When a Mind Is Not Its Own: Mechanisms of Host Behavior Control by Parasitic Fungi

12

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Abstract

Many animal parasites can alter the behavior of animals in ways that benefit their own fitness. Insects are a frequent target for behavior manipulation by various parasites, especially fungi. Despite many decades of observing altered behavior in insects parasitized by fungi, our understanding of the mechanisms underpinning this behavior manipulation remains largely incomplete. This chapter explores our current understanding of the mechanistic basis of behavior manipulations by these so-called zombie fungi, key considerations in continuing to make progress in our understanding, and where I think the next advances will come.

Keywords

Behavior manipulation · Entomopathogens · Zombie fungi · Cadaver transmission · Active host transmission · Summit disease · *Entomophthora* · *Ophiocordyceps* · *Massospora*

12.1 Introduction

Insects are recognized as the largest and most varied group of eukaryotic organisms on the planet, with 900,000 recognized species (Grimaldi et al. 2005) and an expected 5.5 million total species (Stork 2018). The fungal kingdom is also incredibly diverse and species-rich. The number of fungal species has been proposed to be anywhere between 2 and 12 million (Hawksworth and Lücking 2017; Wu et al. 2019) (though this remains a controversial topic of active discussion, see Bhunjun et al. (2022)). It is therefore, perhaps, unsurprising that the majority of fungal phyla contain entomopathogens, species that rely on insects for nutrition (Araújo and Hughes 2016), and that the ability to use insects as food has arisen multiple times over the earth's evolutionary past (Hughes 2011). Indeed, approximately 65% of all insect orders have been observed to be infected by fungi (Araújo and Hughes 2016). Entomopathogenic fungi use a variety of strategies in utilizing insects for nutrition. Some entomopathogenic fungi are generalists: they are able to infect and draw resources from a variety of host species [e.g., *Metarhizium robertsii*, *Beauveria bassiana* (St Leger and Wang 2020; Ortiz-Urquiza 2021)], leveraging the large available insect biomass in their environment. Others are specialists, targeting a narrow range of host species. Many specialist entomopathogenic fungi have evolved to manipulate host behavior. By doing so, the

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Y.-P. Hsueh, M. Blackwell (eds.), *Fungal Associations*, The Mycota 9,
https://doi.org/10.1007/978-3-031-41648-4_12

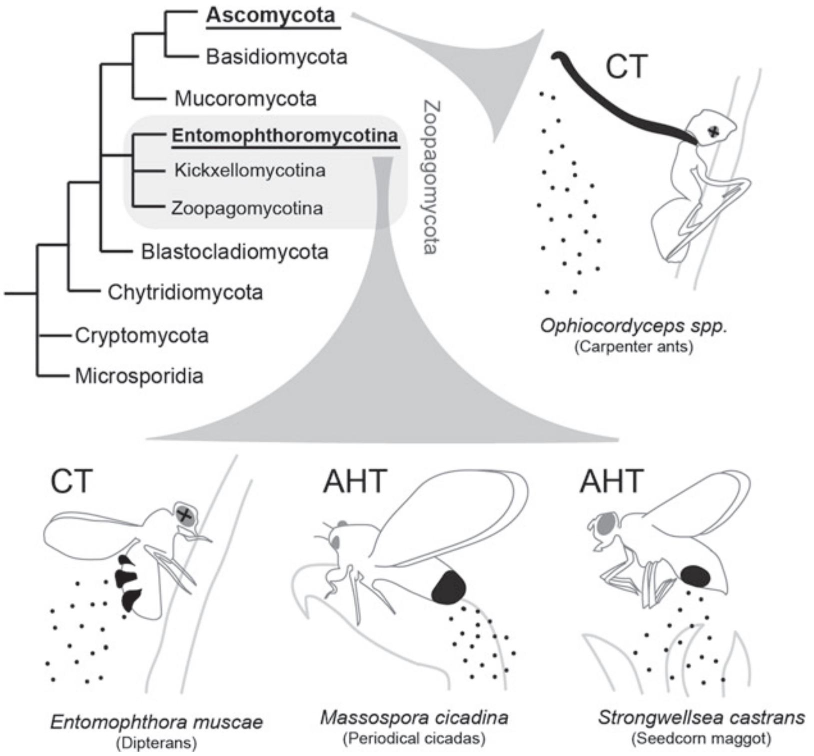

Fig. 12.1 Four examples of zombie fungi, their mode of dispersal and their relative phylogenetic placement within the fungal kingdom. Clockwise from upper right: *Ophiocordyceps* spp., aka zombie ant fungus, infect carpenter ants and spread via cadaver transmission (CT); *Strongwellsea castrans* infects *Delia platura* and spreads via active host transmission (AHT); *Massospora cicadina* infects 13- and 17-year periodical cicadas and spreads via AHT; *Entomophthora muscae* infects various dipterans (true flies) and spreads via CT; fungal phylogeny after Spatafora et al. (2016). Underlined clades (Phylum Ascomycota and Subphylum Entomophthoromycotina) are home to most zombie fungi. Funnels adjacent to these clades indicate taxonomic placement for the examples shown in this figure. *Ophiocordyceps* is an ascomycete fungus (Order Hypocreales); all other examples are in phylum Zoopagomycota (Order Entomophthorales). Tree branch lengths are not proportional to phylogenetic distance

