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Patterns and potential mechanisms of thermal preference in *Entomophthora muscae*-infected *Drosophila melanogaster*

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Abstract

Animals use various strategies to defend against pathogens. Behavioral fever, or fighting infection by moving to warm locations, is seen in many ectotherms. The behavior-manipulating fungal pathogen *Entomophthora muscae* infects numerous dipterans, including fruit flies and house flies, *Musca domestica*. House flies have been shown to exhibit robust behavioral fever early after exposure to *E. muscae*, then switch to prefer cool temperatures in the later stages of infection. Interestingly, no evidence of behavioral fever in response to any investigated pathogen has been found in the fruit fly, *Drosophila melanogaster*. However, they have been found to prefer cool temperatures during infections. To determine if fruit flies utilize behavioral fever, cold-seeking, or both during *E. muscae* infection, we used a two-choice behavioral assay to measure individual temperature preferences of *E. muscae*-exposed and unexposed flies at early (24-72 hour) and late (72-120 hour) infection time points. In contrast with our expectation from house flies, fruit flies did not exhibit behavioral fever. However, we found significant cold temperature-seeking in flies that died from infection on the day of the assay. To investigate whether this cold-seeking behavior was being caused by the fly or the fungus, we tested the effects of temperature on the fitness of the host, *D. melanogaster,* and the pathogen, *E. muscae,* during infection. We found that flies held at low and high temperatures for 24 hours before death from infection laid no eggs at the lower temperature. This could suggest that the fly is not causing the cold-seeking behavior because there is no apparent fitness benefit at low temperatures. Conversely, cadavers sporulating at the low temperature tended to cause more flies to eventually die from infection, indicating that *E. muscae* infects flies more effectively at lower temperatures. Preliminarily, our results support fungal control of temperature preference before

death, though further testing is needed. The idea that *E. muscae* benefits from colder temperatures, and therefore drives cold-seeking behavior in *D. melanogaster* at the end of the host's life, expands our current knowledge about host behavior manipulation by *E. muscae* and provides a fascinating avenue for investigating the mechanisms by which this fungus manipulates complex behaviors in its animal host.

Introduction

Thermoregulation and Immunity

Animals employ a variety of immune strategies in response to infection by pathogens. Due to the negative effects of extreme temperature on pathogen development and survival, a host changing its body temperature during infection is one of the most universal and highly conserved ways in which animals boost immunity (Kluger, 1979). Fevering, or a host increasing their body temperature, is commonly used in both endotherms and ectotherms to slow and kill infection (Kluger, 1979; Moore, 2002; Rakus et al., 2017; Roy et al., 2005). Because ectotherms must regulate their body temperature via their external environment, they use behavioral fever, or moving to warm locations, in response to pathogens. This achieves the same goal of killing or slowing infection that fevering in endotherms accomplishes (de Roode & Lefèvre, 2012; Roy et al., 2005). In contrast to physiologically regulated fevering in endotherms, the regulation of fever in ectotherms via decisions or reactions that change their behavior is not well understood.

An alternative behavioral thermoregulatory immune strategy utilized by ectotherms is cold-seeking. Much like with behavioral fever, spending time at cool temperatures can slow the spread of infection (Moore & Freehling, 2002; Müller & Schmid-Hempel, 1993). The immune benefit of cold-seeking has garnered much less attention in the literature than behavioral fever, but what is documented on the phenomenon demonstrates its adaptive potential for invertebrate ectotherms. The bumblebee *Bombus terrestris* exposes itself to cool temperatures when parasitized by conopid flies by staying outside of the hive at night, prolonging the life of the parasitized worker bees and reducing the fitness of the parasitoid (Müller & Schmid-Hempel, 1993). Cold-seeking has also been observed in cockroaches parasitized by the acanthocephalan

Moniliformis moniliformis, effectively slowing parasite development (Moore & Freehling, 2002). Both behavioral fever and cold-seeking have been shown to increase survival in infected ectotherms when compared to individuals that did not thermoregulate in response to infection (Moore & Freehling, 2002; Ouedraogo et al., 2004).

Fungal pathogens are a useful subject for studying thermoregulatory immune response in ectotherms because they have been shown to elicit behavioral fever and cold-seeking in their hosts. For instance, grasshoppers infected with the entomopathogenic fungus *Beauveria bassiana* have been found to demonstrate behavioral fever, with higher temperatures leading to decreased fungal growth during infection (Inglis et al., 1996). Fruit flies infected with the fungus *Metarhizium robertsii* preferred cooler temperatures, which slowed the growth rate of the fungus, extended the survival time of moribund flies (flies that eventually died from infection), and reduced the mortality rate of infected flies (Hunt et al., 2016). Despite opposing thermoregulatory behaviors, both of these examples demonstrate the adaptive benefit of thermoregulation on immunity to fungal pathogens.

Entomophthora muscae *and Host Thermoregulation*

Fungi in the order Entomophthorales are known to control the behavior of their hosts in ways that benefit the fitness of the fungus (Roy et al., 2005). These fungi mostly parasitize insects, but have been found in other arthropods, such as millipedes (Hodge et al., 2017). Hosts are forced by the fungus to climb high, or "summit," and position themselves such that the fungus can sporulate efficiently (Roy et al., 2005). Once it kills its host, the fungus breaks through the weak points in the host exoskeleton and sporulates by launching spores, or conidia, into the air and striking its next victim (Brobyn & Wilding, 1983). Changes in host behavior by entomophthoralean fungi have been previously studied in house flies using the species *Entomophthora muscae* (Kalsbeek et al., 2001; Krasnoff et al., 1995; Watson et al., 1993). *E. muscae* infects a variety of dipterans and makes them exhibit many of the trademark behaviors caused by Entomophthorales. In addition to forcing its fly host to climb high, *E. muscae* extends the proboscis of the host and attaches it to a substate with fungal holdfasts (hyphae that grow out of the mouthparts), then raises its wings to allow unhindered release of conidia from fungal

conidiophores growing out of the fly's abdomen after death (Krasnoff et al., 1995). Like numerous other entomopthoralean fungi, *E. muscae* consumes its host's internal organs and fat reserves then kills its host just before sunset, likely because lower temperatures and higher humidity at night ensure the most efficient germination (Carruthers & Haynes, 1986; Roy et al., 2005; Watson et al., 1993).

Considering the systemic nature of *E. muscae* infection, it is assumed that infected flies mount a substantial immune response to the fungus. *E. muscae* has been shown to induce behavioral fever and cold-seeking over the course of infection in *Musca domestica* (house flies) (Kalsbeek et al., 2001; Watson et al., 1993). We were interested to see if these behaviors also arose in *Drosophila melanogaster* (fruit flies). Although behavioral fever has, to our knowledge, not been observed in fruit flies infected with any pathogen (Arnold et al., 2015; Ballabeni et al., 1995), the absence of this immune strategy in ectotherms does not always mean that the organism is incapable. Rather, whether an ectotherm employs behavioral fever may depend on its physiology and the nature of the pathogen. Behavioral fever is energetically costly and not intrinsically beneficial to the host (Roy et al., 2005). Thus, animals might choose not to express behavioral fever when doing so would incur greater energetic and fitness costs than benefits or when it would worsen infection (Moore, 2002). Moreover, an animal does not always respond to every pathogen in the same way (de Roode & Lefèvre, 2012). For instance, the cricket *Acheta domesticus* preferred warmer temperatures when infected with the intracellular parasite *Rickettsiella grylli*, but showed no change in thermal preference when infected with the bacterium *Serratia marcescens* (Adamo, 1998). Though unlikely, it is possible that no pathogen that would elicit behavioral fever in fruit flies has been investigated up to this point. Because *E. muscae*-infected house flies have been found to utilize behavioral fever to successfully fight the fungal pathogen, this gave us reason to believe that *E. muscae*-infected fruit flies might also utilize this behavior as an adaptive immune response.

Although behavioral fever has never been observed in fruit flies, fruit flies have been found to exhibit cold-seeking in response to some pathogens. For example, Fedorka et al. found that fruit flies infected with the bacterium *Pseudomonas aeruginosa* preferred cooler temperatures than

uninfected controls (2016). This thermoregulatory response improved the chances that a fly would survive infection (Fedorka et al., 2016), likely because it both slowed bacterial growth and caused an upregulation of immunity-related genes (Linder et al., 2008). Fruit flies have also been found to be more resistant to infection by the fungal pathogen *Beauveria bassiana* when kept at lower temperatures (Le Bourg et al., 2008), indicating that cold-seeking improves immune function.

Interestingly, Watson et al. observed cold-seeking in house flies on the day of death from *E. muscae* infection and suspected it was caused by the fungus (1993). *E. muscae* germinates more efficiently at temperatures lower than the optimal temperature for house flies or fruit flies (Carruthers & Haynes, 1986). Additionally, *E. muscae* is already known to manipulate complex behaviors in flies, potentially by "hijacking" the host nervous system (Elya et al., 2018). Thus, we considered that the fungus could be manipulating host thermal preference as well. This provided an interesting dichotomy for us to investigate whether cold-seeking behavior would arise in *E. muscae*-infected fruit flies like it did in house flies, and whether this behavior, if it arose, would be caused by the fungus or the fly.

To test for both behavioral fever and cold-seeking in *E. muscae*-infected fruit flies, we tested *E. muscae*-exposed flies at multiple post-exposure time points in a thermal preference assay that allowed the flies to wander between a low and high temperature for several hours. Watson et al. only observed behavioral fever in house flies in the middle stages of *E. muscae* infection (1993), and the duration of infection until death is longer in house flies than it is in fruit flies. Therefore, we anticipated that behavioral fever might arise in our fruit flies only until a few days after exposure, before infection becomes too severe. If cold-seeking was utilized by our flies to slow infection instead, this behavior could arise on any day after exposure that we tested. For example, fruit flies exposed to *M. robertsii* expressed cold-seeking within 24 hours of exposure (Hunt et al., 2016). We might see a similarly rapid response in our *E. muscae*-infected flies as well. However, when a fly triggers cold-seeking behavior could depend on the severity or type of infection, so it is possible we would not see fly-induced cold-seeking until late in infection. Alternatively, cold-seeking induced by the fungus would likely only be observed in the final

days of infection, when other marked behaviors are being manipulated and the fungus is preparing to sporulate. We believed our thermal preference assay would help elucidate temperature preference in fruit flies over the course of *E. muscae* infection, either refuting or supporting current evidence that fruit flies use cold-seeking and not behavioral fever in response to infection. Lastly, we were confident that what we found would contribute to a broader understanding of thermoregulatory behavior in ectotherms during host-pathogen interactions.

Thermoregulation and Fecundity in Response to Infection

Many ectothermic invertebrates like insects clearly use alterations in thermal preference as an effective immune strategy. However, the benefits go beyond simply improving host survival. Hindering a pathogen can allow improved lifetime fitness for a host because the host survives for longer or completely overcomes infection (de Roode & Lefèvre, 2012). This is the case with female *D. melanogaster* infected with the fungus *M. robertsii* (Hunt et al., 2016). The longer lifespan resulting from cold-seeking helped these females improve their lifetime fitness by boosting reproductive output later in life. Typically, healthy female fruit flies prefer warm temperatures in order to maximize reproductive output early in life, but flies infected with *M. robertsii* sacrifice this early-age reproduction in order to maximize late-age reproduction while fighting infection (Hunt et al., 2016). We wanted to see if temperature would mitigate the effects of *E. muscae* infection on late-age fitness in moribund females, so we kept female flies at low and high temperatures in the 24 hours before death from infection and counted the number of eggs laid during that time. Interestingly, there is evidence that cold-seeking is only exhibited by females during infection. Female *D. melanogaster* infected with the parasitic nematode *Howardula aoronymphium* were found to prefer cooler temperatures than unparasitized females, while infected male flies tended to prefer warmer temperatures than their healthy counterparts (Ballabeni et al., 1995). Knowing this, we were cognizant of checking differences in temperature preference between sexes during our thermal assay. Differences in thermal preference between sexes might indicate that thermal preference is under fly control, whereas similar preferred temperatures could suggest fungal control. However, it must be considered that males could express cold-seeking because slowing fungal growth allows them more time to mate with

females. It is also possible that the fungus could be more effective at manipulating the thermal preference of females, leading to fungal-manipulated differences in preferred temperatures between sexes.

