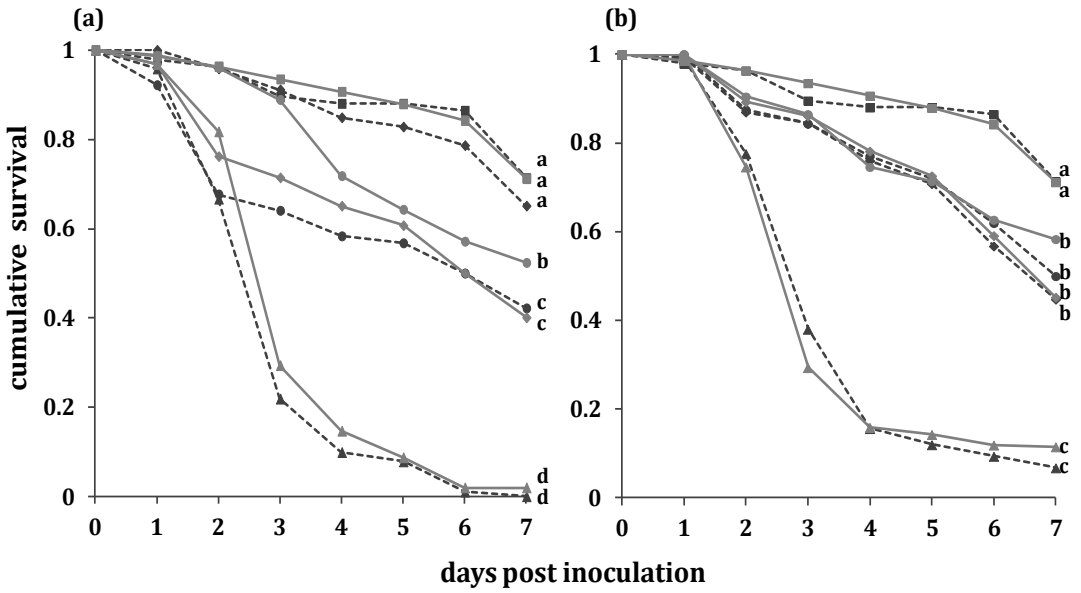

Figure 13: The survival rates over seven days following inoculation of larvae in the post-selection experiment. Each graph shows the different treatment groups for (a) *A. flavus* and (b) *A. phoenicis* parasites for larvae from either the same (black) or different (grey) colonies to those used during the serial passage experiment, and treated with either control suspension (squares), the ancestral isolate (triangles), or derived lines that had been passaged through either a low (diamonds) or high diversity of hosts (circles). The different letters denote significant differences between treatments for each parasite species based on Kaplain-Meier Breslow statistic.

Table 6: The results of the Generalised Estimated Equation analysis of larval mortality and the proportion of cadavers producing conidia observed during 7 days post inoculation in the post selection experiment. Day of death/conidiation were included as the dependent variables and treatment (high host diversity derived, low host diversity derived and ancestral), colony type (SPE and new colonies) and colony of origin and treatment colony interaction as independent variables.

Larval mortality				
Pathogen	Independent variables	Wald *Chi*-squared value	d.f.	*P*
A. flavus	Treatment	447.782	2	0.000
	Colony type	0.439	1	0.507
	Treatment x colony type	62.805	2	0.000
	Colony (colony type)	28.681	8	0.000
A. phoenicis	Treatment	5111.982	2	0.000
	Colony type	0.020	1	0.887
	Treatment x colony type	2.201	2	0.333
	Colony (colony type)	9.586	8	0.295
Conidial production				
A. flavus	Treatment	25.817	2	0.000
	Colony type	0.103	1	0.749
	Treatment x colony type	17.962	2	0.000
	Colony (colony type)	19.671	8	0.012
A. phoenicis	Treatment	147.945	2	0.000
	Colony type	0.001	1	0.981
	Treatment x colony type	0.428	2	0.807
	Colony (colony type)	22.275	8	0.004

