Evolutionary ecology of an obligate and behaviorally manipulating insect- pathogenic fungus, *Entomophthora muscae*

Henrik H. De Fine Licht[1], Sam Edwards[1,2], and Carolyn Elya[3]

[1]Department of Plant and Environmental Sciences, University of Copenhagen
[2]Living Systems Institute, Department of Biosciences, College of Life and Environmental Sciences, University of Exeter
[3]Department of Organismic and Evolutionary Biology, Harvard University

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Abstract

Some insect-pathogenic fungi have evolved the ability to behaviorally manipulate their insect hosts. This has required the fungi to develop intricate mechanisms of infection, proliferation, and behavioral hijacking, which has led to speculation that behaviorally manipulating fungi must only infect a narrow range of hosts. One well-known example is the insect-pathogenic fungus *Entomophthora muscae*, which infects dipterans. Here, we present the different stages of the life cycle of *E. muscae*, focusing on the unique adaptations that allows the fungus to enter and proliferate inside its hosts, the possible ways it manipulates behavior, how the fungus exits the killed host to seek new susceptible hosts, and the ecological implications of these adaptations for determining the host range and intra-specific variation of *E. muscae*. We address the biology of *E. muscae* from an evolutionary ecology perspective and discuss the capacity of the fungus for behavioral manipulation within an extended phenotype framework. We highlight areas where further research is needed to fully develop *E. muscae* as a model system for host-pathogen research, for example to address questions relating to fitness consequences of an infection.

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[1] Department of Plant and Environmental Sciences, University of Copenhagen, 1871 Frederiksberg, Denmark
[2] Living Systems Institute, Department of Biosciences, College of Life and Environmental Sciences, University of Exeter, Exeter, United Kingdom
[3] Department of Organismic and Evolutionary Biology, Harvard University, Cambridge, Massachusetts 02138, United States

Abstract

Some insect-pathogenic fungi have evolved the ability to behaviorally manipulate their insect hosts. This has required the fungi to develop intricate mechanisms of infection, proliferation, and behavioral hijacking, which has led to speculation that behaviorally manipulating fungi must only infect a narrow range of hosts. One well-known example is the insect-pathogenic fungus *Entomophthora muscae*, which infects dipterans. Here, we present the different stages of the life cycle of *E. muscae*, focusing on the unique adaptations that allows the fungus to enter and proliferate inside its hosts, the possible ways it manipulates behavior, how the fungus exits the killed host to seek new susceptible hosts, and the ecological implications of these adaptations for determining the host range and intra-specific variation of *E. muscae*. We address the biology of *E. muscae* from an evolutionary ecology perspective and discuss the capacity of the fungus for behavioral manipulation within an extended phenotype framework. We highlight areas where further research is needed to fully develop *E. muscae* as a model system for host-pathogen research, for example to address questions relating to fitness consequences of an infection.

Introduction

The fungal genus *Entomophthora* is composed of host-specific insect pathogens that can cause epizootics in their various insect hosts (Elya and De Fine Licht, 2021). The type species of the genus is the so-called "zombie fly" fungus *E. muscae* sensu stricto (s.s.), which is well-known for being able to control the behavior of infected flies and forcing them to die and sporulate at elevated positions (Keller *et al*., 1999). The genus *Entomophthora* belongs to the subphylum Entomophthoromycotina within the phylum Zoopagomycota among the early-diverging clades of the Kingdom Fungi (Mycota) (Fig. 1) (Spatafora *et al*., 2016). As such, *E. muscae* is only distantly related to the more widely known fungal phyla Ascomycota and Basidiomycota. While many fungal groups show adaptations to live in or on insects (Humber, 2008), *E. muscae* is perhaps one of the clearest examples of a fungus adapted for obligate insect pathogenesis.
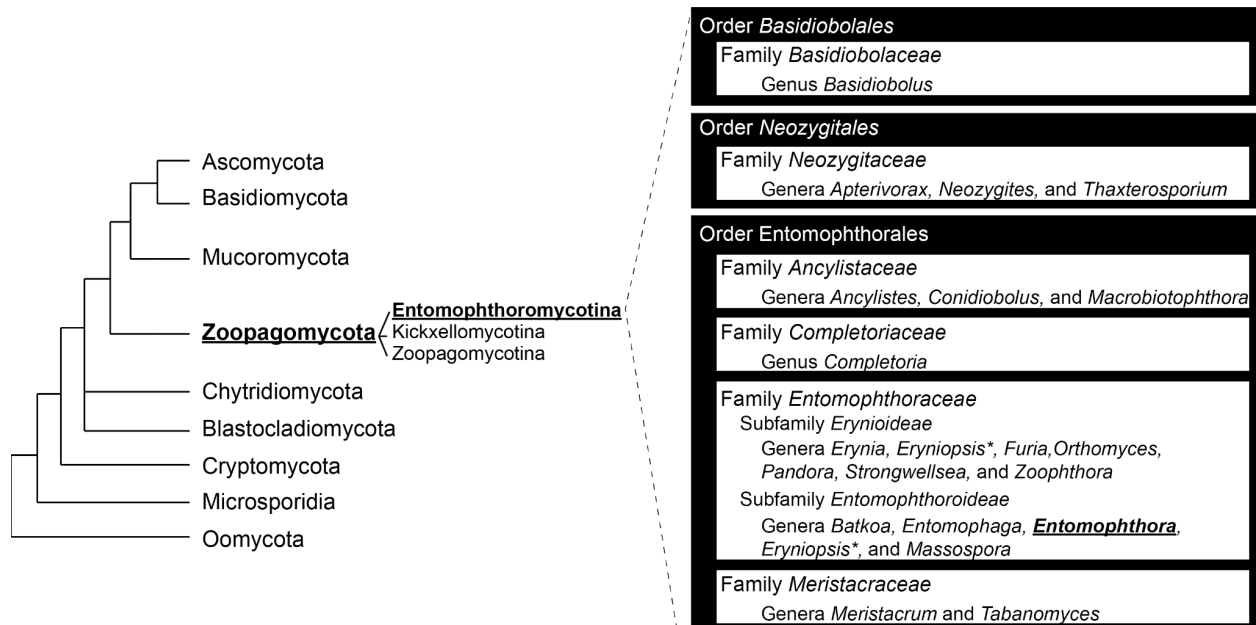
Fig. 1. Phylogenetic placement of the genus *Entomophthora*. To the left, a schematic phylogeny shows the placement of the subphylum Entomophthoromycotina within Zoopagomycota (branch lengths not to scale). To the right, the currently recognized three orders within Entomophthoromycotina: Basidiobolales, Neozygitales, and Entomophthorales, where *E. muscae* is placed within the family Entomophthoraceae of the latter class. The asterisk next to *Eryniopsis* denotes paraphyly (appearance in both Erynioideae and Entomophthoroideae).

The first scientific descriptions of *E. muscae* started to appear more than 150 years ago (Cohn, 1855; Brefeld, 1870, 1871; Thaxter, 1888). Originally described as *Empusa muscae* from infected house flies (*Musca domestica*) (Hall and Bell, 1962), the characteristic sight of a dead fly surrounded by a spore halo had likely not been a rare sight historically but just escaped earlier scientific scrutiny. In the last 150 years, more species of *Entomophthora* have been described and a recent overview of the literature count at least 21 currently recognized species in the genus *Entomophthora* (Elya and De Fine Licht, 2021). Recent taxonomic investigations have revealed that *E. muscae* is part of a species complex, which in addition to *E. muscae* consists of *E. ferdinandii, E. scatophagae,* and *E. schizophorae* (MacLeod *et al*., 1976; Keller, 2002). These species are morphologically highly similar though differ in the range of host species they infect

and in the number of nuclei inside infectious spores (conidia), which have historically been important traits for identification to species (Keller, 2002).