To test whether the temperature preferences we observed in our thermal preference assay were being caused by *E. muscae*, we also measured the fitness of the fungus according to temperature. Counting the number of spores released is considered a viable means of determining fungal fitness (Pringle & Taylor, 2002). As a proxy, we measured the number of new flies infected and killed by *E. muscae* cadavers kept at low or high temperatures while sporulating and germinating. Differences in the number of cadavers produced at each temperature would indicate that *E. muscae* fitness is dependent on temperature. Results from this fitness assay could uncover that behavioral thermoregulation is also under the control of *E. muscae,* a previously unknown manipulation by the fungus.

Regardless of whether our *E.* muscae-infected fruit flies exhibited behavioral fever or cold-seeking, and it benefitted the fitness of the fungus or the fly, the results of this study contribute to a better understanding of thermoregulatory immune response in fruit flies, further discovery on the dynamics of *E. muscae* and its fly hosts during infection, and greater knowledge about the effects of temperature on host and parasite fitness. The focus of this study was on patterns of thermoregulatory behavior and their effect on host and parasite fitness in *E. muscae*-infected *D. melanogaster*. However, we are confident that the molecular and genetic toolkit of *D. melanogaster*, one of biology's most prominent model ectotherms, will allow rapid understanding of the molecular mechanisms behind thermoregulation in fruit flies during *E. muscae* infection. This, in turn, would contribute to a broader understanding of thermoregulation and immunity across the broad range of ectotherm-pathogen interactions.

Methods

Thermal Preference Assay Protocol

Exposure

For both the thermal assay and *E. muscae* infection propagation, we used wild-type CantonS *Wolbachia*-free (CsWF) *D. melanogaster* reared on a cornmeal-based diet (3% weight per volume (w/v) cornmeal, 11% w/v dextrose, 2.3% w/v yeast, 0.64% w/v agar and 0.125% w/v tegosept) with a 12h:12h light:dark cycle at 21℃. We achieved propagation using methods described in Elya et al., 2018. Briefly, we collected fresh *E. muscae*-killed cadavers at 96-168 hours (4-7 days) post exposure to the fungus between Zeitgeber time (ZT) 14 and ZT16 on the day of death, where ZT12 marked the light to dark transition (i.e. sunset). For each vial, we embedded 6 cadavers headfirst in a circle in 5AS medium (5% sucrose, 1.5% agar prepared in Milli-Q water), with wings facing out and tucked into the agar to allow conidia to be effectively launched. We then cut out the cadaver circle with an empty wide-mouth *Drosophila* vial (Genesee, Cat #: 32-112) and placed it in a new wide-mouth *Drosophila* vial to expose experimental flies (Figure 1). We collected fifty healthy flies (25 male, 25 female) at 0 to ~72 hours post-eclosion with CO_2 to be placed in each of the prepared exposure vials. To force close contact with the sporulating cadavers, we confined flies to the bottom 2 cm of the vial using a Droso-plug (Genesee, Cat #: 59-201) (Figure 1). For the first 24 hours of exposure, we kept vials in a humid chamber (~100% humidity, 1L plastic beaker lined with wet paper towels and covered with foil) to encourage sporulation and germination. After 24 hours, we raised the Droso-plugs to the top of each vial and moved all vials to an incubator at 40% humidity and 21℃, where they stayed until the thermal preference assay (Figure 1). For each experiment, we also prepared vials of "mock-exposed" (unexposed control) flies who we subjected to the same treatment as our experimental flies, except for housing them on food without sporulating cadavers.

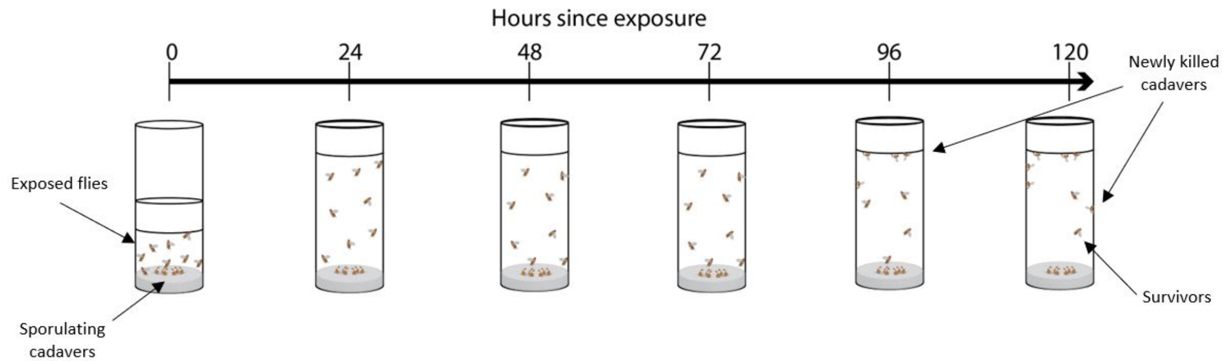


Figure 1. Diagram of general *E. muscae* exposure and infection process for lab-reared CsWF *D. melanogaster*.

Thermal Preference Assay

We used custom thermal preference behavior boxes, courtesy of J. Akhund-Zade, made from black acrylic and enclosing a Blackfly camera (FLIR, Cat#: BFLY-PGE-12A2M-CS) capturing 2 platforms, each with 20 tunnels containing 2 side-by-side Peltier elements (Custom Thermoelectric, Cat#: 12711-5L31-09CQ) for temperature control and covered with clear acrylic coverslips to prevent flies from escaping (Figure 2). A PID temperature controller maintained temperatures of the Peltiers on each side of a platform, allowing us to set each half of the tunnel to a different temperature. Our Peltiers were also connected to a circulating chiller to prevent them from overheating. We ran a few early experiments at 22°C and 32°C, but for most we set the Peltiers on the left side to 18℃ (L) and on the right side to 28℃ (R) such that 1) each fly could achieve an experienced temperature in the range around ~24℃, which is the average preferred temperature of fruit flies (Sayeed & Benzer, 1996), 2) the temperature range would be broad enough to observe differences in preference across groups, and 3) the temperature range would not be so extreme that fruit flies would avoid or risk desiccation on the hot side or cease to move on the cold side.

We loaded individual flies into each tunnel with separate glass aspirators for exposed and unexposed flies and allowed flies to freely navigate the tunnel for 4 hours. We would load all fifty flies from one exposure vial before using the next vial, to ensure we were testing an approximately equal number of males and females. We distributed 10 control flies randomly within the 40 tunnels of each thermal rig, by using a random number generator (MATLAB) to

assign tunnels. For some experiments, we painted the underside of each acrylic coverslip with fresh Sigmacote (Sigma-Aldrich SL2-100ML) to prevent flies from crawling upside down above the Peltiers, possibly resulting in inconsistent temperature exposure. However, not using Sigmacote appeared to have no effect on experiment results.

We ran our 4-hour assay at various time points (~ZT0-ZT4, ~ZT10-ZT14, ~ZT8-ZT12, and ~ZT12-ZT16) on days 1-5 post exposure. The ~ZT8-ZT12 assay is the focus of this paper because it allowed us the opportunity to capture behavioral fever and early- or late-infection cold-seeking. We anticipated the 4 hours leading to sunset to be particularly critical for capturing cold-seeking because evidence suggests that this could strictly be an end-of-life behavior for *E. muscae*-infected flies (Watson et al., 1993). We followed the same protocol for all experiments, 24-120 hours (1-5 days) post exposure, and all tunnels and coverslips were wiped down with 70% ethanol between each experiment. We spread 30 unexposed controls and 90 *E. muscae*-exposed flies evenly across 3 boxes during each of 5 experiments, for a total of approximately 560 tested flies.

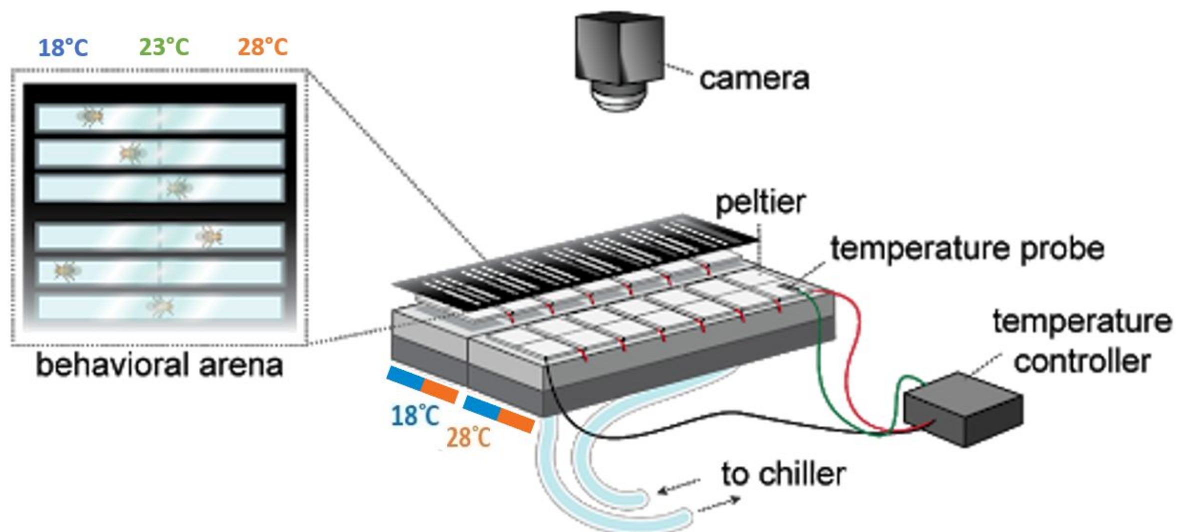


Figure 2. Diagram of behavior rigs used for thermal preference assay. Adapted with permission from J. Akhund-Zade.

Assay Tracking Settings

We used Blackfly cameras (FLIR, Cat#: BFLY-PGE-12A2M-CS) fitted with infrared light filters and captured frames at a rate of 10Hz. We ran our assay illuminated at a 50% duty cycle with far-infrared light (940nm), which is invisible to flies, so the flies navigated the tunnels effectively in the dark. Having limited visible light (the boxes did not completely block out room light) allowed thermal stimulus to be the majority of sensory input for the flies to make their choice. The cameras were connected to the beta version of MARGO, Massively Automated Real-time GUI for Object-tracking software (Werkhoven et al., 2019) in MATLAB 2018a (Mathworks, Inc). This high-throughput ethology platform recorded the x and y positions for each fly for each frame in our assay from which we could extract positional information in subsequent analyses.

Survival Tracking

After each experiment, we turned all Peltiers to 12℃ and used CO_2 to slow the flies and mitigate escape during unloading. We then individually aspirated all flies out of their tunnels and housed them in 200 microliter strip-PCR tubes prepared with 50-100 microliters of 5AS media and air holes punctured into the lids. We housed flies individually so that they could be sexed and monitored until 168 hours (7 days) post exposure. We kept flies at 21°C and recorded their survival status (alive, dead from *E. muscae* infection, or dead for other reasons) daily after ZT14 through 168 hours (7 days) post exposure.

Thermal Preference Data Analysis

Data Grouping

We used survival status data to categorize thermal preference assay data for analysis. We grouped thermal preference and distance traveled behavior data based on the time until death from *E. muscae*. For example, flies that died on the day they were tested (typically 96 or 120 hours (day 4 or day 5) post exposure) would be grouped as “dead in 0 hours.” Thus, we grouped flies dying the next day from *E. muscae* infection as “dead in 24 hours,” and so on. This grouping allowed us to reliably quantify and compare true end-of-life behaviors that may not be

observed if we grouped the flies based on the number of days post exposure that we ran their experiment. Additionally, we grouped flies based on their time since exposure (days 1-5 (24-120 hours post exposure)) when their experiment was run but found that doing so masked thermal preference differences seen when grouping by time until death (Figure 4).

