From So Simple a Beginning: The Evolution of Behavioral Manipulation by Fungi

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Abstract

Parasites can manipulate the behavior of their hosts in ways that increase either their direct fitness or transmission to new hosts. The Kingdom Fungi have evolved a diverse array of strategies to manipulate arthropod behavior resulting in some of the most complex and impressive examples of behavioral manipulation by parasites. Here we provide an overview of these different interactions and discuss them from an evolutionary perspective. We discuss parasite manipulation within the context of Niko Tinbergen's four questions (function, phylogeny, causation, and ontogeny) before detailing the proximate mechanisms by which fungi control arthropod behavior and the evolutionary pathways to such adaptations. We focus on some systems for which we have recently acquired new knowledge (such as the zombie ant fungus, *Ophiocordyceps unilateralis s.l.*), but a major goal is also to highlight how many interesting examples remain to be discovered and investigated. With this in mind, we also discuss likely examples of manipulated spiders that are largely unexplored ("zombie spiders"). Armed with advanced tools in evolutionary biology (from serial block face SEM to RNAseq) we can discover how the fungi, a group of microbes capable of coordinated activity, have evolved the ability to direct animal behavior. In short, we have the ability to understand how the organism without the brain controls the one with the brain. We hope such a goal, coupled with the knowledge that many diverse examples of control exist, will inspire other organismal biologists to study the complex adaptations that have arisen from "so simple a beginning."

1. INTRODUCTION

Fungi have an intimate association with animal life on planet Earth. Life first arose in the sea and subsequently colonized the land. The most diverse groups of animals to have evolved from "so simple a beginning" (Darwin, 1859) are the insects that today have almost one million described species, while spiders are also among the most successful of terrestrial organisms, with over 42,000 known species. As the arthropods were emerging to become the dominant animals in all terrestrial ecosystems, another dominant group of eukaryotes, the Fungi, were also colonizing the land. These two phylogenetically and ecologically diverse taxa (Phylum Arthropoda and Kingdom Fungi) have, over the last 400 million years, evolved a wide array of intimate interactions with one another (Vega & Blackwell, 2005). These interactions run the gamut and include mutualistic endosymbiosis (Suh, Noda, & Blackwell, 2001); fungi as obligate food sources, such as those found in fungus-gardening ants (Mueller, Gerardo, Aanen, Six, & Schultz, 2005); sexually- and behaviorally transmitted parasites, such as Laboulbeniales (DeKesel, 1996); and pathogens that have pronounced effects on host populations (Evans, 1974). Entomopathogens occur in all the major phyla of the Kingdom Fungi and the exploitation of the host body for food has evolved independently and repeatedly (Araújo & Hughes, 2016). Despite this knowledge, fungal—arthropod associations remain an understudied area of fungal biodiversity and likely harbor one of the largest reservoirs of undocumented taxonomic, functional, and genetic diversity within the Fungi (Vega & Blackwell, 2005).

Insects (Class Insecta) belong to the Phylum Athropoda, which includes the familiar spiders (Order Araneae) and mites and ticks (subclass Acari); both taxa are also known to be hosts of fungal parasites (Evans & Samson, 1987; Evans, 2013). The spiders are notable because there are records of very large die off events driven by fungal pathogens (Evans, 2013; Samson & Evans, 1973), which are similar to the graveyard events that occur when fungi infect ants (Pontoppidan, Himaman, Hywel-Jones, Boomsma, & Hughes, 2009). This high occurrence coupled with the observation that many spiders die on the underside of leaves, which is likewise observed in ants and other insects, suggests that as part of the life cycle, fungi that infect spiders may also manipulate host behavior to increase transmission to new hosts.

The observation that, as a Kingdom, Fungi have many parasitic taxa (at the specific, generic, or familial level), does not distinguish them from other major groups. Parasitism is a very common mode of life that has evolved repeatedly and probably more times than predation as a life history strategy (Poulin & Morand, 2000, 2005). What is notable is the apparently high frequency of parasitic fungi that have evolved not just to infect animals, but also to adaptively manipulate animal behavior in ways that increase the fitness of the fungus. In this chapter, we explore the diversity and origins of such behavioral manipulation in insects and spiders before considering the mechanisms by which fungal pathogens control arthropod nervous systems.

2. WHAT IS BEHAVIORAL MANIPULATION?

One of the most distinctive features of animals is their ability to express complex behaviors. Honeybees can use a dance language to signal the location of high quality flowers, wolf packs can act in concert to chase down large prey that an individual pack member could not handle alone, and in peafowl, the peacocks display their genetic quality by parading elaborate tails to potential mates (Alcock, 1993). In recent years, behavioral ecologists, researchers who study the evolution of behaviors within an ecological context, have begun searching for patterns of behavior peculiar to animals that are infected by parasites (Moore, 2002). In many cases, the behavior observed is a general sickness that is the consequence of pathogen growth and development within the animal, and the associated change in either the immune system of the host or its general physiology as it reacts to the stress of parasitism. But in some host–parasite associations, the parasites have gone further than sequestering resources and have evolved the ability to adaptively alter the behavior of the animal in which they live.

It turns out that parasites can be the reason for wholly novel behaviors in animals. Such behaviors can be as complex and novel as the waggle dance of the honeybee (Hughes, 2014). The purpose of such parasite-mediated change of animal behavior is to use the animal as vehicle for parasite genes that are transmitted to either a new host or a new habitat. In all cases, the behaviors are those that the animals would not normally express because such behaviors are costly, and oftentimes fatal, to the animal. Some prominent examples of such behavioral manipulation are *Dicrocoelium dendriticum* (brainworms) that induce ants to bite into leaves to reach the guts of ruminants (Moore, 2002); hairworms causing crickets to jump into water to achieve parasite mating (Thomas et al., 2002); or *Toxoplasma* changing the behavior of rats to induce a fatal feline attraction for the parasite so it reaches its definitive host where it reproduces (Berdoy, Webster, & Macdonald, 2000; Webster, 2001). Such examples of parasites affecting the behavior and morphology of hosts in ways that increase transmission have come to be known as parasite-extended phenotypes (Dawkins, 1982, 1990, 2004, 2012). Here, natural selection has shaped parasite genomes to control host phenotypes and multiple lines of evidence are emerging to illustrate the mechanisms by which parasites achieve this end (Adamo, 2012; Adamo & Webster, 2013; de Bekker, Merrow, & Hughes, 2014; Biron & Loxdale, 2013; , 2013; Lefevre et al., 2009; Van Houte, Ros, & Oers, 2013).

Although parasitism as a life history trait is common, it is not true that behavioral manipulation of animal behavior is also common. It is difficult to estimate what percent of all parasites (in any taxa) have evolved complex control of behavior in their life cycle, but a parsimonious position to take is that it is a small minority (Hughes, 2014). The reasons for this are probably related to the high costs involved in controlling the central nervous system of another organism (Poulin, 1994). This implies that there must have been a strong selective force leading to the evolution of behavioral manipulation. For whatever reasons, these selective forces operate frequently on entomopathogens because the number, range, and diversity of behavioral manipulation of animals by these fungi are high. In the next section, we examine this diversity.

3. DIVERSITY OF FUNGI CONTROLLING ANIMAL BEHAVIOR

There are estimated to be between 1.5 and 5 million species of fungi (Blackwell, 2011; Hawksworth & Rossman, 1997), but only around about 100,000 have been described so far (Kirk, Canon, Minter, & Staplers, 2008). Those are currently organized into seven phyla (Microsporidia, Neocallimastigomycota, Chytridiomycota, Glomeromycota, Entomophthoromycota, Basidiomycota, and Ascomycota), with some groups not assigned to any phylum due to lack of data (Hibbett et al., 2007). Entomopathogenic species are known for all phyla except Neocallimastigomycota, which are anaerobic, inhabiting the rumen of large herbivorous mammals and Glomeromycota, a group formed almost exclusively by arbuscular mycorrhizal fungi, with a single exception *Geosiphon pyriformis* that forms symbiosis with cyanobacteria (Kützing, 1849).