The fungus *E. muscae* is considered a host-specific insect pathogen with a very narrow host range, and likely only naturally infects a single host species per genotype (Jensen and Eilenberg, 2001; Jensen *et al.*, 2001, 2006; Gryganskyi *et al.*, 2013b). Genome-wide comparisons provide further support for the presence of specific *E. muscae* "host-types" associated with, for example, house flies (*Musca domestica*), cabbage flies (*Delia radicum*) (De Fine Licht *et al.*, 2017), and fruit flies (*Drosophila melanogaster*) (Elya *et al.*, 2018). Because the type description of *E. muscae* was from a house fly (Keller *et al.*, 1999), related isolates from house flies should ideally be designated *E. muscae* s.s, whereas isolates from other dipteran species more appropriately should be designated *E. muscae* sensu lato (s.l.) (Keller, 2002).

While the natural host range of *E. muscae* has consistently been found to be narrow, the observed host range, which is the suite of species that are susceptible when artificially exposed to *E. muscae,* is broader (Steinkraus and Kramer, 1987; Gryganskyi *et al.*, 2013b; Becher *et al.*, 2018). Local adaptation of *E. muscae* to specific host species is thus the norm but does not preclude the occasional observation of infection of novel host species in nature (e.g., Skovgård and Steenberg, 2002; Gryganskyi, 2013b). These characteristics have led to many efforts over the years to develop and utilize *E. muscae* as a biological control agent that could be applied as a targeted approach against specific dipteran pest species. However, the highly adapted and obligate life history of *E. muscae* has so far prevented the commercialization of, for example, *in vitro* grown infectious conidia. In addition, insects are increasingly being produced as an environmentally friendly protein substitute compared to conventional meat production, and house flies have been suggested as a way of converting cow manure into protein rich animal feed (Hussein *et al.*, 2017). However, *E. muscae* may be a real concern for house fly production, having already caused severe losses in industry (Eilenberg *et al.*, 2015; S. Edwards, unpubl. data), and a better understanding of this insect disease thus has a more applied perspective. In the remainder of this chapter, we will outline the unusual biology and evolutionary ecology of *E. muscae.*

Life cycle of *Entomophthora muscae*
The life cycle of *Entomophthora muscae* follows the general pattern of infection exhibited by other endoparasites (Fig. 2). First, the fungus enters the body of the host, proliferates inside, and finally leaves the host to seek a new one once the current host's resources are depleted (Hansen and De Fine Licht, 2017; Elya *et al.*, 2018). These processes are described in detail below.
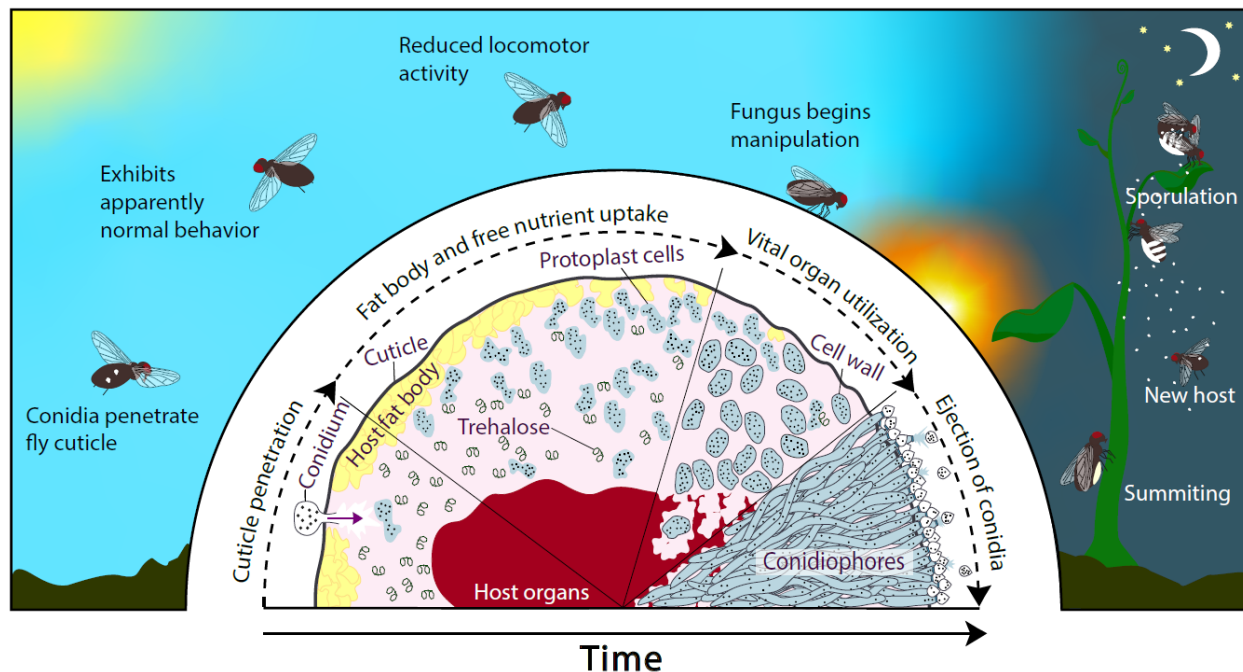
Fig. 2. Schematic illustration of the life cycle of *Entomophthora muscae*. Internal fungus development is depicted inside, and behavioral effects on the host are depicted outside of the half circle, respectively. From left to right: Infectious conidia land on the insect, penetrate the cuticle and release their contents into the fly's hemocoel to begin growth as protoplasts. The protoplasts proliferate inside the insect host using the fat body and trehalose for energy while avoiding destruction of vital organs (e.g., brain, gut, gonads). Initially, the infected fly does not demonstrate overt behavioral changes. The protoplasts proliferate exponentially and continue utilizing host resources. In fruit flies, hosts start to exhibit reduced locomotor activity about 24 hours prior to their death (Elya *et al.*, 2018). By the afternoon on the final day of the host's life (on the sixth or seventh day post conidia penetration in house flies, or fourth or fifth day in fruit flies), non-vital host resources for fungal growth have been exhausted. The protoplasts now invade the internal organs of the host and form cell walls, beginning the transition to conidiophores. Around this time, the fly's behavior changes dramatically: the fly is made to summit to an elevated position, affix its proboscis to the substrate and raise its wings, then dies in a stereotyped death pose. After the fly's death, conidiophores pierce through intersegmental membranes of the abdomen before forcibly ejecting new infectious conidia onto the next unlucky flies.

Cuticle penetration

Entomophthora muscae primary conidia are actively discharged from conidiophores that form within freshly killed hosts (de Ruiter *et al.*, 2019). When a conidium lands on a new suitable host, it germinates, forming a germ-tube through the host cuticle, and releases the cytoplasmic content of the conidium into the host hemocoel (body cavity) (Fig. 2). The process of breaching the host cuticle is achieved through a combination of hydrolytic (digestive enzymes in the form of chitinases and lipases) and mechanical (turgor pressure) forces (Brobyn and Wilding, 1983). Germination takes between two and 24 hours (Brobyn and Wilding, 1983). Conidia are able to enter the fly from any point of the body (thorax, head, legs, wing veins) (Brobyn and Wilding, 1983), but abdominal invasion is ideal as the fungus can immediately encounter the nutrient-replete fat body tissue. Germination of conidia within *Entomophthora* requires high levels of humidity (Kramer, 1980a; Keller *et al.*, 1999; Elya and De Fine Licht, 2021). While most of the enzymes used by *E. muscae* to penetrate the fly cuticle are unknown, genome-wide transcriptome analyses of *E. muscae* have revealed a rich set of cuticle-degrading enzymes (De Fine Licht *et al.*, 2017; Elya *et al.*, 2018). Most notably, *E. muscae* contains a large repertoire of subtilisin-like serine proteases (SLSPs) that degrade chitin-associated proteins in the insect procuticle, the

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chitinized part of the insect cuticle (Arnesen *et al*., 2018). Compared to other ascomycete entomopathogenic fungi, *E. muscae* contains a unique group of SLSPs that are otherwise only known from Bacteria, Oomycota, and other early-diverging fungi such as Cryptomycota and Microsporidia (sensu Strassert and Monaghan, 2022). This particular group of SLSPs has for example not been found in other early-diverging fungal lineages of Kickxellomycotina (Zoopagomycota) and Mucoromycota (Fig. 1), which suggests a unique evolutionary trajectory potentially related to insect adaptation (Arnesen *et al*., 2018).