Calculating Thermal Preference

We calculated the running (frame-wise) average for each individual fly as a mean of temperature preference choices (18°C or 28°C) over a 2-hour sliding window across each frame of the 4-hour experiment. We also calculated the overall average thermal preference for each individual fly, such that we averaged over each frame to calculate one average for the entire experiment. We used the individual running and overall average values to find the running and overall average for each treatment group. We calculated each fly's thermal preference as an average measure of hot and cold side occupancy of the behavior rig tunnels throughout an experiment (Figure 3). We assigned the preferred temperature of a fly in a given frame taken by the camera as either 18℃, 23℃, or 28℃ (or 22°C, 27°C, 32°C for our ~ZT12-ZT16 assay) depending on its position within its tunnel (left, middle zone, or right) (Figure 2). We calculated both total-experiment and hour-interval averages for each fly using its respective instantaneous preferred temperatures. We excluded inactive flies from our analysis, which we defined as flies that traveled a total distance (in pixels) less than 2 standard deviations below the mean travel distance for all flies simultaneously tested. Our goal was to eliminate skewed temperature preference measures because of flies that died (and therefore stopped moving) during an experiment or flies that were abnormally inactive (and therefore not making temperature preference choices). We also checked the by-hour distance traveled for each group to ensure that these variables were not confounding our temperature preference results. Lastly, we removed flies that died for reasons other than *E. muscae* infection and any flies that we lost in the transition between the behavior rigs and housing, the latter because we could not be sure of their survival outcome.

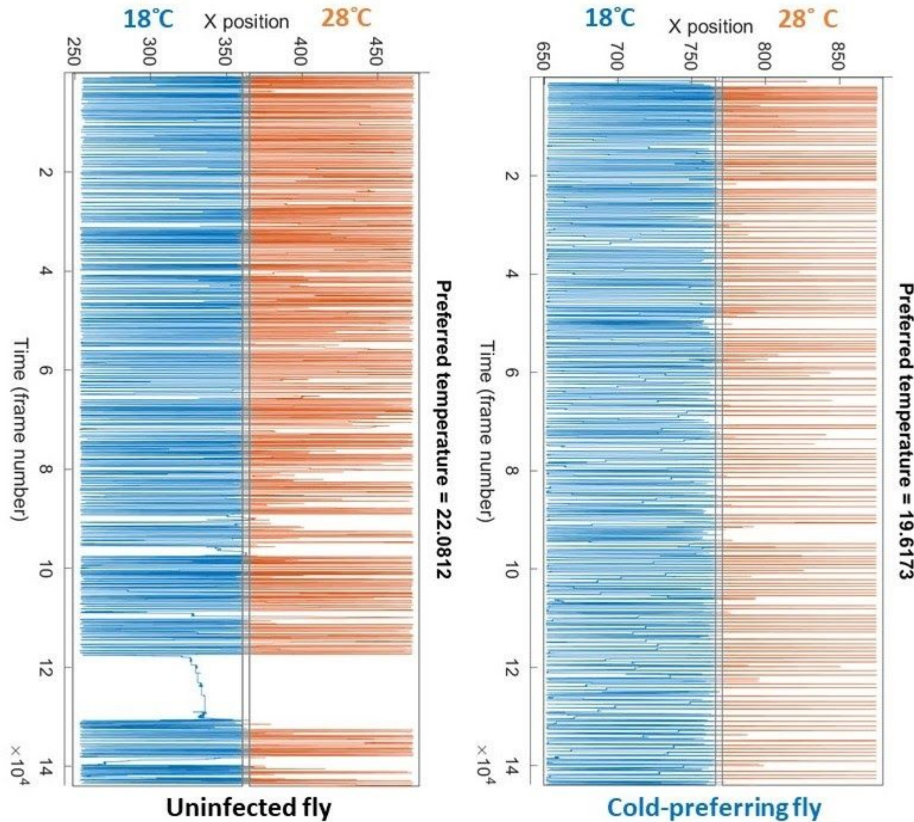


Figure 3. Traces pulled randomly from uninfected mock-exposed (L) and "dead in 0 hours" (R) groups, demonstrating how we used x position within a tunnel (x) during each frame of the assay (y) to quantify thermal preference for individual flies. We assigned thermal preference for frame positions falling in the middle 4% of each tunnel as 23℃.

Data Visualization and Statistics

Because the likelihood of dying from *E. musae* infection drops significantly after 120 hours (5 days) post exposure, we only statistically analyzed and plotted time-until-death results from surviving exposed flies, mock-exposed flies, and flies that died from *E. muscae* infection 0-96 hours (0-4 days) after their respective experiment, despite recording deaths up to 168 hours (7 days) post exposure (Figure 4B). We chose not to include flies in the "dead in 120 hours" (5 days until death, typically 6-7 days post exposure) and "dead in 144 hours" (6 days until death, typically 7 days post exposure) groups, because only 4% of cadavers in our assay died after 120 hours (day 5) post exposure. We ran no statistical analyses on the data grouped by time since exposure. We plotted by-hour individual and group mean and total-experiment group mean temperature preferences for all flies according to outcome (Figure 4). We used the same methods

to plot by-hour individual and group mean distance traveled (Figure S4), as well as by-hour and total experiment thermal preferences from preliminary experiments at different time points and hot and cold side Peltier temperatures (Figure S4).

Because there were greater than sixfold differences in sample sizes and greater than twofold differences in standard deviation across some groups, we used Welch's ANOVA and post hoc Welch's t-test comparisons to analyze the data (Penn, 2020b; Welch, 1951) . We only statistically analyzed group mean temperature preferences within each hour of the ~ZT8-ZT12 assay because this time frame just before sunset allowed us to investigate behavioral fever, early-infection cold-seeking and late-infection cold-seeking. We adjusted alpha values using the Holm-Bonferroni method, allowing us to maintain sufficient power while limiting Type I error (Holm, 1979; Penn, 2020a). Additionally, we scaled each P-value from our Welch's t comparisons to its new corresponding alpha, such that the P-values could be compared to an alpha of 0.05 (Penn, 2020a). All referenced P-values will reflect this alpha of 0.05 scaling (Table S1).

Fitness Assay Protocol

Fly Fitness Assay

Because cold-seeking in fungal-infected *D. melanogaster* has been linked to late-age reproductive success, indicating fly-driven preference for cooler temperatures (Hunt et al., 2016), we investigated reproductive output at low and high temperatures of moribund *E. muscae*-infected flies within 24 hours before death compared to control flies. Following previously mentioned exposure protocol, we prepared wide-mouth Drosophila vials (Genesee, Cat #: 32-112) of 50 exposed or mock-exposed female flies on 5AS medium, held at 21°C until the day of testing. On days 3 and 4 (72 and 96 hours) post exposure, we chose individuals for testing that looked likely to die the next day from infection based on advanced fungal growth in their abdomen because we were specifically interested in moribund fly reproductive behavior. We transferred these exposed flies and mock-exposed flies at ~ZT14 to individual housing (200 microliter strip-PCR tubes). We prepared individual housing with 50-100 microliters of nutrient-rich 5YS medium (5% sucrose, 5% yeast, and 1.5% agar) in order to encourage

egg-laying. We kept separate groups of mock-exposed and exposed flies in incubators at 18°C and 23°C for 15-24 hours. At ~ZT14 on the day following the start of temperature treatment (day 4 or 5 (96 or 120 hours) post exposure), we recorded each fly's survival status (survivor, cadaver, or unexposed control) and number of eggs or larvae produced. We tracked the survival status of flies until 7 days (168 hours) post exposure. For each of 4 experiments we tested 21-56 exposed and 30-56 control flies at the two temperatures. Of tested flies, we only analyzed egg laying information from infected flies that died on the day of egg-counting (day 4 or 5 (96 or 120 hours) post exposure for temperature treatments starting on day 3 or 4 (72 or 96 hours) post exposure, respectively) and unexposed control flies that did not die before egg-counting. We grouped and plotted data by infection status (infected cadaver or uninfected mock-exposed) and temperature (18°C or 23°C) in order to analyze the effects of each of these variables on whether a fly laid eggs during the 15- to 24-hour temperature treatment (Figures 5 and 7). Because the number of eggs laid was considerably skewed toward zero for all groups, we fit our data to multiple logistic regression models to test for the effect of temperature, infection, and the interaction of temperature and infection on whether a fly laid. We settled on the binomial logistic regression model because it had the best fit for our data according to AIC and BIC scores and residual plots. We also graphically represented the number of eggs laid per hour by individual flies and their respective temperature-infection status groups (Figure 5).

Fungus Fitness Assay

In order to investigate the possibility that cold-seeking in moribund flies could be caused by fungal manipulation, we coupled our fly fitness assay with a fungus infectivity assay that allowed us to quantify *E. muscae* fitness at low (18°C) and high (23°C) propagation temperatures based on a cadaver's effectiveness at infecting other flies. We quantified infectivity by tracking the number of *E. muscae*-infection deaths produced by a set of cadavers up to 7 days (168 hours) post exposure. Using fresh cadavers held at 18°C or 23°C for ~24 hours prior to death, we prepared experimental infectivity vials of 50 flies on 5AS medium following previously explained exposure protocol, but decreasing the Droso-plug (Genesee, Cat #: 59-201) distance to 1.5 cm. We made this change because we only used 1-4 cadavers for exposure vials rather than

the standard 6, based on how many cadavers were produced during the fly fitness experiments. We also prepared control infectivity vials, each with 4 cadavers (from fly stocks kept at 21°C) on 5AS media. We kept control and experimental vials in a "humid chamber" at 18°C or 23°C for ~17-22 hours, with the propagation temperature treatment for experimental vials being determined by the temperature that the cadavers were kept at prior to death. Treating cadavers at low and high temperatures for ~24 hours before and after death allowed us to investigate whether cooler temperature exposure before sporulation and germination has an impact on *E. muscae* infectivity, or if cold-seeking might simply be a behavior induced in the fly in order to position it optimally within the environment for later fungus germination. After ~17-22 hours of temperature treatment we raised the Droso-plugs to the top of each vial, replaced the cadaver-embedded 5AS medium with fresh 5AS medium, and moved the vials to 21°C for the remaining 6 days (144 hours) of the experiment. Starting on day 3 (72 hours) post exposure and until day 7 (168 hours) post exposure, we checked experimental and control vials daily at ~ZT14 and recorded the number of cadavers produced that day. We totaled the number of cadavers produced over all 7 days (168 hours) of the assay for each experimental and control vial and calculated the number of cadavers produced per cadaver used to propagate infection. For analyses and plotting, we grouped experimental vial cadaver counts based on temperature treatment before and after propagation-cadaver death and nutrient medium that the propagation-cadaver was kept on during its fly fitness experiment. We grouped control vials by propagation-temperature treatment. Because of unequal variances and N-values across groups, we used Welch's ANOVA and Games-Howell post hoc pairwise comparison to analyze the data (Games & Howell, 2016; Peters, 2019; R Core Team, 2018; Welch, 1951).