fungus increases the likelihood that it will encounter a suitable new host that lies within its target host range. These behavior-manipulating fungi, also referred to as "zombie fungi," have been observed predominantly in two fungal phyla, Ascomycota and Zoopagomycota (Hughes et al. 2016). Like entomopathogenicity itself, the ability to hijack insect behavior has evolved more than once, at least twice within the subphylum Entomophthoromycotina and several times among Ascomycota (Hughes et al. 2016) (Fig. 12.1).

Broadly speaking, zombie fungi utilize one of the two strategies to access new hosts: cadaver transmission (CT) or active host transmission (AHT). In cadaver-transmitting systems, the fungus coerces the host to die in an elevated location and then sporulates from the dead host from this high vantage point. In active host transmitting systems, the fungus alters the abdominal structure of a living host (either completely replacing it with fungal tissue or creating an opening through which fungal tissue can emerge) and uses the movement of the host to its advantage for dispersing spores.

Perhaps the most widely known example of a cadaver-transmitting fungus is the *Ophiocordyceps*-infected zombie ant (Fig. 12.1).

Though often referred to as a single phenomenon, there are actually many different species of *Ophiocordyceps* that each target a different host ant species and elicit their own variation on a behavioral theme (Evans et al. 2011; de Bekker et al. 2014; Loreto et al. 2018; Sakolrak et al. 2018). In these systems, *Ophiocordyceps*-infected ant hosts show abnormal behaviors late in infection. Whereas healthy ants are usually found in the nest or on foraging trails, zombie ants aberrantly leave their nests and wander off the foraging trail to climb vegetation. After their climb, zombie ants affix themselves to the plant substrate, typically by biting down and subsequent "lock-jaw," then die (Hughes et al. 2011). Curiously, death in this manner occurs with specific circadian timing, with zombie ants exhibiting the final climbing and biting behavior specifically around solar noon (Hughes et al. 2011). Over several days, *Ophiocordyceps* erupts from the dead ant's head, producing a fruiting body that will eject infectious fungal spores into the environment (Hughes et al. 2011). Since ants tend to die elevated above foraging trails, this ideally positions *Ophiocordyceps* to infect additional ants. The majority of described behavior-manipulating entomopathogens rely on similar behavior manipulations and cadaver transmission to complete their life cycle.

There are far fewer examples of active host transmitting zombie fungi. Among these is *Massospora cicadina* which infects periodical cicadas, insects that only emerge from dormancy in the soil every 13 or 17 years (Fig. 12.1). Cicada nymphs acquire *Massospora* infection via resting spores deposited in the soil (Lovett et al. 2020). Once the infected adult emerges, *Massospora* grows in its posterior end, ultimately sloughing off the posterior segments of the abdomen and replacing these tissues with a fungal plug (Lovett et al. 2020). *Massospora* then disseminates spores to other cicadas during flight and copulation attempts by the infected individuals, effectively using the host as a mobile transmission vector (Lovett et al. 2020).

Zombie fungi and their pitied insect victims have been documented in the scientific literature since the mid-1800s (Cohn 1855; Fawcett 1886; Thaxter 1888). Ever since this time they have fascinated scholars and non-scholars alike, recently making several appearances in the popular media (Nuwer By Rachel Nuwer on May 27 2021; Yong 2017; Langley 2018; Lu 2019; Zimmer 2019; Iati 2021). Yet despite longstanding fascination in zombie fungi, we are only just now beginning to uncover the mechanisms by which they elicit behaviors in their unwitting hosts.

Call It *Ophiocordyceps*

Once there was a genus of 400 species of insect pathogens, *Cordyceps*, classified in the order Clavicipitales. Use of DNA sequencing and phylogenetic analysis to study the insect associated species of *Cordyceps* revealed that the ecology was more ancient than previously realized and that these insect pathogenic species did not comprise a monophyletic genus. The species previously placed in a single genus now are classified in three families (Clavicipitaceae, Cordycipitaceae, Ophiocordycipitaceae) of Hypocreales reflecting their independent lineages. The phylogenetic classification of these species now includes more than 20 genera and combines ascospore- and conidium-producing species within common genera based on morphological and DNA criteria. Some species now placed in the genus *Ophiocordyceps* are insect pathogens highlighted in this chapter and in current dystopian fiction. Others are known throughout the world in traditional medicine. Less well known are yeast-like members of the *Ophiocordyceps* lineage, including yeast-like symbionts in mutualistic associations with bacteria and insects of the order Hemiptera. The evolutionary pathway from virulent pathogen to obligate symbiont remains to be explained.