5.4.2 *Fitness*

There was a significant effect of treatment on the conidiation rate of *A. flavus* infected larvae (Wald = 25.8, df = 2, P < 0.001), and this effect was dependent on colony type (Wald =17.9, df = 2, P < 0.001). All derived treatments had lower conidiation rates than the ancestral treatments (Fig. 14a), with the lowest being the derived lines that had been passaged through a low diversity of hosts, in which only one individual from the same colonies as used in the SPE producing conidia. There was a significant effect of treatment on the conidation rate of larvae which had been infected with *A. phoenicis* (Wald = 147.9, df = 2, P <0.001; Fig. 14b), which was not influenced by colony type (Wald = 0.001, df = 1, P = 0.981), although there was a main effect of colony (Wald =

22.3, df = 8, P < 0.01). All derived treatments had significantly lower conidiation rates than the ancestral treatments (P < 0.05 in all cases).

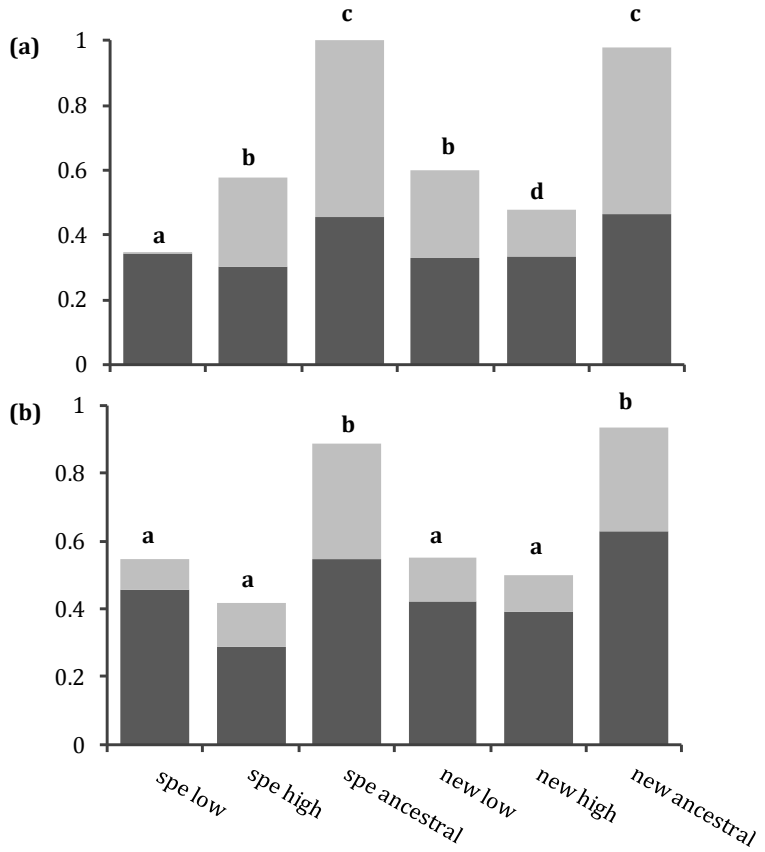

Figure 14: The proportion of larvae for each treatment which died (dark grey) or died and produced conidia (light grey) within seven days after inoculation with either, (a) *A. flavus* and (b) *A. phoenicis* opportunistic parasites in the post-selection experiment. Different letters denote significant differences between treatments within each parasite species based on Kaplain-Meier Breslow statistic (non-conidiating controls were excluded from the graph).

5.5 Discussion

Serial passage experiments are a well-established tool for investigating host-parasite coevolution. Using the *Aspergillus*/honey bee larvae study system we have elucidated some key features of this cryptic host-parasite association. We found no evidence of adaptation in either pathogen over the course of the serial passage experiment. After four passages *A. flavus* and *A. phoenicis* virulence and conidiation rate were reduced in both familiar and unfamiliar host populations and most lines became extinct. This

indicates that, contrary to most other SPEs with other parasites, the *Aspergillus* spp. did not evolve to maximise exploitation of the honey bee larvae.