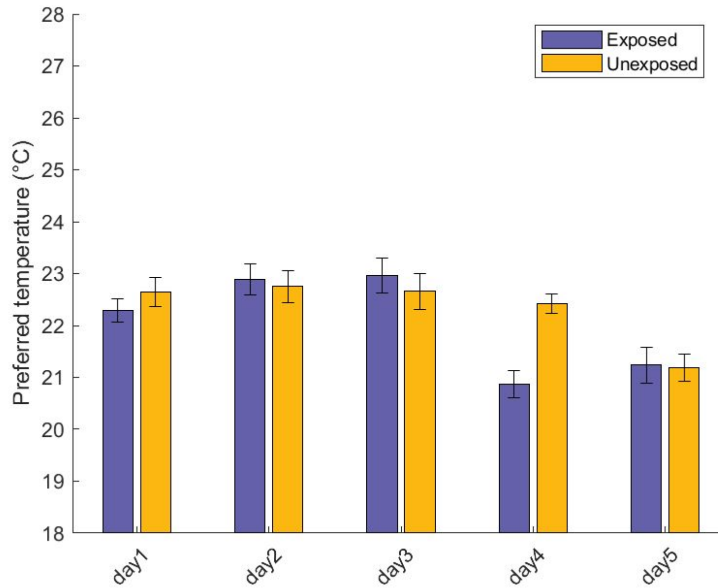
Results and Discussion

What thermal preference do flies exhibit when infected with *E. muscae*?

House flies exposed to *E. muscae* have previously been found to display altered temperature preferences compared to uninfected controls, namely behavioral fever at early infection time points and cold-seeking during late infection (Watson et al., 1993). To see if temperature preferences are also altered in *E. muscae*-exposed *D. melanogaster*, we used a custom,

two-choice thermal behavioral assay to measure temperature preference of individual flies and monitored the survival of tested flies until 7 days (168 hours) post exposure. Under laboratory conditions, fruit flies typically die 96 to 120 hours (4-5 days) after exposure to *E. muscae* (Figure 1), therefore we tested flies at 24-hour time points from 24-120 hours (1-5 days) after exposure. We expected that behavioral fever would occur at 24-72 hours (1-3 days) after exposure to *E. muscae* and any cold-seeking would occur at 72-120 hours after exposure. We initially tested for behavioral fever in *E. muscae*-exposed flies in a ~ZT12-ZT16 assay at 22°C and 32°C, followed by testing for cold-seeking in a ~ZT10-ZT14 assay at 18°C and 28°C. However, we shifted the temperatures to 18°C and 28°C and the time frame of the assay to ~ZT8-ZT12 for both behavioral fever and cold-seeking experiments because most flies did not have preferred temperatures above 28°C in our initial behavioral fever assay and moribund flies were dying during experiments in our initial cold-seeking assay. Changing the time and temperature also allowed us to compare thermal preference trends in *E. muscae*-exposed *D. melanogaster* across the entire typical *E. muscae* incubation period (4-5 days, 96-120 hours), therefore the ~ZT8-ZT12 assay is the focus of our analyses and discussion. As flies vary in time to death after exposure (most flies die at 96 or 120 hours, but can die until 168 hours post exposure), we analyzed our data by both grouping flies 1) by time since exposure (i.e. day 1-5) (Figure 4A) and 2) by time until death (i.e. 120, 96, 72, 48, 24 or 0 hours (died on same day)) (Figure 4B).

(A)



(B)

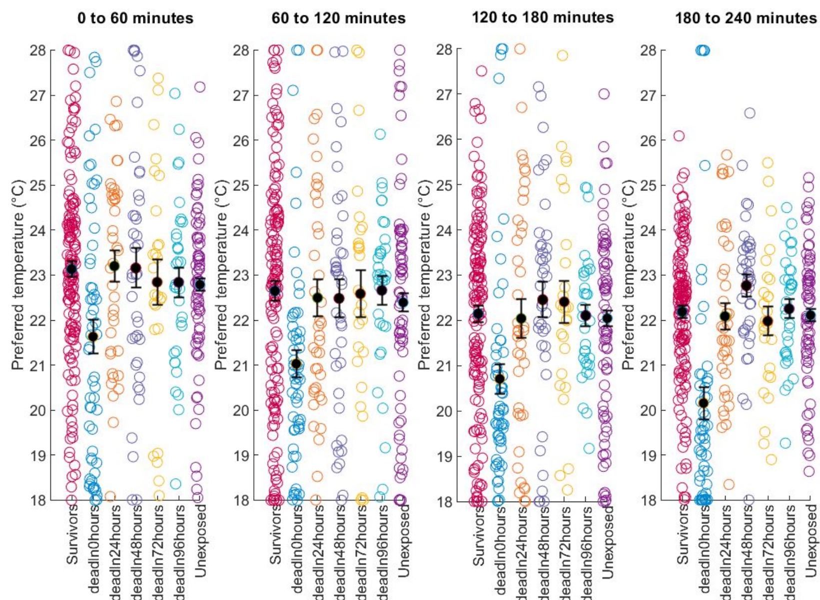


Figure 4. By-hour (~ZT8-ZT12) thermal preference of *E.muscae*-exposed and mock-exposed control *D. melanogaster* tested at 18°C vs 28°C on days 1-5 (24-120 hours) after exposure. Black points show the mean preferred temperature for the respective group in that hour. Error bars show standard error. (A) Groups are color-coded according to time since exposure to *E. muscae* (1-5 days). (B) Groups are color-coded according to time until death from infection (L to R: survived fungal exposure, died 0-96 hours after experiment, or unexposed control).

We found no evidence of flies exhibiting behavioral fever at any point in our ~ZT12-ZT16 assay or ~ZT8-ZT12 assay whether grouped by time since exposure or time until death (Figure 4, Figure S1). However, the “dead in 48 hours” group in our ~ZT10-ZT14 group appeared to prefer warmer temperatures than other groups across each hour of the assay (Figure S2). Although it is possible that flies dying in 48 hours were exhibiting behavioral fever at this time point, it seems unlikely because ~ZT10-ZT14 would be after or just before sunset, when temperatures would not typically be very high in a natural environment. Previous studies reported absence of behavioral fever in *D. melanogaster* (Arnold et al., 2015; Ballabeni et al., 1995), although it is utilized as an immune strategy in a variety of ectotherms to fight pathogens (Moore, 2002), including house flies infected with *E. muscae* (Watson et al., 1993). The absence of behavioral fever could be due to the small body size of *D. melanogaster*, making it energetically costly for flies to maintain their body temperature above ambient temperatures (Ballabeni et al., 1995).

Alternatively, *D. melanogaster* has been found to exhibit cold-seeking in response to pathogens (Ballabeni et al., 1995; Hunt et al., 2016). We observed a clear pattern of colder temperature preference in moribund flies (flies that died on the same day of the experiment) in our ~ZT8-ZT12 assay (Figure 4). The “dead in 0 hours” (moribund) group had a significantly lower mean preferred temperature ($P < 0.05$) than all groups except the “dead in 72 hours” group in the last hour (ZT11-12) of the assay (Table S1). When grouping the data by time since exposure, lower temperature preferences of unexposed flies were apparent on days 4 and 5 (Figure 4A). This is likely because the preference for the exposed group overall was pulled down due to most infected flies tested on these days dying on the day of their assay. What we observed suggests that flies infected with *E. muscae* prefer cooler temperatures in the hours before sunset on the day of death.

Interestingly, moribund “dead in 0 hours” flies had a significantly lower mean preferred temperature than *E. muscae*-exposed “survivors” and mock-exposed (unexposed) flies in every hour of the assay (Figure 4B). However, they did not have a significantly lower thermal preference in every hour of the assay when compared to other *E. muscae*-infected groups (dead in 24-96 hours). This could be due to lower sample size of many of the groups eventually killed

by the fungus, particularly the “dead in 72 hours” group with a sample size less than half that of the “dead in 0 hours” group (Table S2). In the first hour of the assay, any lack of significant difference could arise because moribund flies do not cold-seek until just before death, in the last few hours of our assay. The lack of difference in the first hour could also be caused by flies exploring the thermal assay chamber, and therefore not showing particular preference for one temperature over the other. It is apparent for all groups except the “dead in 0 hours” group that individual mean thermal preferences in the first hour fall consistently around ~23°C, which would be the preferred average if a fly is spending equal time on the 18°C to 28°C sides of its chamber. Throughout the duration of the assay the individual and mean preferred temperatures deviate more from this 23°C middle point (Figure 4B).

Another explanation for the lack of significant difference between the “dead in 0 hours” and other *E. muscae*-killed groups in multiple hours of the assay is that all doomed groups could be cold-seeking as an immune response to infection. A review by Sinclair et al. indicates that exposure to the cold results in greater tolerance to fungal infection and upregulation of immune-related genes by insects (2013). *D. melanogaster* is known to utilize cold-seeking as a behavioral immune response to numerous pathogens (Ballabeni et al., 1995; Fedorka et al., 2016; Le Bourg et al., 2008), including other fungal parasites (Hunt et al., 2016), and *E. muscae* might not be an exception. However, if all *E. muscae*-killed groups were utilizing cold-seeking as an immune response, it raises a couple of questions. First, why did infected flies in our thermal assay still die at a proportion equal to what is typically seen from *E. muscae*-infected *D. melanogaster* using our standard infection protocol mentioned previously (~50-60% typically die from infection)? It is possible that moribund flies did not spend enough time at the low temperatures to have a noticeable impact on survival. This question could also be answered by evidence that *D. melanogaster* might not always utilize cold-seeking at a low enough temperature to optimize infection survival (Fedorka et al., 2016). On average, bacterially-infected flies in a study by Fedorka et al. preferred temperatures only 1°C below the mean thermal preference of healthy, uninfected flies, but a temperature at least 5°C below the healthy fly average was the most optimal for improving infection survival (2016). A similar phenomenon could be occurring in *E. muscae*-infected *D. melanogaster*. But trends in our data

support the interpretation that flies are likely not cold-seeking at all prior to the day of death. No *E. muscae*-killed groups aside from the "dead in 0 hours" group were significantly different from the "unexposed" or "survivors" groups at any point in the assay. Additionally, the means of the other *E. muscae*-killed groups were much closer to the "unexposed" or "survivors" groups, even during hours that they were not significantly different from the moribund "dead in 0 hours" group (Figure 4B). This indicates that any lack of significant difference between the moribund group and other *E. muscae*-killed groups is simply due to chance and not evidence of a true lack of difference between these groups. But it is important to note that sample sizes in *E. muscae*-killed groups were small and standard deviations were not consistent relative to the survivor and unexposed groups, so we will need to repeat testing to elucidate whether cold-seeking is present in *E. muscae*-killed flies before the day of death.

When considering that all *E. muscae*-killed groups in our assay could be cold-seeking as an immune response to infection, we also asked why the "dead in 0 hours" group always preferred temperatures that were considerably lower than other doomed groups. Based on findings from Fedorka et al. about optimal temperatures for fighting bacterial infection in *D. melanogaster* (2016), it could be that moribund flies were choosing much lower temperatures in the final stages of *E. muscae* infection as a last ditch effort to survive. However, it is also possible that this significantly lower thermal preference in moribund flies is being caused by *E. muscae* manipulation of the fly, particularly considering the multiple ways in which *E. muscae* is already known to modify the behavior of *D. melanogaster* within a few hours before death (Elya et al., 2018).

Although moribund flies do not show significantly lower temperature preferences compared to all groups across every hour of our ~ZT8-ZT12 assay, there is a trend toward lower thermal preference ending in significantly lower preferred temperatures for moribund flies compared to all groups during the last hour of our assay (ZT11-12). This supports evidence for cold-seeking only on the day of death from *E. muscae* infection, particularly in the final hours before sunset. At this point, the fungus has effectively taken control of many of the fly's actions and is positioning its host for optimal sporulation by inducing summiting behavior, wing-raising, and

proboscis extension (Elya et al., 2018). It is possible that thermal preference is another behavior manipulation driven by the fungus. Because the optimal germination temperature for *E. muscae* has been found to be ~21°C (Carruthers & Haynes, 1986), Watson et al. speculated that *E. muscae* was inducing the cooler temperature preference observed in house flies on the day of death from infection. Interestingly, infected house flies tended to be congregated at low temperatures for each 2-hour interval of Watson et al.'s 8-hour assay on days 4 and 5 post exposure, not just in the last few hours before death (1993). This could be because Watson et al.'s flies were tested in groups, so social factors could have been influencing where flies were located on the thermal gradient. But if social factors do not have an effect, these findings would support the interpretation that the fruit flies we tested were cold-seeking in all four hours of our ~ZT8-ZT12 thermal preference assay and potentially earlier in the day as well.