12.2 Common Themes in Behavioral Manipulation by Zombie Fungi

There are five major recurring themes in the behaviors elicited by zombie fungi: hyperactivity, summit disease, perimortem postural changes, circadian timing, and sexual attraction (Table 12.1). Here, I will summarize these behaviors and discuss the mechanisms by which they are thought to occur. Where possible, I will discuss occurrences of similar behaviors that are elicited by non-fungal parasites and available mechanistic evidence for these systems.

Table 12.1 Recurring themes in behaviors elicited by zombie fungi

Behavior	Description	Possible host targets	Hypothesized fungal action	Examples	References
Hyperactivity	Increase in locomotion	Nutrient depletion, locomotion circuits	Secretion of fungal effectors (metabolites, SSPs)	*Ophiocordyceps* spp. *Massospora cicadina*	Will et al. (2020), Trinh et al. (2021), Cooley et al. (2018), Boyce et al. (2019)
Summit disease	Climbing to an elevated location right before death	Phototactic and/or gravitactic pathways	Secretion of fungal effectors (metabolites, SSPs)	*Ophiocordyceps spp.* *Entomophthora muscae* *Entomophaga grylli* *Pandora formicae* *Eryniopsis lampyridarum*	Hughes et al. (2011), Krasnoff et al. (1995), Pickford and Riegert (1964), Csata et al. (2021), Steinkraus et al. (2017)
Postural changes	Specific positioning of body parts to maintain cadaver's elevation and/or accommodate fungal dispersal	Invasion and/or restriction of musculature	Mechanical force; modulation of motor neuron activity	*Ophiocordyceps* spp. *Entomophthora muscae* *Entomophaga grylli* *Pandora formicae* *Eryniopsis lampyridarum*	Hughes et al. (2011), Will et al. (2020), Trinh et al. (2021), Krasnoff et al. (1995), Pickford and Riegert (1964), Csata et al. (2021), Steinkraus et al. (2017)
Circadian timing	Behaviors only occur within specific time window relative to environmental cues	Host circadian network, pathways underlying behaviors that occur with circadian timing	Secretion of fungal effectors, perhaps according to fungal molecular clock	*Ophiocordyceps* spp. *Entomophthora* spp. *Entomophaga grylli* *Entomophaga maimaiga* *Erynia neoaphidis*	Hughes et al. (2011), Milner et al. (1984), Krasnoff et al. (1995), Elya et al. (2018), Pickford and Riegert (1964), Nielsen and Hajek (2006), Dustan (1924)
Sexual attraction	Increased frequency of attempted mating with infected individuals	Olfactory and gustatory systems, hormonal axes	Production of volatile chemical attractants, stimulant metabolites, hormonal shifts	*Massospora cicadina* *Entomophthora muscae*	Boyce et al. (2019), Naundrup et al. (2021)

12.2.1 Hyperactivity

Hyperactivity, alternatively referred to as enhanced locomotor activity or ELA, refers to a sustained increase in locomotion and is a behavior observed within hosts infected by both CT and AHT fungi. Carpenter ants infected with *Ophiocordyceps* exhibit hyperactivity prior to their final climb (Trinh et al. 2021). *Massospora*-infected cicadas exhibit hyperactivity after their posterior abdomen has been replaced with a spore-releasing fungal plug (Boyce et al. 2019). In both instances, hyperactivity likely plays a role in positioning the host for spore dispersal. For the ant, hyperactivity may play a role in driving the ant away from its nest and to an elevated location from which it is ideal to eject spores. For the cicada, hyperactivity is hypothesized to increase the number of conspecifics encountered, and thus increase the number of newly infected hosts (Cooley et al. 2018; Lovett et al. 2020).

The molecular drivers of zombie fungus-induced hyperactivity are unclear, but a variety of mechanisms have been proposed. First, hyperactivity could result from more general changes in host physiology. Consistent with their mechanism of action (i.e., consuming host resources for fuel), zombie fungi have been observed to elicit starvation-like states in their host (Elya et al. 2018; Will et al. 2020). As many insects have been observed to increase locomotion during times of starvation (Browne and Evans 1960; Leonard 1970; C. P. Wheater 1991; Yang et al. 2015), hyperactivity could potentially arise from general nutrient depletion.

Alternatively, hyperactivity could be elicited via compounds produced and secreted by the fungus. Genomic, transcriptomic, and metabolomic studies in zombie ants have identified several candidates. In *Ophiocordyceps spp.*-infected ants, two independent studies in distinct experimental systems examined gene expression at the point of manipulation (i.e., biting). The authors observed upregulation of a putative enterotoxin as well as several putative small secreted proteins (SSPs) (de Bekker et al. 2015; Will et al. 2020), the latter of which have been repeatedly implicated in mediating interactions between fungi and their host plants and animals (Kim et al. 2016). Genomic analysis has found that orthologues of the enterotoxin are conserved only among ant-manipulating members of genus *Ophiocordyceps* (de Bekker et al. 2017a). Additionally, many of the SSPs appear to be species-specific, suggesting that they contribute to the unique pathology of these fungi (de Bekker et al. 2017a). These studies also observed a dramatic upregulation of genes in a cluster predicted to synthesize an aflatrem-like alkaloid at the point of manipulation (de Bekker et al. 2015; Will et al. 2020). Aflatrem has known neurobiological activity, often observed to cause tremors and seizures in livestock (Valdes et al. 1985). In cicadas, a recent study investigating the metabolome of *Massospora*-infected individuals found the amphetamine relative cathinone (Boyce et al. 2019). Amphetamines have similar structures to the hormones adrenaline and epinephrine and have been well established to exhibit stimulatory effects on a variety of animals (Randrup and Munkvad 1967). Overall, many exciting compounds have been identified as correlates of hyperactivity. It will be up to future experiments to demonstrate their causality.