The overall effect of the derived treatments was loss of virulence and fitness indicating a lack of adaptation. In SPEs, evolution occurs as a result of either mutation or recombination, or through changes in genotype frequencies by selection and genetic drift (Ebert 2000). When bottlenecks occur in very low doses they impact evolutionary dynamics by reducing the chances of beneficial mutations reaching fixation (Hefernan and Wahl 2002). In finite populations deleterious alleles accumulate in the absence of recombination, a process described as the Muller's ratchet model (Muller 1964, Felsenstein 1974). When the genetic diversity of parasite transfer doses are sufficiently high, selection is likely to be the cause of evolutionary changes and genetic drift can be ruled out. The strength of a bottleneck in our case is determined by the effective population size of the conidia of our *Aspergillus* spp. treatments. These were derived from asexually reproducing, clonal (F4) populations which had been serially sub-cultured on media in optimal conditions and are likely to possess high genetic similarity between variants. As well as the genetic structure of the ancestral population, pathogen fitness will also be determined by the transmission dose (Bergstrom *et al.* 1999, Novella *et al.* 1995, Clarke *et al.* 1993). Previous studies have shown that repeated sub-culture of clonal microbes under non-restrictive culture media conditions can maintain or increase genetic diversity (Scully and Bidochka 2006, Iguchi *et al.* 2002). Subsequent repeated transfer of low doses through the honey bee larva host is likely to generate a Muller's ratchet effect with the *A. flavus* treatments in particular. Genetic drift has been shown to dominate the outcomes of SPEs if the transfer dose is <10 parasites or there are a high number of passages but this will depend on the mutation rates of the parasite involved (Ebert 2000). After seven passages in the *G. mellonella* host, transmission doses of 500 *A. flavus* conidia lead to a significant decrease in genetic diversity which was accompanied by a decrease in virulence and protease production, a known virulence factor in aspergillosis in insects (Leger 1994, Scully and Bidochka 2006). The random nature associated with bottlenecks was also evident by the differences in genetic diversity and make-up between the two lineages studied (Scully and Bidochka 2006). It remains unclear if loss of genetic diversity is the sole explanation for the overall loss of virulence and fitness.

Contrary to our expectation, we found no evidence of increased host specificity in the derived parasite lines in the post-selection assessment. This would have been shown by reduced virulence of the derived lines in larvae from new colonies compared to larvae from the SPE colonies, in which the derived lines had evolved. No effect of

host diversity was observed on the virulence of the *A. phoenicis* derived treatments. Curiously, with the *A. flavus* treatments, transmission between genetically dissimilar hosts was more beneficial. The virulence of *A. flavus* low diversity treatment was significantly reduced and almost extinct in familiar hosts, whilst relatively higher in the unfamiliar host. This may suggest that there was a loss of alleles through genetic drift that would have been beneficial against resistant host genotypes in the low host diversity treatment and the remaining alleles conveyed traits that were still effective against more susceptible genotypes present in the new colony larvae. The hypothesised effect of host genetic diversity on parasite evolution is based on selection. The principle process which appears to have been acting in this SPE is Muller's ratchet, but it would nevertheless be possible that differences would still be seen between parasites passaged through low and high diversity hosts due to the combined effect of Muller's ratchet and selection. Indeed, this combination may well explain the results of a similar SPE in which the *Metarhizium* fungal entomopathogen lost fitness over nine serial passages, but lost it to a lesser extent when the genetic diversity of hosts was low (Hughes and Boomsma 2006). It is possible that the former effect may have been due to Muller's ratchet and the latter effect due to selection. In the current study, only four serial passages were completed and, while this was evidently ample for Muller's ratchet to have an effect, it may well have been insufficient for selection to add its effect. The negative effect of increased host genetic diversity on parasite virulence evolution has been reported in a number of systems including *Daphnia magna*-*Pasteuria ramosa* (Little *et al.* 2006) and in murine nematode and virus models (Dobson and Owen 1977, Kubinak *et al.* 2012). In social insects, *Crithidia bombi* passage through the bumble bee host resulted in decreased infection intensities in unrelated workers (Yourth and Schmidt-Hempel 2006). Further empirical evidence reveals considerable genetic variation for disease resistance in polyandrous social insects (Liersch and Schmid-Hempel 1998, Baer and Schmid-Hempel 1999, Schmid-Hempel and Crozier 1999, Hughes and Boomsma 2004) including honey bees (Tarpy 2003, Tarpy and Seeley 2006, Seeley and Tarpy 2007, Invernizzi *et al.* 2009) and support the role of genetic interactions in coevolutionary outcomes.