It is unclear why the fungus would induce cold-seeking behavior in its host earlier than a few hours before it kills the fly if it truly is controlling the fly's thermal preference on the day of death. This cold-seeking behavior could be a by-product of other behavioral or physiological changes being driven by the fungus, potentially explaining why it arose earlier than necessary in house flies. But cold-seeking earlier than a few hours before death could be helpful for the fungus to prevent behavioral fever in hosts that utilize immune response, like house flies. Watson et al. found that *E. muscae*-infected house flies treated at 40°C on days 4 and 5 post exposure still died from infection but did not sporulate or assume the typical post-mortem posture (wing-raising and proboscis extension) (Watson et al., 1993). Additionally, *E. muscae*-infected fruit flies do not sporulate at temperatures as high as 33°C (Elya, C., personal communication). It is possible that fungus-induced prevention of behavioral fever would not occur until after *E. muscae* has invaded its host's nervous system around 48 hours after exposure, which would explain why behavioral fever is still present in earlier days of infection in house flies and cold-seeking does not arise until later on.

Although our study and past research has not found behavioral fever in *D. melanogaster*, induced cold-seeking by *E. muscae* prior to behavioral changes preparing the host for sporulation and germination on the day of death could still be occurring, like it might be in house flies. Although

E. muscae is thought to be a specialist parasite (De Fine Licht et al., 2017), it is known to infect multiple families of dipterans (Carruthers & Haynes, 1985; Cohn, 1855; Eilenberg, 1987; Elya et al., 2018) and is able to be propagated between individuals of differing Diptera families (Elya et al., 2018). The fungus also tends to induce similar behaviors in each of its host families. Therefore, if *E. muscae* can lower host temperature preference, it is reasonable to assume that it would do so in multiple host species despite differences in immune thermoregulatory behavior across families, namely behavioral fever in house flies and its absence in *D. melanogaster*.

In addition to the strong cold-seeking behavior of fruit flies on their day of death, we also noticed that thermal preference on day 1 post exposure (24 hours after exposure) appeared to trend lower than that of unexposed flies on that day in the ~ZT8-ZT12 assay at 18°C and 28°C, as well as the ~ZT12-ZT16 assay at 22°C and 32°C (Figure 4A, Figure S1). This could suggest cold-seeking in the early stages of infection, but further testing is needed to confirm this. We are also prompted to repeat testing in order to investigate why day 5 unexposed flies preferred temperatures very similar to the low temperature preference of the mostly moribund day 5 exposed flies. This could be coincidental due to small sample sizes. But there also could be a relationship between ageing and thermal preference that we are capturing in healthy flies. Older flies (21 days post-eclosion and older) have been found to exhibit decreased cold sensitivity (Shih et al., 2015), but this explanation seems unlikely for our ~5 day-old flies.

To investigate whether cold-seeking behavior begins prior to the typical end-of-life behaviors on the day of death for *E. muscae*-infected fruit flies (i.e. summiting, proboscis extension, wing-raising), we ran a thermal preference assay at ~ZT0-ZT4 on day 4 (96 hours) and day 5 (120 hours) post exposure. We found inconsistency in temperature preferences across all outcome groups (Figure S3) and believe our results do not show accurate trends of temperature preference at this time point, due to confounding factors producing inactivity in flies. In our ZT0-ZT4 assay testing for the transition to cold-seeking, up to ~8% of flies didn't move after the first hour of the assay and the average distance traveled for all groups was around or lower than that of the least mobile "dead in 0 hours" group in our ~ZT8-ZT12 assay (Table S3, Table S4). This could be caused by the onset of a rest period following the morning activity peak typical in

Drosophila (Grima et al., 2004). Moreover, up to ~36% of moribund flies stopped moving in our ~ZT10-ZT14 cold-seeking assay by the last hour, presumably because they died before the assay ended (Table S5). This is why we changed the assay timing to ~ZT8-ZT12 for our main set of thermal preference experiments.

Regardless of the cause of inactivity, it is important to note its impact on representations of thermal preference in our assay. After an exploratory first hour with more fly activity, the average thermal preference of all groups in our ~ZT0-ZT4 assay was around or below 21°C, close to the average of the obviously cold-seeking but still mobile "dead in 0 hours" group of our ~ZT8-ZT12 assay (Figure 4B, Figure S3). The "dead in 0 hours" group in our ~ZT10-ZT14 assay had a large standard error of thermal preference because of individual preferences at each of our temperature extremes by flies that were already or nearly dead from infection (Figure S3). We tried to prevent inaccuracies of thermal preference by removing flies from analyses that had an average distance traveled less than a certain threshold. However, because the threshold is based on the total mean distance traveled across all flies ran in the experiment, there are a couple of instances in which inactive or dead flies that are presumably not making temperature choices could fail to be removed from the data set: (1) Flies that are inactive or dead might not be eliminated if most flies in the experiment did not move very much. This is because the threshold is based on the total mean distance traveled for that particular experiment, averaging across all flies run in the experiment. (2) If flies are moving a considerable amount during most of the experiment but stop moving for an extended period, either because of inactivity or death, they might not be removed because their mean distance traveled for the whole experiment does not fall below the threshold. Despite these drawbacks with our distance traveled threshold, we did not want to be too stringent with eliminating periodically inactive flies because *E. muscae*-infected flies tend to be more lethargic than healthy flies, particularly as the infection progresses (Elya et al., 2018; Watson et al., 1993). We found that moribund flies in our ~ZT8-ZT12 assay appeared to be considerably inactive when compared to other groups, and their activity tended to decrease by a significant amount in each hour of the assay (Figure S4). We were concerned that many moribund flies stopped moving on the 18°C side of our assay in the last hour and that was falsely lowering the group mean preferred temperature. However, only

~3% of moribund flies stopped moving from ZT11-ZT12. This supports that it is not inactivity that caused a lower preferred temperature in moribund flies. Instead, thermal preference was being mediated by the fly or its fungal parasite.

Control of host thermal preference by *E. muscae* has not been investigated since first observing flies at significantly cooler temperatures on the day of death in house flies (Watson et al., 1993), but field studies on *E. muscae* germination have shown that the optimal germination temperature for the fungus is ~21°C, with a maximum limit for germination around 26-32°C (Carruthers & Haynes, 1986). Interestingly, the optimal range coincides with the mean temperature preferences of 20.2-21.6°C that we found for the "dead in 0 hours" group throughout our *D. melanogaster* assay (Figure 4B), and the maximum limit of germination matches up well with Watson et al. finding all *M. domestica* cadavers at or below 30°C (1993). However, we could not rule out that our flies chose these cooler temperatures independent of fungal control without further investigation of underlying mechanisms.

Does fungus or fly fitness benefit from end-of-life cold-seeking?

Fly Fitness

It has been found that *D. melanogaster* exhibits cold-seeking behavior in response to a variety of pathogens (Fedorka et al., 2016; Hunt et al., 2016; Le Bourg et al., 2008). According to Hunt et al., female *D. melanogaster* infected with the fungal parasite *M. robertsii*, could be using cold-preference as a strategy for optimizing reproductive output in response to pathogens (2016). The cooler temperatures were effectively slowing death from infection and increasing late-age reproductive success in the female fruit flies (Hunt et al., 2016). To investigate whether the cold-seeking behavior we observed in *E. muscae*-infected *D. melanogaster* in the hours before death (ZT8-ZT12) was fly-driven, we conducted a set of experiments testing the fitness benefits of cooler temperatures for female moribund flies. We statistically analyzed whether infected and uninfected flies did or did not lay eggs at low (18°C) and high (23°C) temperatures for ~24 hours before death (Table S6) and graphically represented the number of eggs laid per hour by

individual flies and their respective temperature-infection status groups (Figure 5) to determine if infection status and temperature impacted egg-laying.

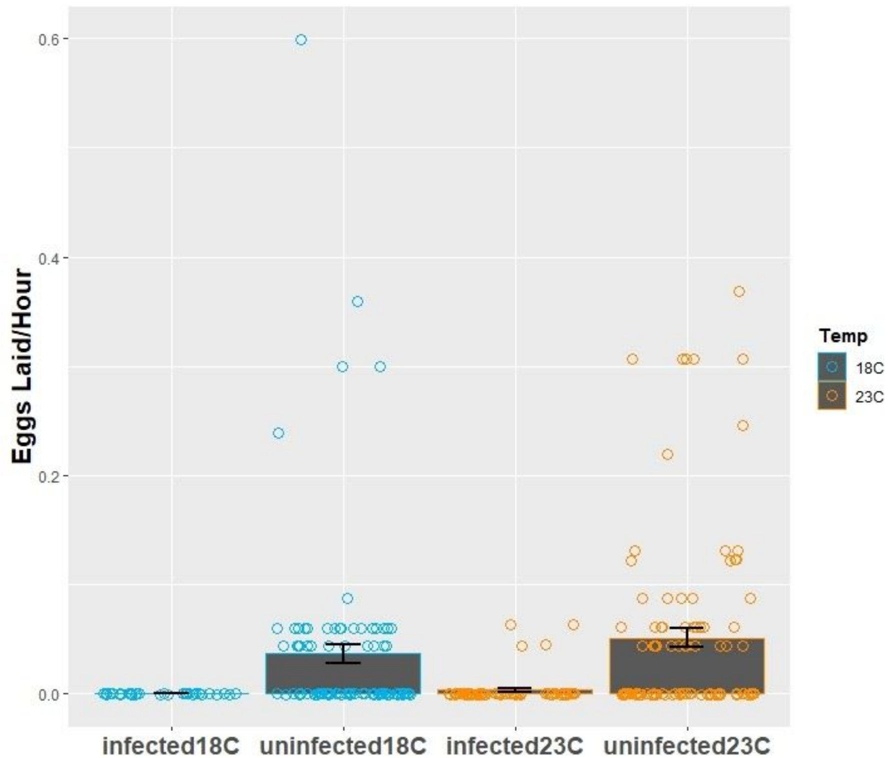


Figure 5. Average number of eggs laid per hour by moribund (dead from infection on the day of egg counting) *E. muscae*-infected *D. melanogaster* and mock-exposed (uninfected) *D. melanogaster*, housed individually at low (18°C) and high (23°C) temperatures for 15-24 hours on 5YS medium directly prior to egg counting. Error bars show standard error.

Large differences in time that our flies had to lay eggs (i.e. up to an 8-hour difference between some experiments) and substantial skewing of the number of eggs laid toward zero prompted us to binarize our egg-laying data (such that egg(s) laid = 1 and no egg(s) laid = 0) and model it using logistic regression to test the effect of temperature, *E. muscae* exposure, and the interaction between temperature and exposure on whether a fly laid. However, we understood that in converting our egg counts for each fly to binary outcomes, we would likely lose any effect of temperature or the interaction of temperature and exposure on egg-laying in our model. This is because we anticipated that temperature would affect the number of eggs laid by a fly, rather than whether a fly laid or not. Indeed, there was no effect of temperature or the interaction of temperature and exposure on egg-laying based on our binomial logistic regression model (Table

S6), despite indication that 1) the mean number of eggs laid per hour at 18°C and 23°C was different and 2) higher temperature potentially mitigated the effects of infection on egg-laying (Figure 5). What is more interesting is that we did not observe an effect of exposure on whether a fly laid eggs or not in our model (Table S6). We expected that flies infected with *E. muscae* would lay fewer eggs than healthy flies and this seems to be supported when looking at the data by eggs laid per hour (Figure 5). The reason why we did not see an effect of exposure in our model could have been due to a poor fit of the model, but the residual deviance demonstrates that our data fits the model well according to the corresponding X^2 test statistic (Table S6).