Hyperactivity is also elicited in insect hosts by several non-fungal parasites. While the specificity of our mechanistic insight varies among these systems, what we have learned so far suggests that several diverse mechanisms, rather than a common pathway, underlie this behavior alteration. In crickets infected with hairworms (Phylum Nematomorpha) and sandhoppers infected with mermithids (Phylum Nematoda), hyperactivity occurs prior to a suicidal plunge into water (Herbison et al. 2019). Proteomics studies in these systems have led to the hypothesis that hyperactivity occurs as a result of dysregulation of energy utilization and/or neurophysiology (e.g., synaptic vesicle packaging and release, maintenance of neuronal compartments) (Herbison et al. 2019). In several species of lepidopteran larvae infected with nucleopolyhedroviruses (NPVs), pre-death hyperactivity has been elegantly demonstrated to depend on a virally-encoded protein tyrosine phosphatase (*ptp*) (Kamita et al. 2005; van Houte et al. 2012). However, a recent study demonstrated that *ptp* is dispensable for hyperactivity in at least one case (Kokusho and Katsuma 2021). Thus, even among NPVs it appears that there may be diverse mechanisms underlying hyperactivity.

In contrast to the increased activity seen in zombie crickets, sandhoppers, and caterpillars, jewel wasps (*Ampulex compressa*) evoke hypoactivity (reduced locomotion) in their cockroach hosts. After an initial non-specific sting that serves to temporarily paralyze its cockroach host, the wasp targets a second injection into the cockroach brain (Haspel et al. 2003). Following this second injection the cockroach engages in approximately 25 min of grooming behaviors and fails to initiate walking behavior (Haspel et al. 2003). Work in this system has found that the venom cocktail for the second injection is a complex mixture of small molecules, peptides and proteins (Arvidson et al. 2019), that specifically targets the insect central complex (a conserved brain region important for coordinating and planning motor output) (Haspel et al. 2003). These behavioral effects are mediated by D1-like dopamine receptors in the cockroach (Nordio et al. 2022), inducing host hyperactivity, and, more broadly, alter host activity levels, apparently, a frequent strategy employed by behavior-manipulating parasites.

12.2.2 Summit Disease

Summit disease, also referred to as tree-top disease or Wipfelkrankheit (Hofmann 1891), refers to the behavior in which an insect climbs to an elevated location immediately prior to its death. The insect then perishes at this high perch, typically held in place by its mouthparts (Hughes et al. 2011; Csata et al. 2021), limbs (Carruthers et al. 1997), and/or fungal holdfasts (Chung et al. 2017). Summit disease is elicited by several fungal parasites including *Ophiocordyceps* (Hughes et al. 2011) and *Entomophthora muscae* (Krasnoff et al. 1995) and is one of the most consistent behavioral motifs observed in cadaver-transmitting fungal parasites (Table 12.1). While the mechanistic basis of this behavior remains to be determined, there is a short list of host pathways hypothesized to serve as fungal targets to elicit this behavior. The first of these is phototaxis, orientation, and movement with respect to light. In field studies, *Ophiocordyceps*-infected ants have been observed to die more frequently in sunny versus shaded regions of observation plots (Andriolli et al. 2019) and to perish with their heads oriented toward opening in the forest canopy (Chung et al. 2017). These observations suggest that light plays an important role in the end-of-life behaviors exhibited by zombie ants. As light appears to influence where ants summit and die, the host phototaxis pathway has been hypothesized to be involved in summiting behavior. Gravitaxis, the orientation and movement in response to earth's gravitational pull, is another pathway proposed to play a role in summit behavior. The hallmark of summit behavior is death following elevation, and achieving elevation requires navigating against the earth's gravitational pull. Thus, it seems reasonable that fungal parasites might drive summiting behavior via altering their host's gravitactic preferences or circuitry.

How fungal parasites might alter phototaxis and gravity sensing in summiting or other host pathways is still unclear, although, again, several hypotheses have arisen from multi-omic approaches in *Ophiocordyceps*-ant systems. As previously discussed, a putative fungal enterotoxin, several SSPs, and a gene cluster responsible for synthesizing a related compound to the tremorgenic aflatrem have been found to be highly expressed during *Ophiocordyceps*-elicited biting behavior in ants (de Bekker et al. 2015; Will et al. 2020). Immediately preceding biting, ants had exhibited both hyperactivity and summiting behaviors. It seems likely that these behaviors likely overlap temporally, thus the compounds implicated in manipulation could be important for driving one or both of hyperactivity and summiting.

