

PREFACE

Insects are the most species-rich group of eukaryotes, and this diversity grants these animals profound power to influence ecosystems. The great research interest in insects arises from their ability to negatively impact human society: as vectors for disease in humans and our livestock and as destroyers of crops and stored products. It is estimated that insects destroy approximately 18% of the world annual crop production, and vector borne diseases kill millions every year. Thus, the diseases, pathogens, and immune responses of insects have been a long-standing research interest. Early interest grew mostly from economic concerns. For example, *Beauveria bassiana* was described by Agostino Bassi in 1835 as the cause of the devastating muscardine disease of silkworm, and it was instrumental in his development of the germ theory of disease (Steinhaus, 1956). In 1880, the pioneer immunologist Elie Metchnikoff was among the first to propose practical methods of microbial biological control of an insect crop pest, initiating trials of the fungus *Metarhizium anisopliae* against grain beetles (Lord, 2005; Mechnikoff, 1879, Fig. 1).

Such applied interest continues today, as we all still have a stake in either lengthening the life expectancy of useful insects or shortening the life expectancy of pestiferous ones. Since fungi are the commonest disease-causing agents in insects and populations of most insects are regulated by density-dependent factors involving pathogens and predators, we need to understand and be able to manipulate the interactions of pest insects with fungi and their other natural enemies in order to feed the world and prevent disease. Most research on fungal-insect pathogens has continued to focus on hypocrealean Ascomycetes from the genera *Beauveria* and *Metarhizium* (family Cordycipitaceae and Clavicipitaceae, respectively). These genera are tractable model species, are readily cultivatable, and have a particularly wide host range allowing them to be applied en masse against vectors of human disease and multifarious agricultural pests. Numerous registered mycoinsecticide formulations are based on *Beauveria* and *Metarhizium* spp. They also have a worldwide distribution with variants adapted from the arctic to the tropics and colonizing an impressive array of environments including forests, savannahs, swamps, coastal zones, and deserts.

The majority of chapters in this volume focus on *Metarhizium* and *Beauveria*, reflecting the preponderance of published data that has utilized