A recent review of these entomopathogenic associations found that approximately 65% of all insect orders (19 of the 30) are known to be infected by fungi (Araújo & Hughes, 2016). Microsporidia infect 14 orders of insects, Ascomycota (mostly species in the order Hypocreales) and Entomophthoromycota infect 13 and 10 orders, respectively, Chytridiomycota infect 3 and Basidiomycota infect 2 orders. Until recently, due to lack of host data (Evans, 2013), such calculations could not be made for spiders (Order Araneae). However, a pioneering study concentrating on one spider-specific fungal genus (*Gibellula*) offers a tantalizing glimpse into the potential diversity of hosts affected by these fungi (Costa, 2014; Evans, unpublished data). Thus far, spiders in 10 families have been recorded as hosts of pathogenic fungi, all in the Order Hypocreales of the Ascomycota, but sharing no common species with the insect pathogens (Evans, 2013). When other spider host records are included, this approximates to over 10% of the 110 known families of spiders (Nentwig, 2013).

Such a broad representation across the taxonomic levels has resulted in heterogeneous ecological groups in many aspects. One example of this heterogeneity is the variation on display in fungal morphology. Chytrids exhibit flagellated zoospores that are adapted to "seek," recognize, and penetrate the host cuticle (Barr & Désaulniers, 1988); whereas, the extremely small spores in some microsporidians (eg, 3 μm long) shoot a harpoon-like structure to inject the protoplasm into the host's cell (Araújo & Hughes, 2016); while complex ascospores (eg, some species of *Ophiocordyceps*) exhibit specific shapes to improve aerodynamics upon dispersion (hosts are often attached to plant material up to 2 m high) and germinate secondary structures once on the forest floor (eg, capilliconidia and capilliconidiophore) (Araújo, Geiser, Evans, & Hughes, 2015; Evans, Elliot, & Hughes, 2011a, 2011b), which are analogous to and demonstrate convergent evolution with the capillispores and capillisporophores of the Entomophthorales. In terms of habitats, we can find equally impressive diversity. Entomopathogenic fungi are found from African deserts (Evans & Shah, 2002) to aquatic environments like ponds, streams, or even leaf axils that collect water (Frances, Sweeney, & Humber, 1989). However, the greatest diversity is found in tropical forests worldwide. There, we find fungi infecting arthropods inhabiting soil (eg, trapdoor spiders) to leaf litter (eg, beetle larvae and caterpillars) to the understory (eg, ants, wasps, bees) to high canopy (eg, homopterans) (Figs. 1–5).

However, one of the most fascinating aspects of these fungal–arthropod associations is the host diversity (Figs. 2–3). The chytrids (the only aquatic group among the entomopathogenic fungi) are known to infect almost exclusively mosquito larvae, including important disease vectors (eg, *Aedes*, *Anopheles*, and *Culex*) with very rare exceptions (eg, *Myiophagus* sp. infecting the purple scale *Lepidosaphes beckii* (Muma & Clancy, 1961)). Although the majority of entomopathogenic microsporidians also infect Diptera, they are known to parasitize a broad range of hosts such as Zygentoma, Ephemeroptera, Odonata, Plecoptera, Orthoptera, Isoptera, Psocoptera, Hemiptera, Coleoptera, Hymenoptera, Siphonaptera, Trichoptera, and Lepidoptera. Although there are more than 32,000 species described for Basidiomycota (Kirk et al., 2008), less than 1% have evolved to live inside the insect body (Araújo & Hughes, 2016), with almost all entomopathogenic species belonging to a single genus, *Septobasidium*, infecting Diaspididae scale insects (Couch, 1938). Entomophthoromycota and Ascomycota are the phyla that exhibit the highest diversity among entomopathogenic fungi. In both phyla, we also see repeated origins of a complex strategy of infection and transmission with the manipulation of host behavior.

Within the Entomophthoromycota, host behavioral manipulation has evolved at least twice and in Ascomycota multiple origins happened over the long evolutionary history of the group. For entomophthoralean fungi, there are several classic examples, *Pandora* infecting the ant genus *Formica* ants (Fig. 2G), *Entomophaga* infecting acridid hosts (especially locusts, Fig. 4A), and *Entomophthoromycota* infecting flies (eg, *Musca domestica* and

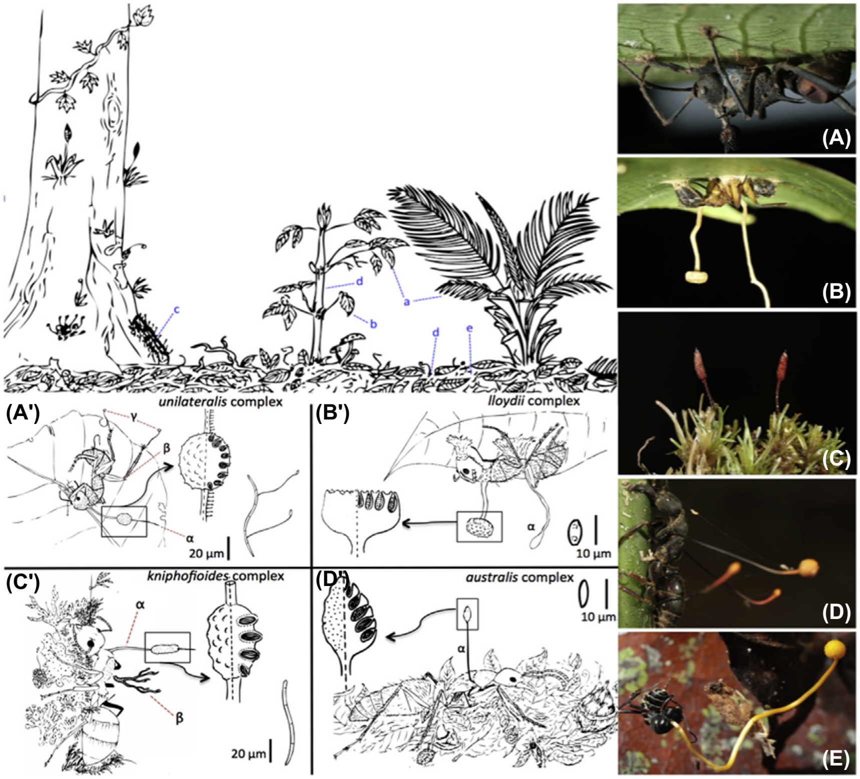

Figure 1 ***Diversity of behavioral manipulation of ants by fungi in a tropical forest setting.*** Represented is some of the extensive taxonomic and functional diversity of *Ophiocordyceps* in ants. Top left shows a composite forest scene with six locations where cadavers are found: under leaves (a = *Ophiocordyceps unilateralis*), (b = *Ophiocordyceps lloydii*), tree bole/bark (c = *Ophiocordyceps kniphofioides*), leaf litter (d = *Ophiocordyceps australis, Ophiocordyceps myrmecophila, Ophiocordyceps irangensis, O. kniphofioides*), and stem (e = *O. australis* in Ghana). Each specific name represents a complex with more than one species. Line diagrams show the functional morphology (represented as a composite, all morphologies do not occur on one species). In (A′) *unilateralis* has one teleomorph (sexual stage) and three anamorphs (asexual, α−γ). The teleomorph (ascoma) shows the outside and inside where ascospores are produced and the ascospore is drawn with capilliconidia (secondary spores on hairs). In (B′) *lloydii* and (D′) *australis* only part spores are produced and they do not produce secondary structures and only one anamorph is found (asexual, α). In *lloydii* the ant is not biting but glued via hyphae from mouth (photo b). The complex (C′) *kniphofioides* (here on ant species *Dolichoderus bispinosus,* which is hidden from view in moss) is related to *unilateralis* and we can see two anamorphs (one does not occur on *Dolichoderus,* but only on *Cephalotes atratus).* The photos A−E show host ants and position of death: A−E *Polyrhachis armata,* Thailand, *Camponotus atriceps*, Brazil, *Dolichoderus bispinosus*, Brazil, *Paltothyreus tarsatus*, Ghana, and *Polyrhachis robsoni*, Australia. Among these are two undescribed fungal species from the following complexes: *unilateralis* (A′) *and australis* (D′).