Our findings, which are contrary to standard virulence evolution predictions, should also be considered from the perspective of the lifestyle of opportunistic parasites. *Aspergillus* species are soil saprobes and the virulence factors associated with aspergillosis in honey bee larvae may originate from antagonistic interactions within the soil environment. Selection has favoured the expression of attributes e.g. mycotoxins and protease production, that protect and convey a survival advantage in

the alternative host. This 'coincidental selection' hypothesis (Brown *et al.* 2012), explains how virulence factors in opportunistic parasites may not be correlated with measures of parasite success in the new host due to being the by-product of adaptations unrelated to parasitism. In the context of our findings where mortality was the measurement of virulence, the derived *Aspergillus* lines did not show greater virulence and the virulence factors associated with honey bee larval infection may not be adapted primarily for the honey bee larval environment. Thus, under the SPE conditions where transmission constraints have been relaxed, virulence does not increase. This has similarly been termed as the 'ready-made virulence' hypothesis and has been proposed as the basis of virulence in other pathogenic environmental fungi e.g. *C. neoformans*, *Histoplasma capsulatum*, *Blastomyces dermatidis* and *Sporothrix schenckii* (Casadevall *et al.* 2003). Like *Aspergillus,* environmental isolates of *Cryptococcus neoformans* are pathogenic in the animal host and suggests virulence factors which confer a survival advantage in the environment also aid infection and disease (Casadevall 1998, Pasqualotto 2009). Intracellular growth of *C. neoformans* in the phagocytic soil amoeba by the formation of polysaccharide-containing vesicles, which accumulate throughout the host cells is similar to the fungal invasion observed in macrophages and provides some evidence for this (Steenbergen *et al.* 2001). As extreme generalists *Aspergillus* fungi are remarkable for their capacity for phenotypic plasticity in response to environmental stress which aids their survival and fitness (Raynor and Coates 1987, Splenecky 2009). Nutritional stress and high osmolarity have been found to induce conidiation through activation of the *brlAβ* gene (Skomne *et al.* 1995, Lee 1996). *Aspergillus* conidiation and mycotoxin biosynthesis can be affected by physical and nutritional environmental factors and are regulated by a G-protein/cAMP/PKA gene signalling pathway (Calvo *et al.* 2002). It is plausible that the honey bee larvae represent suboptimal hosts for *Aspergillus* spp. that in the first encounter stimulates a conidiation response and the reduced conidiation rate in subsequent passages may be the result of a switching off the signal transduction pathway in which the costs to the parasite could outweigh the benefits.

The lack of adaptation by the two *Aspergillus* parasites, and the remarkable reduction in fitness that they showed, demonstrates that the evolutionary outcomes for parasites may not always be as the standard paradigm would predict. In particular, it appears that the effect of Muller's ratchet can be substantial, and much greater than that of selection, and that opportunistic parasites may show quite different evolutionary dynamics from those shown by the specialist parasites that have normally been studied. In addition, to the implications of this for our understanding of host-

parasite evolution, the results also imply that *Aspergillus* infections in honey bees are likely to be acute events, characterised by transient dynamics rather than parasite adaptation, which may explain why these ubiquitous and potentially virulent fungi have been so rarely recorded as causes of high mortality in honey bee colonies.

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Chapter Six

Main Discussion

Microbial virulence and pathogenicity are emergent properties which rely on the characteristics of the environment, host and microbial populations or as a function of interactions between these (Casadevall *et al.* 2011). The expression of virulence factors traditionally distinguishes pathogens from non-pathogenic microbes. This may apply to certain disease causing microbes in the normal host, but a large number of other microbes can cause disease in the immunocompromised host (Casadevall and Pirofski 2001). The work presented in this thesis explored the ecological interactions and evolution of environmental fungi in an insect host using the *Aspergillus* spp.-*Apis mellifera* system. According to the damage-response framework *Aspergillus* species are classed as pathogens that can cause damage primarily at the extremes of both weak and strong immune responses (Casadevall and Pirofski 2003). They are responsible for significant pathogenesis in many immunocompromised organisms and their interaction with honey bees deserves much needed attention. This model system enables research into the susceptibility of the host at the individual, colony and population level under controlled conditions and assists in the unravelling of the complexity of this host-microbe interaction.