There are also several non-fungal parasites that elicit summit disease which can provide insight into the mechanistic underpinnings of this behavior. The trematode *Dicrocoelium dendriticum* infects ants and coerces them to climb to the top of surrounding flora that, like *Ophiocordyceps*-infected ants, they attach via biting (van Paridon et al. 2017). Recent structural analysis of *D. dendriticum*-infected ants revealed that at least one of the infected host's resident worms consistently invades the host's subesophageal ganglion (SOG) (Martín-Vega et al. 2018). Among other functions, the SOG contains descending neurons, neurons that send information from the brain to the ventral nerve cord (the insect analog of the vertebrate spinal cord), that are important for coordinating motor output, such as walking (Hsu and Bhandawat 2016). The location of the worm by itself is unlikely to drive a behavior as complex as summiting but does place the worm in an ideal position for altering the activity of host neurons either through chemical or mechanical action. Notably, *Ophiocordyceps* does not invade ant brain tissue (Fredericksen et al. 2017); however, like *D. dendriticum*, some species of zombie fungi (e.g., *Entomophthora muscae*, *Entomophaga grylli, Pandora formicae*, *Strongwellsea castrans*) have been observed to occupy the host brain (Humber 1976; Brobyn and Wilding 1977, 1983; Funk et al. 1993; Elya et al. 2018; Csata et al. 2021). That brain occupancy is not a consistent feature of behavior-manipulating parasites likely reflects that there are multiple routes (e.g., direct and indirect) to modifying host behavior.

As with parasite-induced hyperactivity, our most thorough understanding of a mechanism underlying parasite-elicited summit disease comes from baculoviruses. Several baculoviruses drive summiting in their larval hosts immediately before death, which is thought to facilitate the enhanced dispersal of viral particles (Vasconcelos et al. 1996). Elegant work in *Lymantria dispar* NPV determined that the gene ecdysteroid uridine 4′-diphosphate glucosyltransferase (*egt*) was necessary and sufficient to elicit summit disease in spongy moths (Hoover et al. 2011). The enzyme encoded by *egt* catalyzes a reaction that leads to the inactivation of the larval hormone 20-hydroxyecdysone (20E) blocking pupation as a result (O'Reilly and Miller 1989). Thus, *egt* is hypothesized to drive summiting behavior in spongy moths partly by blocking the normal occurrence of pupation: infected caterpillars continue feeding on plants rather than returning to the ground to undergo metamorphosis, leading them to be elevated at death. However, *egt* does not appear to be required to drive summiting behavior in other NPV-larval systems (Ros et al. 2015). In addition, various studies have shown that light cues and perception thereof are required for summiting, and that summiting hosts have aberrant expression of circadian genes (Han et al. 2017; Bhattarai et al. 2018). All told, it seems likely that there are several overlapping pathways involved in summit disease that vary even among related viruses.

12.2.3 Perimortem Postural Changes

Many zombie fungus-infected insects exhibit perimortem postural changes ranging from the positioning of mouth parts to the splaying of legs and wings. In cadaver-transmitting systems, these postural changes typically occur after summiting but before death. In *Ophiocordyceps*-infected ants, for example, manipulated individuals maintain elevation by biting onto an available substrate immediately prior to death (Hughes et al. 2016). These animals exhibit lockjaw, which recent work suggests may be achieved by penetration of the muscles by fungal tissue as well as the formation of a fungal cage-like structure surrounding mandibular muscle (Fredericksen et al. 2017; Mangold et al. 2019). In *Entomophthora*-infected flies, a manipulated individual extends its proboscis and is then "glued" where standing via secretions that are thought to be produced by the fungi (Brobyn and Wilding 1983). Proboscis extension has been proposed to arise from mechanical force (i.e., the body cavity has become so full that the proboscis cannot remain contracted due to steric hindrance) (Brobyn and Wilding 1983). More recent observations have reported that the proboscis both extends and retracts in the dying fly, suggesting that proboscis movement could be driven either by impingement upon underlying muscles or by directly or indirectly perturbing the action of motor neurons that directly innervate musculature and drive changes in contractility (Elya et al. 2018).

After summiting and adherence in place, zombie flies and beetles exhibit a further postural change, positioning their wings up and away from the dorsal abdomen (Krasnoff et al. 1995; Steinkraus et al. 2017; Elya et al. 2018). The repositioning of the wings serves to create a direct "line of fire" for the fungal spores that will be ejected from the dorsal abdomen. In the case of flies, wing-raising is rapid, typically fully executed in 15 min (Krasnoff et al. 1995). Wing-raising in zombie flies seems that it could possibly arise by several different mechanisms. The fungus could impinge on musculature directly as it grows in the body cavity or could indirectly alter posture by rapid uptake of remaining liquid (dehydration). Alternatively, the fungus could alter the activity of motor neurons, either directly or indirectly. In the case of *Eryniopsis lampyridarum*-infected beetles with wings that take many hours to raise, a mechanical explanation is the more likely.