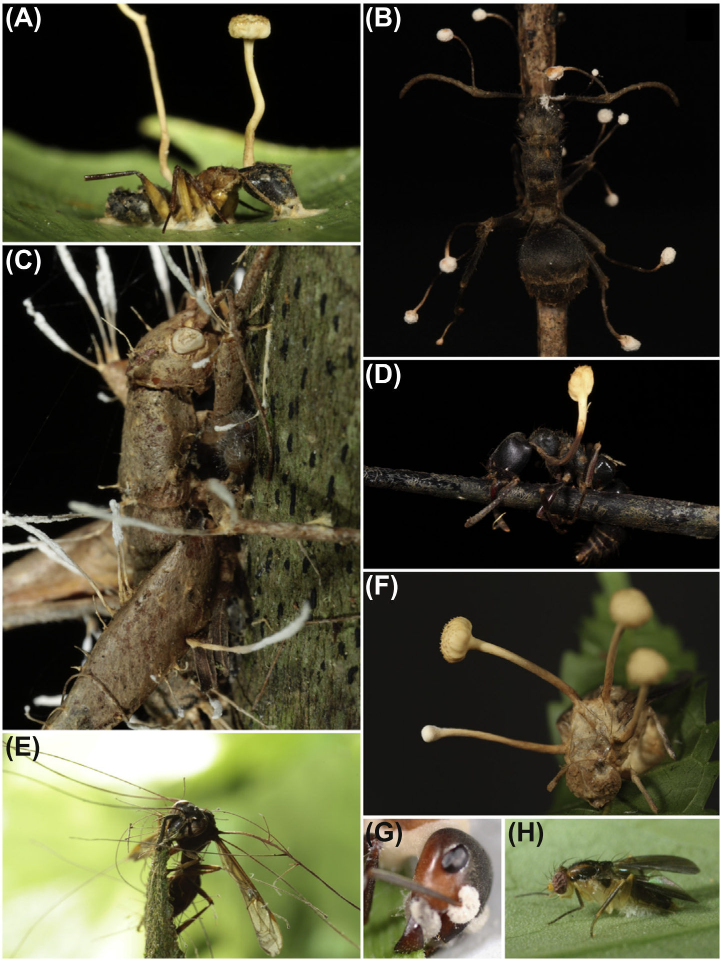

Figure 2 ***Diversity of behavioral manipulation where insects are controlled to die attached to arboreal surfaces*** (A) *Ophiocordyceps lloydii* on *Camponotus atriceps* (Brazilian Amazon); (B) *Stilbella burmensis* on *Polyrhachis cf. militaris* (Ghana); (C) anamorphic Hypocreales on Orthopera (Atlantic rainforest, Brazil); (D) *Ophiocordyceps* sp. on *Pachycondyla impressa* (Brazilian Amazon); (E) *Hirsutella saussurei* (anamorph of *Ophiocordyceps humberti*, Atlantic rainforest in Brazil) on Polistinae wasp; (F) *Ophiocordyceps dipterigena s.l.* (Brazilian Amazon) on unidentified fly; (G) *Pandora formicae* on *Formica* ant (Finland); (H) *Ophiocordyceps dipterigena s.l.* early developmental stage (Atlantic Rainforest in Brazil) on unidentified fly.

Scatophaga stercoraria) (Humber, 1989; Maitland, 1994; Małagocka, Grell, Lange, Eilenberg, & Jensen, 2015). After infection, the fungus proliferates within the host, manipulates the behavior (ie, controlling the host to reach an elevated position on plants, called "summit disease"), kills the host, and in

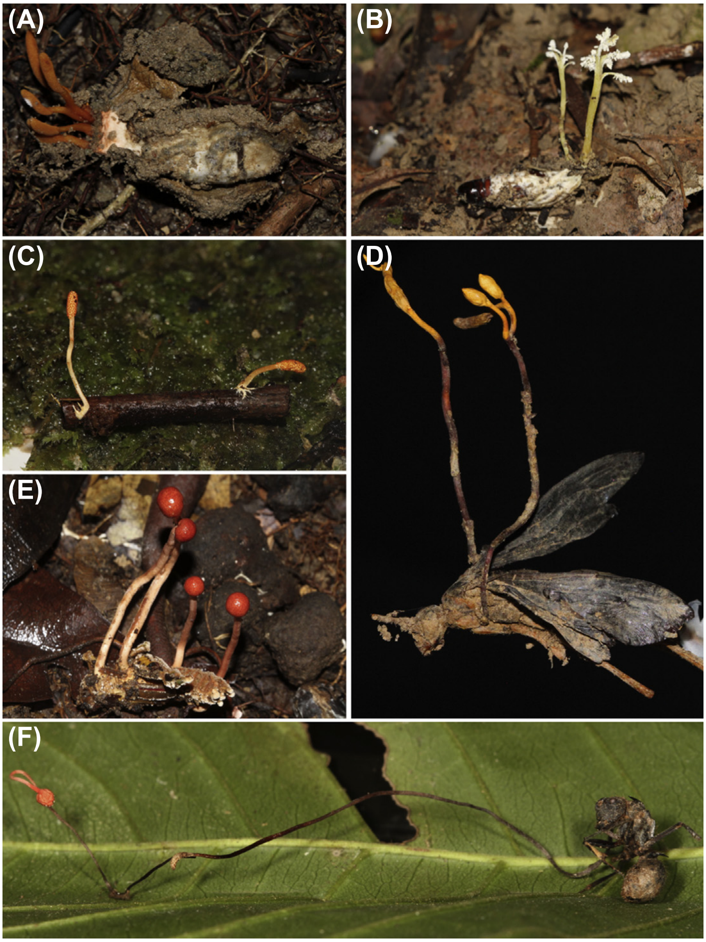

Figure 3 ***Diversity of interactions where insects die in the soil where behavioral manipulation is not assumed to occur*** (A) *Ophiocordyceps* sp. on trapdoor spider (Brazilian Amazon); (B) *Isaria* sp. on Lepidoptera pupa (Brazilian Amazon); (C) *Ophiocordyceps cf. cardinalis* on Coleoptera larva (Brazilian amazon); (D) *Ophiocordyceps* sp. (*Neocordyceps* group) on wasp. (E) *Ophiocordyceps amazonica s.l.* on Orthoptera (Colombia); (F) *Ophiocordyceps australis s.l.* on *Polyrhachis* sp. (Ghana).

certain genera (*Erynia*) creates fungal structures (rhizoids) to attach the host more securely to the substrate (Małagocka et al., 2015) (see Fig. 2G), but more typically this is a "death lock," particularly in grasshoppers and locusts which grasp vegetation with their legs before dying (see Fig. 4A, *Entomophaga grylli*). This precedes fungal growth from the interior to the exterior of the insect followed by sporulation from these exterior structures (Roy,

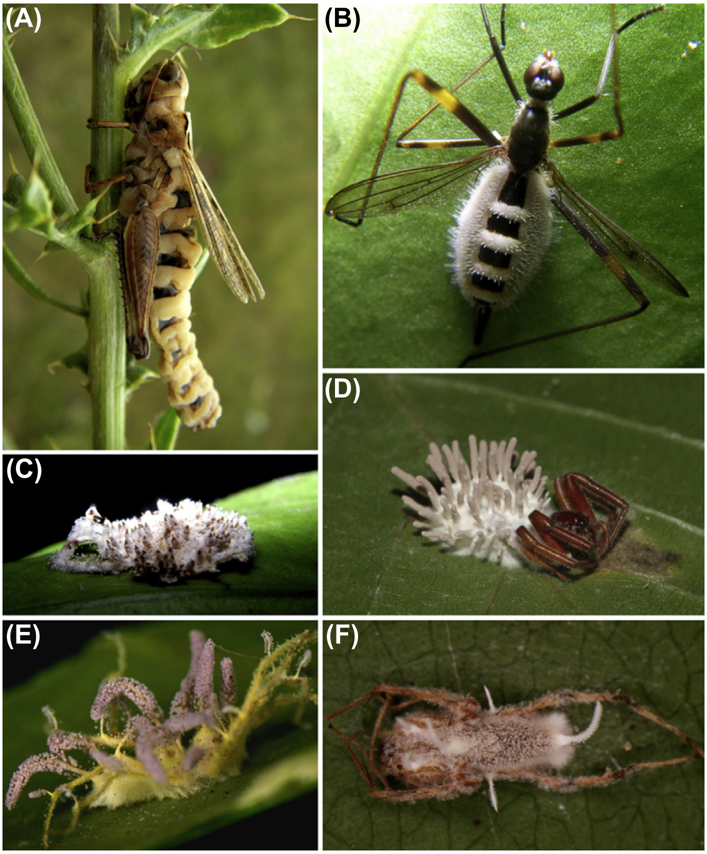

Figure 4 ***Further examples of death on arboreal surfaces*** (A) *Entomophaga grylli* on a locust host, near the summit of a thistle plant, in the grasslands of Outer Mongolia (China). Note the legs clasping the stem and the creamy-white fungal bands bursting from the intersegmental sutures. All the thistles in the immediate area had infected locusts in the same position, mostly with two to three corpses per plant. (B) *Erynia* on an unknown dipteran host, attached to the underside of a shrub leaf by rhizoids, Atlantic rainforest, Mata do Paraíso, Minas Gerais, Brazil. A white spore halo is beginning to form on the leaf surface. (C) *Gibellula* sp. nov. on a huntsman spider (*Caayguara* sp., Sparassidae), underside of shrub leaf, Mata do Paraíso. The asexual stage is nonsynnematal and reduced to sporing heads on the fore legs, while the abdomen bears an abundance of flask-shaped perithecia with prominent necks. The host genus was erected in 2010 and the spider is a fast-moving predator living in tree bark. (D) *Gibellula*, of the *leiopus* group on a spider, on the underside of shrub leaf, Reserva Ducke, near Manaus, Amazonas, Brazil. (E) *Gibellula* sp. nov. on an unknown ghost spider (Anyphaenidae), underside of shrub leaf, Mata do Paraíso. Note the abundance of lilac-colored synnemata arising from the mycelial-covered abdomen. (F) *Gibellula* sp. nov. on a ghost spider (*Iguarima sensoria*, Anyphaenidae), underside of shrub leaf, Mata do Paraíso; showing similar synnematal production to above. This is a fast-moving spider in the forest understory and rests in silken retreats (sacs) in the litter, tree bark, and vegetation.