The exceptional nutritional flexibility and biodeterioration abilities of *Aspergillus* spp. has enabled their global colonisation of both natural and man-made substrates (Baker and Bennett 2008). Their ubiquity is also facilitated by the production of vast quantities of hydrophobic conidia that can be transported long distances across land and water (Hedayati *et al.* 2007). The high natural diversity and prevalences of *Aspergillus* spp. reported in previous microbiological studies is supported by the findings in this thesis which provide further evidence for their ubiquity and diversity in honey bee apiaries (González *et al.* 2005, Gilliam 1997, Gilliam *et al.* 1989, Gilliam and Prest 1987). It was also revealed how the morphologically indistinguishable species from section Nigri, *A. niger* and *A. phoenicis*, differed significantly in pathogenicity towards honey bee larvae, underscoring the importance of molecular identification of microbial pathogen populations. Cryptic species comprise closely related populations that have diverged recently and whose genetic differences may have significant consequences. Accurate and unambiguous pathogen identification is essential to establishing correct biological information upon which host-parasite systems can be studied, and can have important implications for disease management and biosecurity measures in general (Shivas and Cai 2012). True to their description by

Varga and Samson (2008) as 'Janus-faced organisms', the close relationship of *Aspergillus* spp. with honey bees in the colony may range from symbiotic to parasitic (de Souza *et al.* 2013, Gilliam 1997, Gilliam *et al.* 1988). Food stores provide a niche for beneficial and pathogenic microbes in the hive. Fungi, including *Aspergillus* spp., and bacteria produce enzymes, organic acids, lipids and antimicrobial substances that contribute to the conversion of pollen to bee bread (Gilliam 1997). Enzymatic activity on nectar with mechanically driven water evaporation by worker bees produces honey with <18% water content which inactivates microbial growth due to unfavourable osmotic conditions (Winston 1987). Honey, which is hygroscopic, can absorb moisture from the atmosphere and ripening honey consisting of >30% water will ferment suggesting that evaporative behaviour and water homeostasis is critical to the microbial balance of the hive (Anderson *et al.* 2011). Competitive interactions will also impact microbial community assemblages in the hive and within the hosts' themselves. Chapter 4 demonstrated that the strong competitive interactions between *Aspergillus* and *A. apis* resulted in greater within-host growth, but corresponding data revealed the suppression of sporulation illustrating the complexity of inter-pathogen interactions. This raises interesting questions about the fate of multiple infections under natural conditions, as the relationship between within-host competitive ability and transmission may not be straight forward, yet both aspects are crucial for the determination of virulence evolution (Alizon *et al.* 2013). The highly competitive ability of *Aspergillus* spp. was expressed through increased within host growth and conidiation rates, resulting in greater overall virulence and opportunities for transmission for the most competitive pathogen in dual infections. These findings also illustrate the value of measuring multiple parameters towards building a more comprehensive picture when investigating infection patterns in concomitant infection experiments.

Notwithstanding the ubiquity and pathogenicity of *Aspergillus* described in this thesis, reports of stonebrood epidemics appear to be rare (Evans and Schwarz 2011). The findings in Chapter 2 demonstrate the pathogenic potential of fungal species to both adults and larvae sourced from colonies asymptomatic for stonebrood disease. Thus, infections possibly either go unnoticed, or colonies have adaptations which prevent disease outbreaks such that only immunocompromised colonies are open to infection. This thesis highlights two possible explanations for the apparent low prevalences of aspergillosis in bees. *Aspergillus fumigatus*, the most prevalent *Aspergillus* species and the second most common species implicated in stonebrood disease (Gilliam and Vanderberg 1988), was found to be avirulent under standard