Trends of number of eggs laid per hour in moribund flies at low versus high temperature treatments were similar in infected and uninfected groups, such that the average number of eggs laid per hour tended to be less in flies treated at the lower (18°C) temperature (Figure 5). We expected fewer eggs to be laid at lower temperatures because low temperatures generally slow all physiological processes in ectotherms. If cold-seeking was beneficial to the fly, we expected to see that low temperatures would diminish the impact of infection on egg-laying more than higher temperatures would. Thus, in the case where the fly benefited from cold-seeking, the ratio of eggs laid per hour by infected flies to eggs laid per hour by uninfected flies would be greater when the flies were kept at lower temperatures, indicating that there is less of a difference in number of eggs laid between the infected and uninfected groups at low temperatures than at high temperatures. This is not what we found, because the ratio of the mean infected to mean control eggs laid at the low temperature was zero, due to no flies in the 18°C infected group laying eggs. This lack of eggs laid opposes the pattern we were expecting based on Hunt et al.'s observations of increased late-age reproductive output at lower temperatures in fungal-infected *D. melanogaster* (2016). Our results suggest that lower temperatures are not beneficial for fly reproduction at the end of life. Conversely, our data might argue that higher temperatures are actually more beneficial for fly fitness at such a late stage of infection, given that they were able to lay eggs at all. However, there were only 4 flies that laid eggs in our 23°C infected group, so it must be considered that these flies could be outliers. If this is the case, then we could assume that *E. muscae* infection is too severe just before death to allow a sizable increase in host fecundity according to temperature. That being said, we would like to repeat testing with more individuals

and more consistency in time that flies have to lay eggs before making any conclusions on whether or not low or high temperatures are more beneficial for host fecundity during *E. muscae* infection.

It is also important to note that multiple studies on thermal preference response to pathogens in *D. melanogaster* have found that cold-seeking is primarily a sex-dependent immune response occuring only in females (Ballabeni et al., 1995; Zhang et al., 2011). Although it is possible that female flies could have been cold-seeking in our thermal preference assay in a futile attempt to increase late-age reproduction before death, therefore bringing down the mean preferred temperature for the "dead in 0 hours group," our data does not support this because there were not considerable differences in thermal preference between sexes during our assay for any *E. muscae*-infected group (Table S7). In fact, male flies appeared to have a lower average preferred temperature than females in every outcome group except the "dead in 48 hours" group, though we have not confirmed this through statistical analyses. Thus moribund "dead in 0 hours" females are not influencing the cold-seeking temperature disproportionately.

Fungus Fitness

Though it has never been investigated outright, there is evidence consistent with fungal control of cold-seeking on the day of death in *E. muscae*-infected hosts. For example, Watson et al. observed cooler temperature preference on the day of death from *E. muscae* infection in *M. domestica*, a species known to use behavioral fever as an immune response to infection (1993). Moreover, Carruthers and Haynes found the optimal germination temperature for *E. muscae* to be ~21°C (1986), which coincides with the ~20°C-21°C temperature preference of moribund flies in our thermal preference assay. Considering the research of Watson et al. and Carruthers and Haynes, we were interested in investigating whether the cold-seeking we observed in moribund flies on the day of death benefited the fitness of the fungus. If so, this would be a further indication of *E. muscae* control of host thermal preference and warrant investigation of the mechanisms allowing such a host-parasite interaction.

To measure fungal fitness at low and high temperatures, we recorded the infectivity (defined as the number of cadavers produced over 7 days (168 hours) post exposure) of sporulating *E.*

muscae cadavers in vials of previously unexposed flies on 5AS medium held at 18°C or 23°C for the first ~24 hours after initial exposure. The chosen temperatures were meant to approximate what temperatures were generally being preferred by moribund cold-seeking flies versus unexposed, survivor, or non-moribund doomed flies. However, we wanted to make sure the temperatures were different enough to detect any potential effects so we chose a low temperature of 18°C, rather than using 21°C as a more similar approximation to the mean moribund fly preference.

Since growth leading to sporulation and germination occurs before death of the host, we kept morbiund flies at 18°C or 23°C for ~24 hours before death on 5AS medium then used their cadavers to expose new host flies at 18°C or 23°C for 24 hours. We also set up control vials of cadavers kept on 5AS medium with 2 treatments at ~21°C through death to expose new hosts. Thus, we had 4 treatments, with 2 at each temperature (Figure 6). Our experimental groups were tested at low or high temperatures through and after death, while our control groups were tested at low or high temperatures only after death.

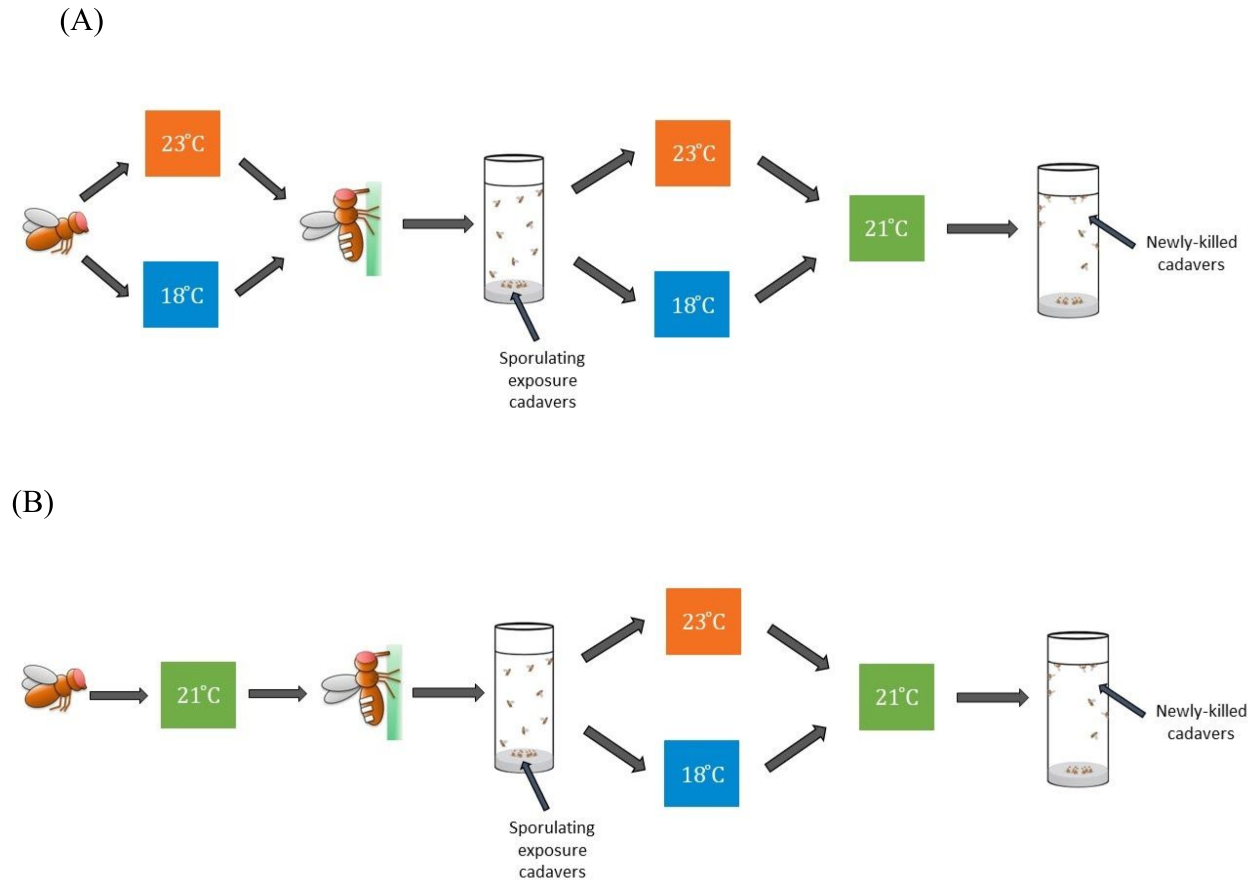


Figure 6. Diagram of how we tested for the effect of temperature on *E. muscae* fitness, as a measure of newly-killed *D. melanogaster* cadavers produced by a sporulating *D. melanogaster* cadaver. (A) To make experimental vials, we kept moribund *E. muscae*-infected *D. melanogaster* on 5AS medium at 18°C or 23°C for ~24 hours before death then used their cadavers to expose new host *D. melanogaster* at 18°C or 23°C for 24 hours. (B) To make control vials, we kept moribund *E. muscae*-infected *D. melanogaster* on 5AS medium at ~21°C through death to expose new host *D. melanogaster*.

We expected that if the fungus was somehow eliciting cold seeking in the host that we would see enhanced infectivity at lower temperatures versus higher temperatures. In contrast to this expectation, temperature did not significantly affect infectivity according to our analyses. The Welch's ANOVA that we ran had a P-value < 0.05, but when we followed up that test with a Games-Howell post hoc, there was no significance with any of our pairwise comparisons (Figure 7, Table S8). We did not expect significant differences between groups tested at the same temperature if pre-death temperature treatment did not have an effect on infectivity. However, we did expect significant differences in infectivity when comparing between temperatures, such

that sporulating cadavers treated at lower temperatures would have higher infectivity than cadavers at higher temperatures. It is important to note that our sample sizes were very low, which could be why we did not see significant differences in infectivity between temperatures as we would expect. The 18°C experimental group (with pre-death temperature treatment) had lower infectivity than the 23°C group. However, the control group (without pre-death temperature treatment) demonstrated higher infectivity for the 18°C group than the 23°C group, as expected (Figure 7, Table S8). This inconsistency between trends in the experimental and control groups could be caused by the low sample size of the 18°C experimental group and the potential for outliers in the 23°C experimental group. It is also possible that treating moribund flies at low or high temperatures for 24 hours before death impacted the growth and eventual germination of *E. muscae*. In particular, the 24-hour 18°C treatment before death could have had substantial effects on cadaver infectivity; such a long incubation at the low temperature could have slowed fungal growth, leading to fewer spores released by a cadaver and lower infectivity. Repeated testing with higher sample sizes and a temperature treatment time more similar to what we were seeing in our thermal preference assay (a few hours before death, rather than 24 hours) is necessary to make solid conclusions with this fitness data. But if trends of temperature effects on infectivity follow what we saw with our control group, this would indicate that lower temperatures benefit *E. muscae* and prompt further investigation of if and how the fungus might be modifying thermal preference behavior in moribund flies.

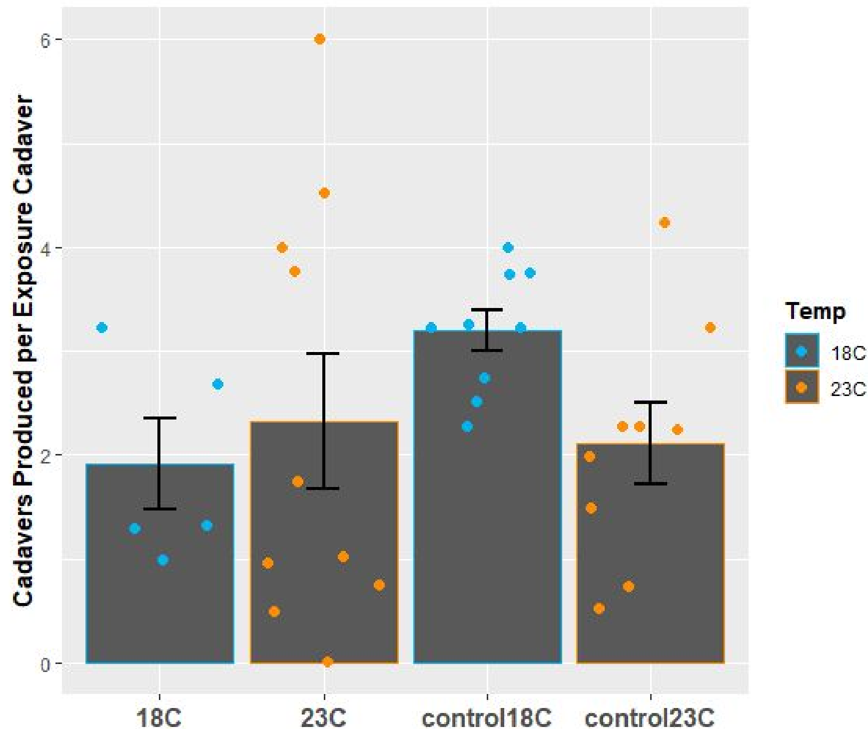


Figure 7. Average number of *E. muscae*-infected *D. melanogaster* cadavers produced per *E. muscae*-infected *D. melanogaster* cadaver used for exposure in vials of flies kept at low (18°C) and high (23°C) temperatures for the first 17-22 hours of exposure. We kept experimental group exposure cadavers at their respective post-death temperatures and on 5AS medium for 15-24 hours directly prior to death. We kept control exposure cadavers at 21°C on 5AS medium prior to death. Error bars show standard error.