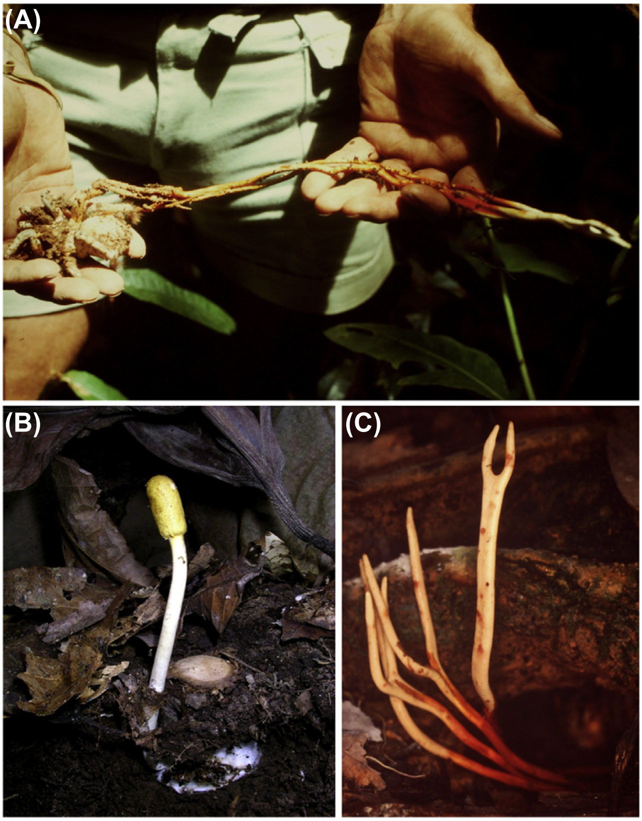

Figure 5 ***Infection of ground dwelling spiders.*** (A) The entire fungus (*Ophiocordyceps caloceroides*) extricated from the deep burrow, showing the mycelial-covered but easily recognizable bird-eating mygalomorph host. (B) *Cordyceps* sp. emerging from the burrow of a trapdoor spider (Ctenizidae), Rio Negro, Amazonian Brazil. The fungal clava has pushed open the trapdoor (right) and formed a yellow perithecial-bearing head (ascostroma), with the host enveloped in a white mycelial mat (C). Clavae or ascomata of *O. caloceroides* (Hypocreales) emerging from the burrow of a *Mygale* spider (Ctenizidae), forest litter, Rio Napo, Amazonian Ecuador. The paler-colored apex contains embedded perithecia and the structures resemble the *Calocera* mushroom genus.

Steinkraus, Eilenberg, Hajek, & Pell, 2006). In some genera, the forcibly discharged primary infective spores (ballistospores) have evolved the ability to form secondary sticky spores (capillispores), if they miss their aerial targets, thereby creating "minefields" around cadavers, potentially, to entrap crawling targets.

Summit disease optimizes both the formation of fungal spores and their subsequent dispersal. Earlier reports also indicate that host manipulation by these "primitive" fungi might be more widespread. For example, sugar-beet aphids that live and feed below ground, emerge when infected by *Erynia aphidis* and ascend the stem to die (Harper, 1958); while *Entomophthora*-infected carrot flies move away from the crop with the females completely altering their soil egg-laying habit so that they deposit their eggs on the foliage of hedgerow trees (Eilenberg, 1986). Such actions would reduce vertical transmission of the parasite within the population, but movement between populations (horizontal transmission) would be enhanced.

For Ascomycota species, the manipulation is well known for some ant—pathogenic species within the genus *Ophiocordyceps* infecting Camponotini ants (Araújo et al., 2015; Evans et al., 2011a, 2011b; Hughes, Anderson, et al., 2011; Hughes, Wappler, & Labandeira, 2011; Loreto, Elliot, Freitas, Pereira, & Hughes, 2014). In this case, about a week after infection, the fungus will induce the host to leave its nest and climb onto the vegetation. Once there, the ant will lock its jaws into the plant tissue and die. After the death of the host, the fungus starts to grow a fruiting body from the back of its head and, in a few weeks of subsequent growth, starts to produce spores that rain down on passing ants as they move on the forest floor or on branches below (Andersen et al., 2009; Hughes, Anderson, et al., 2011; Hughes, Wappler, et al., 2011). As such, the behavioral manipulation functions to provide a platform for the release of forcibly ejected spores (ascospores) that then infect susceptible individuals, thus continuing the cycle (Figs. 1 and 2E represents similar situation in a wasp host). These ascospores, if missing their targets, are capable of producing secondary sticky spores (condia) on long needle-like outgrowths (capilliconidiophore), and it is probably a common occurrence providing an insurance mechanism to ensure successful infection; the spores attaching to the target hosts as they crawl over the substrate. Thus, there is an analogous situation in the genus *Hypocrella* and the Phylum Entomophthoromycota.

The behavioral change that leads infected insects and spiders to die elevated on vegetation prior to host death (Figs. 2 and 4) is not the only complex behavioral manipulation observed. In some cases, the fungus keeps the host alive and controls its flight behavior so that the insect becomes a moving vehicle for spore release. One prominent example is the infection caused by *Strongwellsea castrans* in *Hylemya brassicae* and *H. platura* (Diptera) (Araújo & Hughes, 2016). The fungus causes a large circular hole to develop on the lateral side of the host's abdomen. This hole is filled with fungal tissue and conidiophores (spore-producing cells) that are released during a flight pattern that is described as being stereotypical and centered on a narrow area of fields (R. Humber, personal communication). This presumably aids in spore release to distinct areas of the environment where uninfected flies are present. Another similar case occurs with *Massospora cicadina*, which attack cicadas (Araújo & Hughes, 2016). This fungus also initiates sporulation when the host is still alive (Humber, 1982; Thaxter, 1888). Over time the abdomen falls apart until just the head and thorax of the living insect remain. The ability to fly is retained, increasing dispersion of spores in the environment, especially in the case of infected male cicadas which attempt to attract and copulate with females and even continue to feed (Evans, 1988; Soper, 1963; Soper, Delyzer, & Smith, 1976), which suggests that the central nervous system is functioning normally.

As mentioned previously, an interpretation of such pathogen–host relationships for spiders has not been even remotely possible due to incomplete host identification, especially for the araneomorphs, which tend to be completely overgrown by the fungus, compared to the much larger mygalomorphs (Figs. 4C–F and 5A). Thus, this is unchartered territory and needs to be explored with some urgency given the pivotal position that spiders occupy in ecological networks and the key role that they play in ecosystem functioning (Nentwig, 2013). Now, thanks to a spider taxonomist (Renner L.C. Baptista, Universidade Federal do Rio de Janeiro), new light is being shed onto these associations. In an on-going study, in a fragment of Atlantic rainforest in Minas Gerais (Brazil), almost 80 specimens of spiders infected by the entomopathogenic genus *Gibellula* have been examined: all covered by the fungal stroma and attached to the underside of understory shrubs or small trees. Examination involved excising the upper leaf surface to reveal the under body of the spider—specifically the genital area—to allow for accurate identification, without disturbing the fungal structures. Thus far, 14 genera in 10 families of spiders have been identified; comprising new species of the genus *Gibellula*, as well as new host taxa and only recently described spider genera. Over 50% belong to two families, the Anyphaenidae (ghost spiders) and the Pholcidae (cellar spiders), the former are nocturnal hunters, spending their days in silken retreats ("sleep sacs"), while the latter construct rudimentary webs around leaves and under bark. Other commoner hosts, in the Corinnidae, Salticidae, Sparassidae, Theridiidae, Thomisidae, and Zodariidae, are either fast-moving, free-living predators on vegetation and the forest floor or construct sticky webs or drag lines in the litter. The overall conclusion, therefore, is that, like many of the insect examples, infected spiders move away from their natural habitats to climb and die on understory plants, invariably on the underside of leaves (see Fig. 4C–F). In sharp contrast, the burrow-dwelling mygalomorphs of the family Ctenizidae always die in their underground nests; necessitating the production of complex phototrophic structures (stromata or clubs) by the fungus in order to ensure that the embedded sporulating organs (perithecia) are carried above ground to liberate their spores (see Fig. 5A—C). Conversely, the perithecia of the exposed *Gibellula*-infected spiders are formed directly on the spider cadaver (see Fig. 4C—F).