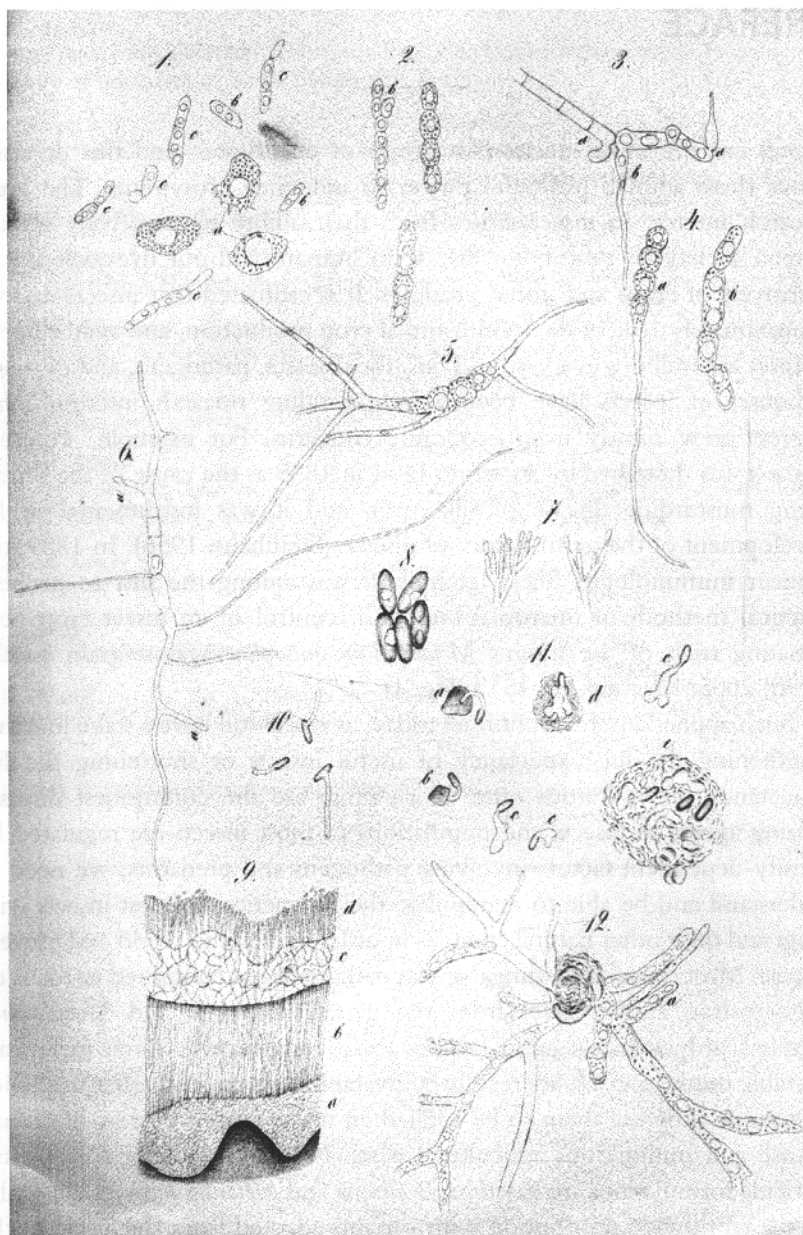


Figure 1 (1) Blood cells and fungal conidia from the blood of dying wheat chafer larvae. Magnified 550 times (Eyepiece 3 + System 9 alone picture). (2) Fungal conidia from the blood of living and apparently healthy larvae. Magnified 860 times (4 + 9). (3) Pieces of subcutaneous mycelial conidiophores formed on fresh cadavers. Magnified

these genera as models for entomopathogenicity. Yet, as clarified by Araújo and Hughes in Chapter 1, most major fungal taxonomic groups have members pathogenic to insects, so fungal-insect pathogens represent lifestyle adaptations that have likely evolved numerous times. Araújo and Hughes emphasize that, despite their ecological importance and potential applications, the entomopathogenic fungi are woefully understudied, especially regarding their biodiversity, which likely harbors one of the largest reservoirs of undocumented fungal species. Their chapter provides a broad overview of host-pathogen relationships by focusing on the impressive diversity of morphologies, ecologies, and interactions between insects and fungi. As host and pathogen are tied together in a very intimate relationship, the presented work strongly argues for empirical studies investigating both sides of the relationship in a much broader range of fungal-insect interactions than has hitherto been conducted. The authors reveal significant differences in host range and pathogenic strategies between the major groups of fungal pathogens. The basal groups, such as aquatic chytrids, infect mostly Diptera, while Microsporidia and Entomophthoromycota infect a wide range of hosts. Basidiomycota infects mostly Hemiptera, while Ascomycota, the most species-rich group, infects a vast number of insect groups. In terms of pathogenic strategies, the ascomycetes, for example, are all hemibiotrophic, switching from a biotrophic phase (parasitism) to a necrotrophic phase, growing on the dead host.

In contrast, the Entomophthoromycota are characteristically biotrophics with no somatic growth after death.

550 times. (4) Conidia from within the same larvae on the day after death. Magnified 550 times. (5) Overgrown conidia on the third day emerging from the larval cadaver in a humid chamber. Magnified 400 times (3 + 8). (6) Conidia and hyphae with two forming conidia (a) in a humidified chamber. Magnified 400 times. (7) Candelabra-shaped stalks with sterigmata forming conidia cultured in a humid chamber. Magnified 400 times. (8) Mature *Entomophthora anisopliae* spores. Magnified 1340 times (Eyepiece 3 + 14 System picture). (9) Cross section of larva covered with fungus. a-conidial layer, b-straight hyphal layer, c-felted layer, d-candelabra layer with sterigmata and conidia. Magnified 90 times (3 + 4). (10) Three conidia grown in sugar water. Magnified 550 times. (11) Germinating spores on larval cuticle. a, b- Spore germ tubes producing yellow spots. c- Bursiform germ linings on the cuticle. d- Further stage of germination. e- Spot on larval cuticle with ungerminated and germinating conidia. Magnified 550 times. (12) Spots on the cuticle with sprouts like rays of radiating hyphae; with notable formation of conidia. Magnified 400 times. This figure is from Elie Metchnikoff's publication discovering *Metarhizium anisopliae* in Odessa in 1879; the legend is a translation of his original Russian legend for the figure.