Similar postural changes have been observed in insects parasitized by non-fungal organisms. Ants infected by *D. dendriticum* also bite down to maintain an elevated position. These ants do not die in place, but instead remain motionless *in situ* for several days (Martín-Vega et al. 2018). This behavior is thought to benefit the trematode by making infected ants more likely to be consumed by their grazing herbivores, which can then spread the parasite via droppings back to the primary snail host (Goater and Colwell 2007). A mechanistic understanding of biting behavior in *D. dendriticum*-infected ants is lacking, although the presence of flukes in the SOG may play a role (Martín-Vega et al. 2018).

12.2.4 Circadian Timing

An extremely common theme among zombie fungi is that they drive changes in host behavior (and ultimately cause death) with stereotyped timing. For example, flies infected with *E. muscae* only exhibit summit disease and subsequent postural changes at sunset (Krasnoff et al. 1995; Elya et al. 2018) and, in the field, as mentioned earlier, ants infected with *Ophiocordyceps* summit and die around solar noon (Hughes et al. 2011). How this timing is achieved is particularly curious as there are likely two molecular clocks at play in each case—one belonging to each the host and the fungus. Molecular clocks are cellular mechanisms that can maintain circadian rhythms in the absence of environmental cues, or Zeitgebers, such as light or temperature. Clocks synchronize, or entrain, to environmental signals to align with the earth's daily cycles and are usually driven by core transcription/translation feedback loops. Clocks have been demonstrated in organisms ranging from bacteria to animals, though their core molecular components vary along mostly phylogenetic lines (Dunlap and Loros 2017). Details of how timing from the clock confers various behaviors and phenotypes are continuously emerging.

In the case of *E. muscae*-infected flies, the favored explanation of how this timing is achieved is that it is not dictated by the host clock. This is derived from the two observations. First, flies infected with *E. muscae* in constant darkness (referred to as "free-running conditions") do not die with rhythmic timing, even though their fly hosts are known to maintain circadian periodicity in the absence of environmental cues (Krasnoff et al. 1995). This suggests that the host clock alone is insufficient to drive the timing of death. Second, flies housed in darkness 72 h after fungal exposure do show rhythmic timing of death, which suggests that there is some machinery present capable of driving timed death in the absence of immediate cues, but that this machinery can only entrain after the onset of infection (Krasnoff et al. 1995). The most likely identity of this mystery machinery is a fungal clock. Though it is very likely that *E. muscae* has a clock of its own, this has not yet been experimentally demonstrated.

On the other hand, the prevailing hypothesis for timing in *Ophiocordyceps*-infected ants is that this is driven by the host clock, which in turn is manipulated by the fungus (de Bekker and Das 2022). Transcriptomic studies have shown that the expression of various clock genes are perturbed at the moment of manipulation (de Bekker et al. 2015; Will et al. 2020) and that foraging rhythms observed in healthy ants are essentially absent in infected individuals (Trinh et al. 2021). The observation that *Ophiocordyceps* continues to produce transcripts in a circadian manner under free-running conditions strongly supports the notion that this fungus has its own molecular timekeeping mechanism (de Bekker et al. 2017b). Details as to how fungal clock activity is linked to host behavior are lacking, but it seems reasonable to hypothesize that host behavior might be altered by the secretion of compounds that alter host physiology, ranging from specifically in neuronal populations to more generally to trigger internal state changes, leading to behavioral outputs.

12.2.5 Sexual Attraction

Some zombie fungi have been observed to alter host sexual behavior, a change that is hypothesized to increase the likelihood of transmission. In the case of *Massospora,* the fungus appears to alter sexual behaviors in infected animals: infected male cicadas produce female-like wing flicks (Cooley et al. 2018) or tolerate physical contact by male conspecifics (Cooley 1999), behaviors that do not occur in healthy males. An increase in sexual behaviors could be an attempt by the host to increase reproductive output before death, but, curiously, these behaviors are only observed in *Massospora*-infected males that actively transmit to another round of hosts and not in infected males that produce resting spores (Cooley et al. 2018). This observation suggests that hypersexuality is driven by the fungus rather than the host. Increased sexual behavior could be related to fungal production of cathinone (Boyce et al. 2019), as amphetamine intake has been associated with increased sexual behaviors in other animals (Frohmader et al. 2010). Alternatively, the fungus could modulate hormonal levels in the host leading to alterations in sexual behavior. At present, the mechanistic basis of this manipulation is unknown.