experimental conditions. Diet manipulation revealed an interaction between nutrition and disease susceptibility that suggest predisposing environmental conditions which compromise hosts' immune defences may prompt the occurrence of natural infections of *A. fumigatus*. The interaction between nutrition and resilience-resistance to parasite challenge has been well established in a number of ruminant-helminth systems as part of integrative approaches to parasite management (Coop and Kryiazakis 1999, Van Houtert and Sykes 1996, Wallace *et al.* 1995). Resistance is the ability of the host to limit the establishment, growth and fitness of the parasite population, and resilience is the hosts' ability to maintain productivity under parasitic challenge (Albers *et al.* 1987). Disease resistance is costly, therefore there are trade-offs against other life history parameters in the host, such as reproduction and longevity. Different types of pathogens can trigger different immune response pathways in the insect host (Brennan and Anderson 2004). Advances in knowledge at the transcription level indicates that the interaction between immune function and nutrition is mediated by nutrient signalling pathways that monitor specific nutrients and metabolites rather than energy status alone (Ponton *et al.* 2012, Duffey and Stout 1996). This suggests that the role of nutritional diversity in healthy immune function is significant, for which the findings in this thesis provide further support. Following serial passage of experimental, pathogenic parasite strains high extinction rates were observed. It was concluded that naturally occurring *Aspergillus* spp. infections are therefore more likely to be acute events rather than epidemics, which may in part explain why *Aspergillus* spp. epidemics in honey bee colonies seem to be uncommon. In asexual pathogens such as *Aspergillus* spp., deleterious alleles occur with each generation, increasing the likelihood of extinction events due to Muller's ratchet and resulting in loss of virulence and fitness (Muller 1964). Under field conditions, parasite population sizes although clonal, are more likely to be aggregated rather than the finite, and fixed transmission doses applied in our experimental setting may only allow populations of adequate size to persist (Novella *et al.* 1995). The discovery of sexual teleomorph phases in a number of *Aspergillus* species suggests that recombination may contribute towards the alleviation of Muller's ratchet effects, but recombination events in *Aspergillus* under natural conditions are thought to be rare (Horn *et al.* 2009, O'Gorman *et al.* 2009, Bidochka and Koning 2001).

Most theoretical studies on virulence evolution assume closed systems consisting of single, obligate parasite infections in one host species (Alizon *et al.* 2009, Anderson and May 1982). Yet only a minority of pathogens are specialists in the sense that they exploit a single host species, and most parasites fail to meet this assumption

being either generalists or opportunistic parasites (Woolhouse *et al.* 2001). Opportunistic parasites are often normally host symbionts or environmental microbes that cause disease either following a perturbation in the host or in immunocompromised non-primary hosts. Unlike obligate pathogens where standard predictions of optimal virulence evolution should apply, multi-environment opportunists are likely to experience non-optimal virulence in a given host (Brown *et al.* 2012). Pathogenicity of obligate parasites is maintained by natural selection and will increase or decrease as an evolutionary response to environmental conditions or transmission opportunity in the host population (Dieckmann *et al.* 2002). Virulence of opportunistic parasites, however, will not be favoured by natural selection as parasite mediated virulence is either coincidental or the product of short-sighted evolution (Levin 1996). Assuming soil is the primary site for virulence factor adaptations in *Aspergillus* (Klich 2002), selection will have generated pre-adaptations for virulent exploitation in the honey bee. If a positive correlation exists between growth in both environments, then virulence factors selected for the soil environment will incidentally confer advantages for growth and transmission in the host. For example, the ability of *A. flavus* conidia to germinate despite phagocytosis by hemocytes (St. Leger *et al.* 2000) may originate as a defence adaptation against predatory soil protists (Steenbergen *et al.* 2001). Alternatively, there may be a negative correlation between growth in each environment where adaptation is to the primary environment and the focal host represents a dead end. If encounters are frequent, specialisation or phenotypic plasticity can be expressed if sufficient genetic variation exists (Brown 2012). Adaptive phenotypic plasticity is the production of a phenotype that lends itself to the optimal value favoured by selection in the new environment and initiates a critical step in adaptation to new environments (Galambor *et al.* 2007, Pigliucci 2001). Therefore if encounters are frequent enough and enough genetic variation exists, opportunities for host specialization may arise.