In their chapter (Chapter 2), De Fine Licht et al. focus on the Entomophthoromycota, as they are a large clade and particularly important natural regulators of insect populations. They relate how many genera in this group contain obligate insect pathogenic species with narrow host ranges, capable of producing epizootics in natural insect populations. The Entomophthoromycota are not easy to mass produce, and most studies have focused on the ecology of these organisms and their role in epizootics. The authors summarize and review the genetic information, albeit limited, which currently exists for Entomophthoromycota in order to provide a foundation that new genomic and transcriptomic information can build upon. Several genome-sequencing projects have been initiated among Entomophthoromycota species with diverse life histories and these will undoubtedly provide new insights into the biology of species and will aid phylogenetic analysis of this basal group within the fungal kingdom.

It is clear that understanding the genetics, biology, and ecology of entomopathogenic fungi is entering a new era. Wang et al. in Chapter 3 show how new insights into the ecological roles that these fungi occupy have been obtained by taking a genomic perspective on the evolution of insect pathogenicity and host range usage. They point out that from a phylogenetic and evolutionary perspective, *Beauveria* and *Metarhizium* are closely related to plant-associated fungi, and they retain many genes you would expect in a plant colonizer (or pathogen), consistent with their continuing abilities to form stable interactions with plants. The genus *Metarhizium* contains species with wide insect host ranges, for example, *Metarhizium robertsii*, and species such as *Metarhizium album*, *Metarhizium acridum* and *Metarhizium majus* show specificity for certain hemipterans, locusts, and beetles, respectively. Wang et al. dissect this observation by discussing the evolution of different components of infection processes. Looking at recognition, signal transduction, and effectors, they show how these differ in their evolutionary trajectories across broad host range and narrow host range species making use of nine whole *Metarhizium* genomes, as well as genomes from broad host range *B. bassiana* and narrow host range *Cordyceps militaris* and *Ophiocordyceps sinensis*. Interestingly, the components of pathogenicity differ not only in how fast they evolve but also in the way in which they change. In specialists, virulence factors show a high degree of amino acid divergence, whereas in generalists, there has been massive expansion of certain gene families. The authors show how the evolution and mechanism of pathogenic strategies and host range is closely linked with the diverse reproductive modes shown by entomopathogenic fungi that often

determine the rates and patterns of genome evolution. These studies emphasize how genomic resources have helped make entomopathogenic fungi ideal model systems for answering basic questions in parasitology, entomology, and speciation.

Despite their long history of use and thousands of publications and patents, the important role of *Beauveria* and *Metarhizium* spp. in forming stable mutualistic interactions with plants, either as plant root colonizers or endophytes, has only become apparent in the last 20 years. Moonjely et al. (Chapter 4) emphasize the need to understand the multiple roles of these fungi as insect pathogens and plant growth promoters if we are to responsibly exploit them as biocontrol agents. The authors challenge the view that interkingdom host jumping occurred from ancestral fungi adapted to plants switching to arthropods and then back to plants. They suggest that many of these fungi evolved to infect insects while maintaining their mutualistic endosymbiosis with plants. They hypothesize that the driving force behind this evolution was the host plant demanding reciprocal nutrient exchange from the fungus in exchange for access to plant carbohydrates in the rhizosphere. Insect pathogenic fungi would be able to provide the plant with a source of nitrogen, or other growth limiting nutrients, derived from insect parasitism. The authors also review the other benefits that plants derive from *Beauveria* and *Metarhizium* endophytes including increases in plant biomass and productivity, alleviation of abiotic stresses (eg, drought, salinity, temperature fluctuations), and improved resistance to biotic stress (eg, herbivory, fungal disease).

Zhao et al. in Chapter 5 argue that as a natural control agent the "slow kill" characteristic of many entomopathogenic fungi allows them to replicate to large numbers, which is adaptive for the pathogen, but a severe limitation for application in modern agriculture or when attempting to control a disease vector. They describe how genetic engineering has proven an efficient tool to improve the efficacy of biofertilizers and mycoinsecticides by improving their tolerance to environmental stresses and increasing their virulence. The addition and expression of genes in ascomycete pathogens has become routine, and the authors describe several successful approaches including (1) inserting one or more foreign genes into the fungal genome whose product alters the pathogen's stress tolerance, alters the physiology of the target host insect or is toxic toward the target host and (2) altering expression of an endogenous gene so as to increase virulence or stress tolerance. Up until now, most "useful" genes for transfer have come from pathogens themselves, host insects, or from arthropods such as spiders that produce insect-specific toxins.

Each of these sources provides a vast array of biologically active metabolites. In addition, the authors describe how some methods appear to mitigate safety concerns regarding genetically engineered entomopathogenic fungi, and they discuss methods that have potential to ensure the success and safety of genetically modified strains.