Within-host processes
Following penetration of the cuticle, the within-host processes of nutrient uptake, exponential growth, and resource depletion begin (Fig. 2). During fungus growth, irregular shaped hyphal bodies multiply throughout the host as multinucleate protoplasts, cells without complete cell walls (Brobyn and Wilding, 1983; Carruthers *et al*., 1985; Carruthers and Haynes, 1985; Eilenberg, 1987a; Boomsma *et al*., 2014; De Fine Licht *et al*., 2016). *Entomophthora muscae*'s protoplastic growth has been hypothesized as a mechanism to evade the fly's immune response (Brobyn and Wilding, 1983; Boomsma *et al*., 2014; De Fine Licht *et al*., 2016). The evasion of the host immune response by *E. muscae* was suggested based on microscopic observations that insect hemocytes did not recognize protoplasts from *Entomophaga aulicae* and *Entomophthora egressa* (Dunphy and Nolan, 1980; Beauvais *et al*., 1989). Although hemocytes do not appear to recognize fungal protoplasts during the infection, the host insects clearly respond to infection by *E. muscae*. This is evident as fruit fly immune gene expression is elevated within 24 hours after infection (Elya *et al*., 2018), but whether this is due to the mechanical injury of having fungal appressoria-like structure(s) penetrate through the cuticle and/or active recognition of growing protoplasts in the hemocoel remains an open question.

Once inside, the host provides *E. muscae* with essential resources for growth. The fungus initially grows exponentially until resources start to become depleted and fungal growth reaches a plateau giving rise to a characteristic logistic growth curve (Hansen and De Fine Licht, 2017). To access and utilize essential nutrients, *E. muscae* uses several enzymes to break down host cell membranes, such as lipases and trehalases, which eventually lead to host starvation (De Fine Licht *et al*., 2017). Other host responses to infection include reduced activity rate of infected house flies (Bick *et al*., 2021) and fruit flies (Elya *et al*., 2018), reduced reproductive fitness of infected house flies (Eilenberg, 1987b; Watson and Petersen, 1993), and decreased expression of metabolic genes (Elya *et al*., 2018). All these observations are consistent with *E. muscae* protoplasts effectively starving their host as they continue to proliferate. Towards the end of *E. muscae* proliferation - after six to seven days in infected house flies and four to five days in infected fruit flies (Hansen and De Fine Licht, 2017; Elya *et al*., 2018) - the depletion of available nutrients triggers the protoplasts to develop cell walls (Gryganskyi *et al*., 2017) (Fig. 2). The formation of cell walls in an infection with *E. muscae* roughly coincides with the onset of host behavior manipulation (see "Extended phenotypes of *E. muscae*" below) and occurs approximately 12–24 hours before the death of the host, depending on the fly species (Krasnoff *et al*., 1995; Hansen and De Fine Licht, 2017; Elya *et al*., 2018).

Host death is thought to occur from tissue consumption and/or by immune collapse due to overwhelming growth of hyphae and not from fungus-produced toxins (De Fine Licht *et al*., 2016). Toxin-producing entomopathogenic hypocrealean fungi (Sordariomycetes), such as *Beauveria*

bassiana and *Metarhizium* species, can infect a variety of insects, including house flies, but do not proliferate extensively throughout the host body cavity until after fungus-released toxins have killed the host (Anderson *et al*., 2011). In contrast, *E. muscae* exhibits a fast growth strategy while the host is still alive to overcome host immune defenses, with very limited fungal growth after the host is dead (Hansen and De Fine Licht, 2017). Biotrophic growth while the host is alive is predicted to correlate with high host specificity (Boomsma *et al*., 2014). In line with this, comparative transcriptomics of two closely related *E. muscae* host-types naturally infecting house flies and cabbage flies (*Delia radicum*), respectively, suggested that evasion of host immune system and intricate nutrient acquisition mechanisms are contributing factors to the evolution of high specificity within the genus *Entomophthora* (De Fine Licht *et al*., 2017).

Infection of new hosts
The moribund fly will raise its wings moments before death; afterwards conidiophores begin to penetrate through the intersegmental membranes of the abdomen (Krasnoff *et al*., 1995; Gryganskyi *et al*., 2017; Elya *et al*., 2018; de Ruiter *et al*., 2019). At the tip of each conidiophore, a conidium forms. The building of pressure by the cytoplasm that accumulates inside conidiophores puts pressure on the cell wall linking the conidium and conidiophore (called a septum), leading to the conidium being forcefully ejected via a water cannon mechanism (de Ruiter *et al*., 2019). For 20–24 hours in *E. muscae*-infected house fly cadavers, thousands of conidia are shot out of the fungal mass exposed from the abdomen of the old host, forming a halo of spores surrounding the cadaver (Mullens, 1985; Carruthers and Haynes, 1986; Elya *et al*., 2018; Naundrup *et al*., 2022) (Fig. 3). Conidial ejection can also be triggered by mechanical stimulation, e.g., touch from a curious live fly (de Ruiter *et al*., 2019). This process of stimulus-based discharge is aided by the fact that *E. muscae* produces compounds that attract flies to inspect the sporulating cadaver (see "Extended phenotypes of *E. muscae* below") (Fig. 3).

Fig. 3. Halo of fungal spores surrounding a fly cadaver killed by *E. muscae*. These two pictures are taken from the inside and show a dead fly attached to the outside of a window. Released *E. muscae* spores forming a halo surrounding the fly cadaver can be seen as a white cloud. Picture was taken through a window on the fifth floor in Lichtenberg Berlin, Germany, January 2023 (Photo: Andrea B. Tiesler).

Conidia that land on a potential host cuticle produce can proceed to germinate. If a conidiophore-launched conidium, referred to as a primary conidium, does not land on a suitable host, it can form a secondary conidium, which is a smaller, potentially more infectious propagule (Bellini *et al*., 1992). Secondary conidia are dispersed via papillar eversion: hydrostatic pressure builds up inside the secondary conidia until the cell wall formed between the primary conidiogenous cell and the

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secondary conidium breaks (Eilenberg *et al*., 1986, 1990; Humber, 2016). This results in propulsion like children's jumping popper toys. Whether or not an infectious conidium is able to penetrate the host cuticle, which is a formidable barrier for fungal pathogens of insects (Humber, 2008), is likely one of the determining factors for the host specificity of *E. muscae.* Naturally-occurring infections are host specific, but in laboratory settings *E. muscae* isolates can infect other dipteran species with varying success (Steinkraus and Kramer, 1987; Jensen *et al*., 2006). For example, an isolate of *E. muscae* s.s. from house flies can infect the spotted wing drosophila, *Drosophila suzukii*, but shows clear developmental and physiological limitations (Becher *et al*., 2018). Host specificity is not only one-sided, as genotype specific infection patterns by an *E. muscae* isolate have also been shown within host species. By comparing *E. muscae* infections of a diverse genotype panel of *D. melanogaster* lines, it has been possible to show that host fly genotypes vary in susceptibility to *E. muscae* infections (Wang *et al*., 2020).