4. TINBERGEN'S FOUR QUESTIONS AS THEY APPLY TO BEHAVIORAL MANIPULATION OF ARTHROPODS BY FUNGI

One of the most important papers in the field of animal behavior is the classic by Niko Tinbergen, (Tinbergen, 1963) "On aims and methods of ethology." Tinbergen was a founding father of the field of animal behavior and, together with Karl Von Frisch and Konrad Lorenz, shared the Nobel Prize in Physiology or Medicine in 1973, awarded for their contributions to animal behavior (Burkhardt, 2005). Tinbergen suggested that animal behavior can be better understood when we ask four complementary questions. We can ask why a behavior exists by studying its (1) function, (2) phylogeny, (3) causation, and (4) ontogeny. In Fig. 6, we place one prominent example of a manipulated behavior, the death grip induced by species in the complex *Ophiocordyceps unilateralis s.l.*, within this four-question framework. This behavior (infected ants biting a leaf) can then be examined from four complimentary approaches. In the next section we examine each of these approaches in turn.

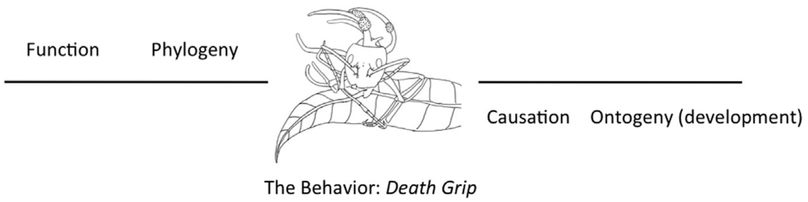

Figure 6 ***The death grip behavior with the framework of Tinbergen's four questions.*** A dead carpenter ant is seen attached to a leaf with the fungus emerging from just behind the head. The manipulated behavior (when the ant was alive) is the product of natural selection acting on fungal genes to control ant behavior (extended phenotype). We can examine this behavior in four complementary ways.

4.1 Function

The first question (function or adaptive value) examines the behavior from the perspective of an organism's fitness. In which way is the behavior adaptive? So, males in some species of birds sing to gain mates or wolves collectively hunt to feed the group. In the case of fungi controlling animal behavior, the framework of the extended phenotype laid out by Richard Dawkins (a student of Tinbergen) argues that the altered behavior in the host benefits parasite genes (Dawkins, 1982, 1990, 2004, 2012). For many examples of parasites controlling behavior, the adaptive value of the altered behavior is inferred. If the behavior is complex, highly stereotyped and not part of the host's repertoire, but possibly benefiting parasite transmission, then we might parsimoniously suggest the altered behavior is an adaptation for the parasite (Poulin, 2011). The death grip behavior in ants infected by *O. unilateralis s.l.* is unusual among examples of parasites controlling behavior because its adaptive value has been tested, twice. In the first test, carried out in a lowland tropical forest in Southern Thailand, the leaves that the manipulated ants bit were experimentally relocated either to the high canopy (15 m), or to the forest floor (0 cm) and, in both cases the fungus failed to develop and thus had zero fitness (Andersen et al., 2009). This experiment supported the claim that manipulating ants to bite into leaves in the narrow understory of the forest was adaptive for the fungus as it provided the parasite with a suitable microhabitat in which to develop. A second experiment, this time in the Atlantic rainforest of Brazil with a species within the *Ophiocordyceps unilateralis* complex (namely *Ophiocordyceps camponoti-rufipedis*) demonstrated that the fungus could not develop inside the ant colony (Loreto et al., 2014). This study offers supportive evidence that fungal manipulated ants leave the colony and bite into leaves which provide species in the complex *O. unilateralis s.l.* with a platform to develop a stalk and release spores that eventually infect other ants. Such stalk formation and spore release, it was shown, could not happen inside the ant nest. While the available evidence would support the conclusion that the complex behavioral changes observed in ants infected by species in the complex *O. unilateralis s.l.* is adaptive for the fungus, it is important to always consider such claims in the context of fungal transmission and specifically spores.

In most examples of fungi infecting insects and spiders, the transmission is direct (from infected animal to susceptible animal). There are examples where the life cycle of the fungus has two different hosts. For example within the Chytridiomycota the species *Coelomomyces psophorae* infects both copepods and mosquito larvae (Whisler, Zebold, & Shemanchuk, 1975). And, there is a recent example from the Ascomycota where an infected Cerambycid beetle larva in its tunnel in a tree produced an outgrowth (synnema), characteristic of the insect–pathogenic genus *Hirsutella,* with additional side branches forming structures typical of the genus *Harposporium*, a pathogen of nematodes with crescent-shaped spores that lodge in the buccal cavity (Evans & Whitehead, 2005). It is supposed that these spores infest the bark and inner wood and are ingested by free-living nematodes. However, most of the life cycles of fungi infecting arthropods are direct, as far as we can ascertain. What is also apparently the default mode is host death as a developmental necessity (but not always, as discussed above). This means that before they can produce transmissible spores the fungal pathogen kills the host insect or spider. Therefore, transmission by the parasite is postmortem for the host. For parasites generally (ie, all taxa and not just Kingdom Fungi), the production of the transmissible stage following host death is rare. It is in fact so rare that a special word, parasitoid, is used to describe the phenomenon (Kuris, 1974). The word parasitoid generally refers to parasitic insects that kill their host arthropod during the course of development. In those cases, the parasite does not transmit from the body of the host it kills, but rather emerges from it to engage upon a free-living stage that often involves feeding, mating, and diapause (Askew, 1971; Godfray, 1994). Technically, fungi that also kill their hosts as a developmental necessity are parasitoids, but the term is generally not used (Andersen et al., 2009). What direct life cycles and postmortem sporulation mean is that where the host animal dies is the point from where the next infection begins. There are two ways then for infection to occur: the susceptible host either touches the cadaver of the insect or spider (becoming infected via contact with spores), or spores are released and the susceptible host encounters them.

We cannot estimate how common transmission to a susceptible host is after that individual touches a sporulating cadaver. It surely occurs because some taxa grow sporodochia, which are infectious spores that are not released and are capped by sticky material that adheres to passing insects. In some cases, the fungus may imitate the smell or visual appearance of sexually receptive female insects to lure males that touch them and become infected, such as within the Entomophthoromycota when the species *Eryniopsis lampyridarum* infects chantarid beetles (Araújo & Hughes, 2016). Another likely unappreciated arena for contact transmission is the soil where burrowing insects encounter the sporulating cadavers and become infected.

For example, cadavers of reproductive ants that die before building a nest have infectious sclerotia that may infect future burrowing reproductive ants (Hughes, Evans, Hywel-Jones, Boomsma, & Armitage, 2009).

The majority of transmission is not via direct contact with a sporulating cadaver, but occurs from spores released from specialized structures such as the sexual ascomata in Phyla Ascomycota or from specialized asexual spore-producing cells (conidiophores). There is a very wide range of sizes, shapes, and masses of entomopathogen spores (Araújo & Hughes, 2016) such that some travel very short distances from the cadaver (millimeters) and others enter the airstream to travel longer distances (meters to presumably kilometers).