Conclusion

Entomopathogenic fungi of the order Entomophthorales are some of nature's most fascinating parasites, manipulating their host's behavior to benefit their own fitness. However, the insects they parasitize are not helpless victims of these resourceful fungal pathogens. Watson et al. demonstrated that house flies infected with *E. muscae* demonstrated behavioral fever and cold-seeking during infection (1993). Both of these thermoregulatory behaviors can serve as immune strategies in ectotherms, helping a host slow the rate of infection or kill off pathogens (de Roode & Lefèvre, 2012). We were interested in whether *D. melanogaster*, or fruit flies, also exhibited these thermoregulatory responses to *E. muscae* infection. We tested our *E. muscae*-infected fruit flies in a two-choice thermal preference assay, in which they could choose between low and high temperatures. In testing on each day after exposure up until most flies

typically died of *E. muscae* infection (days 1-5 after exposure), we were able to capture changes in thermal preference in our flies in each stage of infection. We did not find evidence of behavioral fever, however cold-seeking was present in flies tested on the day they died from infection. Our results aligned with existing literature on thermoregulatory immune response in fruit flies, such that they have never been found to demonstrate behavioral fever (Arnold et al., 2015; Ballabeni et al., 1995) but they do use cold-seeking to slow infection (Ballabeni et al., 1995; Hunt et al., 2016). In short, our results suggest that *E. muscae*-infected *D. melanogaster* do not exhibit behavioral fever at any point during infection. However, we only saw cold-seeking in our flies just before sunset on the day of death from infection, when the fungus has effectively taken control of multiple complex behaviors in the fly and is preparing to position the body of the fly for optimal sporulation and germination. Additionally, lower temperatures are beneficial for *E. muscae* germination (Carruthers & Haynes, 1986) and this is why Watson et al. suspected that the cold-seeking they observed in their house flies on the day of death from *E. muscae* infection is actually being caused by the fungus (1993).

We were eager to investigate whether the cold-seeking we observed in our fruit flies was being caused by the fungus or the fly, so we ran a series of assays testing the benefit of low versus high temperature on the fitness of the fly and fungus. Although our fitness results were inconclusive due to small sample sizes, some of the trends we encountered in our data suggest that lower temperatures might be more beneficial for the fungus than the fly. We would like to do more testing before we make conclusions on the effect of temperature on host and pathogen fitness during *E. muscae* infection, but (1) *E. muscae* cadavers at lower temperatures tended to kill more flies (though our sample sizes are too small to confirm this trend statistically), (2) there was no significant effect of temperature on fecundity in our fly fitness assay, and (3) we did not observe differences in temperature preference between sexes in our thermal preference assay, such that females would be the only flies cold-seeking, effectively pulling down the mean preferred temperature for the entire moribund group. If still consistent after further testing, each of these trends would suggest that the fungus is causing the shift toward cooler temperatures that we observed at the end of host life. Manipulation of thermal preference would be a fascinating addition to the already impressive list of complex behaviors that *E. muscae* controls in its host to

improve its own fitness. We would be intrigued to investigate the neurological mechanisms driving thermal preference manipulation in order to add to a growing body of research on how *E. muscae* and other entomopathogenic parasites control insect behavior to their benefit. Such research is becoming increasingly useful in not only understanding how some parasites control behavior in their hosts, but also understanding the fundamental mechanisms that drive behavior in animals as a whole.

Supplementary Figures and Tables

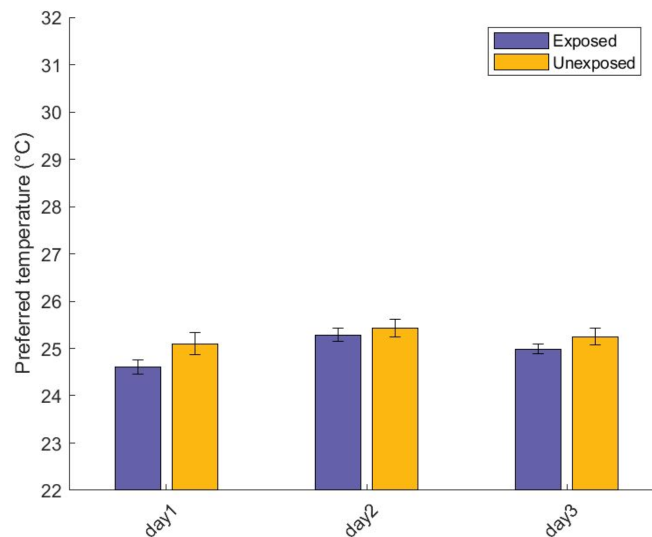


Figure S1. Thermal preference of *E. muscae*-exposed and mock-exposed (unexposed) *D. melanogaster* tested from ~ZT12-ZT16 at 22°C vs. 32°C on days 1-3 (24-72 hours) after exposure. Black points show the mean preferred temperature for the respective group in that hour. Error bars show standard error. Groups are color-coded according to time until death from infection (L to R: survived fungal exposure, died 0-96 hours after experiment, or unexposed control).

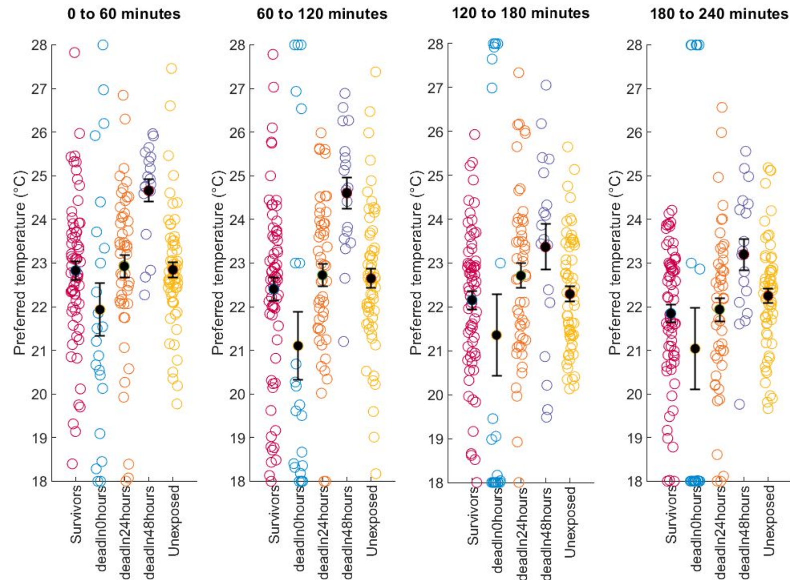


Figure S2. By-hour (~ZT10-ZT14) thermal preference of *E. muscae*-exposed (survivors, dead in 0-48 hours) and mock-exposed (unexposed) *D. melanogaster* tested at 18°C vs. 28°C on days 3-5 (72-120 hours) after exposure. Black points show the mean preferred temperature for the respective group in that hour. Error bars show standard error. Groups are color-coded according to time until death from infection (L to R: survived fungal exposure, died 0-96 hours after experiment, or unexposed control).

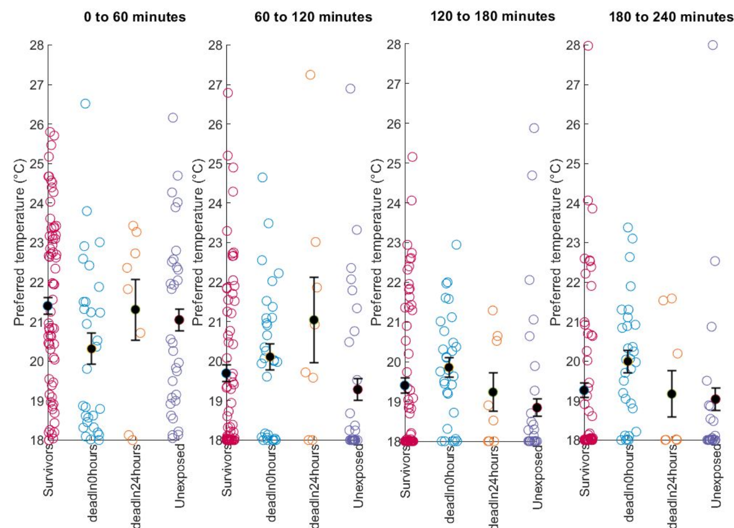


Figure S3. By-hour (~ZT0-ZT4) thermal preference of *E. muscae*-exposed (survivors, dead in 0-24 hours) and mock-exposed (unexposed) *D. melanogaster* tested at 18°C vs. 28°C on days 4 and 5 (96 and 120 hours) after exposure. Black points show the mean preferred temperature for the respective group in that hour. Error bars show standard error. Groups are color-coded according to time until death from infection (L to R: survived fungal exposure, died 0-96 hours after experiment, or unexposed control).

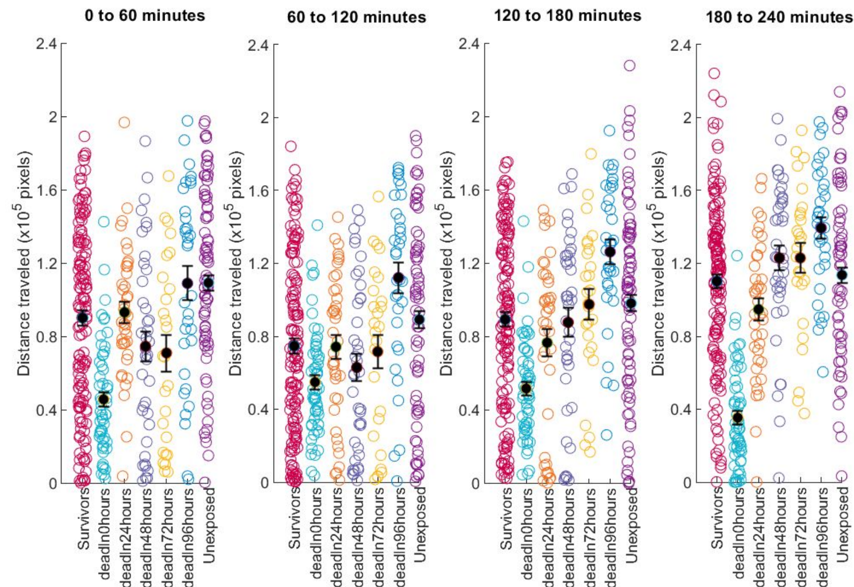


Figure S4. By-hour (~ZT8-ZT12) distance traveled of *E. muscae*-exposed (survivors, dead in 0-96 hours) and mock-exposed (unexposed) *D. melanogaster* tested at 18°C vs. 28°C on days 1-5 (24-120 hours) after exposure. Groups are color-coded according to experimental outcome (L to R: survived fungal exposure, died 0-96 hours after experiment, or unexposed control). Black points show the mean distance traveled for the respective group in that hour. Error bars show standard error.