The fungus *E. muscae* does not always produce conidiophores and conidia. The fungus may also form thick-walled resting spores inside decaying cadavers (Thomsen and Eilenberg, 2000; Thomsen *et al*., 2001). These spores are believed to function as an overwintering stage in temperate regions, as they make their way into the upper soil layers as decaying cadavers fall to the ground and dissolve. Resting spores may or may not be the result of a sexual event but involves the fusion of two hyphae or hyphae-like cells that form a zygospore (Humber, 2016, 2012). It is not well understood what triggers the production of resting spores instead of conidia or how/when resting spores germinate and are able to infect new flies.

Extended phenotypes of *E. muscae*
One of the most notable characteristics of *E. muscae* is its ability to elicit behavioral changes in its host (Krasnoff *et al*., 1995; Roy *et al*., 2006; Lovett *et al*., 2020a; de Bekker *et al*., 2021). The behaviors exhibited by a host who will imminently succumb to death by *E. muscae* are precisely timed, highly stereotyped, and though they are of no obvious use to the dying insect, provide clear benefits for fungal dispersal. Pathogens that take control of host behaviors to increase their own fitness exhibit "extended phenotypes", wherein pathogen genes exert a phenotypic effect outside the organism in which they reside (Dawkins, 1982, 2012). During the final hours of the life of an *E. muscae*-infected fly (about six hours before sunset), the soon-to-be cadaver will exhibit "summit disease" (also known as Wipfelkrankheit or tree top disease), typically climbing to an elevated location in its local environment (Krasnoff *et al*., 1995) (Figs. 2, 3). Once elevated, the fly will cease walking and extend its proboscis, which, upon making contact with the surface that the fly is standing on, will become adhered via sticky secretions (Krasnoff *et al*., 1995; Elya *et al*., 2018). The nature of these secretions is still unresolved (Bałazy, 1984; Brobyn and Wilding, 1989).

If the substrate is narrow, such as a plant stem, the fly will wrap its legs around the stem (Berisford and Tsao, 1974). Finally, attached via legs and/or a tightly glued proboscis, the fly's wings will move up and away from its dorsal abdomen, coming to rest at an acute angle above the fly's back (Krasnoff *et al*., 1995; Elya *et al*., 2018). After striking this final pose, the fly dies and the fungus emerges through the cuticle within hours, eventually launching infectious spores into the surrounding environment to infect new hosts (Elya *et al*., 2018; de Ruiter *et al*., 2019; Naundrup *et al*., 2022). These temporally-gated and highly-specific behavioral changes are distinct from general host response behaviors to sickness (e.g., lethargy, decreased feeding and reproduction) that can be evoked by a variety of pathogens and that either have been demonstrated or could

reasonably be predicted to offer some benefit to the host (Hart, 1988). Thus, it is widely accepted that *E. muscae* drives these behavioral changes in its doomed fly hosts rather than these behaviors being some unintended byproduct of infection (de Bekker *et al*., 2021).

Interestingly, summit disease is a behavior that is elicited by a variety of so-called "mind-control" pathogens, ranging from other entomophthoralean fungi to the hypocrealean *Ophiocordyceps* (the fungi responsible for "zombie ants") to trematodes and even viruses (Lovett *et al*., 2020a; de Bekker *et al*., 2021). That such a phylogenetically diverse suite of pathogens can induce summit disease suggests that (1) the ability to evoke summit disease has several independent origins, and (2) the mechanism to elicit summiting behavior is potentially less complex to evolve and may involve similar pathways across host-pathogen systems (Lovett *et al*., 2020b; de Bekker *et al*., 2021). The fitness benefits of host behavior manipulation are likely large and the ability to cause a host to summit may thus have an outsized effect on pathogen fitness such that this is a highly favored trait in many environments.

Another striking *E. muscae* phenomenon has been observed in house flies infected with *E. muscae* s.s.: healthy males tend to be attracted to and attempt to mate with *E. muscae*-infected female cadavers (Mullens *et al*., 1987; Møller, 1993; Watson and Petersen, 1993; Zurek *et al*., 2002; Hansen and De Fine Licht, 2019). Very recent work has provided additional evidence of this phenomenon and found that volatile compounds (including sesquiterpenes and putative pheromone mimics) that have a likely fungal origin are involved in mediating this fatal attraction (Naundrup *et al*., 2022).

There is evidence suggesting the fungus may be responsible for other behavior effects in infected hosts, though more work remains to be done to confirm that these are true manipulations. Most notably, infected house flies have been shown to alter their thermal preference over the course of infection. Initially, infected house flies prefer warm substrates: flies allowed to wander a thermal gradient within the first 48 hours after exposure demonstrated behavioral fevering (an immune behavior that is not unique to *E. muscae*-infected house flies, but rather elicited by several pathogens in various ectothermic species (Watson *et al*., 1993; Kalsbeek *et al*., 2001). However, as the flies approached the end of life, their preferences shifted and they chose to occupy cooler areas (Watson *et al*., 1993). Cool-seeking has also been demonstrated in fruit flies infected with the generalist pathogen *Metarhizium robertsii*, but in this case the behavior occurs much earlier in the infection (Hunt *et al*., 2015). That this late cool-seeking behavior appears to be unique to moribund *E. muscae*-infected flies, coupled with the fact that cool temperatures are optimal for *E. muscae* growth, suggests that thermal preference may be another host behavior driven by *E. muscae* to enhance fungal fitness. Of course, additional work is needed to test this hypothesis before thermal preference manipulation can be classified as a manipulated behavior and an extended phenotype.

Except for male attraction to infected female cadavers, the mechanistic underpinnings of these extended and putative extended fungal phenotypes remain enigmatic (de Bekker *et al*., 2021). In Figure 3, some potential hypotheses for these behavior alterations are summarized. For the more simplistic of these altered behaviors (proboscis extension and wing raising), it is possible that these could be driven either by mechanical force (e.g., fungus impinging on musculature) or by neuronal manipulation (e.g., fungus altering activity of motor neurons) (Brobyn and Wilding, 1983). The