4.2 Phylogeny

The second question asks how can we understand a behavior by looking at the species displaying that behavior in a phylogenetic context? Perhaps the reason the organism behaves in such a way is because all members of the clade (genus, family etc.) have such a behavior. Previously, Hughes, Wappler, et al. (2011) and Hughes, Andersen, et al. (2011) argued that the death grip behavior observed in *Ophiocordyceps* and *Pandora*, for example, has evolved convergently because these fungi are separated by 500 million years of evolution (Hibbett et al., 2007). The parsimonious explanation is that both examples evolved independently and convergently, as opposed to the hypothesis that the common ancestor of both fungi manipulated insects to bite vegetation and it was subsequently lost in many other taxa.

With increasing resolution in fungal phylogenies, achieved either by better taxon sampling or more genes, we now are in a better position to make a more refined assessment of the role of phylogeny in fungal-extended phenotypes (ie, manipulation of host behavior). For example, the death grip of ants infected by *O. unilateralis s.l.* can be studied in the context of sister taxa. We know from recent studies (Quandt et al., 2014; Sung et al., 2007) that the genus *Ophiocordyceps* infects insects from nine different orders across the Class Insecta, as well as spiders, which are an order themselves. Those in the species complex *O. unilateralis s.l.* are sister to other complexes that infect beetles (eg, *Ophiocordyceps rhizoidea* on beetle larvae) and other ants (*Ophiocordyceps kniphofioides* on ants). In no case is the behavior manipulation as complex as in *O. unilateralis s.l.*, and while further phylogenetic reconstructions that have more taxon sampling will likely rearrange the sister taxa relationships, it is already clear that this approach allows us to better understand the evolutionary pathways to manipulation.

A strong complement to a molecular phylogeny is the use of fossils that can provide calibration points. For fungi infecting insects, an amber fossil of an infected plant feeding insect (Hemiptera) exists from the Cretaceous period *Paleoophiocordyceps coccophagus* (Sung, Poinar, & Spatafora, 2008). This fossil allowed researchers to suggest that the hypocrealean fungi were at least present in the Early Jurassic (193 million years ago with CI of 158–232 million years). Another fossil, this time of a leaf, was used to propose that the complex manipulation of ant behavior by fungi, which leaves telltale marks on leaves, has been occurring since the Eocene, 47 million years ago (Hughes, Andersen, et al., 2011; Hughes, Wappler, et al., 2011). In the future, the discovery of other fossils could provide important data on the evolution of key innovations as fungi both colonized insects and eventually evolved to control them.

4.3 Causation

The third complementary question is causation. How do behaviors occur? In this approach the focus shifts from the ultimate (or evolutionary) scale down to the proximate (or mechanistic) scale. The above two approaches (function and phylogeny) are ultimate in scope and causation and ontogeny (below) are proximate (Fig. 6). How parasites control host behavior is of considerable interest to many areas of biology (Adamo, 2012; Adamo & Webster, 2013). This is because parasites that have evolved the ability to manipulate the nervous systems of their hosts represent independent experiments in evolution. Traditionally, in trying to understand the mechanistic basis of animal behavior we examine genes, chemicals, or neuronal architecture to gain insights into what factors account for complex behaviors. Parasites that evolved to control behavior represent an independent outcome of natural selection acting on a genome to control host behavior. For this reason we can observe the designation of these parasites as neuroengineers (Adamo & Webster, 2013).

Fungi are particularly fascinating because they are microbial. Thus, in examples of fungi controlling animal behavior, it is the brainless organisms controlling the brain. In Section 5, we expand on recent advances that have been made in understanding the question of causation. Here, we would like to stress that the question of causation should be approached using multiple tools. There may be a temptation to rely upon gene expression studies, but the addition of small molecule surveys (metabolomics and proteomics) together with direct visualization will go a long way in helping us understand how fungi control host behavior.

4.4 Ontogeny

Behaviors are typically expressed at relevant points in the organism's life cycle. So, male birds who sing either to establish a territory or to attract a mate only do so when they achieve sexual maturity (Alcock, 1993). Additionally, the timing of singing is important (both during the breeding season and at the correct time of day). From this it is clear that timing is important to the expression of behavior, and it was for this reason Tinbergen included ontogeny in his four complementary approaches. Previous to this, only the triumvirate approach of function, phylogeny, and causation were considered (Tinbergen, 1963). The addition of ontogeny has been crucial to the study of animal behavior, as we can now examine the survival value of a behavior (function), its evolutionary history (phylogeny), or its mechanistic basis (causation) in addition to when in the organism's life cycle the behavior occurs.

The "when" of behavior is especially relevant to studies of parasites altering animal behavior because the novel behavior, typically, is not part of the organism's natural repertoire. The death grip of carpenter ants infected by either *Pandora* or *Ophiocordyceps* (Figs. 1—2) is not something uninfected ants do. So, once infected and before they are killed, the individual ant (or other insect) is taken over by the developing fungus and begins to execute the behavioral change that ultimately enables fungal transmission. At some point in the infection process, the ant stops being a normal member of its colony and becomes a fungus in ant's clothing. Through the process of kin selection, individual worker ants (and other social insects), act altruistically to benefit their colony and themselves via indirect fitness benefits, but at some point in the infection cycle, the behavior of the ant switches from increasing the fitness of the ant colony to increasing the fitness of the colony of fungi growing inside its body. A major frontier in future proximate levels studies will be to understand when ants (and indeed other arthropods) make this switch to being vehicles for fungal fitness.

5. MECHANISMS OF BEHAVIORAL MANIPULATION

Let us return to the major and important question of how do fungi control animal behavior? As we have emphasized, there are a wide diversity of parasites altering the behavior of their hosts ranging from viruses (eg, *Baculovirus*) to bacteria (*Wolbachia*) to fungi (this review) to worms (hairworms, cestodes and nematodes) to insects such as parasitic flies that infect other insects (eg, Phorids). In all cases, a major question is what are the mechanisms by which control occurs? In recent years, the broader field of parasite and host behavior has seen some major advances in our understanding of the proximate mechanisms of parasites controlling host behavior (Adamo, 2012; Adamo & Webster, 2013; Biron & Loxdale, 2013).

Fungi probably represent a special case study in this general field because of several unique factors peculiar to this Kingdom. The first and most prominent is the range and complexity of behavioral manipulation by fungi of arthropods. As reviewed here and by Araújo and Hughes (2016), this ranges from species in the genus *Ophiocordyceps* that control worker ants to seek out and bite leaves near ant trails to the altered flight behavior of living insects (cicadas and flies infected by *Massospora* and *Strongswellsea,* respectively) so that they act as spore dispersal factories. It is difficult and perhaps futile to rank manipulation across different kingdoms of life and argue that fungal manipulation is more complex than that observed when the manipulator is in the Kingdom Animalia (eg, trematodes). However, what is clear is that the diversity of strategies is greater than that observed in other groups. In addition, it is evident that behavioral manipulation has arisen multiple times independently (Hughes, Andersen, et al., 2011; Hughes, Wappler, et al., 2011). These two factors (diversity and multiple origins) offer the researcher interested in the proximate mechanisms by which one organism controls the behavior of another sufficient materials to perform a comparative analysis. For example, the observation that species in the genus *Pandora* and *Ophiocordyceps* both induce ants to bite into leaves and the fact that these two genera are in groups that diverged over 500 million years ago (Hibbett et al., 2007) offers the potential to study independent experiments in evolution. Further, within the genus *Ophiocordyceps* (which contains most of the entomopathogens) the availability of high-quality phylogenetic reconstructions (Quandt et al., 2014), together with detailed field work and analysis of past collections (Fawcett, 1886), allows us to determine that even within this genus, parasite manipulation of host behavior has occurred independently. Here, we discuss some recent advances in understanding the mechanisms of behavioral control and the corresponding changes in the host.

5.1 Molecular Basis of Fungal Control of Insect Behavior (Ants As a Case Study)

The regulation of something as plastic as behavior is rather complex, and, therefore, the mechanisms needed to change it so precisely as done by parasites are likely to be equally complex. Substantial progress on elucidating these mechanisms can be made through the use of RNA and DNA sequencing technology, proteomics and metabolomics to identify the compounds in play, and the development of controlled laboratory infections followed by forward genetics techniques. In addition, advances in microscopy allow us to better visualize the host—parasite interface. Controlled laboratory experiments allow for the elimination of fluctuating environmental factors in the field, which add another level of complexity to the process. Their development is therefore extremely beneficial to tease apart the many aspects that are likely to be involved in behavioral manipulation.