Ortiz-Urquiza and Keyhani, in Chapter 6, show how research on *B. bassiana* has expanded greatly in the last 5 years due to a confluence of robust genetic tools and genomic resources for the fungus, and the recognition of its engagement in ecological interactions with plants and other organisms. Studies on *B. bassiana* currently range from physiological analyses related to its insect biological control potential to addressing fundamental questions regarding the molecular mechanisms of development, virulence, and fungal plant interactions. This work has established *B. bassiana* as an emerging model system, distinct from others in terms of the breadth of questions that can be addressed. The authors discuss generic ways of defining the concept of and genes involved in virulence and review examples of potential virulence determinants based on signaling molecules, effector genes encoding enzymes and toxins, and secondary metabolites. They convincingly show that *B. bassiana* is formidably well armed as an insect pathogen; although, the plethora of insecticidal gene products and their potential redundancy makes it hard to pinpoint genes crucial for pathogenicity that do not also have pleiotropic roles in fungal metabolism. In consequence, the underlying mechanisms of highly virulent *B. bassiana* strains remain elusive, but the functional outcomes are clearly worth studying and likely to lead to insights widely applicable to organisms, as well as those unique to this group of fungi.

The success of insect pathogenic fungi is in the context of insects having robust resistance to infection. The current great interest in insect immunity reflects applied consideration of their intrinsic importance, but, more significantly, insects are excellent models for studying host-pathogen interactions and immune responses. As detailed by Lu and St. Leger (Chapter 7), identification of host immune reactions or immune-related molecules in the best understood insect immune system, that of *Drosophila*, has already greatly benefited human health by assisting strategies for controlling human pathogens. The authors introduce early studies utilizing opportunistic human pathogens, as well as *Beauveria* and *Metarhizium*, which focused on molecules involved in sensing and signaling. They then describe how more recent studies have started to investigate immunity using a more holistic approach to provide a better understanding of behavioral avoidance of

pathogens and how insect immunity integrates into an insect's general physiology to determine whether microbial infection leads to tolerance, resolution, or death. A key issue considered by the authors is that in order to understand the evolution of the insect immune system, with concomitant host-pathogen coevolution and trade-offs, we need to be able to quantify the strength of selection, and very little information is available on natural pathogens and their infection rates in wild populations of *Drosophila*. Similarly, the evolution of pathogen life histories in response to host genotypic variation is poorly understood. These challenges are being met in part by comparative genomics of insect immune defenses of different *Drosophila* species, mosquitoes, and social insects which are beginning to disentangle common themes from specific components of immunity. Likewise, the sequencing of entomopathogens is also beginning to play an important role in the development of insect-fungal model systems for studying host defense mechanisms. However, the authors point to the current focus on generalist pathogens, such as *Beauveria* and *Metarhizium*, as hindering any strong generalizations across the notoriously diverse array of host and parasite life histories. New data across a broad suite of study systems will be necessary to unravel the contributions of different host and pathogen life history traits to generating patterns of coevolution. This message resonates with the one by Araújo and Hughes (Chapter 1).

It also resonates with a review on social immunity in ants by Loreto and Hughes (Chapter 8). It is generally assumed that social life can lead to the rapid spread of infectious diseases and outbreaks. Social insects have limited genetic diversity in their crowded colonies, which should make them particularly prone to disease, as well as a potential model for human groups and their livestock. In practice, disease outbreaks are rare in ants and the expression of collective behaviors is invoked to explain the absence of epidemics in natural populations. For the sake of convenience, most studies on ants have been performed in the laboratory using *B. bassiana* and *Metarhizium* spp. to elicit and study social immunity. The authors discuss the limitations of the protocols in the published studies and argue that the use of generalist pathogens as a tool to understand infectious diseases in ants will better serve us when implemented in natural conditions. The generally held view that an ant nest is a "fortress," well defended against diseases, seems to be corroborated by the absence of outbreaks of *Beauveria* or *Metarhizium* in ant societies. However, as other fungi are commonly found infecting ants, the authors question if the absence of *Beauveria* or *Metarhizium* is, in fact, due to the efficient defenses. Additional studies on parasites known to be

specialized on ants are encouraged as they would significantly complement our current knowledge. Along these lines, Lu and St. Leger (Chapter 7) discuss work with honey bee social immunity that exploits a natural honey bee pathogen, the Chalkbrood fungus.