more complex behaviors (thermal preference, summiting, time of death) almost certainly have a neural basis, though there are many possibilities as to how the fungus drives the circuits underlying these behaviors. *E. muscae* is known to invade the central nervous system of its host well before any of the behavioral alterations occur (Brobyn and Wilding, 1983; Elya *et al.*, 2018). It is possible that this invasion mediates direct distortion or degradation of regions important for controlling circuits of interest and/or permits fungal cells direct access to neurons to manipulate by chemical signaling. Given the behavioral phenotypes observed, the fungus might be predicted to impact gravitactic, circadian, sleep (Lovett *et al.*, 2020b), or thermal processing circuitry, or more broadly influence cell activity in neurosecretory centers. Where and how broadly *E. muscae* affects neural activity is still unclear. In addition, it is also possible that the presence of *Entomophthovirus*, an iflavirus found to infect nearly all of the characterized *E. muscae* isolates, plays a role in driving fungal–host interactions via a thus far undiscovered mechanism (Coyle *et al.*, 2018). Clearly, determining the mechanistic underpinnings of many *E. muscae*-driven behaviors in fly hosts is a rich scientific vein waiting to be tapped.
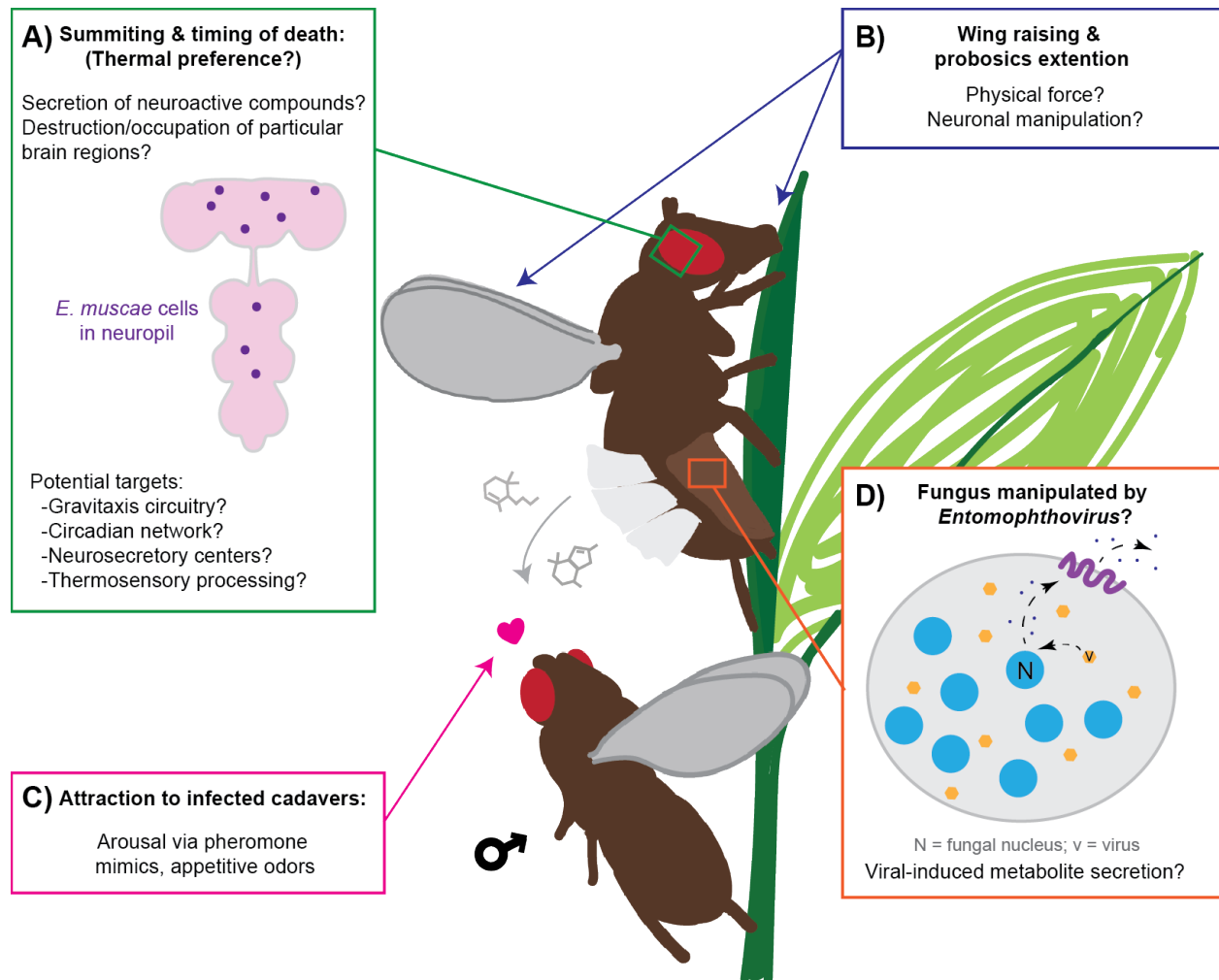
Fig. 3. Summary of hypothesized mechanisms for behavior alterations in *E. muscae*-infected flies. A) Proposed mechanisms underlying summiting, timing of death, and shifts in host thermal preference (the last of which has yet to be conclusively demonstrated to be a manipulated behavior). Cartoon depicts the fly nervous system (brain and ventral nerve cord) showing *E. muscae* cells (purple) present in neuropil. B) Potential mechanisms underlying wing raising and proboscis extension. C) Demonstrated mechanisms of attraction of healthy males to *E. muscae*-killed females

(Naundrup *et al.*, 2022). D) There is yet no specific behavioral alteration in which *Entomophthovirus* is hypothesized to play a role, but the virus could impact *E. muscae* in a variety of ways that in turn affect how *E. muscae* interacts with the host to alter behavior. Diagram of an *E. muscae* cell (gray outline) containing *Entomophthovirus* (v, depicted as yellow hexagons). Blue circles (N) are *E. muscae* nuclei. *Entomophthovirus* could drive production of behavior-altering compounds by the fungal host (dark blue specks), which are depicted as being transported through a transmembrane channel (purple).

Chemical ecology of *E. muscae*

Anyone who has smelled *E. muscae*-killed fly cadavers can attest to their distinct bouquet of odors. Recently the chemical ecology of *E. muscae* has begun to be unraveled. Chemical signaling underlies many insect–fungal interactions, where volatile chemicals can function as either attractants or deterrents. A sporulating, *E. muscae*-killed female house fly female is attractive to healthy males, which in many cases will attempt to mate with such cadavers and become covered in deadly spores in the process (Møller, 1993; Zurek *et al.*, 2002; Naundrup *et al.*, 2022). Both the distinct posture of female cadavers, with wings spread horizontally away from the body, and the increased size of the swollen abdomen, with the fungus characteristically protruding from between the sternites and tergites as conspicuous white fungal bands, provide some visual stimuli to males (Møller, 1993). However, volatile chemicals are clearly also involved in the maladaptive behavioral response of males. An obvious candidate would be the female house fly sex pheromone (Z)-9-tricosene, however it is not involved in the increased male attraction to *E. muscae*-killed females (Zurek *et al.*, 2002).

Using an untargeted gas chromatography mass spectrometry (GC-MS) approach, a recent study provided evidence that a combination of volatile sesquiterpenes produced by *E. muscae* and an increase in certain natural house fly cuticular hydrocarbons mediate this attraction (Naundrup *et al.*, 2022). It is notable that the fungus *E. muscae* contains all genes required for sesquiterpene synthesis and several genes in the fungal pathway are actively expressed in female cadavers (Naundrup *et al.*, 2022). Combining these chemical analyses with behavior data suggest an attraction mechanism whereby males are lured from a distance by a unique fungal bouquet, then drawn in close by altered cuticular hydrocarbon profiles of *E. muscae*-killed females. Since most volatile compounds are not normally encountered in flies, it is still an open question whether the chemical attraction is sexual, as, for example, the male flies may also be lured in closer by the smell of food. However, the physical mating attempts once in the vicinity of a cadaver suggest that the chemical and physical appearance of the cadaver triggers a maladaptive mating response in males.

In the study by Naundrup *et al.* (2022), it was also observed that flies respond to conidia of *E. muscae* and appear to "taste" or eat the conidia by proboscis extension. Both insect attraction and repellency to fungal conidia is known from other insect–fungal interactions (Roy *et al.*, 2006; George *et al.*, 2013). For entomopathogenic fungi, having insects being attracted to conidia would seem like a great advantage, whereas there should be strong selection for insects to avoid such infectious propagules. However, while certainly putting them at a risk of infection by inspecting *E. muscae* conidia with their proboscides, it remains to be shown whether this behavior actually increases the chance of fungal infection.