A substantial leap forward toward learning how certain fungal species can exploit their ant hosts has been the recent publication of the transcriptomes of two fungal entomopathogenic manipulators: *Pandora formicae* and *O. unilateralis s.l.* (de Bekker et al., 2015; Malagocka et al., 2015). For *O. unilateralis s.l.*, this work was paired with the assembly and annotation of a draft genome. These fungi reside within completely different phyla (Zygomycota and Ascomycota) and infect ants from different genera (*Formica* and *Camponotus*). Yet, their manipulated hosts display similar behavioral aspects. In both systems, infected ants are manipulated prior to death to leave the nest, climb up vegetation and fix themselves there with their mandibles (Boer, 2008; Marikovsky, 1962). This elevated position, away from the ant's nest, promotes disease transmission through spore dispersal. In the case of *Pandora,* conidia are actively discharged from soft parts of the ant exoskeleton, while *Ophiocordyceps* shoots sexual ascospores from a fruiting body (ascoma) that has sprouted from behind the ant's head (Fig. 1). Laboratory infections of several *Camponotus* species with *Ophiocordyceps* followed by behavioral observations demonstrated that this entomopathogen could in fact kill all species tested (de Bekker, Quevillon, et al., 2014). However, it was only able to manipulate those species that were under natural conditions. Moreover, *Ophiocordyceps* seemed additionally hampered in its development since the characteristic switch after host death from yeast-like to hyphal growth, and subsequent host mummification, appeared to not take place in those nonmanipulated individuals. This work was accompanied by a metabolomic analysis of ex vivo fungal—ant brain interactions of *Ophiocordyceps* with various ant species. The results suggested a molecular basis for the species-specific manipulation observed as the fungus displayed a significantly heterogeneous secretome upon interaction with the different ant species' brains it was presented with (de Bekker, Merrow, et al., 2014). Unfortunately, similar experiments have not been done with *Pandora* since laboratory conditions for this system have not yet been established.

The transcriptomics studies done on *Pandora* and *Ophiocordyceps* infecting ants had different approaches, making them not directly comparable to one another. Since controlled infections cannot be performed for *Pandora*, field samples were used for RNA extraction. This limited the sampling to ants that recently died and did not display any sporulation yet, and ants carrying infective conidia that died at least one day before sampling (Małagocka et al., 2015). Thus, the manipulation event had already taken place by the time sampling was performed. In the *Ophiocordyceps* system, controlled infection studies could be performed, which made it possible to sample ants one step earlier; during the manipulated biting event. In addition, *Ophiocordyceps* can be cultured in insect cell culture media, so fungal baseline expression levels irrespective of manipulation could be established (de Bekker et al., 2015). Moreover, this allowed for the construction of a good quality draft genome to aid in gene expression analysis and gene annotation. The entomophthoralean fungi unfortunately suffer from a lack of genome and transcriptome data, leaving a large part of the *Pandora* data unannotated at this time. The study into *P. formicae* revealed the various enzymes this entomopathogen employs to rapidly change from growth within the host's body (nonsporulating phase) to the production of infective conidia (sporulation phase). The fungal pathogen goes through an intensive morphological reorganization to establish this. Enzyme production appears to be carefully orchestrated with the upregulation of various pathogenic subtilisin- and trypsin-like serine proteases and catalases protecting against host oxidative defenses during the nonsporulating phase. These catalases have been suggested to be regulating the fungal stress responses. During the following sporulating phase, lipases, chitinases, and GTPases can be found among the highest upregulated protein functions. These enzymes are necessary for the switch to invasive growth with lipases facilitating the switch from yeast to hyphal type of growth (Małagocka et al., 2015). In line with this study, subtilisin- and trypsin-like serine proteases were found to be upregulated during manipulated biting behavior in the *O. unilateralis* study as well. Moreover, many enzymes involved in oxidation—reduction processes were also found during this parasite—host interaction. These processes, together with the secretion of lipocalins might be regulating stress responses in this particular fungus—ant interaction. In addition, clues for morphological reorganization from yeast-like to hyphal cells were found in the form of genes encoding for lectin-like flocculation proteins in this system (de Bekker et al., 2015).

The transcriptomics study on experimentally *Ophiocordyceps*-infected ants centers itself around the event of manipulated biting behavior prior to death and with that proposes mechanisms for fungal control of insect behavior (de Bekker et al., 2015). Generally, genes encoding for proteins involved in sugar metabolism are downregulated during manipulation, while, as expected, pathogenicity-related genes are upregulated. After manipulation has taken place, these pathogenicity features go down again, and sugar metabolism becomes a priority. Similarly, oxidation—reduction processes that are a hallmark for parasite—host interactions are overrepresented among the genes that are active during manipulated biting and inactivated again when death sets in. Differential expression analysis also revealed that the fungal entomopathogen dynamically changes the expression of the genes that encode for secreted proteins depending on the status of infection and manipulation. This is in line with earlier reported metabolomics studies on the secretomes of fungal entomopathogens (de Bekker, Merrow, et al., 2014; de Bekker, Quevillon, et al., 2014). Among the secreted enzymes that are upregulated during manipulated biting behavior are many pathogenicity-related genes such as the lectins and proteases mentioned above. In addition, an aegerolysin with homology to the highly toxic Asp-hemolysin of *Aspergillus fumigatus* was found, as well as 21 (out of a total of 34) genes encoding for enterotoxins. Fungal enterotoxins are not well-described at this point, and genome comparison shows that, while they seem to be present in the genomes of Hypocrealean entomopathogens, fungal plant pathogens of the Phylum Ascomycota do not necessarily have genes encoding for them. The transcriptomics data, however, show that *Ophiocordyceps* dynamically tailors the expression of its secreted enterotoxins. These could be impairing the host's chemosensory pathways by reducing the production of chemosignaling molecules. Enterotoxins could also be complementing the upregulated secretion of the acid sphingomyelinase and, as such, contribute to the extensive muscle atrophy that is observed as a hallmark of *O. unilateralis* infection (Hughes, Andersen, et al., 2011; Hughes, Wappler, et al., 2011). Acid sphingomyelinase is also an important enzyme in sphingolipid metabolism, which determines the composition of biological membranes. Altering the composition of these membranes alters cell signaling, which, in the case of neuron cells, can result in neurological disorders. Additionally, the reported upregulation of different types of alkaloid metabolism can result in signaling issues, since these alkaloids could function as (ant)agonists of various receptors. Such alterations in receptor signaling could result in an altered behavioral output by the brain. An additional possibility is that the fungus targets the peripheral nervous system and in particular the motor neurons (Hughes, Andersen, et al., 2011; Hughes, Wappler, et al., 2011).

Other aspects that were found in this study that are suggestive to changing behavioral outputs are secreted fungal enzymes that could be changing serotonin and dopamine levels, as well as bioactive small secreted proteins, polyketides and nonribosomal proteins that have unknown function at present (de Bekker et al., 2015). Last but not least, this study demonstrated that behavioral manipulators across kingdoms could have mechanisms in common. The secreted enzyme protein tyrosine phosphatase (PTP) was upregulated >110-fold by *Ophiocordyceps*. The gene-encoding PTP in baculoviruses was found to be responsible for the enhanced locomotion activity observed in the caterpillars they infected (van Houte et al., 2012; Kamita et al., 2005). Late in the infection of the so-called "treetop disease" this gene gets activated when caterpillars are moved to the upper plant foliage where they die. *Ophiocordyceps*-infected ants similarly move to elevated positions where they die while biting. This suggests that the induction of enhanced locomotion activity through PTP could also be incorporated in the suite of mechanisms employed by the fungus to control ant behavior. Of course, these mechanisms are all still suggestive, as functional studies are needed to confirm their involvement.

5.2 How Host Brains Are Controlled (Ants As a Case Study)

In parasite manipulation of host behavior there is interplay between host and parasite. Although the abnormal host behavior depends on the parasite genotype, it is expected that the successful manipulation will depend on the host physiology and genotype as well (Lefèvre et al., 2008). Where fungi control host behavior, we have begun to elucidate the parasite genome and transcriptome (as discussed above); however, given that all host–parasite interactions are an interplay of both organisms, it is not possible to completely understand the mechanisms of manipulation without elucidating both parties of the interactions. Manipulative parasites are restricted to their parasitic life and their molecular activity is specialized to infect and manipulate the host. For the host, studying the changes at the molecular level could be more complicated. As in any pathology, the host will display a generalized response to parasite invasion, sickness, and impending death, at the same moment it is being manipulated. This makes it difficult to untangle manipulation effects from pathology responses, especially because they could overlap.