Entomophthora muscae, on the other hand, enhances transmission by appealing to healthy hosts (Moller 1993; Watson and Petersen 1993; Zurek et al. 2002; Naundrup et al. 2021). Male house flies show a remarkable attraction to late-stage female cadavers, an effect that appears to be mediated by the combined effects of fungal-produced compounds (notably sesquiterpenes), visual cues related to the characteristic death pose as well as endogenous female pheromones (Naundrup et al. 2022). Here, there is no host fitness benefit from attracting mates, as the host is dead, strongly arguing that, again, this is a fungus-driven manipulation rather than a host response to infection. Overall, this work is consistent with many previous observations of fungi employing chemical mimicry to attract hosts, to serve as either transmission vectors or prey (Ngugi and Scherm 2006; Matsuura et al. 2009; Hsueh et al. 2017).

Insect sexual behaviors have also been observed to change in the presence of non-fungal parasites, though we have little insight into their mechanistic underpinnings. One likely manipulated behavior occurs in corn earworm female moths infected with the Hz-2V virus with a primary site of replication in reproductive tissues (Burand et al. 2005). These females produce up to seven times the amount of pheromones made by uninfected females and exhibit hypersexual behaviors, continuing to initiate contact with males and resume calling to attract male conspecifics after mating, behaviors which are typically not observed in healthy individuals (Burand et al. 2005). In addition, healthy females that mated with infected males were more likely to resume calling after copulation than if they mated with uninfected males (Burand and Tan 2006), suggesting that there is some alteration to male reproductive chemistry to dampen normal female refractory period post-mating. As copulation requires close physical contact with a partner, these changes in female pheromone production and sexual behavior are likely to drive increased transmission of the virus to male partners and their subsequent consorts. However, it is possible that these effects could be driven by the host in response to infection as an attempt to increase reproductive output before sterilization takes hold. That the host may be driving these changes might be supported by the observation that infection by another virus, Gonad-specific virus (GSV), also leads to increased pheromonal production (Raina et al. 2000), although, unlike Hz-2 V females, GSV-infected females refuse to mate (Raina et al. 2000). There are other instances of infection by parasites being correlated with increased sexual behavior (milkweed beetle *Labidomera clivicollis* and mite *Chrysomelboai labidomerae*, mite *Unionicola ypsilophora* and midge *Paratrichocladius rufiventris,* cricket *Gryllus texensis* and iridovirus IIV-6/CrIV (McLACHLAN 1999; Abbot and Dill 2001; Adamo et al. 2014)), but, again, it is not yet clear which organisms are driving these changes. The argument has been made that any increase in sexual behavior only benefits the parasite, since these parasites induce sterility in their hosts. However, I do not think we can formally exclude the possibility that these sterile hosts fail to "recognize" their impotency and are driving their own futile attempts to reproduce.

12.3 General Considerations for Understanding Manipulated Behaviors

The five themes I introduced early in this chapter (see **Common themes in behavioral manipulation by zombie fungi)** encapsulate many of the behaviors elicited by zombie fungi, but they are by no means an exhaustive list. In addition, there are likely many more parasitic puppeteers to be described in our world, and more manipulated behaviors out there to be discovered. However, as we continue to broaden our understanding in this realm, it is critical to be guided by parsimony. Just because a behavior occurs in an infected animal and has some apparent level of sophistication, we should not assume that this behavior is manipulated by the pathogen. At a minimum, a truly manipulated behavior is one that confers an adaptive advantage to the parasite and is specifically elicited as a result of infection by that parasite, not just a general sickness response of the host. In general, it is wise to remain skeptical that a behavior reflects manipulation unless there is overwhelming evidence to suggest this is the case. As there are already great pieces on this topic (Poulin 1995; Bhattarai et al. 2021), it will not be fully discussed here.

If all signs point to a behavior being truly the result of parasitic manipulation, it is important to check our innate tendencies to ascribe agency or intent of the hijacking organism, and to keep in mind that all these organisms developed their present methods of host behavior manipulation following broad evolutionary principles. That is, while it may be our impulse to look at a behavior hijacking system and be initially struck by its seeming cleverness or insidiousness, we should resist the urge to embrace complex hypotheses explaining manipulated behaviors in favor of considering simpler ones first. While chemical signaling may underlie a behavior in some cases, mechanical forces and physical limitations of the fungus and animal can be just as crucial in driving behavior. Along these lines, *a priori* reasoning predicts that manipulated behaviors are most likely to occur by ectopic activation of existing host circuitry, not by cobbling together novel circuits. Importantly, all the behaviors that a manipulated host exhibits are things the host can already do—for example, a fly can climb, an ant can bite, a cicada can mate. The fungus is not causing new behaviors to occur but altering the timing of these behaviors to align with promoting the fitness of the fungus rather than the host.

In addition to allowing ourselves to be guided by parsimony, it is also important to avoid assuming that just because two behaviors look alike that they are driven by the same or similar underlying mechanisms (de Bekker et al. 2021). That is, parallel behaviors do not necessarily reflect parallel pathways. This does not mean to suggest that each parasite uses a completely different mechanism to drive similar behavior, but it means that we cannot take for granted that just because we have figured out a mechanism in one system that it will be generally applicable to others. Behavior manipulations that look the same but are driven by different parasites probably reflect one of two possibilities: either these behaviors are so advantageous to the pathogen that they are heavily selected for, or these behaviors are relatively easy to elicit (either there are many ways to drive the same behavior, so there are many local minima that a parasite might stumble across and/or the behavior is governed by readily manipulable circuit elements). Understanding the mechanistic underpinnings of similar behaviors in different systems can help us differentiate between these possibilities. As much as possible, research efforts should be geared toward parallel progress in multiple systems to allow for this comparative analysis.