**Temporal and spatial occurrence of *E. muscae***

Our current understanding of *E. muscae*'s global distribution is sparse, owing to relatively limited and sporadic sampling efforts compared to other fungi. Among the richest sources of *E. muscae* sightings, observations reported on the citizen science website iNaturalist (https://www.inaturalist.org/) still show a clear bias in reports of *E. muscae* in western regions, mostly the United States and Europe (Fig. 4). This is almost certainly due to knowledge of *E. muscae* in these regions (most of the scientists who have studied *E. muscae* and related species are based in these locations) and accessibility to this online resource (it is only available in English). This geographic bias is part of a broader phenomenon also observed for other fungal groups such as Leotiomycetes (Quandt and Haelewaters, 2021). Still, *E. muscae* sightings have been reported across South America, sub-Saharan Africa, Asia, and Australia. Combining these data with (1) *E. muscae*'s apparent preference for mild temperatures (summarized in Elya and De Fine Licht, 2021), (2) that observations of *E. muscae* are most frequent during late spring, summer, and early fall, which are the mildest part of the year (Fig. 5; Eilenberg and Philipsen, 1988; Watson and Peterson, 1993; Six and Mullens, 1996), and (3) the known global distribution of dipteran hosts, it does not seem unreasonable to hypothesize that *E. muscae* may be broadly distributed across temperate regions worldwide. Additional environmental sampling is sorely needed to resolve the extent of *E. muscae*'s natural geographical range.
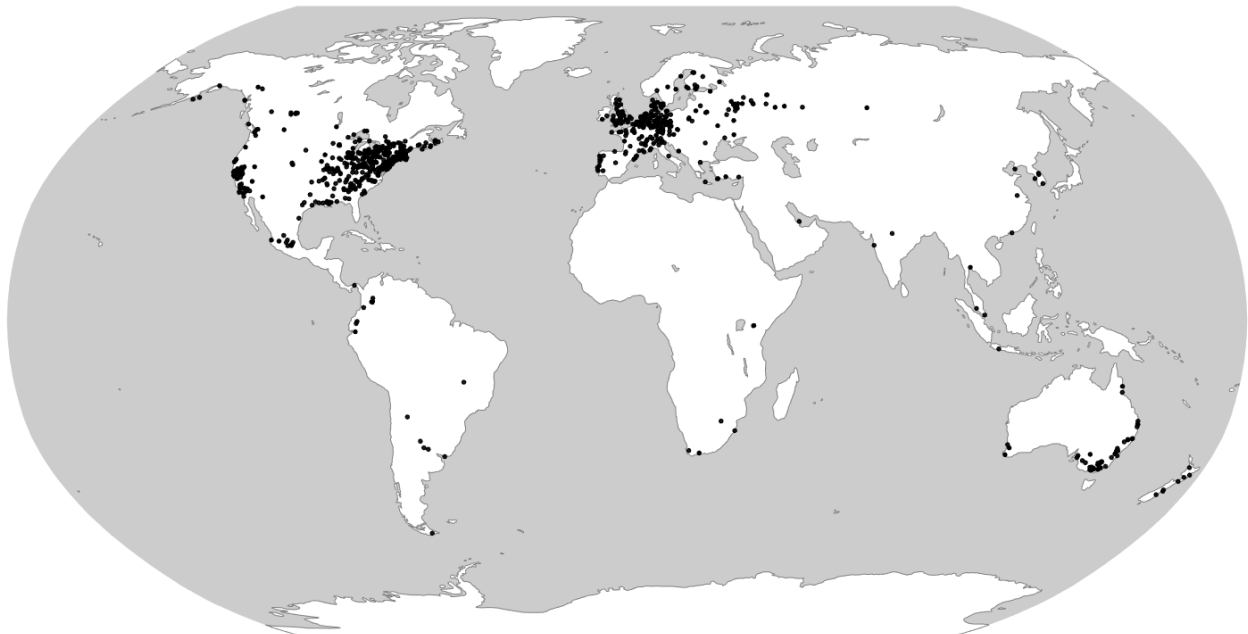
Fig. 4. Global geographical distribution of *Entomophthora muscae*. Each black dot represents one observation of fungus identified as *Entomophthora muscae* from either the Global Biodiversity Information Facility (accessed 8 July 2020, https://doi.org/10.15468/dl.72ww8a), Agricultural Research Service Collection of Entomopathogenic Fungal Cultures (accessed 14 August 2020), or iNaturalist (accessed 28 November 2021).

We have a richer, though still incomplete, understanding of the types of habitats in which *E. muscae* can be found. Field studies have frequently taken place in agricultural sites (e.g., barns, stables, and crop fields), which, unsurprisingly, tend to be sites that support large populations of various dipteran species (Watson and Peterson, 1993; Six and Mullens, 1996; Lihme *et al*., 2009; De Fine Licht *et al*., 2017). *Entomophthora muscae* has also been observed infecting populations

of flies on or near compost piles (Turian and Wüest, 1969), rotting fruit baits (Elya *et al.*, 2018), wineries with open-fermentation vats (C. Elya, pers. obs.), and private residences (Cohn, 1855). Though the prevalence of *E. muscae* observations drops during the colder months, *E. muscae* has been observed to infect hosts indoors during the winter (Kramer, 1980b; Kramer and Steinkraus, 1981; Eilenberg *et al.*, 2013). Collectively, these observations bolster the hypothesis that *E. muscae* can survive as long as it has access to hosts.
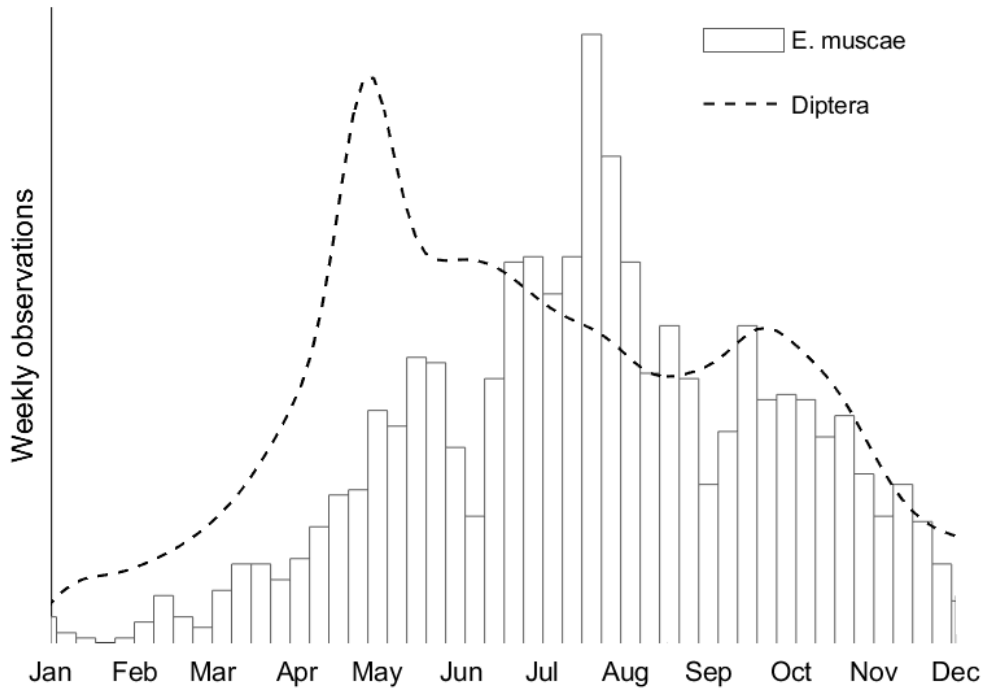
Fig. 5. Seasonal abundance of *E. muscae* and Diptera. White bars show weekly *E. muscae* abundance relative to total *E. muscae* observations (n = 1,713, iNaturalist observations accessed 28 November 2021); dotted line shows kernel density of weekly dipteran abundance relative to total dipteran observations (n = 78,522, iNaturalist observations accessed 4 November 2020). The peak in the Northern Hemisphere's summer (July, August) is likely driven both by known *E. muscae* prevalence in temperate regions and sampling bias.

Cryptic diversity

Fungi identified as *E. muscae* based on morphology have been observed to infect many different dipteran hosts. At first glance, this may seem puzzling given the incredible specificity of the end-of-life behaviors induced by *E. muscae*; it seems unlikely that a single organism could evolve the capacity to manipulate behavior so precisely in a broad range of hosts. Instead, one would expect specialization to come at the cost of generality (Schmid-Hempel, 2011). However, several lines of evidence support the hypothesis that *E. muscae* is a species complex consisting of several morphologically indistinguishable species rather than one monolithic species (Keller, 1984). First, studies have found that strains of *E. muscae* with very similar morphology show different patterns in random amplified polymorphic DNA (RAPD) and restriction length polymorphism (RFLP) assays (Jensen and Eilenberg, 2001; Jensen *et al.*, 2001, 2006). This work was among the first to suggest that strains with overlapping morphologies are heterogeneous at the molecular level and to show that host identity tends to track according to these molecular differences.