All studies in this thesis were conducted under laboratory conditions in the absence of colony level social immunity. As discussed in Chapter 2, nest hygiene and other social immunity behaviours are likely to form a key component of colony-level resistance against stonebrood. Under natural hive conditions in the presence of the full microbial compliment, interactions will also occur between symbionts, pathogens and honey bee immune defences (Anderson *et al.* 2011b). Chapter 4 demonstrates the complexity of interspecific within-host interactions when considering infection outcomes. Growing evidence suggests that bacterial endosymbionts may form an integral part of honey bee disease resistance (Vasquez *et al.* 2012, Evans and

Armstrong 2006). A number of *Bacillus* spp. commonly associated with honey bees have been shown to inhibit growth *in vitro* of the American foulbrood (AFB) bacterium, *Paenibacillus larvae* (Alippi and Reynaldi 2006, Evans and Armstrong 2005, 2006). *Bacillus* and *Paenibacillus* strains have also shown inhibitory effects against the fungal pathogen *A. apis*, and one study found that *Bacillus subtilis* strains isolated from adult bee guts and honey showed inhibitory effects against both *A. apis* and AFB (Sabaté *et al.* 2009, Reynaldi *et al.* 2004, Alipi and Reynaldi 2006). Perturbations in the relative proportions of the phyla constituting the microbial biome may be linked to specific diseases and is termed dysbiosis (Sartor 2008). Metagenomics work confirms a high diversity of endosymbionts in honey bees (Cox-Foster *et al.* 2007). The pathogenic potential of many of these microbes is largely unknown, so future studies including sequence information may provide a broader context for interpreting the ecology of honey bee microbiota.

In the context of the origin of emerging diseases, the endemic pathogen hypothesis suggests that infectious disease emergence may result from the immunological, behavioural or ecological changes in the host or parasite which changes a harmless association into a pathogenic relationship (Carey 1993). Various mechanisms can create a more favourable environment for pathogens and/or stressors in the environment may increase the susceptibility of immunosuppressed hosts. Recent years have seen unprecedented threats to biodiversity and food security due to mycoses (Pennisi 2010, Blehert *et al.* 2009, Fisher *et al.* 2009). Of all the pathogen groups, fungi have the broadest spectrum of host ranges (Fisher 2012). Parasite mediated competition can occur between species that vary in susceptibility (Holdich *et al.* 2009). Few fungi exhibit a truly global distribution like *Aspergillus* species (Ramirez-Camejo *et al.* 2012, Pringle *et al.* 2005). *Aspergillus fumigatus* poses a significant emerging disease threat in human immunocompromised patients whilst *A. sydowii* is responsible for severe declines in gorgonian corals, the dominant reef taxa in the Caribbean (Ader *et al.* 2005, Geiser *et al.* 1998). Population patterns of genetic variation in pathogenic *Aspergillus* species are highly similar such that disease-causing isolates are not genetically distinct from environmental isolates (Rypien 2012, Ramirez-Camejo *et al.* 2012, Debeaupuis *et al.* 1997). This explains the difficulties found in identifying virulence factors involved in aspergillosis and implies that any isolate is capable of causing disease if suitable conditions prevail (Bouchara *et al.* 1995).

In conclusion, the evidence presented in this thesis illustrates the complexity of interactions between *Aspergillus* spp. and *Apis mellifera*, and highlights the significant

influence these common environmental microbes may have on honey bee populations. Important factors which contributed to variation in the observed virulence include environmental effects such as dose-dependence and nutrition, genetic variation, interactions with other microbes and their origin. This research is important for understanding host-microbial interactions in this important group of emerging fungal parasites which have proven ability to cause epidemics with major consequences. Historical reports describing large-scale colony collapses and the subsequent reproduction of symptoms using an *Aspergilllus flavus* isolate suggest that stonebrood epidemics pose a conceivable threat requiring more vigorous investigation (Underwood and vanEngelsdorp 2007). There is an overarching need for further detailed research into social immunity, particularly endosymbiont ecology and antimicrobial prophylactic behaviours which may constitute the primary defence against opportunistic pathogens. The pervasiveness of *Aspergillus* and other opportunistic environmental microbes in the hive suggests an intimate association with interactions which range from beneficial to antagonistic. These interactions are possibly major drivers in the evolution of social immunity and behaviour, perhaps even more so than specialised parasites which may or may not be less prevalent. Expanding our knowledge of host-pathogen dynamics through explorations of social immunity, microbial identification, and experimental tests of host-microbial-environment interactions may facilitate explanations of current patterns of colony declines and allow predictions of future disease dynamics.

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