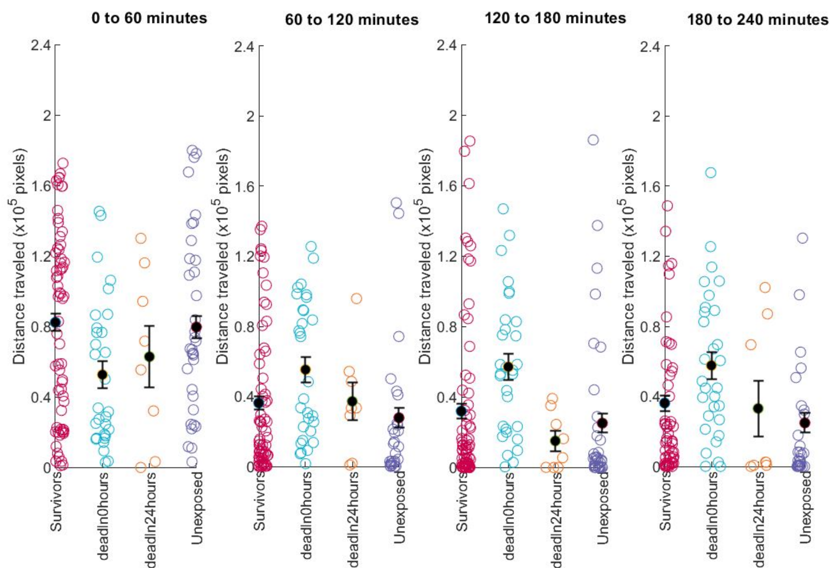


Figure S5. By-hour (~ZT0-ZT4) distance traveled of *E. muscae*-exposed (survivors, dead in 0-24 hours) and mock-exposed (unexposed) *D. melanogaster* tested at 18°C vs. 28°C on days 4 and 5 (96 and 120 hours) after exposure. Groups are color-coded according to experimental outcome (L to R: survived fungal exposure, died 0-96 hours after experiment, or unexposed control). Black points show the mean distance traveled for the respective group in that hour. Error bars show standard error.

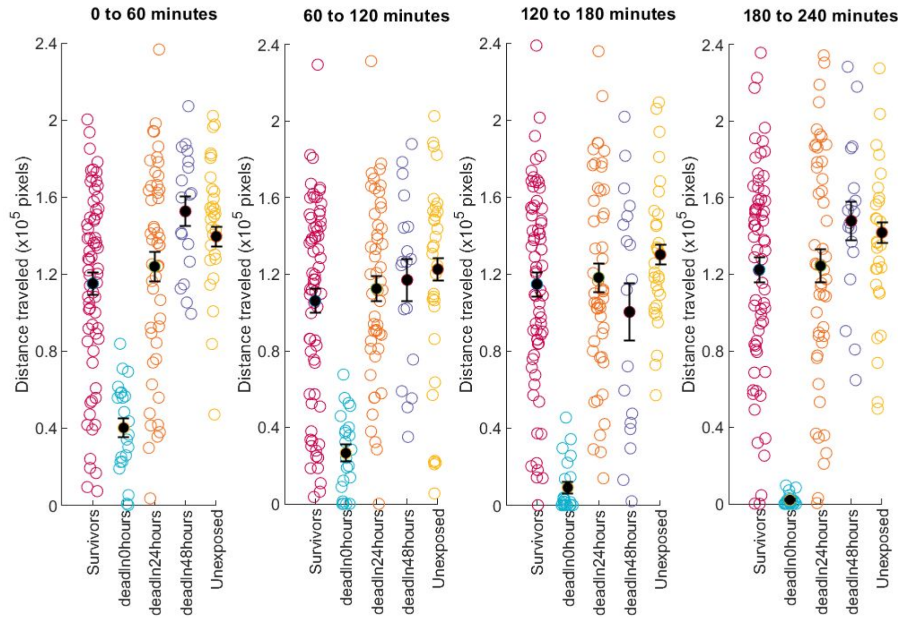


Figure S6. By-hour (~ZT10-ZT14) distance traveled of *E. muscae*-exposed (survivors, dead in 0-48 hours) and mock-exposed (unexposed) *D. melanogaster* tested at 18°C vs. 28°C on days 3-5 (72-120 hours) after exposure. Groups are color-coded according to experimental outcome (L to R: survived fungal exposure, died 0-96 hours after experiment, or unexposed control). Black points show the mean distance traveled for the respective group in that hour. Error bars show standard error.

Table S1. P-values from Welch's t-test comparisons of *E. muscae*-infected "dead in 0 hours" *D. melanogaster* temperature preference to temperature preferences of other *E. muscae*-exposed (survivors, dead in 24-96 hours) and mock-exposed (unexposed) *D. melanogaster* during our ~ZT8-ZT12 thermal preference assay. P-values are scaled to α' = 0.05, according to their respective α-values from the Holm-Bonferroni correction (Penn, 2020a). Groups with significantly higher mean preferred temperatures than the "dead in 0 hours" group are marked with an asterisk (*) for each hour the difference is significant. Hours when a group is not significantly different from the "dead in 0 hours" group are marked with a plus sign (+).

Outcome	Hour 1 (ZT8-9)	Hour 2 (ZT9-10)	Hour 3 (ZT10-11)	Hour 4 (ZT11-12)
Survivors****	0.003	0.005	0.004	> 0.001
deadIn24hrs+++*	0.201	0.187	0.525	0.007
deadIn48hrs++**	0.448	0.207	0.036	> 0.001
deadIn72hrs++++	1.000	0.307	0.155	0.075
deadIn96hrs+*+*	1.000	0.034	0.139	0.002
Unexposed****	0.027	0.012	0.007	> 0.001

Table S2. Descriptive statistics for temperature preference of *E. muscae*-exposed (survivors, dead in 0-96 hours) and mock-exposed (unexposed) *D. melanogaster* from the final hour (~ZT11-ZT12) of our ~ZT8-ZT12 thermal preference assay. We calculated group mean temperature preferences from individual mean temperature preferences, averaging over a fly's instantaneous temperature choices (18°C, 23°C, or 28°C) in each frame (measured at a rate of 10Hz) of the assay. Groups with significantly higher mean preferred temperatures than the "dead in 0 hours" group are marked with an asterisk.

Outcome	Mean Temp. Pref. (°C)	Standard Deviation	N
Survivors*	22.188	1.596	143.000
deadIn0hrs	20.158	2.660	56.000
deadIn24hrs*	22.086	1.843	39.000
deadIn48hrs*	22.769	1.516	39.000
deadIn72hrs	21.979	1.652	26.000
deadIn96hrs*	22.253	1.217	33.000
Unexposed*	22.112	1.566	143.000

Table S3. Descriptive and analytical statistics for distance traveled of *E. muscae*-infected "dead in 0 hours" *D. melanogaster* in each hour of our ~ZT8-ZT12 thermal preference assay. We calculated by-hour mean distance traveled from individual mean distance traveled values, averaging over a fly's distance traveled from frame to frame (taken at a rate of 10Hz) throughout the assay. We ran an ANOVA and Tukey-Kramer test to compare distance traveled in each hour of the assay for the "dead in 0 hours" group. Hours with mean distance traveled values significantly greater than the mean distance traveled during hour 4 (ZT11-ZT12) are marked with an asterisk.

Hour (~ZT8-12)	Mean Dist. Traveled (pixels)	Min. Dist. Traveled (pixels)	Max. Dist. Traveled (pixels)	% Motionless Flies	St. Dev.	P (α = 0.05)
Hour 1*	0.4586	0.0266	1.4276	0.00%	0.2995	0.0029
Hour 2*	0.5486	0.0368	1.4098	0.00%	0.2899	> 0.001
Hour 3*	0.5174	0.0475	1.4305	0.00%	0.2757	0.0016
Hour 4	0.3536	0	1.2426	3.57%	0.2713	---

Table S4. Descriptive statistics combined across all groups for distance traveled of *E. muscae*-exposed (survivors, dead in 0-96 hours) or mock-exposed (unexposed) *D. melanogaster* in each hour of our ~ZT0-ZT4 thermal preference assay. Mean, minimum, and maximum values are averages of all outcome group mean, minimum and maximum values by hour. We calculated by-hour mean distance traveled from individual mean distance traveled values, averaging over a fly's distance traveled throughout the assay.

Hour (~ZT0-4)	Mean of Group Mean Dist. Traveled (pixels)	Mean of Group Min. Dist. Traveled (pixels)	Mean of Group Max. Dist. Traveled (pixels)	% Motionless Flies
Hour 1	0.6952	0.0147	1.6756	0.00%
Hour 2	0.3912	0.0060	1.4525	2.70%
Hour 3	0.3245	0.0015	1.3940	6.31%
Hour 4	0.3796	0	1.6501	8.11%

Table S5. Descriptive statistics for distance traveled of *E.muscae*-infected "dead in 0 hours" *D. melanogaster* in each hour of our ~ZT10-ZT14 thermal preference assay. We calculated by-hour mean distance traveled from individual mean distance traveled values, averaging over a fly's distance traveled throughout the assay.

Hour (~ZT10-14)	Mean Dist. Traveled (pixels)	Min. Dist. Traveled (pixels)	Max. Dist. Traveled (pixels)	% Motionless Flies
Hour 1	0.4022	0.0009	0.8388	0.00%
Hour 2	0.2652	0	0.6759	13.64%
Hour 3	0.0916	0	0.4554	31.82%
Hour 4	0.0203	0	0.0961	36.36%

Table S6. Binomial logistic regression results of egg-laying behavior (egg(s) laid = 1, no egg(s) laid = 0) by moribund (dead on the day of egg counting) *E. muscae*-infected and mock-exposed (unexposed) *D. melanogaster* housed individually at low (18°C) and high (23°C) temperatures for 15-24 hours on 5YS medium directly prior to egg counting. The null deviance is $X^2 = 343.5$ on 281 degrees of freedom, with $P = 0.006$. The residual deviance is $X^2 = 287.19$ on 278 degrees of freedom, with $P = 0.34$.

Coefficients	Estimate	St. Error	z-value	P-value
Exposure	-17.089	722.296	-0.024	0.981
Temperature	0.310	0.295	1.051	0.293
Exposure x Temperature	14.582	722.297	0.020	0.984

Table S7. Mean temperature preference and N-values of male and female *E. muscae*-exposed (survivors, dead in 0-96 hours) and mock-exposed (unexposed) *D. melanogaster* in our ~ZT8-ZT12 thermal preference assay. We calculated group mean temperature preferences from individual mean temperature preferences, averaging over a fly's instantaneous temperature choices (18°C, 23°C, or 28°C) in each frame of the assay.

	Female		*Male*	
Outcome	**Mean Temp Pref (°C)**	**N**	**Mean Temp Pref (°C)**	**N**
Survivors	23.191	72	21.861	71
deadIn0hours	20.911	28	20.855	28
deadIn24hours	22.524	22	22.372	17
deadIn48hours	22.360	23	23.232	16
deadIn72hours	23.184	10	22.000	16
deadIn96hours	22.764	18	22.109	15
Unexposed	22.697	75	21.938	68

Table S8. Games-Howell post hoc comparisons of number of *E. muscae*-infected *D. melanogaster* cadavers produced per *E. muscae*-infected *D. melanogaster* cadaver used for exposure in vials of flies kept at low (18°C) and high (23°C) temperatures for the first 17-22 hours of exposure. We kept experimental group exposure cadavers at their respective post-death temperatures for 15-24 hours on 5AS medium directly prior to death. We kept control exposure cadavers at 21°C on 5AS medium prior to death. The Games-Howell test statistic, q, is synonymous to the Tukey-Kramer test statistic and is determined by Tukey's Studentized range (Toothaker, 1993). Degrees of freedom (df) from the Games-Howell test are based on Welch's degrees of freedom correction (Games & Howell, 2016).

Comparison	q	df	P
23C-18C	0.52	13.00	0.953
control18C-18C	2.65	5.69	0.136
control23C-23C	0.28	14.45	0.992
control23C-control18C	2.49	11.96	0.112

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