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Consistent with these laboratory-based studies, field studies have found evidence for host-specificity among genetically, but not morphologically, differentiable *E. muscae* strains. In 2011-2012, an epizootic event occurred at the start of which *Delia radicum* were most often infected, but later *Coenosia tigrina* became the more common host species (Gryganskyi *et al*., 2013b). Sequencing of several conserved loci from samples collected early and late during this event found evidence for two different fungal haplotypes, one most found in *D. radicum* samples and the other in *C. tigrina*, though occasionally each haplotype was detected in the less common host. Interestingly, fly species other than *D. radicum* and *C. tigrina* were also seen in the region where the epizootic event occurred but were never observed to fall victim to *E. muscae* during this outbreak. Similarly, another study following an *E. muscae* outbreak in a horse stable, found that house flies were the only species observed to die of fungal infection and go on to produce and disperse conidia even though about 40% of the total fly population in this environment consisted of *Stomoxys calcitrans* (Keller, 2002). We expect that as we continue to amass genetic sequence data for different *E. muscae* isolates, we will find that what we now refer to as *E. muscae* is actually a collection of cryptic species.

Evolutionary host–pathogen dynamics
We have an incomplete understanding of what factors determine when and where *E. muscae* epizootics can be observed and only a handful of studies have dealt with measures of seasonal monitoring of infection and prevalence (Jensen and Eilenberg, 2001; Steenberg *et al*., 2001; Gryganskyi *et al*., 2013b). These studies are geographically restricted to open urban lawns in Durham, North Carolina, and several cow stables in Denmark, but show that the number of infections fluctuate over time. Environmental conditions, especially temperature and humidity, have some influence on the number of infected flies sampled in open habitats (Gryganskyi *et al*., 2013b), whereas the size of the house fly population is a strong predictor in human-associated habitats, such as stables (Watson and Peterson, 1993; Six and Mullens, 1996). In the sampled cow stables in Denmark, fly populations build up over the summer with a peak in late summer/early autumn (Skovgård and Steenberg, 2002). Similarly, *E. muscae* infection appears to build up over the summer and can peak with a prevalence of 70–90% in house fly populations in a given cow stable (Steinkraus and Kramer, 1987). These studies also highlight the host specificity of *E. muscae*. House flies often occur sympatrically with the biting fly, *Stomoxys calcitrans,* but very few *E. muscae*-infected *S. calcitrans* were found out of hundreds of flies collected (Kramer and Steinkraus, 1981; Skovgård and Steenberg, 2002).

A high prevalence of *E. muscae* seems detrimental to house fly populations, but the relatively long disease incubation of *E. muscae* in house flies of six to seven days may dampen the negative effects. House flies have an average life span of three to four weeks (Reed and Bryant, 2000; Cooper *et al*., 2004), and are only sexually mature three to four days after emergence as adults from pupae. This implies that the incubation period of *E. muscae* equals 25–33% of the total lifespan of house flies. The disease ontogeny of *E. muscae* results in an exponential build-up of fungal cells inside infected flies (Hansen and De Fine Licht, 2017), which nonetheless allows infected flies to continue reproduction during the first phases of the disease (Watson and Petersen, 1993). Females thus continue to lay eggs and males continue to mate for the initial three to four days post infection, until the *E. muscae* infection has progressed to the extent that the flies are too ill to maintain normal internal homeostasis. Continued reproduction of infected flies thus reduces

the life-time cost of reproduction caused by *E. muscae* infections (Fig. 6). Assuming that the cost of *E. muscae* infection on total lifetime reproduction is highest early in life, the cost of infection later in life gradually decreases since remaining life-time expectancy also reduces expected reproductive output (Fig. 6). Therefore, while a prevalence of *E. muscae* infections approaching 90% in certain house fly populations is certainly high, the negative effect on lifetime reproductive fitness is reduced, compared to an infection that stops host reproduction immediately upon infection.
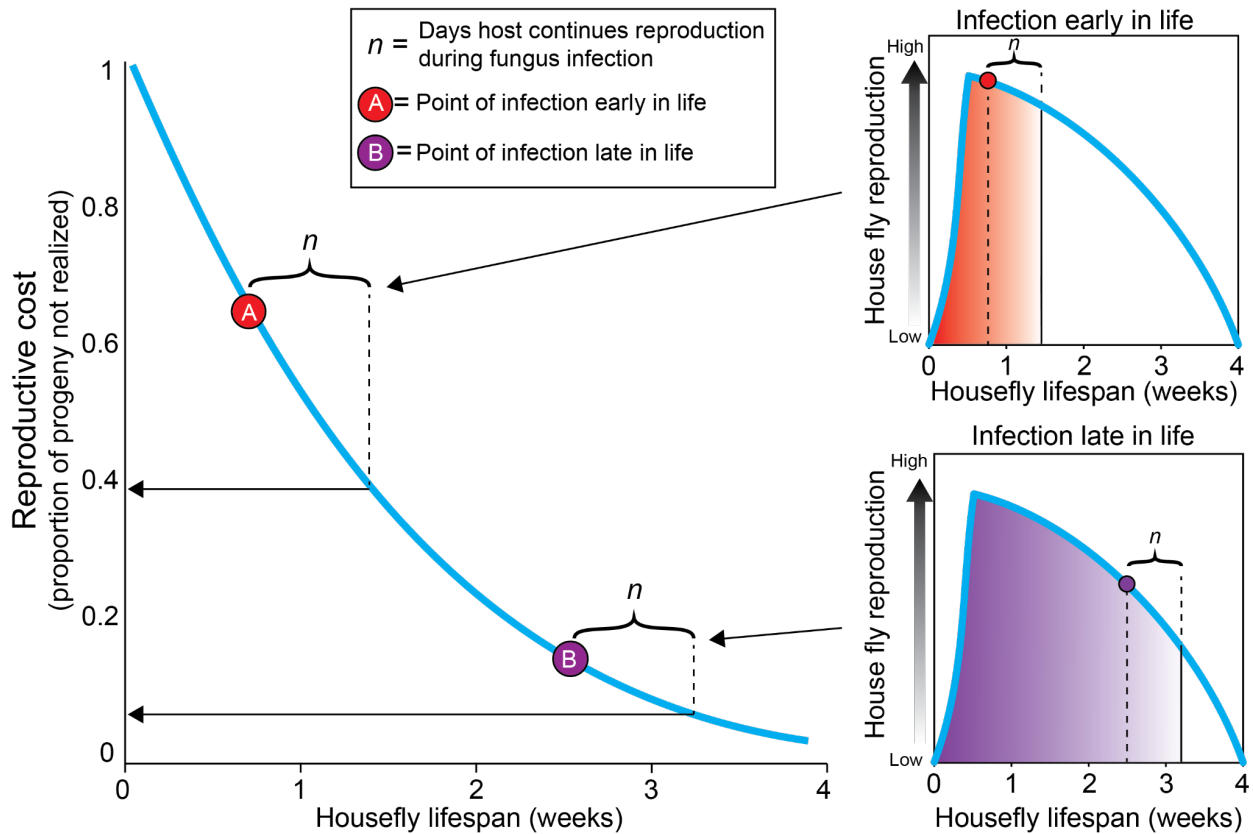
Fig. 6. Hypothesized lifetime reproductive cost to house flies of *E. muscae* infection. The fungus *E. muscae* s.s. has an incubation period of six to seven days between initial exposure and killing of the house fly host. During the initial three to four days of this incubation period, infected males can still mate and infected females can still lay eggs (Watson and Petersen, 1993). The x-axis depicts the house fly lifespan, and the y-axis depicts the lifetime reproductive cost as proportion of progeny not realized. Here we see the relative decrease in total lifetime reproductive output for a healthy fly compared to a fly infected early (A) or late (B) in life, i.e., a high reproductive cost indicates that many potential progeny are not produced; a low cost means that only few potential progeny are lost. As fly fertility declines with age (blue line), reproductive cost is predicted to decrease with increasing fly age at the time of infection with *E. muscae*. The red (A) and purple (B) dots mark the point of infection of a young (A) and old (B) fly, respectively. The horizontal arrows to the y-axis show the lifetime reproductive cost if the fly ceased reproducing at these time points. The later the fly is infected in life, the smaller the reproductive cost is incurred.