An ideal system to explore mechanisms of behavioral manipulation would allow us to study the host under different parasite infections, such as nonmanipulative parasite, manipulative parasite, as well as the healthy condition. However, this approach has not yet been employed, but independent studies already indicate its importance. The infection of ants by the generalist parasitic fungus *Metarhizium brunneun* results in the host upregulating immune-related genes expression (Yek, Boomsma, & Schiøtt, 2013), while the infection of ants by *O. unilateralis s.l.* induced an overall downregulation of these genes (de Bekker et al., 2015). These opposite results could indicate a strategy of the manipulative parasite that requires a longer time to develop inside the host to successful achieve the manipulation. However, as these ants were sampled shortly after infection by *M. brunneun* and shortly prior to being killed by *O. unilateralis s.l.*, an alternative explanation would be the manipulated ant is about to die and there is no more investment in the immune system. The downregulation of the immune system has also been shown in other parasites that do not manipulate the behavior of their hosts (Barribeau, Sadd, du Plessis, & Schmid-Hempel, 2014). Thus, studies on host manipulation, at the molecular level, can be very inconclusive without the suggested approach of controlling for generalized responses to infection.

Molecular approaches can be combined with direct visualization enabled by advances in histology. When a manipulative fungus infects its host, the aberrant behavior is accompanied by other phenotypic alterations as the colony of fungi grow inside the animal (Hughes, Andersen, et al., 2011; Hughes, Wappler, et al., 2011). In the case of ants infected by the fungus *O. unilateralis s.l.*, the fungal cells cause the mandibles of the ant to penetrate the plant substrate. This is accompanied by atrophied mandibular muscles that causes the "lock-jaw" so typical of the death grip phenotype. The function of such behavior is to ensure the fixation of the host after death (Hughes, Andersen, et al., 2011; Hughes, Wappler, et al., 2011). In line with histological observations, a recent study on the transcriptome of the host showed the downregulation of muscle maintenance and integrity-related genes, such as genes encoding collagen, indicating a possible pathway alteration in the host relevant to the manipulation (de Bekker et al., 2015). While the mandibular muscles are atrophied, the brain morphology appears to be preserved, suggesting the importance of the central nervous system of the host for the manipulation. The act of biting is suggested to be related to changes in the dopamine pathways (de Bekker et al., 2015), which is known to mediate aggressive behavior in ants, resulting in opening mandibles and biting (Szczuka et al., 2013). Another characteristic phenotype of ants manipulated by *O. unilateralis s.l.* is the reduced response to external stimuli, which could result from the downregulation of odorant receptors (de Bekker et al., 2015).

This could also explain why the manipulated ants do not follow the foraging trails (Hughes, Andersen, et al., 2011; Hughes, Wappler, et al., 2011). Although very intuitive, the correlation between phenotype and gene expression is not conclusive, and more investigation is necessary to confirm the role the suggested pathways have on the manipulation.

The role of the host responses in the mechanisms of behavioral manipulation has been studied in other systems, such as hairworms manipulating crickets to jump into the water (Biron & Loxdale, 2013). However, the fungal development within the host is substantially different and parallels with other systems like hairworms (an animal) might not be very helpful. On the other hand, many species of *Ophiocordyceps* fungi manipulate other ants (Fig. 1) and wasps to bite (Fig. 2E) as well as fungi in the genus *Pandora,* which also manipulate ants to bite (Fig. 2G). A parallel among these systems would be valuable to identify the convergence on the host histology, physiology, and gene expression that results in the same biting behavior. Additionally, because the fungus takes between 15 and 24 days to manipulate the ant after the infection, another approach to exploit the mechanisms of manipulation is to investigate the changes as the infection progress, for both host and parasite, at different levels. This is the important aspect of causation discussed above. Finally, as mentioned before, the complexity of manipulation by fungal parasites is a spectrum, from precise positioning of the host prior to death to dispersal of spores from the live host (Loreto et al., 2014), and we have just begun to understand one aspect of the total complexity implied by such a spectrum.

6. CAN BEHAVIORAL MANIPULATION BE EVOLVED IN SILICO?

When we consider the complexity of behavioral manipulation of animal behavior by fungi it is often a challenge to understand how it occurs. In Section 5, we discussed how advances are being made in uncovering these proximate mechanisms by focusing on genomic features, transcription, or the production of small molecules that affect behavior, as well direct visualization. A complementary approach to this empirical work is to undertake theoretical experiments asking how complex behavioral changes could result from "so simple a beginning" as killing a host at the point where it was infected. As Fig. 1 shows, there is a diversity of locations where insects are killed prior to the postmortem development of the fungus. If we take the parsimonious position that most entomopathogens kill their insect hosts in the same location as where they were infected (soil, leaf litter, decaying wood), then the evolution of manipulation to the underside of leaves or on other parts of plants represents a derived condition. In evolutionary biology, one approach to understanding the evolutionary pathways to a derived trait is via genetic algorithms. In this section we introduce these and argue that they have utility for understanding the evolution of complex manipulation of animal behavior by fungi.

Genetic algorithms (GAs), a subset of the broader field of evolutionary computation, are an optimization technique that borrows principles from natural selection to adaptively search phenotypic or genotypic space for fitness maximums (Mitchell, 1998). The basic components of a GA consist of a population of solutions ("individuals") that are evaluated for their fitness in solving a particular problem. After their fitness is calculated, the individuals undergo selection and reproduction (ie, the top X% of the population survive and reproduce, or reproduction is fitness proportional). During reproduction, the solutions represented by the parent individuals undergo random mutation and/or recombination to produce offspring, and these offspring and parents merge to form a new population ("replacement"). This new population then repeats the same process of selection, reproduction, and replacement until either convergence to a particular solution is reached or after a target number of generations has occurred. GAs have been used in many different contexts, from finding solutions to the iterated prisoners' dilemma problem (Axelrod, 1987) and optimizing travel routes (Grefenstette, Gopal, Rosmaita, & Van Gucht, 1985) to predicting gene–gene interactions (Hahn, Ritchie, & Moore, 2003). In behavioral ecology, genetic algorithms have been used to model optimal tradeoff decisions between singing for mates and foraging for survival in birds (Sumida, Houston, McNamara, & Hamilton, 1990), and to explore the best antipredator vigilance strategies for animals foraging in groups (Ruxton & Beauchamp, 2008). In the preceding examples, the results from using GAs were similar to those obtained by analytical methods, but GAs have the flexibility to be used for problems that are otherwise analytically intractable.

One potential way to use GAs to explore the evolution of behavioral manipulation in silico would be to represent combinations of fungal fitness components (eg, cadaver placement, spore morphology, production, infectiousness, etc.) as individuals in a population of many different solutions. Fitness could be assessed by competing these individuals in an agent-based model of insect foraging and quantifying the number of insects that they each successfully infect. The most successful individuals of each round would reproduce with some amount of mutation and recombination, and the algorithm would continue until convergence on a particular best individual (combination of fungal traits) or until a given number of generations had been reached. Crucially, by competing these fungal strategies in an agent-based model of insect foraging, we allow for the particular details of host foraging ecology to be included, and, thus, it is likely that we could identify a diversity of "best" strategies for behavioral manipulation.

7. CONCLUSION

Fungi can control arthropod behavior in spectacular and complex ways. In this chapter we sought to present an overview of this complexity and discuss the multiple approaches we can take to study such complex adaptations (eg, Tinbergen questions or in silico genetic algorithms) as well as the advances that have been made so far (eg, mechanisms of host behavior). Despite these advances, we are just at the very tip of what is a considerable iceberg of complex interactions. In recent years, diverse efforts have revealed details of how O. *unilateralis s.l.* controls ant behaviors, but this is just one complex of at least 11 that infect and manipulate ants (Fig. 1). Within the Hymenoptera (the order to which ants belong), there are also wasps and bees that are manipulated. Among the insects, approximately 65% of all orders are infected (Araújo & Hughes, 2016), and in some cases complex manipulation occurs. Outside of the insects, we also suspect spiders are similarly controlled, as evidenced by recent studies (Costa, 2014; Evans, unpublished data). We are thus in a golden age of discovery. Armed with advanced tools in evolutionary biology (from SEM to RNAseq), we are in a position to discover how the fungi, a group of microbes capable of coordinated activity, have evolved the ability to direct animal behavior. In short, we have the ability to understand how the organism without the brain controls the one with the brain. We hope such a goal and the knowledge that many diverse examples of control exist inspires future organismal biologists to study the complex adaptations that have arisen from "so simple a beginning" (Darwin, 1859).

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