Finally, as we pursue a deeper understanding of the mechanistic basis of parasite manipulated behaviors, it's important to keep in mind that these behaviors are likely to be the product of alterations in many parallel pathways or many systems targeted simultaneously (Adamo 2013). That is, while it is tempting to hypothesize simple feed-forward mechanisms, evolution is not a linear process, and the robustness of zombified behaviors is very likely a reflection of redundancy in their mechanisms of action. We may find examples where a single gene appears to have a large effect on behavior [e.g., *egt* in baculovirus (Hoover et al. 2011)], but it is very likely that what remains to be discovered are smaller pieces of the whole manipulation story. Ultimately, though this may be a more challenging course to understanding these compelling phenomena, perhaps it will be more satisfying when we finally crack the case.

12.4 Onward and Upward: Prospects in Understanding Behaviors Driven by Zombie Fungi

Despite our keen interest and immense efforts, we still have a long way to go toward fully understanding how zombie fungi alter the behavior of their hosts. Although demonstrating the mechanistic basis of manipulated behavior has proved challenging for many reasons, the future of the field is brighter than ever before.

Historically, one of the main impediments to our mechanistic understanding of the behaviors elicited by zombie fungi has been the lack of experimentally tractable systems. However, notable progress has been made on at least two fronts: both *Ophiocordyceps* and *Entomophthora muscae* infections can now take place entirely in a laboratory setting (Elya et al. 2018; Will et al. 2020). A recently isolated strain of *E. muscae* infects the fruit fly *Drosophila melanogaster* (Elya et al. 2018), an insect that has been extensively studied and developed as a model organism to study neurobiology and behavior. In the *E. muscae*-fruit fly system, researchers can leverage the *D. melanogaster* genetic toolkit address a virtually endless number of hypotheses, including whether gravitactic or phototactic pathways are involved in summiting behavior and whether the fly's clock is involved in determining the time of its death. In addition, the universal genomic editing tool CRISPR-Cas9 holds great promise to be applied to lab-grown organisms, such as *E. muscae* and *Ophiocordyceps*, to test the causal role of fungal genes in myriad host–pathogen interactions, including manipulated behaviors. Taking advantage of these laboratory systems will be key in demonstrating causal relationships between host and fungal genotypes and behavioral phenotype and allow us to improve our understanding in ways that simply were not possible before.

Even for systems that are not yet laboratory ready, the ongoing improvement of existing and development of new technologies has opened research avenues previously inaccessible. For example, long read and chromatin conformation capture sequencing is enabling the assembly of bloated, repeat-rich entomophthoralean genomes (Hajek et al. 2022), and improved metabolomics detection technology recently allowed the discovery of mind-altering compounds in *Massospora*-infected cicadas (Boyce et al. 2019). In addition, improved imaging methods have offered insight into the structure of fungal and host tissues (Fredericksen et al. 2017) and offer the future discovery of morphological correlates of parasitized insects through additional structural analyses. The recent discoveries in the field have in turn generated a level of excitement that seems likely to translate to expansion of the zombie fungus field. With additional brains and hands working together on these systems, it seems inevitable that we will encounter additional culturing breakthroughs to bring new systems into the laboratory. And so, our efforts will continue to feed forward.

Another challenge within the realm of behavior manipulation has been the difficulty of observing and analyzing zombified behaviors efficiently. Many of these behaviors are not only subtle but also occur clustered in daily bursts, making them challenging to study by traditional analog ethological methods. The widespread use of computational methods to track and analyze animal behavior offers a huge opportunity to help move our understanding forward. For lab-tractable zombie systems, automated ethology can help us with establishing objective criteria for staging organisms, which can allow us to perform comparative analyses of metabolites and gene expression and allow us to run behavioral screens under different abiotic conditions (in non-model hosts) or in various host genotypes (in model hosts). Even for systems that are not yet lab-tractable, we can marry ethological platforms with field work to expand our understanding of typical host behavior (e.g., better understand circadian patterns) and test specific mechanistic hypotheses (e.g., does the application of cathinone or psilocybin to healthy cicadas recapitulate either of the locomotion or mating phenotypes observed in *Massospora*-infected individuals?) We have already begun to see the promise of these approaches in the *Ophiocordyceps*-ant system (Trinh et al. 2021).

Ultimately, the prospect of understanding how zombie fungi elicit behavioral changes offers much more than just satiating scientific curiosity. Taking advantage of "zombie" insects as model systems (i.e., studying hijacked behaviors) will serve to complement traditional neuroscience approaches (i.e., studying intrinsic behaviors) and will serve to enrich our understanding of how behavior is generated and executed in animals.

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