There is no formal evidence for two-sided co-evolution between insects and *E. muscae* (Humber, 2008; Gryganskyi *et al*., 2012, 2013a). However, the many striking adaptations of *E. muscae* to insect infection, such as specialized growth as protoplasts inside infected flies and the multitude of behavioral manipulations, show that the life history of *E. muscae* has been shaped by selection pressures imposed by the host insects (Ebert and Fields, 2020). Such selection is very strong for obligate pathogens that do not have a free-living stage except during transmission (Schmid-

Hempel, 2011). The obligate lifestyle and specialized traits for insect infection suggests that *E. muscae* and the genus *Entomophthora* likely are obvious candidates to look for clear evidence for specific coevolution with insects (Elya and De Fine Licht, 2021).

At present, we have an incomplete understanding of the dynamics of *E. muscae* infections in natural fly populations. To advance our knowledge about *E. muscae* evolutionary dynamics it is useful to structure our knowledge (or lack thereof) according to Tinbergen's (1963) four complementary types of explanations, which are commonly considered to be required for fully understanding a biological phenomenon (Boomsma *et al*., 2014). These are proximate questions of mechanism and development, and ultimate questions of adaptation and phylogeny. Of these, we arguably have the best understanding of the phylogeny of *E. muscae* (Fig. 1), whereas we are only beginning to unravel the many intricate processes of *E. muscae* trait mechanisms and developmental transitions in phenotypes (Elya *et al*., 2018; Naundrup *et al*., 2022). The ultimate question of the adaptive value or function of a given *E. muscae* trait on lifetime reproductive success, which is tightly coupled with transmission for pathogens such as *E. muscae,* is usually only inferred and has rarely been explicitly tested. This is in part due to the difficulty with obtaining or working with *E. muscae* in the laboratory, where slow growth and the requirement of live fly hosts to induce sporulation complicates the use of many standard mycological techniques (Elya and De Fine Licht, 2021).

Conclusions and future perspectives
In this chapter, we provided an overview of the evolutionary ecology of *E. muscae* as well as mechanistic hypotheses for how *E. muscae* achieves manipulation of host behaviors. While some of these alterations of host behavior are conspicuous, others are much more subtle, and there may even be some that have yet to be discovered; disentangling host responses from fungus manipulation is not going to be an easy task. To date, almost nothing is known about the underlying molecular and physiological mechanisms allowing *E. muscae* to manipulate host behaviors. However, "zombie flies" are a tractable system for studying the proximate and ultimate mechanisms of behavioral manipulation (Gryganskyi *et al*., 2017; Lovett *et al*., 2020a; de Bekker *et al*., 2021), not least because the natural host range includes one of the most widely used laboratory organisms, *Drosophila melanogaster* (Elya *et al*., 2018). Many advanced molecular and chemical methods routinely used today have not yet been brought to bear on this system; these have the potential to greatly enhance our understanding of the biology at hand.

The fungus *E. muscae* is not difficult to find if one knows when and where to look, but there are still many aspects of the host–pathogen ecology and population dynamics that are unknown. For example, why can certain dipteran populations suddenly suffer from an *E. muscae* epizootic whereas other populations adjacent in space and/or time are apparently disease free? How big of an impact does *E. muscae* have in shaping fly population dynamics? Why can some species, such as the house fly, have such a high prevalence of *E. muscae* infections, whereas sympatric and abundant species such as *Stomoxys calcitrans* are unaffected? What is the mechanism of this host specificity?

In addition to these ecological and evolutionary open questions, some of the unusual fundamental biological traits of *E. muscae* are also puzzling. Why is *E. muscae* multinucleate throughout all growth forms in its life cycle? Why is the genome apparently so large? Is *E. muscae* haploid or

functionally diploid as some data indicates (De Fine Licht *et al*., 2017)? How often does *E. muscae* sexually reproduce, and does it require co-infections with compatible strains? Many of these traits are likely linked to the ecology and selection imposed by being a highly host-specific and obligate fly pathogen. These fungal traits are interesting but have also hindered the use of many routine mycological laboratory techniques with *E. muscae*. With current and future advances in molecular methods, for example long-read sequencing, unbiased chemical detection and reduced input assays, future *E. muscae* research will surely yield new and useful insights into the evolutionary ecology and general biology of this intriguing fungus.

Glossary

Biotrophic: Feeding on living organisms, parasitic lifestyle.

Conidiophore: Finger-like projection that grows out through the cuticle of dead or moribund host then forms and launches a primary conidium.

Epizootic: The appearance of a particular disease in a large population of animals in the same place and at the same time.

Germ tube: Hyphal-like extension that grows from a conidium during germination. This structure is used by *Entomophthora* species to penetrate the insect cuticle to gain access to the hemolymph.

Hemocoel: The internal body cavity of insects (and arthropods), which is filled with hemolymph. The hemocoel is an open circulatory system where the heart(s) pumps "blood" (**hemolymph**) into the cavity where the fluid surrounds and bathes the organs then returns to the heart(s).

Hemolymph: The "blood" found in arthropods, an interstitial fluid that circulates oxygen and nutrients within the insect body cavity (**hemocoel**).

Hyphae: Filamentous vegetative growth. Usually long and branched, with individual cells connected at septae.

Hyphal body: Vegetative growth occurring in insect hemolymph. May or may not have a cell wall.

Papillar eversion: The mechanism used by *Entomophthora muscae* for the active discharge of a secondary conidium from a primary conidium. The mechanism relies on a septum forming between the primary conidiogenous cell and the secondary conidium by growth of the inner layer of the cell wall. Once the septum is closed and nearly all cytoplasm has been transferred from the primary conidiogenous cell into the secondary conidium, the hydrostatic pressure inside the secondary conidium builds likely because of hydrolysis of storage products. This pressure is subsequently released by the sudden eversion of this papilla resulting in a break of the outer cell wall layer so that the secondary conidium is actively discharged to a considerable distance.

Primary conidium: Asexual spore formed atop a conidiophore and launched into the environment via water cannon mechanism to infect a new host. Contains multiple nuclei; the number of nuclei and dimensions (width, length) have historically been diagnostic for *Entomophthora* species. Can give rise to secondary conidium.

Protoplast: Vegetative growth occurring in insect hemolymph lacking a cell wall. Often irregularly shaped and lacks septae.

Resting spore: A thick-walled structure that allows *Entomophthora* to overwinter. These have been found to be formed in some, but not all, species and strains of *Entomophthora* in response to changing environmental cues or advanced host age. Resting spores can germinate once favorable conditions return, giving rise to germ conidia, which can then infect new hosts.

Secondary conidium: Asexual spore formed from primary conidium and launched via papillar eversion to infect a new host. Slightly smaller than the primary conidium. Under favorable environmental conditions, can give rise to tertiary conidium.

Septum: Internal cross wall between individual cells in a hypha.

Sporulation: The process of forming and ejecting infectious spores (conidia) into the environment. Also referred to as conidiation.

Summit disease: The behavioral syndrome where a host (usually an insect) is forced by an internal pathogen (usually a fungus, virus, or trematode) to crawl upwards and attach itself high up on vegetation such as grass stems and bushes.

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