The chapter by Butt et al. (Chapter 9) considers important questions relevant to the end user of entomopathogenic fungal technology including strain improvement, resistance monitoring, and risk assessment. As the authors note, fungal pathogens currently have a small share in the insect control market due to low virulence (slow kill and high inoculum load) compared to the chemical insecticides with which they compete and due to inconsistencies in their performance; however, as part of an integrated pest management system, entomopathogenic fungi have tremendous potential to impact human health and agriculture. Describing the route of a typical infection, they review the stratagems and tools employed by both the host and pathogen to endure stress, accumulate resources, and ultimately survive. They put these in the context of a microevolutionary arms race between host and pathogen that may work as a double-edged sword for practical purposes of pest control. For example, the known repellency of volatile organic compounds produced by some entomopathogens may reduce the risk of infection to natural predators and other nontarget hosts but interfere with "lure and kill" strategies for pest control.

Both *Beauveria* and *Metarhizium* are well known for producing a large array of biologically active secondary metabolites and secreted metabolites involved in pathogenesis and virulence that have potential or realized industrial, pharmaceutical, and agricultural uses. In Chapter 6, Ortiz-Urquiza and Keyhani include an overview of *Beauveria* secondary metabolites and their potential function in immunosuppression and as antimicrobial reagents. Donzelli and Krassnoff (Chapter 10) provide a comprehensive survey of the profusion of *Metarhizium* secondary metabolites. Notwithstanding the many known *Metarhizium* chemistries, the authors illustrate how the newly available *Metarhizium* genome sequences have revealed an abundance of biosynthetic pathways and a capacity for producing secondary metabolites that far exceeds the known chemistry. Some of the pathways characterized in their survey correspond to known products (eg, serinocyclins, destruxins), some are likely to be responsible for known chemistries (eg, cytochalasins, ovalicin), others are tantalizingly similar to pathways identified in other fungi (ergot, diketopiperazine, resorcylic acid lactones), but candidate products are still unknown from *Metarhizium*, and still others defy attempts to predict the type of molecule they produce. The authors' very reasonable conclusion is that our

understanding of the services that secondary metabolites perform for the producing fungi, especially in interactions with other microbes, insect hosts, and plants is rudimentary at best.

With entomopathogenic fungi producing such complex chemistries, it is no surprise that certain insects have learned to recognize pathogen volatiles and avoid them. However, the hosts are not always the ones pulling the strings when it comes to their behavior; there are parasites that have evolved strategies to seize control of host behavior. In their chapter Hughes and colleagues (Chapter 11) discuss the role of behavior in driving disease in insect societies. With a nod to the chapter by Araújo and Hughes on the diversity of entomopathogens generally, they examine the diverse and stereotyped behaviors of arthropods (including familiar spiders and insects) under fungal entomopathogen manipulation using Niko Tinbergen's four-question paradigm: function, phylogeny, causation, and ontogeny. To explore the proximate causes of behavior manipulation from both host and parasite perspectives, they discuss case studies of a particularly dramatic example: the *Ophiocordyceps* fungi that produce the zombie ant phenomenon where ants are instructed to bite into leaves before dying in a "death grip." Other species induce less severe effects and some related pathogens of beetles do not manipulate at all. Hughes et al. describe how the mechanisms of behavioral control can be unraveled by comparing manipulators and non-manipulators, using tools such as phylogenomics, transcriptomics, and metabolomics. They also discuss how manipulation has evolved because ant societies establish complex conditions for parasite evolution and transmission. In this context, the usefulness of genetics algorithms to understand the evolution of complex behavior modification *in silico* is explored, especially where traditional study systems have not yet been established. The authors rightly describe the study of parasite manipulation of complex host behavior as entering "a golden age of discovery."

The range of articles in this volume covers many interrelated aspects of the genetics, biology, and ecology of these fascinating and useful insect-killing fungi. Although the individual chapters focus on specific issues, the reader should appreciate that each issue is multidimensional, being influenced by a milieu of fungal, insect, and environmental factors. It is the hope of the editors that the chapters untangle the complexity and illuminate the interactions between insects and fungi in a manner that shows the wide-ranging scope of the field and numerous applications of the knowledge it generates. Contained herein is evidence of the progress our field has made since Bassi first cautiously transferred a fungal hypha from a silkworm

cadaver to a healthy insect, but this volume aims to serve as a foundation for future scientists hoping to shine light on our burgeoning field.

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